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Phytochemical, Proximate and Nutrient Composition of *Vernonia calvaona* Hook (Asterecea): A Green-Leafy Vegetable in Nigeria

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Abstract

The leaf of *Vernonia calvaona* was analysed for its phytochemical, proximate, anti-nutrient, mineral elements and vitamin compositions using standard analytical procedures. Flavonoids (7.07 ± 0.43%) were the most dominant plant secondary compound, followed by steroidal saponins (4.42 ± 0.23%), phenolic compounds (3.19 ± 0.05%), and carotenoids (1.62 ± 0.11%). Alkaloids (1.26 ± 0.13%), and sesquiterpene lactones (1.64 ± 0.13%) were also present in the plant. The proximate analysis of the fresh leaf gave a carbohydrate content of 20.80± 0.67 mg/100 g, with a corresponding reducing sugar content of 8.56 ± 0.06 mg/100 g. The sample also gave a protein content of 19.80 ± 0.61 mg/100 g and fat content of 4.17 ±0.15 mg/100 g respectively. The total fatty acid content of the plant was 3.57 ± 0.52 mg/100 g. Overall the green-leafy vegetable of *Vernonia calvaona* which is usually eaten raw and fresh contains a very balanced nutrient composition and provides a total metabolising energy value of 844.49 ± 6.19 KJ/100 g. The plant has a crude fibre content of 7.63 ± 0.22 mg/100 g and an ash content of 10.67 ± 0.33 mg/100 g respectively. The anti-nutrient levels, including oxalates (0.34 ± 0.04 mg/100 g), phytates (0.94 ± 0.04 mg/100 g) and cyanates (0.09 ± 0.01 mg/100 g) were low compared to many known vegetables. The leaf is rich in vitamins (Vit C 11.33 ± 0.88, Vit A 0.61 ± 0.01 and Vit E 0.99 ± 0.13 mg/100 g). The leaf is also rich in vitamins B_1, B_2, B_6, niacin and folic acid. The mineral profile of the leaf sample is also impressive, and includes some key elements such as, Fe, Zn, Ca, Na, K, Mg, P and Se. It may be concluded that the leaves of *V. calvaona* contribute to nutrient intake by the consuming populations in Nigeria and can serve as an antimalarial, antidiabetic, fertility agent, anti-cancer, anti-ulcer and cardioprotective agent.

Keywords: *Vernonia calvaona*, vitamins, mineral elements, secondary compounds

1. Introduction

Vernonia calvaona Hook (*Astereaceae*) is popularly known as "Ekeke leaf" among the indigenous people of the central senatorial district of Cross River State of Nigeria. It is a small shrub of less than 1m tall with petiolate leaves of about 10.0 mm wide which serve as a green-leafy vegetable as well as being used for ethno-medical purposes in Nigeria and Cameroun. It is popularly eaten raw and fresh as a local delicacy with or without palm oil in pepper sauce. It serves as a component of traditional salad among the indigenous consumers. It may also be cooked in native soups and stew, and in the preparation of potatoes, yam and plantain porridge. Its consumption is based on the native belief that the plant as a whole cures heart diseases, blindness, diabetes, malaria, stomach ache and as an anti-helminthic agent. It is eaten to prevent constipation. Most people eat it fresh and raw because the vegetable imparts a sweet taste like sugar in the tongue after its consumption. It is less bitter than the sister plant (*Vernonia amygdalina*), and yet both plants are used for the same ethno-medicinal purposes. The plant is widely distributed in South Western Cameroun and South-Eastern part of Nigeria, just like its close relatives *V. tenoreana*, and *V. amygdalina*. These three species are morphologically similar in many respects, though they can be differentiated using some anatomical features such as the height and broadness of the leaves, as well as the intensity of bitter taste of plant. Of these three species *V. calvaona* is the shortest in height. On the other hand, *V. amygdalina* is the most bitter of the three, followed by *V. tenoreana* and the least bitter is *V. calvaona.* Nothing has been scientifically reported about *V. calvaona,* inspite of its rich phytochemical and nutrient composition which contributes significantly to the dietary nutrient intake by the consuming populations of Eastern Nigeria and South Western Cameroun. Its popular sister species (*V. amygdalina* and *V. tenoreana*) have been thoroughly investigated with respect to human nutrition, *glycaemic effect*, *lipidemic effect*, antimalarial, anthelminthic,

anti-diabetic and antitumorigenic activities (Gyang et al., 2004; Ojiako & Nwanjo, 2006; Igile et al., 1995; Ebong et al., 2008). Considerable attention had been focused on the pharmacodynamic properties of *V. amygdalina* especially its hypoglycaemic activities (Ogbuokiri et al., 1989; Akah & Okafor, 1992). More recently, Abosi and Raseroka (2003) and Izevbigie et al. (2004) discussed antimalarial, anthelminthic and antitumorigenic properties of *V. amygdalina.* Ijeh and Ejike (2011) reviewed the work so far done on the medicinal potential of *V. amygdalina* and hope for the future on the usefulness of this plant for food and medicinal purposes. On the other hand, *V. calvaona* has received very little or no attention in this respect despite its close taxonomic relationship to *V. amygdalina* and its various and potential medicinal and dietary uses.

The importance of the nutritional quality of vegetables to Nigerians has resulted in increased demand for knowledge of the nutrient composition of vegetable foods. Green leafy vegetables are important components of the dietary regime of humans because they provide the necessary vitamin and mineral elements required for growth and maintenance of good health through all ages (Fasuyi, 2006). Green-leafy vegetable foods also contain antinutrients which reduce the bioavailability of important nutrients substances in foods (Akindahunsi & Salawu, 2005; Binita & Khetarpaul, 1997). Aleto and Adeogun (1995), reported that some antinutrients exhibit protective effects thus making them serve dual purposes. For instance, oxalates bind to calcium to form calcium oxalate crystals, which prevent the absorption and utilization of calcium by the body thereby causing diseases such as rickets and osteomalacia (Ladeji et al., 2004). The calcium crystals thus formed may also precipitate around renal tubules causing renal stones. On the other hand phytatic acid combines with some essential elements such as Fe, Ca, Zn and P to form insoluble salts called the phytates which are not absorbed by the body thus making the minerals non-bioavailable. Saponins are plant naturally occurring steroidal or triterpenoidal glycosides found in a wide variety of plants. Some are known to be poisonous to humans, causing cell lysis and hemolysis, teratogenesis, and post–partum hemorrhage when ingested orally or when injected into the blood stream (Igile et al., 1994; Applebaum et al., 1969).Tannins are water soluble phenolic compounds with the ability to precipitate proteins from aqueous solutions. They occur almost in all vascular plants. They combine with digestive enzymes thus making them unavailable for digestion (Agbara, 2003; Binita & Khetapaul, 1997). Despite the fact that vegetables are widely consumed because of their accepted nutritional value, there is lack of sufficient information on the antinutritional factors in some of them, including *V. calvaona*. The present study was therefore undertaken to assess *V. calvaona* for its nutrient value and phytochemical composition, as well as to determine the level of antinutritional factors in the vegetable, with a view to advancing further research into its potential medicinal and pharmacological value to mankind.

2. Materials and Methods

2.1 Source of Plant Material

Vernonia calvaona (Ekeke) green-leafy vegetable was purchased from Assiga daily market in Yakurr LGA of Cross River State of Nigeria and quickly washed and stored under refrigeration. The plant sample was authenticated by Dr. Michael Eko, a Botanist in the University of Calabar, Nigeria, and a voucher specimen (BCH/VC/02) was deposited in the Herbarium of the Department of Biochemistry, University of Calabar, Nigeria.

2.2 Sample Preparation

Five hundred grammes (500 g) of the leaves were washed, cut into small pieces and air-dried at room temperature (27 ± 1.50 ºC) for seven days for phytochemicals and anti-nutrients investigation and for the determination of mineral elements. The samples were ground into powder and stored each in an air tight bottle prior to use for analysis. Fresh leaf samples were used for the analysis of proximate composition and vitamins.

2.3 Phytochemical Analysis

Phytochemical analysis for tannins, phenolics, flavonoids, saponins, carotenoids, sesquiterpenoids, cardiac glycosides and alkaloids were carried out according to known and standard methods.

Tannins were estimated using the Folin-Denis spectrophotometric method (Pearson, 1976). Saponin content was determined using the method of Birk et al. (1963) as modified by Hudson and El Difrawi (1979). Flavonoids, alkaloids and sesquiterpene lactones were determined by ethyl acetate extraction and gravimetric measurement, the alkaline precipitation and gravimetric method, and the double extraction and gravimetric measurement, respectively as described by Harbone (1973).

2.4 Analysis of Anti-Nutrients

Total oxalate was determined according to the procedure of Day and Underwood (1986). Phytate content was determined using the method described by Reddy and Love (1999). Hydrocyanic acid content was determined

using the alkaline titration method of AOAC (1990).

2.5 Vitamin Analysis

The composition of the water-insoluble vitamins, riboflavin, thiamine and pyridoxine, were determined by the method described by Scalar (2000), while ascorbic acid content was determined by the method of AOAC (1980). Vitamin A concentration was determined by the spectrophotometric method described by Pearson (1976).

2.6 Mineral Analysis

Minerals were determined after the dried powdered samples were first digested with nitric acid and perchloric acid and the filtered aliquots were used for the determination of sodium, potassium, calcium, magnesium, phosphorus, iron, copper, zinc, selenium, chromium, cobalt and manganese content. Potassium and sodium were determined by the Flame photometric method. Iron, copper, zinc, manganese, chromium, cobalt, selenium calcium and magnesium were determined by atomic absorption spectrophotometric method described by James (1995; AOAC, 1990).

2.7 Proximate Analysis

The analysis of the proximate composition of *V. calvaona* leaf was carried out using the official methods of analysis of the Association of Official Analytical Chemists (AOAC, 1984) and the FAO (1986).

2.7.1 Determination of % Moisture Content of *V. calvaona*

The leaf sample was ground to a fine form and mixed well. Two grammes (2 g) of the sample was accurately weighed into a moisture dish (in triplicate). The sample was dried for 24 hrs at 105 ºC. After drying, the samples were removed from the oven (Memmert U.27) and placed in a desiccator to cool to constant weight. The percentage moisture content was calculated as follows:

$$\% \text{ Moisture} = \frac{(Y - Z) \times 100}{X}$$

where, X and Y are the sample weight and the weight of dish + sample, respectively prior to drying, and Z the weight of dish + sample after drying. Y – Z is the loss in weight of sample after drying.

2.7.2 Determination of % Ash Content of *V. calvaona*

Five grammes (5 g) of the leaf sample (fine form) was weighed into a porcelain dish that had previously been weighed. This was dried at 105 ºC for three hours in an oven. The dish with content was transferred to a muffle furnace (Heraeus M 110) and ignited at 500 ºC until free from carbon (residue appears greyish-white). This was removed from the oven and the ash moistened with a few drops of water (to expose bits of unashed carbon). The ash was re-dried in the oven at 100 ºC for 3 hours and re-ashed in the furnace at 500 ºC for another one hour. This was removed from the muffle furnace, placed in a desiccator until it cooled, and was then weighed. The percentage ash was calculated as follows:

$$\% \text{ Ash} = \frac{(Y - Z) \times 100}{X}$$

where,

X = sample weight prior to drying

Y = weight of dish and contents after ashing

Z = weight of empty dish.

2.7.3 Determination of % Crude Protein of *V. calvaona*

Two grammes (2 g) of oven dried ground leaf sample was placed into 30 ml Kjeldahl digestion flask (Gerhardt). Fifteen millilitres (15 ml) of concentrated sulphuric acid and 1 g of catalyst mixture were added into the flask. The flask was gently heated on a digestion rack in a fume cupboard until a greenish clear solution appeared. After about 30 minutes when the digest had cleared, the flask was heated for another 30 minutes and allowed to cool. Ten millilitres (10 ml) of distilled water was added to avoid caking. The sample was transferred to the Kjeldahl apparatus (Gerhardt). A 50 ml receiver flask containing 5 ml boric acid-indicator solution was placed under the condenser of the distillation apparatus so that the tip was about 2 cm inside the solution. To the digested sample in the apparatus was added 10 ml of 40% NaOH solution through funnel stopcock. Distillation commenced immediately by closing the steam by-pass and opening the inlet stopcock on the steam jet arm of the distillation apparatus. When the distillate reached the 35 ml mark on the receiver flask, distillation was stopped

by closing inlet stopcock first, then opening the steam by-pass. The condenser tip was rinsed with distilled water. The excess acid was titrated to first pink colour with 0.1N NaOH. The percentage crude protein was calculated as follows:

$$\%\text{Crude protein} = \frac{\text{Titre} \times 14.01 \times \text{Normality of the acid} \times 100 \times 6.25}{1000 \times \text{weight of samp}}$$

Where, 6.25 is a general factor suitable for products in which the portion of specific protein is not well defined. 14.01/1000 is a constant and titre is the volume after titration.

2.7.4 Determination of % Crude Fat (Ether Extract) Content of *V. calvaona*

Five grammes (5 g) of the ground leaf sample was placed in a thimble lined with a circle of filter paper. The thimble with its contents was placed in a 50 ml beaker and dried in an oven for 6 hours at105 ºC .The thimble with its contents was transferred to a Soxhlet extractor. The beaker was rinsed three times with ethyl ether and emptied into the Soxhlet extraction flask. The sample contained in the thimble was extracted with ethyl ether for 6 to 8 hours at a condensation rate of 3 - 6 drops per second. At the completion of the extraction, the fat extract was transferred from the extraction flask into a pre-weighed evaporating dish with several rinsing of ethyl ether. The evaporating dish was placed in a fumehood with the fan on, to evaporate the ethyl ether until no odour was detectable. The dish with its contents was dried in an oven for 30 minutes at 105 ºC, removed from the oven, cooled in a desiccator to constant weight and weighed. The percentage crude fat was calculated as follows:

$$\%\text{Crude fat } \left(\text{ether extract}\right) = \frac{\text{W2-W1} \times 100}{S}$$

where:

W1 = weight of empty evaporating dish

W2 = weight of evaporating dish + content after drying.

S = sample weight before drying.

2.7.5 Determination of %Crude Fibre Content of *V. calvaona*

Five grammes (5 g) of ground sample was weighed and placed in a 1 litre conical flask. A 150 ml pre-heated 0.128 M H_2SO_4 was added and the content boiled for 30 minutes. The content was filtered through fluted funnel and the residue washed three times with hot water. To the digest was added 150 ml preheated 0.15 M KOH and heated to boiling. Some drops of antifoaming agent (n-octanol) was added and the content boiled slowly for 30 minutes, filtered and the residue washed three times with hot water, followed by washing three times with acetone in Cold Extraction Unit (Tecator1615). The resulting residue was dried in the oven at 130 ºC for 1 hr, cooled in a desiccators and weighed, and then ashed at 500 ºC for 30 minutes, cooled in a desiccator and later weighed. The percentage crude fibre was calculated as follows:

where:

$$\%\text{crude fibre} = \frac{\text{W2-W1} \times 100}{\text{W1}}$$

W1 = Sample weight before drying

W2 = Weight of residue after drying

W3 = Weight of residue after ashing

2.7.6 Determination of Nitrogen-Free Extract Content of *V. calvaona*

The nitrogen-free extract of leaf sample was determined by summing up the percentages of moisture, ash, crude protein, fat (ether extract) and crude fibre, and subtracting from 100 (Mcdonald et al., 1973). The difference in value was designated the nitrogen-free extract.

2.7.7 Determination of Carbohydrate Content of *V. calvaona*

The percentage carbohydrate content of the leaf sample was determined by summing up the percentages of moisture, ash, crude protein, fat (ether extract) and subtracting from 100 (Mcdonald et al., 1973). The difference in value was taken as the percentage total carbohydrate content of the leaf sample. The total carbohydrate of the leaf sample is contained in two fractions, the crude fibre and the nitrogen–free extract.

2.7.8 Energy Value

This was calculated in (KJ/100g) using the equation: [(37 x fat) + (17 x carbohydrate) + (17 x protein).

2.8 Statistical Analysis

All determinations were done in triplicates and data were expressed as Mean ± SEM. These data were subjected to analysis of variance (ANOVA).

3. Results

The results of the phytochemical composition, proximate analysis, anti-nutrients, mineral elements and vitamin composition of the vegetable sample are shown in Tables 1-5.

3.1 Phytochemical Composition

Table 1 shows the types and quantities of secondary compounds of metabolism present in the leaf sample analyzed. Flavonoids were shown to be present in appreciable amounts, followed by phenolic compounds and saponins. This suggests that *V. calvaona* is a strongly antioxidative plant.

Table 1. Phytochemical composition of *Vernonia calvaona* leaves (% W/W DMB)

Constituent	Mean	SEM
Tannins	0.67	±0.03
Flavonoids	7.07	±0.43
Alkaloids	1.26	±0.13
Sesquiterpene lactones	1.64	±0.13
Phenolic Compounds	3.19	±0.05
Steroidal Saponins	4.42	±0.23
Cardiac glycosides	1.40	±0.17
Carotenoids	1.62	±0.11

Values are mean ± SEM of three determinations. *DMB = dry matter basis.

3.2 Proximate Composition

Table 2 shows the proximate composition of the leaf sample. The carbohydrate, protein and moisture content occured in appreciable amounts, suggesting that the leaf is a food material. The fibre and ash contents were also high and suggests the high nutritive value of *V. calvaona*.

Table 2. Proximate composition of Fresh leaves of *Vernonia calvaona* (mg/100 g)

Constituent	Mean	SEM
Total Protein	19.80	±0.61
Total Fat	4.17	±0.15
Total Fatty acid content	3.57	± 0.52
Carbohydrate	20.80	± 0.67
Reducing Sugar	8.56	±0.06
Crude Fibre	7.63	±0.22
Ash Content	10.67	±0.33
Moisture content	37.67	±0.33
Energy (KJ/100g)	844.49	±6.19

Values are mean ± SEM of three determinations.

3.3 Anti-Nutrient Composition

Table 3 shows the anti-nutrient composition of the leaf sample. All the three major anti-nutrients (oxalates, phytates and cyanates) gave low and non-toxic values with respect to known vegetables in human nutrition.

Table 3. Antinutrient composition of fresh Leaves of *Vernonia calvaona* (mg/100 g)

Constituent	Mean	SEM
Oxalates	0.34	± 0.04
Phytates	0.94	± 0.04
Cyanates	0.09	± 0.01

Values are mean ± SEM of three determinations.

3.4 Mineral Profile

The mineral composition of the plant sample is presented in Table 4. Iron, sodium, potassium and calcium were present in appreciable quantities. Generally, the plant is rich in vital mineral elements, confirming its use as a multi-purpose plant in nutrition and ethno-medicine.

Table 4. Mineral Composition of fresh leaves of *Vernonia calvaona* (%W/W DMB)

Constituent	Mean	SEM
Fe	1.11	±0.07
Zn	0.54	±0.01
Co	0.40	± 0.01
Cu	0.39	± 0.03
Cr	0.63	±0.05
Na	1.08	±0.09
Mg	0.85	±0.07
Mn	0.25	±0.08
K	2.46	±0.12
Ca	1.04	±0.22
P	0.68	±0.02
Se	0.06	±0.01

Values are mean ± SEM of three determinations.

3.5 Vitamin Composition

Table 5 shows the results of the vitamin composition of the leaf sample of *V. calvaona*. Vitamin C occurred highest followed by vitamins E, B_1 and A respectively. The presence of vitamins A, C and E supports the potential value of this vegetable in anti-oxidation function.

Table 5. Vitamin Composition of fresh leaves of *Vernonia calvaona* (mg/100 g)

Constituent	Mean	SEM
Vitamin C	11.33	±0.88
Vitamin A	0.61	±0.01
Vitamin E	0.99	± 0.13
Vitamin B_1	0.94	± 0.03
Vitamin B_2	0.16	±0.01
Vitamin B_6	0.56	±0.33
Folic Acid	0.26	±0.05
Niacin	0.34	±0.02

Values are mean ± SEM of three determinations.

4. Discussion

4.1 Phytochemicals

The presence of flavonoids in appreciable amount (7.07 ± 0.43 %), inferred that the vegetable has the biological functions such as anti-oxidation, and protection against allergies, inflammation, free radical, platelet aggregation, microbes, ulcers, hepatoxins, viruses and tumour (Okwu, 2004; Farquar, 1996). Flavonoids are potent water soluble antioxidants and free radical scavengers which prevent oxidative cell damage, and have strong anticancer and anti-ulcer activity and protection against the different levels of carcinogenesis (Okwu, 2004). Steroidal saponin content of 4.42±0.23% suggests the usefulness of the vegetable as a potential fertility agent. The saponin level is however low, when compared with the results from other works (Umaru et al., 2007; Ekop, 2007; Nkafamiya et al., 2007; Nkafamiya et al., 2010). Steroidal saponins at low levels < 10% are said to be safe and non-toxic. Saponins are glycosides containing polycyclic aglycone moiety of either C27 steroid or C30 triterpenoids attached to a carbohydrate sugar. High Saponin levels have been associated with gastroenteritsis, manifested by diarrhea and dysentery (Awe & Sodipo, 2001). The presence of appreciable amounts of sesquiterpene lactones (1.64 ± 0.13%), and carotenoids (1.62 ± 0.11%), suggests that the plant may be useful as an anticancer and anti-ulcer agent, a claim that seem to support the traditional use the leaves for ethno-medical purposes.

Alkaloids were present in appreciable amount (1.26 ± 0.13%). Alkaloids are one of the most efficient therapeutically significant bioactive substances in plants. Pure isolated alkaloids and the synthetic derivatives are used as basic medicinal agents because of their analgesic, antispasmodic and bactericidal properties (Stray, 1998).

The low tannin (0.67 ± 0.03%) content in the vegetable implies that the leaf has little or no astringent properties. Tannins quicken the healing of wounds and inflamed mucous membranes (Farquar, 1996). Tannins are water soluble phenolic compounds which precipitate proteins from aqueous solution. They occur in all vascular plants. Tannins bind to proteins making them bio-unavailable (Bagepallis et al., 1993; Aleto, 1993; Sotel et al., 1995). The value in this vegetable is low compared with the findings from other plants (Chinma & Igyor 2007; Ekop, 2007; Abidemi et al., 2009; Amoo et al., 2009; Nkafamiya et al., 2007).

4.2 Proximate Composition

The carbohydrate content was high (20.80 ± 0.67%) suggesting that the vegetable can serve as food. The monosaccharide or disaccharide composition of the carbohydrate may be responsible for the after-sweet-taste of the leaf. The moisture content was also high (37.67 ± 0.33%) indicating that the vegetable is susceptible to spoilage. The high ash content (10.67 ± 0.33%) is an indication of the level of inorganic elements such as calcium, zinc, magnesium, copper, and potassium in the vegetable.

The protein content was also high (19.80 ± 0.61%) and readily available as a macronutrient. Protein is an essential component of human diet needed for the replacement of tissues and for the supply of energy and adequate amount of required amino acids. Protein deficiency causes growth retardation, muscle wasting, oedema, abnormal swelling of the belly and collection of fluids in the body of children (Mounts, 2000). The crude fibre content (7.63 ± 0.22%) was high and may aid digestion, absorption of water from the body and bulk stool. Fibre softens stool and therefore, prevents constipation (Ayoola & Adeyeye, 2009). The vegetable may therefore be very useful in the control of body weight, blood cholesterol and protection against colon cancer. The fat content (4.17 ± 0.15%) of the vegetable was low, and it can therefore be recommended as part of weight reducing diets. Low fat foods are said to reduce the level of cholesterol and obesity (Gordon & Kessel, 2002). The low fat content correlates directly with the low total fatty acid content (3.57 ± 0.52 mg/100 g) in the plant. The metabolising energy content of *V. calvaona* was calculated to be 844.49 ± 6.19 KJ.

4.3 Mineral Composition

Of the minerals analyzed in the vegetable, potassium was the most abundant (2.46 ± 0.12 mg/100 g) element, and this is in agreement with many reports that potassium is the most abundant mineral in Nigerian agricultural products (Afolabi et al., 1995). Potassium helps to maintain body weight and regulate water and electrolyte balance in the blood and tissues (National Research Council [NRC], 1989). The calcium content was determined to be 1.04 ± 0.22 mg/100g. Calcium helps in the regulation of muscle contraction required by children, infants and foetuses for bones and teeth development (Margaret & Vickery, 1997). The concentration of sodium in the sample was also low (1.08 ± 0.09 mg/100 g), and supports the claim by the natives that the vegetable is useful in the treatment of heart related diseases. Excess sodium consumption leads to hypertension (NRC, 1989). The phosphorus content of the vegetable was 0.68 ± 0.06 mg/100 g. This figure is lower than that reported on other

vegetables. Phosphorus plays a vital role in normal kidney functioning and transfer of nerve impulse. The concentration of zinc in the vegetable was given as 0.54 ± 0.01 mg/100 g. Zinc is said to be an essential trace element for protein and nucleic acid synthesis and normal body development (Melaku, 2005). Zinc also stimulates the activity of vitamins, and the formation of red and white blood cells (Claude & Paule, 1979). Zinc plays a role in improving male fertility. The iron content of the vegetable was given as 1.11±0.07 mg/100g, and compares favourably with other vegetables. Iron is said to be an important element in the diet of pregnant women, nursing mothers, infants, convalescing patients and the elderly to prevent anaemia and other related diseases (Oluyemi et al., 2006). The magnesium content of the leaf was found to be 0.85 ± 0.07 mg/100 g. Magnesium plays fundamental roles in most reactions involving phosphate transfer. It is believed to be essential in the structural stability of nucleic acids. It plays a significant role in the intestinal absorption of electrolyte in the body. Its deficiency in man includes severe diarrhoea and persistent migraines (Appel, 1999). Cobalt occurred at a concentration of 0.40 ± 0.01 mg/100 g in this plant, and this value is well below the concentration that is said to be critical (1.5-5.0 mg/100 g) in plant materials (Miroslav & Vladimir, 1999). Cobalt plays an important role as an activating ion in some enzyme reactions (McDonald et al., 1995). The concentration of manganese in the plant sample was determined to be 0.25 ± 0.08 mg/100 g. This compared closely to that of *Nuclea latifolia* 0.21 mg/100 g (Hassan et al., 2004). Consumption of manganese-containing foods is believed to support the immune system. Manganese regulates blood sugar levels, the production of energy and cell reproduction. Deficiency in manganese may result in birth defects if an expectant mother does not get enough of this important element (Anhwange et al., 2004).

4.4 Vitamin Composirion

The vitamin profile of *V. calvaona* is shown in Table 5. Vitamin C was the most abundant element (11.33 ± 0.88 mg/100 g) followed by Vitamin E (0.99 ± 0.13 mg/100 g), Vitamin B_1 (0.94 ± 0.03 mg/100 g), Vitamin A (0.61 ± 0.01 mg/100g) and Vitamin B_6 (0.56 ± 0.33 mg/100 g), in that order. Vitamins A promotes growth, resistance to diseases and delays ageing. It also promotes the health of the eyes, skin, nails and hair (Claude & Paule, 1979). Vitamin B_2 carries oxygen to the cells, while Vitamin B_6 promotes the metabolism of protides and non-saturated fatty acids. Vitamin C (ascorbic acid) promotes the health of teeth and gums, lungs and bronchia, and joints. Vitamin C also aids the purification o blood. Deficiency in ascorbic acid is associated with pains in the joints and defects in skeletal calcification, anaemia, manifestation of scurvy, and haemorrhage from the mucous membranes of the mouth and gastro intestinal tract (Hunt et al., 1980). The presence of ascorbic acid in the vegetable sample suggests that its consumption and use in herbal medicine can prevent against common cold and other diseases like prostate cancer (Okwu, 2004). Folic acid was determined to be 0.26 ± 0.05 mg/100 g and it is essential in preventing birth defects. There is evidence that folic acid may prevent heart disease in both men and women of over 50 years of age. Niacin or nicotinamide is a coenzyme, which is involved in many biochemical processes including the detoxification of foreign compounds in the body (Njoku & Akumefula, 2007).

4.5 Anti-Nutrients

Antinutritive factors limit the use of many plants for food because they elicit deleterious effects in both man and animals (Kubmarawa et al., 2008). Fortunately, the levels of anti-nutrients in this plant were found to be low compared to other vegetables in Nigeria (Agbaire et al., 2012). Oxalate content (0.34 ± 0.04 mg/100 g) found in this study was low and is below the established toxic level. Oxalate tends to render calcium unavailable by binding to plasma calcium ion to form complexes (Al-Rais et al., 1971; Ladeji, 2004, Nkafamiya et al., 2006). The insoluble calcium oxalate complex may precipitate around soft tissues like the kidney, causing kidney stones (Oke, 1969). The phytate value recorded was low (0.94 ± 0.04 mg /100 g) and non-toxic. According to Oke (1969) a phytate diet of 1-6% over a long period of time decreases the bioavailability of mineral elements in mono gastric animals. Phytic acid is a strong chelator, forming protein and mineral-phytic acid complexes thereby decreasing protein and mineral bioavailability (Fasusi et al., 2003; Erdman, 1979). Phytate is associated with nutritional diseases such as rickets in children and osteomalacia in adult humans respectively. The cyanate level in the vegetable sample was also found to be low (0.09 ± 0.01 mg/100 g) and non-toxic to humans and animals. It has been established that excess cyanate in the body inhibits the cytochrome oxidase. This may stop ATP formation and the release of inorganic phosphate to body tissues. Consequently, the body suffers energy deprivation and subsequent death. High level of HCN has been implicated in cerebral damage and lethargy in man and animal.

5. Conclusion

This study has shown the proximate, phytochemical, mineral and vitamin compositions of *Vernonia calvaona* as a balanced and rich source of macro- and micronutrients. The phytochemical profile shows the potential

medicinal usefulness of the plant as an agent capable of ameliorating a myriad of diseases, including diabetes, malarial and cardiovascular problems. The leaf can be seen as a potential source of useful items for food and drugs formulation. Further research work is ongoing to confirm some of the ethno-pharmacological claims on *Vernonia calvaona*.

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Influence of Fat and Moisture Content in the Processing of Light *Requeijão*

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Abstract

The objective of this study was to evaluate the influence of fat and moisture contents in the sensory characteristics and acceptance of light *requeijão*. The experiment followed the Central Composite Rotatable design. Formulations were characterized by Conventional Profile and the acceptability was evaluated by 100 consumers. The results were analyzed by Analysis of Variance, fitting of regression models and Preference Map. The multiple linear regression model showed the best fit for all sensorial attributes. *Requeijão* samples that fat reduction was coupled with increasing moisture demonstrated intermediate intensity of the sensory properties and showed the best acceptance among consumers. Such a result indicated that the reduction in fat levels should be associated to the increase in moisture of the final product so that there is equilibrium in the sensorial properties and the optimization the acceptance of the consumers.

Keywords: descriptive analysis, defatted dry extract, consumers

1. Introduction

Cheese processing technology initiated at the beginning of the 20th century with the need to inhibit microbial and enzymatic processes of Swiss and German cheeses in order to allow for exportation to countries with hot climates. In 1911, Geber and Steller were able to solubilize calcium paracaseinate from raw material by means of heat, utilizing sodium citrate as a fluxing agent, thus obtaining what was referred to as processed cheese. The sodium salt, under constant agitation and heat, promotes internal exchange of ions, transforming the calcium paracaseinate, of unstable hydration, in sodium paracaseinate, whose solution is colloidal and stable (Garrutti, Brito, Brandão, Uchôa & Silva, 2003; Silva et al., 2012c).

Requeijão cremoso is a type of processed Brazilian cheese, fabricated throughout the entire Brazilian territory with some technology variations and is known for presenting an elevated fat content (Gallina, Van Dender, Yotsuyanagi, & Rodrigues De Sá, 2008; Gomes & Penna, 2010).The processed cheese matrix is formed of a continuous protein network, in which the fat and aqueous phases are dispersed (Udyarajan, Horne, & Lucey, 2007). Food products with reduced fat are becoming more and more common. Consumers in general have acquired a greater understanding of the relationship between the diet and health, and are becoming more receptive to products with low levels of fat (Romeih, Michaelidou, Biliaderis & Zerfiridis, 2002; Silva et al., 2012a). However, because fat plays an important role in the flavor, texture and appearance of foods, development of products with reduced fat but with the same sensorial quality as the conventional version is one of the large challenges faced by the food industry (Romeih et al., 2002; Drake & Swanson, 1995; Guinee & O'Callaghan, 2013).

The reduction of fat in *requeijão* cremoso promotes an increase in the percent of dry defatted extract (DDE), which favors protein-protein interactions and makes the casein matrix excessively rigid, resulting in a product with a firm texture (Metzger & Mistry, 1995; Sheehan & Guinee, 2004; Guinee & O'Callaghan, 2013). Various approaches have been used to counteract the adverse effects of fat reduction of cheese. These include homogenization of cheese milk to increase the surface area of the fat phase (Poudaval & Mistry, 1999); the addition of fat replacers to the cheese milk (Rudan, Barbano, Yun, & Kindstedt, 1999), the addition of exopolysaccharide-producing cultures (Perry, Mcmahon, & Oberg, 1998); and/or alterations of the production procedure to decrease the levels of DDE (Metzger, Barbano, Kindstedt, & Guo, 2001). These methods have

resulted in varying degrees of success.

Zalazar et al. (2002) evaluated Argentinian cream cheese with high moisture content and with the additional fat replaced with a protein (Dairy-Lo). In the results, the authors report that the cheeses with reduced fat were a desirable end product. Increasing the moisture content of the final product is presented as a simple strategy to reduce the content of defatted dry extract and restore the quality lost by reducing fat.

In this context, the objective of the present study was to evaluate the influence of the fat and moisture contents on the sensorial properties and acceptability of light *requeijão*.

2. Material and Methods

2.1 Samples

To define the formulations the Central Composite Rotatable Design (CCRD) was used with two factors (fat (F) and moisture (M)) at two levels (2^2), plus four axial points (2 x 2) and the central point. The central point was repeated three times to estimate the pure error, totaling 11 tests (22 + 2 x 2 + 3) (Figure 1).

The fat levels under study were defined so that the formulation with the greatest percentage of fat (18.7%) met the minimal reduction (25%) demanded by legislation to be considered as light (Brasil, 1998). The minimum fat concentration (8.7%) and the high and low moisture concentrations (71.0% and 64.0%) were defined by preliminary testing. The other levels of fat and moisture were stipulated by the statistical design, so that all points were encountered at a distance of α ($\sqrt{2}$) from the central point (CP).

The *requeijão* samples were processed using a mass obtained by direct hot acidification of milk at 70 °C, in accordance with the technology described by Alves, Van Dender, Jaime, Moreno and Pereira (2007). The products were composed of mass (raw skim milk, lactic acid 85% PA), cream (Funarbe), water, whey protein concentrate (2.0% - WPC 34% - Gemacom), NaCl (0.80% - Cisne), emulsifying salts (0.70% - Joha S9) and the preservatives potassium sorbate (0.020% - Gemacom) and nisin (0.017% - Danisco). Processing was conducted in an open pan with mechanical agitation at 50 rpm.

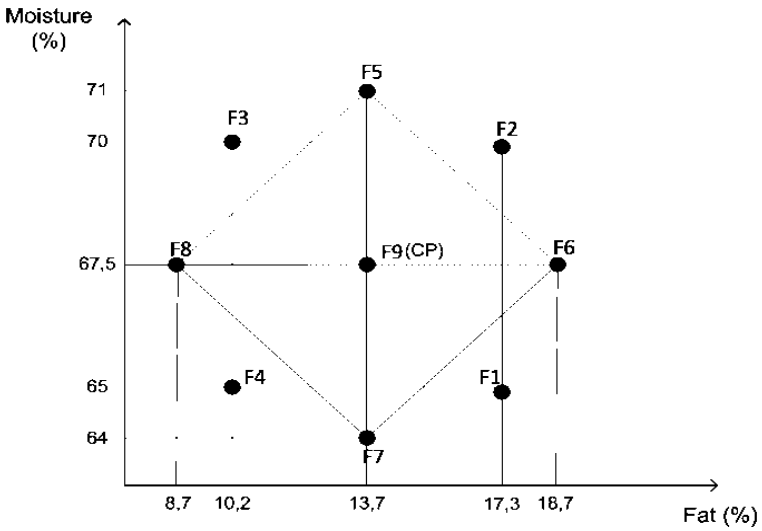

Figure 1. Disposition of the experimental points in the central composite rotatable design (CCRD)

2.2 Centesimal Composition Analysis

The determinations of fat, moisture, proteins and the fixed mineral residue contents of the *requeijão* samples were performed according to the methods described in Brasil (2005). Analyses for the determination of the centesimal composition of the *requeijão* samples were performed in duplicate for each repetition.

The level of carbohydrates and DDE were obtained by the indirect method, as recommended in Brasil (2005). The carbohydrate content was calculated as the percentage difference between the sum of the total content and other nutrients (%Carbohydrate = 100% – [%Protein + %Fat + %Fixed mineral residue + %Moisture]). The level of DDE was calculated as the percentage difference between the total content and the percentage of fat and

moisture formulations (%DDE = %Total - %Fat - %Moisture).

The caloric content was calculated as a function of the composition of the *requeijão* samples in terms of protein, fat and carbohydrates. The conversion factors of 4, 9 and 4 $Kcal.g^{-1}$ of food were used for each nutrient, respectively (United States Department of Agriculture, 1963).

To verify that the levels of fat and moisture from the processed *requeijões* corresponded to the levels set by the experimental design (DCCR), a t-test for independent samples was performed to compare the theoretical value and the value obtained in practice (determining the chemical composition).

2.3 Sensory Profile: Conventional Profile (CP)

To characterize the sensory properties of the *requeijões* was utilized the Conventional Profile, described in Silva et al. (2012b, 2013).

2.3.1 Recruitment of the Evaluator Candidates

Fifty recruitment questionnaires were distributed to those who demonstrated an interest in participating in the sensory tests. Those recruited for pre-selection presented availability, affinity for the product under evaluation, understanding of the descriptive terms, health conditions that would not compromise the analyses and the ability to work with an unstructured scale. The ability to work with unstructured scales was evaluated by means of tests utilizing figures with different levels of filling, as proposed by Meilgaard, Civille and Carr (2006). A maximum variation of 10% from the correct value was accepted.

2.3.2 Pre-Selection

To evaluate the sensory discernment capacity of the candidates, a series of four global differential tests (triangular test) was applied. The criterion for selection was to assert 75% of the tests, as recommended by Meilgaard et al. (2006). The local commercial *requeijão* brand (R_C) and *requeijão* diluted with 10% skim milk (R_{CD}) were used to construct a series of differentiation tests. In preliminary tests, it was found that these products presented a significant difference ($p < 0.05$) among themselves.

2.3.3 Determination of the Descriptive Terminology and Definition of the Reference Material

Development of the descriptive terminology was performed by fourteen pre-selected judges. The previous list technique was used to provide support to the sensory attributes acquisition, as recommended by Damásio and Costell (1991). Thus, open discussion under the supervision of a moderator was used to determine the attributes that characterized the samples, utilizing the previous list of descriptive terms for *requeijão* produced by Garruti et al. (2003). The judges were divided into two groups for the open session with the objective of facilitating discussion and eliciting opinions. The nine formulations were presented together with the list of descriptive terms, and the judges were asked to formulate a list of the descriptive terms that would characterize the products in relation to their appearance, aroma, flavor and texture. The attributes which characterized the *requeijão* samples were: characteristic color, consistency, spreadability, characteristic aroma, characteristic flavor, viscosity and adhesivity.

In a subsequent session, the judges defined each sensory term and the reference materials representing the extremes on the 9-centimeter unstructured scale (Table 1). The judges were also asked to complete an evaluation chart that contained an unstructured 9-centimeter scale associated with each sensory attribute and anchored at the extremities (weak or strong) in relation to the following attributes: characteristic color, consistency, spreadability, characteristic aroma, characteristic flavor, viscosity and adhesibility. Reference materials for the attributes of appearance (except for color) and texture were products with an instrumental firmness of 0.02 N (weak) and 2.34 N (strong). Regarding the attributes of color, aroma and flavor, formulations with combinations of fat and moisture were used, to allow for the acquisition of different sensitivity intensities of the formulations under study.

2.3.4 Training

Seventeen training sessions were held, which were performed in 2 months. In this stage, the judges were instructed to read the definitions of the attributes and subsequently to sample and memorize the reference materials for each sensory attribute. The method of manipulating the samples was also standardized to prevent errors in the interpretation of sensory attributes. Therefore, the judges were clearly exposed to the sensory stimulus for each attribute, clarifying "what" and "how" the sensory stimulus should be evaluated. During the training sessions, seven practical sessions were also held in which panelists were asked to rate the *requeijão* samples using an unstructured 9-centimeter scale. Training was terminated when the judges determined there were no difficulties in evaluating the samples.

Table 1. Sensory attributes determined by the panel of evaluators; their respective definitions and standards define the extremes on the non-structured scale

ATTRIBUTES	DEFINITIONS	REFERENCE MATERIALS
APPEARANCE		
Characteristic color	Color of, varying from whitish-yellow to clear yellow.	**Weak:**cheesecurd containing 17.3%F and 70%M **Strong:**cheesecurd containing 18.7%F and 67.5%M
Consistency	Force necessary to spread the product with a spoon.	**Weak:**cheesecurd containing 17.3%F and 70%M **Strong:**cheesecurd containing 8.7%F and 67.5%M
Spreadability	Ability to spread the cheesecurd on a cracker with a spoon.	**Weak:**cheesecurd containing 8.7%F and 67.5%M **Strong:**cheesecurd containing 17.3%F and 65%M
AROMA		
Characteristic aroma	Aroma of cheesecurd.	**Weak:**cheesecurd containing 8.7%F and 67.5%M **Strong:**cheesecurd containing 18.7%F and 67.5%M
FLAVOR		
Characteristicflavor	Flavor of cheesecurd.	**Weak:**cheesecurd containing 8.7%F and 67.5%M **Strong:**cheesecurd containing 18.7%F and 67.5%M
TEXTURE		
Viscosity	Force necessary to pull the product from a spoon to the mouth.	**Weak:**cheesecurd containing 17.3%F and 70%M **Strong:**cheesecurd containing 8.7%F and 67.5%M
Adhesivity	Force necessary to remove the product which adheres to the palate.	**Weak:**cheesecurd containing 17.3%F and 70%M **Strong:**cheesecurd containing 8.7%F and 67.5%M

F: fat content; M: moisture content.

2.3.5 Evaluation of Performance of the Judges

A preliminary test was performed to verify that the judges were adequately trained. For this purpose, the final evaluation test of the products was simulated, utilizing the evaluation form containing all attributes and the unstructured 9-centimeter scale. The *requeijões* F2 (17.3% F and 70.0% M) and F4 (10.2% F and 65.0% M), encoded with three randomized digits, were presented to the judges according to the Balanced Block Design (BBD) design with four repetitions, with each judge considered as a block. Samples were presented to the judges at the same time, in each repetition. The products were evaluated at a temperature of 7 ± 1 ºC.

To evaluate the discriminatory potential and reproducibility of the results, analyses of variance (ANOVA) were performed with two sources of variation (sample and repetition) per attribute for each judge. The judges that presented a maximal probability of 30% for F_{SAMPLE} ($F_{SAMPLE} < 0.30$) and a minimal probability of 5% for $F_{REPETITION}$ ($F_{REPETITION} > 0.05$) for all attributes were selected for the final stage of the CP. The selection parameters (F_{SAMPLE} and $F_{REPETITION}$) utilized were more rigorous than those proposed by Damásio and Costell (1991). To evaluate the agreement of a judge with the team, the average individual score was compared with the average score of the team, as performed by Richter, Almeida, Prudencio and Benassi (2010). Of the fourteen judges, nine were selected to make up the sensorial team by presenting satisfactory selection parameters.

2.3.6 Final Evaluation of the Products

The samples encoded with three random digits were randomly presented to the nine judges selected utilizing the Balanced Block Design (BBD) design; i.e., each judge evaluated all formulations. Repetitions of the central point were used to estimate the pure error. The samples were served at 7 ± 1 ºC, and the judges received the evaluation form containing all sensory attributes and the nine centimeter scale (serial protocol), as well as the list of definitions of the sensory attributes.

The effect of fat and moisture contents on the sensory properties of the *requeijões* was analyzed by analysis of variance (F test), Principal Component Analysis (PCA) and adjusted regression models. The analysis of variance

with two sources of variation (sample and judge) and the interaction of sample*judge was performed for each attribute separately. To select the best model fit to the data, we assessed the following parameters: no significant lack of fit ($p > 0.10$), significance of regression coefficients ($p < 0.10$) and, finally, the explanation of model by the coefficient of determination R^2.

2.4 Sensorial Acceptability

On the campus of the Universidade Federal de Viçosa, 100 consumers of *requeijão* were recruited. The *requeijão* formulations were served to the consumers both randomly and monadically, in disposable cups containing approximately 10 g of the product. A nine point hedonic scale was used, varying from "extremely liked" (score 9) to "extremely disliked" (score 1) so that the panelists could express acceptance in relation to texture of the products (Meilgaard et al., 2006).

The results of acceptance were analyzed by the Internal Preference Map, where the data acceptance tests (consumer) were organized in a matrix of samples (in rows) and consumers (in columns), and were subject to Principal Components Analysis (PCA), as described in Minim (2010). In addition, Response Surface Analysis (RSA) was used to verify the behavior depending on the sensory acceptance of fat and moisture contents.

2.5 Statistical Analyses

Statistical analyses were performed in SAS (Statistical Analysis System), licensed for use at Universidade Federal de Viçosa in 2013.

2.6 Ethics Committee

This project was analyzed and approved by the Scientific Committee of the Postgraduate Department of Food Technology, Federal University of Viçosa, process nº50717258524/2009, obeying, as outlined, the necessary requirements for its publication.

3. Results and Discussion

3.1 Centesimal Composition of the Light Requeijão Samples

The obtained values of fat and moisture contents of the processed *requeijão* cheeses are shown in Table 2. Theoretical and experimental levels of fat and moisture in the *requeijão* samples did not differ significantly by the t-test, and presented p-values of 0.8670 and 0.7887, respectively. This result indicates that the processed formulations met the requirements stipulated by the design.

Table 2. Centesimal composition of the light *requeijão* samples with the addition of WPC

Formulations	CONSTITUENTS (%)						DDE	CaloricValue
	Moisture	Fat	Protein	Residue Mineral Fixed		Carbohydrate	(%)	(Kcal)
F1	64.90 ± 0.10	16.50 ± 0.20	14.02 ± 0.25	3.14 ± 0.09		1.44 ± 0.14	18.60 ± 0.30	210.34
F2	69.75 ± 0.10	16.40 ± 0.10	10.01 ± 0.20	2.69 ± 0.18		1.15 ± 0.02	13.85 ± 0.00	192.24
F3	69.75 ± 0.10	10.50 ± 0.13	14.35 ± 0.25	3.28 ± 0.46		2.12 ± 0.68	19.75 ± 0.03	165.02
F4	65.47 ± 0.10	11.50 ± 0.32	18.14 ± 0.04	3.19 ± 0.11		1.70 ± 0.28	23.03 ± 0.22	182.86
F5	71.02 ± 0.10	13.00 ± 0.18	12.07 ± 0.30	2.65 ± 0.01		1.26 ± 0.01	15.98 ± 0.28	170.32
F6	67.20 ± 0.00	18.50 ± 0.53	10.54 ± 0.09	2.51 ± 0.02		1.26 ± 0.45	14.31 ± 0.52	213.7
F7	63.50 ± 0.10	13.10 ± 0.08	18.46 ± 0.25	2.96 ± 0.01		1.98 ± 0.06	23.40 ± 0.18	199.66
F8	66.67 ± 0.05	8.45 ± 0.13	19.40 ± 0.17	3.40 ± 0.29		2.08 ± 0.28	24.88 ± 0.18	161.97
F9 R1 (CP)	67.15 ± 0.00	13.60 ± 0.15	15.09 ± 0.20	2.80 ± 0.00		1.37 ± 0.05	19.26 ± 0.15	188.24
F9 R2 (CP)	67.02 ± 0.10	13.75 ± 0.20	15.01 ± 0.05	2.88 ± 0.07		1.34 ± 0.28	19.23 ± 0.30	189.15
F9 R3 (CP)	67.23 ± 0.24	13.20 ± 0.18	15.49 ± 0.24	2.79 ± 0.08		1.29 ± 0.20	19.57 ± 0.26	185.92

F1: 17.3%F e 65% M; **F2:** 17.3%F e 70%M; **F3:** 10.2%F e 70%M**; F4:** 10.2%F e 65%M; **F5:** 13.7%F e 71%M; **F6:** 18.7%F e 67.5%M; **F7:** 13.7%F e 64%M; **F8:** 8.7%F e 67.5%M; **F9 (central point):** 13.7%F e 67.5%M. **%DDE (DeffatedDryExtract):** 100 - %Fat - %Moisture. Standard deviation calculated according to the replicates.

The different combinations of fat and moisture produced *requeijão* samples with different defatted dry extracts (DDE), due to the fact that this constituent (DDE) is calculated by the percent difference between the total content and levels of fat and moisture. Therefore, the reduction in fat and moisture contents resulted in an increase in protein levels (casein) of the *requeijão* samples. Similar results were encountered by Soares et al. (2002) and by Cunha, Viotto and Viotto (2006) when determining the centesimal composition of low fat cheeses.

Thus, *requeijão* samples F2 and F3 presented an increase of 13.85% to 19.76% in DDE when the fat level was reduced from 16.4% to 10.5%. The same behavior was verified for samples F6, F9 and F8 (approximately 67.5% moisture) and also for formulations F1 and F4 (65% M).The moisture content also caused changes in the DDE of the *requeijão*. When comparing the formulations within the same level of fat reduction, it was found that the DDE in formulations F1 and F2 increased from 13.85% to 18.06% when the content moisture decreased from 69.75% to 64.90%. The same was observed for formulations F5, F7 and F9 (13.7% F) and for F3 and F4 (10.2% F).

Therefore, it was identified that the levels of fat and moisture inversely influenced the percentage of DDE in the *requeijão* samples, being that products with different combinations of fat and water (F1, F3 and F9) presented similar DDE levels (approximately 19.30%) due to compensation of water in the DDE content generated by the reduction in fat.

Formulations in which fat reduction did not accompany the increase in water content (F4, F7 and F8) presented elevated protein contents (approximately 23.8% DDE). However the *requeijão* samples containing greater percentages of fat and also presented elevated water contents (F2, F5 and F6) presented low DDE levels (approximately 14.70%).

In regards to the caloric value, samples with less fat and greater water concentrations (F3) presented less energy than the other formulations, which is very interesting for industry since this product can be produced for less money (less utilization of cream and mass) and provides a low caloric content. It also presents the marketing appeal that this *requeijão* has a 65% reduction in fat content of the traditional product (approximately 25% F).

3.2 Sensory Characterization of Light requeijão: Conventional Profile

Nine panelists were selected to make up a sensorial team, who presented the ability of discrimination and repeatability of results, as recommended by Damásio and Costell (1991). The seven attributes evaluated showed to have significant effect ($p < 0.001$) on the sample*panelist interaction. Therefore, the test for the effect of the samples was performed again, using the mean square of interaction as the denominator for all sensorial attributes. The *requeijão* samples differed among themselves ($p < 0.001$) by the F test (versus interaction) in all attributes evaluated.

In the Principal Component Analysis (PCA), Figure 2, the first principal component explained 91.96% of data variation, being sufficient to discriminate the formulations in regards to their sensorial attributes, however only one dimension was considered. The spatial separation of the nine formulations suggests the formation of three distinct groups: the first group formed by samples F4, F7 and F8, the second group composed of formulations F1, F3 and F9, and the other consisting of formulations F2, F5 and F6.

In Figure 2, the sensorial attributes are represented by vectors. Each abscissa and ordinate of a vector is, respectively, a linear correlation between a sensorial attribute and the first and second principal component, respectively. All attributes, except for color, presented correlation ($p < 0.10$) with the first principal component. The color attribute is correlated with only the second principal component, presenting little importance for sensorial characterization of the *requeijão* samples since the second principal component explained only 5.55% of the variation of data. The attribute "characteristic color" showed little importance for sensory description of *requeijão* samples, probability because the samples presented a little difference in color. Therefore, it was not sought to adjust the regression models for the attribute of color.

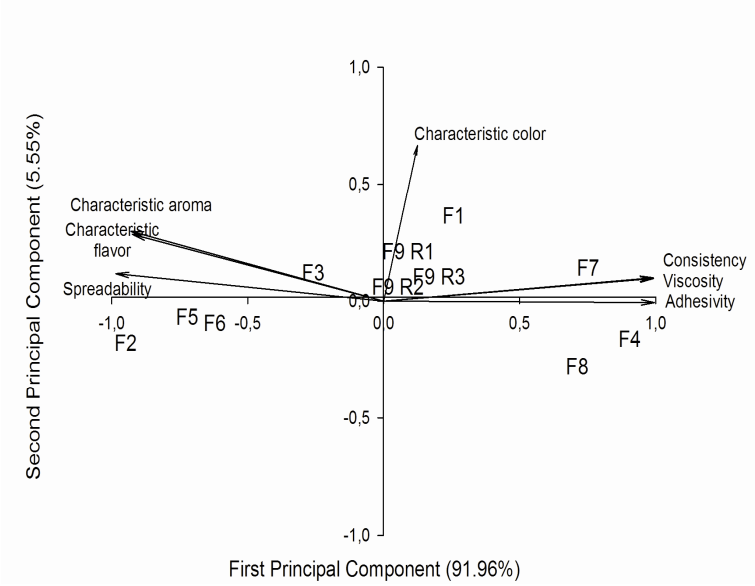

Figure 2. Graphical representation of the sensorial descriptors and the *requeijão* samples in relation to the two principal components

The formulations F4, F7 and F8 showed a greater intensity of the attributes of consistency, viscosity and adhesivity. The *requeijões* F2, F5 and F6 were characterized by the attributes of characteristic aroma, characteristic flavor and spreadability. The group represented by the formulations F1, F3 and F9 showed an intermediate intensity of sensory attributes.

The influence of fat and moisture content on the sensorial attributes was modeled statistically by means of the equations presented in Table 3. The models were tested in regards to the lack of fit and significance of the regression parameters, and also presented a precision coefficient greater than 85%. Effects of second degree and interaction were not significant ($p > 0.10$) by the t-test.

The levels of fat and moisture positively contributed to the intensity of the attributes of aroma, flavor and spreadability, and negatively for the attributes of consistency, viscosity and adhesivity.

The relation of fat content with the attributes of color and flavor is due to the presence of liposoluble pigments, especially carotenoids present in the fat of the milk, which are responsible for the aroma and flavor of lactic products (Fox & Mcsweeney, 1998).With regards to moisture content, the relationship with the flavor attribute is due to the greater contact surface of the *requeijão* with the increased moisture content (less consistent), which provokes better spreadability of the product on the palate and allows for better perception of the flavor attributes. However for the aroma attribute, such a result is due to the greater volatilization of aromas in more aqueous foods (Deibler & Delwiche, 2004). In the PCA (Figure 2), the *requeijão* samples with greatest fat contents, combined with elevate moisture contents (F2, F5 and F6), presented greater intensity of the aroma and flavor attributes, indicating that there is an inverse relation between these constituents (fat and moisture) and the attributes of aroma and flavor.

Considering the attributes of consistency, viscosity and adhesivity, the constituents under study (fat and moisture) presented a negative effect on the intensity of these attributes (Table 3). This is due to the relation of fat and moisture content with the protein content (DDE) of the *requeijão* samples, where formulations with greater levels of fat and moisture showed dilution of DDE causing losses in consistency, viscosity and adhesivity. This behavior was observed in the PCA, Figure 2, where the formulations with elevated fat and moisture contents (F2, F5 and F6) showed lower intensity of these attributes. This relationship is due to the low DDE conferred by high levels of the factors under study (fat and moisture); there are fewer forces, inter- and intra-molecular, associated with casein (Dimitreli & Thomareis, 2004). An inverse behavior was verified for the formulations with greater levels of defatted dry extract (F4, F7 and F8), Figure 2, which was characterized by presenting a greater intensity of the consistency, viscosity and adhesivity attributes than the other formulations. The elevated DDE level provoked the enrichment of the protein matrix due to the elevated number of protein-protein interactions,

causing an increase in the intensity of these texture attributes (Fox, Guinee, Cogan, & Mcsweeney, 2000).

The spreadability attribute showed a positive correlation with fat and moisture contents in regards to its intensity, due to this attribute possessing an inverse relation with the attributes of consistency and viscosity, since the more viscous sample possessed an inferior ability to be spread on a cracker (Garruti et al., 2003). Thus, *requeijão* samples with low levels of DDE (F2, F5 and F6) present greater spreadability. For the formulations with greater DDE percentages (F4, F7 and F8), i.e., lower content of fat and moisture, the opposite behavior was verified.

The formulations with intermediate levels of DDE (F1, F3 and F9) showed intermediately intensities of the sensorial attributes, indicating that the combinations of fat and moisture of these *requeijão* samples permitted the occurrence of an equilibrium in DDE, and consequently, in the texture and flavor of the *requeijão*.

Table 3. Statistical model of the effect of fat and moisture on the sensory properties of light *requeijão* supplemented with WPC

Models	**R^2**
$Y_{AROMA} = -37.5274 + 0.3760_{FAT} + 0.5433_{MOISTURE}$	0.8537
$Y_{CONSISTENCY} = 65.0761 - 0.4254_{FAT} - 0.8070_{MOISTURE}$	0.9399
$Y_{SPREADABILITY} = -56.0184 + 0.5199_{FAT} + 0.7898_{MOISTURE}$	0.9068
$Y_{FLAVOR} = -38.6437 + 0.3606_{FAT} + 0.5650_{MOISTURE}$	0.8547
$Y_{VISCOSITY} = 67.5738 - 0.4437_{FAT} - 0.8423_{MOISTURE}$	0.9496
$Y_{ADHESIVITY} = 65.5538 - 0.5192_{FAT} + 0.8441_{MOISTURE}$	0.9424

Y: response variable; F: fat content; M: moisture content; R^2: coefficient of determination.

3.3 Sensorial Acceptance

In the Internal Preference Map (Figure 3), the first principal component explained 50.67% and the second 21.83%, thus totaling 72.50% of the variance between the nine *requeijão* formulations in regards to acceptance related to overall impression.

The spatial separation of the *requeijão* samples suggests the formation of four distinct groups in accordance with sensorial acceptance, where one group was formed by *requeijão* samples F1, F3 and F9 (first quadrant), another formed by formulations F4, F7 and F8 (second quadrant), a third group composed of the *requeijão* F2 (third quadrant) and the last group formed by the formulations F5 and F6.

The consumers are represented by points where each abscissa and ordinate of a point is, respectively, the linear correlation between the consumer and the principal components. The correlation of the consumers by at least one of the components indicates a difference in the acceptance of the formulations. Therefore, the consumers located in the central region of the graph are not correlated to either of the two components, and therefore do not discriminate the *requeijão* samples in regards to acceptance. As can be observed in Figure 3, few consumers were located in the central region, indicating that the different *requeijão* samples differed well in regards to acceptance.

In this type of graphical representation, the consumers are located near to the products they liked. Thus, formulations F1, F3 and F9 presented a greater acceptance since the majority of consumers are located near these formulations. The requiejão samples F5 and F6 were also accepted, however by a smaller group of consumers; the formulations F2, F4, F7 and F8 (situated in the second and third quadrant) were not accepted by the consumers.

The sensorial dislike of *requeijão* formulations F4, F7 and F8 may be explained due to the elevated DDE level (approximately 24.80%) of these products, which present a very strong protein matrix, not favoring the texture of the samples. However for the *requeijão* F2 the opposite was verified, where the increase in moisture content to 70% combined with 17.3% fat resulted in a high dissolution of the DDE (13.80%), producing in a very flaccid *requeijão* which also caused for a poor acceptance of the product.

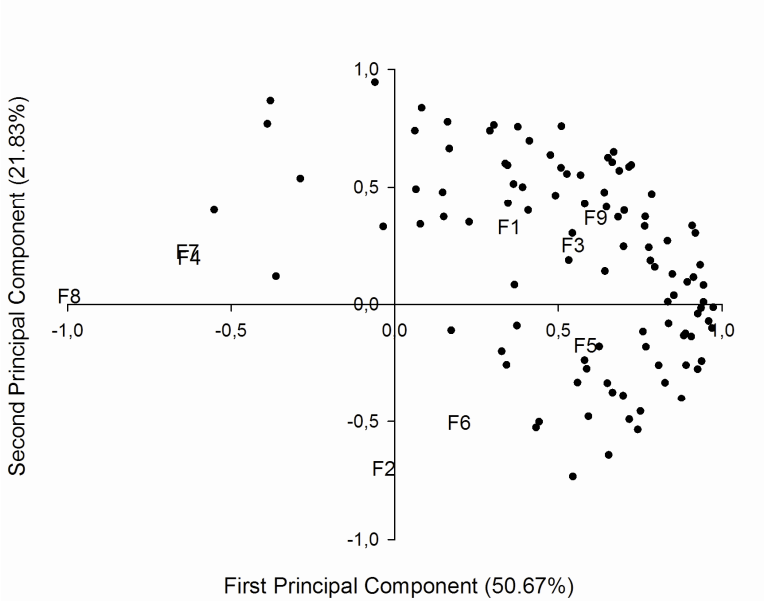

Figure 3. Graphical representation of the *requeijão* formulations and of the consumers in relation to the two principal components in regards to overall impression

It was verified that the *requeijão* samples F1, F3 and F9, formulations with different levels of fat, showed similar sensorial acceptance when combined with different moisture contents, i.e., the *requeijão* with 17.3% fat and 65% moisture (F1) presented sensorial acceptability equivalent to the product with 10.2% fat and 70% moisture (F3), and also equivalent to that with 13.7% fat and 67.5% moisture (F9). It should be emphasized that these formulations presented similar DDE values (approximately 19.30%), indicating that the reduction in *requeijão* fat levels is linked to the increase in moisture content so that there is equilibrium in the sensorial characteristics of the product.

The relationship between the DDE level and sensorial acceptance may be easily visualized in Figure 4, which is a graphical representation of the surface response of sensorial acceptance in function of the fat and moisture levels, where it can be observed that as the level of fat in the *requeijão* decreases, greater should be the moisture content of the product so that the sensorial properties are pleasant to the consumers. From this information, it is possible to determine diverse combinations of fat and moisture, within the studied concentrations, which meet the expectations of consumers.

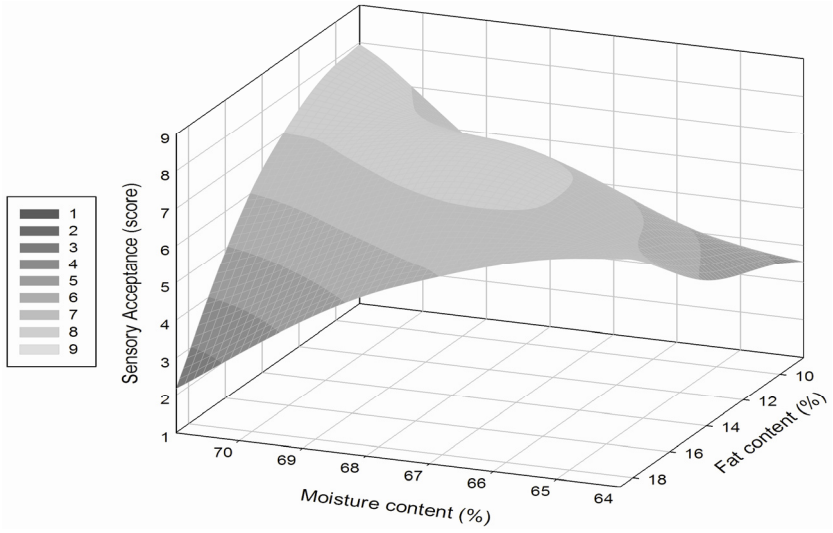

Figure 4. Graphical representation of the Surface Response Analysis for acceptance in the function of fat and moisture content of the *requeijão*

4. Conclusion

The levels of fat and moisture are presented as determining factors sensorial quality in light *requeijão*, indicating the relevance of this study. Different combinations of fat and moisture resulted in *requeijão* samples with similar sensorial profiles, showing that the reduction in fat concentration in *requeijão* should be aligned with increase in moisture content of the final product so that there is an equilibrium in the defatted dry extract (DDE), and consequently in the sensorial quality of the product.

The surface response analysis allowed for identification of innumerous combinations of fat and moisture which meet the expectations of the consumers in regards to sensorial quality of the *requeijão* samples, allowing the development of products with different levels of fat reduction and similar sensorial acceptance.

The conclusions obtained from this research about the influence of the reduction of fat and moisture in the sensory characteristics of *requeijão* are valid only for the concentration ranges studied. The reduction of fat in the *requeijão* must be associated with the increase in moisture of the final product so that it shows a balance in DDE, also generating a balance in the texture of the product and providing improvement in sensory acceptability. However, it is not known what effect on product texture and sensory acceptability for reductions in fat content below 8.7%. The mathematical model established in this study can be used to predict the sensory characteristics of *requeijão*, from fat and moisture content of the product to the concentration range evaluated, which was from 8.7 to 18.7% (fat) and from 64 to 71% (moisture).

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Effect of Moisture Content on Selected Physical and Mechanical Properties of Two Varieties of Tigernut (*Cyperus spp*)

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Abstract

The effect of moisture content on some physical and mechanical properties of two varieties of tigernuts (*Cyperus esculentus*) was investigated. These properties include: geometric dimensions, linear dimensions, 1000 tuber weight, bulk density, tuber size, sphericity, angle of repose, porosity, coefficient of static friction and compressive strength. The moisture content levels used were 20, 25, 30, 35 and 40% wet basis (wb), and the two tigernut varieties used were yellow and brown types. The linear dimension, geometric diameter, sphericity, 1000-tuber weight, bulk density and angle of repose in both varieties increased with increasing moisture content. The average length, width and thickness of the yellow variety increases more than the brown variety at the determined moisture contents. True density of the yellow variety increased while the brown variety decreased with increase in moisture content. The porosity of the yellow variety reduces with increase in moisture content from 45.95 at 20% mc to 42.4 at 40% mc, while the brown variety decreased from 42.72 at 20% mc to 30.77 at 40% moisture content. The yellow variety had bigger size tubers than the brown variety and this has serious implications in packing, handling and transportation issues.

Keywords: moisture content, physical properties, tigernut, mechanical properties

1. Introduction

Tigernut (*Cyperus esculentus*) is an underutilized crop of the family *Cyperaceae* which produces rhizomes from the base and tubers that are somewhat spherical. It is a root crop which grows widely in wet places as a grass and is sometimes cultivated for its small and sweet tubers (Eteshola & Oraedu, 1996). Other common names of tiger nut are; earth almond, chufa, yellow nut sedge and Zulu nuts (Ekeanyanwu & Ononogbu, 2010).

Tigernut grows mainly in the middle belt and northern regions of Nigeria, it is known in Nigeria as 'Ayaya' in Hausa, 'Imumu' in Yoruba and 'Akiausa' in Igbo where three varieties (black, brown and yellow) are cultivated (Osagie & Eka, 1998). Among these, only two varieties, yellow and brown are readily available in the market. The yellow variety is preferred over others because of its inherent properties like its large size, attractive colour and fleshier nature. The yellow variety also yields more milk, contains lower fat and higher protein and less anti-nutritional factors especially polyphenols (Okafor et al., 2003; Belewu & Abodunrin, 2005; Ebringa, 2007).

In Nigeria markets, tigernuts are available in both fresh and dried forms. It's cultivation requires sandy soil and a mild climate. The tubers are planted between April and May and must be continuously irrigated until they are harvested in November and December. The tubers have to be properly dried before storage as the dried tubers possesses lesser weight. The tubers are regarded as a digestive tonic, having a heating and drying effect on the digestive system and enhances urine production (Galedar et al., 2010). The tubers are said to be aphrodisiac, possesses stimulating effect and are also used in the treatment of flatulence, indigestion, colic, diarrhea, dysentery, debility and excessive thirst (Chevallier, 1996).

The tubers contain up to 30% of non-drying oil which is used in cooking, soap making, starch and flour preparation (Carruthers, 1986; Shaker et al., 2009; Muhammad et al., 2011). The selected properties (geometric dimensions, linear dimensions, 100th tuber weight, bulk density, tuber size, sphericity, angle of repose, porosity, coefficient of static friction and compressive strength) are important in many problems associated with the design of machines and the analysis of the behavior of the product during agricultural process operations such as

handling, planting, harvesting, threshing, cleaning, sorting and drying. Solutions to problems in these processes involve knowledge of their physical and engineering properties (Irtwange, 2000; Varnamkhasti et al., 2008; Tavakoli et al., 2009). This study examined the effect of moisture content on the physical and mechanical properties of two varieties of tigernut (yellow and brown) that are useful in the design of harvesting and handling equipment.

2. Materials and Methods

2.1 Sample Preparation

The yellow and brown varieties of tigernut used for this study was obtained at a local market in Akure, Nigeria. The tubers were cleaned manually to remove all foreign matter such as dirt, stones, immature and broken seeds. The initial moisture content of the samples was determined by oven drying method at 103 ºC for 48 hours as used by Sacilik *et al*., (2003). Selected samples were moistened with a calculated quantity of distilled water and conditioned to raise their moisture content to the desired different levels. Equation (1) was used to calculate the quantity of distilled water used according to Coskun et al. (2006).

$$Q = w_i\left(\frac{m_f - m_i}{100 - m_f}\right) \tag{1}$$

Where Q = mass of added water (kg),

w_i is the initial mass of the sample (kg),

m_i is the initial moisture content of the sample (%, *d. b*.) and

m_f is the final moisture content of the sample (%, *d. b*.).

Five levels of moisture contents were obtained for both varieties, 20, 25, 30, 35 and 40% (wb). The samples were then stored in an airtight polythene and kept at 5 ºC in a refrigerator for a week to achieve uniform moisture distribution within the sample.

2.2 Linear Dimensions, Sphericity and 1000 Tuber Weight

Average size was determined using 100 tubers that were randomly picked from each of the five conditioned samples. Three linear dimensions of the tubers namely; length *L*, width *W* and thickness *T* were measured with digital vernier caliper with accuracy of 0.001 mm and their equivalent diameter (D_g) determined obtained using Equation (2) by Sreenarayanan et al. (1985).

$$D_g = (LWT)^{0.333} \tag{2}$$

The sphericity of the tubers was calculated according to Mohsenin, 1978 as shown in Equation (3).

$$\phi = \frac{(LWT)^{0.333}}{L} \tag{3}$$

Where L = length (mm)

W = width and (mm)

T = thickness (mm)

One thousand tuber weight (W_{1000}) was determined using the method described by Baryeh (2002). The 100 seeds were weighed with the aid of an electronic balance and multiplied by 10 to give the mass of 1000 seeds.

2.3 Density, Porosity and Angle of Repose Determination

The true density and volume were determined using the toluene displacement method. Toluene was used in place of water because it is not easily absorbed by the seeds. The volume of toluene displaced was found by immersing a weighed quantity of tigernuts in toluene (Singh & Goswami, 1996). Bulk density was calculated from the mass of bulk tigernuts tubers divided by the volume of the container (Garnayak et al., 2008). The porosity, □ of the tubers was calculated from the values of the bulk and true densities obtained using Equation (4) by Mohsenin, (1970).

$$\varepsilon = \frac{[(\rho_g - \rho_b)100]}{\rho_g} \quad (4)$$

Where ρ_g = bulk density (kg m^{-3})

ρ_b = true or particle density (kg m^{-3})

The static angle of repose is the angle with the horizontal at which the material will stand when piled. This was determined through the use of the apparatus consisting of a plywood box of two plates fixed and adjustable. The box was filled with the sample, and then the adjustable plate was inclined gradually allowing the seeds to flow and assume a natural slope (Tabatabeefar, 2003; Varnamkhasti et al., 2008; Tavakoli et al., 2009).

2.4 Coefficient of Static Friction Determination

The coefficients of static friction were determined on three structural surfaces namely: galvanized steel, glass, stainless steel. The static coefficient of friction was determined using an inclined plane (Suthar & Das, 1996). The friction surface was part of a special construction, which is hinged at one end so that it can be lifted gradually at the unhinged end using a screw device as used by Bart-Plange, and Baryeh (2003). The angle at which the tubers just began to slide down was recorded as the static angle of friction between the tubers and the friction surface. Baryeh (2001, 2002), Dutta et al. (1988), Joshi et al. (1993), Singh and Goswani (1996) and Suthar and Das (1996) used this method for other grains and seeds. The coefficient of friction was calculated using Equation (5) as:

$$\mu = \tan\theta \quad (5)$$

Where μ = coefficient of friction and

θ = angle of tilt in degrees.

2.5 Compressive Strength

The tests were conducted using a universal testing machine (UTM) controlled by a micro-computer where results, statistics and graphs were automatically generated. The breaking force at peak and the Young modulus for the tubers were determined.

3. Results and Discussion

3.1 Effect of Moisture Content on Tigernut Dimensions

Figure 1 shows the variations of average values of the length, width, thickness and geometric diameters of the two varieties of the tiger tubers with increasing moisture content. All the linear dimensions increased with increasing tuber moisture content for both varieties. This is probably due to the air voids trapped in the cell vacuoles as they absorb moisture and thereby making the tubers display appreciable dimension change. This indicates that when the moisture is increased, the tubers increase in length, width, thickness and geometric diameter within the moisture range of 20-40% (w*b*) for yellow and brown varieties. The geometric dimension and their frequencies suggest that sieving will be a good method of separating the tubers from particles.

3.2 Effect of Moisture Content on Sphericity

The values of sphericity were calculated using Equation (3) by using the data on geometric mean diameter and the length of the tigernuts and the results obtained are presented in Figure 2. These figures indicate that the sphericity increased with increasing moisture content in both varieties from 0.86 to 0.89 for the yellow and 0.91 to 0.94 for the brown type.

3.3 Effect of Moisture Content on Mass of Tigernuts

Figure 3 shows the variation of tuber mass with moisture content. The tuber mass increased linearly from 0.32 to 0.42 g and 1.13 to 1.43 g, respectively, for the yellow and brown varieties as the moisture content increases. The figure shows that the lower the moisture content the lower the tuber mass. Transportation of the tuber is therefore advisable at low moisture content because of the reduction in weight.

3.4 Effect of Moisture Content on 1000-Tuber Mass

The variation of 1000-tuber weight with moisture content for both varieties of tigernut is shown in Figure 4. The figures show that the tuber mass increased with tuber moisture content. The variation can be expressed mathematically as W_{1000}=1.28*mc*+87.8 with a correlation coefficient, R^2 of 0.87 in the yellow variety and W_{1000}= 0.52*mc*+20.4 with a correlation coefficient, R^2 of 0.91 in the brown variety. The 1000-tuber mass ranged from 113 g to143 g in the yellow variety and 32 g to 42 g in the brown variety for the sampled moisture content. The result showed that there were significant differences between the mean values of the 1000-tuber weight of the

tigernuts at the 1% probability. Similar patterns have been reported for guna seeds, soyabean, cocoa beans, cumin seeds and bambara groundnuts (Aviara et al., 1999; Baryeh, 2001; Deahpande et al, 1993; Bart-Plange & Baryeh, 2003; Singh & Goswani, 1996; Visvanathan et al., 1996). The weights indicate that blowers can be used to transport the tubers in a processing plant.

3.5 Effect of Moisture Content on Bulk Density

The bulk density increased linearly with increasing moisture content from 590 to 630 kg m^{-3} in the yellow variety and from 600 to 640 kg m^{-3} in the brown variety as show in Figure 5. The bulk density increases in both varieties were very close to each other. The increase in bulk density was due to an increase in mass due to moisture gain in the sample which was higher than the volumetric expansion of the bulk (Pradhan et al., 2008). A similar increasing trend in bulk density has been reported by Baryeh and Mangope (2002) for QP- 38 variety pigeon pea and Kingsley et al. (2006) for dried pomegranate seeds and Nikoobin et al. (2009) for chickpea seeds.

3.6 Effect of Moisture Content on True Density

The change in true density with moisture content for both varieties of tigernut is shown in Figure 5. Here, the density of the yellow type increases in a non-linear manner starting from 1260 kg m^{-3} to 1370 kg m^{-3} as the moisture content increases while the brown type decreases also in a non-linear manner from 1030 kg m^{-3} to 910 kg m^{-3} as the moisture content increases from 20% to 40%. The variation in tuber density may be due to a low decrease in mass of the tuber as well as the smaller sizes of the brown variety compared to its volumetric decrease as tuber moisture content decreases. These results suggest that the tubers are likely to have high terminal velocities because of their densities, making pneumatic separation from lighter particles very feasible.

3.7 Effect of Moisture Content on Porosity

Porosity of materials usually is dependent on the bulk as well on true densities, therefore the magnitude of the varieties were calculated using the data on the bulk and true densities using Equation 4 and the results are presented in Figure 6. The porosity of the yellow variety reduces from 45.9% to 42.4% while that of the brown type reduces from 42.7% to 30.8% as the moisture content increases from 20% to 40%. This has to do with the changes in the mass and density values of the sample as it absorbs more water. High porosity at low moisture content indicates that high numbers of tubers can be stored at low moisture content than at high moisture content due to an increase in the cohesion of the cell structure of the tigernuts as the moisture content increases.

3.8 Effect of Moisture Content on Angle of Repose

Figure 7 shows the effect of moisture increase on the angle of repose for the tigernut varieties. The values were found to increase from 20.3^{o} to 23.7^{o} and 21.3^{o} to 24.3^{o} respectively, for the yellow and brown varieties at the moisture range of 20% to 40%. This increasing trend of angle of repose with moisture content occurs because surface layer of moisture surrounding the particle hold the tigernuts together by the surface tension (Prashan et al., 2008). These results were similar to those reported by Aluntas and Yildiz (2007) and Garnayak et al. (2008) for faba bean grains and jatropha seed, respectively.

3.9 Effect of Moisture Content on Static Coefficient of Friction

The variation of the coefficient of static friction with moisture content for the 2 varieties is shown in Figure 8 for three structural surfaces. At all moisture content levels, the static coefficient of friction was the highest for both varieties on galvanized steel and the least for stainless steel. The least value of static coefficient of friction may be due to smoother and more polished surface of the stainless steel than the other materials used. The reason for the increased friction coefficient at higher moisture content may be due to the water present in the tigernut tubers, offering a cohesive force on the surface of contact. As the moisture content of grains increases, the surface of the samples becomes stickier. Water tends to adhere to surfaces and the water on the moist tuber surface would be attracted to the surface across which the sample is being moved. Other researchers found that as the moisture content increased, the static coefficient of friction increased also (Baryeh & Mangope, 2002; Altunta & Yildiz, 2007; Pradhan et al., 2008). Knowledge of coefficient of friction of agricultural materials on various surfaces has long been recognized by engineers concerned with rational design of grain bins and other storage structures in grain handling (Varnamkhasti et al., 2008; Tavakoli et al., 2009).

3.10 Compressive Strength

The breaking force at peak and the Young modulus for the yellow variety were lower and reduces with increase in moisture content while the brown variety had a slightly higher values which increases at low moisture and then reduces as the moisture increases. Force at peak for the yellow variety reduces from 124.8 N at 20% moisture to 88.1 N at 40% moisture while it was 142.7 N and 167.3 for the same moisture range for the brown

variety. The Young modulus values for the yellow variety reduces from 313.7 N mm^{-1} at 20% moisture to 224.7 Nmm^{-1} at 40% moisture while for the brown type it increase at low moisture (847.7 N mm^{-1} and 20%) and then increase as the moisture level increases (852.4 N mm^{-1} and 40%).

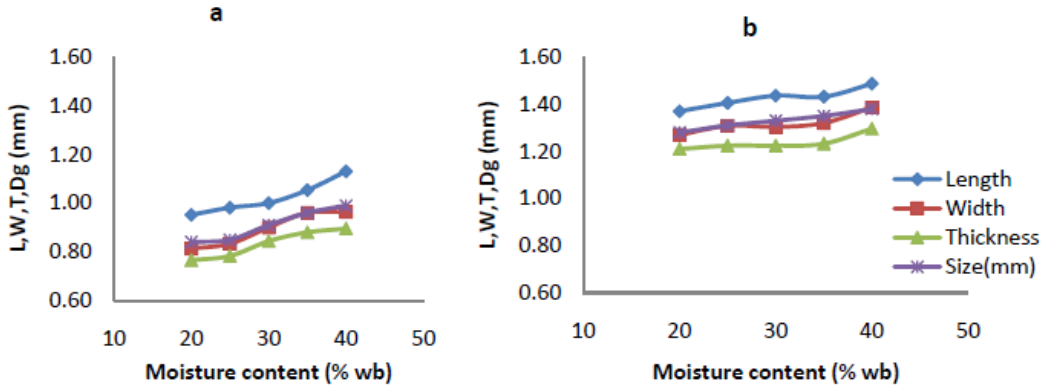

Figure 1. Linear dimensions against moisture content of the yellow (a) and brown (b) varieties of tigernut

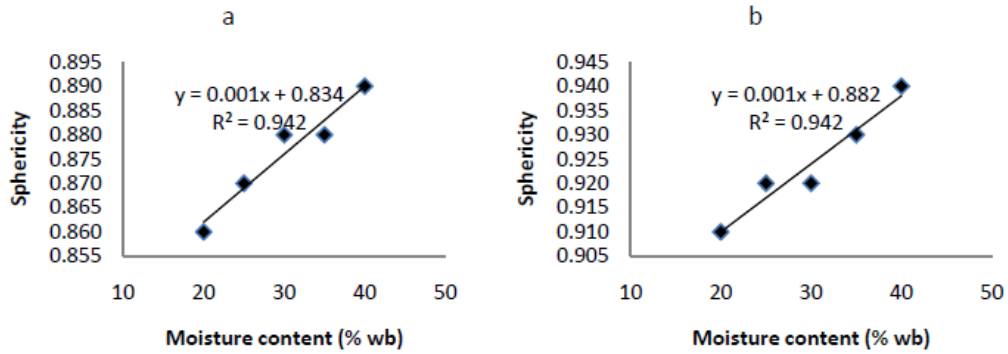

Figure 2. Sphericity against moisture content at different moisture levels of the yellow (a) and brown (b) varieties of tigernut

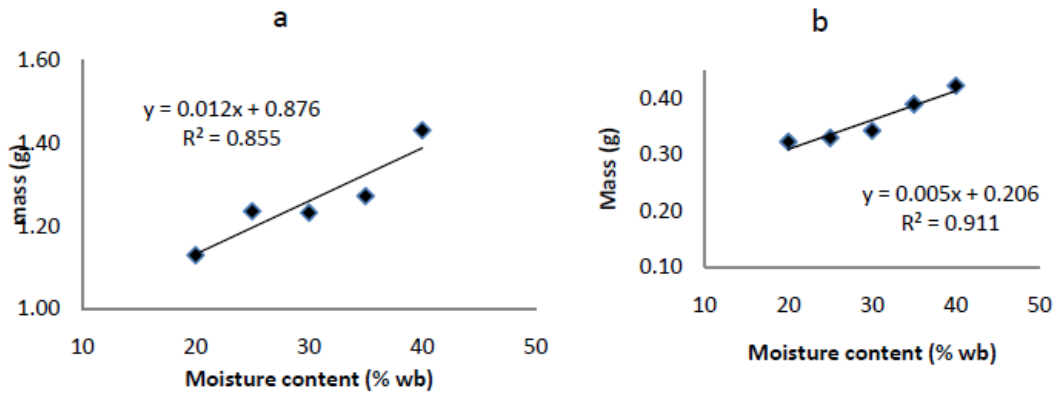

Figure 3. Mass of tuber against moisture content at different moisture levels of the yellow (a) and brown (b) varieties of tigernut

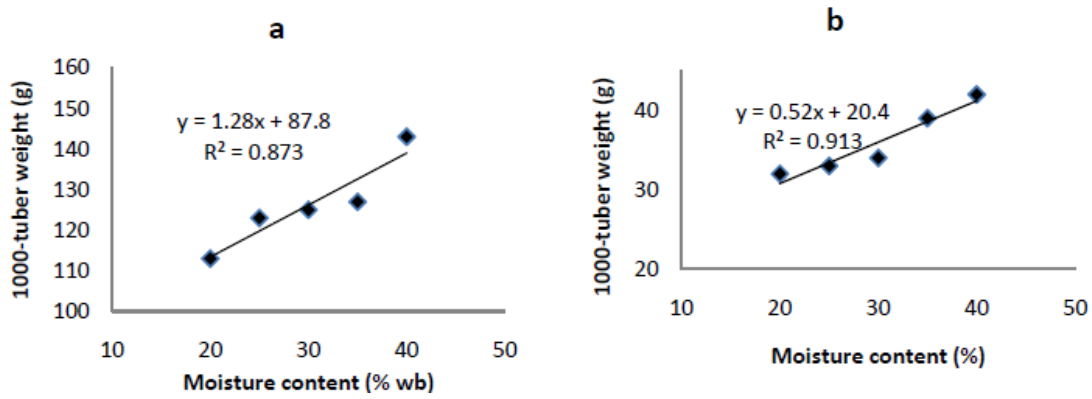

Figure 4. 1000- tuber weight against moisture content at different moisture levels of the yellow (a) and brown (b) varieties of tigernut

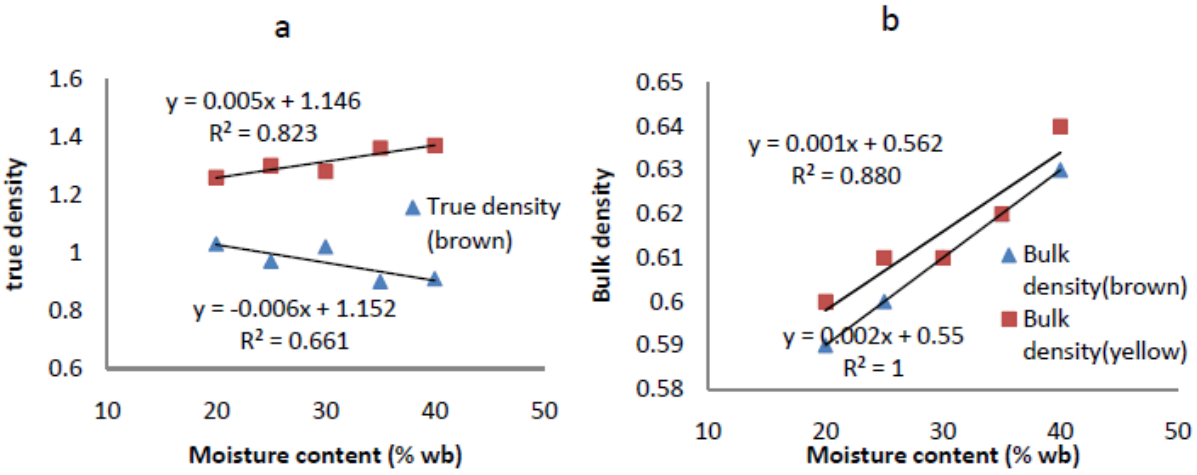

Figure 5. True density and bulk density variations with moisture content at different moisture levels levels of the yellow (a) and brown (b) varieties of tigernut

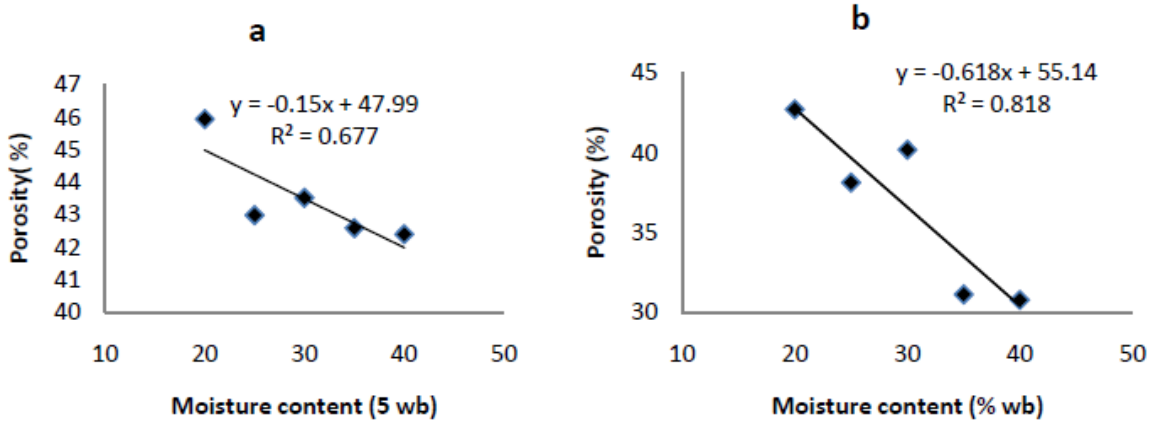

Figure 6. Porosity against moisture content at different moisture levels of the yellow (a) and brown (b) varieties of tigernut

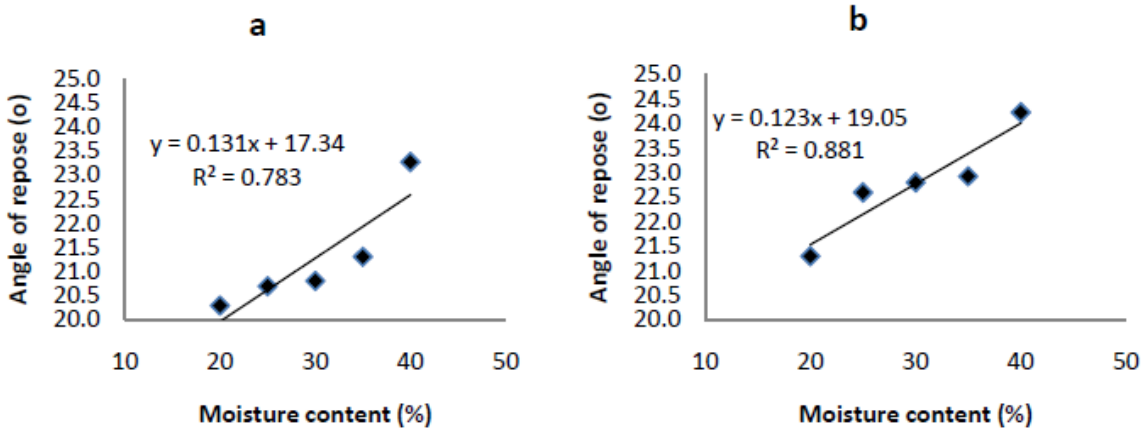

Figure 7. Angle of repose against moisture content at different moisture levels of the yellow (a) and brown (b) varieties of tigernut

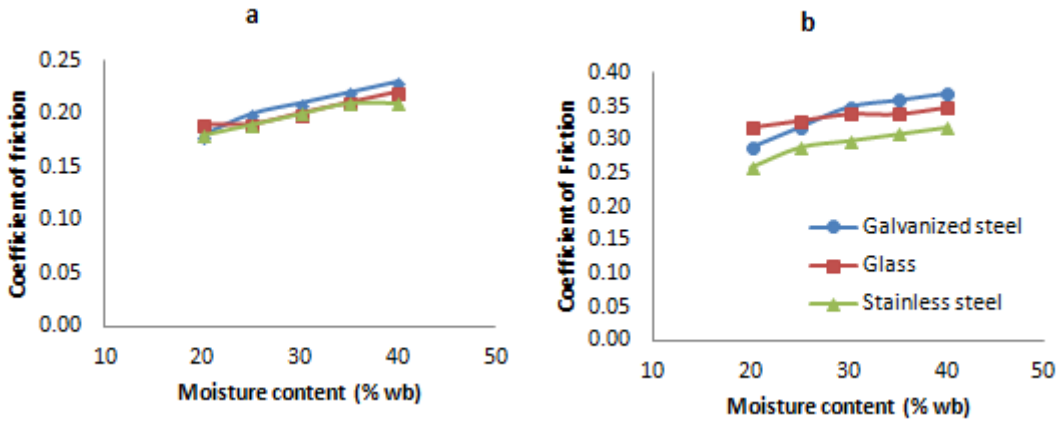

Figure 8. Coefficient of friction on different surfaces against moisture content at different moisture levels of the yellow (a) and brown (b) varieties of tigernut

4. Conclusions

The following conclusions are drawn from the investigations on selected physical and mechanical properties of two varieties of tigernuts (*cyperus spp.*) at five different moisture content levels. Physical and mechanical properties of tigernuts are dependent on their moisture contents. All the linear dimensions increased with increased moisture content for both varieties. The average length, width, thickness, geometric mean diameter, sphericity, volume, bulk density, thousand weight, tuber mass and angle of repose also increased. As the moisture increase in the samples, the porosity values reduce while bulk density increases. True density of the yellow variety increase significantly while the brown variety reduces with increase in moisture of the samples. The variation of the coefficient of static friction for three structural surfaces (glass, galvanized steel and stainless steel) at different moisture levels suggests the flow function of the samples on the materials of construction of the handling equipment. At all moisture content levels examined, the static coefficient of friction was the highest for both varieties on galvanized steel and the least for stainless steel. The least static coefficient of friction may be due to smoother and more polished surface of the stainless steel than the other materials used.

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Synergistic Endo- and Exo-Interactions Between Blueberry Phenolic Compounds, Grape Variety Fractions, Chocolate Covered Strawberries, and Fruit Smoothies

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Abstract

The presence of phytochemicals in fruits and vegetables is considered to be of nutritional importance in the prevention of chronic diseases, such as cancer and cardiovascular disease. Through overlapping or complementary effects, the complex mixture of phytochemicals in fruits and vegetables provides a better protective effect on health than single phytochemicals. Previous studies have shown that synergistic interactions between antioxidants in food result in a higher antioxidant capacity than individually isolated antioxidants. Further work is needed to explore other potential synergistic interactions between antioxidant mixtures within foods (endo-interactions) and between foods (exo-interactions) commonly eaten together. A series of studies examined potential synergy between various components of blueberries, grapes, chocolate covered strawberries, and fruit smoothies using multiple antioxidant assays (ORAC, TEAC and DPPH). At the ratio found in blueberries, significant synergy, antagonism, and patterns were found for many phenolic compound combinations, though they were dependent on the assay. Significant synergy was found in the combinations of skin and juice as well as skin, juice, and seed across three grape varieties. Significant synergy was found in the combination of strawberry and 88% cocoa chocolate in fully dipped strawberries. Fruit smoothies made with blueberries (as opposed to strawberries and raspberries) and soymilk (as opposed to water) exhibited significantly higher antioxidant capacity. The 3 assays measured correlated weakly with each other. This work furthers our understanding of the potential value of complex mixtures and foods in the human diet and is the first to report on the combinations and fractions described.

Keywords: antioxidants, cardiovascular health, oxidative stress, fruit, interactions

1. Introduction

Plants phenolic compounds serve as cell signaling molecules, toxins to invading pests, and antioxidants (Crozier et al., 2006). The phenolic compounds in fruits and vegetables are considered of vital nutritional importance in preventing chronic diseases, such as cancer and cardiovascular disease (Chu et al., 2002a; Chu et al., 2002b; Steinmetz et al., 1996; Van't Veer et al., 2000). The intricate mixture of phytochemicals in fruits and vegetables provides a better protective health effect than single phytochemicals (Eberhardt et al., 2000; Lila & Raskin, 2005).

Research investigating the components of fruit (Robards et al., 1999; Franke et al., 2004; Harnly et al., 2006) has highlighted phenolic compounds. There is an inconsistency between the antioxidant capacity of an individual phenolic compound and the antioxidant capacity of the whole fruit (Miller & Rice-Evans, 1997; Zheng & Wang, 2003); the whole fruit antioxidant capacity is higher. Possible explanations may include unidentified fruit compounds, the summation of many fruit compounds, or various synergistic interactions between phenolic compounds.

Lila and Raskin (2005) discussed additive or synergistic potentiation in terms of endo-interactions, or interactions within a plant that may alter its pharmacological effects, and exo-interactions, which are unrelated plant component and/or drug interactions. Some antioxidant synergy studies through exo-interactions have been

published. Liao and Yin (2000) demonstrated that combinations of alpha-tocopherol and/or ascorbic acid with catechin, epicatechin, caffeic acid, myricetin, quercetin, gallic acid, and rutin had greater antioxidant activity than any of the individual compounds in an Fe^{2+}-induced lipid oxidation system. The combination of quercetin 3-*β*-D-glucoside and an apple extract exhibited synergistic anti-proliferative activity in human breast cancer cells (Yang & Liu, 2009). The combination of acerola cherry extracts and alfalfa and soy phytoestrogen extracts worked to inhibit LDL oxidation synergistically *in vitro* (Hwang et al., 2001). Parker et al. (2010) found synergistic interactions between mixtures of rutin, *p*-coumaric acid, abscisic acid, ascorbic acid, and a sugar mixture using oxygen radical absorbance capacity (ORAC) and electron paramagnetic resonance (EPR). Hidalgo et al. (2010) discussed synergy between mixtures of two phenolic compounds, finding synergy in many of the combinations. Antioxidant synergism occurs in a variety of compounds and extracts.

Endo-interactions within a specific food have not been as widely studied. Seeram et al. (2004) discovered synergy among cranberry phytochemicals against malignant cell lines. Su et al. (1987) found a synergist in *Osbeckia chinensis*. Kayano et al. (2002) found a different synergist in prunes (*Prunus domestica*). Our laboratory published two in vitro studies (Freeman et al., 2010; Reber et al., 2011) and a small human trial (Snyder et al., 2011) that suggest endo-interactions within a fruit are primary contributors to their health benefits. The ratio of antioxidants in fruit plays a significant role in synergy.

The objective of this paper was to explore various endo- and exo-interactions at the chemical, fruit fraction and whole food level in a series of studies. It was hypothesized that synergism could be demonstrated in all three areas. This could help explain the antioxidant capacity difference between whole fruit and individual components, increase our understanding of how fruit fractions interact, and clarify the potential benefits of consuming certain foods together.

2. Materials and Methods

2.1 Chemicals

Trolox, potassium persulfate, quercetin hydrate, perchloric acid, and Folin-Ciocalteu reagent were purchased through Fisher Scientific Inc. (Waltham, MA, U.S.A.). (+)-Catechin hydrate, chlorogenic acid, myricetin, malvidin chloride, fluorescein, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2 diphenyl-1-picrylhydrazyl (DPPH), acetone, *p*-coumaric acid, (-)-epicatechin, gallic acid, dithiothreitol (DTT), *meta*-phosphoric acid (MPA), cupric sulfate pentahydrate and pelargonidin chloride were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) was purchased from Wako Chemicals U.S.A. Inc. (Richmond, VA, U.S.A.). Dimethylformamide (DMF, silylation grade) and N,O-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA w/ 1% TMCS) were purchased from Pierce (Rockford, IL, U.S.A).

2.2 Blueberry Study

2.2.1 Chemical Preparation

The five most concentrated phenolic compounds were chosen and studied at the following ratios: catechin, 7; chlorogenic acid, 5; malvidin, 13; quercetin, 3; and myricetin, 1 (USDA Flavonoid Database, 2007; Zheng & Wang, 2003; Wang et al., 2009). For the TEAC and DPPH assays, all compounds were weighed out, dissolved and diluted in ethanol, and stored at -80 °C. For ORAC, the compounds were dissolved in methanol, then diluted (7:3 acetone:water), and stored (-80 °C). Phenolics were brought to room temperature (RT), vortexed, and then mixed with minimal indirect light.

2.2.2 Mixtures and Extraction Preparation

All possible combinations of 2, 3, and 4 compounds were mixed at the above ratios on the day of their assay. Mixtures were then further diluted to fit a standard curve (ORAC), or give a linear relationship on the concentration-inhibition plot (TEAC and DPPH).

2.2.3 Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay was performed according to Davalos et al. (2004) with some modifications. Briefly, fluorescein, Trolox, and AAPH were diluted to 117.2 nM (phosphate buffer), 80 µM (7:3 acetone:water), and 40mM (phosphate buffer) respectively, final concentration. Fluorescein and AAPH were transferred to all wells of 96-well plates via a Precision Micropipettor (BioTek Instruments, Inc., Winooski, VT, U.S.A.). The standard curve used four concentrations of Trolox (10 µM, 20 µM, 40 µM, 80 µM) and the outer ring of wells were filled, but not used, to maintain temperature uniformity. Fluorescence of all wells was measured at 485/20 nm excitation and 528/20 nm emission every minute for 120 minutes in a BioTek Synergy 2 fluorescence plate

reader (BioTek Instruments, Inc., Winooski, VT, U.S.A.) set at 37 °C after pre-warming. ORAC values were expressed as Trolox Equivalents (TE) per mmol.

2.2.4 Trolox Equivalent Antioxidant Capacity (TEAC)

The TEAC assay was performed according to Re et al. (1999) with slight modifications. Trolox was diluted to 400 μM in ethanol. ABTS was dissolved in double distilled (dd) water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was prepared. Diluted $ABTS^{\bullet+}$ solution (180 μl) was added to 20 μl of antioxidant or Trolox standards (final concentration 5-40 μM) in a 96-well plate via the Precision Micropipettor. The absorbance reading (734 nm) was taken at room temperature exactly 30 min after initial mixing using the Biotek Synergy 2 plate reader. To calculate the TEAC, the slope of percentage inhibition of absorbance vs. concentration is divided by the slope of the plot for Trolox. This gives the TEAC (mM) at 30 min.

2.2.5 2,2-diphenyl-1-picrylhydrazyl (DPPH)

Trolox was diluted to 1.5 mM in ethanol. DPPH was dissolved in ethanol to 0.1 mM. Diluted DPPH solution (200 μl) was added to 50 μl of antioxidant or Trolox standards (final concentration 7.2, 15, 30 μM) in a 96-well plate via the Precision Micropipettor. The absorbance reading (517 nm) was taken at room temperature exactly 40 min after initial mixing using the Biotek Synergy 2 plate reader. To calculate the DPPH value, the slope of percentage inhibition of absorbance vs. concentration is divided by the slope of the plot for Trolox. This gives the DPPH value expressed as Trolox Equivalents (TE) per mmol.

2.3 Grape Study

2.3.1 Component Separation

Black Ribier, Red Globe, and Sugraone varieties of grape were purchased locally (Chilean origin). For the whole grape extract, 10 grapes were weighed and blended. Another 10 grapes were weighed to prepare the skin, seed, pulp, and juice components. Each grape was skinned using a spatula, weighed and blended. The seeds, including the stem connector, were collected and weighed, crushed, and mixed with dd water. The remaining pulp and juice were milled three times. The juice was collected and the pulp was scraped back into the mill, and then collected after the final milling. The juice was weighed. The pulp was weighed and then blended with dd water. All samples were stored at -80 °C.

2.3.2 Extract Preparation

Samples were thawed at 26 °C, then vortexed. A sample was weighed. A mixture of acetone:water:acidic acid (AWA, 70:29.5:0.5, 200 μl) was added, then vortexed for 30 seconds, sonicated (Fischer Scientific FS60D, Pittsburg, PA, U.S.A.) at 37 °C (5 min, 60 degas/minute), and centrifuged (11,000 x g, 1 min). The supernatant was transferred. This was repeated twice more (20,000 x g, 2 min, after third extraction). Combined supernatants were vortexed, centrifuged (10,000 x g, 30 sec) and diluted as necessary.

2.3.3 Combination Preparation

All possible combinations of grape components were then prepared. Components were combined based on the percentage mass of that component found in a grape. All combinations were then stored at -80 °C.

2.3.4 ORAC Assay

The ORAC assay was performed according to the blueberry study, except that AWA was used.

2.3.5 TEAC Assay

The TEAC assay was performed according to the blueberry study except Trolox was diluted in AWA.

2.4 Chocolate Covered Strawberries Study

2.4.1 Preparation

Strawberries (California origin) and 54% cocoa and 88% cocoa chocolate bars were purchased locally. Strawberries were separated into four groups of ten and weighed. Each group of ten strawberries was either fully-dipped or half-dipped in 54% or 88% cocoa chocolate melted at 55-60 °C. Following dipping, each strawberry was dripped (3 sec), frozen (-80 °C, 5 min), and weighed. Average ratios of g chocolate added to g strawberry were determined. Strawberries were pureed and chocolate was aliquoted separately for extraction.

2.4.2 Lipophilic Extraction

Samples were thawed (RT) and extracted with hexane three times via shaking, vortexed (30 sec, 3000 rpm), sonicated (5 min, 37 °C, interrupted once to shake) and centrifuged (60 sec, 10,000 x g) (Davalos et al., 2004; Prior et al., 2003; Wu et al., 2004). On the fourth and final extraction, samples were sonicated (10 min, with

shaking) and centrifuged (2 min, 20,000 x g). Any remaining liquid in the pellets was evaporated under gentle N_2 gas.

2.4.3 Hydrophilic Extraction

Hydrophilic extractions were performed as above, except AWA was used in place of hexane. Chocolate samples required eight AWA extractions; strawberry sample required three. It was assumed that all phenolic compounds were extracted when the supernatant was colorless.

2.4.4 Lipophilic $ORAC_{LP}$ Assay

All steps of the $ORAC_{LP}$ were identical to those described above except hexane was evaporated and Trolox and all lipophilic extracts were re-dissolved in 100% acetone.

2.4.5 Hydrophilic $ORAC_{HP}$ Assay

The $ORAC_{HP}$ assay was performed as in the blueberry study, except AWA was used in place of AW. Strawberry and chocolate AWA extracts were combined to represent the 88% cocoa fully dipped average weight ratio of g chocolate to g strawberry. All combined extractions were diluted with AWA.

2.5 Fruit Smoothies Study

2.5.1 Smoothie Preparation

Fruit and frozen yogurt (Breyers brand, Original) were purchased pre-frozen locally, and then stored (-20 °C) (except soymilk (Silk brand, original flavor) was refrigerated). The following smoothies were prepared: S1-Fruit Combination + Milk (360 ml Soymilk, 120 g frozen yogurt and 60 g ice (milk blend), 60 g each of blueberries, strawberries, and raspberries (fruit blend)), S2- Strawberries + Milk (milk blend and 180 g strawberries), S3- Blueberries + Milk (milk blend and 180 g blueberries), S4- Raspberries + Milk (milk blend and 180 g raspberries), S5- Fruit Combination + Water (360 ml dd water, 120 g frozen yogurt, 60 g ice and fruit blend), C1- Milk + Water (360 ml soymilk, 300 ml dd water, and 60 g ice), C2- Milk + Frozen Yogurt + Water (360 ml soymilk, 120 g frozen yogurt, 180ml dd water, and 60 g ice), C3- Water + Frozen Yogurt (120 g frozen yogurt, 540 ml dd water, and 60 g ice).

Ingredients were weighed, mixed (Waring blender, high, 2.5 min), then sampled every 15 minutes for 45 minutes and stored (-80 °C). Colorimetric analysis was performed before freezing.

2.5.2 Total Phenolics

The total phenolics (TP) assay was performed according to Swain and Hillis (1959) and Serafini et al. (1998). Each smoothie was treated with an acid and base treatment (HCl, NaOH, and *m*-phosphoric acid) to liberate phenolic compounds, followed by vortex, sonication (37 °C, 5 min), centrifugation (11,000 x g, 1 min), extracted with AWA per the chocolate covered strawberries study, and the remaining steps of the Folin-Ciocalteu reaction were performed (final incubation, 25 min, 45 °C). Absorbance was determined in triplicate (Biotek Synergy 2) at 765 nm with a gallic acid standard.

2.5.3 Colorimetric Assay

Color was measured immediately after collection (Hunterlab Colorflex spectrophotometer, Hunter Associates Laboratory, Inc., Reston, VA, U.S.A). L*a*b* values were measured in triplicate according to instrument instructions.

2.5.4 Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay was performed according to the blueberry study, except that AWA was used.

3. Statistics

3.1 Blueberry Study

For combinations of two, a difference was calculated by subtracting the sum of the average ORAC, TEAC or DPPH values for the individual compounds from the resulting average value of the combination of both compounds (Equation 1):

$$\text{Difference} = (\text{combination ab}) - (\text{individual a} + \text{individual b}). \quad (1)$$

Likewise, for combinations of 3 and 4, the difference was calculated by subtracting the average of the individual 3 or 4 compounds from the combination (Equations 2 and 3).

$$\text{Difference} = (\text{combination abc}) - (a + b + c), \quad (2)$$

$$\text{Difference} = (\text{combination abcd}) - (a+b+c+d), \quad (3)$$

Presenting the results in this manner allowed us to easily distinguish those combinations that were at minimum additive, using Fisher's least significant difference (LSD) analysis in the SAS statistical package (version 9.3, SAS Institute Inc., Cary, NC, U.S.A.). Additionally, for combinations of 3 and 4, a difference was calculated by subtracting the sum of the average ORAC values for the combination of 2 or 3, plus 1 individual, from the resulting average ORAC value of the combination of all 3 or 4 compounds (Equations 4 and 5).

$$\text{Difference} = (\text{combination abc}) - (\text{combination ab} + \text{individual c}), \quad (4)$$

$$\text{Difference} = (\text{combination abcd}) - (\text{combination abc} + \text{d}), \quad (5)$$

SAS was used to determine significance of combinations using estimate statistics, which take into account error terms when data are combined. The above described differences were compared through an ANOVA of the individual and combination results, and forming the differences as post hoc tests to determine the effect of combining the individual compounds and combinations. Presenting the results in this manner allowed us to determine which compounds contributed most to a combination.

3.2 Grape Study

Statistical analysis is the same as the blueberry study, except that the analysis was calculated based on grape fraction values.

3.3 Chocolate Covered Strawberries Study

Statistical analysis is the same as the blueberry study, except a combination of 5 was analyzed using all possible combinations.

3.4 Fruit Smoothies Study

Two analyses were carried out. The first ignored time and looked at all combinations of smoothie comparisons in an ANOVA. This was necessary because the control (C1-C3) samples were not measured over time. The initial ANOVA was followed by post hoc tests of pairwise differences. The second analysis was a two way ANOVA with S1-S5 and time (0, 15, 30, and 45 minutes). This was followed by post hoc pairwise tests between S1-S5 and then time.

4. Results and Discussion

4.1 Blueberry Study

4.1.1 ORAC

Only statistically significant synergistic interactions are included in Figure 1. All of the combinations that were synergistic included catechin or malvidin, suggesting they played an important role in determining the synergy of a combination. Combinations that had both catechin and malvidin were more than twice as often synergistic (31.4%) compared to those with only one of the two (12.4%), suggesting their synergy is an important interaction.

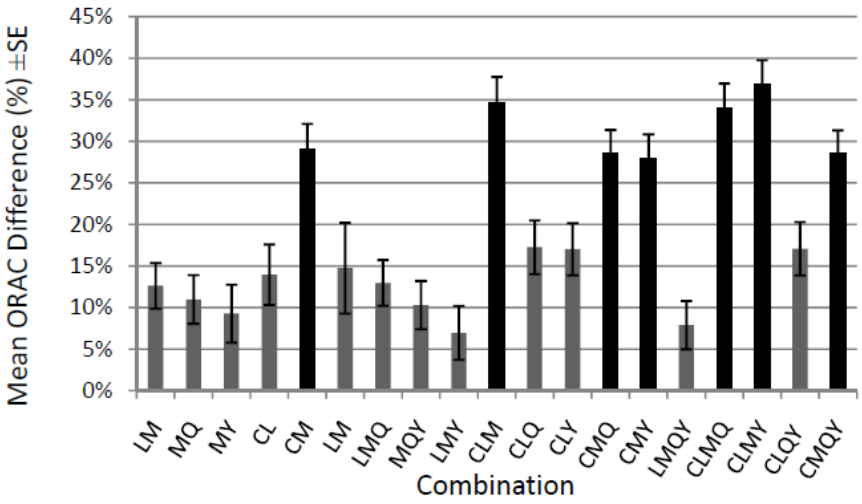

Figure 1. Oxygen Radical Absorbance Capacity (ORAC) differences for combinations minus the individual compounds in the combination (Equation 1 to Equation 3). Only statistically significant ($p < 0.05$) combinations are shown (n=6). C = catechin; L = chlorogenic acid; M = malvidin; Q = quercetin; Y = myricetin. LM indicates the ORAC of the mixture of L and M minus the ORAC of L and the ORAC of M; likewise for the other combinations. The difference was then divided by the average value of the combination, giving a percentage. Gray bars contain catechin *or* malvidin; black bars contain both catechin and malvidin

There appeared to be a positive correlation with the number of compounds in a given mixture with its % synergy. The average synergy of combinations of 2 compounds was 15.1%, of 3 compounds was 19.5%, and of 4 compounds was 24.9%. As the number of compounds increased, the more likely it was that any given combination would have both catechin and malvidin in it.

When looking at all combinations that contained a particular compound, catechin had the highest % synergy. Combinations with catechin had an average % synergy of 25.9%, whereas the average synergy of combinations not containing catechin was 19.0%.

Reber et al. (2011) studied the synergistic effect of 7 phenolic compounds, including catechin, commonly found in strawberries. Their results provide both supporting and refuting evidence. In Reber et al., when catechin was combined with *p*-coumaric acid there was significant synergy. We found a similar result in the catechin+chlorogenic acid combination. However, in a combination of three, catechin+*p*-coumaric acid+pelargonidin, an antagonistic effect was observed by Reber et al. A possible explanation is that the attempt by catechin to donate its electrons to *p*-coumaric acid (synergistic effect) is disrupted by the lack of a catechol group on the pelargonidin. This decreased catechin's effectiveness and resulted in antagonism. We found the opposite with the combination of catechin+chlorogenic acid+malvidin. Structural differences or the ratio of the compounds to each other may account for the differences. Hidalgo et al. (2009) studied the effect of donating electrons from eugenol derivatives; their results support our findings. They found that two eugenol derivatives displayed higher ORAC values due to their ability to stabilize the radical in the ortho (*o*) and para (*p*) positions of an aromatic ring. Three other derivatives had substituents that disrupted the stabilizing effect of the conjugated double bonds.

4.1.2 TEAC

For the TEAC assay, there were 2 combinations of 2 antioxidants that had statistically significant antagonistic interactions, and 3 combinations of 3 antioxidants that had statistically significant synergistic interactions (Figure 2). The myricetin+malvidin combination showed synergy when a 3rd compound was added, suggesting myricetin plays a key role in determining the strength of the interaction. Four out of the 5 combinations with significant interactions contained myricetin.

For the synergistic combinations of 3 in Figure 2, no individual compound or pair of compounds was responsible for the synergy. All corresponding possible 2+1 calculations for synergistic combinations were also synergistic

(CLQ and CMY). Thus there was no indication that any compound was more important than another. In addition, none of the combinations of 4, per eq. 3, were significant.

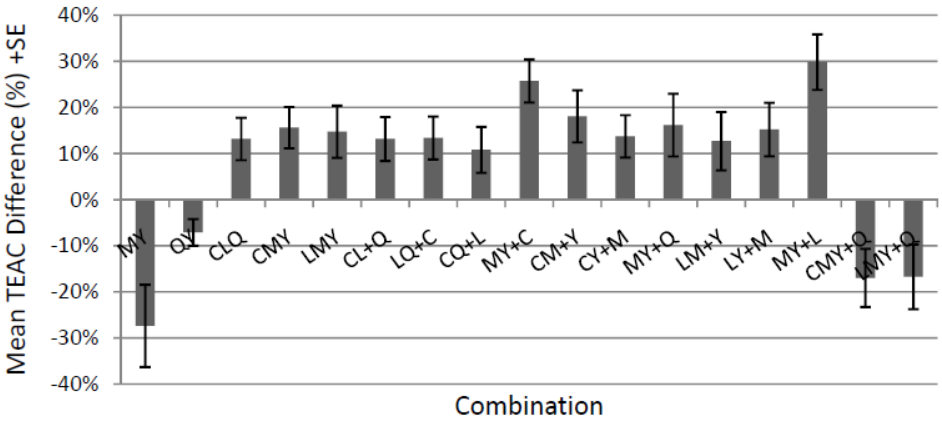

Figure 2. Trolox Equivalence Antioxidant Capacity (TEAC) for combinations minus the individual compounds in the combination (Equation 1 to Equation 3, no plus sign) and combinations of 3 phenolic compounds minus the sum of the 2 + 1 data (Equation 4, plus sign) or 3 + 1 data (Equation 5, plus sign). Analysis of the data by adding one compound at a time elucidates patterns and makes it possible to determine which compound interactions are most influential. Only statistically significant ($p < 0.05$) combinations are shown (n=3). C = catechin; L = chlorogenic acid; M = malvidin; Q = quercetin; Y = myricetin. The difference was then divided by the average value of the combination, giving a percentage

Combinations of 3 that were synergistic (CLQ, CMY, LMY) showed antagonism if quercetin was added as the fourth compound (data not shown). Overall, adding a 3rd compound had a synergistic effect on the mixture, whereas adding a 4th compound had an antagonistic effect. When a 3rd compound was added to MY, a higher level of synergy (23.9%) was found than with other mixtures to which a 3rd compound was added (13.9%). Both significant 3 + 1 calculations yielded antagonistic effects, contained MY, and Q was added.

4.1.3 DPPH

The DPPH assay showed primarily antagonistic interactions (Figure 3). All of the combinations that had statistically significant antagonism included chlorogenic acid in them. There was only one synergistic combination. Adding chlorogenic acid or malvidin to 2 or 3 other compounds had an antagonistic effect. Six out of seven of the antagonistic interactions found when adding a single compound to a combination came from the addition of either chlorogenic acid or malvidin. Wang et al. (2011) found 25% of their tested whole food combinations exhibited an antagonistic DPPH interaction. The synergistic effect of a mixture of two foods within the same category was generally lower than a mixture of two foods from across food categories. This is consistent with our DPPH results as two foods within the same food category are most similar to our data.

4.1.4 Correlation Between ORAC, TEAC, and DPPH Assays

Only two significant correlations occurred between the different assays. When measuring the synergy of antioxidant combinations, we found a significant negative correlation between the ORAC and TEAC assays ($r = -0.296$, $p < 0.05$). When measuring the individual compound antioxidant capacity, we found a significant negative correlation between ORAC and DPPH ($r = -0.308$, $p < 0.05$). Only three combinations showed synergy across multiple assays. Catechin+chlorogenic acid+quercetin, catechin+malvidin+myricetin, and chlorogenic acid+malvidin+myricetin were significantly synergistic in both the TEAC and ORAC assays. None of the compounds that had synergy overlapped with the DPPH assay.

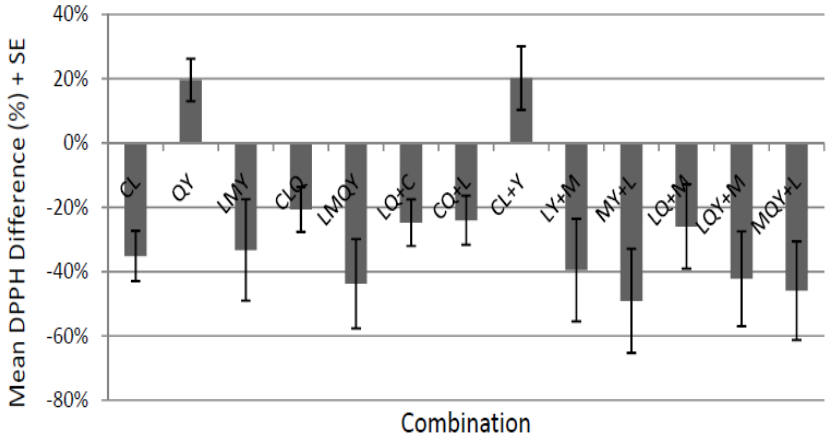

Figure 3. DPPH antioxidant capacities for combinations minus the individual compounds in the combination (Eq. 1 to Equation 3, no plus sign) and combinations of 3 phenolic compounds minus the sum of the 2 + 1 data (Equation. 4, plus sign) or 3 + 1 data (Equation 5, plus sign). Analysis of the data in this plus-one way elucidates patterns and makes it possible to determine which compound interactions are most influential. Only statistically significant ($p < 0.05$) combinations are shown (n=3). C = catechin; L = chlorogenic acid; M = malvidin; Q = quercetin; Y = myricetin. The difference was then divided by the average value of the combination, giving a percentage

The DPPH and TEAC assays used electron-deficient radicals. However, the correlations between them was not high ($r^2 = 0.47$), which is similar to the result reported by Tabart et al. (2009) ($r^2 = 0.36$). Tabart et al. compared the antioxidant capacities (exo-interactions) of several compounds using ORAC, TEAC, and DPPH. After testing 25 compounds, they found little agreement across the varying assays in antioxidant values. Only a few compounds such as myricetin and gallocatechin gave similar results across assays. In the DPPH assay, no antioxidant capacity was observed in quercetin, kaempferol, cyanidin, rutin, gallic acid, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-rutinoside. However, all of these flavonoids exhibited greater activities than Trolox in the TEAC assay. The TEAC assay in general was higher for more compounds than DPPH (68% compared to 52%). This trend is similar to the findings in this paper (76% compared to 15%), though our correlations were based on synergy values rather than individual compound values.

In contrast, Awika et al. (2003) reported a high correlation between TEAC and DPPH with ORAC (R^2= 0.99 and R^2 = 0.97, respectively) while measuring whole foods (i.e. bran bread, bran cookie, etc.). In our measurements of whole food synergy (see below), we did not see this same trend.

Wang et al. (2011) studied the antioxidant capacities of mixtures of legumes, vegetables, and fruits, looking for synergistic exo-interactions. Their results showed that the DPPH assay is more likely to find additive or antagonistic interactions, whereas the ORAC assay is more likely to find additive or synergistic interactions. Our results confirmed this observation.

4.2 Grape Study

4.2.1 ORAC

Individual ORAC values were obtained for each of the five components (whole, juice, seed, skin, and pulp) across three different grape varieties (*Vitis vinifera*, cultivars Black Ribier, Red Globe, and Sugraone). When comparing the cultivars to each other (Table 1), the Black Ribier cultivar had the highest ($p < 0.05$) ORAC value for the whole grape, juice, and skin. The Red Globe variety had the highest ($p < 0.05$) values for seed, and Sugraone had the highest ($p < 0.05$) value for pulp. A possible explanation for the Black Ribier showing greater values in whole, juice, and skin is the greater concentration of anthocyanin antioxidant compounds found in darker colored skins (Gao & Cahoon, 1994). The whole grape also had a higher antioxidant capacity because the skin was included. Black Ribier may have had more soluble antioxidants diffuse into the juice or it produced more of them. Conversely, the seed and pulp components may be more influenced by cultivar. This is consistent with Lutz et al. (2011) who found that blue grape juice (Autumn Royal, Ribier) phenolic content and antioxidant capacity was higher than red grape juice (Red Globe, Crimson Seedless). They also found that other

health-promoting phenolics are abundant mainly in the skin fraction of blue grapes. The juice examined in the present study was extracted directly by milling the grape with no other processing, unlike Lutz et al.

Table 1. ORAC and TEAC values for each component of each grape variety[1]

	ORAC(μmol TE/g)			TEAC(mM)		
Component	Black Ribier	Red Globe	Sugraone	Black Ribier	Red Globe	Sugraone
Whole	22.3 ± 2.0a	6.40 ± 2.0b	7.50 ± 2.0b	1.26 ± 0.2a	0.516 ± 0.2a	0.653 ± 0.2a
Juice	3.34 ± 0.2a	1.43 ± 0.2b	1.72 ± 0.2b	0.370 ± 0.1a	0.09 ± 0.1b	0.09 ± 0.1b
Seed	75.9 ± 7.0a	114 ± 7.0b	79.9 ± 7.0a	0.396 ± 0.03a	1.08 ± 0.03b	0.522 ± 0.03a
Skin	93.3 ± 5.0a	40.8 ± 5.0b	36.0 ± 5.0b	0.89 ± 0.1a	0.99 ± 0.1a	0.59 ± 0.1a
Pulp	3.15 ± 0.3a	2.92 ± 0.3a	4.30 ± 0.3b	0.08 ± 0.03a	0.04 ± 0.03a	0.11 ± 0.03a

[1]Grape components that do not share a lowercase letter are statistically different ($P < 0.05$) within each component row and assay. n=3.

Synergy was found in 55% of all possible component combinations of 2, 3, and 4 (Table 2). More synergistic effects were observed among the combinations of 2 (67%) than were observed among the combinations of 3 (50%) or 4 (0%). This is the opposite of what was found in the blueberry study with individual compounds. The more complex mixtures of antioxidant compounds found in grape components, when blended, may saturate the results.

Combinations that included skin were more often synergistic. All three varieties showed synergism when both skin and juice were combined as well as skin, juice, and seed. The Red Globe skin+juice+seed was significantly more synergistic than the other two varieties. However, adding pulp to a combination nullified the synergistic effect (skin+pulp+juice, all three varieties) and caused an antagonistic effect in some cases (Sugraone skin+pulp+seed and Black Ribier skin+pulp+seed+juice). Again, this may be due to different compounds found in the respective fractions or ratio percentages, which would impact the chemical interactions.

Sandhu and Gu (2010) compared the antioxidant capacity of skin, seed, and pulp of eight different grape cultivars using ORAC. The antioxidant capacity of the phenolic compounds responsible for the antioxidant activity was highest in seed (87.1%), then skin (11.3%), and then pulp (1.6%). This explains our finding that pulp did not induce synergistic activity in combination with other components due to its low phenolic content. It does not explain the antagonistic effect however.

Table 2. ORAC and TEAC differences for combinations minus the individual components in the combination (Equation 1 to Equation 3)[1]

	ORAC(μmol TE/g)			TEAC(mM)		
Combination	Black Ribier	Red Globe	Sugraone	Black Ribier	Red Globe	Sugraone
Sk/P[2]	25.5 ± 3.5*	2.08 ± 4.6	16.0 ± 2.0*	0.21 ± 0.1*	0.23 ± 0.06*	0.04 ± 0.09
Sk/Se	54.2 ± 3.5*	19.3 ± 4.6*	-10.8 ± 2.0*	0.46 ± 0.1*	0.73 ± 0.06*	-0.06 ± 0.09
Sk/J	21.0 ± 3.5*	39.3 ± 4.6*	15.8 ± 2.0*	0.09 ± 0.1	0.89 ± 0.06*	0.15 ± 0.09
P/Se	90.0 ± 3.5*	5.32 ± 4.6	-1.74 ± 2.0	0.81 ± 0.1*	0.33 ± 0.06*	-0.01 ± 0.09
J/Se	1.96 ± 3.5	1.50 ± 4.6	0.16 ± 2.0	-0.06 ± 0.1	-0.001 ± 0.06	0.04 ± 0.09
P/J	6.06 ± 3.5	5.74 ± 4.6	1.03 ± 2.0	-0.07 ± 0.1	0.07 ± 0.06	0.01 ± 0.09
Sk/P/Se	4.87 ± 3.5	6.92 ± 4.6	-3.96± 2.0	0.31 ± 0.1*	0.18 ± 0.06*	0.01 ± 0.09
P/Se/J	1.25 ± 3.5	1.35 ± 4.6	5.10 ± 2.0*	-0.03 ± 0.1	-0.05 ± 0.06	0.03 ± 0.09
Sk/J/Se	11.2 ±± 3.5*	112 ± 4.6*	6.04 ± 2.0*	0.14 ± 0.1	0.98 ± 0.06*	0.14 ± 0.09
Sk/P/J	4.07 ± 3.5	0.51 ± 4.6	3.77 ± 2.0	-0.01 ± 0.1	0.003 ± 0.06	0.09 ± 0.09
Sk/P/Se/J	-0.32 ± 3.5	1.96 ± 4.6	2.50 ± 2.0	-0.16 ± 0.1	-0.04 ± 0.06	0.12 ± 0.09

[1]Each value is the mean ORAC (μmol Trolox/g) or TEAC (mM) difference ± SE. n=3. [2]Sk=Skin, P=Pulp, Se=Seed, J=Juice. *Indicates a statistically significant synergy (positive values) or antagonism (negative values) between the combination and the individual components.

4.2.2 TEAC

The Black Ribier variety showed a statistically higher TEAC value for juice and Red Globe for seeds (Table 1). The other three components were not statistically different between the three varieties.

Surprisingly similar to the ORAC assay, synergy was found in 55% of all possible component combinations of 2, 3, and 4. More synergistic effects were observed among the combinations of 2 (67%) than were observed among the combinations of 3 (50%) or 4 (0%) (Table 2).

4.2.3 Correlation Between ORAC and TEAC

Differences across assays were expected (Tabart et al., 2009). Tabart et al. suggested that the reason for this variance is that ORAC is the only method that uses inhibition time, degree of inhibition, and a completed reaction. Wang et al. (2011) observed that of the 4 assays they used to measure antioxidant activity (ORAC, DPPH, Total Phenolic Content (TPC) and Ferric Reducing Ability of Plasma (FRAP)), generally only one assay would show synergy for any given combination. Our results with grapes also aligned with this finding, with only 3/30 combinations showing synergy in both ORAC and TEAC, despite the similar number of combinations demonstrating synergy across the two assays.

4.3 Chocolate Covered Strawberries Study

4.3.1 ORAC Synergy at the Mixed Whole Food Level

When extracts of each of the chocolates and strawberries were compared statistically to the extracts of the four types of chocolate covered strawberries for potential synergy (Equation 1), a significant interaction was only found in one group; the 88% cocoa chocolate fully-dipped strawberries (88F, data not shown).

4.3.2 ORAC Synergy at the Chemical Level

Chocolate covered strawberries were also analyzed at the chemical level (Figure 4). The $ORAC_{LP}$ values of the lipophilic extracts of both strawberries and chocolate were negligible, so only the $ORAC_{HP}$ values of the hydrophilic extracts were evaluated. All possible combinations of 2, 3, 4, and 5 of (+)-catechin, quercetin, pelargonidin, *p*-coumaric acid, (-)-epicatechin were combined at the ratios found in 88F chocolate covered strawberries. Of all statistically significant combinations, 73% showed synergism. Of the combinations that were statistically significant for synergism, 81% included catechin. Catechin and *p*-coumaric acid showed a strong synergistic relationship, as found in Reber et al. (2011). Ninety five percent of all combinations that included both catechin and *p*-coumaric acid were statistically synergistic. Combinations consisting of only two compounds were more likely to exhibit synergism. It was found that of all statistically significant antagonistic combinations, 70% included quercetin.

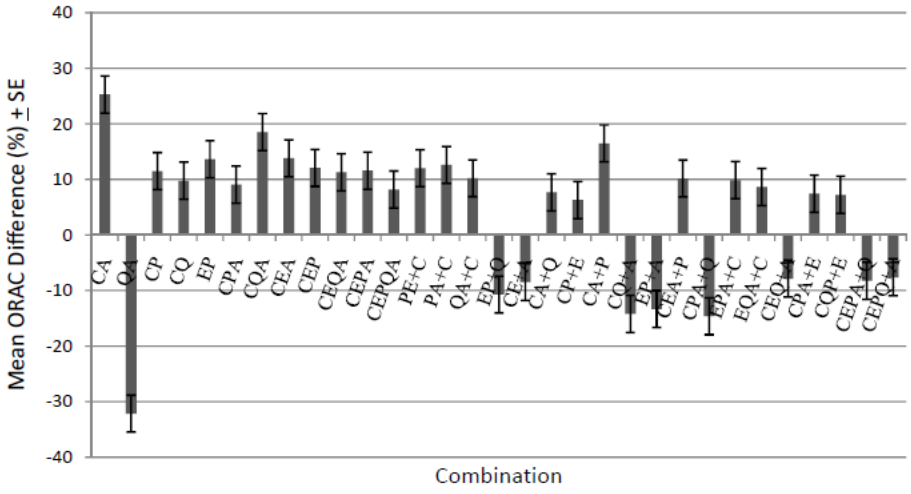

Figure 4. Oxygen Radical Absorbance Capacity (ORAC) differences ± SE for chocolate covered strawberry chemical combinations. Values are antioxidant capacities for combinations minus the individual compounds in the combination (Equation 1 to Equation 3, no plus sign) and combinations of 3 phenolic compounds minus the sum of the 2 + 1 data (Eq. 4, plus sign), 3 + 1 data (Equation 5, plus sign) or 4 + 1 data. Only statistically significant ($p < 0.05$) combinations are shown (n=6). C= (+)-catechin, Q= quercetin, P= pelargonidin, A= *p*-coumaric acid, E= (-)-epicatechin. The difference was then divided by the mean ORAC of the combination, giving a percentage

Parker et al. (2010) reported that more complex combinations of antioxidants tended to show more synergy when measured by the ORAC assay. Our results in this study partially agreed with this. There were fewer combinations of 4 antioxidants that showed synergy than combinations of 2 or 3; however, combinations of 4 that did show synergy tended to have higher percentages of synergy than combinations of 3, which were in turn higher than combinations of 2.

4.4 Fruit Smoothies Study

4.4.1 ORAC

Smoothies 1, 3, 4, and 5 were all statistically different from each other in their antioxidant capacity (Figure 5). Compared to the controls, all fruit-containing smoothies exhibited a much larger antioxidant capacity. Smoothie 3 exhibited the highest antioxidant capacity, showing blueberries increased antioxidant capacity more than raspberries or strawberries. This is consistent with the USDA Database for the Flavonoid Content of Selected Foods (USDA Database for the Flavonoid Content of Selected Foods, 2007) which showed blueberries contained a higher percentage of compounds exhibiting antioxidant capacity than either raspberries or strawberries. Hunter et al. (2012) found that milk which contained fruit and vegetable extracts had a positive effect on antioxidant markers when consumed regularly over a prolonged period compared to milk alone. This is likely due to the phytochemical content of the fruit, and is supported by our data.

Soymilk enhanced the antioxidant capacity of the smoothie. This is seen in the difference between smoothie 1 (soymilk added) and 5 (water only) (Figure 5). A possible explanation is that soymilk has many different components available to interact with the antioxidant compounds found in the fruit. Soy also contains phenolic compounds which could contribute in a positive way to the interaction. This can also be observed in control smoothie 2 vs. 3 where soymilk was removed; the antioxidant capacity decreased.

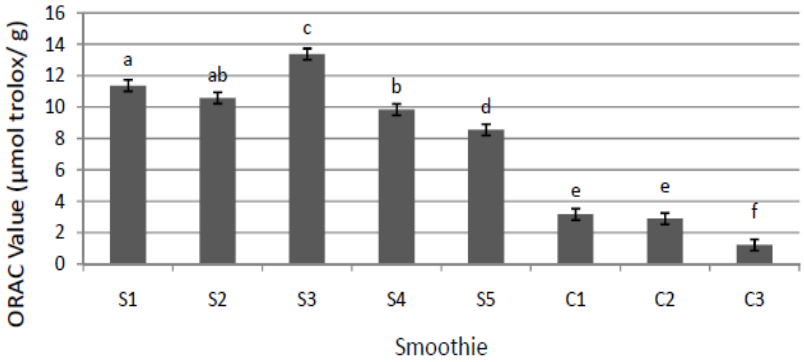

Figure 5. ORAC value (µmol TE/g) of smoothies ± SE. Smoothies that do not share a lowercase letter are statistically different (n=3, $p < 0.05$). The contents of the smoothies are: S1-Fruit Combination + Milk (360 ml Soymilk, 120 g frozen yogurt and 60 g ice (milk blend), 60 g each of blueberries, strawberries, and raspberries (fruit blend)), S2- Strawberries + Milk (milk blend and 180 g strawberries), S3- Blueberries + Milk (milk blend and 180 g blueberries), S4- Raspberries + Milk (milk blend and 180 g raspberries), S5- Fruit Combination + Water (360 ml dd water, 120 g frozen yogurt, 60 g ice and fruit blend), C1- Milk + Water (360 ml soymilk, 300 ml dd water, and 60 g ice), C2- Milk + Frozen Yogurt + Water (360 ml soymilk, 120 g frozen yogurt, 180 ml dd water, and 60 g ice), C3- Water + Frozen Yogurt (120 g frozen yogurt, 540 ml dd water, and 60 g ice)

Antioxidant capacity was measured in all smoothie types at various time intervals after smoothie blending and the averages were calculated (data not shown). This was to determine if fruit enzymatic activity would affect antioxidant capacity over time and if it would be different in more complex exo-interactions. There was a significant difference in the antioxidant capacity between time 0 (time of creation) and 30 min and 45 min, showing a general decreasing trend in antioxidant capacity as time passed. The difference from time 0 to time 45 (a change of 8.8%) may be too small to have a practical significance in decreasing the nutritional value of a smoothie when considering the typical time it takes to consume one.

Total phenolic content decreased significantly from 0 min to 15 min, but did not significantly decrease further at 30 or 45 min (data not shown). Enzymes liberated in the blended fruit may have changed the phenolic

composition of the fruit more initially, and then slowed. However the magnitude of the change from time 0 to 15 was small despite its significance.

The L*a*b color analysis was used to determine changes in color of the smoothies over time (data not shown). Overall, as time passed the smoothies became lighter (statistically different only between times 0 and 15), less red (statistically different at each time interval from time zero), and less blue (statistically different between time 0 and times 30 and 45). Visually, the difference was not noticeable. It may have been due to the ice melting and changing the refractance, as total phenolic content did not change after 15 min. Or, the phenolic changes resulted in the formation of other phenolic compounds which affected the color, but not the total phenolic content.

5. Limitations

Only a limited number of phenolic compounds could be studied in the chemical analysis in the blueberry and chocolate covered strawberries studies. Thus our results do not fully represent the whole food. Only a small sampling of grapes, chocolate and strawberries was purchased, so the results may not extrapolate to common consumption. The differences between mechanisms of the assays used will affect the measurement of the interactions (Tabart et al., 2009). Synergistic combinations of phytochemicals found using one assay did not strongly predict results found using a different assay.

6. Conclusions

6.1 Blueberry Study

When measured by ORAC, combinations that included both catechin and malvidin were more than twice as synergistic as other combinations, suggesting they play important roles in determining the synergy in a combination. There was a positive correlation between the number of compounds in a mixture and its % synergy. There was little correlation across the ORAC, TEAC, and DPPH assays when measuring the synergy of antioxidant combinations.

6.2 Grape Study

Across three grape cultivars (Black Ribier, Red Globe, and Sugraone), the Black Ribier cultivar exhibited the highest whole grape antioxidant capacity. Combinations of components including the skin were more likely to be synergistic. Both the TEAC and ORAC assays resulted in synergy in 55% of all possible combinations of 2, 3, and 4.

6.3 Chocolate Covered Strawberries Study

There was only one synergistic result when looking at synergy at the mixed whole food level (88% cocoa chocolate fully dipped). At the chemical level, catechin and *p*-coumaric acid had the most synergistic relationship and were the most important contributors to antioxidant synergy when both were part of other combinations.

6.4 Fruit Smoothies Study

Fruit smoothies made with blueberries demonstrated higher antioxidant capacity than those made with strawberries or raspberries, or a combination of all three. Also, soymilk showed an apparent synergistic relationship with fruit as compared to water. Overall, as time passed after blending, the smoothies became lighter, less red, and less blue, though with little phenolic change.

6.5 Overall

Little correlation was found among the varying assays in the blueberry, grape, and chocolate covered strawberries studies. In the blueberry and chocolate covered strawberries studies, catechin was present in a majority of synergistic mixtures. In the grape and fruit smoothies studies, the fruit or variety with the darkest skin color exhibited the highest antioxidant capacity (Black Ribier grapes, and blueberries) and skin was present or contributed strongly to apparent synergy. Overall, the complexity of a mixture, whether an endo- or exo-interaction, appears to be the most important factor in the antioxidant capacity of various chemical mixtures, food components or whole foods. Further work is needed to better understand the precise ratios and concentrations that are the most effective producers of synergy in these and other foods.

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Efficacy of Essential Oils From Some African Spices Against Two Strains of *Bacillus cereus* Isolated From Vegetable Salad

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Abstract

Chemical preservatives have been used to preserve our foods against spoilage and pathogenic microorganisms over the years. Consumers now frown at this hence the need to source for preservatives from natural sources. In this investigation fifteen samples of vegetable salad were collected from retail outlets in Lagos, Nigeria dilutions of which were plated on Brilliance *Bacillus cereus* agar. Isolates were identified based on morphological, biochemical characteristics and reactions to API 20E and API 50 CHB/E test kit. Two strains of *Bacillus cereus*; *B. cereus* 1 and *B. cereus* 2 were isolated. The organism was spread evenly on Mueller -Hinton agar and wells of 5mm in diameter were made on the inoculated agar with sterile cork borer. Different dilutions (1, 0.1, 0.01, 0.001 mg/ml) of essential oils extracted from three African spices; *Aframomum melegueta, Xylopia aethiopica* and *Piper guineense* by hydro distillation method were introduced into the wells in triplicate. After incubation diameter of zones of inhibition were measured. The essential oils of the spices inhibited growth of the two strains. *A. melegueta* produced the greatest zone of inhibition (14 to 24 mm) and with the lowest minimum inhibitory concentration (MIC) of 31.25 mg/ml, followed by *X. aethiopica* (10 to 18 mm) with MIC of 62.5 mg/ml and *P. guineense* (11 to 15 mm) and MIC of 125 and 250 mg/ml against *B. cereus* 1 and *B. cereus* 2 respectively. *P. guineense* was the least inhibitory. However, *B. cereus* 1 was more sensitive (inhibition zone of 12 to 24 mm) to the essential oils of the spices than *B. cereus* 2 (inhibition zone of 10 to 17 mm). The higher the concentration of the essential oils the greater the resultant zones of inhibition. The spices especially *A. melegueta* have proved very efficient in the inhibition of *B. cereus* a pathogen obtained from vegetable salad. The spices can therefore help in ensuring food safety.

Keywords: spices, essential oils, zone of inhibition, *Bacillus cereus*, vegetable salad

1. Introduction

According to World Health Organization (2007), consumption of foods contaminated with pathogenic microorganisms is a threat to human health. Friedman et al. (2007) reported that food processors, food safety researchers have been increasingly concerned with the growing number of food borne illness outbreak caused by some pathogens. There have been well-documented reports substantiating the role of *B. cereus* as a food poisoning organism (Whong & Kwaga, 2007). According to Arnesen et al. (2008), *B. cereus* has a wide distribution in nature, frequently isolated from soil and growing plants, but it is also well adapted for growth in the intestinal tract of insects and mammals. It is frequently isolated from milk and dairy products. It is found in rice, rice products, oriental dishes and ingredient. Emetic syndrome caused by *B. cereus* is highly associated with rice and rice products (Narang, 2004).

Reduction in the incidence of food poisoning has been a major challenge to researchers. As a result of this and negative consumer perceptions of artificial preservatives, attention is shifting towards alternatives that are obtained from plant extracts (Mihajilov-Krstev et al., 2010). Dobre et al. (2011) reported that in the variety of techniques available for conservation, food industry is investigating other techniques to replace traditional methods of conservation due to high demand by consumers for tasty and natural food. Spices are very important in human diet. They have been used for many years to improve the flavor, colour and aroma of food. In addition to boosting flavor, spices are also known for their preservative and medicinal value which forms one of the oldest sciences and modern science has started paying attention to the properties of spices (Chaudhry & Tariq 2006; Lai, 2004). According to Levetin and Mc Mahon (2006), there is no evidence of how primitive people first

discovered spices, but it is reasonable to assume that they were attracted to the pleasant aromas of these plants and found many uses of them. According to Ceyhan et al. (2012), medicinal plants such as spices and aromatic vegetable materials, have been used for a wide variety of purposes for many thousands of years in Turkey and all over the World.

According to Delamare et al. (2007), it has long been recognized that some essential oils have antibacterial properties and these have been reviewed in the past as the antibacterial properties of spices but relatively recent enhancement of interest in 'green' consumerism has led to the renewal of scientific interest in these substances. According to Fleischer et al. (2008) essential oils or their constituents are odoriferous substances from plants and are extensively used as medicinal products, in the food industry as flavors and in the cosmetic industry as fragrances. Many of these oils have been shown to exert broad spectrum antimicrobial activity. Although the flavor of spices may have been the initial reason for their use by people, their antimicrobial properties may have been an added benefit for their continued use. Study, which summarized many laboratory tests, has shown compelling evidence that spices inhibit or kill many food-borne bacteria. These researches suggest that food preservation may have been the main reason why spices were incorporated into the human diet (Levetin & Mc Mahon, 2006). Biological activity of essential oils depends on their chemical composition, which is determined by the plant genotype and is greatly influenced by several factors such as geographical origin and environmental and agronomic conditions. Many spices exert antimicrobial activity due to their essential oil fractions. The composition, structure, as well as functional groups of the oils play an important role in determining their antimicrobial activity. The compounds containing phenolic groups are usually most effective (Rota et al., 2004).

Friedman et al. (2007) stated that several antimicrobial wine recipes, each consisting of red or white wine extracts of oregano leaves with added garlic juice and oregano oil were bactericidal against *B. cereus*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica*. Olive powder also exhibited bacteriostatic effect against *B. cereus* (Ferrer et al., 2008). Carvacrol, a natural antimicrobial compound present in the essential oil fraction of oregano and thyme, is bactericidal towards *B. cereus* (Ultee et al., 2002). The use of chemical preservatives in the prevention of pathogenic and spoilage microorganisms in foods has lead to negative health effects. Microorganisms have also been known to have acquired resistance against most of the chemical preservatives over the years, the aim of the present investigation therefore, is to isolate and identify *B. cereus* from vegetable salad and also assess the antibacterial effect of essential oils of some African spices on the isolated *B. cereus* with a view to establishing the possible role of these spices in enhancing food safety.

2. Materials and Methods

2.1 Collection of Vegetable Salad

A total of fifteen samples of vegetable salad were purchased from food outlets at Mushin, Oshodi and Yaba in Lagos, Nigeria. They were taken to the laboratory for analysis without delay.

2.2 Isolation of Bacillus cereus

Using aseptic technique, 50 g of the sample was weighed and introduced into sterilized stomacher, 450 ml sterile distilled water was added and blended for 2minutes at high speed (10,000-12,000 rpm). Serial dilutions were made up to 10^{-6} and approximately, 1ml aliquots of dilutions were surface plated in duplicates on Brilliance *Bacillus cereus* Agar. The plates were incubated at 37 ºC for 24 h. Following incubation, the plates were examined, left at room temperature (25 ºC) for another 24 h and re-examined. Presumptive *Bacillus cereus* colonies were green in colour. Representative colonies of *B. cereus* were gram-stained (Whong & Kwaga, 2007)

2.3 Characterization and Identification of Bacterial Isolates

Pure cultures of bacterial isolates were identified on the basis of their morphology, biochemical characteristics and reactions to API test kits. API 20E was used in conjunction with API 50 CHB/E test kit

2.4 Collection of Spices

Dried fruits of *Aframomum melegueta, Xylopia aethiopica* and *Piper guineense* used for this work were purchased from Mushin market in Lagos State, Nigeria. The spices were identified in Botany Department, University of Lagos, Nigeria.

2.5 Extraction of Essential Oil

Dried fruits of *A. melegueta*, *X. aethiopica, P. guineense* were processed by exposing two hundred grams of fresh plant material to air dry at room temperature (28 ºC ± 2) in the laboratory for 4-5 days. The materials were pulverized in a Waring Moulineax blender for 3-5 minutes and passed through a sieve of 0.1 mm mesh size to standardize particle size. They were kept in brown envelopes at room temperature before extraction of the

essential oil. Extraction of the essential oil was by Hydrodistillation method. Two hundred gram (200 g) of the powdered plant material was hydrodistilled using a modified Clevenger type apparatus. This allowed the oils to be captured in the steam and extracted into n-hexane at 100-110 ºC for 3 h. The oil extracted into 2 ml n-hexane was carefully dispensed, dried with Na_2SO_4 and kept in tinted vials at 4 ºC until further analysis. The n-hexane is volatile and so will esvaporate from the essential oil at the end of extraction and after exposure. The isolation was carried out three (3) times on the plant material for sufficient quantity for bioassay (U.S Pharmacopeia, 2007).

2.6 Antimicrobial Activity

The testing of the bacterial cultures for the inhibitory effect of essential oil of *A. melegueta*, *X. aethiopica, P. guineense* at different concentrations (1, 0.1, 0.01, 0.001 mg/ml) using dimethyl sulfoxide (DMSO) as diluent, was performed by using well diffusion method (Das et al., 2010). The active cell suspension (0.1 ml) of the test organism was spread uniformly with the help of sterile swab stick on Mueller -Hinton agar. Four wells of 5 mm diameter each were made on the inoculated agar medium using sterilized cork borer. Measured quantity (100 µl) of each concentration was introduced into the wells with sterilized pipette. For each test organism, plates were prepared in duplicate per extract. Negative control was prepared using the diluent and extracting solvent. Streptomycin and Chloramphenicol (30 µg/ml) were used as positive control against the test organism. Plates were incubated at 37 ºC for 24 hours before being examined for zones of inhibition (Jeyakumar et al., 2011).

2.7 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of each spice extract was determined by a slight modification of the tube dilution method (Bryant, 1981). In a set of ten sterile capped test tubes using DMSO as diluent, doubling dilutions were made from the different extracts to get graded concentrations in mg/ml of 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95 and a tube containing only the diluent as the sensitivity control. The active cell suspension (0.1ml) of the test organism was spread with the help of sterile swab stick on Mueller - Hinton agar. Wells of 5mm diameter each were made on inoculated agar using sterilized cork borer. Measured quantity (100 µl) of each concentration was introduced into the wells with the help of pipette. For each test organism, plates were prepared in duplicate per extract (Jeyakumar et al., 2011). The wells were sufficiently spaced to avoid overlapping of zones of inhibition. The minimum concentration of the different spice extracts that inhibited the growth of the test organisms was taken as the MIC.

3. Result

The isolates obtained from the vegetable salad were about 5 mm in diameter and had distinctive rough turquoise to peacock blue colour. They were gram positive endospore forming rods, catalase and casein positive.

Bacillus cereus isolates were further identified as *B. cereus* 1 and *B. cereus* 2 using the API 50 CHB medium. *B. cereus* 1 fermented glycerol, D-ribose, D-glucose, D-fructose, D-mannose, N-acetyl glucosamine, arbutin, esculin ferric citrate, salicin, D-celiobiose, D-maltose, D-saccharose, D-trehalose, amidon, glycogen and potassium gluconate. *B. cereus* 2 fermented the above sugars except glycerol, D-mannose and postassium gluconate.

Both strains identified did not ferment erythritol, D-xylose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-cd-mannopyranoside, methyl-ad-glucopyranoside, amygdalin, D-lactose,D-melibiose, inulin, D-melezitose, D-raffinose, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose,L-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate and potassium 5-ketogluconate. These fermentation results helped in the identification of the two strains.

Table 1 shows the plant part, volume of solvent used, amount of spices used and volume of each extract. The colour of the spices oils was yellow, with spicy characteristic odour. The oil yield was found to be 2.05% (v/w) in *A. melegueta*, 4.08% (v/w) in *X. aethiopica* (which is similar to that obtained by Elhassan et al. (2010), and 4.05% (v/w) in *P. guineense. X.aethiopica* appears to be richer in oil than other spices, while *A. melegueta* was the poorest.

Table 1. Yield of extract of spices

Spices	plant part	volume of hexane used (ml)	weight of spices (g)	volume of extract (v/m)	oil colour
A. melegueta	fruits	2	200	2.08	yellow
X. aethiopica	fruits	2	200	4.08	yellow
P. guineense	seeds	2	200	4.05	yellow

The essential oils of *A. melegueta, X. aethiopica and P. guineense* inhibited growth of the two strains, *B. cereus* 1 and *B. cereus* 2 (Tables 2-4). This was shown by the production of zones of inhibition which ranged from 10 to 24 mm, which means that the isolates were sensitive to all the spice extracts with different zones of inhibition at different concentrations. The essential oil of *A. melegueta* had the greatest inhibitory property with zone of inhibition of 14 to 24 mm (Table 2), followed by *X. aethiopica* with inhibition zone of 10 to 18 (Table 3) and *P. guineense* with zone of inhibition of 11 to 15 (Table 4). However, *B. cereus* 1 (with inhibition zone of 12 to 24 mm) was more sensitive to the essential oils than *B. cereus* 2 (with inhibition zone of 10 to 17 mm). The higher the concentration of the essential oils the greater the resultant zones of inhibition. Streptomycin (positive control) also produced zones of inhibition of 21 and 19 mm against *B. cereus* 1 and *B. cereus* 2 respectively. The DMSO (negative control) showed no antimicrobial activity on the test organisms because no zone of inhibition was produced.

Table 2. Antibacterial activity of essential oil from *Aframomum melegueta*

Concentration of spice (mg/ml)	Zones of inhibition (mm)	
	Bacillus cereus 1	*Bacillus cereus* 2
1	24	17
0.1	24	16
0.01	22	14
0.001	20	14
Negative control (Dimethyl sulfoxide)	-	-
Positive control (Streptomycin)	21	19

-: No zone of inhibition.

Table 3. Antibacterial activity of essential oil from *Xylopia aethiopica*

Concentration of spice (mg/ml)	Zones of inhibition (mm)	
	Bacillus cereus 1	*Bacillus cereus* 2
1	18	14
0.1	17	14
0.01	13	12
0.001	12	10
Negative control (Dimethyl sulfoxide)	-	-
Positive control (Streptomycin)	21	19

-: No zone of inhibition.

The result of the minimum inhibitory concentrations of the different spice extracts is shown on Table 5. The MIC ranged from 31.25 to 250 mg/ml. The least minimum inhibitory concentration obtained was 31.25 mg/ml and from *A. melegueta* which also produced the largest zones of inhibition against the test organisms and hence the most inhibitory of the three spices. This was followed by MIC obtained from *X. aethiopica* which was 62.5

mg/ml and this spice was the second most inhibitory of the spices. *P. guineense* produced the highest MIC of 125 and 250 mg/ml against *B. cereus* 1 and *B. cereus* 2 respectively; it was the least inhibitory spice against the test organisms and especially *B. cereus* 2.

Table 4. Antibacterial activity of essential oil from *Piper guineense*

Concentration of spice (mg/ml)	Zones of inhibition (mm)	
	Bacillus cereus 1	*Bacillus cereus* 2
1	15	13
0.1	15	12
0.01	14	11
0.001	12	10
Negative control (Dimethyl sulfoxide)	-	-
Positive control (Streptomycin)	21	19

-: No zone of inhibition.

Table 5. Minimum inhibitory concentration (MIC) of the tested essential oils

	Minimum inhibitory concentration (mg/ml)		
Bacterial strains	*A. melegueta*	*X. aethiopica*	*P. guineense*
B. cereus 1	31.25	62.5	125
B. cereus 2	31.25	62.5	250

4. Discussion

This study has demonstrated that essential oils from these spices have antimicrobial properties against the tested organisms. Different parts of medicinal plants are usually selected for antimicrobial activity. Factors such as antimicrobial efficacy, sensory properties and presence of different components in the essential oils contribute to what part of the plant that is used (Gutierrez et al., 2008). Ntonifor (2011) stated that the plant, especially the spicy edible fruit is used as a spice and flavouring agent in food but also medicinally as well as to control plant pests and diseases. This conforms to the results obtained since the spices were discovered to possess antimicrobial activities against the test organisms.

The activity of the extract against the test organisms reduced with decrease in concentration of the essential oils. Therefore, the antibacterial activities of these extracts may be related to the concentration of the bioactive compounds present which gradually decreased with each successive dilution. This is in line with the work carried out by Ultee et al. (2002) who observed a decrease in the sensitivity of *B. cereus* towards carvacrol after growth in the presence of non-lethal carvacrol concentrations. *A. melegueta* showed the greatest zones of inhibition against the test organisms. This was followed by *X. aethiopica* and *P. guineense* respectively. Considering the test organisms, *B.cereus* 1 was more sensitive than *B.cereus* 2 to the essential oils from these spices. Streptomycin which served as a positive control showed equivalent antimicrobial activity, while dimethyl sulfoxide (DMSO) which served as a negative control showed no antimicrobial activity. The MIC of the plant extracts showed that the MIC of *A. melegueta* for the test organisms was the lowest, followed by that of *X. aethiopica,* and the highest was from *P. guineense*. The MIC obtained from the spices corresponds with their level of inhibitory property towards the test organisms. This proves a possible greater efficacy of *A. melegueta* at a lower concentration than the other spices especially *P. guineense* from which the highest concentration was required to inhibit the test organisms. The results obtained are comparable with previous study (Jeyakumar et al., 2011), where essential oil of *Mentha piperita* was used against *B. cereus*.

5. Conclusion

However, the spices investigated in this study will serve as useful antibacterial agents especially with regards to *B. cereus.* Therefore, they will be useful in ensuring food safety since *B. cereus* is a known food pathogen. The spices are natural preservatives and so they are of advantage over chemical preservatives which consumers now

shy away from because of their negative health effects and the resistance that microorganisms develop towards some of them. The only limitation however, is the very low concentration of these spices applied normally in our foods which may not be enough to give results obtained in this study especially in foods contaminated with this pathogen.

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Optimization of the Rheological and Sensory Properties of Stirred Yogurt as Affected by Chemical Composition and Heat Treatment of Buffalo Milk

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Abstract

The effects of fat content and the supplementation of milk with Sodium Caseinates (SCN) and Whey Proteins Concentrates (WPC) on the rheological and sensory properties of stirred yogurt made from buffalo milk were investigated. Whether the heat treatment of the milk affected the rheological behavior and the sensory characteristics of the samples was also evaluated. Principal Component Analysis (PCA) was used to assess in detail the relative contribution of whey proteins, caseins and fat on the rheological properties and sensory characteristics of the samples. Furthermore, it related the instrumental and objective sensory data to consumer perception (hedonic response of non-trained panelists). The objective acidity and white color intensity were positively correlated and increased with increasing casein content. Fat interacted synergistically with caseins to increase all the hedonic attributes, apart from odor. As far as rheological properties are concerned, elastic modulus (G'), instantaneous elasticity (G_g), retarded elasticity (G_R) and Newtonian viscosity (η_0) were positively correlated with increasing casein content. However, tan δ was negatively correlated with the aforesaid attributes and increased with increasing fat content. Whey proteins in the presence of fat determined the magnitude of flow behavior index (n). The lactic acid concentration (%) and the b component of color (yellow color intensity) were affected positively by SCN and WPC addition but in the absence of fat. In all regression equations the effect of process temperature was found to be insignificant. Finally, the consumer-optimized composition of the fat and the added SCN can be used to formulate a marketable product.

Keywords: buffalo milk, stirred yogurt, whey protein concentrates, sodium caseinates, fat content, rheological properties, sensory properties, mixture design

1. Introduction

Buffalo milk is rich in proteins, fat, calcium and magnesium and has a lower concentration of cholesterol when compared to cow's (C. D. Khedkar, G. D. Khedkar, Patil, & Kalyankar, 2003; Ahmad et al., 2008), goat's or sheep's milk (Agnihotri & Prasad, 1993; Park, Juárez, Ramos, & Haenlein, 2007). Furthermore, buffalo milk is a rich source of most water-soluble and fat-soluble vitamins (Khedkar et al., 2003). The high concentration of nutrients as well as various functional properties (higher emulsifying capacity, increased viscosity, higher curd tension, etc) makes buffalo milk suitable for the manufacturing of a wide range of dairy products including yogurt, butter, ice-cream and certain varieties of cheeses like Mozzarella (Khedkar et al., 2003).

Yogurt is a very popular fermented milk that is consumed all over the world (Sodini, Remeuf, Haddad, & Corrieu, 2004), due to its high nutritional value (Tamine & Robinson, 2007). Its classification can be made based on a variety of criteria like the chemical composition or fat content, the physical nature of the product, its flavor, or the post-fermentation processing. According to its physical nature, yogurt can be divided in two forms, set- and stirred-, with the later type being more popular (Tamine & Robinson, 2007).

The rheological properties as well as the sensory characteristics of stirred-type yogurt are important quality parameters playing a determinant role in consumers' acceptance. Furthermore, the production of high quality products as well as their standardization can be partially fulfilled by relating the perception of texture by the consumers to the instrumental information related to this quality aspect.

Protein fortification and heat treatment are the most important parameters that determine yogurt's rheological

properties (Damin, Alcântara, Nunes, & Oliveira, 2009; Akalin, Unal, Dinkci, & Hayalogut, 2012). Dry dairy ingredients such as Whey Proteins Concentrates (WPC) and Sodium Caseinates (SCN) are commonly used to increase the solids content of yogurt and thus to improve its appearance (whey separation) and physical characheristics. The addition of such ingredients increases the level of proteins in the product, enhances its hydrophilic properties and improves its viscosity (Tamine & Robinson, 2007).

The rheological characterization of yogurt, according to Sodini et al. (2004), requires at least two kinds of measurements to define flow properties and viscoelasticity. For the determination of the flow behavior of yogurt different kinds of viscometers (Skriver, Holstborg, & Qvist, 1999; Ramírez-Santiago, & Vélez-Ruiz, 2013) and rheometers (Skriver et al., 1999; Lee & Lucey, 2006; Ramirez-Santiago, Ramos-Solis, Lobato-Calleros, Pepa-Valdivia, & Vernon-Carter, 2010) have been used. Dynamic testing rheology is an excellent tool in analyzing the viscoelastic nature of yogurt (Karoui & De Baerdemaeker, 2007). Several researchers have used dynamic tests to evaluate the viscoelastic behavior of stirred yogurt as affected by the process variables (Skriver et al., 1999; Lee & Lucey, 2006), the enrichment with different components (Damin et al., 2009; Ramirez-Santiago et al., 2010; Ramírez-Santiago & Vélez-Ruiz, 2013) or the bacterial composition (Skriver et al., 1999). Besides dynamic test, creep-recovery test is a useful tool in providing information about the viscoelasticity of the material and in addition describing the nature of the bonds that predominate between the interlinked molecules of the product. However, creep analysis has not been used so far for the evaluation of the rheological behavior of yogurt.

There are few reports found in the literature concerning the study of the rheological and sensory properties of buffalo milk yogurt. Hanif, Zahoor, Iqbal, Ihsan-ul-haq and Arif (2012) studied the rheological (viscosity, hardness) and sensory (flavor, body and texture, taste, appearance) characteristics of buffalo yogurt milk as affected by the storage time and in comparison with cow milk yogurt. However, the authors did not correlate the rheological properties of the samples with the sensory perception of texture. Simanca, Andrade and Arteage (2013) evaluated the sensory acceptance of buffalo milk yogurt as affected by wheat bran addition and storage time. The effect of preservation of raw buffalo milk, by using the activation of lactoperoxidase system, on the sensory properties (texture, color, taste, flavor, overall acceptability) of the yogurt was studied by Masud, Khalid, Maqsood and Bilal (2010). However, no rheological data have been reported by any of these two research works. The aim of the present work was to study the effect of fat content, Sodium Caseinates (SCN) and Whey Proteins Concentrates (WPC) addition and heat treatment of the milk on the rheological properties and the sensory characteristics of stirred yogurt made from buffalo milk. Rheological properties were evaluated by using large (determination of the apparent viscosity) and small deformation (dynamic analysis, creep-recovery test) tests. Principal Component Analysis (PCA) was used so as to assess in detail the relative contribution of each one of the three components, as well as to relate the objective data (instrumental data and trained panelists) to consumer perception (hedonic response of non-trained panelists). Finally, a consumer-optimized combination of the three components for marketable reasons was attempted.

2. Materials and Methods

2.1 Materials

Full-fat (7.0%) as well as skimmed (0.3%) pasteurized and homogenized buffalo milk was purchased from a local dairy. The starter culture used was of Direct Vat Set (DVS) consisting of the microorganisms *Streptococcus Thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Jointex X3, Dosi 4; CSL Centro Spermentale, de Latte S.P.A, Zelo Buon Persico, Italy). SCN (MIPRODAN 30; Arla Food Ingredients, Viby J., Denmark) and WPC (Hellenic Protein S.A., Athens, Greece) were also used for yogurt production. The composition (% w/w) of the SCN was: moisture ≤6; proteins 88-93.5; fat 1.5; ash 4; lactose 0.3. WPC had the following chemical composition: moisture ≤5; proteins 80; fat 3.5; ash 3; lactose 10.4.

2.2 Preparation of Yogurt Samples

Yogurt samples were prepared from full-fat (7%), reduced-fat (5.25%), low-fat (1.75%) and skimmed buffalo milk. The reduced- and low-fat milk samples were prepared by mixing the full-fat with the skimmed milk. SCN and WPC were added according to the percentage mixture combination of the experimental design (Table 1) at the following concentrations: 0%, 1.75% and 3.5%. Each batch of milk (1400 g) was split into 2 equal parts and placed into 2 sterilized conical flasks that had a maximum capacity of 1 L. SCN and WPC were added to the milk before its heat treatment. After being added to the milk, the additives were dissolved in a Grant GLS400 shaking water-bath (Grant Instruments Ltd, Cambridge, G.B.) under continuous stirring for 20 min at 35 °C. The milk with or without the additives was then heated at 85 °C or 95 °C for 10 min, in the shaking water-bath, and cooled down at approximately 42 °C. Following heat treatment, the milk was inoculated with the starter culture.

Each conical flask (700 g) was inoculated with 26 mg of the starter culture (according the instructions of the manufacturer) and placed in the shaking water-bath at 42 °C for 5 min. The inoculated milk was then transferred into sterilized glass containers that had a maximum capacity of 1.5 kg (each container was filled with the content of the 2 conical flasks of each milk batch) and incubated (Cooled Incubator Series 8000, Termaks AS, Bergen, Norway) at 42 °C until the pH dropped to 4.6. At the end of the fermentation process yogurt samples were cooled down at 15 °C in ice water and the gel was stirred using a prototype viscometer that had a larger capacity (1500 g) than the one used for the determination of the apparent viscosity of the samples (section 2.5.1). Yogurt samples were gently stirred for 1 min at 30 rpm. Stirred yogurt was then cooled at 4 °C and stored in the refrigerator for 1 day before testing.

Table 1. Percentage mixture combinations among three design ingredients at two temperature levels

Treatment	Whey Proteins Concentrates	Sodium Caseinates	Fat	Temperature
1	0.00	0.00	7.00	85 °C
2	3.50	0.00	3.50	85 °C
3	0.00	3.50	3.50	85 °C
4	3.50	3.50	0.00	85 °C
5	0.00	1.75	5.25	85 °C
6	1.75	0.00	5.25	85 °C
7	3.50	1.75	1.75	85 °C
8	1.75	3.50	1.75	85 °C
9	1.75	1.75	3.50	85 °C
10	0.00	0.00	7.00	95 °C
11	3.50	0.00	3.50	95 °C
12	0.00	3.50	3.50	95 °C
13	3.50	3.50	0.00	95 °C
14	0.00	1.75	5.25	95 °C
15	1.75	0.00	5.25	95 °C
16	3.50	1.75	1.75	95 °C
17	1.75	3.50	1.75	95 °C
18	1.75	1.75	3.50	95°C

2.3 Physicochemical Analysis

For the determination of the pH of the yogurt samples a GP353 ATC pH METER (EDT Instruments, Kent U.K.) was used.

The colour measurements were carried out using a tristimulus colorimeter Micro Color LMC (Dr. Bruno Lange GmbH, Dusseldorf, Germany) according to the CIE Lab scale. The system provides the values of three color components according to the Hunter Lab format that is L (brightness), α (+ red to –green component) and b (+yellow to – blue component). Yogurt samples exhibited negative values for the α component and positive for the b.

The acidity of the yogurt samples expressed as lactic acid concentration (% w/w) was determined by titration with a solution of N/10 sodium hydroxide using phenolphthalein as an indicator (AOAC, 2002). Fat content was determined by the Gerber method (ISO, 1976). The chemicals used for the compositional analysis were all analytical grade.

All measurements were conducted in triplicate.

2.4 Experimental Design

The following procedure to detect and precisely determine the texture profile of a new yogurt product was followed:

1) Choice of a three components mixture design including one process variable. The latter is used as a factor with two levels in the experiment and not as a natural part of the mixture, but it may hopefully affect the blending properties of the mixtures.

2) A sensory testing plan adapted to the needs of the mixture design in order to improve its reliability.

3) Itemization of all those attributes that could influence the texture profile of the products and their segregation to four major variable sets: objective sensory, hedonic sensory, rheological and physicochemical, totaling 21 attributes.

Based on the above considerations a refined statistical procedure was followed including three steps:

1) PCA was applied to each variable set aiming to extract the most important variables that could adequately describe and represent the first two major axes (components). The first axis retains and explains a high percentage of the total variability, the second axis a lower percentage and so on. This technique is deemed successful when the first two axes explain a reasonable proportion of the data variability (say > 50%). If so, then the resulting correlation coefficients (loading factors) of the variables with each axis are expected to be high (>|0.600|) thus drafting meaningful effects. These axes were further used as response variables against the mixture ingredients, taken as independent variables, in order to establish substantial relationships between texture profile attributes and the chemical composition of the mixture design by using response contour plots. Thus, instead of conducting numerous equation models, as many as the variables under study, the equations generated by PCA provide a more economic modeling process by choosing only one or only two equations per variable set, while maintaining a high degree of reliability. A contour plot is the projected result of the equation modeling response on a two dimensional area (surface). In accordance with the values of the regression coefficients and the nature of the models (linear, quadratic, cubic) the regression equations provide a particular surface diagram (perigram) which is unique for each dependent variable (PCA axis) considered. Thus, desirable response values and mixture blends can be detected so creating an overall characteristic profile of the products that is confined into the functional ranges of the three selected ingredients.

2) The whole set of attributes, excluding the hedonic ones, was also reworked using PCA to detect meaningful patterns between variables of heterogeneous source.

3) The blending combinations of the three ingredients were thoroughly tested for potential relationships between structure and sensory attributes that could possibly lead to the most acceptable features reflecting particular treatments for product development.

Three components, whey proteins, caseins, and fat, were used in varying proportions to produce a buffalo milk product at two process temperature levels (process variable), 85 °C and 95 °C, according to the scheme shown in Figure 1 and the mixing combinations in Table 1. This design resulted in the following component ranges: 0-3.5% WPC, 0-3.5% SCN and 0-7% fat.

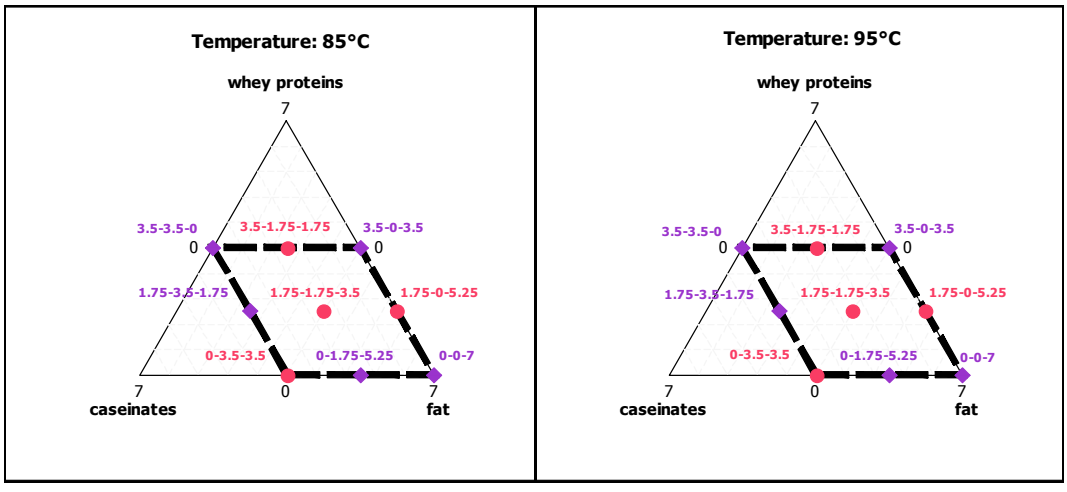


Figure 1. Mixture-amounts with extreme vertices design showing the operating area of the triangles and the points of component combinations at two temperature levels. Red circles indicate mutual reposition between the two amounts designs for the sensory assessment

The mixture design includes 9 combined points per temperature level suggesting the selection, for sensory purposes, of the plan 9B.7 as described by Kuehl (2000) with the following notation: t= 9 treatments, k= 4 treatments per panelist, b= 18 panelists, r= 8 replicates per treatment, λ= 3 pairs of similar treatments. Four randomly chosen points interchanged between the two mixture-amount levels in the sensory plan in order to facilitate equal contribution of the two temperature levels to the panelists (red circled points in Figure 1). Such designs are called Balanced Incomplete Block (BIB) designs (Kuehl, 2000) and are particularly useful when a great number of different treatments are produced and each panelist cannot evaluate so many samples in one session.

2.5 Instrumental Design

2.5.1 Flow Behavior Measurements

The flow behavior of yogurt samples was evaluated using a custom built pneumatic tube rheometer coded TR-1 Rheometer (A.T.E.I. of Thessaloniki, Greece), which has already been used for the rheological evaluation of concentrated starch solutions (Xu & Raphaelides, 1998), processed cheese (Dimitreli & Thomareis, 2004) and kefir (Yovanoudi, Dimitreli, Raphaelides, & Antoniou, 2013).

The yogurt samples were placed inside the sample vessel of the viscometer, under continuous stirring (50 rpm) and the temperature was set to 20 °C. Two capillary tubes with different dimensions, attached to the bottom of the sample vessel were used for the rheological measurements. The first one had an inner diameter of 2.050 mm and a length of 30 mm and the second had dimensions of 0.975 and 35 mm, respectively. The flow rate of the discharged fluid at the exit of the tube at varying pressures was determined so as to obtain the flow curves of the samples, that is to say the apparent viscosity as a function of the shear rate. The flow curves of the samples showed any changes to the actual values of the apparent viscosity or indicated whether the pseudoplastic behavior of the samples was affected by the different conditions. The intensive pseudoplastic behavior or the change towards a Newtonian one was evaluated by the determination of the flow behavior index (n). Rheological measurements were made in triplicate.

The sensory perception of texture can be correlated with the rheological properties related to shearing (apparent viscosity and n), imitating this way the consumption of stirred yogurt from the consumers.

2.5.2 Small Deformation Measurements

Large deformation tests (viscosity measurements) can provide information about the structure of a material, though they cannot describe its viscoelastic behavior. This can be achieved by small deformation tests which allow the study of the chemical bonds that comprise a protein network. For the study of this kind of interaction in stirred yogurt two small deformation tests, dynamic and creep-recovery tests, were applied to the samples using a DMA rheometer (Bohlin C-VOR 150, Malvern Instruments Ltd, Worcestershire, UK).

The rheometer was equipped with a 40 mm diameter plate and a 4° stainless steel cone. A Peltier plate system (-30 to +180 °C) was used for temperature control. Yogurt samples were gently mixed by stirring five times and sufficient amount was placed on the plate of the rheometer using a plastic spatula. After lowering the cone to the measuring position (the gap was set to 150 μm), the excess sample was trimmed off the edges of the cone using the spatula. Silicon oil was applied to the rim of the samples to prevent water evaporation. Rheological measurements were performed at 20 °C in triplicate.

2.5.2.1 Dynamic Test

A frequency sweep from 0.01 to 10 Hz was applied to the samples at strain deformation within the linear viscoelastic region (1.304×10^{-5}) determined by an amplitude test. The amplitude stress sweep test was conducted at a frequency of 1 Hz and stresses ranged from 1 to 100 Pa. Within the linear viscoelastic region both elastic (G') and viscous (G") moduli are stress-independent. From the frequency sweep test the G' and G" as well as the loss tangent (tan δ) were determined. G' is a measure of the energy stored and recovered per cycle, while G" is a measure of the energy dissipated or lost as heat per cycle of sinusoidal deformation (Ferry, 1980). A useful parameter in describing viscoelastic behaviour is tan δ, which give a clear indication of whether the material behaves like a solid or a liquid.

2.5.2.2 Creep-Recovery Test

A stress of 0.2 Pa within the linear viscoelastic region (previously determined) was applied to the samples, allowing them to creep, followed by a recovery time that allowed the material to recover partly its initial structure (viscoelastic material). The duration of stress application was set to maximum 180 s, while the instrument was programmed to automatic acceptance of steady state compliance with the provision that the

acceptance was to be made after the steady state was achieved for 20 s and the creep recovery time was set to 100 s. In all measurements the total strain values attained, ranged from 1×10^{-4} to 1×10^{-5} which again ensured that all samples were tested well within their linear viscoelastic limits. During these tests the instantaneous and retarded compliance as well as the Newtonian viscosity at zero shear rate (η_0) were determined. The instantaneous compliance reflects the strong chemical bonds that have not been destroyed during creep or if they did destroy they reformed again instantly. The retarded compliance reflects the weak chemical bonds that have been destroyed during creep and some of them reform during recovery time. The presence of the Newtonian viscosity (η_0) reveals that the inter-molecular network of a material comprises of entanglements that either remain stable (instantaneous compliance) or reform continuously (retarded compliance) until all the molecules of the network start to move (display of viscosity) due to the application of stress. The creep compliance data were given in the form of elastic moduli data (the reverse of compliance): instantaneous elasticity (G_g); retarded elasticity (G_R).

2.6 Sensory Analysis

Yogurt samples were set to equilibrium at 20 °C, gently mixed by stirring five times and an amount of approximately 20 g was placed in special cups arranged in white plastic dishes and presented to panelists hosted in sensory booths. The order of assessment was randomized within each session. Bottled water and toast were provided to clean the palate between samples in both objective and hedonic tests.

Sensory evaluation was partitioned in two sessions, the objective and hedonic.

The *objective* assessment concerned perception of the intensity of an attribute and was conducted twice. Panelists were selected from the staff and research students of the Faculty. All panelists had regular previous experience of sensory testing milk products and were trained to judge various product formulations of differing textures. Eighteen panelists joined the procedure, were used four times each (18 x 2 process levels x 2 runs = 72) and were trained to assess the following sensory attributes:

- Odor as the typical aroma of the product ascribed mainly to acetaldehyde.
- White color intensity as the brightness.
- Acidity as the intensity of the acid flavor in the mouth.
- Fattiness as the fatty feeling in the mouth and gum.
- Viscosity as the perceived degree of thickness when yogurt was squeezed between tongue and the roof of the mouth and sheared during the back and forth motions of the tongue.

The *hedonic* assessment concerned perception of acceptability of an attribute and used panelists with no previous training or experience in sensory testing. Students from the first two semesters were selected and were taught all the definitions of attributes in order to become familiar with the concept of the experiment. They were then asked to rate the acceptability of odor, white color, fattiness, acidity and viscosity of the samples. The hedonic evaluation was conducted twice using 72 panelists.

Both sensory evaluations use the same definition of attributes (e.g. intensity of viscosity, acceptability of viscosity) following the methodology proposed by Ritzoulis, Petridis, Derlikis, Fytianos and Asteriou, 2010 and Petridis et al. 2012. By this procedure, the overall desirability of a product is substituted for by the acceptability of the attributes assigned, so establishing a direct comparison between the intensity of an attribute and its acceptance. The same attributes were also measured using instrumental methods, rheological and colorimetric, enabling each attribute to be quantitatively linked with the blending components of the mixture experiment. Overall, the hedonic and particularly the objective sensory attributes relate to specific rheological and physicochemical characteristics, which enables the degree of sensory and rheological agreement agreement to be determined, facilitates the construction of the texture profile of the products and finally permit product optimization.

Objective sensory scores per sample were checked for possible outliers using a dotplot in which values far away from the bulk of the data were removed.

Adjusted sensory mean scores of the objective and hedonic variables were calculated from the 9 samples of each run.

2.7 Data Analysis

Regression equations between principal major axes taken as dependent responses and the mixture components considered as independent variables were calculated according to the special cubic multiple regression equation (Piepel & Cornell, 1994):

$$\hat{Y} = b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3 + b_{123} X_1 X_2 X_3 + b_{1V} X_1 V + b_{2V} X_2 V + b_{3V} X_3 V + b_{12V} X_1 X_2 V + b_{13V} X_1 X_3 V + b_{23V} X_2 X_3 V + b_{123v} X_1 X_2 X_3 V$$

where b= regression coefficient, X= mixture component (WPC, SCN, fat) and V= process temperature.

Non-significant terms at 0.05 probability reference level were removed from the equations following the step-by-step backward elimination procedure. The main components were always forced to remain in the final equation form.

A regression model was considered reliable only when the lack of model fit test was not significant ($p > 0.05$), the R^2 values of the determined and predicted coefficients were reasonably high and the determined and predicted coefficients did not differ from each other by more than 20%. Two replicates for the objective variables and two for the hedonic variables were extracted from the sensory experiments. Contour plots were chosen as to elucidate the mixture optimization conditions derived from the aforesaid equation.

3. Results and Discussion

3.1 Principal Component Analysis

3.1.1 Sensory Attributes

PCA was applied to the objective and hedonic data in order to distinguish the most important variables (Sharma, 1996). For the objective attributes, acidity and white color intensity positively form the first major axis (Table 2) which explains 38.3% of the total variation. The second axis retains 28% of data variability and is mainly represented by the viscosity in a negative direction (r = -0.746). Odor and fattiness share equal coefficients between axes thus their effect is not unique. Taking into account the results from the PCA, it is possible to treat the two variables of axis 1 as a single variable. This new variable is now axis 1 and reflects responses of acidity and white color intensity. High values of axis 1 correspond to high values of acidity and white color intensity. The white color of milk, and consequently yogurt, is caused by the light scattering of fat globules and casein micelles (Walstra, Wouters, & Geurts, 2006). Increasing the casein content causes the reflection of the light to increase presumably due to the casein aggregation, which increases the number and size of scattering particles. The increased acidity of yogurt samples is also due to the increased casein concentration. Caseins possess high buffering capacity (Salaün, Mietton, & Gaucheron, 2005). This means that more lactic acid must be produced so as to reduce the pH of the samples to its final value (4.6). Therefore, an increase in the white color intensity of the samples was associated with an increase in their acidity.

Table 2. Correlation coefficients of the four attribute sets with PCA axes 1 and 2

Objective attributes	AXIS1	AXIS2	**Hedonic attributes**	AXIS1	AXIS2
O-acidity	**0.774**	0.433	H-fattiness	**0.934**	0.070
O-odour	0.700	-0.611	H-acidity	**0.914**	0.197
O-color	**0.694**	-0.062	H-viscosity	**0.896**	0.035
O-fattines	0.549	0.528	H-color	**0.813**	0.247
O-viscosity	0.208	**-0.746**	H-odor	0.616	-0.777
Variance	1.916	1.399	Variance	3.551	0.710
Variance %	38.3	28.0	Variance %	71.0	14.2
Rheological attributes	AXIS1	AXIS2	**Physicochemical attributes**	AXIS1	AXIS2
Elastic modulus	**0.958**	-0.049	Fat content	**-0.970**	0.067
Newtonian viscosity	**0.950**	-0.108	Lactic acid concentration	**0.919**	-0.039
Instantaneous elasticity	**0.933**	0.027	b	**0.835**	0.131
Retarded elasticity	**0.933**	0.080	a	0.580	0.757
tanδ	**-0.676**	-0.147	L	-0.665	0.674
Flow behavior index	-0.050	**0.991**			
			Variance	3.260	1.050
Variance	4.021	1.025	Variance %	65.2	21.0
Variance %	67.0	17.1			

Coefficients in bold show high and unique contribution to a particular axis. Each axis explains an explicit percentage of the total variation of the data. The initial capital letters O and H denote objective and hedonic attributes, respectively. Where L, a and b are the three components of color.

All the hedonic attributes, apart from odor, describe efficiently the formation of axis 1 extracting 71% of the whole variance (Table 2). Hedonic acidity and white color intensity were positively correlated, as in the case of the objective variables.

3.1.2 Rheological Properties

The first axis of rheological attributes (67% variance retention) correlates positively with G', G_R, G_g and η_0 and negatively with tan δ (r = -0.676), the second axis is exclusively described by the n (r = +0.991) explaining also another 17.1% of data variability. The increase in G', G_R, G_g and η_0 is due to the increased casein content of the samples, which results in increased protein-protein interactions (increased number and strength), in the presence of extra casein aggregates and in a more dense matrix. According to Sodini et al. (2004) higher protein content increases the elastic character of a gel due to the increased number of protein interactions and bonds. The bonds that comprise the protein matrix are strong secondary bonds (the casein aggregates are linked to each other with secondary bonds rather than chemical ones) like hydrogen, electrostatic and hydrophobic bonds (Lucey & Singh, 1998). Taking into mind that G_g reflects the strong chemical bonds and G_R the weak chemical bonds, G_g could be a measure for the strong secondary bonds of the matrix like hydrogen and electrostatic bonds and G_R a measure for the weak secondary bonds like van der Waals, colloidal and hydrophobic bonds. These bonds contribute to increased elasticity and as a consequence, to reduced viscous behavior of the samples which exhibit lower values of tan δ. Furthermore, as can be seen from the flow curves of the yoghurt in Figure 2 the samples with the highest percentage of SCN addition (3.5%) exhibited the highest values of apparent viscosity. This means that the formed aggregates have increased hydrodynamic volume, and thus an increased resistance to flow resulting in increased values of apparent viscosity and η_0. The heat treatment of the milk did not affect the flow curves of the samples.

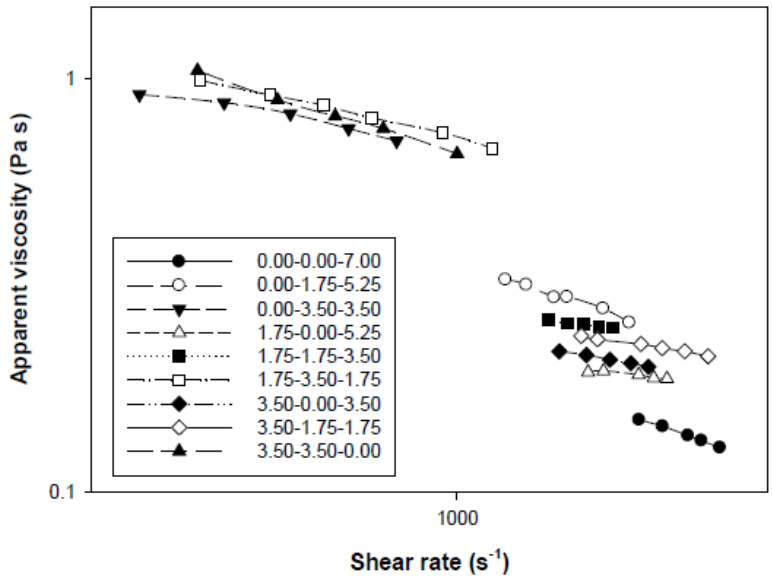


Figure 2. The flow curves of the yogurt samples. The milk for the production of the samples was heated at 85 °C for 10 min (The first number indicates the percentage of Whey Proteins Concentrates, the second the percentage of Sodium Caseinates and the third one the percentage of fat)

3.1.3 Physicochemical Properties

The physicochemical attributes lactic acid concentration (%) and b color component (yellow color intensity) had a strong negative effect the construction of axis 1 (65.2%) whereas the fat content (%) strongly negatively (r = -0.970). The construction of axis is in good agreement with the characterization of the corresponding one of the objective attributes as it concerns acidity, and simultaneously is giving more information about its characterization derived from the colorimetric test. The increased values of b (yellow color intensity) can be explained taking into consideration the combined effect of WPC and SCN but in the absence of fat. WPC exhibit a yellowish color due to the presence of riboflavin (Walstra et al., 2006). Fat globules and caseins causing light

inflection result in the white color of the samples. However, caseins in the absence of fat scatter more blue light (Walstra et al., 2006), which prevails by the yellowish color of WPC. This explains the negative correlation of the fat content with the other attributes of the axis. As it concerns acidity, both whey proteins and caseins increase the buffering capacity of the system (Salaün et al., 2005) resulting in increased lactic acid concentration of the yogurt samples.

3.2 Mixture Regression Equations

The major components were then regressed against the three mixtures components to form specific response equations (Table 3). In all regression equations the effect of process temperature was found to be insignificant thus only a pooled mixture amounts design was drawn. This can be probably attributed to the slow rate of heating during the heat treatment of milk in the water bath. The method of heating affects the degree of whey proteins denaturation (Lucey & Singh, 1998). When the heating rate is slow the amount of the denatured whey protein is increased, meaning that the absolute final value of heating has a secondary effect on the total amount of denatured whey proteins. In agreement with our results, Sodini et al. (2004) reported that the yogurt gel firmness was strongly related to the level of β -lactoglobulin denaturation for up to 60% denaturation. Between 60% and 90% β -lactoglobulin denaturation, the effect of heating intensity became less evident, and no significant differences were observed above 90%. Skriver et al. (1999) also found that the sensory perception of viscosity showed no differences when the milk was heated at different temperatures between 85 °C and 90 °C.

Table 3. Mixture regression equations for each attribute set. The higher the regression coefficient the greater the effect on the response variable

Objective attributes
AXIS1_O = -0.811(WPC) + 0.452(SCN) – 0.060(fat) + 0.206(WPC *fat)
(R^2_d=64.1% R^2_p=54.2% Lack- of- fit test: p=0.636)
AXIS2_O = 0.052(WPC) – 0.476(SCN) + 0.088(fat) + 0.121(WPC * SCN *fat)
(R^2_d=74.6% R^2_p=67.3% Lack-of-fit test: p=0.154)
Hedonic attributes
AXIS1_H = 0.072(WPC) – 0.608(SCN) + 0.010(fat) + 0.221(SCN *fat)
(R^2_d=64.7% R^2_p=54.9% Lack-of-fit test: p=0.093)
Rheological attributes
AXIS1_R = -0.384(WPC) + 0.812(SCN) – 0.188(fat) + 0.098(WPC *fat) – 0.125(SCN *fat)
(R^2_d=92.7% R^2_p=90.2% Lack-of-fit test: p=0.085)
AXIS2_R = -0.493(WPC) – 0.957(SCN) – 0.218(fat) + 0.345(WPC * SCN) + 0.275(WPC *fat) + 0.289(SCN *fat)
(R^2_d=83.7% R^2_p=77.7% Lack-of-fit test: p=0.051)
Physicochemical attributes
AXIS1_PC = 0.264(WPC) + 0.511(SCN) – 0.244(fat) – 0.088(WPC * SCN) – 0.057(SCN *fat)
(R^2_d=97.3% R^2_p=96.4% Lack-of-fit test: p=0.409)

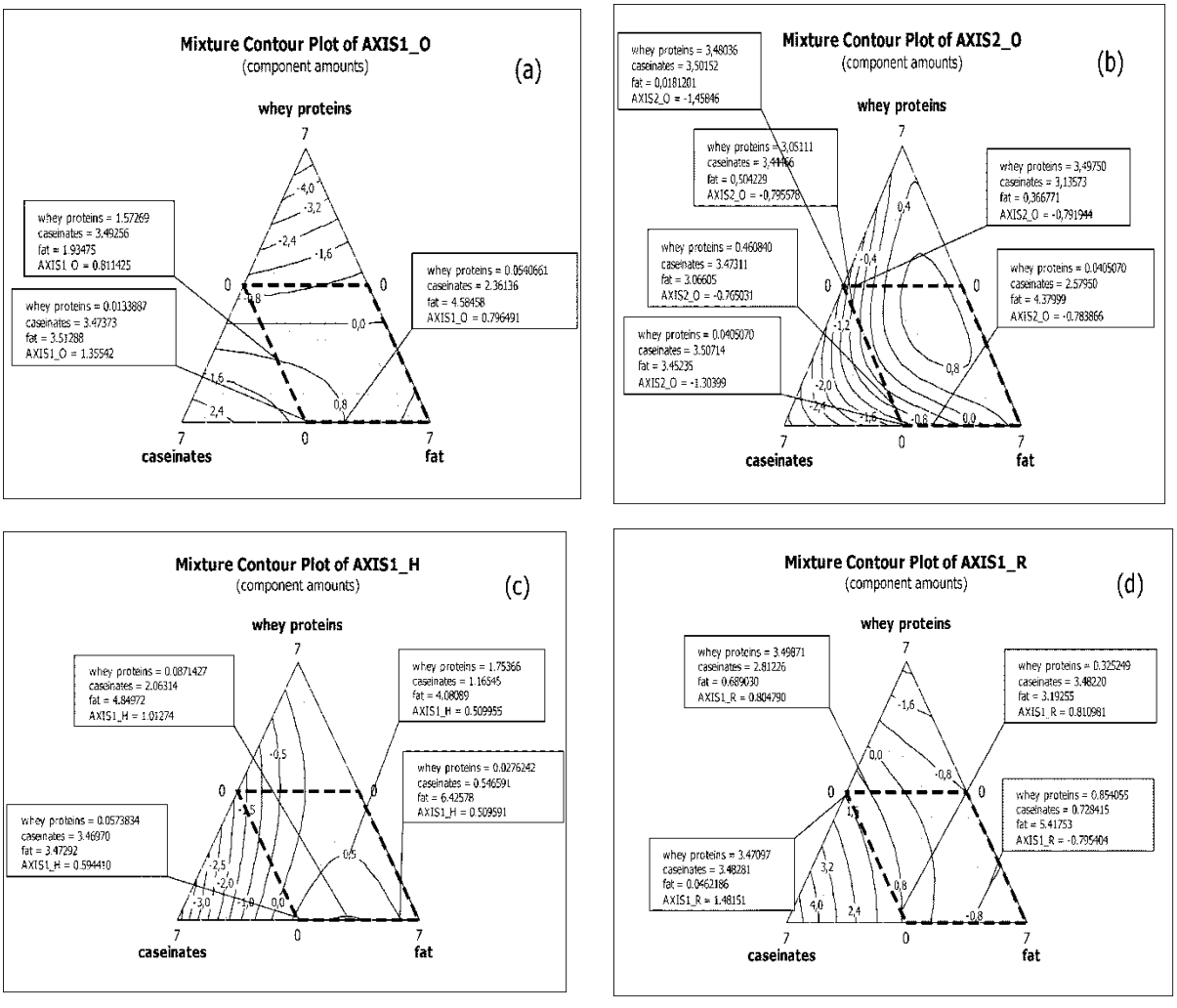
The ending capital letters O, H, R and PC, denote objective, hedonic, rheological and physicochemical origin of PCA axes 1and 2, respectively. Abbreviations are as follows, WPC: Whey Proteins Concentrates, SCN: Sodium Caseinates. R^2_d and R^2_p denote regression coefficients of determination and prediction, respectively.

It is clearly evident from Table 3 that SCN produce the highest coefficients and thereby the greatest effect on all attributes sets apart from the objective axis 1 where WPC reveal a strong negative coefficient (-0.81). This implies that the WPC increase reduces the response of AXIS1_O, that is acidity and color in the mixtures, which is mediated when fat is present (positive interactive effect, +0.206). The decrease in acidity with increasing WPC concentration is due to the lower buffering capacity of whey proteins when compared to caseins (Salaün et al., 2005). Furthermore, the white color of milk and subsequently yogurt, as already mentioned is the result of the light reflection of fat globules and caseins. Thus increasing the WPC addition resulted in reduced acidity and

white color intensity of yogurt samples. The later might be due to the decrease in fat or casein content when the WPC concentration was increased. However in the presence of fat, whey proteins increase the white color intensity. In homogenized milk the native membrane is replaced largely with casein and some whey proteins (Lucey, Munro, & Singh, 1998; Tamine & Robinson, 2007). Denaturation of whey proteins, during milk heat treatment, complexes them with the caseins and whey proteins of the fat globule membrane (Lucey et al., 1998) resulting in increased hydrodynamic volume of the fat and consequently increased light reflection.

3.3 Contour Plots

The performance of all equation terms is explicitly outlined on the response contour plots of Figure 3. The highest response of AXIS1_O (0.8-1.4 standard deviations) is confined by contour limits of WPC 0-1.6%, SCN 2.4-3.5% and fat 1.9-4.6% (Figure 3a). Obviously, caseins participate with their highest amount explaining the increased values of acidity and white color intensity, fat content exhibits medium to reduced values contributing this way to the white color intensity and whey proteins contribute minimally or not at all.



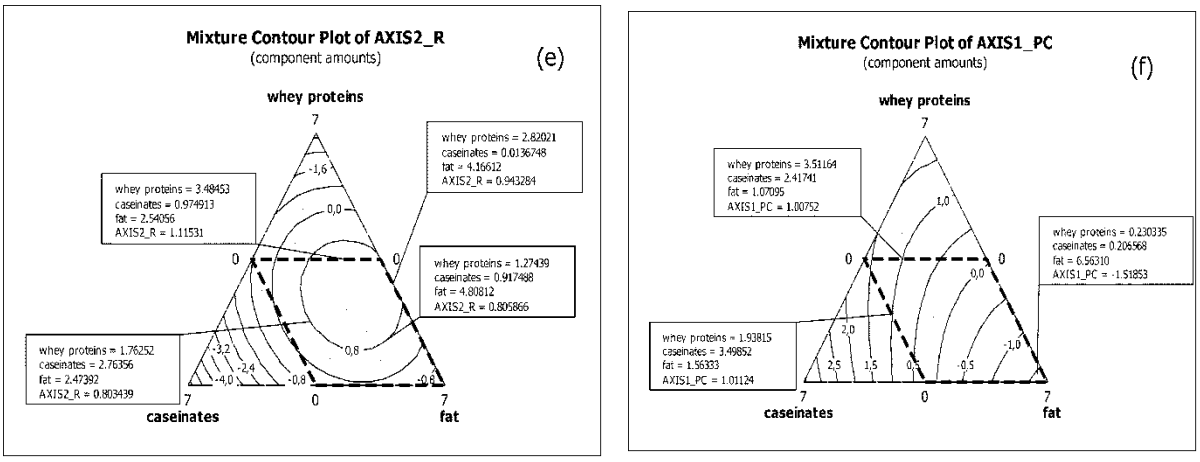


Figure 3. Contour plots of PCA axes responses for the objective (a and b), hedonic (c), rheological (d and e) and physicochemical (f) attributes of the study. The ending capital letters O, H, R and PC, denote objective, hedonic, rheological and physicochemical attributes of PCA axes 1and 2, respectively

The response of AXIS2_O (Figure 3b), which reflects viscosity, increases with SCN concentration (Table 3) because the combination of the two negative signs of component axis (Table 2) and regression coefficient produce a positive effect. Viscosity shows higher values (-0.8-(-1.0)) at two levels of WPC, 0.5% (obtuse angle between SCN and fat) and 3.5% (oblique angle between WPC and SCN), at contour level SCN 2.6-3.5% and fat 3-4.4% (Figure 3b). As can be seen, the SCN participate with their highest amount, the fat content exhibits medium values and the effect of SCN on yogurt viscosity is more pronounced than WPC. The above are in good agreement with the findings of Akalin et al. (2012) who reported that fortification of probiotic yogurt samples with caseinates resulted in increased viscosity when compared with samples made with added WPC. This can be probably attributed to the partition of the caseins into the fat globule membrane of the homogenized milk. During milk acidification, as the isoelectric pH is approached and the net negative charge on casein particles it reduces the caseins on the surface of the fat globule interact with the caseins of the protein matrix, thereby incorporating the fat into the matrix and increasing the volume fraction of fat. Sodini et al. (2004) also reported increased gel firmness of yogurt samples when the fat content was increased due to the interaction of fat globule membrane and the protein matrix.

The varimax rotation of principal axes (Sharma, 1996) proved to enhance the uniqueness of attributes' effect for a particular axis. However, this technique was abandoned since it tended to generate numerous statistically significant regression terms of higher polynomial order, the interpretation of which was excessively complex.

Hedonic fattiness, acidity, viscosity and white color intensity, all positive in PCA AXIS1 (see Table 2), decline with increasing SCN concentration (Figure 3c) covering however at maximal axis response (0.5-1.0) a broad range of SCN in the contour area (0.5-3.5%), a low WPC level (0-1.75) and a high fat content (3.5-6.4%). It is noteworthy that fat interacts synergistically with SCN (positive regression coefficient, (+0.221) increasing thereby the hedonic responses. Although increasing casein content resulted in increased viscosity, in the absence of fat the consistency of the yogurt samples was found to be crumbly, leading to reduced values of hedonic viscosity. This is a valuable indication of the necessity for the differentiation between the objective magnitude of an attribute, which is directly comparable to instrumental rheological properties, and its hedonic counterpart. However, when the increased protein content was combined with high levels of fat, the product hedonic viscosity scored high values. This can be probably attributed to the smooth consistency of the samples due to the presence of fat and its incorporation into the protein matrix. The presence of fat also increased the hedonic fattiness and white color intensity.

SCN increase the response of the rheological attributes presented by AXIS1_R (Table 3) and decrease the effect of tan δ (due to the negative sign, see Table 2). Higher axis activity (0.8-1.5) is depicted at WPC 0.3-3.5%, SCN 2.8-3.5% and fat 0-3.2% (Figure 3d). As it can be seen SCN participate with their highest amount, while WPC and fat range from very low or zero to very high or medium values, respectively. This means that SCN in the presence of WPC or fat contribute to the increased rheological properties of AXIS1_R. The combined effect of SCN and WPC can be attributed to whey protein denaturation. Denatured whey proteins associate with casein

micelles acting as bridging material and increasing the number and strength of bonds between protein particles (Lucey & Singh, 1998) resulting this way in a more dense matrix with increased rheological properties. As far as the combined effect of SCN and fat is concerned is due to the incorporation of the fat into the protein matrix as already mentioned.

The n which forms AXIS2_R (Table 2) responds negatively to a decrease in SCN concentration (Table 3) and that result is best described at levels of WPC 1.3-3.5%, SCN 0-2.8% and fat 2.5-4.8% (Figure 3e). Denatured whey proteins cover the surface of fat globules due to their interactions with the caseins of the fat globule membrane (Lucey et al., 1998), creating a "hairy" appearance. These complexes exhibit increased hydrodynamic volume due to the hydrophilic nature of whey proteins and the increased size of the unfolded denatured molecules. As a result they do not change their shape by the application of high shear rates showing this way a tendency towards Newtonian behavior (increased values of n).

SCN and WPC raise the reaction of lactic acid concentration and b values (AXIS1_PC, Table 3) but lessen the effect of fat content (negative sign in AXIS1_PC, Table 2) due to their increased concentration. Highest axis values (1.0-1.6) are achieved at WPC 1.9-3.5%, SCN 2.4-3.5% and fat 0-1.6% (Figure 3f).

3.4 Overall Product Profile

To elucidate potential interrelationships among attributes of three different datasets and mixture combinations, a PCA biplot of all attributes considered in the study was drawn (Figure 4). The two major components explain 65% of the total variation of the variables (Table 4) and produce a synoptic view of attribute performance: a bundle of attributes on the right part of the Figure 4 identifies a strong positive correlation among rheological variables (G', G_R, G_g and η_0), physicochemical ones (lactic acid concentration, α and b) and viscosity in mixtures where fat is absent and full of WPC and SCN (3.5-3.5-0%). Viscosity correlates strongly positively with G_g, η_0 and a. Caseins and whey proteins are responsible for the increased values of the mentioned rheological properties and objective viscosity. Increasing the casein content results in increased number and strength of the protein-protein interactions (strong and weak secondary bonds), increased hydrodynamic volume and consequently in increased elasticity of the protein matrix. Whey proteins participate in these interactions through their complexes with κ-casein increasing further the density and strength of the protein matrix. According to Figure 2 samples with the formulation 3.5-3.5-0% also exhibited high values of apparent viscosity. Caseins and whey proteins increased the lactic acid concentration and in the absence of fat also increase the values of b component. The increased values of α component (the decrease in the green color intensity due to the negative values of the component) can be attributed to the increased concentration of caseins that reduce the impact effect of WPC: green color due to the presence of riboflavin in the whey serum. Riboflavin is a yellow-green fluorescent compound, which is responsible for the color of milk serum (Fox & McSweeney, 1998).

Table 4. Correlation coefficients between all attributes and PCA axes 1 and 2

Attributes	AXIS1	AXIS2
Elastic modulus	**0.964**	0.107
Lactic acid concentration	**0.912**	-0.164
Fat content	**-0.909**	0.348
Newtonian viscosity	**0.909**	0.295
Retarded elasticity	**0.897**	0.095
Instantaneous elasticity	**0.857**	0.309
b	**0.809**	-0.100
O-viscosity	**0.806**	0.375
a	**0.710**	0.366
tan δ	**-0.708**	0.327
O-acidity	-0.230	-0.101
L	-0.423	**0.802**
O-color	-0.432	**0.701**
flow behaviour index	-0.012	**-0.657**
O-odour	0.200	**0.573**
O-fattines	0.061	-0.149
Variance	7.722	2.669
Variance %	48.3	16.7

Coefficients in bold show high and unique contribution to a particular axis. Both axes explain 65% of the total variation of the data. The capital letter O denotes objective sensory attributes. Where L, a and b are the three components of color.

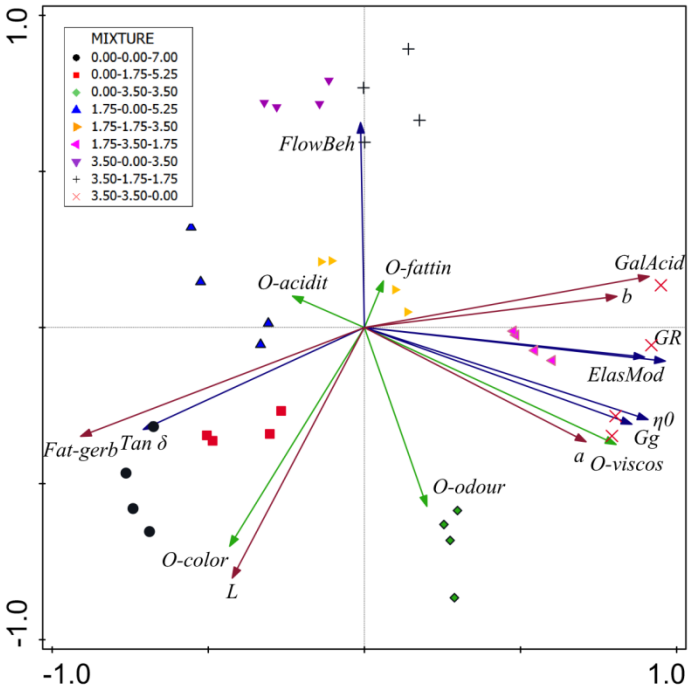


Figure 4. Biplot based on principal component analysis of sensory, rheological and physicochemical profiles at different mixture combinations (the first number indicates the percentage of Whey Proteins Concentrates (WPC), the second the percentage of Sodium Caseinates (SCN) and the third the percentage of fat) The lines indicate the approximate correlation coefficients among variables. Longer arrows denote stronger effects. A small oblique angle formed by two lines shows strong positive correlation, and a wide obtuse angle indicates strong negative correlation. The capital letter O denotes objective sensory attributes. Abbreviations are as follows, FlowBeh: Flow behavior index, ElasMod: Elastic modulus, Gg: instantaneous elasticity, GR: retarded elasticity, η0: Newtonian viscosity, Fat-gerb: Fat content, GalAcid: Lactic acid concentration, viscos: viscosity, fattin: fattiness, acidit: acidity and finally L, a and b are the three components of color

Fat content and tan δ occupy the left part of the graph showing strong positive correlation in mixtures where fat content prevails and a negative one (obtuse angle) with the opposite bundle of attributes. The fat reduces these attributes because it disturbs the rearrangement of protein aggregates that takes place during storage (Sodini et al., 2004) increasing this way their viscous behavior (increased values of tan δ).

Mixtures rich in whey proteins and fat but low in caseins have high values of n (as already explained) which negatively correlates with odor and white color due to protein deficiency but fullness of SCN (0-3.5-3.5%) and richness in fat. The white color correlates strongly positively with brightness L (objective with instrumental variable). The white color is due to the caseins and fat, while the increased values of odor are attributed to the caseins. According to Tamine and Robinson (2007) caseinates contribute to favorable aroma of yogurt.

3.5 Optimization Procedure

The unstructured sensory scale of 0 to 15 cm was further divided into five equally spaced ranks in order to facilitate a better interpretation of sensory intensity:

0-3: not at all

3-6: low

6-9: moderate

9-12: adequate

12-15: very

The fitted values of the hedonic regression equation (AXIS1_H) were sorted in ascending order and tabulated with the corresponding values of the other attributes examined in the study. Table 5 shows the resulting attribute means and the treatments 5, 14, 3 and 12 that comply with the restriction AXIS1_H values > 0.50 which was defined as the lower limit of acceptance in the contour plot (Figure 3c). The two mixtures are characterized by absence of WPC, a medium to maximum level of SCN and medium to high fat content. The process temperature does not influence the two mixtures which are scored as moderate to adequate intensities of objective sensory attributes. Tasters assess hedonic color and acidity as adequate and odor, fattiness and viscosity as moderate to adequate.

Table 5. Mean values of sensory (plus ranking equivalents), rheological and physicochemical attributes as assigned by the hedonic major axis 1 highest fitted scores of standard deviation (> 0.50)

Attributes									
AXIS1_H fits	1.020	1.020	1.020	1.020	0.616	0.616	0.616	0.616	
Treatments	5	14	5	14	3	12	3	12	
WPC	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
SCN	1.75	1.75	1.75	1.75	3.50	3.50	3.50	3.50	
Fat	5.25	5.25	5.25	5.25	3.50	3.50	3.50	3.50	
Temperature	85	95	85	95	95	85	95	85	
									Intensity rank
O-odour	8.84	8.09	7.99	8.32	10.74	9.33	9.66	9.78	moderate to adequate
O-color	10.96	11.08	11.34	10.51	11.92	10.89	11.02	11.21	adequate
O-acidity	8.01	8.45	9.44	9.33	9.22	8.03	8.60	8.29	moderate to adequate
O-fattines	8.43	8.44	9.44	9.03	9.37	8.60	9.15	7.70	moderate to adequate
O-viscosity	8.88	8.61	9.30	9.25	11.26	10.33	10.68	10.62	adequate
H-odour	9.63	8.39	8.52	8.65	9.88	8.40	9.02	8.73	moderate to adequate
H-color	11.63	11.29	11.18	10.69	10.85	10.49	10.33	11.01	adequate
H-acidity	10.92	10.15	10.09	9.30	9.80	8.94	9.47	8.74	adequate
H-fattiness	10.18	9.62	10.30	10.02	9.97	8.21	9.85	8.83	moderate to adequate
H-viscocity	10.32	9.15	10.30	9.68	9.59	6.71	8.88	8.42	moderate to adequate
Elastic modulus	260	564	267	574	1224	1112	1250	1105	
tan δ	0.931	0.736	0.936	0.714	0.776	0.898	0.740	0.862	
Newtonian viscosity	6710	7212	6659	7298	401230	390500	410010	399970	
Retarded elasticity	53	65	55	68	710	664	724	650	
Instantaneous elasticity	690	710	687	708	1398	1375	1378	1354	
Flow behavior index	0.72	0.64	0.73	0.65	0.72	0.73	0.74	0.75	
Fat content	4.85	5.15	5.00	5.10	3.15	3.10	3.30	3.10	
Lactic acid concentration	1.20	1.22	1.21	1.21	1.37	1.36	1.36	1.37	
L	89.4	89.1	90.0	89.1	89.3	89.1	89.2	88.9	
a	-5.1	-3.9	-4.0	-3.9	-4.2	-3.8	-4.2	-4.0	
b	3.9	4.6	3.8	4.6	4.9	5.5	5.0	5.7	

The initial capital letters O and H denote objective and hedonic attributes, respectively. The notation Axis_H refers to the PCA axis 1 of the hedonic attribute set. Where L, a and b are the three components of color. Abbreviations are as follows, WPC: Whey Proteins Concentrates, SCN: Sodium Caseinates.

In absolute values, the mixture with SCN 1.75% and fat 5.25% appears hedonically superior to the mixture 3.5% and 3.5% respectively, with lower objective intensities of viscosity and odor. The first mixture combination gives lower values of G', η_0, G_R and G_g, lower lactic acid concentration and b values, but higher fat content.

According to the panelists, SCN and fat results in the formation of products with favorable attributes. The

absence of WPC from the two mixtures resulted in increased acidity that coincides with adequate hedonic acidity.

Tan δ was significantly ($p < 0.001$) higher in samples heat treated at 85 °C than those heat treated at 95 °C. No other attributes were significantly affected by processing temperature.

4. Conclusion

PCA adequately assessed the contribution of whey proteins, caseins and fat to the rheological properties and sensory characteristics of stirred yogurt samples from buffalo milk. The global profile of the product was best described by the physicochemical variables, fat content (%), lactic acid and b color component, all the rheological attributes considered in the study (elastic modulus, η_0, Gg, GR, tan δ and n), the objective sensory acidity, white color and viscosity, and by nearly all the hedonic sensory attributes (acceptance of fattiness, acidity, viscosity and white color). Mixture components, rheological and physicochemical characteristics were in good agreement with the objective attributes, thereby validating the panelists' sensory evaluation. SCN and fat of medium to high content (1.75-3.5% and 3.5-5.25% respectively) could be used to formulate a commercial stirred buffalo yogurt of marketable acceptability.

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Food Preparation at Home an Example of New Practical Strategies in the Swedish Municipal Food Service - A Qualitative Study

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Abstract

The purpose of the present study was to describe how one of 290 Swedish municipalities improved its FS service by terminating their earlier service of food distribution (FD) which was the delivery of ready cooked meals produced at a central kitchen in the community and introducing food preparation at the client's home. The revised system is referred to as the new FS. This study was performed using an action research approach. The data was collected by individual- and group interviews and through participatory observations. The transcribed interview material was analysed using qualitative content analysis. The (n=30) subjects were recruited The findings of this study revealed that the new FS was experienced as being a good service, that the new municipal FS met individual needs in a better way and that the elderly recipients could participate more actively in planning and preparing their meals. In conclusion, it was found that having their food prepared at home was considered by many of the recipients to be synonymous with individually adjusted help. The results of the study could have implications for nursing, public administration and gerontology.

Keywords: action research, elderly people, home-living, food service, municipality, qualitative research

1. Introduction

In Sweden the municipal food service (FS) for elderly people living at home is a part of the municipal public social and care service. The objective of this service is to ensure proper food intake for persons who are unable to do their own shopping, and prepare their own meals. This means that the Swedish welfare system takes on the responsibility for its citizens when they have a legal social related need of care. Each municipality follows the general guidelines issued by the government but may determine its own way of organising the municipal FS according to its own circumstances. The organisation of the public social and care service in Sweden, of which the food service (FS) is one part, often differs from other welfare states, primarily due to the fact that the public social and care service is based on a State instituted legal obligation to help persons in need, and is financed by taxation (Elmér, 2000). This statutory obligation is regulated by two acts of parliament: The Health and Medical Service Act and The Social Service Act (Grönwall & Holgersson, 2000; Raadu, 2011). Each municipality in Sweden (n=290) has the responsibility to offer this service and may organise it as they wish. The fundamental requirement for a citizen to be granted the municipal FS is that they have a significant need of help as they are unable to fulfill their meal requirements themselves due, for example, to illness related physical or psychological limitations resulting in their being unable to do their own shopping and to prepare their own meals (Pajalic & Westergren, 2013a; Porter, 2007).

Today, about 60000 elderly persons use the community FS service (Berensson, 2009). The FS can be organised in various ways such as the distribution of ready prepared cold meals, warm meals, and the offer of help to buy daily food requirements, mealtime support and preparing food in the home of the client. The distribution of ready prepared meals is the most common way the FS service is used. Based on population prognosis and the increase in the number of persons over 65 years (SCB, 2009) the Swedish social services try to meet future challenges by new ways of thinking related to helping the elderly with their food requirements (Engelheart & Åhlfeldt, 2009). Further, there is demand for each municipality to use its economic resources in the best way and that the funds

should be distributed in accordance with the needs and used in a way that shows respect for good economical housekeeping (Lundgren, 2011). This reality means that the municipalities in Sweden must work hard towards finding sustainable solutions for the future in order to resolve the situation for those with the greatest need of help. This means taking action to deal with the general expansion of the number of clients who have basic social and care requirements (Lagergren et al., 2004). The public home care officers implement a specific assessment strategy to determine what type of FS an individual person requires and how much time should be accorded for each type of service required to help each individual client (Pajalic, 2013a). The range of ready prepared foods available is virtually unlimited and the quality deemed sufficient, a wide range of restaurants and supermarkets offer a large number of different meals ,both hot, cold and frozen. There is an extensive range of ready-made meals in disposable packages that can be supplemented with fresh bread, salad, vegetables and beverage, to make up full meals. This means that today there exists many alternative ways to satisfy the food requirements of the elderly that offer them greater opportunity to decide what they wish to eat and when (Lundgren, 2011).

A literature review revealed that there is no existing research material focusing on how elderly people living at home experience the new food service i.e. having their food prepared in their home.

2. Aim

The aim of the present study was to describe how one Swedish municipality improved their FS in order to put the individual at the centre and to meet their needs in a more holistic way.

3. Method

3.1 Context

The study was conducted within an average-sized municipality of about 120000 inhabitants situated in southern-east part Sweden. Total number of inhabitants in Sweden is about 9000000 (SCB, 2009). Approximately 190 elderly people, living in the municipality, used the new municipal FS where the preparation of meals is done in the recipient's home. Thereby, the earlier service based on the distribution of ready prepared hot meals was terminated. The municipality began their new FS evaluation work during 2004 and concluded that the municipality could not offer the same subsidised service to all citizens. The situation at the time was that the municipality only offered cold meals delivered once a week to elderly citizens living in the town centre and daily distribution of warm meals to those living in rural areas within the community. Distribution of ready-made-meals was perceived as an old fashioned service form that was founded during the period when the range of restaurant sand the availability of ready-made meals at grocery stores was not considered satisfactory. Based on this background the municipality decided that food distribution of ready-made meals should be replaced by food making in the homes of the elderly FS recipients. Those elderly, whose need of help was relatively low were offered help with buying groceries or to visit nearby restaurants if there was a need for this service. The supply of special diets should remain a municipal responsibility backed by medical and nursing evaluation by a district nurse. To cope with the new service, new routines for food and mealtimes were issued to the responsible personnel. The aim with the new routines was to establish good basic hygiene, food hygiene, and offer guidelines for the menus, food purchasing and the preparation of meals with sufficient energy and nutrition content.

3.2 Participants and Data Collection

This study has been carried out in spring 2013. The participants in this study were purposefully selected by a key person employed by the municipality. The selection criteria were that the participants should be personnel involved in the municipal FS at various levels, such as public home care officers, assistant nurses, and decision-makers at both administrative and political levels. Further, the study included two different groups of end users: 1) those who currently have their meals prepared in their home and who had previously had hot meals delivered from a central kitchen, 2) those who currently have municipal assistance with the purchase of prepared dishes and groceries and 3) former users who earlier had their meals supplied through the community distribution service but were re-assessed on the basis that they did not have a great enough need for municipal food support and were therefore required to solve their meal requirements themselves.

The data for this study was collected using semi-structured, individual (Kvale, 2007) and group interviews (Morgan, 1988) and observations (Spradley, 1980). Individual interviews were chosen as the data collection method as the aim was to describe and gain insight into the participant's experiences, to hear them express their point of view in their own words and what they thought was important to them (Kvale, 2007). Participant observations were chosen to gain insight into the FS process and its parts (Spradley, 1980; Stringer & Genat, 2004). The present study was performed using an action research approach (Pajalic & Westergren, 2013b; Stringer & Genat, 2004) with focus on self-reflective aspects and the participants' specific expertise (Pajalic & Westergren,

2013b; Stringer & Genat, 2004). Included in the collected data were individual interviews with elderly persons (n=8), group interviews with various professionals as public home officers and assistant nurses (n=18) and the results from participatory observations during the meal preparation in the elderly people's homes (n=4). The transcribed interview material and notes from observations were analysed using qualitative content analysis (Krippendorff, 2004). The questions used (Table 1) were given to the participants at the start of the interview which took between 30-70 minutes. The data was collected by the author (ZP) using a tape-recorder.

Table 1 Questions used in the interviews

Can you describe the municipal FS as you experience it?
What are the strengths or weaknesses of the new FS service from your point of view?
What is most important point to satisfy with the FS service from your perspective?
How would you wish to see FS organised?

3.3 Data Analysis

For this study manifest content analysis was used for analysis of the transcribed interview texts and notes from the participant observations (Krippendorff, 2004; Pajalic, Persson, Westergren, Berggren, & Skovdahl, 2012; Pajalic, Persson, Westergren, & Skovdahl, 2012b; Spradley, 1980). Qualitative content analysis is an interpretation process that focuses on similarities and differences in various parts of the transcribed text (Krippendorff, 2004). The analysis process began with reading the transcribed interviews and notes from the observations with an open mind in order to get a feeling of the whole and to create ideas for continued analysis with the aim of the study as the basis. The meaning units were then identified and condensed into codes. The analysis process focused on a description of what is clear and visible in the text. The next level of the analysis was to observe the dialectical movements between the whole and the parts and between understanding and explanation. Then a critical reflection over the codes was made and these were compared with aim of the study. Then the subcategories and categories were sorted and discussed in relation to the aim (Pajalic, 2013b). Finally the preliminary results were reconnected to the participants for member checking (Lincoln & Guba, 1985).

3.4 Ethical Considerations

The study was performed in accordance with the Helsinki Declaration (Saif, 2000), and has been examined by the Regional Ethical Review Board in Lund, Sweden (LU12/699). All participants gave their informed consent to participate after having been presented with detailed information about the study and their own participation. They were also informed that they had the right to terminate their participation at any time without it having any consequences for them.

4. Results

4.1 Introduction of the New FS

In the autumn of 2011, the decision makers decided that the new FS system should begin during April 2012. During September-October 2011 all elderly persons involved in the current FS system received written information explaining how the current service would be terminated and replaced with a new FS system based on the preparation of meals in the homes of the elderly clients and that it would require a new needs assessment which would be carried out by a community home care officer during a visit to the client's home

One of the participants noted that: "*In the old food service, all assistance decision has been suspended, we made no reassessments and it could happen to those who needed this service become better but still chose to retain this service. What is new with the new form of service is that we are time-limited aid decision up to a year and do a new review. Further another argument was that we could not offer the same food services to all our citizens. Some were given food once a week and daily. Now it's the same food service to all*".

All those persons who were users of the FS system where sent a letter with a reply form, asking them to choose between four alternatives which were: 1) Do you wish to have a new assessment for your entitlement to the new FS system? 2) Would you prefer to use another means of food supply i.e. from a private source outside the municipal service? 3) Would you like to have assistance from the municipality when shopping for groceries from which you would prepare your own meals? 4) Would you rather not have the municipal food service?

One of the participants described it as: "The *introduction of the new FS i.e. food making at home raised concern,*

there were many who had points of view on how it should be organised. There was nobody interested in preparing clients meals at home. Many of users expressed that they don't want strange people in their kitchen, rather they preferred the distribution of ready prepared meals". Especially, the most skepticism was voiced by those who had food distribution daily and their relatives. Fortunately, although at the beginning many of the participants were skeptical towards the new FS, over time they developed a more positive attitude.

4.2 Strengths of the New FS

All of the participants in the present study expressed that they were positive towards the new FS system and that they really needed this service. Initial negative attitudes had changed and those receiving the new FS service felt satisfied. One of participants described it in the following way: "*Some of them (elderly users and their relatives) had a negative attitude in the beginning but now they signal that the new FS is a good solution and that most of the users are very satisfied. I think that things have become better and more fair as those who really need the service can get it. Now it is the real need that is satisfied and not just a service, the new FS is more based on client participation".*

As additional strengths in the new FS it was put forward that for the client it is positive to be able to have meals prepared in their own home since pre-prepared meals can never replace home cooked food. Food preparation was described by the participants as a social activity where those involved can get together in the planning and implementation of meal preparation. As one of the participants expressed: "*while preparing the clients meal one can ask what do you think? Does it taste and smell good? There is the occasion for conversation while the food is being prepared".* The competence of the cook to prepare food was described as important. One of the participants described the eventual lack of this competence as: "*If the designated person from the community cannot cook then it boils down to frozen meatballs in the microwave*". Conclusively, all the participants agreed that new FS is a service with huge possibilities and that in this matter the municipality had shown great ambition and that new FS is associated with better life-quality.

4.3 Food Preparation at Home Is Synonymous With Proper Food

One of participants expressed that dish variation is presently dependent on the person who prepares the food, their knowledge and fantasy. Mealtimes can be steered to some degree by the personnel. The quality of mealtimes was described as being based on how the food is prepared. If the food is prepared using good quality ingredients it will inevitably lead to significantly better nourishment, and it is the consumers who choose the ingredients. The new FS was described as a service that brings greater variation in nourishment, taste and the appearance of the prepared meal and that was the reason why the end users preferred it. Those persons interviewed, who represented the personnel described how the FS users enjoy having food prepared in their homes especially those who have not had food prepared at home since a long time. Among other things, they enjoyed the smell of cooking. The interviewed participants representing the users who chose the distribution of unsubsidised prepared meals from an external company described how they were satisfied with this service. One of them expressed: "*I do not have anything negative to say about that*". Contrary to this, some expressed that they were disappointed over the municipality's decision to close down the prepared meal service because the alternative was much more expensive for the elderly than the municipal food service.

4.4 Challenges With the New FS

The participants among the FS personnel described how they had learnt much during the practical part of the introduction of the new FS. They described that the new service requires planning with focus on the menus for each day or week and that shopping for groceries demands planning and coordination because these moments involve different persons. Preparation procedures before food making are dependent on which personnel are on the work schedule. They described how they started by planning the first visit to a client in the morning while collecting the ingredients for the day's meal. Sometimes it is task of the client to take the ingredients from the fridge or freezer. Many participants among the FS personnel described how they often need to improvise and find alternative solutions because it often happened that the right ingredients were missing

Regarding experiences related to food making at the clients home the participants described how there was a small group of personnel who do not like to prepare food as they find the task uninteresting. This attitude has a negative influence on the overall quality of the new FS. One participant described it as: "*There are many in our group who cannot prepare food, they think that if there is food in the freezer they need not bother to prepare fresh food*".

One of the participants noted that it was not acceptable that some personnel chose not to cook the clients meal and that this is a matter for the client to decide not the FS staff. It was stressed that the role of the manager was

to ensure that the personnel carried out their instructions fully. Furthermore, the participants agreed that the negative attitudes among some of their colleagues were due to their lack of knowledge and understanding of all the parts involved in servicing home cooking and their difficulty to appreciate the service. If the client chooses an unbalanced diet the staffs tries to add more variety. All the participants agreed that the clients who have their meals prepared in their home eat more. Furthermore, the staff observed that the elderly clients enjoy their company at mealtimes. One of participants described in the following way: *I continue to cook while the client eats; I'm working on making good use of my time.*

To get some idea of the personal results of the meals, weight checks are offered. There is a particular focus on clients who show weight loss. The staff noted that often they will continue to cook while the client eats in order to make good use of their time when preparing the planned courses. One participant noted that it was important not to give the client the impression that they were anxious to be finished and to leave as soon as possible as this can affect the client's appetite.

5. Discussion

5.1 Methodological Considerations

Findings from this study are evaluated in terms of credibility, dependability and transferability (Lincoln & Guba, 1985). Credibility was assured by presenting views from different participants in order to capture their different experiences. Dependability was assured by the fact that the same researcher, the author, carried out all the interviews, observations and transcriptions. The use of a tape-recorder and verbatim transcripts, as well as referring back to and re-reading the transcripts during the analysis process, allowed the researcher to remain close to the text. The citations make it possible to assure conformability. Transferability can be considered to be achieved by the way that the study results can be transferred to different contexts. The preliminary results were reconnected to the participants for member checking (Lincoln & Guba, 1985).

5.2 Discussion of the Results

The present study shows that the introduction of the new FS undertook a process from initial doubt of the personnel to positive evaluation. The positive evaluation of the personnel was shown in another study that the attitudes of professionals in relation to the introduction of something new in their daily routines is related to their professional background (Christensson et al., 2010; Landstrom, Sidenvall, Koivisto Hursti, & Magnusson, 2007; Scott-Samuel & Springett, 2007; Westergren & Hedin, 2010). Those professionals who have a higher educational and professional background and level of possibility to see the big picture and the possible benefits of a new service are more optimistic (Landstrom, et al., 2007). Furthermore Cramm et al. (2012) showed that on-going identification of effective improvements is of importance for the quality of the care for persons with chronic conditions (Cramm, Rutten-Van Molken, & Nieboer, 2012).

Another study showed that nutrition study circles and a nutritional care policy improve nutritional care in both the short- and long-term perspective (Westergren & Hedin, 2010). The present study shows that there is still a need to work on routines and ways of changing any negative attitudes among the personnel related to food preparation as a work task. This result was confirmed in another study that showed the importance of continuously developing professional knowledge in nutrition, inter-professional communication and a comprehensive view over the consequences of FS as a whole (Pajalic, 2013a; Pajalic, Persson, Skovdahl, & Westergren, 2012; Pajalic, Persson, Westergren, & Skovdahl, 2012a). Furthermore it was shown that communication between various professionals is of importance for inter-professional collaboration (Nuno-Solinis, Berraondo Zabalegui, Sauto Arce, San Martin Rodriguez, & Toro Polanco, 2013). Transfer of accurate information related to care users between various organization levels and professionals is needed (Olsen, Hellzen, & Enmarker, 2013). Furthermore Macadam found in her study (2011) that there is need to develop and strength linkage between various care giver organizations in aim to integrate care for the elderly people (Macadam, 2011).

The present study shows that the new FS is more holistic and positively influences the life quality of the receivers. This is confirmed by results from other studies were it is shown that relatives of the municipal FS service users advocate a food preparation service in the home of the recipient reasoning that food preparation at home creates a real sense of homemade food and of being at home. Further, the studies highlighted the importance of the social aspects of the new FS that relieves loneliness and isolation among the recipients living alone and without frequent contact with other people (Pajalic, 2013c; Pajalic et al., 2012b). Another study showed that an individual's experiences are important because these experiences in combination with those of the FS personnel are important when promoting a holistic view on nutrition and better quality of life for individuals requiring help with meal preparation needs related to food intake (Odencrants, 2008).

The present study showed that food prepared at home is more adjusted to the individual. The results of other study results confirmed this and showed that FS receivers needs and rights could be achieved and that systematic work with positive personnel can be of value in the modification of municipal services to better meet individual needs (Pajalic, Skovdahl, Westergren, & Persson, 2013; Pajalic & Westergren, 2013a). Another study showed that a social network including individually adjusted help had a positive influence on the life quality of elderly people living alone (Hellström, 2003). Florin (Florin, 2007) showed in his study that care personnel need to be aware of the care receiver's preferences for participation in social and care services in order that they can plan their actions in accordance with care receivers preferences to the degree preferred by them (Florin, 2007). The results showed that municipal FS should be seen as inter-operability issue. Everyone concerned needs to be involved in the planning for improving the municipal FS, including the receivers. It is important that the involved professionals should have the possibility to emanate from their professional roles when participating in planning for positive development. The involved personnel's role continuously requires the development of their knowledge in nutrition, which also applies to both the involved professionals and the food service receivers. In conclusion, introduction of new routines appeared to arouse initial skepticism which can depend on the professional's attitudes and level of competence. The level of competence appears to be important for the individual's ability to monitor the whole picture of the new FS. It appears that food preparation at home is considered to be synonymous with individually adjusted assistance.

6. Conclusion

This study indicates that the new FS was experienced as good service and that new FS can meet individual need in a better way than distribution of ready meals. The elderly people can participate more actively in the planning and preparation of their meals. The personnel who prepare the food at elderlies home need continuous, updated knowledge regarding nutrition. Further the manager's role was stressed as being important for the continuous development in order to influence the FS personnel's attitudes related to food preparation as being an important task.

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Relevance to Practice

This study indicates that small changes in FS can meet practical and nutritional needs for elderly people. The new service seems to be more holistic in comparison with distribution of ready meals including other benefit as breaking the elderly's social isolation. Furthermore the elderly are actively involved in food making process that my strength their feelings of participation and being part of social context. Furthermore the FS needs to be continuously improved towards offering continuous knowledge development with focus on elderly's nutrition needs.

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Predicting Use of Ineffective Responsive, Structure and Control Vegetable Parenting Practices With the Model of Goal Directed Behavior

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Abstract

This study reports the modeling of three categories of ineffective vegetable parenting practices (IVPP) separately (responsive, structure, and control vegetable parenting practices). An internet survey was employed for a cross sectional assessment of parenting practices and cognitive-emotional variables. Parents (n = 307) of preschool children (3-5 years old) were recruited through announcements and postings. Models were analyzed with block regression and backward deletion procedures using IVPP scales as the dependent variables. The independent variables included validated scales from a Model of Goal Directed Vegetable Parenting Practices (MGDVPP), including: intention, habit, perceived barriers, desire, competence, autonomy, relatedness, attitudes, norms, perceived behavioral control, and anticipated emotions. The available scales accounted for 26.5%, 16.7% and 44.6% of the variance in the IVPP responsive, structure and control subscales, respectively. Different sets of diverse variables predicted the three IVPP constructs. Intentions, Habits and Perceived Behavioral Control variables were strong predictors for each of the IVPP constructs, but the subscales were specific to each IVPP construct. Parent emotional responses, an infrequently investigated variable, was an important predictor of ineffective responsive vegetable parenting practices and ineffective structure vegetable parenting practices, but not ineffective control vegetable parenting practices. An Attitude subscale and a Norms subscale predicted ineffective responsive vegetable parenting practices alone. This was the first report of psychometrically tested scales to predict use of IVPP subscales. Further research is needed to verify these findings in larger longitudinal cohorts. Interventions to increase child vegetable intake may have to reduce IVPP.

Keywords: ineffective vegetable parenting practices, model of goal directed behavior, vegetable, responsive parenting practices, structure parenting practices, control parenting practices

1. Introduction

High vegetable intake appears to lower the risks of heart disease and stroke, probably several cancers(Boeing et al., 2012), and obesity in the adult years (Ledoux, Hingle, & Baranowski, 2011). It appears, however, that the preference for (Birch, 1998) and habit of vegetable intake are established as early as the preschool years (Kudlova & Schneidrova, 2012).

Research suggests parents are important influences on child dietary intake especially in the preschool years (O'Connor et al., 2010). Three categories of food related parenting practices (i.e. parent behaviors intended to influence child food intake) have been broadly conceptualized: structure, demandingness (control), and warmth (responsiveness), and as effective or ineffective practices across those categories (Hughes, O'Connor, & Power, 2008). Effective and ineffective practices loaded independently on separate structures; a three dimensional second level (structure, control and responsiveness) with single dimension first level structure were best fit for

each set of items (Baranowski, et al., 2013). This implies that understanding the influences on effective vegetable parenting practices (EVPP) does not inform the influences on ineffective vegetable parenting practices (IVPP); and knowledge of influences on composite IVPP may not inform influences on its subscales. Parenting change programs likely need to discourage use of IVPP, which may require separate intervention components to discourage use of each IVPP subscale.

Effective interventions are based on understanding influences on a targeted behavior (Baranowski, 2011). Diverse, mostly demographic, variables have been related to food parenting practices (McPhie, Skouteris, Daniels, & Jansen, 2012). Interventions have more commonly been based on cognitive-psychosocial models of behavior (Baranowski, 2011). The Model of Goal Directed Vegetable Parenting Practices (MGDVPP) (Figure 1) obtained high levels of predictiveness of a composite indicator of EVPP (R^2 = 48.6%) (Diep et al., 2013) and of IVPP (R^2 = 40.5%) (Baranowski et al., 2013b). The influences on EVPP were strongly related to habits, while the influences on IVPP were significantly positively associated with habit of controlling vegetable practices, and desire or intrinsic motivation; and significantly negatively correlated with perceived behavioral control of negative parenting practices, the habit of active child involvement in vegetable selection, anticipated negative emotional response to negative child behavior, parent perceived autonomy, attitude about negative effects of vegetables, and descriptive norms. Since the IVPP composite scale has three subscales (Baranowski et al., 2013), it is not clear whether the same set of MGDVPP variables predict each of the subscales, or differences exist across subscales. Differences in predictors might indicate that interventions need to be specific to the type of IVPP targeted. This manuscript reports the modeling of the three IVPP subscales (responsive, structure, control) using validated scales from a MGDVPP (Baranowski et al., 2013a).

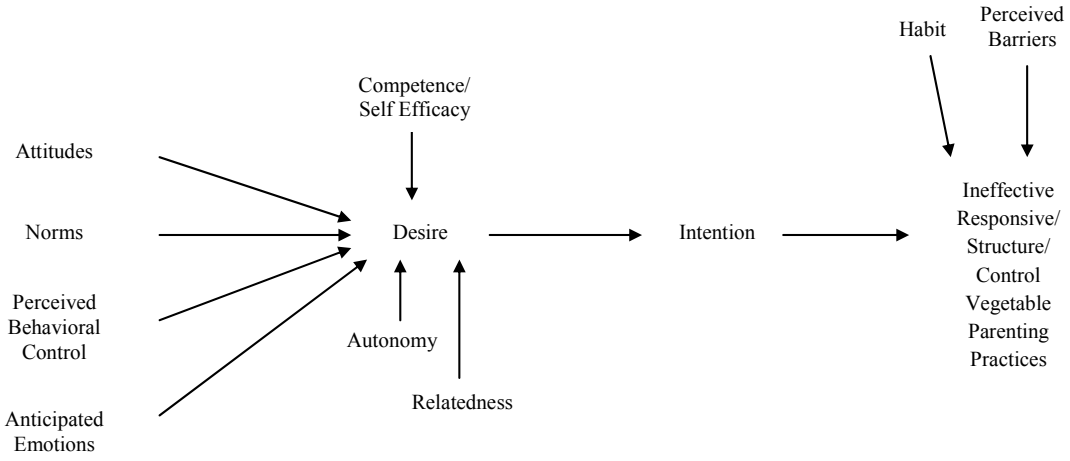

Figure 1. A model of goal directed vegetable parenting practices

2. Methods

2.1 Sample Recruitment

An internet survey was announced in a USDA/ARS Children's Nutrition Research Center (CNRC) newsletter distributed to 25,000 recipients; fliers were posted on participant volunteer billboards around the Texas Medical Center, public libraries and YMCA's in Houston; personal emails were sent to age appropriate members in the CNRC list of research volunteers; and the study was listed on the Baylor College of Medicine volunteer website.

2.2 Measures

An internet survey obtaining parent responses to the IVPP and MGDVPP items was generated using Survey Monkey (Survey Monkey, 2012). Predictor variable items were generated from intensive qualitative interviews using MGDVPP as a guide and verified with cognitive interviews with diverse parents of preschoolers (Hingle et al., 2012). The names, possible ranges, Cronbach's alphas, number of items, means and standard deviations for each scale and subscale (n = 307) appear in Table 1.

Table 1. Possible ranges, cronbach's alphas, number of items, means and standard deviations for all variables in the models predicting component ivpp using mgdvpp variables

Scales	Subscales	Possible Range	Cronbach's alpha	# of Items	Mean	SD
Vegetable Parenting Practices	Ineffective Responsiveness	5-12	0.55	5	10.64	1.30
	Ineffective Structure	5-12	0.50	5	9.16	1.48
	Ineffective Control	7-18	0.63	4	14.63	2.07
Intentions	Authoritative Parenting Intentions	11-18	0.83	6	17.50	1.31
	Active Child Involvement Intentions	6-18	0.84	6	16.05	2.41
	Controlling Parenting Intentions	5-15	0.71	5	9.54	2.59
	Permissive Parenting Intentions	2-6	0.61	2	3.66	1.28
Desire	Intrinsic Motivation	4-12	0.78	4	9.01	2.27
Perceived Barriers	Child Doesn't Like Vegetables	8-24	0.88	8	14.69	4.88
	Respondent Doesn't Like Vegetables	9-26	0.85	9	11.14	3.30
	Cost of Vegetables	5-15	0.67	5	7.53	2.34
Autonomy	Choice	4-9	0.31	3	7.92	1.06
Relatedness	Personal Values	4-12	0.81	4	7.72	2.16
	Child Wellness	3-9	0.61	3	8.26	1.15
Competence/ Self Efficacy	Advanced Competence/Self Efficacy	8-24	0.85	8	19.27	3.87
	Preliminary Competence/Self Efficacy	19-30	0.76	10	27.99	2.50
Habit	Habit of Active Child Involvement in Vegetable Selection	6-18	0.83	6	10.98	3.04
	Habit of Controlling Vegetable Practices	5-15	0.68	5	11.80	2.13
	Habit of Positive Vegetable Environment	3-8	0.67	3	3.59	0.95
	Habit of Positive Vegetable Communications	5-13	0.60	5	6.92	1.74
Anticipated Emotions	Positive Parent Emotional Response to Child Vegetable Refusal	8-23	0.92	8	9.69	2.84
	Negative Parent Emotional Response to ChildVegetable Acceptance	4-11	0.83	4	4.82	1.50
	Negative Parent Emotional Response to ChildVegetable Refusal	8-24	0.79	8	17.90	3.87
	Positive Parent Emotional Response to ChildVegetable Acceptance	4-12	0.66	4	11.38	1.17
Perceived Behavioral Control	Control of Positive Influences on Vegetable Consumption	17-39	0.85	13	34.46	4.37
	Control of Negative Influences on Vegetable Consumption	11-32	0.82	10	16.93	4.29
	Control of Negative Parenting Practices	4-12	0.54	3	7.55	1.80
Attitudes	Health Benefits of Vegetables	9-18	0.72	6	16.14	2.03
	Negative Effects of Vegetables	6-15	0.66	6	7.42	1.73
	Benefits of Vegetables other than Health	7-12	0.66	4	11.58	0.94
Norms	Descriptive Norms	2-6	0.13	3	3.86	0.83
	Normative Expectations	1-18	0.71	3	11.86	5.17

Seventeen of 32 Cronbach's alphas were > 0.7. Scales with lower alphas tended to have few items (e.g. 2-5), but had acceptable item total correlations. Tests of construct validity indicated most scales were bivariately correlated with composite scales of either EVPP or IVPP (Baranowski et al., 2013a). Single dimensions did not acceptably fit the items for most scales (Baranowski et al., 2013a). IVPP (14 items) was submitted to

confirmatory factor analyses and shown to have acceptable model fit with threesecond order and one first order factors (Baranowski et al., 2013b). The three second order factors included ineffective responsive, structure, and control vegetable parenting practices (IVPP-Responsive, IVPP-Structure and IVPP-Control, respectively).

2.3 Analyses

Models were analyzed using block regression procedures with each of the three IVPP scales as the dependent variables, separately. Block regression modeling started with demographic characteristics, then the four intention scales, one desire/intrinsic motivation scale, three barriers scales, one autonomy scale, two relatedness and two competence/self efficacy scales, four habit scales, four anticipated emotion scales, three perceived behavioral control scales, three attitude scales, and two subjective norm scales in separate blocks. Backward deletion was employed at the end of each block entry for any subscales not related to the outcome of at least $p < 0.10$. Demographic variables were retained in all models, but in light of testing three models with the same variables, any other variables not related at $p < 0.0169$ (reflecting a Bonferroni correction) in the final model were deleted. Analyses were conducted using SAS 9.3 (SAS Institute Inc., 2011).

3. Results

Participants (n = 406) provided informed consent, entered our website and initiated our questionnaire. Since the demographic questions were at the end of the survey, we did not have the necessary data to compare the 83 participants who provided incomplete, with the 307 who provided complete, data. Almost 90% of respondents with complete data were female, but more of the children were male (53.1%) (Table 2).

Table 2. Sample demographic characteristics

		n	%
Total		307	100.0
Gender of Parent			
	Male	33	10.7
	Female	274	89.3
Gender of Child			
	Male	163	53.1
	Female	144	46.9
Race/Ethnicity of Parent			
	Black/African American	60	19.5
	White	114	37.1
	Hispanic	31	10.1
	Asian	43	14.0
	Other	59	19.2
Household Highest Educational Attainment			
	HS grad or less	30	9.7
	Technical school	11	3.6
	Some college	67	21.8
	College graduate	96	31.3
	Postgrad study	102	33.2
	Missing	1	0.3
Annual Household Income (2009)			
	< $10K	11	3.6
	$10K - $19K	16	5.2
	$20K – $39K	56	18.2
	$40K - $59K	58	18.9
	≥ $60K	166	54.1

Most respondents were white (37.1%), with representation from all major racial/ethnic groups in Houston (19.5% Black/African American, 10.1% Hispanic, 14.0% Asian, and 19.2% Other). Over half of the sample (64.5%) had at least a college degree or more. Over half the sample (54.1%) had an annual household income of

$60,000 or higher.

In the final model, college graduates (standardized β = 0.153, p = 0.013) were significantly more likely than post graduates, and lower income ($10-$19,999/yr) parents significantly less likely than those making $60,000+ (standardized β = -0.156, p = 0.004) to use IVPP-Responsive. In order of relationship strength, Attitude of Negative Effects of Vegetable (standardized β = -0.244, p < 0.0001), Perceived Behavioral Control of Negative Influences on Vegetable Consumption (standardized β = -0.211, p < 0.001), Negative Parent Emotional Response to Child Vegetable Refusal (standardized β = -0.175, p = 0.001), Habit of Positive Vegetable Communications (standardized β= -0.143, p = 0.010), and Normative Expectations (standardized β = -0.141, p = 0.010) were all negatively related to IVPP-Responsive. This model accounted for 25.5% of the variance in IVPP-Responsive (See Table 3).

Table 3. Predictive models of ineffective vegetable parenting practices subscales (i.e. responsiveness, structure and control) using subscales from the model of goal directed vegetable parenting practices

	Ineffective Responsiveness		**Ineffective Structure**		**Ineffective Control**	
	Parameter Estimates		**Parameter Estimates**		**Parameter Estimates**	
	Standardized Estimate	**Standard Error**	**Standardized Estimate**	**Standard Error**	**Standardized Estimate**	**Standard Error**
Child Age	0.055	0.082	-0.043	0.098	0.052	0.117
Child Gender	0.014	0.135	-0.037	0.160	0.016	0.189
Parent Gender	-0.042	0.212	0.015	0.254	-0.009	0.300
Education						
6th grade or less	-0.013	1.126	-0.058	1.358	0.009	1.614
Attended some high school	-0.030	1.157	-0.002	1.385	0.039	1.637
High school graduate or GED	0.083	0.274	0.016	0.321	-0.007	0.381
Technical school	-0.034	0.371	-0.153**	0.450	-0.066	0.524
Some college	0.105	0.197	-0.055	0.241	-0.113	0.280
College graduate	0.153*	0.168	-0.046	0.204	-0.074	0.241
Reference group: Postgraduate	--	--	--	--	--	--
Family Income						
< $10K	0.022	0.367	0.000	0.434	0.033	0.506
$10K - $19K	-0.156**	0.309	0.102	0.376	0.041	0.447
$20K – $39K	-0.005	0.199	-0.127	0.244	0.068	0.282
$40K - $59K	-0.034	0.179	0.008	0.215	0.001	0.254
Reference group: ≥ $60K	--	--	--	--	--	--
Ethnicity						
Black/African-American	0.130	0.197	-0.006	0.238	-0.001	0.283
Hispanic/Latino	0.079	0.242	-0.065	0.289	-0.021	0.345
Other	-0.067	0.165	-0.123	0.198	0.036	0.248
Reference group: White	--	--	--	--	--	--
Negative Effects of Vegetables(Attitude)	-0.244***	0.040	--	--	--	--
Perceived Behavioral Control of Negative Influences on Vegetable Consumption	-0.211***	0.016	--	--	--	--
Negative Parent Emotional Response to Child Vegetable Refusal	-0.175**	0.018	--	--	--	--
Habit of Positive Vegetable Communications	-0.143*	0.041	--	--	--	--
Normative Expectations	-0.141*	0.013	--	--	--	--

Perceived Behavioral Control of Negative Influences on Vegetable Consumption	--	--	-0.197***	0.020	--	--
Positive Parent Emotional Response to Child Vegetable Acceptance	--	--	-0.184**	0.070	--	--
Habit of Active Child Involvement in Vegetable Selection	--	--	-0.182**	0.028	--	--
Permissive Parenting Intentions	--	--	-0.145*	0.066	--	--
Habit of Controlling Vegetable Practices	--	--	--	--	0.541***	0.056
Controlling Parenting Intentions	--	--	--	--	-0.189**	0.050
Adj R-Sq	0.255		0.167		0.418	

Legend: *p < 0.0169. **p < 0.01. *** p <0.001.

Parents with Technical School as their highest level of education were significantly less likely than post graduates (standardized β = -0.153, p = 0.009) to use IVPP-Structure. Variables significantly negatively related to IVPP-Structure in order of relationship strength included Perceived Behavioral Control of Negative Influences on Vegetable Consumption (standardized β = -0.197, $p < 0.001$), Positive Parent Emotional Response to Child Vegetable Acceptance (standardized β = -0.184, $p < 0.01$), Habit of Active Child Involvement in Vegetable Selection (standardized β = -0.182, $p = 0.002$), and Permissive Parenting Intention (standardized β = -0.145, $p = 0.014$). This model accounted for 16.7% of the variance in IVPP-Structure.

None of the demographic characteristics were significantly related to IVPP-Control. The variable significantly positively related to IVPP-Control was Habit of Controlling VPP (standardized β = 0.541, $p < 0.0001$). The variable significantly negatively related to IVPP-Control was Controlling Parenting Intentions (standardized β = -0.189, $p = 0.003$). The last model accounted for 41.8% of the variance in use of IVPP-Control.

4. Discussion

This was the first report of psychometrically tested scales to predict use of specific IVPP subscales. The available scales accounted for 25.5%, 16.7% and 41.8% of the variance in the responsive, structure and controlling IVPP, respectively, suggesting the models tapped constructs important in IVPP use. Of particular note, with minor exceptions the predictors were largely specific to the type of IVPP, and were not identical with a model predicting a composite IVPP scale (Baranowski et al., 2013b). Thus, understanding the influences on one IVPP subscale would not enhance understanding of the others.

IVPP-Responsive included items such as "I give my child something to eat or drink if they are bored" "I give my child something to eat or drink if they are upset;" and "I get too busy to notice when my child talks about food."A diverse set of variables predicted IVPP-Responsive. College graduates were more likely and those with incomes from $10,000-$19,999 were less likely to use IVPP-Responsive, suggesting an SES gradient, similar to another study (Saxton, Carnell, van Jaarsveld, & Wardle, 2009). The psychosocial variables were all negatively related to IVPP-Responsive, suggesting use of IVPP-Responsive was fairly high with each factor decreasing the generally high use. The mean value of 10.6 out of a possible 12 (Table 1) supports the generally high level of IVPP-Responsive. Parents who believed in more negative outcomes from eating vegetables (an attitude) were least likely to engage in IVPP-Responsive, which may reflect that they did not want their child to eat vegetables and used food (not vegetables) to regulate their child's emotions, or ignored their child's food related issues. Parents who believed it was easy (perceived behavioral control) to use negative influences were also less likely to use IVPP-Responsive, suggesting that these parents were more likely to use the negative than the responsive influences. Parents who negatively emotionally responded to their child refusing vegetables, were less likely to use IVPP-Responsive, perhaps suggesting they used fewer parenting practices in general. Parents with a habit (i.e. an automatically performed behavior) of positively communicating about vegetables to their child, were less likely to use IVPP-Responsive, suggesting parents with good communication habits were less likely to use IVPP-Responsive. Finally, parents reporting higher expectations of significant others (and wanting to please them) to have their child eat vegetables reported lower use of IVPP-Responsive, suggesting the significant others did not encourage use of these particular IVPP-Responsive. Accounting for 26.5% of the variance in

IVPP-Responsive (a behavior) is generally considered respectable for this type of research. The habit of positively communicating about vegetables was strongly positively related to effective responsive vegetable parenting practices (Diep et al., 2013) suggesting that positive (effective) communicators used effective and avoided ineffective vegetable parenting practices. Parents should be trained to use positive parenting communication.

IVPP-Structure included items such as "I let my child watch TV at meals" "I allow my child to drink sweet drinks" "I keep a lot of snack foods in our house;" and "I let my child wander around during a meal." A completely different set of variables predicted IVPP-Structure than IVPP-Responsive, but they were also all negatively related to the parenting subscale. A mean of 9.16 out of a possible 12 also suggests common use of IVPP-Structure, but not as high as IVPP-Responsive. Compared with parents with a post graduate degree, parents with highest education of technical school were least likely to use IVPP-Structure, which may reflect these lower educated families especially understood the ineffectiveness of these practices. The pattern of significant psychosocial predictors, here, also suggested that this sample of generally well educated parents appreciated that these practices were likely to be ineffective. Parents who perceived it easy to use (perceived behavioral control) negative influences were less likely to use IVPP-Structure, suggesting either they did not see structural practices easy to use or they used the negative influences, and didn't need to use the structured influences. Parents who responded positively emotionally to their child eating a vegetable were also less likely to use IVPP-Structure, as were parents who had a habit of actively involving their child in vegetable selection. Parents who intended to treat their child permissively were less likely to employ IVPP-Structure, suggesting they did not see IVPP-Structure as being permissive. Accounting for only 16.7% of the variance in IVPP-Structure is a limitation of this model, suggesting either substantial random error in the independent or dependent variables (making it difficult to detect any relationships), or important other predictor variables have yet to be identified. None of these variables predictive of IVPP-Structure were included in the most predictive model of the IVPP composite scale (Baranowski et al., 2013b), suggesting knowing the influences on the composite scale or on EVPP-Structure would not inform the influences on the ineffective structure subscale.

IVPP-Control included items such as "I promise my child something other than food if they finish their vegetables;" "I keep my child from going to play if they don't eat their vegetables;" and "I reward my child with sweets if they eat their vegetables." None of the variables predictive of IVPP-Control overlapped with predictors of the other IVPP subscales, and no demographic characteristic was significantly associated with IVPP-Control. Habit of Controlling Vegetable Practices was significantly positively related to IVPP-Control, suggesting that parents performed these practices automatically. Controlling Parenting Intentions was negatively related to IVPP-Control, suggesting that parents intended to use less IVPP-Control in the future. Accounting for 41.8% of the variance in IVPP-Control suggests this model tapped two important influences. The Habit of Controlling Vegetable Practices was also a significant predictor of the IVPP composite scale, indicating the very high predictiveness of the subscale accounted for predictiveness of the composite scale. Parents high in Controlling Parenting Intentions were more likely to use EVPP-Control and less likely to use IVPP-Control, indicting that intentions were important in predicting VPP-Control, but not the other IVPP subscales. A recent meta-analysis revealed only a weak relationship between intentions and behavior (Rhodes & Dickau, 2012) indicating intentions may not be as important as once thought.

When the dependent variable is an undesirable behavior (i.e. IVPP), mental gymnastics are needed to clearly state and understand the nature of especially inverse relationships (double negatives). Since respondents considered only single items at a time, they should have been uninfluenced by these mental gymnastics. The likelihood that many parents will not be able to distinguish between effective and ineffective VPP imposes limitations on a predictive cognitive model. Future research would benefit from a knowledge variable assessing the extent to which parents can accurately distinguish effective from ineffective practices.

Most of the predictive variables were generated from Theory of Planned Behavior constructs, a currently highly predictive social cognitive theory (McEachan, Conner, Taylor, & Louton, 2011). Although much of the Theory of Planned Behavior research uses single scale indicators of these constructs, the analyses of this data set indicated single dimensional variables did not fit the data (Baranowski et al., 2013). This suggests that IVPP present a complex set of behaviors requiring differentiated multi component predictors. None of the self determination theory scales or subscales (autonomy, relatedness, competence, intrinsic motivation/desire) predicted any of the IVPP subscales. Alternatively, the innovative anticipated emotion scales predicted both IVPP-Responsive and IVPP-Structure. These findings suggest the many relatively innovative variables related to IVPP would require innovative approaches to reducing IVPP (Baranowski et al., 2013b), which could be tailored to the individual IVPP components. However, further research needs to be conducted using these scales and subscales to better

understand which are the most important influences in which target groups, and assess the effect of moderators, e.g. age of child, sequential position of target child in the family (McEachan et al., 2011).

The strengths of this research include use of a broad theoretical framework and validated indicators of the independent and dependent variables. Limitations include the cross-sectional design, the self-reported nature of all variables, and the mostly higher socioeconomic status of families in the sample. Further research needs to verify the predictiveness of child dietary intake by these IVPP subscales in longitudinal samples; their utility in identifying and targeting components for reducing component IVPP in interventions; and in assessing impact of IVPP intervention programs. Innovative interventions targeting the MGDVPP constructs offer hope of reducing use of IVPP, hopefully thereby leading to increased lifelong child vegetable intake.

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Effect of High-Oxygen Packaging on Respiratory Physiology and Sensorial Qualities of Fresh Shiitake Mushrooms (*Lentinus edodes*)

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Abstract

In this research, the effect of high-oxygen packaging (HOP) with initial 80% and 100% oxygen on fresh shiitake mushrooms was studied. Initial air in package was the control treatment. All the samples were stored at 10 °C with RH 90% for 9 days. Respiration rate, hardness, TSS, and color were determined, and sensory quality was evaluated during the storage. Results indicated that high-oxygen packaging retarded the anaerobic metabolism occurrence and HOP with initial 100% oxygen could maintain the lightness of shitake mushroom better than 80% oxygen. Hardness and TSS did not show significant difference between high-oxygen packaging and control treatment. However, neither initial 100% O_2 nor 80% O_2 could reduce the respiration rate of fresh shiitake mushroom. Sensory quality especially the aroma, cap color and gill color of fresh shiitake mushroom could be better maintained in high-oxygen packaging than control treatment. The acceptability of the shiitake mushrooms after storage was the highest in HOP with initial 100% oxygen. In conclusion, high-oxygen packaging especially with initial 100% oxygen showed the obvious effect maintaining the sensory quality of fresh shiitake mushrooms although it could not reduce the respiration rate at 10 °C.

Keywords: high-oxygen, packaging, shiitake mushrooms, sensorial quality

1. Introduction

Shiitake mushroom (*Lentinus edodes*) is one of the most common edible mushrooms, and its cultivation and consumption have grown continuously (Ares et al., 2006; ÇaglarIrmak, 2007). Shiitake mushrooms have a high nutritional value and contain several nutritive compounds, including polysaccharides, antioxidants, dietary fiber, ergosterol, minerals, vitamin B1, B2 and C (Beluhan & Ranogajec, 2011; ÇaglarIrmak, 2007; Jiang et al., 2010a). However, fresh shiitake mushrooms are very easy to get deteriorate and the short shelf life of mushrooms becomes an impediment to the distribution and marketing of the fresh produce (Parentelli et al., 2007; Antmann et al., 2008). Therefore, the appropriate packaging technology to preserve their quality and prolong the shelf life becomes very important.

At present, fresh shiitake mushrooms packaged in modified atmosphere packaging (MAP) are widely researched. Traditional MAP provided oxygen with low concentration or air as initial atmospheres to reduce the respiration rate of produce, but if the oxygen inside the package is consumed fast by some produce with high respiration rate, the anaerobic metabolism will be soon induced and some fermentation off-odors such as acetaldehyde and ethanol will be released. The accumulation of these off-odors formed through anaerobic respiration may have detrimental effect for storage (Francis, 1996; Oms-Oliu et al., 2008). High oxygen packaging (HOP) as an alternative to traditional MAP has been researched to be effective to reduce the anaerobic fermentation, inhibit the microbiology growth and discoloration on some fruits and vegetables. Pérez and Sanz (2001) found that when strawberries were treated by high oxygen atmospheres (80% O_2/20% CO_2 and 90% O_2/ 10% CO_2) and low oxygen atmosphere (5% O_2, 20% CO_2), fungal growth could be prevented and strawberry firmness was enhanced under both high oxygen and low oxygen atmospheres, but color, titrable acidity, sugars and aroma

were only mildly affected by high oxygen level and more affected by low oxygen atmosphere. Besides, when sliced mushrooms (*Agaricus bisporus*) packaged in high oxygen atmosphere, the shelf life was prolonged compared with low oxygen atmosphere. However, there are also some researches reporting that exposure to high oxygen may simulated, have no effect or reduce the respiration rate of produce depending on the commodity, maturity and ripeness stage, storage temperature and time (Jacxsens et al., 2001). Respiration rate of grapefruit was stimulated under 80 kPa O_2 atmosphere at 14 °C, however exposure of "Bartlett" pear slices to 40, 60, or 80 kPa O_2 decreased their respiration rates during 4 days at 10 °C (Kader & Ben-Yehoshua, 2000). According to the results of previous study, high-oxygen packaging with initial 100% O_2 could retard the anaerobic respiration and do less damage to the tissue membrane of fresh shiitake mushrooms, and showed the potential to maintain the nutritive compounds. However, the respiratory physiology and sensorial quality of shiitake mushrooms under high-oxygen packaging, and optimal oxygen concentration for high-oxygen packaging still call for the further research.

The objective of the study was to investigate the effect of the high oxygen packaging (HOP) with different initial oxygen concentrations on the respiratory physiology and sensorial qualities of fresh shiitake mushrooms (*Lentinus edodes*) during the storage.

2. Materials and Methods

2.1 Samples Preparation, Packaging and Storage Conditions

Fresh shiitake mushrooms were harvested from Ibaraki Prefecture in Japan, and transported to the lab within 24 hours of harvest. The mushrooms were stored before packaging at 10 °C with 90% relative humidity (RH) for 2 hours until the core temperature was the same as the storage chamber.

Shiitake mushrooms (70 ± 5 g) were selected for uniform size and color, and packaged by Low-Density Polyethylene (LDPE) packages of 25 cm × 15 cm × 100 μm. Gas transmission rate of this film was 753.27 $ml \cdot m^{-2} \cdot 24h^{-1} \cdot atm^{-1}$ for O_2 and 3475.54 $ml \cdot m^{-2} \cdot 24h^{-1} \cdot atm^{-1}$ for CO_2. Then different initial gases were injected into the packages after which they were heat sealed and vacuumized: (1) High oxygen packaging 1 (HOP1): 80% O_2 balanced by N_2, (2) High oxygen packaging 2 (HOP2): 100% O_2, (3) Control: air. Then all the samples were stored at 10 °C with RH 90% for 9 days. Then the headspace gas composition, respiration rate, hardness, TSS concentration, color were determined, and sensory evaluation was also carried out on the day 0, 2, 4, 7, 9.

2.2 Gas Composition Analysis

The headspace gas composition (O_2, CO_2, ethanol and acetaldehyde) under all packaging conditions was measured on 0, 2, 4, 7, 9 days of storage. CO_2 and O_2 concentrations in the packages were determined by withdrawing a gas sample (1 ml) from the package headspace and injecting into gas chromatograph (GC-8A, Shimadzu, Japan) with a thermal conductivity detector (TCD) and the packed column ZY-2 consisting of Molecular Sieve 5A column, Porapak Q column and Shimalite Q column. The carrier gas was helium with 0.67 $ml \cdot s^{-1}$ flow rate. Column temperature was 75 °C; injector and detector temperature was 80 °C.

Ethanol and acetaldehyde concentrations were detected by injecting gas samples (1 ml) into a gas chromatograph (GC-14B, Shimadzu, Japan) with a flame ionization detector (FID) and PEG 20M column, and the carrier gases were helium with 0.89 $ml \cdot s^{-1}$ flow rate, hydrogen with 0.67 $ml \cdot s^{-1}$ flow rate and air with 8.17 $ml \cdot s^{-1}$ flow rate. Column temperature was 65 °C, injector temperature was 250 °C and detector temperature was 275 °C.

2.3 Respiration Rate Determination

The respiration rate of fresh produce can be expressed as O_2 consumption rate or CO_2 production rate (Fonseca et al., 2002). The enclosed system was used to determine the respiration rate (Iqbal et al., 2009). In this experiment, the CO_2 production rate was taken to be the respiration rate.

The vegetables were enclosed in 1 L airtight organic glass containers with a rubber stopper for gas sampling. Gas samples of 1ml were withdrawn from the container and injected into the gas chromatography (Shimadzu GC-8A, Japan) with a TCD after 0 hour, 1 hour, and 2 hours. CO_2 production rate (RCO_2) was calculated as the slope of the CO_2 concentration percent versus time curve shown as the equations (1) (Parentelli et al., 2007; Iqbal et al., 2009). It was expressed as $mg \cdot kg^{-1} \cdot h^{-1}$.

$$RCO_2 = (KCO_2 \times V_f) / m \quad (1)$$

Where RCO_2 is carbon dioxide production rate which can be expressed as $mg \cdot kg^{-1} \cdot h^{-1}$, KCO_2 is the slope of carbon dioxide concentration percent versus time curve which can be expressed as $\% \cdot h^{-1}$. V_f is the free volume of container that subtracts the sample's volume from the container volume.

2.4 Hardness, TSS and Color Determination

Hardness, total soluble solids (TSS) concentration and color were measured after the 0, 2, 4, 7, 9 days of the storage. Hardness was assessed in four symmetrical places on the mushroom cap using a hardness tester (Hardmatic Type E, Mitutoyo, Kawasaki, Japan), and mean values were generated. TSS was expressed as the Brix value (%) of the mushroom juice using a digital refractometer (PR-201α, Atago, Tokyo, Japan). Color determination was performed by assessing three equidistant points on the mushroom cap using a Chroma Meter (CR 300, Minolta, Osaka, Japan), and mean L^* (lightness), a^* (red-greenness) and b^* (yellow-blueness) were generated. Three replicates were evaluated of hardness, TSS and color per day of analysis.

2.5 Sensory Evaluation and Acceptability

The sensory quality characteristics of shiitake mushrooms were evaluated by a panel of 5 trained assessors. The sensory quality parameters were discussed by panelists and referred to other publications and decided to score from 1 to 9 points, and scoring method could be described as: (1) Aroma, which was determined by smelling the whole mushroom, and the 9= full shiitake mushroom typical scent or aroma, 7= moderate full aroma, 5= moderate and slight alcohol fermentation smell, 3= slight aroma and obvious smelly odor, 1= severely and unpleasantly smelly odor. (2) Texture was determined by pressing the cap surface with fingers. 9= very firm and resilient, 7= firm, 5= moderately firm, 3= soft and less resilient, 1= very soft and no resilient. (3) Cap color was observed visually, 9= light brown, 7= brown, 5= dark brown, 3= dark brown with black spots, 1= light black. (4) Gill color was also observed gill of shiitake mushrooms visually, in which 9= white, 7= light yellow, 5= yellow, 3= dark yellow, 1= light brown. Then the assessors made the decision that whether they would accept the shiitake mushroom to buy or not, and the acceptability was calculated. The acceptability was calculated as percentage of the accepted samples in total samples.

2.6 Statistical Analysis

All the results were expressed as mean values± standard deviation (SD). All data were analyzed by analysis of variance (ANOVA). Differences between treatments were analyzed by LSD tests and differences at $p \leq 0.05$ were considered to be significantly different.

3 Results and Discussion

3.1 Changes in Headspace Gas Composition and Respiration Rate

Changes of O_2 and CO_2 concentrations in the packages were depicted in Figure 1. Both O_2 and CO_2 concentration changed significantly over time in all packaging conditions. O_2 concentration in initial air packages decreased fast and kept < 1 % from the 2nd day of storage. Till the 4th day, O_2 concentration in initial 80% O_2 packages also decreased to lower than 1 %, which would induce the anaerobic metabolism. Initial 100% O_2 packaging maintained the oxygen concentration >10% till the 4 days of storage although after 7 days decreased lower than 1 %. CO_2 concentration in all the high oxygen packages increased above 40% till the 4th day and then fell down till the end of the storage. CO_2 concentration in air packages increased to about 20% and then decreased mildly to the end of the storage.

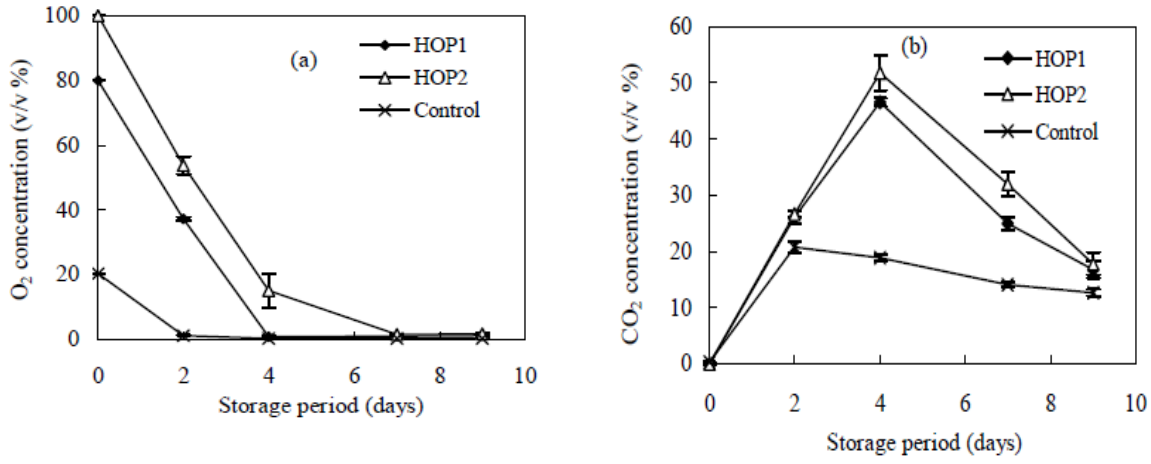

Figure 1. O_2 (a) and CO_2 (b) concentrations in packaged shiitake mushrooms stored at 10 °C. Vertical bars represent standard deviation (n=3)

Respiration rate under all the packaging conditions (showed in Figure 2) reached the climacteric peak on the second day at 10 °C, after that it decreased gradually till the end of the storage. It was also obvious that the respiration rates under 80% and 100% O_2 packaging conditions were significantly higher than air conditions. It could be manifested that respiration rate of fresh shiitake mushroom could be accelerated by increasing oxygen concentration inside the package, which also induced the higher concentration of CO_2 in 100% and 80% O_2 packages.

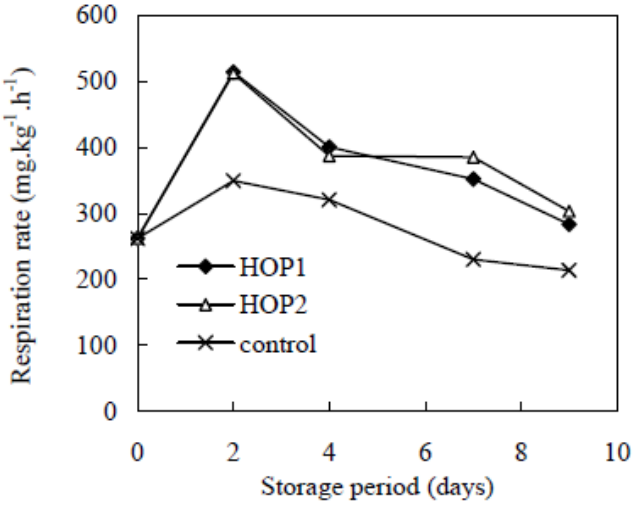

Figure 2. The changes of respiration rate of fresh shiitake mushrooms in packages with different initial oxygen concentrations

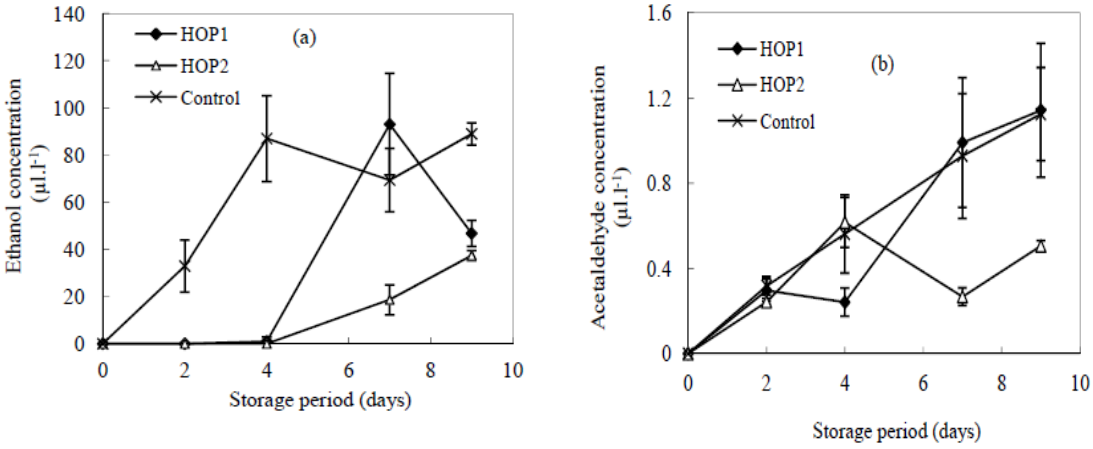

Figure 3. Ethanol and acetaldehyde concentration in packaged shiitake mushrooms stored at 10 °C. Vertical bars represent standard deviation (n=3). (a) Ethanol concentration, (b) Acetaldehyde concentration

Elevated O_2 atmospheres may influence the production and accumulation of some volatile compounds associated with respiratory metabolism such as ethanol and acetaldehyde shown in Figure 3. Ethanol gas could be detected in all high oxygen packages after 4 days when the oxygen concentration decreased to 0%, while in air packages the ethanol concentration has reached beyond 80 $\mu l \cdot l^{-1}$ with an oxygen concentration of 0%. With the storage period prolonged, ethanol and acetaldehyde concentration rose up quickly under 80% O_2 packaging and higher than air condition on Day 7, and ethanol concentration showed a decrease tendency after that. Although the fermentation off-odors could also be detected in 100% O_2 packages, the ethanol and acetaldehyde levels are much lower than 80% O_2 and air packages. Therefore low O_2 atmosphere in the package could promote the production of anaerobic metabolites, and high oxygen packaging could not inhibit the fermentation metabolism

completely, but HOP with initial 100% O_2 concentration reduced the ethanol and acetaldehyde concentration.

3.2 Changes in Hardness, TSS and Color During the Storage

Changes in hardness, total soluble solid (TSS) concentration, color parameters (L^*, a^* and b^*) of fresh shiitake mushrooms under all the high oxygen and AIR packaging conditions at 10 °C throughout 9 days' storage were depicted in Table 1. Samples under all the three packaging conditions decreased in hardness during the storage, but did not have any significant difference between treatments. Until the Day 9, hardness of the HOP1 and HOP2 samples were higher than control samples although there was not any significant difference. This result was different with the high oxygen packaging applied on strawberries which showed that HOP exhibited lower hardness compared to low O_2 MA (Van der Steen et al., 2002). TSS content of shiitake mushrooms did not show any obvious change during the storage at 10 °C, and the different packaging conditions also did not contribute any advantages for TSS content. Colors in all the color parameters did not change dramatically until the 4 days of storage, but on the Day 7, significant higher L^* was observed in HOP1, and significant higher a^* value was observed in both HOP1 and HOP2. At the end of the storage, hardness, TSS content, L^* and b^* values in high oxygen packaging were higher than AIR packaging, which manifested that high oxygen packaging could prevent the browning better than AIR packaging.

Table 1. Changes of hardness, TSS and color (L^*, a^* and b^*) in packaged shiitake mushrooms

	Hardness (N)	TSS (%)	L^*	a^*	b^*
Initial	14.78±1.65	2.28±0.43	42.54±1.86	12.68±0.32	28.55±2.06
Day 2					
HOP1 (80% O_2)	14.03±3.89 ^{a}	1.78±0.21 ^{a}	38.32±2.98 ^{a}	12.11±0.72 ^{a}	22.65±2.26 ^{a}
HOP2 (100% O_2)	19.70±6.81 ^{a}	2.03±0.24 ^{a}	39.91±5.78 ^{a}	12.32±0.99 ^{a}	23.33±2.31 ^{a}
Control(Air)	14.03±2.93 ^{a}	1.95±0.65 ^{a}	41.20±5.25 ^{a}	12.33±1.39 ^{a}	24.73±1.62 ^{a}
Day 4					
HOP1 (80% O_2)	11.63±4.01 ^{a}	1.70±0.29 ^{a}	42.68±2.63 ^{a}	12.30±0.43 ^{a}	25.39±1.77 ^{a}
HOP2 (100% O_2)	12.03±3.92 ^{a}	1.58±0.41 ^{a}	36.59±9.80 ^{a}	12.09±0.64 ^{a}	20.23±5.95 ^{a}
Control (Air)	14.58±5.42 ^{a}	1.93±0.40 ^{a}	41.98±8.63 ^{a}	11.65±0.31 ^{a}	26.20±6.00 ^{a}
Day 7					
HOP1 (80% O_2)	10.00±2.87 ^{a}	3.00±0.47 ^{a}	46.60±5.49 ^{a}	10.59±2.04 ^{ab}	28.71±5.08 ^{a}
HOP2 (100% O_2)	7.58±4.18 ^{a}	2.20±0.27 ^{a}	35.06±3.02 ^{b}	13.36±0.72 ^{a}	23.38±3.28 ^{a}
Control (Air)	9.63±3.37 ^{a}	2.55±0.75 ^{a}	39.99±2.59 ^{b}	12.13±0.98 ^{b}	26.41±2.21 ^{a}
Day 9					
HOP1 (80% O_2)	9.18±2.28 ^{a}	2.98±0.13 ^{a}	45.73±3.72 ^{a}	11.32±1.18 ^{a}	30.16±2.84 ^{a}
HOP2 (100% O_2)	7.38±3.42 ^{a}	2.90±0.41 ^{a}	43.09±5.09 ^{ab}	11.76±0.72 ^{a}	27.32±3.13 ^{ab}
Control (Air)	7.15±1.38 ^{a}	3.05±0.26 ^{a}	37.87±2.17 ^{b}	10.72±0.98 ^{a}	24.23±2.55 ^{b}

Storage temperature: 10 °C.

All the results were expressed as mean values± standard deviation (SD).

Mean values with different letters are significantly different (n=3, $p \leq 0.05$).

3.3 Changes in Sensorial Quality and Acceptability

Sensorial quality of shiitake mushrooms stored at 10 °C was determined during the storage. On the second day, aroma under all the HOP conditions was significant higher than that under AIR condition. HOP with 100% O_2 concentration initially showed higher values in texture, cap color and gill color than other packages after 2 days of storage, but didn't show any significant difference. From the Day 4 to Day 9, aroma scores for mushroom under AIR condition decreased significantly compared with other packaging conditions. However, texture scores under air condition were higher than other conditions and showed significant difference compared with 100% O_2 condition. There were not obvious changes in cap color and gill color during the whole storage period, and significant differences cannot be detected between all the packaging conditions. Therefore, the result indicated that high oxygen packaging could maintain the aroma of fresh shiitake mushrooms, but could not keep the texture better compared with the air atmosphere.

Acceptability has a highest correlation (R^2=0.66) with aroma among the four sensory parameters. Acceptability kept stable 100% in all high oxygen packages till the 4 days of storage. But about 50% samples were rejected from the 2nd day under air packaging condition. After 4 days, more samples could not be accepted and 0% acceptability was showed up in AIR condition, but about 50% in HOP till the end of the storage. Therefore, the in-package atmosphere of air accelerated the decreasing rate of acceptability of shiitake mushrooms and decreased their shelf life, when compared to mushrooms packaged under high oxygen condition during the 9 days' storage period.

Table 2. Sensory scores of packaged fresh shiitake mushrooms

	Aroma	Texture	Cap color	Gill color
Initial	8.77±0.20	8.22±0.19	7.44±0.51	7.22±1.92
Day 2				
HOP1 (80% O_2)	8.00±1.15 ^{a}	7.25±0.96 ^{a}	7.00±0.00 ^{a}	6.50±1.29 ^{a}
HOP2 (100% O_2)	8.00±1.15 ^{a}	8.00±1.15 ^{a}	7.50±1.00 ^{a}	7.50±1.00 ^{a}
Control(Air)	2.50±1.00 ^{b}	7.50±1.00 ^{a}	6.50±1.00 ^{a}	7.50±1.00 ^{a}
Day 4				
HOP1 (80% O_2)	7.50±1.00 ^{a}	6.50±1.00 ^{a}	6.50±1.00 ^{a}	6.00±1.15 ^{a}
HOP2 (100% O_2)	7.00±0.00 ^{a}	6.50±1.00 ^{a}	6.00±1.15 ^{a}	6.50±1.00 ^{a}
Control (Air)	2.50±1.91 ^{b}	7.00±1.63 ^{a}	6.00±1.15 ^{a}	7.25±0.50 ^{a}
Day 7				
HOP1 (80% O_2)	4.50±1.00 ^{a}	4.50±1.91 ^{a}	6.00±1.15 ^{a}	6.00±1.15 ^{a}
HOP2 (100% O_2)	3.50±1.00 ^{ab}	4.50±1.00 ^{a}	5.00±0.00 ^{a}	6.50±1.00 ^{a}
Control (Air)	2.50±1.91 ^{b}	6.00±1.15 ^{a}	6.00±1.15 ^{a}	6.50±1.00 ^{a}
Day 9				
HOP1 (80% O_2)	2.00±1.15 ^{ab}	4.50±1.00 ^{ab}	6.00±1.15 ^{a}	6.00±1.15 ^{a}
HOP2 (100% O_2)	3.00±1.63 ^{a}	3.50±1.00 ^{b}	5.00±0.00 ^{a}	6.00±1.00 ^{a}
Control (Air)	1.00±0.00 ^{b}	5.00±0.00 ^{a}	5.50±1.00 ^{a}	5.50±1.00 ^{a}

Storage temperature: 10 °C.

All the results were expressed as mean values± standard deviation (SD).

Mean values with different letters are significantly different (n=3, $p \leq 0.05$).

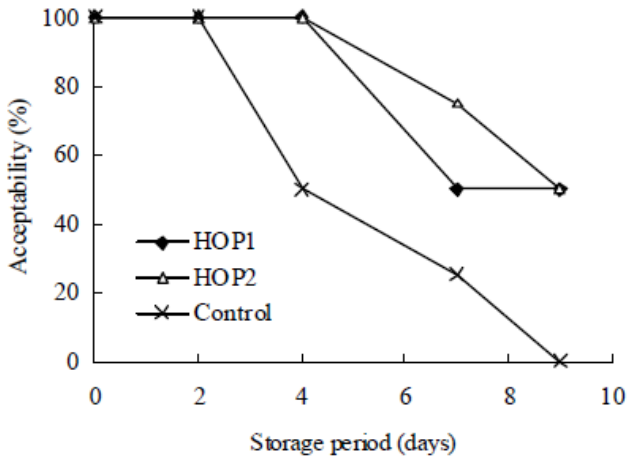

Figure 4. Acceptability of packaged shiitake mushrooms stored at 10 °C

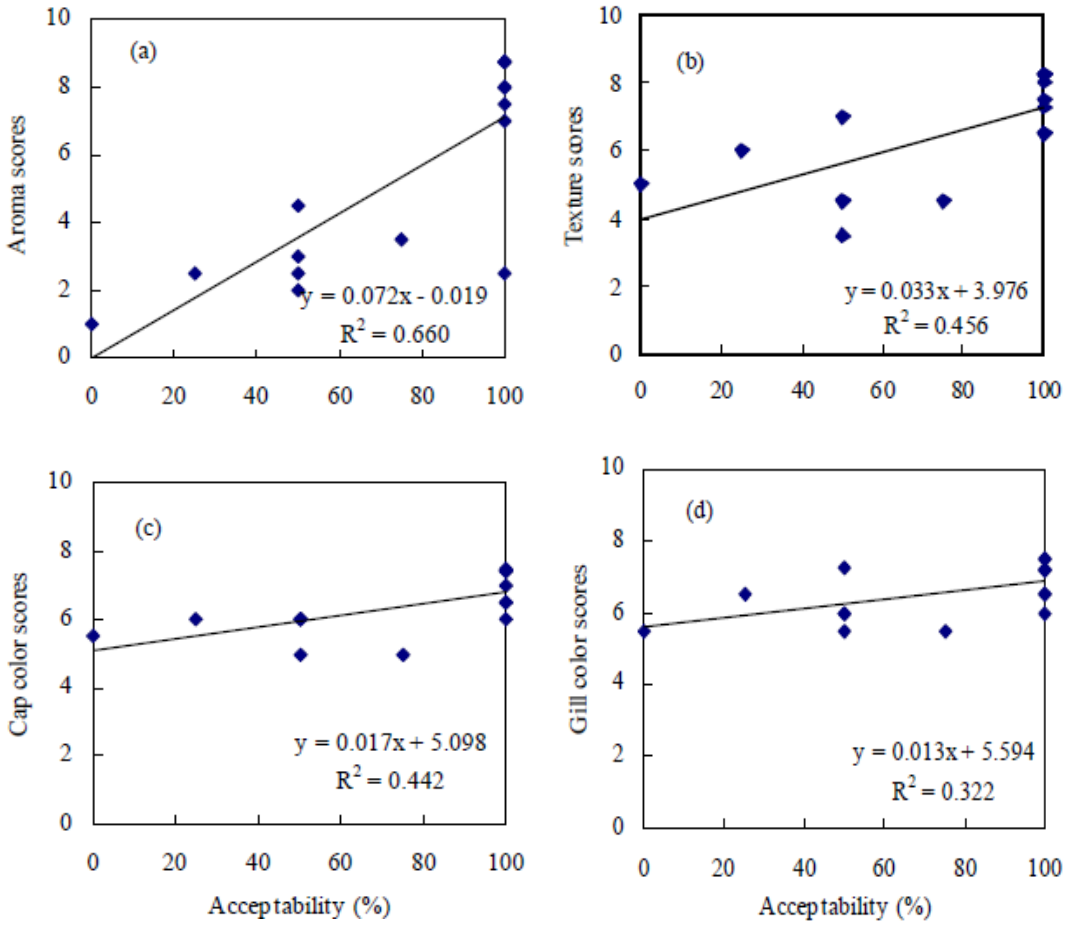

Figure 5. Correlation (R^2) between acceptability and sensory parameters

(a) Aroma, (b) Texture, (c)Cap color, (d) Gill color.

4. Conclusion

High oxygen packaging with initial 100% O_2 and 80% O_2 had beneficial effect on the sensorial quality of fresh shiitake mushrooms by retarding the anaerobic metabolism occurrence and contributing the better aroma scores compared with air packaging. HOP could not reduce the respiration rate or prevent the fermentation metabolism of shiitake mushrooms completely, but HOP with initial 100% O_2 could retard the ethanol and acetaldehyde accumulation. Mushroom's hardness, color parameters of L^* and a^* got significant higher values in HOP than in air packaging after the storage, but TSS content did not show any significant difference between treatments. Aroma played an important role when judging the sensorial quality of fresh shiitake mushrooms, and aroma scores in HOP samples were significantly higher than AIR samples. During the storage, samples stored under HOP condition especially 100% O_2 atmosphere, showed a lower deterioration rate and higher sensory quality than those stored under air condition.

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Optimization of the Lipase Catalyzed Production of Structured Acylglycerols With Polyunsaturated Fatty Acids Isolated From Sardine Oil

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Abstract

In the present work, direct enzyme-catalyzed esterification of n-3 polyunsaturated fatty acids (n-3 PUFA) isolated from sardine oil was optimized to obtain structured acyglycerols. A n-3 PUFA concentrate was prepared by urea crystallization of refined sardine oil and esterification was carried out mixing free fatty acids and glycerol at different molar ratios (M = 0.48, 1.5, 3.0, 4.5 and 5.52 mol/mol), using an immobilized lipase preparation from *Candida antarctica* (NV-435) at different temperatures (T = 38, 45, 55, 65 and 72 °C) and reaction times (t = 0.7, 2.75, 5.75, 8.75 and 10.8 h) in a rotatable central composition design. The degree of esterification was determined by analysis of the acylglycerides produced, using liquid chromatography (HPLC-ELSD). Optimization by response surface methodology (RSM) showed that in order to obtain higher esterification levels of n-3 PUFA to glycerol (99.5%), a molar ratio of 1.3 mol n-3 PUFA/mol glycerol, time 8.3 h and temperature 38 °C, are required. However, results of this work show that it is possible to drive the reaction to any determined product (MAG, DAG or TAG) by modifying the reaction conditions.

Keywords: acylglycerides, enzymatic esterification, polyunsaturated fatty acids, response surface methodology, sardine oil, hplc-elsd

1. Introduction

N-3 polyunsaturated fatty acids (n-3 PUFA) are essential fatty acids because they cannot be synthesized by humans and animals; their tissues lack the enzymatic mechanism to insert double bonds before carbon nine from the end methyl group (Makrides, Neumann, & Gibson, 1996). The well documented health beneficial effects of n-3 PUFA for cardiovascular disease, rheumatoid arthritis, immune function and cancer (Mantzioris, Cleland, & Gibson, 2000; Li, Bode, Drummond, & Sinclair, 2003; Cleland, Caughey, James, & Proudman, 2006) have promoted the rapid development of the nutraceutical and pharmaceutical markets (Young, 2003). One of the most important effects of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids is the prevention of arrhythmias (Sellmayer & Koletzco, 1999); furthermore, researchers have concluded that n-3 PUFA can reduce the amount of triacylglycerides (TAG) in 25 and 30%, with a dose of 2 and 3 g/day (Harris & Isley, 2001).

Fish oil is well known for its high content of n-3 fatty acids (FA), with documented benefits on human health (Nettleton, 1994). DHA has proven capable to reduce the risk of heart disease and inflammatory cytokines (Simopoulos, 2002; Von Schacky, 2007). Additionally, DHA inhibits tumour cells growth (Zhang, Long, Zhang, & Wang, 2007) and it can be consumed as acylglycerol for its moderate absorption and less oxidation compared to the free fatty acid form (Valenzuela, Valenzuela, Sanhueza, & Nieto, 2005). Different methods have been

developed to obtain n-3 PUFA extracts (as acylglicerides or free fatty acids) from marine oils; some of those methods have been combined to increase both efficiency and yield of the extraction (Gamez, Noriega, Medina, Ortega, García, & Angulo, 2003). n-3 PUFA are easily oxidized, for that reason enzymatic reactions have been studied for the production of oils with elevated content of n-3 PUFA, because these reactions are conducted in mild conditions (Haraldsson, Kristinsson, Sigurdardottir, Gudmundsson, & Breivik, 1997). The enzyme-catalyzed enrichment of fish oil with n-3 PUFA have been carried out via transesterification, direct esterification of glycerol with EPA and DHA, interesterification of tributyrin with ethyl esters of EPA and DHA (Haraldsson, Gudmundson, & Almarsson, 1995) and the direct esterification of the free fatty acid (FFA) from tuna oil with alcohols (Shimada, Sugihara, Nakano, Kuramoto, Nagao, Gemba, & Tominaga, 1997). The aim of the present work was the optimization, employing response surface methodology (RSM), of the enzyme-catalyzed esterification of the n-3 PUFA isolated from sardine oil.

2. Methods

To carry out the enzyme-catalyzed esterification, glycerol (reagent, J. T. Baker), molecular sieves (Sigma, 1.6 mm and 4 Å), tert-butylhydroxiquinone (TBHQ, Química Dresen) as antioxidant and nitrogen (ultra high purity, 99.999%), were employed. The commercial immobilized Novozym 435 (Novo Nordisk, Bagsbaerd, Dennmark) from *Candida antarctica* fraction B was employed as the biocatalyst. All the solvents for analysis were HPLC-grade (Fisher, Sigma-Aldrich), and they were filtered (0.45 μm) and degassed before their use. The standards used were trilinolein (C99%), 1,2 dipalmitin (C99%), 1,3 diolein (C99%), 1-monoolein (C99%) and heptadecanoic acid (C99%) that were purchased from Sigma-Aldrich (Mexico).

2.1 Concentrate Extracts of n-3 PUFA

Fresh crude oil from whole sardine (Sardinops sagax caeruleus) was obtained from a fishmeal plant located at Guaymas, Mexico. Refining (R), bleaching (B), and deodorizing (D) of the sardine oil was carried out according to recommended procedures for fish oil (Noriega, Ortega, Angulo, García, Medina, & Gámez, 2009). To obtain the n-3 PUFA from RBD sardine oil, we followed the method described by Gamez, Noriega, Medina, Ortega, García and Angulo (2003). ca. 100 g of RBD sardine oil (containing 0.02 g of TBHQ) were saponified with 200 mL 7M KOH in ethanol (70%) under reflux at 90 °C for 1h. The saponifiable fraction was extracted with distilled water (240 mL) while the unsaponifiable material was extracted with hexane (200 mL) and discarded. The aqueous layer containing the saponified matter was acidified to pH=1.0 with 3 N HCl, for the extraction of FFA with hexane (200 mL, twice), which was further evaporated in a rotary evaporator at 40 °C and 25 mm Hg. Anhydrous Na_2SO_4 was added to dry the concentrate extracts. ca. 15 g of the obtained FFA were placed in Erlenmeyer flasks with urea (25 g) and ethanol (95%, 100 mL) to be heated and stirred until the whole mixture turned into a clear homogeneous solution. The mixture was transferred to centrifugue tubes and rapidly cooled by immersion in cold water, then kept refrigerated (4 °C, 8 h). Crystals were removed by centrifugation (6000x*g*) for 20 min at 5 °C. The supernatant was kept at -30 °C for 12 h then centrifuged again (6000x*g*) at -30 °C for 20 min. Non-complexing supernatant (containing the PUFA) was acidified at pH 4.0 and equal volumes of warm (65 °C) water and hexane were added and stirred thoroughly for 30 min. The n-3 PUFA concentrate was obtained after separation of the phases and evaporation of the solvent (40 rpm, 40 °C, 25 mmHg).

2.2 Analysis of Fatty Acids

FFA were transformed into the corresponding methyl esters with 12% borontrifluoride in methanol (Ce 2-66 AOCS, 2009). A Varian 3400 gas chromatograph, equipped with a flame-ionization detector analyzed the composition of FFA. The column used was Omegawax 250 (30 m x 0.25 mm i.d., 0.25 mm film thickness; Supelco, Inc., Bellefonte, PA). The oven temperature was held at 205 °C for 5 min, then increased to 240 °C at 4 °C/min and held at 240 °C for 8 min. The injector and flame ionization detector were held at 250 and 260 °C, respectively. Nitrogen was used as carrier gas at 20 cm/s flow rate. Identification of the fatty acids was based on a menhaden oil fish standard obtained from Supelco (4-7116). Heptadecanoic acid (C17:0) was used as internal standard.

2.3 Enzyme-Catalyzed Esterification

For all of the esterification reaction trials, different temperatures (T = 38, 45, 55, 65 and 72 °C), substrates molar ratios (M = 0.48, 1.5, 3.0, 4.5 and 5.52 FFA:glycerol) and reaction times (t = 0.7, 2.75, 5.75, 8.75 and 10.8 h) were employed, according to a rotatable central composition design (Montgomery, 2009). 1.0 g of the substrates mixture (containing 0.02% w/w of TBHQ and molecular sieves 20% w/w of substrates) was mixed in a 10 mL glass vials and placed into an incubator with continuous shaking (220 rpm) at constant temperature. To start the reaction, 50 mg of lipase were added. The vials were flushed with nitrogen, sealed with rubber caps and parafilm, and continuously shaken for the trial time.

2.4 Analysis of Acylglicerols

Samples were withdrawn (10 μL) and dissolved with 5 mL of solvent extractor $CHCl_3$:CH_3OH (2:1). Alicuots (1 mL) of this mixture were transferred to 13x100 tubes for solvent evaporation by flushing nitrogen. The remaining matter was redissolved in 1 mL hexane:2-propanol (90:10) to be analyzed by HPLC as reported previously (Liu, Lee, Bobik, Guzman, & Hastilow, 1993). The analysis was carried out using a Varian 9012 HPLC system, fitted with an ELSD 500 (Evaporative Light Scatering Detector; Alltech) using Nitrogen as nebulizer gas at 2.1 bar, and the drift tube temperature was set at 90 °C. The analytical column (250 mm x 4.6 mm ID x 5μm) was a Lichrosorb Si60 (Supelco). A loop of 10 μL was employed to inject of samples into the column. The chromatographic separation was carried out at 40°C using a jacket (Alltech) connected to a recirculation water bath (Thermomix 1420; B. Braun).

2.5 Experimental Design and Statistical Analysis

Experiments were conducted using a central composite design to investigate the linear, quadratic, and cross-product effects of three factors, each varied at five levels and also included four central points for replicates. The three factors chosen were substrates molar ratio (x_1 = 0.48, 1.5, 3.0, 4.5 and 5.52 FFA/glycerol), reaction time (x_2 = 0.7, 2.75, 5.75, 8.75 and 10.8 h) and reaction temperature (x_3 = 38, 45, 55, 65 and 72 °C) for the global esterification (y_1) and the production of monoacylglycerols (y_2), diacylglycerols (y_3) and triacylglycerols (y_4).

The design of the experiments employed is depicted in Table 2. To predict the dependent variables (y_i) a polynomial regression of a second-order model was assumed as follow:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum \sum_{i<j} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where: βo, βi, βii and βij, represent the coefficients for combined, lineal, quadratic and interaction effects of the regression, respectively. In order to screen the effect of independent variables and to further determine the optimal conditions for the enzyme-catalyzed esterification, analysis of variance and RSM were performed respectively, with the statistical program JMPin 4.0.4 (SAS Institute, Inc.).

Table 1. Composition of sardine oil andconcentrated extract of n-3 PUFA (wt%)*

Fatty Acid	Sardine Oil	n-3 PUFA Concentrate
C14:0	5.65	--
C16:0	17.07	--
C16:1	5.71	7.87
C18:0	4.19	0.29
C18:1	9.15	6.82
C18:2 n-6	1.34	--
C18:3 n-3	0.18	--
C18:4 n-3	1.57	10.42
C20:1	2.97	--
C22:1	1.18	--
C20:5 n-3	8.64	18.21
C22:5 n-3	0.50	--
C22:6 n-3	10.16	52.80
Σn-3 PUFA	21.01	81.43

*Total do not add to 100% because some minor peaks of the chromatogram were not identified or concentration was below 0.1%.

Table 2. Experimental setup for enzyme-catalyzed esterification of n-3 PUFA of sardine oil

Experiment	Variables			Responses			
	x_1	x_2	x_3	y_1	y_2	y_3	y_4
1	1.5	2.75	45	81.8	52.0	23.4	6.5
2	1.5	2.75	65	80.3	40.2	33.4	6.7
3	1.5	8.75	45	93.0	50.0	26.7	16.3
4	1.5	8.75	65	71.6	33.4	23.6	14.6
5	4.5	2.75	45	75.8	58.2	12.4	5.2
6	4.5	2.75	65	60.4	34.5	14.8	11.0
7	4.5	8.75	45	68.6	33.2	8.8	26.5
8	4.5	8.75	65	66.1	18.8	8.0	39.4
9	0.48	5.75	55	90.7	41.0	35.3	14.4
10	5.52	5.75	55	58.0	34.0	7.2	16.9
11	3.0	0.7	55	25.4	17.8	4.9	2.7
12	3.0	10.8	55	73.9	31.3	16.4	26.2
13	3.0	5.75	38	72.2	43.3	26.2	2.7
14	3.0	5.75	72	78.4	32.7	20.1	25.6
15	3.0	5.75	55	70.4	35.6	25.3	9.6
16	3.0	5.75	55	73.6	33.4	26.9	13.4
17	3.0	5.75	55	66.0	31.4	23.3	11.3
18	3.0	5.75	55	66.2	37.4	18.1	10.7

x_1=substrate molar ratio (FFA/glycerol), x_2=reaction time (h), x_3=temperature (°C), y_1= global esterification (%), y_2=monoacylglycerols (%), y_3=diacylglycerols (%), y_4=triacylglycerols (%).

3. Results & Discussion

The concentrate of FFA contained 81.43% (w/w) of n-3 PUFA, and consisted of 52.8% DHA, 18.2% EPA and 10.4% octadecatetraenoic acid (Table 1). The remaining matter was composed by monounsaturated fatty acids, mainly oleic (7%). Table 2 lists the experimental parameter settings and the results based on the experimental design.

3.1 Global Esterification (GE)

The extent of esterification reached 93% for a 1.5 FFA/glycerol substrates molar ratio, 8.75 h and 45 °C (Table 2). Esteban, Robles, Jiménez, Ibáñez & Molina (1998), used a cod liver oil concentrate and obtained 68.5% of esterification after 48 h and for the following 48 h, it only increased by 5.3%. In other study, 92.5% of esterification was reported by Robles, Esteban, Giménez, Camacho, Ibáñez & Molina (1999) after 24 h. The second order polynomial regression to experimental data generated the following model for esterification:

$$y_1 = 205.7 - 16.2x_1 + 10.4x_2 - 4.9x_3 + 1.9x_1^2 + 0.098x_1x_2 - 0.45x_2^2 - 0.02x_1x_3 - 0.06x_3x_2 + 0.04x_3^2 \quad (2)$$

where y_1 is the extent of global esterification, $x_1$the substrates molar ratio, x_2 the reaction time and x_3 the temperature.

Figure 1 shows the response surface generated for the experimental data of the global esterification. It can be observed that the greatest extent of global esterification could be obtained with the lower substrates molar ratio (0.48 FFA/glycerol) and temperature (38 °C), after 5 h of reaction. Low temperatures have been suggested for esterification of n-3 PUFA in order to prevent polymerization during the reaction (Kosugy & Azuma, 1994).

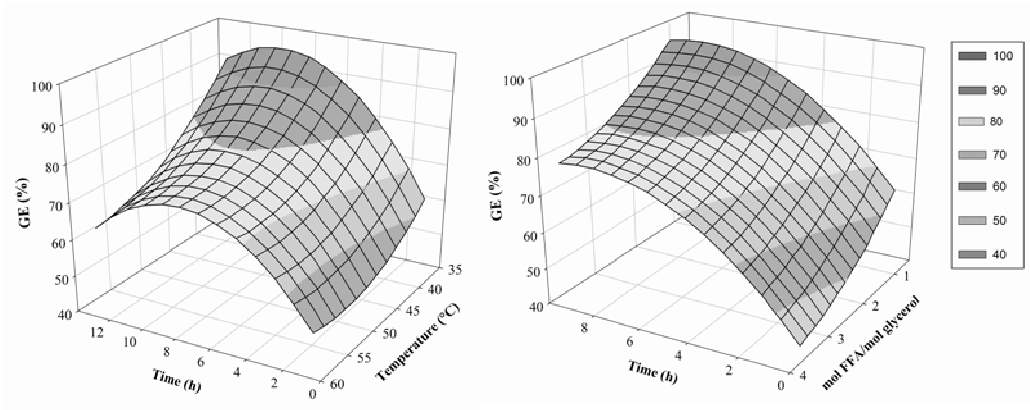

Figure 1. Response surface for global esterification of n-3 PUFA with glycerol

3.2 Production of Triacylglycerols (TAG)

Even when the higher global esterification obtained was 93% (experiment 2), the production of TAG only reached 39% (experiment 8), and this could be due the short reaction time employed (8.75 h) combined with substrate molar ratio (Table 2). Haraldsson, Gudmundson and Almarsson (1995), reported that more than 72 h are required for the production of TAG. Esteban, Robles, Jiménez, Ibáñez and Molina (1998), found that TAG formation can be increased from 26.4% to 59.4% using hexane. In the same work, these authors observed that when trials were allowed to react for 24 h, a higher TAG formation was reached (55%). Mc Neill, Ackman and Moore (1996), obtained more than 90% TAG production after 150 h of reaction. According to the ANOVA, our results shows that substrate molar ratio and the interaction of time with substrates molar ratio have a significant effect ($P < 0.05$) on the TAG production. The resulted mathematical model for the production of TAG was:

$$y_2 = 64.8\text{-}16.4x_1\text{-}2.6\ x_2\text{-}1.5\ x_3\text{+}0.81x_1^2\text{+}0.93x_1x_2\text{+}0.15x_2^2\text{+}0.15\ x_1x_3\ \text{+}0.014\ x_3x_2\text{+}0.012x_3^2 \quad (3)$$

where y_2 is the extent of TAG production, $x_1$the substrates molar ratio, x_2 the reaction time and x_3 the temperature.

The statistical analysis shows that this model can estimate the experimental data with an $F = 0.0030$ and $R^2 = 0.9952$, suggesting that variations could be explained by the fitted model. Figure 2 shows that for higher substrates molar ratio and reaction time, a higher TAG production is obtained. Some reports (Esteban, Robles, Jiménez, Ibáñez & Molina, 1998; Haraldsson, Gudmundson, & Almarsson, 1995) refer that 3 FFA/glicerol is the optimal substrates molar ratio for TAG formation. However, Robles, Esteban, Giménez, Camacho, Ibáñez and Molina (1999), found that an increment of 50% in glycerol concentration, TAG formation was increased.

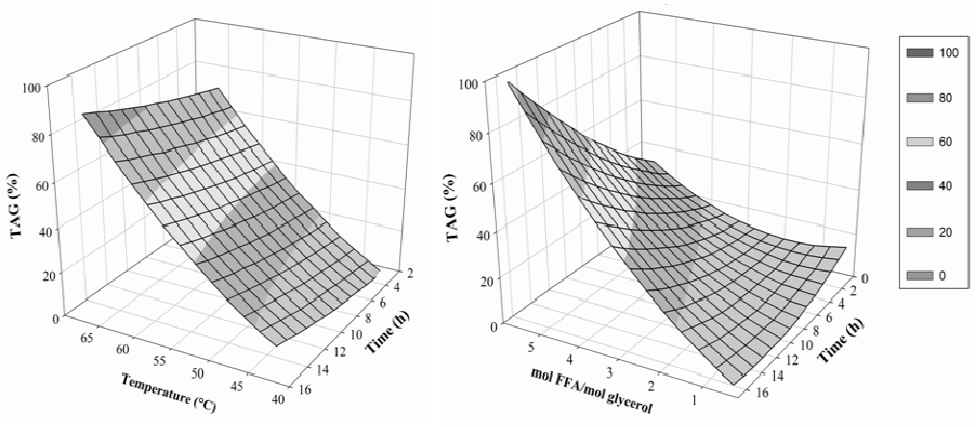

Figure 2. Response surface for TAG formation during esterification of n-3 PUFA on glycerol

3.3 Production of Diacylglycerols (DAG)

We obtained 35% of DAG for a 0.48 FFA/glycerol substrate molar ratio, after 5.75 h at 55 °C (experiment 9). The ANOVA proved that reaction time had a significant effect ($P < 0.05$) on DAG production. The model for the production of DAG is:

$$y_3 = -11.7-0.027x_1+9.6x_2+0.58x_3-0.30x_1^2-0.07x_1x_2-0.46x_2^2-0.05x_1x_3-0.07x_3x_2-0.000x_3^2 \quad (4)$$

where y_3 is the extent of DAG production, x_1 the substrate molar ratio, x_2 the reaction time and x_3 the temperature.

Figure 3 shows a maximum DAG yield production in the middle level of the reaction time (5.75 h). It can also be noted that for low substrate molar ratios, higher DAG production was obtained. It has been reported that the highest level of DAG production (30-35%) is obtained at short reaction times (Haraldsson, Gudmundson, & Almarsson, 1995; Kosuki & Azuma, 1994). Recent works have produced 50% DAG by glycerolysis of oil catalyzed by lipases (Wanget al., 2011; Miranda et al., 2013).

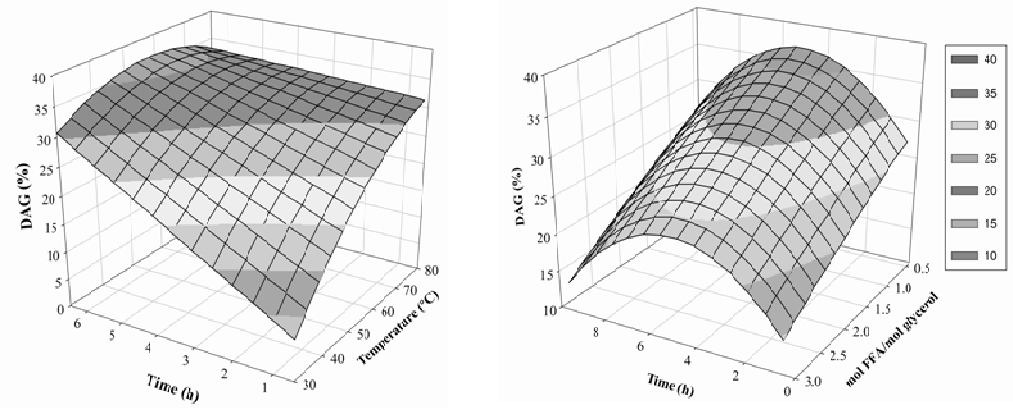

Figure 3. Response surface for DAG formation during esterification of n-3 PUFA on glycerol

3.4 Production of Monoacylglycerols (MAG)

The highest MAG production (58.2%) was obtained with a 4.5 FFA/glycerol substrate molar ratio, after 2.75 h at 45 °C (experiment 5). We observed a rapid MAG formation in the beginning of the reaction, but it decreased with reaction time due to the subsequent formation of DAG and TAG. The model for MAG formation is:

$$y_4 = 152.9+0.15x_1+3.4x_2-3.9x_3+1.4x_1^2-0.77x_1x_2-0.15x_2^2-0.11x_1x_3+0.001x_3x_2+0.033x_3^2 \quad (5)$$

where y_4 is the extent of MAG formation, $x_1$the substrates molar ratio, x_2 the reaction time and x_3 the temperature.

Figure 4 shows that for a very short reaction time and a high substrate molar ratio, a higher amount of MAG was produced. In contrast, Li and Ward (1994) reported that the high production of MAG and DAG is due to the positional specificity of the lipases employed (*Pseudomonas* sp. and *Mucor miehei*).

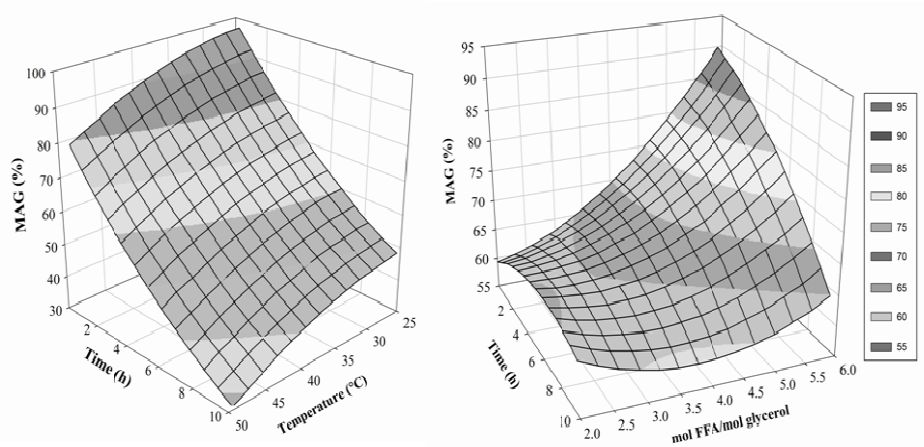

Figure 4. Response surface for MAG formation during esterification of n-3 PUFA on glycerol

3.5 Optimal Conditions for Esterification

Results of this work permited the correct determination of the conditions which produce a maximum yield of the desired acylglycerol as product. According to the RSM analysis, the experimental settings that produced the highest degree of esterification were within those of our experimental trials. As it is shown in Table 3, the optimal conditions for TAG formation (> 95%) were: 4.2 FFA/glycerol substrates molar ratio, 12 h and 72 °C; whereas for MAG formation these conditions were rather contrasting (more than 4.2 FFA/glycerol, less than 2.0 h and 30 °C). DAG production was maximized with a 1.0 FFA/glycerol substrates molar ratio, 5 h and 50 °C.

Table 3. Optimal conditions for the main variables of esterification

Product	Production (%)	Substrate mole ratio (mol FFA/mol Glycerol)	Time (h)	Temperature (°C)
MAG	90	>4.2	<2.0	30
DAG	45	1.0	5.0	50
TAG	95	4.2	>12	72

MAG: monoacylglycerols, DAG: diacylglycerols, TAG: triacylglycerols.

4. Conclusion

In the present work, we successfully estimated by RSM the production of structured acylglycerols with elevated n-3 PUFA using an immobilized lipase from *Candida antarctica* (NV-435). The R^2 (>0.97) and ANOVA suggest that the model represented well the relationship of the main variables and the response. The variables with the highest significant effect on the extent of n-PUFA incorporation were the reaction time and the substrate molar ratio. It is possible to drive the reaction to any desired product (namely MAG, DAG or TAG) with elevated n-PUFA by modifying the reaction conditions of substrate molar ratio, temperature and time.

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Vitamin E Profiles and Triacylglycerol Molecular Species of Colored Rice Bran Cultivars at Different Degree of Milling

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Abstract

The objective of this study was to evalute the tocochromanol distributions, lipid components and molecular species of triacylglycerols (TAG) in three colored rice bran cultivars. The dominant tocochromanol were γ-tocotrienol, α-tocopherol and α-tocotrienol with smaller amounts of γ-tocopherol, δ-tocopherol and δ-tocotrienol. These lipids comprised mainly TAG (78.0-81.6 wt%), free fatty acids (FFA: 5.6-8.8 wt%), and phospholipids (PL: 6.3-7.0 wt%), while other components were present in minor proportions (0.4-2.3 wt%). Sixteen different TAG molecular species were detected and quantified by successive applications of $AgNO_3$-TLC and GC. The major TAG molecular species were SM_2 (6.1-9.8%), S_2D (4.8-7.3%)，M_3 (16.4-18.7%), SMD (6.2-9.2%), SD_2 (6.5-9.5%), SMT (6.3-7.7%), M_2D (12.3-15.5%.), MD_2 (8.4-10.4%), SDT (4.3-5.4%) and D_3 (10.2-15.2%) (where S, M, D, and T denote saturated FA, monoene, diene, and triene, respectively). The results showed that colored rice bran lipids contain large amounts of nutraceutical with proven positive health effects.

Keywords: bran lipids, colored rice, molecular species, tocopherol homologues, triacylglycerols

Abbreviations: FFA, free fatty acids; GC, gas chromatography; HMRB, half-milled rice bran; HPLC, high-performance liquid chromatography; PL, phospholipids; TAG, triacylglycerols; TLC, thin-layer chromatography; WMRB, well-milled rice bran

1. Introduction

Rice (*Oryza sativa* L.) constitutes the world's principal source of food (Yadav & Jindal, 2001). Rice is the stable food of Asia, where 90% of the world's rice crop is produced and consumed. Rice is commonly consumed as milled or white rice, which is produced by removing the hulled and bran layers of the rough rice kernel in dehulling and milling processes, respectively. The colored grain are caused by anthocyanins pigment that gives the dulled rice a red and brownish red color in red rice or a dark purple color in black rice (Oki et al., 2002). These two types of rice were gaining popularity in Japan as functional food and often mixed with rice to enhance the color, flavor, and nutritional value (Itani & Ogawa, 2004). Moreover, rice grain quality is an important economic trait that influences rice production in many rice-producing areas. Although the fat or oil in rice grain is low (i.e., 2-3%) and is concentrated in the germ and bran, it is a key determinant of the processing and cooking quality of rice (Zhou et al., 2002a). For instance, the surface lipid content has been thought to be an indication of the degree of milling (Siebenmorgan, Matsler, & Earp, 2006). The milling efficacy is measured by degree of milling. The amount of bran remaining on the rice kernel after milling affects rice quality, appearance, and texture and rice is thus milled to the end-use preference of different consumers (Perdon et al., 2001; Saleh & Meullenet, 2007). In addition, rice lipid, which frequently forms complexes with starch granules, was shown to affect starch gelatinization, water availability to starch, and rice swelling and thus influences the eating and cooking qualities of rice (Champagne, Marshall, & Goynes, 1990; Marshall, Norma, & Goynes, 1990). Besides dietary consumption, the unique health benefits of rice fat, which includes many unsaturated fatty acids, has drawn much attention (Jennings & Akoh, 2009). A number of studies have shown that rice bran oil reduces the harmful cholesterol (low-density lipoprotein) without affecting the good cholesterol (high-density lipoprotein) in

plasma (Sugano & Tuji, 1997; Wilson et al., 2007). In addition, rice bran oil, which is rich in tocotrienol (Vitamin E), has anti-cancer and anti-radiation effects (Wilson et al., 2007). A combination of tocopherols and tocotrienols, preferably from natural sources, has been suggested to be an essential part of a diet preventing Alzhaimer disease (Morris et al., 2005) or to be aiding in the treatment of Parkinson's syndrone (Itoh et al., 2006). On the other hand, some reports have shown that the hydrolysis and oxidation of rice fat are responsible for rice aging and deterioration of grain flavor during storage, and low-oil rice cultivars are more suitable for storage (Zhou et al., 2002b).

Several studies on lipid fractions of rice brans have been published (Taira et al., 1988; Jennings & Akoh, 2009; Przybylski et al., 2009). However, to the best of our knowledge, no data have been reported on the vitamin E homologues, lipid components and fatty acid compositions of colored rice bran cultivars at different degrees of milling. Therefore, the aim of this study was to investigate the tocochromanol distributions and the molecular species of triacylglycerols (TAG) obtained from the three colored rice bran cultivars, based on differences in the degrees of milling.

2. Materials and Methods

2.1 Raw Materials

Commercially mature rice seeds used were from the three different colored Japanese cultivars: black (*Okunomurasaki*), red (*Beniroman*) and green (*Midorimochi*) harvested in September 2012 at the same district (Takaichigun, Nara Predecture) of Japan. These colored rices were purchased from regional alliance center for industrial technology at Asuka district of Nara prefecture. Black and red rices were nonglutinous rice, and green rice was a glutinous rice. For analysis, seeds were selected for uniformity based on seed weight of 19.8-21.1 mg for black, 20.6-21.7 mg for red and 17.2-18.4 mg for green, respectively. Rices of each cultivar were packed in polyethylene bags under nitrogen gas and placed in a stainless-steel container at -35 ºC until analysis.

2.2 Chemicals and Reagents

All chemicals were of analytical grade (Wako Pure Chemical Inc., Osaka, Japan). TLC-plates were purchased from Merck (Darmstadt, Germany). Standard mixture for TLC, containing monoacylglycerols, diacylglycerols, free fatty acids (FFA), triacylglycerols (TAG), steryl esters and hydrocarbons, was purchased from Wako Pure Chemical. Standard TAG (glyceryl trimyristate, glyceryl tripalmitate, glyceryl tristearate, glyceryl trioleate, glyceryl trilinoleate and glyceryl trilinolenate) was procured from Sigma-Aldrich Co. (St. Louis, Mo, USA). Vitamin E homologues (α, β, γ and δ) were acquired from Sigma-Aldrich Co. All tocopherols were of the D-form (*RRR*-), and their purities were better than 98.8%. Fatty acid methyl esters (FAME) standards (F & OR mixture #3) were obtained from Altech-Applied Science (State College, PA, USA). The internal standards, pentadecane and methyl pentadecanoate, were purchased from Merck, and then 100 mg of each was dissolved in *n*-hexane (20 ml). Boron trifluoride (BF_3) in methanol (14%; Wako Pure Chemical Inc., Osaka, Japan) was used to prepare the FAME.

2.3 Lipid Extractions

Rice bran of each cultivar (500 g) was milled using a domestic miller (BR-CA25, Zojirushi Ltd., Osaka, Japan). Before extraction, each bran prepared from half-milled rice and well-milled rice was defined as HMRB and WMRB, respectively. The degree of milling was 5% milling for HMRB and 10% for WMRB. Total lipids were extracted from 20 g of bran in 300 ml chloroform/methanol (2:1, v/v) with vigorous shaking for 15 min at 0 ºC three times, following the Folch procedure (Folch et al., 1957). The extracted lipids were weighed to determine the lipid content of the rice brans and then transferred to a 25-ml brown-glass volumetric flask with chloroform/methanol (2:1, v/v), flushed by nitrogen, and stored in the dark at -35 °C until further use.

2.4 Tocochromanol Analysis

Determinations of tocochromanols were performed by HPLC according to the methods reported previously (Yoshida, Tomiyama, & Mizushina, 2010). An aliquot (10 μl) from this sample solution was subjected to HPLC analysis, and the amount of each tocochromanol was determined with a fluorescence detector (Shimazdu RF-10 AXL, Kyoto, Japan) as previously reported (Yoshida, Tomiyama, & Mizushina, 2010).

2.5 Lipid Analysis

According to the previous procedure (Yoshida et al., 2013), total lipids were fractionated by TLC into nine fractions using a solvent system of *n*-hexane/diethyl ether/acetic acid (80:20:1, v/v/v). Bands corresponding to hydrocarbons, steryl esters, TAG, unknown, FFA, 1,3-diacylglycerols, 1,2-diacylglycerols, monoacylglycerols and PL were scraped into separate test-tubes. Methyl pentadecanoate (10-100 μg) from a standard solution (5

mg/ml) was added to each tube as the internal standard, except that pentadecane (10 μg) was used as the internal standard for hydrocarbons analysis. FAME was prepared by heating with silica gel for 30 min at 80 ºC in BF_3/methanol (Kitts et al., 2004). The FAME was quantified by gas chromatography (GC, 14B GC Shimadzu) equipped with a hydrogen flame ionization detector (FID) at 350 °C and a polar capillary column (ULBO HE-SS-10 for FAME, fused silica WCOT [no. PSC5481], cyanopropyl silicone, 30 m x 0.32 mm i.d.; Shinwa Chem. Ind., Ltd., Kyoto, Japan) at a column temperature of 180 °C. The component peaks were identified and compared against that of the standard FAME as a previously described method (Yoshida et al., 2013). The detection limit was 0.05 wt% of total fatty acids for each FAME in the FAME mixture, and the results are expressed as wt% of total FAME. The other GC conditions were as in the previously described (Yoshida et al., 2010).

2.6 TAG Analysis

The TAG isolated by TLC was directly analyzed by GC according to a previously described method (Molkentin, 2007), using the Shimadzu Model-14C GC equipped with a hydrogen FID. Helium was used as the carrier gas at a flow rate of 50 ml/min. The injection port and the FID were set at 320 and 350 °C, respectively. The oven temperature was programmed to increase from an initial temperature of 285 °C (5 min hold), rising to 320 °C at a rate of 2 °C/min, which was held isothermally (320 °C) for 20 min. TAG peaks were identified by co-chromatography with known standards. Peak areas were calculated by the addition of a known weight (50 μg) of glyceryl trimyristate as the internal standard, using an electronic integrator (Shimadzu C-R6A).

2.7 TAG Species Composition

Molecular species separation from total TAG was carried out by $AgNO_3$-TLC (Bilyk et al., 1991). TAG classes differing in unsaturation were isolated by $AgNO_3$-TLC using 1.2-2.8% (v/v) methanol in chloroform, depending on the degree of unsaturation (Jham et al., 2005). The plates were streaked with 10-15 mg TAG using the microsyringe, developed with 1.2% (v/v) methanol in chloroform, and S_3, S_2M, SM_2, S_2D, M_3, SMD and SD_2 were easily separated. Furthermore, TAG molecular species such as SMT, M_2D, MD_2, SDT, D_3, MDT, M_2T, MT_2, and D_2T (where S, M, D and T denote saturated FA, monoene, diene, and triene, respectively) were separated by developing the plate with 2.8% (v/v) methanol in chloroform. Each TAG subfraction was identified by comparison with the R_f-values of the TAG standard. Each band was recovered from the plate by extraction with 3.0% aqueous HCl in the purified diethyl ether, and then the solvent was then vaporized under a gentle stream of nitrogen. Methyl pentadecanoate (10-50 μg) of the standard solution (2-10 μl; 5 mg/ml) was added to each tube as the internal standard. After methylation of all TAG sufractions, the relative amount of each TAG fraction was quantified by GC as described in the preceding paragraphs. The other conditions were as previously described (Yoshida et al., 2010).

2.8 Data Analysis

Data in this study were expressed as means ± SD for at least three independent experiments. Differences between the means of individual groups were assessed by one-way analysis of variance and Tukey's multiple range test using the SAS statistical software package (SAS, Cary, NC, USA). Differences were considered significance at $P < 0.05$.

3. Results and Discussion

3.1 The Content of Brans and Their Lipids in Colored Rices

The bran content of the rice black, red and green cultivars (500 g) were 41.9 g (8.4%), 29.6 g (5.9%) and 19.4 g (3.9%) from the HMRB, and then 76.7 g (15.3%), 59.2 g (11.8%) and 37.5 g (7.5%) from the WMRB for black, red and green cultivars, respectively. The bran contents of the HMRB and WMRB were significantly ($P < 0.05$) different among the three cultivars. On the other hand, the lipid contents obtained from these rice brans was 7.4 g (17.7%), 5.4 g (18.2%) and 4.8 g (24.7%) from the HMRB and then 11.0 g (14.4%), 8.9 g (15.1%) and 7.9 g (21.1%) from the WMRB, for black, red and green cultivars. The percentage of lipid contents was significantly ($P < 0.05$) higher in the HMRB than in the WMRB, and was in the rank order: green > red > black in both brans. Therefore, the lipids may be higher in the outer bran layer than in the interior bran layer.

3.2 Tocochromanol Contents in the Rice Bran Cultivars

The lipid content of the rice samples analyzed ranged from 2.2 to 3.7% (data not shown). When comparing tocochromanols among the three cultivars as shown in Table 1, the individual amounts was significantly ($P < 0.05$) lower in the green brans than in the black or red brans. With a few exceptions, the predominant tocochromanol forms in rice bran cultivars were γ-tocotrienol, α-tocotrienol, and α-tocopherol. The content of γ-tocotrienol ranged from 325 to 593 mg/kg, α-tocotrienol was found between 93.6 and 293 mg/kg and

α-tocopherol varied in the range of 78.4-452 mg/kg. In addition to these forms, δ-tocopherol, γ-tocopherol and δ-tocotrienol were also detected in the range of 27.2-56.2 mg/kg, 5.8-89.3 mg/kg and 12.3-25.8 mg/kg, respectively, in all samples. Among individual vitamin E homologues (Heinemann et al., 2008), α-tocopherol, α-tocotrienol, and γ-tocotrienol were the most aboundant components in *japonica* rice, while in *indica* rice, the highest mean level was for γ-tocotrienol, followed by α-tocopherol and α-tocotrienol. Tocochromanol composition was in good agreement with the tocopherol composition of several grape seed oils reported in the literature (Wie et al., 2009; Demirtas et al., 2013). With a few exceptions, the percentage of tocotrienols was significantly ($P < 0.05$) higher in the HMRB than in the WMRB. Therefore, tocotrienols could be present more in the outer bran layer, while tocopherols would be distributed more in the interior layer. The main vitamin E homologue was the α- or γ-isomer among tocopherols or tocotrienols.

Table 1. Tocol composition of colored rice bran cultivars (mg/kg lipid)*

Bran	Lipid class	Cultivar											
		Black				Red				Green			
HMRB	α-Tocopherol	174	±	9.2^{c}	(15.2)	168	±	6^{c}	(16.3)	78.4	±	2.0^{f}	(14.3)
	α-Tocotrienol	252	±	8^{b}	(22.0)	235	±	6^{c}	(22.7)	98	±	2.3^{d}	(17.9)
	β-Tocopherol		nd				nd				nd		
	β-Tocotrienol		nd				nd				nd		
	γ-Tocopherol	89.3	±	2.4^{a}	(7.8)	39.4	±	0.6^{b}	(3.8)	5.8	±	0.2^{e}	(1.1)
	γ-Tocotrienol	563	±	14^{b}	(49.2)	538	±	12^{c}	(52.1)	325	±	8^{e}	(59.4)
	δ-Tocopherol	46.8	±	1.5^{b}	(4.1)	35.8	±	0.4^{c}	(3.5)	27.2	±	0.7^{d}	(5.0)
	δ-Tocotrienol	18.5	±	0.7^{b}	(1.6)	16.8	±	0.4^{b}	(1.6)	12.8	±	0.3^{c}	(2.3)
	ΣTocopherol	310.1	±	7.5^{c}	(27.1)	243.2	±	5.1^{d}	(23.5)	111.4	±	2.0^{f}	(20.4)
	ΣTocotrienol	833.5	±	10.2^{c}	(72.9)	789.8	±	16.3^{d}	(76.5)	435.8	±	937^{f}	(79.6)
	ΣTocol	1143.6	±	21.4^{c}		1033.0	±	20.1^{d}		547.2	±	11.6^{f}	
WMRB	α-Tocopherol	452	±	12^{a}	(31.4)	437	±	8^{b}	(31.6)	113	±	4.2^{e}	(17.8)
	α-Tocotrienol	284	±	2.8^{a}	(19.7)	293	±	7.3^{a}	(21.2)	93.6	±	2.5^{d}	(14.8)
	β-Tocopherol		nd				nd				nd		
	β-Tocotrienol		nd				nd				nd		
	γ-Tocopherol	27.0	±	2.9^{c}	(1.9)	23.5	±	0.7^{d}	(1.7)	7.3	±	0.2^{e}	(1.2)
	γ-Tocotrienol	593	±	17^{a}	(41.3)	557	±	13^{c}	(40.3)	372	±	10^{d}	(58.6)
	δ-Tocopherol	56.2	±	1.8^{a}	(3.9)	47.6	±	0.8^{b}	(3.4)	32.6	±	0.8^{c}	(5.1)
	δ-Tocotrienol	25.8	±	0.7^{a}	(1.8)	24.6	±	0.7^{a}	(1.8)	16.3	±	0.5^{b}	(2.5)
	ΣTocopherol	535.2	±	9.8^{a}	(37.2)	508.1	±	8.9^{b}	(36.7)	152.6	±	2.9^{e}	(24.1)
	ΣTocotrienol	902.8	±	12.2^{a}	(62.8)	874.6	±	12.3^{b}	(63.3)	481.9	±	8.3^{e}	(75.9)
	ΣTocol	1438.0	±	26.0^{a}		1382.7	±	23.0^{b}		634.5	±	12.4^{e}	

HMRB: half-milled rice brans. WMRB: well-milled rice brans. nd: (not detectable) < 0.01 mg/kg.

*Mean values ± standard error. Each value represents the average of three determinations, and is expressed as mg/kg lipid. Values in parentheses are relative wt% contents of the individual tocopherols in HMRB or WMRB. Values in the same row with different superscripts are significantly different between the individual cultivars ($P < 0.05$).

Vitamin E is represented by α-, β-, γ-, and δ-tocopherols, and α-, β-, γ- and δ-tocotrienols, all of which occur in nature, and 14 vitamins are theoretically possible (Bramley et al., 2000). Vitamin E is a term frequently used to designate a family of related compounds, namely, tocopherols and tocotrienols (Amaral et al., 2005), which are important lipophilic antioxidants with essential effects in living system against aging (Agostinucci et al., 2002)

and reducing the risk of cancer (Lee et al., 2000). However, α-tocopherol is regarded as the most active and predominant form (Bender & Mayes, 2003). Tocotrienols have been indicated to suppress the effects of reactive oxygen species more effectively than tocopherols, and different studies of *in vitro* and *in vivo* effects suggest that tocotrienols may lower cholesterol levels and suppress tumor growth (Schaffer, Muller, & Eckert, 2005). Published data related to the tocopherol and tocotrienol content of these colored rice bran lipids is lacking and comparison is not currently possible.

3.3 Lipid Compositions in the Rice Bran Cultivars

Profiles of lipid components were compared among the HMRB and WMRB from the three cultivars (Table 2). Dominant components were TAG (HMRB: 78.4-81.2%; WMRB: 78.0-81.6%), followed by FFA (HMRB: 6.7-7.6%; WMRB: 5.6-8.8%) and PL (HMRB: 6.3-6.8%; WMRB: 6.5-7.0%), accompanied by very small amounts (0.4-2.3%) of other lipid components. When comparing the nine lipid components of the HMRB and WMRB among all three cultivars, the percentage of TAG was significantly ($P < 0.05$) lower in the green cultivar than that in the black or red cultivar, while the percentage of FFA was significantly ($P < 0.05$) higher in the green cultivar than that in the black cultivar. However, with a few exceptions, no substantial differences ($P > 0.05$) in the contents of the lipid components were observed between the values estimated by a combination analysis of TLC and GC using the internal standard (C15:0).

Presumably, the minor components, such as FFA, 1,3- and 1,2-diacylglycerols or monoacylglycerols, may be formed by the partial enzymatic hydrolysis of reserve TAG during the storage of the rice seeds (Aboul-Nasr, Ramadan, & El-Dengawy, 1997). The lipid components resulting from 'fat by hydrolysis' in starch granules were determined, showing the presence of FFA with lysolecithin and lysoglycolipids (Okunishi & Ohtsubo, 2008).

Table 2. Lipid components obtained from colored rice bran cultivars*

Bran	Lipid class	Cultivar											
		Black				Red				Green			
HMRB	Hydrocarbons	29.7	±	0.7^{c}	(0.4)	21.5	±	0.5^{d}	(0.4)	19.2	±	0.4^{d}	(0.4)
	Steryl esters	74.2	±	1.8^{c}	(1.0)	64.6	±	1.6^{d}	(1.2)	52.7	±	1.3^{e}	(1.1)
	Triacylglycerols	5999	±	40^{d}	(80.7)	4374	±	39^{e}	(81.2)	3752	±	38^{f}	(78.4)
	Unknown	59.3	±	1.4^{b}	(0.8)	26.9	±	0.7^{d}	(0.5)	24.0	±	0.5^{e}	(0.5)
	Free fatty acids	497	±	12^{c}	(6.7)	399	±	10^{d}	(7.4)	364	±	9^{e}	(7.6)
	1, 3-Diacylglycerols	118.7	±	2.9^{b}	(1.6)	70.0	±	1.7^{e}	(1.3)	110.2	±	2.8^{b}	(2.3)
	1, 2-Diacylglycerols	111.2	±	2.7^{c}	(1.5)	64.6	±	1.6^{e}	(1.2)	63.9	±	1.5^{e}	(1.3)
	Monoacylglycerols	66.7	±	1.6^{d}	(0.9)	26.9	±	0.7^{e}	(0.5)	76.7	±	1.8^{c}	(1.6)
	Phospholipids	475	±	11^{d}	(6.4)	339	±	8.5^{e}	(6.3)	326	±	8.2^{e}	(6.8)
WMRB	Hydrocarbons	77.3	±	1.9^{a}	(0.7)	71.5	±	1.7^{a}	(0.8)	39.4	±	1.0^{b}	(0.5)
	Steryl esters	154.6	±	3.8^{a}	(1.4)	89.4	±	2.3^{b}	(1.0)	141.7	±	3.5^{a}	(1.8)
	Triacylglycerols	8968	±	65^{a}	(81.2)	7276	±	48^{b}	(81.6)	6124	±	42^{c}	(78.0)
	Unknown	44.2	±	1.1^{c}	(0.4)	71.5	±	1.8^{a}	(0.8)	23.6	±	0.6^{f}	(0.3)
	Free fatty acids	619	±	15^{b}	(5.6)	500	±	12^{c}	(5.6)	693	±	16^{a}	(8.8)
	1, 3-Diacylglycerols	176.7	±	4.3^{a}	(1.5)	98.3	±	2.4^{c}	(1.1)	78.7	±	1.9^{d}	(1.0)
	1, 2-Diacylglycerols	154.6	±	3.8^{a}	(1.4)	134.1	±	3.4^{b}	(1.5)	78.7	±	2.0^{d}	(1.0)
	Monoacylglycerols	88.4	±	2.2^{b}	(0.8)	98.3	±	2.5^{b}	(1.1)	141.7	±	3.5^{a}	(1.8)
	Phospholipids	773	±	19^{a}	(7.0)	581	±	14^{b}	(6.5)	535	±	13^{c}	(6.8)

HMRB: half-milled rice brans. WMRB: well-milled rice brans.

*Mean values ± standard error. Each value represents the average of three determinations, and is expressed as mg lipid class per 500 g of rice. Values in the same row with different superscripts are significantly different between the individual cultivars ($P < 0.05$). Values in parentheses are relative wt% contents of the individual lipids in the total lipids.

3.4 Fatty Acid Composition of Major Lipids in the Rice Bran Cultivars

Fatty acid compositions of total lipids, TAG, FFA and PL in the rice bran lipids were compared among the HMRB and WMRB of the three cultivars (Table 3). The distribution of total unsaturated fatty acids, particularly linoleic (18:2*n*-6) and oleic (18:1*n*-9) acids, which accounted for 77.2-79.3% (total lipids), 77.9-79.9% (TAG), 70.8-73.5% (FFA) and 71.6-76.1% (PL), respectively. These patterns were very similar within total lipids, TAG, FFA or PL among the HMRB and WMRB from all threee cultivars. However, some differences ($P < 0.05$) in fatty acid composition were noted when comparing the four lipid classes (total lipids, TAG, FFA and PL) as shown in Table 3. The percentage of palmitic (16:0) acid was significantly ($P < 0.05$) higher in the FFA (24.1-26.2%) and PL (21.8-25.4%) than that in the total lipids (18.3-19.3%) and TAG (17.1-19.5%) among all three cultivars. With a few exceptions for the green rice cultivar (total, TAG and PL) in both brans, the percentage of oleic (18:1*n*-9) acid was significantly ($P < 0.05$) higher in the black and red bran lipids than in the green bran lipids for the four lipid classes. On the other hand, with a few exceptions of FFA for the HMRB and WMRB, the percentage of linoleic (18:2n-6) acid was significantly ($P < 0.05$) higher in the green rice bran than in the black or red rice bran for total lipids, TAG and PL fractions. It has been demonstrated that there exists distinct differences between nonoglutinous and glutinous types of cereals in lipid content and fatty acid composition (Fujino & Mano, 1972; Taira, 1984; Taira & Lee, 1988). These fatty acid composition for the black and red rice brans (nongulutinous) are very similar to the results observed for rice bran lipids in the cultivars: *Koshihikari, Haenuki, Akitakomachi*, *Hitomibore* and *Sasanishiki* reported in a previous paper (Yoshida et al., 2011). The data for fatty acid distribution of minor lipid components (steryl esters, 1,3- and 1,2-diacylglycerols or monoacylglycerols) in Table 2, were not included in Table 3 because these lipid components were present in too low concentrations to provide reliable results for their fatty acid compositions.

Table 3. Fatty acid distribution of major lipid components obtained from colored rice bran cultivars*

Bran	Lipid class	Cultivar	Fatty acid (wt%)						Total USFA
			16:0	18:0	18:1	18:2	18:3	Others	
HMRB	Total	Black	19.4±1.0^{a}	1.6±0.1^{b}	43.2±1.3^{a}	33.2±1.1^{b}	1.4±0.1^{a}	1.2±0.1^{b}	78.0^{a}
		Red	19.3±1.0^{a}	2.4±0.1^{a}	40.1±1.2^{b}	35.2±1.2^{b}	1.5±0.1^{a}	1.5±0.1^{a}	77.2^{a}
		Green	19.3±1.0^{a}	1.6±0.1^{b}	36.1±1.2^{c}	40.5±1.3^{a}	1.5±0.1^{a}	1.0±0.1^{b}	78.3^{a}
	TAG	Black	18.3±0.8^{b}	1.7±0.1^{d}	46.1±2.1^{a}	31.5±1.2^{d}	1.2±0.1^{b}	1.0±0.1^{b}	79.0^{a}
		Red	17.3±0.7^{b}	1.8±0.1^{b}	43.5±2.0^{b}	34.9±1.3^{c}	1.3±0.1^{b}	1.2±0.1^{a}	79.8^{a}
		Green	19.3±0.8^{a}	1.6±0.1^{b}	35.1±1.2^{c}	41.5±1.5^{a}	1.5±0.1^{a}	1.0±0.1^{b}	79.0^{a}
	FFA	Black	25.2±1.0^{b}	2.7±0.1^{a}	41.5±2.1^{c}	28.7±1.0^{a}	1.2±0.1^{c}	0.7±0.1^{c}	71.6^{b}
		Red	24.1±1.0^{b}	1.9±0.1^{a}	44.9±2.2^{a}	27.8±1.1^{a}	0.6±0.1^{d}	0.7±0.1^{c}	73.5^{a}
		Green	26.1±1.1^{a}	1.8±0.1^{c}	43.8±2.1^{a}	25.4±1.3^{c}	1.6±0.1^{b}	1.3±0.1^{b}	70.9^{b}
	PL	Black	22.3±1.0^{b}	1.3±0.1^{a}	38.6±1.3^{b}	34.6±1.3^{c}	1.5±0.1^{a}	1.7±0.1^{a}	74.9^{b}
		Red	23.1±1.0^{b}	1.1±0.1^{a}	38.9±1.2^{b}	34.7±1.3^{c}	1.5±0.1^{a}	0.7±0.1^{c}	75.4^{a}
		Green	25.4±1.1^{a}	0.8±0.1^{c}	29.9±1.2^{d}	41.8±1.8^{a}	1.3±0.1^{b}	0.8±0.1^{c}	73.2^{b}
WMRB	Total	Black	18.4±0.8^{a}	1.6±0.1^{b}	43.3±1.7^{a}	34.2±1.2^{b}	1.3±0.1^{b}	1.2±0.1^{b}	78.8^{a}
		Red	18.3±0.8^{a}	1.4±0.1^{c}	42.5±2.0^{a}	35.3±1.2^{b}	1.2±0.1^{b}	1.3±0.1^{b}	79.3^{a}
		Green	19.3±1.0^{a}	1.6±0.1^{b}	36.1±1.2^{c}	40.5±2.0^{a}	1.5±0.1^{a}	1.0±0.1^{b}	78.3^{a}
	TAG	Black	18.5±0.8^{b}	1.7±0.1^{b}	46.1±2.0^{a}	31.5±1.2^{d}	1.2±0.1^{b}	1.0±0.1^{b}	79.0^{a}
		Red	17.1±0.8^{b}	2.0±0.1^{a}	43.5±2.0^{b}	34.9±1.2^{c}	1.3±0.1^{b}	1.2±0.1^{a}	79.9^{a}
		Green	19.5±1.2^{a}	1.6±0.1^{b}	37.0±1.3^{c}	39.3±1.4^{b}	1.4±0.1^{a}	1.2±0.1^{a}	77.9^{b}
	FFA	Black	25.3±1.0^{b}	2.2±0.1^{b}	42.4±1.5^{b}	26.7±1.2^{b}	1.8±0.1^{a}	1.6±0.1^{a}	71.4^{b}
		Red	25.5±1.0^{b}	1.6±0.1^{c}	43.6±1.5^{a}	26.8±1.2^{b}	1.1±0.1^{c}	1.4±0.1^{b}	71.8^{b}
		Green	26.2±1.0^{a}	2.1±0.1^{b}	40.9±1.3^{c}	28.6±1.2^{a}	1.0±0.1^{c}	1.2±0.1^{b}	70.8^{b}
	PL	Black	21.8±1.0^{c}	1.3±0.1^{a}	40.7±1.3^{a}	33.7±1.2^{c}	1.2±0.1^{b}	1.3±0.1^{b}	76.1^{a}
		Red	23.3±1.1^{b}	1.3±0.1^{a}	39.2±1.3^{b}	34.0±1.3^{c}	1.2±0.1^{b}	1.0±0.1^{b}	74.7^{b}
		Green	25.4±1.1^{a}	1.3±0.1^{b}	32.1±1.2^{c}	38.2±1.4^{b}	1.3±0.1^{b}	1.7±0.1^{a}	71.6^{c}

HMRB: half-milled rice brans. WMRB: well-milled rice brans. USFA: Unsaturated fatty acids.

*Mean values ± standard error. Each value represents the average of three determinations, and is expressed the relative wt% contents of the individual fatty acids. Values in the same column with different superscript are significantly different between the individual cultivars ($P < 0.05$). "Others" include minor fatty acids such as C14:0, C16:1, C20:0 and C22:0.

3.5 Distribution of TAG Molecular Species

The carbon number (TCN) denotes the total legth of the three acyl-chain present in the TAG. For example, 54 are predominantly composed of 18:0, 18:1, 18:2 and 18:3. These TCN within TAG obtained from the three rice bran cultivars ranged from 48 to 56 as listed in Figure 1. Each value is the mean of triplicate determinations and is expressed as milligram lipid per 20 g brans. Dominant components were 52 (41.3-42.6%) and 54 (47.5-48.6%) TAG, followed by small amounts of 50 (9.5-10.2%), 56 (0.2-0.3%) and 48 (0.2-0.3%) TAG, respectively.

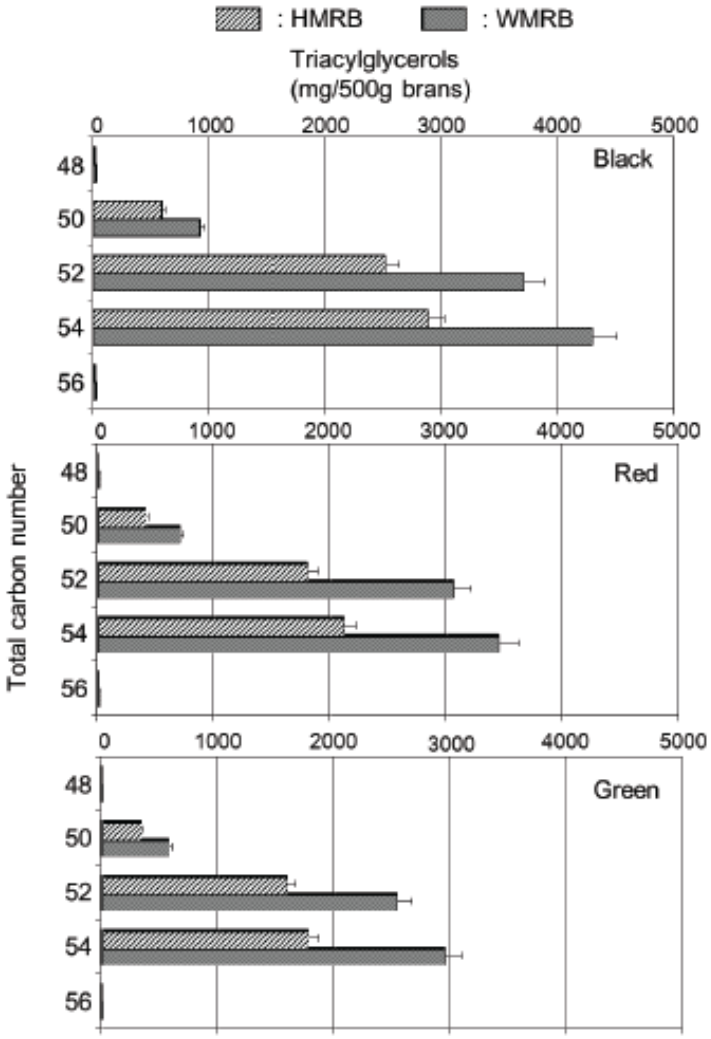

Figure 1. Content of TAG prepared from colored rice bran cultivars. Each value represents the average of three replicates. *Horizontal bars* depict the mean and standard deviation of three determinations

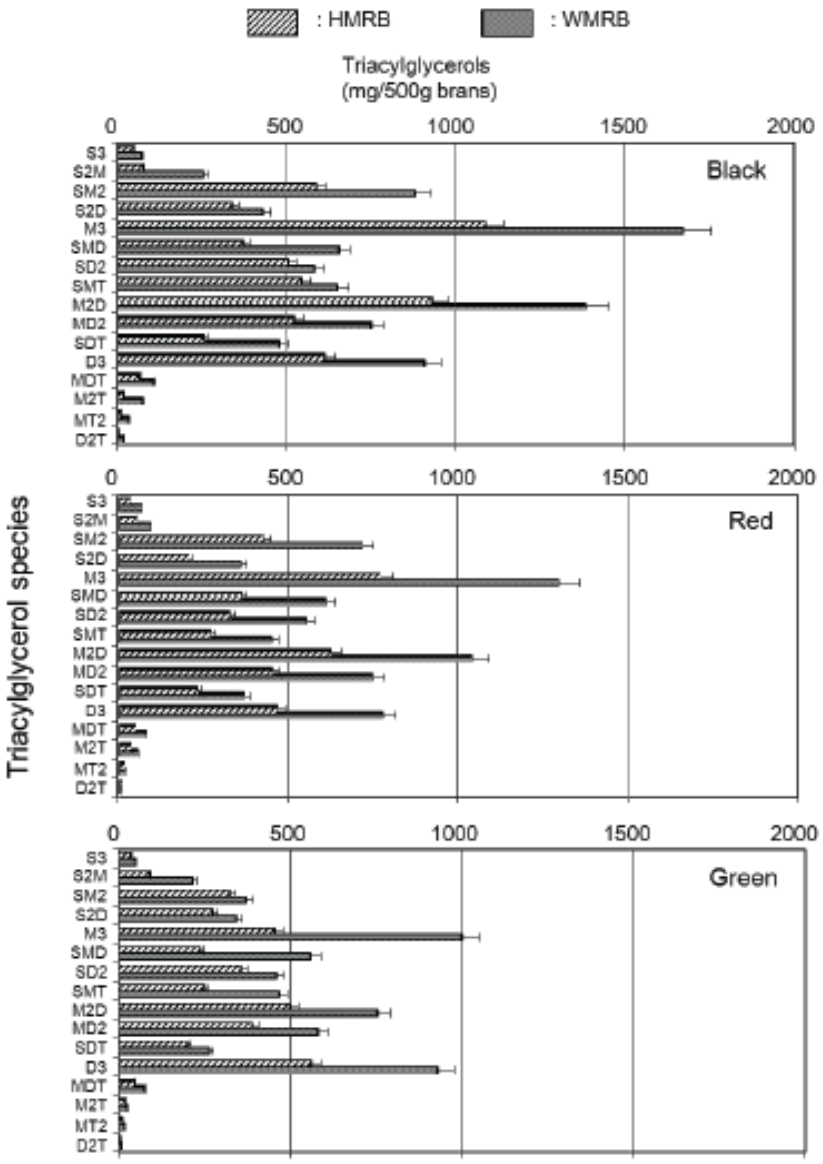

Figure 2. Characteristics of the major molecular species of TAG isolated from colored rice bran cultivars

Saturated FA (S) consists of myristic (14:0), palmitic (16:0), stearic (18:0) and arachidic (20:0) acids. Unsaturated FA, palmitoleic (16:1), oleic (18:1*n*-9), linoleic (18:2*n*-6) and α-linolenic (18:3*n*-3), are denoted as monoene (M), diene (D) and triene (T), respectively. *Horizontal bars* depict the mean and standard deviation of three determinations.

The distribution patterns of the individual TAG molecular species are shown in Figure 2. Sixteen different molecular species were detected among the TAG isolated from these rice bran lipids. These species were arranged according to the degree of unsatiration on the acyl-chain length of TAG (from top to bottom in Figure 2, respectively). In the three cultivars, the major TAG molecular species were SM_2 (POO or StOO), S_2D (PPL or PStL or StStL), M_3 (OOO), SMD (POL or StOL), SD_2 (PLL or StLL), SMT (POL or StOL), M_2D (OOL), MD_2 (OLL), SDT (PLLn or StLLn) and D_3 (LLL) in the three cultivars. On the other hand, the other species (S_3; PPP or PPSt or StStSt, S_2M; PPO or PStO or StStO, MDT; OLLn, M_2T; OOLn, MT_2; OLnLn and D_2T; LLLn) were minor components (less than 3.5%). However, the three-letter designation does not demonstrate regioselective positional isomers of fatty acyl in the TAG: P, palmitic (16:0), St, stearic (18:0), O, oleic (18:1*n*-9); L, linoleic (18:2*n*-6); Ln, α-linolenic (18:3*n*-3) fatty acid moieties. Thus, these distribution patterns in the molecular species of TAG were very similar to each other among the HMRB and WMRB for from the three cultivars.

4. Conclusions

The predominant components were γ-tocotrienol, α-tocopherol, and α-tocotrienol with much smaller amounts of γ- and δ-tocopherols, and δ-tocotrienol. Major lipid components in three different Japanese colored rice bran cultivars were TAG, FFA, and PL, while other components were also present in minor proportions. Sixteen molecular species of TAG were identified in these rice brans. The main components were palmitodiolein or stearodiolein (6.1-9.8%), triolein (16.4-18.7%), palmitoleolinolein or stearoleolinolein (6.3-9.2%), palmitodilinolein or stearodilinolein (6.5-9.5%), palmitoleolinolenin or stearoleolinolenin (6.2-9.2%), dioleolinolein (12.3-15.5%), oleodilinolein (8.4-10.4%) and trilinolein (10.2-15.2%). To the best of our knowledge this is the first report of the TAG composition of colored rice bran cultivars. In general, the distribution patterns were not significantly different ($P > 0.05$) among the HMRB and WMRB from the three cultivars, suggesting similar TAG molecular species and tocochromanol compositions. Currently, the consumer awareness of health food poducts is increasing and food scientists have been searching for interesting sources of healthul natural components. The results showed that rice bran extracts contain large amounts of nutraceticals with proven positive health effects (Ha et al., 2006).

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Chemical Compositions and Nutritional Properties of Popcorn-Based Complementary Foods Supplemented With *Moringa oleifera* Leaves Flour

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Abstract

Cereal gruel is the common complementary foods in developing countries, and it is usually low in energy and protein; hence, responsible for increase in protein-energy malnutrition among underprivileged weaning aged children. Several locally available food materials have been tested in combination for infant food formulations however; popcorn and *Moringa oleifera* leaves combination have not been used. After blanching and fermentation processing, popcorn and moringa leaves were milled into flour and blended to obtain, blanched popcorn-moringa leaves (BPM) (65% popcorn and 35% moringa leaves flour) and fermented popcorn-moringa leaves (FPM) (65% popcorn and 35% moringa leaves flour). Products were analyzed for chemical composition, functional properties and bioassay using standard methods. Protein content of FPM (21.27 ± 0.20 g/100 g) and BPM (15.99 ± 0.14 g/100 g) were higher than *Cerelac* (15.75 ± 0.01 g/100 g) and 'Ogi' (6.52 ± 0.31 g/100 g); while energy values of FPM (393.94 ± 0.39 kcal) and BPM (389.69 ± 1.40 Kcal) were lower than 'Ogi' (418.08 ± 0.47 kcal) and *Cerelac* (431.58 ± 0.01 kcal). Mineral contents of BPM were higher in zinc, iron, potassium, sodium and phosphorous, while FPM sample was higher in copper, calcium and magnesium, and were lower than *Cerelac*. Oxalate, phytate and trypsin inhibitor in FPM were lower than BPM. Biological value and protein efficiency ratio of FPM were higher than BPM and 'Ogi', but lower than *Cerelac*. The albino rats fed with the FPM had higher growth rate when compared with those rats fed with BPM sample and 'Ogi', but lower than those fed with *Cerelac*. Nutrient composition and nutritional profile of popcorn-moringa leaves based complementary foods could be used as substitutes for local complementary foods, which are low in protein and energy.

Keywords: popcorn, *Moringa oleifera* leaves, complementary foods, nutritional profile

1. Introduction

Adequate infant and young child nutrition involves adoption of recommended breastfeeding and complementary feeding practices and access to the appropriate quality and quantity of foods (PAG, 1972). Optimal nutrition in the first year of life is therefore crucial in laying the foundation of good nutrition and health of children. Nutritionally, it has been proven that breast milk is a complete and perfect food for the infant during the first six months of life (Lutter & Rivera, 2003). After 6 months breast milk alone can no longer be sufficient both in terms of quantity and quality to meet the nutritional requirements of infants, hence, appropriate complementary foods should be introduced (UNICEF, 2009).

In developing countries including Nigeria, many families cannot afford commercial complementary foods to wean their infants due to high level of poverty, and thereby engage in weaning the children on cereals gruels (Ikpeme-Emmanuel et al., 2012). Scientific findings have shown that cereal gruels are the common complementary foods in developing countries, which is characterized by low energy and protein density (Hotz & Gibson, 2001; Lutter & Rivera, 2003; Inyang & Zakari, 2008; Igyor et al., 2011) due to large volume of water relative to its solid matter contents during preparation. To increase the energy density of the gruel, more of the solid matter are needed, which will makes the gruel too thick and viscous for infant to eat and too large for their stomach capacity (Ikujenlola & Fashakin, 2005). Infants are therefore unable to fulfill their energy and other essential nutrients requirements (Ikujenlola & Fashakin, 2005), hence, there is increased in protein-energy

malnutrition among weaning aged children.

Improved complementary feeding and breastfeeding practices are essential to achieve the Millennium Development Goals (MDGs) for child survival and prevention of protein-energy malnutrition (Lutter & Rivera, 2003). To achieve this goal, formulation and development of nutritious complementary foods from local and readily available raw food materials have received a lot of attention in many developing countries (Plahar & Annan, 1994), however, locally available popcorn and moringa leaves combination have not been tested for complementary foods.

Cereals are widely utilized as food in African countries than in the developed world (Makinde & Ladipo, 2012). For instance, cereals account for as much as 77% of the total caloric consumption in African countries (Mitchel et al., 1997) and contribute substantially to dietary protein intake in a number of these countries. Compositionally cereals consist of carbohydrate and less in amount of protein and essential minerals. Among the cereals, popcorn (a derived product) contains appreciable amount of protein and minerals when compared with other maize species (Iken & Amusa, 2010). Popcorn, which is grown solely for human consumption in the developed countries, is now becoming popular in Nigeria (Iken & Amusa, 2010).

Moringa oleifera, also known as drumstick originated and widely cultivated in India and it has become naturalized in many locations in the tropics (Fahey, 2005). Moringa is one of the newly discovered vegetable and is gaining wide acceptance as medicine and food in Nigeria (Bamishaiye et al., 2011; Ijeomah et al., 2012). *Moringa oleifera* is the most known and widely cultivated variety of the genus *Moringa,* family *Morigaceae* (Fugile, 2001). *Moringa oleifera* is also known by other common names like Mallungay (Philippines), Benzolive tree (Haiti), Horse raddish tree (Florida), Nebeday (Senegal), Zogale (Hausa), Okwe Oyibo (Igbo), ewé ìgbálè (Yoruba) and Jeghel-agede in Tiv (Fahey, 2005; Bamishaiye et al., 2011). The leaves, seeds and flowers all have good nutritional and therapeutic values, and study has shown that the leaves were used to prevent or treat protein-energy malnutrition and other nutritional related diseases (Tete-Benissan et al., 2012). *Moringa oleifera* leaves are low in fat and carbohydrate but are excellent sources of amino acids (Rajangam et al., 2001), particularly sulphur containing amino-acids, that is, methionine and cystine which are often in short supply in cereals and other plant-based foods (Liu et al., 2007).

Several local cereal-legumes based and commercial complementary foods are available in Nigeria, however, locally available popcorn and *Moringa oleifera* combinations have not been used for infant food. This study was therefore designed to formulate and evaluate nutritional qualities of complementary foods using popcorn and moringa leaves flour.

2. Materials and Methods

2.1 Collection of Food Materials

Popcorn kernels were purchased from Erekesan market, Akure while *Moringa oleifera* leaves were obtained from a botanical garden in Akure, Ondo State, Nigeria.

2.1.1 Processing of Popcorn and *Moringa oleifera* Leaves Flour

Fermented popcorn (*Zea mays averta*) flour: The popcorn kernels were sorted, soaked in hot water for 2 days, washed, wet milled using attrition mill, sieved, fermented for 3 days, decanted, oven dried in hot air oven at a temperature of 60 ºC for 2 days, dry milled using attrition mill, sieved through 0.4 mm wire mesh, packed in a sealed air tight plastic container and stored at room temperature prior to formulations and chemical analysis.

2.1.2 Blanched *Moringa oleifera* Leaves Flour

The *Moringa oleifera* leaves were sorted, washed with distilled water, steam blanched, oven dried at a temperature of 50 ºC, milled using a laboratory blender, sieved through a 0.4 mm wire mesh and stored in airtight container at room temperature prior to formulations and chemical analysis.

2.1.3 Fermented *Moringa oleifera* Leaves Flour

The leaves were sorted, washed with distilled water, steam blanched, tightly wrapped in banana leaves to ferment for 72 hours, oven dried in hot air oven at a temperature of 50 ºC for 3 days, milled using laboratory blender, sieved through 0.4 mm wire mesh, tightly packed in a sealed plastic container and stored at room temperature prior to formulations and chemical analysis.

2.1.4 Food Formulations

Proportions of popcorn flour and *Moringa oleifera* leaves flour in the formulations were determined using NutriSurvey-Linear-Programming Software to obtain the following combinations: Blanched Popcorn-*Moringa*

oleifera leaves flour (BPM) (65% popcorn, 35% *Moringa oleifera* leaves flour) and Fermented Popcorn-Moringa leaves flour (FPM) (65% popcorn, 35% *Moringa oleifera* leaves flour).

2.2 Chemical Analysis

2.2.1 Proximate Analysis

Nutrient composition of the food sample was determined in triplicate using the standard procedures of Association of Official Analytical Chemists - AOAC (2005). Five grams of each formulated complementary foods were used to determine the moisture content in a hot-air circulating oven (Galenkamp). Ash was determined by incineration of 2 grams each of the food samples in a Gallenkamp muffle furnace at 550 ºC (Gallenkamp, size 3) (Method No 930.05) [AOAC, 2005]. Crude fat was determined by exhaustively extracting 10 grams of each sample in petroleum ether (boiling point, 40 to 60 ºC) in a Soxhlet extractor (Method No 930.09). Protein (N × 6.25) was determined by the Kjeldhal method (Method No 978.04) using 0.5 gram each of the formulated complementary foods (AOAC, 2005). Crude fiber was determined after digesting 5 grams each of fat-free complementary food samples in refluxing 1.25% sulfuric acid and 1.25% sodium hydroxide (Method No 930.10) (AOAC, 2005).

2.2.2 Carbohydrate Determination

The carbohydrate content was determined by subtracting the summed up percentage compositions of moisture, protein, lipid, fibre, and ash contents from 100 g of the sample [100%-(moisture% + protein % + fat % and ash %)] (Otitoju, 2009).

2.2.3 Gross Energy

Energy was determined by calculation from fat, carbohydrate and protein content using the Atwater's conversion factor; 4.0 kcal/g for protein, 9.0 kcal/g fat and 4.0 kcal/g for carbohydrate (Iombor et al., 2009).

2.2.4 Minerals Determination

AOAC (2005) methods were used to determine mineral compositions of the samples. One gram of sample was digested with nitric/perchloric/sulphuric acids mixture in ratio 9:2:1 respectively, filtered and the filtrate in a 5 ml volumetric flask was loaded to Atomic Absorption Spectrophotometer, (model703 Perkin Elmer, Norwalk, CT, USA). The standard curve for each mineral (calcium, magnesium, iron, aluminum, lead, copper and zinc), was prepared from known standards and the mineral value of samples estimated against that of standard curve. Sodium and potassium values were determined using Flame photometer (Sherwood Flame Photometer 410, Sherwood Scientific Ltd. Cambridge, UK). The phosphorus was determined using Vanodo-molybdate method.

Procedure: To series of 100 ml volumetric flasks 0.0, 2.5, 5.0, 7.5, 11.0, 15.0, 20.0, 20.0, 40.0, 50.0 ml of the standard phosphate solution was made acidic by addition of 2ml nitric acid (2:1). After which 25 ml of the Vanodo-molybdate reagent was added. The solution was diluted to the mark, mixed thoroughly and allowed to stand for 10 minutes. The optical density was measured at 47 mu.

2.2.5 Amino Acids Determination

A modified method of AOAC (2000) was used for amino acid analysis. Sixty milligrams of freeze-dried sample were hydrolyzed with 8 ml of 6 M HCl under vacuum at 110 ± 3 °C for 24 hours. After cooling, the hydrolysate was washed with distilled water, filtered using Whatman No 1:11µm filter paper and dried at 60 ± 3 ºC (also under vacuum) in a rotary evaporator. The dried sample was then dissolved in 0.01M HCl. The amino acids in the hydrolysate were separated and quantified by injecting 50 µl into a Hitachi 835-50 amino acid analyzer equipped with a 2.6 mm ×150 mm ion exchange column coated with resin 2619#. The column temperature was 53 °C. Sodium citrate buffers (pH 3.3, 4.3, and 6.3) were used as eluents with a flow rate of 0.225 mL min^{-1}. The absorbance of the amino acids was detected by a 166 Detector (Beckman Instruments, California United States) at 570 nm and the amino acids were quantified by calibration curves using standard concentration.

2.3 Determination of Anti-Nutritional Factors

2.3.1 Phytochemical/Antinutrient Determinations

Oxalate content determination: Oxalate content in the food samples was determined using methods of AOAC (2005). One gram of the sample was weighed into a conical flask. 75 mL of 3M H_2SO_4 was added, and the solution was carefully stirred intermittently with a magnetic stirrer for about 1 hour and then filtered using Whatman No 1: 11 µm filter paper. 25 cm^3 of sample filtrated were titrated against a 0.1 N $KMnO_4$ solution to the final point (pink colour) that persisted for at least 30 sec. The oxalate content of each sample was calculated.

Phytate content determination: The phytate content of each sample was determined using the method described

by Latta and Eskin (1980). Two grams were weighed into 250 ml conical flask. 100 mL of 2% conc. HCl were used to soak the samples then it was filtered using Whatman No 1: 11 µm filter paper. Fifty milliliters (50 mL) of each sample filtrate were added to 100 mL of distilled water in a 250 ml beaker to improve acidity. Ten milliliters (10 mL) of 0.3% ammonium thiocyanate solution was added to each sample solution as indicated and titrated with standard iron chloride solution which contained 0.00195g iron/mL and the end point was signified by brownish – yellow colouration that persisted for 5 min. The percentage of the phytic acid was calculated.

Tannin content determination: Tannin contents were determined by the modified vanillin-HCl methods Latta and Eskin (1980). Two grams sample was extracted with 50 ml 99.9% methanol for 20 minutes at room temperature with constant agitation. After centrifugation for 10 min. at 653 rpm, 5 ml of vanillin-HCl (2% Vanilli and 1% HCl) reagent were added to 1 ml aliquots, and the colour developed after 20 min. at room temperature was read at 500 nm. Correction for interference light natural pigments in the sample was achieved by subjecting the extract to the conditions of the reaction, but without vanillin reagent. A standard curve was prepared using Catechin (Sigma Chemical, St. Louis, MO) after correcting for blank, and tannin concentration was expressed in g/100 g.

Determination of Trypsin inhibitor: The Trypsin activity of the samples was determined using the method of Prokopet and Unlenbruck (2002). The inhibitor extract was prepared, the powder samples were defatted with petroleum ether and methanol. One gram of each sample was dispersed in 50 mL of 0.5 M NaCl solution. The mixture was stirred for 30 minutes at room temperature and centrifuged at 1500 rpm for 5 min. The supernatants were filtered and the filtrates used for the assay. Two (2) mL of the standard Trypsin solution was added to 10 mL of the substrate of each sample. The absorbance of the mixture was taken at 410 nm using 10 mL of the same substrate as blank.

2.4 Functional Properties Determinations

Water Absorption Capacity (WAC): Water Absorption Capacity (WAC) was determined using the method of Adebowale et al., 2005. 10 ml of distilled water was added to 1 g of the sample in a beaker. The suspension was stirred using magnetic stirrer for 3 minutes. The suspension obtained was thereafter centrifuged at 3500 rpm for 30 minutes, and the supernatant was measured into a 10 ml graduated cylinder. The water absorbed by the flour was calculated as the difference between the initial volume of the sample and the volume of the supernatant.

$$\text{WAC (\%)} = \frac{Weight\ of\ water\ absorbed \times density\ of\ water \times 100}{weight\ of\ sample}$$

Determination of Least gelation concentration: Gelation property was determined using the method described by Adebowale et al. (2005). Appropriate sample suspensions of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 g were weighed into 10 mL distilled water each to make 20% (w/v) suspension. The test tubes containing these suspensions were heated in a boiling water bath for 1 hour, followed by rapid cooling under running tap water. The test tubes were then cooled for an hour. The least gelation concentration was determined as the concentration when the sample from the inverted test tubes did not slip or fall. The analysis was carried out in triplicate.

$$\text{Least gelation (\%)} = \frac{weight\ of\ sample}{10ml\ of\ water} \times 100$$

Swelling capacity determination: This was determined by the method described by Ikegwu et al. (2010) with modification for small samples. One gram of the flour sample was mixed with 10 ml distilled water in a centrifuge tube and heated at 80 ºC for 30 min under continued shaking. After heating, the suspension was centrifuged at 1000 × g for 15 min. The supernatant was decanted and the weight of the paste taken. The swelling power was calculated as follows: swelling power = weight of the paste / weight of dry flour

Bulk density determination: The procedure of Okaka and Potter (1979) was used to determine the bulk density. A 100 mL graduated cylinder was weighed and recorded as W_1, 15 g of sample were put into the cylinder, tapped hermitically to eliminate air space between the flour, the volume was noted and new mass was recorded as W_2. The bulk density was computed as follows:

$$\text{Bulk Density (BD)} = \frac{Mass\ of\ the\ sample}{Volume\ of\ the\ cylinder}$$

2.5 Nutritional Evaluation of Samples

Forty-two weaning albino rats aged 28-35 days with average initial weight of 33-60 g were obtained from the

Central Animal House, College of Medicine University of Ibadan, Nigeria. The rats were randomly distributed into six groups comprising seven rats each. The rats were housed in individual metabolic cages in a room at 25 ± 2 °C with facilities for urine and fecal collection. The rats were acclimatized for four days and thereafter fed with three experimental diets, basal and control (*Ogi*- a local complementary food and *Cerelac*- a commercial formula) diets with water *ad libitum* daily for 28 days. The feed intake was measured daily and body weight and length at 3 days intervals. The fecal droppings of the rats were collected daily, dried at 85 ± 3.0 °C to a constant weight and then ground into powder for fecal nitrogen determination. Urine samples were collected in sample bottles containing 0.1 N HCl to prevent loss of ammonia and stored in a freezer until analyzed for urinary nitrogen. Data on feed consumption and spilled food were collected by recording the feed measured out for each rat at the beginning and the quantity remaining after feeding. Gain or loss in weights of the rats was also recorded. Feacal and urinary nitrogen of the rats were determined by Kjeldhal method (AOAC, 2005). Protein Efficiency Ratio (PER), Net Protein Utilization (NPU), Biological Value (BV), True Digestibility (TD), Net Protein Retention (NPR) were calculated (FAO/WHO, 1989; AOAC, 2000). The experimental rats were sacrificed with chloroform at the end of 28 days, dissected and blood was collected through cardiac punctured into bijour bottles containing ethylenediaminetetraacetic acid (EDTA). The bottles were immediately capped and the content rocked gently. The blood was used for subsequent hematological studies.

2.6 Determination of Hematological Indices

Determination of packed cell volume (PVC): The blood samples were mixed well but gently for 2 minutes, drawn up a 75 x 1.5 mm capillary tube for $^3/_4$ of the length, one end of the capillary tube was sealed with sealant and then placed in a haematocrit centrifuge ensuring that the sealant is at the outer end before closing the centrifuge lid, the tube was centrifuged at 12,000 rpm for 4 minutes, the tube then placed in a reader and the reading recorded. The reading was expressed as percentage of packed red cells to total volume of the whole blood.

Determination of hemoglobin (Hb): The blood samples were mixed gently for one minute, drawn into 0.2mL pipette to the mark, and then expelled into 4 mL of Drabklin's solution. The pipette was washed thoroughly, re-filled with blood and then expelled into the Drabkin´s solution. The tube was stopped and mixed and allowed to stand for 5 min until a full color was developed. Standard was prepared as above using a blood sample of known hemoglobin concentration. A green (624 nm) filter was used, setting the colorimeter to zero using the Drabkin solution as blank. The sample and standard blood dilutions on the colorimeter were read

$$\text{Hb} = \frac{\text{Absorbance of standard X Standard concentration}}{\text{Absorbance of sample}} (g/dl)$$

Determination of red blood cells count (RBC): The blood samples were mixed thoroughly by repeated inversion; 0.2 ml pipette was used to draw the blood up to the mark before expelling it to a 4 ml of diluting fluid in a bijou bottle and then the pipette washed thoroughly by alternately drawing up and expelling the dilute fluid. The diluted blood were mixed for at least half minute by inversion and a fine Pasteur was used to fill the counting chamber

Determination of white blood cells count (WBC): The white blood cell count samples were diluted in the same way as for the red blood cells but using a 0.05 ml blood pipette and 0.95 ml of diluting fluid.

2.7 Statistical Analysis

The data were analyzed using Statistical Package for Social Sciences (SPSS) software version 16.0. The mean and standard deviations of the analyses were calculated. The analysis of variance (ANOVA) was performed to determine significant differences between the means using Duncan Multiple Range Test at $P < 0.05$.

3. Results and Discussion

3.1 Proximate and Mineral Composition of Popcorn-Moringa Based Complementary Foods

The proximate and mineral composition of blanched and fermented popcorn-*moringa oleifera* flour blends and control samples are presented in Table 1. The moisture content of blanched popcorn-moringa leaves (BPM) formulation (6.73 ± 0.33 g/100 g) was lower than that of fermented popcorn-moringa leaves flour (FPM) formulation (8.02 ± 0.24 g/100 g) and lower when compared with Ogi (8.31 ± 0.57 g/100 g) and Cerelac (11.3 ± 0.50 g/100 g), respectively. This observation indicates that BPM and FPM samples may have longer shelf life, than 'Ogi' and Cerelac because of their lower moisture contents. Studies have shown that moisture content in food products facilitate the growth of microorganisms, which in turns causes spoilage and low nutritional qualities of the food products (Udensi et al., 2012; Oyarekua, 2013). Protein content of FPM (21.27 ± 0.20 g/100

g) had higher values than that of BPM (15.99 ± 0.14 g/100 g) and control samples, which include *Cerelac* (15.75 ± 0.01 g/100 g) and 'Ogi' (6.52 ± 0.31 g/100 g) respectively. However, the protein contents of popcorn-moringa leaves combinations in this present study were higher than FAO/WHO (1991) recommended value for infant complementary food (≥ 15 g/100 g), and also higher than the value reported for complementary foods formulated from sorghum, sesame, carrot and crayfish (Onabanjo et al., 2009). Energy value of FPM blend (393.94 ± 0.39kcal.) was also higher than that of BPM (389.69 ± 1.40 Kcal) sample, however, energy values of formulated diets were lower when compared with those of control samples, that is, '*Ogi*' (418.08 ± 0.47 kcal) and *Cerelac* (431.58 ± 0.01kcal). This observation could be attributed to low carbohydrate contents that were observed in popcorn and moringa leaves flour blends. Nutritionally, The high protein and energy values observed in this study, particularly FPM blend, showed that the formulations are suitable for infants complementary food; and also could be substituted for traditional complementary foods, that is, cereal gruel, which had been implicated as one of the major causes of protein–energy malnutrition among weaning aged children in Nigeria and other developing countries (Anigo et al., 2009). It has been proven that most families in developing countries, including Nigeria, are unable to feed their children with the high cost fortified, nutritious, proprietary complementary foods, due to poverty (Mosha et al., 2000; Amankwah et al., 2009; Bruyeron et al., 2010; Muhimbula et al., 2011). Hence, such families are always depending on low cost family diets or mainly of un-supplemented cereal porridges made from maize, sorghum and millet, which are low in protein and energy density (Eka et al., 2010). To improve nutritional quality of complementary foods, studies have advocated for the use of cereal and legumes or other locally available food materials in combinations (ACC/SCN, 2001; Ibeanu, 2009), which help to increase the protein and energy density of complementary foods fed to young children in developing countries. FAO/WHO (1985) has recommended that foods fed to infants should be adequate in protein and energy dense, because low energy dense foods tend to reduce total energy intake and other essential nutrients in children. Evidence has shown that high-energy foods are necessary for children to cover their energy needs considering the small size of their stomach (Solomon, 2005).

Mineral contents of BPM were higher in zinc, iron, potassium, sodium and phosphorous, while FPM sample was higher in copper, calcium and magnesium. The mineral contents in the formulated diets were comparatively higher than Ogi, but lower than that in *Cerelac*. The lower mineral content observed in the formulated diets when compared with the *Cerelac* could be attributed to the facts that the *Cerelac*, a commercial complementary food, was fortified with micronutrients during its production and such was not applied to the formulated diets in this present study. To compensate for lost of macronutrients and micronutrients in processed foods, a number of researchers have advocated for food fortification, particularly infants foods, during the production process (Rosalind et al., 2000; Lutter & Dewey, 2003). The Ca/P and Na/K molar ratios of popcorn-moringa leaves flour blends ranged from 0.55 to 1.17 and 0.51 to 0.71, respectively. These observations showed that all the samples met the recommended values for Na/K (<1.0) and Ca/P (>2.0), except BPM for Ca/P, which indicate that the formulated diets would support bone and teeth formation in children and also would not pose any danger to heart of the infant whenever taken as complementary food.

Table 1. Proximate composition (g/100g) and mineral composition (mg/100g) of complementary foods formulated from popcorn and *Moringa oleifera* leaf flour

Nutrient	FPC	BPM	FPM	Ogi	Cerelac	Recommended values
Macronutrient composition						
Moisture	5.43 ±0.47^{c}	6.73 ±0.33^{b}	8.02 ±0.24^{b}	8.31 ±0.57^{b}	11.3 ±0.50^{a}	<5
Ash	0.87^{b} ±0.07	4.33 ±0.30^{a}	3.87 ±0.03^{a}	1.09 ±0.01^{b}	3.16 ±0.01^{a}	<3
Protein	14.37 ±0.52^{c}	15.99 ±0.14^{b}	21.27 ±0.20^{a}	6.52 ±0.31^{d}	15.75 ±0.01^{bc}	>15
Fat	5.85 ±1.63^{c}	8.67 ±0.14^{b}	10.51 ±0.11^{a}	5.17 ±0.11^{c}	10.53 ±0.02^{a}	10-25
Fiber	0.81 ±0.21^{b}	2.35 ±0.04^{a}	2.76 ±0.02^{a}	0.85 ±0.01^{b}	2.11 ±0.01^{a}	<5
Carbohydrate	78.09 ±1.25^{b}	61.91 ±0.74^{d}	53.55 ±0.47^{e}	86.38 ±0.21^{a}	68.42 ±0.01^{c}	64
Energy	422.53 ±8.91^{a}	389.69 ±1.40^{b}	393.94 ±0.39^{b}	418.08 ±0.47^{a}	431.58 ±0.01^{a}	400-425
Minerals composition						
Zinc	2.84 ±0.32^{b}	0.16 ±0.01^{c}	0.12 ±0.01^{cd}	0.08 ±0.00^{d}	5.00 ±0.00^{a}	3.2
Iron	4.12^{b} ±0.04	1.83 ±0.01^{c}	1.81 ±0.01^{c}	0.26 ±0.01^{d}	7.50 ±0.01^{a}	16
Potassium	122.59 ±1.68^{c}	270.64 ±0.25^{b}	122.86 ±0.05^{c}	102.39 ±1.01^{d}	635.00 ±0.00^{a}	516
Sodium	140.71 ±0.45^{b}	137.61 ±0.05^{c}	87.62 ±0.07^{d}	14.56 ±0.04^{e}	145.00 ±0.00^{a}	296
Calcium	134.80^{b} ±0.07	45.87 ±0.02^{e}	85.71 ±0.02^{c}	68.66 ±0.35^{d}	600.00 ±0.01^{a}	500
Magnesium	31.44^{d} ±0,96	284.40 ±0.02^{b}	285.71 ±0.01^{a}	34.91 ±0.01^{c}	0.00^{e}	76
Phosphorous	142.51^{b} ±14.62	84.12 ±0.02^{c}	73.28 ±0.01^{d}	85.95 ±0.02^{c}	400.00 ±0.01^{a}	456
Lead		-	-	-	-	-
Na/K	1.15 ±0.02^{a}	0.51 ±0.06^{c}	0.71 ±0.01^{b}	0.14 ±0.01^{e}	0.23 ±0.02^{d}	<1
Ca/P	0.95 ±0.01^{c}	0.55 ±0.0^{e}	1.17 ±0.01^{b}	0.80 ±0.01^{d}	1.50 ±0.01^{a}	1.6-3.6

Mean values with the same superscript in a row are not significantly different (P>0.05), * RV- Recommended values for infant complementary foods (*FAO/WHO, 1991*).

Key: FPM (Fermented Popcorn-Moringa leave flour), BPM (Blanched Popcorn-Moringa leave flour).

3.2 Amino Acids Profile of Popcorn-Moringa Based Complementary Foods

The amino acids profile of the formulated complementary food samples are shown in Table 2. The predominant amino acid in the blends was glutamic acid and the finding agreed with other researchers, who reported that glutamic and aspartic acids are usually the most abundant amino acids in plant based food products (Adeyeye, 2004; Aremu et al., 2006); while methionine was the least concentration. Arginine and histidine which are essential amino acids for infants' growth and development were higher in fermented formulation (FPM) than blanched formulation (BPM). However, these amino acids (arginine and histidine) were lower than the reference egg protein (6.3 g/100 g) and RDA value of (6.6 g/100 g) (FAO/WHO/UNU, 1985). Total essential amino acids in FPM blend (25.99 g/100 g protein) was higher than that of BPM blend (24.56 g/100 g protein), and both were

higher than 'Ogi' (18.32 g/100 g protein), but lower when compared with that of *Cerelac* (31.73 g/100 g protein). The concentration of total essential amino acids observed in FPM blend compared with BPM blend is likely due to the activities of microorganisms, which involves utilizing other nutrients like carbohydrate in the food product to synthesize amino acids which the organisms need for their growth (Cronk et al., 1977).

Table 2. Amino acids (g/100g crude protein) profile of blanched and fermented popcorn-*Moringa oleifera* leaves blends and control samples

Amino acids	FPC	BPM	FPM	Ogi	Cerelac	*RDA
Non essential amino acids (TNEAA)						
Alanine	5.85 ±0.05^{a}	3.09 ±0.01^{d}	3.47 ±0.01^{c}	3.42 ±0.19^{c}	4.42 ±0.02^{b}	
Aspartic acid	7.21 ±0.02^{b}	6.17 ±0.02^{d}	6.88 ±0.01^{c}	6.12 ±0.02^{e}	9.26 ±0.01^{a}	
Serine	0.26 ±0.02^{e}	4.02 ±0.02^{d}	4.22 ±0.02^{c}	4.78 ±0.01^{a}	4.33 ±0.02^{b}	
Glutamic acid	7.59 ±0.01^{e}	14.72 ±0.02^{d}	15.14 ±0.01^{c}	17.64 ±0.01^{b}	19.54 ±0.01^{a}	
Proline	0.55 ±0.01^{e}	2.72 ±0.02^{b}	2.68 ±0.01^{b}	2.49 ±0.01^{c}	3.26 ±0.02^{a}	
Glycine	0.36 ±0.01^{e}	4.79 ±0.01^{c}	5.16 ±0.01^{b}	4.15 ±0.02^{d}	5.88 ±0.01^{a}	
∑NEAA	21.82 ±0.02^{e}	35.52 ±0.07^{d}	37.56 ±0.04^{c}	38.63 ±0.14^{b}	46.73 ±0.04^{a}	
Essential amino acids (TEAA) for infant						
Lysine	2.18 ±0.02^{c}	3.54 ±0.02^{b}	3.65 ±0.02^{b}	1.71 ±0.01^{d}	4.13 ±0.01^{a}	5.8
Arginine	4.06 ±0.02^{c}	1.72 ±0.01^{e}	1.89 ±0.02^{d}	4.84 ±0.02^{b}	9.12 ±0.02^{a}	2
Threonine	1.23 ± 0.01^{d}	4.11 ±0.03^{b}	4.38 ±0.01^{a}	1.09 ±0.01^{d}	3.80 ±0.10^{c}	3.4
Valine	1.38 ±0.01^{e}	3.03 ±0.02^{c}	3.36 ±0.01^{b}	2.69 ±0.01^{d}	4.79 ±0.01^{a}	3.5
TSAA (Meth.+ cystein)	1.10 ±0.00^{e}	3.12 ±0.01^{a}	3.02 ±0.02^{c}	3.08 ±0.01^{b}	2.79 ±0.01^{d}	2.5
Isoleucine	2.27 ±0.01^{d}	2.33 ±0.01^{c}	2.34 ±0.02^{c}	3.56 ±0.01^{b}	4.23 ±0.01^{a}	2.8
Leucine	3.78 ±0.04^{c}	4.96 ±0.02^{b}	5.23 ±0.02^{a}	3.75 ±0.01^{c}	5.25 ±0.01^{a}	6.6
TArAA(Phenyl.+Tyro)	3.27 ±0.01^{c}	6.58 ±0.02^{ab}	6.48 ±0.01^{ab}	6.08 ±0.02^{b}	8.23 ±1.25^{a}	6.3
Histidine	0.55 ±0.05^{e}	1.71 ±0.01^{c}	1.89 ±0.01^{b}	1.57 ±0.01^{d}	2.04 ±0.02^{a}	1.9
Tryptophan	ND	ND	ND	ND	ND	1.1
∑EAA	19.82 ±0.03^{d}	31.13 ±0.08^{b}	32.36 ±0.23^{b}	28.38 ±0.06^{c}	44.51 ±1.38^{a}	
∑AA	41.64 ±0.11^{d}	66.65 ±0.14^{c}	69.92 ±0.26^{b}	67.01 ±0.09^{c}	91.23 ±1.42^{a}	

Mean values with the same superscript in a row are not significantly different (P >0.05),

*FAO/WHO (1991), FPC (fermented popcorn), FPM (Fermented Popcorn-Moringa leave flour), BPM (Blanched Popcorn-Moringa leave flour).

3.3 Anti-Nutrient Factors in Popcorn- Moringa oleifera Leaves Formulations

The concentrations of anti–nutrients in the formulated complementary food samples are shown in Table 3. The values of oxalate, phytate and trypsin inhibitor in fermented popcorn-moringa leaves blend (FPM) were lower when compared with the blanched popcorn-*Moringa oleifera* leaves blend (BPM). It was generally observed in this study that the antinutrient compositions of the formulations were generally low and they are within the tolerable levels. For instance, the oxalate and tannin contents of the formulated complementary foods were lower compared with the complementary food based on Soybean and Tigernut (Ikpeme-Emmanuel et al., 2012). Study has shown that oxalates in large amounts bind with calcium forming calcium oxalate, which is insoluble and not absorbed by the body (Ladeji et al., 2004). Oxalates are considered poisonous at high concentration, but harmless when present in small amounts (Chai & Liebman, 2004). High oxalate level in food has been implicated as the cause of kidney stones because high level of oxalates increases calcium absorption in the kidney (Chai & Liebman, 2004). Tannin has been implicated to form insoluble complexes with proteins thereby reducing digestibility and utilization of food proteins, interference with the absorption of Iron and inhibition of trypsin, chymotrypsin, amylase and lipase (Griffiths & Mosley, 1980; Delumen & Salamat, 1980). Studies have reported that combination of blanching or cooking and fermentation improved the nutritional quality of food products and also reduced the anti-nutritional factors in the food product to a safe level (Paredes-López & Harry, 1988; Obizoba & Atii, 1991).

Table 3. Anti-nutritional content (mg/100g) of complementary foods formulated from *Moringa oleifera* leaves and popcorn

Antinutrients	**BPM**	**FPM**
Oxalate	6.26±0.045	4.95±0.09
Phytate	23.07±0.001	20.19±0.41
Tannin	0.06±0.005	0.09±0.00
Trypsin inhibitor	33.25±0.255	29.79±0.13

Key: FPM (Fermented Popcorn-Moringa leave flour), BPM (Blanched Popcorn-Moringa leave flour).

3.4 Functional Properties of Popcorn-Moringa oleifera Leave Formulations

Functional properties of the complementary foods formulated from popcorn and *Moringa oleifera* leaves are presented in Table 4. Bulk density of BPM and FPM were 0.73 g/cm^3 and 0.70 g/cm^3, respectively. The BPM had higher values in water absorption capacity (68.5 ml/g) and least gellation (0.63%) when compared with FPM blends (55.5 ml/g and 0.62%, respectively), while FPM had higher value in swelling capacity (1.25%) than BPM (1.05%). Comparatively, it was observed that the BPM and FPM blends were higher in bulk density and water absorption capacity than in *Cerelac*, but lower in swelling capacity and least gellation. The higher values of water absorption capacity and bulk density that were observed in this study compared with the control samples do not limit the nutritional advantages of these products. However, these values were similar to other plant-based food products (Masood & Rizwana, 2010; Gernah et al., 2012; Oyarekua, 2013). Higher water absorption capacity indicates higher protein content in the formulations, which absorbs and binds with more water (Otegbayo et al., 2000). Scientific finding has shown that high water absorption capacity indicates that food samples hold large volume of water during cooking into gruels, to yield voluminous low energy and nutrient food (Omueti et al., 2009). According to WHO (2003), appropriate complementary food is the one which produce a gruel or porridge that is neither too thick for the infant to consume nor so thin that energy and nutrient density are reduced. Therefore, low water absorption capacity is desirable in complementary food for making thinner gruels with high caloric density per unit volume. The Bulk Density (BD) is a reflection of the load the sample can carry if allowed to rest directly on one another. The lower the bulk density value, the higher the amount of flour particles that can stay together thereby increases the energy content derivable from such diets (Onimawo & Egbekun, 1998). Evidence has shown that high bulk density limits the caloric and nutrient intake per feed of a child, because of the small capacity of the child's stomach that would not be able to accommodate large volume of food to satisfy their energy and nutrient requirements (Omueti et al., 2009). Besides, bulk density is also important in the packaging requirement and material handling of the complimentary diet (Karuna et al., 1996).

Table 4. Functional properties of complementary foods formulated from *Moringa oleifera* leaves and popcorn

Parameters	Bulk Density (g/cm^3)	Water absorption capacity (ml/g)	Swelling Capacity(%)	Least Gellation (%)
BPM	0.73±0.00	6.9±0.05	1.05±0.05	0.63±0.03
FMP	0.70±0.01	5.6±0.05	1.25±0.05	0.62±0.03
Ogi	0.66±0.01	1.82±0.02	0.90±0.03	9.00±1.11
Cerelac	0.56±0.03	2.31±0.21	2.43±0.03	14.00±1.21

FPM (Fermented Popcorn-Moringa leave flour), BPM (Blanched Popcorn-Moringa leave flour)

3.5 Protein Quality and Heamatological Evaluations

The protein digestibility indices of the formulated diets and control samples are presented in Figure 1. The nutritional indices for evaluating protein digestibility of the formulated diets in rats ranged as follows: 76-88% for food efficiency (FE), 95.9-96.7% biological value (BV), 79.5-84.2% net protein utilization (NPU) and 2.69-4.32 protein efficiency ratio (PER). Comparatively, the popcorn-*Moringa oleifera* leaves sample had higher values in FE, BV, PER than blanched popcorn-*Moringa oleifera* leaves sample and Ogi, but lower when compared with the *Cerelac*. The BV and PER of the formulations met the FAO/WHO (1989) recommended values of 70% and 2.7, respectively. These indicate that the protein content in the formulations were of good qualities and are suitable to support growth and development in infant.

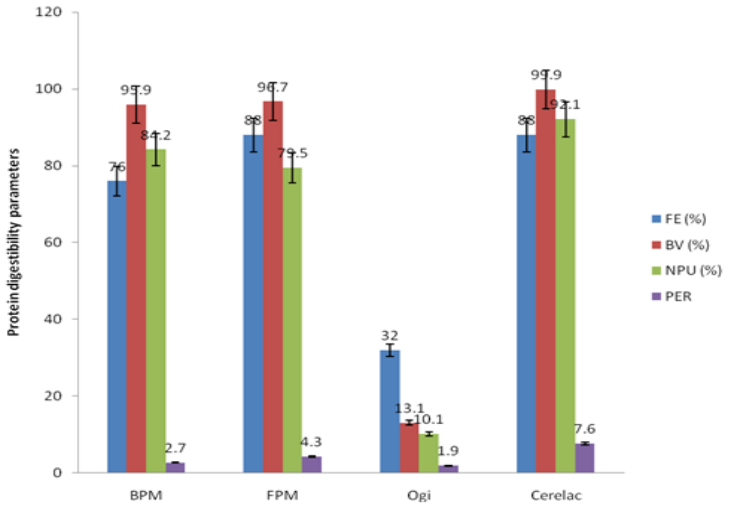

Figure 1. Protein digestibility of formulated diets and weight of organs of Albino rats fed with the diets ($p < 0.05$)

Key: FPM (Fermented Popcorn-Moringa leave flour), BPM (Blanched Popcorn-Moringa leave flour), FE (Food efficiency), BV (Biological value), NPU (Net protein utilization), PER (Protein efficiency ratio)

Growth rates of albino rats fed with the formulated diets and control samples are shown in Figures 2-4. The albino rats fed with the FPM sample had higher growth rate when compared with those rats fed with BPM sample and Ogi, but lower than those rats fed with *Cerelac*. The higher growth rated recorded for those animals fed with the *Cerelac* compared with those fed with formulated diets could be attributed to its food composition, i.e., milk-based and large quantity of food intakes by the rats in *Cerelac* group due to the attractive flavor and taste of the product. This observation agreed with the reports of Sodipo and Fashakin (2011) and Ijarotimi (2006), who reported higher weight gained in animals fed with *Cerelac* and Nutrend, respectively. The weight of the organs, i.e., kidney, liver and heart, of albino rats fed with BPM and FPM diets were well developed than those rats fed with 'Ogi', but comparable with those in *Cerelac* group (Figure 5).

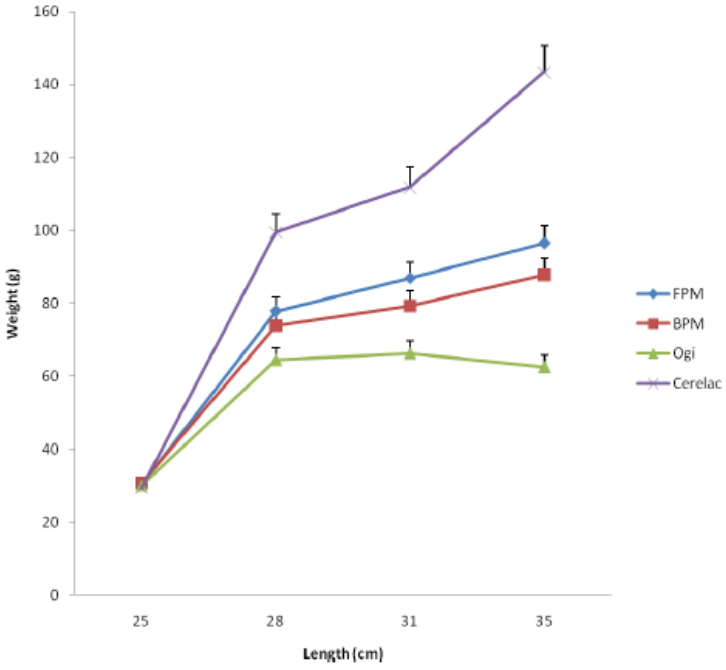

Figure 2. Nutritional status of the Albino rats fed with formulated diets and control food samples (Cerelac and ogi) using weight-for-length nutritional index

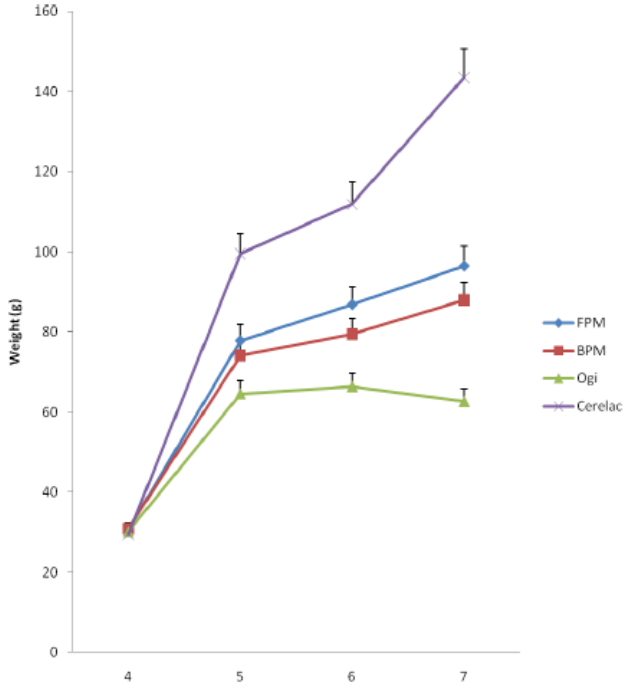

Figure 3. Nutritional status of the Albino rats fed with formulated diets and control food samples (Cerelac and ogi) using weight-for-age nutritional index

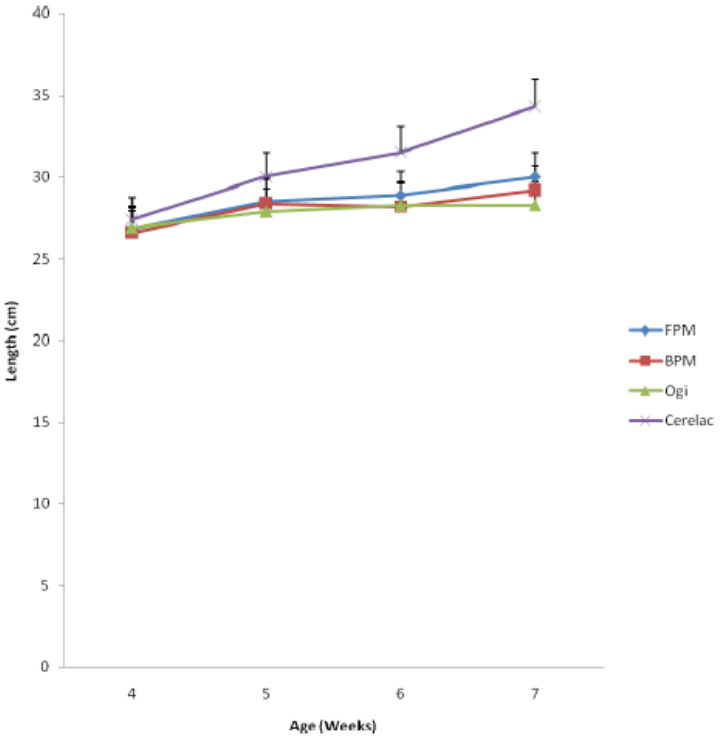

Figure 4. Nutritional status of the Albino rats fed with the formulated diets and control food samples (Cerelac and ogi) using length-for-age nutritional index

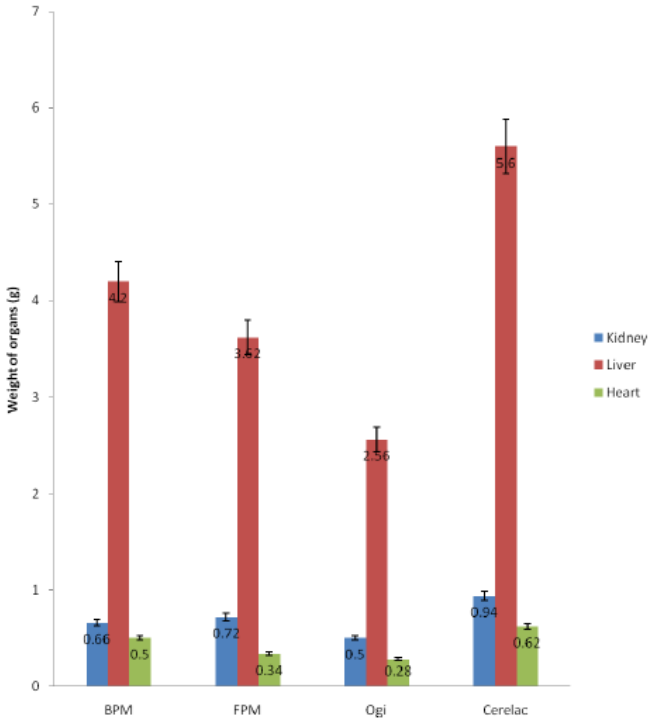

Figure 5. Weight of organs of albino rats fed with the formulated diets and control food samples (*Cerelac* and ogi) ($p<0.05$)

Key: FPM (Fermented Popcorn-Moringa leave flour), BPM (Blanched Popcorn-Moringa leave flour).

The heamatological properties of Albino rats fed with the formulated diets and control samples are presented in Table 5. The pack cell volume (PCV), red blood cells and white blood cells of Albino rats fed with the BPM diet were higher than FPM, and both diets were higher than those rats fed with control samples, except in *Cerelac* group. The growth rate and non-atrophy of livers, kidneys and hearts of rats fed with the formulated diets couple with heamatological values further showed that the popcorn-moringa leaves based diets are good substitutes for 'Ogi' or other family diets used as complementary, which have been implicated as one of the causes of protein-energy malnutrition among weaning aged children in developing countries.

Table 5. Haematological Properties of popcorn-based infant food supplemented with *Moringa oleiera* leaves flour

Heamatological indices	Ogi	*Cerelac*	FPM	BPM	Baseline values
ESR (mm)	1.0^{a}	1.0^{a}	1.0^{a}	1.0^{a}	1.0^{a}
PCV (%)	37.0^{ab}	41.0^{a}	38.0^{ab}	40.0^{a}	36.0^{b}
RBC ($x10^{6}mm^{3}$)	493^{d}	651^{a}	504^{c}	572^{b}	488^{e}
WBC ($x10^{3}mm^{3}$)	221^{b}	186^{d}	234^{a}	208^{c}	213^{c}
Hemoglobin (g/100ml)	12.3^{a}	13.6^{a}	12.6^{a}	13.3^{a}	12.0^{a}
Lymphocytes (%)	60.0^{a}	62.0^{a}	60.0^{a}	64.0^{a}	65.0^{a}
Neutrophils (%)	31.0^{a}	28.0^{b}	32.0^{a}	26.0^{b}	26.0^{b}
Monocytes (%)	5.5^{a}	6.0^{a}	5.0^{a}	6.0^{a}	5.0^{a}
Eosinophils (%)	2.0^{a}	3.0^{a}	2.0^{a}	3.0^{a}	4.0^{a}
Basophils (%)	1.0^{a}	1.0^{a}	1.0^{a}	1.0^{a}	0.0^{b}

Mean values with the same superscript in a row are not significantly different (P>0.05)

Key: FPM (Fermented Popcorn-Moringa leave flour), BPM (Blanched Popcorn-Moringa leave flour).

4. Conclusion

The present study established the chemical compositions and nutritional qualities of infant food formulated from popcorn and *Moringa oleifera* leaves flour combinations. The findings showed that the formulated diets, particularly fermented popcorn-moringa (FPM) blend, were better in terms of protein contents, essential amino acids and ability to support growth and development in experimental animals than local complementary food (Ogi). Hence, these formulations could be used as infant food, particularly for underprivileged children, who cannot have access to qualitative complementary foods.

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