JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

ACCUMULATION OF CADMIUM, LEAD AND MERCURY IN SEEDLINGS OF SELECTED SUGAR BEET VARIETIES AS A RESULT OF SIMULATED SOIL CONTAMINATION

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doi: 10.15414/jmbfs.2016.5.4.351-354

ARTICLE INFO

Received 5. 10. 2015
Revised 3. 11. 2015
Accepted 12. 11. 2015
Published 1. 2. 2016

Regular article

OPEN ACCESS

ABSTRACT

The article presents the findings of a study designed to compare accumulation of cadmium, lead and mercury in seedlings of selected sugar beet varieties, as a result of simulated soil contamination. The analyses included seedlings of eight sugar beet varieties: Alegra C, Delano C, Milton C, Primadonna, Silvetta C, Finezja C, Agnieszka, Janosik, which in the course of growth were fortified with heavy metals. The acquired results were used to calculate Bioconcentration Factors (BCF) and to compare the contents of heavy metals in the examined plants. The greatest capacity to absorb the relevant heavy metals from the soil was found in seedlings of Milton C and Silvetta C varieties for cadmium and lead, and in Silvetta C and Finezja C varieties for mercury. It was determined that in the entire group of plants, the seedlings of Agnieszka and Janosik verities were least susceptible to bioaccumulation of cadmium and lead, while Primadonna variety had the lowest susceptibility to mercury.

Keywords: Heavy metals, soil contamination, sugar beet, bioconcentration factors

INTRODUCTION

The progress of civilization and the increasing pollution of the environment have resulted in numerous irreversible changes affecting natural areas **(Majtkowski *et al.*, 2010)**. Human activity in recent years has led to an increase in quantities and distribution of heavy metals in the atmosphere, soil and aquatic ecosystems **(Gbaruko and Friday, 2007)**. The large-scale gas emissions, combustion of fossil fuels in heat and power generation plants, accumulation of industrial waste as well as utilization of artificial fertilizers and crop protection chemicals in agriculture have significantly contributed to the increased content of heavy metals in the soils and surface waters. Heavy metals taken up by plants from the soils may adversely affect their growth and development **(Majtkowski *et al.*, 2010)**. Accumulation of heavy metals in soil causes an undesirable changes in the soil ecosystems and functioning of plants **(Khan *et al.*, 2008a; Khan *et al.*, 2008b)**. The consequences of exposing plants to heavy metals may include impairments in the processes of flowering, gametophyte development, sprouting and development of seedlings **(Siwek, 2008)**.

Considerable contamination of soils and plants with heavy metals is found in areas located along major roads. Carbon and nitrogen oxides, sulfur dioxide and heavy metals get into the atmosphere along with fumes produced by combustion engines. The use of car tyres and other parts of mechanical vehicles results in the fact that numerous substances, including cadmium and toxic lead compounds find their way into the environment **(Baran *et al.*, 2007)**. Uptake of heavy metals through plants in agricultural areas could pose a serious threat to humans **(Liu, 2007; Yan, 2012)**. With regard to areas used for farming purposes it should be emphasized that the potential sources of heavy metals in the soils include mineral and organic fertilizers, lime as well as waste-based composts. Power plants and industrial facilities generate dust emissions which may spread over large areas, polluting soils at a significant distance from the industrial zones. Air pollution leads to accumulation of such elements as cadmium and lead in the soil. Regardless of the way heavy metals access the soils, once they exceed the acceptable quantities, they may pose significant hazard to plants, animals and people **(D'Amore *et al.*, 2005; Gruca-Królikowska and Wacławek, 2006)**. Importantly, due to the low mobility of heavy metals in soil they are among the most persistent soil pollutants **(Potarzycki *et al.*, 1999)**.

Heavy metals find their way into plants via their root systems and leaf blades. Metals, which in the soil are present in the form of free ions, are more available for plants than element occurring in the form of complexes **(Chaney *et al.*, 1997).** Compounds such as metal chelators and organic acids, released by plant roots, may increase availability of heavy metals occurring in insoluble soil complexes. The degree of heavy metals accumulation in plants depends on the type of metals, their concentration in the soil substrate, the form in which they occur, as well as the plant variety. As for the contents of heavy metals in specific parts of the plant, it is possible to notice a decrease in metal concentration in the following order: root, leaves, stalk, flowers, and seeds **(Ociepa-Kubicka and Ociepa, 2012)**. Excessive quantities of heavy metals in the soil, resulting e.g. from their long-term accumulation, pose significant hazard for living organisms **(Grygierzec, 2013)**. High concentrations of heavy metals may impair physiological and biochemical processes occurring in organisms. The most harmful heavy metals include Pb, Hg, Cd, Sn, Cr, Zn, Cu **(Gosh, 2010)**. Metals which are considered to be most harmful for humans are cadmium and lead **(Sekara *et al.*, 2005)**. Lead is believed to be responsible for many ailments affecting people, e.g. chronic neurological disorders **(Awofolu, 2005)**. In the case of children, lead poisoning may lead to neurological conditions whose symptoms include problems with coordination, loss of short-term memory or learning difficulties **(Padmavathiamma and Li, 2007)**. Toxic activity of cadmium, visible even with low concentrations of the element in the organism, contributes to neoplastic changes, and impairs the functions of liver and kidneys **(Czeczot and Majewska, 2010)**. In plants, cadmium can contribute to disturbances in the photosynthesis process and nutrients collecting **(Kuo *et al.* 2006, Alia *et al.* 2015)**. Cadmium can cause oxidative stress and decrease the rate of a new cells production **(Sandalio *et al.*, 2001; Liu *et al.*, 2004)**. In turn Pb affected to a decline of chlorophyll content and the length of roots and shoots **(Sharma and Dubey, 2005; Joseph *et al.*, 2002)**. Hence, measurements of heavy metals contents in the air, soil and food seem to be a necessary element of monitoring and assessment of pollutants in the environment **(Gruca-Królikowska and Wacławek, 2006)**.

The purpose of the present study was to investigate the level of accumulation of three selected heavy metals (Cd, Pb, Hg) in seedlings of eight sugar beet varieties, with the use of simulated soil contamination. The study was also designed to compare the sugar beet varieties in terms of their ability to take up heavy metals from the soil.

MATERIALS AND METHODS

Experimental design

The materials used in the study included Pb, Cd and Hg (as $Pb(NO_3)_2$, $Cd(NO_3)_2$ and HgO) which were applied to contaminate the soil, as well as eight varieties of sugar beet coming from four companies (a - Alegra C, Silvetta C, b - Delano C,

Milton C, c - Primadonna, Agnieszka, d - Finezja C, Janosik). The experiment was carried out in Petri dishes, with the use of MLR-352 phytotron. For this purpose 30g samples of soil were weighed and transferred to 16 dishes. In the study universal soil based on peat, pH = 6 and salinity not exceed 1.9 $g.dm^{-3}$ NaCl was used. The soil contained a starting dose of compound fertilizers NPK (14-16-18): 0.6 kg/m^3. The study using the optic method of inductively coupled plasma optical emission spectroscopy (ICP-OES) ruled out the presence of cadmium, lead and mercury in the soil. The dishes were divided into two groups: the controls (the substrate contained no heavy metals), and the experimental group, then identical number of seeds was placed in each of the dishes. During the experiment, the dishes were located in a climatic chamber, 5ml portions of demineralized water were added to the control dishes, and solution of cadmium, lead and mercury ions was introduced to the experimental sample. The total of 20mg of the metal ions per 1 kg of the soil was added to each dish. After 10 days of growth in constant conditions, i.e. temperature of 25°C, air humidity at the level of 90% and constant lighting, above-ground parts of the plants were collected for further analyses.

Metal extraction and analysis

The collected plant material was carefully rinsed with demineralized water to remove soil residues. Subsequently, the plant samples were subjected to microwave digestion in Teflon vessels using Milestone ETHOS ONE microwave digestion system. The experimental material was subjected to three digestion processes repeated in parallel. 5 g of fresh plants were accurately weighed in a 120 ml Teflon digestion vessel. Then 8 ml of nitric acid was added and dissolved using microwave mineralization. The acid clear solution were transformed to 50 ml volumetric flasks and diluted with deionized water. The contents of cadmium, lead and mercury were determined using the optic method of inductively coupled plasma optical emission spectroscopy (ICP-OES). All elements were blanked by blind sample with was clear nitric acid mineralized in the same time and conditions. In the calibration step, standard solutions for all elements were prepared from a spectroscopic grade reagent (Thermo) with 3 step curve. A curve fit factor for all elements were above 0,99.

Data and statistical analysis

The findings were used to determine the Bioconcentration Factor (BCF), which shows the capacity of plants to take up heavy metals from the soil and to accumulate them in their tissues. The factor shows the relation of the heavy metal concentration in the plant tissues to the concentration of the same metal in the soil. Bioconcentration Factor was calculated from the formula **(Zhuang *et al.*, 2007)**:

$$BCF = C_{harvested\ tissue} / C_{soil}$$

where $C_{harvested\ tissue}$ means the concentration of the heavy metal in plant tissues, and C_{soil} means the concentration of the metal in the soil from which it was absorbed.

The statistical analysis of the findings was performed using software Statistica, version 10.0. The mean values and standard deviations were computed, and the differences were statistically verified using Student's t-test, at the significance level of $p<0.05$, for $n = 3$.

RESULTS AND DISCUSSION

Figure 1 shows the mean contents of lead and the standard deviation in the examined plants.

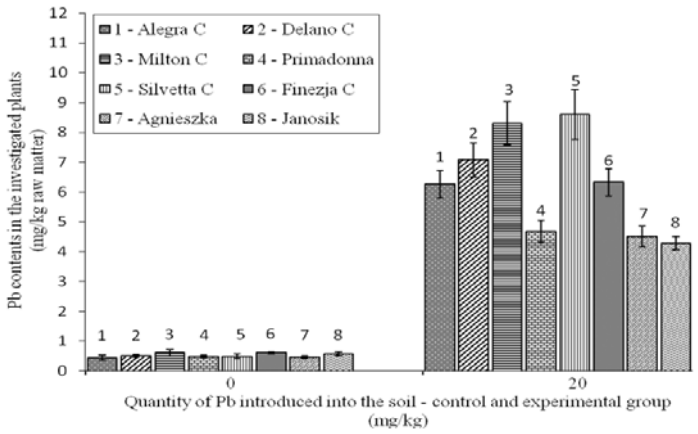

Figure 1 Contents of lead in the experimental and control group depending on sugar beet variety

Concentration of Pb in the investigated plants was significantly higher in all experimental samples, in comparison to the controls (the mean value for the experimental group differs significantly from the control - Student's t-test, $p<0.05$, $n=3$). The highest contents of lead were identified in Milton C and Silvetta C varieties, and the lowest contents of this metal were found in Agnieszka and Janosik varieties.

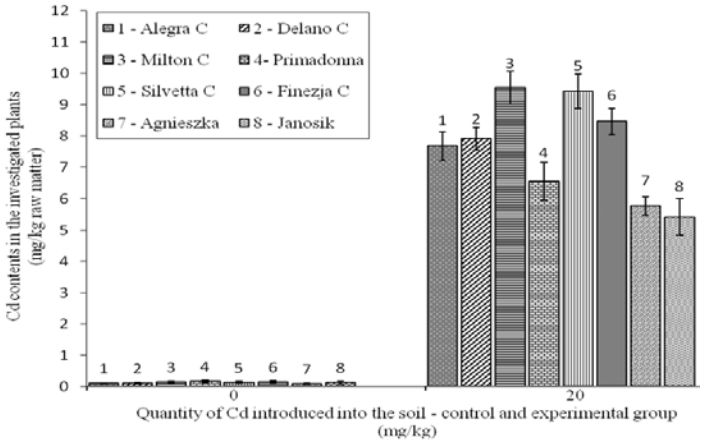

Figure 2 Contents of cadmium in the experimental and control group depending on sugar beet variety

Concentrations of cadmium in all studied varieties of beet were significantly higher, in comparison to the controls (the mean value for the experimental group differs significantly from the control - Student's t-test, $p<0.05$, $n=3$). The highest contents of cadmium in the experimental samples were found in the same plants which had the highest concentrations of lead (Milton C and Silvetta C). The lowest contents of cadmium were again identified in Agnieszka and Janosik varieties. Jankowska *et al.* **(2007)** measured the contents of lead in selected species of dicotyledonous plants growing in the proximity of an express way and they identified varied contents of lead in above-ground parts of the common dandelion, broadleaf plantain, and common sorrel **(Jankowska *et al.*, 2007)**. Differences in the capacity for accumulating lead and cadmium in several varieties of the same plant species were demonstrated by Tyksiński and Kurdubska (2004; 2005) who identified a diverse capacity for accumulating these metals in varieties of lettuce (*Lactuca Sativa* L.) and radish (*Raphanus Sativus* L.). In the case of radish these authors also identified the highest and the lowest concentrations of cadmium and lead in the same plant varieties (**Tyksiński and Kurdubska, 2004; 2005)**.

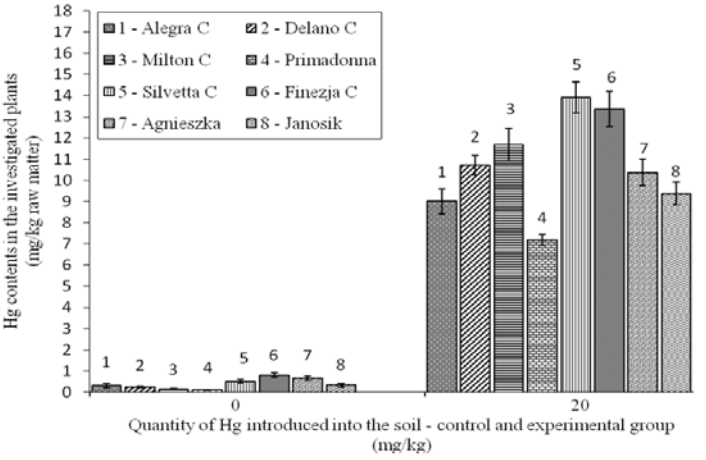

Figure 3 Contents of mercury in the experimental and control group depending on sugar beet variety

Concentrations of Hg in the examined sugar beet varieties were significantly higher in all the experimental groups in comparison to the controls (the mean value for the experimental group differs significantly from the control - Student's t-test, $p<0.05$, $n=3$). The findings showed the highest concentration of mercury in Silvetta C and Finezja C varieties. The lowest concentration of this element was identified in Primadonna variety. Just like cadmium and lead, mercury does not have any nutritional functions for plants, yet it finds its way into plant tissues. Due to the fact that in plant tissues mercury is bound by protein sulfhydryl groups, the element may induce adverse changes in cellular respiration processes, in particular related to enzymatic transformation **(Gworek and Reteńska, 2009)**. As reported by Kabata-Pendias **(1992)** higher plants are not excessively sensitive to the presence of mercury in the environment. In the case of plants which are vulnerable to toxic activity of mercury, e.g. beet, maize, and rose, increased

concentration of the element may injure their roots, and lead to chlorosis **(Kabata-Pendias, 1992)**.

Table 1 shows the calculated values of correlation coefficients. High positive correlation (significant dependence) were identified between mercury and lead (a value of 0.69) as well as mercury and cadmium (a value of 0.69). Practically complete dependence were identified between cadmium and lead value of correlation coefficient 0.96.

Table 1 Correlation between the analyzed elements

	Mercury	Lead	Cadmium
Mercury	1	0.69	0.69
Lead		1	0.96
Cadmium			1

Bioconcentration Factor (BCF) was calculated in order to determine the degree of mobility of the selected heavy metals in the investigated plants. The value of BCF reflects the plants susceptibility to metals infiltrating from the soil and it provides information about the bioaccumulation properties **(Czech *et al.*, 2014)**.

Table 2 Values of Bioconcentration Factor (BCF) for lead, cadmium and mercury

Sugar beet variety	**Bioconcentration Factor (BCF) for the examined part of the plant**		
	Pb	Cd	Hg
Alegra C	0.313	0.384	0.450
Delano C	0.354	0.396	0.535
Milton C	0.415	0.477	0.584
Primadonna	0.234	0.328	0.360
Silvetta C	0.430	0.471	0.696
Finezja C	0.316	0.424	0.668
Agnieszka	0.226	0.289	0.519
Janosik	0.214	0.271	0.469

Table 2 shows the BCF values for lead, cadmium and mercury in the above-ground part of the plants. The present findings suggest that seedlings of Silvetta C variety are most susceptible to accumulation of the relevant heavy metals (Pb, Cd, Hg). The values of BCF for this variety were: 0.430 Pb, 0.471 Cd and 0.696 Hg. The findings also show that all the sugar beet varieties in question are characterized by higher bioaccumulation properties in relation to mercury than in the case of the remaining investigated elements (cadmium and lead).

CONCLUSIONS

It was demonstrated that all the investigated plants have the capacity to absorb heavy metals from the soil and to accumulate them in their tissues. The highest capacity to absorb cadmium and lead from the soil was found in seedlings of Milton C and Silvetta C varieties, and the highest concentrations of mercury were identified in seedlings of Silvetta C and Finezja C varieties. It was determined that in the group of the examined plants Agnieszka and Janosik varieties were least susceptible to cadmium and lead bioaccumulation, while Primadonna variety was least susceptible to mercury. This study show that the seedlings of the specific varieties of sugar beet differed in terms of their capacity to absorb heavy metals from the soil.

Acknowledgments: The study was financed as research project for Young Resarches sponsored by the Polish Ministry of Higher Education.

REFERENCES

Alia, N., Sardar, K., Said, M., Salma, K., Sadia, A., Sadaf, S., Toqeer, A. & Miklas, S. (2015). Toxicity and Bioaccumulation of Heavy Metals in Spinach Spinacia oleracea Grown in a Controlled Environment. *Int. J. Environ. Res. Public Health*, 12,7400-7416. http://dx.doi.org/10.3390/ijerph120707400

Awofolu, O.R. (2005). A survey of trace metals in vegetation, soil and lower animals along some selected major roads in metropolitan city of Lagos. *Environmental Monitoring and Assessment*, 105, 431-447. http://dx.doi.org/10.1007/s10661-005-4440-0

Baran, A., Spałek, I. & Jasiewicz, CZ. (2007). Contents of heavy metals in plants and farming areas adjoining selected petrol stations in Krakow. *Materiały II Krakowskiej Konferencji Młodych Uczonych, Kraków*, 265–272. in Polish.

Chaney, R., Malik, M., LI, Y.M., Brown, S.L., Brewer, E.P., Angle, J.S. & Baker, A.J.M. (1997). Phytoremediation of soil metals. *Current Options in Biotechnology*, 8, 279-284. http://dx.doi.org/10.1016/s0958-1669(97)80004-3

Czech, T., Baran, A. & Wieczorek, J. (2014). Contents of heavy metals in soils and plants from the area of Borzęcin Commune Małopolskie Provinc.. *Inżynieria Ekologiczna*, 37, 89-98. in Polish.

Czeczot, H. & Majewska M. (2010). Cadmium – hazards and health-related effects. *Farm. Pol.* , 66, No. 4, 243-250. in Polish**.**

D'amore, J.J., Al-Abed, S.R., Scheckel, K.G. & Ryan, J.A. (2005). Methods for speciation of metals in soils: A review. *J. Environ. Qual*., 34, 1707–1745. http://dx.doi.org/10.2134/jeq2004.0014

Gbaruko, B.C & Friday, O.U. (2007). Bioaccumulation of heavy metals in some fauna and flora. *Int. J. Environ. Sci. Tech*., 42: 197 – 202. http://dx.doi.org/10.1007/bf03326274

Gosh, S. (2010). Wetland macrophytes as toxic metal accumulators. *International Journal of Environmental Sciences*, 14, 523-528.

Gruca-Królikowska, S. & Wacławek, W. (2006). Metals in the environment, part 2. Impact of heavy metals on plants. *Chemia Dydaktyka Ekologia Metrologia*, 1-2, 41-55. in. Polish.

Grygierzec, B. (2013). Effects of laser stimulation of seeds on bioaccumulation of heavy metals in common meadow-grass Poa pratensis L.. *Rocznik Ochrona Środowiska*, 15: 2412–2424. in Polish**.**

Gworek, B. & Reteńska, J. (2009). Mercury migration in the air-soil-plant system. Ochrona Środowiska i Zasobów Naturalnych, 44, 614-623. in Polish.

Jankowska, J., Sosnowski, J., Ciepiela, G.A. & Jankowski, K. (2007). Contents of lead in selected species of dicotyledonous plants growing in the grasslands in the proximity of an express way. *Ochrona Środowiska i Zasobów Naturalnych*, 30, 99-104. in Polish.

Joseph, L.U., Andrea, L.C. & Mal, T.K. (2002). Effects of lead contamination on the growth of Lythrum salicaria. *Environ. Pollut*., 120, 319–323. http://dx.doi.org/10.1016/s0269-7491(02)00144-6

Kabata-Pendias, A. (1992). Biochemistry of mercury in various environments – ecology and methodology related problems. *Ossolineum*, 7–18. in Polish.

Khan, S., Aijun, L., Zhang, S., Hu, Q. & Zhu, Y.G. (2008a). Accumulation of polycyclic aromatic hydrocarbons and heavy metals in lettuce grown in the soils contaminated with long-term wastewater irrigation. *J. Hazard. Mater*., 152, 506–515. http://dx.doi.org/10.1016/j.jhazmat.2007.07.014

Khan, S., Cao, Q., Zheng, Y.M., Huang, Y.Z. & Zhu, Y.G. (2008b). Health risks of heavy metals in contaminated soils and food crops irrigated with wastewater in Beijing, China. *Environ. Pollut*., 152, 686–692. http://dx.doi.org/10.1016/j.envpol.2007.06.056

Kuo, C.Y., Wong, R.H., Lin, J.Y., Lai, J.C. & Lee, H. (2006). Accumulation of chromium and nickel metals in lung tumors from lung cancer patients in Taiwan. *J. Tox. Environ. Health*, 69, 1337–1344. http://dx.doi.org/10.1080/15287390500360398

Liu, W.J., Zhu, Y.G., Smith, F.A. & Smith, S.E. (2004). Do iron plaque and genotypes affect arsenate uptake and translocation by rice? *J. Exp. Bot*., 55, 1707–1713. http://dx.doi.org/10.1093/jxb/erh205

Liu, W.X., Shen, L.F., Liu, J.W., Wang, Y.W. & Li, S.R. (2007). Uptake of toxic heavy metals by rice Oryza sativa L. cultivated in the agricultural soils near Zhengzhou City, People's Republic of China, *Bull. Environ. Contam. Toxicol*., 79, 209–213. http://dx.doi.org/10.1007/s00128-007-9164-0

Majtkowski, W., Szulc, P.M., Gaca, J. & Mikołajczak, J. (2010). Assessment of Silphium perfoliatum L. and its usefulness in phytoremediation of land contaminated with heavy metals. *Biuletyn Instytutu Hodowli i Aklimatyzacji Roślin*, 256, 163-169. in Polish.

Ociepa-Kubicka, A. & Ociepa, E. (2012). Toxic effects of heavy metals in plants, animals and people. *Inżynieria i Ochrona Środowiska*, Vol. 15, 2: 169 – 180. in Polish.

Padmavathiamma, P.K. & Li, L.Y. (2007). Phytoremediation technology: Hyperaccumulation metals in plants. *Water, Air and Soil Pollution*, 184, 105-126. http://dx.doi.org/10.1007/s11270-007-9401-5

Potarzycki, J. et. al. (1999). Geochemical condition of soils and quality of crops in the area affected by Poznań - Świecko highway. *Rocz. AR. Pozn., CCCX, Melior. Inż. Środ*., 20, 77-85. in Polish.

Sandalio, L.M., Dalurzo, H.C., Gomez, M., Romero-Puertas, M.C., & Rio, L.A. (2001). Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *J. Exp. Bot*., 52, 2115–2126.

Sekara, A., Poniedzialek, M., Ciura, J. & Jedrszczyk E. **(**2005). Cadmium and lead accumulation and distribution in the organs of nine crops: Implications for phytoremediation. *Polish Journal of Environmental Studies*, 14, 509-516.

Sharma, P. & Dubey, R.S. (2005). Lead toxicity in plants. *Braz. J. Plant. Physiol*., 17, 35–52. http://dx.doi.org/10.1590/s1677-04202005000100004

Siwek, M., (2008). Plants in a post-industrial environment contaminated with heavy metals. Part 1. Take up, transport and toxicity of heavy metals trace elements. *Wiadomości Botaniczne*, 52 1/2, 7-22. in Polish.

Tyksiński, W. & Kurdubska J. (2004). Variety-related differences in accumulation of cadmium and lead by radish Raphanus Sativus L.. *Roczniki AR w Poznaniu*, CCCLVI: 209.215. in Polish.

Tyksiński, W. & Kurdubska J. (2005). Differences in cadmium and lead accumulation by lettuce Lactuca sativa l. depending on the cultivar. *Acta Sci. Pol., Hortorum Cultus*, 41, 77-83.
Yan, X., Zhang, F., Zeng, C., Zhang, M., Devkota, L.P. & Yao, T. (2012). Relationship between Heavy Metal Concentrations in Soils and Grasses of Roadside Farmland in Nepal. *Int. J. Environ. Res. Public Health*, 9, 3209-3226. http://dx.doi.org/10.3390/ijerph9093209
Zhuang, P., Yang, Q.W., Wang, H.B. & Shu W.S. (2007). Phytoextraction of heavy metals by eight plant species in the field. *Water, Air and Soil Pollution*, 184, 235-242. http://dx.doi.org/10.1007/s11270-007-9412-2

JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

METABOLITE PROFILES AND ACCEPTABILITY BY CLUSTERS OF DIFFERENT KEFIR TYPES FOR SOUTH AFRICAN CONSUMERS

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doi: 10.15414/jmbfs.2016.5.4.364-368

ARTICLE INFO

Received 26. 8. 2014
Revised 31. 8. 2015
Accepted 25. 11. 2015
Published 1. 2. 2016

Regular article

OPEN ACCESS

ABSTRACT

Kefir is an alternative fermented dairy product for low income South African consumers. However, it was shown that mass culturing of kefir grains leads to a loss of positive sensory attributes. Thus, this paper evaluates the chemical composition and the sensory acceptability for South African consumers of four variants of kefir (traditional kefir, mass-cultured kefir, Candi-kefir and Lacto-kefir). Results showed that all variants of kefir contained key flavour compounds: acetaldehyde (7 – 45 mg.L^{-1}), ethanol (186 – 1774 mg.L^{-1}), diacetyl (5 – 12 mg.L^{-1}), ethyl acetate (1,2 – 30 mg.L^{-1}) and acetic acid (892 – 4 490 mg.L^{-1}); with acidity (TA: 0,85 – 0,96%; pH: 4,13 – 4,25) comparable with ranges reported in literature.
Based on the liking of flavour of the variants of kefir, three clusters of consumers (N=85) were identified using Ward's clustering. Cluster I was negatively sensitive to acidic products as indicated by the low scores (<5,30) given for liking of flavour. For Cluster II consumers, the main driver of liking of flavour was ethanol ($r = 0,963$; $p < 0.05$), therefore giving the best score (7,5) to traditional kefir the most 'yeasty' variant. For cluster III, acidity was a significant driver of liking ($r = 0,999$; $p < 0.05$). Thus, less acidic kefir products obtained better consumer liking scores ranging between 7,09 and 7,63.
The results of this study add to the understanding of sensory attributes which drive consumer preference for kefir. This important information can be used by the South African dairy industry to strengthen the current market through the appropriate production of kefir.

Keywords: Kefir, consumer preference, acidity, flavour compounds, diacetyl to acetaldehyde ratio

INTRODUCTION

Milk and fermented dairy products are widely consumed in South Africa not only because they form a part of the traditional diet of South Africans **(Van Wyk *et al*., 2002)**, but also because of the health benefits associated with their regular consumption. Furthermore, these products have versatile uses and are available in a wide variety of flavours, making them enjoyable and interesting foodstuffs to consume **(Shiratsuchi *et al*., 1994)**.
In recent years, even though new and/or improved fermented dairy products have appeared on the South African market, affordability still remains a problem for the vast majority of the population **(Schönfeldt *et al*., 2010)**. The fact that there has been an increase in the demand for yoghurt and maas **(Coetzee, 2011)** clearly demonstrates that there is a need for affordable good quality fermented dairy products similar to yogurt and maas in the South African dairy market. Maas is a traditional fermented milk made by fermenting raw milk in clays pots or calabashes. It is consumed by the rural inhabitants of South African villages (**Kebede *et al*., 2007**). The industrially made version of maas is also produced and available for people living in urban areas (**Van Wyk *et al*., 2002**).
Kefir, a fermented dairy product originating from the Caucasus Mountains **(Doğan, 2011)**, has the potential to fill that market niche because it has several attributes, such as being home-made, having probiotic properties and having a well-balanced nutritional composition, that would make it an affordable and healthful fermented dairy product for South African consumers. Furthermore, kefir is similar to maas in many aspects and according to **Burger (2010)**, "Kefir would taste similar to the maas connoisseur". Finally, the biggest advantage of this product is that kefir grains, the starter culture from which kefir is made, are endlessly reusable.
Mass production of kefir grains for distribution or selling is possible by using the mass cultivation procedure **(Schoevers and Britz, 2003)**, which yields 'mass cultured kefir grains' (MG). These grains will be a more commercially viable option for kefir production as their culturing will be considerably more cost-effective when compared to traditional grains. The drawback of these grains is that the resultant kefir beverage has an unpleasant flavour when compared the flavour of traditional kefir. The latter has a pronounced buttery flavour and slight to strong yeasty flavour, combined with a distinctive acidity essentially originating from lactic acid **(Bakhshandeh *et al*., 2011)**. Since metabolites are synthesised by the symbiotic activity of the microbial consortium in the kefir grain, it is proposed that the lack of flavour in MG-kefir can be ascribed to an intrinsic microbial imbalance within the MG or the absence of a significant number of flavour forming microbes **(Witthuhn *et al*., 2005)**. Thus enriching mass cultured kefir grains with flavour forming microorganism may improve the sensory character of MG kefir. In that regard, the objective of this study is to evaluate the metabolic profiles and establish the consumer preference of MG kefir, Traditional kefir and variants of MG kefir made from enriched mass cultured kefir grains.

MATERIAL AND METHODS

Preparation of kefir

Two batches of MG were separately enriched by adding *Candida kefyr* 1283 and *Lactococcus lactis* spp. *lactis* biovar. *diacetylactis* 318 to produce two new variants of mass cultured kefir grains, namely MGC (mass cultured grains with *C. kefyr*) and MGL (mass cultured grains with *L. lactis* spp. *lactis* biovar. *diacetylactis*). Kefir was produced from these grains as well as from mass cultured grains (MG), while kefir from traditional grains (TG) served as a control. Thus, it is obtained four variants of kefir.
For the preparation of the kefir variants, 20 g of kefir grains (MG, MGC, MGL and TG) were inoculated into 300 mL pasteurised milk. Milk inoculated with MG, MGC and MGL were incubated at 22ºC, whereas the traditional kefir was prepared under uncontrolled home-style conditions. After an incubation period of 24h, the grains were removed to obtain the final kefir beverages.
The kefir beverages were labelled as follows: Trad-kefir (kefir produced from TG), MG-kefir (kefir produced from MG), Lacto-kefir (kefir made from MGL) and Candi-kefir (kefir made from MGC).

Metabolite profiles and acidity

The measurements of acidity as well as the quantification of short chain fatty acids and volatile organic compounds were done on the four variants of kefir.

Volatile organic compounds determination

Samples were prepared by placing 9.75 mL of kefir in a 20 mL glass vial containing 2.5 g NaCl and 0.25 mL tetrahydrofuran. The vial was crimp-sealed with a silicone-PTFE seal and aluminum cap and incubated in a waterbath for 50 min at 95ºC.

Separation and identification of volatile organic compounds (VOCs = acetaldehyde, ethanol, acetone, diacetyl, 2-butanone and ethyl acetate) present in kefir were determined using a Fisons 8000 Series gas chromatograph (FISONS INSTRUMENTS S.P.A., MILAN, ITALY). Details of the GC settings are fully described in previous works **(Human, 1998; Ntsame Affane, 2012)**.

Quantitative determination of the metabolite compounds was done by integration of the peak areas using an external standard calibration and Borwin Version 1.2 integration software (JMBS DEVELOPMENTS, LE FONTANIL, FRANCE).

Volatile short chain volatile fatty acids determination

Samples were prepared by centrifuging 10 mL of Kefir for 10 min at 10 000 g. The resulting supernatant was filtered through Whatman paper No. 1 filter to remove solid particles and obtain a clear supernatant. One mL of formic acid (35% $v.v^{-1}$) and 2 µL of n-hexanol (as internal standard) were added to 3 mL of the filtered supernatant.

The short chain volatile fatty acids (VFAs) were determined using a Varian 3700 gas chromatograph (Palo Alto, California, USA) equipped with a flame ionisation detector and a 30 m bonded phase Nukol (Supelco, Inc., Belafonte, PA, USA) fused silica capillary column (0.53 mm diameter and 0.5 µm film thickness). The details of the method can be found in previous works (**Sigge *et al*., 2005; Ntsame Affane, 2012**). VFAs were quantified using the Borwin Version 1.2 integration software (JMBS DEVELOPMENTS, LE FONTANIL, FRANCE).

Titratable acidity and pH measurements

The pH values of Kefir were measured with a microprocessor pH meter equipped with a glass electrode and a temperature probe (HANNA INSTRUMENTS model 22x, ANN ARBOR, USA). The titratable acidity (TA) was measured in triplicate by the titration of 10 mL sample with phenolphthalein against 0.11 M NaOH **(James, 1999)**. TA is expressed as % total acids.

Consumer preference analysis

The consumer preference test was conducted with a group of 85 consumers, which regularly consume non-sweetened fermented dairy products such as maas, Greek and Bulgarian yoghurts. Consumers were presented with a set of four 15 mL samples of each variant of kefir, served according to a randomised complete block design. Consumers rated each sample for liking on a 9-point hedonic scale: *Like extremely* (*9), Like very much (8), Like moderately (7), Like slightly (6), Neither like nor dislike (5), Dislike slightly (4), Dislike moderately (3), Dislike very much (2) and Dislike extremely (1)* **(Lawless and Heymann, 2010)**.

Statistical analysis

Consumer sensory data were analysed using SAS® software (Version 9; SAS Institute Inc, Cary, USA) and subjected to the Shapiro-Wilk test for non-normality of the residuals **(Shapiro and Wilk, 1965)**. Then, analysis of variance (ANOVA) was performed and student's t-least significant difference (LSD) was calculated at the 5 % significance level to compare treatment means.

Ward's clustering was performed to cluster individual judges in terms of their liking of the four variants of Kefir with XLStat (Version 7.5.2, Addinsoft, New York, USA). ANOVA was performed, to determine the differences in preference patterns between clusters, with cluster as factor to test for Cluster∗Sample interaction as well as separately for each cluster.

Principal component analysis (PCA) using the correlation matrix was conducted using XLStat (Version 7.5.2, Addinsoft, New York, USA) to visualise and elucidate the relationships between the samples and their attributes.

RESULTS AND DISCUSSION

Metabolite profiles and acidity

The results presented in Table 1 show acetaldehyde (7 – 45 $mg.L^{-1}$), ethanol (186 – 1774 $mg.L^{-1}$), acetone (4,5 – 14 $mg.L^{-1}$), diacetyl (5 – 12 $mg.L^{-1}$), 2-butanone (1 – 4 $mg.L^{-1}$), ethyl acetate (1,2 – 30 $mg.L^{-1}$) and acetic acid (892 – 4490 $mg.L^{-1}$) that were found in all variants of kefir. The differences observed when comparing these results to literature sources originate from differences in the microbial composition of the kefir grains, the inoculum size, the origin (cow, sheep, camel, soya) and type of milk (full cream, low-fat or fat free), the incubation time and the analytical methods used **(Rea *et al*., 1996)**.

In terms of acidity, Trad-kefir was the most acidic product, with a pH of 4,13 and TA of 0,96%. The reason for that could be that Trad-kefir was produced under uncontrolled conditions. For the other kefir types, the pH varied between 4,23 and 4,25; and TA between 0,85% and 0,90% (Tab 1). These values of pH and TA were within the range reported in the literature **(Simova *et al*., 2002; Chen *et al*., 2009)**.

Kefir is known to have an acidic taste originating from the presence of lactic acid, associated with a buttery and slightly yeasty flavour. Although diacetyl plays an important role in the flavour of kefir, it has to be balanced with acetaldehyde. A balanced flavour is weighed by the ratio of diacetyl to acetaldehyde, which in most good cultured dairy products varies between 3 and 5 **(Sandine *et al*., 1972; Kosikowski and Mistry, 1997)**. However in the case of kefir prepared with kefir grains, it appears that a lower ratio is more prevalent in kefir since the ratios found for the four variants varied between 0,12 and 1,07. The results are confirmed by literature sources, which also reported lower ratios (0,0 – 2,57) **(Güzel-Seydim *et al*., 2000; Wszolek *et al*., 2001; Beshkova *et al*., 2003; Ntsame Affane, 2012)**. An exception was an extremely high ratio of 77 obtained in one study **(Liu *et al*., 2002)**. where the concentration of diacetyl after 24 h of incubation at 254 mg.l-1Kefir prepared using soya milk also had higher ratios ranging between 10,5 and 16,6 **(Liu *et al*., 2002; Pourahmad *et al*., 2011).**

Consumer preference analysis

The PCA bi-plot (Fig 1) showed that Trad-kefir, which served as the control sample for this study, was associated with TA, ethanol, ethyl acetate, acetaldehyde and 2-butanone, whereas MG-kefir and Candi-kefir associated strongly with diacetyl and acetone. The figure 1 also shows that Lacto-kefir associated strongly with acetic acid. This distribution originates from the fact that traditional kefir had the highest concentrations in ethanol and ethyl acetate, Candi-kefir and MG-kefir had the highest concentration in diacetyl and acetone whereas Lacto-kefir had the highest concentration of acetic acid (Tab 1). The PCA analysis is in accordance with the obtained results.

Table 1 Metabolite profiles and acidity of the four variants of kefir. Results are expressed as mean ± standard deviation (N=85)

Kefir variants	pH	TA(%)	Acetaldehyde	Diacetyl	Ethanol	Acetone	2-butanone	Ethyl acetate	Acetic acid	Ratio diacetyl to acetaldehyde
			$mg.L^{-1}$							
Trad-kefir	4,13	0,96	45± 0,23	5,5± 1,9	1774 ±252	10± 0,1	4 ±1,0	30 ±7,2	1382± 302	0,12
Candi-kefir	4,25	0,89	16 ±4,3	12,1± 6,7	721± 362	12 ±1,6	1± 0,3	1,5 ±0,4	2431 ±178	0,76
Lacto-kefir	4,23	0,90	7± 1,1	5,1 ±2,2	531 ±150	4,5 ±1,7	1,1± 0,3	1,2± 0,3	4490 ±560	0,73
MG-kefir	4,25	0,85	9 ±0,7	9,6 ±4,4	186 ±68,4	14 ±1,4	1,7 ±0,1	11 ±1,9	892 ±57	1,07

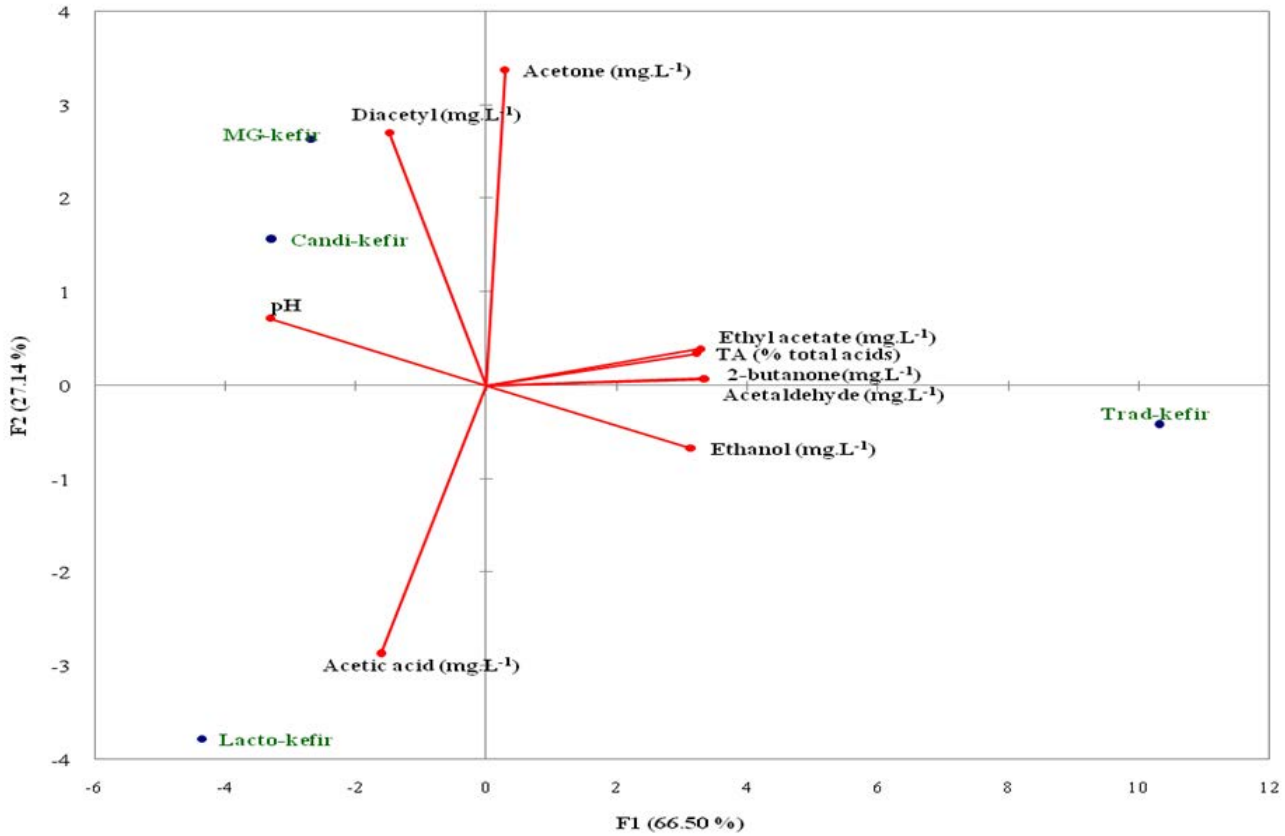

Figure 1 PCA bi-plot indicating the association of chemical constituents in relation to Trad-kefir, Candi-kefir, Lacto-kefir and MG-kefir

Segmentation of consumer acceptability

The composition of the total consumer group was not homogenous but mainly constituted by consumers of Bulgarian yoghurt (39%), Greek yoghurt (31%), buttermilk (57%) and maas 39% than consumers of kefir (16%). Thus, Ward's clustering was applied and three different clusters of consumers were identified, based on their degree of liking of the different variants of kefir.
The data in Table 2 clearly shows that the regular consumption of kefir, maas and buttermilk played a large role in the consumers clustering. After conducting the cluster analysis, the liking scores of each cluster for the flavour of the respective products were superimposed on the chemical data in a second PCA analysis (Fig 2).

Table 2 Demographic information and characteristics of each cluster expressed as percentage

Consumers N=85	Cluster I (%) N=36	Cluster II (%) N=20	Cluster III (%) N=44
Kefir consumption			
NR	100	50	83
R	0	50	17
Greek yogurt consumption			
NR	61	75	66
R	39	25	34
Bulgarian yogurt consumption			
NR	55	69	63
R	45	31	37
Maas consumption			
NR	79	62	43
R	21	38	57
Buttermilk consumption			
NR	48	56	29
R	52	44	71

NR= not regular; R= regular

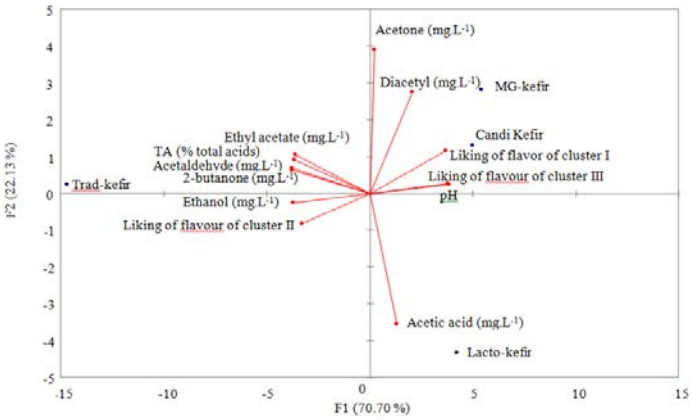

Figure 2 PCA bi-plot indicating the degree of liking of flavour of cluster I, II and III in relation to the four kefir samples and chemical constituents

Cluster I

Cluster I, representing 36% of consumer panel, had the particularity that none of the consumers (0%) were regular consumers of kefir; but rather regular consumers of maas, Greek yoghurt, Bulgarian yoghurt and buttermilk (Tab 2).
This group of consumers gave low scores (2,41 to 5,30) to all the variants of Kefir (Table 3), with the highest score obtained by Candi-kefir (5,30) and the lowest score obtained by Trad-kefir. The most likely reason for the low scores is that this group of consumers has never consumed kefir products prior to the sensory analysis. In addition, less than 25% of this group drinks or uses maas regularly; and they are consequently unfamiliar with the naturally acidic taste of kefir. Furthermore, it could also be hypothesised that this group of consumers may have the tendency to sweeten (with fruits, honey or sugar) plain yoghurts and buttermilk or use them for baking purposes; making this consumer group quite unfamiliar with the natural acidity of traditional fermented dairy products **(Doğan, 2011)**.

Cluster II

Consumers from cluster II represent 20% of the consumer panel and the preference liking of this cluster was significantly driven by ethanol content ($r = 0,963$; $p < 0,05$) (Fig 2), which is responsible for the yeasty flavour in kefir. This yeasty flavour is an essential and definitive character of kefir. Thus, it can be suggested that consumers of cluster II enjoyed the 'yeasty flavour' of the four

variants of kefir. This was confirmed by the results (Table 3), which indicated that the degree of liking of kefir types was associated with the quantity of ethanol present in the different variants of kefir. In keeping with these results, Trad-kefir obtained the best score (7,5), followed by Candi-kefir (6,31), Lacto-kefir (5,94) and MG-kefir (4,87). The concentrations of ethanol in these products were 1774 $mg.L^{-1}$, 720,5 $mg.L^{-1}$; 531 $mg.L^{-1}$ and 186 $mg.L^{-1}$, respectively. In addition, the highest percentage regular consumers of kefir were found in this cluster (Table 2), which offers an additional explanation as to why traditional kefir obtained the best score (7,5). This group of consumers also gave the lowest score to MG Kefir (4,87), which was not surprising since regular kefir consumers would find the taste of MG-kefir untypical.

Different views are held regarding the 'strength' of the yeasty flavour in kefir. Some authors believe that kefir has a strong yeasty flavour **(Marshall, 1984)** whereas others believe that kefir has a weak yeasty flavour **(Vedamuthu, 1977; Güzel-Seydim *et al.*, 2000)**. Thus, considering the results obtained, it appears that consumers from cluster II preferred kefir that exhibited a strong yeasty flavour.

Table 3 Degree (max 9) of liking of flavour for Trad-kefir, Candi-kefir, Lacto-kefir and MG-kefir by cluster I, II and III. Means with different alphabetical letters differ significantly ($p<0.05$).

Samples	Cluster I	Cluster II	Cluster III
Trad-kefir	$2,41^{c} \pm 1,15$	$7,50^{a} \pm 0,97$	$4,91^{c} \pm 1,60$
Candi-kefir	$5,27^{a} \pm 2,03$	$6,31^{ab} \pm 2,24$	$7,63^{a} \pm 0,97$
Lacto-kefir	$4,20^{b} \pm 2,11$	$5,94^{bc} \pm 1,53$	$7,09^{b} \pm 0,96$
MG-kefir	$5,14^{a} \pm 2,15$	$4,87^{c} \pm 1,45$	$7,29^{ab} \pm 1,04$

Cluster III

Cluster III comprised the largest group of consumers (44%) and for this group, pH was the driver of liking ($r = 0,999$; $p < 0,05$) (Fig 2). The preference pattern of cluster III appeared to be similar to that of cluster I. As with cluster I, this cluster indicated the highest preference for Candi-kefir with a rating of 7,63, whereas Trad-kefir obtained the lowest mean score (4,91) (Table 3). This group did, however, give considerably higher scores to all these products (4,91 – 7,63) when compared to consumers from cluster I (2,41 – 5,30) (Table 3).

The high scores for liking of the flavour (7.09 – 7.63) obtained for Candi-kefir, Lacto-kefir and MG-kefir may originate from the fact that these types of kefir were mildly acidic products (pH= 4,23 – 4,25) as opposed to Trad-kefir, which was moderately acidic (pH= 4.13). This study confirmed previous findings that acidity perception generally determines the acceptability of fermented dairy products **(Ott *et al.*, 2000)**.

Furthermore, it is interesting to note that the liking scores obtained for Candi-kefir (7,63), MG kefir (7,29) and Lacto-kefir (7,09) decreased as the content of diacetyl decreased. Diacetyl concentrations were 12 $mg.L^{-1}$, 9,6 $mg.L^{-1}$ and 5 $mg.L^{-1}$ for Candi-kefir, MG-kefir and Lacto-kefir, respectively. Although the driver of liking of flavour for cluster III was not strongly driven by diacetyl ($r = 0,646$; $p > 0.05$), it is possible that as a high proportion of cluster III consumers drink buttermilk regularly (71%), the buttery flavour of kefir types could have prompted the preference ratings of these consumers. Indeed, the buttery flavour imparted by diacetyl, which concentration ranges between 1 and 4 $mg.L^{-1}$ is the prominent flavour found in buttermilk **(Vedamuthu, 2006)**.

CONCLUSION

Amongst the variants of kefir tasted, Candi-kefir was generally preferred by most of the consumer panel. This is an indication that mass cultured kefir grains enriched with *Candida kefyr* (MGC) has the potential to be successfully marketed to South African consumers. However, the other variants of kefir, included Trad-kefir the most acidic variant, would also have a market within the population as shown by the results.

This study has provided some understanding on the preference liking of kefir, a cultured dairy product unknown to South African consumers. It clearly appears different patterns of consumer liking driven by the perception of acidity and the volatile organic compounds diacetyl, and ethanol. Thus, further work on the identification of sensory attributes (eg. sweetness, saltiness, bitterness, astringency, acidity, fruitiness, creaminess), which drive the liking of South African consumers within specific consumer segments will further elucidate the viability of marketing kefir in South Africa.

In addition, this study also showed that drawing conclusions only based on the calculation of ratio of diacetyl to acetaldehyde may be inappropriate and should preferably be associated with a sensory testing.

Acknowledgments: This study was done at the Food Science Department (Stellenbosch University) and supported by the National Research Funding (South Africa).

REFERENCES

BAKHSHANDEH, T., POURAHMED, R., SHARIFAN, A. 2011. Evaluation of flavour and aroma compounds present in kefir. *Journal of Biosciences and Technology*, 1, 11-18.

BESHKOVA, D.M., SIMOVA, E.D., FFRENGOVA, G.I., SIMOV, Z.I., DIMITROV, Zh.P. 2003. Production of volatile aroma compounds by Kefir starter cultures. *International Dairy Journal*, 13(7), 529-535. http://dx.doi.org/10.1016/S0958-6946(03)00058-x

BURGER, L. 2010. Amasi's sweet success. *The Dairy Mail*, August 2010.

CHEN, T-H., WANG, S-Y., CHEN, K-N., LIU, J-R., CHEN, M-J. 2009. Microbiological and chemical properties of kefir manufactured by entrapped microorganisms isolated from kefir grains. *Journal of Dairy Science*, 92, 3002-3013. http://dx.doi.org/10.3168/jds.2008-1669

COETZEE, K. 2011. Australian dairy upbeat – South African situation. *The Dairy Mail*, 18, 7-11.

DOĞAN, M. 2011. Rheological behaviour and physicochemical properties of kefir with honey. *Journal of Consumer Protection and Food Safety*, 6, 327-332. http://dx.doi.org/ 10.1007/s00003-010-0643-6

GRØNNEVIK, H., FALSTAD, M., NARVHUS, J. A. 2011. Microbiological and chemical properties of Norwegian kefir during storage. *International Dairy Journal*, 21, 601-606. http://dx.doi.org/10.1016/j.idairyj.2011.01.001

GÜZEL-SEYDIM, Z., SEYDIM, A.C., GREENE, A.K., BODINE, A.B. 2000. Determination of organic acids and volatile flavour substances in kefir during fermentation. *Journal of Food Composition and Analysis*, 13, 35-43. http://dx.doi.org/10.1006/jfca.1999.0842

HUMAN, M.E. 1998. Optimisation of Maas process parameters to achieve traditional flavour, aroma and texture. MSc in Food Science Thesis, University of Stellenbosch, South Africa.

JAMES, C.S. (1999). Analytical Chemistry of Foods. Great Britain: Chapman & Hall. Pp. 168-169. ISBN 9780834212985.

KEBEDE, A., VILJOEN, B.C., GADAGA, T.H., NARVHUS, J.A., LOURENS-HATTINGH, A. 2007. The effect of container type on the growth of yeast and lactic acid bacteria during production of Sethemi, South African spontaneously fermented milk. *Food Research International*, 40, 33-38. http://dx.doi.org/10.1016/j.foodres.2006.07.012

KOSIKOWSKI, F.V., MISTRY, V.V. 1997. Cheese and Fermented milks. Westport, Connecticut: F.V. Kosikowski, L.L.C. Pp. 61-62. ISBN 9780965645621.

LAWLESS, H. T., HEYMANN, H. 2010. Sensory Evaluation of Food: Principles and Practices. New York: Springer. Pp. 326-328. ISBN 083421752X.

LIU, J-R., LIN, C-W. 2000. Production of kefir from soymilk with or without added glucose, lactose or sucrose. *Journal of Food Science*, 65, 716-719. http://dx.doi.org/10.1111/j.1365-2621.2000.tb16078.x

LIU, J-R., CHEN, M-J., LIN, C-W. 2002. Characterisation of polysaccharide and volatile compounds produced by kefir grains grown in soymilk. *Food Chemistry and Toxicology*, 67, 104-108. http://dx.doi.org/10.1111/j.1365-2621.2002.tb11367.x

MARSHALL, V.M.E. 1984. Fermented milks: new developments in the biochemistry of the starter cultures. *Biochemical Society Transactions*, 12, 1150-1152. http://dx.doi.org/10.1042/bst0121150

NTSAME AFFANE, A.L. 2012. Impact of environmental factors on the metabolic profiles of kefir produced using different kefir grains and subsequent enrichment of kefir prepared with mass cultured kefir grains. PhD in Food Science Dissertation, University of Stellenbosch, South Africa.

OTT, A., HUGI, A., BAUMGARTNER, M., CHAINTREAU A. 2000. Sensory investigation of yogurt flavour perception: mutual influence of volatiles and acidity. *Journal of Agriculture and Food Chemistry*, 48, 44-450. http://dx.doi.org/10.1021/jf990432x

POURAHMAD, R., MOGHIMI, A., DADKHAH, S., ASSADI, M.M. 2011. Evaluation of flavour and aroma compounds amounts in Kefir from soymilk. *World Applied Sciences Journal*, 15, 673-676.

REA, M.C., LENNARTSSON, T., DILLON, P., DRINAN, F.D., REVILLE, W.J., HEAPES, M., COGAN, T.M. 1996. Irish kefir-like grains: their structure, microbial composition and fermentation kinetics. *Journal of Applied Bacteriology*, 81, 83-94. http://dx.doi.org/10.1111/j.1365-2672.1996.tb03286.x

SANDINE, W.E., DALY, C., ELLIKER, P.R., VEDAMUTHU, E.R. 1972. Causes and control of cultured-related flavour defects in cultured dairy products. *Journal of Dairy Science*, 55, 1031-1039. http://dx.doi.org/10.3168/jds.S0022-0302(72)85617-0

SHIRATSUCHI, H., SHIMODA, M., IMAYOSHI, K., NODA, K., OSAJIMA, Y. 1994. Volatile flavour compounds in spray-dried skim milk powder. *Journal of Agriculture and Food Chemistry*, 42, 984-988. http://dx.doi.org/10.1021/jf00040a028

SCHOEVERS, A., BRITZ, T.J. 2003. Influence of different culturing conditions on kefir grain increase. *International Journal of Dairy Technology*, 56, 183-187. http://dx.doi.org/10.1046/j.1471-0307.2003.00104.x

SCHÖNFELDT, H.C., GIBSON, N., VERMEULEN, H. 2010. The possible impact of inflation on nutritionally vulnerable households in a developing country using South Africa as a case study. *Nutrition Bulletin*, 35, 254-267. http://dx.doi.org/10.1111/j.1467-3010.2010.01837.x

SHAPIRO, S.S., Wilk, M. B. 1965. An analysis of variance test for normality (complete samples). *Biometrika*, 52, 591-611. http://dx.doi.org/10.1093/biomet/52.3-4.591

SIGGE, G.O., BRITZ, T.J., FOURIE, P.C., Barnardt, C.A. 2005. The efficiency of ozone as a pre- and post-treatment option for UASB-treated food processing waste waters. *Water Science and Technology*, 52, 167-173.

SIMOVA, E., BESHKOVA, D., ANGELOV. A., HRISTOZOVA, T., FRENGOVA, G., SPASOV, Z. 2002. Lactic acid bacteria and yeasts in kefir grains and kefir made from them. *Journal of Industrial Microbiology & Technology,* 28, 1-6. http://dx.doi.org/10.1038/sj/jim/7000186

VAN WYK, J., BRITZ, T.J., MYBURGH, A.S. 2002. Arguments supporting kefir marketing to the low-income urban African population in South Africa. *Agrekon*, 41, 43-61.

VEDAMUTHU, E.R. 1977. Exotic fermented dairy products. *Journal of Food Protection*, 40, 801-802.

VEDAMUTHU, E.R. 2006. Manufacturing Yoghurt and Fermented Milks. Oxford: Blackwell Publishing. Pp. 89-116. ISBN 9781119967088.

WITTHUHN, R.C., SCHOEMAN, T., BRITZ, T.J. 2005. Characterisation of the microbial population at different stages of kefir production and kefir mass cultivation. *International Dairy Journal*, 15, 383-389. http://dx.doi.org/10.1016/j.idairyj.2004.07.016

WSZOLEK, M., TAMIME, A.Y., MUIR, D.D., BARCLAY, M.N.I. 2001. Properties of kefir made in Scotland and Poland using bovine, caprine and ovine with different starter cultures. *Lebensmittel-Wissenschaft und-Technologie*, 34, 251-261. http://dx.doi.org/10.1006/fstl.2001.0773

JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

AMINO ACID PROFILING OF YEAST CREAM; A POTENTIAL PROTEIN ENRICHED INGREDIENT FOR POULTRY FEED

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doi: 10.15414/jmbfs.2016.5.4.369-373

ARTICLE INFO

Received 2. 4. 2015
Revised 21. 9. 2015
Accepted 25. 11. 2015
Published 1. 2. 2016

Regular article

OPEN ACCESS

ABSTRACT

Yeast cream, an extremely good source of single cell protein (SCP), is formed as a by-product after the fermentation of molasses by *Saccharomyces cerevisiae* in bioethanol industry. A huge amount of yeast cream is produced annualy but seldom has destined as protein source in food industry therefore, produced yeast cream, is discarded due to no utilization especially in under developed countries including Pakistan. The yeast cream can be utilized in various food and feed industries (espcially in poultry industry) in a promising way to solve the protein shortage problems. Although, in previous century, several attempts have been under taken at larger scale to opt SCP as a potential human food ingredient to combat the protein shortage but failed due to its potential hazardous ingredients. However, adopting SCP as a potential poultry feed ingredient can potentially alleviate the such effects. This current study was aimed at deciphering the amino acid level in yeast cream in order to investigate its potential usage in poultry industry . To meet the objectives, three different independent yeast cream samples of two different strains of *Saccharomyces cerevisiae* Instant saf and Thermophilic yeast (Red) were collected from Shakarganj sugar mills distillery, Jhang and their amino acids profiling was determined with the help of HPLC. Our results pinpointed that yeast cream produced by Thermophilic yeast (Red) contains optimal level of essential amino acids. Thus, we hereby recommend that yeast cream produced by Thermophilic yeast (Red) can potentially be employed, as a protein source, in poultry feed. However, careful and sophisticated experimentation is required before adopting yeast cream as a protein substitute in poultry feed.

Keywords: yeast cream, Single cell protein, *Sacchromycescerevisiae*, HPLC, poultry feed

INTRODUCTION

Yeast are unicellular eukaryotes belonging to phylogenetic group of fungi and classified into two groups Ascomycetous and Basidiomycetous **(Gadanho *et al.*, 2003)**. Yeasts are extensively exploited for production of alcoholic beverages, leaven bread dough, enhance or impart a meaty flavour to food products, preparation of soups, gravies, meat products, sauces and in the flavouring of snacks (**Stewart *et al.*, 1998; Walker *et al.*, 1998; Erten & Tanguler., 2006**). *Sacchromyces cerevisiae*, a specie of yeast, is a cheaper source and have been very instrumental in baking, winemaking and brewing since very very long ago **(Stam, 1998).** As a result of fermentation of molasses, a huge amount of yeast cream is produced every year. About ~1300 tons dry matter (DM) of distillery yeast biomass is disposed of annually **(Khan, 2001).** It is considered as a toxic waste and its disposal creat environmental problems due to its some polluting characteristics **(Bustamante, 2008)**. Proximate analysis indicated that yeast cream contains 27 to 29% crude protein that could be utilized as a single cell protein (SCP) for poultry and feed stock for fish diets **(Sharif, 2012).**

Poultry industry is highly dynamic and progressive industry that is expanding with every passing day, it has became a leading protein source for human beings. However, continuous supply of chicken is directly linked to uninterrupted supply of broiler's feed - especially its ingredients. Protein requirements vary according to animal need, for example, poultry broiler chicks need more protein as compared to layers i.e. White Leghorn chicks. Among chief broilers feed ingredients, the (essential) amino acids are of chief importance for chicks development. Various independent studies have unraveled the importance of amino acids in chicken development. U.S. Organic poultry companies concluded that low amount of metheionine in fast growing birds can reduce immune response, poor feathering, cannibalism and feather pecking and increase mortality **(Anne 2008)**. Diet of birds enriched with sulphur containing amino acids (metheionine and cysteine) can increase the antitoxicity which can prevent damage to cells by combating with free radicals **(Anonymous, 2009)** and thus boost defense against invading pathogens **(Diz, 2006)**. Although, in the current scenario, soyabean is being used widely in poultry industry but studies have shown that the relative amount of methionine and cystine is quite low that making it unideal to use in poultry industry. These two amino acids, methionine and cysteine respectively, are major limiting amino acid for birds **(Baize, 2000)**.

To meet the protein demands of broiler's feed industry, different sources including plants, whey, fish meal and feather meal were exploited. However, limitations were observed for example cereals contain only 12-15% protein while, 15-20% of protein is required for growing chicks, additionally, this source is deficient in essential amino acids like methionine and/or lysine.Synthetic herbal products (likeAV/CAP/18) enriched with methionine, choline, lysine and biotin are used in broiler feed which have capability of feed additive as well as hepatoprotective **(Kanuri, 2014)**. Fishmeal, another protnecious source, is relatively deficient in methionine, lysine, histidine, and tryptophan as compared to other source of protein. Feather meal has high content of cysteine, which can inhibit the uptake of both D- and L-methionine in the chick mucosal epithelial membrane **(Saima, 2008)**.

Previous studies have shown that addition of organc acids in poultry feed can effectively increase the absorption of vitamins D and K in chickens, and helps in the formation of soluble salts of calcium and iron (**Teresa, 2009)**. Fresh acid whey mixed with a poor-quality feed effectively declined the mortality rate due to the high amount of amino acids like methionine, lysine and tryptophan. All aforementioned amino acids are, present in high amount in the yeast cream as compared to cereals and these are essential for the growth and health of the animals **(Sudha, 2005)**.

The poultry industry is gaining an importance in developing countries like Pakistan with an annual growth rate about 8 to 10% **(Economic Survey of Pakistan, 2013-2014)**. On the other side, increasing human population have generated the competition between human beings and poultry for feed.

Ultimately the price and scarcity of feedstock create poor performance of poultry industry. It is estimated that poultry feed accounts for 65-75% of total production cost **(Esonu, 2006)**. This has forced the scientist to explore alternate source of protein, especially economical and those one where human are not directly competeing for food with animals. Keeping in mind of such conditions, we investigated the useability of yeast cream, which is by product of distillation process and have no commercial and economical values in Pakistan, as a raw source of protein to replace other sources on which human are directly dependent. To best of our knowledge, no study is available in Pakistan that focus on use of yeast cream as a potential source of amino acids. The study describes here examined with a motive to unravel the nutritional importance of yeast cream in poultry industry. In this purposed study, amino acid profiling along with presence of lactic acid was assessed for commercial use of yeast cream in poultry feed.

MATERIALS AND METHODS

Sample collection and defatting of samples

Batch samples of yeast cream from three independent fermenters were collected carefully from the Shakarganj Sugar Mills Distillery, Jhang, Pakistan. Yeast cream samples produced from commonly used yeast (Saf Instant) was named "S", while yeast cream samples produced by thermophilic yeast (Red Alcohol) were labeled with "T". Collected samples were dried in oven at 60°C for 2 days. The samples were defatted by Soxhelt's apparatus (Pyrex 3740-S, USA) at the Department of Animal Nutrition, University of Agriculture, Faisalabad. To dafat the samples, a known weight of dried sample was extracted with petroleum ether (40 to 60°C) in Soxhlet's apparatus. The extracted material was collected in a petridish and oven dried at 60°C. Samples were stored and used for HPLC analysis.

Crude protein and nitrogen estimation

Crude protein and percentage of nitrogen was estimated with the help of Kjeldahl's apparatus as described by **A.O.A.C, 1990** at Central Hi-Tech Laboratory, University of Agriculture, Faisalabad. A known weight of dried sample was digested in Kjeldahl flask with 30mL of concentrated sulfuric acid and 5g of digestion mixture ($CuSO_4$ 0.5g, $FeSO_4$ 0.5g, K_2SO_4 0.5g and 25mL H_2SO_4 /100mL). The contents of the flask were heated until light green or clear solution was obtained. The digested material was diluted in a 250mL volumetric flask. 10mL of diluted and digested sample solution was transferred into micro Kjeldahl distillation apparatus along with concentrated solution of NaOH (40%). Ammonia was diluted into 10mL of Boric acid (2%) solution containing a few drops of methyl red indicator.The distillate was titrated against N/10 sulphuric acid to light pink colour end point. From the volume of N/10 sulphuric acid, percent nitrogen was calculated by using the following formula:

$$\%\text{Nitrogen} = \frac{\text{Volume of N/10 sulphuric acid used} \times .0014 \times 250 \times 100}{\text{Volume of diluted solution} \times \text{weight of sample}}$$

The percentage of crude protein was worked out by using the following formula
% Crude Protein= % Nitrogen x 6.25

Sample preparation for HPLC analysis

To determine the amino acid profile through High Pressure Liquid chromatography (HPLC, Model 10A, Schimadzu, Japan). To hydrolyze the protein into amino acid 70mg of dried yeast cream was pored into test tube then dissolved in 5mL of 6N HCl and Nitrogen gas was passed through all the samples under vacuum to remove the excess HCl. Test tubes were sealed using a flame produced by a mixture of different gases. Samples were placed in the oven at 110°C for a period of 22 hours. All the samples were cooled to room temperature and test tubes containing the samples were broken from the top. Treated samples of yeast cream were transferred to the China dishes, oven dried at 60°C and mixed thoroughly with 3mL of 0.02N HCl. Samples were then centrifuged at 10,000rpm for 15 minutes (min) and supernatant containing amino acids were separated for final HPLC analysis.

HPLC analysis for amino acid estimation

The concentration of amino acids were assessed in yeast cream samples through HPLC after modifying the method described by Skotty and co-worker **(Skotty et al, 1996)**. The amino acid profiling in yeast cream samples were determined at temperature 30°C by using nonpolar or reversed phase C-18 Octadecyl Silicate (ODS) column having 15cm length, 4.6mM diameter and particle size of 5µM. Solvent triflouroacetic acid (0.1%) and acetonitrile (99.9%) used as a mobile phase with different mixing ratio using a gradient mode of HPLC and flow rate maintained at 1mL/min. The amino acids were detected using spectral detector (SPD-10AV, Schimadzu) at a 280nm wavelength. The amino acids peaks were acquired using CSW32 software and were calculated on amino acid calibration standards. The results of different samples of yeast cream were compared with standards of amino acids for both quantitative and qualitative analysis (Table 1). Retention time and peak areas of standards were noted and calculated respectively. These calculations were employed for estimation of amounts of different amino acids present in the samples of yeast cream. A whole set of experiments was repeated thrice.

RESULTS

Optimization of amino acid standards

All the aforesaid HPLC conditions were used to check the concentration/separation of standards amino acids and found to be satisfactory. Every standard was run in equal concentration (100 µg/mL) and all the parameters (including retention time, peak area and peak height) were determined. These resuts pinpointed that the first amino acid, iso-leucine, was eluted at 2.33 minutes while last amino acid, glutamine, was eluted at 37.50 minutes run time (Table 1).

Table 1 Chromatographic calculations of amino acids (standards)

Sr.#	Amino acid	Concentration (µg/mL)	Retention Time (min)	Peak Area (mV.s)	Peak Area (%)	Peak Height (mV)	Peak Height (%)
1	Phenylalanine	100	3.10	728	95.20	103.45	95.61
2	Tyrosine	100	4.28	342	75.50	32.29	81.10
3	L-Arginine	100	3.35	52	7.00	2.65	24.20
4	Iso-Leucine	100	2.33	125	12.80	6.98	37.60
5	Serine	100	2.40	23	10.90	2.94	58.60
6	Glutamine	100	37.50	1180	33.90	8.62	33.20
7	Lactic Acid	100	15.21	432	30.00	7.21	31.00
8	Methionine	100	20.12	559	21.80	13.57	26.50
9	Histidine	100	17.48	2384	34.50	17.71	21.60
10	Valine	100	2.57	181	76.30	25.45	30.53
11	Threonine	100	32.12	68	85.00	30.40	16.10

Separation of different amino acids through HPLC

The current HPLC based method used for the determination of amino acid gave clear separation of different amino acids from the yeast cream. The sequence of elution of all the test amino acids from both samples were similar to standards. Different amino acids were determined in varying, from low to high, amount thus proving the sensitivity of the method.

Amino acid profiling of S-samples

The opted HPLC method not only separated the different amino acids from S-samples very well (Figure 1) but also showed that different essential amino acids were present in yeast cream in very reasonable amount (Figure 2). Despite a concerted effort, serine and glutamine could not be determined in yeast cream produced from Saf Instant yeast (S-samples). Although, such amino acids could not be detected through HPLC, however, presence of these amino acids in very minute quantity can not be ruled out, that may be due to below the detection limit of HPLC. The most abundant amino acid was iso-Leucine (36%) followed by

arginine (34%) and Threonine (12%). While amino acid present in low amounts were tyrosine (5%), methonine (2%), phenylalanine (2%) repectively (Figure 2). The lactic acid was also present in low amount which is 9%.

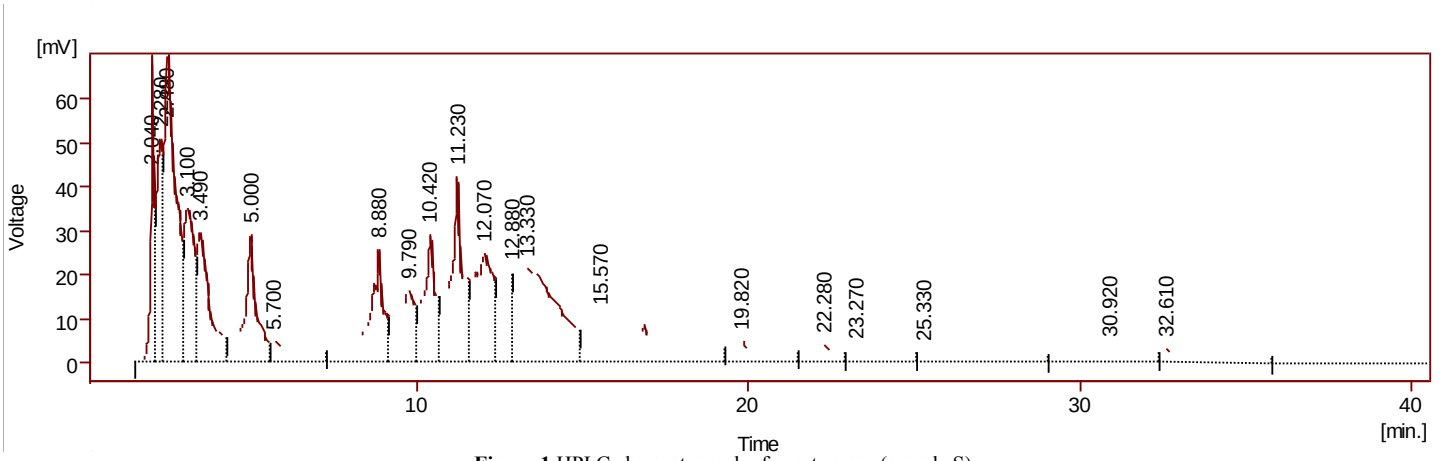

Figure 1 HPLC chromatograph of yeast cream (sample S)

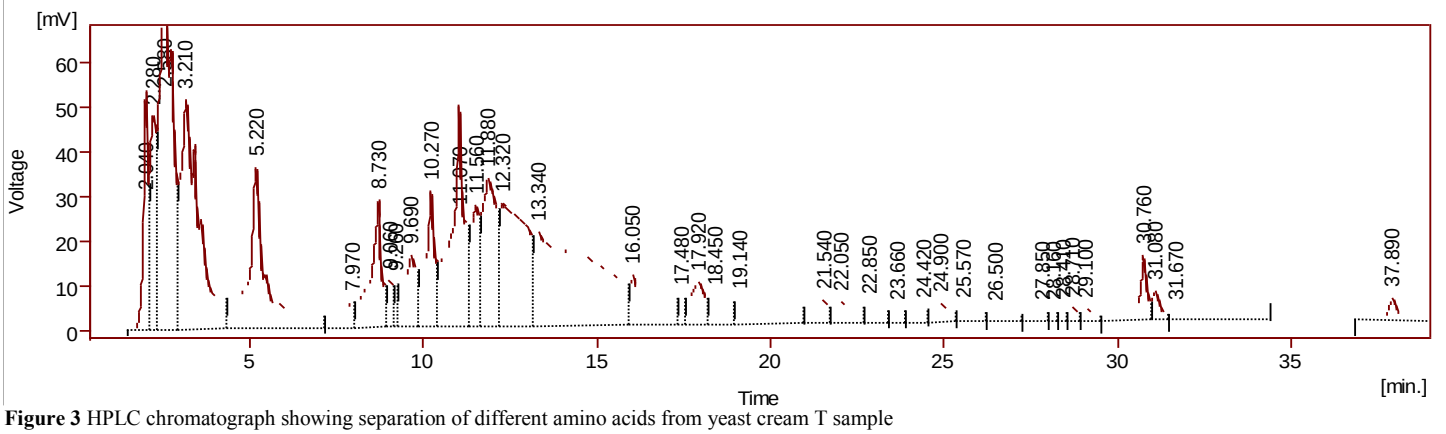

Figure 3 HPLC chromatograph showing separation of different amino acids from yeast cream T sample

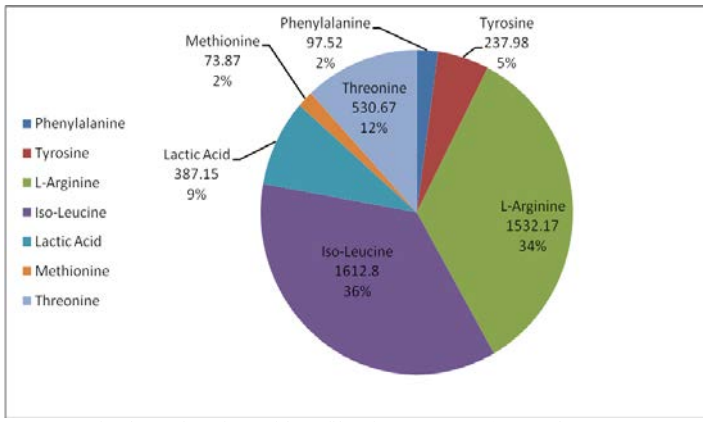

Figure 2 Pie chart of Amino acid profile of yeast cream (S sample)

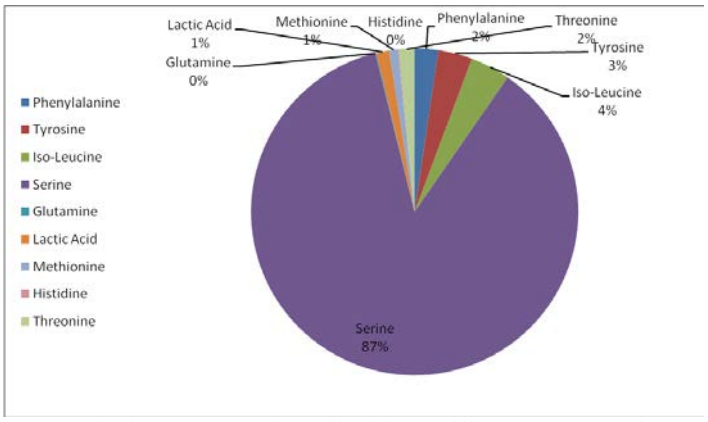

Figure 4 Pie chart of Amino acid profile of thermophilic yeast cream (Red)

Amino acid profiling of T-samples

Separation of different amino acids in T samples were also achieved through this methods and figure 3 is showing the chromatographic separation of different amino acids. Interestinlgy, amino acid profile of yeast cream samples "T" showed that amino acids, absent in S-samples, were found to be present in T-samples (Figure 4).

Serine that could not be detected in S-samples was very high (~87%) in T-samples. Similalry, glutamine percentage was found to be 4% in T-samples. Another, major exception was absence of arginine in these samples that rendered it good.However, other amino acid iso-Leucine (4%), tyrosine (3%), therionine (2%), phenylalanine (1%), methionine (0.1%) and, glutamine were present in very low amount. While lactic acid was found to be 1% (Figure 4).

Crude protein/nitrogen estimation and defating of dried yeast samples

Before HPLC, the yeast samples were subjected to crude protein estimation through Kjeldhal method. This method gave an approximate idea about the percentage of protein. The results showed that concentration of crude protein was higher in T-samples (9.42%) as compared to S-samples (7.31%). However, the quantity of fat was found to be almost same i.e ~8% in both yeast samples.

DISCUSSION

The study described here was stimulated by the increasing demand of protein (as feed) in poultry industry. Such increase in demand is being met from plant and animal sources on which human are dependent, thus, leading towards paucity of

resources. SCP is defined as an alternative source of protein for food and feed which obtained from microbes especially from yeast, bacteria and algae. SCP produced by the yeast has the potential to be opted because of high bioactive compound such as proteins, essential amino acids, vitamins, polysaccharides, lipids, organic acids, phospholipids, polyamines, astaxanthines, β-carotene, trehalose, glutathione, superoxide dismutase, chitinase, amylase and phytase **(Hotmaida, 2013)**. High level of SCP have been achieved through yeast at National research council (NRC) USA and overall crude protein levels were found to be 23, 20, and 18% for starter, grower and finisher phases respectively **(National Research Council, 1994)**. Although, various independent studies have evaluated the SCP ingredients produced by yeast but no such studies have looked at its potential use in poultry industry.

Methionine is considered as an important limiting amino acids in broiler diets which play important role in growth as well as for humoral and cellular immunity of chickens which infected with Newcastle virus **(Tsiagbe, 1987)**. **Gbmegia *et al.*,(2012)** showed that 0.34% methonine and 0.75% play an important for the growth of birds.Amino acid profiling of collected yeast cream showed the presence of methionine in both samples, however, relatively abundance of methionine is high (2%) in S-samples (Fig. 1) while, the percentage of methionine was 1% in T-samples (Fig. 2) **(Gbmegia,2012)**. These findings showed that thermophilic yeast cream have optimum quantity as afeedstock for growing birds.

The percentage value of arginine was quite high in S-samples (34%, Fig. 1), even quite higher than recommended quantity (1-1.44%) of arginine in poultry diet **(Lewis, 1963)**, while arginine could not be detected in samples-T. The arginine has previously been shown to be essential for improving egg production, egg weight as well as improving meat quality by modulating lipid metabolism, which in turn reduces the body fat accumulation and increases the antioxidant defense, furthermore, L-arginine has the ability to alleviate stress, normalise growth performance, reduces mortality under low ambient temperatures, attenuates the adverse effects of heat stress and high stocking density, activates the immune system and enhances its responses to different common diseases in poultry farms **(Fouad, 2012)**. Although, yeast cream is highly enriched with arginine, however, substantiate amount of arginine should be adjusted if yeast cream is opted as feed source.

Threonine is a major component of plasma γ-globulin in animals and it found to 12% in S-samples (Fig. 1), while merely 2% threonine was found to be present in T-samples (Fig. 2). Threonine often appears as the third limiting amino acids, its dietry intake influences the components of the immune system **(Li, 2007)**, although no clear report of a special need of threonine for immunity in poultry is present **(Kidd, 2004)**. L-threonine is the necessary complement of L-lysine for which it optimizes the use by animals for body protein deposition and weight gain. Moreover, L-threonine plays an important role in maintenance processes and more particularly in digestion. **Khan *et al.*, (2006)** experimentally proved that digestible threonine level of 0.73% helps in gaining weight, feed consumptionand improves breast meat yield (**Khan, 2006**). So, again our results are showing that yeast cream produced by thermophilic strain (T-samples) has optimum level of threonine (Fig. 2), hence can be used in poultry feed.

Mehri *et al.*, (2012) worked out the ideal ratios for both methionine and threonine to lysine, of Ross x Ross 308 male broiler chicks, from day 3 to day 16. Response surface methodology was employed using a central composite design. Body weight gain was maximized when Lysine was 1.12% and Methionine was 0.54%, corresponding to a Methionine/Lysine ratio of 48. Similar optimum requirements were noted for feed conversion with Lysine at 1.13% and Methionine at 0.53%, corresponding to ideal ratios for Methionine/Lysine of 47. The S-sample showed methionine and threonine in same concentration i.e 2% (Fig. 1), while T-sample contain methonine 1% and threonine 2% (Fig. 2), which is almost similar ratio which is given by **Mehri *et al.*,(2012)**.Branched-chain amino acid (BCAA, Valine, Isoleucine and Leucine) have been demonstrated vital for the development of immune organs , whereas it appears difficult to dissociate the specific effect of each BCAA (**Li, 2007; Kidd, 2004)**. The lowest Isoleucine:Lysine ratio (0.73:1) was sufficient to ensure satisfactory performance of birds, corresponding to the consumption of 534mg of isoleucine and 731 mg of lysine/day (**Heloisa, 2012)**. No differences were observed in the performance of hens over a wide range of dietary Isoleucine concentrations, but it can be inferred that the lower isoleucine:lysine ratio (0.73:1), can provide satisfactory performance of laying hens (**Etienne, 2011)**. Current results showed that valine and leucine are absent in both Saf instant and extromophile yeast samples. Neverthless, isoleucine is present in higher quantity in S-samples 36% (1.6mg/mL), while in T-samples contain desired level of isoluecine which is about 4% (0.4mg/mL). Previous results showed that the broiler weight gain and feed conversion improved when isoleucine was supplemented to the lowest dietary isoleucine level fed. Supplementation with equal amounts of arginine did not alleviate the dietary isoleucine limitation, thus validating the essentiality and marginality of isoleucine in practical corn-soybean meal diets when at least 2% of meat-and-bone meal is present in diet formulation **(Corzo, 2008)**. The isoluecine level present in S-samples is 36% (Fig. 1), which is much higher than recommended levels while, in T-sample the level of isoleucine level is 4% which is near to recommended level (Fig. 2).

Vegetable protein is not a complete source of protein for Layer's feed. The low amount of protein retards the growth of birds. When the protein level exceeded from optimum level, it will undergo domination which converted protein into uric acid and removed as a fecal nitrogen and cause environmental pollution. On the other hand, in dominating process bird's loses more energy as compared to other process of the body. It is suggested that methionine 0.34% is optimum for growth of poultry **(Gbmegia, 2012)**. **Kalinowski *et al.*, 2012** reported that the total methionine requirement in 0 to 3 week old broiler chicks was 0.50%, regardless as to whether they were slow or fast feathering strains **(Kalinowski, 2003).** However, it was noted that the cysteine requirement was 0.39% for slow-feathering males versus 0.44% of fast-feathering males. Nutri-genomics study revealed that tryptophan is helpful increasing immune response and for better health of broiler **(Trevisil, 2012)**. High concentration of machine and intestinal infection can be decreased by increasing threonine in the diet **(Star, 2012)**.Valine showed significant response when dietary protein is low in broiler feeds **(Magdalena, 2013)**. Our results pinpointed that yeast cream produced by extremophile (T-samples) contains optimum quantity of threonine of 2% while, higher amount of threonine (12%) was present in S-sample (Fig 1 and 2).

Teresa (2009) showed that fresh acid whey or lactic acid in the amount of 4cm^3/L (4mL/L) in poutry feed could effectively prevent the reduction in poultry production by decreasing the mortality risk in chickens as compared to chickens fed with a poor-quality diet (Teresa, 2009). However, a negative effect on broilers production was observed when acidified diet (containing both whey and lactic acid) was given in addition to acidified drinking water, so broiler producers should not use lactic acid or whey as drinking liquid if the chicken are being fed with acidified diet (Teresa, 2009). HPLC analysis of yeast cream showed that both T- and S-samples contain sufficient amount of lactic acid, thus can be adopted as feed source for broilers (Fig. 1 and 2). The high level of serine in amino acid anlaysis is one of the main problem for utilization of yeast cream as a source of feed so, one possible way to deal this sort of problem to add a enzyme serine dehdratase which deaminate the serine into pyruvate. In order to industrilizd and economized this process immobilized serine dehydratase is good option.

CONCLUSION

The overall study indicated that T-sample which is obtained from cream of Thermophilic yeast (Red) has optimum level of amino acids and lactic acid contents, thus it can be opted as a potential ingredient of poultry feed. Some amino acids are present in larger quantity in yeast cream, for example serine, such amino acids should be brings to optimum level to make yeast cream an excellent supplement for broilers. Although, alone yeast cream could not be used as a feed in poultry industry but can be mixed with other feed sources, thus a competition can be reduced for food inbetween human and brids. However, careful analysis and trials should be conducted on broilers before mixing it with other poultry ingredients. This study is a key step for utilization of waste of yeast cream of distillery as a broiler feed which is not being tried yet.

Conflicts of Interest

Authors declares no conflicts of interests.

Authors' Contributions

Zafar Iqbal and Zahid Anwar done statistical anlaysis and drafted manuscript. Shoaib Ali and Mudassar Zafar done chemical analysis and conduct the research. Munir Ahmad Sheikh supervised shoaib Ali, while, Muhmmad Khurshid and Muhammad Irshad coordinate in desingin and drafting the manuscript. The final version of manuscript was studied and approved by all authors.

REFERENCES

GADANHO, M., ALMEIDA, J.M.G.C.F., SAMPAIO J.P. 2003. Assessment of yeast diversity in a marine environment in the south of Portugal by microsatellite-primed PCR. *Antonie van Leeuwenhoek.,* 84(3), 217-227. http://dx.doi.org/10.1023/a:1026038213195

STEWART, R. 1998. An Introduction to Brewing Science and Technology: *Brewer's Yeast*. The Institute of Brewing, London. 108-109. ISBN: 0900489138.

WALKER, G.M. 1998. *Yeast Physiology and Biotechnology*. John Wiley and Sons, Chichester. 350-353.

ERTEN H.,TANGULER H. 2006. The production methods of yeast extract. Feed Info News Service. Fresh water fish species of Northern Nigeria. *Academy Journal of Science*, 41(6), 11-15.

STAM, H., Hoogland M., LAANE, C. 1998. Food flavours from yeast. *Microbiology of Fermented Foods,* 2, 505-542. http://dx.doi.org/10.1007/978-1-4613-0309-1_16

KHAN, M. L. 2001. *Poultry feeds and nutrition*. Kitabistan Publishing Co. 38-Urdu Bazar, Lahore.

BUSTAMANTE, M. A., MORAL, R., PAREDES, C., ESPINOSA, A.P., MORENO-CASELLES, J., PEREZ-MURCIA, M.D. 2008. Agrochemical characterization of the solid byproducts and residues from the winery and distillery industry. *Waste Management,* 28:372-380. http://dx.doi.org/10.1016/j.wasman.2007.01.013

SHARIF, M., SHAHZAD M.A.S., REHMAN S., KHAN R., ALI K.M.L., KHAN, K. 2012. Nutritional Evaluation of Distillery Sludge and Its Effect as a Substitute of Canola Meal on Performance of Broiler Chickens. *Asian-Australasian Journal of Animal Sciences, 25(3),*401-409. http://dx.doi.org/10.5713/ajas.2011.11279

ANNE, F. 2008. Organic Poultry Productionin the United States. A Publication of ATTRA - National Sustainable Agriculture Information Service, 2008. 1-16.

ANONYMOUS, 2009. Amino acids focus at Adisseo seminar. *World Poultry*. Sept. 2.

DIZ, M.S., CARVALHO A.O.R., RODRIGUES A.G., NEVES-FERREIRA M., DACUNHA E.W., ALVES, A.L., OKOROKOVA-FACANHA, M.A., OLIVEIRA, J., PERALES, O.L., MACHADO, G.V.M. 2006. Antimicrobial peptides from chili pepper seeds causes yeast plasma membrane permeabilization and inhibits the acidification of the medium by yeast cells. *Biochimica et Biophysica Acta (BBA) - General Subjects,* 1760(9),1323-1332. http://dx.doi.org/10.1016/j.bbagen.2006.04.010

BAIZE, J.C. 2000. Global soybean meal sampling and analysis activity. United Soybean Board contract#0586 with John C. Baize, Falls Church, Virginia. 2000.

KANURI, A.B., RAVIKANTH, M.J.K., THAKUR, A., MAINI, S. 2014. Comparative efficacy of herbal and synthetic amino acids for growth performance and hepatoprotective action in broiler chickens. *International Journal of Biomedical and Advance Research,* 05(01), 14-18. http://dx.doi.org/10.7439/ijbar.v5i1.589

SAIMA, M., AKHTER, M.Z.U.K., ANJUM, M.I., AHMED, S., RIZWAN, M., IJAZ, M., 2008. Investigation On The Availability Of Amino Acids From Different Animal Protein Sources In Golden Cockerels. *Journal of Animal and Plant Sciences,* 18(2-3),53-55

TERESA, M., KRZYSZTOF, P., KRZYSZTOF, K., KRZYSZTOF, B., PAULIUS, M. 2009. Whey and lactic acid in broiler chickens nutrition. *Veterinary medicine and zootechnics, 47*:69.

SUDHA, K.R., KARTHIKEYAN, S., 2005. Distillery Yeast Sludge (DYS) as an Alternative Feed Resource in Poultry. *International Journal of Poultry Science,*4(10),787-789. http://dx.doi.org/10.3923/ijps.2005.787.789

ECONOMIC SURVEY OF PAKISTAN, 2013-2014. Govt. of Pakistan. Finance Division Economic Advisor's Wing, Islamabad.

ESONU, B.O., OGBONNA, U.D., ANYANWU, G.A., EMENALOM, O.O., UCHEGBU, M.C., ETUK, E.B., UDEDIBIE, A.B.I. 2006. Evaluation of performance, organ characteristics and economic analysis of broiler finisher fed dried rumen digesta. *Int. J. Poultry Science, 5*:1116-1118. http://dx.doi.org/10.3923/ijps.2006.1116.1118

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS (A.O.A.C.) 1990. Official methods of analysis. In:*Association of official Analytical Chemists*. (15th Edition). Arlington Virginia, USA.

SKOTTY, D. R., WON-YONG, L., NIEMAN,T.A.,1996. Determination of dansyl amino acids and oxalate by HPLC with electrogenerated chemiluminescence detection using tris(2,2‘-bipyridyl)ruthenium(ii) in the mobile phase. Analytical *Chemistry,*68(9),1530-1535. http://dx.doi.org/10.1021/ac951087n

HOTMAIDA, S., TRI, A., OSFAR, S. 2013. The amino acid profile of yeasts from ketchup factory waste as a candidate of single cell protein (SCP). *International Journal of* Biosciences. 3(9), 36-43. http://dx.doi.org/10.12692/ijb/3.9.36-43

NATIONAL RESEARCH COUNCIL, 1994. *NutrientRequirements of Poultry*. 9th rev. ed. National Academy Press, Washington, D.C.1994.

TSIAGBE, V.K., COOK, M.E., HARPER, A.E., SUNDE, M.L. 1987. Enhanced immune responses in broiler chicks fed methionine-supplemented diets. *Poultry Science,* 66(7),1147-1154. http://dx.doi.org/10.3382/ps.0661147

GBMEGIA, O.A., SULAIMAN, A.A., FOLUKE, A.A. 2012. The effect of varied dietary crude protein levels with balanced amino acids on performance and egg quality characteristics of layers at first laying phase. *Food and Nutrition Sciences,* 3,526-529. http://dx.doi.org/10.4236/fns.2012.34074

LEWIS, D., SMITH, G.H., PAYNE, C.G. 1963. Dietary requirement for arginine. Arginine in poultry nutrition. *British Journal of Nutrition,* 17(01),415. http://dx.doi.org/10.1079/bjn19630045

FOUAD, A.M., EL-SENOUSEY, H.K., YANG, X.J., YAO, J.H. 2012. Role of dietary L-arginine in poultry production. *International Journal of Poultry Science*. 11,718-729. http://dx.doi.org/10.3923/ijps.2012.718.729

LI, P., YIN, Y-L., KIM, S.W., WU, G. 2007. Amino acids and immune function. *British Journal of Nutrition,* 98(2),237-252. http://dx.doi.org/10.1017/s000711450769936x

KIDD, M.T. 2004. Nutritional modulation of immune function in broilers. *Poultry Science,* 83(4),650-657. http://dx.doi.org/10.1093/ps/83.4.650

KHAN, A.R., NAWAZ, H., ZAHOOR, I. 2006. Effect of different levels of digestible threonine on growth performance of broiler chicks. *Journal of Animal and Plant Sciences,*16,1-2. http://dx.doi.org/10.2141/jpsa.43.388

MEHRI, A., DAVARPANAH, A., MIRZAEI, H.R. 2012. Estimation of ideal ratios of methionine and threonine to lysine in starting broiler chicks using response surface methodology. *Poultry Science,* 91(3),771-777. http://dx.doi.org/10.3382/ps.2011-01818

HELOISA, H.D.C.M., PAULO, C.G., TATIANA, C.D.R., JUAREZ, L.D., RODRIGO, L.D.A., ALLAN, R.T., BRUNO, R.D.C., GABRIEL, D.S.V. 2012. Determination of digestible isoleucine:lysine ratio in diets for laying hens aged 42-58 weeks. *Revista Brasileira de Zootecnia*, 41(5),1313-1317. http://dx.doi.org/10.1590/s1516-35982012000500032

ETIENNE, C., AJINOMOTO, E. J., 2011. (Lohmann AnimalHealth GmbH & Co. KG) valine and Isoleucine. The next limiting amino acids inbroiler diets. 46,59.

CORZO, A., DOZIER, W.A., KIDD, M.T., HOEHLER, D., 2008. Impact of Dietary Isoleucine Statuson Heavy-Broiler Production. *International Journal of Poultry Science. 7(*6),526-529. http://dx.doi.org/10.3923/ijps.2008.526.529

KALINOWSKI, A., MORAN, E.T., WYATT, C., 2003. Methionine and cystine requirements of slow-and fast-feathering male broilers from zero to three weeks of age. *Poultry Science,* 82(9),1423-1427. http://dx.doi.org/10.1093/ps/82.9.1423

TREVISIL, P., CORRENT, E., MESSORI, S., FORMICA, S., PRIORI, D., BOSI, P. 2012. Supplementary tryptophan down regulates the expression of genes induced by the gutmicrobiota in healthy weaned pigs susceptible to enterotoxigenic *Escherichia coli* F4. *Livestock Science,* 147(1-3), 96-103. http://dx.doi.org/10.1016/j.livsci.2012.04.008

STAR, L., ROVERS, M., CORRENT, E., VANDER, K.J.D. 2012. Threonine requirement of broiler chickensduring subclinical intestinal Clostridium infection. *Poultry Science,* 91(3):643-652. http://dx.doi.org/10.3382/ps.2011-01923

MAGDALENA, L., LEITGEB, R., BARTELT, J., CORRENT, E., SCHEDLE, K. 2013. Impact of an increasing true digestible (TD)valine to lysine ratio in a protein reduced dieton the performance of broiler chicks. *Proceeding of the Society of Nutrition and Physiology.* 22,and AEL’s trial report 12AT02.

JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

IMPROVED SHELF LIFE OF BROWN RICE BY HEAT AND MICROWAVE TREATMENT

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doi: 10.15414/jmbfs.2016.5.4.378-385

ARTICLE INFO

Received 18. 5. 2015
Revised 13. 11. 2015
Accepted 4. 12. 2015
Published 1. 2. 2016

Regular article

OPEN ACCESS

ABSTRACT

Widespread marketing and consumption of brown rice is limited by its short shelf life. This study aimed to address this problem by establishing treatments to stabilize brown rice against rancidity, while retaining its antioxidants, for subsequent commercial applications. Three types of treatments, namely dry heat (DH, oven at 60°C), wet heat (WH, steam), and microwave (MW, 800 watts), with different exposure times were tested. Lipase activity was determined for all treatments. Samples were monitored for 0, 4 and 6 months for changes in free fatty acids, antioxidant activity, and total phenolics content; while 0, 2, 4 and 8 months for changes in raw and cooked sensory attributes.

Treatments with longer exposure effectively inactivated lipase enzyme and consequently reduced free fatty acid release, even up to 6 months of storage. Total phenolics content of treated and untreated samples were not significantly different after treatment, but tended to increase during storage. Similar trend was observed on the antioxidant activity of DH treated brown rice, except that of MW and WH treated sample. Initial raw and cooked sensory attributes of treated and untreated samples were comparable. A significant downshift on the raw (aroma, glossiness and off-odor) and cooked (aroma, off-odor and off-taste) sensory attribute scores and acceptability of both untreated and treated brown rice were noted beginning second month, except that of treatments with longer exposure namely DH 25min, MW 90sec and WH 90sec, including MW 60sec. Only treated brown rice samples with longer exposure times (DH 25min, MW 90sec and WH 90sec), including MW 60sec, remained acceptable by the fourth month, and even up to eight months of storage.

Keywords: Brown rice; shelf life; heat treatment; microwave treatment; lipase inactivation

INTRODUCTION

Consumption of whole grain cereals like brown rice helps reduce risks of chronic diseases. One of the whole grain cereals is brown rice. It is more nutritious than milled (polished) rice. Approximately 80% of the minerals and 15% of proteins are taken out when brown rice is milled. A significant amount (68% to 90%) of calcium, phosphorus, riboflavin, and thiamine are removed (**Dykes and Rooney, 2007**). Brown rice is a good source of dietary fiber and antioxidant compounds such as phenolic acids.

Brown rice is also economically important in the Philippines where rice self-sufficiency and supply stability are targeted. Having 10-percentage point milling recovery advantage over milled rice, brown rice can greatly contribute to rice self-sufficiency. Additionally, energy consumption in processing of brown rice is 50-60% lower than milled rice (**Cuyno, 2003**), because polishing and whitening steps are eliminated. Despite its nutritional and economic benefits, brown rice is still not widely consumed and marketed because of its susceptibility to rancidity. The short shelf life of brown rice (2-3 months) is attributed to the rapid reactions of the lipase enzyme, which are released from the breakup of the bran cells during the hulling process, with the lipids in the bran to produce free fatty acids. Hence, brown rice requires suitable storage condition and/or effective control.

Heat treatments have been employed to inactivate lipolytic enzymes in cereals and cereal products, but results have been inconclusive (**Rose *et al.*, 2008**). **Vetrimani and Haridas Rao, (1990)** and **Haridas Rao *et al.*, (1980)** found that heating wheat bran at 175°C for 40 min increased its shelf life from 20 to 90 days and heating wheat germ at 150°C for 25 min increased shelf life from 15 to 180 days, respectively. On the other hand, **Cuendet *et al.*, (1954)** found autoclave treatment not effective in increasing the shelf life of whole wheat flour, while **Lehtinen *et al.*, (2003)** found that the complete inactivation of lipase in oats by extrusion resulted in greater lipid oxidation. As cited by **Rose *et al.*, (2008)**, authors from the two later studies theorized that the increase in lipid oxidation was a result of destruction of antioxidants during heat treatment. Maintaining antioxidants is therefore an important consideration in stabilization of cereals and cereal products.

Several approaches have been utilized to stabilize brown rice against lipolytic hydrolysis. One approach is by inactivating lipase by heating rough rice with moist gas to obtain stabilized products (**Van Ata *et al.*, 1952**) or by parboiling or precooking (**McCabe, 1976**). Another approach is by removing the kernel oil, which serves as the substrate for lipase, through organic solvent extraction (**Kester, 1951**). Finally, by denaturation and inactivation of lipase and lipase-producing bacteria and mold by liquid ethanol (**Champange *et al.*, 1991**) and ethanol vapors (**Champange and Hron, 1992**). However, one drawback to the two latter techniques is the use of chemical solvents that may be harmful to health and the environment.

Likewise, various methods of brown rice storage conditions have been tested to determine the temperature, atmosphere, and kind of packaging material that could improve its shelf life. Storage at low temperature, storage under modified atmospheres such as carbon dioxide, storage under vacuum, storage using polyethylene and nylon films and aluminum pouch, or the combination of different storage conditions and packaging materials have been reported to improve the shelf life of brown rice. However, these storage conditions and packaging materials are costly, hence, may not be suitable for commercial applications.

In effort to address the shelf life problem of brown rice, this study aimed to determine treatment condition for dry and wet heat, and microwave treatment that could stabilize brown rice against lipolytic rancidity during storage, while retaining its antioxidants, for subsequent commercial applications.

MATERIAL AND METHODS

Brown rice preparation

Aromatic and low amylose–gelatinization temperature type rice, Maligaya Special 8 (MS8), previously reported by **Corpuz *et al.*, (2010)** as one of the most acceptable varieties for brown rice consumption, was used in this study. MS8 rough rice sample was obtained from the Income Generation Office of the Philippine Rice Research Institute (PhilRice). Rough rice sample was dehulled using Satake® rice dehuller (Satake Engineering, Tokyo, Japan) to obtain brown

rice. Undehulled and immature grains and other impurities were removed manually before subjecting to different treatments.

Brown rice treatment and storage

One kilogram of brown rice was subjected, in duplicate, to three different types of treatment, as follows: dry heat (DH) for 15, 20 and 25 min using 30 x 20 x 3 cm (length x width x thickness) 150µm screen sieve in an oven (Yamato, Japan) set at 60°C; microwave (MW) treatment for 30, 60 and 90 sec using a 30 cm x 20 cm x 10 cm (length x width x thickness) rectangular microwavable plastic container in an 800 Watts and 2450 MHz commercial microwave oven (American Heritage®) set at high setting; and steam (WH) treatment for 30, 60 and 90 sec using 30 x 20 x 3 cm (length x width x thickness) 150µm screen sieve over a pot of boiling water using a household steamer. These treatments showed no objectionable change in overall appearance compared with untreated brown rice.

Immediately after treatment, samples were placed separately in an aluminum tray and allowed to cool for about 30 min. About 10 g-portion of each sample was obtained, pulverized using Cyclotec® sample mill (Tecator, Sweden), and analyzed for moisture content using Method 44-19 (**AACC International, 2000**). After 24 hours of equilibration, each sample were placed in a separate polyethylene plastic bag and stored together in a metal box at room temperature and ambient humidity. Another 10 g-portion of each sample was obtained, pulverized, and analyzed for lipase activity and moisture content prior to storage. An untreated brown rice was used as control. Moisture content of the brown rice samples immediately after treatment (after 30 min of cooling) progressively decrease ranging from 10.75% to 11.81%, but not on WH treated sample except that of WH 30 sec. While moisture content after 24hr of equilibration at room temperature ranged from 11.35% to 11.91%, which are acceptable levels for storage (data not shown). Temperature and relative humidity during storage ranged from 28-31°C and 42-64%, respectively.

Different exposure times were tested to determine optimum treatment condition for each type of treatment that would effectively reduce lipase activity to consequently stabilize brown rice from rancidity during storage. Mild treatments were employed to maintain important antioxidant compounds such as phenolic acids and to retain physically indistinguishable from the untreated brown rice. Preliminary trials conducted by DH treatment at temperature of 65°C and above for 15-20 min using oven (Yamato DN-83, Japan) resulted in fissuring and discoloration of the brown rice grains (data not shown). Thus, oven temperature at 60°C was employed to inactivate lipase enzymes and different DH treatments were at varied times (0 to 25 min). **Rothe, (1967)** as cited by **Juliano, (1985)** reported that the inactivation temperature for lipase enzyme in rice bran at 14% moisture was 55 ºC, hence 60°C oven treatment may be sufficient for lipase inactivation. For WH treatment, exposure for 100 sec and above under steam resulted in discoloration, while microwave treatment for 100 sec and above caused popping of some of the brown rice grains.

Chemical analysis

The effect of different treatments in lipase activity of the brown rice samples was determined. Changes in free fatty acids level, total phenolics content, and antioxidant activity were monitored for 0, 2, 4 and 6 months of storage at room temperature and ambient humidity using prescribed procedures. All chemical analyses were done in duplicate.

Lipase activity

Lipase activity was measured using the copper soap assay according to the procedure of **Rose and Pike, (2006)**. About 3 g of each ground sample was partially defatted with hexane (1:10 wt/vol) for 30 min on a mechanical shaker. Residual hexane was allowed to evaporate at room temperature (about 10 min), and 1 g of the ground, partially defatted sample was weighed into separate tubes (blank and sample). Olive oil (0.8 mL) and distilled water (0.15 mL) was added on both tubes and were mixed vigorously. The test tube with the blank was immediately extracted using a stepwise procedure. Five mililiters of hexane were added, mixed using vortex, and then centrifuged at 2000 rpm for 3 min using benchtop centrifuge (Clay and Adams, NJ). The hexane was decanted into centrifuge tube and the extraction was repeated twice. The hexane extracts were pooled, and evaporated using a water bath at 40ºC, and the residue was redissolved in 4 mL of isooctane. The test tube with the sample was capped and incubated for 4 hr at 40ºC. After incubation, the test tube with the sample was extracted using hexane as described in the blank. One mililiter of 5% (wt/vol.) cupric acetate (adjusted to pH 6.1 with pyridine) was added and then shaken vigorously for 1 min. Afterwards the tubes were centrifuged at 2000 rpm for 1 min and the absorbance was read at 715 nm using UV-Vis spectrophotometer (Hitachi U-3200, Japan). The absorbance of the sample was compared with the absorbance of oleic acid standard solutions prepared in isooctane (1-10 mM). Lipase activity was expressed as units per gram (U g^{-1}), where 1 U is defined as the micromoles of fatty acid liberated per hour.

$$LA = 1000 * \frac{(4+V) * (Af - Ai)}{stls}$$

Where1000 = conversion factor from mol. L^{-1} to µequiv mL^{-1}, 4 = volume of isooctane used to redissolve lipids (mL), V = volume of olive oil added (mL), Af= absorbance of sample after incubation at 715 nm, Ai = absorbance of blank after incubation at 715 nm, ε = molar absorptivity of oleic acid at 517 nm (M^{-1} cm^{-1}), t = incubation time (h), l = path length (1cm for a standard cuvette), s = mass of sample (g).

Free fatty acids

Lipid degradation during storage as free fatty acids was quantified using the rapid colorimetric method of **Kwon and Rhee, (1986)**. One gram of ground brown rice sample was weighed into test tube and the lipids were immediately extracted similar with the procedure by **Rose and Pike, (2006)**. One ml of 5% (wt/vol.) cupric acetate (adjusted to pH 6.1 with pyridine) was added to the extract and then shaken vigorously for 1 min. Afterwards the tubes were centrifuged at 2000 rpm for 1 min and the absorbance was read at 715 nm using UV-Vis spectrophotometer (Hitachi U-3200, Japan). The sample absorbance was compared with the absorbance of oleic acid standard solutions prepared in isooctane (1-10 mM).

Antioxidant activity (DPPH radical scavenging)

Free radical scavenging capacity of the samples was estimated using 2, 2'-dipheny-1-picrylhydrazyl (DPPH) radical according to the procedure of **Iqbal *et al.*, (2005)**. One gram ground sample was weighed into 15-mL centrifuge tube. Ten mililiters of methanol was then added, shaken for 12 hours, and centrifuged at 2000 rpm for 15 minutes. The supernatant was collected, diluted up to 25 mL with distilled water, and stored at 4°C, until analysis.

An aliquot of the sample extract (3 mL) was added to freshly prepared 0.10 mM solution of DPPH (30 mL) and allowed to stand for 90 min. The absorbance of the DPPH-sample extract mixture was then measured at 517 nm using UV-Vis spectrophotometer (Hitachi U3200, Japan). The DPPH radical scavenging activity was calculated as follows:

$$\% \text{ DPPH radical scavenging activity} = \frac{(A_b - A_s)}{A_b} \times 100$$

Where: A_s = absorbance of the sample and A_b = absorbance of the blank.

Total phenolics content

The total concentration of phenolic acids (soluble and bound) was determined as gallic acid equivalents (GAE) using the Folin-Ciocalteau procedure as cited by **Adom and Liu, (2002)**.

Extraction of soluble phenolics. Ten mililiters of 80% (vol/vol) ethanol was added to 1.0 g of ground sample. The mixture was shaken for 15 min, centrifuged at 2000 rpm for 15 min, and the supernatant was collected. The residue was re-extracted with 80% ethanol twice. The supernatants were combined and the residues were set aside for bound phenolics extraction. The pooled supernatants were oven-dried at 30ºC. After drying, the residue was re-dissolved up to 25.0 mL with distilled water and then store at 4°C, until analysis.

Extraction of bound phenolics. For bound phenolics extraction, the residue was digested for one hour with 25 ml of 2.0 N NaOH with constant shaking using a mechanical shaker. The mixture was then neutralized with an appropriate amount of 6 M HCl and was defatted twice with hexane. The final solution was extracted five times with 30 mL ethyl acetate. The ethyl acetate portion was collected and oven-dried at 30°C. The residue was re-dissolved up to 25.0 ml with distilled water and then stored at 4ºC, until analysis.

Determination of total phenolics content. Five hundred microliters (500µL) of each extract (soluble and bound) was added with 2.5 mL of Folin-Ciocalteau's phenol reagent (1/10 dilution). It was allowed to stand temperature for 15 min at room and then 2.0 mL of 7.5% sodium carbonate was added. After 1 hour of color development, the absorbance of the mixture was measured at 765 nm against a blank and gallic acid standards (0-100 µg mL^{-1}). Phenolics content was calculated based on the standard and values were expressed in gallic acid equivalents per gram sample (GAE g^{-1}).

$$\text{Phenolics acid content (GAE } g^{-1}) = \frac{A \times 25}{\text{Wt. of samples (g)} \times MW_{GA}}$$

Where A= µg g^{-1} gallic acid based on calibration curve and MW_{GA}= gram equivalents of gallic acid (170.2 g eq^{-1}). Total phenolics acid content was then calculated by adding the contents of soluble and bound forms.

Sensory evaluation

Sensory evaluation was conducted at the Sensory Laboratory of the Rice Chemistry and Food Science Division, PhilRice, Science City of Muñoz, Nueva Ecija, Philippines. Raw (aroma, off-odor, color, gloss, and translucency) and cooked (aroma, off-odor, gloss, cohesiveness, tenderness, off-taste, and taste) sensory attributes and acceptability were evaluated for 0, 2, 4, and 8 months of storage by trained internal panels. Attribute intensities and general acceptability were rated using 15-cm unstructured scaled score cards. The scales were anchored at each end: the left side of the scale corresponded to the lowest intensity (0 cm) and the right side to the highest intensity (value 15 cm) of the sensory attribute.

Cooked brown rice was prepared by addition of water to brown rice (1:2 brown rice to water), washing the brown rice for two times by swirling and replacing same amount of decanted water with fresh tap water, soaking the brown rice with water for 25 min, and cooking using 2-cup capacity electric rice cooker (National, Japan) until the audible switches turned off. Raw and cooked brown rice samples were presented to the sensory panels in a blind and randomized 3-digit coded manner for evaluation. None of the staff involved in the study participated as panellist.

Statistical analysis

Analysis of variance (ANOVA) was conducted to detect differences between treatments at $p<0.05$ level. When a significant treatment differences was observed, treatment means were separated using Tukey's honesty significant difference (HSD) test. Statistical analyses of physicochemical and sensory evaluation data were performed using SAS ver. 9.1 for Windows (New York, USA) and STATA ver. 12.1(Texas, USA) software, respectively.

RESULTS AND DISCUSSION

Effects of different treatments on brown rice

Effects in lipase activity and free fatty acids content

Inactivation of lipase enzyme, which catalyzes the hydrolysis of lipids (oil) to produce oxidizable fatty acids that are further converted into carbonyl compounds contributing off-odor and off-taste, is primarily considered in prolonging the shelf life of brown rice. All treated brown rice had significantly lower lipase activity compared with untreated sample (Table 1). Increasing exposure time progressively decrease lipase activity of the brown rice, except that of MW treatment where MW 60sec treatment had lower lipase activity than MW 90sec, but were not statistically significant. Most brown rice samples within each treatment type had comparable lipase activity, except that of DH treatment. For each type of treatment, lowest lipase activity was noted in DH 25 min at 0.29 U g^{-1}, MW 60 sec at 0.31 U g^{-1}, and WH 90 sec at 0.31 U g^{-1}.

Table 1 Lipase activity and free fatty acid content of brown rice samples at different treatments

Sample	Lipase Activity (U g^{-1})	Free Fatty Acid Content (mmole g^{-1})
Control	0.45^{e}	2.57^{g}
DH 10min	0.36^{d}	2.44^{f}
DH 15min	0.34^{cd}	2.36^{e}
DH 25min	0.29^{a}	2.33^{e}
MW 30sec	0.33^{bcd}	2.24^{cd}
MW 60 sec	0.31^{ab}	2.18^{ab}
MW 90 sec	0.33^{bc}	2.17^{ab}
WH 30sec	0.34^{bcd}	2.27^{d}
WH 60sec	0.32^{abc}	2.13^{a}
WH 90 sec	0.31^{ab}	2.20^{bc}

Means within a column with same letters are not significantly different (α=0.05)

All treated brown rice samples had significantly lower free fatty acids content than untreated sample. Increasing treatment exposure of brown rice decreases its lipase activity and consequently reduces its free fatty acids level. MW and WH treated samples have significantly lower free fatty acids content even compared with DH treated brown rice. Free fatty acids level of MW and WH treated brown rice ranged from 2.17-2.24 mmole $g^{-1}$and 2.13-2.27 mmole g^{-1}, respectively; while DH treated brown rice ranged from 2.33-2.44 mmole g^{-1}. This indicates that MW and WH treatments are more effective in inactivating lipase enzymes than DH treatment. This is likely because proteins such as enzymes are more stable against denaturation in a dry environment compared with a wet environment (**Damodara, 1996**). Likewise, **Krugger and Reed, (1988)** and **Vertimani and Haridas Rao, (1990)** as cited by **Rose *et al.*, (2008)** reported difficulty of decreasing lipase activity of wheat flour using dry heat treatment than wet heat and microwave treatments.

The consistent significant decrease in lipase activity and free fatty acids level of treated compared with untreated brown rice (control) confirmed the effectiveness of heat and microwave treatments in inactivating lipase enzymes. Several researchers have utilized heat and microwave treatments to stabilize cereals and cereal products, but limited studies have been conducted for its direct application for brown rice stabilization. Most of the studies on brown rice shelf life improvement or stabilization focused on the use of different storage temperature and packaging materials (**Sharp and Timme, 1986**), storage under modified atmospheres (**Ory *et al.*, 1980**; **Santroprete, 1980**), treatment by ethanol vapors (**Champange and Hron, 1992**), and utilization of an antioxidant or chelator (**Champange and Hron, 1993**; **Champange and Grimm, 1995**).

Effects in total phenolic content and antioxidant activity

Mild treatments employed on brown rice samples aimed to maintain inherent antioxidant compounds such as phenolic acids present that could help inhibit reactive oxygen species (peroxides) that causes rancidity. The presence of these indigenous antioxidants in cereals such as brown rice has a marked effect on the onset of non-enzymatic oxidation due to their capability to quench these reactive molecular species into non-reactive form (**Lehtinen and Laakso, 2004**). This could eventually help improve the storability or shelf life of brown rice. Total phenolic content of all treated brown rice samples did not vary significantly upon treatment (Table 2). Likewise, no significant change was observed on the antioxidant activity of DH treated brown rice compared with untreated sample, except that of MW and WH treated sample. This coincides with the findings of **Rose, *et al.*, (2008)** on the effect of dry heat treatment on the antioxidant activity of wheat flour. On the other hand, contrary to the findings of **Rose, *et al.*, (2008)**, a significant increase in antioxidant activity was noted on MW and WH treated samples. The increase in antioxidant activity on MW and WH treated samples may be attributed to the slight (not significant) increase in total phenolics content in the sample from 2.86 GAE g^{-1} (untreated) to up to 3.41 GAE g^{-1} (WH 60sec), previously noted. Conversely, DH treated brown rice also increase (not significant, $p>0.0$) in total phenolic content, but antioxidant activity did not change. Hence, the increase might be attributed to the type of treatment employed on the brown rice. **Oufnac *et al.*, (2006)** as cited by **Dar and Sharma, (2011)** reported that with rise in extraction temperature more phenolic compounds are released. Likewise, according to **Afoakwah *et al.*, (2012)**, microwave radiation causes disruption of hydrogen bonds which enhance penetration of the solvent into the matrix, allowing dissolution of the components to be extracted. Although exposure of brown rice to MW or WH treatments were not during the extraction process of antioxidants but were employed prior to the analysis, microwave radiation or steam treatments might have caused hydrolysis of other antioxidant compounds present in the sample resulting in the increase of their extractability, thus, higher antioxidant activity by DPPH radical scavenging analysis.

Table 2 Total phenolics content and antioxidant activity of brown rice samples at different treatments

Sample	Total Phenolics Content (Gallic Acid Equivalent g^{-1})	Antioxidant Activity (%DPPH Inhibition)
Control	2.86^{a}	40.45^{a}
DH 10 min	3.11^{a}	41.66^{a}
DH 15 min	3.11^{a}	40.55^{a}
DH 25 min	3.08^{a}	40.77^{a}
MW 30sec	3.05^{a}	54.45^{c}
MW 60 sec	3.24^{a}	54.65^{c}
MW 90 sec	2.90^{a}	51.67^{bc}
WH 30sec	3.08^{a}	51.21^{bc}
WH 60sec	3.41^{a}	51.63^{bc}
WH 90 sec	3.39^{a}	49.62^{b}

Means within a column with same letters are not significantly different (α=0.05)

Effects in raw and cooked sensory attributes

Evaluation by panels perceived no significant change in all raw and cooked sensory attribute scores between treated brown rice and untreated sample (Table 3 and Table 4). However, for each type of treatment, slight increase (not significant) in raw aroma on DH and WH treated samples was perceived by panellists attributed to the release of some aromatic compounds such as 2-acetly-1-pyrroline from the grains caused by heat treatment. On the other hand, raw aroma of MW treated samples was perceived similar with the untreated raw brown rice. Similarly with raw aroma attribute, an increase (not significant) in raw glossiness scores on DH and WH treated samples, while a decrease (not significant) on MW treated brown rice were observed. General acceptability scores of raw and cooked samples ranged from 8.5 to 10.7 and 7.8 to 9.9, respectively. Raw brown rice (untreated and treated) was perceived no off-odor, moderately intense in color, translucent, slightly glossy, and satisfactory general acceptability (liked moderately). For cooked brown rice sample (untreated and treated), assessment by the panelists revealed slight (faint) aroma; no off-odor;

moderate glossy, cohesive, and tender; slightly tasty and no off-taste and; very satisfactory general acceptability (liked).

Table 3 Sensory attribute of raw brown rice samples at different treatments

Sample	Raw Sensory Attribute, Mean Score					
	Aroma[1]	Off-Odor[2]	Color (brown)[3]	Gloss[4]	Translucency[5]	General Acceptability[6]
Control	1.2[a]	0.0[a]	6.5[a]	3.6[abc]	12.8[a]	9.3[a]
DH 10 min	1.7[a]	0.0[a]	4.9[a]	4.9[abc]	12.3[a]	9.0[a]
DH 15 min	1.8[a]	0.0[a]	4.8[a]	5.2[bc]	12.8[a]	10.0[a]
DH 25 min	1.9[a]	0.0[a]	5.8[a]	4.9[abc]	12.4[a]	8.5[a]
MW 30sec	1.0[a]	0.3[a]	5.8[a]	3.8[abc]	12.4[a]	10.0[a]
MW 60 sec	1.0[a]	0.5[a]	5.2[a]	2.8[a]	12.0[a]	9.2[a]
MW 90 sec	1.3[a]	0.2[a]	6.2[a]	2.9[ab]	12.2[a]	9.8[a]
WH 30sec	1.3[a]	0.5[a]	6.4[a]	3.9[abc]	12.9[a]	10.7[a]
WH 60sec	2.8[a]	0.0[a]	5.4[a]	5.6[c]	11.9[a]	9.6[a]
WH 90 sec	2.5[a]	0.0[a]	4.7[a]	5.0[abc]	12.1[a]	8.8[a]

Means within a column with same letters are not significantly different (α=0.05)
[1] 0= none; 3.75= slightly aromatic; 7.5= moderately aromatic; 11.25= aromatic; 15= very aromatic
[2] 0= none; 3.75= slightly perceptible; 7.5= moderately perceptible; 11.25= perceptible; 15= very perceptible
[3] 0= weak; 3.75= slightly intense; 7.5= moderately intense; 11.25= intense; 15= very intense
[4] 0= dull; 3.75= slightly glossy; 7.5= moderately glossy; 11.25= glossy; 15= very glossy
[5] 0= opaque; 3.75= slightly chalky; 7.5= chalky; 11.25= white belly; 15= translucent
[6] 0= dislike extremely; 3.75= like slightly; 7.5= like moderately; 11.25= like; 15= like extremely

Table 4 Sensory attribute of cooked brown rice samples at different treatments

Sample	Cooked Sensory Attribute, Mean Score							
	Aroma[1]	Off-odor[2]	Gloss[3]	Cohesiveness[4]	Tenderness[5]	Taste[6]	Off-taste[7]	General Acceptability[8]
Control	4.7[a]	0.0[a]	7.0[ab]	6.8[a]	5.7[a]	3.7[a]	0.3[a]	9.8[a]
DH 10 min	3.6[a]	0.2[a]	6.8[ab]	6.5[a]	6.1[a]	2.7[a]	0.0[a]	8.0[a]
DH 15 min	3.2[a]	0.0[a]	6.6[ab]	6.8[a]	5.5[a]	2.9[a]	0.0[a]	8.9[a]
DH 25 min	3.6[a]	0.0[a]	7.8[ab]	9.0[a]	7.7[a]	3.0[a]	0.0[a]	9.5[a]
MW 30sec	4.5[a]	0.0[a]	8.5[ab]	8.1[a]	7.9[a]	3.6[a]	0.2[a]	9.9[a]
MW 60 sec	3.4[a]	0.0[a]	6.1[a]	8.4[a]	6.1[a]	3.4[a]	0.2[a]	8.2[a]
MW 90 sec	3.5[a]	0.0[a]	7.5[ab]	7.4[a]	4.9[a]	3.3[a]	0.0[a]	8.0[a]
WH 30sec	3.6[a]	0.0[a]	7.5[ab]	7.8[a]	5.0[a]	2.9[a]	0.6[a]	7.8[a]
WH 60sec	4.0[a]	0.0[a]	8.3[ab]	7.1[a]	6.6[a]	2.9[a]	0.0[a]	8.3[a]
WH 90 sec	5.6[a]	0.0[a]	9.3[b]	8.1[a]	6.4[a]	3.1[a]	0.3[a]	9.1[a]

Means within a row with same letters are not significantly different (α=0.05)
[1] 0= none; 3.75= slightly aromatic; 7.5= moderately aromatic; 11.25= aromatic; 15= very aromatic
[2] 0= none; 3.75= slightly perceptible; 7.5= moderately perceptible; 11.25= perceptible; 15= very perceptible
[3] 0= dull; 3.75= slightly glossy; 7.5= moderately glossy; 11.25= glossy; 15= very glossy
[4] 0= separated; 3.75= slightly cohesive; 7.5= moderately cohesive; 11.25= cohesive; 15= very cohesive
[5] 0= hard; 3.75= slightly tender; 7.5= moderately tender; 11.25= tender; 15= very tender
[6] 0= bland; 3.75= slightly tasty; 7.5= moderately tasty; 11.25= tasty; 15= very tasty
[7] 0= none; 3.75= slightly perceptible; 7.5= moderately perceptible; 11.25= perceptible; 15= very perceptible
[8] 0= dislike extremely; 3.75= like slightly; 7.5= like moderately; 11.25= like; 15= like extremely

Changes on physicochemical properties and sensory attributes during storage

Changes in free fatty acids content

Changes in free fatty acids content of the samples for up to six months of storage is presented in Figure 2. A steady significant increase in free fatty acids level of each treated and untreated brown rice samples was noted during storage, but increase was significantly lower in treated brown rice. **Ory *et al.*, (1980)** reported that free fatty acids level of stored brown rice steadily increased throughout the storage period. A considerable abrupt increase in free fatty acids level occurring from two to four months was observed similar with the findings by **Ramenzanzadeh *et al.*, (1999)** on rice bran. Throughout the storage period, all treated brown rice samples have significantly lower free fatty acid content compared with the untreated brown rice consistent with the lipase activity from the initial analysis (month 0). Lower free fatty acids generation of the samples could be attributed to the reduction of lipase activity caused by different treatments. Effectiveness of lipase inactivation through dry and wet (steam) heat and microwave treatment to subsequently stabilize brown rice against lipolytic rancidity was evident. Hence, this could indicate improvement in storability or shelf life of brown rice samples through these treatments. Treatments with longer exposure times, namely DH 25min, MW 90sec, and WH 90sec were noted to have significantly lowest free fatty acids level after six months of storage.

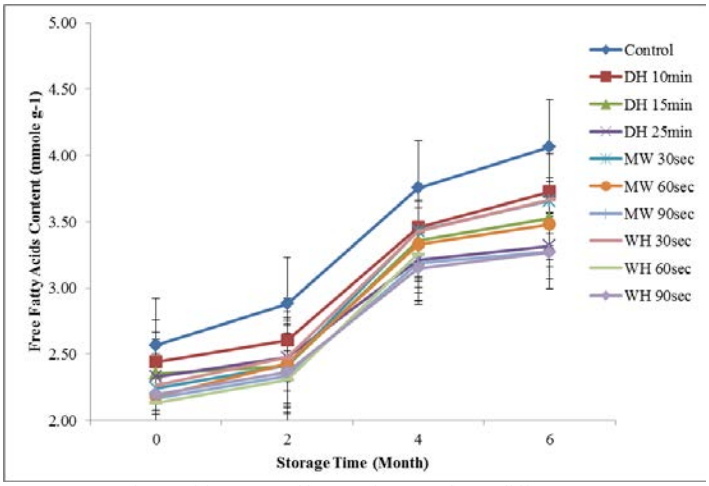

Figure 1 Free fatty acid content of brown rice samples at different treatments during storage

Changes in total phenolics content and antioxidant activity

Changes in total phenolics content was monitored only on samples with longer exposure times namely DH 25min, MW 90sec and WH 90sec since findings on the first month (Month 0) revealed no significant effect of different treatments on the total phenolics content of the brown rice sample. Statistical analysis showed a significant increase in total phenolics content on both treated and untreated brown rice sample from 0 to 2 months, and started to plateau after 2 months of storage (Table 5). Total phenolics content of the samples during 0 month ranged from 2.86 to 3.39 GAE g^{-1}; while starting from 2 months up to 6 months of storage, total phenolics content ranged from 4.14 to 5.52 GAE g^{-1}.This trend on the increase in total phenolics content during storage is similar to the findings of **Tsugita *et al.*, (1983)** as cited by **Juliano, (1985)** in rice grain. **Tsugita *et al.*, (1983)** proposed that bound phenolic acids are released by enzymatic and non-enzymatic reaction during storage to form free phenolic acids.

Table 5 Total phenolics content of brown rice samples at different treatments during storage

	Total Phenolics Content (Gallic Acid Equivalent g^{-1})*			
Treatment	**Month 0**	**Month 2**	**Month 4**	**Month 6**
Control	$2.86^{a\,(a)}$	$4.14^{a\,(b)}$	$4.20^{a\,(b)}$	$5.52^{a\,(c)}$
DH 25 min	$3.08^{a\,(a)}$	$4.23^{a\,(b)}$	$4.70^{a\,(bc)}$	$5.15^{a\,(c)}$
MW 90 sec	$2.90^{a\,(a)}$	$4.31^{a\,(b)}$	$4.60^{a\,(b)}$	$5.11^{a\,(b)}$
WH 90 sec	$3.39^{a\,(a)}$	$5.72^{b\,(b)}$	$4.58^{a\,(ab)}$	$5.49^{a\,(b)}$

Means within a column with same inscriptions are not significantly different (α=0.05)
*Letters inside parenthesis indicate comparison within a row

Similar with the observation on total phenolics content, antioxidant activity of the brown rice samples also increase during storage (Table 6). The increase in DHHP radical scavenging activity may be attributed to the observed increase in total phenolics content of the samples, hence phenolics acids are known to exhibit antioxidative property. A consistent significant increase in antioxidant activity was noted on untreated and DH treated brown rice samples, except that of DH 25min. During the initial months (month 0 to month 2), antioxidant activity of the untreated and DH treated samples (40.45 to 53.47%) were relatively lower than that of MW and WH treated brown rice (49.62 to 62.12%). After 2 months of storage, antioxidant activity of untreated and DH treated brown rice abruptly increase having its peak at month 6. Treatments with shorter exposure time (DH 10min, DH 15min and WH 30sec) had comparable antioxidant activity with the untreated brown rice, except that of MW treated brown rice after 6 months of storage. For MW and WH treated samples, a significant increase in antioxidant activity was observed from 0 to 2 months of storage and started to plateau up to 4 months, except that of MW 30sec; but decrease at 6 months of storage, except that of WH 60sec.

Table 6 Antioxidant activity of brown rice samples at different treatments during storage

	Antioxidant Activity (%DPPH Inhibition)*			
Treatment	**Month 0**	**Month 2**	**Month 4**	**Month 6**
Control	$40.45^{a\,(a)}$	$53.47^{b\,(b)}$	$70.19^{cd\,(c)}$	$76.22^{d\,(d)}$
DH 10 min	$41.66^{a\,(a)}$	$52.12^{ab\,(b)}$	$66.89^{abc\,(c)}$	$73.15^{cd\,(d)}$
DH 15 min	$40.55^{a\,(a)}$	$49.46^{a\,(b)}$	$63.58^{a\,(c)}$	$72.15^{bcd\,(d)}$
DH 25 min	$40.77^{a\,(a)}$	$52.30^{ab\,(b)}$	$64.72^{ab\,(c)}$	$63.49^{a\,(c)}$
MW 30sec	$54.45^{c\,(a)}$	$61.49^{c\,(b)}$	$69.71^{cd\,(c)}$	$67.92^{abc\,(c)}$
MW 60 sec	$54.65^{c\,(a)}$	$62.12^{c\,(ab)}$	$67.31a^{bc\,(b)}$	$64.43^{ab\,(b)}$
MW 90 sec	$51.67^{bc\,(a)}$	$61.68^{c\,(b)}$	$65.07^{ab\,(b)}$	$63.19^{a\,(b)}$
WH 30sec	$51.21^{bc\,(a)}$	$55.15^{b\,(a)}$	$65.61^{ab\,(b)}$	$69.51^{abcd\,(b)}$
WH 60sec	$51.63^{bc\,(a)}$	$61.96^{c\,(b)}$	$71.30^{d\,(c)}$	-
WH 90 sec	$49.62^{b\,(a)}$	$61.87^{c\,(b)}$	$69.23^{cd\,(c)}$	$65.85^{abc\,(bc)}$

*Means within a column with same inscriptions are not significantly different (α=0.05). Letters inside parenthesis indicate comparison within a row.
Note: -, no data (sample deteriorated)

Changes in raw and cooked sensory attributes

Raw and cooked sensory attributes were monitored to assess and compare the quality changes and storability of treated and untreated brown rice samples during storage in addition to monitoring of their physicochemical changes. Sensory evaluation of raw brown rice samples showed a significant decrease in mean scores on aroma and glossiness; while a significant increase in off-odor in both untreated and treated raw brown rice sample during storage (Table 7). Progressive decrease in aroma scores was consistently comparable for each sample throughout the storage period; similar with glossiness scores, except that of DH 25min, MW 60sec, MW 90sec and WH 30 sec. For raw off-odor attribute, a significant increase was perceived on the samples during the second month with the untreated brown rice as the most off-odored sample; but not on DH 10min, DH 25min, MW 60sec and MW 90sec. By the fourth month, a significantly strong and moderate perceptible off-odor were noticed on untreated sample (10.2 score) and DH treated brown rice (5.8 to 5.9 score), respectively, except that of DH 25min; signifying deterioration. These deteriorated samples were then not subjected to the succeeding session of sensory evaluation. MW and WH treated brown rice, including DH 25min treated sample, had significantly lower off-odor scores compared with untreated sample indicative of improvement in storage stability. General acceptability scores of the samples significantly decrease beginning second month of storage, except that of treatments with longer exposure namely DH 25min, MW 90sec and WH 90sec, including MW 60sec. By the fourth month, only treated brown rice sample with longer exposure times (DH 25min, MW 90sec and WH 90sec), including MW 60sec, remained acceptable (liked moderately) even up to eight months of storage. This demonstrates that these treated brown rice had superior storability over the untreated sample.

Table 7 Sensory attribute of raw brown rice samples at different treatments during storage

Sensory Attributes	**Sample, Mean Score**									
	Control	**DH 10min**	**DH 15min**	**DH 25min**	**MW 30sec**	**MW 60sec**	**MW 90sec**	**WH 30sec**	**WH 60sec**	**WH 90sec**
Aroma[1]										
Month 0	1.2^{a}	1.7^{b}	1.8^{b}	1.9^{b}	1.0^{a}	1.0^{a}	1.3^{b}	1.3^{b}	2.8^{b}	2.5^{b}
Month 2	0.5^{a}	0.3^{a}	0.3^{a}	0.6^{a}	0.7^{a}	0.5^{a}	0.3^{ab}	0.3^{a}	0.3^{a}	0.5^{a}
Month 4	0.1^{a}	0.6^{ab}	0.1^{a}	0.3^{a}	0.1^{a}	0.6^{a}	0.4^{ab}	0.5^{ab}	0.7^{a}	0.4^{a}
Month 8	-	-	-	0.0^{a}	-	0.0^{a}	0.1^{a}	-	-	0.1^{a}
Off-odor[2]										

Month 0	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.3[a]	0.5[a]	0.2[a]	0.5[a]	0.0[b]	0.0[a]
Month 2	3.6[b]	1.2[a]	2.0[b]	1.5[ab]	2.6[b]	1.6[a]	1.2[a]	3.0[b]	2.6[a]	2.1[b]
Month 4	10.2[c]	5.9[b]	5.8[c]	3.1[bc]	1.8[ab]	2.1[a]	0.8[a]	1.1[a]	1.8[a]	0.8[ab]
Month 8	-	-	-	4.2[c]	-	4.8[b]	3.8[b]	-	-	4.7[c]
Color[3]										
Month 0	6.5[a]	4.9[a]	4.8[a]	5.8[a]	5.8[a]	5.2[a]	6.2[a]	6.4[ab]	5.4[a]	4.7[a]
Month 2	6.0[a]	5.6[a]	5.4[ab]	6.2[ab]	6.4[a]	4.9[a]	5.9[a]	6.8[b]	6.3[a]	6.3[a]
Month 4	5.4[a]	6.2[a]	6.9[b]	8.5[b]	6.4[a]	5.8[a]	4.6[a]	4.5[a]	5.3[a]	6.4[a]
Month 8	-	-	-	6.1[a]	-	4.8[a]	5.6[a]	-	-	5.3[a]
Glossiness[4]										
Month 0	3.6[b]	4.9[b]	5.2[b]	4.9[b]	3.8[b]	2.8[a]	2.9[a]	3.9[b]	5.6[b]	5.0[c]
Month 2	1.5[a]	2.4[a]	1.4[a]	3.4[ab]	1.8[ab]	3.8[a]	3.2[a]	3.0[ab]	2.2[a]	3.2[b]
Month 4	0.2[a]	1.3[a]	1.3[a]	2.8[a]	0.9[a]	2.8[a]	3.2[a]	2.2[a]	1.5[a]	1.6[ab]
Month 8	-	-	-	2.0[a]	-	1.8[a]	3.1[a]	-	-	1.5[a]
Translucency[5]										
Month 0	12.8[a]	12.3[a]	12.8[a]	12.4[a]	12.4[a]	12.0[a]	12.2[a]	12.9[a]	11.9[a]	12.1[a]
Month 2	11.9[a]	12.7[a]	12.3[a]	12.7[a]	12.4[a]	12.8[a]	12.2[a]	12.2[ab]	11.9[a]	12.0[a]
Month 4	11.4[a]	12.1[a]	11.6[a]	11.9[a]	12.3[a]	12.3[a]	11.6[a]	10.4[a]	11.0[a]	10.9[ab]
Month 8	-	-	-	10.3[a]	-	10.0[a]	10.9[a]	-	-	8.9[b]
General Acceptability[6]										
Month 0	9.3[b]	9.0[b]	10.0[b]	8.5[b]	10.0[b]	9.2[a]	9.8[a]	10.7[b]	9.6[a]	8.8[a]
Month 2	5.3[a]	5.8[a]	5.4[a]	6.1[ab]	5.6[a]	7.0[ab]	7.1[a]	6.0[a]	6.2[b]	6.7[ab]
Month 4	2.4[a]	4.0[a]	3.8[a]	5.9[ab]	4.4[a]	6.9[ab]	7.1[a]	4.8[a]	3.2[c]	6.2[ab]
Month 8	-	-	-	5.5[a]	-	6.7[a]	7.0[a]	-	-	5.4[a]

Means within a row with same letters are not significantly different (α=0.05)
[1] 0= none; 3.75= slightly aromatic; 7.5= moderately aromatic; 11.25= aromatic; 15= very aromatic
[2] 0= none; 3.75= slightly perceptible; 7.5= moderately perceptible; 11.25= perceptible; 15= very perceptible
[3] 0= weak; 3.75= slightly intense; 7.5= moderately intense; 11.25= intense; 15= very intense
[4] 0= dull; 3.75= slightly glossy; 7.5= moderately glossy; 11.25= glossy; 15= very glossy
[5] 0= opaque; 3.75= slightly chalky; 7.5= chalky; 11.25= white belly; 15= translucent
[6] 0= dislike extremely; 3.75= like slightly; 7.5= like moderately; 11.25= like; 15= like extremely
Note: -, no data (sample deteriorated)

On the other hand, sensory evaluation of cooked brown rice samples revealed a significant decrease in aroma during storage and cohesiveness at 2 months; while a significant increase in off-odor and off-taste both at 8 months, except that of untreated sample (Table 8). Previous researches attributed off-flavor (off-taste) and off-odor development to high levels of free fatty acids or phenolics compounds (**Barber, 1972; Tsugita *et al.*, 1983; Molteberg *et al.*, 1996; Zhou *et al.*, 1999; Heinio *et al.*, 2002**); hence sensory evaluation results were consistent with the chemical analysis. No significant change in cooked brown rice attribute score of each sample on glossiness, cohesiveness, tenderness, and taste was observed during storage. This observation is different from the reports of several studies that texture of aged (stored) cooked milled becomes harder (**Moritaka *et al.*, 1971; Villareal *et al.*, 1976; Indudhara *et al.*, 1978; Chrastil, 1990**), where most of the researchers utilize a machine to objectively measure cooked rice hardness. However, **Juliano *et al.*, (1969)** observed similar taste panel scores for tenderness, cohesiveness and gloss for stored (6 months) milled rice, suggesting no appreciably change in the texture of the resulting cooked rice. General acceptability score of each cooked brown rice sample significantly decrease during storage, except that of MW 90sec. By the second month, a significant downshift in general acceptability scores was noted on untreated sample, DH 15 min and MW 30sec; and continued to significantly decrease up to 4 months, except that of DH 25min, MW 90sec, WH 30sec and WH 60sec. After 4 months of storage, only treated brown rice sample with longer exposure times (DH 25min, MW 90sec and WH 90sec), including MW 60sec, lasted and remained acceptable (liked moderately) even up to eight months of storage (Figure 2).

Table 8 Sensory attribute of cooked brown rice samples at different treatments during storage

Attributes	Sample, Mean Score									
	Control	DH 10min	DH 15min	DH 25min	MW 30sec	MW 60sec	MW 90sec	WH 30sec	WH 60sec	WH 90sec
Aroma[1]										
Month 0	4.7[a]	3.6[a]	3.2[a]	3.6[a]	4.5[a]	3.4[b]	3.5[a]	3.6[ab]	4.0[b]	5.6[b]
Month 2	4.9[a]	4.7[a]	5.1[b]	3.7[a]	3.3[a]	2.4[ab]	3.6[a]	4.9[b]	2.9[ab]	2.6[a]
Month 4	2.1[b]	3.4[a]	1.7[c]	2.5[ab]	3.0[a]	2.1[ab]	1.3[b]	2.2[a]	1.5[a]	2.4[a]
Month 8	-	-	-	1.2[b]	-	1.2[a]	1.7[ab]	-	-	1.6[a]
Off-odor[2]										
Month 0	0.0[a]	0.2[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]
Month 2	0.0[a]	0.2[a]	0.0[a]	0.2[a]	0.0[a]	0.1[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]
Month 4	0.3[a]	1.0[a]	0.4[a]	0.7[a]	0.2[a]	0.8[ab]	1.3[b]	0.3[a]	0.3[a]	0.3[a]
Month 8	-	-	-	2.4[b]	-	2.2[b]	1.1[ab]	-	-	1.7[b]
Gloss[3]										
Month 0	7.0[b]	6.8[a]	6.6[a]	7.8[b]	8.5[b]	6.1[a]	7.5[b]	7.5[b]	8.3[a]	9.3[b]
Month 2	5.4[ab]	6.2[a]	5.3[a]	7.8[b]	6.9[ab]	6.0[a]	5.3[ab]	4.8[a]	7.0[a]	6.3[a]

Month 4	4.1[a]	5.2[a]	5.0[a]	5.2[a]	4.6[a]	4.1[a]	4.8[a]	5.7[ab]	4.2[b]	4.7[a]
Month 8	-	-	-	5.1[a]	-	5.0[a]	5.7[ab]	-	-	4.2[a]
Cohesiveness[4]										
Month 0	6.8[b]	6.5[a]	6.8[b]	9.0[b]	8.1[b]	8.4[b]	7.4[b]	7.8[b]	7.1[b]	8.1[b]
Month 2	3.9[a]	5.0[a]	4.4[a]	8.1[b]	5.8[a]	5.1[a]	4.5[a]	3.8[a]	5.6[ab]	5.5[a]
Month 4	4.0[a]	4.9[a]	4.5[a]	4.5[a]	5.1[a]	5.5[a]	5.3[ab]	5.2[a]	4.4[a]	4.4[a]
Month 8	-	-	-	5.5[a]	-	4.2[a]	3.4[a]	-	-	5.0[a]
Tenderness[5]										
Month 0	5.7[a]	6.1[a]	5.5[a]	7.7[a]	7.9[a]	6.1[ab]	4.9[a]	5.0[a]	6.6[a]	6.4[ab]
Month 2	6.9[a]	7.1[a]	6.4[a]	6.2[a]	7.6[a]	5.2[a]	5.3[a]	7.8[a]	5.3[a]	5.9[a]
Month 4	7.4[a]	5.8[a]	7.4[a]	7.8[a]	7.9[a]	6.8[ab]	6.5[a]	7.1[a]	7.0[a]	8.5[ab]
Month 8	-	-	-	8.4[a]	-	8.4[b]	7.5[a]	-	-	8.8[b]
Taste[6]										
Month 0	3.7[b]	2.7[a]	2.9[a]	3.0[a]	3.6[b]	3.4[a]	3.3[b]	2.9[a]	2.9[a]	3.1[a]
Month 2	1.9[a]	1.9[b]	2.8[a]	3.0[a]	2.4[ab]	2.8[a]	3.0[ab]	2.8[a]	2.1[a]	2.1[a]
Month 4	1.5[a]	3.2[ab]	3.2[a]	2.9[a]	1.2[a]	0.5[a]	1.0[a]	3.1[a]	2.1[a]	2.5[a]
Month 8	-	-	-	1.4[a]	-	2.0[ab]	2.1[ab]	-	-	1.8[a]
Off-taste[7]										
Month 0	0.3[a]	0.0[a]	0.0[a]	0.0[a]	0.2[a]	0.2[a]	0.0[a]	0.6[a]	0.0[a]	0.3[a]
Month 2	0.3[ab]	0.9[b]	0.2[a]	0.5[a]	0.4[a]	0.4[a]	0.0[a]	0.4[a]	0.1[a]	0.8[a]
Month 4	1.7[b]	0.2[ab]	0.2[a]	0.2[a]	0.0[a]	0.6[a]	0.3[a]	0.1[a]	0.4[a]	0.7[a]
Month 8	-	-	-	2.3[b]	-	1.7[b]	1.8[b]	-	-	1.3[a]
General Acceptability[8]										
Month 0	9.8[b]	8.0[b]	8.9[b]	9.5[b]	9.9[b]	8.2[b]	8.0[a]	7.8[a]	8.3[a]	9.1[b]
Month 2	5.0[a]	6.0[ab]	6.2[a]	7.4[ab]	5.7[a]	5.7[ab]	6.1[a]	7.0[a]	7.4[a]	7.4[ab]
Month 4	4.6[a]	5.5[a]	4.9[a]	7.5[ab]	5.6[a]	4.7[a]	6.4[a]	6.1[a]	6.7[a]	6.3[a]
Month 8	-	-	-	5.4[a]	-	5.7[a]	5.7[a]	-	-	6.7[a]

Means within a row with same letters are not significantly different (α=0.05)
[1] 0= none; 3.75= slightly aromatic; 7.5= moderately aromatic; 11.25= aromatic; 15= very aromatic
[2] 0= none; 3.75= slightly perceptible; 7.5= moderately perceptible; 11.25= perceptible; 15= very perceptible
[3] 0= dull; 3.75= slightly glossy; 7.5= moderately glossy; 11.25= glossy; 15= very glossy
[4] 0= separated; 3.75= slightly cohesive; 7.5= moderately cohesive; 11.25= cohesive; 15= very cohesive
[5] 0= hard; 3.75= slightly tender; 7.5= moderately tender; 11.25= tender; 15= very tender
[6] 0= bland; 3.75= slightly tasty; 7.5= moderately tasty; 11.25= tasty; 15= very tasty
[7] 0= none; 3.75= slightly perceptible; 7.5= moderately perceptible; 11.25= perceptible; 15= very perceptible
[8] 0= dislike extremely; 3.75= like slightly; 7.5= like moderately; 11.25= like; 15= like extremely
Note: -, no data (sample deteriorated)

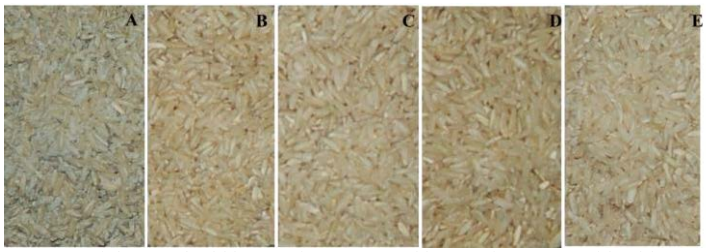

Figure 2 Eight-month old brown rice. Untreated brown rice (A) considerably deteriorated after 2 months of storage compared to 8-month old brown rice exposed to DH for 25min (B), MW for 90sec (C), WH for 90sec (D) and MW for 60sec (E).

CONCLUSION

Findings from this study established heat and microwave treatment conditions that could effectively inactivate lipase enzyme to consequently stabilize brown rice against rancidity, without affecting its antioxidant activity for up to 8 months of storage. Improvement of brown rice storability through these optimum treatment conditions was demonstrated through monitoring the changes in chemical properties (free fatty acids, phenolics content and antioxidant activity), and raw and cooked sensory attributes during storage. Application of these technologies to pilot-scale and subsequently to commercial-scale is being conducted.

Acknowledgments: The authors would like to thank Prof. Dr. Adelina P. Valdez and Prof. Marilene C. Hipolito of the Department of Chemistry, College of Arts and Sciences, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines for their comments and suggestions to improve this manuscript.

REFERENCES

AMERICAN ASSOCIATION OF CEREAL CHEMISTS. 2000. AACC Method 44-15A. Moisture-Air-Oven Methods. Approved Methods of the American Association of Cereal Chemists, St Paul, MN, USA,Vol. 2, 4 p.

ADOM, K.K., LIU, R.H. 2002. Antioxidant activities of grains. *J. Agric. Food Chem,* 50, 6182-6187. http://dx.doi.org/10.1021/jf0205099

AFOAKWAH, A.N., OWUSU, J., ADOMAKO, C., TEYE, E. 2012. Microwave Assisted Extraction (MAE) of antioxidant constituents in plant materials. *Global Journal of Bio-Science and Biotechnology*, 1 (2), 132-140.

BARBER, S. 1972. Milled rice and changes during aging. *In*: Rice Chemistry and Technology, First Ed..; Houston, D.F., Ed., American Association of Cereal Chemistry, Eagan, MN, USA, 215-263.

CHAMPANGE, E.T., HRON SR, R.J., ABRAHAM, G. 1991. Stabilizing brown rice products by acqueous ethanol ethanol extraction. *Cereal Chemistry*, 67, 267-271.

CHAMPAGNE, E.T., HRON SR, R.J. 1992. Stabilizing brown rice to lipolytic hydrolysis by ethanol vapors. *Cereal Chemistry*, 69 (2),152-156.

CHAMPAGNE, E.T., HRON SR, R.J. 1993. Utilizing ethanol containing an antioxidant or chelator to produce stable brown rice products. *Cereal Chemistry*, 70, 562-567.

CHAMPAGNE, E.T., GRIMME, C.C. 1995. Stabilization of brown rice products using ethanol vapors as an antioxidant delivery system. *Cereal Chemistry*, 72 (3), 255-258.

CHRASTIL, J. 1990. Protein-starch interaction in rice grains. Influence of storage on oryzenin and starch. *Journal of Agricultural and Food Chemistry*, 38, 1804-1809. http://dx.doi.org/10.1021/jf00099a005

CORPUZ, H.M., ABLAZA, M.J.C., ROMERO, M.V. 2010. Excellent brown rice from suitable varieties. *The Philippine Journal of Crop Science*, ISSN 0115-463X, Vol. 35 (S1). Available at http://agris.fao.org/agris-search/search.do?recordID=PH2010000575

CUENDET, L.S., LARSON, E., NORRIS, C.G., GEDDES, W.F. 1954. The influence of moisture content and other factors on the stability of wheat flours at 37.5°C. *Cereal Chemistry*, 31, 362-389.

CUYNO, R.V. 2003. The national campaign to combat hidden hunger though brown rice. (Paper presented during Consultative Meeting on Nutritional Aspect of Brown Rice, Sept. 2003, at Food and Nutritional Research Institute, Manila, Philippines).

DAMODARAN, S. 1996. Amino acids, peptides, and proteins. *In*: Fennema O.R. ed., *Food Chemistry* : New York, Marcel Dekker, 321-429.

DAR, B.N., SHARMA, S. 2011. Total phenolic content of cereal brans using conventional and microwave assisted extraction. *American Journal of Food Technology*, 6 (12), 1045-1053. http://dx.doi.org/10.3923/ajft.2011.1045.1053

DYKES, L., ROONEY, L.W. 2007. Phenolic compounds in cereal grains and their health benefits. *Cereal Foods World*, 52 (3),105-111. http://dx.doi.org/10.1094/cfw-52-3-0105

HARIDAS RAO, P., KUMAR, G.V., RANGA RAO, G.C.P., SHURPALEKAR, S.R. 1980. Studies on the stabilization of wheat germ. Lebensm. Wiss. Technology, 13, 302-307

HEINIÖ, R.L., LEHTINEN, P., OKSMAN-CALDENTEY, K.M., POUTANEN K. 2002. Differences between sensory profiles and development of rancidity during long-term storage of native and processed oat. *Cereal Chemistry*, 79 (3), 367-375. http://dx.doi.org/10.1094/cchem.2002.79.3.367

INDUDHARA SWAMY Y.M., SOWBHAGYA C.M., BHATTACHARYA, K.R. 1978. Changes in physicochemical properties of rice with ageing. *J. Sci. Food Agric*., 29, 627-639. http://dx.doi.org/10.1002/jsfa.2740290709

IQBAL, S., BHANGE, M.I., ANWAR, F. 2005. Antioxidant properties and components of some commercially available varieties of rice bran in pakistan. *Food Chemistry*, 93, 265-272. http://dx.doi.org/10.1016/j.foodchem.2004.09.024

JULIANO, B.O. 1985. Rice Chemistry and Technology (2nd Ed.). American Association of Cereal Chemists, St Paul, MN, USA: American Association of Cereal Chemists, Inc. ISBN 0-913250-41-4. http://dx.doi.org/10.1016/0308-8146(87)90086-0

JULIANO, B.O., NAZARENO, M.B., RAMOS, N.B. 1969. Properties of waxy and isogenic nonwaxy rices differing in starch gelatinization temperature. *J. Agric. Foo Chem*., 17, 1364-1369. http://dx.doi.org/10.1021/jf60166a062

KESTER, E.B. 1951. Stabilization of brown rice, US patent 2,538,007

KRUGER, J. E., REED, G. 1988. Enzymes and color. *In* Pomeranz Y. (ed.) *Wheat Chemistry and Technology*, 3rd Ed., Vol. 1. American
Association of Cereal Chemists International, St. Paul, MN, USA, 441-500.

KWON, D.Y., RHEE, J.S. 1986. A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. *Journal of American Oil Chemist Society*, 63(1), 89-92. http://dx.doi.org/10.1007/bf02676129

LEHTINENT, P., LAAKSO, S. 2004. Role of lipid reactions in quality of oat products. *Journal of Agricultural and Food Science*, 13, 88-99. http://dx.doi.org/10.2137/1239099041838085

LEHTINENT, P., KIILIAINENT, K., LEHTOMAKI, I., LAAKSO, S. 2003. Effect of heat treatment on lipid stability in processed oats. *Journal of Cereal Science*, 37, 215-221. http://dx.doi.org/10.1006/jcrs.2002.0496

MCCABE, D. 1976. Process for preparing a quick-cooking brown rice and the resulting product, US patent 3,959,515.

MOLTEBERG, E.L., MAGNUS, E.M., BJØRGE, J.M., NILSSON, A. 1996. Sensory and chemical studies of lipid oxidation in raw and heat-treated oat flours. *Cereal Chemistry*, 73, 579-587.

MORITAKA, S., SAWADA, K., YASUMATSU, K. 1971. Studies on Cereals. VI. Effect of fat extraction on rice quality and storage deterioration of defatted rice. *Eiyo To Shokuryo*, 24, 457-460.

ORY, R.L., DELUCCA, A.J., ST. ANGELO, A.J., DUPUY, H.P. 1980. Storage quality of brown rice as affected by packaging with and without carbon dioxide. *Journal of Food Protocols*, 43, 929.

OUFNAC, D.S. 2006. Determination of antioxidant capacity in corn germ, wheat germ and wheat bran using solvent and microwave-assisted solvent extraction. Louisiana State University (MS Thesis). Available at http://etd.lsu.edu/docs/available/etd-11152006-162056/unrestricted/davidsoufnacthesis.pdf

RAMEZANZADEH, F.M., RAO, R.M., WINDHAUSER, M., PRINYAWIWATKUL, W., TULLEY, R., MARSHALL, W.E. 1999. Prevention of hydrolytic rancidity in rice bran during storage. *Journal of Agricultural and Food Chemistry*, 47 (8), 3050-3052. http://dx.doi.org/10.1021/jf981335r

ROSE, D. J., LOGDEN, V.M., DUNN, L., PIKE, O.A. 2008. Enhanced lipid stability in whole wheat flour by lipase inactivation and antioxidant retention. *Cereal Chemistry*, 85(2), 218-223. http://dx.doi.org/10.1094/cchem-85-2-0218

ROSE, D. J., PIKE, O.A. 2006. A simple method to measure lipase activity in wheat and wheat bran as an estimation of storage quality. *Journal of American Oil Chemist Society*, 83(5), 415-419. http://dx.doi.org/10.1007/s11746-006-1220-0

ROTHE, M. 1967. Hitzeinaktivierung von Ezymen im Zusammenhang mit der Stabilisierung von Reiskleie. *In*: Proc. Meeting, Int. Problems on Modern Cereal Processing and Chemistry 3rd. Part I. Milling. Rice Processing. Inst., Getreideverarbeitung, Bergholz-Rehbrucke, G.D.R., 146-151.

SANTROPRETE, G. 1980. Preservation of slightly milled rice. Riso 29:61.

SHARP, R.N., TIMME, L.K. 1986. Effects of storage time, storage temperature, and packaging method on shelf life of brown rice. *Cereal Chemistry*, 63 (3), 247-251

TSUGITA, T., OHTA, T., KATO, H. 1983. Cooking flavor and texture of rice stored under different conditions. *Agric. Biol. Chem*., 47 (3), 543-549. http://dx.doi.org/10.1080/00021369.1983.10865684

VAN ATTA, G.R., KESTER, E.B., OLCOTT, H.S. 1952. Method of stabilizing rice products, US patent 2,585,978.

VETRIMANI, R., HARIDAS RAO, P. 1990. Changes in the functional characteristics of wheat during soaking and subsequent germination. *Journal of Food Science and Technology*, 27, 332-335.

VILLAREAL, R.M., RESURRECCION, A.P., SUZUKI, L.B., JULIANO, B.O. 1976. Changes in physicochemical properties of rice during storage. *Staerke,* 38, 88-94. http://dx.doi.org/10.1002/star.19760280304

ZHOU, M., ROBARDS, K., GLENNIE-HOLMES, M., HELLIWELL, S. 1999. Analysis of volatile compounds and their contribution to flavor in cereals. *Journal of Agricultural and Food Chemistry*, 10, 3941-3953. http://dx.doi.org/10.1021/jf990428l