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Editor-in-Chief:
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Dipartimento di Scienze Economico-Estimative e degli Alimenti, Università di Perugia,
S. Costanzo, I-06126 Perugia, Italy
Tel. +39 075 5857910 - Telex 662078 UNIPG - Telefax +39 075 5857939-5852067
E-mail: paolofan@unipg.it

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S. Costanzo, I-06126 Perugia, Italy
Tel. +39 075 5857912 - Telex 662078 UNIPG - Telefax +39 075 5857939-5852067
E-mail: ijfs@unipg.it

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Chiriotti Editori s.a.s., Viale Rimembranza 60, I-10064 Pinerolo, Italy
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PAPER

PRODUCTION OF HIGH PROTEIN BREAD USING EXTRUDED CORN AND SOYBEAN FLOUR BLEND

PRODUZIONE DI PANE AD ALTO CONTENUTO PROTEICO UTILIZZANDO UN IMPASTO ESTRUSO DI MAIS E DI SOIA

D. NOVOTNI*, D. CURIC, D. GABRIC, N. CUKELJ and N. CURKO
Faculty of Food Technology and Biotechnology, University of Zagreb,
Pierottijeva 6, 10000 Zagreb, Croatia
*Corresponding author: Tel. +385 1 4605070, Fax +385 1 4605072,
e-mail: dnovotni2@pbf.hr

ABSTRACT

To obtain a high protein bread, wheat flour was enriched with defatted soybean flour (DSF) by adding a 30 or 40% (w/w flour) extruded blend of corn meal (CM) and DSF prepared in two ratios (w/w): 75% CM/25% DSF (blend 1) and 62.5% CM/37.5% DSF (blend 2). All bread samples had high protein content (>14 g/100 g dry matter), high protein digestibility *in vitro* (>93%) and a significantly improved amino acid composition and lysine score. The best nutritional value and

RIASSUNTO

Per ottenere un pane ad alto contenuto proteico la farina di grano è stata arricchita con farina degrassata di soia (DSF) tramite l'aggiunta del 30 o 40% (p/p farina) di miscela estrusa di mais (CM) e DSF preparata in due rapporti (p/p) 75% CM/25% DSF (miscela 1) e 62,5% CM/37,5% DSF (miscela 2). Tutti i campioni di pane presentavano un alto contenuto proteico (>14 g/100 g di sostanza secca), le proteine erano altamente digeribili *in vitro* (>93%) e miglioravano significati-

- Key words: amino acid, bread, extrusion, lysine score, protein digestibility, soybean -

sensory quality were obtained by adding 30% of blend 2.

vamente la composizione in aminoacidi e il contenuto in lisina. Il valore nutritivo ottimale e la qualità sensoriale sono stati raggiunti con l'aggiunta del 30% dell'impasto.

INTRODUCTION

Cereals are a good source of nutrients and are a basic component of a balanced diet (GATENBY *et al.*, 1995). Cereals are rich in complex carbohydrates, low in fat, and contain a significant amount of proteins. In European countries, up to 25% of the daily energy intake is provided by wheat and wheat products which provide up to 25 g of protein per person per day, i.e. about 26% of the daily protein intake (ROSELL, 2003). Cereal proteins, however, are deficient in some essential amino acids such as lysine, threonine and tryptophan, but contain large quantities of sulphur-containing amino acids (methionine and cysteine). In contrast, legume proteins are a relatively rich source of lysine, tryptophan and threonine (BRESSANI *et al.*, 1990). Soybean is the most important legume due to its high protein content and relatively low price. The consumption of soy foods can have beneficial effects on nutrition and health, such as lowering plasma cholesterol, preventing cancer, diabetes and obesity and protecting against bowel and kidney diseases (SINGH *et al.*, 2008). Isoflavones from soybean improve bone strength and are potent antioxidants and free radical scavengers (ENDRES, 2001). In 1999, the Food and Drug Administration (FDA) authorized the use of health claims on the labels of soy protein-containing foods that say that foods containing 6.5 g of soy protein per serving in a diet low in saturated fat and cholesterol may reduce the risk of coronary heart disease by lowering blood cholesterol levels. The addition of soy proteins to bakery products could therefore improve their nutritional quality.

Consumer demand for high protein bakery products is on the rise for various reasons, including nutrition, health and weight control concerns. It is reported that bread protein enrichment usually goes up to 20% although there have been some attempts to obtain bread with 59% protein (MOHAMED *et al.*, 2006). Defatted soy flour is the most widely used ingredient to obtain high protein bakery products (SHOGREN *et al.*, 1981). Use of 1-3% soy flour (dry flour basis) in bread increases absorption and moisture retention which enhances bakery product freshness (SINGH *et al.*, 2008). However, if a large amount of soy protein is incorporated in the bread, the crumb characteristics are poor and loaf volume and acceptability decrease (DHINGRA and JOOD, 2001; DOXASTAKIS *et al.*, 2002; RIBOTTA *et al.*, 2005). BOOKWALTER *et al.* (1987) reported that loaf volume decreased less in extruded soy products than in products made with non-extruded soy flour.

Extrusion cooking has become more popular in the last decades for various reasons including improved product quality, higher productivity and greater energy efficiency (GUY, 2001). In extrusion cooking cereals are usually used in the form of flour or meal and high temperatures are applied for a short time which helps retain many heat-sensitive

food components (GUY, 2001). During the extrusion of cereals, starch undergoes partial gelatinization that results in improved starch digestibility (COLONNA *et al.*, 1989). Extrusion cooking with soybean has been used mostly to produce textured soy protein products. Almost half of the amino acids in soy protein have carboxyl, amino, or amide groups that are potentially free. These may enter into the formation of isopeptide bonds if sufficient energy is provided by the extrusion process (STANLEY, 1989). Therefore, extrusion cooking needs to be optimized so as not to decrease amino acid availability and to obtain the desirable physical properties. Some of the benefits of extrusion cooking with soy flour are: increased protein digestibility (QIN and KWON, 1996), inactivation of antinutritional factors such as trypsin inhibitors, hemagglutinins, goitrogens, antivitamins and phytates (SNYDER *et al.*, 1987), control of bitter flavour and homogeneous bonding to other food ingredients (BJORCK and ASP, 1983).

The aim of the present study was to produce a high protein, easily digestible bread, with excellent sensory quality. Since the use of soybean flour can have a negative impact on the sensory profile of bread, it was blended with corn meal to give it a pleasant aroma and sweet taste. The blend of corn meal and defatted soybean flour was extruded, milled and then added to the wheat flour to be used in bread making. The nutritional and sensory qualities of the enriched breads were evaluated.

MATERIALS AND METHODS

All raw materials were obtained from a local market: wheat flour (WF; protein 13.5%, fat 0.69%, total dietary fibre 0.91%, moisture 13.04%, ash 0.52%) "Podravka", Croatia; corn meal (CM; protein 8.1%, fat 0.46%, total dietary fibre 0.90%, moisture 11.98%, ash 0.32%) "Mlinar", Croatia; defatted soybean flour (DSF; protein 48.6%, fat 1.52%, total dietary fibre 4.76%, moisture 6.54%, ash 6.51%), "Sojaprotein", Serbia.

Wheat flour rheology was determined according to ICC Standard Methods by amylograph (ICC Standard 126/1), extensograph (ICC Standard 114/1) and farinograph (ICC Standard 115/1). The gluten content of wheat flour was determined in compliance with ICC Standard 106/2. For laboratory baking the improver that contained 10% of amylolitic enzymes, monoglycerides and ascorbic acid (Puratos, Groot-Bijgaarden, Belgium), compressed yeast from Kvasac Ltd. (Lesaffre Group, Prigorje Brdovecko, Croatia), salt and tap water was added.

Corn flour and soybean flour blend extrusion process

The extrusion cooking of corn meal and the corn meal/defatted soy flour blend was carried out in a twin-screw co-rotating extruder (APV Baker Type MPF 50:15, Peterborough, UK). Blends of corn flour and defatted soy flour were prepared in two different ratios: 75% CM/25% DSF (w/w) (blend 1) and 62.5% CM/37.5% DSF (blend 2). The High Temperature Short Time (HTST) extrusion cooking was performed with a constant supply of raw materials 76 kg/h, screw speed 370 rpm and two round dies, diameter 2 mm. Screw configuration was 0.25 D Spacer, 6.0 D Feed Screw, 1.0 D 4x60° Forward Paddles, 4.5 D Single Lead Screw, 2.25 D 9×90° Forward Paddles and 1.0 D Single Lead Screw. After cooling at ambient temperature, extruded blends were ground in a vertical centrifugal mill (Ferkar 5, Velenje, Slovenia) and passed through a 0.45 mm mesh sieve.

Laboratory bread-making

Wheat flour was used as the basic raw material and the extruded blends (1 or

2) were added in quantities of 30 or 40% (w/w total flour). Control bread (sample 0) was made of wheat flour plus extruded corn meal (30%, w/w). Farinograph water (60% in bread 0, 1 and 3; 64% in bread 2 and 4), yeast (2.5%), salt (1.8%) and the improver (1.5%) were added to the flour components in the mixer. The dough was mixed in a laboratory mixer (Diosna, Osnabrück, Germany) at 28°C for 6 min at low speed and for 2 min at high speed. Dough was allowed to rest for 30 min (bulk fermentation); divided into 550 g loaves; rounded and placed in baking pans; they were then finally proofed at 35°C and 80% relative humidity for 50 min. Breads were baked at 210°C for 10 min and then at 170°C for 15 min.

After cooling for 1 h at ambient temperature, breads were weighed and the bread volume was determined with the rapeseed displacement method. Dough yield (Dy)% (dough mass expressed in g, prepared from 100 g of flour) and bread volume yield (By) mL/100 g (bread volume expressed in mL, obtained from 100 g of flour) were calculated according to CURIC *et al.* (2002).

Nutritional value

The chemical analyses of raw materials, extruded products and breads were carried out in triplicate following the ICC Standard Methods. The following parameters were determined: moisture content (ICC Standard 110/1), ash content (ICC Standard 104/1), crude protein content (ICC Standard 105/2), fat content (ICC Standard 136) and crude fibre content (ICC Standard 113). The conversion factor of nitrogen to protein was 6.25 for all samples. The bread energy value was calculated. The energy values used for the macronutrients were: protein, 16 kJ/g; carbohydrates, 16 kJ/g and fat, 36 kJ/g.

Protein content, *in vitro* protein digestibility and amino acid composition were determined first in the composite samples and then separately in the crumb and crust of the baked breads.

The amino acid composition of the raw materials and final products were determined using the AAA 400, INGOS automatic amino acid analyzer (Prague, Czech Republic) according to CSAPO *et al.* (2005). Hydrolysis of the samples was performed in the presence of 6M HCl at 110°C for 24 h under nitrogen atmosphere. Tryptophan was determined after alkaline hydrolysis (CSAPO *et al.*, 2005). The amino acid score was calculated using the IOM/FNB 2002 scoring pattern (INSTITUTE OF MEDICINE, FOOD AND NUTRITION BOARD, 2002).

In vitro protein digestibility (IVPD) was determined with pepsin assay according to the method of MALIWAL (1983) with modifications by FAGEER *et al.* (2004). Samples were digested with 2 mg pepsin/g protein (3200-4500 IU/mg, Sigma, Taufkirchen, Germany) at 37°C for 2 h. The reaction was stopped by adding 10% trichloroacetic acid (TCA). After filtration, the nitrogen content in the TCA-soluble fraction was determined by the Kjeldahl method (ICC Standard 105/2). The protein digestibility of the samples was calculated as a ratio of nitrogen content in the filtrate and that in the sample.

Sensory analysis

The sensory properties of the baked breads were evaluated 1 h after baking by six trained panellists; the 5-point-scale of the Deutschen-Landwirtschafts-Gesellschaft (BIB-Ulmer Spatz, 2006) was used. The points ranging from 1 to 5 were multiplied by the significance factors for each of the relevant properties: shape and appearance, significance factor 1; crust and outer surface, significance factor 2; crumb appearance and porosity, significance factor 4; crumb structure and elasticity, significance factor 4; smell and taste, significance fac-

tor 9. To obtain the mean overall sensory score, the points for each bread attribute were multiplied by the factor of significance, and the sum was then divided by 20.

Statistical analysis

All measurements were made at least in duplicate and the results are expressed as the average values. Analysis of variance (ANOVA) was performed at the probability level of $p = 0.05$. The Statistica 7.1 software (StatSoft Inc., Tulsa, USA) was used to analyse the data.

RESULTS AND DISCUSSION

Laboratory baking

The white wheat flour, containing 28.1% wet gluten, had good rheological properties (Table 1), provided high water absorption, a low degree of softening, high dough energy, a good resistance/extensibility ratio and low amylolytic activity. Dough made of high quality wheat flour in combination with the corn meal and soybean flour extruded blend was easy to process, did not stick to the equipment and fermented readily.

While the between-sample dough yield was not significantly different, bread volume yield between samples was significantly different ($p<0.001$) (Table 2). Bread volume yield decreased as the extrusion blend content in the dough increased. The volume decrease was greatest in the bread in which the highest soybean flour blend (blend 2) was added. The significant negative correlation between soybean protein addition and volume yield ($r = -0.969$) is in agreement with other studies (RIBOTTA *et al.*, 2005).

Nutritional value

The amino acid composition of flours and extruded blends is shown in Table 3. As expected, wheat flour and corn meal were deficient in three essential amino acids: lysine, threonine and tryptophan, while the DSF was not. The extrusion process did not influence the amino acid composition of the corn meal, but it increased the protein digestibility signifi-

Table 1 - Wheat flour rheology parameters.

Rheology measurement	Property	Result		
Farinogram	Water absorption,%	60		
	Development time, min	5.0		
	Stability, min	0.5		
	Degree of softening, FU	55		
Amylogram	The beginning of gelatinization, °C	52.6		
	Temperature of the gelatinization maximum, °C	87.6		
	Glue formation time, min	23.2		
	Maximum viscosity, AU	1380		
Extensogram	Time, min	45'	90'	135'
	Energy, cm^2	97	98	101
	Extensability, mm	143	128	132
	Resistance to extension, EU	405	460	460
	Maximum resistance, EU	515	585	610
	Ratio R/E	2.8	3.6	3.5

Table 2 - Dough yield, volume yield and sensory score of the breads (mean value ± standard deviation).

Parameter	Bread 0[a]	Bread 1[b]	Bread 2[c]	Bread 3[d]	Bread 4[e]
Dough yield, g/100 g flour	174±3	170±2	171±2	170±3	171±4
Bread volume yield, cm^3/100 g flour	429±4*	362±2*	341±3*	336±3*	320±5*
Sensory score	4.0±0.1*	4.3±0.1*	4.4±0.1*	4.4±0.1*	3.7±0.1*

*significantly different at p=0.05.
[a]Bread 0: 30% extruded corn meal (w/w wheat flour); [b]bread 1: 30% extruded blend 1 (75% CM/25% DSF) on wheat flour basis (w/w); [c]bread 2: 40% extruded blend 1 (75% CM/25% DSF) on wheat flour basis (w/w); [d]bread 3: 30% extruded blend 2 (62.5% CM/37.5% DSF) on wheat flour basis (w/w); [e]bread 4: 40% extruded blend 2 (62.5% CM/37.5% DSF) on wheat flour basis (w/w).

Table 3 - Amino acid composition (mg/g protein), protein content and *in vitro* protein digestibility (IVPD) of wheat flour, defatted soybean flour (DSF), corn meal (CM) and extruded materials (mean value ± standard deviation).

Sample	Wheat flour	Defatted soybean flour	Native corn meal	Extruded corn meal	Blend 1[a]	Blend 2[b]
Threonine	23 ±0.15	41±0.18	32±0.17	32±0.12	37±0.11	36±0.16
Valine	41±0.21	44±0.35	42±0.19	45±0.36	45±0.27	44±0.21
Isoleucine	34±0.18	41±0.12	35±0.22	35±0.22	37±0.19	39±0.10
Leucine	64±0.26	72±0.26	123±2.35	126±1.81	97±0.94	88±0.89
Lysine	19±0.10	57±0.18	22±0.14	24±0.16	42±0.24	47±0.17
Histidine	20±0.11	25±0.15	27±0.11	26±0.20	27±0.12	26±0.16
Tryptophan	9±0.03	9±0.09	6±0.02	9±0.07	11±0.16	11±0.14
Phenylalanine	47±0.24	49±0.16	50±0.32	49±0.15	48±0.24	49±0.22
Tyrosine	26±0.17	35±0.14	29±0.16	29±0.10	29±0.14	29±0.13
Methionine	12±0.20	10±0.13	25±0.28	26±0.13	15±0.13	13±0.08
Cystine	12±0.09	12±0.10	19±0.13	22±0.15	10±0.09	10±0.07
Aspartic Acid	36±0.16	116±0.48	56±0.25	61±0.15	93±0.64	101±2.26
Serine	46±0.23	52±0.20	48±0.26	47±0.18	51±0.20	53±0.16
Glutamic Acid	351±2.16	204±1.13	204±2.49	198±2.31	206±2.01	207±2.28
Proline	127±1.01	57±0.37	99±0.73	96±0.46	78±0.42	70±0.39
Glycine	34±0.27	44±0.15	29±0.12	34±0.17	42±0.16	43±0.17
Alanine	29±0.19	44±0.21	75±0.34	77±0.14	59±0.19	54±0.16
Arginine	38±0.19	71±0.29	38±0.18	40±0.13	54±0.21	59±0.18
Total essential amino acids	307	395	410	423	398	392
Total amino acids	968	983	959	976	981	979
Proteins, g/100 g db	13.5±0.1	48.6±0.3	8.1±0.2	8.1±0.3	16.1±0.4	21.8±0.6
IVPD, %	89.1±1.5	95.1±1.8	67.9±1.3	82.9±1.7	93.2±2.0	95.9±1.8

[a]Blend 1: extruded blend of corn meal and defatted soy flour 75: 25 (w/w).
[b]Blend 2: extruded blend of corn meal and defatted soy flour 62.5: 37.5% (w/w).

cantly which is in agreement with findings by PÉREZ-NAVARRETE (2007). Extrusion cooking either partially or completely inactivates trypsin and chemotrypsin inhibitors and other antinutritional factors that form insoluble complexes with proteins making them less apt for proteolysis (ALONSO *et al.*, 1998).

CM and DSF extruded blends had high protein contents and improved lysine, tryptophan and threonine scores due to the soybean protein content. While the major difference between the blends was the protein content value, the amino acid composition and IVPD were not significantly different.

A typical mixed corn bread contains 7% protein (USDA, 2006). The average protein contents, IVPD and amino acid composition of the composite bread samples are shown in Table 4. Total amino acid content per protein (mg/g) was not significantly different between bread samples. The breads with added soybean proteins had a higher total essential amino acid content in comparison with the standard bread sample. Enriched breads were only deficient in lysine, while standard bread

Table 4 - Amino acid composition (mg/g protein), lysine score, protein content and digestibility of the bread samples (mean value ± standard deviation).

Parameter	Bread 0[a]	Bread 1[b]	Bread 2[c]	Bread 3[d]	Bread 4[e]
Threonine	22±0.17	27±0.12	29±0.18	28±0.21	30±0.24
Valine	43±0.19	41±0.23	42±0.27	41±0.24	42±0.18
Isoleucine	37±0.20	34±0.15	35±0.14	35±0.01	36±0.01
Leucine	69±0.29	74±0.17	77±0.31	73±0.01	75±0.01
Lysine	21±0.14	26±0.13	28±0.16	30±0.01	33±0.01
Histidine	21±0.16	22±0.18	23±0.16	22±0.01	23±0.01
Tryptophan	8±0.07	10±0.09	10±0.05	10±0.10	10±0.08
Phenylalanine	49±0.31	46±0.19	46±0.24	47±0.01	47±0.01
Tyrosine	19±0.11	26±0.21	27±0.18	27±0.01	27±0.01
Methionine	15±0.14	13±0.16	13±0.15	12±0.01	12±0.01
Cystine	18±0.12	11±0.07	11±0.07	11±0.09	11±0.05
Aspartic Acid	45±0.23	54±0.36	60±0.33	62±0.40	69±0.28
Serine	45±0.18	47±0.22	47±0.21	48±0.19	49±0.27
Glutamic Acid	264±2.41	297±1.92	282±2.28	287±2.57	271±2.11
Proline	111±1.01	108±1.54	103±0.98	102±1.22	95±1.14
Glycine	36±0.15	36±0.17	37±0.18	37±0.17	38±0.19
Alanine	33±0.13	38±0.24	42±0.21	38±0.25	41±0.19
Arginine	37±0.17	43±0.18	44±0.25	46±0.27	48±0.11
Total essential amino acids	322*	331*	341*	336*	345*
Total amino acids	893*	955	956	955	957
Lysine score	0.41*	0.51*	0.55*	0.59*	0.65*
Proteins, g/100 g db	12.47*±0.41	14.52*±0.28	14.91*±0.22	16.26*±0.34	7.55*±0.29
IVPD[f], %	93.9*±0.9	95.6*±0.7	94.1*±1.1	94.8*±0.8	94.6*±1.4
Energy from protein, %	15.08*	17.8*	19.4*	19.9*	21.7*

*significantly different at p=0.05.
[a]Bread 0: 30% extruded corn meal (w/w wheat flour); [b]bread 1: 30% extruded blend 1 (75% CM/25% DSF) on wheat flour basis (w/w); [c]bread 2: 40% extruded blend 1 (75% CM/25% DSF) on wheat flour basis (w/w); [d]bread 3: 30% extruded blend 2 (62.5% CM/37.5% DSF) on wheat flour basis (w/w); [e]bread 4: 40% extruded blend 2 (62.5% CM/37.5% DSF) on wheat flour basis (w/w).
[f]IVPD *in vitro* protein digestibility.

was also deficient in threonine. There was a significant correlation (r = 0.982) between bread protein content and the lysine score. The IVPD between composite bread samples did not differ significantly but the IVPD in the crust was significantly lower than in the crumb ($p<0.001$) (Fig. 1). The IVPD of the bread crumb decreased as the soy protein was added, but not significantly. The lysine score in the crust was significantly lower than in the crumb ($p<0.001$) (Fig. 1); this was probably due to the presence of a free amino group in the lysine that reacted with reducing sugars by means of Maillard browning reaction that occurs mainly in bread crust (MICHALSKA *et al.*, 2008).

The bread energy value derived from proteins is shown in Table 4. Daily requirements for proteins and essential amino acids vary depending on age and body weight (PANEL ON MACRONUTRIENTS, 2002). The proportion of the daily protein, lysine, tryptophan and threonine requirement for adult women (57 kg) that is satisfied by the intake of the breads produced (100 g) is shown in Fig. 2. According to the Codex Alimentarius (CAC/GL 23-1997, Rev 1-2004), bread 1 could be labelled as a "Source" of protein while breads 2, 3 and 4 could be labelled "High" protein bread because they contained more than 20% of the nutrient reference value per 100 g.

Sensory analysis

In the sensory analysis there was a significant difference ($p = 0.00002$) between bread samples, but not between panellists. The results of the mean overall sensory score with the standard deviations are presented in Table 2. The best sensory quality was found for samples 2 and 3. While all the bread samples were uniformly shaped, the bread volume was lower in all the samples with soy protein addition compared to the control bread (sample 0). As the bread volume decreased, the crumb porosity also decreased. The crumbs were intense yellow and darker in the breads with higher soy protein content. They were soft and sufficiently moist. The crumbs were

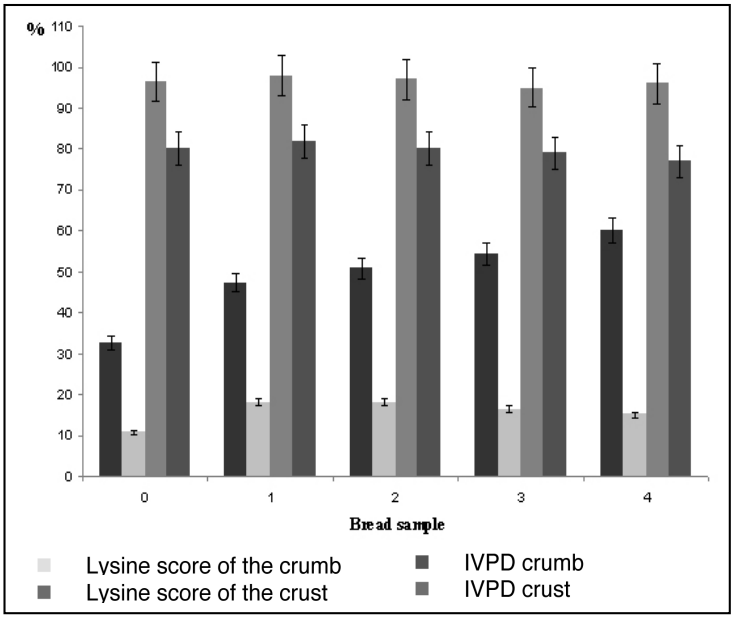


Fig. 1 - Comparison of bread crumb and crust for lysine score (%) and *in vitro* protein digestibility (IVPD, %) of bread samples[a] (mean value ± 95% confidence interval).

[a]Bread 0: 30% extruded corn meal (w/w wheat flour); bread 1: 30% extruded blend 1 (75% CM/25% DSF) on wheat flour basis (w/w); bread 2: 40% extruded blend 1 (75% CM/25% DSF) on wheat flour basis (w/w); bread 3: 30% extruded blend 2 (62.5% CM/37.5% DSF) on wheat flour basis (w/w); bread 4: 40% extruded blend 2 (62.5% CM/37.5% DSF) on wheat flour basis (w/w).

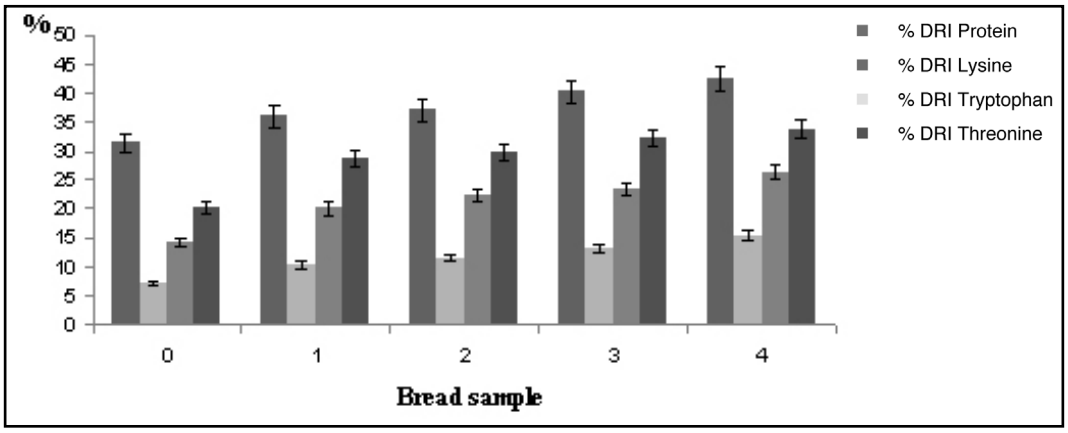


Fig. 2 - Satisfaction of dietary reference intake for protein, lysine, threonine and tryptophan for adult women by consumption of 100 g of bread sample[a] (mean value ± 95% confidence interval).

[a]Bread 0: 30% extruded corn meal (w/w wheat flour); bread 1: 30% extruded blend 1 (75% CM/25% DSF) on wheat flour basis (w/w); bread 2: 40% extruded blend 1 (75% CM/25% DSF) on wheat flour basis (w/w); bread 3: 30% extruded blend 2 (62.5% CM/37.5% DSF) on wheat flour basis (w/w); bread 4: 40% extruded blend 2 (62.5% CM/37.5% DSF) on wheat flour basis (w/w).

less elastic (by finger) in breads with higher soy protein content. The solubility in the mouth was excellent for all the breads. The smell and sweet taste that are typical of corn bread were strongly pronounced in all the bread samples. There was a strong but not dominant beany taste in samples with the higher soy protein content. As the level of soy proteins in the bread increased, a bitter taste became more noticeable in the crust. The beany smell diminished within three hours after baking. The statistical analysis showed that an increase in protein content of more than 16.3% by adding soybean proteins had a strong negative influence on bread sensory quality (Fig. 3).

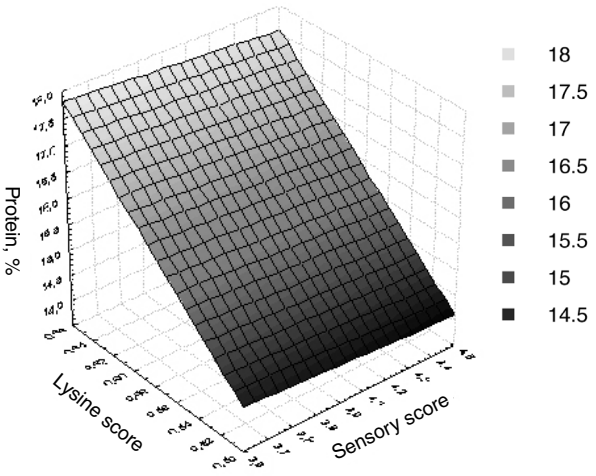


Fig. 3 - Surface plot of bread protein content (%), lysine score and sensory score.

CONCLUSION

The incorporation of 7.5-15% (w/w) defatted soybean flour in the form of an extruded blend with corn meal significantly increased the protein content and improved the amino acid composition of bread. Protein digestibility *in vitro* was high for all the bread samples. The optimal bread sensory characteristics, protein content, essential amino acid composition and IVPD were observed when 30% (w/w wheat flour) extruded blend 2 containing 37.5% of defatted soybean flour and 62.5% corn meal was incorporated into the dough formulation.

The results of this research provide a practical guide for bread manufacturers who want to produce protein-enriched bread. The extruded corn-soybean blends are also suitable for gluten-free bread protein enrichment (CURIC *et al.*, 2007).

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PAPER

IDENTIFICATION AND BIOPRESERVATION POTENTIAL OF *ENTEROCOCCUS* SPP. ISOLATED FROM FULLY RIPENED GRAVIERA, A TRADITIONAL HARD GREEK CHEESE

IDENTIFICAZIONE E POTENZIALE BIOCONSERVAZIONE DI *ENTEROCOCCUS* SPP. ISOLATO DA GRAVIERA STAGIONATA, UN FORMAGGIO A PASTA DURA TRADIZIONALE GRECO

E. GIANNOU[1], A. LIANOU, A. KAKOURI, A. KALLIMANIS[1], C. DRAINAS[1] and J. SAMELIS*
National Agricultural Research Foundation, Dairy Research Institute, Katsikas, 45221 Ioannina, Greece
[1] Laboratory of Biochemistry, Department of Chemistry, University of Ioannina, 45110 Ioannina, Greece
*Corresponding author: Tel. +302 651 094789, Fax +302 651 092523, e-mail: jsam@otenet.gr

ABSTRACT

A total of 86 enterococcal isolates from fully ripened Graviera, a traditional hard Greek cheese made from thermized (60°C; 30 sec) ewe/goat (90:10) milk without the use of commercial starters, were identified using phenotypic traits and sequencing of the 16S rDNA gene of representative strains. The antimicrobial potential of the isolates was also assessed. The en-

RIASSUNTO

Utilizzando i tratti fenotipici e il sequenziamento del gene 16S rDNA di ceppi rappresentativi, sono stati identificati un totale di 86 isolati enterococchici da formaggio Graviera ben stagionato, un formaggio tradizionale greco prodotto con latte di pecora/capra (90:10) termizzato (60°C; 30 sec) senza l'utilizzo di starter commerciali. È stato inoltre valutato il potenziale antimi-

- Key words: *Enterococcus*, enterocins, Graviera cheese, *Listeria monocytogenes* -

terococci levels in the ripened cheeses were 7.8±0.2 log CFU/g. *Enterococcus faecium* (41 isolates) and *E. durans* (35 isolates) were the most prevalent species which share a 99% 16S rDNA homology. Five enterocin-producing (Ent$^+$) *E. faecium* isolates inhibited *in vitro* growth of *L. monocytogenes*. None of the *E. durans* strains or other isolates produced enterocins. These results suggest that some Ent$^+$ *E. faecium* strains may contribute to *Listeria* inhibition in Graviera cheese during ripening. However, before these isolates can be used as bio-protective adjuncts, their overall safety must be assessed in order to ensure a GRAS status at the *E. faecium* strain level.

crobico degli isolati. Il livello di enterococchi nei formaggi stagionati era di 7,8±0,2 log UFC/g. Le specie prevalenti erano l'*Enterococcus faecium* (41 isolati) e l'*E. durans* (35 isolati) che mostravano il 99% di omologia del gene 16S rDNA. Cinque isolati di *E. faecium* producenti enterocina (Ent$^+$) inibivano, *in vitro*, la crescita di *L. monocytogenes*. Nessuno dei ceppi di *E. durans* o degli altri isolate produceva enterocine. Tali risultati suggeriscono che alcuni ceppi di Ent$^+$ *E. faecium* possono contribuire all'inibizione della *Listeria* nel formaggio Graviera durante la stagionatura. Tuttavia, prima che tali isolati possano essere utilizzati come aggiunte bioprotettive, dovrebbe essere valutata la loro sicurezza complessiva allo scopo di assicurare al ceppo di *E. faecium* lo stato GRAS.

INTRODUCTION

Enterococci are commonly associated with traditional cheeses of the Mediterranean area. Their levels range from 10^4 to 10^6 CFU/g in cheese curds and from 10^5 to 10^8 CFU/g in fully ripened cheeses, with *E. faecium* and *E. faecalis* being the dominant species. Enterococci may be predominant in ripened cheeses, such as Manchego, Mozzarella, Cebreiro, Comte and Domiati (as reviewed by FOULQUIE MORENO *et al.*, 2006), and particularly in traditional hard Greek cheeses produced without starters from raw (PRODROMOU *et al.*, 2001) or pasteurized (LITOPOULOUTZANETAKI, 1990) milk. Enterococci are quite heat resistant and have a growth temperature range of 10° to 45°C with an optimum of 37°C. They can prevail in thermized and even pasteurized milk as well as during cooking of curd and ripening of hard cheese. There are still some safety concerns and scientific controversy regarding the risks versus benefits in cheeses with a prevalence of enterococci (GIRAFFA, 2003; FOULQUIE MORENO *et al.*, 2006). Research has shown that enterococcal strains selected on the basis of having desirable technological and/or antagonistic properties but lacking pathogenic traits can be used effectively and safely as cheese adjuncts (NUNEZ *et al.*, 1997; CENTENO *et al.*, 1999; SARANTINOPOULOS *et al.*, 2001, 2002; GIRAFFA, 2003; FOULQUIE MORENO *et al.*, 2003, 2006; FRANZ *et al.*, 2003; BEN OMAR *et al.*, 2004; PIMENTEL *et al.*, 2007).

Gravieras are among the most popular Greek cheese varieties and are traditionally produced in many regions of Greece. Three varieties of Graviera have PDO status; two are produced and named after the islands of Crete (Kriti) and Naxos and the third is produced in the mountain area of Agrafa (ANONY-

MOUS, 2004). In general, Gravieras are hard cheeses with a non-edible, whitish to yellowish rind, which is visibly free from surface flora due to brining (2-5 days in 18-20 Be brine) after moulding, followed by dry salting during ripening and brine washing prior to packaging and distribution. The cheese core should have symmetric, well-developed, evenly distributed "shiny" eyes with no holes or cracks. The cheese should have a pleasant, mild sweet taste and rich aroma. Graviera cheeses are produced from ewe milk only, or ewe milk mixed with 10-30% goat milk depending on the type of cheese, with the exception of PDO Graviera Naxos cheese which is produced from cow milk. The milk is processed raw, pasteurized, or thermized. Graviera cheeses have a maximum water content of 38-40% and a minimum fat content in dry matter of 40%. The addition of rennet, enzymes, edible sea salt, calcium chloride (max 20 g/100 kg) and lactic acid bacteria (LAB) starters in the milk is allowed, but the use of condensed milk, milk powder, milk proteins, casein salts, colourings, and preservatives is prohibited. To enhance whey loss during cheese-making, milk curdling is initiated at 32°-36°C, the curd is then cut into cubes and "cooked" at 48°-52°C before moulding. Cheeses are pressed, drained, brined, and ripened at 12°-18°C and 85-95% RH for at least 3 months before consumption (ANONYMOUS, 2004).

Graviera cheeses are the finest type of traditional hard Greek cheeses. They are very popular and of notable economic importance. Research data on their microbiology however are scarce. The few studies found in the literature describe the microbial composition of the Kritis Graviera PDO cheese (KANDARAKIS *et al.*, 1998; MOATSOU *et al.*, 2004). Studies have recently been undertaken to evaluate and potentially improve the microbiological quality, safety, ecology and traditional ripening programs of Graviera cheese. A preliminary survey (SAMELIS, unpublished data) revealed the occurrence of high (10^7-10^8 CFU/g) enterococcal populations in naturally fermented and ripened Graviera cheeses that were ready for retail distribution. The purpose of the present study was to taxonomically characterise the enterococcal microbiota isolated from these cheeses, and to assess their bio-preservation potential by screening for and evaluating the enterococcal strains that show *in vitro* antibacterial activities.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The enterococcal strains used in this study were isolated from four batches of fully-ripened (90-day-old) Graviera cheese produced in a local dairy plant (Pappas Bros., Filippiada, Epirus) specialised in the production of traditional hard cheeses. All batches were manufactured from thermized (60°C; 30 sec) ewe/goat (90:10) milk without the use of commercial starters. Their natural lactic acid bacteria (LAB) flora was first enumerated; the methods and media described by BONTINIS *et al.* (2008) were used to enumerate the total viable mesophilic or thermophilic LAB populations, including enterococci. Unless otherwise stated, all media and reagents were purchased from Merck (Darmstadt, Germany). For each cheese, all of the colonies derived from one plate of the highest sample dilution on the LAB-selective agar media were sub-cultured in 10 mL MRS broth at 30° or 37°C, and then streaked on kanamycin aesculin azide (KAA) agar to detect which of the isolates were presumptive enterococci. These isolates were then checked for purity by streaking on both KAA and MRS agar plates, and maintained in MRS broth with 20% glycerol at -30°C until further testing. Before experimental use, each

isolate was grown twice in MRS broth at 37°C for 24 h.

Biochemical identification of enterococcal isolates

Isolates were first checked to determine those that were Gram-positive, catalase-negative cocci; these were then subjected to additional tests as reported in Table 1. All tests were done in duplicate for each isolate, according to SAMELIS *et al.* (1994a) and SAMELIS and KAKOURI (2007). Assigning the enterococcal isolates to their respective species was based on the criteria of FACKLAM and COLLINS (1989), particularly the key sugar fermentation reactions that differentiate *E. faecalis*, *E. faecium*, *E. durans* and *E. hirae* (FACKLAM and ELLIOTT, 1995).

SDS-PAGE of whole cell proteins

In order to facilitate the phenotypic differentiation of the most prevalent enterococcal isolates assigned to the *E. faecium* genomic group (see Results), ten representative isolates of the different phenotypic groups were subjected to

Table 1 - Biochemical identification of the species based on sugar fermentation patterns and distribution of 86 *Enterococcus*[a] isolates from four batches of fully-ripened Graviera cheese.

Group	1	2	3	4
No. isolates	41	35	6	4
Fermentation of:				
L- Arabinose	+	-	-	-
Cellobiose	+	+	+	+
Galactose	+	+	+	+
Lactose	+	+	+	+
Maltose	+	+	+	+
Mannitol	+	-	+	+
Melibiose	37/41	19/35	5/6	-
Raffinose	(8/41)	-	-	-
Ribose	+	+	+	+
Sorbitol	18/41	-	+	-
Sucrose	25/41	2/35	+	-
Trehalose	38/41	17/35	+	-
Xylose	-	-	-	-
Biochemical identification	*E. faecium*	*E. durans*	*E. faecalis*	Atypical *E. durans*
Distribution in cheese				
Batch 1	9	16	0	0
Batch 2	8	13	0	2
Batch 3	12	2	3	2
Batch 4	12	4	3	0

Symbols: +, all isolates were positive; -, all isolates were negative; 37/41, 37 out of the 41 isolates were positive; ( ), weak/delayed reaction.

[a]All isolates were Gram-positive, catalase-negative cocci which showed good growth on kanamycin aesculin azide (KAA) agar, did not produce gas from glucose, and were positive to the following tests: ammonia production from arginine, growth at 15° and 45°C, growth in 4 and 6.5% salt, growth at pH 9.6, growth with strong reduction of 0.1% methylene blue milk, survival at 60°C for 30 min.

SDS-PAGE of whole cell proteins, as described by SAMELIS *et al.* (1995). The isolates were selected randomly according to the total number of isolates in each phenotypic group of *E. faecium* or its related phylogenetic species (Table 1). Five isolates (KE61, KE67, KE86, KE112 and KE120) made up group 1, four isolates (KE66, KE91, KE94 and KE110) made up group 2 and one isolate (KE98) made up group 4. Cell-free extracts were prepared by mixing 0.8 mL of concentrated (100-fold) cell suspensions of each strain washed in 0.9% saline with 1.5 mL zirconium beads (0.15 mm) and 0.4 mL Tris HCl buffer (pH 8.0). The mixture was then beaten in a MiniBead-beater (TM Biospec Products, Bartlesville, Oklahoma, USA). The cell debris suspension was centrifuged (12,000 g, 12 min at 4°C), and 20 μL portions of the supernatant fluid were loaded on the gels. After gel de-staining, the profiles of the ten isolates were compared macroscopically to detect the main differences in the protein bands.

Molecular identification of enterococcal isolates

Three of the representative isolates, KE98, KE110 and KE112, each representing one phenotypic group within the *E. faecium* genomic group of species in Table 1 (see Results), were subjected to 16S rDNA gene sequencing to validate their biochemical identification. The isolates were grown in MRS broth at 37°C for 3 h, the cells were then collected by centrifugation (6,000 g, 15 min at 25°C). Cellular DNA was extracted according to JGI protocol using CTAB and then purified (POSPIECH and NEUMANN, 1995). Mixtures of the cellular DNA extracts, Taq polymerase (4u/μL, Finnzymes), Primer 16SF (5'- AAG GAG GTG ATC CAG CC-3', Tm=46.2) 10 pmol, Primer 16SR (5'- AGA GTT TGA TCC TGG CTC AG-3', Tm=48.3) 10 pmol (STACKENBRANDT and LIESACK, 1993), dNTPs 10 mM, $MgCl_2$ 50 mM and Buffer 10X were prepared and subjected to thermal cycling. The thermal cycling program was as follows: 5 min denaturating at 95°C, 29 cycles of 1 min at 95°C, 3 min annealing at 60°C, 3 min at 72°C with a final extension of 10 min at 72°C, and cooling at 4°C. Nucleospin Extract II protocol was used to purify the PCR products, which were sequenced (Macrogen, Seoul, Korea). The isolates were compared with other aligned sequences and a phylogenetic identification tree was constructed using MEGA 3.1 software (KUMAR *et al.*, 2004).

Assessment of antibacterial activity

Enterococcal isolates were screened for antilisterial activity by a well diffusion assay (SAMELIS *et al.*, 1994b) that was slightly modified as follows: all isolates were grown in MRS broth at 30°C; all indicator strains (Table 2) were grown in Brain Heart Infusion (BHI) broth (LAB M, Bury, Lancashire, UK) at 30°C for 18-22 h; TSAYE plates inoculated (0.2% v/v) with each of the indicator strains were used for the assay; on each plate the 6-mm diameter wells were filled with 50-60 μL of filter-sterilised MRS culture supernatants of each isolate. Initially, all isolates were tested against two indicator strains of *Listeria monocytogenes*, Scott A and N-7143. Only the isolates which gave MRS culture filtrates that retained antilisterial activity after their pH adjustment to 6.0-6.5 were further tested against the additional *Listeria* spp. and *S. aureus* indicator strains (Table 2).

Bacteriocin detection and activity

To ensure that the observed inhibition zones around the wells were due to proteinaceous compounds, such as bacteriocins, active pH-adjusted culture filtrates of the inhibitory isolates were treated with (i) 300 units/mL catalase (Fluka, Sigma Aldrich Chemie GmbH, Steinheim, Germany) to eliminate a po-

Table 2 - Antimicrobial activity of the pH-adjusted culture filtrates of selected *Enterococcus faecium* isolates from Graviera cheese as evaluated against *Listeria* spp. and *Staphylococcus aureus* strains by well diffusion assays.

Indicators[a]	Producers[b]			
	KE64	KE82	KE118	K295
L. monocytogenes Scott A (clinical isolate, serotype 4b)	+ (6.9)	+ (7.5)	+ (7.5)	+ (7.1)
L. monocytogenes N-7143 (meat isolate, serotype 3a)	+ (7.3)	+ (8.0)	+ (7.3)	+ (7.5)
L. monocytogenes N-7144 (meat isolate, serotype 1/2b)	+ (3.3)	+ (4.0)	+ (3.5)	+ (3.8)
L. monocytogenes N-7155 (meat isolate, serotype 1/2b)	+ (7.3)	+ (7.8)	+ (7.8)	+ (7.0)
L. monocytogenes No. 10 (non-virulent meat isolate, serotype 4ab)	+ (7.0)	+ (7.5)	+ (6.8)	+ (6.5)
L. monocytogenes ISS G79 (soft cheese isolate, serotype 1/2b)	+ (5.0)	+ (6.0)	+ (6.0)	+ (5.5)
L. monocytogenes ISS G185 (blue cheese isolate, serotype 1/2a)	+ (5.5)	+ (6.3)	+ (6.0)	+ (6.3)
L. monocytogenes WSLC 1294 (goat cheese isolate, serotype 3b)	+ (5.0)	+ (6.3)	+ (6.0)	+ (6.3)
L. monocytogenes WSLC 1482 (soft cheese isolate, serotype 1/2b)	+ (5.0)	+ (5.8)	+ (5.8)	+ (5.8)
L. innocua LMG 11387	+ (6.8)	+ (7.0)	+ (6.8)	+ (6.8)
S. aureus ISS 465 (clinical isolate, enterotoxin type A)	-	-	-	-
S. aureus ISS 464 (cow cheese isolate, enterotoxin type A)	-	-	-	-
S. aureus ISS 23 (sheep milk isolate, enterotoxin type C)	-	-	-	-

[a]*L. monocytogenes* strain Scott A and strains of meat origin were obtained from Colorado State University, USA (Dr. John N. Sofos); *L. monocytogenes* strains ISS G79 and ISS G185 and *S. aureus* strains were kindly provided by Dr. Paolo Aureli (Instituto Superiore di Sanita, Rome, Italy); *L. monocytogenes* strains WSLC 1294 and WSLC 1482 were kindly provided by Dr. Klaus Neuhaus (Technical University of Munchen, Germany).
[b]Presence (+) or absence (-) of zones of inhibition; numbers in parentheses correspond to the width of the inhibition zones (when observed) in mm, which represents the radius of inhibition calculated as follows: diameter of the total zone minus the diameter of the well divided by 2.

tential inhibitory effect of hydrogen peroxide, (ii) proteinase K from *Tritirachium album* (Sigma, Sigma Aldrich Chemie GmbH, Steinheim, Germany), (iii) α-chymotrypsin, type II from bovine pancreas (Sigma), (iv) trypsin from porcine pancreas (Sigma), and (v) lipase from *Candida rugosa* (Sigma), each at a final concentration of 1 mg/mL. Samples with and without added enzymes were filter-sterilised and incubated at 37°C for 3 and 24 h; residual inhibitory activity was determined by well diffusion assay against *L. monocytogenes* N-7143, which was shown to be the most sensitive indicator strain.

Bacteriocin excretion and activity were further evaluated in broth challenge experiments. Each of the most active enterococcal isolates was co-inoculated in 50 mL of MRS broth with a cocktail of *L. monocytogenes* strains, both at a level of ca. 5 log CFU/mL. Two different cocktails were challenged against each active isolate in separate flasks. One was a 5-strain cocktail consisting of the four meat strains plus the clinical strain Scott A, and the other was a 4-strain cocktail consisting of the dairy strains (Table 2). Flasks inoculated with the cocktails only, or inoculated with the cocktails and the non-bacteriocin-producing enterococcal isolates showing high phenotypic similarity with the bacteriocin-producing isolates being tested, served as controls. Flasks were incubated at 30°C and sampled at 0, 8 and 24 h post-inoculation. Populations of *Listeria monocytogenes* were enumerated on Agar Listeria Ottaviani Agosti (ALOA) (AES Laboratories, Bruz, France), incubated at 37°C for 24-48 h, while the enterococci were enumerated on KAA agar. *In situ* bacteriocin excretion was detected at 8 and 24

h by well assay testing of pH-adjusted filtrates against *L. monocytogenes* Scott A and N-7143.

Challenge experiments were carried out in duplicate for each isolate against each of the cocktails. Microbial counts were converted to log CFU/mL and subjected to analysis of variance (SPSS for Windows, version 15.0; Microsoft; Chicago, IL, USA; 2007). Means and standard deviations were calculated, and the mean significant differences were determined at the $P<0.05$ level.

RESULTS AND DISCUSSION

The mean population value of enterococci, selectively enumerated on KAA agar, in the four batches of Graviera cheese was 7.8±0.2 log CFU/g, while the corresponding mean population value of the total mesophilic bacteria (TVC), enumerated on CASO agar, was 8.7±0.3 log CFU/g. Population values of mesophilic lactic acid bacteria (LAB) (MRS agar; 30°C), thermophilic LAB (MRS agar; 45°C), mesophilic cocci (M-17 agar; 22°C) and thermophilic cocci (M-17 agar; 42°C) were 8.5±0.3, 8.0±0.3, 8.1±0.1 and 7.8±0.3 log CFU/g, respectively. The enterococci comprised a significant part of the LAB populations in the ripened cheeses, considering that the LAB species selectivity of the MRS and M-17 agars is low, and that the populations on KAA agar were similar to those on the above media, especially when incubated at 42° or 45°C. The pH of the fully ripened Graviera cheese samples was 5.6±0.2.

A total of 86 Gram-positive, catalase-negative coccoid isolates from the MRS, M-17 and KAA plates of the highest sample dilution showed abundant growth on KAA agar, and had the main phenotypic characteristics of *Enterococcus* (footnoted in Table 1). These isolates were collected from all four Graviera batches: 25 from batch 1, 23 from batch 2, 19 from batch 3, and 19 from batch 4. They were also collected from all LAB enumeration media. All of the isolates from the MRS agar plates at 45°C, most isolates from M-17 plates at 42°C, and some isolates from MRS at 30°C and M-17 at 22°C were assigned to *Enterococcus* together with all of the isolates from the KAA agar. The 86 enterococcal isolates were then grouped on the basis of their sugar fermentation patterns (Table 1). Based on their reactions with four key sugars (arabinose, mannitol, sorbitol and raffinose) (FACKLAM and ELLIOTT, 1995), four groups were formed, three of which were clearly assigned to known enterococcal species. The majority of the isolates (41) were identified as *E. faecium* (group 1: arabinose +; mannitol +, sorbitol- and raffinose-variable) followed by *E. durans* in group 2 (35 isolates) (negative with all four key sugars), and *E. faecalis* in group 3 (6 isolates) (arabinose -, mannitol +, sorbitol +, raffinose -). Identification of the isolates of group 4 was doubtful (arabinose -, mannitol +, sorbitol -, raffinose -, sucrose -); they were intermediate to the other three groups. FACKLAM and COLLINS (1989) reported a few atypical mannitol-positive strains in the *Enterococcus* Group III which included *E. durans*, *E. hirae* and asaccharolytic variants of *E. faecalis*. The group 4 isolates did not ferment raffinose and sucrose, and thus, were not *E. hirae*. They were considered to be atypical *E. durans* isolates that require molecular identification. In general, it is difficult to differentiate between the *Enterococcus* spp. using only phenotypic criteria because sugar fermentation patterns and other biochemical traits include many variable reactions. So strains in phenotypic enterococcal groups are often intermixed and are not always in accord with their PCR-based genotypic identification (FRANZ *et al.*, 2003, VELASCO *et al.*, 2004). Variable reactions even occur within intra-species genomic groups, such as *E. faecium* (VANCANNEYT *et al.*, 2002).

The 16S rRNA gene sequencing anal-

ysis is one of the most powerful tools for determining the phylogenetic relationship of LAB species, including enterococci (FRANZ *et al.*, 2003; FOULQUIE MORENO *et al.*, 2006), and thus, it is often used in cheese ecology studies (MOREA *et al.*, 1998; RANDAZZO *et al.*, 2002; FORTINA *et al.*, 2003; DOLCI *et al.*, 2008). Based on 16S rRNA data, enterococci are divided into 8 groups, with *E. faecium* and *E. faecalis* being the most abundant species in two of the groups, which are well separated on the 16S rRNA-based phylogenetic tree (FRANZ *et al.*, 2003). *E. durans* belongs to the genomic group of *E. faecium* together with *E. hirae*, *E. mundtii* and a few other species, and thus, the 16S rRNA gene sequence similarity between *E. faecium* and *E. durans* can be as high as 99%. Oftentimes these closely related LAB species can not be further distinguished by other identification tools, such as the SDS-PAGE of whole-cell protein profiles (PIRAINO *et al.*, 2006). Since most (93%) of the Graviera cheese isolates were phenotypically assigned to the *E. faecium/durans* genomic group, the SDS-PAGE and 16S rRNA gene sequencing analyses were applied to random representatives of groups 1, 2 and 4 (Table 1). The whole-cell protein profiles of the representative *E. faecium* (group 1) isolates (KE61, KE67, KE86, KE112 and KE120), *E. durans* (group 2) isolates (KE66, KE91, KE94 and KE110) and the atypical *E. durans* KE98 (group 4) isolate were very similar; there were only slight differences in the location of their cell protein bands (data not shown). Next it was confirmed that the representative isolates KE112 (group 1) and KE110 (group 2) were highly related, and share a 99% 16S rRNA homology with *E. durans* and *E. faecium* reference strains (Fig. 1). In addition, the atypical isolate

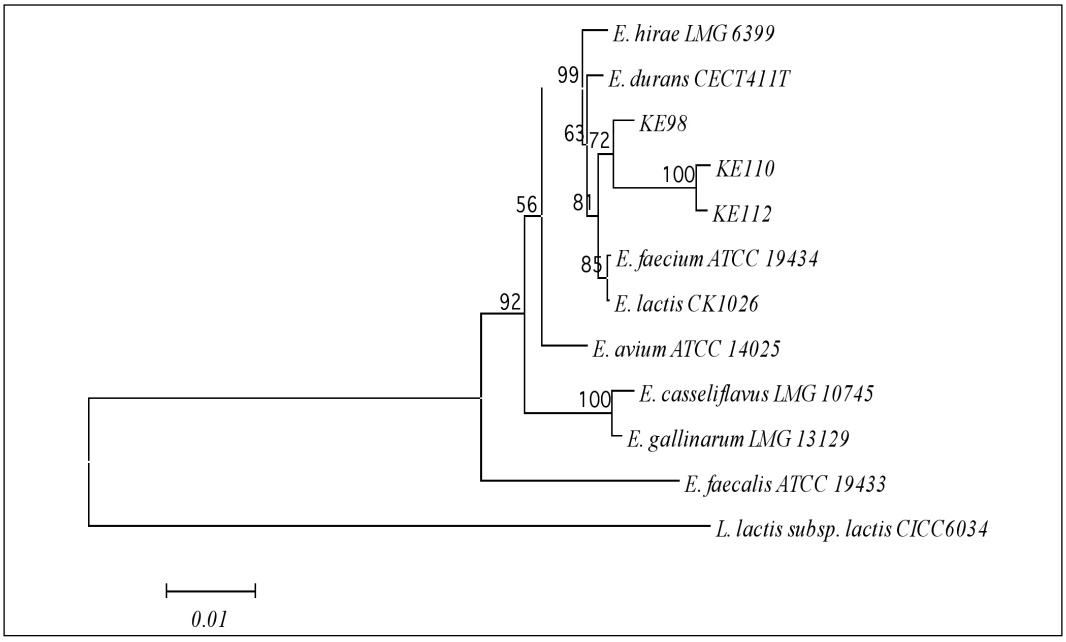


Fig. 1 - 16S rRNA-based tree showing the relationship of *Enterococcus* isolates KE98, KE110 and KE112 from Graviera cheese with reference strains of *Enterococcus* spp. that are commonly associated with dairy foods. Numbers show the percentage sequence similarities of the different species on the branches. The scale bar indicates the number of substitutions per nucleotide position. *Lactococcus lactis* subsp. *lactis* CICC6034 was used as an out-group reference strain.

KE98 was clearly distant from *E. faecalis*, but clustered close to the *E. durans* reference strain (Fig. 1). Thus, the atypical mannitol-positive isolates of group 4 belong to the *E. faecium* group, probably *E. durans*, in accord with their sugar pattern-based identification (Table 1). Given the high 16S rRNA similarity of these two species, the sugar fermentation patterns were more valuable than SDS-PAGE profiles for distinguishing the *E. faecium* isolates from those of *E. durans* in Graviera cheese. Additional RAPD-PCR, AFLP and PFGE analyses (DE ANGELIS *et al.*, 2001; MOSCHETTI *et al.*, 2001; VANCANNEYT *et al.*, 2002) are needed to differentiate the enterococci strains in Graviera cheese at the species level and below.

The distribution of the enterococcal isolates in each of the fully ripened Graviera batches is shown (Table 1). Isolates belonging to the two prevalent species, *E. faecium* and *E. durans*, were isolated from all four batches; *E. durans* prevailed in batches 1 and 2, whereas *E. faecium* was more prevalent in batches 3 and 4. Interestingly, *E. faecalis* was not prevalent in any of the batches, and was not even isolated in batches 1 and 2 (Table 1).

With regard to antilisterial activity, five promising enterococcal isolates were detected by screening: KE64, KE67 and KE82 from batch 1, KE118 from batch 2 and K295 from batch 3. All five isolates belonged to *E. faecium* (group 1) (Table 1). Since isolates KE64 and KE67 came from the same batch and had identical phenotypic profiles, they were considered replicates of potentially the same strain. Thus, only isolate KE64 was studied further. All *L. monocytogenes* indicator strains, as well as *L. innocua* LMG 11387, were inhibited by the pH-adjusted culture filtrates of the four *E. faecium* isolates, with strain N-7144 being the most resistant. In contrast, none of the *S. aureus* indicator strains were inhibited by any of the four isolates (Table 2). While activity against *L. monocytogenes* N-7143 was maintained when pH-adjusted culture filtrates of all the selected isolates were treated with lipase or catalase, all activity was lost after treatment with proteinase K, trypsin, or α-chymotrypsin at 37°C for 3 or 24 h. Thus, the antiisterial activity of *E. faecium* isolates is not due to acid, but is attributable to proteinaceous compounds, probably enterococcal bacteriocins, known as enterocins (FOULQUIE MORENO *et al.*, 2006). Conversely, no *E. durans* (groups 2 and 3) or *E. faecalis* (group 4) isolates demonstrated any antilisterial potential in the well diffusion assays.

The four enterocin-producing (Ent^+) *E. faecium* isolates showed antilisterial activity in broth challenge experiments against two different cocktails of *L. monocytogenes* strains (Table 3). All of the Ent^+ isolates (KE64, KE82, KE118 and K295) exerted a strong listeriostatic effect ($P>0.05$). As a result, the *in vitro* growth in MRS broth of either cocktail of *L. monocytogenes* was inhibited ($P<0.05$) in the presence of all of the Ent^+ isolates in contrast to the prolific growth of the pathogen in their absence. On the other hand, *E. faecium* KE85, an enterocin-negative (Ent^-) co-isolate of the Ent^+ strain KE82, did not prevent *L. monocytogenes* growth, but caused a much slower, restricted growth ($P<0.05$) compared to the pure control (LM1 and LM2) cultures of the pathogen (Table 3). When cell-free, pH-adjusted supernatants of all the above cultures were tested for antilisterial activity by well-assay, inhibition zones were always detected in the cultures in which *L. monocytogenes* was co-inoculated with the Ent^+ *E. faecium* isolates. In contrast, inhibition zones were not observed in the pure cultures or in cultures in which the pathogen was mixed with isolate KE85. The inhibition zones were usually smaller at 8 than at 24 h (data not shown). These results indicated that *in situ* enterocin was secreted in amounts that were sufficient to cause

Table 3 - *In vitro* growth inhibition of the populations (log CFU/mL) of two different cocktails (LM1 and LM2)[a] of *Listeria monocytogenes* strains by enterocin-producing (Ent$^+$) *Enterococcus faecium* isolates from fully ripened Graviera cheese when in co-culture in MRS broth at 30°C.

Listeria monocytogenes cocktail	*E. faecium* isolate in co-culture	Incubation time (h)		
		0	8	24
LM1	None	5.45 (0.01) Ca	6.71 (0.05) Bb	9.34 (0.01) Ac
LM1	KE85 Ent$^-$ [b]	5.52 (0.06) Ba	6.60 (0.01) Ab	6.91 (0.11) Ab
LM1	KE64 Ent$^+$	5.48 (0.03) Aa	5.22 (0.09) Aa	5.14 (0.25) Aa
LM1	KE82 Ent$^+$	5.45 (0.10) Aa	5.32 (0.11) Aa	5.15 (0.13) Aa
LM1	KE118 Ent$^+$	5.40 (0.06) Aa	5.20 (0.16) Aa	5.35 (0.06) Aa
LM1	K295 Ent$^+$	5.43 (0.02) Aa	5.22 (0.01) Aa	5.30 (0.01) Aa
LM2	None	5.35 (0.06) Ca	7.12 (0.06) Bb	9.11 (0.01) Ac
LM2	KE85 Ent$^-$[b]	5.47 (0.03) Ba	6.87 (0.18) Ab	6.64 (0.05) Ab
LM2	KE64 Ent$^+$	5.33 (0.19) Aa	5.21 (0.56) Aa	5.09 (0.10) Aa
LM2	KE82 Ent$^+$	5.41 (0.05) Aa	5.41 (0.64) Aa	5.14 (0.24) Aa
LM2	KE118 Ent$^+$	5.37 (0.11) Aa	5.32 (0.71) Aa	5.34 (0.46) Aa
LM2	K295 Ent$^+$	5.27 (0.14) Aa	5.38 (0.66) Aa	5.32 (0.45) Aa

Values (log CFU/mL) are the means of duplicate experiments; SD values are in brackets.
Means in the same row bearing different capital letters are significantly different (P<0.05).
Means in the same column bearing different lowercase letters are significantly different (P<0.05).
[a]LM1 was a 5-strain cocktail consisting of four meat strains (N-7143, N-7144, N-7155, N°10) plus the clinical strain Scott A; LM2 was a 4-strain cocktail consisting of the dairy strains ISS G79, ISS G185, WSLC 1294 and WSLC 1482; strains origin and serotypes are given in Table 2.
[b]KE85 was an enterocin-negative (Ent$^-$) *E. faecium* isolate that originated from the same Graviera cheese sample and shared a high phenotypic similarity with the Ent$^+$ *E. faecium* KE82 isolate.

inhibition already at 8 h and increased after 24 h of incubation.

Enterocin production is common and is one of the most important functions of enterococci in cheese (GIRAFFA, 2003). Interestingly, the vast majority of known enterocins are produced by either *E. faecium* or *E. faecalis* strains. There have been sporadic reports on enterocin production by other species, such as *E. mundtii* (reviewed by FOULQUIE MORENO *et al.*, 2006) and more recently *E. durans* (BATDORJ *et al.*, 2006). Consistent with this natural enterococcal trend, only Ent$^+$ *E. faecium* and no Ent$^+$ *E. durans* were isolated during this study although the latter species was present in all of the Graviera batches and was the prevalent species in batches 1 and 2.

Given that all four Ent$^+$ *E. faecium* Graviera isolates were active against *Listeria* spp. (Tables 2 and 3), but inactive against *S. aureus* (Table 3), and that their pH-independent activity was suppressed by α-chymotrypsin and proteinase K but not by lipase, they may produce class IIa enterocins (FOULQUIE MORENO *et al.*, 2006). The class IIa antilisterial enterocins, also known as pediocin-like enterocins, such as enterocin A (AYMERICH *et al.*, 1996), are the most common in *E. faecium* of dairy origin (DE VUYST *et al.*, 2003; BEN OMAR *et al.*, 2004). Additional studies are in progress to characterise the enterocin gene/s of the Ent$^+$ *E. faecium* isolated from Graviera cheese.

The prevalence of enterococci, especially *E. faecium*, in fully ripened Graviera cheese was in agreement with previous data on the overall ecology of cheese (SARANTINOPOULOS *et al.*, 2001; GIRAFFA, 2003), particularly in naturally fer-

mented hard cooked Greek cheeses (LITOPOULOU-TZANETAKI, 1990; PRODROMOU *et al.*, 2001). Enterococcal populations were also higher in matured PDO Graviera Kritis cheese that was made without starters compared to the same product made with commercial starters (KANDARAKIS *et al.*, 1998). In starter-free Kefalotyri cheese, which is dryer and saltier than Graviera, the enterococci accounted for 47.6 and 52.7% of the LAB flora after 60 and 120 days of ripening, respectively; *E. faecium* was the predominant species comprising 28.6 and 35.6% of the isolates, respectively (LITOPOULOU-TZANETAKI, 1990). The Graviera cheeses of this study, and other hard cooked Greek cheeses of previous studies, had higher populations of *E. durans* and lower populations of *E. faecalis* than those generally reported for other cheese types (SARANTINOPOULOS *et al.*, 2001, GIRAFFA, 2003). In Kefalotyri, *E. durans* made up 11.1 and 9.2% of the LAB isolates at 60 and 120 days of ripening, while *E. faecalis* made up 7.9 and 7.9%, respectively (LITOPOULOU-TZANETAKI, 1990). Given the prevalence of enterococci, the multifunctional role of *E. faecium* and *E. durans* in Graviera-type cheeses should be investigated, as has been done for other Mediterranean cheese types (NUNEZ *et al.*, 1997; CENTENO *et al.* 1999; SARANTINOPOULOS *et al.*, 2001, 2002; FOULQUIE MORENO *et al.*, 2003).

CONCLUSION

The results of this study indicate that enterococci are important LAB in traditionally ripened Graviera cheese. The inhibition of the *in vitro* growth of *L. monocytogenes* by Ent$^+$ *E. faecium* strains is common. The production and ripening conditions that favour enterococci and their technological and potentially bioprotective role in Graviera cheese deserve further detailed investigations. Ent$^+$ *E. faecium* strains may contribute to the *in situ* inactivation of natural *Listeria* contaminants during Graviera cheese ripening, and therefore, show some promise for use as a protective culture. However, the present results also raise questions regarding appropriate measures to control enterococci that could potentially produce biogenic amines in cheese. These are also some safety concerns given the opportunistic pathogenic nature of several *Enterococcus* spp., including *E. faecium* strains. Before Ent$^+$ *E. faecium* Graviera cheese isolates can be used as bio-protective adjuncts, their overall safety must be assessed in order to ensure a GRAS status at the strain level (OGIER and SERROR, 2008).

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PAPER

FATTY ACID COMPOSITION OF LIPID CLASSES IN COMMERCIAL BREAST MUSCLE OF CAPONS AS A MARKER OF THE DIETARY FAT SOURCE

LA COMPOSIZIONE IN ACIDI GRASSI DELLE CLASSI DEI LIPIDI IN PETTI DI CAPPONE COMMERCIALI QUALE INDICE DELL'ORIGINE DEI GRASSI NELLA DIETA

F. CARCIONE, L.M. CHIESA, D. BALLABIO[1#], S. SONCIN, P.A. BIONDI*, P. CATTANEO and C. CANTONI

Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza Alimentare, Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy
[1]Dipartimento di Scienze e Tecnologie degli Alimenti e Microbiologia, Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy
[#]Present address: Dipartimento di Scienze Ambientali, Università di Milano-Bicocca, P.zza della Scienza 1, 20126 Milano, Italy
*Corresponding author: Tel. +39 02 50317891, Fax +39 02 50317890, e-mail: pierantonio.biondi@unimi.it

ABSTRACT

Breast muscle samples from capons fed with diets containing either soy oil or lard were analysed in order to identify the added fat source and thus recognise a possible fraud. Among extracted total lipids, neutral (triacylglycerol and free fatty acids) and polar (phosphatidyl-ethanolamine, -choline and -serine+inositol) fractions were separated by thin-layer chromatography and

RIASSUNTO

Sono stati raccolti sul mercato ed analizzati campioni di petto ottenuti da capponi alimentati con una dieta contenente olio di soia o lardo in modo da identificare la fonte lipidica supplementare e rivelare eventuali frodi. A partire dall'estratto lipidico totale sono state separate in cromatografia su strato sottile le frazioni dei lipidi neutri (triacilgliceroli ed acidi grassi liberi) e po-

- Key words: breast muscle, capon, dietary fat source, fatty acid analysis, lipid classes, thin-layer chromatography -

their fatty acid percentages were determined. The data, subjected to principal component analysis, demonstrated that capons can be distinguished according to the diet only by the fatty acid content of the phosphatidyl-choline fraction.

lari (fosfatidil-etanolammina, -colina e -serina+inositolo) ed è stato determinato il loro contenuto percentuale in acidi grassi. I dati ottenuti, sottoposti all'analisi delle componenti principali, hanno dimostrato che i capponi possono essere distinti in funzione della dieta solo dal contenuto di acidi grassi della frazione della fosfatidil-colina.

INTRODUCTION

The influence of dietary fat source on lipid composition in poultry meat has been extensively investigated (BAVELAAR and BEYNEN, 2003; CORTINAS *et al.*, 2004; PINCHASOV and NIR, 1992; VILLAVERDE *et al.*, 2005). Broiler chickens have been the most studied species. Different dietary fat sources have been evaluated in order to obtain data on the different fatty acid compositions in various tissues, such as abdominal fat and breast muscle (AZMAN *et al.*, 2004; NEWMAN *et al.*, 2002; SKRIVAN *et al.*, 2000). Due to the relative abundance of polar lipids in poultry muscle, meat can be enriched in polyunsaturated fatty acids (PUFA) (MOUROT and HERMIER, 2001). In the experiments reported the diets were characterised by a marked variety in classes of fatty acids (saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA)), unless appropriate mixtures had been prepared (WALDROUP and WALDROUP, 2005). Furthermore it has been demonstrated that the various lipid fractions in the breast tissue are influenced differently by dietary fat source (SANZ *et al.*, 1999).

Capon meat, which is less marketed than broiler meat, has an important place in human nutrition and its greater tenderness is its main feature (MAST *et al.*, 1981). In comparison with cocks, capons show more intermuscular fat (TOR *et al.*, 2002) and an increased MUFA content in dissected muscle meat (TOR *et al.*, 2005). The main difference between broilers and capons is the slaughtering age, 35-52 days and about 200 days, respectively. Different capon breast meat types are available on the national markets depending on the diet, either a totally vegetable diet (VD), supplemented with soy oil, or a cheaper conventional diet (CD), supplemented with fat from an animal source, such as lard. These diets do not differ markedly with respect to fatty acid composition, in contrast with the more wide-ranging diets reported in studies on broilers. Therefore there may not be a consequent significant difference in fatty acid composition of capon meat.

The purpose of this work was to examine whether the routine determination of fatty acid percentages carried out on capon breast muscle samples would allow recognition of meat obtained from animals fed with slightly different diets, present on the market with different labels and sold at different prices. With this aim, the various lipid fractions were initially separated by thin layer chromatography (TLC), their fatty acids were derivatised to fatty acid methyl esters (FAME) and analysed by gas-chromatography (GC) and the data were evaluated by principal component analysis (PCA).

MATERIALS AND METHODS

Samples

Commercial breast portions kept at +1°C were supplied by Progeo Mangimi (Reggio Emilia, Italy) two days after slaughtering. Twenty-four specimens were taken from 210-day-old capons (Hi-Line Brown) fed with a diet containing soy oil as fat source (VD) and twenty-four specimens were taken from subjects raised under the same conditions with the exception that lard was used as the dietary fat source (CD). Breast portions of the first group were sent to market with an "Only vegetable" label and the second group was sent without any label. The samples were frozen in liquid nitrogen (-80°C), milled to obtain fine particle size powder and stored at -20°C until further analysis. In addition to the fat source, the other ingredients of the two diets were corn, soy flour, extruded soy, calcium hydrogenphosphate, calcium carbonate, sodium chloride, sodium hydrogencarbonate, L-lysine, D,L-methionine and vitamin-trace mineral premix; no other information was furnished about the quantitative composition. A sample of each diet was supplied by Progeo Mangimi for analysis of the chemical composition. They were frozen, milled and stored under the same conditions as the breast portions.

Chemicals

A FAME standard mixture (Restek Food Industry FAME Mix, cat. 35077) was purchased from Superchrom (Milan, Italy); trioleylglycerol, dioleylphosphatidylcholine, dipalmitoylphosphatidylethanolamine, dipalmitoylphosphatidylserine and dipalmitoylphosphatidylinositol were purchased from Sigma-Aldrich (Buchs, Switzerland); TLC plates 20x20 cm, 250 μm film thickness, pore particle size 60 A, were purchased from VWR International (Milan, Italy); all other solvents and reagents were of analytical grade and purchased from Sigma-Aldrich (Buchs, Switzerland).

Extraction and lipid class separation

Total lipids were extracted from the feed (1 g) and muscle (10 g) samples according to the method of BLIGH and DYER (1959). The extract was brought to dryness under reduced pressure and subsequently diluted to 100 mg/mL with chloroform.

The lipid fractions were separated by TLC according to CHRISTIE (2003). Briefly, the triacylglycerol (TAG) and free fatty acid (FFA) fractions were separated using a mixture of hexane: diethyl ether: formic acid 80:20:2 (v/v); while the phospholipid fractions, phosphatidyl choline (PC), phosphatidyl ethanolamine (PE) and phosphatidyl inositol (PI), containing also phosphatidylserine (PS), were separated with a mixture of methyl acetate: 2-propanol: chloroform: methanol: aqueous KCl 0.25% (w/v) 25:25:25:10:9 (v/v). The bands corresponding to the lipid classes were detected by spraying them with 2',7'-dichlorofluorescein in MeOH:H_2O 95:5 (0.1%w/v) and observing them under a UV lamp at 254 nm. The identification of the bands was carried out by comparison with standard compounds. The fractions were collected by scraping the corresponding bands (TAG and FFA in typical Rf ranges of 0.58-0.48 and 0.17-0.13, respectively, PE, PI+PS and PC in typical Rf ranges of 0.44-0.37, 0.28-0.21 and 0.14-0.07, respectively). The TAG and FFA fractions were eluted from silica with chloroform:methanol 98:2 (v/v), while the phospholipid fractions were eluted with chloroform:methanol:water 5:5:1 (v/v). Before the derivatisation, the extracts were brought to dryness in PTFE-lined screw-capped tubes.

FAME preparation

A direct transesterification method based on one previously described (LEPAGE and ROY, 1986) was followed. Brief-

ly, the dried residues of extracted lipid fractions were treated at 100°C for 60 min with a methanol-toluene mixture (4:1) (v/v) (2 mL) after adding acetyl chloride (0.2 mL). Aqueous potassium carbonate (6% (w/v), 5 mL) was added to the reaction mixtures which were then vigorously shaken. The organic upper phase was injected into the GC.

Chromatographic analysis

FAME GC analysis was carried out on a TraceGC apparatus (Thermo Finnigan, Milan, Italy) equipped with a split/splitless (SSL) injector and a flame ionisation detector (FID). The fused-silica capillary column (100 m x 0.2 mm ID) contained a cyanopropyl-phenylpolysiloxane RT-2560 stationary phase (0.25 μm film thickness) (Superchrom, Milan, Italy). Nitrogen was used as carrier gas, at a flow rate of 0.5 mL/min, and as make-up gas at 30 mL/min. The column temperature was held at 70°C for 3 min, increased to 240°C at 3°C/min and finally held at 240°C for 30 min. The injector and detector temperatures were set at 250° and 300°C, respectively. The injections were performed in split mode with a 1:30 split ratio. The chromatographic profiles were elaborated by Azur (version 3.0) software (Analytical Technology, Brugherio, Italy). Identification of fatty acids was accomplished by comparing the retention times with those of FAME standard mixtures. Only the area percentages of the main peaks (area percentage not less than 0.1%) were considered; total area of the unidentified peaks was always less than 5.23%. In order to test the precision of the procedure, a sample of breast muscle was analysed five times and the variation coefficient for each fatty acid was never higher than 5.5%.

Chemometric analysis

The fatty acid percentages of breast muscle lipid fractions were analysed by means of Principal Component Analysis (PCA) using SCAN (Software for Chemometric Analysis), (Minitab Inc., State College, PA). In the matrix, the breast samples of each group of capons made up the 48 specimens, while the fatty acid percentages of TAG, FFA, PC, PE and PI+PS fractions, their SFA, MUFA and PUFA grouping and their MUFA/SFA, PUFA/SFA, PUFA/MUFA ratios made up the 23 variables. Each lipid fraction (TAG, FFA, PC, PE and PI+PS) was analysed separately. The SPSS 14.0 software was used to do the statistical analysis; the comparisons among means were performed by ANOVA at the 1% confidence limit, using the Student-Newman-Keuls post hoc method.

RESULTS AND DISCUSSION

The principal fatty acid area percentages and the total area percentages of fatty acid groups, obtained by the GC analysis of the two diets, are shown in Table 1. The SFA content was the same and the MUFA and PUFA contents were only slightly different, with higher CD and VD values respectively. These two diets correspond to those that are currently used to feed subjects destined for the market. These diets differ markedly from those used in previous studies performed on broilers where marked differences in the fatty acid contents characterised the experimental diets. The small difference between the two diets in the present study was comparable only to a previous study on mixed diets (where the fat source was obtained by blending lard and soy) (WALDROUP and WALDROUP, 2005) carried out on male broilers. In that study the differences in the 18:1 and 18:2 fatty acid contents (main components of MUFA and PUFA, respectively) in adipose tissue were not significant. On the other hand, when various fat sources were used to study the influence of the dietary fatty acid composi-

Table 1 - Fatty acid analysis of the vegetable (VD) and conventional (CD) diets.

Principal fatty acids and their groups.		
	VD	CD
16:0	22.24[a]	18.68
18:0	4.14	8.58
18:1 n-9	27.29	31.68
18:2 n-6	39.62	35.36
18:3 n-3	3.28	2.62
total SFA	28.25[b]	28.70
total MUFA	28.80	32.95
total PUFA	42.95	38.35

[a]Area percentage of the corresponding FAME peak. The values are means of two determinations. The maximum mean deviation was <9.6%.
[b]Total area percentage of the FAME peaks corresponding to the fatty acids of the group.

tion on the main lipid fractions in broiler breast muscle (SANZ *et al.*, 1999), only neutral lipids clearly reflected the dietary fat source. The changes produced by various fat sources in broilers were more pronounced in the adipose tissue than in breast muscle (SKRIVAN *et al.*, 2000). Based on these findings the lipid classes were separated by TLC before the GC fatty acid analysis, in order to better investigate an eventual influence of the slightly different diets on the breast muscle samples.

Among the neutral lipids, only TGA and FFA were studied, while all of the main classes of polar lipids, PC, PE and the TLC zone containing both PI and PS, were collected. The FAMEs were well separated on a long capillary column containing a polar stationary phase and the data regarding the resulting area percentages of the corresponding peaks were used for the final chemometric analysis.

Tha large volumes of data were analysed by PCA in order to show any data that would be distinctive among the two groups fed with the different diets. In Fig. 1 the score plots relative to the neutral, PE and PS+PI fractions are shown. In these lipid classes, no definite clusters appear for the two capon groups. In Fig. 2 the score plot relative to the PC fractions recovered from breast muscle is shown. The variance in the first and second principal components was 48.2 and 14.1%, respectively. The subjects fed with the two diets are grouped into different clusters. The fatty acid area percentages obtained by GC analysis of the PC fractions of the two capon groups are reported in Table 2. The large variability found in specimens from the same group indicates that most of fatty acid percentages are not significantly different; only 18:1n-7, 22:0, 24:0, 24:1n-9 were significantly higher in the CD group and 22:4n-6 in the VD group.

As previously reported for chickens (CORTINAS *et al.*, 2004), by increasing the PUFA content in the diet there is an increase in PUFA and a decrease in MUFA in breast muscle; on the other hand SFA, equally concentrated in the diets used in the present study, did not lead to any change in their content in the meat samples. Differences in the MUFA and PUFA contents in CD and VD groups, as well as all the ratios of FAME classes were significant. In the PC fractions of breast muscle lipids the high concentration of arachidonic acid (20:4n-6) is noteworthy. This acid is not contained in the diet and presumably was synthetised from linoleic acid. This finding agrees with the results obtained with broilers (NEWMAN *et al.*, 2002).

With regard to the lipid fractions that were most influenced by the diets, it was found that almost none of the fractions were influenced by the different diets. Only a polar fraction, namely PC, showed a fatty acid composition that was distinctive to the dietary fat source. These results differed greatly with respect to a previous study carried out on broilers (SANZ *et al.*, 1999). The marked

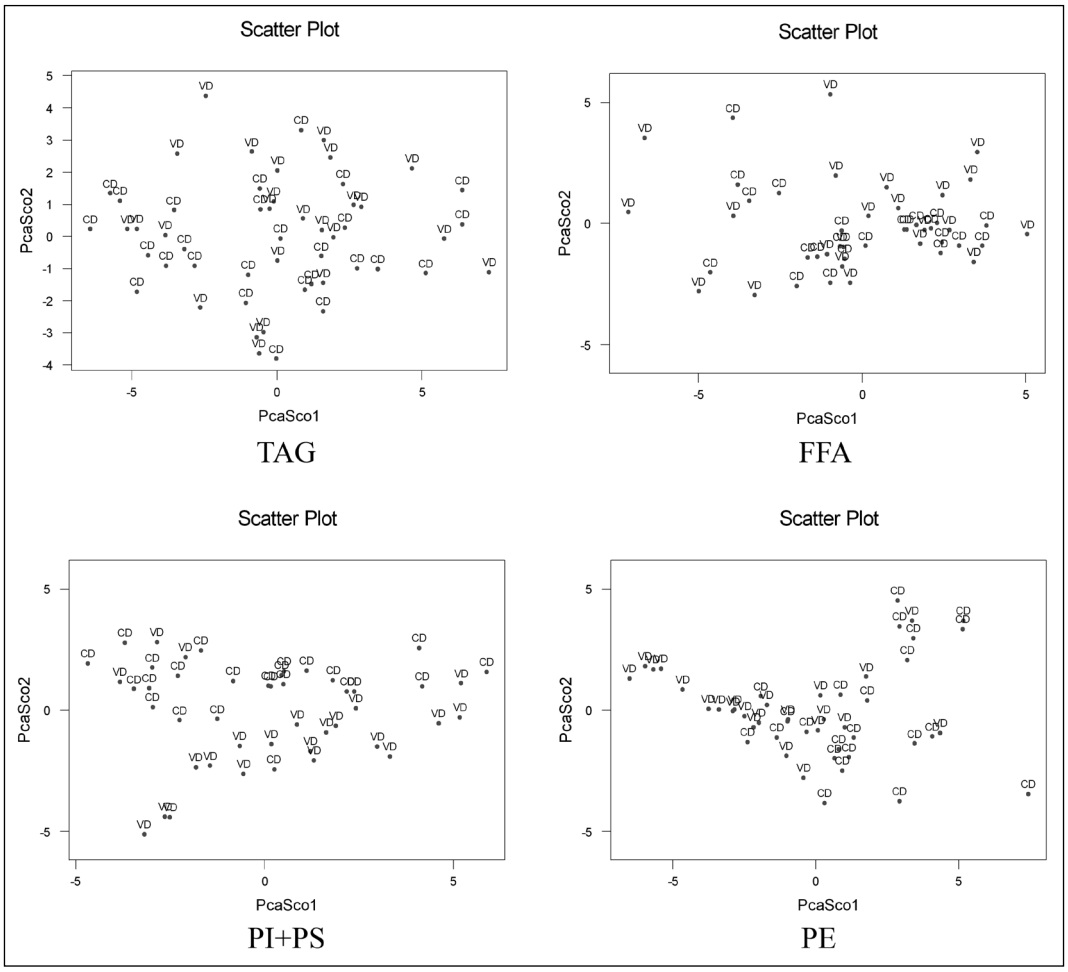


Fig. 1 - Score plots of PCA analysis of the FAME percentages of TGA, FFA, PE and PS+PI fractions in breast muscle samples obtained from capons fed with two different diets, a vegetable diet (VD) and a conventional one (CD).

difference in slaughtering age between broilers and capons can probably explain this finding; a more uniform fatty acid content is probably produced over a longer adaptation time. Chain elongation to give long-chain (C20, C22 and C24) saturated and unsaturated fatty acids appears to be the main feature of the fatty acid metabolism in the longer lived capons.

Regarding the main aim of the present work, which was to determine if it is possible to distinguish between capon breast muscle samples sold at different prices on the market, it is concluded that routine GC analysis of fatty acids is helpful, on condition that a previous separation of polar lipid fractions is performed. This study was an explorative analysis on the diagnostic capability of fatty acid composition. In the future the number of samples could be increased in order to develop a really predictive classification method. Finally, for

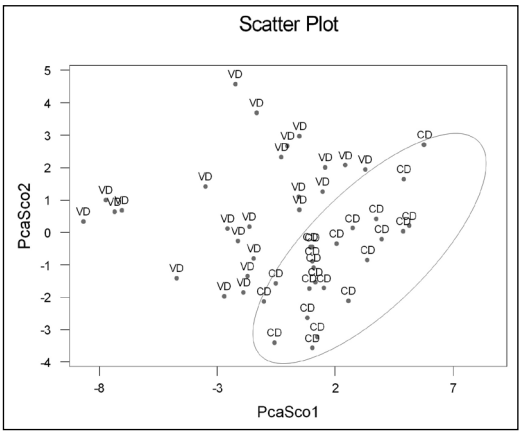


Fig. 2 - Score plot of PCA analysis of the fatty acid percentages of PC fractions in breast muscle samples obtained from capons fed with two different diets, a vegetable diet (VD) and a conventional one (CD).

Table 2 - FAME area percentages of PC fractions in breast muscle samples from capons fed with the vegetable (VD) and conventional (CD) diets.

Fatty acid	VD[a] mean (SD)	CD[b] mean (SD)
14:0	0.15 (0.05)	0.13 (0.05)
16:0	28.46 (2.42)	28.89 (2.37)
18:0	7.02 (1.12)	6.86 (1.83)
18:1n-9	19.20 (3.86)	22.55 (3.13)
18:1n-7	2.05 (0.62)[x]	2.91 (0.55)[y]
18:2n-6	15.73 (2.15)	14.48 (1.82)
20:0	0.11 (0.04)	0.13 (0.04)
20:2n-6	0.48 (0.10)	0.45 (0.08)
22:0	0.22 (0.07)[x]	0.31 (0.09)[y]
20:3n-6	0.65 (0.22)	0.53 (0.10)
20:4n-6	18.77 (3.57)	16.84 (3.57)
24:0	0.23 (0.07)[x]	0.35 (0.10)[y]
20:5n-3	0.10 (0.03)	0.09 (0.03)
24:1n-9	0.12 (0.04)[x]	0.19 (0.05)[y]
22:4n-6	1.56 (0.44)[x]	0.42 (0.13)[y]
22:5n-3	2.77 (0.77)	2.60 (0.58)
22:6n-3	2.38 (0.74)	2.27 (0.63)
SFA	36.19 (2.62)	36.67 (2.27)
MUFA	21.37 (4.20)[x]	25.6 (3.20)[y]
PUFA	42.44 (5.70)[x]	37.68 (3.93)[y]
MUFA/SFA	0.59 (0.12)[x]	0.70 (0.08)[y]
PUFA/SFA	1.17 (0.24)[x]	1.03 (0.17)[y]
PUFA/MUFA	1.98 (0.72)[x]	1.47 (0.37)[y]

[a] Mean (standard deviation) of the FAME area percentage obtained from 24 specimens fed the vegetable diet (VD).
[b] Mean (standard deviation) of the FAME area percentage obtained from 24 specimens fed the conventional diet (CD).
[x,y] Different letters as superscript indicate statistically significant differences (P<0.01).

the first time, a preliminary study on the influence of the dietary fat source on the fatty acid content of breast muscle was carried out in capons.

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PAPER

ANALYSIS OF FACTORS AFFECTING CUSTOMER RETAILER LOYALTY IN THE TURKISH FOOD MARKET: THE CASE STUDY OF ERZURUM

ANALISI DEI FATTORI CHE INFLUENZANO IL RAPPORTO FIDUCIARIO TRA RIVENDITORE E CONSUMATORE NEL MERCATO ALIMENTARE TURCO: IL CASO DI ERZURUM

Y. TOPCU* and A.S. UZUNDUMLU
Department of Agricultural Economics, Collage of Agriculture,
Ataturk University, 25240 Erzurum, Turkey
*Corresponding author: Tel. +90 442 2311393, Fax +90 442 2312678,
e-mail: ytopcu@atauni.edu.tr

ABSTRACT

Increasing competition and slow growth in the Turkish food market have increased the need for retailers to use marketing strategies focused on retaining current customers and attracting new ones. To reach these aims, retailers have been trying to enhance their customer loyalty through retention programs and relationship marketing strategies. The aim of this study was to determine the most salient factors affecting customer retailer loyalty by using factor analysis and then to identi-

RIASSUNTO

L'incremento della concorrenza e la lenta crescita del mercato alimentare turco hanno accresciuto la necessità dei rivenditori di adottare strategie di mercato focalizzate sul mantenimento della clientela attuale e sull'attirare nuova clientela. A tale scopo, i rivenditori hanno tentato di migliorare la fedeltà dei propri consumatori attraverso programmi di mantenimento e strategie di relazioni di mercato. Lo scopo di questo studio era quello di determinare, utilizzando un fattore di analisi, i

- Key words: customer retailer loyalty, factor analysis, k-means cluster analysis, market segmentation -

fy consumer group profiles by k-means cluster analysis. The results showed that satisfaction with the product and store image in Cluster 1, satisfaction with the sales process and perceived service quality in Cluster 2, and variety seeking in Cluster 3 were the most prominent retailer attributes that affected consumer retailer loyalty. The results of this study provide insights into understand interrelationships among retailer attitudes, preference values and attitudes of Turkish consumers, and could be used to develop marketing strategies and more effective policies to improve customer retailer loyalty in Turkey.

principali fattori che influenzano la fedeltà del consumatore al rivenditore, e quello di identificare profili di gruppi di consumatori attraverso l'analisi dell'algoritmo di clustering. I risultati ottenuti mostravano che i principali attributi del rivenditore che influenzavano la fedeltà del consumatore erano: la soddisfazione per il prodotto e l'aspetto estetico del negozio per il cluster 1; la soddisfazione per il processo di vendita e la percezione della qualità del servizio per il cluster 2; la ricerca della varietà (scelta) per il cluster 3. I risultati di tale studio forniscono una visione approfondita per comprendere le interrelazioni tra le attitudini dei rivenditori, le preferenze e le attitudini dei consumatori turchi, e potrebbero essere utilizzate per lo sviluppo di strategie di mercato e politiche più efficaci per migliorare la fedeltà al rivenditore da parte del consumatore in Turchia.

INTRODUCTION

Increasing competition and slow growth in the Turkish food market have stimulated retailers to use marketing strategies that are focused on retaining current customers and attracting new ones (TOPCU and ISIK, 2007). However, a strategy that is effective for acquiring new customers may not be the most effective for retaining existing customers. Today many retailers are trying to enhance their customer loyalty through retention programs and relationship marketing strategies (YI and LA, 2004). Customer loyalty is very important to the retailer, because it provides a continuous flow of profit, reduces marketing costs and increases referrals and price premiums. Loyal customers are not easily convinced by the promotional efforts of competitors (REICHHELD, 1996; SIROHI *et al.*, 1998; REINARTZ and KUMAR, 1999; YI and JEON, 2003).

Most importantly, loyal customers form a stable pool for the products or services of a retailer. A small shift in customer retention rates can make a large difference in earnings, and this influence accelerates over time (WALLACE *et al.*, 2004). Most research to determine customer loyalty characteristics have defined loyalty as usually choosing the same brand, a high incidence of a positive word of mouth, frequent repurchase intention and willingness to pay higher prices. There is a strong link between loyalty and profitability. Practically, there is a difference between the cost of getting new customers and the cost

of keeping existing customers; it has often been demonstrated that it is important to enhance existing customer loyalty (WRIGHT and SPARKS, 1999; YI and LA, 2004).

In past decades, retailers used manufacturer brands to generate consumer interest, sponsors and loyalty. However with increased competition, retailers have begun to compete with manufacturers by offering their own store brand in order to enhance their relative marketing power and acquire a share in the total profit pie (TOPCU *et al.*, 2007). The increase in retailer brands has become one of the most important trends in Turkish food retailing.

This store-brand strategy could help retailers attract customers and create loyalty to the store by offering exclusive product lines and premium products (CORSTJENS and LAL, 2000). This strategy could also help retailers to project a lower-price image, increase their bargaining power with manufacturers and producers of major national brands, and have more control over their shelf space (DUNNE and NARASIMHAN, 1999). With the overwhelming success of some store brands in Europe (e.g. Marks and Spencer, Asda, Tesco, Carrefour, Migros, etc.), there is great interest in strong store brands. For example, retailer concentration and share of strong store brands are 86% and 45% in Switzerland, 65% and 30% in Germany, 80% and 25% in Belgium and 89% and 17% in Denmark, respectively, while there are 26% and 4% in Turkey (ACNIELSEN, 2005; TOPCU, 2006). Therefore, the cumulative power carried by these retailers and their store brands is very significant.

Customer loyalty encompasses loyalty to the retailer as well as brand loyalty; which has been extensively studied (RAJU *et al.*, 1990; OLIVER, 1997, 1999; ODEKERKEN *et al.*, 2003; GREWAL *et al.*, 2004). In contrast, little research has been conducted on the critical role of customer loyalty to the retailer (JOHNSON *et al.*, 1997; HOMBURG and GIERING, 2001; YI and LA, 2004; LIU, 2007; OLSEN, 2007).

Customer loyalty to the retailer is of extreme interest to merchants, because high customer acquisition costs are difficult to recover without repeat purchasing. Due to increased competition and minimal customer switching costs, it has become increasingly difficult to retain customers (SRINIVASAN *et al.*, 2002).

In light of these current developments, retailers have been trying to develop marketing strategies that create loyalty in existing and new customers within the Turkish food sectors. It is important for retailers to measure the effectiveness of these strategies on customer loyalty and to apply the ones that are appropriate for the target customer group.

In this study, the relationships between retailers and loyal customers in the Turkish food market, have been determined and the factors that influence customers have been investigated. The target market has been divided into segments according to the socioeconomic and demographic characteristics of the customers.

MATERIAL AND METHODS

The preliminary data of the present research were obtained from a survey conducted in Erzurum[1], Turkey. To determine the sample size, while minimizing sample bias and representing the population correctly, the city centre was divided into four districts: the east-side (Kazimkarabekir district) with 17,976 households, the west-side (Dadaskent district) with 6,562 households, the north-side (Palandoken district) with 30,022

[1] Erzurum (39°45'N latitude and 41°15'E longitude) is on the Silk Road, and is located in the north-eastern region of Turkey. Erzurum is one of the biggest provinces in the region with a total population of 962,000; 402,000 live in the city centre.

households, and the south-side (Yakutiye district) with 26,099 with households (ANONYMOUS, 2007).

To determine the sample size for each district, the following formula was used (TOPCU, 2006; YILDIZ *et al.*, 2006; TOPCU and ISIK, 2008):

$$n = \frac{Z^2 * p * (1 - p)}{c^2} = 250$$

Where;

n = sample size;

Z = Z value (1.96 used for 95% confidence level);

p = percentage making a choice (0.8 used for sample size needed);

c = confidence interval (used 0.05=±5).

Then, based on the population of each district, the weighted sample size and survey distribution for each district were determined proportionally. The number of questionnaires allocated to the Dadaskent, Kazimkarabekir, Yakutiye and Palandoken districts were 20, 56, 81 and 93, respectively, for a total of 250.

A simple random sampling method (i.e., each member of the population has an equal chance of being chosen) was used to select the consumer households in each district that received the final questionnaire. Each district was divided into sub-districts and then, a face-to-face survey method was used with randomly selected heads-of-households (either wife or husband) in various parts of the sub-districts. The survey was conducted in the home or at the shopping centres.

Survey participants were asked to respond to each statement, indicating the level of significance the food attribute had for them, using a Likert-format 1-5 scale (where 1 refers to the least important, and 5 refers to the most important attributes). Of the 29 attributes, four were related to the shopping environment (store traffic, position of food departments, better service and indoor design), nine were related to the store facilities (the number of check-out counters, the possibility for group shopping, the presence of a cafe, restaurant, recreation place and children's playground, store size, packaging service, opening and closing hours, availability of parking and store location), three were related to the increasing sales activities (brand/store promotion and advertisement, advantage/club-card and possibility of payment by installment), five were related to individual preferences (product freshness, alternatives of price and brand, price-quality relationship and customer experience) and eight were related to store image (natural, hygienic, health and product quality, store-brand, brand names, product diversification and customer profile).

Each question was reduced to a single statement to which responses were collected using the Likert scale described above. Demographic and socioeconomic information (gender, age, occupation, education, consumer food expenditure and income levels) was also gathered in the survey.

After editing and coding, the data were first analyzed by factor analysis to determine the main factors affecting customer loyalty. Cluster analysis was then used to classify the target consumer group using their demographic and socioeconomic characteristics. SPSS statistical software was used to perform these two analyses.

Factor analysis is a data reduction technique that reduces the number of variables used in an analysis by creating new variables (called factors) that combine redundancy in the data (SPSS 15.0, 2006).

The first step is to determine the number of relevant factors. Therefore, the factor analysis in this study reduced 29 attributes to five factors that had eigenvalues greater than 1.0 (determined by Principal Component Analysis[2] using Varimax rotation method[3]). Factor analysis

was used initially to identify the underlying dimensions that could explain the correlation among a set of retailer attributes and the associated preference values. In addition, the factor analysis was conducted to identify the attributes that accounted for a relatively large proportion of variance in the sample. This subset was then used for consumer segmentation.

In the second step of the analysis, the k-means cluster analysis was used. Clustering algorithms can be classified into two categories: hierarchical and non-hierarchical. Hierarchical clustering algorithms start with "n" clusters, equal to the number of observations, and proceed until all observations are in one cluster. In non-hierarchical clustering, the researcher specifies the number of clusters in the data set *a priori* (SPSS 15.0, 2006). In this study, preference values between five factors and three customer clusters were identified. Since, the number of clusters could be specified; non-hierarchical k-means clustering was used.

The final step was to use cross-tabulations to examine the distribution of the clusters in relation to the three-food expenditures and income levels, and the demographic and socioeconomic attitudes. A χ-square independence test was conducted for each of the clusters to determine whether there was a relationship between the personal characteristics and their positions in each group. If $p \geq 0.05$, the null hypothesis was accepted, which means that the demographic characteristics and their position levels were statistically independent (YILDIZ *et al.*, 2006).

RESULTS

Factors affecting customer loyalty and preference values

Kaiser Normalization compares partial correlation coefficients with the observed ones. The value was calculated to be 0.92, which means that data sets were perfect for factor analysis because the test score was greater than 0.50.

The results of principal component analysis for the first factors with eigenvalues greater than 1.0 are shown (Table 1). The first five factors accounted for 65.32% of the total variance. Factor 1, which accounted for 40.5% of the total variance, was dominated by attributes that related to store traffic, position of food department, the number of checkout counters, store promotion, better service, indoor design and group shopping possibility. Hence, Factor 1 is associated with the satisfaction with the sales process. Factor 2 accounted for 10.26% of the total variance, and was dominated by the presence of a café, restaurant, recreation place and children's playgrounds, store size, packaging service, store opening and closing hours, availability of parking, customers' good experiences about the store and location. Factor 2, therefore, is associated with the perceived quality of service.

Factor 3 accounted for 6.20% of the total variance, and was dominated by six attributes: natural, hygienic, safe, and fresh food, sensory quality and perceived food value. This factor is more related to customer satisfaction with the product. Factor 4 accounted for 4.68% of the total variance; it was dominated by the attributes of price and brand alternatives, and diversification of food products. Factor 4, therefore, was related to variety seeking. Factor 5 is related to store image consisting of store brand, customer

[2] A factor extraction method was used to form uncorrelated linear combinations of the observed variables. The first component has maximum variance. Successive component explain progressively smaller portions of the variance and are all uncorrelated with each other. Principal component analysis is used to obtain the initial factor solution. It can be used when a correlation matrix is singular.

[3] This method is an orthogonal rotation method that minimizes the number of variables that have high loading on each factor. It simplifies the interpretation of the factors.

Table 1 - Factors and correlated variable loadings.

Variables	Factors				
	F_1	F_2	F_3	F_4	F_5
Shopping convenience	**0.787**	0.228	0.299	0.014	-0.014
Food department position	**0.756**	0.289	0.279	0.073	0.151
The number of adequate check-out counters	**0.727**	0.255	0.145	0.042	0.296
Store promotion	**0.704**	0.192	-0.067	0.269	0.355
Better service presentation	**0.660**	0.164	0.337	0.097	0.083
Indoor design of store	**0.624**	0.508	0.029	0.230	0.181
Group shopping possibility	**0.525**	0.272	0.227	0.174	0.233
Availability of cafe, restaurant and recreation place	0.131	**0.823**	0.012	0.045	0.248
Availability of children's playground	0.131	**0.797**	0.010	0.111	0.282
Store size	0.021	**0.675**	0.204	0.367	0.176
Packaging service	0.444	**0.633**	0.306	0.022	0.149
Store opening and closing hours	0.360	**0.604**	0.216	-0.028	0.111
Availability of parking	0.421	**0.588**	0.138	0.192	0.083
To have good experience about the store	0.435	**0.574**	0.368	0.152	0.188
Location of the store	0.517	**0.522**	0.122	0.207	-0.112
Advantage card and instalment sale possibility	0.413	0.421	-0.122	0.151	0.355
Natural or biological products	0.061	0.152	**0.748**	0.045	0.288
Quality-price relation (perceived value)	0.108	-0.031	**0.746**	0.108	0.173
Hygienic product	0.192	0.137	**0.712**	0.266	0.005
Food safety and business guarantee	0.285	0.043	**0.691**	0.198	0.051
Product freshness	0.285	0.076	**0.690**	0.280	-0.019
Product quality (sensory quality)	-0.025	0.180	**0.552**	-0.073	0.523
Alternative prices for food products	0.020	-0.014	0.145	**0.866**	0.128
Alternative brand (store and manufacturer)	0.173	0.188	0.222	**0.701**	0.094
Product diversification	0.322	0.134	0.310	**0.556**	0.016
The store's own brand submission	0.213	0.301	-0.003	0.036	**0.766**
Customer profile	0.272	0.297	0.325	-0.079	**0.644**
Store or brand advertisements	0.415	0.301	0.081	0.096	**0.583**
Store name and prestige	0.427	0.338	0.198	0.112	**0.578**
Eigen-value	***11.736***	***2.974***	***1.799***	***1.357***	***1.075***
Share of explained variance (%)	***40.468***	***10.257***	***6.204***	***4.681***	***3.709***
Cumulative share of explained variance (%)	***40.468***	***50.725***	***56.929***	***61.610***	***65.319***
KMO (Kaiser-Meyer-Olkin) statistic					**0.916**

* Bold numbers indicate the largest loading for each variable.

profile, store-brand advertisement and store name and prestige and accounted for 3.71% of the total variance. These factors demonstrate different preference values related to customer loyalty.

The question arose as to whether certain consumer segments could be associated with these preference values and if so, how many consumers were in each segment and how were these segments related to the preference values listed above? These questions are addressed in the following section.

K-means clustering and customer segmentation

Three clusters had group means that could be explained reasonably. The final cluster centres and the number of cases in each cluster are shown in Ta-

ble 2. The total number of cases was 250. Cluster 1 (C1) was the largest group with 149 (59%) consumers. Consumers in this group cared more about satisfaction with the product (F3) and store image (F5). Cluster 2 (C2) was the second largest group, made up of 84 (34%) consumers. Satisfaction with the sales process (F1) and perceived service quality (F2) were the dominant factors for consumers in this group. As for Cluster 3 (C3), there were 17 (7%) consumers in this group, and variety seeking, including alternative brands and prices in the same product line and production diversification was the most important factor in their preference decisions.

Cross-tabulation between the consumer demographic and socioeconomic characteristics and the three cluster groups are shown in Table 3. These characteristics include gender, age, education, occupation, monthly food expenditure and income.

The results show that there were more female than male consumers in C1 and C2. C1 and C2 had the highest female/male ratios with 53% and 64%, respectively. The distribution of consumers based on age indicates the 25-34 and over 55 age-groups had the highest and lowest portions in all clusters, respectively. C2 had the largest ratio of the 15-24 age group (35%), while C1 and C3 had the largest ratios of the 25-34 age group (42% and 41%, respectively). As for the education levels of consumers, the lowest ratio in all the clusters was made up of literate (i.e., those who can read and write but do not have a diploma) people. Consumers with a college degree dominated C1 (44%) and C3 (35%), while high school graduates dominated in C2.

The occupational distribution among the clusters is as follows: blue-collar state workers, pensioners and the unemployed made up the lowest ratios in all the clusters. Students made up the second highest ratio in all the clusters, whereas white-collar state employees, housewives and small-scale retailers made up the highest ratios in C1 (24%), C2 (32%) and C3 (30%), respectively. The results related to household food expenditures show that those whose monthly food expenditures were between € 125 and € 250 dominated in all the clusters. As for income levels, those with more than € 750 income dominated in C1, whereas the respondents in C2 had an

Table 2 - Final cluster centres and the number of cases in each cluster.

Factor interpretation	Clusters[a]		
	C1	C2	C3
Satisfaction with the sales process (F1)	0.224	**0.341**	0.158
Received service quality (F2)	0.112	**0.854**	0.035
Satisfaction with the product (F3)	**2.117**	0.277	0.149
Variety Seeking (F4)	0.250	-0.154	**-1.356**
Store image (F5)	**0.615**	0.264	0.038
Number of cases in each cluster	***149***	***84***	***17***
% of total cases in each cluster	***59%***	***34%***	***7%***

* Final cluster centre scores are at 0.01 significant levels.
* Bold numbers indicate the largest final cluster centre scores for each factor.
The total number of cases (n): 250.
[a] The numbers in this table indicate final cluster centres. The final cluster assignment and Euclidian distance between the case and cluster centre used to classify the case are given for each case.

income less than € 375 and those in C3 had an income between € 375 and € 750. According to the χ-square test of independence, all of the demographic characteristics (except food expenditure) and their positions are statistically independent since $p \geq 0.05$ in all the groups for all the variables.

Table 3 - Demographic characteristics and cluster number of cross-tabulation cases.

Demographic characteristics		Number of cases in each cluster					
		C1 High-income professionals		C2 Low-income housewives		C3 Mid-income retailers	
		Number	**%**	**Number**	**%**	**Number**	**%**
Gender	Male	70	47	30	36	9	**53**
	Female	*79*	***53***	*54*	***64***	*8*	*47*
	χ^2 ***test of independence***			***(χ^2 =3.420 df = 2, p: 0.181)***			
Age Groups	15-24 age	38	26	29	**35**	5	29
	25-34 age	*63*	***42***	*17*	*20*	*7*	***41***
	35-54 age	37	25	28	33	3	18
	55- + age	*11*	*7*	*10*	*12*	*2*	*12*
	χ^2 ***test of independence***			***(χ^2 = 12.598, df = 6, p: 0.055)***			
Education	Literate	4	3	3	4	1	6
	First school	*29*	*19*	*19*	*23*	*5*	*29*
	High school	51	34	33	**39**	5	29
	College	*65*	***44***	*29*	*35*	*6*	***35***
	χ^2 ***test of independence***			***(χ^2 = 3.170, d f= 6, p: 0.787)***			
Occupation	White-collar state employee	9	**24**	3	19	2	1
	Blue-collar state worker	*36*	*7*	*16*	*5*	*2*	
	Businessman	10	6	4	4	0	12
	Small-scale retailer	*13*	*9*	*4*	*5*	*1*	***30***
	Pensioner	10	7	4	4	1	6
	Housewife	*4*	*23*	*26*	***32***	*1*	*16*
Student	35	21	25	29	5	18	*Unem-*
ployed	*32*	*3*	*2*	*4*	*5*	*6*	
	χ^2 ***test of independence***			***(χ^2 = 9.708, df = 14, p: 0.783)***			
Food Expenditure	Less than €125**	11	7	10	12	4	29
	Between €125 and €250	*92*	***62***	*45*	***54***	*8*	***38***
	More than €250	46	31	29	35	5	32
	χ^2 ***test of independence***			***(χ^2 = 9.821, df = 4, p: 0.044)****			
Income Groups	Less than €375**	35	4	45	**54**	0	0
	Between €375 and €750	*49*	*45*	*33*	*40*	*12*	***65***
	More than €750	65	**51**	6	6	5	35
	χ^2 ***test of independence***			***(χ^2 = 4.185, df = 4, p: 0.381)***			

** The prices of the products were converted from New Turkish Lira (NTL) to Euro (€) using the exchange rate on May 15, 2008. The conversion rate used was 1.95 NTL/€.
* $p<0.05$.

Characteristics of the cluster groups

The cluster profiles were determined on the basis of the final cluster centres of the factors and demographic characteristics of the consumers in each cluster. The main demographic characteristics of the C1 group are young people (25-34 age group) with higher education (college degree) and white-collar state employees with high income. This cluster is referred to as "young highly-educated and high income professionals". Satisfaction with the product, and store image were the most influential factors on retailer loyalty for those in C1.

The dominant demographic characteristics in C2 are the youngest age group (15-24 years old), high-school educated, and housewives with a low-income. C2 is referred to as "young educated low-income and housewives". The most important factors in C2 were satisfaction with the sales process and perceived service quality, which is a more specific judgment and a component of customer satisfaction. This factor has often been examined as an antecedent of repurchase intention. These factors focus on satisfaction with the service (OLIVER, 1993; IACOBUCCI *et al.*, 1995; YI and LA, 2004).

Finally, the respondents in the C3 group were mostly young (35-54 age group) and highly-educated and mid-income people and small-scale retailers. This group is referred to as "young highly educated and mid-income retailers". Variety seeking was the most important factor in this cluster.

DISCUSSION

The factors related to product and retailer satisfaction

The results show that there are cognitive and affective differences between customer satisfaction and retailer loyalty. Whereas satisfaction with the product and sales process, perceived service quality and store image have a positive influence on customer retailer loyalty, variety seeking has a negative effect on it.

Due to the positive relationship between satisfaction with the sales process and perceived service quality, these factors could be referred to as only one factor, satisfaction with the service (BITNER, 1990; CRONIN and TAYLOR, 1992; IACOBUCCI *et al.*, 1995). Since service quality and satisfaction with the sales process are antecedent to service satisfaction, this has a significant effect on repurchase intention. It might be useful to investigate the roles of service quality received and satisfaction with the sales process simultaneously (CRONIN and TAYLOR, 1992; YI and LA, 2004).

While satisfaction with the service had a positive relationship not only with satisfaction with the product and store image but also with retailer loyalty in all the clusters, particularly in C2, satisfaction with the product had a much stronger effect than satisfaction with the service in all the clusters, especially C1. These findings are supported by previous studies related to satisfaction with the product and service (ANDERSON *et al.*, 1994; RUST *et al.*, 1995; HALLOWEL, 1996; PATTERSON *et al.*, 1997; PRITCHARD *et al.*, 1999; HOMBURG and GIERING, 2001; YI and LA, 2004; OLSEN, 2007).

This study also provides same new insights about how store image and variety seeking affect customer retailer loyalty. The retailer's image and reputation have a strong positive effect on customer evaluation, purchase intention and satisfaction (DHAR and HOCH, 1997; GREWAL *et al.*, 1998; AHLUWALIA and GURHAN, 2000). In other words, store image has a positive effect on the link between customer satisfaction and loyalty. When compared with the results of

earlier research (CORSTJENS and LAL, 2000; AILAWADI *et al.*, 2001; AILAWADI and HARLAM, 2004), the results of this study show that store image had a positive influence on customer retailer loyalty, especially in C1.

The phenomenon of variety seeking is a much-discussed topic. The basic idea behind the concept of variety seeking is that under certain conditions everyone needs variety in daily life. As customers seek variety, they have desires and needs that cannot be filled by a single brand. Therefore, customers seek variety in product choices in order to avoid monotony and boredom (FEINBERG *et al.*, 1992; MENON and KAHN, 1995; HOMBURG and GIERING, 2001).

In light of this theoretical information, variety seeking has a negative effect on the other factors. The impact of satisfaction with the service for variety is weaker than satisfaction with the product for people with a strong drive. This finding is consistent with the current interpretation of variety seeking as an essentially motivated phenomenon; this means that the customer switches brands just for the sake of variety, irrespective of satisfaction with the current brand. Hence, customer drive for variety could influence the relationship between product satisfaction and loyalty. Because variety seeking is regarded as a product-related phenomenon, customers with a strong need for variety could express this intrinsic motivation in some product categories. No moderating effect of variety seeking is expected on the linkages between service satisfaction and loyalty.

Factors with respect to consumer demographic characteristics

Demographic characteristics are very important determinants of customer retailer loyalty. For example, when a consumer prefers a retail store for food products, the demographic characteristics reflect their psychological state, cultural values and buying attitudes. Many studies in the areas of consumer attitude and behaviour have neglected demographic factors and have shifted attention to psychological constructs (KALYANAM and PUTLER, 1997; ARENI *et al.*, 1998; REINARTZ and KUMAR, 1999). There is no doubt that these constructs can explain many of the individual-level phenomena better than general demographic factors. However, this study indicates that demographic characteristics such as gender, age, education, occupation and income play an important role in moderating the relationships between psychological and behavioural constructs.

Regarding the impact of gender on customer retailer loyalty, the results show that the purchasing behaviour of women is to be strongly influenced by their evaluation of personal interaction processes. Compared to men, women are more involved in purchasing activities and pay more attention to satisfaction with the service which involves consulting quality services of the sales personnel related to the sales process, to store image, and satisfaction with the product. In contrast, males are less involved in buying activities and are involved with taking care of variety seeking. A woman's decision to buy a food product at the same retailer is strongly influenced by her satisfaction with the product and service, and store image. On the other hand, a man's loyalty to a certain retailer is determined by alternative price and brand, and product diversification. These findings are supported by many other studies (SLAMA and TASHLIAN, 1985; ZEITHAML, 1985; JASPER and LAN, 1992; GILBERT and WARREN, 1995).

The results of this study show that age has an effect on customer retailer loyalty. Whereas the youngest customers focus on satisfaction with the service, mature and older customers are more influenced by satisfaction with the product and store image. Thus, when mak-

ing a buying decision, to purchase older respondents are likely to focus on their experience-based evaluation of the product's key attribute. Younger people do not rely so much on their satisfaction with the product itself; but rather, seem to base their decision primarily on the information provided by the sales person. These findings are consistent with other studies related to age (GILLY and ZEITHAML, 1985; ROEDDER and COLE, 1986; SMITH and BALTES, 1990; LIGHT, 1991; SORCE, 1995).

The relationship between retailer loyalty and satisfaction with the product and store image is stronger for respondents with high income than for those with mid-income. However, the relationship between satisfaction with the service and loyalty is weaker for customers with high income than for those with mid-income. In contrast, low-income people focus on variety seeking and their retailer loyalty is very low and has a negative effect. Hence, product satisfaction and store image is much more important for high income people. One reason could be the financial and health risk associated with the purchase of a poor-quality product. Another reason could be associated with social status and living standard. As a result people with a higher income have a higher level of education and a better job and they want to live on a higher social scale (ZEITHAML, 1985; MURRAY and SCHLACTER, 1990; SPENCE and BRUCKS, 1997). There is a strong correlation among income, education, and occupation (JACOBY and KYNER, 1973; SCHANINGER and SCIGLIMPAGLIA, 1981; SPENCE and BRUCKS, 1997).

CONCLUSIONS

In this study, the major factors affecting customer retailer loyalty in Erzurum, Turkey, were examined. Factor and k-means cluster analyses were used to determine the main factors that affect customer retailer loyalty and to cluster the customers based on the relative homogeneity of their attitudes. The results of this study show that satisfaction with the product including food quality and food safety attributes and store image are the factors that have the strongest effect on customer retailer loyalty in all the clusters but especially in C1. Satisfaction with the service derived from a blend of satisfaction with sales process and perceived service quality is the second-most important factor in all the groups, particularly in C2. The third most important factor, variety seeking, has a negative effect on retailer loyalty especially in C2 and C3.

Some demographic characteristics also affect consumer retailer loyalty. Using multiple group causal analysis, significant differences were found for each of the factors across the subgroups. While satisfaction with the product and service, and store image positively affected customer retailer loyalty, variety seeking had a negative effect on it. In addition, gender, age and income with a strong correlation between education and occupation had an important influence on retailer loyalty. The results of this study show the importance of the relationship between main factors and demographic characteristics in determining retailer loyalty.

In an attempt to build customer loyalty retailers should concentrate on providing food safety, sensory and perceived quality, hygiene and freshness, and engaging strategies such as private labelled products, and store/brand advertisements to satisfy the respondents of C1, (educated women, with high-income) who look for satisfaction with the product. Retailers should provide stronger service policies related to the sales process and service quality focused to retain current and attract new customers (C2), particularly women with low-income and average education who pay more attention to satisfaction with the service. The retailer should focus on more effective

price, brand and product diversification strategies for (C3) (highly-educated and mid-income men). Consequently, retailers should develop and implement marketing tactics and strategies that pay more attention to the relationships between the factors affecting customer loyalty and the demographic characteristics of the target customers.

Even though this study has some scientific merit for the academic, policy-making, and food manufacturing and retailing community, it also has some limitations. One limitation is that the survey was conducted in only one city that could not possibly represent all Turkish consumers of food products. The city of Erzurum does, however, give some indication of the direction in which other cities are moving. In future studies, this model could be expanded to include more specific foods and factors.

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PAPER

EFFECT OF PULSED ELECTRIC FIELDS ON GERMINATION TUBE ELONGATION AND SPORE GERMINATION OF *BOTRYTIS CINEREA* INOCULATED INTO SOUR CHERRY JUICE, APRICOT AND PEACH NECTARS

EFFETTO DI CAMPI ELETTRICI PULSATI SULLO SVILUPPO DEI TUBULI GERMINATIVI E SULLA GERMINAZIONE DELLE SPORE DI *BOTRYTIS CINEREA* INOCULATA IN Ssucco DI CILIEGIA E IN NETTARI DI ALBICOCCCA E PESCA

G. AKDEMIR EVRENDILEK*, F.M. TOK[1], E.M. SOYLU[1] and S. SOYLU[1]
Department of Food Engineering, Faculty of Engineering and Architecture,
Abant Izzet Baysal University, Golkoy Campus 14280 Bolu, Turkey
[1]Department of Plant Protection, Faculty of Agriculture,
Mustafa Kemal University, 31034 Alahan Hatay, Turkey
*Corresponding author: Tel. +90 374 2534640, Fax +90 374 2534558,
e-mail: gevrendilek@ibu.edu.tr or gevrendilek@yahoo.com

ABSTRACT

Inactivation of *Botrytis cinerea* inoculated into sour cherry juice, apricot and peach nectars was determined by pulsed electric fields (PEF) based on the measurement of germination tube elongation, spore germination rate and light and scanning electron (SEM) mi-

RIASSUNTO

È stata studiata, sulla base di misurazioni dello sviluppo di tubuli germinativi, della velocità di germinazione delle spore e di osservazioni sia alla luce, sia al microscopio elettronico a scansione (SEM), l'inattivazione di *Botrytis cinerea* inoculata in un succo di cilie-

- Key words: *Botrytis cinerea*, fruit juice, germination tube elongation, pulsed electric fields, spore germination rate -

croscopic observations. With increasing electric field strength and processing time, germination tube elongation and spore germination were completely inhibited ($P \leq 0.05$). Microscopic observations revealed considerable morphological alterations such as cytoplasmic coagulation, vacuolations, hyphal shriveling and protoplast leakage. PEF processing of juice/nectars was very effective to inactivate *B. cinerea*. This method could be used to prevent product loss in fruit juice/nectars due to *B. cinerea* contamination.

gia acida e in nettari di albicocca e pesca per mezzo di campi elettrici pulsati (PEF). Con l'incremento della forza del campo elettrico e del tempo di trattamento, lo sviluppo dei tubuli germinativi e la germinazione delle spore risultavano completamente inibiti ($P \leq 0.05$). Le osservazioni al microscopio rivelavano considerevoli alterazioni morfologiche come la coagulazione del citoplasma, la vacuolazione, il raggrinzimento delle ife e la perdita del protoplasto. Il trattamento PEF di succhi e nettari è risultato altamente efficace nell'inattivazione della *B. cinerea*. Questo metodo potrebbe essere utilizzato per prevenire la perdita di prodotto nei succhi e nettari di frutta dovuta a contaminazione da *B. cinerea*.

INTRODUCTION

Non-thermal food preservation technologies, especially pulsed electric fields (PEF) have become important for scientists, the food industry and consumers because they are safe and have positive effects on the quality and sensory attributes of food products (ZHANG *et al.*, 1994). PEF processing of foods has been applied most frequently to fruit juices (JIN and ZHANG, 1999; RASO *et al.*, 1998; SEN GUPTA *et al.*, 2005), milk and milk products (CALDERON-MIRANDA *et al.*, 1999; EVRENDILEK *et al.*, 2004a, 2004b; FERNANDEZ-MOLINA *et al.*, 2006) and liquid egg (HERMAWAN *et al.*, 2004; MALICKI *et al.*, 2004) in order to inactivate microorganisms, obtain quality measurements and extend shelf-life.

While inactivation of different microorganisms including bacteria, yeasts and molds that are pathogenic and cause food spoilage have been studied with PEF, studies related to the inactivation of different molds are limited. The use of PEF to inactivate *Byssochlamys fulva* conidiospores and *Neoartoria fischeri* ascospores suspended in apple, orange, pineapple, cranberry, grape, and tomato juices (RASO *et al.*, 1998) as well as *Byssochlamys nivea* ascospores has been studied (GRAHL and MARKL, 1996). Recently PEF inactivation of *Penicillum expansum* inoculated into sour cherry juice, peach and apricot nectars was investigated (EVRENDILEK *et al.*, 2008). While *P. expansum*, *B. fulva*, *N. fischeri* and *B. nivea* have been inactivated by using PEF, there is a lack of information regarding the use of PEF treatments to inactivate fungal pathogens, especially *Botrytis cinerea*. Grey mold disease caused by *B. cinerea* is one of the most serious diseases that affect a wide range of crops worldwide. Post harvest losses are very high due to the development of grey mold during storage and distribution of harvested fruits and vegetables (DAFERERA *et al.*, 2003). The

post-harvest *B. cinerea* contamination of fruit causes product loss in the fruit juice industry due to the production of a wide range of cell wall degrading enzymes, toxins and other low-molecular-weight compounds such as oxalic acid (WILLIAMSON *et al.*, 2007). Depending on the concentration of these compounds, they can affect the color, viscosity, clarity, taste and safety of fruit juices. Therefore, to insure product quality and safety it is important to inactivate *B. cinerea* in the fruit juice/nectar. In the fruit juice industry *B. cinerea* is currently inactivated but this method causes undesirable changes in the physical, chemical and sensory characteristics of the product. While different attempts have been made to inactivate *B. cinerea* in fruit and fruit juices (MARQUENIE *et al.*, 2002), the effect of PEF has not been reported. The objective of this study was to determine the level of inactivation of *B. cinerea* inoculated into sour cherry juice, apricot and peach nectars by using PEF. Electric field strengths and treatment times were varied and the level of inactivation was determined by measuring germination tube elongation and spore germination rate, and by light and scanning electron (SEM) microscopic observations. Given that spores are the primary source of initial mold contamination, the rate of spore germination was determined. In fungi, the conidia germination and germ-tube elongation are initial requisites for disease development; subsequent processes, including host penetration, ultimately contribute to the spread of disease and plant death. Germination was measured by germination tube elongation and the rate of spore germination.

MATERIALS AND METHODS

Test microorganism

Botrytis cinerea was isolated from infected strawberry fruit and maintained on potato dextrose agar (PDA) (Merck, Darmstadt, Germany). The culture was stored at 4°C and sub-cultured once a month. A spore suspension was prepared from a 2-week-old PDA culture. The spores were removed from the surface of the culture, and then suspended in 1,000 mL of sour cherry juice, apricot or peach nectar. Spore concentration was determined using a haemocytometer and adjusted to 10^5-10^6 spores mL^{-1} (SOYLU *et al.*, 2006).

Food samples

Freshly squeezed sour cherry, apricot and peach concentrates were obtained from Dimes Gıda Sanayii ve Tic. A.Ş. (Tokat, Turkey) in aseptic pouches. Sour cherry concentrate was diluted to 13.5°Brix, while apricot and peach concentrates were diluted to 11° and 10°Brix, respectively. The conductivity of sour cherry juice, apricot and peach nectar were measured as 3.64±0.17, 1.67±0.02 and 1.15±0.01 mS cm^{-1}, respectively. After preparation, the fruit juice/nectars were immediately subjected to PEF processing.

Pulsed electric field processing unit

A bench scale continuous PEF system [OSU-4A] was used to treat the inoculated samples (The Ohio State University, Columbus, OH, USA). Six treatment chambers connected in series were integrated into the PEF system. Each treatment chamber was constructed with a treatment zone of 0.29 cm in diameter and 0.23 cm in gap distance. The electrode material was stainless steel, and the insulator was made of Delrin® (Cole Palmer, Columbus, OH, USA). The contact between the electrode and insulator was air tight and designed specifically to prevent the sample from being trapped. The OSU-4A bench scale PEF generator provided square wave bipolar pulses. There was one positive and

one negative square wave pulse with a 20 μs pulse delay in between. The pre- and post-treatment temperatures (t_2-t_1, t_4-t_3, and t_6-t_5) at the inlet and outlet of each pair of treatment chambers were monitored by using K-type dual channel digital thermocouples (Fisher Scientific, Pittsburgh, PA) attached to the outer surface of the thin walled stainless steel tubing. The K-type thermocouple probe that was used had a time constant of 0.1 s and a response time of 0.5 s. Its resistance at 20°C was 24.08 Ω and the measuring range was -200° to 982°C (EVRENDILEK *et al.*, 2004b).

The treated sample was cooled after each pair of chambers by cooling coils submerged in a water bath at 12°C (Model RTE-111, NESLAB Instruments Inc., Newington, NH, USA). A trigger generator (Model 9300 series, Quantum Composers, Inc., Bozeman, MT, USA) was used to control the pulse duration time, pulse delaying time and pulse repetition rate. The voltage and current applied were monitored by a two-channel digital oscilloscope (Model TDS 320, Tektronix Inc., Beaverton, OR, USA). Specifications of the OSU-4A bench scale PEF units were 12,000 max output voltage, 60 A max output current, 10,000 pulse per second (pps) max repetition rate, 200-1,200 Ω load resistance and 16 J energy storage in the pulse generator when fully charged. Preliminary tests were performed to determine the PEF treatment conditions for sour cherry juice and peach and apricot nectars (EVRENDILEK *et al.*, 2004b).

Before and after each trial, tap water was pumped at a flow rate of 40 mL min^{-1} for 5 min to clean the system and remove all of the remaining sample. A solution of household bleach diluted with water at a 1:10 ratio was pumped through the system until the pHydrion Micro Chlorine strip (LaMotte, Chestertown, MD, USA) dipped into the sample outlet port indicated that the chlorine concentration was more than 200 ppm. The system was then soaked with this bleach solution for at least 5 min. Sterile de-ionized water was pumped through the system until the pHydrion Micro Chlorine strip dipped into the sample outlet port indicated that the chlorine concentration was zero ppm. The uninoculated sample was pumped through the system to further clean any remaining chlorine residues. The entire system was then flushed and filled with the uninoculated sample (EVRENDILEK *et al.*, 2004b).

PEF-processing parameters were applied as a function of electric field strength and treatment time. For this purpose, 0 (control), 17, 20, 23, 27, 30 and 34 $kVcm^{-1}$ electric field strength were applied under the following conditions: 45 mL min^{-1} flow rate, 3 μs pulse duration, 500 pulses per second (pps) frequency, and 163 μs treatment time. Similarly, 0 (control), 62, 94, 123, 163, 198 and 218 μs treatment times were applied with a 40 mL min^{-1} flow rate, 3 μs pulse duration, and 17 kV cm^{-1} electric field strength.

pH measurement

The pH measurements were performed on 10 mL samples at room temperature taken in triplicate using an Orion perpHect logR meter (Inolab WTW, Weilheim, Germany).

Determination of germination tube elongation and spore germination rate

Aliquots of 1,000 μL of control and PEF-treated sour cherry juice, peach and apricot nectars containing *B. cinerea* conidial suspensions at a concentration of 10^5 spores mL^{-1} were poured into Petri plates (ca. 20 mL/plate) containing potato dextrose agar (PDA, Difco, Darmstadt, Germany). All inoculated Petri plates were incubated for 24-48 h, at 22±2°C. Approximately 200 *B.*

cinerea spores were evaluated for germination tube elongation (μm) and the spore germination rate for each treatment within each triplicate using a microscope eyepiece graticule. A conidium was considered germinated if the germ tube length was 1-1.5 times the length of the conidium. Each treatment was replicated three times and the experiment was repeated twice. The growth values were obtained and then converted into the inhibition percentage of spore germination and germ tube elongation in relation to the control treatment (SOYLU *et al.*, 2006).

Light microscopy observation

Inoculated control and PEF-treated samples were collected, and 1 mL of samples was transferred onto PDA. After incubation of the PDA plates at 22±2°C for 24-48 h, a thin layer of agar disc was aseptically removed at one-day intervals and processed for light microscopy. Agar discs (1 mm thick) were cut from the growing edges, placed in a drop of glycerol on a microscope glass slide, and subsequently covered with a glass cover. Observations were made under a light microscope (Olympus BX-51, Tokyo, Japan).

Scanning electron microscopy observation

B. cinerea-inoculated control and PEF-treated samples were collected and centrifuged for 5 min at 3,000 rpm (Hettich Universal 32R D78532 model, Tuttlingen, Germany). After centrifugation, the conidial cells were collected and attached to double-sided carbon tape on stubs (1 cm in diameter). The fixed material was on stubs coated with gold/palladium in a sputter coater system in a high-vacuum chamber (Polaron SC7620, Watford, UK) for 150 s at 9 mA. The samples were examined, and digital images were captured using a JEOL JSM 5500 SEM (Croissy-sur-Seine, FR) at an accelerating voltage of 5 kV (SOYLU *et al.*, 2006).

Data analyses

Minitab software, version 12.1 (Minitab, Inc., State College, PA, USA) was used for the data analysis. One-way analysis of variance (ANOVA) was performed to compare the mean differences among the control and treated samples with respect to germination tube elongation and spore germination rate at 95% confidence interval. Duncan's Multiple Range Test was performed to determine differences among the treatments.

RESULTS AND DISCUSSIONS

PEF processing of sour cherry juice and apricot and peach nectars was carried out at electric field strengths of 0, 17, 20, 23, 27, 30 and 34 $kVcm^{-1}$ with 45 mL min^{-1} flow rate, 3 μs pulse duration, 500 pulses per second (pps) frequency, and 163 μs treatment time. Similarly, treatment times of 0, 62, 94, 123, 163, 198 and 218 μs were applied at 40 mL min^{-1} flow rate, 3 μs pulse duration, and 17 kV cm^{-1} electric field strength. Changes in the sample temperatures during PEF treatment were measured before and after each pair of treatment chambers. With the application of the highest electric field strength, 34 kV/cm, the initial sample temperature of 10°C increased 11°C and reached 21°C. Since the temperature increase was controlled by cooling coils, the PEF processing treatment was carried out at a temperature less than 22°C. Therefore, the whole treatment was non-thermal.

The pH values of the control samples were measured as 3.1±0.0, 3.8±0.0, and 3.9±0.0 for sour cherry juice, apricot and peach nectars, respectively. The increase in electric field strength did not cause

Table 1 - Germination tube elongation (μm) of *Botrytis cinerea* inoculated into sour cherry juice, apricot and peach nectars treated by pulsed electric fields as a function of electric field strength (kV/cm).

	Electric field strength (kV/cm)						
Food sample	**0 (control)**	**17**	**20**	**23**	**27**	**30**	**34**
Sour cherry juice	462±36^{a}	218±62^{b}	0.0±0^{c}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}
Apricot nectar	460±32^{a}	283±45^{b}	217±30^{c}	0.0±0.0^{d}	0.0±0.0^{d}	0.0±0.0^{d}	0.0±0.0^{d}
Peach nectar	440±34^{a}	387±21^{b}	333±38^{b}	219±30^{c}	0.0±0.0^{d}	0.0±0.0^{d}	0.0±0.0^{d}

*Data in the same row with different superscript letters are significantly different ($P \leq 0.05$)
**Processing parameters were 45 mL min^{-1} of flow rate, 3 μs of pulse duration, 500 pulse per second (pps) of frequency and 163 μs of treatment time.

any significant difference in the initial pH values of the samples ($P>0.05$). Similarly, increased treatment time did not cause any significant change in the initial pH values of the sour cherry juice and apricot and peach nectars ($P>0.05$).

The measurement of the germination tube elongation of *B. cinerea* in the controls of sour cherry juice and apricot and peach nectar samples were 462.4±36.6, 460.4±32.1 and 440.3±34.0 μm, respectively. As the electric field strength increased, the germination tube elongation greatly diminished ($P \leq 0.05$). No growth was observed in the sour cherry juice after 20 kV cm^{-1}, whereas, in apricot and peach nectars no growth was measured after 23 and 27 kV cm^{-1}, respectively. There were significant differences among sour cherry juice, peach nectar and apricot nectar with respect to the inhibition of germination tube elongation ($P \leq 0.05$) (Table 1).

With regards to the effect of treatment time, the germination tube elongation in the control samples of sour cherry juice and apricot and peach nectars were 412±63, 445±40 and 462±16 μm, respectively. These initial measurements decreased to zero when the treatment time was increased to 218 μs. No growth was observed at 94 μs treatment time in sour cherry juice, and at 123 and 198 μs treatment times in peach and apricot nectars, respectively ($P \leq 0.05$). There were significant differences among the sour cherry juice, apricot and peach nectars with respect to the inhibition of germination tube elongation ($P \leq 0.05$) (Table 2).

Table 2 - Germination tube elongation (μm) of *Botrytis cinerea* inoculated into sour cherry juice, apricot and peach nectars treated by pulsed electric fields as a function of treatment time (μs).

	Treatment time (μs)						
Food sample	**0 (control)**	**62**	**94**	**123**	**163**	**198**	**218**
Sour cherry juice	412±63^{a}	220±41^{b}	0.0±.0.0^{c}	0.0±.0.0^{c}	0.0±.0.0^{c}	0.0±.0.0^{c}	0.0±.0.0^{c}
Apricot nectar	445±40^{a}	393±35^{b}	209±31^{c}	0.0±.0.0^{d}	0.0±.0.0^{d}	0.0±.0.0^{d}	0.0±.0.0^{d}
Peach nectar	462±16^{a}	396±15^{a}	349±30^{b}	336±38^{c}	269±34^{c}	153±47^{c}	0.0±0.0^{d}

*Data in the same row with different superscript letters are significantly different ($P \leq 0.05$)
**Processing parameters were 40 mL min^{-1} of flow rate, 3 μs of pulse duration and 17 kV cm^{-1} of electric field strength.

Table 3 - Spore germination rate (%) of *Botrytis cinerea* inoculated into sour cherry juice, apricot and peach nectars treated by pulsed electric fields as a function of electric field strength (kV/cm).

	Electric field strength (kV/cm)						
Food sample	**0 (control)**	**17**	**20**	**23**	**27**	**30**	**34**
Sour cherry juice	100.0±0.0^{a}	4.2±2.3^{b}	0.0±.0.0^{c}	0.0±.0.0^{c}	0.0±.0.0^{c}	0.0±.0.0^{c}	0.0±.0.0^{c}
Apricot nectar	100.0±0.0^{a}	24.1±3.0^{b}	4.1±1.2^{c}	0.0±.0.0^{d}	0.0±.0.0^{d}	0.0±.0.0^{d}	0.0±.0.0^{d}
Peach nectar	100.0±0.0^{a}	82.5±2.7^{b}	51.9±2.3^{c}	11.7±2.1^{d}	0.0±.0.0^{e}	0.0±.0.0^{e}	0.0±.0.0^{e}

*Data in the same row with different superscript letters are significantly different ($P \leq 0.05$)
**Processing parameters were 45 mL min^{-1} of flow rate, 3 µs of pulse duration, 500 pulse per second (pps) of frequency and 163 µs of treatment time.

Applied electric field strength completely inhibited the spore germination rate in the control samples of sour cherry juice, apricot and peach nectars (100.0±0.00%). Complete inhibition was recorded at 20 kV cm^{-1} in sour cherry juice, 23 kV cm^{-1} in apricot nectar and 27 kV cm^{-1} in peach nectar ($P \leq 0.05$) (Table 3).

The rate of spore germination decreased as treatment time increased; eventually, spore germination was completely inhibited. The spore germination rates in the control samples of sour cherry juice and apricot and peach nectars were 99.8±0.4, 100.0±0.0 and 100.0±0.0%, respectively. No spore germination was recorded after 123 µs in sour cherry juice and apricot nectar, and 198 µs in peach nectar ($P \leq 0.05$) (Table 4).

The microscopic observation of PEF-treated sour cherry juice and peach and apricot nectars containing *B. cinerea* conidial suspension showed degenerative changes in the conidial morphology. Following exposure of the fungal conidia to the most efficient treatment (30 kV cm^{-1}), the non-germinated conidia had a degraded appearance, with large vesicles inside the cell walls. With PEF treatment, germination of the fungal conidia was suppressed and their structure was modified (Fig. 1). SEM observations revealed cytoplasmic coagulation, shrinkage, and a degraded morphology of the fungal spores after PEF treatments of 17 (Fig. 2B), 20 (Fig. 2C), 23 (Fig. 2D), 27

Table 4 - Spore germination rate (%) of *Botrytis cinerea* inoculated into sour cherry juice, apricot and peach nectars treated by pulsed electric fields as a function of treatment time (µs).

	Treatment time (µs)						
Food sample	**0 (control)**	**62**	**94**	**123**	**163**	**198**	**218**
Sour cherry juice	99.8±0.4^{a}	10.7±11.0^{b}	1.4±1.6^{c}	0.0±.0.0^{d}	0.0±.0.0^{d}	0.0±.0.0^{d}	0.0±.0.0^{d}
Apricot nectar	100.0±0.0^{a}	31.3±2.8^{b}	4.2±1.3^{c}	0.0±.0.0^{d}	0.0±.0.0^{d}	0.0±.0.0^{d}	0.0±.0.0^{d}
Peach nectar	100.0±0.0^{a}	100.0±0.0^{a}	90.8±2.8^{b}	56.6±6.1^{c}	44.7±3.1^{d}	16.1±2.5^{e}	0.0±0.0^{f}

*Data in the same row with different superscript letters are significantly different ($P \leq 0.05$)
**Processing parameters were 40 mL min^{-1} of flow rate, 3 µs of pulse duration and 17 kV cm^{-1} of electric field strength.

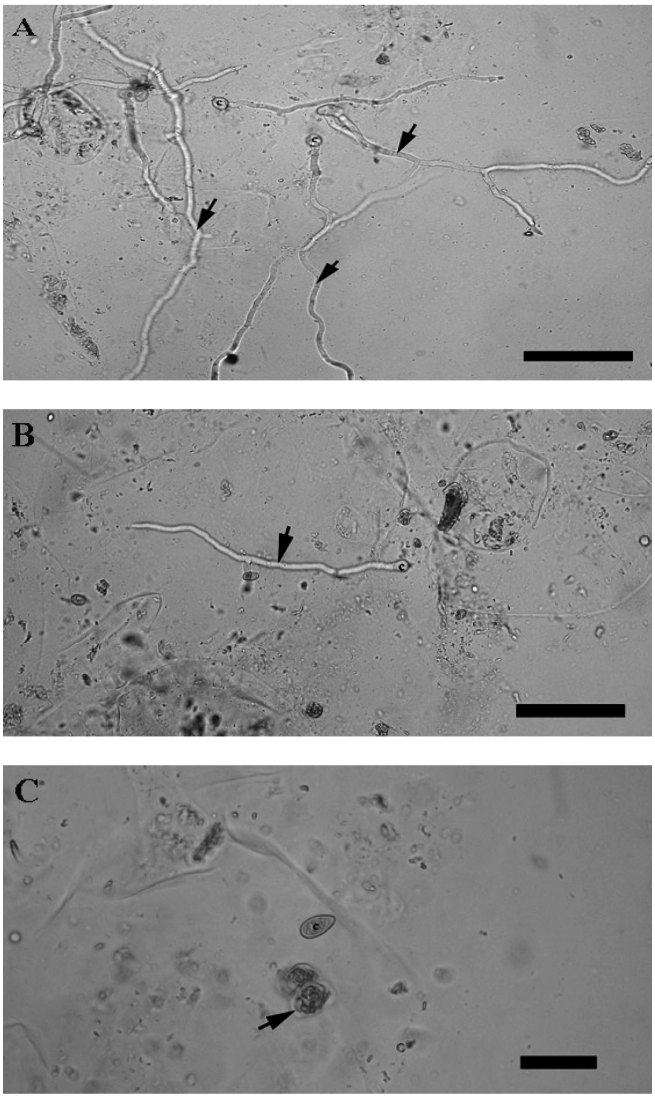


Fig. 1 - Effect of the different electric field strengths on conidial germination and fungal morphology of *B. cinerea* under light microscope. (A) Germinated conidia (c) with long healthy hyphae (arrows) in untreated control medium. Inhibitory effects of different electric currents (17 kV cm^{-1}) on hyphal elongation (B) and morphology of conidia (treated by 30 kV cm^{-1}) (C). Bars, A=10 μm; B and C=50 μm. PEF processing parameters 45 mL min^{-1} of flow rate, 3 μs of pulse duration, 500 pulse per second (pps) of frequency and 163 μs of treatment time.

(Fig. 2E) and 30 (Fig. 2F) kV cm^{-1} (Fig. 1C) when compared to the control samples (Fig. 2A). With PEF, fungal growth was suppressed and the hyphal structure displayed several morphological changes. In contrast to the control samples, in which the surfaces of the conidia were smooth, the surfaces of the PEF-treated conidia were very rough and caved in on many sides (Fig. 2).

The degree of inhibition of germination tube elongation and spore germination rate as a function of electric field strength and treatment time was in the order of sour cherry juice ≥ apricot nectar > peach nectar. Inhibition of *B. cinerea* germination tube elongation and spore germination rate as a function of electric field strength and treatment time in sour cherry juice was higher than that in both apricot and peach nectars.

It has been reported that microbial inactivation by PEF is affected by the pH and conductivity of the food matrix. Increased acidity causes an increase in microbial inactivation (VEGA-MERCADO *et al.*, 1996). The low electrical conductivity increases the difference in the electrical conductivity between the medium and the microbial cytoplasm. This increased difference in the electrical conductivity weakens the membrane structure of microorganisms due to an increased flow of ionic substances across the membrane during PEF treatment (RASO *et al.*, 1998). It has been reported that lower electrical conductivity causes higher electric field strength and, thus, the higher microbial inactivation. The pH values of the sour cherry juice, apricot and peach nectars were 3.1±0.0, 3.8±0.0 and 3.9±0.0, respectively. Al-

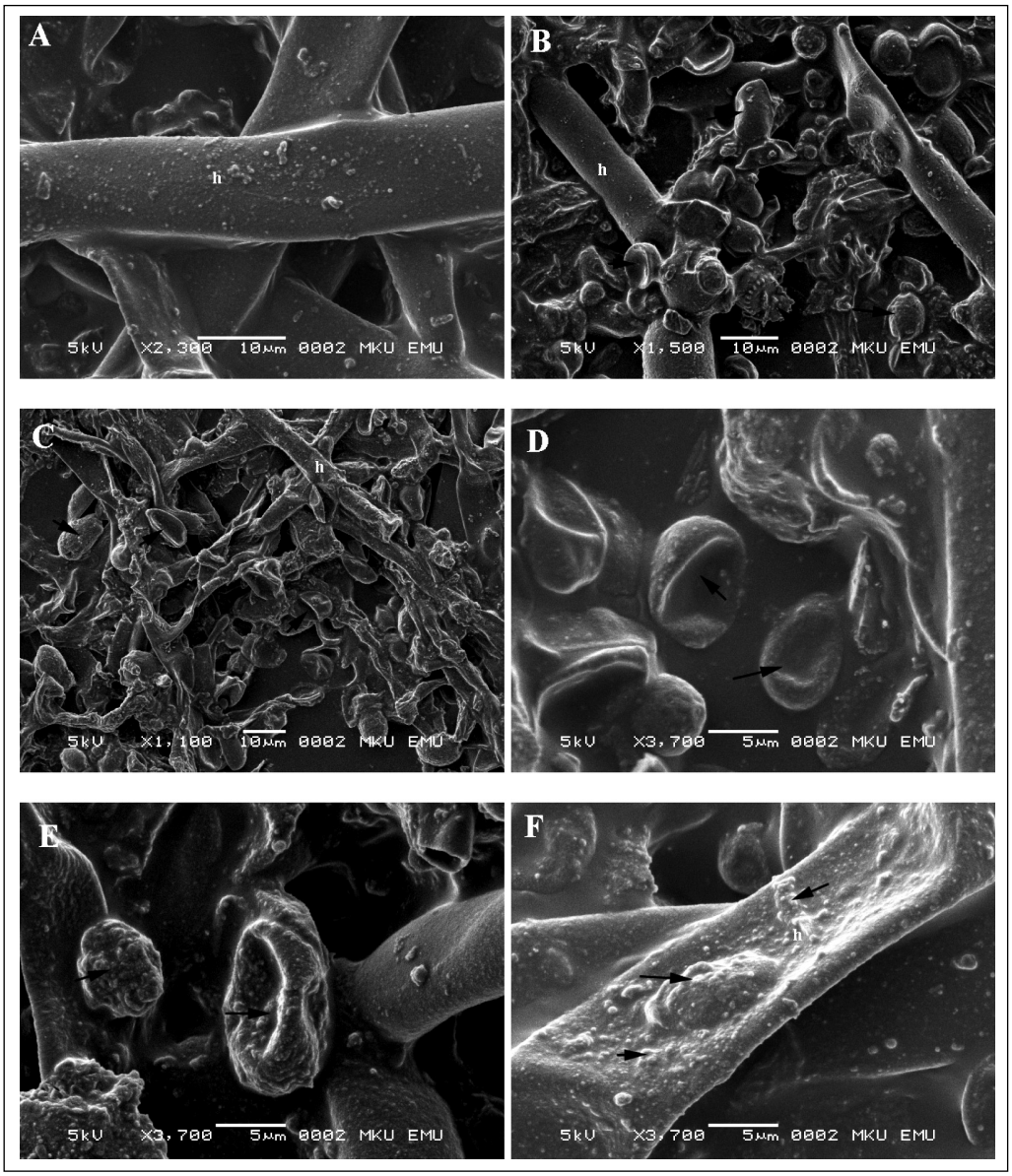


Fig. 2 - Effect of the different electric field strengths on conidial germination and fungal morphology of *B. cinerea* under scanning electron microscope. (A) Germinated conidia (c) with long healthy germ tube (arrow) in untreated control medium. Inhibitory effects of different electric currents on germination tube elongation and fungal morphology for 17 kV cm^{-1} (B), 20 kV cm^{-1} (C), 23 kV cm^{-1} (D), 27 kV cm^{-1} (E) and 30 kV cm^{-1} (F). PEF processing parameters were 45 mL min^{-1} of flow rate, 3 µs of pulse duration, 500 pulse per second (pps) of frequency and 163 µs of treatment time.

though sour cherry juice, apricot and peach nectars are highly acidic foods, the pH of the sour cherry juice was lower than that of the apricot and peach nectars. Due to the lower pH of sour cherry juice, the inactivation of *P. expansum* in sour cherry juice was higher than that the apricot and peach nectars.

This is the first report of a study that used germination tube elongation and spore germination rate of *B. cinerea* inoculated into sour cherry juice, peach and apricot nectars processed by PEF. Except for the inactivation of *P. expansum*, most previous studies using PEF have been related to the inactivation of different bacteria, yeasts and molds. Inactivation by PEF processing of *Byssochlamys fulva* conidiospores and *Neosartoria fischeri* ascospores suspended in different fruit juices showed that the *B. fulva* conidiospore inactivation increased when the number of pulses applied was increased and the hierarchy of effectiveness for conidiospore inactivation was cranberry > grape > pineapple > orange > apple > tomato. There was, no inactivation of *N. fischeri* in cranberry juice, even after 40 pulses of treatment at 51.0 kV cm^{-1} (RASO *et al.*, 1998).

In contrast to the present study, previous studies dealing with the inactivation of different molds have focused mainly on the inactivation of conidiospores and/or ascospores. Therefore, the results cannot be compared easily. However, the results of this study are parallel to the inactivation of *P. expansum* in that with increased electric field strength and treatment time there was complete inactivation of the germination tube elongation and spore germination rate in the fungus (EVRENDILEK *et al.*, 2008).

Similar effects were reported with PEF-treated microbial cells as observed by microscopic examinations. The physical effects of PEF on the inactivation of diarrhoeagenic *Bacillus cereus* cells suspended in 0.1 g L^{-1} peptone water were examined by transmission electron microscopy (TEM). Those investigations revealed an irreversible rupture of the cell membrane at a number of locations, which resulted in the apparent loss of intracellular contents (ROWAN *et al.*, 2000).

PEF treatments of *Escherichia coli* subjected to 60 pulses at 41 kV cm^{-1} were examined by TEM and SEM. Changes in the cytoplasm were observed and the cell surface appeared rough. The outer membranes of the cells were partially destroyed which resulted in leakage of cell cytoplasm (DUTREUX *et al.*, 2000). SEM and TEM observations of *S. aureus* cells inoculated in a model food and subjected to 64 pulses at 20, 40 and 60 kV cm^{-1} presented ultrastructural changes. In addition, SEM observations revealed surface roughness after treatment with electric field (POTHAKAMURY *et al.*, 1997). Our conclusions were similar to those reported in the literature; PEF treatment causes structural damage in microbial cells.

Light and SEM observations of *P. expansum* were similar to those obtained in this study. PEF treatment caused marked damage to the *P. expansum* conidia compared to non-treated controls. When electric field strength was applied there were morphological alterations in the hyphal morphology, shrinkage, shriveled hyphal aggregates, reduced hyphal diameters and lyses of hyphal wall (EVRENDILEK *et al.*, 2008).

CONCLUSIONS

Processing of different fruit juices and/or nectars is important to evaluate the potential use of PEF technology. The study on the inactivation of plant pathogen fungi such as *B. cinerea* provides information that can be used to extend PEF technology in fruit juice processing. In this study the effect of PEF on the spore germination rate, germination tube elongation and structural changes of *B. cinerea* was explored. More studies are needed on the inactivation of different molds in fruit juices/nectars with inactivation kinetics, germination tube elongation and spore germination rate, and on the changes in the physical, chemical and sensory properties of fruit juices/nectars at the electric field strengths

that provide microbial inactivation. This approach could be an alternative way to eliminate *B. cinerea* contamination in the fruit juice industry.

While these results demonstrate the degenerative changes caused by PEF treatment on conidia, the mechanisms of action have not been well documented. The general changes in the morphology of the hyphae and conidia could be due to the loss of cell wall integrity. Consequently plasma membrane permeability could be affected, which would explain the changes in the morphology and size of the internal organelles. Further studies are needed to explain the effect of the PEF mechanism on fungal hyphae and conidia.

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PAPER

ANTIOXIDANT ACTIVITIES OF THE ESSENTIAL OILS AND EXTRACTS OF PORTUGUESE *THYMBRA CAPITATA* AND *THYMUS MASTICHINA*

ATTIVITÀ ANTIOSSIDANTE DI OLI ESSENZIALI ED ESTRATTI DI *THYMBRA CAPITATA* E *THYMUS MASTICHINA* PORTOGHESI

J. BENTES, M.G. MIGUEL*, I. MONTEIRO[1], M. COSTA[1], A.C. FIGUEIRED, J.G. BARROSO[2] and L.G. PEDRO[2]

Faculdade de Engenharia de Recursos Naturais, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

[1]Direcção Regional de Agricultura e Pescas do Algarve, Apartado 282, Braciais, Patacão, 8001-904 Faro, Portugal

[2]Universidade de Lisboa, Faculdade de Ciências de Lisboa, DBV, Centro de Biotecnologia Vegetal, C2, Campo Grande, 1749-016 Lisbon, Portugal

*Corresponding author: Tel. +351289800900, Fax +351 289818419, e-mail: mgmiguel@ualg.ptxy

ABSTRACT

The antioxidant activities of the essential oils (EO), the remaining water-soluble hydrodistillation-aqueous phase (RHAPhW) and chloroform-soluble phases (RHAPhC) and the hexane (HEX), dichloromethane (DCM) and methanol (MeOH) extracts from the dried flowering aerial parts of *Thymbra capitata* and *Thymus mastichina* were

RIASSUNTO

Sono state valutate le attività antiossidanti degli oli essenziali (OE), della fase idrosolubile residua dell'idrodistillazione (RHAPhW) delle fasi solubili in cloroformio (RHAPhC) e degli estratti in esano (HEX), diclorometano (DCM) e metanolo (MeOH) di parti aeree essiccate di *Thymbra capitata* e *Thymus mastichina*. Gli EOs sono stati analizzati

- Key words: antioxidant activity, essential oils, hydrodistillation-aqueous phase, *Thymbra capitata*, *Thymus mastichina* -

evaluated. The EOs were analyzed by GC and GC-MS. The total phenol content was determined by the Folin-Ciocalteu method. The antioxidant activity was evaluated by DPPH, TBARS, reductive potential, metal chelating and by superoxide anion scavenging activity. Carvacrol (75%) and 1,8-cineole (44%) were the main components of the *T. capitata* and of *Th. mastichina* EOs, respectively. The DCM extracts of *T. capitata* and the MeOH extracts of *Th. mastichina* had the highest phenol contents. The RHAPhWs of both plant species were the best free radical scavengers. The HEX and MeOH extracts of *T. capitata* prevented lipid peroxidation most effectively. RHAPhWs and MeOH extracts of *T. capitata* and *Th. mastichina*, respectively, had the strongest reducing activity. Only the MeOH extracts of both species were able to chelate Fe(II) and only RHAPhWs and MeOH extracts were able to scavenge superoxide radicals.

via GC e GC-MS. il contenuto in polifenoli totali è stato determinato con il metodo Folin-Ciocalteu. L'attività antiossidante è stata valutata per mezzo di DPPH, TBARS, potenziale riducente, metalli chelanti e per mezzo di attività di scavenging di anioni superossido. Il carvacrolo (75%) e il 1,8-cineolo (44%) erano, rispettivamente, i maggiori rappresentanti degli EOs della *T. capitata* e della *Th. mastichina*. Le RHAPhWs di entrambe le tipologie di piante erano le migliori radical scavengers. Gli estratti HEX e MeOH della *T. capitata* prevenivano molto efficacemente la perossidazione lipidica. Le RHAPhWs e gli estratti MeOH della *T. capitata* e della *Th. mastichina*, rispettivamente, presentavano le più alte attività riducenti. Solo gli estratti metanolici di entrambe le specie presentavano la capacità di chelare il Fe (II) e solo le RHAPhWs e gli estratti MeOH erano capaci di attività di scavenging dei superossi radicali.

INTRODUCTION

Lipid oxidation in foodstuffs is undesirable because it negatively affects the quality due to the formation of unpleasant volatile compounds. In order to extend the shelf life of a product, the food industry uses antioxidants that are chain-breaking inhibitors of lipid peroxidation. Many synthetic antioxidants are currently used, such as butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), gallates and *tert*-butylhydroquinone (TBHQ), since they provide good protection for unsaturated fats and oils at low cost and high stability (TSIMIDOU and BOSKOU, 1994; ÖZCAN and AKGÜL, 1995; DAPKEVICIUS *et al.*, 1998; BELITZ and GROSCH, 1999). There is also increasing interest in the food industry to find natural antioxidants because they are potentially less toxic. The search for natural antioxidants includes spices and aromatic plants because they are known to improve the organoleptic properties in products as well as contribute to their preservation (STASHENKO *et al.*, 2002; POLITEO *et al.*, 2006; BOZIN *et al.*, 2006).

Eleven species of *Thymus*, with a total of fourteen taxa, can be found in Portugal. These taxa belong to five of the eight sections of this genus. *Thymbra capitata* (L) Cav. from the genus *Thymbra* is also largely distributed throughout Portugal. The ability of the essential oils from Portuguese thyme species and *T. capitata* to prevent lipid peroxidation has been evaluated (FIGUEIREDO *et al.*, 2008 and references therein). These studies demonstrated that the chemical composition of the essential oils and the type of lipid

substrates were responsible for the antioxidant activities of the oils. Data concerning the antioxidant activity of extracts from these Portuguese plants is scarce or non-existent.

In the present study, the antioxidant activities of the essential oils, and the remaining hydrodistillation-aqueous phase extracts and Soxhlet extracts isolated from *T. capitata* and *Th. mastichina* were assessed by using five different methods. The results were then compared.

MATERIAL AND METHODS

Plant material

The aerial parts (leaves + flowers + stems) of *T. capitata* and *Th. mastichina*, collected in the flowering phase, were kindly provided by Direcção Regional de Agricultura e Pescas do Algarve (Portugal), in June 2004, and dried in the dark, at room temperature, at the University of Algarve (Portugal).

Essential oil isolation (EO)

The essential oils were isolated from the dried plant material (100 g) by hydrodistillation for 3 h using a Clevenger-type apparatus according to the European Pharmacopoeia method (ANONYMOUS, 1996). The essential oils were stored at -20°C in the dark prior to analysis.

Preparation of the extracts

Remaining hydrodistillation-aqueous phase extracts (RHAPh)

After completion of hydrodistillation, the remaining hydrodistillation-aqueous phase (RHAPh) was collected and concentrated under vacuum at 60°C. This extract was re-dissolved in water and fractionated with chloroform to obtain water-soluble (RHAPhW) (4.1 and 9.7%, w/w, for *T. capitata* and *Th. mastichina*, respectively) and chloroform-soluble (RHAPhC) fractions (0.3 and 0.5%, w/w, for *T. capitata* and *Th. mastichina*, respectively).

Preparation of the hexane (HEX), dichloromethane (DCM) and methanol (MeOH) extracts

A portion of dried plant material was extracted with HEX (1.9 and 4.0%, w/w, for *T. capitata* and *Th. mastichina*, respectively), followed by DCM (2.4 and 9.0%, w/w, for *T. capitata* and *Th. mastichina*, respectively), and MeOH (13.2 and 21.2%, w/w, for *T. capitata* and *Th. mastichina*, respectively) in a Soxhlet apparatus (6 h for each solvent); the temperature never exceeded 40°C. All extracts were kept in the dark at -4°C until use.

Determination of total phenols

The total phenol contents in the hydrodistillation-aqueous phase extracts and Soxhlet extracts were determined using the Folin-Ciocalteu reagent and gallic acid as standard as described by SLINKARD and SINGLETON (1977). The sample (0.5 mL) and 2 mL of sodium carbonate (75 g/L) were added to 2.5 mL of 10% (v/v) Folin-Ciocalteu reagent. After 30 min of reaction at room temperature, the absorbance was measured at 765 nm in a Shimadzu 160-UV (Tokyo, Japan) spectrophotometer. The results are given as gallic acid equivalent /g extract (GAE/g extract).

Essential oil analysis

Gas Chromatography (GC)

Gas chromatographic analyses were performed using a Perkin Elmer Autosystem XL (Perkin Elmer, Shelton, CT, USA) gas chromatograph equipped with two flame ionization detectors (FIDs), a data handling system and a vaporizing injector port into which two columns of

different polarities were installed: a DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm) (J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column (30 m x 0.25 mm i.d., film thickness 0.15 μm) (J & W Scientific Inc.). Oven temperature was programmed, 45°-175°C, at 3°C/min, subsequently at 15°C/min up to 300°C, and then held isothermal for 10 min; injector and detector temperatures, 280° and 300°C, respectively; carrier gas, hydrogen, was adjusted to a linear velocity of 30 cm/s. The samples were injected using split sampling technique, ratio 1:50. The volume of injection was 0.2 μL of a pentane-oil solution. The percentage composition of the oils was computed by the normalization method from the GC peak areas, calculated as mean values of two injections from each oil, without using correction factors.

Gas chromatography-mass spectrometry (GC-MS)

The GC-MS unit consisted of a Perkin Elmer Autosystem XL (Perkin Elmer, Shelton, CT, USA) gas chromatograph, equipped with a DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm) (J & W Scientific, Inc.), and interfaced with a Perkin-Elmer Turbomass mass spectrometer (software version 4.1, Perkin Elmer, Shelton, CT, USA). Injector and oven temperatures were as above; transfer line temperature, 280°C; ion trap temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; ionization current, 60 μA; scan range, 40-300 u; scan time, 1 s. The identity of the components was assigned by comparison of their retention indices, relative to C_9-C_{16} *n*-alkane indices and GC-MS spectra from a home-made library, constructed based on the analyses of reference oils, laboratory-synthesised components and commercially available standards.

Antioxidant activity evaluation

From each sample, different concentrations of essential oils and extracts were prepared in methanol (10-1,000 mg/L). The antioxidant activity of each sample was evaluated using five different methods: free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH), thiobarbituric acid reactive substances assay (TBARS), reductive potential, metal chelating and by superoxide anion scavenging activity. All determinations were performed in triplicate. The results are reported as the mean ± standard deviation.

Free radical scavenging activity (DPPH)

A methanolic stock solution (50 μL) of each sample [essential oils, extracts and positive control (α-tocopherol)] at different concentrations (10-1,000 mg/L) was placed in a cuvette, and 2 mL of 60 μM methanolic solution of DPPH was added (BRAND-WILLIAMS *et al.*, 1995). Absorbance measurements were made at 517 nm using a Shimadzu 160-UV spectrophotometer (Tokyo, Japan) after 5 min of reaction at room temperature. Absorption of a blank sample containing the same amount of methanol and DPPH solution acted as negative control. The percentage inhibition of the DPPH radical by the samples was calculated according to the following formula:

$$\text{Scavenging effect \%} = [(A_0 - A_1) / A_0] * 100$$

where A_0 was the absorbance of the blank sample and A_1 was the absorbance in the presence of the sample.

Thiobarbituric acid reactive substances (TBARS)

The experiment was based on a modified thiobarbituric acid (TBA) reactive substances assay (TBARS) to measure the antioxidant ability of the samples

and that of α-tocopherol (positive control). Egg yolk homogenate was used as lipid-rich media obtained as described by DORMAN *et al.* (1995), that is, an aliquot of yolk material was made up to a concentration of 10% (w/v) in KCl (1.15%, w/v). The yolk was then homogenized for 30 s followed by ultrasonication for 5 min. Five hundred μL of the homogenate and 100 μL of sample, dissolved in methanol, were added to a test tube and made up to 1 mL with distilled water; 1.5 mL 20% acetic acid (pH3.5) and 1.5 mL 0.8% (w/v) TBA in 1.1% (w/v) sodium dodecyl sulphate (SDS) were then added. This mixture was stirred in a vortex and heated at 95°C for 60 min. After cooling at room temperature, 5 mL of *n*-butanol were added to each tube, stirred and centrifuged at 3,000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm using a Shimadzu 160-UV spectrophotometer. All the values are expressed as antioxidant index (AI%); the control was completely peroxidized and each oil and tested substance demonstrated a comparative percentage of antioxidant protection. The AI% was calculated using the formula: *(1–t/c)x100*, c being the absorbance value of the fully oxidized control and t, the absorbance of the tested sample.

Reductive potential

The reductive potential was determined according to the method previously described (OYAIZU 1986). Each sample or the positive control (ascorbic acid) was mixed with phosphate buffer (2.5 mL, 0.2 M, pH6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 3,000 rpm. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm in a Shimadzu 160-UV spectrophotometer.

Chelating activity

The Fe^{2+}-chelating ability of all samples was determined according to the method of CARTER (1971). One hundred μL of each sample or positive control (EDTA) were incubated with 100 μL of $FeCl_2$ and 3.7 mL of methanol. The reaction was initiated by the addition of 100 μL of ferrozine and, after the mixture had reached equilibrium (10 min), the absorbance at 562 nm was read with a Shimadzu 160-UV spectrophotometer. The percentage of inhibition of the complex ferrozine-Fe^{2+} was calculated using the following equation:

$$\text{Inhibition \%} = [(A_0 - A_1) / A_0] * 100$$

where A_0 was the absorbance of the control (without sample: essential oil or extract) and A_1 was the absorbance in the presence of the sample.

Superoxide anion scavenging activity

Measurements of superoxide anion scavenging activity of samples and positive control (ascorbic acid) were based on the method described by SOARES (1996). Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system by oxidation of NADH and assayed by reduction of nitroblue tetrazolium (NBT). The superoxide anion was generated in 3 mL of phosphate buffer (19 mM, pH7.4), containing NBT (43 μM) solution, NADH (166 μM) solution and different concentrations of extracts. The reaction was started with the addition of PMS solution (2.7 μM) to the mixture. The reaction mixture was incubated at 20°C for 7 min and absorbance at 560 nm was recorded against blank samples in a Shimadzu 160-UV spectrophotometer. The percentage of inhi-

bition was calculated using the following equation:

$$\text{Inhibition } \% = [(A_0 - A_1) / A_0] * 100$$

where A_0 was the absorbance of the control (without sample) and A_1 was the absorbance in the presence of the sample.

RESULTS AND DISCUSSION

Essential oils composition

The essential oils isolated by hydrodistillation from the dried flowering aerial parts of *T. capitata* and *Th. mastichina* were obtained in average yields of 4% (w/w).

Carvacrol (75%) and 1,8-cineole (44%) were the most representative components in the essential oils of *T. capitata* and *Th. mastichina*, respectively. The carvacrol precursor *p*-cymene (10%) and γ-terpinene (4%) were the next most important components in *T. capitata* oil, whereas in *Th. mastichina* oil, camphor (10%), borneol (7%), camphene (7%), α-pinene (6%), α-terpineol (5%) and β-pinene (4%) were also present in relatively high amounts.

The level of carvacrol present in *T. capitata* oil was in accord with that previously reported for the same species collected in different places, isolated from different plant parts and at different developmental stages (BOUNATIROU *et al.*, 2007).

The dominance of 1,8-cineole is a typical characteristic of *Th. mastichina* oils in the Iberian Peninsula (SALGUEIRO *et al.*, 1997; FIGUEIREDO *et al.*, 2008 and references therein).

Total phenols

The total amounts of phenols in the extracts of *T. capitata* and *Th. mastichina* were determined using the Folin-Ciocalteu method. With the exception of the DCM Soxhlet extract of *Th. mastichina*, the lowest amounts of phenolic compounds were found in the extracts derived from the remaining hydrodistillation-aqueous phase.

The highest phenolic content in *T. capitata* was found in the DCM and MeOH extracts (297 and 139 GAE/g extract, respectively). The MeOH extract of *Th. mastichina* had the highest amount of phenolic compounds (206G AE/g extract). The quantification of phenols in the HE extracts was not possible due to the formation of a precipitate in the reaction system. In spite of the inability to determine the phenol content in the HE extracts, it was possible to detect antioxidant activity determined by DPPH, TBARS and iron(III) to iron(II)-reducing assays, which indicated the presence of compounds that were able to prevent oxidation. Oleoresin could have been responsible for such activity. According to TEPE *et al.* (2005) oleoresin, which is present in hexane extracts, may possess moderate antioxidant activities.

Antioxidant activity

The antioxidant activities of the essential oils, as well as those of the different extracts of *T. capitata* and *Th. mastichina* were screened by five different methods: DPPH free radical scavenging, thiobarbituric acid reactive substances (TBARS) for measuring the inhibition of lipid peroxidation, iron(III) to iron(II)-reducing activity, iron(II) chelating activity and superoxide radical scavenging. This approach is crucial because extracts and essential oils are complex systems and different mechanisms may contribute to the oxidizing processes. In the iron(III)-reduction assay, the general ability of the extracts to donate electrons is evaluated, while in the DPPH assay hydrogen atoms are also involved. Transition metal ions serve as catalysts in Fenton reactions and therefore one important mechanism of antioxidant action may be

the chelation of iron(II) ions. During the first step of oxidation, polyunsaturated fatty acids produce hydroperoxides that decompose giving rise to diverse components: aldehydes, ketones, hydroxyacids, hydrocarbons and polymers. One mechanism of antioxidant action is to prevent the formation of these secondary components; this can be evaluated by the TBARS method. Different reactive oxygen species (ROS) may be generated and several target structures such as lipids, proteins and carbohydrates can be affected. Superoxide radical anion is one of the ROS that can be generated and one mechanism of antioxidant action is to scavenge it.

DPPH free radical scavenging

DPPH$^{\bullet}$ is a violet stable nitrogen-centered free radical that turns yellow after being reduced, by either hydrogen- or electron-donation. When a compound can perform this reaction it is considered to be an antioxidant and therefore a radical scavenger (BRAND-WILLIAMS *et al.*, 1995).

Of the assayed samples, the *T. capitata* essential oil (EO) had the least capacity to scavenge DPPH free radicals (Fig. 1a); at 100 mg/L, the lowest concentration assayed for the essential oils, the inhibition percentage was only 11%, followed by the HEX extract (24%). Only at 1,000 mg/L did the EO show a comparable activity to that of RHAPhC, DCM and MeOH extracts at 100 mg/L. The remaining hydrodistillation-aqueous water-soluble (RHAPhW) extract showed the best activity, with percentages ranging from 15 to 73%, at 10 and 100 mg/L, respectively. All samples showed inhibition percentages that were noticeably lower than those observed for the positive control (α-tocopherol). In the same concentration range of 10-100 mg/L, α-tocopherol showed a free radical scavenging activity between 30 and 93%.

When evaluated by this methodology, the *Th. mastichina* essential oil was almost totally ineffective as an antioxidant (Fig. 1b), in contrast to the RHAPhW extract that showed similar activity to that of α-tocopherol, particularly at higher concentrations (75 and 100 mg/L). This result indicates that a considerable portion of the antioxidant substances was retained in the remaining hydrodistillation-aqueous water. This extract was better at free radical scavenging than the corresponding one from *T. capitata*. The free radical scavenging activity of RHAPhW- and MeOH extracts of *Th. mastichina* was higher than that recorded for the remaining extracts. TEPE *et al.* (2004) also reported that the polar extracts of *Thymus eigii* had a stronger antioxidant capacity than the apolar ones.

TBARS assay

TBARS assay evaluates the secondary lipid peroxidation product, malonaldehyde. This compound reacts with thiobarbituric acid (TBA) producing a pink pigment that can be determined spectrophotometrically at 532 nm (BERSET and CUVELIER, 1996).

The antioxidant indexes (AI%) of the RHAPhW- and RHAPhC extracts of *T. capitata* and *Th. mastichina* could not be evaluated by this method due to the formation of a deep red color that prevented the quantification. BURITS *et al.* (2001) also reported the production of red and yellow chromogens when the antioxidant activity of *Artemisia abyssinica* was determined. According to the authors, the formation of these pigments was due to the presence of aldehydes such as 2-butenal, 2-hexen-1-al or octadecenal. In the present case, other components may be responsible for the formation of the red color, since this type of aldehyde would have been isolated during hydrodistillation.

The essential oil of *T. capitata* had a stronger antioxidant activity than that of *Th. mastichina* when evaluated by this

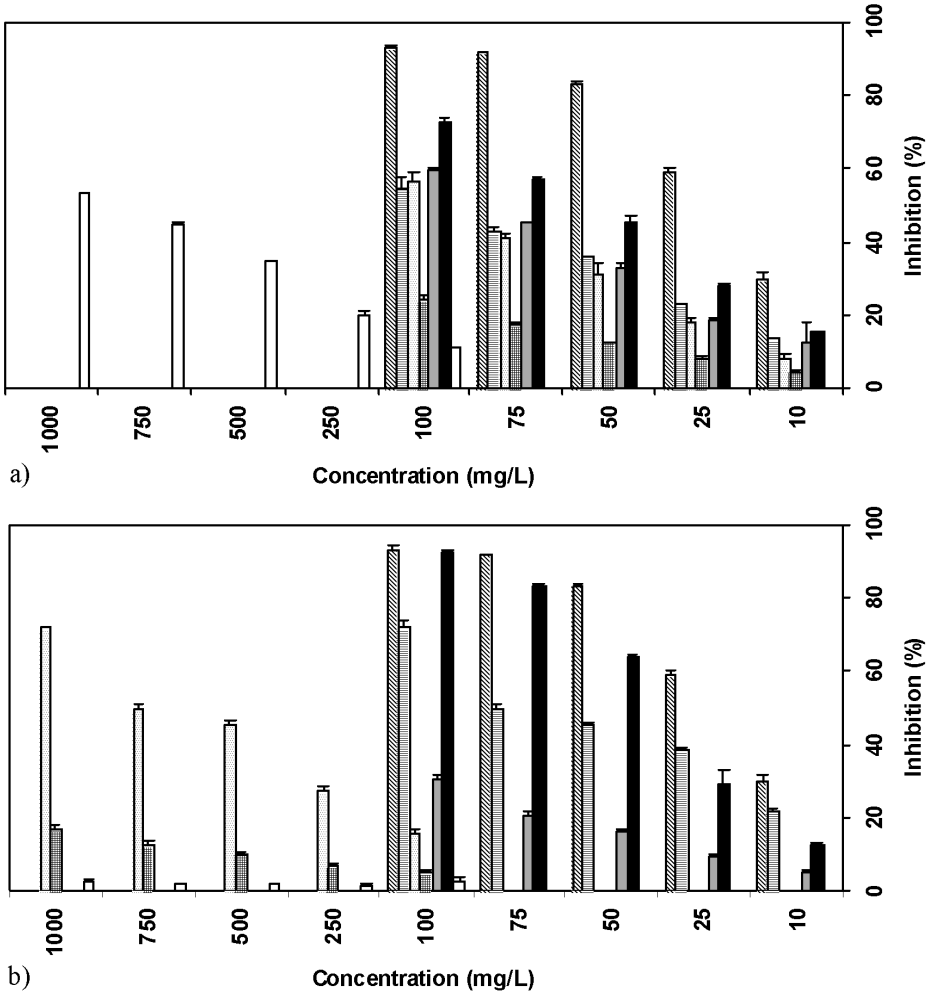


Fig. 1 - DPPH free radical scavenging activity of the essential oils (□), of the remaining hydrodistillation-aqueous water-soluble (■) and chloroform-soluble phases (▤) and of the hexane (⊞), dichloromethane (⊡) and methanol (▥) Soxhlet extracts of *T. capitata* (a), *Th. mastichina* (b) and the positive control (α-tocopherol, ▨) at different concentrations. The bars indicate ± SE (n=3).

method (Fig. 2a and b). The percentages of inhibition obtained with both essential oils agree with those already reported for the same species also collected in Portugal, particularly at the higher concentrations (MIGUEL *et al.*, 2007; FALEIRO *et al.*, 2005).

The HEX and MeOH Soxhlet extracts of *T. capitata* were more effective in preventing lipid peroxidation than the corresponding extracts from *Th. mastichina* (Fig. 2a and b). At 100 mg/L, the MeOH extract of *T. capitata* showed an AI of 52%, while the MeOH extract of *Th. mastichina* showed no activity. At concentrations higher than 500 mg/L, the AIs of the DCM and MeOH extracts of *Th. mastichina* decreased.

Reductive potential

Fe(III) reduction can be used as an indicator of electron-donating activity and therefore reflects an important mecha-

nism of the phenolic antioxidant action (YILDIRIM *et al.*, 2001).

In this study, the reducing power was evaluated by the ferric-ferrous transformation monitored at 700 nm. The reducing ability generally increased with increasing sample concentration (Fig. 3a and b). The essential oils of *T. capitata* had a higher reducing activity than the essential oils of *Th. mastichina*. These results showed a positive correlation between DPPH radical scavenging ability and the reducing power assay, which indicated that the reducing ability of the *T. capitata* oils contributed in part to the antioxidant activity, as already reported by some authors (CHANG *et al.*, 2002; DUAN *et al.*, 2007). However, the correlation between DPPH and the reducing activity assays was not as clear in the evaluation of the different extracts. Whereas the RHAPhW extracts of both plant species were the best antioxidants when evaluated by DPPH, the MeOH extracts from *Th. mastichina* had a greater capacity for reducing Fe(III) than the RHAPhW extract (Fig. 3b). HINNEBURG *et al.* (2006) also reported the absence of any correlation in the evaluation of antioxidant activities of extracts from selected culinary herbs and spices.

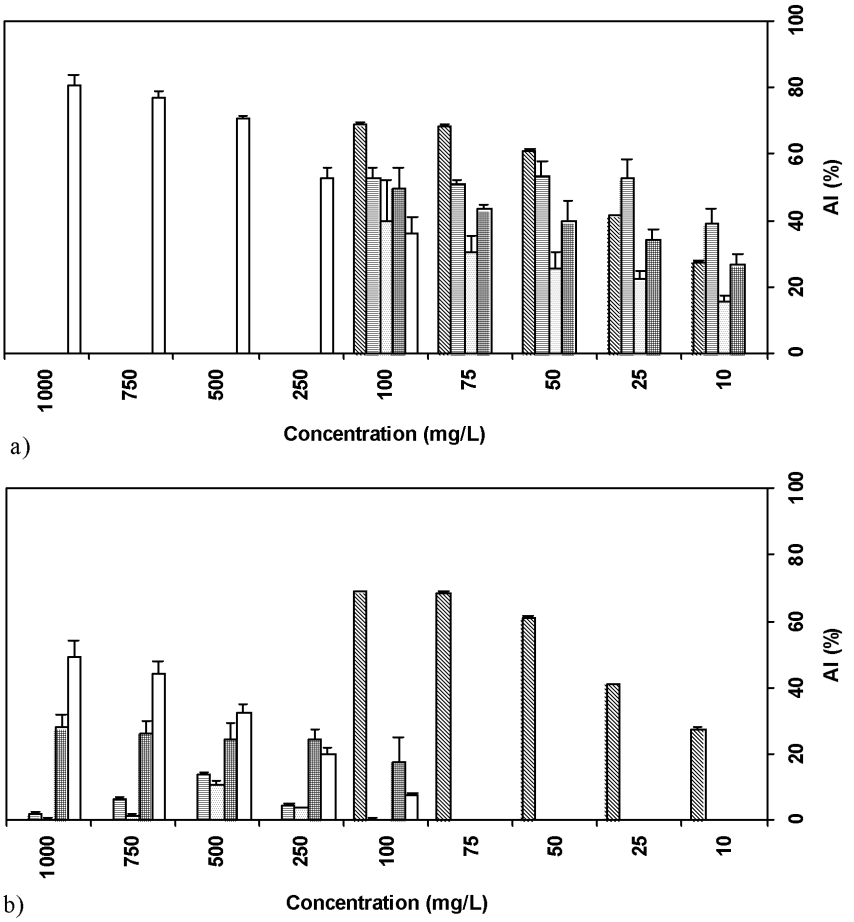


Fig. 2 - TBARs antioxidant indices of the essential oils (□) and of the hexane (▦), dichloromethane (▩) and methanol (▥) Soxhlet extracts of *T. capitata* (a), *Th. mastichina* (b) and the positive control (α-tocopherol, ▨) at different concentrations. The bars indicate ± SE (n=3).

Chelating activity

Bivalent transition metal ions can lead to the formation of hydroxyl radicals and hydroperoxide decomposition reactions via Fenton chemistry (HINNEBURG *et al.*, 2006). Iron is the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron can stimulate lipid peroxidation by the Fenton reaction, and also accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that, in turn, remove hydrogen, perpetuating the chain reaction of lipid peroxidation (PEKSEL *et al.*, 2006). Ferrozine, a chelating agent, was used to indicate the presence of a chelator in the samples. Ferrozine forms a red complex with the free Fe(II) ions, but not with those bound by the extracts. In the presence of chelating components, the complex formation of Fe(II) and ferrozine is disrupted, which causes the red color of the complex to diminish. The reduction of red color can be spectrophotometrically determined by

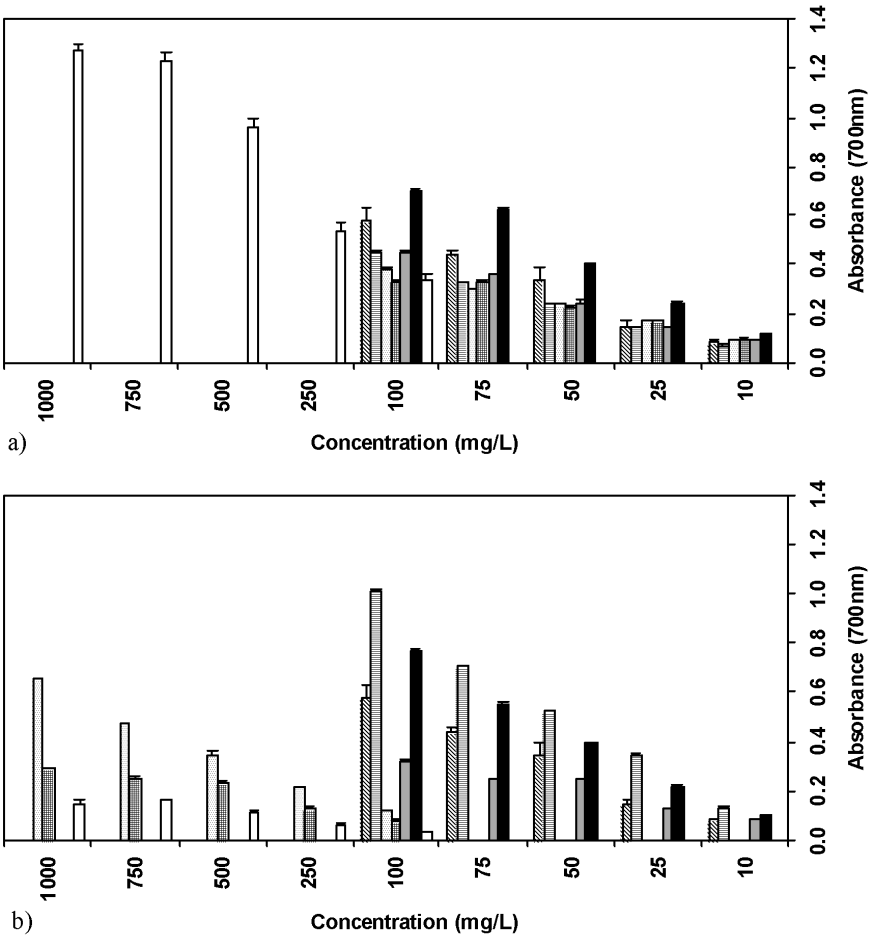


Fig. 3 - Reducing activities of the essential oils (□), of the remaining hydrodistillation-aqueous water-soluble (■) and chloroform-soluble phases (▤) and of the hexane (⊞), dichloromethane (⊡) and methanol (▥) Soxhlet extracts of *T. capitata* (a), *Th. mastichina* (b) and the positive control (ascorbic acid, ▨) at different concentrations. The bars indicate ± SE (n=3).

measuring the chelating activity of the sample (YAMAGUCHI *et al.*, 2000).

Only the MeOH Soxhlet extracts of *T. capitata* and *Th. mastichina* were able to chelate Fe(II) (Fig. 4). Lower concentrations (100-750 mg/L) of *Th. mastichina* extracts were more efficient in forming complexes with Fe(II) than the *T. capitata* extracts. Nevertheless, at a higher concentration (1,000 mg/L) this difference was not as evident (around 80% in both samples). At 100 mg/L the *T. capitata* extracts did not show any chelating activity.

Superoxide radical scavenging activity

Superoxide radical anions contribute to tissue damage and several diseases. Therefore, it is important to find components that can scavenger superoxide radical anions. There are several methods for evaluating this capacity; one of them is that in which the superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm, due to the presence of an antioxidant, measures the consumption of the superoxide anion in the reaction mixture (JUNTACHOTE and BERGHOFER, 2005; PEKSEL *et al.*, 2006).

In the two plant species, only the RHAPhW and MeOH extracts were able to scavenge superoxide radicals (Fig. 5a and b); the RHAPhW extracts were the most effective. The superoxide radical scavenging capacity of these extracts was even higher than that of the positive control (particularly at 500 mg/L). *Th. mastichina* MeOH extracts were better superoxide radical scavengers than those of *T. capitata*.

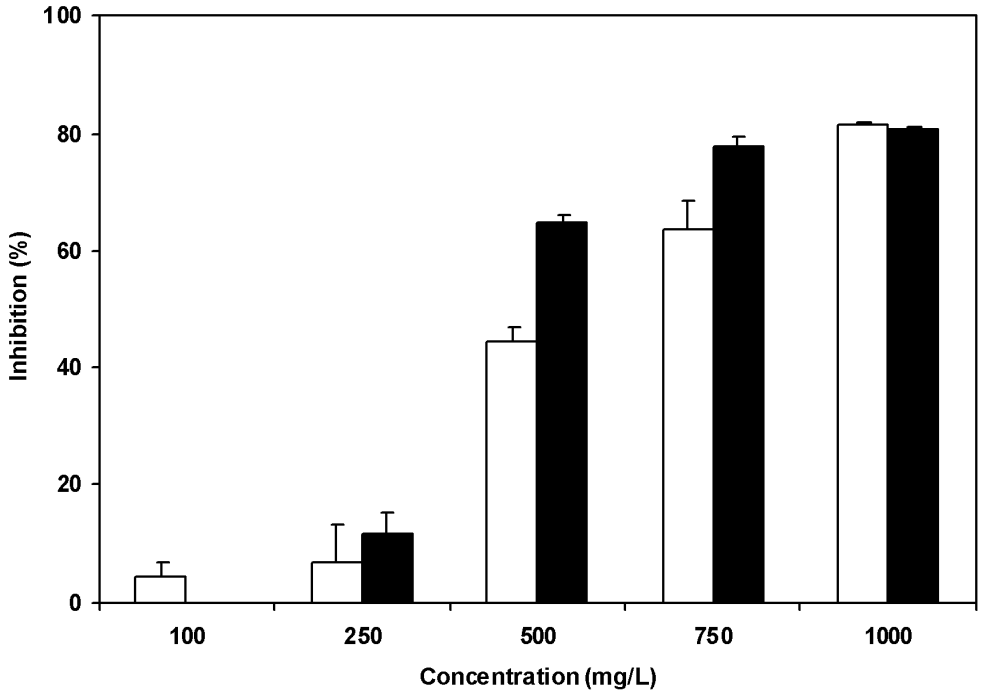


Fig. 4 - Chelating activities of the methanol Soxhlet extracts of *T. capitata* (□) and *Th. mastichina* (■) at different concentrations. The bars indicate ± SE (n=3).

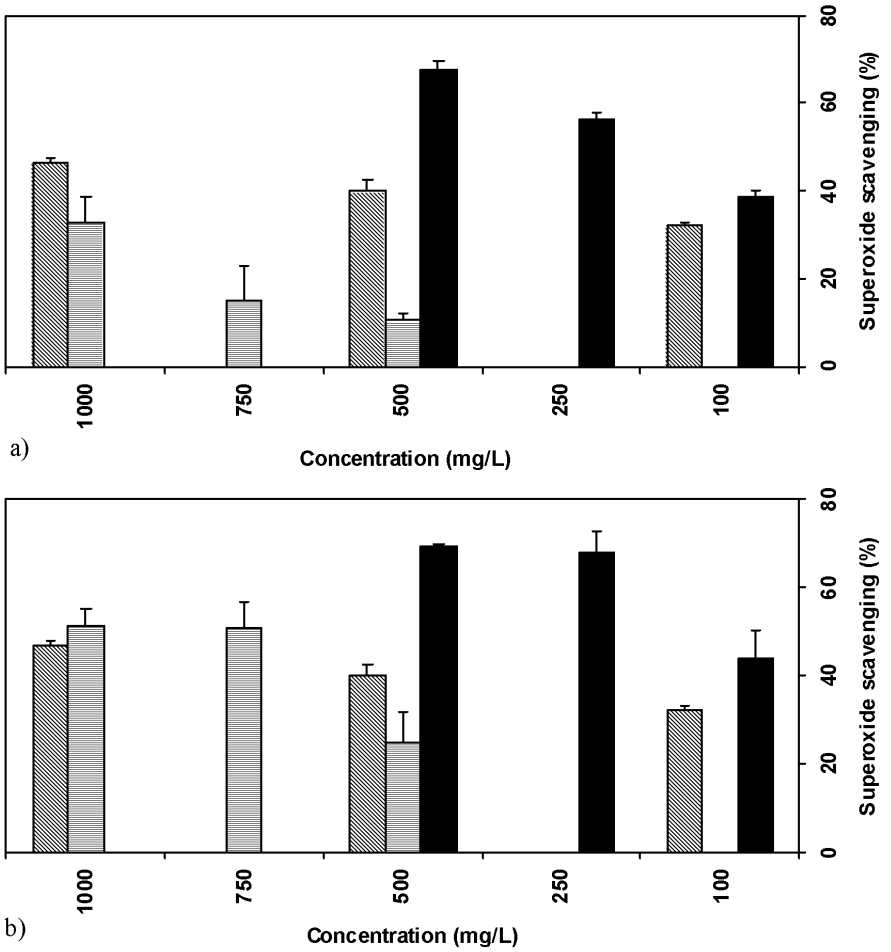


Fig. 5 - Superoxide radical scavenging activities of the remaining hydrodistillation-aqueous water-soluble phase (■) and of the methanol (▥) Soxhlet extracts of *T. capitata* (a), *Th. mastichina* (b) and the positive control (ascorbic acid, ▨) at different concentrations. The bars indicate ± SE (n=3).

CONCLUSIONS

To the best of our knowledge, this is the first report on the antioxidant activity of the remaining hydrodistillation-aqueous phase and of solvent extracts of *T. capitata*, and particularly *Th. mastichina*. Since no correlation was found between the phenol content and the antioxidant activity, the active compounds of these extracts must be purified and identified in order to obtain a better understanding of the protective mechanisms involved.

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PAPER

EFFECT OF HEAT AND ENZYME TREATMENT ON YIELD, PHENOLIC CONTENT AND ANTIOXIDANT CAPACITY OF JUICES FROM CHOKEBERRY MASH

EFFETTO DEL CALORE E DEL TRATTAMENTO ENZIMATICO SULLA RESA, SUL CONTENUTO FENOLICO E SULLA CAPACITÀ ANTIOSSIDANTE DI Ssucchi DI POLPA DI *ARONIA* SPP.

E.J. BOROWSKA*, A. SZAJDEK[1] and S. CZAPLICKI
Chair of Food Plant Chemistry and Processing,
University of Warmia and Mazury, Pl. Cieszyński 1, 10-957 Olsztyn, Poland
[1]Chair of Human Nutrition, University of Warmia and Mazury,
Pl. Cieszyński 1, 10-957 Olsztyn, Poland
*Corresponding author: e-mail: eulalia.borowska@uwm.edu.pl

ABSTRACT

The objective of the present study was to determine the effect of different methods of chokeberry mash treatment prior to juice pressing on the yield, phenolic content, anthocyanin content and antioxidant capacity of the juice. Juice yields ranged from 63.3 to 73.9%, with maximum yield rendered by combined treatment (heat treatment +85°C for 5 min, followed by mash maceration

RIASSUNTO

L'obiettivo del presente studio era la determinazione dell'effetto di differenti metodi di lavorazione della polpa di *Aronia* spp., prima della spremitura a succo, sulla resa, sul contenuto in composti fenolici ed antociani e sulla capacità antiossidante del succo. La resa di succo variava tra il 63,3 ed il 73,9%, la massima resa è stata ottenuta attraverso un trattamento combinato (tratta-

- Key words: anthocyanins, chokeberry, DPPH$^{\cdot}$ radical scavenging activity, juice, juice yield, mash maceration, OH$^{\cdot}$ inhibition, polyphenols -

with Pectinex BE Colour, Pectinex BE XXL, or Gammapect LC Color). Combined treatment and mash maceration with the use Gammapect LC Color was highly effective with respect to yield, polyphenol content and DPPH˙ and OH˙ radical scavenging activity. The highest anthocyanin content was obtained in juices produced by enzymatic maceration, particularly using Gammapect LC Color. Cyanidin-3-galactoside was the predominant anthocyanin in all juice types. The polyphenol and anthocyanin content correlated with the DPPH˙ and OH˙ radical scavenging activity.

mento termico +85°C per 5 minuti, seguito dalla macerazione della polpa con Pectinex BE Color, Pectinex BE XXL, o Gammapect LC Color). Il trattamento combinato e la macerazione della polpa con l'impiego di Gammapect LC Color sono risultati altamente efficaci in termini di resa, contenuto in polifenoli ed attività di radical scavenging nei confronti dei radicali DPPH˙ ed OH˙. Il più alto contenuto in antociani è stato osservato nei succhi ottenuti per macerazione enzimatica, utilizzando il Gammapect LC Color. La cianidina-3-galattoside è risultata l'antocianina predominante in tutte le tipologia di succhi di frutta. Il contenuto in polifenoli e antociani è stato correlato all'attività di radical scavenging DPPH˙ e OH˙.

INTRODUCTION

The interest in plant products that are rich in health-protecting secondary metabolites has been increasing (COHEN *et al.*, 2000; ARA, 2002; DIETRICH *et al.*, 2004; BOROWSKA *et al.*, 2005). These bioactive substances, such as polyphenols, have strong antioxidant activities and include anthocyanins, flavanols, flavanones, proanthocyanidins and phenolic acids. Other plant compounds known for their antioxidant properties are vitamin C, carotenoids, readily assimilable simple sugars and mineral salts (KALT *et al.*, 1999; KÄHKÖNEN *et al.*, 2001; MOYER *et al.*, 2002; DIETRICH *et al.*, 2004; BOROWSKA and SZAJDEK, 2005; SLIMESTAD *et al.*, 2005). Epidemiological studies have shown that diets rich in natural antioxidants can help slow down the aging process and prevent degenerative diseases, such as cancer and cardiovascular diseases (KNEKT *et al.*, 1991; COHEN *et al.*, 2000). Clinical trials have demonstrated that the antioxidant potential of juice compounds is superior to that of pharmaceutical preparations. One of the reasons for this phenomenon may be the synergy between various compounds in the juices (LIAO and YIN, 2000; BENVENUTI *et al.*, 2004; CURADI *et al.*, 2005).

Antioxidant-rich juices and drinks made from berries, including chokeberry, bilberry, black currant and cranberry, are believed to be particularly beneficial. The compounds responsible for their antioxidant properties are anthocyanins, catechins, and proanthocyanidins, and vitamin C in black currant juice (PRIOR *et al.*, 1998; MOYER *et al.*, 2002; DIETRICH *et al.*, 2004; LANDBO and MEYER, 2004; BOROWSKA and SZAJDEK, 2006). In many berries (e.g. black currant, chokeberry, whortleberry), phenolic compounds, including anthocyanins, are located primarily in the peel

of the fruit (MEYER, 2002; MIKKELSEN and POLL, 2002). Their release into the juice during processing is largely dependent on the processing parameters, such as fruit grinding, mash heating, mash maceration with enzyme preparations and juice pressing (OSZMIANSKI and SOZYNSKI, 1989; BOYLES and WROLSTAD, 1993; PŁOCHARSKI and MARKOWSKI, 2003; LANDBO and MEYER, 2004; BAGGER-JØRGENSEN and MEYER, 2004; BUCHERT *et al.*, 2005; BOROWSKA and SZAJDEK, 2006). Fruit mash maceration prior to juice pressing plays a key role and involves heat treatment or the addition of highly specific enzyme preparations that act on cell wall polysaccharides (HELBIG, 2001; URLAUB, 2002; HILZ *et al.*, 2005). This process facilitates juice extraction and improves the extractability of the phenolic compounds (VERSARI *et al.*, 1997; PŁOCHARSKI and MARKOWSKI, 2003; LANDBO and MEYER, 2004). However, in some cases the activities of the accompanying enzymes can destroy these bioactive substances, which decrease the antioxidant capacity and cause undesirable changes in color and flavor (KADER *et al.*, 1999; SKREDE *et al.*, 2000; HELBIG, 2001). The presence of β-galactosidase or β-glucosidase in some preparations can promote the hydrolysis of anthocyanins and the formation of aglycones, which are easily transformed into brown-colored polymers or colorless compounds (VERSARI *et al.*, 1997). According to SKREDE *et al.* (2000), the activity of endogenous fruit enzymes in these phenomena cannot be ruled out. Therefore, selecting the appropriate enzyme preparation is not easy, particularly since manufacturers offer a wide range of such products for the fruit processing industry.

A new generation of pectinases has recently been developed. They are produced from genetically-modified microorganisms, which show one or two types of enzymatic activity: pectin lyase and pectinesterase plus polygalacturonase, without side activities. In some cases, single, pure enzyme preparations can be added during processing in order to enrich traditional pectinase activity (GRASSIN *et al.*, 2005). PŁOCHARSKI and MARKOWSKI (2003), BAGGER-JØRGENSEN and MEYER (2004) and LANDBO and MEYER (2004) demonstrated that the type of enzyme preparation used for the mash maceration during black currant juice production significantly affects the efficiency of anthocyanin and phenolic compounds. The enzyme maceration of fruit mash is generally considered to be more important than the heat treatment.

The objective of this study was to evaluate the effect of different methods of chokeberry mash treatment prior to juice pressing on juice yield, the release of polyphenols (including anthocyanins) into the juice, and 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$) and hydroxyl radical ($OH^{\cdot}$) scavenging activity. Relationships between the polyphenol and anthocyanin content and the radical scavenging activity of the juices were also evaluated.

MATERIALS AND METHODS

Chemicals and standards

$DPPH^{\cdot}$, gallic acid and deoxyribose were purchased from Sigma-Aldrich Co GmbH (Steinheim, Germany). Folin-Ciocalteu reagent was purchased from Merck KGaA (Darmstadt, Germany). Cyanidin-3-galactoside was purchased from Polyphenols Laboratories AS (Sandnes, Norway). All other chemicals were purchased from PPH "POCh" (Gliwice, Poland). The enzyme preparations Pectinex BE XXL and Pectinex BE Colour were from Novozymes A/S (Bagsvaerd, Denmark); Gammapect LC Color was from Gamma Chemie GmbH (Darmstadt, Germany).

Plant material

Juice was made from chokeberries (*Aronia melanocarpa* E.) grown in the Experimental Garden of the University of Warmia and Mazury in Olsztyn in 2005. Chokeberry fruit was collected in early September at full ripeness.

Juice production

Chokeberry juice was produced under laboratory conditions. Mash maceration conditions are given in Table 1 and the characteristics of the enzyme preparations are described in Table 2. Chokeberry fruits were washed, dried on filter paper, and crushed in a laboratory food processor (type ZM Mesko, Skarżysko-Kamienna, Poland). The mash was divided into eight samples (each weighing approximately 250 g) and processed by various methods of maceration: heat treatment (method 2), enzymatic maceration (methods 3, 4, 5) or combined treatment-enzymatic maceration preceded by heat treatment (methods 6, 7, 8). Fruit mash that was not subjected to heat or enzymatic treatment (method 1) served as a control.

Enzyme preparations for mash maceration were added in the amounts recommended by the manufacturers. Fruit mashes 2, 6, 7, and 8 were heated at 85°C for 5 min and then cooled to 50°C. Enzyme preparations were added to mash samples 6, 7 and 8, and maceration was carried out at 50°C for 1.5 h. Then, the fruit mash was heated at 85°C for 2 min in a water bath to inactivate the enzymes and pressed using a laboratory hydraulic press (ZPBB, Bydgoszcz, Poland). The juices were weighed, poured into 200 mL glass containers, pasteur-

Table 1 - Methods of chokeberry mash treatment.

Method treatment*	Heat maceration**	Enzymatic preparation	Enzymatic addition***	Preparation
1	-	-	-	-
2	+	-	-	-
3	-	+	Pectinex BE Colour	0.0375
4	-	+	Pectinex BE XXL	0.0625
5	-	+	Gammapect LC Colour	0.0375
6	+	+	Pectinex BE Colour	0.0375
7	+	+	Pectinex BE XXL	0.0625
8	+	+	Gammapect LC Colour	0.0375

*85°C, 5 min;
**50°C, 90 min;
***mL of enzymatic preparation/250 g of mash; before being added to the mash, the preparations were diluted with redistilled water by a factor of 10.

Table 2 - Characteristics of enzyme preparations used in the experiment.

Enzymatic preparation	Producer	Declared activity	Main and side activities
Pectinex BE Colour	Novozymes A/S	3600 MOE/mL	main: polygalacturonase
Pectinex BE XXL	Novozymes A/S	30000 UPTE/mL	main: pectin lyase
Gammapect LC Colour	Gamma Chemie GmbH	30000 AJDU/mL	main: polygalacturonase, pectin esterase, pectin lyase side: cellulase, hemicellulase

ized in a water bath at 100°C for 10 min, cooled under running water and refrigerated at 15°C. The juices were stored for 1 week prior to analysis.

Juice yield was determined as follows:

$$Y = (A/B) \times 100$$

where Y is juice yield (%), A is juice weight (g) and B is mash weight (g).

The entire experiment was conducted twice and mash treatments (methods 1-8) were performed in triplicate each time.

Analytical methods

Juice separation into fractions

The juices were separated into fractions using ethyl acetate, as described by GHISELLI *et al.* (1998). Multiple liquid/liquid extractions were performed to obtain several fractions containing different classes of polyphenols. Fifty mL of juice was extracted three times with 50 mL of ethyl acetate (pH = 2). The aqueous fraction contained primarily anthocyanins (fraction I – test sample) and the organic fraction contained catechins, procyanidins, phenolic acids and flavonols. Ethyl acetate was evaporated from the organic phase and the residue was dissolved in 50 mL of distilled water. The pH was adjusted to 7 and the fraction was extracted three times with ethyl acetate (50 mL) to obtain an aqueous phase containing phenolic acids and an organic phase containing catechins, procyanidins and flavonols. Ethyl acetate was evaporated from the organic phase; the residue was dissolved in methanol to a volume of 50 mL (fraction II – test sample). The aqueous phase containing ionized phenolic acids was adjusted to pH 2 and extracted three times with 50 mL of ethyl acetate to obtain a fraction containing mostly phenolic acids. Ethyl acetate was evaporated; the residue was dissolved in methanol to a volume of 50 mL (fraction III – test sample). The total phenolic content (SINGLETON and ROSSI, 1965), the $DPPH^{\cdot}$ scavenging activity (BRAND-WILLIAMS *et al.*, 1995) and the $OH^{\cdot}$ inhibition activity (CHU *et al.*, 2000) were determined for each of the fractions.

Determination of total phenolic and anthocyanin content

The total phenolic content was determined for juices produced by methods 1-8 using the Folin-Ciocalteu method according to SINGLETON and ROSSI (1965). Total phenolics were expressed as mg of gallic acid equivalents (GAE) per L of juice. Total anthocyanin content was determined by a differential pH method according to WROLSTAD (1976). Absorbance was measured using a spectrophotometer (Unicam UV/VIS Spectrometer UV2-100, ATI Unicam, Cambridge, UK) at 530 nm and at 700 nm in buffers at pH 1.0 and 4.5, using $A = [(A_{530} - A_{700})_{pH1.0} - (A_{530} - A_{700})_{pH4.5}]$ with a molar extinction coefficient of cyanidin-3-galactoside of 30,200. The results are expressed as mg of cyanidin-3-galactoside equivalents per L of juice. In order to calculate the yield of polyphenols and anthocyanins, their content was also determined in the control mash sample (method 1). The yield of polyphenols and anthocyanins in fruit mash macerated by methods 1-8 was determined as follows:

$$Y_1 = (B_1/A_1) \times 100$$

where Y_1 is the yield of polyphenols or anthocyanins (%), A_1 is the weight (g) of polyphenols or anthocyanins in the control mash sample (method 1) and B_1 is the weight (g) of polyphenols or anthocyanins in juices produced by methods 1-8.

HPLC analysis

Eight types of juice were assayed for the presence of anthocyanins, according to GOIFFON *et al.* (1999). The analyt-

ical HPLC system consisted of a Hewlett-Packard 1050 (Hewlett-Packard, Palo-Alto, CA, USA) liquid chromatograph equipped with a diode array detector. The wavelength was set to 520 nm. A 10 μL sample was analyzed on a stainless steel column LiChrospher C_{18} (250x4.6 mm inner diameter) packed with 5 μm diameter silica particles (Merck). The temperature of the oven column was 30°C. The samples were isocratically eluted at a constant flow of 1.1 mL/min in a mobile phase containing 81% water, 9% acetonitrile and 10% formic acid.

Anthocyanins were quantified using cyanidin-3-galactoside as an external standard. Standard curves were prepared daily. The day-to-day repeatability and within-day variation of the results, linearity ranges of the standard curves and the detection and determination limits of anthocyanins were determined.

Determination of 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

DPPH$^{\cdot}$ scavenging activity was determined according to BRAND-WILLIAMS *et al.* (1995). The juices were diluted 5-fold to 300-fold (7 dilutions). Solutions of different antioxidant concentration (100 μL) were added to 3.9 mL of DPPH$^{\cdot}$ (0.025 g L^{-1}) in methanol, prepared fresh daily. The absorbance at 515 nm was measured at different time intervals until the reaction reached a plateau (steady state). DPPH$^{\cdot}$ scavenging activity was expressed as EC_{50} in μL of juice. DPPH$^{\cdot}$ scavenging activities in fractions I, II and III were determined in the same way.

Determination of hydroxyl radical scavenging activity

OH$^{\cdot}$ scavenging activity was measured by the deoxyribose method (CHU *et al.*, 2000). One hundred μL of juice (diluted 40-fold) was added to 690 μL 10 mM phosphate buffer (PBS) at pH 7.4 containing 2.5 mM 2-deoxyribose. Then, 100 μL of 1.0 mM iron ammonium sulfate premixed with 1.04 mM EDTA was added. Samples were kept in a water bath at 37°C and the reaction was started by adding 100 μL of 1.0 mM ascorbic acid and 10 μL of 0.1 M H_2O_2. Samples were kept at 37°C for 10 min and then 1.0 mL of cold 2.8% trichloroacetic acid was added, followed by 0.5 mL of 1% thiobarbituric acid. Samples were boiled for 8 min then cooled; the absorbance was measured at 532 nm. The degree of OH$^{\cdot}$ inhibition was expressed as a percentage. OH$^{\cdot}$ scavenging activities in fractions I, II and III were determined in the same way.

Statistical analysis

The experiment was conducted twice and each analysis was repeated in triplicate. The figures and tables report mean results. The results were verified statistically by a one-factor analysis of variance. The significance of differences between mean values was estimated by Duncan's test at $p<0.05$. Correlation analysis was also performed. Statistical analysis was performed using Statistica 6.0 software (Statsoft Inc, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Juice yield

Juice yields ranged from 63.3 to 73.9%, depending on the method of mash treatment (Fig. 1). The lowest yield was achieved with method 2, when fruit mash was heated at 85°C for 5 min prior to juice pressing. The highest yields were achieved with methods 6, 7, and 8; fruit mash was first heated (85°C, 5 min) and then macerated using one of three enzyme preparations (Pectinex BE Colour, Pectinex BE XXL or Gammapect LC Color). Differences in juice yield for methods 6, 7 and 8 were not statistically significant ($p<0.05$). It is not surpris-

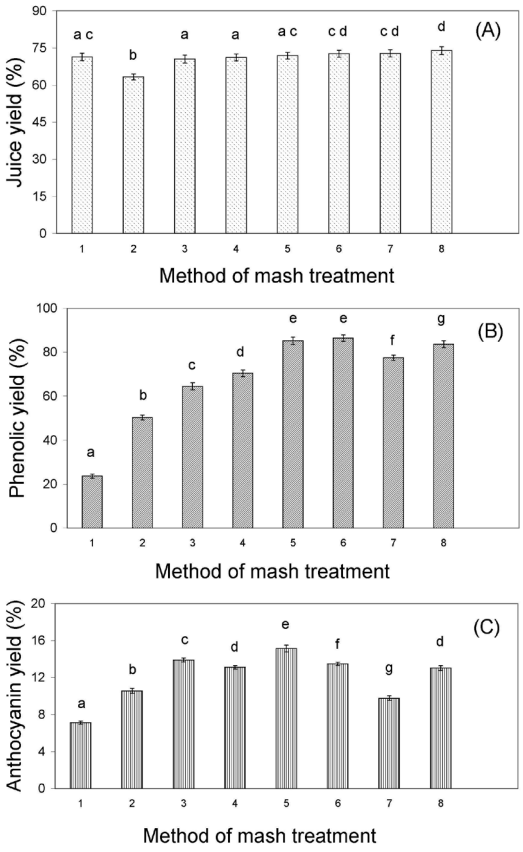


Fig. 1 - Effect of chokeberry mash maceration treatment on the juice yield (A), phenolic compound yield (B), and anthocyanin yield (C). Method 1, control; method 2, heat only; method 3, Pectinex BE Colour; method 4, Pectinex XXL; method 5, Gammapect LC Color; method 6, heat then Pectinex BE Colour; method 7, heat then Pectinex BE XXL; method 8, heat then Gammapect LC Color. Various letters (a, b, c…) indicate significant differences at $p<0.05$. Data are reported as mean ± standard deviation ($n=6$).

ing that the highest juice yields resulted from combined treatment of fruit mash because partial thermal hydrolysis of the cell wall polysaccharides facilitates penetration of enzymes into the substrate. It should be stressed that an integral part of these enzyme preparations is their side activity that supports the main activity. Comparing chokeberry juice yields with the yield of juices made from other berry species, it was found that chokeberry, similar to bilberry, releases juice readily (SZAJDEK and BOROWSKA; unpublished data). A high yield of chokeberry juice (75%) produced by heat treatment alone was previously reported by OSZMIANSKI and SOZYNSKI (1989). According to these authors, the thick peel of the chokeberry fruit contains a relatively large amount of pectin in an insoluble form, which facilitates juice pressing; similar observations have been reported (BOROWSKA and SZAJDEK, 2005). Pectin is believed to act as a drainage agent, facilitating juice release during pressing.

Polyphenols and anthocyanins in juice

The polyphenol content in chokeberry juice varied depending on the method of mash treatment (Table 3). The control sample (method 1) had the lowest polyphenol content, 3,172 mg GAE L^{-1}. Heating the fruit mash (method 2) increased the concentration of polyphenols in the juice to 5,480 mg GAE L^{-1}. Juice made from fruit mash macerated with Gammapect LC Color was the richest source of polyphenols, while juices made from fruit mash subjected to combined treatment (methods 6, 7, 8) had a slightly lower polyphenol content. The concentration of polyphenols in these juices was more than twice that of the control sample, ranging from 6,824 mg GAE L^{-1} to 7,282 mg GAE L^{-1} (Table 3). The highest concentration of anthocyanins was found in juices produced by enzymatic maceration (methods 3, 4, 5; Table 3). The best results were obtained when fruit mash was macerated with Gammapect LC Color; the anthocyanin content in juice made according to method 5 was about two-fold higher than found in the control sample.

The effects of particular mash treatments on the final concentrations of anthocyanins and polyphenols in juices were reflected in the anthocyanin/phe-

Table 3 - Total phenolic content, total anthocyanin content and anthocyanin/phenolic ratio in chokeberry juices.

Treatment of mash (method)	Phenolics*	Anthocyanins**	Anthocyanin/ Phenolic
Control (1)	$3172^{a}\pm21$	$508^{a}\pm6$	0.16
Heat (2)	$5480^{b}\pm34$	$869^{c}\pm7$	0.16
Pectinex BE Colour (3)	$6067^{c}\pm29$	$1027^{g}\pm9$	0.17
Pectinex BE XXL (4)	$6424^{d}\pm37$	$957^{f}\pm6$	0.15
Gammapect LC Colour (5)	$7340^{f}\pm43$	$1087^{h}\pm11$	0.15
Heat, Pectinex BE Colour (6)	$7282^{f}\pm38$	$946^{e}\pm8$	0.13
Heat, Pectinex BE XXL (7)	$6823^{e}\pm25$	$701^{b}\pm6$	0.10
Heat, Gammapect LC Colour (8)	$7173^{f}\pm34$	$925^{d}\pm9$	0.13

Different letters in the same column indicate significant differences, $p<0.05$; (±SD, $n=6$).
*Expressed in mgL^{-1} as gallic acid equivalent.
**Expressed in mgL^{-1} as dominant cyanidin-3-galactoside equivalent.

nol ratio (Table 3). The lowest ratios resulted from maceration using methods 6, 7 and 8 (0.13, 0.10 and 0.13, respectively), indicating that the yield of polyphenols was substantially higher than the yield of anthocyanins.

Previous studies have indicated that the mash treatment conditions have a strong influence on the concentration of phenolic compounds in black currant juice (BAGGER-JØRGENSEN and MEYER, 2004; LANDBO and MEYER, 2004; CZYŻOWSKA and POGORZELSKI, 2004). A weaker effect of enzymatic maceration on the anthocyanin content of raspberry and strawberry juices was reported by VERSARI *et al.* (1997). The authors investigated seven different enzyme preparations and found that the concentration of anthocyanins varied from 369 to 470 mg L^{-1} in raspberry juice and from 264 to 323 mg L^{-1} in strawberry juice.

In this study, HPLC analysis revealed that cyanidin-3-galactoside was the predominant anthocyanin in all juice types (319.4 to 506.1 mg L^{-1}). Cyanidin-3-arabinoside, cyanidin-3-glucoside and cyanidin-3-xyloside were present in smaller quantities (Table 4). Juice made according to method 5 (maceration with

Table 4 - Anthocyanin content in chokeberry juices (HPLC method).

Treatment of mash (method)	Cyanidin-3-galactoside*	Cyanidin-3-glucoside*	Cyanidin-3-arabinoside*	Cyanidin-3-xyloside*
Control (1)	$349.4^{c}\pm2.3$	$24.0^{a}\pm0.3$	$122.4^{c}\pm1.3$	$22.5^{d}\pm0.2$
Heat (2)	$389.3^{f}\pm 2.1$	$27.3^{d}\pm0.3$	$125.0^{d}\pm1.3$	$19.8^{a}\pm0.1$
Pectinex BE Colour (3)	$416.3^{g}\pm2.3$	$29.7^{e}\pm0.4$	$141.1^{e}\pm1.2$	$28.1^{f}\pm0.3$
Pectinex BE XXL (4)	$364.4^{e}\pm1.9$	$33.0^{f}\pm0.4$	$144.8^{f}\pm1.3$	$21.3^{b}\pm0.1$
Gammapect LC Colour (5)	$506.1^{h}\pm2.0$	$43.7^{h}\pm0.4$	$178.7^{h}\pm1.5$	$29.4^{g}\pm0.2$
Heat, Pectinex BE Colour (6)	$356.4^{d}\pm1.8$	$36.1^{g}\pm0.4$	$148.3^{g}\pm1.2$	$24.2^{e}\pm0.2$
Heat, Pectinex BE XXL (7)	$322.0^{b}\pm1.6$	$26.7^{c}\pm0.2$	$118.7^{b}\pm1.2$	$21.8^{c}\pm0.1$
Heat, Gammapect LC Colour (8)	$319.4^{a}\pm1.3$	$26.1^{b}\pm0.2$	$110.1^{a}\pm1.2$	$21.9^{c}\pm0.2$

Different letters in the same column indicate significant differences, $p<0.05$; (±SD, $n=3$).
*Expressed in mgL^{-1}; quantification of anthocyanins was carried using cyanidin-3-galactoside as standard.

Gammapect LC Color) had the highest concentrations of cyanidin-3-galactoside and the other three anthocyanins. One peak on the chromatogram of the juice from method 5 remained unidentified. Fig. 2 shows an example of a juice chromatogram (method 8), where "1" denotes an unidentified peak with a maximum of absorption at 279 nm and two smaller maxima at 513 and 327 nm. It is possible that this peak is a product of interaction between anthocyanins and other compounds. DIETRICH *et al.* (2004) suggested that during extraction, clarification and pasteurization of black currant juice, anthocyanins might interact with one another, as well as with other phenolic compounds (such as flavonoids), resulting in the formation of adducts with different absorption maxima. It is quite likely that such interactions occurred during this experiment.

Yields of polyphenols and anthocyanins

Polyphenol yields ranged from 23.7 to 86.4% (Fig. 1), reaching the highest values following combined treatment with Pectiney BE Colour. Differences in polyphenol yield were statistically significant ($p<0.05$) for most of the mash treatment methods (except between methods 5 and 6). According to MEYER (2002) and HILZ *et al.* (2005), mash heating causes partial degradation of the pectin contained in the cell wall matrix and the middle lamella; however, a significant increase in polyphenol release was observed only after pectolytic enzymes and accompanying enzymes with side activities were used (such as hydrolyzing cellulose and hemicelluloses). A marked effect of enzymatic maceration on the yield of phenolic compounds in black currant juice was described by LANDBO and MEYER (2004) and by BAGGER-JØRGENSEN and MEYER (2004). In both studies, the authors noted a positive correlation between the degree of enzymatic degradation of cell wall polysaccharides and the amount of phenolic compounds released into the juice.

The yield of anthocyanins was substantially lower than that of phenols and was the highest (15.2%) when fruit mash was macerated with Gammapect LC Color (method 5). It should be emphasized that this preparation has a wide spectrum of pectinolytic activities and side hemicellulase and cellulase activities. OSZMIANSKI and SOZYNSKI (1989) reported that the mash maceration conditions had a considerable influence on anthocyanin yield in chokeberry juice. They found the highest anthocyanin yield (2.2-times higher than the control) following fruit mash heating at 80°C for 5 min. When maceration was carried out with Drum Pectinase 263, Pektopol PT and Rohalase HT, the anthocyanin yields were 8.6, 7.7 and 2.2% lower, respectively. According to the authors, the lower anthocyanin yield could have been due to the activity of the additional enzymes in Drum Pectinase 263 that hydrolyze the esters of the phenolic compounds.

DPPH$^{\cdot}$ scavenging activity and OH$^{\cdot}$ inhibition

The chokeberry juices differed with regard to DPPH$^{\cdot}$ scavenging activity and OH$^{\cdot}$ inhibition (Table 5). The control juice had the lowest activity with respect to both types of radicals, while the highest activity was reported for juices made from fruit mash subjected to combined treatment (methods 6, 7, 8) and enzymatic maceration with Gammapect LC Color (method 5). There were strong correlations between polyphenol content and DPPH$^{\cdot}$ scavenging activity ($R = -0.96$), as well as between polyphenol content and OH$^{\cdot}$ inhibition ($R = 0.92$). Weaker correlations were noted for anthocyanins and radical scavenging activity, reaching -0.74 for DPPH$^{\cdot}$ and 0.48

Table 5 - Antioxidant activity (DPPH· scavenging and OH· radicals inhibition) of juices and their corresponding fractions.

Treatment of mash (method)	Juices		Fraction I of juice		Fraction II of juice		Fraction III of juice	
	DPPH·(EC_{50})*	OH·**	DPPH·(EC_{50})*	OH·**	DPPH·(EC_{50})*	OH·**	DPPH·(EC_{50})*	OH·**
Control (1)	2.63^{f}	31.2^{a}	5.38^{g}	42.1^{a}	19.5^{f}	14.1^{a}	100.5^{f}	7.6^{a}
Heat (2)	1.18^{e}	35.5^{b}	7.18^{h}	46.1^{b}	14.2^{d}	19.6^{e}	72.0^{e}	9.3^{e}
Pectinex BE Colour (3)	0.87^{d}	36.0^{c}	1.42^{e}	47.7^{c}	23.9^{h}	15.2^{b}	79.8^{e}	8.8^{c}
Pectinex BE XXL (4)	0.83^{d}	37.0^{d}	1.51^{f}	47.5^{c}	20.0^{g}	15.8^{c}	75.5^{d}	9.0^{d}
Gammapect LC Colour (5)	0.72^{c}	39.1^{e}	1.14^{c}	49.7^{d}	18.4^{e}	18.6^{d}	65.0^{b}	10.0^{f}
Heat. Pectinex BE Colour (6)	0.67^{a}	42.1^{h}	1.05^{a}	52.2^{e}	12.9^{c}	20.2^{f}	50.5^{a}	11.2^{g}
Heat. Pectinex BE XXL (7)	0.65^{a}	41.0^{g}	1.33^{d}	51.8^{e}	9.8^{b}	23.9^{g}	67.5^{b}	9.9^{f}
Heat. Gammapect LC Colour (8)	0.76^{b}	40.2^{f}	1.11^{b}	53.1^{f}	8.2^{a}	25.1^{h}	83.0^{e}	8.3^{b}

Different letters in the same column indicate significant differences. $p<0.05$; (n=3).
Fraction I-containing anthocyanins; Fraction II-containing catechins and several flavonols; Fraction III-containing mostly phenolic acids.
*EC_{50} expressed as µL of juice;
**OH· inhibition expressed as %.

for OH· (Fig. 3). The above cited differences in correlation values could indicate that phenolic compounds other than anthocyanins could have a greater antioxidant activity. Similar relationships between polyphenol concentration and the antioxidant capacity of the berry fruits and berry products have been described by other investigators (PRIOR *et al.*, 1998; MOYER *et al.*, 2002), who stressed that antioxidant activity was more strongly correlated with the total phenolic content than with the anthocyanin concentration.

In order to evaluate the antioxidant capacity of various groups of phenolic compounds, the DPPH· scavenging activity and OH· inhibition activity were determined for fractions I, II and III that were separated as described by GHISELLI *et al.* (1998). Regardless of the maceration method, fraction I exhibited the highest activities, while fraction III showed the lowest activities with respect to both radicals (Table 5). For DPPH·, the activities of particular fractions were lower than the activities of the corresponding juice, which supports the hypothesis that there is a synergism that occurs between individual juice compounds that is manifested in the high antioxidant capacity (LIAO and YIN, 2000).

Fig. 2 - HPLC chromatogram of anthocyanins in chokeberry juice prepared according to method 8 (520 nm). Peak 1, unidentified form; peak 2, cyanidin-3-galactoside; peak 3, cyanidin-3-glucoside; peak 4, cyanidin-3-arabinoside; peak 5, cyanidin-3-xyloside.

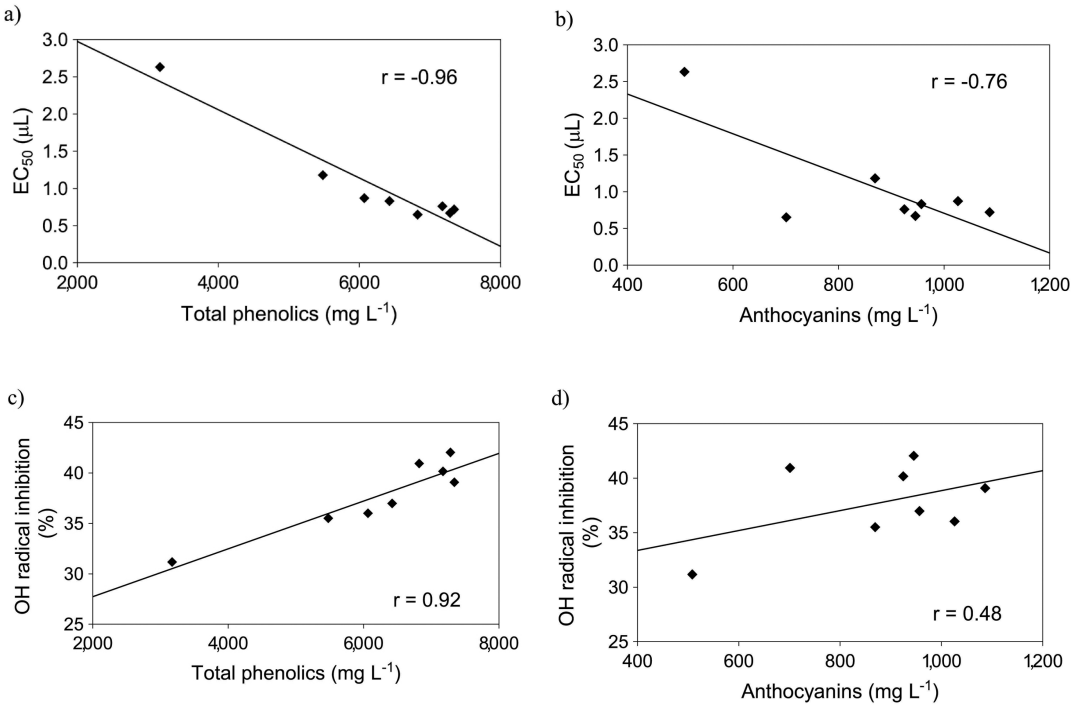


Fig. 3 - Correlation between a) total phenolic compound content and EC_{50}; b) anthocyanin content and EC_{50}; c) total phenolic compound content and $OH^{\cdot}$ inhibition; d) anthocyanin content and $OH^{\cdot}$ inhibition.

CONCLUSIONS

These data indicate that different methods of chokeberry mash maceration have significant effects on total juice yield, the yield and content of polyphenols and anthocyanins, and the antioxidant properties of the juice. Juice yields ranged from 63.3 to 73.9% and were highest when heat treatment of the fruit mash was followed by enzymatic maceration. This mash treatment method was also the most effective with respect to the yield and content of polyphenols in the juices. Comparable results were obtained for mash maceration with Gammapect LC Color. The highest anthocyanin content occurred in juices produced by mash maceration with enzyme preparations, particularly with Gammapect LC Color. Juices produced by combined treatment with Gammapect LC Color had the highest $DPPH^{\cdot}$ and $OH^{\cdot}$ scavenging activities. There was a strong correlation between polyphenol content and EC_{50} (R = -0.96) and between polyphenol content and $OH^{\cdot}$ inhibition (R = 0.92). A weaker correlation was noted between anthocyanin content and antioxidant activity.

In conclusion, heat treatment followed by enzymatic maceration of chokeberry mash prior to juice pressing, in particular using Gammapect LC Color, is recommended for maximizing juice yield, phenolic compound content and the antioxidant properties of chokeberry juice.

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PAPER

HACCP IN THE MALTING AND BREWING PRODUCTION CHAIN: MYCOTOXIN, NITROSAMINE AND BIOGENIC AMINE RISKS

L'HACCP PER IL CONTROLLO DELLE MICOTOSSINE, NITROSAMMINE E AMMINE BIOGENE NELLA FILIERA PRODUTTIVA DEL MALTO E DELLA BIRRA

M. ERZETTI[2], O. MARCONI[1], E. BRAVI[1], G. PERRETTI[1]*, L. MONTANARI[1] and P. FANTOZZI[2]

[1]Dipartimento di Science Economico-Estimative e degli Alimenti, Università degli Studi di Perugia, Via S. Costanzo, 06126 Perugia, Italia

[2]Centro di Eccellenza per la Ricerca sulla Birra (CERB), Università degli Studi di Perugia, Via S. Costanzo, 06126 Perugia, Italia

*Corresponding author: Tel. +39 075 5857922, Fax +39 075 5857939, e-mail: perretti@unipg.it

ABSTRACT

This paper reports the management of Hazard Analysis and the identification of the Critical Control Points for specific contaminants, i.e. mycotoxins, N–nitrosamines and biogenic amines, that can occur in small and medium-sized enterprises in the malting and brewing production chain. Following the principles of the Hazard Analysis and the Critical Control Points system, this paper describes: step 1, how to conduct the hazard analysis, step 2, how

RIASSUNTO

Questo lavoro descrive un sistema di gestione, basato sul metodo dell'HACCP, che può essere applicato alle piccole e medie imprese della filiera produttiva del malto e della birra al fine di prevenire, ridurre e tenere sotto controllo le problematiche derivanti dalla presenza di micotossine, nitrosammine e ammine biogene. Seguendo i principi dell'HACCP, questo lavoro descrive come: punto 1, condurre l'analisi del rischio; punto 2, identificare i punti critici di con-

- Key words: biogenic amines, brewing, HACCP, malting, mycotoxins, nitrosamines -

to determine the critical control points, step 3, the critical limits, step 4, how to establish the monitoring system, step 5, how to establish the corrective action, step 6, how to establish procedures to verify the effectiveness of the system and step 7 how to document all procedures and records for the correct application of the system. This methodology will help assist malters and brewers to understand the malting and brewing safety aspects and how to manage the risks related to the malting and brewing processes. The method was elaborated on conventional malting and beer production. The schemes are somewhat simplified for the sake of clarity. The beer production scheme illustrates the production of a lager beer with bottom fermenting yeast.

trollo; punto 3, definire i limiti critici; punto 4, stabilire le adeguate misure di controllo dei punti critici; punto 5, stabilire le opportune azioni correttive; punto 6, stabilire le procedure di verifica del sistema; punto 7, elaborare appropriate procedure e moduli di registrazione per la corretta applicazione del sistema. Questa metodologia sarà di supporto ai produttori di malto e di birra per capire meglio gli aspetti di sicurezza e come gestire i rischi relativi ai processi di maltaggio e di birrificazione. Questo metodo è stato elaborato su una produzione convenzionale di malto e birra.

INTRODUCTION

Food safety involves several factors: legislation (to establish the minimum hygiene requirements); official controls (to check food business operators' compliance) and food business operators (to establish and manage food safety programmes and procedures based on the Hazard Analysis and Critical Control Point (HACCP) principles). The HACCP system is a tool to help food business operators attain a higher standard of food safety and to identify, evaluate, and control hazards in order to ensure the safety of food (LEE and HATHAWAY, 1998; ROPKINS and BECK, 2000; EFSTRATIADIS and ARVANITOYANNIS, 2000; MORTIMORE, 2001; FAO/WHO, 2001; Reg. 852/2004; SENIRES and ALEGADO, 2005; SPERBER, 2005; RUSH, 2006).

HACCP is a tool to assess hazards and establish control systems by focusing on prevention rather than relying mainly on end-product inspection. Any HACCP system is capable of accommodating change, such as advances in equipment design, processing procedures and technological development (CODEX ALIMENTARIUS, 1997).

Food safety management systems such as HACCP, and the pre-requisite programs of Good Manufacturing Practices (GMP), Good Hygiene Practices (GHP), Good Agricultural Practices (GAP) and Good Storage Practices (GSP) provide the operators in the food supply chain with excellent tools for designing and implementing a specific food manufacturing process in a proper and rigorous way (FAO/IAEA, 2001; GORRIS, 2005). The HACCP principles are incorporated into food safety legislation, and are a reliable component of the standardization of international food quality control and assurance practices (ROPKINS and BECK, 2000).

The pursuit of a high level of protec-

tion of human life and health is one of the fundamental objectives of European food laws, as defined in EC Regulation N. 178/2002. Council Directive 93/43/EEC of the 14th of June 1993 defined the general rules of hygiene for foodstuffs, and the procedures for verifying compliance with these rules.

EC Regulation N. 852/2004 requires food business operators to establish, implement and maintain permanent procedures based on the HACCP principles. An integrated approach is necessary in order to ensure food safety from the site of primary production of the raw materials and ingredients up to and including the distribution and sales on the market. Every food business operator along the food chain must ensure that food safety is not compromised.

Beer may be contaminated by biological, chemical and physical substances at various stages within the process. Adopting the HACCP approach, the operators can limit this possibility; the HACCP program is recognized worldwide as a preventive, systematic approach to beer safety; it addresses the risk through prevention rather than finished product inspection (EBC, 2006). Over the last years, a number of investigators have demonstrated that mycotoxins (SALAS *et al.*, 1999; BOEIRA *et al.*, 2000; CREPPY, 2002; BOEIRA *et al.*, 2002; MEDINA *et al.*, 2006; WOLFHALL, 2007), nitrosamines (SMITH, 1994; LONGO *et al.*, 1995; IZQUIERDO-PULIDO *et al.*, 1996a; SEN *et al.*, 1996; GLORIA *et al.*, 1997; YURCHENKO and MOLDER, 2005; VLB, 2006; MARCONI *et al.*, 2007) and biogenic amines (IZQUIERDO-PULIDO *et al.*, 1996b; SHALABY, 1996; GLORIA and IZQUIEDRO-PULIDO, 1999; HALÁSZ *et al.*, 1999; IZQUIEDRO-PULIDO *et al.*, 2000; KALAČ *et al.*, 2002; KALAČ and KŘIŽEK, 2003; LORET *et al.*, 2005; CORTACERO-RAMIREZ *et al.*, 2007; MARCONI *et al.*, 2007) are present and are risks in the beer chain. Therefore, taking into account EC Regulation N. 852/2004, it is important to set up specific procedures to assure food safety in the beer production chain for current and emerging risks.

Naturally occurring moulds grow easily on grain during malting or under high moisture storage conditions. Moulds such as *Aspergillus flavus, Penicillum parasiticus, Fusarium graminearum, F. culmorum, F. roseum* and *F. moniliforme* are known to produce aflatoxins (AFLs), trichothecenes, fumonisins (FBs), ochratoxin A (OTA), zearalenone (ZEA), and other mycotoxins (BOEIRA *et al.*, 2000; CREPPY, 2002; MBUGUA and GATHUMBI, 2004; MEDINA *et al.*, 2006). Mycotoxin contamination in very common in naked cereal grain such as maize, wheat, sorghum and millet, which, unlike barley, are not protected by the presence of husks (MBUGUA and GATHUMBI, 2004). Therefore, when maize is used as an adjunct (a typical practice in lager beer production in Italy) this hazard is particularly important. The most important mycotoxins in beer production are: AFLs (AFB1, B2, G1, G2), OTA, trichothecenes (mainly deoxynivalenol - DON, or vomitoxin), ZEA and FBs (B1, B2) (EBC, 2000; BOIVIN, 2005).

Nitrosamines are formed when secondary or tertiary amines react with a nitrosating agent (MANGINO and SCANLAN, 1981; POOCHAROEN *et al.*, 1992; LIJINSKY, 1999; KALAČ and KŘIŽEK, 2003; YURCHENKO and MOLDER, 2005). The same reactions can develop in several natural matrices.

Biogenic amines are commonly found in foods such as cheese, meat and fish products, wine, beer, and other fermented foods (STRATTON *et al.*, 1991; HALÁSZ *et al.*, 1994). These amines can have aliphatic, aromatic or heterocyclic structures, and most of them can be generated by microbial decarboxylation of free amino acids (BRINK *et al.*, 1990). The presence of biogenic amines in foods and alcoholic beverages is important from a toxicological point of view (KALAČ and KŘIŽEK, 2003; YURCHENKO and MOLDER, 2005).

This paper presents an HACCP-based approach for evaluating the specific risk of mycotoxins, nitrosamines and biogenic amines in the malting and brewing industries. The aim of this study was to identity the Critical Control Points (CCPs) for mycotoxins, *N*-nitrosamines and biogenic amines that could occur in malting and brewing processes and which may cause the end-product to be unsafe for human consumption. This HACCP study should then be applied to small and medium-sized enterprises (SMEs) because of particular needs in terms of raw material management, processing and logistics.

METHOD

The method used applied the seven basic principles of HACCP that have been internationally accepted and published by the CODEX ALIMENTARIUS COMMISSION (1997). The study followed the scheme of EC Regulation N. 852/04. The key HACCP components include identifying potential problems that may cause the product to be unsafe for consumption; establishing and monitoring targeted control points to prevent, eliminate or reduce such problems (in this case mycotoxins, nitrosamines and biogenic amines); and documenting the results (SENIRES and ALEGADO, 2005).

RESULTS AND DISCUSSION

The results of the study are reported following the seven steps of the HACCP system (CODEX ALIMENTARIUS, 1997).

Step 1 Hazard analysis (HA). Identify hazards and assess the risks associated with them at each step within the commodity system

Hazard identification is based on experience and qualitative reasoning (MCMEEKIN *et al.*, 2006). The first step of hazard analysis is a description of the product, and identification of the hazards associated with the raw materials. In hazard analysis it is very important to define and describe the process and product specifications in a flow-chart diagram (LEE and HATHAWAY, 1998; MORTIMORE, 2000; EBC, 2006).

Italian legislation defines beer as the product made by the alcoholic fermentation of a wort obtained from malt (barley or wheat), water, hops and adjuncts (maximum 40%) such as maize, rice, sorghum or wheat using *Saccharomyces cerevisiae* and/or *Saccharomyces carlsbergensis* yeast (D.P.R., 1998).

The flow-chart diagrams for malting and beer production are reported in Figs. 1 and 2, respectively, and include the parameters for producing a generic lager beer by using adjuncts (SUNIER, 1988; HARDWICK, 1995; KUNZE, 2004). There are many variations in the malting and brewing process depending on the specific types of malt and beer. Lager beer produced by using adjuncts is typical in the Italian beer producing industry. The flow diagram was used to manage the hazards identified.

HA for mycotoxins

Mycotoxins are chemical substances synthesized by certain filamentous fungi that cause a toxic response, mycotoxicosis, when introduced in a natural pathway in relatively low concentrations for higher vertebrates and other animals. The European Commission has laid down and will lay down maximum limits for the presence of mycotoxins in cereal and cereal product. The maximum levels are reported in the EC Regulation N. 1881/2006 and modification. Mycotoxins have many toxic effects: carcinogenic, nephrotoxic, teratogenic and immunotoxic effects (LOPEZ-GARCIA *et al.*, 1999; CREPPY, 2002; BOIVIN, 2005).

The contamination of cereal grains by mycotoxins can occur in the field, during

storage or during the malting process. The production of mycotoxins is not homogeneous: for instance, OTA contamination often occurs in "spot" during storage in silos (MELOTTE, 2004). The use of raw materials contaminated by mycotoxins, e.g. contaminated barley for malting and contaminated cereal adjunct (maize, wheat, rice) in brewing, lead to possible contamination of finished beer (BOEIRA *et al.*, 2000). It is reported that mycotoxins survive the major beer production process as malting, mashing, boiling and fermentation (SCOTT, 1996).

HA for nitrosamines

The main carcinogenic *N*-nitroso compounds found in food are nitrosodimethylamine (NDMA), nitrosodiethylamine (NDEA), nitrosopyrrolidine, nitrosopiperidine, and occasionally nitrosodi-n-butylamine. Nitrite and nitrogen oxides play a key role in forming N-nitroso compounds by interacting with secondary and tertiary amino compounds. Foods are therefore examined for the presence of *N*-nitroso compounds, and those that have been found have almost exclusively been in those foods containing nitrite or which have been exposed to nitrogen oxides. Beer does not usually contain large amounts of NDMA. The levels are commonly around 5-10 μg/L, but some exceptions have been reported for a few brands of German beers in which as much as 70 μg/L of NDMA have been detected. Nitrosamine levels have been declining during the past three decades, as a result of greater control of exposure of malt to nitrogen oxides in beer-making and an overall reduction of nitrite use in food (LIJINSKY, 1999; MARCONI *et al.*, 2007).

The highest NDMA concentrations have been found in dark beers that were high in malt content. The origin of the NDMA depends on the presence of the alkaloids hordenine and gramine and perhaps others in barley and hence, in the malt. These alkaloids are derivatives of dimethylamine and are easily nitrosated, in this case, by nitrogen oxides as the nitrosating agent. The nitrogen oxides are in the flue gases from the burning of the fuel used to dry (heat) the malt and are allowed to come in contact with the latter (SEN *et al.*, 1996).

Microbiological nitrate reduction is the principal pathway of N-nitrosamine formation in beer brewing (SMITH, 1994).

HA for biogenic amines

Biogenic amines are natural health hazard substances, that are important from a hygienic point of view because they have been implicated as the causative agents in a number of food-poisoning episodes. Amines are produced by bacteria (e.g. Enterobacteriaceae and lactic acid bacteria) that are capable of decarboxylating amino acids (SHALABY, 1996). The biogenic amines in beer originate from the raw materials and the fermentation phases of beer production (JURKOVÁ *et al.*, 2005). Beer has been reported to be a possible health risk for some consumers due to the presence of biogenic amines (IZQUIEDRO-PULIDO *et al.*, 1994; TAILOR *et al.*, 1994; SHALABY, 1996; SHULMAN *et al.*, 1997; HALÁSZ *et al.*, 1999).

High biogenic amine intake levels are not usually the result of high amine levels in individual beers, but rather are caused by excessively high beer consumption. These compounds arise from the bacterial decarboxylation of the corresponding amino acids: histamine (HIS) from histidine, tyramine (TYR) from tyrosine, tryptamine (TRY) from tryptophan, β-phenylethylamine (PHE) from phenylalanine, agmatine (AGM) from arginine, cadaverine (CAD) from lysine, putrescine (PUT) from ornithine and agmatine, spermidine (SPD) and spermine (SPM) from the biochemical reaction of putrescine by the addition of aminopropyl moieties catalyzed by spermidine and spermine synthases. PUT, SPD, SPM and AGM can be considered as natural beer constitu-

ents that originate from the malt, while HIS, TYR and CAD usually indicate activity of contaminating lactic bacteria during brewing. TYR is the main biogenic amine that has been identified in beer and has caused adverse effects on human health (KALAČ and KŘIŽEK, 2003; MARCONI *et al.* 2007). While PUT, SPD, SPM and CAD have no direct adverse effect on human health, they may react with nitrite to form carcinogenic nitrosamines (ÖNAL, 2007).

Step 2: Determination
of the Critical Control Points (CCPs)

Determination of CCPs is a step or a procedure in the brewing process where it is essential to have a control in order to prevent, eliminate or reduce a hazard to an acceptable level. The World Health Organisation (WHO) recommends that CCPs should be determined by using the HACCP decision tree established by FAO/WHO (2001). If a control can be implemented easily and a hazard can be easily prevented, then the process step is merely identified as a control point (CP) (SENIRES and ALEGADO, 2005). It is very important to introduce a step where only a control or a CP help to control the process and prevent the formation of hazards. When multiple steps address a hazard, the CCP shall be the step nearest the end of the process, if this step provides adequate control (RUSH, 2006).

CCPs for mycotoxins

The first line of defence against the introduction of mycotoxins is at the farm level and starts with the implementation of Good Agricultural Practices (GAP) to prevent infection. Where appropriate, GAP should be adopted to make sure that the harvested commodity will not present a food hazard along the chain. GAP helps farmers understand and anticipate how and where the product may be contaminated, in the farming and production cycle.

GAP are general procedures to reduce hazards related to product safety at the farm level during the operations which precede processing: cultivation, development, harvest and storage. These are considered to be a prerequisite for implementing a HACCP plan at the farm level. Preventive strategies must be implemented from the pre- harvest through the post-harvest stages (MURPHY *et al.*, 2006).

Intake and Storage
(Malting and Brewing)

Cereals must be inspected for any possible mould infections, and for the presence of any mycotoxins already formed (VLB, 2004). The breweries perform careful controls of barley and other cereals before accepting them for malting, because mouldy cereals will spoil the flavour and reduce the quality of the beer, e.g., by causing gushing (KROGH *et al.*, 1974).

Malting process

The malting process provides the ideal temperature and moisture conditions for the growth of natural microbial barley contaminants. If malting barley is contaminated with mycotoxin-producing fungi, these fungi will grow during steeping, germination and the first step of kilning and will produce mycotoxins that are leached out during steeping (BOIVIN, 2005).

Brewing process

No mycotoxins are produced during the brewing process but sometimes they occur during the storage of raw materials (see above) (BOIVIN, 2005).

CCPs for nitrosamines

Malting process (kilning)

Malted barley is the primary source of NDMA. Only malt dried by direct-fired kilning, where nitrogen oxides could react with certain alkaloids, could account for the NDMA levels found in the

final beer (MANGINO and SCANLAN, 1985; IZQUIERDO-PULIDO et al., 1996a; YURCHENKO and MOLDER, 2005).

NDMA is formed at high temperatures but now the kilns are heated indirectly rather than directly and the hot nitrogen oxide-rich (NO_X) gas used for kilning no longer passes through the malt. In the production of smoked malt, smoke is passed through the malt before kilning, to give it a scratchy flavour; smoke does not cause the formation of NDMA (VLB, 2004).

The NDMA concentration in malt depends on the type of drying technique used. High combustion temperatures yield high concentrations of NDMA. Green malt is usually dried with hot air at a temperature less then 100°C, and the malt germination is very short; the germ and root of the malt are usually very small. This reduces the quantities of alkaloids in the malt (YURCHENKO and MOLDER, 2005).

Brewing process

Studies on beer ingredients have shown that NDMA is not normally present in significant quantities in any processing aid, additive or ingredient other than malted barley, although small amounts have been found in hops. Because NDMA in hops is diluted during brewing, its contribution is negligible. The NDMA content of water is quite variable and charcoal filters adsorb it. Experience suggests that NDMA in the water supply is not a common source of NDMA in beer. The most convincing evidence is that NDMA is not normally formed during beer fermentation, or during mashing or wort boiling (WAINWRIGHT, 1986a).

The occurrence of apparent total N-nitroso compounds (ATNC) in beer is due to the metabolism of certain groups of bacteria and is thus indicative of hygienic problems at some stage in the brewing process (SMITH, 1994).

Hordenine and gramine are possible sources of dimethylamine (DMA), which is formed in the barley kernels during malting. NDMA could be formed during the brewing process if traces of nitrosating agents were available at that stage; nitrite could be formed from nitrate during processing or nitrogen oxides could be present. The NDMA concentration depends on the percent of alcohol content since ethanol is an inhibitor of nitrosation (YURCHENKO and MOLDER, 2005). Except during malt kilning, when gaseous N_2O_3 or N_2O_4 is present, NDMA is not usually formed during malt or beer production or storage because the concentrations of NO_2 and nitrite ions are too low at the temperature and/or pHs involved (WAINWRIGHT, 1986b).

CCPs for biogenic amines

Data on biogenic amine levels in malt, hops or hop derivatives as well as pitching yeast, is very limited (KALAČ and KŘIŽEK, 2003). Malt is a source of agmatine, putrescine, spermidine and spermine, while tyramine, histamine and cadaverine are formed during the main fermentation by contaminating lactic acid bacteria (KALAČ *et al.*, 2002).

Malting

No amines should be present in the production water used during malting (KALAČ and KŘIŽEK, 2003).

The increase of PUT, SPD, SPM and AGM in beer was observed during five days of barley germination. Malting conditions, such as germinating intensity, kiln temperature and barley variety will affect the final amine levels in malt (KALAČ and KŘIŽEK, 2003). Decarboxylating enzyme activity of micro-flora causes amine formation during barley malting (KALAČ *et al.*, 1997).

Brewing

No amines should be present in the water used for the production of beer (KALAČ and KŘIŽEK, 2003). In brewing, the types of amines are dependent on the raw materials, as well the meth-

od of brewing, and any microbial contamination (lactic acid bacteria activity) that occurs during the brewing process or during storage. Amines can also be formed by the activity of lactic acid bacteria during the storage of beer in bottles (GLORIA and IZQUIEDRO-PULIDO, 1999; KALAČ and KŘIŽEK, 2003).

Step 3: Establish the critical limits

One or more prescribed tolerances (critical limits; maximum or minimum values; applicable targets) must be met to ensure that the CCP effectively controls the hazard (CODEX ALIMENTARIUS, 1997). Critical limits must be set for each CCP identified. The critical limits define the difference between a safe and an unsafe process, and it is not necessarily the legal limit of the contaminant in the product. In the case of mycotoxins, nitrosamines and biogenic amines, the critical limits are connected with physical and chemical parameters that affect the occurrence of the hazard. In particular, the moisture of cereal grains, the reaction of nitrogen oxides with certain alkaloids during the direct-fire drying step of the malting process or the bacterial activity can be exploited in different ways to define specific critical limits for mycotoxins, nitrosamines and biogenic amines. The limit applies to the control measure and not the hazard, and must be able to be measured quickly and simply to enable prompt corrective action (FAO/IAEA, 2001; RUSH, 2006). Critical limits must be stated in terms of the safety requirements of the process, not overall process requirements (RUSH, 2006). Moreover, Commission Regulation (EC) N. 1881/2006 and following modifications set maximum levels for certain contaminants in foodstuffs.

Critical limits for mycotoxins

The most effective mycotoxin control measure is to dry the commodity such that the water activity (a_w) is too low to support mould growth and/or prevent mycotoxin production. To prevent the growth of most moulds, a_w should be ≤ 0.70, which corresponds to a moisture content of approximately 14% for maize (FAO/IAEA, 2001).

Critical limits for nitrosamines

A level ≤ 5 ppb is generally "accepted" in beer (IZQUIERDO-PULIDO *et al.*, 1996a; LIJINSKY, 1999). The voluntary limit of the industry is 0.5 ppb (SMITH, 1994). In Germany there is a technical reference concentration of 2.5 ppb for malt and 0.5 ppb for beer (VLB, 2006).

Critical limits for biogenic amines

Moderate amounts of biogenic amines (about 50 mg/kg food) can be ingested with food without affecting consumer health. Upon intake of high loads of biogenic amines with food, the human detoxification system is unable to process them sufficiently. LORET *et al.* (2005) developed the Beer Biogenic Amine Index (Beer BAI). It consists of the ratio of the biogenic amine concentration of bacterial origin over the natural biogenic amines found in the malt:

$$\frac{[CAD] + [HIS] + [TYR] + [PUT] + [PHE] + [TRY]}{1 + [AGM]}$$

Beer BAI allows the quality of the production process to be assessed; a BAI ≥10 corresponds to a beer showing vasoactive biogenic amine values >10 mg/L which could cause health problems in certain types of consumers.

Different investigations on the presence of biogenic amines in beer have been carried out (BUIATTI *et al.*, 1995; IZQUIERDO-PULIDO *et al.*, 1996b, GLORIA and IZQUIEDRO-PULIDO, 1999; KALAČ and KŘIŽEK, 2003; FUMI and DONADINI, 2005, LORET *et al.*, 2005) and report results about different kinds of beer from various countries (Europe, Brazil, Canada and Cuba). AGM and PUT were always present in all beers and were the

prevailing amine in the tasted beers; TYR and PUT were present at relatively high levels, while the levels of the others, including HIS, were usually lower and probably of less toxicological importance. SPM and SPD were not always present, but when they were, their contents were generally low.

Step 4: Establish
a monitoring system

After the critical limit has been set, the preventive or control measures should also be established. These measures include any action or activity that can be used to control an identified hazard, such that it is prevented or eliminated or reduced before it enters the process or finds its way to the finished product. Preventive or control measures provide guidance on how to meet the critical limits. The measures can also include prerequisite programmes (e.g. staff training for a particular operation, covered by GAP, GMP and GHP, cleaning and sanitation procedures, pest control).

Monitoring is the scheduled measurement or observation at a CCP to assess whether the step is under control, e.g. within the critical limit(s) specified in step 3 (FAO/IAEA, 2001).

Monitoring procedures specify how a critical limit is to be measured. Monitoring procedures should give the specific method (or include a reference to the method, the responsibility, and the frequency of monitoring) (RUSH, 2006). A monitoring procedure can be in-line, on-line or off-line. Process parameters such as temperature, time and moisture should be controlled within the limits required (CODEX ALIMENTARIUS, 1997).

Monitoring system for mycotoxins

It is essential to require the sanitary inspections (HACCP report) from the malt suppliers/manufactures on a pre-crop year basis for barley and malt.

Intake and storage
(malting and brewing)

A policy on housekeeping and cleaning standards for cereal storage and processing should be established along the malting and brewing food chain (EBC, 2006). The stored product shall not be exposed to environmental conditions, thereby avoiding moisture that can promote mould growth (PARK *et al.*, 1999). Empty silos shall be swept or vacuumed to remove grain residues and may be fumigated or treated with suitable insecticides according to company policy (MAGB, 2006).

The use of formalized management systems (e.g. Quality Management System), with defined working procedures for GHP and GMP are required for the quality control of raw materials. Finally an analytical control is required.

Malting process

In this process, parameters such as temperature and moisture cannot be changed without having an adverse effect on the organoleptic quality of the final product. It is absolutely necessary to control the temperature and the humidity, using formalized management systems (e.g. Quality Management System), with defined working procedures for GMP and GHP.

Monitoring system for nitrosamines

Malting process

Good Agricultural Practices (GAP) are required for the malt suppliers. Use of GMP (do not use direct-firing in kilning; use natural gas, not oil as the kiln fuel source and use indirect malt drying techniques) (IZQUIERDO-PULIDO *et al.*, 1996a; SEN *et al.*, 1996).

The use of sulphur dioxide in the kiln air stream seems to reduce or prevent the formation of undesirable nitrosamines in malt; high application of bromates at the beginning of malting, or spraying or steeping green malt with dilute nitric acid before kilning

has also been proposed (BRIGGS *et al.*, 1981).

Moreover, the NDMA concentration can be reduced by controlling the exposure of malt to nitrogen oxides. This stops the nitrosation of the alkaloids in the malt (LIJINSKY, 1999).

The use of a formalized management system, GHP and GMP, is also required for nitrosamines.

Beer production

Malt suppliers/manufactures are required to carry out NDMA analysis for each malting batch.

The use of a formalized management system (e.g. qualified supplier conforms to brewers' specifications; application of the GHP) is useful. Cleaning-In-Place (CIP) procedures should be used to reduce potential bacterial contamination.

Monitoring system for biogenic amines

The histamine content in beer is a good indicator of the hygienic conditions of barley storage and malting and brewing, because the histamine content of the product does not originate from the barley or malt (HALÁSZ *et al.*, 1999).

Malting process

Malt coming from barley grown in soils with low potassium or sodium or with high ammonia levels should not be used for brewing beer (IZQUIEDRO-PULIDO *et al.*, 1994).

In the GMP for the malting process, barley variety, storage, germinating intensity and kiln temperature will affect the final amine levels in the malt (HALÁSZ *et al.*, 1999; KALAČ and KŘIŽEK, 2003).

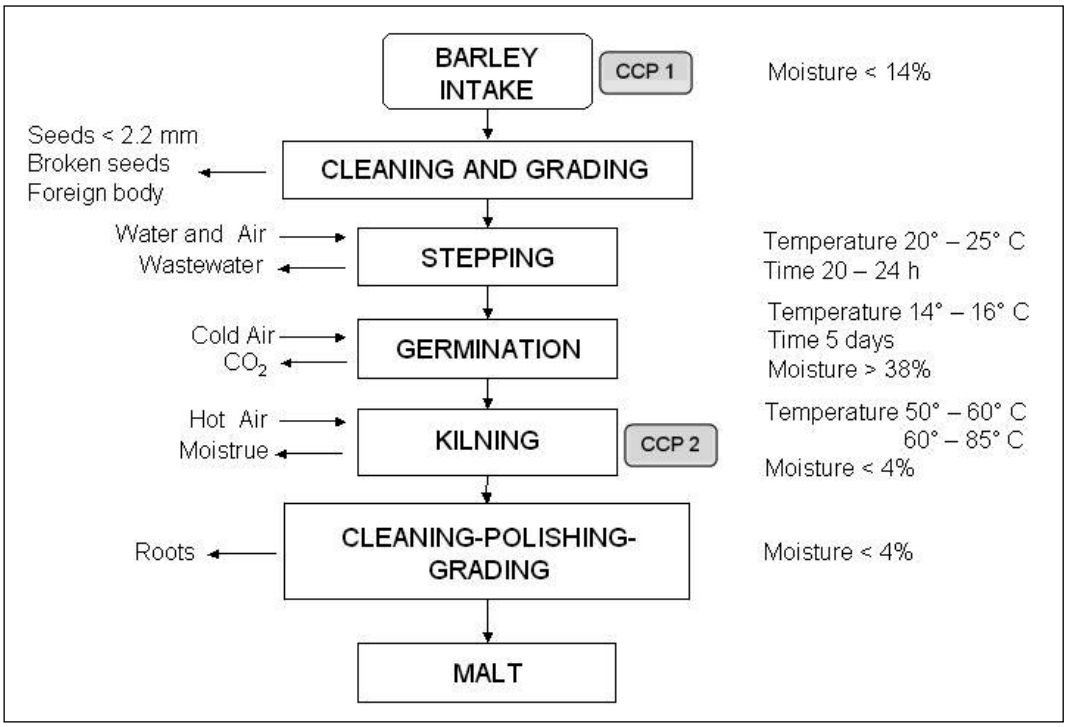


Fig. 1 - Example of the malting process.

Brewing process

TYR, HIS and CAD formation has been observed during the main fermentation due to the presence of lactic acid bacteria (e.g. *Pediococcus* spp., mainly *P. Damnosus*, and species of *Lactobacillus*, *L. Frigidus*, *L. Brevissimilis* and *L. Brevis*) (IZQUIEDRO-PULIDO *et al.*, 2000; KALAČ and KŘIŽEK, 2003).

The use of a formalized management system is required to monitor biogenic amine formation.

Step 5: Establish procedures for corrective action

A procedure for corrective action and for correction must be adopted when monitoring a CCP because it indicates a deviation from an established critical limit (FAO/IAEA, 2001).

Corrective actions are steps that are taken to eliminate the causes of an existing non-conformity or other undesirable situations; the corrective action process includes cause analysis and is designed to prevent recurrence. A correction is any action that is taken to eliminate nonconformity (ISO 22000:2005). The corrective action must provide assurance that the CCP had been brought under control; it must also describe the proper actions to be taken in disposing of the affected product (SENIRES and ALEGADO, 2005). Corrections and corrective actions must be established for each CCP. For the HACCP, the corrective action must address the actions taken to deal with the product produced while the critical limit was being violated and what steps (corrections) are necessary in order that the process return to a state of control and the operation can continue (RUSH, 2006). Corrective action procedures and responsibilities need to be specified (EFSTRATIADIS and ARVANITOYANNIS, 2000).

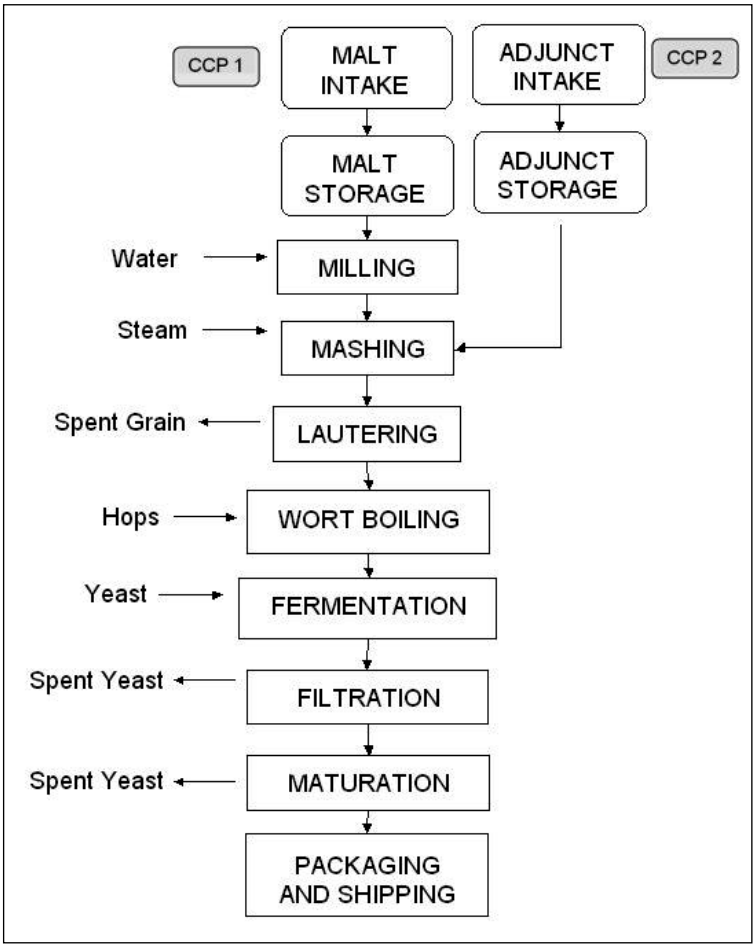


Fig. 2 - Example of the brewing process.

Corrective actions and corrections for mycotoxins

Storage

Corrective action: GMP, GHP and GSP have to be in place before and during storage. Intake procedures should be established with the control parameters for the inspection of grain (e.g. visual check, odour check, moisture). Correction: segregate the batch and reject it if it contains an unacceptable level of mycotoxins (FAO/IAEA, 2001).

Malting process

Corrective action: monitoring the presence of moulds during the process. GMP have to be in place; procedures should be established with the control parameters (e.g. moisture, time). Correction: when moulds are present during this process it is important to segregate the batch and reject it; then CIP measures should be adopted.

Corrective actions and corrections for nitrosamines

Malting process

Corrective action: GMP have to be in place (e.g. monitoring the presence of NDMA during the process by analytical control of malt); new process technologies have to be implemented (indirect malt drying techniques); sulphur dioxide should be used in the kiln air stream.

Correction: use of sulphur dioxide in the kiln air stream to reduce the formation of undesirable nitrosamines in malt; high levels of bromates applied at the beginning of malting, or spraying or steeping green malt with dilute nitric acid before kilning (BRIGGS *et al.,* 1981). The NDMA concentration can be reduced by controlling the exposure of malt to nitrogen oxides, thereby stopping nitrosation of the alkaloids in the malt (LIJINSKY, 1999).

Corrective actions and corrections for biogenic amines

Brewing process

Corrective action: GMP and GHP have to be in place (e.g., washing the pitching yeast with phosphoric acid to reduce the number of *Pediococcus* spp. bacteria and consequently reduce the TYR content of beer (KALAČ and KŘIŽEK, 2003). Special attention is required in controlling the presence of lactic acid bacteria contamination (IZQUIEDRO-PULIDO *et al.*, 2000).

Correction: GHP (CIP) should be in place if bacterial contamination is found.

Step 6: Establish procedures for verification

Each industry has to establish procedures to verify and confirm the effectiveness of the HACCP plan. Such procedures should include auditing the HACCP plan to review deviations and product disposition as well as random sampling and checking to validate the whole plan (FAO/IAEA, 2001).

Each industry has to establish the frequency (e.g., annual; every 6 months; every month; every delivery) of the action used to monitor the system. Following are some examples:

- Process: malting process; Hazard: NDMA; Control Measures: analytical control; Frequency: every batch.

- Process: malt intake; Hazard: mycotoxins; Control Measures: visual inspection of grain; Frequency: every delivery.

It is essential that all HACCP-related activities be accurately and efficiently documented. This includes creating records of the hazards and their methods of control, the monitoring of safety requirements, and the action taken to correct problems. All actions must also be properly recorded for the traceability requirements (SENIRES and ALEGADO, 2005). The results of analytical control are part of the verification process for the contaminants evaluated.

Step 7: Establish documentation concerning all procedures and records appropriate to these principles and their application

Verifying whether HACCP works effectively is extremely important in order to determine if it is necessary to modify or alter the HACCP plans adopted and in operation, and the relevant procedures associated with it. Validation, on-going verification, reassessment, and audits are the tools implemented to verify the effectiveness of the HACCP system. These tools can detect if the preventive measures, corrective actions, critical limits, and risk assessments are sufficient to address the problems (SENIRES and ALEGADO, 2005). The results of monitoring activities during the execution of the HACCP plan must be recorded (RUSH, 2006).

For example, the results of the hazard analysis and the study of CCP, CP and the overall management of mycotoxins in malting and brewing processes are reported in Tables 1 and 2. The control measures, critical limits and corrective actions are reported for mycotoxins and moulds at different steps within the malting and brewing processes.

Storage is the most critical post-harvest phase in the beer chain. An inappropriate storage facility, improper packaging and/or condition of the product can cause mycotoxin contamination during storage. An accumulation of moisture and heat and/or physical damage to the product foster fungal invasion, which can lead to the occurrence of mycotoxins. Stored products should not be exposed to environmental conditions (e.g. moisture) that promote mould growth.

CONCLUSIONS

An innovative HACCP-based approach for assessing, evaluating, and controlling the specific risk of mycotoxins, nitrosamines and biogenic amines in malting and brewing industries has been presented. The main Critical Control Points (CCPs) for mycotoxins, *N*–nitrosamines and biogenic amines have been identified. The specific concerns regarding the application of this HACCP study to small and medium-sized enterprises (SMEs) have been addressed and clarified.

It is clear that the proposed HACCP plan, when correctly and systematically applied, offers a real and powerful tool for identifying and assessing any possible chemical and microbiological hazard.

In particular, many factors that influence mycotoxin contamination of cereals, such as weather and insects, are environmentally-related and are difficult to control. This is the main reason why the HACCP system should be adopted at the field/farm level. This choice would strongly enhance the safety of harvested commodities and prevent food hazards along the whole food chain. GMP and GAP are indispensable pre-requisites for implementing HACCP at this level.

Moreover, post-harvest storage, transport and processing are all important stages, which must be monitored and managed with the GAP and GMP. Food safety must be assured by controlling the whole process and not just by testing the finished product.

This paper has given input on how to develop HACCP programmes based on GMP and GAP for mycotoxins, nitrosamines and biogenic amines in the brewing production chain. Since HACCP, GAP, and GMP are the major components of safety management systems along the whole food supply chain, this paper could be used as an example for other correlated chains, such as those that use cereals as raw materials (bread, cookies, pasta, etc.).

Finally, it is essential to take into consideration the specific needs of small enterprises (microbreweries, micro pubs, etc.). They are quite often associated with

Table 1 - E.g.: List of identified CCP, CP and the overall management of mycotoxins in malting.

CCP and CP No.	Process Step	Hazard Description	Critical Limit	Monitoring Procedures	Correction/Corrective Actions	Verification Procedure/Frequency	Record keeping
CP 1	Barley intake	Presence of mycotoxins & mould	Malter specifications	Qualification of suppliers	Reject supplier Qualify new supplier Request farmer to implement GAP and encourage growers to use GAP.	Evaluate the suppliers every year	YES
CCP 1	Barley intake	Presence of mycotoxins & mould	European Legislation: Regulation (EC) n. 1881/2006 setting maximum levels for certain contaminants in foodstuffs, and modification European Legislation: Regulation (EC) n. 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs.	Quality control: - careful inspection with adequate sampling techniques - visual inspection must take place for insects, foreign materials and mould - the smell of barley before the intake will reveal if it has been stored under good conditions - documents must accompany every delivery of barley to malts, indicating whether or not a pesticide has been applied by the growers/seller post harvest - deliveries shall conform to malters' specifications, which will take into account the customers' requirements - analytical control	Reject the contaminated batch. No delivery will be accepted without an accompanying document.	Every batch	YES
CP 2	Barley storage	Growth of mycotoxins & mould	European Legislation: Regulation (EC) n. 1881/2006 Moisture < 13%; Temperature <15°C; Time	Green barley shall not be stored for longer than 2 weeks at moisture levels at or above 18% GHP: have to be in place before storage: - storage area must be clean, dry and well ventilated; - all parts of the storage area, including	Stored products should not be exposed to environmental conditions, such as moisture, that promote mould growth. Once a contaminated product has been	Green barley moisture control every 2 weeks (moisture not above 18%)	YES

Table 1 (continued).

CCP and CP No.	Process Step	Hazard Description	Critical Limit	Monitoring Procedures	Correction/Corrective Actions	Verification Procedure/Frequency	Record keeping
				the inside surface and others that will be in contact with the grain, may be treated and left to dry. GMP: have to be maintained during the malting production: - first in-first out practices; - cereal must be managed during storage; - maintain segregation of the lots; - regular inspection of the grain during storage (visual control); - temperature and moisture management is vital to prevent spoilage in stored grain.	identified, clean-up, and separation of the batch are the actions to be put in place. Destruction of the contaminated lot and cleaning the storage area. Empty silos or storage area have to be swept or vacuumed to the sites cleaning schedule to remove grain residues, and may be fumigated or treated with suitable insecticides (only conform to National legislation) according to company policy		
CP 2	Malting process: steeping; germination and storage	Growth of mycotoxins & mould	Temperature Time Moisture	It is necessary to control the temperature and moisture, using a formalized management system with defined working procedures. separation of the batch are the first actions to be GHP: have to be in place before the process. GMP: have to be maintained during the malting production (first in-first out practices; segregation of the lot)	Once a contaminated product has been identified, clean-up, and shall be controlled within the limits put in place.	Process parameters such as temperature, time and moisture required for the type of malt being made, daily.	YES

Table 2 - List of identified CCP, CP and the overall management of mycotoxins in brewing.

CCP and CP No.	Process Step	Hazard Description	Critical Limit	Monitoring Procedures	Correction/Corrective Actions	Verification Procedure/Frequency	Record keeping
CP 1	Raw material intake (Malt and Adjuncts)	Presence of mycotoxins & mould	Brewer's specifications	Qualification of suppliers	Reject supplier Qualify new supplier	Evaluate the suppliers every year;	YES
CCP 1	Raw material intake (Malt and Adjuncts)	Presence of mycotoxins & mould	European Legislation: Regulation (EC) n. 1881/2006 setting maximum levels for certain contaminants in foodstuffs, and modification. European Legislation: Regulation (EC) n. 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs.	Quality control: - careful inspection with adequate sampling techniques - visual inspection must take place for insects, foreign materials and mould; - the smell of malt before the intake will reveal if it has been stored in under good conditions - deliveries shall conform to brewers' specifications, which will take into account the customers' requirements and any regulatory limits - documents must accompany every delivery to brewers - analytical control is required for a large amount of raw materials intake. No delivery will be accepted without an accompanying document.	Reject the contaminated bulk Request the missing document from the suppliers	Every batch	YES

Table 2 (continued).

CCP and CP No.	Process Step	Hazard Description	Critical Limit	Monitoring Procedures	Correction/Corrective Actions	Verification Procedure/Frequency	Record keeping
CP 2	Raw materials storage	Growth of mycotoxins & mould growth	European Legislation: Regulation (EC) n. 1881/2006 setting maximum levels for certain contaminants in foodstuffs, and modification. Moisture <13%; Temperature <15°C;	GHP have to be in place before storage: - storage area must be clean, dry and well ventilated; - all parts of the storage area, including the inside surface and others which will be in contact with the grain, may be treated and left to dry. GMP: have to be maintained during beer production: - first in-first out practices; - cereal must be managed during long storage; - maintain the segregation of lots; - regular inspection of the grain during long storage (visual control); - temperature and moisture management is vital to prevent spoilage in long storage insecticides (only conform to national legislation) according to company policy	Stored products shall not be exposed to environmental conditions, such as moisture, that promote mould growth. Once a contaminated product has been identified, clean-up, and separation of the batch are the actions to put in place. Destruction of the contaminated lot and cleaning the storage area. Empty silos or storage area have to be swept or vacuumed to the sites cleaning schedule to remove grain residues, and may be fumigated or treated with suitable	Store quality control: once a year Quality control during long period of storage: visible inspection once/month.	YES

the use of non-standardized lots of barley or malt. Therefore, particular attention must be given to potentially dangerous factors that affect mould growth. Moreover, water, malt or adjuncts with unknown nitrogen contents are often used and therefore proper hygiene cannot always be effectively controlled and standardized.

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SHORT COMMUNICATION

THE FATTY ACID COMPOSITION OF BRAZILIAN FISHERY BY-PRODUCTS

COMPOSIZIONE IN ACIDI GRASSI DEI SOTTOPRODOTTI DEL PESCATO BRASILIANO

G.F. OLIVEIRA, F.C. DESCHAMPS[1] and M.L. PESSATTI*
Centro de Ciências Tecnológicas da Terra e do Mar,
Universidade do Vale do Itajaí (CTTMAR/UNIVALI), Itajaí, SC, Brasil
[1]Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina S.A. (EPAGRI), Itajaí, SC, Brasil
*Corresponding author: e-mail: pessatti@univali.br

ABSTRACT

The total lipids and their fatty acid composition were evaluated in different by-products from fish processing plants in the State of Santa Catarina, Brazil. By-products from *Katsuwonus pelamis*, *Thunnus albacares*, *Sardina pilchardus*, *Sardinella brasiliensis*, *Ophistonema oglinum*, *Harengula clupeola*, *Micropogonias furnieri*, *Cysnoscion leiarchus* and toasted fish meal were evaluated. The highest levels of lipids (dry matter) were found in *K. pelamis* and *O. oglinum*, while by-products

RIASSUNTO

Sono stati valutati i lipidi totali e la loro composizione in acidi grassi in differenti sottoprodotti della lavorazione del pesce nello stato di Santa Caterina in Brasile. Sono stati analizzati sottoprodotti di *Katsuwonus pelamis*, *Thunnus albacares*, *Sardina pilchardus*, *Sardinella brasiliensis*, *Ophistonema oglinum*, *Harengula clupeola*, *Micropogonias furnieri*, *Cysnoscion leiarchus* e di alimenti tostati a base di pesce. I più alti livelli di lipidi (riferiti al peso secco) sono stati determinati nei sottoprodot-

- Key words: fatty acids, fishery, fishery by-products, gas chromatography, lipids -

from *K. pelamis, S. brasiliensis* and *S. pilchardus* had the highest concentrations of polyunsaturated fatty acids (PUFAs). Many by-products from industrial fish processing are potential sources of PUFAs.

ti di *K. pelamis* e *O. oglinum*, mentre i sottoprodotti di *K. pelamis, S. brasiliensis* e *S. pilchardus* presentavano le concentrazioni più alte di acidi grassi poliinsaturi (PUFAs). Molti sottoprodotti della lavorazione industriale del pesce sono potenziali fonti di PUFAs.

INTRODUCTION

During production the fish processing industry can generate more than 50% by-products, which have social, environmental and economic implications (OETTERER, 1994; RIVERA, 1994; TIMOFIECSYK and PAWLOWSKY, 2000). The loss can begin on board the fishing vessel due to several factors, such as storage temperature and packaging conditions (volume, time) (ANDRADE, 1998; OETTERER, 1989).

Despite the importance of the fishing industry in Brazil, the by-products usually have little value. In one of the largest industrial parks in southern Brazil (which unloads about 97 thousands tons of fish/year (20% of the national industrial production), some 30 thousand tons of by-products are generated annually, which are used mainly for the production of fish meal (PESSATTI, 2001; STORI, 2000). Some products for human consumption can be made from these fishery by-products, such as fish oil and surimi (BIMBO, 1987; MORALES-ULLOA and OETTERER, 1995).

It has been demonstrated that up to 50% of these by-products are fit for human consumption and contain high levels of lipids and proteins (PESSATTI, 2002). These by-products could provide an important source of essential fatty acids, since the consumption of fish oil has been highlighted as a growing market (FAO, 2000).

Many studies have demonstrated the abundance of essential fatty acids in fish, particularly from the omega-3 (n-3) and omega-6 (n-6) series (BIMBO, 2000; HEARN *et al.*, 1987; KINSELLA, 1986; PACHECO *et al.*, 1991; PIGOTT and TUCKER, 1987; STANSBY *et al.*, 1990). There is strong evidence of the benefits to human health related to the consumption of polyunsaturated fatty acids (PUFAs). The role that eicosapentaenoic (EPA, C20:5) and docosahexaenoic (DHA, C22:6) acids play in the prevention of cardiovascular diseases, and their effects on the vascular and haemostatic systems, the brain, the retina and other body tissues, have been investigated (STANSBY *et al.*, 1990; VISENTAINER *et al.*, 2000). Other studies have demonstrated the anti-inflammatory properties and benefits to the immunological system, with particular effectiveness against asthma and rheumatic arthritis (BADOLATO *et al.*, 1991). PUFAs, mainly of the n-3 family, have been used in margarine, milk, eggs and feed supplements (BADOLATO *et al.*, 1991; BIMBO, 1987; CHAPMAN and REGENSTEIN, 1997). The aim of this work was to characterize the lipid fraction of fish by-products (fish heads, viscera, backbone and muscle), particularly from sardine and tuna, because these are the most commonly processed species in the region where the study was conducted.

MATERIALS AND METHODS

The fishing fleet that supplies the processing plant operates in the southern

Table 1 - Fish species and possible by-products based on fatty acid characterization.

Family	Generous/species	Common names	By-products
Scombridae	*Katsuwonus pelamis* (LINNAEUS, 1758)	Skipjack tuna	Crude visceras, toasted visceras, skin, crude head, cooked head and dark muscle.
Scombridae	*Thunnus albacares* (BONNATERRE, 1788)	Yellowfin tuna	Crude head and visceras.
Clupeidae	*Sardinella brasiliensis* (STEINDACHNER, 1789)	Brazilian sardinella	Crude head, visceras and tail.
Clupeidae	*Opisthonema oglinum* (LESUER, 1818)	Atlantic thread herring	Crude ventral keel and head
Clupeidae	*Sardina pilchardus* (WALBAUM, 1792)	European pilchard, Sardine	Crude head and visceras.
Sciaenidae	*Cynoscion leiarchus* (CUVIER, 1830)	Smouth weakfish	Crude head, visceras, flippers and backbones.
Sciaenidae	*Micropogonias furnieri* (DESNAREST, 1823)	Whitemouth croaker	Crude head.
Sciaenidae	*Harengula clupeola* (CUVIER, 1829)	False herring	Whole.
–	Several	Fish flour	Several

and southeast, regions of the Brazilian coast. To know the origin of the by-products, samples of the fish were collected and identified before processing (FIGUEREDO and MENEZES, 1978, 2000; MENEZES and FIGUEREDO, 1980; WHITEHEAD, 1985) (Table 1).

After being unloaded, the fish were kept for a maximum of 3 days in a cooling tunnel at 0°C, until processed. An exception was the *Sardina pilchardus*, which was kept frozen at -23°C, for about 1 month prior to processing, since they were imported from Morocco. Samples for this study were obtained from fish immediately after processing.

The by-products were collected from four different production lines: tuna loin, sardine viscera and flattened fish fillets. Since the by-products are generally donated to flour factories, a sample of the flour, comprised of a mixture of fish by-products, was also collected.

Samples of the by-products, taken from about 20 specimens, were ground in a manual grinder and homogenized. Half of each sample was used to determine moisture and lipid content. The other half was used to extract the lipids which were then analyzed to determine the fatty acid profile. The samples of mixed flour, toasted viscera and cooked heads of skipjack tuna (*Katsuwonus pelamis*), white whitemouth croaker (*Micropogonias furnieri*) and smouth weakfish (*Cynoscion leiarchus*) were frozen for one month prior to analysis.

The moisture content was determined in triplicate, after heating in an oven at 105°C, until constant weight. The quantity of total lipids was determined in duplicate (average of two samples), using a Soxhlet extractor and a mixture of petroleum and ethyl ether (1:1) as extraction solvent.

To analyze the fatty acid profile, the lipid extraction was carried out according to BLIGH and DYER (1959), with the addition of 0.1% butylated hydroxytoluene (BHT). After solvent evaporation under nitrogen, the samples were stored at -25°C under nitrogen atmosphere and protected from light. The derivation of fatty acids to their respective methyl esters (FAMEs, fatty acid methyl esters) was carried out using the HARTMAN and LAGO (1973) method. The FAMEs were analyzed by gas chromatogra-

phy (GC), in a Shimadzu gas chromatograph (model 17A, Shimadzu do Brasil, São Paulo, SP, Brazil), with an automatic injector, using a flame ionization detector and a capillary column (SP 2340; 60 m x 0,25 mm i.d. x 0,2 μm film, Supelco, São Paulo, SP, Brazil). The injector was operated at 250°C and the detector (FID) at 260°C. Helium was used as the mobile phase, with a flux of 17 cm/s, with 1 μL of sample volume injected. The initial temperature was 120°C, with increasing heat at 4°C/min until 240°C. The total run time was 45 min.

The separated FAMEs were identified by comparising their retention times with those of previously analyzed standards (Fame Mix Supelco 37 Components, Sigma-Aldrich Brazil, São Paulo, SP, Brazil). For quantification, the percentage of peak areas was calculated in relation to the total area of detected peaks, including non-identified peaks.

RESULTS AND CONCLUSIONS

The composition of the by-products derived from the fish processing industry shows some variations. The generation of by-products depends on the production line in operation, as well as other variables in the fish capture technology, as well as on the characteristics of the fish processing plant (ACKMAN, 1989; BADOLATO *et al.*, 1994; BOTTA *et al.*, 1987; SILVA *et al.*, 1993; STANSBY, 1981, 1990). Despite variations, the results show that the various by-products have a high lipid content (Table 2). Moreover, the fatty acid composition, in general, is very interesting with an abundant n-3 content, especially EPA and DHA (Table 3).

Of the by-products analyzed, tuna skin had a high concentration of lipids and polyunsaturated fatty acids (PUFAs), even when cooked (Tables 2 and 3). These results could be related to nat-

Table 2 - Moisture and lipid content of the by-products.

By-products	Moisture *	Lipids *
Scombroids		
Crude viscera of *T. albacares*	75.58±0.66	5.19±0.15
Crude head of *T. albacares*	71.13±0.87	11.53±0.11
Crude viscera of *K. pelamis*	74.92±0.37	15.70±0.81
Cooked viscera of *K. pelamis*	8.91±0.76	14.73±1.52
Crude head of *K. pelamis*	69.26±1.61	19.09±0.23
Cooked head of *K. pelamis*	62.14±1.94	6.77±0.25
Cooked skin of *K. pelamis*	48.99±1.23	42.81±2.00
Cooked dark muscle of *K. pelamis*	66.78±0.95	10.01±0.04
Clupeids		
Crude head of *O. oglinum*	69.99±0.35	14.97±1.57
Crude ventral keel of *O. oglinum*	62.24±0.28	37.11±0.86
Crude head/viscera of *S. pilchardus*	74.16±0.49	16.64±0.07
Crude head/viscera of *S. brasiliensis*	75.62±0.28	16.55±0.02
Others		
Crude head of *M. furnieri*	69.57±1.08	15.32±0.70
Crude head/viscera of *C. leiarchus*	74.28±1.09	10.10±0.24
Crude backbone of *C. leiarchus*	74.45±0.26	17.04±0.51
H. clupeola crude whole	71.12±1.10	9.52±2.39
Fish flour	10.10±1.57	1.68±0.43

* The results represent the means of triplicates (moisture) or duplicates (lipids) with their respective standard deviations.

ural antioxidants, such as tocopherols, and some amino acids, which can protect PUFAs from oxidation. Moreover, it is known that some proteins complex metals, which blocks their pro-oxidant effect (ACKMAN, 1989; CHEFTEL and CHEFTEL, 1992; SILVA *et al.*, 1993).

Compared to the raw samples, the cooked and toasted samples, in general, had lower PUFA concentrations and higher saturated fractions. These results were similar to those reported in other studies (ACKMAN, 1989; BELTRAN and MORAL, 1991; GALL *et al.*, 1983). Some studies, however, did not show any changes in the fatty acid composition of the fish products after processing (BELTRAN and MORAL, 1991; SILVA *et al.*, 1993), including smoking (MUSTAFA and MEDEIROS, 1985). This demonstrates that there is no clearly-defined standard of interference of physical conditions on the PUFA content and profile. An example of this apparent paradox was the cooked tuna heads (*K. pelamis*), which showed higher levels of PUFAs than the raw sample. As observed by BELTRAN and MORAL (1991), samples of frozen and cooked sardine (*S. pilchardus*) showed higher C20:5 and C22:6 (EPA and DHA, respectively) values, when compared with raw fillet, which had values similar to those observed in this work for tuna. Those authors reported that some possible causes of this phenomenon, at least for the frozen material, could have been an increased susceptibility of these fatty acids to extraction by organic solvents, due to the weakening of lipoprotein bonds as a result of freezing. This hypothesis is reinforced in the present work, since the sample of cooked tuna (*K. pelamis*) heads was stored for one month prior to extraction. Cooking, on the other hand, can induce the release of structural fatty acids, as result of the application of heat.

The method of extraction may also affect the yield and composition of these lipids. IVERSON *et al.* (2001) compared two classic methods of cold extraction, those of BLIGH and DYER (1959) and FOLCH *et al.* (1957), and concluded that the first underestimates the lipid content compared to the second.

The PUFA content was around 30% for the majority of by-products. Nevertheless, non-identified peaks, making up around 15% of the total, may have affected the results. These peaks represent other volatile molecules, such as non-identified fatty acids and alcohols, which do not have corresponding molecules in the FAME standards used.

Another important result is that the samples with higher lipid contents were not always those with a higher PUFA content, as in the case with the ventral keel of sardine (*O. oglinum*). Like all pelagic and filtrator species (BELVÈZE and ERZINI, 1983; SACCARDO, 1983), the ventral and subcutaneous regions are the main sites of lipid deposits.

The DHA and EPA levels were high (28.4%) in the dark muscle of tuna, although it was lower than the previously reported 34.6% (BRUSCHI, 2001). The sample in that study was not processed and was frozen prior to analysis, while the sample used in the present work was cooked and processed immediately afterwards.

The EPA:DHA (C20:5n3:C22:6n3) ratio was lower than one most of the species studied which shows the potential of these by-products as sources of DHA. The exceptions were sardine (*S. pilchardus*) and white mouth croaker (*M. furnieri*) by-products, which have been proven to be good sources of EPA. For sardine *S. brasiliensis*, the ratio established was as low as 0.5.

The results of this study corroborate the potential use of by-products from the fishing industry, as a source of fish oil and PUFAs. It should be stressed, however, that the by-products that had the highest levels of PUFAs were those from sardine and tuna, which are the most frequently processed species in the region of the study.

Table 3 - Fatty acid composition of the fish by-products.

		SCOMBROIDS				
		Crude viscera *T. albacares*	Crude head *T. albacares*	Crude viscera *K. pelamis*	Toasted viscera *K. pelamis*	Crude head *K. pelamis*
Fatty acid						
Capric	C10:0	1.47	2.20	0.42	1.88	3.75
Myristic	C14:0	0.74	3.16	2.87	4.36	-
Pentadecanoic	C15:0	0.46	0.79	0.85	1.02	0.90
Palmitic	C16:0	14.84	17.36	20.51	25.24	20.38
Palmitoleic	C16:1	0.76	3.20	2.74	3.51	3.82
Heptadecanoic	C17:0	0.83	1.07	1.08	1.34	1.05
Cis - 10 - Heptadecanoic	C17:1	-	0.46	-	-	-
Estearic	C18:0	7.07	6.04	7.81	7.61	5.99
Oleic	C18:1n9c	4.39	15.05	10.28	15.74	15.82
Linoleic	C18:2n6c	0.46	1.31	1.30	1.38	1.46
Arachidic	C20:0	-	-	-	-	-
Cis - 11 – Eicosanoic	C20:1	0.41	2.05	0.88	1.47	2.63
Linolenic	C18:3n3	-	0.64	0.78	-	0.88
Cis - 11, 14, 17 - Eicosadienoic	C20:2	-	-	-	0.68	-
Arachidonic	C20:4n6	5.16	2.27	3.23	1.80	1.54
Lignoceric	C24:0	-	-	-	-	-
Cis - 5,8,11,14,17- Eicosapentaenoic EPA	C20:5n3	4.35	4.89	6.02	5.01	5.76
Norvonic	C24:1	0.36	0.89	0.77	1.16	0.92
Cis - 4,7,10,13,16,19 - Docosahexaenoic DHA	C22:6n3	20.89	21.59	20.49	16.61	22.77
Total non-identified		37.81	17.05	19.96	11.18	12.31
Total saturated		25.41	30.62	33.54	41.45	32.08
Monounsaturated		5.92	21.65	14.68	21.89	23.20
Polyunsaturated		30.87	30.68	31.83	25.48	32.41
Total ω-3		25.25	27.11	27.30	21.61	29.41
Total ω-6		5.62	3.57	4.53	3.18	3.00
Total ω-9		4.39	15.05	10.28	15.74	15.82

BY-PRODUCTS/RESIDUES											
			SARDINES - CLUPEIDS				OTHERS				
Cooked head *K. pelamis*	Cooked skin *K. pelamis*	Cooked dark muscle *K. pelamis*	Crude head *O. oglinum*	Crude ventral keel *O. oglinum*	Crudes head / visceras *S. pilchardus*	Crudes head / tail *S. brasiliensis*	Crudes head / visceras *C. leiarchus*	Crudes backbone / tail *C. leiarchus*	Crudes head / visceras *M. furnieri*	H. clupeola whole crude	Fish flour
			%								
-	-	1.37	-	-	1.66	1.42	-	1.79	1.14	-	-
3.45	-	3.29	6.00	6.15	7.75	5.76	3.71	4.23	4.10	5.42	7.27
1.13	1.02	0.74	1.46	1.61	-	1.34	0.53	0.56	0.86	1.29	-
21.89	21.19	21.48	25.14	27.66	19.42	23.13	21.88	23.49	25.69	22.54	30.51
4.28	4.18	2.50	4.31	4.07	7.99	3.61	7.44	7.83	12.83	3.49	7.88
1.30	1.04	1.00	1.51	1.57	-	1.38	0.55	0.56	1.16	1.27	-
-	0.58	-	-	-	-	-		-	-	-	6.92
6.48	4.19	6.73	7.36	7.34	4.22	6.79	5.01	4.86	8.18	6.10	-
13.25	16.58	17.58	7.49	8.77	7.08	6.33	20.78	22.76	11.07	7.70	3.95
1.44	1.63	1.46	1.39	1.38	3.09	1.40	1.06	1.25	-	1.32	-
-	0.39	-	0.44	0.65	-	0.81	-	-	-	-	-
1.00	1.24	1.47	0.54	0.77	1.50	0.98	0.79	1.11	1.00	-	-
-	1.03	0.72	1.42	1.58	0.64	1.47	0.60	0.73	1.28	1.23	-
-	-	-	-	-	-	1.53	-	-	-	-	-
1.88	1.59	1.25	2.27	1.94	1.59	2.18	1.97	1.95	2.33	2.34	-
-	-	-	-	-	-	-	-	-	-	-	6.68
5.91	7.25	4.26	7.28	6.71	19.52	10.08	5.95	5.92	9.16	5.99	-
-	0.55	2.20	0.49	0.37	0.63	0.56	0.51	0.65	-	-	-
27.69	24.47	24.11	20.46	17.84	11.42	19.98	11.06	11.13	6.06	20.15	-
10.30	13.18	9.86	12.43	11.57	13.50	11.26	18.16	11.17	15.14	21.16	36.78
34.25	27.54	34.61	41.93	44.99	33.04	40.63	31.68	35.50	41.13	36.62	44.47
18.53	22.88	23.73	12.83	13.98	17.20	11.48	29.52	32.35	24.90	11.19	18.75
36.92	35.60	31.80	32.82	29.46	36.25	36.64	20.65	20.98	18.83	31.03	-
33.60	32.41	29.10	29.16	26.13	31.57	31.53	17.61	17.78	16.50	27.37	-
3.32	3.19	2.70	3.66	3.33	4.68	3.58	3.03	3.20	2.33	3.66	-
13.25	16.41	17.58	7.49	8.77	7.08	6.33	20.78	22.76	11.07	7.70	3.95

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THE MONIQA NETWORK: AN OVERVIEW ON LEGAL CONSTRAINTS AND FACTORS LIMITING RESEARCHER MOBILITY AND INTEGRATION WITHIN THE NETWORK

LA RETE DI ECCELLENZA EUROPEA MONIQA: LIMITI LEGALI ED ALTRI FATTORI LIMITANTI LA MOBILITÀ DEI RICERCATORI E L'INTEGRAZIONE ALL'INTERNO DELLA RETE

M. CARCEA* and F. MELINI
Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione, INRAN, Via Ardeatina 546, 00178 Roma, Italy
*Corresponding author: carcea@inran.it

ABSTRACT

The following is an overview of the state of researcher mobility in food science and technology within and outside of Europe. The laws and rules which currently regulate researcher recruitment, publishing of job vacancies, restrictions of access, preferential channels, health and safety in the workplace, Intellectual Property Rights issues and mobility in the 31 MoniQA Consortium partner are reported. MoniQA – "Towards the harmonisation of analytical methods for

RIASSUNTO

Questo lavoro è una panoramica sullo stato attuale della mobilità dei ricercatori che lavorano nell'ambito delle Scienze e Tecnologie alimentari in Europa e nei paesi extra-europei. In particolare, vengono presentate le leggi e le normative europee che regolano attualmente il reclutamento dei ricercatori, la pubblicazione dei posti di lavoro, le restrizioni d'accesso e i canali preferenziali per certe posizioni scientifiche, la salute e la sicurezza nel posto di lavoro, i Diritti di Proprietà Intellettuale, nonché la mo-

- Key words: MoniQA Network of Excellence, researcher recruitment, Intellectual Property Rights, mobility, network integration -

monitoring food quality and safety in the food supply chain" – is a European Network of Excellence that was started in 2007. Regarding researcher mobility, the possible length of the mobility period, the extent to which bureaucracy hampers mobility, the economic issues regarding staff on leave (salaries, support for housing and living expenses, grants and/or scholarships) and any procedure to be followed so that a researcher can be hosted by a research institution/centre are reported. Some factors that limit researcher mobility: language (especially applying through websites, vacancy advertisements and understanding of documents in general), limited funding and the lack of contacts in the hosting institution.

bilità dei ricercatori nell'ambito delle 31 Istituzioni che fanno parte della rete di eccellenza europea MoniQA – "Towards the harmonisation of analytical methods for monitoring food quality and safety in the food supply chain" – finanziata dalla Commissione Europea a partire dal febbraio 2007. Per quanto riguarda la mobilità dei ricercatori, questa indagine ha analizzato soprattutto i limiti applicabili alla durata del periodo di mobilità del ricercatore, quanto la burocrazia costituisce un ostacolo alla mobilità, gli aspetti economici (stipendio, vitto e alloggio, erogazione/disponibilità di borse di studio), nonché le procedure da seguire nel caso in cui un ricercatore voglia essere ospitato da un istituto di ricerca/università. I fattori principali che limitano o che potrebbero limitare la mobilità dei ricercatori sono costituiti dalla lingua, dal limitato finanziamento, dalla mancanza di contatti tra il ricercatore e l'istituto ospitante.

INTRODUCTION

The aim of the EC-funded Project MoniQA, "Towards the harmonisation of analytical methods for monitoring food quality and safety in the food supply chain" (www.moniqa.org) is to establish long-lasting cooperation among leading food research institutions, food and biotechnology industries, as well as small- and medium-sized businesses to ensure food quality and safety for consumers. Two years after the kick-off meeting, progress has been made and new steps have been taken to coordinate the analytical methods for monitoring food quality and safety in the food supply chain. Meetings have been organised on a regular basis so that the 33 partners from the EU and non-EU countries could discuss any improvements and future challenges. Moreover, workshops have been held to provide the scientific and administrative staff with training on several topics in order to share knowledge and to network with organisations that are not part of the MoniQA.

Databases have been established to overcome the current fragmentation in food science and technology and to implement the planned harmonisation. The "research database" currently allows MoniQA partners to search for analytical methods for specific analyses including information on the degree of validation, legal requirements, legal limits and availability. The "consortium database", on the other hand, provides information about expertise, special equipment and participant expertise within MoniQA. The latter, in particular, provides an overview of available skills and

expertise within the consortium. By providing key information, it can facilitate close cooperation and coordination of activities of entire research groups and/or departments in an ambitious attempt to create a "virtual laboratory". Another database currently organises the outcome of the survey on "legal constraints and factors that limit researcher mobility". The survey was carried out by the Italian National Institute for Research on Food and Nutrition (INRAN) in collaboration with 31 of the 33 MoniQA partners from 20 countries (Austria, Belgium, Bulgaria, China, Egypt, Finland, Germany, Greece, Holland, Hungary, Indonesia, Israel, Italy, New Zealand, Norway, Poland, Spain, Turkey, the United Kingdom and Vietnam).

INRAN SURVEY OF "LEGAL CONSTRAINTS AND FACTORS LIMITING RESEARCHER MOBILITY"

This paper gives a significant overview of the state of research integration in food science and technology within and outside of Europe as far as researcher mobility is concerned. Given the number and typology of participants, it can be considered to be representative of the world of research in food science and technology, at least in Europe. The laws and rules which currently regulate researcher recruitment, restrictions of access, preferential channels, health and safety in the workplace, issues of Intellectual Property Rights (IPR) and mobility of 31 MoniQA partners are reported.

Within the countries participating in the MoniQA Network, research in food science and technology is carried out in universities and in university-related-centres of excellence, in public institutions under the auspices of specific Ministries, in private research institutions and in scientific and technological parks. These categories are responsible for varying percentages of research in the different countries. In most of the network, researcher recruitment is rarely planned in advance. Only 3 out of 31 partners plan recruitment so that it takes place at the same time each year. For 17 partners, recruitment depends on the specific needs at the moment, on the projects or when there is a vacancy. Nine partners reported that personnel recruitment is planned only when it concerns higher scientific positions.

In general, job vacancies are posted and publicised so that any researcher interested in a specific vacancy can submit an application. However, it was found that only 22 of the 31 partners publish vacancies on the web. Six partners only publish vacancies in official national journals and newspapers, which limits its access to foreign researchers unless they know the specific official journal and/or newspaper. In most cases, the job vacancy is not publicised in English but in the language of the specific country. Only 10 partners advertise vacancies in English, 4 of which are English mother-tongue partners. Therefore, language is a limiting factor that limits researcher mobility.

RESEARCHER RECRUITMENT

Researcher recruitment is currently regulated by both internal laws, which are specific to each institution, and by national laws which provide the legal framework which regulates the recruitment procedures, contracts, probationary periods and career development for scientific personnel in each country.

Recruitment of both permanent and temporary staff is generally conducted in the same manner which includes job advertising, receipt of applications, assessment and decision-making (14 countries out of 20). Assessment is usually based on personnel interviews, and only occasionally on written tests. In six countries, different laws apply for the recruitment

of permanent and temporary staff. Permanent positions are usually regulated by national laws which require a national selection process with a written assessment, whereas recruitment of temporary staff is almost always regulated by rules which are internal to each university and/or research institution.

The investigation also showed the existence of restricted access and preferential channels for scientific staff in the recruitment process. In 12 of the 20 consortium countries, no restricted access was noted for either permanent or temporary staff due to language, citizenship, curriculum or qualifications awarded in another country. In 7 countries (Belgium, Bulgaria, China, Finland, Italy and the United Kingdom) language is or could be an obstacle. A command of the language of the country which hosts the researcher is required, especially for some positions. In 5 countries, citizenship and qualifications awarded in another country limit the recruitment of researchers for specific positions. The equivalency of qualifications hampers or could hamper recruitment in countries such as Greece (1), Italy (2), Poland and Turkey. In these countries the academic qualifications awarded in another country must be equivalent to the national academic qualifications.

Recruitment also takes place by means of preferential channels which mainly apply to scientists with outstanding, internationally recognised scientific qualifications. Countries such as China, Finland, Germany, Greece, Israel, Italy, Poland, Turkey, United Kingdom and Vietnam welcome people with outstanding scientific qualifications and have created preferential channels for them. Some examples of this are the "Finland Distinguished Professor" (3) funding programme which aims at strengthening scientific knowledge and know-how in Finland, and the "Fast Track" (4) scheme in the UK and other similar initiatives in the above-mentioned countries.

The extent to which "gender" might be a limiting factor to researcher mobility was also investigated. The survey results indicate that gender is not a discriminating factor in recruitment. No form of discrimination between genders was noted among the participants in this network of excellence. Recruitment is mainly based on ability. It is interesting to note that the equal gender-based share of positions, despite being mentioned, is seldom planned. An exception is BOKU University (Austria), where its statutes specify set objectives for achieving gender equality for recruiting more female applicants.

Safety and health in the workplace of researchers working for all the MoniQA partners are safeguarded by national laws. These laws are usually based on the European Regulations on Safety and Health Requirements for the Workplace and are contained in Ministerial and/or Presidential Decrees, Labour Codes, Employment Acts, and Health and Safety Acts. They include the safety aspects related to hazardous working conditions, use of chemicals, risk of accidents and long-term exposure, and state researcher rights.

INTELLECTUAL PROPERTY RIGHTS

Most MoniQA partners (28 out of 31) have an Intellectual Property Rights policy that regulates the ownership and protection of data and the intellectual property rights of researchers. In most cases, a distinction is made between inventions which are made within a contract where the invention activity is the object of the contract itself, and the case of a researcher in the university or public administration with research purposes. In the first case, the rights coming from the invention belong to the employer, whereas in the second case the researcher has the exclusive rights over the patented invention which he/she has invented. The

latter rule does not apply if the invention is made within the framework of a specific research project which is financed by public or private funds. When a researcher works within a financed project, an agreement is usually drawn up among the partners regarding the exploitation of the research results including the names on the possible publication. In this type of agreement, it is usually stated that before any exploitation of results, including publication, there must be a multilateral or other type of agreement. When this issue applies to researchers some MoniQA partners agree on IPRs prior to a visit by an external researcher. Three partners do not have any explicit policy regarding IPRs.

RESEARCHER MOBILITY

Researcher mobility towards other research organisations is regulated by the specific rules/laws of each single organisation. Sixteen of 31 MoniQA partners currently set time limits: in 9 partners, a researcher cannot leave his institution for more than 12 months. A long-term leave, longer than one year, is possible for researchers in 7 partners, provided that they come back to the employer institution. For example, if a researcher does not return to the research institution he/she will be asked to pay all the expenses plus the legal interest rate. In 15 partners, mobility is a case-by-case decision. It is usually subject to a satisfactory business arrangement being made and consequently an official time limit regarding the length of absence is not set. The length of the leave depends on the tasks, position, responsibilities and obligations that the person has in his/her organisation. It is usually easier for junior members to go for longer periods of time.

The extent to which bureaucracy hampers mobility and, specifically, the time needed for a researcher to get permission to leave his/her own institution were investigated. In 9 partners, authorisations are granted in a matter of days or a few weeks, whereas in 7 it takes up to two months. For most of the Consortium partners, permission to leave the Institution mainly depends on the length of time, the funding programme and the nature of the project, the host institution and the field in which the researcher works. Bureaucracy, apparently, is never a constraint to researcher mobility.

Regarding economic issues, in 28 out of 31 partners, the staff on leave keep their own salaries while being on leave; in only very few cases the researchers do not keep their salaries. For most of the Consortium partners, the question of keeping a salary could be a limitation depending on the length of time and financial contribution of the hosting institution. In general, the longer is the leave period, the higher is the cut in the current salary.

When researchers go outside their institution for training or to work on a project that is of interest to their employer, 19 of the 31 Consortium partners give additional economic support from the institution to be used for housing and living expenses. In 12 MoniQA partners, researchers do not receive any additional support while on leave.

Researchers on leave often rely on the economic support of the European Commission or national bodies such as the "Austrian International fund" of BOKU, the Austrian Federal Ministry for Science and Research, the Austrian FFG-österreichische Forschungsförderungsgesellschaft, the Foreign Ministry of Austria and the Institute of Advanced Studies of the University of Bologna (5) or the Italian National Research Council (CNR).

The survey also investigated the procedures that a researcher has to follow in order to do research in a research institution different from his/hers. Beforehand, a researcher must have an idea of the objectives of his/her research

that must fit within the research project which is undertaken at the hosting institution. For 11 partners, if a researcher is interested in being hosted by a specific research institution and/or university, the researcher does not usually need to know anyone in the research institution. He/she can check on the website of the research institution/university to see if there is any possibility of doing research there and just send the "Application form". In contrast, in 9 MoniQA partners a researcher needs a personal acquaintance or a mentoring researcher who is willing to look after the guest.

CONCLUSIONS

To conclude, the results of this survey present a comprehensive and interesting picture of the management and regulation of research personnel in institutions that deal with food science and technology within and outside of Europe, with special attention having been given to the legal constraints and factors that limit researcher mobility.

A few factors have been identified that seem to limit the free mobility and integration of researchers. Language is an important issue, because efficient communication and access to information is often hampered if websites, documents, vacancy advertisements, and codes of conducts, are published in the national language. In some cases, specific citizenship is required for certain positions and, in the public sector of several countries there is also a problem with the recognition and equivalency of qualifications awarded in another country. Mobility is sometimes hampered by limited funding. Even if a researcher keeps his/her own salary while on leave, it might not be enough to cover the expenses in another country. Lack of contacts can also be a limiting factor, because in several cases a "local" contact is needed who can take care of the newcomer. This is stressed by the fact that a satisfactory business/research case of mutual interest facilitates mobility.

This survey did not consider the fulfilment of immigration requirements which is a prerequisite for researcher mobility.

More information about the database on legal constraints and factors that limit researcher mobility can be found on the MoniQA website (http://www.moniqa.org/).

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Dipartimento di Scienze Tecnologie Agroalimentari (D.I.S.T.A.),
Facoltà di Agraria,
Università degli Studi della Tuscia, Viterbo — Fax +39-0761-357498

Dipartimento di Ingegneria e Tecnologie Agro-Forestali,
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Dipartimento di Scienze degli Alimenti,
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Dipartimento di Scienze e Tecnologie Agroalimentari e
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Dipartimento di Scienze e Tecnologie Alimentari
e Microbiologiche (DI.S.T.A.M.), 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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