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Editor-in-Chief:
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Università di Milano, Milano, Italy

HEALTH QUALITY AND NUTRITIONAL VALUE OF RYE BREAD PRODUCED ON A SMALL AND LARGE SCALE IN POLAND

M. RADZYMINSKA*, B. GARBOWSKA and D. JAKUBOWSKA
Chair of Commodity Science and Food Research, University of Warmia and Mazury in Olsztyn
*Corresponding author: mradz@uwm.edu.pl

ABSTRACT

This study is a small part of a project aimed at performing comprehensive evaluation of the quality of local raw materials and products in the region of Warmia and Mazury in Poland. This study evaluates selected determinants of the health quality and nutritional value of rye bread in relation to origin (small and large scale production). The analytical procedure included determination of the content of acrylamide, organochlorine pesticides and polychlorinated biphenyls, n-methylcarbamates, ochratoxin A, acidity, moisture, protein, glucose, fat, salt, Cu, Mn, Fe, Zn, Ca, and Mg. The quality of bread produced on a small scale, compared to that produced in large bakeries, differed only in terms of some determinants. Rye bread from local producers contained statistically significantly less ($P \leq 0.05$) acrylamide and statistically significantly more copper, manganese and zinc.

- Keywords: traditional products, acrylamide, organochlorine pesticides, polychlorinated biphenyls, n-methylcarbamates, ochratoxin A, Cu, Mn, Fe, Zn, Ca and Mg -

INTRODUCTION

Now that globalization of the food economy has made the market of food products predominated by mass products whose quality parameters frequently fail to meet consumers' expectations, local agri-food initiatives, proposed as sustainable alternatives, are gaining importance both for small-scale producers and environmentally-aware consumers (SELFA and QAZI, 2005; O'HARA and STAGL, 2001; HINRICKS, 2000). The level of consumers' trust in food and perception of risks vary, but purchasing decisions are increasingly often associated with seeking safe food products (YEUNG and MORRIS, 2001; VERBEKE *et al.*, 2007; ANGULO and GIL, 2007) with well-known and tested places of origin (ESPEJEL *et al.*, 2007; DU PLESSIS and DU RAND 2011). The region of origin is beginning to play an important role in decisions of food products purchase (LOBB and MAZZOCCHI, 2007). Those from rural areas are perceived as products of high quality (HENCHION and MCINTYRE, 2000).

"Traditional food" refers to products made from specific raw materials, and/or with a recipe known for a long time, and/or with a specific process (CAYOT, 2007). The current EU legal regulations (COUNCIL REGULATION No 2081/92; No 2082/92; No 510/2006; No 509/2006) enable the protection of original agricultural products and food characteristic of the place of origin and traditional methods of production. These include: Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Speciality Guaranteed (TSG). Quality of food, resulting from the method of its production and processing, as well as the raw materials used, should be the most important attribute of regional food and the base its positive image should be built on. The term "traditional" is, however, not adequately defined. Moreover, it is often intentionally or unintentionally misused. Therefore, the important issues of registration and standardization of traditional foods arise in order for these products (1) to be protected against imitations, (2) to be of high quality and (3) to conform to contemporary rules of appropriate and safe production. A way to ensure authenticity and high quality of traditional food products is to establish criteria for their registration that will thereafter determine standards for their commercial production (TRICHOPOULOU *et al.*, 2007). Reproduction of traditional food, especially today, in the era of globalisation, entails a number of questions about the possibility of obtaining components, reconstructing a recipe and a process, the nutritional value, specificity or typicality of the products. CAYOT (2007) has pointed out that the industrialization of food production, European laws on food safety and even the development of innovative products necessitate the characterization of even the typical sensory traits of the traditional products.

Bread made from whole and brown rye flours is a major component of diet in Northern and Eastern European countries (MICHALSKA *et al.*, 2008). As bread is a staple food to many populations, its quality is of outmost importance. Being a component of an everyday diet, bread should have high nutritional quality (DEWETTINCK *et al.*, 2008). It has been shown to be a source of nutrients, especially carbohydrates, fiber, proteins and some minerals (magnesium, phosphorus, iron) (KOPEĆ *et al.*, 2011). As reported by MICHALSKA *et al.* (2008), rye products based on wholemeal rye flour may be considered to be nutritious and healthy food.

The chemical composition, nutritional value and sensory quality of bread depend on the type and extract of rye flour used for its production, on the technological process, water content and technological additives (KIHLBERG *et al.*, 2004) which impart the product specific functional properties (JONES and JEW, 2007). Modification of the recipe by the addition of some ingredients, for example, dietary fiber and sour dough, may affect the nutritional value of the finished product (LOPEZ *et al.* 2003; POUTANEN *et al.*, 2009; TUCKER *et al.*, 2010).

An increasing number of products are appearing on the market with names given by producers and implying that they were "home-made". By information provided on packages, the producers suggest that the food was manufactured with traditional methods, according to traditional recipes.

There are only scarce data on local food. Information about products manufactured on a small scale, from local raw materials and with traditional methods, are frequently only uncorroborated press reports. The aim of this study was to compare: 1) health quality (acrylamide, Σ DDT, γ HCH, PCB, N-methylcarbamates, ochratoxin A, and 2) nutritional value (protein, glucose, Cu, Mn, Fe, Zn, Ca and Mg) of rye bread produced by micro-companies and larger producers.

EXPERIMENTAL PROCEDURES

Samples

During the first stage, an inventory was performed of bread produced by agri-tourist farms and micro-companies in the bakery industry, located in the region of Warmia and Mazury in Poland (Fig. 1). Seventy-five personal interviews with representatives of the entities under study were conducted. The questionnaire contained three groups of questions: 1) concerning the types of products made, 2) focusing on raw materials and methods of production, 3) aimed at providing a qualitative description of products made from local raw materials. 20% of the

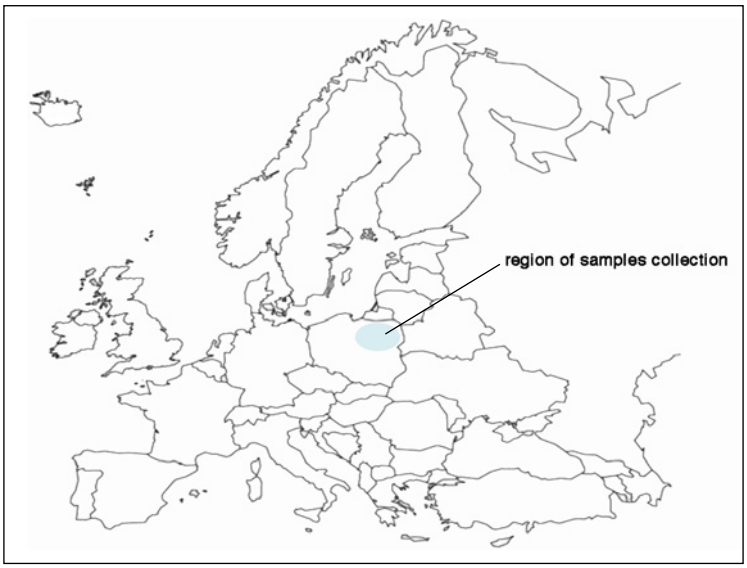


Fig. 1 - Map of rye bread collection

companies under study were found to use only flour purchased from local, regional producers. 3% of the companies used materials originating from their own production. The place of origin of materials was an important factor, taken into account in purchasing decisions for 45% of the companies. The analyzed products were those with higher-than-average quality, resulting from traditional methods of production and materials of local origin used in the production process.

Of all the products under study, samples of rye bread from 22 manufacturers were collected (the samples were labelled as traditional food). Moreover, bread labelled as "wholemeal bread" was bought on the Warmia and Mazury market (Poland) from 8 large-scale manufacturers (the samples were labelled as conventional).

Health quality

Health quality of rye bread was determined based on selected indicators: acrylamide, Σ DDT (1.1.1. - trichloro-2.2-bis(4-chlorophenyl)ethane - as a total sum of DDT + DDE +DDD), γ HCH (γ-hexachlorocyclohexane), polychlorinated biphenyls (PCBs), N-methylcarbamates and ochratoxin A.

The content of acrylamide in the samples was determined with the RP-HPLC method (WENZL *et al.*, 2003; ZHANG *et al.*, 2005). The 4.0-g sample after acrylamide extraction using 80% methanol in water and de-fatting using hexane was cleaned up by adding acetonitrile (20 mL) and Carrez I (500 μL) and Carrez II (500 μL). The aliquot was centrifuged for 10 min at 4,500 g at 4°C and the clear supernatant was cleaned up with Oasis HLB SPE cartridges purchased from Waters (Milford, MA). The final sample was filtered through a membrane filter (0.45 μm) and analyzed with the RP-HPLC method. The analyses were carried out using Shimadzu LC-20AD series for HPLC equipped with UV-Vis 190-600 nm diode array detector (DAD, Shimadzu) set at 200 nm, a vacuum degasser, binary pumps, thermostated autosampler and temperature-controlled column oven. The column used was C18, 4.0 mm guard column and Synergi 4 Hydro-RP 80 A 250 x 4.6 mm LC column from Phenomenex (Torrance, CA); mobile phase: isocratic mixture of 5 mM heptasulfonic acid; flow rate 1.0 mL/min; injection volume: 100 μL, column temperature 25°C, autosampler temperature 4°C; UV-Vis detector set at 200 nm. Acrylamide elutes near 8-9 min. Total time between injections was 30 min. Peak identification was based on the retention time by comparison of the ratio of UV spectra with that of a standard commercial compound. The acrylamide standard was obtained from Sigma-Aldrich Chemical Company (USA). Stock standards were prepared in 80% methanol. The stock solution of the standard was 20, 10, 5, 2, 1, 0.5, 0.25, 0.10, 0.05 and 0.01 μm/mL. All standard solutions were stored at 4°C in glass light-resistant bottles. The linearity of the standard curve was expressed in terms of the correlation coefficient (R_2), the plot of the integrated peak area (% of UV spectra) against standard concentration. A linear regression equation was found with linearity (R_2=0.9996, n=6) over the range of 0.01 - 20 μg/mL. This was used to determined acrylamide concentration in food samples. Repeatability and precision of the method were determined by repetitive analyses of the content of acrylamide in 10 one-batch samples of rye bread and calculating the RSD (relative standard deviation). The RSD (n=10) of one-batch samples was 4.7% which is a satisfactory level in food quality control. The mean recovery was 105%.

Organochlorine pesticides (DDT and γ HCH) and polychlorinated biphenyls (PCBs) were extracted from the samples with petroleum ether and acetone together with fat. The identification and quantitative determination of the analyzed compounds were carried out with gas chromatography using a PYE Unicam 4600 apparatus with an EC detector.

N-methylcarbamates were determined with the HPLC method (DIONEX CORPORATION 2007; ISO 1998). The method of sample preparation involves a salting-out extraction step with acetonitrile, NaCl, and $MgSO_4$; and a SPE clean-up step using primary secondary amine (PSA) resin to extract carbamates and remove interfering substances from the samples. 5 g of the sample were put into a tube and then 5 ml of acetonitrile were added. After 1 min of vortexing, 2 g of $MgSO_4$ and 0.5g NaCl were added and the sample was mixed for 1 min using vortex. Then, it was centrifuged for 10 min (rpm 3,000) and 1 mL of supernatant was pipetted into 1.5 mL and 100 mg $MgSO_4$ and 50 mg PSA were added. Afterwards, the sample was vortexed for 1 min and centrifuged for 5 min

(10,000 rpm). Then, 100 μL of the supernatant were mixed with 900 μL of distilled water and analyzed with the HPLC. The following separation conditions were applied: Shimadzu LC-20AD liquid chromatograph; column: guard column: C18 Acclaim Carbamate 3.0 x 10 mm, 3 μm, analytical column: Acclaim Carbamate 3.0 x 150 mm, 3 μm; column temperature: 50°C; mobile phase: methanol-water in gradient; flow rate 0.9 mL/min; injection volume: 50 μL; detector fluorescent (excitation: 330 nm, emission: 465 nm). Peak identification was based on the retention time by comparison with the standard.

Ochratoxin A was determined with the HPLC method with bicarbonate clean up according to ISO 15141-2 (ISO 1998). Extraction of ochratoxin A was made using SPE-cartridges purchased from Waters (Milford, MA). After extraction, the level of OTA was determined with HPLC using Shimadzu LC-20AD series for HPLC Chromatograph with reversed phase column (250 x 4.6 mm, 5 μm C_{18}). A mixture of acetonitrile, water and glacial acetic acid at the ratio of 49·5:49·5:1 and the flow rate of 1 mL/min was used in the analysis. A fluorimetric detector was used as well (excitation wavelength 330 nm, emission wavelength 460 nm). Qualitative identification was performed by comparing the retention times of the standard with that of the sample under study and quantitative determination was performed by comparing the areas of peaks of the standards with those of the sample under study.

Nutritional value

Nutritional value of rye bread was determined based on selected indicators: protein, glucose, fat, Cu, Mn, Fe, Zn, Ca, and Mg.

Protein content in bread was determined according to Kjeldahl's method. This method consists in mineralization (burning) of the examined organic substance in concentrated sulfuric acid, at boiling temperature. The examined substance oxidizes to carbon dioxide and water, and the nitrogen it contains gets separated in the form of ammonia. About 2 g of the sample were mineralized and distilled. The mineralization and distillation process was performed in the FOSS apparatus. After distillation, the samples were titrated with 0.1 M of hydrochloric acid. Protein content in bread was expressed in grams per 100 g of the product.

Glucose content was determined using the Lane-Eynon method. The principle for determining sugars according to this method is copper reduction in Fehling I and II (because of sugars present in the examined sample) and complete conversion of bivalent into monovalent copper (by titrating with a standard solution of saccharose) in the presence of the indicator (methylene blue).

Determination of fat content was carried out using the Weibull-Stoldt method. The principle of the method consists in extracting fat substances using petroleum benzine and in gravimetric determination of the amount of fat collected in the receiver of the Soxhlet apparatus.

Determination of calcium and magnesium contents were conducted according to the method described by WHITESIDE and MIMER (1984). The samples were mineralized in a mixture of nitric and perchloric acids (03:01). Mineralization was conducted in an electric aluminium heating block with temperature programming (VELP DK 20, manufactured by VELP Scientifica, Italy). Contents of Ca and Mg in the mineral residue were determined using flame atomic absorption spectroscopy (acetylene-air flame). Measurements were carried out with the use of atomic absorption spectrometer Unicam 939 Solar - Great Britain, equipped with an Optimus data station, background correction (deuterium discharge lamp) and an appropriate cathode lamp. A 10% aqueous lanthanum chloride solution, in amounts ensuring a final concentration of La^{3+} at 1%, was added to all measured solutions to determine Ca content. Standards of calcium and magnesium (1 mg/cm^3), diluted with 0.1 M solution of HNO_3, were prepared on the basis of BDH standards (Germany).

In addition, bread was subjected to the evaluation of its physicochemical parameters including: acidity, salt content and moisture content.

Determination of potential acidity was specified by titration of the examined solution with a base of a known concentration, in the presence of phenolphthalein as an indicator.

Determination of salt content in bread was performed with the Mohr's method. The principle of the method consists in titrating an aqueous extract of the pulp with silver nitrate, in the presence of potassium chromate as an indicator.

Determination of bread moisture was performed with the application of a drying method. Such determination consists in specifying the loss of water from a sample while drying it at 130°C for 60 minutes.

Data analysis

Data obtained were analyzed statistically with the use of basic statistics, i.e. mean and standard deviation and relative standard deviation. Differences between health quality and nutritional value categories of bread (traditional and conventional) were determined with the use of one-way analysis of variance (ANOVA). The significance of differences was tested at a significance level of 0.05.

RESULTS AND DISCUSSION

Average concentrations of selected indicators of bread quality in all examined samples in relation to their origin (traditional and conventional) are presented in Table 1.

Table 1 - Average values of selected determinants in the bread samples under study.

		Bread			
		Traditional n= 66		Conventional n= 40	
		$\bar{x}$ ± SD	RSD	$\bar{x}$ ± SD	RSD
Acrylamide	µg/kg	119.62± 37.11[b]	31.02	163.35±26.22[a]	16.05
Σ DDT	mg/kg of fat	0.032±0.034	106.25	0.028±0.029	103.57
γ HCH	mg/kg of fat	0.001±0.001	100	0.002±0.001	50
PCB	mg/kg of fat	0.007±0.003	42.86	0.005±0.004	80
N-methylcarbamate	mg/kg	No found		No found	
Ochratoxin A	mg/kg	No found		No found	
Acidity	°	8.41±1.02[a]	12.13	5.55±1.62[b]	29.19
Moisture	%	45.69±1.93	4.22	46.90±2.27	4.84
Protein	%	5.25±0.91	17.33	5.21±0.55	10.56
Glucose	mg/kg-dw	1.17±0.32	27.35	1.57±0.77	49.04
Fat	mg/kg	0.91±0.31	34.06	0.96±0.20	20.83
Salt	mg/kg	2.25±0.37	16.44	2.58±0.51	19.77
Cu	mg/kg	1.86±0.52[a]	27.96	1.44±0.17[a]	11.80
Mn	mg/kg	18.49±9.32[a]	50.40	11.24±3.49[b]	31.05
Fe	mg/kg	25.15±11.53	45.84	17.58±3.06	17.41
Zn	mg/kg	17.60±8.24[a]	46.82	10.67±1.33[b]	12.46
Mg	mg/kg	451.42±187.83	41.61	330.77±54.31	16.42
Ca	mg/kg	403.59±246.93	61.18	321.40±81.30	25.29

n – samples number; $\bar{x}$ ± SD - average value; SD – standard deviation; [a,b] – statistically significant difference at the level $p<0.05$; RSD - relative standard deviation.

Health quality

Maillard reactions are the most important chemical events occurring during the manufacture of bakery products. During these reactions, healthy and potentially-harmful compounds are produced. Among the harmful ones, acrylamide has received a great attention in recent years. It is classified as a 'probable human carcinogen' and defined as a compound with the potential to cause a spectrum of toxic effects. Acrylamide was observed to form mainly from asparagine and reducing sugar trough Maillard reactions. Several factors, such as initial concentration of the precursors, their ratio, heating temperature, time of processing, pH, and water activity of a product, have been reported to influence acrylamide content in heated food (CLAUS *et al.*, 2008; HEDEGAARD *et al.*, 2008; CAPUANO *et al.*, 2009; GÖKMEN *et al.*, 2009; BARTKIENE *et al.*, 2012; SERPEN *et al.*, 2012).

Statistically significant differences in acrylamide content (Table 1) were recorded between bread from small-scale and large-scale production. A significantly lower content ($P \leq 0.05$) was found in samples of traditional bread (119.62 ± 37.11 µg/kg of bread) as compared to the conventional samples (163.35 ± 26.22 µg/kg of bread).

SVENSSON *et al.* (2003) showed that the content of acrylamide in soft wheat bread was in the range of < 30-160 µg/kg of fresh bread. According to MUSTAFA *et al.* (2009), the main precursors of acrylamide in cereal products are asparagine and reducing sugars, with asparagine being the limiting precursor. It was shown that yeast fermentation had an important role in controlling the formation of acrylamide at low levels of asparagine, such as those generally present in bread raw materials. HEDEGAARD *et al.* (2008) found that rosemary was suppressing acrylamide formation when added to dough prior to baking. In an experiment by MUSTAFA *et al.* (2009), it was found that the yield of alkali-extractable acrylamide in rye crisp bread was on average 37% higher than the yield of water-extractable acrylamide. The storage of samples for 20 months led to a decrease in the acrylamide content but did not change the correlation between the acrylamide yield from neutral and alkaline extraction. According to research conducted by CAPUANO *et al.* (2009), after 30 min of toasting wheat, whole-wheat and rye bread samples at 180°C, the final concentration of acrylamide was 262.3, 291.0 and 301.0 µg/kg, respectively. The higher concentration of acrylamide in rye bread is due to a higher free asparagine content in rye flour, which is one of the factors influencing acrylamide amount in bakery products. A similar correlation between free asparagine level of flour and acrylamide concentration in rye bread slices was reported by GRANDY *et al.* (2009). MUSTAFA *et al.* (2008) demonstrated that the concentration of acrylamide was affected by asparagine content. In bread produced from flour with a lower (0.44 g/500 g flour) content of asparagine, the content of acrylamide was less than 50 µg/kg, whilst a higher concentration of asparagine (3.08 g/500 g flour) raised acrylamide content to 600 µg/kg product. According to the International Agency on Research on Cancer (1994), acrylamide was classified as

a potential factor likely to accelerate cancer development in man. And although bread does not pose great risk to man, owing to a relatively low content of this compound compared to French fries, chips or crackers, it is consumed every day and therefore measures should be undertaken to reduce acrylamide content of bread.

The average content of chlorinated organic pesticides (Σ DDT, γ HCH) and polychlorinated biphenyls (PCBs) was low in both traditional and conventional bread. No differences were reported in this respect between samples of traditional and conventional bread ($P \leq 0.05$). No residuesof N-methylcarbamates or ochratoxin A were found in the analyzed samples. These results seem to suggest that, in the analyzed region, N-methylcarbamates and OTA contamination is well under control and unlikely to represent a threat to consumer health. It has been found that Ochratoxin A (OTA) has a variety of potentially deadly toxic effects. Bread samples collected from commercial outlets across Portugal were tested for OTA and all samples were found to be compliant with the European Commission. OTA content reached a maximum of 0.49 ng/g in Algarve and 0.43 ng/g in Bragança, and was thus below the 3 ng/g maximum limit established by European legislation for bread (BENTO *et al.*, 2009).

Nutritional value

The average protein content in the bread under study was 5.24 ± 0.82% – 5.25 ± 0.91% in samples of traditional bread and 5.21±0.55% in those of conventional bread. A study by MICHALSKA *et al.* (2008) showed that both carbohydrate and protein concentrations were affected by milling and baking processes. Protein content decreased from 9.34 ± 0.04 to 7.85 ± 0.02 g/100 g of flour sample during milling, thus lowering its nutritional quality. These results may be explained by a loss of grain material containing protein during milling. Baking did not affect the protein concentration as much as milling did. The obtained data did not correspond with results reported by KOPEĆ *et al.* (2011). Those authors showed that baking technology impacted protein quality. The differences in protein quality were due to the baking technology applied.

The content of glucose (1.17±0**.**32 mg/kg-dw) and fat (0.91±0.31 mg/kg) in traditional bread was similar to that in bread produced by large bakeries. LAPPI *et al.* (2010) reported lower proportions of fibre (3.3) and fat (1.5) in a white wheat bread portion compared with wholemeal bread portions (8.4 and 2.6, respectively).

Samples of traditional bread were found to contain slightly (but statistically significantly) more ($P \leq 0.05$) copper, manganese and zinc, equal to 18.6 ± 0.52; 18.49 ± 9.32; 17.60 ± 8.24 mg/kg, respectively. Samples of traditional bread contained more iron, calcium and magnesium with varied concentrations of those elements (for iron SD = 11.53, RSD = 45.84, for magnesium SD = 187.83, RSD = 41.61, for calcium SD = 246.93, RSD = 61.18). Some data suggest that sourdough bread is a better source of magnesium, iron and zinc compared to the breads produced with other methods (LOPEZ *et al.*, 2003; CHAOUI *et al.*, 2006). A study by DEMIROZU *et al.* (2003) showed that the mean values of iron, copper, zinc, lead and cadmium levels were 19.2 ± 8.1 mg/kg-dry weight (dw), 2.1 ± 1.0 mg/kg-dw, 10.0 ± 3.0 mg/kg-dw, 86.8±176.0 mg/kg-dw, and 12.2 ± 6.1 mg/kg-dw, respectively.

Acidity, salt and moisture content

The samples' place of origin (small or large companies) had no impact on values determined for concentration of moisture and salt. Acidity of rye bread in samples of traditional bread (table 1) was significantly higher (8.41° ± 1.02° of acidity), compared to the samples of mass-produced bread (5.55° ± 1.62° of acidity). The results suggest that the production process of rye bread made on a large scale does not include the fermentation phase or that the phase was shortened in order to, for example, reduce production costs.

CONCLUSIONS

In general, the health quality and nutritional value of rye bread produced by micro-companies localized in the province of Warmia and Mazury (Poland), compared to bread of the same kind manufactured in large bakeries, varied only with respect to some determinants. The only exceptions were the content of acrylamide, copper, manganese and zinc. The reported levels of acrylamide were relatively low to pose health risk. The acidity of bread produced by large bakeries suggests that it does not have the features which are typical of bread made from sourdough, which may imply that the producers who claim their bread is made with traditional methods are misleading consumers.

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PAPER

ANTHOCYANIN ESTERIFICATION IN SANGIOVESE GRAPES

L. RUSTIONI*, M. ROSSONI, O. FAILLA and A. SCIENZA
Università degli Studi di Milano,
CIRIVE, Centro Interdipartimentale di Ricerca per l'Innovazione in Viticoltura ed Enologia,
Via Celoria 2, 20133 Milano, Italy
*Corresponding author: laura.rustioni@unimi.it

ABSTRACT

The traceability of the origin of a variety using the anthocyanins profile was recently discussed for Sangiovese wines, producing an important market impact. The variability of the anthocyanin profile of Sangiovese was studied over three years in grapes and wines. In the same vineyard, five clones, two bunch exposure conditions and three ripening times were evaluated following a full factorial experimental design. HPLC profiles showed a wide range in variability, significantly affected by environmental and cultural factors. The grapes profile changes obtained in the vineyard were maintained in the wines. Also the relative proportions of the acetic and *p*-cumaric acids involved in the esterification were significantly affected.

- Keywords: acylated pigments, anthocyanins, Brunello di Montalcino, origin evaluation, phenotype, Toscana, traceability -

INTRODUCTION

The traceability of the varietal origin was recently discussed for Sangiovese grapes used for the production of "Brunello di Montalcino", when in 2008 some wine stocks were considered fraudulent due to the higher than expected proportion of acylated pigments. The alarm for the hypothetical scandal had a high impact on the market and on the economy. Following the events, experts and scientists discussed the use of anthocyanin profile as an index of the varietal purity of the wine.

Even though the anthocyanin biosynthetic pathways have a cultivar specificity (MATTIVI *et al.*, 2006), many factors may influence the molecule proportions in ripe berries. Significant differences related to the varietal clones were observed by ROGGERO *et al.* (1986) in Syrah, by FERRANDINO *et al.* (2007) in Nebbiolo, and by LA NOTTE and ANTONACCI (1985) in Uva di Troia grapes. FERRANDINO *et al.* (2007) also found that the cultivation region has a significant influence on the acylated anthocyanins in Nebbiolo grapes. Many environmental factors can influence the pigments profile. HILBERT *et al.* (2003) and DO and CORMIER (1991) showed an effect of soil nitrogen availability on the pigments accumulation. Light exposure affects the anthocyanin profile as reported by TARARA *et al.* (2008); DOWNEY *et al.* (2004); RUSTIONI *et al.* (2011); HASELGROVE *et al.* (2000); RISTIC *et al.* (2007). The variation of light exposure can be related to the *terroir* characteristics or also to the vineyard management (shoot positioning and defoliation). Other viticultural techniques affecting pigments profile are bunch thinning (BUCELLI and GIANNETTI, 1996) and harvesting time (HILBERT *et al.* 2003, ROGGERO *et al.* 1986). Also the seasonal weather course affects the accumulation of anthocyanin (BUCELLI and GIANNETTI, 1996, GUIDONI *et al.*, 2008).

Beside the viticultural factors, the impact of the winery technology on the wine's anthocyanin profile has been demonstrated. FISCHER *et al.* (2007) underlined the impact of enzyme treatment, temperature during thermovinification and duration of skin contact on the anthocyanin composition (sum of acylated anthocyanins and ratio of acetylated/coumaroylated pigments), with the objective of preventing false alarms, and of protecting wine producers, merchants and consumers.

Other authors (LA NOTTE and ANTONACCI, 1985; VERSARI *et al.*, 1999; GARCÍA-BENEYTEZ *et al.*, 2002) studied the effect of the extraction technique on the pigments profile, concluding that the anthocyanin fingerprints of wines only reflect in part the anthocyanin fingerprints of fresh grapes. Also the effect of SO_2 on monomeric anthocyanin extraction changes in relation to the molecular structure (DALLAS and LAUREANO, 1994). The ratios between the different molecules can also change during aging (REVILLA *et al.*, 2001; LA NOTTE and ANTONACCI, 1985; GARCÌA-PUENTE RIVAS *et al.*, 2006).

Finally it is important to consider the anthocyanin profile variability related to sampling protocols, analytical techniques, molecular stability and to polymerization/degradation/oxidation reactions (CASASSA and CATANIA, 2006; RIBÉREAU-GAYON, 2000; GARCÌA-PUENTE RIVA *et al.*, 2006; LA NOTTE and ANTONACCI, 1985; DOWNEY *et al.*, 2007; FONG *et al.*, 1971; HOSHINO *et al.*, 1980; MORI *et al.*, 2005).

The knowledge of the anthocyanin profile in relation to its typicality and variability is a useful tool for wine growers and oenologists. The aim of this work was mainly to underline the variability of the anthocyanin esterification, by providing a numerical evaluation of the range of variation of the content of acylated pigments in Sangiovese grapes, to give a contribution for preventing further crisis in the wine market.

MATERIALS AND METHODS

Plant material

The variability of anthocyanin profile was studied over a period of three years (2008, 2009, 2010), focusing on acylations, in Sangiovese cultivated in Montalcino (Toscana, Italy).

The vineyard was located at an elevation of 115 m a.s.l., on an hillside facing South-East. The soil was clay textured. Plants were spaced at 2.4 x 1 m for about 4,160 plants/ha, and trained to the Spur Cordon system, leaving 12 buds/vine after winter pruning. The interrow soil was kept weed-free by two glyphosate herbicide treatments yearly.

In the same vineyard, five clones (one row/clone planted in succession), under two bunch exposure conditions, harvested in three ripening times were studied in triplicate. The clones checked were: Janus 50; Tin 10; Janus 10; Janus 20 (grown on 420A rootstock) and VCR 4 (grown on 1103P rootstock). To evaluate the effect of the harvesting time, anthocyanins profile was analyzed at commercial harvest (0), about 10 days before (-10), and 20 days after the commercial ripening (+20). Exposed bunches were obtained managing the canopy, and shaded grapes were obtained closing bunches in white paper bags opened on the bottom. The experiments were conducted selecting vines according to the correct vine balance, evaluated by visual inspection and able to assure a proper grape ripening according to the local experience (generally more than one square meter of exposed leaves per expected kilogram of grapes). In order to take into proper consideration the differences due to the individual physiological status of each vine, as well as to minimize the

possible disturbance of the slight leaf removal around the exposed bunches, every treatment (i.e. sun exposed and leaf shaded) was replicated on each vine. Air temperature and rainfall data were collected by a standard meteorological station closely located to the vineyard. All the treatments were done at veraison (corresponding to the onset of grape ripening), and maintained during the ripening stages until late harvesting.

Microvinifications

Three clones (Janus 10, Janus 20 and VCR4), ripened under the two exposure conditions, were selected for microvinification tests in 2009 and 2010, to be analyzed after fermentation, 6 months and 1 year of aging. For winemaking, four rows were selected for each clone and two of them were defoliated, while the others were covered by a white nonwoven fabric applied over the canopy zone of the bunches. All the treatments were done at véraison and maintained during all the ripening period, until harvest. Wines were made from 500-1,200 kg of grapes, depending on the productions. Vinifications were controlled concerning yeast inoculation, fermentations, temperature, and wine conservation.

Sample preparation and HPLC analysis

Grape samples for the HPLC analysis were obtained from 20 representative whole berry skins (analyzed in three replications) and extracted in 100 mL of pure methanol for 24 hours at room temperature. The extract was than filtered, evaporated and re-suspended in 50 mL of 7% formic acid.

The solvents that were used were HPLC grade methanol, formic acid and acetonitrile obtained from Chemsupply (Gillman, SA, Australia).

Analyses were performed on a Varian Prostar 240I HPLC (Mulgrave, Vic, Australia) using a Hypersil ODS 5 μm C18 110A 250 x 4.6 mm column (Lane Cove, NSW, Australia), with a Phenomenex Security Guard column, operated at a temperature of 35°C. The modified method of BALDI *et al.* (1995) involved a gradient elution with a mobile phase comprising by water:formic acid (93:7 v/v) eluent A, and water:formic acid:acetonitrile (60:7:30 v/v/v), eluent B, and a gradient program as follows: from 25 to 55% B (30 min), from 55 to 80% B (5 min), constant 80% B (8 min), from 80 to 20% B (7 min). The flow rate was 0.8 ml/min. The elution was monitored by a Varian Prostar 335 photodiode array detector (Mulgrave, Vic, Australia) at 520 nm. The proportions between the picks were used to define the anthocyanins profile, while their sum indicated the total monomeric anthocyanic quantification.

Statistical analysis

About 300 anthocyanins HPLC profiles (from grapes and wines) were evaluated, and all the data were analyzed with SPSS statistical software (version PASW Statistics 18, SPSS Inc. Chicago, Illinois). The effects were statistically tested by a general linear model which included year, bunch exposure, clone and harvesting time and their interactions as factors, followed by the expected components of the variance estimation. Treatment means were separated by LSD test (P = 0.05). The possible relationships among anthocyanins accumulation and percentage of acylation were detected by ordinary Pearson's correlation and regression models.

RESULTS AND CONCLUSIONS

As expected, the genetic control of the anthocyanin biosynthetic pathway produced a typical pigments profile. The average of the 255 grape extracts analyzed by HPLC indicated a medium profile with the following characteristics:

- considering the non-esterified pigments, the proportions between delphinidin-3-O-glucoside, cyaniding-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside and malvidin-3-O-glucoside were respectively 11.85, 17.75, 13.92, 16.89, and 39.60%;
- the esterification confirmed that Sangiovese is characterized by a low acylated pigments percentage. In particular, our samples showed an average of 96.48% of non-esterified malvidin, 1.03% of malvidin acetate, and 2.49% of malvidin p-cumarate.

Nevertheless it should be noted that Sangiovese is a very unsettled grape cultivar (PONI, 2000), and few papers also reported grape samples with a high percentage of acylated compounds (LA NOTTE and ANTONACCI 1985; RUSTIONI *et al.*, 2011). At the same time, according to our survey, Sangiovese demonstrated a wide range of variability in response to environmental and cultural factors.

The total monomeric anthocyanin content ranged between 196.81 and 2262.83 mg/kg of grapes, with an average value of 805.87 mg/kg, a median 731.26 mg/kg, and a standard deviation of 395.90.

Sangiovese demonstrated to be a very plastic cultivar considering the anthocyanin profile. Regarding the non-esterified pigments:

- the tri-substituted molecules percentage ranged between 34.53 and 85.38%, with an average value of 65.36% and a standard deviation of 8.94;
- the methoxylation involved a minimum of 38.10% and a maximum of 89.09% of the molecules, with an average value of 70.40% and a standard deviation of 8.39;
- the *ortho*-diphenol molecules ranged between

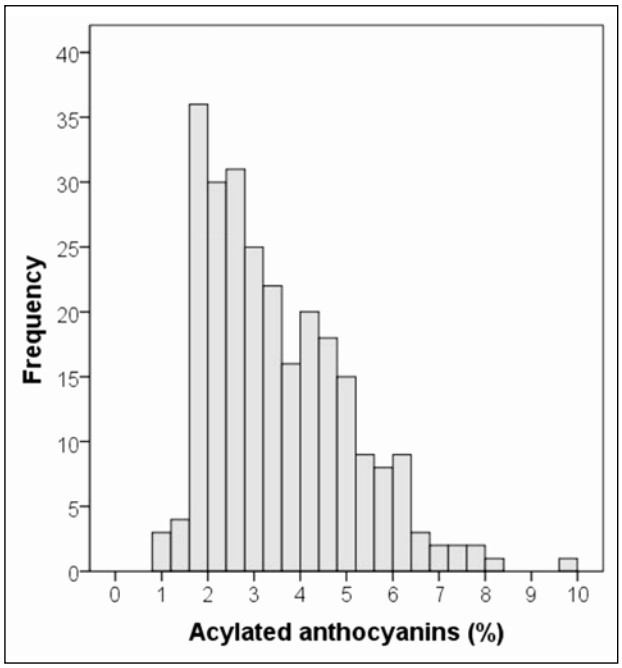


Fig. 1 - Frequency distribution of the percentage of acylated anthocyanins recorded in the three years.

18.14 and 73.73%, with an average of 43.51% and a standard deviation of 9.79.

The HPLC profiles also showed a high variability in the percentage of acylated malvidin, ranging between 1.00 and 9.84% (Fig. 1), with an average value of 3.52%, a median 3.17%, and a standard deviation of 1.53. The vintage was responsible for 49% of the explained variability, bunch exposure for 8%, harvesting time for 6%, clone for 5%, in addition the interactions vintage-bunch exposure and vintage-harvesting time explained respectively 9 and 14% of the variability. The characteristics of Sangiovese clones can vary in the environmental and cultural growing conditions (STORCHI *et al.*, 2000). This is in agreement with the importance shown by the effect of the interactions between different factors considered in our survey.

The three years covered different weather conditions during the growing season (Figures 2 and 3): 2009 was the warmest both in spring time (April and May) and during the first part of the grape ripening period (August and September); 2010 was the less warm through all the season; 2008, compared to the other two years showed

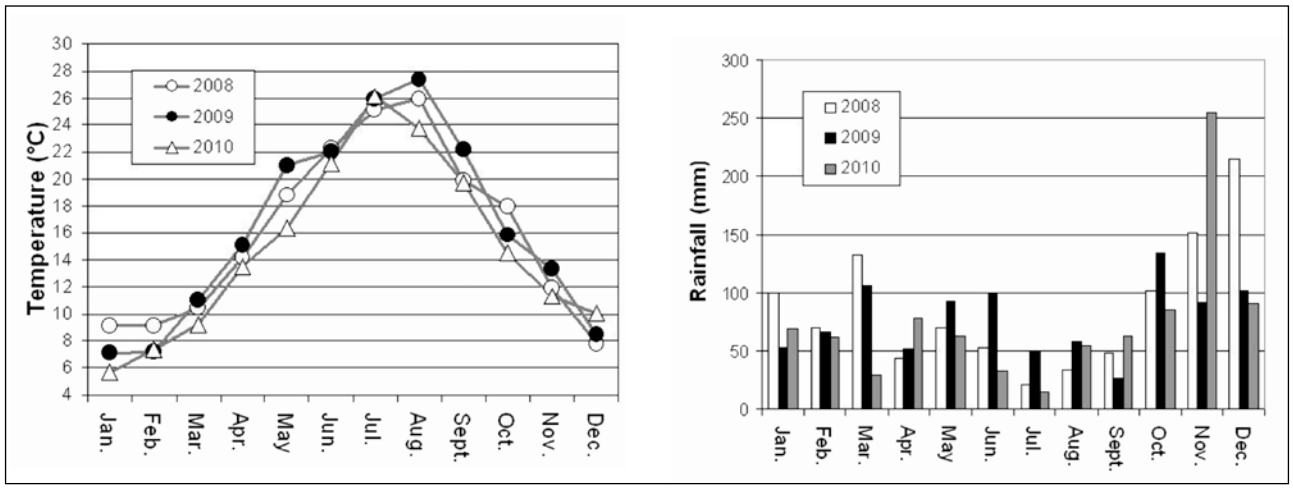


Fig. 2 - Monthly average temperature and total rainfall recorded in the three years survey.

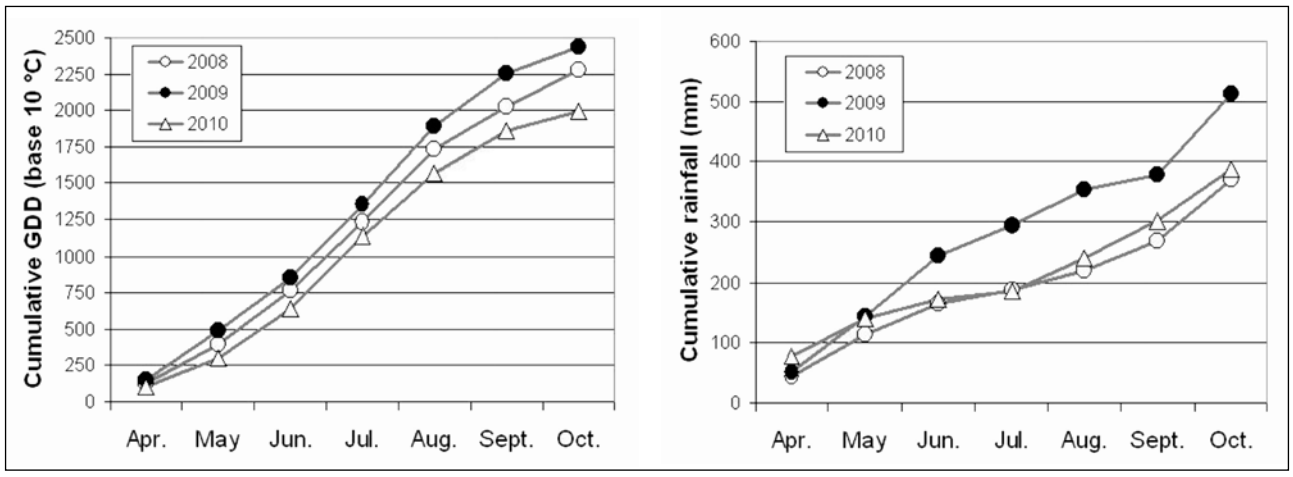


Fig. 3 - Active temperature summation (base 10°C) and rainfall from April to October recorded in the three years survey.

Table 1 - Anthocyanin acylation (% esterified malvidin/total malvidin) variability in response to environmental and cultural factors.

a) Year effect.

Year	Acylated anthocyanins (%)
2008	4.06 b[a]
2009	4.45 a
2010	2.11 c
Average	3.52

[a]Values followed by the same letter are not statistically different per P = 0.05.

b) Clone per year interaction effect.

	Clone				
	Janus 50	Janus 10	Tin 10	Janus 20	VCR 4
Year	Acylated anthocyanins (%)				
2008	3.58 b[a]	4.18 ab	3.70 b	4.62 a	4.21 ab
2009	4.27 b	4.54 b	3.52 c	5.24 a	4.99 a
2010	2.03 bc	1.93 c	1.92 c	2.25 ab	2.34 a
Average	3.32 cd	3.52 bc	3.09 d	3.92 a	3.77 ab

[a]In each line values followed by the same letter are not statistically different per P = 0.05.

c) Harvest time per year interaction effect.

	Harvesting time		
	Early (- 10 days)	Normal (0)	Late (+ 20 days)
Year	Acylated anthocyanins (%)		
2008	3.17 c[a]	5.25 a	3.77 b
2009	3.78 c	4.67 b	5.06 a
2010	1.92 b	2.16 a	2.25 a
Average	2.96 c	4.00 a	3.59 b

[a]In each line values followed by the same letter are not statistically different per P = 0.05.

d) Bunch exposure per year interaction effect.

	Bunch exposure	
	Shaded	Exposed
Year	Acylated anthocyanins (%)	
2008	4.22 a[a]	3.74 b
2009	5.30 a	3.64 b
2010	2.21 a	2.02 b
Average	3.92 a	3.03 b

[a]In each line values followed by the same letter are not statistically different per P = 0.05.

an alternant course. In August, temperatures were intermediate compared to the other vintages, September was similar to 2010 and higher than 2008. Rainfall courses during the growing season were very similar in 2008 and 2010; 2009 had a relatively higher rainfall course. The percentage in acylated forms seemed to be related to the thermal course of the ripening period (Table 1a), with higher values in 2009, the warmest season, intermediate in 2008, and lower in 2010, the cooler season. Figure 4 shows the correlation between the anthocyanins profile indexes (as average of the data obtained each year) and the thermal course expressed in cumulative GDD. While the percentage of acylated malvidin shows a direct correlation, the ratio of acetic esters and p-coumaric esters of malvidin

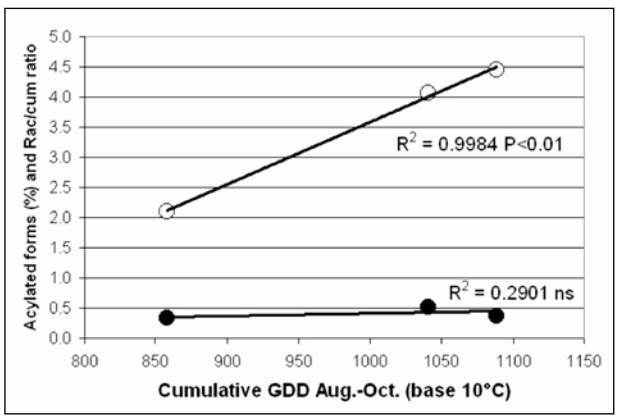


Fig. 4 - Relationship between active temperature summation (base 10°C) from August to October and the percentage in acylated forms (o) and Rac/cum (•) (ns = not significant).

Table 2 - Rac/cum (ratio malvidin acetate/malvidin p-coumarate) variability in response to environmental and cultural factors.

a) Year effect.

Year	Rac/cum ratio
2008	0.518 a[a]
2009	0.376 b
2010	0.329 c
Average	0.410

[a]Values followed by the same letter are not statistically different per P = 0.05.

b) Clone per year interaction effect.

	Clone				
Year	Janus 50	Janus 10	Tin 10	Janus 20	VCR 4
	Rac/cum ratio				
2008	0.541 a[a]	0.532 a	0.526 a	0.510 a	0.482 a
2009	0.361 b	0.356 b	0.311 c	0.447 a	0.429 a
2010	0.323 b	0.321 b	0.324 b	0.361 a	0.320 b
Average	0.410 ab	0.407 ab	0.390 b	0.440 a	0.407 ab

[a]In each line values followed by the same letter are not statistically different per P = 0.05.

c) Harvest time per year interaction effect.

	Harvesting time		
Year	Early (-10 days)	Normal (0)	Late (+ 20 days)
	Rac/cum ratio		
2008	0.452 b[a]	0.841 a	0.261 c
2009	0.424 a	0.353 b	0.344 b
2010	0.345 a	0.324 b	0.318 b
Average	0.408 b	0.504 a	0.303 c

[a]In each line values followed by the same letter are not statistically different per P = 0.05.

d) Bunch exposure per year interaction effect.

	Bunch exposure	
Year	Shaded	Exposed
	Rac/cum ratio	
2008	0.497 a[a]	0.560 a
2009	0.354 b	0.398 a
2010	0.308 b	0.349 a
Average	0.401 a	0.421 a

[a]In each line values followed by the same letter are not statistically different per P = 0.05.

(Rac/cum) had the highest values in 2008, the intermediate from the thermal point of view (Table 2a). Anyway, the relative proportions of the acids involved in the esterification were significantly (P=0.05) affected by the year and by the vineyard management. The percentage of esterification with acetic acid compared to the sum of the esterified forms showed a high variability, ranging between 15.84 and 57.00%; the median was 25.88%, and the standard deviation 8.043. In general, the Rac/cum index showed an average of 0.41. The variability of the Rac/cum is evidenced in Figure 5 and Table 2, showing the effect of the year, the clones, the harvesting time and the bunches exposure on the Rac/cum.

The clones Janus 20 and VCR 4 always showed the highest proportion of acylated malvidin, whereas the Tin 10 clone had a smaller

percentage of these compounds (Table 1b). As regards the Rac/cum index, with the exception of 2008, when no significant differences were detected, Janus 20 had the highest, and Tin 10 the lowest, acetic esters proportion (Table 2b). In our study, the clone affected both the percentage of acylated malvidin and the ratio of acetic and p-cumaric esters. This is in agreement with the high intra-varietal genetic diversity of Sangiovese, attested by several authors. COSTACURTA (2000) cited Sangiovese as an example for a largely diffused grape variety, which differentiated in particular areas, showing populations with different characteristics. The high cultivar variability is also discussed by EGGER *et al.* (2000) and by SCALABRELLI and LORETI (2000), which also underlined the mismatching obtained by comparing ampelographyc data and anthocyanic profile. Moreover a large variability in the anthocyanin profile has to be expected following the results of FILIPPETTI *et al.* (2005) that called "Sangiovese-like biotypes" some grape populations, largely known as Sangiovese, but genetically different from each other and not closely related to the cv. Sangiovese assumed as true to type (clone SG12T).

Early harvesting resulted in a lower proportion of acylated malvidin (Table 1c), while the lowest Rac/cum was found in the latest harvests (Table 2c). Concerning the acylation percentage, the late harvesting time showed a different behavior in the three years.

Significantly higher percentage of acylated malvidin was found in shaded bunches all the tested years (Table 1d), with the smallest differences among the treatments in 2010. As regards the Rac/cum index, even though the exposure didn't show a significant effect in the average of the three years, in two years over three the exposed bunches presented a higher ratio (Table 2d).

The effect of bunch exposure was already underlined in Sangiovese (RUSTIONI *et al.*, 2011) and in other cultivars (TARARA *et al.*, 2008, SPAYD *et al.*, 2002, DOWNEY *et al.*, 2004), and it is generally in agreement with our data which shows that bunch exposure decreases the percentage of acylated malvidin. Anyway, different results can be obtained with other cultivars: RISTIC *et al.* (2007) did not find any significant difference in the behavior of Syrah.

Considering the three years data, an increase of the pigment accumulation in berries corresponded to a decrease in the percentage of acylated malvidin (Fig. 6). Also the Pearson's correlation indicated, for each year, a negative trend between the monomeric pigments content and the percentage in acylated malvidin: -0.332 in 2008, -0.465 in 2009 and -0.720 in 2010 respectively (all significant per $P<0.01$). We suppose that this negative correlation can be related to the fact that the anthocyanins acylation is one of the last steps of the biosynthetic pathway. The environmental conditions and the external factors can stimulate the pigments synthesis, without affecting the last reactions. This hypothesis can be supported also by the enzyme localization. The enzyme involved in the flavonoid biosynthesis are all located on the endoplasmic reticulum (JØRGENSEN *et al.*, 2005), whereas the acyltransferase seems to be located in the cytosol (FUJIWARA *et al.*, 1998).

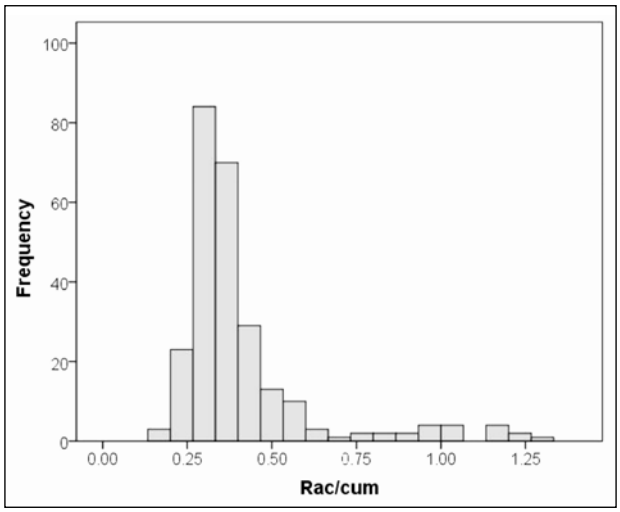


Fig. 5 - Frequency distribution of Rac/cum (ratio of acetic esters and p-coumaric esters) recorded in the three years survey variability.

In 2010, the skin pigments profiles were analyzed during the maceration (data not shown). No specific extraction trends were underlined between acylated and non esterified malvidin, indicating a non specific extraction of the acylat-

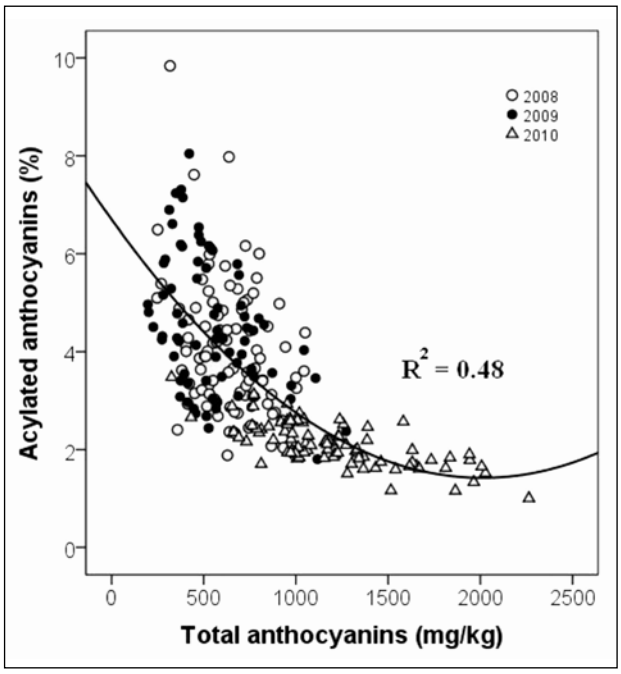


Fig. 6 - Relation between pigments accumulation in berries and percentage of acylated compound.

Table 3 - Anthocyanin acylation variability in wines.

Bunch exposure	2010	2009			Av.
	0 months	0 months	6 months	12 months	
Shaded	1.97 a[a]	1.79 a	2.2 a	1.85 a	1.91 a
Exposed	1.47 b	1.55 a	1.57 a	1.53 b	1.53 b

[a]In each column values followed by the same letter are not statistically different per P = 0.05.

ed molecules, when compared to the non esterified ones. However, during winemaking, the differences related to the vineyard management were maintained, at least in the first year of wine maturation (Table 3). Considering the Rac/cum index, the average increased at 0,81, compared to the 0.41 value of the grape skins. The Rac/cum has also been used as a varietal indicator, but the average increase underlined in our data, suggests a modification of this value during vinification. This can be related to the different extractability during maceration (in opposition with our maceration results), or to the synthesis/substitution of acetic acid during fermentation and aging.

In conclusion, the varietal specificity of the anthocyanins biosynthetic pathway can be a useful instrument for vine growers and oenologists to better understand the grapes characteristics, managing, if possible, varietal lack. Consequently, by understanding the sources of variability it is possible to suggest ways to obtain an anthocyanin profile which is more useful for the enological objectives. Keeping in mind that phenolic compounds are secondary metabolites, and, thus, they represent the first plant answer to the changes in environmental conditions, significant profile variation can be obtained with different vineyard management. As a consequence, the use of the anthocyanin profiles for the traceability of the grape varietal origin should be carefully considered, and the hypothetical limits should take into account the cultivar plasticity. Sangiovese is a very reactive grape variety. Thus, a legal limit for esterified pigments in wines, to ascertain the varietal purity, which would take into account the varietal properties should be, for Sangiovese, relatively high and therefore quite useless to ascertain possible blending with wines or grapes from different varieties. On the other hand, the improved knowledge of the variability could be an important support for the grape and wine industry.

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PAPER

EFFECT OF TECHNOLOGICAL FACTORS ON GLYCOALKALOIDS AND NITRATES CONTENT IN DEHYDRATED POTATO

E. RYTEL
Department of Food Storage and Technology,
Wrocław University of Environmental and Life Science,
Chełmońskiego 37/41 Str., 51-630 Wrocław, Poland
Corresponding author: Tel. +4971 207716, Fax +4971 207768,
email: elzbieta.rytel@wnoz.up.wroc.pl; rytel.elzbieta@gmail.com.pl

ABSTRACT

The influence of technological factors such as blanching and pre-drying temperatures applied to dried diced potato was studied. Used parameters influenced on glycoalkaloids and nitrates content in semi- and ready products. The more fine material, the lower content of glycoalkaloids and nitrates. Blanching temperatures used in the experiment contributed to further losses of glycoalkaloids in semi-products. There were no significant changes in the nitrate content in the dice depending on the applied temperature of blanching. Dice pre-dried at the lowest temperature (120°C) contained about 5% more glycoalkaloids than the dice dried at 140°C and 12% more than dice dried at 160°C. Whereas the higher the temperature during pre-drying, the lower the nitrate content in the final product. The dice dried at the lowest temperature still had 49% of the initial nitrate content found in the raw material, at 140°C – it was 41% and 160°C - 33%.

- Keywords: technological factors, dehydrated potato, glycoalkaloids, nitrates -

INTRODUCTION

Potatoes are the raw material from which we may obtain a wide range of products, e.g. potato chips, French fries, and dehydrated potato products. The latter are used as finished products or semi-products in the production of further processed foods such as frozen vegetables, potato concentrates, chips from potato dough such as "Pringels" or "Chipsletten".

In many well-developed Countries consumption of less-processed food decrease in favor of semi-products and finished products, hence the ever-increasing interest in fried or dehydrated potato products. Regardless of the type of product, processing potatoes in the food industry involves a number of similar technological steps. These include the initial processes such as washing, peeling, slicing and rinsing of tubers. This prepared material is then subjected to blanching and processes specific to the type of product, e.g. drying during the production of dehydrated potatoes, or frying in the production of chips and French fries (LISIŃSKA *et al.*, 2009; RYTEL *et al.*, 2011).

Finished potato products contain the same ingredients as the raw material, only in changed quantities or proportions. Like most plants, these are not only nutritional components but also antinutritional or even toxic substances, e.g. nitrates and glycoalkaloids. In potatoes used for consumption or for food processing, the amount of these compounds generally does not exceed accepted limits: 20 mg $100g^{-1}$ fresh weight for glycoalkaloids and 200 mg kg^{-1} fresh weight for nitrates (HILL 1999; LESZCZYŃSKI 2000; MURAWA *et al.*, 2008; KNUTHSEN *et al.*, 2009; GINZBERG *et al.*, 2009). However, the levels of these compounds in potatoes must be constantly monitored as they may vary significantly due to adverse conditions during the growing season, transport or storage. There are a considerable number of reports on the effects of technological factors in the production of chips and French fries on the contents of antinutritional and toxic compounds in the raw material, intermediates and final products (FRIEDMAN and DAO 1992; CIEŚLIK 1992; CIEŚLIK 1997; TAJNER-CZOPEK *et al.*, 2008; RYTEL *et al.*, 2005; ZGÓRSKA *et al.*, 2006; PĘKSA *et al.*, 2006; RYTEL, 2012). However, there is little data on dehydrated potato products. Differences between dehydrated and fried potato products do not only concerning the method of slicing and blanching, but also the applied temperature, time of exposure to high temperatures, drying or frying method, and many others, which to a varying degree may affect the content of toxic compounds.

Based on literature data, it can be concluded that the size of the loss of nitrates and glycoalkaloids in potato processing is related to the specific distribution of these compounds in potato tubers. Most anti-nutritional compounds (nitrates V) and toxins (glycoalkaloids) in potatoes are located just below the skin or in the vicinity of the eyes, which is why pre-treatment, the peeling and additional cleaning of tubers, results in the removal of a considerable proportion of these compounds (CIEŚLIK 1997; PĘKSA *et al.*, 2006; FRIEDMAN 2006; TAJNER-CZOPEK *et al.*, 2008; OSTRY 2010; RYTEL, 2010). Depending on the size of the tubers, peeling technique and the potato variety, the decrease in potato total glycoalkaloids (TGA) can reach even 80-96%, while after incomplete removal of the skin it may drop by 20-35% (CIEŚLIK, 1997; PĘKSA *et al.*, 2006; FRIEDMAN, 2006; TAJNER-CZOPEK *et al.*, 2008; TAJNER-CZOPEK *et al.*, 2011). However, the process of peeling may be insufficient during the processing of varieties with a bitter taste in the flesh, i.e. large amounts of glycoalkaloids (LESZCZYŃSKI, 2000; RYTEL *et al.*, 2011).

Likewise, the content of nitrate V is significantly reduced, by about 30%, in the process of peeling the potatoes (BECKA *et al.*, 1992; CIEŚLIK, 1992; RYTEL *et al.*, 2005; PĘKSA *et al.*, 2006). Further stages of technological processes affect changes in these compounds to a lesser extent. The next stages which may contribute to the loss of toxic or antinutritional compounds are thermal processes. The amount of glycoalkaloids decreases only slightly compared with the process of peeling, which can be explained by the thermostability of these compounds (FRIEDMAN and MCDONALD, 1997). By contrast, nitrate is water soluble and as a result of blanching, particularly in the case of finely fragmented material (e.g. sliced chips) the amount of nitrate in the processed material may drop by an average of 20% (CIEŚLIK, 1992; PĘKSA *et al.*, 2006).

Understanding all the factors contributing to the reduction in anti-nutritional or toxic compounds naturally occurring in potatoes still seems a valid and important problem.

Hence the aim of this study was to determine the effect of grinding the raw material, and different temperatures of blanching and drying, on the content of glycoalkaloids (α-chaconine and α-solanine) and nitrates in semi-finished and finished product.

MATERIAL AND METHODS

Raw material

The potatoes used for the study were collected from the store house of a potato food processing plant in 2009-2010. Potatoes were stored at 6°C in the dark. The results represent the average of three technical replicates.

Potato sample preparation for analysis

Potatoes were washed and peeled, using a laboratory carborundum peeler (to a depth of 1.5

mm). In order to study the effect of the dice size on the content of glycoalkaloids and nitrates, the peeled tubers were cut into cubes of three sizes: 5x5x5, 10x10x10, and 15x15x15 mm. Then, in order to determine the effect of temperature blanching on the content of these substances in the samples, 10x10x10 mm dices were selected for further analysis and blanched for 5 min. at three temperatures: 60°, 75° and 90°C. In order to determine the effect of drying temperature on the content of glycoalkaloids and nitrates in semi-finished potato products, further analysis involved 10x10x10mm dices blanched at 75°C. Dices after blanching were pre-dried for 1 hour, at temperatures of 120°, 140° and 160°C, and redried at 50°C for 12 h to a moisture content of 10-11%.

The diagram of the laboratory studies:



* Samples selected for further analysis.

The concentrations of α-solanine and α-chaconine

Apparatus

A high-pressure liquid chromatograph HPLC (pro Star) was used (Varian, Walnut Creek, CA, USA). The HLPC was equipped with a UV detector – 310 type, Microsorb NH2 analytical column (25x46 cm LD) (Rainin Instrument, Woburn, Ma, USA), and a computer system for monitoring the chromatograph (Varian Chromatography System).

Conditions of glycoalkaloid separation

A mixture of tetrahydrofuran (Merck, Germany), acetonitrile and water 50:20:30 + KH_2PO_4 (1.02 g) per litre was used as an eluent. The process was carried out at a temperature of 35°C, with a speed of flow of 2 $cm^3 min^{-1}$ and pressure of 11.3 MPa, applying a light wavelength of 208 nm.

Sample preparation for chromatographic analysis

Dried material (1 g) was homogenized with 4 cm^3 of water and 30 cm^3 of methanol (Labscan, Ireland) for 2 minutes, followed by filtration. The filtrate was brought to a final volume of 50 cm^3 with methanol. A 5 cm^3 aliquot of extract was cleaned up on the SPE column (Bond Elut C18; 500mg; 6.0 cm^3, Varian, USA). Glycoalkaloids were rinsed with methanol and evaporated to dryness in a vacuum at a temperature of 50°C. The resultant residue was dissolved in 1 cc of THF:ACN:H_2O – 50:20:30. Before application into the column, the sample was cleaned using 0.45μm filters. The volume of the injection was 10 μL.

Standard solutions (1 mg/cm^3) were prepared by dissolving 10 mg of α-solanine and α-chaconine (Sigma) in 10 cm^3 of methanol. Standard solution was dissolved to obtain samples containing from 1 to 50 μg/cm^3 of both α-solanine and α-chaconine. On the column 10 μL of these solutions was injected.

The content of nitrates

A reflectometric method with test strips was used, at a measurement range from 5 to 225 mg kg^{-1} NO_3. According to the principle of reflectometry (remission photometry), the reflected light from the strip was measured. In classical photometry, the difference in the intensity of emitted and reflected light allows a quantitative determination of the concentration of specific analysis.

Sample preparation for nitrates analysis

Nitrate concentrations were determined by reflectometry using a Rqflex analyser (Merck). Determinations were made in 20 g of distilled water solution containing 5g of a dry sample. In the solution nitrate concentrations were measured using test strips.

Analytical methods

The nitrate content of the potato tubers was determined by reflectometry method in the potato tubers, intermediates and finished products (RYTEL *et al.*, 2005). The quantities of α-solanine and α-chaconine were determined using the methods by PĘKSA *et al.* (2002) and SAITO *et al.* (1990). All the analyses were carried out twice.

Statistical analysis

The results obtained in the experiment were subjected to statistical calculations using Statistica 9.0 software. We applied a multi-way analysis of variance and Duncan's test ($P \leq 0.05$) for the determination of the significance of differences between means. All experiments were performed in three technological replications from two years of investigation and the present results show the mean of all data combined.

RESULTS AND DISCUSSION

In Table 1 are presented results of glycoalkaloids content determination in potatoes before and after peeling. In this study, potatoes were peeled using a laboratory carborundum peeler, removing the skin to a depth of 1.5 mm. After this process there was still about 70% of the initial content of these compounds in the raw material (Table 1).

Glycoalkaloids are located in larger quantities in the area of phellem cells, the parenchyma cells of the periderm and outer cortex of the tuber. The content of glycoalkaloids in the central pith and the inner cortex was very low. More glycoalkaloids are detected in the bud end than in the stem end of the tuber (FRIEDMAN and MCDONALD 1997). Generally, higher concentrations of these compounds are found in the skin and outer layer of potato tubers (to a depth of about 1.5 mm) which is why the peeling of potatoes can remove a considerable proportion of the glycoalkaloids. Depending on the depth of peeling, different amounts of these compounds may remain in the pulp. According to PĘKSA *et al.* (2006), peeling with a mechanical peeler removes about 30% of the initial content of glycoalkaloids in potato tubers, and hand-peeling about 60% (TAJNER-CZOPEK *et al.*, 2008)

Subsequent processes such as slicing, blanching or cooking were less intense in lowering content of glycoalkaloids in processed potatoes (TAKADI *et al.*, 1990; PĘKSA *et al.*, 2006; FRIEDMAN and LEVIN, 2009; DONALD, 2008). This is mainly due to the nature of these compounds. Glycoalkaloids are welder soluble in water, and according to DONALD (2008) solanine is almost insoluble. In Table 2, we presented the results for glycoalkaloid content in potatoes cut into dices of different sizes and rinsing in water.

The more fine material, the lower content of glycoalkaloids. The 5x5x5 mm dices contained almost 14% less of these compounds than the large dices (15x15x15 mm) and 10% less than the mid-sized dices (10x10x10 mm) (Table 2). During the shredding and rinsing in water of potatoes we observed greater drops in chaconine than solanine. In industrial practice, dices are usually 10x10x10 mm in size, hence our further analysis concerned this size. The next process was blanching.

Blanching is the first stage of thermal preparation of dried potato dices. In the study there were used three different temperatures: 60°, 75°, and 90°C. Blanching temperatures utilized in the experiment contributed to further losses of glycoalkaloids in semi-products. Greater reductions in these compounds occurred after blanching the dice at 90°C. Dice blanched at this temperature contained on average 20% less glycoalkaloids than the dice blanched at 60° or 75°C (Table 3). α-Chaconine to a greater extent than α-solanine reacted to the temperature, and

Table 1 - The content of glycoalkaloids and nitrates in dry matter unpeeled and peeled potatoes.

Analyzed compounds	2010		2011	
	Unpeeled	Peeled	Unpeeled	Peeled
α-chaconie [mg 100 g^{-1}]	14.8[a]	9.92[b]	17.9[a]	11.7[b]
α-solanine [mg 100 g^{-1}]	8.18[a]	6.08[b]	8.77[a]	5.85[b]
nitrate [mg $NaNO_3$ kg^{-1}]	149[a]	140[a]	181[a]	157[b]

[a,b]: row indicate significant differences (Duncan test, $p<0.05$).

Table 2 - Total glycoalkaloids content (mg 100 g^{-1} d.m.) in potato dice of different cube sizes.

Cube size	2010		2011	
	α-chaconine	α-solanine	α-chaconine	α-solanine
5x5x5 mm	9.75[a]	4.64[a]	9.01[a]	4.61[a]
10x10x10 mm	9.90[b]	5.10[b]	10.7[b]	5.31[b]
15x15x15 mm	10.3[b]	5.20[b]	11.4[c]	5.48[b]

[a,b,c]: columns indicate significant differences (Duncan test, $p<0.05$).

Table 3 - Total glycoalkaloids content (mg 100 g^{-1} d.m.) in potato dice of 10x10 mm cube sizes, blanching in different temperatures.

Blanching temperatures	2010		2011	
	α-chaconine	α-solanine	α-chaconine	α-solanine
60°C	7.27[b]	4.82[b]	8.10[b]	4.31[b]
75°C	7.93[b]	4.11[a]	7.85[b]	4.09[b]
90°C	6.48[a]	4.20[a]	6.16[a]	3.16[a]

[a,b]: columns indicate significant differences (Duncan test, $p<0.05$).

Table 4 - Total glycoalkaloids content (mg 100g^{-1} d.m.) in dried potato dice of 10x10 mm cube sizes, blanching in 75°C and pre-drying in different temperatures.

Pre-drying temperatures	2010		2011	
	α-chaconine	α-solanine	α-chaconine	α-solanine
120°C	7.03[b]	3.98[b]	7.53[b]	4.06[a]
140°C	6.94[a]	3.96[b]	6.70[a]	4.00[a]
160°C	6.51[a]	2.09[a]	7.34[b]	3.99[a]

[a,b]: columns indicate significant differences (Duncan test, $p<0.05$).

hence its content dropped more - on average by 28%, with α-solanine decreasing by 19% (Table 3) compared with samples after slicing.

There were no significant differences in the content of glycoalkaloids (α-chaconine and α-solanine) in blanched samples at 60° and 75°C (Table 3). Generally, blanching of potato cubes contributes to about a 25% drop in glycoalkaloids compared to samples after cutting. Glycoalkaloids are thermostable and hydrophobic (BUSHWAY and PONNAMPALAM, 1981; FRIEDMAN and DAO, 1992; CIESLIK, 1997; FRIEDMAN and LEVIN, 2009; DONALD, 2008), hence thermal processes including blanching or cooking decrease the content of glycoalkaloids by about 20-30% (RYTEL *et al.*, 2005; PĘKSA *et al.*, 2006; RYTEL, 2012). According to CIEŚLIK (1997) cooking of whole potatoes in water results in a glycoalkaloids drop of only 14% on average; the drop was greater in peeled potatoes than unpeeled ones.

For farther analysis samples blanched in 75°C were chosen. It is enough temperature for enzyme inactivating, which are active in potato flesh darkening and the improving of final product colour. Higher temperature of blanching can lead to collapse of dice but lower - 60°C could be not enough for enzyme inactivating.

After blanching, the dice was pre-dried at three different temperatures for 1 hour and redried for 8 hours at 50°C by convection. Pre-drying during the preparation of potato dice is to reduce the total drying time by removing excess water from the surface of the dice and to improve the color and consistency of the finished product. Irrespective of the temperature of pre-drying, losses of the examined compounds were not significant (Table 4). Dice pre-dried at the lowest temperature (120°C, redried in 50°C for 12 hours) contained about 5% more glycoalkaloids than the dice dried at 140°C (redried in 50°C for 12 hours) and 12% more than dice dried at 160°C (redried in 50°C for 12 hours). This could have resulted from insufficient time (1 hour) of exposure to higher temperature during drying of potato cubes. Glycoalkaloids are thermostable and in a dryer they are subject to only high temperature, with no factor that would result in leaching or degradation of these compounds. The finished product contained on average 7.01 α-chaconine mg 100 g^{-1} and 3.68 α-solanine mg 100 g^{-1} (Table 4). Larger changes in glycoalkaloid content were observed after frying potatoes. According to PĘKSA *et al.* (2006), frying French fries reduces the amount of glycoalkaloids by 35% and according to RYTEL *et al.* (2005) - by 40%. During frying the fragmented material is subject not only to high temperature but also to the factor contributing to the leaching of these compounds - oil.

This study also investigated the content of nitrates. Similar to glycoalkaloids, nitrates accumulate mostly in the skins of potatoes. According to CIEŚLIK (1992; 1995), after peeling, 70% of nitrates still remain in the tuber, and according to PĘKSA *et al.* (2006) 72-75%, depending mainly on the peeling method. In our study, potatoes were peeled mechanically, which resulted in a reduction of nitrate content in the analyzed raw material by 10% (Table 1). During technological processing or cooking of potatoes, these compounds change their content in tubers. In general, due to the high solubility of nitrates, their

Table 5 - Nitrates content ($mgNaNO_3^- kg^{-1}$ d.m.) in potato dice of different cube sizes.

Cube sizes	2010	2011	mean
5x5x5 mm	103[a]	142[a]	122[a]
10x10x10 mm	112[ab]	149[ab]	130[ab]
15x15x15 mm	128[b]	156[b]	142[b]

[a,b]: columns indicate significant differences (Duncan test, $p<0.05$).

Table 6 - Nitrates content ($mgNaNO_3^- kg^{-1}$ d.m.) in potato dice of 10x10 mm cube sizes, blanching in different temperatures.

Blanching temperatures	2010	2011	mean
60°C	83[a]	134[a]	108[a]
75°C	79[a]	114[b]	96[a]
90°C	70[a]	109[b]	90[a]

[a,b]: columns indicate significant differences (Duncan test, $p<0.05$).

Table 7 - Nitrates content ($mgNaNO_3^- kg^{-1}$ d.m.) in dried potato dice of 10x10 mm cube sizes, blanching in 75°C and pre-drying in different temperatures.

Pre-drying temperatures	2010	2011	mean
120°C	68[b]	97[b]	82[b]
140°C	52[a]	82[ab]	67[ab]
160°C	40[a]	69[a]	54[a]

[a,b]: columns indicate significant differences (Duncan test, $p<0.05$).

content in solid products decreases and increases in brine or broth (CIEŚLIK, 1995).

After the cutting of raw material and rinsing in water, there were significant changes in the content of these compounds (Table 5). The lowest amount of nitrates was found in the smallest dice (5x5x5 mm), which contained on average 14% less nitrates than the thick dice (15x15x15 mm) and 6% less than the mid-sized dice (10x10x10 mm) (Table 5). Blanching helped further reduce the amount of nitrates in the dice, on average approximately 26% compared with the samples after slicing (Table 6). There were no significant changes in the nitrate content in the dice depending on the applied temperature of blanching. By increasing the temperature from 60° to 90°C, nitrate content dropped by about 17% (Table 6). Lengthening the blanching time probably could bring better results, but this in turn could have affected the quality of the final product (breaking-up of the dice during blanching). According to CIEŚLIK (1997) nitrate drop during potato blanching is the result of many factors such as temperature and time of its operation, the amount of water used in relation to raw material, water absorption capacity and the degree of fragmentation. CIEŚLIK (1997) reports that cooking potato dice using traditional methods reduces the amount of nitrate by 71% and cooking in a microwave oven by only about 30%. In studies by CIEŚLIK (1997) cooking whole unpeeled potato tubers decreased nitrate content by only 16%. The presence of skins greatly impeded the transfer of nitrates to the brew. According to PĘKSA *et al.* (2006) blanching of potatoes cut into chips resulted in a loss of nitrate by 19-20%, and from RYTEL *et al.* (2005) blanching potatoes in the form of strips reduced the amount of nitrates by 18%.

In this study, blanching of potato dice gave much better results than drying. The higher the temperature during pre-drying, the lower the nitrate content in the finished product. Dice dried at 160°C contained 54 mg $NaNO_3^-$ and at 120°C - 82 $mgNaNO_3^-$ (Table 7). Pre-drying dice for 1 hour at the temperature 40°C higher (160°C) than the temperature most frequently used in the food industry (120°C), contributed to a 34% nitrate drop. The dice dried at the lowest temperature still had 49% of the initial nitrate content found in the raw material, at 140°C – it was 41% and 160°C - 33% (Table 7).

CONCLUSIONS

Used technological parameters influenced on glycoalkaloids and nitrates content in semi- and ready products. The more fine material, the lower content of glycoalkaloids and nitrates. The 5x5x5 mm dices contained almost 14% less of these compounds than the large dices (15x15x15 mm) and about 10% less than the mid-sized dices (10x10x10 mm). Blanching temperatures used in the experiment contributed to further losses of glycoalkaloids in semi-products. Diced potato blanched at 75°C contained on average 20% less glycoalkaloids than the dice after cutting and rinsing in water. Dice pre-dried at the lowest temperature (120°C), redried in 50°C contained about 5% more glycoalkaloids than the dice dried at 140°C (redried in 50°C) and 12% more than dice dried at 160°C (redried in 50°C). Where as the higher the temperature during pre-drying, the lower the nitrate content in the finished product. The dice dried at the lowest temperature still had 49% of the initial nitrate content found in the raw material, at 140°C – it was 41% and 160°C - 33%.

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PAPER

INFLUENCE OF CHEMICAL COMPOSITION AND STRUCTURE OF STRAWBERRY GELS ON THE CHOOSEN PHYSICAL PROPERTIES OF FREEZE-DRIED FINAL PRODUCT

A. CIURZYŃSKA, A. LENART and W. TRACZYK
Faculty of Food Sciences, Department of Food Engineering and Process Management, Warsaw University of Life Sciences, SGGW 159c Nowoursynowska St., 02-787 Warsaw, Poland
*Corresponding authorTel. +48 22 5937577, Fax +48 22 5937576,
email: agnieszka_ciurzynska@sggw.pl

ABSTRACT

This study undertook an assessment of the influence of the modification of composition on selected physical properties of strawberry jellies preserved via lyophilization. Moreover, the study presents an attempt to elaborate the technology of lyophilization-enabled strawberry jelly manufacture based on strawberry powder.

Six strawberry jelly recipes were obtained. Selected features of the final product were regulated by the addition of glucose and/or chokeberry juice concentrate. The jelly made from 7% strawberry powder and 5.2% chokeberry juice concentrate was selected as the product with the best quality features, the most profitable color and the highest rehydration degree.

- Keywords: lyophilization, jelly, strawberry, sodium alginate, calcium lactate -

1. INTRODUCTION

Improved living standards and the continuously decreasing amount of time spent on meal preparation have meant that consumers very often choose so-called convenience food, which may be quickly and easily prepared for consumption. Developments in food technology are being directed at product improvement so that it is both of a constant quality, and convenient and functional at the same time (DHANSEKHARAN *et al.*, 2005).

A large proportion of the ingredients included in "convenience food" are dried products, e.g. fruits, vegetables, mushrooms or more often meat. Therefore, the production of such kinds of food is constantly increasing and, as reported by RZĄCA and WITROWA-RAJCHERT (2007), the production of dried products in Poland increased from 17.7 thousand tons in 2000 to 27.9 thousand tons in 2004. Dried fruits and vegetables are characterized by elongated shelf life, higher stability, and also low mass and decreased volume, which facilitates transport (SAGAR and SURESH KUMAR, 2010). Additionally, the development of microorganisms is limited and the course of biochemical reactions is decelerated (CIURZYŃSKA and LENART, 2010a).

It is commonly known that drying in a vacuum from frozen state (freeze-drying) allows the product to be obtained with minimal changing, and after moistening it almost entirely recovers its properties. Such a method of drying is the most effective and progressive manner of wet product protection (NOVIKOVA *et al.*, 2003; LIAPIS and BRUTTINI, 2006). Lyophilization (freeze-drying) involves water removal from material which has previously been frozen. This process proceeds via ice sublimation, i.e. direct transition from solid to gaseous phase, omitting the liquid state. It may proceed properly only at low temperatures and with considerably lowered pressure (MARQUES *et al.*, 2006; LIU *et al.*, 2008).

Numerous studies on the application of freeze-drying have been conducted around the world, starting from studies on the application of lyophilisation for the preservation of strawberries, other fruits and vegetables, the preservation of meat, e.g. from reindeer, hen eggs or orange juice (LIAPIS and BRUTTINI, 2006; MARQUES *et al.*, 2006; CHAN *et al.*, 2009; HUANG *et al.*, 2009; CIURZYŃSKA and LENART, 2010a). Lyophilized products are most often encountered as ingredients of "instant" type dishes, but also of soup concentrates, breakfast or dinner ready meals and some confectionery (RATTI, 2001; PAN *et al.*, 2008; CHAN *et al.*, 2009).

Strawberries are a good source of vitamin C, folians, potassium and also other mineral components in smaller amounts, e.g. iron, magnesium or phosphorus. Unfortunately, these fruits have low durability and are not suitable for storing for a longer time without processing (CIURZYŃSKA and LENART, 2009b; HUANG *et al.*, 2009). Processed strawberries in the form of jellies (fruit gels) are used in the production of stuffed bakery products and confectionery, and also as products for direct consumption. Dried strawberries in turn can be used as a filling for dairy products, in cereals, teas or instant desserts, such as fruit creams or puddings (EL-BELTAGY *et al.*, 2007; HAMINIUK *et al.*, 2007).

A by-product in the form of strawberry powder is formed during dried strawberry production, and especially dried product grinding and this may also be used for the manufacture of valuable products. Both the market and consumer demand force the creation of new recipes (CIURZYŃSKA and LENART, 2010c).

The jelly is a gel product obtained from a solution of sugar and starch syrup with the addition of gelling agents, flavor-aroma, or if required also coloring substances. The consistency of the jelly should be gelatinous, elastic and dry matter content should not be lower than 74% (PN-A-88000:1993/A1 and PN-A-88108:1998).

Fruit gels are used the most often as fruit fillings for the manufacture of various kinds of products. In baked goods they are fillings of confectionery products processed thermally or not subjected to temperature activity. In confectionery, fruit fillings are used for pastry and chocolate products. In the dairy industry they are widely used for milk products such as yoghurts, milk drinks and desserts. Freeze-dried hydrocolloid gels could be useful as carriers for many food snacks, non-food matrices and biotechnological operations (NUSSINIVITCH and ZVITOV-MARABI, 2008).

Application of various kinds of fruit gel stabilizers is used for fruit processing due to the need for their preparation as a product for further processing. The kind of fruit and the destination is conditioned by the additive used for structure formation support. Selecting the gel composition and preparation conditions enables the creation of sponges with controlled microstructures and mechanical properties (NUSSINOVITCH *et al.*, 2000).

The aim of the study was to determine the influence of the modification of strawberry jelly composition on selected physical properties of the freeze-dried final product. Moreover, the study presents an attempt to elaborate the technology of powder-based strawberry jellies preserved via lyophilization.

The scope of the study included:

- determination of the appropriate amounts of: lyophilized strawberry powder, sodium alginate as a thickener and calcium lactate,
- an attempt at the regulation of strawberry jelly properties via the addition of glucose and/or chokeberry juice concentrate,
- an analysis of selected properties of strawberry jellies preserved via lyophilization.

2. MATERIALS AND METHODS

2.1. Materials

The raw material for the study was lyophilized strawberry powder obtained from lyophilized strawberry mix, stored in a dark place at room temperature manufactured by Department of Food Engineering and Process Management in Wuls-SGGW in Warsaw. The composition of the examined jellies also included sodium alginate and calcium lactate (both manufactured by Hortimex). The flavor and color of strawberry jellies were modified by the addition of crystalline glucose manufactured according to the PN-84/A-74771 standard (manufactured by JAR, Jaskulski Aromaty), and 67.5% chokeberry juice concentrate (manufactured by ALPEX Sp. Z o.o.).

2.2. Technological methods

The first stage of the study included an attempt at the determination of lyophilized strawberry powder content in jelly recipes, which would allow samples of the specified properties and parameters to be obtained (Table 1). Then, the optimum proportions of sodium alginate and calcium lactate were examined. At the next stage of the study, the properties of jellies obtained were regulated by the addition of glucose and/or chokeberry concentrate.

Then, analysis of strawberry jellies after lyophilisation was conducted. In some of the samples the examined physical properties were regulated by the addition of glucose and/or chokeberry juice concentrate, which were added before the introduction of thickening substances. After gelling, the jellies were cut into cubes of 1 cm sides.

The strawberry jellies were frozen in a National Lab GmbH freezer (ProfiMaster Personal Freezers PMU series) at a temperature of -70°C for 2 hours. The frozen cubes were next moved to a Christ Company ALPHA 1 – 4 LDC – 1 m lyophilizer with contact heating of the raw material. The process was conducted with constant parameters: pressure 63 Pa, safety pressure 137 Pa, temperature of lyophilizer heating shelves 30°C, time 24 hours.

After the lyophilisation process the jelly cubes were moved to glass vessels which were tightly closed and stored in a dark place at room temperature up to the time of analyses.

2.3. Analytical methods

2.3.1. Water activity determination

Water activity was determined in a Rotronic Hygroscop DT apparatus according to the manufacturer's manual at a temperature of 25°±1°C. Determination was conducted in four repetitions.

2.3.2. Dry matter content determination

Dry matter content for strawberry jellies was determined before and after lyophilization. Gel cubes were crumbled in a mortar. The crushed samples were weighed on analytical scales with an accuracy up to 0.001 g, about 3 g of homogenate to glass weighing bottles. The drying process was conducted for about 24 hours in a convection dryer at a temperature of 60°C until solid mass was obtained.

2.3.3. Color determination

Color was determined using a Minolta Company (Austria) Chroma – Meter CR – 300 apparatus in a LAB system. The examination was conducted with diffused lightning at an angle of 0°, the diameter of the measurement hole was 8 mm, standard light of C type. The measurements were conducted in five repetitions. Mean values were determined for the results obtained. Also, color coefficients were calculated. Diagrams were produced for the values obtained and standard deviations were determined as well.

Color coefficients were determined on the basis of equations:

$$H = \text{arctg}\,(b^* \times a^{(-1)})$$

H: hue angle
a*: red color coefficient
b*: yellow color coefficient

2.3.4. Rehydration property determination

Determination of rehydration properties involved holding strawberry jellies preserved via lyophilization in water for specified periods of time. The measurements were done at temperatures of 20° and 80°C for 10 minutes for all kinds of strawberry jellies in three replications (WITROWA-RAJCHERT and LEWICKI, 2006).

2.3.5. Mechanical properties determination

Determination of mechanical properties involved measurement of compressive force using a TA-XT2i Texture Analyzer device at a room temperature of 20°±2°C in five replications. The cubes were subjected to compression with a constant head speed of 6 mm/min up to the moment a 50% deformation in the initial height of the sample was obtained. Diagrams of compression curves were produced, and the work performed during 50% cube deformation was determined (CIURZYŃSKA and LENART, 2010b).

2.4. Statistical methods

The statistics package Statgraphics Plus v. 4.1 and Microsoft Excel 2002 were used to conduct the statistical analysis of the results. Standard

Table 1 - Recipes of strawberry jellies.

Lp	Liophilized strawberry powder [%]	Sodium alginate [%]	Calcium lactate [%]	Glucose [%]	Chokeberry concentrate [%]	Water [%]
1	10	1.5	0.05	0	0	88.45
2	7	1.5	0.05	0	0	91.45
3	7	1.5	0.05	5.2	0	86.25
4	7	1.5	0.05	0	5.2	86.25
5	7	1.5	0.05	5.2	5.2	81.05
6	7	1.5	0.01	0	0	91.49

deviations were determined for the averaged results, and an analysis of variance using the Fischer test was conducted to verify the hypothesis of the equality of object means, while the Tukey test was used for homogenous group determination.

3. RESULTS AND DISCUSSION

3.1. The influence of the participation and modification of lyophilized strawberry powder and calcium lactate on the properties of strawberry jellies preserved by freeze-drying

Strawberry jellies preserved by lyophilization of a basic composition, differing only in terms of the amount of lyophilized strawberry powder (jelly 1 and 2) and the amount of calcium lactate (jelly 2 and 6) were compared (Table 1).

3.1.1. Changes in water content and water activity

It was observed that for strawberry jellies preserved by freeze-drying a smaller addition of strawberry powder (2) led to a statistically significant decrease in water content (Fig. 1a).

A decrease in the amount of calcium lactate added up to 0.01% (6) statistically significantly influenced the increase in water content with respect to jelly containing 0.05% of calcium lactate (2) (Fig. 1a).

A decrease of up to 7% (2) in the amount of strawberry powder added, resulted in a statistically significant increase in water activity in the samples (2) in comparison to jelly (1) (Fig. 1b).

It was also demonstrated that the jelly preserved by lyophilization and of a lowered calcium lactate content of up to 0.01% (6) resulted in statistically significantly lower water activity (Fig. 1b).

3.1.2. Changes in color

The amount of strawberry powder added influenced the lightness coefficient L* of the surface of the freeze-dried final product. A decrease in the proportion of strawberry powder from 10% (1) to 7% (2) caused a darkening of the sample surface. Differences were not significant statistically (Fig. 2a). The results obtained confirm that the color of jellies preserved by lyophilization and with a lower strawberry powder content (2) is close to the natural color of freeze-dried strawberries (CIURZYŃSKA and LENART, 2009a).

A decrease in the amount of calcium lactate added of up to 0.01% (6) does not induce statistically significant changes in the lightness coefficient L* of the surface of the freeze-dried final

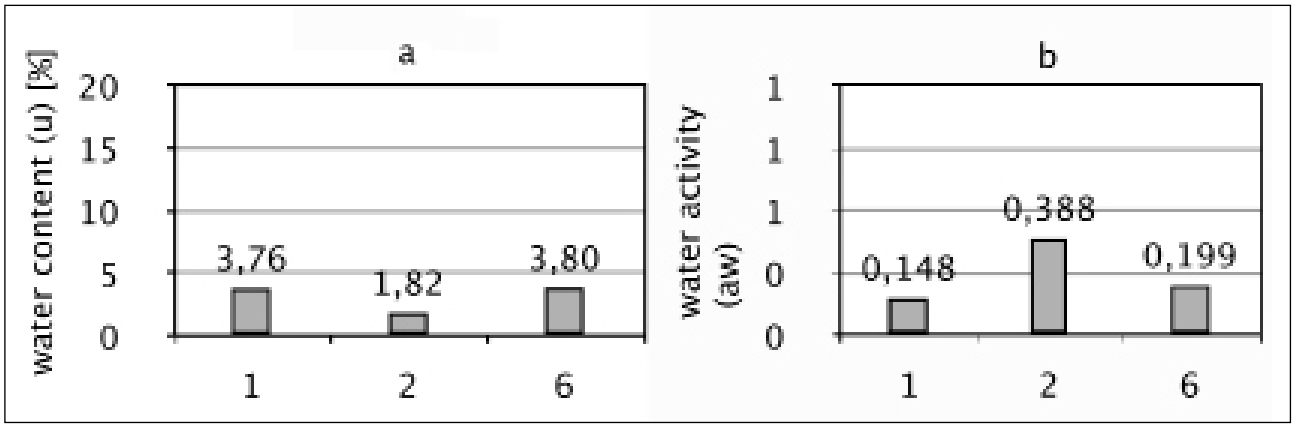


Fig. 1 - Water content (a) and water activity (b) of strawberry jellies in dependence on amount of added strawberry powder and calcium lactate. Designations in Table 1.

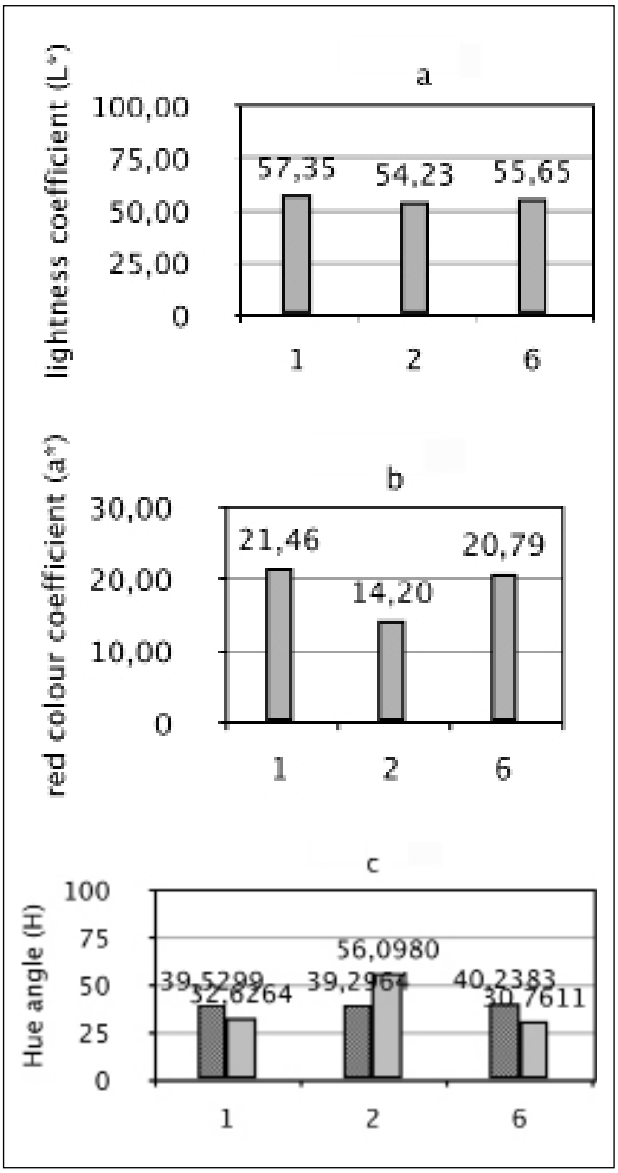


Fig. 2 - Lightness coefficient (L*) (a), red colour coefficient (a*) (b) and hue angle (H) (c) of strawberry jellies surface in dependence of amount of added strawberry powder and calcium lactate. Designations in Table 1.

product. No statistically significant changes in the examined index were noted after a lowering of the amount of calcium lactate added (6) (Fig. 2a). Appearance is an important factor, which gives quality to gelled products. Up to now, little work has been done to assess the appearance (color and transparency, the property of the material in which a negligible portion of the transmitted light undergoes scattering) of gelled products (CALVO and SALVADOR, 1997).

A decrease in strawberry powder content from 10% (1) to 7% (2) caused a statistically significant decrease in the red color coefficient (a*) of the surface of strawberry jellies preserved by freeze-drying (Fig. 2b), but a decrease in the amount of calcium lactate added - up to 0.01% (6) – induced a statistically significant increase in the red color coefficient (a*) of the surface of samples with respect to jelly containing 0.05% of calcium lactate (2) (Fig. 2b).

The hue angle (H) has the most significant correlation with visual scores and gives a good indication of the amount of color (SHISHEHGARHA *et al.*, 2002).

A decrease in the strawberry powder addition from 10% (1) to 7% (2) caused statistically significant changes in the hue angle (H) of the surface of strawberry jellies preserved by lyophilization (Fig. 2c). An approximately 23 unit lower value of the hue angle (H) was obtained for jelly with a 10% of strawberry powder content (1) when compared to the sample with 7% of the strawberry powder. The decrease in the value of the (H) index for lyophilized final samples with a 10% (1) addition of strawberry powder was influenced by an increase in the value of the red color coefficient (a*) and a decrease in the yellow color coefficient (b*).

An application of an amount of calcium lactate lowered by up to 0.01% (6) caused a statistically significant decrease in the value of the hue angle (H) in comparison with the jelly containing 0.05% of calcium lactate (2) (Fig. 2c).

3.1.3. Changes in mechanical properties

The lyophilization process influenced the distinct strengthening of the structure of strawberry jellies (Fig. 3). The course of the curve of compression for the jelly preserved by lyophilization with an addition of 10% strawberry powder (1) differs distinctly from the course of the curve of compression for the jelly with a 7% addition of strawberry powder (2). The differences are significant statistically (Table 2). The compression curve for jelly no 1 is characterized by a less smooth course when compared to jelly no 2, and a higher force is required during the whole compression time to deform it. A previous study by CIURZYŃSKA and LENART (2010b) on lyophilized strawberries gave results similar to those obtained for jelly after lyophilization with an up to 7% decreased content of strawberry powder (2), which confirms that sample no 2 possesses the features closest to the lyophilized strawberry.

The jelly with lactate content lowered by up to 0.01% (6) was characterized by a very delicate structure. The compression test could not be conducted, since the jelly cubes broke up immediately under the pressure of the texture meter head.

The alginate gel is considered a semi-solid material, and the junction zones at which the alginate polymers are bound represent the solid state. Reacting soluble sodium alginate with calcium results in the production of gels with various consistencies. To achieve a stiff texture, a small amount of calcium may be sufficient. In fact, the stoichiometric amount is 0.75 mg cal-

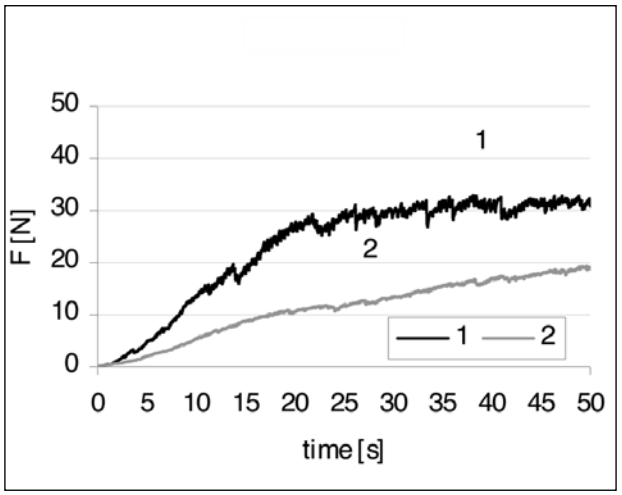


Fig. 3 - Mechanical properties (compression test) of strawberry jellies in dependence of amount of added strawberry powder. F – force necssery to 50% sample deformation. Designations in Table 1.

cium per gram of algin (depending on the viscosity grade of the selected sodium alginate). Optimal complexing of the alginate and calcium is obtained by using approximately 40% of the stoichiometric amount, which varies with calcium source and the presence of other soluble solids and pH, among other things (NUSSINOVITCH, 1997).

It was observed that when deforming jelly of a basic composition with a 10% strawberry powder content (1) 50% more compression work when compared to the sample containing 7% strawberry powder (2) must be performed (Table 3). A decrease in the lyophilized strawberry powder content from 10% (1) to 7% (2) weakened the structure of the samples and meant that about 50% less work was needed to be performed during the jelly deformation. The values for the work for the lyophilized jelly containing 7% (2) strawberry powder are similar to the values for compression work for the lyophilized strawberries (CIURZYNSKA and LENART, 2010b).

Table 2 - LSD test results for compression curves of strawberry jellies. Designations in Table 1.

LSD test; Statistical significant differences in significant level p < 0,05 bold.		
	1	**2**
1		**0,00**
2	**0,00**	

Table 3 - Average work value (W_{av}) necessary to 50% deformation of strawberry jellies. Designations in Table 1.

Freeze-dried strawberry jellies	Average work, W_{av} [J]
1	$1{,}12*10^{-1}$
2	$5{,}51*10^{-2}$

3.1.4. Changes in rehydration properties

Rehydration capacity and rate, in relation to drying, are the quality attributes which are the most often assessed in the literature (MEDA and RATTI, 2005).

The decrease in a contribution of strawberry powder from 10% (1) to 7% (2) influenced statistically significant increase in an amount of water absorbed by the strawberry jellies preserved by liophilization subjected to rehydration in water of a temperature of 20° and 80°C (Fig. 4a).

Statistically significant decrease in an ability for rehydration in water of a temperature of 20° and 80°C was noted for the strawberry jellies of lowered calcium lactate content up to 0.01% (6) with respect to the sample containing 0.05% of calcium lactate (2) (Fig. 4a).

Similar relationships were demonstrated during the examination of an influence of change in strawberry powder addition on the content of dry matter after rehydration of lyophilized final samples in water of a temperature of 20° and 80°C. The decrease in a contribution of strawberry powder from 10% (1) to 7% (2) influenced statistically significant improvement in rehydration properties of the dried materials. The jellies preserved by freeze-drying containing 7% of the strawberry powder absorbed more water (Fig. 4b).

Decrease in calcium lactate content from 0.05% (2) to 0.01% (6) caused deterioration of rehydration properties of the samples. They absorbed statistically significantly lower amount of water when compared to the sample no 2 during rehydration in water of a temperature of 20° and 80°C (Fig. 4b).

3.2. An influence of glucose and/or chokeberry juice concentrate addition on the changes in properties of strawberry jellies preserved by liophilization

An influence of glucose and/or chokeberry juice concentrate addition on the changes in selected physical properties of strawberry jellies was examined for the following samples: of a basic composition (2), with an addition of 5.2% of glucose (3), with an addition of 5.2% of chokeberry juice concentrate (4), and with an addition of 5.2% of glucose and 5.2% of chokberry juice concentrate (5) (Table 1).

3.2.1. Changes in water content

An increase in water content was obtained for all strawberry jellies preserved by lyophilization after an addition of glucose and/or chokeberry juice concentrate (3-5) with respect to the sample of a basic composition (2) (Fig. 5a), but only for the samples with glucose addition (3) the differences were statistically significant when compared to the sample no 2.

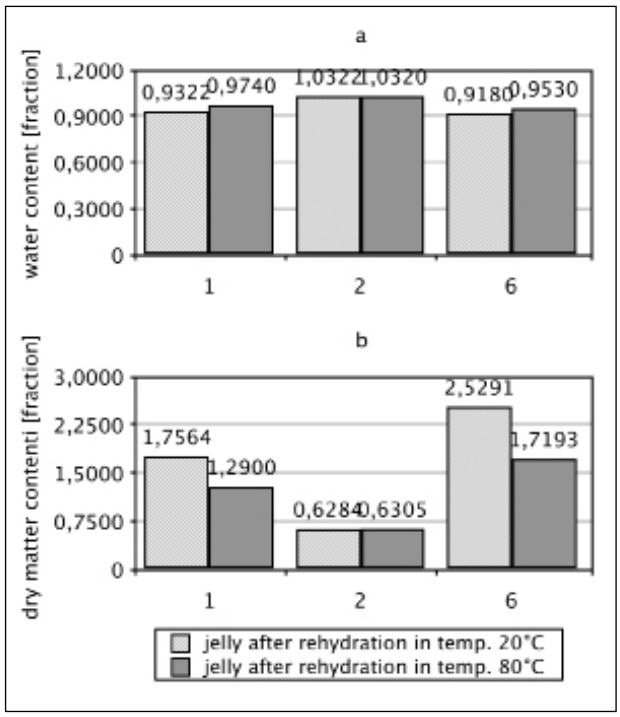


Fig. 4 - Changes in water (a) and dry matter content (b) during rehydration of strawberry jellies in water in temperature 20° and 80°C, in dependence of amount of added strawberry powder and calcium lactate. Designations in Table 1.

CIURZYNSKA and LENART (2007) in turn, in the study on lyophilized strawberries, demonstrated that an application of an osmotic dehydration of fruits in glucose solution influenced an increase in water content in dried material when compared to non dehydrated samples.

Statistically significant decrease in water activity in all examined gels kinds (3, 4 and 5) was noted in compared to the lyophilized strawberry jelly of a basic composition (2). The lowest value of water activity was obtained for the sample with an addition of 5.2% of glucose and 5.2% of concentrate (5) (Fig. 5b).

CIURZYNSKA and LENART (2010c) also observed in their study a decrease in water activity value for all strawberry jellies preserved by lyophilization with an addition of glucose and/or chokeberry concentrate with respect to the sample of a basic composition.

3.2.2. Changes in color

An application of an addition of chokeberry juice concentrate (4) or glucose together with concentrate (5) influenced lowering of the analyzed index, the surface of the jellies was darker when compared to the jelly of a basic composition (2). An increase in the lightness coefficient (L*) of the surface was observed for the jelly with only a glucose addition (3), but the differences were not significant statistically with respect to the jelly of a basic composition (2) (Fig. 6a). CIURZYŃSKA and LENART (2010c) demonstrated that the lightness coefficient (L*) is subject to decrease with the addition of glucose and/or chokeberry concentrate to the strawberry jellies. For freeze-dried strawberry dehydrated in glucose in turn, CIURZYŃSKA *et al.* (2011) noted that the lightness coefficient (L*) was increased when compared to non-dehydrated samples.

A statistically significant influence of glucose and/or chokeberry concentrate addition (3, 4 and 5) on the increase in the red color coefficient (a*) of the surface of strawberry jellies preserved by lyophilization with respect to the sample of a basic composition (1) was demonstrated. The highest value of the examined index, about 24 units, was obtained for the jelly with 5.2% chokeberry concentrate addition (3) (Fig. 6b).

CIURZYŃSKA *et al.* (2011) demonstrated that the red color coefficient (a*) of lyophilized strawberries dehydrated in glucose decreases when compared to non-dehydrated, freeze-dried strawberries. CIURZYŃSKA and LENART (2010c) confirmed, for the lyophilized strawberries, that an addition of glucose only to the lyophilized strawberry jellies causes an increase in the value of the red color coefficient (a*), but the addition of glucose and chokeberry concentrate causes a decrease in the examined coefficient.

It was observed that the addition of glucose and /or chokeberry juice concentrate in the case

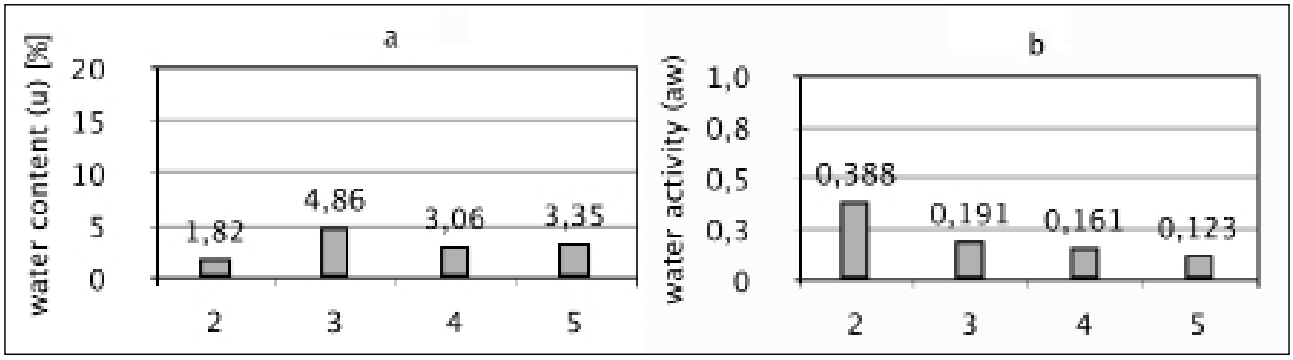


Fig. 5 - Water content (a) and water activity (b) of strawberry jellies in dependence on addition of glucose and/or chokeberry juice concentrate. Designations in Table 1.

Table 4 - LSD test results for compression curves of strawberry jellies. Designations in Table 1.

LSD test; Statistical significant differences in significant level p < 0,05 bold.				
	2	**3**	**4**	**5**
2		**0,00**	**0,00**	**0,00**
3	0,00		**0,00**	**0,00**
4	0,00	**0,00**		0,81
5	0,00	**0,00**	0,81	

of the three jellies under consideration (3, 4 and 5) caused statistically significant changes in hue angle (H) when compared to the jelly of a basic composition (2). An addition of glucose and/or chokeberry juice concentrate (3-5) caused a statistically significant decrease in hue angle (H) in comparison to the samples (2). The differences were 20, 42 and 41 units (Fig. 6c).

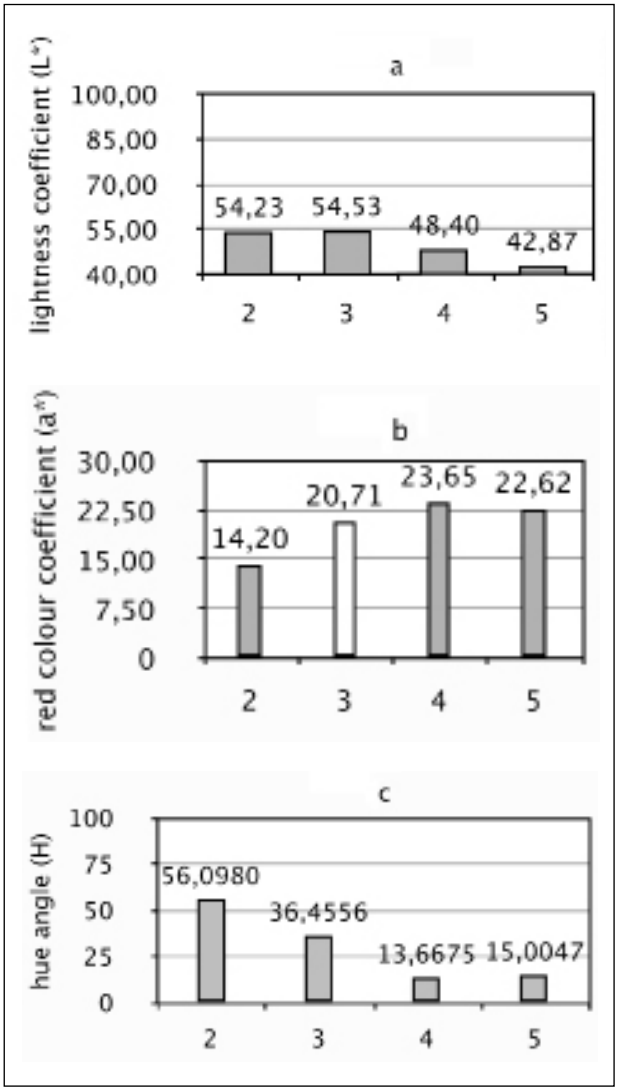


Fig. 6 - Lightness coefficient (L*) (a), red colour coefficient (a*) (b), hue angle (H) (c) of strawberry jellies surface in dependence of amont of added glucose and/or chokeberry juice concentrate. Designations in Table 1.

Modification of strawberry jelly composition by the addition of glucose and/or chokeberry juice concentrate caused the value of hue angle of the samples to move towards the value of that index for lyophilized strawberry from the study by CIURZYŃSKA *et al.* (2011), which was about 20 units.

3.2.3. Changes in mechanical properties

Distinct strengthening of the structure of the samples with an addition of glucose (3), chokeberry juice concentrate (4) and glucose with concentrate (5) was demonstrated for jellies preserved by lyophilization when compared to the jelly of a basic composition (2) (Fig. 7). It was demonstrated that the course of the curves is not smooth, as in the case of compression for jelly no 2. During the experiment, the jellies of a modified composition cracked and required the application of a higher force to cause 50% sample deformation with respect to the sample of a basic composition (2). The highest strengthening of the structure was noted for the sample with an addition of glucose and chokeberry juice concentrate (5) (Fig. 7) (Table 4).

NUSSINOVITCH *et al.* (2001) investigated the mechanical and acoustic properties of agar, gellan and kappa carrageenan based dried gels and assessed the effect of admixed sucrose and starch. It was found that the influence of these added ingredients on the dried gel density was well beyond their stochiometric contribution, i.e. their presence had a qualitative effect on the solid matrix properties. As expected, low density dried gels with an open cellular structure with thin walls are very brittle and fragile. Consequently, their compressive force-displacement curve, or mechanical signature, is irregular (jagged) and unreproducible.

Also, NUSSINOVITCH *et al.* (2000) showed that the incorporation of sugar into three gum matrices (agar, K-carrageenan and gellan gels) increases the brittleness of their dried gels. This resulted in a measurable increase in the jaggedness of the dried gels' mechanical signatures. Added sugar increased the dried gel stiffness, as expressed in the stress levels at comparable strains, but this was at least partly due to the higher density of the sugar containing products. The magnitude of the density increase could not have been predicted on the basis of stoichiometric relations, which indicates that the sugar in the gum-sugar matrix is not an inert "filler". It seems that added sugar can be a means to control, or produce, a desired brittleness. This seems to be a limit to how much sugar can be added, which in this work was around 10%. If greatly exceeded, it will cause the collapse of the sample during the gel drying.

It was observed for the strawberry jellies preserved by freeze-drying (3, 4, 5) that the work performed during 50% deformation of the sample is about 2 to 6 fold higher than the work

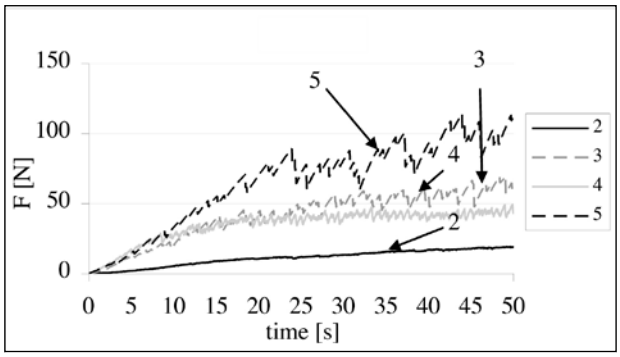


Fig. 7 - Mechanical properties (compression test) of strawberry jellies in dependence of addition glucose and/or chokeberry juice concentrate. F – force necssery to 50% sample deformation. Designations in Table 1.

needed for deformation of the jelly of a basic composition (2) (Table 5). An addition of glucose and/or chokeberry juice concentrate (5) causes the highest strengthening of the structure of the lyophilized final samples.

In their investigations NUSSINOVITCH *et al.* (2001) showed that the presence of starch and/or sugar in the solid matrix altered the solid matrix at a molecular level, i.e. it created solids with new mechanical properties. However while the presence of starch, at least at the concentrations studied, always stiffened the structure, the presence of sucrose could sometimes have the opposite effect.

It may be concluded from the study by CIURZYŃSKA and LENART (2009b) conducted on the lyophilized strawberries that the osmotic dehydration of fruits in glucose solution strengthens the structure of the dried material with respect to the strawberries not subjected to osmotic processing.

3.2.4. Changes in rehydration properties

Few papers deal with the rehydration of freeze-dried products (MEDA and RATTI, 2005; LEE *et al.* 2006; CIURZYŃSKA and LENART, 2010a). Freeze-dried foods are usually rehydrated by soaking them in water or other liquids, such as fruit or vegetable juices, cider, milk, glucose solution, etc., for a period of up to 2 h. During rehydration, the water uptake was faster during the initial stage and then the rate decreased gradually as the moisture content approached equilibrium when water nearly filled up all the pores and the fruit regained a considerable percentage of its original moisture content. Dehydrated products are usually rehydrated prior to their use (LEE *et al.*, 2006).

Modification of the composition of jellies preserved by lyophilization caused a deterioration of the rehydration properties of the samples (3, 4 and 5) when compared to the sample of a basic composition (2). It was demonstrated that the differences were significant statistically for those jellies subjected to rehydration in water at a temperature of 20°C. Application of a temperature of 80°C in turn led to an improvement in the rehydration degree of the samples with an addition of chokeberry juice (4) and glucose and chokeberry juice (5), between which significant statistically differences were not observed (Fig. 8a). After rehydration in water at temperatures of 20° and 80°C, the content of water in cubes of jellies no 3, 4 and 5 was to a smaller degree close to the content of water in samples before lyophilisation. The jelly with glucose addition (3) is characterized by the lowest degree of rehydration at temperatures of 20° and 80°C. The most profitable ability for rehydration among the jellies after lyophilization of the modified composition was obtained for the sample with the addition of chokeberry juice concentrate (4).

However, NUSSINOVITCH *et al.* (2000) maintain that the presence of sugar also increases the dried gel hygroscopicity, but this phenomenon was not investigated in this study.

Analysis of the changes in dry matter content resulting from rehydration also demonstrated a lower degree of reconstruction of final samples preserved via lyophilization with an addition of glucose and/or chokeberry juice concentrate (3-5) with respect to the sample of a basic composition (2) (Fig. 8b). The differences were significant statistically both for rehydration in water at temperatures of 20° and 80°C. Only the differences between jellies no 4 and 5 hydrated at a temperature of 80°C were not significant.

4. CONCLUSIONS

A decrease in the addition of strawberry powder from 10 to 7% caused higher water activity without significant changes in the water content of strawberry jellies preserved via liophilization. Also, a deterioration of the color coefficients, together with an increase in the ability of sample rehydration was obtained.

A decrease in the addition of calcium lactate from 0.05 to 0.01% to strawberry jellies caused lower values for water activity and an increase in the water content of the final product preserved via liophilization. Also, a deterioration of the color coefficients and mechanical properties (the samples were breaking up) and a lower rehydration capacity was obtained.

Modification of composition by the addition

Table 5 - Average work value (W_{av}) necessary to 50% deformation of strawberry jellies. Designations in Table 1.

Strawberry jellies	Average work, W_{av} [J]
2	$5{,}51*10^{-2}$
3	$2{,}02*10^{-1}$
4	$1{,}17*10^{-1}$
5	$3{,}20*10^{-1}$

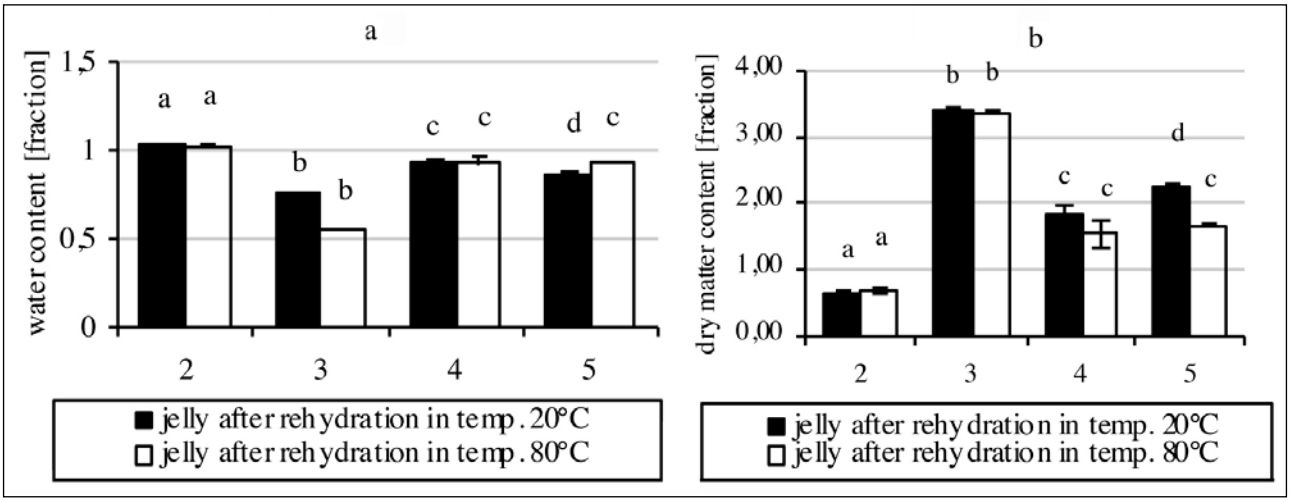


Fig. 8 - Changes in water content (a) and dry matter content (b) during rehydration of strawberry jellies in water in temperature 20° and 80°C, in dependence on addition glucose and/or chokeberry juice concentrate. Designations in Table 1.

of glucose and/or chokeberry juice concentrate to strawberry jellies preserved via lyophilization caused lower water activity values and increased water content. Also, a deterioration of the color coefficients was obtained for those jellies with an addition of glucose or an addition of glucose and chokeberry concentrate. Strengthening in the structure and deterioration of rehydration properties was noted for all the examined lyophilized fruit jellies.

Based on the results of this study and observations performed while obtaining strawberry jellies preserved via lyophilization, it was demonstrated that the sample with 7% strawberry powder and 0.05% calcium lactate, enriched with an addition of 5.2% chokeberry juice concentrate is the product with the best quality features.

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PAPER

ULTRASOUNDS APPLICATION IN WINEMAKING: GRAPE MACERATION AND YEAST LYSIS

P. FERRARETTO, V. CACCIOLA, I. FERRAN BATLLÓ and E. CELOTTI*
Department of Food Science, University of Udine, Via Sondrio 2/A, 33100 Udine, Italy
*Corresponding author: Tel. +39 0432 558140, Fax +39 0432 558130,
email: emilio.celotti@uniud.it

ABSTRACT

The chemical and mechanical effects of ultrasound (US) are interesting in the food industry and this work attempts to study new applications on grapes as the raw material and then in the vinification process.

Phenolic compounds extracted from grapes by means of disruption of the cell wall due to pressure alternance and cavitation were studied. The tests carried out showed an improvement in the extraction of polyphenolic substances, with a reduction in the duration of classic maceration.

Also the lytic effect of the US on yeast cell structures, in order to accelerate the process of ageing over lees was evaluated, with the liberation of different fractions in the wine.

- Keywords: ultrasound, extraction, maceration, phenolic compounds, lees, yeast -

INTRODUCTION

The Ultrasound technology is based on mechanical waves at a frequency higher than the upper limit of human hearing (> 16kHz). Being sound waves, ultrasound is transmitted through any substance, solid, liquid or gas, which possesses elastic properties. These waves travel either through the bulk of a material or on its surface at a speed which is characteristic of the nature of the wave and the material through which it is propagating (POVEY *et al.*, 1988; MASON, 1998; KNORR *et al.*, 2004). In the food industry, ultrasound can be divided into two frequency ranges: High frequency ultrasound (100 kHz - 1 MHz) and Power ultrasound (16-100 kHz).

High frequency ultrasound is commonly applied as an analytical technique to provide information on the physicochemical properties of food such as ripeness, sugar content, acidity, etc (DEMIRDÖVEN and BAYSAL, 2009). The application of Power ultrasound is so large that it is used to alter either physical or chemical properties of food instead (MCCLEMENTS, 1995). This Power ultrasound has been used for many years to generate emulsions, disrupt cells and disperse aggregated materials; more recently some applications have been identified with greater potential for future development, e.g. modification and control of crystallization processes, degassing of liquid foods, enzymes inactivation, enhanced drying and filtration and the induction of oxidation reactions (KNORR *et al.*, 2004; MCCLEMENTS, 1995; ROBERTS, 1993, ZHENG and SUN, 2006).

The fundamental effect of ultrasound on a continuum fluid is to impose acoustic pressure (P_a) in addition to the hydrostatic pressure already acting on the medium. The combination of factors such as heat, pressure and turbulence, is used to accelerate mass transfer in chemical reactions, create new reaction pathways, break down and dislodge particles or even generate different products from those obtained under conventional conditions (SUSLICK, 1988).

The use of ultrasonics in industrial processes requires two main factors: a liquid medium (even if the liquid element forms only 5% of the overall medium) and a source of high-energy vibrations (the ultrasound).

Over the last few years, ultrasonic devices suitable for characterizing foods have been developed.

The interaction of acoustic energy with a food is mainly substantiated through a liquid medium because the cavitation and cavitation-induced physical and chemical actions play an important role in the food quality alterations in an ultrasound-processed food.

At a solid and liquid interface, the water jet formed by an imploding transient cavitation is also a factor that may contribute to some changes in the overall properties of a food product. It is noteworthy that all the chemical and physical effects of ultrasounds are microscopic. However, the interplay of these cavitation-induced chemical and physical activities with a food, is manifested through macroscopic changes that can be perceived by consumers and may be expressed through terms such as texture, color and flavor (FENG and LEE, 2011).

The properties of power ultrasound waves have aroused interest in the food industry, since the promotion of physical and chemical reactions can lead to a strategic advantage in processing. Since the '90s, ultrasound treatments have become a widely applicable alternative in the processing industry (BATES and PATIST, 2010); ultrasound is applied in food technology for its mechanical and/or chemical effects on homogenization, mixing, extraction, filtration, crystallization, dehydration, fermentation, degassing, defoaming, particle size reduction, temporary or permanent changes of viscosity, modulation of growth of living cells, cells disruption and aggregate materials dispersion, inactivation of microorganisms and enzymes, sterilization of equipment (MASON *et al.*, 1996; CHEMAT *et al.*, 2010; CHANDRAPALA *et al.*, 2012). For these reasons, the effects of this technology are interesting in the food industry, but there has not been much research done on fermented beverages, especially regarding the winemaking process. Some research concerning the wine sector has been conducted, but it was not designed for an application in the winemaking process, considering collateral aspects, such as the antioxidant effects and barrels sanitation (GHAFOOR and CHOI, 2009; PALACIOS *et al.*, 2012; MORELLI and PRADO 2012; LIEU and LE, 2010; GONZÁLES *et al.*, 2006; TIWARI *et al.*, 2010). A possible application of ultrasound in the wine industry could be an important technological innovation that fastens some thermodynamically possible, but kinetically slow reactions required in the winemaking process.

This work attempts to study new applications on grapes as the raw material and in the vinification process. Firstly, the extraction of phenolic compounds from grapes obtained by the disruption of the cell wall due to pressure alternance and cavitation was studied. Studying the release of these compounds was the starting point of this work. The second part regarding application on lees, had the objective of evaluating the lytic effect of US on yeast cell structures, with liberation of different fractions into the medium in order to accelerate the process of ageing over lees.

MATERIALS AND METHODS

Ultrasound (US) treatment

An ultrasonic processor (SONOPLUS HD 2200, Bandelin electronic, Berlin, Germany) with 13 mm sonotrode probe made of titanium was

used for sonication. Samples were processed in a continuous sonication at a constant frequency of 20 kHz. The energy input was controlled by setting the amplitude of the sonicator probe; the total nominal output was 200 W. The ultrasound probe was submerged to a depth of 50 mm in the sample.

The amplitude levels and processing times were varied on the basis of the experiments, and based on tests carried out prior to this study. The final treatment temperature was measured for all samples.

Phenolics extraction from the grape berry skin

Samples

For the trial regarding the extraction from grapes, a wide set (No. 40) of different varieties of red grapes sampled at technological maturity was evaluated for the tests conducted in laboratory. They were sampled directly upon delivery in the cellar, in order to have a representative and real sampling in relation to the ripening degree of the grapes delivered to the cellar. A representative sample (250 g), stalk removed and manually crushed, was put in a 250 mL beaker and subjected to the treatment. To obtain a wide statistical basis, it was decided to increase the number of varieties in a single test to assess the extent of the extraction. The polyphenols extracted were evaluated in the liquid obtained by centrifugation from the treated grapes.

Treatment conditions

The conditions promising the best results in terms of extraction following a preliminary test (treatment time from 1 to 5 minutes at three different levels of amplitude – 30, 60 and 90%) were chosen for the laboratory tests. The treatment conditions selected to study phenolics extraction were 3 and 5 minutes at the amplitude of 90%. At 5 minutes of treatment time, the maximum sample temperature reached was 47.6°C for the amplitude level of 90%.

Micro-vinification trials

Samples

In order to perform maceration and fermentation trials, it was decided to use some crushed samples (800 g) of the same grapevine variety (Merlot), but with a different polyphenolic content, replicated in three series (series 1 and 3 at the same level and series 2 with a higher anthocyanins content) in the cellar. They were previously subjected to ultrasonic treatment (every sample was divided in 4 parts and treated separately in a 250 mL beaker, in order to allow the use of the probe; after the treatment the 4 parts were all mixed together), and then micro-vinified and racked sequentially.

A reference sample was prepared for all the tests in order to have the sample that simulated the traditional maceration without the US treatment compared to the one that was treated.

All samples, including the reference, were placed in open containers (2 L) and placed in equal conditions of maceration-fermentation in order to make comparable the phenolics extraction.

The maceration conditions provided were: a constant temperature of 25°C, 3 manual punching down of the cap a day, 60 ppm SO_2, inoculum of dry yeast for oenological use *Sacch. Cerevisiae* on the first day and at the end of fermentation the separation of the solid parts was carried out with a manual press.

The effect on wine color was studied on micro-vinification trials of the samples performed directly in a cellar due to the easy access to different grape samples having reached the technological maturity. The maceration time for the ultrasound (US) treated samples was 2, 3 and 4 days, while for the control sample (untreated) it was 5 days.

Ultrasound treatment conditions

A treatment of 5 minutes at the amplitude level of 80% was carried out to assess ultrasound effects on the maceration kinetics. At 5 minutes of treatment time, the maximum sample temperature reached was 46.9°C for the amplitude level of 80%.

Analytical methods

Analysis of Total Phenolics Index - Abs 280 nm (Ribéreau-Gayon 1970), colour intensity (Glories 1984), hue (Sudraud 1958) and anthocyanins content (Ribéreau-Gayon and Stonestreet 1965) (using 2% Chloridric acid, 0.1% acidified Ethanol and 20% Potassium Metabisulphite solutions as reagents) were conducted on grape samples right after the crushing (reference value) and right after the treatment (test results) once centrifuged at 3,000 rpm for 10 minutes.

To be able to compare the increase in compounds extracted versus the classical vinification, a reference sample followed extraction with a wine-like solution (5 g/L Tartaric Acid (Carlo Erba, Milano, Italy), 12% (v/v) Ethanol (Carlo Erba, Milano, Italy), pH was adjusted to 3.2 with NaOH) or Methanol (Carlo Erba, Milano, Italy) (1% acidified with Chloridric Acid) for 4 hours, then centrifuged and analyzed.

Anthocyanins content (RIBÉREAU-GAYON and STONESTREET, 1965) and total polyphenols index - Abs 280 nm (RIBÉREAU-GAYON, 1970) of micro-vinified and reference samples were analyzed daily. In addition to these analyses, tannins content (Bate-Smith, 1954) (using Acid Bu-

tanol) and polymerized pigments index (Glories, 1978) (using buffer alcoholic solution, water and 20% Potassium Metabisulphite solution) were analyzed after racking (after 2, 3 and 4 days for the treated sample and 5 days for the control).

Lees treatment

Ultrasound treatment

The same treatment was used for the extraction tests, the only difference is that in this case the probe was submerged to a depth of 30 mm in the 250 mL beaker containing the sample.

The optimization of the best conditions of lees extraction (soluble colloids and proteins extraction, particle size, surface electrical charge and Peak Height 16.5 kDa (RI)) was carried out using an RMS (Response Surface Methodology) design. The experimental design employed was a central composite design for two independent variables each at five levels using Modde statistical software (Modde version 8.0.2). The experimental order was randomized. The levels of independent variables, namely amplitude level and sonication time, were selected based on the values obtained in preliminary experiments. The amplitude level was varied between 30 and 90% and the sonication time between 1 and 5 min. The optimized design described in eleven experiments (8 + 3 replicates in the center point) the three-dimensional curvature of the response surface.

The validity of the model was determined by comparing the experimental and predicted values.

An analysis of variance (ANOVA) with 95% confidence level was then carried out for each response variable in order to test the model significance and suitability.

Treatment conditions

The conditions provided by the experimental design were 1, 3 and 5 min at 30, 60 and 90% of amplitude, with 3 replicates of the test at 60% for 3 minutes.

Samples

Samples to evaluate the lytic effect caused by ultrasound on yeast cell structures, tests were carried out on lees samples (250 mL) collected after the second racking, three months after vinification, from a Chardonnay wine fermented with commercial active dry yeast *Sacch. Cer.* The samples were added to a wine-like solution in order to obtain liquid lees with 50% of humidity.

Analytical methods

After centrifugation (3,000 rpm for 15 min) and microfiltration (0.8 and 0.45 μm), both the reference and the tests were analyzed in terms of soluble colloids (using Chloridric Acid - 1:1 and 96% Ethanol) (Usseglio-Tomasset and Castino, 1975) and soluble proteins (Lowry method: using 2% Na_2CO_3 in 0.1 M NaOH, 1% $CuSO_4 \times 5H_2O$ in distilled water, 2% Sodium Tartrate in distilled water and Folin-Ciocalteau reagent) (Regenstein and Regenstein, 1984), before and after adding PVPP (Sigma-Aldrich LLC, St. Louis, MO, USA) to the sample. Other specific analyses were conducted: the particle size distribution of the supernatant directly and filtered at 0.8 μm was determined by laser diffraction using a Submicron Particle Sizer – NICOMP (Santa Barbara, USA), with 1.33 as the refractive index of the colloidal dispersed phase (Celotti *et al.*, 2003); and the surface electrical charge with the Particle Charge Detector – MÜTEK PCD-02 (Germany) (FERRARINI *et al.*, 1996).

Molecular mass determination

To evaluate the modification of the composition of the colloidal fraction, molecular mass determination was carried out by gel permeation high pressure liquid chromatography (HPLC) using a TSK-GEL G2000SWXL (Tosoh Bioscience LLC, PA, USA) column and pre-column, alcohol tartaric buffer as eluent with pH 3.2 and flow rate 0.6 mL/min. Two detectors were used: UV lamp (JASCO 875) to measure the phenolic and protein fraction; refractive index (VARIAN 350 RI) for the colloidal carbohydrate fraction. The samples were filtered on nylon (Whatman – Little Chalfont, UK) at 0.45 μm and immediately injected.

Calibration curves were constructed using standard proteins of well defined molecular weight in the range of 14 to 70 kDa for globular proteins (Lisozyme WT 14 kDa, β-Lactoglobulin 36 kDa, Ovoalbumin 44 kDa e Bovine Serum Albumin (BSA) 67kDa, Sigma-Aldrich LLC, St.Louis, MO, USA).

The values were estimated on the basis of the corresponding elution volumes.

RESULTS AND DISCUSSION

Phenolic compounds extraction (from grape)

Ultrasonic treatment, with high-frequency pressure waves and the cavitation phenomenon in a solid-liquid medium, such as pressed crushed grapes, was responsible for accelerating the release and the dissolution of phenolic matrix contained in epidermal tissues.

The total polyphenols index, the anthocyanins and the colour intensity, related to the control sample extracted in a wine-like solution and in acidified methanol, proportionally increased (50% or more) with the time and amplitude of treatment for all grapevine varieties included in this work (Figs. 1 and 2). Under the

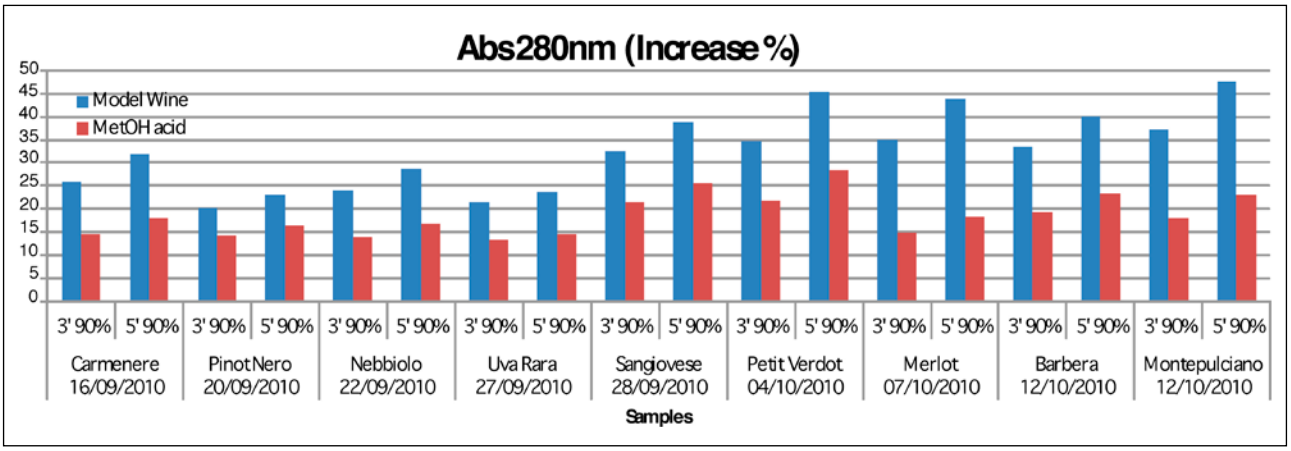


Fig. 1 - Percentage Increase of Abs 280 nm of some red grapes varieties treated with US (3 and 5 minutes at 90% of amplitude) compared to the control extracted in model wine and in acidified methanol, for the phenolics extraction from the grape berry skin trials.

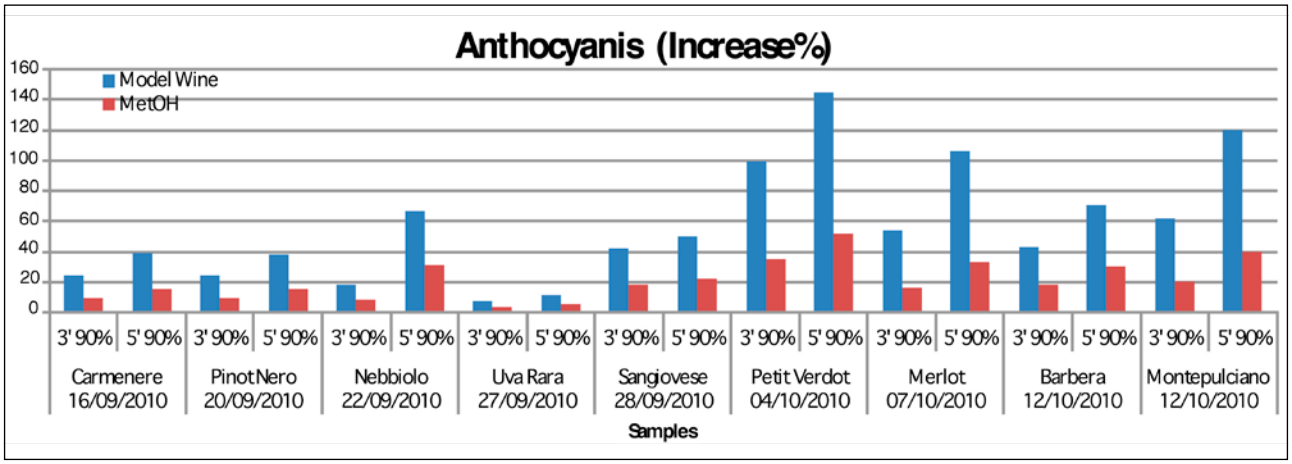


Fig. 2 - Percentage Increase of Anthocyanins of some red grapes varieties treated with US (3 and 5 minutes at 90% of amplitude) compared to the control extracted in model wine and in acidified methanol, for the phenolics extraction from the grape berry skin trials.

same treatment conditions, the total polyphenols index and the anthocyanins proved different depending probably on the characteristics (cell wall maturity, pectin content) of the different grapes varieties.

The tests carried out to evaluate the immediate effect on the color showed an important interaction between the cavitation phenomena and skin cell wall, leading to a higher color intensity and anthocyanins content. The compound extraction increases directly with the amplitude level (r^2=0.915) (Table 1).

Table 1 - Change in Colour Intensity (CI), hue and anthocyanins content of the sample treated with ultrasound at different amplitudes (20, 40, 60 and 80%) for the same time (2') for the phenolics extraction from the grape berry skin trials.

Samples	C.I.	Hue	Anthocyanins (mg/kg)
Control (untreated)	2.77	1.52	23
2' 20%	6.13	1.59	42
2' 40%	7.50	1.58	60
2' 60%	9.01	1.49	69
2' 80%	9.44	1.52	81

Micro-vinification trials

The micro-vinification of samples treated with ultrasound and racked after 2, 3 and 4 days respectively, compared to the control sample with classic maceration and racked after 5 days, provided interesting results (Fig. 3).

The crushed treated vinified samples had a higher polyphenol content in comparison with the reference at the beginning of maceration. The same was found at the end of the process and it influenced the total polyphenols index more than the anthocyanins content.

The three different series gave different results, especially series 2 with a higher initial phenolic content. Generally, it is possible to affirm that the variability of the US treatment effect depends on the quality and the polyphenols content of the raw material. Focusing on process

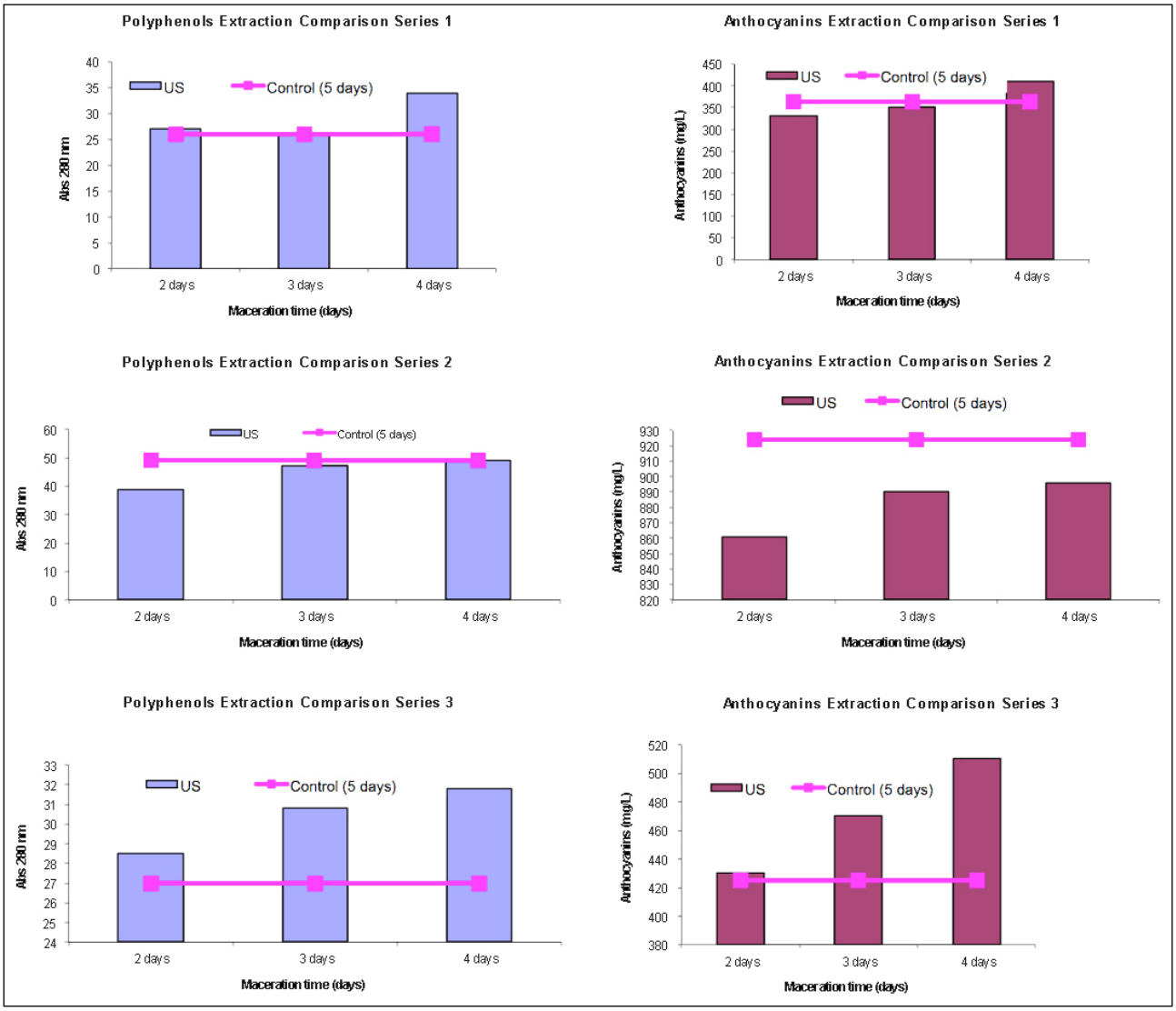


Fig. 3 - Comparison of the extraction of polyphenols and anthocyanins on wine after 2, 3 and 4 days of maceration (Merlot) with respect to the control (5 days of maceration) for the series 1, 2 and 3, in the micro-vinification trials.
Control = reference sample; US = samples treated with ultrasound

optimization, a maximum of three days maceration time could be saved with respect to the five days in the classical vinification (Fig. 3).

The results have shown a reduction from 1 to 3 days of maceration time for both the phenolic and anthocyanins content. Consequently, the US treatment could be considered an auxiliary technology during maceration.

Series 3 treated with US and 4 days maceration showed a higher polyphenols index compared to the reference probably due to the significant tannin extraction from grapes. Tannin extraction could be a positive index to improve color stability (data not shown). The results highlight the important effect on the total tannic fraction. This aspect definitely makes the use of US attractive in vinification, not only to extract color, but also stabilizing factors such as tannins.

Anthocyanins polymerization was also analyzed and gave good results for series 1 and 3, although there were no significant differences.

Lytic effect of US on yeast cell structures

Trials on lees proved efficient in increasing the soluble colloids, which are a function of the duration and wave amplitude. The extraction was significant for short times and intermediate amplitudes (Fig. 4). Another result was that the increase in soluble proteins after the PVPP treatment was proportional to time, regardless of the amplitude used.

HPLC-gel permeation confirmed the same results. With Refractive Index Detector, three different fractions were highlighted with 86.6, 16.7 and 15.4 KDa. On the other hand, the UV detector showed a higher diversity of fractions (phenolic and protein type). The most interesting peak (with 16.7 kDa) was found with both

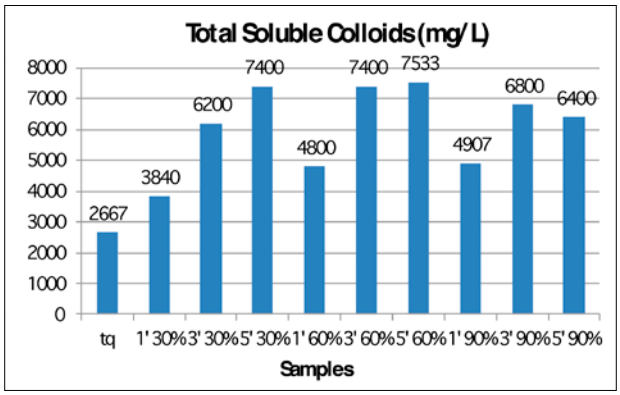


Fig. 4 - Effect of US treatment on the extraction of soluble colloids from fine lees.
tq = reference sample.

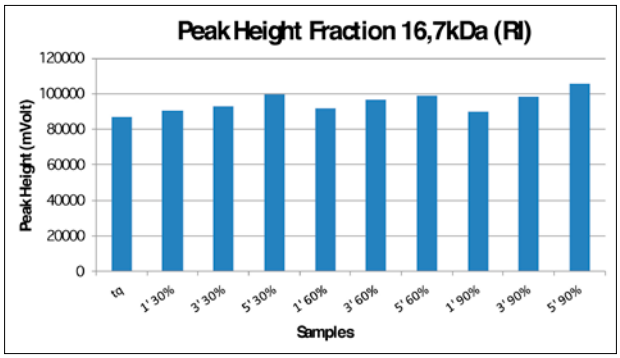


Fig. 6 - Effect of US treatment on lees: Change in peak height of the 16.7 kDa mass fraction detected with RI after US treatment.
Tq = reference sample.

detectors and is supposed to be a glycoprotein fraction which is in agreement with the kind of substances released by wine lees. The consequence of the cavitation phenomena on lees is the hypothesized release of glycoproteins and polysaccharides.

The hypothesis above is also confirmed by the chromatogram in Fig. 5, where this fraction did not change after a treatment with PVPP, gelatin and carbon.

The treatment entailed a concrete enrichment of the medium in colloidal fractions, presumably glycoproteins, as a result of the cavitation effect on yeast cells present in the lees.

The fractions detected with the RI also showed an increase, which leads to hypothesizing an increment of polysaccharides (Figs. 6 and 7).

A more accurate technique, such as the HPLC-gel permeation, for the evaluation of the release of colloidal substances, resulted comparable with the determination of soluble colloids. This analysis becomes therefore a valid indication of the effect of cavitation on the yeast cells and about the effectiveness of the treatment.

Model fitting

The experimental results obtained as a function of ultrasound amplitude level (%) and sonication time (min) are shown in Table 2.

Time and amplitude have significant positive effects treatment process yield. The enhancement of soluble colloids extraction is interesting for all the different amplitude levels considered, also for short times.

As a result, application of ultrasounds in lees treatment accelerates the enrichment of the media more than the traditional treatment and the process time was shortened over a few months.

During the sonication treatment an increase in the values of the negative surface electrical charge, compared with the untreated sample, was observed, under the different treatment conditions, with the longer times and higher levels of amplitude (60 and 90%) having a higher impact.

This increase of the negative surface electrical charge, consequently to the treatment, could suggest a release of polysaccharide and phenolic component, but also a possible breaking of the

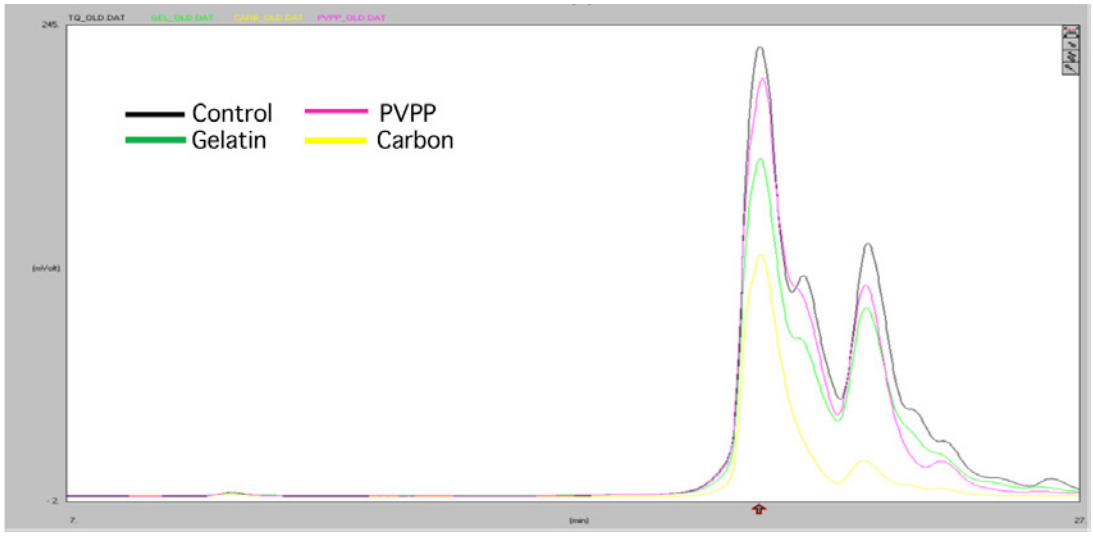


Fig. 5 - HPLC - gel permeation: UV profile change (λ 280 nm) of wine (control) after a treatment with PVPP, gelatin and carbon.

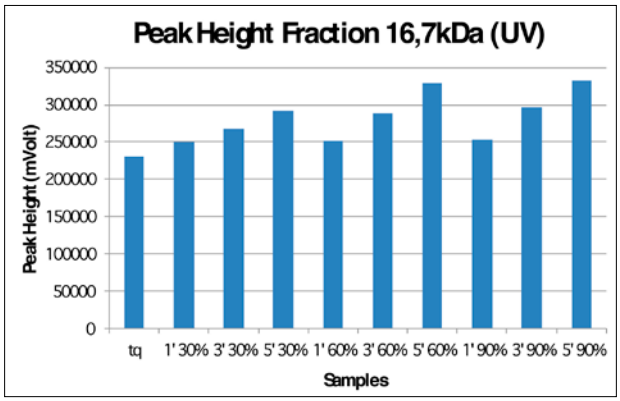


Fig. 7 - Effect of US treatment on lees: Change in peak height of the 16.7 kDa mass fraction detected with UV after US treatment.
Tq = reference sample.

protein polypeptide chains, with a release and an increase in their negative functional groups.

Regarding the particle size, the values confirm that the ultrasonic treatment causes a reduction of the particle diameter, as reported in MASON *et al.* (1996) and PATIST and BATES (2008) with a minimal impact at the short times, independently from the percentage of amplitude.

The statistical parameters of the response surface model are shown in Table 3. The model presented showed high correlation coefficients for all the parameters considered, except for the protein content. This model demonstrated to be an effective technique for investigating extraction as a function of amplitude level and sonication time for lees. The coefficients of determination for predicted parameters showed good correlation with the experimental data at the 95% confidence level. The work demonstrates that sonication significantly influences the yield extraction.

CONCLUSIONS

The tests carried out on different grape varieties showed how a few minutes of treatment at different amplitude levels can achieve an improvement in the extraction of polyphenolic substances, and in terms of time, a progressive reduction of up to 30% of the classic maceration duration.

This represents a possibility to optimize fermentation technology and, therefore, a better management of red grape vinification. Furthermore, application of ultrasound can be considered as a continuous pre-treatment of crushed red grapes before loading the vinification tank, in view of the very short treatment times.

The treatment of fermentation lees due to the yeast cell disorganization and the easier particu-

Table 2 - Experimental design matrix and responses for the lees trial.

Exp No.	Run order	Temperature (°C)	Amplitude (%)	Time (min)	Total Colloids (mg/L)	Proteins (mg/L BSA)	RI Peak Height (16.5 kDa)	(-) Surface electrical charge (mq/L)	Size Ø (nm)
1	11	23.7	30	1	3,840	12,202	92,464	4.39	865
2	7	23.2	90	1	4,907	11,101	94,748	4.10	799
3	5	33.1	30	5	7,400	15,400	98,788	5.69	367
4	1	42.4	90	5	6,400	14,623	112,314	6.02	605
5	2	24.2	30	3	6,200	11,968	96,741	4.72	613
6	8	39.5	90	3	6,800	12,494	98,658	5.80	513
7	9	25.0	60	1	4,800	11,706	94,291	4.24	798
8	3	38.6	60	5	7,533	12,936	103,678	6.18	546
9	6	33.1	60	3	7,440	13,097	97,809	5.65	395
10	10	32.7	60	3	7,280	11,864	100,493	5.74	496
11	4	32.0	60	3	7,480	11,927	99,691	5.70	332
Control (untreated lees)					2,667	10,475	88,657	0.039	1,140

Table 3 - Statistical parameters related to the experimental design for the lees trial.

Coefficient	Total Colloids (mg/L)	Proteins (mg/L BSA)	RI Peak Height (16.5 kDa)	(-) Surface electrical charge (mq/L)	Size Ø (nm)
F	336.467	4.33459	11.7391	64.3699	10.9615
P	0.000	0.090	0.017	0.001	0.019
R^2	0.997629	0.845159	0.938178	0.987742	0.942299
R^2 Adj.	0.994664	0.65161	0.860901	0.972418	0.870172
Q^2	0.865605	0.331127	0.679921	0.849586	0.599272
Model Validity	0.855729	0.758821	0.615231	0.295293	0.917044
Reproducibility	0.994003	0.734097	0.940171	0.996695	0.813579

late cell leakage promotes the lysis of yeast with a rapid release of colloids, polysaccharides and mannoproteins, providing also a possible reduction in wine ageing on lees.

The results obtained confirm the extractive and lytic effects of the ultrasonic treatment on food matrices and could be applied on a technological level to optimize some technological processes in the wine industry.

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PAPER

ANTIOXIDATIVE PEPTIDES DERIVED FROM DENATURATED EGG WHITE PROTEIN

A. ZAMBROWICZ, M. POKORA, E. ECKERT, J. CHRZANOWSKA, M. SZOŁTYSIK, A. DĄBROWSKA and T. TRZISZKA
Wroclaw University of Environmental and Life Sciences, Department of Animal Products Technology and Quality Management, Chelmonskiego 37/41, 51-630 Wroclaw, Poland
*Corresponding author: Tel. +48 71 320 7773,
email: graszkiewicz@o2.pl

ABSTRACT

The antioxidant activity of denaturated egg white protein preparation (EP) was evaluated by enzymatic hydrolysis. The EP treated with digestive and microbial proteases resulted in different degradation products confirmed by degree of hydrolysis (DH) and free amino groups concentration (FAG) measurement. The highest free radical scavenging activity reached 0.21 μmol $trolox_{eq}$/mg and was exhibited by tryptic hydrolysate. EP hydrolysates showed significant, enzyme-dependent ferric reducing activity. The highest chelating activity was observed for protein preparations hydrolyzed with neutrase (397.0 μg Fe^{2+}/mg). Antioxidative peptide fractions were isolated from tryptic hydrolysate by reserved phase high pressure liquid chromatography (RP-HPLC). Among the obtained fractions, we identified: ISQAVHAHAEINEAGR, SVL, QL and AP, KVR antioxidative peptides corresponded to ovalbumin and ovotransferrin fragments, respectively.

- Keywords: antioxidant, DPPH, hydrolysis, ovalbumin, peptide -

INTRODUCTION

Biologically active proteins from eggs, like lysozyme and cystatin which can be used as nutraceuticals in the production of functional food or therapeutic agents, are very attractive substances for both the food and pharmaceutical industries (MINE and KOVACS-NOLAN, 2006; TRZISZKA *et al.*, 2004). In the process of their isolation according to SOKOŁOWSKA *et al.* (2007), ethanol denaturated egg white protein preparation of low functional properties is produced as by-product.

One of the best methods of modifying such protein is enzymatic hydrolysis (CHEN *et al.*, 2011; SATYA *et al.*, 2011). This process leads to the improvement of functional and biological properties of the protein due to release from its sequence a number of peptides which would have a beneficial physiological effect in the organism through interaction with the circulatory, nervous, digestive and immune systems (IWANIAK and MINKIEWICZ, 2007; MIGUEL *et al.*, 2006). There are many reports about the biological activity of hydrolysates and peptides obtained from ovalbumin, ovotransferrin, ovomucin or lysozyme digestion (TSUGE *et al.*, 1997; MINE and KOVACS-NOLAN, 2006; MIGUEL *et al.*, 2006). Special attention has been given to peptides with antioxidant activity as a new source of natural antioxidants that inhibit oxidation both in food and in the human body (GALLEGOS-TINTORÉ *et al.*, 2010; SAKANAKA *et al.*, 2004 and 2006). Antioxidants occurring in the diet play an important role in combating oxidative stress in the body (SUETSUNA *et al.*, 2000). Due to the participation in lipid peroxidation, protein and DNA modifications, free radicals may damage cells and tissues (SUETSUNA *et al.*, 2000). It has been shown that they are also involved in the occurrence of many chronic diseases such as cardiovascular diseases, diabetes, neurological disorders and even Alzheimer's disease.

Lipid oxidation is also a significant problem for the food industry, as it leads to the development of undesirable off-flavors, dark colors and potentially toxic reaction products (GRASZKIEWICZ *et al.*, 2007, SAKANAKA *et al.*, 2004; JE *et al.*, 2007).

Therefore, the benefits of antioxidants, which inhibit lipid oxidation in food storage is of great interest in research (SAKANAKA *et al.*, 2004 and 2006).

Non-hydrolyzed proteins, due to their compact design, have minimal antioxidant activity. It is generally observed that the antioxidant potency of proteins drastically increases upon hydrolysis (KONG and XIONG, 2006). The mechanisms of antioxidative action of proteins, peptides and individual amino acids include radical scavenging or product reduction, metal chelating, and enzyme inhibition (SARMADI and ISMAIL, 2010).

HIROSE and MIYASHITA (1999) suggested that protein hydrolysates and biopeptides not only stand as an electron donor to free radicals, but can also create droplets of lipid membrane protecting against oxidation processes.

The egg white protein preparation, a by-product of lysozyme and cystatin extraction from egg white with ethanol, was characterized by a high degree of denaturation and hence does not have technologically attractive functional properties (GRASZKIEWICZ *et al.*, 2010).

The aim of the present research was to obtain valuable product exhibiting antioxidant activity from denaturated egg white protein preparation degraded with proteinases of different specificity. Furthermore, the study aimed at the isolation and characterization of antioxidant peptides from obtained hydrolysates.

MATERIAL AND METHODS

Materials

The egg white protein preparation (EP), a by-product of lysozyme and cystatin isolation, was obtained at our laboratory (SOKOŁOWSKA *et al.*, 2007) and used as a substrate. Proteinases from *Aspergillus melleus* and *Bacillus amyloliquefaciens* (neutrase), bovine trypsin TPCK-treated, bovine chymotrypsin type II, porcine pepsin type A, 5% picrylsulfonic acid, trinitro-benzene sulfonic acid (TNBS), bovine serum albumin, 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical, Trolox™, Nα-benzoyl-DL-arginine-p-nitroanilide, N-succinyl-l-Ala-l-Ala-l-Pro-l-Phe-p-nitroanilide and 2,4,6,tripyridyl-s-triazine (TPTZ) were obtained from Sigma Chemicals Co., trichloroacetic acid was obtained from Ubichem, acetonitrile was obtained from Lab-Scan, and trifluoroacetic acid (TFA) was obtained from Fluka.

Determination of protein content

The total protein (N × 6.25) in insoluble substrates was determined using the Kjeldahl method. Protein content in hydrolysates was determined by LOWRY *et al.*, method (1951).

Determination of proteolytic activity

Proteolytic activity of microbial proteinases form *Aspergillus melleus* and from *Bacillus amyloliquefaciens* (neutrase) was determined in reaction with 1% casein as a substrate at pH 7.5 and the enzyme to substrate ratio: 1:140 (w/w). The substrate with the enzyme was incubated for 10 minutes at 45°C. After this time, the reaction was stopped by the addition of 10% trichloroacetic acid (TCA). The tubes were then centrifuged (3,145 g, 15 min, 20°C). The absorbance of supernatants was measured at λ = 280 nm.

Pepsin activity was determined in a reaction with 2% acid-denaturized hemoglobin as a substrate according to CHRZANOWSKA and KOŁACZKOWSKA (1998) at E:S 1: 250 (w/w), pH

3.0 and temperature 37°C. After 10 min incubation, the reaction was stopped by the addition of 10% trichloroacetic acid. The tubes were then centrifuged (3,145 g, 15 min, 20°C). The absorbance of the supernatants was measured at 280 nm.

Proteolytic activity was determined with the synthetic substrates Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA) (ERLANGER *et al.*, 1961) and N-succinyl-l-Ala-l-Ala-l-Pro-l-Phe-p-nitroanilide (AAPFpNa) (NAKAJIMA *et al.*, 1979) for trypsin and chymotrypsin, respectively. The enzymes were dissolved in 0.1 mol/L Tris; 20 mmol/L $CaCl_2$; 5% dimethyl sulfoxide to a final protein concentration of 4 to 20 μg/mL and reaction was started by applying 10 μL 0.1 mol/L substrates. The enzyme to substrate ratio was: 1: 170 (w/w) and 1: 220 (w/w) for trypsin and chymotrypsin, respectively. After 10 min, the reaction was stopped by the addition of 33% acetic acid. The absorbance was measured at 410 nm.

One unit of enzymatic activity (U) was defined as the amount of enzyme producing an increase in absorbance at 280 nm of 0.1 under reaction conditions.

Egg white protein hydrolysis

The hydrolysis of egg-white protein precipitate with all enzymes without pepsin was carried out in 0.2 mol/L Tris- HCl buffer, pH 8.0 at 37°C for 24 hours using a dose of enzyme of 10 U/mg. Hydrolysis with pepsin was conducted in Gly-HCl buffer at pH 3.0. The reactions were terminated by heating at 100°C for 15 min. The hydrolysates were cooled and centrifuged (3,145 g, 10 min, 10°C). The supernatants were lyophilized and stored at 4°C until use.

The degree of hydrolysis (DH)

Degree of hydrolysis (DH%) was expressed as the percentage ratio of protein soluble in 5% trichloroacetic acid (TCA) to total protein content (SPELLMAN, 2003). The samples were mixed with 10% TCA (1:1) (v:v). After 30 min the samples were centrifuged (3,145 g, 10 min, 10°C). The concentration of acid-soluble product in the supernatant was measured spectrophotometrically at λ = 280 nm.

The content of free amino acid groups

The concentration of the free amino groups was determined according to KUCHROO *et al.* (1983). 2 mL of the hydrolysate diluted with 0.1 mol/L borate buffer was mixed with 50 μL of TNBS reagent (0.03 mol/L). The samples were incubated in the dark for 2 hours at room temperature. The reaction was stopped by adding 2 mL of 0.1 mol/L sodium phosphate containing 1.5 mmol/L sodium sulfate and the absorbance was measured at 420 nm. A blank was prepared with water instead of hydrolysate. The results were expressed as μmol Gly/g hydrolyzed protein by reference to a standard curve prepared with defined concentrations of glycine.

Reversed - phase high performance liquid chromatography (RP-HPLC)

Peptide separation was performed using a Zorbax XDB-C_{18} Agilent column (4.6 x 250 mm) equilibrated with 0.1% TFA in water (trifluoroacetic acid) (phase A). The samples were dissolved in phase A, applied on the column and eluted after 5 min analysis with an increasing percentage (2%/min) of phase B (0.1% TFA in acetonitrile). The operative conditions were injection volume 100 μL; analysis time 55 min; T = 30°C; flow rate 1 mL/min; Ten peptide fractions were collected and lyophilized.

Rechromatography of collected antioxidant fractions was conduced at the same operative conditions like in below except flow rate and gradient. The flow rate of 0.4 mL/min. Gradient of B presented in Fig. 7 finally reached 25%.

Determination of the molecular weight distribution of the hydrolysates

The molecular weights of the peptides were determined by gel filtration chromatography in a HPLC system equipped with a Zorbax GF-250 Agilent column (4.6 × 250 mm) at 30°C. An aliquot of 0.1 mL was injected and eluted using 0.02 mol/L phosphate buffer (pH 7.2) containing 0.2% NaCl at 0.5 mL/min. The absorbance was monitored at 230 nm. Bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa), trypsinogen (24 kDa), β-lactoglobulin (18.4 kDa), lysozyme from chicken egg white (14.3 kDa), aprotonin (6.5 kDa) and bovine insulin, chain B (3.5 kDa) were obtained from Sigma Chemicals and used as standards.

Amino acid composition

Amino acid analysis was performed at BioCentrum Ltd. (Kraków, Poland). The protein samples were hydrolyzed in gas phase using 6 mol/L HCl at 115°C for 24 h. The liberated amino acids were converted into phenylthiocarbamyl (PTC) derivatives and analyzed by high-pressure liquid chromatography (HPLC) on a PicoTag 3.9 x150 mm column (Waters, Milford, MA, USA). The amino acid concentration was expressed as nmol/10 μg of protein.

Molecular mass identification by mass spectrometry (MS) (via the) coupled with electrospray ionization (ESI)

The peptide fractions were analyzed using an Esquire HCT quadrupole ion trap instrument (Bruker, Germany). The samples were injected

into the mass spectrometer via the electrospray interface (activation time: 1 min, flow: 220 μL/min). Spectra were recorded over the *m/z* range of 100 to 2,200. Analysis was performed in the laboratory of BioCentrum Ltd. (Kraków, Poland).

Their share in the mixture is expressed as relative concentration (%) of particular peptide in relation to the most intensive peak. If only one peak was detected, the relative concentration was established in relation to the residual spectrum.

The amino acid sequence

The amino acid sequence analysis was performed at BioCentrum Ltd. (Krakow, Poland) by the Edman reaction with phenylisothiocyanate. Then the final products: N-terminal phenylthiohydantoin amino acid derivatives were analyzed by liquid chromatography.

Determination of antioxidant activity as the ability to scavenge DPPH free radicals

Antioxidant activity of obtained hydrolysates was assessed on the basis of the radical scavenging effect of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical activity according to YEN and CHEN (1995), with modifications. The tested samples were dissolved in water to a final volume of 1 mL and mixed with 1 mL of ethanol (98%). The reaction was started by adding 0.5 mL of 0.3 μmol/L DPPH in ethanol. The mixtures were left for 30 min at room temperature and the absorbance of the resulting solution was measured at 517 nm. Aqueous solutions of known Trolox concentration ranged from 2 to 20 μg (able to scavenge 500 μL of 0.3 mmol/L DPPH radical solution) was used for calibration. Radical scavenging activity of the peptides was expressed as μmol $trolox_{eq}$/mg protein.

FRAP method

The FRAP method (Ferric Reducing Antioxidant Power) was used to determine the antioxidative capacity in the hydrolysates according to Benzie and Strain (1996). 3 mL of FRAP working solution (300 mmol/L acetate buffer pH 3.6; 10 mmol/L 2,4,6,tripyridyl-s-triazine (TPTZ); 20 mmol/L $FeCl_3$ x 6 H_2O (10:1:1 v/v)) was mixed with a 1 mL sample. After 10 min of reaction, the absorbance was measured at 593 nm. Aqueous solution of known Fe (II) concentration was used for calibration (in a arrange of 100 to 1,000 μg). Results were expressed as μg Fe^{2+}/mg protein.

Determination of Fe (II) ion chelation

Chelation of iron ions by hydrolysates was estimated by the method of XU *et al.*, 2007, with modifications. A 250 μL sample was mixed with 1,250 μL H_2O and 110 μL 1 mmol/L $FeCl_2$. After 2 min, 1 mL of 500 μmol/L ferrozine aqueous solution was added and the mixture was allowed to react for 10 min. The absorbance of ferrous iron-ferrozine complex was measured spectrophotometrically at 562 nm. A known concentration of $FeCl_2$ (0-20 μg) was used as a standard curve and the ability to chelate iron ions was expressed as μg Fe^{2+}/mg protein.

Data analysis

All experiments were carried out in triplicates. The data obtained were subjected to multi-factor variance analysis (ANOVA), followed by the Duncan's multiple range test to determine the significant difference between sample at $p<0.05$ level using Statistica v. 9.0.

RESULTS AND DISCUSSION

In this study, the degradation of the EP was carried out using two microbial proteases from *Aspergillus melleus* and *Bacillus amyloliquefaciens* (neutrase) and digestive enzymes: trypsin, chymotrypsin and pepsin. Both types of enzymes were effectively used in hydrolysis of egg white proteins (WANG and WANG, 2009; CHEN *et al.*, 2011, CIGIĆ and ZELENIK-BLATNIK, 2004; GRASZKIEWICZ *et al.*, 2007 and 2010). The course of protein degradation was monitored quantitatively by determining the degree of hydrolysis (DH) (Fig. 1) and the increment of free amino groups (Fig. 2). The type of enzyme, its specificity and reaction time have the greatest impact on the degree of EP hydrolysis. DH is an important parameter in the enzymatic modification of proteins and might be a factor controlling the composition and properties of the modified proteins (GE and ZHANG, 1993). In all variants, more than 40% DH after 24 h reaction was observed. Pepsin was the most efficient enzyme in EP degradation (DH = 85%). Whereas 24 h hydrolysis of this protein using proteinase from *Bacillus amyloliquefaciens* and bovine trypsin resulted in lower DH than for pepsin, about 5 and 30% less, respectively.

The highest level of free amino acid groups (5190.1 μmol/g) was also determined in the hydrolysate obtained with porcine pepsin after 24 h of reaction. The level of free amino groups determined in the EP degraded with microbial proteases was about two times lower than in the pepsin hydrolysate. The use of bovine trypsin resulted in slower degradation of the EP. The content of amino acid groups released after degradation of EP with this protease was finally 671.2 μmol/g.

Our results confirm the observation of other authors that pepsin is an efficient enzyme in egg-white protein degradation. MIGUEL *et al.* (2004) hydrolyzed whole egg white. In their study, DH was 53.9% after 3 h of enzymatic reaction and increased to 74.3% after 24 h. Moreover, high

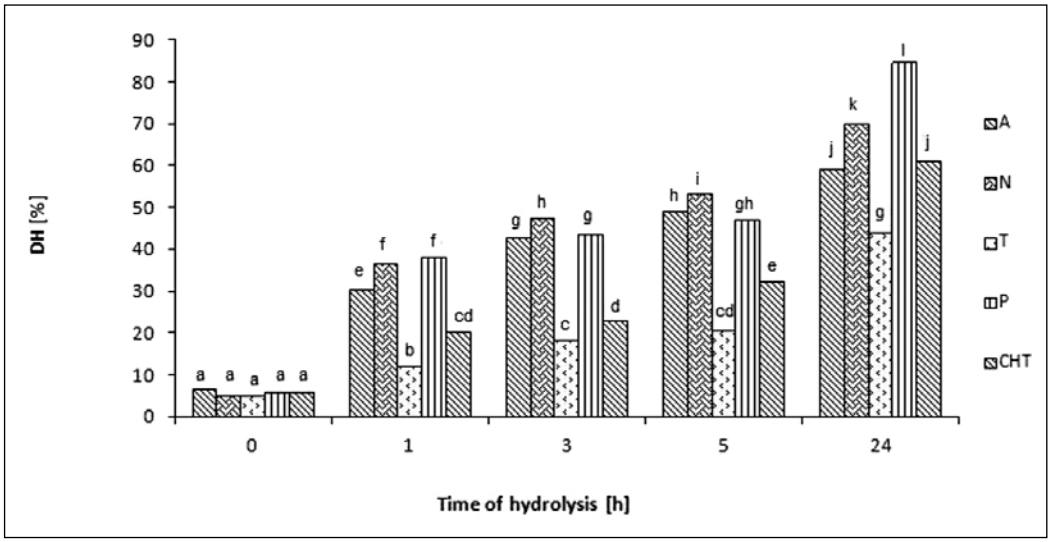


Fig. 1 - Effect of time on the degree of hydrolysis (DH) during hydrolysis of egg white protein preparation by proteases from *A. melleus* (A) and *B. amyloliquefaciens* (neutrase) (N), bovine trypsin (T), porcine pepsin (P) and bovine chymotrypsin (CHT). a-l – means followed by the same letter in figure do not differ significantly ($p \leq 0.05$).

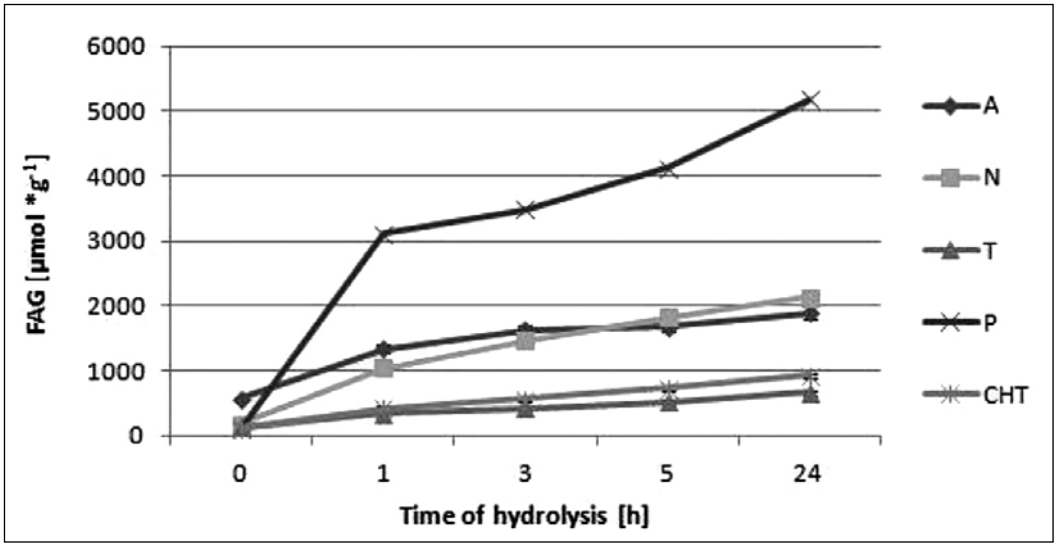


Fig. 2 - Effect of time on the free amino groups contents (FAG) during hydrolysis of egg white protein preparation by proteases form *A. melleus* (A) and *B. amyloliquefaciens* (neutrase) (N), bovine trypsin (T), porcine pepsin (P) and bovine chymotrypsin (CHT).

activity of enzymes from microbial sources including: *Bacillus ssp.* and *Aspergillus ssp.* towards the protein substrates was also shown by JE *et al.* (2007) who used hydrolysis as a method of disposing of waste proteins in the fishing industry and achieved almost total degradation of the substrate.

In this study, the antioxidant activity of enzymatically degraded EP measured by the determination of the ability to scavenge DPPH free radicals (Fig. 3), to reduce the oxidation of Fe (III) (FRAP) (Fig. 4) and to chelate Fe (II) (Fig. 5) was assessed.

It was found that all the analyzed hydrolysates were able to scavenge DPPH radicals. The highest free radical scavenging activity was observed for tryptic hydrolysate (0.21 µmol $trolox_{eq}$/mg) and for hydrolysate obtained with neutrase (0.17 µmol $trolox_{eq}$/mg) after 24 h of reaction. It was revealed that even shorter hydrolysis with these enzymes (5 h) generated the protein degradation products that exhibited activity against DPPH radicals at a level of 0.15 and 0.1 µmol $trolox_{eq}$/mg, respectively. Free radical scavenging activity of final hydrolysates obtained with *A. melleus* proteinase, porcine pepsin and chymotrypsin was two times lower than for trypsin and neutrase. In previous studies GRASZKIEWICZ *et al.* (2007; 2010) also showed that the proteolytic action of trypsin led to release peptides from egg-white proteins which exhibited stronger free-radical scavenging activity than peptides obtained

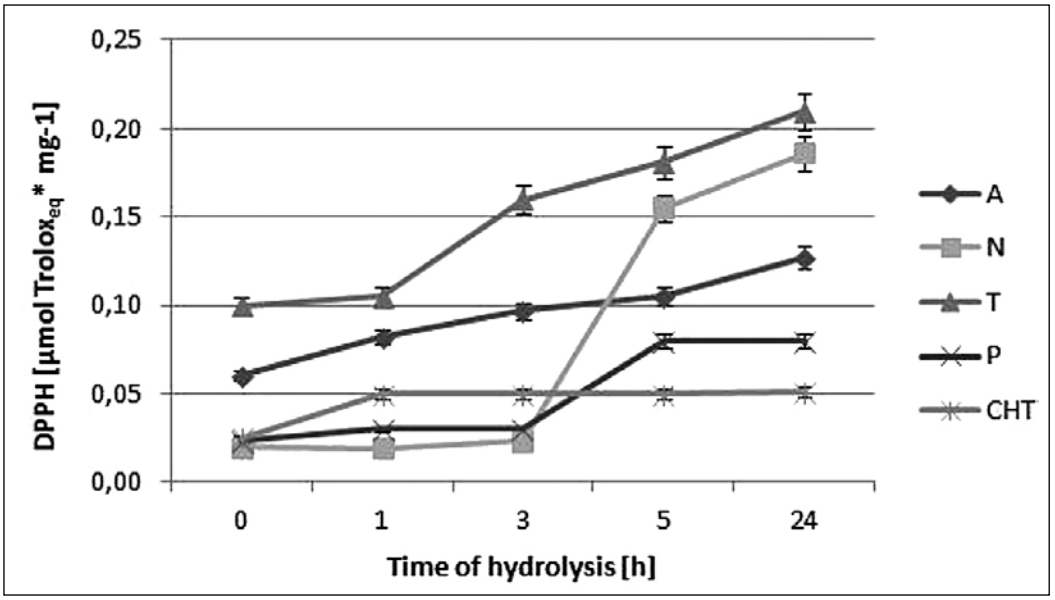


Fig. 3 - DPPH scavenging activities of egg white preparation hydrolysates.

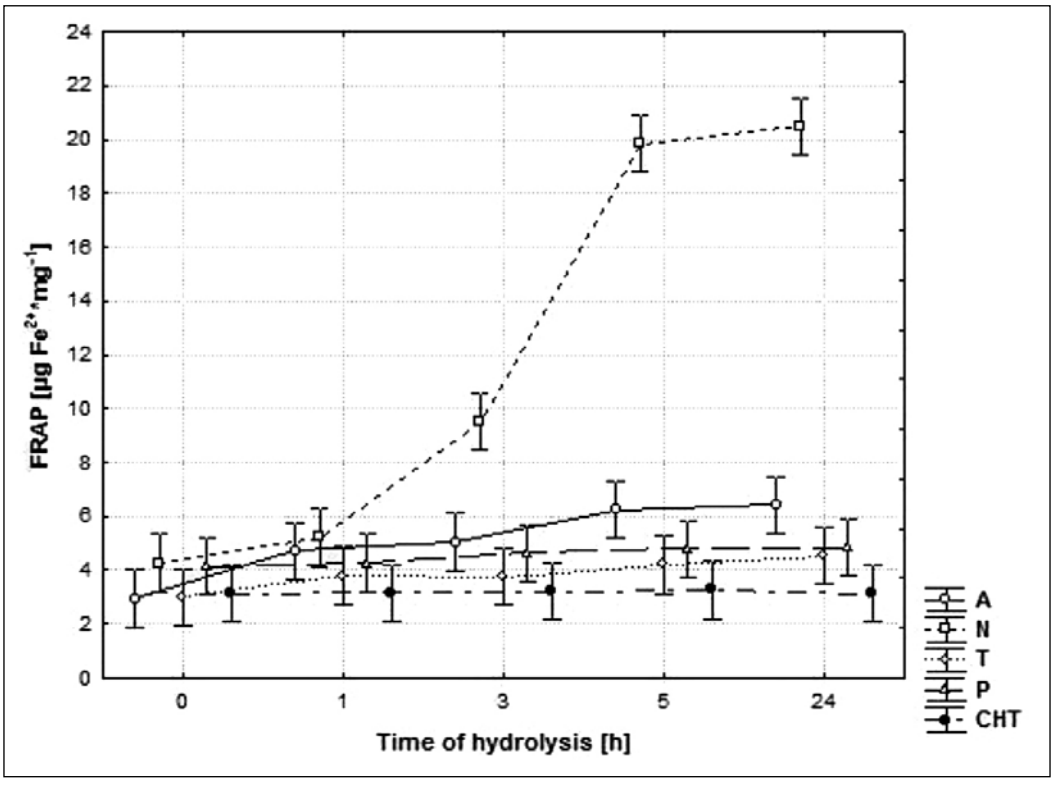


Fig. 4 - The Ferric Reducing Antioxidant Power (FRAP) of egg white protein preparation hydrolysates.

with other proteolytic enzymes. After 7 h degradation of EP with trypsin, the peptides exhibited antioxidant activity of 0.38 µmol trolox$_{eq}$/ mg.

The result of this study also revealed the relationship between DPPH radical scavenging activity, the degree of hydrolysis and the type of enzyme. DPPH activity of investigated hydrolysates increased with increasing DH. Certainly, this results from an increase in the availability of hydrogen ions (electron donors) and increasing concentrations of carboxyl and amine groups. The evidence of the higher level of antioxidant activity (54.3% of scavenged free radicals) at DH of 14.7% than the level of antioxidant activity (29.2% of scavenged free radicals) at DH of 5.2% from enzymatic hydrolysates obtained by CHEN *et al.* (2012), supports this hypothesis. LIU *et al.*, (2010) in their study also showed an increase of antioxidant activity as a result of progress of hydrolysis. Longtime hy-

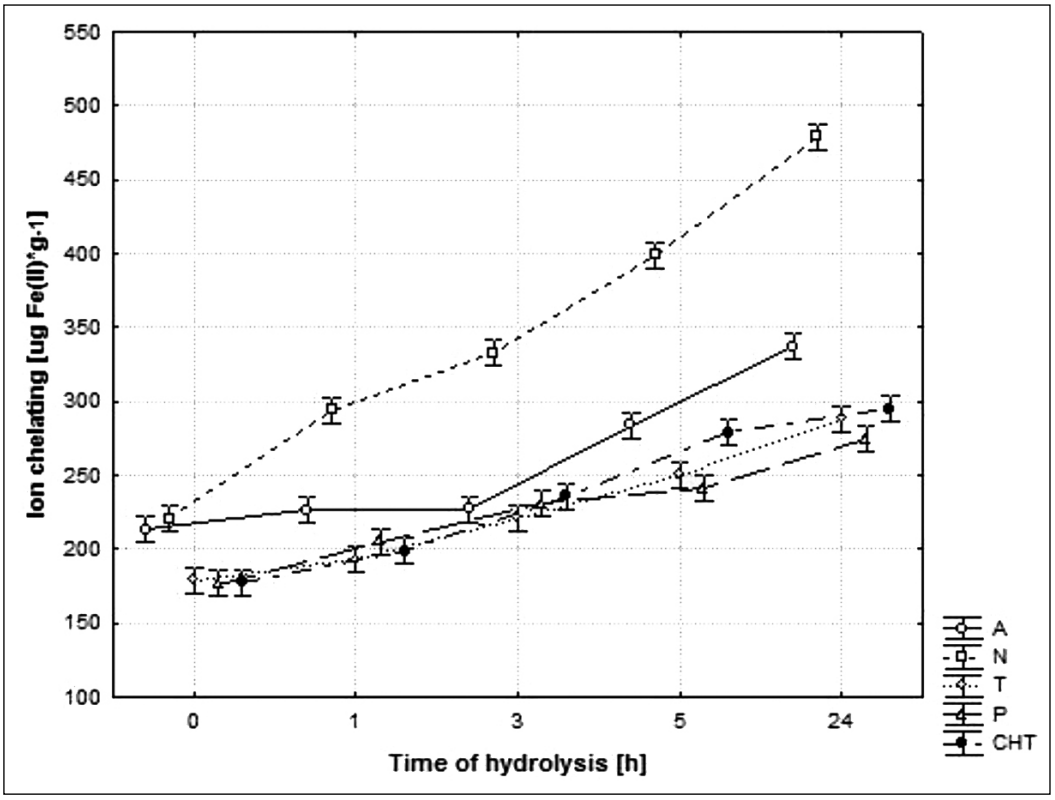


Fig. 5 - Ferrous ion-chelating activities of egg white protein preparation hydrolysates.

drolysis of porcine plasma by alcalase led to obtain peptides exhibiting higher antioxidative activity than products of partial hydrolysis. In that study, the value of scavenging activity expressed in percentage amount of scavenged free radicals by hydrolysates at the concentration 40 mg/mL was 21.7, 31.3, 45.4 and 76.8% corresponding to 0, 6.2, 12.7 and 17.6% of DH, respectively.

Potential antioxidant activity of EP hydrolysates, in terms of their reducing capacity, was expressed by the ability to decrease oxidation of Fe (III) to Fe (II). The relationship between the reducing power as well as time and the degree of protein degradation was observed. These results were similar to the results obtained by MOURE *et al.* (2006) who reported that the reducing power showed a clear influence of protein size and degree of hydrolysis. Moreover, all hydrolysates showed enzyme-dependent ferric reducing activity. The highest values of this activity were observed for hydrolysates obtained after 24 hours of reaction with use of microbial enzymes. The use of neutrase resulted in a four-fold increase in FRAP activity (21.0 μg Fe^{3+}/mg) compared to *A. melleus* proteinase, trypsin, pepsin and chymotrypsin which showed 5.9, 4.5, 4.7 and 2.9 μg Fe^{3+}/mg, respectively.

Enzymatic hydrolysates have also been studied in terms of their ability to chelate on iron ions (II). Owing to this chelating property, peptides exert strong antioxidant activity and may be carriers of metals such as Ca, Fe and Zn, which are essential elements in human nutrition (TORRES-FUENTES *et al.*, 2012).

All hydrolysates obtained in this research showed chelating activity, increasing with time and the degree of protein degradation. The highest ability to bind Fe (II) ions was observed for protein preparations hydrolyzed with neutrase, yielding a maximum level of 397.0 μg Fe^{2+}/mg. In hydrolysates obtained with other enzymes, the iron ion chelation value was lower than in hydrolysates obtained with neutrase, amounting to a maximum level of 241.2; 250.3; 278.1 and 282.0 μg Fe^{2+}/mg for pepsin, trypsin, chymotrypsin and protease from *A. melleus,* respectively. Antioxidant activity of protein hydrolysates obtained from egg white using a variety of proteolytic enzymes has also been shown by DAVALÕS *et al.* (2004) and HUANG *et al.* (2010).

Both the enzymatic hydrolysis *in vitro* and the *in situ* by microbial fermentation of food proteins give rise to mixtures of peptides and, in particular situations, each biopeptide might require a specific process protocol. In our study, 24 h tryptic hydrolysate of EP, which exhibited the strongest free radical scavenging activity, was resolved by means of RP-HPLC according to procedure described by GRASZKIEWICZ *et al.* (2007). The mixture of peptides was applied on Zorbax XDB- C_{18} column and ten fractions were collected (Fig. 6). Peptides eluted between 20 and 25% acetonitrile exhibited the strongest free radical scavenging activity (0.18 - 0.21 μmol

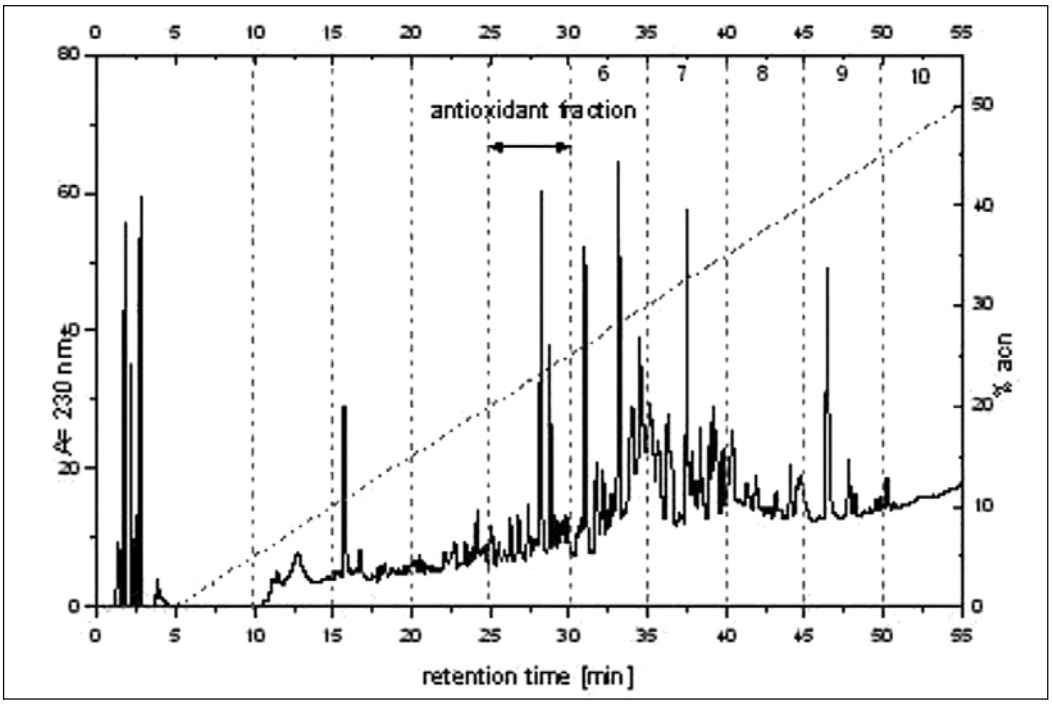


Fig. 6 - RP-HPLC – peptide profile of tryptic 24-hour hydrolysate of egg white protein preparation. Ten fractions were collected.

trolox$_{eq}$/mg). The rechromatography of this fraction was performed on the same column under modified conditions (Fig. 7). The highest levels of free radical scavenging activity were estimated in subfractions No. 1 and No. 2 and approximated 1.2-1.4 and 1.0-1.2 μmol trolox$_{eq}$/mg, respectively. These levels were 6.7 and 5.7 times higher, respectively, than those reported for 24 h tryptic hydrolysate.

RP-HPLC is widely used as a peptide purification method (SAIGA *et al.*, 2003; ADEBIYI *et al.*, 2008). ADEBIYI *et al.* (2008) fractionated rice

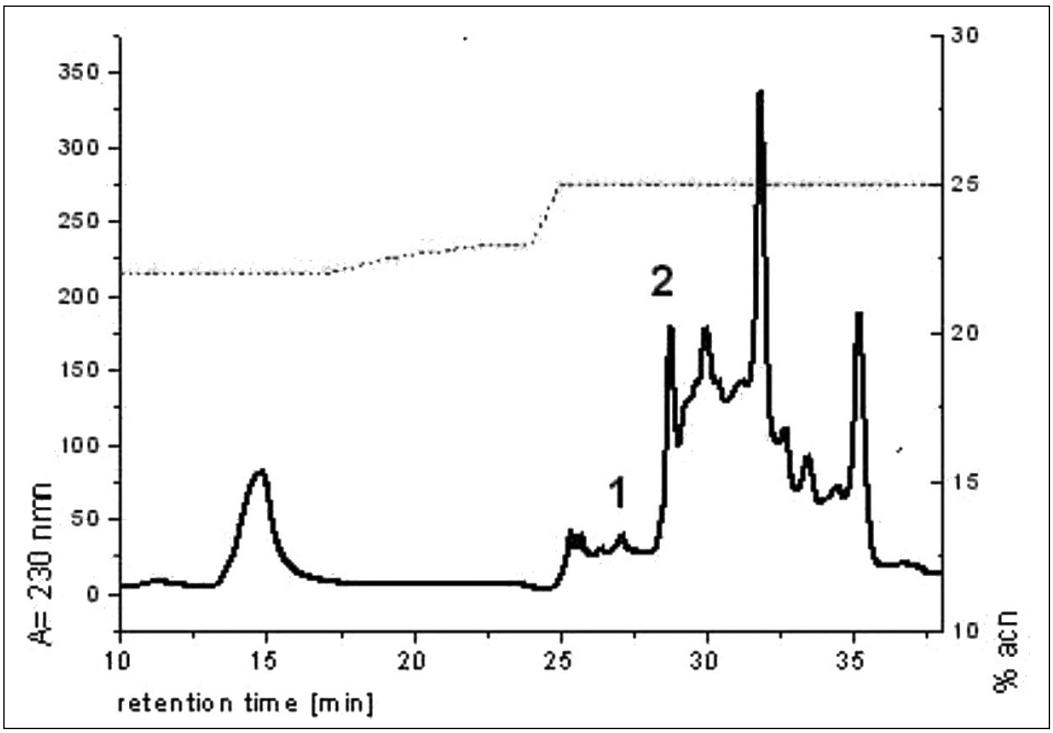


Fig. 7 - RP-HPLC peptide profile of fraction (eluted between 25-30 min analysis) isolated from tryptic 24-hour hydrolysate of egg white protein preparation.

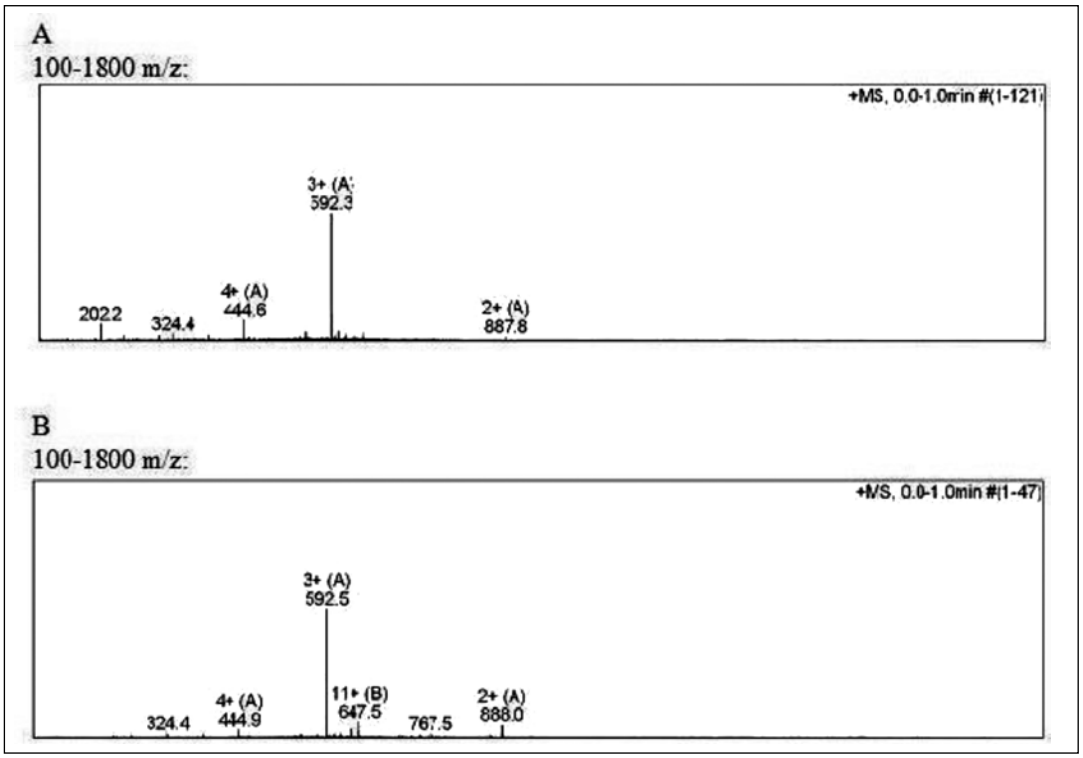


Fig. 8 - Molecular mass identification (MS-ESI) (100- 1,800 m/z) of peptide fractions (A- subfraction no 1; B- subfraction no 2) isolated from tryptic 24-hour hydrolysate.

bran hydrolysates to pure, antioxidative peptides by RP-HPLC on Kaseigel ODS resin, using a stepwise gradient of aqueous ethanol only.

The molecular masses and amino-acid composition of the two obtained fractions were analyzed (Fig. 8; Table 1). The molecular masses analysis by MS-ESI indicated that subfraction No. 1 was relatively homogenous and one peptide with molecular mass of 1.77 kDa was confirmed (Fig. 8). The main component of subfraction No. 2 was the same as peptide in fraction No. 1 (89.3%). Furthermore, the peptide with molecular mass of 7.11 kDa (10.7%) was also detected for this subfraction. Generally, many authors (PARK *et al.*, 2001; DÁVALOS *et al.*, 2004, TSUGE *et al.*, 1991) demonstrated that the antioxidant activity was mainly attributable to peptides with low molecular masses. However, numerous long- chain peptides with antioxidant activity have been described. For example, peptides composed of 13 and 16 amino- acids isolated from *Allasca Pollack* skin hydrolyzed with Profaze E exert antioxidant activity (PILANTO, 2006).

The amino-acid composition of these subfractions was analyzed. Subfraction No.1 had a high concentration of glutamic acid and glutamine (4.85 nmol/10 μg) as well as of alanine (2.88 nmol/10 μg). However, low concentration of histidine residues (0.49 nmol/10 μg) in this fraction was estimated. Similar contribution of these amino acids concentration for subfraction No. 2 was observed. The concentrations of glutamic acid and glutamine, alanine and histidine amounted to: 23.83, 17.04 and 3.19 nmol/10 μg, respectively. It is known that the main components of the antioxidative peptides are the amino acids known for their antioxidative properties such as histidine, tyrosine, methionione, lysine and tryptophan (PARK *et al.*, 2001). Not only the composition of amino acids, but their

Table 1 - Amino acid composition (nmol/10 μg) of peptide fractions isolated from tryptic 24-hour hydrolysate of egg white protein preparation.

Amino acid	Composition (nmol/10 μg)	
	F1	F2
Asx	1.85[a]	7.37[b]
Glx	4.85[a]	23.83[b]
Ser	2.33[a]	10.97[b]
Gly	2.14[a]	8.5[b]
His	**0.49[a]**	**3.19[b]**
Arg	1.94[a]	8.94[b]
Thr	**1.55[a]**	**6.45[b]**
Ala	**2.88[a]**	**17.04[b]**
Pro	1.46[a]	6.52[b]
Tyr	1.08[a]	4.7[b]
Val	2.2[a]	11.92[b]
Met	**0.42[a]**	**3.23[b]**
Ile	1.31[a]	7.31[b]
Leu	1.5[a]	7.42[b]
Phe	0.68[a]	3.35[b]
Lys	**1.25[a]**	**5.91[b]**

[a-b], means followed by the same letter in line do not differ significantly ($p \leq 0.05$).

sequence and configuration as well also affect the antioxidative properties of peptides (IWANIAK and MINKIEWICZ, 2007; PARK *et al.*, 2001; DÁVALOS *et al.*, 2004).

Following amino acid sequence analysis identified the same peptide: ISQAVHAHAEINEAGR in subfractions No. 1 and No. 2, which matched 324-340 residues of the ovalbumin (1-386). Furthermore, peptides: SVL, QL matched (296-298), (255-256) residues ovalbumin and AP, KVR matched (70-71; 186-187;217-218;489-490), (646-648) residues ovotransferrin were detected in fraction No. 2.

DAVALŌS *et al.* (2004) also isolated and characterized a few peptides exhibiting free radical scavenging activity the amino acid sequence of which corresponded to ovalbumin fragments.

The presence of 4 alanine and 2 histidine residues in 16- amino- acid fragment of ovalbumin confirmed its antioxidant activity. Furthermore, ovotransferrin-derived peptides obtained in this study contained a leucine residue at their N- therminal position, what is essential for antioxidant activity. This is in line with the results obtained by PARK *et al.* (2001). They isolated two peptides from lecitin-free egg yolk hydrolysate which showed antioxidant activity in a linoleic acid model system. These peptides, composed of 10 and 15 amino acid residues, both contain a leucine residue at their N- thermal positions.

Isolation of antioxidant peptides from 24 h hydrolysate of EP obtained with neutrase which revealed the highest values of reducing capacity and ability to chelate on iron ions (II) was also performed. Peptide fractions were separated by size exclusion chromatography (Zorbax GF-250). Four fractions were collected and the antioxidant activity was determined in each of them (Fig. 9). The level of antioxidant activity varied considerably between the obtained fractions. Fraction labeled N1 exhibited the strongest reducing capacity (21.8 μg Fe^{3+}/mg) and ability to chelate on iron ions (II) (912.2 μg Fe^{2+}/mg).

Degradation of EP with neutrase resulted in the peptides which exhibited molecular weights from 3.21 to 33.33 kDa. However, in the most active fraction (N1), the peptides with the highest molecular masses from 13.89 to 33.33 kDa were confirmed. The obtained results are opposite to the thesis that peptides with low molecular size exhibit biological activity (PAMPANIN *et al.*, 2012). As a matter of fact, not only molecular size, but also chemical properties, such as hydrophobicity and electron transferring ability of amino acid residues in the sequence determined biological activity of peptides. The hydrophobic property of the peptides plays an important role in antioxidant activity, especially in the scavenging effect of free radicals. In addition, as hydrophobicity of antioxidants is important for accessibility to hydrophobic targets, it is presumed that the presence of hydrophobic amino acids in purified peptides may contribute to antioxidant activity by increasing solubility of peptides in non-polar medium, exp. lipids and thereby facilitating better interaction with radical species (SUN *et al.*, 2012).

Fraction N1 obtained from ion exchange chromatography was further subjected to RP-HPLC

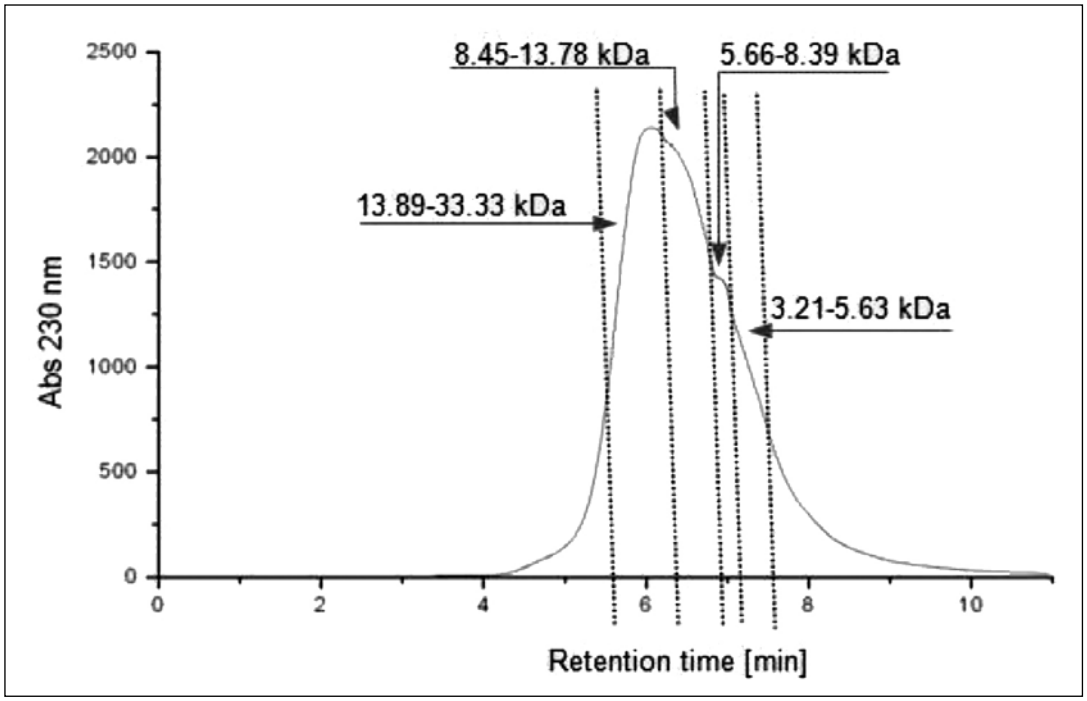


Fig. 9 - Gel filtration chromatogram of EP24h degraded with neutrase, showing the peptide molecular weight repartition.

chromatography. In the obtained peptide profiles, the presence of numerous peaks, differing in hydrophobicity was observed (data not shown). Further separation of pure peptides using this method would be difficult in this case. It is necessary to carry out another purification protocol to obtain peptides responsible for specific actions.

CONCLUSIONS

A denaturated protein by-product (EP), obtained during lysozyme and cystatin isolation from egg white, was converted into more useful products with antioxidant activity through hydrolysis with the use of proteinases of different specificity. Enzymatic hydrolysis of EP proved that peptides exhibited antioxidant activity. Bovine trypsin and neutrase were enzymes effective in producing peptides with free radical scavenging activity and Fe^{2+}chelating activity, respectively. Peptide fraction isolated from tryptic hydrolysate by RP-HPLC exhibited strong free radical scavenging activity. Among the obtained fractions, we identified ISQAVHAHAEINEAGR, SVL, QL and AP, KVR antioxidative peptides corresponding to ovalbumin and ovotransferrin fragments, respectively.

ACKNOWLEDGEMENTS

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PAPER

PRELIMINARY CHARACTERIZATION OF A BACTERIOCIN-LIKE SUBSTANCE PRODUCED BY A *BACILLUS SUBTILIS* ISOLATED FROM ARGENTINEAN VEGETABLE FOOD

M.F. FANGIO*[1,2] and R. FRITZ[2]
[1]CONICET, Consejo Nacional de Investigaciones Científicas y Técnicas, Departamento de Química, Facultad de Ciencia Exactas y Naturales, Universidad Nacional de Mar del Plata, Argentina
[2]UNMDP, Universidad Nacional de Mar del Plata,
Funes 3350, Mar del Plata, Provincia de Buenos Aires, Argentina
*Corresponding author: Fax +54 223 4753150,
email: mfangio@mdp.edu.ar

ABSTRACT

Bacillus spp. produces a large number of antimicrobial peptides and includes a variety of species of industrial importance. The aim of this study was to characterize the antimicrobial activity associated with a *Bacillus subtilis* strain. The activity of the crude bacteriocin-like substance (CBLS) was dependent on the components of the culture media and was detected in the exponential phase of growth. The CBLS was stable at a variable pH range and at 100°C, but it was inactivated by sterilization conditions. It was also stable to storage at refrigeration and freezing temperatures for a considerable time. The mode of action of the antimicrobial activity was bacteriostatic and the effect on the cell membrane was determinated by Fourier transform infrared (FTIR) spectroscopy.

- Keywords: antimicrobial peptides, *Bacillus cereus*, *Bacillus subtilis*, bacteriocins -

INTRODUCTION

Due the concern on reducing the use of chemical additives in food, since some of them have been proven harmful to human health as well as the environment, the necessity of more natural and safe food products has been recognized (XU *et al.*, 2013). Bacteriocins are an alternative for natural food preservation, as well as for human therapy as possible additive or replacements for currently used antibiotics (ABO-AMER, 2007).

Bacteriocins are proteinaceous compounds bioactive to other bacteria than the producing strain (JOERGER, 2003). The activity spectrum of bacteriocins can be narrow and limited to closely related species, or it can be relatively extensive and comprise many different bacterial species (OSCARIZ and PISABARRO, 2000).

The isolation of new strains also allows to discover new bacteriocin produced by them, since different kinds of bacteria produce different types of bacteriocins. Consequently due to the nascent field of biopreservation there is a demanding need to explore and isolate more and more bacteria from new sources capable of producing novel bacteriocins and characterize them to be added to food (SHARMA and GAUTAM, 2008).

Bacillus spp. is of major interest in bacteriocin research since this genus produces a diverse array of antimicrobial peptides with several different basic chemical structures (CLADERA-OLIVERA *et al.*, 2004; XIE *et al.*, 2009; ABRIOUEL *et al.*, 2010). The *B. subtilis* group is one of the major producers of these substances (MARTIRANI *et al.*, 2002; CLADERA-OLIVERA *et al.*, 2004; LISBOA *et al.*, 2006; ANTHONY *et al.*, 2009; HAMMAMI *et al.*, 2009; XIE *et al.*, 2009; KINDOLI *et al.*, 2012), being *B. subtilis* one of the most prolific. In addition, *B. subtilis* is generally recognized as safe organisms (GRAS) by the Federal Drug Administration (FDA) for specific applications such as enzyme production (APETROAIE-CONSTANTIN *et al.*, 2009).

The extensive screening of nearly 100 strains of *Bacillus* spp. isolated from vegetable food from Argentina led to the selection of several strains which demonstrated antibacterial activity (FANGIO *et al.*, 2010).

This paper reports a preliminary characterization of the biological and physicochemical properties of the antimicrobial substances produced by a strain isolated from rice, *B. subtilis* R1.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacillus subtilis R1 isolation and bacteriocin-like identification have been described elsewhere (FANGIO *et al.*, 2010). Indicator and producer strains were kept frozen in 20% (v/v) glycerol at –20°C until use. *Bacillus* spp. were growth in nutritive agar and *Paenibacillus larvae* was cultured in Mueller-Hinton, yeast extract, glucose, sodium pyruvate and agar (MYPGP) supplemented with nalidixic acid at 37°C for 48 h.

Preparation of crude extracts

The bacteriocin-like producing strain was grown in brain-heart infusion (BHI) at 37°C for 24 hours. After incubation, the bacterial cells were removed by centrifugation at 10,000 g for 15 min. The supernatant was collected and filtered with membrane 0.22 µm (Titan syringe filters; Sri Scientific Resources Inc., USA). This supernatant was designated as crude bacteriocin-like substance (CBLS) (XIE *et al.*, 2009).

Reagents and media

Mueller-Hinton Agar (MH), nutrient broth (NB) and brain-heart infusion (BHI) were from Britania (Buenos Aires, Argentina). All other media and reagents were from Merck (Darmstadt, Germany). Mueller-Hinton, yeast extract, glucose and piruvato of sodium agar (MYPGP) were prepared according to composition reported by DINGMANN and STAHLY (1983).

Bacteriocin-like activity assay

Antimicrobial activity of the supernatant was evaluated by the agar well-diffusion method (FANGIO *et al.*, 2010). *P. larvae* was chosen as indicator strain because of its high sensitivity to the antimicrobial substance (FANGIO *et al.*, 2010). In addition, a *B. cereus* strain was selected as indicator strain since it is an important foodborne pathogen. The CBLS titre was determined by the serial two-fold dilution method described in LISBOA *et al.* (2006) and expressed as activity units (AU) per milliliter.

Kinetics of growth strains and antimicrobial activity production

To study the kinetics of production of the antimicrobial substance, the producing strain was seeded in 5 mL of brain heart broth and incubated 18 hours at 35°C. Fifty ml of BHI were inoculated with 1 mL (2 %) of the preinoculated broth and incubated at 35°C. Samples were taken at different times. To monitor the cell growth viable cells count was performed on nutrient agar plate, while production of antimicrobial substance of the CBLS was measured by the agar well-diffusion method (CHERIF *et al.*, 2001).

Effect of culture media

Different commercial culture media (brain heart infusion (BHI), lactose broth, Mueller-Hinton broth and nutrient broth) were analyzed as substrates for the production of antagonis-

tic substances. The strain was seeded in 5 mL of BHI and incubated 18 hours at 35°C. An aliquot of this preinoculated culture was added in every culture media and incubated at 35°C for 18 hours. The antimicrobial activity of the CBLS obtained from every culture media was analyzed using the agar diffusion assay (DOMINGUEZ *et al.*, 2007).

Effects of pH and heat on antimicrobial activity

To determine the stability of the antimicrobial substances to pH, CBLS was diluted in different buffers and incubated at room temperature for 2 hours. To analyze the heat stability of the substances, CBLS were subjected to the following treatments: 20°, 40°, 60° and 80°C for 30 min, 100°C for 5, 10, 15, 20, 30, 40, 50 and 60 min and 121°C at 1 atm for 15 minutes. After treatments, the antimicrobial activities were evaluated using the agar diffusion method (LISBOA *et al.*, 2006).

Stability in different storage conditions

CBLS was stored under refrigeration (4°C) and freezing (-20°C). At different time intervals (10 and 30 days) the stored material was evaluated for antimicrobial activity by agar diffusion method (ABO-AMER, 2007).

Kinetics of antimicrobial action

To study the mode of action of the antimicrobial substance, CBLS (75 AU/mL) was added to fresh cultures of *B. cereus* in brain heart broth in the logarithmic phase of growth. Samples were taken at different times and recorded both cell biomass (measured as OD_{600nm} with a spectrophotometer UV-Vis Spectrophotometer Scanning Shimadzu (UV-2101PC)) and viable cells count on nutrient agar plate (XIE *et al.*, 2009).

Phospholipase C activity and capacity for erythrocyte hemolysis

Phospholipase C activity and capacity for erythrocyte hemolysis of CBLS were verified according to BIZANI *et al.* (2005).

Fourier transform infrared (FTIR) spectroscopy

A bacterial suspension of the indicator bacteria *Paenibacillus larvae* (10^8 cfu/mL) was added to an equal volume of CBLS. After incubation for 1.5 hours, both treated and control cells were washed three times with sterile distilled water. Twenty µL of each bacterial sample was applied onto a ZnSe optical plate, dried and then analyzed by FTIR spectroscopy. All IR spectra (4,000-400 cm^{-1}) were obtained according to BIZANI *et al.*, 2005. To minimize the variation due to the difference between different biomass samples, the spectra were normalized according to DEAN *et al.* (2010).

Concentration of inhibitory substance

CBLS was concentrated by extraction with organics solvent using 1-butanol (BIZANI *et al.*, 2005). After evaporation of the 1-butanol, the extract was suspended in phosphate buffer pH 7.2 and stored at 4°C until use. The measure of the activity of the extract was determined by the agar diffusion method.

Statistical analysis

The antimicrobial activity of strains in the different treatments were compared using analysis of variance (ANOVA) with Tukey's test (ZAR, 2009) using SPSS 15.0 (SPSS Inc., Chicago, Illinois, USA) for Windows.

RESULTS AND DISCUSSION

In a previous paper (FANGIO *et al.*, 2010) we reported the identification of a new bacteriocin-like substance produced by *B. subtilis* R1. In this report, the proteinaceous nature of this substance produced by *B. subtilis* R1 was established. However, its biochemical characterization was not completed.

The antibacterial activity was detected in the mid-exponential phase with a maximum in the stationary phase (Fig. 1), which suggests that the antimicrobial peptide is produced as a primary metabolite (CLADERA-OLIVERA *et al.*, 2004). The kinetics of this antimicrobial peptide was similar to the bacteriocin produced by *B. subti-*

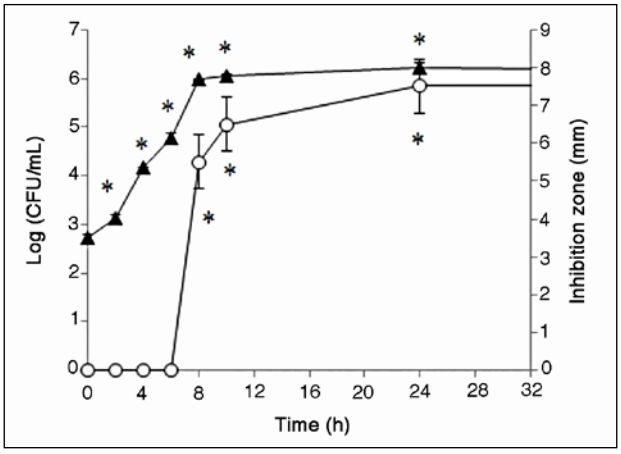


Fig. 1 - Kinetics of antimicrobial substance production during the growth of *B. subtilis* R1 in BHI at 37°C for 32 h. Viable cell count (log CFU/mL) of *B. subtilis* R1 (black triangles) and antimicrobial activity expressed as inhibition zones (mm) against *B. cereus* (open circles). An asterisk indicates statistically significant difference ($p < 0.05$) with initial values.

lis LFB112 (XIE *et al.*, 2009) which antibacterial activity was detected in half of the rapid growth phase and peaked in early stationary phase. The comparative study of the kinetics of growth of the strain and production of the antimicrobial substance is important to identify the most effective production stage.

Nutrient selection and determination of their concentrations in the culture media is an important step in cell growth and production of useful metabolites produced from microorganisms. To optimize the conditions of the culture medium is especially important for products that are required in large amounts as for technological uses (DOMINGUEZ *et al.*, 2007). The inhibitory activity of CBLS obtained with different culture media showed that CBLS obtained from the growth of *B. subtilis* R1 in brain heart broth, produced a higher activity ($p<0.05$) that those obtained from the other culture media studied (Fig. 2). Brain heart broth is a rich culture medium that differs from other media analyzed for possessing large quantities of beef brain heart infusion. An antimicrobial substance produced by a strain of *B. amyloliquefaciens* presented important activity when is grown in brain heart broth, but not in peptone broth, suggesting that the biosynthesis of the peptide requires a rich medium (LISBOA *et al.*, 2006). By contrast, peptone seems to be a key nutrient for the production of antifungal compounds by *B. amyloliquefaciens* RC-2 (YOSHIDA *et al.*, 2001). Mueller-Hinton broth, also has large quantities of beef extract and caseine hydrolysate, but instead of glucose, the main source of carbohydrate is starch. In studies of production of bacteriocins of lactic acid bacteria has been assumed that the supply of amino acids promotes the production of bacteriocins (AASEN *et al.*, 2000) and also the lack of glucose has been considered a limiting factor for the production of bacteriocins (RUSSELL and MANTOVANI, 2002). In addition, JATINDER and JANA (2009) observed that a concentration increased from 10 to 15 g/L of starch and from 2 to 5 g/L of peptone has negative effects on cell concentration of a *Bacillus licheniformis* strain.

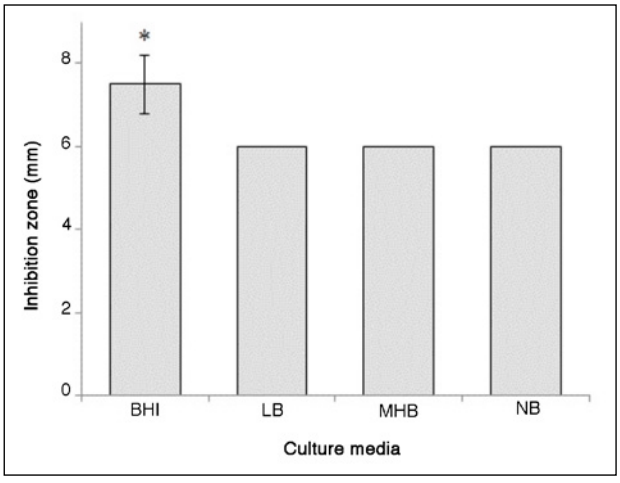


Fig. 2 - Effect of culture media on the production of antagonistic substances: brain heart infusion (BHI), lactose broth (LB), Mueller-Hinton broth (MHB) and nutrient broth (NB). The antimicrobial activity of different extracts was measured by the agar diffusion method against *B. cereus* and expressed as inhibition zones (mm). An asterisk indicates statistically significant difference ($p< 0.05$).

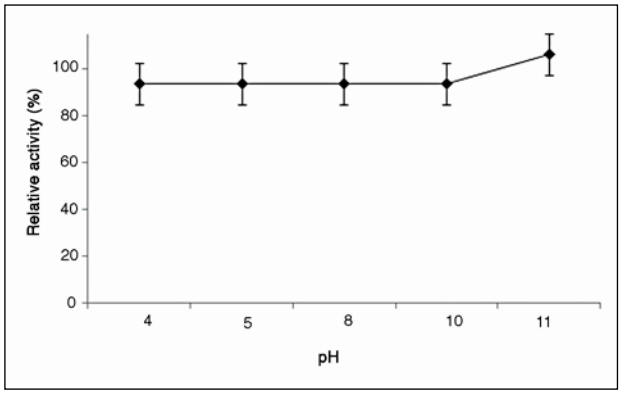


Fig. 3 - Effect of pH on the antimicrobial activity of CBLS. The antimicrobial activity is expressed as the percentage of relative activity against *B. cereus* of the buffered extracts compared with the untreated extracts of pH 7. An asterisk indicates statistically significant difference ($p< 0.05$).

CBLS produced by strains of *B. subtilis* R1 was also active over a wide pH range (Fig. 3), showing not significant differences ($p<0.05$). It was observed that this CBLS was stable at pH 4 to 11, without showing reductions of activity in the extreme pH. The bacteriocin Bac 14B produced by a *B. subtilis* strain was studied in the pH range 2 to 12, showing an optimal activity at pH 7 and a significantly reduction of the activity to less than 2.5 times that of the control at pH 9 (HAMMAMI *et al.*, 2009). In some cases, the effect of pH has been associated with changes in the activity of enzymes involved in post-translational modification or pH-dependent release of bacteriocins from the cell surface (RUSSELL and MANTOVANI, 2002). The stability of bacteriocins at different pHs is a great advantage from the industrial point of view, since the pH of many foods varies from acidic to very basic (MARTIRANI *et al.*, 2002). These observations increase the probability that these compounds are suitable for the food conservation and personal care applications, because they can adapt and function in a variety of environments (SUTYAK *et al.*, 2008). The importance of pH on bacteriocin activity has been explained due to its impact on the stability and solubility of the bacteriocins (LIU and HANSEN, 1990). For example, nisin has shown that it is 228 times more soluble at pH 2 than at pH 8 (LISBOA *et al.*, 2006) and has a much higher activity at pH values below 6.

The antimicrobial substance produce by *B. subtilis* R1 was partially stable to heat treatments; the activity was maintained during treatments up to 100°C and disappeared only after

15 min of autoclaving at 121°C (Figs. 4 and 5). Studies of thermal effect on the antimicrobial activity revealed that CBLS activity resisted high temperatures. At 100°C the CBLS remained active despite it does not resist sterilization conditions. Similar results were obtained for bacteriocin substance of *B. subtilis* 14 whose activity resisted up to 2 hours at 100°C (HAMMAMI *et al.*, 2009) or the bacteriocin of *B. amyloliquefaciens* described by SUTYAK et al. (2008) that resists heat treatment up to 100°C for 60 min. However, a CBLS produced by *B. subtilis* strain loses most of its activity at 100°C for 10 min (KINDOLI *et al.*, 2012). The effect of temperature on the antimicrobial activity of these substances is important for their potential use as food additives. The technological processes applied to foods can affect the inhibitory activity of these compounds. Food tolerates a variety of heat treatments during preparation, so it is imperative that antimicrobial peptides were resistant to heat when food production demands this kind of treatment.

The effect of both pH and temperature on the antimicrobial substance showed that CBLS maintain the antimicrobial activity at high temperatures and mainly acidic pHs (Fig. 6).

The crude extracts produced by *B. subtilis* R1 exhibited antimicrobial activity under conditions of freezing and cooling after 10 and 30 days of storage in the refrigerator. CBLS maintained an activity above 80% compared to the initial activity (Fig. 7). The antimicrobial activity of CBLS was stable to storage at refrigeration and freezing temperatures for a considerable time. ABO-AMER (2007) obtained similar results in storage studies of plantaricin AA135 produced by *L. plantarum* AA135 determining that this bacteriocin crude extract stored at -20° and 4°C conserved its activity without substantial loss for a minimum of 100 days. The fact that this substance show stability after freezing has a technological importance, since the demand of frozen food is increasing, which is associated with problems as maintaining freezing temperature during storage and distribution. These results suggest that this antimicrobial substance could be applied to foods that have a prolonged durability at refrigerating or freezing temperatures, without showing a reduction of its antimicrobial action.

The mode of action of bacteriocins may be bactericidal or bacteriostatic, determining death or extension of lag phase, respectively (SETTANI and CORSETTI, 2008). Indicator bacteria cells in exponential growth are subjected to different concentrations of bacteriocins, assessing the viability and cell biomass. A reduction in viable cell counts and OD_{600nm} of *B. cereus* treated with the CBLS was showed (Fig. 8). After 2 hours of treatment the viable cell counts showed a significant reduction, compared to the control at the same time of incubation. Also it was measured as a reduction of OD_{600nm} of the treated cells compared with the control at 6 h of treatement. CBLS produced by *B. subtilis* R1 showed inhibitory activity against closely related species (FANGIO *et al.*,

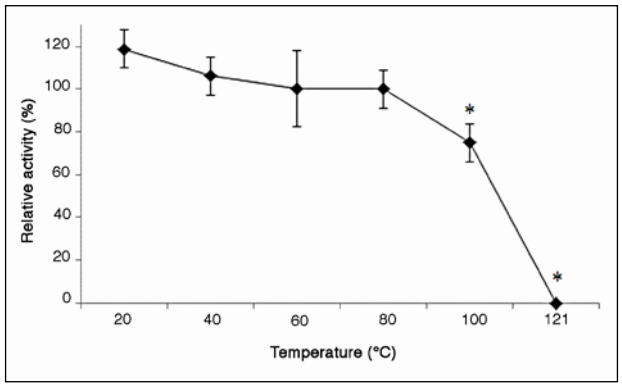


Fig. 4 - Effect of temperature on antimicrobial activity. The antimicrobial activity is expressed as the percentage of relative activity against *B. cereus* of the extracts incubated 30 minutes at different temperatures and 15 minutes at 121°C compared with untreated extracts incubated at 37°C. An asterisk indicates statistically significant difference ($p < 0.05$).

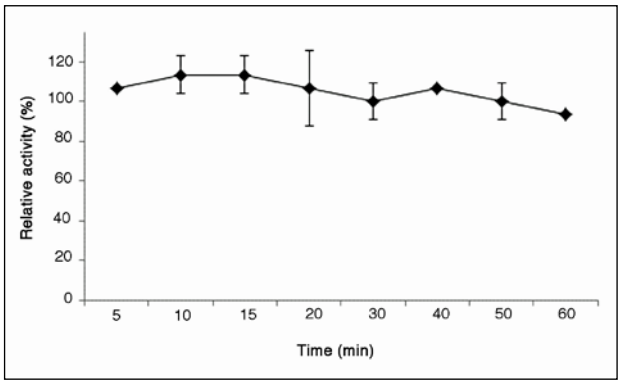


Fig. 5 - Effect of exposure time at 100°C on the antimicrobial activity. The antimicrobial activity is expressed as the percentage of residual activity against *B. cereus* of extracts incubated at different times at 100°C compared to the antimicrobial activity of extracts. An asterisk indicates statistically significant difference ($p < 0.05$).

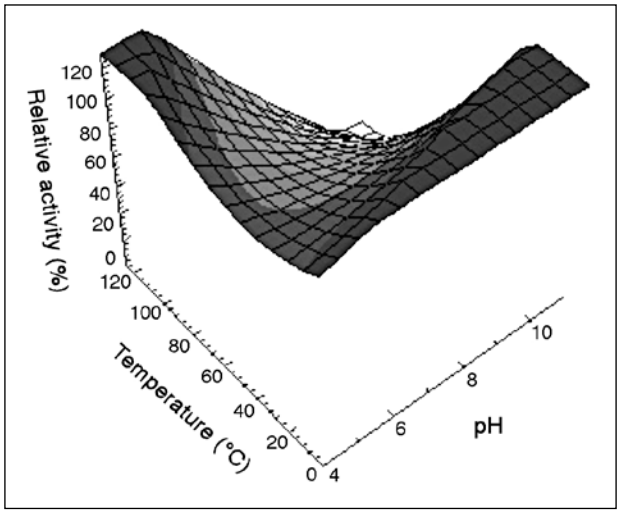


Fig. 6 - Effect of pH and temperature on the stability of *B. subtilis* R1 bacteriocin-like substance.

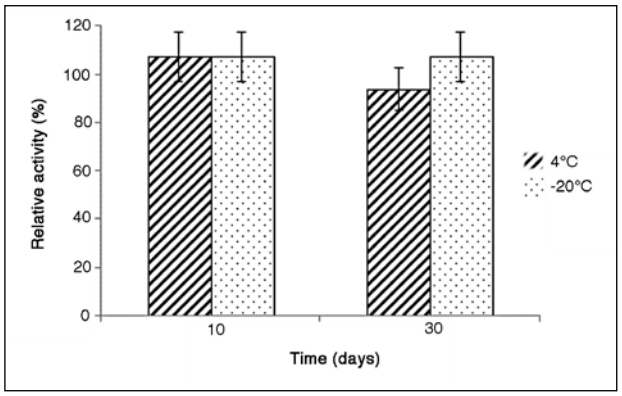


Fig. 7 - CBLS stability in different temperatures and storage times. The antimicrobial activity is expressed as the percentage of relative activity of the extracts stored compared to untreated extracts against *B. cereus*. An asterisk indicates statistically significant difference ($p < 0.05$).

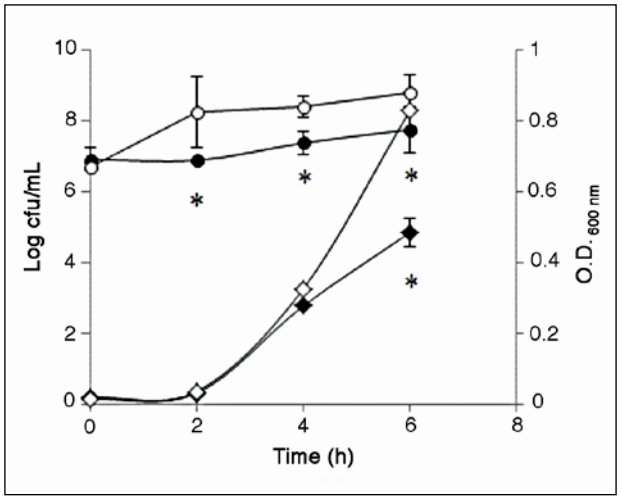


Fig. 8 - Effect of CBLS on the growth of *B. cereus*. Optical density (diamonds) and log CFU/mL (circle) of *B. cereus* untreated (open) and treated (closed) with CBLS. An asterisk indicates statistically significant difference ($p < 0.05$) between control and treated cells.

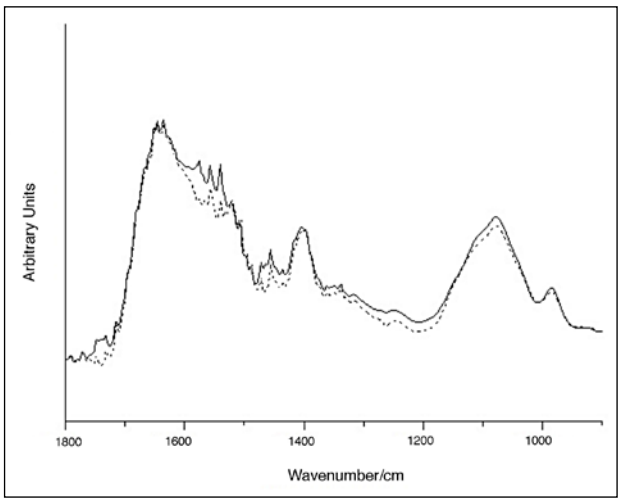


Fig. 9 - Spectroscopy Fourier Transform Infrared (FTIR) of *P. larvae*. The normalized infrared spectra (1,800-900 cm^{-1}) of treated (dashed line) or untreated (solid line) biomass were recorded using the ATR method.

2010). In this study viable cell counts of the indicator strain *B. cereus*, were immediately affected by the presence of the supernatant tested, showing a reduction in the rate of growth of *B. cereus* compared to the untreated control. Consequently our results suggest that this substance has a bacteriostatic effect against *B. cereus*. However, the effect could be subject to the particular assay conditions, such as the quantity and purity of the antimicrobial substance, the indicator strain, and its cellular concentration. Indeed, some bacteriocin has showed a bacteriostatic activity in a low dose while a bactericidal and bacteriolytic activity was observed at high concentration of the bacteriocin (BOUCABEILLE *et al.*, 1997).

The hemolytic activity of CBLS R1 assayed against erythrocytes was negative. In addition, negative reactions for phospholipase C were also observed. Gram-positive bacteria produce a variety of extracellular molecules that show antibacterial activity, including hemolysins, phospholipases and muramidases (OSCARIZ and PISABARRO, 2000). Some bacteriocins produced by bacteria isolated from food, have hemolytic activity but this activity is not associated with antimicrobial activity (BIZANI *et al.*, 2005). On the other hand class III bacteriocins include megacins A-216 and A-19 213 large proteins (430 kDa) with phospholipase activity as produced by strains of *B. megaterium*. BIZANI *et al.* (2005) determined the lack of phospholipase activity associated with the proteolitic enzyme inactivation suggesting the protein status of the cerein 8A.

The effect of the antimicrobial substance over macromolecular structures of the cell was studied by FTIR spectroscopy using *P. larvae* as testing strain. The cells of *P. larvae* treated with the CBLS and the control showed differences in the absorbance in the band corresponding to the region of fatty acids (3,000 to 2,800 cm^{-1}) (data not showed), in the band assigned among other to ester carbonyl and carboxyl groups (1,800-1,500 cm^{-1}), and in the band assigned among others to the phospholipids (1,500-1,200 cm^{-1}) (NIETO *et al.*, 2004). FTIR was used to obtain a better understanding of the molecular mechanisms responsible for the antimicrobial activity of the CBLS. FTIR spectroscopy applied to cultures of *P. larvae* treated with the CBLS shows significant changes in symmetrical bonds of C=O (1,400 cm^{-1}), antisymmetrical stretching of bonds CH_2 and CH (2,918 cm^{-1}) and antisymmetrical stretching of bonds of CH and CH_3 (2,955 cm^{-1}) in the region corresponding to fatty acids, essential components of the membranes. Some authors proposed that the effects on cell membranes eventually translate into the loss of vesicular contents across the local destabilization of lipid packing, or the formation of "pores". This effect would modify the barrier properties of the membranes due to damage in areas where the antimicrobial substance interacts with the phospholipids. This would cause

structural fluctuations that may well explain the main mode of action that quickly affects membrane integrity instead of other vital processes. Similar results were obtained by BIZANI *et al.* (2005) when they studied the mechanism of action of cerein 8A by FTIR, proposed that it could be a peptide that forms pores.

The CBLS was partially purified by extraction with solvents, in this case, 1-butanol, resulting in a concentrated solution. The concentrated extract showed higher activity that the crude extract against *B. cereus* and *P. larvae* (Table 1). The titration of the antimicrobial activity of the concentrated extract against *B. cereus* was measured as 400 AU/mL. Many methods have

Table 1 - Antimicrobial activity of extracts.

	B. cereus	P. larvae
CBLS	8,3±0,6[a]	15,7±0,6[a]
Concentrated extract	14,5±0,7[b]	34±1,41[b]

*Antimicrobial activity of different extracts is expressed as inhibition zones (mm). Values against the same indicator strains (vertical columns) with different superscripts differ significantly ($P < 0.05$).

been used to concentrate and purify the bacteriocins, some of which combine several procedures (LI *et al.*, 2006), while others use just a few steps of purification (OSCARIZ and PISABARRO, 2000; ABO-AMER, 2007). When these methods were performed, the recovery of antimicrobial activity should take into account, since in each step of concentration and purification material loss occurs. As a result, we obtain a concentrated extract which antimicrobial activity is greater than the original crude extract. TEN BRINK *et al.* (1994) determined that the bacteriocin acidocin B is a hydrophobic peptide due to recovery with 1-butanol extraction from cell-free neutralized supernatants, being a feature shared with other bacteriocins (KLAENHAMMER, 1993).

CONCLUSIONS

The activity of the crude bacteriocin-like substance (CBLS) was dependent of the components of the culture media and was detected in the exponential phase of growth. The CBLS was stable at a variable pH range and temperature of pasteurization, being inactivated by sterilization. In addition, CBLS was stable to storage at temperature of refrigeration and freezing. The mode of action of the antimicrobial activity was bacteriostatic and changes of the bands in the infrared region also make evident that the effect occurs on the cell membrane by FTIR. These results indicate that the BLS produced by *B. subtilis* R1 would have a technological interest in food preservation.

ACKNOWLEDGEMENTS

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PAPER

QUALITY OF BREAD CONTAINING *POTENTILLA ANSERINA* L. CULTIVATED IN CHINA

YING JI, JIE GANG and WENZHONG HU*
Key Laboratory of Biotechnology & BioResources Utilization, College of Life Sciences, Dalian Nationalities University, 18 Liaohe Road West, Dalian 116600, China
*Corresponding author: jyapple@gmail.com

ABSTRACT

The *Potentilla anserina* L. is a traditional Chinese functional food. The objective of this study was to exploit the use of this nutrient-rich food in bread by replacing wheat flour at 1-9% level.

Breads prepared with *P. anserina* were analyzed for dough rheology properties, loaf volume, crumb texture, micro-structural and selected sensory attributes. Dough rheological properties were investigated using farinograph. Upon addition of *P. anserina*, significant increase in water absorption, development time and stability, and decrease in the mixing tolerance index was recorded. Loaf volumes decreased significantly from 5.32 to 3.85 cm^3/g as the *P. anserina* supplementation increased from 0 to 9%. Hardness and total phenolic contents significantly increased with the addition of *P. anserina* powder. The microstructure of the breads was characterized by scanning electron microscopy (SEM). The addition of *P. anserina* to wheat flour distinctly destroyed gluten matrix in the SEM images and produced a continuous sheet of gelatinized starch. Sensory evaluations revealed that *P. anserina* can be added to flour at the level of 5%, as did not deteriorate the bread sensory evaluation.

The acceptable quality bread could be made with up to 5% *P. anserina*, as did not deteriorate the bread sensory evaluation. Therefore, the addition of *P. anserina* could be an effective way to produce functional wheat flour bread.

As the role of diet in the prevention of non-communicable diseases has become more evident, there is a growing demand for a new generation of healthier food products. Since limited references are published in the area of *P. anserina* enriched breads, studies on the development of new products with acceptable physico-chemical characteristics are essential for exploiting value-added uses of *P. anserina*.

- Keywords: *Potentilla anserina* L., dough property, rheology, bread quality -

INTRODUCTION

Nowadays, there is a growing demand for a new generation of healthier food products which at the same time have excellent sensory qualities and consumers do not want artificial or synthetic additives to be used for breadmaking. With appropriate formula modifications or process optimization, breads can be made with the addition of non-traditional ingredients. A variety of wheat flour substitutions have been tried in bread formulations; for example, soy or corn flours (ALPASLAN and HAYTA, 2006; RYAN *et al.*, 2002), soybean flour (JUNQUEIRA *et al.*, 2008), sweetpotato flour (HATHORN *et al.*, 2008) flaxseed (KOCA and ANIL, 2007), wheat germ (SIDHU *et al.*, 2001), and sunflower seed (SKRBIC and FILIPCEV, 2008).

The silverweed cinquefoil (*Potentilla anserina* L.) is distributed widely in the western areas of China, especially in Qinghai-Tibetan Plateau. For thousands of years, it has been used frequently as a crude substance, taken orally as a functional food and folk medicine. It is documented in the Chinese Tibetan Material Medical that the major biologically components of *P. anserina* contain carbohydrates, protein, fat, tannin, flavonoids, lysine, histidine, etc. (CHEN *et al.*, 2000). For thousands of years, it has been used frequently as a crude substance, taken orally as folk medicine in Hepatitis B virus treatment (QIAO *et al.*,1992).

In response to the promotion of the health benefits of consumption, a variety of plant sources have been used in bread to improve the nutrition of the final product (FILIPOVIC *et al.*, 2007; HATHORN *et al.*, 2008; PERESSINI and SENSIDONI, 2009). Since limited references are published in the area of *P. anserina* enriched breads, studies on the development of new products with acceptable physico-chemical characteristics are essential for exploiting value-added uses of *P. anserina*. The main objectives of this study were to assess the rheological properties of wheat doughs and the final quality of the resulting breads added with *P. anserina* at different levels.

MATERIALS AND METHODS

Materials

The *P. anserina* was donated by Dalian Fusheng Pharmaceutical Co., Ltd. (China). The whole *P. anserina* was ground in a grinder (shanghai shibang machinery Co., Ltd, China) and passed through a 150 μm sieve to a fine powder.

General

The *P. anserina* powder was 1, 3, 5, 7 and 9% in wheat flour respectively. The raw materials for bread-baking were weighed according to the formula proportions 1, 3, 5, 7 and 9 g *P. anserina* was incorporated in 99, 97, 95, 93 and 91 g wheat flour, respectively. A commercial blends of wheat flour (14.6% moisture, 11.3% protein and 0.61% ash) was used. Amounts of other ingredients were similar in these different formulations i.e. 60 g sugar, 15 g salt, 15 g baker's yeast, 30 g butter, 40 g non-fat dry milk and 600 g water.

Dough characteristics

The effect of the *P. anserina* powder on dough rheology during mixing was determined by a Farinograph (Brabender, Duisburg, Germany), following the AACC Method (2000). A 300 g of flour sample supplemented with the major components of the *P. anserina* formulations were loaded in the farinograph. The parameters determined were: water absorption or percentage of water required to yield dough consistency of 500 BU (Brabender Units), dough development time (time to reach maximum consistency in minutes), stability (time dough consistency remains at 500 BU) and mixing tolerance index (MTI, consistency difference between height at peak and that 5 min later, BU).

Baking tests

A straight dough bread making process was performed. Basic dough formula on 100 g flour basis the amount of water required to reach 500 BU of consistency. The doughs were optimally mixed, fermented for 30 min, then dough pieces (100 g) were divided, hand-moulded and sheeted. Doughs were proofed at 35°C up to optimum volume increase, and baked at 190°C for 20 min. The bread quality attributes were evaluated after cooling for 1 h at room temperature.

Bread texture

Texture profile analysis (TPA) was performed using a TA-XT2i texturometer (Stable Microsystems, Surrey, UK) equipped with a plug (P/36R). Crumb slices of 2 cm were 50% compressed. The probe speed during the test was 2 mm/s and the compression distance 10 mm. The resulting peak force was measured in grams. Crumb slices of 2 cm were 50% compressed. Four replicates from two different sets of baking were analysed and averaged. The parameters recorded were hardness, chewiness, cohesiveness, springiness and resilience.

Loaf volume of bread

Breads were allowed to cool for 1 h at room temperature before the measurement of the loaf volume by a rapeseed displacement method (AACC, 2000).

Scanning electron microscopy analysis

The baked sample pieces were mounted and dried in a forced convection oven (at 100°C for

1h). Then, the dried samples were kept in a desiccator until further use. The samples were spatter-coated with palladium: platinum by ion coater (Hitachi, E-1030), and observed by scanning electron microscope (SEM, Hitachi, S-4500). Samples were observed at a magnification level of 600x.

Determination of total phenolic contents

Total phenolics were determined using the Folin-Ciocalteau method (SINGLETON, ORTHOFER and LAMUELA-RAVENTOS, 1999). 1 g turmeric powder or powdered bread was homogenised in 80% ethanol at room temperature, centrifuged at 10,000 g for 15 min and the supernatant was saved, respectively. The residue was extracted twice again with 80% ethanol and supernatants were pooled. Extract was evaporated at room temperature until dry. Residue was dissolved in 5 mL distilled water. Hundred microliters of this extract was diluted to 3 mL using distilled water and mixed with 0.5 mL Folin-Ciocalteau reagent. After 3 min, 2 mL of 20% sodium carbonate was added followed by thorough mixing. Solutions were heated in a 40°C water bath for 30 min. Absorbance was measured at 765 nm using spectrophotometer (Optima 2000DV, Perkin-Elmer, Waltham, MA, USA). Gallic acid was used as a standard and results were expressed as mg gallic acid equivalent/100 g (mg GAE/100 g).

Sensory analysis

Sensory evaluations of bread were conducted by 40 panellists, consisting of Food Engineering College staff and students using a one to nine hedonic rating scale for the overall acceptability, where nine means extreme satisfaction and one extreme dissatisfaction (1: dislike extremely, 5: neither like nor dislike, 9: like extremely). Samples were placed on white plates and identified with random three-digit numbers. Panellists evaluated the samples in a testing area and were instructed to rinse their mouths with water between samples to minimise any residual effect.

Statistical analysis

The experimental data obtained were analyzed statistically for variance (SOKAL and ROHLF, 1981) and inference reported at the appropriate places. For the results, the mean values and standard deviations were calculated.

RESULTS AND DISCUSSION

Effect of *P. anserina* on the farinograph characteristics of wheat flour

The farinograph results of *P. anserina* supplemented dough and the reference dough (without *P. anserina* addition) were shown in Fig. 1 and Table 1. As the flours contained *P. anserina*, these materials were considered to disturb the continuous gluten network structure in the wheat dough.

Water absorption was increased by *P. anserina* addition in agreement with previous data (ROSELL *et al.*, 2006; WANG *et al.*, 2002). The water absorption linearly increased from 63.2% in the reference to 71.9% in the sample with 9% *P. anserina*. This is likely caused by the great number of hydroxyl groups existing in the fibre structure of *P. anserina*, which allow more water interactions through hydrogen bonding. Dough development time and stability value are indicators of the flour strength, with higher values suggesting stronger dough. Dough development time and stability value trend to increase as a consequence of addition *P. anserina* from 0 to 9%, but an increase of the dough stability time and a decrease of the development value when adding 3% *P. anserina* was found, which is in agreement with LAURIKAINEN *et al.* (1998). The addition of *P. anserina* to wheat flours improved the strength of the doughs and the effect was especially prominent in the case of weaker flour. ZAIDUL *et al.* (2004) thought that dough development time was related to water absorption percentage of the flours. An increase in water absorption will often increase development time. The mixing tolerance index was reduced by the addition of the tested *P. anserina*, and the extent of the decrease depended on the *P. anserina* considered. These results could be explained by the interactions between fibres of *P. anserina* and gluten.

Influence of *P. anserina* on bread quality evaluation

To demonstrate the effect of *P. anserina* on bread qualities, the additional baking test was conducted using increased amount of *P. anserina*. Loaf volume is regarded as the most important bread characteristic since it provides a quantitative measurement of baking performance (Tronsmo *et al.*, 2003). A significant decrease in bread volume was noted with an increase in the *P. anserina* powder level. Bread loaf volumes decreased significantly ($P < 0.05$), from 5.32 to 3.85 cm^3/g, as the *P. anserina* powder increased from 0 to 9% (Table 2). Partial replacement of wheat flour with non-glutinous flour has been shown to result in lower bread volumes, as reported by GÓMEZ *et al.* (2003) who observed a significant decrease in baked volume of bread made with purified dietary fibres. The decrease in loaf volume was expected as a result of gluten-free *P. anserina* powder in bread formulation and the addition of *P. anserina* powder reduced the ratio of wheat flour responsible for the formation of network structure.

The hardness values increased with increasing levels of *P. anserina*, from 509.524 g for the control bread to 2254.205 g in 9% *P. anserina* bread (Table 3). The addition of *P. anserina* pow-

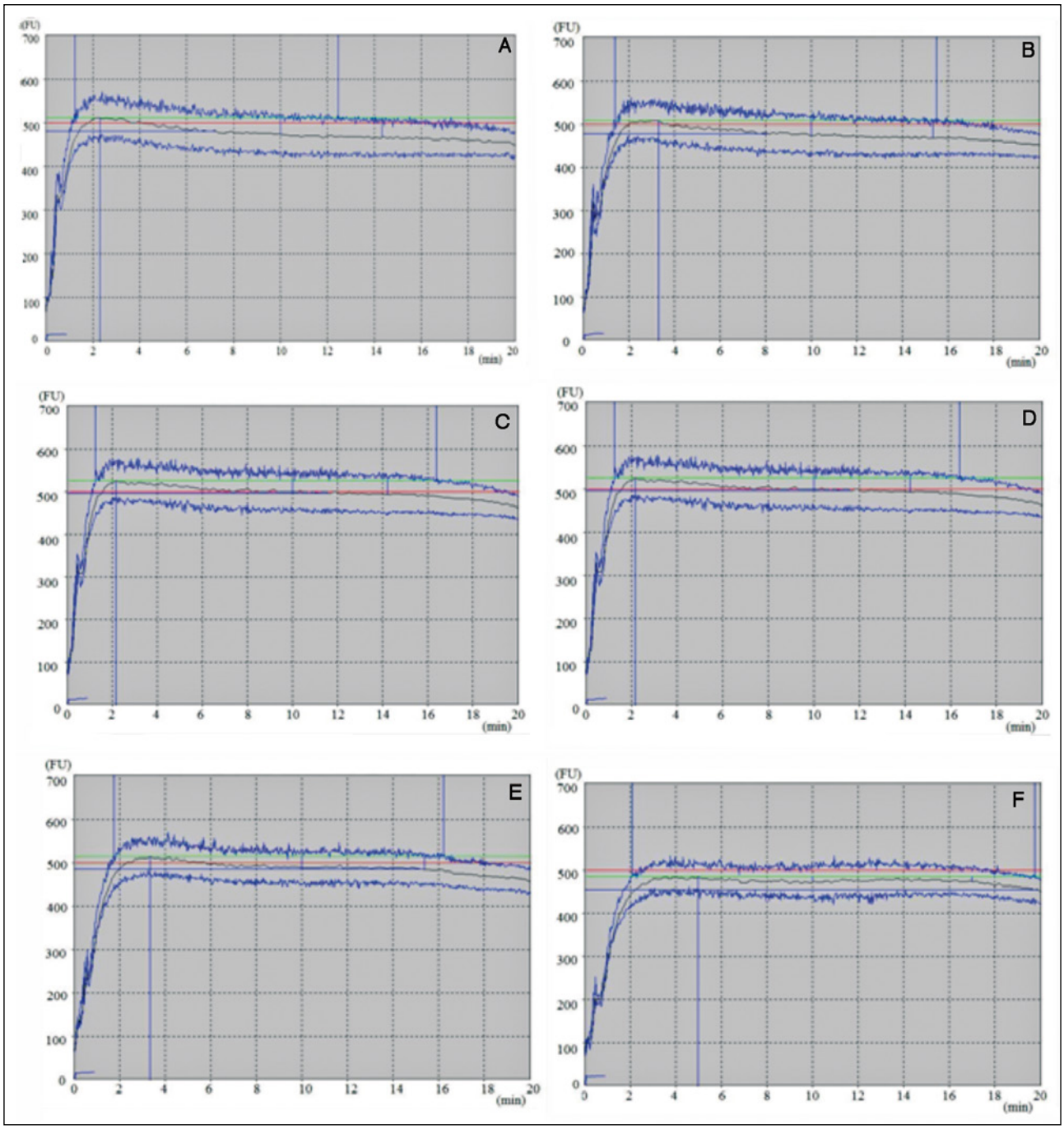


Fig. 1 - Farinograph curves of wheat flour enriched with *P. anserina* at 0%(A), 1%(B), 3%(C), 5%(D), 7%(E) e 9(F).

Table 1 - Effect of *P. anserina* addition on water absorption, arrival time, peak time, stability time, development time and mixing tolerance index of doughs at farinograph.

P. anserina (%)	Water absorption (%)	Arrival Time (min)	Peak Time (min)	Stability Time (min)	Development time (min)	Mixing Tolerance Index (BU)
0	63.2	1.27	2.36	11.2	2.3	38
1	64.2	1.36	3.27	14.1	3.3	35
3	65.5	1.25	2.15	15.1	2.2	27
5	67.7	1.64	3.13	15.0	3.2	23
7	70.0	1.76	3.38	14.5	3.4	27
9	71.9	2.08	5.02	17.7	5.0	12

Table 2 - Effect of *P. anserina* addition on the volume of wheat bread (mean ± S.D.).

***P. anserina* %**	**0**	**1**	**3**	**5**	**7**	**9**
Loaf volume(cm^3/g)	5.32 ± 0.28^{c}	5.39 ± 0.41^{a}	5.28 ± 0.33^{b}	5.13 ± 0.23^{cd}	4.52 ± 0.35^{b}	3.85 ± 0.17^{d}

Means followed by the same letter were not significantly different (P < 0.05).

Table 3 - Effect of *P. anserina* addition on crumb texture values (mean ± S.D.).

P. anserina **(%)**	**Hardness (g)**	**Gumminness (g)**	**Chewiness (g)**	**Stickiness (g)**	**Springness (g)**	**Cohesiveness (g)**	**Resilience (g)**
0	509.524 ± 47^{b}	432.585 ± 27^{c}	414.133 ± 32^{b}	60.872 ± 14^{b}	0.980 ± 0.41^{b}	0.849 ± 0.32^{b}	0.319 ± 0.13^{bc}
1	782.698 ± 35^{d}	652.770 ± 38^{ab}	629.923 ± 25^{c}	129.357 ± 13^{bc}	0.965 ± 0.33^{c}	0.834 ± 0.37^{a}	0.377 ± 0.24^{a}
3	1488.377 ± 39^{cd}	1224.934 ± 41^{a}	1185.736 ± 37^{a}	196.108 ± 15^{b}	0.968 ± 0.35^{c}	0.823 ± 0.28^{bc}	0.365 ± 0.15^{b}
5	1194.716 ± 56^{a}	939.046±35b	885.520 ± 22^{c}	230.915 ± 18^{a}	0.943 ± 0.24^{d}	0.786 ± 0.25^{c}	0.326 ± 0.11^{c}
7	1399.607 ± 40^{c}	1153.276 ± 30^{c}	1099.072 ± 19^{cd}	132.555 ± 11^{c}	0.953 ± 0.45^{a}	0.824 ± 0.31^{b}	0.331 ± 0.16^{b}
9	2254.205 ± 33^{d}	1812.381 ± 33^{b}	1705.450 ± 26^{c}	386.583 ± 17^{a}	0.941 ± 0.31^{c}	0.804 ± 0.23^{c}	0.323 ± 0.18^{b}

Means followed by the same letter were not significantly different (P < 0.05).

der retained more moisture thus increasing the hardness values of the bread and changed the quantity of protein in wheat flour *P. anserina* powder system and thereby affected the uniform structure in *P. anserina* bread. The addition of *P. anserina* also had an effect on the parameters of gumminess, chewiness and stickiness, while the other parameters from the TPA did not show important changes. ALPASLAN and HAYTA (2006) reported that the ground flaxseed, soy and corn flours had a significant effect on the textural parameters of flaxseed containing breads.

Microstructure of bread

Scanning electron microscopy was used in order to investigate the structural integrity of bread crumb at various levels of *P. anserina* powder. As shown in Fig. 2 (A), SEM of the bread without *P. anserina* covered most starch

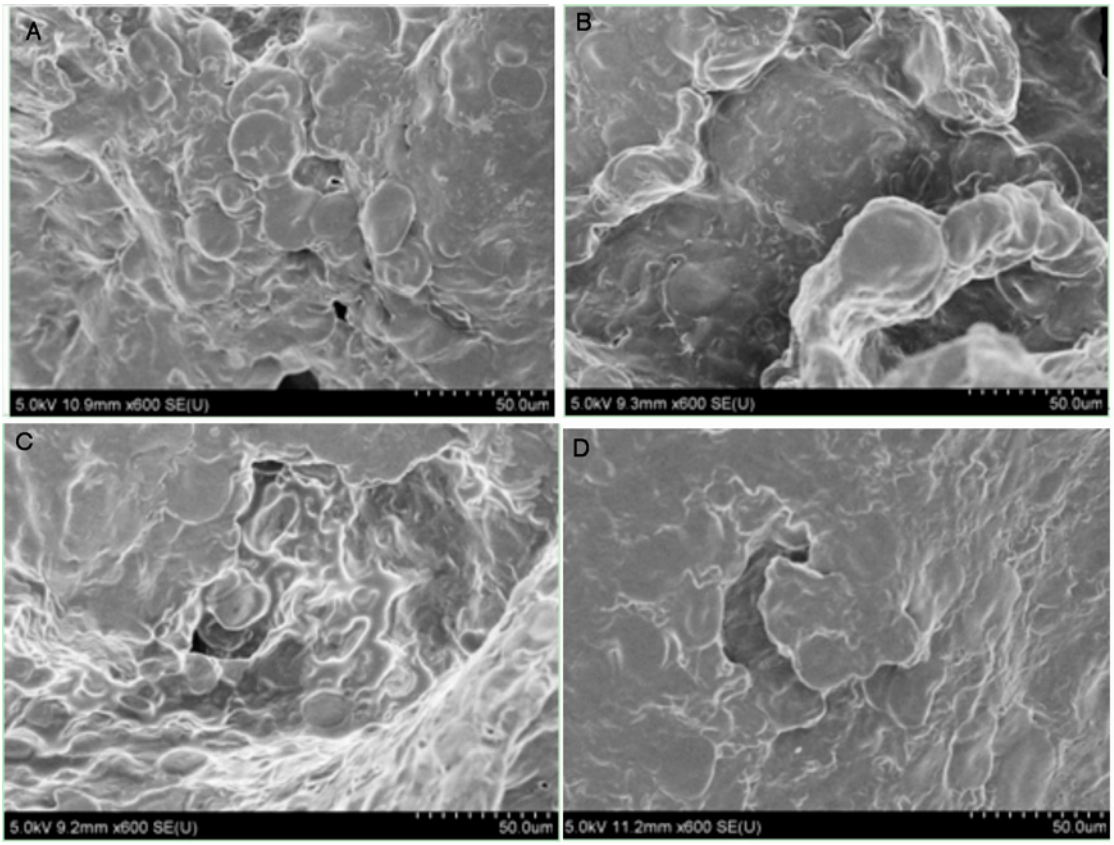


Fig. 2 - Scanning electron micrograms (x600) of the crumb of bread with *P. anserina* at 0%(A), 1%(B), 5%(C) e 9%(D).

granules coated by gluten network to form a continuous film. The addition of 1% *P. anserina* had no detectable effects on the microstructure observed in the bread samples as shown in Fig. 2(B). Fig. 2(C) shows the samples with a *P. anserina* level of 5%. As shown in figure 2(C), some granules in the breads also appeared to be swollen and slightly elongated, possibly due to gelatinization during the baking process. Fig. 2(D) shows 9% *P. anserina* powder-supplemented bread. The inner surface starch granules of *P. anserina* bread have being completely distorted and having lost their identity and formed a continuous sheet of gelatinized starch, suggesting a greater starch gelatinisation and amylose/amylopectin leakage. Furthermore, a continuous gluten film was not observed. According to SEETHARAMAN *et al.* (1997) doughs containing 8% of soluble fiber had poor gluten development and extensive starch gelatinization during baking.

Total phenolics contents

The total phenolic contents of *P. anserina* powder and bread samples were analysed and results are shown in Fig. 3. The phenolic content increased significantly with increasing *P. anserina* powder levels in bread and the highest was found in bread with 9% substitution with *P. anserina* powder. The phenolic content

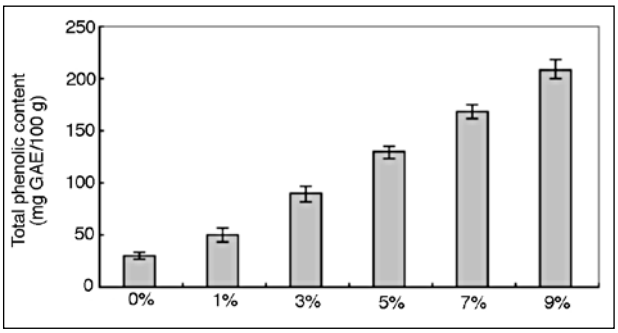


Fig. 3 - Total phenolic contents of breads containing *P. anserina*.

of *P. anserina* powder was 3,365 mg GAE/100 g (data not shown). Whereas the bread prepared with 9% *P. anserina* powder had 208.86 mg GAE/100 g of those. Phenolics are quite heat unstable and reactive compounds (CHEYNIER, 2005) and the baking process might have resulted in some heat damage to phenolic compounds. However, despite the loss of total phenol content after baking, all breads containing *P. anserina* showed significantly higher phenolic compounds when compared with the control. ALVAREZ-JUBETE *et al.* (2010) reported that wheat seeds and wheat bread contained 53.1 and 29.1 mg GAE/100 g of total phenols, respectively.

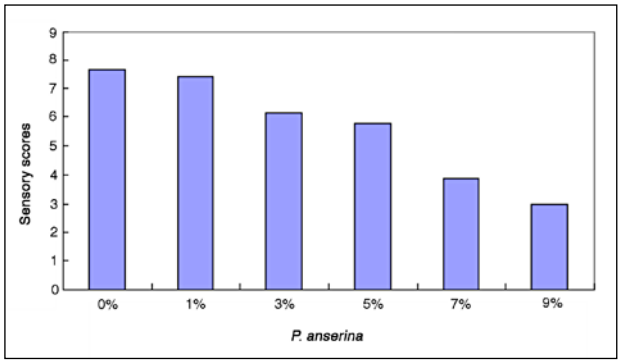


Fig. 4 - Sensory scores for overall acceptability of bread supplemented with *P. anserina*.

Sensory evaluation of bread

The effects of *P. anserina* addition on bread overall sensory score are presented in Fig. 4. The overall acceptability of breads with *P. anserina* at substitution is quite evident in breads with up to 9% *P. anserina*. In general, panellists preferred bread without *P. anserina* added. Nevertheless, all the breads were acceptable except when the *P. anserina* powder addition at the level of 7 and 9%. Scores were markedly lower in these cases probably due to the original properties of the *P. anserina*, since the colour of *P. anserina* was dark yellow. As can be seen in Fig. 4, Sensory expert analysis carried out in bread texture showed that wheat bread with up to at least 5% wheat flour by *P. anserina* could be substituted without any detrimental effect on the texture characteristics of bread. We may explain that a daily intake of 50 g *P. anserina* bread having 5% wheat flour substitution with *P. anserina* powder can deliver approximately 64.63 mg GAE of total phenolic compounds which can render additional health benefits to human body.

CONCLUSIONS

From the overall results, it could be concluded that the addition of *P. anserina* to wheat flour modifies the rheological properties of the dough. As a result, the substitution of *P. anserina* significantly decreased the loaf volume as compared with the wheat bread. The results of this study showed that bread hardness increased as the level of *P. anserina* increased from 0 to 9%. Our results further support that acceptable quality bread could be made with up to 5% *P. anserina*, as did not detrimental effect on the texture characteristics of bread. The higher levels (>5%) of *P. anserina* powder addition are not recommended as most quality attributes were shown to have affected negatively at the level of 7 and 9%. Optimizing the bread formulations and/or altering processing conditions could overcome any of the negative effects of *P. anserina* powder

addition at higher level. This study demonstrated that the addition of *P. anserina* could be an effective way to produce functional wheat flour bread. Further studies are needed to evaluate changes in *P. anserina* enriched bread characteristics during storage.

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PAPER

MODIFICATION OF THE RHEOLOGICAL BEHAVIOUR OF SODIUM ALGINATE BY CHITOSAN AND MULTIVALENT ELECTROLYTES

BASIM ABU-JDAYIL[1]* and DEEB ABU FARA[2]
[1]Chemical & Petroleum Engineering Department, U.A.E. University, Al-Ain, P.O. Box 17555, U.A.E.
[2]Chemical Engineering Department, University of Jordan, Amman, 11942, Jordan
*Corresponding author: babujdayil@uaeu.ac.ae

ABSTRACT

The rheological behaviour of sodium alginate/chitosan blends was examined at several mixture ratios. While both polymers exhibited nearly the same viscosity at low concentrations (1.0 and 2.0 w%), chitosan solutions had higher viscosity and greater deviation from Newtonian behaviour at high solution concentrations (3.0-5.0 w%). The viscosity of alginate/chitosan mixtures was substantially increased. Blends containing predominantly one component were of higher viscosity than blends with approximately equal proportions of both polymers. The viscosity of alginate solutions was strongly affected by the addition of electrolytes, with $BaCl_2$ exerting the greatest effect on the alginate viscosity and shear thinning behaviour.

- Keywords: alginate, chitosan, electrolyte, rheology, shear thinning -

1. INTRODUCTION

Over the past decade, there has been a surge of interest in natural polymers obtained from renewable sources. Approximately 30,000 metric tons/year of sodium alginates are currently used in the food, cosmetic, pharmaceutical, textile, and paper industries as thickening, stabilizing, and gelling agents. Alginate is a structural biopolymer obtained from brown seaweeds, with a wide range of applications in food, pharmacy, agriculture, and environmental science due to its natural origin and non-toxicity (PAMIES *et al.*, 2010). Alginates are naturally derived linear copolymers of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid residues (G). The way in which these M and G units are arranged in the chain and the overall M/G ratio in a chain varies between seaweed species (OUWERX *et al.*, 1998; LU *et al.*, 2006).

The utility of alginates is based on three main properties. The first is their ability, when dissolved in water, to increase the viscosity of aqueous solutions. The second is their ability to form gels when a calcium salt is added to a solution of sodium alginate in water (MOE *et al.*, 1995; LU *et al.*, 2006). The third property is the ability to form films of sodium or calcium alginate and fibers of calcium alginate (RIBEIRO *et al*, 2011). Alginates have a wide range of applications, including the controlled release of medicinal drugs and other chemicals (AL-MUSAA *et al.*, 1999; ALBARGHOUTHI *et al.*, 2000, JOSEF *et al.*, 2010) and as thickeners in the food industry (GOMEZ-DIAZ and NAVAZA, 2004).

Chitosan is a derivative of chitin (a natural polysaccharide) which may be prepared by *N*-deacetylation of chitin in an alkaline medium (RINAUDO, 2006; KEMPE *et al.*, 2008). Chitin is the second most abundant natural polymer in the world and is biorenewable, biocompatible, environmentally friendly, biodegradable, and biofunctional (RINAUDO, 2006; HUE *et al.*, 2010). Chitosan is frequently used in biomedical, food, drug, and cosmetic applications (CHO *et al.*, 2006; KEMPE *et al.*, 2008; PHUOC *et al.*, 2011).

When alginate is mixed with chitosan, strong ionic interactions occur between the carboxyl residues of the alginate and the amino terminals of the chitosan, forming a polyelectrolyte complex. This complex does not dissolve in the presence of anti-gelling cations, and thus may be used to stabilize the gel and reduce the porosity of alginate beads (ALBARGHOUTHI *et al.*, 2000). Recently, oral controlled-release systems involving alginate microspheres, sometimes coated with chitosan to improve mechanical strength, have been tested as a way of delivering various drugs (CHATCHAWALSAISIN *et al.*, 2004; COPPI and IANNUCCELLI, 2009; FINOTELLI *et al.*, 2010; CHÁVARRI *et al.*, 2010; LUCINDA-SILVA *et al.*, 2010; XING *et al.*, 2010). In addition, the alginate/chitosan polyelectrolyte system has been used to produce fibrous scaffolds (WANG *et al.*, 2010).

The application and acceptability of foods and pharmaceuticals are often dependent on the flow properties of the final product. Rheological measurements are an important means of determining the flow and deformation behaviour of materials and can not only improve efficiency in processing but can also help formulators and end users design products that are optimal for their individual needs.

Although many researchers have investigated the rheology and flow properties of alginate-based (CLEMENTI *et al.*, 1998; OUWERX *et al.*, 1998; GOMEZ-DIAZ and NAVAZA, 2004; SIMEONE and GUIDO, 2004; TAYLOR *et al.*, 2005; FUNAMI *et al.*, 2009; VALLÉE *et al.*, 2009; De CELIS ALONSO *et al.*, 2010; PAMIES *et al.*, 2010; RIBEIRO *et al.*, 2011) and chitosan-based (JIANG *et al.*, 1999; MONTEMBAULT *et al.*, 2005; CHO *et al.*, 2006; KEMPE *et al.*, 2008; HU *et al.*, 2011; LI *et al.*, 2010; MIR *et al.*, 2011) materials, only limited studies of the flow properties of alginate/chitosan blends have been reported (SHON *et al.*, 2007).

In this study, the flow properties of sodium alginate, chitosan, and their blends at several ratios were studied. In addition, the effect of divalent and trivalent electrolytes ($CaCl_2$, $BaCl_2$, and $AlCl_3$) on the flow behaviour of alginate was characterized.

2. MATERIALS AND METHODS

2.1 Materials

The materials used in this study were supplied by the Jordanian Pharmaceutical Manufacturing Co. (Naor, Jordan). Sodium alginate with a molecular weight of 14,000-200,000 (Protanal LF120, $C_6O_7H_6Na$) was obtained from Protan Biopolymers. Chitosan (molecular weight 8,000-12,000) was obtained from Qingdao Rich Waters Industrial Ltd, China. The metal salts used as crosslinking materials were calcium chloride dihydrate (Riedel de Haen analytical grade), barium chloride dihydrate (Merck analytical grade $BaCl_2 \cdot 2H_2O$), and aluminium chloride hexahydrate (Merck analytical grade $AlCl_3 \cdot 6H_2O$).

2.2 Rheological measurements

The rheological properties of the prepared gel solutions were determined using a concentric cylinder Chan 35 viscometer, which is equipped with an inner cylinder rotating inside a stationary outer cylinder. The shear stress (τ) of the samples was measured as a function of shear rate ($\dot{\gamma}$) at a constant temperature of 25°±0.1°C. The shear rate was varied from 1.70 to 1002 s^{-1}. To predict the apparent viscosity (η) the following equation was used:

$$\tau = \eta\dot{\gamma} \qquad (1)$$

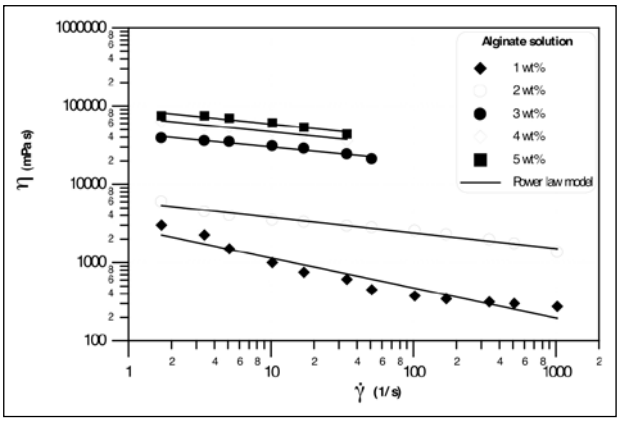


Fig. 1 - The apparent viscosity of alginate solution at different concentrations.

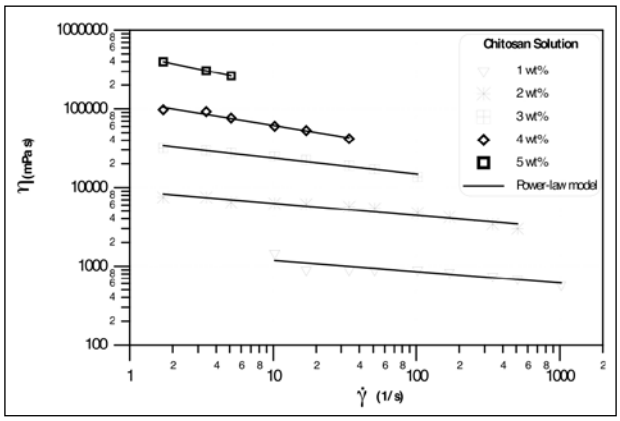


Fig. 2 - The apparent viscosity of chitosan solution at different concentrations.

Rheological experiments were carried out in duplicate with an average reproducibility of ±5%. The average values were used for analysis.

2.3 Preparation of solutions

Solutions of alginate and chitosan were prepared based on mass/volume percentage (0.5 – 5.0% w/v) using an electronic balance (METTLER, 200) with a precision of ± 0.1 mg. The alginate was dissolved in distilled water while the chitosan was prepared in an acidic medium. The chitosan solution was prepared by mixing an appropriate amount of chitosan into distilled water containing 1.0 vol.% acetic acid and stirring using a magnetic stirrer until the solutes were completely dissolved. Alginate-chitosan (ALG-CS) blends were prepared at various volume ratios (4:1, 3:2, 2:3, and 1:4) by mixing appropriate volumes of 1.0 wt % alginate and chitosan solutions.

In the crosslinking experiments, 1.0 g of alginate was dissolved in 80 mL of distilled water and various amounts of salt (0.05-0.50 g) were dissolved in 20 mL of water. The salt solution was added dropwise to the alginate solution to obtain a concentration of 0.05-0.5 g electrolyte/g alginate.

3. RESULTS AND DISCUSSION

Pure solutions of alginate and chitosan displayed shear thinning behaviour at all concentrations studied, in which the apparent viscosity decreased with increasing shear rate (Figs. 1 and 2). This behaviour is expected as the shearing of the polymer chains causes disruption of the three dimensional structure through breaking of primary and secondary bonds. In addition, Figs. 1 and 2 indicate the increase in alginate and chitosan solution viscosity with increasing polymer concentration.

The non-Newtonian behaviour of alginate and chitosan solutions was modelled using the two-parameter power-law model, which is the most frequently used model for alginate (MANCINI *et al.*, 1996; CLEMENTI *et al.*, 1998; GOMEZ-DIAZ and NAVAZA, 2004) solutions. It is given by:

$$\eta = m\dot{\gamma}^{n-1} \qquad (2)$$

where η is the apparent viscosity, m is the consistency coefficient, $\dot{\gamma}$ is the shear rate, and n is the flow behaviour index.

As shown in Figs. 1 and 2, the rheological behaviour of alginate and chitosan can be well described by the power law model. The parameters of power-law model obtained by non-linear regression at different polymer concentrations are reported in Table 1.

Referring to Table 1, the chitosan solutions displayed greater deviation from Newtonian behaviour (n = 1) than the alginate solutions at high concentrations. In addition, the shear thinning behaviour of chitosan increased with concentration, while it was nearly constant in the case of alginate solutions. PAMIES *et al.* (2010) observed that the shear thinning effect in alginate solutions is due to disentanglement of the polymer chains. Both solutions have comparable viscosity at low concentration, while chitosan solutions had greater viscosity at concentrations of 4.0 and 5.0 wt %. This is also apparent in the m values reported in Table 1 and plotted in Fig. 3 as a function of concentration, where a signif-

Table 1 - Regressed parameters of power-law model for alginate and chitosan solutions.

Solution Conc. (w/v %)	m (mPa)		n (-)		R^2	
	Alginate	Chitosan	Alginate	Chitosan	Alginate	Chitosan
1.0	2,753	1,296	0.61	0.90	0.965	0.997
2.0	5,972	8,203	0.80	0.86	0.998	0.991
3.0	44,945	37,603	0.82	0.80	0.998	0.998
4.0	71,459	122,552	0.82	0.70	0.999	0.996
5.0	90,193	488,860	0.82	0.62	0.996	0.999

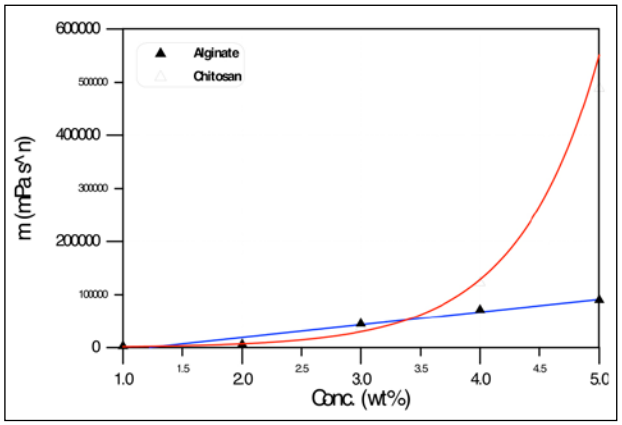


Fig. 3 - Dependence of m values of alginate and chitosan on solution concentration.

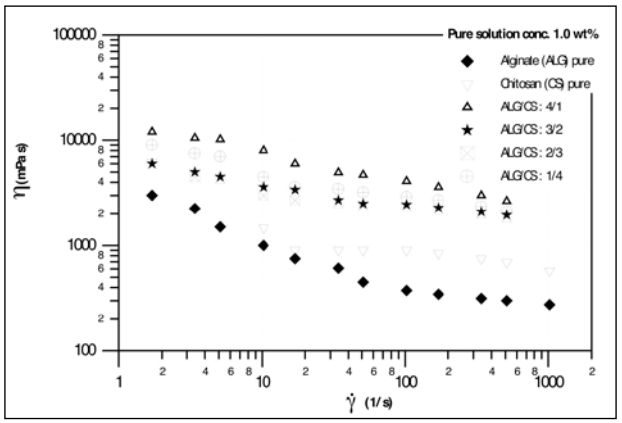


Fig. 4 - The apparent viscosity of alginate-chitosan blends.

icant increase in m values was detected in the chitosan solutions. Fig. 3 also indicates that the consistency coefficient of the alginate solutions increased linearly with increasing solution concentration, while the dependence of the m value of chitosan on the solution concentration is described by an exponential relationship. This is because for very dilute solutions, the polymer coils are widely separated and do not overlap. At a critical concentration marking the transition from the extremely dilute to the dilute concentration region, the hydrodynamic volumes of individual coils begin to touch. As the concentration is increased further, the coils begin to overlap, eventually resulting in the formation of entanglements that significantly increase viscosity.

In the second part of this study, alginate-chitosan blends containing 1.0 wt % total polymer concentration were prepared and their rheological behaviour was compared with the behaviour of pure solutions (Fig. 4). All blends exhibited higher viscosity than the pure solutions. There was no general trend in the viscosity increase, but blends predominantly containing either alginate or chitosan were of higher viscosity than blends with approximately equal proportions of both polymers. SHON *et al.* (2007) reported that mixtures of alginate and chitosan had substantially increased viscosities, and that this increase is related to the formation of a polyelectrolyte complex (PEC). This theory explains the light turbidity observed upon mixing the two polymers. A polyelectrolyte complex is formed by the association of two or more polymers through electrostatic interactions, for example the polycationic chitosan interacting with polyanionic alginate through proton transfer (SHON *et al.*, 2007). MENG *et al.* (2009) observed that films formed from alginate-chitosan blends exhibited different mechanical properties depending upon the composition ratio of the two polysaccharides.

Table 2 reveals a significant enhancement in m values in the alginate/chitosan blends. In addition, the mixing process alters the rheological behaviour of the solution, with the flow behaviour indices (n) of the blends varying between those of pure chitosan and pure alginate. This phenomenon indicates that crosslinking of alginate with chitosan significantly increases the viscosity of the solution while at the same time reducing the shear thinning behaviour.

It is known that alginates exhibit characteristic ion binding to multivalent cations and that this forms the basis for their gelling properties. Cation binding leads to the formation of covalent bonds and precipitation of an insoluble hydrogel. The crosslinking process stiffens and roughens the polymer and reduces swelling in solvents (ALMUSAA *et al.*, 1999). In the final portion of this investigation, 1.0 wt % pure alginate solutions were crosslinked using various concentrations (0.05 – 0.50 g crosslinker/g alginate) of $CaCl_2$, $BaCl_2$, and $AlCl_3$. Adding salts to alginate solutions resulted in an increase in the solution viscosity. PAMIES *et al.* (2010) demonstrated that addition of salts produces large changes in the viscosity of alginate solutions. The viscosity increased with the addition of small amounts of.... due to hydrogel formation, but this effect was reduced when NaCl was added instead. In this study, barium chloride exerted the greatest effect on alginate viscosity in the concentration range under examination (Fig. 5). The addition of 0.05 g of $BaCl_2$ to one gram of alginate increased the solution viscosity by a factor of 2.5,

Table 2 - Regressed values of power law model for alginate – chitosan blends of 1.0 w % polymer solutions.

Blend Ratio Alginate/Chitosan	m (mPa)	n (-)	R^2
Pure alginate (1.0 w %)	2,753	0.61	0.965
Pure chitosan (1.0 w %)	1,296	0.90	0.997
4/1	14,322	0.73	0.990
3/2	6,032	0.81	0.989
2/3	5,466	0.81	0.971
1/4	9,222	0.75	0.986

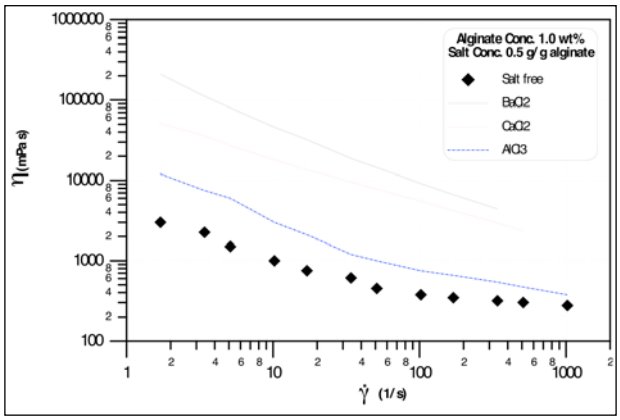


Fig. 5 - The effect of different salts on the apparent viscosity of alginate solution.

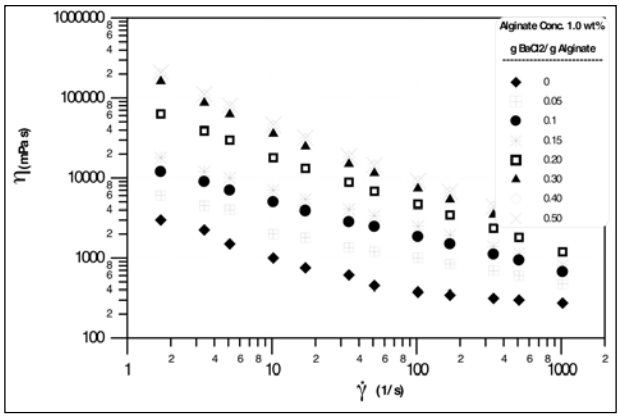


Fig. 6 - The effect of $BaAl_2$ concentration on the apparent viscosity of alginate solution.

and adding 0.5 g of $BaCl_2$ increased the viscosity by a factor of 25. $CaCl_2$ exhibited a greater crosslinking effect than $AlCl_3$ (Fig. 5). OUWERX *et al.* (1998) found that the Young's modulus of alginate beads in the presence of Ba cations is greater than in the presence of Ca cations. It has been reported in the literature (PAMIES *et al.*, 2010) that when divalent cations are added to a solution of sodium alginate, a polymer network is formed. The properties of these networks are dependent on the quantity of cations added. This reversible gelation is due to the capacity of divalent ions to link with alginate chains and induce a conformational change. Divalent cations link with GG blocks of alginate according to the egg-box model (FUNAMI *et al.*, 2009). The cations are located between the polymer chains in a 3-dimensional structure in which the polymers are ordered and form gaps in a manner similar to an egg-box and in which the cations play the role of the eggs. Based on this model, the strong effect of Ba^{+2} on the alginate viscosity could be attributed to the larger size of Ba^{+2} cations relative to and ^{3}cations (BURGESS, 1978), which reduces the gap size between the polymer chains and causes greater resistance to flow.

Increasing the concentration of $BaCl_2$ resulted in a substantial increase in the degree of crosslinking in the alginate solution (Fig. 6), causing a corresponding large increase in solution viscosity. As the barium content is increased, the probability of cation-mediated association increases, causing greater resistance to flow (PAMIES, 2010). Changes in $CaCl_2$ and $AlCl_3$ concentrations did not produce the clear effect on the degree of alginate crosslinking as did $BaCl_2$. In the case of $AlCl_3$, no increase in alginate viscosity was observed at concentrations of up to 0.20 g salt/g alginate. The effect of salt concentration on the rheological parameters of 1.0 wt % alginate solution is presented in Table 3. Increasing the salt concentration increased the consistency coefficient value (m) of the alginate solution and decreased the flow behaviour index (n). Increasing the barium chloride concentration led to a substantial increase in the consistency coefficient (m) which reflects the solution viscosity, and to a significant increase in the shear thinning behaviour, i.e. an increase in the deviation from Newtonian behaviour. This is due to the increase in the degree of bonding in the crosslinked solution as the shearing of the polymer chains caused breaking of primary and secondary bonds and produced a significant decrease in the apparent viscosity with increasing shear rate.

4. CONCLUSIONS

Alginate and chitosan solutions (0.5-5.0 wt % concentration) behaved as shear thinning materials. Blending alginate and chitosan modified the rheological behaviour, increasing the viscosity and altering the shear thinning behaviour to an intermediate value between the behaviour of pure alginate and pure chitosan. Addition of divalent and trivalent salts produced large changes in the viscosity of alginate solutions. The viscosity increased significantly with the addition of small amounts of $BaCl_2$, but the effect was reduced when the added salt was $CaCl_2$ or $AlCl_3$. The increase in viscosity was highly dependent on the salt concentration and substantially increased the deviation from Newtonian behaviour.

Table 3 - Regressed parameters of power law model for crosslinked 1.0w % alginate solution.

g salt/ g alginate	m (mPa)			n (-)		
	$BaCl_2$	$CaCl_2$	$AlCl_3$	$BaCl_2$	$CaCl_2$	$AlCl_3$
0.0		2,753			0.61	
0.05	6,348	3,989	3,016	0.61	0.58	0.70
0.1	14,574	4,017	4,230	0.55	0.59	0.63
0.15	21,730	4,129	5,006	0.53	0.62	0.60
0.2	79,678	5,286	8,626	0.39	0.57	0.50
0.3	198,671	6,655	10,030	0.30	0.60	0.48
0.4	252,242	14,230	10,210	0.28	0.60	0.47
0.5	266,282	65,418	12,110	0.27	0.46	0.45

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PAPER

ENVIRONMENTAL ASSESSMENT OF THE CITRUS FRUIT PRODUCTION IN SICILY USING LCA

A. LO GIUDICE[1]*, C. MBOHWA[1], M.T. CLASADONTE[2] and C. INGRAO[3]
[1]Department of Quality and Operations Management,
Faculty of Engineering and the Built Environment, University of Johannesburg,
South Africa Doornfontein 2028, Johannesburg, South Africa
[2]Department of Economics and Business, University of Catania, Corso Italia 55,
95129, Catania, Italy
[3]External collaborator of the Department of Economics and Business, University of Catania, Italy
*Corresponding author: a.logiudice@unict.it

ABSTRACT

Citrus production is one of the most important sectors of Sicilian agriculture. In particular, Sicilian orange production is of the best quality, producing mainly pigmented or blood oranges (*cv* Tarocco, Moro, and Sanguinello). No other region in the Mediterranean area or on the American Continent produces, on a large scale, blood oranges. The Sicilian blood oranges have some special flavours and organoleptic and functional characteristics. The aim of this paper is to quantify the total environmental impacts of the life cycle of *Tarocco* oranges - integrated production. The study was conducted in accordance with the ISO standards 14040:2006 and 14044:2006. The analysis of the input and output materials and energy flows indicates that the production phase has the most environmental impacts, causing almost half of the total damage. In terms of the substances having the most environmental impacts, the study shows that: the emission to air of Nitrogen oxides, particulates, Sulphur dioxide and carbon dioxide; the emission into soil of zinc; and the consumption of resources such as crude oil, uranium and natural gas have the most impacts. These results enable us to propose different ways to reduce the impact of the production of the oranges on the environment.

- Keywords: agri-food, citrus fruit production, Life Cycle Assessment, environmental hotspots -

INTRODUCTION

The worldwide citrus fruit production in 2010 was about 122 million tons, with oranges representing 56% of that production. In particular, the greatest citrus producers were China and Brazil with a share of about 36% of the world total. As far as Europe is concerned, the areas of production are concentrated in the Mediterranean Countries: Spain and Italy accounted for 86% of the total European production, in 2010, at 10 million tons. Italy occupied the second place after Spain (FAOSTAT, 2010).

In Italy citrus fruit production was more than 3.8 million tons, produced mainly in Sicily and Calabria. Out of this total, almost 2.4 million tons were oranges (ISTAT, 2010; CSO, 2010; INEA, 2011). Most of the oranges produced were the pigmented ones or the bood oranges (*cv* Tarocco, Moro, and Sanguinello).

Pigmented oranges are mostly produced in eastern Sicily where they account for around 60-70% of the total oranges harvested (LO GIUDICE, 2002; INGALLINERA *et al.*, 2004; CORERAS, 2006; GIARÈ *et al.*, 2008). This is the area (in the south and south-west of Mount Etna), characterized by special pedoclimatic features, where the production of high quality pigmented oranges is most suitable. These oranges have high aroma and antioxidant activity. Their most striking feature is the red colour which is of varying intensity and prevalence. It is caused by the anthocyanins whose accumulation depends on the environment in which they are grown. The intensity of the colour depends on the cultivar but also on the ripeness and climatic conditions. The blood orange varieties require strong day-night temperature to enable intense colour formation in the fruit flesh. Although blood oranges are grown in other countries, in some years entire harvests are lost due to the non-optimal conditions during the ripening phase of the fruit. When they are cultivated in Brazil or Florida couloration is generally weak, absent and unreliable (INGALLINERA *et al.*, 2004; BUTELLI *et al.*, 2012).

It has recently been discovered that a gene called "Ruby" plays a key role in the pigmentation of the blood orange (BUTELLI *et al.*, 2012).

The application of the Life Cycle Assessment (LCA) methodology in the food-processing field has increased in the last decade because it can be used to find new and alternative methods of agricultural production which can reduce environmental impacts, thus increasing product sustainability (MILÀ I. CANALS *et al.*, 2006; FFTC 2007; CLASADONTE *et al.*, 2010). Three studies carried out respectively in 2007 (FFTC 2007), in 2008 (MEISSNER SCHAU *et al.*, 2008) and in 2009 (ROY *et al.*, 2009) are some of the main LCA studies of agricultural and food production conducted between 1998 and 2008.

In particular, with regard to the assessment of the environmental profile of the citrus fruit sector, on an international level a number of studies have been conducted (SANJUAN *et al.*, 2005; YOSHIKAWA *et al.*, 2008; COLTRO *et al.*, 2009, RIBAL *et al.*, 2009; MORDINI *et al.*, 2009; RUVIARO *et al.*, 2012). Some studies have also been conducted on the environmental impacts of orange juice production (COLTRO *et al.*, 2008; MORDINI *et al.*, 2009; SPREEN *et al.*, 2010; KNUDSEN *et al.*, 2011; DWIVEDI *et al.*, 2012).

In Italy, a number of studies have also been done on the same subject (LA ROSA *et al.*, 2008; BECCALI *et al.*, 2009 and 2010).

It is in this regard that this paper discusses the life cycle assessment with a view to contributing to the field within the Italian orange production industry and adding value to international discourse. The aim of this paper is to quantify the total environmental impacts associated with the life cycle of the integrated production of oranges *cv* Tarocco.

MATERIALS AND METHODS

1. Goal and scope and system boundaries

The aim of this work was to quantify the total environmental impacts of oranges *cv* Tarocco - integrated production using a LCA approach. The study scope included the evaluation of possible improvements in the production methodology on the basis of the obtained results.

The idea was not to study the complete current Sicilian oranges integrated cultivation or to develop a study by using average data on a regional scale, but to conduct oranges production life cycle assessment based on a chosen pieces of land with a view to refine and improve LCA application to this type of study. This was done by clearly defining the system boundaries and using specific data provided by a few firms, which showed an interest in taking part in the study.

The main system boundaries include the phases of *production*, *processing* and *end of life* of the oranges, as well as the *transportation* by trucks of these oranges from the field to the processing plant and from the processing plant to the distribution centre in the north of Italy. This essentially follows the "cradle-to-gate" approach (CERUTTI *et al.*, 2001). The cultivation phase in the nursery was excluded from the system boundaries. This was outside the scope intended for the study and would enabled comparison of methods and results with other studies (BECCALI *et al.*, 2009).

Details of the production system studied are shown in Fig. 1, with the functional unit represented by 1 ton of oranges. This functional unit was chosen because it enables measurements of inventories to be easier so that the cut-off point of inventories to be considered is higher than when a kilogram or 100 kilograms was considered. That way a broader spectrum of invento-

ries could be considered. A co-product of 100 kg of non-edible parts of oranges, to be composted, was also considered. This approach is also in agreement with a previous research as reported by CERUTTI *et al.* (2011) in which, for fruit products, typical functional units of 1 kg or 1 ton of fruit were used.

2. Inventory analysis

The Life Cycle Inventory (LCI) analysis quantifies the use of resources and energy and environmental releases associated with the system being evaluated. In the following tables LCI data have been listed, displaying the results obtained for raw materials and water consumption, electricity, and solid waste generation. The related inventory data are shown in Table 1.

Citrus production phase

The production phase was considered for a citrus grove of a reference farm (part of an association of citrus producers) in full production located in the province of Catania (Italy).

The farm demonstrated a high level of environmental awareness since it has already adhered for a long time to the Global Gap standard for producing and marketing certified and guaranteed products.

The plantation for *cv* Tarocco oranges considered has a frame of 5x5 m, thus resulting in a density of 400 trees/ha. It was assumed that the citrus grove cultivation lasts about 50 years, during which there is full production for 30 years and that the production is conducted using the common techniques of Integrated Production (IP). Integrated Production/Farming is a farming system that produces high quality food and other products by using natural resources and regulating mechanisms to replace polluting inputs in order to ensure sustainable farming. Emphasis is placed on a holistic systems approach regarding involving the entire farm as the basic unit; on the central role of agro-ecosystems; on balanced nutrient cycles, and on the welfare of all species in animal husbandry. The preservation and improvement of soil fertility and of a diversified environment in ensured. In addiction the observation of ethical and social criteria is an essential component. Biological, technical and chemical methods are balanced carefully taking into account the protection of the environment, profitability and social requirements

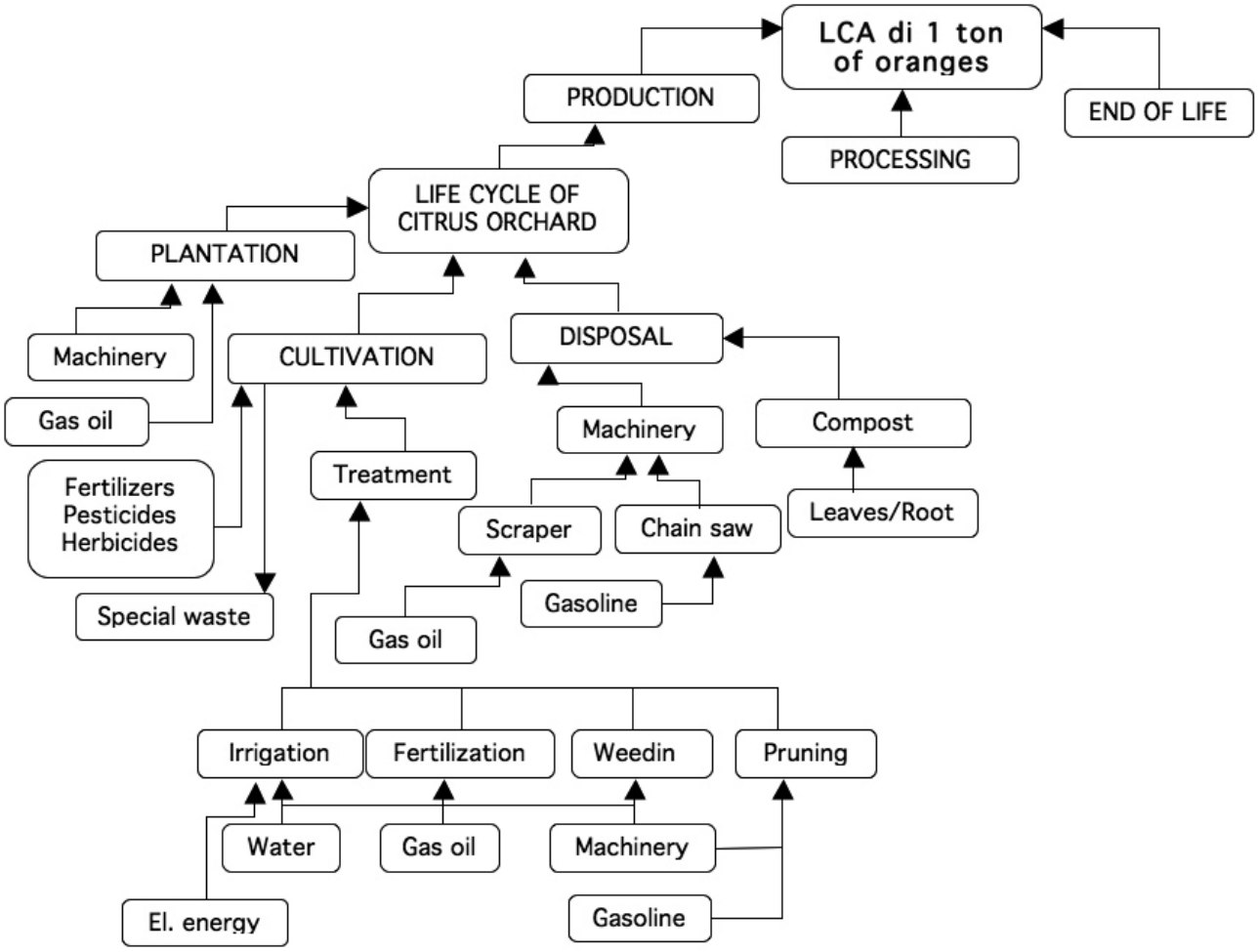


Fig. 1 - Flow chart related to citrus life cycle.

Table 1 - Input data 1 ton oranges life cycle.

Functional Unit		1	ton	Oranges
Co-product		100	kg	Oranges Non-edible part to be composted
Input flow		**Physic amount**	**Measure unit**	**Comment**
Production		8.148E-5	p	This phase has been represented by creating the oranges orchard life cycle and then computing it within the oranges life cycle for the share associated to 1 ton of them.
Processing		7.628E-6	p	Even in this case, the warehouse life cycle has been implemented considering the amount of oranges processed every year. Then, the warehouse life cycle has been computed within the oranges one for the share associated to 1 ton of them.
End of life		100	kg	This phase is simply represented by the oranges eating and the peel discarding. In developing this phase, it has been decided to make the assumption that, being in a context of separate waste collection, the peel, together with the other organic waste, is treated by composting. As a matter of fact, in the inventory phase has been considered the compost plant with the association of the amount of peels in 1 ton of oranges.
Transport	A	55	ton*km	Transport from the field to the processing plant. Knowing the kind of trucks commonly used for such operations, in order to develop a study being as real as possible, an euro 3 truck with a lorry of 3.5-7.5 ton has been computed (EURO 3).
	B	80	ton*km	Transport from the processing plant to the distribution plant around Sicily. Euro 4 truck with a lorry 16-32 ton (EURO 4).
	C	600	ton*km	Transport from the processing plant to the distribution plant in the North of Italy. By the provided data it results that the truck used is an euro 5 having a 16-32 ton lorry (EURO 4).

(IOBC/WPRS Commission "IP-Guidelines and Endorsement, 2004).

The citrus grove has an average yield of 270 tons (t) per year (corresponding to an average yield of 25 ton/ha) and the irrigation of the field occurs by drip irrigation with an average yearly consumption of about 4,600 m^3/ha of water. The water is phreatic that is ground water below the water-table and it is brought to the surface using an electric pump. The yearly treatments done to the soil consist of the following: two fertilization treatments using, as the main nutrients, Nitrogen (N), Phosphorus (P) and Potassium (K); three pesticide treatments (phosphorus compounds); two treatments with herbicides (glyphosate and MCPA). The data collected almost agrees with the ones reported by SANJUAN *et al.* (2005) and BECCALI *et al.* (2009).

The pruning of the trees is done once or twice on alternate years using a chain saw and pruning scissors, according to the Integrated Production normative. The fronds, after being ground, are left on the soil surface so to be decomposed and become organic fertilizer, while the branches are used as firewood.

The picking of the fruit is done by hand; the product is put into crates and is transported to the loading areas using a towed vehicle.

The cultivation phase data was taken into account considering the "citrus grove life cycle". For the citrus grove life cycle a Functional Unit (FU) of 1 p it was chosen. This is equal to a production of oranges in the orchard life time of 50 years, which for the same period is equal to a total production of 13,500 t.

The citrus grove life cycle was considered including, inside the system boundaries, the phases of: Planting; Cultivation; Harvesting and transportation; and the Uprooting of the trees and the end of life of the citrus orchard. In particular, the following phases have been taken into consideration: the production of chemical products for input such as fertilizers, pesticides and herbicides and their transportation into the farm; irrigation, fertilization, herbicide, insecticide as well as fungicide treatments. The pruning of the trees and the harvesting of the oranges, including the machinery and the related consumption of resources, raw materials and electric power were also considered; incineration of the special waste produced; the transportation of oranges to the specific plant; the uprooting of grove, the recovery of the tree trunks as firewood, and of the fronds, branches and roots as compost.

The "Inventory data of citrus grove life cycle" are shown in Table 2.

In this assessment it was also necessary to create the "citrus grove end of life" choosing, as the FU, 1 p. It was considered that this phase occurs in the 50th year of the citrus grove lifetime and it is composed of by three processes: pruning, eradication and trunks cutting.

Table 3 shows the inventories for the input data for citrus grove orchard end of life.

Citrus processing phase

The processing warehouse assessed is a small and medium scale enterprise located in Palagonia, in the province of Catania, which was used to consider the processing stage of the Sicilian oranges. Starting from the life time of the plant and the average quantity of oranges processed in a year, the life cycle of the processing warehouse has been constructed taking, as the functional unit 1 p which corresponds to the amount of oranges processed during the warehouse life time of 30 years. This gives an amount of about 144,000 t.

In particular, the following were taken into consideration: all the processing phases, starting from the moment when the oranges are received from the farm till they are packaged and trans-

Table 2 - Input data oranges orchard life cycle.

Functional Unit	1	p	1 p corresponds to a production of oranges in the orchard life time (50 years) equal to 13,500 ton
Co-product	420	ton	Leaves from the orchard end of life phase to be composted
Input flow	**Physic amount**	**Measure unit**	**Comment**
		Resources	
Occupation, permanent crop, fruit, extensive	10.8	ha*yr	
Transformation, to permanent crop, fruit, extensive	10.8	ha	
Transformation, from pasture and meadow	10.8	ha	
Water, process, unspecified natural origin	2723.25	m^3	Water requirements for all the fertilization and pesticide treatments in the orchard life time.
		Raw materials and fossil fuels	
Fertilizer N	127.44	ton	
Fertilizer P_2O_5	94.5	ton	
Fertilizer K_2O	94.5	ton	
Pesticides, unspecified at regional storehouse	2.572	ton	
		Electric and thermal energy	
Planting	11.32	ha	The planting phase occurs after five years in the nursery. The transport of plants from the nursery to the orchard has not been taken into account, because the nursery phase has not been included in the system boundaries.
Irrigating	2484000	m^3	The amount of water used for irrigating has been taken into account and is equal to 2484000 m^3 in the citrus grove life time.
Fertilizing	2700	ha	
Pesticide treatments	1620	ha	
Transport	3.2	kton*km	Transport of the raw materials for the orchard maintenance treatments (distance= 10 km).
Power saw	3	p	Tree pruning phase. No waste treatments considered as in this case the leaves are burned on-site at the citrus grove edges; non calculable carbon dioxide emissions in air.
Petrol unleaded at refinery	367.2	kg	Petrol unleaded consumption for the pruning treatment
Diesel at refinery	11560	kg	Diesel amount for pneumatic shears and for milling
Lubricating oil	226.95	kg	Lubricating oil for pneumatic shears
Sawmill	4.2E-5	p	It is used for the brunches crushing after the pruning phase
Transport, lorry 16-32 ton, EURO 3	15	ton*km	Special waste
Orchard end of life	1	p	
		Emission in water	
Nitrogen, total	38.232	ton	
Phosphorus, total	4.123	ton	
Potassium	23.535	ton	
		Waste treatments	
Disposal, hazardous waste, 25 % water, to hazardous waste incineration	600	kg	Cultivation phase hazardous waste

Table 3 - Input data oranges orchard end of life.

Functional Unit	1	p	This phase occurs at the 50th year of the citrus grove life time and it is composed by three treatments: pruning, eradication and trunks cutting
Avoided product	346	m^3	Logs obtained by the citrus grove end of life at disposal for every possible use
Input flow	**Physic amount**	**Measure unit**	**Comment**
		Raw materials and fossil fuels	
Power saw	4	p	For pruning and trunks cutting
Excavation, hydraulic digger	14175	m^3	Tree eradication
Compost at plant	420	ton	Leaves composting

Table 4 - Input data oranges warehouse life cycle.

Functional Unit	1	p	1 p is associated to the amount of oranges being processed during the warehouse life time (30 years); it is equal to about 144,000 ton. All the input data, listed in the present table, has been referred to the warehouse life cycle and so multiplied per 30 years.
Co-product	1350	ton	Low quality oranges not to be able to be processed and then treated by composting
Input flow	**Physic amount**	**Measure unit**	**Comment**
		Resources	
Water, process, unspecified natural origin	6,000	m^3	Firm water requirements
		Raw materials and fossil fuels	
Propane/butane at refinery	52.83	ton	
Polypropylene, granulate, at plant	1009.26	ton	Oranges plastic packaging bags
Cardboard cellulose	26100	kg	Cardboard labels matched to the oranges plastic packaging bags
Packaging carton	1204.83	ton	Carton box
		Electric and thermal energy	
Electricity HV use in I + import	2,040	MWh	Every year, firm electric energy requirements is equal to 68,000 kWh
Conveyor belt, at plant	157	m	
Wooden platforms life cycle	15	p	
Printing colour, offset, 47,5% solvent, at plant	1,500	kg	
Tanker	55.50	ton*km	Transport of propane (distance = 35 km)
Transport, lorry 3.5-7.5t, EURO 4	34.65	ton*km	Transport of the oranges plastic packaging bags (distance = 35 km)
Transport, lorry 3.5-7.5t, EURO 4	2400	ton*km	Transport of the oranges carton packaging box (distance = 2 km)
Transport, lorry 3.5-7.5t, EURO 4	39150	ton*km	Cardboard labels transport
Transport, lorry 3.5-7.5t, EURO 4	550	ton*km	Wooden platform
Plastic box life cycle	1934598.75	p	1 p represents one box in its life cycle. Every year, 515893 plastic boxes are used within the processing phase; every box useful life is equal to 8 years, which means that in 30 years 1934598.75 units are used.
Transport, lorry 7.5-16t, EURO4	162.51	kt*km	Transport of the plastic boxes used in all the processing system life cycle
Conveyor belt, at plant	157	m	Replacing of the conveyor belt after 15 years of life
Compost at plant	1350	ton	Low quality oranges being treated in an industrial compost plant
Building, multi-storey	400	m^3	Shed where the oranges processing takes place. The physic amount is referred to the concrete used in the shed construction phase
		Waste treatments	
Sanitary landfill	1204.83	ton	Carton box
	26100	kg	Carton labels
Polypropylene recycling	1009.26	ton	Oranges plastic packaging bags
Wood recycling	1854	ton	Wooden platforms

ferred to the distribution centre. These steps were considered taking into account the consumption of raw materials, resources and electric power associated with them. The warehouse is made of reinforced concrete. The waste associated with this process was noted. It is necessary to specify that the machinery and equipment used, with the exception of the conveyor belts, are specifically made for the processing of citrus fruit and, as a result, they are not present in the database of the software used. It was therefore considered within the calculation of the total consumption of electric power. Table 4 shows the inventory data of oranges warehouse life cycle.

Functional unit end of life phase

The end of life phase is simply represented by the consumption of oranges and the composting or disposal of the peel. In developing this phase, it was decided to make the assumption that, being in a context of separate waste collection, the peel and the other organic waste are treated by composting. As a matter of fact, the inventory phase has considered the compost plant using peels from 1 ton of oranges.

Data quality

The inventory data were collected from various sources. The data regarding the agriculture phase were collected from people working in the sector and also from publications. It was thus possible to measure the quantity of fertilizers, water and plant protection products used and to know exactly which methods of cultivation were used. Furthermore it was possible to estimate the consumption of fuel and electricity. The Ecoinvent database was also used for assessing the impact of fertilizer and pesticides-production and fuel production. The data for the processing phase were collected directly from a processing warehouse, which processes citrus fruit. It was thus possible to obtain information about electricity and water consumption and the amount of input resources and quantities related to the packaging. We made sure that the quality of the data also complied with the quality requirements of the European Platform on Life Cycle Assessment (EUROPEAN COMMISSSION, 2006). It is also noted that all the collected data were compared with that from literature and with that evaluated by experts in terms of quality and reliability.

Table 5 - Damage and impact categories (IMPACT 2002+).

Damage category	Impact category
Human Health	Human toxicity Respiratory inorganics Ionizing radiations Ozone layer depletivo Photochemical oxidation
Ecosystem Quality	Acquatic ecotoxicity Terrestrial ecotoxicity Terrestrial acidification/nutrification Acquatic acidification Acquatic eutrophication Land occupation
Climate Change	Global warming
Resources	No-renewable Energy Mineral extraction

Source: Joillet *et al*., 2003.

RESULTS AND DISCUSSION

1. Life cycle impact assessment (LCIA)

The study was conducted in accordance with the guidelines and requirements of the ISO Standards 14040:2006 and ISO 14044:2006. The damage assessment phase was carried out including both the mandatory elements (Classification, Characterization and Damages Evaluation) and the optional ones (Normalization and Weighing), as indicated in the standards, in order to express the results with equivalent numerical parameters so as to be able to represent quantitatively the environmental effects of the system analysed.

The environmental effects were given in quantitative measure. All the collected data were processed by Simapro 7.0, the most internationally used and widespread software, while Impact 2002+ was chosen as the impact assessment method. This method has the following advantages: it calculates the non-renewable energy consumption which, in the agricultural sector, represents a fundamental aspect to be considered. It recognizes Carbon dioxide as having the greatest responsibility for the greenhouse effect and climate change, during characterization. Table 5 shows the damage and the impact categories. The impact categories represent the negative effects to the environment ranging from damage due to an emitted substance to resource usage level. The damage categories are obtained by grouping the impact categories and represent the environmental compartments suffering from the damage (JOLLIET *et al.*, 2003, ARPA Sicilia, 2009).

It was found that the total damage corresponds to 0.101 pt and it can be attributed to the following:

35.60% to the cultivation phase;

25.50% to the processing phase;

23.00% to the transportation of the oranges from the warehouse to the distribution platform in the North of Italy;

10.10% to the transportation of the oranges form the field to the warehouse;

3.23% to the transportation of the oranges from the warehouse to Sicilian platform;

Table 6 - Damage evaluation, weighting and total damage distribution (%) for each damage categories.

Damage Categories	Weighing (pt)	Damages Evaluation		Phases of 1 ton oranges life cycle (Impact percentage)					
				Cultivation	Processing	Transport			Oranges non-edible part composting (Oranges end of life)
						A	B	C	
		Value	Measure Unit						
Resources	0.0364	5.53E3	MJ primary	35	30.3	8.5	2.96	22.9	0.759
Human health	0.0319	0.000227	DALY	36.1	26.5	11.5	2.98	17.9	5.01
Climate change	0.0259	257	Kg CO_2 eq	34.6	20.4	11	3.7	28.1	2.14
Ecosystem quality	0.00725	99.3	PDF*m^2*yr	39.9	15.3	9.06	4.02	29.5	2.25

Transport A: from field to the warehouse;
Transport B: from warehouse to Sicilian platform (40% of 1 ton);
Transport C: from warehouse to platform in Florence (60% of 1 ton).

Table 7 - Substances causing the most environmental impacts for Damage Category: Human Heath.

HUMAN HEALTH			
Substance	*Emission sector*	*Quantity*	*Measure Unit*
Nitrogen oxides	air	939	g
Particulates <2.5 micron	air	50.8	g
Aromatic hydrocarbons	air	2.89	g
Sulphur dioxide	air	117	g

2.56% to the peel composting (oranges end of life).

In terms of damage categories, the total damage is divided as follows:

35.80% Resources;

31.50% Human Health;

25.50% Climate Change;

7.20% Ecosystem Quality.

In table 6 each environmental damage category has been allocated a corresponding weighing point.

The damage assessment value and the percentage impact by phases are shown in Tables 7 to 10. The substances and resources causing most of the environmental impacts are listed for each damage category.

Fig. 2 shows a histogram in which all the life cycle phases of the production of oranges are linked to each impact category.

Table 11 shows the environmental impact categories with the highest damage values. They have been given a weighting score, a characteri-

Table 8 - Substances causing the most environmental impacts for Damage Category: Resources.

RESOURCES		
Raw material	*Quantity*	*Measure Unit*
Oil, crude, in ground	64.32	kg
Uranium, in ground	1.92	g
Gas, natural, in ground	22.4	m3

Table 9 - Substances causing the most environmental impacts for Damage Category: Climate Change.

CLIMATE CHANGE			
Substance	*Emission sector*	*Quantity*	*Measure Unit*
Carbon dioxide, fossil	air	187	kg

Table 10 - Substances causing the most environmental impacts for Damage Category: Ecosystem Quality.

ECOSYSTEM QUALITY			
Substance	*Emission sector*	*Quantity*	*Measure Unit*
Zinc	soil	1.11	mg

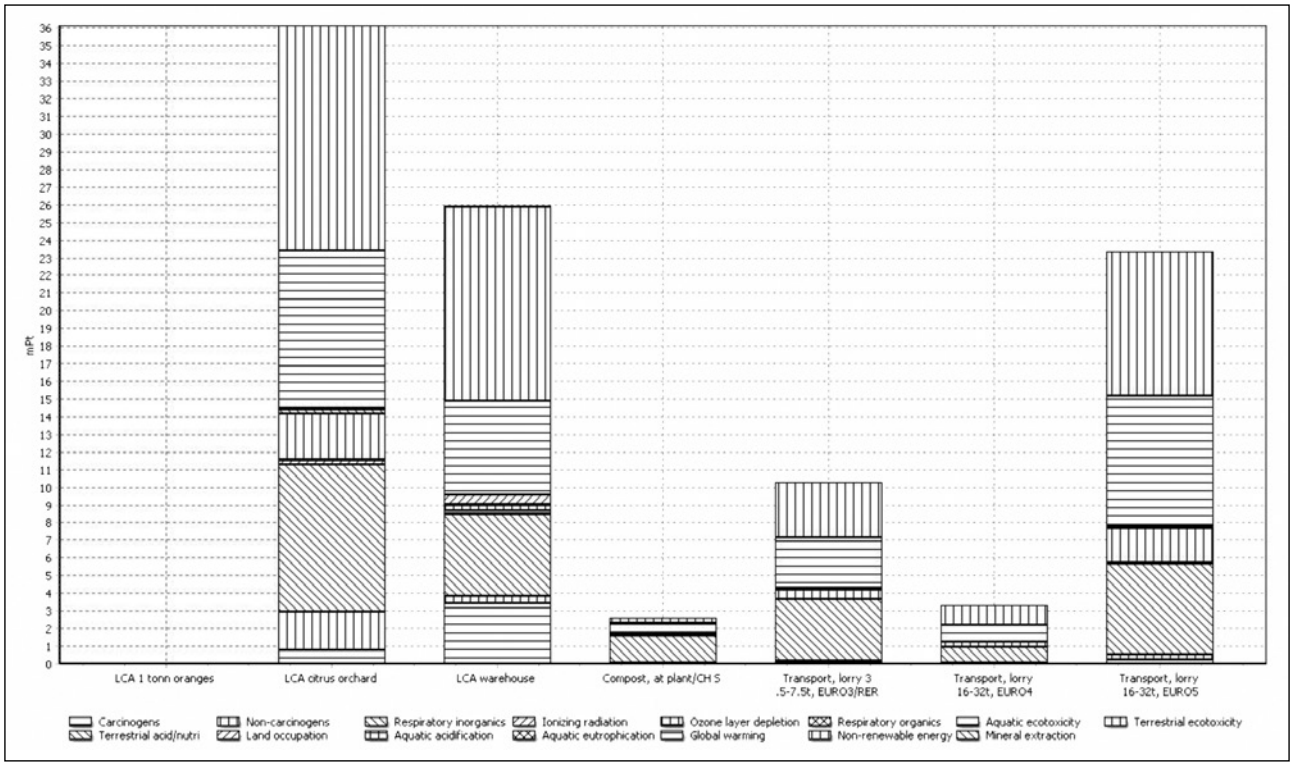


Fig. 2 - Oranges life cycle – single score evaluation per impact categories (Impact 2002+).

zation value and a damage percentage also classified according the life cycle phase of the orange production process.

2. Improvement hypothesis

By assessing the oranges life cycle, it can be asserted that the oranges production and the oranges processing phase, as well as the processed oranges transportation to the north of Italy, contribute most of the environmental impacts causing about 80% of the total damage. Based on these results, the following solutions are proposed in order to reduce, as much as possible, the total damage associated with the oranges life cycle.

Orange production phase

This phase is characterized by high consumptions of resources, principally due to the irrigation process. The most consumed resources are crude oil, uranium and natural gas. These are all raw materials associated with the cultivation treatments. Efforts to minimize the environmental impact should concentrate on im-

Table 11 - Characterization, weighting and total damage distribution (%) for the most relevant impact categories.

Impact Categories	Weighing (pt)	Characterization		Phases of 1 ton oranges life cycle (Impact percentage)					
				Cultivation	Processing	Transport			Oranges non-edible part composting (Oranges end of life)
						A	B	C	
		Value	Measure Unit						
Non renewable energy	0.0364	5.52E3	MJ Primary	35	30.3	8.5	2.96	22.4	0.759
Respiratory Inorganics	0.024	0.244	Kg PM2.5 eq	34.6	19.2	14.5	3.65	21.5	6.52
Global warming	0.0259	257	Kg CO_2 eq	34.6	20.4	11	3.7	29.1	2.14

Transport A: from field to warehouse;
Transport B: from warehouse to Sicilian platform (40% of 1 ton);
Transport C: from warehouse to platform in Florence (60% of 1 ton).

proving the necessary cultivation techniques. Attention can be paid to the reduction of waste and the optimization of water consumption. The optimization of water is fundamental objective, given that Sicily is characterized by long periods of drought. Water wastage and optimization can be achieved by use of more efficient irrigation techniques, such as localized underground irrigation carried out by means of micro-droppers under the soil. Using manure for fertilizing the orange trees can reduce the environmental impacts that are associated with the production of nitrogen and phosphorus based mineral fertilizers. Concurrently, more rational use of chemicals could lead to a reduction of the amount of pesticides used. This reduction of the use of plant protection products can improve the natural defence systems of the crop in addition to reducing environmental impacts.

Oranges processing phase

Concerning the oranges processing phase, the Life Cycle Impact Assessment (LCIA) shows that the production of the plastic boxes required for the packaging and transportation of oranges contributes most (70%) of the environmental impacts. The use of the 100% recycled polypropylene (PP) granulate for these containers, in accordance with the new Italian standards, can reduce the total impact by 40-50%. Additional environmental reductions can be obtained by the installation of a photovoltaic plant. This can guarantee a total impact reduction of about 50% when the environmental impact of this plant is measured and analysed from a life cycle perspective.

Transportation of oranges was found to cause environmental impacts which, as shown by this assessment, are significant. These are due to the consumption of diesel; the construction and maintenance of the vehicles used; and the environmental impacts of building and maintaining the infrastructure necessary for transportation. Therefore, in light of the significant environmental impacts attributable to the transportation of oranges to the North of Italy, it is suggested that rail transportation be considered. Rail transportation is the second most energy efficient mode of both passenger and freight transportation after maritime transportation. Specific CO_2 emissions of rail transport have decreased by about 14% from 1995 to 2009 mainly due to shifting from diesel to electric trains, as a result of technological improvements and due to increased load factors. (EEA, 2010). It is estimated that the use of rail transportation can results in about 75% CO_2 emission reductions (AICQ, 2008).

INTERPRETATION

The study results show that:

The most environmental impacts are due to the production and processing phases as well as the transportation of oranges, especially the transportation to the north of Italy):

The most environmental impact damages are from the category "Resources":

The most significant impact categories for the environmental assessment are: "Non Renewable Energy (NRE)", Respiratory Inorganics (RI) and Global Warming (GW):

In the category NRE, the main contributions (35 and 30.3%) are due to the "oranges cultivation and processing phases"; in the category RI the main contribution (34.6%) is due to the "cultivation phase"; the last category (GW) the main contributions (34.6 and 29.1%) are due to the "cultivation phases" and to the "transportation C".

CONCLUSIONS

The analysis has highlighted the main environmental hot spots to be linked to the cultivation and processing phases within the oranges life cycle, painting a clearer picture of their environmental performance. Regarding the production phase, the most important environmental problem can be attributed to the fertilizers and pesticides used in the cultivation phasel. Furthermore, the results obtained in the LCIA phase show that the orchard life cycle consumes a lot of water due to irrigation. This also consumes a lot of electricity and fossil fuels to power agricultural machinery further contributing to environmental degradation.

These environmental impacts and resource consumption patterns are a major concern. BECCALI *et al.* (2009) and MUELLER *et al.* (2012) have also stated similar concerns about the increased consumption of fertilizers and water due to the intensification of agricultural practises linked to the increasing human demand for food. Meeting the food security and sustainability challenges of the coming decades is possible, but will require considerable changes in nutrient and water management.

Food production is so wholly dependent on water that agriculture can use 75 to 90% of freshwater (THE WORLD WATER ORGANIZATION, 2010). In particular, water is a fundamental resource for the cultivation of oranges. It is important, therefore, to find alternative and more sustainable ways of irrigation in order to conserve water. For example using treated wastewater from neighbouring plants or purified water can be considered in future (BECCALI *et al.*, 2010).

Concerning the oranges processing phase, the most serious problem is the use of a large amount of plastic packaging systems which cause non - renewable energy consumption as well as a consequent greenhouse gas emissions. In this context, the solution of replacing PP granulate with 100% recycled container has been proposed.

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PAPER

EFFECT OF UV-C LIGHT IN THE PRESERVATION OF RAW FERMENTED BEVERAGES

M. BORCAKLI[1], J. LUCAS[2], L. CAPUTO[3*], T. OZTURK[1], F. BARUZZI[3], V. FUSCO[3], G.M. QUERO[3], L. QUINTIERI[3] and M. HOUGHTON[2]

[1]TÜBİTAK Marmara Research Center, Food Institute, P.O. Box 21, 41470 Gebze/Kocaeli, Turkey

[2]Department of Electrical Engineering & Electronics, University of Liverpool, Brownlow Hill, L69 3GJ, Liverpool, United Kingdom

[3]Institute of Sciences of Food Production, National Council of Research of Italy, Via G. Amendola 122/O, 70126 Bari, Italy

[*]Corresponding author: Tel. +39 080 5929323, Fax +39 080 5929374, email: leonardo.caputo@ispa.cnr.it

ABSTRACT

The aim of this work was to check the efficacy of an annular ultraviolet reactor for stabilizing the growth of lactic acid bacteria (LAB), the main components of the microbiota of three raw fermented beverages, Boza, Ayran and Matsoni during cold storage. The ultraviolet (UV) dose of 260 mJ cm^{-2} caused a reduction of 2.6 and 1 log CFU mL^{-1} in the LAB loads of Boza and Ayran samples, respectively, whereas multiple UV treatments were required for Matsoni; they caused the decrease of 3 log CFU mL^{-1} in LAB counts. Twenty-two bacterial strains, belonging to 11 species, were identified in the natural microflora of the three beverages before and after the UV treatments. Only a minimal post-acidification process was registered up to day 30 in all the three beverages. This ultraviolet device is at disposal of beverage manufacturers, who could apply it for producing microbially-stable drinks with an extended shelf-life.

- Keywords: fermented beverages, lactic acid bacteria, mild technologies, molecular typing, post-acidification, ultraviolet thin film system -

INTRODUCTION

Many fermented foods are still produced around the world by empirical processes based on spontaneous fermentation of different raw materials.

Some raw fermented beverages produced in Eastern European Countries as Boza, Ayran and Matsoni have a very short shelf-life.

Boza is an ancient cereal-based fermented beverage originating in Mesopotamia 8000-9000 years ago and still produced in Turkey, Bulgaria, Albania and Romania under traditional processing conditions. Recent papers have investigated the production of bacteriocins from natural LAB occurring in Boza (TODOROV and DICKS, 2004; 2005; TODOROV, 2010) and their probiotic properties (TODOROV *et al.*, 2008). However, some interactions among the autochthonous microorganisms could cause uncontrolled fermentations that lead to a variable product quality and a shelf-life less than two weeks under refrigeration condition (HANCIOĞLU and KARAPINAR 1997; GOTCHEVA *et al.*, 2000; GENÇ *et al.*, 2002; BOTES *et al.*, 2007). Currently, producer companies do not apply any preservation methods other than sorbate amendment.

Ayran is a yoghurt beverage produced in Turkey, traditionally manufactured by adding salted water to yoghurt at a level of 30-50% (ANONYMOUS, 1982). Quality of Ayran deteriorates during storage, due to acidity development and whey syneresis, thus limiting its shelf-life to 10-15 days at refrigeration temperatures (ANONYMOUS, 1982).

Matsoni is a Georgian bottle-fermented beverage based on pasteurized milk, back-slopped with an inoculum made up of thermophilic streptococci and different species of lactobacilli, leading to traces of alcohol and carbon dioxide (UCHIDA *et al.*, 2007). The shelf-life of Matsoni is 5-7 days at refrigeration temperatures, after which the product becomes too sparkling and sour.

In these circumstances manufacturers need procedures suitable for stabilizing microbial loads and extending the shelf-life of these drinks without completely suppressing their inherent microorganisms, and yet still preserving their taste and texture. Moreover, these drinks are not suitable for severe procedures (heat, high pressure) and for the addition of preservatives and stabilizers, subjected to public concern and ever tighter legislative control.

Interest is being shown in alternative, so-called "physical" treatments such as UV-light as a result of increasing consumer demands for fresh-like quality food products (ZHU *et al.*, 2005).

Many studies, carried out on disinfection of apple juice and microbial inactivation of beers and ciders using UV-light equipment, have shown the reduction of bacterial populations to undetectable limits (KOUTCHMA *et al.*, 2004; GEVEKE, 2005; LU *et al.*, 2010a; 2010b). Recently, LU *et al.* (2011) set up a novel UV apparatus based on the Dean vortex technology for sanitizing milk highly contaminated by pathogenic bacteria. MANZOCCO *et al.* (2011) applied a UV-C light treatment to fresh-cut melons and showed that UV-light exposure could be a potential novel technology for surface decontamination of ready-to-eat foods.

For these reasons, a UV-C light system was applied in this study with the aim of preventing the outgrowth of lactic acid bacteria in raw fermented beverages as Boza, Ayran and Matsoni during their cold storage. Molecular analyses were performed on bacterial populations to understand how UV-C treatments affect the microbial composition of these beverages.

MATERIALS AND METHODS

Beverage samples

Boza was manufactured following the Turkish recipe reported by HANCIOĞLU and KARAPINAR (1997) requiring 2% inoculum with a back-slopping starter obtained from the previous production. Boza samples were provided by Deniz Ticaret A.Ş. (Bursa, Turkey).

Ayran was produced by mixing home-made yoghurt and 0.5-1% NaCl solution according to the validated Turkish method of the Turkish Institute of Standards (ANONYMOUS, 1982). Ayran samples were provided by Aygın Süt A.Ş. (Konya, Turkey).

Matsoni was manufactured following the traditional Georgian recipe using cow's milk pasteurized at 90°C for ten min, cooled up to 45°C and inoculated with a 3% back-slopping obtained from the batch production of the previous day. Fermentation was carried out in glass bottles at 45°C until reaching the pH value of 4.6 (after 5-7 hours). Then, samples were placed in a cooling cell and stored at 6°C. Matsoni samples analyzed in this work were manufactured by Amaltea-Didube Milk (Tbilisi, Georgia).

All beverage samples were immediately transported under refrigerated condition to the laboratory where they were processed by the UV-system described below and then kept at 4°C for sixty days.

The UV-light system

The system consisted of a UV ozone-free lamp (LUCAS and AL-SHAMMA'A, 2003), which emitted energy at two wavelengths (254 and 185 nm) at high power levels (1 kW). The lamp was operated at a constant power of 30 W and was covered by a quartz sleeve situated in a stainless steel chamber. The liquid to be treated was placed into the liquid sample tank which held up to 3 L and had a liquid level indicator. As shown in Fig. 1,

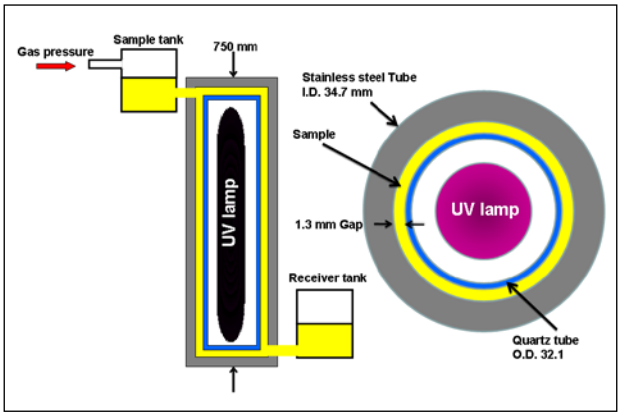


Fig. 1 - Schematic diagram of the annular UV-light system and cross section of the UV tube. Drawings are not to scale.

the liquid was flowed vertically from the sample tank into the UV reactor, as a thin film well exposed to the UV-light, and finally pumped to a receiver tank. The flow rate was set and altered by varying the gas pressure (P) within the liquid tank and allowing the liquid to flow through an adjustable needle valve. The gas pressure was fed from the laboratory source (5 bar) and was regulated to about 1 bar.

Cleaning of the system

The system was cleaned between each trial by running tap water 3 times through the instrument at a flow rate of 0.5 L min^{-1}, then passing 2 L of 5% hypochloride 3 times at room temperature for disinfection. Finally, sterile distilled water was passed through the system until microbial growth was no longer detected.

Single UV-C treatments

Two different doses of UV-light (66 mJ cm^{-2}, flow rate 2 L min^{-1} and 260 mJ cm^{-2}, flow rate 0.47 L min^{-1}) were applied to 2-litre samples of Boza, Ayran and Matsoni 1 day after their production. During UV treatments, the beverage temperature did not undergo any appreciable changes.

Microbial loads, pH and total titratable acidity (TTA) were recorded for all samples immediately before, after UV treatment and during cold storage at 7, 14, 30 and 60 days. Each experiment was carried out in triplicate.

Serial UV-C treatments of Matsoni

As single UV doses resulted ineffective in decreasing microbial loads in Matsoni, multiple UV-C light treatments were applied to these drink samples. In particular, two different experiments were carried out. In the first experiment, samples were treated with three successive increasing doses of UV-light (87, 135 and 206 mJ cm^{-2}), whereas in the second trial, six successive UV doses (266, 223, 238, 166, 231 and 240 mJ cm^{-2}) were applied. Microbial loads were determined at the end of each treatment. Each experiment was carried out in triplicate. UV treatment of beverages did not cause any appreciable changes in temperature.

Microbiological analyses of beverage samples

Samples were collected from each beverage before and after UV treatment in glass vials and used immediately for counting the microbial viable cells. These samples were stored at 4°C for further analysis. Ten grams of each beverage sample were dispersed in 90 g of 2% sodium citrate solution, homogenised in sterile bags via a stomacher, and serially diluted in sterile 0.1% buffered peptone water. The appropriate dilutions were plated in triplicate on different media: M17 with 0.5% lactose (LM17, Oxoid S.p.A., Garbagnate, Milan, Italy) and de Man, Rogosa and Sharpe (MRS, Oxoid) agar for counting presumptive Gram positive cocci and rods, respectively; Violet Red Bile lactose Agar (VRBA, Difco, Difco Laboratories, Detroit, MI) for enumerating total coliforms; Potato Dextrose Agar (PDA, Difco), supplemented with chloramphenicol (0.1 g L^{-1}) for isolating yeasts and moulds. LM17 and MRS plates were incubated for 48 h under anaerobic conditions (AnaeroGene, Oxoid S.p.A.) at 30°C (for Boza sample) or 37°C (for Ayran and Matsoni samples), whereas VRBA plates were incubated at 37°C for 48 h and PDA plates at 25°C for 7 days under aerobic conditions. The microbial content of each experiment was analyzed in triplicate.

For each beverage sample, 10-15 well-isolated bacterial colonies, randomly picked up from MRS and LM17 agar plates seeded with the highest sample dilutions, were inoculated in MRS and M17 broths, respectively, and incubated under appropriate conditions. All isolates, harvested by centrifugation (6,000 x *g* for 5 min), were suspended in the same medium containing 20% glycerol, frozen at - 80°C and stored for further characterization.

Molecular analyses of microbial isolates

Total DNA for molecular analyses was extracted from bacterial isolates using the Wizard-Genomic DNA Purification kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. DNA quantity and quality were determined by electrophoresis with known amounts of a molecular weight marker (Marker VI, Roche Diagnostics, Milan, Italy) as a standard.

Bacteria strain typing was performed using the "two-step RAPD PCR" protocol, as described by BARUZZI *et al.* (2000), including control reaction mixtures lacking DNA template in each

experiment. XD9 (MOSCHETTI *et al.*, 1998) and Coc1 (COCCONCELLI *et al.*, 1995) primers were used, respectively, for rod- and cocci-shaped LAB.

PCR amplifications were carried out in a Thermal Cycler 9700 (Perkin-Elmer, Alameda, CA, USA). Taq polymerase and deoxynucleoside triphosphates were purchased from Sigma-Aldrich S.r.l. (Milan, Italy), gel-filtration purified oligonucleotides from Sigma-Genosys Ltd. (Cambridge, UK), whereas DNA molecular weight markers were purchased from Qiagen SpA (Milan, Italy). The amplified fragments, separated by electrophoresis in 2.0% agarose gel, were sized using the Quantity One 4.3.1 software (Bio-Rad Laboratories S.r.l., Milan, Italy). When different isolates gave the same electrophoretic pattern, they were grouped. The ratio ($P = n/t$) between the number of colonies grouped for each strain (n) and total colonies, analyzed (t) from the same medium for each sample, represented the percentage (P) of the presence of that strain in the total population. The product of viable cell count value and the percentage (P) of each isolate made it feasible to calculate the viable cell count for each strain. One isolate from each group was chosen as the representative strain and stored for further analyses.

Each representative strain from RAPD-PCR groups was identified by amplifying and sequencing the 16S rRNA gene, as previously reported (WIESBURG *et al.*, 1991). The DNA sequences were obtained using an ABI PRISM Big Dye Terminator Cycle Sequencing Kit ver3.1 (PE Applied Biosystems, Inc., Foster City, CA, USA) with both the forward and reverse primers being used. The reaction products were analyzed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The Applied Biosystems software package (Sequencing Analysis ver. 3.3 and MT Navigator PPC ver. 1.0.2) was used for the analysis and comparison of DNA sequences. Taxonomic strain identification was performed, comparing the rDNA sequences of beverage isolates with the sequences present in the Basic BLAST Search, as described by ALTSCHUL *et al.* (1997). Taxonomic identification of *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus delbrueckii* subsp. *bulgaricus, Enterococcus faecium*, *Leuconostoc mesenteroides* and *Streptococcus thermophilus* was confirmed by species-specific PCRs (LICK *et al.*, 1996; SONG *et al.*, 2000; TORRIANI *et al.*, 2001; FURET *et al.*, 2004; BENSALAH *et al.*, 2006).

pH value and total titratable acidity determinations

The pH of beverage samples was determined by direct insertion of a pHmeter (Oakton Benchtop pH 510 meter, Cole-Parmer, Vernon Hills, Illinois, USA).

Total titratable acidity (TTA) was measured in the following way: 10 g of beverage sample were weighed in a 250 mL Erlenmeyer flask, and distilled water was added up to 50 mL including a few drops of phenolphthalein. The mixture was titrated with 0.1 N NaOH according to the AOAC method no. 947.05 (AOAC 2000) and expressed as percentage of lactic acid in 100 grams of sample.

Statistical analysis

Statistical analysis of data was carried out using the Statistica 6 software package (StatSoft Italia, Vigonza, Italy). Unpaired two-tailed Student's t test was used to analyze statistical differences in incidence ratios of bacterial biotypes and in the total viable cell counts of the treated beverage samples and controls with respect to each beverage, taking $P \leq 0.05$ as significant. One-way analysis of variance (ANOVA) followed by the Duncan's multiple test range method was applied to verify whether there were significant differences ($P \leq 0.05$) in pH and TTA mean values for each beverage before UV treatment and throughout its cold storage.

RESULTS AND DISCUSSION

The first practical applications of UV-C were in disinfection of surfaces and water sterilisation by setting up suitable UV equipment that are currently well accepted in the food industry (AL-SHAMMA'A *et al.*, 2001; HOYER, 1998).

In the last 20 years, UV-light has been widely used for controlling foodborne pathogens as *Escherichia coli* O157:H7 and enterotoxigenic *Staphylococcus aureus*, *Salmonella typhimurium* and *Listeria monocytogenes* in pure culture suspensions, stainless steel and foods (MCKILLIP *et al.*, 1998; KIM *et al.*, 2002; KRISHNAMURTHY *et al.*, 2004; YAUN *et al.*, 2004).

A lab-scale UV apparatus was applied to three raw fermented beverages (Boza, Ayran and Matsoni) in order to stabilize the viable counts of microbial groups such as lactic acid bacteria (LAB) responsible for the phenomenon of beverage post-processing acidification during cold storage.

Microbiological and molecular analyses of untreated fermented beverages

Since UV-C light treatments were applied in order to reduce but not destroy the natural microflora of each beverage, it was necessary to monitor the dominant bacterial strains occurring before and after UV-C light treatments.

At the end of the manufacturing process, all beverage samples contained high microbial loads of lactic acid bacteria (7.8 log CFU/mL, on average); while fungi and yeasts were undetectable; during cold storage, bacterial counts in the

untreated samples of the three drinks remained quite stable till day 60 (Fig. 2). The coliform counts in the three beverage samples were below the detection threshold.

Molecular analyses in the untreated Boza samples revealed complex and heterogeneous populations made up of nine different bacterial strains belonging to seven species (Table 1). These results are in accordance with those reported by other authors (GOTCHEVA, 2000; HANCIOĞLU and KARAPINAR, 1997; BOTES *et al.*, 2007).

Microbiological and molecular analyses of Ayran showed, in addition to the characteristic microflora of yoghurt, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, as previously reported (ANONYMOUS, 1982), even one single strain of *Lactobacillus plantarum* (Table 2).

As concerns Matsoni, microbiological analyses allowed to ascertain that, in the untreated samples, Gram-positive cocci viable counts resulted dominant, accounting for more than 8 log CFU mL^{-1}, followed by Gram-positive rods with more than 5 log CFU mL^{-1} (Fig. 2). Molecular analyses confirmed the results previously reported by UCHIDA *et al.* (2007), who, analysing 26 Matsoni samples collected throughout Georgia, found that the microbiota of this beverage was mainly made up of *S. thermophilus* and *L. delbrueckii* spp. *bulgaricus*. In addition, our molecular analyses revealed the dominance of *S. thermophilus* on *L. delbrueckii* subsp. *bulgaricus* strains in the samples (Table 3), confirming the results of the microbiological investigations (Fig. 2).

Effect of UV-C light treatment on beverages

The UV-C dose of 66 mJ cm^{-2} was ineffective on all beverages, whereas promising results were obtained treating Boza and Ayran samples with the UV-C dose of 260 mJ cm^{-2} and 0.47 L/min. A reduction of about 2 log CFU mL^{-1} was registered in both Gram-positive rods and cocci of Boza samples immediately after UV treatment (Fig. 2); the same values of microbial counts were registered at the end of cold storage.

Molecular analyses showed that some strains, occurring at high amounts before the UV-light treatment, were not isolated from the same sample after UV treatment; on the other hand, some new strains were recovered after UV-light treatment, suggesting they occurred as sub-populations in the untreated Boza samples (Table 1).

In Ayran samples, the 260 mJ cm^{-2} UV

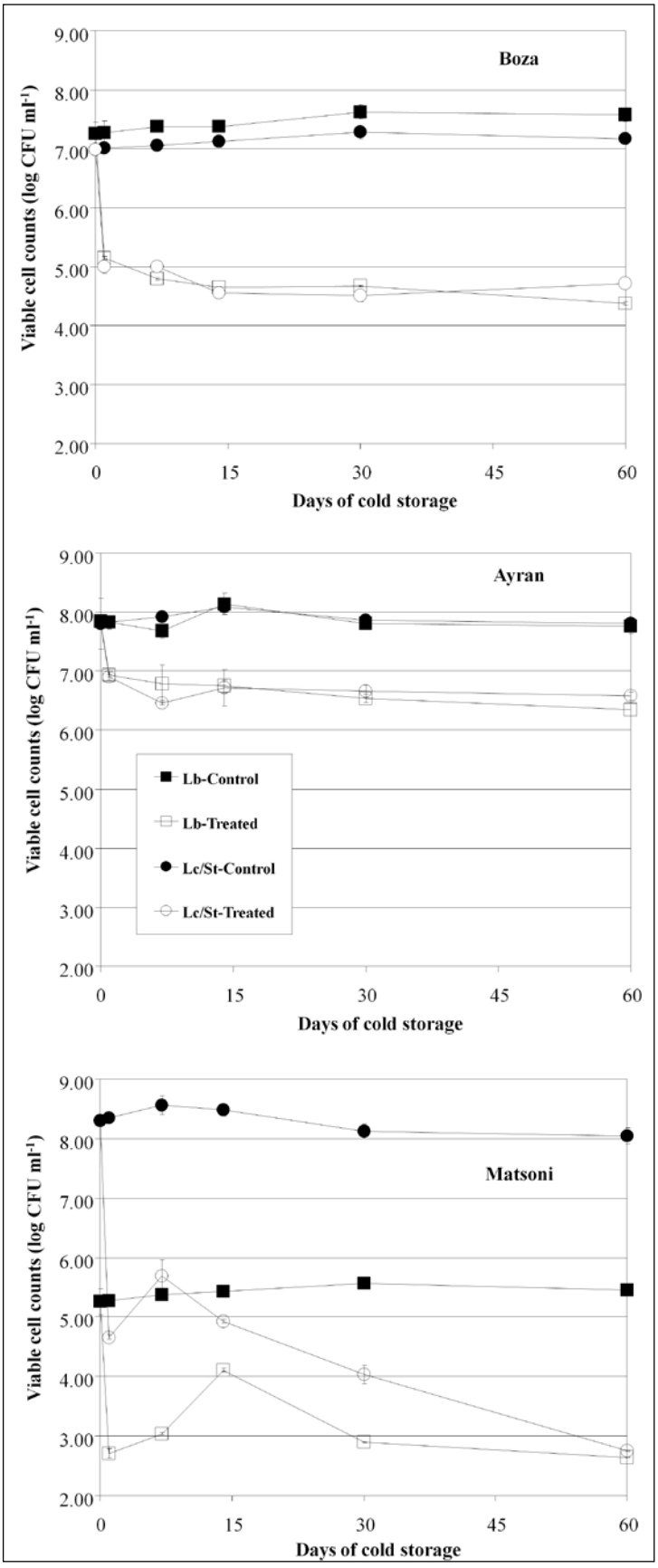


Fig. 2 - Total viable cell counts of presumptive lactobacilli and lactococci/streptococci from untreated (Lb-Control and Lc/St-Control, respectively) and UV-treated (Lb-Treated and Lc/St-Treated, respectively) fermented beverages: Boza and Ayran treated with UV dose of 260 mJ cm^{-2}; Matsoni treated with multiple UV doses (87, 135 and 206 mJ cm^{-2}). Values are means of three replicates ± SD.

Table 1 - Percentage of dominant strains within bacterial populations from Boza samples before and after ultraviolet treatment[a].

Boza			Strains (%)		
Medium	Strain	Species	Before UV treatment	After UV treatment	Sig.[b]
MRS	B41	*Lactobacillus plantarum*	25.00	50.00	**
	B44	*Weissella confusa*	25.00	16.67	*
	B34	*Lactobacillus paracasei*	25.00	16.66	*
	B38	*Lactobacillus plantarum*	25.00	0	**
	B23	*Leuconostoc mesenteroides*	0	16.67	**
	Total		100.00	100	n.s.
	Total viable cell counts (log CFU mL^{-1})		7.26±0.25	5.02±0.10	**
M17	B2(b)	*Enterococcus faecium*	12.50	8.25	*
	B3	*Enterococcus durans*	25.00	8.25	**
	B5	*Weissella confusa*	12.50	24.75	*
	B19	*Leuconostoc lactis*	37.50	0	**
	B18	*Lactococcus lactis* subsp. *cremoris*	12.50	0	**
	B4	*Leuconostoc lactis*	0	34.00	**
	B1	*Lactobacillus plantarum*	0	8.25	**
	B2(a)	*Lactobacillus coryniformis*	0	8.25	**
	B11	*Lactobacillus paracasei*	0	8.25	**
	Total		100.00	100.00	n.s.
	Total viable cell counts (log CFU mL^{1})		6.99	5.00	**

[a]Values represent the average ratios between each strain and the total viable cell counts grown on MRS or M17 agar.
[b]Significant differences in incidence percentages of biotypes and in total viable cell counts before and after UV treatment (*: $P \leq 0.05$; **: $P < 0.01$; n.s.: not significant; two-tailed Student's test, N = 3).

C dose caused a decrease of about 1 log cycle in both Gram-positive rods and cocci viable loads; lactic acid bacteria (LAB) counts remained quite stable till day 60 (Fig. 2). LU *et al.* (2010b) registered a similar reduction value in total LAB by applying the 4.8 mJ cm^{-2} UV dose to beer samples, using a thin film apparatus equipped with quartz optical fibres. Molecular analyses of Ayran UV-treated samples revealed that LAB strains belonged to *S. thermophilus* and *L. plantarum*, whereas *L. delbrueckii* subsp. *bulgaricus* 401, recovered from the untreated samples, was not retrieved (Table 2).

Single UV doses were ineffective in decreasing microbial loads in Matsoni. GUERRERO- BELTRÀN and BARBOSA-CÀNOVAS (2004) found that milk solids may limit UV penetration and thereby decrease the efficiency of UV treatments.

Table 2 - Percentage of dominant strains within bacterial populations from Ayran samples before and after ultraviolet treatment[a].

Ayran			Strains (%)		
Medium	Strain	Species	Before UV treatment	After UV treatment	Sig.[b]
MRS	A4	*Lactobacillus plantarum*	12.51	50.00	**
	A13	*Streptococcus thermophilus*	85.32	50.00	*
	401	*Lactobacillus delbrueckii* subsp. *bulgaricus*	2.17	0	**
	Total		100.00	100	n.s.
	Total viable cell counts (log CFU mL^{-1})		7.84	6.93	*
M17	A13	*Streptococcus thermophilus*	100.00	100.00	n.s.
	Total		100.00	100.00	n.s.
	Total viable cell counts (log CFU mL^{-1})		7.81	6.93	*

[a]Values represent the average ratios between each strain and the total viable cell counts grown on MRS or M17 agar.
[b]Significant difference in incidence ratios of biotypes and in total viable cell counts before and after UV treatment (*: $P \leq 0.05$; **: $P < 0.01$; n.s.: not significant; two-tailed Student's test, N = 3).

Table 3 - Percentage of dominant strains within bacterial populations from Matsoni samples before and after the ultraviolet treatment[a] with multiple UV doses (87, 135 and 206 mJ cm^{-2}).

Ayran Medium	Strain	Species	Strains (%) Before UV treatment	Strains (%) After UV treatment	Sig.[b]
MRS	A	*Lactobacillus delbrueckii* subsp. *bulgaricus*	90.00	60.00	**
	B	*Lactobacillus delbrueckii* subsp. *bulgaricus*	10.00	40.00	*
	Total		100.00	0	**
	Total viable cell counts (log CFU mL^{-1})		5.26	2.71	**
M17	C	*Streptococcus thermophilus*	100.00	70.00	**
	D	*Streptococcus thermophilus*	0	20.00	**
	E	*Lactococcus lactis*	0	10.00	**
	Total		100.00	100.00	n.s.
	Total viable cell counts (log CFU mL^{-1})		8.34	4.66	**

[a]Values represent the average ratios between each strain and the total viable cell counts grown on MRS or M17 agar.
[b]Significant differences in incidence percentages of biotypes and in total viable cell counts before and after UV treatment (*: $P \leq 0.05$; **: $P < 0.01$; n.s.: not significant; two-tailed Student's test, N = 3).

This behaviour could explain why one single UV-C treatment was more effective in reducing LAB loads in Boza than in Ayran. Thus, in the milk-based beverage Matsoni two different experiments were carried out: in the first one, samples were subjected to three successive UV-light treatments with increasing doses, whereas in the latter experiment six successive UV-C doses were applied to the drink samples, as previously described. As concerns the first UV experiment, Gram-positive rods were more resistant to UV-C treatment in comparison with cocci during cold storage (Fig. 2, BOZA and AYRAN). A reduction of more than 3.5 log CFU mL^{-1} was registered in Gram-positive cocci viable counts immediately after UV treatment; they increased by 1 log after one week of cold storage, decreasing to 2.8 log at day 60 (Fig. 2). The same UV-C dose caused a reduction of about 2.5 log CFU mL^{-1} in Gram-positive rod viable counts immediately after UV treatment; this bacterial population increased to over 4 log at day 15, decreasing to about 3 log at day 60 of cold storage (Fig. 2). Molecular analyses revealed that the UV-treated samples contained the same two strains of *L. delbrueckii* subsp. *bulgaricus* retrieved in the original sample, with one dominant biotype, suggesting that this latter strain was more resistant to UV treatment (Table 3); as concerns *St. thermophilus* biotypes, the same RAPD-PCR pattern found in the untreated sample was retrieved as dominant, together with a second profile. In addition, one single *Lactococcus lactis* biotype was found after UV treatment (Table 3). The second UV-C experiment carried out on Matsoni samples caused the complete disappearance of LAB (data not shown); therefore this treatment was considered too drastic for the purpose of the present work.

Values of pH and total titratable acidity determinations of untreated and treated beverage samples

In accordance with the increase in microbial counts, pH decreased markedly while TTA increased in the untreated cold-stored Boza and Matsoni samples (Table 4). The marked decrease in pH in both untreated beverages during cold storage was probably due to the increase in TTA, which in turn depends on microbial fermentation (ROBINSON *et al.*, 2006). Some bacterial species isolated from Boza as *Leuconostoc lactis* and *Leuconostoc lactis* subsp. *cremoris* displayed a psychrophilic behaviour that allowed them to grow at low temperatures and produce different types of organic acids (lactic and acetic acids) that have a direct influence on acidity and the final flavour of the beverage. ZORBA *et al.* (2003) reported the results of pH and TTA values in Boza during fermentation time; however, to the best of our knowledge, no reports on the analysis of these values during cold storage of this beverage are available so far.

Interestingly, in our work, pH and TTA in UV-treated Boza samples changed slightly during cold storage unlike the pH of the untreated samples (Table 4).

As in yoghurt, in the milk-based beverages Ayran and Matsoni heterofermentative bacteria as *L. delbrueckii* subsp. *bulgaricus* are responsible for the phenomenon of post-processing acidification, causing a dangerous drop in pH below 4.3, which destabilizes the casein clot and weaving of beverages in retail (ROBINSON *et al.*, 2006).

No increase in TTA values was registered in all three UV-treated beverages after 2 months of cold storage. Thus, the phenomenon of beverage

Table 4 - Values of pH and total titratable acidity (TTA) of untreated and UV-C light treated beverage samples: Boza and Ayran treated with UV dose of 260 mJ cm^{-2}; Matsoni treated with multiple UV doses (87, 135 and 206 mJ cm^{-2}).

	Before UV treatments		After UV treatments under refrigeration condition (days)									
			0		7		15		30		60	
Boza												
pH (untreated)	4.90	**a**	4.91	**a**	4.00	**C**	3.40	**e**	3.60	**d**	3.80	**d**
pH (UV-treated)	4.89	**a**	4.96	**a**	4.68	**B**	4.70	**b**	4.55	**bc**	4.10	**c**
TTA (untreated)	0.12	**c**	0.11	**c**	0.16	**B**	0.19	**b**	0.28	**a**	0.25	**a**
TTA (UV-treated)	0.11	**c**	0.12	**c**	0.04	**D**	0.05	**d**	0.04	**d**	0.11	**c**
Ayran												
pH (untreated)	4.44	**d**	4.45	**d**	4.10	**E**	4.09	**e**	4.10	**e**	4.12	**e**
pH (UV-treated)	4.67	**c**	4.71	**c**	4.77	**B**	4.74	**c**	4.71	**c**	4.80	**a**
TTA (untreated)	0.25	**c**	0.26	**c**	0.86	**A**	0.84	**a**	0.81	**a**	0.83	**a**
TTA (UV-treated)	0.26	**c**	0.24	**c**	0.24	**C**	0.35	**b**	0.21	**c**	0.2	**c**
Matsoni												
pH (untreated)	4.75	**a**	4.75	**a**	4.00	**C**	3.90	**c**	3.80	**c**	3.60	**c**
pH (UV-treated)	4.76	**a**	4.76	**a**	4.61	**B**	4.48	**b**	4.58	**b**	4.49	**b**
TTA (untreated)	0.25	**d**	0.26	**d**	0.45	**C**	0.67	**b**	0.79	**a**	0.83	**a**
TTA (UV-treated)	0.25	**d**	0.24	**d**	0.24	**D**	0.24	**d**	0.24	**d**	0.27	**d**

Values are means of three replicates. Same superscript letters denote no significant differences ($P > 0.05$; Duncan's multiple range test).

post-processing acidification did not occur up to day 30 in comparison with that of the untreated drinks. The best results were achieved in Matsoni, whose shelf-life is usually shorter than one week under refrigeration condition.

CONCLUSIONS

As an alternative to drastic treatments (heat, high pressure) and the addition of antimicrobials, here we used an annular UV-C system in which the fermented beverages were flushed as a thin film. This treatment did not affect the reciprocal relationships among LAB, even though reduced the microbial loads in the beverage samples. As these drinks usually contain viable microorganisms contributing to their typical quality and taste, special attention was paid to the choice of the appropriate UV doses in order to obtain a mild reduction but not the total inactivation of the beverage microbiota.

Promising results were obtained with Boza by applying a single UV-C dose of 260 mJ cm^{-2} that caused a suitable reduction in viable counts of both Gram-positive cocci and rods; then they remained stable till day 60 of cold storage. The same UV-C dose applied to Ayran was less effective, lowering bacterial loads by about 1 log CFU mL^{-1}; however, microbial counts did not increase during two months under refrigeration condition.

Interestingly, a significant reduction in bacterial populations was obtained in Matsoni after UV-C treatment stabilizing the acidity at least up to one month. Thus, the application of UV-C light by an annular device, as an alternative to more drastic methods, experienced in this study, could enable beverage manufacturers to produce microbially-stable drinks with an extended shelf-life.

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PAPER

DIFFERENT STABILIZATION TREATMENTS OF RICE BRAN ADDED TO WHEAT FLOUR DETERMINE DIFFERENT PROPERTIES IN PARTIALLY BAKED WHEAT BREAD

J. QUILEZ[1,2,*], M. ZATOR[3], J. SALAS-SALVADÓ[1,2] and L. ALVAREZ[4]
[1]Human Nutrition Unit, School of Medicine, IISPV, Universitat Rovira i Virgili,
Sant Llorenç 21, 43201-Reus, Spain
[2]CIBER Fisiopatologia de la Obesidad y Nutrición (CIBEROBN),
Instituto de Salud Carlos III, Madrid, Spain
[3]Departament de Tecnologia, EUROPASTRY S.A., Xavier Cugat 2,
08174-Sant Cugat del Vallès, Spain
[4]Departament d'Enginyeria Agroalimentària i Biotecnologia, Universitat Politècnica de Catalunya,
Castelldefels, Spain
*Corresponding author: joan.quilez@urv.cat

ABSTRACT

In the present study, wheat flour was replaced by 3, 6 and 9% of rice bran (RB) previously exposed to two types of heat treatment: extrusion (EXT) and steam cooking (STC). The aim was to investigate how RB affected the properties of wheat-flour dough and partially baked wheat bread, as a final product. The results of the study indicate that neither of the RB treatments had a negative influence on the rheological characteristics of the dough, but that its fermentation capacity and gas retention improved, especially in case of EXT-RB, which was exposed to more intensive heat treatment. In the final bread product, there was also a positive increase in the specific total volume for the EXT-RB bread, and a decrease for the STC-RB bread. Nevertheless, sensory analysis showed that the score for the RB-enriched bread decreased when RB content increased. We concluded that low concentrations of RB exposed to a more intensive heat stabilization process improves several rheological properties of the dough and the physical properties of the partially wheat bread.

- Keywords: rice bran, stabilization, partially baked, wheat bread -

INTRODUCTION

Rice is the second most cultivated cereal grain in the world, and the most important cereal crop of Asia, Australia and South America. In Europe rice is grown mainly in Italy and Spain. Rice bran (RB), which amounts to 8% of the total rice grain, is a by-product of white rice, the most common form for consumption. After its production, RB deteriorates very rapidly because of lipid hydrolysis caused by the action of endogenous lipases. This poses a serious problem if it is to be consumed by both animals and humans and means that widespread consumption of RB is unacceptable. In order to disable the enzyme action and ensure that RB is not damaged, different types and intensities of heat stabilization treatment have been assayed (DA SILVA *et al.*, 2006). The annual production of rice in Spain is around 750 million kg, which means approximately 60 million kg of RB. Since RB is not subjected to stabilization processes and cannot be consumed immediately, there is an enormous amount of potential waste and its disposal is an environmental problem.

Unlike other brans consisting mainly of fiber, RB does contain insoluble fiber but also significant amounts of protein and fat. RB has been extensively characterized in the past, especially in its lipid fraction (WARREN and FARRELL, 1990), and it has shown interesting properties from the nutritional point of view. RB is rich in tocopherols and especially in γ-oryzanol. It mainly consists of ferulic acid esters, sterols and triterpenic alcohols (GARCIA-LERMA *et al.*, 2009), all the important nutrients with potential health properties. RB intake has been shown to be effective at decreasing plasma LDL-cholesterol in hypercholesterolemic individuals (GERHARDT and GALLO, 1998). The lipid fraction of RB has also been shown to be responsible for decreasing cholesterol in humans (MOST *et al.*, 2005). No differences in the glycemic index have been observed after the consumption of corn bread and corn bread supplemented with RB (SCHNELL *et al.*, 2005). However, after the long-term consumption of whole RB or its fractions, the glucose metabolism in patients with type I and II diabetes mellitus has been shown to improve (QURESHI *et al.*, 2002). Finally, the antioxidant activity of RB is high in both lipophilic and hydrophilic fractions, much higher than has been observed for most other plant products (WU *et al.*, 2004).

For many years, there has been increasing interest in adding RB to bread (CARROLL, 1990; SHARP and KITCHENS, 1990). Most research has focused on increasing the nutritional value of bread, basically the protein, fiber and mineral content (AJMAL *et al.*, 2006; SADAWARTE *et al.*, 2007). Nevertheless, the reported levels of RB added were generally very high and many different types of bread were used, so it is difficult to extrapolate these data to other sorts of bread. In the present study, small doses of RB, exposed to two types of heat treatment were added to wheat flour so that we could assess the effects of RB addition on the properties of wheat flour dough and partially baked wheat bread, widely consumed in Europe.

MATERIALS AND METHODS

The RB used was of the Bomba variety and cultivated in the delta of the Ebro River (Spain). The RB obtained was ground and thermally treated by two different methods within 24 hours of procurement. One of the methods was an extrusion cooking (EXT) procedure (Bühler AG, Switzertland) and RB was extruded at 115°C and an end-plate pressure of 12.1 bar. The other method was a steam cooking (STC) procedure (FSH; Bühler AG, Switzerland) in which RB was stabilized by being subjected to steam at 100 °C and a subsequent residence time of at least 1 minute. After stabilization and cooling, RB was stored in closed paper bags until it was used.

Analysis of wheat flour and rice bran

The proximate composition of wheat flour (La Meta, Lleida, Spain) and the two types of RB (EXT and STC) was analyzed. Humidity was determined by drying the sample at 130°C for 2 h. Ash was analyzed by burning the sample at 525°C for 5 h. Protein was analyzed by the Kjeldahl AOAC method 920.87 (AOAC, 2005) in a semiautomatic system (Büchi, Switzerland). Total lipid content was determined by the Soxhlet AOAC method 920.39c (AOAC, 2005). Total, insoluble and soluble dietary fiber was analyzed using the AOAC Official Method 991.43 (AOAC, 2005). The protein solubility of RB was determined according to SHARMA *et al.* (2004).

The particle size distribution of wheat flour and two types of RB (EXT and STC) was analyzed using an electromagnetic sieve shaker (CISA, Barcelona, Spain) with four meshed sieves with size openings of 500, 300, 150, and 125 μm. In each case 500 g of the sample was placed on the top screen (500 μm openings) and then shaken for 20 minutes. In order to determine the amount of sample retained on each screen, the screens were subsequently weighed.

All analyses were run in triplicate, and the average values were adopted.

Experimental conditions

In order to investigate the properties of dough and baked bread, mixtures of wheat flour replaced with 3, 6 and 9% of both types of RB (EXT and STC) were prepared. The dough and baked bread made with wheat flour without added RB were used as a control.

Dough characteristics

For each experimental condition, the viscoelastic behavior of the dough was determined by the Alveograph MA82 (Tripette and Renaud, France) according to the AACC method 54-30.02 (AACC, 2009). The parameters measured were: the strength or deformation energy (W), tenacity or resistance to extension (P) and dough extensibility (L). Proteolytic degradation was determined by comparing the W value right after analysis and after two hours of dough resting time (W2h) (ROSELL *et al.,* 2002)

The mixing behavior of the dough was determined using the Farinograph 810-105 (Brabender, Germany), according to the AACC method 54-21.01 (AACC, 2009). The following parameters were determined: water absorption (%), dough development time (DDT), stability and the decay at 10 minutes.

The gelatinization properties, measured as the gelatinization maximum or the peak viscosity in AU (Amylographic Units), were analyzed using the Amylograph E (Brabender, Duisburg, Germany), according to the AACC method 22-10.01 (AACC, 2009).

Fermentation behavior was measured by the Rheofermentometer F3 (Tripette and Renaud, France) according to the supplier's specifications. The following parameters of dough development were measured: maximum dough fermentation height (H_m) and the time at which dough reaches maximum height (T_1). The gas parameters measured were: the time of maximum gas formation (GT_1), dough permeability when gas starts to escape from the dough (T_x), the volume of gas produced throughout fermentation (V_{CO2}), and the gas retained in the dough at the end of the assay (R), in both milliliters (ml) and percentage (%) of gas retained.

All assays were performed in triplicate, and the average values were adopted.

Breadmaking process

The wheat bread used for the test was produced industrially using a Sancassiano single spiral kneading system (15 min; 23°C final dough temperature) (Sancassiano, Alba, Italy) and a Mecatherm production line (Mecatherm, Barembach, France) in a straight process that takes into account the various experimental conditions described above. Water (57%), yeast (2%), salt (1.8%), and commercial bread improver (0.5%) with ascorbic acid, DATEM, α-amylase and xylanase (Leag-Eurogerm, Barcelona, Spain), were added to the wheat flour or mixtures of wheat flour with RB (flour basis; see section on experimental conditions). After kneading and make-up, the dough underwent a 90 min fermentation process and prebaking was performed for 12 min at 185°C. After the cooling phase (20 min), the partially baked bread was deep-frozen at -18°C, packed in plastic bags in a cardboard box, and kept in frozen storage at -18°±2°C. It was then stored in the freezer for 15 days until analyses had been completed. Before analysis, the bread was defrosted for 45 min and second baking was performed (16 min at 190°C) in a Salva convection oven (Salva, Lezo, Spain). Samples were analyzed after cooling at room temperature for two hours.

Bread loaf volume

Bread volume was determined using a laser sensor, with the volume analyzer BVM-L 370 (TexVol Instruments, Sweden). The value of volume index was measured following the AACC method 10-91 (AACC, 2009) according to GOMEZ *et al.* (2003). Specific volume was calculated as the ratio between the volume of bread and its weight. The final value obtained for each substitution level was an average of 20 repetitions (5 loaves x 4 pieces).

Sensory analysis

The sensory analysis was carried out following the ISO-8587 Standards (ISO, 2006). A total of 30 people, habitual consumers of bread, were asked to test seven samples (control sample and six bread samples containing RB) and rank them from the one they liked the most to the one they liked the least. In this case, the high number of samples made the sensory analysis difficult for untrained panelists and the evaluation parameters had to be simplified to overall taste preferences only. Once the samples had been sorted according to the panelists' palatability, they were each scored on a scale of 7 (like the most) to 1 (like the least).

Statistical analysis

Analysis of variance (ANOVA) was applied to specific volume and sensory analysis data to evaluate the differences between the seven kinds of tested bread. The substitution level of wheat flour by two types of RB (EXT and STC) was considered. In order to discriminate among means, Fisher's least significant differences (LSD) test was used (95% confidence interval). The software used for statistical analysis was Statgraphics Centurion XVI V.16.0.08 provided by StatsoftInc., USA.

RESULTS

Wheat flour and rice bran characteristics

Table 1 summarizes the proximate composition of wheat flour and the two types of RB (EXT and STC). Our results were in agreement with previously reported results for this kind

Table 1 - Chemical composition and particle size of wheat flour, extrusion cooking (EXT) and steam cooking (STC) rice bran.

	Wheat flour	EXT Rice bran	STC Rice bran
Chemical composition (g/100 g)			
Moisture	13.8	7.3	6.1
Protein	9.8	14.9	15.4
Fat	1.3	15.9	17.0
Ash	0.6	9.4	9.5
Total fibre	4.4	23.7	23.5
- *Insoluble fiber*	3.0	21.8	21.5
- *Soluble fiber*	1.4	1.9	2.0
Carbohydrates[a]	70.1	28.8	28.5
Bran soluble protein		2.3	3.4
Particle size (%)			
>0.50 mm	0.1	31.7	20.9
0.50-0.30 mm	11.9	51.7	65.6
0.30-0.25 mm	18.4	12.5	9.7
0.25-0.20 mm	31.3	3.7	3.6
0.20-0.125 mm	35.1	0.4	0.2
<0.125 mm	3.2	0.0	0.0

[a]Calculated by difference. Values are means from triplicate determinations.

of product (CARROLL, 1990; PACHECO DE DELAHAYE *et al.*, 2009). RB has a high content of soluble albumins and globulins and their denaturation during the heat treatment led to a decrease in its soluble form (PRAKASH and RAMANATHAN, 1994). According to the findings reported by SHARMA *et al.* (2004), the very low content of soluble protein in EXT-RB is due to the high temperature applied during the heat treatment.

Influence of rice bran on dough properties

The viscoelastic characteristics of the dough are shown in Table 2. The deformation energy (W) tends to decrease when the RB content of the mixture increases. This effect is greater than expected due to the dilution of gluten, especially in RB-STC. For a given W value, the P/L ratio is a good predictor of dough behavior. This kind of behavior was specific for each mixture and determined by interactions between different components of wheat flour and RB. In studies carried out on mixtures of wheat flour and fibers, the alveographic parameters were observed to depend heavily on the type of fiber (GOMEZ *et al.*, 2003; WANG *et al.*, 2002). Another important aspect is the degradation of dough due to proteolysis, expressed as the loss of dough strength after 2 hours of dough formation (W2h). The value for wheat flour was 22% and was not affected by the addition of RB, which suggests that the heat treatment of RB was intense enough to prevent any proteolytic activity.

To study the effect of RB addition on dough mixing properties, the main farinograph parameters were measured. The results in Table 2 show that addition of RB to the wheat flour did not substantially change the mixing properties of the dough under the experimental conditions outlined above. Water absorption slightly decreased for all the concentrations and types of RB studied, but it was not significant from a functional point of view. Therefore, all bread-making trials were conducted at a fixed water concentration (57% wheat flour basis) indepen-

Table 2 - Effect of RB addition to the wheat flour on alveographic, farinographic and amylographic analysis of dough (abbreviations are specified in section *Dough characteristics*).

	Alveograph					Farinograph				Amylograph
	W (J)	P (mm)	L (mm)	P/L	W 2 h (%)	Absorption (%)	DDT (min)	Stability (min)	Decay 10 m (BU)	Peak Viscosity (AU)
Wheat flour	211	82	76	1.08	-22	57.4	2.2	8.2	42	1204
Substitution of 3% wheat flour by rice bran										
EXT	196	89	65	1.37	-19	57.2	1.6	8.5	41	1203
STC	194	90	60	1.50	-20	57.1	1.8	9.7	27	1191
Substitution of 6% wheat flour by rice bran										
EXT	191	91	58	1.57	-18	56.8	1.9	8.9	40	1193
STC	186	95	52	1.83	-22	57.0	1.5	9.8	31	1181
Substitution of 9% wheat flour by rice bran										
EXT	173	97	47	2.06	-15	56.9	1.6	9.7	32	1154
STC	163	96	45	2.13	-19	57.0	1.8	9.6	26	1177

Values are means from triplicate determinations. EXT: Extrusion cooking; STC: Steam cooking.

Table 3 - Effect of RB addition on the fermentation characteristics of wheat flour (abbreviations are specified in section "Dough characteristics").

	Dough development		Gas behaviour				
	H_m (mm)	T_1 (min)	GT_1 (min)	T_x (min)	V_{CO2} (mL)	R (mL)	R (%)
Wheat Flour	52.5	2.59	1.02	0.57	1576	1235	78.4
Substitution of 3% wheat flour by rice bran							
EXT	60.1	2.41	1.08	1.06	1614	1423	88.2
STC	63.5	2.58	1.10	1.21	1630	1302	79.9
Substitution of 6% wheat flour by rice bran							
EXT	58.9	2.20	1.16	1.11	1652	1356	82.1
STC	65.8	2.58	1.18	1.21	1582	1268	80.2
Substitution of 9% wheat flour by rice bran							
EXT	66.4	3.00	1.19	1.18	1578	1282	81.2
STC	64.5	2.29	1.16	1.15	1574	1225	77.8

Values are means from triplicate determinations. EXT: Extrusion cooking; STC: Steam cooking.

dently of the dough formulas used, and always at the same consistency of the dough. It should be pointed out that dough stability, which indicates the tolerance of dough to kneading, increased with the presence of RB, inversely to the decay value. These results supported the hypothesis that there were no negative interactions between the components of wheat flour and RB. These data were similar to those obtained by KIM *et al.* (1997) but not consistent with those of other studies (GHUFRAN-SAEED *et al.*, 2009; SUDHA *et al.*, 2007). However in these latter studies the basic conditions, (e.g. wheat flour characteristics, RB composition and concentrations) were different.

The amylogram parameters give information about the viscosity generated by the gelatinization of starch. The most important of these parameters is the peak viscosity shown in Table 2. In the case of the wheat flour used in this study, the peak viscosity value was very high (1204 AU). The addition of RB reduced it slightly, although not significantly from functional point of view. Analyzing the Falling Number, another test measuring the starch viscosity, GUFRAN-SAEED *et al.* (2009) made similar findings. The peak viscosity of starch is considerably reduced by α-amylase activity and, in this case, neither RB contributed to any amylase activity. Therefore, for all experimental conditions studied, breadmaking trials were conducted in which the same dose of bread improver was added as the amylase contributor. Throughout the fermentation process the dough behavior was recorded by the rheofermentometer. The influence of RB addition on the fermentation characteristic is shown in Table 3. The maximum dough height (H_m) increased with the addition of RB, regardless of its type and concentration. The time required to reach the maximum dough height was not affected. With respect to gas behavior a backlog in porosity development (T_x) was observed and, except at high concentrations of RB (9%), gas production and retention was higher, and more pronounced in the case of EXT-RB. The only data available for this kind of analysis have been provided by WANG *et al.* (2002), who added different fibers to the wheat flour and the results depended on the type of fiber used. In the main, it can be assumed that adding cellulose or bran to wheat flour decreased the gas holding capacity.

From the overall results it can be concluded that supplementing wheat flour with RB, mainly at concentrations of 3 and 6%, did not influence the formulation and mixing properties of the dough and improved the fermentation and breadmaking process of the partially baked wheat bread.

Influence of rice bran on bread quality

Apart from shape and color, the specific volume of bread is also an important element in consumer selection criteria. The specific volume data are shown in Fig. 1. Adding RB-EXT at concentrations of 3 and 6% led to a significant increase in the specific volume and only at the highest concentration (9%) did it slightly decrease, in comparison to the control. On the contrary, the specific volume obtained for STC-RB bread at concentrations of 3 and 6% did not change in comparison to the control, and decreased sharply at 9%. Several authors have reported a progressive decrease in specific volume when RB content is increased (SEKHON *et al.*, 1997; SHARMA and CHAUAN, 2002; SOARES *et al.*, 2009). However, other authors have observed an increase in specific volume at low concentrations of RB which decrease at higher concentrations (LIMA *et al.*, 2002; PARK *et al.*, 2008). LIMA *et al.* (2002) also observed

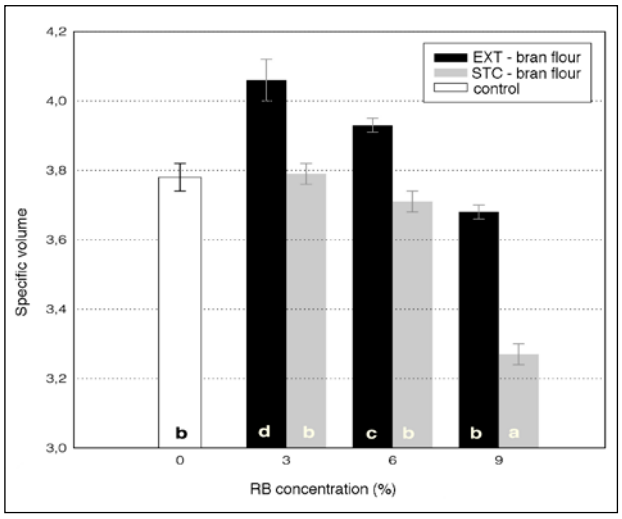


Fig. 1 - Effect of RB addition to the wheat flour on the specific volume of breads.
Means with different letters were significantly different (P<0.05).
EXT: Extrusion cooking; STC: Steam cooking.

differences related to the origin, and therefore the fiber content, of the RB variety: lower fiber contents result in higher volume. When the RB fiber is added directly to the bread, volume decreases (ABDUL-HAMID and LUAN, 2000). When the hemicellulose fraction and insoluble fraction of the RB fiber are added separately, the volume remains unchanged in the first case and decreases in the second (HU *et al.*, 2009). These findings confirm the hypothesis that insoluble RB fiber has an important effect on the specific volume. In the case of wheat bran, the heat treatment counteract the negative effects mentioned above. Wheat bran added to the flour strengthened the gluten matrix (AMIRKAVEEI *et al.*, 2009) and restored the initial bread volume (DE COOK *et al.*, 1999).

The results obtained in our study, consistent with the above, show that a more intensive heat treatment of EXT-RB helps to stabilize the gluten network and, therefore, to retain gas, especially at low bran concentrations (3 and 6%). This is probably due to the oxidation of reducing compounds and is consistent with the higher rate of gas retention in EXT-RB dough. Thus, adding low concentrations of RB that has previously been exposed to high heat treatment acts as a bread improver.

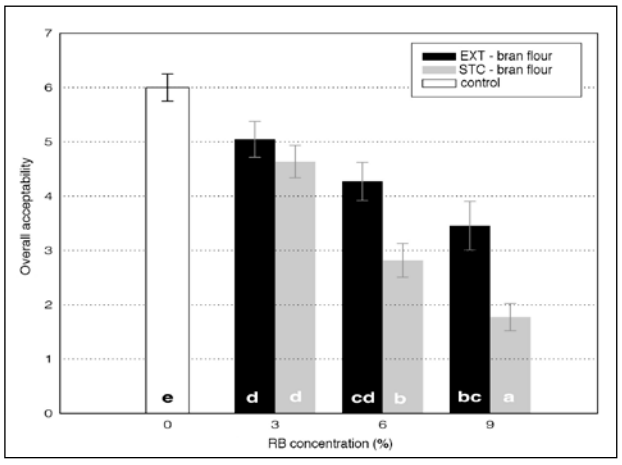


Fig. 2 - Effect of RB addition to the wheat flour on the sensory evaluation of breads.
Means with different letters were significantly different (P<0.05).
EXT: Extrusion cooking; STC: Steam cooking.

Sensory evaluation

Fig. 2 shows the average sensory evaluation of overall acceptability. The bread without RB (control) was given the highest score, which was significantly different from the rest. The score given to breads with added RB decreased when the RB concentration increased and the lowest scores were given to STC-RB supplemented breads. The difference between the two types of RB becomes significant at concentrations of 6% and 9%. From the qualitative point of view, breads with 9% of RB were described by several panelists as having off-flavors.

Good sensory acceptance is one of the key aspects of using complementary or alternative ingredients in wheat bread. Several studies have been made of RB at different concentrations and in different bread types. The findings are in good agreement with those of our study: that is to say, the highest RB concentration was given the lowest score (SEKHON *et al.*, 1997; SHARMA *et al.*, 2004; SUDHA *et al.*, 2007; GHUFRAN-SAEED *et al.*, 2009). The same thing happened for RB derivatives (ABDUL-HAMID *et al.*, 2000; SADAWARTE *et al.*, 2007; HU *et al.*, 2009). However, no significant differences were detected between 7.5% RB bread and the control by SOARES *et al.* (2009). PACHECO DE DELAHAYE *et al.* (2009) even showed sensory preferences for 5% RB bread. One possible explanation for this is the places these studies were carried out: the former were from Asia while the latter were from South America, so there might be some variations in the susceptibility to the flavors added by RB to the bread. This is why sensory evaluation results are difficult to extrapolate, because cultural and culinary habits, and the different types of breads consumed need to be taken into account. Our study of a Spanish population indicates that the addition of RB to partially baked wheat bread would be limited to special RB bread with sensory characteristics that are different from those of the traditional bread.

CONCLUSIONS

The present paper studies how substituting wheat flour by two types of RB, thermally treated by two different methods (EXT and STC), at concentrations of 3, 6 and 9%, affects the rhe-

ological properties of the dough and the quality of breads obtained in an industrial process of partially baked production. The variables analyzed in the dough showed that RB addition had a very slight or no effect on the wheat flour and even improved gas retention. These findings were more evident at low concentrations of RB, and for EXT-RB addition. The characteristics of the breads obtained depended on the RB type (EXT or STC) and were clearly superior for EXT-RB. These differences were due to the different heat treatment intensities applied to the two types of RB, in which the destruction of heat-sensitive constituents might lead to less protein-fiber interferences during the formation of the gluten network. However, further investigations are required if this hypothesis is to be confirmed.

In conclusion, bread supplemented with low concentrations of EXT-RB may be a way to increase the intake of dietary fiber in the population, as well as of other important compounds present in the lipid fraction of RB. In Spain, however, these breads do not have the same sensorial acceptance as the traditional baguette and should not be treated as a white bread substitute, but as a different specialty with added nutritional value.

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PAPER

PREDICTION OF THE MAJOR FATTY ACIDS IN THE BACKFAT FROM IN- AND OUTDOOR IBERIAN PIGS AT SLAUGHTER

A. DAZA[1], M.A. LATORRE[2]* and C.J. LÓPEZ-BOTE[3]
[1]Departamento de Producción Animal, Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica de Madrid, Spain
[2]Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, Spain
[3]Departamento de Producción Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Spain
*Corresponding author: Tel. +34 876554168,
email: malatorr@unizar.es

ABSTRACT

The objective was to predict the proportion of the major fatty acids (FA; C16:0, C18:0, C18:1n-9 and C18:2n-6) at slaughter (approximately 150 kg of body weight, BW) from fat samples taken at the beginning (100 kg BW) of the finishing period in Iberian pigs under indoor (eating feedstuffs) and outdoor (eating acorns and grass) conditions. For each production system, different linear regression equations were found between the FA percentages at the beginning of the finishing phase and the variation of the FA proportions with respect to the BW gain during that period. It might be very useful for pig producers.

- Keywords: backfat, Iberian pig, fatty acids, prediction -

INTRODUCTION

Iberian is an autochthonous breed of fatty pigs located in the South West of Iberian peninsula. Animals are slaughtered at 150-160 kg of body weight (BW) and can be reared indoor, feeding feedstuffs, or outdoor, making use of the natural resources, mainly acorns and grass (LÓPEZ-BOTE, 1998). The meat from Iberian pigs is widely known because of high amount of intramuscular fat, great concentrations of heme pigments and high levels of monounsaturated fatty acids (FAs) (RODRÍGUEZ-SÁNCHEZ *et al.*, 2010) showing excellent sensorial characteristics (VENTANAS *et al.*, 2006). The carcasses from Iberian pigs are valued in the market according to the proportions of the major FAs in subcutaneous backfat; a high quality is related to at least 54% of oleic (C18:1n-9) and a proportion of palmitic (C16:0), stearic (C18:0) and linoleic (C18:2n-6) bellow 21, 9.5 and 9.5%, respectively (DAZA and LÓPEZ-BOTE, 2007). Fresh meat and dry-cured and elaborated products obtained from outdoor Iberian pigs reach higher prices in the market than those from indoor Iberian pigs.

The proportion of the major FAs in subcutaneous backfat at slaughter depends on factors such as breed and crossbreed, gender, BW gain during the finishing phase (from 100 to 150 kg BW approximately), age and BW at slaughter or feeding system (DAZA *et al.*, 2005a). To know the relationship between the FA percentages at the beginning of the finishing phase and the variation of FA proportions with respect to the BW gain during that phase might be very interesting for pig producers. Farmers could estimate the necessary BW gain of the pigs during fattening period to reach the FA proportions required at slaughter by the market (DAZA *et al.*, 2005b). This aspect has not been studied until now.

Therefore, the main objective of this trial was to predict the final proportion of the major FAs in backfat of Iberian pigs reared under different systems (indoor vs. outdoor) during finishing period. Moreover, this work evaluates the differences of rearing-feeding system during the fattening phase on FA profile of subcutaneous fat of those pigs.

MATERIAL AND METHODS

Experimental design and sampling

All the experimental procedures used in the trial were in compliance with the Spanish guidelines for the care and use of animals in research (BOLETÍN OFICIAL DEL ESTADO, 2007). Two trials were carried out and are described below.

In the Experiment 1, the aim was to study the relationship between the FA percentages in subcutaneous fat at the beginning of the outdoor and indoor fattening period and the variation of the FA proportions with respect to the BW gain during that period. For it, a total of 96 Torbiscal barrows (CIA "El Dehesón del Encinar", Toledo, Spain) were used. Torbical is a synthetic line from the crossbreed of several lines from Iberian breed. A total of 48 pigs were reared outdoor from 100.2±2.7 to 155.1±3.1 kg BW and fed exclusively acorns and grass and the other 48 pigs were reared indoor from 99.3±3.5 to 153.9±2.6 kg BW and fed a commercial feedstuff (Table 1).

In the experiment 2, the aim was to study the accuracy of the regression equations obtained in the previous trial. For it, other three groups of pigs of the same genetic line were used: i) ten pigs were reared outdoor from 99.6±2.5 to 154.4±2.6 kg BW and fed exclusively acorns and grass, ii) twelve pigs were reared indoor from 101.2±3.0 to 154.7±3.1 kg BW and fed the same feedstuff as described in Experiment 1 (named feedstuff 1), and iii) twenty four pigs were reared indoor from 98.1±2.0 to 152.8±2.8 kg BW and fed a different feedstuff (named feedstuff 2) (Table 2).

In both Experiments, during the growing period (approximately from 30 to 100 kg BW, 70 and 74 days for Experiment 1 and 2, respectively), all pigs were reared indoor and ate the same feedstuff (around 2.0 kg/day, 3,000 kcal of metabolizable energy/kg and 15.6% of crude protein).

At the beginning of the fattening period, a backfat biopsy sample was taken from each pig using a cylindrical biopsy device with a diameter of 8 mm under local anesthesia with 2% lidocaine-HCL (Alphacaine, Fendigo). After the biopsy removal, the wound was desinfected locally with 2 mL of penicillin (300,000 IU/mL, Depocilline, Mycofarm). When animals achieved the slaughter BW, they were moved to a local abattoir, electrically stunned, slaughtered and eviscerated according to standard commercial procedures. Afterwards, a sample of backfat was taken from each carcass and samples were individually identified and stored until de laboratorial analyses.

Chemical analyses

At least 1 kg of acorns was collected from 10 different holm oak trees (Quercus ilex) randomly selected. Approximately 2 kg of grass were taken directly from the ground choosing at random four different areas. The concentrate diets were sampled in the feedstuff plant. The analysed nutrient value (AOAC, 2000) of all feeds used in the Experiments 1 and 2 are shown in Tables 1 and 2, respectively. The FAs were extracted and quantified by the one-step procedure as described SUKHIJA and PALMQUIST (1988) in lyophilised samples. Pentadecenoic acid (C15:1) (Sigma, Alcobendas, Madrid) was used as internal standard. Previously methylated FA samples were identified by gas chromatography as described elsewhere (LÓPEZ-BOTE et al., 1997) using a 6890 Hewlett Packard gas chromatograph

Table 1 - Nutrient content of the feeds used during the fattening period (from 100.2 to 155.1 kg of body weight) in Experiment 1[a].

	Indoor pigs	Outdoor pigs	
	Feedstuff[b]	Acorns	Grass
Dry matter (DM, g/kg)	896.2	661.7	239.8
Crude protein (g/kg DM)	140.7	48.3	132.5
Ether extract (g/kg DM)	40.3	98.1	36.8
Crude fibre (g/kg DM)	44.8	8.5	164.1
Ash (g/kg DM)	46.8	20.1	135.7
Free-nitrogen extractives (g/kg DM)	727.4	825.0	530.9
Fatty acids (g/100 g of total fatty acids)			
C16:0	13.8	22.5	21.0
C18:0	5.4	4.6	2.6
C18:1n-9	40.4	59.9	3.2
C18:2n-6	37.9	12.4	12.9
C18:3n-3	1.8	0.61	51.0

[a]Analysed according to AOAC (2000).
[b]Supplied per kg of diet: 532.8 g barley, 310.5 g wheat, 50.2 g high-oleic sunflower seed, 81.0 g soybean meal-44, 8.3 g calcium carbonate, 9.2 dicalcium phosphate, 4 g sodium chloride, 4 g mineral and vitamin premix.

Table 2 - Nutrient content of the feeds used during the fattening period (from 99.6 to 154.4 kg of body weight) in Experiment 2[a].

	Indoor pigs	Indoor pigs	Outdoor pigs	
	Feedstuff 1[b]	Feedstuff 2[c]	Acorns	Grass
Dry matter (DM, g/kg)	896.2	892.0	649.9	246.2
Crude protein (g/kg DM)	140.7	143.1	50.2	142.1
Ether extract (g/kg DM)	40.3	70.2	91.9	25.8
Crude fibre (g/kg DM)	44.8	61.7	9.5	168.3
Ash (g/kg DM)	46.8	55.7	18.1	121.1
Free-nitrogen extractives (g/kg DM)	727.4	669.3	830.3	542.7
Fatty acids (g/100 g of total fatty acids)				
C16:0	13.8	9.9	23.1	20.4
C18:0	5.4	4.1	5.1	2.2
C18:1n-9	40.4	56.8	57.9	2.9
C18:2n-6	37.9	20.2	13.0	14.1
C18:3n-3	1.8	0.35	0.51	55.3

[a]Analysed according to AOAC (2000).
[b]Supplied per kg of diet: 532.8 g barley, 310.5 g wheat, 50.2 g high-oleic sunflower seed, 81.0 g soybean meal-44, 8.3 g calcium carbonate, 9.2 dicalcium phosphate, 4 g sodium chloride, 4 g mineral and vitamin premix.
[c]Supplied per kg of diet: 472.0 g barley, 200 g wheat, 80 g corn, 160 g sunflower meal, 50 g high-oleic sunflower oil, 12.0 g calcium carbonate, 20 g dicalcium phosphate, 4 g sodium chloride, 2 g mineral and vitamin complex.

and a 30 m x 0.32 mm x 0.25 μm cross-linked polyethylene glycol capillary column. A temperature program of 170° to 245°C was used. The injector and detector were maintained at 250°C. The carrier gas (helium) flow rate was 3 mL/min.

Lipids from subcutaneous backfat were extracted according to the procedure proposed by BLIGH and DYER (1959). Fat extracts were methylated in the presence of sulphuric acid and analysed as has been described for feeds.

Statistical analyses

Data were analysed using the statistical package SAS (1999).

In Experiment 1, a linear regression procedure was carried out. For each FA evaluated, the intercepts and slopes of regression equations obtained from indoor and outdoor pigs were compared by Student's t-test. In Experiment 2, a paired t-test (SWANTEK *et al.*, 1999) was used and the correlation coefficients between actual and predicted values of major FA percentages in subcutaneous backfat were calculated. In both Experiments, ANOVA analyses were carry out to study the average daily gain and the major FA proportions in subcutaneous backfat comparing rearing and feeding systems.

RESULTS AND DISCUSSION

The average daily gain during the fatting period was similar for indoor and outdoor pigs irrespective of the trial because the feed supply for indoor animals was calculated accord-

Table 3 - The proportions (%) of major fatty acids in subcutaneous backfat at the beginning and at the end of the fattening period (100 and 154 kg of body weight, respectively) of pigs under different feeding-rearing systems.

	Experiment 1				Experiment 2				
	Indoor[a]	Outdoor[b]	SEM[c]	p[d]	Indoor[a]	Indoor[e]	Outdoor[b]	SEM[c]	p[d]
Beginning									
C16:0	21.65	21.27	0.16	NS	21.13	21.14	21.60	0.24	NS
C18:0	10.58	10.53	0.15	NS	10.21	10.48	10.79	0.26	NS
C18:1n-9	44.71	44.59	0.35	NS	43.95	44.56	44.12	0.31	NS
C18:2n-6	11.62	11.90	0.32	NS	10.98	11.27	10.89	0.33	NS
End									
C16:0	18.25	19.84	0.13	***	20.09^{x}	18.39^{y}	18.91^{y}	0.30	*
C18:0	8.55	9.28	0.13	**	9.67^{x}	8.87^{xy}	8.70^{y}	0.28	*
C18:1n-9	54.34	51.06	0.24	***	50.69^{z}	52.19^{y}	53.89^{x}	0.38	***
C18:2n-6	9.10	9.10	0.14	NS	9.98	9.24	9.16	0.29	NS

[a]Feeding based on feedstuff. It supplied per kg of dry matter of the feed: 140.7 g crude protein, 40.3 g ether extract, 44.8 g crude fibre.
[b]Feeding based on acorns and grass.
[c]SEM: Standard Error of the Mean.
[d]p: Level of statistical significance. In Experiment 2, different superscripts (x, y, z) in the same raw indicate differences. NS: Not Significant; *p<0.05; **p<0.01; ***p<0.001.
[e]Feeding based on feedstuff. It supplied per kg of dry matter of the feed: 143.1 g crude protein, 70.2 g ether extract, 61.7 g crude fibre.

ing to the growth rate of outdoor animals (data not shown). The results for the main FA proportions found in subcutaneous backfat in Experiments 1 and 2 are shown in Table 3. As expected, at the beginning of the fattening period, in both trials, no effect of rearing system was detected for any FA because all pigs had eaten the same feedstuff previously, during the growing period. However, at slaughter of the Experiment 1, the fat from outdoor pigs had lower C16:0 (p<0.001) and C18:0 (p<0.01) and higher C18:1n-9 (p<0.001) proportions than that from indoor pigs without any difference in C18:2n-6. At slaughter of the Experiment 2, the fat from indoor pigs fed the feedstuff 1 had higher C18:0 (p<0.05) and lower C18:1n-9 (p<0.001) percentages than that from outdoor pigs with results from indoor pigs fed the feedstuff 2 (rich in C18:1n-9) being intermediate. In addition, C16:0 content was higher in fat from indoor pigs fed the feedstuff 1 than in fat from the other treatments.

The results of the present trials are in agreement with those reported by CARRAPISO *et al.* (2003), REY *et al.* (2006) and DAZA *et al.* (2007) who observed that, in backfat from Iberian pigs reared under free-range conditions, C16:0 and C18:0 proportions were lower and C18:1n-9 proportion was higher than in Iberian pigs reared in confinement eating feedstuff. When indoor pigs received a diet rich in C18:1n-9, the retention of that FA in subcutaneous fat was higher than that in pigs fed the other diet and it also carried out a reduction in C16:0 content. These results agree with data from GONZALEZ *et al.* (2005). SANZ *et al.* (2007) showed that it was possible obtain a similar FA profile in indoor Iberian pigs fed feedstuffs rich in C18:1n-9 during the fattening period to that obtained under free-range conditions eating acorns and grass.

According to the t test results, the initial and slaughter BW of pigs used to calculate and validate the regression equations were similar in both production systems (p>0.05).

The Table 4 shows the relationship between the percentage of the major FAs in the outer subcutaneous backfat layer per kg of BW gain (Y) and the proportion of each FA at the beginning of the finishing period (X) under indoor (eating feedstuff) or outdoor (eating acorns and grass) conditions. The relationships between Y and X were linear and significant in both management systems. According to these results, irrespectively of the feeding/rearing system, the greatest decrease rate was observed (p<0.001) for the proportions of C16:0, C18:0 and C18:2n-6 acids. In addition, the smallest increase rate was detected (p<0.05) for the proportion of C18:1n-9 during free-range period. Other authors (FONTANILLAS *et al.*, 1998; DAZA *et al.*, 2007) also observed an increase of C16:0, C18:0 and C18:2n-6 and a reduction of the C18:1n-9 proportions with higher levels of those FAS at the beginning of the finishing period.

For outdoor pigs, the initial proportion of C16:0, C18:0, C18:1n-9 and C18:2n-6 acids observed in subcutaneous backfat at the beginning of fattening period accounted for 47, 56, 20 and 88% of the variation, respectively, which is in agreement with results found by DAZA *et al.*, (2005b) in Iberian pigs raised also under free-range conditions. For indoor pigs, the initial proportion of C16:0, C18:0, C18:1n-9 and C18:2n-6 detected in subcutaneous backfat at the beginning of fattening period accounted for 24, 25, 51 and 78% of the variation, respectively.

It is interesting to note that the slopes of the regression equations calculated for C16:0 and C18:0 proportions were different ($p<0.05$) when both fattening systems were compared (0.0245 vs. 0.0143 for C16:0; and 0.0249 vs. 0.0123 for C18:0 for outdoor and indoor, respectively). As increased the initial C16:0 and C18:0 proportions, the reduction of C16:0 and C18.0 per kilogram of BW gain was higher in outdoor than in indoor pigs. However, no significant differences ($p>0.05$) were observed for the C18:2n-6 reduction speed and C18:1n-9 increase speed according to finishing system (free-range *vs.* confinement). It would indicate that, at the beginning of the finishing period, the C16:0 and C18.0 contents in the subcutaneous backfat from pigs finished under outdoor conditions can be higher than in pigs finished under indoor conditions. This aspect must be taken into account by the producers in order to formulate adequately the feed to provide during the growing period (before fattening phase).

The Table 5 shows the results obtained, by paired test t and correlation analysis, for each FA under the three kind of feeding systems stud-

Table 4 - Regression equations between the major fatty acids in the subcutaneous backfat at the beginning of the fattening period (X, in %) and their variation with respect to live weight gain during that period (Y, in %/kg) in pigs reared indoor or outdoor (Experiment 1).

	Regression equation	R^{2a}	RSD[b]	p[c]
Outdoor				
C16:0	$Y = (0.458^{x} \pm 0.0975) - (0.0245^{x} \pm 0.00445) \cdot X$	0.47	0.023	***
C18:0	$Y = (0.276^{x} \pm 0.0489) - (0.0299^{x} \pm 0.00451) \cdot X$	0.56	0.020	***
C18:1n-9	$Y = (0.932^{x} \pm 0.2510) - (0.0164^{x} \pm 0,00576) \cdot X$	0.20	0.061	*
C18:2n-6	$Y = (0.154^{x} \pm 0.0142) - (0.0173^{x} \pm 0.00112) \cdot X$	0.88	0.013	***
Indoor				
C16:0	$Y = (0.261^{y} \pm 0.0821) - (0.0143^{y} \pm 0.00371) \cdot X$	0.24	0.018	***
C18:0	$Y = (0.122^{y} \pm 0.0327) - (0.0123^{y} \pm 0.00310) \cdot X$	0.25	0.116	***
C18:1n-9	$Y = (0.869^{y} \pm 0.0110) - (0.0175^{x} \pm 0.00250) \cdot X$	0.51	0.031	***
C18:2n-6	$Y = (0.212^{y} \pm 0.0180) - (0.0179^{x} \pm 0.00141) \cdot X$	0.78	0.018	***

[a]R^2: Determination coefficient.
[b]RSD: Residual standard deviation.
[c]p: Level of statistical significance. For each fatty acid, between rearing systems, intercepts and slopes with different superscripts (x, y in lower case letter) are significantly different. *p<0.05; ***p<0.001.

Table 5 - Predicted vs. actual values (means ± standard deviation) of the major fatty acids (%) in the subcutaneous backfat of pigs reared under different feeding-rearing systems during fattening period (Experiment 2).

	Value	Predicted value	Actual-Predicted value	t value	p[a]	r[b]	p[c]
Outdoor							
C16:0	0.097 ± 0.040	0.091 ± 0,015	0.006	0.57	NS	0.64	*
C18:0	0.053 ± 0.022	0.058 ± 0,013	- 0.005	0.82	NS	0.45	NS
C18:1n-9	0.243 ± 0.078	0.216 ± 0,030	0.027	1.29	NS	0.58	NS
C18:2n-6	0.054 ± 0.047	0.057 ± 0,033	- 0.003	0.48	NS	0.93	***
Indoor[d]							
C16:0	-0.059 ± 0.014	-0.062 ± 0.007	0.003	0.088	NS	0.38	NS
C18:0	-0.017 ± 0.004	- 0.015 ± 0.005	- 0.002	1.31	NS	0.25	NS
C18:1n-9	0.082 ± 0.043	0.089 ± 0.018	- 0.007	0.83	NS	0.84	***
C18:2n-6	0.017 ± 0.030	0.009 ± 0.002	0.008	1.42	0.09	0.71	**
Indoor[e]							
C16:0	- 0.026 ± 0.014	- 0.044 ± 0.014	0.018	9.7	***	0.79	***
C18:0	- 0.023 ± 0.016	-0.008 ±0.012	-0.015	5.7	***	0.64	***
C18:1n-9	0.120 ± 0.029	0.092 ± 0.017	0.028	6.7	***	0.74	***
C18:2n-6	-0.052 ± 0.019	- 0.003 ± 0.017	-0.049	21.2	***	0.81	***

[a]p: Level of statistical significance between actual vs. predicted means. NS: Not Significant; ***p<0.001.
[b]r: Correlation coefficient between actual and predicted values.
[c]p: Level of statistical significance of r. NS: not significant, *p<0.05; **p<0.01; ***p<0.001.
[d]Data obtained from pigs which fed feedstuff 1.
[e]Data obtained from pigs which fed feedstuff 2.

ied. For the outdoor pigs, the regression equations calculated were especially accurate for C16:0 ($p<0.05$) and C18:2n-6 ($p<0.001$). No significant differences ($p>0.05$) between actual and predicted values were observed for any of the four FAs studied.

For the indoor pigs that consumed the diet with same feed composition as that fed by the pigs used to calculate the regression equations pattern, the regression equations obtained were also accurate, although a high t value for C18:2n-6 was detected ($t = 1.42$, $p<0.09$). For this group of pigs, no difference between actual and predicted values of the FA proportions was found. Nevertheless, for the indoor pigs that ate different concentrate diet as that consumed by the pigs used to calculate the regression equations pattern, the regression equations obtained were not accurate for the four major FAs studied. The paired test t detected differences ($p<0.0001$) between actual and predictedvalues for C16:0, C18:0, C18:1n-9 and C18:2 n-6 acids.

CONCLUSIONS

It is concluded that fat from outdoor pigs had higher proportion of C18:1n-9 and lower of C16:0 than fat from indoor pigs. The supply of a feedstuff rich in C18:1n-9 led a higher deposition of that FA and lower of C16:0 proportion in backfat. The use of the regression equations obtained in the present study might be very useful for the producers of indoor and outdoor Iberian pigs because knowing the FAs at the beginning of the finishing period and the FAs required at slaughter, the BW gain can be calculated and put in practice.

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SHORT COMMUNICATION

MINERAL CONTENT OF A MOSS TEA FOR HYPERTENSION

B. PEJIN[1]*, D. BELIC[2], G. VUCKOVIC[2], Y. KIEN-THAI[3] and B. STANIMIROVIC[2]
[1]Department of Life Sciences, Institute for Multidisciplinary Research,
University of Belgrade, Serbia
[2]MOL a.d., Belgrade, Serbia
[3]Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia
*Corresponding author: borispejin@imsi.rs, brspjn@gmail.com

ABSTRACT

The content of sodium, potassium, magnesium, calcium, selenium, phosphorus, nitrite and nitrate in the moss *Rhodobryum ontariense* and its tea was investigated for medicinal reason. The aim of this study was preliminary estimation of *R. ontariense* minerals' contribution to the treatment of hypertension, claimed for the moss tea by Traditional Chinese Medicine. Majority of cations were determined by EPA methods, while phosphorus was determined with an appropriate ISO method. The anions nitrite and nitrate were determined by ion chromatography. Potassium was the most abundant mineral in the investigated tea (5895.20 ± 1.22 mg/kg) and might be speculated as one of its beneficial ingredients for the treatment of elevated blood pressure conditions.

- Keywords: heart disease, minerals, potassium, *Rhodobryum ontariense*, traditional Chinese medicine -

Hypertension is a major contributing factor for cardiovascular and cerebrovascular diseases. Optimal blood pressure is therefore of great importance and can be regulated with the reduction of alcohol intake, weight loss, and limiting dietary sodium to <100 mmol/day (SARAFIDIS and BAKRIS, 2008). However, the present average sodium intakes, approximately 3,000-4,500 mg/day in various industrialised populations, are 2-3-fold higher in comparison with the current Dietary Reference Intake (DRI) of 1,500 mg. The sodium intakes markedly exceed even the level of 2,500 mg, which has been recently given as the maximum that is likely to have no effects on blood pressure or otherwise. By contrast, the present average potassium, calcium, and magnesium intakes are remarkably lower than the recommended intake levels (DRI). Indeed, there is convincing evidence which indicates that such intakes of the aforementioned minerals produce and maintain elevated blood pressure in a big proportion of the population. Hence, the so-called Dietary Approaches to stop Hypertension, which has shown an excellent blood pressure lowering effect, insists on decreased intakes of sodium and increased intakes of potassium, calcium, and magnesium (KARPPANEN *et al.*, 2005).

On the other hand, it is known that under specific conditions inorganic nitrite and nitrate can be recycled *in vivo* to form nitric oxide which induces hypotension. Furthermore, nitrate itself reduces blood pressure as well (TANG *et al.*, 2009).

Traditional Chinese Medicine (TCM) suggests that mosses of *Rhodobryum* species can cure cardiovascular diseases as crude drugs in form of medicinal tea (ASAKAWA, 2008; HARRIS and YANG, 2009). The name for the genus *Rhodobryum* in Yunnan Province is HuiXinCao (回心草), a name referring to the heart-healing properties of this plant; the name literally means "return-the-heart-herb" (HARRIS, 2008). PEJIN *et al.* (2011a,b; 2012a,b,c,d,e) investigated chemical composition and biological activity of the moss *Rhodobryum ontariense* for the first time finding that its lyophilised aqueous extract (100 mg/kg b.w. dissolved in 0.2 mL of saline and injected intravenously) significantly and quickly normalised arterial blood pressure and reduced pulse pressure, thus decreasing the risk for cardiovascular events in the group of spontaneously hypertensive rats (PEJIN, 2011b). Therefore, the aim of this study was preliminary estimation of *R. ontariense* minerals' contribution to the treatment of hypertension.

The sample of *Rhodobryum ontariense* (Kindb.) Kindb. (Bryaceae) originated from the Fraser's Hill (Malaysia, August 2010). Voucher specimen has been deposited in the Herbarium of the Institute of Biological Sciences, Faculty of Science, University of Malaya (KT Yong 7635).

Before extraction the moss was carefully inspected for contaminants: soil and plant material were completely removed. The gametophyte tips were used for the extraction. Air-dried parts of *R. ontariense* (2 g) were ground and extracted with hot water for 30 min at room temperature. The extract (tea) was filtered and concentrated by lyophilisation to give 0.14 g of residue (yield, 7 %) which was stored at +4°C for further use.

The cations were determined by adequate EPA/sodium (EPA M 273.1), potassium (EPA M 258.1), magnesium (EPA M 242.1), calcium (EPA M 215.1) and selenium (EPA M 270.2)/and ISO/phosphorus (SRPS EN ISO 6878)/methods, while the anions nitrite and nitrate were determined by ion chromatography. All analyses were done in triplicate. The data are given as mean ± uncertainty of measurement (Table 1).

The content of all examined minerals in both moss samples were below the safety levels for human consumption (HATCHOCK, 2004). Potassium, calcium and magnesium were the most abundant cations in *R. ontariense* tea (5895.20 ± 1.22 mg/kg, 803.57 ± 0.85 mg/kg and 259.28 ± 0.48 mg/kg, respectively), while selenium was the least abundant one (2.13 ± 0.02 mg/kg). Moreover, the content of sodium in the same sample (224.34 ± 0.53 mg/kg) was much lower than potassium one. On the other hand, nitrates in the tea (147.86 ± 0.014 mg/kg) were higher than nitrites (< 0.05 mg/kg). Notably, many clinical studies support the blood pressure-lowering effects of potassium supplementation (FRANZONI *et al.*, 2005; WHELTON *et al.*, 1997). Indeed, maintenance of adequate dietary potassium intake shows promise in the prevention and treatment of hypertension and should be thought as part of the armamentarium of lifestyle changes. Concerning hypertension, the obtained results primarily indicate a potential importance of potassium. Furthermore, the nitrate content can be hypothesised to contribute to nitric oxide bioactivity potential of *R. ontariense* tea (TANG *et al.*, 2009). However, new analyses of various samples of the moss should be performed in order to claim the beneficial role of any mineral for the treatment of elevated blood pressure conditions.

Table 1 - Mineral content of *Rhodobryum ontariense* samples.

Mineral	Moss (mg/kg)	Tea[a] (mg/kg)
Na	254.73±0.62	224.34±0.53
K	13868.49±1.75	5895.20±1.22
Mg	1080.00±0.85	259.28±0.48
Ca	3420.92±1.18	803.57±0.85
P	310.22±0.15	102.95±0.10
Se	3.32±0.03	2.13±0.02
NO_2^-	<0.05	<0.05
NO_3^-	214.07±0.016	147.86±0.014

[a] calculated on dried moss material.

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SHORT COMMUNICATION

FIRST DATA ON THE ANTIMICROBIAL ACTIVITY OF *YUCCA FILAMENTOSA* L. BARK EXTRACTS

M. BONONI, G. GUGLIELMI[1], P. ROCCHI[1] and F. TATEO*
Dipartimento di Scienze Agrarie e Ambientali, Università degli Studi di Milano,
Via Celoria 2, 20133 Milano, Italy
[1]Rocchi dr. Eugenio s.r.l., Via J.F. Kennedy 3, 40053 Bazzano, BO, Italy
*Corresponding author: Tel. +39 02 50316540, Fax +39 02 50316539,
email: fernando.tateo@ .it

ABSTRACT

The aim of this study was to investigate the antimicrobial activity of two *Yucca filamentosa* L. extracts produced by hydro-alcoholic extraction under different operational conditions. The extracts were concentrated and de-alcoholised, and were added to media to test their effects on target microorganisms. Both extracts displayed equivalent specific growth inhibitory activity against *Saccharomyces cerevisiae*. No other antimicrobial activity was found. This anti-fermentative activity may open the doors to new possibilities for using *Yucca filamentosa* L. extracts in the food industry. These findings are of particular interest, since yucca extracts are already classified as being generally recognized as safe (GRAS).

- Keywords: *Yucca filamentosa* L., antimicrobial activity, yucca extracts -

INTRODUCTION

The various *Yucca* species (fam. Asparagaceae, sub-fam. Agavoideae) are tropical or sub-tropical plants with a tree-like growth habit. They are found in arid or semi-arid South-Western regions of the US and Mexico (PELLMYR, 2003). In traditional medicine of the Native Americans, yucca-derived extracts were used against a variety of diseases, including arthritis and rheumatism (MUKHERJEE *et al.*, 2001); these extracts have GRAS (generally recognized as safe) status and are therefore accepted by the US FDA for use in humans. Yucca powder and sap are derived from the logs of the plant; such extracts can be produced by mechanical squeezing and subsequent evaporation of the sap, and are widely used in food, cosmetics, and pharmaceutics (MATTIA and COLUZZI, 2005). Yucca powder and extracts are also used as animal feed additives due to their beneficial anti-inflammatory effects (WENZIG *et al.*, 2008), which have been linked to their high content of phenolic compounds.

Many studies have also examined the saponin-rich extracts of different *Yucca* species for antibacterial activity (CHEEKE *et al.*, 2006; FAVEL *et al.*, 2005; KILLEN *et al.*, 1998; KOWALCZYK *et al.*, 2011; MILGATE and ROBERTS, 1995; MIYAKOSHI *et al.*, 2000; MONTORO *et al.*, 2008; PIACENTE *et al.*, 2005; WENZIG *et al.*, 2008). Saponins are glycoside compounds of a fat-soluble nucleus (aglycone) that can be either a triterpenoid (C_{30}), as in soybean, alfa-alfa, quillaja, and guar (HASSAN *et al.*, 2010); or an alkaloid steroid (C_{27}), as in yucca, tomato, and oats. One or more side chains or water-soluble sugars (glycone) are attached through ester linkages to the aglycone nucleus at different carbon sites (KANEDA *et al.*, 1987). Saponins have shown haemolytic (KHALIL and EI-ADAWY, 1994) and antibacterial activities (SEN *et al.*, 1998), but not all saponins have the same biological activities (HASSAN *et al.*, 2010, KANEDA *et al.*, 1987). It has been demonstrated that yucca bark accumulates polar bidesmosidic saponins; the bark powder samples are dominated by high polarity, while steroidal glycosides with mid- and short-length saccharide chains are predominant in the steam.

A large number of papers have determined that *Yucca schidigera* contains steroidal saponins with anti-yeast activity, and therefore such extracts have been investigated for use as new agents to prevent food deterioration (MIYAKOSHI *et al.*, 2000). Our preliminary experiments show similar properties exhibited by root extracts of *Yucca filamentosa* L. from the USA, also studied previously addressing the importance of chemistry and geographic variation of floral scent (SVENSSON *et al.*, 2005). In the present study, we tested the efficiencies of producing *Yucca filamentosa* L. extract using different extraction solvents, and then tested the antimicrobial activities of these extracts *vs.* several target microorganisms. We found evidence that the extracts had considerable microbicidal activity exclusively against *Saccharomyces cerevisiae* and not against other target microorganisms.

MATERIAL AND METHODS

Extract production for microbiological testing

During the extraction, and particularly during separation of the solid waste, we attempted to avoid introducing technologically differentiated parameters for the various extracts by adopting the simple spontaneous drainage, without affecting by pressure the release of active principles from solid. The dry vegetable material of *Yucca filamentosa* L. was cut into pieces with an average diameter of 10 mm. Preliminary extractions were performed under varying conditions, in order to identify the parameters that produced the best results in terms of percentage of the total soluble solids in the extracts.

Extractions were performed by seven days of infusion of dry materials with solvent in a solid:liquid ratio of 1:6.7; the extraction mixture was shaken three times per day. Three different solvents were used: (A) water, (B) water/95% ethanol (50:50 v/v), and (C) 95% ethanol. In theory, derivation using different types of solvents could result in the extracts having different compositions of the active substances.

Extraction efficiencies were measured (Table 1). Solvent B was determined to produce the best extractive yield. A new extract was produced using solvent B, this time cutting the dry yucca to pieces with an average diameter of 1-2 mm. This extraction product was concentrated in a rotovapor (2:1); the obtained product was named extract B and contained a concentration of total soluble solids equal to 14.0 g 100 mL^{-1}. We also combined the original extracts produced with solvents A, B, and C, in a ratio of 1:1:1. This mixture was concentrated in a rotovapor (4:1) to produce an extract with total soluble solids concentration of 14.2 g 100 mL^{-1}, similar to that of the concentrated extract B. Both concentrated products had a residual ethanol content of no more than 0.01%. The concentrated extracts B and ABC were next tested for their activity on microorganisms.

Microbiological verifications

For testing the antimicrobial activity of the yucca extracts, the following species were used as target microorganisms: *Aspergillus brasiliensis* (NCPF 2275/ATCC 16404), *Escherichia coli* (NCTC 12241/ATCC 25922), *Staphylococcus aureus* (NCTC 12981/ATCC 25923), *Listeria monocytogenes* (NCTC 11994), *Bacillus cereus* (NCTC 7464/ATCC 10876), and *Saccharomyces Cerevisiae* (NCPF 3178). These strains

Table 1 - Preliminary results of testing three different extraction solvents for *Yucca filamentosa* L.

	Yucca (g)	95% EtOH (mL)	H_20 (mL)	Extract (mL)	Total Soluble Solids (g 100 mL^{-1})
Solvent A	888	6,000	---	4,330	0.56
Solvent B	878	3,000	3,000	3,800	5.32
Solvent C	880	---	6,000	2,400	3.58

were obtained from TCS Biosciences Ltd. (Botolph Claydon, Buckingham, UK), and are the standards used for quality control testing in the Laboratory "Rocchi dr. Eugenio s.r.l", Bazzano (Bologna, Italy).

Solid media used included Sabouraud dextrose agar (Biokar diagnostic) for *Aspergillus brasiliensis* and *Saccharomyces cerevisiae*, and tryptone soy agar (Oxoid) for all other target microorganisms. Each solid medium was prepared in the following different versions: undiluted, with the addition of 1 mL ABC extract 100 mL^{-1} of medium, and with the addition of 1 mL B extract 100 mL^{-1} of medium. Testing verified that the addition of extracts did not significantly modify the pH of the medium; the pH remained in the interval of 7.1 ± 0.02 for tryptone soy agar, and of 5.5 ± 0.05 for Sabouraud dextrose agar.

The stock suspensions for the inoculum were produced by collecting a loopful (with a 1 µL loop) of target microorganisms from slant cultures, and dispersing the microorganisms into 10 mL of sterile Ringer 1\4 solution (Biokar diagnostic). From these stock suspensions, decimal dilutions were prepared up to the third. Then 50 µL of the third and the second decimal dilutions were used to inoculate four replica plates, undiluted on the surface of the media with a Don Whitley spiral plater. Incubation conditions were chosen in accordance with the nature of each target microorganisms as follows: *Aspergillus brasiliensis*, 25°C for 5 days; *Escherichia coli*, 37°C for 24 hours; *Staphylococcus aureus*, 37°C for 48 hours; *Listeria monocytogenes*, 37°C for 48 hours; *Bacillus cereus*, 30°C for 48 hours; *Saccharomyces cerevisiae*, 25°C for 5 days.

Table 2 - Results concerning growth values as cfu mL^{-1} derived from sowing stock suspension on undiluted media and media modified with two yucca extracts (ABC and B). Counts on tryptone soy agar (t.s.a.) and Sabouraud dextrose agar (s.a.) media are reported. The values are the average of four replicas, calculated according to ISO 8199:2005 Standard (8.4.2); standard deviation (S) and *p* values are reported for the significant growth reduction of *S. cerevisiae.*

	Medium	cfu mL^{-1}	95% Confidence Interval (cfu mL^{-1})		Log cfu mL^{-1}	95% Confidence Interval (Log cfu mL^{-1})			
Bacillus	t.s.a.	1.456×10^6	1.451×10^6	1.461×10^6	6.163	6.162	6.165		
cereus	t.s.a. + ABC	1.475×10^6	1.470×10^6	1.480×10^6	6.169	6.167	6.170		
	t.s.a. + B	1.341×10^6	1.337×10^6	1.346×10^6	6.127	6.126	6.129		
Escherichia	t.s.a.	23.249×10^6	23.159×10^6	23.339×10^6	7.366	7.365	7.368		
coli	t.s.a. + ABC	34.424×10^6	34.290×10^6	34.558×10^6	7.537	7.535	7.539		
	t.s.a. + B	24.324×10^6	24.236×10^6	24.411×10^6	7.386	7.384	7.388		
Staphylococcus	t.s.a.	4.236×10^6	4.221×10^6	4.250×10^6	6.627	6.625	6.628		
aureus	t.s.a. + ABC	4.252×10^6	4.237×10^6	4.268×10^6	6.629	6.627	6.630		
	t.s.a. + B	5.026×10^6	5.005×10^6	5.047×10^6	6.701	6.699	6.703		
Listeria	t.s.a.	26.424×10^6	26.322×10^6	26.526×10^6	7.422	7.420	7.424		
monocytogenes	t.s.a. + ABC	20.999×10^6	20.916×10^6	21.082×10^6	7.322	7.320	7.324		
	t.s.a. + B	28.126×10^6	28.013×10^6	28.238×10^6	7.449	7.447	7.451		
Aspergillus	s.a.	31.500×10^3	30.706×10^3	32.294×10^3	4.498	4.487	4.509		
brasiliensis	s.a. + ABC	29.500×10^3	28.732×10^3	30.268×10^3	4.470	4.458	4.481		
	s.a. + B	33.500×10^3	32.681×10^3	34.319×10^3	4.525	4.514	4.536		
Saccharomyces	s.a.	68.333×10^4	67.358×10^4	69.307×10^4	5.835	5.828	5.841	S	p
cerevisiae	s.a. + ABC	3.000×10^2	2.452×10^2	3.548×10^2	2.477	2.390	2.550	0.12	<0.0005
1st test	s.a. + B	2.500×10^2	2.000×10^2	3.000×10^2	2.398	2.301	2.477	0.17	<0.0005
Saccharomyces	s.a.	19.365×10^5	19.306×10^5	19.425×10^5	6.287	6.286	6.288	S	p
cerevisiae	s.a. + ABC	5.500×10^3	5.265×10^3	5.735×10^3	3.740	3.721	3.758	0.10	<0.0005
2nd test	s.a. + B	25.250×10^3	24.748×10^3	25.752×10^3	4.402	4.394	4.411	0.29	<0.0005

RESULTS AND CONCLUSIONS

At the end of the incubation, a relative count was performed for each microorganism, enabling comparison of the growth on control media with that on media modified by adding the two extracts, ABC and B. The calculations took into account the results obtained from all dilutions and replicas sown with each target microorganism, and applied the formula as per the ISO 8199:2005 standard at point 8.4.2. The 95% confidence interval was also determined for each count, as indicated by the aforementioned ISO standard. Preliminary sterility evaluations were made on media loaded with the extracts ABC and B, and showed that the extracts had negligible bacteria content of 560 cfu mL^{-1} for the ABC extract and 640 cfu mL^{-1} for the B extract. Such values confirm the non-existence of interference from microbial content peculiar to the extracts.

Table 2 reports the results for each target microorganism; values express the growth as colony forming units (cfu) per mL, derived from sowing the stock suspensions on either the undiluted media or the media modified with the two examined yucca extracts. Table 2 also presents the data expressed in log cfu mL^{-1} and the 95% confidence intervals. Data show that yucca extracts had a significant microbial growth reduction effect only on *S. Cerevisiae*, statistically confirmed by *p*-values (<0.0005) as reported in Table 2; it is very clear that the effect of ABC and B extracts doesn't exist against other target microorganisms at the applied 1% concentration (no inhibition is readable on logarithmic scale). Data clearly show the effects of both extracts on *Saccharomyces cerevisiae* compared to the unaltered media; the activity of the two *Yucca filamentosa* L. extracts led to inhibition by more than 3 logarithmic scales. The activity is confirmed by a 2nd test performed ten days later: the results are reported in Table 2.

The Student's *t*-test was used to analyse the data, confirming that a normal distribution could be assumed. This statistical analysis showed that except in the case of *S. cerevisiae*, non-significant differences were detected in the target microorganism counts among the undiluted media and the media modified with extracts ABC and B. The statistically significant effect on *S. cerevisiae* growth was repeatable between the four replicas, in two quantitative tests performed using a basic medium from two different batches.

The present reported, specifically growth inhibitory activity of *Yucca filamentosa* L. extracts against yeast opens a wide range of possible applications for using these extracts in the food sector. Further studies should be performed to investigate the effectiveness of these extracts on other microorganisms with a fermentative metabolism.

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RESEARCH INSTITUTES

Dipartimento di Scienze e Tecnologie Agroalimentari e
Microbiologiche (DI.S.T.A.A.M.),
Università del Molise, Campobasso Fax +39-0874-404652

DeFENS, Department of Food, Environmental
and Nutritional Sciences, Università di Milano, Milano Fax +39-02-50316601

Dipartimento di Valorizzazione e Protezione delle Risorse
Agroforestali (DI.VA.P.R.A.), Sezione Microbiologia
ed Industrie Agrarie, Università di Torino, Grugliasco Fax +39-011-6708549

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