ISSN 1927-0887 (Print)
ISSN 1927-0895 (Online)

Journal of Food Research

Vol. 6, No. 4 August 2017

Canadian Center of Science and Education®

JOURNAL OF FOOD RESEARCH

An International Peer-reviewed and Open Access Journal of Food Research

Journal of Food Research is an international, double-blind peer-reviewed, open-access journal. JFR is published by the Canadian Center of Science and Education in both print and online versions. JFR aims to promote excellence through dissemination of high-quality research findings, specialist knowledge, and discussion of professional issues that reflect the diversity of this field.

The scopes of the journal include:

- History and Culture of Food
- Novel Foods and Sensory Analysis
- Food Quality and Safety
- Food Chemistry and Toxicology
- Food Microbiology
- Food Nutrition and Health
- Nutraceuticals and Functional Food
- Food Engineering and Physical Properties
- Food Laws and Regulations
- Food Economics and Marketing
- Concise Reviews and Hypotheses in Food Science

The journal is included in:

- AGRICOLA
- BASE
- Chemical Abstracts Service (CAS)
- Global Health
- InfoBase Index
- JournalFinder
- JournalTOCs
- SHERPA/RoMEO
- Ulrich's
- WorldCat

Open-Access Policy

We follow the Gold Open Access way in journal publishing. This means that our journals provide immediate open access for readers to all articles on the publisher's website. The readers, therefore, are allowed to read, download, copy, distribute, print, search, link to the full texts or use them for any other lawful purpose. The operations of the journals are alternatively financed by publication fees paid by authors or by their institutions or funding agencies.

Copyright Policy

Copyrights for articles are retained by the authors, with first publication rights granted to the journal/publisher. Authors have rights to reuse, republish, archive, and distribute their own articles after publication. The journal/publisher is not responsible for subsequent uses of the work.

Submission Policy

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the authorities responsible where the work was carried out. However, we accept submissions that have previously appeared on preprint servers (for example: arXiv, bioRxiv, Nature Precedings, Philica, Social Science Research Network, and Vixra); have previously been presented at conferences; or have previously appeared in other "non-journal" venues (for example: blogs or posters). Authors are responsible for updating the archived preprint with the journal reference (including DOI) and a link to the published articles on the appropriate journal website upon publication.

The publisher and journals have a zero-tolerance plagiarism policy. We check the issue using the plagiarism prevention tool and a reviewer check. All submissions will be checked by iThenticate before being sent to reviewers.

This is an 'Open Access' journal published under a creative commons license. You are free to copy, distribute, display, and perform the work as long as you clearly attribute the work to the authors.

JFR accepts both Online and Email submission. The online system makes readers to submit and track the status of their manuscripts conveniently. For any questions, please contact jfr@ccsenet.org.

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

EDITORIAL TEAM

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

CONTENTS

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Bacterial Transfer Associated with Blowing Out Candles on a Birthday Cake

Paul Dawson[1], Inyee Han[1], Danielle Lynn[1] , Jenevieve Lackey[1], Johnson Baker[1] & Rose Martinez-Dawson[2]

[1]Department of Food, Nutrition and Packaging Sciences, [2]Department of Mathematical Sciences, Clemson University, Clemson, SC 29634, USA

Correspondence: Paul Dawson, Depertment of Food, Nutrition and Packaging Sciences, Clemson University, Clemson, SC 29634, USA. Tel: 1-864-656-1138. E-mail: pdawson@clemson.edu

Received: March 24, 2017 Accepted: April 10, 2017 Online Published: May 22, 2017

doi:10.5539/jfr.v6n4p1 URL: https://doi.org/10.5539/jfr.v6n4p1

Abstract

This study examined the potential spread of bacteria when blowing out candles on a birthday cake. Preliminary tests of blowing on nutrient agar indicated that bioaerosols in human breath expelled from the mouth may be a source of bacteria transferred to cake surfaces. To test aerosol transfer to cake, icing was spread evenly over foil then birthday candles were placed through the foil into a Styrofoam™ base. After consuming pizza, test subjects were asked to extinguish the candles by blowing. Icing samples were sterilely recovered then surface plated, to determine the level of bacterial contamination. Blowing out the candles over the icing surface resulted in 1400% more bacteria compared to icing not blown on. Due to the transfer of oral bacteria to icing by blowing out birthday candles, the transfer of bacteria and other microorganisms from the respiratory tract of a person blowing out candles to food consumed by others is likely.

Keywords: birthday candles, aerosolized bacteria, blowing, bacterial transfer

1. Introduction

1.1 Blowing Out Birthday Candles

The tradition of blowing out birthday candles has different theories as to its origin. Some theorize the practice began in Ancient Greece related to bringing cakes with lit candles to the temple of the goddess of the hunt, Artemis. Other ancient cultures believed the smoke from candles carried their wishes and prayers to the gods. A written account reported of birthday candles matching the age of Count Ludwig Von Zinzendorf being presented at the Count's birthday celebration in Germany in 1700's (Frey, 1753). This tradition has become commonplace in many parts of the world.

1.2 Spread of Bacteria

Bacteria are an unavoidable part of life, present in and on almost everything humans contact. Whether benign or pathogenic, it is important to understand how bacteria are transferred and become familiar with measures for avoiding contamination. Illnesses related to pathogenic bacteria, which can spread rapidly throughout the population, are a major public health concern in today's society. Bioaerosols and poor air hygiene can have adverse effects on human health (Douwes, Thorne, Pearce & Heederik., 2003; Xu et al., 2011). Respiratory droplets expelled by coughing and sneezing are sources of normal human flora, as well as pathogenic bacteria (Obeng, 2008; 1970; Houk, 1980) and viruses (Loosli, Hertweck, & Hockwald, 1970). The respiratory tract can be colonized with pathogenic organisms that can then be aerosolized in the breath of an infected individual (Couch, Knight, Gerone, Cate, Douglas, 1969; Knight, 1973). The spread of respiratory diseases including SARS (Yu et al., 2004) and H1N1 avian influenza (Baker et al., 2010) have been attributed to oral airborne transmission. In fact, influenza virus particles were detected in the exhaled breath of infected individuals through coughing, breathing and talking (Fabian et al., 2008; Stelzer-Braid et al., 2009; Huynh, Oliver, Stelzer, Rawlinson & Tovey, 2008; Lindsley et al., 2010). When respiratory droplets are released, they may spread infection directly from person-to-person or by contamination of surfaces then touched by others (Obeng, 2008). The bacteria may have originated from either respiratory droplets expelled directly onto surfaces or indirectly as droplets coating hands that are transferred by hands to surfaces. In fact, exhaled breath contained 693 to 6,293 CFU of bacteria/m^3 (Xu et al., 2012) and Qian, Hospodsky, Yamamoto, Nazaroff & Peccia, (2012) reported that human occupants are

significant contributors to indoor air bacteria and that humans emit bacteria at a rate of about 37 million gene copies per person per hour. Thus when a person forcibly exhales, as with blowing out birthday candles, bacteria or viral particles are aerosolized from the respiratory tract of that individual.

1.3 Research Objective

The purpose of this research was to evaluate the level of bacterial transfer transferred to the top of a cake when blowing out the candles on a birthday cake. Scientific data from our investigation may help raise awareness of possible health risks associated with birthday celebrations and encourage others to take steps toward preventing the spread of bacteria.

2. Methods

2.1 Blowing Out Candles

A sheet of foil (Bakers & Chefs, Bentonville, AR) cut in the shape of a circle with a diameter of 149 mm placed on a Styrofoam™ disc (Styrofoam Brand Foam, Floracraft, Ludington, MI), of the same size then 18g of icing (Betty Crocker Rich & Creamy Vanilla Frosting, General Mills, Minneapolis, MN) was spread in a thin layer on the foil. Seventeen candles (Best Occasions, Bentonville, AR) (3.2 mm in diameter, 50.8 mm high, and set in plastic holders 19.0 mm high) were evenly spaced into the Styrofoam, passing through the icing and foil layers. Each test subject was asked to smell and consume a piece of hot pizza to simulate a meal-dessert sequence. After lighting the candles, test subjects were instructed to blow until all of the candles were extinguished on the mock cake (Figure 1). For each testing session a control sample was collected where the procedure was followed for the test sample except candles were not blown out.

2.2 Enumeration of Bacteria

After lit candles were blown out (blow) or not blown out (no-blow) the candles and holders were removed from the Styrofoam™ base, without touching the icing. Using sterile forceps, the foil was folded in half with the layer of icing inside. Then, the foil was placed in a stomacher bag (Classic 400, Seward, UK) and unfolded inside the bag.

Figure 1. Styrofoam™ base and candle apparatus with icing used to test bacterial transfer when blowing out candles

Fifty ml of 0.1% sterile peptone solution were poured into the stomacher bag over the iced surface of the foil. The stomacher bag was placed in a stomacher (Stomacher 400, Seward, UK) at 230 rpm for 1 min. Duplicate samples of 1 ml and 0.1 ml volumes were aseptically removed from the stomacher bag (Classic 400, Seward, UK), serially diluted and surface plated on plate count agar (Difco Plate Count Agar, Sparks, MD) in petri dishes. Samples were spread evenly on the agar and incubated at 37°C for 48 hours. Colony forming units (CFU) were counted on plates containing 25-250 colonies and converted to CFU per sample and $\log_{10}$ of CFU per sample.

2.3 Research Design and Statistical Analysis

The experiment was replicated 3 times on separate days by 11 subjects yielding 33 observations per treatment (blow or no blow). The effect of blowing vs. not blowing candles out on bacterial counts in the frosting was determined using the proc univariate command of SAS (2010) to obtain mean, median, range and standard deviation. The student's t-test was also performed and proc glm and pdiff commands were used to determine if significant differences existed between the blowing and non-blowing treatments.

3. Results and Discussion

Blowing out candles over icing resulted in 15 times more and statistically higher number of bacteria recovered from icing compared to icing that did not have candles blown out (Table 1). Also, the variation (range) in bacteria recovered from icing was 100 times greater for icing exposed to the blow compared to the no blow treatment. Furthermore, the median and maximum transfer of bacteria increased 300 and 12,000 %, respectively, due to blowing out candles. Studies on airborne droplet size from the oral cavity are found as early as 1899 (Flugge, 1899) and by several others before the mid 20th century (Hutchison, 1901; Winslow, 1910; Strausz, 1922; Lange & Nowoselsky, 1925; Hamburger, 1944; Duguid, 1946). These early studies came to varying conclusions but found droplets were released into the atmosphere surrounding humans that are breathing, coughing and sneezing. One study reported that 90% of bacteria-carrying droplets remaining airborne for 30 minutes in still air and that some smaller droplets remained for up to 30 hours (Duquid, 1946). More recently, Wan et al. (2014) established that up to over 2,000 moisture particles were released per breath, all less than 5 um in diameter. The particle size is an important factor since bioaerosols will carry both bacteria and viruses in small particle droplets generated by breathing, blowing and coughing. The average size of expelled particles generated by couching and speaking was found to be much larger (13.5 um for coughing and 16.0 um diameter for speaking) by measurement at the mouth opening thus minimizing the effect of evaporation on droplet (particle) size (Chao et al., 2008) which may be a factor in other studies using droplet condensation methodology. Chao et al. (2008) also found that there were between 1000 to 2000 in number and 2 to 5 ml in volume of droplets per cough and even 0.2 ml of moisture droplets during speaking. Therefore the size of droplets in expelled air are large enough to carry bacteria as well as viruses. Normal respiratory aerosols can include *Staphylococcus* spp., *Streptococcus* spp., *Corynebacterium* spp., *Haemophilus* spp., and *Neisseria* spp. (Madigan, Martinko, Dunlap, & Clark, 2009). Madigan et al. (2009) also found certain pathogenic species, such as *Streptococcus pneumonia* and *Staphylococcus aureus*, may cause illness when spread through surface contamination via oral aerosols. Considering contagious diseases such as influenza, some researchers have concluded that airborne transmission is a likely pathway (Weder & Stilianakis, 2008; Wein & Atkinson, 2009). Fabian et al. (2008) and Stelzer-Braid et al. (2009) detected viral influenza in the exhaled breath of infected patients. To this point, Fabian et al. (2008) reported that 60% of patients with influenza A had detectable levels of the virus in exhaled breath with 87% of exhaled particles less than 1 um in diameter. In another study, Lindsley et al. (2010) reported that 81% of influenza patients had influenza RNA in their breath and that 65% of the influenza were found in aerosol particles 4 um in diameter or smaller.

Verifying that bacterial cells as well as viruses are carried on human bioaerosols, Fennelly et al (2004) reported that 25% of tuberculosis patients exhaled from 3-633 CFU per cough of *Mycobacterium tuberlosis* in expelled air particles.

Birthday celebrations routinely include the ceremonial blowing out of candles on top of a cake. Some food safety concern exists in light of previous research on bioaerosols generated by breathing, coughing and speaking supported by the results of the present study finding that bacterial levels averaged 15 times higher in icing due to blowing out candles.

Table 1. Mean, median, range and standard deviation of the bacterial counts for cake icing exposed to blowing out candles and not blowing out candles

	No blow[1]	Blow[2]	Increase from No-blow to blow	
	CFU/sample[3] (log CFU/sample)[4]		CFU/sample[5]	(%)[6]
Mean	183[b] (2.2)[b]	2889[a] (3.5[a])	2706	1479
Median	150 (2.2)	600 (2.8)	450	300
Maximum	300 (2.5)	37,450 (4.6)	37150	12383
Standard deviation	112 (2.1)	6620 (3.8)	6508	5811

[1]*No-blow = cake icing not exposed to blowing out candles*

[2]*Blow = cake icing exposed to blowing out candles*

[3] *CFU/sample = colony forming units per cake icing sample. N = 33.*

[4] *Log CFUsample = log_{10} of colony forming units per cake icing sample*

[5] CFU/sample Increase = CFU/sample from samples blow on - CFU/sample from samples not blown on

$$[6]\%\text{Increase} = \frac{(\text{CFU/sample from samples blow on} - \text{CFU/sample from samples not blown on})}{\text{CFU/sample from samples not blown on)}} \text{ X } 100$$

[a,b] means with different superscripts are significantly different ($p \leq 0.0001$).

Acknowledgments

This research was supported by the Creative Inquiry Program at Clemson University. ***Technical Contribution No. 6547 of the Clemson University Experiment Station***.

References

Brankston, G., Gitterman, L., Hirji, Z., Lemieux, C., & Gardam, M. (2007). Transmission of influenza A in human beings. *The Lancet Infectious Diseases, 7*, 257-265. https://doi.org/10.1016/S1473-3099(07)70029-4

Bright, K. R., Boone, S. A., & Gerba, C. P. (2010). Occurrence of Bacteria and Viruses on Elementary Classroom Surfaces and the Potential Role of Classroom Hygiene in the Spread of Infectious Diseases. *Journal of School Nursing, 26*(1), 33-42. https://doi.org/10.1177/1059840509354383

Chao, C. Y. H., Wan, M. P., Morawska, L., Johnson, G. R., Ristovski, Z. D., … Katoshevski, D. (2009). Characterization of expiration air jets and droplet size distributions immediately at the mouth opening. *J Aerosol Sci., 40*(2), 122-133. https://doi.org/10.1016/j.jaerosci.2008.10.003

Couch, R., Knight, V., Gerone, P., Cate, T., & Douglas, R. (1969). Factors influencing response of volunteers to inoculation with Coxsackie virus A type 21. *American Review of Respiratory Disease, 99*, 24-30. http://www.atsjournals.org/doi/abs/10.1164/arrd.1969.99.1.24#readcube-epdf

Douwes, J., Thorne, P., Pearce, N., & Heederik, D. (2003). Bioaerosol health effects and exposure assessment: Progress and prospects. *Annals of Occupationanl Hygiene, 3*, 187-200. https://doi.org/10.1093/annhyg/meg032

Duguid, J. P. (1946). The size and the duration of air-carriage of respiratory droplets and droplet nuclei. *Epidemiology and Infection, 44*(6), 471-479 https://doi.org/10.1017/S0022172400019288

Fabian, P., McDevitt, J. J., DeHaan, W. H., Fung, R. O. P., Cowling, B. J., Chan, K. H., Leung, G. M., & Milton, D. K. Influenza virus in human exhaled breath: an observational study. *PLoS ONE, 3*, e2691. https://doi.org/10.1371/journal.pone.0002691

Fennelly, K. P., Martyny, J. W., Fulton, K. E., Orme, I. M., Cave, D. M., & Heifets, L. B. et al. (2004). Cough-generated aerosols of Mycobacterium tuberculosis: a new method to study infectiousness. *American Journal of Respiratory and Critical Care Medicine, 169*(5), 604-609. http://dx/doi.org/10.1164/rccm.200308-1101OC

Flugge, C. (1899). Die Verbreitung der Phthise durch staubförmiges Sputum und durch beim Husten verspritzte Tröpfchen. *Zeitschrift fur Hygiene und Infektionskrankheiten, 30*(1), 107-124. http://dx/.doi.org/10.1007/BF02198683

Frey, A. (1753) A True and Authentic Account of Andrwe Frey. https://books.google.com/books?id=VIoUAAAAQAAJ&pg=PA15&hl=en#v=onepage&q&f=false

Hamburger, M. (1944). Studies on the transmission of hemolytic Streptococcus infections: I Cross infection in army hospital wards. *Journal of Infectious Diseases, 75*(1), 58-70. https://doi.org/10.1093/infdis/75.1.58

Houk, V. N. (1980) Spread of tuberculosis via recirculated air in a naval vessel: the Byrd study. *Annals of the New York Academy of Sciences, 353*, 10-24. https://doi.org/10.1111/j.1749-6632.1980.tb18901.x

Hutchison, R. F. (1901). Die Verbreitung von Keimen durch gewöhnliche Luftströme. *Zeitschrift fur Hygiene und Infektionskrankheiten, 36*(1), 223-253. https://doi.org/ 10.1007/BF02141226

Huynh, K. N., Oliver, B. G., Stelzer, S., Rawlinson, W. D., & Tovey, E. R. (2008). A new method for sampling and detection of exhaled respiratory virus aerosols. *Clin Infect Dis, 46*, 93-95. https://doi.org/10.1086/523000

Knight, V. (1973). Airborne transmission and pulmonary deposition of respiratory viruses. In: Hers JF, Winkles KC, editors. Airborne transmission and airborne infections. VIth Int. Symp. on Aerobiology. New York, NY: Wiley, pp. 175-182.

Lange, B., & Nowoselsky, W. (1925). Experimentelle Untersuchungen über die Bedeutung der Staubinfektion bei der Tuberkulose. *Medical Microbiology and Immunology, 104*(1-2), 286-307. https://doi.org/10.1007/BF02175004

Lindsley, W. G., Blachere, F. M., Thewlis, R. E., Vishnu, A., Davis, K. A., Cao, G., & Palmer, J. E. (2010) Measurements of airborne influenza virus in aerosol particles from human coughs. PLoS ONE 5: e15100. https://doi.org/10.1371/journal.pone.0015100 .

Loosli, C., Hertweck, M., & Hockwald, R. (1970) Airborne influenza PR8-A virus infections in actively immunized mice. *Arch Envir Hlth, 21*, 332-346. https://doi.org/10.1080/00039896.1970.10667248

Madigan, M. T., Martinko, J. M., Dunlap, P. V., & Clark, D. P. (2009). Brock: Biology of Microorganisms, 12th ed. San Francisco: Pearson. 1061 p.

Obeng, C. S. (2008). Personal Cleanliness Activities in Preschool Classrooms. *Early Childhood Education Journal, 36*, 93-99. http:// doi.org/10.1007/s.10643-008.0253.4.

Qian, J., Hospodsky, D., Yamamoto, N., Nazaroff, W. W., & Peccia, J. (2012) Sizeresolved emission rates of airborne bacteria and fungi in an occupied classroom. Indoor Air, http://doi.org/10.1111/j.1600-0668.2012.00769 .

SAS (2010). Statistical Analysis Institute User's Guide :Basics, 9.1 ed., SAS Institute Inc. Cary, NC.

Stelzer-Braid, S., Oliver, B. G., Blazey, A. J., Argent, E., Newsome, T. P., Rawlinson, W. D., & Tovey, E. R. (2009). Exhalation of respiratory viruses by breathing, coughing, and talking. *Journal of Medical Virology, 81*, 1674-1679. http://doi.org/10.1002/jmv.21556

Strausz, W. (1922). Versuche über beim Sprechen verschleuderte Tröpfchen. *Zeitschrift fur Hygiene und Infektionskrankheiten, 96*(1), 27-47 https://doi.org/ 10.1007/BF02183834

Tellier, R. (2009) Aerosol transmission of influeza A virus: a review of new studies Journal of the Royal Society Interface. Published online 1-8. http://doi.org/10.1098/rsif.2009.0302.focus

Wan, G. H., Wu, C. L., Chen, Y. F., Huang, S. H., Wang, Y. L., & Chen, C. W. (2014) Particle Size Concentration Distribution and Influences on Exhaled Breath Particles in Mechanically Ventilated Patients. *PLoS ONE, 9*(1), e87088. http://doi:10.1371/journal.pone.0087088

Weber, T. P., & Stilianakis, N. I. (2008) Inactivation of influenza A viruses in the environment and modes of transmission: a critical review. *Journal of Infection, 57*, 361-373. https://doi.org/10.1016/j.jinf.2008.08.013

Wein, L. M., & Atkinson, M. P. (2009) Assessing infection control measures for pandemic influenza. *Risk Analysis, 29*, 949-962. http://doi.org/10.1111/j.1539-6924.2009.01232.x

Winslow, C. E. A. (1910).An investigation of the extent of the bacterial pollution of the atmosphere by mouth spray. *The Journal of Infectious Diseases, 7*(1), 17-37. http://dx/doi.org/ 10.1093/infdis/7.1.17

Xu, Z., Wu, Y., Shen, F., Chen, Q., Tan, M., & Yao, M. (2011) Bioaerosol science, technology, and engineering: past, present, and future. *Aerosol Science and Technology, 45*, 1337-1349. https://doi.org/10.1080/02786826.2011.593591

Xu, Z., Shen, F., Li, X, Wu, Y., Chen, Q., Jie, X., & Yao, M. (2012). Molecular and microscopic analysis of bacteria and viruses in exhaled breath collected using a simple impaction and condensing method. *Plos One, 7*(7), 1-8. Published online www.plosone.org

Yu, I. T., Li, Y., Wong, T. W., Tam, W., Phil, M., Chan, A. T., Lee, J., Leung, D., & Ho, T. (2004) Evidence of airborne transmission of the severe acute respiratory syndrome virus. *New England Journal of Medicine, 351*, 1731-1739. https://doi.org/10.1056/NEJMoa032867

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Human Health Risk Assessment of Heavy Metals in Kampala (Uganda) Drinking Water

Michael Bamuwamye[1, 2], Patrick Ogwok[1], Vivian Tumuhairwe[1], Richard Eragu[2], Henriettah Nakisozi[1] & Patrick E. Ogwang[3]

[1]Kyambogo University, Department of Food Technology, P. O. Box 1, Kyambogo, Kampala

[2]Ministry of Health, Natural Chemotherapeutics Research Institute (NCRI), P. O. Box 4864, Kampala, Uganda

[3]Mbarara University of Science and Technology, P. O. Box 1410, Mbarara, Uganda

Correspondence: Patrick Ogwok, Kyambogo University, Department of Food Technology, P. O. Box 1, Kyambogo, Kampala. E-mail: ogwokp@yahoo.com

Received: April 23, 2017 Accepted: May 20, 2017 Online Published: June 4, 2017

doi: 10.5539/jfr.v6n4p6 URL: https://doi.org/10.5539/jfr.v6n4p6

Abstract

Levels of aluminium, arsenic, cadmium, chromium, copper, iron, mercury, manganese, nickel, lead and zinc in tap water, groundwater-fed protected spring and bottled water were determined. The cancer and non-cancer risks associated with ingestion of heavy metals (HM) were also assessed for both children and adults. Forty seven water samples obtained from five divisions of Kampala city were analyzed using atomic absorption spectrophotometry. Cancer and non-cancer risks were determined using incremental lifetime cancer risk (ILCR) and non-carcinogenic hazard quotient (HQ), respectively. Lead content was higher than permissible limits (PL) according to East African Standard, World Health Organization, European Union and United States Environmental Protection Agency (USEPA). Arsenic showed minor exceedances above guideline values in tap water and groundwater-fed protected spring, whereas mercury, manganese and nickel were higher than PL. Levels of aluminium, cadmium, chromium, copper, iron, and zinc were below the PL. The lifetime risk of developing cancer through the oral route was greater than the USEPA acceptable level for both children and adults, revealing that exposure to HM in drinking water posed an unacceptable potential cancer risk. Arsenic contributed ca. 90% of the ILCR in tap water and groundwater-fed protected spring. The combined non-cancer risk of the HM expressed as hazard index (HI) was greater than one, with values for children being higher than those for adults. Lead contribution towards HI was in all cases above 90%. These results demonstrate the presence of alarming non-cancer risks for children.

Key words: heavy metal, risk, cancer, non-cancer, drinking water

1. Introduction

Environmental contamination and human exposure to heavy metals (HM) such as mercury (Hg), cadmium (Cd), lead (Pb), arsenic (As) and nickel (Ni), is a serious problem throughout the world (Orisakwe, 2014). Heavy metals accumulate in the environment through emissions from industries, industrial effluents, use of leaded gasoline and paints, agricultural activities, indiscriminate disposal of municipal wastes and incineration of toxic substances (Muwanga & Barifaijo, 2006). African countries, including Uganda, are nowadays faced with a crisis of industrial waste management due to absence or weak guidelines on HM pollution and environmental management (Okot-Okumu & Nyenje, 2011). Generated waste, in Uganda, is estimated between 1.2 and 3.8 kg/capita/day. Barriers to proper urban waste management also include lack of funds and poor governance (Oosterveer & Van Vliet, 2010). Therefore, reforms to build institutional capacity in order to mitigate the rapid buildup of HM in aquatic ecosystems in African countries are essential. So far, decentralization has been proposed to involve local actors in the environmental and natural resource management in Africa (Oosterveer & Van Vliet, 2010).

Human exposure to toxic metals is a global environmental health burden. Arsenic, Pb, Hg and Cd are systemic environmental toxicants that have been implicated in causing cancer, neurological and cardiac problems, and kidney damage (Fernández-Luqueño et al., 2013). In Uganda, Pb and Cd levels in excess of WHO limits have been reported in edible vegetables from the Lake Victoria basin wetlands (Mbabazi, Wasswa, Kwetegyeka, &

Bakyaita, 2010). Lead and Hg have been detected in African fish eagles, Marabou storks and Nile perch (Hollamby et al., 2004; Ogwok, Muyonga & Sserunjogi, 2009). Further, high levels of Pb and Cd have been reported in milk from farms in Wakiso district (Nyakairu, Muhwezi & Biryomumaisho, 2011). Based on the adverse effects of HM, information on their levels in drinking water is required to guide policy.

Access to safe drinking water contributes directly to good health, food security, poverty eradication and the long-term socio-economic development of a country (Opio, 2012). Many studies have been conducted in reference to the concentration and potential health risks associated with toxic metals, as well as necessary elements in surface, ground and bottled waters around the world (Hadiani, Dezfooli-manesh, Shoebi, Ziarati, & Khaneghah 2014; Kolawole & Obueh, 2015). However, there is limited data on both the chemical water quality monitoring and human health risk assessment (HHRA) of HM in drinking water in Uganda. Therefore, the objectives of this study were to determine the concentration of aluminium (Al), chromium (Cr), iron (Fe), manganese (Mn), As, Cd, Cu, Hg, Ni, Pb and Zn contained in tap water, groundwater-fed protected spring and selected brands of bottled water in Kampala city. The cancer and non-cancer risks associated with ingestion of HM from the daily water consumption were also assessed.

2. Materials and Methods

2.1 Study Area

The study area was Kampala, the Capital City of Uganda. The city lies at latitude 0.3476°N and longitude 32.5825°E, and 1223 m (4012 ft) above sea level. It covers a total area of 189 square kilometers (73 sq. miles) with a radius of 6 km. Kampala has a tropical climate with an average annual temperature of 21.3 °C, and average annual rainfall of *ca.* 1,400 mm, with peaks in April and November.The resident population of Kampala is estimated to be 1,936,080 (0.78 male: 1.0 female) inhabitants of whom 16% are children 5 to 9 years of age (UBOS, 2016). Sixty percent of Kampala's population resides and or works within the peri-urban areas of the city. Most peri urban areas are low lying (mostly reclaimed wetlands) with a high water table (< 1.5 m).

2.2 Materials

Analytical grade nitric acid (Sigma-Aldrich) was used in the study. Distilled water was used to prepare solutions and for dilution purposes. All glassware were washed and dried in the oven at 105°C. Bottles for collecting water samples were cleaned by soaking in dilute nitric acid (10%) and rinsed several times with distilled water prior to sample taking. Water samples were obtained from public water-taps and groundwater-fed protected springs around Kampala. Bottled water was purchased from different places in the city.

2.3 Sample Collection

Samples of water were randomly obtained from 8 public water taps and 12 groundwater-fed protected springs in the five divisions of Kampala, within a radius of 5 km from the city center. Three samples, each of 500 ml, were obtained per sampling point. Also, 3 samples of each of 9 bottled water brands were purchased from local shops, supermarkets and public transport stations. Sampling was done between September and November 2015.

2.4 Determination of Heavy Metals

Levels of metals in water were determined on a Shimadzu Electro-thermal Graphite Furnace Atomic Absorption Spectrophotometer (GF-AAS) equipped with High-speed Deuterium (BGC-D2) and Self-Reversal method background correction (BGC-SR) along with an ASC6100 auto-sampler (Shimadzu Corporation, Japan). Analysis was done at the Natural Chemotherapeutics Research Institute (NCRI) chemistry laboratory. Standard solutions of the respective metals were prepared at 5 different concentrations and the absorbance (A) determined. A calibration curve was in each case generated. The calibration curves were linear within the range of concentrations used, with regression coefficients (R^2) > 99.9%. Each of the water samples was aspirated into the AAS and the absorbance measured was used to determine the concentration of the metal from the calibration curve. This procedure was repeated three times and the average concentration obtained. Analysis was done at wavelength (λ) 309.3, 193.7, 228.8, 357.9, 324.8, 248.3, 253.7, 279.5, 232.0, 283.3, and 213.9 nm for Al, As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn, respectively. The metal concentrations expressed as mg/L, were compared with permissible limits for HM in drinking water set by East African Community (EAC), United States Environment Protection Agency (USEPA), World Health Organization (WHO) and the European Union (EU) shown in Table 1.

Table 1. Permissible limits of heavy metal concentrations (mg/L) in drinking water for different international agencies

Agency	Element										
	Al	As	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn
EU (1998)	0.2	0.01	0.005	0.05	2	0.2	0.001	0.05	0.02	0.01	-
WHO (2008)	0.2	0.01	0.003	0.05	2	0.3	0.006	0.4	0.07	0.01	3
USEPA (2009)	0.2	0.01	0.005	0.1	1	0.3	0.002	0.05	0.1	0.015	5
UNBS (2014)	0.2	0.01	0.003	0.05	1	0.3	0.001	0.1	0.02	0.01	5

2.5 Human Health Risk Assessment

Human health risk assessment (HHRA) involves estimation of the nature and magnitude of adverse health effects in humans who may be exposed to hazards in contaminated environmental media. Risk assessment consisted of hazard identification, exposure assessment, dose-response (toxicity) and risk characterization (Adamu, Nganje, & Edet, 2015). The health risk assessment of each potentially toxic HM was done based on the quantification of the risk level and expressed in terms of cancer and non-cancer health risks (Sun, Zhang, Ma, & Chen, 2015). Two toxicity risk indices reported were the cancer slope factor (CSF) for cancer risk characterization and the oral reference dose (RfD) for non-cancer risk characterization (Adamu, Nganje, & Edet, 2015).

2.5.1 Exposure Assessment

In order to assess both non-cancer and cancer risks for children and adults from ingestion of drinking water, the chronic daily intakes (CDI) of toxic metals, which represents the lifetime average daily dose (LADD) of exposure to a chemical contaminant were used (Yu, Wang, & Zhou, 2014). The CDI ($mgkg^{-1}day^{-1}$) of toxic metals via water was calculated using equation 1:

$$CDI = (C \times IR \times EF \times ED)/ (BW \times AT) \quad (1)$$

where: CDI is the chronic daily intake (mg/kg/day); C is the concentration of the contaminant in tap, groundwater-fed protected spring and bottled water (mg/L); IR is the ingestion rate per unit time (1L/day for child and 2L/day for adult); ED is the exposure duration (6 years for child and 30 years for adult); EF is the exposure frequency (365 days/year); BW is body weight (15 kg for child and 70 kg for adult); AT is the averaging exposure time (for carcinogens, AT=70×365=25550 days for both children and adults; for non-carcinogens, AT=ED×365 which equals 2190 days and 10950 days for children and adults, respectively) (USEPA, 1989).

2.5.2 Non-cancer Risks

Non-cancer risks due to non-carcinogenic effects of HM in drinking water were determined by the non-cancer hazard quotient using equation 2:

$$HQ = CDI/RfD \quad (2)$$

where: HQ = non-cancer hazard quotient; CDI = chronic daily intake (mg metal/kg/day); RfD = chronic oral reference dose, which is an estimate of a daily oral exposure level for the human population, including sensitive subpopulations, that is likely to be without an appreciable risk of deleterious effects during a lifetime (Bamuwamye, Ogwok, & Tumuhairwe, 2015).

Potential risk to human health through more than one HM, was measured by the chronic hazard index (HI), which is the sum of all HQ calculated for individual HM (Li, et al., 2013). A value of HQ or HI < 1 implies no significant non-cancer risks; a value ≥ 1 implies significant non-cancer risks, which increase with increasing value of HQ or HI (Wei, et al., 2015).

2.5.3 Cancer Risks

Cancer risks were expressed in terms of incremental lifetime cancer risk (ILCR), which is the probability that one may develop cancer over a 70-year lifetime due to a 24 hour exposure to a potential carcinogen (Adamu, Nganje, & Edet, 2015). Cancer risk was calculated as the product of CDI (mg/kg-day) and cancer slope factor (CSF) measured in $(mg/kg/day)^{-1}$. The latter is the risk produced by a lifetime average dose of 1 mg/kg body weight/day of a contaminant. Cancer risk was calculated as follows (Adamu, Nganje & Edet, 2015):

$$ILCR = CDI \times CSF \quad (3)$$

where: ILCR = incremental lifetime cancer risk; CDI = chronic daily intake (mg/kg BW/day); CSF= cancer slope factor $(mg/kg/day)^{-1}$.

The total cancer risk as a result of exposure to multiple contaminants due to consumption of a particular type of water was assumed to be the sum of the individual metal incremental risks (∑ILCR, n=1 to n). The USEPA considers the minimum or acceptable cancer risk for regulatory purposes within the range of 1×10^{-6} to 1×10^{-4} (Li et al., 2013).

3. Results and Discussion

3.1 Heavy Metal Concentration in Drinking Water

3.1.1 Tap Water

Levels of Pb in tap water ranged from 0.017 to 0.31mg/L (Table 2). Arsenic was detected in substantial amounts (0.005 to 0.014 mg/L) while Cu concentrations (0.006 to 0.036 mg/L) were low. Lead and As concentrations were above the 0.01 mg/L limit prescribed for either metal by WHO, EAC and EU. Manganese concentration ranged from not detected to 0.337mg/L. The levels of Mn in tap water were therefore below the maximum allowable limit set by WHO. However, two sampled water taps had Mn level above EU, EAC, and USEPA recommendations (Table 1). Nickel concentration (not detected to 0.17 mg/L) was higher than all maximum allowable limits considered in this study. Mercury concentration in tap water was below WHO recommendations but above USEPA, EU and EAC limits. The concentrations of Fe and Zn were rather low. Aluminium and Cd were not detected in all the samples.

Lead levels in tap water in Kampala were lower than previously reported in the East Africa region (Table 5). Lead in tap water is possibly a result of corrosion of older fixtures or from the solder that connects pipes (Gaur, Singh, & Saxena, 2011). Polyvinyl chloride (PVC) pipes, used on the national water supply grid in Uganda, and for domestic water supply around Kampala, also contain Pb compounds as cost effective form of stabilizer. Lead can leach into the water when it flows through leaded pipes for several hours. Similarly, Ni has its origin in pipes and installations (Sankar & Rao, 2014). This explains the observed levels of Pb and Ni in the tap water in Kampala city. Arsenic, Mn and Hg have been reported in high concentrations in drinking water in Africa (Ahoulé, Lalanne, Mendret, Brosillon, Maiga, 2015). In Kampala, As, Mn and Hg found in water may be related to sand mining operations, agricultural drains, improper waste disposal, and incineration of toxic wastes around Lake Victoria (Muwanga & Barifaijo, 2006; Okot-Okumu & Nyenje, 2011). Tap water contamination by Hg could be linked to gold mining reported along Lake Victoria shores (Van Straaten, 2000). On the other hand, drinking water naturally contains very low amounts (0.01 to 0.05 g/L) of Cr except for regions with substantial deposits (Mebrahtu & Zerabruk, 2011). Drinking water seldom contains Zn at concentrations > 0.1 mg/litre, but high levels in tap water can be due to leaching from older galvanized plumbing materials. Low amounts of Cd in tap water could be due to coagulation or precipitation used in municipal water treatment that reduces Cd concentration to levels ≤ 0.002 mg/litre. These factors support the observed levels of Cr, Zn and Cd in tap water in Kampala.

Table 2. Concentration (mg/L) of heavy metals in Tap water (mg/L) in Kampala

Metal	Water tap (n=08)								Minimum	Maximum
	1	2	3	4	5	6	7	8		
Al	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
As	0.014	0.005	0.009	0.011	0.011	0.013	0.010	0.011	0.005	0.014
Cd	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cr	n.d.	n.d.	n.d.	n.d.	0.026	0.034	n.d.	n.d.	n.d.	0.034
Cu	0.008	0.036	0.014	0.024	0.017	0.019	0.032	0.006	0.006	0.036
Fe	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.029	0.029	n.d.	0.029
Hg	0.002	n.d.	n.d.	0.001	0.002	0.002	0.001	0.003	n.d.	0.003
Mn	n.d.	0.337	n.d.	n.d.	n.d.	n.d.	0.297	n.d.	n.d.	0.337
Ni	0.096	n.d.	n.d.	0.029	0.058	0.170	0.163	0.04	n.d.	0.170
Pb	0.178	0.192	0.266	0.192	0.178	0.017	0.310	0.090	0.017	0.310
Zn	0.756	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.756

n.d.: not detected

3.1.2 Groundwater-fed Protected Spring

Lead and As were in concentrations ranging from 0.105 to 0.412 mg/L and 0.008 to 0.014 mg/L, respectively (Table 3). Arsenic showed minor exceedances above guideline values in groundwater-fed protected spring while Pb was well above the prescribed limit of 0.01mg/L according to WHO, USEPA, EU and EAC (Table 1).

Mercury concentration of not detected to 0.004mg/L implies that all springs had Hg level below the WHO limit of 0.006 mg/L. However, the majority of the springs had mercury level above EU and EAC limits. The concentration (not detected to 1.501 mg/L) of Mn in most groundwater-fed protected springs exceeded recommended limits (Table 1). Nickel had a maximum concentration of 0.373 mg/L with half of the springs having values above WHO, USEPA, EU and EAC limits. Levels of Pb, As, Hg, Mn, and Ni in groundwater generally exceeded prescribed limits. Chromium, Cu, Fe Al, Cd and Zn were in low amounts in groundwater. Chromium was below detection limit with the exception of one spring, which had Cr concentration above WHO, EAC and EU recommendations. Similarly, the Cu concentrations (not detected to 0.062 mg/L) and that of Fe ranging from not detected to 0.084 mg/L were low. Aluminium, Cd and Zn were not detected in groundwater.

Groundwater-fed protected spring contained Pb, Cu, Ni and Mn concentrations within ranges previously reported for groundwater in Africa (Table 5). On the other hand, Cr, Fe, Al, Cd, Zn and As levels were far lower than reported elsewhere. Despite the high toxicity of Hg, it is not widely studied in Africa. However, surface water and borehole water from Nigeria has been reported to contain Hg concentrations (2.179 to 3.148 and 1.38 to 2.806 mg/L, respectively) far above recommended limits (Abdullahi et al., 2016). An increased level of HM in groundwater in Kampala can largely be attributed to indiscriminate disposal and incineration of toxic wastes, large-scale application of agrichemicals, use of lead-based gasoline and dissolution from rocks (Muwanga & Barifaijo, 2006; Okot-Okumu & Nyenje, 2011). Solid waste streams including domestic, industrial, healthcare, and commercial sources that contribute to urban waste load in Kampala are poorly managed (Okot-Okumu & Nyenje, 2011). Lack of appropriate waste disposal, and improper waste management in Kampala allows leakage of heavy metals into groundwater. A national strategy to control inappropriate waste disposal and use of agrichemicals to reduce influx of HM in water is therefore required.

Table 3. Concentration (mg/L) of heavy metals in groundwater-fed protected spring around Kampala

Metal	Groundwater-fed protected spring (n=12)												Minimum	Maximum
	1	2	3	4	5	6	7	8	9	10	11	12		
Al	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
As	0.011	0.009	0.008	0.01	0.01	0.009	0.01	0.013	0.008	0.014	0.012	0.008	0.008	0.014
Cd	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cr	n.d.	n.d.	n.d.	n.d.	0.098	0.005	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.098
Cu	0.035	0.05	0.049	0.02	n.d.	0.032	0.38	0.062	0.049	0.051	0.038	0.047	n.d.	0.062
Fe	n.d.	n.d.	0.046	0.080	n.d.	n.d.	0.084	0.058	0.005	n.d.	n.d.	0.037	n.d.	0.084
Hg	0.003	0.004	0.004	n.d.	n.d.	0.002	n.d.	0.004	0.001	0.002	0.002	n.d.	n.d.	0.004
Mn	n.d.	n.d.	0.188	n.d.	0.712	n.d.	0.970	0.570	1.501	0.884	1.213	0.987	n.d.	1.501
Ni	n.d.	n.d.	n.d.	0.18	0.078	n.d.	0.185	n.d.	0.21	0.179	n.d.	0.373	n.d.	0.373
Pb	0.280	0.105	0.266	0.266	0.266	0.383	0.207	0.134	0.207	0.134	0.354	0.412	0.105	0.412
Zn	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.: not detected

3.1.3 Bottled Water

Of the eleven elements considered in this study, Pb and Cr were detected in bottled water in Kampala city. Lead concentration was in the range of 0.091 to 0.241 mg/L (Table 4). Chromium was detected in most bottled water brands with concentration of up to 0.107 mg/L. Levels of Pb in bottled water exceeded prescribed limits by WHO, USEPA, EU and EAC. On the contrary, except for two bottled water brands, the amount of Cr was below permissible limits. In Uganda, bottled water is obtained from a variety of sources including protected underground springs, wells and municipal supplies. The water is filtered through multi-barrier filtration systems, reverse osmosis and micro-filtration. Further treatment may include exposure to ultraviolet light or ozonation. These methods remove up to 80% of HM, which explains why most elements were not detected in bottled water. However, the methods may probably not remove Pb and Cr to that magnitude. Lead levels in this study are within the range of 0.008 to 0.253 mg/L previously reported for bottled water in Kampala (Semuyaba, Segawa, & Wamala, 2014).

Table 4. Concentration (mg/L) of heavy metals in bottled water in Kampala

Metal	Bottled water Brand (n=27)									Minimum	Maximum
	1	2	3	4	5	6	7	8	9		
Al	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
As	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cd	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cr	0.0168	n.d.	n.d.	0.03	0.107	0.006	n.d.	0.031	0.065	n.d.	0.107
Cu	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Fe	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Hg	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mn	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ni	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pb	0.195	0.180	0.169	0.172	0.2	0.241	0.146	0.091	0.182	0.091	0.241
Zn	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.: not detected

Table 5. Levels (mg/L) of heavy metals in drinking water from selected African environments

Country	DW source	Element											Reference
		Al	As	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn	
Uganda	TW	n.d.	0.005-0.014	n.d.	n.d.-0.034	0.006-0.036	n.d.-0.105	n.d.-0.003	n.d.-0.337	n.d.-0.017	0.017-0.31	n.d.-0.7558	This study
	GW	n.d.	0.008-0.014	n.d.	n.d.-0.098	n.d.-0.062	n.d.-0.084	n.d.-0.004	n.d.-1.501	n.d.-0.373	0.105-0.412	n.d.	
	BW	n.d.	n.d.	n.d.	n.d.-0.107	n.d.	n.d.	n.d.	n.d.	n.d.	0.091-0.241	n.d.	
Uganda	TW	-	-	-	-	-	-	-	-	-	0.9-1.9	-	Mghweno, et al. (2008)
	SW	-	-	-	-	-	-	-	-	-	3.2-6.1	-	
Uganda	BW	-	-	-	-	-	-	-	-	-	0.008- 0.253	-	Semuyaba, et al. (2014)
Uganda	SW	-	-	0.09-0.11	0.01-0.02	1.0-2.1	15.0-21.1	-	-	-	0.91-1.64	0.20-0.50	Fuhrimann, et al. (2015)
Kenya	SW	-	-	-	0.23 –0.79	0.69 – 0.94	-	-	0.05–3.276	-	0.26–0.99	0.22	Oyoo-Okoth, et al. (2010)
Tanzania	GW	-	-	-	-	n.d.-0.013	-	-	-	-	0.01-0.35	0.01-0.28	Mkude (2015)
Rwanda	Well	-	-	-	-	-	0.02-0.45	-	0.003-0.42	-	-	0.02-0.15	Nigatu et al. (2015)
Ethiopia	DW	-	0.39-1.06	n.d.	n.d.	n.d.	0.134-0.307	-	0.025-0.031	n.d.	0.052-1.347	0.439-5.055	Mebrahtu & Zerabruk (2011)
Egypt	SW	0.136-0.864	-	-	-	0.003-0.012	0.160-4.03	-	0.001-0.474	-	n.d.	-	El-Sayed & Salem (2015)
	GW	0.125-1.69	-	-	-	0.0001-0.018	0.40-1.88	-	0.017-1.095	-	0.028-0.179	-	
Nigeria	SW	-	-	0.004-0.011	0.16-1.65	-	0.351-12.0	-	0.10-5.15	-	0.02-2.5	-	Afiukwa (2013)
	GW	-	-	0.004-0.012	0.208-0.29β	-	0.40-1.88	-	0.10-3.34	-	0.028-0.179	-	
Nigeria	Well	-	-	n.d.-0.003	-	n.d.-0.003	n.d.-3.428	2.179-3.148	0.057-0.175	n.d.-0.006	n.d.-0.021	-	Abdullahi, et al. (2016)
	Borehole	-	-	n.d.-0.056	-	n.d.-0.193	0.228-23.256	1.38-2.806	0.035-1.787	n.d.-0.03	n.d.-0.194	-	

TW: Tap water; BW: Bottled water; SW: Surface water; GW: Ground water; DW: Drinking water; n.d.: not detected

3.2 Human Health Risk Assessment

Cancer and non-cancer risks were determined based on the mean concentrations of carcinogenic and non-carcinogenic metals using the incremental lifetime cancer risk (ILCR) and the non-cancer hazard quotient (HQ), respectively (Liu, et al., 2013). Heavy metal mean concentrations, and oral reference doses and cancer slope factors used in the assessment are presented in Table 6.

Table 6. Mean metal concentrations, oral reference doses and cancer slope factors of the respective heavy metals

Metal	Al	As	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn
Tap water (mg/L)	-	0.011	-	0.03	0.019	0.054	0.002	0.079	0.07	0.178	0.094
GWPS (mg/L)	-	0.010	-	0.051	0.043	0.059	0.002	0.585	0.087	0.251	-
Bottled water (mg/L)	-	-	-	0.025	-	-		-	-	0.175	-
RfD (mg/kg/day)**	0.14	0.0003	0.0005	1.5	0.04	0.7	0.0003	0.14	0.02	0.0004	0.3
CSF $(mg/kg/day)^{-1}$**	0.2	1.50	6.30	42	-	-	-	-	-	0.0085	-

GWPS: Groundwater-fed protected spring; RfD: Oral reference dose; CSF: Cancer slope factor (**Source: USEPA, 2016).

3.2.1 Non-cancer Risks

Tap water, groundwater-fed protected spring and bottled water had hazard quotient (HQ) values showing unacceptable risk (HQ > 1) for Pb in both children and adults (Table 7). Arsenic in tap water and groundwater-fed protected spring also showed unacceptable risk among children and potential risk in adults. The potential health risk of Cr was minimal (HQ < 0.001) in all the three water types for both adults and children in comparison to other HM investigated (Guerra, Trevizam, Muraoka, Marcante, & Canniatti-Brazaca, 2012). Hazard quotients for Al, Cd, Cr, Cu, Fe, Hg, Mn, Ni and Zn were < 1 for tap water, groundwater-fed protected spring and bottled water signifying that the population would not experience non-cancer risks due to exposure to these metals in drinking water. Hazard Index (HI) values of the heavy metals for groundwater-fed protected spring, tap water and bottled water for children were 50.226, 36.372, and 32.409, respectively. For adults, HI values of 21.525 for groundwater, 15.588 for tap water and 13.889 for bottled water were obtained. Hazard indices >1 were obtained in all water samples, indicating unacceptable risk for non-carcinogenic adverse health effect.

Lead contributed most towards exposure to non-cancer risks in the exposed population followed by As. A HQ value of 1 < HQ < 5 indicates a level of concern while a value of 10 < HQ < 100 suggests need for additional data gathering. This study therefore shows that As was in a level of concern in tap water and groundwater, while Pb needed further data collection. Hazard indices for children were higher than those for adults meaning that children would experience more non-cancer risks than adults. Young children absorb chemicals four times more than adults (Akkus & Ozdenerol, 2014). Similar observations have been reported (Guerra et al., 2012; Bamuwamye, Ogwok, & Tumuhairwe, 2015).

Table 7. Chronic daily intakes and Non-cancer hazard quotients for children and adults, by metal and water-type for drinking water in Kampala city

Metal	CDI-Children			CDI-Adults			HQ-Children			HQ-Adults		
	TW	GWPS	BW	TW	GWPS	BW	TW	GWPS	BW	TW	GWPS	BW
Al	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
As	0.001	0.001	0.000	0.000	0.000	0.000	2.444	2.222	0.000	1.048	0.952	0.000
Cd	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Cr	0.002	0.003	0.002	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001
Cu	0.001	0.003	0.000	0.001	0.001	0.000	0.032	0.072	0.000	0.014	0.031	0.000
Fe	0.004	0.004	0.000	0.002	0.002	0.000	0.005	0.006	0.000	0.002	0.002	0.000
Hg	0.000	0.000	0.000	0.000	0.000	0.000	0.444	0.444	0.000	0.190	0.190	0.000
Mn	0.021	0.059	0.000	0.009	0.025	0.000	0.151	0.418	0.000	0.065	0.179	0.000
Ni	0.006	0.012	0.000	0.003	0.005	0.000	0.310	0.580	0.000	0.133	0.249	0.000
Pb	0.012	0.017	0.012	0.005	0.007	0.005	32.963	46.481	32.407	14.127	19.921	13.889
Zn	0.006	0.000	0.000	0.003	0.000	0.000	0.021	0.000	0.000	0.009	0.000	0.000
∑HQ = HI							36.372	50.226	32.409	15.588	21.525	13.890

TW: Tap water; GWPS: Groundwater-fed protected spring; BW: Bottled water; CDI: Chronic daily intakes; HQ: Hazard quotient; HI: Hazard Index

3.2.2 Cancer Risks

Lifetime cancer risk through ingestion of Pb and As was 9.90×10^{-5} and 2.12×10^{-4} for tap water; 9.79×10^{-5} and 2.10×10^{-4} for groundwater; 8.50×10^{-6} and 1.82×10^{-5} for bottled water (Table 8), for children and adults, respectively. These results indicate higher cancer risks for adults than children. Arsenic contribution towards

ILCR for both children and adults was 91.3% and 87.6% in tap water and groundwater, respectively. Bottled water had a significantly ($p < 0.05$) lower ILCR, compared with tap water and groundwater, attributable to extremely low levels of As in bottled water. There was no pronounced difference between tap water and groundwater in terms of cancer risks. The USEPA proposed a one out of one million chance of additional cancers as a management goal for risks posed by environmental contaminants (Qu, Li, Wu, Wang, & Giesy, 2015). Risks ranging from 1 out of 10,000 to 1 out of 1,000,000 are considered as acceptable, depending on the circumstances (Qu et al., 2015). A risk of 1×10^{-3} will absolutely require protective measures (Pawełczyk, 2013). Compared with this risk range, the results of this study imply intolerable cancer risks for both children and adults due to heavy metals in drinking water over a lifetime. The ILCR method for calculating cancer risk estimates the incremental increase in risk for the exposed populations over a lifetime, but does not consider when the cancer will occur (Charnley & Putzrath, 2001). Exposure to cancer-causing chemicals in food, water, air, and consumer products early in life can lead to cancer later in life (Carpenter & Bushkin-Bedient, 2013). Therefore, prevention of early life exposure to cancer agents is essential.

Table 8. Incremental lifetime cancer risks for the children and adult populations of Kampala city through consumption of drinking water

Element	Tap water		Groundwater-fed protected spring		Bottled water	
	Children	adults	Children	Adults	Children	Adults
As	9.03×10^{-5}	1.94×10^{-4}	8.57×10^{-5}	1.84×10^{-4}	0	0
Pb	8.63×10^{-6}	1.85×10^{-5}	1.22×10^{-5}	2.61×10^{-5}	8.50×10^{-6}	1.82×10^{-5}
∑ILCR	9.90×10^{-5}	2.12×10^{-4}	9.79×10^{-5}	2.10×10^{-4}	8.50×10^{-6}	1.82×10^{-5}

ILCR: Incremental lifetime cancer risks

4. Conclusion

Tap water, groundwater-fed protected spring and bottled drinking water in Kampala was contaminated with heavy metals making it a health concern. Bottled water had low Pb levels compared to groundwater-fed protected spring and tap water. The concentration of other elements detected in drinking water was too low to present a health risk to consumers. All drinking water sources showed high hazard indices indicating unacceptable risk of non-carcinogenic adverse health effects. Compared with other elements, lead contribution towards the hazard index was highest in all cases. Excess lifetime cancer risks via the oral route revealed intolerable cancer risks for both children and adults due to heavy metals in drinking water over a 70-year lifetime. Arsenic, in particular, contributed most to the total incremental lifetime cancer risks in tap water and groundwater-fed protected springs. It is therefore conclusive that children, pregnant women and women of childbearing age in Kampala are at high risk of heavy metal poisoning from drinking water. There is need to update the current National policy on environmental management in order to control the influx of heavy metals in drinking water sources. Government water bodies and water bottling companies should adopt multi-purpose water and wastewater treatment procedures for effective purification of drinking water.

References

Abdullahi, S., Ndikilar, C. E., Suleiman, A. B., & Hafeez, H. Y. (2016). Assessment of Heavy Metals and Radioactivity Concentration in Drinking Water Collected From Local Wells and Boreholes of Dutse Town, North West, Nigeria. *Journal of Environment Pollution and Human Health, 4*(1), 1-8. https://doi.org/10.12691/jephh-4-1-1

Adamu, C., Nganje, T., & Edet, A. (2015). Heavy metal contamination and health risk assessment associated with abandoned barite mines in Cross River State, southeastern Nigeria. *Environmental Nanotechnology, Monitoring & Management, 3*, 10-21. http://dx.doi.org/10.1016/j.enmm.2014.11.001

Afiukwa, J. N. (2013). Evaluation and correlation study of heavy metals load in drinking water and update of water-related disease cases in Ebonyi State from 2001 –2011. *American Journal of Scientific and Industrial Research, 4*(2), 221-225. https://doi.org/10.5251/ajsir.2013.4.2.221.225

Ahoulé, D. G., Lalanne, F., Mendret, J., Brosillon, S., & Maïga, A. H. (2015). Arsenic in African Waters: A Review. *Water, Air, & Soil Pollution, 226*, 302. http://dx.doi.org/10.1007/s11270-015-2558-4

Akkus, C., & Ozdenerol, E. (2014). Exploring Childhood Lead Exposure through GIS: A Review of the Recent Literature. *International Journal of Environmental Research and Public Health, 11*, 6314-6334. http://dx.doi.org/10.3390/ijerph110606314

Bamuwamye, M., Ogwok, P., & Tumuhairwe, V. (2015). Cancer and Non-cancer Risks Associated With Heavy Metal Exposures from Street Foods: Evaluation of Roasted Meats in an Urban Setting. *Journal of Environment Pollution and Human Health, 3*(2), 24-30. https://doi.org/10.12691/jephh-3-2-1

Carpenter, D. O., & Bushkin-Bedient, S. (2013). Exposure to Chemicals and Radiation During Childhood and Risk for Cancer Later in Life. *Journal of Adolescent Health, 52*, S21-S29. http://dx.doi.org/10.1016/j.jadohealth.2013.01.027.

Charnley, G., & Putzrath, R. M. (2001). Children's Health, Susceptibility, and Regulatory Approaches to Reducing Risks from Chemical Carcinogens. *Environmental Health Perspectives, 109*, 187-192. http://ehpnet1.niehs.nih.gov/docs/2001/109p187-192charnley/abstract.html

El-Sayed, M., & Salem, W. M. (2015). Hydrochemical assessments of surface Nile water and ground water in an industry area–South West Cairo. *Egyptian Journal of Petroleum, 24*, 277-288. https://doi.org/10.1016/j.ejpe.2015.07.014

European Union (EU). (1998). Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. *Official Journal of the European Communities* (OJ L), 330, 42-45

Fernández-Luqueño, F., López-Valdez, F., Gamero-Melo, P., Luna-Suárez, S., Aguilera-González, E. N., Martínez, A. I., Álvarez-Garza, M. A. (2013). Heavy metal pollution in drinking water - a global risk for human health: A review. *African Journal of Environmental Science and Technology, 7*(7), 567-584. http://dx.doi.org/10.5897/AJEST12.197

Fuhrimann, S., Stalder, M., Winkler, M. S., Niwagaba, C. B., Babu, M., Masaba, G., & Cissé, G. (2015). Microbial and chemical contamination of water, sediment and soil in the Nakivubo wetland area in Kampala, Uganda. *Environmental Monitoring and Assessment,* 187(475), 15. http://dx.doi.org/10.1007/s10661-015-4689-x

Gaur, S., Singh, N., & Saxena, S. (2011). Status of lead present in ground drinking water samples of Uttarakhand (Garhwal Region) in India. *Asian Journal of Biomedical and Pharmaceutical Sciences, 1*(1), 32-38.

Guerra, F., Trevizam, A. R., Muraoka, T., Marcante, N. C., & Canniatti-Brazaca, S. G. (2012, January/February). Heavy metals in vegetables and potential risk for human health. *Scientia Agricola, 69*(1), 54-60. http://dx.doi.org/10.1590/S0103-90162012000100008

Hadiani, M. R., Dezfooli-manesh, S., Shoeibi, S., Ziarati, P., & Khaneghah, A. M. (2014). Trace elements and heavy metals in mineral and bottled drinking waters on the Iranian market. *Food Additives & Contaminants: Part B*, 07. http://dx.doi.org/10.1080/19393210.2014.947526

Hollamby, S., Afema-Azikuru, J., Sikarskie, J. G., Kaneene, J. B., Stuht, J. N., Fitzgerald, S. D., … Rumbeiha, W. K. (2004). Clinical Pathology And Morphometrics of African Fish Eagles In Uganda. *Journal of Wildlife Diseases, 40*(3), 523-532. http://dx.doi.org/10.7589/0090-3558-40.3.523

Kolawole, S. E., & Obueh, H. O. (2015). Evaluation of the minerals, heavy metals and microbial compositions of drinking water from different sourcesin Utagba-Uno, Nigeria. *ISABB-Journal of Health and Environmental Sciences, 2*(2), 6-10. http://dx.doi.org/10.5897/ISAAB-JHE2015.0017

Li, P.-H., Kong, S.-F., Geng, C.-M., Han, B., Lu, B., Sun, R.F., Bai, Z.-P. (2013). Assessing the Hazardous Risks of Vehicle Inspection Workers' Exposure to Particulate Heavy Metals in Their Work Places. *Aerosol and Air Quality Research, 13*, 255-265. http://dx.doi.org/10.4209/aaqr.2012.04.0087

Liu, X., Song, Q., Tang, Y., Li, W., Xu, J., Wu, J., Brookes, P. C. (2013). Human health risk assessment of heavy metals in soil–vegetable system: A multi-medium analysis. *Science of the Total Environment*, 463-464, 530-540. http://dx.doi.org/10.1016/j.scitotenv.2013.06.064

Mbabazi, J., Wasswa, J., Kwetegyeka, J., & Bakyaita, G. K. (2010). Heavy metal contamination in vegetables cultivated on a major Urban wetland inlet drainage system of Lake Victoria, Uganda. *International Journal of Enviromental studies, 67*(3), 333-348. http://dx.doi.org/10.1080/00207231003612613

Mebrahtu, G., & Zerabruk, S. (2011). Concentration of Heavy Metals in Drinking Water from Urban Areas of the Tigray Region, Northern Ethiopia. *Momona Ethiopian Journal of Science, 3*(1), 105-121. http://dx.doi.org/10.4314/mejs.v3i1.63689

Mghweno, L. R., Makokha, A. O., Magoha, H. S., Wekesa, J. M., & Nakajugo, A. (2008). Environmental lead pollution and food safety around Kampala City in Uganda. *Journal of Applied Biosciences, 12*, 642-649.

Mkude, I. T. (2015). Comparative analysis of heavy metals from groundwater sources situated in Keko and

Kigogo residential areas, Dar es Salaam. *Journal of Water Resources and Ocean Science, 4*(1), 1-5. https://doi.org/10.11648/j.wros.20150401.11

Muwanga, A., & Barifaijo E (2006). Impact of industrial activities on the heavy metal loading and their physico-chemical effects on wetlands of Lake Victoria basin (Uganda). *African Journal of Science and Technolology, 7*(1), 51-63. http://dx.doi.org/10.4028/3-908158-03-6

Nigatu, W., Umuhire, C. A., Nsengimana, J., Nsabimana, A., & Dieudonne, S. (2015). Quantitative Assessment of the Chemical Safety of Groundwater Wells in Two Selected Districts of Rwanda. *International Journal of Environmental Protection and Policy, 3*(4), 104-110. https://doi.org/10.11648/j.ijepp.20150304.14

Nyakairu, G. W., Muhwezi, G., & Biryomumaisho, S. (2011). Assessment of Heavy Metals in Milk from Selected Dairy Farms and Shops in Wakiso District, Uganda. *SUZA Journal of Natural and Social Science, 1*(1), 36-52.

Ogwok, P., Muyonga, J. H., & Sserunjogi, M. L. (2009). Pesticide residues and heavy metals in Lake Victoria Nile perch, Lates niloticus, belly flap oil. *Bulletin of Environmental Contamination and Toxicology, 82*(5), 529-33. http://dx.doi.org/10.1007/s00128-009-9668-x

Okot-Okumu, J., & Nyenje R. (2011). Municipal solid waste management under decentralisation in Uganda. *Habitat International, 35*, 537-543. *http*://dx.*doi*. *org*/*10.1016*/*j.habitatint.2011.03.003*

Oosterveer, P., & Van-Vliet, B. (2010). Environmental Systems and Local Actors: Decentralizing Environmental Systems and Local Actors: Decentralizing. *Environmental Management,* 45, 284-295. http://dx.doi.org/10.1007/s00267-009-9423-4

Opio, C. (2012). Building effective drinking water management policies in rural Africa: Lessons from Northern Uganda. *Discussion paper series No. 6*. Ontario, Canada: Africa Initiative and The Centre for International Governance Innovation (CIGI). Retrieved from www.africaportal.org

Orisakwe, O. E. (2014). Lead and Cadmium in Public Health in Nigeria: Physicians Neglect and Pitfall in Patient Management. *North American Journal of Medical Sciences, 6*(2), 61-70. http://dx.doi.org/10.4103/1947-2714.127740

Oyoo-Okoth, E., Wim, A., Osano, O., Kraak, M. H., Ngure, V., Makwali, J., & Orina, P. (2010). Use of the fish endoparasite Ligula intestinalis (L., 1758) in an intermediate cyprinid host (Restreneobola argentea) for biomonitoring heavy metal contamination in Lake Victoria, Kenya. *Lakes & Reservoirs: Research and Management, 15*, 63-73. http://dx.doi.org/10.1111/j.1440-1770.2010.00423.x

Pawełczyk, A. (2013). Assessment of health risk associated with persistent organic pollutants in water. *Environmental Monitoring and Assessment, 185*, 497-508. http://dx.doi.org/10.1007/s10661-012-2570-8

Qu, C., Li, B., Wu, H., Wang, S., & Giesy, J. P. (2015). Multi-pathway assessment of human health risk posed by polycyclic aromatic hydrocarbons. *Environmental Geochemistry and Health*. http://dx.doi.org/10.1007/s10653-014-9675-7

Sankar, T. R., & Rao, P. T. (2014). Heavy metal assessment in industrial groundwater in and around vijayawada, andhra pradesh, india. *European Chemical Bulletin, 3*(10), 1008-1013. http://dx.doi.org/10.17628/ecb.2014.3.1008-1013

Semuyaba, A. S., Segawa, I., & Wamala, A. (2014). Potential Risk of Lead Toxicity from Bottled Water in Uganda. *Makerere Pharmaceutical Journal, 12*(1), e13-20.

Sun, C., Zhang, J., Ma, Q., & Chen, Y. (2015). Human Health and Ecological Risk Assessment of 16 Polycyclic Aromatic Hydrocarbons in Drinking Source Water from a Large Mixed-Use Reservoir. *International Journal of Environtal Research and Public Health, 12*, 13956-13969. http://dx.doi.org/10.3390/ijerph121113956

UBOS. (2016). The National Population and Housing Census 2014 – Main Report. Kampala: Uganda Bureau of Statistics.

UNBS. (2014, 10 15). *Uganda Standard* . US EAS 12: 2014 (Potable water Specification), First Edition. Kampala: Uganda National Bureau of Standards. Retrieved from www.unbs.go.ug

USEPA. (1989). Risk Assessment Guidance for Superfund Volume 1: Human Health Evaluation (Part A). Springfield: US Department of Commerce, National Technical Information Service.

USEPA. (2009). *United States Environmental Protetion Agency*. Retrieved from Ground Water and Drinking

Water: National Primary Drinking Water Regulations: https://www.epa.gov/ground-water-and-drinking-water/national-primary-drinking-water-regulations

USEPA. (2016). *United States Environmental Protection Agency (EPA)*. Retrieved from IRIS Chemical Assessment Quick list: https://cfpub.epa.gov/ncea/iris_drafts/simple_list.cfm?list_type=alpha

Van Straaten, P. 2000. Mercury contamination associated with small scale gold mining in Tanzania and Zimbabwe. *Science of the Total Environment, 259*, 105-113. *http*://dx.*doi.org*/*10.1016*/*S0048-9697*(*00*)*00553-2*

Wei, H., Le, Z., Shuxian, L., Dan, W., Xiaojun, L., Lan, J., & Xiping, M. (2015). Health risk assessment of heavy metals and polycyclic aromatic hydrocarbons in soil at coke oven gas plants. *Environmental Engineering and Management Journal, 14*(2), 487-496. http://omicron.ch.tuiasi.ro/EEMJ/

WHO. (2008). *Guidelines for drinking-water quality [electronic resource]: incorporating 1st and 2nd addenda, Vol.1, Recommendations. 3rd edition.* Geneva: World Health Organization (WHO).

Yu, B., Wang, Y., & Zhou, Q. (2014). Human Health Risk Assessment Based on Toxicity Characteristic Leaching Procedure and Simple Bioaccessibility Extraction Test of Toxic Metals in Urban Street Dust of Tianjin, China. *PLoS ONE, 9*(3), e92459. *http*://*doi.org*/*10.1371*/*journal.pone.0092459*

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

A Novel Functional Fruit/Vegetable Beverage for the Elderly: Development and Evaluation of Different Preservation Processes on Functional and Enriched Components and Microorganisms

Juliano De Dea Lindner[1], Daiane Paggi[2], Vinícius Duarte Pinto[3], Douglas Soares[4], Maressa Danieli Dolzan[5], Daniele Bevacqua[6], Gustavo Amadeu Micke[7] & José Vladimir Oliveira[4]

[1]Food Science and Technology Department, Federal University of Santa Catarina, Florianópolis, Brazil

[2]Research and Development Department, Organic Bios, Capinzal, Brazil

[3]Research and Development Department, R&S Blumos, Campinas, Brazil

[4]Food Engineering Department, Federal University of Santa Catarina, Florianópolis, Brazil

[5]Federal Institute of Santa Catarina, Canoinhas, Brazil

[6]EA-unite UR 1115 PSH, Institut National Recherche Agronomique, Avignon, France

[7]Department of Chemistry, Federal University of Santa Catarina, Florianópolis, Brazil

Correspondence: Juliano De Dea Lindner, Federal University of Santa Catarina (UFSC), Agricultural Science Center (CCA), Food Science and Technology Department (CAL), Rodovia Admar Gonzaga, 1346. Itacorubi. Florianópolis, SC, Brazil. Tel: 55-483-721-6183. E-mail: juliano.lindner@ufsc.br

Received: April 30, 2017 Accepted: May 24, 2017 Online Published: June 4, 2017

doi:10.5539/jfr.v6n4p17 URL: https://doi.org/10.5539/jfr.v6n4p17

Abstract

Despite lower energy intakes with age, elderly have higher requirements for several nutrients, making them vulnerable to deficiencies that further aggravate aging chronic conditions. To manage this problem, new food formulations are needed to address the nutritional needs of the worldwide growing aging population. In the present work, we develop an innovative functional fruit/vegetable beverage for the elderly using coconut water, orange juice and carrot juice. The beverage was enriched with vitamin A and C and supplemented with omega-3 fatty acids and inulin to provide important nutritional elements to the target consumers of this study. Osmolality classified the beverage as hypertonic. Heat (TT), supercritical carbon dioxide (SC-CO_2) and dimethyl dicarbonate (DMDC) treatments were applied as preservation methods, and the responses of the functional and enriched components and enzymes were evaluated. *Alicyclobacillus acidoterrestris* ATCC 49025 and a surrogate *Escherichia coli*, ATCC 25922, were inoculated in the beverage to induce spoilage and verify the effectiveness of inactivation processes on the cells. The DMDC process reduced *E. coli* to undetectable levels and exhibited a greater reduction of *A. acidoterrestris* vegetative cells, molds and yeasts. The conditions applied in the process with SC-CO_2 did not show satisfactory results in reducing juice microbiota. Loss of vitamin C and A was mainly associated with the SC-CO_2 treatment. The beverage received a positive acceptability evaluation among the tasters in the sensory analysis and may be introduced as a new vehicle for the consumption of functional compounds, especially by vegans and/or vegetarians and lactose-intolerant elderly.

Keywords: coconut water, dimethyl dicarbonate, microbiological challenge testing, non-dairy beverage, orange juice, supercritical carbon dioxide, carrot juice

1. Introduction

Consumers are looking for nutrition that goes beyond the status quo, either in terms of extra nutrition or condition-specific benefits. They desire ready-to-drink (RTD) beverages with “better for you” ingredients and simple “clean” labels, and they seek products with functional benefits and great taste. Beverage companies are meeting new consumer demands by introducing group-specific (*e.g.,* gluten-free, muscle recovery, bone health) beverages. Functional beverages can be specifically targeted to particular sectors of the population, focusing, *e.g.,* on women or the elderly. The increase in the elderly population is a general global trend. The leading worldwide cause of death among the elderly is associated with chronic disease, and there is great potential for the prevention of these diseases through diet (United States Department of Health and Human Services and World

Health Organization, 2011). Fruit/vegetable-based beverages represent an easy and convenient way of consuming important sources of compounds such as vitamins, phenolics and fibers, so necessary for this group of population.

In spite of the many nutritional properties and bioactive compounds described for coconut water (Yong, Ge, Ng, & Tan, 2009), orange juice (Agvan, Akyildiz, & Akdemir Evremdilek, 2014) and carrot juice (Corona et al., 2016), there are no reports on the state-of-the-art technological development of an orange/carrot functional beverage based on coconut water. Recently, Camargo Prado et al. (2015) reported a closely related topic on the development of an innovative functional beverage using coconut water as the main ingredient and cited its natural hydrating qualities, functional health properties and nutritional benefits.

To commercialize an RTD fruit/vegetable beverage, appropriate preservation process and subsequent storage methods are necessary to guarantee the stability of the nutritional and functional compounds, as fruits and vegetables are susceptible to oxidative, enzymatic or microbial spoilage. Heat treatments are the most widely used preservation method for coconut water (Tan, Cheng, Bhat, Rusul, & Easa, 2014) and fruit juices (Bilek & Bayran, 2015), as high temperatures lead to inactivation of microbial and enzymatic activities. However, excessively high process temperatures lead to loss of nutritional and healthy compounds as well as the sensory properties of food (Cappelletti et al., 2015).

Non-thermal food processing technologies for preservation and safety have gained widespread acceptance throughout the food industry (Rawson et al., 2011) but still need to be evaluated for effectiveness in different matrices that have not been wet-tested. Among these technologies, particular attention has been given to the use of antimicrobials such as dimethyl dicarbonate (DMDC) and supercritical carbon dioxide (SC-CO_2). DMDC, a dicarbonic acid ester approved by U.S. FDA (United States Food and Drug Administration, 2001a), is a microbial control agent inhibitor of yeasts used in beverages at a maximum limit of 250 mg/L. Numerous studies have demonstrated that DMDC treatment efficiently causes lethality in many pathogenic microorganisms in fruit juices and has few effects on sensory properties and nutritional value (Yu et al., 2013). High-pressure carbon dioxide (HPCD) process that applies SC-CO_2 is being used to inactivate enzymes and pathogenic microorganisms, resulting in minimal degradation of thermo-labile nutrients and bioactive compounds of foods and preserving sensory and nutritional characteristics (Zhou, Wang, Hu, Wu, & Liao, 2009). Many studies have demonstrated that HPCD treatment at moderate pressure and temperature can effectively inactivate microorganisms in foods (Damar & Balaban, 2006).

Microbiological challenge testing continues to be a useful tool for the validation of processes that are intended to deliver some degree of lethality against a target food microorganism. *Alicyclobacillus acidoterrestris* is an acidothermophilic spore-forming bacterium of great concern to the fruit beverage industry that cause spoilage by producing taint compounds responsible for off-flavors (*e.g.*, 2-methoxyphenol "guaiacol") (Molva & Baysal, 2015). Thermal resistance studies have demonstrated the ability of this microorganism to survive pasteurization applied to acidic fruit juices (Oteiza, Soto, Alvarenga, Sant'Ana, & Gianuzzi, 2015). Additionally, pathogens from known foodborne outbreaks should be included in challenge tests to ensure that the formulation and process is sufficiently robust to inhibit those organisms. Pathogenic strains of *Escherichia coli* were involved in outbreaks linked to the consumption of contaminated unpasteurized fruit juices (Besser et al., 1993). Outbreaks have indicated that low pH (< 4.0) of fruit beverages cannot safeguard against the survival of *E. coli* O157:H7 (Leyer, Wang, & Johnson, 1995). Thus, an ideal surrogate *E. coli* strain applied in the challenge must have, for example, similar thermal and acidic inactivation kinetics as pathogenic *E. coli* strains (U.S. FDA, 2001b).

In this context, the present study was performed to develop an innovative functional fruit/vegetable beverage using coconut water as the main ingredient. Preservation processes were tested in order to correlate losses in nutritional ingredients with the treatments. The beverage was inoculated with *A. acidoterrestris* and nonpathogenic *E. coli* to induce spoilage and to verify the effectiveness of inactivation processes on the cells using microbial challenge testing criteria. Although the formulation of juices has been widely explored, the developed beverage can be introduced as a new nutritional vehicle for the consumption of functional and enriched components, especially by vegans and/or vegetarians and lactose-intolerant elderly.

2. Method

The fruit/vegetable beverage was produced for this study to evaluate the relationship between its chemical, physicochemical, functional composition and its sensory acceptance after three different preservation processing technologies. To evaluate the effectiveness of the treatments, the beverage was microbiologically challenged.

2.1 Extraction of Raw Materials and Beverage Formulation

Green coconuts (*Cocos nucifera* L. var. *nana*) between 6 and 8 months of age and oranges (*Citrus x sinensis* var. *bahia*) and carrots (*Daucus carota* subsp. *sativus*) at commercial maturity were purchased at the local market in Florianópolis (SC, Brazil). The coconut water, orange juice and carrot juice used in this work were extracted after the fruits were brushed and washed with water containing 100 ppm active chlorine. The coconut water and fresh-squeezed juices were homogenized, filtered under vacuum using filter paper (Whatman, UK) and maintained at -20 °C to prevent any microbial or enzymatic activity.

The fruit/vegetable beverage formulation was previously developed after pre-trials using not structured sensory tests (data not shown), and production was carried out at the Federal University of Santa Catarina (UFSC), Food Science and Technology Department Pilot Plant. The formulation was composed of 48% (w/v) coconut water, 40% (w/v) orange juice and 10% (w/v) carrot juice. Enriched and functional ingredients (vitamins A and C, omega-3 fatty acids and inulin) were added in the amount necessary to achieve, in the final formulation, 100% of the RDI (Recommended Daily Intake for adults) in Brazil (Brasil, 2005; Brasil, 2008). In total, forty-five mg of vitamin C as ascorbic acid (SweetMix, São Paulo, Brazil), 600 µg of vitamin A as retinyl acetate (M. Cassab, São Paulo, Brazil), 1.5 g of an omega-3 mixture from natural fish oil concentrate with EPA (10%) and DHA (8%) (Vana-Sana EPA/DHA 10/8 ES, FrieslandCampina Kievit, Meppel, the Netherlands) and 1.5 g of inulin as soluble fiber from the chicory root (Cargill, São Paulo, Brazil) were added to compose a dose. After weighing and mixing the components in a tank under agitation (600 rpm) for 10 min, the pH of the beverage was adjusted to 3.50 (HI9321 pH meter, Hanna Instruments, São Paulo, Brazil) with citric acid (Cargill, São Paulo, Brazil), and total soluble solid content was determined directly using a digital refractometer (PR-101, ATAGO, Tokyo, Japan) at 25 °C, resulting in 8.30 °brix. The beverage was degassed under vacuum (-0.65 mbar) and bottled in 250-ml glass bottles previously sanitized by immersion in peracetic acid solution (1800 ppm) for 10 min. After filling, the headspace was flushed with N_2 flux at 1 bar, and the bottles were sealed with sanitized metallic caps and stored at 4 °C in total darkness.

2.2 Preservation Processing Technologies

The analyses performed in this work were carried out on fresh untreated matrix (F) and thermally (TT), supercritical carbon dioxide (SC-CO_2)- and dimethyl dicarbonate (DMDC)-treated beverages.

2.2.1 Thermal Treatment (TT)

Heat pasteurization equipment consisted of a water bath (Dubnoff Bath TE-0532, Tecnal, Piracicaba, Brazil) with agitation into which 250-mL bottles of beverage were placed. The pasteurization was performed at 85 °C for 5 min. The process conditions were chosen based on Cappelletti et al. (2015). After treatment, the bottles were cooled and exhausted in an ice water bath and subsequently stored at 4 °C.

2.2.2 Supercritical Carbon Dioxide Treatment (SC-CO_2)

Treatment was conducted employing the static-synthetic method in a high-pressure variable-volume reactor as described by Silva et al. (2013). The experimental setup consisted of a variable-volume reactor with a maximum internal volume of 27 mL, two sapphire windows for visual observation, an absolute pressure transducer (Smar LD301) with a precision of ~0.03 MPa, a portable programmer (Smar HT201) for pressure data acquisition and a syringe pump (ISCO 260D). The reactor contained a movable piston that permits pressure control inside the cell without allowing exchange of fluids.

Initially, an amount of 15 mL of beverage was loaded into the cell with help of a sterile syringe. The juice/CO_2 mass ratio was 1:08. The charge of CO_2 was performed with the help of the syringe pump. Then, the cell content was kept at continuous agitation with the help of a magnetic stirrer and a Teflon-coated stirring bar. A metallic jacket surrounds the cell and water from a thermostatic bath was used as heating fluid, which flows through the jacket, so that the cell was kept at the temperature of 33 °C. The pressure system was increased at pressurization rate of 10 MPa/min using CO_2 as pressurizing fluid from its vapor pressure at room temperature (~5 MPa) up to 8 MPa, which was considered the initial working pressure. At this point the system was hold for a short period (~1 min) to allow system stabilization, and then the pressure was increased until 20 MPa. After the procedure, the system pressure was reduced to 8 MPa at the rate of depressurization pre-established. At the end of this process the pressure was manually reduced from 8 MPa to atmosphere pressure. All runs were conducted using one pressure cycle for 120 min (Fig. 1).

Figure 1. Supercritical carbon dioxide (SC-CO_2) treatment conditions adopted, shown in terms of time and pressure

2.2.3 Dimethyl Dicarbonate Treatment (DMDC)

The beverage was vigorously mixed in bottles by inversion for 2 min after the addition of 200 μL/L DMDC (Velcorin, Lanxess, Cologne, Germany) and kept for 4 h at 25 °C. The bottles were cooled and maintained at 4 °C until analysis. The final concentration of DMDC added to the beverage was 250 mg/L.

2.3 Assessment of Composition and Physicochemical Properties of the F Matrix and TT Beverage

The following analyses were performed to characterize the F matrix and TT beverage.

2.3.1 Physicochemical

The pH was measured directly using a model HI9321 pH meter (Hanna Instruments, São Paulo, Brazil). Acidity was measured by titrimetric analysis using the TT beverage diluted 1:50 (v/v) in distilled water. The diluted solution was neutralized with 0.1 M NaOH (Merck, Germany) using an alcoholic solution of phenolphthalein (1 % v/v) as an indicator. Total soluble solids content was determined directly using a digital refractometer (PR-101, ATAGO, Tokyo, Japan) at 25 °C. The concentration of reducing sugars was measured using the 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959), and total sugar content was measured using acid hydrolysis (Association of Official Analytical Chemistry, 2000). The protein content was determined according to Lowry, Rosebrough, Farr and Randall (1951).

2.3.2 Antioxidant Activity

The F and TT samples were analyzed for antioxidant activity (free radical scavenging capacity), which was examined by the reduction of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) as described by Ramadan, Kroh and Moersel (2003) with modifications. Five mL of a 20 mg/mL DPPH solution in methanol were added to 5 mL of a methanolic solution of the beverage (1:10, v/v). Absorbance was determined spectrophotometrically (UV-1601PC Spectrophotometer, Shimadzu, Japan) at 517 nm after 30 min, and scavenging activity was calculated as the percent radical reduction. The percent inhibition was defined as [(A517 blank − A517 sample) A517 blank−1]·100−1 (%). Ascorbic acid was used as a reference component. The analysis was performed in triplicate, and the standard deviation of the mean values was calculated.

2.3.3 Total Phenolic Content

The Folin-Ciocalteu method (Singleton & Rossi, 1965) was used with some modifications to quantify total phenolic compounds using a gallic acid calibration curve (0 to 500 mg/L). A total of three hundred μl of diluted F matrix and TT beverage in a ratio of 1:100 with methanol:water (6:4) was mixed with 1.5 mL of 10-fold-diluted Folin-Ciocalteu's phenol reagent and 1.2 mL of 0.1 M sodium carbonate solution. The mixture was maintained in the dark for 90 min at 25 °C before absorbance was measured at 760 nm using a UV-1601PC spectrophotometer (Shimadzu, Japan). Dilutions were performed in triplicate, and the results are expressed as mg of gallic acid equivalents (GAE)/L of sample.

2.3.4 Omega 3 Fatty Acid

The total lipid content of the F matrix and TT beverage was determined by extracting with organic solvent in a Soxhlet extractor (Tecnal Equip., Piracicaba, Brazil) (AOAC, 2005). The samples had been previously homogenized and subjected to acid hydrolysis with 8 M HCl. From the extract obtained in the determination of total lipids, esterification of fatty acids was performed according to a procedure developed by Hartman and Lago (1973). The methyl esters of the fatty acids were determined by gas chromatography with a flame ionization detector (GC-FID) (GC-2014, Shimadzu, Kyoto, Japan) using an Rtx-2330 fused silica capillary column (105 m,

0.25 mm i.d., 0.20 μm film thick, Restek Corp., Bellefonte, PA, USA). The operating conditions were: initial temperature of the column, 140 °C for 5 min; increasing to 240 °C at a rate of 2.5 °C/min; and remaining at 240 °C for 15 min. The detector was isothermally maintained at 260 °C. The fatty acids were identified using GC Solution software (Shimadzu, Kyoto, Japan) by comparing retention times with those of standards (F.A.M.E. Mix C4-C24, Supelco Analytical, Bellefonte, PA, USA). Nitrogen was used as the carrier gas at a flow rate of 26 mL/min with a split ratio of 20:1.

2.3.5 Osmolality

The osmolality of the TT beverage was determined according to Henriques and Rosado (1999). The freezing point of the sample was measured using a cryoscope (MK 540L, ITR, Esteio, RS, Brazil) and the following equation was applied to determine the osmolality: molal concentration of the sample=Δtc/Kc, where Δtc is the cryoscopic decrease, and Kc is the cryoscopic constant of water (1.86 °C/mol/kg). One osmole is equal to 1 mole for osmotic effects, which is the osmolality of the solution at the freezing point.

2.3.6 Microbiological Analysis

Microbiological analysis of TT beverages was based on Brazilian criteria (Resolution RDC 12) that establish presumptive tests for total and thermo-tolerant coliform Most Probable Number (MPN) and the absence of *Salmonella* sp. in a 25-mL sample (Brasil, 2001). For the MPN of total and thermo-tolerant coliforms and the determination of presence or absence of *Salmonella* sp., the APHA (American Public Health Association, 1992) recommendations were followed. Suspected *Salmonella* colonies were submitted to standard biochemical and serological tests (agglutination test performed with polyvalent flagellar antiserum) (Probac, São Paulo, Brazil).

2.3.7 Sensory Evaluation

Sensory evaluation was approved by the Human Research Ethics Committee (CEPSH) at UFSC, human ethics approval number 1076963. Sensory evaluation of the TT beverage was performed after 15 days of storage at 4 °C. Sensory characteristics of the TT beverage were compared with those of the leading commercial pasteurized orange and carrot juices. Sensory hedonic testing of the beverages was performed according to Ferrari Pereira Lima, De Dea Lindner, Thomaz Soccol, Parada and Soccol (2012) by a group of 57 non-trained testers (elderly participants greater than 60 years old) from the Sector of Studies of Elderly (NETI) from UFSC. The participants were regular fruit juice consumers who judged the color, odor, taste and overall acceptability using a hedonic rating scale from 1 to 9 (1: extremely dislike; 2: dislike very much; 3: moderately dislike; 4: slightly dislike; 5: neither like nor dislike; 6: slightly like; 7: moderately like; 8: like very much; 9: extremely like) (Meilgaard, Civille, & Carr, 2007). Sensory tests were performed in individual booths under white light in the morning (9:00-11:30 a.m.). Samples were served refrigerated at 5 °C in transparent glass cups. The data obtained were analyzed by ANOVA and Tukey's test according to Monteiro (1994) using Assistat version 7.5 software (Assistat, Brazil). To verify the acceptability of the tested beverages, an acceptability factor (*AF*) (Dutcosky, 1996) using standardized criteria was calculated to evaluate each sensory analyzed attribute: $AF = A \bullet 100/B$, where A is the mean value obtained for each attribute, and B is the maximum mean value ascribed to each attribute.

2.4 Comparison of Preservation Process Technologies and Stabilization of the Beverage Components

2.4.1 Vitamin A

An LC10AT high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) equipped with a diode array detector (DAD) was used to measure vitamin A in the samples tested. Chromatographic separations were performed using an Eclipse XDB-C_{18} column - 150 mm x 4.6 mm, 5 μm (Agilent Technologies, Palo Alto, CA, USA). The mobile phase was composed of 10% acetonitrile and 90% methanol/water 98/2, and the device was operated in the isocratic mode with a flow rate of 1 mL/min. A total of 20 μL of sample was injected, and the analyte was monitored at 330 nm. The analyte was confirmed by enrichment of sample using a pharmaceutical standard of retinol and its UV-spectra. The retention time of the analyte was 4.4 min under the experimental conditions. For this assay, the peak area of vitamin A observed for the F matrix was considered 100 % concentration; the percentage of the analyte presented in the treated beverages could thus be estimated.

Before injection into the HPLC system, all samples were prepared as follows: 0.5 mL of chloroform was added to 2 mL of sample in a Falcon tube, and the mixture was vortexed for 1 min and centrifuged for 10 min. Thereafter, 200 μL of the organic phase was transferred to a microtube and dried using an N_2 flow. The sample was resuspended using 100 μL of acetonitrile and injected into the chromatographic system.

2.4.2 Vitamin C

A 7100 capillary electrophoresis (CE) system (Agilent Technologies, Palo Alto, CA, USA) equipped with a DAD was used in this assay. For all analyses, a fused silica capillary with 32 cm total length (23 cm of effective length) x 75 μm internal diameter x 375 μm outer diameter was used. Samples were injected hydrodynamically by applying 50 mbar for 3 s, and the analyte was monitored at 266 nm. The separation voltage was 25 kV under positive polarity at the injection side, and the temperature was maintained at 25 ˚C. To minimize instrumental errors, sorbic acid was used as internal standard. The background electrolyte (BGE) was composed of 40 mM tris(hydroxymethyl)aminomethane (TRIS) and 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), buffered at pH 8.2.

To quantify the concentration of vitamin C in the samples, a calibration curve was constructed using an analytical standard of ascorbic acid from 10.1-40.6 mg/L using 7 concentration levels. The limit of detection and quantification of the method were 0.4 mg/L and 1.35 mg/L, respectively, calculated by the signal-to-noise ratio of 3:1 and 10:1, respectively.

Before injection into the CE system, 1 mL of sample was centrifuged, and the supernatant was collected and properly diluted to 1 mL of deionized water. Both the calibration curve and samples were prepared in duplicate, with a final internal standard concentration of 25 mg/L.

2.4.3 Enzymatic Activity

Peroxidase (POD, EC 1.11.1.7) activity was determined according to the method described by Abreu and Faria (2007) and Campos, Souza, Coelho and Glória (1996), with modifications. A total of 7 mL of 0.2 M buffer solution of monobasic sodium phosphate (pH 5.8), 1.5 mL 0.05% guaiacol (phenolic substrate) and 0.5 mL 0.1% hydrogen peroxide were added to a test tube maintained in a water bath at 35 °C. After stabilization of the temperature, 1 mL of the treated beverage samples was added to the tube. The mixture was homogenized, and changes in absorbance at 470 nm were measured in a U-1800 UV/VIS Spectrophotometer (Hitachi, Berkshire, United Kingdom).

Polyphenol oxidase (PPO, EC 1.10.3.1) activity was determined according to the method described by Abreu and Faria (2007) and Campos et al. (1996), with modifications. Volumes of 5.5 mL of 0.2 M buffer solution of monobasic sodium phosphate (pH 6.0) and 1.5 mL of 0.2 M pyrocatechol solution (phenolic substrate) were added to a test tube maintained in a water bath at 25 °C. After stabilization of the temperature, 1 mL of the treated beverage samples was added to the tube. The mixture was homogenized, and changes in absorbance at 425 nm were measured in a U-1800 UV/VIS Spectrophotometer.

The absorbance values were acquired after 10 min for both POD and PPO enzymes. The blank for analysis of both enzymes was a mixture of all the reactants, but replacing the beverage sample with distilled water. The enzyme activity was expressed in units/mL. One unit is equivalent to a change in absorbance of 0.001/min/mL of sample. The following equation was used: Enzymatic activity=$(A10_{sample}-A0_{sample})-(A10_{blank}-A0_{blank})/0.01$, where A10 is the absorbance after 10 min of the reaction, and A0 is the initial absorbance.

2.4.4 Microbiological Challenge Testing

To evaluate the effectiveness of the preservation processes used to deliver some degree of lethality against target potential pathogenic and/or spoilage microorganisms, a surrogate *E. coli*, ATCC 25922, and *A. acidoterrestris* ATCC 49025 were separately inoculated into the F matrix before treatment.

The *E. coli* stock culture was maintained on Brain-Heart Infusion agar (BHI) (Acumedia, Lansing, Michigan, USA) slants at 4 °C. The cultures for the experiments were subcultured twice from the stock culture in nutrient broth (NB) (Oxoid, Basingstoke, Hampshire, England) incubated at 35 °C for 18 h. The culture was inoculated at a concentration of $1.5x10^6$ CFU/mL in the F matrix. To evaluate the effect of the treatments on microbial inactivation, the enumeration of viable *E. coli* cells, after serial dilutions of the treated beverages in buffered peptone water, was obtained using violet red bile agar with 4-methylumbelliferyl-β-D-glucuronide (VRBA MUG) (Acumedia, Lansing, Michigan, USA) at 45 °C/24 h. Replicate counts in CFU were converted to log values and expressed as averaged log numbers.

A. acidoterrestris was cultivated in yeast glucose starch (YGS) medium pH 3.7 at 45 °C for 5 days according the methodology described by Alberice, Funes-Huacca, Barreto Guterres and Carrilho (2012). The spores obtained from the culture were spread onto YGS agar and incubated at 45 °C for 5 days. After reaching more than 90% of sporulation, as confirmed by microscopy following staining with malachite green, spores were collected with a sterile swab and resuspended in sterile distilled water. The spores collected were centrifuged at 4.500*g* for 15 min at 4 °C, washed two times with sterile distilled water, resuspended in acid sterile distilled water (pH 3.7) and

stored at −20 °C until use. The bacterial spore suspensions were activated by heat-shock at 80 °C for 10 min immediately prior to inoculation at a concentration of approximately $4x10^4$ CFU/mL in the F matrix. The number of viable *A. acidoterrestris* cells was evaluated from serial dilutions of the treated beverages in YGS agar pH 3.7 (45 °C/5 days). Counts in CFU were obtained in the same manner as described above for *E. coli.*

Plate count agar (PCA) (Himedia, Mumbai, India) at 30 °C for 48 h and dichloran rose bengal chlortetracycline (DRBC) (Acumedia, Lansing, Michigan, USA) at 25 °C for 5 days were used to enumerate the presence of uninoculated (naturally present in the F matrix) viable mesophilic aerobic bacteria and yeasts and molds, respectively, in the untreated and treated samples for each preservation process. Counts for CFU were performed in duplicate, and the mean values were calculated.

3. Results and Discussion

3.1 Characterization of the Beverage

3.1.1 Physicochemical and Composition Characterization

The characterization of the TT beverage, presented in Table 1, is consistent with those reported for coconut water and orange juices. The obtained pH values classified the beverage as low-acid, and the high carbohydrate content could contribute to the development of food-borne pathogen contamination (Walter, Kabuki, Esper, Sant'Anan, & Kuaye, 2009). A beverage pH below 3.8 is significant for determining microbiological stability (Battey & Schaffner, 2001) and is directly correlated with the product's taste. However, the thermo-acidophilic spore-forming bacteria *Alicyclobacillus sp*. (TAB) can not only survive the normal pasteurization procedure applied to fruit juices and beverages but also germinate and proliferate in acidic products (Smit, Cameron, Venter, & Witthuhn, 2011).

Table 1. Characteristics of the TT beverage

Parameter	Mean ± SD
pH	3.50 ± 0.0
Acidity*	7.30 ± 0.01
Soluble solids*	8.33 ± 0.58
Reducing sugars*	42.90 ± 0.05
Total sugars*	66.00 ± 0.11
Proteins*	0.66 ± 0.07

Note. SD= standard deviation; *g/L.

The sugars that are known to contribute to the sweet taste in coconut water and orange juice are fructose, glucose and sucrose (Bilek & Bayram, 2015; Camargo Prado et al., 2015). Trace amounts of protein were found in the beverage. The presence of free amino acids and reducing sugars may result in the occurrence of Maillard reactions during thermal processing, which would leads to nutritional and sensory changes in the beverage (Cappelletti et al., 2015).

3.1.2 Vitamin C, Antioxidant Activity and Total Phenolic Content

The concentrations of vitamin C as ascorbic acid in the F and TT beverage are shown in Table 2. Of this total, 180 mg/L was added to the beverage during formulation as a technological antioxidant and vitamin enrichment component. With this amount, the TT beverage achieves 162% of the RDI recommended in Brazil. Both vitamin C concentration and total phenolic content are strongly correlated with antioxidant capacity, as determined by the reduction of DPPH (Gardner, White, McPhail, & Duthie, 2000). The DPPH assay has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors. Santos et al. (2013) detected and quantified ascorbic acid in several varieties of coconut water (25.8 mg/L). In cell culture, the green dwarf variety of coconut water was efficient in protecting against oxidative damage induced by hydrogen peroxide. Vitamin C is highly bioavailable and is consequently one of the most important water-soluble antioxidants in cells (Halliwell, 1996).

Table 2. Ascorbic acid content, antioxidant activity, total phenol content, omega 3 fatty acid content and osmolality of F (fresh untreated matrix) and TT (thermally treated beverage)

	F	TT
Ascorbic acid (mg/L)*	291.73 ± 0.68[a]	285.23 ± 1.45[b]
Antioxidant activity (% inhibition of DPPH)*	63.48 ± 2.41[a]	68.48 ± 2.00[b]
Total phenol content (mg/L)*	952.75 ± 5.69[a]	962.50 ± 2.06[b]
Omega 3 fatty acids (mg/L)	516.40	530.10
Osmolality (mOsmol/L)	ND	545.00

Note. *Mean ± standard deviation. Values with different capital letters in the same line are significantly different ($P < 0.05$). ND= not determined.

The F matrix and TT beverage exhibited high ascorbic acid content due to the high ascorbic acid content in the orange juice (403.15 mg/L) and coconut water (23.10 mg/L), which were added to 40% and 48%, respectively, in the beverage formulation. The consumption of fruit juice-based beverages contributes to overall antioxidant intake, with potentially beneficial biological effects for human health. Coconut water and orange juice were shown to have antioxidant activity, which was positively correlated with the concentration of flavanones and kinetin in orange juice and coconut water, respectively (Sánchez-Moreno et al., 2005; Yong et al., 2009). The F and TT beverage showed different results for antioxidant activity (Table 2). Comparison of the beverage with that in the study conducted by Ferrari Pereira Lima et al. (2012) showed that the anti-radical performance towards DPPH radicals was just below that of a fermented herbal mate beverage rich in phenolic compounds. According to Qin, Jin and Park (2010), individual phenolic compounds with high antioxidant activity might be produced during the fermentation process. Compared with other antioxidant beverages (Ramadan-Hassanien, 2008), especially those rich in caffeine, the TT beverage has high antioxidant capacity, slightly less than that of tea with lemon, green tea, black tea and soluble coffee.

The total phenolic content in the TT beverage was slightly above that of F (Table 2). The major contribution to phenolics in the beverage came from the orange juice. In a study conducted by Gardner and White (2000), the level of phenolic compounds in orange juice was 755 mg GAE/L. As mentioned above, the addition of 40% of orange juice, increased the total phenolic content of the beverage. The use of orange juice in the formulation is thus important as a source of phenolic compounds. The coconut water, present in the formulation in the range of 46%, did not contribute significantly to the total phenolic content; Santos et al. (2013) and Tan et al. (20140 found a low content of phenolics in coconut water (99.40 and 54.00 mg/L, respectively).

3.1.3 Omega 3 Fatty Acids

The concentration of omega-3s (EPA+DHA) in the F and TT beverage is shown in Table 2. In this amount, a dose of 250 mL of beverage achieves 51.3% of the FAO minimal recommendation per day. Decreases in DHA status are associated with cognitive decline in the elderly and those with Alzheimer's (Abubakari, Naderali, & Naderali, 2014). Human studies suggest that an adequate dietary intake of omega-3s could decrease age-related cognitive decline, may protect against senile dementia and provide better cognitive performance (Denis, Potier, Vancassel, & Lavialle, 2013; D'Ascoli et al., 2016). García-Alonso, Jorge-Vidal, Ros and Periago (2012) compared the effects of consumption of omega-3-enriched tomato juice on serum lipid profiles and levels of biomarkers related to antioxidant status and cardiovascular disease (CVD) risk in healthy women. Stronger positive amelioration of CVD risk factors was observed following the intake of enriched juice, suggesting a possible synergistic action between omega-3s and tomato antioxidants (362 mg/L). In comparison, the TT beverage had 2.65-fold more total phenolics in almost the same amount of omega-3s. Hawthorne, Abrams and Heird (2009) evaluated the effects of providing a supplement of micro-encapsulated algal DHA in orange juice. They demonstrated that DHA supplementation of juice at either 50 mg/day or 100 mg/day for 6 weeks was effective in increasing the plasma phospholipid DHA contents of children.

3.1.4 Osmolality

The osmolality of the beverage, expressed in mOsmol/L, depends on its osmotic pressure. Osmolality is a crucial determinant of the physiological acceptance of the beverage. The desired osmolality should be < 700 mOsm (Klang, McLymont, & Ng, 2013). An appropriate osmolar load is necessary for the beverage be tolerated by a eutrophic elderly consumer, ensuring the successful assimilation of the formulation components. The osmolar concentration found in the TT beverage (Table 2) was 545.00 mOsm/L, classifying the product as a hypertonic

beverage (> 330 mOsmol/L). Fruit juices are considered typical hypertonic beverages, with osmolality values in the range of 600-700 mOsmol/L (Henriques & Rosado, 1999).

3.1.5 Microbiological Analysis and Sensory Evaluation

Microbial evaluation of total, thermo-tolerant coliforms and *Salmonella* sp. was performed (data not shown) in accordance with Brazilian criteria to ensure safe consumption of the TT beverage, indicating that efficient heat treatment occurred after a satisfactory hygienic process. Then, a panel of 57 non-trained elderly volunteers, each greater than 60 years old and a regular fruit juice consumer, participated in the evaluation. The acceptance levels for the TT beverage and the leading commercial pasteurized orange/carrot juice are presented in Table 3. No significant differences in global evaluation, taste, color or aroma were obtained.

Table 3. Acceptance (average values ± SD) and acceptability factors (AFs) for TT (thermally treated beverage) and commercial pasteurized orange/carrot juice

Attributes (AF)	TT beverage	Commercial pasteurized juice
Global evaluation	6.89 ± 1.50^{a} (100)	5.61 ± 2.20^{a} (81)
Taste	6.81 ± 1.70^{a} (100)	5.19 ± 2.30^{a} (76)
Color	7.12 ± 1.80^{a} (100)	5.47 ± 2.10^{a} (77)
Aroma	6.70 ± 1.70^{a} (100)	5.42 ± 2.10^{a} (81)

Note. [a] Means ± standard deviation with the same letter in the same line are not significantly different ($P > 0.05$).

These findings were in accordance with Camargo Prado et al. (2015), who evaluated a fermented coconut-based beverage supplemented with sucrose and artificial coconut flavor. Figure 2 shows that the TT beverage received the best overall rating from the panel. For the overall evaluation, the average values for the sensory analysis and acceptability factors were kept within the acceptance range. According to Dutcosky (1996), AF ≥ 70% represented good acceptability for the attribute analyzed in a sensory analysis. Therefore, product acceptance was good and was comparable to that of similar commercial beverages available at the local market.

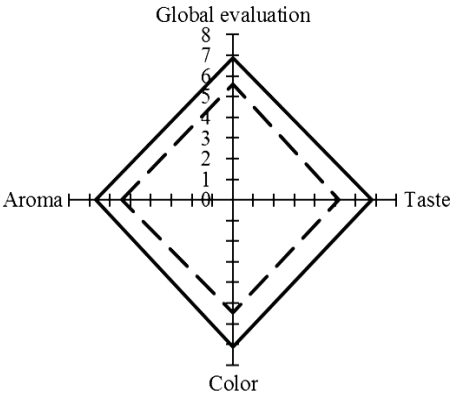


Figure 2. Characteristics of the acceptance profile of the TT beverage (thermally treated beverage) (solid line) and the leading commercial pasteurized orange/carrot juice (dashed line)

3.2 Comparison of Preservation Process Technologies and Stabilization of the Beverage Components

Thermal processes induce undesirable changes in beverages, such as micronutrient losses, through chemical reactions and leads to losses in perceived freshness (Rodríguez-Roque et al., 2015). Non-thermal beverage preservation technologies such as SC-CO_2 and DMDC have been developed as an alternative. Previous studies have reported that both technologies inactivate microorganisms without compromising the nutritional and sensory attributes of food (Yu, Xiao, Xu, Wu, & Wen, 2014; Cappelletti et al., 2015).

3.2.1 Vitamins A and C and Enzymatic Activity

The analyses used to measure the reduction of vitamin content and enzymatic activity during the processes are described in table 4.

Table 4. Vitamin A and ascorbic acid levels and peroxidase and polyphenol oxidase activities of coconut water (CW), fresh untreated matrix (F), thermally treated beverage (TT), supercritical carbon dioxide-treated beverage (SC-CO_2) and dimethyl dicarbonate-treated beverage (DMDC)

			Preservation process			
	Orange juice	Coconut water	F	TT	SC-CO_2	DMDC
Vitamin A (%)	n.d.	n.d.	100	144	92	106
Ascorbic acid (mg/L)*	403.15±0.78	23.10±3.0	291.73±3.68^{a}	285.23±1.45^{b}	245.69±0.52^{c}	279.30±8.86^{ab}
Peroxidase (U/mL)	7.40	66.30	3.90	ND	ND	ND
Polyphenol oxidase (U/mL)	ND	14.7	ND	ND	ND	ND

Note. *Means ± standard deviation. n.d.= not determined, ND= not detectable. ^{a,b,c} Values with different capital letters are significantly different ($P < 0.05$).

The TT beverage showed higher vitamin A values (increased 44% in relation to F). This result was in agreement with a report by Sánchez-Moreno, Plaza, De Ancos and Cano (2003) that showed an increase in extractable carotenoids due to thermal treatment of orange juice. According to Schieber and Carle (2005), the availability of carotenoids may increase due to rupture of the food matrix by heat or mechanical treatment. Thermal processes increase the chemical extractability of carotenoids and other substances.

According to Zhou et al. (2009), who investigated the effect of SC-CO_2 on the quality of carrot juice, the SC-CO_2 treatment resulted in an 8% decrease in the vitamin A concentration, and they observed that carotenoids in the treated juices were stable. Carotenoids are bound to protein and membrane lipids. Processes that apply high pressure are known to affect food macromolecular structures. Sánchez-Moreno et al. (2005) found that in orange juice, high pressure processes increased the release of carotenoids from the suspended pulp particles, making them more accessible to extraction and leading to increased carotenoid release and vitamin A values. Our result, in accordance to Johannsen and Brunner (1997), may be related to the solubility of fat-soluble vitamins after supercritical CO_2 interaction that is correlated with differences in the temperature and density of CO_2 applied.

Compared to F, the DMDC treatment showed a 6% increase in the vitamin A concentration. There is little known about the interaction between this molecule and vitamin A. More research could clarify the mechanism of interaction between DMDC treatment and vitamins.

The content of ascorbic acid from the treatments analyzed is reported in Table 4. Before the addition of ascorbic acid to the beverage formulation, ascorbic acid was measured as 23.10 and 403.15 mg/L for coconut water and orange juice, respectively. Significant differences among the preservation processes were detected. In contrast, Rodríguez-Roque et al. (2015) and Sánchez-Moreno et al. (2005) showed that the greatest losses of this bioactive compound were found in TT fruit juice beverages (by 31% and 9%, respectively, compared with untreated matrixes) subjected to different preservation technologies. Vitamin C is a heat-sensitive nutrient and is vulnerable to enzyme-catalyzed oxidation by oxidoreductases (*e.g.,* ascorbate oxidase and POD) (Davey et al., 2000). In our work, the treatments applied may have partially eliminated some of the enzymes responsible for vitamin C loss. Thermal treatments with higher temperature tended to result in a higher decrease in the content of vitamin C, but in our case, the binomial time-temperature applied in the TT process demonstrated no significant difference from non-thermal treatments.

There was no significant degradation of ascorbic acid after treatment with DMDC (Table 4). Yu et al. (2014) treated fermented litchi juice with DMDC and observed, contrary to our results, a 58.1% reduction in the ascorbic acid content relative to the fresh sample. Leong and Oey (2012) reported that ascorbic acid was minimally reactive with DMDC and that the loss of the compound could be attributed to ascorbic acid oxidases in the juice during DMDC treatment. In our case, depleting dissolved oxygen in the deaeration process and filling the bottle headspace with N_2 both inhibited the oxidation of ascorbic acid.

SC-CO_2 treatment using 20 MPa showed the greatest decrease in ascorbic acid content, a more than 15% reduction compared with F (Table 4). According to Fabroni, Amenta, Timpanaro and Rapisarda (2010), the addition of CO_2 to orange juice is probably beneficial for ascorbic acid retention due to the displacement of dissolved oxygen from the liquid matrix. Fabroni et al. (2010) applied CO_2 over 23 MPa for 15 min, and 6% ascorbic acid in the juice was degraded, but at lower pressures (13 MPa), the concentration remained unchanged. They concluded that lower operative pressures are likely to be beneficial for the retention of ascorbic acid, and

the change in CO_2 concentration did not appear to have an influence.

Higher concentrations of POD and PPO were found in coconut water (Table 4), the major component of the beverage. In orange juice and F, the high concentration of ascorbic acid and the pH of 3.5, respectively, were sufficient to inhibit enzyme activities. These results are in accordance with Abreu and Faria (2007), who reported that the addition of ascorbic acid was effective in the inactivation of PPO in coconut water before heat treatment. Campos et al. (1996) showed that 20 mg of ascorbic acid per 100 mL of coconut water was sufficient to reduce the activity of both oxidoreductases. Cultivar differences and component matrix variations during fruit maturity are key elements in the variations in enzymatic value.

No enzyme activity was detected in the TT, SC-CO_2 or DMDC beverages (Table 4), which could be related to the synergic effect of the treatments, the presence of ascorbic acid in the formulation and/or the low pH of the beverage. Campos et al. (1996) and Tan et al. (2014) reported that thermal treatment had a pronounced effect on the activities of POD and PPO in coconut water, with a drastic decrease observed at higher heating temperatures. The results showed that complete inactivation of POD was achieved with heat treatment at 90 °C and a holding time of 2.5 min. The cited works also reported a similar result; PPO was more heat-resistant than POD.

Yu et al. (2014) used DMDC in litchi juice and observed that the content of ascorbic acid increased in *Lactobacillus casei*-fermented juice; this result was attributed to the deoxidization of oxidized ascorbic acid. During a four-week period, no large variations in color were observed, demonstrating the stability of the enzyme activities in the DMDC-treated juice. According to Wang et al. (2013), DMDC can inhibit the activity of PPO and POD, contributing to the maintenance of ascorbic acid content.

Depending on the processing parameters (*e.g.*, temperature, pressure and depressurization rate), SC-CO_2 may negatively affect the enzyme activity due to conformational changes caused by gas in the secondary and tertiary structure. Gui et al. (2007) observed that cloudy apple juice, when exposed to SC-CO_2 at 30 MPa (55 °C for 60 min), presented a reduction in PPO activity of over 60%, which was greater than that observed under atmospheric conditions at the same temperature (27.9% reduction) and indicated a combined effect of pressure, temperature and time after SC-CO_2 treatment. Four years later, Xu et al. (2011) studied the SC-CO_2-mediated inactivation of enzymes in the same matrix. PPO was completely inactivated after 10 min of treatment at 22 MPa and 60 °C. Liu, Hub, Zhaoc and Song (2012) studied the effect of SC-CO_2 on watermelon juice and obtained 95.8% reductions in PPO activity and 57.9% in PDO when 30 MPa pressure was applied at 50 °C for 50 min. The cited works reported that the major effect of SC-CO_2 on enzymes occurred during the first minutes of treatment, in accordance with our results.

3.2.2 Microbiological Challenge Testing

The inoculum level used in a microbiological challenge depends on the objective of the study. In our case, the level was determined for validation of process lethality, and 10^6 CFU/mL for *E. coli* was used to demonstrate the extent of reduction in the challenge. In the USA, juice processors are required to demonstrate a 5 log reduction in relevant hazardous microorganisms in their products (5 D performance standard) (U.S. FDA, 2016).

Several pathogenic bacteria may resist the inherent acidity of fruit juice and develop adaptive mechanisms that enhance their survival and sometimes even their ability to grow in acidic environments. Analysis of the data (Table 5) showed that the TT and DMDC processes could deliver the required level of lethality according to the pre-determined performance standard of a 5 log reduction for juices (U.S. FDA, 2016). The SC-CO_2 treatment demonstrated a 3.38 log reduction. Our results are in agreement with those of (Basaran-Akgul, Churey, Basaran, & Worobo, 2009), who challenged apple cider (pH 4.0) by inoculating 10^6-10^7 CFU of three different strains of *E. coli* O157:H7 per mL of juice. A greater than 5 log reduction was achieved at room temperature with 250 ppm DMDC after incubation for 6 h. Treatment with DMDC may offer a viable alternative to TT for the production of safe juice.

Table 5. Viable cell counts (log orders CFU/mL) performed in duplicate. The results are presented as the mean values for *E. coli* ATCC 25922, *A. acidoterrestris* ATCC 49025, mesophilic aerobic bacteria, yeasts and molds in the fresh untreated matrix (F), thermally treated beverage (TT), supercritical carbon dioxide-treated beverage (SC-CO2) and dimethyl dicarbonate-treated beverage (DMDC)

		Preservation process			
	Medium	F	TT	SC-CO_2	DMDC
E. coli ATCC 25922	VRBA MUG	6.08	ND	2.70	ND
A. acidoterrestris ATCC 49025	YGS	4.60	2.60	2.78	2.18
Mesophilic aerobic bacteria*	PCA	2.60	ND	1.70	2.18
Yeasts and molds*	DRBC	3.48	2.70	3.30	2.30

Note. ND= colonies not detected - counts below the detection limit (1 CFU/mL). *Enumeration of uninoculated microorganisms (naturally present in the matrix).

E. coli ATCC 25922 demonstrated some level of resistance to the conditions applied in the SC-CO_2 treatment (Table 5). Silva et al. (2013) used SC-CO_2 to build an inactivation curve for the same ATCC 25922 strain. The researchers obtained considerable log reductions using pressures of 10 MPa/25 min and found that microbial inactivation increases with increasing supercritical CO_2 pressure cycles and system pressure. The food matrix can cause variations in the ability of pressurized CO_2 to physicochemically act (four mechanisms described by Spilimbergo and Bertucco, 2003) to lead to loss of cell viability and the resistance and recovery of the microorganism (Debs-Louka, Louka, Abraham, Chabot, & Allaf, 1999).

Detectable taint production in fruit juice is generally reported when the levels of *A. acidoterrestris* reach approximately 4 to 5 log CFU/mL (Molva & Baysal, 2015); using this information, we chose to inoculate 4 log. TT reduced the concentration of *A. acidoterrestris* in the beverage by 2 log (Table 5). The heat resistance of *A. acidoterrestris* was reported in several studies. According to Bevilacqua, Sinigaglia and Corbo (2008), it is necessary to correlate heat with pH and soluble solids concentration to achieve a significant reduction in thermoacidophilic bacteria (TAB) because the resistance of endospores is strain-dependent. Alberice et al. (2012) investigated heat treatment and incubation time in orange juice inoculated with 4 log CFU/mL of *A. acidoterrestris* spores to evaluate the best temperature for inactivation. At 87 °C, counts of cell viability decreased slowly within the first 50 min of incubation. The best inactivation was obtained with a one and two minutes at 99 °C.

Viable *A. acidoterrestris* cells demonstrated resistance to inactivation with DMDC treatment (Table 5). The reduction observed was only 2.18 log. According to Chen, Harte, Davison and Golden (2013), DMDC (250 ppm) reduced the initial vegetative cell population by 2 log CFU/mL in *Bacillus acidoterrestris* thermophilic broth and significantly increased the time to reach stationary phase. During the hydrolysis period, DMDC demonstrated activity, achieving some level of reduction for vegetative cells and spores, but after hydrolysis occurred, considerable growth of cells was detected. Therefore, the hydrolysis time was not sufficient to affect spores structured to resist environmental stresses. Although DMDC treatment may help control the vegetative cells of *A. acidoterrestris*, it may not provide adequate overall control for spores. Based on these results, the use of other antimicrobial agents for long shelf-life products is recommended.

The SC-CO2 treatment also failed to achieve the expected effectiveness for *A. acidoterrestris*; the reduction observed was only 1.82 log (Table 5). This technique combines many variables, such as pressure, pressurization/depressurization rate and cycles, temperature and process time, which are necessary to apply an experimental design to evaluate the behavior of conditions with the juice matrix. According to Garcia-Gonzalez et al. (2009), both Gram-positive and Gram-negative bacteria can be sensitized by 10 MPa SC-CO_2 at 35 °C for 20 min, but yeasts and the vegetative cells of *A. acidoterrestris* in apple juice show higher resistance (reduction of 2.0 and 0.3 log, respectively).

In the present study, negative results were recorded for *A. acidoterrestris* in all treatments tested even though viable spores may be present in the beverage. Consequently, defective batches of juice may enter the filling line after treatment. Therefore, the fate of *A. acidoterrestris* spores in our beverage is worthy of investigation. No shelf life tests were conducted to further explore the prevalence of the strain with time after treatment.

Viable mesophilic aerobic bacteria, yeasts and molds were tested in terms of resistance to inactivation by the applied treatments (Table 5). Thermal treatment was able to reduce the total cultivable mesophilic aerobic bacteria naturally present in the matrix, in accordance with the results of Cappelletti et al. (2015). SC-CO_2 and DMDC were

not able to act similarly. Furthermore, our results demonstrate that the applied treatments were not adequate to reduce naturally present yeasts and molds. Many fungi have the ability to produce ascospores that survive heat treatment, germinating within the packaging and causing deterioration. Salomão, Muller, Couto do Amparo and Falcão de Aragão (2014) evaluated molds and yeasts in the apple juice heat concentration process and identified heat-resistant molds such as *Byssochlamys fulva*. Consistent with our results, Yu et al. (2014) achieved a small reduction of 3.5 and 1.6 log for yeast and molds, respectively, in fermented litchi juice by applying DMDC (250 mg/L) at 30 °C. Generally, molds are more resistant to DMDC treatment than are yeasts and bacteria.

In conclusion, preventive interventions through dietary modification are attractive strategies for promoting the health of the elderly. RTD beverages are a great vehicle for healthful ingredients for elderly consumers' dietary needs, vulnerabilities, preferences and restrictions because of their convenience and availability. The developed beverage can contribute to vitamin C, antioxidant, phenolic, omega-3 and fiber intake. The osmolality tests indicate that the beverage is hypertonic and appropriate for tolerance by eutrophic elderly consumers, ensuring the assimilation of the formulation components. The study demonstrated that TT was more effective than the SC-CO_2 and DMDC treatments in preserving bioactive compounds in the fresh beverage, but the alternative non-thermal preservation methods represented a promising alternative to thermal processing for microbiologically stabilizing beverages without altering their quality attributes. The beneficial effects of the beverage as a healthy product should be explored in further studies, including trials with the elderly. Such research will provide insights into the use of the beverage as a non-dairy substrate and its introduction as a new vehicle for the consumption of functional beverages, especially by vegans and/or vegetarians and lactose-intolerant elderly.

Acknowledgments

The authors thank CNPq for financial support and scholarships, Lanxess for the Velcorin (DMDC) and NETI (UFSC) for sensory evaluation support.

References

Abreu, L. F., & Faria, J.A.F. (2007). Temperature and ascorbic acid effects in physico-chemical stability and enzimatic activity of coconut water (*Cocos nucifera* L.) aseptic filled. *Food Science and Technology*, *2*, 226-232. https://doi.org/10.1590/S0101-20612007000200003

Abubakari, A. R., Naderali, M. M., & Naderali, E. K. (2014). Omega-3 fatty acid supplementation and cognitive function: are smaller dosages more beneficial? *International Journal of Genetical Medicine*, *19*, 463-473. https://doi.org/10.2147/IJGM.S67065

Agcam, E., Akyıldız, A., & Akdemir Evrendilek, G. (2014). Comparison of phenolic compounds of orange juice processed by pulsed electric fields (PEF) and conventional thermal pasteurisation. *Food Chemistry*, *143*, 354-361. https://doi.org/10.1016/j.foodchem.2013.07.115.

Alberice, J. V., Funes-Huacca, M. E., Barreto, Guterres, S., & Carrilho, E. (2012). Inactivation of *Alicyclobacillus acidoterrestris* in orange juice by saponin extracts combined with heat-treatment. *International Journal of Food Microbiology*, *159*, 130-135. https://doi.org/10.1016/j.ijfoodmicro.2012.08.004

AOAC. (2000). Association of Official Analytical Chemistry, Official methods of analysis. Washington, USA.

AOAC. (2005). Association of Official Analytical Chemistry, Official methods of analysis. Washington, USA.

APHA. (1992). Standard Methods for the Examination of Water and Wastewater. 18th edition, American Public Health Association (APHA), American Water Works Association (AWWA) and Water Pollution Control Federation (WPCF). Washington, USA.

Basaran-Akgul, N., Churey, J. J., Basaran, P., & Worobo, R. W. (2009). Inactivation of different strains of *Escherichia coli* O157:H7 in various apple ciders treated with dimethyl dicarbonate (DMDC) and sulfur dioxide (SO2) as an alternative method. *Food Microbiology, 26*, 8-15. https://doi.org/10.1016/j.fm.2008.07.011

Battey, A. S., & Schaffner, D. W. (2001). Modelling bacterial spoilage in cold filled ready to drink beverages by *Acinetobacter calcoaceticus* and Gluconobacter *oxydans. Journal of Applied Microbiology*, *91*, 237-247. https://doi.org/10.1046/j.1365-2672.2001.01381.x

Besser, R. E., Lett, S. M., Weber, J. T., Doyle, M. P., Barret, T. J., Wells, J. G., & Griffin, P. M. (1993). An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh pressed apple cider. *JAMA*, *269*, 2217-2220. https://www.ncbi.nlm.nih.gov/pubmed/8474200

Bevilacqua, A., Sinigaglia, M., & Corbo, M.R. (2008). *Alicyclobacillus acidoterrestris*: new methods for inhibiting spore germination. *International Journal of Food Microbiology, 125*, 103-110. https://doi.org/10.1016/j.ijfoodmicro.2008.02.030

Bilek, S. E., & Bayram, S. K. (2015). Fruit juice drink production containing hydrolyzed collagen. *Journal of Functional Foods, 14*, 562-569. https://doi.org/10.1016/j.jff.2015.02.024

Brasil. (2001). ANVISA - Agência Nacional de Vigilância Sanitária. Resolução RDC 12. Regulamento técnico sobre padrões microbiológicos para alimentos.

Brasil. (2005). ANVISA - Agência Nacional de Vigilância Sanitária. Resolução RDC 269. Regulamento técnico sobre a ingestão diária recomendada (IDR) de proteína, vitaminas e minerais.

Brasil. (2008). ANVISA - Agência Nacional de Vigilância Sanitária. Alimentos com Alegações de Propriedades Funcionais e ou de Saúde, Novos Alimentos/Ingredientes, Substâncias Bioativas e Probióticos. IX - Lista de alegações de propriedade funcional aprovadas.

Camargo Prado, F., De Dea Lindner, J., Inaba, J., Thomaz-Soccol, V., Kaur Brar, S., & Soccol, C. R. (2015). Development and evaluation of a fermented coconut water beverage with potential health benefits. *Journal of Functional Food, 12*, 489-497. https://doi.org/10.1016/j.jff.2014.12.020

Campos, C. F., Souza, P. E. A., Coelho, V., & Glória, M. B. A. (1996). Chemical composition, enzyme activity and effect of enzyme inactivation on flavour quality of green coconut water. *Journal of Food Processes and Preservation***,** *20*, 487-500. https://doi.org/10.1111/j.1745-4549.1996.tb00761.x

Cappelletti, M., Ferrentino, G., Endrizzi, I., Aprea, E., Betta, E., Corollaro, M. L., Charles, M., Gasperi, F., Spilimbergo, S. (2015). High Pressure Carbon Dioxide pasteurization of coconut water: A sport drink with high nutritional and sensory quality. *Journal of Food Engineering, 145*, 73-81. https://doi.org/10.1016/j.jfoodeng.2014.08.012

Chen, W., Harte, F. M., Davidson, P. M., & Golden, D. A. (2013). Inactivation of *Alicyclobacillus acidoterrestris* using high pressure homogenization and dimethyl dicarbonate. *Journal of Food Protection, 76*, 1041-1045. https://doi.org/10.4315/0362-028X.JFP-12-443

Corona, O., Randazzo, W., Miceli, A., Guarcello, R., Francesca, N., Erten, H., Moschetti, G., & Settanni, L. (2016). Characterization of kefir-like beverages produced from vegetable juices. *LWT - Food Science and Technology*, *66*, 572-581. https://doi.org/10.1016/j.lwt.2015.11.014

Damar, S., & Balaban, M. O. (2006). Review of dense phase CO2 technology: microbial and enzyme inactivation, and effects of food quality. *Journal of Food Science, 71*, 1-11. https://doi.org/10.1111/j.1365-2621.2006.tb12397.x

D'Ascoli, T. A, Mursu, J., Voutilainen, S., Kauhanen, J., Tuomainen, T. P., & Virtanen, J. K. (2016). Association between serum long-chain omega-3 polyunsaturated fatty acids and cognitive performance in elderly men and women: The Kuopio Ischaemic Heart Disease Risk Factor Study. *European Journal of Clinical Nutrition, 70*, 970-975. https://doi.org/10.1038/ejcn.2016.59

Davey, M. W., Van Monagu, M., Inze, D., Sanmartin, M., Kanellis, A., Smirnoff, N., … Fletcher, J. (2000). Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing. *Journal of Science Food and Agriculture, 80*, 825-860. https://doi.org/10.1002/(SICI)1097-0010(20000515)80:7<825::AID-JSFA598>3.0.CO;2-6

Debs-Louka, E., Louka, N., Abraham, G., Chabot, V., & Allaf, K. (1999). Effect of Compressed Carbon Dioxide on Microbial Cell Viability. *Applied Environmental Microbiology, 65*, 626-631. http://aem.asm.org/content/65/2/626.short

Denis, I., Potier, B., Vancassel, S., & Lavialle, H.C. (2013). Omega-3 fatty acids and brain resistance to ageing and stress: Body of evidence and possible mechanisms. *Ageing Research Review***,** *12*, 579-594. https://doi.org/10.1016/j.arr.2013.01.007

Dutcosky, S. D. (1996). Análise sensorial de alimentos. Champagnat, Curitiba, Brasil, pp.123.

Fabroni, S., Amenta, M., Timpanaro, N., & Rapisarda, P. (2010). Supercritical carbon dioxide-treated blood orange juice as a new product in the fresh fruit juice market. *Innovative Food Science & Emerging Technology, 11*, 477-484. https://doi.org/10.1016/j.ifset.2010.02.004

Ferrari Pereira Lima, I., De Dea Lindner, J., Thomaz Soccol, V., Parada, J. L., & Soccol, C. R. (2012). Development of an Innovative Nutraceutical Fermented Beverage from Herbal Mate (*Ilex paraguariensis*

A.St.-Hil.) Extract. *International Journal of Molecular Science*, *13*, 788-800. https://doi.org/10.3390/ijms13010788

García-Alonso, F. J., Jorge-Vidal, V., Ros, G., & Periago, M. J. (2012). Effect of consumption of tomato juice enriched with n-3 polyunsaturated fatty acids on the lipid profile, antioxidant biomarker status, and cardiovascular disease risk in healthy women. *European Journal of Nutrition, 51*, 415-424. https://doi.org/10.1007/s00394-011-0225-0

Garcia-Gonzalez, L., Geeraerd, A. H., Elst, K., Van Ginneken, L., Van Impe, J. F., & Devlieghere, F. (2009). Influence of type of microorganism, food ingredients and food properties on high-pressure carbon dioxide inactivation of microorganisms. *International Journal of Food Microbiology, 28*, 253-263. https://doi.org/10.1016/j.ijfoodmicro.2008.12.005

Gardner, P. T., White, T. A. C., McPhail, D. B., & Duthie, G. G. (2000). The relative contributions of vitamin C, carotenoids and phenolics to the antioxidant potential of fruit juices. *Food Chemistry*, *68*, 471-474. https://doi.org/10.1016/S0308-8146(99)00225-3

Gui, F., Wu, J., Chen, F., Liao, X., Hu, X., Zhang, Z., & Wang, Z. (2007). Inactivation of polyphenol oxidases in cloudy apple juice exposed to supercritical carbon dioxide. *Food Chemistry, 100*, 1678-1685. https://doi.org/10.1016/j.foodchem.2005.12.048

Halliwell, B. (1996). Vitamin C: antioxidant or pro-oxidant in vivo? Free Radical Research*, 25*, 439-454. https://doi.org/10.3109/10715769609149066

Hartman, L., & Lago, R. C. A. (1973). Rapid preparation of fatty acid methyl esters from lipids. *Laboratorial Practice*, 175-176. https://www.researchgate.net/publication/18441624_Rapid_preparation_of_fatty_acids_methyl_esters

Hawthorne, K. M., Abrams, S. A., & Heird, W. C. (2009). Docosahexaenoic Acid (DHA) Supplementation of Orange Juice Increases Plasma Phospholipid DHA Content of Children. *Journal of American Dietology Association, 109*, 708-712. https://doi.org/10.1016/j.jada.2008.12.024

Henriques, G. S., & Rosado, G. P. (1999). Formulação de dietas enterais artesanais e determinação da osmolalidade pelo método de crioscópio. *Revista Nutrição, 13*, 225-232. https://doi.org/10.1590/S1415-52731999000300003

Johannsen, M., & Brunner, G. (1997). Solubilities of the fat-soluble vitamins A, D, E, and K in supercritical carbon dioxide. *Journal of Chemistry & Engineering Data*, *42*, 106-111. https://doi.org/10.1021/je960219m

Klang, M., McLymont, V., & Ng, N. (2013). Osmolality, pH, and Compatibility of Selected Oral Liquid Medications With an Enteral Nutrition Product. *Journal of Parenteral and Enteral Nutrition, 37*, 689-694. https://doi.org/10.1177/0148607112471560

Leong, S. Y., & Oey, I. (2012). Effect of endogenous ascorbic acid oxidase activity and stability on vitamin C in carrots (*Daucus carota* subsp. *sativus*) during thermal treatment. *Food Chemistry*, *134*, 2075-2085. https://doi.org/10.1016/j.foodchem.2012.04.002

Leyer, G. J., Wang, L. H., & Johnson, E. A. (1995). Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. *Applied Environmental Microbioliology, 61*, 3752-3755. https://www.ncbi.nlm.nih.gov/pubmed/7487011

Liu, Y., Hub, X., Zhaoc, X., & Song, H. (2012). Combined effect of high pressure carbon dioxide and mild heat treatment on overall quality parameters of watermelon juice. *Innovative Food Science & Emerging Technology, 13*, 112-119. https://doi.org/10.1016/j.ifset.2011.11.001

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, *193*, 265-275. http://devbio.wustl.edu/InfoSource/ISPDFs/Lowry%201951.pdf

Meilgaard, M., Civille, G. V., & Carr, B. T. (2007). Sensory Evaluation Techniques. CRC Press, Boca Raton, USA.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Analitical Chemistry*, *31*, 426-428. http://download.bioon.com.cn/upload/month_1002/20100202_79e2638a4a8db64734c5QyCZjgBzadbY.attach.pdf

Molva, C., & Baysal, A. H. (2015). Effects of pomegranate and pomegranate-apple blend juices on the growth

characteristics of *Alicyclobacillus acidoterrestris* DSM 3922 type strain vegetative cells and spores. *International Journal of Food Microbiology*, *200*, 52-56. https://doi.org/10.1016/j.ijfoodmicro.2015.01.019

Monteiro, C. L. B. (1994). Técnicas de Avaliação Sensorial. UFPR/CEPPA, Curitiba, Brazil, pp. 101.

Oteiza, J. M., Soto, S., Alvarenga, V. O., Sant'Ana, A. S., & Gianuzzi, L. (2015). Fate of *Alicyclobacillus spp*. in enrichment broth and in juice concentrates. *International Journal of Food Microbiology, 210,* 73-78. https://doi.org/10.1016/j.ijfoodmicro.2015.05.021

Qin, Y., Jin, X., & Park; D. H. (2010). Comparison of antioxidant activities in black soybean preparations fermented with various microorganisms. *Agriculture Science China, 9*, 1065-1071. https://doi.org/10.1016/S1671-2927(09)60191-7

Ramadan, M. F., Kroh, L. W., & Moersel, J. T. (2003). Radical scavenging activity of black cumin (*Nigella sativa* L.), coriander (*Coriandrum sativum* L.) and niger (*Guizotia abyssinica* Cass.) crude seed oils and oil fractions. *Journal of Agriculture and Food Chemistry, 51*, 6961-6969. https://doi.org/10.1021/jf0346713

Ramadan-Hassanien M. F. (2008). Total antioxidant potential of juices, beverages and hot drinks consumed in Egypt screened by DPPH in vitro assay. *Grasas y Aceites*, *59*, 254-259. https://doi.org/http://dx.doi.org/10.3989/gya.2008.v59.i3.516

Rawson, A., Patras, A., Tiwari, B. K., Noci, F., Koutchma, T., & Brunton, N. (2011). Effect of thermal and non-thermal processing technologies on the bioactive content of exotic fruits and their products: review of recent advances. *Food Research International*, *44*, 1875-87. https://doi.org/10.1016/j.foodres.2011.02.053

Rodríguez-Roque, M. J., de Ancos, B., Sánchez-Moreno, C., Pilar Cano, M., Elez-Martínez, P., & Martín-Belloso, O. (2015). Impact of food matrix and processing on the *in vitro* bioaccessibility of vitamin C, phenolic compounds, and hydrophilic antioxidant activity from fruit juice-based beverages. *Journal of Functional Foods, 14*, 33-43. https://doi.org/10.1016/j.jff.2015.01.020

Salomão, B. C. M., Muller, C., Couto do Amparo, H., & Falcão de Aragão, G. M. (2014). Survey of molds, yeast and Alicyclobacillus spp. from a concentrated apple juice productive process. *Brazilian Journal of Microbiology, 45*, 49-58. https://doi.org/10.1590/S1517-83822014000100008

Sánchez-Moreno, C., Plaza, L., De Ancos, B., & Cano, M. P. (2003). Effect of high-pressure processing on health-promoting attributes of freshly squeezed orange juice (*Citrus sinensis* L.) during chilled storage. *European Food Research and Technology*, *216*, 18-22. https://doi.org/10.1007/s00217-002-0625-8

Sánchez-Moreno, C., Plaza, L., Elez-Martínez, P., De Ancos, B., Martín-Belloso, O., & Cano, M. P. (2005). Impact of high pressure and pulsed electric fields on bioactive compounds and antioxidant activity of orange juice in comparison with traditional thermal processing. *Journal of Agriculture and Food Chemistry*, *53*, 4403-4409. https://doi.org/10.1021/jf048839b

Santos, J., Bispo, V.S., Filho, A.B.C., Pinto, I.F.D., Dantas, L.S., Vasconcelos, D.F., ... Matos, H.R. (2013). Evaluation of Chemical Constituents and Antioxidant Activity of Coconut Water (*Cocus nucifera* L.) and Caffeic Acid in Cell Culture. *Annals da Academia Brasileira de Ciências, 85*, 1235-1246. https://doi.org/10.1590/0001-37652012105312

Schieber, A., & Carle, R. (2005). Occurrence of carotenoid cis-isomers in food: technological, analytical, and nutritional implications. *Trends in Food Science & Technology, 16*, 416-422. https://doi.org/10.1016/j.tifs.2005.03.018

Silva, J. M., Rigo, A. A, Dalmolin, I. A., Debien, I., Cansian, R. L., Oliveira, J. V., & Mazutti, M. A. (2013). Effect of pressure, depressurization rate and pressure cycling on the inactivation of *Escherichia coli* by supercritical carbon dioxide. *Food Control*, *29*, 76-81. https://doi.org/10.1016/j.foodcont.2012.05.068

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, *16,* 144-158. http://www.ajevonline.org/content/16/3/144

Smit, Y., Cameron, M., Venter, P., & Witthuhn, R. C. (2011). *Alicyclobacillus* spoilage and isolation — a review. *Food Microbiology*, *28*, 331-349. https://doi.org/10.1016/j.fm.2010.11.008

Spilimbergo, S., & Bertucco, A. (2003). Non-thermal bacteria inactivation with dense CO2. *Biotechnology and Bioengineering*, *84*, 627-638. https://doi.org/10.1002/bit.10783

Tan, T., Cheng, L., Bhat, R., Rusul, G., & Easa, A. M. (2014). Composition, physicochemical properties and thermal inactivation kinetics of polyphenol oxidase and peroxidise from coconut (*Cocos nucifera*) water

obtained from immature, mature and overly-mature coconut. *Food Chemistry*, *142*, 121-128. https://doi.org/10.1016/j.foodchem.2013.07.040

U.S. Department of Health and Human Services and World Health Organization. (2011). Global health and aging. NIH publication number 11-7737.

U.S. Food and Drug Administration. (2001a). United States Food and Drug Administration: 21 Code of Federal Regulations Part 172.133. Dimethyl dicarbonate.

U.S. Food and Drug Administration. (2001b). Report from Institute of Food Technologists. Evaluation and Definition of Potentially Hazardous Foods.

U.S. Food and Drug Administration. (2016). Guidance for Industry: Juice HACCP Hazards and Controls Guidance First Edition; Final Guidance.

Walter, E. H. M., Kabuki, D. Y., Esper, L. M. R., Sant'Ana, A. S., & Kuaye, A. Y. (2009). Modelling the growth of *Listeria monocytogenes* in fresh green coconut (*Cocos nucifera* L.) water. *Food Microbiology*, *26*, 653-657. https://doi.org/10.1016/j.fm.2009.04.003

Wang, C., Chen, Y., Xu, Y., Wu, J., Xiao, G., Zhang, Y., & Liu, Z. (20130. Effect of dimethyl dicarbonate as disinfectant on the quality of fresh-cut carrot (*Daucus carota* L.). *Journal of Food Processing and Preservation*, *37*, 751-758. https://doi.org/10.1111/j.1745-4549.2012.00718.x

Xu, Z., Zhang, L., Wang, Y., Bi, X., Buckow, R., & Liao, X. (2011). Effects of high pressure CO2 treatments on microflora, enzymes and some quality attributes of apple juice. *Journal of Food Engineering*, *104*, 577-584. https://doi.org/10.1016/j.jfoodeng.2011.01.020

Yong, W. J. W. H., Ge, L., Ng, Y. F., & Tan, S. N. (2009). The chemical composition and biological properties of coconut (*Cocos nucifera* L.). *Molecules*, *14*, 5144-5164. https://doi.org/10.3390/molecules14125144

Yu, Y., Wu, J., Xiao, G., Xu, Y., Tang, D., Chen, Y., & Zhang Y. (2013). Combined effect of dimethyl dicarbonate (DMDC) and nisin on indigenous microorganisms of litchi juice and its microbial shelf life. *Journal of Food Science*, *78*, 1236-1241. https://doi.org/10.1111/1750-3841.12215

Yu, Y., Xiao, G., Xu, Y., Wu, J., & Wen, J. (2014). Effects of Dimethyl Dicarbonate (DMDC) on the Fermentation of Litchi Juice by *Lactobacillus casei* as an Alternative of Heat Treatment. *Journal of Food Science*, *79*, 947-954. https://doi.org/10.1111/1750-3841.12428

Zhou, L., Wang, W., Hu, X., Wu, J., & Liao, X. (2009). Effect of high pressure carbon dioxide on the quality of carrot juice. *Innovattive Food Science & Emerging Technology*, *10*, 321-327. https://doi.org/10.1016/j.fset.2009.01.002

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Effectual Gold Nanoprobe Sensor for Screening Horse Adulteration in Meat Products

Dimitra Panagiotis Houhoula[1], Meropi Kouzilou[1], Constantine Tzogias[1], Vassiliki Kyrana[1], Constantine Sflomos[1], John Tsaknis[1] & Vladimiros Pavlos Lougovois[1]

[1]Department of Food Technology, Technological Educational Institution of Athens, Greece

Correspondence: Dimitra Panagiotis Houhoula, Department of Food Technology, Technological Educational Institution of Athens, Greece. E-mail: itsahouhoula@gmail.com

Received: May 3, 2017 Accepted: May 24, 2017 Online Published: June 4, 2017

doi:10.5539/jfr.v6n4p34 URL: https://doi.org/10.5539/jfr.v6n4p34

Abstract

A gold nanoparticle (AuNP) probe strategy for testing meat authenticity was developed, which relies on the colorimetric differentiation of a particular DNA sequence, due to the differential aggregation profiles exhibited by the AuNPs in the presence or absence of specific target hybridization. Gold nanoparticles were conjugated with thiolated oligonucleotides for specifically identifying a 69 bp fragment of the horse *cytochrome b* gene. In the presence of a complementary target preventing aggregation of the AuNPs when acid was added, the reaction mixtures retained the original pink colouration of the colloidal particles, whereas they turned purple in the opposite event. Fresh meatballs, prepared using pure bovine meat, were used as blanks, producing a purplish coloured solution with a peak at ≥570nm. Horse meat was used as positive control and the pink colour obtained after hybridization exhibited maximum absorption at 524 nm. Both the specificity and sensitivity of the tests performed were 100%. Visual observations and spectroscopic data indicated that the coloration produced by the AuNPs (positive-pink, negative-purple) was very stable, showing no change under normal laboratory conditions. The use of AuNPs for the colorimetric detection of DNA targets from undeclared species in meat products provides an inexpensive and easy-to-perform alternative to common molecular assays. The technology described here can be further developed to accommodate detection of many cases of adulteration and fraudulent practices.

Keywords: Gold nanoparticles, food authentication, horse meat adulteration

1. Introduction

Fraudulent practices involving substitution of high-priced meats by lower commercial value species or undeclared non-meat ingredients are becoming a serious problem in the meat processing sector. Although generally sporadic, these practices seem to have escalated in recent years, challenging consumers' confidence in the integrity and safety of the meat supply chain, as well as impacting purchasing and eating behaviour (Barnett et al., 2016; Stanciul, Stanciul, Dimistrascu, Ion, & Nistor, 2013). Ground meat products and precooked, ready-to-eat meat items seem to be more prone to adulteration, due to the lack of external diagnostic traits for species identification. Several factors appear to contribute to this growing phenomenon, including economical gain, increasing diversity of products, complexity of international food trading networks, deficiencies in traceability systems for ethnic foodstuffs, as well as lack of effective methods to identify meat sources, particularly in thermally processed products (Rahmati, Julkapli, Yehye, & Basirun, 2016; Armani et al., 2015; Ercsey-Ravasz, Toroczkai, Lakner, & Baranyi, 2012). Regardless of the motive or the level at which adulteration takes place, the repercussions of this act are manifold, far exceeding economic fraud. A multitude of practical, ethical, religious, and safety issues may be introduced as a result of fraudulent meat substitution in the processing and merchandising chain (Iammarino, Marino, & Albenzio, 2017; Rahmati et al., 2016; Ali, Razzak, & Hamid, 2014). Combating mislabelling requires that sensitive and inexpensive diagnostic tests for rapidly identifying meat species are developed and integrated within sector-specific and generic traceability systems. Most of the methods currently in use for monitoring food authenticity are based on the detection of species-specific proteins and DNA analyses. Molecular methodologies are especially useful in food authentication testing, thanks to the ubiquitous presence of DNA molecules in all biological tissues, and their stability under the production and processing operations applied along the food-chain (Asensio, González, García, & Martin, 2008; Taylor et al., 2012). DNA-hybridization techniques used for identifying sequences in the

genomic DNA provide a far more effective approach in authentication tests (Rahmati et al., 2016). Thus, Chen, Wei, Chen, Zhao, & Yang (2015) developed a duplex PCR assay for the identification of horse, donkey and mule species in raw and heat-processed meat products, based on the amplification of a fragment of the mitochondrial DNA. According to the authors, the target meat species could be detected at a level of 1%. Similarly, Ali et al. (2015) and Ilhak and Arslan (2007) were able to determine the origin of several meat species (beef, sheep, pork, goat, horse, cat, dog, monkey, and rat) by multiplex PCR assays, using amplified species-specific fragments of the mitochondrial and *cytochrome b* genes. Currently, a new challenge in the identification of meat origin in foods has emerged, which involves the development of specific nanoparticle-based probes, capable of detecting several-fold shorter DNA target sequences found in highly processed products (Ali et al., 2014). Designing DNA hybridization detection assays that do not depend on target-amplification by the PCR reaction offers higher flexibility and greater multiplexing capabilities, as well as ignoring the requirement for time-consuming electrophoresis that sometimes needs self-authentication by RFLP-analysis, sequencing, or blotting (Hill & Mirkin, 2006). Therefore, the development of a simplified, cost-effective and accurate procedure for detection of trace amounts of fraud, not requiring complex instrumentation, seem to be imperious in order to overcome the time delay and allow rapid and sensitive detection. Nanotechnology and, more specifically, gold nanoparticle chemistry provides revolutionary opportunities for the rapid and simple diagnosis of authenticity, being able to detect trace amounts of fraud, due to their unique optical properties. Gold nanoparticles have been used successfully as colorimetric sensors for visually identifying pork adulteration in beef and chicken meatball preparations (Ali, Hashim, Mustafa, Che Man, & Islam, 2012). The aim of the present study was to design and construct functionalized gold nanoparticles (AUNPs) that could be incorporated into an easily applicable DNA detection methodology for the identification of horse meat adulteration in meat products.

2. Method

2.1 Meat Products and Controls

A total of 40 meat items were collected from local super markets and fast food restaurants in the area of Athens. The sampled products included fresh meat cuts (5 items), slices of roasted beef and pork (14 items), ready to eat meatballs (8 items), and country style sausages (13 items). Fresh meatballs prepared in the laboratory using pure bovine meat were used as blanks, whereas horse meat was used as positive control. Bovine meatballs containing different levels (0.1-50%, w/w) of horse meat were also analyzed, in order to determine the assay's detection limit of horse meat adulteration.

2.2 Preparation of Gold Nanoparticle Probes

Twenty nm gold colloid nanoparticles (AuNPs) were purchased from BBI Solutions (Cardiff, UK). The oligonucleotides specified sense GAA GCA TAA TAT TCC GG and Antisense primer TTA GTG TCA GTA AGT CTG CC were used (specific for the identification of the *cytochrome b* gene). All oligonucleotides were thiolated (modified with 10 dATP in the 5' end of the primer). The AuNP merging with the oligonucleotides was performed by adding 1 ml of an aqueous solution of AuNPs to 4 nmol of the thiolated oligonucleotides, specific for the *cytochrome b* gene, using a previously described protocol (Li & Rothberg, 2004). Briefly, the thiol modified oligonucleotides were initially incubated with the AuNPs overnight, using an orbital shaker at room temperature. The solution was then brought to 9 mM phosphate buffer (pH 7) and SDS solution (0.1%, w/v) was added to prevent aggregation. The total volume of salting buffer (2 M NaCl in 10 mM PBS) required to bring the AuNPs solution to a final concentration of 0.3 M NaCl was divided into six doses that were added over the next 48 hours. After centrifugation, the precipitate was washed with 500 μl of 10 mM PBS (pH 7.4), 150 mM NaCl, and 0.1% SDS, followed by centrifugation and re-suspension in 500 ml of the same buffer. The gold nanoprobes were placed in glass vials and stored in the dark, at room temperature (Hill & Mirkin, 2006).

2.3 DNA Isolation

DNA extraction from horse meat (designated positive control) and commercial meat products was performed using the NucleoSpin Food kit (Macherey-Nagel, GmbH & Co. KG, Germany), according to the manufacturer's instructions. The extracted DNA was quantified spectrophotometrically at 260 nm, and serial tenfold dilutions were prepared (C_1=1,23 ng/μL, C_2=123 pg/μL, C_3=12,3 pg/μL, C_4=1,23 pg/μL, KC_5=123 fg/μL, KC_6=12,3 fg/μL) for evaluating the analytical sensitivity of the DNA extraction method. DNA samples from bovine and pork meat were used for specificity confirmation (designated specificity negative controls).

2.4 PCR Amplification

PCR was performed according to a previously published protocol (Chisholm, Conyers, Booth, Lawley, & Hird, 2005) in 50 μl final volume solutions, using the GoTaq Hot Start Master Mix (Promega Gmbh, Mannheim,

68199, Germany), 1 mM each of the primers Forward: GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA and Reverse: CTC AGA TTC ACT CGA CGA GGG TAG TA amplifying a 439 bp gene, and 10 µl of eluted DNA. PCR products were separated in 2% agarose gel, stained with ethidium bromide (0.5 µg/ml) and documented under UV illumination.

2.5 Direct Hybridization and Colour Detection in Food Samples

In order to obtain an indication of the method's performance on food samples, the optimized assay was applied for detecting horse meat adulteration in the food samples referred to above. For the detection of horse meat, 15 µl of the DNA extracted from the food were added to 10 µl of the AuNP-oligonucleotide solution, followed by two incubation steps, one denaturation step at 95°C for 5 min, and one annealing step at 55°C for 5 min. The presence of complementary DNA prevented aggregation of the nanoprobes when 2 µl 0.1 N HCl were added in the reaction mixture, followed by incubation for 5 min at room temperature, so that the solution remained pink; in the opposite event (no presence of complementary DNA), the reaction mixture turned purple. The colour change could be detected visually and it was further confirmed by absorption spectra. The solutions were photographed after standing for 5–15 min at room temperature for full colour development. All experiments were performed in triplicate and the results were compared with those obtained by PCR assays. To assess the repeatability of the method for the specific types of samples, testing with the proposed assay was repeated three times for each DNA extract.

3. Results and Discussion

Spherical gold nanoparticles in the size range of 13–20 nm, showing absorbance peak at around 520 nm, have been employed in biosensors due to ease of synthesis. The AuNP-oligonucleotide solution exhibits a pink colour because of surface plasmon resonance at an absorbance peak of ~525 nm. In the absence of the specific DNA target, the addition of HCl enhances aggregation of the AuNP-oligonucleotide probes, leading to a change in colour from pink to purple. The visually detected changes are strongly supported by the remarkable features in UV-Vis spectrum (Li & Rothberg, 2004). Using horse meat (positive control), the proposed assay produced positive results, with the sensitivity being 100%. Serial 10-fold dilutions of the positive control DNA, starting from 1.23 ng/µL (C_1), indicated that the lower detection limit (LOD) of the assay was 12.3 fg/µL of DNA, as compared to the 123 fg/µL LOD of the PCR method, thus indicating that the AuNP-oligonucleotide assay performed better results than the PCR in the range of one 10-fold dilution. PCR-based methods need comparatively longer targets, which are known to break down during chemical and physical stresses induced by food processing, causing template crisis in the PCR assay (Ali et al., 2011). The UV-Vis spectroscopic data obtained supported the hypothesis of aggregation-induced visual discrimination of the samples. As shown in Figure 1, samples containing horse genomic DNA exhibited the characteristic absorbance peak of the AuNPs at 520 nm, caused by the collective excitation of the free conduction band electrons of the dispersed particles, known as the surface plasmon resonance. On the other hand, the wide absorbance spectrum exhibited by negative (bovine and/or pork meat) samples is indicative of the peak shift towards longer wavelengths (≥ 550nm), due to the coupling in the surface plasmons of the particles in the aggregates (Figure 2). Thus, the collective plasmon peak was intensified and appeared in a new position, between 560 and 800 nm, depending on the degree of aggregation and concentration of the AuNP-oligonucleotides. The specificity of the assay was 100% (true negative rate).

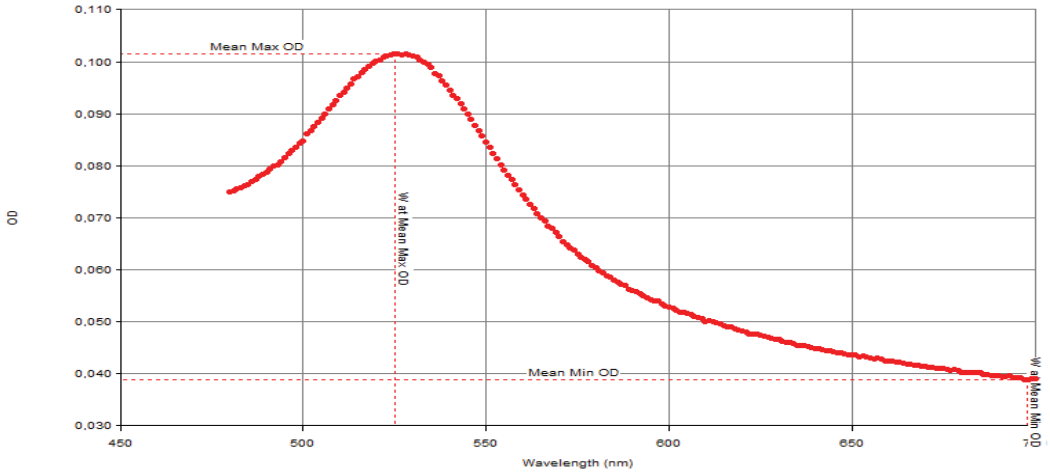


Figure 1. UV-Vis spectrum from sample containing horse genomic DNA, showing characteristic absorbance peak of AuNP at 520 nm

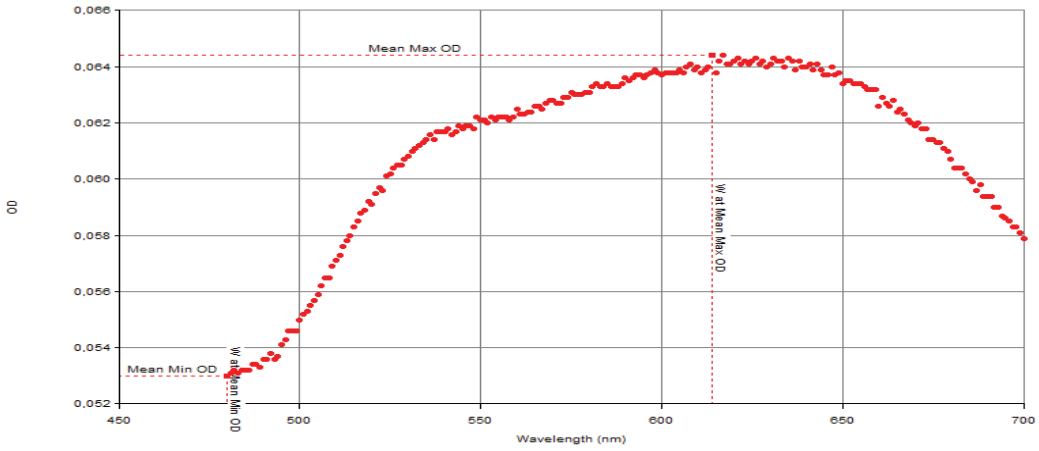


Figure 2. Negative sample exhibiting wide absorbance spectrum longer wavelength (≥600nm)

The method was repeatable, producing identical results all three times it was performed. Interestingly enough, when left overnight at room temperature, positive samples retained their colour, indicating long time stability of the AuNP-oligonucleotide probe hybridization with the target sequence. This feature can be particularly useful when prolonged read-out capability is required for high-throughput applications. The colouration exhibited by AuNP-oligonucleotides obtained from bovine meatballs containing different levels (0.1-50%) of horse meat, is shown in Figure 3. It was clear from visual observations and spectroscopic data that the colloidal particles retained their original coloration practically unaffected, regardless of the horse meat level. By contrast, the pinkish-red colour of the AuNP-oligonucleotides turned into purple, when only beef was present, indicating aggregation of the AuNP-oligonucleotide probes. This was confirmed by the appearance of a collective plasmon peak near 535 nm and a considerably stronger absorption between 550 and 650 nm. Of the 40 items tested in this study, 16 (40%) were positive by the AuNP-oligonucleotide assay; the horse meat gene was detected in 6 out of the 8 (75%) meatball items, as well as in 10 out of the 13 (77%) country style sausages. The sensitivity of the assay was 100% (true positive samples). When using the PCR assay, only 10 out of the 40 items (25%) were positive. The sensitivity of the PCR assay was 62.5%, probably due to the inefficiency of the method when applied to highly degraded samples.

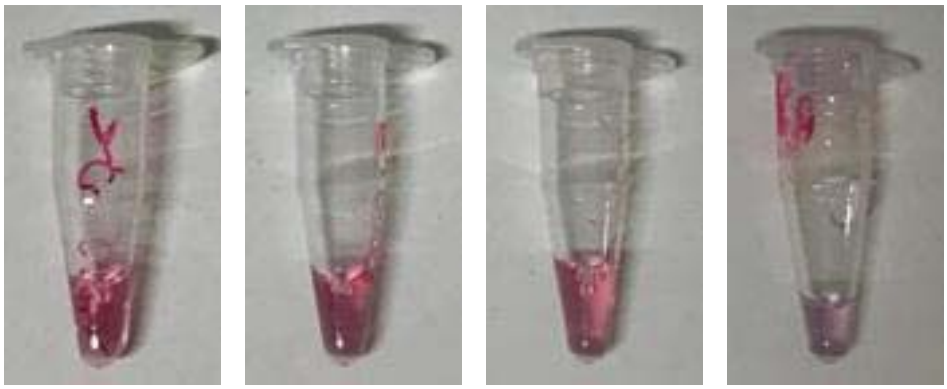


Figure 3. Horse-beef mixtures in 0.1: 99.9, 10: 90 and 50: 50 ratios (w/w), along with a negative control (100% beef)

Acknowledgments

The present work was financed by the Special Research Fund Account of the Technological Educational Institution (TEI) of Athens (Project No. 80251/2015).

References

Ali, M. E., Hashim, U., Mustafa, S., Che Man, Y. B., Adam, T., & Humayun, Q. (2014). Nanobiosensor for the detection and quantification of pork adulteration in meatball formulation. *Journal of Experimental Nanoscience*, *9*, 152-160. https://doi.org/10.1080/17458080.2011.640946

Ali, M. E., Hashim, U., Mustafa, S., Che Man, Y. B., & Islam, Kh, N. (2012). Gold Nanoparticle Sensor for the Visual Detection of pork adulteration in meatball formulation. *Journal of Nanomaterials,* 7. https://doi.org/10.1155/2012/103607

Ali, M. E., Hashim, U., Mustafa, S., Che Man, Y. B., Yusop, M. H. M., Bari, M. F., ... Hasan, M. F. (2011). Nanoparticle sensor for label free detection of swine DNA in mixed biological samples. *Nanotechnology*, *22*(19), 8. https://doi.org/10.1088/0957-4484/22/19/195503

Ali, M. E., Razzak, M. A., & Hamid, S. B. A. (2014). Multiplex PCR in species authentication: probability and prospects – a review. *Food Analytical Methods*, *7*, 1933-1949. https://doi.org/10.1007/s12161-014-9844-4

Ali, M. E., Razzak, M. A., Hamid, S. B. A., Rahman, M. M., Al Amin, M., & Rashid, N. R. A. (2015). Multiplex PCR assay for the detection of five meat species forbidden in Islamic foods. *Food Chemistry*, *177*, 214-224. https://doi.org/10.1016/j.foodchem.2014.12.098

Armani, A., Guardone, L., La Castellana, R., Gianfaldoni, D., Guidi, A., & Castigliego, L. (2015). DNA barcoding reveals commercial and health issues in ethnic seafood sold on the Italian market. *Food Control*, *55*, 206-214. https://doi.org/10.1016/j.foodcont.2015.02.030

Asensio, L., González, I., García, T., & Martin, R. (2008). Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA). *Food Control*, *19*, 1-8. https://dx.doi.org/10.1016/j.foodcont.2007.02.010

Barnett, J., Begen, F., Howes, S., Regan, A., McConnon, A., Marcu, A., … Verbeke, W. (2016). Consumers' confidence, reflections and response strategies following the horsemeat incident. *Food Control*, *59*, 721-730. https://doi.org/10.1016/j.foodcont.2015.06.021

Chen, A., Wei, C., Chen, G., Zhao, Y., & Yang, S. (2015). Duplex PCR approach for the detection and quantification of donkey, horse and mule in raw and heat-processed meat products. *International Journal of Food Science and Technology*, *50*, 834-839. https://doi.org/10.1111/ijfs.12720

Chisholm, J., Conyers, C., Booth, C., Lawley, W., & Hird, H. (2005). The detection of horse and donkey using real-time PCR. *Meat Science*, *70*, 727-732. https://dx.doi.org/10.1016/j.meatsci.2005.03.009

Ercsey-Ravasz, M., Toroczkai, Z., Lakner, Z., & Baranyi, J. (2012). Complexity of the international agro-food trade network and its impact on food safety. *PLoS One, 7*(5), e37810. https://dx.doi.org/10.1371/journal.pone.0037810

Hill, H. D., & Mirkin, C. A. (2006). The bio-barcode assay for the detection of protein and nucleic acid targets

using DTT-induced ligand exchange. *Nature Protocols*, *1*(1), 324-336. https://doi.org/10.1038/nprot.2006.51

Iammarino, M., Marino, R., & Albenzio, M. (2017). How meaty? Detection and quantification of adulterants, foreign proteins and food additives in meat products. *International Journal of Food Science and Technology*, *52*, 851-863. https://doi.org/10.1111/ijfs.13350

Ilhak, O., & Arslan, A. (2007). Identification of meat species by Polymerase Chain Reaction (PCR) technique. *Turkish Journal of Veterinary and Animal Sciences*, *31*(3), 159-163.

Li, H., & Rothberg, L. J. (2004). Label-free colorimetric detection of specific sequences in genomic DNA amplified by the polymerase chain reaction. *Journal of the American Chemical Society*, *126*(35), 10958-10961. https://doi.org/10.1021/ja048749n

Rahmati, S., Julkapli, N. M., Yehye, W. A., & Basirun, W. J. (2016). Identification of meat origin in food products – A review. *Food Control*, *68*, 379-390. https://doi.org/10.1016/j.foodcont.2016.04.013

Stanciu, S., Stanciuc, N., Dimistrascu, L., Ion, R., & Nistor, C. (2013). The effects of horse meat scandal on Romanian meat market. *Cross-Cultural Management Journal*, *XV*; *SEA Practical Application of Science*, *1*(1), 174-181.

Taylor, A. W., Coveney, J., Ward, P. R., Dal Grande, E., Mamerow, L., Henderson, J., & Meyer, S. B. (2012). The Australian Food and Trust Survey: Demographic indicators associated with food safety and quality concerns. *Food Control*, *25*, 476-483. https://doi.org/10.1016/j.foodcont.2011.11.003

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Consumer Sensory Acceptance of Standard Pre-cooked Hamburger Patties versus Premium Patties

Peter L. Bordi[1], Hyojin C. Cho[1], & Jessica M. MacMartin[1]

[1]Center for Food Innovation, School of Hospitality Management, The Pennsylvania State University, 124 Mateer Building, University Park, Pennsylvania, 16802 USA

Correspondence: Peter L. Bordi, Center for Food Innovation, School of Hospitality Management, The Pennsylvania State University, 124 Mateer Building, University Park, Pennsylvania, 16802, USA. Tel: 814-863-3579. E-mail: plbjr@psu.edu

Received: April 6, 2017 Accepted: May 13, 2017 Online Published: June 7, 2017

doi:10.5539/jfr.v6n4p40 URL: https://doi.org/10.5539/jfr.v6n4p40

Abstract

Consumers' increasing concerns toward nutrition, health, and sustainable food have influence food industry. Practitioners in the meat product industry and retailers are focusing on premium labeled meat products, such as Certified Angus Beef and grass-fed beef, to meet consumers' demand. Although many consumers assume the premium has better taste and texture, there is little research comparing the sensory attributes of the premium and non-premium burgers. This study compared the sensory attributes of three different hamburger patties: flame broiled pre-cooked beef (non-premium, standard patties), Angus beef, and grass-fed beef patties (premium patties). The results show that participants prefer pre-cooked hamburger patties significantly than Angus and grass-fed patties in initial taste and flavor. Also, this pre-cooked hamburger patties are significantly preferred compared to grass-fed patties in overall quality and overall liking attributes. Other sensory attributes, such as appearance, texture, juiciness, and seasoning, show no significant difference among three different patties. This indicates that the pre-cooked hamburger patties can be preferred than (or compatible to) Angus or grass-fed patties.

Keywords: sensory acceptance, hamburger patties, pre-cooked, premium, Angus, grass-fed

1. Introduction

Burgers have been and continue to be a staple of the American diet. A foodservice market researchers estimated that more than 9 billion burgers were sold in the United States to consumers at restaurants and other foodservice outlets in 2014 (McLynn, 2015). At casual dining restaurants, consumers choose burgers over higher priced beef entrees (McLynn, 2015). There are numerous choices of burgers given the different combinations of hamburger patties, toppings and sauces. More recently, consumers' increasing concerns over nutrition and health have focused attention on the patties itself: consumers are questioning the breed, origin, and diet and raising of the cattle from which their burgers are made. (Caldwell, 2014).

This relatively recent curiosity has arisen in the wake of larger cultural concerns about the food we eat and how we treat the animals who provide it. The meat industry must be prepared to deal with consumer needs for ethical decision making when it comes to diet. As part of the response toward consumers' concerns about health and sustainability, meat industry principals and their retail customers are focusing on premium labeled meat products (Weber, Heinze, & DeSoucey, 2008).

One important variety of premium beef is Angus. Angus is a Scottish breed, formerly called Aberdeen Angus. Angus cattle are the one of most common breeds in America because of their high ratio of muscle-to-carcass weight and distinctive marbling (Beam, 2009). The brand "Certified Angus Beef" is not synonymous with Angus beef. Certified Angus beef must meet certain United States Department of Agriculture (USDA) criteria including phenotype or genotype, as well as marbling, rib eye area, hot carcass weight, fat thickness and more (USDA, 2014). Less than 8% of all beef sold in the United States earns this premium name (Chang, 2009). Retail prices for Certified Angus Beef are approximately 10-15% higher than non-Angus USDA choice-graded beef. (Chang, 2009).

Grass-fed beef also commands a premium. "Grass-fed" was first used as a technical term to describe meat that

had not followed standard feeding practice – i.e. fattening cattle with corns or grain. Once considered inferior, because it didn't follow the standard feeding process, grass-fed meat has been perceptually re-positioned so it's now considered to a high-end, premium beef by consumers and retailers with the increase of health and sustainable food consumption. Grass-fed beef is known to have a different nutrient composition than corn- or grain-fed beef, specifically high levels of 18:3 α-linoleic acids and n-3 long-chain poly unsaturated fatty acids. This is reflected in the relative price: As of October 2016, the average price for a pound of grass-fed beef patties is $10.27 (USDA, 2016a). For corn- or grain-fed beef, approximately $5.63 per pound (USDA, 2016b).Some consumers pay a premium for the "grassy" flavor resulting from the difference in feeding practices (Wood, Richardson, Nute, Fisher, Campo, Kasapidou, Sheard, Enser, 2004).

Many fast food chains, family restaurants, and convenience stores continue to use non-premium, pre-cooked, corn-fed hamburger patties. Since the patties are pre-cooked, they are easily and quickly prepared, to further reduce cook time and labor costs, some food services now use rapid-cooking ovens. A rapid-cooking oven uses a combination of convection and microwave technologies to reduce cook time by 30% or more (Moyer, 2005).

Although the premium hamburger patties (e.g. Angus, grass-fed) have captured consumers' attention, there is little research comparing the sensory attributes of premium and non-premium burgers. The present study investigates if the demand for premium hamburgers and specialty beef is based on improved sensory attributes compared to non-premium hamburger patties. This research aims to discover if Angus beef hamburgers, grass-fed hamburgers and pre-cooked regular hamburgers possess specific desirable sensory attributes, and if significant differences in consumer preference among the three types of patties exists.

2. Method

This study compared three different hamburger patties: flame broiled pre-cooked patties, Angus patties and grass-fed patties. We sought to determine whether the flame broiled pre-cooked patties (standard pre-cooked patties) would hold its own against the two premium competitors (Angus and grass-fed patties). This study offered a half portions of all three burgers to each participant. Participants rated each burger on a variety of sensory attributes, as summarized in Table 2.

2.1 Equipment

The pre-cooked patties were prepared using a rapid-cook oven, the TurboChef Tornado (TurboChef, Carrollton, TX, United States). The Tornado is able to reach temperatures of 422° Celsius (792° Fahrenheit) to cook the patties completely in a short amount of time (Turbochef Tornado, 2013). Suggested cooking times can be found in the TurboChef Tornado manual. Frozen pre-cooked burgers can be prepared in the oven in one minute and 25 seconds for one patty, or one minute and 45 seconds for two patties prepared at the same time. If the pre-cooked burgers are thawed, they can be placed in the TurboChef oven for 40 seconds for one patty, or one minute for two patties.

Raw burgers were cooked on a standard Vulcan char-broiler grill (model number HMCB34-SEFACV, Baltimore, MD, United States). The Angus and the grass-fed patties were placed onto a hot grill and cooked for three minutes on each side. All patties were checked to ensure they reached 74°C (165°F).

2.2 Materials

All hamburger patties were originally frozen, then defrosted one night prior to the study. The pre-cooked flame-broiled patties weighed 4 ounces and had the following ingredients: beef, seasoning salt (salt, dextrose, natural flavors, and spice), and natural flavoring. The Angus patties were 1" thick, 12 ounce raw frozen 100% Certified Angus Beef ground chuck patties. The grass-fed patties were ¾" thick, 8 ounce frozen 100% natural Angus patties. Both the Angus and the grass-fed patties decreased in weight and size when grilled. The reduction of weight in an 80% lean raw burger when grilled is approximately 31% (U.S. Department of Agriculture, 2012), resulting in an 8.28 ounce Angus burger and 5.52 ounce grass-fed burger.

2.3 Participants

50 untrained panelists were chosen to participate in this research from a listserv at a large university in the northeast. Untrained panelists are ideal for rating preferences amongst hedonic attributes (Lawless & Heymann, 2010). A majority of the participants were male (60%) and between the ages of 25-34 (56%). Age breakdowns are offered in Table 1.

Table 1. Age groups of participants

Age Group	Frequency	Percent
18-24	9	18.0%
25-34	28	56.0%
35-45	13	26.0%
Total	50	100%

2.4 Methods

Hamburgers were prepared as described above, either in a TurboChef oven (pre-cooked burger patties) or on a grill (Angus and grass-fed patties). Researchers prepared the burgers, put them on buns, cut them in half and served them without any condiments or vegetables. Each participant received a tray with one-half of each hamburger and a cup of water to cleanse their palate before starting the test and in between each sample. Ten panelists were able to test at a time in separate booths, answering questions about each individual hamburger on a computer. Participants assessed the following attributes: appearance, initial taste, flavor, texture, juiciness, liking of seasoning, overall quality, and overall liking. Participants were requested to evaluate each hamburger attribute on a 9-point hedonic scale from dislike extremely to like extremely. Participants were also asked to rank the three hamburger types in order from their favorite to least favorite.

3. Results

A one-way within subjects analysis of variance (ANOVA) was conducted to verify the difference of sensory attributes three different hamburger patties; pre-cooked, Angus, and grass-fed. Pre-cooked patties are significantly different from Angus and grass-fed patties in initial taste ($M_{pre\text{-}cooked}$=6.36, M_{angus}=5.48, $M_{grass\text{-}fed}$=5.42, F=5.17, p<.01) and in flavor ($M_{pre\text{-}cooked}$=6.28, M_{angus}=5.36, $M_{grass\text{-}fed}$=5.32, F=6.53, p<.01). Pre-cooked and Angus patties are significantly different from grass-fed patties in overall quality ($M_{pre\text{-}cooked}$=6.18, M_{angus}=5.56, $M_{grass\text{-}fed}$=5.32, F=3.94, p<.05) and overall liking ($M_{Pre\text{-}cooked}$=6.28, M_{angus}=5.48, $M_{grass\text{-}fed}$=5.26, F=5.26, p<.01). However, there is no significant differences in three patties in liking of appearance ($M_{pre\text{-}cooked}$=5.62, M_{angus}=5.66, $M_{grass\text{-}fed}$=5.38, F=.57, p=.57), texture ($M_{Pre\text{-}cooked}$=5.84, M_{angus}=5.38, $M_{grass\text{-}fed}$=5.20, F=1.86, p=.17), juiciness ($M_{Pre\text{-}cooked}$=6.16, M_{angus}=5.78, $M_{grass\text{-}fed}$=5.88, F=.97, p=.38), and seasoning ($M_{pre\text{-}cooked}$=5.94, M_{angus}=5.38, $M_{grass\text{-}fed}$=5.22, F=2.89, p=.064). The summary of sensory attribute data can be found in Table 2.

Table 2. Attribute† Mean Comparison among Pre-cooked, Angus, and grass-fed Hamburger Patties

	pre-cooked *M (SD)*	Angus *M (SD)*	grass-fed *M (SD)*	F	p-value
Appearance	5.62 [a] (1.63)	5.66 [a] (1.59)	5.38 [a] (1.60)	.568	.567
Initial Taste	6.36 [a] (1.52)	5.48 [b] (1.54)	5.42 [b] (1.81)	5.173	.009*
Flavor	6.28 [a] (1.36)	5.36 [b] (1.40)	5.32 [b] (1.87)	6.534	.002*
Texture	5.84 [a] (1.72)	5.38 [a] (1.68)	5.20 [a] (1.88)	1.855	.167
Juiciness	6.16 [a] (1.57)	5.78 [a] (1.53)	5.88 [a] (1.71)	.969	.381
Seasoning	5.94 [a] (1.66)	5.38 [a] (1.48)	5.22 [a] (1.79)	2.89	.064
Overall Quality	6.18 [a] (1.49)	5.56[a] (1.64)	5.32[b] (1.93)	3.939	.026*
Overall Liking	6.28 [a] (1.46)	5.48[a] (1.68)	5.26[b] (1.94)	5.258	.009*

† Nine-point hedonic scale: 1=dislike extremely, 2=dislike very much, 3=dislike moderately, 4=dislike slightly, 5=neither like nor dislike, 6=like slightly, 7=like moderately, 8=like very much, 9=like extremely

* $p < 0.05$

[ab] Means within a row with different superscripts are significantly different ($p < 0.05$)

4. Discussion/Conclusion

This study investigated the differences in sensory acceptance among three different hamburger patties; pre-cooked, Angus, and grass-fed. The results show that participants preferred pre-cooked hamburger patties to Angus and grass-fed patties in: initial taste, flavor, overall quality and overall liking. Other sensory attributes, such as appearance, texture, juiciness, and seasoning, show no significant difference among the three types of patties. This indicates that the pre-cooked hamburger patties cooked in a rapid-cook oven are preferred more than or equally to Angus or grass-fed patties cooked on a conventional grill. These results show that consumers may not recognize (or care about) the superior sensory attributes of the premium burger patties.

5. Limitation/Future Studies

Since this study focuses on three different hamburger patties used in restaurants, the preparation and cooking conditions were designed to resemble the industry's general practice. The pre-cooked patties currently used in food industry are mostly seasoned while premium raw burger patties are not seasoned. Thus this study used seasoned pre-cooked patties and unseasoned premium burger patties. Also, a rapid-cook oven was used for cooking the pre-cooked patties, while a conventional grill was used for cooking the raw premium patties. Since the cooking mirrored industry practice, this study result would be helpful for practitioners. However, for more accurate comparisons, future researchers should control the material and cooking procedure differences.

Funding Sources/Conflict of Interest

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

References

Beam, C. (2009, October 7). Holy Cow: What's So Good About Angus Beef? Slate, Retrieved from http://www.slate.com/articles/news_and_politics/explainer/2009/10/holy_cow.html

Caldwell, M. (2014). The rise of the gourmet hamburger. *Contexts, 13*, 72-74. https://doi.org/10.1177/1536504214545765

Chang, A. (2009). Angus beef can be found in your local grocery store and many fast food chains, but is it worth the higher cost? The Street. Retrieved from https://www.thestreet.com/story/12803768/1/should-you-pay-extra-angus-beef.html

Lawless, H. T., & Heymann, H. (2010). Sensory evaluation of food: Principles and practices (2nd ed.). New York: Springer. https://doi.org/10.1007/978-1-4419-6488-5

McLynn, K. (2015, January 27). Restaurant Burgers Had A Banner Year in 2014, Reports NPD. Retrieved from https://www.npd.com/wps/portal/npd/us/news/press-releases/2015/restaurant-burgers-had-a-banner-year-in-2014

Moyer, L. (2005). Fast-cook ovens cook food in minutes. Retrieved from http://www.nbcnews.com/id/9839623/ns/technology_and_science-tech_and_gadgets/t/fast-cook-ovens-cook-ovens-cook-food-minutes

TurboChef. (2013). Tornado: Tornado and Tornado 2 (NGC) Ventless Submittal Information. Retrieved from http://www.turbochef.com/filemanager/36/NGC_Ventless_Submittal_Package.pdf

United States Department of Agriculture (2016a). National Monthly Grass Fed Beef Report. Retrieved from https://www.ams.usda.gov/mnreports/lsmngfbeef.pdf

United States Department of Agriculture (2016b). Meat Price Spreads. Retrieved from https://www.ers.usda.gov/data-products/meat-price-spreads.aspx

United States Department of Agriculture, Agricultural Research Service. (2012). USDA Table of Cooking Yields for Meat and Poultry. Nutrient Data Laboratory Home Page. Retrieved from https://www.ars.usda.gov/ARSUserFiles/80400525/Data/retn/USDA_CookingYields_MeatPoultry.pdf

United States Department of Agriculture. (2014). USDA Specification for Certification of Beef Carcass Quality and Yield Characteristics of Officially Graded Beef. Retrieved from https://www.ams.usda.gov/sites/default/files/media/G-133%20--WR%20Reserve%20Premium%20Black%20Angus%20Natural%20Beef.pdf

Weber, K., Heinze, K. L., & DeSoucey, M. (2008). Forage for thought: Mobilizing codes in the movement for grass-fed meat and dairy Products. *Administrative Science Quarterly, 53*(3), 529-567. https://doi.org/10.2189/asqu.53.3.529

Wood, J. D., Richardson, R. I., Nute, G. R., Fisher, A. V., Campo, M. M., Kasapidou, E., ... & Enser, M. (2004). Effects of fatty acids on meat quality: a review. *Meat science, 66*(1), 21-32. https://doi.org/10.1016/S0309-1740(03)00022-6

Copyrights

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Selection of Lactic Acid Bacteria for the Optimized Production of Sheep's Milk Yogurt with a High Conjugated Linoleic Acid Content

Gabriela Christina Kuhl[1], Ana Paula Gusso[2], Brenda Lee Simas Porto[3], Carmen Maria Olivera Müller[1], Ricardo Ruiz Mazzon[4], Marcone Augusto Leal de Oliveira[3], Neila Silvia Pereira dos Santos Richards[2] & Juliano De Dea Lindner[1]

[1]Food Science and Technology Department, Federal University of Santa Catarina, Florianópolis, Brazil

[2]Food Science and Technology Department, Federal University of Santa Maria, Santa Maria, Brazil

[3]Chemistry Department, Federal University of Juiz de Fora, Juiz de Fora, Brazil

[4]Microbiology, Immunology and Parasitology Department, Federal University of Santa Catarina, Florianópolis, Brazil

Correspondence: Juliano De Dea Lindner, Federal University of Santa Catarina (UFSC), Agricultural Science Center (CCA), Food Science and Technology Department (CAL), Rodovia Admar Gonzaga, 1346. Itacorubi. Florianópolis, SC, Brazil. Tel: 55-483-721-6183. E-mail: juliano.lindner@ufsc.br

Received: May 2, 2017 Accepted: June 1, 2017 Online Published: June 15, 2017

doi:10.5539/jfr.v6n4p44 URL: https://doi.org/10.5539/jfr.v6n4p44

Abstract

Thirty-five different lactic acid bacteria (LAB) strains were screened for their conjugated linoleic acid (CLA) isomers (C18:2 *cis*-9, *trans*-11 and C18:2 *trans*-10, *cis*-12) producing ability from linoleic acid (LA) in sheep's milk. Preliminary experiments revealed that *Lactobacillus delbrüeckii* ssp. *bulgaricus* 2230 and *Streptococcus thermophilus* St 360 among the screened strains had the highest CLA-producing ability. This two strains were assayed in an 11-run fractional factorial design to investigate the effect of four variables included glucose, powdered sheep milk, LA and inoculum ratio on CLA production in a sheep's milk yogurt. The optimum conditions for producing the highest levels of CLA (42.86%) were obtained by adding 10.00 mg/mL of glucose, 30.00 mg/mL of powdered sheep milk, 0.90 mg/mL of LA and a 1:2 (*St:Lb*) ratio of bacterial strains in the inoculum. This CLA-rich sheep's milk yogurt could be an important supplementary food source for increasing the CLA in the human diet.

Keywords: dairy product, functional food, *Lactobacillus delbrüeckii* ssp. b*ulgaricus, Streptococcus thermophilus*

1. Introduction

Bovine milk is the most commonly consumed type of milk. However, in many countries, milk from other animal species is a significant share of the milk consumed. Ewe milk, for example, has been proposed as an alternative milk source to feed infants who are allergic to milk from other species (Balthazar et al., 2017). Compared to bovine and caprine milk, ovine milk is richer in fat, proteins, ash, calcium, iron, manganese, phosphorus, zinc, all essential amino acids, most vitamins, medium-chain fatty acids and monounsaturated fatty acids (MUFA) (Balthazar et al., 2015; Park, 2007).

Ovine milk is favored in terms of fatty acids (FA); they have a high proportion of saturated fatty acids (SFA) with both short and medium chains (Corrêa, Rohenkohl, & Osório, 2014; Mayer & Fiechter, 2012). Ovine milk has also the highest conjugated linoleic acid (CLA) content (*i.e.* 1.08% of ovine against 1.01% and 0.65% of bovine and caprine milks respectively) and 3.21% of linoleic acid (LA) (Jahreis et al., 1999).

The acronym CLA is used to describe a family of isomers of octadecadienoic acid (18:2), which have a pair of conjugated double bonds along the alkyl chain (Garcia et al., 2017). Recent studies have shown that isomers of CLA present in dairy products improve human health at a dosage of 1.00 to 3.00 g/day (MacDonald, 2000), through their anti-cancer (Thuillier et al., 2013), anti-diabetic (Malinska, Hüttl, Oliyarnyk, Bratova, & Kazdova, 2015), anti-atherosclerotic (Stachowska et al., 2012) and anti-osteoporosis (DeGuire et al., 2012) properties, as well as their ability to prevent increased body fat (Chen et al., 2012) and stimulate of the immune system (Bassaganya-Riera et al., 2012). We recently discussed these properties in a review (Kuhl and De Dea Lindner, 2016).

CLA may be formed in the rumen by anaerobic bacteria as an intermediate in the biohydrogenation of LA and/or in the mammary glands through desaturation of vaccenic acid (*trans*-11 octadecenoic acid) from Δ9-desaturase (Serafeimidou, Zlatanos, Laskaridis, & Sagredos, 2012). Biohydrogenation pathway of LA was firstly demonstrated by Kepler, Hirons, and Tove (1966) using *Butyrivibrio fibrisolvens.* LA was firstly isomerized to a conjugated 18:2 and then hydrogenated, leaving a *trans*-18:1 as the final product. Two categories of LA isomerase enzymes involved in this process were characterized to date. The C12 isomerase, which was predominantly found in *Lactobacillus* species, catalyzes the conversion of LA to C18:2 *cis*-9, *trans*-11 isomer. While the C9 isomerase, found mainly in *Propionibacterium*, catalyzes LA to C18:2 *trans*-10, *cis*-12 isomer (Luo et al., 2013).

The production of CLA by rumen bacteria led to the speculation that other microorganisms may also be able to synthesize this metabolite (Lucatto, Brandão, & Drunkler, 2014). Several studies reviewed by Gorissen, Leroy, De Vuyst, De Smet, and Raes (2013) reported the production of CLA during lactic fermentation by lactic acid bacteria (LAB). According to these studies, some strains of bacteria were able to change the FA profile of milk, in addition to producing functional FAs during fermentation as a result of its metabolism.

There are several important factors that can lead to the increased production of CLA by LAB. Among them, studies have cited variables such as fermentation time and temperature, as well as protein concentration and LA source (Gholami & Khosravi-Darani, 2014; Khosravi, Safari, Khodaiyan, & Gharibzahedi, 2015; Terán et al., 2015). According to Kim and Liu (2002) a glucose source is more efficient for CLA production than sucrose or lactose supplementation. Besides, the presence of LA isomerase and its activity in the culture may improve CLA production by adding LA to the medium (Dahiya & Puniya, 2017). Lin, Lin, and Lee (1999) reported the addition of 1.00 mg/mL (w/v) LA to the medium led to an effective CLA conversion during fermentation by *Lactobacillus delbrüeckii* ssp. *bulgaricus* and *Streptococcus thermophilus* in a single culture. The addition of dairy-based additives such as sodium caseinate, whey powder, or nonfat milk powder were described to CLA concentration enhancing in dairy products (Shantha & Decker, 1993). Overall, the CLA conversion by lactic acid fermentation may be influenced by numerous factors like bacterial strain, cell density, incubation time and cell age (Gholami & Khosravi-Darani, 2014).

The purpose of the present study was to evaluate strains of *L. bulgaricus* and *S. thermophilus* for their ability to produce CLA from LA. It is well documented that these strains are able to synthesize CLA using LA under single culture conditions. However, there is little information on CLA production during co-culture. Therefore, the effect of processing variables (glucose, powdered sheep's milk, LA and inoculum ratio) was investigated for the first time with regards to production of a sheep's milk yogurt with a high CLA content.

2. Materials and Methods

2.1 Bacterial Strains and Growth Conditions

A total of 35 LAB strains obtained from the Food Microbiology Laboratory collection culture of the Food and Drug Science Department (University of Parma, Italy) were used in this study (Table 1). All of the employed strains are potential starter cultures for milk fermentation. The initial screening of strains aimed to determine their ability to produce CLA from free LA in supplemented sheep's milk. *L. bulgaricus* strains were cultured in Man Rogosa Sharpe (MRS) medium (Merck, Darmstadt, Germany) and *S. thermophilus* strains were cultured in M17 medium (HiMedia, Mumbai, India) with incubation at 37 ºC and 42 ºC, respectively, for 48 h under anaerobic conditions. Stock solutions for maintenance of the strains were prepared with 1 mL of bacterial suspension in 0.5 mL of glycerol solution (1:1 v/v) and kept at -80 ºC.

Table 1. Strains used in screening for their ability to produce CLA from LA in sheep's milk.

Strain	Origin
Lactobacillus delbrüeckii* ssp. *bulgaricus	
2214; 2259; 2260; 2230	Grana Padano cheese
1865; Lb 260; Lb 261; Lb 263; Lb 264; Lb 265; Lb 308; Lb 309; Lb 313	Unknown
Streptococcus thermophilus	
1688; 1689	Parmigiano Reggiano cheese whey starter
St 360; St 362; St 356	Valtellina Casera cheese milk starter
St 393	Gorgonzola cheese milk starter
St 410	Provolone cheese whey starter
St 383	Provolone cheese
St 233; St 234	Grana Padano whey starter
St 451	Pecorino Toscano cheese whey starter
St 257; St 258; St 508; St 509	Pecorino Toscano cheese
St 357; St 361; St 363; St 366; St 387; St 388; St 390	Unknown

2.2 Screening of Strains for CLA Production

Raw sheep's milk was provided by the Pinheiro Seco Farm (Bom Retiro, SC, Brazil). The milk originated from Lacaune × Texel sheep crosses was pasteurized at 82 ºC for 15 min in batches, hot filled in bottles previously sanitized and immediately cooled to 4 ºC in an ice bath before being frozen until further use.

The stock solution of LA (30.00 mg/mL) was composed by 1.20 g of pure LA (Neon, São Paulo, Brazil), 40.00 mL of sheep's milk with 1.00% (v/v) Tween 80 (polyoxyethylene sorbitan monooleate) (Synth, Diadema, Brazil) to improve its solubility (Ando, Ogawa, Kishino, & Shimizu, 2004; Van Nieuwenhove, Oliszewski, González, & Pérez Chaia, 2007). The strains were reactivated overnight under the same conditions described above. Bacterial cells were harvested by centrifugation (4000 × g, 10 min) and suspended in 0.10% (w/v) peptone water until reaching a 0.10 OD at 600 nm as assessed by a SP-2000 UV spectrophotometer (Spectrum, Shanghai, China).

For the screening, excepted in control trial (none strain added), each strain was inoculated in milk supplemented with 1.66 mL of previously prepared LA stock solution, reaching a concentration of 0.50 mg/mL LA, 1.00% (w/v) glucose and 5.00% (w/v) ovine skim milk powder. These mixtures were incubated at 37 ºC (*L. bulgaricus*) or 42 ºC (*S. thermophilus*), until reaching pH 4.65 and maintained at -20 ºC until FA analysis.

2.3 Fatty Acid Analysis

Fermented milk samples were collected to determine the FA profile by gas chromatography (GC). Samples were carried out in duplicate, expressing results as mean value. Lipids were extracted following the Hara and Radin (1978) method using a hexane/isopropanol solution (3:2, v/v) and derivatized to methyl ester using methyl acetate and sodium methoxide (1 M) in a methanol solution at room temperature, according to (Christie, 1982).

Fatty acid methyl esters (FAME) were analyzed by a GC (model GC 2010-Plus, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector, an automatic injector (model AOC-i-20) and a CP-SIL 88 (100 m × 0.25 mm × 0.2 μm) GC capillary column (Agilent Technologies, Palo Alto, USA). The injection volume was 1.00 μL in a split mode 1:50 ratio. GC operating conditions were set according to Cruz-Hernandez et al. (2007): injector and detector temperature set to 250 ºC, initial oven temperature (45 ºC) increased to 175 ºC at 13 ºC/min and held for 27 min, temperature increased to 215 ºC at a rate of 4 ºC/min and held for 35 min. Hydrogen was used as a carrier gas at a flow rate of 1.5 mL/min. Fatty acids were identified by comparison with the retention times of methylated standards of Supelco 37 Component FAME Mix (Sigma, St Louis, MO, USA). A mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 octadecadienoic acid methyl esters was used as CLA standard (Sigma).

Data were calculated as normalized area percentages of fatty acid and converted to mg/mL of fermented milk by considering the total fatty acids in 1 mL of fermented milk as reference. Because milk supplemented with LA was used as a fermentation medium, total FAs were quantified and CLA production was calculated by subtracting the natural CLA content (Van Nieuwenhove et al., 2007). Percentages increase in CLA was calculated by using equation 1. Only one strain of each *L. bulgaricus* and *S. termophilus* which showed the greatest abilities to produce CLA, was selected for the next steps of this work.

$$[(CLAs \times 100/CLAm) - 100] \quad (1)$$

Note. CLAs= mg of CLA in the sample, CLAm= naturally present mg of CLA in the sheep's milk.

2.4 Influence of LA Concentration on Bacterial Growth

To evaluate the inhibitory potential and ability of bacteria to grow in the presence of LA, overnight activated strains were inoculated in MRS or M17 broth, according to specie. These broths were chosen to avoid turbidity interference in the OD measurement from milk matrix. The media were supplemented with 0.25, 0.50, 1.00, 1.50 or 2.00 mg/mL LA and incubated for 12 h at 37 ºC or 42 ºC under anaerobic conditions. The growth of selected strains was determined by measuring OD values at 600 nm using a spectrophotometer. Cultures without LA supplementation served as controls.

2.5 Experimental Design

After the initial screening, the selected strains that demonstrated major ability to produce CLA from LA were chosen for fermentation by protocooperation in co-culture to produce yogurt with a high CLA content. *S. thermophilus* St 360 and *L. bulgaricus* 2230 were assayed in a fractional design to evaluate the effect of four independent variables, including LA (Neon, São Paulo, Brazil); powdered sheep's milk, provided by Universidade Regional Integrada do Alto Uruguai e das Missões (Erechim, RG, Brazil); glucose (Sigma-Aldrich, Saint Louis, USA); and inoculum ratio, on CLA production (Table 2).

Table 2. Level of coded and real values for fractional factorial design.

Factors	Level		
	-1	0	1
Glucose (mg/mL) (X_1)	10.00	20.00	30.00
Powdered sheep's milk (mg/mL) (X_2)	10.00	20.00	30.00
LA (mg/mL) (X_3)	0.10	0.50	0.90
Inoculum ratio (*St*:*Lb*) (X_4)	1:2	1:1	2:1

The experiments were performed according to Table 3 to optimize the parameters. Experimental data were fitted to a quadratic polynomial model, and the model proposed for predicting the response variables was explained by the equation 2.

$$Y = A_0 + \sum_{i=1}^{n} A_i X_i + \sum_{i=1}^{n} \sum_{j=i+1}^{n} A_{ij} X_j X_i \quad (2)$$

Note. Where **Y**= predicted response, $\mathbf{A_0}$= regression coefficients for the intercept, **Ai**= linearity, $\mathbf{A_iX_i}$= square, $\mathbf{A_{ij}}$= interaction, $\mathbf{X_i}$ and $\mathbf{X_j}$= coded independent factors.

The data were analyzed using Statistic software version 7.0 (StatSoft Inc., Tulsa, OK, USA), and the coefficients were interpreted using the *F*-test. Analysis of variance (ANOVA), regression analysis and plotting of the function that correlated CLA production with the evaluated factors (glucose content, powdered sheep's milk concentration, LA concentration and inoculum ratio) were performed to establish the optimum conditions for CLA production.

2.6 Yogurts Production

The eleven runs (Table 3) were performed by adding specific proportions of each variable to the milk according to the experimental design (Table 2). Before the treatment at 90 ºC for 5 min, glucose and powdered sheep's milk were added to the milk in each batch. Afterwards, the mixtures were cooled to 42 ºC in an ice bath for LA addition (from previously prepared stock solution) and culture inoculation in the ratios described within the experimental design. The mixtures were incubated at 42 ºC for 15 h. After the fermentation period, the yogurts were stirred and stored at 4 ºC until further analysis.

Table 3. Experimental design used to test the influence of X_1 (glucose), X_2 (powder sheep's milk), X_3 (LA) and X_4 (Inoculum ratio).

Run	Coded Variables			
	X_1	X_2	X_3	X_4
1	-1	-1	-1	-1
2	1	-1	-1	1
3	-1	1	-1	1
4	1	1	-1	-1
5	-1	-1	1	1
6	1	-1	1	-1
7	-1	1	1	-1
8	1	1	1	1
9	0	0	0	0
10	0	0	0	0
11	0	0	0	0

2.7 Identification and Quantification of FAs

Lipid extraction, derivatization of FA to FAME and the GC conditions were the same as described above. As an internal standard, tricosanoic acid (C23:0 Me) was added to the medium to a final concentration of 1.00 mg/mL. Identification of the FAME peaks was performed by comparing the retention times of the standard Supelco 37 Component FAME Mix and mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 octadecadienoic acid methyl esters. C18:2 *cis*-9, *trans*-11 isomer concentrations in the yogurt were calculated, as described by Visentainer (2012), and expressed as mg/g of total lipids.

2.8 Physico-chemical Analysis

Moisture content within the different yogurts was determined by weight loss using an oven at 102 ºC and weighed until constant weight (Brazil, 2006). Ashes were determined by the incineration method in a muffle at 550 ºC according to the AOAC 945.46 method (AOAC, 2007). Crude protein was determined via the micro Kjeldahl method with nitrogen (%) × 6.38 following the Brazilian normative instruction No. 68 (Brazil, 2006). The pH was measured using a pH meter (model DM-22, Digimed, São Paulo, Brazil). Acidity was assayed by a simple titration method (0.1 M NaOH) according to the AOAC 947.05 method (AOAC, 2007). The analyses were performed in triplicate and the values reported are the mean ± standard deviation. The analysis of variance (ANOVA) and Tukey's studentized range test (5% significance) were carried out to identify significant differences between the results. The data were analyzed using Statistic software.

3. Results and Discussion

3.1 Screening of Strains for CLA Production

Strains tested in our study either increased or decreased CLA levels (Table 4). Jiang, Björck, and Fondén (1998) found similar results using an *in vitro* screening for dairy starter cultures, by varying the concentration of LA free in medium. The depletion of the milk's natural CLA content observed for some strains could be potentially associated with lipolytic metabolism or explained by fermentation temperature, as observed by Gorissen et al. (2011). To optimize microorganism ability to grow at different temperature, a thermal control system allows the regulation of the phospholipid membrane fluidity by incorporating unsaturated fatty acids (Hernandez-Mendoza, Lopez-Hernandez, Hill, & Garcia, 2009; Kishino, Ogawa, Omura, Matsumura, & Shimizu, 2002).

Table 4. Increase or decrease in CLA concentrations in sheep's milk fermented by LAB strains.

Specie	Strain	CLA (mg/mL)	Increase/Decrease (%)
Lactobacillus delbrüeckii ssp. *bulgaricus*	2214	0.82	25
	2259	0.61	-7
	2260	0.88	34
	2230	1.15	74
	1865	0.84	27
	Lb 260	0.88	34
	Lb 261	0.79	20
	Lb 263	1.03	56
	Lb 264	0.94	42
	Lb 265	0.71	7
	Lb 308	1.01	53
	Lb 309	0.90	36
	Lb 313	0.80	21
Streptococcus thermophilus	1688	0.50	-24
	1689	0.57	-14
	St 360	1.02	54
	St 362	0.95	44
	St 356	0.85	30
	St 393	0.84	28
	St 410	0.64	-3
	St 383	0.53	-20
	St 233	0.77	17
	St 234	0.92	39
	St 451	0.88	34
	St 257	0.38	-42
	St 258	0.57	-13
	St 508	0.60	-9
	St 509	0.53	-20
	St 357	0.89	35
	St 361	0.75	14
	St 363	0.64	0
	St 366	0.77	17
	St 387	0.71	8
	St 388	0.74	12
	St 390	0.82	24

Wang, Delettre, Guillot, Corrieu, and Béal (2005) used a low fermentation temperature for *Lactobacillus acidophilus* and *S. thermophilus* and the result showed an increased incorporation of unsaturated fatty acids in the cell membrane. Furthermore, Jenkins and Courtney (2003) reported a preference for the incorporation of CLA to LA into the cell membrane, which may account in part for the depletion of CLA naturally present in milk by "homeoviscous adaptation" (Ernst, Ejsing, & Antonny, 2016).

According to Hernandez-Mendoza et al. (2009), a part of the isomerized product from LA could be incorporated into cell membranes while another part could be released into the growth medium. Besides, because the enzyme responsible for bioconversion of LA to CLA (linoleate isomerase - EC 5.2.1.5) is anchored to the cell membrane, authors suggests that the range of biohydrogenation may decrease if some membrane damage occurs or the exposure to a low pH, for example.

Among the microorganisms screened in our work, *L. bulgaricus* 2230 and *S. thermophilus* St 360 demonstrated the highest potential for producing CLA in sheep's milk; the total CLA quantified was 1.15 mg/mL (74.00% increase from the original CLA content in the milk) and 1.02 mg/mL (54.00% increase), respectively (Table 4).

Several authors reported the ability of different LAB strains to produce CLA (Jiang et al., 1998; Kim & Liu, 2002; MacOuzet, Lee, & Robert, 2009; Rodríguez-Alcalá, Braga, Xavier Malcata, Gomes, & Fontecha, 2011;

Terán et al., 2015). Lin et al. (1999) demonstrated the production of CLA by *S. thermophilus* and *L. bulgaricus* as single strains and observed an increase in levels by adding 1.00 mg/mL LA in a culture medium containing 12.00% (w/v) skim milk powder, during a 24 h fermentation at 37 ºC. *L. bulgaricus* and *S. thermophilus* strains produced 0.09 and 0.07 mg/mL of CLA from LA under these conditions, respectively. In addition, Van Nieuwenhove et al. (2007) demonstrated a conversion of 30.30% by *S. thermophilus* in fermented buffalo milk supplemented with 0.80 mg/mL of LA, while *L. bulgaricus* did not exhibit any ability to produce CLA from LA.

3.2 Influence of LA Concentration on Bacterial Growth

To evaluate the inhibitory potential and ability of bacteria to grow in the presence of LA, the selected *L. bulgaricus* and *S. thermophilus* grew in MRS and M17 media in the presence of different concentrations of LA for 12 h (Figure 1). Both strains tested (data not shown for *S. thermophilus* St 360) suffered inhibition of growth in the presence of more than 1.00 mg/mL LA. As stated by Van Nieuwenhove et al. (2007), different strains tolerate different concentrations of LA. Additionally, the inhibitory dose depends on the availability of the FA (Gorissen, Raes, De Smet, De Vuyst, & Leroy, 2012; Trigueros & Sendra, 2015). According to Kim and Liu (2002), the growth of *Lactococcus lactis* was completely inhibited at LA concentrations higher than 0.50 mg/mL. In a similar study, *Bifidobacterium animalis* ssp. *lactis* was tested for growth in a MRS-cysteine medium supplemented with 0.20 - 1.00 mg/mL LA. Higher concentrations of LA (0.80 and 1.00 mg/mL) significantly inhibited bacterial growth (Terán et al., 2015). According to Lin et al. (1999) the inhibitory effect of high LA concentrations could be due to an antimicrobial effect of free LA.

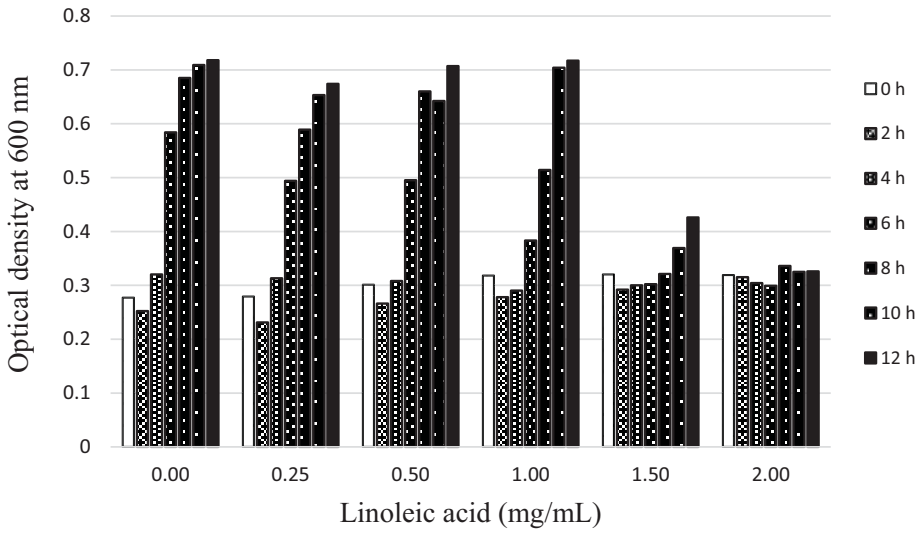


Figure 1. Inhibition of *L. bulgaricus* 2230 growth in MRS broth by the presence of different concentrations of LA (mg/mL). OD at 600 nm was determined during 12 h.

3.3 Optimization of CLA Production in Yogurt

The optimal mixture for producing CLA by selected bacterial strains consisted of adding 10.00 mg/mL (w/v) glucose, 30.00 mg/mL (w/v) powdered sheep's milk, 0.90 mg/mL (w/v) LA and a 1:2 (*St:Lb*) (v/v) ratio of bacterial strains (Run 7) (Table 5). The highest CLA level found (70.41 mg/g fat) was increased 42.86% over those in raw sheep's milk. Table 5 shows the actual response values obtained from experimental data and the predicted response values based on the quadratic polynomial model.

Table 5. Microbial CLA production (%) in yogurt under different conditions based on a fractional factorial design.

Run	CLA experimental production (%)	CLA predicted production (%)
1	36.82	36.16
2	26.92	26.26
3	01.74	01.07
4	11.34	10.67
5	04.32	03.66
6	30.23	29.57
7	42.86	42.19
8	38.66	37.99
9	19.60	23.45

Analysis of variance (ANOVA) (Table 6) showed that the quadratic polynomial model was significant ($p < 0.015$), revealing a viability of the constructed model and also was sufficient to represent the actual relationship between the response and significant parameters. A high value of R^2 (0.97) is an indication that the fitted model can be used for prediction with reasonable precision. The *F*-value (20.16) implies that the model is significant at a 95.00% confidence level. The value of the lack of fit test (0.99) was higher than 0.42, which is not significant relative to the pure error, and indicates that the fitting model is adequate to describe the experimental data. The variation for CLA synthesis according different treatments might be linked to the independent variables.

Table 6. Analysis of variance (ANOVA) for the fitted factorial polynomial model for optimization of CLA production and model equation.

Model	$Y (\% CLA) = 23.44+2.68X_1 + 4.91X_3 - 6.20X_4 + 12.20X_1X_4$				
Source	Sum of Squares (SS)	Degree of Freedom (DF)	Mean of Squares (MS)	*F*-value	*p*-Value
Model	1838.14	7	142.09	20.16	<0.015
Lack of fit	12.89	1	12.89	0.99	0.42
Pure error	25.87	2	12.94		

Note. $R^2 = 0.97$.

CLA production was shown to be significant ($p < 0.05$) depending on glucose concentration (X_1), inoculum ratio (X_4) and LA content (X_3). Powdered sheep's milk as a protein source does not appear to be a significant variable in CLA production. Second order interactions between independent variables were not found to be significant, except for glucose and the inoculum ratio represented in the response surface methodology (RSM) (Figure 2). The interaction of glucose and inoculum ratio in minimum levels enhances the CLA percentage production in a confidence level of 95.00%. The RSM showed that CLA production increase with inoculum ratio inversion (2:1 to 1:2) during the decreasing of glucose content.

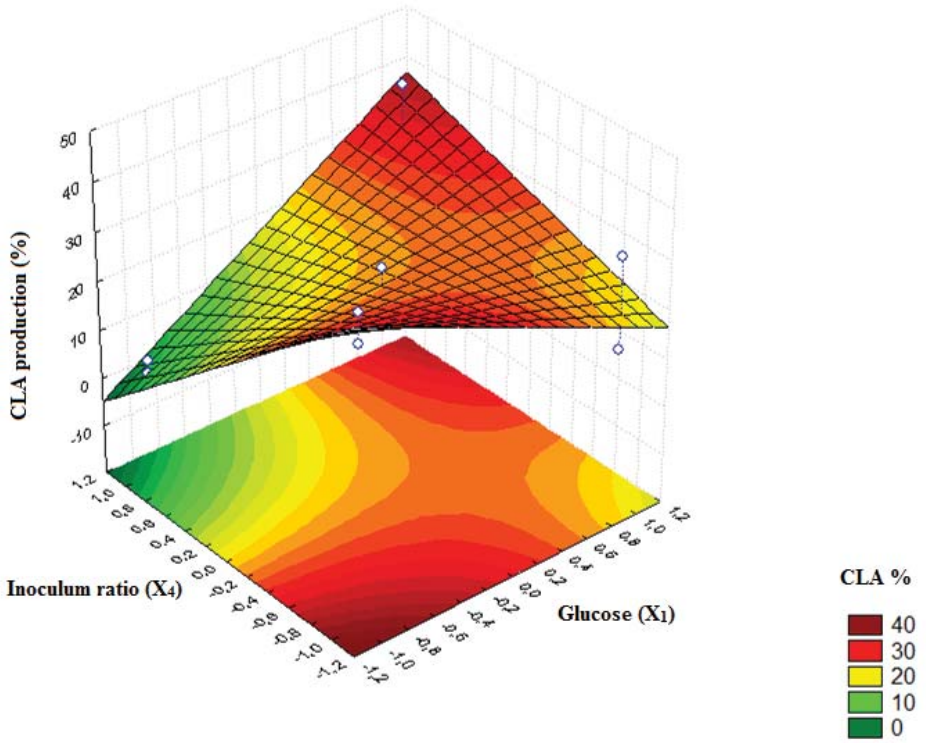


Figure 2. Response surface plots showing the significant ($p < 0.05$) interaction effects (glucose and inoculum ratio) on the CLA produced (%) by yogurt co-culture.

Kim and Liu (2002), in a previous research, had used different model systems to optimize conversion conditions from LA to CLA by diverse bacterial species. Recent studies also used independent variables in the production of fermented milk to determine the best conditions for increasing CLA levels in products. Khosravi-Darani, Reihani, and Feili (2014) analyzed the effect of processing variables with regards to CLA production in probiotic yogurt containing strains of *L. acidophilus*, *Bifidobacterium bifidum* and *Propionibacterium freudenerchii*. Variables included the addition of supplements, fermentation conditions and both inoculum size and viability. The highest concentration of CLA (11.03 mg/g fat) was obtained by the addition of 4.00% (w/v) whey powder, 4.00% (v/v) grape seed oil, inoculation of 0.80% (v/v), 36 h of incubation and 27 h of fermentation at 35 °C. The research showed that under the optimized conditions, the amount of CLA in probiotic yogurt increased by 40.00% from an average of 8.01 mg/g fat.

Khosravi et al. (2015) evaluated the ability of different *Lactobacillus* strains to produce CLA from LA. Experiments revealed that *Lactobacillus plantarum* had the highest CLA-producing potential (0.09 mg/mL). The results showed that the use of yeast extract and glucose could significantly increase cell growth and CLA production. RSM was also applied to investigate the effects of three independent variables (LA, yeast extract concentrations and inoculum size) on CLA formation. The optimum conditions to achieve the highest CLA production (0.24 mg/mL) were obtained using 3.00 mg/mL LA, 4.00 mg/mL yeast extract and a 4.00% (v/v) inoculum size.

The highest CLA content was produced by adding 0.90 mg/mL LA. According to Gorissen et al. (2011), the conversion of CLA depends on the amount of free LA in the medium. Gorissen et al. (2012) used *Bifidobacteria* and *Lactobacillus sakei* strains, which are able to produce CLA *in vitro,* as starter cultures for milk fermentation, and no significant increase in CLA content was observed, even with sufficient amounts of LA. This result is in accordance with the prior study (Gorissen et al., 2011), suggesting that the availability of free FA was likely too low. Xu, Boylston, and Glatz (2004) tested *Propionibacterium freudenreichii* ssp. *shermanii* inoculum in a model system containing milk-hydrolyzed soybean oil to obtain CLA. They obtained 1.45 mg of CLA (C18:2 *cis*-9, *trans*-11 isomer) per gram of fat after 24 h of fermentation. Lin et al. (1999) evaluated six lactic cultures for their ability to generate CLA from LA. The results showed no significant differences in CLA levels without added LA, suggesting that addition of a LA source is effective at enhancing CLA conversion during fermentation by LAB.

In the present study, we showed for the first time that *L. bulgaricus* and *S. thermophilus* could be co-cultured to produce CLA in yogurt. Surprisingly, a substantial increase in the amount of CLA was obtained by using an inoculum ratio of 1:2 (*St:Lb*). A 1:1 (*St:Lb*) ratio might not be effective for CLA synthesis as observed for these two strains. In yogurt production technology, if the 1:1 ratio is not respected, the ratio is generally unbalanced in coccus favor that drives the early stages of fermentation. Ye et al. (2013) found that a 1:1 ratio is optimal for the co-culture of *L. acidophilus* and *S. thermophilus* using hydrolyzed safflower oil as a substrate in a skim milk-based medium. However, a co-culture of *L. acidophilus* and *L. plantarum* led to an even greater production of CLA, which suggests that different factors influence CLA production.

Glucose was also an important variable in the production of CLA in yogurt samples. According to Kim and Liu (2002) supplementation of glucose improves CLA production even than compared to sucrose or lactose sources, which authors have associated with biohydrogenation pathway activation or bacterial cell growth by an easier energy source.

The addition of the lowest tested concentration (10.00 mg/mL) of glucose had a positive effect on CLA production. As shown in Figure 2, the variation in the CLA yield could be explained as a polynomial function of the glucose concentration and inoculum ratio. Two hypotheses can be used to explain why the low glucose concentration in the model positively affects the production of CLA. The first one is that the high availability of glucose in the mixture can cause metabolic stress by the generation of osmotic pressure. The second hypothesis comes from the presence of LA in high amounts, up to a specific limit that might potentially activate a metabolic response (detoxification) to convert LA into a less toxic molecule for cell growth. A better understanding of these two hypotheses will be investigated in future studies using comparative proteomics. Lin (2000) tested *L. bulgaricus* and *S. thermophilus* cultures for the effects of different carbohydrates (sucrose, lactose and fructose) at 60.00 mg/mL in skim milk medium on CLA (C18:2 *cis*-9,*trans*-11 isomer) production. All carbohydrates at this concentration inhibited CLA produced by *L. bulgaricus*.

3.4 Fatty Acid Composition of Yogurts

The C18:2 *cis*-9, *trans*-11 was the most abundant isomer in all different runs (Table 7). According to Jenkins, Wallace, Moate, and Mosley (2008), C18:2 *cis*-9, *trans*-11 isomer is usually considered the main health-promoting CLA for human consumption. The FAs involved in the biohydrogenation indicate an interesting pattern of oscillation between C18:2 *cis*-9, *trans*-11 and C18:2 *trans*-10, *cis*-12 isomers, and the amount of LA added to the yogurts appears to influence this ratio. Treatments containing 0.10 mg/mL LA (runs 1 to 4), disfavored the synthesis of the C18:2 *trans*-10, *cis*-12 isomer, which was 0.00-10.00% of the total CLA quantified. Whereas, treatments containing 0.90 mg/mL LA (runs 5 to 8) favored the C18:2 *trans*-10, *cis*-12 isomer, which was 22.00-25.00% of the total CLA quantified. However, in both cases, C18:2 *cis*-9, *trans*-11 was the major isomer produced. Furthermore, it was observed that the common factor between the two treatments able to form C18:2 *trans*-10, *cis*-12 isomer at low LA concentrations, was a 2:1 inoculum ratio of *St:Lb.* Thus, the production of these two isomers depend not only the enzyme sort, but also LA concentrations and appears to be strain dependent.

Table 7. Profile of the FAs involved in the biohydrogenation from different treatments used to produce sheep's milk yogurt (mg/g fat). Runs 1 to 9 are correspondent to the experimental design.

Fatty Acid	Run								
	1	2	3	4	5	6	7	8	9
Vaccenic acid (C18:1 t11)	157.45	125.30	121.10	130.49	149.01	146.34	131.34	174.85	115.95
LA (C18:2n6c)	26.93	105.25	78.74	94.45	88.88	113.32	133.05	119.53	86.11
CLA (c9, t11)	67.43	62.55	50.15	54.87	51.41	64.18	70.41	68.34	58.94
CLA (t10, c12)	0.00	4.90	5.0	0.00	14.59	21.42	24.18	19.64	9.25

Among the FAs involved in LA biohydrogenation, a purely empirical observation showed an inverse proportion to variations corresponding to LA and vaccenic acid levels, which can be explained by the reversible characteristic of the metabolic pathways (isomerization and hydrogenation processes). However, this issue has not been fully explored and needs further clarification.

To determine whether the storage time affects the fatty acid composition, especially in light of the CLA concentrations, in yogurt produced under different treatment conditions, the percent of FA in different samples was analyzed on the first day of fermentation and after 14 days of storage at 5 ºC (Table 8). The FA composition was classified into saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) FA according to the

saturation degree. In addition, the levels of C18:2 *cis*-9, *trans*-11 were represented in relation to the storage period as well.

Table 8. CLA value (mg/g fat) and FA percentage (% total FAME) from yogurts at day one of fermentation and after 14 days of storage at 5 ºC.

Run	SFA (%)		MUFA (%)		PUFA (%)		CLA (mg/g) (*cis*-9, *trans*-11 isomer)	
	1 day	14 day	1 day	14 day	1 day	14 day	1 day	14 day
1	71.70	71.30	25.11	24.55	03.17	04.15	67.43	55.22
2	72.77	71.37	23.80	24.40	03.43	04.24	62.55	48.42
3	72.20	71.48	23.82	24.40	04.00	04.15	50.14	50.42
4	72.54	71.07	23.53	24.67	03.93	04.25	54.87	48.20
5	73.47	71.80	22.78	23.94	03.75	04.28	51.41	48.26
6	72.02	71.21	23.76	24.60	04.21	04.20	64.18	55.17
7	72.15	71.27	23.70	24.47	04.13	04.26	70.41	47.95
8	72.56	71.20	23.51	24.54	03.92	04.26	68.34	47.02
9	72.16	71.15	23.81	24.60	04.03	04.25	58.94	49.31

Note. SFA= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids.

The PUFA levels ranged from 03.17% to 04.26%. Serafeimidou et al. (2012) observed lower PUFA levels (02.80%) in sheep's milk yogurt, while in a similar study, Serafeimidou, Zlatanos, Kritikos, and Tourianis (2013) found equivalent PUFA levels (04.50%). In our work, the percentage of CLA generally decreased during cold storage in the samples analyzed. Kim and Liu (2002) suggested that this behavior might be related to the activation of reduction steps in the biohydrogenation pathway. Florence et al. (2012) suggested a possible strategy to increase the amount of CLA during cold storage by co-inoculating probiotic bacteria with yogurt starter cultures. The authors found a significant increase in relative CLA levels after 7 days of storage at 4 ºC in fermented milk with a co-culture of *B. animalis* ssp. *lactis, S. thermophilus* and *L. bulgaricus*. In accordance with these results, Xu, Boylston, and Glatz (2005) screened four probiotic bacteria in single or co-culture with traditional yogurt cultures to evaluate CLA production in fermented milk. The combination of most bacteria with the yogurt cultures produced higher levels of CLA isomers than yogurt culture alone after 14 days of storage, which suggests that incorporation of probiotic bacteria could be key to keeping the CLA levels stable during storage periods of 14 days.

In a similar study, Serafeimidou et al. (2013) described changes in the CLA concentrations, FA profile and chemical composition of yogurt from cow's and sheep's milk, produced using traditional methods, during 14 days of storage at 5 °C. Refrigerated storage resulted in a significant decrease in CLA in cow's milk yogurt, while in sheep's milk yogurt there was the opposite trend, indicating a contradiction compared to results obtained in the present study.

The influence of storage time on CLA levels in dairy products is still controversial (Serafeimidou et al., 2013). Some authors have suggested that the accumulation of lactic acid has a detrimental effect on CLA levels (Khosravi-Darani et al., 2014; Kim & Liu, 2002). In our work, a co-culture of *L. bulgaricus* 2230 and *S. thermophilus* St 360 provided a more suitable condition for acid production (post-acidification), which may explain the two-week storage results. Dairy products can be impacted by several physical and chemical characteristics resulting from a modified acid and FA profile because the risk of oxidation and off-flavors increases with a high concentration of unsaturated FAs (Collomb, Schmid, Sieber, Wechsler, & Ryhänen, 2006).

3.5 Physico-chemical Analysis of Yogurts

The analytical results of the physical-chemical characteristics of sheep's milk yogurts are shown in Table 9. Moisture values ranged between 75.38 and 81.79%. Although there were no significant differences ($p > 0.05$) in the moisture content, this variation appears to be associated with glucose content into the samples. The incorporation of glucose as an ingredient in the prepared yogurt, leads to an increase in the osmolarity which would attract water to the yogurt-forming casein micelles. Serafeimidou et al. (2013) measured similar values in sheep's milk yogurt (79.37%), and no significant change was observed during storage. Prandini, Sigolo, Tansini, Brogna, and Piva (2007) found no correlation between CLA levels and the moisture of yogurt in their studies.

Table 9. Physico-chemical characteristics of sheep's milk yogurts.

Run	Moisture (%)	Ash (%)	Protein (%)	pH	Titratable acidity (% lactic acid)
1	81.79±0.58^{a}	1.02±0.00^{a,b}	5.96±0.17^{a,b}	4.34±0.07^{a,b}	1.64±0.07^{a,b,c}
2	75.38±6.16^{a}	1.00±0.02^{a,b,c}	5.72±0.26^{a,b}	4.33±0.15^{a,b}	1.59±0.06^{a,b}
3	76.28±1.38^{a}	1.08±0.03^{a}	6.25±0.27^{a,b}	4.28±0.31^{a,b}	1.58±0.00^{a}
4	77.01±3.98^{a}	1.03±0.03^{a,b}	6.11±0.22^{a,b}	4.58±0.13^{a,b}	1.35±0.01^{d}
5	81.27±0.27^{a}	0.92±0.06^{b,c}	5.70±0.14^{a}	4.64±0.21^{b}	1.75±0.01^{a,b,c}
6	77.93±1.04^{a}	0.89±0.07^{c}	5.06±0.99^{a}	4.16±0.20^{a}	1.76±0.08^{b,c}
7	79.96±0.57^{a}	0.99±0.06^{a,b,c}	6.84±0.62^{b}	4.26±0.17^{a,b}	1.64±0.01^{a,b,c}
8	76.95±0.15^{a}	1.06±0.02^{a}	6.15±0.12^{a,b}	4.47±0.05^{a,b}	1.60±0.00^{a,b,c}
9	79.73±0.50^{a}	1.02±0.02^{a}	5.90±0.10^{a,b}	4.31±0.14^{a,b}	1.77±0.07^{c}

Note. ^{a,b,c} Means of three replicates ± SD with different letters in the same column are significantly different ($p < 0.05$).

There were significant ($p < 0.05$) differences in ash between yogurt samples. The highest ash levels were noted in run 3 (1.08%), whereas the lowest were found in run 6 (00.89%). Bano, Abdullah, Nadeem, Babar, and Khan (2011) used different concentrations of goat's and sheep's milk to develop a functional yogurt. The ash content increased significantly for all levels of sheep's milk added to the yogurt.

Generally, there were no significant ($p > 0.05$) differences for protein content in treatments of the yogurt samples, except between run 7 (06.84%) and runs 5 (05.70%) and 6 (05.06%). Katsiari, Voutsinas, and Kondyli (2002) and Voutsinas, Katsiari, Pappas, and Mallatou (1996) found similar protein levels in sheep's milk yogurt. Based on the results of Khosravi-Darani et al. (2014), the addition of 4.00% whey powder to whole milk increased CLA concentrations. The authors suggest that this occurs due to proteins acting as hydrogen donors during the first step of biohydrogenation, improving isomerization of LA and facilitating CLA formation. Kim and Liu (2002) also observed enhanced CLA production with the addition of nonfat dry milk powder. LAB partially hydrolyzes proteins, which increases the amount of free amino acids in fermented dairy products, making yogurt proteins more easily digestible than the proteins found in liquid milk, although the amount of proteins in each are similar (Hossain, 2015).

The analytical results showed a significant ($p < 0.05$) difference between run 5 (04.64%) and run 6 (04.16%) in terms of pH values. This finding is in agreement with Balthazar et al. (2015) who produced a sheep's milk yogurt with a pH of 04.41. These authors also found an acidity (lactic acid % w/v) of 00.94, similar to our results.

Significant ($p < 0.05$) differences in titratable acidity were also found in some yogurt samples. The highest lactic acid was observed in run 9 (01.77%), whereas the lowest was found in run 4 (01.35%). According to Jay (2005), *S. thermophilus* can produce approximately 00.50% lactic acid, while *L. bulgaricus* can produce 00.60 to 00.80% (pH 04.20-04.50). However, if the incubation time is longer, the pH may fall, increasing lactic acid to 02.00%.

In conclusion, among screened *L. bulgaricus* strains, 12 exhibited an ability to produce CLA (C18:2 *cis*-9, *trans*-11 isomer) in sheep's milk. The percentage of CLA produced varied from 07.00 to 74.00%. For the *S. thermophilus* strains, 13 of them showed an increase in CLA (C18:2 *cis*-9, *trans*-11 isomer) levels (from 8.00 to 54.00%). Therefore, when placed together in co-culture, *L. bulgaricus* 2230 and *S. thermophilus* St 360 should elevate the CLA content in sheep's milk yogurt. The optimum conditions for producing the highest CLA levels in sheep's milk yogurt consisted of adding 10.00 mg/mL (w/v) glucose, 30.00 mg/mL (w/v) powdered sheep's milk, 0.90 mg/mL (w/v) LA and a 1:2 (*St:Lb*) (v/v) ratio of bacterial strains. Nevertheless, the CLA levels in sheep's milk yogurt decreased after storage at 5 ºC for 14 days.

To confer health benefits to humans, one must consume 1.00 to 3.00 g of CLA per 70 kg of human body weight per day. To reach this recommended value, approximately four servings per day of 250 mL each of the high-CLA yogurt produced in this work would be sufficient. To estimate the number of recommended dose, calculation was based on total fat (4.28%) and total CLA (70.41 mg/g fat) of treatment 7. However, further investigation into the ingestion of CLA is necessary considering the fact that there are other sources of CLAs in the human diet. Further studies are also needed to characterize the screened CLA producer strains to explore their potential functional properties, which could be used to the benefit of consumers. Furthermore, the model obtained in this work may allow for optimization in the development of CLA-rich yogurts by the dairy industry, although an expansion to a proper technological and sensory evaluation is required.

Acknowledgments

The authors acknowledge Professor Erasmo Neviani from the University of Parma for providing the LAB strains, Paulo Gregianin from Pinheiro Seco Farm for the milk, Professor Juliana Steffens from the Universidade Regional Integrada do Alto Uruguai e das Missões for the sheep's milk powder and CAPES/CNPq for scholarships.

References

AOAC Official Method 945.46. (2007). In: HORWITZ, W. (Ed.), *Official Methods of Analysis of AOAC International*. (18th ed, revision 2). Gaithersburg, Maryland: AOAC International.

AOAC Official Method 947.05. (2007). In: HORWITZ, W. (Ed.), *Official Methods of Analysis of AOAC International*. (18th ed, revision 2). Gaithersburg, Maryland: AOAC International.

Ando, A., Ogawa, J., Kishino, S., & Shimizu, S. (2004). Conjugated linoleic acid production from castor oil by Lactobacillus plantarum JCM 1551. *Enzyme and Microbial Technology*, *35*(1), 40-45. http://doi.org/10.1016/j.enzmictec.2004.03.013

Balthazar, C. F., Gaze, L. V., Azevedo da Silva, H. L., Pereira, C. S., Franco, R. M., Conte-Júnior, C. A., … de Oliveira Silva, A. C. (2015). Sensory evaluation of ovine milk yoghurt with inulin addition. *International Journal of Dairy Technology*, *68*(2), 281-290. https://doi.org/10.1111/1471-0307.12189

Balthazar, C. F., Pimentel, T. C., Ferrão, L. L., Almada, C. N., Santillo, A., Albenzio, M., … Cruz, A. G. (2017). Sheep Milk: Physicochemical Characteristics and Relevance for Functional Food Development. *Comprehensive Reviews in Food Science and Food Safety*, *16* (Wendorff 2001), 247-262. https://doi.org/10.1111/1541-4337.12250

Bano, P., Abdullah, M., Nadeem, M., Babar, M. E., & Khan, G. A. (2011). Preparation of functional yoghurt from sheep and goat milk blends. *Pakistan Journal of Agricultural Sciences*, *48*(3), 211-215.

Bassaganya-Riera, J., Hontecillas, R., Horne, W. T., Sandridge, M., Herfarth, H. H., Bloomfeld, R., & Isaacs, K. L. (2012). Conjugated linoleic acid modulates immune responses in patients with mild to moderately active Crohn's disease. *Clinical Nutrition*, *31*(5), 721-727. https://doi.org/10.1016/j.clnu.2012.03.002

Brazil. Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa no 68, de 12 de dezembro de 2006. Estabelece métodos analíticos fisico-químicos oficiais para leite e produtos lácteos. Diário Oficial da República Federativa do Brasil, Brasília, p. 8, Seção 1, 14 de dezembro de 2006.

Chen, S.-C., Lin, Y.-H., Huang, H.-P., Hsu, W.-L., Houng, J.-Y., & Huang, C.-K. (2012). Effect of conjugated linoleic acid supplementation on weight loss and body fat composition in a Chinese population. *Nutrition (Burbank, Los Angeles County, Calif.)*, *28*(5), 559-65. https://doi.org/10.1016/j.nut.2011.09.008

Christie, W. W. (1982). of Glycerolipids and Cholesteryl Esters. *Journal Of Lipid Research*, *23*, 1072-1075.

Collomb, M., Schmid, A., Sieber, R., Wechsler, D., & Ryhänen, E. L. (2006). Conjugated linoleic acids in milk fat: Variation and physiological effects. *International Dairy Journal*, *16*(11), 1347-1361. https://doi.org/10.1016/j.idairyj.2006.06.021

Corrêa, G. F., Rohenkohl, J. E., Osorio, M. T. M. (2014). Produção e Qualidade do Leite Ovino. In: Selaive-Villarroel, A. B., Osorio, J. C. S. (Org.). Produção de ovinos no Brasil. Roca, São Paulo, pp. 485-499.

Dahiya, D. K., & Puniya, A. K. (2017). Optimisation of fermentation variables for conjugated linoleic acid bioconversion by *Lactobacillus fermentum* DDHI27 in modified skim milk. *International Journal of Dairy Technology*, *70*, 1-10. https://doi.org/10.1111/1471-0307.12375

DeGuire, J. R., Makarem, N., Vanstone, C. A., Morin, S., Duque, G., & Weiler, H. A. (2012). Conjugated linoleic acid is related to bone mineral density but does not affect parathyroid hormone in men. *Nutrition Research*, *32*(12), 911-920. https://doi.org/10.1016/j.nutres.2012.08.006

Ernst, R., Ejsing, C. S., & Antonny, B. (2016). Homeoviscous Adaptation and the Regulation of Membrane Lipids. *Journal of Molecular Biology*, *428*(24), 4776-4791. https://doi.org/10.1016/j.jmb.2016.08.013

Florence, A. C. R., Béal, C., Silva, R. C., Bogsan, C. S. B., Pilleggi, A. L. O. S., Gioielli, L. A., & Oliveira, M. N. (2012). Fatty acid profile, trans-octadecenoic, α-linolenic and conjugated linoleic acid contents differing in certified organic and conventional probiotic fermented milks. *Food Chemistry*, *135*(4), 2207-2214. https://doi.org/10.1016/j.foodchem.2012.07.026

Garcia, C., Duby, C., Catheline, D., Toral, P. G., Bernard, L., Legrand, P., & Rioux, V. (2017). Synthesis of the suspected trans-11, cis-13 conjugated linoleic acid isomer in ruminant mammary tissue by FADS3-catalyzed Δ13-desaturation of vaccenic acid. *Journal of Dairy Science*, *100*(1), 783-796. https://doi.org/10.3168/jds.2016-11455

Gholami, Z., & Khosravi-Darani, K. (2014). An overview of conjugated linoleic acid: microbial production and application. *Mini Reviews in Medicinal Chemistry*, *14*(9), 734-746.

Gorissen, L., Leroy, F., De Vuyst, L., De Smet, S., & Raes, K. (2013). Bacterial production of conjugated linoleic and linolenic acid in foods: a technological challenge. *Critical Reviews in Food Science and Nutrition*, (January 2015), 37-41. https://doi.org/10.1080/10408398.2012.706243

Gorissen, L., Raes, K., De Smet, S., De Vuyst, L., & Leroy, F. (2012). Microbial production of conjugated linoleic and linolenic acids in fermented foods: Technological bottlenecks. *European Journal of Lipid Science and Technology*, *114*(4), 486-491. https://doi.org/10.1002/ejlt.201100239

Gorissen, L., Weckx, S., Vlaeminck, B., Raes, K., de Vuyst, L., de Smet, S., & Leroy, F. (2011). Linoleate isomerase activity occurs in lactic acid bacteria strains and is affected by pH and temperature. *Journal of Applied Microbiology*, *111*(3), 593-606. https://doi.org/10.1111/j.1365-2672.2011.05087.x

Hara, A., & Radin, N. S. (1978). Lipid extraction of tissues with a low-toxicity solvent. *Analytical Biochemistry*, *90*(1), 420-426. https://doi.org/10.1016/0003-2697(78)90046-5

Hernandez-Mendoza, A., Lopez-Hernandez, A., Hill, C. G., & Garcia, H. S. (2009). Bioconversion of linoleic acid to conjugated linoleic acid by Lactobacillus reuteri under different growth conditions. *Journal of Chemical Technology & Biotechnology*, *84*(2), 180-185. https://doi.org/10.1002/jctb.2021

Hossain, N. (2015). *Development of improved quality yogurt in terms of texture, flavor, food value and low cost*, 2015. M.S dissertation - department of mathematics and natural sciences, BRAC University. Bangladesh, pp. 127. http://123.49.46.157/bitstream/handle/10361/4232/MNS.pdf?sequence=1

Jahreis, G., Fritsche, J., Möckel, P., Schöne, F., Möller, U., & Steinhart, H. (1999).The potential anticarcinogenic conjugated linoleic acid,cis-9, trans-11 C18: 2, in milk of different species: cow, goat, ewe, sow, mare, woman. *Nutrition Research*, *19*(10), 1541-1549.

Jay, J. M. (2005). Microbiologia de Alimentos, sixth ed. Artmed, Porto Alegre, pp. 711.

Jenkins, J. K., & Courtney, P. D. (2003). Lactobacillus growth and membrane composition in the presence of linoleic or conjugated linoleic acid. *Canadian Journal of Microbiology*, *49*(1), 51-7. https://doi.org/10.1139/w03-003

Jenkins, T. C., Wallace, R. J., Moate, P. J., & Mosley, E. E. (2008). Board-Invited Review: Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. *Journal of Animal Science*, *86*(2), 397-412. https://doi.org/10.2527/jas.2007-0588

Jiang, J., Björck, L., & Fondén, R. (1998). Production of conjugated linoleic acid by dairy starter cultures. *Journal of Applied Microbiology*, *85*(1), 95-102. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9721660

Katsiari, M. C., Voutsinas, L. P., & Kondyli, E. (2002). Manufacture of yoghurt from stored frozen sheep's milk. *Food Chemistry*, *77*(4), 413-420. https://doi.org/10.1016/S0308-8146(01)00367-3

Kepler, C. R., Hirons, K. P., & Tove, S. B. (1966). Intermediates and Products of the Biohydrogenation of Linoleic Acid by Intermediates of Linoleic and Products of the Biohydrogenation Acid by Butyrivibrio fibrisolvens. *Journal of Biological Chemistry*, *241*(6), 1350-1354.

Khosravi, A., Safari, M., Khodaiyan, F., & Gharibzahedi, S. M. T. (2015). Bioconversion enhancement of conjugated linoleic acid by Lactobacillus plantarum using the culture media manipulation and numerical optimization. *Journal of Food Science and Technology*, *52*(9), 5781-5789. https://doi.org/10.1007/s13197-014-1699-6

Khosravi-Darani, K., Reihani, F. S., & Feili, R. (2014). Bioproduction of conjugated linoleic acid in yogurt by probiotic bacteria. *International Journal of Biotechnology for Wellness Industries*, *3*(2), 62-68.

Kim, Y. J., & Liu, R. H. (2002). Increase of conjugated linoleic acid content in milk by fermentation with lactic acid bacteria. *Journal of Food Science*, *67*(5), 1731-1737. https://doi.org/10.1111/j.1365-2621.2002.tb08714.x

Kishino, S., Ogawa, J., Omura, Y., Matsumura, K., & Shimizu, S. (2002). Conjugated Linoleic Acid Production from Linoleic Acid by Lactic Acid Bacteria. *Jaocs*, 159-163.

Kuhl, G. C., & Lindner, J. D. D. (2016). Biohydrogenation of Linoleic Acid by Lactic Acid Bacteria for the Production of Functional Cultured Dairy Products : A Review, 1-11. https://doi.org/10.3390/foods5010013

Lin, T. Y. (2000). Conjugated linoleic acid concentration as affected by lactic cultures and additives. *Food chemistry*, *69*(1), 27-31. https://doi.org/10.1016/S0308-8146(99)00218-6

Lin, T. Y., Lin, C. W., & Lee, C. H. (1999). Conjugated linoleic acid concentration as affected by lactic cultures and added linoleic acid. *Food Chemistry*, *67*(1), 1-5. https://doi.org/10.1016/S0308-8146(99)00077-1

Lucatto, J. N., Brandão, S. N. T. G. de, & Drunkler, D. A. (2014). Ácido Linoleico Conjugado: Estrutura Química, Efeitos Sobre a Saúde Humana E Análise Em Lácteos. *Revista Do Instituto de Laticínios Cândido Tostes*, *69*(3), 199. https://doi.org/10.14295/2238-6416.v69i3.282

Luo, X., Zhang, L., Li, H., Zhang, S., Jiao, Y., Wang, S., … Fan, R. (2013). Comparison of enzymatic activity of two linoleic acid isomerases expressed in E. coli. *Molecular Biology Reports*, *40*(10), 5913-5919. https://doi.org/10.1007/s11033-013-2698-9

MacDonald, H. B. (2000). Conjugated linoleic acid and disease prevention: a review of current knowledge. *Journal of the American College of Nutrition*, *19*(2), 111S-118S. https://doi.org/10.1080/07315724.2000.10718082

MacOuzet, M., Lee, B. H., & Robert, N. (2009). Production of conjugated linoleic acid by probiotic Lactobacillus acidophilus La-5. *Journal of Applied Microbiology*, *106*(6), 1886-1891. https://doi.org/10.1111/j.1365-2672.2009.04164.x

Malinska, H., Hüttl, M., Oliyarnyk, O., Bratova, M., & Kazdova, L. (2015). Conjugated linoleic acid reduces visceral and ectopic lipid accumulation and insulin resistance in chronic severe hypertriacylglycerolemia. *Nutrition*, *31*(7-8), 1045-1051. https://doi.org/10.1016/j.nut.2015.03.011

Mayer, H. K., & Fiechter, G. (2012). Physical and chemical characteristics of sheep and goat milk in Austria. *International Dairy Journal*, *24*(2), 57-63. https://doi.org/10.1016/j.idairyj.2011.10.012

Park, Y. W. (2007). Rheological characteristics of goat and sheep milk. *Small Ruminant Research*, *68*(1-2), 73-87. https://doi.org/10.1016/j.smallrumres.2006.09.015

Prandini, A., Sigolo, S., Tansini, G., Brogna, N., & Piva, G. (2007). Different level of conjugated linoleic acid (CLA) in dairy products from Italy. *Journal of Food Composition and Analysis*, *20*(6), 472-479. https://doi.org/10.1016/j.jfca.2007.03.001

Rodríguez-Alcalá, L. M., Braga, T., Xavier Malcata, F., Gomes, A., & Fontecha, J. (2011). Quantitative and qualitative determination of CLA produced by Bifidobacterium and lactic acid bacteria by combining spectrophotometric and Ag+-HPLC techniques. *Food Chemistry*, *125*(4), 1373-1378. https://doi.org/10.1016/j.foodchem.2010.10.008

Serafeimidou, A., Zlatanos, S., Kritikos, G., & Tourianis, A. (2013). Change of fatty acid profile, including conjugated linoleic acid (CLA) content, during refrigerated storage of yogurt made of cow and sheep milk. *Journal of Food Composition and Analysis*, *31*(1), 24-30. https://doi.org/10.1016/j.jfca.2013.02.011

Serafeimidou, A., Zlatanos, S., Laskaridis, K., & Sagredos, A. (2012). Chemical characteristics, fatty acid composition and conjugated linoleic acid (CLA) content of traditional Greek yogurts. *Food Chemistry*, *134*(4), 1839-46. https://doi.org/10.1016/j.foodchem.2012.03.102

Shantha, N. C., & Decker, E. A. (1993). Conjugated linoleic acid concentrations in processed cheese containing hydrogen donors, iron and dairy-based additives. *Food Chemistry*, *47*(3), 257-261. https://doi.org/10.1016/0308-8146(93)90158-C

Stachowska, E., Siennicka, A., Baśkiewcz-Hałasa, M., Bober, J., Machalinski, B., & Chlubek, D. (2012). Conjugated linoleic acid isomers may diminish human macrophages adhesion to endothelial surface. *International Journal of Food Sciences and Nutrition*, *63*(1), 30-5. https://doi.org/10.3109/09637486.2011.593505

Terán, V., Pizarro, P. L., Zacarías, M. F., Vinderola, G., Medina, R., & Van Nieuwenhove, C. (2015). Production of conjugated dienoic and trienoic fatty acids by lactic acid bacteria and bifidobacteria. *Journal of Functional Foods*, *19*, 417-425. https://doi.org/10.1016/j.jff.2015.09.046

Thuillier, P., Pande, N. T., Ghena, A., Song, S., Lawrence, Y., Shridhar, V., … Olson, S. (2013). Dietary Conjugated Linoleic Acids Arrest Cell Cycle Progression and Prevent Ovarian Cancer Xenografts Growth Suggesting a Trans-10 Cis-12 Isoform Specific, *2013*(May), 33-42.

Trigueros, L., & Sendra, E. (2015). Fatty acid and conjugated linoleic acid (CLA) content in fermented milks as assessed by direct methylation. *LWT - Food Science and Technology*, *60*(1), 315-319. https://doi.org/10.1016/j.lwt.2014.09.053

Van Nieuwenhove, C. P., Oliszewski, R., González, S. N., & Pérez Chaia, A. B. (2007). Conjugated linoleic acid conversion by dairy bacteria cultured in MRS broth and buffalo milk. *Letters in Applied Microbiology*, *44*(5), 467-474. https://doi.org/10.1111/j.1472-765X.2007.02135.x

Visentainer, J. V. (2012). Aspectos analíticos da resposta do detector de ionização em chama para ésteres de ácidos graxos em biodiesel e alimentos. *Quimica Nova*, *35*(2), 274-279. https://doi.org/10.1590/S0100-40422012000200008

Voutsinas, L. P., Katsiari, M. C., Pappas, C. P., & Mallatou, H. (1996). Production of yoghurt from sheep's milk which had been concentrated by reverse osmosis and sensory and physical characteristics of yoghurt, *29*, 411-416.

Wang, Y., Delettre, J., Guillot, A., Corrieu, G., & Béal, C. (2005). Influence of cooling temperature and duration on cold adaptation of Lactobacillus acidophilus RD758. *Cryobiology*, *50*(3), 294-307. https://doi.org/10.1016/j.cryobiol.2005.03.001

Xu, S., Boylston, T. D., & Glatz, B. A. (2005). Conjugated linoleic acid content and organoleptic attributes of fermented milk products produced with probiotic bacteria. *Journal of Agricultural and Food Chemistry*, *53*(23), 9064-72. https://doi.org/10.1021/jf051030u

Xu, S., Boylston, T. D., & Glatz, B. A. (2004). Effect of Lipid Source on Probiotic Bacteria and Conjugated Linoleic Acid Formation in Milk Model Systems. *Journal of the American Oil Chemists' Society [H.W. Wilson - AST]*, *81*(6), 589-595.

Ye, S., Yu, T., Yang, H., Li, L., Wang, H., Xiao, S., & Wang, J. (2013). Optimal culture conditions for producing conjugated linoleic acid in skim-milk by co-culture of different Lactobacillus strains. *Annals of Microbiology*, *63*(2), 707-717. https://doi.org/10.1007/s13213-012-0523-7

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Chromatographic Methods for Coffee Analysis: A Review

Alexander Yashin[1], Yakov Yashin[1], Xiaoyan Xia[2] & Boris Nemzer[2,3]

[1]International Analytical Centre of Zelinsky Institute of Organic Chemistry of Russian, Academy of Science, Moscow, Russia

[2]Department of Research & Development, VDF FutureCeuticals, Inc., Momence, IL, USA

[3]Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Correspondence: Boris Nemzer, FutureCeuticals, Inc., 2692 N. State Rt. 1-17, Momence, IL, USA. Tel: 812-507-1427. E-mail: bnemzer@futureceuticals.com

Received: April 27, 2017 Accepted: June 2, 2017 Online Published: June 17, 2017

doi:10.5539/jfr.v6n4p60 URL: https://doi.org/10.5539/jfr.v6n4p60

Abstract

Coffee has been one of the most commercialized food products and most widely researched beverage in the world for decades. It is considered a functional food, primarily due to its high content of compounds that exert antioxidant and other beneficial biological properties. This review summarized the data from analysis of coffee components, both volatile constituents and non-volatile high-molecular weight compounds performed by various chromatographic methods. A list of compounds identified by gas chromatography with mass spectrometry which define the aroma of coffee is provided. Publications on the measurement of methylxanthines (caffeine, theobromine, and theophylline), chlorogenic acids, diterpenes, sugars, amino acids, gamma-aminobutyric acid, dibasic acids, anions, and other compounds by HPLC and UHPLC-MS are reviewed. An overview of publications on the determination of organic contamination in coffee (PAHs, acrylamides, mycotoxins, etc.) and ways to reduce contamination through production technology and brewing methods are presented. Finally, an overview of the literature on authentication assessment for different grades of coffee grown in different regions is provided.

Keywords: coffee, chemical composition, chlorogenic acid, caffeine, GC, HPLC

1. Introduction

Coffee has been studied for many years and the research varied depending on the needs: pests, nutrition, pruning, harvesting, processing and quality. Recent studies have shown that the moderate intake of coffee reduces body fat and decreases oxidative damage-related diseases, such as type 2-diabets, cardiovascular, Alzheimer's and Parkinson's disease (Bakuradze et al., 2011; Freedman et al., 2012, O'Keefe et al., 2013). Erdem et al. (2016) indicated that cinnamic acid derivatives have been related with different biological effects including anti-inflammatory, antioxidant, anticarcinogenic, or neuroprotective activities. Coffee has a complex chemical composition and the compounds which include volatile and non-volatile compounds, diterpenes, sugar, amino acids and organic pollutants are important for coffee flavor, quality and health effects. This review article will focus on the chemical composition of coffee and their analytical techniques majorly by different chromatographic methods. We will also introduce some compounds found from different coffee processing procedures and their analytical techniques.

2. Chromatographic Methods Used to Determine the Chemical Composition of Coffee

Coffee contains over two thousand components—from volatile low-molecular weight to high-molecular weight compounds (Morton and Macleod, 1986). Various chromatographic methods have been used to separate and identify all kinds of mixtures, from gases and volatile compounds to high-molecular weight compounds, as well as mixtures of organic and inorganic ions (cations and anions). Therefore, almost all components of coffee can be detected by chromatographic methods. The components in a mixture are first separated on columns by different adsorption rates, and then the separated components are registered at the column outlet with detectors.

Gas chromatography has been used to separate and identify volatile and semi-volatile compounds in coffee. It is important that these compounds do not decompose during the evaporation process. Compounds with molecular

weights up to 500-600 can be identified by gas chromatography. Liquid chromatography has been used to separate and identify non-volatile high-molecular weight compounds at temperatures close to room temperature. Therefore, even unstable compounds can be determined by liquid chromatography. For example, six quality markers in coffee, which are caffeine, trigonelline, nicotinic acid, N-methylpyridinium, 5-caffeoylquinic acid, and 5-hydroxyfurfural were simultaneously detected for three coffee matrices—green, roasted, and soluble—with the limit of quantification 0.069-0.71 μg/ml by HPLC-DAD. This technique is useful for routine determination of the thermal degradation rate during the roasting process (Gant, Leyva, Gonzalez & Maruenda, 2015). Ion chromatography and ion exchange chromatography methods utilize columns filled with ion exchangers to separate ion mixtures. In thin layer chromatography, separation occurs not on columns but on plates made of glass, metal, or polymer coated with a thin layer of adsorbent material. Capillary electrophoresis techniques employ quartz capillaries with an inner diameter of 0.1 mm, or less. Mixtures may be separated due to the electrophoretic mechanism alone (capillary zone electrophoresis), or due to electrophoretic and chromatographic mechanisms at the same time. In the latter case, a layer of adsorbent is applied on the internal walls of the capillary, or sorbent (adsorbent) is added to the mobile phase.

Gas chromatography with mass spectrometry (GC-MS) and liquid chromatography with mass spectrometry (HPLC-MS) have recently come into wide use to identify the components of the separated compounds (i.e., to conduct qualitative analysis). Table 1 provides a list of chromatography methods used to analyze coffee (based on publications).

Table 1. Chromatographic methods used for the analysis of coffee

No.	Methods	References
1.	Gas chromatography (GC)	Lercker et al, 1995; Kolling-Speer et al., 1999; Holscher and Steinhart, 1992; Sanz et al., 2001; Bicchi et al., 1997.
2.	Gas chromatography with mass spectrometry	Dirinck et al., 2001; Costa-Freitas et al., 2001.
3.	Two-dimensional gas chromatography	Ryan et al., 2004
4.	Gas chromatography in coupled with head-space analysis	Semmeirich and Grosh, 1995; Maetzu et al., 2001; Procida et al., 1997; Bücking and Steinhart, 2002; Rocha et al., 2004.
5.	Pyrolysis gas chromatography	Harada et al., 1987
6.	High performance liquid chromatography (HPLC)	de Andrade et al., 1995; Casal et al., 2002; Bispo et al., 2002; Meger et al., 1996; Dias et al., 2010.
7.	HPLC-mass spectrometry	Kurzrock and Speer, 2001; Ventura et al., 2003.
8.	HPLC-MS/MS	Trugo, 1984
9.	Gel-filtration chromatography	Daglia et al., 2004.
10.	Micellar chromatography	Perez-Martinez et al., 1995
11.	Ion chromatography	Gennano and Abrigo, 1992
12.	Thin layer chromatography	Levi, 1975

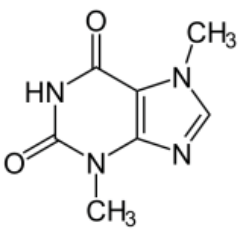
In addition to individual chromatographic methods, coffee has been analyzed by various combinations of chromatography methods, such as HPLC and GC, capillary electrophoresis, spectrophotometric methods, and ‘electronic nose’ and ‘electronic tongue’ systems. Table 2 provides a list of compounds which were identified in coffee (except the volatile compounds). Various estimates suggest that coffee contains 2000-3000 compounds. Table 2 shows only useful components that determine nutritional value of coffee: sugars, amino acids, fatty acids, vitamins, antioxidants, trace elements etc.

Table 2. List of compounds identified in coffee and typical analytical methods

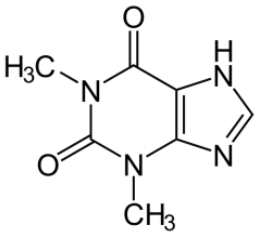
No.	Compounds	Typical concentration	Comment	Analytical methods	References
1	Chlorogenic acids	0.6-26.4%	More than 10 types of acids	HPLC, LC/MS	Trugo et al., 1984; Clifford, 2000; Mullen et al., 2011
2	Caffeine, theobromine, theophylline	3-350μg/mL		HPLC, LC/MS	Wanyika et al., 2010; Bispo et al., 2002; Eanyika et al, 2010
3	Trigonelline	3-10 mg/g		HPLC, LC/MS	Ky et al., 2001; Casal et al., 2000
4	Carbohydrates	3.41-9.43%	Saccharose, glucose, fructose, arabinose, galactose	IC	Knopp et al., 2006
5	Amino acids	4.4-1075 mg per 100g coffee powder	16 amino acids	IC, HPLC, LC/MS	Nakhmedov et al., 1984; Bytof et al., 2005
6	Vitamins	less than 3mg	Vitamin B_1, riboflavin (B_2), nicotinic acid (PP), pyridoxine (B_6), tocopherol (E), vitamin B_{12}	HPLC	Clarke, 1985; O'Driscoll, 2014
7	γ-aminobutyric acid	30-1860 mg/kg		HPLC	Bytof et al., 2005, Kramer et al., 2010
8	Serotonin	about 10 mg/g dry weight	'happiness hormone'	HPLC	Kele and Ohmacht, 1996
9	Organic acids	about 1%	Citric, malic, oxalic, acetic acids, etc.	HPLC	Kele and Ohmacht, 1996; Mabrok and Deatheroge, 1956
10	Anions	phosphates 0.2%, sulfates 0.1%	Fluoride, chloride, nitrate, sulfate, phosphate	IC	Mabrok and Deatheroge, 1956
11	Oxyaromatic acids	3-6%	Ferulic, n-coumaric, 3,4-dimethoxycinnamic, 3,4,5-trimethoxycinnamic, sinapic acids	HPLC	Clarke, 1985; Clifford, 2000; Murata et al., 1995
12	Tannins	3.6-7.7%		Spectrophotometry	Clarke, 1985; Savolainen, 1992
13	Polysaccharides	over 12%	Cellulose, pectic substances, fibers	IC, HPLC	Clarke, 1985; Moreira et al., 2012
14	Melanoidins	5-60 g/100g	Dark brown natural coloring agent	HPLC, DPPH assay	Clarke, 1985; Moreira et al., 2012; Pérez-Hernández et al., 2012
15	Mineral substances	3-4.5%	Potassium, magnesium, calcium, sodium, iron, manganese, zinc, copper	ICP/MS	Clarke, 1985; Abdulmadjid et al., 2017

3. Structural Formulas and Primary Compounds in Coffee

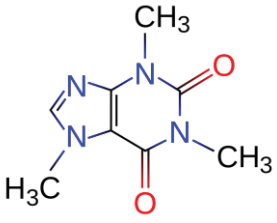
The structures of primary compounds in coffee are presented below (Fig. 1): caffeine, theobromine, theophylline; Trigonelline; caffeic, ferulic, protocatechuic acids; chlorogenic acids; gamma-isobutyric acid and diterpenes.



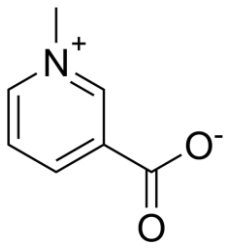
Theobromine



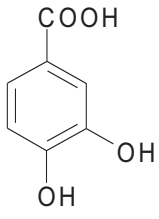
Theophylline



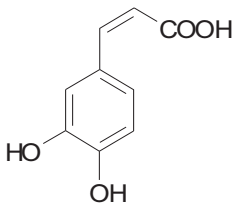
Caffeine



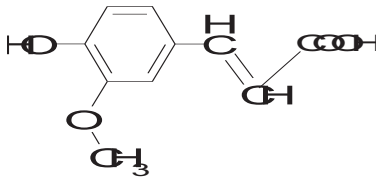
Trigonelline



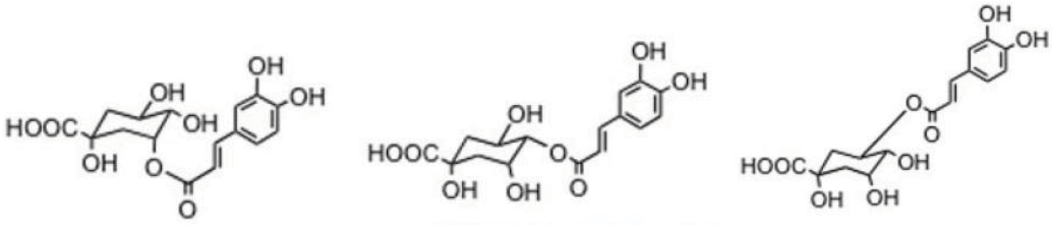
Protocatechuic acid



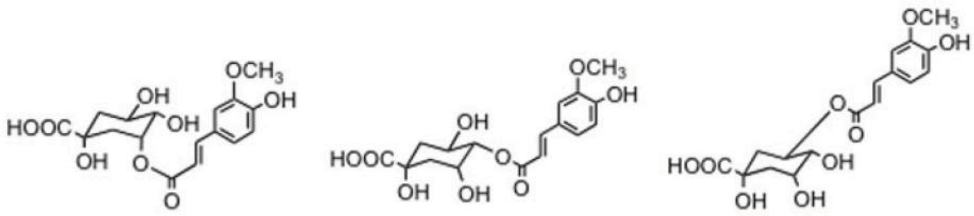
Caffeic acid



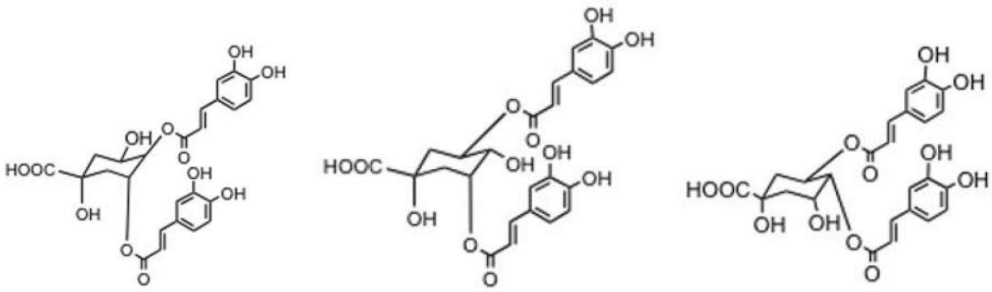
Ferulic acid



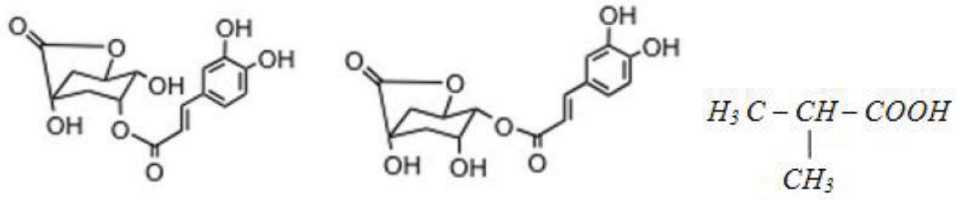
3-O-caffeoylquinic acid 4-O-caffeoylquinic acid 5-O-caffeoylquinic acid



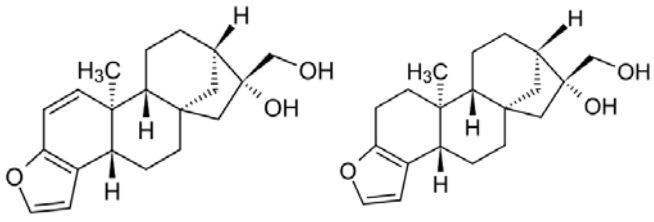
3-O-feruloylquinic acid 4-O-feruloylquinic acid 5-O-feruloylquinic acid



3,4-O-dicaffeoylquinic 3,5-O-dicaffeoylquinic 4,5-O-dicaffeoylquinic



3-O-caffeoyl-1,5-quinate 4-0-caffeoyl-1,5-quinate Gamma-isobutyric acid



Kahweol Cafestol

Figure 1. The structures of primary compounds in coffee

As one of the major primary compounds, chlorogenic acid was first isolated from coffee beans in 1908, and their structure was determined in 1932. Chlorogenic acids are mono- or diesters of quinic and cinnamic acids. Coffee contains more than fifteen chlorogenic acids along with their derivatives (lactones). Recent research indicated that the chlorogenic acid profiles of whole coffee fruits were influenced by the extraction procedures (Mullen et al., 2011; Craig et al., 2016)

Coffee is one of the richest sources of chlorogenic and other oxyaromatic acids. Due to its health effects, chlorogenic acid consumption rate can be up to 1 g per day (Murata, Okada & Homma, 1995). Eleven chlorogenic acids have been detected by three-dimensional HPLC. Farah et al. (2008) studied chlorogenic acids in extracts of green and decaffeinated coffee and identified 9 major and 17 minor components. Shan et al. (2016) simultaneous determined different chlorogenic acids in green coffee bean extracts with effective relative response factors. Köseoglu Yilmaz & Kolak (2017) used a new solid phase extraction method (hydrophilic-lipophilic balance cartridges) combined with HPLC analysis to perform chlorogenic acid analysis recently. According to some publications, coffee is a major nutritional source of oxyaromatic acids for its consumers (Clifford, 2000; Murata, Okada, & Homma 1995). Besides chlorogenic acid isomers (main isomer is 5-caffeoylquinic acid) and their diesters, some other hydroxycinnamic acid conjugates were also identified (Clifford, 2000) in coffee. The recognized chlorogenic acids and their derivatives, as well as their content in coffee are provided in Tables 3, 4 and 5.

Table 3. List of main chlorogenic acids (Clifford, 2000)

No.	Proper Chemical Names of Acids	Simplified Chemical Names of Acids
1.	Caffeoyl-3-quinic	Chlorogenic
2.	4,5-dicaffeoylquinic	Isochlorogenic A
3.	3,4-dicaffeoylquinic	Isochlorogenic B
4.	3,5-dicaffeoylquinic	Isochlorogenic C
5.	Caffeoyl-5-quinic	Neochlorogenic

Table 4. List of main chlorogenic acids and their derivatives contained in coffee (Clifford, 2000)

No.	Acid Name	Typical value, %
1	5-0-caffeoylquinic	26.4
2	4-0-caffeoylquinic	17.3
3	3-0-caffeoylquinic	16.0
4	5-0-feruloylquinic	14.9
5	4-0-feruloylquinic	4.9
6	3-0-feruloylquinic	4.4
7	5-0-n-coumaroylquinic	0.7
8	4-0-n-coumaroylquinic	0.9
9	3,4-0-dicaffeoylquinic	1.3
10	3,5-0-dicaffeoylquinic	0.6
11	3-0-caffeoylquinic acid lactone	7.5
12	4-0-caffeoylquinic acid lactone	5.1

Table 5. Content of the main chlorogenic acids in extracts of green and decaffeinated coffee (Farah et al., 2008)

No.	Chlorogenic Acids and Their Derivatives	μmol per 0.4g
1	5-caffeoylquinic	119.8±0.23
2	3-caffeoylquinic	103.3±0.14
3	4-caffeoylquinic	97.4±1.2
4	3,4-dicaffeoylquinic	16.8±0.01
5	4,5-dicaffeoylquinic	16.2±0.32
6	3,5-dicaffeoylquinic	10.2±0.06
7	5-feruloylquinic	22±0.02
8	3-feruloylquinic	20.7±0.69
9	4-feruloylquinic	16.4±0.30
10	5-n-coumaroylquinic	1.7±0.01
11	3-n-coumaroylquinic	1.1±0.28
12	diferuloylquinic (1 isomer)	1.3±0.01
13	caffeoylferuloylquinic acid (6 isomers)	11.8±1.33
14	caffeoyltryptophan	10.5±0.15
15	4-caffeoylquinate	0.3±0.01
16	3- feruloylquinate	0.3±0.01
17	4- feruloylquinate	0.1±0.03
18	3,4-dicaffeoylquinate	0.03±0.004

4. Composition of Volatile Components in Coffee

In the early twentieth century little was known about coffee aromatic components. Later, different compounds such as methylamine, ammonia, trimethylamine, pyrrole, pyridine, acetone, formic and valeric acids, furfural, furfuryl alcohol were identified by classic chemical methods.

In the 1920s, 29 more compounds were detected with the financial support of the Government of Switzerland (Morton and McLeod, 1986).The most typical compounds were furfuryl mercaptan, α-diketones and alkylpyrazines.

Twenty-seven extra compounds were identified between 1929 and 1962. From 1963 to 1982, more than 600 components were identified by gas capillary chromatography. Different classes of compounds (Table 6) which determine aroma of roasted coffee were identified by Morton & MacLeod (1986). Primary compounds which determine coffee aroma have been study by Ky et al. (2001) (Table 7).

Table 6. Identified classes of compounds which determine aroma of roasted coffee (Morton & MacLeod, 1986)

No.	Classes of Compounds	Number of identified compounds
1.	Furans	99
2.	Pyrazines	79
3.	Ketones	70
4.	Pyrroles	67
5.	Hydrocarbons	50
6.	Phenols	42
7.	Esters	29
8.	Aldehydes	28
9.	Thiazoles	28
10.	Oxazoles	27
11.	Amines and nitrogen-containing compounds	24
12.	Thiophenes	26
13.	Acids	20
14.	Alcohols	20
15.	Sulfides and sulfur-containing compounds	16
16.	Pyridines	13
17.	Lactones	8
18.	Other compounds	9
	Total:	655

Table 7. Primary compounds which determine coffee aroma (Ky et al., 2001)

No.	Compounds	No.	Compounds
1.	β-myrcene	31.	2-thiophenecarbaldehyde
2.	limonene	32.	3-methyl-2-thiophenecarbaldehyde
3.	n-cumene	33.	5-methyl-2-thiophenecarbaldehyde
4.	naphthalene	34.	2-acetylthiophene
5.	1-methylnaphthalene	35.	2-propionylthiophene
6.	2-methylnaphthalene	36.	2-acetyl-3-methylthiophene
7.	2-ethylnaphthalene	37.	2-acetyl-5-methylthiophene
8.	biphenyl	38.	1-(2-thienyl)-1,2-propanedione
9.	3-methylbiphenyl	39.	phenyl formate
10.	methylol	40.	methyl 2-thiophenecarboxylate
11.	α-terpineol	41.	4,5-dihydro-2(3H)thiophenone
12.	2-methylbenzaldehyde	42.	4,5-dihydro-2(2H)thiophenone
13.	2-heptanone	43.	2-methyl-4,5-dihydro-3(2H)-thiophenone
14.	2-undecanone	44.	2,4-dimethyloxazole
15.	3-methyl-2-hydroxy-2-cyclo-pentane-1-one	45.	4,5-dimethyloxazole
16.	geranylacetone	46.	2,4,5-trimethyloxazole
17.	β-damascenone	47.	2-ethyl-4,5-dimethyloxazole
18.	catechol	48.	2-methylbenzoxazole
19.	hydroquinone	49.	2-phenyloxazole
20.	guaiacol	50.	5-acetyl-2- methyloxazole
21.	n-methylguaiacol	51.	5-acetyl-2,4-dimethyloxazole
22.	n-ethylguaiacol	52.	methanethyl
23.	n-vinylguaiacol	53.	dimethylsulfide
24.	eugenol	54.	dimethyl disulfide
25.	isoeugenol	55.	dimethyl trisulfide
26.	thiophene	56.	3,3-dimethyl-1,2-dithiolane
27.	3-methylthiophene	57.	3,3-dimethyl-1,2-dithiolane-4-one
28.	4-ethyl-2-methylthiophene	58.	2-methyl-3-oxa-8-thiabicyclo[3,3,0]-1,4-octadiene
29.	benzothiophene	59.	2,4-dimethyl-3-oxa-8-thiabicyclo[3,3,0]-1,4-octadiene
30.	thiopheno [3,2-δ] thiophene		

In one study (Maeztu et al., 2001), aroma of espresso coffee from different botanical varieties and types of roast were investigated. Aroma components were determined by gas chromatography with mass spectrometry, volatile components were extracted by static headspace. Seventy-seven compounds were identified in all samples (Table 8). Among them, 13 key odorants have the greatest effect on coffee aroma (Maeztu et al., 2001) (Table 9). Chromatographic analyses were comparable with the sensory flavor profile. Aldehydes were found to correlate with a fruity aroma, diones with buttery aroma, and pyrazines with earthy burnt aroma.

Similar studies were conducted on samples of coffee Arabica (Colombia) and coffee Robusta (Indonesia) (Bücking & Steinhart, 2002). Analyses were performed by gas chromatography, flame ionization detection (FID), mass spectrometry, olfactometry, and specialized headspace.

These investigations showed that approximately 30 volatile compounds (Table 10) were substantially responsible for the coffee aroma. Most typical volatile compounds are provided in the literature (Vitenberg & Ioffe, 1984). The study also investigated how milk added to coffee influenced coffee aroma. The aroma intensity decreased due to milk lipids, proteins, and carbohydrates, however, aroma profile remained the same.

In recent years, a combination of GC-GC-FID with solid-phase microextraction was used for analysis of volatile compounds. In one study (Ryan et al., 2004), 44 volatile compounds were identified by two-dimensional chromatography (the first column with a polar phase and the second with a non-polar phase). Coffee was also analyzed by a time-of-flight mass spectrometer in this study. The coffee aroma was found to contain various classes of chemical compounds (ketones, tyrosines, furans, phenols, pyrroles, etc.). Bressanello et al. (2017) correlated the chemical composition of the coffee volatile fraction to its sensory properties. The chemical information concerning coffee aroma and flavor obtained with HS-SPEM of the ground coffee and in-solution

Table 8. Volatile compounds identified in aroma of coffee espresso (Maeztu et al., 2001)

No.	Compound name	No.	Compound name
	Alkenes		Ketones
1.	1,3-pentadiene	31.	2-propanone
	Sulfur compounds	32.	2-butanone
2.	Methanethiol	33.	2,3-butanedione
3.	Dimethylsulfide	34.	3-hexanone
4.	Dimethyl disulfide	35.	2,3-pentanedione
	Aldehydes	36.	3,4-hexanedione
5.	Acetaldehyde		Alcohols
6.	Propanal	37.	2-methyl-1-propanol
7.	2-methylpropanal	38.	2-methylbutan-1-ol
8.	Butanal	39.	3-methyl-3-buten-1-ol
9.	3-methylbutanal	40.	3-methyl-2-buten-1-ol
10.	2-methylbutanal	41.	2-ethyl-1-hexanol
11.	Hexanal		Thiophenes
	2-methyl-2-butanal	42.	Thiophene
12.	Esters	43.	2-methylthiophene
	Formic acid methyl ester		Pyrroles
13.	Acidic acid methyl ester	44.	1-methylpyrrole
	Acidic acid ethyl ester	45.	1-ethyl-1H-pyrrole
14.	Propionic acid methyl ester	46.	2,5-dimethylpyrrole
	1-hydroxy-2-propanone acetate		Pyridines
15.	Furans	47.	Pyridine
	Furan	48.	2-methylpyridine
16.	3-methylfuran	49.	3- ethylpyridine
	2-methylfuran		Pyrazines
17.	2,5-dimethylfuran	50.	Pyrazine
18.	2-vinylfuran	51.	2-methylpyrazine
19.	2-vinyl-5-methylfuran	52.	2,5-dimethylpyrazine
20.	2-methoxymethylfuran	53.	2,6-dimethylpyrazine
21.	2-methyltetrahydrofurol-3-one	54.	Ethylpyrazine
22.	2-furancarboxaldehyde	55.	2,3-dimethylpyrazine
23.	2-furfurylmethylsulfide	56.	N-propylpyrazine
24.	furfuryl formate	57.	2-vinylpyrazine
25.	2-acetylfuran	58.	2-methyl-6-vinylpyrazine
26.	2- furfurylfuran		Thiazoles
27.	Furfuryl alcohol	59.	1,3-thiazole
28.	Furfuryl acetate	60.	4-methylthiazole
29.	5-methylfurfural		Acids
30.	2-furfurylfuran	61.	Acetic acid
			Lactones
		62.	γ-butyrolactone
			Phenolic compounds
		63.	2-methoxyphenol (guaiacol)

Table 9. Relative ratio of key odorants in coffee espresso (Maeztu et al., 2001)

Compound name	% (relative)	
	Arabica	Robusta
Sulfur compounds		
Methanethiol	0.13 ± 0.01	0.08 ± 0.01
Aldehydes		
Acetaldehyde	0.36 ± 0.02	0.35 ± 0.04
Propanal	0.32 ± 0.07	0.50 ± 0.07
2-methylpropanal	1.80 ± 0.25	2.55 ± 0.35
3-methylpropanal	1.25 ± 0.11	2.33 ± 0.34
3-methylbutanal	2.61 ± 0.29	3.33 ± 0.56
Hexanal	0.05	0.06
Ketones		
2,3-butanedione	0.42 ± 0.04	0.36 ± 0.04
2,3-pentanedione	0.63 ± 0.04	0.42 ± 0.03
Pyrazines		
2-ethyl pyrazine	0.10 ± 0.02	0.17 ± 0.01
2-ethyl-6-methylpyrazine	0.06 ± 0.01	0.13 ± 0.02
2-ethyl-3,5-dimethylpyrazine	0.04 ± 0.01	0.07 ± 0.01
Phenolic compounds		
Guaiacol	0.11 ± 0.01	0.09 ± 0.01

Table 10. Identified coffee odorants (Bücking and Steinhart, 2002)

No.	Compound Name	Type of Aroma
1.	Methanethiol	putrefactive
2.	Dimethylsulfide	putrefactive
3.	2-methylpropanal	aroma of cocoa
4.	2,3-butanedione	buttery
5.	3-methylbutanal	malt-like
6.	2-methylbutanal	fruity
7.	2,3-pentanedione	oily
8.	Hexanal	aroma of leaves
9.	2,3-methylbutyric acid	sweetish
10.	3- methyl-2- buten-1 thiol	putrefactive
11.	Methional	potato-like
12.	2-furfurylthiol	aroma of roasted coffee
13.	1-octen-3-one	mushroom-like
14.	2,3,5-trimethylpyrazine	frying
15.	3-mercapto-2-methylbutyl-formate	putrefactive
16.	phenylacetaldehyde	honey-like
17.	2-ethyl-3,5-dimethylpyrazine	earthy/frying
18.	Guaiacol	phenolic/burnt
19.	2-isopropyl 3-methoxypyrazine	earthy/frying
20.	(E)-2 nonenal	cucumber-like
21.	2-isobutyl-2- methoxypyrazine	aroma of sweet pepper

SBSE/SPME sampling combined with GC-MS to evaluate their compatibility with the cupping evaluation for quality control purposes. Yang et al. (2016) found that different temperature profiles for roasting can affect volatile aroma compounds associated with roast coffee defects (light, scorched, dark, baked and underdeveloped) by GC-MS with headspace solid phase micro extraction.

Volatile thiols are among the compounds that have the greatest impact on the flavor of coffee. Due to their extremely low odor thresholds, they have a significant sensory impact even at very low concentration. Thiols are formed during coffee roasting and are the key odorants influencing the sensory characteristics of coffee. Gas chromatography coupled to mass spectrometry is most frequently used technique for the analysis of coffee thiols.

On-column injection at low temperature has been applied in order to prevent thermal degradation of the thiols (Czerny, Mayer & Grosch, 1999, Dulsat-Serra, Quintanilla-Casas & Vichi, 2016)

5. Determination of Non-Volatile Components in Coffee

5.1 Simultaneous Determination of Caffeine, Theobromine, and Theophylline

Methylxanthines, i.e. 1,3,7-trimethylxanthine (caffeine), 3-dimethylxanthine (theobromine), and 1,3-dimethylxanthine (theophylline) in coffee were determined by reversed-phase chromatography HPLC with UV detector at 273 nm and a Bondesil C_{18} column (15×0.4 cm, particle size 5 μm) (Bispo et al., 2002). A mixture of methanol-water-acetic acid or ethanol-water-acetic acid in a ratio of 20:75:5 was used as an eluent. Optimum flow rate of the eluent was 0.7 mL min^{-1}. The detection limit achieved under these conditions was $1 \cdot 10^{-11}$g. Separation time was 10 min, the yield sequence was theobromine, theophylline, and caffeine. In the same study, the content of methylxanthines was determined in some beverages (coffee, tea, cocoa, and mate) and in human urine after consumption as well. Caffeine was determined to be within 0.1 pg (350 μg mL^{-1}), theobromine within 0.1 pg (32 μg mL^{-1}), and theophylline within 0.1 pg (47 μg mL^{-1}).

Table 11 shows the values of methylxanthines contained in various drinks and urine, as determined by HPLC.

Table 11. Concentrations of methylxanthines in beverages and urine (Bispo et al., 2002)

No.	Analyzed medium	Concentration, μg mL^{-1}		
		Caffeine	Theobromine	Theophylline
	Beverages			
1.	Roasted coffee	350.0	17.0	$<1 \cdot 10^{-7}$
2.	Decaffeinated coffee	26.0	13.0	47.0
3.	Instant coffee	122.0	12.0	15.0
4.	Black Tea	217.0	12.0	$<1 \cdot 10^{-7}$
5.	Cocoa	4.0	17.0	$<1 \cdot 10^{-7}$
6.	Mate	62.0	32.0	21.0
	Urine			
7.	Coffee	3-71	$1 \cdot 10^{-7}$	$1 \cdot 10^{-7}$

The International Olympic Committee (IOC) banned the use of beverages in such amounts which results in excess of the maximum permitted urinary caffeine level of 12 μg mL^{-1}. Caffeine at high concentrations leads to various disorders, such as increased secretion of acid gastric juice, kidney dysfunctions, nervous system disorders, arrhythmias, etc. The IOC does not restrict the use of theobromine and theophylline because these compounds have been used to treat asthma.

In one of the most recent studies (Wanyika, Gotebe, Gitu, Ngumba & Maritim, 2010), caffeine content was determined in certain instant coffee (Africafe, Nescafe and Dormans) by HPLC with a spectrophotometric detector at a wavelength of 278 nm determined. The 250×4.6 mm column with ODS sorbent was used; methanol-acetic acid-water (20:0.1:79.9) as mobile phase and flow rate was 1 mL min^{-1}. The results are Dormans 1.64%, Africafe 3.42% and Nescafe 3.12%. The caffeine content was also determined by the spectrophotometric method—the results were about twice as high. Demissie et al. (2016) used UV/VIS spectrometer to determine caffeine in green coffee beans from hararghe, Ethiopia, using beer-lamberts's law and integrated absorption coefficient techniques.

Caffeine is known to be a pharmacologically active compound which stimulates the central nervous system. Caffeine does not accumulate in the body and is excreted in the urine within a few hours after consumption.

In one study (Rajkovic et al., 2004), quality of coffee was analyzed by different analytical methods, and the content of caffeine, heavy metals and aflatoxins was determined. Caffeine was additionally determined by HPLC in accordance with the recommendations of US Food and Drug Administration. The conditions used were: 150×2.1 mm column with Symmetry Shield RP18, particle size 3 μm; acetonitrile-water (30:70) as eluent, flow rate 0.3 cm^3 min^{-1} (Rajkovic, Vukoivc & Moriera, 2004). 1.38%, 1.59%, and 1.61% of caffeine was detected in three different coffee samples. The total content of caffeine, phenolic compounds, and chlorogenic acids is shown in Table 12 (Trugo, de Maria, Moriera & Petracco, 1995).

Table 12. Composition of coffee samples (methanolic extracts) (Trugo et al., 1995)

Coffee samples	Total content of phenolic compounds, %	Content of caffeine, %	Content of chlorogenic acids, %
Instant coffee 1	15.14	2.36	23.8
Instant coffee 2	14.50	2.99	23.0
Light roasted coffee	5.41	1.37	16.9
Medium roasted coffee	5.25	1.62	19.2
Dark roasted coffee	5.70	1.48	15.5

Using ion chromatography and ion exchange chromatography, both inorganic (chloride, nitrate, sulfate, phosphate) and organic anions (acetate, formate, tartrate, oxalate, citrate) were determined in decaffeinated coffee on AS19 capillary column (250×0.4 mm), gradient eluent KOH, 30ºC, eluent flow rate 10 μL min^{-1}, conductometric detector and IonPak ICE-AS6 column, conductometric detector separately (Mabrok & Deatheroge, 1956).

5.2 Determination of Diterpenes (Kahweol and Cafestol) in Coffee

Many studies related to the effects of coffee consumption on human health are aimed at investigating its lipid composition such as coffee oil (Boekschotem, Engberink & Katan, 2003). Coffee oil contains pentacyclic diterpene alcohols. Coffee diterpenes are primarily esterified by various fatty acids. Fourteen cafestol derivatives and twelve kahweol derivatives were detected (Kurzrock & Speer, 2001). These compounds are partially preserved during the roasting process (Roos, Van Der Weg & Urgert, 1997). But they are also dehydrated which results in the formation of dehydrated derivatives along with other decomposition products, such as kahweol, cafestol, isokahweol, and dihydroisokahweol. Cafestol was found in both coffee Arabica and in coffee Robusta whereas kahweol was found only in coffee Arabica (Roos, Van Der Weg & Urgert, 1997). These diterpenes may contribute to the decomposition of toxic substances and provide a protective effect, particularly against aflatoxin B1 (Cavin, Holzhaeuser & Scharf, 2002). Moreover, they were noted to have anticarcinogenic, antioxidant, anti-inflammatory, and hepatoprotective properties (Kim, Hwang & Jeong, 2007; Lee, Choi & Jeong, 2007; Lee & Jeong, 2007). These beneficial effects of coffee oil create great opportunities for its use in the food and cosmetics industries. The use of coffee oil as a sunscreen agent in cosmetics has already been patented (Grollier & Plessis, 1998). However, the diterpenes, mainly cafestol, were also reported to have an undesired effect which tends to increase cholesterol levels (Kurzrock & Speer, 2001; Kim Hwang & Jeong, 2009; Urgert, Van Der Weg & Kosmeijer-Schuil, 1995). A reliable analytical method is necessary for the determination of synthetic metabolic pathways of these diterpenes. In addition, the determination of diterpene content can be used for classification of coffee mixtures, in particular as a means of establishing the difference between Arabica and Robusta.

Diterpene content in coffee drinks strongly depends on the way they were prepared. Espresso coffee may contain 5-10 times as much diterpenes as in filtered coffee (Urgert, Van Der Weg & Kosmeijer-Schuil, 1995; Ruiz del Castrillo, Herraiz & Blanch, 1999). The Arabica beans have the highest concentration of cafestol—11 mg per 100 g (Kölling-Speer, Strohschneider & Speer, 1999). In one study, diterpene content was determined in different parts of the coffee bean tissues (pericorp, perisperm, endosperm) (Dias, Campanha & Veira, 2010). Several methods were used for analysis and identification of diterpenes, i.e., Raman spectroscopy (Rubayiza & Meurens, 2005), GC (Urgert, Van Der Weg & Kosmeijer-Schuil, 1995; Ruiz del Castillo, Herraiz & Blanch, 1999; Pettitt, 1987), and HPLC (Kurzrock & Speer, 2001; Ruiz del Castillo, Herraiz & Blanch, 1999; Pettitt, 1987; Araujo & Sandi, 2006). The HPLC was used most often because this method allows the direct determination of components without derivatization, avoiding the decomposition of other lipid compounds. In HPLC, the 250×4.6mm reversed-phase columns filled with C_{18}, 5 μm, were used. Acetonitrile-water mixture at a ratio of 55-45% were used as eluents. A UV detector was used for detection; the absorption maximum was set at 290 nm for kahweol and at 230 nm for cafestol. Prior to the analysis, the diterpenes were extracted from coffee beans using organic solvents, such as n-hexane, diethyl ether, petroleum ether, followed by saponification by an alcoholic KOH solution. Diterpene compounds were also determined in Arabica coffee by HPLC-DAD (Erny, Moeenfard & Alves, 2015). In Arabica and Robusta coffee beverages, previously unknown compounds affecting their flavor were identified by high resolution HPLC-MS (Nascimento et al., 2015).

5.3 Determination of Sugars in Coffee

The content of low molecular weight sugars was investigated in green Arabica coffee beans which were obtained after flesh of the berries was separated from the seeds by wet or dry methods (Knopp, Bytof & Selmar, 2006).

Low content of fructose and glucose was noted in washed coffee beans (wet method). The content of these sugars was higher when coffee beans were dry processed. Laboratory models of these processing methods have confirmed the above results. Table 13 shows the content of monosaccharides in coffee samples of several manufactures from various countries. As can be seen from these data, saccharose typically has the highest content level.

Table 13. Concentration of the most common sugars in commercial green coffee samples (Knopp, Bytof & Selmar, 2006)

Type of green coffee and processing method	Country/Province	Content of sugars, %		
		Saccharose	Fructose	Glucose
Arabica (dry)	Brazil 1	9.25	0.14	0.04
	Brazil 2	8.70	0.15	0.04
	Brazil 3	7.60	0.02	0.01
	Ethiopia 1	8.26	0.17	0.04
	Ethiopia 2	6.30	0.01	0.01
	Mexico	9.64	0.05	0.03
	Honduras	6.92	0.05	0.03
Arabica (wet)	Colombia 1	8.76	0.06	0.04
	Colombia 2	8.07	0.01	0.01
	Colombia	7.16	0.20	0.01
	El Salvador	9.89	0.05	0.02
	Peru	8.21	0.05	0.02
	Kenya	9.31	0.06	0.03
	Cameroon	5.87	0.04	0.01
Robusta (dry)	Vietnam	3.15	0.16	0.10
	Ivory Coast	3.27	0.18	0.03
	Uganda	4.55	0.11	0.03
	Indonesia	4.85	0.18	0.06

Saccharose content is significantly lower in the Robusta than the Arabica. In the Arabica coffee, the saccharose content remains basically the same and does not depend either on the processing method or location where it was grown. A similar conclusion can be drawn with respect to glucose. However, the fructose content does depend both on the processing method and the location where it was grown. The fructose content in the Robusta coffee is higher compared with the Arabica, yet the glucose content is approximately the same in both grades. Typical content of sugars is provided in Table 14. Coffee processing technology can be ascertained by the content of fructose and glucose.

Table 14. The content of different sugars in the Arabica coffee beans (Brazil, Province Acaia) obtained by the dry processing method (Knopp, Bytof & Selmar, 2006)

Kind of Sugar	Content, %
Saccharose	7.07
Fructose	0.39
Glucose	0.23
Raffinose	0.06
Stachyose	0.04
Galactose Arabinose Rhamnose Mannose	0.01
Total:	7.79%

The content of sugars was determined by ion exchange chromatography on the Dionex PA20 column with amperometric detector at gold electrodes; NaOH solution as eluent and the flow rate was 0.5 mL min^{-1}.

5.4 Determination of Amino Acids in Coffee

Amino acids in coffee have been determined by a classic ion exchange chromatography in the form of

derivatives as well as by a direct method with amperometric detection (Nakhmedov, 1984). Amino acids were also separated by HPLC and ion-pair chromatography in the form of derivatives with ortho-phthalic dialdehyde by other researchers (Bytof et al., 2005). The following amino acids were identified in green coffee: glutamic acid, aspartic acid, serine, histidine, glycine, arginine, alanine, tyrosine, methionine, valine, norvaline, tryptophan, phenylalanine, isoleucine, leucine, lysine, etc with concentration range 4.4-1075 mg per 100g of coffee dry powder. The content of protein amino acids from different process methods are different. It is higher in dry processed coffee than wet processed coffee.

As a non-protein amino acid, Gamma-aminobutyric acid (γ-aminobutyric acid, GABA) is an inhibitory neurotransmitter which reduces brain activity, especially during sleep, and reduces depression. The World Health Organization believes that by 2020 depression will be the second most common disease after cardiovascular diseases. γ-aminobutyric acid (GABA) is contained in the Gabaron tea grade which was specially cultivated in Japan. GABA is also contained in coffee.

Drinks containing GABA are considered to be medicinal, they have the following basic biological properties:

- Help cure stress-related insomnia and improve sleep quality;
- Reduce risk of cardiovascular diseases;
- Have anti-hypertensive properties;
- Help cure alcoholism (alcoholics have decreased GABA content in the blood);
- Help in treatment of diabetes, Alzheimer's disease and Parkinson's disease;
- Retard the aging process.

In one study, the impact of coffee technological processing on the GABA content was investigated (Bytof, Knopp & Schieberle, 2005). Significant amounts of GABA were found in green coffee obtained by dry method. Coffee beans produced by the wet method had significantly less GABA.

GABA and other amino acids were determined by high performance liquid chromatography (de Andrade, Pinheiro & Lopes, 1995; Granvogl & Schieberle, 2007; Casal, Oliveira & Ferreira, 2002, Bispo et al., 2002; Meger, Ngiruwonsanga & Henze, 1996; Dias, Campanha & Veira, 2010). It was reported that GABA is produced in plants by α-decarboxylation of glutamic acid.

5.5 Determination of Heavy Metals

As for heavy metals, in accordance with the Regulation of Health Food, coffee may contain only trace amounts. In one study, less than 0.1 μg kg^{-1} of lead and less than 0.05 μg kg^{-1} of arsenic was detected by atomic absorption spectrophotometry (Rajkovic, Vukovic & Demin, 2004). Maximum permissible concentration of these elements is below 1μg kg^{-1}.

6. Determination of Organic Pollutants in Coffee

There are some compounds undesirable for flavor and bioactivity of the brew which occur due to inappropriate harvesting, weather conditions during processing or improper storage of coffee. Most of these compounds are microbial by products such as ochratoxin and specific biogenic amines. Acrylamide and polycyclic aromatic hydrocarbons could be formed by high roasting temperature.

6.1 Determination of Polycyclic Aromatic Hydrocarbons (PAHs)

The PAHs were determined in coffee by HPLC with different detection methods and GC-MS. (Shi et al., 2016; Guatemala-Morales et al., 2016; Ventura et al., 2003; Pissinatti et al., 2015). Seven types of PAHs were detected in coffee by HPLC with fluorimetric detection (Ventual et al., 2003) (benzo(a)anthracene, benzofluoranthene, benzo(a)pyrene, benzoperylene, dibenzoanthracene, indenopiren). Separation was performed on a 100×4.6mm column filled with Chrom Sep with gradient elution. Excitation wavelengths of the fluorimetric detector were set at 274, 296, and 300 nm, emission wavelengths were 414, 406, and 470 nm, respectively. The PAH detection limit was in the range of 0.2 ng kg^{-1}, the linear range was between 0.2-10 μg L^{-1}. Ten polyaromatic hydrocarbons were determined in the roasted coffee by GC-MS on 24 commercial samples at levels of 1.00-11.29 μg kg^{-1} (Pissinatti et al., 2015).

The concentrations of PAHs in different grades of coffee are different, with lower content in green coffee. Benzo(a)pyrene was not found in green coffee at all. The most amount of benzo(a)pyrene was determined in

instant coffee and in roasted coffee. It was shown that the content of benzo(a)pyrene depends on the roasting degree (Kayali-Sayadi et al., 1999; Badolato, et al., 2006). Typically, benzo(a)pyrene served as an indicator of the overall contamination of coffee with PAHs.

6.2 Determination of Acrylamide in Coffee

Acrylamide exerts a carcinogenic effect (Tarcke, Rydberg, Karlsson & Tomnqvist, 2002). Acrylamide is synthesized when carbohydrate-rich foods are heated (Taeymans et al., 2004). One of the possibilities is that acrylamide synthesis occurs via Maillard reaction when amino acids react with carbonyl compounds during heating. Significant amounts of acrylamide can be formed during coffee roasting process, therefore coffee may become a source of acrylamide in people's every day diet. Reliable and quick method to measure acrylamide level in coffee is needed to optimize the technology of making coffee with minimal acrylamide level. As is well-known, some compounds which are formed in the roasting process give coffee unmatched aroma and pleasant taste. At the same time, some undesirable and even harmful compounds could also be formed in the roasting process.

Measuring acrylamide level in coffee is a challenging task because it requires pre-extraction from a complex matrix. Gas chromatography, liquid chromatography, GC-MS (Surma, Sadowska-Rociek, Cieślik & Sznajder-Katarzyńska, 2017; Ono et al., 2003; Biedermann et al., 2002) and LC-MS (Khan et al., 2017; Becalski, Lau, Lewis & Seaman 2003; Roach, Andrzejewski, Gay, Nortrup & Musser, 2003; Zyzak et al., 2003) are used most often. The acrylamide contents are different in some commercial coffee from several manufactures in different countries, with 42-338 ng g^{-1} in instant coffees and about 50ng g^{-1} in filtered coffee.

The effect of temperature and roasting time on the acrylamide content in coffee was also studied (Senyuva & Gökmen, 2005). The same sample was roasted at 150ºC, 200ºC, and 225ºC for 30 min. At 150ºC, the acrylamide content was increasing during all roasting period, whereas at 200ºC and 225ºC, the acrylamide content was increasing only during the first 10 minutes, after that, the acrylamide content was continuously decreasing during the remaining roasting time (up to 30 min). The level of acrylamide cut down to 5%.

Coffee also changed its color during roasting process. Nonlinear correlation between the color of coffee, as measured by Minolta's CM-3600d spectrophotometer, and acrylamide content in it was noted for 9 types of coffee. The highest acrylamide level, 338 ng g^{-1}, was noted in Jacobs Monarch coffee.

LC-MS method used for measuring acrylamide was quite fast, reliable, and accurate (Senyuva & Gökmen, 2005). Prior to the analysis, samples were extracted with methanol and purified.

6.3 Determination of ochratoxin in coffee

Ochratoxin is one of the carcinogenic mycotoxins which may develop in foods and beverages under inadequate production processes and storage conditions. In many countries, the maximum allowable levels of ochratoxin are set within 2-50 ng g^{-1}.

The EU Commission plans to establish standards for ochratoxin levels in green and roasted coffee. AOAC current method for the determination of ochratoxin in coffee was first published in 1975 (Levi, 1975). This method is based on the thin-layer chromatography with an insufficiently sensitive detection (maximum permissible concentration 20 ng g^{-1}).

It has been planned to develop a new method for the determination of ochratoxin in coffee. In recent years, dozens of articles were published on this subject. In one study, it was suggested to use reversed-phase HPLC with fluorimetric detection. The presence of ochratoxin in coffee was confirmed by mass spectrometry. The detection limit was 0.1 ng g^{-1}. Twenty samples of coffee from different countries, such as Brazil, Colombia, Zimbabwe, India, and Indonesia, were analyzed. Extraction was carried out with 1% $NaHCO_3$. A polymer-based column (Oasis MAX) which employs both reversed-phase and ion-exchange mechanisms of retention was used in this study. Using such a state-of-the-art technique, ochratoxin in the amounts exceeding the MPC was not detected either in green or in roasted coffee.

Another publication describes a method for measuring an ochratoxin level in green coffee by immunoaffinity column cleanup followed by HPLC separation and analysis (Vorgas, Dos Santos & Pittet, 2005). This technique was tested in 8 countries as part of preparation for establishing it as the EU official method.

A new HPLC-MS-MS method was developed for simultaneous determination of 21 mycotoxins in coffee beverages. Mycotoxins were detected at the μg kg^{-1} level. Ochratoxin—a mycotoxin regulated in coffee in Europe—was measured in two samples at the maximum allowable level (García-Moraleja, Font, Mañes & Ferrer, 2015).

Eight types of aflatoxins (B_1, B_2, G_1, G_2, M_1, M_2, GM, GM_2) could be formed out of the three structural variants of aflatoxins. Determining aflatoxin B_1 is the most important goal since it is the precursor of all types of aflatoxins (Rajkovic, Vukovic & Demin, 2004).

Aflatoxins were determined by HPLC with fluorimetric detection at the detection limit of 2.5 μg kg^{-1}, excitation wavelength of 366 nm and emission wavelength 460nm. In all samples of Grand and Don coffee varieties (Golex Product), the total amount of B_1 and G_1 aflatoxins was less than 2.5 μg kg^{-1} whereas the maximum permissible concentration is 5.0 μg kg^{-1} (Regulation of Health Food).

The harmful substances in coffee can be eliminated or reduced to a minimum (below MPC) by adherence to the adequate technology for gathering and processing green coffee beans, and proper storage conditions of the finished product. It is worth to mention people should avoid to consume coffee or tea along with foods containing significant amount of nitrates since hydrogen cyanide may be formed (Seto et al., 2008).

7. Identification of Coffee

Identifying characteristics of coffee at different stages of its production are determined by: the shape and size of coffee beans, its organoleptic characteristics, element compositions (Krivan et al., 1993; Martin et al., 1998 and 1999; Weckerle et al., 2002; Serra et al., 2001; Wieser et al., 2002; Rodrigues et al., 2010), aroma analysis (Dirinck et al., 2001), physical and chemical composition (Rocha et al., 2004; Martin et al., 1998; Ky et al., 2001; Casals et al., 2000; Guerrero et al., 2001; Carrera et al., 1998; Valdenbro et al., 1999 ; Bertrand et al., 2005; Martin, et al., 2001; Gonzales et al., 2001; Anderson et al., 2002; Kemsley et al., 1995; Dupuy et al., 1995; Suchanek et al., 1996; Downey et al., 1996). For identification of botanical species of coffee beans, assessment of the outward anatomy and morphology may sufficient. Arabica and Robusta coffee beans differ in shape and size. Green Arabica coffee beans have an elongated shape 6-15mm long. Green Robusta coffee beans have rounded shape 4-9mm long. After roasting, the bean size is increased by 25-50%.

Coffee Arabica has richer aroma than coffee Robusta. Arabica coffee tastes slightly sour whereas Robusta coffee has a more bitter taste. Experts can organoleptically—by taste and aroma—distinguish natural roasted Arabica and Robusta coffee.

Modern physicochemical methods, primarily chromatography, provide the most reliable criteria for identification of coffee authenticity at all production and preparation stages because the main varieties of coffee Arabica and Robusta differ in content of sugars, caffeine, theobromine, theophylline, trigonelline, chlorogenic acids and other compounds (Table 15).

Table 15. Overall typical chemical composition of Arabica and Robusta coffee

No.	Compounds	Arabica	Robusta
1	Water-soluble extractive substances	19-20%	24-27%
2	Chlorogenic acids (more than 10)	5.5-8%	9-11%
3	Caffeine	0.6-1.2%	1.8-3%
4	Theophylline	1-4 %	10-100 ppm
5	Theobromine	1.3-2.5 %	0.1-0.67%
6	Trigonelline	1-1.2%	0.6-0.7%
7	Diterpene glycosides	290-340 mg/kg	10-45 mg/kg
8	Carbohydrates. Polysaccharides, cellulose Monosugars	 Over 12% 0.7-1%	 Over 12% 0.7-1%
9	Protein substances (by amino nitrogen)	1.55-1.63%	1.36-1.72%
10	Amino acids (free)	Over 1.6%	Over 1.6%
11	Tannins	3.6-7.7%	2.2-6.6%
12	Free fatty acids (linoleic, palmitic, oleic, myristic, linolenic, etc.)	0.5-3%	1.2-3.8%
13	Organic acids (citric, malic, maleic, oxalic, acetic, etc.)	About 1%	About 1%
14	Vitamins B1, B2, B6, pantothenic acid, nicotinic acid (PP), tocopherol (E).	Less than 3 mg	Less than 3 mg
15	Mineral substances (Na, Mg, K, Fe, Mn, Zn, etc.)	3-4.5%	3-4.5%

Note: Coffee contains polyamines and gamma-aminobutyric acid. The table shows average values—the actual values depend on coffee variety and growing area.

Recently the elemental detection of green bean of arabica and robusta coffee from Gayo Highland, Aceh-Indonesia, has been identified by using fundamental Nd-YAG Laser for distinguishing the characteristics of both coffees. It is noticed that the order of elements concentration from highest to lowest are Ca>K>CN>Na>C for arabica and K>Ca>CN>Na>N for robusta. The ratio of intensities of these elements to C intensity could be used as a marker to discriminate kind of coffee for the purpose of authentication (Abdulmadjid, Meilina, Hedwig & Kurniawan, 2017).

The state-of-the-art equipment, namely, multicollector inductively coupled plasma mass spectrometer (MC-ICP-MS) and isotope ratio mass spectrometer (IRMS), were used to determine coffee authenticity and its origin (Bertrand, Etienne, Lashermes, Guyot & Dovrieux, 2005). Sixty samples from twenty different geographical areas were analyzed. The $^{87}Sr/^{86}Sr$ isotope ratio and average value of the $^{18}O_2$ isotope were determined in the samples. The measurement results showed a high degree authenticity of coffee origin, especially for countries in South America.

Different compounds from coffee have been used to assess the grade of coffee and its origin. Samples of coffee (fruit, beans, husks) from China, India, and Mexico were analyzed with regards to the content of chlorogenic acids, caffeine, and total polyphenols (Mullen et al., 2013). Among chlorogenic acids (69 chlorogenic acids are currently identified in green coffee beans), 5-O-caffeoylquinic acid was prevalent. The analysis was performed by UHPLC coupled with a QE Orbitrap MS. The samples' antioxidant capacity was also determined. The content of chlorogenic acids in coffee from India and Mexico was similar (18.8 and 22.9 mg g^{-1} in Arabica coffee beans; 27.3 and 27.4 mg g^{-1} in Robusta coffee beans, respectively), while it was significantly lower in coffee beans from China (1.7 and 7.2 mg g^{-1}). Also, coffee from China contained a third to a half of the amount of polyphenols in coffee from India and Mexico, although the total content of flavonoids was about the same. Antioxidant activity measured by FRAP was 2-2.5 times lower in coffee from China than in coffee from India and Mexico (112, 227 and 267 μmol g^{-1}, respectively, for Arabica coffee). Coffee husk turned out to be a rather rich source of procyanidins, flavanols, and flavonols—about 115-130, 18-30, 260+ μg g^{-1}, respectively. Mehari et al., (2015) simultaneously determined the alkaloids in green coffee beans from Ethiopia using chemometric method to evaluate the geographical origin of coffee. High performance liquid chromatography was applied and the limits of detection for the method were established. Study showed that the application of linear discriminant analysis provided 75% correct classification of samples into the respective production regions, with a 74% prediction success rate. The moderate classification efficiency obtained when using alkaloid data demonstrated the potential of using this class of compounds in discriminant models for determination of the geographical origin of green coffee beans from Ethiopia.

8. Conclusion

Coffee is one the most popular beverage and consumed by millions of people all over the world. Numerous factors throughout the coffee production chain, from plant to cup, have been shown to have effects on the color, aroma, and flavor of coffee. This article reviews publications on qualitative and quantitative determination of volatile and non-volatile compounds in green and roasted coffee which determine its aroma, taste and quality, majorly by chromatographic methods.

Using these methods, the following antioxidants and nutrients were identified in coffee: oxyaromatic acids, chlorogenic acids, vitamins, amino acids, sugars, diterpene glycosides, trace minerals, etc. Knowing the chemical composition of coffee will facilitate the study of coffee impact on human health and disease prevention.

It has been reliably proven by now that moderate consumption of coffee reduces cardiovascular risk, prevents the development of type 2 diabetes, protects the liver and even helps combat liver cirrhosis, reduces the risk of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases, and protects against asthma. In general, coffee intake decreases oxidative stress which precedes many dangerous diseases.

References

Abdulmadjid, S. N., Meilina, H., Hedwig, R., & Kurniawan, K. H. (2017). Elemental detection of Arabica and Robusta green bean coffee using laser-induced plasma spectroscopy. *AIP Conference Proceeding, 1801*, 050001. https://doi.org/10.1063/1.4973099

Anderson, K. A., & Smith, B.W. (2002). Chemical profile to differentiate geographic growing origins of coffee. *J. Agric. Food Chem, 50*, 2068-2075. https://doi.org/10.1021/jf011056v

Araujo, J. M. A., & Sandi, D. (2006). Extraction of coffee diterpenes and coffee oil using supercritical carbon dioxide. *Food Chem, 101*, 1087-1094.

Badolato, E. S. G., Martins, M. S., Aued-Pimentel, S., Alaburda, J., Kumagai, E. E., Baptista, G. G., & Rosenthal, A. (2006). Sistematic Study of Benzo[a]Pyrene in Coffee Samples. *J. Brazilian Chem. Soc., 117*, 989-993. https://doi.org/10.1590/s0103-50532006000500025

Bakuradze, T., Boehm, N., Janzowski, C., Lang, R., Hofmann, T., Stockis, J. P., Albert, F. W., Stiebitz, H., Bytof, G., Lantz, I., Baum, M., & Eisenbrand, G. (2011). Antioxidant-rich coffee reduces DNA damage, elevates glutathione status and contributes to weight control: results from an intervention study. *Mol. Nutr. Food Res. 55*(5), 793-797

Becalski, A., Lau, B. P. Y., Lewis, D., & Seaman, S. W. (2003). Acrylamide in foods. Occurrence, sources and modeling. *J. Agric and Food Chem, 51*, 802-808. https://doi.org/10.1021/jf020889y

Bertrand, B., Etienne, H., Lashermes, P., Guyot, B., & Dovrieux, F. (2005). Can near-infrared reflectance of green coffee be used to detect introgression in coffea Arabica cultivars? *J. Sci. food Agric., 85*, 955-962. https://doi.org/10.1002/jsfa.2049

Bicchi, C. P., Panero, O. M., Pellegrino, G. M., & Vanni, A. C. (1997). Characterization of roasted coffee and coffee beverages by solid-phase micro-extraction/Gas Chromatography and Principal Component Analysis. *J. Agric. Food Chem, 45*, 4680-4686. https://doi.org/10.1021/jf9704827

Biedermann, M., Biedermann-Brem, S., Noti, A., & Grob, K. (2002). Two GC-MS methods for the analysis of acrylamide in foodstuff. *Mitteilung Lebens mittel und Hygiene, 93*, 638-652.

Bispo, M. S., Veloso, M. C., Pinheiro, H. L. de Oliveira, R. F. S., Reis, J. O. N., & de Andrade, J. B. (2002). Simultaneous determination of caffeine, theobromine and theophylline by High-Performance liquid chromatography. *Chromatogr. Science, 40*, 45-48. https://doi.org/10.1093/chromsci/40.1.45

Boekschotem M.V., Engberink M.F., Katan M.B., & Schouten E.G. (2003). Reproducibility of the serum lipid response to coffee oil in healthy volunteers. *Nutr. J.* v. 2, p. 1-8. https://doi.org/10.1186/1475-2891-2-8

Bressanello, D., Liberto, E., Cordero, C., Rubiolo, P., Pellegrino, G., Ruosi, M. R., & Bicchi, C. (2017). Coffee aroma: Chemometric comparison of the chemical information provided by three different samplings combined with GC-MS to describe the sensory properties in cup. *Food Chemistry, 214*, 218-226. https://doi.org/10.1016/j.foodchem.2016.07088

Bücking, M., & Steinhart, H. (2002). Headspace and Sensory Analysis Characterization of the Influence of Different Milk Additives on the Flavor Release of Coffee Beverages. *J. Agric. Food Chem, 50*, 1529-1534. https://doi.org/10.1021/jf011117p

Bytof, G., Knopp, S.-E., Schieberle, P., Teutsch, I., & Selmar, D. (2005). Influence of processing on the generation of γ-aminobutyric acid in green coffee beans. *Eur. Food Res. Technol., 220*, 245-250.

Carrera, F., Leon-Camacho, M., Pablos, F., & Gonzalez, A. G. (1998). Authentication of green coffee varieties according to their sterolic profile. *Anal. Acta., 370*, 131-139. https://doi.org/10.1016/s0003-2670(98)00303-1

Casal, S., Oliveira, M. B. P. P., & Ferreira, M. A. (2002). Determination of biogenic amines in coffee by an optimized liquid chromatographic method. *J. Liquid Chromatogr. And Related Technologies, 25*, 2535-2549. https://doi.org/10.1081/jlc-120014273

Casal, S., Oliveira, M. B. P. P., Alves, M. R., & Ferreira, M. A. (2000). Discrimination analysis of roasted coffee varieties for trigonelline, nicotinic acid and caffeine content. *J. Agric Food Chem., 48*, 3420-3424. https://doi.org/10.1021/jf990702b

Cavin, C., Holzhaeuser, D., Scharf, G., Constable, A., Huber, W. W., & Schilter, B. (2002). Cafestol and kahweol, two coffee specific diterpenes with anticarcinogenic activity. *Food Chem. Toxicol., 40*, 1155-1169. https://doi.org/10.1016/s0278-6915(02)00029-7

Clarke, R. J., & Mocrae, R. Coffee vol. 1. Chemistry. Elsevier, New York 1985.

Clifford, M. N. (2000). Chlorogenic acids and other cinnamates-nature, occurrence, dietary burden, absorption and metabolism. *J. Sci. Food Agr., 80*, 1033-1043. https://doi.org/10.1002/(sici)1097-0010(20000515)80:7%3C1033::aid-jsfa595%3E3.3.co;2-k

Costa-Freitas, A. M., Parreira, C., & Vilas-Boas, L. (2001). Comparison of two SPME fibers for differentiation of coffee by analysis of volatile compounds. *Chromatographia, 54*, 647-652.

Craig, A. P., Fields, C., Liang, N., Kitts, D. & Erickson, A. (2016). Performance review of a fast HPLC_UV

method for the quantification of chlorogenic acids in green coffee bean extracts. *Talanta, 154*, 481-485. https://doi.org/ 10.1016/j.talanta.2016.03.101

Czerny, M., Mayer, F., & Grosch, W. (1999). Sensory study on the character impact odorants of roasted Arabica coffee. *Journal of agricultural and Food Chemistry, 47*, 695-699

Daglia, M., Racchi, M., Papetti, A., Lanni, C., Govoni, S., & Gazzani, G. (2004). In vitro and ex vivo antihydroxyl Radical Activity of Green and Roasted Coffee. *J. Agric. Food Chem., 52*, 1700-1704. https://doi.org/10.1021/jf030298n

de Andrade, J. B., Pinheiro, H. L. C., Lopes, W. A., Martins, S., Amorin, A. M. M., & Brandao, A. M. (1995). Determination of caffeine in beverages by HPLC. *Quimica Nova., 18*, 379-381.

Demissie, E. G., Woyessa, G. W., & Abebe, A. (2016). UV/VIS spectrometer determination of caffeine in green coffee beans from hararghe, Ethiopia, using beer-lamberts's law and integrated absorption coefficient techniques. *Chemistry & Chemical Engineering, Biotechnology, Food Industry, 17*(2), 109-123

Dias, R. C. E., Campanha, F. G., Vieira, L. G. E., Ferreira, L. P., Pot, D., Marraccini, P., & Benassi, M. D, T. (2010). Evaluation of kahweol and cafestol in Coffee Tissues and roasted coffee by a new high-performance Liquid Chromatography Methodology. *J. Agric. Food Chem., 58*, 88-93. https://doi.org/10.1021/jf9027427

Dirinck, I., van Leuven, I., & Dirinck, P. (2001). SDE-GC-MS analysis and titratable acidity for objective flavour characterization of coffee. *Spec. Publ. – R. Soc. Chem., 269*, 248-252.

Dirinck, M. I., Van Lenven, I., & Dirinck, J. P. (2001). Analytical flavor characterization and classificztion of Arabica anf robusta coffees from different origins. *Proceedings of 19th ASIC Colloquium (Trieste) ASIC*, Paris, France.

Downey, G., & Boussion, J. (1996). Authentication of coffee bean variety by near-infrared reflectance spectroscopy of dried extract. *J. Sci. Food Agric., 71*, 41-49. https://doi.org/10.1002/(sici)1097-0010(199605)71:1%3C41::aid-jsfa546%3E3.0.co;2-i

Dulsat-Serra, N., Quintanilla-Casas, B., & Vichi, S. (2016). Volatile thiols in coffee: A review on their formation, degradation, assessment and influence on coffee sensory quality. *Food Research International, 89*, 982-988. https://doi.org/ 10.1016/j.foodres.2016.02.008

Dupuy, N., Huvenne, J. P., Duponchet, L., & Legrand, P. (1995). Classification of green coffees by FTIR analysis of dry extract. *Appl. Spectrosc, 49*, 580-585. https://doi.org/10.1366/0003702953964174

Erdem, S. A., Senol, F. S., Budakoglu, E., Orhan, I. E., & Sener, B. (2016). Exploring in vitro neurobiological effects and high-pressure liquid chromatography-assisted quantitation of chlorogenic acid in 18 Turkish coffee brands. *Journal of Food and Drug Analysis, 24*, 112-120. https://doi.org/10.2016/j.jfda.2015.08.001

Erny, G. L., Moeenfard, M., & Alves, A. (2015). Liquid Chromatography with Diode Array Detection Combined With Spectral Deconvolution for the Analysis of Some Diterpene Esters in Arabica Coffee Brew. *J Sep Sci., 38*(4), 612-620. https://doi.org/10.1002/jssc.201401095

Farah, A., Monteiro, M., Donangelo, C. M., & Lafay, S. (2008). Chlorogenic acids from green coffee extract are highly bioavailable in humans. *J. Nutr., 138*, 2309-2315

Freedman, N. D., Park, Y., Abnet, C. C., Hollenbeck, A. R., & Sinha, R. (2012). Association of coffee drinking with total and cause-specific mortality. *N. Engl. J. Med., 366*(20), 1891-1904

Gant, A., Leyva, V. E., Gonzalez, A. E., & Maruenda, H. (2015). Validated HPLC-Diode Array Detector for Simultaneous Evaluation of Six Quality Markers in Coffee. *Journal of AOAC International, 98*(1), 98-102(5). https://doi.org/10.5740/jaoacint.14-113

García-Moraleja, A., Font, G., Mañes, J., & Ferrer, E. (2015). Simultaneous determination of mycotoxin in commercial coffee. *Food control, 57*, 282-292. https://doi.org/10.1016/j.foodcont.2015.04.031

Gennano, M. C., & Abrigo, C. (1992). Caffeine and theobromine in coffee, tea and cola beverages simultaneous determination by reversed-phase ion interaction HPLC. *Frenesius J. Anal. Chem., 343*, 523-525. https://doi.org/10.1007/bf00322162

Gonzalez, A. G., Pablos, F., Martin, M. J., Leon-Camacho, M., & Valdenebro, M. S. (2001). HPLC analysis of tocopherols and triglycerides in coffee and then use as authentication parameters. *Food Chem., 73*, 93-101. https://doi.org/10.1016/s0308-8146(00)00282-x

Granvogl, M., & Schieberle, P. (2007). Quantification of 3-aminopropionamide in cocoa, coffee and cereal products. *Eur. Food Res. Technol., 225*, 857-863. https://doi.org/10.1007/s00217-006-0492-9

Grollier, J. F., & Plessis, S. (1998). Use of coffee bean oil as a sun filter. U.S. Patent 4793990.

Guatemala-Morales, G. M., Beltrán-Medina, E. A., Murillo-Tovar, M. A., Ruiz-Palomino, P., Corona-González, R. I., & Arriola-Guevara, E. (2016). Validation of analytical conditions for determination of polycyclic aromatic hydrocarbons in roasted coffee by gas chromatography-mass spectrometry. *Food Chemistry, 197*, 747-753. https://doi.org/10.1016/j.foodchem.2015.10.135

Guerrero, G., Suarez, M., & Moreno, G. (2001). Hydroxycinnamic acids as genotype discrimination criteria for green coffee beans. *ASIC 19th Colloquium*, Trieste, Italy.

Harada, K., Nishimura, O., & Mihara, S. (1987). Rapid analysis of coffee flavour by gas chromatography using a pyrolyzer. *J. Chromatography, 391*, 457-600.

Holscher, N., & Steinhart, H. (1992). Investigation of roasted coffee freshness with on improved headspace technique. Z. Lebensm.-Unters. *Forsch., 195*, 33-38. https://doi.org/10.1007/bf01197836

Ioffe, B., & Vitenberg, A. G. (1984). Headspace analysis and related methods in gas chromatography. A Willey-Interscience Publication. New York

Kayali-Sayadi, M. N., Rubio-Barroso, S., Cuesta-Jimenez, M. P., & Polo-Diez, L. M. (1999). A new method for the determination of selected PAHs in coffee brew samples by HPLC with fluorimetric detection and solid-phase extraction. *J. Liquid Chromatogr. And Related Techn., 22*, 615-627.

Kele, M., & Ohmacht, R. (1996). Determination of serotonin released from coffee wax by liquid chromatography. *J. Chromatography, 730*, 53-62. https://doi.org/10.1016/0021-9673(95)01186-2

Kemsley, E. K., Ruault, S., & Wilson, R. H. (1995). Discrimination between coffea Arabica and canephora variant robusta beans using infrared spectroscopy. *Food Chem., 54*, 321-326.

Khan, M. R., Alothman, Z. A., Naushad, M., Alomary, A. K., Alfadul, S. M., Alsohaimi, I. H., & Algamdi, M. S. (2017). Occurrence of acrylamide carcinogen in Arabic coffee Qahwa, coffee and tea from Saudi Arabian market. *Nature*. 1-7. http://dx/doi.org/10.1038/srep41995

Kim, H. G., Hwang, Y. P., & Jeong, H. G. (2009). Kahweol blocks STAT3 phosphorylation and induces apoptosis in human lung adenocarcinoma A549 cells. *Toxicol. Lett., 187*, 28-34. https://doi.org/10.1016/s0278-6915(02)00029-7

Knopp, S., Bytof, G., & Selmar, D. (2006). Influence of processing on the content of sugars in green Arabica coffee beans. *Eur. Food Res. Technol., 223*, 195-201. https://doi.org/10.1007/s00217-005-0172-1

Kölling-Speer, I., Strohschneider, S., & Speer, K. (1999). Determination of free diterpenes in green and roasted coffees. J. High Resol. *Chromatogr, 22*, 43-46. https://doi.org/10.1002/(sici)1521-4168(19990101)22:1%3C43::aid-jhrc43%3E3.3.co;2-g

Köseoglu, Yilmaz, P., & Kolak, U. (2017). SPE-HPLC determination of chlorogenic and phenolic acids in coffee. *J Chromatogr Sci., 18*, 1-7. http://dx/doi.org/10.1093/chromosci/bmx025

Kramer, D., Breitensteub, B., Kleinwächter, M., & Selmar, D. (2010). Stress metabolism in green coffee beans (coffea Arabica L.): expression of dehydrins and accumulation of GABA during drying. *Plant & Cell Physiology, 51*(4), 546-553. http://dx/doi.org/10.1093/pcp/pcq019

Krivan, V., Barth, P., & Morales, F. (1993). Multielement analysis of green coffee and its possible use for the determination of origin. *Mikrochim. Acta., 110*, 217-236. https://doi.org/10.1007/bf01245106

Kurzrock, T., & Speer, K. (2001). Identification of kahweol fatty acids esters in Arabica coffee by means of LC/MS. *J. Sep. Sci., 24*, 843-848. https://doi.org/10.1002/1615-9314(20011101)24:10/11%3C843::aid-jssc843%3E3.0.co;2-9

Ky, C. L., Lonarn, J., Dussert, S., Guyot, B., Hamon, S., & Noirot, M. (2001). Caffeine, trigonelline, chlorogenic acids and sucrose diversity in wild coffee arabica L., C. canephora P., accessions. *Food Chem., 75*, 223-230. https://doi.org/10.1016/s0308-8146(01)00204-7

Lee, K. J., & Jeong, H. G. (2007). Protective effects of kahweol and cafestol against hydrogen peroxide-induced oxidative stress and DNA damage. *Toxicol. Lett., 173*, 80-87. https://doi.org/10.1016/j.toxlet.2007.06.008

Lee, K. J., Choi, J. H., & Jeong, H. G. (2007). Hepatoprotective and antioxidant effects of the coffee diterpenes kahweol and cafestol on carbon tetrachloride induced liver damage in mice. *Food Chem. Toxicol., 45*,

2118-2125.

Lercker, G., Frega, N., Bocci, F., & Rodriguez-Estrada, M. T. (1995). High resolution gas chromatographic determination of diterpenic alcohols and sterols in coffee lipids. *Chromatographia., 41*, 29-33. https://doi.org/10.1007/bf02274191

Levi, C. P. (1975). Determination of ochratoxin A in coffee by thin-layer chromatography. *J. Assoc. Anal. Chem., 58*, 258-262.

Mabrok, A. F., & Deatheroge, F. E. (1956). Organic acid in brewed coffee. New York.

Maeztu, L., Sanz, C., Andueza, S., Paz, De Peña, M. , Bello, J., & Cid, C. (2001). Characterization of Espresso Coffee Aroman by Static Headspace GC-MS and Sensory Flavor Profile. *J. Agric. Food Chem., 49*(11), 5437-5444. https://doi.org/10.1021/jf0107959

Martin, M. J., Pablos, F., & Gonzalez, A. G. (1998). Characterization of green coffee varieties according to their metal content. *Anal. Chim. Acta., 358*, 103-104. https://doi.org/10.1016/s0003-2670(97)00610-7

Martin, M. J., Pablos, F., & Gonzalez, A. G. (1998). Discrimination between Arabica and robusta green coffee varieties according to their chemical composition. *Talanta, 46*, 1259-1264. https://doi.org/10.1016/s0039-9140(97)00409-8

Martin, M. J., Pablos, F., & Gonzalez, A. G. (1999). Characterization of Arabica and robusta roasted coffee varieties and mixture resolution according to their metal content. *Food Chem., 66*, 565-570. https://doi.org/10.1016/s0308-8146(99)00092-8

Martin, M. J., Pablos, F., Gonzalez, A. G., Valdenebro, M. S., & Leon-Camacho, M. (2001). Fatty acid profiles as discriminant parameters for coffee varieties differentiation. *Talanta., 54*, 291-297. https://doi.org/10.1016/s0039-9140(00)00647-0

Meger, A., Ngiruwonsanga, T., & Henze, G. (1996). Determination of adenine, caffeine, theophylline and theobromine by HPLC with amperometric detection. ~~*Frenesius J.*~~ *Anal. Bioanal. Chem., 356*, 284-287. https://doi.org/10.1007/s0021663560284

Mehari, B., Redi-Abshiro, M., Chandravanshi, B. S., Atalbachew, M., Combrinck, S., & McCrindle, R. (2015). Simultaneous determination of alkaloids in green coffee beans from Ethiopia: Chemometric evaluation of geographic al origin. *Food anal. Methods*. https://doi.org/10.1007/s12161-016-0340-2

Moreira, A. S. P., Nunes, F. M., Domingues, M. R., & Coimbra, M. A. (2012). Coffee melanoidins: structures, mechamisms of formation and potential health impacts. *Food Funct., 3*, 903. https://doi.org/10.1039/c2fo30048f

Morton, I. D., & Macleod, A. D. (1986). Food Flavours: Part B, The flavor of beverages-Part 2. ISSN 0167-4501, Elsevier.

Mullen, W., Nemzer, B., Ou, B., Stalmach, A., Hunter, J., Clifford, M. N., & Combet, E. (2011). The Antioxidant and Chlorogenic Acid Profiles of Whole Coffee Fruits Are Influenced by the Extraction Procedures. *Journal of Agricultural and Food Chemistry, 59*, 3754-3762. https://doi.org/10.1021/jf200122m

Mullen, W., Nemzer, B., Stalmach, A., Ali, S., & Combet, E. (2013). Polyphenolic and Hydroxycinnamate Contents of Whole Coffee Fruits from China, India and Mexico. *Journal of Agricultural and Food Chemistry, 61*, 5298-5309. https://doi.org/10.1021/jf4003126

Murata, M., Okada, H., & Homma, S. H. (1995). Hydroxycinnamic Acid-Derivatives and P-Coumaroyl-(L)-Tryprophon a Novel Hydroxycinnamic Acid-Derivative, from Coffee Beans. Biosci. *Biotechnol. Biochem, 59*, 1887-1890. https://doi.org/10.1271/bbb.59.1887

Nakhmedov, F. G. (1984). Technology of Coffee Products. Moscow. *Consumer Goods and Food Industry*, 184.

Nascimento, E. A., Morais, S. A. L., Chang, R., Aquino, F. J. T., Alves, B. H. P., & Afonso, R. J. C. F. (2015). Looking for Unknown Molecules in Arabica and Robusta Coffee Brews by High Performance Liquid Chromatography Coupled to High Resolution Mass Spectrometry and Differentiating Both Coffee Species by this Technique. *International Journal of Applied Science and Technology, 5*(1), 26-37.

O'Driscoll, D. J. (2014). Analysis of coffee bean extracts by use of ultra-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. *MethodsX, 1*, 264-268. https://doi.org/10/1016/j.mex.2014.10.006

O'Keefe, J. H., Bhatti, S. K., Patil, H. R., DiNicolantonio, J. J., Lucan, S. C., Lavie, C. J. (2013) Effects of

habitual coffee consumption on cardiometabolic disease, cardiovascular health, and all-cause mortality. *J. Am Coll. Cardiol, 62*(12), 1043-1051.

Ono, H., Chuda, Y., Ohnishi-Kameyama, M., Yoda, H. Ishizaka, M., Kobayashi, H., & Yochida, M. (2003). Analysis of acrilamide by LC-MS/MS and GC-MS in processed Japanese foods. *Food Additives and Contaminants, 20*, 215-220.

Perez-Martinez, L., Sagrado, S., & Medina-Hermandez, M. J. (1995). A rapid procedure for determination of a caffeine, theophylline and theobromine in urine by miscellar liquid chromatography and direct sample injection. *Anal. Chim. Acta., 304*, 195-201. https://doi.org/10.1016/0003-2670(94)00619-w

Pérez-Hernández, L. M., Chávez-Quiroz, K., Medina-Juárez, L. Á., & Meza, N. G. (2012). Phenolic characterization, melanoidins, and antioxidant activity of some commercial coffees from coffea Arabica and coffea canephora. *J. Mex. Chem. Soc., 56*(4), 430-435

Pettitt, B. C. (1987). Identification of the diterpene esters in Arabica and canephora coffees. *J. Agric. Food Chem., 35*, 549-551. https://doi.org/10.1021/jf00076a027

Pissinatti, R., Nunes, C. M., de Souza, A. G., Junqueira, R. G., & de Souza, S. V. C. (2015). Simultaneous analysis of 10 polycyclic aromatic hydrocarbons in roasted coffee by isotope dilution gas chromatography-mass spectrometry: Optimization, in-house method validation and application to an exploratory study. *Food Control., 51*, 140-148. https://doi.org/10.1016/j.foodcont.2014.11.003

Procida, G., de Palo, D., & Bogoni, P. (1997). Analysis of the volatile constituents of coffee by headspace GC-MS. *Riv. Ital. EPPOS Spec.*, 251-261.

Rajkovic, M. B., Vukovic, G., Peric, L, Demin, M., Lalicic, J., & Kovacevic, D. (2004). Analyzing of coffee quality with different methods. *J. Agricultural Science, 49*, 87-96. https://doi.org/10.2298/jas0401087r

Roach, J. A., Andrzejewski, D., Gay, M. L., Nortrup, D., & Musser, S. M. (2003). Rugged LC-MS/MS survey analysis for acrylamide in foods. *J. Agric. And Food Chem., 51*, 7547-7554. https://doi.org/10.1021/jf0346354

Rocha, S., Maeztu, L., Barros, A., Cid, C., & Coimbra, M. A. (2004). Screening and distinction of coffee brews based on head space solid-phase microextraction/gas chromatography/principal component analysis. *J. Sci. Food Agric., 84*, 43-51. https://doi.org/10.1002/jsfa.1607

Rodrigues, C., Maguas, C., & Prohaska, T. (2010). Strontium and oxygen isotope fingerprinting of green coffee beans and its potential to proof authenticity of coffee. *Eur. Food Res. Technol*.

Roos, B., Van Der, Weg, G., Urgert, R., Bovenkamp, P., Charrier, A., & Katan, M. (1997). Levels of cafestol, kahweol and related diterpenoids in wild species of the coffee plant. *J. Agric. Food Chem., 45*, 3065-3069. https://doi.org/10.1021/jf9700900

Rubayiza, A. B., & Meurens, M. (2005). Chemical discrimination of Arabica and robusta coffees by Fourier transform Raman spectroscopy. *J. Agric. Food Chem., 53*, 4654-4659.

Ruiz, del Castillo, M. L., Herraiz, M., & Blanch, G. P. (1999). Rapid analysis of cholesterol - elevating compounds in coffee brews by off-line high-performance liquid chromatography/high resolution gas chromatography. *J. Agric. Food Chem., 47*, 1525-1529.

Ryan, D., Shellie, R., Tranchida, P., Casilli, A., Mondello, L., & Marriott, P. (2004). Analysis of roasted coffee bean volatiles by using comprehensive two-limensional gas chromatography-time-of-flight mass spectrometry. *J. Chromatogr, 1054*, 57-65. https://doi.org/10.1016/s0021-9673(04)01408-6

Sanz, C., Ansorena, D., Bello, J., & Cid, C. (2001) Optimizing headspace temperature and time sampling for Identification of Volatile Compounds in Ground Roasted Arabica Coffee. *J. Agric. Food Chem., 49*, 1364-1369. https://doi.org/10.1021/jf001100r

Savolainen, H. (1992). Tannin content of tea and coffee. *J apple toxicol.*, *12*(3), 191-2. https://doi.org/10.1002/jat.2550120307

Semmeirich, P., & Grosh, W. (1995). Analysis of roasted coffee powders and brews by Gas Chromatography-olfactomertry of headspace samples. *Food Sci. Technol., 28*, 310-313. https://doi.org/10.1016/s0023-6438(95)94411-7

Senyuva, H. Z., & Gökmen, V. (2005). Study of acrylamide in coffee using an improved LC-MS method: Investigation color changes and acrylamide formation in coffee during roasting. *Food Additives and*

Contaminants, 22, 214-220. https://doi.org/10.1080/02652030500109834

Serra, F., Guillou, C. G., Reniero, F., Ballarin, L. Cantagallo, M. I., Wieser, M., IYer, S. S., Héberger, K., & Vanhaecke, F. (2005). Determination of the geographical origin of green coffee by principal component analysis of carbon nitrogen and boron stable isotope ratios. *Rapid Commun. Mass Spectrom, 19*, 2111-2115.

Seto Y., Ohmori T., Kanamori-Kataoka M., Tsuge K., & Ohsawa I. (2008). Artifactual production of cyanide from coffee and tea drinks in the presence of nitrite. *Annales de Toxicologic Analytique, 20*, 155-160.

Shan, Y., Jin, X., Cheng, Y., & Yan, W. (2016). Simultaneous determination of chlorogenic acids in green coffee bean extracts with effective relative response factors. *International Journal of Food Properties*, http://www.tandfonline.com/doi/abs/10.1080/10942912.2016.1230746

Shi, Y., Wu, H., Wang, C., Guo, X., Du, J., & Du, L. (2016). Determination of polycyclic aromatic hydrocarbons in coffee and tea samples by magnetic solid-phase extraction coupled with HPLC-FLD. *Food Chemistry, 199*, 75-80. https://doi.org/10.1016/j.foodchem.2015.11.137

Suchanek, M., Filipova, H., Volka, K., Delgadillo, I., & Davies, A. N. (1996). Qualitative analysis of green coffee by infrared spectrometry. *Fresenius J. Anal. Chem., 354*, 327-332. https://doi.org/10.1007/s0021663540327

Surma, M., Sadowska-Rociek, A., Cieślik, E., & Sznajder-Katarzyńska, K. (2017). Optimization of QuECHERS sample preparation method for acrylamide level determination in coffee and coffee substitutes. *Microchemical Journal, 131*, 98-102. https://doi.org/10.1016/j.microc.2016.11.021

Taeymans, D., Wood, J., Ashby, P., Blank, I., Studer, A., Stadler, R. H., Gondé, P., Van Eijck, P., Lalljie, S., Lingnert, H., Lindblom, M., Matissek, R., Müller, D., Tallmadge, D., O'Brien, J., Thompson, S., Silvani, D., & Whitmore, T. (2004). A Review of acrylamide: An industry perspective on research, analysis, formation and control. *Crit. Reviews in Food Science and Nutrition, 44*, 323-347.

Tarcke, E., Rydberg, P., Karlsson, S., Eriksson, S., & Törnqvist, M. (2002). Analysis of acrylamide, a carcinogen formed in heated foodstuffs. *J. Agric. And Food Chem., 50*, 4998-5006. https://doi.org/10.1021/jf020302f

Trugo, L. (1984). High Performance Liquid Chromatography in coffee analysis. Ph.D.Thesis. University of Reading, England.

Trugo, L., Macrae, R., & Dick, J. (1984). Chlorogenic acid composition of instant coffee. *Analyst, 109*, 263-266. https://doi.org/10.1039/an9840900263

Trugo, L. C., De Maria, C. A. B., Moreira, F. R. A., & Petracco, M. (1995). Simultaneous determination of total chlorogenic acid, triginelline and caffeine in green coffee by high-performance gel filtration chromatography. *Food Chem., 52*, 447-449. https://doi.org/10.1016/0308-8146(95)93298-6

Urgert, R., Van Der, Weg, G., Kosmeijer-Schuil, T., van de Bovenkamp, P., Hovenier, R., & Katan, M. (1995). Levels of the cholesterol-elevating diterpenes cafestol and kahweol in various coffee brews. *J. Agric. Food Chem., 43*, 2167-2172. https://doi.org/10.1021/jf00056a039

Valdenbro, M. S., Leon-Camacho, M., Pablos, F., Gonzalez, A. G., & Martin, M. J. (1999). Determination of the Arabica/robusta composition of roasted coffee according to their sterolic content. *Analyst, 124*, 999-1002. https://doi.org/10.1039/a902245g

Ventura, M., Vallejos, C.,.Anaya, I. A., Broto-Puig, F., Agut, M., & Comellas, L. (2003). Analysis of ochratoxin A in Coffee by Solid-Phase Cleaning and narrow-bore liquid Chromatography-Fluorescence detector-Mass spectrometry. *J. Agric. Food Chem., 51*, 7564-7567. https://doi.org/10.1021/jf034856+

Vitenberg, A. G., & Ioffe, B. V. (1982). Gas Extraction in Chromatographic Analysis. Leningrad, Chemistry, 240.

Vorgas, E. A., Dos, Santos, E. A., & Pittet, A. (2005). Determination of ochratoxin A in Green Coffee by Immunoaffinity Column Cleaning and Liquid Chromatography: collaborative study. *J. AOAC Intern, 88*, 773-779.

Wanyika, H. N., Gotebe, E. G., Gitu, L. M., Ngumba, E. K., & Maritim, C. W. (2010). Determination of caffeine content of tea and instant coffee brands found in the Kenyan market. *African Journal of Food Science, 4*, 353-358.

Weckerle, B., Richling, E., Heirinch, S., & Schreier, P. (2002). Origin assessment of green coffee (coffea Arabica) by multi-element stable isotope analysis of caffeine. *Anal. Bioanal. Chem., 374*, 886-890. https://doi.org/10.1007/s00216-002-1560-z

Wieser, E. M., Iyer, S. S., Krouse, H. P., & Cantagallo, M. I. (2001). Variations in the boron isotope composition of coffea Arabica beans. *Appl. Geochem, 16*, 317-322. https://doi.org/10.1016/s0883-2927(00)00031-7

Yang, N., Lui, C., Liu, X., Degn, T. K., Munchow, M., & Fisk, I. (2016). Determination of volatile marker compounds of common coffee roast defects. *Food Chemistry, 211*, 206-214. https://doi.org/10.1016/j.foodchem.2016.04.124

Zyzak, D. V., Sonders, R. A., Stojanovich, M., Tallmadge, D. H., Eberhart, B. L., Ewald, D. K., Gruber, D. C., Morsch, T. R., Strothers, M. A., Rizzi, G. P., & Villagran, M. D. (2003). Acrylamide formation in heated foods. *J. Agric. And Food Chem., 51*, 4782-4787. https://doi.org/10.1021/jf034180i

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Omega-3 Fatty Acid Oil Enhancement of a Protein-Based Recovery Beverage: *Sensory Analysis with Athletes*

Jessica MacMartin[1], W. E. Buckley[2], David Cranage[3], Tim Bream[4] & Peter Lawrence Bordi[1]

[1]Center for Food Innovation, School of Hospitality Management, The Pennsylvania State University, Univesrity Park, Pennsylvania, USA

[2]Department of Kinesiology, The Pennsylvania State University, University Park, Pennsylvania, USA

[3]School of Hospitality Management, The Pennsylvania State University, University Park, Pennsylvania, USA

[4]Athletic Deparment, The Pennsylvania State University, University Park, Pennsylvania, USA

Correspondence: Peter Lawrence Bordi, Center for Food Innovation, School of Hospitality Management, The Pennsylvania State University, 124 Mateer Building, University Park, Pennsylvania, 16802, USA. Tel: 814-863-3579. E-mail: plbjr@psu.edu

Received: April 7, 2017 Accepted: May 13, 2017 Online Published: June 21, 2017

doi:10.5539/jfr.v6n4p83 URL: https://doi.org/10.5539/jfr.v6n4p83

Abstract

Essential omega-3 fatty acids must be consumed through the diet to meet the body's nutrition requirement. Daily-recommended intake of omega-3 fatty acids for adults is 270 milligrams/day. These fatty acids are commonly consumed through fish, but it is known that the United States population at large is not meeting their recommended daily intake. Supplements containing these important acids should be considered to close the gap between recommendations and actual intake. To create a product with these beneficial acids, sensory analysis was conducted to see if non-trained male and female athlete panelists could notice the difference in several key sensory characteristics (appearance, initial taste, color, sweetness, consistency, chocolate flavor, aftertaste, overall quality and overall liking) in a chocolate protein-based recovery beverage. The sensory-neutral oil was added into the beverage and athletes (n=95) were asked to taste the omega-3 and original beverage and rank each characteristic on hedonic and just-about-right scales. Color of the drink, aftertaste, overall quality and overall liking were rated significantly higher for the omega-3 added drink. Overall, the addition of the omega-3 fatty acids improved the beverage in several key attributes and can be added into the final formulation of the product.

Keywords: athlete, beverage enhancement, omega-3 fatty acid, sensory analysis

1. Introduction

The American Heart Association (2017) states that omega-3 polyunsaturated [omega-3] fatty acids have been shown in research to benefit the heart in various ways, such as decreasing the risk of arrhythmias, decreasing triglyceride levels, slowing the rate of growth for atherosclerotic plaque, and lowering blood pressure. Researchers such as Hu et al. (2002), Hu, Cho, Rexrode, Albert, & Manson (2003), Oomen et al. (2000) and Dewailly, Blanchet, Gingras, Lemieux, & Holub (2003) have provided well-known observational studies, which have laid the foundation for drawing a connection between omega-3 fatty acids and heart benefits. In addition, the National Institutes of Health (NIH) reviewed current omega-3 fatty acid research to see if supplementation is effective in the treatment of various health conditions. Omega-3 fatty acids are currently being studied as a complementary health approach for cardiovascular disease, rheumatoid arthritis, various diseases of the brain and of the eyes, allergies, asthma, Crohn's disease, cystic fibrosis, diabetes, kidney disease, lupus, obesity, and osteoporosis, among many others. Although hard conclusions cannot currently be drawn about omega-3s and their benefits, additional research is still being conducted and funded at a national level (National Center of Complementary and Integrative Health [NCCIH], 2015).

Scientists began to study the health benefits of long chain omega-3 fatty acids over three decades ago. They found that the Greenland Eskimos had less incidence of heart disease than other ethnic groups despite their high fat diet, which included blubber from marine animals (Bang & Dyerberg, 1980). Multitudes of recent health and nutrition articles have focused on long chain omega-3 polyunsaturated fatty acids and their benefits for human health. These include both improved heart health and stronger immune function (Ruxton & Derbyshire, 2009).

The current scientific evidence points to improved heart health with the addition of omega-3 into diets through prevention of atrial fibrillation, reduction of hypertension, reduction of fatal ventricular arrhythmias and altered production of prostaglandins. Together, these result in reduced inflammation and improved endothelial and platelet function (Cao et al., 2015).

A growing body of evidence (Fontani et al., 2005; Campoy, Escolano-Margarit, Anjos, Szajewska, & Uauy, 2012; Hu et al., 2002; Hu et al., 2003) strongly suggests that including or increasing omega-3 intake is important to a healthy diet. Current United States guidelines suggest a combined minimum of 270 milligrams/day for adults of two particular types of omega-3 acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Institute of Medicine, 2005). One way to ensure that minimum requirements for EPA and DHA are met is through the American Heart Association's recommendation of eating fatty fish at least two times (two servings of 3.5 ounces cooked) per week. Although it is preferred to consume omega-3 fatty acids through food, supplementation may be necessary for those with coronary heart disease who need more than they can reasonably consume through diet alone, or for those who need to be careful of mercury or other environmental contaminants intake ("Fish and Omega-3 Fatty Acids," 2015).

Of special interest are EPA and DHA, two long chain omega-3 polyunsaturated fatty acids (LC3PUFA). As a country, we chronically under-consume these essential acids; what we do get is provided through aquatic life and fish (Forchielli & Walker, 2011). Rich sources of LC3PUFA include salmon, trout, herring, mackerel, lake trout, and sardines ("Fish and Omega-3 Fatty Acids," 2015). Other common sources of omega-3 fatty acids other than fish and marine life include some vegetable oils, walnuts, chia and hemp seeds, and soy foods, such as edamame or soybeans (Denny, 2015). There is moderate evidence that the addition of fatty fish to one's regular diet is beneficial, but the benefits of omega-3 supplementation are still unclear (NCCIH, 2016). Although the demand for marine omega-3 fatty acids have been increasing steadily, the production of these oils is stable and not expected to increase due to sustainability concerns (Tur, Bibiloni, Sureda, & Pons, 2012).

National Health and Nutrition Examination Survey (NHANES) reports the average consumption of omega-3 fatty acids in the population of 19 years and older in the United States. NHANES found that the average intake of fish high in omega-3 fatty acids was 0.15± 0.03 ounces per day (Papanikolaou, Brooks, Reider, & Fulgoni, 2014). This is well below the recommendation from the American Heart Association (2016) of approximately one ounce on average per day. Considering that heart disease is the leading cause of death in the United States, accounting for one in four deaths or 600,000 deaths per year, it affects individuals across the country, regardless of their gender or ethnicity ("Heart Disease Facts," 2015). It was estimated that in 2011, cardiovascular disease cost $320.1 billion dollars for treatment, medications, and lost productivity (Mozaffarian et al., 2015).

Omega-3 fatty acid benefits have been studied in athletes. Atashak et al. (2013) studied their positive effects that daily omega-3 supplementation had on blood levels of oxidative stress, muscle damage, and inflammation markers in athletes. Hasadsri et al. (2013) reviewed several studies on omega-3 fatty acids as a potential treatment for traumatic brain injuries (e.g. concussions). Additional research needs to be completed for evidence to be conclusive.

There is suggestive but not conclusive evidence linking omega-3 fatty acid supplementation with various benefits (Balk et al., 2016). As the American Cancer Society (2015) points out, supplements are not required to go through rigorous testing like pharmaceuticals and thus may pose a risk to health. This may be a concern, as a few studies have potentially found correlations between omega-3 fatty acid blood levels to prostate cancer (Brett, 2013).

This study aims to discover if 50 milligrams of essential omega-3 fatty acid oil can be added into a serving of a protein-based recovery drink and remain sensory-neutral from a consumer's standpoint. Sensory analysis for this purpose can be conducted as a product comparison test, as it is an effective way to gauge consumer liking and what factors may be driving that liking (Sensory Analysis Center, 2015). Conducting sensory analysis is important to ensure that the product is still acceptable after the reformulation, as it is known that liking a food item has a strong positive correlation with intake (Byrnes & Hayes, 2013). If the sensory properties were degraded, the formulation would need to be reconsidered, as the negative attributes may sway individuals to not consume the beverage, regardless of health benefits. In addition, as Lawless and Heymann (2010) point out, studying the sensory characteristics and attributes of a product by utilizing consumer's opinion and their point of view is useful information to the developers of the product. In this case, a chocolate protein-based recovery beverage enhanced with omega-3 fatty acids will be compared against the original chocolate beverage without the fatty acids.

2. Methodology and Materials

Data collection was conducted on iPads in the athletic training rooms of male and female athletes at a large university in the northeastern United States. Athletes were given both the original recovery drink and the omega-3 enhanced recovery drink in 5-ounce plastic cups at the same time, individually labeled with three-digit blinding codes. Participants were asked to taste and rank each beverage for several sensory characteristics and select their overall preference.

2.1 Beverage Development

The original beverage has been formulated with a research-based protein formula intended for athletic recovery and was available for athletes and all consumers at the university. The current protein formula has been developed to ease soreness, accelerate muscle growth, and prevent injuries (Reidy et al., 2014). The beverage was produced on the university campus and was tested extensively for quality and consumer preferences. The omega-3 enhanced recovery beverage was also formulated at the university, with the addition of omega-3 fatty acids. As mentioned previously, 50 milligrams of omega-3 oil have been added into each serving. Both of the beverages nutritional information has been detailed in Table 1 and Table 2.

The omega-3 enhanced beverage is nearly identical to the original beverage when considering the nutrition and the composition. All ingredients were kept constant, with the exception of the milk solids. Milk solids are composed of fat (International Dairy Foods Association, 2017), and thus were pulled out to offset the addition of fat from the omega-3 oil. The goal of this reformulation was to keep the nutritional components nearly identical while replacing the fat from the milk solids with a fat that has health benefits.

Table 1. Nutritional Information for Original Recovery Beverage

Original Beverage, 12 oz	
Calories	338
Calories from Fat	68
Total Fat	8 g
Saturated Fat	5 g
Trans Fat	0 g
Cholesterol	26 mg
Sodium	473 mg
Total Carbohydrate	41 g
Dietary Fiber	0 g
Sugars	39 g
Protein	26 g

Table 2. Nutritional Information for Omega-3 Enhanced Recovery Beverage

Omega-3 Enhanced Beverage, 12 oz	
Calories	310
Calories from Fat	45
Total Fat	5 g
Saturated Fat	3 g
Trans Fat	0 g
Cholesterol	25 mg
Sodium	340 mg
Total Carbohydrate	40 g
Dietary Fiber	2 g
Sugars	37 g
Protein	23 g

2.2 Testing Procedure

Individuals were pre-screened for potential allergies and intolerances and informed of potential risks before participation. Samples of each drink were served in 5-ounce clear plastic cups for the taste test. Compusense Cloud software (Compusense Cloud, Compusense Inc., Canada) was utilized to collect data on the tablets and run initial statistical analysis. The Institutional Review Board (IRB) assigned the following submission approval number: IRB #00004465.

2.3 Participants

A total of 95 male and female athlete participants were recruited on a volunteer basis from various athletic teams across the university. Age and gender breakdowns are offered in Table 3 and 4. Most of the participants work out 6-7 times per week (78 participants; 82.11%), with the minimum number of workouts for a participant being 3 times per week (1 participant, 1.05%). Sport involvement for the participants included football, lacrosse, field hockey, tennis, rugby, soccer and swimming, and is offered in a breakdown by gender in Table 4. A majority of the recruited participants were male football players because this demographic most commonly consumes the recovery beverage at the university. Athletes were separated during testing to ensure their peers did not influence their opinions.

Table 3. Participants' Age Distribution

Age Group	Frequency	Percent
18-19	36	37.89%
20-21	48	50.53%
22-23	10	10.53%
24-25	1	1.05%
Total	**95**	**100.00%**

Table 4. Participants' Sport Involvement

Sport	Male	Female
Football	45	0
Lacrosse	17	10
Field Hockey	0	12
Tennis	0	6
Rugby	0	3
Soccer	0	1
Swimming	0	1
Total (N=95)	**62 (65.26%)**	**33 (34.74%)**

2.4 Sensory Evaluation

Participants were requested to rate the following sensory characteristics on a 9-point hedonic scale: appearance, initial taste, aftertaste, overall quality and overall liking. Color of the drink, sweetness, consistency and chocolate flavor were rated on a 5-point JAR (just about right) scale to determine the appropriateness of the level of each specific attribute. In addition, participants were asked to indicate their preference between the two samples

3. Results and Discussion

To compare sensory differences between the plain recovery protein beverage and the omega-3 supplemented beverage, paired t-tests were employed. The means, standard deviations, calculated t-value and resulting p-value are shown in Table 5 for all participants, while male and female data are broken down in Table 6.

Table 5. Mean Comparison Between Original and Omega-3 Added

	Original		Omega-3 Added		Differences	
	Mean	SD	Mean	SD	T	p-value
Appearance [a]	7.24	1.12	6.84	1.19	3.38	**0.00****
Initial Taste [a]	7.02	1.44	7.07	1.31	-0.27	0.79
Color of Drink [b]	2.87	0.33	2.99	0.31	-2.46	**0.02***
Sweetness [c]	2.94	0.60	2.81	0.49	1.65	0.10
Consistency [d]	3.01	0.56	3.05	0.51	-0.58	0.57
Chocolate Flavor [e]	2.68	0.64	2.78	0.53	-1.22	0.23
Aftertaste [a]	5.65	1.68	6.61	1.63	-5.13	**0.00****
Overall Quality [a]	6.85	1.25	7.18	1.18	-2.27	**0.03***
Overall Liking [a]	6.87	1.42	7.29	1.23	-2.40	**0.02***

[a] Nine-point hedonic scale, where 1=dislike extremely, 2=dislike very much, 3=dislike moderately, 4=dislike slightly, 5=neither like nor dislike, 6=like slightly, 7=like moderately, 8=like very much, 9=like extremely

[b] Five-point just about right (JAR) scale: 1= too light, 2= slightly too light, 3=just about right, 4=slightly too dark, 5=too dark

[c] Five-point JAR scale: 1= not sweet, 2= slightly not sweet, 3=just about right, 4=slightly too sweet, 5=too sweet

[d] Five-point JAR scale: 1= too thin, 2= slightly too thin, 3=just about right, 4=slightly too thick, 5=too thick

[e] Five-point JAR scale: 1= too weak, 2= slightly too weak, 3=just about right, 4=slightly too strong, 5=too strong

* $p < 0.05$, ** $p < 0.01$

Table 6. Mean Comparison Between Original and Omega-3 Added for Males and Females

	Males						**Females**					
	Original		**Omega-3**		**Differences**		**Original**		**Omega-3**		**Differences**	
	Mean	SD	Mean	SD	T	p-value	Mean	SD	Mean	SD	T	p-value
Appearance[a]	7.16	1.10	6.84	1.18	2.22	**0.03***	7.39	1.14	6.85	1.23	2.67	**0.01****
Initial Taste[a]	7.29	1.11	6.98	1.41	1.42	0.16	6.52	1.84	7.24	1.09	-1.94	0.06
Color of Drink[b]	2.87	0.34	2.98	0.38	-1.72	0.09	2.88	0.33	3.00	0.00	-2.10	**0.04***
Sweetness[c]	2.97	0.60	2.79	0.49	2.02	**0.05***	2.88	0.70	2.85	0.36	0.21	0.84
Consistency[d]	2.94	0.54	3.03	0.51	-1.14	0.26	3.15	0.57	3.09	0.52	0.44	0.66
Chocolate Flavor[e]	2.74	0.60	2.82	0.56	-0.84	0.40	2.58	0.71	2.70	0.47	-0.89	0.38
Aftertaste[a]	5.68	1.68	6.42	1.74	-3.01	**0.00****	5.61	1.71	6.67	1.33	-5.16	**0.00****
Overall Quality[a]	7.06	1.07	7.08	1.15	-0.11	0.92	6.45	1.46	7.36	1.22	-3.29	**0.00****
Overall Liking[a]	7.05	1.18	7.18	1.18	-0.69	0.49	6.55	1.75	7.52	1.30	-2.80	**0.01****

[a, b, c, d, *, **] Scales above are the same used in Table 5

When comparing the original protein beverage to the omega-3 protein beverage amongst all participants, appearance, color of the drink, aftertaste, overall quality and overall liking were rated significantly different. Appearance and aftertaste were the only two characteristics that consistently had significant findings for both genders. It is possible that the difference in appearance was found due to the omega-3 enhanced beverage appearing to be more "glossy" due to the addition of the oil. Taste, consistency, and chocolate flavor were unaffected by the addition of omega-3's, thus characteristics key to athlete/consumer acceptance were comparable between beverages. Indeed, means for the omega-3 drink were higher for these attributes: color, aftertaste, quality and liking.

The gender breakdown in Table 6 shows that females rated the overall quality and the overall liking of the omega-3 enhanced beverage higher over the original beverage, while males did not. While is documented that men and women differ in their taste perceptions (Leshem, Haliwa, Hochman, & Manasherov, 2008), it is important to consider the sample size and proportion of women in this study ($n_{Women} = 33$, $n_{Total\ participants} = 95$). It is possible that if more females participated in this study, the same results would not occur. This warrants additional investigation on the differences in taste perception between males and females, and how those perceptions influence their final decisions on how to rate key sensory attributes.

At the end of the test, individuals were asked which of the two drinks they preferred. Table 7 details the individual response rates within the group and between males and females. The analysis was carried out using the total number of responses, with both genders grouped together. As suggested by Lawless & Heymann (2010), because the "no preference" option has below 20% of responses (actual percentage 16.84%), the no-preference option is eliminated for the purposes of analysis. For the, remaining responses (n=79), the minimum value required for a significant difference with an alpha criterion is 49 (Lawless & Heymann, 2010). Among those expressing a preference, there was a significant preference for the omega-3 beverage. When looking at whether there was a statistically significant difference amongst the male responses, 34 individuals would have had to indicate a preference for one of the beverages, but only 22 males preferred the original beverage and 29 preferred the omega-3 beverage. However, there was a significant difference amongst the female responses. This required a minimum of 20 (Lawless & Heymann, 2010) females to express a preference for one drink. Because the omega-3 added drink had 21 females responding that they favored it, there was a significant difference in preference for females.

Table 7. Preference Test

Preference	Male Responses	Female Responses	Total Number of Responses
Original	22 (35.48%)	7 (21.21%)	29 (30.53%)
Omega-3 Added	29 (46.77%)	21 (63.64%)	50 (52.63%)
I prefer both equally	11 (17.74%)	5 (15.15%)	16 (16.84%)

4. Conclusion

Before finalizing any new product reformulation changes, it is important to verify that the major sensory attributes are not negatively affected by the addition or subtraction of ingredients. The present study showed that it is possible to enhance a milk-based protein recovery beverage with omega-3 fatty acids and not have the sensory results degraded. In fact, several attributes important to consumers when choosing a beverage were improved, including the color of the drink, aftertaste, overall quality and overall liking. This confirms that incorporating omega-3 fatty acid oils into the beverage is advantageous, due to the presumed health benefits gained without hurting (indeed improving) the quality of the drink. It is key to realize that liking of a food has a strong, positive correlation with intake (Byrnes & Hayes, 2013), indicating that the significant difference found between overall liking in this experiment is very important, as it favors the omega-3 containing beverage. Although not all of the attributes came out as favoring the omega-3 beverage, overall liking is perhaps the most important.

Because of the need to incorporate more essential fatty acids into American's diets, researchers and product developers should continue to test the addition of omega-3 acids into a larger range of beverage and food products.

Funding Sources/Conflict of Interest

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

References

American Cancer Society. (2015). Risks and side effects of dietary supplements. Retrieved from https://www.cancer.org/treatment/treatments-and-side-effects/complementary-and-alternative-medicine/dietary-supplements/risks-and-side-effects.html

American Heart Association. (2016). Fish and omega-3 fatty acids. Retrieved from http://www.heart.org/HEARTORG/HealthyLiving/HealthyEating/HealthyDietGoals/Fish-and-Omega-3-Fatty-Acids_UCM_303248_Article.jsp#.WK-JmLYrI0Q

American Heart Association. (2017). Heart and stroke encyclopedia: Omega-3 fatty acids. Retrieved from http://www.heart.org/HEARTORG/Encyclopedia/Heart-Encyclopedia_UCM_445084_Encyclopedia.jsp?levelSelected=15&title=omega%203%20fatty%20acids

Atashak, S., Sharafi, H., Azarbayjani, M. A., Stannard, S. R., Goli, M. A., & Haghighi, M. M. (2013). Effect of omega 3 supplementation on the blood levels of oxidative stress, muscle damage and inflammation markers after acute resistance exercise in young athletes. *Kinesiology, 45*(1), 22-29. Retrieved from https://www.ebscohost.com/academic/sportdiscuss-with-full-text/

Balk, E. M., Adam, G. P., Langberg, V., Halladay, C, Chung, M., Lin, L., & Trikalinos, T. A. (2016). Omega-3 Fatty Acids and Cardiovascular Disease: An Updated Systematic Review. Evidence Report/Technology Assessment No. 223. (Prepared by the Brown Evidence-based Practice Center under Contract No. 290-2012-00012-I.) AHRQ Publication No. 16-E002-EF. Rockville, MD: Agency for Healthcare Research and Quality. Retrieved from www.effectivehealthcare.ahrq.gov/reports/final.cfm

Bang, H. O., & Dyerberg, J. E. (1980). Lipid metabolism and ischemic heart disease in Greenland Eskimos. In H. H. Draper (Ed.), *Advances in Nutritional Research*, 1-22. https://doi.org/10.1007/978-1-4757-4448-4_1

Brett, A. S. (2013). Higher [omega]-3 fatty acid levels are associated with risk for prostate cancer. *NEJM Journal Watch General Medicine, 105*(1132). https://doi.org/10.1056/nejm-jw.NA31812

Byrnes, N. K., & Hayes, J. E. (2013). Personality factors predict spicy food liking and intake. *Food Quality Preference, 28*(1), 213-221. https://doi.org/10.1016/j.foodqual.2012.09.008

Campoy, C., Escolano-Margarit, M. V., Anjos, T., Szajewska, H., & Uauy, R. (2012). Omega 3-fatty acids on child growth, visual acuity and neurodevelopment. *The British Journal of Nutrition, 107*, S85-S106.

https://doi.org/10.1017/S0007114512001493

Cao, Y., Lu, L., Liang, J., Liu, M., Li, X., Sun, R., … Zhang, P. (2015). Omega-3 fatty acids and primary and secondary prevention of cardiovascular disease. *Cell Biochemistry and Biophysics, 72*(1), 77-81. https://doi.org/10.1007/s12013-014-0407-5

Compusense Cloud [Computer software]. 2017. Retrieved from https://www.compusense.com

Denny, S. (2015, May 19). What are Omega-3 fatty acids? Retrieved on August 14, 2016 from http://www.eatright.org/resource/food/vitamins-and-supplements/types-of-vitamins-and-nutrients/what-are-omega-3-fatty-acids

Dewailly, E., Blanchet, C., Gingras, S., Lemieux, S., & Holub, B. J. (2003). Fish consumption and blood lipids in three ethnic groups of Québec (Canada). *Lipids, 38*(4), 359-365. https://doi.org/10.1007/s11745-003-1070-4

Fish and Omega-3 Fatty Acids. (2015). Retrieved from http://www.heart.org/HEARTORG/HealthyLiving/HealthyEating/HealthyDietGoals/Fish

Fontani, G., Corradeschi, F., Felici, A., Alfatti, F., Migliorini, S., & Lodi, L. (2005). Cognitive and physiological effects of Omega‐3 polyunsaturated fatty acid supplementation in healthy subjects. *European Journal of Clinical Investigation, 35*(11), 691-699. https://doi.org/10.1111/j.1365-2362.2005.01570.x

Forchielli, M. L., & Walker, W. A. (2011). [Omega]-3 fatty acids: Impact on cognitive development and inflammation. *Nutrition Today, 46*(5), 224-234. https://doi.org/10.1097/NT.0b013e3182303fc4

Hasadsri, L., Wang, B. H., Lee, J. V., Erdman, J. W., Llano, D. A., Barbey, A. K., & Wang, H. (2013). Omega-3 fatty acids as a putative treatment for traumatic brain injury. *Journal of Neurotrauma, 30*(11), 897-906. https://doi.org/10.1089/neu.2012.2672

Heart Disease Facts. (2015, August 10). Retrieved on August 17, 2016 from http://www.cdc.gov/heartdisease.facts

Hu, F. B., Bronner, L., Willett, W. C., Stampfer, M. J., Rexrode, K. M., Albert, C. M., … Manson, J. E. (2002). Fish and omega-3 fatty acid intake and risk of coronary heart disease in women. *Journal of the American Medical Association, 287*(14), 1815-1821. https://doi.org/10.1001/jama.287.14.1815

Hu, F. B., Cho, E., Rexrode, K. M., Albert, C. M., & Manson, J. E. (2003). Fish and long-chain omega-3 fatty acid intake and risk of coronary heart disease and total mortality in diabetic women. *Circulation, 107*(14), 1852-1857. https://doi.org/10.1161/01.CIR.0000062644.42133.5F

Institute of Medicine. (2005). Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein and Amino Acids. Food and Nutrition Board. The National Academies Press: Washington, DC. https://doi.org/10.17226/10490

International Dairy Foods Association. (2017). *Definitions*. Retrieved from http://www.idfa.org/news-views/media-kits/milk/definition

Lawless, H. T., & Heymann, H. (2010). Sensory evaluation of food: Principles and practices (2nd ed.). New York, NY: Springer.Leshem, M., Haliwa, M., Hochman, A., Manasherov, M., & Yaccobi, A. (2008). Gender differences in basic taste perception. *Appetite, 51*(2), 381. https://doi.org/10.1016/j.appet.2008.04.146

Mozaffarian, D., Benjamin, E. J., Go, A. S., Arnett, D. K., Blaha, M. J., Cushman, M., … Turner, M. B. (2015). Heart disease and stroke statistics-2015 update: A report from the American Heart Association. *Circulation, 131*(4), e29-e322. https://doi.org/10.1161/CIR.0000000000000152

National Center for Complementary and Integrative Health. (2015). Omega-3 supplements: In depth. (NCCIH Publication No. D482). Washington, DC: U.S. Government Printing Office.

Oomen, C. M., Feskens, E. J., Räsänen, L., Fidanza, F., Nissinen, A. M., Menotti, A., … Kromhout, D. (2000). Fish consumption and coronary heart disease mortality in Finland, Italy, and the Netherlands. *American Journal of Epidemiology, 151*(10), 999-1006. https://doi.org/10.1093/oxfordjournals.aje.a010144

Papanikolaou, Y., Brooks, J., Reider, C., & Fulgoni, Victor, L. F. (2014). U.S. adults are not meeting recommended levels for fish and omega-3 fatty acid intake: Results of an analysis using observational data fromNHANES 2003-2008. *Nutrition Journal, 13*(31). https://doi.org/10.1186/1475-2891-13-31

Reidy, P. T., Walker, D. K., Dickinson, J. M., Gundermann, D. M., Drummond, M. J., Timmerman, K. L., … Rasmussen, B. B. (2014). Soy-dairy protein blend and whey protein ingestion after resistance exercise

increase amino acid transport and transporter expression in human skeletal muscle. *Journal of Applied Physiology, 116*(11). https://doi.org/10.1152/japplphysiol.01093.2013

Ruxton, C. H. S. & Derbyshire, E. (2009). Latest evidence on omega-3 fatty acids and health. *Nutrition and Food Science, 39*(4), 423-438. https://doi.org/10.1108/0034665910976293

Sensory Analysis Center. (2015). Consumer Product Comparison Tests. Retrieved from http://www.sensoryanalysis.com/services/consumer-evaluation/product-comparison-tests.html

Tur, J. A., Bibiloni, M. M., Sureda, A., & Pons, A. (2012). Dietary sources of omega 3 fatty acids: Public health risks and benefits. *The British Journal of Nutrition, 107*, S23-S52. https://doi.org/10.1017/S0007114512001456

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Development and Testing of Gluten-Free Pasta Based on Rice, Quinoa and Amaranth Flours

Sarah Makdoud[1] & Kurt A. Rosentrater[1]

[1]Agricultural and Biosystems Engineering, Food Science and Human Nutrition, Iowa State University, United States

Correspondence: Kurt A. Rosentrater, Agricultural and Biosystems Engineering, Food Science and Human Nutrition, Iowa State University, United States. E-mail: karosent@iastate.edu

Received: January 13, 2017 Accepted: June 16, 2017 Online Published: June 26, 2017

doi:10.5539/jfr.v6n4p91 URL: https://doi.org/10.5539/jfr.v6n4p91

Abstract

The goal of this study was to make high quality gluten-free pasta using amaranth, quinoa and rice flours, water and eggs using extrusion processing, and to compare these with gluten-free pasta already commercialized. The difficulty was to reproduce the texture provided by the gluten network without using gluten. To do that, an experimental design was created in order to make samples with different quantities of each grain, egg whites and water. Samples were manufactured and various tests (e.g., color analysis, water activity, cooking loss, texture, etc.) were carried out in order to find the best formulation, namely the formulation which was closest to Barilla or Andean dream gluten-free commercial pasta. With Rcommander software, results were analyzed and it was determined that the best pasta formulation was 10% amaranth flour, 40% quinoa flour, and 50% rice flour, with 18% eggs whites and 39% water. This optimal formulation was manufactured and subjected to sensory analysis with other commercial samples (Barilla, Andean Dream). Statistical analyses were conducted and it was shown that, even though this formulation did not quite achieve Barilla or Andean Dream pastas quality, it approached closely in some parameters. Indeed, 80% of consumers did not refuse to eat this pasta again, and with addition of tomato sauce, no differences were seen between the spaghettis. However, individual sample analysis did indicate that consumers did not appreciate the formulation's sticky texture, thus this parameter would have to be reworked to achieve higher quality.

Keywords: celiac disease, gluten-free, quinoa, amaranth, rice, pasta, extrusion

1. Introduction

Celiac disease (CD), also known as celiac sprue and gluten sensitive enteropathy, is a permanent intolerance to gluten and one of the most frequent food intolerance worldwide. It may be defined as an inflammatory disease of the upper small intestine in genetically susceptible individuals, in both children and adults, triggered by ingestion of wheat, rye, barley, and possibly oat products. Indeed, the precipitating factor of CD are the storage proteins of these cereals, found in the triticeae tribe of grains and widely called gluten, which are harmful for the sensitive consumers (Wieser and Koelher, 2007).

For these people, eating gluten causes the villi of the intestine to atrophy, which prevents food from being absorbed and produces an inflammatory reaction (Broz and Horn, 2007).

Many different symptoms are associated with CD, which can be divided into intestinal features (chronic diarrhea, vomiting, abdominal distension, etc.) and into the results of malabsorption (deficiencies of vitamins and minerals, loss of weight, etc.). Currently, the only effective treatment for CD is the strict lifelong renunciation of gluten containing foods. Unfortunately, most common foods and beverages, such as bread, biscuits, beer, pizza and pasta, are made from cereals containing gluten such as wheat. Wheat contains two proteins, glutenin and gliadin, which during mixing and kneading develop into gluten. Gluten is responsible for the protein-starch interaction that provides specific viscoelastic properties in products.

Since the discovery of Dicke (1950) that the ingestion of wheat was responsible for the symptoms of CD, numerous reviews appeared in order to meet the demand for gluten-free products such as pasta. Indeed, pasta is a highly convenient food product, consumed all over the world. The term usually refers to unleavened extruded wheat dough, composed simply of flour and water, sometimes egg. Pasta provides significant quantities of

complex carbohydrates, proteins, B-vitamins, and iron and is low in sodium, amino acids, and total fat. However, a significant part of the human population cannot tolerate gluten, hence, there is a high demand for gluten-free pasta. Furthermore, there is also a growing segment of the population choosing to follow gluten-free diet for nonmedical reasons. These people may have family or friends with gluten intolerance or they may simply feel better on a gluten-free diet. Indeed, switching from refined wheat products to nutritionally more valuable grains could bring benefits regarding health and well-being (Hager et al., 2012).

Thus, the target for development is to develop gluten-free pasta suitable for the CD patient with a taste and texture similar to those of pastas made with whole wheat flour. That is a big challenge for food research and development, because the network forming ability of gluten needs to be substituted by other means, in order to achieve products with satisfying quality (Schoenlechner et al., 2010).

Though wheat, rye, barley and possibly oats are harmful, corn, rice, amaranth, quinoa, buckwheat and teff are not and can be used in gluten-free pasta formulation. Furthermore, these ancient grains have nutrition, antioxidants, and textural qualities suitable for functional foods.

Amaranth (*Amaranthus caudatus*) and Quinoa (*Chenopodium quinoa*) contain about thirty percent more protein than cereals such as rice, sorghum and rye. They are a good source of the essential amino acid, lysine, which is low in other grains, and dietary minerals including calcium, magnesium, and phosphorus. Quinoa, also called super food because of its remarkable nutritional value, contains antioxidant phytonutrients (polyphenols and phytosterols) and flavonoid in concentrated amounts with possible nutraceutical benefits. Moreover, quinoa starch has useful physicochemical properties, such as viscosity and freeze stability (Inglett et al., 2015). Because these grains have unique characteristics, studies focused on the manufacture and characterization of gluten-free pastas have been conducted. They evaluated Quinoa and Amaranth's pasting properties, water holding capacities, phenolic contents, and antioxidant activities. In each case, the spaghetti samples were manufactured by means of a pilot plant equipped with an extruder and a spaghetti nozzle, for the production of fresh-extruded spaghetti, and a dryer for the production of the dry spaghetti. Different type of grains and flour were used to prepare the dough. Some samples were manufactured with a mixture of pre-gelatinized flour and conventional flour, whereas others samples were only manufactured with conventional flour. Pre-gelatinization is done using a steam cooker, where a quantity of water was mixed with another quantity of flour and heated for about 1 h and then cooled. Once pasta made, samples were taken for physicochemical measurements such as dough rheological properties, carotenoid determination, chemical determination, spaghetti cooking quality (optimal cooking time, cooking loss, swelling index and water absorption), texture, determination of gelatinization degree, in vitro digestion, sensory analysis, etc.) (Padalino et al., 2012).

Studies show that there is an unbalanced intake of carbohydrates, protein, and fat, as well as limited intake of certain essential nutrients in celiac subjects compared to a control group of people on a normal diet. Indeed, commercial gluten-free pasta often shows significantly lower protein contents compared to wheat-containing counterparts. Hence, the utilization Amaranth and Quinoa is beneficial as they contain higher amounts of protein than many other flours, as vegetable flour or wheat flour. Moreover, apart from the utilization of high-quality raw materials, enrichment with additional protein ingredients is another approach to improve the nutritional value but also the texture of gluten-free products. An obvious ingredient to increase the protein content of pasta is egg. Eggs are traditionally used in pasta mainly to achieve flavor effects, but can also aid structure formation. Egg proteins facilitate the formation of a tighter protein network, yielding a harder product, both before and after cooking. In addition, the tighter protein network reduces penetration by water and hence starch granule swelling during cooking.

Gluten-free dough is often more fragile, less elastic and more susceptible to overworking; thus, the production of pasta based solely on gluten-free flours has largely been unsuccessful. Several publications report that cooking loss is increased for gluten-free products, due to the absence or interruption of the gluten network Indeed, a single ingredient that will replace gluten does not exist. This is in agreement with previous authors who also found that additional structuring agents are necessary to obtain extrudable dough. Chillo et al. (2007) were not successful in the production of oat pasta unless carboxymethylcellulose and pre-gelatinized starch were added. Moreover, Sabanis and Tzia (2011) showed that a major role in the formation of a tight protein network is played by ovalbumin, the main protein of albumin.

According to Engleson and Atwell (2008), the common approach to improve the texture of gluten-free product is to assemble a mixture of starches, hydrocolloids, fibers and dairy ingredients to replace all the functionalities of pasta with gluten. In addition to proteins, others structuring agents can be used like carboxymethylcellulose, transglutaminase and pre-gelatinized starch to mimic the viscoelastic properties of gluten in gluten-free pasta.

Also, the fatty nature of emulsifiers enables them to act as a lubricant in the extrusion process, resulting in less nozzle wear and tear and thus making production easier. Hager et al. (2012) found that mono- and diglycerides of fatty acids form complexes with amylose, thereby preventing the passage of starch into the cooking water, reducing cooking loss and stickiness.

More specific research was carried out to study the effect of three non-grasses cereals, amaranth, quinoa and buckwheat, in gluten-free pasta production. The results demonstrated that pasta produced from amaranth decreased texture firmness, cooking time and cooking tolerance, while pasta from quinoa were better agglutinated but showed increased cooking loss. In buckwheat pasta the least negative effects were observed.

By combination of all three raw materials to one flour blend (60% buckwheat, 20% amaranth and 20% quinoa), dough matrix and nutritional properties were improved. The quality of the product was more improved with addition of xanthan, egg or emulsifier. In order to replace the missing gluten in amaranth, quinoa and buckwheat flour a further aim of investigation was the addition of different protein isolates. The isolates selected were egg white powder, soy protein isolate and casein. The egg addition had positive effects on gluten-free pasta quality: higher texture firmness and lower cooking loss (except for buckwheat pasta). Cooking time was reduced in the pasta with soy protein and casein added pasta, demonstrating that the dough matrix was not strong enough to prevent the pasta from disintegration. Dough moisture is also recognized to have a major influence on pasta quality. High dough moisture toughened the dough, which adhered to the screw of the pasta machine and the produced pasta were very sticky, disintegrated during boiling and therefore showed very low texture firmness. Too low dough moisture resulted in noodles, which showed surface cracks. Additionally, dough moisture content had a significant effect on cooking loss, which was minimized with lower moisture content. After several trials, optimum dough moisture content to increase texture firmness and minimize cooking loss was determined to be 30%. Then, addition of emulsifier (DATEM and DMG) in gluten-free pasta was studying. Generally, according to the food additive legislation, it is not allowed to add emulsifiers to dry pasta, but an exception can be made for gluten-free pasta. Results show that only the addition of DMG (1,2%) improved dough matrix in all three pastas: texture firmness and cooking weight were increased and cooking loss decreased.

In sum, by addition of an increased amount of egg white powder (up to 6% of flour) and the emulsifier DMG (1.2% of flour) at optimum dough moisture (30%), the cooking quality parameter for all produced pasta were within a satisfying range. Cooking time could be defined to 8 min for all pasta, which corresponds to the average cooking time for wheat pasta. Furthermore, pastas were much better agglutinated, showed good texture firmness and low cooking loss.

However, compared to wheat pasta, the gluten-free pasta produced from non-grasses differs in terms of color, elasticity and sensory properties. Thus, in order to optimize these parameters, still more research will be necessary (Schoenlechner et al., 2010).

Thus the goal of this research was to make high quality gluten-free pasta using a mixture of grains (amaranth, quinoa, and rice), water and egg whites. To do this, an experimental design was created and different samples were made with a Kitchen Aid mixer. Several tests were carried out in order to find the optimal recipe using a suitable proportion of water, eggs and flour. After that, a sensory analysis and consumer acceptance were performed on the best formulation and compared to Barilla and Andean Dream gluten-free pasta, which were controls in this study.

2. Materials and Methods

2.1 Raw Materials

Amaranth grains were provided by Nutricity LLC (Scottsdale, Arizona, United States). Rice flour (Bob's Red Mill) and quinoa grains (Full Circle) were obtained at a local market (Hyvee, Des Moines, Iowa, United States). All trial pastas were compared with Barilla and Andean dream gluten-free pasta also found in the same local market.

Grains were milled into particles of diameter 0.7mm or less, using a grinding machine (Magic Bullet, MB 1001)

2.2 Pasta Production

2.2.1 Pasta Dough Formulation

The doughs were made by combining flours, eggs and water. The addition of water is the most critical part of the dough manufacture. Actually, if the moisture content is too high, the dough will be too sticky to be extrude. On the contrary, if the moisture content is too low, it will be difficult to knead it and it will be impossible to extrude. Also, according to Schoenlechner et al. (2010), the dough moisture is an important parameter because it has an

influence on pasta quality; if the dough moisture is too high, the pasta will be too sticky and they will disintegrate during cooking.

N	X1	X2	X3	X4
1	0	0	0	0
2	1	0	0	0
3	0,5	0,866	0	0
4	0,5	0,2887	0,8165	0
5	0,5	0,2887	0,2041	0,7906
6	-1	0	0	0
7	-0,5	-0,866	0	0
8	-0,5	-0,2887	-0,8165	0
9	-0,5	-0,2887	-0,2041	-0,7906
10	0,5	-0,866	0	0
11	0,5	-0,2887	-0,8165	0
12	0,5	-0,2887	-0,2041	-0,7906
13	-0,5	0,866	0	0
14	0	0,5774	-0,8165	0
15	0	0,5774	-0,2041	-0,7906
16	-0,5	0,2887	0,8165	0
17	0	-0,5774	0,8165	0
18	0	0	0,6124	-0,7906
19	-0,5	0,25887	0,2041	0,7606
20	0	-0,5774	0,2041	0,7606
21	0	0	-0,6124	0,7906

X1	Quinoa (%)
-1	10
-0,5	20
0	30
0,5	40
1	50

X2	Amaranth (%)
-0,866	5
-0,5774	10
-0,2887	15
0	20
0,2887	25
0,5774	30
0,866	35

X3	White eggs (%)
-0,8165	10
-0,6124	11
-0,2041	13
0	14
0,2041	15
0,6124	17
0,8165	18

X4	Moisture Content
-0,7906	33
0	36
0,7606	39

Figure 1. Experimental matrix using a Doehlert experimental design

In order to find the best recipe, different samples were made according to a formal experimental design. We choose to use a Doehlert experimental design with 4 factors: quantity of amaranth, quinoa, eggs and water. Rice quantity was adjusted with the others. Thus, 21 samples were prepared. The experimental design matrix is detailed in Figure 1.

To make pasta dough, flours were mixed together and adequate amounts of water and egg whites were slowly added during mixing using a laboratory scale mixer (Kitchen Aid KSM75WH) with a speed of 2 for a duration of 5 min. Then, the dough was kneaded by hand during 10 min and stored in a refrigerator for 15 min.

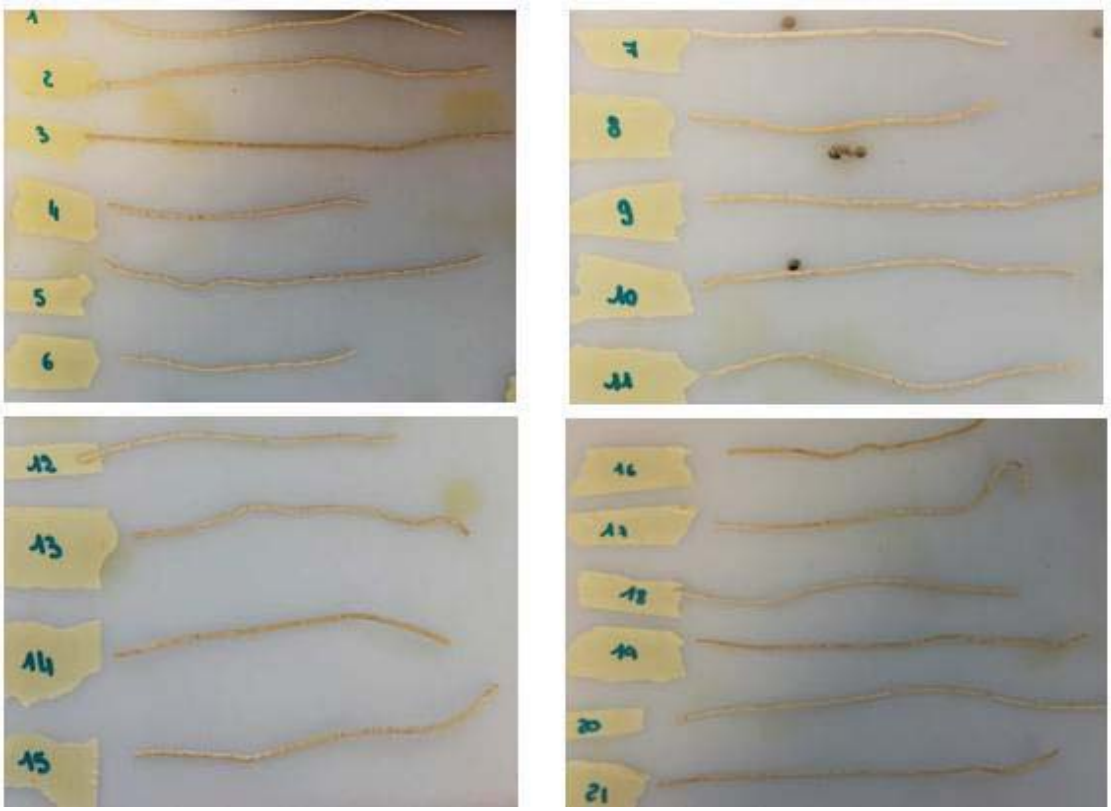


Figure 2. Pictures of all pasta samples (experimental treatments are detailed in Figure 1)

2.2.2 Pasta Manufacture

After 15 min in the refrigerator, the extrusion processing was performing using a Stand-Mixer Pasta-Extruder Attachment (Kitchen Aid KPEXTA) with a speed of 4. The diameter of the holes of the disc die was ≈ 1.65 mm. Extruded spaghettis were immediately manually separated and air-dried for 24 h at ambient temperature (around 25°C), then they were stored in airtight bags. Pictures of all trial pastas are presented in Figure 2.

2.3 Physical Analyses of Pasta

2.3.1 Moisture Content

Moisture content was determined according to the AACC method 44-19 (AACC, 2000). 2g dried spaghettis were put into the oven (Heratherm General Protocol Ovens, ThermoFisher Scientific, Waltham, Massachusetts, United States) at 135°C during 2 h. The mass difference between the initial and final weights gives the moisture content of samples:

$$MC\ (\%) = \frac{DPW - OPW}{DPW} \times 100$$

DPW = dried pasta weight (g); OPW = original (wet) pasta weight (g)

2.3.2 Unit Density

According to Rosentrater et al. (2005), unit density was determined on dried spaghettis as the ratio of the mass (m) to the volume (V). The volume was calculated by measuring the length (L) and the diameter (D) of the sample thanks to the Traceable Electronic Digital Caliper (Fisher Scientific, Pittsburg, Pennsylvania 15275, United States). Spaghetti were assumed to be a cylinder shape, so the following formula can be used:

$$V = \pi \times \frac{D^2}{4} \times L$$

Unit density (UD) was expressed in kg/m^3.

$$UD = \frac{m}{V}$$

2.3.3 Color Analysis

Uncooked spaghetti color was measured using the Chroma Meter CR-410 colorimeter (Konica Minolta Optics, Inc. Chroma meter, Ramsey, New Jersey, USA) equipped with a xenon lamp. Samples were put in a petri dish and the measure was made by direct contact between the sensing head of the colorimeter and the samples. Spaghettis were characterized by three parameters: L* (lightness, scale of 100; 0 matches with black and 100 with white), a* (greenness/redness) and b* (blueness/yellowness). The colorimetric difference ΔE between two samples can be estimated with the following formula:

$$\Delta E = \sqrt{(\Delta L*)^2 + (\Delta a*)^2 + (\Delta b*)^2}$$

If the colorimetric difference is lower than 2, then an unexperienced observer cannot visibly see the difference between two samples in terms of color (Mokryzcki and Tatol, 2011).

2.3.4 Water Activity

Water activity was measured at room temperature using a calibrated water activity meter (AquaLab, series 3TE, model 0800753, Decagon Devices, Inc., Pullman, Washington, USA). The bowl of the water activity meter was filled with cut samples and placed in the measuring chamber of the instrument for measuring the water activity.

2.3.5 Optimal Cooking Time

The optimal cooking time (OCT) was determined according to the AACC Approved Method 66-50, where 5-g dried spaghetti samples were boiled in 200mL of distilled water. Each 30 sec, a spaghetti was removed from boiling water and squeezed between two pieces of Plexiglas. Spaghettis were considered cooked when the center core disappeared.

2.3.6 Water Absorption and Cooking Loss

Water absorption (WA) and cooking loss (CL) were determined according to the AACC Approved Method 66-50. 10g dried spaghettis samples were pre-weighed and boiled in 300mL of water during the cooking time previously determined. Then, spaghettis were removed and weighed: the weight difference before and after cooking was used to calculate the water absorption.

$$WA\ (\%) = \frac{CPW - \mathrm{DPW}}{DPW} * 100$$

CPW= cooked (wet) pasta weight (g)

CPW = dried pasta weight (g)

Solids particles that diffuse from pastas into the cooking water are known as CL. CL was measured by putting cooked pasta in an oven at 50°C for 48 h (using the same units as described previously):

$$CL\ (\%) = \frac{DPW - OPW}{DPW} \times 100$$

2.3.7 Texture Analysis

Dried pasta texture was measured using three replicate pastas for each treatment with a texture analyzer (Autograph AGS-J, SHIMADZU). The test included a three-point bending fixture, which measured the force required to break each pasta sample. For each measurement, a single strand of pasta of about 3cm in length was placed in the center of the fixture (the gap between the supports was 24 mm). Then, the test probe moved with a speed of 1 mm/min until breakage occurred. The probe movement caused deformation of the pasta sample until the sample fractured. The texture analyzer software recorded force and stroke data, with which the stress-strain relationship could be obtained. A typical curve is shown in Figure 3. Stress and strain were calculated according to the formulae:

$$Stress\ (N/mm^2) = \frac{F*L}{\pi r^3}$$

F = force (N); L = gap between the supports; r = spaghetti radius (mm)

$$Strain = \frac{6 * D * d}{L^2}$$

D = course (mm); d = spaghetti diameter (mm)

The stress-strain relationship (Figure 3) during the matrix compression is divided into three distinct regions. The first involves elastic deformation of the intact structure. The second (A) comprises at break and irreversible structural collapse. Finally, the compression of the material completes the cycle (fracture point) (Gibson and Ashby, 1988).

From this curve, Young's modulus (calculation of the slope) and toughness (area under the curve) were calculated.

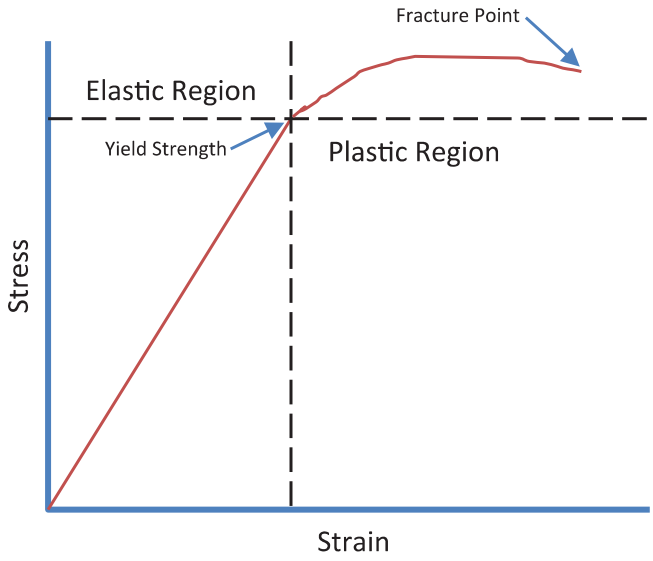


Figure 3. Typical Stress-Strain curve relationship

2.4 Sensory Analysis

The sensory test panel consisted of 10 untrained panelists (5 males and 5 females). Each of them was in an individual box to taste samples. A glass of water and a spittoon were at their disposal. Three different samples were tested: best formulation pastas made with the Kitchen Aid and gluten-free pastas from Andean Dream and Barilla. Spaghetti samples were cooked in tap water to optimum cooking time and served warm. The sensory analysis was divided into three different parts. The first part was a descriptive test of each spaghetti separately. It focused on different scale of perception, such as eyesight (color), odor, taste, texture, and overall acceptability. Descriptors were marked on scale of 5. After that, consumers had to answer three questions: What did you like? What did you dislike? Will you be ready to consume this product again? Secondly, the three samples were distributed at the same time, and consumers had to choose their favorite formulation. To finish, the same test was reiterated with tomato salsa (Prego, Italian traditional) in pastas.

Results were compared using Microsoft Excel 2016 in order to find significant differences or similarities among samples. With the results of the ranking test, a Friedman test was realized. After classification of samples in ascending order, rank sum was calculated: the value given to each sample was relative to it's ranking. For example, if the sample A is found 7 times in first position, 2 times in second position and 1 time in third position, the sum of ranks is equal to 7*1 + 2*2 + 3 = 14. From these data, the Friedman coefficient could be calculated with the following formula:

$$F = \left(\frac{12}{N.k.(k+1)}\right).\sum R^2 - (3.N.(k+1))$$

N=number of panelists; k=number of samples; R=sum of ranks

To determine if the different pastas were perceived differently with or without sauce, the Friedman coefficient was compared with the critical value from the Friedman table (since N < 14 and k < 6). A level of significance of 0.05 was used for all statistical analyses.

3. Results and Discussion

All tests were analyzed both one by one, and all in one, via the experimental design.

3.1 Moisture Content

The moisture content is the percentage of water contained in dried pasta. Results of this measurement are illustrated in Figure 4.

In Figure 4 we can see that Barilla's MC was closer to our samples' MC, although it was higher in the experimental pasta. The moisture content depends on initial dough MC and it also depends on the drying process. Sample number 7 had the lowest MC even though our drying process was not as good as that in industry. According to Bustos et al. (2015), the pasta MC level had to be equal to or less than 12.5% after drying in order to avoid contamination by microorganisms. Here, all samples MC was under 12% so they were good concerning MC level.

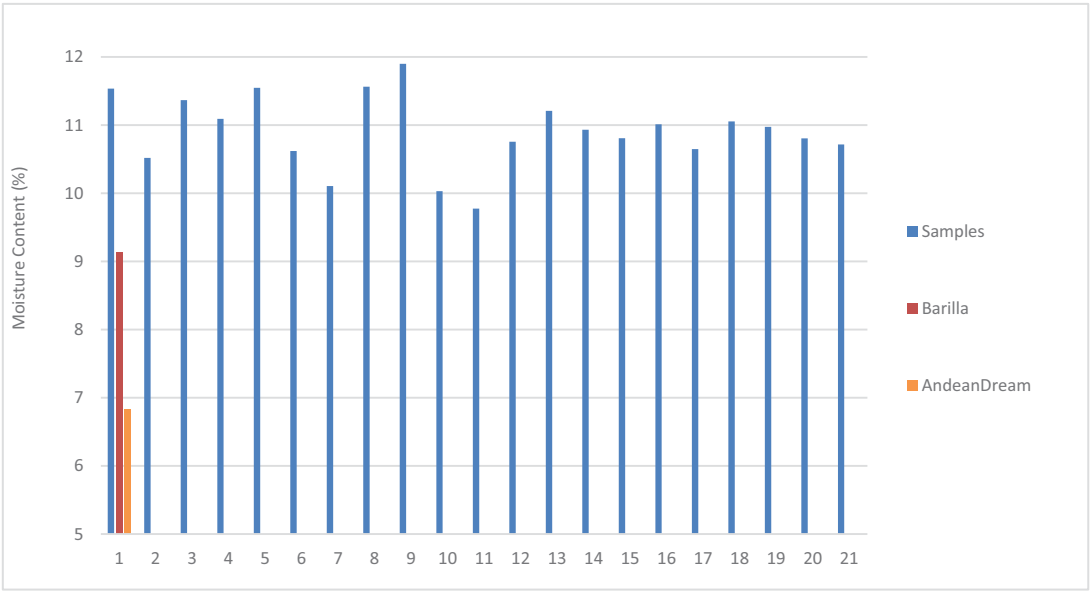
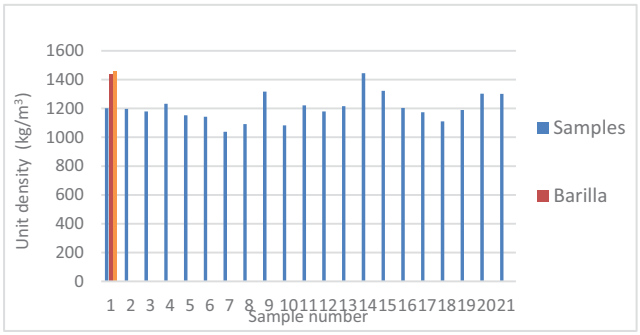


Figure 4. Moisture content of trial pastas and control pastas

Data analysis via Rcommander software showed that none of factors, including added water, had an effect on moisture content. The adjusted R^2 was by 93% so the model seemed to be well suited. Leaving the samples dry in the open air and the amount of water added, thanks to the preliminary study, varying little (33 to 39%); it is not surprising that water had no effect on the moisture content of the final product.

3.2 Unit density

Unit density measurement is used to understand the entrapped air in pastas. If there is a lot of air, spaghetti will have a low weight and a high volume, which means a low unit density. Results are shown below in Figure 5.




```
                Estimate Std. Error t value  Pr(>|t|)
(Intercept) 1235.2880    23.2034 53.2374 1.269e-15 ***
X1            10.2197    34.7615  0.2940   0.77378
X2            78.7306    34.8218  2.2610   0.04314 *
X3           -54.6408    34.7465 -1.5726   0.14180
X4             1.9688    35.0714  0.0561   0.95616
X2^2        -128.2956    66.7525 -1.9220   0.07867 .
X2:X3       -218.1307    97.9995 -2.2258   0.04595 *
X2:X4       -142.6991   105.3898 -1.3540   0.20068
X3:X4         90.3143   100.0987  0.9023   0.38468
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Multiple R-squared:  0.5914, Adjusted R-squared:  0.3191
F-statistic: 2.171 on 8 and 12 DF,  p-value: 0.1092
```

Figure 5. Unit density of trial pastas and control pastas; and Rcommander software results using the FO(X1,X2,X3,X4)+TWI(X2,X3,X4)+PQ(X2) design

According to Figure 5, unit density of the samples were lower than those of commercial pasta, but they were greater than 1000 kg/m^3, which means that the trial pastas will not float in water. So, it seems that particle bonding was good. Regarding the Rcommander results, even adjusting the best model, R^2 remained low, with a value by 32%. Based on these data, it appears that amaranth level and interaction with egg whites had an influence on the value of US, but the R^2 was low. Although UD value can be influenced by ingredients, it also depends on the preparation process and above all, dough kneading. Indeed, air can be entrapped during this step. According to the previous figure, it seems that UD values were almost the same and suitable for all samples. Thus, this setting was acceptable and did not have to be improved.

3.3 Color Analysis

Colorimeter gives four results: L*, a*, b* and ΔE. All samples had a good lightness (Figure 6), L* was always higher than 60 but some were lighter than others. Results showed that an increase in lightness values (L*) can be observed in samples containing more rice than quinoa and amaranth. According to Rosa et al. (2015), even if some consumers could accept dark pastas if they are healthier, lighter colored pastas most frequently have a better acceptance because consumers are accustomed to eat wheat pastas; thus it was important to add rice to our pasta in order to increase lightness.

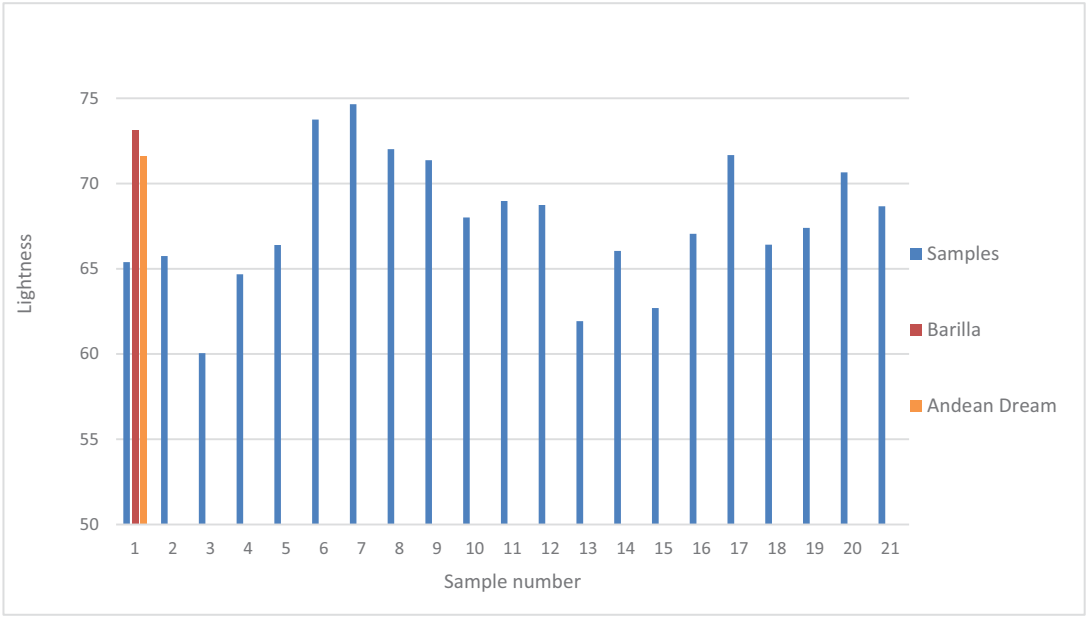


Figure 6. Lightness (L*) color results of trial pastas and control pastas

Regarding the a* and b* color, results are shown in Table 1.

Table 1. Red/green (a*) and yellow/blue (b*) color results of trial pastas and control pastas

	a*		b*	
	Mean	Standard deviation	Mean	Standard deviation
Samples	2.95	0.49	14.14	1.28
Barilla	0.54	0.1	49.11	2.4
AD	-1.06	0.24	24.46	1.8

Even if they were a bit higher, a* value of the samples was approximately the same as the a* value of commercial pasta. Samples' b* value was lower than pasta commercialized b* value. Although the addition of amaranth contributes to the yellowness of the pasta because of the amount of carotenoid pigment and enzymatic reactions (Islas-Rubio et al., 2014), our samples did not have a very high b* value because they contained a little amount of amaranth. According to Belton and Taylor (2010), it also can be due to oxidation during processing: the enzyme lipoxygenase bleaches the yellow carotenoid pigments by oxidation.

The ΔE variation between the samples show the difference of color (Figure 7). The graph shows that samples colors were more similar to Andean Dream color because ΔE values were lower. Indeed, Barilla pasta were yellower, whereas Andean Dream were whiter, compared to our pastas. ΔE analysis via Rcommander showed significant influence ($P<0.05$) due to quinoa and amaranth on the product color. Despite the various combinations tested, R^2 value was low (35%), but the software results confirm the assumptions made above. These cereals make the product more or less dark depending on the quantity used.

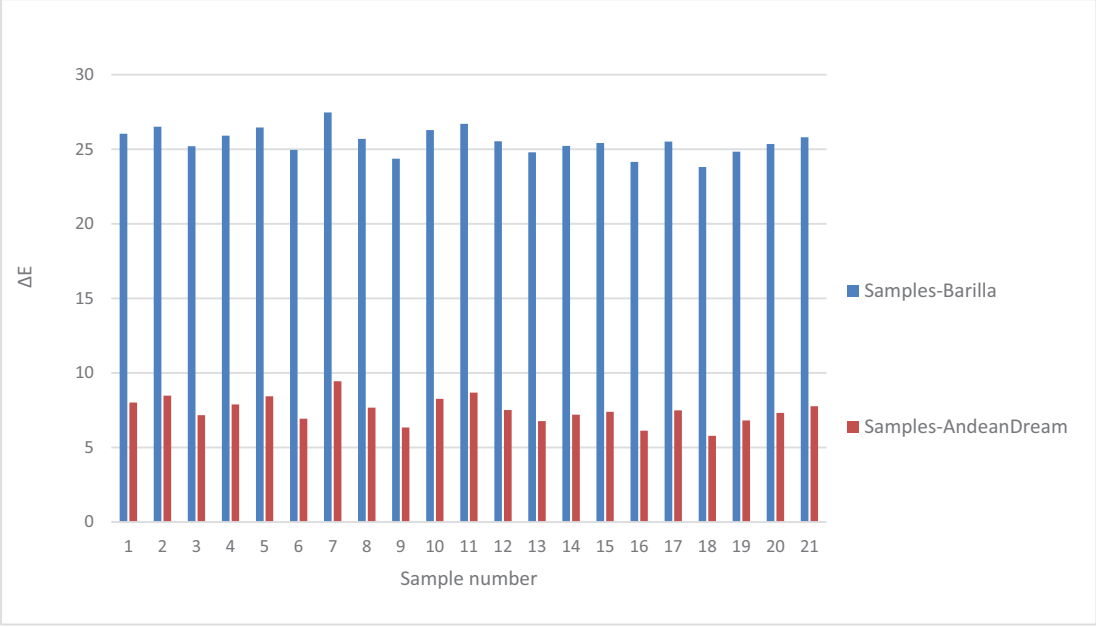


Figure 7. Variation of ΔE between our samples and Barilla pastas, or between our samples and Andean Dream pastas

Nevertheless, the parameters L *, a *, b * were analyzed separately in order to reduce the Type I error risk (Figures 8 and 9).

First of all, luminance analysis confirmed the significant influence of amaranth and quinoa ($P <0.001$) as well as their interaction ($P<0.05$). Eggs and their interaction with amaranth also had an influence, although lower ($P<0.1$) and therefore not taken into account here. This confirms our assumption since $R^2=94\%$.

	Estimate	Std. Error	t value	Pr(>|t|)	
(Intercept)	65.33783	0.90014	72.5862	4.601e-10	***
X1	-3.35301	0.40291	-8.3220	0.0001633	***
X2	-6.06204	0.40358	-15.0208	5.485e-06	***
X3	-0.79922	0.40271	-1.9846	0.0944160	.
X4	0.63136	0.40697	1.5514	0.1717956	
X1:X2	2.73965	1.04009	2.6340	0.0388491	*
X1:X3	-0.55332	1.16226	-0.4761	0.6508619	
X1:X4	0.18619	1.21832	0.1528	0.8835435	
X2:X3	-2.64488	1.16204	-2.2761	0.0631387	.
X2:X4	1.51906	1.23757	1.2275	0.2656198	
X3:X4	1.34450	1.21226	1.1091	0.3098566	
X1^2	4.40423	1.10282	3.9936	0.0071709	**
X2^2	-0.35929	1.10263	-0.3259	0.7555996	
X3^2	3.58940	1.07030	3.3536	0.0153502	*
X4^2	2.42482	1.06761	2.2713	0.0635570	.

Figure 8. Luminance results via Rcommander using SO() design

	Estimate	Std. Error	t value	Pr(>|t|)	
(Intercept)	2.949009	0.050116	58.8441	4.879e-14	***
X1	0.075064	0.102708	0.7308	0.48164	
X2	0.873611	0.102891	8.4906	6.965e-06	***
X3	0.267688	0.102664	2.6074	0.02616	*
X4	-0.020718	0.103634	-0.1999	0.84556	
X1:X2	-0.101257	0.265173	-0.3819	0.71056	
X1:X3	-0.069287	0.296321	-0.2338	0.81984	
X1:X4	0.122889	0.310404	0.3959	0.70049	
X2:X3	0.016022	0.281092	0.0570	0.95567	
X2:X4	-0.128903	0.306407	-0.4207	0.68288	
X3:X4	-0.317387	0.291438	-1.0890	0.30168	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Multiple R-squared: 0.8905, Adjusted R-squared: 0.7811

	Estimate	Std. Error	t value	Pr(>|t|)	
(Intercept)	14.33439	0.19285	74.3284	< 2.2e-16	***
X1	-0.25125	0.29763	-0.8442	0.41276	
X2	1.92574	0.29813	6.4594	1.499e-05	***
X3	0.65447	0.29751	2.1998	0.04512	*
X4	-0.78836	0.30029	-2.6254	0.01997	*
X1^2	-0.80866	0.53292	-1.5174	0.15141	
X1:X2	-1.22585	0.69657	-1.7598	0.10026	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Multiple R-squared: 0.8098, Adjusted R-squared: 0.7284

Figure 9. a* results with SO() design on the left, and b* results with FO(X1,X2,X3,X4)+TWI(X1,X2)+PQ(X1) design on the right

The a* value, which represent the range of colors from green to red, would appear to be influenced by the amount of amaranth (P <0.001) and egg white (P <0.05). Regarding b* value (color range from blue to yellow), it would appear to be influenced by the amount of amaranth (P<0.001), egg whites and water individually (P<0.05). These results are consistent with those obtained in Belton and Taylor (2010) which has shown that the addition of amaranth gives a yellow color to the dough due to the intake of carotenoid. Egg whites also influence color since it provides a whitish hue. Thus, it is quite logical to see the influence of these two factors.

3.4 Water Activity

Water activity (a_w) represents the water available in the product. Free water is the part of the whole water contained in a product which can participate to biochemical reactions or which can be used by microorganisms. Thus, the higher the water activity value is, the higher the product can be contaminated. Table 2 shows the measurement results.

Table 2. Water activity of trial pastas and control pastas

	a_w	
	Mean	Standard deviation
Samples	0.59	0.02
Barilla	0.62	0.01
AD	0.57	0.01

For our samples, a_w were between Barilla and Andean Dream a_w. It seems that our pasta a_w was adequate, and thus they were shelf stable. Moreover, according to Ayadi et al. (2011) a product is considered stable with long storage stability with a water activity equal to or less than 0.6. Only three samples (number 4,8,9) had an a_w higher than 0.6, thus the other samples which had low water activity could support a long storage times without the risk of fast spoilage (Islas-Rubio et al., 2014).

Data analysis via Rcommander confirmed the hypothesis that none of factors had an influence on samples water activity value, with a Type I error of 0.05%. Furthermore, with a Type I risk by 0.01%, interactions between quinoa and eggs and amaranth and eggs seem to influence this value. But, we choose to focus only on risk by 0.05% or less, so it appeared that none of factors had particular influence on the water activity of the final product. This was readily determined from the previous graph, since the a_w values were very close to each other.

3.5 Optimal Cooking Time

The method used to determine optimum cooking time for pastas samples is quite uncertain so the OCT was determined in three duplicates for each sample to refine the best cooking time interval. The cooking time for all samples was lower (about 7 min) than the commercial gluten-free pastas (14 min for Barilla and 17-18 min for Andean Dream pastas). Gluten network seems to be more reproduced in commercial pasta than in ours. Consequently, a protective layer formed on the surface of spaghetti. Thus, the water needs more time to reach the spaghetti matrix, mainly the spaghetti center. That is why the optimal cooking time is longer with commercial gluten-free spaghetti.

Analysis of our experimental design has shown that the quantity of water and egg whites had an influence on the cooking time. Moreover, the interaction between grains and water also had an effect ($P < 0.05$). Indeed, depending on the amount of each factor the dough had a protein structure more or less similar to that present in traditional pasta. For example, the addition of eggs reduces the penetration of water into dough and thus increases the cooking time.

3.6 Water Absorption

During drying, denaturalization, polymerization and aggregation of proteins allowed to create a solid film on spaghetti surface. But during boiling, this film is perturbed by cooking water which penetrates into the protein matrix. Moreover, in the other hand, starch granules absorb water and swell. Consequently, the volume of pasta increased. According to Bustos et al. (2015), they are considered acceptable if they absorb 150-200g of water/100g pasta namely a water absorption between 150 and 200%. Nevertheless, Donnelly (1979) reported that a mass increase could be comprised between 200 and 250% and Hummel (1966) cited a minimum value of 100%.

Andean Dream pasta showed a WA of 94% (Figure 10), which was not very acceptable. But, according to Rosa et al. (2015), the mass increase of the pasta depends upon cooking time, shape, and quality. Barilla WA was 155 % which was clearly acceptable. Our samples range between 108 and 190% with an average of 151%, so we can say that they were all acceptable because there were all between 100 and 200%.

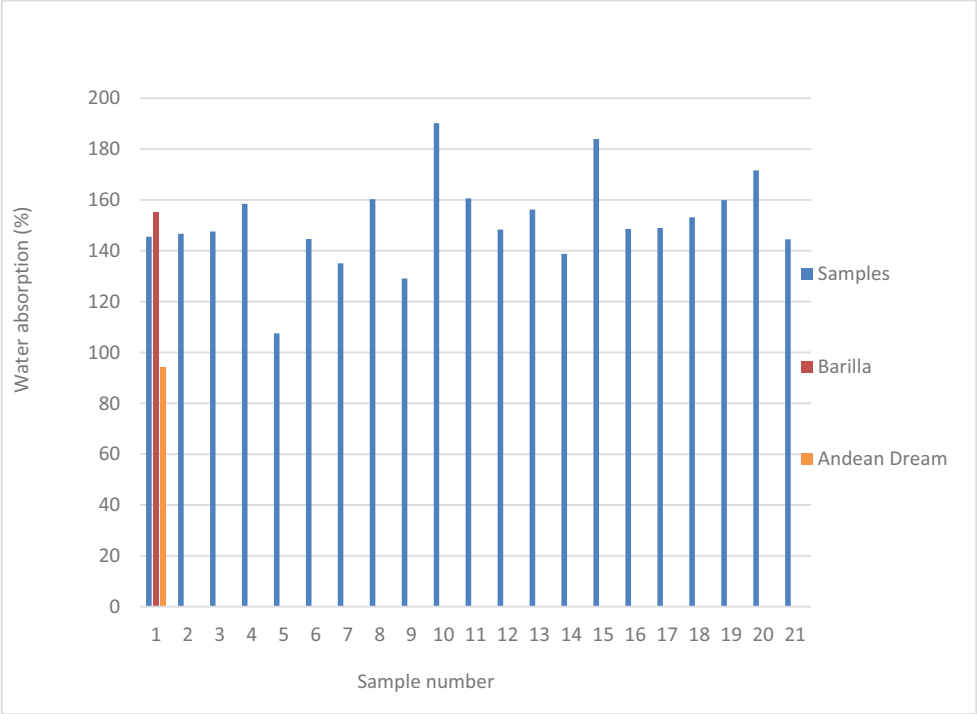


Figure 10. Water absorption (WA) of trial pastas and control pastas

Data processing via Rcommander showed that water absorption was influenced by the interaction between amaranth and quinoa and between quinoa and water ($P<0.05$) but it was more greatly influenced by the interaction between amaranth and water (0.001%). Indeed, as mentioned above, the protein network was less strong in the case of gluten-free pasta and this influence the water penetration during cooking (protein film disturbed during penetration of water). Data suggest that the protein network was more or less acceptable depending on the amount of different grains. Indeed, the greater the rice quantity, the greater the water absorption. It was the opposite for amaranth and quinoa.

3.7 Cooking Loss

The quantity of solids which migrates into the water during cooking is also an important factor for quality pasta products (Figure 11). Main structuring agents of pastas are starch and proteins, and the original point of cooking losses is often due to excessive starch swelling. Good quality pastas are made of a protein network able to withstand starch swelling during cooking. Delcour et al. (2012) showed that an optimal cross-linked protein network functions as a continuous framework, starch is trapped so starch swelling is restricted and cooking losses are minimized. To sum up, the more the protein network is developed, the lower the CL.

Barilla spaghettis seems to be good quality pastas because they had low CL (Figure 11) (10.3%) whereas, once again, Andean Dream pasta could be considered poor quality pasta because of their high CL (27.5%). All samples had different CL value, but the majority range between 15 and 20%. Only three samples had a CL greater than 20% (number 7, 9 and 17) and should not be choose as best formulation.

In wheat pasta the cooking loss is typically lower than 8%, but the lack of gluten in gluten-free pasta can cause an increase in CL and a decrease in firmness because the starch polymers are less linked to the matrix (Marti et al., 2014). Also, Tudorica et al. (2002) showed that increasing of cooking loss could be caused by fibers which use a higher water quantity. Thus, there would be less water available for the starch swelling. CL values for our samples seems to be acceptable according to literature.

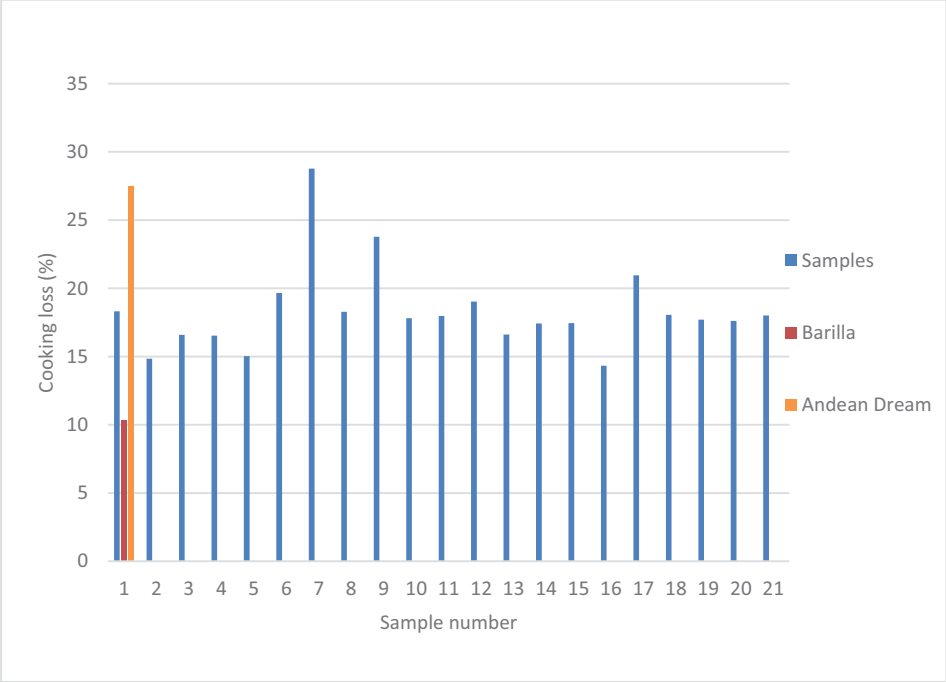


Figure 11. Cooking loss of trial pastas and control pastas

Data analysis via Rcommander software showed which variables influenced cooking losses. The results are represented in Figure 12. We can see that factors X1, X2 individually but also in interaction (P<0.01) influence cooking losses. Indeed, depending on the type of grains the amount of starch is different and it is primarily responsible for cooking losses. Typically, FAO (1993) data that rice contains 90 % starch while amaranth and quinoa contain 60%, so there should be more cooking losses when there is rice. The quantity of water also seems, less importantly, to influence cooking losses (P <0.01).

```
            Estimate Std. Error t value  Pr(>|t|)
(Intercept) 17.50050    0.47805 36.6079 1.106e-13 ***
X1          -2.60355    0.73072 -3.5630 0.0039023 **
X2          -3.63409    0.73201 -4.9645 0.0003283 ***
X3          -0.70593    0.73040 -0.9665 0.3528684
X4          -1.60315    0.73726 -2.1745 0.0503895 .
X1:X2        6.12313    1.78955  3.4216 0.0050636 **
X1:X4       -0.85532    2.18294 -0.3918 0.7020607
X2:X4        3.22392    2.17583  1.4817 0.1641965
X2^2         3.41220    1.34340  2.5400 0.0259394 *
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Multiple R-squared:  0.8391, Adjusted R-squared:  0.7319
```

Figure 12. Cooking loss analysis via Rcommander software using FO(X1,X2,X3,X4)+TWI(X1,X2,X4)+PQ(X2) design

3.8 Texture Analysis

Texture is a main concern of consumers, with sticky, soft pasta being generally unacceptable. A firm but elastic product is desired, that is, pasta that is “al dente” (Hager et al., 2012).

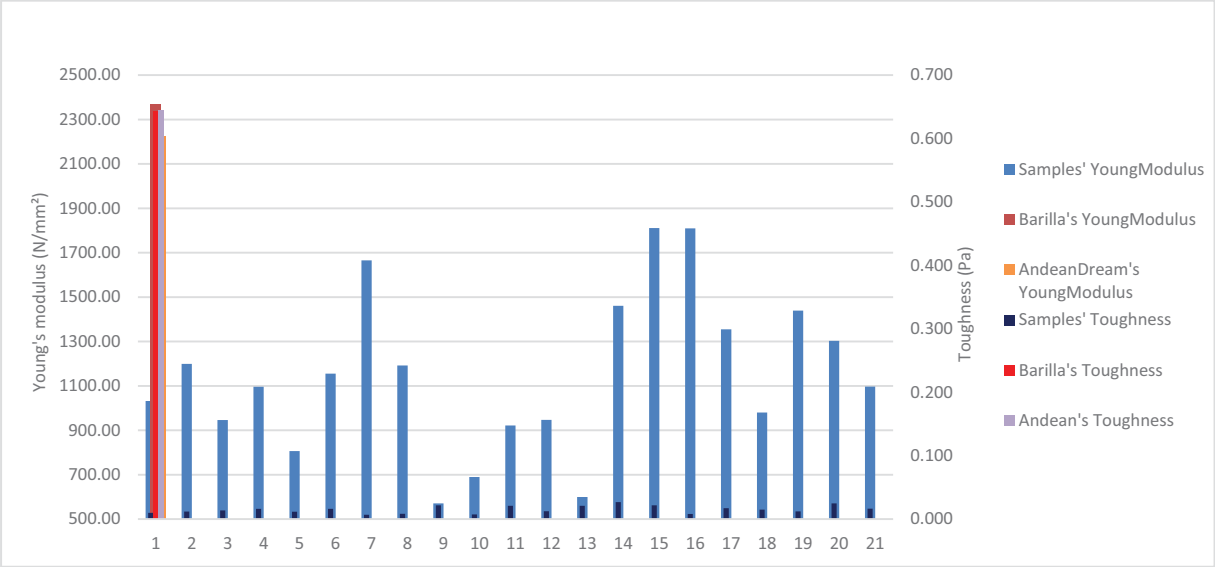


Figure 13. Young's modulus and toughness of trial pastas and control pastas

As we can see (Figure 13), there were large texture differences between our samples and commercialized pastas. Barilla and Andean Dream's spaghettis had similar texture, indeed, their Young's modulus and toughness values were close. Nevertheless, Young's modulus was lower in our samples: it lasts from 560 to 1811 N/mm² whereas it was about 2369 ± 353 N/mm² and 2265 ± 1000 N/mm² for Barilla and Andean Dream, respectively. Regarding the elasticity, it appears that samples 7, 14, 15, 16 and 19 were closest to commercialized pastas' elasticity. Furthermore, samples' toughness was really low compared to Barilla and Andean Dream pastas (difference of 97%). Thus, this parameter needs to be reconsidered. One solution might be to add an emulsifier in the recipe, indeed, the study of Schoenlechner et al. (2010) has shown that adding an emulsifier besides egg whites improve the texture. Considering elasticity and toughness, it seems that sample 15 was the one which had the best texture.

Only the Young's modulus (R^2=33%) was selected after data analysis via Rcommander because results for toughness were not reliable with a R^2 by 1%. Young's modulus analysis is presented below in Figure 14.

It seems that factors had no impacts individually, but X2:X4 and X1:X2 interactions had a significant influence ($P<0.05$) on the elasticity of the product. However, these results remain uncertain since the R^2 was low. Moreover, eggs should normally have an influence on product's texture: indeed, eggs start to "gelified" at a certain temperature (60°C). Furthermore, it is possible that the ambient air drying was not suitable for it's processing, and perhaps we should practice a shorter drying at higher temperature.

```
             Estimate Std. Error t value  Pr(>|t|)
(Intercept) 1055.682     83.856 12.5892 5.113e-07 ***
K1          -175.663    130.286 -1.3483   0.21052
K2            14.268    130.518  0.1093   0.91535
K3           106.446    130.230  0.8174   0.43482
K4            53.419    131.461  0.4063   0.69398
K1:X2        764.994    336.374  2.2742   0.04902 *
K1:X3       -542.953    375.885 -1.4445   0.18250
K1:X4       -759.143    393.749 -1.9280   0.08595 .
K2:X3       -213.835    356.568 -0.5997   0.56350
K2:X4       -880.708    388.680 -2.2659   0.04970 *
K3:X4        364.490    388.178  0.9390   0.37226
K3^2         381.052    229.789  1.6583   0.13164
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Multiple R-squared:  0.6993, Adjusted R-squared:  0.3317
```

Figure 14. Young's modulus analysis using FO(X1,X2,X3,X4)+TWI(X1,X2,X3,X4)+PQ(X3) design

3.9 Best Formulation Choice

Doehlert's experimental design belongs to plans for response surface; thus, graphical analysis allows numerous

additional information. In view of the previous results, it seems that CL, texture, color and Oct were the only parameters that had to be considered to optimize the recipe. In fact, other parameters do not seem to be influenced by any of the factors X1, X2, X3, X4. In order to find the best formulation, different graphs have been developed. The optimal values for each factor could be determined step by step, testing the various possible formulations and observing their impact on the value of the parameters. The study began with cooking losses (major parameter for quality assessment) followed by texture, color, and cooking time. The 3D graph X1=f(X2) was traced for CL, which must be minimized (nearly 10%), since these two parameters influence them. Each time, the values of X3 and X4 factors varied from +1 to -1 in order to visualize the impact. All combinations were tested and results show that to have minimal cooking losses, X4, which correspond to the amount of water added, must be equal to 1, which corresponds to 39%. These data did not allow to define an optimal value for the others factors. Nevertheless, X2 seems to be between -0.5 and -1.

Color graphical analysis (L*, a*, b*) was carried out by testing the various possible settings. b* results showed that X3 value should always be maximum. Thus, in addition to a maximum amount of water (39%), the optimal formulation must also have a maximum amount of egg whites (18%). However, to maximize this parameter, it should have a maximum X2, which was contrary to previous analyzes. Maintaining X4 and X3 maximum and X2 between -1 and -0.5, luminance results indicate that X1 should be between -1 and -0.5. The X1 and X2 values were therefore still unknown.

The elasticity should be maximized to reach a value of 2300 N/mm². To visualize how factors should vary, X1=f(X2) and X2=f(X4) were plotted. Results showed that, for maximum X4 and X3, X2 value must be between -0.5 and -1 in good agreement with the results obtained for cooking losses. X1 value would seem to be between 0 and -1. However, these values cannot be guaranteed by 100% since the R^2 was only 33 %.

Finally, the graphical analysis of the setting "optimal cooking time" was conducted by plotting X1=f(X4) and X2=f(X4). Results demonstrated that to maximize it, the formulation would have X1 [-1; -0.5], with X2 between -1 and -0.5 and X3 and X4 equal 1.

Thus, according to this study, the ideal formulation should consist of 39 % water and 18% of eggs but grains ratio was still unknown. In order to define it, the different graphics selected for each parameter were plotted and compared. The parameters b * and a * in the colorimetric analysis provided no information at the X1 and X2 values. Results are shown in Figure 15.

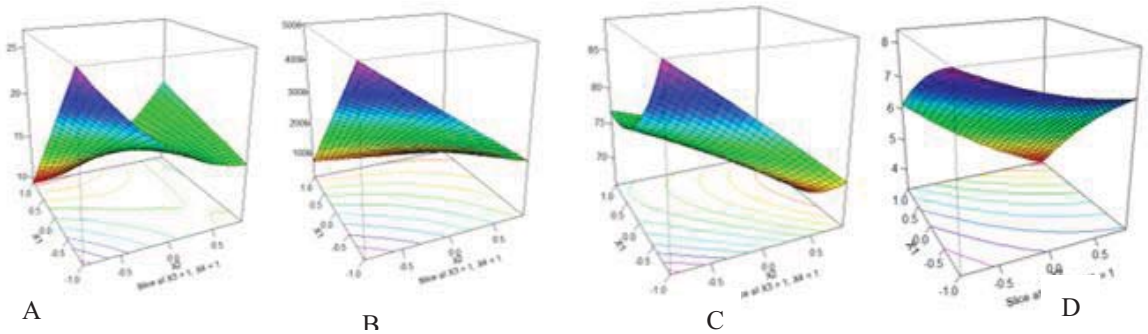


Figure 15. Graph X1=f(X2) with X3 and X3 equal 1. A=CL, B=Young's modulus, C=Luminance, D=OCT

To minimize CL, it would have a large amount of X1 (equal to 1) and a very small quantity of X2 (equal to -1). Regarding Young's modulus (about 2300 N/mm²), maintainning X2 between -1 and -0.5, X1 should be between -1 and 0.5. Having a luminance of about 73, would require an amount of X2 between -1 and -0.5 regardless of the X1 value. Finally, to maximize the cooking time, it would have amounts of amaranth and quinoa very low. Thus, all data taking into account, the selected optimal value of X1 was 0.5, which means 40% quinoa. This value meets the texture and color settings while it was slightly lower than provided value to minimize CL and larger than desired value to maximize the cooking time. Indeed, the latter parameter was not the most important for quality assessment. The same reasoning was done for X2 factor; the selected optimal value was 0.5 that means 10% of amaranth.

In conclusion, the ideal formulation was composed of 50 % rice, 40% quinoa, 10% amaranth, 18% egg whites,

and 39% water. Once prepared the ideal formulation prepared, its physical and chemical characteristics were measured and compared with those of Barilla pastas (Table 3).

Table 3. Physical property comparisions between Barilla pasta and our optimal formulation

	Optimal Formulation	Barilla
a_w	0.52	0.62
OCT (min)	7.0	13.8
L*	65.32	73.12
a*	3.18	0.54
b*	17.23	49.11
Delta E	56.1	32.9
Unit density (kg/m^3)	1272	1436
Young's modulus (N/mm^2)	2137	2369
WA (%)	161	155
CL (%)	14.0	10.3
MC (%)	11.83	9.13

Both spaghettis had similar properties, except for the OCT which was divided by 2 in our formulation. This parameter in itself was not critical and did not appear to influence the other, so a 7-minute cooking time was deemed acceptable. b* value could be from -120 to +120, so the difference was supposed to be negligible. Regarding water activity, that of our preparation was lower than 0.6 (Ayadi et al., 2011). Compared to this parameter, it would seem that gluten-free Barilla pastas were not acceptable. Regarding color, both had high L* value: therefore, they had a light color that should appeal to consumers. Best formulation's unit density was slightly lower than Barilla's UD which means that these pastas contain less air and this could influence the texture in the mouth. Nevertheless, this small difference was not significant. Regarding Young's modulus values, they were all close. However, a texture difference was clearly visible to the naked eye and to the touch: it therefore appears that this was not the elasticity but the product firmness should be improved. Water absorption was higher than 150 %. Similarly, pastas were acceptable according to their moisture content, which were lower than 12.5% (Bustos et al., 2015). The cooking losses were reduced (they were between 15 and 20% formerly) and were thus suitable.

3.10 Sensory Analysis

First part of the sensory analysis was to assess pastas according to different parameters. Results, after statistical analysis, are shown in Table 4.

Table 4. Statistical analysis of hedonic tests conducted on Barilla and Andean Dream pastas vs. our best formulation

		Barilla	Andean Dream	Best formulation
Appearance	Mean	3.7	3.8	3.6
	Standard deviation	1.3	1.2	0.7
Color	Mean	3.8	3.6	3.5
	Standard deviation	1.4	1.2	1.1
Odor	Mean	3.4	3.7	3.1
	Standard deviation	1.1	1.1	1.1
Taste	Mean	3.7	4.0	3.4
	Standard deviation	1.1	0.8	1.3
Texture	Mean	4.4	3.8	3.3
	Standard deviation	1.1	1.0	1.4
Overall acceptability	Mean	3.8	3.8	3.2
	Standard deviation	1.1	0.8	1.1

Differences between our formulation and commercialized pasta were not very large. Regarding product appearance, results were close and best formulation's standard deviation was lower than those of Barilla and Andean Dream pastas. So it seems that the product made with rice, quinoa and amaranth was visually pleasing,

as well at its color. In terms of smell and taste, those of our pastas were slightly less preferred, this probably stems from the fact that they were composed of unusual cereals. Indeed, some consumers found that smell and taste were too herbaceous. Nevertheless, standard deviations were rather high: the subjects being untrained, thus their rating varies according to their personal taste.

Obviously, the texture rating for the best formulation was lower. This was entirely predictable in view of the results of physicochemical analyzes. Consumers think that the texture was too viscous. Finally, the overall acceptability score was identical to both commercialized pastas with a value of 3.8/5 while ideal formulation achieves a score of 3.2/5. Thus, according to the ratings, it appears that the main criterion to improve was the texture. The taste and smell may also be improved, but opinions were very diverse about these parameters.

To analyze the ranking test, Friedman coefficient were calculated and compared to the Friedman critical value. Results are shown in Table 5.

Table 5. Results of the ranking test. Friedman coefficient calculated and Friedman critical value using a Type I error rate of 5%

	With sauce	Without sauce
Friedman coefficient	0.5	11.4
Friedman critical value	6.2	

Without sauce, pastas were perceived differently as Friedman coefficient calculated was higher than Friedman critical value, read directly in the table. Nevertheless, Fcalculated < Fcritical when pastas were served with tomato sauce: consumers fail to perceive a difference between spaghettis. Indeed, the sauce can mask the grassy taste and reduce the stickiness' sensation in the homemade product.

Each panelist also had to indicate if they were ready to eat these different pastas again. The results are illustrated in Figure 16.

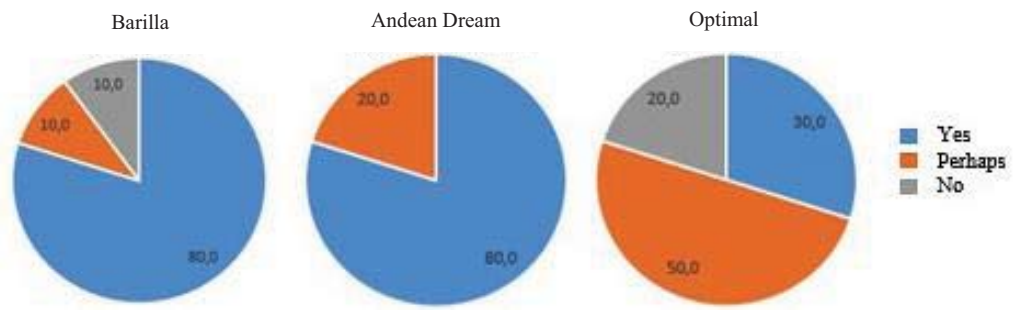


Figure 16. Pie chart illustrating the percentage of consumers ready (or not) to eat the products again

It appears that 80% of consumers would be willing to consume again Barilla or Andean Dream pastas while the result was only by 30% for the ideal formulation. But, 50% these pastas still had a mixed review. Furthermore, this question was asked in the first part of the test, but it would have been wiser to ask it at the end, after consumers had tasted the pastas with tomato sauce: the number of positive responses would certainly have been higher.

In conclusion, it seems that opinions were divergent for the optimal formulation: it pleases some while others do not appreciate it. However, with the addition of sauce, opinions were unanimous and the optimal formulation was placed at the same level as commercialized pastas. Some settings must be improved, especially the texture of the product. If the project continues, it would be interesting to add additives to achieve a firmer and less sticky product.

4. Conclusions

The goal of this study was to formulate good quality gluten-free spaghettis made up of rice, quinoa and amaranth. Optimizing the best formulation, quality product with acceptable parameters was obtained with the following formulation: 50% of rice, 40% of quinoa, 10% of amaranth, 18 % of egg whites and 39% of eggs. Indeed, a

significant amount of rice achieves white spaghettis that attract consumers. Nevertheless, it was necessary to mix the rice with other grains in order to reduce cooking loss. Amaranth and quinoa have both interesting nutritional properties but amaranth had a stronger taste and can bring a musty taste in proportion (Schoenlechner et al., 2010), so the amount was minimal. The addition of egg whites was essential since it allows production of better texture.

With this formulation, the physicochemical parameters were acceptable and were closer than those obtained for Barilla gluten-free pastas. However, some parameters still need to be improved, especially the texture. First of all, in order to ameliorate this setting, we should use flour with a finer grinding. We could choose to only keep flour with a diameter under 500 nm: indeed, when we buy flour in store the diameter was never greater than 500 nm. Secondly, adjusting drying process could improve pastas texture. Instead of drying pastas 24 h at ambient temperature, it could be better to use a drying process with a higher temperature during a short time. Indeed, studies have shown that a drying process in an oven can improve pastas texture: 42°C for at least 9 h (Schoenlechner et al., 2010) or 20 min at 55°C + 10 h at 75°C + 40 min at 60°C + 20 min at 45°C + 14 h at 40°C (Padalino et al., 2012). We might also consider adding one or several types of emulsifier to the dough (like guar gum or monoglycerides). Indeed, coupled with eggs, it appears in Schoenlechner et al. (2010) study that the addition of an emulsifier allows to obtain spaghettis with better texture (firmer) and more stability during cooking (less losses). Also, the extrusion process using extruder could improve protein structure and thus texture of spaghetti since the energy imparted to the dough would be greater than that applied during the extrusion process via the Kitchen Aid.

According to sensory analysis, it appears that the optimal formulation (made up of rice, quinoa and amaranth), even if it had characteristics similar to those of marketed gluten-free pastas, still needed improvement. Indeed, consumers think that the texture was too sticky and not enough firm and soft. In contrast, in terms of taste, the ideal formulation was acceptable: with the addition of tomato sauce, no difference between the three types of spaghettis was perceived.

Thus, according to this study, we can say that gluten-free pastas composition was not easy to find. Indeed, each gluten-free grain has different properties which may influence the quality. In order to find best gluten-free pastas formulation, it was important to mix different cereals in order to try to just keep their advantages and to limit bad consequences. The water quantity added in the recipe was important and it must not be chosen at random. Also, the recipe must contain egg whites since they offer a better texture to the product. Nevertheless, it was really difficult to obtain a firm and soft product, indeed, in traditional pastas this texture was create by the gluten which provides a good protein network. Without its use, we were able to have a good elasticity but not a good toughness in the product. This last parameter must be improved.

References

Ayadi, F. Y., Muthukumarappan, K., Rosentrater, K. A., & Brown, M. L. (2011). Twin-screw extrusion processing of rainbow trout (*Oncorhynchus mykiss*) feeds using various levels of corn-based distillers dried grains with solubles (DDGS). *Cereal Chemistry, 88*(4), 363-374).

Belton, P., & Taylor, J. (2010). *Pseudocereals and Less Common Cereals – Grain Properties and Potential Utilization*. Berlin, Germany: Springer.

Broz, R., & Horne, T. (2007). Gluten-free product development. *Cereal Foods World, 52*(3), 148-149.

Bustos, M. C., Perez, G. T., & Leon, A. E. (2015). Structure and quality of pasta enriched with functional ingredients. *RSC Adv. 5*, 30780-30792.

Delcour, J. A, Joye, I. J., Pareyt, B., Wilderjans, E., Brijs, K., & Lagrain, B. (2012). Wheat gluten functionality as a quality determinant in cereal-based food products. *Annual Rev Food Sci Technol, 3*, 469-492.

Dicke, W. K. (1950). Celiac disease investigation of the harmful effects of certain types of cereal on patient with celiac disease. Ph. D. Thesis, State University of Utrecht.

Donnelly, B. J. (1979). Pasta products: raw material, technology, evaluation. *The Macaroni Journal, 61*(1), 6-7, 10, 12, 14-15, 18.

Engleson, J., & Atwell, B. (2008). Gluten-free product development. *Cereal Foods World, 53*(4), 180-184.

FAO. (1993). Rice in human nutrition. chapter 3. Grain structure, composition and quality. Rome, Italy: Food and Agriculture Organization of the United Nations. Available online: http://books.irri.org/9251031495_content.pdf.

Chillo, S., Laverse, J., Falcone, P. M., & Del Nobile, M. A. (2007) Effect of carboxymethylcellulose and

pregelatinized corn starch on the quality of amaranthus spaghetti. *Journal of Food Engineering, 83*, 492-500.

Gibson, L. J., & Ashby, M. F. (1988). Cellular solids : Structure & properties. *Advances in Polymer Technology, 9*(2), 165-166.

Hager, A. S., Lauck, F., Zannini, E., & Arendt, E. K. (2012). Development of gluten-free fresh egg pasta based on oat and teff flour. *European Food Research and Technology, 235*(5), 861-871.

Hummel, C. (1966). *Macaroni Products*. London: Food Trade Press.

Inglett, G. E, Chen, D., & Sean, X. L. (2015). Antioxidant activities of selective gluten free ancient grains. *Food and Nutrition Sciences, 6*, 612-621.

Islas-Rubio, A. R., Calderon De La Barca, A. M., Cabrera-Chavez, F., Cota-Gastellum, A., & Beta, T. (2014). Effect of semolina replacement with a raw: popped amaranth flour blend on cooking quality and texture of pasta. LWT - *Food Sciences and Technology, 57*, 217-222.

Marti, A., Barbiroli, A., Marengo, M., Fongaro, L., Iametti, S., & Pagani, M. A. (2014). Structuring and texturing gluten-free pasta: egg albumen or whey proteins*? European Food Research and Technology, 238*, 217-224.

Mokryzcki, W., & Tatol, M. (2011). Color difference Delta E – A survey. *Machine Graphics and Vision, 20*(4), 383-411.

Padalino L, Mastromatteo, M., Lecce, L., Cozzolino F., & Del Nobile, M. A. (2012). Manufacture and characterization of gluten-free spaghetti enriched with vegetable flour. *Journal of Cereal Science, 57*(3), 333-342.

Rosa, C. S., Prestes, R. C., Tessele, K., & Crauss, M. (2015) Influence of the different addition levels of amaranth flour and rice flour on pasta buckwheat flour. *International Food Research Journal, 22*(2), 691-698.

Rosentrater, K. A., Richard, T. L., Bern, C. J., & Flores, R. A. (2005). Small-scale extrusion of corn masa by-products. *Cereal Chemistry, 82*, 436-446.

Sabanis, D., & Tzia, C. (2011). Effect of hydrocolloids on selected properties of gluten-free dough and bread. *Food Science and Technology International, 17*(4), 279-291.

Schoenlechner, R., Drausinger, J., Ottenschlaeger, V., Jurackox K., & Berghofer, E. (2010). Functional properties of gluten-free pasta produced from amaranth, quinoa and buckwheat. *Plant Foods Hum Nutr., 65*(4), 339-349.

Tudorica, C. M., Kuri, V., & Brennan, C. S. (2002). Nutritional and physicochemical characteristics of dietary fiber enriched pasta. *Journal of Agricultural and Food Chemistry, 50*, 347-356.

Wierser, H., & Koelher, P. (2007). The biochemical basis of Celiac disease. *Cereal Chem., 85*(1), 1-13.

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Transfer of *Escherichia Coli* to Lemons Slices and Ice during Handling

Paul Dawson[1], Inyee Han[1], Ahmet Buyukyavuz[1], Wesam Aljeddawi[1], Rose Martinez-Dawson[2], Rachel Downs[1], Delaney Riggs[1], Carrrie Mattox[1], Alejandro Kurtz[1], Mary MacInnis[1], Jacob Freeland[1], Seth Garrison[1], Taylor May[1], James McClary[1], Frank Monitto[1], Trinh Nguyen[1], Kelly Polte[1], Matthew Suffern[1], Zachary Tanner[1], Alana Thurmond[1] & Virginia Ellis[1]

[1]Department of Food, Nutrition and Packaging Sciences, [2]Department of Mathematical Sciences, Clemson University, Clemson, SC 29634, USA

Correspondence: Paul Dawson, Depertment of Food, Nutrition and Packaging Sciences, Clemson University, Clemson, SC 29634, USA. Tel: 1-864-656-1138. E-mail: pdawson@clemson.edu

Received: May 1, 2017 Accepted: June 9, 2017 Online Published: June 28, 2017

doi:10.5539/jfr.v6n4p111 URL: https://doi.org/10.5539/jfr.v6n4p111

Abstract

The objective of this study was to determine the transfer and survival of bacteria during the handling and storage of lemons and transfer of bacteria during handling of ice. Ice and lemon slices are handled and stored in public eating places and used in beverages. During handling and storage the contamination and growth of bacteria may occur leading to the spread of disease. To fulfill the objective, hands were inoculated with *Escherichia coli* prior to handling of wet and dry whole lemons and in a separate experiment, ice cubes were handled. *E. coli* transferred to whole lemons or ice after handling were determined. The CFU per lemon and percentage of *E. coli* transferred were greater for wet lemons -6123 cfu and 4.62% compared to 469 cfu and .2% for dry lemons. The second experiment found from 2 to 67% of the bacteria on hands were transferred to ice by hands and from 30 to 83% of the bacteria on scoops were transferred to ice. In a third experiment, lemons were inoculated with *E. coli*, then sliced and stored at 4 or 22C and tested at 0, 4 and 24 hr. Lemons stored at room temperature (22°C) had an increase in *E. coli* population after 24 hour while those stored under refrigeration had a decrease even though bacteria did survive on lemons in either case.

Keywords: lemons, lemon slices, ice, *E. coli*, handling, storage

1. Introduction

The non-alcoholic beverage market is an $841 billion industry (Bailey, 2014) with the annual alcoholic beverage market at $494 billion (beer), $319 billion (wine) and $637 billion (spirits) (Marketrealist, 2014). Beverages are often prepared with the addition of ice and cut fruit slices such as lemons, limes and oranges. These drink items are also often handled by a server or the individual consuming the drink which offers an opportunity for contamination which is a possible source of contamination leading to foodborne illness.

1.1 Foodborne Illness

Thirty-one major pathogens cause 9.4 million cases of foodborne illness and about 2,612 deaths from tainted food annually (CDC, 2011). The Economic Research Service reports that foodborne illness costs $6.9 billion in medical expenses, lost productivity and deaths (USDA, 2014). Hand cleanliness or lack thereof, plays a major role in transmission of infectious disease in various public sectors including the food industry (Jumma, 2005).

1.2 Ice

The US Food and Drug Administration defines ice as food (FDA, 2010) and the World Health Organization has stated that ice coming in contact with food should have the same level of safety and quality as drinking water (WHO, 1997). Ice used for human consumption can be contaminated with pathogenic organisms and be a vector for spreading foodborne illness (Falcao, Dias, Correa & Falcao, 2002; Gerokomou et al., 2011). In 1987, the Centers for Disease Control reported a 4-state outbreak of Norwalk virus in Pennsylvania, Delaware and New Jersey from contaminated ice estimated to have affected more than 5,000 people (Levine, Stephenson & Craun, 1990). Contaminated ice was also a prominent transmission vector for spreading the 1991 cholera epidemic in Peru causing 7922 illnesses, 17 deaths and also expanding throughout Latin America (Ries et al., 1992). More recently,

diarrheagenic *E. coli* has been found in commercial ice produced in Brazil (Falcao, Falcao & Gomez, 2004). In the past, pathogens have been detected in ice from ice making machines (Stout, Yu & Muraca, 1985; Panwalker & Fuhse, 1986; Laussucq, Baltch, Smith, Smithwick, Davis et al., 1988; Wilson, Hogg & Barr, 1997). In a survey of over 3,500 samples of ice used to cool drinks, Nichols, Gillespie & Louvois, (2000) found that 9% contained coliforms at greater than 100 cfu/100 ml and 11% had total aerobes at greater than 1000 cfu/ml. Ice produced at retail outlets in Nigeria were contaminated with more than 1000 cfu/ml and isolates displayed 100, 67 and 87% resistance to Ampicillin, Erythromycin and Tetracycline, respectively (Lateef, Oloke, Guegium-Kana & Pacheco, 2006).

1.3 Lemons

Food establishments often place lemons in open containers at room temperature throughout the day for consumer access allowing consumers and food service workers to handle lemons for cutting and when serving slices with beverages. Lemons naturally contain bacteria that provide a symbiotic relationship with the fruit (Gardner, Feldman, & Zablotowicz, 1982). Similarly, human hands naturally contain bacteria such as *Micrococcus luteus* and *Serratia. rubidea* that transfer from humans to other objects. Transfer is particularly high when fingers contact lips (Rusin, Maxwell, & Gerba, 2002). Martinez-Gonzalez et al. (2003) found that orange surfaces inoculated with 2.3, 3.6 and 4.4 $\log_{10}$CFU/cm^2 *Salmonella* Typhimurium, *E. coli* and *Listeria monocytogenes*, respectively resulted in 1.0, 2.3 and 2.7 $\log_{10}$CFU/ml for these organisms in orange juice prepared from the inoculated oranges. Cut lemons are located at self-service drink stations for consumers to handle which increases the number of people touching lemon slices, many who are not food service workers and not subject to hand washing regulations. Lemons are held without refrigeration and to reduce waste, leftover lemons are sometime placed under refrigeration overnight for use the next day. Thus lemon slices are exposed to numerous opportunities for contamination and held unrefrigerated to allow microbial growth prior to use by consumers.

1.4 Hand Sanitation and Cross Contamination

Cross contamination in food service may play an important role in foodborne illness (Fendler, Dolan & Williams, 1998). During food preparation, bacteria on hands can be transferred to raw foods from hands and indirectly from other surfaces (Montville, Chen & Schaffner, 2002). Hands can also be a source for contamination from food workers that may be ill by not have overt symptoms who shed pathogens (Rocourt & Cossart, 1997; Rose & Slifko, 1999). Numerous studies have examined the transfer of bacteria to food from food contact surfaces including stainless steel (Kusumaningrum, Riboldi, Hazelberger & Beumer, 2003; Moore, Sheldon & Jaykus, 2003; Rodriguez & McLandsborough, 2007; Kesiken, Todd & Ryser, 2008), fabrics (Marples & Towers, 1979; Sattar et al. , 2001; Scott & Bloomfield, 1990), gloves (Legg, Khela, Madie, Fenwick, Quynh & Hedderley, 1999; Heal et al., 2003; Montville et al., 2001; Blom, Gozzard, Heal, Bowker, & Estela, 2002; Gill & Jones, 2002; Mackintosh & Hoffman, 1984; Patrick, Findon, & Miller, 1997; Scott & Bloomfield, 1990; Shale, Jacoby & Plaatjies, 2006) and hands (Scott & Bloomfield, 1990; Legg et al., 1999; Merry, Miller, Findon, Webster & Neff, 2001). A scoop, hands or other utensil is often used to deliver ice to a beverage offering the opportunity for cross contamination. The objectives of this study were to determine 1) to what extent bacteria is transferred to lemons when handled with contaminated hands; 2) the degree of bacterial transfer to ice when handled with contaminated hands or scoops; and 3) if bacterial numbers will increase during the storage of contaminated lemons.

2. Methods

2.1 Bacterial Inoculum

An *Escherichia coli* ampicillin-resistant strain with a fluorescent gene was used for the bacterial transfer and survival studies. A non-pathogenic *E. coli* strain JM109 was labeled with jellyfish green fluorescent protein according to the following protocol as described previously (Jiang *et al*., 2002). The competent bacterial cells were electroporated in a Gene Pulser II (Bio-Rad) with plasmid vector pGFPuv (ClonTech, Palo Alto, CA). Transformants were selected from isolated colonies grown on Luria-Bertani agar (LB) plates containing 100 g ampicillin/mL. The resulting ampicillin-resistant transformants emitted bright green fluorescence under UV light. The stability of GFP label in the *E. coli* strain was determined by streaking on trypticase soy agar (TSA) plates containing 100 g ampicillin/mL for several generations. The *E. coli* JM 109 culture was held in a −80˚C freezer in vials containing tryptic soy broth (TSB) (Becto™, Becton Dickinson and Company Sparks, MD, USA) supplemented with 20% (v/v) glycerol (Sigma, St. Louis, MO, USA). The frozen vial was thawed at room temperature prior to culturing. From this thawed vial, 0.1 mL of culture was transferred to 10 mL TSB containing 0.5% ampicillin (Sigma, St. Louis, MO, USA) in 2 loosely screw-capped tubes and then the tubes were incubated for 16 - 18 h at 37˚C with vigorous shaking (Thermolyne Maxi-Mix III type 65,800,

Barnstead/Thermolyne, Dubuque, IA). The second transfer was prepared from this first transfer culture by adding 0.1 mL from the first transfer tube to another fresh 10 mL TSB (DIFCO) with 0.5% ampicillin (Sigma), and again incubated for 16 - 18 h at 37˚C with shaking. After incubation, the cells were harvested by centrifugation at 3000 rpm (1200 g) (IEC HN-SII Centrifuge, International Equipment CO., Inc., Needham Heights, MA, USA), then the pellet re-suspended in 10 mL of sterile peptone solution (0.1%) (Bacto peptone, Becton Dickinson) to obtain a population of approximately 6-7 log CFU/mL. Initial cell populations were verified by enumeration of the cells following surface plating in TSA containing 0.5% ampicillin (DIFCO™, Becton Dickinson and company Sparks, MD, USA) and incubation at 37˚C for 24 h.

2.2 Experiment 1: E. Coli Transfer from Hands to Whole Lemons

2.2.1 E. Coli on Hands

Each subject washed their hands with warm water and soap, allowed their hands to air dry, and then 1 mL of the *E. coli* inoculum was deposited in the center of their dominate hand. The *E. coli* was applied by rubbing hands together for 30 sec, and then hands were allowed to air dry for 30 sec. To enumerate bacteria on subject's hands, both hands (separately) were placed into a sterile stomacher bag with 20 mL of sterile 0.1% peptone and rinsed for 30 seconds, covering all fingers, palm, and back of the hand. Next, 1 mL of the peptone solution was removed from the stomacher bag, placed into 9 mL of sterile 0.1% peptone and serially diluted. A 0.1 ml aliquot from sample dilutions were pipetted and spread onto TSA plates containing 100g ampicillin/mL. Plates were held for 5-10 minutes and were then inverted and placed in an incubator at 37^{o}C for 24 hours. The next day the plates were inspected under UV light and plates with 25 to 250 CFU/plate were counted and then multiplied by the dilution number then converted to CFU/hand and log CFU/hand based on serial dilutions.

2.2.2 E. Coli Transferred to Lemons

Four treatments were employed to determine bacterial transfer from hands: 1. Un-inoculated hands handling dry lemons, 2. inoculated hands handling dry lemons, 3. Un-inoculated hands handling wetted lemons, and 4. Inoculated hands handling wetted lemons. Each subject washed their hands then handled a lemon for 30 seconds by rolling the lemon between hands.

For the inoculated treatments (2 and 4) the procedure was repeated as described for un-inoculated hands only instead, hands were inoculated as described for section 2.2.1, then lemons were handled for 30 seconds. Lemons handled by inoculated or un-inoculated hands were placed into separate filter stomacher bags, each with 20 mL of sterile 0.1% peptone solution. The lemon and peptone were mixed for 30 sec in the bag. Then 1 mL samples of the liquid from the bags were taken in duplicate, serially diluted as previously described then plated on TSA. Samples were incubated and counted as described for the hand sample.

Serial dilutions were then prepared, plated, and spread in duplicate and plates were incubated and counted as previously described. Bacteria were counted 24 hours after plating by identifying colonies under a UV light then converted to CFU/lemon and log CFU/lemon as described for hands (2.3.1).

The % transfer of *E. coli* from hands to lemons was calculated using (1):

$$\%\ transfer = \frac{CFU\ recovered\ from\ lemons}{CFU\ recovered\ from\ hands + CFU\ recovered\ from\ lem} \text{ X } 100$$

2.3 Experiment 2: Transfer of E. Coli to Ice from Hands and Metal Scoops

The bacterial inoculum was prepared for the ice transfer experiments in the same manner as described under 2.1 for lemons.

2.3.1 Inoculation of E. Coli on Hands and Scoops

Each subject will wash their hands with warm water and soap, allow their hands to air dry, and then 1 mL of the *E. coli* inoculum will be deposited in the center of their dominate hand. The *E. coli* will be applied by rubbing hands together for 30 sec, and then hands will be allowed to air dry for 30 sec. For scoops, a sanitized scoop was inoculated by placing 1 mL of *E. coli* inoculum in the center of the scoop then spread across the scoop surface using a sterile glass rod then allowed to dry for 30 seconds.

2.3.2 E. Coli Transferred To Ice from Scoops and Hands

Four treatments were employed to determine bacterial transfer from hands to ice: 1. Un-inoculated hands handling ice, 2. inoculated hands handling ice, 3. Un-inoculated scoop handling ice, and 4. Inoculated scoops

handling ice. Each subject washed their hands then picked up a handful of ice, the immediately placed the ice in a filter stomacher bag containing 20 mL of sterile 0.1% peptone solution. This procedure was repeated with inoculated hands and both inoculated and un-inoculated scoops. The ice and peptone water were mixed for 30 sec in the bag. To enumerate bacteria on subject's hand the dominant hand used to pick up ice were placed into a sterile stomacher bag with 20 mL of sterile 0.1% peptone and rinsed for 30 seconds, covering all fingers, palm, and back of the hand. For inoculated scoops, 5 pieces of ice were placed into the scoop and gently moved back and forth for 5 seconds then the ice was immediately placed into a filter stomacher bag containing 20 ml of sterile 0.1% peptone solution.

Next, 1 mL of the peptone solution was removed from each stomacher bag, placed into 9 mL of sterile

0.1% peptone and serially diluted. Nine ml test tubes of sterile peptone solution (0.1%) were used for serial dilution of samples. 0.1 ml from sample dilutions were pipetted and spread onto TSA plates containing 100g ampicillin/mL. Plates were held for 5-10 minutes and will be then inverted and placed in an incubator at 37^{o}C for 24 hours. The next day the plates were inspected under UV light and appropriate petri dishes will be chosen for counting. Plates with a number of colonies ranging from 25 to 250 CFU/plate were counted and converted to CFU/ml based on the dilution. Plates were examined under the UV light and only the fluorescent bacteria counted. Bacterial counts were converted to CFU/hand or scoop and log CFU/hand or scoop based on the amount of rinse solution used. Percentage of *E. coli* transferred from hands to ice will be calculated using (1):

$$\%\ transfer \cdot \frac{CFU\ recovered\ from\ ice}{CFU\ recovered\ from\ hands\ (scoops)\ +\ CFU\ recovered\ fro} \quad X \quad = \quad (1)$$

Another experiment (2-1) was conducted to determine the transfer of E. coli from metal scoops to ice at 4 different times after inoculation (0, 1hr, 1.5hr and 2hr) and for three sequential times using the same scoop. Scoops were inoculated with the E. coli ampicillin-resistant strain with a fluorescent gene as described in section 2.3.1. prior to exposure to ice. Bacteria were enumerated using the method that was previously described in section 2.2.2. at each of the storage times and for each of the sequential exposure to ice.

2.4 Experiment 3: Survival of E. Coli on Sliced Lemons

Survival of E. coli on lemon slices was tested at three different time intervals (0, 4, and 24hr) and at refrigerated (4±2°C) and room (21±2°C) temperatures. Lemons were inoculated with the E. coli ampicillin-resistant strain with a fluorescent gene as described in section 2.1 by placing each lemon in sterile bag containing 20 ml of a ~6 log CFU/mL of E. coli solution which was shaken for 30 sec then the lemon removed and allowed to dry for 5 min. Lemons were then sliced into quarters. One set of slices were enumerated for E. coli after 10 min while other lemons were stored for 4 and 24hr at room or refrigerated temperature. Bacteria were enumerated using the method that was previously described in section 2.2.1. at each of the storage times.

2.5 Statistical Analysis

All three experiments were conducted as completely randomized designs and simple mean, standard deviation, minimum and maximum values were determined for treatment using the Statistical Analysis System (SAS, 2014). Experiment 1 (bacterial transfer from hands to lemons) had 11-13 subjected per each of 3 replications with each observation duplicated for a total of 70 observations per treatment (wet or dry lemons). Experiment 2 (bacterial transfer from hands or scoops to ice) had 11 subjects per each of 3 replications with each observations duplicated for a total of 66 observations per treatment (hand or scoop). In a separate experiment 2.1 (bacterial transfer from 3 sequential scoops held for up to 2 hours) was conducted using three replications having 2 variables of (1) 1-3 scoops in sequence and (2) holding time (0, 1, 1.5 and 3 hours). Two scoops were utilized for each of 3 replications and each scoop was analyzed in duplicate yielding 12 observations per treatment. Experiment 3 (bacterial survival on stored lemons) had 2 variables of (1) storage temperature (room or refrigerated) and (2) storage time (0, 4 and 24 hours) with duplicates for each of 5 replications for a total of 10 observations for each combined storage temperature and storage time treatment combination. Treatments were subjected to an analysis of variance, and since the treatments had a significant effect ($p > 0.05$), were separated using the pdiff command of SAS (2014).

3. Results and Discussion

3.1 Experiment 1: E. Coli Transfer from Hands to Whole Lemons

No fluorescent *E. coli* were recovered from lemons handled with un-inoculated hands. One interesting finding was that all (100%) of the lemons that were wet prior to handling with inoculated hands showed bacterial

transfer while only 30.3% of lemons that were dry prior to handling with inoculated hands had bacteria detected after handling. The average CFU per lemon when wet was 6123 with an average transfer of 4.82% (Table 1). Conversely, the dry lemons had an average CFU of 469 (Table 1) and a transfer of 0.2%.

Table 1. Mean, median, range of the population and % transfer of bacteria on lemons from hands inoculated with *E. coli*

	CFU/lemon		LogCFU/lemon		%CFU transferred	
	Dry lemons	Wet lemons	Dry lemons	Wet lemons	Dry lemons	Wet lemons
Mean	469[b] (1502)	6912[a]	2.67[b] (3.2)	3.84[a]	0.20[b] (0.65)	4.86[a]
Stand Error	223	1644	0.16	0.09	0.07	0.8
Median	0	2180	0	3.34	0	2.48
Maximum	11720	62400	4.07	4.8	2.57	29.52
Minimum	0	40	0	1.60	0	0.02

[a,b]means with different superscripts are significantly different ($p \leq 0.05$). n=70

(values in parenthesis are calculated from only the 30% having transfer)

Patrick, Findon & Miller (1997) also found the wetness of hands (degree of drying) was directly related to the percentage of bacteria transferred to food. In the current study, the transfer of bacteria was greater when lemons were wet. Perez-Rodriquez et al. (2008) summarized the modelling of bacterial transfer between recipient and donor surfaces including intrinsic factors of bacterial hydrophilicity/hydrophobicity and biofilm development, and environmental factors including contact time, pressure, surface roughness and surface moisture. Based previously reviewed research, Perez-Rodriquez et al. (2008) concluded that increased moisture increased bacterial transfer from surfaces to food. Kusumaningrum et al. (2003) found a greater transfer of bacteria to cucumber slices than roasted chicken from inoculated stainless steel indirectly supporting the finding that moisture facilitates bacterial transfer. Shale, Jacoby & Plaatjies (2006) reported that transfer of *Staphylococcus* spp. was greater between meat and surfaces than between airborne bacteria in meat abattoirs. Furthermore, there was no difference in the degree which bacteria adhered to hands or gloves according to Legg et al. (1999).

Moore et al. (2003) found varying results for transfer from inoculated stainless steel to wet or dry lettuce. For example, transfer of *Salmonella* Typhimurium from stainless steel to dry lettuce ranged from 6 to 66% (depending on how long the bacteria were on the surface before lettuce contact) and from 23 to 31% for wet lettuce. Also for *Campylobacter jejuni* transfer for was from 16 to 38% for dry lettuce and from 15 to 27% for wet lettuce. Gill & Jones (2002) reported greater transfer of *E. coli* from meat to gloves and from gloves to meat when gloves were wet with between 2 to 4 log cfu/piece of meat transferred from hands and gloves contaminated by handling inoculated meat.

3.2 Experiment 2. Transfer of E. Coli to Ice from Hands and Metal Scoops

Ice is a known transmission vector of pathogenic microorganisms in human foodborne illness (Levine, Stephenson & Craun, 1990; Reis et al., 1992; Falcao et al. 2004). In these studies the pathogen was carried in water used to create the ice however, cross contamination due to handling food and ice is also a cause of foodborne illness (Fendler, Dolan & Williams, 1998; Montville, Chen & Schaffner, 2002). Bacteria can reside on hands (Rocourt & Cossart, 1997; Rose & Slifko, 1999) and stainless steel food contact surfaces (Kusumaningrum, Riboldi, Hazelberger & Beumer, 2003; Moore, Sheldon & Jaykus, 2003; Rodriguez & McLandsborough, 2007; Kesiken, Todd & Ryser, 2008) and transfer bacteria to food. In the current study an average of 19.5 % of the bacteria on hands were transferred to ice and 66.2% of bacteria on scoops were transferred to ice (Table 2). The higher level of transfer from scoops compared to hands is expected due to the lack of attachment on stainless steel compared to skin.

Table 2. Mean, median, range of the population and % transfer of bacteria on ice from hands and scoops inoculated with *E. coli*

	CFU/hand or scoop		LogCFU/hand or scoop		%CFU transferred	
	Hand Ice	Scoop Ice	Hand Ice	Scoop Ice	Hand Ice	Scoop Ice
Mean	23676[b]	506800[a]	4.1[b]	5.6[a]	19.5[b]	66.2[a]
Stand Error	3284	60773	0.07	0.04	1.9	1.2
Median	16698	369380	4.2	5.6	13.9	67.8
Maximum	1102465	1900030	5.0	6.3	67.0	82.5
Minimum	607	93610	2.8	0.3	1.9	30.0

[a,b]means with different superscripts are significantly different ($p \leq 0.05$). n=66.

Transfer of bacteria from contaminated ice holding bins or scoops to ice may be an issue since Hampikyan, Bigol, Cetin & Colak (2017) reported finding *E. coli* in 6.7 %of ice samples, 22 % of ice chest samples but no positive *E. coli* from water samples used to make the ice.

In a separate experiment, the time after inoculation and sequential scoops were evaluated as factors affecting *E. coli* transfer to ice. A significant effect ($p \leq 0.05$) of holding scoops after inoculation and for taking multiple ice samples in sequence using the same scoop was found on the population of E. coli transferred to ice (Figure 1). Less bacteria was transferred in the second scoopful of ice at 0 and 1 hour after inoculation but relatively high levels of bacteria were still transferred from the scoop to the ice in the third scoopful. The percentage of bacteria transferred at 0 hours for scoop sample 1, 2, and 3 was 71, 53 and 49% respectively. This trend held for all of the holding times with a decreasing number of bacteria as holding time increased. The overall difference in % transfer between the 3 scoopfuls taken in sequence was 11% between scoopful 1 and 2 and 16% between scoopful 1 and 3. Overall the percentage of bacteria transferred to ice was significantly different between scoopfuls 1 and 2 and between 1 and 3 but not 2 and 3 ($p \leq 0.05$). . This repeated transfer of *E. coli* from scoops to ice is supported by previous research that reported bacteria residing on surfaces could shed during repeated contact with other surfaces (Moore et al., 2003).

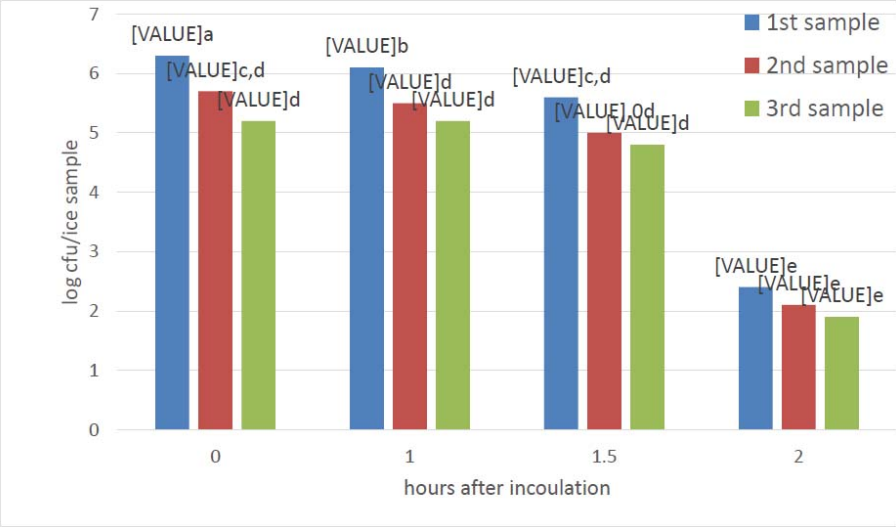


Figure 1. The population of *E. coli* recovered from ice exposed to scoops for 5 seconds then held for different times after inoculation and then exposed three sequential times using the same scoop

[a-e]means with different superscripts are significantly different ($p \leq 0.05$). n=12. Standard error for the 12 treatments ranged from 0.16 to 0.35 logcfu/ice sample.

3.3 Experiment 3. Bacterial Survival during Holding of Lemons

During the storage of lemons, bacterial population was highest for refrigerated lemons at time t=0. Lemons held at room temperature lemons had the highest E. coli populations at 0 and 24 hours (Figure 2). Refrigeration reduced E. coli populations from about 5 logs cfu/lemon to about 2 logs after 4 hours which did not further diminish after 24 hours of refrigerated storage. Beumer & Kusamaningrum (2003) found that leftover foods stored at 10°C increased by 2-3 log cycles in 3 days. Overall, to prevent growth and transfer, lemons should be handled dry and kept refrigerated. Enteropathogenic bacteria (*Shigella* and *Salmonella* spp.) increased in population several log cycles in 6 hours on cut papaya and watermelon when stored at 25-27°C and the application of lemon juice to the fruit surface reduced the population of *S.* typhii but the bacteria began to increase in population after 2 hours (Escartin, Ayala & Lozano, 1989). Cross contamination in food service environments is a major factor in many foodborne illness outbreaks (Bloomfield & Scott, 1997; Guzewich & Ross, 1999).

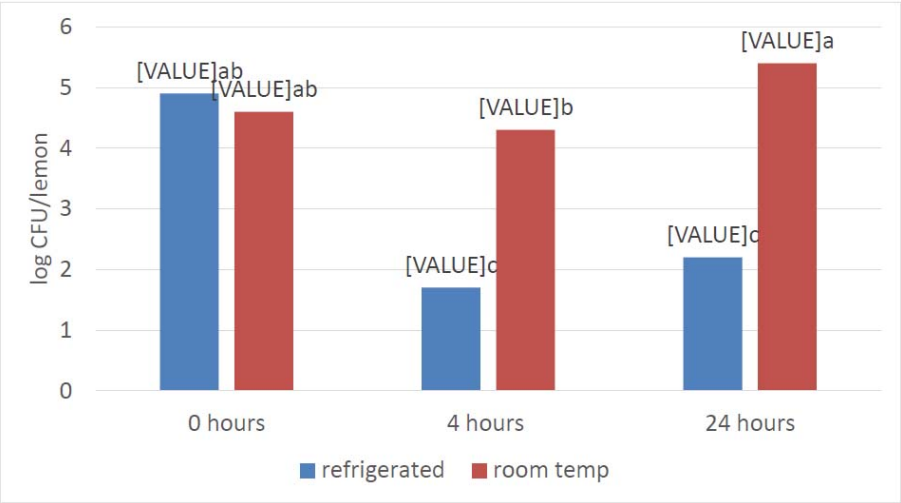


Figure 2. Population of *E. coli* on lemons after different storage times held at room temperature or under refrigeration

[a,b]means with different superscripts are significantly different ($p \leq 0.05$). n=10. Standard deviation for the 6 treatments ranged from 0.26 to 1.9.

Chen, Jackson, Chai & Schaffner (2001) modeled the transfer of a surrogate bacterium *Enterobacter aerogenes,* starting from raw chicken to hands, then after washing of hands, the transfer of bacteria remaining of hands to lettuce. These researchers found transfer rates as high as 100% with over 3 logs recovered on lettuce from an initial inoculation of 8 logs on chicken despite the two transfer steps and hand washing before touching lettuce. Chen et al (2001) also demonstrated touching other surfaces such as spigots to turn on water would create surfaces that could, in turn be sources of contamination.

3.5 Bacteria in Beverages

Harmful bacteria can be added to beverages by handling of ice and other garnishes. In fact Loving & Perz (2007) found 69.3% of lemon slices put in drinks in 21 different restaurants were carrying bacteria or fungi many of which are associated with human contamination. A common fallacy is that acidic and alcoholic beverages will protect the consumer from harmful microorganisms. In fact, Dickens, DuPont & Johnson (1985) found that 4 pathogens frozen in ice and allowed to melt for 30 minutes in cola, soda, Scotch (80 proof), a mixture of Scotch/soda and Tequila (86 proof) were not eliminated. The following percentages were recovered in each of the following; 100% in club soda, 55-74% in cola, 64-94% in scotch and soda, 11-16% in pure Scotch and 5-10% in pure Tequila. Two other studies found a slight inhibitory effect of drink acidity but only when the bacteria were exposed to acidic drinks for a day or more. In a study to determine the best beverage to consume to avoid "traveler's diarrhea" or "Montezuma's revenge." Sheth, Wisniewski & Franson (1988) reported that wine, diet cola and sour mix eliminated Salmonella and *E. coli* after 48 hours of exposure while beer and regular cola had a strong inhibitory effect but did not eliminate these bacteria. A second study examined orange drinks with pH levels of 3.0, 4.9 and 6.8 finding that only the 3.0 pH drink reduced *E. coli* and *Salmonella* spp. populations but only to low levels after 30 hours of exposure (Massa, Facciolongo, Rabasco & Caruso, 1998). In these studies, extremely long exposure times were used, a scenario not likely to occur with drinks served with ice or fresh cut lemons. Thus the contamination of ice or cut fruit such as lemons to drinks could be a potential vehicle to transmit bacteria. Food service workers are the primary of contamination of food with norovirus, hepatitis A and *Shigella* spp. and can transfer other pathogens such as *E. coli* and *Salmonella* spp. to food (Lynch, Tauxe & Hedberg, 2009) thus sanitation of surfaces contacting ice and lemons served in beverages should be considered in minimizing food contamination.

References

Bailey, S. (2014) A guide to the non-alcoholic beverage industry. Market Realist. November 20, 2014 http://marketrealist.com/2014/11/guide-non-alcoholic-beverage-industry/ accessed 4-18-2017.

Beumer, R. R., & Kusumaningrum, H. (2003). Kitchen hygiene in daily life. *International Biodeterioration & Biodegradation, 51*(4), 299-302. https://doi.org/10.1016/S0964-8305(03)00041-6

Blom, A. W., Gozzard, C., Heal, J., Bowker, & Estela, C. M. (2002). Bacterial strike-through of re-usable surgical drapes: the effect of different wetting agents. *Journal of Hospital Infection, 52*(1), 52-55. https://doi.org/10.1053/jhin.2002.1262

Bloomfield, S. F., &Scott, E. (1997). Cross-contamination and infection in the domestic environment and the role of chemical disinfectants. *Journal of Applied Microbiology, 83*, 1-9. https://doi.org/10.1046/j.1365-2672.1997.00199.x

CDC (2011). http://www.cdc.gov/foodborneburden/ accessed 2/28/2017.

Chen, Y., Jackson, K. M, Chea, F. P., & Schaffner, D. W. (2001). Quantification and variability analysis of bacterial cross-contamination rates in common food service tasks. *Journal of Food Protection, 64*(1), 72-80. https://doi.org/10.4315/0362-028X-64.1.72

Falcao, J. P., Dias, A. M. G., Correa, E. F., & Falcao, D. P. Microbiological quality of ice used to refrigerate foods. *Food Microbiology, 19*, 269-276. http://dx.doi.10.1006/yfmic.490

Dickens, D. L., DuPont, H. L., & Johnson, P. C. (1985). Survival o bacterial enteropathogens in the ice of popular drinks. *Journal of the American Medical Association, 253*(21), 3141-3143. https://doi.org/10.1001/jama.1985.03350450113034

Escartin, E. F., Ayala, A. C., & Lozano, J. S. (1989). Survival and growth of Salmonella and Shigella on sliced fresh fruit. *Journal of Food Protection, 52*(7), 471-472. https://doi.org/10.4315/0362-028X-52.7.471

FDA, (2010). US Food and Drug Association, FDA regulates the safety of packaged ice. International Packaged Ice 390 Association. Available at: http://www.packagedice.com/downloads/FDA%20Regulates%20the%20Safety%20of%20Pa392 ckaged%20Ice.pdf. accessed on 4-23-2017.

Falcao, J., A. Dias, E. Correa, & Falcao, D. (2002). Microbiological quality of ice used to refrigerate foods. *Food Microbiology, 19*, 269-276 https://doi.org/10.1006/fmic.2002.0490

Falcao, J. P., Falcao, D. P., & Gomez, T. A. T. (2004). Ice as a vehicle for diarrheagenic Escherichia coli. *International Journal of Food Microbiology, 91*(1), 99-103. https://doi.org/10.1016/S0168-1605(03)00327-1

Fendler, E. J., Dolan, M. J., & Williams, R. A. (1998). Hand washing and gloving for food protection. Part I. Examination of the evidence. *Dairy and Food Environmental Sanitation, 18*, 814-823.

Gardner, J. M., Feldman, A. W., & Zablotowicz, R. M. (1982). Identity and Behavior of Xylem-Residing Bacteria in Rough Lemon Roots of Florida Citrus Trees. *Applied and Environmental Microbiology, 43*(6), 1335-1342.

Gerokomou, V., Voidarou, C. Vatopoulos, A., Velonakis, E., Rozos, G., Alexopoulos, A., Plessas, S., Stavropoulou, E., Bezirtzoglou, E., Demertzis, P. G., & Akrida-Demertizi, K. (2011). Physical, chemical and microbiological quality of ice used to cool drinks and foods in Greece and its public health implications *Anaerobe, 17*(6), 351-353. https://doi.org/10.1016/j.anaerobe.2011.06.005

Gill, C. O., & Jones, T. (2002). Effects of wearing knitted or rubber gloves on the transfer of Escherichia coli between hands and meat. *Journal of Food Protection, 65*(6), 1045-1048. https://doi.org/10.4315/0362-028X-65.6.1045

Guzewich, J. J., & Ross, M. P. (1999). Evaluation of risks related to microbiological contamination of ready-to-eat food by food preparation workers and the effectiveness of interventions to minimize those risks. http://vm.cfsan.fda.gov/;ear/rterisk.html.

Hampikyan, H., Bingol, E. B., Cetin, O., & Colak, H. (2017). Microbiological quality of ice and ice machines in food establishments. *Journal of Water and Health, 15*(2), available online; https://doi.org/10.2166/wh.2017.159

Heal, J. S., Blom, A. W., Titcomb, D., Taylor, A., Bowker, K., & Hardy, J. R. W. (2003). Bacterial contamination of surgical gloves by water droplets spilt after scrubbing. *Journal of Hospital Infection, 53*(2), 136-139. https://doi.org/10.1053/jhin.2002.1352

Jumma, P. A. (2005). Han hygiene. Simple and complex. *International Journal of Infectious Diseases, 9*, 3-14. https://doi.org/10.1016/j.ijid.2004.05.005

Keskinen, L. A., Todd, E. D. & Ryser, E. T. (2008). Transfer of surface-dried Listeria monocytogenes from

stainless steel knife blades to roast turkey breast. *Journal of Food Protection, 71*(1), 176-181. https://doi.org/10.4315/0362-028X-71.1.176

Kusumaningrum, H. D., Riboldi, G., Hazelberger, W. C., & Beumer, R. R. (2003). Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. *International Journal of Food Microbiology, 85*, 227-236. https://doi.org/10.1016/S0168-1605(02)00540-8

Lassucq, S., Baltch, A. L., Smith, R. P., Smithwick, R. W., Davis, B. J., Desjardin, E. K., Silcox, A., Spellacy, A. B., Zeimes, R. T., Gruft, H. M., Good, R. C., & Cohen, M. L. (1988). Nosocomial Mycobacterium foruitum colonization from a contaminated ice machine, *American Review of Respiratory Disease, 138*(4), 891-894. https://doi.org/10.1164/ajrccm/138.4.891

Lateef, A., Oloke, J. K., Gueguim Kana, E. B., & Pacheco, E. (2006). The microbiological quality of ice used to cool drinks and foods in Ogbomoso Metropolis, Southwest, Nigeria. *Internet Journal of Food Safety, 8*, 39-43.

Legg, S. J., Khela, N., Madie, P., Fenwick, S. G., Quynh, V., & Hedderly, D. I. (1999). A comparison of bacterial adherence to bare hands and gloves following simulated contamination from a beef carcass. *International Journal of Food Microbiology, 53*(1), 69-74. https://doi.org/10.1016/S0168-1605(99)00153-1

Levine, W. C., Stephenson, W. T., & Craun, G. F. (1990). Waterborne Disease Outbreaks, 1986-1988. https://www.cdc.gov/mmwr/preview/mmwrhtml/00001596.htm accessed 4-23-2017.

Loving, A.L. & Perz, J. 2007. Microbial flora on restaurant beverage lemon slices. Journal of Environmental Health 70(5): 18-22.

Lynch, M. F., Tauxe, R. V., & Hedberg, C. W. (2009). The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. *Epidemiology and Infection, 137*(3), 307-315. https://doi.org/10.1017/S0950268808001969

Mackintosh, C. A., & Hoffman, P. N. (1984). An extended model for transfer of micro-organisms via the hands: differences between organisms and the effect of alcohol disinfection. *Journal of Hygiene, 92*, 345-355. https://doi.org/10.1017/S0022172400064561 .

Marketrealist. (2014). http://marketrealist.com/2014/11/guide-non-alcoholic-beverage-industry/accessed 4-18-2017.

Marples, R. R., & Towers, A. G. (1979). A laboratory model for the investigation of contact transfer of micro-organisms. *Journal of Hygiene, 82*(2), 237-248. https://doi.org/10.1371/journal.pone.0071438

Martinez-Gonzalez, N. E., Hernandez-Herrera, A., Martinez-Chavez, L., Rodriguez-Garcia, M. O., Torres-Vitela, M. R., Mota de la Garza, L., & Castillo, A. (2003). Spread of bacterial pathogens during preparation of freshly squeezed orange juice. *Journal of Food Protection, 66*(8), 1490-1494. https://doi.org/10.4315/0362-028X-66.8.1490

Massa, S., Facciolongo, M., Rabasco, E., & Caruso, M. (1998)> Survival of indicator/pathogenic bacteria in orange soft drink. *Food Microbiology, 15,* 253-257. https://doi.org/10.1006/fmic.1997.0168

Merry, A. F., Miller, T. E., Findon, G., Webster, C. S., & Neff, S. P. W. (2001). Touch contamination levels during anesthetic procedures and their relationship to hand hygiene procedures: a clinical audit. *British Journal of Anaesthesia, 87*, 291-294. https://doi.org/10.1093/bja/87.2.291

Montville, R., Chen, Y., & Schaffner, D. W. (2002). Risk assessment of hand washing efficacy using literature and experimental data. *International Journal of Food Microbiology, 83*, 305-313. https://doi.org/10.1016/S0168-1605(01)00666-3

Montville, R., & Schaffner, D. W. (2003). Inoculum size influences bacterial cross contamination between surfaces. *Applied and Environmental Microbiology, 69*(12), 7188-7193. https://doi.org/10.1128/AEM.69.12.7188-7193.2003

Moore, C. M., Sheldon, B. W., & Jaykus, L-E. (2003). Transfer of Salmonella and Campylobacter from stainless steel to Romaine lettuce. *Journal of Food Protection, 66*(12), 2231-2236. https://doi.org/10.4315/0362-028X-66.12.2231

Nichols, G., Gillespie, I., & de Louvois, J. (2000). The microbiological quality of ice used to cool drinks and ready-to-eat food from retail and catering premises in the United Kingdom. *Journal of Food Protection, 63*(1), 78-82. https://doi.org/10.4315/0362-028X-63.1.78

Panwalker, A. P., & Fuhse, A. L. (1986). Nosocomial Mycobacterium gordonae pseudoinfection from contaminated ice machines. *Infection Control and Hospital Epidemiology, 7*(2), 67-70. https://doi.org/10.1017/S0195941700063918

Patrick, D. R. Findon, G., & Miller, T. E. (1997). Residual moisture determine the level of touch-contact-associated bacterial transfer following hand washing. *Epidemiology and Infection, 119*(3), 319-325. http://www.jstor.org/stable/4617422

Perez-Rodriguez, F., Valero, A., Carrasco, E., Garcia, R. M., & Zurera, G. (2008). Understanding and modelling bacterial transfer to foods: a review. *Trends in Food Science and Technology, 19*(3), 131-144 https://doi.org/10.1016/j.tifs.2007.08.003

Ries, A. A., Vugia, D. J., Beingolea, L., Palacios, A. M., Vasquez, E., Wells, J. G., Baca, N. G., & Swerdlow, D. L. (1992) Cholera in Piura, Peru: a modern urban epidemic. *Journal of Infectious Disease, 166*(6), 1429-1433. https://doi.org/10.1093/infdis/166.6.1429

Rocourt, J. B., & Cossart, P. (1997). *Listeria monocytogenes*. In: Food Microbiology-Fundamentals and Frontiers, (Doyle, M.P., Beuchat, L.R. & Montville, T.J. Editors). ASM Press, Washington, DC, pages 337-352.

Rodriguez, A., & McLandsborough, L. A. (2007). Evaluation of the transfer of Listeria monocytogenes from stainless steel and polyethylene to bologna and American cheese. *Journal of Food Protection, 70*(3), 600-606. https://doi.org/10.4315/0362-028X-70.3.600

Rose, J. B., & Slifko, T. R. (1999). *Giardia, Cryptosporidium* and *Cyclospora* and their impact on foods: a review. *Journal of Food Protection, 62*, 1059-1070. https://doi.org/10.4315/0362-028X-62.9.1059

Rusin, P., Maxwell, S., & Gerba, C. (2002). Comparative surface‐to‐hand and fingertip‐to‐mouth transfer efficiency of gram‐positive bacteria, gram‐negative bacteria, and phage. *Journal of Applied Microbiology*, *93*(4), 585-592. https://doi.org/10.1046/j.1365-2672.2002.01734.x

Sattar, S. A., Springthorpe, S., Mani, S., Gallant, M., Nair, R. C., Scott, E., et al. (2001). Transfer of bacteria from fabrics to hands and other fabrics: development and application of a quantitative method using Staphylococcus aureus as a model. *Journal of Applied Microbiology, 90*, 962-970. https://doi.org/10.1046/j.1365-2672.2001.01347.x

Scott, E., & Bloomfield, S. F. (1990). The survival and transfer of microbial contamination via cloth, hands and utensils. *Journal of Applied Microbiology, 68*(3), 271-278. https://doi.org/10.1111/j.1365-2672.1990.tb02574.x

Shale, K., Jacoby, A., & Plaatjies, Z. (2006). The impact of extrinsic sources on selected indicator organisms in a typical deboning room. *International Journal of Environmental health Research, 16*(4), 263-272. https://doi.org/10.1080/09603120600734162

Sheth, N. K., Wisniewski, T. R., & Franson, T. R. (1988). Survival of enteric pathogens in common beverages: An in vitro study. *The American Journal of Gastroenterology, 83*(6), 658-660.

Stout, J. E., Yu, V. L., & Muraca, P. (1985). Isolation of Legionellla pneumophila from the cold water of hospital ice machines: Implications for origin and transmission of the organism. *Infection Control and Hospital Epidemiology, 6*(4), 141-146. https://doi.org/10.1017/S0195941700062937

USDA (2014). http://www.ers.usda.gov/data-products/cost-of-foodborne-illnesses.aspx accessed 2/28/2017.

WHO, (1997). World Health Organization. Guidelines for drinking-water quality. *Surveillance and 436 Control of Community Supplies, 2*(3), Available at: http://www.who.int/water_sanitation_health/dwq/gdwqvol32ed.pdf. Accessed on 4-23-2017

Wilson, I. G., Hogg, G. M., & Barr, J. G. (1997). Microbiological quality of ice in hospital and community. *Journal of Hospital Infection, 36*(3), 171-180. https://doi.org/10.1016/S0195-6701(97)90192-4

Technical Contribution No. 6670 of the Clemson University Experiment Station.

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

In-Vitro Antioxidant Capacity and Bioactive Compounds Preservation Post-Drying on Berrycacti (*Myrtillocactus geometrizans*)

Priscila D. Santiago-Mora[1], Anaberta Cardador-Martínez[1], Carmen Téllez-Pérez[1], José Gerardo Montejano-Gaitán[1] & Sandra T. Martín del Campo[1]

[1]Escuela de Bioingenierías, Escuela Nacional de Ingeniería y Ciencias, Tecnológico de Monterrey, Campus Querétaro, Querétaro, México

Correspondence: Sandra T. Martín del Campo, Escuela de Bioingenierías, Escuela Nacional de Ingeniería y Ciencias, Tecnológico de Monterrey, Campus Querétaro, Epigmenio González 500, Fracc. San Pablo. Querétaro, Querétaro, 76130, México. Tel: 52-442-238-3223. Email: smartinde@itesm.mx

Received: May 4, 2017 Accepted: May 31, 2017 Online Published: June 30, 2017

doi:10.5539/jfr.v6n4p121 URL: https://doi.org/10.5539/jfr.v6n4p121

Abstract

Berrycactus is a cactus which does not require special agronomic attention, the berries are consumed locally and its commercialization is rather scarce because of the extremely short shelf-life. The significance of the application of any drying methods used to extend the shelf-life on the berrycacti is currently unknown. The aim of this work was to preserve berrycacti (*Myrtillocactus geometrizans*) and test the bioactive compounds and antioxidant capacity using two distinctive drying methods, freeze-drying (FD) and Instant Controlled Pressure Drop (DIC). Ripe berrycacti was chosen for the drying procedures because the antioxidant capacity and levels of soluble phenols and betalains were at their peak. Colour, phenols, non-extractable polyphenols, tannins, betalains, and antioxidant capacity were considered as factors to determine drying efficacy. Only colour parameters could discriminate between FD and DIC, concluding that both methods are suitable and efficient for preservation of antioxidant properties and retention of bioactive compounds. Both drying methods demonstrated higher in-vitro antioxidant capacity compared to the fresh fruit; highlighting the increase of non-extractable polyphenols and condensed tannins, and good retention of betalains and ascorbic acid after the drying treatments. This research points to use this sustainable crop to provide added value to berrycacti while considering this fruit as functional food due to the antioxidant capacity present even after being processed.

Keywords: antioxidant capacity, berrycactus, drying, freeze-drying, Instant Controlled Pressure Drop (DIC), ripening stages

1. Introduction

Myrtillocactus geometrizans (berrycactus) is a perennial Cactaceae plant native to Central Mexico, approximately 2.0 m tall with curved and thorny branches (Guzmán-Maldonado, Villordo, González-Chavira, Pons-Hernández, & Hernández-López, 2012). The cactus has white flowers and produces dark purple berry like fruit with ellipsoid dimensions about 2.8 cm by 2.0 cm (Arias, 2010). These fruits have a very thin skin and the flesh is rich in color with gelatinous pulp and miniature black seeds (Herrera-Hernández, Guevara-Lara, Reynoso-Camacho, & Guzmán-Maldonado, 2011). *Myrtillocactus geometrizans* grows in arid and semiarid regions and the cactus does not require special agronomic attention. The berries are consumed locally in rural areas (Perez-Gonzalez, 1995). Berrycactus commercialization is rather scarce because of the extremely short shelf-life, which is approximately 2 days at room temperature and 5 days under refrigeration (Hernández-López, Vaillant, Reynoso-Camacho, & Guzman-Maldonado, 2008). The significance of the application of any drying methods used to extend the shelf-life on the berrycacti is currently unknown.

Berrycactus fruit contains about 2.3 mg of betalains per 100 g of fresh fruit (Hernández-López and others 2008). Betalains have shown potent antiradical-scavenging activity in-vitro (Butera et al., 2002; Cai, Sun, & Corke, 2003; Pavlov, Kovatcheva, Georgiev, Koleva, & Ilieva, 2002). Human bioavailability studies showed evidence for oxidative stress prevention through intestinal absorption (Tesoriere, Butera, Pintaudi, Allegra, & Livrea, 2004). Wu et al. (2006) concluded that the peel of red pitaya, which contains the same betalainic pigments, had high antioxidant activity and showed strong inhibitory *in-vitro* melanoma cell proliferation. Reynoso-Camacho, Martinez-Samayoa, Ramos-Gomez, Guzmán y Salgado (2015) tested the hypoglycemic and antioxidant effects

of *Myrtillocactus geometrizans* berries in streptozotocin-induced diabetic rats, highlighting the importance of berrycactus fruit betalains as adjuvant in the treatment of diabetes and renal complications.

Preserving the beneficial properties of betalain compounds in food during processing can be challenging if high temperatures are applied. However, if the thermal process is neither extensive nor extreme, this degradation is partially reversible (Castellar, Obón, Alacid, & Fernández-López, 2003). Also, different forms of radioactive light have been shown to degrade these compounds. Under dark conditions, stability is at least double that of normal visible light conditions (Delgado-Vargas, Jiménez, & Paredes-López, 2000). Finally, using minimal water in the process of extracting betalains has been shown to stabilize these molecules (Gokhale & Lele, 2014).

Drying is an ancient preservation method which extends product shelf-life by reducing or eliminating microbiological spoilage. Many drying methods cause flaws in the food, such as hardening, woody-texture, low rehydration capacity, loss of cellular integrity, and loss of nutritional quality. Chemical degradation enzymatically or by the Maillard reaction along with many other processes, degrade the quality and potential benefits of foods (Barbosa-Cánovas & Vega-Mercado, 2000). For that reason, the study of different drying technologies is important to improve the quality of these foods.

Freeze-drying has been shown to be a good alternative to preserve labile and photooxidable compounds in food, as well as creating a less compact product with good rehydration capacities (St. George & Cenkowski, 2009). Détente Instantanée Contrôlée (DIC, French for Instant Controlled Pressure Drop) is a hydro-thermo-mechanical pre-treatment involving high pressures, followed by an instant pressure drop that changes the texture in the product with a less compact structure that facilitates further extraction, drying, freezing, and other unit operations (Ratti, 2009).

Marginal research has been published on the drying of cactus-fruits, nevertheless showing the impact on the betalainic pigments and antioxidant capacity. This research identifies the changes occurring in berrycacti, while using freeze-drying or Instant Controlled Pressure Drop, advancing the knowledge in the field of dried foods. This research highlights proximal analysis, color parameters, bioactive compounds and in-vitro antioxidant capacities of the berrycacti after the application of the drying treatment.

The novelty of this work highlights berrycacti and its betalainic pigments and antioxidant capacity present even after being processed. On the other hand, this work aims to use a sustainable crop to provide added value to berrycacti.

2. Materials and Methods

2.1 Reagents and Solvents

Reagents like 2,2-diphenyl-1-picrylhydrazyl (DPPH), l, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulfate, potassium phosphate-buffered saline (PBS), sodium carbonate, acetate buffer, 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ), chlorhydric acid, ferric chloride, Folin Ciocalteu reagent, gallic acid, vanillin, catechin, metaphosphoric acid, acetic acid and indophenol were supplied by Sigma-Aldrich (St. Louis, MO, USA), ACS reagent and ≥98 % purity.

Solvents like methanol and ethanol were HPLC grade and supplied by KARAL (Leon, Gto., Mexico).

2.2 Biological Material

Berrycactus fruit (*M. geometrizans*) was the biological material, harvested from the community of Pie de Gallo, located in Querétaro, México (20º 47′ 28.8″ N, 100º 30′ 09.5″ W) between May and July 2013, at different maturation stages for the determination of an optimal (section 2.3).

Only the ripe berrycacti were washed after harvest, and flower buds were removed. The other berrycacti were discarded. The ripe fruit samples were separated into three batches. Batch 1 was stored at -40 ºC until physicochemical quantifications and in-vitro antioxidant capacity assays were performed. Batch 2 was separated into individual trays at -40 ºC in preparation to further freeze-drying treatments. Batch 3 was used immediately for Instant Controlled Pressure Drop drying treatments. All analyses were performed in triplicate to minimize the experimental error as determined by standard deviation calculations.

2.3 Determination of Optimal Degree of Maturation

Samples of berrycacti collected in different maturation stages ranging from unripe (green berry), changing (purple with some green hue notes), ripe (purple but firm fruit) to overripe (purple and soft to touch) were compared.

Weight of berrycacti (g) was recorded using an O´Haus Pioneer scale. Diameter and length were measured using a millimetric Vernier (Westward). Moisture content, using the AOAC (2012) 934.01 method. pH was measured, after mashing some berrycacti, with a Thermo Scientific pH benchtop potentiometer. Total titratable acidity (TTA) was determined potentiometrically by addition of NaOH 0.1N until pH 8.6 was achieved, this value was expressed as a percentage of TTA. Total soluble solids (TSS) were determined with an ABBE refractometer (Model 1211, Atago Co., LTD, Japan) expressed in ºBrix.

Soluble phenol analysis was carried out on an extract prepared by weighting 20 g of fresh berries and homogenized with 20 mL (1% HCl) acidified methanol, that was centrifuged for 10 minutes at 6000 rpm in a Hermle-Z 383K centrifuge (Equipar, Germany) and kept in absence of light at 4 °C. The phenol content was measured using a modified (Tavares et al., 2010) Folin-Ciocalteu procedure using a microplate and measured at 765 nm. The results were expressed in mg of gallic acid equivalents per mL of berrycactus extract.

Antioxidant capacity was determined by the scavenging test (Fukumoto & Mazza, 2000) with 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical measured at 515 nm using a visible-UV microplate reader (X Mark Microplate Reader, Bio-Rad Laboratories, Inc., Japan). A calibration curve was prepared with different concentrations of Trolox as standard and results were expressed as micromole Trolox equivalents/100 g (μmol T eq/100 g). Total betalain content of berrycactus extracts were spectrophotometrically measured at 538, 700 and 480 nm (Nilsson, 1970). The betalain content was calculated using the equations (Eq.1 and 2) from Cai y Corke (1999). Then, betacyanins were calculated as equivalents of betanin (mg BN eq/kg), and betaxanthins as equivalents of indicaxanthin (mg IX eq/kg); total betalain content were calculated by the mathematical addition of these two and were expressed in mg/kg.

$$\text{Betacyanin } \left(\frac{\text{mg}}{\text{L}}\right) = \frac{(A_{538} \times DF \times M_W \times 100)}{(\varepsilon \times L)} \quad (1)$$

Where:

A_{538}= absorption value at betanin λ max (538 nm) corrected by the absorption at 700 nm

DF= dilution factor

Mw= betanin molecular weight (550 g mol^{-1})

ε= betanin molar extinction coefficient (60,000 L mol^{-1} cm^{-1})

L= path length (1 cm) of the cuvette

$$\text{Betaxanthin } \left(\frac{\text{mg}}{\text{L}}\right) = \frac{(A_{480} \times DF \times M_W \times 100)}{(\varepsilon \times L)} \quad (2)$$

Where:

A_{480}= absorption value at indicaxanthin λ max (480 nm) corrected by the absorption at 700 nm

Mw= indicaxanthin molecular weight (308 g mol^{-1})

ε= indicaxanthin molar extinction coefficient (48,000 L mol^{-1} cm^{-1}).

L= path length (1 cm) of the cuvette

The optimal maturation stage was determined by comparing measured parameters such as TSS, acidity and antioxidant capacity. This stage was used for further drying experimental testing.

2.4 Drying Techniques

2.4.1 Freeze-drying (FD)

FD was applied to berrycacti previously frozen at -40 ºC in trays for at least 24 hours. Experiments were carried out in a LabConco FreeZone Triad (Kansas City, USA) where the freeze-dryer plate reached an initial freezing temperature of -50 ºC followed by sublimation and desorption performed at constant vacuum.

Central composite rotatable design was employed to evaluate the effect of FD operating parameters. The independent variables (n=2) were the plate´s final temperatures (PFT) (26, 30, 40, 50 and 54 ºC) and heating rates (HR) (1.6, 2.0, 3.0, 4.0 and 4.4 ºC/h).

2.4.2 Instant Controlled Pressure Drop (DIC)

Pre-drying of berrycacti was performed inside a cabinet dryer UNB 800 Model (Memmert, Germany), with air at 50 ºC, 265 Pa partial pressure of vapor and 1.2 m⁄s of air flux. This pre-drying treatment was carried out until

initial moisture content was achieved (10, 14, 20, 26 or 30% dry basis (DB)). The pre-dried fruit samples were then placed inside a chamber in the DIC equipment (LABIC0.1, ABCAR-DIC Process, La Rochelle, France). An initial vacuum of 0.003 MPa was established. Saturated steam was injected to the reactor (0.10, 0.17, 0.28, 0.38 or 0.45 MPa); once the chamber was pressurized, it was maintained for a given time (5, 13, 25, 37 or 45 seconds) and instantly decompressed towards vacuum of 0.003 MPa. After a vacuum stage period time, pressure was released toward the atmospheric pressure and fruit samples were removed from the reactor. A post-drying stage was performed inside the cabinet dryer at 50 ºC until the berrycactus samples achieved a constant weight, also known as an equilibrium where there is no more loss of moisture.

Central composite rotatable design was employed to evaluate the effect of DIC operating parameters. The independent variables (n=3) were saturated steam pressure (P), processing time (t) and initial moisture content of the sample (M), ranging from 0.1 to 0.45 MPa, 5 to 45 seconds, and 10 to 30% DB, respectively.

2.5 Proximal Analysis and Color Parameters

Moisture (934.01), ash (940.26), ether extract (920.85) and nitrogen (960.52) were measured according to the Association of Official Analytical Chemists (AOAC) standards using the AOAC (2012) methods. The total protein was determined using the conversion factor of 6.25 multiplied by the nitrogen measurements. Soluble fiber was determined by the enzymatic-gravimetric method from Prosky, Asp, Schweizer, DeVries y Furda (1988). The remaining percentage represented carbohydrates. All results were expressed as percentage in a dry basis (DB). The color parameters of the dried berry fruits, L*, a*, b*, chroma and hue were determined using a Minolta CM508D colorimeter (Metro Lab International, Japan).

2.6 Antioxidant Capacity Assays

For quantifications of antioxidant capacity as well as soluble phenols, condensed tannins, betacyanins and betaxanthins, a methanolic berrycactus extract was prepared from the dried samples as previously mentioned in section 2.3.

For all the dried samples, in-vitro antioxidant capacity was determined by three methods. The first determination was performed using DPPH radical as described in section 2.3. The second method, used ABTS radical as described by Re et al. (1999). The last antioxidant capacity method was the ferric reducing antioxidant power (FRAP) as described by Benzie y Strain (1996).

2.7 Bioactive Compounds Quantification

Non-extractable polyphenols (NEPP) were determined according to Saura-Calixto (1998) as a treatment of the residue from the initial extraction with acidified methanol with butanol/HCl (97.5:2.5, v/v) at 100 ºC for 60 min in the presence of $FeCl_3$, followed by spectrophotometry measurement at 555 nm using a Genesis 2100 UV/Vis spectrophotometer (Model G105, Thermo Fischer Scientific, China) using catechin as standard. Results were expressed in mg catechin equivalents/kg (mg CT eq/kg).

For soluble phenols quantification, an aliquot of the initial methanolic extract was used with the Folin Ciocalteu reagent (Singleton, Orthofer, & Lamuela-Raventos, 1999) and measured at 760 nm using the UV/Vis spectrophotometer after comparing the readings with a standard curve of gallic acid. Results were expressed in mg of gallic acid equivalents/ kg of sample (mg GA eq/kg).

To quantify the condensed tannins, an aliquot of the supernatant from the initial methanolic extract was treated with vanillin/methanol (1%) and samples were measured at 500 nm according to the vanillin method (Deshpande, Cheryan, Salunkhe, & Luh, 1986); results were reported as mg catechin equivalents/kg (mg CT eq/kg) after comparing readings with a catechin standard curve.

The determination of betacyanins and betaxanthins in the initial extract was performed as previously described in section 2.3; while ascorbic acid (967.21) was determined following the AOAC (2012) methods.

2.8 Water Holding Capacity and Texture Analysis for Dried Products

Water holding capacity (WHC) was evaluated on powdered FD and DIC dried berrycacti. On a 30-mL centrifuge plastic tube, 22.5 mL of water were added to 2.5 g of berrycactus powder at room temperature. Sample tubes were hand shaken vigorously for 1 minute and then incubated for 1 hour at room temperature. Then, they were centrifuged at 3500 rpm for 30 minutes in a Hermle-Z 383K centrifuge with partial intervals every 5 minutes to remove the supernatant resulted throughout the centrifugation process. The final moisture content was calculated as the cake weight loss and expressed as percentage of water holding capacity (DB) (Larrauri, Rupérez, Borroto, & Saura-Calixto, 1996).

Texture properties of dried berrycactus samples were evaluated in a MTS-QTEST11 (Universal Testing Machine,

Eden Prairie, MN, USA) using the Kramer shear cell (53 mm x 56 cm) with ten shear cutting blades, alternating (65 mm) which are loosely suspended from a blade holder. The compression ratio used was 50% deformation from the initial height at a rate of 200 mm/min. Results were obtained from the Tension Zero ver. 1.0 software (Hernández & Sariñana, 2007) showing the tenderness of the dried berry reported as the peak force (N).

2.9 Statistical Analysis

Statistical analysis was performed using the Statistica software v 13 (Dell, Inc., Tulsa, OK, USA). For each drying method data sets, analysis of variance (ANOVA) was applied to evaluate any significant differences ($p < 0.05$). The Fisher Least Square Difference (LSD) test was conducted for all the significant factors. A new data set was constructed by dividing the final value of each evaluated parameter with its initial value getting a Delta of this change. Then, ANOVA and LSD were applied to evaluate significant differences ($p < 0.05$) between drying methods for each individual parameter.

General Discriminant Analysis (GDA) was applied to the data to discriminate between the maturation stages and secondly between the dehydration processes. The forward stepwise method (p inclusion 0.05, p exclusion 0.05) was performed to minimize the model sizes.

3. Results and Discussions

3.1 Determination of Optimal Degree of Maturation

Samples of berrycacti with four different maturation stages showed significant changes in moisture content, Total Titratable Acidity (TTA), Total Soluble Solids (TSS), soluble phenols, antioxidant capacity (DPPH) and total betalains (Table 1). Moisture content and TTA reduced significantly, 10.38% and 30.95% respectively, as the berrycactus matures; on the other hand, TSS (19.08%), soluble phenols (85.92%) and total betalains (44.43%) increased significantly, comparing the overripe to the unripe berrycacti. Antioxidant capacity in the berrycacti demonstrated an increase between the unripe and ripe stages (32.61%). However, the values slightly decreased while achieving the overripe stage (26.27%) compared to the unripe.

Table 1. Physicochemical properties of berrycactus

	P-value	Unripe	Changing	Ripe	Overripe
Weight (g)	0.446	0.707^{a}	0.643^{a}	0.557^{a}	0.590^{a}
Diameter (cm)	0.596	1.000^{a}	0.967^{a}	0.867^{a}	0.900^{a}
Length (cm)	0.596	1.300^{a}	1.233^{a}	1.200^{a}	1.333^{a}
Moisture content (% DB)	0.003**	0.780^{b}	0.760^{b}	0.712^{a}	0.699^{a}
pH	0.233	4.75^{a}	4.89^{a}	4.70^{a}	4.71^{a}
Total titratable acidity (%)	0.002**	12.18^{c}	9.88^{b}	9.73^{ab}	8.41^{a}
Total soluble solids (^{o}Brix)	0.000**	29.67^{a}	31.33^{b}	33.33^{c}	35.33^{d}
Soluble phenols (mg GA eq/ mL)[1]	0.000**	6.89^{a}	9.63^{b}	11.74^{c}	12.81^{d}
DPPH (μmol T eq/100 g) [1]	0.000**	7215.3^{b}	8334.0^{c}	9568.5^{a}	9110.6^{a}
Total betalains (mg/kg)	0.000**	64575^{a}	73062^{b}	84519^{c}	93264^{d}

[1]μmol T eq/100 g: μmol of Trolox equivalents/100 g of DB. mg GA eq/kg: mg of gallic acid equivalents/kg of DB.

*Significance at $p \leq 0.01$ is marked with two asterisks

Means without a common letter through rows are significantly different ($p \leq 0.05$)

Coria Cayupán, Ochoa y Nazareno (2011) found very variable TSS in the pulp of *Opuntia megacantha*, *Opuntia ficus-indica* and *Opuntia* spp. during the fruit development. However, particularly *O. megacantha* betalain concentration, soluble phenols, antioxidant capacity (DPPH and ABTS) increased in the pulp and peel while ripening, similar to the results of this study. Coria Cayupán et al. (2011) also conclude that antioxidant capacity increases could be attributed to the increase in ascorbic acid and other active compounds as polyphenols and betalains.

Herrera-Hernández et al. (2011) prepared fractions for total phenols, betacyanins and betaxanthins and observed a decrease in berrycactus total phenols (81.5%) in ripe fruit compared to the unripe fruit contrary to what was observed in this research. Betacyanins in ripe (44.9%) and overripe (92.0%) fruits increased compared to the unripe berrycacti, opposite, betaxanthins diminished as the fruit ripened. The same authors also reported the antioxidant activity in their three fractions as Trolox Equivalent Antioxidant capacity, obtaining a decrease in the ripe and overripe berrycacti, compared to the unripe for total phenols (45.3% and 72.9%, respectively) and betacyanin fractions (57.4% and 66.5%, respectively); meanwhile, for the betaxanthin fraction, the values

obtained were less affected compared to the unripe fruit.

General Discriminant Analysis (GDA) was performed to separate samples per the maturation stages. Forward stepwise analysis led to the selection of two parameters: total betalains and antioxidant capacity (DPPH). With the selected parameters, 100% of the samples were correctly classified according to the maturation stage (Fig. 1). This confirms the selection, the ripe berrycactus stage, to optimize the highest antioxidant capacity and betalain content for further dehydration.

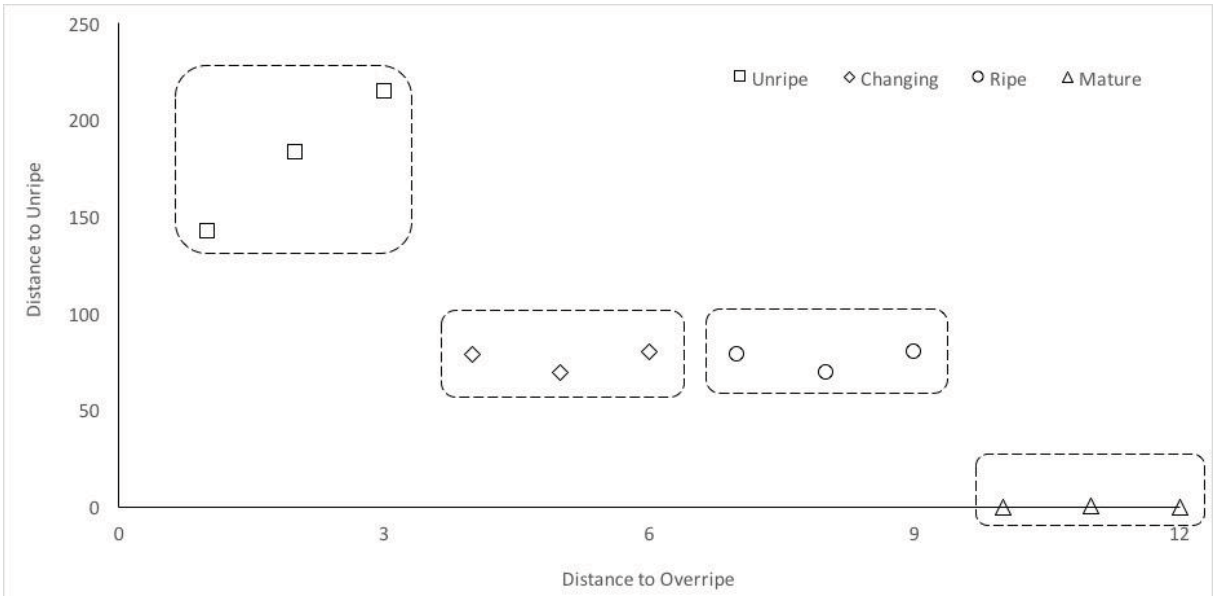


Figure 1. Cooman´s graph for maturation stages observed in berrycactus

The graph illustrates the Mahalanobis distances between the maturation stages in berrycacti, showing all four stages were clearly different among each other and samples can be correctly classified as unripe, changing, ripe and overripe berrycacti.

3.2 Freeze-drying ANOVA

ANOVA did not show significant differences ($p>0.05$) for any proximal analysis parameters.

However, when analyzing the color changes, there was a significant difference for the a* parameter ($p=0.040$) which describes the redness of the fruit while changing the HR for the FD treatment. For the antioxidant assays, ABTS showed significant differences ($p=0.015$) for the interaction between the PFT and the HR while for FRAP significant differences ($p=0.004$) were observed when changing the HR. Furthermore, quantification of bioactive compounds such as soluble phenols, NEPP, condensed tannins, betacyanins, betaxanthins and ascorbic acid showed no significant differences neither for WHC nor for tenderness (data not shown). Fig. 2 shows three graphs on the fitted surface response for the significant parameters during the FD treatments over the tested conditions; color parameter a* (Fig. 2.a), antioxidant capacity measured with ABTS (Fig. 2.b) and FRAP (Fig. 2.c). The research concludes that the optimal conditions for a FD treatment was plate´s final temperature of 26 ^{o}C with a constant heating rate of 3^{o}C/h.

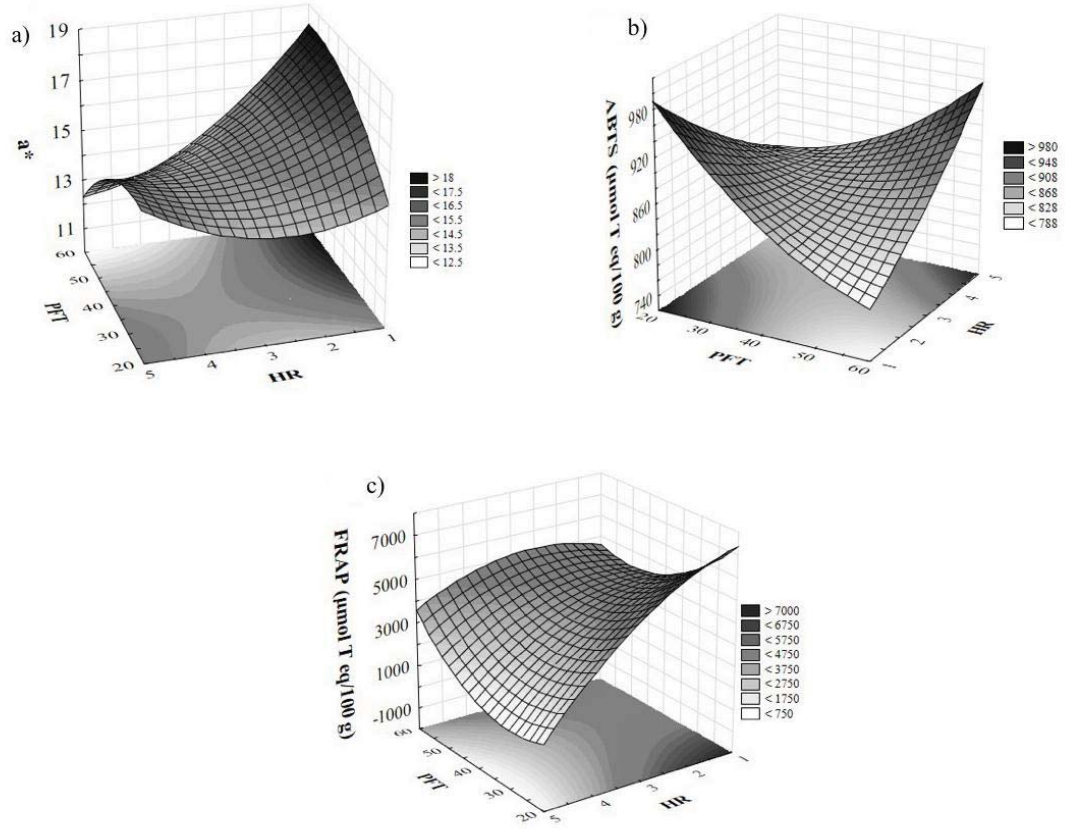


Figure 2. Fitted surface response for significant parameters during FD treatments

a) Color parameter a*. b) ABTS antioxidant capacity. c) FRAP antioxidant capacity.

a) The surface response for the color parameter a* (redness) shows that higher the PFT, the loss of redness is also higher in the berrycacti.

b) The surface response for ABTS antioxidant capacity shows that higher PFT, higher the loss of in-vitro antioxidant capacity in the berrycactus.

c) FRAP antioxidant capacity surface response graphs shows that higher HR, higher the in-vitro antioxidant capacity in the berrycactus.

Viloria-Matos, Corbelli-Moreno, Moreno-Álvarez y Belén (2002) evaluated the betalain stability in prickly pear (*Opuntia boldinghii*) pulp after freeze-drying under conditions of 12 hours of processing, 70 mmHg of pressure, -20 ºC of chamber´s temperature and 20 ºC of plate´s constant temperature. They reported good stability for betalain pigment with this method resulting in a good shelf life. Liaotrakoon, De Clercq, Lewille y Dewettinck (2012) observed in red-flesh dragon fruit (*Hylocereus polyrhizus*), a good retention of vitamin C and a slight increase in L* and a* and decrease in b*.

3.3 DIC ANOVA

ANOVA showed significant differences ($p<0.05$) only for the interactions between the factors tested. Individual factors did not show significant differences for any evaluated parameter (Fig. 3).

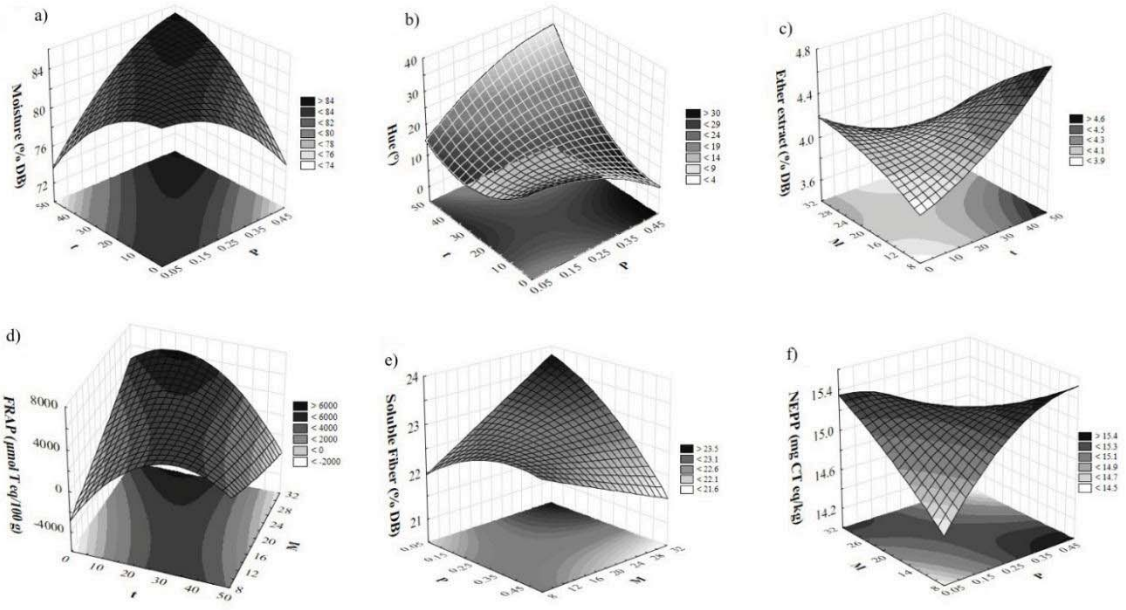


Figure 3. Fitted surface response for significant parameters during the DIC treatments

a) Moisture (% DB). b) Color hue. c) Ether extract (% DB). d) FRAP antioxidant capacity (μmol T eq/100 g). e) Soluble fiber (% DB). f) NEPP (mg CT eq/kg).

a) The surface response for the residual moisture content (%DB) shows that higher t, there is less moisture present in the berrycacti after dehydration.

b) Color hue surface response shows that higher t, there is less hue in the berrycacti after dehydration.

c) Surface response for ether extract shows that higher t, there is better extraction of the fat content in the berrycacti.

d) The surface response for FRAP antioxidant capacity shows that higher M (initial moisture of the berrycacti), lower the loss of FRAP in-vitro antioxidant capacity in the berrycactus.

e) Surface response graphs for shows that higher M, lower content of soluble fiber available in the berrycacti.

f) Surface response graphs for shows that higher M, lower content of NEPP available in the berrycacti.

The interaction between P and t was significant for the final moisture content (p=0.047; Fig. 3.a) and the color hue (p=0.024; Fig. 3.b), this are meaningful results due to a better extraction of moisture after the process as well as a better shade obtained in the berrycactus color. The interaction between t and M was significant for the ether extract (p=0.042; Fig. 3.c) and the antioxidant capacity reported with FRAP (p=0.030; Fig. 3.d), meaning again, a better extraction of the fat content in the berrycacti and higher antioxidant capacity with this in-vitro analysis. Finally, the interaction between P and M was significant for the fiber content (p=0.006; Fig. 3.e) and NEPP (p=0.005; Fig. 3.f) suggesting better availability of these compounds in the fruit.

This comparative analysis shows neither WHC nor tenderness were significantly different between the FD and DIC methods. DIC has been tested in different fruits (Alonzo-Macías, Cardador-Martínez, Mounir, Montejano-Gaitán, & Allaf, 2013; Haddad, Mounir, Sobolik, & Allaf, 2008; Mounir, Allaf, Berka, Hassani, & Allaf, 2014) where authors report shorter drying and rehydration times, as well as lower final water content in the fruits due to the rapid removal of water as the diffusivity was improved. Alonzo-Macías et al. (2013) reported an increase of antioxidant activity in strawberries with DIC compared to hot air drying and freeze-drying. Research articles have not indicated any significant changes in the soluble fiber, non- extractable polyphenols, such as proanthocyanidins or color changes for fruits using the DIC technology.

Based upon the parameters showing the fitted surface response (Fig. 3 A-F), and particularly highlighting the antioxidant capacity (FRAP), NEPP and the color hue of the berry after the DIC process, concluding that the optimal conditions are P of 0.45 MPa, t of 25 s and an M of 20% DB.

3.4 Variation of Berrycactus Parameters after Dehydration

Overall effect of FD and DIC treatments were compared against fresh fruits over the parameters shown in Table 2. The two drying methods used throughout the process demonstrated higher antioxidant activities than the fresh fruit, regardless of the methodologies used for its in-vitro determination, highlighting the increase in FRAP determination for FD (33.6%) and for DIC (41.5%). Furthermore, for condensed tannins, such increase was 9.0% and 13.5% for FD and DIC, respectively. Retention of betalains after FD was 95.1% and DIC was 87.1% due to the higher retention of betacyanins, which are the most abundant pigment in berrycacti. The retention of ascorbic acid was greater than 98% for both methods despite of the thermal exposure in the DIC treatment.

Table 2. Comparison of fresh and dried FD and DIC berrycacti

	Fresh	*p*-value	Δ for FD[1]	Δ for DIC[1]
Moisture (% DB)	80.29±2.63	0.022*	0.986[a]	1.029[b]
Ash (% DB)	3.69±0.33	0.816	1.009[a]	1.000[a]
Ether extract (% DB)	4.06±0.19	0.719	1.000[a]	1.000[a]
Protein (% DB)	4.99±0.28	0.583	0.990[a]	0.989[a]
Soluble fiber (% DB)	22.52±0.39	0.010**	1.004[a]	1.018[b]
Carbohydrates (% DB)	64.83±0.29	0.185	1.002[a]	0.997[a]
L*	32.98±6.80	0.483	1.034[a]	1.055[a]
a*	14.66±0.62	0.000**	0.906[a]	1.171[b]
b*	4.12±1.25	0.033*	1.488[a]	1.239[b]
Chroma	16.60±0.46	0.627	0.994[a]	0.955[a]
Hue (°)	10.84±1.93	0.667	1.430[a]	1.474[a]
DPPH (μmol T eq/100 g) [1]	8572.7±1034.9	0.597	1.011[a]	1.025[a]
ABTS (μmol T eq/100 g) [1]	830.3±24.5	0.009**	1.037[a]	1.007[b]
FRAP (μmol T eq/100 g) [1]	2901.3±1202.7	0.913	1.336[a]	1.415[a]
Soluble phenols (mg GA eq/kg) [1]	10070±500	0.059	1.000[a]	0.974[a]
Non-Extractable Polyphenols (mg CT eq/kg) [1]	14.94±0.21	0.010**	1.004[a]	1.018[b]
Condensed tannins (mg CT eq/kg) [1]	2.20±0.66	0.733	1.090[a]	1.135[a]
Betacyanins (mg BN eq/kg) [1]	81.77±12.75	0.072	0.951[a]	0.871[a]
Betaxanthins (mg IX eq/kg) [1]	0.188±0.086	0.027*	0.814[a]	0.560[b]
Total betalains (mg/kg)	81.96±12.77	0.071	0.951[a]	0.871[a]
Ascorbic acid (mg AA eq/kg) [1]	248.09±13.82	0.804	0.982[a]	0.981[a]

[1]FD: Freeze-drying. DIC: Instant Controlled Pressure Drop. μmol T eq/100 g: μmol of Trolox equivalents/100 g of DB. mg GA eq/kg: mg of gallic acid equivalents/kg of DB. mg CT eq/kg: mg of catechin equivalents/kg of DB. mg BN eq/kg: mg of betanin equivalents/kg of DB. mg IX eq/kg: mg of indicaxanthin equivalents/kg of DB. mg AA eq/kg: mg of ascorbic acid equivalents/kg of DB.

*Significance at $p \leq 0.05$ is marked with an asterisk; $p \leq 0.01$ is marked with two asterisks.

Means without a common letter are significantly different ($p \leq 0.05$)

ANOVA and LSD tests were applied to the delta value for each evaluated parameter. Final moisture content, soluble fiber, color parameter a*, and NEPP showed significant differences between FD and DIC, where FD showed lower delta values. On the other hand, the delta values for color parameter b*, ABTS antioxidant capacity and betaxanthins were significantly lower for DIC treatments. The remaining evaluated deltas were not significant between the two drying methods.

The color parameter a* exemplifies the color changes observed after the treatments, where FD shows a loss in "redness" compared to DIC where products retain and boost their red color. The b* parameter, describing the yellowness, increased for both methods compared to the fresh berries. FD showed higher yellowness than DIC due to a possible correlation with the betaxanthin retention.

NEPP are phenolic compounds that are bond to the soluble fiber (Pérez-Jiménez & Torres, 2011). While comparing the drying treatments, identical increments were noted for NEPP and soluble fiber, validating the correlation establish by the previously mentioned authors. In both cases for FD and DIC, there was an increase of the NEPP compared to the fresh berrycacti. However, this variation was higher for those berrycacti treated with DIC suggesting that DIC increases the availability of these compounds because of the expansion of the cell structure.

Betaxanthins were another bioactive compound which showed a significant difference between the two drying

methods. Although this parameter decreased after the treatment, the retention was better with FD.

The statistical analysis concluded that there were no significant differences between FD and DIC dehydration. General Discriminant Analysis (GDA) was performed to separate samples according to the drying method. Forward stepwise analysis led to the selection of two parameters: a* and b*. With the selected parameters, 95.8% of the total samples were correctly classified per the drying method (Fig. 4). Only one of the DIC samples was misplaced with the FD samples. Overall they can be perfectly classified into two separated groups. Only color parameters could discriminate between FD and DIC samples. In conclusion, both methods are suitable for preservation of the fruit and its bioactive compounds as well as the antioxidant capacity of the berrycacti.

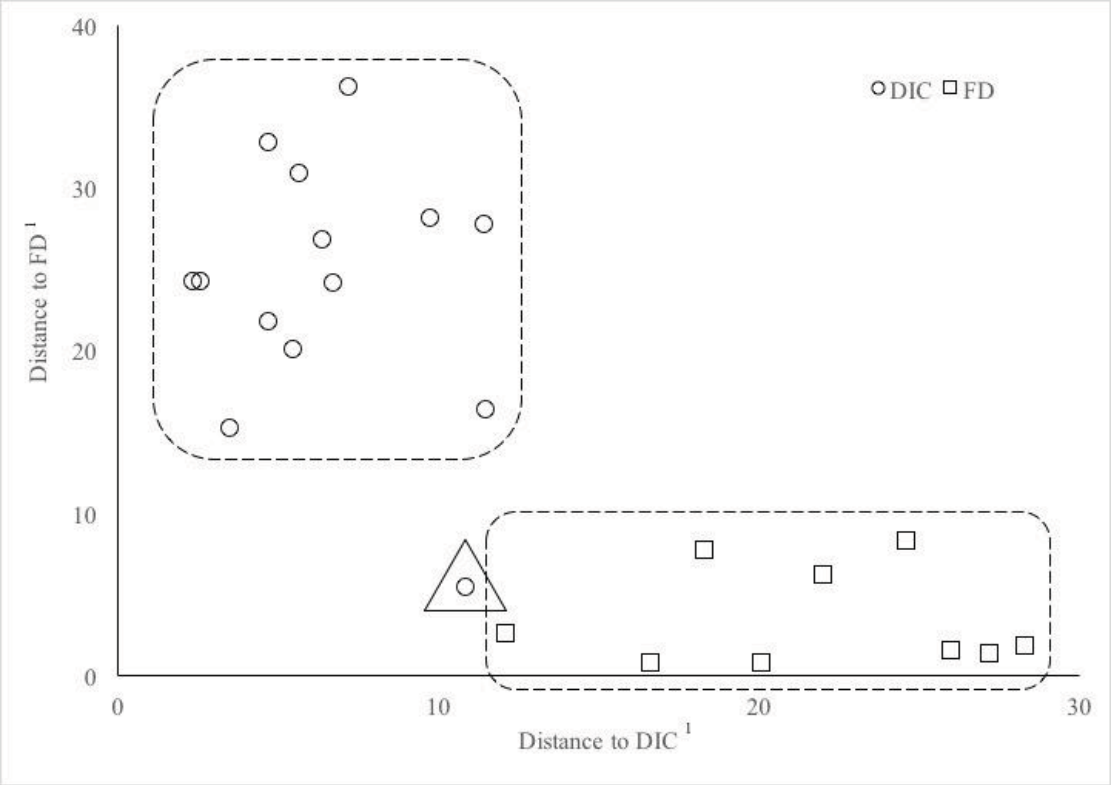


Figure 4. Cooman´s graph for dehydration techniques applied to berrycactus

[1]FD: Freeze-drying. DIC: Instant Controlled Pressure Drop.

The graph illustrates the Mahalanobis distances between FD and DIC, showing that the dehydration techniques applied to berrycacti can be differentiated among them and samples could potentially (95.8%) be classified correctly according to the drying method applied.

4. Conclusions

Based on the DPPH antioxidant capacity and betalain content, the ripe maturation stage was determined as the optimal stage for performing the comparative drying methods on berrycacti.

The comparative analysis of the two drying methods showed only minimal differences in the proximal analysis, color parameters and bioactive compounds content of the berrycacti. Both drying methods demonstrated high antioxidant activities regardless of the methodologies used for its in-vitro determination. Overall, FD and DIC were efficient drying methods for the berrycacti due to good quality attributes of the final products. Moreover, the preservation of the antioxidant properties and the retention of bioactive compounds such as soluble phenols, NEPP, tannins, betacyanins, betaxanthins and ascorbic acid was achieved. These compounds were present after drying treatments possibly aiding as beneficial health promoters in the prevention of chronic diseases such as hypoglycemia. Areas for future research may include the evaluation of individual bioactive compounds in berrycacti during the drying process as well as clinical trials using the dried berrycacti impact on human health.

Acknowledgments

Mrs. Priscila Santiago-Mora is grateful for the financial support of Consejo Nacional de Ciencia y Tecnología (CONACYT) through doctoral scholarship 269441.

The authors thank Dr. Klaus Tenbergen, Dr. Joseph M. Ryan and Michelle Teschky for the review of this manuscript.

References

Alonzo-Macías, M., Cardador-Martínez, A., Mounir, S., Montejano-Gaitán, G., & Allaf, K. (2013). Comparative Study of the Effects of Drying Methods on Antioxidant Activity of Dried Strawberry (*Fragaria* var. Camarosa). *Journal of Food Research, 2*(2), 92. https://doi.org/10.5539/jfr.v2n2p92

AOAC. (2012). *Official Methods of Analysis* (G. W. Latimer Ed. 19th edition ed.). Washington, D.C.: Association of Official Analytical Chemists

Arias, S. (2010). Diversidad de cactáceas y su aprovechamiento en México. México, D.F.: Instituto de Biología, Jardín Botánico UNAM.

Barbosa-Cánovas, G. V., & Vega-Mercado, H. (2000). *Deshidratación de Alimentos* (A. Ibarz Ribas, Trans. E. Acribia Ed.). Zaragoza, España: Aspen Publishers.

Benzie, I. F., & Strain, J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. *Analytical Biochemistry, 239*(1), 70-76. https://doi.org/10.1006/abio.1996.0292

Butera, D., Tesoriere, L., Di Gaudio, F., Bongiorno, A., Allegra, M., Pintaudi, A. M., Kohen, R., & Livrea, M. A. (2002). Antioxidant Activities of Sicilian Prickly Pear (*Opuntia ficus indica*) Fruit Extracts and Reducing Properties of its Betalains: Betanin and Indicaxanthin. *Journal of Agricultural and Food Chemistry, 50*(23), 6895-6901. https://doi.org/10.1021/jf025696p

Cai, Y., & Corke, H. (1999). Amaranthus Betacyanin Pigments Applied in Model Food Systems. *Journal of Food Science, 64*(5), 869-873. https://doi.org/10.1111/j.1365-2621.1999.tb15930.x

Cai, Y., Sun, M., & Corke, H. (2003). Antioxidant Activity of Betalains from Plants of the Amaranthaceae. *Journal of Agricultural and Food Chemistry, 51*(8), 2288-2294. https://doi.org/10.1021/jf030045u

Castellar, R., Obón, J. M., Alacid, M., & Fernández-López, J. A. (2003). Color Properties and Stability of Betacyanins from *Opuntia* Fruits. *Journal of Agricultural and Food Chemistry, 51*(9), 2772-2776. https://doi.org/10.1021/jf021045h

Coria Cayupán, Y. S., Ochoa, M. J., & Nazareno, M. A. (2011). Health-promoting substances and antioxidant properties of *Opuntia sp.* fruits. Changes in bioactive-compound contents during ripening process. *Food Chemistry, 126*(2), 514-519. https://doi.org/10.1016/j.foodchem.2010.11.033

Delgado-Vargas, F., Jiménez, A., & Paredes-López, O. (2000). Natural Pigments: Carotenoids, Anthocyanins, and Betalains - Characteristics, Biosynthesis, Processing, and Stability. *Critical Reviews in Food Science and Nutrition, 40*(3), 173-289. https://doi.org/10.1080/10408690091189257

Deshpande, S. S., Cheryan, M., Salunkhe, D., & Luh, B. S. (1986). Tannin analysis of food products. *Critical Reviews in Food Science and Nutrition, 24*(4), 401-449. https://doi.org/10.1080/10408398609527441

Fukumoto, L., & Mazza, G. (2000). Assessing Antioxidant and Prooxidant Activities of Phenolic Compounds. *Journal of Agricultural and Food Chemistry, 48*(8), 3597-3604. https://doi.org/10.1021/jf000220w

Gokhale, S., & Lele, S. (2014). Betalain Content and Antioxidant Activity of *Beta vulgaris*: Effect of Hot Air Convective Drying and Storage. *Journal of Food Processing and Preservation, 38*(1), 585-590. https://doi.org/10.1111/jfpp.12006

Guzmán-Maldonado, S. H., Villordo, E., González-Chavira, M., Pons-Hernández, J. L., & Hernández-López, D. (2012). Estudio preliminar de la diversidad genética del género *Myrtillocactus* en el Estado de Guanajuato *La Biodiversidad en Guanajuato: Estudio de Estado* (Vol. II, pp. 524). México: Comisión Nacional para el Conocimiento y Uso de la Biodiversidad/ Instituto de Ecología del Estado de Guanajuato.

Haddad, M. A., Mounir, S., Sobolik, V., & Allaf, K. (2008). Fruits & Vegetables Drying Combining Hot Air, DIC Technology and Microwaves. *International Journal of Food Engineering, 4*(6). https://doi.org/10.2202/1556-3758.1491

Hernández-López, D., Vaillant, F., Reynoso-Camacho, R., & Guzman-Maldonado, S.-H. (2008). *Myrtillocactus* (*cactaceae*): botanical, agronomic, physicochemical and chemical characteristics of fruits. *Fruits, 63*(05), 269-276. https://doi.org/10.1051/fruits:2008042

Hernández, M., & Sariñana, A. (2007). *Diseño Integral de Máquina General de Ensayos de Materiales.* . Paper presented at the 5^0 Congreso Internacional Sobre Investigación y Desarrollo Tecnológico (CIINDET),

Cuernavaca, Morelos, Mexico.

Herrera-Hernández, M. G., Guevara-Lara, F., Reynoso-Camacho, R., & Guzmán-Maldonado, S. H. (2011). Effects of maturity stage and storage on cactus berry (*Myrtillocactus geometrizans*) phenolics, vitamin C, betalains and their antioxidant properties. *Food Chemistry, 129*(4), 1744-1750. https://doi.org/10.1016/j.foodchem.2011.06.042

Larrauri, J. A., Rupérez, P., Borroto, B., & Saura-Calixto, F. (1996). Mango Peels as a New Tropical Fibre: Preparation and Characterization. *LWT-Food Science and Technology, 29*(8), 729-733. https://doi.org/10.1006/fstl.1996.0113

Liaotrakoon, W., De Clercq, N., Lewille, B., & Dewettinck, K. (2012). Physicochemical properties, glass transition state diagram and colour stability of pulp and peel of two dragon fruit varieties (*Hylocereus* spp.) as affected by freeze-drying. *International Food Research Journal, 19*(2), 743-750.

Mounir, S., Allaf, T., Berka, B., Hassani, A., & Allaf, K. (2014). Instant Controlled Pressure Drop technology: From a new fundamental approach of instantaneous transitory thermodynamics to large industrial applications on high performance–high controlled quality unit operations. *Comptes Rendus Chimie, 17*(3), 261-267. https://doi.org/10.1016/j.crci.2013.10.019

Nilsson, T. (1970). Studies into the Pigments in Beetroot (*Beta vulgaris L. ssp. vulgaris var. rubra L.*). *Lantbrukshogskolans Annaler, 36*, 179-219.

Pavlov, A., Kovatcheva, P., Georgiev, V., Koleva, I., & Ilieva, M. (2002). Biosynthesis and Radical Scavenging Activity of Betalains during the Cultivation of Red Beet (*Beta vulgaris*) Hairy Root Cultures. *Zeitschrift für Naturforschung C, 57*(7-8), 640-644. https://doi.org/10.1515/znc-2002-7-816

Perez-Gonzalez, S. (1995, July 30- August 3, 1995). *Agroecological study and determination of yield potential of garambullo (Myrtillocactus geometrizans) in Queretaro, Mexico*. Paper presented at the Annual Meeting of the American Society for Horticultural Science, Montréal, Québec, Canada.

Pérez-Jiménez, J., & Torres, J. L. s. (2011). Analysis of Nonextractable Phenolic Compounds in Foods: The Current State of the Art. *Journal of Agricultural and Food Chemistry, 59*(24), 12713-12724. https://doi.org/10.1021/jf203372w

Prosky, L., Asp, N.-G., Schweizer, T., DeVries, J., & Furda, I. (1988). Determination of Insoluble, Soluble, and Total Dietary Fiber in Foods and Food Products: Interlaboratory Study. *Journal of AOAC International, 71*(5), 1017-1023.

Ratti, C. (2009). Novel Food Dryers and Future Perspectives. In C. Ratti (Ed.), *Advances in Food Dehydration* (pp. 447-462). USA: CRC Press.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. *Free Radical Biology and Medicine, 26*(9), 1231-1237. https://doi.org/10.1016/S0891-5849(98)00315-3

Reynoso-Camacho, R., Martinez-Samayoa, P., Ramos-Gomez, M., Guzmán, H., & Salgado, L. M. (2015). Antidiabetic and Renal Protective Properties of Berrycactus Fruit (*Myrtillocactus geometrizans*). *Journal of Medicinal Food, 00*(0), 1-7. https://doi.org/10.1089/jmf.2013.0164

Saura-Calixto, F. (1998). Antioxidant Dietary Fiber Product: A New Concept and a Potential Food Ingredient. *Journal of Agricultural and Food Chemistry, 46*(10), 4303-4306. https://doi.org/10.1021/jf9803841

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent. *Methods in Enzymology, 299*, 152-178. https://doi.org/10.1016/S0076-6879(99)99017-1

St. George, S. D., & Cenkowski, S. (2009). Dehydration Processes for Nutraceuticals and Functional Foods. In C. Ratti (Ed.), *Advances in Food Dehydration* (pp. 285-314). USA: CRC Press.

Tavares, L., Carrilho, D., Tyagi, M., Barata, D., Serra, A. T., Duarte, C. M. M., Duarte, R. O., Feliciano, R. P., Bronze, M. R., & Chicau, P. (2010). Antioxidant Capacity of Macaronesian Traditional Medicinal Plants. *Molecules, 15*(4), 2576-2592. https://doi.org/10.3390/molecules15042576

Tesoriere, L., Butera, D., Pintaudi, A. M., Allegra, M., & Livrea, M. A. (2004). Supplementation with cactus pear (*Opuntia ficus-indica*) fruit decreases oxidative stress in healthy humans: a comparative study with vitamin C. *The American Journal of Clinical Nutrition, 80*(2), 391-395.

Viloria-Matos, A., Corbelli-Moreno, D., Moreno-Álvarez, M., & Belén, D. (2002). Estabilidad de betalaínas en pulpa de tuna (*Opuntia boldinghii* Br. et R.) sometidas a un proceso de liofilización. *Revista de la Facultad de Agronomía de la Universidad de Zulia*, 324-331.

Wu, L., Hsu, H., Chen, Y., Chiu, C., Lin, Y., & Ho, J. (2006). Antioxidant and antiproliferative activities of red pitaya. *Food Chemistry, 95*(2), 319-327. https://doi.org/10.1016/j.foodchem.2005.01.002

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

A Simple Gold Nanoprobe Assay for the Identification of Staphylococcus Aureus, Listeria Monocytogenes and Salmonella Enteritidis in Food Specimens

Dimitra Panagiotis Houhoula[1], Ekatherina Charvalos[2], Spyros Konteles[1], Stamatis Koussissis[1], Vladimiros Lougovois[1] & Joseph Papaparaskevas[3]

[1]Department of Food Technology, Technological Educational Institution of Athens Agiou Spyridonos St, 12210, Aegaleo, Greece

[2]2Central Labs, Iaso Hospital, 12152, Greece

[3]Department of Microbiology, Medical School, National and Kapodistrian University of Athens, 11527, Athens, Greece

Correspondence: Dimitra Panagiotis Houhoula, Department of Food Technology, Technological Educational Institution of Athens Agiou Spyridonos St, 12210, Aegaleo, Greece. E-mail: itsahouhoula@gmail.com

Received: May 2, 2017 Accepted: June 13, 2017 Online Published: July 2, 2017

doi:10.5539/jfr.v6n4p134 URL: https://doi.org/10.5539/jfr.v6n4p134

Abstract

In the present study, we developed a gold nanoprobe assay, which relies on the colorimetric differentiation of specific DNA sequences, based on different aggregation profiles. We evaluated the assay on DNA extracted from pathogen cultures and from contaminated food specimens. The targets used were the *fem*A gene for the identification of *Staphylococcus aureus*, the *hly* gene of the *Listeria monocytogenes* listeriolysin and the *invA* sequence for *Salmonella* spp. Comparison was performed with the reference assay, as described in the respective ISO guidelines for each pathogen, and a direct PCR amplification and detection. The minimum detection limit of the assay was defined at 123 fg/μL DNA, for all species, lower than PCR detection. Specificity was 100%. Sensitivity was 100%, as compared το the reference method, whereas the sensitivity of PCR was 93.3%. The evaluated assay could be used as a sensitive screening method, which does not require major infrastructure, for the detection and identification of pathogens in food specimens. In addition, it can accommodate detection of multiple species, thus allowing multiplexing and expedite turnaround time.

Keywords: gold nanoparticles, foodborne pathogens, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* spp.

1. Introduction

Food borne bacterial infections are a continuing challenge to human health world-wide. Over the past two decades, changes of the epidemiology of food borne infections have been reported as a consequence of changes in the social environment and the ability of pathogens to adapt to new niches (Newell et al., 2010). In that respect, the continuous monitoring and surveillance of the pathogens is vital for food safety, clinical diagnosis and therapeutic decisions, and prevention strategies. To date, the existing technology for the detection of food-borne pathogens only meets the criteria of speed, sensitivity, and specificity when it uses large, laboratory-based, sophisticated and costly equipment (Hoorfar, 2011) Therefore, the development of a simplified, cost–effective and accurate procedure for detection of trace amounts of bacterial pathogens, which does not require complex instruments, seems to be imperious in order to overcome the time delay and allow rapid and sensitive detection (McGrath, Elliot, & Fodey, 2012) in infrastructure deprived environments. Nanotechnology, and more specifically gold nanoparticle chemistry, provides opportunities for the rapid and simple diagnosis of many infectious diseases, being able to detect trace amounts of bacteria, due to their uniqueoptical properties (Agasti et al., 2010; Syed & Bokhari, 2011). In the present study we developed and evaluated a gold nanoprobe assay, which does not require the amplification of the target DNA, but relies on the hybridization of the pathogen's DNA with gold nanoprobes with subsequent colorimetric differentiation. Subsequently, we compared the assay for sensitivity, specificity, user-friendliness, and infrastructure-independency, to the reference ISO detection method of *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* spp. in food samples, and

furthermore to PCR detection and identification of these pathogens.

2. Method

2.1 Food Specimens

During the study period, a total of 60 food specimens from the food industry were collected. All samples were positive by a reference microbiological conventional culture (ISO 6573:2002 for *Salmonella* spp., ISO 6888-1:1999 for *S. aureus,* and ISO 11290-1:1996 for *L. monocytogenes*) assay for at least one of these pathogens causing food-borne infections.

2.2 Gold Nanoparticle Preparation

Gold nanoparticles (AuNPs) size 20 nm, were purchased commercially (BBI Solutions, Cardiff, UK). The following oligonucleotides pairs (OLG) were used: (a) 5'-CTT-ACT-TAC-TGG-CTG-TAC-CTG-3' and 5'-ATG-TCG-CTT-GTT-ATG-TGC-3', corresponding to a 686 bp fragment of the *fem*A gene of *S. aureus*, (b) 5'-CAT-TAG-TGG-AAA-GAT-GGA-ATG-3' and 5'-GTA-TCC-TCC-AGA-GTG-ATC-GA-3', corresponding to a 732 bp fragment of the *hly* gene encoding the pore-forming listeriolysin of *L. monocytogenes* and (c) 5′-TAT-CGT-ACT-GGC-GAT-ATT-GGT-GTT-TA-3′ and 5′-GGA-CAA-ATC-CAT-ACC-ATG-GCG-AGT-CA-3′, corresponding to a 540 bp fragment of the *inv*A sequence of *Salmonella* spp. (Vannuffel et al., 1995; Gouws & Liedeman, 2005; Vasquez-Novel et al., 2005). All OLG were thiolated (modified with 10xdATP) in the 5'-end of the primer. The AuNP merging with the OLG was performed by adding 1 ml of an aqueous solution of AuNPs to 4 nmol of the thiolated OLG pair (forward and reverse primer) using a previously described protocol (Hill & Mirkin, 2006). Briefly, each pair of thiol modified OLG was incubated with the AuNPs at room temperature, overnight. The solution was then brought to phosphate buffer 9 mM, pH 7, and subsequently a 0.1% (w/v) solution of Sodium Dodecyl Sulfate (SDS - Sigma-Aldrich, Life Science Chemilab S.A., Greece) was added. The total salting buffer, 2 M NaCl in 10 mM Phosphate-buffered Saline (PBS - Sigma-Aldrich, Life Science Chemilab S.A., Greece) was divided in six doses, and was added over the next 48 hours. After centrifugation, the precipitate was washed with 500 μl of 10 mM PBS (Sigma-Aldrich, Life Science Chemilab S.A., Greece) pH 7.4, 150 mM NaCl, 0.1% SDS (Sigma-Aldrich, Life Science Chemilab S.A., Greece), and it was re-centrifuged and re-suspended in 500 ml of the same buffer. In that respect, three different AuNP-OLG solutions were prepared, corresponding to each one of the three pathogens, and were stored at room temperature (Hill & Mirkin, 2006).

2.3 DNA Extraction from the Isolates

DNA extraction was performed from a 24h culture of the strains *S. aureus* ATCC 29213, *S. aureus* ATCC 25923, *L. monocytogenes* NCTC 10357 and a clinical *S. enteritis* strain isolated from a patient with gastrointestinal infection (fully characterized at the National Salmonella Reference Centre), using the NucleoSpin Tissue kit (Macherey-Nagel, GmbH and Co. Germany) according to the manufacturer's instructions, with the addition of a Proteinase K overnight incubation step at 65^{o}C. The extracted DNA was designated positive control and was quantified using a spectrophotometer at 260 nm and subsequently serial ten-fold dilutions were prepared in order to evaluate the analytical sensitivity of the PCR method. Extracted DNA (as described previously) from a 24h culture of *Escherichia coli* ATCC 25922, and a clinical *Yersinia enterocolitica* strain (designated specificity controls), was used for specificity confirmation. Extraction was additionally performed using an equivalent volume of 10 mM PBS solution (designated negative extraction control).

2.4 DNA Extraction from the Food Specimens

The food specimens were subjected to DNA extraction using the NucleoSpin Food kit (Macherey-Nagel, GmbH & Co. Germany) according to the manufacturer's instructions, with the addition of a Proteinase K overnight incubation step at 65ºC, followed by DNA quantification, as described in the previous section.

2.5 PCR Amplification

PCR was performed according to previously published protocols (Vannuffel et al., 1995; Gouws & Liedeman, 2005; Vasquez-Novel et al., 2005) in 50 μl final volume, using the GoTaq Hot Start Master Mix (Promega Gmbh, 68199, Germany), 1mM each of the primers and 10 μl of eluted DNA. PCR products were separated in a 2% agarose gel, stained with ethidium bromide (0.5 μg/ml) and documented under UV illumination.

2.6 Hybridization and Colour Detection

Hybridization on the extracted DNA from the isolates with the AuNP-OLG solution was performed by adding 10 μL of eluted DNA to 10 μl of the solution, followed by two incubation steps, five minutes at 95ºC, and five minutes at 55ºC for *S. aureus*, *L. monocytogenes* and 60ºC for *Salmonella* spp. The presence of complementary

DNA prevents aggregation with the addition of 2 μl HCl 0.01 N after 5 min of incubation at room temperature (the solution remains pink), whereas in the opposite event (no presence of complementary DNA) the color turns into purple. Colour detection was performed visually and was confirmed with an absorption spectrum using Ultraviolet–visible spectroscopy (UV-vis spectrum - Epoch Spectrophotometer, BioTek, 74177, Germany). The solutions were kept for 5–15 min at room temperature until colour was developed and then they were photographed. All experiments were performed in triplicate.

2.7 Direct Application on Food Samples

After optimization using DNA extracted from the isolate cultures, as described in the previous paragraphs, the assay was applied on DNA extracted from food samples, which were found to be contaminated with pathogens causing food-borne infections. More specifically, 15 μl of the extracted DNA from the food was added to 10 μl of the AuNP-OLG solution, as described above. The colour change was evaluated visually and confirmed with an absorption spectrum. The results were compared to those obtained by PCR assays. In order to assess the repeatability of the method for the specific type of samples, testing with the proposed assay was repeated five times for each DNA extract.

3. Results and Discussion

Spherical gold nanoparticles in the size range of 13–20 nm with absorbance peak around 520 nm have been employed in biosensors due to their ease of synthesis. The AuNP-OLG solution exhibits a pink colour because of surface plasmon resonance at an absorbance peak of ~525 nm. The addition of HCl enhances the aggregation of AuNP-OLG because of the absence of the specific target of DNA, leading to a change of colour from pink to purple. The changes in colour can be visually detected but also confirmed by aUV-vis spectrum (Willets & Van Duyne, 2007; Verma, Rogowski, Jones, Frank, & Gu, 2015). We used this approach and designed an assay that was evaluated in a two-step process, (a) on pure cultures of isolated bacteria (positive controls) and subsequently (b) on food specimens that were found by a reference method to be contaminatedby pathogens causing food-borne infections.

Regarding the pure culture evaluation (first step), the assay produced positive results with all three pathogens tested (*S. aureus*, *L. monocytogenes*, and *S. enteritidis*), and the sensitivity was 100%. We tested our assay in comparison to PCR using serial 10-fold dilutions of the culture positive control DNA, starting from C_1=12.3 ng/μL. The lower detection limit (LOD) of our assay was defined at 123 fg/μL of DNA, whereas the respective LOD of the PCR was 1.23 pg/μL, thus indicating that the AuNP-OLG assay performed better than the PCR in the range of one 10-fold dilution.

UV-Vis spectroscopic data for samples (Figure 1) supported the hypothesis of aggregation-induced visual discrimination of the sample and showed an absorbance peak at 520 nm, which was due to the collective excitation of the free conduction band electrons of the dispersed particles, known as the surface plasmon resonance. In contrast, the negative sample's wider absorbance spectrum is indicative of peak shift towards longer wavelength, due to the coupling in the surface plasmons of the particles in the aggregates (Figure 2). Negative results were produced after testing the negative control bacteria *E. coli* and *Y. enterocolitica* (Figure 2). The collective plasmon peak was intensified and appeared in a new position between 560 and 800 nm, depending on the degree of aggregation and concentration of AuNPs. The method was repeatable and produced the same results all five times it was performed. The colour density was concentration depended, and final turnaround time was less than 30 minutes.

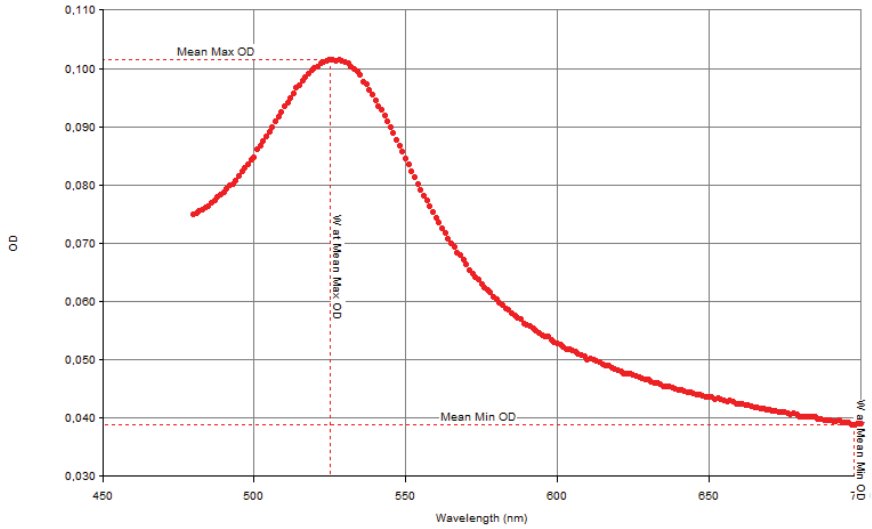


Figure 1. Representative spectrum from the positive sample containing *L. monocytogenes* genomic DNA, showing characteristic absorbance peak at 520 nm

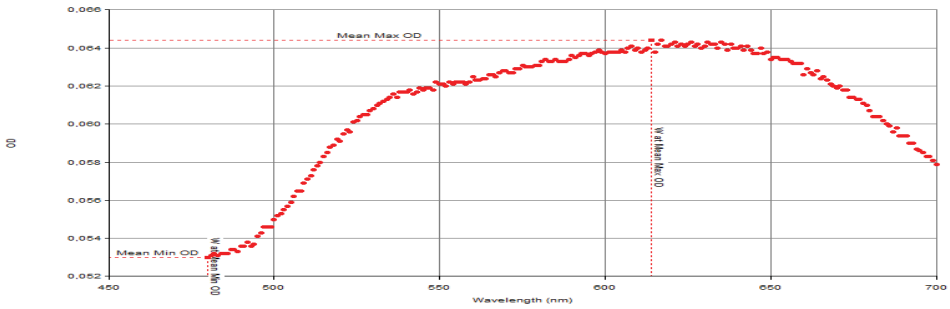


Figure 2.Representative spectrum from a negative sample exhibiting wide length of absorbance and a peak beyond 600nm

There have been only a few studies focusing on direct detection of bacterial food-borne pathogens using gold nanoprobes that compare different detection methods. A previously described nanoprobe-based study indicated comparable results to those presented here; thus, size 23 nm gold nanoparticles were able to produce a detection limit of one log lower than the PCR targeting the *stn* gene of *Salmonella* spp. (Hitchins, Jinneman, & Chen, 1998), a difference that was similar to the one observed in our study. In addition, there have been reports about the detection of *L. monocytogenes* and *S. enterica* in samples of food and/or beverage by targeting the *hly* and *hut* genes, respectively, and in contaminated milk samples using 13 nm gold nanoparticles (Fu, Zhou, & Xing, 2013), with similar sensitivity as in the present study. Also, the AuNP-OLG method indicated that it could detect mycobacteria in a reliable and highly specific manner (Liandris et al., 2009; Hussain, Samir, & Azzazy, 2013), and no crossreactions with related bacteria were observed, as demonstrated effectively after application on faecal samples harbouring mixed bacterial populations. Finally, very recently, gold nanoparticles were combined with ligase-based amplification, resulting in highly specific detection of food-borne pathogens (Wang, Ying, Wei, & Yuan, 2017), although this approach required additional steps and infrastructure (ligase-based amplification and micro-array detection), whereas the present one is infrastructure-independent.

It should be noted however, that the evaluation in most of these studies was performed mainly on culture isolates. In contrast, our assay was additionally evaluated on 60 food specimens (second step of evaluation). These specimens were identified by a reference microbiological assay to be contaminated with *Salmonella* spp., *S. aureus*, or *L. monocytogenes*. All these specimens were found to be contaminated with a single pathogen each; more specifically, 15 meat products were contaminated with *Salmonella* spp., 35 milk products were

contaminated with *S. aureus*, and finally five cheese pies and five ready-made salads were contaminated with *L. monocytogenes*.

All 60 food specimens were positive by the AuNP-OLG assay method and no discrepancies with the reference method were detected (100% sensitivity). In contrast, discrepant results were obtained by the PCR assay, which was used as a comparison to the nano-probe assay; two dairy products were negative for *S. aureus* and two meat specimens were negative for *Salmonella* spp. In that respect, PCR did not detect a total of four specimens (6.7%), resulting in 93.3% sensitivity. All positive food samples produced the assay's characteristic absorbance peak at 520 nm, with gradual decrease in absorbance relative to the concentration of genomic DNA present in the test sample, whilst the negative ones exhibited the red shift in their spectra, with the absorbance peak being shifted to longer wavelength (≥600 nm), coupled by a peak decrease in 520 nm. An interesting feature of this assay is that the positive samples, when kept overnight at room temperature, continued to retain their colour (data not shown), suggesting the stability of the AuNP-OLG probe hybridization with the DNA sequence, a particularly useful feature for prolonged read-out capability.

One limitation of the present method is that it cannot quantify the bacterial load, whereas culture, or Real-Time PCR using the TaqMan chemistry, can do. Nevertheless, culture has a long turnaround time, and RT-PCR requires expensive instruments and infrastructure. In contrast, UV-vis spectroscopy is usually available in laboratories and can further confirm the visually identified results of the nanoparticles (Jung, Jung, Parab, Li, & Park, 2010). It should be noted also, that the platform can accommodate detection of many bacterial species simultaneously, allowing multiplexing, high throughput screening and expedite turnaround time of clinical, veterinary and food specimens.

In conclusion, the present study evaluated a highly sensitive and specific assay, on pure culture isolates but also on contaminated food samples, and indicated similar performance to the reference method and better performance than PCR amplification, without the need of using complex instruments and infrastructure.

Acknowledgments

The present work was supported by the Special Account for Research Grants of the Technological Educational Institution (TEI) of Athens (Project No. 80251/2015).

References

Agasti, S. S., Rana, S., Park, M-H., Kim, C. K., You, C-C., & Rotello, V. M. (2010). Nanoparticles for detection and diagnosis. *Advanced Drug Delivery Reviews, 62*(3), 316-328. https://doi.org/10.1016/j.addr.2009.11.004

Fu, Z., Zhou, X., & Xing, D. (2013). Rapid colorimetric gene-sensing of food pathogenic bacteria using biomodification-free gold nanoparticle. *Sensors and Actuators B: Chemical*, *182*, 633-641. https://doi.org/10.1016/j.snb.2013.03.033

Gouws, P. A., & Liedemann, I. (2005). Evaluation of diagnostic PCR for the detection of *Listeria monocytogenes* in food products. *Food Technology and Biotechnology, 43*, 201-205.

Hill, H. D., & Mirkin, C. A. (2006). The bio-barcode assay for the detection of protein and nucleic acid targets using DTT-induced ligand exchange. *Nature Protocols*, *1*(1), 324-336. http://dx.doi.org/10.1038/nprot.2006.51

Hitchins, A. D., Jinneman, K., & Chen, Y. (1998). Detection of *Listeria monocytogenes* in Foods an Environmental Samples, and Enumeration of *Listeria monocytogenes* in Foods. Bacteriological Analytical Manual, 8th Edition, Revision A. U.S. Food and Drug Administration. https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm

Hoorfar, J. (2011). Rapid detection, characterization, and enumeration of foodborne pathogens. *APMIS, 119*(Supplement s 133), 1-24. https://doi.org/10.1111/j.1600-0463.2011.02767.x

Hussain, M. M., Samir, T. M., & Azzazy, H. M. E. (2013) Unmodified gold nanoparticles for direct and rapid detection of *Mycobacterium tuberculosis* complex. *Clinical Biochemistry, 46*(7-8), 633-637. https://doi.org/10.1016/j.clinbiochem.2012.12.020

Jung, Y. L., Jung, C., Parab, H., Li, T., & Park, H. G. (2010). Direct colorimetric diagnosis of pathogen infections by utilizing thiol-labeled PCR primers and unmodified gold nanoparticles. *Biosensors and Bioelectronics, 25*(8), 1941-1946. https://doi.org/10.1016/j.bios.2010.01.010

Liandris, E., Gazouli, M., Andreadou, M., Čomor, M., Abazovic, N., Sechi, L. A., & Ikonomopoulos, J. (2009).

Direct detection of unamplified DNA from pathogenic mycobacteria using DNA-derivatized gold nanoparticles. *Journal of Microbiological Methods, 78*(3), 260-264. https://doi.org/10.1016/j.mimet.2009.06.009

McGrath, T. F, Elliott, C. T, & Fodey, T. L. (2012). Biosensors for the analysis of microbiological and chemical contaminants in food. *Analytical and Bioanalytical Chemistry, 403*(1), 75-92. https://doi.org/10.1007/s00216-011-5685-9

Newell, D. G., Koopmans, M., Verhoef, L., Duizer, E., Aidara-Kane, A., Sprong, H., ... Kruse, H. (2010). Food-borne diseases – The challenges of 20 years ago still persist while new ones continue to emerge. *International Journal of Food Microbiology, 139*(S3-S15). https://doi.org/10.1016/j.ijfoodmicro.2010.01.021

Syed, M. A., & Bokhari, S. H. A. (2011). Gold Nanoparticle Based Microbial Detection and Identification. *Journal of Biomedical Nanotechnology*, *7*(2), 229-237. https://doi.org/10.1166/jbn.2011.1281

Vannuffel, P., Gigi, J., Ezzedine, H., Vandercam, B., Delmee, M., Wauters, G., & Gala, J. L. (1995). Specific detection of methicillin resistant *Staphylococcus* species by multiplex PCR. *Journal of Clinical Microbiology*, *33*(11), 2864-2867.

Vasquez-Novel, M. D., Pazos, A. J., Abad, M., Sanchez, J. L., & Perez-Paralle, M. L. (2005). Eight-hour PCR-based procedure for the detection of *Salmonella* in raw oysters. *FEMS Microbiology Letters, 243*(1), 279-283. https://doi.org/10.1016/j.femsle.2004.12.016

Verma, M. S., Rogowski, J.L., Jones, L., Frank, X., & Gu, F. X. (2015). Colorimetric biosensing of pathogens using gold nanoparticles. *Biotechnology Advances, 33*(6), 666-680. https://doi.org/10.1016/j.biotechadv.2015.03.003

Wang, X., Ying, S., Wei, X., & Yuan, J. (2017). Development of a gold nanoparticle-based universal oligonucleotide microarray for multiplex and low-cost detection of foodborne pathogens. *International Journal of Food Microbiology, 253*, 66-74. https://doi.org/10.1016/j.ijfoodmicro.2017.05.005.

Willets, K. A., & Van Duyne, R. P. (2007). Localized surface plasmon resonance spectroscopy and sensing. *Annual Review of Physical Chemistry, 58*, 267-97. https://doi.org/10.1146/annurev.physchem.58.032806.104607

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Effects of Palm Oil Consumption on Lipid Profile among Rural Ivorian Youth

Ake Ake Alexandre[1, 2], Monde Absalome[2], Edjeme-Ake Angèle[3, 4], Bahi Gnogbo Alexis[4], Djaman Allico Joseph[4] & Yapo Angoué Paul[1]

[1]Laboratory of Animal Physiology, Pharmacology and Pharmacopoeia, Department of Natural Sciences, Nangui Abrogoua University, Abidjan, Côte d'Ivoire

[2]Laboratory of Medical Biochemistry, Department of Medical Sciences, University of Félix Houphouet Boigny, Abidjan, Côte d'Ivoire

[3]Laboratory of Biochemistry, Department of Pharmaceutical and biological sciences, University of Félix Houphouet Boigny, Abidjan, Côte d'Ivoire

[4]Laboratory of Medical and Fundamental Biochemistry, Institut Pasteur, Abidjan, Côte d'Ivoire

Correspondence: Monde Absalome, Laboratory of Medical Biochemistry, Department of Medical Sciences, University of Félix Houphouet Boigny, Abidjan, Côte d'Ivoire. Tel: 225-0573-7067. E-mail: monde_abs@yahoo.fr

Received: May 31, 2017 Accepted: June 25, 2017 Online Published: July 11, 2017

doi:10.5539/jfr.v6n4p140 URL: https://doi.org/10.5539/jfr.v6n4p140

Abstract

As palm oil has been qualified as atherogen, we have studied the impact of its consumption on changes of lipid and lipoprotein profiles of young Ivorian healthy subjects living in rural areas. It is a descriptive cross-sectional analytical study of about 120 Ivorian subjects aged 18 to 30 years, including 65 regular consumers of palm oil and 55 subjects consuming that oil periodically as control subjects. Serum concentrations of triglycerides, total cholesterol, HDL, LDL cholesterols and lipoprotein (a) were measured by enzyme conventional methods. The TC serum varied not significantly in both subjects' groups as the triglycerides and HDL-C did. In addition, 58.46% of palm oil consumers had hypoLDLemia. The serum concentration of lipoprotein (a) was not significantly elevated ($p > 0.05$) with consumers compared to controls: 33.85% versus 29.09%, $p = 0.55$. The percentage of subjects with normal serum concentrations is higher in all the studied parameters, with both that is the consumers and the controls, except LDL cholesterol, of which the percentage of subjects with a lower value is the highest (58.46% for consumers and 52.73% for controls). This study has shown that the consumption of palm oil did not alter the lipid and lipoprotein profile of the consumer, on the contrary, this consumption revealed a decrease in cholesterol levels with these subjects.

Keywords: HDL-Cholesterol, LDL-Cholesterol, palm oil, total cholesterol, triglycerides

1. Introduction

Palm oil, which is extracted from the *Elaeis guineensis* oil palm fruit, is the most widely consumed vegetable oil in the world since 2005 (Maatschappelijk Verantwoord Ondernemen [MVO], 2010). Apart from the phytonutrients called minors (vitamin E, carotenoids), it is composed of 50% unsaturated fatty acids and 50% saturated fatty acids, of which 44% palmitic acid (Edem, 2002), making it an oil Which is known to be pro-atherogenic and which would be responsible for cardiovascular complications (Aranceta & Perez-rodrigo, 2012; Assmann et al., 2014; Berger, 2014; Mancini et al., 2015). Almost 60% of all deaths in 2005 were related to these diseases, 80% of which occurred in developing countries (Kennedy, 2005; Organisation Mondiale pour la Santé [OMS], 2005). Several studies attribute the increased risk of cardiovascular disease to high serum cholesterol and its fractions (Keys, Anderson, & Grande, 1965; Kromhout et al., 1995; Odia, Ofori, & Maduka, 2015). This elevation of cholesterol seems to derive from edible fats and oils including palm oil, which is the most cited. Indeed, Tholstrup et al. (2011) reported that palm oil significantly increased LDL cholesterol ($p < 0.001$) with 32 subjects compared with olive oil (Tholstrup, Hjerpsted & Raff, 2011). However, this oil is common in African and Asian food habits, mainly in rural areas where it is used in raw form (Ong & Goh, 2002; Oluba & Oyeneke, 2009; Onyeali, Onwuchekwa, Monago & Monanu, 2010; Oyewole & Amosu, 2010). We

conducted a study with 2240 healthy subjects aged 18 years and more to verify the effect of palm oil consumption on anthropometric parameters (weight and height). It was found that the regular and normal consumption of palm oil by this population did not increase the weight of the subjects consuming it (Aké Aké et al., 2015). In addition, several studies have also shown the health benefits of palm oil consumption (Hornstra, 1988; Zhang, Ping, Chunrong, Shou, & Keyou, 1997; Sundram, Sambanthamurthi & Tan, 2003; Lecerf, 2013). Thus, Voon et al. (2011) found a non-significant difference ($p > 0.05$) between the impact of palm oil consumption and olive oil consumption on serum concentrations of LDL cholesterol (Voon, Ng, Lee, & Nesaretnam, 2011). In Côte d'Ivoire, we studied changes in lipid profile in farm chickens fed with different varieties of vegetable oils, including palm oil which had a cholesterol-lowering effect (Mondé et al., 2016). This study also indicated that consumption of this oil decreased serum levels of triglycerides and LDL cholesterol but increased HDL cholesterol. Another study carried out on 120 subjects suffering from ischemic heart disease reported no disturbance of lipid and lipoprotein parameters with these subjects who consumed palm oil after four weeks (Mondé et al., 2017). Several other works of our team on the benefits of palm oil have been carried out for the most part in hypertensive, obese, diabetic, cancerous subjects or suffering from ischemic cardiopathies (Djohan et al., 2010; Béké, 2015; Coulibaly, 2015; Léga, 2015; Yapo, 2015; Mondé et al., 2017), but not with healthy subjects. Knowing that some apparently healthy subjects have an apprehension about palm oil, we found it interesting to study the impact of palm oil consumption on healthy young people in rural areas in order to see whether there are or not lipid and lipoprotein anomalies related to this consumption.

2. Subjects, Material and Methodology

2.1 Subjects and Palm Oil Consumption Criteria

It is a descriptive cross-sectional and analytical study carried out with 120 apparently healthy young volunteers aged between 18 and 30 years with the same dietary habits apart from the palm oil quantity consumed. The recruitment of the study population was carried out following the results of a food survey carried out in Grand-Alepé, a village about 35 km north-east from Abidjan, the economic capital of Côte d'Ivoire. In this survey, we identified 535 young people aged 18 to 30 years as our target population. Among these 535 young people, we selected 120 subjects (22.43%) who accepted to participate to the study to whom we determined the lipid parameters. Regarding the oil consumption, the recommended intakes were calculated as previously described (Mondé et al, 2017). According to the nutritional recommendations, the average adult (total metabolism of 2,500 kcal) should consume about 100 grams of fat per day (i.e. 20% to 30% of the total daily intake). Indeed, 65 active young people consuming an average of 5 tablespoons of palm oil corresponding to 65 g per day and per subject during six weeks (Apports Nutritionnels de Référence [ANREF], 2006; Agence Nationale de Sécurité Sanitaire de l'alimentation [ANSES], 2016) and 55 others (controls) with an average palm oil consumption of 2 tablespoons meaning 25 g of palm oil per day and per subject, during the same period.

2.2 Material and Methodology

2.2.1 Food Survey

The previous food survey showed that palm oil was a major part of the diet in this rural population as described above.

2.2.2 Blood Sampling

In each subject, a blood sampling by venipuncture at the elbow crease was performed after 12 hours of fasting. The blood was collected in 5 ml (red cap) vacutainer tubes (*IMPROVE* ^{R}) and immediately sent to the laboratory of the Alepe General Hospital to obtain the serum after centrifugation at 3000 rpm for 5 min. The serum aliquots collected were stored by freezing (≤ -20 ° C) until the number of samples required for the study was reached. Once the required number of samples was reached, they were sent to the Laboratory of Medical and Fundamental Biochemistry of the Institute Pasteur of Côte d'Ivoire for analysis.

2.2.3 Anthropometry

The weight of all subjects studied was measured with an electronic scale (*SCALE* ^{R}). Measurement of the size was made using a tape measuring more than 2 m. The body mass index was calculated from the following formula:

$$\textbf{BMI (kg/m}^2\textbf{) = Subject mass (kg) / [Subject size (m)]}^2$$

The limits of the BMI used were according to the official classification accepted by the World Health Organization (OMS, 2003): 18.5-24.9 kg / m^2 for normal subjects, <18.5 Kg / m^2 for the undernourished and ≥ 25.0 kg / m^2 for overweight and obese subjects.

2.2.4 Determination of Biochemical Parameters

Serum concentrations of total cholesterol and triglycerides were determined by Trinder's enzyme colorimetric method (Abell, Levy & Kendall, 1958; Trinder, 1969). HDL cholesterol was assayed by direct method (Sugiuchi, 1995). LDL cholesterol was estimated using the Friedewald formula (Friedewald, Levy & Fredickson, 1972):

LDL Cholesterol (g / L) = Total Cholesterol - HDL Cholesterol - Triglycerides / 5

The lipoprotein (a) was determined by immuno-turbidimetry (Simó *et al.,* 2003). The atherogenicity index (AI) was calculated by the ratio:

IA = Total Cholesterol / HDL Cholesterol

The determination of the biochemical parameters was carried out on the Cobas C 311 automaton (*Roche* ^{R}).

2.2.5 Ethical Considerations

We obtained informed consent from all subjects who participated in the study.

2.2.6 Statistical Analysis

The results were expressed by means associated with their standard deviation. For the study parameters, the Student t-test and the Mann-Whitney non-parametric test were used to compare averages by using the Graph Pad Prism 6 software. The different proportions of the biochemical parameters observed were compared by the likelihood test G or test log Likehood ratio with the software R.2.0.1 version Windows. The significance threshold was defined for a value of p less than 0.05 (Ihaka & Gentleman, 1996; Statsoft, 2005).

3. Results

3.1 Anthropometric Parameters

The analysis of the anthropometric characteristics summarized in Table 1 indicates that the population studied generally has a normal BMI average which is 22.09 ± 3.68 kg / m^2 and also about age that is 23, 35 ± 4.74 years. The major consumers of palm oil weight average was higher but it was not statistically significant compared to controls: 61, 90 ± 10, 53 kg versus 59,55 ± 11,60 kg, p=0,163. The controls had an average value of BMI not significantly greater than the one of the major consumers: 22,16 ± 3,92 Kg/m^2 versus 22,02 ± 3,50 Kg/m^2, p=0,820.

Table 1. Anthropometric characteristics of the general population

Anthropometric Parameters	Consumers (n=65)	Controls (n=55)	p-value
Ages (years) 23,35 ± 4,74	23,49 ± 4,89	23,18 ± 4,60	0,83 (NS)
Height (m) 1,66 ± 0,08	1,68 ± 0,08	1,64 ± 0,09	0,015 (S)
Weight (Kg) 60,82 ± 11,05	61,90 ± 10,53	59,55 ± 11,60	0,163 (NS)
BMI (Kg/m^2) 22,09 ± 3,68	22,02 ± 3,50	22,16 ± 3,92	0,820 (NS)

BMI: Body mass index n: effectif S: Student-Fisher ($p < 0,05$) NS: Student-Fisher ($p > 0,05$)

Consumers: 450 kcal / day via palm oil Controls: 150 kcal / day via palm oil n: real

3.2 Lipid and Lipoprotein Parameters

3.2.1 General State of Lipid and Lipoprotein Parameters

Mean serum concentrations of all lipid and lipoprotein parameters (Table 2) in the two study groups were normal except for LDL cholesterol (1.00 ± 0.31 g / L) which was lower than the normal values with consumers. The results of comparison between the two groups showed that, overall, mean concentrations varied not-significantly ($p > 0.05$) for all parameters. In this sense, triglycerides were higher in palm oil consumers than controls: 0.81 ± 0.42 g / L versus 0.77 ± 0.32 g / L, p = 0.746. Conversely, serum total cholesterol, HDL cholesterol and LDL cholesterol levels were 1.67 ± 0.46 g / L, 0.43 ± 0.19 g / L and 1.08 ± 0, 39 g / L, with controls compared to consumers with values of 1.58 ± 0.37 g / L, 0.42 ± 0.15 g / L and 1.00 ± 0.31 g / L, respectively. The index of atherogenicity in the two groups was on average normal with a non-significant superiority with the consumers compared to the controls: 4.18 ± 1.57 versus 4.17 ± 1.24, p = 0.978. The mean concentration of lipoprotein (a) was not significantly higher with palm oil consumers compared to controls: 0.29 ± 0.2 g / L versus 0.25 ± 0.18 g / L, p = 0.093.

Table 2. Comparison of Lipid and Lipoprotein parameter Means of the Two Groups of the Study population

Lipid and lipoprotein parameters	**Consumers (n=65)**	**Controls (n=55)**	p-value **(p<0,05)**
TC (g/L)	1,58 ± 0,37	1,67 ± 0,46	0,348
Trigl (g/L)	0,81 ± 0,42	0,77 ± 0,32	0,746
HDL C (g/L)	0,42 ± 0,15	0,43 ± 0,19	0,971
LDL C (g/L)	1,00 ± 0,31	1,08 ± 0,39	0,251
AI= TC/ HDL C (g/L)	4,18 ± 1,57	4,17 ± 1,24	0,978
Lp (a) [g/L]	0,29 ± 0,2	0,25 ± 0,18	0,093

n: real TC: total Cholestrol AI: atherogenicity Index LP(a): Lipoprotein (a) Trigl: Triglycerides HDL C: High Density Lipoprotein cholesterol LDL C: Low Density Lipoprotein cholesterol p: Student-Fisher

3.2.2 Comparison of the Proportions of the Lipid and Lipoprotein Parameters According in the Two Groups

3.2.2.1 Distribution of the Proportions of the BMI

Analyses of the comparative proportions of the biochemical parameters by the G-test revealed general variations in both groups of studies. It was found that those with a lower BMI were 10.77% and 10.91%, respectively, and those with a normal BMI were 81.54% and 70.91%. The subjects suffering from over-nutrition were 7.69% and 18.18% respectively in consumers and controls (Figure 1).

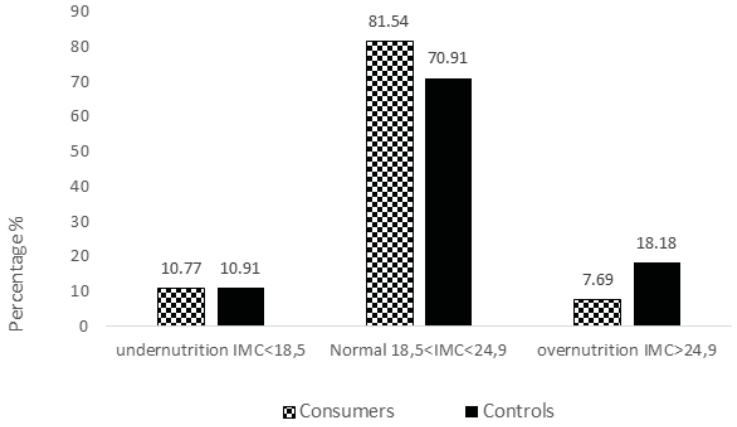


Figure 1. Distribution of BMI proportions

3.2.2.2 Distribution of the Proportions of Total Cholesterol and Its Fractions Hdl / Ldl

96.92% of consumers had normal serum total cholesterol compared with 92.72% of controls (Figure 2A) and 67.7% and 58.18%, respectively, with normal HDL cholesterol values (Figure 2B). Total hypercholesterolemia observed with controls (3.64%) was statistically higher (p = 0.024) than that of consumers (0%). No consumer had serum total cholesterol (Figure 2A) and higher than normal HDL cholesterol (Figure 2B). Furthermore, LDL cholesterol had a different behavior with 58.46% and 52.73% respectively of consumers and controls below normal compared with 35.38% and 38.18%, respectively, with normal serum concentrations. The percentage of controls with a high LDL cholesterol value was not significantly (p = 0.451) higher than that of consumers (Figure 2C).

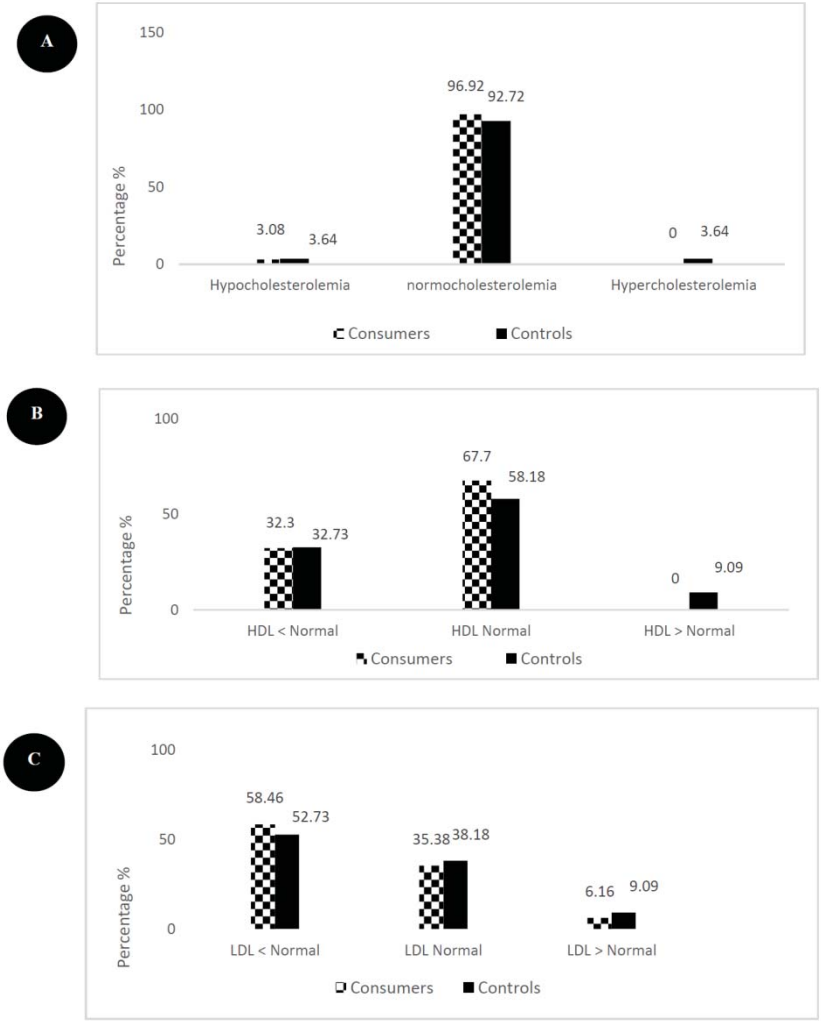


Figure 2 (A, B, C). Distribution of Serum total Cholesterol (A) and its fractions HDL (B) / LDL (C) levels according to the two groups

3.2.2.3 Distribution of Triglyceride Proportions

90.77% of consumers and 96.36% of controls had normal serum concentrations of triglycerides and 7.69% and 3.64% respectively had hypertriglyceridemia. This hypertriglyceridemia was not-significantly ($p = 0.223$) higher with consumers than controls (Figure 3).

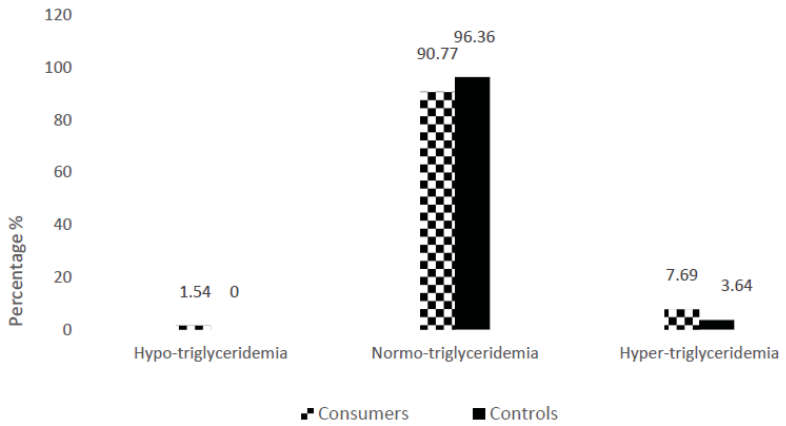


Figure 3. Distribution of triglyceride proportions

3.2.2.4 Distribution of the Proportions of the Ratio CT / HDL-C and the Lipoprotein (A)

The CT / HDL-C ratio (AI: atherogenicity index) varied the same way with the two groups. 83.08% of consumers had a normal atherogenicity index compared with 80% in controls (Figure 4A). 66.15% of consumers had normal serum lipoprotein (a) compared to 70.91% in controls. The elevated serum concentration of lipoprotein (a) was significantly higher (p = 0.548) with consumers compared to controls: 33.85% versus 29.09%, p = 0.55 (Figure 4B).

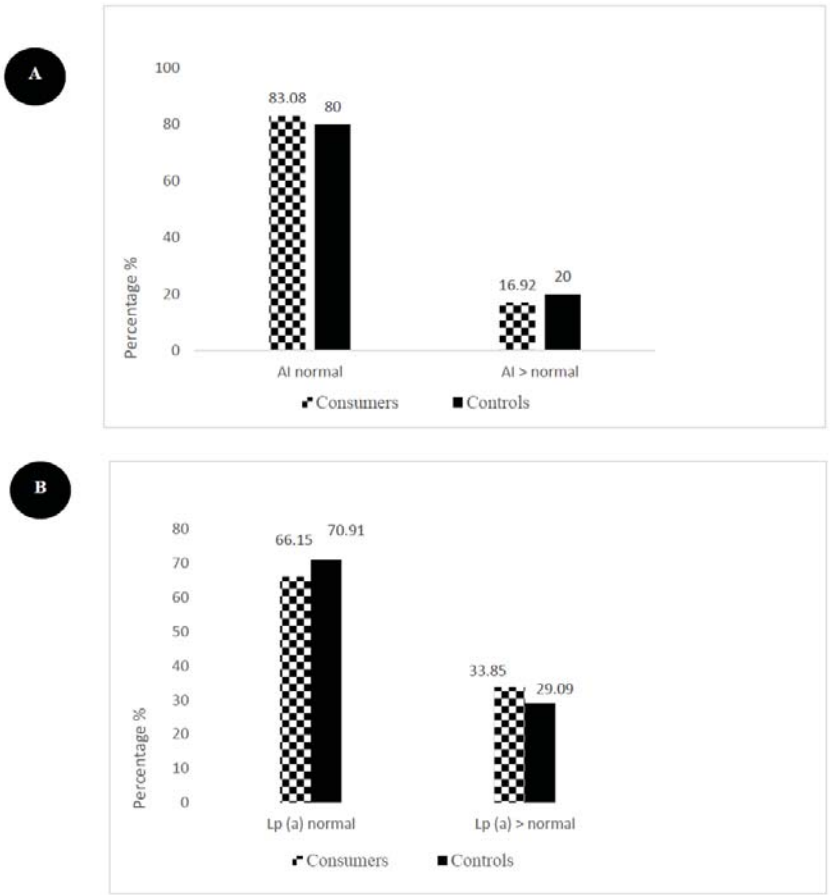


Figure 4 (A, B). Distribution of CT / HDL-C ratio (AI) and lipoprotein (a) [Lp(a)]

4. Discussion

In this study, the effect of regular consumption of palm oil was studied on serum lipid and lipoprotein levels of young subjects in a rural area in Côte d'Ivoire. The anthropometric parameters of the study population showed that the weight of palm oil consumers did not vary significantly compared to controls. Knowing that a high BMI is a major risk factor for chronic diseases (Yessoufou et al., 2012), the study population as a whole appeared not to be a population at risk for these diseases. Hornstra (1988), studying the effects of palm oil and cardiovascular disease observed that this oil was cholesterol-lowering. This observation corroborates our findings, which showed that no total and HDL hypercholesterolemia were observed with consumers compared to controls of whom 3.64% and 9.09% respectively suffered. In addition, the percentage of consumers with normal total cholesterol was higher than the one of controls. Furthermore, the results of our study showing a high serum concentration of bad cholesterol (LDL) with controls compared to consumers would suggest that the controls would be more exposed to atherosclerosis. These results are opposed to other authors' work (Tholstrup et al., 2011; Voon et al., 2011), who indicated that palm oil increased LDL cholesterol compared to olive oil.

Indeed, the excess LDL cholesterol is captured by the macrophages of the vascular wall thus inducing the formation of foam cells which play an essential role in atherogenesis (Hennen, 1996; Febbraio, Podrez & Smith, 2000; Leoni, 2001; Ducobu, Heller & Van, 2003). Another study carried out on 120 Chinese consumers of palm oil showed a decrease in LDL cholesterol (Zhang et al., 1997). In addition, Temme et al. (1996) showed that a diet rich in palmitic acid increased LDL cholesterol (Temme, Mensink & Hornstra, 1996) compared to oleic acid.

The palmitic acid content of nearly 44% in palm oil (Sambanthamurthi, Sundram & Tan, 2000; Sundram *et al.,* 2003) may therefore be responsible of this LDL cholesterol elevation. However, other authors have shown that the bioavailability of palmitic acid was less than 15% of the fatty acids in palm oil (Zock, De Vries, De Fouw, & Katan, 1995; Graille, 2005; Forsythe, French, Goh, & Clandinin, 2007). This would explain the high percentage (58.46%) of low LDL cholesterol observed in palm oil consumers of our study. It would therefore be important to be careful in the statement of the problematic about the hypercholesterolemia effect of palm oil basing on its high concentration of saturated fatty acids. Hypertriglyceridemia and elevated lipoprotein (a) were not significantly higher with consumers than cwith ontrols. These results could lead us to assert that the consumption of palm oil would therefore have no influence or would have a negligible impact on triglyceridemia and lipoproteinemia (a) in these consumers. This would be in favor of non-atherogenicity of palm oil, contrary to the observations of certain studies (Anitschkow, 1983; Tholstrup et al., 2011; Voon et al., 2011). Our work is comparable to that reported by Lecerf (2013), showing that palm oil consumption induced a stable lipid profile compared to partially hydrogenated vegetable fats (Lecerf, 2013). In addition, another study showed that palmitic acid is not hypercholesteremic, starting with a threshold of more than 4.5% of linoleic acid. On the other hand, total cholesterol and its fractions (LDL and HDL) increase if linoleic acid is low (French, Sundram, & Clandinin, 2002), fortunately, palm oil contains 10% (Sambanthamurthi et al., 2000; Sundram et al., 2003). This could explain that slight increase of the mean concentration in these parameters, observed with controls compared to consumers of palm oil in our study. The consumption of that palm oil at low cost, accessible and with undeniable nutritional qualities due to its recognized antioxidant properties, should be encouraged (Chow, 1992; Sutapa & Analava, 2009; Mondé et al., 2010).

5. Conclusion

There is a relevant dissension between several authors about the consumption of palm oil due to its poorly or partially known nutritional aspect. This study showed the beneficial influence of palm oil consumption on the lipid and lipoprotein profile of a young population living in rural areas. It was found that the percentage of subjects with normal serum concentrations was higher with TC, HDL-C, triglycerides, Index Atherogenicity and lipoprotein (a), in both consumers and controls, except LDL cholesterol of which the percentage of lower than normal value was the highest (58.46% for consumers and 52.73% controls). The lipid and lipoprotein profile with consumers of palm oil was not disturbed with the subjects studied. On the other hand, the effects of palm oil on health can not only be summarized by saturated fatty acid effects, but especially by its antioxidant and nutritional properties.

Acknowledgments

We thank the Director of Alépé Health Department for the present study authorization. We also gratefully acknowledge technical staff of Institut Pasteur in Abidjan for their assistance during various manipulations. Designed research (MA, YAP, DAJ); wrote the paper (AAA, MA); conducted research (AAA, EAA, BGA), analyzed data or performed statistical analysis (AAA, MA). All authors read and approved the final manuscript.

References

ANREF (2006). Les apports nutritionnels de référence. Institute of Medicine (IOM), National Academies Press, Washington D.C, 560p.

ANSES (2016). Agence nationale de sécurité sanitaire de l'alimentation (anciennement AFSSA). https://www.ansese.fr/.

Abell, L. L., Levy, B. B., & Kendall, F. E. (1958). Cholesterol in serum. *In: Seligon D (ed.). Standard Methods of Clinical Chemistry, 2*, 26-33. https://doi.org/10.1016/b978-1-4831-9683-1.50008-3

Aké Aké, A., Mondé, A. A., Yapo, A. P., Akpolé, K. J., Bléyéré, N. M., & Camara-Cissé, M. (2015). Etude des paramètres anthropométriques d'une population rurale consommatrice d'huile de palme en Côte d'Ivoire. *Cahier de santé publique, 10*, 140-149.

Anitschkow, N. (1983). Classics in arteriosclerosis research: On experimental cholesterin steatosis and its significance in the origin of some pathological processes by Anitschkow N and Chalatow S, translated by Mary Pelias Z, 1913. *Arteriosclerosis, 3*, 178-182. https://doi.org/10.1161/01.ATV.3.2.178

Aranceta, J., & Perez-rodrigo, C. (2012). Recommended dietary reference intakes, nutritional goals and dietary guidelines for fat and fatty acids: A systematic review. *Br. J. Nutr, 107*, S8-S22. https://doi.org/10.1017/S0007114512001444

Assmann, G., Buono P., Danielle, A., Della, V. E., Farinaro, E., Ferns, G., … Merino, J. (2014). Functional foods

and cardiometabolic diseases. International Task Force for Prevention of Cardiometabolic Diseases. *Nutr. Metab. Cardiovasc. Dis, 24*, 1272-1300. http://dx.doi.org/10.1016/j.numecd.2014.10.010

Béké, P. (2015). *Evaluation des connaissances sur les valeurs nutritionnelles de l'huile de palme chez les patients ayant l'hypertension artérielle à l'Institut de Cardiologie d'Abidjan* (unpublished doctoral dissertation). University Felix Houphouet Boigny, Abidjan, Côte d'Ivoire.

Berger, N. A. (2014). Obesity and cancer pathogenesis. *Ann. N. Y. Acad. Sci, 1311*, 57-76. https://doi.org/10.1111/nyas.12416

Coulibaly, F. (2015). *Evaluation de la connaissance sur les valeurs nutritionnelles de l'huile de palme chez les patients diabétiques reçus au service d'Endocrinologie-Diabétologie et au CADA* (unpublished doctoral dissertation). University Felix Houphouet Boigny, Abidjan, Côte d'Ivoire.

Djohan, Y. F., Mondé, A. A., Djinhi J. *et al.,* (2010). Effets antioxydants de l'huile de palme rouge traditionnelle chez la femme ménopausée en côte d'Ivoire. *Rev CAMES-Série A*, (11), 22-25

Ducobu, J., Heller, E., & Van, G. L. (2003). *Recommandations pour le Diagnostic et le Traitement des Dyslipidémies : Guide de Poche* (4ème ed). *Belgian Lipid Club*, 8-12.

Edem, D. O. (2002). Palm oil: Biochemical, physiological, nutritional, hematological, and toxicological aspects: A review. *Plant Foods for Human Nutrition, 57*, 319-341. https://doi.org/10.1023/A:1021828132707

Febbraio, M., Podrez, E. A., & Smith, J. D. (2000). Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. *J Clin Invest, 105*, 1049-1056. https://dx.doi.org/10.1172%2FJCI9259

Forsythe, C. E., French, M. A., Goh, Y. K., & Clandinin, M. T. (2007). Cholesterolaemic influence of palmitic acid in the sn-1, 3 v. the sn-2 position with high or low dietary linoleic acid with healthy young men. *Br J Nutr, 98*, 337-44. https://doi.org/10.1017/S0007114507704993

Chow, C. K. (1992). Fatty Acids in Foods and their Health Implications. *New York: Marcel Dekker Inc.*, 237-262.

French, M. A., Sundram, K., & Clandinin, M. T. (2002). Cholesterolaemic effect of palmitic acid in relation to other dietary fatty acids. *Asia Pac J Clin Nutr, 11*(suppl. 7), S401-7. https://doi.org/10.1046/j.1440-6047.11.s.7.3.x

Friedewald, W. T., Levy, R. I., & Fredickson, D. S. (1972). Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentriguge. *Clin Chem, 18*, 499-502

Graille, J. (2005). L'huile de palme alimentaire. Un lipide de plus en plus consommé dans le monde. www.groupesifca.com/pdf/huile_palme_alimentaire.pdf

Hennen, G. (1996). Biochimie humaine. Introduction biochimique à la médecine interne. *Paris: DeBoeck université*.

Hornstra G. (1988). Dietary lipids and cardiovascular disease: effects of palm oil. *Oleagineax, 43*, 75-81.

Ihaka, R., & Gentleman, R. (1996). A language for data analysis and graphics. *Journal of computational and graphical statistics, 5*, 299-314. https://doi.org/10.1080/10618600.1996.10474713

Kennedy, E. T. (2005). La situation globale de la nutrition : Que peuvent faire les états et l'industrie. *Nutrition News for Africa J Nutr, 135*, 913-15. http://www.hki.org/research/nna_storage/nna_abst_fr_avr15-05.htm.

Keys, A., Anderson, J. T., & Grande, F. (1965). Serum cholesterol response to changes in the diet: IV. Particular saturated fatty acids in the diet. *Metabolism, 14*, 776-787. https://doi.org/10.1016/0026-0495(65)90004-1

Kromhout, D., Menotti, A., Bloemberg, B., Aravanis, C., Blackburn, H., Buzina, R., … Jansen, A. (1995). Dietary saturated and trans fatty acids ans cholesterol and 25-year mortality from coronary heart disease: the seven countries study. *Prev. Med., 24*, 308-315. https://doi.org/10.1006/pmed.1995.1049

Lecerf, J. M. (2013). L'huile de palme : aspects nutritionnels et métaboliques. Rôle sur le risque cardiovasculaire *OCL, 20*(3), 147-159. https://doi.org/10.1051/ocl.2013.0507

Léga E. S. (2015). *Evaluation des connaissances sur les valeurs nutritionnelles de l'huile de palme chez les patients obèses et en surpoids au service de Nutrition INSP Adjamé* (unpublished doctoral dissertation). University Felix Houphouet Boigny, Abidjan, Côte d'Ivoire.

Léoni, J. (2001). Physiopathologie de l'athérosclérose. Mécanismes et prévention de l'athérothrombose. *Thèse*

d'exercice pour l'obtention du Diplôme d'Etat de Docteur en Pharmacie, Université de Franche-Comté, UFR de Pharmacie, Besançon, France; www.123bio.net Biologie et recherche.

Mancini, A., Imperlini, E., Nigro, E., Montagnese, C., Aurora, D., Orrù, S., & Buono, P. (2015). Biological and nutritional properties of palm oil and palmitic acid: effects on health. *Molecules, 20*, 17339-17361. https://doi:10.3390/molecules200917339

Mondé, A., Carbonneau, M. A., Diabaté, S., Konan, E., Sess, D., & Cristol, J. P. (2010). Effets des extraits polyphénoliques d'huiles de palme dans l'oxydation in vitro des LDL par le cuivre. *J Sci Pharm Biol*., *11*(1-2010), 47-56

Mondé, A. A., Lohoues, E. C., Gauze, G. A. C., Camara-Cissé, M., Diomandé, M. I., Djessou, S. P., & Sess, E. D. (2016). Relationship between Lipid Assessment and Arterial Lesions Observed in Farm Chickens Fed on Different Vegetable Oils. *Journal of Food and Nutrition Sciences, 4*(5), 126-130. https://doi:10.11648/j.jfns.20160405.12

Mondé, A. A., Camara-Cissé, M., Konan, G., Coulibaly, I., Adeoti, M., Gauze, G. A. C., … Hauhouot-attoungbre, M. L. (2017). Effects of palm oil consumption on Lipidic and Lipidoproteinic Profiles with Patients Suffering from Ischemic Heart Pathologies. *International Journal of Nutrition and Food Sciences, 6*(1), 6-11. https://doi:10.11648/j.ijnfs.20170601.12

Sutapa, M., & Analava, M. (2009). Health effects of palm oil. *J Hum Ecol, 26*(3), 197- 203

MVO, (2010), Fact sheet palm oil. http://www.mvo.n1/link/click.aspx?fileticket=jsFVMZwZzkc%3D

Odia, O. J., Ofori, S., & Maduka, O. (2015). Palm oil and the heart: A review. *World J Cardiol, 7*(3), 144-9. https://doi.org/10.4330/wjc.v7.i3.144

Oluba O. M., & Onyeneke, C. E. (2009). Effects of palm oil supplementation on lipid peroxidation and glutathione peroxidase activity in cholesterol fed rats. *Internet journal of cardiovascular research, 6*, 1

OMS, (2003), Obésité: Prévention et prise en charge de l'épidémie mondiale : série de rapports techniques, 894, 196-200.

OMS, (2005). Poursuite du dialogue entre l'OMS et les responsables du secteur agro-alimentaire à propos de l'alimentation et des maladies chroniques. http://www.who.int/mediacentre/news/notes/2003/np21/fr.

Ong, A. S., & Goh, S. H. (2002). Palm oil: a healthful and cost-effective dietary component. *Food Nutr Bull, 23*, 11-22. https://doi.org/10.1177/156482650202300102

Onyeali, E. U., Onwuchekwa, A. C., Monago, C. C., & Monanu M. O. (2010). Plasma lipid profile of wistar albino rats fed palm oil supplemented diets. *Int J Biol Chem Sci., 10*, 1-7.

Oyewole, O. E., & Amosu, A. M. (2010). Public health nutrition concerns on consumption of red palm oil (RPO): the scientific facts from literature. *Afr J Med Sci., 39*, 255-265.

Sambanthamurthi, R., Sundram, K., & Tan, Y. A. (2000). Chemistry and biochemistry of palm oil. *Progress in lipid Research, 39*, 507-558. https://doi.org/10.1016/S0163-7827(00)00015-1

Simó, J. M., Camps, J., Gómez, F. *et al.,* (2003). Evaluation of a Fully Automated Particle-enhanced Turbidimetric Immunoassay for the Measurement of Plasma Lipoprotein(a). Population-Based Reference Values in an Area with Low Incidence of Cardiovascular Disease. *Clin Biochem Mar, 36*(2), 129-134

Statsoft, (2005). Statistica (Data Analysis Software System). Version 7.1 available at *www.statsoft.com.*

Sugiuchi, H., Uji, Y., Okabe, H., Irie, T., *et al.,* (1995). Direct Measurement of High Density Lipoprotein

Cholesterol in Serum with Polyethylene GlycolModified Enzymes and Sulfated α-Cyclodextrin. *Clin Chem May, 41*(5), 717-723.

Sundram, K., Sambanthamurthi, R., & Tan, Y. A. (2003). Palm fruit chemistry and nutrition. *Asia Pac J Clin Nutr, 12*, 355-62.

Temme, E. H., Mensink, R. P., & Horntra, G. (1996). Comparison of the effects of diets enriched in lauric, palmitic, or oleic acids on serum lipids and lipoproteins with healthy women and men. *Am J Clin Nutr, 63*, 897-903.

Tholstrup, T., Hjerpsted, J., & Raff, M. (2011). Palm olein increases plasma cholesterol moderately compared with olive oil with healthy individuals. *Am J Clin Nutr, 94*, 1426-32. https://doi.org/10.3945/ajcn.111.018846

Trinder, P. (1969). Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Ann Clin Biochem, 6*, 24-27. https://doi.org/10.1177/000456326900600108

Voon, P. T., Ng T. K. W., Lee V. K. M., & Nesaretnam, K. (2011). Diets high in palmitic acid (16 :0), lauric and myristic acids (12 :0 + 14 :0), or oleic acid (18 :1) do not alter postprandial or fasting plasmahomocysteine and inflammatory markers in healthy Malaysian adults. *Am J Clin Nutr, 94*, 1451-7. https://doi.org/10.3945/ajcn.111.020107

Yapo, S. (2015). *Evaluation des connaissances sur les valeurs nutritionnelles de l'huile de palme chez les patients porteurs de cardiopathies ischémiques à l'Institut de Cardiologie d'Abidjan* (unpublished doctoral dissertation). University Felix Houphouet Boigny, Abidjan, Côte d'Ivoire.

Yessoufou, G., Attakpa, E., Baba-Moussa, F., Hounhouigan, J. D., Baba-Moussa, L., et Sezan, A. (2012). Prévalence et rôle des lipides dans l'avènement de l'obésité au Bénin. *Int J Biol Chem Sci*., 6(4), 1695-1702. https://doi.org/10.4314/ijbcs.v6i4.26

Zhang, J., Ping, W., Chunrong, W., Shou C. X., & Keyou, G. (1997). Nonhypercholesterolemic effects of palm oil diet in Chinese adults. *J Nutr, 127*, 509S-13S.

Zock, P. L., De Vries, J. H., De Fouw, N. J., & Katan, M. B. (1995). Positional distribution of fatty acids in dietary triglycerides: effects on fasting blood lipoprotein concentrations in humans. *Am J Clin Nutr., 61*, 48-55.

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

A Retrospective Study on Changes in Food Preferences of Japanese High School Students from Childhood to the Present Day

Tomoko Osera[1, 2], Mitsuyo Awai[3], Setsuko Tsutie[4], Misako Kobayashi[2] & Nobutaka Kurihara[1]

[1]Hygiene and Preventive Medicine, Graduate School of Life Science, Kobe Women's University, Japan

[2]Takakuradai Kindergarten attached to Kobe Women's University, Japan

[3]School of Nursing, Kansai University of Nursing and Health Sciences 1456-4 Shichi, Awaji-city, 656-2131, Japan

[4]Clinical Nutrition Management, Graduate School of Life Science, Kobe Women's University, Japan

Correspondence: Nobutaka Kurihara, MD, Graduate School of Life Science, Kobe Women's University, 2-1 Higashisuma-Aoyama, Suma, Kobe, Japan. Tel: 81-78-737-2417. E-mail: kurihara@suma.kobe-wu.ac.jp

Received: May 13, 2017 Accepted: June 17, 2017 Online Published: July 28, 2017

doi:10.5539/jfr.v6n4p150 URL: https://doi.org/10.5539/jfr.v6n4p150

Abstract

Background: To conduct a retrospective study for investigating changes in food preferences of high school students from childhood to the present day. Methods: The study included 1,300 students aged 16–18 years who responded to a questionnaire regarding food items that they disliked at present and in their childhood; they selected a list of 55 foods and responded to 35 questions regarding their food habits. The distribution was categorized into four patterns of food preferences based on whether a particular student had disliked a particular food item during childhood (+) and during high school at present (+). Food preference at present was examined for all other items using logistic regression analysis after adjusting for gender and age. Results: In total, 66.9% of the subjects reported (+) to (+), 12.5% reported (+) to (−), 6.5% reported (−) to (+), and 14.1% reported (−) to (−). Even in the (+) to (+) group, a significant decrease was observed in the number of disliked foods from childhood (5.5 ± 5.4) to the present day (4.2 ± 4.1) ($P < 0.001$, ANOVA). No dislike for any food item at present was related to no dislike for any food item during childhood [odds ratio (OR), 12.57; 95% confidence interval (CI), 8.3–19.1]] and talking positively about food (OR, 1.28; 95% CI, 1.11–1.49) but inversely related to the limited use of smartphone while eating (OR, 0.86; 95% CI, 0.75–0.98). Conclusion: Decreasing the dislike for foods at present as well as no dislike for any food item during childhood may be crucial for developing future good food habits in high school students. In addition, to improve current food preferences, students may need to eat together.

Keywords: food preferences, disliked food, high school students, childhood

1. Introduction

Early childhood is the most important period for establishing healthy eating habits and controlling the food preferences of children. Childhood is a crucial period for developing food acceptance patterns (Nicklas et al., 2001, Ilingworht & Lister, 1964, Cashidan, 1994). Some investigators have insisted that preferences are shaped by a combination of genetic and environmental factors (Wardle & Cooke, 2008). The development and long-term health of children is linked to food habits established from early childhood (Scaglioni et al, 2008). The correct food habits may continue in the future of children's life. In addition, children are exposed to unhealthy food choices, which may have greatly contributed to the increase in the prevalence of overweight observed among their youth in the past several years (St-Onge MP, 2003). Therefore, it is important to take correct food habits during childhood.

On the other hand, adolescence is a crucial period for the development of health status (Mechanic & Hansell, 1987, Vingilis, Waed, & Seeley, 2002, Vingilis, Wade, & Seeley, 2007). During this period, health-related behaviors including smoking, alcohol consumption, drug use, nutrition-related behavior, and physical activities, are established (Sharma et al, 2016). Further, in this period, health is strongly affected by social factors, including income inequality, family support, school environment, and peer influence (Viner et al., 2012). The

food group consumption varies by socioeconomic, demographic, and life style factors in young adults (Deshmukh-Taskar P, 2007).

However, it is unclear how food preferences during childhood influence those during high school. In this retrospective study, we investigated changes in the food preferences of high school students from childhood to the present day.

2. Method

2.1 Participant (Subject) Characteristics

This was a cross-sectional study. From May to November 2015, 1,300 students aged 16–18 years from 11 high schools in Japan were included in the study. Of all students enrolled in the study, 1,296 (99.7%) completed and returned the questionnaire, and all provided informed consent for its use.

2.2 Questionnaire

The students answered a questionnaire regarding foods they disliked at present and in their childhood. They selected a list of 55 foods and answered 35 questions regarding their food habits.

This questionnaire is a revised version of the one originally created by the Japan Sports Council that was used to determine the food habits of high school students (Japan Sports Council, 2010, Osera et al., 2016a). Further questionnaire items addressed 'respect for food', utilising a 5-point rating scale. In addition, Self-rated heath (SRH) measures generally included questions, such as "How would you rate your overall health?," and offered five response categories that ranged from excellent to poor: Excellent, Very good, Good, Fair, and Poor (Joffer et al., 2016, Warnoff, et al., 2016, Wu et al, 2013).

The foods student disliked, which were chosen by themselves from a list of 55foods; Noodle, Rice, Bread, Konjaku, Sweet potato, Potate, Azuki beans, Soybeans, Freeze-dried tofu, Tofu, Deep-fried tofu, Sesame, Pampkin, Peas, String beans, Carrot, Leak, Green pepper, Broccoli, Spinach, Japanese mustard spinach, Cabbage, Cucumber, Burdock, Japanese radish, Onion, Corn, Eggplant, Chinese cabbage, Tomato, Cherry tomato, Banana, Tangerine, Apple, Pineapple, Enoki mashroom, Shimeji mashroom, Dried shitake, Toasted laver, Hijiki, Seaweed, Squid, Shrimp, Fish paste cake, Spanish mackerel, Sermon, Liver, Beef, Chicken, Pork, Cheese, Yogurt, Milk, Egg, Quail egg. The foods on the list were selected from what were available at regular school lunches and often disliked by children as shown in our previous study (Osera et al, 2016a).

The distribution was categorized into four patterns of food preferences based on whether a particular student had disliked a particular food item during childhood (+) or not (−), and during high school days at present (+) or not (−). For example, (+) to (−) means that the student disliked some foods during childhood while as high school students they had no dislikes, (+) to (+) means that the students disliked some foods as childhood and that they disliked some foods as high school days, (−) to (+) means that the students disliked no foods as childhood and that as high school days they disliked some foods, (−) to (-) means that the students disliked no foods as childhood and that they disliked no foods as high school days.

2.3 Statistics and Data Analysis

The analysis of variance (ANOVA) was used to compare each number of disliked foods between the time of childhood and at present groups in they have food preference. The chi-square test or Fisher's exact test was used to evaluate associations between independent variables and food preferences at the time of childhood and at high school age. McNemar test was used to assess the food preferences change from childhood to high school age. The percentage of high school students who disliked each food was significantly different from that of them in childhood. A probability (P) value < 0.05 was considered statistically significant (Figure 2).

All significant variables in the bivariate analysis were then entered into a logistic regression analysis. Food preference at present, the differences between those that disliked foods at present and they those that did not dislike any food at present, were examined for all the other items using logistic regression analysis after adjusting for gender and age. A dichotomy regression analysis was performed by setting a stepwise method. The sample data were entered and analyzed using SPSS for Windows, Version.23 (IBM, New York, NY).

2.4 Ethics

Students were informed as to the objects and methods of this study and answered the questionnaire only if they so desired, in the absence of any compelling force and with the right of free withdrawal. Individual privacy was strictly protected thorough the investigation. This study was approved by the leader of the high school. Under these conditions, students agreed with their corporation for the scientific investigations in the high schools, including this study, when their students entered. This study was approved by the Kobe Women's University

Ethics Committee Regarding Human Subjects.

3. Results

3.1 Relationship between Food Preferences and Physical Characteristics, Self-Rated Health

Fisher's exact probability test and ANOVA revealed that the relationship between food preferences in childhood and at high school age and physical characteristics, self-rated health was statistically significant (Table 1). In males, a relationship between height, weight, and food preferences in childhood was observed ($P < 0.001$); however, such a relationship was not observed at high school age (P = N.S). Notably, no significant difference was observed in BMI in either gender during childhood and at high school age (Table 1).

3.2 Relationships between Food Preferences during Childhood to High School Age

Fisher's exact probability test revealed that the relationship between food preferences in childhood and current food preferences by gender was statistically significant (Table 2). In total, about 30% of the students answered "I do not know" or "I do not remember." In total, 66.9% of subjects reported (+) to (+), 12.5% reported (+) to (−), 6.5% reported (−) to (+), and 14.1% reported (−) to (−) (Table 3). Significant differences by age were observed.

Table 1. Comparison in characteristics of high school students between presence and absence of preferences

	Variable	During childhood					The present day				
		Presence (+)		Absence (−)		P value	Presence (+)		Absence (−)		P value
All	Height (m)	665	162.1±8.2	167	165.0±8.9	0.000^{+++}	857	161.5±8.2	308	165.1±8.5	0.000^{+++}
	Weight (kg)	615	54.3±9.2	158	57.3±11.1	0.000^{+++}	791	54.1±9.4	297	56.4±10.2	0.000^{+++}
	BMI (m/kg^2)	616	20.5±2.7	157	20.9±2.8	N.S	791	20.6±2.7	296	20.6±2.9	N.S
Male	Height (m)	280	169.2±6.3	88	171.3±6.7	0.006^{++}	311	169.4±6.1	174	170.4±6.4	N.S
	Weight (kg)	277	59.7±9.3	88	63.0±11.0	0.006^{++}	310	60.3±9.9	173	60.5±9.9	N.S
	BMI (m/kg^2)	277	20.8±2.8	87	21.5±3.2	N.S	309	21.0±3.0	172	21.0±3.0	N.S
Female	Height (m)	385	156.9±5.0	79	158.1±5.0	N.S	546	156.9±5.3	134	158.1±5.1	0.027^{+}
	Weight (kg)	338	49.9±6.3	70	50.3±6.0	N.S	481	50.1±6.5	124	50.2±6.6	N.S
	BMI (m/kg^2)	339	20.2±2.5	70	20.2±2.1	N.S	482	20.3±2.5	124	20.1±2.6	N.S
Distribution of BMI											
All	Over 25.0	27	4.4%	8	5.1%	N.S	16	5.4%	41	5.2%	N.S
	18.5-25.0	479	77.8%	130	82.8%		228	77.0%	610	77.1%	
	Under 18.5	110	17.9%	19	12.1%		52	17.6%	140	17.7%	
Self-rated health											
All	Excellent	236	34.4%	102	58.3%	0.000^{***}	301	33.9%	157	48.6%	0.000^{***}
	Very good	328	47.8%	51	29.1%		441	49.6%	112	34.7%	
	Good	76	11.1%	13	7.4%		87	9.8%	33	10.2%	
	Fair	40	5.8%	8	4.6%		53	6.0%	18	5.6%	
	Poor	6	0.9%	1	0.6%		7	0.8%	3	0.9%	
Male	Excellent	82	28.9%	55	59.8%	0.000^{***}	101	32.0%	79	44.4%	N.S
	Very good	140	49.3%	27	29.3%		157	49.7%	67	37.6%	
	Good	36	12.7%	8	8.7%		33	10.4%	20	11.2%	
	Fair	21	7.4%	2	2.2%		20	6.3%	10	5.6%	
	Poor	5	1.8%	0	0%		5	1.6%	2	1.1%	
Female	Excellent	154	38.3%	47	56.6%	0.004^{**}	200	34.9%	78	53.8%	0.000^{***}
	Very good	188	46.8%	24	28.9%		284	49.6%	45	31.0%	
	Good	40	10.0%	5	6.0%		54	9.4%	13	9.0%	
	Fair	19	4.7%	6	7.2%		33	5.8%	8	5.5%	
	Poor	1	0.2%	1	1.2%		2	0.3%	1	0.7%	

Note. Significance tested using a Fisher's exact probability test;* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Significance tested using a t-test;+ $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ 'Presence' (+) means that students dislike more than one foods, 'Absence' (−) means that students disliked no food.

Even in the (+) to (+) group, a significant decreased was observed in the number of disliked food items from childhood (5.5 ± 5.4) to the present day (4.2 ± 4.1) ($P < 0.001$, ANOVA; Figure 1). In male, a significant decreased was observed in the number of disliked food items from childhood (4.8 ± 4.9) to the present day (3.6 ± 4.0) ($P < 0.001$, ANOVA; Figure 1). In female, a significant decreased was observed in the number of disliked food items from childhood (6.0 ± 5.7) to the present day (4.5 ± 4.0) ($P < 0.001$, ANOVA; Figure 1).

Table 2. Relationship between during childhood and at present by gender

	During childhood								
	Presence (+)		Absence (−)		I do not know		I did not remember		P value
	N	(%)	N	(%)	N	(%)	N	(%)	
Male	284	(51.9)	92	(16.8)	44	(8.0)	127	(23.2)	0.016*
Female	405	(53.9)	83	(11.1)	80	(10.7)	183	(24.4)	
	The present day								
Male	316	(57.9)	178	(32.6)	52	(9.5)			0.000***
Female	575	(76.7)	146	(19.5)	29	(3.9)			

Note. The relationship between preference and gender were assessed using a Fisher's exact probability test; * p < 0.05, ** p < 0.01, *** p < 0.001 'Presence' (+) means that students dislike more than one foods, 'Absence' (−) means that students disliked no food.

Table 3. Student's preference of during childhood to high school in relation with gender

	(+)→(+)		(+)→(−)		(−)→(+)		(−)→(−)		
	N	(%)	N	(%)	N	(%)	N	(%)	*P* value
Male	213	(59.7)	54	(15.1)	23	(6.4)	67	(18.8)	0.000***
Female	345	(72.3)	50	(10.5)	31	(6.5)	51	(10.7)	
All	558	(66.9)	104	(12.5)	54	(6.5)	118	(14.1)	

Note. Food preferences of children during childhood to high school in terms of gender were assessed using Fisher's exact probability test; * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001

(−) means that students disliked no food, (+) means that students dislike more than one foods, (+) to (+), means that the students disliked some foods as childhood and that they disliked some foods as high school days, (+) to (−), means that the students disliked some foods as childhood and that as high school days they disliked no foods, (−) to (+), means that the students disliked no foods as childhood and that as high school days they disliked some foods, (−) to (−), means that the students disliked no foods as childhood and that they disliked no foods as high school days.

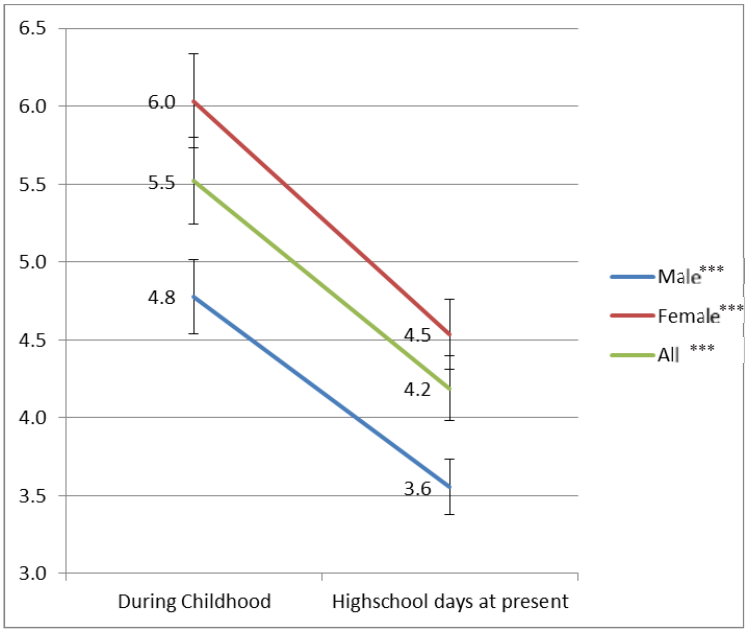


Figure 1. Relationship between the number of their disliked food childhood and high school

Note. The number of their disliked food during childhood to high school in terms of gender were assessed using two way ANOVA; * P < 0.05, ** P < 0.01, *** P < 0.001

The foods student disliked, which were chosen themselves from a list of 55 foods.

3.3 Relationships between Food Preferences during Childhood to High School Age

The students' food preferences at present and in childhood exhibited significant relevance for 18 types of vegetables, three types of mushrooms, three types of seafood, and two types of beans ($P < 0.001$, by McNemar test). However, grains, fruits, and meat group were not significantly relevant. Green pepper (41.8%) was the food preference with the highest improvement rate of 23.8%. Liver and shrimp topped the list of disliked food items reported by the students, but did not exhibit significant changes in preference compared to childhood. In childhood, the top five most disliked foods were green pepper (41.8%), peas (41.6%), liver (40.7%), eggplant (40.0%), and dried mushroom (32.3%). The highest rate was liver 38.8%. Liver exhibited significant changes from childhood to high school age. Now, in high school, the number of disliked food items decreased; green pepper (23.8%), eggplant (17.9%), and peas (17.3%) had the highest improvement rates ($P < 0.001$). The most disliked food was vegetables in both childhood and in high school. If children dislike some types of food, when they grow up, they may be improving their preferences (Figure 2).

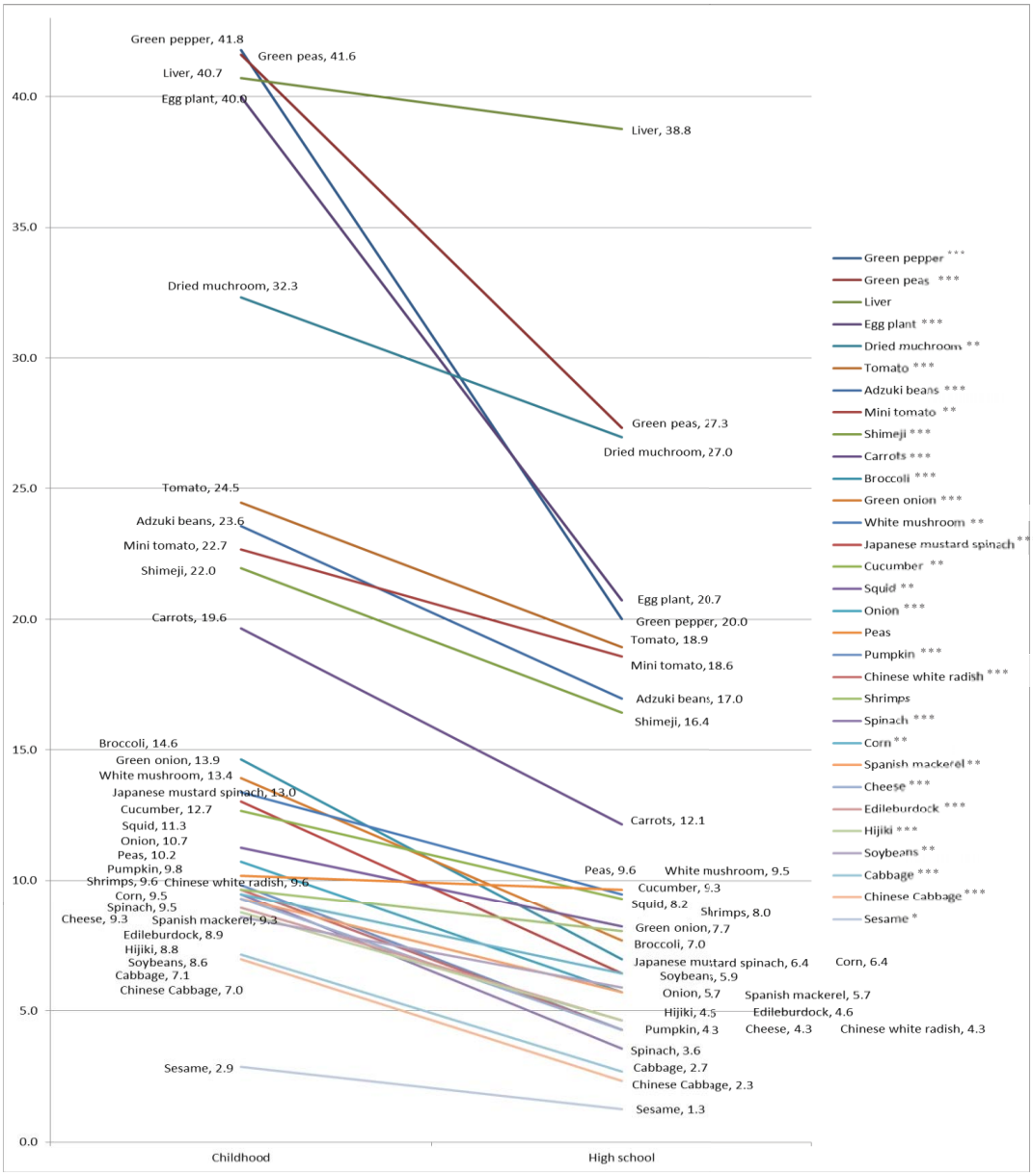


Figure 2. In the detail of each food, relationship between their disliked food childhood and high school.

Note. The percentage of food preferences of children during childhood to high school were assessed using. The foods student disliked, which were chosen themselves from a list of 55 foods. McNemar test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

3.4 Multiple Logistic Regression Analysis of Factors Affecting Students' Preferences

No dislike for any food item at present was related to no dislike for any food item during childhood [odds ratio (OR), 12.57; 95% confidence interval (CI), 8.3–19.1)], talking positively about food (OR, 1.28; 95% CI, 1.11–1.49), but inversely related to limited use of smartphone while eating (OR, 0.86; 95% CI, 0.75–0.98) (Table 4).

Table 4. Means and 95% confidence intervals of food habits based on current preferences in the high school students after covariate adjustment

	OR	(95% CI)	*P* value
No dislike for any food item during childhood	12.57	(8.29, 19.05)	0.000
Talking about food	1.28	(1.11, 1.49)	0.013
Limited use of smartphone while eating	0.86	(0.75, 0.98)	0.022

OR, Odds ratio; CI, confidence interval Multiple regression analysis by setting a stepwise method

'Talking about food' increases if students talk with their family during meals. Adjusted for gender and age.

4. Discussion

According to Tables 1 and 2, food preference influences on health, exhibiting significant differences by gender at high school age. A relationship between height, weight, and food preferences in childhood was observed ($P < 0.001$). It is important evidence to correct food preference during childhood. In addition, SRH is a measure of the relationship between during childhood and high school age. The utility of SRH assessments has been shown in persons with moderate-to-severe chronic kidney disease (Robinson-Cohen et al., 2014). SRH assessment among adolescents is a good measure of enduring self-concepts of health. It is strongly associated with general well-being and psychosomatic symptoms (Vingilis, Waed, & Seeley, 2002, Boardman, 2006, Breidablik, Meland, & Lydersen, 2008, Piko, 2007). SRH was also influenced by gender, family support, lifestyle and psychological factors (Sharif et al., 2016). The result revealed that children's preferences in childhood may be connected with their health when they are at high school age.

In total, 66.9% of subjects reported (+) to (+), 12.5% reported (+) to (−), 6.5% reported (−) to (+) and 14.1% reported (−) to (−) (Table 3). A previous study on children aged 4–6 years in kindergartens revealed similar trends (Osera et al., 2014). The changes in the people's food preference are almost same percentages in childhood and childhood to high school ages.

No dislike for any food item at present was related to no dislike for any food item during childhood (OR, 12.57; 95% CI, 8.3–19.1), talking positively about food (OR, 1.28; 95% CI, 1.11–1.49), but inversely related to limited use of smartphone while eating (OR, 0.86; 95% CI, 0.75–0.98) (Table 4). "Talking about food" and "limited use of smartphone while eating" may be connected with cultural upbringing. Three previous studies of ours suggested that mothers' attitude toward food and eating may have profound effects on children's food preferences (Osera et al., 2012, Osera et al., 2016b). At high school age, it may be important that home and family food environment is similar to that as in childhood. In most cases, the mothers make the dinner (data not shown). So, the mother's role is very important. "Talking about food" rephrase "they eat together." Studies have shown that the meal pattern of skipping breakfast or having "breakfast and lunch together" is related to a less healthy lifestyle and food choices leading to a poorer nutritional intake (Sjoberg et al., 2003). In addition, Stratton described that eating together with the family at meal time was very important for children's development (Stratton, 2003). Taken together, it is suggested that eating while talking to the family positively affects food preference at high school age. In addition, using smartphones is associated with several obesity risk factors (Kenny & Gortmaker, 2017). Another researcher suggested that depression, anxiety, and daytime dysfunction scores were higher in the high smartphone use group than in the low smartphone use group (Demirci et al, 2015). Therefore, it is suggested that long time using smartphones is related with not only food preferences but also health.

Figures 1 and 2 show that the number of preferences may decrease from childhood to high school age. In the (+) to (+) group, a significant decrease was observed the number of disliked foods from childhood to the present day by gender. Our data described that female have the number of their disliked food over male. The finding corresponds with the previous data about gender influences on food preferences reported by Caine-Bish and Scheule (2009). Gender differences should be considered (Figure 1).

In addition, green pepper and eggplant could be changed (Figure 2). People whose diet comprises more vegetables and fruits have greater longevity and are also protected from heart disease and cancer Bazzano LA (2006). Eating fruits and vegetables has been found to help prevent illness such as cancer and obesity (Ness &

Powles, 1997). The more children are exposed to a variety of vegetables, the more likely they are to like those vegetables (Birch & Marline, 1982). Therefore, it is good for children and adolescents to eat vegetable. According to Figure 1 and Figure 2, as they grew up, their disliked food may be decreased. If children and high school students would like to reduce dislike of food, we found that when the children's "Respect" and "Concern" levels increased, the percentage of children who disliked each of the top 10 disliked foods significantly decreased, showing a similar tendency in the number of foods which children disliked in relation to "Respect" and "Concern" reported by others (Osera et al., 2016a). Importantly, during high school age, children may decrease their preference especially for vegetables.

There are several inherent limitations of the present analysis. A limitation of the current study is that about 30% of the students answered "I do not know" or "I do not remember." It was also a limitation that it was a retrospective study due to recall bias. In addition, we only reported associations. We could not clearly demonstrate a cause and effect relationship. This study suggested a relationship between children's food preference and subsequent high school SRH and height and weight in males, but we do not know the strength of the health effects. In the next study, we will try to do the investigation or cohort study.

In conclusion, less dislike for certain foods in high school students was related to eating with somebody at present day as well as a lower number of dislike food items in childhood. Decreasing the dislike for foods at present as well as no dislike for any food item in childhood may be critical to developing good food habits in the future of high school students.

Acknowledgments

We thank all the students and teachers in Japan for their contributions to this study. We are also grateful to the teachers for their assistance.

Author contributions

Tomoko Osera, Setsuko Tsutie, and Nobutaka Kurihara developed the standardized protocol and structured questionnaire. Mitsuyo Awai, Misako Kobayashi conducted the focus group research in the high school. Tomoko Osera drafted the manuscript. All authors critically revised the article for important intellectual content and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Bazzano, L. A. (2006). The high cost of not consuming fruit and vegetables. *Journal of American Diet Association, 106*, 1364-1368. http://dx.doi.org/10.1016/j.jada.2006.06.021

Birch, L. L., & Marlin, D. W. (1982). I don't like it; I never tried it: Effects of exposure on two-year-old children's food preferences. *Appetite, 3*, 353-360. https://doi.org/10.1016/S0195-6663(82)80053-6

Boaedman, J. D. (2006). Self-rated health among US adolescents. *Journal of Adolescent Health, 38*, 401-408. http://dx.doi.org/10.1016/j.jadohealth.2005.01.006

Breidablik, H. J., Meland, E., & Lydersen, S. (2008). Self-rated health in adolescence: a multifactorial composite. *Scandinavian Journal of Public Health, 36*, 12-20. http://dx.doi.org/10.1177/1403494807085306

Caine-Bish, N. L. & Scheule, B. (2009). Gender differences in food preferences of school-aged children and adolescents. *Journal of School Health, 79*, 532-540. http://dx.doi.org/10.1111/j.1746-1561.2009.00445.x

Cashidan, E. (1994). A sensitive period for learning about food. *Human Nature, 5*, 279-291. http://dx.doi.org/10.1007/BF02692155

Demirci, K., Akgonul, M., & Akpinar, A. (2015). Relationship of smartphone use severity with sleep quality, depression, and anxiety in university students. *Journal of Behavioral Addictions, 4*, 85-92. http://dx.doi.org/10.1556/2006.4.2015.010

Deshmukh-Taskar, P., Nicklas, T. A., Yang, S., Berenson, G. S. (2007). Does Food Group Consumption Vary by Differences in Socioeconomic, Demographic, and Lifestyle Factors in Young Adults? The Bogalusa Heart Study. *Journal American Diet Association, 107,* 223-234. http://dx.doi.org/10.1016/j.jada.2006.11.004

Ilingworht, R. S., & Lister, J. (1964). The critical or sensitive period, with special reference to certain feeding problems in infants and children. *The journal of Pediatrics, 65*, 839-848. http://dx.doi.org/10.1016/S0022-3476(64)80006-8

Japan Sports Council. The report of food habits of elementary school children and junior high school students. (2010). http://www.jpnsport.go.jp/anzen/anzen/_school/tyosakekka/tabid/1490/Default.aspx (accessed 5 April 2017)

Joffer, J., Jerden, L., Ohman, A., & Flacking, R. (2016). Exploring self-rated health among adolescents: a think-aloud study. *BMC Public Health, 16*, 156. http://dx.doi.org/10.1186/s12889-016-2837-z

Kenney, E.L., & Gortmaker, S. L. (2016). United States Adolescents' Television, Computer, Videogame, Smartphone, and Tablet Use: Associations with Sugary Drinks, Sleep, Physical Activity, and Obesity. *The Journal of Pediatrics, 182*, 144-149. http://dx.doi.org/10.1016/j.jpeds.2016.11.015

Mechanic, D., & Hansell, S. (1987). Adolescent competence, psychological well-being, and self-assessed physical health. *Journal of Health and Social Behavior, 28*, 364-74.

Ness, A. R., & Powles, J. W. (1997). Fruit and vegetables, and cardiovascular disease: A review. *International Journal of Epidemiology, 26*, 1-13. https://doi.org/10.1093/ije/26.1.1

Nicklas, T. A., Baranowski, T., Baranowski, J. C., Cullen, K., Rittenberry, L., & Olvera, N. (2001). Family and child- care provider influences of preschool children's fruit, juice, and vegetables consumption. *Nutrition Reviews, 59*, 224-235. http://dx.doi.org/10.1111/j.1753-4887.2001.tb07014.x

Osera, T., Tsutie, S., Kobayashi, M., & Kurihara, N. (2012). Relationship of mothers' food preferences and attitudes with children's preferences. *Food and Nutrition Sciences, 3*, 1461-1446. http://dx.doi.org/10.4236/fns.2012.310190

Osera, T., Tsutie, S., Kobayashi, M., & Kurihara, N. (2014). A retrospective study on the relationship of changes in likes/dislikes with food habits in 4- and 6-year-old children. *European Journal of Nutrition & Food Safety, 4*, 604-613. http://dx.doi.org/10.9734/EJNFS/2014/10604

Osera, T., Tsutie, S., Kobayashi, M., & Kurihara, N. (2016a). Associations between children's food preferences and food haibts towards healthy eating in Japanese children. *Journal of Children & Adolescent Behavior, 4*, 1000292. http://dx.doi.org/10.4172/2375-4494.1000292

Osera, T., Tsutie, S., Kobayashi, M., Segawa, Y., Kajiwara, C., Hashimoto, H., et al. (2016b). The effect of mothers' and fathers' food preferences on children's preferences with their attitude. *European Journal of Nutrition & Food Safety, 6*, 93-100. http://dx.doi.org/10.9734/EJNFS/2016/24085

Piko, B. F. (2007). Self-perceived health among adolescents: the role of gender and psychosocial factors. *European Journal of Pediatrics, 166*, 701-708. http://dx.doi.org/10.1007/s00431-006-0311-0

Robinson-Cohen, C., Hall, Y. N., Reindl, M., & Vega, W. A. (2014). Validity of self-rated health aong lation(a)s. *American Journal of Epidemiology, 155*, 755-759. https://doi.org/10.1093/aje/155.8.755

Scaglioni, S., Salvioni, M., & Galimberti, C. (2008). Influence of parental attitudes in the development of children eating behavior.British *Journal of Nutrition, 99*(Supple 1), S22-25. http://dx.doi.org/10.1017/S0007114508892471

Sharif, M. Z., Rizzo, S., Marino, E., Belin, T. R., Glik, D. C., Kuo, A. A., et al. (2016). The association between self-rated eating habits and dietary behavior in two neighborhoods: Findings from Proyecto Mercado FRESCO. *Preventive Medicine Reports, 3*, 270-275.

Sharma, B., Nam, E. W., Kim, D., Yoon, Y. M., Kim, Y., & Kim, H. Y. (2016). Role of gender, family, lifestyle and psychological factors in self-rated health among urban adolescents in Peru: a school-based cross-sectional survey. *BMJ open*, e010149. http://dx.doi.org/10.1136/bmjopen-2015-010149

Sjoberg, A., Hallberg, L., Hoglund, D., & Hulthen, L. (2003). Meal pattern, food choice, nutrition intake and lifestyle factors in the Hoteborg adolescence study. *European Journal of Clinical Nutrition, 57,* 1569-1578. http://dx.doi.org/10.1038/sj.ejcn.1601726

St-Onge, M. P., Keller, K. L., Heymsfield, S. B. (2003). Changes in childhood food consumption patterns: a cause for concern in light of increasing body weights. *American Journal of Clinical Nutrition, 78*, 1068-1073.

Stratton, P. (2003). Contemporary families as contents for development. In: Valsiner J, Connolly, KJ (ed.) Handbook of developmental psychology. London: SAGE pp: 333-357.

Viner, R. M., Ozer, E. M., Denny, S., et al. (2012). Adolescence and the social determinants of health. *Lancet, 379*, 1641-52. http://dx.doi.org/10.1016/S0140-6736(12)60149-4

Vingilis, E., Wade, T., & Seeley, J. (2002). Predictors of adolescent self-rated health. Analysis of the National Population Health Survey. *Canadian Journal of Public Health, 93*, 193-197.

Vingilis, E., Wade, T., & Seeley, J. (2007). Predictors of adolescent health care utilization. *Journal of Adolescence, 30*, 773-800. https://doi.org/10.1016/j.adolescence.2006.10.001

Warde, J & Cooke, L. (2008). "Genetic and environmental determinants of children's food preference. *Journal of Nutrition, 99*, S15-21.

Warnoff, C., Lekander, M., Hemmingsson, T., Sorjonen, K., Melin, B., & Andreasson, A. (2016). Is poor self-rated health associated with low-grade inflammation in 43110 late adolescent men of the general population? A cross-sectional study. *BMJ Open, 6*, e009440. http://dx.doi.org/10.1136/bmjopen-2015-009440

Wu, S., Wang, R., Zhao, Y., Ma, X., Wu, M., Yan, X., et al. (2013). The relationship between self-rated health and objective health status: a population-based study. *BMC Public Health, 13*, 320. http://dx.doi.org/10.1186/1471-2458-13-320

Journal of Food Research; Vol. 6, No. 4; 2017
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Reviewer Acknowledgements

Journal of Food Research wishes to acknowledge the following individuals for their assistance with peer review of manuscripts for this issue. Their help and contributions in maintaining the quality of the journal are greatly appreciated.

Journal of Food Research is recruiting reviewers for the journal. If you are interested in becoming a reviewer, we welcome you to join us. Please find the application form and details at http://recruitment.ccsenet.org and e-mail the completed application form to jfr@ccsenet.org.

Reviewers for Volume 6, Number 4

Afef Janen, Alabama A&M University, United States

Alexandrina Sirbu, Constantin Brancoveanu University, Romania

Amira Mohamed Elkholy, Suez Canal University, Egypt

Antonella Santillo, University of Foggia, Italy

Arulmozhi Yuvaraj, Bharathiar University, India

Asima Asi Begic-Akagic, Faculty of Agriculture and Food Sciences, Bosnian

Beatriz Sevilla-Moran, INIA-Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Spain

Cristina Damian, Stefan cel Mare University of Suceava, Faculty of Food Engineering, Romania

Diego A. Moreno-Fernández, CEBAS-CSIC, Spain

Eduardo Esteves, Universidade do Algarve and Centre of Marine Sciences, Portugal

Elsa M Goncalves, Instituto Nacional de Investigacao Agrária (INIA), Portugal

Haihan Chen, University of California, United States

J. Basilio Heredia, Research Center for Food and Development, Mexico

Jose Maria Zubeldia, Gestión Sanitaria de Canarias – Gobierno de Canarias, Spain

Lenka Kourimska, Czech University of Life Sciences Prague, Czech Republic

Luis Patarata, Universidade de Trás-os-Montes e Alto Douro, Portugal

Marco Iammarino, Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, Italy

Marta Mesias, Spanish National Research Council, Spain

Marwa Ibrahim Abd El Hamid, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Paa Akonor, Council for Scientific and Industrial Research-Food Research Institute, Ghana

Palmiro Poltronieri, National Research Council of Italy, Italy

Shalini A. Neeliah, Ministry of Agro-industry and food security, Mauritius

Sonchieu Jean, Higher Technical Teachers Training College (HTTTC), University of Bamenda, Cameroon

Tinna Austen Ng'ong'ola-Manani, Lilongwe University of Agriculture & Natural Resources, Malawi

Winny Routray, McGill University, Canada

Xinyin Jiang, Brooklyn College, United States

ACADEMIC AND OPEN ACCESS

Canadian Center of Science and Education

CCSENET.ORG

➢ CALL FOR MANUSCRIPTS

Journal of Food Research (JFR) is a peer-reviewed, open access journal, published by Canadian Center of Science and Education. It publishes original research and applied articles in all areas of food studies. Authors are encouraged to submit complete unpublished and original works, which are not under review in any other journals. We are seeking submissions for forthcoming issues. All manuscripts should be written in English. Manuscripts from 3000–8000 words in length are preferred. All manuscripts should be prepared in MS-Word format, and submitted online, or sent to: jfr@ccsenet.org

Paper Selection and Publishing Process

a) Submission acknowledgement. If you submit manuscript online, you will receive a submission acknowledgement letter sent by the online system automatically. For email submission, the editor or editorial assistant sends an e-mail of confirmation to the submission's author within one to three working days. If you fail to receive this confirmation, please check your bulk email box or contact the editorial assistant.

b) Basic review. The editor or editorial assistant determines whether the manuscript fits the journal's focus and scope. And then check the similarity rate (CrossCheck, powered by iThenticate). Any manuscripts out of the journal's scope or containing plagiarism, including self-plagiarism are rejected.

c) Peer Review. We use a double-blind system for peer review; both reviewers' and authors' identities remain anonymous. The submitted manuscript will be reviewed by at least two experts: one editorial staff member as well as one to three external reviewers. The review process may take two to four weeks.

d) Make the decision. The decision to accept or reject an article is based on the suggestions of reviewers. If differences of opinion occur between reviewers, the editor-in-chief will weigh all comments and arrive at a balanced decision based on all comments, or a second round of peer review may be initiated.

e) Notification of the result of review. The result of review will be sent to the corresponding author and forwarded to other authors and reviewers.

f) Pay the publication fee. If the submission is accepted, the authors revise paper and pay the publication fee.

g) Send printed journals by post. After publication, two hard copies of the journal will be sent to the corresponding author.

h) Publication notice. The authors and readers will be notified and invited to visit our website for the newly published articles.

More Information

E-mail: jfr@ccsenet.org

Website: http://jfr.ccsenet.org

Paper Submission Guide: http://submission.ccsenet.org

Recruitment for Reviewers: http://recruitment.ccsenet.org

➢ JOURNAL STORE

To order back issues, please contact the editorial assistant and ask about the availability of journals. You may pay by credit card, PayPal, and bank transfer. If you have any questions regarding payment, please do not hesitate to contact the editorial assistant.

Price: $40.00 USD/copy Shipping fee: $20.00 USD/copy

ABOUT CCSE

The Canadian Center of Science and Education (CCSE) is a private for-profit organization delivering support and services to educators and researchers in Canada and around the world.

The Canadian Center of Science and Education was established in 2006. In partnership with research institutions, community organizations, enterprises, and foundations, CCSE provides a variety of programs to support and promote education and research development, including educational programs for students, financial support for researchers, international education projects, and scientific publications.

CCSE publishes scholarly journals in a wide range of academic fields, including the social sciences, the humanities, the natural sciences, the biological and medical sciences, education, economics, and management. These journals deliver original, peer-reviewed research from international scholars to a worldwide audience. All our journals are available in electronic form in conjunction with their print editions. All journals are available for free download online.

Mission

To work for future generations

Values

Scientific integrity and excellence

Respect and equity in the workplace

CONTACT US

General

Tel: 1-416-642-2606

Fax: 1-416-642-2608

E-mail: info@ccsenet.org

Website: www.ccsenet.org

Mailing Address

1120 Finch Avenue West

Suite 701-309

Toronto, ON., M3J 3H7

Canada

Visiting Address

9140 Leslie St., Suite 110

Richmond Hill, Ontario, L4B 0A9

Canada

The journal is peer-reviewed
The journal is open-access to the full text
The journal is included in:

AGRICOLA	Global Health	SHERPA/RoMEO
AGRIS	Google Scholar	Standard Periodical Directory
BASE	InfoBase Index	Ulrich's
CAB Abstracts	JournalFinder	WorldCat
Chemical Abstracts Service	JournalTOCs	
Electronic Journals Library	PKP Open Archives Harvester	

Journal of Food Research

Bimonthly

Publisher Canadian Center of Science and Education
Address 1120 Finch Avenue West, Suite 701-309, Toronto, ON., M3J 3H7, Canada
Telephone 1-416-642-2606
Fax 1-416-642-2608
E-mail jfr@ccsenet.org
Website http://jfr.ccsenet.org

ISSN 1927-0887