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Relationship Between Viscoelastic and Physicochemical Parameters in Cebreiro Cheese (PDO)

Inmaculada Franco[1], Verónica Bargiela[2], Javier Carballo[1] & Clara A. Tovar[2]

[1] Área de Tecnología de los Alimentos, Universidad de Vigo, Facultad de Ciencias de Ourense, Ourense, Spain

[2] Departamento de Física Aplicada, Universidad de Vigo, Facultad de Ciencias de Ourense, Ourense, Spain

Correspondence: Inmaculada Franco, Área de Tecnología de los Alimentos, Universidad de Vigo, Facultad de Ciencias, Ourense 32004, Spain. Tel: 34-988-387-055. E-mail: inmatec@uvigo.es

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Abstract

Rheological and physicochemical analyses of *Cebreiro* cheese were carried out. Four samples of each of three batches of cheese from different commercial producers were analyzed. Mechanical spectra data were obtained from frequency sweeps at 20ºC, a quality factor *Q* and fractal dimension *Df* were obtained from these rheological data. Moisture content, physicochemical (pH and titratable acidity) and proteolysis-related parameters were also measured. Analysis of the temperature dependence of the complex viscosity also enabled characterization of the activation energy of the three batches. The different viscoelastic parameters were highly consistent and structural differences between the different batches were observed. As regards the chemical composition level, high correlations between moisture, titratable acidity, nitrogen fractions and several viscoelastic parameters were observed. In general, casein networks were more extensive and elastic in batches with higher titratable acidity and moisture.

Keywords: acid coagulation, casein network, fractal dimension, loss tangent, *Cebreiro* cheese, quality factor

1. Introduction

Cebreiro is a traditional variety of cheese made in the north-east of Spain from pasteurized cow's milk. The cheese, which was awarded Protected Denomination of Origin (PDO) status in 2004 (Xunta de Galicia, 2004), is soft and creamy, with no holes, intensely crumbly and melts on the palate. Manufacture of this cheese is characterized by a predominantly acidic coagulation and prolonged whey drainage (Lafuente, Carballo, González Prieto, & Martín Sarmiento, 1995).

The physical properties of cheese are influenced by a number of factors including: milk composition, milk quality, temperature, rate and extent of acidification by the starter bacteria, pH history of cheese from coagulation process to final ripening, concentration of Ca salts (proportions of soluble and insoluble forms), extent and type of proteolysis and other ripening reactions (Lucey, Mishra, Hassan, & Johnson, 2005).

Cheese is a viscoelastic dairy gel, and the texture of the product is important because it is this property by which the consumer first identifies and judges the specific variety. The total Ca content, pH and proteolysis are critical parameters that influence the textural and physical properties of cheeses (Lawrence, Creamer, & Gilles, 1987; Lucey & Fox, 1993; Watkinson et al., 2001; Lucey, Johnson, & Horne, 2003; Sheehan & Guinee, 2004). The texture of a cheese is determined primarily by its pH and the ratio of intact casein to moisture (Antoniou, Petridis, Raphaelides, Ben Omar, & Kesteloot, 2000). The pH directly determines the number and balance of the charge distribution of amino acids on casein molecules (Swaisgood, 1993). The amount of insoluble Ca in cheese plays a key role in controlling the texture as it has a direct influence on casein–casein interactions (Lucey et al., 2003). Caseins possess important structural features that affect gelation, such as the variation in the degree of phosphorylation, glycosylation, hydrophobicity and amphipathic structures (Rao, 2007).

At the molecular level, cheese gelation involves the formation of a continuous network of casein molecules, in which the stress–resisting bulk properties (solid-like behaviour) are imparted by a framework of protein chains that extend throughout the gel phase. Acidification causes two major processes to occur. Firstly, the calcium and phosphate are dissolved out of the micelle as a result of protonation of the ionized phosphate groups, and the structure of the micelle is profoundly altered (Damodaran & Paraf, 1997). When the isoelectric point is

approached, caseins precipitate isoelectrically, and a three-dimensional solid-like network is formed by charge neutralization. The network formed may be designated as being fine-stranded or particulate (Rao, 2007). The microstructure of particulate gels consists of large aggregates, which are opaque and have a low water holding capacity. Acid milk gels are examples of "particulate gels" and have been described as fractal in nature.

To date, no information has been reported regarding the influence of physicochemical parameters on the viscoelastic properties of the *Cebreiro* cheese (PDO). The objectives of the present study were therefore: (a) to characterize the rheological properties of batches of *Cebreiro* cheese made by different manufacturers, and (b) to relate these properties with to physicochemical and proteolytic parameters.

Apart from helping to improve the quality of this variety of cheese, the results of this study will contribute data on the biochemical and rheological characters of fresh acid curd cheeses in general.

2. Materials and Methods

2.1 Samples

Three batches of *Cebreiro* cheese manufactured by three different producers following the industrial method described by Lafuente et al. (1995), in compliance with PDO status, were analyzed in the present study. Four samples were taken from each batch on different days. Each cheese sample consisted of one whole cheese. Samples were transported to the laboratory under refrigeration (below 4ºC) and were subjected to rheological test measurements on the day of sampling.

2.2 Moisture, pH, Titratable Acidity and Nitrogen Fractions

The water contents of the cheese samples were determined following the FIL-IDF 4A: 1982 standard method. The pH and titratable acidity were measured following the 14.022 AOAC method (1980). The total nitrogen content (TN) was determined by the Kjeldahl method, as described in the FIL-IDF 20B: 1993 standard. Water-soluble nitrogen (WSN) was determined by fractionation of the cheese samples with water, according to Kuchroo and Fox (1982). The trichloroacetic acid soluble nitrogen (TCASN) fraction was prepared by adding 7.5 mL of an aqueous solution of 48% (w/v) trichloroacetic acid to 22.5 mL of water-soluble extract; the mixture was allowed to stand at room temperature for 30 min and then filtered through Whatman No. 42 filter paper. The phosphotungstic acid soluble nitrogen (PTASN) test was carried out by adding 14 mL of 3.95 M sulphuric acid and 6 mL of 33.3% (w/v) phosphotungstic acid to 20 mL of water soluble extract; the mixture was allowed to stand at 4ºC overnight and subsequently filtered through Whatman No. 542 filter paper. Aliquots from both fractions were then analyzed by the Kjeldahl method. All measurements were made in duplicate.

2.3 Dynamic Rheological Measurements

All rheological measurements were performed with a Bohlin CVO controlled-stress rheometer (Bohlin Instruments, Inc. Cranbury, NJ). The measurement system used was a 20 mm parallel-plate. Cheese samples were cut into cylindrical samples of 20 mm diameter and 1 mm thick. Samples were placed between the parallel plates of the rheometer; the upper plate was lowered and stopped at a final gap of 1.0 mm from the lower plate. Any excess sample protruding beyond the upper plate was carefully removed. Samples were allowed to rest for 15 min before analysis to ensure both thermal and mechanical equilibrium at the time of measurement. A thin film of Vaseline® petroleum jelly (purissimum Codex) was gently applied to the edge of each exposed sample to prevent moisture loss. No evidence of specimen slippage at the bottom plate was detected. The temperature of the lower plate was maintained at 20.0 ± 0.1ºC in a Bohlin Rheology fluid circulating bath (also from Bohlin Instruments, Inc.) and controlled by use of a computer.

To determine the linear viscoelastic (LVE) region, a stress sweep (angular frequency 6.28 rad/s) was performed in the range of 10-2000 Pa at 20ºC. Changes in storage (*G'*) and loss modulus (*G''*) were recorded. The procedure for determining the limit values of stress (σ_{max}) and strain (γ_{max}), was explained in a previous study in which creep and recovery tests were used to corroborate the amplitude of LVE range (Campo-Deaño & Tovar, 2009). Frequency sweeps were carried out on the samples in the 0.1–10 Hz range at 0.5% strain, and changes in *G'* and *G''* moduli were recorded at 20ºC.

The values of complex viscosity ($\mu^* = G^*/\omega$) for all samples from the three batches were obtained at five temperatures, at ω = 6.3 rad/s and a fixed strain of 0.5% (within the LVE range). The selected temperatures were 20, 22, 24, 26, 28, and 30ºC.

2.4 Statistical Analyses

Significantly different means ($P < 0.05$) were compared by the least squares difference (LSD) test by use of the statistical software Statistica 5.1 for Windows (StatSoft Inc., 1996, Tulsa, OK, USA). Statistical correlations between

the biochemical and the rheological parameters were determined by multiple regression with confidence intervals of 95% ($P < 0.05$), 99% ($P < 0.01$) and 99.9% ($P < 0.001$), by use of the Statistica 5.1 software.

3. Results and Discussion

3.1 Biochemical Parameters

The values of the Total Solid (TS) contents, physicochemical parameters and nitrogen fractions of *Cebreiro* cheese are shown in Table 1. The moisture content is consistent with values reported for the same cheese, by other authors (Lafuente et al., 1995; Fresno, Prieto, Urdiales, Martín Sarmiento, & Carballo, 1995). The TS values were significantly lower ($P < 0.05$) in batch B samples than in batch *A* and *C* samples. This variation may be caused by the differences in intensity of whey drainage used by the different cheesemakers. Rearrangement of casein micelles during the whey draining process causes shrinkage of the casein matrix and subsequent expulsion of whey from curd. The rate and extent of whey drainage controls the moisture and lactose contents of curd (Castillo, Lucey, & Payne, 2006), which effects the moisture and pH of the cheese, and consequently the texture.

The low pH of *Cebreiro* cheese is due to the acid coagulation processes involved in production of the cheese (Lafuente et al., 1995). The pH values were similar in the three batches studied and are within the range described by other authors (Lafuente et al., 1995; Fresno et al., 1995).

Taking into account the observed pH values, similar values of titratable acidity would be expected, however the pH of batch *A* samples was significantly lower ($P < 0.05$) than the pH of batch *B* and *C* samples. This may be attributed to different draining intensities. The TS content and the titratable acidity were negatively and significantly correlated ($P < 0.01$, $r = -0.72$) (Table 5). The long coagulation periods with intense acidification induce extensive demineralization of the casein micelles. The large mineral losses in the whey (greater drainage in batch *A* samples) may be related to the buffering capacity of the mass, which would be lower in batch *A*, resulting in lower values of titratable acidity.

The values of the different nitrogen fractions in *Cebreiro* cheese indicate that this cheese undergoes a low level of proteolysis. These values are within the range of those observed in other unripe Spanish cheese and similar to those determined by other authors for this variety of cheese (Lafuente et al., 1995). The rennet that remains in the curd after whey drainage is responsible for the initial attack on caseins, forming large-sized peptides which are later degraded by the action of microbial and milk autochthonous enzymes. The low values of WSN and TCASN may be related to the small quantities of rennet used in the elaboration of this cheese.

The WSN and TCASN contents were positively and significantly correlated ($P < 0.05$) with moisture content ($r = 0.77$ and $r = 0.87$, respectively) and titratable acidity values ($r = 0.78$ and $r = 0.79$, respectively) (Table 5). The values of WSN and TCASN were significantly lower ($P < 0.05$) in the batch *A* cheeses than in batch *B* and *C* cheeses. This appears to be affected by the moisture content and therefore by the rennet retained in the curd.

Table 1. Changes in total solids, pH, titratable acidity and nitrogen fractions in three batches of *Cebreiro* cheese

	A	B	C
Total Solids*	43.78 ± 0.88^a	38.23 ± 2.37^b	41.41 ± 1.97^a
pH	4.31 ± 0.06^a	4.21 ± 0.05^a	4.22 ± 0.13^a
Titratable acidity**	1.06 ± 0.08^a	1.34 ± 0.13^b	1.25 ± 0.05^b
TN**	2.37 ± 0.13^a	2.27 ± 0.13^a	2.24 ± 0.08^a
WSN***	1.31 ± 0.13^a	1.76 ± 0.21^b	1.72 ± 0.29^b
TCASN***	0.64 ± 0.06^a	1.02 ± 0.09^b	0.89 ± 0.06^c
PTASN***	0.12 ± 0.01^a	0.11 ± 0.01^a	0.12 ± 0.01^a

[a] Means in the same file not followed by the same letter differed significantly ($P<0.05$);

* Expressed as g /100 g cheese;

** Expressed as g /100 g Total Solids*;

*** Expressed as g/100 g Total Nitrogen.

3.2 Oscillatory Tests at Small Strain and Constant Temperature

3.2.1 Amplitude Sweeps

These tests enable discrimination of two different regions: a linear viscoelastic (LVE) region, in which the viscoelastic moduli: complex modulus, (G^*) storage modulus (G') and viscous (G'') are almost constant, and a non linear region in which G' began to decrease while G'' began to increase, and finally both moduli tended to cross over).

As long as the γ-amplitudes are still below the limiting value γ_L, the structure of the sample remains stable under these conditions, and viscoelastic parameters possess physical meaning (Barnes, Hutton, & Walters, 1998). The limiting values of σ_L and γ_L in batches *A*, *B* and *C* are similar and statistically undistinguishable ($P < 0.05$) at 20ºC (Table 2). Moreover in all samples $\gamma_L < 1\%$, a finding that is consistent with the corresponding γ_L values for other Galician cheeses produced by enzymatic coagulation, and with a short ripening time (Tovar, Franco, Riveiro, Romaní, & Carballo, 2004a).

The G^* values for the three batches were not able to be distinguished because of the degree of experimental uncertainty (Table 2). G^* is related to the rigidity of the material, and can be used as a measure of the gel strength in different protein gels and their derivative products (Campo-Deaño & Tovar, 2009). Cheese appears to have a three-dimensional network, but it also flows, and therefore G^* is a measure of the "strength" of the interactions between the flowing units (Gabriele, de Cindio, & D'Antona, 2001) such as micelle aggregates. Thus the firmness of all samples of *Cebreiro* cheese was high and similar, since it is an unripe cheese, and therefore the degree of proteolysis was low (Table 1).

However it was possible to distinguish the loss tangent ($tan\delta$) within the LVE region $tan\delta = G''/G'$ is the ratio of the viscous to elastic properties, and is associated with relaxation of bonds in the gel during deformation (Lucey, 2002). The value of $tan\delta$ was higher in batch *A* than in batch *B* and *C* cheeses (Table 2). This may be attributed to a partial loosening of bonds within and between casein molecules in the gel network of batch *A* cheeses. On the contrary $tan\delta$ was significantly lower in batches *B* and *C*, in which the titratable acidity is significantly higher (Table 1), indicating that their casein matrices are more elastic than in batch *A*. The Horne model suggests that acidification of milk leads to solubilization of colloidal calcium phosphate (*CCP*), which loosens the bridging between caseins, which also disturbs the delicate balance between attraction and repulsion that governs micellar integrity (Horne, 1998). Moreover, solubilization of *CCP* appears to alter the balance between viscous and elastic components (Lucey, 2002), but not the overall rigidity of the network; this may explain the significant influence of titratable acidity on $tan\delta$ but not on G*. The low losses of *CCP* in *B* and *C* batches due to the lower degree of demineralization during whey drainage fortified the internal structure of the micelles; as a result the solid-like character of casein networks increased and $tan\delta$ decreased (Table 2).

Table 2. Linear Interval Limit values: shear stress, shear strain, complex modulus and loss tangent, for the three batches of *Cebreiro* cheese, from stress sweep test at 1 Hz and 20°C

	A	**B**	**C**
G* (kPa)	25.0 ± 9.3^a	27.2 ± 6.0^a	42 ± 15^a
σ_{lim} (Pa)	125 ± 12^a	154 ± 15^a	154 ± 15^a
% γ_{lim}	0.55 ± 0.22^a	0.59 ± 0.14^a	0.40 ± 0.16^a
tanδ	0.316 ± 0.011^a	0.281 ± 0.012^b	0.290 ± 0.001^b

[a] Values in the same file not followed by the same letter differed significantly ($P<0.05$).

3.2.2 Frequency Sweeps

The frequency dependence of G' and G'' can provide valuable information about the structure of a gel. A material whose G' and G'' are frequency-independent, with $G' > G''$, is a true gel. In contrast strong frequency dependence suggests a material structure with molecular entanglements (Ross-Murphy, 1984).

Mechanical spectra for batches *A*, *B* and *C* are shown in Figure 1. By performing a series of frequency sweep tests at small strain amplitude (0.5%), it was possible to confirm that the all samples of *Cebreiro* cheese behaved as solid-like materials ($G' > G''$), with G' one order of magnitude greater than G'' (Figure 1); this was consistent with the findings of Fernández-Abalat, Fernández, and Méndez (2006).

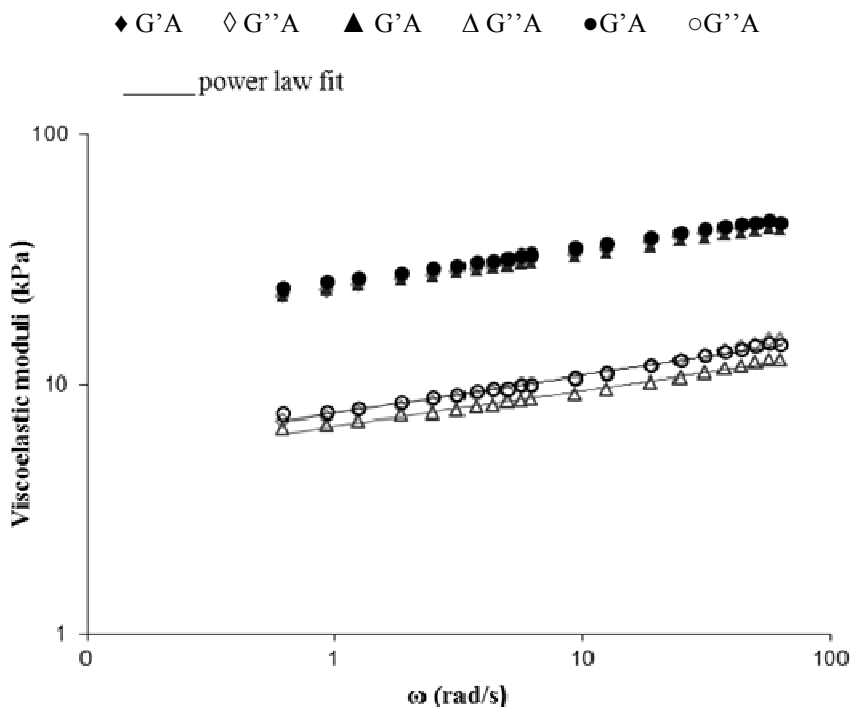

Figure 1. Mechanical spectra data for the three batches of *Cebreiro* cheese at 20ºC

Frequency sweep tests were used to fit *G'* and *G''* to the power law (Equations 1 and 2). *G'* is a measure of the deformation energy stored by the sample during the shear process, and represents the elastic behavior of a material. *G''* is a measure of the deformation energy used up by the sample during the shear process, and so lost by the sample; it represents the viscous behavior of the material.

$$G' = G_0^{'}\omega^{n'} \quad (1)$$

$$G'' = G_0^{''}\omega^{n''} \quad (2)$$

$$G^* = A_n\omega^n \quad (3)$$

where $A_n = (G_0^{'2}+G_0^{''2})^{1/2}$. The values of *G'* and *G''* were similar in all three batches at all range of frequencies; thus the overall rigidity of the casein networks is statistically indistinguishable (Table 3 and Figure 1). However there were significant differences in the *n'* and *n''* parameters (Table 3). The difference between the strong (true) gel and the weak gel can be established by mechanical spectroscopy (Clark & Ross-Murphy, 1987). In the former, the molecular rearrangements within the network were reduced over the time scales analyzed; *G'* and *G''* are almost independent of ω (low and similar *n'* and *n"* parameters). According to this, the gel characteristics of batches *B* and *C* (with lower *n'* and *n''* parameters) were better and these were therefore stronger gels than batch *A* (Table 3). Moreover the *n'* and *n''* parameters were negatively and significantly correlated ($P < 0.01$) with titratable acidity values ($r = -0.72$ and $r = -0.71$, respectively). This may be related to the fact that acidity values and moisture content were higher in batches *B* and *C* (Table 1), since the titratable acidity and moisture of cheese markedly affects its rheology due to its influence on paracasein hydration. Hydration of paracasein decreases as the pH is reduced further to 4.6 (Creamer, Lawrence, & Gilles, 1985), thus the casein aggregation increases, and the frequency dependence of the *G'* and *G''* diminishes (Table 3).

Table 3. Power law parameters of equations 1 and 2 for the three batches of *Cebreiro* cheese at 20°C

	A	B	C
Go'·(kPa·s^n)	23.7 ± 5.9[a]	23.7 ± 6.6[a]	25.3 ± 9.1[a]
n'	0.149 ± 0.001[b]	0.136 ± 0.001[a]	0.139 ± 0.001[a]
Go''·(kPa·s^n)	7.3 ± 1.5[a]	6.8 ± 1.8[a]	7.6 ± 2.7[a]
n''	0.170 ± 0.004[c]	0.142 ± 0.003[b]	0.151 ± 0.003[c]
r^2	0.9989	0.9993	0.9986

[a] Values in the same file not followed by the same letter differed significantly (P<0.05).

The striking structural differences among the three batches are also quantifiable in terms of the *quality factor Q*, a parameter frequently used in mechanical oscillatory systems. It is a dimensionless magnitude that is related to the degree of damping of an oscillator. Taking into account the oscillatory character of frequency sweeps, using equations 1 and 2, the following Equation (4) was proposed to discriminate the rheological quality of different food protein gels (Campo-Deaño, Tovar, & Borderías, 2010):

$$Q = 2\pi \frac{G_0^{'}}{G_0^{''}} \omega^{(n'-n'')} \tag{4}$$

The Q factor unifies parameters that provide different kinds of structural information: $G_0^{'}$ and $G_0^{''}$ are related to the strength of the intermolecular interactions and *n'* and *n''* to the extent and stability of the protein network.

The Q values were higher (P < 0.05) in batches *B* and *C* than in batch *A* (Figure 2), thus confirming that the casein networks have better gel characteristics in samples with higher titratable acidity and higher water content (Table 1). According to this, the Q factor was negatively and significant correlated (P < 0.05) with TS (Table 5), since if a total solid diminishes, the fluidity of protein networks increases, then the viscous losses decrease and Q increases. This result is consistent with the lower *tanδ* values in batches *B* and *C* determined by stress sweeps (Table 2); in these batches less *CCP* was solubilized and the interactions increased as a result of the greater number of crosslinks (Lucey et al., 2003), thus the elastic character of the different samples increased and loss tangent values decreased.

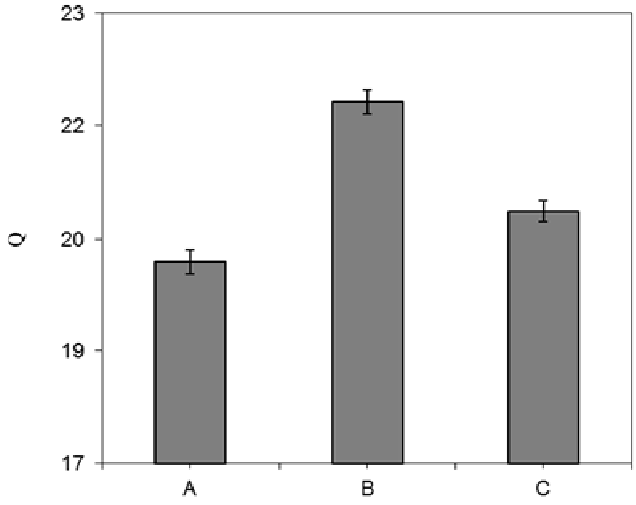

Figure 2. Comparison of quality factor values for the three batches of *Cebreiro* cheese at 1 Hz and 20ºC

3.2.3 Fractal Dimension

The concept of fractal geometry is a quantitative tool used to measure the complexity of gel structures by showing a pattern of self-similarity characterized by non-integer dimension: the fractal dimension (D_f). This

parameter reflects the degree of polymerization in the three-dimensional casein network, since casein micelles become aggregated on acidification, by charge neutralization, to form networks of chains and clusters (Heertje, Visser, & Smits, 1985). The fractal dimension can be calculated by rheological equations, starting from the power law frequency dependence of the complex modulus *n* values (Equation 3). The structure may be described by a fractal dimension D_f according to Equation 5 (Campo-Deaño et al., 2009):

$$n = \frac{d\left(d + 2 - 2D_f\right)}{2\left(d + 2 - D_f\right)} \tag{5}$$

where d is the spatial dimension, and D_f relates the molecular weight of the protein aggregate, M, to its cluster size R, by $R^{Df} \approx M$ (Celli et al., 2007). In general, when there is sufficient protein aggregation, the fractal aggregates grow until they occupy the entire liquid volume, at which point a three-dimensional continuous network or gel is formed.

Fractal dimension values were derived from Equation 5 to evaluate the different reticular extension of casein networks in batches *A*, *B* and *C*. The three D_f values calculated (≈ 2.4) are similar to those reported for other acid casein gels (Bremer, Van Vliet, & Walstra, 1989).

The value of parameter D_f at 20ºC was higher and similar in batches *B* and *C*, in which the titratable acidity values were undistinguishable ($P < 0.05$) (Table 1). The higher D_f values can be explained by taking into account that, when the pH decreases from 4.5 to 4, stronger gels are produced as a result of charge neutralization (Xiong, Aguilera, & Kinsella, 1991), leading to the formation of chains and clusters that are linked together to form more dense clusters of aggregated casein particles, which in turn aggregate to form a fractal aggregate. Also, parameter D_f was positively and significantly correlated ($P < 0.01$) with titratable acidity values (Table 5). It was recently suggested that cheese texture is determined by the balance between repulsive and attractive interactions in the caseins that form the protein network. Attractive interactions include *CCP* crosslinks, hydrogen bonds, and hydrophobic interactions (Lucey et al., 2003). In batches *B* and *C* there was an increase in attractive interactions since the moisture contents were higher than in batch *A* (Table 1), and demineralization lower, hence the number of *CCP* crosslinks among the casein micelles was higher, thus allowing reticular expansion and higher D_f.

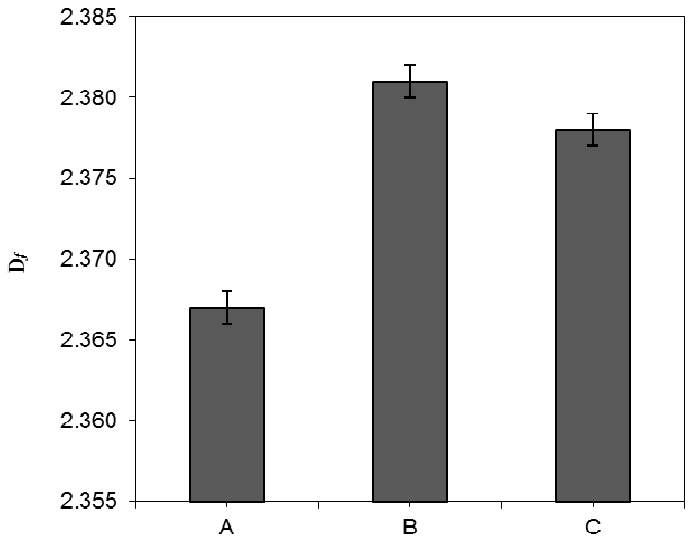

Figure 3. Comparison of fractal dimensions for the three batches of *Cebreiro* cheese at 20ºC

3.3 Oscillatory Tests at Small Strain at Different Temperatures

The complex viscosity (μ*) values for the three batches at 0.5% strain are shown in Table 4. A decrease in complex viscosity was observed with increasing temperature. For each cheese, a plot of μ* against the reciprocal of absolute temperature (1/T) produced a straight line that followed the Arrhenius equation (Equation 6):

$$\mu^* = A \cdot e^{Ea / RT} \quad (6)$$

Where A is the pre-exponential factor, E_a is the activation energy, and R is the gas constant (8.314 J/mol·K). The Ea values were obtained from the data in Table 4 and using equation 6 (45 ± 11, 61 ± 5 and 59 ± 7 kJ/mol for batches *A*, *B* and *C* respectively). The higher E_a values for the *B* and *C* batches indicate that their micellar networks break down less easily than in *A* batch (Tunick, Nolan, Shieh, Basch, & Thompson, 1990). This is consistent with the higher values of parameters D_f and Q, since their casein networks possess a large number of attractive interactions, and hence higher reticular extent and elastic character (Figures 2 and 3). Moreover, parameters Q and D_f were positively and significantly correlated ($P < 0.05$) with proteolysis data (Table 5). This is in apparent contradiction with the physical meaning of the three rheological parameters. However taking into account the values of the different nitrogen fractions (Table 1), it is possible to affirm that *Cebreiro* cheese undergoes a low level of proteolysis, which does not disturb the structural integrity of their micellar networks, resulting in lower and statistically undistinguishable values of PTASN in batches *A*, *B* and *C* (Table 1).

Table 4. Values of complex viscosity (kPa·s) at different temperatures for the three batches of *Cebreiro* cheese at 1 Hz

T (ºC)	A	B	C
20	5.0±1.0	5.1±1.0	4.7±1.3
22	3.4±0.9	3.9±0.6	3.4±0.5
24	3.7±0.7	3.7±0.6	3.4±0.8
26	3.57±0.5	3.2±0.6	3.0±0.5
28	3.07±0.4	2.6±0.5	2.4±0.3
30	2.26±0.3	2.1±0.3	1.9±0.3

Table 5. Some significant correlation coefficients obtained from the correlation matrix for variables analysed in *Cebreiro* cheese (n = 12)

Variables	r
TS – Titratable acidity	– 0.72**
TS – WSN	– 0.77**
TS – TCASN	– 0.87***
TS – Q	– 0.68*
pH – n’	0.71*
pH – n’’	0.64*
pH – D_f	– 0.71*
Titratable acidity – n’	– 0.72**
Titratable acidity – n’’	– 0.71**
Titratable acidity – Q	0.60*
Titratable acidity – D_f	0.73**
TCASN – WSN	0.82**
TCASN – n’	– 0.75**
TCASN – n’’	– 0.88***
TCASN – Q	0.77**
TCASN – D_f	0.78**
Ea – D_f	0.71**
Ea – Q	0.73**

* P<0.05; **P<0.01; ***P<0.001.

4. Conclusions

A detailed physicochemical and rheological study of *Cebreiro* cheese is presented. The viscoelastic parameters of all cheese samples, and consequently their textural properties, were significantly affected by the titratable acidity values and total solid contents. Several chemical parameters, such as titratable acidity, moisture content and nitrogen fractions were found to be correlated with different physical magnitudes, such as quality factor and fractal dimension.

Although the firmness of all samples was high and similar, the structural influence of demineralization effect in casein micelles was observed, in terms of the behaviour of the loss tangent in their fractal aggregates. Taking into account the overall physicochemical and dynamic oscillatory measurements, it was concluded that batches *B* and *C* possess better gel properties than batch *A*. Further research should focus on analyze the effect of heating process on the viscoelasticity under small deformations.

Acknowledgments

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The Association Between Ready-to-Eat Cereal Consumption, Nutrient Intakes of the Canadian Population 12 Years and Older and Body Weight Measures: Results From a Nationally Representative Canadian Population

Ann M. Albertson[1], Sandra G. Affenito[2], Julie M. Culp[1], Pierrette Buklis[3] & Nandan A. Joshi[4]

[1] Bell Institute of Health and Nutrition, General Mills Inc., Minneapolis,USA

[2] School of Health and Natural Sciences, Univeristy of Saint Joseph, West Hartford,USA

[3] General Mills Inc.,Canada

[4] General Mills India Pvt. Ltd., Mumbai,India

Correspondence: Ann M. Albertson, Principle Nutrition Research Scientist, General Mills: Bell Institute of Health and Nutrition, James Ford Bell Technical Centre, 9000 Plymouth Avenue North, Minneapolis, MN 55427, Tel: 763-764-4133. E-mail: ann.albertson@genmills.com

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Abstract

Background: To examine the relationship between ready-to-eat (RTE) cereal consumption habits and body mass index (BMI) of a nationally representative sample of Canadians. **Methods:** Population-based survey of Canadians aged 12 years and older. Participants provided 7-day self-reported food diary records during the data collection period of October 2003 through September 2004. Height and weight of the respondents was also reported. Main outcome measures included frequency of RTE cereal consumption, Body Mass Index (BMI), and nutrient intakes. The sample population of 2926 aged 12 years and older was divided into three groups by frequency of RTE cereal consumption over the 7-day period: 0-1 serving, 2-3 servings and 4+ servings. **Results:** The RTE cereal intake ranged from 0 to greater than 8 servings over the 7 days. Males who consumed 4+ servings of RTE Cereal had significantly lower mean BMI measures than the ones who consumed 0-1 serving ($P < 0.006$). Significantly lower proportion of Canadians who consumed 4+ serving of RTE cereal were classified as overweight or obese than those who consumed 0-1 servings in seven days ($p = 0.011$). Higher cereal intake group also had favourable nutrient intake profiles than the lower cereal intake group and were more likely to meet micronutrient intake recommendations. **Conclusion:** Self-reported RTE cereal consumption is related to lower BMI and improved nutrient intake in Canadians aged 12 years and older.

Keywords: ready-to-eat (RTE) cereal, nutrient intake, body mass index (BMI)

1. Introduction

In recent decades, overweight and obesity are increasing health-related concerns among the adolescent and adult populations in Canada (Langlois, Garriguet, & Findlay, 2009; Vanasse, Demers, Hemiari, & Courteau, 2006). Between 1985 and 1998, there was an almost threefold increase in obesity among Canadian adults, from 5.6% to 14.7%, respectively (Katzmarzyk, 2002). In addition, the 2004 Canadian Community Health Survey documented a 23.1% prevalence of obesity among individuals greater than eighteen years of age. These data demonstrate that obesity increased with age, but did not differ between men and women. However, it is noteworthy that the rates of obesity vary across Canadian health regions (Vanasse et al., 2006). These differences in obesity rates may be related to a number of factors including socioeconomic status, household income, education, or amendable lifestyle behaviours such as dietary intake, eating habits, and physical activity (Vanasse et al., 2006; Langlois et al., 2009; Garriguet, 2009; McLaren, Godley, & MacNairn, 2009). The etiology for obesity is complex and is influenced by modifiable lifestyle habits, such as eating patterns and physical activity, environmental and psychosocial factors and non-modifiable genetic determinants (Bray & Champagne, 2005). Similar to the results

of studies conducted in the United States (U.S.) (Kant & Graubard, 2006; Bowman & Vinyard, 2004; Howarth, Huang, Roberts, Lin, & McCrory, 2007) empirical research demonstrates that eating habits are changing among Canadians. Canadians are consuming more meals away from home (Woodruff & Hanning, 2009), with increased consumption of high energy, nutrient poor foods (Slater et al., 2009) and reduced intake of fresh fruit, vegetables and fibre (Langlois et al., 2009). These dietary changes likely negatively impact the body weight of Canadians. For example, empirical data have shown an association between persons who consume diets high in fibre and healthy body weights (Burgess-Champoux, Larson, Neumark-Sztainer, Hannan, & Story, 2010; Williams, Grafenauer, & O'Shea, 2008; Steffen et al., 2003) and, when adjusted for covariates, increased total energy and reduced fibre intake were significantly associated with obesity among men (Langlois et al., 2009).

In addition to consuming more meals away from home, there is evidence which points to the unfavourable association between breakfast omission and regulation of body weight. Research has shown that persons who skip breakfast are at increased risk for overweight (Williams, O'Neil, Keast, Cho, & Nicklas, 2009; Keski-Rahkonen, Kaprio, Rissanen, Virkkunen, & Rose, 2003; Cho, Dietrich, Brown, Clark, & Block, 2003; Ma et al., 2003). Breakfast consumption may be an indicator for associated healthful lifestyle behaviours including adequate physical activity patterns and enhanced nutrient intake and a high quality dietary intake (Song et al., 2006; Barton et al., 2005; Cho et al., 2003), thus facilitating body weight regulation (Song et al., 2005; Barton et al., 2005; Keski-Rahkonen et al., 2003). Across age groups, a commonly preferred breakfast selection is ready-to-eat (RTE) cereal because of its accessibility, taste, convenience, cost, and availability (Nicklas, McQuarrie, Fastnaught, &O'Neil, 2002; Williams et al., 2009).

There are over 160 varieties of ready-to-eat (RTE) cereal available across Canada. Many cereals are made from whole grains and provide fibre. A typical serving of Canadian RTE cereals is 3/4 c (175 mL) to 1c (250 mL). This is true of the most commonly consumed varieties, although nutrition information among these leading choices is presented in two reference amounts-either 30 g or 55 g. Nearly all RTE cereals in Canada are fortified according to the voluntary standard permitted by Health Canada, where it is possible to add, singly or in combination a specifically limited amount of only the following vitamins and minerals: thiamine, niacin, vitamin B_6, folic acid, pantothenic acid, magnesium, iron and zinc. Breakfast cereals are also typically low in fat and contain < 10 g of sugar per serving.

Ready-to-eat (RTE) cereal has the potential tonot only increase micronutrient, fibre, and whole grain intake, but to aid body weight control (Albertson et al., 2009; Albertson et al., 2008; Albertson Anderson, Crockett, &Goebel, 2003; Barton et al., 2005; Gibson, 2003; Berkey, Rockett, Gillman, Field, & Colditz, 2003). Indeed, studies conducted in the U.S. have documented an inverse association between the frequency of RTE cereal consumption and body weight (Albertson et al., 2003; Albertson et al., 2008; Albertson et al., 2009; Barton et al., 2005; Cho et al., 2003). To date, these data are not available for the Canadian population. Therefore, this research investigated the impact of RTE cereal consumption patterns on BMI and nutrient intake in Canadians ages 12+ using 7-day food intake methodology.

2. Methods

2.1 Dietary Intake Assessment

To determine the contribution of food consumption patterns on nutrient intake, a unique, proprietary nutrient assessment methodology was developed at the General Mills Bell Institute of Health and Nutrition (Albertson et al., 2003). This methodology combines National Eating Trends (NET) food diary data from the NPD Group (Canada, Toronto) with portion size estimates derived from six years of National Health and Nutrition Examination Survey (NHANES) collection, 1999-2004 (CDC) and nutrient data from the University of Minnesota's Nutrition Data System for Research (NDS-R) version 2008 (Nutrition Coordinating Centre, Minneapolis, MN). The resulting integrated database is processed and analysed using SAS® Version 9.2 (SAS Institute, Cary, NC).

2.1.1 Food Consumption Data/Participants

The current study uses the comprehensive food consumption data set made available from the NPD Group, a marketing information company, with their National Eating Trends® (NET) service. NET has been continuously tracking the eating habits of Americans since 1980 and Canadian population since 1998. The present research presents results on food intake from approximately 1700 households representing approximately 4,000 individuals. The sample is weighted to reflect the Canadian census based on region, household size, income, homemaker age/employment and ethnicity. The diary and reporting procedures are the same as in the U.S.

This study utilized NET data collected from October 2003 through September 2004, as documented by the Canadian Census Bureau database, which is balanced according to demographics and geographics. The variables are reported at the household level and include the ages of panel participants as well as household data such as income level, size, and age of head of household. Employment status and race are also reported. NET data are provided for fifty-two sub samples whereby nearly forty households record all food and beverages consumed weekly by all household members. Seasonal variations in food intake are considered as well, as the reporting is distributed evenly throughout the year. A daily "eating diary" for one week is maintained by each household. The person most responsible for meal preparation is instructed to record the name and brand of each food and beverage consumed by all members of the household, including all additives, ingredients and cooking aids.

The diary consists of separate sections for each meal and snack situation, and collects food names, flavour descriptors, brand names, package types, product forms, appliances used in preparation, and any special nutritional attributes, among other details. The same information is collected on ingredient and additive items used to create dishes or meals in the home. At the end of each day, the recorder is instructed to mail the daily diary back to The NPD Group. After all seven daily diaries are received from a household; they are coded and made ready for data processing. The sample of 1700 households (approximately 4,300 subjects, including 3531 individuals ages 12 and older) used for this study was weighted to match Canadian demographics (Table 1). Out of the 3531 individuals ages 12 and older, 2926 had complete BMI information and did not meet criteria for underreporting and, thus, were used for analysis. These persons were categorized according to their gender and RTE cereal consumption pattern.

Table 1. Canadian Demographics from Statistics Canada, 2005

Demographic		% Census
Female Head Age	18-24	4.1%
	25-34	16.2%
	35-49	32.6%
	50-64	26.3%
	65+	20.9%
Household Income[1]	<20	14.1%
	20-34	16.1%
	35-59	24.1%
	60+	45.6%
Household Size	1 Member	26.8%
	2 Member	33.6%
	3 Member	15.9%
	4 Member	14.7%
	5+ Member	8.9%
Presence of Children	Under 12	14.3%
	12 to 18	10.7%
	Both	5.7%
	No Kids	69.3%

[1]Household income reported in thousands of dollar.

2.1.2 Portion-Size Data

Canadian NET panellists record the foods and beverages consumed by household members but not the quantities. This procedure is standard for panel surveys to minimize recorder burden and thus increase reliability. Because national Canadian portion size data were unavailable, age and gender specific mean portion weights derived from NHANES 1999-2004 were assigned to each food recorded in the diary. Serving weights for individual food codes were aggregated and then collapsed for like-foods to strengthen cell sizes, and smoothed to eliminate outliers. Age and gender- specific mean serving weights were thereby determined for over 800 food types; these portions were subsequently assigned to each food recorded and coded in the NET diary.

2.1.3 Nutrient Data

Using the recipe component of the Nutrition Data System for Research (NDS-R) software, the investigators recorded and coded the nutrient values as documented in the NET diary. The NDS-R software analysis is precise and provides a complete nutrient values for 160 nutrients for more than 18,000 foods, including many brand-name products. Each food or recipe was entered into NDS-R per 100 grams of that food, and closely matched to the description provided in the NET diary. Recipes were created to account for foods with special nutritional attributes (i.e. low fat, fat-free, low cholesterol, calcium fortified, low sodium, or reduced sodium). Because NDS-R reports nutrient values for the U.S. food supply, considerable adjustment were made "recipes" to match nutrient values reported in Canadian Nutrient File (CNF) (Health Canada, Canadian Nutrient File, 2007). Particular attention was made to enriched and fortified foods such as ready-to-eat cereals and dairy products.

2.2 Data Tabulation

In order to estimate the potential effect of RTE cereal consumption on body weight measures and nutrient intakes, the frequency of RTE cereal consumption in 7 days was recorded for each participant. Intake of cereal was considered as a categorical variable. Because the distribution of cereal is not symmetrical and somewhat truncated, quartiles were not used to categorize the data. Rather, the population was classified into three groups based on cereal consumption patterns during the 7-day data collection period: 0-1, 2-3 servings and 4 servings and more servings. For the purposes of this analysis, a "serving" is a record of cereal consumption and is assigned an age/gender appropriate mean serving amount.

2.3 Body Mass Index

Individual, self-reported heights and weights were recorded in the diary for each respondent and used to calculate BMI according to the formula

$$BMI = weight\ (kilograms)/height\ (meters)^2 \quad (1)$$

Overweight was defined as a BMI $\geq$ 25 and obese was defined as a BMI $\geq$ 30 (29). Study sample participants who did not record height and/or weight (n=605) were excluded from the analysis. Final sample size of the study was 2926 adults (1246 men and 1680 women).

Table 2. Distribution of sample population by age, gender and BMI

Age-group	% Distribution
12-18	6.1
19-50	48.6
51-70	33.4
71+	11.9
Gender	% Distribution
Male	42.6
Female	57.4
Quantile	BMI Distribution
100%	54.8
99%	43.9
95%	37.4
90%	33.7
75%	29.3
50%	25.8
25%	22.8
10%	20.6
5%	19.4
1%	17.2
0%	12.7

2.4 Statistical Analysis

Means or proportions were computed by age and gender and according to RTE intake category. Analysis of variance was used to determine if BMI, per cent overweight/obese differed by age and RTE consumption categories. Pair wise t-tests were performed where differences were found among the categories. Logistic regression was used to analyse the association between total energy, macronutrient intake, RTE consumption pattern and intake. The contrasts were examined between the possible pairs of cereal consumption categories using the Wald chi-square. An alpha level of 0·05 was used to determine significance for the analysis of variance comparisons except where otherwise noted. All analyses were performed using SAS® version 9.2 (SAS Institute, Cary, NC).

3. Results

Over the seven days of dietary collection, intake ranged from zero to greater than eight servings of RTE cereal (Figure 1).

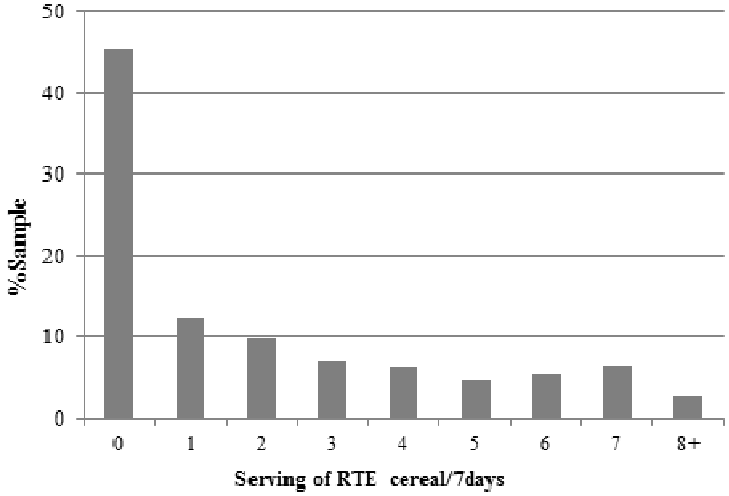

Figure 1: The distribution of ready-to-eat (RTE) cereal consumption among Canadians aged 12 years and older (n = 2,926) over the 7-day collection period

An inverse relationship between BMI and frequency of RTE cereal consumption for the total sample was directionally favorable but did not reach statistical significance (p = 0.062) (Table 3).

Table 3. Mean body mass index (BMI) of Canadians age 12 and older (n = 2,926) by gender and cereal consumption tertiles[1]

	0-1 Servings		2-3 Servings		4+ Servings		Total		p-value
	Mean± SEM	n	Mean± SEM	n	Mean± SEM	n	Mean± SEM	n	
Male	27.4 ± 0.19^{a}	744	26.7 ± 0.35^{ab}	196	26.0 ± 0.25^{b}	306	27.0 ± 0.14	1246	0.006
Female	26.6 ± 0.20	954	26.5 ± 0.39	298	26.3 ± 0.30	428	26.5 ± 0.16	1680	0.835
Total	27.0 ± 0.14^{a}	1698	26.5 ± 0.27^{ab}	494	26.2 ± 0.21^{b}	734	26.7 ± 0.11	2926	0.062

[1]Means within the same row with different letters are statistically significantly different ($p < 0.05$).

This relationship was statistically significant when males were considered alone ($p < 0.006$); however, did not hold true for females ($p = 0.835$). A statistically significant inverse relationship also existed for the proportion of overweight or obese and frequency of cereal consumption (Table 4). The proportion of Canadians aged 12 and older who were overweight or obese were significantly lower for those consuming four or more servings of RTE cereal during a seven day period than those consuming 0-1 servings in seven days ($p=0.011$) (Table 4). This relationship was also directionally favorable by gender, but did not reach statistical significance.

Table 4. Percentage of Canadians aged 12 (n = 2,926) or older categorized as overweight or obese by gender and the frequency of cereal consumption[1]

	0-1 Servings		2-3 Servings		4+ Servings		Total		p-value
	% Overweight/ Obese	n	% Overweight/ Obese	n	% Overweight / Obese	n	% Overweight/ Obese	n	
Male	67.5[a]	744	61.2[ab]	196	59.5[b]	306	64.5	1246	0.028
Female	53.9	954	50.0	298	50.2	428	52.3	1680	0.314
Total	59.8[a]	1698	54.5[b]	494	54.1[b]	734	57.5	2926	0.011

[1] Percentages within the same row with different letters are statistically significantly different ($p < 0.05$).

Total daily energy intake differed across cereal consumption tertiles (Table 5). There was a positive association between cereal consumption frequency and energy intake ($p < 0.0001$). Despite this association, there were no differences in total daily fat, saturated fat, trans fat, or cholesterol intake among cereal consumption tertiles (Table 5).Daily intakes of fibre, potassium, vitamin A, vitamin C, vitamin E, thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, calcium, vitamin D, magnesium, iron and zinc all increased with increasing consumption of RTE cereal (Table 5), and each of these relationships was statistically significant.

Table 5. Mean Nutrient Daily Intake of Canadians aged 12 years and older (n = 2,926) by Cereal Consumption Tertiles[1]

	Cereal Consumption				
Nutrients	0-1 Servings n=1698	2-3 Servings n=494	4+ Servings n=734	Total n=2926	p-value
Energy (kcal)	1853.4 ± 751.6[a]	1970.3 ± 734.9[b]	2083 ± 800.8[c]	1930.7± 767.5	<0.001
Total CHO (g)	222.5 ± 101.2[a]	250.1 ± 96.8[b]	274.6 ± 108.3[c]	240.2 ± 104.6	<0.001
Total Fat (g)	74.2 ± 31.2[a]	75.3± 31.2[a]	76.2 ± 33.6[a]	74.9 ± 31.8	0.351
Saturated Fat (g)	25.3 ± 11.5[a]	25.8 ± 11.6[a]	26.1 ± 12.7[a]	25.6 ± 11.8	0.293
Trans Fat (g)	5.8 ± 2.9[a]	5.9 ± 3.1[a]	5.7 ± 3.3[a]	5.8 ± 3.1	0.650
Cholesterol (mg)	257.5± 134.5[a]	260 ± 128.8[a]	249.1 ± 123[a]	255.8 ± 130.7	0.254
Total Protein (g)	71.5 ± 26.8[a]	75.6 ± 27.5[b]	80 ± 28.3[c]	74.3 ± 27.5	<0.001
Total Sugar (g)	100.2 ± 64[a]	114.3 ± 60.3[b]	126.2 ± 63.6[c]	109.1 ± 64.2	<0.001
Dietary Fiber (g)	13.6 ± 6.1[a]	16.3 ± 6.6[b]	20 ± 9.5[c]	15.6 ± 7.7	<0.001
Sodium (mg)	3355.7± 1316.9[a]	3551.3± 1341.1[b]	3548.3 ± 1340.5[b]	3437.1± 1330	0.001
Potassium (mg)	2407.1 ± 941[a]	2682 ± 1007.1[b]	3015 ± 1050[c]	2606 ± 1013.4	<0.001
Vitamin A (mcg rae)	613.8 ± 503.6[a]	688 ± 508.2[b]	735 ± 518.2[b]	656.7 ± 510.6	<0.001
Vitamin C (mg)	76.6 ± 62.4[a]	88 ± 63.5[b]	103.4 ± 67[c]	85.3 ± 64.7	<0.001
Vitamin E (mg α-tocopherol)	5.4 ± 3[a]	5.6 ± 2.8[ab]	5.9 ± 3.1[b]	5.5 ± 3	0.001
Thiamin (mg)	1.6 ± 0.6[a]	1.9 ± 0.7[b]	2.4 ± 1[c]	1.8 ± 0.8	<0.001
Riboflavin (mg)	1.9 ± 0.8[a]	2.1 ± 0.8[b]	2.3 ± 0.9[c]	2 ± 0.8	<0.001
Niacin (mg)	20.4 ± 7.9[a]	21.4 ± 8.3[b]	22.9 ± 8.4[c]	21.2 ± 8.2	<0.001
Vitamin B6 (mg)	1.4 ± 0.6[a]	1.6 ± 0.6[b]	1.8 ± 0.7[c]	1.6 ± 0.6	<0.001
Folate (mcg)	439 ± 179.3[a]	464.7 ± 180.5[b]	488.1 ± 184.6[c]	455.7 ± 182	<0.001
Vitamin B12 (mg)	4.1 ± 2.9[a]	4.6 ± 3[b]	4.9 ± 3.1[b]	4.4 ± 3	<0.001
Calcium (mg)	713 ± 363.8[a]	818.2 ± 379.2[b]	909.2 ± 401.9[c]	780 ± 385.4	<0.001
Vitamin D (mcg)	4.2 ± 2.8[a]	5.1 ± 2.8[b]	5.9 ± 3.2[c]	4.8 ± 3	<0.001
Magnesium (mg)	232.4 ± 94.2[a]	266.1 ± 100.5[b]	308.8 ± 121.1[c]	257.2 ± 107.5	<0.001
Iron (mg)	11.6 ± 4.5[a]	14.1 ± 4.7[b]	17.3 ± 6.9[c]	13.5 ± 5.8	<0.001
Zinc (mg)	9.0 ± 4.8[a]	9.7 ± 3.8[b]	10.5 ± 3.9[c]	9.5 ± 4.5	<0.001

[1] Means within the same row with different letters are statistically significantly different ($p < 0.05$).

The percentage of the population consuming less than 100% of their EARs was also analyzed by cereal consumption tertile. A high proportion of the sample did not meet their EARs for vitamin E (96.2%), magnesium (69.1%) and vitamin A (51.5%) (Table 6). The proportion of Canadians not meeting their EARs for vitamin A, vitamin C, thiamin, riboflavin, niacin, vitamin B6, vitamin B12, folate, magnesium, iron and zinc was significantly greater in the lowest tertile of cereal consumption (0-1 servings) when compared to the highest (4+ servings) (Table 6).

Table 6. Percentage of Canadians aged 12 and older (n = 2,926) not meeting their estimated average requirement (EAR) by cereal consumption tertiles[1]

	Cereal Consumption				
Nutrients	0-1 Servings n=1698	2-3 Servings n=494	4+ Servings n=734	Total n=2926	p-value
Vitamin A (mcg rae)	57.54[a]	49.39[b]	39.10[c]	51.54	<0.001
Vitamin C (mg)	54.53[a]	43.52[b]	33.51[c]	47.40	<0.001
Vitamin E (mg α-tocopherol)	96.70[a]	97.57[a]	94.28[b]	96.24	0.004
Thiamin (mg)	13.07[a]	2.63[b]	1.36[b]	8.37	<0.001
Riboflavin (mg)	9.42[a]	3.85[b]	3.00[b]	6.87	<0.001
Niacin (mg)	9.36[a]	5.87[b]	3.68[b]	7.35	<0.001
Vitamin B6 (mg)	38.99[a]	27.33[b]	16.08[c]	31.27	<0.001
Folate (mcg)	26.44[a]	21.46[b]	15.53[c]	22.86	<0.001
Vitamin B12 (mg)	15.31[a]	7.49[b]	6.54[b]	11.79	<0.001
Magnesium (mg)	78.21[a]	63.77[b]	51.77[c]	69.14	<0.001
Iron (mg)	13.25[a]	2.83[b]	0.27[c]	8.24	<0.001
Zinc (mg)	42.05[a]	32.79[b]	22.62[c]	35.61	<0.001
Calcium	71.32[a]	63.16[b]	53.95[c]	65.58	<0.0001
Vitamin D	95.58[a]	94.53[a]	88.15[b]	93.54	<0.0001

[1] Percentages within the same row with different letters are statistically significantly different ($p < 0.05$).

4. Discussion

To date, this research is the first to present the findings of RTE cereal consumption patterns on body weight measures and nutrient intake in Canadians aged twelve years or greater, using validated 7-day food intake methodology. Similar to previous studies conducted among persons in the U.S. (Albertson et al., 2003; Albertson et al., 2009; Barton et al., 2005; Cho et al., 2003; Song et al., 2005), this research demonstrated a positive association between consumption of RTE cereal and body weight measures. However, unlike past studies (Albertson et al., 2003; Albertson et al., 2009; Barton et al., 2005; Cho et al., 2003), the association between RTE cereal consumption and BMI differed by gender and weight status. An inverse assocation between BMI and frequency of RTE cereal consumption was statistically significant for males, as well as for the proportion of overweight or obese persons in the full sample population. Other research has shown that there are gender- and age-related differences in regularity in breakfast consumption, and hence RTE cereal intake. More specifically, evidence demonstrates that females omit breakfast more often than males, and this pattern of intake begins as children transition through adolescenceand is often related to the desire to lose weight (Woodruff & Hanning, 2009; Rampersaud et al., 2005; Berkey et al., 2003) Further, in efforts to lose weight, those persons who are overweight or obese omit the breakfast meal more often compared with those of normal weight (Alexander et al., 2009; Williams et al., 2008; Song et al., 2005). Thus, the relationship between RTE cereal consumption and body weight is complex, but it appears that RTE cereal may assist with control of body weight (Crockett & Affenito, 2012).

In addition to the potential contribution of RTE cereal consumption to regulation of body weight, findings of the current study point to the favorable association between RTE cereal consumption and nutrient intake. Those persons who consumed RTE cereal most often had improved nutrient intakes and were more likely to meet the dietary reference standard, compared with those who do consume RTE cereal less often or not at all. These results are in agreement with past research conducted in the U.S.(Deshmukh-Taskar et al., 2010; Albertson et al., 2003; Song et al., 2006; Barton et al., 2005; Gibson, 2003; Albertson et al., 2008; Cho et al., 2003), reflecting the significant contribution of RTE cereal to diet quality. However, despite increased nutrient intake in cereal consumers, intake of several nutrients, such as vitamin E, magnesium, and vitamin A, calcium, vitamin D, and potassium, remained below the reference standard. In studies assessing micronutrient intake among Canadians, the prevalence of reduced intake of calcium was found to be undesirably high across all age groups (Poliquin, Joseph, &Gray-Donald, 2009). Over recent years, calcium fortification and supplement use have reduced the prevalence of inadequacy, yet mean intakes remain below the Dietary Reference Intakes (DRIs), particularly for women over 50 years. Findings of the current study also parallel data from a rigorous study employing dietary assessment interviews of indigenous adults of 44 representative communities of Yukon First Nations (n=797), Dene/Métis, (n = 1007) and Inuit (n = 1525). Using the EAR cut-point method, nutrients of concern, which were defined to fall significantly below the goal value included magnesium, folate, vitamin A, vitamin C and vitamin E. In addition, for those nutrients in which Adequate Intake (AI) reference standards are available, under desirably high nutrient intakes were documented forfiber, n-6 fatty acids and calcium(Kuhnlein, Receveur, Soueida, & Berti, 2008). Further, intake of vitamin A was found to be suboptimal, particularly among the younger generations of Inuit men and women (Egeland et al., 2004).These results may reflect a reduced intake of fresh fruits and vegetables and dairy products (Garriguet, 2009) as well as increased consumption of convenience foods, snack foods, and meals eaten away from home (Woodruff & Hanning, 2009). Thus, nutrition education efforts are necessary to target these shortfall nutrients and to encourage healthful eating patterns at and away from the home environment.

Limitations of the current study should be kept in mind when interpreting these data. Dietary data were collected by self-report, a method that is subject to recall errors and underreporting (Garriguet, 2008). However, instructions provided for panellists to fully describe food intakes and return of daily food intake diaries may offset this limitation. A second limitation is related to the estimates of portion size, which were applied based on the average serving size for age 20 and gender groups reported in national surveys. When estimating the portion size, the estimates assumed that the average serving size applies to all individuals of same age and gender. This assumption clearly provides errors of the estimate for the individual. However, when applied to the total sample it would be expected that mean intakes would approximate estimates of intake provided by dietary survey data (Albertson et al., 2003). This appears to be true because of the agreement with previously published population-based surveys (Cho et al., 2003; Albertson et al., 2003). In addition, it should be noted that differences in the mean comparisons across categories of RTE cereal consumption cannot be explained by an error in accuracy of the estimate of total intake, an error which would be present across all categories (Albertson et al., 2003). Finally, nutrient intake was assessed by using data adjusted for day-to-day variation, which may have reduced error when estimating the prevalence of intakes below a specified reference value (Jahns, Arab, Carriquiry, & Popkin, 2005).

5. Conclusions

Canadians who frequently consume RTE cereals have healthier BMI and are less likely to be overweight or obese compared to those who seldom or never consume RTE cereals. In addition, RTE cereal consumption was related to improved nutrient intakes in Canadians age 12 years and older. As part of a healthful eating pattern, RTE cereal consumption should be encouraged to promote the maintenance of healthy body weight and positive nutrient intake.

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Microwave Irradiation for Dry-Roasting of Hazelnuts and Evaluation of Microwave Treatment on Hazelnuts Peeling and Fatty Acid Oxidation

Loredana F. Ciarmiello[1], Pasquale Piccirillo[1], Carmela Gerardi[2], Filippo Piro[3], Antonio De Luca[1], Francesco D'Imperio[4], Valerio Rosito[4], Palmiro Poltronieri[2] & Angelo Santino[2]

[1] Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Unità di Ricerca per la Frutticoltura, Caserta (CE), Italy

[2] CNR-Istituto di Scienze delle Produzioni Alimentari, Unità di Supporto di Lecce, Lecce (LE), Italy

[3] Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Centro di Ricerca per l'Orticoltura, Pontecagnano (SA), Italy

[4] EMITECH srl, Molfetta (BA), Italy

Correspondence: Pasquale Piccirillo, CRA-Unità di Ricerca per la Frutticoltura, Via Torrino 3, Caserta (CE) 81100, Italy. Tel: 39-0823-256-201. E-mail: pasquale.piccirillo@entecra.it

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Abstract

European hazelnut is an important nut crop in Italy, where about 121,750 tons of in-shell nuts are produced every year. Roasting is the most important practice for hazelnut preservation and commonly is carried out in commercial electrical ovens at 120-160°C for 10-20 min. This needful practice is time and energy expensive, so the development of new processing methods is required to reduce processing costs and to obtain top quality roasted nuts. The aim of this study was to develop a simple microwave treatment for hazelnuts peeling and roasting.

With this aim, some physical (colour, temperature, moisture) and chemical (taste, lipoxygenase activity, fatty acids, vitamins, sensory attributes) features of inshell nuts and kernels of three Italian hazelnut varieties (Tonda di Giffoni, Tonda Romana and Nocchione) after conventional oven or microwave roasting were evaluated.

Results showed that microwave roasting of kernels for 450 s gave a higher peeling score than the conventional oven treatment. This paralleled with better colour and taste scores for microwaved roasted kernels. Furthermore, a 360-450 s microwave roasting was able to inactivate almost completely lipoxygenases, avoiding adverse effects on fatty acids hydroperoxides and PUFA content. A shorter microwave treatment (360 s) was enough to obtain good peeling and sensory scores of inshell hazelnuts.

Taken together our results indicated that microwave technology can be successfully applied to both kernels and inshell hazelnuts to obtain suitable peeling and high quality roasted nuts.

Keywords: *Corylus avellana* L., hazelnut roasting, microwave, LOX activity, fatty acid

1. Introduction

European hazelnut (*Corylus avellana* L.) is the fifth important nut crop in the world and Italy has an average of 121,750 tons in-shell annual production (ISTAT, 2008). About 90% of the world crop is shelled and sold as kernels, whereas the remaining 10% is sold inshell for table consumption (Valentini et al., 2006).

Hazelnuts are a rich source of essentials minerals, sterols, tannins, free phenolic acids, sugars, organic acids and phenolic compounds (i. e. gallic acid), all greatly contributing to its peculiar sensory properties (Cristofori et al., 2008; Alasalvar et al., 2009; Alasalvar et al., 2010; Jakopic et al., 2011; Schmitzer et al., 2011). Due to their high polyphenols content, hazelnuts are recognised as a good source of natural antioxidants (Solar & Stampar, 2011).

Furthermore, hazelnuts contain unsaturated fatty acids, α-tocopherol and carotenoids which are reported to lower the risk of chronic diseases (Özdemir et al., 2001; Amaral et al., 2006; Köksal et al., 2006; Kornsteiner et al., 2006).

During storage, hazelnuts suffer significant changes in their chemical, physical, structural and sensorial properties with a consequent loss in their nutritional and quality value. Roasting is the most important practice for preservation improvement (Basaran & Akhan, 2010).

Conventional roasting of hazelnut is currently carried out by commercial electrical ovens at 120-160°C for 10-20 min depending on the shell thickness. This thermal process reduces moisture content from 4-6% to 1-3%; therefore contributing to reduce possible microbial contaminations and the activity of enzymes involved in lipid peroxidation (Demir et al., 2003). Roasting involves a number of physico-chemical changes including dehydration and chemical reactions. Maillard reactions in particular give rise to brown pigments and pyrazine compounds associated with the development of typical roasted flavour and of a light golden colour, while dehydration develops a crispy and crunchy texture (Saklar et al., 2001; Sagrero-Nieveso, 2006; Wang et al., 2011).

Flavor development by roasting is also a basic processing step of the multibillion dollar nut industry. The principal flavor component of the hazelnut is filbertone, which is formed during roasting (Özilgen & Özdemir, 2001).

Burdack-Freitag and Schieberle (2010) showed that roasting induced changes in the key volatile compounds of cv Tonda Romana and identified 46 aroma compounds in roasted hazelnuts.

The effects of roasting on enzymes involved in fatty acid oxidation, i.e. lipase and lipoxygenase, have not yet been fully elucidated. As reported for peanuts, oven roasting reduced oxidative activity of lipase and lipoxygenase (Adelsberg & Sanders, 1997; Schirack et al., 2006). In another work (Sanders et al., 1999), the same process was reported to slightly increase these enzymatic activities with little impact on shelf life.

Tensions generated by roasting at the interface between kernel and skin ease subsequent skin removal (peeling) by mechanical brushing. Commonly, a first heating step of 45 min at 85-90°C is used for peeling and, after peeling, a second step of 40-60 min at 120-160°C is carried out for roasting (Ory et al., 1992; Adelsberg & Sanders, 1997; Sanders et al., 2002; Alamprese et al., 2009). The percentage of pellicle removal increases proportionally with temperature increase.

Several other peeling methods have been proposed, even though they often require relatively long processing times, thereby increasing processing costs. Therefore, there is a need to develop new processing methods to obtain top quality roasted nuts.

As alternative, infrared heating was successfully used for dry-roasting and pasteurization of almonds (Yang et al., 2010). Infrared (IR) radiation is an energy in the form of electromagnetic wave with a more rapid heat transfer than convectional conduction mechanisms. IR heating has been found to be more effective compared to conventional heating, but requires an initial high capital cost (Krishnamurthy et al., 2008).

Dielectric processes of radiofrequency (RF) and microwave (MW) are among the fastest growing applications in food processing (Akgul et al., 2008). Microwave radiation is between common radio and infrared frequencies, being usually at 2.45 gigahertz (GHz)-or, in large industrial/commercial ovens, at 915 megahertz (MHz). Water, fat, and other substances in the food absorb energy from the microwaves in a process called dielectric heating. The frequency range of MW (300 MHz-3 GHz) corresponds to quantum energies that can be absorbed by the polar materials and as a result the food gets warmer.

There has been a great deal of research on the application of MW to food for a variety of purposes e.g., drying, cooking, fruits and vegetables blanching, pasteurization and disinfection (Brody, 1992; Ramesh et al., 2002; Akgul et al., 2008). Microwave was successfully used for drying of lettuce cubes, vegetable soup, carrot and apple chips (Cui et al., 2008; Wang et al., 2009; Feng et al., 2012).

Microwave treatment can be an attractive alternative to traditional processing methods because the treatment is faster than conventional ones, and heating takes place only in the food material and not in the surrounding medium, reducing energy costs (Giese, 1992).

During conventional roasting processes, moisture is initially removed from the surface of the nut and water moves from the interior of the product to the dried surface through a diffusion process. This phenomenon is time and energy consuming. In a microwave drying system, the product heating is associated with a volumetric heat generation, which leads to higher internal temperature resulting in an increase in internal vapour pressure which helps to push liquid flow towards the surface, producing higher drying rates (Akkarachaneeyakorn & Birlouez-Aragon, 2010). In a conventional roaster, the temperature increase to 120-160°C in nuts needs about one hour whereas microwave heating requires few minutes.

Indeed, as reported by Schirack et al. (2006) in their experiment on peanuts peeling, shorter heating times also lead to greater nutrient retention and better quality characteristics such as texture and flavour. Indeed, no changes in walnut, hazelnut and almond kernel lipids were detected after microwave roasting at full power for 180 s (Momchilova & Nikolova-Damyanova, 2007).

Microwave treatment of inshell hazelnuts has been found effective for reducing contamination of the aflatoxin producing micro-organism *Aspergillus parasiticus* without any noticeable change in nutritional and sensory properties of nuts (Basaran & Akhan, 2010).

The aim of this study was to assess the impact of microwave heating on peeling and some hazelnut quality characteristics and therefore, the development of a simple technique for hazelnut roasting. With this aim, a Microwave oven with 2.45 GHz radiation was applied directly to hazelnuts roasting to test a range of exposure times on both kernels and inshell nuts of three Italian varieties.

2. Materials and Methods

2.1 Plant Materials

Three cultivars of hazelnut (*C. avellana* L.) from the Fruit Tree Research Unit's collection (Pignataro Maggiore-Caserta, Italy) were chosen for their different peeling features: cv Tonda di Giffoni (cv T. Giffoni), high; cv Nocchione, average; cv Tonda Romana (cv T. Romana), low (Manzo & Tamponi, 1982; Farinelli et al., 2001; Bioversity International, 2008). Nut and kernel characteristics, including nut and kernel weight, shell thickness, kernel yield (%), kernel fibre were also evaluated for each cultivar (Table 1) (Bioversity International, 2008).

Table 1. Nut and kernel characters of the three cultivars. Carpological traits were evaluated from three years data

Cultivar	Nut Shape	Shape Ratio	Shell Seal	Shell Colour	Shell Tickness	Nut Weight (g)	Kernel Weight (g)	Kernel Yield (%)	Kernel Fibre Texture
Nocchione	sub-elliptic	spheroidal	smooth	light brown striated	high	2,7	1,1	40,7	strongly corky
Tonda Romana	sub-spherical	spheroidal	smooth	light brown striated	thin	2,7	1,3	48	lightly corky
Tonda di Giffoni	sub-spherical	spheroidal	smooth	brown striated	relatively thin	3	1,5	50	lightly corky

2.2 Hazelnuts Processing

Microwave treatments with exposures times of 240, 300, 360, 450 and 600 s, selected after preliminary trials, were performed and were compared with a conventional oven treatment of 20 min at 120°C. Exposures time was measured by a stopwatch.

The different treatments were applied to kernel and inshell nut samples from the three selected cultivars. Each treatment consisted of five replicates of 20 nuts. Samples, about 100 g, were placed in the rotating device of the microwave input area.

Nut samples from the three selected cultivars, harvested two months before, had been dried at 43°C to prevent quality deterioration and rancidity, frozen with carbon dioxide to destroy insect pests and stored according to good handling practices in clean, closed vials at room temperature.

All experimental treatments were performed in triplicate. Before processing, nut moisture was determined on samples of inshell nuts and kernels by oven drying for 10 hours at 130°C, using the modified procedure described by Walton and Wallace (2010) and expressing the moisture lost as a percentage from the original weight. Average temperatures of nuts before treatments, evaluated using thermal imaging, were on average 24°C.

Microwave applications were performed at the Emitech S.R.L (Molfetta-Bari, Italy) factory, using an ALTER microwave device with a microwave frequency of 2.45 GHz, producing 6 kW of microwave power, provided with a special fanning system to achieve homogeneous heat distribution.

The microwave system consisted of a metal structure and an inner metal surface that reflects microwaves into the cell. The surface was made of stainless steel (compatible with food products) with an asymmetric geometry

specifically designed to optimize the electromagnetic reflection. Access was through a high efficiency screening door. Glare surface inside the cell was further enhanced by the continuous rotation of a wave stirrer which reflected the microwave thus helping the generation of a statistically uniform electromagnetic field and consequently a uniform heating.

The microwave source consisted of an ALTER microwave generator with a maximum power of 6 kW connected to the cavity via an asymmetric type of WR340 waveguide. In order to improve the uniformity of dielectric heating, a device has been installed in the static device to enable the continuous mixing of the food matrix, simulating a dynamic treatment, obtaining more uniform temperature profiles.

Conventional roasting was performed in a Binder ED 23-720 oven (Binder GmbH-Tuttlingen, Germany).

A single layer of nuts was placed on a wire net, at half height of the oven chamber. Oven temperature was continuously registered by means of a thermo probe 80 T-150 UA (Fluke Corporation) placed at 10 cm above hazelnuts.

At the end of treatments kernels and inshell nuts were cooled at room temperature for 2 min, and hand-peeled. Hazelnuts were stored under vacuum at 4°C and analyzed within three days from treatment. Hazelnuts used for colour analyses, were kept at room temperature and analyzed within 24 h after roasting.

Before and after roasting experiments, external temperatures, heat distribution and maximum temperature of the hazelnut were steadily measured using an infrared camera whose emissivity was fixed at a value of 0.95 (Fluke IR Thermal Imager TI20).

2.3 Peeling and Sensory Evaluation

The roasted nuts were peeled by manual brushing the day after treatment and assessed for peeling, taste and colour by an industry expert. Peeling was expressed in percentage; colour assessment was classified in three classes (RSH, 1966, 1986, 1995); taste assessment was in five classes: raw, lightly roasted, roasted, darkly roasted and burnt. Taste and colour (creamy, tanned, browned) classes were combined in a sensory index by geometric averaging after transformation in percent scores (Wang et al., 2009). An overall grade index was synthesized combining the peeling and sensory scores, also by geometric averaging.

2.4 Protein Extraction and Lipoxygenase Activity

Soluble protein samples were extracted from hazelnut kernels as previously reported (Santino et al., 2003). Lipoxygenase (LOX) activity was assayed spectrophotometrically, monitoring the increase in A_{234} of the conjugated-diene structures as previously reported by Santino et al. (2003). Linoleic acid was used as substrate in a reaction mixture (1.0 mL) consisting of 100 mM sodium phosphate buffer pH 6.0 containing 0.3 mM substrate and different amounts of protein samples. The enzymatic activity (expressed as nmol of hydroperoxide formed min^{-1} mg^{-1} of proteins) was calculated by measuring absorbance changes at 234 nm using a molar extinction coefficient of 25000 M^{-1} cm^{-1}. Proteins were quantified by the Bradford dye-binding method (1976) using bovine serum albumin (BSA, Sigma) as a standard.

2.5 HPLC Analysis of Hydroperoxy Fatty Acids

Free fatty acids (FFA) and polyunsaturated fatty acids (PUFA) hydroperoxides were extracted with chloroform/methanol (2:1, v/v) from grounded hazelnut kernels as previously reported by Mita et al. (2007). FFA were dried and resuspended in methanol before monitoring the absorbance at 234 nm. An aliquot of the extracts was submitted to RP-HPLC with a C18 Ultrasphere column (Beckman, 0.46 x 25 cm) and a solvent system of methanol: water: acetic acid (85:15:0.1). Detection of hydroperoxy fatty acids (HFA) was carried out recording the absorbance at 234 nm (indicating the conjugated diene system).

2.6 Tocopherol Extraction and Quantification

Hazelnut kernels (0.1 g) were frozen in liquid nitrogen and ground in a mortar. Samples were incubated in a screw-capped tube with 10 mL 12% potassium hydroxide, 20% (v/v) ethanol, 0.1% (w/v) sodium chloride and 3% (w/v) pyrogallol. After alkaline digestion at 70°C for 30 min and subsequent cooling, 15 mL 1% (w/v) sodium chloride solution was added. The sample was then extracted twice with 15 mL n-hexane:ethyl acetate (9:1). The organic phase was collected, evaporated and the dry residue was dissolved in 1 mL 98% (v/v) methanol. A sample volume of 20 µL was separated by reverse phase (RP)-HPLC. Chromatographic separation was performed using a Beckman HPLC Analytical System. An aliquot of the extracts was submitted to RP-HPLC with a C18 Ultrasphere column (Beckman, 0.46 x 25 cm) in methanol (98%, v/v). Detection of tocopherols was carried out recording the absorbance at 289 nm. The tocopherol content was calculated by means of standard calibration curves.

2.7 Determination of Fatty Acids

Fatty acids methyl esters were prepared according to standard methods (Ichihara & Fukubayashi, 2010) and analysed on a GC-MS system (Shimadzu 17A) fitted with a DB-5 capillary column (30 m x 0.25 mm ID and 0.25 µm thickness). Oxygen-free nitrogen was used as carrier gas at a flow rate of 1.0 $mL \cdot min^{-1}$. Other conditions were as follows: initial oven temperature, 80ºC, ramp rate $10ºC \cdot min^{-1}$ up to 150ºC, than $5ºC \cdot min^{-1}$ up to 250ºC. Compounds were identified by using online NIST-library spectra and published MS data. The FA composition was reported as a relative percentage of the total peak area. Nonadecanoic acid was used as an internal standard.

2.8 Statistical Analysis

Statistical summaries of treatment effects on the peeling, sensory and overall response indexes were calculated applying a general linear model to the set of all factor combinations and a mixed model with random effects for varieties to the treatment x shell condition combinations, with average batch values as observation units, using the logits of the indexes after scaling to proportions, in order to satisfy model requirements and preserve score boundaries. Expected values and their predictive intervals for treatment combinations, back-transformed to the percent scale, were obtained by simulated distributions of model coefficients. Computations and graphical presentations of their results were performed with the *R* environment (Holleczek & Brenner, 2009; R Development Core Team, 2010; Holleczek & Brenner, 2012) and functions of the *arm* (Gelman et al., 2010), *lme4* (Bates & Maechler, 2010), *rms* (Harrell, 2012) and *ggplot2* (Wickham, 2008) packages.

3. Results and Discussion

3.1 Effect of Microwave Treatments on Hazelnut Roasting and Taste

The effects of microwave and oven roasting on taste, colour and peeling were compared for all combinations of treatments and hazelnut samples (Figure 1). Relative frequencies of different taste and colour scores after oven (1200 s) and microwave (240, 300, 360, 450, 600 s) treatments were compared for both inshell hazelnuts and kernels of the selected three cultivars (Figure 1).

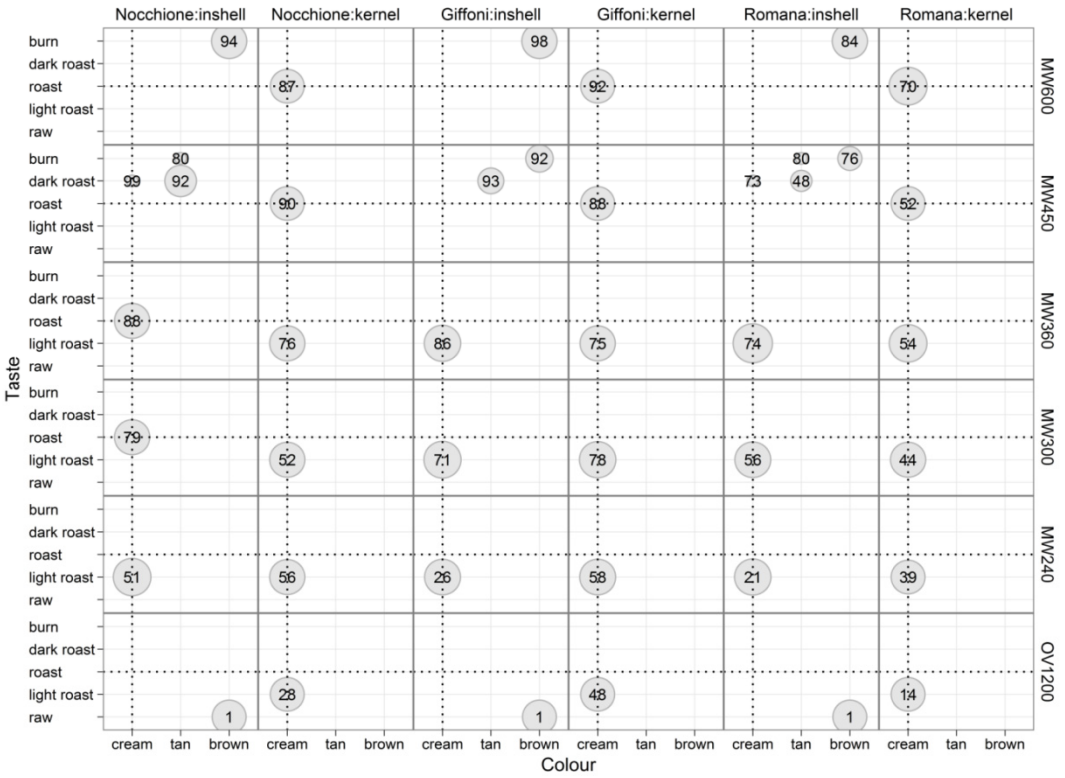

Figure 1. Relative frequency of taste and color classes (size of circles) and percentage of skin removal (numbers in circles) of inshell nut and kernels from three hazelnut varieties after conventional oven roasting for 1200 s (OV1200) and microwave oven roasting for 240 to 600 s (MW). The best outcome is marked by the crossing of the dashed lines

The best taste score was recorded with 450 s microwave treatment for kernels of Nocchione and T. Giffoni cultivars and with 600 s treatment for cv T. Romana (Figure 1). Conventional oven roasting gave creamy, lightly roasted nuts in roughly similar proportions for all cultivars.

In the case of unshelled hazelnuts, a smaller roasting time (360 s for cv Nocchione and cv T. Giffoni; and 450 s for cv T. Romana) was enough to obtain the best taste score. Longer exposures (450-600 s) resulted in darkly roasted or burned inshell nuts from all cultivars.

Inshell nuts showed, after conventional oven treatment, a peeling score of 0% and a higher score for kernels, with the highest average score observed for cv T. Giffoni (48%).

The peeling score increased with exposure time both for kernels and inshell nuts of all cultivars, though inshell nuts tended to peel better than kernels with longer microwave exposures. Varieties differences in peeling ability still persisted, since cv Nocchione and cv T. Giffoni showed a peeling score higher than cv T. Romana.

The comparison between the peeling and sensory scores between the conventional oven and the 240 s microwave treatment is summarized in boxplots (Figure 2). Boxplot gives a good sense of data distribution (median, minimum and maximum) relative to different processing methods and gives a good idea about the distance of the data to the extremes if they lie near the median (Upton Gand Cook, 1996).

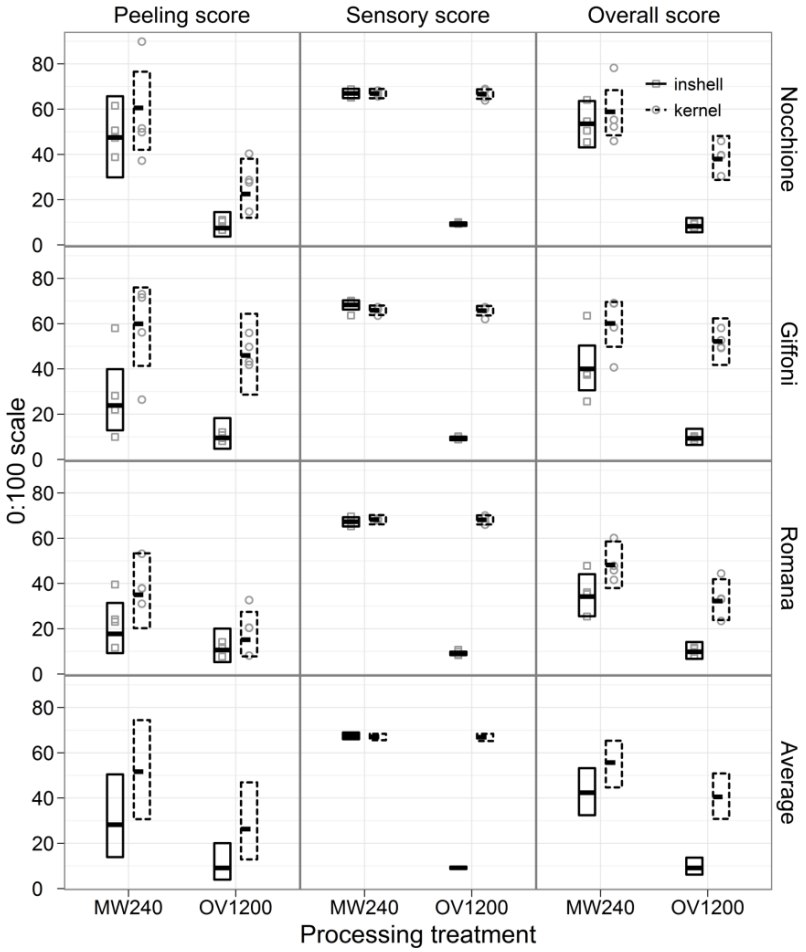

Figure 2. Effect of roasting by microwave treatment for 240 s (MW240) vs conventional oven for 1600 s (OV1600) of inshell nuts and kernels on peeling score (peeling), sensory (combination of color and taste) score and their combination (overall) score for three hazelnut varieties (cv Nocchione, cv T. Giffoni, cv T. Romana) and averaged over varieties. Boxplots displaying the extremes, the upper and lower values, and the median of the maximum difference within a category. Observed values (symbols) and means with 95% confidence intervals (crossbars). Black lines represent confidence intervals for inshell nuts; black dashed lines represent confidence intervals for kernels trends

Peeling was generally higher for kernels and microwave treatment, with the highest score (60%) observed for kernels of cv Nocchione and T. Giffoni. Remarkably, cv Nocchione showed a significant higher peeling score either for kernels and inshell nuts after microwave treatment in comparison with traditional oven (Figure 2; peeling score). The sensory scores were similar without significant differences after both treatments either for kernels as for inshell nuts (Figure 2; sensory scores). The overall score, obtained combining peeling and sensory scores, was significant higher for microwaved inshell nuts than oven roasted samples (Figure 2; overall score). The overall score for microwaved and oven roasted kernels were comparable, even though a higher score was recorded from microwaved samples.

Taken together these results indicated that the microwave treatment of 240 s outperformed the conventional oven treatment on both the peeling and sensory indexes, showing a significantly higher overall score for inshell nuts (Figure 2).

The response of peeling and sensory scores to different microwave exposure times is reported in Figure 3.

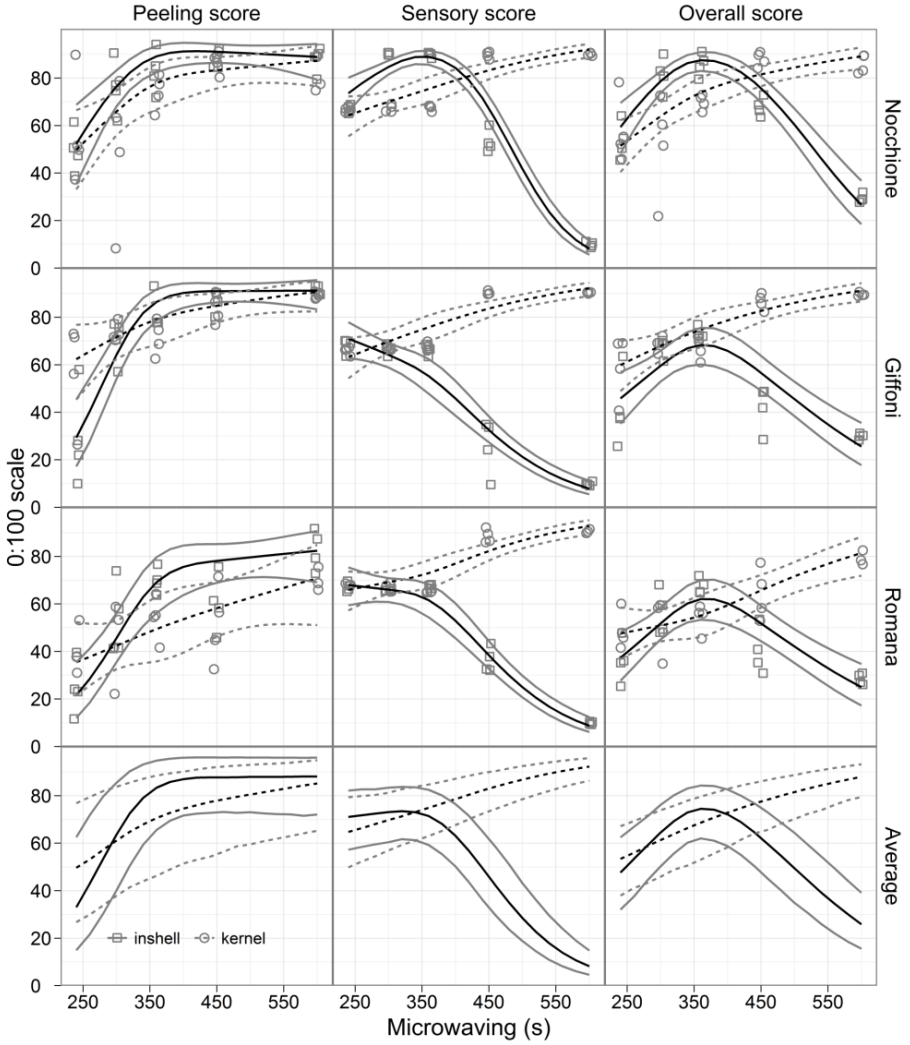

Figure 3. Effect of microwave roasting (from 240 to 600 s) on inshell nuts and kernels on peeling score, sensory score (combination of color and taste) and their combined (overall) scores for three hazelnut varieties (cv Nocchione, cv T. Giffoni, cv T. Romana) and averaged over varieties. Observed values (symbols) and smoothed trends with 95% confidence bands. Gray lines are minimum and maximum trends for inshell nuts; gray dashed lines are minimum and maximum trends for kernels. Black lines represent medium trends for inshell nuts; black dashed lines represent medium trends for kernels

A smooth fit was preferred over other fitting choices with the aim of showing essential trends. The peeling scores increased slightly with the increase of the microwave exposure from 240 to 360 s in inshell nuts of all three cultivars. In particular, the recorded peeling percentage reached 90% at 360 s from 20% at 240 s in the cv Nocchione, from 30% to 90% in the cv T. Giffoni and from 20% to 70% in the cv T. Romana (Figure 3, peeling score). At longer exposure times, the peeling score reached a plateau and did not show any further increase. A similar trend was observed with kernels whose peeling scores showed only a marginal increase at MW exposure times longer than 360 s. At the 240-360 s MW treatment range, the sensory scores were similar either in inshell nuts and kernels (Figure 3, sensory scores). At longer exposures, the recorded trend of sensory scores was opposite, since it increased in kernels and sharply decreased in inshell nuts. The overall score allowed to identify the best MW processing time for inshell nuts and kernels, i.e. 360 s and 450/600 s, respectively (Figure 3, overall score).

According to Mitcham et al. (2004), the diverging behaviour in the sensory scores recorded for kernels and inshell nuts after microwave treatments longer than 360 s, may be due to different water contents and consequently different temperatures were reached during treatment. Humidity content, determined before treatment application, was 2.2 times higher for inshell nuts (6.4% and 2.9% for inshell and kernels, respectively). The recorded humidity level of kernels was adequate for storage and comparable to levels found in peanuts subjected to peeling trials (Sanders et al., 2002). Improved peeling of peanuts after microwave treatment (Adelsberg & Sanders, 1997) was positively related to a higher water content. The temperatures of hazelnuts batches calculated on the average of temperatures of single nut, were higher for inshell nuts (about 3°C, 8°C, 14°C for cv T. Romana, T. Giffoni and Nocchione, respectively; Figure 4).

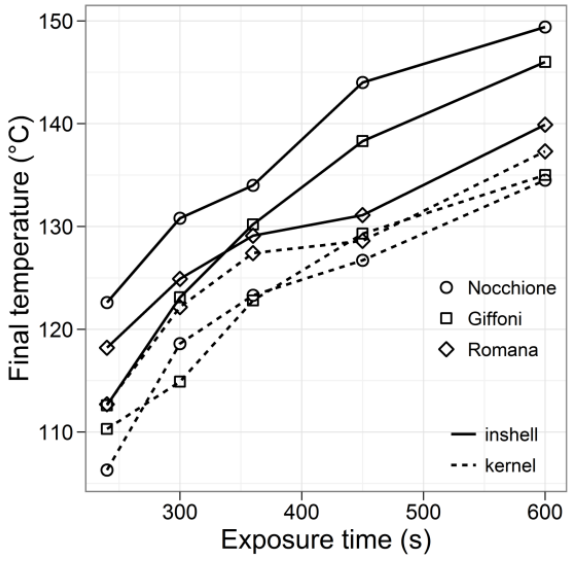

Figure 4. Temperature recorded in inshell hazelnuts and kernels of three varieties after roasting by microwave and traditional oven. Dots, rectangles and rhomboids represent cv Nocchione, cv T. Giffoni and cv. T. Romana respectively. Black lines represent inshell nuts; black dashed lines represent kernels

Shell thickness and the presence of fibres around the kernel, two well known cultivar related genetic traits, might affect the maximum temperature of the inshell nuts. Indeed, a thick shell might preserve a higher temperature inside the shell for longer times compared to a thin shell, with a consequent negative effect on the taste and the colour of the kernels. However, temperatures higher than 135°C, as those reached by inshell kernels with microwave exposures longer than 360 s, resulted in an excessive roasting with a negative impact on sensory scores.

Moreover, according to other authors (Giese, 1992; Akkarachaneeyakorn & Birlouez-Aragon, 2010) microwave treatment reduces process energy costs. Energy consumption for microwave drying (about 0.6 kW for the longest tested treatment) was about a half of that of the conventional oven (1.34 kW).

3.2 Effect of Microwave on Hazelnut Quality

Lipoxygenase (LOX) activity was assayed in kernels and inshell hazelnuts after microwave exposition ranging from 240 to 600 s using untreated dry hazelnuts and oven roasted kernels as controls. Data recorded were summarized in boxplots (Figure 5A).

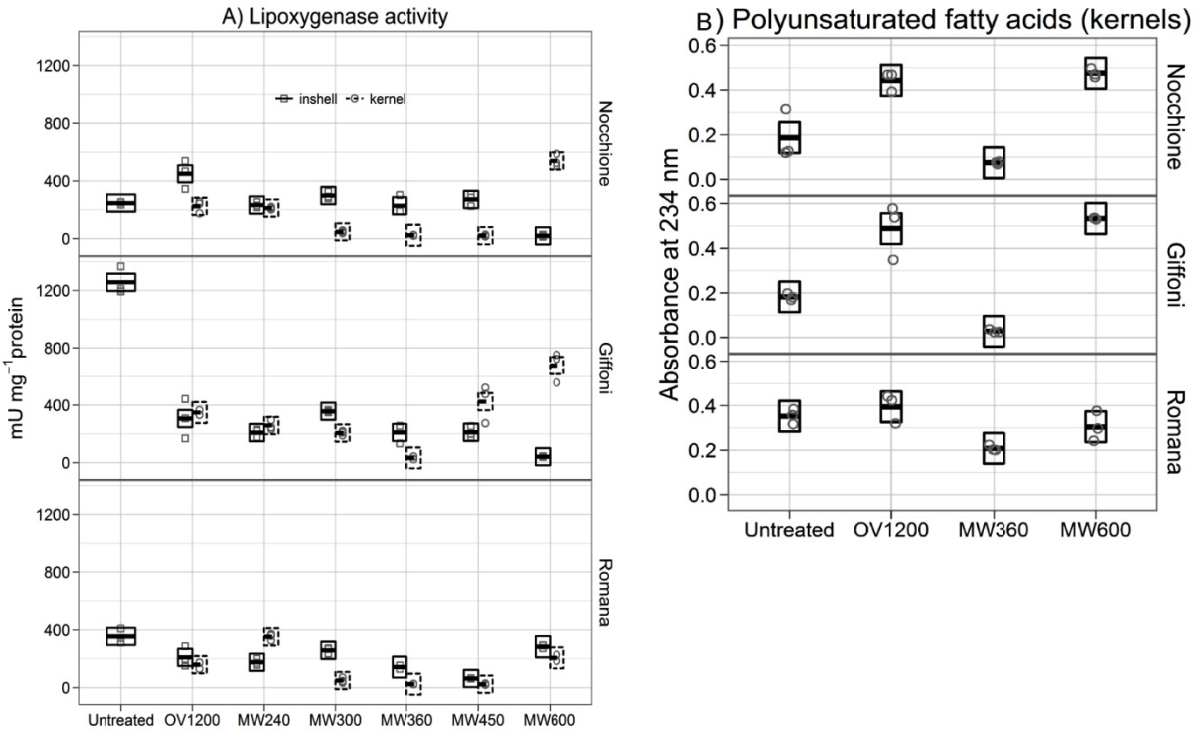

Figure 5. Effect of roasting treatment by microwave oven for 240 s up to 600 s of inshell and kernels of three hazelnut varieties (cv Nocchione, cv T. Giffoni, cv T. Romana) on lipoxygenase activity (A), polyunsaturated fatty acids (PUFA) hydroperoxides (Abs_{234} nm; B). Observed values (symbols) and means with 95% confidence intervals (bars)

Hazelnuts belonging to cv T. Romana and Nocchione showed a similar LOX activity in all the conditions here considered. A higher LOX activity was recorded in untreated samples from cv T. Giffoni (Figure 5 A). These results may indicate some variability in LOX activity among different hazelnut cv. Roasting with conventional or microwave oven was able to reduce LOX activity in most the samples analysed. In general, LOX activity in hazelnut samples after microwave treatment was lower than that recoded from samples roasted with conventional oven. The best results in term of LOX inhibition were obtained with kernels after microwave roasting of 360 s. In these conditions, LOX activity was almost undetectable. However, increasing the roasting time to 600 s, resulted in a rapid increase of LOX activity. This was more evident in kernels samples from cv T. Giffoni and T. Romana.

To confirm these results, the absorbance of free fatty acids (FFA) at 234 nm, indicative of polyunsaturated fatty acids (PUFA) hydroperoxide content, was monitored. A net increase in the hydroperoxide content was observed in hazelnuts roasted with a traditional oven or microwaved for 600 s. On the other hand, microwave roasting for 360 s resulted in a significant decrease in hydroperoxides (Figure 5 B). These results confirmed that microwave roasting for 360 s was able to inactivate LOX activity and consequently the production of PUFA hydroperoxides.

The colour of oil extracted from roasted hazelnut showed an absorbance peak at 485 nm (Abs_{485}) and was used as a parameter of the quality of the roasting process. The Abs_{485} recorded from oils of hazelnuts microwaved for 240 s and 360 s was lower than that recorded from traditionally roasted hazelnut. However, it increased significantly in oils obtained from hazelnuts microwaved for 600 s (Figure 6 A), turning from light yellow to

yellow/brown. The browning of substances in many heat-treated food can result from Maillard-type non enzymatic reactions, from caramel or phospholipids degradation and it commonly increases with roasting time.

We also evaluated the effects of roasting on tocopherol content. α-tocopherol is the main form, representing about 98-99% of the total α-tocopherols in hazelnut kernels. In agreement with results already published by Amaral et al. (2006b), our results indicated that, the roasting process either with a traditional oven or microwave resulted in a decrease in the α-tocopherol content (about 20-30%) compared to untreated hazelnuts of cv Nocchione and T. Romana. No significant reduction was found in α-tocopherol in roasted hazelnuts of cv T. Giffoni (Figure 6 B).

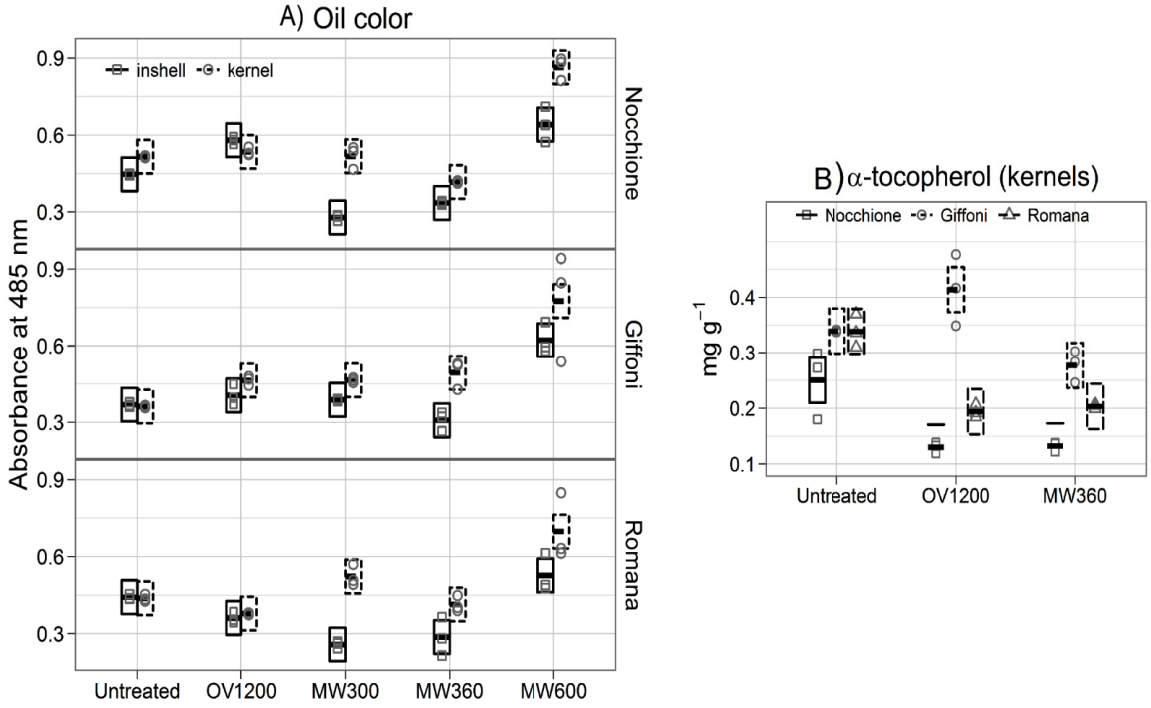

Figure 6. Effect of roasting treatment by microwave oven for 240 s up to 600 s of inshell and kernels of three hazelnut varieties (cv Nocchione, cv T. Giffoni, cv T. Romana) on oil color (Abs_{485} nm; A); alpha-tocopherol content (B). Boxplots displaying the extremes, the upper and lower values, and the median of the maximum difference within a category. Observed values (symbols) and means with 95% confidence intervals (bars)

PUFA content is another parameter of stability of oils during roasting, since higher PUFA content parallels with a higher oxidation rate of the oils. The main fatty acids composition of hazelnut oil from cv T. Giffoni kernels after roasting is shown in Figure 7.

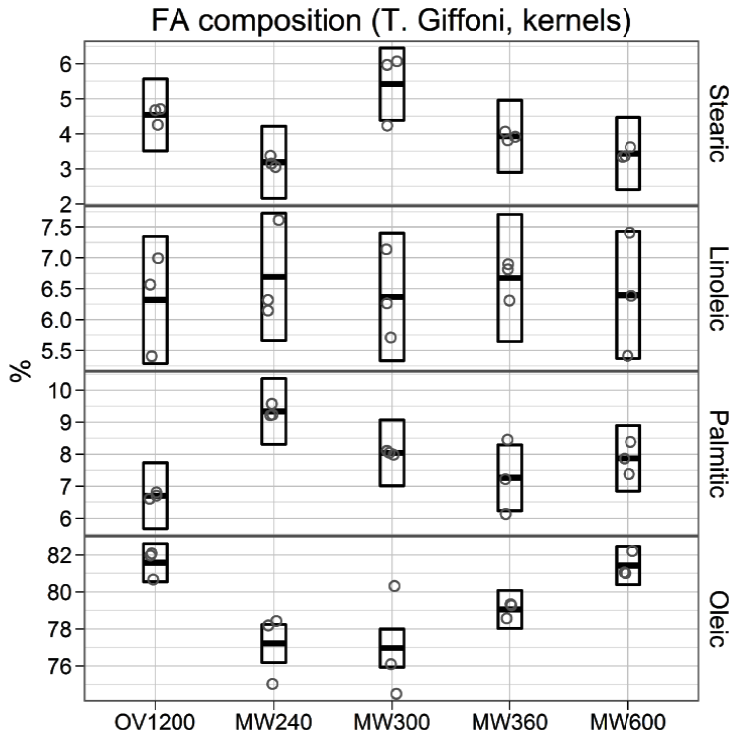

Figure 7. Effect of roasting treatment by microwave oven for 240 s up to 600 s of cv T. Giffoni kernels on main fatty acids content. Boxplots displaying the extremes, the upper and lower values, and the median of the maximum difference within a category. Observed values (symbols) and means with 95% confidence intervals (bars)

Similar values of linoleic and linolenic acids were found in all the tested oils. A lower oleic acid content was recorded in the oils from microwaved kernels (240-300 s). This paralleled with a higher content in palmitic acid at the same conditions (Figure 7).

4. Conclusion

Results show that microwave roasting for 360 and 450 s (for inshell nuts and kernels, respectively) allowed high peeling, good colour and taste of hazelnuts. Moreover, the low energy input required, the short process times and the easy process control indicate that the process here reported could be considered suitable for hazelnut processing. Indeed, adverse effects on texture, aroma and nutritional properties were minimised, due to the short times of heat exposure.

Furthermore, in contrast to conventional oven treatment, which needs to be applied to kernels with consequent unsatisfactory peeling scores, microwave roasting can be successfully applied to both kernels and inshell hazelnuts, giving in both cases, with appropriate exposure times, very good peeling and the desired roasted colour.

Taken together these results confirmed the potential of microwave technology for nuts processing in a context of sustainable agro-food industry.

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Development of an Encapsulation System for the Protection and Controlled Release of Antimicrobial Nisin at Meat Cooking Temperature

Khadidja Boualem[1,2], Muriel Subirade[2,3], Yves Desjardins[2,4] & Linda Saucier[1,2]

[1] Department of Animal Science, Pavillon Paul-Comtois, Université Laval, Quebec, QC, Canada

[2] Institute of Nutrition and Functional Foods (INAF), Université Laval, Quebec, QC, Canada

[3] Department of Food Science and Nutrition, Pavillon Paul-Comtois, Université Laval, Quebec, QC, Canada

[4] Department of phytology, PavillonEnvirotron, Université Laval, Quebec, QC, Canada

Correspondence: Linda Saucier, Department of Animal Science, Pavillon Paul-Comtois, Université Laval, Quebec, QC G1V 0A6, Canada. Tel: 1-418-656-2131 ext. 6295. E-mail: linda.saucier@fsaa.ulaval.ca

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Abstract

Nisin is an antimicrobial peptide produced by *Lactococcus lactis* spp. *lactis* widely investigated for use in foods as a natural antimicrobial. However, its effective use in meat products is restricted notably by its reaction with meat constituents (including glutathione) in raw meat. The purpose of this study was to develop an encapsulation system that would optimize nisin activity when used in meat. To achieve this goal, an encapsulation in dipalmitoylphosphatidylcholine (DPPC) liposomes was developed. DPPC liposomes were formed in phosphate buffer with or without nisin. The encapsulation efficiency of nisin in liposomes was greater than 46 ± 2%. The median size of nisin-loaded liposomes was 495 nm, compared to 170 nm for empty liposomes. The liposomes containing nisin were stable for up to 7 days at 4°C but a zone of inhibition was observed afterwards. Stability of the liposome to heat was also tested and demonstrated that above 37°C nisin was released from the melted liposomes to form zones of inhibition. Activity of free and encapsulated nisin was tested in raw and cooked ground beef (71°C). Free nisin lost its activity in raw beef but DPPC-encapsulated nisin remained active and was released upon melting of the liposome during heat treatment.

Keywords: antimicrobial, delivery system, encapsulation, liposome, meat, nisin

1. Introduction

Bacteriocins are antimicrobial peptides, which are produced by bacteria to inhibit the growth of other closely related organisms and can be potentially used as natural preservative (Cleveland, Montville, Nes, & Chikindas, 2001; de Arauz, Jozala, Mazzola, & Penna, 2009). Nisin is a 3.5 kDa cationic peptide produced by *Lactococcuslactis* subsp. lactis (O'Sullivan, Ross, & Hill, 2002). It is used as a food preservative because of its antimicrobial activity against several pathogenic Gram-positive bacteria that can be present in food, such as *Listeria monocytogenes*, *Staphylococcus aureus*, spores of *Bacilluscereus* and *Clostridium botulinum* (Najjar, Chikindas, & Montville, 2007). However, nisin is not active against Gram-negative bacteria, yeast or molds (Delves-Broughton, Blackburn, Evans, & Hugenholtz, 1996) unless a chelating agent is used concurrently (Cutter & Siragusa, 1995). Nisin antibacterial activity targets the cytoplasmic membrane where it inhibits peptidoglycan synthesis and supports the formation of pores (Bonev, Chan, Bycroft, Roberts, & Watts, 2000; Wiedemann et al., 2001). Nisin is a permitted food additive in more than 50 countries, including the US and Europe, where it is used notably in processed cheeses, dairy products and canned foods (Delves-Broughton et al., 1996).

Despite its extended uses, addition of nisin to meat products has been largely unsuccessful, unless used at very high concentrations (Stevens, Sheldon, Klapes, & Klaenhammer, 1991) or post-treatment (Davies et al., 1999; Rose, Sporns, Stiles, & McMullen, 1999). Several reports attribute the inactivation of nisin in meat as being mainly due to reactionwith meat components (Chung, Dickson, & Crouse, 1989), sensitivity to food enzymes (Shiba, Krushna, & Supratim, 2011), poor solubility at the pH of meat (Scannell, Hill, Buckley, & Arendt, 1997),

high bacterial loads (Scott & Taylor, 1981) and interaction with phospholipids (Henning, Metz, & Hammes, 1986). These difficulties are primarily linked to the hydrophobic nature of nisin and its poor solubility at neutral pH (Henning et al., 1986; Scott & Tylor, 1981; Stringer, Dodd, Morgan, & Waites, 1995; Delves-Broughton et al., 1996). At room temperature, nisin A is relatively stable in the pH range of 2 to 6, with highest stability at pH 3 (Rollema, Kuipers, Both, De Vos, & Siezen, 1995). A sharp decrease in stability is observed at pH 7 and 8. The pH of fresh meat is in the range of 5.5 to 5.8 depending on the animal species. Furthermore, dark, firm and dry (DFD) meats, and some processed meats, like bologna, have pH values above 6 (Faucitano et al., 2010; Viuda-Martos, Ruiz-Navajas, Fernándes-López, & Pérez-Álvarez, 2010). So not all added nisin is expected to be solubilized in meat matrices.

Nisin is inactivated by the presence of proteases, titanium dioxide, sodium metabisulfite (Delves-Broughton, 2005) or by enzymatic reaction with glutathione, a low molecular mass (307 Da) thiol compound found in meat tissues (Rose et al., 1999; Rose, Palcic, Sporns, & McMullen, 2002). In contrast, when nisin is applied to cooked meat, there is no inactivation. The concentration of glutathione in beef, chicken and pork is generally high with 156 to 627 nmol/g wet weight compared to raw fish (21 nmol/g wet weight; Jones et al., 1992). Under optimal reaction temperatures three glutathione molecules can bind to one nisin molecule and multiple dehydro-residues are involved, resulting in the loss of antimicrobial activity (Rose et al., 2002). Tilokavichai, Jindaprasert, Pilasombut, Sethakuland Swetwiwathana (2011) showed that nisin A and nisin Z is inactivated by an enzymatic reaction with 250 mM glutathione at a temperature of approximately 30 to 32°C. On the contrary, the same authors showed that glutathione does not affect the activity of pediocin PA-1 and plantaricin W at 30 to 32°C and 4°C (Tilokavichai, Jindaprasert, Pilasombut, Sethakul, & Swetwiwathana, 2012).

In this study, we investigated the possibility of protecting nisin by encapsulation in liposomes to provide a temperature controlled release system that enhances the efficacy and stability of nisin added to raw meat (here raw beef), similar to what is achieved during the direct acidification of meat (e.g., pepperoni; Barbut, 2005). Encapsulation in liposomes allows for the timely liberation of acids as the liposomes melt during cooking. This approach has already been successfully applied to protect nisin in a cheese matrix (Benech, Kheadr, Laridi, Lacroix, & Fliss, 2003) and other food systems (Malheiros, Micheletto, da Silveira, & Brandelli, 2010). Liposomes are spherical bi-layer vesicles formed by the dispersion of polar lipids in aqueous solvents and may consist of single or multiple bilayers composed of polar lipids (Mertins, Sebben, Pohlmann, & da Silveira, 2005). The most commonly used phospholipid for liposome formation is lecithine but its transition phase occurs at 25°C (Mertins, Sebben, Schneider, Pohlmann, & da Silveira, 2008). However, other phospholipids, like dipalmitoylphosphatidylcholine (DPPC) with a transition phase at 42°C, are more convenient for meat cooking applications. Before nisin can be used to its full potential in various meat systems, it is important to develop strategies that can effectively protect it from inactivation.

2. Materials and Methods

2.1 Bacterial Cultures and Growth Conditions

Several bacterial strains were tested as indicator organism of nisin activity (Table 1). The two strains of *Listeria monocytogenes* were kindly provided by Health Canada and were originally isolated from meat. The strains of *L. innocua* and *Pediococcus acidilactici* UL5 were obtained from the Department of Food Science and Nutrition and the strain of *Clostridium sporogenes* ATCC19404 from the Department of Biochemistry and Microbiology at Université Laval. Stock cultures were stored at -80°C in Lactobacilli de Man, Rogosa and Sharpe broth (MRS; BD Difco, Franklin Lakes, New Jersey, USA; pH 6.7 ± 0.2) for *P. acidilactici* UL5, in Brain Heart Infusion (BHI; BD Difco; pH 7.2 ± 0.2) for *Listeria* spp. and in BHI supplemented with 5 g/l yeast extract, 0.1% L-cysteine (BHIS; pH 6.7 ± 0.2) for *C. sporogenes* ATCC19404. All frozen cultures were supplemented with 20% glycerol (FisherBiotech, Fairlawn, NJ, USA) as a cryoprotectant. Prior to experimental use, working cultures were individually thawed and subcultured (1% (v/v)) daily in their respective broth media described above for a minimum of two and a maximum of seven consecutive days. Cultures were incubated overnight at 30°C for *Listeria* spp. and *P. acidilactici* UL5, and for 4 days at 37°C in anaerobic condition for *C. sporogenes* ATCC19404 (Forma Anaerobic System Model Covered 1025 S/N 13930-475, Thermo Scientific, Inc., Marietta, OH, USA).

Table 1. Antimicrobial activity of nisin against different strains

Strains	Nisinactivity[b]		
	AU/ml	Diameter (mm)[c]	MIC (μg/ml)[d]
C. sporogenes ATCC19404	800	13.1 ± 0.9	10.4 (5.2 - 10.4)
L. innocua HPB13	400	14.9 ± 2.0	5.2
L. monocytogenes HPB2371	400	13.7 ± 0.5	2.6 (2.6 - 5.2)
L. monocytogenes HPB2569	400	10.4 ± 1.2	2.6 (2.6 - 5.2)
P. acidilactici UL5	3200	28.8 ± 3.3	0.7

[a] The concentration of pure nisin in the solution used to produce the liposome is 83.3 μg/ml.

[b] All experiments were repeated three times.

[c] Zone of inhibition produced by the undiluted nisin solution used to produce the liposome (83.3 μg/ml).

[d] MIC was determined as the lowest concentration required for complete growth inhibition of the target. microorganism and are expressed as the median, the range of values are in parentheses. In the first well, after proper dilution with cells and media, nisin concentration was 41.7 μg/ml.

2.2 Antimicrobial Activity

Nisin activity was determined by the agar diffusion methods as previously described by Gratia (1946, cited by Dajani, & Wannamaker, 1976; Rose et al., 1999). Minimum inhibitory concentrations (MIC) were determined using the microplate assay previously described by Mota-Meira, LaPointe, Lacroix andLavoie (2000). For the agar diffusion method, 15 ml of the appropriate soft agar (0.75% (w/v) agar) was melted, inoculated at 1% (v/v) with a fully grown culture of the indicator organism, mixed and poured onto a pre-solidified agar plate (1.5%). A 20 μl aliquot of the antimicrobial solution to be tested was spotted at different concentration on the inoculated soft agar and was dried in a biosafety cabinet (highest concentration of nisin tested was 83.3 μg/ml). Activity was expressed as arbitrary activity units (Ahn & Stiles, 1990a, b); the dilution factor was multiplied by 50 to bring the arbitrary activity unit to 1 ml. Zones of inhibition were measured using a caliper (Mitutoyo Corporation., Ltd., Aurora, IL, USA) after 18 h of incubation for *Pediococcus* and *Listeria* and after 24 h to 48 h for *Clostridium*, under the same growth conditions as described above. For the microplate dilution method, 125 μl of each medium was added to each well of a sterile in 96-well U-bottom plate (Dynex Technologies LTD., Guernsey, Channel Islands, UK). The same volume (125 μl) of the nisin solution (83.3μg/ml) to be tested was added to the first well and serial dilutions (1:2) were made. The optical density (OD_{600}) of the overnight cultures was adjusted to 0.1 with fresh broth (Benchmark, Bio-Rad, Hercules, CA, USA) and corresponds to a 0.5 McFarland standard (1-2 x 10^8 CFU/ml). The microplates were incubated for 18 h for *Pediococcus* and *Listeria* and after 24 h to 48 h for *Clostridium*. Absorbance was read (OD_{600}) with a spectrophotometer (Thermo-Spectronic, UV-1 model, Thermo Electron Corporation, USA). The MIC was determined as the lowest concentration required for complete growth inhibition of the target microorganism determined by comparing OD_{600} value with the negative control without cells. Since *P. acidilactici* UL5 was the most sensitive strains amongst those evaluated, it was selected to follow nisin activity (Table 1).

2.3 Liposome Preparation and Encapsulation Efficiency

Liposomes were prepared from DPPC (Avanti Polar Lipids, Alabaster, AL, USA) using the method of Taylor, Gaysinsky, Davidson, Bruce and Weiss (2007) with the following modifications. The lipids were first dispersed in chloroform and dried under N_2 to form a lipid film on the wall of glass reaction tubes. Samples were desiccated overnight under vacuum to remove solvents. For nisin-loaded liposomes, Nisaplin (2.5% (w/w) in NaCl and denatured milk solids) was kindly provided by DuPont (formerly Danisco, New Centery, KS, USA) and dissolved in 20 mM HCl (pH 2) to obtain an acidic stock solution of pure nisin at a concentration of 250 μg/ml. Throughout this manuscript, all levels of nisin are reported as pure nisin. The nisin stock solution was immersed in boiling water for 5 min, filter sterilized through a 0.22 μm surfactant-free cellulose acetate filter (SFCA, 28 mm syringe filter; Corning Inc., Corning, NY, USA). Lipid films were rehydrated with nisin diluted in 0.1% phosphate buffered saline (PBS; 0.017 M KH_2PO_4, 0.05 M Na_2HPO_4, and 1.5 M NaCl at pH 7.4; Biowhittaker, Rockland, ME, USA) to a final nisin and lipid concentration of 83.3 μg/ml and 10 mM, respectively. After rehydration, liposomes were frozen in liquid nitrogen for 10 s, then gently thawed in water (25°C) for 10 s, and immediately immersed in a 50°C water bath for 15 s. This thermal cycle was repeated four

times to favor encapsulation. Liposomes were then held at 50°C for 20 min to ensure that phospholipids were above their gel-liquid crystalline phase transition temperature (T_m) before sonication to promote uniform sizes and to reduce the size of the vesicles. Multilamellar vesicles (MLV) were exposed to five cycles of sonication (Sonic Dismembrator, Model 500, Fisher Scientific, Pittsburgh, PA, USA) for 1 min, followed by 3 min of cooling on ice (Malheiros et al., 2010). The sample remained on ice for 15 min and then the liposomes were separated from unencapsulatednisin by ultracentrifugation (model L8-70 M ultracentrifuge; Beckman, Palo Alto, CA, USA) at 85 000 x g for 1 h at 20°C, washed twice, and recentrifuged (Benech et al., 2002). The pelleted liposomes were dissolved in 0.1% PBS at pH 7.4 and the supernatants were retained to determine the encapsulation efficiency by protein concentration analysis. To determine the activity of encapsulated nisin, the pellet was heated above the transition temperature of the lipid (60°C) to release nisin and the preparation was centrifuged to keep only the released nisin in the supernatant. Total protein was determined using the BCA Protein Assay Kit-Reducing Agent Compatible (Thermo Scientific, Rockford, IL, USA).

2.4 Stability of Liposomes Stored at 4°C

The liposomes were stored at 4°C and their stability was followed for 7 days by measuring the zone of inhibition produced on a lawn of *P. acidilactici* as described above. The size distribution of empty and nisin-loaded DPPC liposomes was analyzed by photon correlation spectroscopy using a Nicomp Submicron particle sizer apparatus (Model 370, HIAC/ROYCO Instruments, Menlo Park, CA, USA) equipped with a 25 mW helium/neon laser. Different temperatures (4, 25, 37, 42, 50, 63 and 71°C) were tested for the liberation of nisin. Liposomes were heated for 30 min. Nisin liberation was detected by the inactivation of the indicator organism as described above.

2.5 Activity of Free and Encapsulated Nisin Incorporated in Raw and Cooked Beef

To determine the activity of nisin in extra lean ground beef (≤ 10% fat; pH 5.61 ± 0.03) free (control) and encapsulated nisin were added to the meat samples (83.3 µg/g or 3200 AU/ml). The meat was hand massaged to evenly distribute the added nisin in the meat matrix. Raw beef containing free or encapsulated nisin was tested immediately after mixing or after storage at 4°C for various lengths of time (15, 30 and 60 min, and 24h) to allow the reaction of nisin with raw beef constituents. Residual nisin activity in the raw beef was evaluated by inhibition of the indicator strains as described below. Residual nisin activity was also evaluated after the raw beef was cooked to a core temperature of 71°C (no holding time). For cooking, samples of raw beef (7 g) containing free or encapsulated nisin were placed in glass tubes 1 cm in diameter and were heated in a high precision (± 0.001°C) circulating programmable water bath (Cole-Palmer Polystat Heated Circulating Bath, Cole-Parmer Canada Inc., Anjou, QC, Canada) set at 80°C. Empty liposome without nisin was also evaluated as a negative control. The temperature of the meat was followed using a data logger equipped with a type T thermocouple (Food tracker MultiPaq21, Datapaq Inc., Wilmington, MA, USA). After treatment, samples were cooled in an iced water bath (4°C). To detect residual nisin activity when added to raw beef, before or after cooking, 50 ml of MRS agar (1.5% (w/v) agar) was poured into a 100 ml beaker. A sterile glass tube was used to punch a hole in the center of the solidified agar to create a well of 1.5 cm in diameter and 4 cm deep. The raw or cooked meat sample was placed in the well and covered with 8 ml of soft MRS agar (0.75% (w/v) agar) inoculated with the indicator organism.The beaker was then incubated for 24 h at 30 ± 1°C. The size of the inhibition zone obtained was measured with a caliper.

3. Results and Discussion

3.1 Liposome Encapsulation Efficiency

As determined by protein dosage, the encapsulation efficiency of nisin in DPPC liposomes was greater than 46 ± 2% and higher than the efficiency previously determined for commercial proliposome H (34.6%), as reported by Laridi et al. (2003). Previous studies demonstrated that encapsulation efficiency was greater in phosphatidylcholine (PC) prepared liposomes than in phosphatidylglycerolprepared liposomes (Laridi et al., 2003; Were, Bruce, Davidson, & Weiss, 2004). Images obtained by light microscopy before and after sonication (Figures 1A and B, respectively) indicated that liposomes varied greatly in size; after sonication, liposomes became smaller. Encapsulation of nisin resulted in an increase in liposome size, confirming its insertion in the liposome. The median size of nisin-loaded liposomes was 495 nm, compared to 170 nm for empty liposomes formed in PBS buffer 0.1%, pH 7.4 without nisin. Taylor et al. (2007) showed that encapsulation of pure nisin in phosphatidylcholine liposomes resulted in vesicles with an approximate size of 310 nm, compared to 103 nm for empty liposomes.

3.2 Liposome Stability

Liposomes containing nisin were stored at 4°C and the release of nisin was monitored over time to evaluate the liposome stability. Nisin remained within the liposome for up to 7 days at 4°C as demonstrated by the absence of an inhibition zone when spotted on a lawn of the indicator organism (Figure 1C). After 7 days at 4°C, nisin began to be released from the liposome in its active form as indicated by the zone of inhibition observed (Figure 1D). Nisin acts as a cationic detergent leading to the formation of pores in the lipid bilayers of membranes (Harris, Daeschel, Stiles, & Klaenhammer, 1989) and this characteristic suggests that nisin may be able to form pores in the liposome bilayer similar to those observed in the cytoplasmic membranes of sensitive organisms. Because nisin is released in its active from the liposome after a certain time, it may be advantageous for use in a food system for controlled release during storage. Malheiros et al. (2010) have demonstrated that the decrease in antimicrobial activity of nisin containing liposomes is time dependent and up to 25% of the activity can remain inside the liposome after 10 days at 4°C.

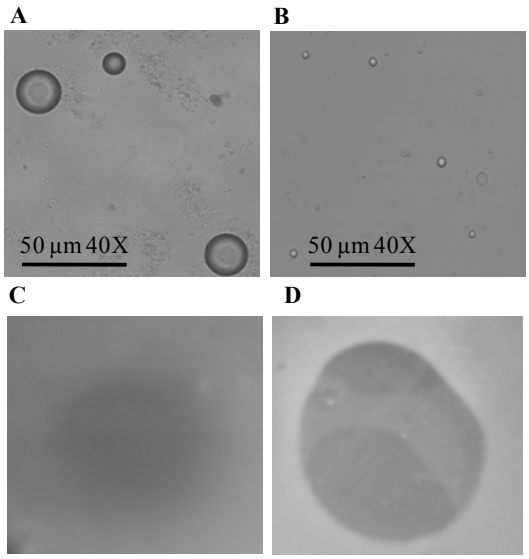

Figure 1. Optical microscopy images and stability of liposomes stored at 4°C

Images include liposomes (A) before and (B) after sonication, respectively. After sonication, liposomes became smaller. No clear zone of inhibition was observed from liposome containing nisin up to 7 days of storage at 4°C suggesting that if nisin was released, it was not enough to inhibit the indicator strains, *P. acidilactici,* (C) but after 7 days, zones of inhibition were observed suggesting that sufficient quantity of nisin began to be released from the liposome in its active form to inhibit the indicator strains, *P. acidilactici,* (D). Photographs C and D were taken against a black background.

3.3 Temperature Stability of Liposomes

The controlled release of nisin from DPPC liposomes was tested at different temperatures (4, 25, 37, 42, 50, 63 and 71°C). Liposomes (500 µl) were heated for 30 min and 20 µl was spotted on inoculated soft agar plates (Figure 2). At 4 and 25°C, nisin was completely retained as no inhibition zone was observed (Figures 2A and B). When the temperature was increased to 37°C and above, nisin was released, resulting in zones of growth inhibition (Figure 2C-F). These results show that temperatures ≥ 37°C alter the surface properties of the liposomes to allow nisin to be effectively released.

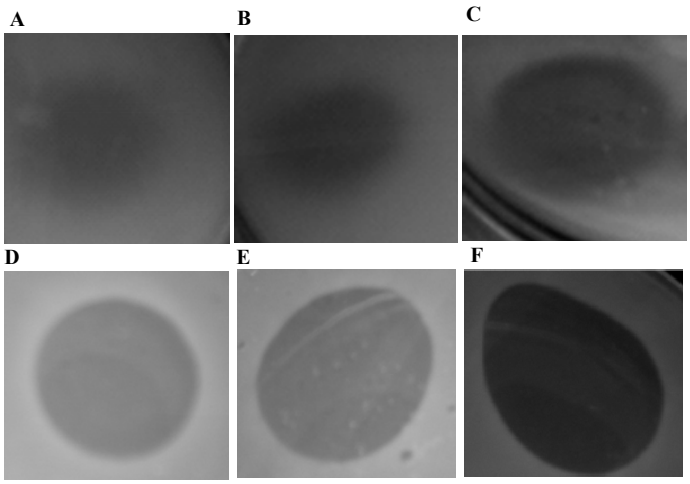

Figure 2. Liposome stability after heat treatments at different temperatures

The release of nisin from liposomes was tested at 4 (A), 37 (B), 42 (C), 50 (D), 63 (E) and 71°C (F). Liposomes were heated and then the solution spotted on the inoculated soft agar plates. At 4 and 25°C, no clear inhibition zone was observed suggesting that if nisin was released, it was not in sufficient quantity to inhibit the indicator strains, *P. acidilactici,* (data not shown for 25°C). When the temperature was ≥ 37°C, nisin was released, resulting in zones of growth inhibition. Experiments were repeated three times. Photographs were taken against a black background.

3.4 Protection of Nisin Activity in a Beef Model

Empty liposome as well as free and encapsulated nisin were mixed into ground beef and stored at 4°C for various lengths of time before heating to a core temperature of 71°C. Nisin activity was detected as a growth inhibition of the indicator organism. For a free nisin concentration of 83.3 μg/g (3200 AU/g), no inhibition zone was observed with raw or cooked beef after 30 to 60 min of contact with the ground beef (Figures 3A and B). No zone of inhibition was observed with the empty liposome either before or after heat treatment (data not shown) indicating that HCl at the concentration used in our experiments had no contribution to the inhibitory effect observed. A minimum time of contact was required before the free nisin added to raw beef was completely inactivated. As indicated above, previous reports indicated that the loss of free nisin activity in meat could be due, notably, to the composition of the meat, such as the presence of proteases (Delves-Broughton, 2005), and the reaction of nisin with meat constituents such as glutathione (Rose et al., 1999). Encapsulated nisin incubated with raw beef did not produce inhibition zones of the indicator strains, no matter the length of exposure (Figure 3C). After cooking to a core temperature of 71°C, sufficient quantity of nisin was released in cooked beef to form inhibition zones of the indicator strains (Figure 3D). The size of inhibition zones varied from 20 to 25 mm (zone edge to opposite zone edge). Similar results as Figure 3 line C and D were observed even after 24 h of incubation before and after cooking (data not shown). These results suggest that nisin was protected inside the liposomes and that the cooking temperature releases it upon liposome melting as was observed in the temperature stability experiment (see section 3.3). The observed activity after cooking supports the results of Rose et al. (1999) who found that nisin remained active when added to cooked meat. DPPC encapsulated nisin retained its activity in cooked beef. The temperature increase during cooking likely led to the timely denaturation of glutathione and other meat constituents involved in nisin inactivation (Freeman, Huntley, Meredith, Senisterra, & Lepock, 1997) concurrent with the release of nisin from the melted liposome (fusion temperature of DPPC is 63°C). Degnan and Luchansky (1992) monitored the activity of free pediocinAcH in slurries of beef tallow and beef muscle (25% in dH_2O). Significant loss of pediocin AcH activity was observed, but more activity was retained in heated slurries (100°C, 3 min) compared to unheated slurries. They suggested that enzymes degrading pediocin were inactivated by the heat treatment or that reaction substrates were denatured and no longer able to react with pediocin.

Concerns regarding the high levels of nitrite in cured meat have resulted in the research for alternatives. Although, unlike nitrite, nisin has no functionality in meat with respect to color, antioxidant properties, flavor etc., its antimicrobial activity, especially towards sporeformers, may allow the reduction of the nitrite/nitrate concentration currently used in meat systems since most of it is added to fulfil microbial preservation (Saucier,

1999). Its production by a dairy starter culture, *L. lactis* spp. *lactis,* is attractive since it could meet consumer demand for more natural additives for use in processed foods. However, the inactivation of nisin in raw meat systems is well documented and has been a major setback, delaying its wider application as an antimicrobial for use in meat systems. In this study, we developed the production of liposomes in hydrated film to encapsulate nisin and to protect it against inactivation prior to cooking, therefore, allowing it to retain its activity in cooked meat although initially added to raw beef. Figure 4 is a schematic of how the encapsulation system works. Free nisin in meat becomes inactive through reaction with meat constituents, including glutathione, as previously reported (Rose et al., 1999; Rose et al., 2002). By liposome encapsulation, nisin is protected inside the liposome and is released with increasing temperature. Increasing temperature destabilizes the liposome membrane and likely denatures meat constituents, including glutathione, which are then no longer able to react with nisin, leaving nisin in its active form in the meat matrix.

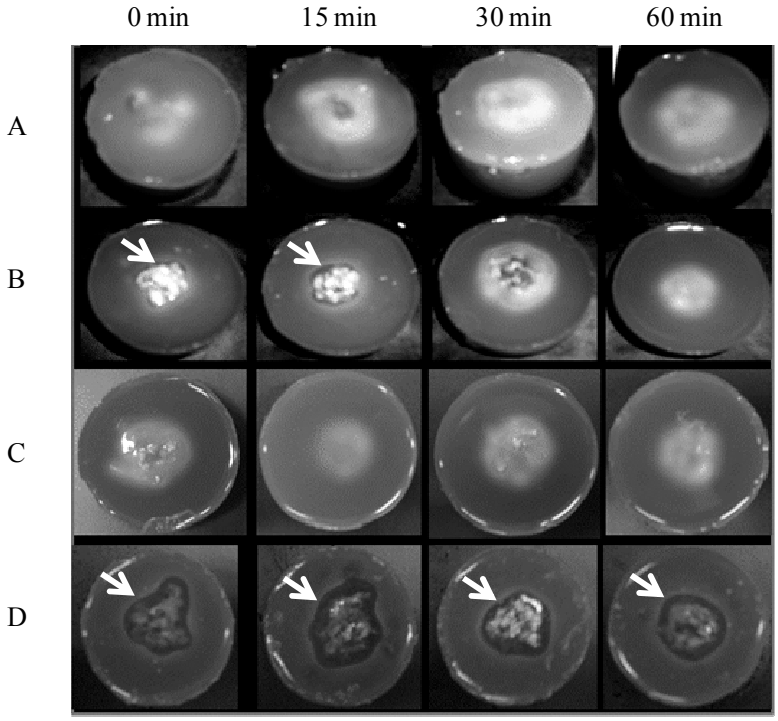

Figure 3. Activity of free (A, B) and DPPC encapsulated nisin (C, D) in ground beef

Nisin was tested at 83.3 µg/g (3200 AU/ml) of meat. Free and encapsulated nisin were tested in raw beef immediately after mixing or after 15, 30 and 60 min of contact prior (A, C) or after heat treatment to a core temperature of 71°C (B, D). A minimum time of contact (30 min and more) was required before the free nisin added to raw beef was completely inactivated (B). Encapsulated nisin incubated with raw beef did not produce inhibition zones no matter the length of exposure (C) indicating that nisin remained encapsuled until the heat treatment was applied and the liposome melted (D). Arrows indicate zones of inhibition. The experiments were repeated three times.

The results obtained in this study suggest that other sensitive molecules could be protected from heat inactivation by encapsulation in liposomes provided that their chemical characteristics are compatible with the liposome constituents. Now that we have demonstrated that this strategy works in beef, further studies are required to improve level of encapsulation, to develop procedures to produce liposomes without the use of solvent (here chloroform), and to validate the efficacy of encapsulated nisin to control spoilage and pathogenic organisms in various meat systems.

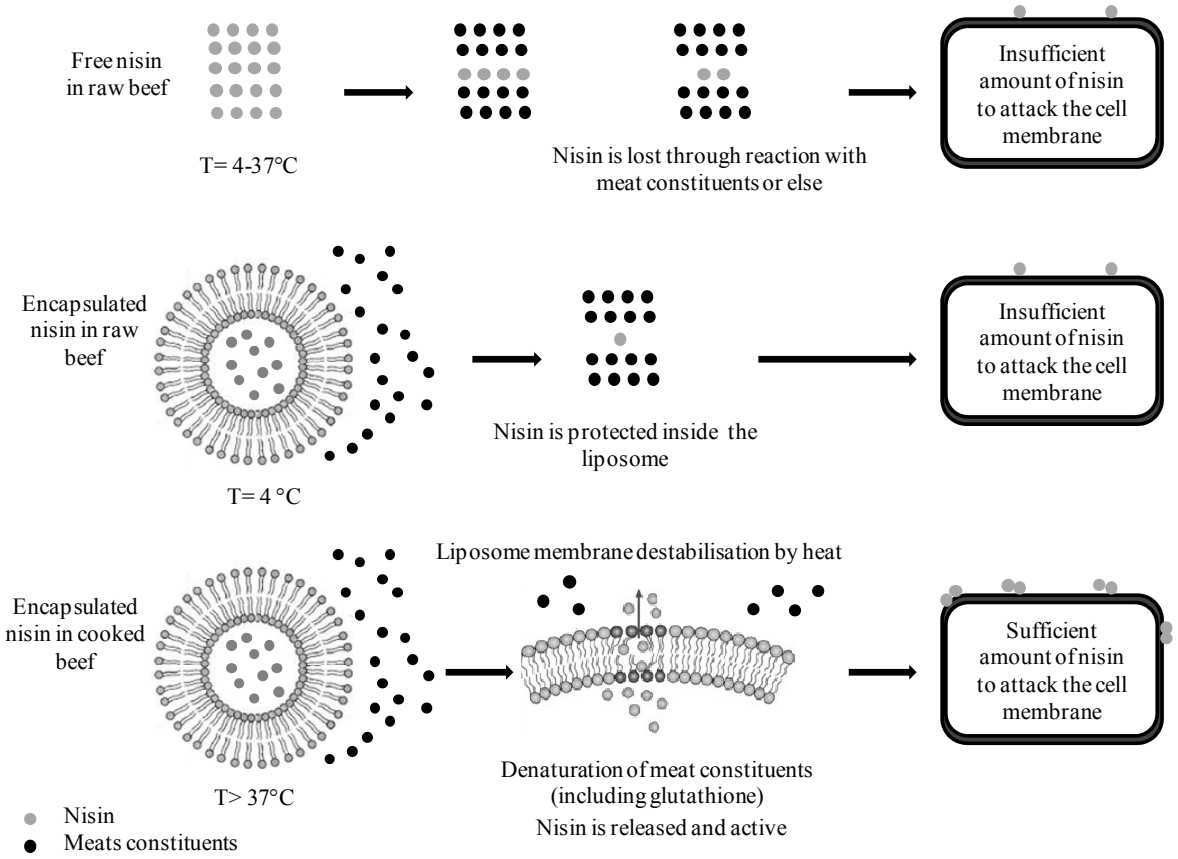

Figure 4. Schematic of nisinprotection mechanism by encapsulation in liposomes

Free nisin in raw beef is inactivated, resulting in an insufficient amount of nisin to attack the cell membranes of target microorganisms. Nisin is protected inside the liposome until cooking is applied. Upon heat treatment, nisin is released from the liposome in its active form so the target organisms can be inactivated.

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Enhancing Social Support System for Improving Food Security Among the Elderly Headed Household in Communal Areas of Zimbabwe

Kefasi Nyikahadzoi[1], Byron Zamasiya[2], George Alex Muchinako[1] & Charles Dziro[1]

[1] School of Social Work, University of Zimbabwe, Harare, Zimbabwe

[2] Socio-economist, International Centre for Tropical Agriculture, Harare, Zimbabwe

Correspondence: Kefasi Nyikahadzoi, School of Social Work, University of Zimbabwe, P. Bag 66022, Kopje, Harare, Zimbabwe. Tel: 263-772-240-861. E-mail: knyika@gmail.com

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Abstract

The study sought to establish factors that contribute towards food security among elderly headed households and then seek ways of enhancing them. The study was conducted in Mudzi District in Mashonaland East Province of Zimbabwe. Data was collected from wards 11, 12 and 16. The study used cross sectional household data collected using a structured questionnaire. Two measures of food security are used; namely household food insecurity access score and household dietary diversity score. The results showed that food insecurity access score was statistically higher for elderly headed household when compared to those headed by younger people. The study revealed that social capital, remittances, and off farm income generating projects can increase the elderly headed household's likelihood of being food secure. The study also showed that public assistance is not making a positive contribution towards food security of elderly headed household. This paper argues that it is important for government and civil society to promote social capital and support channels of remittances to elderly headed households in communal areas.

Keywords: food security, elderly, social support, dietary diversity, Zimbabwe

1. Introduction

According to Zimbabwe Statistical Agency, people aged above 60 years constitute 7% of the projected population of 12.4 million Zimbabweans (Zimbabwe National Statistical Agency, 2012). The majority of these people reside in communal areas. Studies by Huisman (2005) have shown that 51% of the chronically ill are the elderly people above the age of 60. Huge medical bills, coupled with physical, functional and cognitive impairment make the elderly people vulnerable to food insecurity. Being food secure in our context implies having access to enough food for good health and active life through socially accepted ways. At minimum, food security should include readily availability of balanced diet and being assured to acquire acceptable food in a socially acceptable manner (Eicher & Staatz, 1985). This rule out being food secure through emergency relief, scavenging, stealing or any other coping strategies (Woltil, 2012; Rukuni et al., 1990).

The elderly and elderly headed households are particularly vulnerable and prone to food insecurity (Kaseke, 1998). Given their deteriorating health, elderly people have unique dietary requirements that if not adequately met could exacerbate existing health complications. Inadequate food intake resulting in low feeding frequency with insufficient energy and other important nutrients may increase disease frequency. Quandt et al. (2001) note that hunger and food insecurity among the elderly, exacerbates acute chronic diseases and speed up the onset of degenerative diseases. Age related biological factors increase the older adults' risk of nutritional deficiencies (Quandt et al., 2001). These combine to decrease the quality and length of life among the elderly. Unless factors that enhance the elderly headed household's food security are known and ways of promoting them are devised, inadequate access to food and poor intake of nutritious food will continue to compromise the overall health of elderly people in communal areas. It is therefore important to investigate best practices used to enhance food security within the communal areas and then discuss how these can be out-scaled. This paper uses the institutional perspective to identify the social, economic, cultural and political factors that determine food security among the elderly people in rural areas. Once these factors are identified, the paper then uses theoretical

arguments and empirical evidence, to show how civil society and government can promote food security among elderly headed households through enhancing factors that explain food security.

1.1 Theoretical and Empirical Factors That Enhance Food Security

Literature identified several factors that constrain elderly headed households from accessing enough food for active health life. Quandt et al. (2001) note that rural elderly have lower incomes and hence poor health than their counterparts in urban areas. They noted that the cost of food in rural areas is much higher and with limited selection in rural areas. According to Kaseke (1998), public assistance programs are not evenly distributed in rural areas and lack of knowledge of these limit rural elderly from accessing them. Even in cases where these facilities are available, distance to distributing points and lack of public transportation limit the elderly from accessing public assistance. As noted by Quandt et al. (2001) pride and reluctance to use services considered to be welfare as inhibiting elderly to secure food security.

Other studies found out that elderly people with social support systems such as family, and friends tend to be food secure (Quandt, 2001). In a study carried out in the USA, Woltil (2012) finds that having a spouse helps in preventing or reducing food insecurity. The author also identifies physical and cognitive health problems, limited resources, availability of reliable social support systems and the social environment as determinants of food security. Another research study on food security among elderly people in the USA finds that public and private food programs, personal savings, proximity of children and family members and food storage skills as major determinants of food security (Wolfe et al., 2012). The identified factors seem to be country specific and it is therefore important to identify factors that contribute to food security among the elderly headed households.

In Zimbabwe, most of the food insecure elderly headed households are in communal areas where they depend on peasant agriculture for own food production (Kaseke, 1998). Often, the elderly headed households own land and livestock and they use these for food production to enhance their food security (Kaseke et al., 1998). There are other factors that influence food production by the peasant farmers, these include the rainfall pattern and availability, the soils – some lands have been farmed for more than fifty years and cannot produce much without yield enhancing inputs like fertilizers which the peasants cannot easily afford (Kaseke et al., 1998). However, the availability of these inputs and even favorable agroecological conditions for agriculture are known to enhance the food security of non-elderly headed households. Kaseke et al. (1998) attributed this to the reduced ability to work in their own fields due to old age.

To mitigate the problem of food insecurity among vulnerable elderly headed households, the Government introduced food support programs like the free food distribution to the elderly (Kaseke et al., 1998). This does not however cover all the needy elderly headed households because of resource constraints on the Government part (Kaseke et al., 1998). Sometimes there is politicization of food distribution, corruption in the distribution system and ignorance of the bureaucratic system by the elderly households. Other government programs intended to benefit the elderly headed households include the Grain Loan Scheme (the elderly are not expected to repay the grain) and Public Assistance administered to the elderly aged 60+ years (Kaseke et al., 1998). With the Public Assistance system, it is assumed that the elderly face challenges of accessing offices of the Department of Social Services and other accompanying costs (transport and food for the elderly and their escorts) of collecting the meager amounts they receive under this program (Mbanje, 1991). It is thus important to empirically determine factors that enhance food security among the elderly headed households and then promote their functioning. This study uses the Ordinary Least Squares methods to investigate factors that determine food security among the elderly headed households in Zimbabwe.

2. Research Methods

The study was conducted in Mudzi district, which is in Mashonaland East Province of Zimbabwe. The study sites lie in natural farming region IV, which is semi-arid zone at an altitude of 500-900 metres above sea level. The major economic activity within the study area is predominantly farming. This natural farming region is an agro-ecologically low potential zone with high incidences of droughts and frequent long mid-season dry spells. The mean annual rainfall in Mudzi district ranges from 450 to 500 mm while the mean annual temperature is 23°C. Due to the high aridity, maize yield in Mudzi district is about 0.5 tones/ha.

2.1 Sampling and Data analysis

Data for this study was collected from wards 11, 12 and 16. This study uses cross sectional household data from the baseline survey collected through structured interviews. Simple random sampling was used to select the wards and the households for interviewing from the lists that were provided by resident agricultural extension officers. During the survey, any selected households that were inaccessible were replaced by the next eligible

household on the household list. Data collection for this study was done in December 2011 through face-to-face administration of questionnaires.

The data collection involved a household survey using a questionnaire with semi structured and structured questions. The survey collected information on household demographics and socioeconomic characteristics, 24-hour recall dietary assessment, dietary diversity, food consumption from major food groups, food insecurity, crop production and marketing. A sample of 120 households is actually greater than 74, an apriori power analysis computed using G power (Franzel et al., 2007). It therefore means that the sample provides acceptable statistical power (that is 0.80) for moderate correlation r = 0.30, at two tailed 0.05 level of significance.

2.2 Conceptual/Empirical Framework

$$Y_1 = B_o + B_1X_1 + B_2X_2 + B_3X_3 + B_4X_4 + \ldots\ldots\ldots + e \quad (1)$$

Y_1 is the dependent variable. In our case, it was in two forms; (a) Household Food Insecurity Score and (b) Household Dietary Diversity Score. The X_i sare independent variables. These are listed in Table 1.

2.2.1 Dependent Variables

(a) Household Food Insecurity Access Score (HFIAS)

The HFIAS is a continuous measure of the degree of food insecurity (access) in the household in the past 30 days. According to Deitchler et al. (2011) the HFIAS reflects the three universal domains of household food insecurity that is anxiety about household food insecurity, insufficient quality and insufficient quantity of food supplies. This indicator captures the household's perception about their diet regardless of its nutritional composition (Coates, Swindale, & Bilinsky, 2005). This food insecurity measure focuses on consumption related strategies and captures the household's behavioral and psychological responses to food insecurity or perceived food insecurity. The HFIAS is based on the assumption that households' experiences of food insecurity cause predictable reactions and responses that can be captured and quantified through a survey and then summarized into a score.

During the survey, the respondents were asked nine occurrence questions that consist of a generally increasing level of food insecurity. Specifically, the respondents were asked whether a specific condition associated with the experience of food insecurity ever occurred during the past 30 days. The questions included the occurrences of (i) worrying about food adequacy; (ii) eating the kinds of less preferred foods; (iii) eating limited variety; (iv) inability to eat less preferred foods; (v) eating smaller meal than needed; (vi) eating fewer meals in a day; (vii) failing to get food of any kind; (viii) sleeping at night hungry and; (ix) going the whole day or night without eating anything. Respondents were asked to either say yes = 1 if event occurred or no = 0 if the event did not occur. Each severity question is followed by a frequency-of-occurrence question, which asks how often a reported condition occurred during the previous four weeks. There are three response options representing a range of frequencies (1 = rarely, 2 = sometimes, 3 = often). As suggested by (Coates, Swindale, & Bilinksy, 2007), the HFIAS is computed as follows: HFIAS (0-27) = summation of the occurrence multiplied by frequency-of-occurrence during the past 30 days for the nine food insecurity related conditions.

At a household level, a high HFIAS shows that a household is very food insecure while a low score shows that a household is less food insecure. The minimum HFIAS is zero and occurs when a household responds 'no' to all questions on the household food insecurity access scale. Alternatively, 27 is the maximum HFIAS and is obtained by summing up of all frequencies on the frequency on the frequency of occurrence questions when a household responds yes to occurrence question and 'often' as frequency of occurrence to the nine frequency-of-occurrence questions.

(b) Household dietary requirements

Dietary diversity is a qualitative measure of food consumptions that reflect household's access to wide variety of foods and it's a proxy of nutrient adequacy of the diet for household. It is meant to reflect, in a snapshot, the economic ability of a household to consume a variety of foods. That is, respondents were asked if they consumed the following food staff in the last twenty-four hours.

> A = Cereals; B = Vitamin rich vegetables and tubers; C = Root and tubers; D = Dark green leafy vegetables; E = other vegetables; F = Vitamin A fruits; G = Other fruits; H = Meat, poultry, offal; I= Eggs; J = Fish and seafood; K = Pulses/legumes/nuts; L= Milk and milk products; M = Oil/fats; N = Sugar/honey.

Expected responses were yes = 1 or no = 0.

A-M represents the food groups consumed by members of the household. Values for A through M will be either 0 or 1. Long reference periods were deliberately avoided as these could result in less accurate information due to imperfect recall. According to Swindale and Bilinsky (2005) the household dietary diversity score is the calculated as follows:

$$HDDS\ (0\text{-}14) = Sum\ (A + B + C + D + E + F + G + H + I + J + K + L + M + N) \quad (2)$$

This reflects the total number of food groups consumed by members of the elderly households.

2.2.2 Independent Variables

Table 1 gives a summary of variables that determine food security among the elderly headed households.

Table 1. Independent Variables

Explanatory Variables	Description and type of variable
Age	Age of the respondent (years)
Household size	Number of family members in a household
Farming experience	Number of years a household has been farming
Dependence ratio	Number of dependence over number in active group
Ownership cattle	Number of cattle owned
Total land size	Total size of land holding under cultivation 2008/9
Labour	Number of members providing labour
Level of education	Years in school
Marital status	Married (1 = Yes, 0 = No)
Gender	Gender of household head (1 Yes, 0 No)
Sale of crops	Whether household sold (1 = Yes, 0 = No)
Sale of livestock	Whether household sold livestock (1 = Yes, 0 = No)
Extension	Visited by or visited an extension agent (1 yes, 0 if not)
Remittances	Participated in research activities (1 yes, 0 if not)
Social capital	Membership to a network (group, or association)
Public assistance	Whether household received public assistance (1 = yes, 0 if no)
Own an ox cart	Whether the household own an ox cart (1 yes, 0 if no)

2.3 Data Analysis

Data were analysed using STATA version 11.2. The descriptive analysis covers means, proportions and standard deviations to provide distribution across contextual variables. The results are presented in Table 2. Linear regression analysis was used to identify the factors that affect Household Food Insecurity Access Scale Score and the household dietary requirements. Before running the analysis, a test of multi-collinearity was conducted to determine if there were highly correlated independent variables. For those that were correlated, one of them was dropped from the model. The default cut off correlation value of 0.7 was used.

3. Results

Results of this study are presented in Tables 2, 3, 4 and 5. Tables 2 and 3 provide descriptive statistics of the dependent and independent variables respectively. Specifically, Table 2 compares food security situations of elderly and non-elderly headed households. Tables 4 and 5 provide determinants of household food insecurity access and dietary diversity respectively.

Table 2. Comparison of food security between elderly and non-elderly headed households

Variable	Elderly	Non elderly	*t*statistics	*p*value
HIAS	15.282	8.494	-4.758	0.0000***
	(8,700)	(6.563)		
HDDS	5.282	6.321	3.500	0.0005***
	(1.773)	(1.331)		

Level of Significance: *** = 1%; ** = 5%; * = 10%.

Results in Table 2 show a significantly higher average household food insecurity score of 15.282 for elderly headed households compared with 8.494 for their counterparts. The mean household food insecurity access score is statistically higher for elderly headed households when compared to those households headed by younger people. The results also show that in a recommended basket of fourteen food items, the elderly and non-elderly headed households consume 5.282 and 6.321 respectively. The dietary diversity score is also statistically and significantly lower among elderly headed households compared to the younger household heads. According to Styen et al, (2006), lower dietary diversity scores imply that elderly headed households eat more of starch giving foods and less of protein rich foods. There is a high probability that of the elderly headed households consumed foods that are rich in carbohydrates but lack the essential micronutrients necessary for a health life.

Table 3. Socio economic characteristics of the sample

Variable	**Mean**	**Std. Dev**	**Minimum**	**Maximum**
Head's Marital status	0.7692	0.4268	0.00	1.00
Social capital	0.2308	0.4268	0.00	1.00
Dependency ratio	1.0185	0.9060	0.00	4.00
Head's Sex	0.7949	0.4091	0.00	1.00
Head Age	69.5385	8.7895	60.00	99.00
Household size	5.8718	2.3190	2.00	12.00
Household Labour	3.1579	1.6689	0.00	7.00
Head's Farming experience	30.9167	10.6191	4.00	50.00
Total land size	3.1136	1.8992	0.20	8.00
Sale of crops	112.2821	200.4768	0.00	700.00
Sale of livestock	51.0256	112.7770	0.00	600.00
Off farm activities	11.7179	38.3659	0.00	192.00
Remittances	194.8718	431.0628	0.00	2000.00
Extension	0.5641	0.5024	0.00	1.00
Number of cattle	2.8718	2.7737	0.00	12.00
Own ox cart	0.5128	0.5064	0.00	1.00

The average household head age of the elderly headed households is 69.5 with over 80% of them being males. The mean household size is 5.87. About 70% of the elderly headed households have adequate household labour to work in their fields. The mean farming experience of the elderly is 50 years and the mean land size is 8 acres. On average, the most important source of income are remittances, followed by sale of crops then sale of livestock and lastly income from other non-farm activities such as selling of charcoal, mushrooms etc. The dependence ratio of 1.08 is slightly higher than 2.5 cited by Nyikahadzoi et al. (2012) in the same area for non-elderly headed households. This is contrary to Mutangadura's (2005) findings that elderly headed households have higher dependence ratio due to the high death rates among 15 – 35 age group, which left many children under the care of grandparents.

Table 4. Factors affecting household food insecurity access scores

	Beta	Std. Error	*t*	p value
Constant		8.2240	4.0430	0.001**
Social capital	-0.037	5.3800	-.1460	0.885
Head's Education	-0.421	6.2190	-1.8220	0.087*
Marital status	0.938	10.7360	1.7210	0.104
Sex	-0.767	10.7730	-1.4570	0.164
Household size	-0.104	1.2370	-0.4060	0.690
Labour	-0.408	1.5730	-1.4190	0.175
Farming experience	-0.113	0.1590	-0.5850	0.567
Total land size	-0.177	1.2350	-0.7220	0.481
Sale of crops	-0.437	0.0110	-1.6960	0.109
Sale of livestock	0.064	0.0180	0.2850	0.780
Off farm activities	-0.15	0.0400	-0.940	0.39
Remittances	-0.395	0.0040	-2.1370	0.048*
Extension	0.423	4.0370	1.8400	0.084*
Public assistance	0.413	10.3650	2.0390	0.058*
Number of cattle	-0.081	0.6610	-0.3750	0.712
Own Ox cart	-0.297	4.5790	-1.1440	0.269
Dependency ratio	-0.086	1.744	-0.4700	0.645

$R^2 = 0.56$; Level of Significance: *** = 1%; ** = 5% ; * = 10%.

Variables that are known to reduce anxiety over likely occurrence food insecurity in a household include education level of the elderly head of the household, engagement in some off farm activities and remittances. Results in Table 4 show that public assistance and extension services are positively related to household food insecurity access scores. This probably implies that elderly headed households that already worry most about food insecurity status of the households tend to seek extension services and public assistance. Ownership of what Chipanhura (2010) describes as minimum bundle (that is scotch cart, and draft power), that delineates the poor and the rich does not impact on household food insecurity access of elderly headed households.

Table 5. Factors affecting Household dietary diversity

Model	Coefficients	Std. Error	*t*-statistic	Sig.
(Constant)		1.649	2.4170	0.028
Education	0.2690	1.0890	1.3780	0.187
Marital status	-0.0030	0.8740	-0.0160	0.988
Dependency ratio	0.0500	0.3480	0.2860	0.779
household size	0.0840	0.2450	0.3470	0.733
Labour	0.3790	0.3100	1.3890	0.184
Farming experience	-0.3530	0.0310	-1.9250	0.072*
Total land size	0.0270	0.2580	0.1110	0.913
Sale of crops	0.3220	0.0020	1.2990	0.212
Sale of livestock	-0.1720	0.0040	-0.7660	0.455
Off farm activities	-0.0053	0.0090	-0.3000	0.068*
Remittances	0.4390	0.0010	2.4620	0.026*
Extension	-0.2820	0.8540	-1.2040	0.246
Public assistance	-0.4860	2.0910	-2.4700	0.025*
Number owned cattle	0.3190	0.1370	1.4890	0.156
Own ox cart	0.1450	0.9160	0.5810	0.570
Social capital	0.4260	0.9950	1.9070	0.075*

$R^2 = 0.61$; Level of Significance: *** = 1%; ** = 5% ; * = 10%.

Variables that were found by Nyikahadzoi et al (2012) to be the main determinants of household dietary diversity among smallholders in communal areas of Zimbabwe are not significant in explaining diversity among the elderly headed households. These variables include availability of household labour, land size, ownership of draft power and farming experience. In fact, the results show that elderly headed households that get more involved in farming are nutritionally insecure. This confirms Ziliak and Gundersen's (2011) observations that as smallholders get older, they have less energy to work in the fields. Results in Table 5 show that they become more dependent on social networks, off farm activities and remittances. It is however surprising that public assistance is contributing negatively to household dietary diversity. The reason could be that public assistance is only provided to sustain life and one cannot buy adequate food.

Elderly headed households with high social capital are food secure. According to Putnam (1995) social capital helps the elderly heads of households to access information, influence, power and claims for support from others. Social capital allows the elderly headed households to have access to resources and services that would not otherwise be readily available to them. Putnam (1995) argues that social capital promotes network-based relations that are important in facilitating in-kind assistance for the elderly poor relatives. Remittances and other off farm income generating activities are a source of income for elderly headed households that help them to be nutritionally secure. The household uses the money to increase their dietary diversity by purchasing other food items that they do not produce.

4. Discussion

Discussions in this section are based on theoretical and empirical evidence to recommend how government and civil society can improve upon the variables that significantly explain food security among the elderly headed households. Variables that government and civil society can influence to improve food security among elderly headed households include social capital; off farm income generating activities, and remittances.

4.1 Social Capital

Social capital can increase the elderly headed households' likelihood of accessing various forms of social support during times of need. Martin et al. (2004) observe that households that know and trust neighbors may be more likely to borrow food. In our study area, social capital acquired over years of staying in a neighborhood or community is helping the elderly headed households to access food. In this regard, it is important therefore for civil society and government working in these communities to come up with innovative ways of supporting interpersonal relationships and supporting communities in which elderly people live in order to improve their access to food. We agree with Martin et al. (2004) that developmental agents that promote increased access to food could incorporate social capital by linking their services to neighborhood or community based activities. The government should encourage culturally oriented values such as "adopt-a-granny" concepts, which would see communities adopting elderly people in their communities.

4.2 Promoting of off Farm Income Generating Projects

Our results show that off farm income generating projects improve elderly people's dietary diversity. It is important therefore to introduce new income generating projects that are not labour intensive. These will include mushroom production, bee keeping and cattle rearing. Such technology should be age sensitive. According to Nyanguru (2008), elderly people in Zimbabwe are famous for making beads, mats and, woven baskets from local materials for income generation. Civil society could facilitate this effort through the provision of low interest micro finance schemes for the elderly to boost their business or even start new ones. Efforts should also be made to help industrious elderly in communal areas to find a market for their commodities. This is particularly important in making the elderly headed households to be self-sufficient and independent.

4.3 Public Assistance

Results from our study show that public assistance is not making a positive contribution towards improving food security of elderly headed households. Admittedly, public assistance has the potential to enhance the food security of elderly headed households especially if the amounts are substantial and transaction costs of accessing the assistance is low. Martin et al. (2004) argue that transaction costs of collecting public assistance can be too high compared to the benefit and hence discouraging. Transaction costs for accessing Public Assistance might include high transportation cost to the distribution point and long queues and time spent before being served. Therefore, to be effective in improving food security, public assistance interventions must deal with possible challenges that elderly people might face in accessing it.

4.4 Remittances

Remittance is the single variable that promotes both food access and dietary diversity. In Zimbabwe, remittances are either from relatives working within the country or from those in the diaspora. For resident family members remitting money and other items to the elderly, government should consider introducing some tax concessions. This may include reducing their taxable income upon proof that they have an elderly person under their direct care.

Government should also create an enabling environment that promotes people in the Diaspora to remit money back home. These include reduction in remittance transaction costs and even extending financial services to rural areas where the bulk of the elderly live. The government and civil society can make efforts to broaden the number of people who remit money from the Diaspora by availing information on employment opportunities in other countries.

5. Conclusion

In this study we note that government policies that seek to improve agricultural production in communal areas fail to improve food security situations for elderly headed households. In fact, the results show that such an effort would make the elderly headed households even more vulnerable to food insecurity. For the elderly headed households, it has to be accepted that food security is not necessarily an agricultural problem. As people get older, household produce for own consumption becomes less important as a source of food security and income from other sources becomes more important.

The household and the local community are very important units for elderly people in rural areas. They provide social protection systems for elderly people in communal areas. At household level, the elderly can assess financial, material and human resources of other family members. There is also an urgent need for a paradigm shift in the focus of development through the provision of social services whereby Non Governmental Organizations and even government should provide services to the elderly as a method of instilling self-reliance and not to provide as a way of hand to mouth so that the services give explicit economic and social value to elderly people's contribution to their families and communities.

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Importance of Espresso Coffee Machine Parameters on the Extraction of Chlorogenic Acids in a Certified Italian Espresso by Using SPE-HPLC-DAD

Giovanni Caprioli[1], Manuela Cortese[1], Luigi Odello[1], Massimo Ricciutelli[1], Gianni Sagratini[1], Giacomo Tomassoni[1], Elisabetta Torregiani[1] & Sauro Vittori[1]

[1] School of Pharmacy, University of Camerino, Camerino, Italy

Correspondence: Sauro Vittori, School of Pharmacy, University of Camerino, Camerino, Italy. Tel: 39-073-740-2266. E-mail: sauro.vittori@unicam.it

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Abstract

Chlorogenic acids (CGA) are a group of phenolic acid derivatives, which are commonly found in coffee at concentrations reaching 0.1-0.2%. A method based on high performance liquid chromatography-diode array detector (HPLC-DAD) is proposed for the simultaneous determination of three chlorogenic acids, i.e. 3-*O*-caffeoylquinic acid (3-CQA), 5-*O*-caffeoylquinic acid (5-CQA) and 3,5-di-O-caffeoylquinic acid (3,5-diCQA), in time portions of espresso coffee. Two different espresso coffee machines working with different pressure and temperature curves, and two different blends (i.e. Arabica and Robusta) were used. The method presents good linearities (correlation coefficient greater than 0.99) and recoveries (in the range 67-99%) for the 3 chlorogenic acids. The concentration of total CGAs in a cup of Certified Italian Espresso ranged from 1522.5 to 2223.4 mg kg^{-1} and CGA isomer contents were, in decreasing order, 5-CQA > 3-CQA > 3,5-diCQA.

The concentration of total chlorogenic acids was higher in Espresso coffee (EC) prepared with Aurelia machine rather than with Leva; Arabica blend possessed higher level ot total chlorogenic acids than Robusta samples.

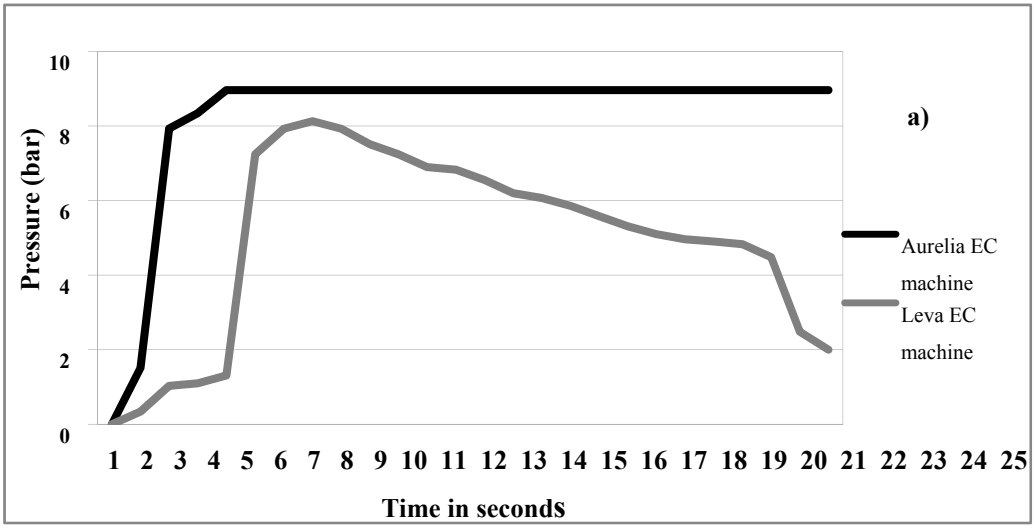
Keywords: coffee, certified Italian espresso, chlorogenic acids, antioxidant, SPE-HPLC-DAD, espresso coffee machines

1. Introduction

Brew coffee is a very popular beverage in the world (Parliament & Stahl, 1995). Consumers appreciate this drink for its enjoyable aroma, appreciable directly through the nose. Espresso coffee (EC) has specific aroma characteristics, mainly due to the presence of foam, which traps the volatilized aromas and doses their emission into the atmosphere (Blumberg, Frank, & Hofmann, 2010; Illy & Viani, 1995; Maeztu et al., 2001a). EC preparation is affected by factors related to the quality of coffee and water, and to settings of the machine. Water is brought to the desired pressure (normally about 9 atmosphere) and then passed through a heat exchanger, which gives to the water the set temperature (normally between 91°C and 96°C). Then, water is sprayed over the coffee and the extraction of the espresso components begins (Caprioli et al., 2012; Odello & Odello, 2006). The Certified Italian Espresso is the drink-in-a-cup conforming to the strict production specifications issued by the Italian Espresso National Institute and approved by a third-party Body operating in conformity with ISO standard 45011, and it is safeguarded and promoted through a product certification (certificate of product conformity Csqa n. 214-24 September 1999, DTP 008 Ed.1). To prepare a Certified Italian Espresso the following technical conditions are reported in literature, though only those would not be sufficient to completely fulfill quality requirements: needed portion of ground coffee of 7 g ± 0.5, temperature of water coming out from the unit of 90°C ± 2°C, coffee temperature in the cup of 67°C ± 3°C, water pressure of 9 bar ± 1, percolation time of 25 seconds ± 2.5, viscosity at 45°C > 1.5 mPas, total fat > 2 mg/ml, caffeine < 100 mg/cup, volume in the cup (inclusive of foam) 25 ml ± 2.5 (Odello & Odello, 2006). Chlorogenic acid is an ester formed between caffeic acid and quinic acid, and it is a phenolic compounds present in coffee that possess important biological effects since some previous findings reported that these phenolic compounds can acts as antioxidant, antitumor, antimutagenic and anticarcinogenic agents (Cetto & Wiedenfeld, 2000; Jiang et al., 2000; Moseira, Spitzer, Schapoval, & Schenkel, 2001). Caffeoylquinic acid derivatives, like 3-*O*-caffeoylquinic acid (3-CQA),

5-*O*-caffeoylquinic acid (5-CQA) and 3,5-di-O-caffeoylquinic acid (3,5-diCQA), are natural pohenolic compounds that have been isolated from a variety of traditional medicine plants and present a broad spectrum of pharmacological properties, including antioxidant, hepatoprotectant, antibacterial, antihistaminic and other biological effects (Basnet, Matsushige, Hase, Kadota, & Namba, 1996; Kwon, Jung, & Shin, 2000). Recently, it has been demonstrated that caffeoylquinic acid derivatives possess also neuroprotective effects (Hur, Soh, & Kim, 2001; Soh, Kim, Sohn, Lee, & Kim, 2003). More, it is reported that 3,5-diCQA exhibited neuroprotective properties against neuronal cell death that can be applied in the development of brain protection, as well as in the treatment of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and ischemia (Kim, Park, Jeon, Kwon, & Chun, 2005). Several authors have suggested a relationship between the composition of the CGA fraction and the quality of the beverage; addition of dicaffeoylquinic acids was negative for coffee flavor, whereas addition of mono caffeoylquinic acids brought about positive results (Ohiokpehai, Brumen, & Clifford, 1982). Tressl (1977) reported a more direct link between CGA fraction and beverage quality, through the influence on beverage aroma of organoleptically significant CGA degradation products such as caffeic acid, quinic acid and others. Clifford (Clifford & Ohiokpehai, 1983) and Naish (Naish, Clifford, & Birch, 1993) investigated the astringency of dicaffeoylquinic acid (DCQA) in depth; they reported a response for 5CQA similar to that of tannic acid and grape seed tannin, which traditionally are associated to astringency. Ohiokpehai (Ohiokpehai, Brumen, & Clifford, 1982) reported that the caffeoylquinic acid CQA/DCQA molar ratio on green coffee beans may influence the pleasantness of obtained beverage. Moreover, Nagel (Nagel, Herrick, & Graber, 1987) demonstrated that CQA does not show the bitter taste when its acid character is masked. There have been many reports on the presence of CGA in green coffee beans (Clifford, 1979; Van der Stegen & Van Duijin, 1980) or in brew coffee (Fujioka & Shibamoto, 2008) but not in ECs. For example, the content of CGA in various green coffee beans (21 species) from Cameroon and Congo ranged from 0.8% to 11.9% on a dry matter basis (Campa, Doulbeau, Dussert, Hamon, & Noirot, 2005), while it was ~5-15 mg g^{-1} in brew coffee (Fujioka & Shibamoto, 2008). The CGA content in brewed coffee is influenced by the kind of coffee beans used, because Arabica beans contain less CGA than Robusta ones (Ky et al., 2001). Most commercial brands of coffee are, however, made up of both Arabica and Robusta beans. The roasting method might also play an important role in the CGA content of the final coffee product. In fact, in the only article available on the CGA concentration in espresso coffee, it is reported that the CGA content is higher in Arabica rather than in Robusta EC samples (Ludwig et al., 2012). Various analytical methods have been proposed for the quantification of chlorogenic acids in coffee: HPLC (High-performance liquid chromatography) is the most common method, coupled with UV detector (Bicchi, Binello, Pellegrino, & Vanni, 1995; Fujioka & Shibamoto, 2008; Ky, Noirot, & Hamon, 1997; Maeztu et al., 2001) or with a mass spectrometer (Blumberg, Frank, & Hofmann, 2010). Other procedures, such as the simultaneous determination of total CGA and caffeine in coffee by high performance gel filtration (HPGF) chromatography has also been reported (De Maria, Trugo, & Moreira, 1995).

To the best of our knowledge, to date, no quantification of chlorogenic acids in espresso coffees wasperformed by comparing two EC machines working on different principles with the combination of two different blends. In fact, the aim of our work was to quantify the three most concentrated chlorogenic acids, i.e. 3-*O*-caffeoylquinic acid (3-CQA), 5-*O*-caffeoylquinic acid (5-CQA) and 3,5-di-O-caffeoylquinic acid (3,5-diCQA), in time portions of espresso coffees, comparing two different espresso machines, working with different pressure and temperature curves, and two different blends, Arabica and Robusta. The kinetic extraction of the Aurelia Competizione (A) EC machine was compared with that of the Leva Victoria Arduino (B). In this case, the settings of machine A were 92°C and 9 bar while espresso machine B is unsettable (Figure 1).



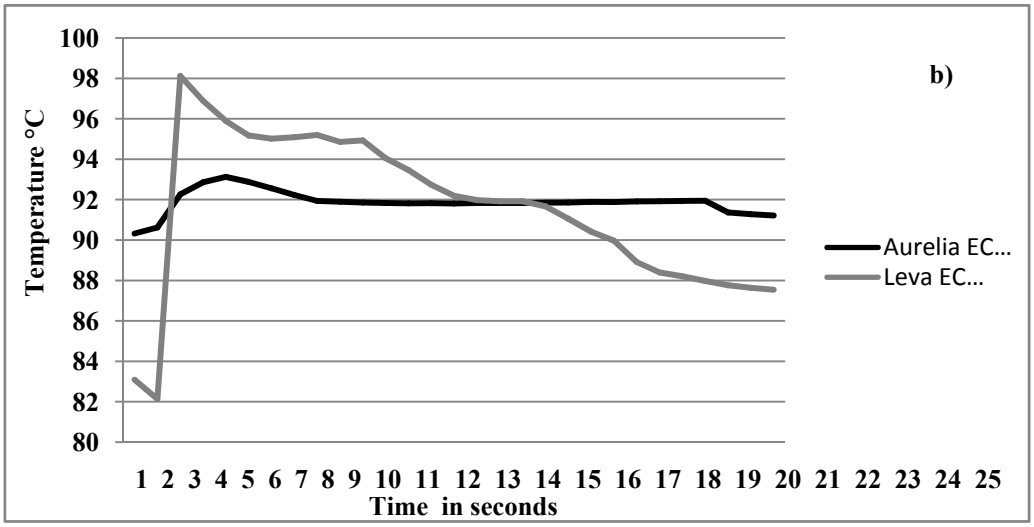


Figure 1. Curves of pressure (a) and temperature (b) on the coffee filter exhibited by Aurelia Competizione and Leva "Victoria Arduino" EC machines

2. Methods

2.1 Materials and Standards

The analytical standards of 5-*O*-caffeoylquinic acid (5-CQA), 3-*O*-caffeoylquinic acid (3-CQA), and 3,5-di-O-caffeoylquinic acid (3,5-diCQA) were purchased from Sigma-Aldrich (Milano, Italy).

Individual stock solutions were prepared by dissolving 100 mg of each compound in 100 ml of methanol (HPLC-grade, 99.9%; Sigma-Aldrich, Milano, Italy) and stored in glass-stoppered bottles at 4°C. Standard working solutions, at various concentrations, were daily prepared by appropriate dilution of aliquots of the stock solutions in methanol.

HPLC-grade formic acid was supplied by Merck (Darmstadt, Germany). Deionized water (> 8 MΩ cm^{-1} resistivity) was obtained from the Milli-Q SP Reagent Water System (Millipore, Bedford, MA). All the solvents and solutions were filtered through a 0.45 µm PTFE filter from Supelco (Bellefonte, PA, USA) before use. Cartridges SPE Strata-X 6 ml, 200 mg, were purchased from Supelco (Bellefonte, PA, USA).

2.2 Coffee Types and Espresso Machines

2 Kind of coffee, Arabica (pure *Coffea Arabica* from Colombia) and Robusta (95:5 blend of *Coffea canephora* and *Coffea Arabica*) and two EC machines, the Aurelia Competizione and the Leva Victoria Arduino working

with peculiar and different curves concerning pressure and temperature (Figure 1), were a gift from a local company (Nuova Simonelli, Belforte del Chienti, Italy). The variance in settings derives from the production method of the two EC machines. Aurelia is equipped with an electric pump and a heat exchanger, while in Leva pressure is given by a spring and water is brought to the desired temperature by passing it through a boiler.

2.3 Certified Italian Espresso Sample Preparation

Powdered coffee was made using a coffee grinder set in such a way that the obtained EC had a volume of 25 ml in 25 seconds of extraction in the coffee machine. Toasted coffee beans were ground immediately before each preparation; 7.5 g of the obtained coffee powder was used for each EC. After in-depth studies performed from our research group on the quality of EC, 9 bar of water pressure and 92°C were chosen as settings for the EC machine A for preparation and analysis of 7 time portions of the EC sample (0-10, 11-15, 16-20, 21-25, 26-30, 31-35, 36-40 sec.), with the aim at comparing these results with those from the same analysis performed using EC B. The conditions of EC "B" (T and P) are unsettable: pressure and temperature change as reported in Figure 1.

2.4 Sample Purification: Solid Phase Extraction (SPE)

The solid phase extraction was carried out using SPE Strata-X extraction cartridge (200 mg, 6 ml). The SPE cartridge was conditioned with methanol (3 ml) followed by water (6 ml). A volume of 0.5 ml of espresso coffee diluted with 2 ml of water was loaded onto each cartridge using a vacuum pump and collected. The elution was performed using methanol (7.5 ml). The eluent was evaporated under vacuum (60 mbar) at 30°C by a Büchi apparatus (Büchi R200, Labortechnik, Flawil, Switzerland); 10 ml of methanol were added to the residue, the solution filtered (0.45 μm nylon membrane) and transferred to a glass vial before injection.

2.5 LC/DAD Analysis

The separation was achieved on an analytical column Polar-RP 80Å (150 x 4.6 mm I.D., 4 μm) from Phenomenex (Chesire, U.K.). The mobile phase for LC/DAD analysis was water (A) and methanol (B), both containing 0.1% of formic acid at a flow rate of 1 ml min^{-1}. The solvent composition varied from 0-5.5 min: 25% B (v/v); 5.5-8 min: 50% B (v/v); 8-13.5 min: 50% B (v/v); 13.5-18 min: 25% B (v/v). The injection volume was 5 μl.

LC/DAD experiments were performed using a Hewlett Packard (Palo Alto, CA, USA) HP-1090 Series II, made of an autosampler and a binary solvent pump, equipped with a diode-array detector (DAD). LC/DAD analysis were performed monitoring two different wavelengths: 325 nm for 5-*O*-caffeoylquinic acid (5-CQA) and 330 nm for 3-*O*-caffeoylquinic acid (3-CQA), and 3,5-di-O-caffeoylquinic acid (3,5-diCQA).

2.6 Statistical Analysis

Analysis of variance (ANOVA) was performed using the SPSS software package, Chicago, IL, USA) for Windows. Values of $p < 0.05$ were considered as statistically significant.

3. Results and Discussion

3.1 Pressure and Temperature Curves From the Two EC Machines

Considering the pressure curve (Figure 1a), values increased up to a maximum of 9 and 8 bar for EC machines Aurelia and Leva, respectively. After that, the pressure curve in machine Aurelia remained unchanged, due to being equipped with an electric pump, while in machine Leva the pressure decreased until it reached 2 bar after 26 s.

Considering the temperature curve (Figure 1b), in the first 3 seconds of extraction the values increase up to a maximum of 93°C for Aurelia and 98°C for Leva. Due to the differences in construction, for Aurelia, the temperature value remains fairly constant, while for Leva the temperature drops about 10 degrees during extraction (Figure 1).

3.2 Comparison of Chlorogenic Acids Content in Coffee Samples Using Two EC Machines

Different trends of concentration have been obtained for ECs prepared by using two different blends, i.e. Arabica and Robusta (Figure 2).

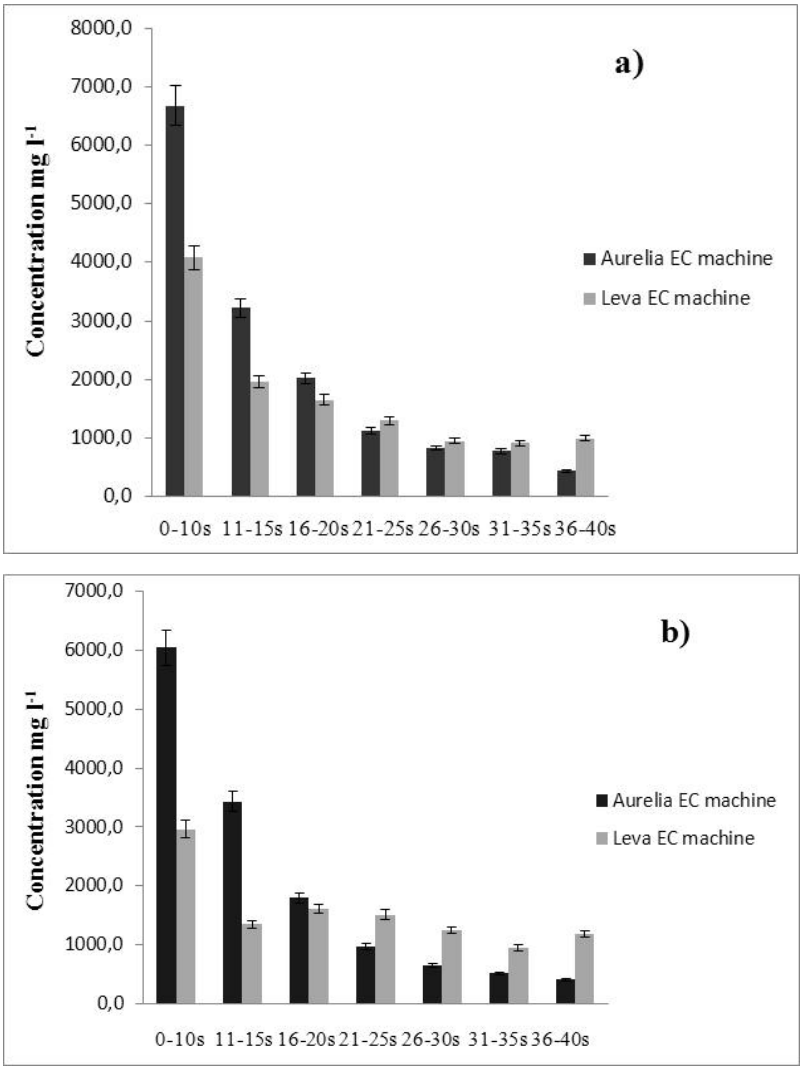


Figure 2. Trend of concentration (mg l^{-1}) of chlorogenic acids in time portions of ECs in Arabica (a) and Robusta (b) with the two EC machines (n = 3 RSD% < 5.7)

The concentrations of the three chlorogenic acids in each time portions is constantly decreasing. By using Leva EC machine, the chlorogenic acids concentration is greatly higher in Arabica rather than in Robusta in the first three portions, meanwhile in the other fractions an opposite trend is observed.

Similar trends of concentration have been obtained for ECs prepared by using two different EC machines, i.e. Aurelia and Leva, with a fixed blend (Figure 2). By comparing the trends of chlorogenic acids concentration obtained with Robusta blend, the chlorogenic acid concentration is greatly higher in samples obtained with Aurelia rather than with Leva in the first three fractions. These concentration values displayed large differences, especially in the first two fractions (0-10 sec. and 11-15 sec.). In fact, we found a concentration of chlorogenic acids of 6041.1 and 3431.7 mg l^{-1} in Aurelia, and of 2958.5 and 1349.7 mg l^{-1} Leva EC samples, respectively, showing great differences and high relative Aurelia/Leva ratio (2.042 and 2.543). Also by comparing the trends of chlorogenic acids concentration obtained with Arabica, the total concentration is higher in samples obtained using Aurelia rather than Leva in the first three fractions. In detail, the concentration of chlorogenic acid in the first two fractions (0-10 sec. and 11-15 sec.) is of 6684.7 and 3217.7 mg l^{-1} in Aurelia and of 4083.5 and 1966.3 mg l^{-1} in Leva EC samples, showing a relative ratio of 1.637 and 1.636, respectively.

As can be seen in Figures 2a and 2b, the extraction of chlorogenic acid, both in terms of concentration and of content (*data not show*) is higher in Aurelia than in Leva EC machines in the first three fraction (i.e. 0-10 s, 11-15 s, and 16-20 s).

In Figure 3 it is reported an overlapping of an HPLC-DAD chromatograms referred to a Robusta coffee sample (0-10 s of extraction time) prepared with Aurelia (gray) and Leva (black) in which it is clearly evident the most effective extraction of chlorogenic acid with Aurelia EC machine.

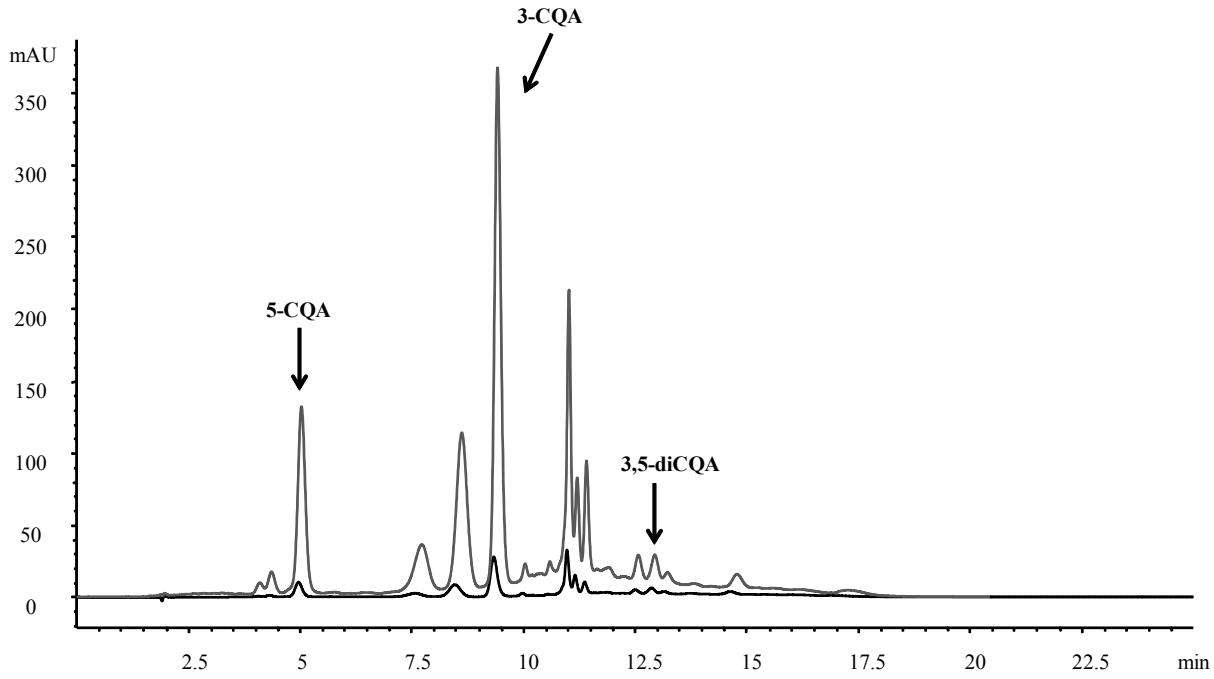


Figure 3. Overlapping of an HPLC-DAD chromatograms referred to a Robusta coffee sample (0-10s of extraction time) prepared with Aurelia (gray) and Leva (black)

These results can be explained by considering the temperature, higher in Leva respect to Aurelia up to 15 seconds (Figure 1). A lower temperature value seems to be more suitable to extract chlorogenic acids. As clearly visible in Figure 2, chlorogenic acids extraction is more efficient in the first fractions. In detail, for both Arabica and Robusta, the extraction of CQAs in the first fractions is more efficient for Aurelia than Leva. Because of this, concentration is: a) higher in coffees made with Aurelia in the first 2 fractions, b) very similar between the coffees made with the two EC machines in the central fractions, c) higher in EC made with Leva in the last fractions.

As shown in Figure 1, the water pressure in Aurelia (9 bar) is always higher than that in Leva EC machine. This value of 9 bar is reported to be the best extraction pressure condition for 5-CQA (Andueza et al., 2002). Probably, in Leva EC machine, because of the previous considerations, the pressure parameter seems to be not highly influent for chlorogenic acid extraction.

In our work, we have screened chlorogenic acid content in a long time of extraction for EC (40 sec, including a total of 7 fractions), in such a way to investigate all the type of preparation of EC worldwide.

By considering that chlorogenic acids play a beneficial role for human health, due to their antioxidant action, the concentration and the content of the three compounds in a cup of espresso (25 ml, i.e. the volume of a Certified Italian Espressso) have been considered (Table 1).

Table 1. Concentration (mg l^{-1}) and content (mg) of each chlorogenic acids in a cup of espresso coffee (i.e. 25 ml) (n = 3 RSD% < 5.0).

EC machine	Blend	3-CQA (mg l^{-1})	3-CQA (mg)	5-CQA (mg l^{-1})	5-CQA (mg)	3,5-diCQA (mg l^{-1})	3,5-diCQA (mg)	Total concentration in a cup of coffee	Total content in a cup of coffee
Leva	Arabica	422.8	10.6	1220.2	30.5	90.7	2.3	1733.7	43.3
	Robusta	394.9	9.9	986.1	24.7	141.5	3.5	1522.5	38.1
Aurelia	Arabica	555.8	13.9	1559.9	39.0	107.8	2.7	2223.4	55.6
	Robusta	529.3	13.2	1416.1	35.4	177.1	4.4	2122.5	53.1

RSD: Relative Standard Deviation; CQA: CaffeoylQuinic Acid.

CGA isomer concentration were in decreasing order 5-CQA > 3-CQA > 3,5-diCQA, with a concentration ranging from 986.1 to 1559.9 mg l^{-1}, 394.9 to 555.8 mg l^{-1}, and 90.7 to 177.1 mg l^{-1}, respectively. The amount (mg) in a cup of EC ranged from 24.7 to 39 mg, 9.9 to 13.9 mg and 2.3 to 4.4 mg for 5-CQA, CQA and 3,5-diCQA, respectively.

The most concentrated CGA was 5-CQA and was found in Arabica coffee prepared with Aurelia EC machine with a higher concentration of 1559.9 mg l^{-1}, in agreement with previous findings (Ludwig et al., 2012). The highest level of 3-CQA was also detected in Arabica coffee sample prepared with Aurelia EC machine (555.8 mg l^{-1}). On the contrary, the highest concentration of 3,5-di-CQA was found in Robusta coffee sample prepared with Aurelia EC machine (177.1 mg l^{-1}).

The total concentrations of the three compounds in a cup of coffee (25 ml) ranged from 1522.5 to 2223.4 mg l^{-1} and the total amount ranged from 38.1 to 55.6 mg (Table 1). Coffee samples made with Arabica blend and Aurelia EC machine displayed the highest concentration and content of total chlorogenic acids; however, samples obtained by using Robusta with the same EC machine (Aurelia) showed also the highest total concentration (2122.5 mg l^{-1}) and highest total content (53.1 mg). On the contrary, coffees made with Leva EC machine displayed a lower concentration and a lower content of total chlorogenic acid compounds, with a concentration of 1733.7 and 1522.5 mg l^{-1} and a content of 43.3 and 38.1 mg, for Arabica and Robusta, respectively.

3.3 Method Validation

The method was validated by determining linearity, recovery at three fortification levels, repeatability and with-in reproducibility, limits of detection (LODs) and limits of quantification (LOQs) (instead of CCs alpha and CCs beta).

Calibration curves of the analyzed compounds were constructed injecting 5 µl of standard solutions at seven different concentrations, i.e. 1, 2.5, 5, 7.5, 10, 25 and 50 mg l^{-1} in HPLC/DAD. Five replicates for each concentration were performed and the relative standard deviations (RSDs) ranged from 1.10 to 1.48 % for run-to-run precision, and from 2.25 to 4.33 % for day-to day precision. All the calibration curves of the analyzed compounds showed a correlation coefficient greater than 0.99.

In the HPLC/DAD analysis of espresso coffee samples, the recoveries, obtained by spiking the beverage solution with a final concentration of 10, 20 and 50 mg l^{-1} with a standard mixture of the three chlorogenic acids, were in the range 67-99% for all analyzed compounds (Table 2).

Table 2. Percent recovery and repeatability of the method evaluated by HPLC-DAD on ECs at three fortification levels (n = 5)

Compounds	Spiked Concentration in matrix (mg l^{-1})	Recovery %	RSD%	LOD (mg l^{-1})	LOQ (mg l^{-1})
5-CQA	**200**	89	3.94		
	400	87	3.71	0.08	0.25
	1000	99	0.73		
3-CQA	**200**	88	4.93		
	400	67	4.04	0.1	0.3
	1000	80	1.46		
3,5-diCQA	**200**	97	1.3		
	400	90	0.79	0.1	0.3
	1000	99	0.15		

RSD: Relative Standard Deviation;

LOD: Limits Of Detection;

LOQ: Limits Of Quantification.

The repeatability of the method was calculated on fortified samples at 10, 20 and 50 mg l^{-1} (n = 8), giving RSD% that were in a range 1.3-3.94%, 0.79-3.25 % and 0.15-1.46%, respectively.

The Limits Of Detection (LOD) and the Limits Of Quantification (LOQ) of the three chlorogenic acids, expressed in mg kg^{-1}, calculated in the matrix, were estimated on the basis of 3:1 and 10:1 S/Ns. LODs and LOQs of chlorogenic acids were in the range 0.08-0.1 and 0.25-0.3 mg l^{-1}, respectively.

Retention time stability was utilized to demonstrate the specificity of the method. Reproducibility of the chromatographic retention time for each compound in coffee samples was examined five times over a 5-day period (n = 25). The retention times using this method were stable, with a percent RSD value ≤ 1.38%.

4. Discussion

From the results obtained, it is clear that similar trends for the concentration of CGA have been obtained for different coffee blends made with the same espresso machine, even if the total concentration of the three compounds is slightly higher in Arabica than in Robusta especially in the first three fractions. Regarding the peculiarity of the two different EC machines, the total concentration and content of the three chlorogenic acids is higher with Aurelia EC machine rather than with Leva, very likely due to differences in extraction temperature; pressure seems to be not very influent on this process. The selection of EC machine utilized in preparing coffee seems to influence the extraction of chlorogenic compounds more than the selection of blends. This data concerning the comparison of two different EC machine are, to the best of our knowledge, the first available in literature. The total concentration of the three chlorogenic acids in a cup of coffee (i.e. 25 ml as reported above) ranged from 1522.5 to 2223.4 mg l^{-1} meanwhile the total amount ranged from 38.1 to 55.6 mg. These data are of great interest considering that the consumption of coffee is a great source of antioxidants for many populations that worldwide daily consumption of coffee is currently increasing and considering the importance of these compounds for their health benefits in the prevention of many illnesses, especially related to central nervous system.

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Use of Essential Oils as Natural Food Preservatives: Effect on the Growth of *Salmonella* Enteritidis in Liquid Whole Eggs Stored Under Abuse Refrigerated Conditions

Djamel Djenane[1], Javier Yangüela[2], Pedro Roncalés[2] & Mohammed Aider[3,4]

[1] Faculty of Biological Sciences and Agronomical Sciences, Department of Biochemistry and Microbiology, University Mouloud Mammeri, BP, Tizi-Ouzou, Algeria

[2] Department of Producción Animal y Ciencia de los Alimentos (Animal Production and Food Sciences), University of Zaragoza, C/ Miguel Servet, Zaragoza, Spain

[3] Department of Soil Sciences and Agri-Food Engineering, Université Laval, Quebec, QC, Canada

[4] Institute of Nutrition and Functional Foods (INAF), Université Laval, Quebec, QC, Canada

Correspondence: Mohammed Aider, Department of Food Engineering, Laval University, Quebec, QC G1V0A6, Canada. E-mail: mohammed.aider@fsaa.ulaval.ca

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Abstract

The steam distillation-extracted essential oils (EOs) of three aromatic plants from the Kabylie region of Algeria (*Eucalyptus globulus*, *Lavandula angustifolia*, and *Satureja hortensis*) were analyzed by gas chromatography coupled with mass spectrometry (GC/MS). The primary compounds from these EOs were 1,8-cineole (81.70%) for *Eucalyptus globulus*, 1,8-cineole (37.80%) and β-caryophyllene (20.90%) for *Lavandula angustifolia*, and carvacrol (46.10%), p-cymene (12.04%), and γ-terpinene (11.43%) for *Satureja hortensis*. To test the antibacterial properties of the EOs, agar diffusion and microdilution methods were used for *Salmonella enterica* serovar Enteritidis CECT 4300. The results revealed that all of the EOs possessed a significant anti-Salmonella activity. The inhibition diameters for *Lavandula angustifolia* and Eucalyptus globulus were 41.30 and 35.26 mm, respectively, whereas the essential oil (EO) of *Satureja hortensis* showed a stronger anti-Salmonella activity (51.15 mm) when compared to the two other EOs. The minimum inhibitory concentration values ranged from 1 to 8 μL/mL, and the MIC value of the *Lavandula angustifolia* EO was the lowest (1 μL/mL). Moreover, the anti-Salmonella activity of the EOs added at various concentrations to liquid whole eggs was investigated, and the results showed that the antibacterial effect is proportional to the quantity of EO added to the product. Based on the observed anti-Salmonella activity, the EOs tested are promising natural alternatives for the preservation of liquid whole eggs stored at 7 ± 1ºC to simulate Algerian refrigeration conditions.

Keywords: essential oils, *Eucalyptus globulus*, *Lavandula angustifolia*, *Satureja hortensis*, antibacterial activity, liquid eggs, *Salmonella* enteritidis

1. Introduction

Eggs are a staple diet for many populations throughout the world and represent an important source of proteins and phospholipids of high nutritional value. Egg and the derived products are used in the preparation of a large number of foods, such as mayonnaise, pastries, sauces, tortillas, and pastas. In the case of industrial applications, the egg is exploited for its various functional properties, such as its aromatic, hydrating, viscous, coloring, and emulsifying properties. However, changes in the consumption habits, modes of consumption, and the development of fast-food restaurants have changed the demand for egg products to include more elaborated products.

The infectious diseases transmitted by foods have remained a major concern of public health and an important economic problem in many countries over the past two decades, and Salmonella poisoning is one of the most important examples of a foodborne disease. The link between the increase in infection from *Salmonella enterica* serovar Enteritidis and the consumption of food preparations that use raw eggs or eggs that are not fully cooked is demonstrated by several studies. The risk associated with egg-derived infections remains high, but this risk

also varies as a function of the production conditions of the egg product and the technologies applied to its transformation and usage (European Commission, 2003). The importance of egg products in the human diet requires methods that ensure the safety of these products. The presence of Salmonella in eggs and its derived products, particularly the presence of *Salmonella* Enteritidis, is closely related to the prevalence of this bacterium during the entire process of primary production (Guard-Petter, 2001). Although sanitary plans, good hygienic and fabrication practices, and the application of hazard analysis-critical control points (HACCP) are imposed during food processing by competent authorities in developed countries, the success of these tools for the reduction of the incidence of human Salmonella infection always requires improvement. In the case of less-developed countries, these measures are inefficient because of their inadequate implementation. Consequently, it is important to focus on efforts to identify the best strategies to decrease the risk of Salmonella infection throughout the food chain to reduce the incidence of *S*. Enteritidis in egg products.

One of the modern ways to improve the hygienic safety of manufactured food products is to exploit the antimicrobial properties of natural plant extracts, allowing for the reduction of the use of chemical antimicrobial agents, which constitute a potential human health hazard. In this regard, the antimicrobial properties of EOs have been known for a long time and continue to be the subject of several studies that evaluate their microbial potential as alternatives to chemical agents in Food industries (Hammer, Carson, & Riley, 1999; Djenane et al., 2009; Djenane et al., 2012b). Thus, the objective of this study was to determine the in vitro anti-Salmonella activity of the EOs extracted from the leaves of *E. globulus* (Eucalyptus), *L. angustifolia* (Lavender), and *S. hortensis* (Summer savory) in relation to their chemical compositions and to evaluate the antibacterial activity of these EOs when added to liquid whole eggs stored at 7 ± 1ºC (to simulate Algerian abuse refrigeration conditions) under aerobic conditions for 6 days.

2. Materials and Methods

2.1 Essential Oils

2.1.1 Plant Material and Extraction of Essential Oils

The EOs was extracted from the aerial parts of *E. globulus*, *L. angustifolia*, and *S. hortensis* specimens collected in Tizi-Ouzou Province (Algeria) from March to May of 2009. These plant materials were deposited at the Herbarium of the Faculty of Biological Sciences and Agronomical Sciences of the University Mouloud Mammeri of Tizi-Ouzou (Algeria). The material was dried for 10 days at room temperature (25-30°C) in a dark room. The EOs were extracted using water steam distillation with a semi-pilot Clevenger apparatus (SAIDAL Pharmaceutical Group, Biotic Company, Algeria) and stored in the dark at 2°C in sealed tubes.

2.2 Essential Oil Analyses

2.2.1 Chromatography

The EOs were analyzed using a Hewlett-Packard 6890 chromatograph equipped with a Stabilwax capillary column (polyethylene glycol, PEG) (length of 30 m, internal diameter of 0.32 mm), with a film thickness of 1 μm (polysilphenylene-siloxane, by SGE), a split/splitless injector, and a flame ionization detector (FID) (Center for Technical and Scientific Research in Physical-Chemical Analysis, Alger, Algeria). The carrier gas was helium, and the auxiliary gas was hydrogen; gases were filtered to remove organic impurities. The analytical conditions were as follows: an injector temperature of 250°C; a detector temperature of 280°C; and an oven temperature of 60°C (8 min), increasing from 60 to 280°C (2°C/min), with a constant temperature of 280°C for 30 min.

2.2.2 Gas Chromatography (GC)/Mass Spectrometry

The EOs were analyzed using a Hewlett-Packard 6890 gas chromatograph system (Agilent Technologies) equipped with a capillary column (5% methylsiloxane phenyl, length of 30 m, internal diameter of 0.25 mm, film thickness of 0.25 μm) coupled to a Hewlett Packard 5973 quadrupole mass spectrometer with an electron impact detector, 70 eV, scanning 30-550 units of atomic mass (CRAPC, Algeria). The analytical conditions were as follows: an injector temperature of 250°C; a detector temperature of 280°C; and an oven temperature of 60°C (8 min), increasing progressively to 280°C at 2°C/min, with a constant temperature maintained for 30 min. The carrier gas was helium at a flow rate of 1.5 mL/min. For all of the analyses, a mixture of oil-hexane was injected (0.2 μL) in split mode. Each EO sample was injected three times, as was the internal calibration solution containing a mixture of C7-C29 n-alkanes. The various compounds of the EOs were identified by comparing their mass spectra to those of the compounds in the Willet and National Institute of Standards and Technology-NIST 98 databases for gas-phase chromatograph/mass spectrometry (CPG/MS) and the Adams spectral databases. The identification of the molecules was confirmed by comparing their retention indices to

those from the literature (Adams, 2001). The retention indices of the compounds were calculated using the time of retention of an n-alkane series with a linear interpolation.

2.3 Antibacterial Activity

2.3.1 Bacterial Strain and Culture Conditions

The *Salmonella enterica* serovar Enteritidis CECT 4300 strain used in this study was obtained from the Colección Española de Cultivo Tipo - CECT (Spanish Type Culture Collection). This strain was cultivated for 12 h at 37 ± 1°C on Mueller Hinton agar (MHA, Oxoid, Basingstoke, UK). Two successive inoculations were performed for 24 h at 37 ± 1°C in tubes containing 9 mL of Brain Heart Infusion Broth (BHIB; Oxoid, Basingstoke, UK). After 48 h, 100 µL of the bacterial suspension was used to inoculate BHI broth. The cultures were then incubated at 37 ± 1ºC for 12 h to obtain a fresh bacterial solution of approximately 2-3 × 10^5 colony-forming units (cfu)/mL, as determined by the transmission at 600 nm (Spectrophotometer: Spectronic 20 Bausch & Lomb). The *S*. Enteritidis strain was stored at -80ºC and re-isolated before each test.

2.3.2 Test of Agar Diffusion

The assessment of the antibacterial activity of the EOs was determined using the technique of agar diffusion described by Hazzit, Baaliouamer, Veríssimo, Faleiro, and Miguel (2009). Petri dishes containing 15 mL of molten Mueller Hinton agar were placed under a vertical laminar flow hood and were allowed to solidify and dry at 25 ± 1°C for 30 min. A 0.1 mL aliquot of the standardized inoculum suspension (2-3 × 10^5 cfu/mL) was applied and uniformly spread on each plate. All of the plates were allowed to dry for 5 min. The EOs were dissolved in a 0.5 % (v/v) solution of dimethyl sulfoxide (DMSO) (Sigma Aldrich®-Química, S.A.), and 5 µL of the EO was applied to sterile paper disks (6 mm in diameter, Filter LAB ANOIA, Barcelona, Spain) using a capillary micropipette (Finnpipette®, Thermo Fischer Scientific Inc.). The Petri dishes were incubated for 15 min at 25 ± 1°C and then at 37 ± 1°C for 24 h. The antibacterial activity was evaluated using a caliper (Wiha dialMax® ESD-Uhrmessschieber, CH) by measuring the diameter of the clear zones (mm) that developed around the disks (∅ of the disk is included: 6 mm). The sensitivity of *S*. Enteritidis to the different EOs was classified based on the diameter of the inhibition halos (Ponce et al., 2003): ∅ < 8 mm, insensitive; 9 < ∅ < 14 mm, sensitive; 15 < ∅ < 19 mm, very sensitive; and ∅ > 20 mm, highly sensitive. A negative control was used with the same solvent in which the EOs was dissolved. A standard antibiotic, chloramphenicol (10 µg/disk), was used as a positive control. Each test was repeated three times.

2.3.3 Minimum Inhibitory Concentration: Microdilution Test

The minimum inhibitory concentration was also determined for *S*. Enteritidis. The inoculum of *S*. Enteritidis was obtained from a pre-culture incubated for 12 h, and the microbial charge was adjusted to 5 × 10^3 cfu/mL using the McFarland 0.5 turbidity standard. The EOs were dissolved in DMSO (0.5%), and ½ serial dilutions were prepared in a concentration range of 32 to 0.3125 µL/mL in sterile test tubes containing Mueller Hinton (MH) broth. The MICs of the various EOs against *S*. Enteritidis were determined using the well microdilution method: 96-well plates (Iwaki brand, Asahi Techno Glass, Japan) were prepared by adding 95 µL of MH broth and 5 µL of inoculum to each well. A 100-µL aliquot of each EO solution previously prepared at a concentration of 32 µL/mL was added to the first well of each plate, and 100 µL of each serial dilution was then added to each successive well. The last wells were used as the negative controls and contained 195 µL of nutritive broth without EO and 5 µL of inoculum. The final volume of each well was 200 µL.

Levofloxacin, a standard antibiotic, was used as the positive control, with Levofloxacin concentrations (32-0.3125 µL/mL) prepared in MH broth. The same protocol as that for the EOs was used. All contents of each well were then homogenized (300 rpm/20 s), and the plates were placed at 37 ± 1°C for 18-24 h in an incubator with shaking. After the incubation, each well was examined, and the MIC (µL/mL) was determined by taking into account the lowest concentration of EO that inhibits all bacterial growth (no turbidity). The DMSO solution (0.5%) used to dissolve the EOs was used as the negative control. Each test was repeated twice.

2.4 Application to Liquid Whole Eggs

2.4.1 Preparation of Liquid Whole Eggs

Industrial chicken eggs were purchased from a local producer in Tizi-Ouzou (Algeria) and stored at 4 ± 1°C; eggs with blood spots or that were dirty or cracked were discarded. Before the beginning of the experiment, the egg shells were rinsed in 70% ethanol (Monfort et al., 2010) and air-dried. The disinfected eggs were then broken and placed in sterile Stomacher bags (Tekmar Co. Cincinnati, Ohio, U.S.A). The bags were then homogenized for 2 minutes at 300 rpm using a Stomacher 400 circulator (Tekmar Co. Cincinnati, Ohio, U.S.A).

The liquid whole eggs obtained were centrifuged at 10^2 × g for 2 min using a Heraeus Megafuge 1.0R to eliminate any residual air.

2.4.2 Treatments of Liquid Whole Eggs

Before the inoculation with *S*. Enteritidis, samples of the liquid whole eggs were analyzed for the presence/absence of *S*. Enteritidis. A 50-g sample of the liquid whole eggs was placed in a sterile glass container and inoculated with approximately 5×10^3 cfu of *S*. Enteritidis/g. Prior to the inoculation; various concentrations of the studied EOs were added to the samples, except for the negative controls. A total of 128 samples of 50 g each were prepared to examine the growth of *S*. Enteritidis in the presence of various EO concentrations (1 × MIC, 4 × MIC, 8 × MIC, and 16 × MIC). All of the samples were placed at 7 ± 1°C under aerobic conditions for 6 days. The counting of *S*. Enteritidis was performed every other day for the duration of the storage period.

2.5 pH Measurement

The pH of the liquid whole eggs was measured using a micro pH-meter model 2001 (Crison Instruments, Barcelona, Spain) after homogenizing 3 g of the product in 27 mL of distilled water for 10 s at 1300 rpm using a Ultra-Turrax T25 macerator (Janke & Kunkel, Staufen, Germany). Each value was the average of three measurements.

2.6 Sensory Analysis

Samples of liquid whole eggs were evaluated for freshness odor by a sixth-member trained panel. Panelists were selected among students and staff of the department and trained according to the guidelines of Djenane et al. (2001, 2012).

Three open-discussion sessions were held to familiarise panelists with the attributes and the scale to be used. The attribute "EO odor" referred to the intensity of perceptible EO odor after sample opening: 1 = none; 2 = slight; 3 = small; 4 = moderate; and 5 = extreme. A score of 3 or higher in any of the attributes denoted that liquid whole eggs were unacceptable for sale or consumption.

2.7 Bacteriological Analysis

In between each analysis, 25 g of samples was placed in sterile bags, and 225 mL of buffered peptone water was added aseptically. The bags were homogenized in a Stomacher for 1 min at 25ºC. Serial decimal dilutions were prepared in sterile peptone water (0.1%), and 0.1 mL of each dilution was inoculated onto Petri dishes containing the appropriate medium for *S*. Enteritidis (Salmonella-Shigella Agar - SS Agar, BD, 274500). The dishes were then incubated at 37ºC for 24 h. The results are expressed in $\log_{10}$ cfu/g.

2.8 Statistical Analysis

The variance was evaluated to determine significant differences between the treatments using the software SPSS (SPSS for Windows, 6.1.2., SPSS Inc., Chicago, IL). The differences between means were tested using the least significant difference (LSD). The values were considered significantly different when $p < 0.05$.

3. Results and Discussion

3.1 Chemical Composition of the Essential Oils

Hydro-distillation is currently the preferred industrial method to extract EOs. However, despite the fact that the extraction by supercritical carbon dioxide (CO_2) under high pressure produces EOs with natural organoleptic profiles, the use of this method in industry is limited due to its low economic profitability. The chemical analysis (Table 1) revealed a set number of compounds for the three EOs, representing 94.63%, 96.35%, and 95.57% for *E. globulus*, *L. angustifolia* and *S. hortensis*, respectively.

Table 1. Chemical composition (%) of the EOs obtained from the leaves of *E. globulus*, *S. hortensis* and *L. angustifolia*

Compound	*E. globulus*	*S. hortensis*	*L. angustifolia*
α-Pinene	2.32	0.07	0.51
Camphene	-	2.06	-
Sabinene	-	0.08	0.72
β-Myrcene	-	-	2.83
p-cymene	1.07	**12.04**	3.81
Limonene	-	-	**11.20**
β-Phellandrene	-	-	2.30
1,8- Cineole	**81.70**	-	**37.82**
Cis-β-Ocimene	-	-	0.50
β-Pinene	-	0.06	0.22
α-Phellandrene	0.08	0.20	-
α-Terpinene	0.02	3.70	-
γ-Terpinene	8.50	**11.43**	**-**
α-Terpinolene	-	0.34	1.11
Linalool	-	-	0.53
Borneol	-	0.37	0.60
2-Méthylbuterate	-	-	-
Terpinene-4-ol	0.08	0.90	-
Bornyl acetate	-	-	0.40
Linalyle acetate	-	-	0.22
α-terpinyl acetate	0.10	-	-
Carvacrol acetate	-	9.57	-
Carvacrol	-	**46.10**	-
α-Caryophyllene	-	5.06	-
β-Caryophyllene	0.04	-	**20.90**
Allo-aromadandrene	-	0.08	-
β-Cubebene	-	0.38	-
Ledene	-	0.09	-
β-Bisabolene	-	2.65	-
Cadinene	-	0.27	7.14
Spathulenol	-	0.07	-
Caryophyllene Oxyde	-	0.05	2.00
Camphre	-	-	1.73
Bergamotenes	-	-	1.81
Total identified(%)	94.63%	95.57%	96.35%

The EO of *E. globulus* was characterized by a high percentage of 1,8-cineole (81.70%) and γ-terpinene (8.50%). In contrast, the EO of *L. angustifolia* displayed a notable quantity of 1,8-cineole (37.82%), β-caryophyllene (20.90%), and limonene (11.20%), followed by cadinene (7.14%), p-cymene (3.81%), and β-myrcene (2.83%). The results from the analysis also showed that the EO of *S. hortensis* is rich in carvacrol (46.10%), p-cymene (12.04%), γ-terpinene (11.43%), and carvacrol acetate (09.57%), followed by β-caryophyllene (5.06%), γ-terpinene (3.70%), β-bisabolene (2.65%), and camphene (2.06%). However, the percentages of most of the identified compounds in the EOs vary significantly in the literature. These differences in composition could be due to seasonal variations in the different compounds of the plant's EO that are linked to changes throughout the life cycle of the plant. The environmental conditions in the Mediterranean region could also have an impact.

Indeed, environmental factors, such as geography, temperature, day length, and nutrients, have been examined and are thought to play a role in the chemical composition of EOs (Masotti, Juteau, Bessière, & Viano, 2003; Angioni, Barra, Coroneo, Dessi, & Cabras, 2006; Gardeli, Vassiliki, Athanasios, Kibouris, & Komaitis, 2008). These factors influence the biosynthetic pathways of the plant and, consequently, play a role in the relative proportions of the major characteristic compounds, leading to the existence of different chemotypes that distinguish EOs from different regions. Our data analysis showed that the EO chemical profiles were different from those of the same EOs derived from plants from different areas. Moreover, quantitative differences in individual compounds exist. For example, the EO of *E. globulus* from the municipality of Sobral in Brazil has been characterized by 1,8-cineole (83.89%), limonene (8.16%), and α-pinene (4.15%) (Maciel et al., 2010), whereas in the central area of the Congo in Africa, the chemotype of eucalyptus EO was reported to be mostly 1,8-cineole (44.30%), with high amounts of camphene (23.10%) (Cimanga et al., 2002). However, *E. globulus* from Portugal contains 1,8-cineole (63.80%) and α-pinene (14%) as the major compounds of its EO (Silvestre, Cavaleiro, Delmond, Filliatre, & Bourgeois, 1997).

According to the presence and quantity of the major compounds, the EO of L. angustifolia tested in the present study produced results that are different from those previously published. The EO of *L. angustifolia* from Iran contains 1,8-cineole (65.40%), borneol (11.50%), and camphor (9.50%) as the most abundant compounds (Hajhashemi, Ghannadi, & Sharif, 2003). However, the EO derived from *L. angustifolia* grown in Greece is characterized by linalool (20.10%), linalyl acetate (13.30%), and eucalyptol (12.40%) (Hassiotis, Tarantilis, Daferera, & Polissiou, 2010), whereas 1,8-cineole is the primary compound (38.40%), followed by cis-verbenol (4.30%) and cymene-8-ol (3.80%) in the EO of *L. angustifolia* from the Cherchel region (North Algeria) (Dob, Dahmane, Tayeb, & Chelghoum, 2005). Finally, the oil of *L. angustifolia* from the South of France is characterized by a high percentage of linalool (42.52%), 1,8-cineole (14.40%), and borneol (9.38%) (Sahraoui, Vian, Bornard, Boutekedjiret, & Chemat, 2008).

Several analyses were undertaken to determine the chemical profile of the EOs extracted from species of savory from various sources. Based on the presence and quantity of the major compounds, the EO of *S. hortensis* from our region contains the same percentage of carvacrol (46%) as the EO from an Iranian source (Sefidkon, Abbasi, & Khaniki, 2006). However, qualitative and quantitative differences were noted relative to other compounds. In a recent study, Hadian, Ebrahimi, and Salehi (2010) analyzed the EO of *S. hortensis* from several areas in Iran, and the primary compounds were found to be carvacrol (42-83.30%), γ terpinene (0.50-28.50%), and p-cymene (1-17.10%). Güllüce et al. (2003) determined the chemical composition of the EO of *S. hortensis* obtained by hydro-distillation using gas chromatography/flame ionization detection (GC/FID) and GC/MS, and 22 compounds representing 99.9% of the EO were identified, with thymol (29.0%), carvacrol (26.50%), γ-terpinene (22.60%), and p-cymene (9.30%) being the major compounds. Bakkali, Averbeck, Averbeck, and Idaomar (2008) noted that EOs is a very complex natural mixture that can contain approximately 20-60 compounds at very different concentrations. EOs are generally characterized by a few major compounds at relatively high concentrations (20-70%), with other compounds present in minute quantities, and the major compounds determine the biological properties of the EO. In conclusion, the various chemical compositions of the EOs could be correlated with the geographic origin of the plants and the ecological conditions under which the plants develop. Indeed, several authors suggest the possible existence of new chemotypes.

3.2 Antibacterial Activity (Agar Diffusion Method)

The in vitro tests of the antimicrobial activity of the different EOs against *S.* Enteritidis, a common pathogenic bacteria associated with foodborne diseases, were assessed qualitatively and quantitatively by the presence or absence of inhibition zones and the determination of the MICs. According to the results, the different EOs showed strong anti-Salmonella activities that were comparable to those obtained with chloramphenicol, the antibiotic used as the positive control.

The inhibition diameters obtained (for *S.* Enteritidis) were 51.15 ± 2.20, 41.30 ± 0.90, and 35.26 ± 3.20 mm, for the EOs of *S. hortensis*, *L. angustifolia*, and *E. globulus*, respectively; the inhibition zone of chloramphenicol was 33.25 ± 0.80 mm. The strong antimicrobial activity of these three EOs was confirmed by the microdilution method, showing MIC values against *S.* Enteritidis close to 1, 2, and 8 µL/mL for the EOs of *L. angustifolia*, *S. hortensis*, and *E. globules*, respectively.

Vilela et al. (2009) reported similar results for the EO of *E. globulus* tested against *Aspergillus flavus* and *Aspergillus parasiticus*. The authors pointed out that the EO of *E. globulus*, applied either by direct contact or by using the microatmosphere method, provided a total inhibition of these two fungi. The antimicrobial effects observed in the present study are for the most part comparable to those reported in the literature. Vagionas,

Graikou, Ngassapa, Runyoro, and Chinou (2007) recorded MIC values for the EO of savory against certain pathogenic bacteria that are very similar to our observations (0.04 to 0.10%). Many authors also reported that the EO of savory plants was among the most powerful EO in terms of its antimicrobial properties (Ciani et al., 2000), as was confirmed in the present study. The EOs of many species of the Satureja genus (savory) are known to have antibacterial and antifungal properties, such as *S. montana*, *S. hortensis*, *S. thymbra*, *S. biflora*, *S. masukensis*, and *S. pseudosimensis* (Ciani et al., 2000; Özcan & Boyraz, 2000; Özcan & Erkmen, 2001). In a recent study, Hanamanthagouda et al. (2010) evaluated the antimicrobial activity of lavender EO against certain bacteria (Gram positive and Gram negative) and molds and reported that lavender oil has a considerable antibacterial activity against Gram-positive bacteria, with MIC values ranging from 0.5 to 2 µg/µl and from 2 to 4 µg/µl for bacteria and molds, respectively. According to the same authors, lavender EO is characterized by an antibacterial activity that is stronger than its antifungal activity. The antibacterial activity attributed to these EOs could be due their elevated levels of compounds known for their antimicrobial activity, such as linalool (Sonboli, Eftekhar, Yousefzadi, & Kanani, 2005), p-cymene (Bagamboula, Uyttendaele, & Debevere, 2004), β-myrcene (De-Oliveira, Ribeiro-Pinto, & Paumgartten, 1997), 1,8-cineole (Sonboli, Babakhani, & Mehrabian, 2006), carvacrol (Botelho et al., 2007), and thymol (Wendakoon & Sakaguchi, 1995). Confirming previous reports, it has been noted that the power and spectrum of the antimicrobial activity differ among the species of Satureja and type of bacteria (Gram positive or Gram negative) studied. However, Gram-positive bacteria are generally more sensitive to the effects of EOs, whereas Gram-negative bacteria have a higher overall resistance because of their external phospholipid membrane that is almost impermeable to lipophilic compounds. The absence of this barrier in Gram-positive bacteria allows the direct contact of the hydrophobic compounds from EOs with the phospholipid bilayer of the bacterial cellular membrane. Thus, these compounds can be effective and lead to an increase in the ion permeability and the release of vital intracellular constituents or an alteration of the bacterial enzyme systems (Wendakoon & Sakaguchi, 1995).

Furthermore, the antimicrobial activity of EOs is difficult to correlate to a specific compound because of their complexity and variability. Nevertheless, some researchers have mentioned that there is a tight relationship between the chemical composition of the most abundant elements and the antimicrobial activity. For example, 1,8-cineole (abundant in the *E. globulus* and *L. angustifolia* EOs tested in this study) is well known for its antimicrobial potential (Sonboli, Eftekhar, Yousefzadi, & Kanani, 2005). Lis-Balchin and Deans (1997) showed that EOs with high levels of 1,8-cineole are better antibacterial agents than EOs that have no 1,8-cineole. Accordingly, strong antimicrobial activities of the EOs found in this study could be attributed to their chemotypes. Moreover, numerous reports have mentioned that carvacrol and thymol and their precursors (p-cymene and γ-terpinene) are biologically and functionally tightly associated (Ultee, Bennik, & Moezelaar, 2002). Within this context, p-cymene was more abundant in the EO of *S. hortensis* (12.04%) than in the EOs of *E. globulus* and *L. angustifolia*. The MIC values show that the EOs of *L. angustifolia* and *S. hortensis* were more efficient than that of *E. globulus*. Kim, Marshall, and Wei (1995) indicated that, because of the variation in the diffusion and solubility properties of the various EOs in different media, the results obtained using the disk method are not directly comparable to those obtained using the microdilution method. Nevertheless, our results showed that the EOs that resulted in large inhibition zones of *S.* Enteritidis is those that generated the lowest MIC values.

3.3 Application to Liquid Whole Eggs and Antibacterial Efficacy

Because of the use of such plants as eucalyptus, lavender, and savory in the cuisine and traditional medicine of the Kabylie region, the aim of this study was first to determine the in vitro antibacterial efficacy of the EOs obtained from these plants in this region of Algeria. Second, this study aimed at assessing the application of EOs to liquid whole eggs to test their anti-Salmonella efficacy. Figures 1-4 show the results of the *S.* Enteritidis growth in liquid whole eggs stored at 7 ± 1ºC in the presence of various concentrations of the EOs from *E. globulus*, *L. angustifolia*, and *S. hortensis*. These results show that the antibacterial effect generated was proportional to the concentration of each EO added to the product. Figures 1-4 also show that the number of Salmonella in the liquid whole eggs used as the controls (no EO added) reached 4.8 $\log_{10}$ cfu/g after the 4th day of storage; two days later (6^{th} day), this concentration reached a value of 5.4 $\log_{10}$ cfu/g. These results show that a significant antibacterial effect ($p < 0.05$) resulted from the EOs being applied to the product at a concentration equal to 1 × MIC (Figure 1), as compared to the samples with no treatment (controls). This effect was obvious from the 4th day of storage. Indeed, a decrease of 2.40, 2.55, and 1.96 $\log_{10}$ cfu/g (decreases of 44.50, 47.20, and 36.30%) was recorded on the last day of storage for the EOs of eucalyptus, lavender, and summer savory, respectively.

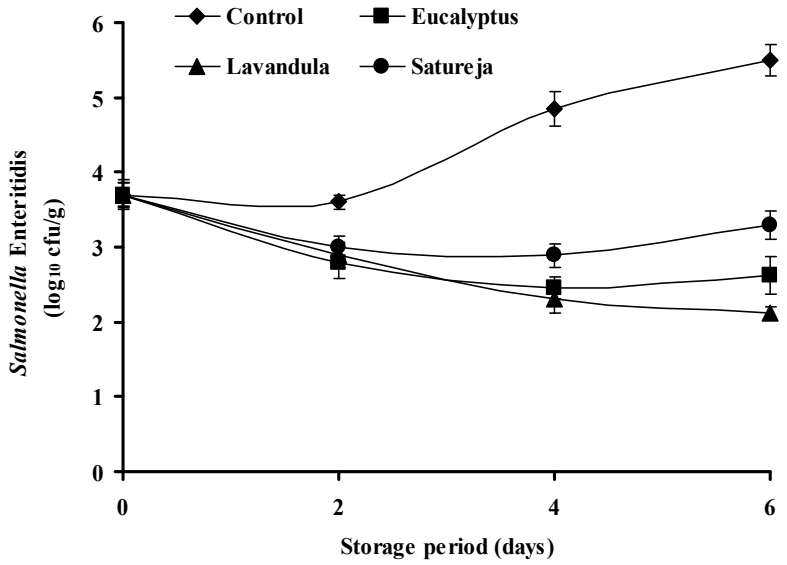

Figure 1. Inhibition of *S. Enteritidis* by the EOs added at a concentration equal to the MIC value in liquid whole eggs stored at 7 ± 1ºC. (♦) Control; (■) *E. globulus*; (▲) *L. angustifolia*; (●) *S. hortensis.* The error bars represent standard deviation

Figure 2 summarizes the antibacterial effect of the EOs applied to the liquid whole eggs at a concentration of 4 × MIC. According to the results, these EOs were all potent against Salmonella ($p < 0.05$), but the effect was dependent on the concentration.

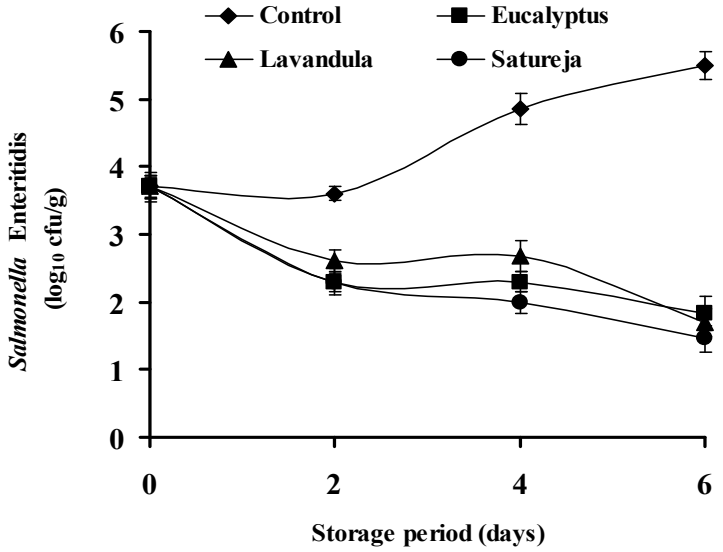

Figure 2. Inhibition of *S. Enteritidis* by the EOs added at a concentration equal to 4 times the MIC in liquid whole eggs stored at 7 ± 1ºC. (♦) Control; (■) *E. globulus*; (▲) *L. angustifolia*; (●) *S. hortensis.* The error bars represent standard deviation

In comparison with the previous results (1 × MIC), an increase in the inhibition rate on the order of 35, 35.08 and 53.20% (decreases of 1.05, 1, and 1.83 $\log_{10}$ cfu/g) was recorded on the last day of storage for the EOs of eucalyptus, lavender, and summer savory, respectively. Nevertheless, an application on the order of 8 × MIC (Figure 3) generates a stronger decrease in the bacterial growth when compared to that of the previous two treatments. Indeed, growth reductions of 0.98, 1.13 and 1.04 $\log_{10}$ cfu/g were recorded on the 4th day of storage for the 4 × MIC treatment for the EOs of eucalyptus, lavender, and summer savory, respectively.

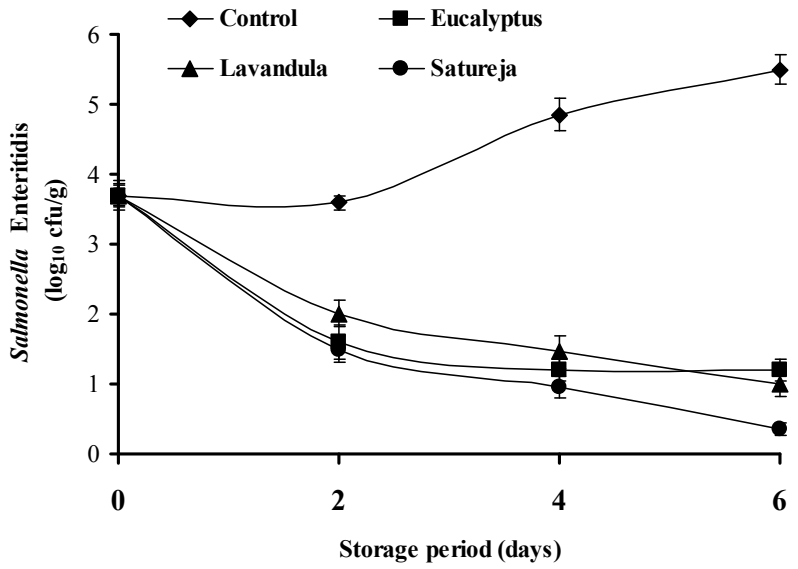

Figure 3. Inhibition of *S. Enteritidis* by the EOs added at a concentration equal to 8 times the MIC in liquid whole eggs stored at 7 ± 1ºC. (♦) Control; (■) *E. globulus*; (▲) *L. angustifolia*; (●) *S. hortensis.* The error bars represent standard deviation

However, the treatment of 8 × MIC generated a stronger growth reduction when compared to that of the 1 × MIC treatment. In this case, the growth reductions were 1.71, 1.54 and 2.30 $\log_{10}$ cfu/g and 1.96, 2.01 and 2.97 $\log_{10}$ cfu/g on the 4th and 6th storage days, respectively, for the same EOs. From our results, it is clear that an application on the order of 16 × MIC allowed a stronger bacterial reduction when compared to that of the other treatments. Figure 4 shows that, from day 4 of storage, the bacterial concentration reached values less than 1 $\log_{10}$ cfu/g for all of the EOs tested. Indeed, in control samples, inhibition rates of 81.55, 88.90 and 62.60% (reductions of 4.40, 4.80 and 5 $\log_{10}$ cfu/g) were recorded on the last storage day for the eucalyptus, lavender, and summer savory EOs, respectively.

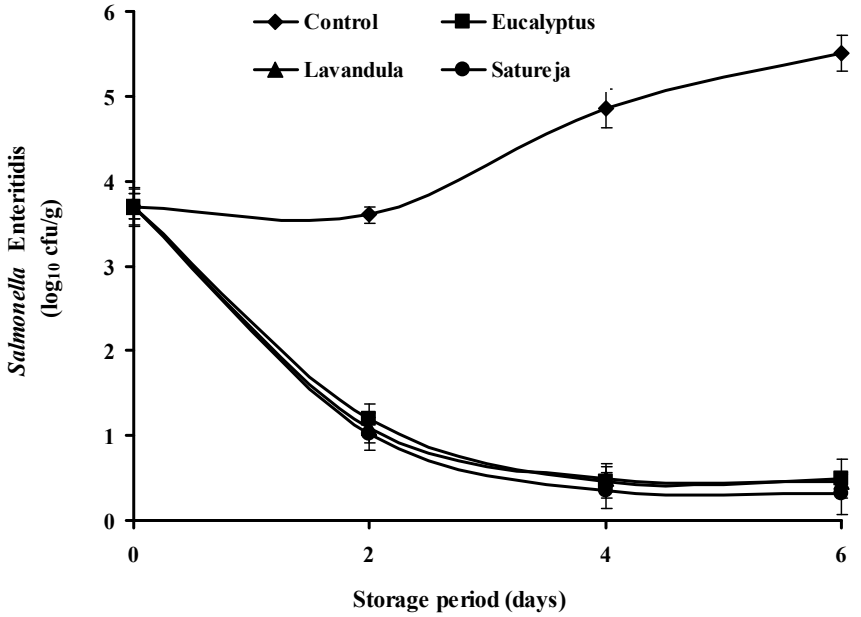

Figure 4. Inhibition of *S. Enteritidis* by the EOs added at a concentration equal to 16 times the MIC in liquid whole eggs stored at 7 ± 1 º C. (♦) Control; (■) *E. globulus*; (▲) *L. angustifolia*; (●) *S. hortensis*. The error bars represent standard deviation

This study shows that, when EOs are applied to a product at a low concentration, the observed effect is bacteriostatic. However, the bactericide activity was pronounced for all of the EOs, particularly for *S. hortensis*, when they are applied at higher concentrations. Several recent studies have successfully shown the potential application of EOs to reduce or control the presence of pathogenic agents in food products, such as milk (Karatzas, Kets, Smid, & Bennik, 2001), fish (Mejlholm & Dalgaard, 2002), fruits (Roller & Seedhar, 2002), and meat (Tsigarida, Skandamis, & Nychas, 2000). The antimicrobial activity of *S. hortensis* observed in this study could be attributed to the presence of higher concentrations of carvacrol, γ-terpinene, and p-cymene, three monoterpenes considered to be potential antibacterial (Oussalah, Caillet, Saucier, & Lacroix, 2007) and antifungal (Sefidkon, Abbasi, & Khaniki, 2006) compounds. Lis-Balchin and Deans (1997) showed that EOs with high quantities of 1,8-cineole are good antibacterial agents. The high antimicrobial activity of the EOs of eucalyptus and lavender could also be associated with their high levels of 1,8-cineole.

With regard to their antibacterial properties, EOs are considered a complex mixture of numerous chemicals. Thus, their antibacterial effects could be the result of a synergy among all of the molecules or due the major molecules that are present. The mechanisms by which microorganisms are inhibited by EOs seem to imply that there are different pathways involved. The phenolic compounds present in EOs have been recognized by several authors for their antimicrobial activity (Tajkarimi, Ibrahim, & Cliver, 2010). In addition, the necessity of increasing the EO concentrations used in food is linked to the more complex nature of food matrices (the presence of fat and proteins). The pH of liquid whole eggs is an important factor that affects the activity of EOs: at a low pH, the hydrophobicity of some EOs increases, and, consequently, they distribute properly in the lipid phase of the product. The EOs can also more easily dissolve in the lipid phase of the bacterial membrane, thus reinforcing their antimicrobial activity. The initial pH of the liquid whole eggs was 7.4 ± 0.2 and decreased to approximately 7.2 after the treatment with the EOs. The pH values were not significantly different ($p > 0.05$) among all of the samples during storage. The fact that the initial pH of the product slowly decreased in the presence of the EOs and that there was no significant difference ($p > 0.05$) between the samples could be explained by the buffering power of the product. As a general rule, the sensitivity of bacteria to the antimicrobial effect of EOs seems to increase when a decrease is noted in the pH of the food, the storage temperature, and the amount of oxygen (O_2) present around the product (Tsigarida, Skandamis, & Nychas, 2000). Concerning the nature of the food matrix, Holley and Patel (2005) found that some EOs were more efficient in products containing lower amounts of lipids. In agreement with these findings, Smith-Palmer, Stewart, and Fyfe (2001) showed that the active compounds present in different EOs generated a higher antimicrobial activity against *Listeria monocytogenes* in products low in fats when compared to products with high amounts of fats. The above-mentioned aspects should be considered for any optimized application of EOs in food products. Generally speaking, the major compounds in EOs are the reflection of their antibacterial activity. Thus, the synergic functions of the different molecules within EOs, as opposed to the action of one or two major compounds in the oil, seem arguable. However, it is possible that the activity of those major compounds is modulated by other minor molecules. In fact, the synergic effects of the diversity of the major and minor compounds present in EOs should be taken into consideration to understand their biological activity. According to studies investigating the mechanisms of the antimicrobial action of those molecules, it appears that, following the breakdown of the bacterial cellular membrane caused by these substances, the release of intracellular metabolites leads to cellular death (Rasooli, Rezaei, & Allameh, 2006; Cristani et al., 2007). Longara-Delamare, Moschen-Pistorello, Artico, Atti-Serafini, and Echeverrigaray (2007) attributed the high antibacterial activity of EOs to the presence of caryophyllene, a compound found in the lavender (20.90%) used in the present study. In our study, various EO concentrations were evaluated to test the effect of the concentration on the organoleptic properties of the product. The application of the three EOs to liquid whole eggs at the indicated concentrations seemed to improve the antimicrobial efficacy of the EOs. It is important to note that a panel of consumers did not detect any particular odor linked to the presence of the EOs in the liquid whole eggs (Table 2).

Table 2. Mean sensory scores (1–5) for EOs odor of liquid whole eggs stored for 6 days at 7 ± 1°C (1 = none; 2 = slight; 3 = small; 4 = moderate; and 5 = extreme. A score of 3 or higher denoted that product was unacceptable for sale or consumption)

Parameter	Treatment	Days of storage			
		0	2	4	6
Eucalyptus odor	Control	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
	1×MIC	2.17±0.41	2.33±0.52	1.00±0.00	1.00±0.00
	4×MIC	2.33±0.52	2.17±0.41	2.00±0.00	1.00±0.00
	8×MIC	2.50±0.55	2.50±0.84	2.17±0.41	2.00±0.00
	16×MIC	3.00±0.00	2.33±0.52	2.17±0.41	2.17±0.41
Lavender odor	1×MIC	2.00±0.00	2.66±0.82	1.00±0.00	1.00±0.00
	4×MIC	2.17±0.41	2.17±0.41	2.00±0.00	1.00±0.00
	8×MIC	3.00±0.00	2.33±0.52	2.17±0.41	2.00±0.00
	16×MIC	4.00±0.00	2.50±0.55	2.17±0.41	2.33±0.52
Summer savory odor	1×MIC	2.33±0.52	2.17±0.41	1.00±0.00	1.00±0.00
	4×MIC	2.67±0.52	2.50±0.84	2.00±0.00	1.00±0.00
	8×MIC	2.83±0.75	2.17±0.41	2.17±0.41	2.00±0.00
	16×MIC	3.00±0.63	2.33±0.52	2.17±0.41	2.17±0.41

Consequently, the sensorial properties of the liquid whole eggs treated with these EOs were acceptable to the panelists. Preliminary studies showed that a mixture of various EOs , or EOs mixed with other compounds, had a stronger antimicrobial efficacy against food-borne pathogenic agents (Gutierrez, Barry-Ryan, & Bourke, 2008; Djenane et al., 2011a,b). However, additional studies are necessary to evaluate the efficacy of an EO mixture and the use of active packaging to assess their performance as natural antimicrobial agents for preservation and food safety (Ponce, Fritz, del Valle, & Roura, 2003; Monfort et al., 2010).

4. Conclusion

The results of the antibacterial tests and the very interesting chemical profile of the EOs support their potential use as natural preservation agents to help reduce *Salmonella* in liquid whole eggs. Sensory evaluation revealed that the aroma of liquid whole eggs treated with EOs was acceptable by panellists at the levels used.

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Moisture Adsorption Isotherms of African Arrowroot Lily (*Tacca involucrata*) Tuber Mash as Influenced by Blanching and Natural Fermentation

B. D. Igbabul[1], C. C. Ariahu[1] & E. U. Umeh[2]

[1] Department of Food Science & Technology, University of Agriculture, Makurdi, Nigeria

[2] Department of Biological Sciences, University of Agriculture, Makurdi, Nigeria

Correspondence: B. D. Igbabul, Department of Food Science & Technology, University of Agriculture, Makurdi, Nigeria. E-mail: bibidoo2009@yahoo.com

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Abstract

The adsorption isotherms of African arrowroot lily (*Tacca involucrata*) as influenced by blanching and natural fermentation were investigated at 10, 20, 30, and 40°C using gravimetric method. The BET, GAB, Oswin, Halsey, Henderson equations were fitted to the experimental sorption data and Clausius-Clapeyron equation was used to determine the isosteric heat of sorption. The equilibrium moisture content of the products at a given water activity is decreased by blanching and fermentation. The developed models, GAB, Oswin, Halsey and Henderson equations in that order provided good fit to experimental data. The BET monolayer moisture contents (M_o) values were lower than the values predicted by the GAB model. The surface areas for monolayer moisture sorption decreased with increasing temperature and blanching and fermentation reduced these values. Isosteric heat of sorption decrease at the average temperatures investigated with increase in moisture content. Blanching and fermentation offer perspectives to improve the shelf life of the product.

Keywords: blanching, fermentation, moisture adsorption, monolayer moisture content

1. Introduction

The African arrowroot lily (*Tacca involucrata*) is a perennial plant that belongs to the family *Araceae* of the order *Arales*. The plant is native to tropical Africa and is widely distributed in most parts of the forest and savannah regions of Nigeria. The African arrowroot lily produces underground swollen stems or tubers, which are the main food reserve. The tubers are spherical in shape and measures up to 5 cm in diameter. The tuber known as "gbache" and "onu-umwah" respectively by the Tiv and Etulo people is one of the unconventional and less exploited sources of food for human and animal nutrition. In Benue, Nassarawa and Plateau States of Nigeria, the tubers are processed into thick gels, which are eaten with soups, stews and beniseeds. The starch is used as a thickening agent for soups and in local textile industries for improving the strength and shine of clothing. There is increasing attention to the cultivation and utilization of the plant since it acts as a "bridge tuber crop" between the planting and harvesting periods of the conventional roots and tubers such as cassava, potato and yam. Unfortunately, the African arrowroot lily tubers are highly perishable and therefore require adequate and prompt preservation or processing for shelf life extension. Several preservation processes have been developed in order to extend the shelf life of foodstuffs by lowering the availability of water to microorganism and inhibiting some chemical reactions. Water activity (a_w) describes water availability and mobility in foods while moisture content is an important criterion to judge food quality (Arslan & Togrul, 2006). To understand the stability of foodstuffs, the relationship between moisture content and water activity known as moisture sorption isotherm has to be established. This stability is mainly a consequence of the relationship between the equilibrium moisture content of the food material and its corresponding water activity at a given temperature (Myhara et al., 1998). An addition or removal of water changes the composition and dimensions of products and may induce phase changes (Irzyniec & Klimezak, 2003).

Sorption isotherms of foodstuffs are essential for design, modeling and optimization of many processes such as aeration, drying and storage (Labuza, 1968; Bala, 1991; Ariahu et al., 2006). Knowledge of sorption isotherms is also important for predicting stability and quality changes during packaging and storage of dried

foods and for ingredient mixing prediction (Jamali et al., 2006). The knowledge of sorption isotherms at different temperatures allows the isosteric heat of sorption to be defined. The heat of sorption determines the interaction between an adsorbent and adsorbate. Water availability in the reactions of food degradation depends both on water content and on the properties of the diffusion surface, which is the thermodynamic function of sorbed water. The thermodynamic functions of several foods have been evaluated using experimental sorption data (Al-Muhtaseb et al., 2004; Mcminn & Magee, 2003). However, information is unavailable on the equilibrium moisture content (EMC) of African Arrowroot lily tubers at various relative humidity and temperatures. The world's energy demand for human nutrition and industrial purposes is growing very rapidly. The present cassava initiative and emphasis by the Federal Government of Nigeria is a response to the search for cheaper sources of starch for domestic and international industrial markets. Starch is used for production of ethanol, glucose, sugar syrup, artificial honey and various pharmaceuticals. Their demands have resulted to increase in the price of the conventional staples. There is therefore the urgent need to shift emphasis to the less exploited and unconventional tuber crops such as *Tacca involucrata* for domestic and industrial uses. Such a shift will reduce the current competition demand and pressure on the common staples like yam, potatoes and cassava.

There is the need to study the equilibrium moisture content of African arrowroot lily tubers in order to understand its drying characteristics and storage behavior. The present study was carried out with the objective of determining moisture adsorption isotherms of the African arrowroot lily lily in order to investigate whether pretreatments like blanching and fermentation could have an effect on the storage stability. The net isosteric heat of sorption was calculated from the experimental data. Moisture sorption models in literature were used to analyse the isotherms. Efforts were also made to find out the most suitable model describing the isotherms of African arrowroot lily.

2. Materials and Methods

2.1 Sources of Materials and Preliminary Handling

Mature (4-5 months) tubers (5-6 cm, diameter); 76% (wet basis) moisture content were harvested from farm lands in the University of Agriculture, Makurdi; and transported to the laboratory in jute bags. The tubers were promptly sorted and washed with tap water to remove adhering soil and other foreign matter. Air tight 500 ml capacity plastic containers (12.5 cm diameter x 11.8 cm height each) and wire gauze were purchased from a local market.

The flow chart for the production the various *Tacca involucrata* tuber products are shown in Figure 1. Essentially, the tubers were peeled and sliced to 0.5 cm thickness using stainless steel knives. The slices were divided into four sub-lots/ sub-lot I was blanched, wet milled and the mash subjected to accelerated natural fermentation 'involves back-sloping' as described below. Sub-lot II was blanched but non-fermented. Sub-lot III was non-blanched, mashed and subjected to accelerated natural. Sub-lot IV, which served as control in this research, was non-blanched and non-fermented.

Adequacy of blanching was verified using peroxidase test strips (Sigma Company, California, USA). Accelerated natural fermentation was achieved using the methods described by Ariahu et al. (1999). In this process, 120 g of mash of each of sub-lots I and III was placed in a covered 500 ml glass beaker at room temperature (30 ± 2°C) for 24 hours. At the end of this period, 50% of the fermenting mash was used as starter for a new fermentation cycle. During this process, the pH and titratable acidity (an index of lactic bacteria activities) were monitored (Results not shown). The fermentation process was continued until pH of the concentrates stabilized by remaining constant with further increase in fermentation cycles. The pH of the concentrates was 4.00, 4.30, 6.20 and 6.50 for BF, NBF, BNF and NBNF respectively (Igbabul et al., 2012). As starch gelatinizes at temperatures above 60°C (Gevauden et al., 1989), the various sub-lots were dried at 55°C in a vacuum oven to minimize the influence of heating especially on the non-blanched samples. The dried samples were milled using a bench-top hammer mill (Brook Compton series 200, Christy Hunt England) to pass through a sieve size of 500 µm. Each powder was packed in 10 g sachets in polyethene bags and sealed with an electric impulse sealing machine (model 210-8E, Clamco Corporation, Cleveland, Ohio). Sub-lots I, II, III and IV were designated as blanched - fermented (BF), blanched - non fermented (BNF), non blanched fermented (NBF) and non blanched - non fermented (NBNF) flours respectively. The packaged products were stored on a dry shelf in air- tight metal containers until used and moisture adsorption studies.

2.2 Sorption Isotherm Studies

Sorption studies were as described by Ariahu et al. (2006) with some modifications. Sulphuric acid solutions were used to provide water activities ranging from 0.08-0.93 as described by Ruegg (1980). About 100 ml of each acid solution were introduced into 500 ml airtight plastic containers. In each plastic container, wire gauze was

forced into place over the sulphuric acid solution to form support for the samples. Triplicate samples, each of 0.5 g of the dried products were weighed in crown corks and placed on the wire gauze above the sulphuric acid solutions for adsorption isotherm studies. The containers were covered tightly with lids and allowed to equilibrate in a thermostatically controlled cooled incubator (Gallenkamp model no GB 4043 U.K) at the selected temperature of 10, 20, 30 and 40°C respectively. The samples were removed and weighed every 2 days until difference between consecutive reading were < 0.5% of each sample weight. The total time for removal and putting back in the air tight containers was about 2-5 min as recommended by the Cooperative Project, COST 90 (Gal, 1988). This minimized atmospheric moisture sorption or desorption during weighing (The adsorption results are presented in this paper). The EMCs were determined by material balance from the initial moisture contents (Toledo, 1980).

2.3 Modeling of Sorption Isotherms

The equilibrium moisture data were fitted using the BET, GAB, Oswin, Halsey Henderson models (Iglesias & Chirife, 1978; Rizvi, 1995; Lomauro et al., 1985a, 1985b; Iglesias & Chirife, 1982). These models (Equations (1)-(5)) in Table 1 were chosen for their versatility, relatively simple mathematical computations and their reported fits for starchy foods. Monolayer moisture contents were evaluated using the BET (a_w up to 0.48) and GAB (a_w up to 0.83) models. The sorption data were analyzed using analysis of variance (ANOVA), least square linear regression and non-linear regression equations. ANOVA was by multiple range F-test (Gupta, 1978). The derivatives and constants of Oswin, BET, Henderson and Halsey models were obtained by least square linear regression from their respective equations. The non-linear regression (NLR) using window release 6.0 of SPSS software (SPSS Inc., 1993).

The goodness of fit of the different models were evaluated with the percentage root mean square of error (% RMS) between the experimental (M_{obs}) and predicted (M_{est}) moisture contents as described by Iglesias and Chirife (1976a), Mok and Hettiarachchy (1990), and Wang and Brennan (1991):

$$\%RMS = \sqrt{\frac{\sum\left(\frac{M_{obs} - M_{est}}{M_{obs}}\right)^2}{n}} \times 100$$

Where M_{obs} and M_{est} are experimental and predicted moisture values respectively and n is number of experimental data.

2.4 Isosteric Heat of Sorption

The net isosteric heat of sorption gives a measure of the water-solid binding strength. The determination of the sorption of the differential molar quantities derived from the temperature dependence of the sorption isotherm was calculated by applying Clausius-Clapeyron equation:

$$\ln a_w = C_{st}\frac{1}{RT}$$

Where ΔH_{st} = net isosteric heat, C_{st} = constant related to entropy of sorption, T = temperature at absolute condition (°C) and R= molar gas constant (0.008314 kJ/mol °C)

To the isosteres obtained at constant moisture contents up to 30 g H_2O/100 g solids following the procedure reported by Iglesias and Chirife (1976b), MCMinn and Magee (2003) and Ariahu et al. (2006). Re-plotting the experimental sorption isotherm in the form In (a_w) versus I/T, for a specific moisture content, ΔH_{st} was determined from the slope ($-\Delta H_{st}$ /R). This procedure is based on the assumption that ΔH_{st} is invariant with temperature and requires measurement of the sorption isotherms at more than two temperatures (Tsami et al., 1990a).

The predictive models for the relationship between moisture content and net isosteric heats was proposed by Tsami et al. (1990b) using an empirical equation to describe the relationship between ΔHst and the equilibrium moisture content:

$$\Delta Hst = \Delta Ho \exp.(M/Mc)$$

where M is the equilibrium moisture content, ΔHo is the isosteric heat of sorption of first molecule of water; Mc is a characteristic moisture content of the food material. For the adsorption isotherms of NBNF, BF, NBF and BNF *Tacca involucrata* products, the following models are proposed:

$$\Delta H_{st} = 66.1797 \exp. 0.0660M \ (r^2 = 0.988)$$

$$\Delta H_{st} = 42.3724 \exp. 0.0838M \ (r^2 = 0.967)$$

$$\Delta H_{st} = 38.4425 \exp. 0.7920M \ (r^2 = 0.983)$$

$$\Delta H_{st} = 43.7348 \exp. 0.0693M \ (r^2 = 0.986)$$

3. Results and Discussion

3.1 Sorption Isotherms

The adsorption isotherms of Tacca involucrata at 10, 20, 30 and 40°C are shown in Figures 2-5. These temperatures were selected as possible storage conditions. The isotherms have a sigmoidal shape depicting an increase in the equilibrium moisture content with a_w. This is typical of type II isotherms (Brunauer et al., 1940), and has been reported for starchy products such as potato and wheat starch (Van den Berg, 1981), potato starch gel (McMinn, 1996), cookies and corn snacks (Palou et al., 1997).

It can be seen from the curves, that the equilibrium moisture contents (EMCs) increased with increase in water activity and were lower as the temperature increased. The effect of temperature on food isotherms is extremely important in food technology. During storage, a dry food product stored in sealed package may be exposed for long periods of time to temperatures higher than the temperature at which it was package. Consequently, the activity of the water vapour at constant moisture content increases thereby altering the food's quality (Dural & Hines, 1993). The water activity shift of food isotherms at constant moisture content with respect to temperature variation has been shown to be directly related to the rates of food deteriorative reactions (Van den Berg & Bruin, 1981; Iglesias & Chirife, 1982; Labuza, 1984). Hill and Rizvi (1982) noted that generally, increasing the temperature results in an increase in water activity. For most dry foods, an increase in water activity of 0.1 may decrease the shelf life by a factor of two to three. Therefore, the greater influence of temperature on the water sorption isotherms of the native NBNF indicates that pretreatments such as blanching and fermentation would enhance storage stability of the products.

As temperature is increased at constant a_w, the equilibrium moisture content decreases. The amount of adsorbed water by the Tacca involucrata tuber products therefore decreased with increase in temperature at constant relative humidity. Thus, the products became less hygroscopic at higher temperatures.

The results obtained from the present study therefore implied that the equilibrium moisture content of the products at a given water activity is decreased by blanching and fermentation, and these treatments offer perspectives to improve shelf life. Comparison of Figures 2-5 and especially Figures 3 and 4 suggests that these treatments have a stronger effect on moisture adsorption than blanching. Further examination of the isotherms indicated that the pretreatments (blanching and fermentation) had significant ($p < 0.05$) effects on the moisture sorption isotherms. Generally, the native (NBNF) samples had higher positioned isotherms and hence higher moisture sorptive capacity followed by BNF, then NBF and lowest for the BF at the temperatures investigated. This observation indicates that blanching and fermentation treatments could have affected the sorption sites of the flour. The heat may have damaged some active binding sites while fermentation may have modified the tissue structures thereby reducing the number of active binding sites that are available for water molecules. The higher sorptive capacity of the NBNF samples could be due to availability of relatively more undamaged and unoccupied binding sites. Studies by Johnson and Brennan (2000) indicated that the sorptive capacity of a product can be influenced by treatment or denaturation processes like heating, desalting and pH changes. As reported by Kapsalis (1984) and Johnson and Brennan (2000), heat treatment may change the polar and other groups that bind water along with changes in capillary and other configurations of the food structure thereby affecting the moisture sorption of the food product.

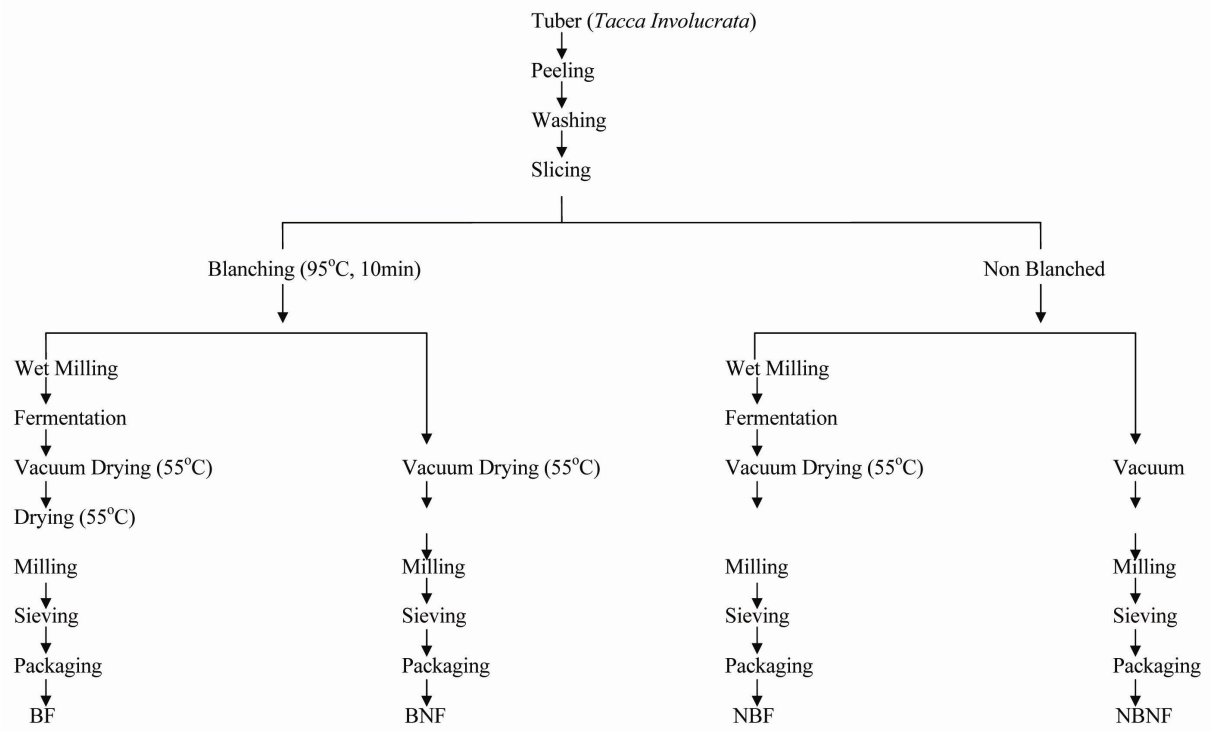

Figure 1. Flow chart for production of various *Tacca involucrata* tuber products

BF = Blanched fermented, BNF = Blanched non-fermented, NBF = non-blanched fermented and NBNF = Non-blanched non-fermented.

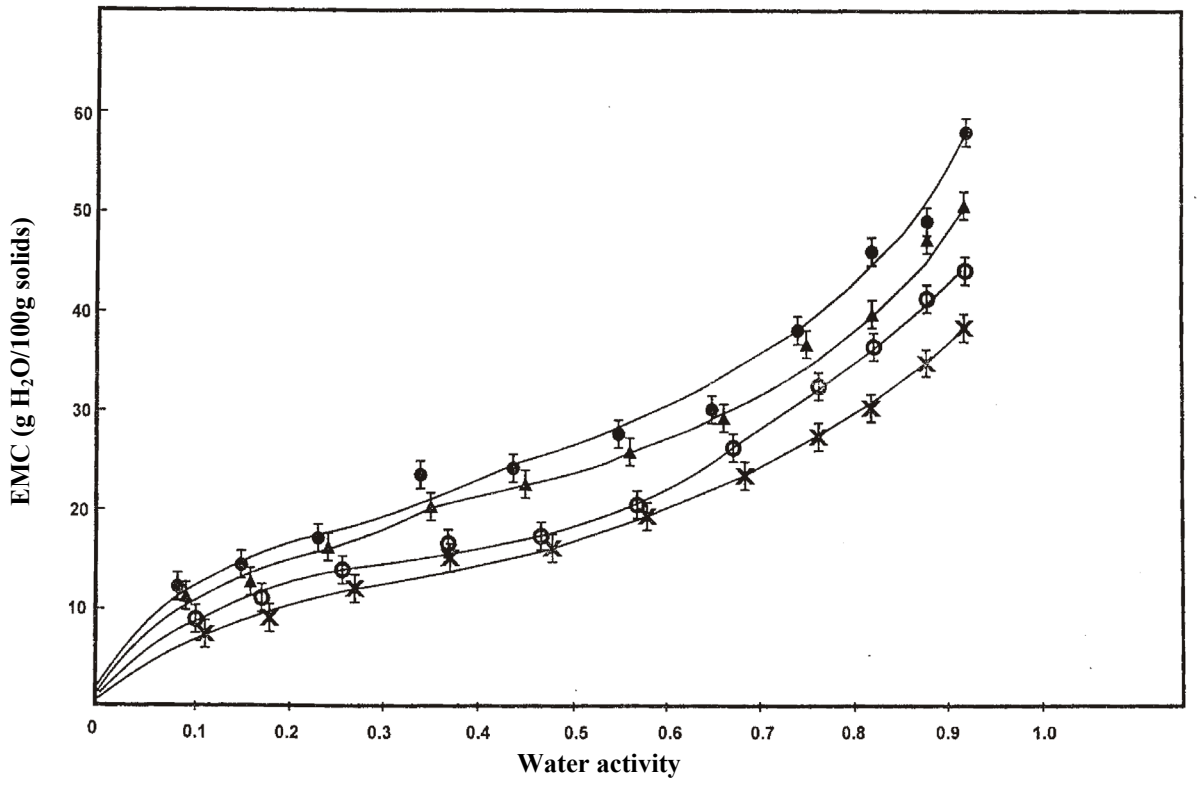

Figure 2. Moisture isotherms of non-blanched non-fermented *Tacca involucrata* tuber product at 10°C (●), 20°C (▲), 30°C (O), and 40°C (**X**)

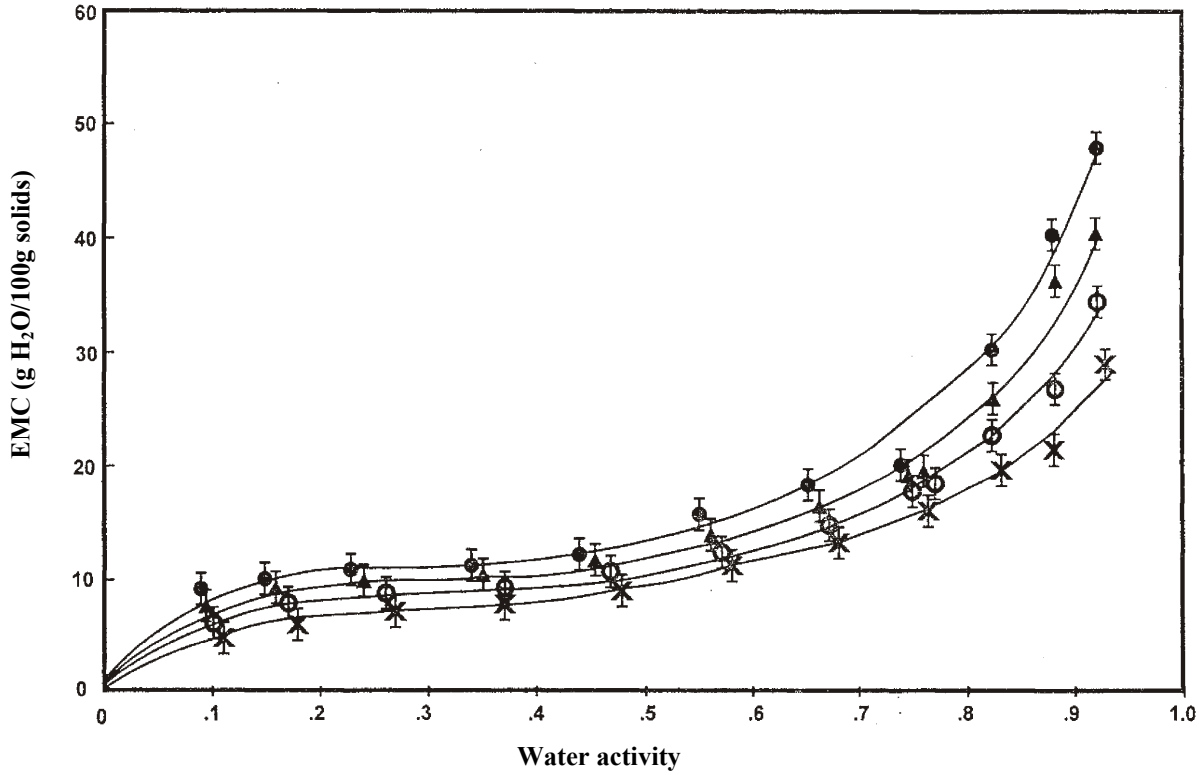

Figure 3. Moisture adsorption isotherms of blanched non-fermented *Tacca involucrata* tuber product at 10°C (●), 20°C (▲), 30°C (O) and 40°C (X)

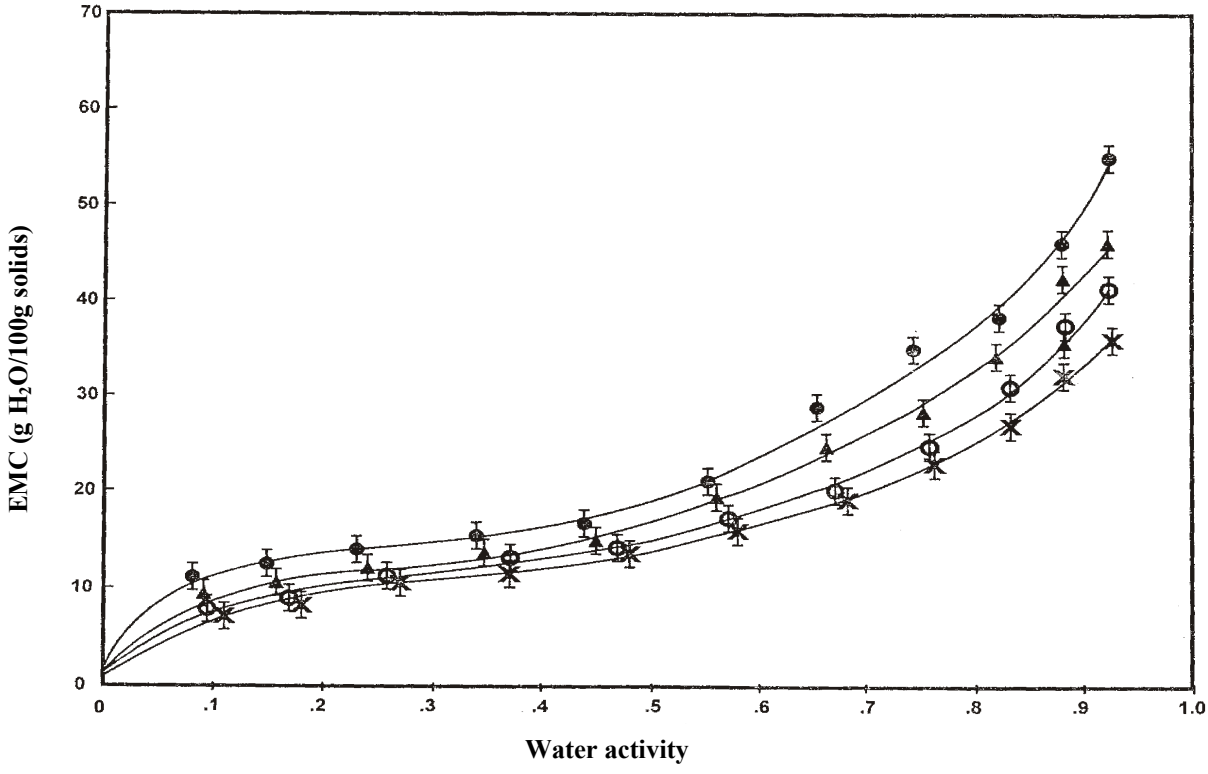

Figure 4. Moisture adsorption isotherms of blanched non-fermented *Tacca involucrata* tuber product at 10°C (●), 20°C (▲), 30°C (O) and 40°C (X)

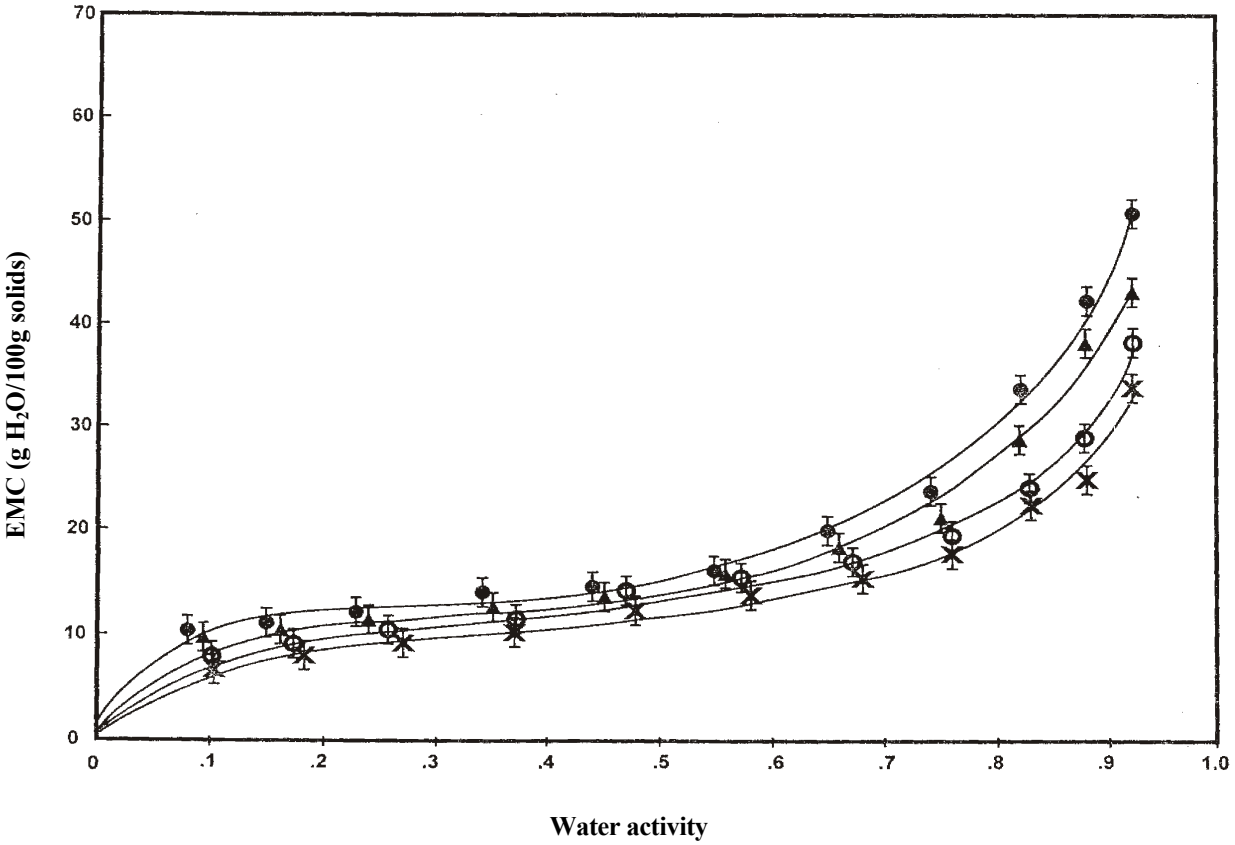

Figure 5. Moisture adsorption isotherms of blanched and fermented *Tacca involucrata* tuber product at 10°C (●), 20°C (▲), 30°C (O) and 40°C (X)

3.2 Comparisons of Sorption Models

The percent root mean square of error (% RMS) of the various models tested in this study is presented in Table 2. According to Wang & Brennan (1991), percent root mean square of error (% RMS) values of ≤ 10% indicate a reasonably good fit for practical purposes. The BET isotherm is usually valid for a_w between 0.1 and 0.5 (Chirife and Iglesias, 1978). This is considered a disadvantage when compared with the GAB model which predicts EMCs up to a_w of 0.90 (Rizvi, 1995). Generally, the GAB model and the predictive equations generated in this study (Table 3) gave the best fit to the overall data analysed. The Halsey and Oswin models also described the sorption data adequately. These results are in conformity with the observations of Bizot (1983), Lomauro et al. (1985a, 1985b) and Palou et al. (1997) that the GAB model fits best in various foods of plant and animal origin. The Henderson model gave an $r^2 \geq 0.869$ for the sorption of the water by *Tacca involucrata* products. In comparison with the other models investigated, the Henderson equation gave the lowest r^2 values.

3.3 Monolayer Moisture Contents and Derivative

The monolayer moisture content (M_o) is a measure of the moisture content for maximum stability of a food material (Rockland & Nishi, 1980). The M_o values calculated using the BET and GAB models (Tables 4 and 5) indicated that the blanched and fermented samples gave the least monolayer values in both the adsorption and desorption models. This could be an indication that pretreatments (blanching and fermentation) might have destroyed some active sites and/or affected the tissues leading to a decrease in its sorptive ability and subsequently the monolayer moisture contents. The GAB equation gave higher monolayer values than BET model. Studies by other workers showed similar variations in M_o values between the GAB and BET equations. The results obtained in this present study are consistent with those of Labuza et al. (1985) who reported that the M_o values for corn and fish flour calculated by the GAB and BET models were different. Similar trends were reported by Van den Berg (1985) for starch, Mok and Hettiarachcy (1990) for sunflower products and Palou et al. (1997) for cookies and corn snacks. Dural and Hines (1993a, 1993b) and also Timmermann et al. (2001) reported that the M_o values determined from the GAB equation were 3 to 18% higher than values predicted by the BET equation.

The results for the surface areas of sorption (S_o) obtained from this study were higher than the values of 100-250 m^2/g solid exhibited by most foods as reported by Labuza (1968). The higher magnitude of surface area exhibited by the Tacca involucrata tuber products could be attributed to the fact that water molecules are able to plasticize the various long chain polymers that make up the structural matrix, thus exposing more sites for sorption. In addition, since the water molecule is smaller, it is able to enter smaller pores and crevices thus increasing the surface area more than surface area predicted using nitrogen in the BET theory (Labuza, 1968).

3.4 Isosterics Heat of Sorption

The net isosteric heats of sorption (Figure 6) increased until a maximum and then decreased with increase in moisture content until it finally leveled off. The increase in isosteric heats at low moisture contents can be explained considering that the sorption of water by the dry matrix lead to swelling of the food polymers resulting in the exposure of sorption sites of higher binding energies not previously available. After the maximum, the decrease in isosteric heat with the amount of water sorbed, can be qualitatively explained considering that initially, sorption occurred on the most active available sites giving rise to greatest energy interaction. As these sites became occupied, sorption occurred on the less active site giving lower heats of sorption. (Palou et al., 1997; Dural and Hines (1993) explained that such curves are indication of surface heterogeneity. The trend observed in this study conformed to the report of Wang and Brennan (1991) and Loong et al. (1995).

The maximum bet isosteric heat were higher than those reported by Iglesias and Chirige (1976b) for tapioca (12.6 kJ/mol), Benado and Rizvi (1985) for sorghum (17.0 kJ/mol); Labuza et al. (1985) for cornflour (18.6 kJ/mol), and Palou et al. (1997) for cookies (between 6.7-10.1 kJ/mol) and cornsnacks (7.5 kJ/mol). The differences in the isosteric heats could be due to differences in the chemical and structural compositions of the food materials. Palou et al. (1997) advanced some reason for the variation in the net isosteric heats of sorption between their work and that of others. These workers attributed their low net isosteric heats of sorption to the extensive heat treatment that the studied products received. In this present study, the heat treatment may have damaged some sorptive sites resulting in lower net isosteric heats. This is possibly the reason for the highest net isosteric heats observed for othe NBNF (native) samples that did not receive any pre-treatment. If this reasoning holds, it then implies that the pre-treatment that affected the sorption sites most was fermentation followed by blanching.

4. Conclusions

The moisture sorption isotherms of Tacca involucrata tuber flour follow the type II isotherm pattern for the temperature range of 10 to 40°C. Temperature has effect on the sorption behavior at higher a_w values being observed at the same moisture content when temperature is increased. Among the models evaluated, the Henderson model proved the least adequate function to describe the experimental data. The other models fit the data reasonably well, with the best fit being the GAB model. The GAB monolayer values are higher than the BET values for the African arrowroot lily powder. Blanching and fermentation reduce the hygroscopic and other thermodynamic properties of the dehydrated products, with the BF sample having the least EMCs, monolayer moisture values, surface area of sorbent and net isosteric heats of sorption.

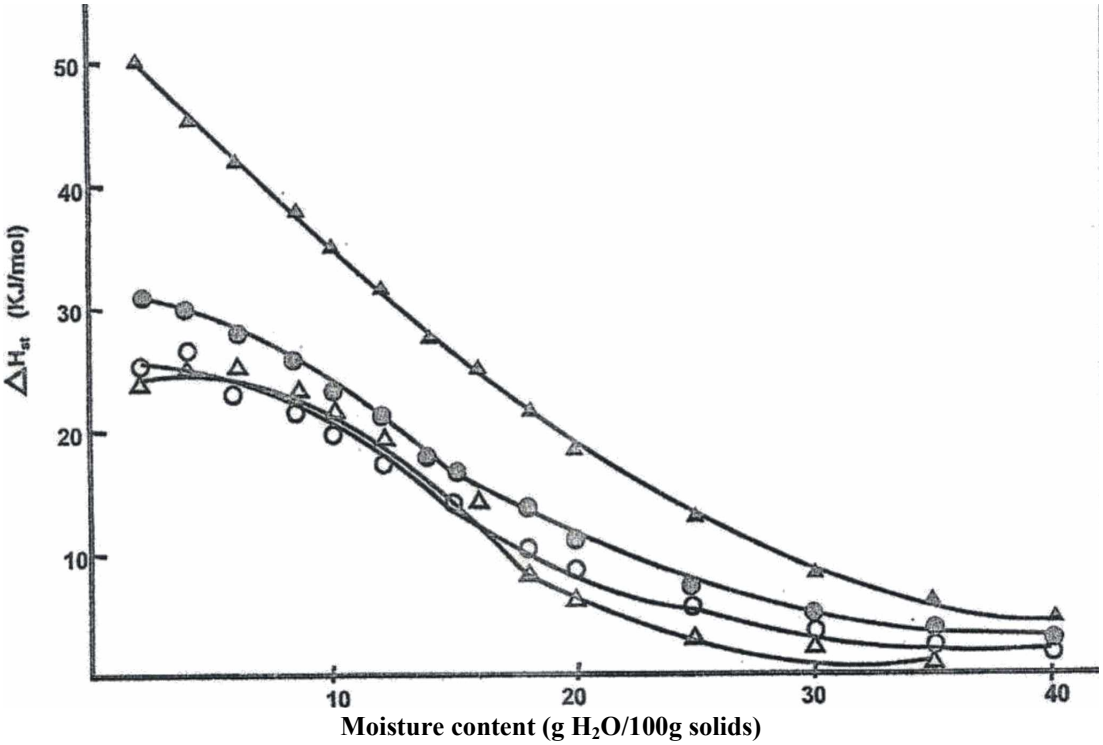

Figure 6. Isosteric heats (ΔH_{ST}) of moisture adsorption in blanched and fermented (△), blanched non-fermented (●), non-blanched fermented (O) and native (A) *Tacca involucrata* tuber products

Table 1. Sorption model equations used for dehydrated African arrowroot lily

Model	Mathematical expression
Brunauer-Emmett-Teller (BET) (Brunauer et al, 1938)	$\frac{a_w}{(1-a_w)M} = \frac{1}{M_0 C} + \frac{C-1}{M_0 C} a_w$
Guggenheim-Anderson-de Boer (Bizot 1983)	$\frac{M}{M_0} = \frac{GKa_w}{(1-Ka_w)(1-Ka_w+GKa_w)}$
Halsey (1948)	$a_w = \exp\left[\frac{A}{RT\theta^r}\right]$
Oswin(1946)	$M = A\left[\frac{a_w}{1-a_w}\right]^B$
Henderson (1952)	$\ln(1-a_w) = -ATM^B$

M = moisture content (g H_2O/100g solids), Mo = monolayer moisture content (g H_2O/ 100g solids), a_w = water activity, C = BET constant, G and K = GAB constants, A, B and r are constants, $\theta = \frac{M}{M_0}$, T= Temperature (^oK).

Table 2. Predictive equations developed for moisture adsorption by *tacca Involucrata* tuber products

Product	Temperature (^oC)	Equation	r^2
BNF	10	$M = 173.05_a^3 – 203.92a_w^2 + 99.63a_w$	0.984
	20	$M = 161.12\ a_w^3 – 188.13a_w^2 + 87.79a_w$	0.9915
	30	$M = 145.8a_w^3 - 172.65a_w^2 + 79.90a_w$	0.9969
	40	$M = 115.17a_w^3 - 138.03a_w^2 + 68.45a_w$	0.9972
NBF	10	$M = 242.30a_w^3 – 284.40a_w^2 + 111.06a_w$	0.9932
	20	$M = 204.15a_w^3 - 244.37a_w^2 + 98.577a_w$	0.9893
	30	$M = 165.95a_w^3 - 205.10a_w^2 + 86.979a_w$	0.9864
	40	$M = 122.44a_w^3 - 151.73a_w^2 + 68.365a_w$	0.9878
BF	10	$M = 225.86a_w^3 - 258.18a_w^2 + 98.041a_w$	0.9899
	20	$M = 188.37a_w^3 - 219.09a_w^2 + 85.88a_w$	0.9909
	30	$M = 137.87a_w^3 - 164.00a_w^2 + 69.405a_w$	0.9929
	40	$M = 104.94a_w^3 - 126.75a_w^2 + 56.321a_w$	0.9814
	10	$M = 204.95a_w^3 - 266.85a_w^2 + 134.49a_w$	0.9902
NBNF	20	$M = 152.07a_w^3 - 197.96a_w^2 + 10855a_w$	0.9956
	30	$M = 122.02a_w^3 - 148.24a_w^2 + 81.998a_w$	0.9899
	40	$M = 106.71a_w^3 - 134.30a_w^2 + 74.439a_w$	0.9966

[1]M = moisture content (gH2O/100g solids), a_w = water activity, r^2 = coefficient of determination. BNF = blanched non fermented, NBF = non blanched fermented, BF= blanched fermented and NBNF = non blanched non fermented (control) *Tacca involucrata* tuber products.

Table 3. Goodness of fit of various sorption models to moisture adsorption data of *Tacca involucrata* tuber product

Product	Sorption Model	%RMS				
		Temperature (^oC)				
		10	20	30	40	Mean
BNF	GAB[1]	8.07	6.47	9.41	6.78	7.68
	Oswin[2]	10.44	11.45	5.24	4.81	7.99
	Henderson[3]	16.60	15.25	12.93	10.52	13.54
	Halsey[4]	7.04	5.69	5.64	6.32	6.17
	Experimental[5]	6.84	9.15	6.21	3.21	6.35
NBF	GAB	3.03	8.49	8.83	5.79	6.54
	Oswin	14.89	11.29	7.14	4.31	9.41
	Henderson	22.40	18.93	13.66	10.52	16.38
	Halsey	8.55	8.12	4.16	15.29	9.03
	Experimental	7.21	8.39	5.54	5.89	6.75
BF	GAB	6.81	6.62	9.95	6.50	7.82
	Oswin	15.71	13.31	8.04	5.23	10.59
	Henderson	24.44	21.23	15.49	9.77	17.73
	Experimental	9.53	7.44	2.89	5.05	6.22
NBNF	GAB	6.82	5.81	5.36	7.60	6.40
	Oswin	5.40	4.22	6.32	3.70	4.91
	Henderson	9.30	12.60	10.78	14.41	12.29
	Nelsey	7.05	5.81	8.77	4.52	6.54
	Experimental	6.11	5.16	4.14	4.11	4.63

I. GAB equation (Bizot 1983), 2. Owsin (1946). 3. Henderson (1952); 4. Halsey (1948), 5. This work, %RMS = percentage root mean square of error. BNF= blanched non fermented, NBF = non blanched fermented, BF= blanched fermented and NBNF = non blanched non fermented (control) Tacca involucrata tuber products.

Table 4. BET parameters and derivatives for moisture adsorption by Tacca involucrata tuber products

Product	Temp (°C)	A	B	C	Mo (gH_2O/100 g)	S_o (m^2/g solid)
BNF	10	-2.1874 E-3	0.1097	-49.14	9.3032	326.89
	20	-1.989 E-3	0.1250	-64.13	8.1263	285.51
	30	-2.4344 E-4	0.1267	521.29	7.8800	276.86
	40	1.6772 E-3	0.1326	80.06	7.4473	261.65
NBF	10	-2.8242 E-3	0.1256	-43.46	8.1462	386.20
	20	-3.4801 E-3	0.1391	-38.96	7.3758	259.14
	30	-1.2330 E-4	0.335	1083.92	7.4821	252.88
	40	-1.8967 E-3	0.1448	77.31	6.8120	239.57
BF	10	-4.3328 E-3	0.1533	-34.38	6.7126	235.84
	20	-4.0398 E-3	0.1624	-39.20	6.3155	221.89
	30	-2.1043 E-3	0.1732	-81.29	5.8460	205.39
	40	-14033 E-4	0.1926	1373.11	5.1897	182.34
NBNF	10	-13891 E-3	0.1677	49.30	14.6014	513.34
	20	-2.8214 E-3	0.720	26.53	13.3591	469.36
	30	-3.5100 E-3	0.1054	301.34	9.6086	332.18
	40	-1.6193 E-4	0.1145	71.73	5.1897	302.45

A and B = intercept and slope coefficients respectively where A = //MoC and B= (C-1)/MoC; C = BET constant, M_o = monolayer moisture contents, S_o = apparent, surface area of sorbent. BNF = blanched non fermented, NBF = non blanched fermented, BF = Blanched fermented and NBNF = non blanched non fermented Tacca involurata tuber products.

Table 5. GAB parameters and derivatives for moisture adsorption by *Tacca involucrata* tuber products

Product	Temp (°C)	G	K	M_o (g H2O/100g solids)
BNF	10	3.5680	0.8112	11.4994
	20	3.4984	0.8336	9.7224
	30	2.5793	0.8408	8.8278
	40	1.9898	0.8665	8.5914
NBF	10	6.1371	0.8213	8.1940
	20	4.9047	0.8294	7.6983
	30	4.8570	0.8414	7.8494
	40	4.2934	0.8658	7.4860
BF	10	5.9524	0.8123	7.3938
	20	5.3942	0.8455	6.5798
	30	5.0725	0.8652	6.3742
	40	4.2545	0.8754	5.9236
NBNF	10	3.3698	0.7513	16.4201
	20	2.2331	0.7662	15.8653
	30	1.8074	0.7671	11.5263
	40	1.7523	0.7707	9.7245

G and K = GAB constants, M_o monolayer moisture content, BNF = blanched non fermented, NBF = non blanched fermented, BF = blanched fermented and NBNF = non blanched non fermented Tacca involucrate tuber products.

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The Potential of Baobab (*Adansonia digitata* L.) Extracts as Biocontrol on the Growth and Aflatoxin Production by *Aspergillus flavus* and *A. parasiticus*

Saifeldin A. F. El-Nagerabi[1], Abdulkadir E. Elshafie[2], Suleiman S. AlKhanjari[1], Saif N. Al-Bahry[2] & Mohamed R. Elamin[3]

[1] Department of Biological Sciences and Chemistry, College of Arts and Sciences, University of Nizwa, Oman

[2] Department of Biology, College of Science, Sultan Qaboos University, Oman

[3] Department of Chemistry, Sudan Academy of Science, Khartoum North, Sudan

Correspondence: Saifeldin A. F. El-Nagerabi, Department of Biological Sciences and Chemistry, College of Arts and Science, University of Nizwa, Birkat Al Mouz, Nizwa, P. O. Box 33, PC 616, Oman. Tel: 968-9636-5051. E-mail: nagerabi@unizwa.edu.om; nagerabi@hotmail.com

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Abstract

Moulds and associated mycotoxins, especially aflatoxins, are important factors that advesely affect food and feed produced from contaminated plant and animal prodcuts. They are lethal to humans and animals, which emphasizes the great concern in food and feed production. In this study, the effects of baobab (*Adansonia digitata*) extracts on the vegetative growth and aflatoxin secretion by *A. flavus* (SQU21) and *A. parasiticus* (CBS921.7) strains were exzmined. Different concentrations of baobab fruit extract (1.5, 3, 5, and 7% w/v) and essential oil (0.5, 1, 3 and 5% v/v) was used. Fruit extract of baobab apparently inhibited the total aflatoxin secretion up to 20.4-68.5% for *A. flavus* and 11.9-69.1% for *A. parasiticus,* whereas the inhibition of aflatoxin B_1 production ranged between 29.9-79.2% and 13-68% for the two strains, respectively. The highest inhibition levels of total aflatoxin and aflatoxin B_1 secretion by *A. flavus* (47.2-95.7%; 28.1-89.7%) and *A. parasiticus* (42.7-93.3%; 25.9-80.2%) were obtained with essential oil extracted from baobab seeds. The two extracts significantly reduced the vegetative growth and the mycelial dry weights of selected fungi. This indicates the antifungal activity and inhibitory effect of baobab on the growth and aflatoxin production by the two toxigenic strains. Thus, fruit extract and essential oil of *A. digitata* can be suggested as potentially effective biocontrol and biopreservative substrates against food and feed contamination by aflatoxigenic moulds.

Keywords: *Adansonia digitata*, *Aspergillus flavus*, *A. parasiticus,* baobab fruit, essential oil

1. Introduction

Adansonia digitata L. (Baobab) of the family Malvaceae is a large iconic deciduous and stem-succulent tree indigenous to the dry regions of Africa. It is found in many countries of South Africa (Zimbabwe, Mozambique, South Africa), West Africa (Mali, Benin, Senegal, the Ivory Cost, Cameron, Burkino Faso), and East Africa Kenya, Uganda, Sudan, Tanzania) (Sidibé & Williama, 2002; Wickens & Lowe, 2008; Kamatou, VermaaK, & Viljoen, 2011; Vermaak, Kamatou, Komane-Mofokeng, Viljoen, & Beckett, 2011; De Smedt, Sanchez, Van den Bilcke, Simbo, Potters, & Samson, 2012). In the past decade, different parts of the baobab tree have been reported to be useful and this has attracted the interest of pharmaceutical companies and scientists. This is due to its various traditional uses as medicinal, nutritional and cosmetic plant (Igboeli, Addy, & Salami, 1997; Wickens & Lowe, 2008; Buchmann, Prechsler, Hartl, & Vogl, 2010; Kamatou et al., 2011). Recently, the European Commission authorized the importation of baobab fruit pulp as a novel food for human consumption (Buchamann et al., 2010). In 2009, it was approved by the Food and Drug Adminstration (FDA) as a food ingredient in the United States of America (Addy, 2009). The dry pulp is commonly used to prepare fruit juice with higher levels of vitamin C than orange, and calcium than milk (Assogbadjo, Chadare, Kakari, Fandohan, & Baidu-Forson, 2012). Various plant parts such as leaves, bark, and fruit pulp have been traditionally used as immuno-stimulant, anti-inflammatory, analgesic, and pesticide, and in the treatment of fever, diarrhoea, cough, dysentery, haemoptysis, tuberculosis, microbial infection and worms (Wickens & Lowe, 2008; Kamatou et al.,

2011; Vermaak et al., 2011). The seeds are used as roasted snacks, fermented and used as a thickening and flavouring agent in soup (Igboeli et al, 1997). The oil extracts are used as food, fuel, medicine, cosmetic applications and topical treatment of various conditions such as dandruff, muscle spasms, varicose veins and wounds (Chivandi, Davidson, & Erlwanger, 2008; Kamatou et al., 2011; Vermak et al., 2011).

Mycotoxins are toxic secondary metabolites of fungal origin and natural contaminant of agricultural commodities under both pre- and post-harvest conditions (Wagacha & Muthomi, 2008; Herzallah, 2009; Salim & Ahmad, 2010). The species of the genus *Aspergillus*, *Fusarium*, and *Penicillium* are the major mycotoxin producing fungi. The most important mycotoxins are aflatoxins, fumonisins, and ochratoxins (Kumar, Basu, & Rajendran, 2008). Aflatoxigenic fungi are the most devastating contaminants of different plants and animals products (Payne, 1998; Elshafie, Al Rashdi, Al-Bahry, & Bakheit, 2002; Abdulkadir, Al-Ali, Al-Kildi, & Jedah, 2004; Santacrose, Conversano, Casalino, Lai, Zizzadoro, & Centoducati, 2008; El-Nagerabi, Al-Bahry, Elshafie, & AlHilali, 2012). Aflatoxins in general and aflatoxin B_1 in particular are mutagenic and hepatocarcinogenic secondary metabolites secreted by *Aspergillus flavus*, *A. parasiticus*, *A. nominus* and *A. pseudotamorii* are pose serious effects on human and animal health (Sidhu, Chandra, & Behl, 2009; Elshafie, ElMubarak, El-Nagerabi, & Elshafie, 2010; Liu & Wu, 2010; El-Nagerabi et al., 2012).

The vegetative growth and associated aflatoxin production by *A. flavus* and *A. parasiticus* were found to be affected by many extracts from different plant parts due to their fungicidal and fungistatic properties (Soliman & Badeaa, 2002; El-Nagerabi et al., 2012). This includes dry leaves and calyx extracts of *Hibiscus sabdariffa* (Al-Shayeb & Mabrook, 1984; El-Nagerabi et al., 2012), herbal compounds (Gowda, Malathi, & Suganthi, 2004), and fruit rinds of *Garcinia cowa* and *G. pendunculata* (Joseph, Jayaprakasha, Seli, Jena, & Sakariah, 2005). Leaf extract from *Syzigium aromaticum*, *Cucuma longa*, *Allium sativum*, and *Ocimum sanctum* showed significant antifungal activities and inhibit aflatoxin B_1 production by *A. flavus* and *A. parasiticus* (Reddy, Reddy, & Muralidharan, 2009). Similar effects were observed with essential oils from medicinal and herbal plants such as anise, caraway, cinnamon, black cinum, and fennel (Bullerman, Lieu, & Seier, 1977; Farag, Daw, & Abo-Raya, 1989; Soher, 1999; Patkar, Usha, Shetty, Poster, & Lacey, 1993; Hasan, 1994; Montes-Belmont & Carvajal, 1998; Soliman & Badeaa, 2002). Oil of *Nigella sativa* at concentration of 1-3% completely inhibited aflatoxin production (Maraqa, Alsharoa, Farah, Albjeirami, Shakya, & Sallal, 2007; El-Nagerabi et al., 2012).

Many researchers worldwide are continuously assessing different detoxification methods and inhibition techniques on aflatoxin secretion by aflatoxigenic fungi (Gandomi, Misaghi, Basti, Bokaei, Khosravi, Abbasifar, & Javan, 2009; Kumar, Shukla, Singh, & Dubey, 2009; Oguz, 2011; El-Nagerabi et al., 2012). Reduction or inactivation of aflatoxin by various decontamination procedures using different physical and chemical methods have been studied extensively together with microbiologial degradation (Alberts, Gelderblom, Botha, & Van Zyl, 2009; Kumar et al., 2009). Nevertheless, these synthetic chemicals are hazardous to humans and domestic animals as well as the environment (Szczerbanik, Jobling, Morris, & Holford, 2007; Gandomi et al., 2009; Kumar et al., 2009; Prakash, Shukla, Sigh, Mishra, Dubey, & Kharwar, 2011). This prompted us to search for simple, safe, and environment friendly antifungal and growth inhibitors from biological sources. Nonetheless, the antifungal, inhibitory, and detoxification effects of *A. digitata extracts* on the fungal growth and aflatoxin production had not been screened. Thus, there is high potential for extracts from *A. digitata* to inhibit the fungal growth and aflatoxin production by these aflatoxigenic fungi. The present investigations aim to evaluate the effects of fruit pulp powder and oil extracted from seeds of baobab on the fungal growth and aflatoxin secretion of two aflatoxigenic strains of *A. flavus* (SQU21) and *A. parasiticus* (CBS921.7). This will contribute with international efforts to fill the gap in our knowledge about the antimicrobial properties of baobab and possibly lead to developments in the food industry related to preparation, preservation, storage, and consumption.

2. Materials and Methods

2.1 Fungal Isolates

Two strains of high aflatoxin-producer fungi of *Aspergillus flavus* (SQU21) and *A. parasiticus* (CBS921.7) [NRR22999] were obtained from the culture collections of Sultan Qaboos University, Oman. These isolates were cultivated on Czapek Dox Agar (CDA) and described taxonomically using the manual prepared by Raper & Fennel (1965). These strains were used as inoculum in this study.

2.2 Source and Properties of Adansonia digitata Extracts

The fruit powder of *A. digitata* pulp was purchased from AlNaser Company, Khartoum, Sudan. Numerous studies were carried on the nutritional constituents of baobab parts (Sidibé & Williams, 2002; Chadare, Hounhouigan, Linnemann, Nout, & Van Boekel, 2009; Assogbadjo et al., 2011). Biochemical analysis indicated that baobab parts (pulp, leaves and seeds) are rich in several microelements such as iron, vitamin C, A, E and F

in addition to calcium, potassium, magnesium, zinc, proteins and lipids (Chadare et al., 2009). The oil extract of this plant was obtained from Chemistry for Life Company, Muscat, Oman. The chemical nature of the essential oil extracted from the seeds was reported by researchers. The oil is extremely stable with a half life of between 2 to 5 years, a high saponiofication value compared to other edible oils, and the iodine value is 87.9 g/100 g as non-drying oil, with 33% saturated, 36% monosaturated and 31 polysaturated fatty acids in addition to palmitic and oleic acids as major constituents (Vermaak et al., 2011).

2.3 Inoculation of Aspergillus Starins on Media Containing A. digitata Extracts

A. flavus (SQU21) and *A. parasiticus* (CBS921.7) were inoculated onto Potato Dextrose Agar (PDA) and incubated at ambient temperature of 25 ± 2°C for 10 days. Sterile thin glass tubes of 5 mm in diameter were used to cut several discs from the growing cultures. Two discs of 5 mm in diameter were added aseptically to each flask containing 200 ml sterile yeast malt broth with 1.5, 3, 5, and 7 g/100 ml of *Adansonia digitata* fruit pulp extract and 0.5, 1, 3 and 5 ml/100 ml oil extract. As a control, fruit pulp extract and oil extract were mixed with yeast malt broth and without any fungal inoculation. Three inoculated flasks from each treatment were incubated at 25 ± 2°C for two weeks. Similarly inoculated flasks were used to determine the mycelial dry weight of the two fungal strains.

2.4 Effect of A. digitata Fruit Extract and Oil on Pure Aflatoxin B_1

Aflatoxin B_1 powder (Sigma Company) was added to 100 ml sterile distilled water which gave an aflatoxin B_1 concentration of 870 ppb. The highest concentrations from *A. digitata* fruit pulp (7 g/100 ml) and oil (5 ml/100 ml) were chosen. For this, 7 grams of *A. digitata* fruit pulp and 5 ml of oil were added to the different flasks of aflatoxin B_1. The flasks were incubated at 25 ± 2°C for 10 days. The aflatoxin concentration was measured.

2.5 *Extraction and Detection of Aflatoxin by Afla Test-P Affinity Column*

For aflatoxin extraction, similar method used in our previous study on the effect of *Hibiscus sabdariffa* extract and *Nigella sativa* oil on the growth and aflatoxin B1 of *Aspergillus flavus* and *A. parasiticus* strains was adopted (El-Nagerabi et al., 2012). For measuring the concentration of aflatoxin, calibrated Vicam fluorometer (Series-4EX) from Vicam Company, Milford, MA, USA was used. The fluorometer was set at excitation wavelength of 360 nm and emission wavelength of 440 nm (Elshafie & Al-Shally, 1998).

2.6 *Statistics and Data Analysis*

To assess the variation between the effects of fruit and oil extracts of *A. digitata* extracts on the vegetative and aflatoxin production, one way ANOVA test (correlation coefficient) was used. The statistical package software SPSS (version 11.0) was used.

3. Results and Discussion

3.1 Effects of Fruit Pulp Extract of Baobab on Fungal Growth and Aflatoxins Production

The effects of various concentrations of *Adansonia digitata* (Baobab) fruit pulp extract on the total aflatoxin (Figure 1a), aflatoxin B_1 (Figure 1b), and mycelia dry weight (Figure 1c) of *A. flavus* (SQU21) and *A. parasiticus* (CBS921.7) were recorded. The total aflatoxins and aflatoxin B_1 production by the two *Aspergillus* strains were significantly ($p < 0.05$) reduced by the tested concentrations of baobab fruit extract (1.5, 3, 5, and 7 g/100 ml) compared to the control. Similarly, the mycelial dry weight of the two fungal strains was significantly ($p < 0.05$) reduced by the different concentrations of baobab fruit pulp extract comparable to the control.

The antifungal activities and detoxification properties of different plant extracts were investigated by many researchers (Gandomi et al., 2009; Kumar et al., 2009; Oguz, 2011; El-Nagerabi et al., 2012). Nonetheless, based on the available literature, the antifungal ability and detoxification properties of fruit extract of *A. digitata* on the fungal growth and aflatoxins production by *Aspergillus* species had not been evaluated before. To our knowledge, this is the first study on the biological activities of different extracts from this plant. However, extracts from fruit rind of *Garcinia cowa* and *G. penduculata* completetely inhibited the growth and aflatoxin B_1 production by *A. flavus* (Joseph et al., 2005). Threrefore, it is possible that fruit and other extracts from *A. digitata* could reveal similar inhibitory effects on the fungal growth and aflatoxins secretion by the two aflatoxigenic strain of *A. flavus* and *A. parasiticus*. Hence, it is evidently important to evaluate the inhibitory effect of various extracts from *A. digitata* against the fungal growth and aflatoxin production by aflatoxigenic fungi and compared with the similar studies which used different extracts from herbal and medicinal plants. In the present investigations, the concentrations of baobab fruit extract (1.5-7%) apparently inhibited total aflatoxin production by 20.4-68.5% for *A. flavus* (SQU21) and 11.9-69.1% for *A. parasiticus* (CBS921.7), whereas the inhibition of aflatoxin B_1 production ranged between 29.9-79.2% and 13-68% for the two strains as suggested by Joseph et al. (2005)

using similar extract from fruit rind of *G.cowa* and *G. penduculata.* Also neem seed cake and leaf extract of *Azadirchta indica* inhibited the fungal growth and aflatoxin production by *A. flavus* and *A. parasiticus*. Other studies showed similar inhibition of the fungal growth and aflatoxin production. For example, aqueous extracts from mature leaves of *Vernonia amygdalina*, *Sena elata* and *Cymbopogon citrulus* (Suleiman, Emua, & Taiga, 2008), plant extract of *Syzigium aromaticum*, *Curcuma longa*, *Allium sativum* and *Ocimum sanctum* (Reddy et al., 2009), herbal compounds (Gowda et al., 2004), and dry leaves and calyx extracts of *Hibiscus sabdariffa* (A-Shayeb & Mabrook, 1984; El-Nagerabi et al., 2012). Cinnamon extract concentrations of 0.02-20% inhibit aflatoxin production by 25-100%, and 2% of cinnamon led to 97% inhibition of aflatoxin secretion by aflatoxigenic fungi (Bullerman et al., 1977). About 91.5-97.9% reduction in aflatoxin B_1 production by *A. flavus* and *A. parasiticus* was caused by leaf and calyx extracts (5-12.5%) of *H. sabdariffa* (El-Nagerabi et al., 2012; Al-Shayeb & Mabrook, 1984). Our results showed that the highest inhibition levels of total aflatoxin (68.5-69.1%) and aflatoxin B_1 (68-79.1%) were reported at 7% concentration of baobab fruit extract. Therefore, it is possible that various growth inhibitors present in this plant extracts would affect aflatoxin secretion by aflatoxigenic fungi. On the other hand, inoculation of *A. flavus* (SQU21) and *A. parasiticus* (CBS921.7) strains on yeast malt broth containing different concentrations of baobab fruit extract (1.5, 3, 5, and 7 g/100 ml) significantly inhibited the fungal growth and mycelial dry weights of the two strains. Similarly, extract from the dried leaves of *H. sabdariffa* evidently retarded the growth and vigour of different fungi (Guerin & Revillere, 1984). On the contrary, calyx extract (5-12.5%) from *H. sabdariffa* did not show any effect on the mycelial growth of *Aspergillus* species (El-Nagerabi et al., 2012). Some herbal drugs and medicinal plants inhibit the mycelial growth of *A. flavus* and *A. parasiticus* while others improved mycelial growth, but retarded aflatoxin secretion (Bahk & Marth, 1983; Gowda et al., 2004; Joseph et al., 2005; Suleiman et al., 2008; Reddy et al., 2009; Da Costa et al., 2010). Cinnamon at the concentrations of between 0.02-2.0% inhibited aflatoxin biosynthesis and the growth of *A. parasiticus* by 16-100% (Bullerman et al., 1977). The leaf extracts cassia and bay enhance the mycelial growth of *A. parasiticus* and inhibit the mycelial growth and aflatoxin production by *A. flavus* (Paranagama, Abeysekera, Abeywickrama, & Nugaliyadde, 2003; Krishnamsrthy, & Shashikala, 2006; Sandosskumar, Karthikeya, Mathiyazhaga, Mohankumar, Chandrasekar, & Velazhahan, 2007). Therefore, it is evident that *A. digitata* fruit extract showed antifungal activities and inhibitory effect on aflatoxin production by *A. flavus* and *A. parasiticus.*

3.2 Effects of Essential Oil of Baobab on Fungal Growth and Aflatoxins Production

The uses of essential oils (EOs) extracted from herbal, medicinal and aromatic plants against the fungal growth and aflatoxin production of *A. flavus* and *A. parasiticus* have been suggested by many researchers (Maraqa et al., 2007; El-Nagerabi et al., 2012). They had different fungistatic activities (Gandomi et al., 2009; Shukla et al., 2012). *Nigella sativa* oil at 3% completely inhibited (Maraqa et al., 2007). At concentrations of 1-3%, this oil caused 47.9-58.3% reduction in aflatoxin B_1 for *A. flavus* and 32-48% for *A. parasiticus* strains (El-Nagerabi et al., 2012). Oil of cassia and bay leaves reduced aflatoxin B_1 (98%) and stimulated fungal growth, whereas coriander oil had no effect on the fungal growth and its toxigenicity (Attanda, Akqan, & Oluwafemi, 2007). Aflatoxin B_1 production by NKD-208 isolates of *A. flavus* was strongly inhibited at lower fungistatic concentrations of essential oil of *Callistemon lanceolatus* (Shukla et al., 2012). All concentrations of *Zataria multifora* essential oil exhibited significant inhibition of fungal growth as well as spore production (Gandomi et al., 2009). *Ocimum gratissimum* oil shows better efficacy as a fungitoxicant than prevailing fungicide Wettasul-80 (Prakash et al., 2011). The essential oils of *T. eriocalyx* and *T. x-porlock* were evidently fungicidal and inhibitory to aflatoxin production (Rasooli & Abyaneh, 2004). Of the 96 plant extracts, EOs proved to be the most effective extract controlling aflatoxigenic strains (Bluma, Amaiden, & Etcheverry, 2008). Frankincense of *B. carteri* at 2% (v/v) showed the strongest mycelium inhibition against *A. flavus* and other pathogenic fungi (Udomsilp et al., 2009). In this investigation, the effects of different concentrations of *A. digitata* essential oil (0.5, 1, 3, and 5 ml/100 ml) on total aflatoxin secretion (Figure 2a), aflatoxin B_1 (Figure 2b) and the mycelial growth (Figure 2c) of *A. flavus* (SQU21) and *A. parasiticus* (CBS921.7) were reported. The results showed that the oil of baobab significantly ($p < 0.05$) inhibited total aflatoxin secretions up to 47.2-95.7% for *A. flavus* and 42.7-93.3% for *A. parasiticus,* whereas aflatoxin B_1 showed inhibition of 28.1-89.7% and 25.9-80.2%, respectively. The mycelial dry weights of the *Aspergillus* strains were significantly ($p < 0.05$) reduced by the tested concentrations of *A. digitata* oil. This indicates the antifungal and inhibitory effects of baobab essential oil against the growth and aflatoxin production by the two strains of *A. flavus* (SQU21) and *A. parasiticus* (CBS921.7). Similar findings were reached by many authors using different oils from *Nigella sativa* (El-Nagerabi et al., 2012), cassia and bay (Attanda et al., 2007), *Cymbopogon flexuous* (Kumar et al., 2009), *Callistemon laceolatus* (Shukla et al., 2012), *Zataria multifora* (Gandomi et al., 2009), and *Ocimum gratissimum* (Prakash et al., 2011).

3.3 Detoxification of Aflatoxins B_1 by Fruit Extract and Essential Oil of Baobab

Detoxifications with biological factors offer promising alternatives for aflatoxin elimination and maintaining the quality and safety of food and feed (Alberts et al., 2009; Oguz, 2011; Prakash et al., 2011). The ability of some herbal and medicinal plants as detoxifying agents was suggested by many researchers (Sandosskumar et al., 2007; El-Nagerabi et al., 2012). This includes Garlic (*Allium sativum* L. x) and onion (*Allium cepa* L.) roots extracts which cause 58.5% reduction in aflatoxin B_1. Seed extract of *Trachyspermum ammi* degraded 90% of aflatoxin G_1 by altering the ring structure of lactone (Velazhahan, Vijayanandraj, Vijayasamundeeswari, Parandidharan, Samiyappan, Iwamoto, Friebe, & Muthukrishnan, 2010). The presence of inactivation factors in *T. ammi* seed extract was responsible from 80% reduction of total aflatoxin content (Hajare, Haijare, & Sharma, 2006). In the present evaluation, the strains of the two selected *Aspergillus* species are aflatoxin-producers and secreting different level of aflatoxins. In the present study, we investigated the effect of the the highest concentrations of fruit pulp (7% w/v) and oil extract (5% v/v) of *A. digitata* on 780 ppb aflatoxin B_1 incubated at 25-29°C for 10 days. The results showed that the two extracts (7% fruit pulp 774 ppb; 5% oil 776 ppb) have no detoxification effect on pure aflatoxin B_1 comparison with the control (780 ppb). This suggests the non-detoxification properties of these extracts on aflatoxin B_1. Therefore, it is apparent that fruit and oil extracts of *A. digitata* had antifungal and inhibitory effect on aflatoxin secretion by *Aspergillus* strains (*A. flavus* SQU21 and *A. parasiticus* CBS921.7).

4. Conclusion

This paper describes the effects of fruit pulp extract and essential oil from the seeds of *A. digitata* (Baobab) on the growth and inhibition of aflatoxin production of *A. flavus* (SQU21) and *A. parasiticus* (CBS921.7). As far as we know, this is the first report on the biological activities of baobab on the fungal growth and aflatoxin secretion by aflatoxigenic fungi. The overall results demonstrate that both fruit extract and essential oil of this plant inhibited the mycelial dry weights and aflatoxins production by the two strains of *Asppergillus* species. The two extracts did not detoxify pure aflatoxin B_1. This indicates the antifungal activities and inhibitory effect of *A. digitata* extracts against moulds contamination. Therefore, baobab fruit and its essential oil can be suggested as plant additives and biopreservatives which enhance the nutritive value, quality, and protection against aflatoxin contamionation as well as storage life. More phytochemical analysis is needed to identify the active chemical ingredients and testing their antimicrobial activites against different microorganisms and mycotoxins invasion. This will bring to the literature useful information which eveually promotes the quality of food and feed products and related agricultural and pharmaceutical industries.

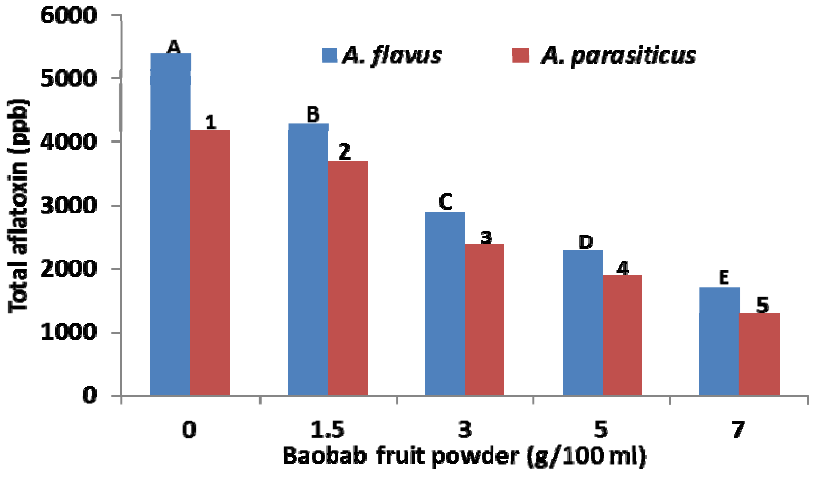

Figure 1a. Total aflatoxin production of *A. flavus* strain SQU21 and *A. parasiticus* strain CBS921.7 at different concentrations of *A. digitata* fruit extract (Identical numbers and letters indicate no significant diffrence, $p < 0.05$)

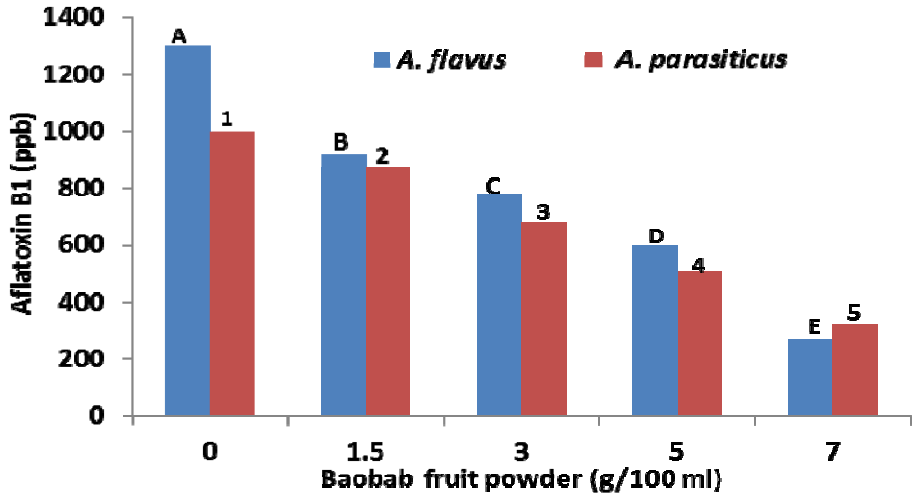

Figure 1b. Aflatoxin B_1 production of *A. flavus* strain SQU21 and *A. parasiticus* strain CBS921.7 at different concentrations of *A. digitata* fruit extract (Identical numbers and letters indicate no significant diffrence, $p < 0.05$)

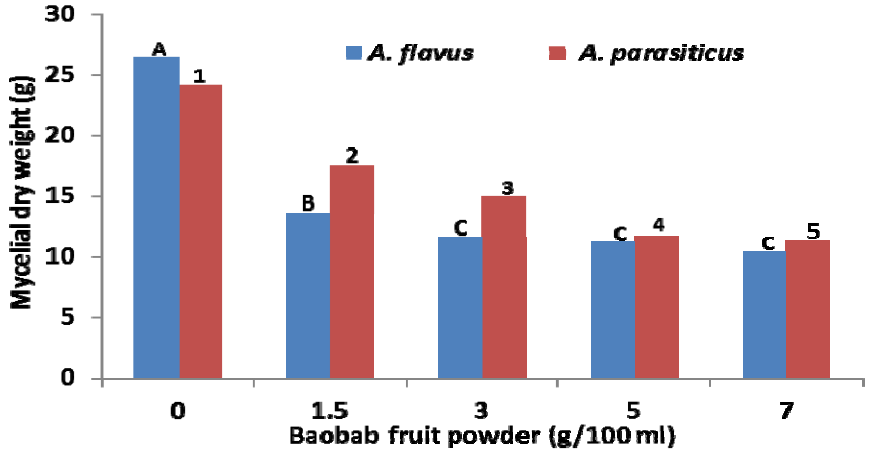

Figure 1c. Mycelial dry weight of *A. flavus* strain SQU21 and *A. parasiticus* strain CBS921.7 at different concentrations of *A. digitata* fruit extract (Identical numbers and letters indicate no significant diffrence, $p < 0.05$)

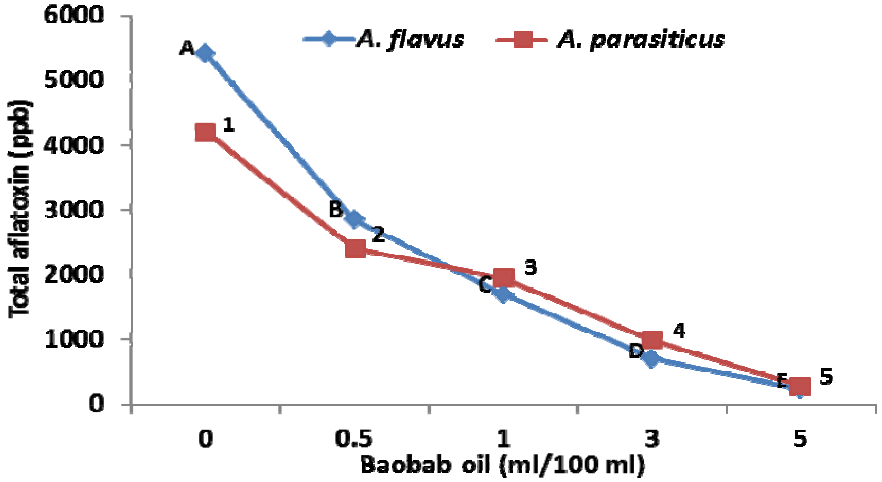

Figure 2a. Total aflatoxin production of *A. flavus* strain SQU21 and *A. parasiticus* strain CBS921.7 at different concentrations of *A. digitata* oil extract (Identical numbers and letters indicate no significant diffrence, $p < 0.05$)

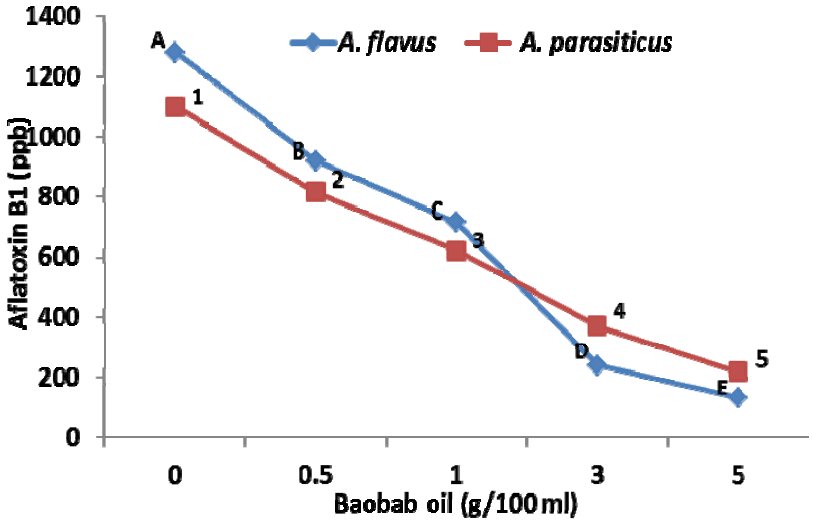

Figure 2b. Aflatoxin B_1 production of *A. flavus* strain SQU21 and *A. parasiticus* strain CBS921.7 at different concentrations of *A. digitata* oil extract (Identical numbers and letters indicate no significant diffrence, $p < 0.05$)

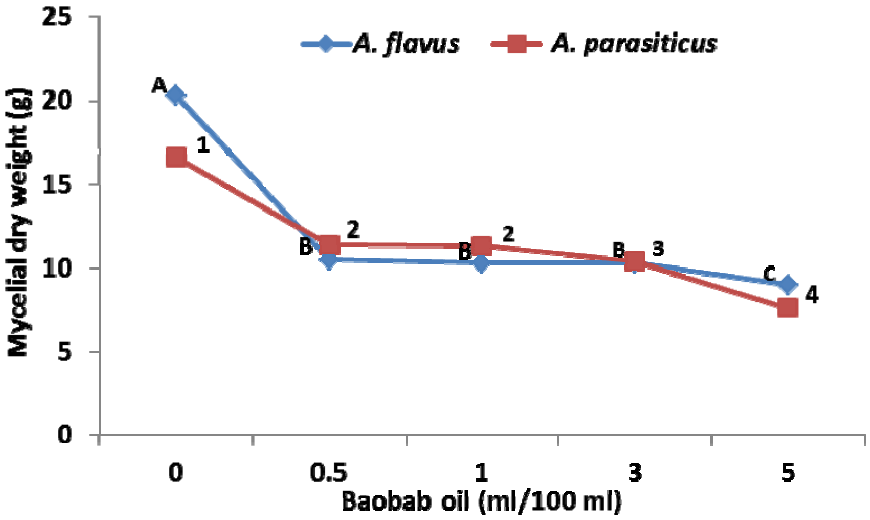

Figure 2c. Mycelial dry weight of *A. flavus* strain SQU21 and *A. parasiticus* strain CBS921.7 at different concentrations of *A. digitata* oil extract (Identical numbers and letters indicate no significant diffrence, $p < 0.05$)

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Evaluation of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry for Rapid Identification of Bacteria in Processed Soybean Products

Yuko Furukawa[1]*, Mitsuru Katase[1]* & Kazunobu Tsumura[1]

[1] Quality Assurance Department, Fuji Oil Co., Ltd., Osaka, Japan

* Both authors contributed equally to this work

Correspondence: Kazunobu Tsumura, Analytical Center for Food Safety, Quality Assurance Department, Fuji Oil Co., Ltd., 1 Sumiyoshi-cho, Izumisano, Osaka 598-8540, Japan. Tel: 81-72-463-1123. E-mail: tsumura.kazunobu@so.fujioil.co.jp

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Abstract

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has recently been demonstrated as a rapid and reliable method for identifying bacteria in colonies grown on culture plates. Rapid identification of food spoilage bacteria is important for ensuring the quality and safety of food. To shorten the time of analysis, several researchers have proposed the direct MALDI-TOF MS technique for identification of bacteria in clinical samples such as urine and positive blood cultures. In this study, processed soybean products (total 26 test samples) were initially conducted a culture enrichment step and bacterial cells were separated from interfering components. Harvested bacterial cells were determined by MALDI-TOF MS and 16S rRNA gene sequencing method. Six processed soybean products (23%) were increased bacterial cells after culture enrichment step and they were successfully obtained the accurate identification results by MALDI-TOF MS-based method without colony formation.

Keywords: bacterial identification, MALDI-TOF MS, food spoilage

1. Introduction

Processed soybean products are an important part of diet not only in East Asia but also worldwide because of their beneficial health effects, particularly because they are an excellent source of high-quality proteins, isoflavones, and vitamins (Hettiarachchy & Kalapathy, 1997; Jacobsen et al., 1998). The potential health benefits of soy have been reported against cancer, heart diseases, and diabetes. However, processed soybean products are also associated with health hazards because of the incidence of food-borne illnesses caused by the presence of pathogenic bacteria (Fang et al., 1999; No et al., 2002). In addition, microbial spoilage of products is an important economic problem that discourages manufacturers.

The microbiota of soybean products are closely associated with the condition of raw materials, level of bacterial contamination, and preservation methods, which affect the growth and survival of bacteria. Thus, food-borne pathogenic and spoilage bacteria can be present as resident microbiota or can be introduced as contamination during processing. It should be noted that the bacterial species also play an important role in soybean food-borne illness and spoilage. Thus, quality and safety of processed soybean products is important (Katase & Tsumura, 2011; Tsumura & Tsuboi, 2012).

Based on the ribosomal proteins profiles of different bacteria, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been demonstrated as a rapid and reliable method for bacterial identification (Lay, 2001; Mazzeo et al., 2006). Databases of various pathogenic bacteria have been generated for use of this method in routine bacterial identification from plate cultures (Seng et al., 2009; Bizzini et al., 2010). However, this method generally requires at least 24-48 h to obtain colonies before MALDI-TOF MS. In addition, it is difficult to harvest sufficient cells because of cell damage or some cells may be viable but non-culturable (Rowan, 2004). Nevertheless, direct identification of bacteria may increase the utility of MALDI-TOF MS because it can significantly shorten the time required for bacterial identification (Ferreira et al., 2010).

The aim of the present study was to evaluate the potential usefulness on the direct identification of bacteria in processed soybean products using MALDI-TOF MS.

2. Materials and Methods

2.1 Samples

Twenty six of processed soybean products (soy milk; 14, tofu; 4, frozen tofu; 4, dried tofu; 4) were purchased from different retail outlets in the Osaka region of Japan and stored at 4°C until analysis. Except dried tofu, all samples were examined within 2 days of purchase. Soy milk (3.6% fat) for the contamination experiment was obtained from Fuji Oil Co., Ltd. (Osaka, Japan).

2.2 Bacterial Strains and Culture Media

Escherichia coli NBRC 3301, *Klebsiella pneumoniae* NBRC 14940 and *Citrobacter freundii* NBRC 12681 were were purchased from Biological Resource Center (NBRC), National Institute of Technology and Evaluation (Chiba, Japan) and used for model bacteria in the contamination experiment. Plate Count Agar (PCA, Nissui Seiyaku, Tokyo, Japan) was used for colony counts. Bacto™ Tryptic Soy Broth (TSB) was purchased from Nippon Becton Dickinson Company, Ltd. (Tokyo, Japan).

2.3 Contamination Experiment Using Soy Milk

To determine the identification of bacteria at low contamination levels using MALDI-TOF MS, soy milk was inoculated with *E. coli*, *K. pneumoniae*, or *C. freundii*, which were incubated in PCA at 35°C for 24 h before each experiment. Subsequently each bacterial strain was grown in TSB at 35°C for 24 h and harvested by centrifugation at 11,000 × *g* for 5 min (Himac CR22, Hitachi Koki, Tokyo, Japan). Sequential serial dilutions were made using fresh soy milk to achieve aliquots with bacterial counts ranging from 10^7 to 10^9 CFU/ml. About 100 µl of concentrated hydrochloric acid (36%, Kanto Kagaku,Tokyo, Japan) was added to 40 ml of each of these inoculated soy milk samples at pH 5 and incubated at 45°C for 5 min.Subsequently, most of soy proteins were coagulated by isoelectric precipitation method and the coagula were filtered using stomacher bags (Tempo bag, bioMérieux, Lyon, France). Aliquots were then filtered through 10-µm pore size syringe filters (PALL Life Sciences, Ann Arbor, Michigan) and centrifuged at 9,000 ×g for 5 min (Model 3500, Kubota Corporation, Tokyo, Japan). Bacterial cells were recovered and suspended in 400 µl of sterile water. A cell suspension (300 µl) was used for identification using MALDI-TOF MS, as described below. Total bacteria count was estimated using PCA at 35°C for 48 h.

2.4 Sample Preparation From Commercially Available Processed Soybean Products

We introduced a culture enrichment step before cell recovery because processed foods generally contain less viable bacterial cells. Twenty six samples of commercial soybean products were evaluated by culture enrichment prior to direct MALDI-TOF MS method. Increased bacterial cells in 6 of the 26 samples in total were determined by direct MALDI-TOF MS and 16S rRNA gene sequencing. Commercially available soy milk (8 ml) was mixed with 32 ml of sterile TSB and incubated at 35°C for 8 h. After this culture enrichment, bacterial cells were harvested according to the method described above and the cell suspensions were used for MALDI-TOF MS. In addition, the suspension was serially diluted and plated on PCA. The plate was incubated at 35°C for 48 h. All colonies grown in the plate were used for 16S rRNA sequence-based identification. All the experiments were performed in duplicate samples, and data shown are means values.

Ten grams of another processed soy product (i.e., tofu, frozen tofu and dried tofu) was mixed with 90 ml of sterile 0.9% NaCl solution and incubated at 35°C for 24 h. Next, 50 ml of this suspension was filtered through a stomacher bag (Tempo bag, bioMérieux) and centrifuged at 9,000 ×g for 5 min (Model 3500, Kubota Corporation). The harvested bacterial cells were suspended in 0.5 ml of sterile water and used for MALDI-TOF MS and 16S rRNA gene sequencing as the same manner.

2.5 MALDI-TOF MS

MALDI-TOF MS analysis was performed according to standard procedures (La Scola & Raout, 2009). A bacterial cell suspension (300 µl) was mixed with 900 µl absolute ethanol (99.5%, Kanto Kagaku, Tokyo, Japan) and centrifuged at 13,000 ×g for 2 min (Model 3615, Kubota Corporation). The supernatant was discarded and residual ethanol was removed after repeated centrifugations. Subsequently, 10 µl of formic acid (70%, Kanto Kagaku, Tokyo, Japan) was added to the pellet and mixed thoroughly by pipetting before the addition of 10 µl acetonitrile (98%, Kanto Kagaku, Tokyo, Japan). This mixture was centrifuged at 13,000 × g for 2 min. The supernatant (1 µl) was placed on a spot of the steel target and air dried at room temperature. The sample spots were overlaid with 1 µl of matrix solution [saturated solution of HCCA (α-cyano-4-hydroxycinamic acid) in an organic solvent (50% acetonitrile and 2.5% trifluoroacetic acid)] and air dried at room temperature.

Measurements were performed using the Autoflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Leipzig, Germany) equipped with a 20-Hz nitrogen laser. Spectra were recorded in the linear, positive mode at a laser frequency of 20 Hz within a mass range from 2-20 kDa The IS1 voltage was 20 kV, the IS2 voltage was maintained at 18.5 kV, the lens voltage was 6 kV, and the extraction delay time was 250 ns. For each spectrum, 300 laser shots were collected and analyzed. The spectra were calibrated externally using the standard calibration mixture (Bruker Daltonics). For automated data analysis, raw spectra were processed using MALDI Biotyper 3.0 software (Bruker Daltonics) at default settings. To identify unknown bacteria, each peak that was generated was matched directly against reference libraries (3,995 strains) using the integrated pattern-matching algorithm incorporated in the Biotyper 3.0 software. Identifications obtained using MALDI-TOF MS were evaluated according to modified scores (ranging from 0 to 3) proposed by the manufacturer. A score of more than 1.7 indicated probable identification, and a score of less than 1.7 indicated no reliable identification.

2.6 16S rRNA Gene Sequencing Analysis

Genomic DNA was isolated from a pure colony. In brief, the bacterial cells were lysed with the PrepMan Ultra Reagent (Applied Biosystems, Foster City, California, USA) and then heated for 10 min at 98°C. Extracted DNA was amplified by 500 bp to the 5' end of the 16S rRNA gene using the MicroSeq 500 16S rDNA Bacterial Identification PCR kit (Applied Biosystems). PCR was performed according to standard procedures (Arosio et al., 2008). Amplicons were purified with Amicon ultra-centrifugal filters (Millipore, Bedford, Massachusetts, USA). The sequencing reactions were performed with MicroSeq 500 16S rDNA Bacterial Identification Sequencing kit (Applied Biosystems) and PCR products were purified using Performa DTR Gel Filtration Cartridges (Edge Bio, Gaithersburg, Maryland, USA).

The cycle sequencing products were analyzed on an ABI 3130 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. The Basic Local Alignment Search Tool (BLAST) program in the DNA Data Bank of Japan (DDBJ) was used for sequence homology analysis.

3. Results and Discussion

To determine the identification of bacteria at low inoculum levels for accurate identification using MALDI-TOF MS, the recovery of bacteria from artificially contaminated soy food sample (e.g., soy milk) was investigated. As a model of bacteria,we used *Enterobacteriaceae* family and *Coliform* bacteria which are one of the most important indicators of sanitary quality of foods. Soy milk has many components, such as proteins, lipids and sugars, which interfere with MALDI-TOF MS analysis. In this study, most of the soy proteins were removed by isoelectric precipitation method that is commonly applied for production of soy protein isolate (Petenate & Glatz 1983; Tsumura et al., 2004) and the resulting supernatants were centrifuged to harvest bacterial cells. Good separation of bacteria from soybean food components was achieved with recovery rates between 50% and 90% (data not shown). Bacteria used for contamination experiment could be identified with adequate identification scores, providing a high reliability of these results (Table 1). In our contamination experiment, we obtained accurate identification results using soy milk samples that were contaminated with high bacterial count ($> 10^8$ CFU/ml). No significant differences in bacterial count range were observed between the test strains. These results coincided with previous studies that the bacterial count was an important factor for identification scores, and more than 10^7-10^8 CFU/ml bacterial cells were needed (Drancourt, 2010; Kroumova et al., 2011). At a lower bacterial count ($< 10^6$ CFU/ml), background peaks became more prominent and affected spectrum matching.

Table 1. MALDI-TOF MS scores for different inoculated bacterial counts in soy milk

Microorganisms	Inoculated bacterial count (CFU/ml)	MALDI-TOF MS score
E. coli NBRC 3301	3×10^9	2.0
	5×10^8	2.2
	5×10^7	NI
C. freundii NBRC 12681	2×10^9	2.4
	5×10^8	2.2
	7×10^7	NI
K. pneumoniae NBRC 14940	1×10^9	2.0
	1×10^8	1.8
	1×10^7	NI

NI, no reliable identification.

Although MALDI-TOF MS may become a popular identification method in food microbiology, there is little information on direct identification of bacteria in processed food products using MALDI-TOF MS (Angelakis et al., 2011; Böhme et al., 2011; Hochel et al., 2012). The separation of bacteria is conducted to harvest cells from complex food matrices that impede accurate detection (Benoit & Donahue 2003; Stevens & Jaykus, 2004). In the present study, a rapid and simple procedure to recover bacterial cells from contaminated soybean products was conducted and the bacterial cells obtained could be directly identified using MALDI-TOF MS.

To determine the feasibility of the proposed direct identification of bacteria using MALDI-TOF MS, processed soybean products such as soy milk and tofu were used. The most frequently occurring bacteria deduced in these samples using the 16S rRNA gene sequencing method were correctly identified by MALDI-TOF MS with adequate identification scores (Table 2).

Table 2. Comparison between MALDI-TOF MS and 16S rRNA gene sequencing identification techniques for bacteria in the processed soybean foods

Product	CFU/ml		MALDI-TOF MS [1)]	16S rRNA gene sequencing analysis [2)]
	Initial bacterial count	Bacterial count after culture enrichment		
Soy milk-1	3×10^3	9×10^8	*Leuconostoc lactis* (2.0)	*Leuconostoc lactis (57%)* *Lactococcus sp., Serratia sp.*
Soy milk-2	9×10^2	6×10^8	*Serratia liquefaciens* (2.1)	*Serratia liquefaciens (50%)* *Lactococcus sp., Enterobacter sp., Aeromonas sp.*
Tofu-1	$<1\times10^1$	1×10^8	*Bacillus cereus* (2.5)	*Bacillus cereus (100%)*
Tofu-2	2×10^3	7×10^7	*Leuconostoc lactis* (1.8)	*Leuconostoc lactis (100%)*
Frozen tofu	4×10^1	9×10^7	*Raoultella planticola* (2.3)	*Raoultella planticola (85%)* *Lactococcus sp., Enterobacter sp.*
Dried tofu	$<1\times10^1$	1×10^8	*Bacillus cereus* (2.4)	*Bacillus cereus (83%)* *Enterococcus sp.*

[1)] MALDI-TOF MS scores indicate in parentheses.

[2)] The ratio of identified bacteria indicates in parentheses. The names in the lower berth show the other bacteria identified.

Although pure cultures are generally employed for identification using MALDI-TOF MS, it has been reported that a mixed culture can be analyzed if the predominant bacterial count is sufficiently large (Maier & Kostrzewa, 2007; Christner et al., 2010). Moreover, improved software identification algorithms may increase the concordance scores with spectra from 2 or more different species (Wenzel et al., 2011).

Most frequently isolated bacteria were belonged to *Bacillus*, *Leuconostoc*, and *Enterobacteriaceae*, which are ubiquitous microbes commonly found in spoiled soybean food (Fang et al., 1999; No et al., 2002). The microbiota of soybean products are closely associated with the condition of raw materials, level of bacterial contamination, and preservation methods, which affect the growth and survival of bacteria.So It is assumed that these bacteria survived from raw materials or contaminated while processing or preservating. Processed soybean products examined in the present study were probably sterilized during manufacturing. *Enterobacteriaceae* do not usually survive under these conditions, although there may still be a risk of accidental cross-contamination during manufacturing, and the food may inevitably be contaminated during some stages of the process. Increased bacterial cell numbers were rare in most of the samples tested in the present study. This was probably the result of good manufacturing practices and strict hygiene procedures during manufacturing. However, it must be noted that only a limited number of samples were tested in this study.

4. Conclusion

MALDI-TOF MS used in the present study rapidly identified frequently occurring bacteria in contaminated food

samples. Thus, from a rapid quality assessment point of view, direct identification using MALDI-TOF MS could contribute to significant progress in the quality and safety of the food industry.

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Quality Assessment of Lightly Salted Atlantic Salmon Fillets Injected With Brine Solutions Containing Sodium Bicarbonate

Magnus Åsli[1,2], Marit Rødbotten[1], Gjermund Vogt[1], Sergey Afanasyev[1,3] & Turid Mørkøre[1,2]

[1] Nofima AS, Osloveien 1, 1432 Ås, Norway

[2] Department of Animal and Aquacultural Sciences (IHA), Norwegian University of Life Sciences, 1432 Ås, Norway

[3] SechenovInstitute of Evolutionary Physiology and Biochemistry, St Petersburg, Russia

Correspondence: Magnus Åsli, Nofima AS, Box 210, 1431 Ås, Norway. Tel: 47-4063-8912. E-mail: magnus.asli@nofima.no

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Abstract

The objective of this study was to produce lightly salted Atlantic salmon (*Salmo salar* L.) fillets with improved technical and sensory attributes. Brine containing 0, 50, 150 or 250 g/L NaCl with or without additional 25 g/L sodium bicarbonate ($NaHCO_3$) was injected into the fillets. 24 hours after injection, the muscle NaCl concentration ranged from 0.2 to 2.4%, and pH ranged from 6.18 to 6.48. Untreated fillets lost 1% weight, whereas the weight increase was 4% of the fillets injected with NaCl or a combination of NaCl and $NaHCO_3$. Liquid loss (LL) during storage at 4°C for three days were similar for the untreated fillets and the fillets injected with 50 g/L NaCl (LL 12%), while LL was reduced to 7.5% with the addition of $NaHCO_3$ to the 50 g/L brine. LL was the lowest for the groups injected with 250 g/L NaCl. Injection of NaCl resulted in higher lipid oxidation compared with untreated fillets, determined as doubled levels of alkanals (4.3 vs. 10.4 ng/g) and pentenols (8.0 vs. 15.1 ng/g), but addition of $NaHCO_3$ counteracted the action of NaCl as a pro-oxidant. Furthermore, $NaHCO_3$ addition of the 50 g/L brine significantly improved the color of raw and cooked fillets (higher a*-value, Salmo Fan score, red/orange color tone). Sensory assessment of cooked fillet revealed that brine added $NaHCO_3$ gave superior odor (less rancid), flavor (less metallic) and higher scores for tenderness. In conclusion, addition of $NaHCO_3$ to the brine solutions improved liquid retention, storage stability, color, odor and flavor of lightly salted salmon fillets.

Keywords: Salmon, fish quality, lightly salted, color, taste, smell, water holding capacity (WHC), yield

1. Introduction

The global supply of salmonids exceeded three million tonnes in 2010, of which approximately two thirds was farmed (Food and Agriculture Organization of the United Nations [FAO], 2013). Improved efficiency in the aquaculture industry has resulted in declining real prices, making farmed salmon products an attractive source of protein (Asche & Guttormsen, 2009; Norwegian Seafood Council, 2012). However, value added products are not exploited to their full potential, especially in regard to the retail sector, where consumers demand tasty and easily prepared products (Bergersen & Iversen, 2011; Norwegian Seafood Council, 2012).

Historically, fish products have been heavily salted for preservation purposes, whereas currently, lightly salted fish products (< 6% NaCl) are common due to the sensory enhancing properties of sodium chloride (NaCl) (Albarracín, Sánchez, Grau, & Barat, 2011; Gillette, 1985; Huss, 1994; Thorarinsdottir, Bjørkevoll, & Arason, 2010). With exception of the European Union, Russia and Japan are the major importers of Norwegian farmed salmonids (Norwegian Seafood Council, 2013). In Russia, nearly all processed salmon products are lightly salted and consumed uncooked (60-80%) or smoked (20-40%) (Johannesen, 2012; Tribilustova & Aandahl, 2007). Japan is the world's largest market for salmonids, and over 40% of the processed products are salted (Japanese Ministry of Internal Affairs and Communications [MIC], 2013). Lightly salted salmon is commonly broiled and eaten for breakfast or sold as ready-to-eat food products such as "bento" (packed lunch) or sushi (Nakamoto, 2000). However, the publics increased awareness of negative health associated with excessive dietary intake of sodium (Na) (e.g. elevated blood pressure and cardiovascular diseases), has escalated the demand for low Na

products. The recommended maximum dietary intake of Na per day for healthy adults is 2000 mg according to the World Health Organization (WHO); yet the average consumption is higher in most industrialized countries (WHO, 2006).

Methods for reducing the Na content in food typically encompass substitution of Na with other ions, or a gradual temporal reduction of NaCl content so that consumers have time to adjust to a lower salt flavor (Pedro & Nunes, 2007). However, both strategies have disadvantages. Partial replacement of NaCl with other salts such as KCl, $MgCl_2$ or $CaCl_2$ often has a negative effect on either flavor, liquid retention or texture properties (Albarracín et al., 2011; Gelabert, Gou, Guerro, & Arnau, 2003; Martinez-Alvarez, Borderias, & Gomez-Guillen, 2004; Weinberg, Regenstein, & Baker, 1984), while reducing the salty flavor result in declining consumer acceptability (Pedro & Nunes, 2007).

Quality traits including color, texture, liquid retention, odor and flavorare considered to be of great importance to the aquaculture industry due to consumer desirability and their attributes for secondary processing (Alfnes, Guttormsen, Steine, & Kolstad, 2006; Robb, 2001; Sveinsdottir, Hyldig, Martinsdottir, Jorgensen, & Kristbergsson, 2003). In salmonids, a post mortem rapid reduction inmuscle pH in addition to a low ultimate pH may result in poor quality attributes such as pale flesh (Robb, Kestin, & Warriss, 2000; Richards & Hultin, 2000), reduced liquid retention anddeteriorated texture (Kiessling, Espe, Ruohonen, & Mørkøre, 2004; Robb, 2001).

Sodium chloride improves water holding capacity (WHC) in meat with a maximal effect at approximately 6% NaCl (Fennema, 1990; Offer & Trinick, 1983). Additionally, increasing the pH above the protein iso-electric point (*pI*), results in swelling of the muscle (Offer & Trinick, 1983).

Atlantic salmon is a fatty species high in health beneficial polyunsaturated fatty acids (PUFA). However, PUFAs are very susceptible to peroxidation, which is a primary cause of deprived sensory quality (Refsgaard, Brockhoff, & Jensen, 1998; Undeland, Hall, & Lingnert, 1999). While NaCl may promote oxidation of PUFA (Pedro & Nunes, 2007), elevating an acidic pH may reduce lipid oxidation (Richards & Hultin, 2000).

Several studies have shown that treating meat with an alkali brine containing sodium bicarbonate ($NaHCO_3$) results in higher pH, darker color, improved liquid retention and texture including Atlantic cod (Åsli & Mørkøre, 2012), poultry (Sen, Naveena, Muthukumar, Babji, & Murthy, 2005), pork (Kauffman et al., 1998; Wynveen et al., 2001) and beef (Sultana et al., 2008).

Given the challenges facing the food industry to reduce dietary Na consumption, this project aimed to develop a method for production of low-salt salmon fillets, while simultaneously improving fillet quality and sensory characteristics.

2. Materials and Methods

2.1 Raw Materials and Salting

Atlantic salmon (*Salmo salar* L.) were raisedat the Nofima research station (Averøy, Norway) (Group A, n = 54), and at a commercial fish farm (Bremnes Seashore AS, Bremnes, Norway) (Group B, n = 18). The fish were killed with a blow to the head, filleted within 30 minutes after slaughtering, and stored on ice for three days prior to injections with brine solutions. The mean fillet weight of the salmon studied were 1.6 kg (SD 0.25). Group A fillets were weighed and labeled subsequent to being randomly divided into nine groups of six fillets. Brine solutions at concentrations of 0, 50, 150 or 250 g/L NaCl wereprepared using refined NaCl (99.8% NaCl, CG Rieber Salt AS, Ålesund, Norway) with or without the addition of 25 g/L $NaHCO_3$ (Ph.Eur., VWR, Haasrode, Belgium). The solutions (4°C) were injected into the fillets using a salt injection machine (16/64F, Fomaco Food Machinery Co. A/S, Køge, Denmark) with an injection pressure of 80 kPa. The salt content of the respective brine solutions and their acronyms are given in Table 1. Subsequent to injection, the fillets were air-dried for 10 minutes and individually packed in plastic bags. One group was randomly assigned as an untreated Control. The fillets were stored on ice and analyzed 24 hours following injection.

Fillets for sensory assessment (Group B) were injected using the same procedures and equipment as described above. The brines used for injection were 50 g/L NaCl, with or without the addition of 25 g/L $NaHCO_3$. Untreated fillets were used as Control (n = 6/treatment).

Table 1. Composition of brine solutions injected into farmed Atlantic salmon (*Salmo salar* L.) fillets

Abbreviation	NaClg/L	$NaHCO_3$ g/L	Injected
Control	0	0	-
0-NaCl*	0	0	X
50-NaCl	50	0	X
150-NaCl	150	0	X
250-NaCl	250	0	X
0-$NaHCO_3$	0	25	X
50-$NaHCO_3$	50	25	X
150-$NaHCO_3$	150	25	X
250-$NaHCO_3$	250	25	X

*Injected with water only.

2.2 Fillet Yield

The fillets were weighedprior to (W_1), immediately after (W_2), and 24 hours after injection salting (W_3). The volume injected was calculated as the increase from W_1 to W_2 (%), and the yield was calculated 24 hours after injection (W_1 to W_3).

2.3 Liquid Loss (LL)

Approximately 15 g muscle was stored for three days at 4°C on a cellulose absorber pad in a sealed polyethylene bag as described by Mørkøre, Netteberg, Johnson and Pickova (2007). The liquid loss was determined gravimetrically as the amount of weight lost from the muscle during storage.

2.4 Muscle pH

The fillet pH was measured using a muscle electrode (Schott pH-elektrode, Blueline 21, Schott instruments, Mainz, Germany) and a temperature probe (TFK 325, WTW, Weilheim, Germany) connected to a 330i pH meter (WTW, Weilheim, Germany).

2.5 Chloride and Sodium Determination

The NaCl concentration of all the fillets were determined stoichiometrically as water soluble Cl^- with the use of a Corning 926 Chloride Analyzer (Corning Medical and Scientific, Halstead, U.K.), as described by Engdahl and Kolar (1993).

The content of sodium in selected treatments (Control, 50-NaCl, 50-$NaHCO_3$, 150-NaCl and 150-$NaHCO_3$, n = 5/treatment) were determined by the use of atom absorption spectroscopy according to the standard method described in AOAC (2003). Sodium and chloride analyses were determined as an average of three measurements/sample.

2.6 Color and Fat

Triplicate color analyses were performed photometrically on the dorsal section of the fillet using the PhotoFish™ aparatus as described by Folkestad et al. (2008). The fillet lightness is presented as L* (100 = white, 0 = black), a* descring color intensity on the red/green axis (a* > 0 = red, a* < 0 = green), and b* describing the color intensity on the yellow/blue axis (b* > 0 = yellow, b* < = blue). The visual SalmoFan™ color score and fat content of the Control is based on predicted values in the PhotoFish™ software.

2.7 GC-MS Headspace Volatiles

Gas chromatogram phymass spectrometry (GC-MS) was used to identify and quantify volatile organic components as described by Olsen, Vogt, Veberg, Ekeberg and Nilsson (2005). Samples from 150-NaCl, 150-$NaHCO_3$ and Control (n = 6/treatment) were frozen at -80°C, thawed, pooled and homogenized before they were stored on ice for five days prior to analysis. The samples were analyzed in duplicate, and thevolatile components were identified according to retention time and mass spectra of the sample peaks using heptanoic acid ethyl ester as an internal standard.

2.8 Sensory Analysis

Fillets injected with 50 g/L NaCl with or without the addition of 25 g/L $NaHCO_3$, and untreated Control fillets (Group B) were assessed by a sensory panel consisting of tenmembers trained according to ISO guidelines 8586-1 (1993), and with a minimum of four years' experience in sensory evaluation. A modified quantitative descriptive profile method, ISO 6564 (1985), was used by the assessors, and the evaluations were carried out according to the guidelines in ISO 8589 (1988)in a sensory laboratory with separate booths and electronic registration of data (CSA, Compusense Five, Version 4.6, Guelph, Ontario, Canada, 1999). Cutlets of 20 mm thickness were vacuum packed in coded bags prior to heating (75°C) for 10 minutes. When served to the assessors, the sample temperature was approximately 60°C, and each assessor was served samples from the same region of the evaluated fillets. Prior to the sensory analysis, the assessors were calibrated using cooked salmon fillets that were injection salted with or without the addition of $NaHCO_3$. The samples were evaluated in random order. Each bag was individually opened by the assessor for immediate registration of sample odor, while appearance, flavor and texture attributes were recorded when the sample was removed from the bag. A computer transformed the responses into numbers between 1 and 9 for low and high intensity, respectively. The heated samples were evaluated for 20 attributes (Table 2).

Table 2. Sensory attributes of farmed Atlantic salmon fillets

Attribute	Definition
Odor	
Fresh	Fruity/fresh and sour/sweet. Also known as acidic
Metallic	Ferro sulphate, blood, iron
Seawater	Fresh, salty, sea, ocean
Rancid	Oxidized fat (like hay, stearin, paint)
Chemical	Chemical
Appearance	
Gloss	Shiny surface
Color tone	Red/orange
Taste	
Saltiness	Typical salt flavor e.g. sodium chloride
Bitter	Like quinine
Flavor	
Fresh	Fruity/fresh and sour/sweet. Also known as acidic
Metallic	Ferro sulphate, blood, iron
Seawater	Fresh, salty, sea, ocean
Rancid	Oxidized fat (like hay, stearin, paint)
Chemical	Chemical
Texture	
Hardness	Force required to bite through sample
Juiciness	Moist, perception of water released during chewing
Tenderness	Effort needed to prepare the sample ready for swallowing
Fatty	Oily mouth feeling
Fibrousness	Perception of long particles oriented in the same direction
Flakiness	Visual gaping of fillet when cut with a knife

As shown in Table 2, attributes of farmed Atlantic salmon fillets evaluated by a trained sensory panel after heating to 75°C. The fillets were untreated or injected with 50 g/L NaCl brines with or without addition of 25 g/L $NaHCO_3$, and stored for 24 hours on ice prior to heating.

2.9 Statistical Analyses

The effect of NaCl inclusion wasanalyzed using one-way ANOVA (0-NaCl to 250-NaCl and 0-$NaHCO_3$ to 250-$NaHCO_3$), while the effect of adding $NaHCO_3$ to the respective brine concentrations was analyzed using a paired t-test. Differences among means were separated by Tukey`sstudentized range test (HSD) or the t-test, $P < 0.05$ (SAS software, SAS Inst. Inc., Cary, N.C., U.S.A). To identify the most informative parameters for description of differences between experimental and Control groups (50-NaCl, 50-$NaHCO_3$ and Control), linear discriminant analysis was performed using Unscrambler v.9.8 (Camo Process A/S, Oslo, Norway). The best combinations with minimum values of Wilkins lambda were selected from all possible subsets of the sensory and instrumentally measured data. Each subset included 3 to 15 variables. This procedure resulted in 130 combinations of variables, and frequency of representation in this list (%) was determined for each variable.

3. Results

3.1 Fillet Yield

The untreated fillets had a weight reduction of 1.1% during the first 24 hours of storage (Table 3). In comparison, the yield of the injected groups ranged from 1.7 to 4.2% with significant differences between treatments. Adding $NaHCO_3$ to the water that was injected into the fillets (0-$NaHCO_3$) resulted in 1.5 % greater weight increase compared with injecting water only (0-NaCl). No significant effects were observed when $NaHCO_3$ was added to brine solutions.

Table 3. Yield, pH, NaCl and color parameters of salmon fillets

		Control	NaCl concentration in brine, g/L				ANOVA	
			0	50	150	250	Pooled SE	Effect of [NaCl]
Yield (%)	NaCl	-1.1	$1.7^{\dagger b}$	$3.7^{\dagger a}$	$4.1^{\dagger a}$	$3.8^{\dagger a}$	0.3	**
	$NaHCO_3$		$3.2^{\dagger b}$	$3.9^{\dagger ab}$	$4.2^{\dagger a}$	$3.8^{\dagger ab}$	0.2	**
	Effect of $NaHCO_3$		**	*ns*	*ns*	*ns*		
Muscle pH	NaCl	6.46	$6.35^{\dagger a}$	$6.31^{\dagger a}$	$6.22^{\dagger b}$	$6.18^{\dagger b}$	0.02	**
	$NaHCO_3$		6.41^{ab}	6.48^{a}	$6.38^{\dagger b}$	$6.32^{\dagger b}$	0.02	**
	Effect of $NaHCO_3$		*	**	**	**		
NaCl (%)	NaCl	0.2	0.2^{c}	0.6^{c}	$1.5^{\dagger b}$	$2.4^{\dagger a}$	0.2	**
	$NaHCO_3$		0.2^{c}	$0.7^{\dagger c}$	$1.5^{\dagger b}$	$2.3^{\dagger a}$	0.2	**
	Effect of $NaHCO_3$		*ns*	*ns*	*ns*	*ns*		
L-value*	NaCl	41.3	42.4	$43.7^{\dagger}$	$43.7^{\dagger}$	42.2	0.5	*ns*
(lightness)	$NaHCO_3$		42.2	41.1	41.8	41.2	0.6	*ns*
	Effect of $NaHCO_3$		*ns*	**	*	*ns*		
a- value*	NaCl	29.1	28.6	$27.3^{\dagger}$	$26.6^{\dagger}$	$26.7^{\dagger}$	0.6	*ns*
(redness)	$NaHCO_3$		27.7	29.5	27.6	27.6	0.6	*ns*
	Effect of $NaHCO_3$		*ns*	*	*ns*	*ns*		
b-value*	NaCl	22.9	23.6^{a}	22.8^{ab}	$21.8^{\dagger bc}$	$20.8^{\dagger c}$	0.4	**
(yellowness)	$NaHCO_3$		22.6^{ab}	23.2^{a}	22.4^{ab}	$21.6^{\dagger b}$	0.3	**
	Effect of $NaHCO_3$		*ns*	*ns*	*ns*	*ns*		
Visual color	NaCl	27.8	27.5	27.1	$26.8^{\dagger}$	27.2	0.3	*ns*
score	$NaHCO_3$		27.3	28.0	27.2	27.4	0.3	*ns*
	Effect of $NaHCO_3$		*ns*	*	*ns*	*ns*		

As shown in Table 3, yield, pH, NaCl and color parameters of raw salmon (*Salmo salar* L.) fillets injected with brines containing 0-250 g/L NaCl or the aforementioned brine solutions added 25 g/L $NaHCO_3$ (n = 6 fillets/treatment).* denotedifferences of $P < 0.05$, ** denotesignificant differences of $P < 0.01$. ns = no significant differences. † indicate significant difference from Control. Different letters indicate significant differences between NaCl concentration [NaCl] in brines.

3.2 Liquid Loss (LL)

The injected samples had a LL ranging from 4.6% to 12% relative to the initial sample weight (Figure 1), and wasnegatively correlated to NaCl concentration in the muscle ($r^2 = 0.67$). The 50-NaCl treatment did not differ significantly from the Control that had an average LL of 12.3%. The addition of $NaHCO_3$ to the brine solution containing 50 g/L NaCl resulted in lower LL compared to both the 50-NaCl and the Control. The lowest observed LL of 4.6% was found in the 250-$NaHCO_3$ treatment; however it was not significantly different from the 250-NaCl treatment.

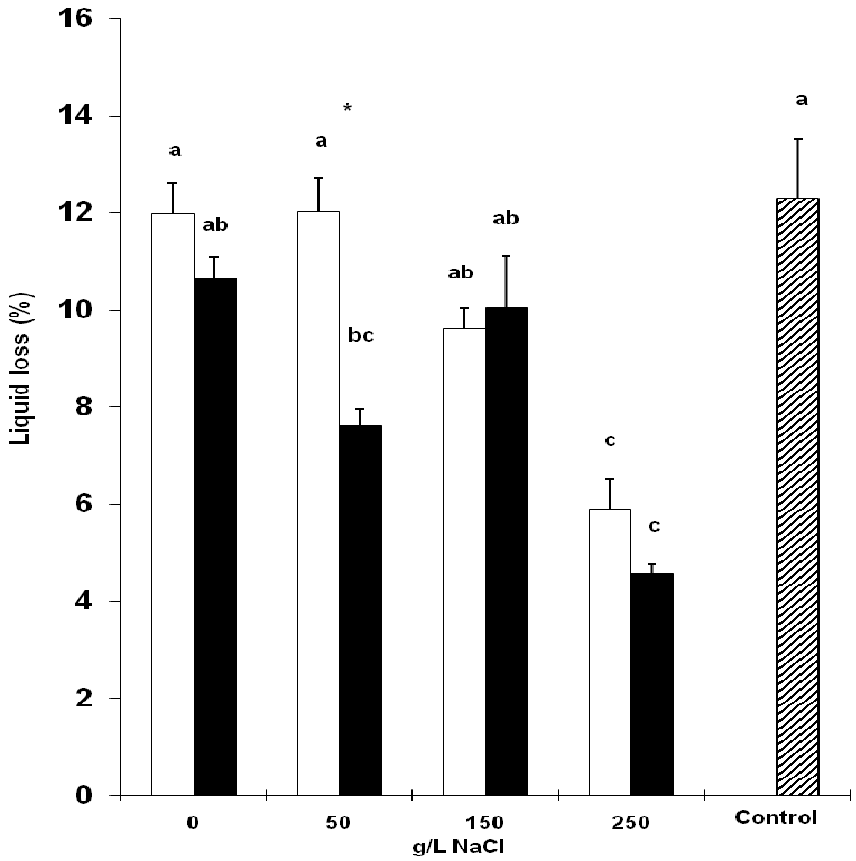

Figure 1. Liquid loss (LL) of farmed Atlantic salmon muscle during three days of storage at 4°C

The fillets were previously injected with NaCl brines (0, 50, 150 or 250 g/L) with (■) or without (□) addition of 25 g/L $NaHCO_3$, orstored as untreated Control(◪). Different letters indicate significant differences ($P < 0.05$) between brine concentrations (0-250 g/L NaCl), and * denotesignificant differences ($P < 0.05$) between fillets injected with NaCl and $NaHCO_3$ or NaCl only within brine concentration.

3.3 Muscle pH

The muscle pH ranged from 6.18 to 6.48 with significant differences between treatments (Table 3). The pH decreased with increasing brine concentration. Adding $NaHCO_3$ to the solutions resulted in significantly higher pH of all treatment groups. The most pronounced effect of the $NaHCO_3$ addition was seen between the 50-NaCl and 50-$NaHCO_3$ treatments, where the pH increasedby 0.17 units.

3.4 Chloride and Sodium Determination

The Cl^- analyzescorresponded to a NaCl level ranging between 0.2 to 2.4%, and was significantly affected by brine concentration (Table 3). Adding $NaHCO_3$ to the brine resulted in similar NaCl concentrations compared to fillets salted with NaCl brine only. No significant differences were observed between the Na content of the 50-NaCl and 50-$NaHCO_3$ (220 vs. 264 mg/100 g), or between 15-NaCl and 15-$NaHCO_3$ (601 vs. 640 mg/100 g). The Na concentration of the Control was 37 mg/100 g.

3.5 Colorand Fat

Injecting fillets with brine containing only NaCl increased L-*value (lightness), and reduced a*-value (redness) compared with the Control, while the addition $NaHCO_3$ to the brines counteracted this color shift (Table 3). Inclusion of $NaHCO_3$ in the brines resulted in significantly lower L*-valuesfor the 50-$NaHCO_3$ and 150-$NaHCO_3$ treatments compared with 50-NaCl and 150-NaCl. The a*-values were higher for brine injected fillets with NaCl in combination with $NaHCO_3$, although only significantly higher for the 50-$NaHCO_3$ treatment. The b*-value decreased significantly with increasing brine concentration, while addition of $NaHCO_3$ to the brine had no significant effect. The visualcolor scores, SalmoFan™ values, showed higher scores for fillets injected with NaCl added $NaHCO_3$ compared with NaCl only (significant between 50-NaCl and 50-$NaHCO_3$). The fat content of the Control group was 18.9% (SE ± 0.4).

3.6 GC-MS Headspace Volatiles

The levels of volatile components were similar for the Control and the 150-$NaHCO_3$ groups, except from nonanal which was significantly higher of the 150-$NaHCO_3$ treatment (Table 4). 1-penten-3-ol was 41-44% higher of the 150-NaCl treatment, whereas the content of hexanal was 70-82% higher than the Control and 150-$NaHCO_3$ treatment. Moreover, the sum of alkanals and pentenols were higher in the 150-NaCl treatment compared to the 150-$NaHCO_3$ treatment and the Control (Figure 2).

Table 4. Volatiles (ng/g) identified by GC-MS in salmon fillets

	Control	*Treatment*	
		NaCl	NaCl & $NaHCO_3$
Volatiles ng/g			
Propanal	0.4±0.4[b]	4.4±0.3[a]	0.6±0.6[b]
Butanal	0.3±0.0	0.5±0.1	0.3±0.0
Hexanal	1.0±0.1[b]	3.3±0.0[a]	0.6±0.6[b]
Heptanal	0.0±0.0	0.2±0.0	0.1±0.1
Nonanal	2.5±0.4[b]	2.0±0.1[b]	3.8±0.9[a]
2-penten-1-ol	0.3±0.3[b]	2.0±0.3[a]	0.8±0.2[b]
1-penten-3-ol	7.7±1.0[b]	13.1±1.2[a]	7.3±0.2[b]

As shown in Table 4, volatiles identified by GC-MS in untreated salmon fillets, or fillets injected with 150 g/L NaCl brine with or without the addition of 25 g/L $NaHCO_3$. Prior to analysis, samples (n = 6/treatment) were frozen at -80°C, thawed, pooled, homogenized and stored on ice for five days. Different letters denotesignificant differences between groups.

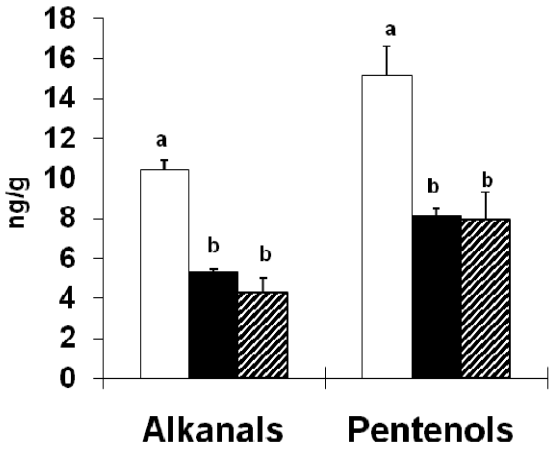

Figure 2. Volatile components (ng/g) identified by Gass chromatography-mass spectrometry in salmon fillets injected with brines with (■) or without (□) the addition of 25 g/L $NaHCO_3$, or untreated Control(▨). Alkanals include propanal, butanal, hexanal, heptanal and nonanal, whereas the pentenols encompass 1-penten-3-ol and 2-penten-1-ol. Different letters denote significant differences ($P < 0.05$) between treatments

3.7 Sensory Evaluation

Significantly stronger rancid odor was found in the Control compared to the 50-$NaHCO_3$ treatment (Table 5). The saltiness was highest for the injection salted samples, while metallic flavor was highest of the Control, and lowest of the 50-$NaHCO_3$ treatment. The 50-$NaHCO_3$ treatment scored significantly higher on glossiness, juiciness, and tenderness, and lower on hardness compared to the Control. Further, adding $NaHCO_3$ to the 50 g/L NaCl brine gave a higher red/orange color tone.

Table 5. Sensory attribute scores for salmon fillets

Attribute	Control	*Treatment*	
		NaCl	NaCl & $NaHCO_3$
Odor			
Fresh	2.9±0.1	3.2±0.1	2.9±0.1
Metallic	4.0±0.1	4.1±0.1	4.0±0.1
Seawater	2.5±0.1	2.7±0.1	2.5±0.1
Rancid	2.8±0.1^{a}	2.5±0.2^{ab}	2.3±0.2^{b}
Chemical	1.9±0.1	1.9±0.2	1.8±0.1
Taste			
Saltiness	2.1±0.1^{b}	3.1±0.2^{a}	3.1±0.1^{a}
Bitter	4.6±0.1	4.4±0.1	4.5±0.1
Flavor			
Fresh	3.2±0.1	3.5±0.2	3.4±0.1
Metallic	4.4±0.1^{a}	4.2±0.0^{b}	4.1±0.0^{c}
Seawater	2.6±0.1	2.9±0.2	2.7±0.1
Rancid	3.0±0.1	2.6±0.2	2.9±0.3
Chemical	1.8±0.1	1.6±0.2	1.7±0.1
Appearance			
Glossy	3.1±0.1^{b}	3.1±0.1^{b}	3.4±0.1^{a}
Color tone (red/orange)	5.2±0.1^{ab}	5.1±0.1^{b}	5.3±0.1^{a}
Texture			
Hardness	4.4±0.1^{a}	4.2±0.1^{ab}	4.1±0.1^{b}
Juiciness	5.0±0.1^{b}	5.3±0.1^{ab}	5.5±0.1^{a}
Tenderness	5.2±0.2^{b}	5.4±0.1^{ab}	5.6±0.1^{a}
Fatty	4.4±0.1	4.4±0.1	4.5±0.1
Fibrousness	5.6±0.1	5.5±0.1	5.4±0.1
Flakiness	4.8±0.2	4.7±0.2	5.0±0.1

As shown in Table 5, mean sensory attribute scores for odor, flavor, appearance and texture on Atlantic salmon (*Salmo salar* L.) injected with 50 g/L NaCl brines with or without the addition of 25 g/L $NaHCO_3$. Fillets were stored for 24 hours on ice prior to analysis.Different letters denotesignificant differences between groups.

3.8 Discriminant Analysis

Multivariate statistical analysis of the 50-NaCl, 50-$NaHCO_3$ and the Controlrevealed that metal flavor gave the greatest contribution in discrimination of treatments (81%), followed by pH (62%), liquid loss (60%), photometrically measured color (53%) and tenderness (46%).

4. Discussion

The physiochemical changes observed in this study were connected to dissimilar ionic concentrations in the muscle, elevated muscle pH, and possibly a favorable interaction between NaCl and $NaHCO_3$.

Increasing NaCl concentration in the muscle reduced liquid loss (LL), which was expected as NaCl induce swelling of the myofibrils (Offer & Trinick, 1983). The highest LL was seen in untreated fillets and the fillets injected with water, probably because of the low NaCl concentration (< 0.5%) (Fennema, 1990).

pH values above the proteins iso-electric point correlate positively with liquid retention due to an increased negative charge of the myofibrillar proteins, causing repulsion of the myofilaments and an enlarged myofilament lattice (Hamm, 1986; Offer & Trinick, 1983; Regenstein, Jauregui, & Baker, 1984). LL is most affected by NaCl and pH changes when the ionic strength and muscle pH is low (Bertram, Kristensen, & Andersen, 2004; Ofstad, Kidman, Myklebust, Olsen, & Hermansson, 1995). In this study, the effect on LL was more pronounced in fillets injected with low salt concentrations compared to fillets injected with higher NaCl concentrations. The difference in LL between the NaCl injected groups and the $NaHCO_3$ groups are probably caused by differences in pH, and also a cooperative effect of NaCl and $NaHCO_3$, similar to NaCl and phosphates as explained by Offer and Trinick (1983). Kaufmann et al. (1998) argue that lower LL in meat treated with phosphates or $NaHCO_3$ is caused by increased protein solubility, while Wynveen et al. (2001) suggest that $NaHCO_3$ and phosphates may work with different mechanisms. Future research is needed to elucidate the impact of $NaHCO_3$ on protein solubility in salmon muscle, andto further examine possibilities for greater reductions of Na.

Improved color of raw and cooked salmon fillets demonstrate that the addition of $NaHCO_3$ to brine may counteract the negative effect on appearance caused by brine injection. Low pH may give a lighter color of fish fillets due to changes in the protein conformation, resulting in altered light reflection pattern (Stien et al., 2005; Robb et al., 2000). Further, low pH accelerate the oxidation of myoglobin, and metmyoglobin has been coupled with deterioratedcolor of salmonids (Ottestad, Sørheirm, Heia, Skaret, & Wold, 2011; Richards & Hultin, 2000). The characteristic pink color of salmon flesh is a major determinant for consumers preferred choice of product (Alfnes et al., 2006). The findings in this study show that the practical implication of adding $NaHCO_3$ to brine may be of great importance for the salmon farming and processing industry; also to avoid quality downgrading due to pale flesh (Michie, 2001).

The lower levels of alkanals and pentenols in the $NaHCO_3$ treated fillets suggesta preservative effect on NaCl induced lipid oxidation. 1-penten-3-ol and hexanal are volatile components known to correspond with early lipid oxidation and off-odor/flavor (Alghazeer, Saeed, & Howell, 2008; Olsen, Vogt, Veberg, Ekeberg, & Nilsson, 2005). For n-3 PUFA oxidation, the level of 1-penten-3-ol was halved, while for n-6 FA oxidation, the reduction of hexanal indicated an even greater effect of the added $NaHCO_3$. Åsli and Mørkøre (2012) found similar levels of alkanals and pentenols in cod fillets injected with brine added $NaHCO_3$, but the relevance of reducing oxidation may be of greater importance for the shelf-life of a high fat species such as salmon.

Unpleasant odors and flavorswere lower of the $NaHCO_3$ treated fillets, and coincide with studies on sow meat (Sindelar et al., 2003) and pork (Sheard & Tali, 2004). In line with a study on cod (Åsli & Mørkøre, 2012), the $NaHCO_3$ treatment significantly altered the texture with higher scores for juiciness and tenderness, and lower scores forhardness compared with the untreated fillets. These attributes areprobably connected with increased protein solubility of the $NaHCO_3$ treated fillets.

A wider range of low sodium products is required to meet the WHO recommendations regarding a reduced dietary sodium intake (maximum 2000 mg Na/day) (WHO, 2006). The Na content (mg/100 g) of smoked salmonis typicallyin the range of 780-1372, beef burgers 290-590, and low sodium ham 969 (Bannerman & Horne, 2001; Desmond, 2007; Pedro & Nunes, 2007). In comparison, injecting a 50 g/L or 150 g/L brine with or without the addition of $NaHCO_3$ resulted in a sodium content of 220-263 mg/100 g and 601-640 mg/100 g respectively, and would comply with consumer demands of lowered sodium content in salted salmon products.

5. Conclusion

Injecting salmon fillets with brine added sodium bicarbonate is an efficient and economically beneficial method of producing attractive low salt products with improved flavorand color.

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Plackett-Burman Experimental Design for Investigating the Effect of Porcine Plasma Protein, Trehalose and Bovine Meat Protein Isolate on Cook Yield and Texture of Minced Bovine Meat

Vareltzis Patroklos[1], Petridis Dimitris[1], Argiro Petropoulou[1] & Tsamitropoulou Fani[1]

[1] Department of Food Technology, ATEI of Thessaloniki, Thessaloniki, Greece

Correspondance: Vareltzis Patroklos, Department of Food Technology, ATEI of Thessaloniki, PO Box 141, Thessaloniki 57400, Greece. Tel: 30-231-067-4779. E-mail: p.k.vareltzis@gmail.com

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Abstract

A common additive to counter the cooking losses considerations in the muscle food industry is sodium tripolyphosphate (STP), which however may pose health risk for certain segments of the population. This paper employs a Plackett-Burman design to investigate the effect of porcine plasma protein (PPP), protein isolate from bovine meat (PI), trehalose and pH of the additive mixture on cook yield and textural characteristics of minced beef during frozen storage. Results show that PI and PPP significantly ($p < 0.05$) increase the cooking yield throughout the storage period compared to the control sample. Fresh samples with PPP, PI and STP had ~90% cooking yield, while control sample had only 75%. PI and PPP can improve the cooking yield of minced beef for the first two months of storage by at least 20%, in a similar and comparable pattern with STP. Upon optimization the optimal values were achieved at PI concentration 2.3-5.0% and PPP levels 1.7-4.7%.

Keywords: porcine plasma protein, trehalose, Plackett-Burman, cook yield, STP, protein isolate

1. Introduction

1.1 Cook Losses in the Meat Industry

The major changes occurring in meat during cooking are shrinkage, toughening of tissues, releasing of meat juice and colour changes (Dreeling et al., 2000; Vittadini et al., 2005). These are caused by the changes in muscle proteins, e.g., denaturation of muscle proteins and shrinkage of collagen due to the increase in temperature. All these leads to changes in textural properties, decrease in the water-holding capacity of proteins, and hence cook losses, which are important economic considerations. Therefore, minimization of cook losses to a certain safety level would be desirable (Erdog˘du et al., 2001).

1.2 Limitations of Polyphosphate Salts

The meat processing industry uses phosphates to improve textural properties and to reduce cook losses. Action mechanism of phosphates, which exhibit a pronounced polyionic character, is based on the ionization of protein molecules, increasing the interaction between protein and water molecules due to increased pH and ionic strength, and reducing the interaction among the proteins (Cheftel et al., 1985; Martin et al., 2002; Unal et al., 2006). Sodium tripolyphosphates (STPs) are actually the most popular form of phosphates used in the meat industry (Dzeizak, 1990; Lampila, 1992). However, sodium tripolyphosphate is a suspected neurotoxin according to the National Institute for Occupational Safety and Health's (NIOSH) Registry of Toxic Effects of Chemical Substances. Several studies which focused on the effect of the addition of phosphates on consumer health have been published and these studies have given contradictory results. The kidneys easily control the blood phosphorus level and efficiently excrete any excess of phosphorus; hence, up to now, there is no evidence that higher phosphate intakes are detrimental to bone health or to bone calcium excretion in the urine in healthy adults not having problems with kidneys (Fenton et al., 2009; Whybro et al., 1998). However, in the study of Huttunen et al. (2006) with adult rats, excessive intake of dietary phosphate without the company of calcium caused rise in concentration of serum parathyroid hormone and hindered mineral deposition into cortical bone, leading to lower bone mineral density. High dietary phosphorus also has significant effects on cardiac fibrosis and arterial wall thickening, relevant for increased cardiac risk especially in haemodialysis and chronic kidney disease patients (Amann et al., 2003; Foley et al., 2009; Sherman & Mehta, 2009). These controversies over

human health, as well as increasing consumer concerns about additives in foodstuff justify the interest in replacing polyphosphates with natural ingredients.

1.3 Previous Work on Substistution of Polyphosphate Salts

Other ingredients can be introduced in meat products to obtain polyphosphate-free-products without losing yield or sensory quality. Several additives and non-meat ingredients have been used to enhance the water-binding capacity of cooked sausages. Atughonu et al. (1998) reported that sodium caseinate, soy protein isolate, whey protein concentrate, and wheat germ flour increased the cooking yield in frankfurters. Most of the ingredients that can help in binding water, stabilizing emulsions and increasing firmness of meat products are proteins. Egg, milk, soya and wheat proteins may pose health concerns because of potential allergenicity.

Blood plasma from slaughtered animals has been shown to be a good food ingredient due to its gelling properties as well as its ability to improve water holding capacity, and emulsion stability (Cofrades et al., 2000; Dàvila et al., 2007; Parés & Ledward, 2001). Also, the use of blood proteins in the food industry is a good way to upgrade this by-product and hygienically collected blood is a raw material that is a cheap food ingredient which has not been shown to be allergenic. Moreover, since blood is naturally present in meat, the introduction of blood derivatives in meat products does not involve the addition of an unnatural ingredient.

Protein isolates derived by a pH-shift process have been shown to have improved functional characteristics (Tahergorabi et al., 2012a). Protein isolates, besides myosin and actin, are enriched in sarcoplasmic proteins. These proteins have been shown to improve textural characteristics of cooked sausages (Farouk et al., 2001). The pH-shift method has been extensively applied to various fish-species and fish processing byproducts (Nolsoe & Undeland, 2009). However, there is little literature data on protein isolates from other type of muscles such as chicken (Tahergorabi et al., 2011; Tahergorabi et al., 2012b), turkey (Liang & Hultin, 2003) and especially beef muscle used as an enhancer in meat products.

To protect myofibrillar proteins from freeze-denaturation during frozen storage and to maintain its possible high processability, cryoprotectants, such as disaccharides, polysaccharides, polyalcohols or polyphosphates are generally added (Park et al., 1988; Mac Donald & Lanier 1991). Trehalose (d-glucopyranosyl-α(1→1)-d-glucopyranoside) is a non-reducing disaccharide with a low calorific value and low sweetness, i.e. only 45% of that of sucrose (Colaco et al., 1994). Because of its ability to form strong hydrogen bonds with the polar group of biomolecules and a very high glass transition temperature, trehalose has superior preservation properties as compared to other sugars (Patist & Zoerb, 2005). Kovacevic and Mastanjevic (2011) showed that trehalose has a better cryoprotective effect than maltose on washed and frozen beef meat. So far, no information regarding the effect of trehalose on comminuted beef meat has been reported.

1.4 Aim of This Study

This work aims to investigate the effect of a) porcine plasma protein (PPP), b) protein isolate from beef muscle (PI), c) trehalose and d) the pH of the addition mixture on cook yield, water holding capacity and textural properties of fresh and frozen ground beef muscle and then compare them to the polyphosphates effect. Since, all the investigations using full factorial design are tedious and multivariate methods, which is a collection of statistical and mathematical techniques, has been proved to be an effective way for the desired purpose (Petridis et al., 2013; Murphy et al., 2004; Rincón et al., 2008; Pereira et al., 2011; Kim et al., 2013), a Plackett-Burman design was chosen for the experimental design and analysis. The selected response variables were water holding capacity, cook yield and texture parameters such as hardness, firmness, gumminess, chewiness, cohesiveness.

2. Materials and Methods

2.1 Materials

Lean beef muscle was purchased from local retail shop and was transferred to the laboratory in an ice box. All chemicals were of ACS grade and were obtained from Sigma-Aldrich SA.

2.2 Sample Preparation

Lean beef muscle tissue was trimmed from any visible fat and connective tissue and minced using a Kitchen Aid ultra power grinder (model KSM90, St. Joseph MI) through a 5 mm perforated plate. To all mince samples the following ingredients were added:

a. NaCl 2% (w/w)
b. Trehalose (TR): 0% or 3% (w/w)
c. Added distilled water: 10% (w/w)

d. Sodium Lactate 23.3% (w/v) of the added distilled water

e. Protein isolate (PI): 1% or 5% (w/v) calculated based on protein concentration of the added water

f. Porcine Plasma Protein (PPP): 1% or 5% (w/v) of the added water

Sodium lactate, TR, PI, and PPP were mixed in the added water volume prior to the addition to the mince. The pH of the added solution was adjusted to 9 or 11 with 1 N NaOH.

Two more samples were prepared: the blank, which contained only 10% (v/w) distilled water, NaCl and sodium lactate and the blank+STP sample, which additionally contained 3% (w/v) STP in the added water.

The mince samples (30 g) were packed in polyethylene cylindrical bags and either heated immediately in a water bath at 80°C to achieve an internal temperature of 72°C or placed in the freezer at -20°C for 4 months. Each month, samples were taken out, thawed under cold running water and then cooked under the same conditions as the freshly prepared samples.

2.2.1 Preparation of Porcine Plasma Protein (PPP)

The procedure followed, was adapted from Benjakul et al. (2001). Porcine blood was collected from a slaughterhouse in Kalohori, Greece. Mean composition in% (w/w) of the plasma was: moisture 90.2 ± 0.004, ash 1.66 ± 0.01, and protein 7.66 ± 0.06. Plasma was then freeze-dried and kept at 4°C until used. The dry powder was referred to as porcine plasma protein (PPP).

2.2.2 Preparation of protein Isolate (PI)

Proteins from lean beef meat were isolated by applying a pH-shift method (Vareltzis et al., 2008). Specifically, beef tissue was trimmed from any visible fat and connective tissue and minced using a KitchenAid ultra power grinder (model KSM90, St. Joseph MI) through a 5 mm perforated plate. Minced samples were transferred to a beaker and placed in a container filled with crushed ice and were homogenized in 9 volumes of cold distilled-deionized water for 40 s at 14000 rpm using an Ultra Turrax T18 Basic homogenizer (IKA, Taquara, RJ, Brazil). The pH of the homogenate was brought to10.8 (alkaline solubilization) with 2 N NaOH. The solubilized homogenate was filtered through three layers of commercial cheesecloth. The filtrate was readjusted to pH 5.3 to precipitate the solubilized proteins, which were collected after filtration through 3 layers of cheesecloth.

The collected proteins, called protein isolate (PI), were analysed for moisture content and protein concentration by the Biuret method (Gornall et al., 1949). Mean composition of protein isolate was (w/w): moisture content 89.1 ± 0.4%, protein 10 ± 0.2%, fat 0.5 ± 0.02% and ash 0.5 ± 0.01%.

2.3 Experimental Design and Statistical Analysis

A particular Plackett-Burman experimental design for screening significant effects on chemical and mechanical variables was chosen (Montgomery 2001). The design included 12 runs of two- leveled four factors (see Table 1 and sample preparation) and was repeated five times following the experimental freezing periods: 0, 1, 2, 3, 4 months, with 0 freezing period representing the immediate cooking of the 12 samples that were prepared from fresh minced beef.

Table 1. Layout of the Plackett-Burman experimental design (uncoded values)

Sample	pH of added solution	PPP (% w/v)	TR (% w/w)	PI (%w/v)
1	9	1	0	1
2	9	5	0	5
3	11	5	3	5
4	11	5	0	1
5	11	1	0	5
6	9	1	3	1
7	11	5	0	5
8	11	1	3	1
9	9	1	0	5
10	11	1	3	5
11	9	5	3	1
12	9	5	3	1

Analysis of variance was performed at different freezing times on the variables under study testing the potential effect of four factors, pH and three additives (PPP, PI, TR). Optimized combinations between additive levels of the most important attributes were attempted. The additives' effects were compared to those produced by polyphosphates and blank material by freezing month. The 95% confidence intervals of the means, produced from each Placket-Burman design, were used to compare with individual values of polyphosphate and blank material since the latter factors were not included in the experimental design. Means whose 95% intervals do not overlap denote pairwise statistically significant difference.

Finally, a Principal Component Analysis (PCA) was applied on the correlation matrix of attributes to detect the most important variables emerging at various additive combined levels at progressive freezing times.

All statistical designs and analysis were performed using the statistical software Minitab v.16 (Minitab Inc.).

2.4 Measurements

2.4.1 Cook Yield (CY) and Water Holding Capacity (WHC)

The mince samples (30 g) were placed in polyethylene cylindrical bags and heated in a water bath at 80°C to achieve an internal temperature of 72°C. During cooking, the temperatures in the centre of the meat samples were monitored with a glass thermometer inserted in the centre of the sample. The drip was drained from the sample. The cooked mass was cooled and subsequently weighted to determine weight loss. Cook yield was determined as 100 x (final weight/initial weight).

Water holding capacity was determined by centrifuging 2 g of cooked samples in the presenceof Na_2SO_4 at 3000 x g for 10 min at room temperature. After blotting dry, the weight was recorded. WHC was calculated as 100x(initial weight/final weight) (Abdullah & Al-Najdawi, 2005)

2.4.2 Texture Analysis

Experiments were performed using a TA-XT texture Analyzer (TA instruments, New Castle, DE), as described before by Ritzoulis et al. (2010). The analyzer was equipped with a 75-mm-diameter aluminiumcylinder, operating with a compression rate of 5 mm/sec. Samples, 20 mm in length, were cut using a dedicated template ring, and axially compressed to 40% of their original height. The capacity of the load cell used was 30 g. All tests were performed at least three times. TPA was performed 10 min aftecooking. The load cell was calibrated prior to use. TPA parameters were: hardness, cohesiveness, gumminess, chewiness.

The OriginPro 8.0 2 (OriginLab Corporation, USA) computer program was chosen to obtain graphic displays of the texture analysis data and perform the necessary calculations.

2.4.3 Moisture Content, pH and our

The moisture content was determined in triplicate by AOAC method 950.46 B (AOAC, 1990). The pHvalues of the samples were measured in duplicate (Thermo Orion 3STAR pH meter equipped with SN 9099083 electrode) on a homogenate of 1 g sample in 9 ml distilled water.

The colour of the meat samples was measured using a HunterLab spectrocolourimeter (Hunter Lab Ltd., Reston, VA, USA) and expressed as CIE L*(lightness), a* (redness) and b* (yellowness) values.

3. Results and Discussion

3.1 pH and Colour

The pH of the samples varied between 5.8 and 6.2 (reported values are the mean of three readings) as it can be seen in Table 2. Even though the pH of the added solution was adjusted to two different values (9 and 11), there were only slight differences in the final pH of the samples before cooking (samples 1-12), probably due to the buffering capacity of the meat itself, as well as the added proteins. After cooking, the pH of all samples did not change significantly ($p > 0.05$).

Certain treatments of minced beef exhibited significant effects ($p < 0.05$) on colour parameters when compared to the blank sample (Table 2). It is observed that samples 10, 11 and 12 have significantly higher L* and a* values compared to the blank. These samples have 2 characteristics in common: high trehalose and high protein (either PPP or PI) content in the added solution. Since the protein source was either the blood plasma or the bovine meat, these differences can be attributed to residual haemoglobin/myoglobin. These observations suggest that porcine plasma and/or protein isolate can be used as replacers for artificial pigments.

Table 2. Colour of fresh samples and pH before and after cooking (mean ± SD, n = 3)

Samples	L*	a*	b*	pH before cooking	pH after cooking
blank	28.5 ± 0.98	16.0 ± 1.13	8.0 ± 2.5	5.78	5.91
Blank+STP	26.9 ± 3.18	17.63 ± 1.58	9.3 ± 1.60	5.91	5.93
1	38.5 ± 2.76	14.9 ± 0.28	5.5 ± 0.20	5.82	5.92
2	28.5 ± 0.57	17.2 ± 0.86	8.7 ± 0.21	5.81	6.01
3	23.3 ± 2.37*	18.5 ± 1.44	7.9 ± 0.69	6.13	6.22
4	27.8 ± 2.76	16.76 ± 1.09	9.1 ± 0.26	6.11	6.15
5	29.8 ± 0.65	16.1 ± 0.703	9.4 ± 0.58	6.30	6.21
6	25.3 ± 0.65	17.3 ± 0.66	8.3 ± 0.69	6.25	6.40
7	24.9 ± 2.6	15.2 ± 0.49	8.4 ± 1.87	6.16	6.24
8	27.7 ± 3.3	16.7 ± 0.89	6.9 ± 1.03	6.12	6.14
9	33.1 ± 4.45	18.3 ± 0.047	9.5 ± 1.60	6.21	6.54
10	30.8 ± 1.20*	19.8 ± 1.23*	10.6 ± 0.84	6.19	6.14
11	32.1 ± 0.81*	21.1 ± 1.14*	10.5 ± 0.87	5.93	6.00
12	33.9 ± 1.81*	20.5 ± 0.8*	11.3 ± 0.82	6.20	6.12

*denotes significant difference from the blank ($p < 0.05$).

3.2 Significant Effects

The Plackett-Burman's analysis of variance revealed significant effects of all additives on cook yield, moisture content, springiness and cohesiveness (Table 3). Specifically, Table 3 summarizes only the significant effects of the process variables (pH, PI, PPP and Trehalose (TR)) on the physicochemical parameters of the samples for every month of freezing (samples 1-12). Cook yield increased at 5% PPP addition and in the two following freezing months. A similar pattern was observed for the other two factors considered (pH, PI). Porcine plasma protein has been shown to exhibit high hydrophilic character compared with dairy or meat proteins (Alting et al., 2002; Howell & Lawrie, 1984; Zayas, 1985). PPP has been used to substitute STP and caseinate in frankfurters, where it was found that neither moisture nor WHC, as well as cooking losses showed significant differences (Hurtado et al., 2012). An increase in WHC had been observed by Cofrades et al. (2000) in meat products containing bovine plasma. The WHC increases with the concentrations of plasma protein only at concentrations lower than 8%, while plasma proteins have a pH of around 7.3-7.5 that raises the meat pH, leading to increased WHC (Chen & Lin, 2002; Feiner, 2006).

In a similar manner, protein isolate from bovine muscle increased cook yield, achieving the highest value at the 5% level of addition. Furthermore, protein isolate increased springiness and cohesiveness at low concentration levels (1%). It has been shown that pH treatment of myofibrillar proteins significantly increased their solubility compared to native proteins. Good myofibrillar protein solubility is believed to be a prerequisite for many functional properties, including gelation and emulsification, and this is why salt is typically added to muscle-derived products to obtain desirable quality characteristics (Kristinsson & Hultin, 2003). Furthermore, protein isolates are enriched in sarcoplasmic proteins. These proteins have been shown to improve textural characteristics of cooked sausages (Farouk et al., 2001). In addition, the fact that the pH of the solution was adjusted to 9 and 11 should further enhance the protein's ability to hold water and therefore minimize cook losses.

Trehalose decreased the moisture content when added at 3% for months 0 and 1. On the contrary, trehalose increased springiness at the 3% level of addition for month 1. These results can be explained by taking into account how trehalose stabilizes the proteins. According to the water replacement theory, which was presented to explain the unique properties of trehalose, the disaccharide is thought to substitute water around the proteins, maintaining their three-dimensional structure by providing sites with hydrogen-bonding species. This substitution also leads to a decrease in the solvation layer around the protein and restricts the mobility of the biological macromolecule (Jain & Ipsita, 2009; Sola-Penn & Meyer-Fernandes, 1998). Therefore, proteins actually bind less water, which then can be lost during cooking.

Table 3. Statistically significant effects of additives on physicochemical and mechanical attributes of minced meat during freezing time. 95% confidence intervals of means based on pooled standard error and exact probability value are shown

Month	Variables	Additives	Level	Means	-95%	+95%	P
0	%CY	PPP	1	85,4	82,9	88,0	0,0323
			5	89,8	87,1	92,4	
0	%MC	TR	0	71,7	70,9	72,4	0,0262
			3	70,3	69,5	71,0	
1	%CY	pH	9	86,4	84,7	88,1	0,0313
			11	87,8	86,2	89,4	
1	%CY	PPP	1	85,3	83,6	87,0	0,0100
			5	88,7	87,1	90,4	
1	%CY	PI	1	85,9	84,3	87,5	0,0117
			5	88,7	87,0	90,4	
1	%MC	PPP	1	69,9	69,4	70,5	0,0088
			5	70,9	70,4	71,4	
1	%MC	TR	0	71,0	70,5	71,5	0,0034
			3	69,9	69,3	70,4	
1	Springiness	TR	0	1,06	0,72	1,39	0,0082
			3	1,62	1,27	1,97	
1	Springiness	PI	1	1,45	1,11	1,79	0,0444
			5	1,15	0,80	1,50	
1	Cohesiveness	PI	1	0,54	0,46	0,62	0,0334
			5	0,44	0,36	0,52	
2	%CY	PPP	1	81,0	79,1	82,9	0,0235
			5	84,4	82,5	86,2	

Additives did not exert any significant effect on the parameters water holding capacity, soluble protein, hardness, gumminess and chewiness. Also, no particular effect resulted for all attributes during the third and the fourth freezing month. Increasing protein content several textural characteristics, such as hardness, chewiness and cohesiveness are also increased (Hayes et al., 2005; Hughes et al., 1997). However, in the present study the addition of PPP and PI to fresh minced beef samples did not have a significant effect on these attributes compared to the STP sample, but only when compared to the blank one. This can be partially explained by the fact that the addition of these proteins took place at high pH values (9 or 11), where they have increased solubility. When the proteins are introduced back to the minced beef, they partially refold. This new conformation of the proteins has been shown in other studies to exhibit good functional properties, including solubility and emulsification (Hultin & Kelleher, 1999; Undeland et al., 2002; Kristinnson & Hultin, 2003; Liang & Hultin, 2003). The fact that no significant difference was observed in the pH of the cooked minced samples and additionally that pH was in all samples well above the isoelectric point of the major proteins in meat (pH 5-5.5) can explain why water holding capacity showed no marked differences (Gault, 1984).

3.3 Principal Component Analysis

Principal Component Analysis (PCA) on both variables and additive factors, the latter treated as dummy variables, resulted in the formation of two major axes explaining 46.5% of the total variation. The first axis is composed mostly by the variables hardness (HARD), cook yield (CY), moisture content (MC) and freezing months 0 and 2 (Freeze_0 and Freeze_2) due to their high correlation values with this axis (Table 4, Figure 1). The second axis is formed by high correlation values of springiness, cohesiveness and secondarily by pH and trehalose 3% (TR_3).

Table 4. Correlation coefficients between PCA axes 1 and 2 and variables (additive factors are shown as dummy variables). The two axes explain 46.5% of the total variation.c

Variable	Axis 1	Axis 2
HARD	0,937	-0,083
Freeze_2	-0,814	-0,037
CY	0,791	0,168
Freeze_0	0,767	0,052
MC	0,734	0,343
PPP_5	0,411	-0,336
PI_1	-0,148	0,103
SPRING	0,081	-0,785
COHES	-0,241	-0,642
WHC	0,553	-0,567
pH_11	0,011	0,493
TR_3	-0,122	-0,426
%SolPro	-0,209	0,280
Variance	3,912	2,133
Variance %	30,1	16,4

According to the findings of Figure 1, moisture, cook yield and hardness show positive strong correlation with the time soon as the samples had been cooked (month 0) and negative strong correlation after freezing for two months, indicating the strong negative effect of freezing at -20°C for two months. This is also in agreement with the data on Figure 2, where it can be observed that after 2 months of freezing the cook yield, and textural characteristics of PPP and PI are rapidly deteriorating. Freezing, and especially at slow rate (-20°C) can have adverse effects on meat textural characteristics, cook yield and WHC. Most of these effects are attributed to protein denaturation. When meat is frozen at a slow rate or warmer temperatures, there is considerably more damage done to the fibres and myofibrils (Petrovic et al., 1993). PPP and PI, individually, at levels 5% were able to maintain a high cooking yield of the minced samples compared to the blank for all four months of frozen storage. However, cohesiveness (COHES) and hardness (HARD) were not maintained at high values after 1 month of storage. Springiness and cohesiveness correlate strongly and positively in the addition of 3% trehalose but strongly negatively when the added solution was adjusted to pH 11. A possible explanation for this is that trehalose solutions resist degradation between pH 3.5 and 10 (Teramoto et al., 2008). A higher pH, such as 11 that was used in the added solution containing trehalose could alter trehalose structure and therefore alter its functionality. A loose positive correlation occurs between water holding capacity (WHC) and 5% addition of porcine plasma protein.

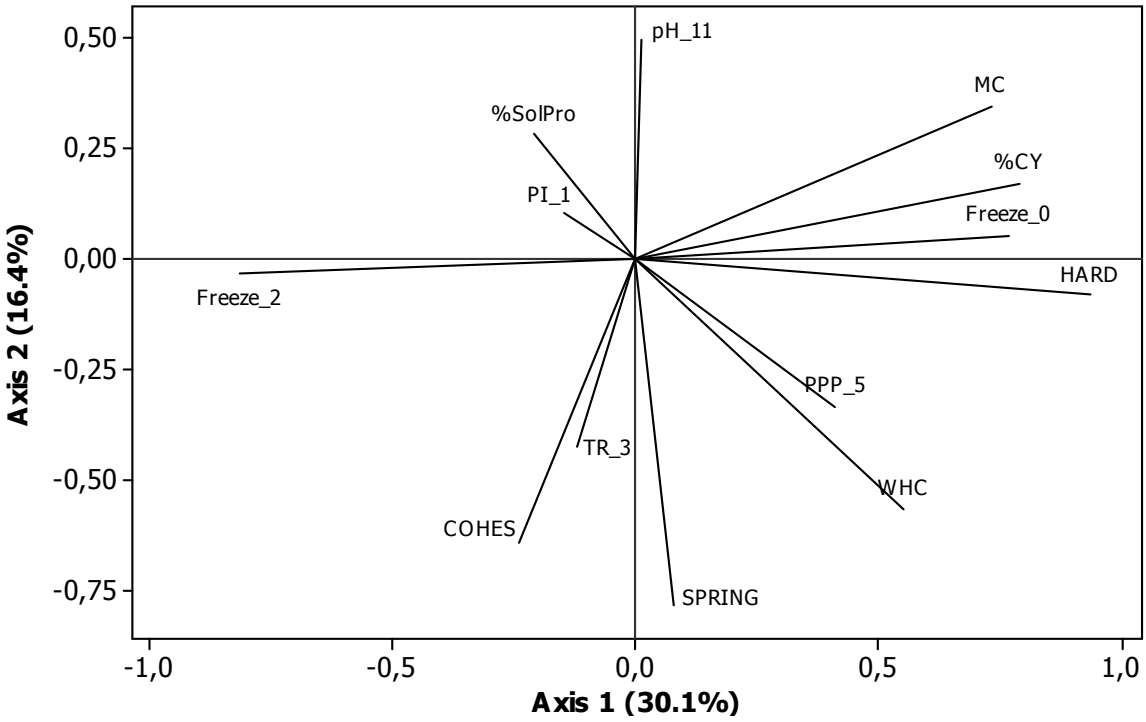

Figure 1. Correlation biplot of physicochemical, mechanical and dummy additive variables by the principal component analysis

3.4 Comparison With Sodium Polyphosphate

Statistical analysis showed that the two additives that had a significant positive effect on cook yield and on some of the textural parameters were PPP and PI. The additives' effects were compared to those produced by polyphosphates and blank material by freezing month. Freezing time affects drastically the shift of some of the attributes when additives such PPP and PI are present in the minced meat (Figures 2 and 3). Cook yield, at PPP addition, remains steady until the first month of freezing declining thereafter and reaching the lowest values in the fourth month (Figure 2). A comparable shift is obvious for the blank +STP sample. On the other hand the absence of PPP or STP from the mince (blank sample) results in an abrupt drop of the cook yield after freezing for one month which afterwards remains more or less steady by time. The same pattern is observed for PI addition as well (Figure 3).

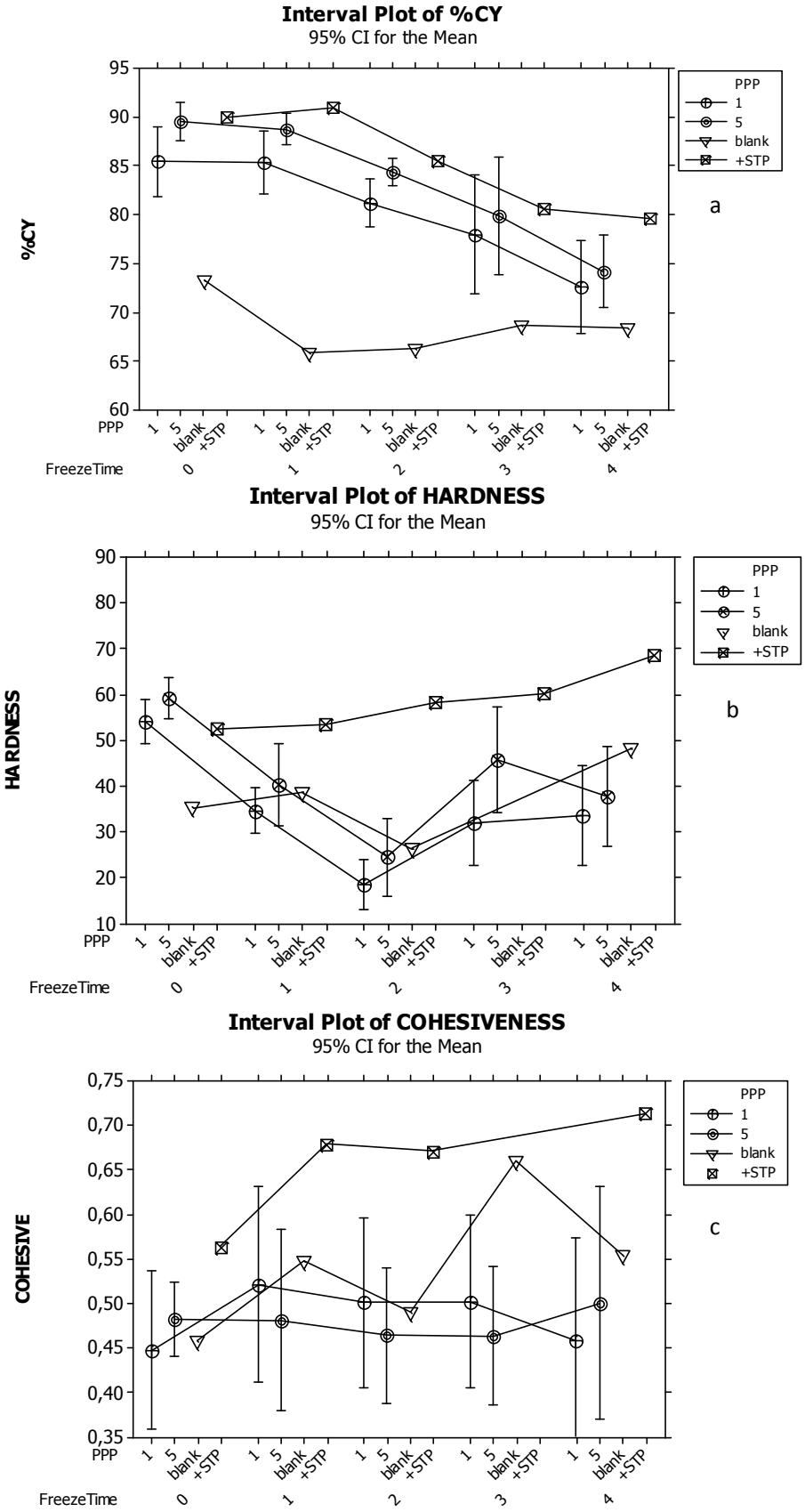

Figure 2. Monthly mean changes of (a) cook yield, (b) hardness and (c) cohesiveness at two PPP addition levels, without additives (blank) and with addition of polyphosphates (+STP). Vertical lines denote the 95% confidence intervals of the means

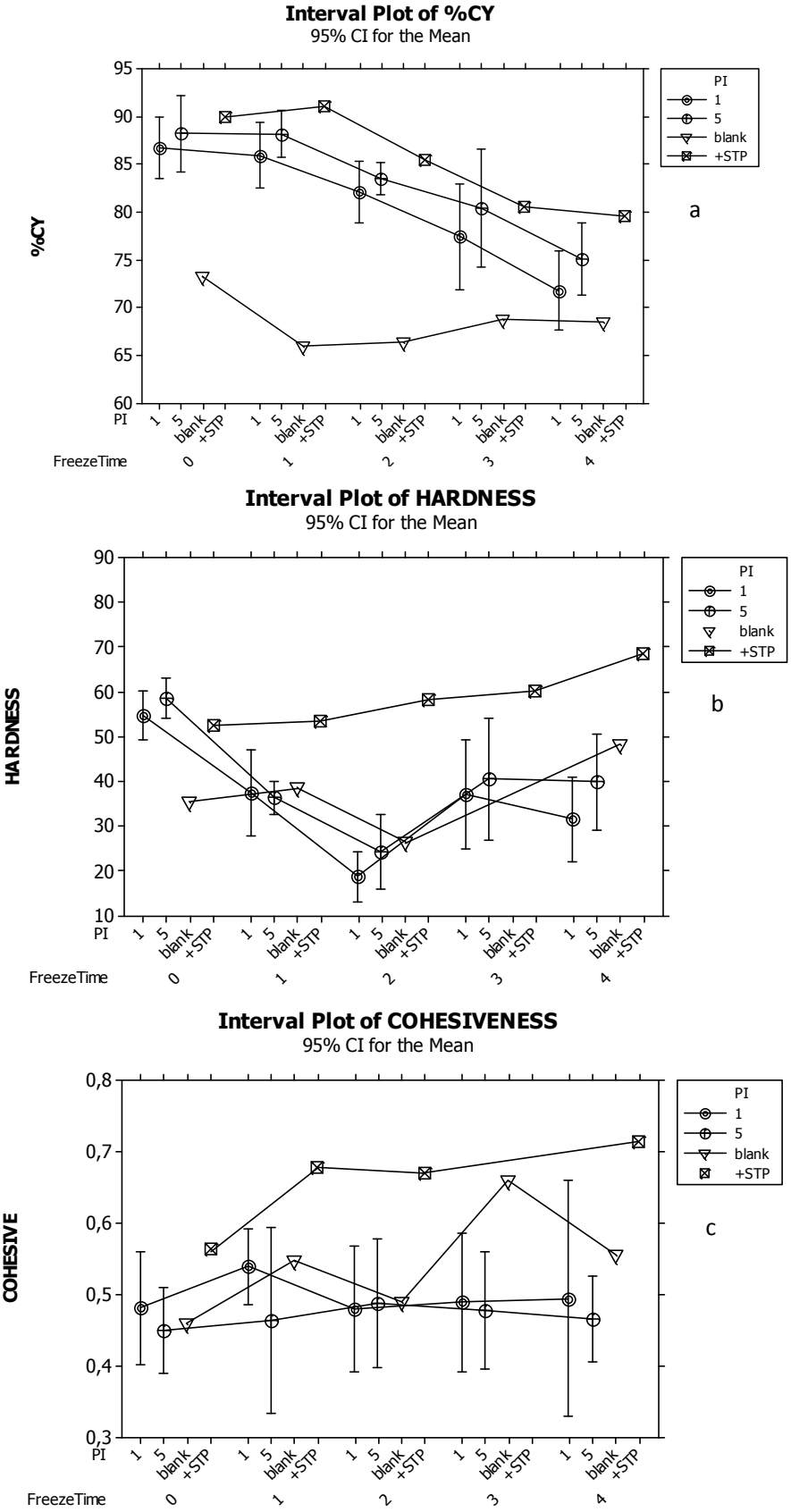

Figure 3. Monthly mean changes of (a) cook yield, (b) hardness and (c) cohesiveness at two PI addition levels, without additives (blank) and with addition of polyphosphates (+STP). Vertical lines denote the 95% confidence intervals of the means

At PPP addition, the mechanical hardness decreases with time approaching a minimum in the second month and then stabilizes in a position a bit upwards in the following two months (Figure 2). Blank sample follows a same direction. The addition of STP though results in a steady, slightly gradual increase in hardness. At PI addition, a similar pattern takes place (Figure 3).

Mechanical cohesiveness remains steady without undulations by freezing time for both additives present, as the blank sample does too. However the shift of cohesiveness for the STP sample increases markedly with time approximating higher values than those of additives (Figures 2 and 3).

Conclusively, it can be said that both PPP and PI individually, and especially at the 5% level of addition, can increase cook yield of fresh and frozen minced beef to the same extent as the polyphosphates do. On the other hand, the effect of PPP and PI on mechanical hardness is similar to polyphosphates only for the fresh samples (0 freeze time), while they exert no effect on cohesiveness.

3.5 Optimization

Optimization procedure was based on those variables that were mostly influenced by the factors under study (see ANOVA results, Table 3).

Thus, by setting the chemical variables, cook yield and moisture content; at ranges near their optimal performance, that is their maximal, the overlaid contour plots as affected by PI and PPP were obtained while holding the pH and trehalose levels at 11 and 3% respectively (Figure 4). The white region of the plot is the feasible region, or the area that satisfies the criteria for all responses. The optimal chemical conditions were achieved at PI concentration 2.3-5.0% and PPP levels 1.7-4.7% which both define performance of 90% cook yield and 71.6% MC.

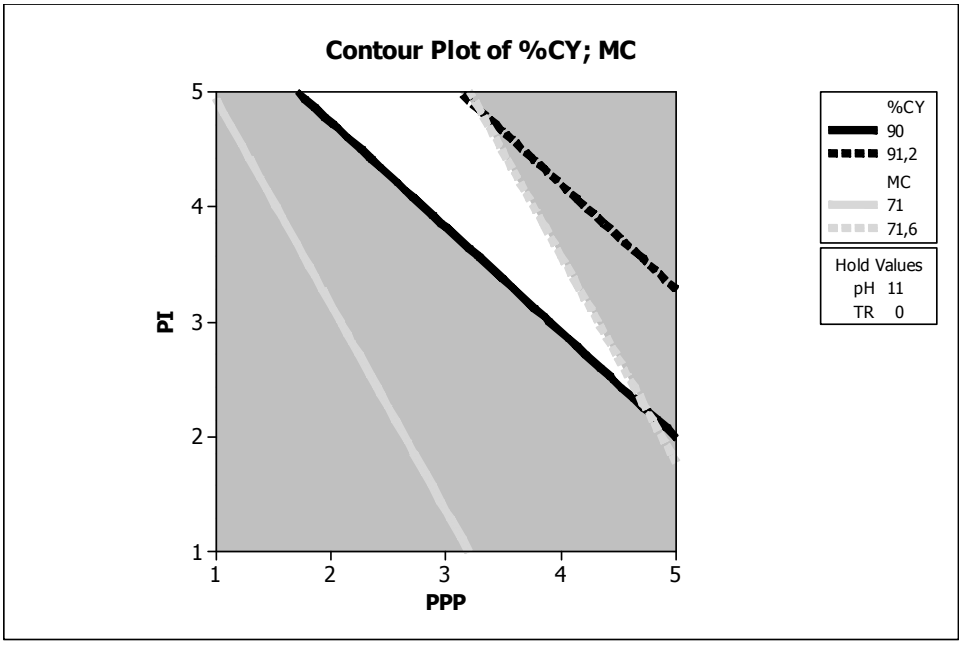

Figure 4. Overlaid contour plot of cook yield and moisture content at varying levels of PI and PPP concentration

Regarding the mechanical springiness, its optimal performance is obtained in the area defined by the narrow stripe shown in Figure 5 that corresponds to springiness 1.65-1.75, PI 3-3.7% and TR 1.5-1.9% while holding pH and PPP at midrange levels 10 and 3%.

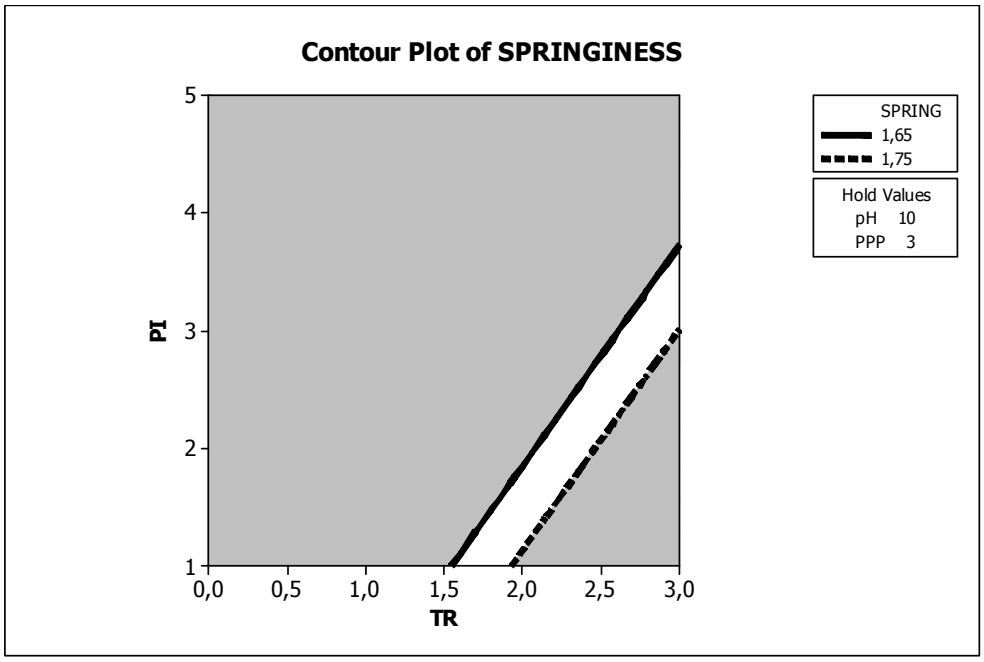

Figure 5. Overlaid contour plot of springiness at varying levels of PI and TR concentration

4. Conclusions

PCA reveals two bundles of variables with a distinguishing but uniform behaviour: moisture content, cook yield and mechanical hardness start their maxima before freezing commences (freezing time 0, first bundle) and mechanical springiness and cohesiveness show high values at 3% trehalose addition which occurs one month after freezing (second bundle). Trehalose did not enhance cook yield of fresh or frozen minced beef samples. Porcine plasma protein and protein isolate from bovine meat were able to increase cook yield of beef samples to a similar extend as the STP did. PPP and PI did not alter colour or the pH of the samples compared to STP, nor affected textural parameters especially for the fresh samples and for the samples that were frozen for one month. Therefore, PPP and PI can be used as a polyphosphate alternative in the meat industry.

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Phytochemicals in Edible Wild Mushrooms From Selected Areas in Kenya

T. W. Wandati[1], G. M. Kenji[1] & J. M. Onguso[2]

[1] Department of Food Science, Jomo Kenyatta University of Agriculture and Technology Nairobi, Kenya

[2] Institute of Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology Nairobi, Kenya

Correspondence: T. W. Wandati, Department of Food Science, Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000-00200, Nairobi, Kenya. E-mail: wan.wanda@yahoo.com/twandati@gmail.com

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Abstract

Mushrooms have been used as food for centuries all over the world because of their characteristic soft texture and mild flavor. They are documented as being good source of nutrients and bioactive compounds that are beneficial to the human body (Chang, 2011). While the exotic varieties have been extensively analyzed, local edible wild mushrooms have not and yet they are consumed by communities living near the forests. This research aimed at screening and determination of phytochemicals compounds in wild edible mushroom found in selected areas in Kenya Two commercially grown mushrooms, oyster (*Pleurotus florida*) and button (*Agaricus bisporus*), and ten edible wild mushrooms species were collected from different locations of the country. They were analyzed for total polyphenols, flavonoids and radical scavenging activity using standard methods. All the analysis was done in triplicate. Phytochemical screening showed presence of saponins, polyphenols and terpenoids. Total polyphenols values obtained ranged between 210-1614 mg Gallic Acid equivalent (GAE)/100g, dry weight basis (dwb) and flavonoids 214-1695 mg Quercetin Equivalent (QE)/100 g dwb. Total polyphenols ($R^2 = 0.82$, $P \leq 0.05$) and flavonoids values showed a positive correlation with the radical scavenging activity.

The results show that cultivated and wild edible mushrooms are rich in health-promoting phytochemical compounds.

Keywords: mushrooms, phenolic compounds, flavonoids, radical scavenging activity

1. Introduction

Mushroom has been defined as ‘a macro-fungus with a distinctive fruiting body, which can be hypogenous or epigeous, large enough to be seen with the naked eye and to be picked by hand (Chang & Miles, 1989) They are documented as being rich in proteins, minerals, vitamins while they are low in lipids (Pathak et al., 1997).

Documented literature indicates that mushrooms have photochemicals and other compounds which are strong antioxidants (Fang et al., 2002; Liu, 2004**)**. Phenolic compounds, alkaloids, saponins, flavonoids, tannins, sterols, triterpenes, coumarins and cyanogenic glycosides have been detected in wild mushrooms analyzed in Sudan and in Nigeria (Adebayo et al., 2012; Egwim et al., 2011; Ehssan & Saadabi, 2012).

The compounds seem to mop the free radicals generated in the normal natural metabolism of aerobic cells, mostly in the form of reactive oxygen species (ROS). These include superoxide (O_2^-) and hydroxyl (OH^-) radicals among several others. Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents and pesticides (Barja, 2004). Once in circulation, most of the free radicals are neutralized by cellular antioxidant defense enzymes e.g. Superoxide dismutase (SOD) or catalase (CAT). Non-enzymatic molecules like ascorbic acid and carotenoids are reported to be present in mushrooms and they also act as antioxidants (Fang et al., 2002; Isabel et al., 2004). Maintenance of equilibrium between free radicals production and antioxidant defenses is an essential condition for normal organism functioning (Hollman & Arts, 2000). The disequilibrium, excess free radicals in the system, is known as oxidative stress. It interferes with cell integrity hence normal functioning is altered leading to many stress-related diseases like cancers and diabetes.

Mushroom nutriceuticals describe a new class of compounds extractable from either the mycelium or fruit body of mushrooms and embodies both their nutritional and medicinal features. They are consumed as a dietary supplement which has potential therapeutic applications (Chang & Miles, 1989). Mushroom Nutraceuticals are

enriched food materials which are used for Maintenance of healthy diet. These are part of a meal (Chang & Miles, 1989; Shiuan, 2004). Infusion of mushrooms has been used to prevent beriberi. In addition, the decoction has been used for the treatment of abscesses and wounds (Yu et al., 2009).

2. Materials and Methods

2.1 Materials

Mushrooms used in this research comprised of wild mushrooms (Figure 1) collected from natural habitat and included 7 species from Arabuko Sokoke, one species from Aberdares and Mt Elgon forests, 2 species from Kisumu and Kakamega counties. Two cultivated species; oyster (*Preurotus florida*) and button (*A*garicus *biporus*) were included for comparison. To determine the effect of maturity and mushroom parts on phytochemicals, oyster was segregated into young and mature fruit bodies while *oruka* was segregated into caps (pilei) and stipes Samples of fresh wild mushrooms were taken to the Museums of Kenya, Botany/Herbarium department for scientific identification which is still in progress. Consequently, local mushroom names will be used in this report for uniformity.

Figure 1. Some of the wild mushrooms collected: A Mkundu wa nyani; B, Masikiro meruhe; C, Rerema; D, Mariondonic/makunu ma mutitu; E, Oruka; F, Joga muhama; G, Kadzodzo; H, Malombo

2.2 Sample Preparation

At the university, Jomo Kenyatta University of Agriculture and Technology (JKUAT), they gently brushed off dirt and were all dried at 70°C, ground using laboratory mill. The Milled mushroom was put in labeled air-tight bottles and stored at 4°C.

2.3 Preparation of Mushroom Extracts

Extraction, screening and quantification was done according to the method described by Harbone (1998) with minor modifications Known weight of the dried mushroom powder was mixed with 100 ml ethanol in a conical flask. The content was put on a shaker for 24 hours at room temperature. The liquid part was decanted and stored at 4°C. The solid was re-suspended in ethanol and procedure repeated. The liquid was then combined, filtered using whatman paper no. 4. The filtrate was concentrated in vacuum evaporator to 10 ml. This was put in sample bottles and stored at 4°C to await further analysis. Similarly, known weights of mushroom powder were mixed with hot water (50°C), put on a shaker for 24 hours. The rest of the procedure was as described.

2.5 Quick Tests for Phytochemicals

Ethanolic and water extracts were subjected to preliminary phytochemical screening for the identification of various classes of active chemical constituents using standard methods (References).

2.5.1 Quick Test for Saponins' Presence

Foam test: To 1 ml of the extracts 5ml distilled water was added and shaken vigorously. Formation of foam indicated presence of saponins.

2.5.2 Quick Test for Total Polyphenols

Ferric Chloride test: To 1 ml of the extract, 2 ml of distilled water, 3 drops of 10% aqueous ferric chloride ($FeCl_3$) and 3 drops of potassium ferrocyanide were added. Formation of blue or green color showed the presence of polyphenols.

2.5.3 Quick Test for Anthraquinones

Weighed mushroom powder, 0.5 g, was boiled in 10% hydrochloric acid and filtered hot. To this, 2 ml chloroform and 10% ammonia solution each were added .Formation of pink color in the aqueous layer indicated presence of anthraquinones.

2.5.4 Quick Test for Terpenoids

Water extract, 5 ml, was mixed with 2 ml chloroform followed by sulfuric acid along the tube wall. Formation of brown color at interface was a positive indicator.

2.5.5 Quick Test for Tannins

To 3 ml ethanolic extract was added 3 ml 10% ferric chloride ($FeCl_3$). Formation of blue/black color was a positive indicator.

2.5.6 Quick Test for Alkaloids

On silica gel-coated plates, 10 µl extract was spotted equidistance from each other and eluted with methanol-sulfuric acid solution. The dried plates were sprayed with Dragendr off reagent. Formation of red-brown color was positive indicator.

2.6 Determination of Total Polyphenols

Phenolic compounds in the mushroom extracts were estimated by a colorimetric assay, based on standard procedures described by Harbone (1998) with minor modifications.

To 5 ml distilled water was added 0.5 ml Folin Ciocalteu's reagent. After 3 min, 1 ml 7.5% sodium carbonate solution, 1 ml extract were added to the mixture and made to 10 ml with distilled water. The mixture was kept in water bath maintained at 50°C for 16 minutes. UV Visible spectrophotometer (UV-Vis Shimadzu) was used to read the absorbance at 765 nm. Gallic acid was prepared in different concentrations and the absorbance equally read at 765 nm. The values obtained were used to generate the standard curve against which polyphenols in the mushrooms were calculated and expressed as Gallic acid equivalents (GAEs) per 100 g dwb.

2.7 Determination of Flavonoids as Quecetin Equivalent

Flavonoids contents in the extracts were determined by standard colorimetric method with minor modifications. To 1 ml mushroom extract was added 0.3 ml 5% sodium nitrite; 4 ml distilled water and held for 5 minutes. To the mixture 0.3 ml 10% aluminium chloride was added and held for 6 minutes. Finally 2 ml 1 M sodium

hydroxide was added and the content made to 10 ml with distilled water. Using UV spectrophotometer, (UV-Vis) the intensity of pink color was measured at 415 nm. Pure quercetin was prepared in different concentrations and absorbance read at same wavelength. The readings were used to make standard curve against which flavonoids in the sample were calculated and expressed as mg of quercetin equivalents (QE)/100 g dwb.

2.8 Determination of Radical Scavenging Activity (RAS) Using DPPH

The radical scavenging activities of the mushroom extracts against 2, 2-Diphenyl-1-picryl hydrazyl radical were determined by UV visible spectrophotometer, UV-Vis-SDD-10AV SHIMADZU, at 517 nm. Radical scavenging activity was measured using standard procedures. The following concentrations of the extracts were prepared, 0.05, 0.1, 0.5, 1.0, 2.0 and 5 mg/ml in ethanol. Ascorbic acid was used as the antioxidant standard at concentrations of 0.02, 0.05, 0.1, 0.2, 0.5 and 0.75 mg/ml.

To 1 ml of the extract in a test tube 3 ml ethanol was added followed by 0.5 ml 1 mM DPPH in ethanol. Incubation was done for 5 minutes. A blank solution was prepared containing the same amount of ethanol and DPPH. The absorbance of the blank was read at 517 nm and a standard curve generated using the values. The absorbance of solutions with extract was similarly read at 517 nm.

The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation:

% RSA = [(ADPPH-AS)/ADPPH] 100, where AS is the absorbance of the solution when the sample extract has been added at a particular level, and ADPPH is the absorbance of the DPPH solution.

The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph.

2.9 Statistical Analysis

Results are given as mean of triplicate ± SD. Correlation coefficient between total polyphenols and radical scavenging activity was done by regression using Genstat statistical software version 14.

3. Results and Discussion

3.1 Mushrooms Collected

Result of the mushroom collected and analyzed is shown in Table 1.

Collection is normally done early in the morning. A major problem encountered in collection is competition with wild animals like the baboons. It is common to find they started earlier and have eaten them, so one comes back empty handed. Encounter with dangerous animals like elephants is a reality which makes it very risky to the communities that would benefit from this freely available food. Entry to the forest is therefore highly restricted by Kenya Wildlife Service (KWS). The volume picked is therefore very low and no conservation is done. Where collection is from disturbed land e.g. Kakamega and Kisumu, drying is done and it is possible to buy dry mushroom in far off places.

Table 1. Mushrooms collected and analyzed

Botanical name	Common/Local name (community)	Region	Habitat
Agaricus bisporus	button/makunu (kikuyu)	Central	Commercially grown
Preurotus florida	Oyster/Makunu (kikuyu)	Central	Commercially grown
Termitomyces sp	Makunu ma mutitu(kikuyu)	Central	Grows on farms/ forest/ anywhere
Termitomyces sp	Mariondonik (Sabaot)	Rift valley	Grows on farms/ forest/ anywhere
-	Olando (Dholuo)	Nyanza	Grows on farms
-	Obulando (Luhya)	Western	Grows on in farms
Termitomyces sp	Oruka (Dholuo)	Nyanza	Grows on ant-hills
-	Joga muhama (Giriama)	Coast	Grows on disturbed land.
	Joga misinga (wadtha)		
Termitomyces sp	Joga utuwe (Giriama)	Coast	Grows on ant -hills
	Dugo dhinthu (Wadtha)		
-	Malombo (Giriama)	Coast	Mixed & Brachystegia forests

Russula Compressa	Mkundu wa nyani (Giriama)	Coast	Brachystegia forests
-	Joga kadzonzo (Giriama)	Coast	Mixed & Brachystegia forests
Amanita zambiana	Rerema (Giriama)	Coast	Mixed & Brachystegia forests
-	Masikiro meruhe (Giriama)	Coast	Mixed & Brachystegia forests

- Name not identified.

Key-Mixed forest is composed of *Afzelia quanzensis, Manikara sansibarensis, Hymenaea verrucosa.* Trees;

Brachystegia forest is composed of *Brachystegia speciformis, Manikara sansibarensis and Hymenaea verrucosa and Patches of grass present.* ASF: Arabuko Sokoke Forest in Kenyan coast;

Olando of Kisumu same species as Obulando of Kakamega;

Makunu ma mutitu of Aberdares =same species with mariondonik of Mt Elgon.

3.2 Quick Assays for Presence of Phytochemical Compounds

The results of phytochemical screening are given in Table 2. The screening indicated presence of saponins, polyphenols and terpenoids. Alkaloids, tannins and anthraquinons were absent in all the species. The Phenolic compounds include different subclasses (flavonoids, phenolic acids, stilbenes, lignans, tannins, oxidized polyphenols) that display a large diversity of structures (Nijveldt, 2001).

The compound detected will depend on the method used, the pH and the interaction with other compounds. As a result it is not possible to conclude that those that were absent would not be detected using different reagents or methods.

Saponins comprise a large family of structurally related compounds containing a steroid or triterpernoid aglycone. They are reported to have a wide range of pharmacological properties that exert various benefits, such as anti-inflammatory and anti-diabetic properties (Lee et al., 2012).

Terpenoids (isoprenoids) are secondary metabolites with molecular structures containing carbon backbones made up of isoprene. The compounds have been reported to show a wide range of pharmacological benefits that include anti-malarial, anti-inflammatory and anti-cancer among others (Beattie, 2011; Roslin & Annular, 2011).

Table 2. Phytochemical compounds detected in the mushrooms

Mushroom	Saponins	Polyphenols	Alkaloids	Tannins	Terpenoids	Anthra-quinones
A bisporus	+++	+	-	-	+	-
P florida	++	+	-	-	+	-
Makunu-ma mutitu	+	+	-	-	+	-
Mariondonik	+	+	-	-	+	-
Obulando	+	+	-	-	+	-
Olando	++	+	-	-	+	-
Oruka	+++	+	-	-	+	-
Joga Muhama	+	+	-	-	+	-
Joga utuwe	++	+	-	-	+	-
Malombo	+++	+	-	-	+	-
Mkundu wa nyani	+++	+	-	-	+	-
Joga kadzonzo	++	+	-	-	+	-
Rerema	+++	+	-	-	+	-
Masikiro maruhe	+++	+	-	-	+	-

+ = Presence; ++ = increasing concentration; - = absence;

Olando of Kisumu same species as Obulando of Kakamega;

Makunu ma mutitu of Aberdares =same species with mariondonik of Mt Elgon.

3.3 Polyphenols, Flavonoids and Radical Scavenging Activity Values Are Shown in Table 3

The figures obtained for polyphenols range between 210-1614 mg GAE/100g, dwb. Flavonoids values obtained are 214-1695 mg QE/100 g and the RSA values obtained ranged between 58.07-458.01

The total polyphenols content show a positive correlation with flavonoids, high polyphenols accompanied by high flavonoids although not in direct proportionality ($P \leq 0.05$). The levels of these compounds are influenced by species, substrate on which mushrooms grew, maturity and the part of the mushroom analyzed **(**Oboh & Shodehinde, 2009). High levels of flavonoids were accompanied by high RSA (low value). The radical scavenging activity of phenolic compounds has been correlated to their chemical structures (Nijveldt, 2001). RSA is also influenced by other factors, such as presence of other H-donating groups like –NH or –SH. Other compounds which have RSA include ascorbic acid, tocopherols, and carotenoids. All these are reported to be present in mushrooms at different levels **(**Barros et al., 2007). This probably explains why there is no obvious trend that relates levels of TP and TF with RSA. The antioxidant level would therefore be influenced by the nature and levels of compounds elucidated The RSA values obtained range between 58.07-458.01. The reported values are in the same range of 76-1000 mg/100 g dwb in Portuguese wild mushrooms (Barros et al., 2007).

The Young Fruit Bodies (YFB) have higher TP and total flavonoids (TF) than the mature fruit bodies (MFB). This is in conformity to reported figures (Isabel et al., 2004). The author suggested that the compounds in mature stages could be involved in defense mechanism as a result of the aging process; hence reduced content on extraction.

The TP, TF and RSA values are dependent on the part of the mushroom analyzed. This is exemplified by values obtained for *oruka* cap and stipe. The TP, TF and RSA mean values in cap were 1332.24, 1511.08 and 58.07 respectively. Those for the stipe were 872.57, 648.20 and 59.59 respectively. Similar but lower figures have been reported for total polyphenols in cap 677-1066 and 400-760 for stipe (Isabel et al., 2004). Other reports indicate that polyphenol extracts from the stipes had a significantly ($p < 0.05$) higher free radical scavenging ability and reducing power than those from the caps (pilei) (Oboh & Shodehinde, 2009). Whole *oruka* values were 788.52, 979.64 76.65 for TP, TF and RSA respectively. This indicates that the values obtained for the whole mushroom depend on the proportion of stipe or cap present in the analyzed sample. This research used a whole mushroom.

When all factors that influence the nature and content of phytochemical compounds are considered, the differences are expected. However, although the stipe may have lower TP and TF the RSA may be same or higher than that of cap (Barros et al., 2007). This is the case where values obtained for RSA in cap and stipe is 58.07 and 59.59 respectively. The reason may be that different compounds or compounds with different structures accumulate in stipe but contribute to RSA

All mushrooms from Arabuko sokoke forest exhibited high levels of total polyphenols than the rest from highlands. The flavonoids levels compare well with the mushrooms from other places. However the RSA is not high. This seems to suggest that these compounds are involved in stress-related reactions rather than RSA. This would suggest that the geographic region of coast has influenced the synthesis and bioaccumulation of compounds different from elsewhere. The high temperatures and salt concentration may have influenced production of high levels of polyphenols to cope with environmental stress.

Table 3. Polyphenols, flavonoids and radical scavenging activity values of the mushrooms

Mushroom	TP GAE (mg/100g)	TF QE (mg/100g)	RSA IC_{50} (mg/100g)
Button	460.42±1.02	801.34±0.50	156.83±0.89
Oyster-MFB	675.56±0.97	890.87±0.90	61.86±0.56
Oyster-YFB	836.2±0.59	1129.75±0.33	62.64±0.32
****Makunu ma mutitu***	798.57±1.03	730.20± 0.55	68.62±0.48
****Mariondonik***	728.05±1.05	798.66±0.65	70.02±0.21
#Obulando	432.66±0.41	740.77±0.14	185.10±0.14
#Olando	773.43±1.54	726.36±0.20	205.05±0.05
Oruka whole	788.52±0.45	979.64±0.60	76.65±0.57
Oruka cap	1332.24±0.67	1511.08±0.85	58.07±0.67
Oruka stipe	872.57±0.90	648.20±0.79	59.59±0.60

Muhama	1543.22±1.22	921.30±1.02	68.29±0.45
Joga utuwe	1580.08±0.66	944.55±0.58	122.45±0.55
Malombo	1080.45±0.50	528.52±1.56	112.65±0.56
Mkundu wa nyani	947.95±0.75	463.50±0.46	116.25±0.85
Joga kadzodzo	1250.24±0.56	748.01±0.55	379.10±0.70
Rerema	1058.05±0.54	433.10±0.73	99.15±0.65
Masikiro meruhe	331.54±0.91	214.15±0.57	458.01±0.85

Values expressed as means, mg/100 g ± SD of triplicates on dry weight basis. Correlation coefficient between TP and RSA = 0.82;

TP = Total polyphenols; GAE=Gallic Acid Equivalent; TF = Total flavonoids; QE = Quercetin Equivalent; RSA = Radical scavenging activity; IC_{50} = Inhibition concentration for 50%; YFB = Young fruit body; MFB = Mature fruit body;

*Makunu ma mutitu of Aberdares = same species with mariondonik of Mt Elgon; #Olando of Kisumu same species as Obulando of Kakamega.

4. Conclusion

The data obtained from this research clearly show that exotic and wild mushrooms contain phytochemical compounds that are necessary for a healthy body.

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