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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 - Telefax +39 075 5857939-5857943 - e-mail: paolofan@unipg.it

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Chiriotti Editori srl, Viale Rimembranza 60, I-10064 Pinerolo, Italy - Tel. +39 0121 393127 - Fax +39 0121 794480
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OPINION PAPER

LINGUA FRANCA ENGLISH AND FOOD SCIENCE EDUCATION

E. CIANFLONE[1]*, G. DI BELLA[2], and G.MO DUGO[3]
[1]Corso di Laurea in Scienze Gastronomiche,
Dipartimento di Scienze degli Alimenti e dell'Ambiente, Università degli Studi di Messina,
Facoltà di Scienze MM.FF.NN, Contrada Papardo, 98100 Messina, Italy
[2]Dipartimento di Sanità Pubblica Veterinaria, Facoltà di Medicina Veterinaria,
Università degli Studi di Messina, Polo Universitario S.S. Annunziata, 98100 Messina, Italy
[3]Dipartimento di Scienze degli Alimenti e dell'Ambiente "G. Stagno d'Alcontres",
Università degli Studi di Messina, Facoltà di Scienze MM.FF.NN, Contrada Papardo,
98100 Messina, Italy
*Corresponding author: ecianflone@unime.it

ABSTRACT

English is the most widely used medium of scientific communication. In contexts where it is a foreign language, this situation impacts university syllabi since language courses should aim at developing field-specific communicative skills in line with this Anglicised trend. This educational goal can be met by language and content-teacher collaboration, as is the case of a pilot project carried out within the undergraduate degree in *Gastronomic Sciences* at the University of Messina (Italy). The co-joined work has so far resulted in the implementation of a Food Specification template to serve English classes.

- Key words: collaboration, food specification, Food Science education, lingua franca English -

Today English is widely employed as the *lingua franca* of international communication among speakers of different nationalities (JENKINS, 2007; WIDDOWSON, 2003) and as the medium of election for research and scholarship (FERGUSON, 2007; HYLAND, 2009). Available data show that 68% of the scholarly periodicals use English as their official language of publication (HYLAND, 2009), a prevalent condition in Europe and Japan (SWALES, 2004), as confirmed by the editorial policy of this same journal.

This almost English-driven situation is not an hindrance to research. The global use of English should be seen as a beneficial medium to unfold results within the international audience by means of a common idiom, or *lingua franca*, serving the communicative needs of speakers from different countries. This means that in Higher Education, and where this idiom is a foreign language, the leadership of English asks language teachers to promote learning activities pivoted on the specific communicative practices required for study or workplace purposes.

To reach such specific learning outcomes, blending foreign language and contents by means of teacher collaboration is a solution highly recommended (Dudley-Evans and St. Johns 1998) since experts in the field can help language practitioners cope with an educational dilemma. This consists of language teachers' training, mainly based on foreign language teaching theory and praxis, and their engagement with topics and the technical vocabulary of specific disciplines (ALMAGRO ESTEBAN and VALLEJO MARTOS, 2002; DUDLEY-EVANS and St. JOHNS, 1998).

To bridge the gap, that is to solve the educational dilemma and to develop English classes cut on students' needs, a pilot project of collaboration has been implemented for the undergraduate degree in *Gastronomic Sciences* at the University of Messina (Italy) among the language teacher and the Dipartimento di Scienze degli Alimenti e dell'Ambiente "G. Stagno d'Alcontres". The first result from the co-joined activity is a Food Specification (FS) template adapted for foreign language teaching purposes (CIANFLONE *et al.*, 2010).

As an educational genre describing foodstuffs or agricultural products, FSs are an essential tool to future food-and-wine experts to acquire proficiency when labelling foodstuff. For this same reason, FSs are a suitable topic to exploit in English classes to facilitate the acquisition of written/oral skills to describe products in a foreign language and to assist language teachers in developing the highly specialised English vocabulary practitioners will need.

In our pilot, students were offered a blank FS template, developed to this aim with the concourse of content experts, where the product's main features, the geographical production area, the method to obtain or to grow it, together with relevant information on its use, its selling season and packaging details were described. In a second time, a model on Sicilian red oranges was given and discussed to let learners have a specimen to be used in language classes. Students were, then, asked to surf the Internet to look for typical Sicilian products, to describe and to examine them with the group in a workshop session.

The trial workshop gave positive results, FSs were seen by the students as a motivating means to learn English. The model, in fact, offered active involvement in the discovery and the production of texts pertaining to their field of interest. It also helped the language practitioner with written and presentation skills, by exploiting discourse conventions to be used once in the profession. Additionally, FS assisted the acquisition and correct use of technical vocabulary (e.g. vitamin, anthocyanins), specific terms of general use (e.g. peel, skin, rind) and their correct collocation (orange peel, banana skin or lemon rind) while revising grammar (e.g. countable and uncountable nouns, the passive voice or the past tenses).

Although the project is at present in its pilot stage, this co-joined work was a benefit to the English syllabus because of the specific know-how, in the form of the FS, brought by content specialists and rarely addressed in the teaching materials commercially available. Furthermore, language drills taken from university contents enhanced learning since students were motivated by the direct involvement in producing, in the target language, exemplars pertaining to their academic studies and to revise grammar in a different way. These issues are in line with *lingua franca* use of English since at university this idiom should be read with the practical aim of allowing future practitioners to enter their communities of practice as proficient speakers/users of English (CIANFLONE *et al.*, 2010).

NOTES

The degree in Gastronomic Sciences, formerly Mediterranean food-and-Wine Sciences and Health, is a course established under the reform promoted by the Italian Ministry of Education University and Research to align Italian Higher Education to European standards. The course, pivoted on Food Science and food-related majors, wants to train experts interested in starting a career in food production and distribution, food inspection and evaluation, in catering or working as niche-products dealers.

REFERENCES

A. Almagro Esteban, M.C. Vallejo Martos. 2002. A case study of collaboration among the ESP practitioner, the content teacher, and the students. Revista Alicantina de Estudios Ingleses, 15:7-21.

E. Cianflone, G. Di Bella, G. Dugo. 2010. Food specifica-

tions as a language learning tool in ESP classes. English for Specific Purposes World, 9:1-7.

T. Dudley-Evans, M.J. St. John. 1998. Developments in English for specific purposes. Cambridge: Cambridge University Press.

G. Ferguson. 2007. The global spread of English, scientific communication and ESP: questions of equity, access and domain loss. Iberica, 13: 7-38.

K. Hyland. 2009. Academic discourse. London: Continuum.

J. Jenkins. 2007. English as a lingua franca: Attitude and identity. Oxford: Oxford University Press.

J.M. Swales. 2004. Research genres. Explorations and applications. Cambridge: Cambridge University Press.

H.G. Widdowson. 2003. Defining Issues in English Language Teaching. Oxford: Oxford University Press.

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PAPER

CHANGES IN SENSORY AND INSTRUMENTAL FEATURES OF INDUSTRIAL SICILIAN BREAD DURING STORAGE

C.M. LANZA*, A. MAZZAGLIA, A. SCACCO and B. PECORINO[1]
DOFATA, Sezione di Tecnologie Agroalimentari, Università di Catania,
Via S. Sofia 98, 95123 Catania, Italy
[1]DISEAE, Facoltà di Agraria, Università di Catania, Via S. Sofia 98, 95123 Catania, Italy
*Corresponding author: cmlanza@unict.it

ABSTRACT

Industrial bread ("fresh line" with a shelf life of five days and "long life" with a shelf life of forty days) was evaluated through instrumental and sensory analyses. Assays were made considering moisture %, degree of acidity, digital image analysis, resistance to cutting and sensory profile. Data were statistically analysed by Analysis of Variance and correlated by Principal Component Analysis. It is possible to conclude that the correlation between sensory and instrumental parameters constitutes a useful tool to determine the qualitative deterioration of this bread and the potential for its introduction into markets.

- Key words: bread, instrumental parameters, sensory analysis, shelf life -

INTRODUCTION

Industrial bread constitutes a valid alternative to fresh bread. While still having great appeal in Italy, fresh bread has a continuously decreasing market share owing to the inconvenience of having to purchase it on a daily basis. In Italy, the fresh bread sector consists of handcrafted bakeries (63%), industrial bread making (25%) and "in-store" bakeries (12%), with a production of over 250 types of bread. In 2007, the market amounted to 3.5 million tons in volume and 6 million euros in value, with a per-capita consumption of 55 kilograms per annum. Thus bread is increasingly produced in industrial bakeries in order to diversify the offer, favour the concentration of purchases and to serve vast numbers of consumers.

Bread staleness, a complex phenomenon describing the time-dependent loss in its sensory quality, is decisive for consumer acceptance. The problem of defining the shelf life of bakery products remains unsolved, despite extensive study. The present study was prompted by the consideration that along with physicochemical and digital image analyses (RIVA, 2003), sensory evaluation may be the key to determining bread quality (GÁMBARO *et al.* 2002; 2004; GIMENEZ *et al.*, 2007). Since 1976, a cooperative of durum wheat producers in the Province of Enna has employed strategies for the development of new product typologies (fresh and long-life bread) using suitable packaging to increase the shelf life and to increase its market (3,700 tons in 2000 to over 6,000 tons in 2007). This success is due to the raw material (Sicilian durum wheat), traditional methods (use of sourdough) (LOTONG *et al.*, 2000) and an innovative continuous production process. The product leader is fresh bread (sliced and uncut loaves), although the marketing of long-life bread has increased to approximately 10% of the total.

In order to improve the marketability of industrial Sicilian bread, the aging process responsible for important economic losses for both the industry and consumers was studied through sensory and instrumental parameters.

MATERIALS AND METHODS

Fresh loaf (FL) and sliced fresh bread (FS), produced daily with a shelf life of five days, was packaged (Cryovac Sealed Air Corporation) in polyolefin micro-perforated film (OPT1330). For long shelf life bread (LSL), with a shelf life of 40 days, different packaging (Cryovac Sealed Air Corporation) was used: a multi-layer co-extruded film for the top (TM PLY T9250 permeability O_2 45 bar $cm^3/m^2/24$ h, moisture vapour transmission rate (MVTR) ≤10) and a multi-layer co-extruded film (TM PLY T6010 B permeability O_2 1 bar $cm^3/m^2/24$ h MVTR ≤10) for the base.

The fresh bread was checked daily for five days (t1, t2, t3, t4, t5) and repeated for three successive weeks, while samples of different "long life" breads were analysed over six weeks (wk1, wk2, wk3, wk4, wk5, wk6).

The samples were assessed, in triplicate, in terms of moisture percentage, degree of acidity, digital image analysis, resistance to cutting and sensory profile. Determination of moisture % and degree of acidity were performed according to AOAC methods (1990). Digital image analysis (expressed as percentage pore area) was performed using a scanner (Cannon), PC Pentium running Windows XP Professional and ImageJ software. Resistance to cutting was determined on cubes of bread using a Warner Bratzler (Instron 4411) and results are expressed as kg/cm^2. Cubes, 1 cm x 1 cm x 1 cm, were obtained by slicing the bread using a knife; the loaf of bread, preliminarily cut into slices, was then cut into cubes. In order to define the attributes characterizing the product, a sensory profile (UNI 10957, 2003) was constructed by a trained panel of 10 judges submitted to further training over 4 weeks to develop a common vocabulary and to generate attributes using handmade and industrial breads, following the definitions given by RAFFO *et al.* (2003). Assessors were requested to evaluate, by assigning a score between 1 (absence of the sensation) and 9 (extremely intense), the intensity of the twenty sensory descriptors selected on the basis of frequency (%): colour, alveolar structure, elasticity and moisture of crumb, aroma and flavour of bread, aroma and flavour toasted, aroma and flavour wheat, aroma and flavour yeast, sweet, salt, acid, bitter, soft, cohesive, off-odour and off-flavour. The evaluations were conducted in the sensory laboratory (UNI ISO 8589, 1990) of the DOFATA Department equipped with specific software (FIZZ Biosystèmes), randomizing the order of presentation among judges and sessions.

RESULTS AND CONCLUSIONS

The results of the instrumental analyses (Table 1) show that the moisture content of long-life samples did not change over the 6 weeks of production, while in the FL and FS samples, moisture % increased from t2 due to the micro-perforated film used as packaging. On the contrary, the degree of acidity for all the samples remained constant. The resistance to cutting of LSL samples increased up to wk2 of shelf-life, maintaining high values thereafter. In FS and FL samples, resistance to cutting increased at t3 and t4, respectively. Digital image analysis did not provide any data correlated to the aging process.

Statistical analysis of the sensory data (for samples FL and FS at t1, t3, t5; for fresh bread and LSL at wk1, wk4, wk6) for each attribute

Table 1 - Mean values and standard deviation of the physicochemical parameters of the breads studied.

Samples	Humidity %	Degree acidity	Resistance to cut kg/cm^2	Pore area %
LSL wk1	35.0±0.3	4.5±0.4	0.75±0.1	31.90±2.7
LSL wk2	32.2±0.4	4.6±0.2	1119±39	23.55±0.5
LSL wk3	34.2±0.4	4.3±0.2	1110±56	18.95±2.3
LSL wk4	34.2±0.4	4.2±0.5	1115±2	19.60±1.3
LSL wk5	34.0±0.5	4.7±0.4	1117±12	26.10±1.1
LSL wk6	33.4±0.2	4.5±0.2	1012±0.7	25.63±5.9
FS t1	22.5±0.1	4.1±0.3	0.63±0.1	28.75±0.6
FS t2	32.2±0.2	4.0±0.4	0.67±0.03	18.60±1.3
FS t3	29.7±0.2	4.3±0.5	1099±0.7	23.40±0.8
FS t4	32.2±0.2	4.3±0.4	1074±44	21.60±3.4
FS t5	30.7±0.1	4.6±0.4	1053±19	26.0±3.8
FL t1	20.8±0.1	4.1±0.5	0.76±0.01	28.75±0.3
FL t2	34.4±0.2	4.4±0.5	0.65±0.02	26.55±0.2
FL t3	33.2±0.2	4.1±0.4	0.77±0.06	25.65±0.8
FL t4	33.0±0.3	4.2±0.5	1133±72	28.67±0.2
FL t5	30.6±0.1	4.4±0.5	1082±24	22.40±0.6

was performed using Analysis of Variance (ANOVA) on samples, judges and replicates, and their respective interactions JxS, SxR, and JxR. The significance of these effects was tested with the F test. Data analysis was carried out using Statgraphics plus statistics software.

The results of ANOVA for fresh FL and FS (Table 2) show significant differences among samples for many of the attributes evaluated, except for colour, alveolar structure and wheat flavour for FL samples, aroma/flavour yeast and wheat aroma for FS samples and attributes salt, acid, bitter and aroma toasted in both types of bread. The judges found significant differences for all attributes considered, while the replicates showed good reliability for all attributes. The SxJ interaction revealed significant differences for all attributes except for crumb moisture and bitterness in FS samples and for the attributes of alveolar structure, acid and bitterness for FL samples. The SxR interaction showed a good homogeneity of the samples throughout the replicates except for yeast flavour for FL samples. Finally, the JxR interaction showed the good reliability of the responses given by the judges except for sweet and wheat flavour for FS samples.

The results of ANOVA for LSL bread (Table 3) showed significant differences among samples evaluated at wk1, wk4 and wk6 for all attributes, except colour, yeast aroma, off-odour, salt and bitter. The judges found significant differences for all attributes considered, while the replicates showed good reliability for all attributes except alveolar structure, soft, cohesive and bread flavour. The SxJ interaction revealed significant differences for all attributes. The SxR interaction showed good homogeneity of the samples except alveolar structure, wheat aroma and acid. The JxR interaction indicated good reliability of the responses given by the judges except for salt, bread flavour and off-flavour.

The mean values were analysed using the multiple comparison test applying the LSD (Least Significant Difference) procedure to determine which attributes differentiate the samples (Table 4). Many sensory attributes of fresh bread samples showed significant deterioration after three days. Only elasticity and crumb moisture behaved differently in FL samples where they decreased at t5. Instead, the results for LSL bread showed deterioration after four weeks of storage only for alveolar structure, elasticity and acid.

Principal Component Analysis (PCA) was also applied to the sensory and instrumental mean values (except for digital image analysis results) in order to interpret the differences among samples.

Fig. 1 shows the Bi-plot of fresh bread, overlapping the Score plot (six samples for 3 replications) and Loading plot, representing the position of the more significant variables considered. The variables chosen were three physicochemical and sixteen sensory ones; salt, acid, bitterness and toasted aroma were excluded because they were not significant in either FS or FL samples, with an explained variance of 69.47% by the first two principal components (PC). The Bi-plot shows the good reliability of replications and a clear distinction between samples at the different time points. Along with the first component (PC1) which explains 57.77% of variance, samples FL and FS at t1 on the right correlate positively to elasticity, crumb moisture, soft, cohesive, aroma/flavour bread and negatively to resistance to cutting, moisture %, off-odour and off-flavour. Samples FS and FL at t5 are differently placed; the first (lower left) is characterised by off-odour, off-flavour, degree of acidity and resistance to cutting, while FL samples (up-

Table 2 - Influence of samples (FS, FL), judges (10), replications (3) and their interactions on the twenty attributes.

	Fresh Sliced F Values						Fresh Loaf F Values					
Attributes	Samples	Judges	Replications	S x J	S x R	J x R	Samples	Judges	Replications	S x J	S x R	J x R
Colour	12.52***	30.82***	0.67 n.s.	4.09***	1.14 n.s.	1.77 n.s.	0.72 n.s.	39.21***	1.85 n.s.	1.95*	0.53 n.s.	1.15 n.s.
Alveolar structure	3.29*	10.75***	0.12 n.s.	2.45**	1.90 n.s.	0.44 n.s.	3.07 n.s.	2.13*	0.43 n.s.	0.85 n.s.	1.11 n.s.	0.68 n.s.
Elasticity	23.64***	2.67*	0.23 n.s.	2.37**	0.68 n.s.	1.28 n.s.	35.22***	5.49***	1.31 n.s.	11.97***	0.88 n.s.	1.26 n.s.
Humidity	25.72***	7.75***	0.00 n.s.	1.69 n.s.	0.77 n.s.	0.86 n.s.	95.01***	10.52***	1.01 n.s.	6.16***	0.26 n.s.	1.52 n.s.
Bread aroma	34.89***	24.38***	1.62 n.s.	10.15***	1.09 n.s.	0.92 n.s.	25.31***	11.64***	0.78 n.s.	4.54***	0.50 n.s.	1.07 n.s.
Yeast aroma	0.05 n.s.	10.39***	0.74 n.s.	3.40***	0.81 n.s.	1.04 n.s.	11.84***	10.06***	2.52 n.s.	3.08***	0.91 n.s.	0.71 n.s.
Toasted aroma	2.09 n.s.	17.78***	0.70 n.s.	7.01***	1.26 n.s.	0.89 n.s.	0.57 n.s.	10.18***	0.67 n.s.	3.54***	0.59 n.s.	0.94 n.s.
Wheat aroma	3.08 n.s.	51.21***	0.86 n.s.	8.83***	2.32 n.s.	1.02 n.s.	5.08*	16.22***	0.61 n.s.	5.42***	0.24 n.s.	1.55 n.s.
Off-odour	17.69***	40.05***	0.16 n.s.	5.42***	0.82 n.s.	0.95 n.s.	8.92***	29.26***	0.44 n.s.	9.51***	1.84 n.s.	1.22 n.s.
Sweet	7.44**	35.54***	1.08 n.s.	6.76***	2.01 n.s.	2.66**	11.45***	31.43***	0.99 n.s.	5.06***	0.62 n.s.	0.89 n.s.
Salt	0.55 n.s.	5.58***	1.10 n.s.	1.91*	0.66 n.s.	0.69 n.s.	0.31 n.s.	8.57***	0.31 n.s.	2.07*	0.27 n.s.	0.67 n.s.
Acid	2.67 n.s.	6.58***	1.77 n.s.	2.54**	0.49 n.s.	0.75 n.s.	0.83 n.s.	5.73***	0.13 n.s.	0.76 n.s.	0.74 n.s.	0.48 n.s.
Bitter	1.38 n.s.	2.76**	0.35 n.s.	1.27 n.s.	0.70 n.s.	0.58 n.s.	1.73 n.s.	3.81***	0.14 n.s.	0.99 n.s.	2.39 n.s.	0.49 n.s.
Soft	45.78***	8.74***	1.49 n.s.	6.02***	0.12 n.s.	0.84 n.s.	50.86***	6.20***	0.50 n.s.	3.14***	0.49 n.s.	1.21 n.s.
Cohesive	24.69***	8.69***	0.19 n.s.	2.96**	1.39 n.s.	0.55 n.s.	20.27***	11.44***	0.50 n.s.	3.42***	0.35 n.s.	1.60 n.s.
Bread flavour	8.10***	11.13***	0.58 n.s.	5.03***	0.94 n.s.	0.96 n.s.	13.40***	7.66***	0.22 n.s.	1.92*	1.67 n.s.	0.44 n.s.
Yeast flavour	0.77 n.s.	4.18***	0.89 n.s.	3.57***	0.18 n.s.	0.93 n.s.	5.60**	8.86***	1.56 n.s.	2.85**	3.93**	1.28 n.s.
Toasted flavour	4.78*	25.74***	0.79 n.s.	5.34***	1.23 n.s.	0.71 n.s.	9.07***	17.38***	0.24 n.s.	3.25***	1.40 n.s.	1.16 n.s.
Wheat flavour	11.99***	10.06***	0.31 n.s.	4.63***	0.05 n.s.	1.83*	1.13 n.s.	7.97***	0.85 n.s.	4.35***	1.82 n.s.	1.03 n.s.
Off-flavour	8.90***	15.67***	0.20 n.s.	4.66***	0.79 n.s.	0.51 n.s.	7.31**	11.98***	2.37 n.s.	7.22***	1.38 n.s.	0.98 n.s.

*** significant difference for $p \leq 0.001$; ** significant difference for $p \leq 0.01$; * significant difference for $p \leq 0.05$; n.s. no significant difference.

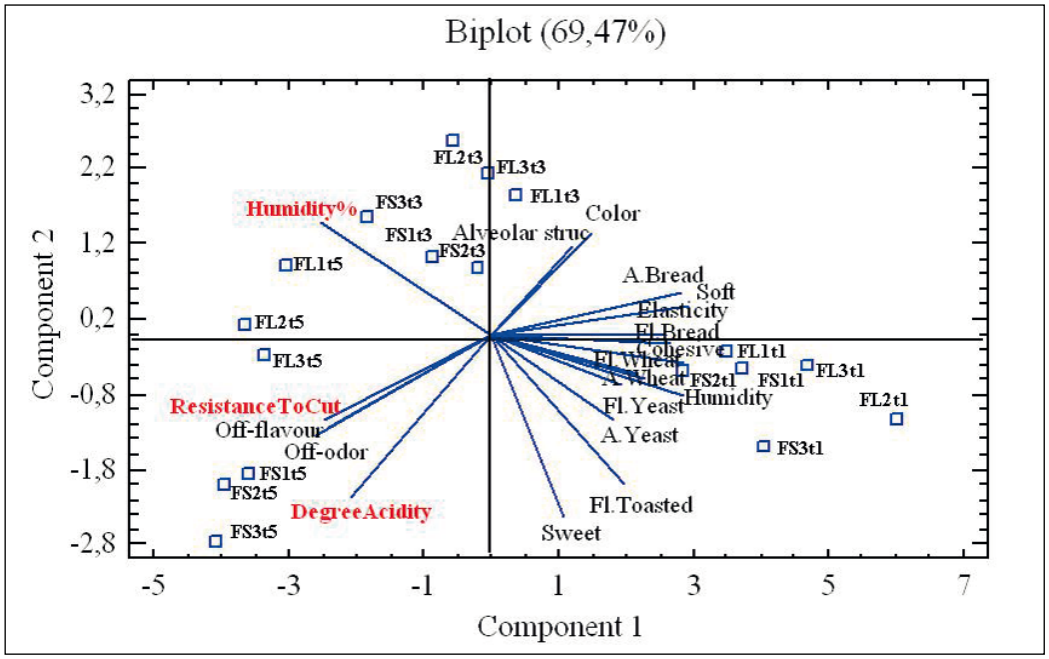


Fig. 1 - Bi-plot: overlapping score (six samples for 3 replications) and loading plot (3 instrumental and 16 sensory variables).

per left) show high moisture %. Along with PC2 (explaining 11.70% of variance), at a higher position, the samples at t3 correlate positively to alveolar structure and colour and negatively to sweet, toasted flavour, off-odour and off- flavour.

The Bi-plot of LSL samples at wk1, wk4, wk6 (Fig. 2) shows a variance of 79.48%, explained by the first two PCs. Along with the first PC (45.89%), the samples at wk1, lower right, positively correlate to elasticity, crumb moisture, sweet, soft and % moisture. The samples at wk6, lower left, show high values of resistance to cut, flavour toasted and off-flavour. Along with second component, the samples at wk4, placed higher, positively correlate to aroma and bread flavour, aroma and wheat flavour, and yeast flavour, negatively to alveolar structure, and degree of acidity.

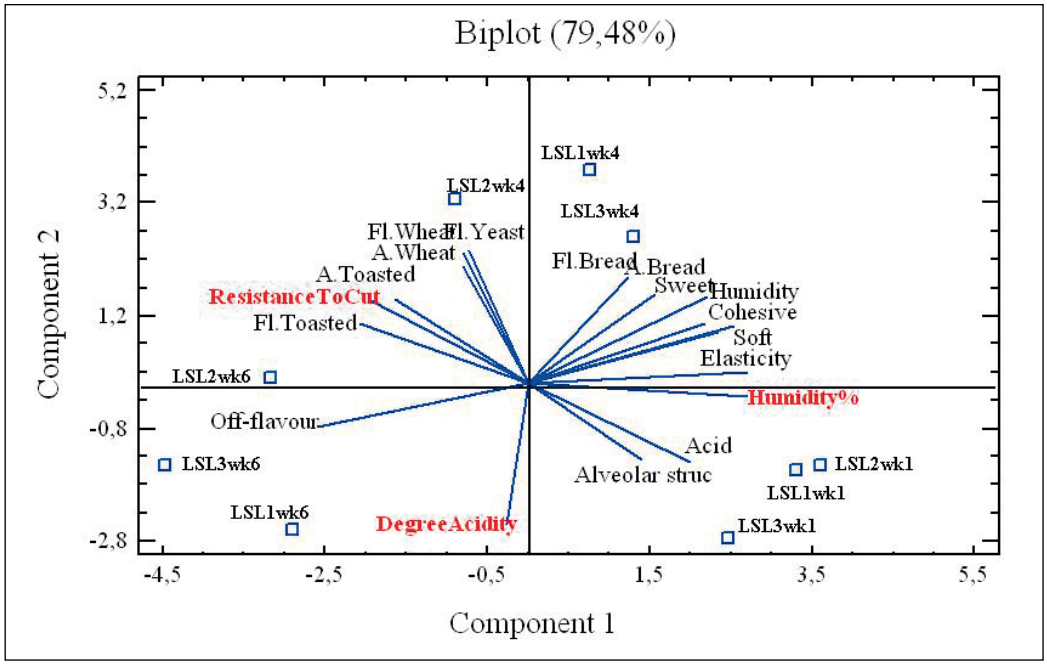


Fig. 2 - Bi-plot: overlapping score (three samples for 3 replications) and loading plot (3 instrumental and 15 sensory variables).

Table 3 - Influence of samples (LSL), judges (10), replications (3) and their interactions on the twenty attributes.

	F values					
Attributes	Samples	Judges	Replications	S x J	S x R	J x R
Colour	1.64 n.s.	22.76***	0.58 n.s.	2.01*	0.45 n.s.	0.72 n.s.
Alveolar structure	15.14***	8.04***	10.21***	5.03***	3.06*	1.32 n.s.
Elasticity	25.82***	6.83***	0.17 n.s.	3.22***	1.13 n.s.	0.57 n.s.
Humidity	51.79***	7.41***	1.29 n.s.	5.71***	0.57 n.s.	0.95 n.s.
Bread aroma	3.92*	10.88***	1.83 n.s.	3.22***	1.05 n.s.	0.58 n.s.
Yeast aroma	0.60 n.s.	8.25***	0.30 n.s.	2.97**	1.08 n.s.	0.84 n.s.
Toasted aroma	17.31***	16.99***	2.60 n.s.	5.17***	2.28 n.s.	0.99 n.s.
Wheat aroma	9.17***	21.94***	0.32 n.s.	4.40***	2.59*	0.96 n.s.
Off-odour	2.77 n.s.	18.77***	1.16 n.s.	6.53***	2.57 n.s.	1.13 n.s.
Sweet	12.47***	29.41***	1.31 n.s.	5.0***	0.64 n.s.	1.38 n.s.
Salt	1.03 n.s.	13.56***	0.44 n.s.	4.30***	0.20 n.s.	2.10*
Acid	12.71***	15.63***	0.17 n.s.	4.66***	2.83*	0.61 n.s.
Bitter	1.85 n.s.	11.49***	0.41 n.s.	4.69***	0.57 n.s.	0.64 n.s.
Softness	20.60***	3.65***	4.58*	7.70***	1.59 n.s.	0.57 n.s.
Cohesiveness	15.32***	15.20***	3.27*	3.02***	1.77 n.s.	0.95 n.s.
Bread flavour	8.91***	20.48***	3.49*	9.19***	1.20 n.s.	2.0*
Yeast flavour	7.63**	10.65***	0.22 n.s.	4.85***	0.43 n.s.	1.34 n.s.
Toasted flavour	5.08*	11.34***	1.10 n.s.	2.58**	0.96 n.s.	0.66 n.s.
Wheat flavour	13.41***	10.63***	0.11 n.s.	3.04***	1.53 n.s.	1.01 n.s.
Off-flavour	31.66***	31.28***	2.44 n.s.	13.75***	2.20 n.s.	2.26*

*** significant difference for $p \leq 0.001$; ** significant difference for $p \leq 0.01$; * significant difference for $p \leq 0.05$; n.s. no significant difference.

Table 4 - Mean score of the 20 sensory attributes for Fresh loaf (FL), Fresh Sliced (FS) and Long Shelf Life bread (LSL) at the different time points.

	Mean values								
	FS			FL			LSL		
Attributes	t1	t3	t5	t1	t3	t5	wk1	wk4	wk6
Colour	5.02^{b1}	5.08^{b}	4.22^{a}	4.72	4.75	4.52	4.86	4.77	5.13
Alveolar structure	4.91^{b}	4.44^{ab}	4.27^{a}	6.94	6.33	6.16	5.25^{b}	4.22^{a}	4.27^{a}
Elasticity	6.22^{b}	4.50^{a}	4.44^{a}	5.66^{b}	5.52^{b}	4.00^{a}	6.72^{c}	6.00^{b}	4.75^{a}
Humidity	5.47^{b}	3.83^{a}	3.86^{a}	5.94^{c}	4.22^{b}	3.16^{a}	5.55^{b}	5.55^{b}	3.55^{a}
Bread aroma	6.22^{c}	5.58^{b}	4.80^{a}	6.86^{c}	5.88^{b}	5.38^{a}	5.97^{ab}	6.33^{b}	5.61^{a}
Yeast aroma	4.97	4.88	4.91	5.33^{c}	4.75^{b}	4.08^{a}	4.91	5.13	4.83
Toasted aroma	5.50	5.16	5.00	4.88	4.61	4.58	4.44^{a}	5.63^{b}	5.61^{b}
Wheat aroma	4.94	4.66	4.52	5.05^{b}	4.50^{a}	4.33^{a}	4.61^{a}	5.47^{c}	5.05^{b}
Off-odour	1.72^{a}	1.97^{a}	2.58^{b}	1.75^{a}	1.88^{a}	2.30^{b}	1.77	1.72	2.02
Sweet	4.19^{b}	3.52^{a}	3.86^{ab}	4.13^{b}	3.94^{b}	3.30^{a}	4.72^{b}	4.91^{b}	3.94^{a}
Salt	3.77	3.97	4.11	3.47	3.27	3.47	4.33	4.27	4.05
Acid	2.16	1.94	1.75	2.19	1.86	1.91	2.61^{b}	1.97^{a}	1.97^{a}
Bitter	2.08	1.69	1.69	2.02	1.52	1.69	2.30	1.94	2.02
Soft	5.58^{c}	4.47^{b}	3.58^{a}	6.13^{c}	4.94^{b}	3.61^{a}	4.94^{b}	4.83^{b}	3.50^{a}
Cohesive	5.88^{b}	4.75^{a}	4.27^{a}	5.94^{b}	4.83^{a}	4.63^{a}	5.69^{b}	5.58^{b}	4.55^{a}
Bread flavour	5.72^{b}	5.11^{a}	4.77^{a}	6.55^{b}	5.44^{a}	4.97^{a}	5.91^{b}	6.25^{b}	5.52^{a}
Yeast flavour	4.13	3.77	3.83	4.55^{b}	4.02^{ab}	3.63^{a}	3.63^{a}	4.50^{b}	3.94^{a}
Toasted flavour	5.16^{b}	4.41^{a}	4.61^{a}	4.94^{b}	4.05^{a}	3.75^{a}	4.27^{a}	5.11^{b}	5.16^{b}
Wheat flavour	4.80^{b}	3.75^{a}	3.69^{a}	4.47	4.02	4.13	3.91^{a}	5.19^{b}	4.41^{a}
Off-flavour	1.69^{a}	2.05^{a}	2.47^{b}	1.47^{a}	1.80^{b}	2.05^{b}	1.44^{a}	1.50^{a}	2.02^{b}

[1]The values marked with different letters in the same row are significantly different ($p \leq 0.05$) according to LSD Multiple Comparison Test.

A large vocabulary of twenty sensory attributes was developed for these industrial breads, showing a good prediction capacity for their sensory quality. The results obtained for the sensory attributes fixed the qualitative decay of sliced and loaf fresh bread at t3. The fresh loaf, however, differentiated to t5 for attributes off-odour, off-flavour, degree of acidity and resistance to cut, confirming its greater stability. For the long life bread, significant changes were evident over 4 weeks. The limits determined in the present study are, therefore, lower than those indicated on the packaging. The shelf lives indicated on the label coincide with those determined by consumers, whose choice depends on the acceptability of the food. It would be interesting therefore to define these parameters by a specific methodology such as Survival Analysis to establish when deterioration of the products renders the bread unacceptable, and then use these results in bread making and packaging processes to improve quality. The sensory profile of the fresh bread loaf, which has recently obtained the DOP as "Pagnotta del Dittaino", is an important instrument able to distinguish this product from the others.

REFERENCES

AOAC. 1990. "Official Methods of Analysis of the A.O.A.C." (15th Ed.). Association of Official Analytical Chemists, Inc., K. Helrich Ed., Arlington, VA.

Gámbaro A., Varela P. and Giménez A. 2002. Textural quality of white pan bread by sensory and instrumental measurements. J. Texture Stud. 33: 401.

Gámbaro A., Fiszman S., Giménez A., Varela P. and Salvador A. 2004. Consumer acceptability compared with sensory and instrumental measures of white pan bread: Sensory shelf-life estimation by Survival Analysis. J. Food Sci. 69: 401.

Gimenez A.,Varela P., Salvador A., Ares G., Fiszman S. and Garitta L. 2007. Shelf life estimation of brown pan bread: A consumer approach. Food Quality Pref. 18: 196.

Lotong V., Chambers IV E. and Chambers D.H. 2000. Determination of the sensory attributes of wheat sourdough bread. J. Sensory Stud. 15: 309.

Raffo A., Pasqualone A., Sinesio F., Paoletti F., Quaglia G. and Simeone R. 2003. Influence of durum wheat cultivar on the sensory profile and staling rate of Altamura bread. Eur. Food Res. Technol. 218: 49.

Riva M. 2003. Analisi dell'immagine dei prodotti da forno. Tecnologie Alimentari 4: 30.

UNI ISO 8589. 1990. Analisi sensoriale. Criteri generali per la progettazione di locali destinati all'analisi. Ente Nazionale Italiano di Unificazione. Milano, Italy.

UNI 10957. 2003. Analisi sensoriale - Metodo per la definizione del profilo sensoriale degli alimenti e bevande. Ente Nazionale Italiano di Unificazione. Milano, Italy.

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PAPER

BLOOD N-3 FATTY ACIDS INCREASE AFTER CONSUMPTION OF AN ENRICHED YOGHURT

P. RISÉ*, C. COLOMBO, S. GHEZZI, N. MAURO, G. ORLANDINI[1] and C. GALLI
Dipartimento di Scienze Farmacologiche, Università degli Studi di Milano,
Via Balzaretti 9, 20133 Milano, Italy
[1]Dipartimento di Medicina Sperimentale, Università di Parma, Via Volturno 39, 43100 Parma, Italy
*Corresponding author: Tel. +39 02 50318366, Fax +39 02 50318284,
e-mail. patrizia.rise@unimi.it

ABSTRACT

The effects of a n-3 fatty acid (FA) enriched yoghurt on blood FA were investigated in 23 females and 23 males, who consumed 2 yoghurts/day for 6 weeks, providing 64 mg EPA and 74 mg DHA. Blood samples were collected before treatment, at the beginning, end, and after wash out. Yoghurt intakes increased blood EPA (35%) and DHA (11%) with different trends of changes in women versus men. In conclusion, the daily consumption of a yoghurt containing small amounts of n-3 FA significantly increases blood n-3 FA, providing a useful approach to reach the recommended intakes in populations.

- Key words: FA incorporated in yoghurt, gender differences, omega-3 -

INTRODUCTION

There is a vast amount of literature supporting the favourable impact of n-3 long chain polyunsaturated fatty acids (LC PUFA) in the prevention and treatment of chronic diseases, especially of the cardiovascular (CV) system (YASHODHARA *et al.,* 2009; GALLI and RISE', 2009). Studies on the effects of the administration of n-3 fatty acid (FA) preparations, mainly in the form of capsules, are generally based on doses in the order of at least 1 g/day, and the low doses mentioned in some investigations refer to at least 600 mg/day (BAYS, 2007). The nutritional features of these compounds, in relation to human physiology at different ages, the patho-physiological processes at the basis of chronic diseases, and the changes in dietary habits (individual and age-related), clearly indicate that the intake of essential and bioactive nutrients, such as n-3 LC PUFA, should be maintained throughout one's entire life (FLEITH and CLANDININ, 2005; CALDER, 2007). Since these FA are rather precious and are present in very small amounts in most diets (HIBBELN *et al.,* 2006), formulations that optimize their bioavailability and facilitate strategies aimed at maintaining optimal intakes should be developed. The ingestion of n-3 FA as components of foods rather than formulated preparations allows greater bioavailability (VISIOLI *et al.,* 2003; ELVEVOLL *et al.,* 2006; GALLI and MARANGONI, 2006). This type of approach has been used, e.g. through the intake of milk enriched with n-3 FA (VISIOLI *et al.,* 2000), giving significant increments of the circulating levels of these FA, even with relatively low intake. Milk-derived products such as yoghurt, a rather popular food item in most countries, can also represent a potential form of administration of n-3 LC PUFA, contributing to optimizing the intake of these compounds, especially in the case of subjects who do not generally consume fish or may not appreciate this type of food.

HLAVATY *et al.* (2008) reported increments of circulating n-3 FA after 3 weeks of consumption of a yoghurt providing about 600 mg/day of n-3 LC PUFA; these amounts were lower than those used by DAWCZYNSKI *et al.* (2009), i.e. 1.2 g/day of n-3 LC PUFA, a dose with favourable effects on the cardiovascular system and on rheumatoid arthritis. However, the consumption of the yoghurt containing 600 mg/day of n-3 LC PUFA (HLAVATY *et al.,* 2008), in addition to increasing the proportion of n-3 FA in serum lipids, prevented unfavourable changes in FA composition of serum lipid classes in obese females under a low caloric diet.

On the basis of the above considerations we planned to specifically investigate the effects of a newly designed yoghurt preparation with low amounts of n-3 FA, i.e. 184 mg/day, lower than those used by HLAVATY *et al.* (2008), on the FA profile in whole blood of volunteers. This study was conducted for six weeks, a time period that is followed in most nutritional interventions.

MATERIALS AND METHODS

Subjects and study design

Subjects (46) were recruited by the Department of Experimental Medicine, University of Parma, from people living in the Province of Parma. The informed consent was obtained from all volunteers participating in the study.

At the beginning of the study, serum total cholesterol and triglycerides were assessed by enzymatic methods using commercially available kits.

At T0 the subjects began to consume 2 yoghurts/day, providing 184 mg/day of total n-3 FA, of which 64 mg were eicosapentaenoic acid (20:5 n-3, EPA) and 74 mg were docosahexaenoic acid (22:6 n-3, DHA). This regimen was maintained for 6 weeks (T6) and then followed by 4 weeks of washout, for a total duration of 10 weeks. Since the study was aimed exclusively at assessing the changes in blood FA profiles during the consumption of a LC n-3 FA-enriched yoghurt, a control group consuming a preparation without these FA was not included.

Blood samples for FA analysis were collected at the following times: 2 weeks before initiation of treatment (run-in period, i.e. T -2), at the beginning of treatment (T0), at the end of 6 weeks of treatment (T6) and after 4 weeks of wash-out (T10). During the run-in period, recommendations were given to the subjects to avoid foods rich in linoleic acid (LA, 18:2 n-6), namely seed oils with high LA levels, e.g. corn, soy bean, sunflower, and especially foods rich in n-3 FA, such as walnuts and spinach containing ALA (alpha linolenic acid, 18:3 n-3), and fish providing EPA and DHA.

The yoghurt used in the study (yoghurt Omega 3 Plus® produced by Parmalat, Parma, Italy) consisted of a 115 g preparation/dose, containing (g/100 g) 1.6 g fat, 60 mg EPA + DHA, 0.1 g dietary fiber, 4 mg Vitamin E, 0.4 μg Vitamin B12 and 80 μg folate. The detailed FA composition of the yoghurt is presented in Table 1. The enriched yoghurt was obtained by adding 0.25% of fish oil (ROPUFA ® '30') that contained n-3 FA as triglycerides.

Whole blood FA analysis

Whole blood drops, obtained from a fingertip, were collected on a special adsorbent paper embedded with the antioxidant butyl hydroxyl toluene both contained in a specific kit (Sigma-Aldrich, St. Louis, MO). The FA analysis of whole blood was then carried out as previously described (MARANGONI *et al.,* 2004). FA methyl esters (FAME) were directly prepared by transes-

Table 1 - Percentage levels of major fatty acids in the yoghurt enriched with omega 3.

FA	% *(w/w)*
C4:0	3.38
C6:0	2.25
C8:0	1.20
C10:0	2.75
C12:0	3.23
C14:0	11.35
C14:1	0.94
C15:0	1.08
C16:0	32.20
C16:1	2.29
C17:1	0.19
C18:0	8.70
C18:1 n-9	16.48
C18:1 n-7	0.96
C18:1 n-5	0.26
C18:2 n-6	1.21
C18:2 *trans*	0.20
CLA	0.29
C18:3 n-3	0.59
C18:4 n-3	0.32
C20:0	0.15
C20:1 n-9	0.50
C20:4 n-6	0.14
C20:4 n-3	0.26
C20:5 n-3	1.91
C22:5 n-3	0.32
C22:6 n-3	2.22

Values are the average of data obtained on several samples. FA, fatty acids; CLA, conjugated linoleic acid.

terification with 600 μL of 3N HCl/methanol and maintained in a dry bath at 90°C for 1 h. Then, after extraction with a KCl solution (2 mL) and hexane (400 μL), FAME were analysed by gas liquid chromatography (Shimadzu FAST GC 2010), equipped with a PTV injector (220°C) and a FID detector (270°C). An Agilent DB-FFAB capillary column (15 m length, 0.10 mm I.D., 0.10 mm film thickness) was used with helium as carrier gas. Oven temperature was programmed from 150° to 248°C. FA from 16 to 24 carbon atoms were identified by the use of pure reference compounds, and were quantified as relative percentages of total FA.

Statistical analysis

It was calculated that the sample size, established also on the basis of previous data (VISIOLI *et al.*, 2003), and allowing a type I error (α) level of 0.05 and a power of the test of 90% with the expected SD for EPA and DHA (30% of mean levels), would allow a change of 10% in variation with the same power to be detected. Data analysis was performed with the statistical software SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Results are expressed as mean±SD; one-way ANOVA univariate between groups and Tukey post-hoc for multiple comparisons were applied and a value of $p<0.05$ was considered significant. The significance of differences for FA analyses between males and females, at different times, was assessed using the unpaired Student T-test.

RESULTS

The characteristics of the subjects are shown in Table 2. The average ages of men and women were rather similar and ranged from 40 to 50 years. Anthropometric data reflected average values of the population in the area, also including a certain percentage of smokers (> 5 cigarettes/day). A rather small percentage of subjects were moderately hypercholesterolemics (average value of total cholesterol 250±22 mg/dL) and the proportion of hypertriglyceridemics (average value of triglycerides 360±170 mg/dL) was even lower. A certain percentage of subjects occasionally took some drugs (antibiotics, anti-inflammatory), which however do not affect lipid metabolism.

The FA profiles of whole blood lipids, expressed as percentage values of total FA, at the different time periods are reported in Table 3. At the various time points, there was a significant difference in the trend of changes, especially for EPA, docosapentaenoic acid (DPA) and DHA. This is reflected in changes of SFA, PUFA and of the unsaturation index (U.I., it is the sum of the percentage of each FA x its number of double bonds) over time. Also total n-6 and n-3 PUFA, n-6 and n-3 HUFA were significantly different at the different time points.

In particular, there were significant increments in the n-3 LC PUFA, while the precursor ALA, present in very small amounts in the yoghurt, decreased at T6 versus T0. In fact, there was an increase of EPA from 0.40 to 0.54% (35%) between T0 and T6, followed by a decline from T6 and T10 (13%), although the final value (0.47%) remained higher than that at T0. As for DHA, the increments followed a different

Table 2 - Characteristics of the subjects.

	All n=46	Females n=23	Males n=23
Age (y)	43.2±7.0	42,2±6.7	44.3±7.3
Weight (kg)	73.3±14.8	64.7±11.4	82.0±12.8
Height (cm)	170.8±8.7	164.7±5.3	177.0±6.8
BMI	25.0±4.1	24.0±4.7	26.1±3.2
% Smokers	13	13	13
% Hyperchol	15	17	13
% Hypertrigl	6	9	4
% Drugs	24	30	17

BMI, body mass index (the ratio between the weight and the squared height in meters).

Table 3 - Whole blood fatty acid profiles of healthy subjects (n=46), consuming a yoghurt rich in n-3 fatty acids.

% FA *(w/w)*	T-2	TO	T6	T10
16:0	25.72±1.51	25.49±1.60	25.91±1.86	24.95±1.61§
18:0	11.46±0.88	11.25±0.85	11.28±0.98	10.91±0.75§
16:1	1.38±0.66	1.44±0.65	1.48±0.67	1.39±0.56
18:1 n-9	20.32±2.45	20.61±2.98	19.88±3.95	20.29±2.66
20:3 n-9	0.13±0.03	0.13±0.04	0.13±0.04	0.13±0.04
18:2 n-6	18.24±1.63	18.55±2.07	18.26±2.17	18.96±2.00
18:3 n-6	0.20±0.07	0.23±0.08	0.26±0.11	0.24±0.09
20:3 n-6	1.38±0.26	1.38±0.30	1.46±0.30	1.45±0.27
20:4 n-6	7.46±1.19	7.67±1.25	7.91±1.36	8.24±1.05
22:4 n-6	0.96±0.23	0.96±0.22	1.00±0.25	1.02±0.20
22:5 n-6	0.21±0.07	0.21±0.06	0.22±0.06	0.23±0.06
18:3 n-3	0.29±0.19	0.29±0.09	0.24±0.06	0.28±0.11
20:5 n-3	0.43±0.14	0.40±0.11	0.54±0.20	0.47±0.17#
22:5 n-3	0.62±0.13	0.63±0.13	0.70±0.15	0.71±0.14#
22:6 n-3	1.78±0.45	1.76±0.40	1.95±0.50	2.03±0.59§
SFA	41.60±1.53	40.96±1.71	41.21±1.90	39.77±1.75#
MUFA	26.68±2.44	26.83±3.01	26.05±3.75	26.40±2.65
PUPA	31.57±2.21	31.99±2.68	32.57±3.28	33.67±2.31#
U.I.	119.90±5.84	121.29±5.93	123.48±7.11	126.87±5.57#
n-6	28.46±2.05	29.00±2.69	29.10±2.85	30.15±1.93§
n-3	3.12±0.62	3.07±0.57	3.43±0.75	3.48±0.80#
n-3 HUFA	2.84±0.65	2.79±0.57	3.19± 10.76	3.20±0.81#
n-6 HUFA	10.02±1.45	10.23±1.55	10.59±1.70	10.94±1.26§

T-2 is the start of run in period; after 2 weeks the start of treatment is T0; T6 (after 6 weeks) the end of treatment and T10 the end of wash out (a 4 week period). During the treatment, volunteers consumed 2 yoghurt/day that provided 202 mg/day of n-3 FA. Values are mean ±SD; one-way ANOVA univariate between groups (different time periods) is applied and the significant differences are §: $p<0.05$; #: $p<0.01$.
FA, fatty acids, SPA, saturated FA; MUFA, monounsaturated FA, PUPA, polyunsaturated FA; U.I. unsaturation index; HUFA, highly unsaturated FA.

trend and were of lower magnitude: from T0 to T6 there was an 11% increment, but the value was further increased after wash-out (15% versus T0). As for the changes in DPA, the intermediate compound in the conversion of EPA to DHA, there was an increment especially during consumption of the yoghurt, of the same magnitude as that of DHA (11%), and the increment was substantially maintained after wash-out (12.6%).

A further type of evaluation concerns differences between females and males in the response to the consumption of the yoghurt. At T0 the EPA and DHA levels were similar in the 2 groups with a tendency to be higher in females than in males. By comparing the changes of EPA and DHA levels from T0 to T10 it appears that increments of both DHA and EPA were greater in women than in men (Fig. 1). In fact, EPA increments in women, over values at T0 were 49% at T6 and 24% at T10, and those of DHA were 16% at both T6 and T10, while in men the changes for EPA were 21 and 13% and for DHA were 5 and 17%, respectively. In addition, increments of DHA in women took place mainly in the T0 to T6 time period, and remained constant during wash out, while in men increments occurred mainly during wash-out.

DISCUSSION

Increments in the consumption of n-3 LC PUFA are presently widely accepted as part of an effective strategy for optimizing physiological processes and preventing major diseases (HOOPER *et al.,* 2004). The relationship between intake of fish or fish oil and relative risk of death from cardiac heart disease shows that the modest consumption of 250 mg/day EPA and DHA appears sufficient for primary prevention (MOZAFFARIAN and RIMM, 2006). A contribution to this strategy may be provided by the use of food items containing these compounds or food items enriched with n-3 FA, and generally consumed by large population groups.

Among natural foods, milk is characterized by highly dispersed features such as fat globules (ranging from 0.1 to 10 μm in diameter), a condition that favours the digestion and intestinal absorption of fats and FA. These characteristics are retained in yoghurt, a type of milk-derived product that is widely consumed.

The intake of 2x115 g of partially defatted yoghurt (1.6% fat *versus* 3.2% in a standard yoghurt) fortified with omega 3 and providing only 64 mg EPA and 74 mg DHA/day resulted in significant increments of both FA in blood lipids

after 6 weeks. The increments were statistically significant and especially concerned EPA, as predictable on the basis of lower starting values of this FA, that is also mainly incorporated into metabolically less stable lipid pools (RISE' *et al.,* 2007). This last consideration partly explains the decline of EPA during the wash-out, in agreement with the trend towards a reduction of the FA during the run-in period. Reduction of blood EPA levels during the wash-out period was also observed in a previous study (GALLI and MARANGONI, 2006). Conversion of the supplemented EPA to its derivatives, supported also by the increments of DPA and especially of DHA even during wash-out, may contribute to the reduction of EPA after its intake is interrupted. We observed a similar trend, i.e. increments of blood DHA levels at the end of wash-out following a 3 weeks intake of LC n-3 PUFA through either fish consumption or ingestion of encapsulated preparations in male subjects and this increment was greater when the FA were ingested through food (GALLI and MARANGONI, 2006).

HLAVATY *et al.* (2008) showed combined increments of EPA + DHA of about 60% from basal values, after the daily intakes of an n-3 FA (620 mg/day) enriched yoghurt. Interestingly in our study, the increment of 15% in these FA in blood was obtained with a dose of about 25% (138 mg/day) of that in the study by HLAVATY *et al.* (2008). On the other hand, the basal values of DHA, which in a previous comparative assessment in plasma versus whole blood lipids were very similar (RISE' *et al.,* 2007), were significantly higher in our study than in the Czech population, possibly reflecting the low intake of fish in this population, as mentioned in the article (HLAVATY *et al.,* 2008).

Another interesting finding concerns the different patterns of EPA and DHA changes, i.e., greater in women than in men. In addition, during washout EPA declined in women more than in men while DHA remained stable in women and increased in men, although remaining lower than in women. Higher circulating levels of DHA associated with lower levels of the precursor DPA, suggesting more efficient conversion to the end product, have been previously reported in pre-menopausal women versus men (MARANGONI *et al.,* 2007). These differences have been attributed to estrogenic effects (GILTAY *et al.,* 2004) and greater efficiency in the conversion of ALA to DHA has been described using stable isotope techniques (BURDGE and WOOTTON, 2002). In addition gender-related differences have been described in the kinetics of various "exogenous" compounds, such as drugs (bioavailability, distribution, metabolism and elimination) (GHANDI *et al.,* 2004). The gender differences in blood increments with the same doses of n-3 FA should, however, be more carefully explored, although they were recently described (METHEREL *et al.,* 2009), also in term of functional responses to the different increments, in order to optimize the supplementation of these compounds in women versus men.

Our data show that the consumption, for a relatively short duration, of a low fat yoghurt containing small amounts of n-3 FA, compared to the generally administered doses in most supplementation studies, resulted in appreciable increments in blood levels of these compounds, thus providing a useful approach to reach the recommended intake in large population groups consuming this type of food item.

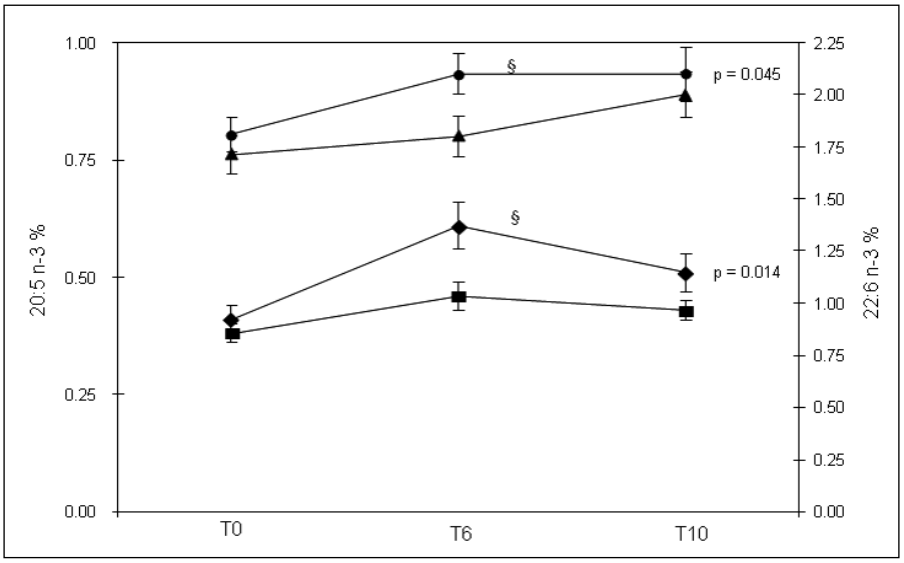


Fig. 1 - 20:5 n-3 and 22:6n-3 levels in whole blood of male and female subjects.
Values represent the mean±SEM; -■- 20:5 n-3 male; -◆- 20:5 n-3 female; -▲- 22:6 n-3 male; -●- 22:6 n-3 female.
One-way ANOVA univariate between groups (p values) was applied; differences between males and females, at different times, were assessed using the unpaired Student T-test. The differences were significant for §: $p<0.05$.

REFERENCES

Bays H.E. 2007. Safety considerations with omega-3 fatty acid therapy. Am. J. Cardiol. 99: 35C.

Burdge G.C. and Wootton S.A. 2002. Conversion of a linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. Br.J. Nutr. 88: 411.

Calder P.C. 2007. Immunomodulation by omega-3 fatty acids. Prostaglandins Leukot. Essent. Fatty Acids 77: 327.

Dawczynski C., Shubert R., Hein G., Muller A., Eidner T., Vogelsang H., Basu S. and Jahreis G. 2009. Long-term moderate intervention with n-3 long-chain PUFA-supplemented dairy products: effects on pathophysiological biomarkers in patients with rheumatoid arthritis. Br. J. Nutr. 101: 1517.

Elvevoll E.O., Barstad H., Breimo E.S., Brox J., Eilersten K.E., Lund T., Olsen J.O. and Osterud B. 2006. Enhanced incorporation of n-3 fatty acids from fish compared with fish oils. Lipids 41: 1109.

Fleith M. and Clandinin M.T. 2005. Dietary PUFA for preterm and term infants: review of clinical studies. Crit. Rev. Food Sci. Nutr. 45: 205.

Galli C. and Marangoni F. 2006. N-3 fatty acids in the Mediterranean diet. Prostaglandins Leukot. Essent. Fatty Acids 75: 129.

Galli C. and Risé P. 2009. Fish consumption, omega 3 fatty acids and cardiovascular disease. The science and the clinical trials. Nutr. Health 20: 11.

Gandhi M., Aweeka F., Greenblatt R.M. and Blaschke T.F. 2004. Sex differences in pharmacokinetics and pharmacodinamics. Ann. Rev. Pharmacol. Toxicol. 44: 499.

Giltay E.J., Gooren L.J., Toorians A.W., Katan M.B. and Zock P.L. 2004. Docosahexaenoic acid concentrations are higher in women than in men because of estrogenic effects. Am. J. Clin. Nutr. 80: 1167.

Hibbeln J.R., Nieminen L.R., Blasbalg T.L., Riggs J.A. and Lands W.E.M. 2006. Healthy intakes of n-3 and n-6 fatty acids: estimations considering worldwide diversity. Am. J. Clin. Nutr. 83:1483S.

Hlavatý P., Kunesová M., Gojová M., Tvrzickà E., Vecka M., Rounbal P., Hill H., Hlavatà K., Kalouskovà P., Hainer V., Zàk A. and Drbohlav J. 2008. Change in fatty acid composition of serum lipids in obese females after short-term weight-reducing regimen with the addition of n-3 long chain polyunsaturated fatty acids in comparison to controls. Physiol. Res. 57: S57.

Hooper L., Thompson R.L., Harrison R.A., Summerbell C.D., Moore H., Worthington H.V., Durrington P.N., Ness A.R., Capps N.E., Davey Smith G., Riemersma R.A. and Ebrahim S.B. 2004. Cochrane Database Syst. Rev. 4:CD003177.

Marangoni F., Colombo C. and Galli C. 2004. A method for the direct evaluation of the fatty acid status in a drop of blood from a fingertip in humans: applicability to nutritional and epidemiological studies. Anal. Biochem. 326: 267.

Marangoni F., Colombo C., Martiello A., Negri E. and Galli C. 2007. The fatty acid profiles in a drop of blood from a fingertip correlate with physiological, dietary and lifestyle parameters in volunteers. Prostaglandins Leukot. Essent. Fatty Acids 76: 87.

Metherel A.H., Armstrong J.M., Patterson A.C. and Stark K.D. 2009. Assessment of blood measure of n-3 polyunsaturated fatty acids with acute fish oil supplementation and washout in men and women. Prostaglandins Leukot. Essent. Fatty Acids 81: 23.

Mozaffarian D. and Rimm E.B. 2006. Fish intake, contaminants, and human health: evaluating the risk and the benefits. JAMA 296: 1885.

Risé P., Eligini S., Ghezzi S., Colli S. and Galli C. 2007. Fatty acid composition of plasma, blood cells and whole blood: relevance for the assessment of the fatty acid status in humans. Prostaglandins Leukot. Essent. Fatty Acids 76: 363.

Visioli F., Risé P., Barassi M.C., Marangoni F. and Galli C. 2003. Dietary intake of fish vs formulations leads to higher plasma concentrations of n-3 fatty acids. Lipids 38: 415.

Visioli F., Risé P., Plasmati E., Pazzucconi F., Sirtori C.R. and Galli C. 2000. Very low intakes of n-3 fatty acids incorporated into bovine milk reduce plasma triacylglycerol and increase HDL-cholesterol concentrations in healthy subjects. Pharmacol. Res. 41: 571.

Yashodhara B.M., Umakanth S., Pappachan J.M., Bhat S.K., Kamath R. and Choo B.H. 2009. Omega-3 fatty acids: a comprehensive review of their role in health and disease. Postgrad. Med. J. 85: 84.

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PAPER

DETERMINATION OF DIMETHYL SULPHIDE IN BREWERY SAMPLES BY HEADSPACE GAS CHROMATOGRAPHY MASS SPECTROMETRY (HS-GC/MS)

A. STAFISSO, O. MARCONI*, G. PERRETTI and P. FANTOZZI
Italian Brewing Research Centre (CERB), Via San Costanzo, 06126 Perugia, Italy
*Corresponding author: Tel./Fax +39 075 585 7926,
e-mail: omarconi@unipg.it

ABSTRACT

Dimethyl sulfide (DMS) is an important beer flavour compound and when there is more than 100 μg/L as free DMS, it is considered an off-flavour because it may give an undesirable cabbage flavour and smell to the beer. The aim of this work was to set up a gas chromatography-mass spectrometry (GC-MS) method, coupled with headspace, to determine the DMS present in the beer chain. This could then be useful for setting up the quality control of parameters in malting and brewing. A case study for application of the proposed method is reported for barley variety screening.

- Key words: barley, beer, dimethyl sulfide, gas chromatography-mass spectrometry, malt -

INTRODUCTION

A wide variety of sulphur compounds has been reported to occur in beer (NYKÄNEN and SUOMALINEM, 1983). Even if the malt, hops and water used in brewing may all be possible sources of sulphur compounds, the principal volatile and semi-volatile compounds do not come directly from the raw materials, but they mainly originate during malting and fermentation (HILL and SMITH, 2000; WALKER and SIMPSON, 1992;). The main volatile sulphur compound in beer is dimethyl sulfide (DMS), a thioether which plays an important role in beer flavour (BAMFORTH, 1998; HILL and SMITH, 2000; KUNZE, 2004). The contribution of DMS in beer is well known (YANG and SCHAWARZ, 1998a). Its threshold level in beer is about 30-50 μg/L (BAMFORTH, 1998; ZHAO *et al.*, 2006). In the range of 50-100 μg/L, DMS contributes to the taste of lager-type beers, whereas above 100 μg/L it may give an undesirable vegetable-like or cabbage flavour and smell to the beer. The control of DMS formation, or its removal, is therefore desirable.

Two DMS precursors (DMS-p) or two routes of DMS formation during malting and brewing have been proposed and different techniques have been used to identify the precursors: S-methyl-L-methionine (SMM) and dimethyl sulfoxide (DMSO). Both of these precursors originate from malt but they form DMS in different ways (YANG and SCHAWARZ, 1998a).

SMM, which is synthesized in the seed germ and rootlets during barley germination, is converted into DMS under high temperatures during malt kilning and wort boiling. DMSO, instead, is reduced to DMS by yeast during wort fermentation (ANNESS and BAMFORTH, 1982).

It has widely been accepted that the final DMS level in beer will depend mainly on the SMM content of the malt. However, it is also believed that the level of DMSO in wort plays a major role in determining the DMS level in the final beer (KUNZE, 2004; ZHAO *et al.*, 2006; YANG and SCHAWARZ, 1998a).

During steeping and germination some attempts have been made to transform SMM as little as possible into active free DMS (KUNZE, 2004). The residual part of SMM is thermally degraded into DMS mainly during malt kilning, and a small amount is lost to the atmosphere with the evacuation of gases.

During the kilning process, part of the SMM is degraded into DMS, and, at the same time, some DMS is oxidized to DMSO. During wort fermentation, DMSO can be transformed into DMS and water (DMSO → DMS + H_2O) through metabolic reduction by brewer's yeast, catalyzed by DMSO-reductase in a NADPH-dependent manner (BAMFORTH, 1980). The amount of DMS produced by yeasts changes from strain to strain (WALKER and SIMPSON, 1992) and this kind of DMS production is one of the main causes of beer off-flavours. The reduction of DMSO is probably affected by the formation of methionine sulfoxide (MetSO). MetSO competitively inhibits DMSO-reductase because it is the preferred substrate of the enzyme. The affinity of DMSO reductase for MetSO is higher than that of DMSO (ANNESS and BAMFORTH, 1982; HANSEN *et al.*, 2002).

Intensive wort boiling causes the DMS concentration in the final beer to be reduced (HERTEL and SOMMER, 2008; KUNZE, 2004; VAN DEN EYNDE, 1991).

The DMS produced from DMSO during fermentation is removed together with the other volatile compounds present by mean of stripping with CO_2. (VAN DEN EYNDE, 1991).

Given the importance of DMS in beer flavour, and taking into account the influence of technical parameters of malting and brewing on the DMS content of beer, a sensitive instrumental method of analysis for the routine determination of this compound is required (COGHE *et al.*, 2004).

The study and determination of DMS is difficult principally due to its low concentration in beer. Various techniques have been reviewed: amperometry, titrimetry (GARZA-ULLOA, 1980), colorimetry (INSTITUTE OF BREWING, 1982), fluorimetry (BLOCKMANS *et al.*, 1981) and potentiometry (NARZISS *et al.*, 1985) but none of them provide the level of sensitivity and selectivity usually required.

Actually, the simplest analytical methods involve headspace sampling of the beer followed by direct injection into a gas cromatograph (GC) (WALKER, 1991), but this type of analysis is not useful when the concentration of the sulphur compounds is low or when they are partially volatile. Generally, in these cases methods, which involve "purge and trap", whereby the volatile components in the beer headspace are concentrated onto a suitable adsorbent material, have been described (LEPPANEN, 1979). Furthermore, it is possible to set up an analytical method using columns and detectors specific for separating and identifying sulphur compounds (MUNDY, 1991; HILL and SMITH, 2000). Several researchers have indicated the use of headspace gas chromatography or sometimes Gas -Liquid Chromatography (GLC) equipped with a Flame Photometric Detector (FPD) or a Sulphur Chemiluminescent Detector (SCD) or a Pulsed Flame Photometric Detector (PFPD) or a Flame Ionization Detector (FID) as possible analytical devices, with or without preliminary Solid-Phase Micro Extraction (SPME) (BAKER, 1988; DROZD *et al.*, 1990; DUPHIRE, 1999; HILL and SMITH, 2000; VAS, 1997; WALKER, 1992; YANG and SCHWARZ, 1998b). SPME has already been applied in many areas of the food and beverage industries with important results (HILL and SMITH, 2000).

Little information is available, however, on the use of gas chromatography coupled with Mass Spectrometry (MS) for DMS analysis in barley, malt, wort and beer.

The aim of the present work was to demonstrate the possibility of setting up a Headspace-Gas Chromatography-Mass Spectrometry (HS-GC-MS) method able to identify and quantify DMS as free DMS, total-DMS and DMS precursors (DMS-p) (total DMS - free DMS) in barley, malt, wort and beer, in order to control the malting and brewing process.

With GC, DMS in beer can be sampled and at the same time, coupling GC with MS, it is possible to use the high selectivity, precision and sensitivity of MS to identify the substance of relevance from barley and malt as well as from elaborate matrices such as wort and beer.

Different varieties of barley and their respective malts, worts and beers were analysed as a case study for the application of the proposed method.

MATERIALS AND METHODS

Reagents

DMS and ethyl methyl sulfide (EMS) standards were purchased from Sigma-Aldrich (Milwaukee, WI). Ethanol 99% (v/v) was purchased from VWR International S.R.L. (Milano, IT). Sodium hydroxide was purchased from J.J. Baker (Deventer, NL). Distilled water was of high purity.

Barley samples

Six two-row varieties of barley were used: *Henley*, *Cheri*, *Scarlett, Barke*, *Regina* and *Puffin*. The first four were spring and the last two were autumnal. All samples were grown in the experimental fields of the University of Perugia.

Malting process

For the evaluation of barley malts, a standard malting program was followed. The steeping, germination and drying programs were suitable for the production of "pilsner" type malt. The barley samples were malted in a micro-malting pilot-plant (Custom Laboratory Product, Keith, Scotland, UK) consisting of 4 independent steeping/germination tanks, each having a 5 kg capacity.

The steeping phase lasted 20 hours and was divided into 3 steps:

- Step 1: 5 h of soaking in water at 18°C followed by 7 h of resting at 17°C.
- Step 2: 2 h of soaking in water at 16°C followed by 5 h of resting at 16°C.
- Step 3: 1 h of soaking in water at 16°C before starting of germination.

The germination phase lasted 4 days, during which time the temperature was regularly decreasing: 16°C during the first day, 15°C during the second day and 14°C for the last two days. The green malts were dried using a drying plant (Custom Laboratory Product, Keith, Scotland, UK) consisting of 4 units and applying the following drying temperatures:

- 55°C for 15 h;
- 72°C for 4 h and 30 min;
- 82°C for 3 h and 30 min;
- Cooling (to room temperature).

Brewing process

Mashing

Each sample of barley malt was processed in a pilot-scale plant (Braumaister, Feltre, I) programmed in order to produce 25 litres of wort suitable for "pilsner" type beer. Five kilograms of malt were milled in a two-roller mill (120 kg/h capacity, ENGL maschinen Gmbh, Schwebheim, Germany,) with a 0.5 mm gap between the crushing rollers. The grist was then mixed with 23 L of water. The properties of the brewing liquor were: 0.5 Millival, 15 mg/L of carbonate (CO_3^{-2}), 30.5 mg/L of hydrogen-carbonate (HCO_3^{-2}), 11 mg/L of dissolved CO_2 and pH 6.5. The milled malt was mashed in the brewing liquor at 52°C for 30 min to allow protein breakdown. The temperature was increased to 65°C in 15 min and maintained for 45 min for development of the beta-amylase activity. The temperature was increased to 72°C in 5 min and maintained at this temperature for 20 min to allow the alpha-amylase activity to develop. The efficiency of saccharification was tested by the iodine solution test.

The last temperature step was a short rest at 76°C to inactivate the enzymes. Afterwards, the mash was transferred to a 30 L lauter-tun vessel. The first wort was collected from the lauter-tun, then the spent grains were sparged with 12 L of water (78°C) to wash out the second wort. The total amount of wort was collected in a heated 30 L kettle for boiling; the temperature was raised to 100°C and maintained for 75 min. 100 grams of hops pellets (Saaz variety, 2.1% of α-acids) were added: 70% of hops was added at the beginning of boiling, and 30% was added 15 min before the end of boiling.

Fermentation

The wort was cooled to 18°C and transferred to a 30 L fermentation vessel (Spadoni, Orvieto, I). 11.5 grams of ale yeasts (*Safale S-04*, Fermentis, Marcq-en-Baroeul Cedex - France) were activated with 100 mL of sterile wort. The fermentation temperature was maintained at 18°C for 5 days. When the gravity of the wort reached the limit of attenuation (85%), the beer was bottled to continue fermentation at 18°C. At the end of maturation, the bottles were kept at 4°C for two weeks and then analysed.

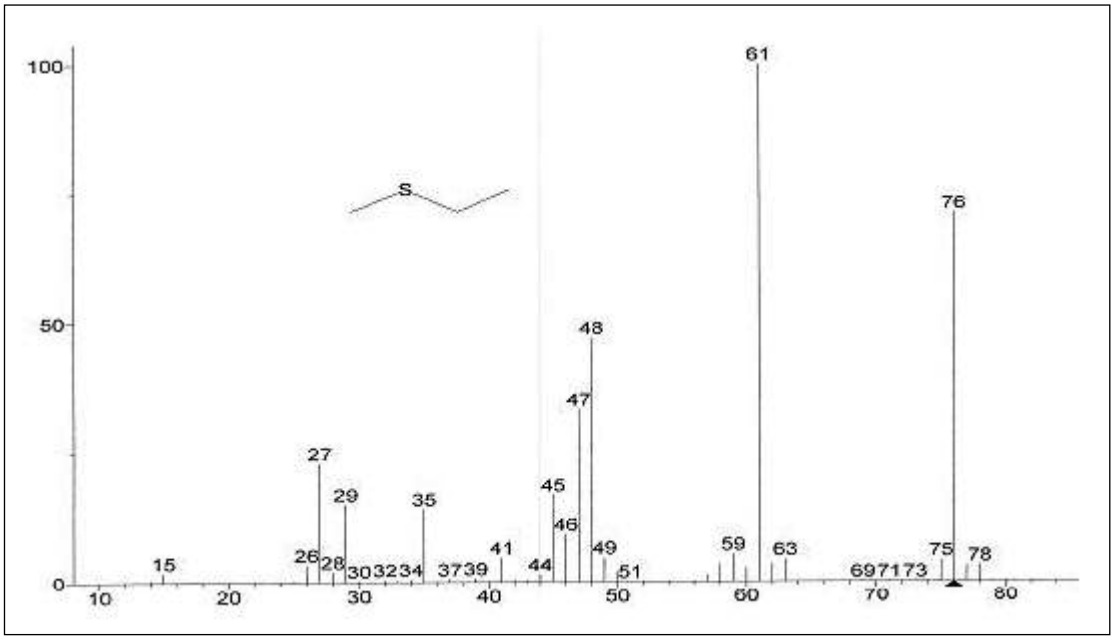


Fig. 1 - EMS molecular fragmentation.

Apparatus

The DMS concentration was determined in static headspace with an Agilent Technologies 6850 gas chromatograph equipped with an Agilent Technologies Mass Spectrometer 5975C (Santa Clara, CA) coupled with Maestro Autosamples Gerstel Multi Purpose Sampler (MPS) (Baltimore, MD).

GC-MS conditions

The gas chromatograph-mass spectrometer was fitted with a DB-5MS capillary column, 60 m X 0.32 mm X 1 μm (J&W Scientific Inc, Folsom, CA) consisting of cross-linked 5% phenyl methyl siloxane. Helium was the carrier gas at a flow rate of 1.2 mL/min. The injection port temperature was 250°C, and the detector was set at 280°C. The source and the quadrupole temperature were 230° and 150°C, respectively. The ionization energy was 70eV. Detection and data acquisition was performed in Selected Ion Monitoring (SIM) mode.

The target molecular ions for EMS were selected to be (*m/z*) 48, 61 and 76 (Fig. 1) and those for DMS were selected to be 47 and 62 (Fig. 2).

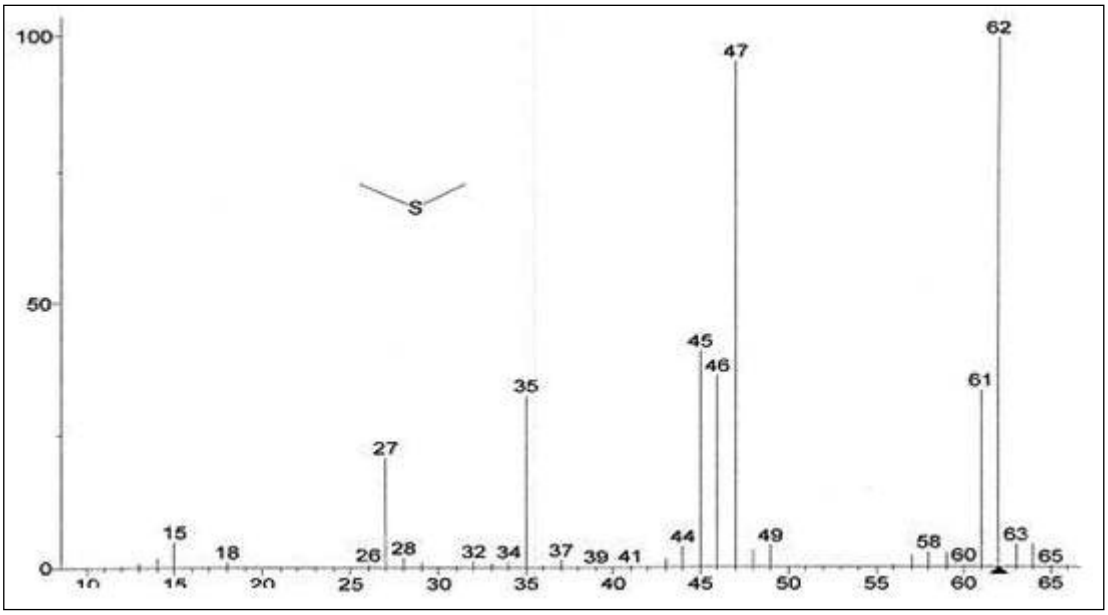


Fig. 2 - DMS molecular fragmentation.

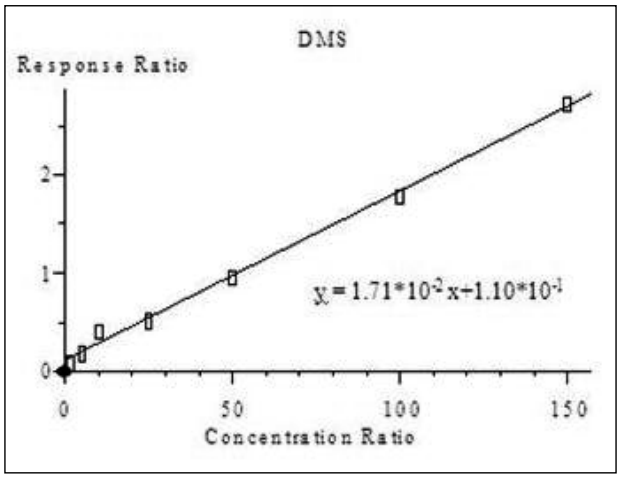


Fig. 3 - Calibration curve for DMS.

The oven temperature program used was 40°C for 2 min, followed by an increase of 3°C/min to 100°C and 10°C/min to 150°C. The final temperature was held for 3 min. The headspace conditions were 30 min of incubation at 50°C on unrest (250 rpm). The shaker temperature was 60°C, the syringe capacity was 2.5 mL, and the temperature was 70°C. The volume injected was 1 mL.

Standard solutions and calibration

DMS stock solution was prepared at 2,553 mg/L in ethanol whereas the DMS working solution was prepared at 25.53 mg/L in ethanol. EMS was used as internal standard (IS). The EMS stock solution was prepared at a concentration of 8,422 mg/L in ethanol and the working standard solution at 5 mg/L in ethanol.

DMS calibration curve (Fig. 3) was determined for five different concentrations in a range from 1.66 μg/L to 150.00 μg/L and with EMS as IS (0.05 mg/L). The calibration solutions were prepared from the working standard solution. Each concentration was analyzed three times.

Sample preparation

The sample preparation was carried out following European Brewery Convention (EBC) and Mebak methods with some modifications. The free DMS concentration of barley and malt samples was determined on a cold water extract of 10 +/-0.01 g of ground barley and malt, respectively, mixed with 100 mL of water under magnetic agitation at 150 rpm for 35 min. at room temperature (20°C). Then, 5 mL of the extract were mixed with 5mL of water and 100 μL of IS, and the solution was directly analysed by HS-GC-MS. The total DMS concentration was determined by adding 1 mL of 1N NaOH, 6.5 mL of water and 100 μL of IS to 2.5 mL of the barley and malt extract. Then the solutions obtained (10.1 mL), with an IS concentration of 0.05 mg/L, were boiled and analyzed by HS-GC-MS.

The free DMS concentration in wort and beer was quantified directly in 10 mL of sample, adding 100 μL of IS solution. The total DMS concentration was determined by adding 2.5 mL of 1N NaOH, 1 mL of water and 100 μL of IS solution to 6.5 mL of wort sample and boiling them for 60 min. The sample solutions were prepared in 20-mL vials in duplicate and then analyzed by HS-GC-MS. The beer samples were not degassed to remove the carbon dioxide in order to avoid removing of DMS, because of its high volatility.

The total and free DMS concentration in barley, malt, wort and beer was obtained by mean of the calibration curve (Fig. 3). The DMS-p was calculated by subtracting free DMS from tot-DMS.

RESULTS AND DISCUSSION

The first step was the setting up of an innovative analytical method by GC-MS to identify and quantify DMS in different kinds of samples after elimination of interferring substances. Barley and malt samples for the analysis of the DMS content were mainly prepared following the official methods, but with some modifications (ANALYTICA-EBC 3.39; HILL and SMITH, 2000; MEBAK ROHSTOFFE 3.1.4.17; WALKER, 1991; YANG and SCHWARZ, 1998b). Filtration of the extract was excluded because it causes DMS losses. Fig. 4 shows that a satisfactory separation of DMS was achieved in a short time.

The performance of the GC-MS method was evaluated taking into account the calibration curve, the linearity, the repeatability, the limit of detection (LOD) and the limit of quantification (LOQ) (Table 1). The confidence interval of

Table 1 - Concentration range, repeatability, slope (a), intercept (b), and coefficient of determination (r^2) of calibration plots *, limit of detection (LOD) and limit of quantification (LOQ) of the optimized method for determination of DMS in barley, malt, wort and beer.

Concentration range (μg/L)	Repeatability† ± RSD (%)	a	b	r^2	LOD† (μg/L)	LOQ† (μg/L)
1.66-150.00	4.96	$1.71*10^{-2}$	$1.10*10^{-1}$	0.996	0.09	0.23

† *n*=10; * y=ax+b; RSD= relative standard deviation.

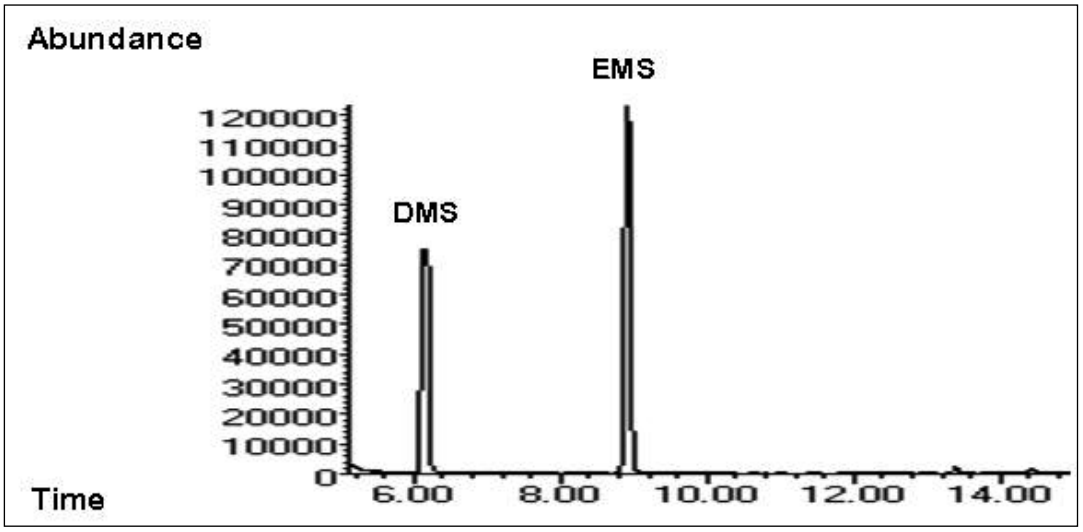


Fig. 4 - DMS and EMS GC-MS chromatogram of a beer sample.

the calibration curve included the origin of the axes, and the coefficient of determination was r^2=0.996. The LOD was calculated as s_b+3s, where s_b was the average signal of ten blank injections and s the standard deviation. The LOQ was calculated as s_b+10s, where s_b was the average signal of ten blank injections and s the standard deviation (EURACHEM GUIDE, 1998). The blank sample was obtained by boiling the beer for 30 min to evaporate all free DMS. The LOD was 0.09 μg/L and the LOQ was 0.23 μg/L.

The repeatability of the method was measured by performing the determination of the same beer sample with a free DMS concentration of 25 μg/L 10 times on the same day and estimating their RSD (%) (EURACHEM GUIDE, 1998). The RSD calculated on each concentration used to work out the calibration curve was 4.96%.

The accuracy of the method for DMS in malt was verified by participating in the International Proficiency MAPS test (Malt Analytes Proficiency Testing Scheme) (LGC Standards Proficiency Testing, Lancashire, UK) every three months. The results of the participation in the MAPS rounds are reported in Table 2. The accuracy of the method for DMS in beer was verified by participating in the International Proficiency BAPS test (Brewing Analytes Proficiency Scheme). The results of the participation in the BAPS rounds are reported in Table 3.

The efficiency of the proposed method was evaluated by performing the recovery test on beer samples with the addition of three different DMS concentrations: 25, 50 and 75 μg/L. The recovery test results are reported in Table 4. The recovery ranged from 69 to 101% with a mean value of 80%. The data showed good results at the lowest spiked level which corresponds to the acceptable range for free DMS in beer. Therefore, the recovery test and the MAPS results confirmed the efficiency of the method.

The analytical screening of different samples allowed the development of DMS to be studied during malting and brewing. A comparative study of the results enables the following remarks to be made.

The majority of total DMS in barley was represented by DMS-p which was more than 80% in each case. The *Regina, Puffin* and *Barke* varieties showed a similar DMS-p concentration, with values of 0.23, 0.14 and 0.20 mg/kg, respectively, confirming that a low amount of DMS-p

Table 2 - Results obtained by participating in 4 rounds of a Malt Analytes Proficiency Testing Scheme (MAPS test).

	Round 1			Round 2			Round 3			Round 4		
	Assigned value (mg/kg)	Result (mg/kg)	Z score	Assigned value (mg/kg)	Result (mg/kg)	Z score	Assigned value (mg/kg)	Result (mg/kg)	Z score	Assigned value (mg/kg)	Result (mg/kg)	Z score
DMS-p	3.60	4.10	0.83	11.30	13.50	0.65	0.60	0.49	-0.18	1.90	1.44	-0.83
Free DMS	5.70	6.00	0.30	3.60	3.00	-0.60	1.20	0.78	-0.43	2.00	1.71	-0.30
Total DMS	9.60	10.20	0.50	14.55	16.51	1.34	1.80	1.27	-0.44	4.10	3.15	-0.75

|z|≤ 2.00 Satisfactory result; 2.00 < |z| < 3.00 Questionable result; |z|≥ 3.00 Unsatisfactory result.

is present in barley (Table 5) (YANG and SCHAWARZ, 1998a). The differences between the six barley varieties are probably due to their different genetic characteristics.

Table 6 reports the free DMS, total DMS and DMS-p contents of the malts analysed. All malts contained DMS and DMS-p. It is well known that during germination the level of SMM increases and then kilning which leads to an increase in the temperature until 82°C, causing an enhancement of free DMS because of the thermodegradation of SMM to DMS.

The *Cheri* variety malt showed the highest amount of DMS-p, while the other five varieties showed similar concentrations ($p<0.05$). Among the six varieties, *Scarlett* had a significant concentration of free DMS with 8.47 mg/kg. This can be explained by the hypothesis that a high level of SMM in *Scarlett* green malt was produced during germination and then it was broken down during kilning to produce DMS. *Scarlett* is probably the variety with the highest amount of enzymes involved in SMM synthesis (ANNESS and BAMFORTH, 1982). Therefore, considering that all the barley samples were subjected to the same malting process, the different DMS concentration should be related to the variety and its genetic factors. It is interesting to focus on the reaction of the six different barley types to the heat treatment.

The free DMS concentration in wort ranged from 9.91 μg/L for *Scarlett* to 83.59 μg/L for *Barke* (Table 7). The autumn varieties *Regina* and *Puffin* had a DMS-p concentration similar to *Henley;* among the spring varieties only *Scarlett* and *Barke* were similar. The concentration of DMS-p in *Cheri* was different from the other five varieties. It is important to take into account the

Table 3 - Result obtained by participating in the Brewing Analytes Proficiency Scheme (BAPS).

	Round 1		
	Assigned Value (μg/L)	Result (μg/L)	Z score
Free DMS	14.60	12.00	-0.59

|z|≤ 2.00 Satisfactory result; 2.00 < |z| < 3.00 Questionable result; |z|≥ 3.00 Unsatisfactory result.

Table 4 - Recovery test performed by adding three different amounts of DMS to a beer sample.

Original DMS (μg/L)	Added (μg/L)	Recovery (%)	s.d.
40	25	101	7
40	50	69	8
40	75	71	5

n=2; s.d.=standard deviation.

Table 5 - The DMS concentration in barley samples.

	DMS-p (mg/kg)	free DMS (mg/kg)	total DMS (mg/kg)
Henley	0.07[a]	0.02[a]	0.09[a]
s.d.	*0.02*	*0.01*	*0.02*
Cheri	0.13[a,c]	0.01[a, b]	0.14[a, b]
s.d.	*0.03*	*0.01*	*0.03*
Scarlett	0.13[a, c]	0.01[c]	0.13[a, b]
s.d.	*0.01*	*0.01*	*0.01*
Barke	0.20[b]	0.01[b, c]	0.21[b, c]
s.d.	*0.01*	*0.01*	*0.01*
Regina	0.23[b]	0.01[c]	0.24[c]
s.d.	*0.02*	*0.01*	*0.02*
Puffin	0.14[b, c]	0.01[b, c]	0.15[a, b, c]
s.d.	*0.01*	*0.01*	*0.01*

n=2;
s.d.= standard deviation.
Samples in the same column with the same letters are not statistically different ($p \leq 0.05$).

Table 6 - The DMS concentration in malt samples.

	DMS-p (mg/kg)	free DMS (mg/kg)	total DMS (mg/kg)
Henley	3.35[a]	5.70[a]	9.05[a]
s.d.	*0.46*	*0.13*	*0.52*
Cheri	6.82[b]	7.25[b]	14.07[b]
s.d.	*0.64*	*0.18*	*0.46*
Scarlett	4.94[a]	8.47[c]	13.41[b]
s.d.	*0.52*	*0.56*	*0.04*
Barke	4.31[a]	5.32[a]	9.64[a]
s.d.	*0.38*	*0.04*	*0.34*
Regina	4.93[a]	5.48[a]	10.41[a]
s.d.	*0.01*	*0.15*	*0.14*
Puffin	3.28[a]	4.97[a]	8.25[a]
s.d.	*0.20*	*0.13*	*0.33*

n=2;
s.d.= standard deviation.
Samples in the same column with the same letters are not statistically different ($p \leq 0.05$).

Table 7 - The DMS concentration in wort samples.

	DMS-p (μg/L)	free DMS (μg/L)	total DMS (μg/L)
Henley	78.04[a]	32.75[a]	110.78[a]
s.d.	*5.59*	*9.81*	*8.26*
Cheri	131.75[c]	46.80[a]	178.55[b]
s.d.	*5.31*	*0.10*	*5.31*
Scarlett	102.38[b]	9.91[b]	112.29[a]
s.d.	*9.41*	*0.10*	*9.32*
Barke	113.30[b]	83.59[c]	196.89[b]
s.d.	*4.80*	*0.95*	*3.84*
Regina	90.92[a, b]	41.24[a]	132.16[a]
s.d.	*2.36*	*0.14*	*2.22*
Puffin	61.54[a]	15.49[b]	77.02[c]
s.d.	*4.90*	*0.11*	*4.79*

n=2;
s.d.= standard deviation.
Samples in the same column with the same letters are not statistically different ($p \leq 0.05$).

Table 8 - The free DMS concentration in beer samples.

	free DMS (µg/L)
Henley	138.63[a]
s.d.	*2.31*
Cheri	48.80[b]
s.d.	*0.13*
Scarlett	84.40[c]
s.d.	*1.40*
Barke	148.61[d]
s.d.	*2.66*
Regina	61.95[e]
s.d.	*1.07*
Puffin	38.34[f]
s.d.	*0.74*

n=2;
s.d.= standard deviation.
Samples in the same column with the same letters are not statistically different ($p \leq 0.05$).

value of DMS-p in wort, as DMSO, because the concentration of free DMS in the beer may depend on it (KUNZE, 2004; VAN DEN EYNDE, 1991). In fact, the level of SMM in the wort does not influence the production of DMS in the beer because the yeast is not able to produce DMS from SMM (ANNESS and BAMFORTH, 1982).

The beer samples were Pilsner type, and traditionally they should be characterized by a concentration of free DMS around 50-60 µg/L, but not higher than 100 µg/L. Table 8 reports the free DMS concentration of the beer analyzed, which ranged from 38.34 µg/L to 148.61 µg/L. Brewers should pay attention to the level of free DMS as the off-flavour of beer is caused by the free DMS (KUNZE, 2004). This phenomenon may be the consequence of yeast activity during fermentation which produces free DMS from the DMSO.

The beer samples had acceptable concentrations of free DMS: out of the six beers, *Cheri*, *Scarlett* , *Regina* and *Puffin* were within limits. *Cheri* and *Regina* barley varieties produced beers with low concentrations of free DMS despite the high concentrations of total DMS in the barleys. This means that the brewing process influences the free DMS concentration in the final product. *Henley* and *Barke* samples showed a significant concentration of free DMS (138.63 µg/L and 148.61 µg/L, respectively). However, these data are acceptable because they are in the same range of concentration as the threshold. The results about free DMS concentration in beer showed that the six samples were statistically different ($p \leq 0.05$). Taking into account the apparent DMS production during fermentation, as the difference between the DMS concentration of the final beer and the wort, just 2 µg/L of DMS were produced by yeast during fermentation of the *Cheri* wort, while 106 µg/L were produced for the *Henley* wort. Hence, it was hypothesized that the DMS-p in *Cheri* wort were mainly represented by SMM, while in *Henley* wort they were mainly by DMSO. It also seemed that DMS-p in the wort obtained from the autumnal variety malts were mainly constituted by SMM.

All the barley samples showed low levels of total DMS and precursors because DMS is produced from SMM by heating. Therefore, DMS is not present in barley, but it increases during germination. In the majority of malt samples, DMS was constituted by a significant amount of DMS-p. The wort samples in each case had a small concentration of free DMS. At the end of the brewing process, in the final beer, the DMS concentration was mainly represented by free DMS as reported by YANG and SCHWARZ (1998a) and VAN DEN EYNDE (1991).

CONCLUSIONS

The Head-Space Gas Chromatography-Mass Spectrometry (HS-GC-MS) method set up in this study provides an important tool for the study of DMS development during malting and brewing processes. The precision, accuracy and repeatability of the analytical method allows the quality of raw materials to be controlled, as well as the choice of materials in order to produce a better quality beer. It requires routine and versatile equipment, and therefore it can be easily adopted by laboratories worldwide.

The proposed method enables the presence and concentration of DMS to be checked during the brewing process. The screening of different barley varieties demonstrated the applicability of the proposed HS-GC-MS method. Considering DMS concentrations, the results showed that the six varieties studied produced different beers ($p \leq 0.05$), indicating that the variety has to be considered due to its influence on beer DMS concentration. This confirms data and knowledge already available (YANG and SCHWARZ, 1998a). The best results in term of free DMS concentration were obtained from the autumn barley samples, *Regina* and *Puffin*, probably due to their lower content of nitrogen compounds suggesting that they are suitable for beer production; among the spring varieties *Cheri* is suggested.

Some of the data reported were presented at the 1st Mass Spectrometry Food Day, 2-3 December 2009, Parma, Italy.

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REFERENCES

Analytica-EBC/European Brewery Convention 3.39 "Dimethyl Sulfide and other Lower Boiling Point Volatile Compounds in Beer by Gas Chromatography", EBC Committee: Fachverlag Hans Carl 2000 p. 219. Nurnbergh, Germany.

Anness B.J. and Bamforth C.W. 1982. Dimethyl sulphide-A review. J. Inst. Brew. 88: 244.

Baker C.D. 1988. Determination of dimethyl suplhide in beer by gas chromatography – collaborative trial. J. Inst. Brew. 95: 325.

Bamforth C.W. 1980. Dimethyl sulphoxide reductase of *Saccharomyces* spp. FEMS Microbiol. Lett. 7:55.

Bamforth C.W. 1998. "Beer: Tap into the Art and Science of Brewing." Plenum Press, New York, Usa.

Blockmans C., Meersche J., Masschelein C.A. and Devreux A. 1981. Proceedings of the European Brewery Convention Congress, p. 347. Copenhagen, Denmark.

Coghe S., Martens E., D'Hollander H., Dirinck P.J. and Delvaux F.R. 2004. Sensory and instrumental flavour analysis of wort brewed with dark specialty malts. J. Inst. Brew. 110: 94.

Drodz J., Havlová P. and Novák V. 1990. Quantitative evaluation of headspace determination of dimethyl sulphide in malt. J. Inst. Brew. 96: 69.

Duphire S. 1999. Determination of DMS and other volatile compounds in beer by headspace capillary gas chromatography and flame ionisation detection. J. Inst. Brew. 105: 265.

Eurachem Guide 1998. "The Fitness for Purpose of Analytical Methods", LGC, Teddington. LTD. Middlesex, UK.

Garza-Ulloa H. 1980. Analytical control of sulphur compounds in beer. A review. Brew. Digest. 55: 20.

Hansen J., Bruun S.V., Bech L.M. and Gjermansen C. 2002. The level of MXR1 gene expression in brewing yeast during beer fermentation is a major determinant for the concentration of dimethyl sulfide in beer. FEMS Y. Res. 2: 137.

Hertel M. and Sommer K. 2008. La bollitura del mosto. Birra e malto 97-98: 4.

Hill P.G. and Smith R.M. 2000. Determination of sulphur compounds in beer using headspace solid-phase microextraction and gas chromatographic analysis with pulsed flame photometric detection. J. Chrom. A. 872:203.

Institute of Brewing. 1982. "Recommended Methods of Analysis". p. 40. London, UK.

Kunze W. 2004. Malt production. Ch. 2. In: "Technology Brewing and Malting". Ch. 11. 3rd International Ed., VLB (Versuch-und Lehranstalt für Brauerei in Berlin), p. 97. Berlin, Germany.

Leppanen O., Denslow J. and Ronkainen P. 1979. Estimation of volatile sulphur compounds in beer. J. Inst. Brew. 85:350

Mebak Rohstoffe 3.1.4.17 Gerste-Rohfrucht-Malz-Hopfen und Hopfenprodukte-Einführung und Validierung von Analysemethoden. Freising, Germany.

Mundy A.P. 1991. The determination of dimethyl sulphide in beer by headspace gas chromatography – A collaborative investigation of precision. J. Inst. Brew. 97: 45.

Narziss L., Miedaner H. and Kattein U. 1985. Behavior of sulfur-containing aroma components during the brewing process. Monatsschr. Brauwiss., 38: 439.

Nykänen L. and Suomalinem H. 1983. "Aroma of Beer, Wine and Distilled Alcoholic Beverages". Ch.16. Akademie Verlag, p. 233. Berlin, Germany.

Van den Eynde E. 1991. The D.M.S. story from malt to beer. Cerevisiae Biotech. 16: 45.

Vas G. 1997. Characterization of beer sample, using SPME/capillary GC Analysis. Supelco, 16:7.

Walker B.D. 1991. Estimation of volatile sulphur compounds in beer. J. Inst. Brew. 98: 283.

Walker M.D. and Simpson W.J. 1992. Production of volatile sulphur compounds by ale and lager brewing strains of *Saccharomyces cerevisiae*. Lett. App. Microbiol. 16; 40.

Yang B. and Schwarz P. 1998a. Factors involved in the formation of two precursors of dimethylsulfide during malting. J. Am. Soc. Brew. Chem. 56: 85.

Yang B. and Schwarz P.B. 1998b. Application of nitrogen-purging of malt extracts to measure two dimethylsulfide precursors by headspace gas chromatography. J. Am. Soc. Brew. Chem. 56:81.

Zhao F.J., Fortune S., Barbosa V.L., McGrath S.P., Stobart R., Bilsborrow P.E., Booth E.J., Brown A. and Robson P. 2006. Effects of sulphur on yield and malting quality of barley. J. Cereal Sci. 43, 369.

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PAPER

SACCHAROMYCES HYBRIDS AS A TOOL FOR IMPROVING THE QUALITY OF MOSCATO DI SIRACUSA DOC WINE

C. RESTUCCIA*, G. MURATORE, S. MUCCILLI, C.L. RANDAZZO, C. CAGGIA, A. MAZZAGLIA, C.M. LANZA, F. LICCIARDELLO and P. GIUDICI[1]

DOFATA-Sezione Tecnologie Agroalimentari, Università degli Studi di Catania, Via Santa Sofia 98, 95123 Catania, Italy

[1]Dipartimento di Scienze Agrarie e degli Alimenti, Università degli Studi di Modena e Reggio Emilia, Via G. Amendola 2 Padiglione Besta, 42100 Reggio Emilia, Italy

*Corresponding author: Tel. +39 095 7580219, Fax +39 095 7141960, e-mail: crestu@unict.it

ABSTRACT

The study aimed to evaluate four *Saccharomyces cerevisiae* strains, one intraspecific *S. cerevisiae* hybrid, and five interspecific *S. cerevisiae*×*S. uvarum* hybrids with respect to the quality of Moscato di Siracusa DOC wine by comparing them with a commercial *Saccharomyces cerevisiae* strain.

Most of the interspecific hybrids maintained volatile acidity (VA) at very low levels, produced high concentrations of glycerol, malic and succinic acid, and yielded the highest concentration of positive sensory attributes.

On the basis of the results of these experimental fermentation trials, a real opportunity to produce special wines employing *S. cerevisiae*×*S. uvarum* hybrids is proposed.

- Key words: acidic components, intraspecific and interspecific *Saccharomyces* hybrids, Moscato di Siracusa DOC wine, sensory analysis -

INTRODUCTION

The fermentation of high-sugar grape musts, such as in the production of iced or dried grape wines, can give rise to stuck or sluggish fermentations due to the high osmotic pressure and toxicity of ethanol to yeast cells (BISSON, 1999; BISSON and BUTZKE, 2000). When the fermentation of these musts stops prematurely, wines of low quality and stability are produced due to high volatile acidity (VA) from the growth of acetic acid bacteria, heterofermentative lactic acid bacteria, non-*Saccharomyces* yeasts (CARIDI *et al.*, 1999; FLEET and HEARD, 1993), and/or *Saccharomyces cerevisiae* wine yeasts used as starter cultures (MURATORE *et al.*, 2007). The latter seems to be a major contributor of VA in high °Brix musts because previous studies demonstrated that sugar stress regulates the expression of structural genes involved in the synthesis of acetic acid from acetaldehyde (SHIMAZU and WATANABE, 1981; ATTFIELD, 1997; BAUER and PRETORIUS, 2000; ERASMUS *et al.*, 2003), and the production of VA is inversely correlated with the maximum cell concentration and the assimilable nitrogen concentration (BELY *et al.*, 2003). Concerning this problem, MURATORE *et al.* (2007) demonstrated the successful use of a *S. uvarum* strain as a starter culture in the production of Malvasia delle Lipari wine; '*uvarum*-type' strains found in the fermenting yeast biota of sweet wines, which are usually osmotolerant and psychrotolerant, are frequently able to overgrow *Saccharomyces cerevisiae* by the end of the fermentation (SIPICZKI *et al.*, 2001; NAUMOV *et al.*, 2002). As an alternative approach, a recent study of BELY *et al.* (2008) suggested the use of a mixed culture of *Torulaspora delbrueckii* and *S. cerevisiae* as the best combination for improving the analytical profile of sweet wine, particularly volatile acidity and acetaldehyde production.

However, the degree of acetic acid formation is yeast-strain dependent (REMIZE *et al.*, 1999) because different yeast strains react to the same osmotic pressure by producing different concentrations of acetic acid and glycerol. For this reason, the selection and genetic improvement of the starter yeast should consider other more specific properties in addition to the basic oenological traits for the type of wine desired.

At present, wine yeast selection is based mainly on screening wild yeast populations. However, the likelihood of identifying a strain expressing all of the optimal properties for winemaking is very low. An alternative approach to obtaining a strain with numerous oenological properties without using recombinant DNA technology is breeding. Crossing *Saccharomyces* species is considered to be a useful tool for obtaining improved wine yeast strains combining fermentative features of both parents (ROMANO *et al.*, 1985; ZAMBONELLI *et al.*, 1997; RAINIERI *et al.*, 1998; MARULLO *et al.*, 2004; 2006; GIUDICI *et al.*, 2005). Interspecific hybrids between cryotolerant *S. uvarum* and non-cryotolerant *S. cerevisiae* strains have been successfully employed in oenology because they possess higher fermentation competitiveness than the parental strains as well as characteristics of the parents in new and interesting combinations (CASTELLARI *et al.*, 1994; ZAMBONELLI *et al.*, 1997). However, a noteworthy study by SOLIERI *et al.* (2008) demonstrated that the type of mtDNA is an important trait for constructing new improved hybrids for winemaking. Hybrids with *S. uvarum* mtDNA have a higher tendency to ferment and a lower tendency to respire than those with *S. cerevisiae* mtDNA, suggesting that mtDNA type and fermentative:respiratory performance are correlated in *S. cerevisiae*×*S. uvarum* hybrids.

The aim of this study was to compare four *S. cerevisiae* yeast strains, one intraspecific *S. cerevisiae* hybrid, and five interspecific *S. cerevisiae*×*S. uvarum* hybrids with respect to the acidic component, glycerol formation, ethanol tolerance, and sensory characteristics in a very high sugar content must for the production of Moscato di Siracusa from sun-dried grapes, which is one of the most ancient wines produced in Italy. In such a high osmotic must, the practice of inoculating a *Saccharomyces* strain to carry out the vinification requires the previous selection of a suitable wine yeast strain, both for maintaining volatile acidity below the legal limit fixed by COUNCIL REGULATION (EC) No. 479/2008 and for improving overall quality (2008).

MATERIALS AND METHODS

Yeast strains and fermentation trials

Four strains of *S. cerevisiae*, one *S. cerevisiae*×*S. cerevisiae* hybrid, and five *S. cerevisiae*×*S. uvarum* hybrids bearing *S. cerevisiae* mtDNA (SOLIERI *et al.*, 2008) were used. The list of the strains and origins is reported in Table 1. The strains were cultured for 48 h at 25°C on fresh YPD medium (w/v: yeast extract 1%, peptone 2%, glucose 2%, agar 2%) before their use in fermentation experiments.

The strains' ability to perform alcoholic fermentation of special wines was tested in Muscat grape must. Moscato di Siracusa wine is produced from dried grapes in Sicily in the Siracusa area, and its production is regulated by the controlled origin wine appellation system, known in Italy as DOC (Denominazione di Origine Controllata), which was instituted in 1973 (DPR, OFFICIAL GAZETTE OF ITALIAN REPUBLIC, 1973).

Lightly dried Muscat Blanc grapes produced on sandy soil and siliceous clay in the Siracusa area and surroundings (Sicily, Italy), undamaged and without *Botrytis cinerea*, were used.

Table 1 - *Saccharomyces* strains used in this study.

Strain	Species	Origin	Characteristics
LS3	*S. cerevisiae*×*S. uvarum*	Dipartimento di Scienze Agrarie e degli Alimenti (University of Modena and Reggio Emilia, Italy)	*S. cerevisiae* mtDNA
LS6 (4003-1A×7877-10A)	*S. cerevisiae*×*S. uvarum*	"	*S. cerevisiae* mtDNA
LS7 (4003-1B×7877-10B)	*S. cerevisiae*×*S. uvarum*	"	*S. cerevisiae* mtDNA
LS8 (6167-3A×7877-9B)	*S. cerevisiae*×*S. uvarum*	"	*S. cerevisiae* mtDNA
LS9 (6167-8C×7877-6C)	*S. cerevisiae*×*S. uvarum*	"	*S. cerevisiae* mtDNA
FRENCH	*S. cerevisiae*	"	Parental A3B
16003	*S. cerevisiae*	"	Parental A3B
AL41	*S. cerevisiae*	DOFATA (University of Catania, Italy)	β-glucosidase positive strain (RESTUCCIA *et al.*, 2002)
522	*S. cerevisiae*	Davis (California)	Commercial reference strain
A3B	*S. cerevisiae*×*S. cerevisiae*	Dipartimento di Scienze Agrarie e degli Alimenti (University of Modena and Reggio Emilia, Italy)	High fermentation power

The grape berries were crushed and clarified by pressurised filtration through a sack-filter (Spagni s.n.c., Reggio Emilia, Italy); then, 1.5 L was poured into 2-L glass fermentors and inoculated in triplicate with a 48-h yeast pre-culture (5% of volume) that had been prepared in the same sterilised must. Pre-cultures were inoculated independently with the different yeasts to reach an initial population of 7 log CFU mL^{-1}; yeast population densities were estimated by direct count using a haemocytometer.

Fermentation was carried out in a conditioned room where the temperature was maintained at 18°±1°C. The fermentation was monitored by daily measuring of the weight loss associated with the liberation of carbon dioxide. Fermentation was considered complete when the weight loss was negligible.

At the end of fermentation, the wine was filtered, poured into 0.375-L glass bottles, corked, and stored at 4°C.

Analytical determinations

Ethanol content, total acidity, volatile acidity, reducing sugars, and pH were determined for the musts and wines according to the official methods of the Office International de Vigne et du Vin (OIV, 1990).

Wine colour was determined using a spectrophotometer set at 420 nm.

L-malic acid, succinic acid, and glycerol were quantified using enzymatic assay kits (K-LMALR, K-SUCC and K-GCROL, Megazyme International Ireland Ltd, Bray, Co. Wicklow, Ireland).

All analyses were performed in triplicate. The data shown are the average of all repetitions with standard deviations.

Principal component analyses (PCA) and analysis of variance (ANOVA) were performed by STATGRAPHICS® Plus version 4.0 (Manugistics, Scottsdale, AZ, USA) on chemical and physical data to ascertain significant differences among mean values.

Sensory analysis

To define the attributes of the products and to investigate the differences among the samples, the sensory profile method (ISO 13299, 2003) was used. The wines were evaluated by a panel of ten (six female and four male) trained judges experienced in wine sensory analysis and ranging in age between 20 and 30 years selected from among the Food Science and Technology Department staff members of the University of Catania.

A preliminary session was performed using several commercial Moscato di Siracusa wines to develop a common vocabulary, and this allowed the assessors to use the same terms for describing their perceptions. Descriptors with at least a 70% frequency of citation were chosen. The nineteen descriptors used included: two for appearance (yellow intensity and golden reflection), seven for aroma (fruity, exotic fruit, raisins, wood, honey, sourdough, alcohol), two for taste (sour and sweet), one for mouth feel (sharp), one for rheological properties (viscosity), and six for flavour (fruity, exotic fruit, raisins, wood, honey, sourdough). All evaluations were conducted in individual testing booths at the sensory laboratory (ISO 8589, 1988) at 20°C, asking the judges to quantify the intensity of each attribute by assigning a score between 1 (absence of perception) and 9 (extremely intense). A data collection program was used (FIZZ Software® solutions for sensory Analysis and consumer Tests, Ver.2, Biosystemes, Couternon, France). Samples were evaluated in five sessions (two by two) using 20

mL of wine in approved wine glasses (ISO 3591, 1977) labelled using a 3-digit code and covered with a plastic lid to minimise the loss of volatile compounds.

Data were statistically processed using STATGRAPHICS® Plus version 4.0 (Manugistics, Scottsdale, AZ, USA). Each attribute was analysed by one way analysis of variance (ANOVA) to verify significant differences among the samples. The significance was evaluated by means of the F test; the mean values were subjected to the multiple comparison test using the LSD procedure (least significant difference), which allows the attributes differentiating the samples to be determined. Principal component analysis was applied to the sensory data.

RESULTS AND DISCUSSION

Different yeast strains were screened for their vinification properties in must from partially dried Muscat Blanc grapes to select the most appropriate strain for this kind of product. Fermentations of high-sugar grape musts are often sluggish, taking months to reach the desired ethanol level, and they usually have high levels of volatile acidity.

The sugar content of freshly squeezed Muscat must was 319 g L^{-1}, comparable with those reported by NICOLOSI ASMUNDO *et al.* (1990) for similar grape musts (280-340 g L^{-1}). Total acidity was 7.0 g L^{-1} of tartaric acid; malic acid content was estimated at 0.5 g L^{-1}, and the pH was 3.34.

Fermentation with the different yeast strains resulted in widely variable residual sugar content. The sample inoculated with strain LS9 presented the highest sugar content (98.3 g L^{-1}), while the strains LS7, 522, and AL41 showed a strong ability to reduce the initial amount, and therefore to perform alcoholic fermentation, in musts with high sugar content (Table 2).

Except for the LS9 strain, which probably was influenced by both ethanol and sugar stressors (TROLLMO *et al.*, 1988; PIPER, 1995), ethanol content (Table 2) was about 15% vol. for all the hybrid strains, as required by the disciplinary regulations of production; among them, LS7 yielded the highest value (16.2% vol.).

In spite of the very low levels of reducing sugars at the end of the alcoholic fermentation, strains AL41 and 522 exhibited lower ethanol levels (14.3 and 14.6% vol., respectively) than the interspecific hybrids, which could be explained by the diversion of sugar metabolism into by-products other than ethanol.

Except for LS9 and LS8, which produced the highest total acidity values, the total acidity of the wine samples at the end of fermentation was largely unaffected by the identity of the yeast strain (Table 2). The ability to increase the acidic component is considered particularly favourable, especially for wines produced in warm climates such as southern Italy, because advanced ripening leads to products with very low acidity that lack the sensory characters of freshness and vivacity. In addition, acidity contributes resistance to oxidative and microbial spoilage.

VA content was below 1 g L^{-1} for most of the wine samples, except for LS9 and 16003, which produced the highest VA levels (2.06 and 1.14 g L^{-1} respectively). In particular, strains LS3, LS6, and LS7 showed a marked ability to maintain volatile acidity at very low levels (Table 2). VA has a negative effect on the quality of wines and is considered one of the principal problems for the marketability of wines produced from dried grapes. The results of the present study are particularly interesting if compared with those found by MALACRINÒ *et al.* (2005) in wines from partially dried Corvina grapes fermented with selected *S. cerevisiae* (0.81-1.24 g L^{-1}), and with those reported by MURATORE *et al.* (2007) in Malvasia delle Lipari wines fermented with *S. uvarum* strains during two consecutive vintages (0.64-0.85 g L^{-1}).

The malic acid content of the grape must was very low due to the over-ripening of the grapes. The production of malic acid during the fermentation process was variable among the *S. cerevisiae* strains, with a minimum content of 0.89 g L^{-1} and a maximum of 1.46 g L^{-1}. On the other hand, the interspecific hybrids produced higher amounts of malic acid, with LS9 producing the highest level of 2.00 g L^{-1} (Table 2).

Succinic acid is not usually present in grape must, and its origin is related to the yeast strain employed. *S. cerevisiae* strains produced the lowest concentrations, with a minimum of 0.23 g L^{-1}. The interspecific hybrids, except for LS9, showed a good ability to synthesise succinic acid as reported in the literature (ZAMBONELLI *et al.*, 1997; RAINIERI *et al.*, 1998), with a peak content of 1.20 g L^{-1} for LS8 (Table 2). This was probably due to stimulation of succinic acid production by sugar stress, as the transcription of all genes involved in the production of succinic acid is enhanced under these conditions (ERASMUS *et al.*, 2003).

Enzymatic assessment of glycerol levels showed wide variability among the strains. The lowest values were synthesised by the *S. cerevisiae* strains and the intraspecific hybrid A3B. The highest concentrations of glycerol were produced by the interspecific hybrids, as demonstrated by EUSTACE and THORTON (1987), with a maximum value of 11.63 g L^{-1} for LS8 (Table 2). This is a considerable amount if compared with what is usually formed by *S. cerevisiae* in wine, which is in the range of 4-9 g L^{-1} (RIBEREAU-GAYON *et al.*, 1972; GRAZIA *et al.*, 1995). This finding confirms a previous study that demonstrated a direct correlation between succinic acid and glycerol production (GIUDICI *et al.*, 1995). Glycerol is a non-volatile compound with no aromatic properties, but it significantly contributes to wine quality by providing sweetness and fullness

Table 2 - Chemical and physical parameters of the Moscato di Siracusa DOC wine samples.

	16003	522	A3B	AL41	FRENCH	LS3	LS6	LS7	LS8	LS9
Sugar (g L^{-1})	46.00^{c}±5.00	2.00^{e}±0.00	18.58^{d}±4.00	2.90^{e}±0.25	43.40^{c}±3.00	19.65^{d}±2.00	48.40^{c}±6.00	3.20^{e}±0.50	60.40^{b}±8.00	98.30^{a}±4.00
Ethanol (% vol.)	14.80^{ab}±0.00	14.55^{bc}±0.95	14.01^{c}±0.81	14.30^{c}±0.20	14.12^{c}±0.92	15.20^{ab}±0.50	15.85^{ab}±0.25	16.20^{a}±0.00	14.58^{bc}±0.99	12.50^{d}±0.30
Total acidity	8.50^{bc}±0.00	6.45±0.05^{e}	7.23±0.53^{de}	6.70±0.00^{e}	8.08±0.40^{c}	6.85±0.75^{e}	7.80±0.20^{cd}	7.90±0.00^{cd}	9.25±0.45^{b}	10.90±0.00^{a}
Volatile acidity (g L^{-1})	1.14^{b}±0.02	0.51^{cd}±0.13	0.60^{cd}±0.18	0.65^{cd}±0.01	0.81^{bc}±0.12	0.38^{ef}±0.15	0.44^{de}±0.06	0.25^{f}±0.02	0.88^{bc}±0.29	2.06^{a}±0.26
Malic acid (g L^{-1})	1.05^{ef}±0.04	0.89±0.01^{fg}	0.72±0.05^{g}	1.29±0.06^{cd}	1.46±0.13^{c}	1.21^{de}±0.16	1.39^{cd}±0.09	1.72^{b}±0.06	1.74^{b}±0.14	2.00^{a}±0.12
Succinic acid (g L^{-1})	0.23^{d}±0.08	0.50^{c}±0.09	0.51^{c}±0.02	0.49^{c}±0.03	0.46^{c}±0.09	0.54^{c}±0.09	0.74^{b}±0.06	1.15^{a}±0.10	1.20^{a}±0.09	0.45^{c}±0.11
Glycerol (g L^{-1})	6.71^{de}±0.70	6.61^{de}±0.61	6.37^{de}±0.93	5.71^{e}±0.65	7.90^{bc}±1.00	7.53^{cd}±0.19	9.60^{ab}±0.35	9.24^{ab}±1.05	10.39^{a}±1.05	9.47^{ab}±0.71
Colour intensity (Abs)	0.37^{c}±0.04	0.53^{b}±0.03	0.23^{d}±0.04	0.21^{d}±0.05	0.26^{d}±0.03	0.23^{d}±0.06	0.37^{c}±0.06	0.19^{d}±0.01	0.67^{a}±0.04	0.47^{b}±0.05

Means in rows followed by the same letter are not significantly different ($p \leq 0.05$).

Table 3 - Analysis of variance of sensory attributes (F values). Mean scores of the nineteen sensory attributes for the ten samples.

		LSD Samples									
Attribute	**F value**	**16003**	**522**	**A3B**	**AL41**	**FRENCH**	**LS3**	**LS6**	**LS7**	**LS8**	**LS9**
Yellow colour	10.19***	7.0b	4.9^{a}	6.9b	4.9^{a}	6.8b	4.1^{a}	6.8b	4.8^{a}	7.6b	7.7b
Golden reflection	2.47*	4.2^{abcd}	3.1^{a}	5.3^{cde}	4.2^{abcd}	4.3^{abcde}	3.3^{ab}	5.2^{bcde}	3.7^{abc}	6.1^{de}	6.2^{e}
Fruity aroma	1.11 n.s	5.1	4.7	5.4	5.0	5.2	6.0	4.3	3.4	5.1	4.7
Exotic fruit aroma	1.79 n.s.	4.5	4.1	4.6	4.8	4.8	5.8	4.6	2.7	5.5	4.4
Raisin aroma	3.36**	5.2^{b}	4.8^{b}	6.0^{bc}	4.4^{ab}	5.2^{b}	4.3^{ab}	5.3^{b}	2.8^{a}	5.7^{bc}	7.4^{c}
Wood aroma	1.25 n.s.	3.7	3.2	3.5	3.1	4.5	3.7	5.0	4.7	5.1	5.0
Honey aroma	2.20*	4.7^{bc}	3.7^{ab}	4.4^{bc}	3.9^{bc}	4.2^{bc}	3.4^{ab}	4.2^{bc}	2.2^{a}	4.4^{bc}	5.3^{c}
Sourdough aroma	2.18*	2.9^{a}	3.2^{a}	2.9^{a}	3.0^{a}	2.6^{a}	3.8^{ab}	3.9^{ab}	5.2^{b}	3.4^{a}	2.7^{a}
Alcohol aroma	0.59 n.s.	4.8	5.0	4.9	4.4	4.7	5.0	6.0	5.2	5.3	4.7
Sourness	3.11**	4.8^{abc}	6.0^{cd}	4.4^{abc}	5.4^{bcd}	4.1^{ab}	6.0^{cd}	4.5^{abc}	6.6^{d}	4.5^{abc}	3.3^{a}
Sweetness	13.03***	4.5^{b}	2.1^{a}	4.9^{bc}	2.3^{a}	5.5^{bc}	2.7^{a}	5.3^{bc}	2.4^{a}	5.9^{cd}	7.0^{d}
Sharpness	1.70 n.s.	4.6	4.9	3.8	3.8	5.1	5.3	4.0	5.4	3.7	2.7
Viscosity	3.38**	4.0^{abcd}	3.4^{ab}	3.9^{abc}	2.7^{a}	4.1^{abcd}	3.3^{a}	4.8^{bcd}	2.9^{a}	5.2^{cd}	5.4^{d}
Fruity flavour	2.22*	4.0^{bcd}	3.5^{ab}	3.7^{abc}	4.0^{bcd}	5.2^{d}	4.0^{bcd}	4.3^{bcd}	2.5^{a}	5.1^{cd}	4.6^{bcd}
Exotic fruit flavour	1.23 n.s.	4.1	3.4	3.2	4.0	4.5	3.6	4.3	2.9	4.9	3.6
Raisin flavour	4.32***	5.3^{cde}	4.3^{abcd}	4.4^{bcd}	3.4^{ab}	5.4^{cde}	3.8^{abc}	5.9^{de}	2.6^{a}	6.0^{de}	6.8^{e}
Wood flavour	1.10 n.s.	3.5	3.5	4.4	2.6	4.1	3.7	4.8	4.4	3.9	5.1
Honey flavour	5.09***	4.0^{cde}	2.9^{abc}	3.8^{cde}	3.5^{bcd}	5.0^{ef}	2.4^{ab}	4.4^{def}	2.0^{a}	4.7^{def}	5.4^{f}
Sourdough flavour	0.69 n.s.	4.1	3.1	2.9	3.2	2.9	3.6	3.2	4.4	3.2	3.0

Values for each parameter followed by different lower-case letters indicate differences according to the Student-Newman-Keuls test ($p \leq 0.05$).
*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; n.s. not significant.

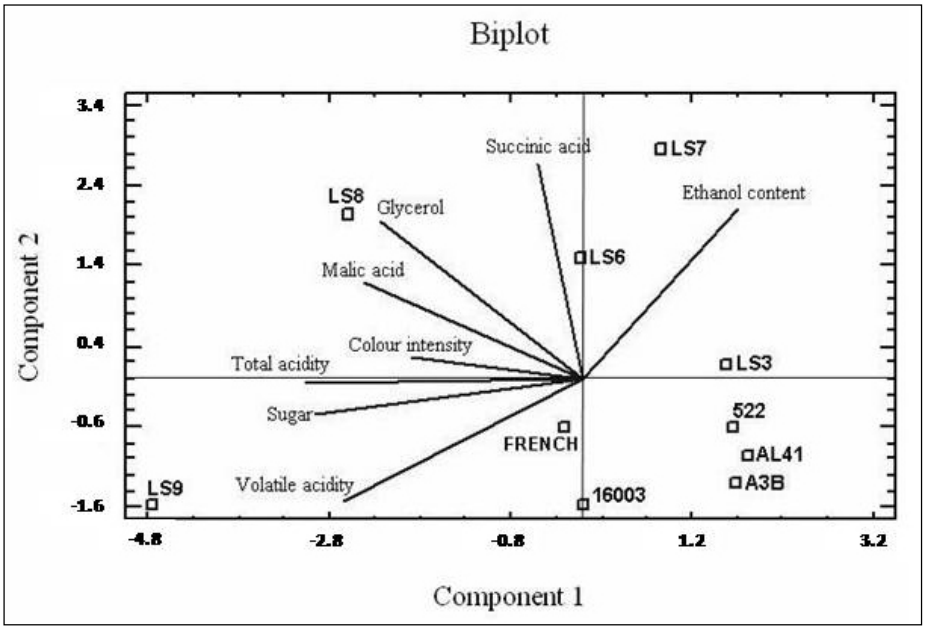


Fig. 1 - Principal component analysis of mean chemical data from the Moscato di Siracusa DOC wine samples.

(MALACRINÒ *et al.*, 2005; RIBEREAU-GAYON *et al.*, 1972). Wine yeasts generally adapt to increased osmotic stress by enhanced production of intracellular glycerol, which is the main compatible solute that counter-balances the osmotic pressure (NEVOIGT and STAHL, 1997). Due to the favourable impact of glycerol on wine quality, the benefits of increasing glycerol production to improve the sensory characteristics of wines lacking in body have been emphasised (PRETORIUS and VAN DER WESTHUIZEN, 1991; BARRE *et al.*, 1993; DEGRE, 1993; BISSON, 1996).

The absorbance values at 420 nm, indicating the influence of the yeast strains on the yellowness of the wines, are reported in Table 2. The ability of the yeast strains to delay browning has recently been investigated by several authors such as LOPEZ-TOLEDANO *et al.* (2006), who ascribed this characteristic to an inhibitory effect on the formation of coloured compounds. Spectrophotometric analysis revealed a different yellow intensity for each sample. The results of the experimental trials indicated no correlation between browning prevention and the type of yeast strain, as strains LS7, AL41, and A3B, which all showed a good ability to maintain the pale yellow colour of Moscato di Siracusa wine, are (respectively) an interspecific hybrid, a *Saccharomyces cerevisiae*, and an intraspecific hybrid.

The relationships among the chemical parameters are shown in Fig. 1, where the first two principal components explained 82.76% of the variance. Sugar, total acidity, and volatile acidity had the highest negative loading on PC1 (explained variance 53.48%), and the sample LS9 had the highest values for these parameters. Ethanol content, succinic acid, and glycerol had the highest positive loading on PC2 (explained variance 29.28%); in particular, the sample produced by the strain LS7 (unlike the other samples) was characterised by a high level of ethanol and low sugar content. Except for the interspecific hybrid LS3, all the *S. cerevisiae* strains and the intraspecific hybrid were grouped as a separate cluster, which was characterised by lower levels of acid and glycerol.

Among the 19 sensory attributes considered, only 11 (Table 3) significantly contributed to the character of the wines: yellow intensity ($p \leq 0.001$), golden reflection ($p \leq 0.05$), raisin aroma ($p \leq 0.01$), honey aroma, sourdough aroma ($p \leq 0.05$), sour aroma ($p \leq 0.01$), sweetness ($p \leq 0.001$), viscosity ($p \leq 0.01$), fruity flavour, ($p \leq 0.05$), raisin flavour, and honey flavour ($p \leq 0.001$).

The relationships among the eleven significant attributes were explicated by PCA analysis; the first two principal components explained 90.43% of the variance. As can be observed from the PCA plot (Fig. 2), the wines were distinct in terms of sensory attributes. Moving from left to right along the first component (explained variance 80.72%), the samples produced with LS3, 522, and AL41 are distinct from the others. The second component (explained variance 9.71%) distinguishes the LS7 sample (in the upper left corner) from the other wines. Raisin aroma, sweetness, raisin flavour, and honey flavour (positive loading), as well as acid (negative loading) are on PC1; sourdough aroma and golden reflection had the highest positive loading on PC2, while the attribute viscosity was equally positively loaded on PC1 and PC2.

The sample fermented with LS7 was characterised by a more intense sourdough aroma, while LS6, LS8, and LS9 provided wines with intense yellow colour and golden reflection, high

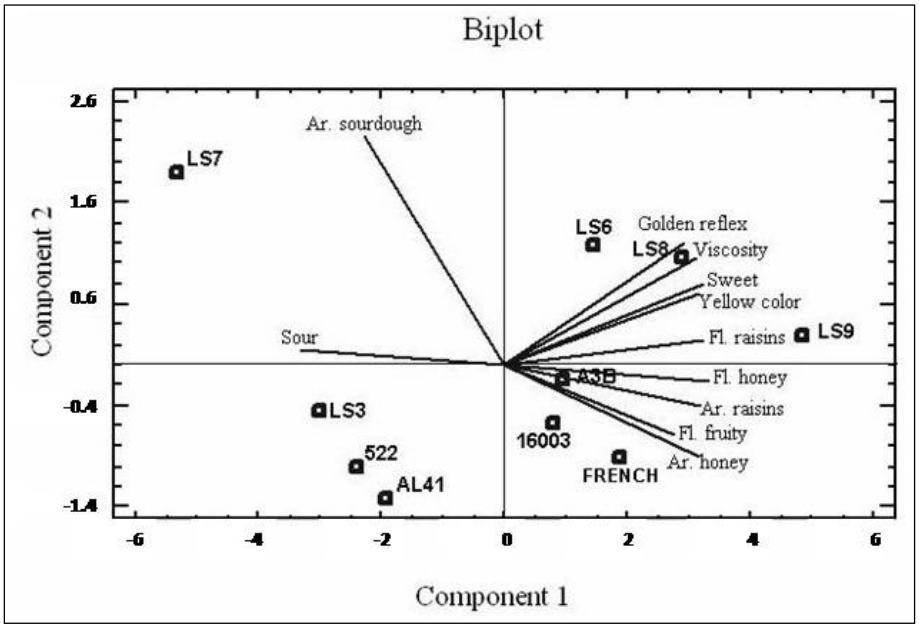


Fig. 2 - Principal component analysis of mean significant sensory attributes of the Moscato di Siracusa DOC wine samples.

viscosity and sweetness, raisin and honey flavours, and a negative correlation with undesirable attributes.

The sensory profiles of six of the wine samples selected on the basis of the previous PCA (those fermented with 522, 16003, A3B, FRENCH, LS6, and LS8) were defined by the mean values of the eleven significant attributes. Except for the sour attribute, the wine fermented with the commercial 522 strain had the least intense sensory attributes in comparison with the other five samples, while strains LS8 and LS6 showed a richness of aroma and flavour compounds in addition to intense yellow colour, golden reflection, and high viscosity. An intermediate sensory profile was found for the A3B sample.

CONCLUSIONS

To make wines of high quality, starter cultures are usually used to induce effective and rapid fermentation. Thus, strains of *S. cerevisiae* characterised with respect to their principal technological traits are normally used. However, in some cases, such as in the production of special wines from dried grapes, strains that match the specific characteristics of the particular wine should be selected. The results of the present study demonstrate that hybrids between cryo- and non-cryotolerant *Saccharomyces* strains may be technologically promising for the production of Moscato di Siracusa wine.

Their ability to perform alcoholic fermentation in grape must with high sugar content was comparable (LS8) or higher (LS3, LS6, LS7) than that of a *Saccharomyces cerevisiae* reference strain. In addition, they were able to increase total acidity, malic acid, succinic acid, and glycerol, and they strongly reduced VA.

The sensory analysis performed on the wine samples by experienced judges revealed (especially for the interspecific hybrids LS6 and LS8) a greater richness of aroma and fruity, raisin, and honey flavour compounds relative to the commercial yeast strain, in addition to intense yellow colour, golden reflection, and high viscosity; most of these attributes are reported in the production disciplinary of Moscato di Siracusa DOC as characterising this precious wine.

The PCA plot of chemical and sensory parameters revealed that the *Saccharomyces* strains employed in this study strongly differ in their chemical and sensory properties.

Among the interspecific hybrids, LS9 produced the highest VA content, while LS7 and LS3 yielded a sourdough aroma and a sour taste, respectively, that were considered to be negative traits. Thus, LS6 and LS8 provided the best fit and might be of use for the production of this quality wine.

This study proved and clearly confirmed the contribution that newly selected yeast strains can make to improve the quality of wine produced according to the most ancient traditions.

REFERENCES

Attfield P.V. 1997. Stress tolerance: the key to effective strains of industrial baker's yeast, Nat. Biotechnol. 15: 1351.

Barre P., Vezinhet F., Dequin S. and Blondin B. 1993. Genetic improvement of wine yeasts. Ch. 9. In: "Wine Microbiology and Biotechnology". G.H. Fleet (Ed.), p. 265. Harwood Academic, Chur, Switzerland.

Bauer F.F. and Pretorius I.S. 2000. Yeast stress response

and fermentation efficiency: how to survive the making of wine – a review. S. Afr. J. Enol. Vitic. 21: 27.

Bely M., Rinaldi A. and Dubourdieu D. 2003. Influence of assimilable nitrogen on volatile acidity production by *Saccharomyces cerevisiae* during high sugar fermentation. J. Biosci. Bioeng. 96: 507.

Bely M., Stoeckle P., Masneuf-Pomarède I. and Dubourdieu D. 2008. Impact of mixed *Torulaspora delbrueckii-Saccharomyces cerevisiae* culture on high-sugar fermentation. Int. J. Food Microbiol. 122: 312.

Bisson L. 1999. Stuck and sluggish fermentations, Am. J. Enol. Viticult. 50: 107.

Bisson L. and Butzke C.E. 2000. Diagnosis and rectification of stuck and sluggish fermentations. Am. J. Enol. Viticult. 51: 168.

Bisson L.F. 1996. Genetic engineering of yeast for wine production. Agro Food Industry Hi-Tech: 26.

Bonilla F., Mayen M., Merida J. and Medina M. 2001. Yeast used as fining treatment to correct browning in white wines. J. Agric. Food Chem. 49: 1928.

Caridi A., Crucitti P. and Ramondino D. 1999. Winemaking of must at high osmotic strength by thermotolerant yeast. Biotechnol. Lett. 21: 617.

Castellari L., Ferruzzi A., Magrini A., Giudici P., Passarelli P. and Zambonelli C. 1994. Unbalanced wine fermentation of cryotolerant *vs* non-cryotolerant *Saccharomyces* strains. Vitis 33: 49.

Council Regulation (EC) No 479/2008 of 29 April 2008 on the common organisation of the market in wine, amending Regulations (EC) No 1493/1999, (EC) No 1782/2003, (EC) No 1290/2005, (EC) No 3/2008 and repealing Regulations (EEC) No 2392/86 and (EC) No 1493/1999. Official Journal of the European Communities, L148/1 (2008).

Degre R. 1993. Selection and commercial cultivation of wine yeast and bacteria. Ch. 15. In: "Wine Microbiology and Biotechnology". G.H. Fleet (Ed.), p. 421. Harwood Academic, Chur, Switzerland.

DPR, Decree of the President of the Republic of 26 June 1973. Published on "Official gazette of Italian Republic of 06 December 1973, No 315.

Erasmus D.J., Van der Merwe G.K. and Van Vuuren H.J.J. 2003. Genome-wide expression analyses: metabolic adaptation of *Saccharomyces cerevisiae* to high sugar stress. FEMS Yeast Res. 2: 375.

Eustace R. and Thornton R.J. 1987. Selective hybridization of wine yeasts for higher yields of glycerol. Can. J. Microbiol. 33: 112.

Fleet G.H. and Heard G.M. 1993. Yeasts-growth during fermentation. Ch. 2. In: "Wine Microbiology and Biotechnology". G.H. Fleet (Ed.), p. 42. Harwood Academic, Chur, Switzerland.

Giudici P., Solieri L., Pulvirenti A.M. and Cassanelli S. 2005. Strategies and perspectives for genetic improvement of wine yeasts. Appl. Microbiol. Biot. 66: 622.

Giudici P., Zambonelli C., Passarelli P. and Castellari L. 1995. Improvement of wine composition with cryotolerant *Saccharomyces* strains. Am. J. Enol. Viticult. 46: 143.

Grazia L., Iorizzo M., Venditti M. and Sorrentino A. 1995. The yeasts during the ripening of the grapes. Industrie delle Bevande 24: 589.

ISO 13299 (2003). Sensory analysis - Methodology - General guidance for establishing a sensory profile. International Organization for Standardization, Geneva.

ISO 3591 (1977). Sensory analysis – Apparatus – Wine – Tasting glass. International Organization for Standardization, Geneva.

ISO 8589 (1988). Sensory analysis – general guidance for the design of test rooms. International Organization for Standardization, Geneva.

Lopez-Toledano A., Mayen M., Merida J. and Medina M. 2006. Yeasts used to delay browning in white wines. Food Chem. 97: 498.

Malacrinò P., Tosi E., Caramia G., Prisco R. and Zapparoli G. 2005. The vinification of partially dried grapes: a comparative fermentation study of *Saccharomyces cerevisiae* strains under sugar stress. Lett. Appl. Microbiol. 40: 466.

Marullo P., Bely M., Masneuf-Pomarede I., Aigle M. and Dubourdieu D. 2004. Inheritable nature of enological quantitative traits is demonstrated by meiotic segregation of industrial wine yeast strains. FEMS Yeast Res. 4: 711.

Marullo P., Bely M., Masneuf-Pomarede I., Pons M., Aigle M. and Dubourdieu D. 2006. Breeding strategies for combining fermentative qualities and reducing off-flavor production in a wine yeast model. FEMS Yeast Res. 6: 268.

Muratore G., Nicolosi Asmundo C., Lanza C.M., Caggia C., Licciardello F. and Restuccia C. 2007. Influence of *Saccharomyces uvarum* on volatile acidity, aromatic and sensory profile of Malvasia delle Lipari wine. Food Technol. Biotech. 1: 101.

Naumov G.I., Naumova E.S., Antunovics Z. and Sipiczki M. 2002. *Saccharomyces bayanus* var. *uvarum* in Tokaj wine-making of Slovakia and Hungary. Appl. Microbiol. Biot. 59: 727.

Nevoigt E. and Stahl U. 1997. Osmoregulation and glycerol metabolism in the yeast *Saccharomyces cerevisiae.* FEMS Microbiol. Rev. 21: 231.

Nicolosi Asmundo C., Cataldi Lupo M.C., Campisi S. and Russo C. 1990. La Malvasia delle Lipari: influenza delle tecnologie di produzione sui componenti volatili dell'aroma. Vignevini 4: 33.

Office International de la Vigne et du Vin (OIV). Récueil des méthodes internationales d'analyse des vins et des moûts (Collection of the international methods of analysis of wines and musts), Paris (1990).

Piper P.W. 1995. The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. FEMS Microbiol. Lett. 134: 121.

Pretorius I.S. and Van der Westhuizen T.J. 1991. The impact of yeast genetics and recombinant DNA technology on the wine industry. A Review. S. Afr. J. Enol. Vitic. 12: 3.

Rainieri S., Zambonelli C., Tini V., Castellari L. and Giudici P. 1998. The enological traits of thermotolerant *Saccharomyces* strains. Am. J. Enol. Viticult. 49: 319.

Remize F., Roustan J.L., Sablayrolles J.M., Barre P. and Dequin S. 1999. Glycerol overproduction by engineered *Saccharomyces cerevisiae* wine yeast strains leads to substantial changes in byproduct formation and to a stimulation of fermentation rate in stationary phase. Appl. Environ. Microb. 65: 143.

Restuccia C., Pulvirenti A., Caggia C. and Giudici P. 2002. A β-glucosidase positive strain of *Saccharomyces cerevisiae* isolated from grape must. Ann. Microbiol. 52: 47.

Ribereau-Gayon J., Peynaud E., Sudraud P. and Ribereau-Gayon P. 1972. In: "Traité D'oenologie. Sciences et Techniques du Vin", p. 353. Dunod, Paris, France.

Romano P., Soli G., Suzzi G., Grazia L. and Zambonelli C. 1985. Improvement of a wine *Saccharomyces cerevisiae* strain by a breeding program. Appl. Environ. Microb. 50: 1064.

Shimazu Y. and Watanabe M. 1981. Effects of yeast strains and environmental conditions on formation of organic acids in must during fermentation. J. Ferment. Technol. 59: 27.

Sipiczki M., Romano P., Lipani G., Miklos I. and Antunovics Z. 2001. Analysis of yeasts derived from natural fermentation in a Tokaj winery. Anton. Leeuw. Int. J. G. 79: 97.

Solieri L., Antúnez O., Pérez-Ortín J.E., Barrio E. and Giudici P. 2008. Mitochondrial inheritance and fermentative: oxidative balance in hybrids between *Saccharomycescerevisiae* and *Saccharomyces uvarum*. Yeast 25: 485.

Trollmo C., André L., Blomberg A. and Adler L. 1988. Physiological overlap between osmotolerance and thermotolerance in *Saccharomyces cerevisiae.* FEMS Microbiol. Lett. 56: 321.

Zambonelli C., Passarelli P., Rainieri S. and Bertolini L. 1997. Technological properties and temperature response of interspecific *Saccharomyces* hybrids. J. Sci. Food Agr. 74: 7.

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PAPER

SENSORY PROFILE OF A SPECIALTY SICILIAN CHOCOLATE

C.M. LANZA*, A. MAZZAGLIA and E. PAGLIARINI[1]
DOFATA, Sezione Tecnologie Agrolimentari, Università di Catania,
Via S. Sofia 98, 95123 Catania, Italy
[1]DISTAM, Sezione Tecnologie Alimentari, Università di Milano, Via Celoria 2, 20133 Milano, Italy
*Corresponding author: cmlanza@unict.it

ABSTRACT

In this study a sensory profile definition by a trained panel and instrumental measures determination (pH, acidity, reducing sugar content) were carried out on the Sicilian chocolate known as "Modica" that is a niche cacao product spiced (cinnamon, vanilla) so as to obtain the Protected Geographical Indication (PGI) branding for such a product. Sensory and instrumental data were also submitted to statistical analysis. The chocolate of Modica has shown a sensory profile different from other industrial products; in fact it is unique for the presence of sugar crystals and its gritty texture resulting from the manner of its production.

- Key words: chocolate, instrumental determination, sensory profile, Modica, spicy -

INTRODUCTION

Chocolate originates from Mexico where the Mayas, Incas and Aztecs cultivated the cacao tree (*Theobroma cacao*) preparing this product only on special occasions (COE and COE, 1996). While we eat chocolate the pleasure centres of our brain are activated. One typical quality of chocolate is its melt point; it is solid at ambient temperature but it melts in the mouth and it is dissolved in saliva allowing a clear final assessment of its texture.

Particle size distribution and ingredient composition of chocolate (sugar and cocoa about 70% total in a continuous fat phase) play an important role in shaping its rheological behaviour and sensory perception (AFOAKWA *et al.*, 2007).

In the past chocolate was seen only as a food of sensual pleasure with negative effects on health, however, today chocolate has been revaluated positively, thanks to greater nutritional information that discredits many fallacies. Despite high lipid and sugar content, its consumption has some beneficial effects on the human diet; cocoa is rich in antioxidants, above all polyphenols and minerals such as potassium, magnesium, copper and iron, so its intake may be useful in dietary deficiencies or may balance low levels of neurotransmitters involved in the regulation of food intake (serotonin and dopamine) (BRUINSMA, 1999). Thus it is not surprising that chocolate has always been the most commonly and intensely craved food in western cultures (WEINGARTEN and ELSTON, 1990; OSMAN and SOBAL, 2006).

Switzerland ranks first among the world's consumers of chocolate, it is a "chocolate heavy user country" followed by Belgium and Denmark; Italy only ranks 12th place with a constant growing trend, in fact, the per capita consumption passed from 3.2 kg in 1997 to 4.3 kg in 2006 (+36.4%) (BOMMEZZADRI, 2007).

In the choices of the consumer, tradition has prevailed: as for bars, milk chocolate is always the best seller, and there is a continuing rise in dark chocolate sales, while white chocolate sales are declining (BOMMEZZADRI, 2007).

Differences in the sensory characters of chocolate can be ascribed to the use of different cocoa types, i.e. flavour quality of chocolate usually depends on the origin of the cocoa beans (JINAP *et al.*, 1995), variations in ingredient proportions and in the processing methods (JACKSON, 1999) that differ in relation to national consumer preferences and producer company practices (BECKETT, 2000; WHITEFIELD, 2005). In this panorama there are niche chocolates that offer variety in sensory characteristics. For these products it is useful to define the standard of sensory identity through analyzing the characteristics of locally crafted chocolates. In this research the chocolate of Modica has been characterized.

During the Spanish domination (1516-1713), an Aztec recipe to prepare the "xocoatl", cocoa mass mixed with vanilla or cinnamon and sugar, was transferred to the inhabitants of the county of Modica (Sicily) as a gift of fidelity. The formula of this chocolate was lost and only through historical sources the original recipe has been recovered. Until 1992 the chocolate of Modica was quite unknown, it was mainly consumed at a local level with a production of a few thousand bars/year. Unlike other chocolate commercial products, this type of chocolate has often been considered as a new product (CIUFFOLETTI and CRESTI, 2004).

Today some techniques and ingredients are employed to prepare a primitive type of chocolate bar whose ancient formula may be exploited to put on the market as a new product. Winning strategies of correct visibility regarding marketing and packaging gave a new status to the product. In fact, 300.000 bars/year of production have entered into national distribution only in specialized shops. One of the greatest producers of chocolate of Modica has had a 25% growth trend in 2006 and the chocolate spiced with cinnamon/vanilla has been the most sold with a 10% growth trend (BOMMEZZADRI, 2007).

With reference to the chocolate production technology (roasting of cocoa beans, mixing, conching, and tempering) the chocolate of Modica processing method is very simple.

For the production of chocolate of Modica, the Disciplinary (2003) compiled by the Consortium of Guardianship foresees that a mass of cocoa heated to 45°C and mixed with sugar and spices (vanilla, cinnamon, chilli) without addition of emulsifiers is manually worked with stone tools at a constant temperature that does not allow the melting of sugar crystals. By subsequent cold tempering the cocoa butter consolidates and the product is ready to be formed in rectangular shape (www.cioccolatomodica.it).

Since there are no scientific publications about this product and considering that the characteristics of chocolate have been assessed by sensory and instrumental measures (GUINARD and MAZZUCCHELLI, 1999), the aim of this study is to underline the diversity of this chocolate percepted by the consumers safeguarding its identity. The memory of this chocolate was lost and only through historical sources the original recipe has been discovered. For this ancient chocolate that is produced by a number of craft-made producers, the definition of the sensory profile as well as the analytical measures of pH, titratable acidity and reducing sugar on the cinnamon and vanilla chocolate of Modica can constitute a fundamental step to identify the parameters that should be included in the PGI disciplinary (Reg. CEE, 1992). This step constitutes a winning strategy so that the memory becomes enterprise giving a new status to the product.

MATERIALS AND METHODS

Sampling

Samples analyzed were: 3 craft-made chocolates of Modica spiced with cinnamon (Bc, DAc, Sc), 5 craft-made chocolates of Modica spiced with vanilla (Bv, Ev, Cv, Rv, Sv) obtained from different producers, a craft-made chocolate of Modica without spices (BNA), a sample of dark chocolate by intermediate sensory characteristics due to muscovado sugar (D), and a commercial chocolate (N) (extra bitter Italian chocolate with 72% of cocoa), indicated as Nc and Nv in the sensory evaluation, respectively of cinnamon and vanilla chocolate of Modica. The chocolate samples were brought to room temperature (25°±0.5°C) prior to chemical and sensory analyses.

Sensory evaluation

A descriptive panel of 8 judges (5 females and 3 males, aged between 25 and 40 years) selected from students and university staff was utilized to define the sensory profile (UNI 10957, 2003). The judges were trained in 12 sessions, each approximately 1 h in duration, using both commercial and Modica craft-made chocolate, in order to develop a common vocabulary for the description of the sensory attributes of dark chocolate samples and to familiarize the panellists with scales and procedures. Each attribute term was extensively described and explained to avoid any doubt about the relevant meaning. The panel had agreed on attributes utilized including a list of reference standards for each participant (Table 1). These attributes correspond to the highest intensity score on the rating scale used, and were established according to a previous study (LANZA *et al.*, 2004).

Experimental design

To reduce perception fatigue of the judges, the working plan fixed the sensory evaluation of the three cinnamon and the five vanilla chocolate in triplicate at different times. In every session, the set submitted to judges was composed of four samples: the sample to evaluate and as reference standards chocolate of Modica without spices, chocolate with muscovado sugar and commercial chocolate.

Random samples, prepared by cutting the chocolate bar into squares were evaluated in triplicate; this was performed by assigning to every attribute a score between 1 (absence of corresponding sensation) and 9 (extremely intense) in individual booths under incandescent white lighting in the sensory laboratory of the DOFATA Department. Within each session the design was balanced for carry over effect among samples and session (PAGLIARINI, 2002).

Water at room temperature was used to rinse after sample tasting. A computerized data collection program was used (FIZZ Software Solutions for sensory Analysis and Consumer Tests, Biosystemes, Couternon, France).

pH and titratable acidity determination

The pH and titratable acidity were determined, in triplicate, as described by JINAP and DIMICK (1990): 10 g samples were pulverized in 90 mL boiling water.

The extract was filtered and the pH was measured.

Titratable acidity was determined on the extract with 0.10 M sodium hydroxide to an endpoint of pH 8.0.

Reducing sugars

The concentration of reducing sugar was determined by the Fehling titration method on the extract after the inversion of sucrose with sulphuric acid (AOAC, 1990).

Statistical analysis

The sensory data for each attribute were submitted to Analysis of the Variance (ANOVA) with samples (S), judges (J), replicates (R) and their relevant interactions J x S, S x R, J x R as effects by using SAS/STAT® statistical software package version 9.1 (SAS Institute Inc., Cary, USA). The significance of these effects was tested with the F test. The mean values were submitted to the multiple comparison test using the procedure LSD (Least Significant Difference) that enables the determination of the attributes that differentiate the samples.

Principal Component Analysis (PCA) was also applied to sensory and instrumental means data in order to interpret differences among chocolate samples using THE UNSCRAMBLER® statistical software package version 9.2 (Camo As, Trondheim, Norway).

RESULTS AND DISCUSSION

The mean values of the instrumental data are reported in Table 2. The different values of pH and titratable acidity among samples can be ascribed to the different origins of the cocoa mass utilized by the different producers. The large range in reducing sugar values for these products are indicative of the absence of a standardized process for producing chocolate of Modica.

During their training period the judges produced a list of attributes that were useful to define the sensory profile. Among the terms generated, those with a percentage of elicitation greater than 70% are included in the evaluation card: Colour uniformity, Bright and Presence of crys-

Table 1 - List of sensory attributes evaluated, their definitions and corresponding standards.

Attribute	Definition	Reference
Appearance		
Bright	Perceived color of an object indicating the relationship between reflected and absorbed light	Dark chocolate(Perugina*®)
Presence of crystals	Amount of sugar crystals present in the surface of sample (appearance)	Chocolate of Modica
Aroma		
Butter aroma	Characteristic aroma of butter perceived with the sense of smell	White chocolate (Milka®)
Cocoa	Characteristic aroma of cocoa perceived with the sense of smell	Cocoa powder (Perugina)
Cinnamon/	Characteristic aroma of cinnamon perceived with the sense of smell	Dark chocolate (Perugina) with 1% of cinnamon aroma
Vanilla	Characteristic aroma of vanilla perceived with the sense of smell	Dark chocolate (Perugina) with 1% of vanilla aroma
Chocolate	Characteristic aroma of chocolate perceived with the sense of smell	Dark chocolate (Perugina)
Taste		
Sour	One of the four basic tastes caused by aqueous solutions of acid compounds perceived on the tongue	Dark chocolate (Perugina) with 5% of citric acid
Bitter	One of the four basic tastes caused by aqueous solutions of bitter compounds perceived on the tongue	Dark chocolate (Lindt**®)
Sweet	One of the four basic tastes caused by aqueous solutions of sweet compounds perceived on the tongue	Dark chocolate (Perugina) with 10% of sucrose
Flavour		
Cocoa	Characteristic flavour of cocoa perceived with the swallowing	Cocoa powder (Perugina)
Cinnamon	Characteristic flavour of cinnamon perceived with the swallowing	Dark chocolate (Perugina) with 1% of cinnamon/vanilla aroma
Vanilla	Characteristic flavour of vanilla perceived with the swallowing	Dark chocolate (Perugina) with 1% of vanilla aroma
Chocolate	Characteristic flavour of chocolate perceived with the swallowing	Dark chocolate (Perugina)
Mouthfeel		
Astringent	Sensory perception in the oral cavity that may include drying sensation, and roughing of the oral tissue	Dark chocolate (Perugina) with 1% of tartaric acid
Texture		
Firm	Strength required to compress a substance between the molars	Chocolate of Modica
Cohesive	Degree of compression (between the teeth) obtained prior to breaking of the product	Dark chocolate (Lindt)
Adhesive	Strength required to remove product completely from palate using tongue, after compression of the sample between tongue and palate	Dark chocolate (Lindt)
Melting	A phase change in the mouth due to the increasing of temperature in oral cavity	Dark chocolate (Perugina)
Friable	Strength with which a product crumbles and flakes	Dark chocolate (Perugina) with 20% of cereal powder
Gritty	Amount of small particles perceived in the mouth when biting the sample	Dark chocolate (Perugina) with 20% of “Pavesini”® biscuits

*dark chocolate with 60% of cocoa mass; ** dark chocolate with 99% of cocoa mass.

Table 2 - Instrumental means data.

Sample	pH	Std. dev.	Tritatable acidity Meq NaOH/g	Std. dev.	Reducing sugar g/100 g	Std. dev.
N	5.70	±0.18	4.30	±0.35	28.00	±0.05
BNA	5.92	±0.04	2.73	±0.02	55.44	±3.09
D	5.51	±0.01	4.32	±0.00	40.97	±0.01
Cinnamon						
Bc	5.74	±0.02	3.03	±0.02	40.41	±0.79
Dac	6.78	±0.06	1.21	±0.12	67.11	±1.56
Sc	5.77	±0.02	2.73	±0.02	41.68	±0.24
Vanilla						
Bv	5.76	±0.08	2.81	±0.14	54.90	±0.49
Ev	6.92	±0.08	0.99	±0.08	62.17	±0.01
Cv	6.94	±0.06	1.28	±0.11	58.48	±1.38
Rv	6.97	±0.10	1.07	±0.06	64.42	±2.03
Sv	6.08	±0.00	2.12	±0.07	62.35	±1.26

tals (appearance), Butter Aroma, Cocoa Aroma, Chocolate Aroma (olfactive), Sour, Bitter, Sweet (gustative), Cocoa Flavour, Chocolate Flavour (flavour), Astringent (mouthfeel), Firm, Cohesive, Adhesive, Melting, Friable, Gritty (texture), for the cinnamon samples while for the vanilla chocolates they were classified by their vanilla aroma and flavour, however for the vanilla samples the judges did not elicit the attributes Colour uniformity, Bright, Sour, Astringent, and Friable.

The results of ANOVA for cinnamon chocolates (Table 3) showed significant differences among the samples for all the attributes with the exception of Cocoa flavour and Melting. The judge effect indicated significant differences for many attributes however the replication effect was not significant except for Cocoa aroma attribute. The interactions SxJ revealed significant differences with the exception of Colour uniformity, Bright, Sweet, Cinnamon flavour, Adhesive. The interactions S x R showed a good homogeneity of the samples during replicates, with the exception of Bright and Cinnamon aroma, and finally, the interactions J x R underlined a good reliability of the answers furnished by the judges.

ANOVA of vanilla chocolate (Table 4) showed significant differences among samples for all of the attributes, except that for Cocoa aroma and flavour and Melting. The judge effect indicated significant differences for all attributes however the replication effect was not significant except for the Presence of crystals attribute. The interactions SxJ reveal significant differences, except that for Chocolate aroma and flavour.

Table 3 - Influence of samples (6), judges (8) and replications (3) on the nineteen descriptors for cinnamon chocolate.

	F value					
Attributes	Samples	Judges	Replications	SxJ	SxR	JxR
Colour uniformity	110.45***	0.89 n.s.	1.90 n.s.	1.07 n.s.	1.23 n.s.	1.38 n.s.
Bright	97.10***	1.29 n.s.	2.26 n.s.	1.12 n.s.	1.97*	0.94 n.s.
Presence of crystals	119.25***	3.60**	0.42 n.s.	3.21***	0.27 n.s.	0.58 n.s.
Cocoa aroma	2.52*	4.37***	3.73*	1.67*	0.97 n.s.	0.50 n.s.
Chocolate aroma	15.60***	1.57 n.s.	0.05 n.s.	1.65*	0.50 n.s.	0.48 n.s.
Cinnamon aroma	109.54***	0.53 n.s.	0.42 n.s.	2.51***	2.12*	1.40 n.s.
Butter aroma	4.12**	0.93 n.s.	0.46 n.s.	2.31**	1.04 n.s.	0.76 n.s.
Sweet	28.13***	1.46 n.s.	0.44 n.s.	1.08 n.s.	0.42 n.s.	0.30 n.s.
Sour	7.30***	0.68 n.s.	0.32 n.s.	3.20***	1.44 n.s.	0.80 n.s.
Cocoa flavour	0.72 n.s.	2.18*	0.62 n.s.	1.99**	1.24 n.s.	0.43 n.s.
Chocolate flavour	14.51***	3.98***	0.73 n.s.	1.83*	1.20 n.s.	0.53 n.s.
Cinnamon flavour	76.50***	0.75 n.s.	0.36 n.s.	1.45 n.s.	1.21 n.s.	0.52 n.s.
Astringent	10.44***	2.34*	0.39 n.s.	3.64***	0.40 n.s.	1.43 n.s.
Firm	5.31***	0.90 n.s.	1.33 n.s.	2.34**	0.83 n.s.	0.62 n.s.
Cohesive	8.93***	2.68*	0.29 n.s.	1.98**	1.10 n.s.	0.52 n.s.
Adhesive	6.11***	2.00 n.s.	1.46 n.s.	1.38 n.s.	0.49 n.s.	0.72 n.s.
Melting	2.25 n.s.	2.95**	2.09 n.s.	1.82*	0.85 n.s.	0.97 n.s.
Friable	71.12***	4.02***	0.09 n.s.	1.60*	0.49 n.s.	0.37 n.s.
Gritty	112.62***	4.04***	0.20 n.s.	3.69***	0.54 n.s.	1.06 n.s.

*** significant difference for $p \leq 0.001$; ** significant difference for $p \leq 0.01$; * significant difference for $p \leq 0.05$; n.s. no significant difference.

Table 4 - Influence of samples (8), judges (8) and replications (3) on the fourteen descriptors for vanilla chocolate.

	F value					
Attributes	**Samples**	**Judges**	**Replications**	**SxJ**	**SxR**	**JxR**
Presence of crystals	131.31***	38.64***	4.98**	4.50***	20.80***	0.87 n.s.
Cocoa aroma	1.73 n.s.	5.09***	0.71 n.s.	2.72***	1.67 n.s.	1.35 n.s.
Chocolate aroma	22.32***	12.29***	1.04 n.s.	1.29 n.s.	0.80 n.s.	1.91*
Vanilla aroma	7.01***	11.09***	0.19 n.s.	2.58***	2.50**	0.85 n.s.
Butter aroma	3.14**	12.80***	1.22 n.s.	2.15***	1.44 n.s.	0.60 n.s.
Sweet	25.03***	27.60***	1.64 n.s.	2.53***	10.63***	0.61 n.s.
Cocoa flavour	1.40 n.s.	10.90***	0.03 n.s.	2.74***	0.99 n.s.	0.76 n.s.
Chocolate flavour	10.90***	10.38***	0.79 n.s.	1.40 n.s.	1.16 n.s.	1.09 n.s.
Vanilla flavour	9.75***	11.82***	1.39 n.s.	2.65***	2.48**	0.91 n.s.
Firm	2.91**	17.98***	0.83 n.s.	1.78**	0.62 n.s.	0.61 n.s.
Cohesive	3.33**	17.10***	0.06 n.s.	2.17***	0.81 n.s.	2.06*
Adhesive	5.57***	19.51***	0.88 n.s.	2.04**	1.25 n.s.	1.65 n.s.
Melting	1.40 n.s.	7.54***	0.61 n.s.	2.16***	1.92*	1.12 n.s.
Gritty	93.85***	36.90***	1.14 n.s.	5.26***	10.88***	1.14 n.s.

*** significant difference for $p \leq 0.001$; ** significant difference for $p \leq 0.01$; * significant difference for $p \leq 0.05$; n.s. no significant difference.

The interactions S x R did not show a good homogeneity of the samples during replicates, and finally, the interactions J x R underlined a good reliability of the answers furnished by the judges with the exception of Chocolate aroma and Cohesive.

The mean values of the samples' sensory data submitted to the multiple comparison test (LSD) were useful to determine what attributes differentiate the samples (Tables 5 and 6).

With respect to the cinnamon chocolate, the commercial sample (Nc) shows the highest mean score as far as Color uniformity, Bright, Butter aroma and Astringent attributes are concerned, while with respect to the vanilla samples, the commercial sample (Nv) shows the highest mean scores for Aroma and Chocolate Flavour, Butter Aroma and Adhesive attributes. Even if the samples of Modica have different rheological characteristics (Presence of crystals, Gritty

Table 5 - Mean of the score among the 19 sensory attributes for cinnamon and no spicy samples (6).

Attributes	**Bc**	**BNA**	**DAc**	**Sc**	**Dc**	**Nc**
Color uniformity	1.60^{a1}	2.54^{b}	5.10^{c}	6.85^{d}	7.33^{d}	8.42^{e}
Bright	1.58^{a}	1.95^{a}	4.85^{b}	6.29^{c}	6.46^{c}	7.43^{d}
Presence of crystals	7.90^{e}	7.42^{de}	6.54^{c}	6.98^{cd}	4.46^{b}	1.00^{a}
Cocoa aroma	4.52^{abc}	5.17^{c}	4.06^{a}	4.29^{ab}	4.88^{bc}	5.07^{bc}
Chocolate aroma	4.10^{bc}	2.83^{a}	3.46^{ab}	3.90^{bc}	4.58^{c}	6.31^{d}
Cinnamon aroma	4.83^{b}	1.00^{a}	5.71^{c}	6.13^{c}	1.00^{a}	1.00^{a}
Butter aroma	1.85^{a}	2.33^{a}	1.92^{a}	1.85^{a}	2.33^{a}	3.10^{b}
Sweet	7.06^{c}	6.42^{c}	6.79^{c}	6.44^{c}	4.25^{b}	3.28^{a}
Sour	2.42^{ab}	2.88^{b}	2.06^{a}	2.73^{ab}	4.00^{c}	3.10^{b}
Cocoa flavour	4.56	4.75	4.44	4.56	5.08	4.74
Chocolate flavour	4.08^{b}	2.79^{a}	3.92^{b}	3.88^{b}	4.46^{b}	6.11^{c}
Cinnamon flavour	4.42^{b}	1.00^{a}	5.52^{c}	6.35^{d}	1.00^{a}	1.00^{a}
Astringent	2.38^{a}	2.67^{a}	2.58^{a}	3.04^{a}	4.38^{b}	4.08^{b}
Firm	4.02^{a}	4.46^{ab}	5.40^{c}	5.50^{c}	5.42^{c}	5.07^{bc}
Cohesive	2.92^{a}	4.17^{b}	3.94^{b}	4.13^{b}	4.96^{c}	5.31^{c}
Adhesive	3.23^{a}	4.54^{c}	3.52^{ab}	4.06^{abc}	4.42^{bc}	5.57^{d}
Melting	4.77	4.92	5.40	5.42	5.29	5.83
Friable	6.86^{d}	5.63^{c}	6.90^{d}	6.71^{d}	3.96^{b}	1.39^{a}
Gritty	7.56^{d}	6.88^{c}	7.00^{cd}	6.73^{c}	4.33^{b}	1.14^{a}

[1]The values marked with different letters in the same line are significantly different ($p \leq 0.05$).

Table 6 - Mean of the score among the 14 sensory attributes for vanilla and no spicy samples (8).

Attributes	BNA	Bv	Ev	Cv	Dv	Rv	Sv	Nv
Presence of crystals	7.42^{e1}	6.84^{e}	7.33^{e}	5.44^{c}	5.04^{bc}	4.81^{b}	6.31^{d}	1.08^{a}
Cocoa aroma	5.17	5.33	4.81	4.60	5.63	5.00	4.94	4.92
Chocolate aroma	2.83^{a}	4.60^{cd}	3.71^{b}	4.33^{bc}	5.00^{d}	4.48^{cd}	4.96^{cd}	6.58^{e}
Vanilla aroma	1.00^{a}	2.35^{d}	2.02^{cd}	2.10^{cd}	1.71^{bc}	2.25^{d}	2.15^{cd}	1.50^{b}
Butter aroma	2.33^{a}	2.38^{a}	2.27^{a}	2.56^{a}	2.54^{a}	2.29^{a}	2.38^{a}	3.54^{b}
Sweet	6.42^{d}	6.10^{cd}	6.42^{d}	5.50^{b}	4.29^{a}	5.73^{bc}	6.34^{d}	3.79^{a}
Cocoa flavour	4.75	5.33	4.83	4.60	5.25	4.94	4.58	4.96
Chocolate flavour	2.79^{a}	4.58^{c}	3.65^{b}	4.17^{bc}	4.46^{c}	4.54^{c}	4.73^{c}	5.54^{d}
Vanilla flavour	1.00^{a}	2.19^{c}	1.81^{bc}	2.17^{c}	1.63^{b}	2.17^{c}	1.98^{bc}	1.13^{a}
Firm	4.46^{a}	5.54^{c}	4.81^{ab}	5.06^{abc}	5.54^{c}	5.46^{bc}	5.57^{c}	5.42^{bc}
Cohesive	4.17^{ab}	3.73^{a}	3.96^{ab}	4.34^{b}	4.13^{ab}	4.46^{b}	4.02^{ab}	5.17^{c}
Adhesive	4.54^{cd}	3.21^{a}	3.58^{ab}	4.04^{bc}	3.92^{bc}	4.19^{bc}	3.90^{abc}	5.13^{d}
Melting	4.92	5.73	5.65	5.46	5.21	5.38	5.79	5.17
Gritty	6.88^{d}	6.56^{d}	7.04^{d}	5.04^{b}	5.33^{bc}	4.83^{b}	5.65^{c}	1.04^{a}

[1]The values marked with different letters in the same line are significantly different ($p \leq 0.05$).

and Friable), they are not significantly different from commercial chocolate samples as for the intrinsic attributes of chocolate that is Melting and Cocoa flavour.

The next step regards the principal component analysis of all of the sensory and instrumental data of the cinnamon and vanilla samples in order to identify the importance of various attributes to discriminate among the samples obtaining a multidimensional space. Figures 1 and 2 report the principal component score plot and the principal component loading plot from chocolate sensory and analytical data, respectively.

The variance explained by the first two principal components was 75%. Figure 1 (score plot) shows the position of the samples in the 3 replicates. Since the distance among the replicates of the same product is very little, it can be affirmed that the results are reliable and that the judges have furnished their judgments in a consistent way. Furthermore, chocolate samples appear to be well separated in the space.

Moving left to right along the first component (explained variance 54%), commercial chocolate samples (N and D) are distinct from the Modica chocolate. The second component (explained variance 21%) distinguishes the cinnamon and not spicy samples from the vanilla chocolate.

Principal components loading were examined in order to identify the importance of various attributes in discriminating among the samples.

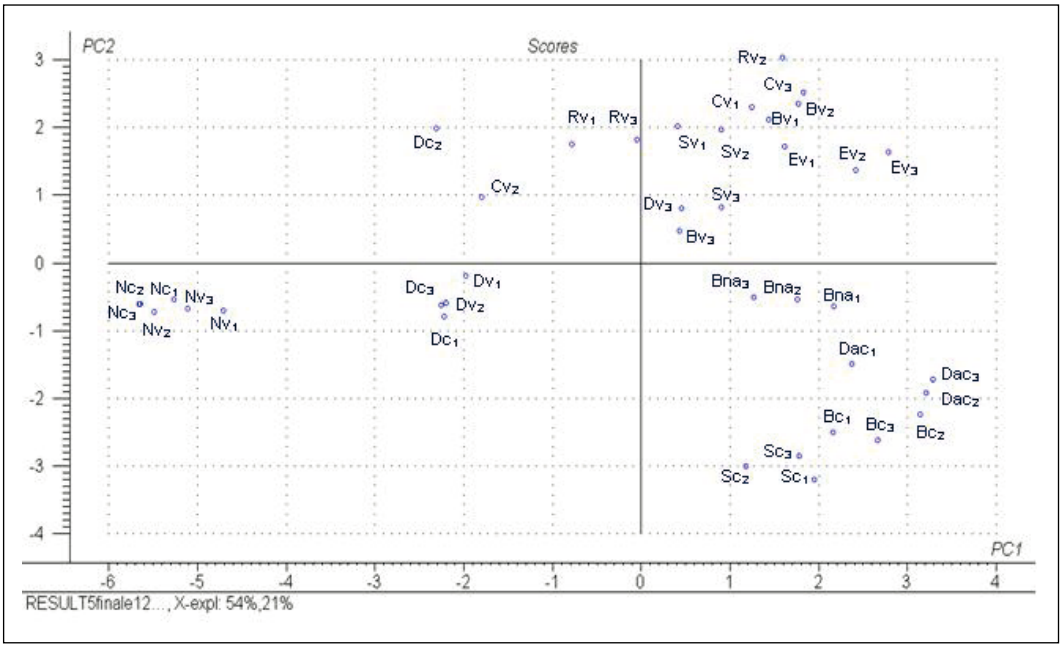


Fig. 1 - Score plot of chocolate samples.

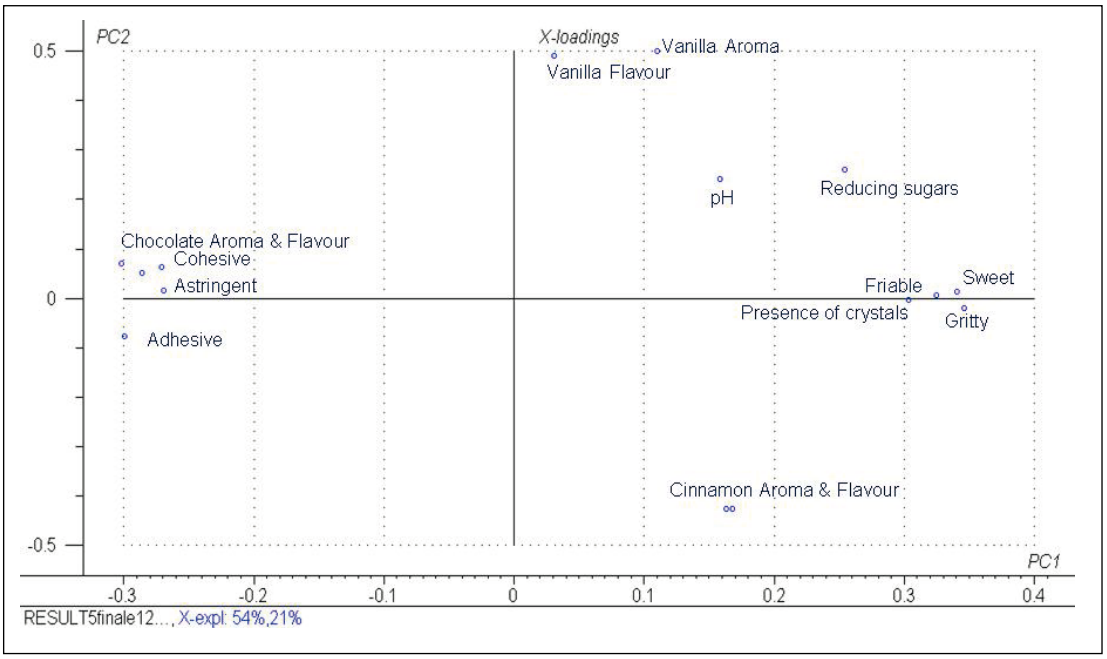


Fig. 2 - Loading plot of chocolate samples.

Figure 2 represents the plot of the sensory and physico-chemical parameters in the plane defined by the first two components.

The commercial samples (N and D) on the left of the first PC in Fig. 1 are negatively correlated to the Presence of Crystal, Friable, Gritty and Sweet, and positively correlated to Chocolate Aroma and Flavour, Cohesive and Astringent. The results from PCA show that the sensory profile of the chocolate of Modica is very different from that of the other samples. In general, the second dimension makes the difference between the chocolate of Modica and commercial products.

CONCLUSIONS

The results obtained in this research show the peculiar sensory profile of the chocolate of Modica, a fact that differentiates it from the commercial dark chocolate, and principally the chocolate of Modica's attributes of appearance (Presence of crystals), taste (Sweet), and texture (Gritty), that are a result of its production technology. Despite its diversity, the chocolate of Modica did not lose two sensory attributes that are typical characteristics of chocolate: melting and cocoa flavour.

If aroma makes the difference between the cinnamon and vanilla references, it is not clear why some attributes (Colour uniformity, Bright, Sour, Astringency, and Friable) of the cinnamon samples are not present in the sensory profile of vanilla samples. This result underlines the scarce homogeneity among the samples examined. In fact, each producer of the Association has "interpreted" the original recipe, varying the ingredients in their quantities and modifying some phases of the workmanship to shorten the production process. Therefore a follow up study is necessary to set a range of chemico-physical and sensory parameters to conform and standardize the production, so as to achieve the identification with the PGI brand. In fact, cacao and its products are still developing and there are some avant-gardists with strangest tastes who like orange, chilli, cinnamon, ginger, and even absinthe flavoured chocolate. This study made it possible to sort out the sensory dimensions on an ancient chocolate helping to get relevant and useful information to guide product development. It is nevertheless a fact that the industry and marketing must better understand the consumer sensory development in order to satisfy his needs.

REFERENCES

Afoakwa E.O., Paterson A. and Fowler M. 2007. Factors influencing rheological and textural qualities in chocolate - A review. Trends in Food Science & Technology 18:290-298.

AOAC 1990. Official Methods of Analysis of the A.O.A.C. (15th Ed.). Association of Official Analytical Chemists, Inc., Kenneth Helrich Ed., Arlington, VA.

Beckett S.T. 2000. The Science of Chocolate. London: Royal Society of Chemistry.

Bommezzadri M. 2007. Dossier Cioccolato. Food 17(10):131-168.

Bruinsma K. and Taren D.L. 1999. Chocolate: Food or drug?. Journal of the American dietetic Association 99:1249-1256.

Ciuffoletti Z. and Cresti C. 2004. Dolceamaro: storia e storie dal cacao al cioccolato. Alinari IDEA, pag. 87. Firenze, Italy.

Coe S.D. and Coe M.D. 1996. The true history of chocolate. New York: Thamer and Hudson Ltd.

Guinard J.X. and Mazzucchelli R. 1999. Effects of sugar and fat on the sensory properties of milk chocolate: descriptive analysis and instrumental measurements. Journal of the Science of Food and Agriculture 79:1331-1339.

http://www.cioccolatomodica.it

Jackson K. 1999. Recipes. In: Beckett S.T. Editor. Industrial chocolate manufacture and use. 3rd Ed. Oxford: Blackwell Science. p. 36-56.

Jinap S. and Dimick P.S. 1990. Acidic characteristics of fermented and dried cocoa beans from different countries of origin. Journal of Food Science 55:547-550.

Jinap S., Dimick P.S. and Hollender R. 1995. Flavour evaluation of chocolate formulated from cocoa beans from different countries. Food Control 6(2):105-110.

Lanza C.M., Pagliarini E., Mazzaglia A. and Laureati M. 2004. Sensory characterization of the typical chocolate of Modica. European Conference on Sensory Science of Food and Beverages "A Sense of Identity" 26-29 September, Firenze, Italy.

Osman J.L. and Sobal J. 2006. Chocolate craving in American and Spanish individuals: Biological and cultural influences. Appetite 47:290-301.

Pagliarini E. 2002. Metodi discriminanti. In: Valutazione sensoriale: aspetti teorici, pratici e metodologici. 1st Ed. Milano (Italy): Hoepli. p 59.

Reg. CEE n° 2081/92.1992. Gazzetta Ufficiale, n. L 208 del 24/07.

UNI 10957 2003 Sensory analysis – Method for establishing a sensory profile in foodstuffs and beverage. Milano: Ente Nazionale Italiano di Unificazione.

Weingarten H. and Elston D. 1990. The phenomenology of food craving. Appetite 15:231-246.

Whitefield R. 2005. Making chocolates in the factory. London, UK: Kennedy's Publications Ltd.

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PAPER

THE APPLICATION OF HACCP SYSTEM IN MEAT PRODUCTS FOR CATERING INDUSTRY A PRACTICAL GUIDE FOR PORK LOIN CHOP

A. GRAMZA-MICHALOWSKA*, B. NOWAK and J. KORCZAK
Faculty of Food Science and Nutrition, Department of Food Service and Catering,
University of Life Sciences, Wojska Polskiego 31, 60-624 Poznan, Poland
*Corresponding author: angramza@up.poznan.pl

ABSTRACT

The HACCP system is based on an assumption that the virtual hazard and irregularities in food processing may be detected before or during technological processes, so that their risk is minimalised. Preventing health hazards is a major objective of the HACCP system, which is currently considered as one of the most efficient tools available in this respect.

The aim of the present study is to elaborate selected sections of the HACCP system for meat products, to adapt and verify it in the production chain. The study involved a description of selected representative meat product – Pork Loin Chops, the production design, designation of critical control points (CCP) in the process, the hazard and the development of a hazard identification analysis sheet. A monitoring system, prevention and correction measures were also planned. The HACCP system was practically verified by testing its introduction and implementation during the production process.

- Key words: catering, critical control point, HACCP, food safety, meat products, pork loin chops -

PRACTICAL APPLICATIONS

Present investigations aimed at the minimization of contamination risk as the HACCP system are based on hazard identification during all stages of the food chain process. The system's main goal is the prevention of problems, facilitating the elimination of hazards originating from raw materials, equipment, human resources and accessories. Presented results are guidelines for modern catering industry, which may not work properly without quality control systems. The preparation of the presented HACCP system included a representative meal, starting with raw material acceptance and ending with consumption by consumers. Biological, physical and chemical hazards had been formulated and located. Quality control systems in catering business turned out to be beneficial on the condition that all actions are systematically controlled.

INTRODUCTION

Today's consumers' confidence in food safety is ensured by applied quality control systems. The Hazard Analysis Critical Control Point (HACCP) system is based on the control focused on operation points critical for food safety (CODEX ALIMENTARIUS, 2003). The other standards widely used include the Danish Standard (2002) or the new ISO 22000 Standard (2005). The main system's advantages are monitoring, preventiveness and easiness (HENSON *et al.,* 1999). Another positive aspect of the HACCP system is managing the resources, food hygiene, improved productivity and profitability and reduced production costs (WORSFOLD, 2001; WORSFOLD and GRIFFITH, 2003). On the other hand, also negative reasons why HACCP system incorrectly implemented would fail (MITCHELL, 1998). The most important of these are: no business and customers' demand, constraints on financial and human resources, and the absence of legal requirements (ENGEL, 1998; WORLD HEALTH ORGANIZATION, 1999). The first step in the implementation of food safety and quality systems along the entire food production chain is the GHP and GMP practice (CHARISIS, 2004; USDA, 2005; FDA, 2008). Good Hygiene Practice (GHP) refers to procedures that must be undertaken and hygiene conditions that must be fulfilled and monitored at all stages of production or trade in order to guarantee food safety, while Good Manufacturing Practice (GMP) is focused on actions, conditions that must be fulfilled and materials admissible for contact with food, that are properly executed, guarantying food safety for human consumption.

The accession of Poland to the European Union was a milestone for the development of food and catering industry. According to the Council Regulation (EC) No 852/2004, since 1st of January 2006 the implementation of the HACCP system is obligatory for all sectors of food business that participate in food production and distribution processes. The HACCP system was introduced and extended by the government aiming at enhancing food safety (WORLD HEALTH ORGANIZATION, 2003.). Free trade exchange within the European Union requires the unification of food legislation and quality standards within the EU. The HACCP system was primarily recommended to food exporters, then it became a required system for all food industry units as a food safety assurance tool (HEALTH & CONSUMER PROTECTION D.G., 2005.). As the living standards in Poland are improving and more foreigners visit our country every year, it has become very important to increase food product safety and thus welfare of the domestic consumer.

Catering and restaurant meals have been mainly implicated in foodborne disease outbreaks (TAYLOR, 2008; COMMUNITY SUMMARY REPORT, 2009; WIENEKE *et al.,* 1993). Main contributors to general outbreaks are *Clostridium perfringens, Salmonella, Staphylococcus aureus, Listeria monocytogenes* and *Bacillus cereus* (DeROEVER, 1999; SORIANO *et al,.* 2002). Research showed that the introduction of a HACCP system facilitated an improvement of microbiological quality of meals (BRYAN, 1990; MARTINEZ-TOME *et al.,* 2000; LITTLE *et al.,* 2002; WALKER and JONES, 2002). The application of the HACCP system also makes it possible for producers to take correct and safe actions in case of governmental inspection. The benefits of quality control systems would be manifested on the condition that all food chain actions are systematically controlled. Hazard analysis and critical control points system is applied both in basic production and at all stages of the food chain, starting with farms and finishing with consumer safety (SUN and OCKERMAN, 2005; BAS *et al.,* 2006; KNAFLEWSKA and POSPIECH, 2007).

Practically no research has been conducted in this field, especially on practical guidelines and examples of implementation of the HACCP system in Poland. Although current literature shows many aspects of HACCP and advantages and disadvantages of its implementation, there is still a limited body of literature showing the manner of applying the quality assurance system particularly in food production, e.g. grocery products (GRAMZA-MICHALOWSKA and KORCZAK, 2008; VARZAKAS and ARVANITOYANNIS, 2008).

The objective of this study is to elaborate selected aspects of the HACCP system based on a representative meat product, i.e. pork loin. Research involved a description of the selected meat product, production design, designa-

tion of critical control points (CCP) in the processes, the hazard and the identification analysis sheet. On the basis of received data selected chapters of the HACCP manual for catering meat products were created. Research also showed the complexity and precision that is required for the HACCP system for its correct adoption and satisfactory operation.

MATERIALS AND METHODS

The subject of the study is the implementation of the HACCP system in food production at a restaurant. The menu of the restaurant was checked and corrected following the actual technological process, all the HACCP system procedures were presented and adapted. Pork loin was selected as a representative of meat products, and then technological processing and its production flow diagram were elaborated, corrected and verified according to its daily application. Afterwards hazard identification, its controlling procedures and critical control points identification were evaluated. Identification of CCP was carried out using the HACCP decisive tree (CODEX ALIMENTARIUS, 2003). Subsequently parameters and inspection characteristics for critical limit assessment, CCP monitoring and controlling methods and corrective measures have been elaborated. The last step of HACCP implementation in practice was to conduct the documentation and HACCP system verification processes.

RESULTS AND DISCUSSION

The implementation of basic sanitary and HACCP system requirements allows food producers to evaluate a complete HACCP manual. The most important step is product specification, so not the sensory value, but consumer safety should be the most important aspect (Table 1). A conventional product description should include: materials, recipe, basic storage conditions (label), and expiration date or instruction for use. It is also very important to foresee all possible uses and evaluate a potential negative effect on consumers' health, including other preparation methods or a possible presence of allergens.

The next step is the elaboration of technological process design (Fig. 1). The technological process flow diagram should be drawn by technologists or cooks that know best the specific production steps. All production flow diagrams need to be verified in practice, otherwise they will never be adequate for a correct operation of HACCP systems. The verification process makes it possible to find potentially forgotten side processes and procedures that play an essential role in integral food production. This step is not concerned with any hazard analysis, but it includes only specific processes.

The most common mistakes in the evaluation of hazard analysis include confusing causes and effects, or even ignoring other hazards that might occur during the production chain. A standard procedure for the hazard analysis sheet is to create a table consisting of process stages, hazard identification, description, category (biological, physical and chemical), prevention procedures and corrective measures (Table 2). The next step is to evaluate the importance of control points with the use of a decisive tree. A series of different questions and answers leads to the determination of critical control points. According to the HACCP system regulations, the CCP is a part of procedure or step which should be controlled and where food safety hazard can be prevented, eliminated or reduced to acceptable levels. The most common critical control points in catering production are shown to be: the acceptance of materials and products, storage, heat processing or cooling, and washing of consumers' utensils. An average CCP list consists of too many points determined. Usually it is done by thinking forward (commonly called "just in case"), which is surely not a good manner of HACCP implementation. The number of marked CCPs is clearly reversely proportional to the modernity of a given catering institution and employees' knowledge. The next step of HACCP implementation is the designation of parameters for critical CCP values (Table 3). Critical values should be described as easy to measure, especially since the catering process is very fast and it could be a problem to conduct microbiological analysis of products. The monitoring system should be defined as a specific and detailed information source, concerning who and how should monitor CCPs. The possibility of losing control over a CCP (exceeding critical limits) should be easy to identify, while produced food needs to be condemned, specifically labeled, protected and separated from other food products. Such an inadequate food product should be marked and considered as an incompatible product. The verification process should be continuous with the use of audit or laboratory evaluations (e.g. levels of pathogens or chemicals), especially when the food process is modernized. The HACCP system verification is mainly based on the correct critical control point monitoring and efficient sanitary procedures. Apart from actual practical evaluations, the correctness of HACCP system documentation should also be continuously checked. When a food safety assurance system is regularly working the Quality Manual is introduced to

Table 1 - Product description "Pork loin chops".

PRODUCT'S DESCRIPTION	
PORK MEATS	
SAFETY/ PRODUCT'S CLEANNESS/ ORGANOLEPTIC AND ANALYTICAL REQUIREMENTS	
Basic requirements	
Raw material and technological processes during the production should be in agreement with obligatory Food Law Regulations and Good Manufacturing Practice (GMP) principles.	
Product's name	Recipe
"Pork loin chop"	**Quantity of the individual meal portion**: 200 g **Raw material constituents:** 100 g pork loin without bones, 20 g crumbs, 20 g margarine, 1 egg, 2 g salt, 1 g pepper. **Preparation method:** Frozen meat portions should be defrosted in microwave conditions for 3 min before the production. Egg needs to be sterilized before use. The next step is meat's smashing, spicing with salt and pepper. Then meat should be coated with egg and crumbs, afterwards is fried on hot margarine until light brown colour is obtained. Meal is finally decorated, distributed to the consumer's room, or packaged and used for the catering.
Consumer assignment	Product ready to eat for all people, except for health contraindications and infants.
Distribution to the consumer	Serving directly after preparation.
Storage conditions	Meal for direct serving, not appropriate for repeated warming.
Consumption period	No longer than 24 hours, including thermal processing and consumption time.
Organoleptic requirements	
Overall apperance	Pork loin – no deterioration signs; margarine – no colour changes; egg – typical; crumble – typical.
Consistency	Characteristic for adequate raw materials.
Colour	Characteristic for adequate raw materials.
Taste and aroma	Characteristic for adequate raw materials with no rancid aftertaste.
Physicochemical and microbiological requirements	
According to the Polish Ministry of Health decree, 30 April 2004 L.B. 120 position 1257	

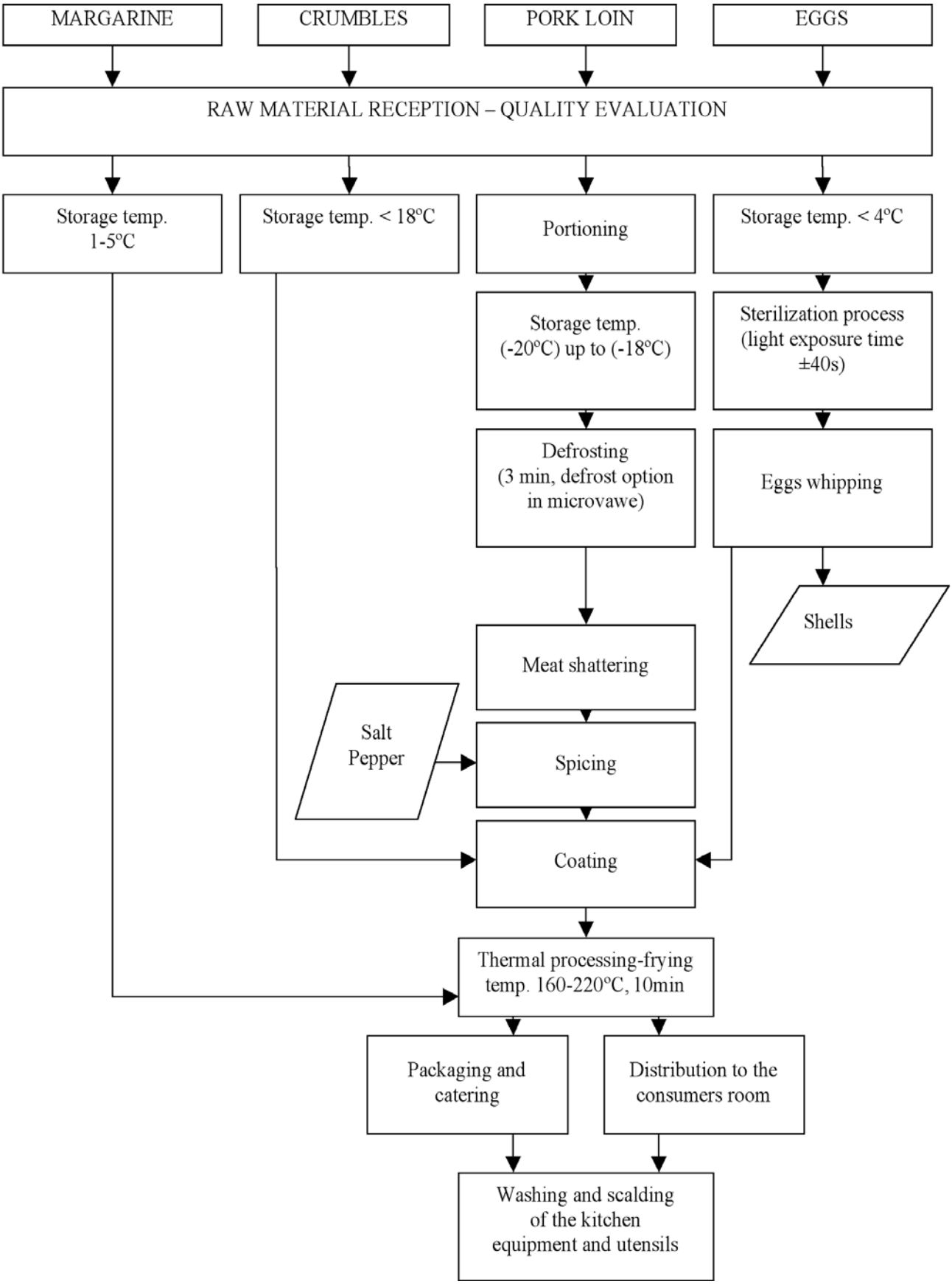


Fig. 1 - The description of technological process of "Pork loin chops".

the auditor for the system documentation approval.

CONCLUSIONS AND IMPLICATIONS

Consumer protection as the last step of the food chain is the most essential step during the HACCP quality control system evaluation. The application of the HACCP system facilitates a faster and simpler execution of adequate control measures and as a result the elimination of hazards. It promotes consumer confidence in food safety and quality.

Restaurants or catering business with the applied HACCP system proved its correct and safe operation in case of governmental inspection. Quality control systems may turn out to be beneficial on the condition that all actions are systematically controlled. The catering industry should first work on the basis of GHP and GMP systems, creating an accurate and safe environment for the correct realization of the production process. The preparation of the presented HACCP system included pork loin chop production, starting with raw material acceptance and ending with consumption by clients. The present study formulated and located biological (e.g. pathogens), physical (e.g. bones), and chemical hazards (e.g. cleaning liquid residues). For meat products two critical control points were identified. The most important is the control of storage conditions, but heat processing is crucial for meat product safety and quality. Aiming at the minimization of contamination risk the HACCP system is based on hazard identification in the technological process, during all stages of the food chain process. The system's main goal is the prevention of problems, facilitating the elimination of hazards originating from raw materials, equipment, human resources and accessories. Modern catering industry may not work properly without quality control systems following government and European Union regulations and directives. Moreover, today's conscious consumers demand high quality food products provided with meal safety assurance.

Table 2 - Hazard identification and inspection methods - "Pork loin chops".

INGREDIENT / PROCESSING STAGE	HAZARD IDENTIFICATION			PREVENTIVE MEASURES		CCP/CP
	HAZARD	DESCRIPTION	REASONS	PROCEDURES	CONTROL MEASURES	
1	2	3	4	5	6	7
RAW MATERIAL RECEPTION						
- pork loin meat	P	Bones	Ineffective bones removal process	Raw material reception & suppliers inspection procedures	Visual supplies inspection	CP
	M	Pathogenes	Inadequate transport & storage conditions	Raw material reception & suppliers inspection procedures	Deteriorated portion elimination, visual supplies and transport inspection	CP
- eggs	M	Pathogenes	Inadequate transport & storage conditions	Raw material reception & suppliers inspection procedures	Deteriorated portion elimination, visual supplies and transport inspection	CP
- margarine	M	Pathogenes & rancidity	Inadequate transport & storage conditions	Raw material reception & suppliers inspection procedures	Visual supplies and transport inspection	CP
- crumbs	P	Mould	Inadequate transport & storage conditions	Raw material reception & suppliers inspection procedures	Visual supplies and transport inspection	CP

Table 2 - Continued.

PORTIONING						
- meat	CH	Washing & disinfection resources residue	Ineffective washing process	Washing procedure	Visual inspection of equipment cleanness	-
	P	Metal inclusions	Inadequate production realization process	Initial preparing conditions procedure	Visual inspection of equipment cleanness	-
	M	Pathogenes	Inadequate production realization process	Initial preparing conditions procedure	Deteriorated portions elimination	-
STORAGE						
-pork loin meat	M	Pathogenes & rancidity	Inadequate transport and storage conditions, washing manner residues	Raw material reception & suppliers specification procedures, temp inspections	Visual supply and store portions inspection. Deteriorated portions elimination	CCP 1
- eggs	M	Pathogenes	Inadequate storage conditions, temp	Raw material reception & suppliers specification procedures, temp inspections	Visual supply and store portions inspection. Deteriorated portions elimination	CP
- margarine	M	Pathogenes & rancidity	Inadequate storage conditions	Raw material reception & suppliers specification procedures	Visual supply and store portions inspection	-
- crumbs	P	External contaminations	Inadequate transport and storage conditions, suppliers equipment contamination	Raw material reception & suppliers specification procedures	Visual supply and store portions inspection	-
STERILIZATION OF EGGS	M	Time and temp.	Inadequate sterilization conditions	Sterilization instruction	Sterilization process inspection	CP
MEAT CHOPS DEFROST	CH	Washing & disinfection resources residue	Ineffective cleaning process	Washing instruction	Completed washing process inspection	-
	M	Pathogenes	Not effective meat defrost process	Defrosting procedure	Defrosting stage inspection	CP

Table 2 - Continued.

MEAT COATING WITH EGGS	P	Egg shells & bones	Not efficient shells and bones removal process	Shells and bones removal procedure	Visual inspection	-
THERMAL PROCESSING						
- pork loin chops	CH	Process – induced chemicals	Not correct thermal processing	Thermal processing procedure	Visual inspection	-
	CH	Washing & disinfection resources residue	No efficient meat washing process	Washing procedure	Visual inspection after washing process	-
	M	Pathogenes	Not efficient thermal processing	Thermal processing procedure	Cooking time for a standardized meat thickness inspection	CCP 2
- margarine	P	Temp. & time	Not correct thermal processing	Thermal processing procedure	Visual inspection	-
MEAL'S PACKAGING	P	Package	No certifitations	Everyday package stage inspection procedure (certificate, cleanness, damage)	Removal of packages not facile with the procedure needs	CP
WASHING AND SCALDING OF THE KITCHEN EQUIPMENT AND UTENSILS	CH	Washing & disinfection resources residue	Ineffective washing & scalding process	Washing, scalding kitchen equipment & utensils procedure	Water temp inspections before washing process. Visual inspection of kitchen equipment and untensils cleanness	CP
	M	Pathogenes and viruses	Ineffective washing process	Washing, scalding kitchen equipment & utensils procedure	Water temp inspections before washing process. Visual inspection of kitchen equipment and untensils cleanness	CP
TRANSPORT	M	Suppliers equipment contamination, inefficient temp & time	No hygiene abidance, transport's contamination, inefficient transport temp in adequate time	Food products transport procedure and hot holding step	Visual inspection of transport, its technical condition, hygiene abidance, periodical medical examinations inspection	CP

CH – chemical hazard; P – physical hazard; M – microbiological hazard; CP – control points, CCP – critical control points

Table 3 - Critical limits of parameters, control, monitoring and correction characteristics for "Pork loin chops".

	Critical Control Points / Control Points	Parameters for critical value	Monitoring	Corrective actions
1.	Raw material reception			
	pork loin meat	temperature: raw material cooled, sensory at 6 - 8°C cleannes visually evaluated	temperature control of raw material visual cleanness control of transport supplier's control	delivery refusal when transport is in insanitary condition delivery refusal when product's temp. > 10°C supplier's informing about abnormalities
	eggs	cleanness visually evaluated		delivery refusal when transport is in insanitary condition supplier's informing about abnormalities
	margarine	temperature: raw material cooled, sensory at 6 - 8°C		
	crumbs	cleanness visually evaluated		
2.	Storage			
	pork loin meat	storage time below the expiry date temperature: frezer (-20°C) – (-18°C).	temperature control of freezer, at least three times daily visual control of store - rooms cleanness	daily triple temperature measurement inside irregularly working freezer raw material transport into a clean and working correctly freezer over the expiry date portion removal
	eggs	storage time below the expiry date temperature: cooler (1 - 5°C)	temperature control of cooler, at least three times daily visual control of store - rooms cleanness	daily triple temperature measurement inside irregularly working cooler raw material transport into a clean and working correctly cooler over the expiry date portion removal
3.	Defrosting			
	pork loin meat	temperature inside the product after defrosting (2 - 6°C) time from defrosting to thermal treatment – 24 h.	defrosting temperature and time control cleanness visual evaluation of processing room and utensils	preserving the appropriate defrost conditions (cooler defrosting, use of defrost water retaining tools, defrost products should not be frozen again) keeping sanitary clean tools used for defrosting
4.	Thermal processing			
	pork loin meat	temperature and processing time: ▪ frying (160 – 220°C, 10 min.) ▪ temperature inside of product min. 75°C, >2min.	temperature of frying process (inside the product) and processing time control	processing time extension for designed parameters obtaining – temp. inside of product min. 75 °C
5.	Washing and scalding of consumer utensils			
	Consumer utensils and dishes	temperature and processing time: ▪ washing and sterilizing (60 – 90°C), according to equipment's procedures.	temperature of washing and sterling process and processing time control	processing time extension for designer parameters obtaining temp. inside of cleaning chamber ± 90°C

REFERENCES

Bas M., Ersum A.S. and Kivanc G. 2006. Implementation of HACCP and prerequisite programs in food businesses in Turkey. Food Control, 17(2): 118-126.

Bryan F.L. 1990. HACCP systems for retail food and restaurant operations. Journal of Food Protection 53, 978-983.

Charisis N. 2004. Hazard Analysis & Critical Control Point Systems. Concepts and Applications. Draft. MZCC, Athens, Greece 2004; http://www.mzcp-zoonoses.gr/pdf/HACCPBook.pdf-

Codex Alimentarius Commission. Food Hygiene Basic Texts 2003. CAC/RCP – 1969, Rev. 4 – 2003, FAO/WHO.

Community Summary Report 2009. Food-borne outbreaks in the European Union in 2007. The EFSA Journal, 271; http://www.efsa.europa.eu/en/scdocs/doc/271r.pdf.

Danish Standards Association, 2002. Management of food safety based on HACCP (Hazard Analysis and Critical Control Points) – Requirements for a management system for food producing organizations and their suppliers.

Deroever C. 1999. Microbiological safety evaluations and recommendations on fresh produce. Food Control 10: 117-143.

Engel D. 1998. Teaching HACCP – Theory and practice from the trainer's point of view. Food Control 9(2-3): 137-139.

FDA. Managing Food Safety: A Manual for the Voluntary Use of HACCP Principles for Operators of Food Service and Retail Establishments. 2008; http://www.fda.gov/downloads/Food/FoodSafety/RetailFoodProtection/ManagingFoodSafetyHACCPPrinciples/Operators/UCM077957.pdf

Gramza-Michalowska A. and Korczak J. 2008. Vegetable products as HACCP system subject in modern gastronomy. Acta Scientiarum Polonorum, Technologia Alimentaria 7(3): 47-53.

Health & Consumer Protection D.G. 2005. Guidance document on the implementation of procedures based on the HACCP principles, and on the facilitation of the implementation of the HACCP principles in certain food businesses. Brussels. http://ec.europa.eu/food/food/biosafety/hygienelegislation/guidance_doc_haccp_en.pdf.

Henson S., Holt G. and Northen J. 1999. Costs and benefits of implementing HACCP in the UK dairy processing sector. Food Control 10: 99-106.

ISO 22000:2005. Food Safety Management Systems – Requirements for any organization in the food chain.

Knaflewska J. and Pospiech E. 2007. Quality assurance systems in food industry and health security of food. Acta Scientiarum Polonorum, Technologia Alimentaria 6(2): 75-85.

Little C.L., Barnes J. and Mitchell R.T. 2002. Microbiological quality of take-away cooked rice and chicken sandwiches: effectiveness of food hygiene training of the management. Communicable Disease and Public Health 5: 289-294.

Martinez-Tome M., Vera A.M. and Antonia Murcia M. 2000. Improving the control of food production in catering establishments with particular reference to the safety of salads. Food Control 11: 437-445.

Mitchell R.T. 1998. Why HACCP fails. Food Control 9(2-3): 101.

Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs 2004, Corrected version in Official Journal of the European Union L 226 of 25 June 2004.

Soriano J.M., Rico H., Molto J.C. and Manes J. 2002. Effect of introduction of HACCP on the microbiological quality of some restaurant meals. Food Control 13: 253-261.

Sun Y.M. and Ockerman H.W. 2005. A review of the needs and current applications of Hazard Analysis and Critical Control Point (HACCP) system in foodservice areas. Food Control 16: 325-332.

Taylor E. 2008. A new method of HACCP for the catering and food service industry. Food Control 19 (2): 126-134.

USDA, Food and Nutrition Service & National Food Service Management Institute. - HACCP based standard operating procedures (SOPs). The University of Mississippi, 2005; http://sop.nfsmi.org/HACCPBasedSOPs/HACCPBasedSOPs.pdf.

Varzakas T.H. and Arvanitoyannis I.S. 2008. Application of ISO 22000 and comparison to HACCP for processing of ready to eat vegetables: part 1. International Journal of Food Science and Technology 43: 1729-1741.

Walker E. and Jones J. 2002. An assessment of the value of documenting food safety in small and less developed catering businesses. Food Control 13: 307-314.

Wieneke A.A., Roberts D. and Gilbert R.J. 1993. Staphylococcal food poisoning in the United Kingdom, 1969-90. Epidemiology Infection 110: 519-531.

World Health Organization, 1999. Strategies for of implementing HACCP in small land/or less developed businesses. A report of a WHO consultation in collaboration with the Ministry of Health, Welfare and Sports, the Netherlands.

World Health Organization, 2003. Recommended International Code of Practice - General Principles of Food Hygiene CAC/RCP 1-1969, Rev. 4.

Worsfold D. 2001. A guide to HACCP and function catering. Journal of the Royal Society for the Promotion of Health 121: 224-229.

Worsfold D. and Griffith C.J. 2003. A survey of food hygiene and safety training in the retail and catering industry. Nutrition and Food Science 33: 68-79.

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PAPER

EVALUATION OF ANTIOXIDANT EFFICIENCY OF POTATO AND ORANGE PEEL AND APPLE POMACE EXTRACTS IN SUNFLOWER OIL

H. YALCIN*, S. KARAMAN and I. OZTURK
Erciyes University, Food Engineering Department, 38039 Kayseri, Turkey
*Corresponding author: Tel. (90 352) 4374901/32731, Fax (90 352) 4375784,
e-mail: hsnyalcin@hotmail.com

ABSTRACT

In this study, potato peel (PPE), orange peel (OPE) and apple pomace (APE) ethanolic extracts were used as natural antioxidants in sunflower oil and their antioxidant activity was evaluated during 45 days storage at 40°C. The peroxide value (POV), free fatty acid (FFA) and iodine values (IV) of oil samples were determined and the results were compared to the control sample and butylated hydroxytoluene (BHT), a synthetic antioxidant. After storage sunflower oil containing 1,500 ppm APE showed lower POVs (123.85 meqO_2/kg oil) among the natural extracts than the control sample (140.82 meqO_2/kg oil). The lowest POV was observed in the sample containing BHT (93.11 meqO_2/kg oil). The FFA values of the control sample were determined in the range of 0.16-0.28% during storage but samples containing natural extracts showed lower FFA values. The IVs of samples decreased with increasing storage time because of oxidation. The addition of natural extract inhibited the decrease in IV. These results showed that PPE, OPE and APEs have strong antioxidant activity and can be used as a natural antioxidant agent for the inhibition of deteriorative oxidation in vegetable oils or other food products.

- Key words: potato peel, orange peel, apple pomace, ethanolic extract, antioxidant, sunflower oil -

INTRODUCTION

One of the principal reasons for edible oils, quality deterioration is lipid peroxidation (GORDON, 1991). Oxidative reactions occur in food because of O_2 caused lipid peroxidation and because of this loss of nutritional value, flavor, aroma and texture degradation may develop. In addition, high levels of oxidation can cause health problems like tumors, heart failure, cataract and brain dysfunction by means of acting cytotoxically (MANIAK and TARGONSKI, 1996). Edible vegetable oils are a popular and beneficial food consumed by humans in their daily diet because of cholesterol lowering effects; however, their preservation is rather difficult. They are rather susceptible to oxidation and easily deteriorate when they react with air oxygen (MATALGYTO and ALKHALIFA, 1998). The addition of antioxidants to food formulations is a popular, effective method to prevent oxidative reaction in food and increases the shelf life of lipids, especially oils, and lipid containing foods (HALLIWELL *et al.*, 1995). In the food industry, synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butyl hydroquinone (TBHQ) are commonly used to prevent oxidation in food formulation. However, there are some questions about the use of synthetic antioxidants because of issues related to their toxicity and carcinogenicity (ITO *et al.*, 1985). REHMAN *et al.* (2004) reported that synthetic antioxidants may cause liver swelling and influence liver enzyme activity. For these reasons, there is an increased demand for natural antioxidants due to consumer concerns about the safety of synthetic antioxidants; there is also a need for natural antioxidants to prevent the deterioration of food, especially oils, because of the health effects. There are many scientific studies which report that natural antioxidants obtained from natural sources have strong antioxidant activity (GAMEZ-MEZA *et al.*, 2009). In addition, extracts obtained from spices, herbs and hulls by different methods of solvent extraction have varying degrees of antioxidant activity. Moreover, some of these natural extracts, generally obtained from natural organic substances, have greater antioxidant activity than some synthetic antioxidants (REHMAN *et al.,* 2004).

Fruits are a good source of phenolic compounds (EBERHARDT *et al.*, 2000). It is known that the concentration of total phenolic compounds is much greater in the peel of fruits than in the flesh (ESCARPA and GONZALEZ, 1998; LI *et al.*, 2006; GORINSTEIN *et al.*, 2001). The structure and distribution of these phytochemicals between the flesh and the peel of the fruit is also different. The peel possesses additional flavonoids not found in the flesh (VAN DER SLUIS *et al.*, 2001). It has been noted that in all fruits and vegetables peels have high phytochemical concentrations or high antioxidant activity (TALCOTT *et al.*, 2000a; GIL *et al.*, 2000; TALCOTT *et al.*, 2000b). This suggests that the peels alone would be even more effective (WOLFE *et al.*, 2003). In the food industry, large amounts of byproducts, such as skin, peel, seed and pomace, are generated during food processing (BOCCO *et al.*, 1998). The fruit peels are typically used for unimportant purposes. They are often pressed to make fruit juice or vinegar, pressed into a cake for livestock feed, or used as fertilizer. Sometimes they are used as a source of pectin (WOLFE *et al.*, 2003). It seems, therefore, that fruit peels are a rich source of natural antioxidants and worthy of further study (LI *et al.*, 2006). For that reason, the extracts obtained from waste materials containing different antioxidant compounds can be used to prevent oxidative deterioration in food, especially edible oils, and also for inhibiting oxidative damage in living systems by scavenging oxygen free radicals (MAKRIS *et al.*, 2007). Apple pomace, orange peel and potato peel are waste materials obtained from the food processing industry and many studies have shown that they are a valuable source of natural antioxidants and bioactive compounds. Due to their having different antioxidant compounds, it was determined in earlier studies that apple pomace (LU and FOO, 2000; ĆETKOVIĆ *et al.*, 2008; SUÁREZ *et al.*, 2010), orange peel (JOHN, 2004; ANAGNOSTOPOULOU *et al.*, 2006; CHERIF *et al.*, 2006; LONDOŃO-LONDOŃO *et al.*, 2010) and potato peel (REHMAN *et al.*, 2004; SINGH and RAJINI 2004; KANATT *et al.*, 2005; SEVCIK *et al.*, 2009; MOHDALY *et al.*, 2010) extracts showed strong antioxidant or antiradical activity.

Although it is known that these byproducts have more antioxidative activity, there are very few studies on the use of these waste materials for increasing the stabilization of edible oils. The aim of the present study was to evaluate the antioxidant efficiency of PPE, OPE and APEs as natural antioxidants in sunflower oil in view of the fact that they are rich sources of phenolic compounds with antioxidant capacity.

MATERIALS AND METHODS

Materials

Refined, bleached and additive-free sunflower oil was purchased from the Zade vegetable oil factory (Konya-Turkey). Apple pomaces were supplied from Gülsan-Meysu Juice Co. (Kayseri-Turkey). Orange and potato peels were obtained from the student cafeterias at Erciyes University. Butylated hydroxytoluene (BHT), a synthetic antioxidant, was purchased from Merck (Darmstadt, Germany).

Preparation of natural extracts

The apple pomace and orange and potato peels (washed) were dried at 45°C for 48 hours in a hot

air oven (Nuve FN 120, Turkey) and ground to a fine powder by a grinder (Waring Blender, USA). A powdered sample (10 g) was then extracted with 100 mL ethanol (80%) (Merck-Darmstadt, Germany) at 30°C for 2 h in an ultrasonic water bath (Bandalin Sonore RK100H, Germany). The extract was filtered and the residue was re-extracted under the same conditions. After that, all of the combined extracts were concentrated by using a rotary evaporator (Buchi, R-200, Switzerland) under vacuum at 50°C to obtain the crude extracts.

Incorporation of natural extracts into sunflower oil

The sunflower oil without extracts and those containing 1,500 ppm APE, OPE or PPE were separately prepared. Each 100 mL prepared sample was placed into an open beaker and for a comparative study, a sample containing BHT at its legal limit of 200 ppm was prepared. Afterwards, prepared oil samples were stored at 40°C in a hot air oven (Nuve EN 120, Turkey) exposed to constant sunlight and air during storage for 45 days. Analyses of the samples were then carried out periodically at 15-day intervals.

Antioxidant efficiency test procedures

Peroxide value (POV)

The POVs of the samples were determined according to the procedure described by the AOAC (2000). For this purpose approximately 5 g oil were weighed into an erlenmeyer flask (250 mL) and dissolved in 10 mL chloroform. Then, 15 mL acetic acid and 1 mL saturated potassium iodine (KI) were added and mixed. The mixture was left in the dark at room temperature for 5 min. Distilled water (75 mL) was then added and the mixture was shaken vigorously. 1 mL prepared starch solution (1%) was added and the resulting solution titrated with 0.01 N sodium thiosulphate ($Na_2S_2O_3$) until the color became clear. The peroxide value was calculated using the following formula:

$$POV = [(V_1 - V_0)\ N]/M$$

where V_1 is the amount of $Na_2S_2O_3$ used for titration (mL), V_0 is the amount of $Na_2S_2O_3$ used for the blank (mL), N is the normality of $Na_2S_2O_3$ and M is the weight of the sample (g). POV was expressed as milliequivalents (meq) of active oxygen per kg of oil.

Free fatty acids (FFA)

The FFA values of the samples were determined according to the procedure described by the AOAC (2000). For this purpose, a range of 5-10 g oil was weighed into an erlenmeyer flask and dissolved in 100 mL ethanol:diethylether (1:1 v/v). Next, a few drops of phenol-phthalein (1%) were added and the resulting solution titrated with 0.1 N sodium hydroxide (in ethanol prepared NaOH) until the color became pink. The free fatty acid value was calculated using the following formula:

$$FFA\% = [(V x 2.82]/M$$

where V=titration volume of NAOH (mL); 2.82: gram molecule of oleic acid; M=weight of sample (g). FFA% was expressed as oleic acid.

Iodine value (IV)

The IVs of the samples were determined according to the procedure described by the AOAC (2000). Approximately 0.2 g oil was weighed into an erlenmeyer flask (500 mL) and dissolved in 15 mL cyclohexane-acetic acid (1:1 v/v). Then, 25 mL Wijs solution (Merck, Germany) was added and the mixture was left in the dark at room temperature for 60 min. Next, 20 mL KI solution and distilled water (150 mL) were added respectively. 1 mL prepared starch solution (1%) was added and the resulting solution titrated with 0.1 N sodium thiosulphate ($Na_2S_2O_3$) until the color became clear. The iodine value was calculated using the following formula:

$$IV = [(V_1 - V_2)\ x N x 1.269]/M$$

where V_1=titration volume of $Na_2S_2O_3$ for blank (mL); V_2=titration volume of $Na_2S_2O_3$ for sample (mL); N=normality of $Na_2S_2O_3$ solution; M=weight of sample (g)

Statistical analysis

Statistical analyses were conducted using the Statistical Analysis System (SAS) (SAS, 1988). The effect of extract addition and storage period and their interactive effect on POV, FFA and IVs were determined using a two-way Analysis of Variance (ANOVA) with the general linear model (GLM) procedure.

RESULTS AND DISCUSSION

The POVs of sunflower oil samples containing different antioxidant agents are illustrated in Fig. 1. As shown in figure, the POV of the control sample increased rapidly during storage at 40°C. It was determined as 2.19 meqO_2/kg oil at day 0^{th}, but because of intense light and oxygen exposure during storage, oxidation was accelerated and peroxide values increased to 140.82 meqO_2/kg oil on the 45^{th} day. Exposure to air (ISABEI and MARIANO, 2001; KRISTINA and HANS, 1998), heat (MATALGYTO and ALKHALIFA, 1998; WITTING *et al.*, 1999) and light (XIAOYING and AHN, 1998) accelerated the oxidation process. The addition of different ex-

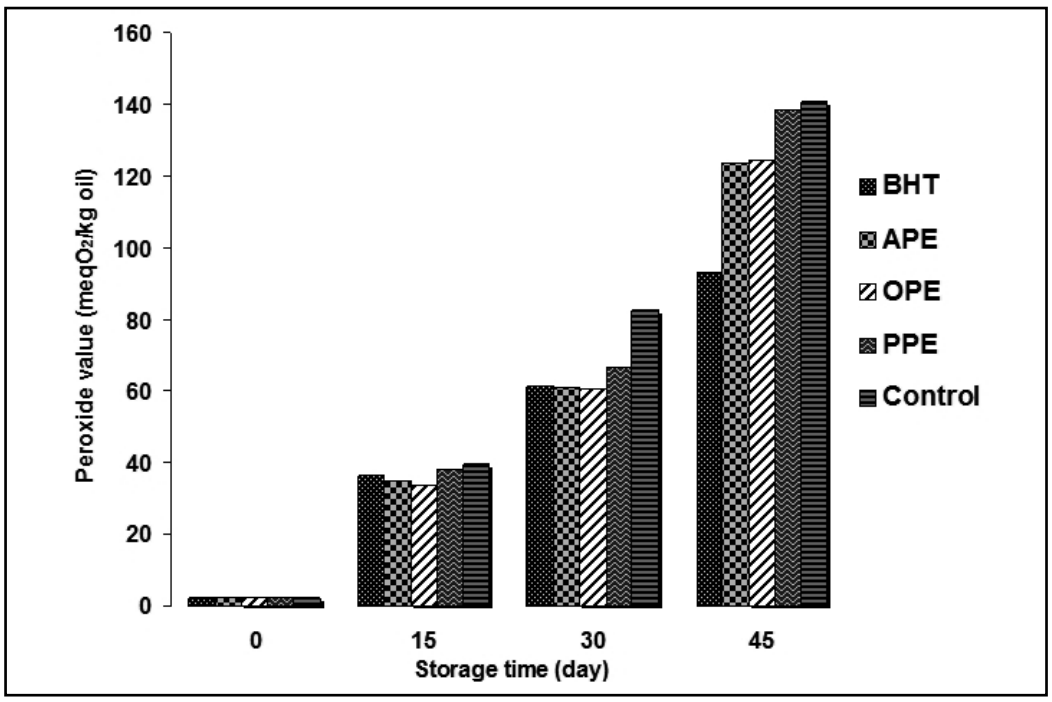


Fig. 1 - Peroxide values of samples.
BHT: Butylated hydroxytoluene; APE: Apple Pomace Extract; OPE: Orange Peel Extract; PPE: Potato Peel Extract.

tracts as natural antioxidants provided a significant reduction in the POV and FFA values of sunflower oil during 45 days storage at 40°C. As can be seen from Fig. 1, the addition of extracts to the oil provided stability against oxidative reactions in the sunflower oil. In general, natural extracts slightly inhibited peroxide formation compared to the control sample and the POV decreased in the sample containing natural extract. After 45 days storage at 40°C, the POV of the samples with PPE, OPE and APE were 138.27, 124.79 and 123.85 meqO_2/kg oil, respectively. The POVs of the sunflower oil samples with extracts were found to be lower compared to the control sample after 45 days storage. The reduction of POV in the sunflower oil samples treated with different natural extracts during storage was found to be statistically significant ($p<0.01$). In addition, the effect of storage period, extract type and their combined effect were found to be statistically significant ($p<0.01$). The POV of samples at different storage periods was statistically different ($p<0.01$) but in treatment with a natural extract, there was no different antioxidant effect between OPE and APE during the storage period ($p<0.01$). It can be said that OPE and APE were generally the most effective extracts against oxidation compared to the other natural extracts. Different phenolic compounds are responsible for the antioxidant properties of APE and OPE. Hesperidin is an abundant phenolic compound found in citrus peels and has strong antioxidant activity. Moreover, it improves vascular integrity and decreases capillary permeability. It has been reported that hesperidin can inhibit copper-induced low density lipoprotein (LDL) oxidation (VALENSI *et al.*, 1996; CIRICO and OMAYE, 2006; LONDOŃO-LONDOŃO *et al.*, 2010). There are many important bioavailable phenolic compounds such as flavonols (e.g., quercetin, hyperin, monomeric and oligomeric flavanols), dihydrochalcones (e.g., phloridzin), anthocyanidins, p-hydroxycinnamic (e.g., caffeic, chlorogenic acid) and p-hydroxybenzoic acids in apple pomace (ESCARPA and GONZALEZ, 1998; ĆETKOVIĆ *et al.*, 2008; SUÁREZ *et al.*, 2010) The POVs of samples treated with OPE were 33.65 and 60.43 meqO_2/kg oil on the 15th and 30th storage day while they were 39.65 and 82.56, 35.03 and 61.08, 38.24 and 66.84 meqO_2/kg oil for the control sample and samples treated with APE and PPE, respectively. BHT, a synthetic antioxidant used in the food industry, was found to be more effective compared to all the other extracts used as antioxidants in sunflower oil. The reduction in POV was more pronounced in samples treated with BHT. The POV of the sample with added BHT was 93.11 meqO_2/kg oil at the end of the storage period while the control sample's POV was 140.82 meqO_2/kg oil. REHMAN *et al.* (2004) studied the use of the petroleum ether extract of potato peel in soybean oil and compared the efficiency of this natural extract with BHT and BHA. They found that after 45 days storage, the POV of the soybean oil sample treated with BHT at 45°C was 4.80 meqO_2/kg oil while the POVs of soybean oil treated with PPEs at different concentrations were 23.0, 7.0 and 6.70 meqO_2/kg oil for samples containing 800, 1,600 and 2,400 ppm natural extract, respectively. As seen from the reported results, synthetic antioxidants such as

BHT and BHA are generally more effective than natural antioxidants, but the concentrations of natural extracts are important. The concentrations of synthetic antioxidants should not exceed 200 ppm. If the concentrations of natural antioxidants used in refined oils are increased, the efficiency of natural antioxidants would be higher than synthetic antioxidants. In another study carried out by the same author, the antioxidant efficiency of BHT was more prominent than that of citrus peel extract. The POV of BHT at 45°C after two months storage was 4.3 meqO_2/kg oil while the POV of corn oil samples containing 1200 ppm citrus peel extract was 5 meqO_2/kg oil at the same conditions. However, increasing the citrus peel extract concentration in oil decreased the POV of the oil. It was 3.5 and 3.0 meqO_2/kg oil in samples containing 1,600 and 2,000 ppm citrus peel extract, respectively (REHMAN, 2006). In the present study, our samples were exposed to sun light and oxygen. Therefore our results were found to be greater than those of other studies. In one study, the POV of additive-free sunflower oil was reported as 0.5 meqO_2/kg oil, but after 6 months of storage at 45°C, the POV increased to 198 meqO_2/kg oil (REHMAN *et al.*, 2003). In another study carried out by the same researchers, the POV of the soybean oil control sample was 0.32 meqO_2/kg oil, but after 60 days storage at 45°C, the POV values of the sample increased to 79 meqO_2/kg oil (REHMAN *et al.*, 2004).

The FFA values of all samples can be seen in Fig. 2. As illustrated in the figure, the FFA values of the control sample increased a little. Initially the FFA value of the control sample was 0.16%, and it increased slowly and reached 0.28% at the end of the storage period (Fig. 2). The differences among the FFA values of the control sample at different storage periods was statistically significant ($p<0.05$). Also, the effect of extract type and its combined effect with storage period were found to be statistically significant ($p<0.01$) on FFA values. The FFA values of samples treated with natural antioxidants were found to be lower than the control sample's FFA content. Using natural antioxidants reduced the formation of FFA during the storage period compared to the control sample. It was found that the FFA value of the control sample was 0.28% after 45 days storage. The FFA values of samples containing APE, OPE and PPE were 0.26, 0.23 and 0.23%, respectively. The reduction in FFA in different samples was statistically significant ($p<0.05$) on the last storage period. However, there was no significant difference in the FFA values of all samples at the end of the 45th storage day ($p>0.05$). Storage period had a significant effect on the FFA content of the control sample ($p<0.05$). The addition of BHT did not have an important positive effect on the FFA value but reduced the formation of FFA compared to the control (Fig. 2). REHMAN *et al.* (2003) reported that the FFA value of sunflower oil not containing any extract was 0.12% after three months storage at 45°C. In the same study, the FFA values of samples containing BHT, 1,600 and 2,400 ppm of ginger extract were 0.043, 0.052 and 0.049%, respectively. In another study, citrus peel extracts were added to corn oil and the FFA values for the control and samples containing 1,600 and 2,000 ppm citrus peel extract were determined as 5.0, 1.2 and 1.0% at 45°C for two months storage. The effect of citrus peel extracts

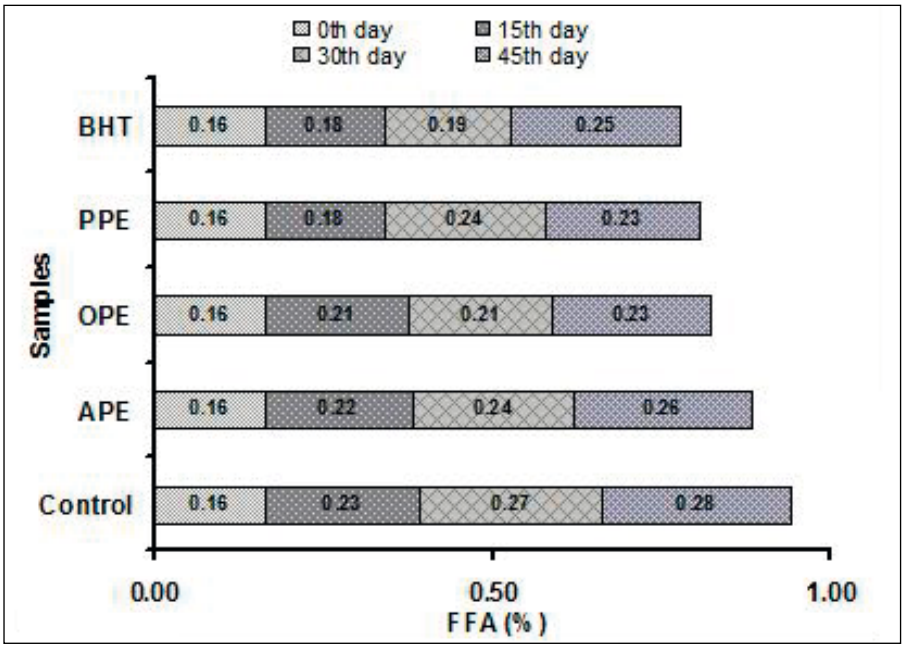


Fig. 2 - Free Fatty Acid values of samples.
BHT: Butylated hydroxytoluene; APE: Apple Pomace Extract; OPE: Orange Peel Extract; PPE: Potato Peel Extract.

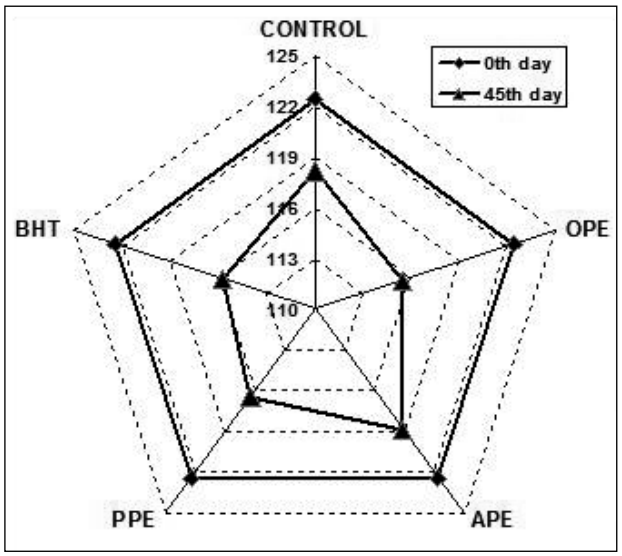


Fig. 3 - Iodine values of samples.
BHT: Butylated hydroxytoluene; APE: Apple Pomace Extract; OPE: Orange Peel Extract; PPE: Potato Peel Extract.

on the reduction of corn oil samples FFA values was found to be statistically significant ($p<0.05$) (REHMAN, 2006).

The IV of the control sample decreased with increasing storage time as expected (Fig. 3). IV is seen as an indicator of unsaturation. The most important chemical reaction is oxidation and because of this reaction, the double bonds of unsaturated fatty acid in the oil are broken during storage and the iodine value decreases (NOOR and AUGUSTIN, 1984; REHMAN *et al.*, 2004). The value of iodine was 122.46 at the beginning of the storage period for the control sample and it decreased to 118.15 after 45 days storage at 40°C (Fig. 3). The decrease in IV from 122.46 to 118.15 was found to be statistically significant ($p<0.05$). Also, the IV of samples decreased with increasing storage time because of oxidation. The effect of storage time and its interactive effect with extract type on the IVs of the sample was found as significant ($p<0.05$). While the IV of the control sample was 118.15 after 45 days storage, the IVs of samples containing APE, PPE and OPE were 118.82, 116.41 and 115.41, respectively. The samples containing natural antioxidative extracts showed lower IVs than the control sample. Similar results were reported by REHMAN *et al.* (2003). They found that the IV of control samples was 109 at 45°C while the IV of the sample containing 800 ppm ginger extract was 96. In another study, the IV of the control soybean oil sample was found as 99 at 45°C after 15 days storage and the samples containing PPE had a lower IV than the control and it was determined as 85, 88 and 90 for samples containing 800, 1,600 and 2,400 ppm PPE, respectively (REHMAN *et al.*, 2004). Many studies have shown that edible and non-edible plant materials have antioxidant activity due to the presence of phenolic compounds in their structure.

CONCLUSIONS

It can be said that potato peel, orange peel and apple pomace ethanolic extracts, waste materials which were used as natural antioxidants in model oil samples, showed strong antioxidant activity during storage. In general the use of natural extracts reduced the formation of oxidative reactions and so decreased the peroxide value. When compared to natural antioxidants, the synthetic antioxidant (BHT) showed better antioxidative properties. However, it is a fact that good results can be obtained by using natural antioxidants by increasing the level of natural waste extracts. Thus, it is suggested that natural extracts obtained from food waste such as apple pomace, orange peel and potato peel, can be used as a safe alternative to synthetic antioxidants.

REFERENCES

Anagnostopoulou M.A., Kefalas P., Papageorgiou V.P., Assimopoulou A.N. and Boskou D. 2006. Radical scavenging activity of various extracts and fractions of sweet orange peel (*Citrus sinensin*). Food Chem., 94: 19-25.

AOAC, 2000. Analyses code 965.33. In: Official Methods of Analysis of AOAC International. Washington DC, Association of Official Analytical Chemists 17th Ed. 2(41): 8-13.

Bocco A., Cuvelier M.A., Richard H. and Berset C. 1998. Antioxidant activity and phenolic composition of citrus peel and seed extracts. J. Agric. Food Chem., 46 (6): 2123-2129.

Ćetković G., Čanadanović-Brunet J., Djilas S., Savatović S., Mandić A. and Tumbas V. 2008. Assessment of polyphenolic content and *in vitro* antiradical characteristics of apple pomace. Food Chem., 109: 340-347.

Cherif J.K., M'Rabet I., El-Habiri M., Abidi R. and Albrecht-Gray M. 2006. Measurement of the antiradical efficiency of Tunisian orange juice and skins using the DPPH radical. Fruits, 61 (2): 99-107.

Cirico T.L. and Omaye S.T. 2006. Additive or synergetic effects of phenolic compounds on human low density lipoprotein oxidation. Food Chem. Toxicol., 44(4): 510-516.

Eberhardt M.V., Lee C.Y. and Liu R.H. 2000. Antioxidant activity of fresh apples. Nature, 405: 903-904.

Escarpa A. and Gonzalez M. 1998. High-performance liquid chromatography with diyote-array detection for the performance of phenolic compounds in peel and pulp from different apple varieties. J. Chromatog. A., 823: 331-337.

Gamez Meza N., Noriega-Rodriguez J.A., Leyva-Carrillo L., Ortega-Garcia J., Bringas-Alvarado L., García H.S. and Medina-Juarez L.A. 2009. Antioxidant activity comparison of Thompson grape pomace extract, rosemary and tocopherols in soybean oil. J. Food Process Pres., 76: 1445- 1447.

Gil M.I., Tomas-Barberan F.A., Hess-Pierce B., Holcroft D.M. and Kader A.A. 2000. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. J. Agric. Food Chem., 48: 4581-4589.

Gordon M.H. 1991. Oils and fats: Taint or flavor. Chemistry in Britain (November), 1020-1022.

Gorinstein S., Martín-Belloso O., Park Y., Haruenkit R., Lojek A., Ciz M., Caspi A., Libman I. and Trakhtenberg S.

2001. Comparison of some biochemical characteristics of different citrus fruits. Food Chem., 74: 309-315.

Halliwell B., Murcia M.A., Chirico S. and Aruoma O.I. 1995. Free radicals and antioxidants in food and in vivo: What they do and how they work. Crit. Rev. in Food Sci., 35: 7-20.

Isabei P. and Mariano M. 2001. Study of the thermal stability of edible vegetable oils in different environments. Afinidad, 58(493): 190-196.

Ito N., Fukushima S. and Tsuda H. 1985. Carcinogeneity and modification of the carcinogenic response by BHA, BHT, and other antioxidants. CRC. Crit. Rev. Toxicol., 15, 109-150.

John I.M. 2004. Fractionation of orange peel phenols in ultra filtered molasses and mass balance studies of their antioxidant levels. J. Agric. Food Chem., 52: 7586-7592.

Kanatt S.R., Chander R., Radhakrishna P. and Sharma A. 2005. Potato peel extract - a natural antioxidant for retarding lipid peroxidation in radiation processed lamb meat. J. Agric. Food Chem., 53:1499-1504.

Kristina A. and Hans L. 1998. Influence of oxygen and copper concentration on lipid oxidation in rapeseed oil. J. Am. Oil Chem. Soc., 75(8): 1041-1046.

Li Y., Guo C., Yang J., Wei J., Xu J. and Cheng S. 2006. Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. Food Chem., 96: 254-260

Londoño- Londoño J., Rodrigues de Lima V., Lara O., Gil A., Pasa T.B.C., Arango G.J. and Pineda J.R.R. 2010. Clean recovery of antioxidant flavonoids from citrus peel: Optimizing an aqueous ultrasound-assisted extraction method. Food Chem., 119: 81-87.

Lu Y. and Foo Y. 2000. Antioxidant and radical scavenging activities of polyphenols from apple pomace. Food Chem., 68: 81-85.

Makris D.P., Boskou G. and Andrikopoulos N.K. 2007. Polyphenolic content and *in vitro* antioxidant characteristics of wine industry and other agri-food solid waste extracts. J. Food Compos Anal., 20: 125-132.

Maniak B. and Targonski Z. 1996. Przeciwutleniacze naturalnie wystepujace w ywnosci [Antioxidants naturally occur in food]. Przem Ferm Owoc Warz, 4: 7-9 [in Polish].

Matalgyto F.S. and Al-Khalifa A.S. 1998. Effect of microwave oven heating on stability of some oil and fats. Arab Gulf J. Sci. Res., 16(2): 21-40.

Mohdaly A.A.A., Sarhan M.A., Smetanska I. and Mahmoud A. 2010. Antioxidant properties of various solvent extracts of potato peel, sugar beet pulp and sesame cake. J. Sci. Food Agric., 90: 218-226.

Noor N. and Augustin M.A. 1984. Effectiveness of antioxidant on the stability of banana chips. J. Sci. Food Agric., 35: 805-812.

Rehman Z.U. 2006. Citrus peel extract-A natural source of antioxidant. Food Chem., 99: 450-454.

Rehman Z.U., Habib F. and Shah W.H. 2004. Utilization of potato peel extract as a natural antioxidant in soybean oil. Food Chem., 85: 215-220.

Rehman Z.U., Salariya A.M. and Habib F. 2003. Antioxidant activity of ginger extract in sunflower oil. J Sci Food Agric., 83: 624-629.

SAS. 1988. SAS/STAT User's Guide (6.03), SAS Institute Inc., Cary, NC.

Sevcik R., Kvasnicka F. Jiruskova M., Vacek J., Hamouz K., Voldrich M., Cizkova H., Kondrashov A., Holasova M. and Fiedlerova V. 2009. Influence of potato varieties and culinary treatment on their antioxidation capacity Vyziva a Potraviny, 64: 161-163.

Singh N. and Rajini P.S. 2004. Free radical scavenging activity of an aqueous extract of potato peel. Food Chem., 85: 611-616.

Suárez B., Álvarez A.L., García Y.D., Barrio G., Lobo A.P. and Parra F. 2010. Phenolic profiles, antioxidant activity and in vitro antiviral properties of pomace. Food Chem., 120: 339-342.

Talcott S.T., Howard L.R. and Barnes S. 2000a. Contribution of periderm material and blanching time to the quality of pasteurized peach puree. J. Agric. Food Chem., 48: 4590-4596.

Talcott S.T., Howard L.R. and Brenes C.H. 2000b. Antioxidant changes and sensory properties of carrot puree processed with and without periderm tissue. J. Agric. Food Chem., 48: 1315-1321.

Valensi P.E., Behar A., de Champvallins M.M., Attalah M., Boulakia F.C. and Attali J.R. 1996. Effects of a purified micronized flavonoid fraction on capillary filtration in diabetic patients. Diab. Med., 13(10): 882-888.

Van Der Sluis A.A., Dekker M., de Jager A. and Jongen W.M.F. 2001. Activity and concentration of polyphenolic antioxidants in apple: effect of cultivar, harvest year, and storage conditions. J. Agric. Food Chem., 49: 3606-3613.

Witting P.K., Detlef M. and Roland S. 1999. Assessment of prooxidant activity of vitamin E in human low-density lipoprotein and plasma. Methods in Enzym., 299: 362-375.

Wolfe K., Wu X. and Liu R.H. 2003. Antioxidant activity of apple peels. J. Agric. Food Chem., 51: 609-614.

Xiaoying C. and Ahn D.U. 1998. Antioxidant activities of six natural phenolics against lipid oxidation induced by Fe^{+2} or ultraviolet light. J. Am. Oil Chem. Soc., 75(12): 1717-1721.

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PAPER

ANTIOXIDANT PROPERTIES OF EXTRA VIRGIN OLIVE OIL FROM *CERASUOLA CV* OLIVE FRUIT: EFFECT OF STONE REMOVAL

D. RESTUCCIA*, U.G. SPIZZIRRI, S. CHIRICOSTA[1], F. PUOCI, I. ALTIMARI and N. PICCI
Dipartimento di Scienze Farmaceutiche, Università della Calabria,
Via P. Bucci Edificio Polifunzionale, 87036 Arcavacata di Rende (CS), Italy
[1]Dipartimento di Studi e ricerche Economico-aziendali ed Ambientali,
Università degli Studi di Messina, Via dei Verdi 75, 98121 Messina, Italy
*Corresponding author: Tel. +39 0984 493296, Fax +39 0984 493298,
e-mail: donatella.restuccia@unical.it

ABSTRACT

This research investigated the effect of stone removal before processing on the antioxidant activity of monovarietal extra virgin olive oil (EVOO) in comparison with the non-destoned counterpart. The antioxidant activity of the polar compounds in the EVOO extract from destoned olives and the scavenging properties against 2,2'-diphenyl-1-picrylhydrazyl radical showed high efficiency in preventing the damages induced by free radicals. Moreover, polar extract showed the presence of flavonoic compounds and the ability to preserve β-carotene from products of linoleic acid lipidic peroxidation. The comparison of the antioxidant properties of the destoned EVOO polar extract with the non-destoned EVOO extract proved an increased antioxidant activity by stone removal.

- Key words: antioxidant properties, destoned fruit, extra virgin olive oil, *Cerasuola Cv* -

1. INTRODUCTION

Olive oil is obtained from the fruit of *Olea europaea*, a traditional tree crop of the Mediterranean Basin of the family Oleaceae. As a consequence, virgin olive oil is one of the edible fats most highly prized by the Mediterranean people and therefore it constitutes a fundament of their diet. Moreover, it is commonly used in cooking, cosmetics and pharmaceuticals. The beneficial effect of the consumption of virgin olive oil on human health is well known and it is related either to the characteristic fatty acid composition or to the presence of minor components, such as squalene, phytosterols and antioxidant molecules as tocopherols and other phenolic compounds (OWEN *et al.*, 2000). A large increase in demand for high-quality virgin olive oil during the past few years can be attributed not only to its potential health benefits, but also to its particular organoleptic properties. The quality of extra virgin olive oil (EVOO) is strictly related with both agronomic and technological parameters (RANALLI *et al.*, 2001) such as cultivar, harvesting systems and oil extraction techniques (crushing, the olives, olive paste kneading, oil phase separation, etc). Different kinds of crushers used to produce olive pastes and the essential malaxation operation (ANGEROSA, 1995; MONTEDORO *et al.*, 1992) modify phenolic compound contents and are also responsible for the changes of volatiles because of the activation of enzymes involved in the lipoxygenase pathway. Also the preparation of the olive paste is one of the most important stage of the process where oil is mechanically extracted from the fruits. In all of the Mediterranean countries the more currently used EVOO extraction processes require grinding of the whole olives and malaxation of the paste, followed by separation of the oil from the solids and vegetation water by centrifugation or pressure. In this sense, a new technological procedure is being developed that includes stone removal before the olive oil extraction process (AMIRANTE *et al.*, 2001).

The ancient Latin writer Columella (1st century A.D.) firstly described an alternative process for olive oil production, using destoned olives for oil production (COLUMELLA, 1997). Although destoning lowers olive oil yields, manufacturing plants for stone removal and milling of pulp have been recently set up, showing good oil yield. Interest in this technology is increasing, and some producers believe that oils obtained from destoned olives are of better quality than oils extracted from the whole fruit (BACCIONI, 2001). Nevertheless, reports on this issue are contradictory. The first study by FREGA *et al.* (2001) showed that oils extracted from destoned olives had a greater oxidative stability, as measured by the Rancimat test, and better sensory properties than oils obtained from the traditional milling of entire fruits. Accordingly, ANGEROSA *et al.* (1999) showed that oils obtained from olive pulp had a higher content in volatiles arising from the lipoxygenase pathway than oils obtained by the whole olives. BENTIVENGA *et al.* (2001) identified the presence of α-copaene and α-murolene in the volatile fraction of an oil extracted from destoned olives, but these compounds were not present in oils obtained from whole olives. SERVILI and MONTEDORO (2002) found that total phenolic concentration can be improved by extracting oil from destoned olives. On the contrary, PATUMI *et al.* (2003) found no effect of destoning on olive oil quality, as measured by acidity, peroxide number, spectrophotometric indices, phenolics, and volatiles. As documented by these studies, destoning had no influence on the fatty acid and sterol composition of olive oil.

The aim of the present work is to verify the influence of the employment of the de-stoner on the oil quality by measurement of the antioxidant properties of the polar fraction produced by hot extraction with methanol both from EVOO and destoned EVOO. The evaluation of the antioxidant activity of the produced polar fractions was performed by determination of total antioxidant activity, polyphenol and flavonoid contents. In addition, the scavenging activity of the hydrophilic parts and their ability to preserve β-carotene from linoleic acid lipidic peroxidation products were also reported. The extraction procedure proposed in this paper allows the obtaining of an alcoholic extract of EVOO that could be largely used either in cosmetic or in food industry.

2. MATERIALS AND METHODS

2.1. Materials and instrumentation

(+)-Catechin hydrate (CT), Gallic acid (GA), 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH), Folin-Ciocalteu reagent, sodium carbonate, sulphuric acid (96% w/w), trisodium phosphate, ammonium molybdate, β-Carotene, linoleic acid, Tween 20, were obtained from Sigma-Aldrich (Sigma Chemical Co., St Louis, MO, USA). Ethanol, chloroform, methanol were HPLC-grade and provided by Fluka Chemika-Biochemika (Buchs, Switzerland). UV-Vis absorption spectra were recorded with a Jasco V-530 UV/Vis spectrometer.

2.2. Olives and olive oil production

Extra-virgin olive oil (EVOO) from organic agriculture was made from pitted olives of *Cerasuola* variety, located in the Partinico in Palermo country area. EVOO was obtained from immediately cold pressed olives which were harvested by hand on M. Layla Fatta's farm from the end

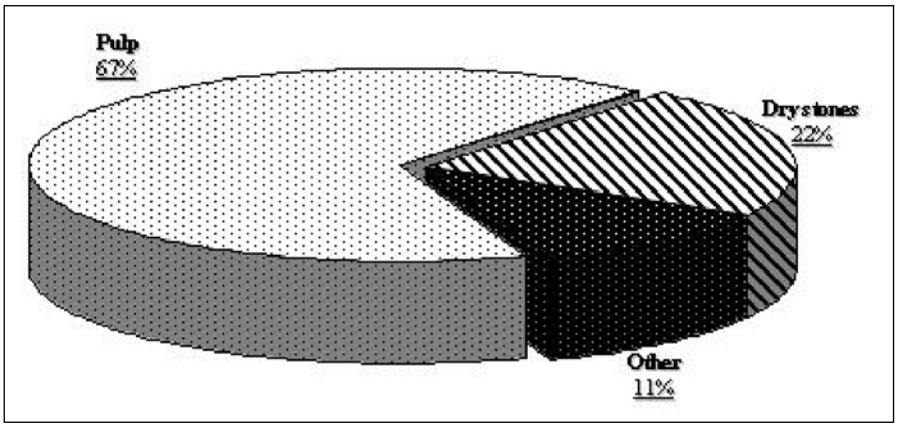


Fig. 1 - Olive fruit composition of *Cerasuola cv.*

of September to mid-October. The crushing operation, for the traditional process, was carried out by a hammer crusher model GR 32 (Rapanelli Fioravante S.p.a., Foligno, Italy). The olives were subjected to a separation of the stone from the pulp by a Toscana Enologica Mori (Firenze, Italy) destoner. The malaxation was carried out at 25°C for 30 min, and the oil separation was obtained using a three phase decanter at low water addition (0.2:1 v:w), RAMEF model 400 ECO-G (Rapanelli Fioravante S.p.a., Foligno, Italy). The oil was not filtred and for this reason some deposits may be found on the bottom of the bottle indicating that the oil was actually from the year written on the label. Immediately afterward, the oil was poured into large containers and was left to deposit. The oil was then bottled. The EVOO samples were stored in the dark at 13°C until analysis.

2.3. Extraction procedures of polar compounds

The stones represent a relevant part of the total weight of the fruit (Fig. 1) and starting from the olive fruit, the stone was removed from the drupe, without significantly pulp modifications. In order to extract polar compounds from the EVOOs, the procedure reported in Fig. 2 was employed, according to the literature with some modifications (OWEN *et al.*, 2000). The extraction procedure of the compounds from the EVOOs

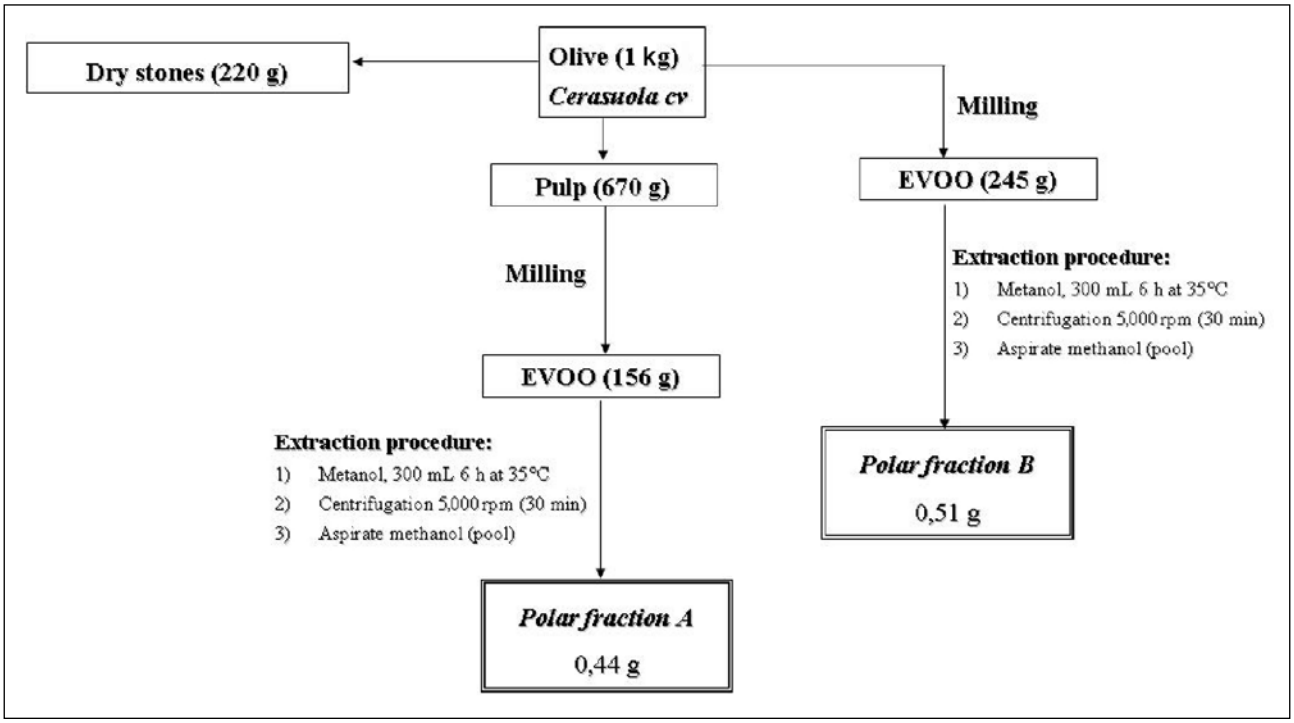


Fig. 2 - Extractive procedure of polar extract from olive drupe.

Table 1 - Antioxidant properties of polar extracts from EVOO obtained from stoned and destoned *Cerasuola cv* olives.

	ANTIOXIDANT ACTIVITY				
	Total antioxidant activity µmol equ GA/ g polar extract	**Disposable phenolic groups** µmol equ GA/ g polar extract	**Total flavonoids** µmol equivalent CT/ g of polar extract	**Scavenging effect on DPPH radical** IC_{30} (mg polar extract/mL)	**β-Carotene-Linoleic Acid assay** IC_{30} (mg polar extract/mL)
Destoned EVOO Polar Fraction (A)	3.66±0.11[a]	2.65±0.07[a]	0.38±0.02[a]	$9.7^{x}10^{-3}$±0.0001[a]	0.043±0.002[a]
EVOO Polar Fraction (B)	3.15±0.13[a]	1.53±0.05[b]	0.09±0.01[b]	$2.4^{x}10^{-2}$±0.0008[b]	0.090±0.003[b]

[a-b]Within the same treatment, values not sharing a letter were significantly different ($P < 0.05$; Fisher's test).

was carried out employing methanol at 35°C. The mixture was then centrifuged and the aspiration of alcoholic fractions provided the polar extracts from destoned EVOO (A) and non-destoned EVOO (B).

2.4. Measurement of antioxidant activity

2.4.1. Evaluation of disposable phenolic equivalents by Folin-Ciocalteu procedure

The amount of total phenolic equivalents was determined using Folin-Ciocalteu reagent procedure, according to the literature with some modifications (PAN *et al.*, 2007). A 2 mL aliquot of four different alcoholic extracts was mixed thoroughly with 1 mL of Folin-Ciocalteu reagent in a volumetric flask. After 3 min, 1 mL of Na_2CO_3 (7.5% w/w) were added, and then the mixture was allowed to stand for 2 h with intermittent shaking. The final concentrations of extracts in the samples were 0.02, 0.05, 0.08, 0.20 mg/mL, respectively. The absorbance of the solutions was measured at 760 nm to record a calibration curve and the correlation coefficient (R^2), slope and intercept of the regression equation obtained were calculated by the method of least square. The amount of total phenolic compounds in the extracts was expressed as mean (micromoles of gallic acid equivalents per gram of polar extract) ± SD for five replications, by using the equation obtained from the calibration curve (R^2 = 0.996) of the antioxidant. This one was recorded by employing five different gallic acid standard solutions in the same conditions reported for the olive stone extracts. The final concentrations of gallic acid in the test tubes were 0.8, 2.4, 3.2, 4.0 µM, respectively.

2.4.2. Determination of scavenging effect on DPPH radicals

In a standard procedure alcoholic extracts was allowed to react with a stable free radical, 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH), with the aim of evaluating their free radical scavenging properties (PARISI *et al.*, 2009). A solution of DPPH in ethanol (200 µM) was prepared freshly. A 8.0 mL aliquot of this solution was mixed with 12.0 mL of six different extract solutions to obtain the final concentrations of 2.0, 4.0, 10, 20, 30, 40 mg/mL, respectively. The solutions in the test tubes were shaken well, incubated in the dark for 30 min at room temperature and the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The scavenging activity of the extracts reported in Table 1 was measured as the decrease in absorbance of the DPPH and it was expressed as percent inhibition of DPPH radicals calculated according to the following equation:

$$Inhibition\% = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 is the absorbance of a standard that was prepared in the same conditions, but without extracts, and A_1 is the absorbance of extract samples.

2.4.3. Determination of Total Antioxidant Activity

The total antioxidant activity of alcoholic extracts was evaluated according to the method reported in literature (CURCIO *et al.*, 2009). Briefly, in five test tube, 0.3 mL of five different extract solutions were mixed with 1.2 mL of reagent solution (0.6 M H_2SO_4, 28.0 M Na_3PO_4, and 4.0 M $(NH_4)_2MoO_4$) to rise the final concentrations of 0.02, 0.05, 0.08, 0,11, 0,16 mg/mL, respectively. Then the reaction mixture was incubated at 95°C for 150 min and, after cooling to room temperature, the absorbance of the mixture was measured at 695 nm to record the calibration curve. The total antioxidant activity of the extracts was expressed as mean (micromoles of gallic acid equivalents per gram of polar extract) ± SD for five replications, by using the equation obtained from the calibration curve of the antioxidant. This one was recorded by employing five different gallic acid standard solutions with the same procedure (R^2). The final concentrations of gallic acid in the test tubes were 8.0, 16.0, 24.0, 32.0, and 40.0 µM, respectively.

2.4.4. β-Carotene-Linoleic Acid Assay

The antioxidant properties of alcoholic extract were evaluated through measurement of percent inhibition of peroxidation in linoleic acid system by using the β-carotene bleaching test (SPIZZIRRI *et al.*, 2009). Briefly, 1.0 mL of β-carotene solution (3.0 mg/mL in chloroform) was added to 7 μL of linoleic acid and 70 μL of Tween 20. The mixture was then evaporated at 40°C for 10 min in a rotary evaporator to remove chloroform. After evaporation, the mixture was immediately diluted with 35 mL of distilled water. The water was added slowly to the mixture and agitated vigorously to form an emulsion. The emulsion (5 mL) was transferred to five different test tubes and 1.5 mL of extract was added to rise the final concentrations of 0.015, 0.050, 0.090, 0.130, 0.180 mg/mL, respectively. The tubes were then gently shaken and placed in a water bath at 45°C for 60 min. The absorbance of the filtered samples and control was measured at 470 nm against a blank, consisting of an emulsion without β-carotene. The measurement was carried out at the initial time (t = 0) and successively at 60 min. The antioxidant activity (AA), reported in Table 1, was measured in terms of successful bleaching of β-carotene using the following equation:

$$AA = (1 - \frac{A_0 - A_{60}}{A_0^o - A_{60}^o})$$

where A_0 and A_0° are the absorbance values measured at the initial incubation time for samples and control, respectively, while A_{60} and A_{60}° are the absorbance values measured in the samples and in control, respectively, at t = 60 min.

2.4.5. Determination of flavonoids

A slightly modified version of the spectrophotometric method was used to determine the flavonoid contents of samples (DEWANTO *et al.*, 2002). Briefly, in a graduate flask 0.7 mL of five different alcoholic extract solutions were mixed with 2.0 mL of distilled water followed by addition of 0.3 mL of a $NaNO_2$ solution (5% w/w). After 6 min, 600 μL of a $AlCl_3$ x $6H_2O$ solution (10% w/w) were added and allowed to stand for another 5 min before 2.0 mL of 1 M NaOH was added. The mixture was brought to 10 mL with distilled water and mixed well. The final concentrations of extract in the samples were 0.10, 0.25, 0,030, 0,045, 0,060 mg/mL, respectively. The absorbance was measured immediately against the blank at 510 nm to record the calibration curve. The amount of total flavonoids in the starting material was expressed as mean (micromoles of catechin equivalents per gram of initial material) ± SD for five replications, by using the equation obtained from the calibration curve of the antioxidant (R^2). This one was recorded by employing five different catechin standard solutions with the same procedure. The final concentrations of catechin in the test tubes were 10, 25, 50, 75, 100 μM, respectively.

2.4.6. Statistical analysis

The results are presented as the average of five experiments and standard deviation (± SD). Data in Table 1 were analyzed using one-way analysis of variance (ANOVA). When significant values were found ($P < 0.05$), post-hoc comparison of means was made using the Fisher's test. The inhibitory concentrations 30% (IC_{30}) were obtained by interpolation from linear regression analysis.

3. RESULTS AND DISCUSSION

3.1. Extraction procedure

The antioxidant composition of EVOO is the result of a number of variables acting before oil extraction (such as olive variety, environmental, climatic, soil and cultivation conditions, olive ripeness, olive health) and during EVOO extraction and storage (VELASCO *et al.*, 2002). In order to test antioxidant activity of the oils, as reported in Fig. 2, the EVOO and destoned EVOO of *Cerasuola* variety were treated with methanol at 35°C to extract from the oils all the polar compounds, mainly with a phenolic structure, potentially useful as antioxidant molecules (OWEN *et al.*, 2000). The extraction procedure allows obtaining two polar extracts from destoned EVOO (Fraction A) and EVOO without stone removal (Fraction B). These fractions represent about 0.3 and 0.2% of the pulp and 0.05 and 0.06% of the fruit, respectively.

3.2. Measurement of Antioxidant Activity

3.2.1. Total phenolic content

The olive drupe contains high concentrations of phenolic compounds that can range between 1-3% of the fresh pulp weight. The main classes of phenols present in olive fruit are phenolic acids, phenolic alcohols, flavonoids, lignans and secoiridoids (SERVILI and MONTEDORO, 2002). The (3, 4-dihydroxyphenyl) ethanol (3,4-DHPEA) and (p-hydroxyphenyl) ethanol (p-HPEA) are the most abundant phenolic alcohols in the olive fruit. The flavonoids include flavonol glycosides such as luteolin-7-glucoside and rutin as well as anthocyanins, cyanidin and delphinidin glycosides, in particular. Secoiridoids including aglycon derivatives of oleuropein, demethyloleuropein and ligstroside, that are present in olive fruit, are the most abundant phenolic antioxidants of EVOO.

The Folin-Ciocalteu reagent is used to obtain a crude estimate of the amount of total phenolic compounds present in the sample (SPIZZIRRI *et*

al., 2010). They undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the Folin-Ciocalteu reactant. The color development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic/phosphotungstic acid complexes to form chromogens in which the metals have lower valence. By comparing the data with the GA calibration curve, the amounts of GA equivalent were 2.65 and 1.53 μmol GA/g polar extract for fractions A and B, respectively.

The results confirm previous researches (AMIRANTE *et al.*, 2002; AMIRANTE *et al.*, 2001; SERVILI *et al.*, 2000). In particular SERVILI *et al.* (2007) studied olive stoning during the VOO mechanical extraction process, to show the effect on the phenolic and volatile composition of the oil. To study the impact of the constitutive parts of the fruit in the composition of olive pastes during processing, the phenolic compounds and several enzymatic activities such as polyphenoloxidase, peroxidase, and lipoxygenase of the olive pulp, stone, and seed were also studied. The olive pulp showed large amounts of oleuropein, demethyloleuropein, and lignans, while the contribution of the stone and the seed in the overall phenolic composition of the fruit was very low. It was found that the occurrence of crushed stone in the pastes, during malaxation, increased the peroxidase activity in the pastes, reducing the phenolic concentration in VOO and, at the same time, modifying the composition of volatile compounds produced by the lipoxygenase pathway. The oil obtained from stoned olive pastes contained higher amounts of secoiridoid derivatives such as the dialdehydic forms of elenolic acid linked to (3,4-dihydroxyphenyl)-ethanol and (p-hydroxyphenyl)ethanol (3,4-DHPEA-EDA and p-HPEA-EDA, respectively) and the isomer of the oleuropein aglycon (3,4-DHPEA-EA) and, at the same time, did not show significant variations of lignans.

More recently, RANALLI *et al.* (2010) analysed destoned and organic-destoned virgin olive oils coming from all the Italian olive-growing regions. It was found that destoning significantly increased the concentration of biophenols in the pulp oil, such as lignans, flavonoids, phenol acids, phenol alcohols and natural and oxidised oleuropein and ligstroside aglycons. These new crop oil kinds, owing to the soft action of destoner, also were richer in α- and γ-tocopherol and in α- and γ-tocotrienol, which are considered to be other very bioactive compounds.

All obtained results seem to confirm the relationships between the control of oxidative reactions during processing and the phenolic concentration in the oil because peroxidase is highly concentrated in the olive seed. The soft action of destoner on olive drupes results in lower thermal quinonisation of phenols; moreover, since the kernel tissues are richest in polyphenoloxidase and peroxidase enzymes, there is lower enzymatic oxidation of phenols (RANALLI *et al.*, 2007). The destoning process, excluding the olive seed before malaxation, partially remove the peroxidase activity in the pastes and consequently can reduce the enzymatic degradation of the hydrophilic phenols in the oils during the process thus improving their concentration and oil oxidative stability and sensory properties (SERVILI *et al.*, 1999). Although it is a well-known circumstance that other olive processing steps (crushing and malaxing noticeably) can enhance the concentration of biophenol and volatile compounds in the oil phase (RANALLI *et al.*, 2003; 2009), authors (RANALLI *et al.*, 2010) believe that destoning is more effective compared to them (RANALLI *et al.*, 2008).

3.2.2. Determination of Total Antioxidant Activity

The assay is based on the reduction of Mo (VI) to Mo (V) by antioxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at acid pH. As can be seen in Table 1, total antioxidant activity of extract A is about 1.2 times that the extract B. Comparing this data with total phenolic content of the extracts, it appears as the antioxidant properties of extracts A are mainly due to phenolic compounds (72% of the molecules showing antioxidant efficiency), while the contribution of these molecules at the total antioxidant activity significantly decreased in the extract B (48% of the molecules showing antioxidant efficiency). It follows that the total antioxidant activity is phenol concentration-dependent.

3.2.3. Total flavonoids content

It has frequently been reported that flavonoids are a particular class of compounds associated with antioxidant activity (DEWANTO *et al.*, 2002). Thus, for the characterization of the antioxidant activity of the polar extracts, $AlCl_3$ assay was employed to have a direct determination of the total flavonoid content of the olive-product expressed as μmol catechin equivalent per g of polar extract. Catechins are one of main classes of flavonoids and present in large amounts in green and black tea, in red wine, chocolate, fruits, etc. They are potentially beneficial to human health as they are strong antioxidants, anti-carcinogens, anti-inflammatory agents, and inhibitors of platelet aggregation *in vivo* and *in vitro* studies (VAN ACKER *et al.*, 1996). In particular, this assay allowed to estimate for A extract a flavonoid amount of 0.38 μmol of CT/g polar extract, while in the extract B the flavonoid compounds were 0.09 μmol of CT/g polar extract. In addition, it is important to note as in the extract A, the flavonoids represent about the 10% of antioxidant molecules with phenolic groups while this percentage decreases 4 times in the extract B. It could be inferred from our results that there was a positive correlation be-

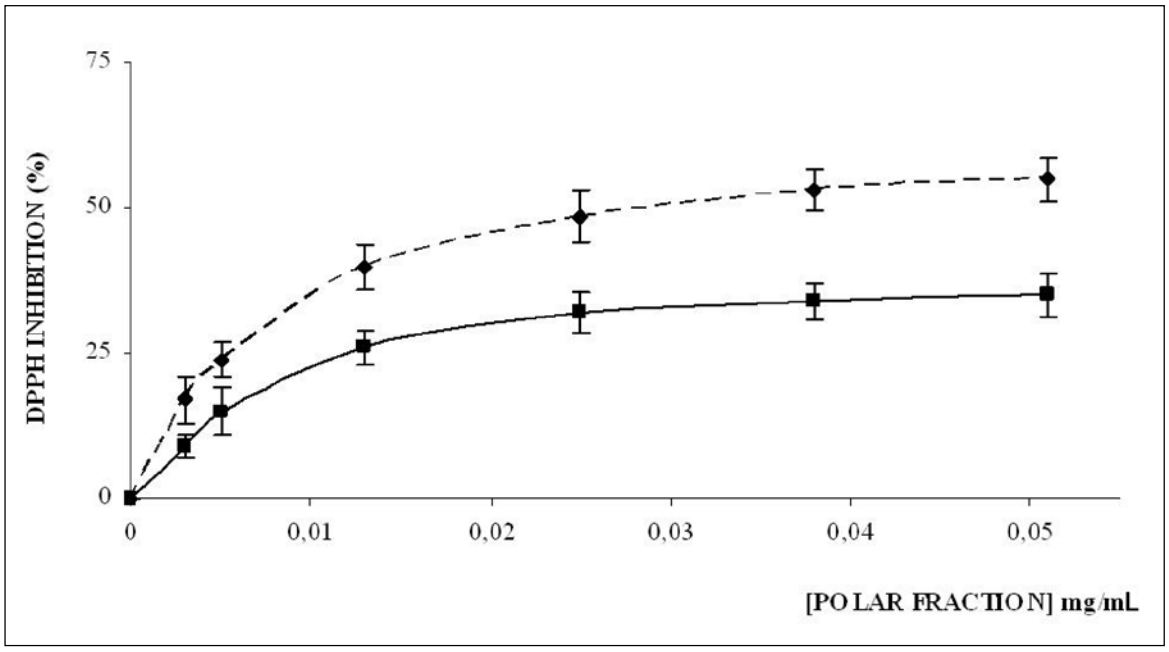


Fig. 3 - Scavenging activity of polar extract from destoned EVOO (- - - -) and EVOO (—).

tween flavonoid content and antioxidant activity, as a higher activity of the methanol fraction could be related to a higher flavonoids content.

3.2.4. Determination of scavenging effect on DPPH radicals

The antioxidant activity of phenolics of different sources is well known (GARROTE *et al.*, 2009). Compounds such as benzoic acids and hydroxicinnamic acids have been assayed for antioxidant activity using different tests, but none of the available methods provides an absolute measurement of the phenomena involved (FRANKEL *et al.*, 2000).

The scavenging of hydrogen radicals is one of the important mechanisms of antioxidation. In this study, DPPH was used to determine the free radical scavenging activity of the alcoholic extracts obtained by olive stone. The DPPH radical is a stable organic free radical with an absorption maximum band around 515-528 nm and, thus, it is a useful reagent for evaluation of the antioxidant activity of compounds. In the DPPH test, the antioxidants reduce the DPPH radical to a yellow compound, diphenylpicrylhydrazine, and the extent of the reaction depends on the hydrogen-donating ability of the antioxidants. It has been documented that cysteine, glutathione, ascorbic acid, tocopherols, and polyhydroxy aromatic compounds (e.g., ferulic acid, hydroquinone, catechin, gallic acid) reduce and decolorize 1,1-diphenyl-2-picrylhydrazine (DPPH) by their hydrogen-donating capabilities. EVOO extracts (A and B) scavenger property was evaluated in terms of DPPH reduction using different alcoholic extract concentration. Data are expressed as percent of inhibition, as shown in Fig. 3. The IC_{30}, which is the efficient concentration required to reduce initial DPPH concentration by 30% of sample, was also calculated as was found to be 9.7×10^{-3} and 2.4×10^{-2} mg polar extract/mL for extracts A and B, respectively (Table 1). These data show as the scavenging activity of the chemical compounds in the polar extracts A is about 2.5 times higher than the compounds in the B fraction. In addition, the scavenger activity of the polar extracts was also calculated as micromoles of gallic acid equivalents per volume of solution and micromoles of catechin equivalents per volume of solution. In this way, it was possible to assess whether the different antioxidant activities of the extracts were due to differences in the concentration of the phenolics/flavonoids compounds or could be related to differences in the antiradical activity of the molecular mixture irrespective of their phenolic/flavonoid content. In particular, for the extract A the scavenger activity was 2.6×10^{-5} µmol equivalent GA/mL and 3.7×10^{-6} µmol equivalent CT/mL, while the scavenger activity of the extract B was 3.6×10^{-5} µmol equivalent GA/mL and 2.3×10^{-6} µmol equivalent CT/mL.

The results indicate that the extracts possess phenol concentration-dependent antiradical activity, as already reported for total antioxidant activity. It is worth commenting on the relationship between the total phenolic content and scavenging activity against DPPH, since phenolics contribute to the antiradical activity. Fractions rich in total phenolics are more effec-

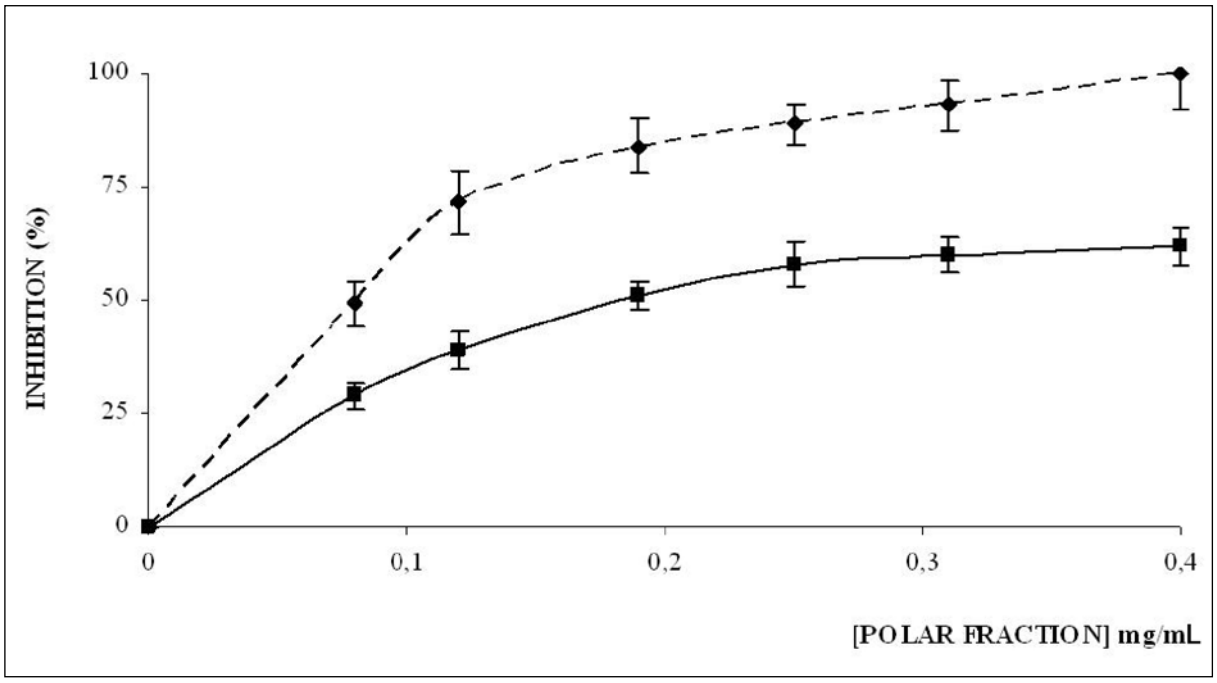


Fig. 4 - Linoleic acid-β-carotene assays of polar extract from destoned EVOO (- - - -) and EVOO (——).

tive scavengers of DPPH radicals than phenol poor fractions. However, the inhibition of DPPH radical scavenging by different extracts is not strictly proportional to the concentration of total phenolics, since it has been reported that fractions possessing similar antioxidant properties show different concentration of phenolics (XANTHOPOULOU *et al.*, 2009). This may be attributed to the different quality of phenolics they contain, and, consequently, to the different antioxidant activity they posses, but also to the different content of other constituents (carbohydrates, phospholipids, fatty acids), that may contribute to the antioxidant activity as well.

It's well known that antioxidant properties depend on several structural features of the molecule of polyphenols in its base structure and are primarily attributed to the high reactivity of hydroxyl substituent. Among EVOO phenolics, compounds with the presence of a 3,4-dihydroxy moiety linked to an aromatic ring were more active than those with only one hydroxyl group (CIOFFI *et al.*, 2010; MORELLO *et al.*, 2005); the glycosidation decreased the antioxidant activity. Oleuropein derivatives, which share an *o*-diphenolic structure, are reported to be mainly responsible for the effects potentially indicative of possible health effects of EVOO on cells rather than ligstroside derivatives, as studied in vitro by different cell or cell-free systems (VISIOLI *et al.*, 2002; VISIOLI and GALLI, 2002; BALDIOLI *et al.*, 1996; LAVELLI, 2002; VISIOLI *et al.*, 1998). In particular, LAVELLI and BORDESAN reported in 2005 a positive relation between changes in the contents of dialdehydic form of decarboxymethyl oleuropein aglycone and variation of the DPPH radical scavenging ability of EVOOs, although data were influenced also by genetic factors and by ripening stage, as well as by the presence or absence of the stone during oil extraction.

3.2.5. β-Carotene-Linoleic Acid Assay

In this model system, β-carotene undergoes rapid discoloration in the absence of an antioxidant, which results in a reduction in absorbance of the test solution with reaction time. This is due to the oxidation of linoleic acid that generates free radicals (lipid hydroperoxides, conjugated dienes and volatile byproducts) that attacks the highly unsaturated β-carotene molecules in an effort to reacquire a hydrogen atom. When this reaction occurs, the β-carotene molecule loses its conjugation and, as a consequence, the characteristic orange colour disappears. The presence of antioxidant avoids the destruction of the β-carotene conjugate system and the orange colour is maintained (AMIN *et al.*, 2004). The inhibition percentages of lipidic peroxidation by alcoholic extracts was evaluated at different concentration as shown in Fig. 4 and the IC_{30} value was found. In our experiments, after one hour of incubation the IC_{30} of the extracts were found to be 0.043 and 0.090 mg polar extract/mL for extracts A and B, respectively (Table 1). These data clearly show as the activity of the compounds in the polar extracts A is about 2 times higher than the compounds in the B fraction. In addition, in order to correlate the different ability of the extracts to preserve β-carotene

from products of linoleic acid lipidic peroxidation with their differences in the concentration of the phenolics/flavonoids, the activity of the polar extracts was also calculated as micromoles of gallic acid equivalents per volume of solution and micromoles of catechin equivalents per volume of solution. In particular, for the extract A the activity was 1.2×10^{-4} µmol equivalent GA/mL and 1.7×10^{-5} µmol equivalent CT/mL, while the activity of the extract B was 1.6×10^{-4} µmol equivalent GA/mL and 8.3×10^{-6} µmol equivalent CT/mL. The data confirm as the ability of the extracts to preserve β-carotene from products of linoleic acid lipidic peroxidation due to differences in the concentration of the phenolics/flavonoids in the extracts.

The antioxidant tests carried out on the destoned EVOO and on the EVOO of *Cerusola Cv* proved that for this variety of olive fruit the effect of olive stone removal before milling process increase the antioxidant properties of the extract suitable for use in food, cosmetic, nutraceutical and pharmaceutical applications.

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REFERENCES

Amin I., Zamaliah M.M. and Chin W.F. 2004. Total antioxidant activity and phenolic content in selected vegetables. Food Chem., 87: 581.

Amirante P., Catalano P., Amirante R., Clodoveo M.L., Montel G.L., Leone A. and Tamborrino A. 2002. Prove sperimentali di estrazione di oli extravergini di oliva da paste snocciolate. Olivo & Olio, 5: 16.

Amirante P., Catalano P., Amirante R., Montel G.L., Dugo G., Lo Turco V., Baccioni L., Fazio D., Mattei A. and Marotta F. 2001. Prove sperimentali di estrazione di olio extra vergine da paste snocciolate. Olivo & Olio, 4: 48.

Amirante R., Cini E., Montel G.L., Pasqualone A. 2001. Influence of mixing and extraction parameters on virgin olive oil quality. Grasas y Aceites 52: 198-201.

Angerosa F., Basti C., Vito R. and Lanza B. 1999. Effect of fruit stone removal on the production of virgin olive oil volatile compounds. Food Chem. 67: 295.

Angerosa F., D'Alessandro N., Konstantinou P. and Di Giacinto L. 1995. GC-RIS evaluation of phenolic-compounds in virgin olive oil. J. Agric. Food Chem. 43: 1802.

Baccioni L. 2001. Aspetti impiantistici e tecnologici. In: "Innovazione Tecnologica per l'Estrazione di Olio ExtraVergine da Paste di Olive Snocciolate", I Georgofili Quaderni 2001-IV; Società Editrice Fiorentina, Firenze, Italy.

Baldioli M., Servili M., Perretti G. and Montedoro G.F. 1996. Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil. J. Am. Oil Chem. Sci. 73: 1589.

Bentivenga G., D'Auria M., De Luca E., De Bona A. and Mauriello G. 2001. The use of SPME-GC-MS in the analysis of flavor of virgin olive oil. Riv. Ital. Sostanze Grasse 78: 157-162.

Cioffi G., Pesca M.S., De Caprariis P., Braca A., Severino L. and De Tommasi N. 2010. Phenolic compounds in olive oil and olive pomace from Cilento (Campania, Italy) and their antioxidant activity. Food Chem. 121: 105.

Columella L.G.M. 1997. "L'Arte dell'Agricoltura e Libro sugli Alberi", Calzecchi Onesti R., Carena C. (Ed.). Giulio Einaudi Editore, Torino, Italy.

Curcio M., Puoci F., Iemma F., Parisi O.I., Cirillo G., Spizzirri U.G. and Picci N. 2009. Covalent insertion of antioxidant molecules on chitosan by a free radical grafting procedure. J. Agric. Food Chem. 57: 5933.

Dewanto V., Wu X., Adom K.K. and Liu R.H. 2002. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J. Agric. Food Chem. 50: 3010.

Frankel E.N. and Meyer A. 2000. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. J. Sci. Food Agric. 80: 1925.

Frega N., Caglioti L. and Mozzon M. 1997. Composizione chimica e parametri di qualità degli oli estratti da olive snocciolate. Riv. Ital. Sostanze Grasse 74: 241.

Garrote G., Cruz J.M., Moure A., Domìnguez H. and Parajo J.C. 2004. Antioxidant activity of byproducts from the hydrolytic processing of selected lignocellulosic materials. Trends Food Sci. Technol. 15: 191.

Lavelli V. 2002. Comparison of the antioxidant activities of extra virgin olive oils. J. Agric. Food Chem. 53: 1102.

Lavelli V. and Bondesan L. 2005. Secoiridoids, tocopherols, and antioxidant activity of monovarietal extra virgin olive oils extracted from destoned fruits. J. Agric. Food Chem. 50: 7704.

Montedoro G., Servili M., Baldioli M. and Miniati E. 1992. Simple and hydrolyzable phenolic-compounds in virgin olive oil. 1.Their extraction, separation, and quantitative and semiquantitative evaluation by HPLC. J. Agric. Food Chem. 40: 1571.

Morello J.R., Voureola S., Romero M.P., Motilva M.J. and Heinonen M. 2005. Antioxidant activity of olive pulp and olive oil phenolic compounds of the arbequina cultivar. J. Agric. Food Chem. 53: 2002.

Owen R.W., Giacosa A., Hull W.E., Haubner R., Spiegelhalder B. and Bartsch H. 2000. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. Eur. J. Cancer 36: 1235.

Owen R.W., Mier W., Giacosa A., Hull W.E., Spiegelhalder B. and Bartsch H. 2000. Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignans and squalene. Food Chem. Toxicol. 38: 647.

Pan Y., Zhu J., Wang H., Zhang X., Zhang Y., He C., Ji X. and Li H. 2007. Antioxidant activity of ethanolic extract of Cortex fraxini and use in peanut oil. Food Chem. 103: 913.

Parisi O.I., Puoci F., Iemma F., De Luca G., Curcio M., Cirillo G., Spizzirri U.G. and Picci N. 2009. Antioxidant and spectroscopic studies of crosslinked polymers synthesized by grafting polymerization of ferulic acid. Polym. Adv. Technol. 21: 774.

Patumi M., Terenzani S., Ridolfi M. and Fontanazza G. 2003. Effect of fruit stoning on olive oil quality. J. Am. Oil Chem. Soc. 80: 249.

Ranalli A. and Contento S. 2010. Analytical assessment of destoned and organic-destoned extra-virgin olive oil. Eur. Food Res. Technol. 230: 965.

Ranalli A., Benzi M., Gomes T., Delcuratolo D., Marchegiani D. and Lucera L. 2007. Concentration of natural pigments and other bioactive components in pulp oils from de-stoned olives. Innov. Food Sci. & Emerg. Tech. 8: 437.

Ranalli A., Cabras P., Iannucci E. and Contento S. 2001. Lipochromes, vitamins, aromas and other components of virgin olive oil are affected by processing technology. Food Chem. 73: 445.

Ranalli A., Gomes T., Delcuratolo D., Marchegiani D. and Pardi D. 2008. Producing nutraceutical virgin olive oil with innovative processing technologies Ital. Food Bev. Tech. 53: 10.

Ranalli A., Marchegiani D., Contento S., Pardi D., Girar-

di F. and Kotti F. 2009. Evaluation of Functional Phytochemicals in Destoned Virgin Olive Oil. Food Bioprocess Tech. 2: 27.

Ranalli A., Pollastri L., Contento S., Lucera L. and Del Re P. 2003. Enhancing the quality of virgin olive oil by use of a new vegetable enzyme extract during processing. Eur. Food Res. Technol. 216: 109.

Servili M. and Montedoro G.F. 2002. Contribution of phenolic compounds to virgin olive oil quality. Eur. J. Lipid Sci. Technol. 104: 602.

Servili M., Baldioli M., Begliuomini A.L., Selvaggini R., Montedoro G.F. and Lanzotti V. 2000. Dordrecht, The Netherlands: Kluwer Academic Publishers, 163.

Servili M., Baldioli M., Mariotti F., Montedoro G.F., Metzidakis I.T. and Voyiatzis D.G. 1999. Phenolic composition of olive fruit and virgin olive oil: distribution in the constitutive parts of fruit and evolution during the oil mechanical extraction process. Acta Horticul., 474: 609.

Servili M., Taticchi A., Esposto S., Urbani S., Selvaggini R. and Montedoro G.F. 2007. Effect of olive stoning on the volatile and phenolic composition of virgin olive oil. J. Agric. Food Chem. 55: 7028.

Spizzirri U.G., Iemma F., Puoci F., Cirillo G., Curcio M., Parisi O.I. and Picci N. 2009. Synthesis of Antioxidant Polymers by Grafting of Gallic Acid and Catechin on Gelatin. Biomacromolecules 10: 1923.

Spizzirri U.G., Parisi O.I., Iemma F., Cirillo G., Puoci F., Curcio M. and Picci N. 2010. Antioxidant-polysaccharide conjugates for food application by eco-friendly grafting procedure. Carbohyd. Polym. 79: 333.

Van Acker S.A.B.E., Van den Berg D.J., Tromp M.N.J.L., Griffioen D.H., Van Bennekom W.P., Van der Vijgh W.J. and Bast A. 1996. Structural aspects of antioxidant activity of flavonoids. Free Radic. Biol. Med. 20: 331.

Velasco J. and Dobarganes C. 2002. Oxidative stability of virgin olive oil. Eur. J. Lipid Sci. Technol. 104: 661.

Visioli F. and Galli C. 2002. Biological properties of olive oil phytochemicals. Crit. Rev. Food Sci. Nutr. 42: 209.

Visioli F., Bellomo G. and Galli C. 1998. Free radical-scavenging properties of olive oil polyphenols. Biochem. Biophys. Res. Commun. 247: 60.

Visioli F., Galli C., Galli G. and Caruso D. 2002. Biological activities and metabolic fate of olive oil phenols. Eur. J. Lipid Sci. Technol. 104: 677.

Xanthopoulou M.N., Nomikos T., Fragopoulou E. and Antonopoulou S. 2009. Antioxidant and lipoxygenase inhibitory activities of pumpkin seed extracts. Food Res. Int. 42: 641.

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PAPER

MORTADELLA SAUSAGE PRODUCED WITH SOYBEAN OIL INSTEAD OF PORK FAT

M.A. TRINDADE[1]*, J.M. DE OLIVEIRA[1], N.M.G. NOGUEIRA[1], P.R.C. OLIVEIRA FILHO[1], S.M. DE ALENCAR[2] and C.J. CONTRERAS-CASTILLO[2]

[1]College of Animal Sciences and Food Engineering, University of São Paulo,
C.P. 23, 13635-900 Pirassununga, São Paulo, Brazil

[2]Luiz de Queiroz Agriculture School, University of São Paulo,
C.P. 9, 13418-900, Piracicaba, São Paulo, Brazil

*Corresponding author: Tel. +55 19 3565 4245, Fax +55 19 3565 4284,
e-mail: trindadema@usp.br

ABSTRACT

This study examines the effects of partially or completely replacing pork backfat with soybean oil in mortadella production. Mortadella sausages of various formulations showed no differences ($P > 0.05$) in any of the technological and physico-chemical parameters evaluated (process yield, shear force, pH, water activity and proximate composition). When compared to products made with pork backfat, those made with vegetable oil had a higher unsaturated fatty acid content ($P \leq 0.05$) and a similar cholesterol content ($P > 0.05$). Products made with vegetable oil scored lower ($P \leq 0.05$) than those made with pork fat on all of the evaluated sensory attributes (colour, odour, flavour, texture and overall acceptability).

- Key words: Sausage, soybean oil, fatty acids, sensory evaluation -

INTRODUCTION

High dietary intakes of saturated fatty acids increase total cholesterol and LDL cholesterol. In the treatment of hypercholesterolemia, patients are advised to reduce their dietary consumption of saturated fat. Animal fats (except those from fish), palm and coconut oils are rich in saturated fatty acids. It is therefore advisable to limit the consumption of these fats, particularly animal fat in fatty meats and sausages. Dietary cholesterol increases blood cholesterol in several animal species, including humans. Replacing saturated fatty acids with polyunsaturated fatty acids reduces total cholesterol and blood LDL (SOCESP, 2006). The American Heart Association recommends a daily intake of 300 mg of cholesterol for humans (AHA, 2009). Oleic and linoleic acids increase HDL (good cholesterol) and decrease LDL (PEREIRA *et al.*, 2000).

Soybean oil is very rich in polyunsaturated fatty acids, particularly linoleic acid (55.62%). It also has the highest PUFA percentage of any oil (59.70%, compared to 30.08% for canola oil) and the largest PUFA/SFA ratio (MAIA *et al.*, 2006). According to the Brazilian Association of Vegetable Oil Industries (ABIOVE, 2009), Brazil produces about 28% of the world's supply of soybeans, with the 2006 harvest estimated to be 57 million tons. Brazil is the second-largest world producer and exporter of soybeans, soy meal and soybean oil. In 2008, Brazil produced 6.19 million tons of soybean oil. There are several advantages to replacing animal fat with soybean oil in meat products; soybean oil can be produced in large volumes and is healthier like olive, corn and sunflower oils. Soybean oil is also a relatively inexpensive alternative to animal fat; the price of soybean oil in Brazil is almost half the price of pork backfat (weight by weight).

Meat products such as hamburgers, mortadella and salami are popular with consumers and are a major market for the sector industries. Their consumption is affected by many factors, including the product's sensory and nutritional characteristics, safety, price and convenience (JIMENEZ-COLMENERO *et al.*, 2001). The relatively high cholesterol content and low ratio of polyunsaturated to saturated fatty acids (PUFA/SFA) presented by animal fats play a negative role in the development of several diseases, including coronary artery disease. Pork lard, the main source of animal fat used in the development of sausages, contains 38% saturated fatty acids, while soybean oil contains only 16% saturated fatty acids (MAIA *et al.*, 2006; BRAGAGNOLO and RODRIGUEZ-AMAYA, 2002).

Mortadella, one of the most commonly consumed meat products, is produced from a meat emulsion that may contain pork backfat and other ingredients. This emulsion is wrapped in natural or artificial casings, shaped and submitted to appropriate heat treatment (BRAZIL, 2000). Animal fat may be primarily responsible for the development of an acceptable aroma, taste, texture and succulence in meat products. YILMAZ *et al.* (2002) replaced animal fat with sunflower oil and found significant differences in texture, odour and succulence, but not in flavour. However, HSU and YU (2002) showed that replacing animal fat with vegetable oils from various sources did not significantly change the texture, colour, odour, flavour and acceptability of meatballs.

The objective of this study was to evaluate the nutritional, technological and sensory characteristics of mortadella sausages produced with soybean oil instead of the traditional pork backfat.

MATERIALS AND METHODS

Sausage manufacture

Three types of mortadella were produced: a "Pork" formulation made with 100% pork backfat; a "50/50" formulation made with 50% pork backfat and 50% soybean oil; and an "Oil" formulation made with 100% soybean oil.

The raw animal materials included beef meat that had been cleaned of excess fat and collagen and pork backfat that had been skinned, ground and homogenised before freezing. Commercial soybean oil (Liza brand) was used. The levels of protein, lipid and moisture of the meat and pork backfat were determined, and the vegetable oil composition was considered to be 100% fat. Based on the composition of the raw materials, each of the three formulations was developed to ensure equal levels of protein (12.5%) and fat (15%) and a moisture to protein ratio of 5:1. Two batches (two replicates) of 15 kg were processed for each treatment. Table 1 shows each of the three formulations used in the first replication of the experiment. Other ingredients used in identical proportions in all of the treatments included: 1.5% mortadella seasoning mix (Aglomix S100-T - DiCARNE) containing an antioxidant (sodium erythorbate) and emulsifier (sodium polyphosphate); 1.6% table salt (Cisne); 0.35% cure salt (Standard, DiCARNE); 1% isolated soy protein (Supro 500L - Solae); 0.5% crushed garlic; 0.1% ground white pepper; and 0.1% monosodium glutamate (Ajinomoto). A combination of starch was also used in a ratio of 1.5% OSA starch (RD 474™) to 3.5% waxy maize starch (AMISOL™ 4000); both starches were obtained from Corn Products. Added fat and/or oil was homogenised with the meat and other ingredients in a cutter for approximately 10 minutes according to the following procedure: the meat (at an initial temperature of around 1°C) was combined with the regular salt, cure salt and seasoning mix and comminuted for two minutes. Next, half of the ice was added; after one minute, the vegetable oil and/or pork fat was added. The fat was added directly, which

Table 1 - Formulations used in mortadella preparation (percentages of 15 kg formulations).

Raw material	Oil	50/50	Pork
Vegetable oil (%)	13.00	6.50	---
Beef (%)	56.85	56.03	55.60
Pork backfat (%)	---	7.62	15.22
Added ice (%)	20.00	19.70	19.03
Subtotal (%)	89.85	89.85	89.85
Other ingredients (%)	10.15	10.15	10.15
Total (%)	100	100	100

means that neither the oil nor the pork fat was pre-emulsified. The mass was then chopped for two minutes, and the isolated soy protein was added. After one additional minute, the rest of the ice was added. The mass was chopped for one more minute and the starches were added. Finally, the entire mass was chopped for about two minutes and was removed with a temperature of around 14°C. The emulsion was stuffed into 70 mm diameter cellulosic casings; each unit was approximately 500 g. The mortadellas were heat-processed in a smokehouse for about 4 hours until the internal temperature of the coolest product reached 72°C. Temperature was measured with thermocouples placed in the centre of three sausages located in different areas of the smokehouse. The temperature of the smokehouse varied from an initial 55° to 85°C at the end of the cooking process. After they were cooked, the mortadellas were cooled in a cold water shower, vacuum packed and stored at 0°C (±1°C) until analysis. The sausages were analysed between five and seven days after they were processed. The sausages were kept under vacuum to avoid dehydration during cold storage that can result from the use of cellulosic casing that are permeable to water vapour. This method of packaging (cellulosic casing plus vacuum storage) is also used by the leading commercial mortadella producer in Brazil.

Physical and chemical determinations

The official AOAC methodology (CUNNIF, 1998) was used to determine moisture, protein, lipid and ash contents. These determinations were made in triplicate for each treatment.

Two tests were conducted to characterise the texture of the products; both tests used a texturometer (TA.XT2i, SMS) at 25°C. Ten cylinders of each treatment were tested as follows:

- To measure hardness (compression strength: maximum force required to compress the samples), the products were cut into 20x20 mm cylinders and submitted to compression tests with a 20 mm cylindrical probe moving at 500 mm/min until 50% deformation was achieved.

- To measure tenderness (shear force: maximum force required to shear the samples on their round surface), the products were cut into cylinders with a diameter of 14 mm and a height of 20 mm and sheared with a Warner-Bratzler probe moving at a speed of 500 mm/min.

- To calculate the process yield, stuffed mortadellas were weighed before they entered the smokehouse and immediately after they were cooked and cooled. The yield was calculated using the following equation: Yield (%) = (final weight / initial weight) x 100.

- To assess water activity, mortadellas were crushed and homogenised. Water activity was measured by means of the Aqualab apparatus (Series 3TE) at 25°C. This analysis was performed in triplicate for each treatment.

- To determine pH values, the electrode of a portable pH meter (model F-1002) was immersed in a solution created with 200 g of mortadella sample per litre of water.

Fatty acid composition and cholesterol determination

A three-stage process was used to determine the fatty acid composition. Lipid extraction was performed using the method described by FOLCH *et al.* (1957), and fatty acid esterification was performed using the method described by HARTMAN and LAGO (1973). Finally, gas chromatography was performed. The hexanic fraction containing the esters was analysed in a gas chromatograph (HP 5890 Series II) equipped with a capillary column (DB-23 from Agilent) with dimensions of 60 m x 0.25 mm x 0.25 μm; the capillary column was coupled to a flame ionisation detector. The gas flow included 1 mL/min of helium, 10 mL/min of hydrogen and 100 mL/min of air. The following temperature gradient was used: 130°C for 1 minute, from 130° to 170°C at 6.5°C/min, from 170° to 215°C at 2.8°C/min, 215°C for 12 minutes, from 215° to 230°C at 40°C/min, and 230°C for 3 minutes. The temperature in the injector was 270°C and the temperature in the detector was 280°C. Saturated and unsaturated fatty acids with 6, 8, 10, 12, 14, 15, 16 (*cis* and *trans*), 17, 18 (*cis* and *trans*), 20, 22 and 24 carbon atoms were analysed through comparison with data obtained from the GC authentic standards (SUPELCO - Catalog. No 18920-1AMP) that were methylated and eluted under the same conditions. The calculations were made by a computer coupled to the gaschromatograph using the GC Solution software.

Cholesterol content was determined in two stages. Cholesterol was first extracted and then submitted to high-performance liquid chromatography (HPLC). The analysis was conducted, with some adjustments, according to the method described by SALDANHA *et al.* (2004). For the cholesterol extraction, 2 g of KOH and 50 mL of alcohol were added to 2 g of the homogenised

sample. Flasks containing this solution were placed in a water bath at 90°C for 30 minutes and then cooled under running water and ice; 50 mL of distilled water were added. The solution was transferred to a separation funnel and 20 mL of hexane were added. The funnels were agitated and left to rest until phase separation. The lower phase was collected in another separation with the addition of hexane and was discarded. The collected solutions (hexane phases) were washed with distilled water. The filtrate was evaporated, first in a water bath at 90°C and then with N_2. A liquid chromatograph (SHIMADZU) with a photodiode array detector was used for the high-performance liquid chromatography. A C18 (250 mm x 4.6 mm x 5 μm) Regis analytical column was used. The mobile phase consisted of acetonitrile and isopropanol in a ratio of 85:15; the flow rate was 1.0 mL/min and the analysis was conducted for 12 minutes at 40°C. Cholesterol was identified by comparing the retention time of the samples with the standard (SIGMA C8667-1G) and quantified through the corresponding peak areas by internal standardisation.

Sensory evaluation

To evaluate the sensory characteristics of the mortadellas, acceptance tests were performed to evaluate the differences perceived by consumers in the various formulations. Sixty consumers were recruited from students, faculty and staff at the Sao Paulo University (USP), Pirassununga, Brazil; consumers were recruited based on their affinity for the product. The samples were sliced into approximately 1.5 mm thick pieces just before the beginning of the tests and kept in a refrigerator (8°±1°C) during the entire sensory evaluation (about 3 hours). Consumers were accommodated in individual tasting booths where they received instructions about the use of the scale, the nature of the products and the type of evaluation to be carried out. Samples were served under white light in a randomised and monadic manner. The samples were served on small plastic plates coded with three-digit random numbers and with a plastic fork. For each sample, two superposed slices were served.

The consumers were asked to use a nine-point hedonic scale to evaluate the samples with regard to colour, odour (which should be evaluated by raising one of the slices of mortadella and smelling between the slices), flavour, texture and overall quality.

Statistical analyses

A completely random design with 3 treatments and 2 replicates was used. The data on physicochemical and technological parameters were analysed with an analysis of variance (ANOVA) to compare results among the treatments; means were compared using Tukey's test. Consumer acceptance data were analysed by ANOVA, using a mixed model, considering the random effect for panelist and the fixed effect for treatment. To evaluate the differences among the samples, a comparison of the means was carried out using Student's t test. Significance was defined at $P \leq 0.05$. SAS® (©2000, SAS Inst. Inc.) software was used for the statistical analyses.

RESULTS AND DISCUSSION

Physical and chemical characteristics

There were no significant differences ($P > 0.05$) in the moisture, protein, fat and ash contents of the various treatments (Table 2); these findings show that the calculations used to determine the proximate composition of the formulations were adequate and that the emulsification and cooking processes did not affect the final composition of the products. Other researchers have also evaluated the use of vegetable oil instead of animal fat in sausages and have found no differences in the proximate composition of the products (BLOUKAS *et al.*, 1997; VALENCIA *et al.*, 2008; YILDIZ-TURP and SERDAROGLU, 2008). The fat, protein and moisture contents (Table 2) of the final products were in accordance with Brazilian legislation (BRAZIL, 2000) establishing the "Identity Standards and Quality of Mortadella"; these standards set maximum and minimum levels for some of these contents (maximum moisture of 65%, maximum fat of 30% and minimum protein of 12%). In addition to meeting current legal regulations, the mortadella formulations also simulated conditions normally found in the market. Some national commercial mortadella, made without apparent pork backfat pieces, contain 12.5% protein and 15% fat.

The hardness (compression strength) and tenderness (shear force) did not change significantly ($P > 0.05$) when animal fat was replaced with soybean oil (Table 2). Similarly, when 60% of the pork backfat was replaced with olive oil in a cooked meat batter with a total fat content of approximately 11%, FERNANDEZ-MARTÍN *et al.* (2009) found no difference in the hardness of the products (values were around 24-26 N). On the other hand, AMBROSIADIS (1996) found that the shear force values of frankfurter sausage made with 19.5% pork backfat were higher (1.7 kg) than those of frankfurters made with 19.5% soya oil (1.2 kg). The shear force values observed in this experiment (between 1.6 and 1.7 kg) were higher than those found by TRINDADE *et al.* (2005) for mortadella prepared with mechanically separated chicken meat (between 0.3 and 0.7 kg). The higher shear force values found in this experiment may be due to the use of cellulosic casing, which is susceptible to moisture

Table 2 - Averages (and standard deviations) of the physico-chemical parameters of different mortadella formulations.

	Oil	50/50	Pork
Moisture (%)	60.49±0.27[a]	59.71±0.91[a]	59.30±0.091[a]
Fat (%)	13.99±0.61[a]	14.84±0.48[a]	15.16±0.25[a]
Protein (%)	14.03±0.30[a]	14.58±0.13[a]	14.85±0.25[a]
Ashes (%)	3.81±0.15[a]	3.80±0.06[a]	3.88±0.07[a]
Process yield (%)	88.06±2.47[a]	88.00±3.07[a]	88.98±2.60[a]
Water activity	0.95±0.01[a]	0.94±0.01[a]	0.95±0.01[a]
pH	6.21±0.02[a]	6.22±0.04[a]	6.21±0.06[a]
Texture parameters			
Hardness (N)	61.50±6.40[a]	63.00±7.10[a]	58.00±1.40[a]
Tenderness (kg)	1.75±0.07[a]	1.65±0.49[a]	1.60±0.71[a]

Oil: mortadella made with 100% soybean oil.
50/50: mortadella made with 50% pork backfat and 50% soybean oil.
Pork: mortadella made with 100% pork backfat.
[a] Averages with the same letters on the same row are not significantly different (0.05).

loss and may lead to dehydration in the cooking process.

No significant differences ($P > 0.05$) were observed in the process yields of the various treatments (Table 2). Because oils have a lower melting point than animal fat, greater losses due to dripping oil during the cooking process could adversely affect of the process yield; however, this effect was not confirmed. Emulsion stability and consequent losses due to cooking can be influenced by several factors, including the functionality of myofibrillar proteins, temperature, size of the fat droplets, pH, viscosity and the amount of salt used in the formulation (PARDI, 1994; COFRADES *et al.*, 1998). All of these factors, combined with the addition of starch and soy protein in the various formulations, ensured stability and adequate yield despite the use of vegetable oil instead of animal fat (MATULIS *et al.*, 1995). PAPPA *et al.* (2000) presented results similar to those of the present study; these researchers found no change in the process yield of low-fat frankfurters when pork backfat was replaced with olive oil (0-100%). HSU and YU (2002) evaluated an emulsified meatball (Kung Wan) prepared with various vegetable oils in place of pork fat and found no differences in the yield assessments during cooking between the control product (prepared with pork fat) and the product prepared with soybean oil.

All of the water activity values were approximately 0.95; there were no significant differences ($P > 0.05$) among the treatments (Table 2). This finding was expected because the formulations were calculated to present the same moisture contents; in addition, the same ingredients were used in all of the treatments. Furthermore, the source of variation between treatments (vegetable oil or pork fat) does not influence water activity. The water activity values found in the present work are similar to those found by other researchers examining mortadella Bologna-type sausages; CÁCERES *et al.* (2008) found that sausages with 1-6% fish oil instead of pork backfat had a water activity value of 0.96-0.98, and NOWAK *et al.* (2007) found that sausages with 3-12% inulin instead of pork backfat had a water activity value of 0.97.

The pH value of the mortadellas in this study was 6.2 and did not differ ($P > 0.05$) among the various treatments (Table 2). Our data agree with those of PAPPA *et al.* (2000), who also did not observe differences in the pH values (about 6.5) of low-fat frankfurters prepared with olive oil (0-100%) instead of pork backfat. The pH value may have an effect on proteins, which reach their maximum emulsifying capacity when the pH is close to neutral (MOHLER, 1982; MATULIS *et al.*, 1995). The results of this study indicate that the meat proteins in the tested mortadellas show a good emulsifying capacity because the pH values were reasonably close to neutral.

Fatty acid profile and cholesterol content

The "Oil" treatment contained 51.32% polyunsaturated FA (PUFA) and 26.40% saturated FA (SFA), the "50/50" treatment contained 33.69% PUFA and 34.89% SFA and the "Pork" treatment contained 16.00% PUFA and 46.60% SFA. The PUFA/SFA ratios of the three treatments were 1.94, 0.97 and 0.34, respectively (Table 3). The World Health Organization (WHO, 2010) recommends a PUFA/SFA intake ratio of one or higher; the Oil treatment exceeds this recommendation and the 50/50 treatment almost achieves it. The linoleic and α-Linolenic acid contents were higher ($P \leq 0.05$) in mortadella made with 100% soybean oil, and mortadella with 50% soybean oil gives an intermediate value that does not differ significantly ($P > 0.05$) from that of mortadella made with 100% soybean oil or with 100% pork backfat. On the other hand, the pork formulation shows a higher ($P \leq 0.05$) content of oleic acid. MUGUERZA *et al.* (2003) evaluated "Chorizo de Pamplona" and observed that, as pork backfat

Table 3 - Fatty acid profile (%) and cholesterol levels (mg/100 g) in different mortadella formulations.

Fatty acid	Oil	50/50	Pork
Myristic (C14:0)	0.95±0.35[a]	1.59±0.68[a]	2.70±1.18[a]
Palmitic (C16:0)	16.89±2.16[a]	21.90±3.88[a]	30.15±5.77[a]
Palmitoleic (C16:1)	0.60±0.22[a]	2.07±0.58[a]	3.79±1.09[a]
Margaric (C17:0)	0.52±0.16[a]	0.66±0.20[a]	0.75±0.22[a]
Stearic (C18:0)	8.03±1.38[a]	10.75±0.25[a]	13.00±1.63[a]
Oleic (C18:1)	20.83±0.72[a]	28.44±0.65[a,b]	32.47±2.61[b]
Linoleic (C18:2)	46.67±3.90[a]	31.36±5.04[a,b]	16.00±2.88[b]
α-Linolenic (C18:3)	4.15±0.36[a]	2.33±0.63[a,b]	0.93±0.32[b]
CLA[1]	0.50±0.02[a]	0.41±0.05[a,b]	0.27±0.09[b]
CLA[2]	0.26±0.08[a]	0.21±0.03[a]	*
Arachidic (C20:0)	0.44±0.01[a]	0.35±0.02[b]	0.29±0.06[b]
Gadoleic (C20:1)	0.25±0.05[a]	0.37±0.20[a]	0.80±0.27[a]
SFA	26.40	34.89	46.60
UFA	72.74	64.20	52.25
MUFA	21.42	30.51	36.26
PUFA	51.32	33.69	15.99
ω-3	4.15	2.33	0.93
ω-6	46.67	31.36	15.99
PUFA/SFA	1.94	0.97	0.34
ω-6/ω-3	11.25	13.48	17.19
Cholesterol	33.34±2.71[a]	36.26±3.01[a]	39.78±3.82[a]

Mean±SE.
Oil: mortadella made with 100% soybean oil.
50/50: mortadella made with 50% pork backfat and 50% soybean oil.
Pork: mortadella made with 100% pork backfat.
[1]Conjugated linoleic acid (*cis* 9, *trans* 11); [2]Conjugated linoleic acid (*trans* 10, *cis* 12).
SFA = total of saturated fatty acids; UFA = total of unsaturated fatty acids; MUFA = total of monounsaturated fatty acids; PUFA = total of polyunsaturated fatty acids.
*Values not identified through gas chromatography.
[a, b]Averages with the same letters on the same row are not significantly different (0.05).

was increasingly replaced with soybean oil (0-25%), SFA, and particularly palmitic acid (23.7 for 18.8 g/100 g) and myristic acid (1.4 for 1.0 g/100 g), decreased while PUFA increased (15.2 for 23.9 g/100 g).

As shown in Table 3, the 50/50 treatment contained approximately 56% of the ω3 content found in the Oil treatment; this finding shows that the amount of ω3 was proportional to the amount of soybean oil added. Vegetable oils are the main source of ω6 fatty acids. The amount of ω6 in the three formulations declined considerably with the amount of soybean oil (i.e., Oil treatment > 50/50 treatment > Pork treatment). The ω6 content was reduced by approximately 30.7% in mortadellas made with 100% pork backfat when compared to the mortadellas made with 100% soybean oil. In the 50/50 treatment, the ω6 content was reduced by exactly 49.85% of this 30,7% reduction, showing that the ω6 reduction results exclusively from the reduction in the soybean oil content. According to MOREIRA and MANCINI-FILHO (2004), the proportions of ω6 and ω3 fatty acids should be balanced. Long-chain polyunsaturated fatty acids (EPA and DHA) are formed from the elongation and desaturation of linoleic and α-linolenic acids. When they are insufficient in diet, the body has to synthesise them from counterparts with a higher number of carbon atoms. Excessive linoleic acid will compete with the α-linolenic acid for desaturases, possibly leading to a low production of eicosapentaenoic acid. The World Health Organization (WHO, 2010) recommends that ω6 fatty acids make up between 5-8% of the total daily fat intake for healthy individuals and that ω3 fatty acids make up between 1-2% of the total daily fat intake. It could therefore be inferred that a ω6 to ω3 ratio of about 4:1 is necessary to maintain a balanced diet. In this study, the Oil treatment showed this proportion with a value of 11.25 (Table 3); this value is higher than the recommended value but is lower than the value shown by the Pork treatment (17.19).

The Oil treatment showed a higher ($P \leq 0.05$) CLA[1] content than the Pork treatment (Table 2). When we measured the total trans conjugated linoleic acid content (CLA[1] + CLA[2]), we found that the Oil and 50/50 treatments showed high amounts of total CLA (0.76 and 0.62%, respectively) when compared with the Pork treatment (0.27%). CLA is a mixture of positional and geometric isomers of linoleic acid found primarily in dairy and meat products. Several studies indicate that CLA is a powerful compound that affects several different kinds of cancers, metastases, atherosclerosis, diabetes, immunity, and body fat/protein composition. This type of fat-

ty acid is unique because it is present in food from animal sources, and its anticancer efficacy is expressed at concentrations close to human consumption levels (KRAMER *et al.*, 2004; PARIZA *et al.*, 2001).

The average cholesterol content of the three treatments did not differ significantly ($P > 0.05$) and were approximately 33 mg/100 g and 40 mg/100 g of sample for the Oil and Pork treatments, respectively (Table 3). This minor difference in the cholesterol content of the two treatments is probably because the beef meat contributes most of the cholesterol to the mortadellas. This type of meat, with 3% fat, contains approximately 52 mg of cholesterol per 100 g of meat (ALMEIDA *et al.*, 2006). The tested mortadellas contained 54-58% beef meat (which contained approximately 3.5% fat); it is therefore possible that approximately 30-35 mg of the cholesterol present in every 100 g of mortadella were from the beef. A small contribution from pork backfat to the cholesterol content of mortadellas could also be expected. The cholesterol level in pork backfat is around 53 mg per 100 g (BRAGAGNOLO and RODRIGUEZ-AMAYA, 2002), and the 50/50 and Pork treatments included only 7.5% and 15% pork backfat, respectively. MUGUERZA *et al.* (2003) found that cholesterol levels in Chorizo de Pamplona made with soybean oil (15-25%) instead of pork backfat were higher (92.96-87.71 mg/100 g) than those found in the present study; this higher cholesterol level was probably due to the higher level of fat (30%) added to the Chorizo de Pamplona. In general, the cholesterol levels found in all of the mortadella formulations were relatively low; a 100 g mortadella would contribute little more than 10% of the American Heart Association's (2009) recommended maximum daily intake of 300 mg of cholesterol.

Sensory evaluation

When sensory characteristics were evaluated (i.e., colour, odour, flavour, texture and overall acceptability), mortadellas made with 100% pork backfat scored significantly better ($P \leq 0.05$) on a number of variables than those made with 100% soybean oil (Table 4). Mortadellas made with 100% pork backfat and 50/50 pork fat and soybean oil had similar ($P > 0.05$) colour and texture attributes, although the pork formulation was more acceptable ($P \leq 0.05$) than the 50/50 formulation in the other attributes. However, while the Oil and 50/50 formulations had a similar odour and flavour, the 50/50 mortadella was significantly more acceptable ($P \leq 0.05$) than the Oil mortadella in colour, texture and overall acceptability. AMBROSIADIS *et al.* (1996) observed a similar decrease in the sensory acceptability of emulsified cooked meat products when pork fat was replaced with vegetable oil (soybean, sunflower, cotton-seed and corn); however, their products maintained good quality for consumption. BROCE *et al.* (2007) evaluated the acceptability of the odour and taste of low fat chicken frankfurters that had a 10% fat content and were made with pork fat (control) or vegetable oil (canola, sunflower, olive or a mixture). These researchers found that the hedonic scores for the control sausage were highest and those for the sausage made with sunflower oil were the lowest; however, neither product showed scores that were significantly different from those attributed by the untrained panel to the rest of the vegetable oil sausages. CÁCERES *et al.* (2008) added pre-emulsified fish oil (1-6%) to mortadella as a source of PUFA n-3 and evaluated the sensory acceptability of the products using a hedonic scale. Even with the addition of 6% fish oil, no changes were observed in colour, texture and overall acceptability and all of the batches were rated as generally acceptable.

All of the treatments in our study were acceptable; the Oil treatment received an average score of about 6 (liked moderately) while the Pork treatment received an average score of about 7. Moreover, absolute differences among the scores obtained for the three products, for all parameters, were small (less than 0.9 on a nine-point hedonic scale). For example, the difference in the overall acceptability scores between the Pork and 50/50 treatments was smaller than 0.5. When developing this kind of product, the meat industry should consider the need to balance a loss in the sensory profile with a gain in the nutritional profile.

CONCLUSIONS

The use of vegetable oil instead of animal fat produced a healthier product with an improved fatty acid profile without affecting the physicochemical and technological characteristics of mortadella sausage. However, products with vegetable oil had less acceptable sensory characteris-

Table 4 - Averages (and standard errors) of the sensory acceptance results for the different mortadella formulations.

Attribute	Sensory score*		
	Oil	50/50	Pork
Colour	6.19±0.25^{b}	6.65±0.24^{a}	6.73±0.27^{a}
Odour	6.13±0.24^{b}	6.22±0.27^{b}	6.96±0.21^{a}
Flavour	6.38±0.24^{b}	6.76±0.20^{b}	7.20±0.18^{a}
Texture	6.04±0.26^{b}	6.54±0.23^{a}	6.85±0.19^{a}
Overall acceptability	6.18±0.20^{c}	6.62±0.20^{b}	7.07±0.20^{a}

Oil: mortadella made with 100% soybean oil.
50/50: mortadella made with 50% pork backfat and 50% soybean oil.
Pork: mortadella made with 100% pork backfat.
$^{a, b, c}$Values in the same row followed by same letter do not differ significantly at the level of 5%.
*Average of 60 responses.

tics than those made with the traditional animal fat. Replacing 50% of the pork fat with soybean oil is more acceptable, representing a good balance between nutritional gains and sensory biases.

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REFERENCES

ABIOVE. Associação Brasileira das Indústrias de Óleo Vegetal. Estatística mensal de soja e derivados 2005/2006. [Acessed: 2009 dec 21]. Available from: http://www.abiove.com.br/estatistica_br_0809.html.

AHA. American Heart Association. Cholesterol. [Acessed: 2009 dec 21]. Available from: http://www.americanheart.org/presenter.jhtml?identifier=4488.

Almeida J.C., Perassolo M.S., Camargo J.L., Bragagnolo N. and Gross J.L. 2006. Fatty acid composition and cholesterol content of beef and chicken meat in Southern Brazil. Rev. Bras. Cienc. Farm. 42:109-117.

Ambrosiadis J., Vareltziz K.P. and Georgakis S.A. 1996. Physical, chemical and sensory characteristics of cooked meat emulsion style products containing vegetable oils. Int. J. Food Sci. Technol. 31:189-194.

Bloukas J.G., Paneras E.D. and Fournitzis G.C. 1997. Effect of replacing pork backfat with olive oil on processing and quality characteristics of fermented sausages. Meat Sci. 45(2):134-144.

Bragagnolo N. and Rodriguez-Amaya D.B. 2002. Teores de colesterol, lipídios totais e ácidos graxos em cortes de carne suína. Ciênc Tecnol. Aliment. 22:98-104.

Brasil. Leis, decretos, etc. Instrução Normativa nº 4 de 31 de mar.2000 da Secretaria de Defesa Agropecuária do Ministério da Agricultura e do Abastecimento. Aprova os Regulamentos Técnicos de Identidade e Qualidade de Carne Mecanicamente Separada, de Mortadela, de Linguiça e de Salsicha. Diário Oficial, Brasília, 05 abr. 2000, Seção 1, p. 6-10.

Broce K.D., González-Sanjosé M.L., Jaime I., Linssen J.P.H. and Rovira J. 2007. Low fat chicken frankfurters manufactured with vegetable oils. 53rd International Congress of Meat Science and Technology, Beijing, China. Book of Abstracts 567-568.

Cáceres E., García M.L. and Selgas M.D. 2008. Effect of pré-emulsified fish oil – as source of PUFA n-3 – on microstructure and sensory properties of mortadella, a Spanish bologna-type sausage. Meat Sci. 80:183-193.

Cofrades S. and Jiménez-Colmenero F. 1998. Protein molecular interactions involved in the formation of frankfurters: Effect of fat level and heating rate. Meat Sci. 49 (4):411-423.

Cunnif P. (Ed.). Official Methods of Analysis of AOAC International. Arlington V.A. AOAC International, 16th Ed. 4th revision. 1998. Chapter 16, p. 26-27.

Fernandez-Martín F., López-López I., Cofrades S. and Jiménez Colmenero F. 2009. Influence of adding Sea Spaghetti seaweed and replacing the animal fat with olive oil or a konjac gel on pork meat batter gelation. Potential protein/alginate association. Meat Sci. 83:209-217.

Folch J., Lees M. and Stanley G.H.S. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 22:497-509.

Hartman L. and Lago R. 1973. Rapid preparation of fatty acid methyl esters from lipids. Lab Practice 22:475-476.

Hsu S.Y. and Yu S.H. 2002. Comparisons on 11 plant oil fat substitutes for low-fat Kung-wans. J. Food Eng. 51:215-220.

Jimenez-Colmenero F., Carballo J. and Cofrades S. 2001. Healthier meat and meat products: their role as functional foods. Meat Sci. 59:5-13.

Kramer J.K.G., Cruz-Hernandez C., Deng Z., Zhou J., Jahreis G. and Dugan M.E.R. 2004. Analysis of conjugated linoleic acid and *trans* 18:1 isomers in synthetic and animal products. Amer. J. Clin. Nutr. 79, 1137S-1145S.

Maia F.J., Branco A.F., Mouro G.F., Coneglian S.M., Santos G.T., Minella T.F. and Guimarães K.C. 2006. Inclusão de fontes de óleo na dieta de cabras em lactação: produção, composição e perfil dos ácidos graxos do leite. R. Bras. Zootec. 35:1504-1513.

Matulis R.J., Mckeith F.K., Sutherland J.W. and Brewer M.S. 1995. Sensory characteristics of frankfurters as affected by fat, salt and pH. J. Food Sci. 60(1):42-47.

Mohler K. 1982. El curado. Zaragoza: Acribia. 116 p.

Moreira A.V.B. and Mancini-Filho J. 2004. Influência dos compostos fenólicos de especiarias sobre a lipoperoxidação e o perfil lipídico de tecidos de ratos. Rev. Nutr. 17:411-424.

Muguerza E., Ansorena D. and Astiasarán I. 2003. Improvement of nutritional properties of Chorizo de Pamplona by replacement of pork backfat with soy oil. Meat Sci. 65:1361-1367.

Nowak B., Von Mueffing T., Grotheer J., Klein G. and Watkinson B.M. 2007. Energy content, sensory properties and microbiological shelf life of German bologna-type sausages produced with citrate or phosphate and with inulin as fat replacer. J. Food Sci. 72(9):629-638.

Pappa I.C., Boukas J.G. and Arvanitoyannis I.S. 2000. Optimization of salt, olive oil and pectin level for low-fat frankfurters produced by replacing pork backfat with olive oil. Meat Sci. 56:81-88.

Pardi M.C. 1994. Ciência, higiene e tecnologia da carne. 2nd Ed. Goiânia: CECRAF-UFG. 1107 p.

Pariza M.P., Park Y. and Cook M.E. 2001.The biologically active isomers of conjugated linoleic acid. Prog. Lipid Res. 40:283-98.

Pereira N.R., Tarley C.R.T., Matsushita M. and Souza N.E. 2000. Proximate composition and fatty acid profile in Brazilian poultry sausages. J. Food Comp. Anal. 13:915-920.

Saldanha T., Mazalli M.R. and Bragagnolo N. 2004. Avaliação comparativa entre dois métodos para determinação do colesterol em carnes e leite. Ciênc Tecnol Aliment 24:109-113.

SOCESP. Sociedade de Cardiologia do Estado de São Paulo. Os diferentes tipos de gordura no sangue. [Accessed: 2006 nov 20). Available from: http://www.socesp.org.br/espaco_leigo/colesterol_fator_risco.asp.

Trindade M.A., Contreras C.J.C. and Felício P.E. 2005. Mortadella sausage formulations with partial and total replacement of beef and pork backfat with mechanically separated meat from spent layer hens. J. Food Sci. 70:236-241.

Valencia I., O`Grady M.N., Ansorena D., Astiasarán I. and Kerry J.P. 2008. Enhancement of the nutritional status and quality of fresh pork sausages following the addition of linseed oil, fish oil and natural antioxidants. Meat Sci. 80:1046-1054.

WHO, 2010. World healthy organization. Population nutrient intake goals for preventing diet-related chronic diseases. Available from: http://www.who.int/nutrition/topics/5_population_nutrient/en/print.html. Acessed: April 23, 2010.

Yildiz-Turp G. and Sedaroglu M. 2008. Effect of replacing beef fat with hazelnut oil on quality characteristics of sucuk – A Turkish fermented sausage. Meat Sci. 78:447-454.

Yilmaz I., Simsek O. and Isikli M. 2002. Fatty acid composition and quality characteristics of low-fat cooked sausages made with beef and chicken meat, tomato juice and sunflower oil. Meat Sci. 62:253-258.

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PAPER

A MATHEMATICAL MODEL TO DESCRIBE MALOLACTIC FERMENTATION

A. ZINNAI, F. VENTURI, M.F. QUARTACCI and G. ANDRICH*
Dipartimento di Biologia delle Piante Agrarie, Università di Pisa,
Via del Borghetto 80, 56124 Pisa, Italy
*Corresponding author: Fax +39 50 2216636,
e-mail: gandrich@agr.unipi.it

ABSTRACT

The study aimed at determining mathematical models able to describe the kinetics of substrates consumptions (L-malate and D-glucose) and synthesis of end-products involved in malolactic and lactic fermentations promoted by *Oenococcus oeni* ML 34. The strain was added to aqueous solutions of L-malate or/and D-glucose and to a white wine. The time evolution of bacterial cells and concentrations of both substrates and final products were evaluated by mathematical equations based on a same kinetic approach which involved the introduction of a reduced number (five) of functional parameters characterised by a well identified biological meaning, namely bacterial charge, substrate consumption and accumulation of end-products.

- Key words: kinetic models, malolactic fermentation, lactic fermentation, *Oenococcus oeni* -

INTRODUCTION

Because of the effects induced by malolactic fermentation (MLF) on sensorial characteristics of wines, this secondary fermentation is particularly desirable for structured red or white products (VERSARI *et al.*, 1999), but it can produce deleterious effects on microbial stability and organoleptic properties of not-structured wines produced in warm climatic zones, which are often characterised by low malic acidity (HERVÉ *et al.*, 2004).

The MLF is promoted by some Gram+ Eubacteria, not sporigens (*Oenococcus, Leuconostoc, Pediococcus, Lactobacillus*) which prefer anaerobic conditions but can proliferate also in air (O_2 tolerant), and involves the decarboxilation of L(-) malic acid to produce mainly α, D- and L-lactic acid (GRANCHI *et al.*, 2005; LONVAUD-FUNEL, 1999; SAAYMAN and VILJOEN-BLOOM, 2006; VOLSCHENK *et al.*, 2006). When the MLF is promoted by *Oenococcus oeni*, the L form largely prevails on the other isomer (LIU, 2003).

The synthesis of D-lactic acid may occur either by direct racemisation (GARVIE, 1980) or reduction of the intermediate pyruvate catalysed by D-lactic acid dehydrogenase. In its turn, pyruvate can be produced by L-lactate dehydrogenation (LIU, 2003) or by reduction of L-malate to give oxalacetate which, after decarboxylation, produces pyruvate which is subsequently reduced by L- or D-lactic acid dehydrogenase to give L or D-lactate (LAFON-LAFOURCADE, 1975).

When reducing environmental conditions are ensured, *O. oeni* converts D-glucose to give, via the pentose phosphate pathway, ethanol, CO_2 and mainly D-lactic acid together with more reduced amount of the L-isomer.

Although the conversion of L-malate to L/D-lactate has been widely investigated (ZAPPAROLI *et al.*, 1998; LIU, 2003; COUCHENEY *et al.*, 2005) more information on the kinetics involved in this transformation is needed to allow a better control of this fundamental process. Due to the importance of MLF in winemaking, the development of a mathematical model based on few parameters characterised by a clear biological meaning might represent a valid tool to allow a better control of this fundamental process involved in winemaking of many important wines. Moreover, the kinetic approach followed and the related mathematical algorithms obtained have not been previously considered in this context.

In an attempt to reduce the large number of possible variables which could affect the MLF kinetics, in this study the time course of malic acid conversion was investigated by aqueous buffered (pH = 3.4) model solutions of L-malate or/and D-glucose into which heterofermentative lactic acid bacteria (*O. oeni* ML 34 strain) were inoculated. To validate the MLF kinetic model developed on the basis of the experimental data collected using the solutions, the experimental points concerning the time evolution of L-malic acid in a white wine were compared with the theoretical values calculated by the model.

MATERIALS AND METHODS

The experimental kinetic runs used to identify the values to be assigned to the different equation parameters were carried out utilising a batch reactor able to ensure anaerobic (N_2) and sterile conditions. The characteristics of this bioreactor, realised at the Dipartimento di Biologia delle Piante Agrarie of the University of Pisa, were already reported in a previous paper (ANDRICH *et al.*, 1988). Briefly, the bioreactor was formed by a three bottle-neck flask with a central neck joined to a bubble cooling column. A lateral neck was joint to a suitable apparatus which allowed the sampling of homogenous reaction medium aliquots under sterile and reduced condition obtained by an overpressures of N_2 previously sterilised by filtration. A second lateral neck was used to inoculate *O. oeni* bacteria previously separated from their growth media by filtration. The fermentation temperature was maintained by an heat exchanger, whereas the homogeneity of the reaction medium was ensured by a magnetic stirrer.

O. oeni ML 34 cells (Collection of the Department of Crop Biology, Pisa) were grown in sterile conditions in a modified MRS medium (DE MAN *et al.*, 1960) (Fluka, Milan, Italy), named MRS-TJC and containing 20 g l^{-1} Tomato Juice Broth (Fluka), 5 g l^{-1} L-malic acid (Fluka) adjusted to pH 5.

The bioreactor was initially filled with 500 mL of an acetate buffer aqueous solution (pH = 3.4) containing L-malic acid or D-glucose at the concentrations of 10 and 8 g L^{-1}, respectively. These solutions, before being added to the flask, were sterilised by filtration (0.2 μm). The bacterial cells were separated from their growth medium by filtration, repeatedly rinsed with the sterile acetate buffer solution and then added to the reaction medium by an inverse flow of the sterile buffer solution. The inoculation represented the initial time (t=0) of the fermentation process, which the following sampling time points refer to. The experimental runs were carried out under inert atmospheres (100% N_2 or CO_2). At each time point a sample of the reaction media was collected by the sampling apparatus and divided in two aliquots. The first aliquot was used to determine the number of bacterial viable cells by a total plate count on tomato juice agar (incubation at 30°C for 7 days), while the second aliquot was previously heated to inactivate the *O. oeni* ML 34 cells and then analysed to determine the concentrations

of substrates (L-malate or D-glucose) and final products (L/D-lactate, ethanol) using specific commercial (Megazyme) enzymatic kits (ANDRICH *et al.*, 1988). In order to validate the kinetic model, the same experimental procedure above cited was carried out filling the bioreactor with both L-malate and D-glucose (run f) or using a sterilised 'Trebbiano toscano' white wine (pH=3.18±0.01; titrable acidity=9.1±0.2 g L^{-1} tartaric acid; malic acid=4.12±0.02 g L^{-1}; lactic acid (D- + L-)= 0.66±0.1 g L^{-1}; volatile acidity = 0.56±0.03 g L^{-1} acetic acid; SO_2 =not detectable; alcoholic degree = 10.5±0.1; residual sugars < 2 g L^{-1}) added with malic acid to reach a concentration equal to 4 g L^{-1} (run w).

The identification of the best values to be assigned to the model parameters was carried out by a specific statistical programme (BURENL) able to identify in a space of j-dimensions (where j is equal to the number of model parameters) the minimum value of the F function, which is given by the sum of squares of differences occurring among experimental ($Y_{i,\ exper.}$) and calculated ($Y_{i,calc.}$) data:

$$F = \sum_{i=1}^{N} (Y_{i,calc.} - Y_{i,exper.})^2$$

In this expression N represents the total number of experimental determinations. The values assumed by the model parameters at the minimum of the F function represent the desired best values.

For each experimental run the calculation of the two parameters related to the time evolution of the bacterial cells (k_B = kinetic constant of bacterial population growth [min^{-1}]; B_0 = bacteria concentration [CFU·mL^{-1}] at the initial run time) was carried out utilising the experimental data deriving from the determination of the bacteria concentration (e.g. run 1 reported in Table 1 involved 10 experimental determinations for k_B and B_0 determinations, whereas run a shown in Table 2 involved 9 determinations). To evaluate the kinetic constants related to the time evolution of L-malic acid ($[M]_{t=0}$ = concentration of L-malate initially added; k_M = specific kinetic activity shown by a single cell [L·CFU^{-1}·min^{-1}]) the experimental data related to L-malic acid decrease and L/D-lactic acid accumulation were used (e.g. run 1 reported in Table 1 involved 10 experimental determinations of L-malic acid plus 10 determinations for L- and D- lactic acid, respectively, for a total of 30 experimental values). The $R_{L,M}$ parameter (constant related to the percentage of L-lactic acid produced for unit of L-malic acid converted) was evaluated utilising 20 experimental values (10 for L- and 10 for D-lactic acid, respectively; see run 1 shown in Table 1). To evaluate the kinetic constants related to the time evolution of D-glucose ($[G]_{t=0}$ = concentration of D-glucose initially added; k_G = specific kinetic activity shown by a single cell [L·CFU^{-1}·min^{-1}]) the experimental points related to D-glucose decrease and both ethanol and L-/D-lactic acid productions were used (e.g. run a reported in Table 2 involved 9 experimental determinations for D-glucose plus 9 determinations for ethanol and L/D- lactic acid, respectively, for a total of 36 experimental values). The $R_{L,G}$ parameter (constant related to the percentage of L-lactic acid produced for unit of D-glucose converted) was evaluated utilising 18 experimental points (9 of L- and 9 of D- lactic acid, respectively; see run a reported in Table 2). As regards the two different atmospheres tested (100% N_2 or CO_2), five replications were performed for MLF (Table 1), whereas three replications for N_2 and two for CO_2 were performed for lactic fermentation (Table 2).

Table 1 - Parameters (mean ± CI) and corresponding square values of the correlation coefficients of the kinetic model adopted to describe the time evolution of the bacterial population (B) and L-malate (M), L-lactate (L) and D-lactate (D) concentrations during malolactic fermentation.

Run No.	e.a.†	T (°C)	k_B 10^4 (min^{-1})	B_0 10^{-11} (CFU·L^{-1})‡	k_M 10^{16} (L·CFU^{-1}·min^{-1})	M_0 (mmol·L^{-1})	$R_{L,M}$	r_B^2	r_M^2	r_L^2	r_D^2
1 (10)*	N_2	21.5	0.47±0.01**	0.94±0.02	9.47±8.92	33.1±0.2	0.77±0.03	0.58	0.98	0.96	0.96
2 (9)	N_2	23.5	-2.45±0.01	0.60±0.01	9.13±8.32	39.5±0.2	0.82±0.01	0.71	0.90	0.65	0.79
3 (9)	N_2	23.0	-5.86±0.02	6.35±0.01	9.60±10.5	34.7±0.1	0.82±0.01	0.60	0.98	0.98	0.92
4 (8)	N_2	23.0	1.27±0.03	1.65±0.01	9.86±11.5	68.7±0.2	0.79±0.02	0.44	0.95	0.88	0.98
5 (10)	N_2	23.5	-2.36±0.02	3.86±0.01	8.92±9.30	86.4±0.2	0.76±0.03	0.63	0.98	0.98	0.90
6 (11)	CO_2	22.0	-0.09±0.03	0.53±0.50	8.97±10.3	103.1±0.1	0.92±0.01	0.80	0.98	0.98	0.92
7 (7)	CO_2	21.5	1.42±0.04	1.12±0.42	9.71±12.4	82.3±0.2	0.91±0.02	0.07	0.97	0.96	0.98
8 (8)	CO_2	24.0	-2.63±0.02	1.48±0.02	9.94±11.1	36.7±0.1	0.93±0.02	0.20	0.93	0.97	0.74
9 (8)	CO_2	22.0	0.06±0.01	1.55±0.02	8.43±9.53	34.8±0.2	0.88±0.01	0.12	0.97	0.95	0.59
10 (11)	CO_2	21.5	-0.50±0.02	0.71±0.01	8.38±4.45	77.4±0.1	0.91±0.01	0.46	0.98	0.98	0.84

* number of experimental points; ** $P < 0.05$; † environmental atmosphere (100% N_2 or CO_2); ‡ CFU = colony forming units.
k_B, kinetic constant of bacterial population evolution; B_0, concentration of bacteria at the initial time; k_M, kinetic constant of L-malate conversion; M_0, concentration of L-malate at the initial time; $R_{L,M}$, accumulation rate of L-lactate.

Table 2 - Parameters (mean ± CI) and corresponding square values of correlation coefficients of the kinetic model adopted to describe the time evolution of the bacterial population (B) and D-glucose (G), ethanol (E) and L/D-lactate (L, D) concentrations during lactic acid fermentation.

Run No.	e.a.†	T (°C)	$k_B \cdot 10^4$ (min^{-1})	$B_0 \cdot 10^{-11}$ ($CFU \cdot L^{-1}$)‡	$k_G \cdot 10^{16}$ ($L \cdot CFU^{-1} \cdot min^{-1}$)	G_0 ($mmol \cdot L^{-1}$)	$R_{L,G}$	r_B^2	r_G^2	r_E^2	r_L^2	r_D^2
a (9)*	N_2	23	1.15±0.01*	11.51±0.01	0.68±1.63	42.7±0.1	0.40±0.01	0.88	0.98	0.98	0.98	0.94
b (11)	N_2	24	2.54±0.01	12.69±0.01	0.70±0.35	20.5±0.1	0.40±0.01	0.82	0.86	0.82	0.07	0.86
c (9)	N_2	25	1.33±0.01	3.84±0.01	0.65±0.45	27.1±0.1	0.40±0.01	0.31	0.98	0.91	0.49	0.63
d (7)	CO_2	23	0.15±0.01	2.82±0.01	0.71±1.65	42.8±0.1	0.41±0.01	0.04	0.93	0.93	0.95	0.89
e (8)	CO_2	24	3.01±0.10	6.16±0.01	0.50±0.42	61.4±0.1	0.44±0.01	0.89	0.49	0.42	0.52	0.52

* number of experimental points; ** $P < 0.05$; †environmental atmosphere (100% N_2 or CO_2); ‡CFU = colony forming units.
k_B, kinetic constant of bacterial population evolution; B_0, concentration of bacteria at the initial time; k_G, kinetic constant of D-glucose conversion; G_0, concentration of D-glucose at the initial time; $R_{L,G}$, accumulation rate of L-lactate.

RESULTS AND DISCUSSION

A kinetic model to describe malolactic fermentation

MLF, promoted by the malolactic enzyme, involves the direct decarboxylation of L(-)-malate to produce L(+)-lactate and CO_2 (ARTHURS and LLOYD, 1999; KUNKEE, 1991; LABARRE *et al.*, 1996; LIU, 2003; GRANCHI *et al.*, 2005). Fig. 1 reports the decrease of concentration of L-malate ($[M]_{t=t}$) and the increase in the two stereo isomeric forms L(+) and D(-) of α-lactic acid with time. As together with the predominant presence of the L stereo isomer ($[L]_{t=t}$) also a more reduced amount of the D form ($[D]_{t=t}$) was always detected in the reaction medium, different concomitant and competitive reactions must be involved (LAFON-LAFOURCADE, 1975; LONVAUD *et al.*, 1977; LIU, 2003).

According to the stoichiometry of MLF, the sum of the experimental values determined for the three species involved in this transformation ($[M]_{t=t}$, $[L]_{t=t}$ and $[D]_{t=t}$) did not change significantly with reaction time, to assume values quite constant and statistically equivalent to the concentration of L-malate ($[M]_{t=0}$) initially dissolved inside the bioreactor (Fig. 1):

$$[M]_{t=0} = [M]_{t=t} + [L]_{t=t} + [D]_{t=t} \quad (1)$$

so that no significant accumulation of any other possible intermediate involved in this fermentation process seemed to take place. From equation 1 the following relation can be easily obtained:

$$[M]_{t=0} - [M]_{t=t} = [L]_{t=t} + [D]_{t=t}$$

which, after derivatization respect to the run time, can be written as:

$$-d[M]_{t=t}/dt = [L]_{t=t}/dt + [D]_{t=t}/dt$$

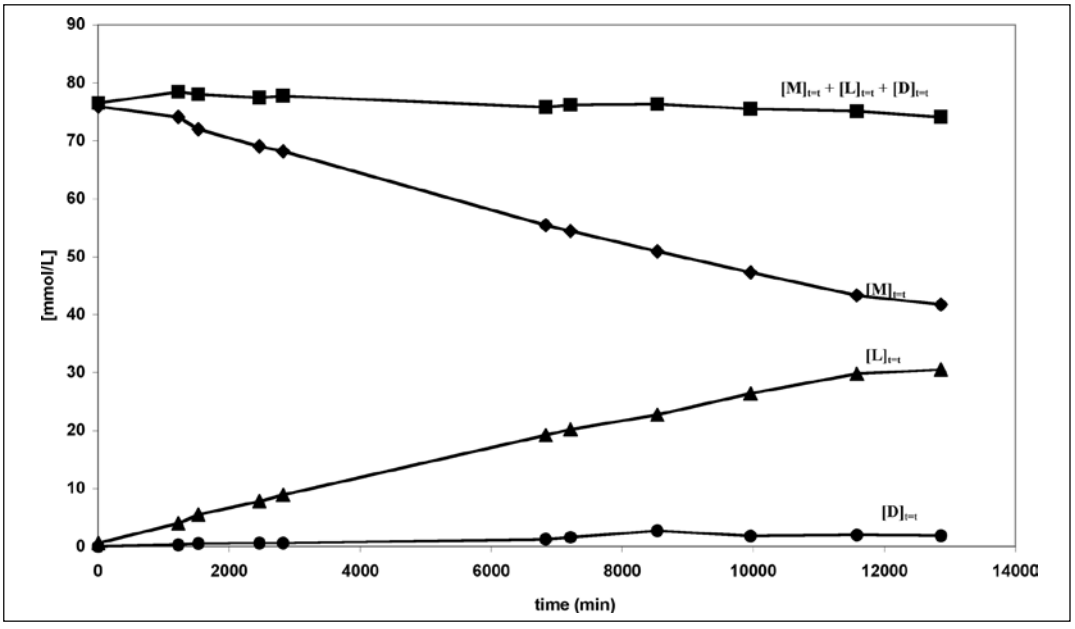


Fig. 1 - Time evolution of the chemical species involved in malolactic fermentation.
M = malic acid; L = L-lactic acid; D = D-lactic acid.

It follows that the rate of L-malate consumption is always equal to the sum of the accumulation rates of L- and D-lactate. If the time evolution of L-malate ($[M]_{t=t}$) decrease is described by a first order kinetics, the following mathematical equation can be written:

$$-d[M]_{t=t}/dt = k' \cdot [M]_{t=t}$$

where $-d[M]_{t=t}/dt$ is the rate of L-malate conversion, $[M]_{t=t}$ is the concentration of L-malate dissolved in the reaction medium at a random time t=t, and k' represents the first order kinetic constant. Assuming the concentration of the enzyme involved in this transformation to be proportional to the density of lactic bacteria cells ($[B]_{t=t}$) in the reaction medium, the following relation can be drawn:

$$k' = k_M \times [B]_{t=t}$$

where the proportional factor k_M is related to the kinetic activity shown by a single active cell of the bacterial population. If the rate of cells density evolution ($d[B]_{t=t}/dt$) during time can be evaluated as the difference between the bacteria replication (r_R) and inactivation (r_D) rates, this expression can be obtained:

$$d[B]_{t=t}/dt = r_R - r_D$$

and assuming that both r_R and r_D rates follow a first order kinetics, the below reported relations can be deduced:

$$d[B]_{t=t}/dt = r_R - r_D = k_R \cdot [B]_{t=t} - k_D \cdot [B]_{t=t} = \\ = (k_R - k_D) \cdot [B]_{t=t} = k_B \cdot [B]_{t=t}$$

where the kinetic constant k_B is equal to the difference between the kinetic constant of the bacteria replication rate and that of their inactivation ($k_B = k_R - k_D$). Thus, to calculate the time course of L-malate concentration in the reaction medium, the following system of two differential equations must be solved:

$$\begin{cases} -d[M]_{t=t}/dt = k' \cdot [M]_{t=t} = k_M \cdot [B]_{t=t} \cdot [M]_{t=t} \\ d[B]_{t=t}/dt = k_B \cdot [B]_{t=t} \end{cases}$$

and this mathematical relation can be obtained:

$$[M]_{t=t} = [M]_{t=0} \cdot e^{k_M/k_B \cdot B_{t=0} \cdot (1 - e^{k_B \cdot t})}$$

where $[M]_{t=0}$ is the L-malate concentration initially (t=0) present in the reaction medium, k_M the kinetic constant of L-malate conversion expressed for active cell, k_B the kinetic constant related to the time evolution of the active bacterial population, and $[B]_{t=0}$ the number of active cells initially present in the unit volume of the reaction medium. This expression is able to satisfy the two limit conditions, namely run time equal to zero (t=0, initial point) and run time tending to infinite ($t \to \infty$) so to ensure the complete transformation of L-malate ($[M]_{t=0}$). Indeed:

$$\lim_{t \to 0} [M]_{t=t} = \lim_{t \to 0} [M]_{t=0} \cdot e^{k_M/k_B \cdot B_{t=0} \cdot (1 - e^{k_B \cdot t})} = \\ = [M]_{t=0} \cdot e^0 = [M]_{t=0}$$

$$\lim_{t \to \infty} [M]_{t=t} = \lim_{t \to \infty} [M]_{t=0} \cdot e^{k_M/k_B \cdot B_{t=0} \cdot (1 - e^{k_B \cdot t})} = \\ = [M]_{t=0}/e^{\infty} = 0$$

Apart from the sign assumed by the kinetic constant k_B ($k_B > 0$ when the bacterial population increases and $k_B < 0$ when it decreases), the exponential expression:

$$k_M/k_B \cdot B_{t=0} \cdot (1 - e^{k_B \cdot t})$$

is always negative. Indeed, if $k_B > 0$ then $1 - e^{k_B \cdot t}$ will be negative and so the whole exponential expression as well, while on the contrary if $k_B < 0$ then $1 - e^{k_B \cdot t}$ will be grater than 0 but the total exponential factor will assume the same sign of k_B and then will be still negative. Thus, the possible increase or decrease of the lactic acid bacterial population affects only the degree of L-malate conversion but obviously cannot change the direction of this transformation.

On the basis of the relation previously reported:

$$[M]_{t=0} - [M]_{t=t} = [L]_{t=t} + [D]_{t=t}$$

and dividing both members of this equation by the amount of substrate converted ($[M]_{t=0} - [M]_{t=t}$) the following expression can be obtained:

$$1 = [L]_{t=t}/([M]_{t=0} - [M]_{t=t}) + [D]_{t=t}/([M]_{t=0} - [M]_{t=t}) = \\ = R_{L,M} + R_{D,M}$$

where $R_{L,M}$ and $R_{D,M}$ represent the accumulation rates of the two isomeric forms of lactic acid derived from L-malate. Assuming that the values of $R_{L,M}$ and $R_{D,M}$ do not change in relation to L-malate conversion, it can be written:

$$R_{L,M} = [L]_{t=t}/([M]_{t=0} - [M]_{t=t})$$

and

$$[L]_{t=t} = R_{L,M} \cdot ([M]_{t=0} - [M]_{t=t}) = \\ = R_L \cdot [M]_{t=0} \cdot (1 - e^{k_M/k_B \cdot B_{t=0} \cdot (1 - e^{kB \cdot t})})$$

while

$$[D]_{t=t} = R_{D,M} \cdot ([M]_{t=0} - [M]_{t=t}) = (1 - R_{L,M}) \cdot [M]_{t=0} \cdot \\ \cdot (1 - e^{k_M/k_B \cdot B_{t=0} \cdot (1 - e^{kB \cdot t})})$$

In this way, with five parameters (k_B, $B_0 = [B]_{t=0}$, k_M, $M_0 = [M]_{t=0}$, and $R_{L,M}$) it is possible to describe the time evolution of the bacterial population, of malate decrease and of L/D-lactic acid accumulations (Fig. 2).

The identification of the best values to assign to the five functional parameters of the

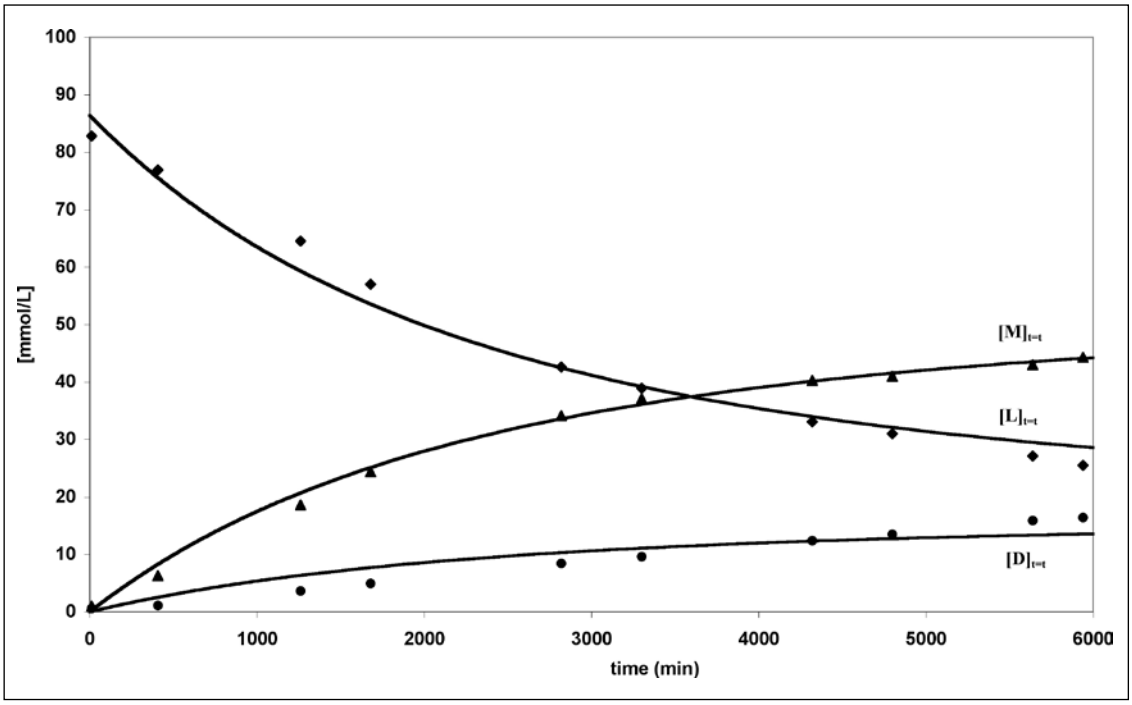


Fig. 2 - Experimental data points and calculated time evolution of chemical species involved in malolactic fermentation using model solutions as reaction medium. M = malic acid; L = L-lactic acid; D = D-lactic acid.

kinetic model was carried out utilising the experimental data related to the time courses of the bacterial population ($[B]_{t=t}$), L-malate ($[M]_{t=t}$) consumption and those of L ($[L]_{t=t}$) and D ($[D]_{t=t}$)-lactate accumulation (Table 1). The kinetic constant of the bacterial population evolution (k_B) was affected by a high variability so that the correlation coefficient showed a remarkable variation passing from 0.27 to 0.89. The number of bacterial cells did not change significantly during the experimental runs with malate as substrate, so that there was a very low correlation between cell number and run time. However, together with negative (Table 1) also positive values were obtained for k_B so that in some cases the bacterial population showed a slightly increase also when L-malate was the only substrate present in the reaction medium. According to the literature (PILONE and KUNKEE, 1972; ANDRICH *et al.*, 1988; COX and HENICK-KLING, 1989, 1995; PASSOS *et al.*, 2003), our experimental evidence allows to claim that only MLF can produce the necessary amount of ATP able to ensure a sufficient energetic contribution to maintain the microbial population.

The values assumed by the k_M constant, connected with L-malate decrease and D/L-lactate increases, were not affected by the different initial concentrations of L-malate (M_0), the different amount of bacterial population (B_0) and its time evolution (k_B). Although k_M was affected by a high variability as well – as shown by the confidence intervals – , it was possible to calculate a mean value ($k_{M,mean} = 9.24 \cdot 10^{-16}$ [L· CFU^{-1}· min^{-1}]) with a low confidence interval (± $1.10 \cdot 10^{-16}$ [L· CFU^{-1}· min^{-1}]; p=0.05). The high square values of the correlation coefficients (r_M, r_D and r_L) related to the time evolution of L-malate, D-lactate and L-lactate concentrations (Table 1), confirm the suitability of the kinetic equations as well as the validity of the suggested hypothesis.

The low values found for the confidence intervals related to the production of L-lactic acid isomer ($R_{L,M}$) during L-malate conversion underline how during conversion the ratio between the concentrations of the two products, L- and D-lactate, remained almost unchanged. On the contrary, this not dimensional factor was remarkably affected by the composition of the surrounding atmosphere (100% N_2 or CO_2). In particular, when an inert atmosphere of N_2 was ensured, a value of $R_{L,M,mean}$ equal to 0.79 ± 0.03 (p=0.05) was obtained, while an $R_{L,M,mean}$ value of 0.91 ± 0.02 was found when only gaseous CO_2 was present.

A kinetic model to describe the lactic fermentation of D-glucose promoted by heterofermentative lactic acid bacteria (*O. oeni* ML 34)

The same approach followed to describe the kinetics of L-malate fermentation was adopted to study the time evolution of the compounds involved in the heterolactic fermentation of D-glucose, carried out by *O. oeni* ML 34 strain.

Analogously to what already reported for L-malate, the following two relations can be written:

$$[G]_{t=0} = [G]_{t=t} + [E]_{t=t} = [G]_{t=t} + [L]_{t=t} + [D]_{t=t}$$

$$[G]_{t=0} - [G]_{t=t} = [E]_{t=t} = [L]_{t=t} + [D]_{t=t}$$

from which, after derivatization respect to run time, the following relation can be obtained:

$$-d[G]_{t=t}/dt = d[E]_{t=t}/dt = d[L]_{t=t}/dt + d[D]_{t=t}/dt$$

The rate of D-glucose consumption is equal to that of ethanol accumulation and to the sum of those related to the two forms (D/L) of lactic acid produced. Thus, to calculate the time course of D-glucose concentration, the system of two differential equations must be solved:

$$\begin{cases} -d[G]_{t=t}/dt = k' \cdot [G]_{t=t} = k_G \cdot [B]_{t=t} \cdot [G]_{t=t} \\ d[B]_{t=t}/dt = k_B \cdot [B]_{t=t} \end{cases}$$

from which the following mathematical relation can be obtained:

$$[G]_{t=t} = [G]_{t=0} \cdot e^{\,k_G/k_B \cdot B_{t=0} \cdot (1 - e^{kB \cdot t})}$$

where $[G]_{t=0}$ represents the D-glucose concentration initially (t=0) present in the reaction medium, k_G the kinetic constant of D-glucose conversion expressed for active cells, k_B the kinetic constant related to the time evolution of the active bacterial population, and $[B]_{t=0}$ the concentration of bacteria cells initially present in the medium. According to the relations previously reported and adopting the same procedure used for L-malate, the below reported kinetic equations can be written:

$$[E]_{t=t} = [G]_{t=0} - [G]_{t=t} = [G]_{t=0} \cdot (1 - e^{\,k_G/k_B \cdot B_{t=0} \cdot (1 - e^{kB \cdot t})})$$

$$[G]_{t=0} - [G]_{t=t} = [L]_{t=t} + [D]_{t=t}$$

$$1 = [L]_{t=t}/([G]_{t=0} - [G]_{t=t}) + [D]_{t=t}/([G]_{t=0} - [G]_{t=t}) = = R_{L,G} + R_{D,G}$$

$$[L]_{t=t} = R_{L,G} \cdot ([G]_{t=0} - [G]_{t=t}) = R_{L,G} \cdot [G]_{t=0} \cdot \cdot (1 - e^{\,k_G/k_B \cdot B_{t=0} \cdot (1 - e^{kB \cdot t})})$$

and

$$[D]_{t=t} = R_{D,G} \cdot ([G]_{t=0} - [G]_{t=t}) = R_{D,G} \cdot [G]_{t=0} \cdot \cdot (1 - e^{\,k_G/k_B \cdot B_{t=0} \cdot (1 - e^{kB \cdot t})})$$

Also for D-glucose transformation, the identification of the best values to be assigned to the five functional parameters of the kinetic model (k_B, $B_0 = [B]_{t=0}$, k_G, $M_0 = [M]_{t=0}$, $R_{L,G}$) was carried out utilising the experimental data related to the time evolutions of bacterial population ($[B]_{t=t}$) and D-glucose ($[G]_{t=t}$) concentration, together with those of ethanol ($[E]_{t=t}$) and L ($[L]_{t=t}$)/D ($[D]_{t=t}$) lactate (Table 2).

For what concerns the kinetic constant k_B (Table 2), the same considerations previously reported for L-malate can be repeated. This parameter was affected by a high variability so that the correlation coefficient varied widely passing from 0.19 to 0.95. The number of cells did not change significantly during the experimental runs and this justifies the low correlation coefficients found. Also in this case, together with negative also positive values of k_B were obtained. The k_G constant, which is connected to the decrease of D-glucose and the increases in ethanol and D/L-lactate, was not remarkably affected by the other reaction variables (initial concentration of D-glucose, different amounts of bacterial population, etc.). Thus, it was possible to calculate a mean value of k_G with a rather restricted confidence interval ($k_{G,mean} = 0.65 \pm 0.17$). The kinetic constant $k_{G,mean}$ ($0.65 \cdot 10^{-16}$ [L·CFU^{-1}] ·min^{-1}) of D-glucose conversion was much lower ($k_{G,mean}/k_{M,mean} = 0.07$) (ANDRICH *et al.*, 1993) than that found for L-malate ($k_{M,mean} = 9.24 \cdot 10^{-16}$ [L·CFU^{-1}]· min^{-1}). Being the rates of lactic fermentation of D-glucose very low, it needed a lot of time to detect sensible reductions of this monosaccharide during the experimental runs. This increased the experimental errors and made more difficult to obtain correlation coefficients comparable to those found using L-malate as substrate of fermentation (Table 1).

Contrary to what found for L-malate, the conversion factor $R_{L,G}$ ($R_{L,G,mean} = 0.41 \pm 0.04$), which rules the accumulation of the two isomeric forms L/D of α-lactic acid, was not affected by the presence of CO_2 in the atmosphere. Thus, it is possible to exclude that carbon dioxide could selectively inhibit the two enzymes (D- and L- dehydrogenases) involved in the pyruvate reduction to produce the two lactic acid isomers. Indeed, pyruvate is an intermediate of the lactic fermentation of D-glucose and its hydrogenation allows the production of D/L-lactate.

The accumulation of D-lactate during MLF could occur by hydrogenation of pyruvate coming directly from L-malate by a single reaction step (dehydrogenation and decarboxylation) (LONVAUD *et al.*, 1977) or by a previous dehydrogenation of L-malate to produce oxalacetate (LAFON-LAFOURCADE, 1975), which is successively decarboxylated to give pyruvate. In this case, increasing concentrations of CO_2 in the atmosphere would be able to inhibit the decaboxylation step and then the accumulation of pyruvate as well, with a consequent reduced production of D-lactate. On the contrary, the L-isomer deriving from the direct decarboxylation of L-malate did not seem to be inhibited by increasing amounts of CO_2 as shown by the k_M values reported in Table 1.

Table 3 - Values assigned to model parameters to calculate the theoretical evolution of the chemical species (L-malate, D-glucose, ethanol, L- and D-lactate) involved in the L-malate and D-glucose transformations promoted by *Oenococcus oeni* ML 34 strain.

Parameters	Run f (8)	Run w (6)
Gas composition	N_2 (100%)	N_2 (100%)
Temperature (°C)	22	24
$[B]_{t=0}$ (CFU·L^{-1})†	$0.60 \cdot 10^{11}$	$2.93 \cdot 10^{11}$
k_B (min^{-1})	$3.9 \cdot 10^{-5}$	$6.9 \cdot 10^{-5}$
$[M]_{t=0}$ (mmol·L^{-1})	38.4	30.6
$[G]_{t=0}$ (mmol·L^{-1})	12.6	-
k_M (L·CFU^{-1}·min^{-1})	$9.24 \cdot 10^{-16}$	$9.24 \cdot 10^{-16}$
$R_{L,M,mean}$	0.79	0.79
k_G (L·CFU^{-1}·min^{-1})	$0.65 \cdot 10^{-16}$	-
$R_{L,G,mean}$	0.41	
B_0,det (CFU·L^{-1})	$0.58 \cdot 10^{11}$	$3.03 \cdot 10^{11}$

†CFU = colony forming units;
B_0,det = the experimentally determined bacterial charge initially present in the reaction medium.

Validation of the kinetic model

Two different experimental runs were performed to test the suitability of the kinetic model. In the first case (run f), the time evolution of the concentrations of D-glucose, L-malate, ethanol and the two isomeric forms D/L of lactic acid were determined following *O. oeni* ML 34 strain addition to an aqueous solution of both the substrates (L-malate and D-glucose). In the second run (run w), the same lactic bacterium was added to a white wine (Trebbiano toscano) previously sterilised by filtration and containing malic acid at a concentration of 4 g L^{-1}. In Table 3 are reported the initial concentrations of the two substrates used in these runs, the values of the two kinetic constants $k_{M,mean}$ and $k_{G,mean}$ as well as the two kinetic parameters $[B]_{t=0}$ and k_B necessary to describe the evolution of the microbial population during each experimental run.

Fig. 3 reports the time evolution of experimental and calculated points related to the concentrations of the species (L-malate, D-glucose, ethanol, L- and D-lactate) involved in L-malate and D-glucose transformations promoted by *O. oeni* ML 34 lactic acid strain. The theoretical evolutions of substrates (L-malate and D-glucose) and products (ethanol, L- and D-lactate) were calculated assigning the values reported in Table 3 (run f) to the parameters present in the kinetic model. The rather good degree of overlapping occurring between experimental and calculated values, gives a measure of the suitability of the model to describe the time evolutions of the main chemical compounds involved in the MLF.

The time courses of metabolites directly involved in MLF (L-malate, L- and D-lactate) utilising wine as reaction medium are reported in Fig. 4. Also in this case, the points calculated assuming as model parameters the values reported in Table 3 (run w) did not remarkably differ from the corresponding experimental values.

As during the experimental runs the time evo-

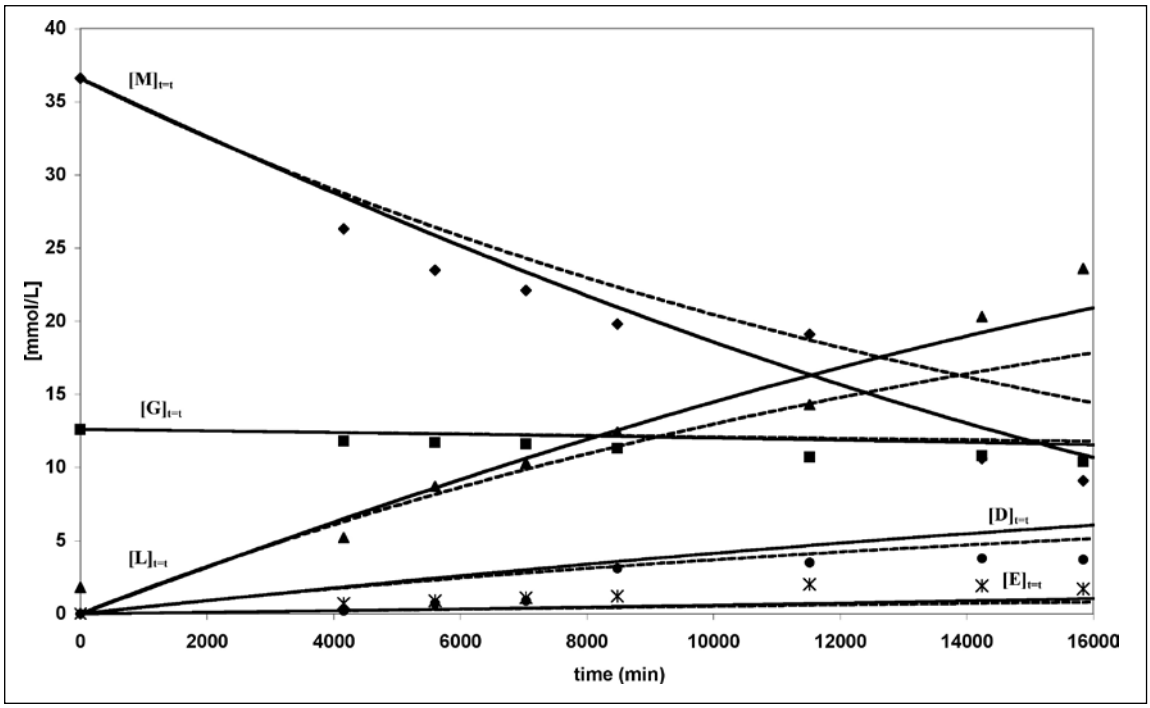


Fig. 3 - Experimental data points and calculated time evolution of chemical species involved in L-malate and D-glucose transformations. M = malic acid; L = L-lactic acid; D = D-lactic acid; G = glucose; E = ethanol. Continuous line, complete version of kinetic model; dotted line, simplified version of kinetic model.

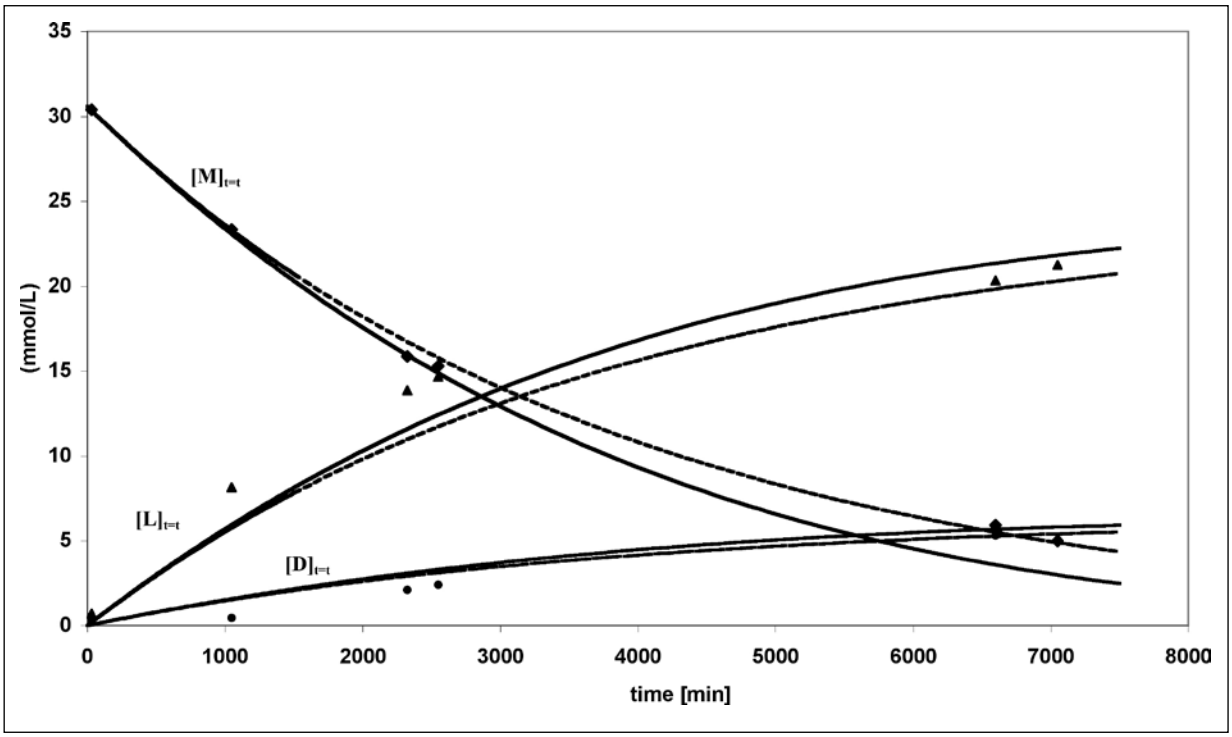


Fig. 4 - Experimental data points and calculated time evolution of chemical species involved in malolactic fermentation using a real wine as reaction medium. M = malic acid; L = L-lactic acid; D = D-lactic acid. Continuous line, complete version of kinetic model; dotted line, simplified version of kinetic model.

lution of the microbial population was very slow so to assume a nearly constant value, it can be possible to reduce the complexity of the kinetic model in order to make its practical utilisation easier. Indeed, assuming that the evolution of the microbial population can be disregarded, the following relation would be obtained:

$$-d[S]_{t=t}/dt = k_S \times B_0 \times [S]_{t=t}$$

where $[S]_{t=t}$ represents the substrate concentration (L-malate = $[M]_{t=t}$ or D-glucose = $[G]_{t=t}$) in the reaction medium at a generic time t=t, while k_S is the kinetic constant of the substrate transformation (k_M or k_G of Table 3). B_0 represents the microbial concentration (CFU·L^{-1}) initially present in the reaction medium which remained practically unchanged during all the experimental run. The differential equation above reported can be integrated to obtain the following expression:

$$[S]_{t=t} = [S]_{t=0} \cdot e^{-k_S \cdot B_0 \cdot t}$$

while the accumulation of the product P (ethanol, L- and D-lactate) in the reaction medium can be described by the relation:

$$[P]_{t=t} = R_{P,S} \cdot ([P]_{t=0} - [P]_{t=t}) = R_{P,S} \cdot [S]_{t=0} \cdot (1 - e^{-k_S \cdot B_0 \cdot t})$$

where $R_{P,S}$ represents the conversion factor related to the accumulation of the product P deriving from L-malate or D-glucose.

Table 3 reports the number of active cells of *O. oeni* ML 34 strain initially determined in the reaction medium ($B_{0,}$det), while the curves outlined by dotted lines (Figs. 3 and 4) show the theoretical evolutions of the chemical species involved in this microbial transformation and calculated by the simplified form of the kinetic model. A notable increase in the microbial population occurred during both these two experimental runs, as shown by the positive sign assumed by the kinetic constant k_B (Table 3). The concentrations of the chemical species calculated by the simplified form of the model (Figs. 3 and 4, dotted lines) were higher for the substrate (L-malate) and lower for products (L- and D-lactate) compared to those evaluated using the complete version of the kinetic model (Figs. 3 and 4, full lines). As the differences do not seem to be relevant, the simplified version of this model could be usefully utilised, at least to have a preliminary roughly picture of the evolution of MLF.

Moreover, if the experimental value related to the number of active cells of *O. oeni* ML 34 strain would be not available, it could be roughly evaluated being known some experimental data connected to the decrease of the substrates S (L-malate or D-glucose) or to the accumulation of the products P (ethanol, L- and D-lactate) with time, by the following relations:

$$B_0 = 1/(k_S \cdot t) \cdot \ln([S]_{t=0}/[S]_{t=t})$$
$$B_0 = 1/(-k_S \cdot t) \cdot \ln(1 - 1/R_{P,S} \cdot [P]_{t=t}/[S]_{t=0})$$

This represents only one of many possible applications of this kinetic model which could also utilised to better determine the kinetic behaviour of a lactic acid bacterial population able to promote MLF. In particular a better and a more reliable comparison of the kinetic activities shown by different bacterial populations could be more easily obtained.

REFERENCES

Andrich G., Casella S., Fiorentini R. and Spettoli P. 1988. A tentative model to evaluate the kinetics of malolactic fermentation. Annals of the New York Academy of Science 542: 356.

Andrich G., Zinnai A., Fiorentini R. and Casella S. 1993. Kinetics and thermodynamics of L-malate and D-glucose bioconversion promoted by *Leuconostoc oenos*. Abstract Book of the 6th European Congress on Biotechnology, Firenze, Italy, TU008.

Arthurs C.E. and Loyd D. 1999. Kinetics, stereospecificity and expression of malolactic enzyme. Applied Environmental Microbiology 65: 3360.

Coucheney F., Desroche N., Bou M., Tourdot-Maréchal R., Dulau L. and Guzzo J. 2005. A new approach for selection of *Oenococcus oeni* strains in order to produce malolactic starters. International Journal of Food Microbiology 105: 463.

Cox D.J. and Henick-Kling T. 1989. Chemiosmotic energy from malolactic fermentation. Journal of Bacteriology 171: 5750.

Cox D.J. and Henick-Kling T. 1995. Proton motive force and ATP generation during malolactic fermentation. American Journal of Enology and Viticulture 46: 319.

De Man J.C., Rogosa M. and M.E. Sharpe 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23: 130.

Garvie E.I. 1980. Bacterial lactate dehydrogenases. Microbiological Reviews 44: 106.

Granchi L., Guerrini S. and Vincenzini M. 2005. I batteri lattici e la fermentazione malolattica". In: Microbiologia del vino. Eds. M. Vincenzini, P. Romano and G.A. Farris (Casa Editrice Ambrosiana: Milano) pp. 261.

Hervé A., Costello P.J., Remize F., Guzzo J. and Guilloux-Benatier M. 2004. *Sacchaomyces cerevisiae-Oenococcus oeni* interactions in wine: current knowledge and perspectives. International Journal of Food Microbiology 93: 141.

Kunkee R.E. 1991. Some roles of malic acid in the malolactic fermentation in winemaking. FEMS Microbiological Reviews 88: 55.

Labarre B., Guzzo J., Cavin J.F. and Divies C. 1996. Cloning and characterization of genes encoding the malolactic enzyme and malate permease of *Leuconostoc oenos*. Applied and Environmental Microbiology 62: 1274.

Lafon-Lafourcade S. 1975. Factors of malolactic fermentation of wine. In: Lactic acid bacteria in beverage and food. Eds. J.G. Carr, C.V. Cutting and G.C. Whiting. (Academic Press: London) p. 43.

Liu S.Q. 2003. Practical implications of lactate and pyruvate metabolism by lactic acid bacteria in food beverage fermentations. International Journal of Food Microbiology 83: 115.

Lonvaud M., Lonvaud-Funel A. and Ribereud-Gayon P. 1977. Le mecanisme de la fermentation malolactique des vins. Connaissance de la Vigne et du Vin 11: 73.

Lonvaud-Funel A. 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. Antonie Van Leeuwenhoek 76: 317.

Passos F.V., Fleming H.P., Hassan H.M. and McFeeters R.F. 2003. Effect of malic acid on the growth kinetic of *Lactobacillus plantarum*. Applied Microbiology and Biotechnology 63: 207.

Pilone G.J. and Kunkee R.E. 1972. Characterization and energetics of *Leuconostoc oenos* ML 34. American Journal of Enology and Viticulture 23: 61.

Saayman M. and Viljoen-Bloom M. 2006. The biochemistry of malic acid metabolism by wine yeasts – A review. South African Journal of Enology and Viticulture 27: 113.

Versari A., Parpinello G.P. and Cattaneo M. 1999. *Leuconostoc oenos* and malolactic fermentation in wine: a review". Journal of Industrial Microbiology and Biotechnology 23: 447.

Volschenk H., van Wuuren H.J.J. and Viljoen-Bloom M. 2006. Malic acid in wine: origin, function and metabolism during vinification. South African Journal of Enology and Viticulture 27: 123.

Zapparoli G., Torrioni S., Pesente P. and Dellaglio F. 1998. Design and evaluation of malolactic enzyme gene targeted primers for rapid identification and detection of *Oenococcus oeni* in wine. Letters in Applied Microbiology 27: 243.

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PAPER

PRESSURIZED SOLVENT EXTRACTION OF FLAVONOIDS FROM GRAPE POMACE UTILIZING ORGANIC ACID ADDITIVES

K. SRINIVAS[1], J.W. KING[1]*, J.K. MONRAD[2], L.R. HOWARD[2] and D. ZHANG[1]
[1]Ralph E. Martin Department of Chemical Engineering, University of Arkansas,
3202 Bell Engineering Center, Fayetteville, Arkansas 72701, USA
[2]Department of Food Science, University of Arkansas,
2650 North Young Avenue, Fayetteville, Arkansas 72704, USA
*Corresponding author: Tel. 01 (479) 575 5979, Fax 01 (479) 575 7926,
e-mail: jwking1@uark.edu

ABSTRACT

Flavonoids present in freeze-dried Sunbelt (*Vitis labrusca L.*) grape pomace were extracted at a pressure of 10.3 MPa and temperatures between 60° and 140°C using pressurized hydroethanolic solvents (0, 40, 80% v/v ethanol in water) acidified with organic acids (formic, acetic, citric and tartaric acid at a pH of 2.5). Acetic acid had a significant effect in maximizing anthocyanin yield from grape pomace when using 80% aqueous ethanol as a solvent at 80°C (1028.9 mg/100 g DW). However, organic acids did not have a significant effect on the extraction of flavonols from grape pomace. The maximum amount of flavonols extracted from grape pomace using 80% aqueous ethanol as the solvent at 120°-140°C was 9.65 mg/100 g DW. Surface response regression analysis of the experimental data indicated the optimum temperature for extraction of a maximum amount of anthocyanins from grape pomace was 85.4°C and that for flavonols was 124°C. These optimized conditions obtained from this study performed on an analytical scale can be used in scale-up and design of a dynamic subcritical fluid extraction system for extraction of flavonoids from food processing waste, such as grape pomace.

- Key words: anthocyanin, flavonoid, grape pomace, pressurized solvent extraction -

INTRODUCTION

The grape pomace consists of the skins, stems and seeds that are left behind after the pressing of the grapes (MOURE *et al.*, 2001). Studies have estimated that the about 20 wt% of the processed grapes are left behind as pomace (SCHIEBER *et al.*, 2001) with approximately 60-70 wt% of total phenolics concentrated in grape seeds, and the rest in the grape skins (GARCIA-MARINO *et al.*, 2006). The localization of such phenolic compounds may vary with the type of grapes, climate and other environmental conditions as well as the maturity of the grape during cultivation (PINELO *et al.*, 2006). Sunbelt grape (*Vitis labrusca L.*), a juice grape variety designed for growth in the State of Arkansas, is a type of juice grape that contains a large amount of anthocyanin diglucosides, unlike wine grapes which contain larger amounts of anthocyanin monoglucosides (REVILLA *et al.*, 1999).

Flavonoids are a group of polyphenolic compounds that are present in many natural products and have been studied extensively due to their antioxidant, anti-microbial and antiproliferative activities (HARBORNE and WILLIAMS, 2000; LURTON, 2003; BOZIDAR, 1995; HALPERN *et al.*, 1998). Studies have indicated that a greater amount of anthocyanins, flavonols (quercetin) and flavan-3-ols (catechin/epicatechin) are concentrated in the grape skins while the grape seeds contain greater amounts of procyanidins (BRAIDOT *et al.*, 2008). Apart from their health beneficial and nutraceutical value, anthocyanins are also used as food colorants (FRANCIS and MARKAKIS, 1989). Fundamental studies have indicated that at different pH levels, the anthocyanins undergo a structural transformation that is responsible for their color change in solution (HEREDIA *et al.*, 1998). The most stable form for anthocyanin in grape pomace is the flavylium cation which exists at pH less than 4 (CLIFFORD, 2000). Studies have indicated that with an increase in pH, there is rapid decrease in the color and an increase in its antioxidant capacity (LAPIDOT *et al.*, 1998).

SPIGNO and DE FAVERI (2007) and other research groups around the world have proposed the use of agricultural wastes such as grape pomace as a low-cost source for the extraction of antioxidant compounds. Studies have indicated that about 10.5-13.1 million tonnes of grape pomace is produced in the world each year (DE CASTRO *et al.*, 2008) Extraction of flavonoids from grape pomace has been traditionally done using alcoholic solvents such as methanol and ethanol with water as a cosolvent (LAPORNIK *et al.*, 2005; METIVIER *et al.*, 1980).

Usually, a low concentration of acid such as hydrochloric acid (HCl) is added to the solvent to increase the efficiency of the extraction to yield maximum amount of anthocyanins (JU and HOWARD, 2003). The addition of the acid denatures the cellular membranes present in the grape pomace matrix, thereby facilitating flavonoid dissolution in the extraction solvent (JACKMAN *et al.*, 1987). The concentration of the acid in the extraction solvent should be very low since the addition of excess acid can cleave the acylated and sugar groups of the anthocyanins during the extraction and concentration steps, resulting in formation of undesirable components. REVILLA *et al.* (1998) have previously reported on the partial hydrolysis of the aceylated malvidin glucosides when 1% concentration (by weight) of 12N HCl was added to methanol used as a solvent. It has been proposed that weaker organic acids such as formic, acetic, citric and tartaric acids or extremely low concentrations of stronger acids such as HCl and trifluoroacetic acid can be used to solve this problem (DE CASTRO *et al.*, 2008; JU and HOWARD, 2003). Studies performed by BLEVE *et al.* (2008) indicated that the flavonoids extracted from grape pomace using hydroethanolic solvents acidifed with 0.2% trifluoroacetic acid were easily available for further purification compared to those extracted in the absence of the acid. However, studies performed by DOWNEY *et al.* (2007) on the stability of the anthocyanins and flavonols extracted from grape pomace using methanol acidified with HCl, acetic, formic, citric and maleic acids indicated that the flavonoid content decreased rapidly with increasing temperature or residence time of the pomace. Moreover, in the afore-mentioned studies, the total contact time between the solvent and the pomace matrix was very high (~ 1 day or more).

A number of studies have been performed on the extraction of flavonoids from grape pomace using hydroethanolic solvents. SAVOVA *et al.* (2007) indicated that the maximum amount of flavan-3-ols could be extracted from grapes using 50% ethanol in water at 65°C. Another study on the extraction of phenolic compounds from winery wastes indicated maximum recovery of these phenolic compounds using 50% ethanol in water (LAFKA *et al.*, 2007). Recently, similar studies performed by MONRAD *et al.* (2010a,b) indicated that 50-70% v/v ethanol in water at temperatures between 80° and 120°C was optimal for the recovery of anthocyanins and procyanidins from grape pomace.

Water at temperatures above its boiling point and under sufficient pressure (also referred to as "subcritical water") has been used as a solvent in recovery of polar compounds from natural products (KING and SRINIVAS, 2009). The enhanced dissolution and diffusion rates of such compounds in water at elevated temperatures is due to a decrease in the hydrogen bonding propensity of water as measured by the total solubility parameter as a function of temper-

ature (SRINIVAS *et al.*, 2009). Subcritical water at temperatures between 100° and 150°C have been found optimal for recovering maximum amount of anthocyanins from berry substrates (KING *et al.*, 2003), grape skins (JU and HOWARD, 2006) and black currants (CACACE and MAZZA, 2002). However, at these temperatures there is a greater possibility for thermal degradation of the flavonoid compounds during their extraction from grape pomace. However, by optimizing the extraction time, it is possible to minimize the loss of flavonoid recovery due to thermal degradation (MONRAD *et al.*, 2010a). Pressurized liquid extractions can be performed using an accelerated solvent extraction (or ASE). Using this technique, extractions can be performed at higher temperatures (40°-200°C) abetted by pressure using very short extraction times (1-20 min) (PALMA *et al.*, 2001; JU and HOWARD, 2003). Such rapid rate extraction conditions can improve recovery of the polyphenolic solutes thereby avoiding thermal degradation of the solutes.

Studies performed on black carrots using water acidified with citric and lactic acids at temperatures between 50° and 100°C and 50 bar pressure yielded the maximum amount of anthocyanins, particularly when lactic acid was used as the acidulant (GIZIR *et al.*, 2008). Greater thermal degradation of the anthocyanin extracts occurred at 100°C along with higher concomitant browning when sulfuric acid was used as an acidulant. In this study, the high temperature extractions were performed in 10 minutes, whereas the sulfuric acid extractions were performed at room temperature over a period of 12 hours.

In this study, an ASE system was used to investigate the effects of temperature, solvent composition and the presence of various organic acids on the extraction of flavonoids (anthocyanins and flavonols) from Sunbelt grape pomace.

EXPERIMENTAL SECTION

Grape and pomace samples

The materials used in this study are similar to those described previously (MONRAD *et al.* 2010a,b). Sunbelt grapes harvested at the University of Arkansas' Agricultural Experimental Station Farm (Fayetteville, AR) in 2006 were crushed and de-stemmed followed by pressing in a 70 L Enrossi bladder press (Enoagricola Rossi s.r.l., Calzolaro, Italy). The recovered pomace was then freeze-dried in VirTis Genesis freeze-dryer (Gardner, NY) and stored at -70°C in a Thermo Scientific Ultra-Low Freezer (Waltham, MA). The freeze-dried pomace was then ground to a homogeneous powder (500 μm) using an Udy Cyclone Sample Mill (Fort Collins, CO) for extraction purposes.

Reagents

Formic acid (CAS# 64-18-6; Lot# K37957664; 98% ACS grade), glacial acetic acid (CAS# 64-19-7; Lot# 200733306; ACS grade), citric acid (CAS# 77-92-9; Lot# 10132195; Stock# A10395) as well as L-(+)-tartaric acid (CAS# 87-69-4; Lot# J25S046; Stock# L12804) were obtained from VWR Corporation (Batavia, IL). Absolute ethanol (CAS# 64-17-5; 200 proof; ACS grade) was also obtained from VWR Corporation. HPLC grade methanol was obtained from EMD Chemicals (Gibbstown, NJ). Water (1-5 ppb TOC, 18.2 MΩcm) was purified using a Milli-Q Synthesis A10 system (Millipore, Ballerica, MA, USA).

The anthocyanin HPLC standards used in this study consisted of a mixture of malvidin, delphinidin, peonidin, cyanidin, petunidin and pelargonidin monoglucosides which were purchased from Polyphenol Labs (Sandes, Norway). The flavonols were expressed as quercetin-3-β-D-glucoside (CAS# 21637-25-2; Lot# 1373210; Filling code: 11908159; ≥ 90% HPLC) equivalents. The quercetin-3-β-D-glucoside was obtained from Sigma-Aldrich (St. Louis, MO). The HPLC-grade solvents used in the analysis were acquired from VWR (Batavia, IL).

Pressurized acidified solvent extraction of anthocyanins from grape pomace

The extractions of flavonoids from grape pomace were performed using a Dionex ASE-200 extractor system (Dionex Corp., Sunnyvale, CA). 0.5 g of freeze-dried grape pomace was placed in an 11 mL extractor cell with a cellulose-loaded filter placed at the bottom of the cell. Solvents consisted of mixtures of ethanol in water (0, 40 and 80% v/v). The solvents were acidified to a pH of 2.5 using the following organic acids: formic, acetic, citric and tartaric acids. The acidification of the solvent was done by adding the organic acids to the solvent while measuring the pH using a Mettler Toledo SevenEasy S-20 pH meter fitted with an InLab Expert Pro "3-in-1" pH electrode (Columbus, OH). The initial pH of 0, 40 and 80% ethanol (v/v) solutions in water were found to be 6.85 (±0.5), 5.35 (±0.5) and 4.86 (±0.5) units, respectively.

In the ASE system, heating of the solvent is performed using an aluminum block heater placed in the ASE system. The solvent is allowed to flow into the cell and the cell heated to the set point temperature value within 5-7 minutes. The oven temperature was set at 60°, 80°, 100°, 120° and 140°C for the subsequent extractions. The static extraction time after equilibration to the set experimental time was set at 1 min. An external pressure of 10.3 MPa was applied to the extractor cell during the static extraction period. After the static extraction time, the extract was allowed to flow into a collection vial and the cell flushed using a quantity of sol-

vent equivalent to 70% of the cell's volume, followed by purging the contents of the extraction cell with nitrogen for 110 seconds.

After extraction, the volumes were adjusted to 50 mL with water and centrifuged for 10 minutes at 8,000 rpm using a Beckman GS-15R centrifuge (Calbiochem, San Diego, CA) to remove any insoluble solids. The centrifuged extracts were then mixed with methanol in 1:1 (v/v) ratio and analyzed by HPLC. Triplicate measurements were made.

Conventional extraction

Conventional extraction of anthocyanins and flavonols from grape pomace, as described by HAGER *et al.* (2008), was used to determine the efficiency of the ASE extractions. This method was also used by MONRAD *et al.* (2010a). In this method, 2 g of ground pomace were homogenized with 20 mL of methanol/water/formic acid (60:37:3, v/v/v) using an Ika T18 Ultra-Turrax tissuemizer (Wilmington, NC) for 30 s at room temperature conditions. The homogenate was filtered through Miracloth (Calbiochem, San Diego, CA) and the filtrate collected. The residue was collected, and the procedure repeated two more times. The filtrates were combined and adjusted to 100 mL with extraction solvent and centrifuged to remove insoluble solids. The supernatant was collected for analysis and stored at -20°C prior to analysis. The conventional extractions were also performed in triplicate.

HPLC analysis

HPLC analysis of the anthocyanins and flavonols in the grape extracts was performed using the method described by CHO *et al.* (2004). A Waters Alliance Model 2695 HPLC system (Waters Corp., Milford, MA) was used. 5% formic acid in water (v/v) (A) and methanol (B) were used as solvents in this HPLC method The HPLC gradient program was executed as follows: 98% A to 40% A in 60 min, 40% A to 98% A in 65 min followed by a 10 min re-equilibration period. The entire HPLC run time was 80 minutes using a flow rate of 1 mL/min.

The resultant grape extracts were filtered before HPLC assay using a 0.5 μm PTFE filter (Varian Inc., Palo Alto, CA), and 100 μL of the filtered extract was injected onto a 250 x 4.6 mm Waters Symmetry C_{18} column (Waters Corp., Milford, MA). The anthocyanins were detected using a Waters 2996 Photodiode array detector (Waters Corp., Milford, MA) at a 510 nm, and the peaks were compared with the retention times of a standard grape pomace extract analyzed by HPLC-MS. Calibration curves were obtained using a mixture of anthocyanin monoglucosides. The anthocyanins in the extracts were reported based on these monoglucoside equivalents. The amount of anthocyanins extracted from grape pomace is expressed in terms of mg/100 g (of dry weight pomace).

The flavonols extracted from grape pomace were detected at a 364 nm and the peaks were quantified as quercetin-β-D-glucoside equivalents. The amount of flavonols is also expressed in terms of mg/100 g (of dry weight pomace).

HPLC-MS analysis of anthocyanins in grape pomace

The HPLC-MS analysis of the anthocyanins present in a grape pomace extract was done using the method described by CHO *et al.* (2004). The grape extract was injected into the Waters Symmetry C_{18} column connected to a Hewlett Packard 110 series HPLC (Agilent Technologies, Wilmington, DE) with an auto sampler, binary HPLC pump, and an UV/Vis detector. The solvents and the gradient program were set as stated above. The anthocyanin peaks were detected at 510 nm and flavonol peaks were detected at 364 nm. The mass spectrophotometric analysis was done using a Bruker Esquire LC-MS (Billerica, MA) ion trap mass spectrometer connected to the Hewlett-Packard 1100 system. The eluting peaks showing absorptivity at 520 nm were analyzed using the LC-MS system and its attendant software. The mass spectrophotometer parameters set for the anthocyanin analysis were as follows: positive ion electrospray mode with a capillary voltage of 4 kV; nebulizing pressure of 0.21 MPa; drying gas flow rate of 0.9 mL/min; temperature of 300°C. The flavonols were analyzed in the mass spectrophotometer under same conditions as discussed above but in a negative ion electrospray mode. The data were collected over the mass range of *m/z* 50 through 1,000 in full scan mode at 1.0 s/cycle.

Experimental design for statistical analysis

The experimental design was a full factorial (3x5x5) treatment completely randomized design with three replications. The statistical design consists of three solvents (0, 40 and 80% (v/v) ethanol in water), five acid conditions (no acid, formic, acetic, citric, and tartaric acids) and five temperatures (60°, 80°, 100°, 120° and 140°C). The linear statistical model (MONRAD *et al.*, 2010a,b) used for the analysis is as given below:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (\beta\gamma)_{jk} + (\alpha\gamma)_{ik} + (\alpha\beta\gamma)_{ijk} + \varepsilon_{ijkl} \quad (1)$$

where Y_{ijkl} is the observed measured response of the l^{th} replication of the i^{th} solvent with the j^{th} acid condition on the k^{th} temperature and μ is the overall population average response. α_i is the i^{th} solvent main effect ($\sum_{i=1}^{3} \alpha_i = 0$), β_j is the j^{th} acid main effect ($\sum_{j=1}^{5} \beta_j = 0$), γ_k is the k^{th} tempera-

ture main effect ($\sum_{k=1}^{5}\gamma_k = 0$), $\alpha\beta_{ij}$ is the solvent-acid interaction effect ($\sum_{i=1}^{3}\sum_{j=1}^{5}(\alpha\beta)_{ij} = 0$), $\beta\gamma_{jk}$ is the acid-temperature interaction effect ($\sum_{j=1}^{5}\sum_{k=1}^{5}(\beta\gamma)_{jk} = 0$), $\alpha\gamma_{ik}$ is the solvent-temperature interaction effect ($\sum_{i=1}^{3}\sum_{k=1}^{5}(\beta\gamma)_{jk} = 0$), $\alpha\beta\gamma_{ijk}$ is the solvent-acid-temperature interaction effect ($\sum_{i=1}^{3}\sum_{j=1}^{5}\sum_{k=1}^{5}(\alpha\beta\gamma)_{ijk} = 0$) and $\varepsilon_{ijk} \sim N(0, \sigma^2)$ is the unobserved ijkth random error effect. The errors are assumed to be independent, identically, and normally distributed with mean zero and common variance σ^2. The two-way ANOVA representing the aforementioned statistical design is calculated using JMP software (ver. 8; Cary, NC). The Student's t-test was used to analyze the significance of the main and interaction effects evaluated at 95% confidence interval ($P<0.05$).

In order to assess the variability in the extraction of anthocyanins and flavonols from grape pomace using the pressurized acidified solvents, a surface response regression analysis was used. This method is based on the principle that in a three-component system, if two of the factors can be specified, than the third factor affecting the optimization of the anthocyanin yield can be fixed (TOYOMIZU *et al.*, 1982). The general model for a linear regression equation is given below:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \\ + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{23} X_2 X_3 + \beta_{13} X_1 X_3 \quad (2)$$

where, $\beta_{1\text{-}6}$ are parameter estimates for the best regression equation that yield maximum amount of anthocyanins at the optimized conditions, X_1 refers to solvent composition (0.0, 0.4 and 0.8); X_2 refers to temperature (60°, 80°, 100°, 120° and 140°C); and X_3 refers to acid (1- no acid, 2- formic acid, 3- citric acid, 4- tartaric acid, and 5- acetic acid). 'Y' refers to the derived response for the particular model. In this model, X_{12}, X_{23} and X_{13} refers to the effect due to interactions between the different factors.

RESULTS AND DISCUSSION

Pressurized liquid extraction of grape pomace by ASE uses pressures ranging between 3.45 MPa and 20.68 MPa. Studies have indicated that pressures sufficient to maintain the solvent in its liquid state at temperatures above its boiling point (i.e. equal to or above its vapor pressure at a particular temperature) are required for performing such pressurized liquid extractions (SHARIF *et al.*, 2010). In case of subcritical water, this pressure requirement would be < 2 MPa at temperatures between 100° and 150°C. Studies performed by LUTHRIA (2008) and MUKHOPADHYAY *et al.* (2006) have indicated that pressure does not have a significant effect on the extraction of phenolic compounds from natural products. Hence, in this study, a constant pressure of 10.3 MPa was used for extracting anthocyanins and flavonols from grape pomace using the described subcritical fluids.

A typical chromatogram obtained from the HPLC analysis of the anthocyanins and flavonols present in the Sunbelt grape pomace is presented in Figs. 1a and b. The anthocyanin peaks are numbered from 1-12 and flavonol peaks are numbered from 1-4 as detected using the HPLC-MS system. Mass spectrophotometric data are given in Table 1.

Peaks 2 [Malvidin-3, 5-O-diglucoside] and 8 [Peonidin-3-(6-O-coumaroyl)-5-O-diglucoside + Malvidin-3-(6-O-p-coumaroyl)-5-O-diglucoside] are the most prominent anthocyanins present in Sunbelt grape pomace extract (Fig. 1a). An elaborate discussion on the identification of the anthocyanins in Sunbelt grape pomace can be found in our previous work (MONRAD *et al.*, 2010a). It can also be seen that the anthocyanins can form acyl derivatives with acetic acid and coumaric acids with the coumaroyl derivatives predominating in Sunbelt grape pomace. Studies have indicated that the anthocyanin acyl derivatives can be by-products of hydrolysis of the anthocyanins due to the presence of acylating acids which include ferulic, caffeic, malic, succinic and tartaric acids (ANDERSON *et al.*, 1970).

The HPLC profile for the flavonols detected at 364 nm in Sunbelt grape pomace is shown in Fig. 1b. There is no data in the literature on the mass spectrophotometric analysis of flavonols in Sunbelt grape pomace, but the identification of flavonols in Sunbelt grape pomace was comparable with that reported for various grape genotypes (CHO *et al.* 2004). Although, a number of peaks were detected by HPLC-MS, only 4 major peaks: myricetin-3-glucoside, quercetin-3-glucuronide, quercetin-3-glucoside and quercetin-3-rutinoside were positively identified. It should also be noted that quercetin-3-glucoside and quercetin-3-galactoside have identical *m/z* values and co-eluted using this method. Even though quercetin-3-glucuronide and quercetin-3-glucoside/galactoside have similar retention times, their mass spectra vary slightly (Table 1). Studies performed by SOUQUET *et al.* (2000) indicate that quercetin-3-glucuronide is a major phenolic component in grape stems and is easily assimilated by the human body compared to corresponding glucosides/galactosides. In order to avoid any confusion, the total amount of flavonols discussed in this study refers to the sum of the 4 identified peaks and the unidentified flavonols have not been taken into account.

Solvent and temperature optimization

Optimization of the solvent and temperature conditions to maximize anthocyanin yield from grape pomace using subcritical fluid extraction

Table 1 - Retention times and mass spectral data of the anthocyanins and flavonols detected in the Sunbelt grape pomace extract.

Peak No.	Retention Time (min)	Anthocyanin in the extract*	(m/z) values	
			M+	fragments
1	23.2	Pnd-3, 5-O-diglucoside	625	463, 301
2	25.7	Mvd-3, 5-O-diglucoside	655	493, 331
3	28.2	Ptd-3-O-monoglucoside	479	317
4	30.6	Pnd-3-O-monoglucoside +	463	301
		Ptd-3-O-(6-acetyl)-5-O-diglucoside	683	317
5	32.1	Mvd-3-O-(3-acetyl)-5-O-diglucoside	697	535, 493, 331
6	38.9	Dpd-3-(6-O-p-coumaroyl)-5-O-diglucoside	773	611, 465, 303
7	41.7	Ptd-3-(6-O-p-coumaroyl)-5-O-diglucoside	787	625, 479, 317
8	43.7	Pnd-3-(6-O-coumaroyl)-5-O-diglucoside +	771	609, 463, 301
		Mvd-3-(6-O-p-coumaroyl)-5-O-diglucoside	801	639, 493, 331
9	44.9	Dpd-3-O-(6-O-p-coumaroyl) monoglucoside	611	303
10	47.1	Cyd-3-O-(6-O-p-coumaroyl) monoglucoside	595	287
11	48.0	Ptd-3-O-(6-O-p-coumaroyl) monoglucoside	625	317
12	50.3	Mvd-3-O-(6-O-p-coumaroyl) monoglucoside	639	331
Peak No.	**Retention Time (min)**	**Flavonol in the extract**	**M-**	**Fragments**
1	32.8	Myricetin-3-glucoside	479	317
2	38.7	Quercetin-3-glucuronide	477	301
3	39.4	Quercetin-3-glucoside/galactoside	463	301
4	43.6	Quercetin-3-rutinoside	609	301

*The anthocyanin abbreviations pnd refers to peonidin, mvd refers to malvidin, ptd refers to petunidin, dpd refers to delphinidin, and cyd refers to cyanidin.

is based on the sum of all the anthocyanins detected in the grape extracts as previously discussed by MONRAD *et al.* (2010a). The statistical analysis performed using the general linear model given in Equation 1 indicated that there was a significant effect of the solvent (or ethanol composition in water) ($P<0.001$) and temperature ($P=0.0457$) on the amount of total anthocyanins extracted from the grape pomace. The data, as previously discussed, were analyzed with a 95% confidence interval. There was, however, an insignificant effect of the interaction between the solvent and the temperature ($P=0.3519$) on the anthocyanin yield.

A simple Student's t-test at the 95% confidence level indicated that 80% (v/v) ethanol in water was marginally better in extracting maximum number of anthocyanins from grape pomace when compared to 40% (v/v) aqueous ethanol. This hydroethanolic solvent mixture, however, extracted a significantly greater amount of anthocyanins from grape pomace relative to using neat water as a solvent. Similar trends were noted by MONRAD *et al.* (2010a), i.e., significant amount of anthocyanins were extracted using 50 - 70% aqueous ethanol as solvent.

The total amount of anthocyanins extracted by hydroethanolic solvents from Sunbelt grape pomace are shown in Table 2. The standard deviations for the anthocyanin data as presented in Table 2 were calculated using Equation 3.

$$RMSD = \sqrt{\frac{\sum_{i=1}^{n}\left(C_{g,i} - \overline{C_g}\right)^2}{(n-1)}} \qquad (3)$$

where $C_{g,i}$ = amount of total anthocyanins (mg/100 g DW) extracted from Sunbelt grape pomace for i^{th} replicate at a particular temperature; = mean of the total anthocyanin (mg/100 g DW) extracted from sunbelt grape pomace at a particular temperature for all replicates and; n = number of replicates.

It can be seen from Table 2 that peaks 4 (Pnd-3-O-monoglucoside + Ptd-3-O-(6-acetyl)-5-O-diglucoside) and 10 (Cyd-3-O-(6-O-p-coumaroyl) monoglucoside), though identified through HPLC-MS, were not found in the ASE extracts except when acetic acid was added to the hydroethanolic solvent. This can be due to the acylation of the anthocyanins in presence of the acidified solvents or the reduction in polarity of solvent which facilitated extraction of these compounds that might already be present in grape pomace.

The flavonols extracted from Sunbelt grape pomace as a function of solvent composition and temperature is shown in Table 3. The standard deviations for the flavonol data were also calculated using Equation 3. Statistical analysis indicated that there was a significant solvent ($P<0.001$) and temperature ($P<0.001$) effect on the extraction of flavonols from the grape pom-

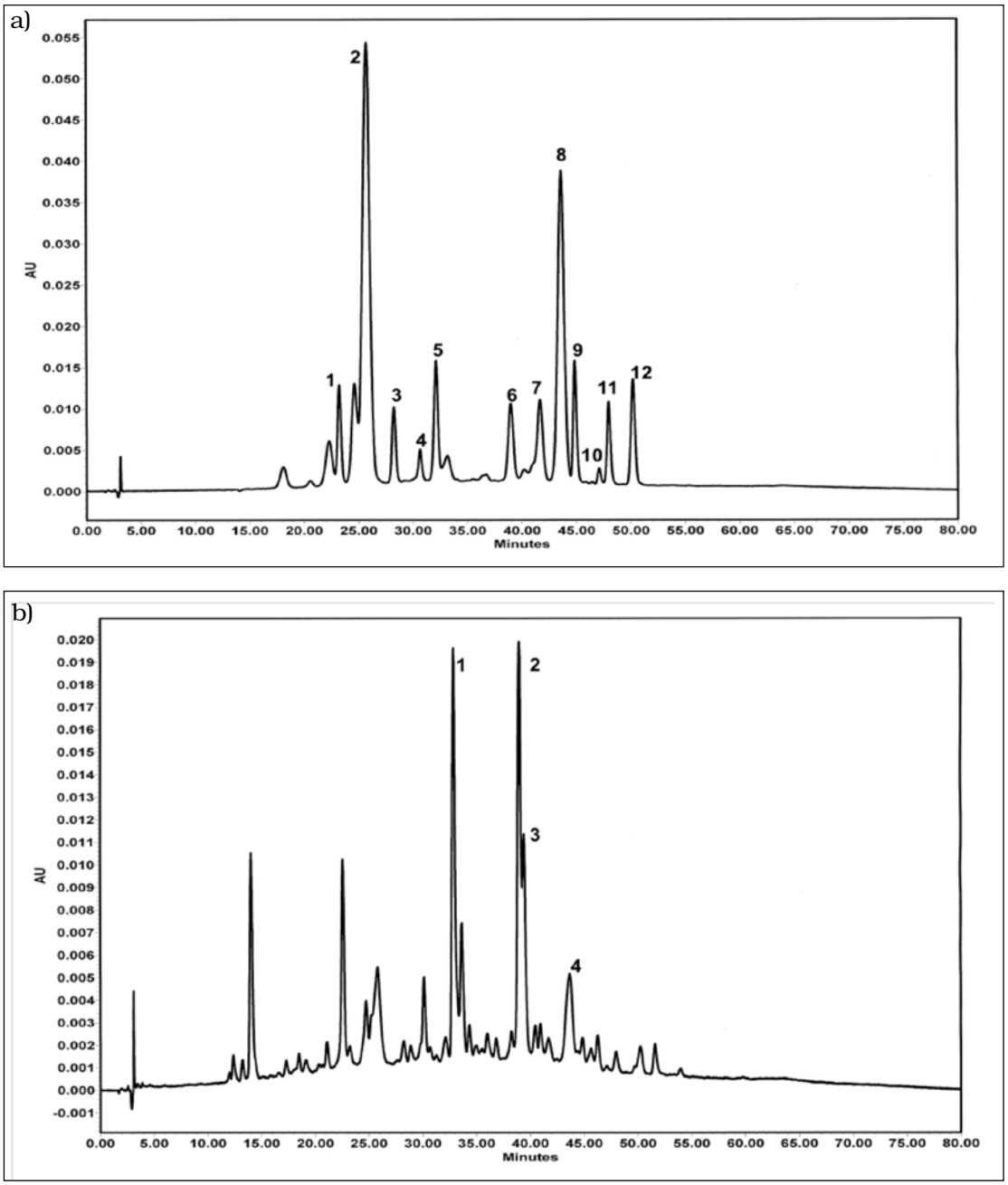


Fig. 1 - A typical HPLC chromatogram for the (a) anthocyanins (λ=510 nm) and (b) flavonols (λ=364 nm) extracted from grape pomace. The peaks are identified in Table 2.

ace, while, the solvent-temperature interaction effect was insignificant (P=0.1889). It can be seen from Table 3 that almost an equal amount of myricetin and quercetin glycosides were extracted from the grape pomace. A Student's t-test performed at 95% confidence level showed that the efficacy of the solvent in extracting flavonols from grape pomace is as follows: 80% (v/v) aqueous ethanol > 40% (v/v) aqueous ethanol > water. Comparing the results for anthocyanin and flavonol extraction from grape pomace, it can be seen that 80% (v/v) aqueous ethanol is the best solvent composition for extracting flavonoids from Sunbelt grape pomace.

While temperatures between 80° and 120°C were found to be optimum for extracting anthocyanins from grape pomace, higher temperatures (120°-140°C) were better for extracting the maximum amount of flavonols from Sunbelt grape pomace. The difference in the temperature conditions required for maximum anthocyanin versus flavonol yield can be due to lower solubility of flavonols such as quercetin at lower temperatures (SRINIVAS *et al.*, 2010). Solubility measurements made by SRINIVAS *et al.* (2010) indicated that there was an exponential increase in the solubility of quercetin aglycone at temperatures above the boiling point of water. Another reason

Table 2 - Effect of ethanol composition and temperature on the concentration of anthocyanins(mg/100 g DW) extracted from grape pomace using subcritical water. RMSD calculated from data obtained from three replicate measurements*.

Solvent***	Temp (°C)	Peak 1	Peak 2	Peak 3	Peak 4**	Peak 5	Peak 6	Peak 7	Peak 8	Peak 9	Peak 10**	Peak 11	Peak 12	Total Anthocyanins (mg/100 g DW)
0% Ethanol	60	31.6 (±4.5)	200 (±11)	4.48 (±2.2)	-nd-	26.5 (±7.2)	16.9 (±4.3)	16.0 (±9.7)	96.8 (±1.0)	6.34 (±4.5)	-nd-	0.200 (±0.3)	8.00 (±4.7)	312(±49)
	80	33.0 (±1.8)	186 (±15)	5.82 (±1.3)	-nd-	24.5 (±4.0)	19.1 (±1.8)	15.2 (±2.7)	95.5 (±0.3)	17.3 (±4.5)	-nd-	3.44 (±0.8)	15.2 (±1.2)	321(±28)
	100	35.7 (±1.4)	188 (±11)	6.03 (±1.0)	-nd-	28.3 (±0.9)	20.2 (±2.3)	18.6 (±4.4)	97.5 (±0.4)	22.2 (±4.5)	-nd-	6.23 (±1.7)	18.4 (±2.2)	345(±29)
	120	37.8 (±2.2)	210 (±11)	7.59 (±1.2)	-nd-	34.2 (±2.2)	23.6 (±1.5)	22.7 (±3.4)	115 (±0.3)	26.6 (±4.5)	-nd-	9.02 (±0.8)	22.3 (±1.4)	396(±25)
	140	30.3 (±3.2)	169 (±12)	4.14 (±0.8)	-nd-	28.6 (±2.0)	20.2 (±2.0)	18.3 (±3.1)	105 (±0.3)	22.0 (±4.5)	-nd-	6.51 (±0.7)	20.2 (±1.1)	321(±26)
40% Ethanol	60	41.8 (±4.5)	201 (±20)	8.92 (±2.3)	-nd-	35.0 (±5.0)	27.0 (±5.2)	24.3 (±8.0)	120 (±0.8)	33.0 (±4.5)	-nd-	12.5 (±8.7)	26.7 (±11)	413(±79)
	80	42.5 (±4.3)	216 (±19)	10.8 (±1.9)	-nd-	39.1 (±3.6)	29.8 (±3.4)	26.1 (±4.0)	130 (±0.4)	45.1 (±4.5)	-nd-	19.2 (±2.8)	35.0 (±3.5)	466(±46)
	100	38.2 (±0.4)	218 (±8.9)	10.7 (±0.7)	-nd-	38.7 (±3.2)	28.9 (±1.0)	28.0 (±4.8)	131 (±0.5)	45.0 (±4.5)	-nd-	19.6 (±1.1)	35.2 (±1.6)	465(±23)
	120	38.5 (±3.4)	235 (±16)	11.6 (±1.8)	-nd-	43.0 (±4.2)	31.8 (±2.2)	28.8 (±2.1)	140 (±0.2)	48.3 (±4.5)	-nd-	21.1 (±1.9)	37.9 (±2.7)	499(±38)
	140	28.3 (±2.7)	210 (±8.1)	8.82 (±0.8)	-nd-	37.6 (±2.8)	29.6 (±1.5)	29.4 (±3.3)	137 (±0.3)	44.2 (±4.5)	-nd-	19.1 (±1.3)	35.8 (±1.7)	445(±21)
80% Ethanol	60	65.6 (±31)	207 (±38)	3.72 (±6.5)	-nd-	24.2 (±18)	34.4 (±0.3)	34.0 (±3.0)	153 (±0.3)	50.6 (±4.5)	-nd-	22.2 (±2.2)	38.4 (±2.0)	484(±27)
	80	99.7 (±4.5)	173 (±31)	0.44 (±0.8)	-nd-	14.3 (±1.8)	35.2 (±5.1)	33.4 (±7.7)	160 (±0.8)	57.0 (±4.5)	-nd-	25.2 (±2.0)	41.7 (±2.5)	484(±54)
	100	116 (±22)	153 (±4.7)	-nd-	-nd-	13.1 (±0.1)	30.6 (±0.3)	28.2 (±0.3)	152 (±0.0)	54.9 (±4.5)	-nd-	24.0 (±1.0)	40.1 (±0.9)	463(±29)
	120	123 (±1.5)	152 (±17)	-nd-	-nd-	13.3 (±1.1)	30.2 (±2.6)	27.1 (±2.5)	154 (±0.2)	54.7 (±4.5)	-nd-	24.3 (±0.7)	40.7 (±0.6)	468(±26)
	140	120 (±6.8)	144 (±7.8)	-nd-	-nd-	12.5 (±0.2)	27.5 (±2.4)	25.4 (±1.2)	147 (±0.1)	51.2 (±4.5)	-nd-	22.5 (±1.0)	38.7 (±1.4)	445(±8)
Conventional Solvent	25	40.1 (±5.4)	167 (±35)	23.5 (±3.3)	43.5 (±5.2)	44.0 (±7.4)	39.1 (±6.2)	43.7 (±6.1)	108 (±21)	69.8 (±7.7)	2.10 (±0.37)	26.5 (±3.6)	38.2 (±6.6)	646(±100)

*RMSD values calculated using Equation 3; Anthocyanins peaks 1-12 are as given in Table 2.

**nd means the peaks were non-determined.

***Hydroethanolic solvents were prepared in various concentrations of ethanol/water in a volume by volume basis.

Table 3 - Effect of ethanol composition and temperature on the concentration of flavonols(mg/100 g DW) extracted from grape pomace using subcritical water. RMSD calculated from data obtained from three replicate measurements*.

Solvent**	Temperature (°C)	Peak 1	Peak 2	Peak 3	Peak 4	Total Flavonols (mg/100 g DW)
0% Ethanol	60	1.27(±0.149)	1.42(±0.282)	1.07(±0.152)	1.19(±0.090)	4.95(±0.672)
	80	1.67(±0.031)	1.73(±0.081)	1.23(±0.041)	1.19(±0.080)	5.83(±0.221)
	100	1.87(±0.105)	1.91(±0.131)	1.33(±0.069)	1.26(±0.120)	6.38(±0.425)
	120	2.13(±0.075)	2.19(±0.065)	1.52(±0.039)	1.35(±0.107)	7.19(±0.272)
	140	1.97(±0.102)	2.12(±0.122)	1.58(±0.105)	1.40(±0.075)	7.07(±0.404)
40% Ethanol	60	1.54(±0.200)	1.89(±0.409)	1.35(±0.221)	1.41(±0.135)	6.19(±0.957)
	80	2.00(±0.056)	2.26(±0.146)	1.57(±0.066)	1.54(±0.082)	7.36(±0.346)
	100	2.19(±0.086)	2.29(±0.080)	1.60(±0.050)	1.54(±0.040)	7.62(±0.252)
	120	2.53(±0.098)	2.53(±0.087)	1.78(±0.059)	1.67(±0.053)	8.50(±0.295)
	140	2.41(±0.133)	2.48(±0.141)	1.81(±0.124)	1.66(±0.104)	8.36(±0.501)
80% Ethanol	60	2.39(±0.173)	2.47(±0.152)	1.87(±0.064)	1.79(±0.059)	8.53(±0.440)
	80	2.61(±0.226)	2.73(±0.117)	2.00(±0.134)	1.72(±0.260)	9.05(±0.222)
	100	2.68(±0.065)	2.79(±0.069)	1.97(±0.060)	1.92(±0.062)	9.36(±0.249)
	120	2.86(±0.164)	2.89(±0.063)	1.95(±0.059)	1.96(±0.017)	9.65(±0.189)
	140	2.69(±0.008)	2.87(±0.195)	1.94(±0.042)	1.98(±0.077)	9.48(±0.243)
Conventional Solvent	25	12.5(±2.40)	27.9(±4.39)	16.7(±0.880)	12.8(±2.29)	69.9(±8.82)

*RMSD values calculated using Equation 3; Flavonol peaks 1-4 are as given in Table 2.

**Hydroethanolic solvents were prepared in various concentrations of ethanol/water in a volume by volume basis.

for this recorded difference could relate to a difference in the relative thermal degradability of flavonols and anthocyanins. Studies performed by TANCHEV and JONCHEVA (1973) have indicated that degradation of flavonoid glucosides increased with an increase in temperature and the thermal degradation kinetics of the flavonoids is also dependent on the pH of the solvent. PETERSSON *et al.* (2010) have shown that flavonol glycosides in red onions started to degrade at around 8 minutes at 110°C in a static batch extractor. The thermal degradation of flavonoid glycosides was found to result in breaking of the sugar-flavonoid bonds, thereby, forming aglycones and monoglucosides (KIM *et al.*, 1991).

Effect of organic acids on subcritical fluid extraction of flavonoids from grape pomace

The statistical analysis of the anthocyanin extraction data indicated that there is a significant effect of the solvent, temperature and acid ($P<0.0001$) on anthocyanin and flavonol yield from grape pomace. It was observed that two-way interaction and the three-way interaction effects of solvent, temperature and acid on the extraction of anthocyanins from Sunbelt grape pomace can also be significant. Similar results were obtained on the statistical analysis of the results on the flavonols extracted from grape pomace in this study.

The total anthocyanins and flavonols in mg/100 g dry weight (DW) of grape pomace extracted by the acidified solvent as a function of temperature are given in Table 4. A standardized Student's t-test on the effect of organic acids on the subcritical fluid extraction of anthocyanins from grape pomace indicated that acetic acid addition provided a significantly higher total anthocyanin yield relative to using the other acids. However, acetic acid was less efficient in extracting flavonols from grape pomace, and the addition of any acid to the pressurized fluid, actually decreased the extraction efficiency of flavonols. Similar trends were seen by DOWNEY *et al.* (2003) where 50-60% methanol (v/v) in water was found to be a better solvent for extracting flavonols, such as quercetin glycosides, from grape pomace, in the absence of acid addition. DOWNEY *et al.* (2003) also indicated that a mineral acid (such as HCl) had a marginally greater benefit followed by organic acid addition, to hydromethanolic solvent used for the extraction of anthocyanins from grape pomace.

Examining the two-way and three-way interaction effects mentioned previously, 80% (v/v) aqueous ethanol acidified with acetic acid at 80°C extracted a significantly higher amount of anthocyanins from grape pomace. Additionally, 80% (v/v) aqueous ethanol was an optimal solvent composition for the extraction of flavonols from grape pomace in the temperature range of 80° and 140°C and in the absence of organic acids. However when acetic acid and tartaric acid were added to 80% (v/v) aqueous ethanol a significantly greater amount of flavonols was extracted from grape pomace at 80° and 120°C respectively.

A negative effect of acids on the extraction of flavonols from grape pomace was also noticed by DOWNEY *et al.* (2003) for 50% (v/v) aqueous methanol extraction of flavonol glycosides in absence of acid. Moreover, these studies also indicated that formic and acetic acids increased the amount of flavonols extracted with increased time of extraction. This negative effect of acid addition on extraction of flavonol glycosides from grape pomace can be related to the effect of pH on thermal degradation of the flavonols. Studies performed by BUCHNER *et al.* (2006) indicated that flavonols such as quercetin and rutin, in aqueous acidified solution at pH=5.0 (with HCl) showed excessive thermal degradation leading to a loss of about 75% relative to the original total concentration at 5 hours for 100°C. Similar studies performed by GIZIR *et al.* (2008) showed thermal degradation of anthocyanins extracted from black carrots when acetic, sulfuric and lactic acids were added to subcritical water used as a solvent at temperatures greater than 100°C. In this study, the total amount of anthocyanins and flavonols extracted from Sunbelt grape pomace was found to not decrease until a temperature of 140°C was reached, which suggests the conditions used in this study are preferably to those cited above.

The total anthocyanins and flavonols extracted from Sunbelt grape pomace using acidified hydroethanolic mixtures were compared with that extracted using a conventional extraction solvent consisting of (60:37:3 (v/v) methanol:water:formic acid). The amount of anthocyanins and flavonols extracted with this solvent is assumed to be a baseline value in order to determine the efficiency of the ASE extractions. It was found that hydroethanolic mixtures (either in presence or absence of organic acids) extracted only about 12-14% of the total flavonols present in grape pomace when compared to the conventional solvent extraction method. On comparing the results using 0, 40 and 80% (v/v) aqueous ethanol to the conventional solvent extraction results, approximately 68.0, 90.5 and 95.8% of total anthocyanins were recovered respectively.

Studies performed by MONRAD *et al.* (2010a) indicated that 50-70% (v/v) aqueous ethanol extracted about 103-105% of total anthocyanins present in grape pomace when compared with the conventional solvent extraction method. In this present study, when formic, acetic, citric and tartaric acids were added to 80% (v/v) aqueous ethanol, these solvent compositions extracted approximately 92.3, 120, 96.7 and 97.7% of total anthocyanins relative to the conventional

Table 4 - Concentrations (mg/100 g DW) of total amount of anthocyanins and flavonols extracted from grape pomace using a subcritical fluid as a function of solvent composition, temperature and organic acid added to the solvent. RMSD calculated from the data obtained from three replicate measurements*.

Solvent**	Temperature (°C)	Total Anthocyanins (mg/100 g DW)	Total Flavonols (mg/100 g DW)	Solvent	Temperature (°C)	Total Anthocyanins (mg/100 g DW)	Total Flavonols (mg/100 g DW)
Formic acid				**Tartaric acid**			
0% Ethanol	60	344(±69.8)	4.41(±0.608)	**0% Ethanol**	60	456(±57.2)	5.55(±0.667)
	80	380(±97.4)	5.27(±0.384)		80	381(±40.5)	5.56(±0.313)
	100	497(±53.6)	6.37(±0.451)		100	494(±49.4)	6.29(±0.546)
	120	373(±40.3)	5.84(±0.295)		120	503(±86.0)	6.55(±0.629)
	140	406(±90.0)	6.31(±0.531)		140	371(±23.7)	6.12(±0.149)
40% Ethanol	60	509(±70.5)	6.23(±0.826)	**40% Ethanol**	60	525(±81.5)	6.25(±0.660)
	80	646(±7.67)	7.59(±0.103)		80	617(±31.1)	7.20(±0.319)
	100	604(±50.1)	7.70(±0.665)		100	614(±24.3)	7.40(±0.293)
	120	629(±12.5)	8.09(±0.136)		120	545(±20.9)	7.06(±0.159)
	140	554(±14.2)	7.49(±0.030)		140	521(±23.8)	7.27(±0.011)
80% Ethanol	60	650(±13.2)	7.64(±0.168)	**80% Ethanol**	60	591(±48.1)	7.70(±0.229)
	80	637(±5.93)	8.08(±0.179)		80	639(±24.8)	8.29(±0.025)
	100	587(±78.0)	8.24(±0.340)		100	626(±24.4)	8.71(±0.176)
	120	589(±56.4)	8.35(±0.146)		120	768(±167)	9.30(±0.530)
	140	517(±17.2)	8.25(±0.128)		140	530(±57.9)	8.97(±0.174)
Citric acid				**Acetic acid**			
0% Ethanol	60	478(±32.6)	5.35(±0.357)	**0% Ethanol**	60	346(±49.7)	4.45(±0.606)
	80	493(±39.7)	6.13(±0.413)		80	450(±17.3)	5.77(±0.113)
	100	508(±17.4)	6.60(±0.157)		100	504(±15.4)	6.66(±0.123)
	120	369(±30.2)	5.98(±0.178)		120	425(±46.9)	6.24(±0.323)
	140	409(±11.1)	6.20(±0.480)		140	350(±3.69)	6.33(±0.104)
40% Ethanol	60	547(±33.8)	6.18(±0.372)	**40% Ethanol**	60	588(±53.3)	7.00(±0.719)
	80	517(±53.2)	6.56(±0.381)		80	640(±38.3)	7.81(±0.251)
	100	575(±46.6)	7.17(±0.167)		100	612(±52.1)	7.91(±0.371)
	120	605(±39.6)	7.91(±0.433)		120	661(±11.9)	8.69(±0.109)
	140	554(±17.8)	7.77(±0.284)		140	538(±26.1)	7.97(±0.080)
80% Ethanol	60	653(±31.3)	7.39(±0.281)	**80% Ethanol**	60	757(±22.5)	7.78(±1.48)
	80	677(±29.2)	8.15(±0.110)		80	1,020(±157)	9.47(±1.48)
	100	606(±70.1)	8.33(±0.321)		100	763(±49.0)	8.37(±0.623)
	120	652(±14.6)	8.95(±0.178)		120	702(±35.5)	8.76(±0.071)
	140	534(±27.8)	8.74(±0.031)		140	624(±13.2)	8.17(±0.436)

*RMSD values calculated using Equation 3.
**Hydroethanolic solvents were prepared in various concentrations of ethanol/water in a volume by volume basis.

solvent extraction method, respectively. Under optimal conditions, acetic acid added to 80% (v/v) aqueous ethanol at 80°C, actually extracted approximately 158% of the total anthocyanins compared to conventional solvent extraction method, indicating that the conventional solvent failed to extract all of the anthocyanins from grape pomace.

Surface regression models on the anthocyanin and flavonols extractions performed on the grape pomace were performed as discussed in the Experimental section. The surface response regression method used in this study is purely empirical, since the acids were assigned numerical values in performing the linear regressions. Hence, this regression model is restricted to this study and should be used only to estimate the optimal conditions to maximize anthocyanin and flavonol yield under these extraction conditions for this grape pomace.

The parameters for the generalized linear response model obtained from JMP are given in Table 5. It can be seen from the R^2 value for the goodness of fit given in Table 5 that the predicted equation is in good agreement with the experimental values (70.1% for anthocyanins and 85.3% for flavonols). However, a regression (R^2) value greater than 90% is usually sought to indicate a very good correlation between the surface regression model and the experimental data. From the interaction profiler plots for optimizing the effect of solvent, temperature and acid on the extraction of anthocyanins (Fig. 2a) and flavonols (Fig. 2b) from grape pomace, it can be seen that 80% (v/v) aqueous ethanol was the most desired solvent. Moreover, while maximum

Table 5 - Parameters for general linear surface response model given in Equation 2 for optimization of the subcritical fluid extraction of anthocyanins and flavonols from grape pomace in presence of organic acid additives.

Coefficient*	Value for Anthocyanin data	Value for Flavonol data
β_0	432	4.74
β_1	262	3.22
β_2	-0.414	0.0155
β_3	37.1	-0.0373
β_{11}	-261	-0.879
β_{22}	-0.0421	-0.000369
β_{33}	-8.76	0.118
β_{12}	-1.41	-0.00852
β_{23}	-0.259	-0.00232
β_{13}	56.5	-0.0652
R^{2**}	0.727	0.853
S.E. of estimate**	70.1	0.505

*Coefficient of the surface regression model given by Equation 2.
**The R^2 value for the goodness of fit and the standard error (SE) of the estimates was obtained from JMP.

anthocyanin yield was obtained at 85.4°C using acetic acid as additive, the maximum flavonol yield was obtained at 124°C in absence of any organic acid added to the solvent. The optimized conditions were reported at three significant digits due to the error reported in the prediction model.

Similar studies performed by MONRAD *et al.* (2010a) indicated the optimal conditions for the extraction of anthocyanins from Sunbelt grape pomace using hydroethanolic solvent mixtures without the addition of acids was 103.7°C and 70% (v/v) aqueous ethanol as solvent.

Extraction of acylated and non-acylated flavonoids from grape pomace using pressurized solvents

Statistical analysis on the anthocyanin and flavonol extraction data revealed that water was the least desirable solvent for the optimal extraction of flavonoids from grape pomace when compared to the hydroethanolic solvents. Similar trends were also noticed by MONRAD *et al.* (2010a) and JU and HOWARD (2003; 2005). In the

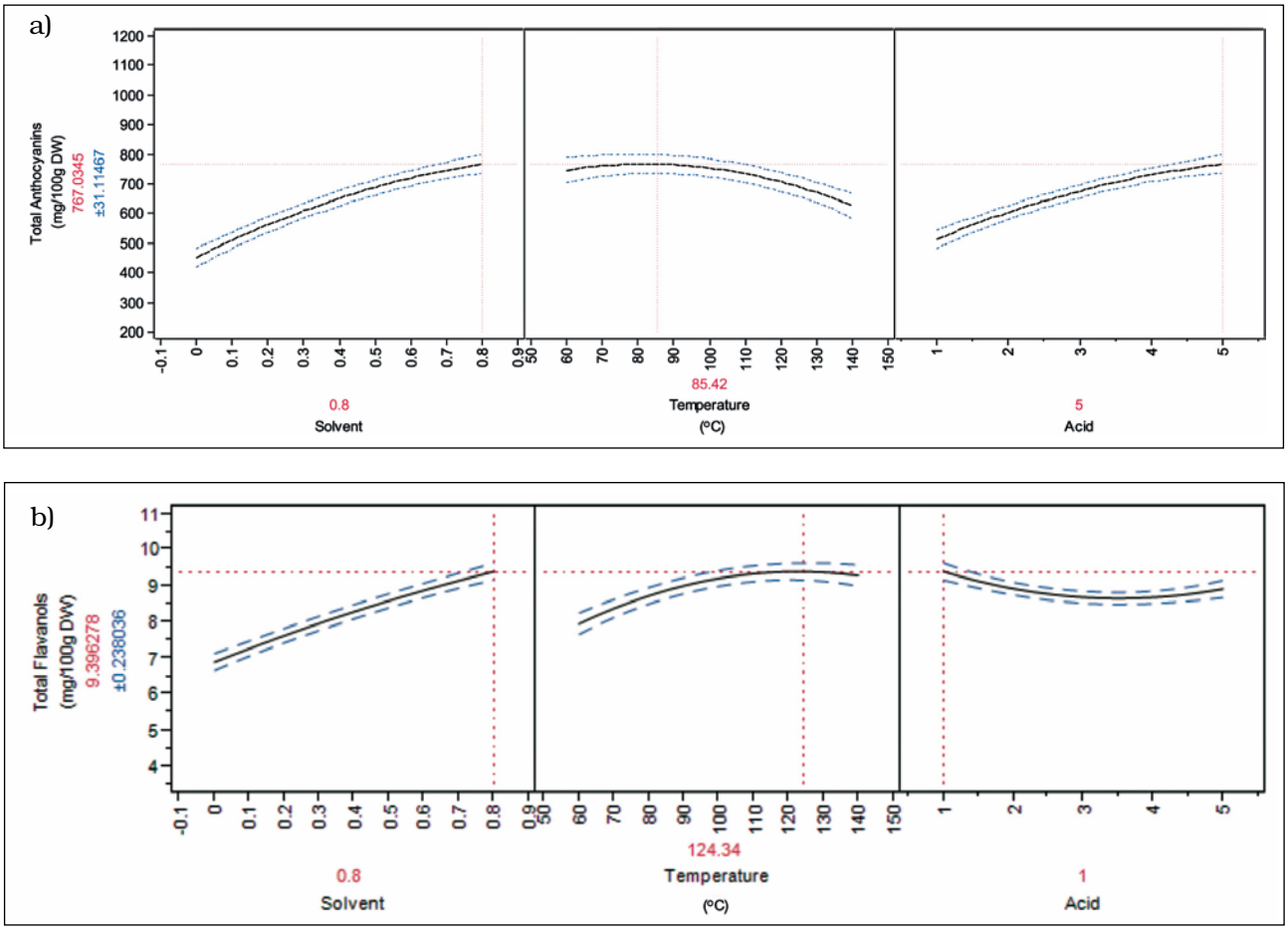


Fig. 2 - Optimal extraction conditions for (a) total anthocyanins; (b) total flavonols expressed in terms of mg/100 g DW as analyzed by the surface response regression method. The abscissa indicates solvent compositions (mole fraction of ethanol in water ranging between 0.0 and 0.8), temperature (60°, 80°, 100°, 120° and 140°C), and organic acid added to the extraction solvent (1- no acid, 2- formic acid, 3- citric acid, 4- tartaric acid, and 5- acetic acid).

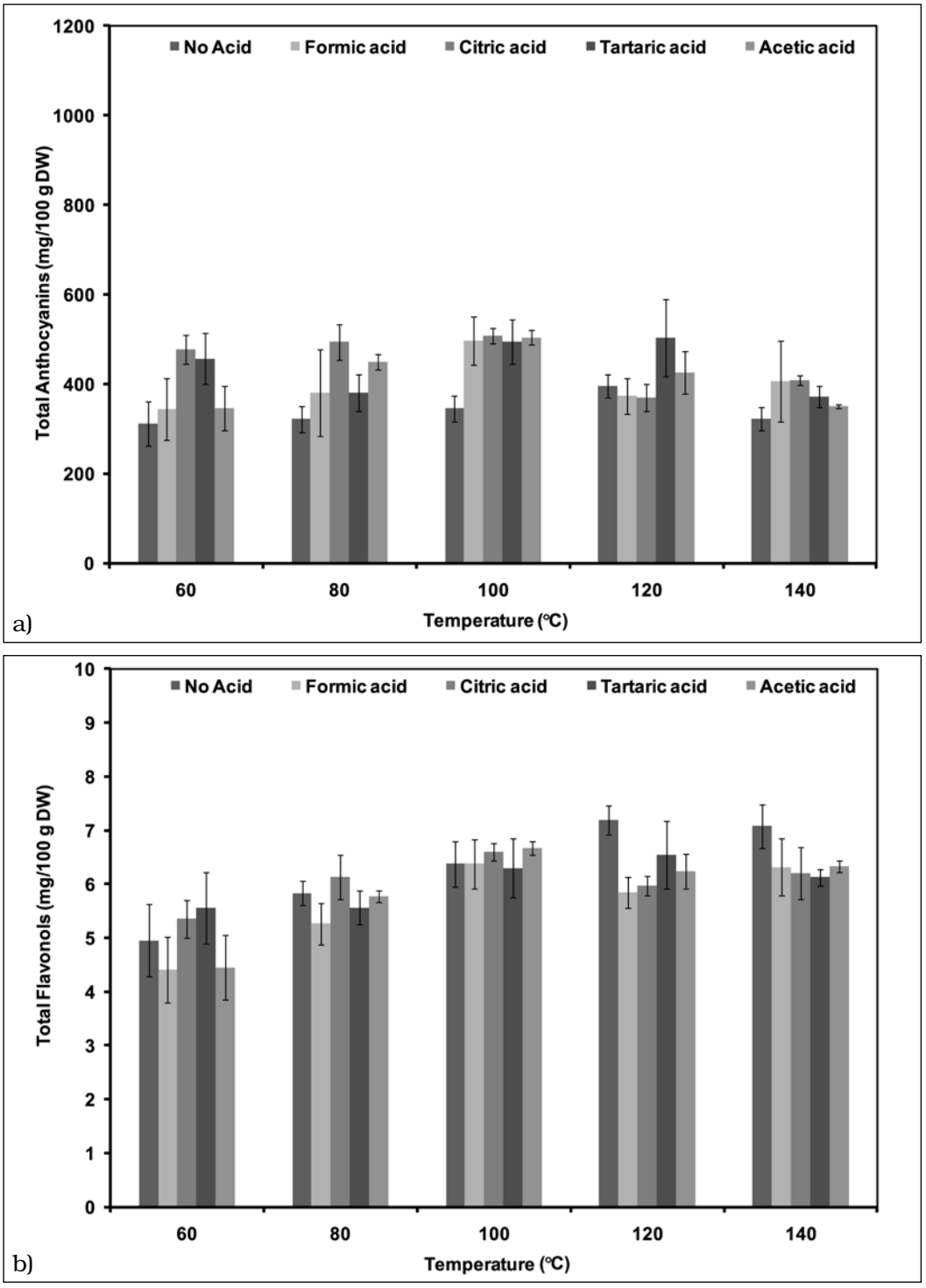


Fig. 3 - Effect of organic acids on the subcritical water extraction of (a) anthocyanins and (b) flavonols from grape pomace. The amount of anthocyanins and flavonols are expressed in terms of mg/100 g dry weight (DW). Error bars represent standard deviation calculated using Equation 3 (n=3).

studies performed by JU and HOWARD (2005), it was found that sulfured water was a better solvent than neat, subcritical water in extracting phenolic compounds from red grape skins and this was attributed to the possible formation of a sodium bisulfate complex which increased the stability of the phenolic compound. It can be seen from Fig. 3a, that addition of organic acids had a significant effect on extraction of anthocyanins from Sunbelt grape pomace while there was no significant effect of acid additions to subcritical water on extraction of flavonols from grape pomace (Fig. 3b). Moreover, there was no significant difference between the total amounts of anthocyanins extracted from grape pomace using subcritical water acidified with the different organic acids. While acetic acid added to subcritical water decreased the concentration

of total anthocyanins extracted above 100°C, citric and tartaric acids showed a minimum at 120°C followed by a slight increase in total anthocyanins extracted at 140°C.

Studies performed by JU and HOWARD (2003) indicated that acidified water extracted an equivalent amount of anthocyanins from grape pomace at 80°-100°C when compared to acidified 60% (v/v) aqueous methanol at around 120°C. They also reported that there was a greater recovery of anthocyanin mono- and di-glucosides from grape pomace when acidified water was used as extraction solvent. Studies have indicated that while non-acylated anthocyanin glycosides are water-soluble and easily assimilated by the human body (MARKAKIS, 1992), the more non-polar acylated anthocyanins are more stable to pH changes, heat treatment and light exposure (DANGLES *et al.*, 1993; FRANCIS, 1992). This high stability of the acylated anthocyanin glycosides was attributed to higher inter- and intra-molecular copigmentation due to glycosylation and acylation, metal complexing and presence of inorganic salts (GIUSTI and WROLSTAD, 2003). Studies by KURILICH *et al.* (2005) indicated that while acylated anthocyanins have relatively higher stability, their bioavailability is less compared to non-acylated polar anthocyanins. The study performed on human urine and plasma of the intake of acylated and non-acylated anthocyanins from carrots indicated that the polar non-acylated anthocyanins were easily absorbed in the human plasma while the acylated anthocyanins were easily excreted through human urinary system (KURILICH *et al.*, 2005). These studies illustrate the significance and applications of the both acylated and non-acylated anthocyanins in nutraceutical and food industries.

The total amount of acylated and non-acylated anthocyanins extracted from Sunbelt grape pomace as a function of solvent, temperature and acid added to the solvent is given in Table 6. The non-acylated anthocyanins represent the sum of peaks 1-3 while the acylated anthocyanins represents the sum of all the other peaks 4-12 (as given in Table 1). The data given in Table 6 that water had a greater tendency to extract the more polar non-acylated anthocyanins from Sunbelt grape pomace. The ratio of the non-acylated anthocyanins to acylated anthocyanins extracted from Sunbelt grape pomace using water as a solvent varied between 1.5 and 3 while that by hydroethanolic solvents was less than or equal to 1.5. Additionally, the amount of acylated anthocyanins extracted from grape pomace using water as solvent increased with an increase in the extraction temperature, thereby reducing the ratio of non-acylated to acylated anthocyanins. This tendency of subcritical water (either in presence or absence of organic acids) in extracting more polar non-acylated an-

Table 6 - Effect of organic acids on the relative amounts of total mono & di-glucosides and the acylated glucosides extracted from grape pomace using pressurized solvents.

Solvent**	Temperature (°C)	Total mono & di-glucosides (mg/100 g DW)*	Total acylated glucosides (mg/100 g DW)*	Ratio of total mono & di-glucosides to total acylated glucosides
		No acid		
0% Ethanol	60	236(±18)	75.5(±32)	3.13
	80	225(±17)	96.2(±12)	2.34
	100	229(±14)	116(±15)	1.98
	120	255(±15)	141(±11)	1.81
	140	204(±16)	118(±10)	1.73
40% Ethanol	60	252(±26)	161(±53)	1.56
	80	270(±24)	197(±22)	1.37
	100	266(±10)	198(±13)	1.34
	120	285(±21)	214(±17)	1.34
	140	247(±10)	199(±13)	1.24
80% Ethanol	60	276(±14)	207(±14)	1.33
	80	273(±31)	210(±23)	1.30
	100	269(±26)	194(±3.5)	1.39
	120	275(±16)	193(±9.4)	1.43
	140	264(±5.9)	180(±2.1)	1.47
		Formic acid		
0% Ethanol	60	201(±29)	142(±42)	1.41
	80	206(±50)	175(±47)	1.18
	100	261(±25)	236(±29)	1.10
	120	186(±24)	187(±19)	0.991
	140	198(±53)	208(±38)	0.953

Table 6 - Continued.

Solvent**	Temperature (°C)	Total mono & di-glucosides (mg/100 g DW)*	Total acylated glucosides (mg/100 g DW)*	Ratio of total mono & di-glucosides to total acylated glucosides
40% Ethanol	60	246(±26)	263(±46)	0.933
	80	292(±6.4)	354(±1.4)	0.825
	100	278(±23)	327(±27)	0.850
	120	284(±8.4)	346(±5.0)	0.820
	140	243(±7.6)	312(±7.5)	0.778
80% Ethanol	60	270(±11)	380(±3.5)	0.711
	80	257(±11)	379(±4.8)	0.678
	100	241(±51)	346(±28)	0.696
	120	241(±48)	348(±8.2)	0.693
	140	213(±3.2)	304(±17)	0.700
		Citric acid		
0% Ethanol	60	264(±11)	213(±30)	1.24
	80	257(±17)	237(±23)	1.08
	100	257(±7.7)	251(±9.7)	1.03
	120	177(±17)	192(±13)	0.923
	140	203(±3.4)	206(±10)	0.982
40% Ethanol	60	252(±13)	295(±21)	0.852
	80	239(±21)	278(±32)	0.860
	100	267(±21)	308(±26)	0.868
	120	281(±22)	324(±18)	0.866
	140	247(±6.9)	307(±11)	0.805
80% Ethanol	60	259(±9.0)	393(±22)	0.660
	80	268(±12)	409(±17)	0.656
	100	256(±37)	350(±33)	0.732
	120	282(±8.6)	370(±6.3)	0.763
	140	216(±16)	318(±11)	0.677
		Tartaric acid		
0% Ethanol	60	251(±11)	205(±46)	1.22
	80	200(±19)	181(±22)	1.10
	100	254(±24)	239(±26)	1.06
	120	254(±43)	249(±43)	1.02
	140	177(±13)	194(±11)	0.914
40% Ethanol	60	252(±33)	273(±49)	0.925
	80	290(±14)	328(±17)	0.884
	100	289(±11)	325(±13)	0.887
	120	255(±11)	290(±9.8)	0.881
	140	236(±15)	285(±9.1)	0.830
80% Ethanol	60	229(±27)	362(±22)	0.632
	80	254(±17)	385(±7.8)	0.659
	100	249(±21)	376(±3.6)	0.663
	120	264(±13)	504(±18)	0.523
	140	211(±37)	319(±21)	0.660
		Acetic acid		
0% Ethanol	60	208(±19)	138(±37)	1.50
	80	245(±8.2)	205(±9.5)	1.19
	100	265(±4.6)	239(±11)	1.11
	120	217(±24)	208(±21)	1.04
	140	162(±3.6)	188(±2.7)	0.858
40% Ethanol	60	273(±16)	315(±38)	0.864
	80	288(±18)	352(±21)	0.817
	100	275(±22)	338(±31)	0.814
	120	293(±7.7)	368(±6.0)	0.795
	140	221(±15)	318(±12)	0.695
80% Ethanol	60	338(±16)	419(±8.3)	0.807
	80	461(±50)	560(±11)	0.824
	100	328(±10)	435(±42)	0.754
	120	305(±21)	397(±16)	0.769
	140	272(±12)	353(±1.6)	0.770
Conventional	25	231(±43)	415(±59)	0.553

*RMSD values calculated using Equation 3.

**Hydroethanolic solvents were prepared in various concentrations of ethanol/water in a volume by volume basis.

thocyanin glycosides from Sunbelt grape pomace makes it a desirable extraction solvent for nutraceutical applications.

A similar trend, i.e., increase in the amount of non-acylated anthocyanins extracted from grape pomace with an increase in the extraction temperature was also seen by JU and HOWARD (2003) and was attributed to a decrease in polarity of subcritical water resulting in the extraction of the more non-polar anthocyanins. From Table 6, it can also be seen that the addition of organic acid to subcritical water also increased the total amount of acylated anthocyanins extracted from grape pomace. Of all the organic acids added to the subcritical fluids, citric and tartaric acids extracted a larger amount of acylated anthocyanins relative to the amount of non-acylated anthocyanins, thereby exhibiting a lower non-acylated to acylated anthocyanin ratio as a function of solvent and temperature when compared to extraction using non-acidified solvents.

When 40% (v/v) aqueous ethanol was used as a solvent, there was no significant difference between the non-acylated to acylated anthocyanin ratio using different organic acid and temperature conditions. The acidified hydroethanolic mixtures, however, are given greater preference by the food industry due to their tendency to extract greater amounts of acylated anthocyanins from natural products which have greater color, stability to temperature and light (GIUSTI and WROLSTAD, 2003) and can be used as natural colorants.

CONCLUSIONS

In this study, the effect of ethanol as co-solvent, temperature and organic acids on the subcritical water extraction of anthocyanins were analyzed. The solvent pH was maintained at 2.5 units by using formic, acetic, citric and tartaric acids. 80% (v/v) ethanol/water was found to be the most optimal solvent for extracting a larger amount of anthocyanins (in presence of acetic acid) and flavonols (in absence of organic acids added to the solvent) from Sunbelt grape pomace. Surface regression analysis of the anthocyanin and flavonol data indicated that the optimum extraction temperature for maximum anthocyanin yield was 85.4°C and that for maximum flavonol yield as 124°C. Under these optimal conditions, approximately 158% of the total anthocyanins and 14% of total flavonols were extracted from Sunbelt grape pomace when compared to the conventional solvent extraction method (60:37:3 (v/v) methanol:water:formic acid). Subcritical water, in the presence or absence of organic acids, extracted a greater amount of polar non-acylated anthocyanins from grape pomace when compared to acidified hydroethanolic mixtures. The hydroethanolic mixtures (especially acidified with formic or acetic acid) extracted greater amounts of acylated anthocyanins from Sunbelt grape pomace.

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REFERENCES

Anderson D.W., Gueffory D.E., Webb A.D. and Kepner R.E. 1970. Identification of acetic acid as an acylating agent of anthocyanin pigments in grapes. Phytochemistry 9: 1579.

Bleve M., Ciurlia L., Erroi E., Lionetto G., Longo L., Rescio L., Schettino T. and Vasapollo G. 2008. An innovative method for the purification of anthocyanins from grape skin extracts by using liquid and sub-critical carbon dioxide. Sep. Purif. Technol. 64: 192.

Borges G., Degeneve A., Mullen W. and Crozier A. 2010. Identification of flavonoid and phenolic antioxidants in black currants, blueberries, raspberries, red currants and cranberries. J. Agric. Food Chem. 58: 3901.

Bozidar S. 1995. The role of polyphenols as chemopreventers. Polyphenol Actualities 13: 24.

Braidot E., Zancani M., Petrusa E., Peresson C., Bertolini A., Patui S., Macri F. and Vianello A. 2008. Transport and accumulation of flavonoids in grapevine (*Vitis vinifera L.*). Plant Signal Behav. 3: 626.

Buchner N., Krumbein A., Rohn S. and Kroh L.W. 2006. Effect of thermal processing on the flavonoids rutin and quercetin. Rapid Commun. Mass Spectrom. 20: 3229.

Cacace J.E. and Mazza G. 2002. Extraction of anthocyanins and other phenolics from black currants with sulfured water. J. Agric. Food Chem. 50: 5939.

Cho M.J., Howard L.R., Prior R.L. and Clark J.R. 2004. Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. J. Sci. Food Agric. 84: 1771.

Clifford M.N. 2000. Anthocyanins – nature, occurrence and dietary burden. J. Sci. Food Agric. 80: 1063.

Dangles O., Saito N. and Brouillard R. 1993. Anthocyanin intramolecular copigment effect. Phytochemistry 34: 119.

de Castro M.D.L., Luque-Rodriguez M.L. and Japon-Lujan R. 2008. Exploitation of residues from vineyards, olive groves, and wine and oil production to obtain phenolic compounds of high-added value. In: "Methods of Analysis for Functional Foods and Nutraceuticals" Hurst W.J. (Ed.) p. 207. CRC Press, Boca Raton, FL.

Downey M.O., Mazza M. and Krstic M.P. 2007. Development of a stable extract for anthocyanins and flavonols from grape skin. Am. J. Enol. Vitic. 58: 3.

Francis F.J. 1992. A new group of food colorants. Trends Food Sci. Technol. 3: 27.

Francis J. and Markakis P.C. 1989. Food colorants: anthocyanins. Crit. Rev. Food Sci. Nutr. 28: 273.

Garcia-Marino M., Rivas-Gonzalo J.C., Ibanez E. and Garcia-Moreno C. 2006. Recovery of catechins and proanthocyanidins from winery by-products using subcritical water extraction. Anal. Chem. Acta. 563: 44.

Giusti M.M. and Wrolstad R.E. 2003. Acylated anthocyanins from edible sources and their applications in food systems. Biochem. Eng. J. 14: 217.

Gizir A.M., Turker N. and Artuvan E. 2008. Pressurized acidified water extraction of black carrot (*Daucus carota ssp. Sativus var. artrorubens Alef.*) anthocyanins. Eur. Food Res. Technol. 226: 363.

Hager T.J., Howard L.R. and Prior R.L. 2008. Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of pressed blackberry products. J. Agric. Food Chem. 56: 689.

Halpern M.J., Dahlgren A-L., Leakso I., Seppanen-Laakso T., Dahlgren J. and McAnulty P.A. 1998. Red-wine polyphenols and inhibition of platelet aggregation: possible mechanisms, and potential use in health promotion and disease prevention. J. Intl. Med. Res. 26: 171.

Harborne J.B. and Williams C. 2000. Advances in flavonoid research since 1992. Phytochem. 55: 481.

Heredia, F.J., Francia-Aricha, E.M., Rivas-Gonzalo, J.C., Vicario, I.M. and Santos-Buelga C. 1998. Chromatic characterization of anthocyanins from red grapes I. pH effect. Food Chem. 63: 491.

Jackman R.L., Yada R.Y., Tung M.A. and Speers R.A. 1987. Anthocyanins as food colorants – a review. J. Food Biochem. 11: 201.

Ju Z. and Howard L.R. 2006. Subcritical water and sulfured water extraction of anthocyanins and other phenolics from dried red grape skin. J. Food Sci. 70: S270.

Ju Z.Y. and Howard L.R. 2003. Effect of solvent and temperature on pressurized liquid extraction of anthocyanins and total phenolics from dried red grape skin. J. Agric. Food Chem. 51: 5207.

Kim Y. C., Higuchi R., Kitamura Y. and Komori T. 1991. Thermal degradation of glycosides, IV. Degradation of flavonoid glycosides. Liebigs Annalen der Chemie, 1991: 1285

King J.W. and Srinivas K. 2009. Multiple unit processing using sub- and supercritical fluids. J. Supercrit. Fluids. 47: 598.

King J.W., Gabriel R.D. and Wightman J.D. 2003. Subcritical water extraction of anthocyanins from fruit berry substrates. Proceedings of the 6th International Symposium on Supercritical Fluids, Tome 1, April 28-30, Versailles, France, p. 409.

Kurilich A.C., Clevidence B.A., Britz S.J., Simon P.W. and Novotny J.A. 2005. Plasma and urine responses are lower for acylated *vs* nonacylated anthocyanins from raw and cooked purple carrots. J. Agric. Food Chem. 53: 6537.

Lafka T-I., Sinanoglou V. and Lazos E.S. 2007. On the extraction and antioxidant activity of phenolic compounds from winery wastes. Food Chem. 104: 1206.

Lapidot T., Harel S., Akiri B., Granit R. and Kanner J. 1998. pH-dependent forms of red wine anthocyanins as antioxidants. J. Agric. Food Chem. 47: 67.

Lapornik B., Prosek M. and Wondra A.G. 2005. Comparison of extracts prepared from plant by-products using different solvents and extraction time. J. Food Sci. 71: 214.

Lurton L. 2003. Grape polyphenols: a new powerful health ingredients. Innovations Food Tech. 18: 28.

Luthria D.L. 2008. Influence of experimental conditions on the extraction of phenolic compounds from parsley (*Petroselinium crispum*) flakes using a pressurized liquid extractor. Food Chem. 107: 745.

Markakis P. 1992. Stability of anthocyanins in foods. In "Anthocyanins as Food Colors". Markakis P. (Ed.). Chapter 6. Academic Press, New York.

Metivier R.P., Francis F.J. and Clydesdale F.M. 1980. Solvent extraction of anthocyanins from wine pomace. J. Food Sci. 45: 1099.

Monrad J.K., Howard L.R., King J.W., Srinivas K. and Mauromoustakos A. 2010a. Subcritical solvent extraction of anthocyanins from dried red grape pomace. J. Agric. Food Chem. 58: 2862.

Monrad J.K., Howard L.R., King J.W., Srinivas K. and Mauromoustakos A. 2010b. Subcritical solvent extraction of procyanidins from dried red grape pomace. J. Agric. Food Chem. 58: 4014.

Moure A., Cruz J.M., Franco D., Dominguez J.M., Sineiro J., Dominguez H., Jose Nunez M. and Parajo C. 2001. Natural antioxidants from residual sources. Food Chem. 72: 145.

Mukhopadhyay S., Luthria D.L. and Robbins R.J. 2006. Optimization of extraction process for phenolic acids from black cohosh (*Cimicifuga racemosa*) by pressurized liquid extraction. J. Sci. Food Agric. 86: 156.

Murthy K.N.C., Singh R.P. and Jayaprakasha G.K. 2002. Antioxidant activities of grape (*Vitis vinifera*) pomace extracts. J. Agric. Food Chem. 50: 5909.

Palma M., Pineiro Z. and Barroso C.G. 2001. Stability of phenolic compounds during extraction with superheated solvents. J. Chromatogr. A. 921: 169.

Petersson E.V., Liu J., Sjoberg P.J.R., Danielsson R., and Turner C. 2010. Pressurized hot water extraction of anthocyanins from red onion: a study on extraction and degradation rates. Anal. Chim. Acta 663: 27.

Pinelo M., Arnous A. and Meyer A.S. 2006. Upgrading of grape skins: significance of plant cell-wall structural components and extraction techniques for phenol release. Trends Food Sci. Tech. 17: 579.

Revilla E., Ryan J-M. and Martin-Ortega G. 1998. Comparison of several procedures used for the extraction of anthocyanins from red grapes. J. Agric. Food Chem. 46: 4592.

Ruberto G., Renda A., Daquino C., Amico V., Spatafora C., Tringali C. and De Tommasi N. 2007. Polyphenol constituents and antioxidant activity of grape pomace extracts from five Sicilian red grape cultivars. Food Chem. 100: 203.

Savova M., Kolusheva T., Stourza A. and Seikova I. 2007. The use of group contribution method for predicting the solubility of seed polyphenols of *Vitis Vinifera L.* within a wide polarity range in solvent mixtures. J. Univ. Chem. Technol. Metallur. 42: 295.

Schieber A., Stintzing F.C. and Carle R. 2001. By-products of plant food processing as a source of functional compounds - recent developments. Trends Food Sci Technol. 12: 401.

Sharif A., Saim N., Jasmani H. and Ahmad W.Y.W. 2010. Effects of solvent and temperature on the extraction of colorant from onion (*Allium cepa*) skin using pressurized liquid extraction. Asian J. Appl. Sci. 3: 262.

Souquet J-M., Labarbe B., Le Guerneve C., Cheynier V. and Moutounet M. 2000. Phenolic composition of grape stems. J. Agric. Food Chem. 48: 2076.

Spigno G. and De Faveri D.M. 2007. Antioxidants from grape stalks and marc: influence of extraction procedure on yield, purity and antioxidant power of the extracts. J. Food Eng. 78: 793.

Srinivas K., King J.W., Howard L.R. and Monrad J.K. 2010. Determination of solubility of quercetin and quercetin dihydrate in subcritical water using a dynamic flow technique. J. Food Eng. 100: 208.

Srinivas K., King J.W., Monrad J.K., Howard L.R. and Hansen C.M. 2009. Optimization of subcritical fluid extraction of bioactive compounds using Hansen solubility parameters. J. Food Sci. 74: E342.

Tanchev S.S. and Joncheva N. 1973. Kinetics of the thermal degradation of cyaniding-3-rutinoside and peonidin-3-rutinoside. Z. Lebensm. Unters-Forsch 153: 37.

Toyomizu M., AAkiba Y., Horiguchi M. and Matsumoto T. 1982. Multiple regression and response surface analyses of the effects of dietary protein, fat and carbohydrate on the body protein and fat gains in growing chicks. J. Nutr.112: 886.

Wang H., Cao G. and Prior R.L. 1997. Oxygen radical absorbing capacity of anthocyanins. J. Agric. Food Chem. 45: 304.

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MOLECULAR CHARACTERIZATION OF *L. MONOCYTOGENES* ISOLATED FROM READY-TO-EAT SEAFOOD IN ITALY

A. MUREDDU, M. CONTER[1], D. MELONI*, F. PIRAS, A. IANIERI[1] and R. MAZZETTE
Dipartimento di Biologia Animale, Facoltà di Medicina Veterinaria, Università di Sassari,
Via Vienna 2, 07100 Sassari, Italy
[1]Dipartimento di Produzioni Animali, Biotecnologie Veterinarie,
Qualità e Sicurezza degli Alimenti, Università di Parma, 43100 Parma, Italy
*Corresponding author: Tel. +39 079 229 447, Fax +39 079 229 458,
e-mail: dmeloni@uniss.it

ABSTRACT

Detection and enumeration of *L. monocytogenes* have been carried out in 92 Ready-to-Eat (RTE) seafood products, manufactured in different countries and marketed in Italy. Fifty-one strains were characterised by serotyping, multiplex PCRs for the presence of virulence genes and PFGE. Of the samples tested, 8.6% were contaminated at a level of less than 100 CFU/g in 98% of the samples. Isolates were serotyped as 1/2a, 1/2b, 1/2c and 4e, and presented all the virulence genes considered. PFGE revealed a high similarity among the isolates, suggesting the existence of a widespread population of *L. monocytogenes* clones in different geographical areas.

- Key words*: Listeria monocytogenes,* multiplex PCR, PFGE, ready-to-eat, seafood products -

INTRODUCTION

L. monocytogenes, an ubiquitous microorganism in the environment, is found in a wide range of raw foods and is able to adapt to different conditions of pH and temperature (including refrigeration) as well as high salt concentrations (FARBER, 2000). *L. monocytogenes* is also the etiologic agent of listeriosis, which occurs in both humans and animals. Two main forms of listeriosis have been described in humans: gastro-enteric and invasive, with the latter posing a serious problem to public health. In fact, invasive human listeriosis is a rare but severe infection, typically causing septicemia, encephalitis and meningitis (VAZQUEZ-BOLAND *et al.*, 2001b; SWAMINATHAN and GERNER-SMIDT, 2007). The categories at high-risk are the so-called "YOPI": young, old, pregnant, and immunocompromised individuals (FARBER and PETERKIN, 1991). Since the beginning of the 1980s *L. monocytogenes* has been recognized as an emerging food-borne pathogen after several sporadic and epidemic cases of listeriosis occurred in Europe and the USA (KATHARIOU, 2002). Food such as milk, dairy products, vegetables, and meat products are most frequently associated with listeriosis. Seafood products have also been reported to be contaminated with *L. monocytogenes*, however, few reports of outbreaks or sporadic cases have been linked to these products (JOHANSSON *et al.*, 1999; MEDRALA *et al.*, 2003). In recent years epidemiological evidence has indicated that listeriosis is often associated with "ready-to-eat" (RTE) seafood products, and in particular with cold or hot-smoked salmon, "gravad" salmon, shrimps, mussels, fermented fish, fish and seafood salads (BEN EMBAREK, 1994; ROCOURT *et al.*, 2000; GOMBAS *et al.*, 2003). The prevalence of *L. monocytogenes* has been widely analyzed and varies from zero (BEN EMBAREK *et al.*, 1997) to about 30% (HOFFMAN *et al.*, 2003; MIETTINEN and WIRTANEN, 2005) in raw fish and from zero to 75% in cold-smoked salmon (DAUPHIN *et al.*, 2001). The contamination level is normally low (<100 colony forming units-CFU/g) but, in favourable conditions, *L. monocytogenes* may exceed the level of 100 CFU/g. This was the case in a Finnish outbreak associated with cold-smoked rainbow trout, where levels of 190 x 10^3 CFU/g were detected (MIETTINEN *et al.*, 1999). Commercially prepared seafood salads made by food processors may be contaminated with *L. monocytogenes*, but usually the addition of acidulants and inhibitors can retard its growth. In contrast, fresh seafood salads prepared in retail outlets may not contain preservatives and could support the growth of *L. monocytogenes* to high numbers (ILSI, 2005). Many factors influence the sources of *L. monocytogenes* contamination of seafood products: the use of contaminated raw materials, the efficacy of cleaning and processing procedures and the existence of surface-persistent *L. monocytogenes* subtypes in processing facilities. There have been reports of characteristic "in-house" *L. monocytogenes* strains that persistently colonize the production environments of particular plants (AUTIO *et al.*, 1999; RØRVIK, 2000). The molecular pathogenesis of *L. monocytogenes* is determined by multiple key virulence factors, such as internalins, haemolysin, phosphatidylinositol-specific phospholipase C (PI-PLC), phosphatidylcholine-specific phospholipase C (PC-PLC) and actin polymerization protein (VAZQUEZ-BOLAND *et al.*, 2001a). The expression of these virulence factors is directly modulated by the regulator gene *prfA* (KREFT and VAZQUEZ-BOLAND, 2001). It is essential to characterize *L. monocytogenes* strains in order to carry out epidemiological studies and to trace the sources of contamination in the food chain (VITAS *et al.*, 2004). Several phenotypic and molecular methods are used, including serotyping, multilocus enzyme electrophoresis (MLEE), pulsed-field gel electrophoresis (PFGE), ribotyping and DNA sequencing-based subtyping techniques such as multilocus sequence typing (MLST) (LIU *et al.*, 2005; JIANG *et al.*, 2008). PFGE is one of the most powerful methods in subtyping *L. monocytogenes* strains in epidemiological studies and investigations on outbreak sources (MEAD *et al.*, 2006; GERNER-SMIDT *et al.*, 2006). It has been shown to be a very accurate and reproducible method and has been widely used for identifying the existence of persistent clones in the processing environments and for investigating sources and routes of contamination in processing plants (BILLE and ROCOURT, 1996; CHOU and WANG, 2006; THÉVENOT *et al.*, 2006; LOMONACO *et al.*, 2009). PFGE is still currently the "gold standard" for *L. monocytogenes* subtyping due to its high reproducibility, robustness and high discriminating power (GRAVES and SWAMINATHAN, 2001; ZHANG *et al.*, 2007). The aims of this study were to: i) investigate the prevalence and level of contamination by *L. monocytogenes* in RTE seafood products marketed in Italy, ii) to define the pathogenic profile of the isolates, iii) to analyze the intraspecies diversity, iv) to determine whether there are predominant clones and how widespread they are in RTE seafood products.

MATERIALS AND METHODS

Sample collection

During a 7-month period (September 2004 - March 2005), a total of 92 RTE seafood products were collected from different retail outlets in Sardinia (Italy). The samples analyzed were 50 sliced cold smoked fish from 26 different manufacturers and 42 seafood salads. Concerning the smoked products, 90% of the fish were caught or reared in FAO fishing area 27 (Atlantic, Northeast), 6%

were from FAO fishing area 31 (Atlantic, Western-central), 2% from area 77 (Pacific, Eastern-central), and 2% from area 87 (Pacific, Southeast). The products were processed in plants located in Norway (50%), Spain (18%), UK (16%), Ireland, France (6% each), and Denmark (4%). All the products were vacuum packaged and stored at refrigeration temperatures (0°-4°C). In 76% of the products there was no evidence of the shelf life set by the producer, because of the lack of the production date. In the remaining 24%, the (mean ± S.D.) shelf life was of 75±23.93 days. With regard to seafood salads, over 80% of the products were processed in Italy and the rest in other European countries. All the products were packaged in modified atmosphere (MAP) and stored at refrigeration temperatures (0°-4°C). In all the products there was no evidence of the shelf life set by the producer (Table 2). All the items of RTE seafood products were kept in ice boxes at 3°C during transport and were immediately analyzed upon arrival at the laboratory. Details on product type, country and shelf life of the samples are shown in Tables 1 and 2.

Chemical-physical analysis

pH and a_w were determined in order to assess if the RTE seafood products were able to support the growth of *L. monocytogenes.* These values were compared to those stated in EC Regulation 2073/2005: products with pH ≥ 4.4 or a_w ≥ 0.92, products with pH ≥ 5.0 and a_w ≥ 0.94). The pH was measured in duplicate, using a GLP 21 pH-meter (Crison, Carpi, MO, Italy) according to APHA methods (2001). A_w was measured in duplicate using a CX3 AquaLab (Decagon Devices, Inc., Pullman, Washington, USA). The final pH and a_w values were obtained by taking the average of the two measurements.

Detection and enumeration of *Listeria* spp and *L. monocytogenes*

Detection and enumeration of *Listeria* spp and *L. monocytogenes* were carried out according to methods of the International Organisation for Standardization: EN ISO 11290-1:1996 (ANONYMOUS, 1996) and 11290-2:1998 (ANONYMOUS, 1998). Briefly, 25 g of solid samples were homogenized with 225 mL of Fraser broth base (Biolife, Milan, Italy) and incubated at 20°C for 1 h. *L. monocytogenes* was enumerated by spreading a volume of 1 mL on 3 Palcam agar plates (Oxoid, Milan, Italy), incubated at 37°C for 24-48 h. For the detection of *L. monocytogens*, the homogenates were supplemented with the Half Fraser (Biolife) selective supplement (first enrichment) and incubated at 30°C for 24 h. For the second enrichment, a volume of 0.1 mL was inoculated in 10 mL of Fraser selective supplement (Biolife) and incubated at 37°C for 48 h. Plates of Oxford agar (Oxoid) and Agosti Listeria Ottaviani Agar (ALOA, Biolife) incubated at 30° and 37°C for 48 h, respectively, were used as solid isolation medium from both enrichments. Up to five suspected colonies of *Listeria* spp were selected from each selective medium and streaked for isolation on Tryptone Soya Yeast Extract Agar plates (TSYEA, Oxoid). After incubation at 37°C for 24 h, isolates were submitted to Gram staining, catalase and oxidase tests. For the confirmation of *L. monocytogenes*, all *Listeria* spp suspected colonies were submitted to haemolytic activity and CAMP test on sheep blood agar plates (37°C/24 h) and to biochemical characterization by using the API Listeria identification system (bioMérieux, Marcy l'Etoile, France).

Serotyping

Serotyping of *L. monocytogenes* strains was carried out by the agglutination method using commercial *Listeria* antisera (Denka Seiken, Ltd, Swadlincote, UK) for somatic antigens (O) according to manufacturer's instructions. Detection of flagellar antigens (H) was carried out using the procedure described by UEDA *et al.* (2002).

PCR analysis of virulence factors

L. monocytogenes isolates were grown on Tryptone Soya Broth (TSB, Oxoid) at 37°C for 16-18 h. The cells were pelleted by centrifugation at 10,000 rpm for 1 h at 4°C and washed twice in phosphate-buffered saline (PBS). DNA was extracted using the "High Pure PCR Template Preparation Kit" (Roche, Basel, Switzerland), according to the manufacturer's instructions. Three multiplex PCRs were standardized in order to detect the following genes: multiplex PCR 1): *rrn* (16S rRNA), *hlyA* (haemolysin), *actA* (actine) and *prfA* (regulatory gene); multiplex PCR 2): *inlA* (internalin A), *inlB* (internalin B) and *iap* (protein p60); multiplex PCR 3): *plcA* (phosphatidylinositol-specific phospholipase C), *plcB* (phosphatidylcholine-speciphic phospholipase C) and *mpl* (zinc metalloprotease) by modifying the protocols of BORDER *et al.* (1990) and JARADAT *et al.* (2002). All amplification reactions were performed in a final volume of 50 µL, containing 2 µL of DNA, 5U of Taq polymerase (Roche diagnostics, Milan, Italy), 0.2 m-mol l-1 of each deoxynucleoside triphosphate (dNTP), 1X PCR buffer (1.5 m- mol l-1 $MgCl_2$, 50 m-mol l-1 KCl, 10 m-mol l-1 Tris-HCl, pH 8.3). Table 4 lists the concentration of each primer (Eurogentec, Seraing, Belgium) used in the three multiplex PCRs. All amplification reactions were performed in a Techne TC-412 thermocycler (Techne, Cambridge, UK) programmed as follows: for multiplex PCR 1, denaturation at 94°C for 80 s, annealing at 55°C for 90 s and extension at 72°C for 120 s, followed by a final extension peri-

Table 1 - Characteristics, prevalence and level of contamination by *Listeria* spp and *L. monocytogenes* of the cold smoked fish (n. 50).

Sample code	Food product	Country	Shelf life (days)	pH (Mean±s.d.)	a_w (Mean±s.d.)	Presence of *Listeria* spp	Presence of *L. monocytogenes*	*L. monocytogenes* cfu/g
A1	CSS[a]	NOR	*nd*[c]	6.23±0.04	0.95±0.00	-	-	-
A2	CSS	NOR	*nd*	5.98±0.00	0.92±0.00	+	+	<100
A3	CSSW[b]	SPA	*nd*	5.58±0.02	0.95±0.00	+	+	<100
A4	CSS	SPA	*nd*	5.99±0.01	0.94±0.00	-	-	-
A5	CSS	NOR	*nd*	5.98±0.04	0.93±0.00	-	-	-
A6	CSS	FRA	*nd*	5.98±0.00	0.95±0.00	-	-	-
A7	CSS	NOR	*nd*	6.14±0.01	0.95±0.00	-	-	-
A8	CSS	NOR	90	7.04±0.03	0.95±0.00	-	-	-
C1	CSS	UK	*nd*	5.99±0.01	0.94±0.00	+	-	-
C2	CSS	NOR	*nd*	6.09±0.01	0.96±0.00	-	-	-
C3	CSS	IRE	60	6.05±0.02	0.95±0.00	-	-	-
D1	CSS	NOR	*nd*	6.07±0.01	0.96±0.00	-	-	-
E1	CSS	NOR	*nd*	6.14±0.01	0.95±0.00	-	-	-
F1	CSS	UK	*nd*	6.08±0.00	0.96±0.00	-	-	-
F2	CSS	UK	*nd*	6.08±0.01	0.95±0.00	-	-	-
F3	CSS	FRA	*nd*	6.09±0.03	0.91±0.00	-	-	-
F4	CSS	IRE	60	6.14±0.04	0.95±0.00	-	-	-
F5	CSS	NOR	*nd*	6.21±0.01	0.97±0.00	+	+	<100
F6	CSS	UK	*nd*	6.37±0.01	0.95±0.00	+	+	1.2×10^4
G1	CSS	IRE	60	6.07±0.01	0.95±0.00	-	-	-
H1	CSS	UK	*nd*	6.11±0.01	0.95±0.00	-	-	-
L1	CSS	NOR	*nd*	6.03±0.01	0.97±0.00	-	-	-
M1	CSS	NOR	*nd*	5.60±0.01	0.92±0.00	-	-	-
M2	CSS	UK	*nd*	6.07±0.01	0.93±0.00	-	-	-
M3	CSS	UK	*nd*	6.18±0.01	0.95±0.00	-	-	-
N1	CSSW	SPA	*nd*	5.69±0.01	0.95±0.00	-	-	-
N2	CSS	NOR	*nd*	5.99±0.01	0.94±0.00	-	-	-
N3	CSS	SPA	*nd*	5.69±0.01	0.95±0.00	-	-	-
N4	CSS	NOR	90	6.01±0.01	0.95±0.00	+	-	-
N5	CSS	NOR	30	6.03±0.03	0.95±0.00	-	-	-
N6	CSSW	SPA	*nd*	5.76±0.01	0.96±0.00	-	-	-
N7	CSS	NOR	*nd*	6.18±0.05	0.81±0.00	-	-	-
N8	CSS	NOR	*nd*	6.19±0.04	0.81±.0.00	-	-	-
N9	CSS	NOR	*nd*	6.21±0.01	0.81±0.00	-	-	-
N10	CSS	NOR	*nd*	6.22±0.01	0.81±0.00	-	-	-
N11	CSS	NOR	*nd*	5.99±0.01	0.92±0.00	-	-	-
N12	CSS	NOR	*nd*	6.00±0.01	0.96±0.00	+	-	-
N13	CSS	NOR	*nd*	6.21±0.00	0.92±0.00	-	-	-
N14	CSS	SPA	90	6.02±0.00	0.92±0.00	-	-	-
N15	CSS	FRA	*nd*	6.19±0.01	0.96±0.00	-	-	-
N16	CSS	NOR	*nd*	6.06±0.01	0.95±0.00	-	-	-
N17	CSS	NOR	90	5.62±0.01	0.95±0.00	+	+	<100
N18	CSS	NOR	60	6.19±0.01	0.96±0.00	-	-	-
N19	CSS	NOR	*nd*	5.84±0.01	0.96±0.00	-	-	-
O1	CSSW	SPA	90	5.53±0.00	0.93±0.00	-	-	-
O2	CSSW	SPA	120	5.47±0.01	0.93±0.00	-	-	-
O3	CSSW	SPA	90	6.32±0.01	0.93±0.00	-	-	-
P1	CSS	DEN	*nd*	6.03±0.01	0.96±0.00	-	-	-
Q1	CSS	UK	*nd*	6.06±0.04	0.95±0.00	+	+	<100
R1	CSS	DEN	60	6.08±0.01	0.95±0.00	+	-	-

[a]CSS, cold smoked salmon; [b]CSSW, cold smoked swordfish; [c]*nd*, not determined.

od at 72°C for 10 m. For multiplex PCR 2 and 3, cycles were as follows: initial denaturation at 94°C for 3 m, 35 cycles of denaturation at 94°C for 60 s, annealing at 60°C for 120 s, and extension at 72°C for 60 s, followed by a final extension at 72°C for 5 m. The amplified fragments were separated by 1.3% agarose gel electrophoresis (Roche diagnostics, Milan, Italy) in 1X TAE buffer and stained with ethidium bromide (10 mg/mL). The gels were observed and digitalized by the Gel-Doc UV trans-illuminator (Bio-Rad, Hercules, CA, USA).

DNA macrorestriction and PFGE analysis

DNA macrorestriction and PFGE analysis were carried out using the standardized PulseNet protocol for *L. monocytogenes* (GRAVES and SWAMINATHAN, 2001), submitting the isolates to restriction with *Apa*I and *Asc*I (New England Biolabs, Beverly, MA, USA) and to PFGE in a CHEF Mapper XA system (Bio-Rad, Hercules, CA, USA). After electrophoresis, the gels were stained for 15-45 m in 400 mL of deionized water containing 0.4 mL of ethidium bromide (10 mg/mL) and

Table 2 - Characteristics, prevalence and level of contamination by *Listeria* spp and *L. monocytogenes* of the seafood salads (n. 42).

Sample code	Food product	Country	Shelf life (days)	pH (Mean±s.d.)	a_w (Mean±s.d.)	Presence of *Listeria* spp	Presence of *L. monocytogenes*	*L. monocytogenes* cfu/g
T1	SS[a]	ITA	*nd*[b]	4.05±0.00	0.99±0.00	-	-	-
T2	SS	ITA	*nd*	3.98±0.02	0.99±0.00	-	-	-
T3	SS	ITA	*nd*	4.04±0.00	0.95±0.00	-	-	-
T4	SS	ITA	*nd*	4.10±0.03	0.98±0.00	-	-	-
T5	SS	ITA	*nd*	3.94±0.00	0.98±0.00	-	-	-
T6	SS	ITA	*nd*	4.45±0.00	0.98±0.00	-	-	-
T7	SS	NED	*nd*	4.29±0.00	0.98±0.00	+	-	-
T8	SS	ITA	*nd*	5.86±0.00	0.97±0.00	-	-	-
T 9	SS	FRA	*nd*	5.19±0.00	0.98±0.00	-	-	-
T 10	SS	ITA	*nd*	7.15±0.00	0.98±0.00	+	-	-
T 11	SS	ITA	*nd*	5.10±0.00	0.97±0.00	-	-	-
T14	SS	ITA	*nd*	3.93±0.04	0.98±0.00	-	-	-
T15	SS	NED	*nd*	4.00±0.05	0.96±0.00	-	-	-
T17	SS	ITA	*nd*	4.27±0.02	0.98±0.00	+	+	<100
T18	SS	ITA	*nd*	7.56±0.01	0.97±0.00	-	-	-
T19	SS	NED	*nd*	3.85±0.01	0.98±0.00	-	-	-
T22	SS	NED	*nd*	4.02±0.00	0.96±0.00	-	-	-
T23	SS	NED	*nd*	4.38±0.00	0.99±0.00	-	-	-
T25	SS	ITA	*nd*	5.59±0.00	0.98±0.00	-	-	-
T26	SS	ITA	*nd*	5.81±0.00	0.98±0.00	-	-	-
T27	SS	ITA	*nd*	5.61±0.00	0.98±0.00	-	-	-
T28	SS	ITA	*nd*	5.41±0.02	0.98±0.00	-	-	-
T29	SS	ITA	*nd*	4.02±0.06	0.94±0.00	-	-	-
T30	SS	ITA	*nd*	4.36±0.01	0.98±0.00	-	-	-
T31	SS	ITA	*nd*	4.18±0.00	0.99±0.00	-	-	-
T32	SS	ITA	*nd*	4.28±0.00	0.99±0.00	-	-	-
T33	SS	ITA	*nd*	4.25±0.03	0.99±0.00	-	-	-
T34	SS	ITA	*nd*	4.70±0.00	0.99±0.00	-	-	-
T49	SS	ITA	*nd*	4.31±0.02	0.99±0.00	-	-	-
T50	SS	ITA	*nd*	4.18±0.00	0.99±0.00	+	+	<100
U2	SS	NED	*nd*	4.36±0.00	0.98±0.00	-	-	-
U3	SS	ITA	*nd*	4.28±0.00	0.99±0.00	-	-	-
U4	SS	ITA	*nd*	3.97±0.00	0.95±0.00	-	-	-
U5	SS	ITA	*nd*	4.70±0.00	0.99±0.00	-	-	-
U6	SS	ITA	*nd*	3.95±0.00	0.98±0.00	-	-	-
U8	SS	ITA	*nd*	3.86±0.00	0.98±0.00	-	-	-
U9	SS	NED	*nd*	4.28±0.00	0.99±0.00	-	-	-
V1	SS	ITA	*nd*	4.12±0.00	0.99±0.00	-	-	-
V2	SS	ITA	*nd*	4.23±0.00	0.98±0.00	-	-	-
V4	SS	ITA	*nd*	4.28±0.01	0.99±0.00	-	-	-
V5	SS	ITA	*nd*	4.20±0.00	0.99±0.00	-	-	-
V7	SS	ITA	*nd*	4.01±0.00	0.99±0.00	-	-	-

[a]SS, seafood salad; [b]*nd*, not determined.

Table 3 - Composition of the subset of 51 *L. monocytogenes* isolates submitted to phenotypical and molecular characterization.

Sample code	Origin of the isolates						
	Enumeration method	Detection method				Total of the isolates	Selected isolates No. (%)
		1st enrichment		2nd enrichment			
	Palcam agar	*Oxford agar*	*Aloa agar*	*Oxford agar*	*Aloa agar*		
A2	-	5 (2)	-	5 (3)	5 (3)	15	8 (53)
Q1	-	-	-	-	5 (3)	5	3 (60)
F5	-	-	5 (2)	-	5 (2)	10	4 (40)
F6	5 (3)	5 (3)	5 (3)	5 (3)	5 (3)	25	15 (60)
N17	-	5 (2)	-	5 (2)	-	10	4 (40)
A3	-	-	5 (3)	-	5 (3)	10	6 (60)
T17	-	-	5 (2)	-	5 (3)	10	5 (50)
T50	-	5 (3)	-	5 (3)	-	10	6 (60)
		Total				95	51 (53)

Table 4 - Primer pairs used for the amplification of *L. monocytogenes* virulence genes.

PCR	Primers		Sequence (5'-3')	Concentration of each primer	Product size (bp)
1	*rrn*	F	CAG CAG CCG CGG TAA TAC	*0.9 µM*	938
		R	CTC CAT AAA GGT GAC CCT		
	hlyA	F	CCT AAG ACG CCA ATC GAA		702
		R	AAG CGC TTG CAA CTG CTC		
	actA	F	GAC GAA AAT CCC GAA GTG AA		268 or 385
		R	CTA GCG AAG GTG CTG TTT CC		
	prfA	F	CTG TTG GAG CTC TTC TTG GTG AAG CAA TCG		1060
		R	AGC AAC CTC GGA ACC ATA TAC TAA CTC		
2	*inlA*	F	CCT AGC AGG TCT AAC CGC AC	*0.9 µM*	255
		R	TCG CTA ATT TGG TTA TGC CC		
	inlB	F	AAA GCA CGA TTT CAT GGG AG		146
		R	ACA TAG CCT TGT TTG GTC GG		
	iap	F	ACA AGC TGC ACC TGT TGC AG		131
		R	TGA CAG CGT GTG TAG TAG CA		
3	*plcA*	F	CGA GCA AAA CAG CAA CGA TA	*0.9 µM*	129
		R	CCG CGG ACA TCT TTT AAT GT		
	plcB	F	GGG AAA TTT GAC ACA GCG TT		261
		R	ATT TTC GGG TAG TCC GCT TT		
	mpl	F	TTG TTC TGG AAT TGA GGA TG		502
		R	TTA AAA AGG AGC GGT GAA AT		

destained for 60 m in 400 mL of deionized water. Gel images were visualized and captured using the Gel-Doc UV trans-illuminator (Bio-Rad, Hercules, CA, USA). The banding patterns for each enzyme were assigned by visual analysis of the restriction profiles.

PFGE pattern analysis

*Asc*I and *Apa*I macrorestriction patterns were analyzed using BioNumerics software (Applied Maths, Sint-Martens-Platen, Belgium). Isolates were designated to be genetically indistinguishable (same pulsotype) when their restriction pattern had the same number of bands of apparently the same size (TENOVER *et al.*, 1995). PulseNet protocols have established that even a single fragment difference between two restriction patterns is significant and that the two patterns should be assigned to different pulsotypes (GRAVES *et al.*, 2005). Cluster analysis was performed by the unweighted pair group method using arithmetic averages (UPGMA) as clustering method and the Dice coefficient as measure of band based similarity. The threshold of the similarity measure was fixed at 0.70%. The discriminatory power of PFGE based on Simpson's index of diversity (SID) was determined as described by HUNTER and GASTON (1988).

Table 5 - A summary of the phenotypic and genotypic characteristics of the 51 *L. monocytogenes* strains submitted to phenotypical and molecular characterization.

Strain no.	Sample code	Food product	Country	Serotype	Multiplex PCRs			PFGE patterns		Cluster profile
					1[d]	2[e]	3[f]	*Asc*I	*Apa*I	
L1.A.001	A2	CSS[a]	NOR	1/2c	+	+	+	A6	P6	A
L1.A.002	A2	CSS	NOR	1/2c	+	+	+		P12	D
L1.A.003	A2	CSS	NOR	1/2c	+	+	+		P12	D
L1.A.004	A2	CSS	NOR	1/2c	+	+	+		P6	A
L1.A.005	A2	CSS	NOR	1/2c	+	+	+		P12	D
L1.A.006	A2	CSS	NOR	1/2a	+	+	+		P6	A
L1.A.007	A2	CSS	NOR	1/2a	+	+	+		P6	A
L1.A.008	A2	CSS	NOR	1/2a	+	+	+		P6	A
L1.A.009	Q1	CSS	UK	1/2a	+	+	+	A7	P7	B
L1.A.010	Q1	CSS	UK	1/2a	+*	+	+		P13	E
L1.A.011	Q1	CSS	UK	1/2a	+	+	+		P7	B
L1.A.012	F5	CSS	NOR	1/2a	+	+	+	A10	P10	D
L1.A.013	F5	CSS	NOR	1/2a	+	+	+	A9	P9	A
L1.A.014	F5	CSS	NOR	1/2a	+	+	+		P11	
L1.A.015	F5	CSS	NOR	1/2a	+	+	+		P9	
L1.A.016	F6	CSS	UK	1/2a	+	+	+	A5	P5	C
L1.A.017	F6	CSS	UK	1/2a	+	+	+			C
L1.A.018	F6	CSS	UK	1/2a	+	+	+			F
L1.A.019	F6	CSS	UK	1/2a	+	+	+			C
L1.A.020	F6	CSS	UK	1/2a	+	+	+			B
L1.A.021	F6	CSS	UK	1/2a	+	+	+			C
L1.A.022	F6	CSS	UK	1/2a	+	+	+			C
L1.A.023	F6	CSS	UK	1/2a	+	+	+			C
L1.A.024	F6	CSS	UK	1/2a	+	+	+			C
L1.A.025	F6	CSS	UK	1/2a	+	+	+			C
L1.A.026	F6	CSS	UK	1/2a	+	+	+			C
L1.A.027	F6	CSS	UK	1/2a	+	+	+			C
L1.A.028	F6	CSS	UK	1/2a	+	+	+			C
L1.A.029	F6	CSS	UK	1/2a	+	+	+			C
L1.A.030	F6	CSS	UK	1/2a	+	+	+			C
L1.A.031	N17	CSS	NOR	4e	+	+	+	A4	P15	G
L1.A.032	N17	CSS	NOR	4e	+	+	+		P4	
L1.A.033	N17	CSS	NOR	4e	+	+	+		P4	
L1.A.034	N17	CSS	NOR	4e	+	+	+		P4	
L1.B.001	A3	CSSW[b]	ES	1/2a	+	+	+	A8	P14	E
L1.B.002	A3	CSSW	ES	1/2a	+*	+	+		P8	B
L1.B.003	A3	CSSW	ES	1/2a	+	+	+		P8	B
L1.B.004	A3	CSSW	ES	1/2a	+	+	+		P8	B
L1.B.005	A3	CSSW	ES	1/2a	+	+	+		P8	B
L1.B.006	A3	CSSW	ES	1/2a	+	+	+		P8	B
L1.D.001	T17	SS[c]	IT	1/2b	+	+	+	A2	P2	F
L1.D.002	T17	SS	IT	1/2b	+	+	+		P1	H
L1.D.003	T17	SS	IT	1/2b	+	+	+		P2	F
L1.D.004	T17	SS	IT	1/2b	+	+	+		P1	H
L1.D.005	T17	SS	IT	1/2b	+	+	+		P2	F
L1.D.006	T50	SS	IT	1/2b	+	+	+		P5	C
L1.D.007	T50	SS	IT	1/2b	+	+	+		P2	F
L1.D.008	T50	SS	IT	1/2b	+	+	+		P2	F
L1.S.001	T50	SS	IT	1/2b	+	+	+	A1	P2	F
L1.S.002	T50	SS	IT	1/2b	+	+	+			
L1.S.003	T50	SS	IT	1/2b	+	+	+	A3	P3	-

[a]CSS, cold smoked salmon; [b]CSSW, cold smoked swordfish; [c]SS, seafood salads; [d]Multiplex PCR 1: *rrn, hlyA, prfA, actA*; [e]Multiplex PCR 2: *inlA, inlB, iap;* [f]Multiplex PCR 3: *plcA, plcB,mpl; *L1.A.010 and L1.B.002 showing an actA 268bp product.*

Table 6 - Distribution of pulsotypes and cluster profiles according to food product of the 51 strains of *L. monocytogenes* submitted to phenotypical and molecular characterization. L1.S.003 was not included in any cluster.

Food product	Number of strains	Number of different pulsotypes		Cluster profile *Asc*I + *Apa*I
		*Asc*I	*Apa*I	
Smoked salmon	34	A4 (4), A5 (15), A6 (8), A7 (3), A9 (3), A10 (1)	P4 (3), P5 (15), P6 (5), P7 (2), P10 (1), P11 (1), P9 (2), P12 (3), P13 (1), P15 (1)	A (8), B (2), C (14), D (4), E (1), F (1), G (4),
Smoked swordfish	6	A8 (6)	P8 (5), P14 (1)	B (5), E (1)
Seafood salad	11	A1 (2), A2 (8), A3 (1)	P1 (2), P2 (7), P3 (1), P5 (1)	C (1), H (2), F (7)

RESULTS

Chemical-physical analysis

All RTE seafood samples were able to support the growth of *L. monocytogenes* (EC Regulation 2073/2005). The lowest mean pH value (3.85±0.01 S.D.) was observed in some seafood salads, for which the use of acidulants and inhibitors (0.1% benzoate or 0.1% sorbate) was declared. Seafood salads also showed the highest mean pH value (7.56±0.01 S.D.). The highest mean pH value in smoked fish was 7.04 (±0.03 S.D.). Mean water activity values were higher in seafood salads (0.98±0.02 S.D.) than in smoked products (0.93±0.00 S.D.). Details on mean values of pH and a_w are reported in Tables 1 and 2.

Detection and enumeration of *Listeria* spp and *L. monocytogenes*

The overall prevalence of *Listeria* spp was 15.21%. In the sliced cold smoked fish products and in the seafood salads, it was 20 and 9.52%, respectively. *L. monocytogenes* was found in 8 of the 92 samples tested (8.69%). The highest prevalence (12%) was found in sliced cold smoked fish products. Only 2 (4.76%) of the seafood salads were contaminated. The contamination level was <100 CFU/g in all but one sample of sliced cold smoked fish, showing a *L. monocytogenes* contamination level of 1.2×10^4 CFU/g. Results are summarized in Tables 1 and 2. The mean ± S.D. pH and a_w values of the positive *L. monocytogenes* samples were, respectively, 6.01±0.18 and 0.94±0.01 in the smoked fish products and 4.30±0.70 and 0.98±0.00 in the seafood salads. Altogether, from all *Listeria* spp positive samples, one hundred twenty six isolates were isolated: 95 were *L. monocytogenes*, 12 were *L. innocua*, 7 were *L. welshimeri*, 5 were *L. seeligeri*, 4 were *L. grayi*, and 3 were *L. ivanovii*. Concerning *L. monocytogenes* strains, a subset of n. 51 was selected from all strains isolated from the eight positive samples. Table 3 lists the origin of isolates and whether they were isolated from enumeration or detection media. In both cases, 40 to 60% of all isolates were selected for further phenotypical (serotyping) and molecular characterization (PCR analysis of virulence factors and PFGE). In particular, PFGE was aimed at finding genetic similarity among the strains isolated from the different food products and highlighting the possible presence of different pulsotypes in isolates from the same food product. A summary of the phenotypic and genotypic characteristics of the *L. monocytogenes* isolates is reported in Table 5.

Serotyping

Four different serotypes were detected: 1/2a, 1/2b, 1/2c, and 4e (Table 5). Thirty-one (77.5%) *L. monocytogenes* strains isolated from the smoked products belonged to serovar 1/2a, 5 (12.5%) to serovar 1/2c and 4 (10%) to serovar 4e.

PCR analysis of virulence factors

The amplification products of the virulence genes analyzed were found in all 51 isolates identified as *L. monocytogenes*. Primers used for the detection of *actA* allowed the amplification of two different products (268-bp and 385-bp) showing a polymorphism for this gene. Only two isolates out of 51 (3.9%) showed the 268-bp amplification band (Fig. 1).

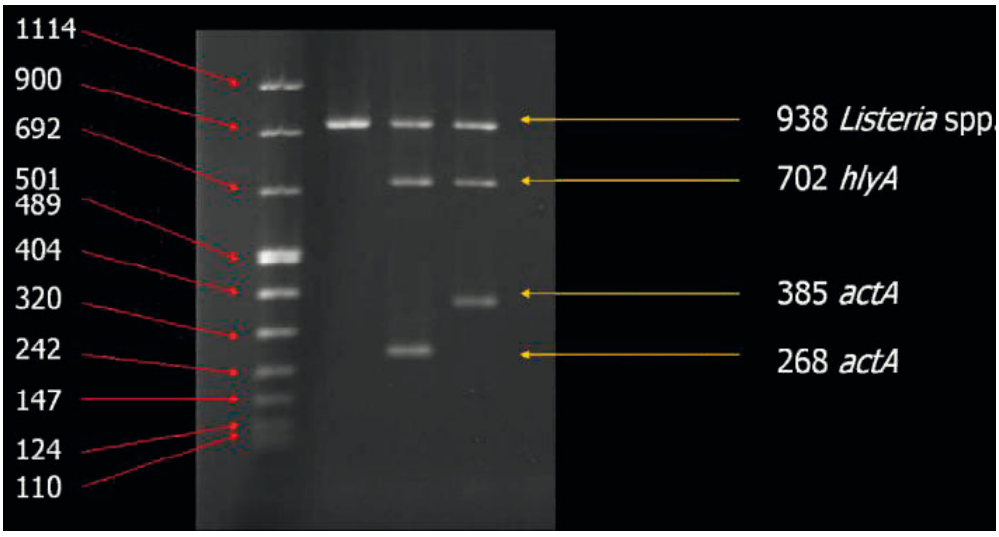


Fig. 1 - Multiplex PCR showing the polymorphism of *actA* gene. The lanes from left to right are: marker; *L. innocua*; *L. monocytogenes* (268 bp *actA*); *L. monocytogenes* (385 bp *actA*).

PFGE pattern analysis

Restriction with *Apa*I differentiated the 51 isolates into 15 different patterns, while restriction with the *Asc*I enzyme produced 10 different patterns (Table 6). *Apa*I and *Asc*I showed a SID value of 0.87 and 0.85, respectively. The combination of both enzymes increased the discriminatory power to 0.91. Regarding *Apa*I restriction, 23 isolates (45%) were divided into two pulsotypes: P5 with 16 isolates and P2 with 7 isolates. The remaining 28 isolates (55%) were grouped into 13 pulsotypes: P3, P10, P11, P13, P14, P15 (1 isolate); P1, P7, P9, (2 isolates); P4, P12 (3 isolates) and P6, P8 (5 isolates). *Asc*I restriction grouped 37 isolates (72%) into 4 pulsotypes: A5 (15 isolates); A2, A6 (8 isolates); A8 (6 isolates). The remaining 14 isolates (28 %) were divided into 7 pulsotypes: A3, A10 (1 isolate); A1 (2 isolates); A7, A9 (3 isolates); A4 (4 isolates). Both restriction enzymes were able to highlight the presence of different pulsotypes in strains isolated from different enrichments or selective mediums of the same food product, confirming the usefulness of submitting 40-60% of all isolates retrieved from each positive sample to restriction analysis. By the cluster analysis performed on the *Apa*I and *Asc*I macrorestriction patterns, 8 PFGE clusters (A-H) were obtained. One isolate (L1.S.003) was not assigned to any cluster, because of the low similarity threshold (30%) with the nearest clusters (A-B-C). The distribution of isolates in the different clusters is shown in Fig. 2. Cluster analysis grouped isolates with the same serotype (1/2a) from different origin and food products: cluster B included 7 isolates from cold smoked fish processed in Norway and the United Kingdom and cold smoked swordfish processed in Spain. Cluster E included 2 isolates from cold smoked salmon processed in the United Kingdom and cold smoked sword-

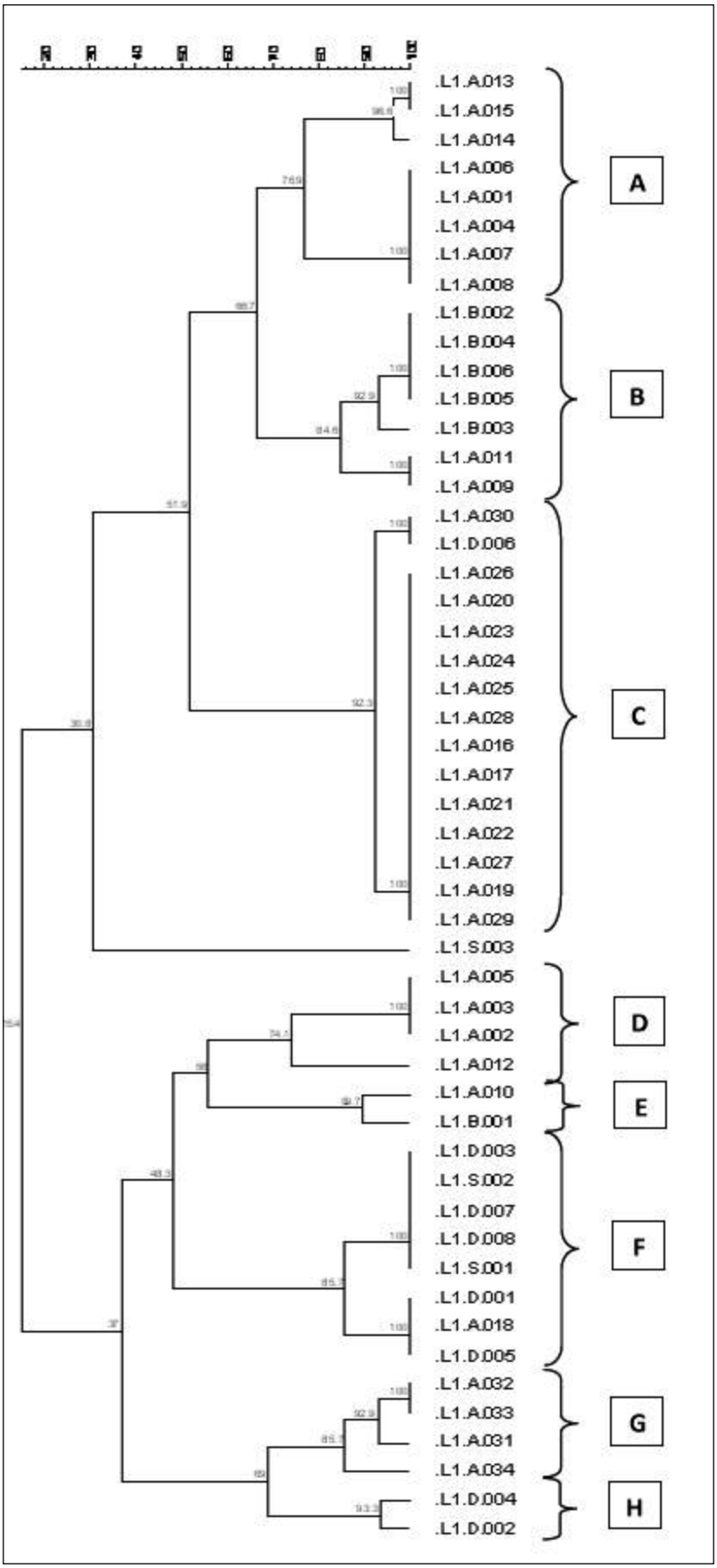


Fig. 2 - Combined dendrogram of *Asc*I and *Apa*I macrorestriction patterns of the 51 strains of *L. monocytogenes* submitted to cluster analysis.

fish processed in Spain. Isolates from different origin and food products belonging to different serotypes were grouped into clusters C and F: in cluster C, 13 isolates (serotype 1/2a) from cold smoked fish processed in the United Kingdom and 2 (serotype 1/2b) from seafood salads processed in Italy. Cluster F included 1 isolate (serotype 1/2a) from cold smoked fish processed in the United Kingdom and 7 (serotype 1/2b) from seafood salads processed in Italy. Isolates in other clusters (A and D) had the same origin (Norway) and were isolated from the same product (cold smoked salmon) but belonged to different serotype: 1/2a and 1/2c in both clusters. In clusters G and H, isolates from the same product, with the same origin and belonging to the same serotype were grouped: 4 isolates (serotype 4e) from cold smoked salmon processed in Norway were grouped in cluster G and isolates (serotype 1/2b) from seafood salad processed in Italy were grouped in cluster H 2.

DISCUSSION AND CONCLUSIONS

The present survey provides a contribution to the knowledge of prevalence, enumeration, pathogenic potential and traceability of *L. monocytogenes* in RTE seafood produced in different countries and marketed in Italy. The prevalence of *L. monocytogenes* was higher in the sliced cold smoked fish products (12%). This finding is in agreement with published data, where its prevalence in smoked fish ranged from 10 to 20% (GNANOU BESSE *et al.,* 2004). Cold smoked fish is one of the most perishable food products: in fact, cold smoking processing does not eliminate the possibility of contamination of raw materials. Moreover, refrigeration and vacuum storage are not able to prevent the growth of *L. monocytogenes* (DUFFES, 1999). For these reasons, cold chain management during the shelf life becomes very important to minimize the growth of the pathogen and shortening of the best-before date is sometimes useful to reduce the risk of causing food-borne listeriosis (ILSI, 2005). In fact, the ability of *L. monocytogenes* to grow to a significant level also at refrigeration temperature, given sufficient time must be taken into account (LITTLE *et al.,* 2007). Although the growth of *L. monocytogenes* at refrigeration temperatures is typically slow, temperature abuse may be a factor in growth to high numbers unless prolonged storage has occurred (ILSI, 2005). All but one RTE samples positive for *L. monocytogenes* contamination had a contamination level lower than 100 CFU/g, fulfilling the *food safety criteria* laid down by the Commission Regulation EC No. 2073/2005 for RTE foods able to support the growth of *L. monocytogenes*.

As expected, given the low discriminatory power, serogroup 1/2 was confirmed as the most common among the *L. monocytogenes* strains isolated from RTE foods. Nevertheless, serotyping has been widely used and it still remains the traditional and routinely used typing method as pre-requisite for other different techniques or in case of outbreaks (AARNISALO *et al.,* 2003). In particular, serotype 1/2a was prevalent in smoked fish and 1/2b in seafood salads, which agrees with previous studies (RØRVIK, 2000; NORTON *et al.,* 2001; VITAS *et al.,* 2004; CORCORAN *et al.,* 2006). These two serotypes have been associated with epidemic and sporadic cases of listeriosis in humans (NAKAMURA *et al.*, 2004).

The molecular findings revealed the presence of all the considered virulence genes, suggesting that all the isolates could be potentially dangerous for public health. Nevertheless, from a risk analysis perspective, it is important to remark that 10-20% of strains isolated from the food industry are weakly or non-pathogenic both *in vivo* and *in vitro* tests (TABOURET *et al.,* 1991; ROCHE *et al.,* 2009). In this research, PCR products of the virulence genes were not polymorphic, except for the *actA* gene. In particular, 49 isolates produced the expected 385-bp *actA* fragment, while the remaining 2 showed a fragment of 268-bp. Some authors (WIEDMANN *et al.,* 1997; JIANG *et al.*, 2006) hypothesized that a deletion of nucleotides in this gene, causing the polymorphism described, might be related to different virulence properties among the isolates. Recent studies have reported no statistical correlation between the presence of the 268-bp *actA* band and the ability to invade HeLa cells multiplying intracellularly. For this reason, the role of *actA* as marker of unambiguous identification of foodborne isolates which are attenuated or non-virulent should be revised (CONTER *et al.,* 2009). PFGE results strongly support the in-depth study of the ecology of *L. monocytogenes* in RTE seafood. In particular, PFGE using restriction endonucleases *Asc*I and *Apa*I makes it possible to point out that a single band difference, which shows a single genetic event, is determinant to assign an isolate to a specific profile (YDE and GINECOT, 2004). Our findings confirm that PFGE is capable of revealing not only differences among apparently similar isolates (i.e. same serotype or same food product) but also similarities among apparently different strains (i.e. different serotype). No correlation was found between PFGE types and serotypes, as described in other studies (GIANFRANCESCHI *et al.,* 2002). Overall, the results of the present study demonstrate a great similarity (> 70%) among strains isolated from cold smoked fish and seafood salads of different origin, as also reported by AUTIO *et al.* (2002). This allowed the existence of a widespread population of highly similar *L. monocytogenes* in different geographical areas to be confirmed (PIFFARETTI *et al.,* 1989; RASMUSSEN *et al.*, 1995; WIEDMANN *et al.,* 1997). Considering the wide distribution of *L. monocytogenes*,

it is very important to fully understand the relation between PFGE types and human disease-associated isolates (FUGETT *et al.,* 2007). In the USA, PulseNet plays an essential role in the surveillance of foodborne illness outbreaks. Also in Europe, extensive databases are maintained by institutions such as the "Pasteur Institute" in France and the "Istituto Superiore di Sanità" in Italy. To improve *L. monocytogenes* surveillance and to trace-back foodborne infections at European and worldwide levels, direct access to comparable typing data for human isolates as well as for food and animal isolates is necessary. A large-scale database of *L. monocytogenes* subtypes is needed to increase the effectiveness of this typing method in the investigations of human listeriosis and its sources. All this information can facilitate risk assessment, outbreak surveillance and control of this pathogen (GIANFRANCESCHI *et al.*, 2009).

REFERENCES

Aarnisalo K., Autio T., Sjoberg A.M., Lunden J., Korkeala H. and Suihko M. L. 2003. Typing of *Listeria monocytogenes* isolates originating from the food processing industry with automated ribotyping and pulsed-field gel electrophoresis. J. Food Prot. 66:249.

American Public Health Association (APHA) 2001. Measurement of water activity (A_w) and acidity. Ch. 8 In: "Compendium of Methods for the Microbiological Examination of Foods", 4th Ed., F. Pouch Downes and K. Ito (ED.), p. 135. American Public Health Association publication, Washington, DC, USA.

Anonymous 1996. Microbiology of food and animal feeding stuffs-horizontal method for the detection and enumeration of *Listeria monocytogenes*. Part 1: Detection method, 1st Ed. ISO method 11290-1. International Organization for Standardization, Genève, Switzerland.

Anonymous 1998. Microbiology of food and animal feeding stuffs-horizontal method for the detection and enumeration of *Listeria monocytogenes*. Part 2: Enumeration method, 1st Ed. ISO method 11290-2. International Organization for Standardization, Genève, Switzerland.

Autio T., Hielm S., Miettinen M., Sjöberg A.M., Aarnisalo K., Bjorkroth J., Mattila-Sandholm T. and Korkeala H. 1999. Sources of *Listeria monocytogenes* contamination in a cold-smoked rainbow trout processing plant detected by pulsed-field gel electrophoresis typing. App. Environ. Microbiol. 65: 150.

Autio T., Lundén J., Fredriksson-Ahomaa M., Björkrotha J., Sjöbergb A.M. and Korkealaa H. 2002. Similar *Listeria monocytogenes* pulsotypes detected in several foods originating from different sources. Int. J. Food Microbiol. 77: 83.

Ben Embarek P.K. 1994. Presence, detection and growth of *Listeria monocytogenes* in seafoods: a review. Int. J. Food Microbiol. 23: 17.

Ben Embarek P.K., Hansen L.T., Enger O. and Huss H.H. 1997. Occurrence of *Listeria* spp. in farmed salmon and during subsequent slaughter: comparison of Listertestk Lift and the USDA method. Food Microbiol. 14: 39.

Bille J. and Rocourt J. 1996. WHO international multicenter *Listeria monocytogenes* subtyping study - rationale and set-up of the study. Int. J. Food Microbiol. 32: 251.

Border P.M., Howard J.J., Plastow G.S. and Siggens K.W. 1990. Detection of *Listeria* and *Listeria monocytogenes* using polymerase chain reaction. Lett. Appl. Microbiol. 11: 158.

Chou C.H. and Wang C. 2006. Genetic relatedness between *Listeria monocytogenes* isolates from seafood and humans using PFGE and REP–PCR. Int. J. Food Microbiol. 110: 135.

Conter M., Vergara A., Di Ciccio P., Zanardi E., Ghidini S. and Ianieri A. 2009. Polyporphism of *act*A gene is not related to *in vitro* virulence of *Listeria monocytogenes.* Int. J. Food Microbiol. 137:100.

Corcoran D., Clancy D., O' Mahony M., Grant K., Hyland E., Shanaghy N., Whyte P., McLauchlin J., Moloney A. and Fanning S. 2006. Comparison of *Listeria monocytogenes* strain types in Irish smoked salmon and other foods. Int. J. Hyg. Environ. Health 209: 527.

Dauphin G., Ragimbeau C. and Malle P. 2001. Use of PFGE typing for tracing contamination with *Listeria monocytogenes* in cold-smoked salmon processing plants. Int. J. Food Microbiol. 64: 51.

Duffes F. 1999. Improving the control of *Listeria monocytogenes* in cold smoked salmon. Trends Food Sci. Technol. 10: 211.

European Commission (EC) 2005. Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off. J. Eur. Union L 338:1.

Farber M.J. 2000. Present situation in Canada regarding *Listeria monocytogenes* and ready-to-eat seafood products. Int. J. Food Microbiol. 62: 247.

Farber M.J. and Peterkin P.I. 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Mol. Biol. Rev. 55:476.

Fugett E.B., Schoonmaker-Bopp D., Dumas N.B., Corby J. and Wiedmann M. 2007. Pulsed-Field Gel Electrophoresis (PFGE) analysis of temporally matched *Listeria monocytogenes* isolates from human clinical cases, foods, ruminant farms, urban and natural environments reveals source-associated as well as widely distributed PFGE types. J. Clin. Microbiol. 45:865.

Gerner-Smidt P., Hise K., Kincaud J., Hunter S., Rolando S., Hyytia-Trees E., Ribot E.M., Swaminathan B. and the PulseNet Taskforce 2006. PulseNet USA: a five-year update. Foodborne Path. Dis. 3:9.

Gianfranceschi M., Pourshaban M., Gattuso A., Wedell-Neergaard C. and Aureli P. 2002. Characterization of *Listeria monocytogenes* strains isolated from food and humans in Italy by pulsed-field gel electrophoresis. Food Microbiol. 19:47.

Gianfranceschi M.V., D'Ottavio M.C., Gattuso A., Bella A. and Aureli P. 2009. Distribution of serotypes and pulsotypes of *Listeria monocytogenes* from human, food and environmental isolates (Italy 2002-2005). Food Microbiol. 26:520.

Gnanou Besse N., Audinet N., Beaufort A., Colin P., Cornu M. and Lombard B. 2004. A contribution to the improvement of *Listeria monocytogenes* enumeration in cold-smoked salmon. Int. J. Food Microbiol. 91:119.

Gombas D.E., Chen Y., Clavero R.S. and Scott V.N. 2003. Survey of *Listeria monocytogenes* in ready-to-eat foods. J. Food Prot. 66:559.

Graves M.L. and Swaminathan B. 2001. PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. Int. J. Food Microbiol. 65:55.

Graves M.L., Hunter S.B., Ong A.R., Schoonmaker-Bopp D., Hise K., Kornstein L., DeWitt W.E., Hayes P.S., Dunne E., Mead P. and Swaminathan B. 2005. Microbiological aspects of the investigation that traced the 1998 outbreak of listeriosis in the United States to contaminated hot dogs and establishment of molecular subtyping-based surveillance for *Listeria monocytogenes* in the PulseNet network. J. Clin. Microbiol. 43:2350.

Hoffman A., Gall K.L., Norton D.M. and Wiedmann M. 2003. *Listeria monocytogenes* contamination patterns for the smoked fish processing environment and for raw fish. J. Food Prot. 66:52.

Hunter P. and Gaston M.A. 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. 26:2465.

Ilsi Research Foundation-Risk Science Institute, Expert Panel on *Listeria monocytogenes* in foods 2005. Achieving continuous improvement in reductions in foodborne listeriosis- a risk-based approach. J. Food Prot. 68:1932.

Jaradat Z.W., Schutze G.E. and Bhunia A.K. 2002. Genet-

ic homogeneity among *Listeria monocytogenes* strains from infected patients and meat products from two geographic locations determined by phenotyping, ribotyping and PCR analysis of virulence genes. Int. J. Food Microbiol. 76:1.

Jiang L., Chen J., Xu J., Zhang X., Wang S., Zhao H., Vongxay K. and Fang W. 2008. Virulence characterization and genotypic analyses of *Listeria monocytogenes* isolates from food and processing environments in eastern China. Int. J. Food Microbiol. 121: 53.

Jiang L.L., Xu J.J., Chen N., Shuai J.B. and Fang W.H. 2006. Virulence phenotyping and molecular characterization of a low-pathogenicity isolate of *Listeria monocytogenes* from cow's milk. Acta Biochim. Biophys. Sin. 38:262.

Johansson T., Rantala L., Palmu L. and Honkanen-Buzalski T. 1999. Occurrence and typing of *Listeria monocytogenes* strains in retail vacuum packed fish products and in a production plant. Int. J. Food Microbiol. 47:111.

Kathariou S. 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. J. Food Prot. 65:1811.

Kreft J. and Vázquez-Boland J.A. 2001. Regulation of virulence genes in *Listeria*. Int. J. Med. Microbiol. 291:145.

Little C.L., Taylor F.C., Sagoo S.K., Gillespie I.A., Grant K. and McLauchlin J. 2007. Prevalence and level of *Listeria monocytogenes* and other Listeria species in retail prepackaged mixed vegetable salads in the UK. Food Microbiol. 24:711.

Liu D., Lawrence M.L., Ainsworth A.J. and Austin F.W. 2005. Comparative assessment of acid, alkali and salt tolerance in *Listeria monocytogenes* virulent and avirulent strains. FEMS Microbiol. Lett. 243:373.

Lomonaco S., Decastelli L., Nucera D., Gallina S., Bianchi D.M. and Civera T. 2009. *Listeria monocytogenes* in Gorgonzola: Subtypes, diversity and persistence over time. Int. J. Food Microbiol. 128:516.

Mead P.S., Dunne E.F., Graves L., Wiedmann M., Patrick M., Hunter S., Salehi E., Mostahari F., Craig A., Mshar P., Bannerman T., Sauders B.D., Hayes P., Dewitt W., Sparling P., Griffin P., Morse D., Slutsker L. and Swaminathan B. 2006. Nationwide outbreak of listeriosis due to contaminated meat. Epidemiol. and Infect, 134:744.

Medrala D., Dabrowski W., Czekajlo-Kolodziej U., Daczkowska-Kozon E., Koronkiewicz A., Augustynowicz E. and Manzano M. 2003. Persistence of *Listeria monocytogenes* strains isolated from products in a Polish fish processing plant over a 1-year period. Food Microbiol. 20:715.

Miettinen H. and Wirtanen G. 2005. Prevalence and location of *Listeria monocytogenes* in farmed rainbow trout. Int. J. Food Microbiol. 104:135.

Miettinen M.K., Siitonen A., Heiskanen P., Haajanen H., Bjorkroth K.J. and Korkeala H.J. 1999. Molecular epidemiology of an outbreak of febrile gastroenteritis caused by *Listeria monocytogenes* in cold-smoked rainbow trout. J. Clin. Microbiol. 37: 2358.

Nakamura H., Hatanaka M., Ochi K., Nagao M., Ogasawara J., Hase A., Kitase T., Haruki K. and Nishikawa Y. 2004. *Listeria monocytogenes* isolated from cold-smoked fish products in Osaka City, Japan. Int. J. Food Microbiol. 94:323.

Norton D.M., McCamey M.A., Gall K.L., Scarlett J.M., Boor K.J. and Wiedmann M. 2001. Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. Appl. Environ. Microbiol. 67:198.

Piffaretti J.C., Kressebuch H., Aescenbacher M., Bille J., Bannerman E., Musser J.M., Seelander R.K. and Rocourt J. 1989. Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease. Proc. Natl. Acad. Sci. U.S.A., 86:3818.

Rasmussen O.F., Skouboe P., Dons L., Rosen L. and Olsen J.E. 1995. *Listeria monocytogenes* exists in at least three evolutionary lines: evidence from flagellin, invasive associated protein and listeriolysin O gene. Microbiology 141:2053.

Roche S.M., Gracieux P., Velge P. 2009. Poor detection of low-virulence field strains of *L. monocytogenes* is related to selective agents in selective media and is unrelated to PrfA. Food Microbiol. 26:21.

Rocourt J., Jacquet C. and Relly A. 2000. Epidemiology of human listeriosis and seafoods. Int. J. Food Microbiol. 62:197.

Rørvik L.M. 2000. *Listeria monocytogenes* in the smoked salmon industry. Int. J. Food Microbiol. 62:183.

Swaminathan B. and Gerner-Smidt P. 2007. The epidemiology of human listeriosis. Microbes Infect. 9:1236.

Tabouret M., Rycke J., Audurier A. and Poutrel B. 1991. Pathogenicity of *Listeria monocytogenes* isolates in immunocompromised mice in relation to listeriolysin production. J. Med. Microbiol. 34:13.

Tenover F.C., Arbeit R.D., Goering R.V., Mickelsen P.A., Murray B.E., Persing D.H. and Swaminathan B. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. 33:2233.

Thévenot D., Delignette-Muller M.L., Christieans S., Leroy S., Kodjo A. and Vernozy-Roland C. 2006. Serological and molecular ecology of *Listeria monocytogenes* isolates collected from 13 French pork meat salting-curing plants and their products. Int. J. Food Microbiol. 112:153.

Ueda F., Sugamata M., Aota M., Mochizuki M., Yamada F. and Hondo R. 2002. Swift and definite serotyping for isolated *Listeria monocytogenes* strains. Microbiol. 25: 165.

Vázquez-Boland J.A., Dominguez-Bernal G., Gonzalez-Zorn B., Kreft J. and Goebel W. 2001a. Pathogenicity islands and virulence evolution in *Listeria*. Microbes Infect. 3:571.

Vázquez-Boland J.A., Kuhn M., Berche P., Chakraborty T., Domínguez-Bernal G., Goebel W., González-Zorn B., Wehland J. and Kreft J. 2001b. *Listeria* pathogenesis and Molecular Virulence Determinants. Clin. Microbiol. Rev. 14:584.

Vitas A.I., Aguado V. and Garcia-Jalon I. 2004. Occurence of *Listeria monocytogenes* in fresh processed foods in Navarra (Spain). Int. J. Food Microbiol. 90:349.

Wiedmann M., Bruce J.L., Keating C., Johnson A.E., McDonough P.L. and Batt C.A. 1997. Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. Infect. Immun. 65:2707.

Yde M. and Ginecot A. 2004. Use of PFGE to characterize clonal relationships among Belgian clinical isolates of *Listeria monocytogenes*. J. Med. Microbiol. 53:399.

Zhang Y., Yeh E., Hall G., Cripe J., Bhagwat A.A. and Meng J. 2007. Characterization of *Listeria monocytogenes* isolated from retail foods. Int. J. Food Microbiol. 113:47.

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Acknowledgments. Acknowledgments of assistance are appropriate provided they are not related to analyses or other services performed for a fee. Financial support, thanks for assistance, artic1e number or thesis fulfilment may be included.

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(Anonymous)

Anonymous. 1982. Tomato product invention merits CTRI Award. Food Technol. 36(9): 23.

(Book)

AOAC. 1980. "Official Methods of Analysis" 13th ed. Association of Official Analytical Chemists, Washington, DC.

Weast, R.C. (Ed.). 1981 "Handbook of Chemistry and Physics" 62nd ed. The Chemical Rubber Co. Cleveland, OH.

(Bulletin, circular)

Willets C.O. and Hill, C.H. 1976. Maple syrup producers manual Agric. Handbook No. 134, U.S. Dept. of Agriculture, Washington, DC.

(Chapter of book)

Hood L.F. 1982. Current concepts of starch structure. Ch. 13. In "Food Carbohydrates". D.R. Lineback and G.E. Inglett (Ed.), p. 217. AVI Publishing Co., Westport, CT.

(Journal)

Cardello A.V. and Maller O. 1982. Acceptability of water, selected beverages and foods as a function of serving temperature. J. Food Sci. 47: 1549.

IFT Sensory Evaluation Div. 1981a. Sensory evaluation guide for testing food and beverage products. Food Technol. 35 (11): 50.

IFT Sensory Evaluation Div. 1981b. Guidelines for the prepaation and review of papers reporting sensory evaluation data. Food Technol. 35(4): 16.

(Non-English reference)

Minguez-Mosquera M.I., Franquelo Camacho A, and Fernandez Diez M.J. 1981. Pastas de pimiento. Normalizacion de la medida del color. Grasas y Aceites 33 (1): 1.

(Paper accepted)

Bhowmik S.R. and Hayakawa, K. 1983. Influence of selected thermal processing conditions on steam consumption and on mass average sterilizing values. J. Food Sci. In press.

(Paper presented)

Takeguchi C.A. 1982. Regulatory aspects of food irradiation. Paper No. 8, presented at 42nd Annual Meeting of Inst. of Food Technologists, Las Vegas, NV, June 22-25.

(Patent)

Nezbed R.I. 1974. Amorphous beta lactose for tableting U.S. patent 3,802,911, April 9.

(Secondary source)

Sakata R., Ohso M. and Nagata Y. 1981. Effect of porcine muscle conditions on the color of cooked cured meat. Agric. & Biol. Chem. 45 (9): 2077. (In Food Sci. Technol. Abstr. (1982) 14 (5): 5S877).

Wehrmann K.H. 1961. Apple flavor. Ph. D. thesis. Michigan State Univ., East Lansing. Quoted in Wehrmann, K.H. (1966). "Newer Knowledge of Apple Constitution", p. 141, Academic Press, New York.

(Thesis)

Gejl-Hansen F. 1977. Microstructure and stability of Freeze dried solute containing oil-in-water emulsions Sc. D. Thesis, Massachusetts Inst. of Technology, Cambridge.

(Unpublished data/letter)

Peleg M. 1982. Unpublished data. Dept. of Food Engineering., Univ. of Massachusetts, Amherst.

Bills D.D. 1982. Private communication. USDA-ARS. Eastern Regional Research Center, Philadelphia, PA.

CONTRIBUTORS

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ASSOCIATIONS and COMPANIES

Associazione Italiana di Tecnologia Alimentare (A.I.T.A.) - Milano	Fax +39-02-2365015 www.aita-nazionale.it
Società Italiana di Scienze e Tecnologie Alimentari (S.I.S.T.Al) - Perugia	Fax +39-075-5857939 www.sistal.org
Soremartec Italia srl - Alba	Fax +39-0173-313966

RESEARCH INSTITUTES

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 Scienze degli Alimenti,
Università di Udine, Udine Fax +39-0432-558130

Dipartimento di Scienze e Tecnologie Agroalimentari e
Microbiologiche (DI.S.T.A.A.M.),
Università del Molise, Campobasso Fax +39-0874-404652

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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