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PAPER

ANALYSIS OF THE COLLABORATION BETWEEN THE US AND THE EU IN VITICULTURE AND OENOLOGY

R. ALEIXANDRE-BENAVENT[1]*, J.L. ALEIXANDRE TUDÓ[2], G. GONZÁLEZ ALCAIDE[3] and J.L. ALEIXANDRE[2]

[1]IHMC López Piñero, Spanish Research Council-CSIC, Universidad de Valencia-UISYS, Plaza Cisneros 4, 46003 Valencia, Spain

[2]Departamento de Tecnología de Alimentos, Universidad Politécnica de Valencia, Camino de Vera s/n, Valencia 46022, Spain

[3]Departamento de Historia de la Ciencia y Documentación, Universidad de Valencia, Blasco Ibáñez 10, 46010 Valencia, Spain

*Corresponding author: +34 697881288,
email: rafael.aleixandre@uv.es

ABSTRACT

The network of scientific collaboration in viticulture and oenology between the United States and the European Union was studied for the period 1991-2010. A total of 498 articles were published collaboratively during this time. The most collaborative institutions in the US were the University of California Davis and Cornell University (New York), and the most collaborative institutions in the EU were Institut Nationale de la Recherche Agronomique (France), the Italian universities of Milan, Bologna and Udine, and the Spanish University of Barcelona. We note a considerable increase in collaboration in recent years, with the University of California Davis situated in a central position in the network.

- Keywords: biblometric indicators, collaboration networks, European Union, scientific publications, United States of America, viticulture and oenology research -

INTRODUCTION

The European Union is the largest producer of wine in the world. It is also the largest exporter and importer of wine in the world, highlighting the socio-economic importance of the wine sector in this area. Wine consumption in the United States has increased over the last ten years at a constant rate of approximately 3% per year, which is slightly lower than the consumption in France and Italy. In the coming years, the United States is expected to overtake these Countries to become the largest wine market in the world. Although the total consumption of wine is considerable, consumption per capita in the United States remains below Countries with older traditions of winemaking. This phenomenon may be considered an indicator of growth potential (FERNÁNDEZ *et al.*, 2006).

Wine imports have increased significantly in recent years from the European Union, which is the main supplier of wine to the United States. Three quarters of the wine consumed in the United States is of domestic origin, whereas imported wines have a quota of 25% (CBI, 2009). These commercial transactions make scientific and technical cooperation between the European Union and the United States increasingly more important. Extensive collaboration in research in this area has produced large numbers of collaborative journal articles on the wine trade.

This study provides a bibliometric analysis of collaborative research on wine and grapes published in the United States of America and the European Union during the 1991-2010 period. According to GLÄNZEL and VEUGELERS (2006), BEAVER and ROSEN (1979) and LUUKKONEN *et al.* (1992), international collaboration allows researchers to exchange scientific information with their international colleagues on the latest scientific developments, to gain access to the best equipment, facilities, and talent, and to participate in large-scale research projects that are beyond the financial capabilities of individual Countries.

METHODS AND DATA SOURCES

The articles under analysis were obtained from the Science Citation Index Expanded (SCIE) database, accessed via the Web of Knowledge platform from terminals at the Universidad Politécnica of Valencia (Spain). Only "citable documents" were considered (that is, papers published as articles, letters, notes or reviews), excluding documents such as book reviews, summaries of communications at conferences, reprints, news and bibliographic articles.

To define the field of "wine research", we used the same strategy employed by GLANZEL and VEUGELERS (2006) in a previous paper and combined three components: a) specific search words, b) institutional addresses, and c) European Union Countries combined with the United Sates.

First, for the specific search words, we used the following strategy:

TS = (grapevin* or wines or "wine grap*" or "wine pro*" or "red wine*" or "white wine*" or winemaking or enolog* or viticult* or oenolog* or "wine cell*" or "wine yeast*" or winery or wineries).

The search was restricted to the field "topic", which retrieved records if the above-mentioned terms were included in the titles, keywords or abstracts of articles.

Second, for institutional addresses, we used the following strategy:

AD = (enolog* or viticult* or oenol*), where AD was the label of the authors' institutional address.

Finally, we limited the search to articles that were signed by at least one European Union Country and United States in the 'address' field of the search options of the SCIE database. The current European Union Countries are Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, and the United Kingdom (which includes England, Scotland, Wales, and Northern Ireland).

For some terms, the root was used (truncating the word with an asterisk) to obtain all of the documents associated with the derived words (e.g., by using the truncation enolog*, the SCIE database finds enology, enological, enologist, and so on).

The analysis was limited to 20 years (the period from 1991 to 2010). In order to identify the institutions and Countries with high and increasing citation rates, the citation/document rates by 5-year period were calculated.

The final records were exported to a relational database using the proprietary software Bibliometrics. Variants of the names of authors or institutions were unified because this information does not always appear standardised in SCIE. The following step was to analyse the information to obtain bibliometric indicators and to identify all combinations of pairs of authors and institutions in each paper to quantify the number of co-authorships (ie, the presence of joint signatures of authors or institutions in different articles). We examine publication productivity using a normal count or full counting scheme, where equal credit is given to all contributors; one full unit is assigned to each author, or each institution or Country involved in a scientific paper, in opposition to the fractional count (adjusted count), where each co-authored paper are divided by the number of co-authors. This information formed the basis for identifying research groups. The software Pa-

jek, designed for the analysis and visualisation of networks, was used for the construction and graphical representation of the research groups (BATAGELJ and MRVAR, 2001).

RESULTS

1. General data: annual productivity and journals

During the 1991-2010 period, 498 articles were published: 452 original articles (90.76%), 34 reviews (6.83%), 8 notes (1.6%), and 4 letters (0.8%). Most of them (43%) were published in the last five years, making 2009 the most productive year (n=51) (Fig. 1). In contrast, 73% of papers were published during the 2001-2010 decade, and the remaining 27% were published in the 1990s.

Papers were published in 237 different journals. The American Journal of Enology and Viticulture was the most productive journal, with 50 papers published, followed by the Journal of Agricultural and Food Chemistry, with 33. Both of these journals were edited in the United States. The VITIS German journal published 29 articles, and 3 other journals published more than 10 articles: Phytopathology (n=14), Plant Disease (n=14) and Applied and Environmental Microbiology (n=11). Table 1 shows the 33 journals with three or more published articles distributed by five-year periods and includes the 2009 impact factor and Country of publication.

The journals with the largest impact factors were the Proceedings of the National Academy of Sciences of the United States of America (FI=9,771), followed by BMC Genomics (FI=4,206), edited in United Kingdom, and Journal of Chromatography A (FI=4,194), edited in The Netherlands. As shown in the table, the most productive journals were published in the United States (8 journals), the United Kingdom (4 journals), Germany (3 journals), The Netherlands (2 journals) and Italy (one journal).

2. Publication output and citation impact by Country

For the comparative analysis of national publication activity and citation impact, Table 2 shows the Countries with more than one published paper and lists the number of papers published during the period, citations and the rate of citations per document. The United States, which had the highest share of publication output and citations (498 papers and 13,630 citations), was followed by Italy, France, Spain and Germany, with 146, 117, 73 and 50 papers, respectively. This ranking is similar for citations, with the exception that Austria and the United Kingdom had more citations than Germany. Among the most productive Countries, the highest rate of citations per papers belonged to Austria (102.3), followed by Spain (46.6). The UK is the Country with the highest increase in the rate of citations per document in the 2006-2010 period.

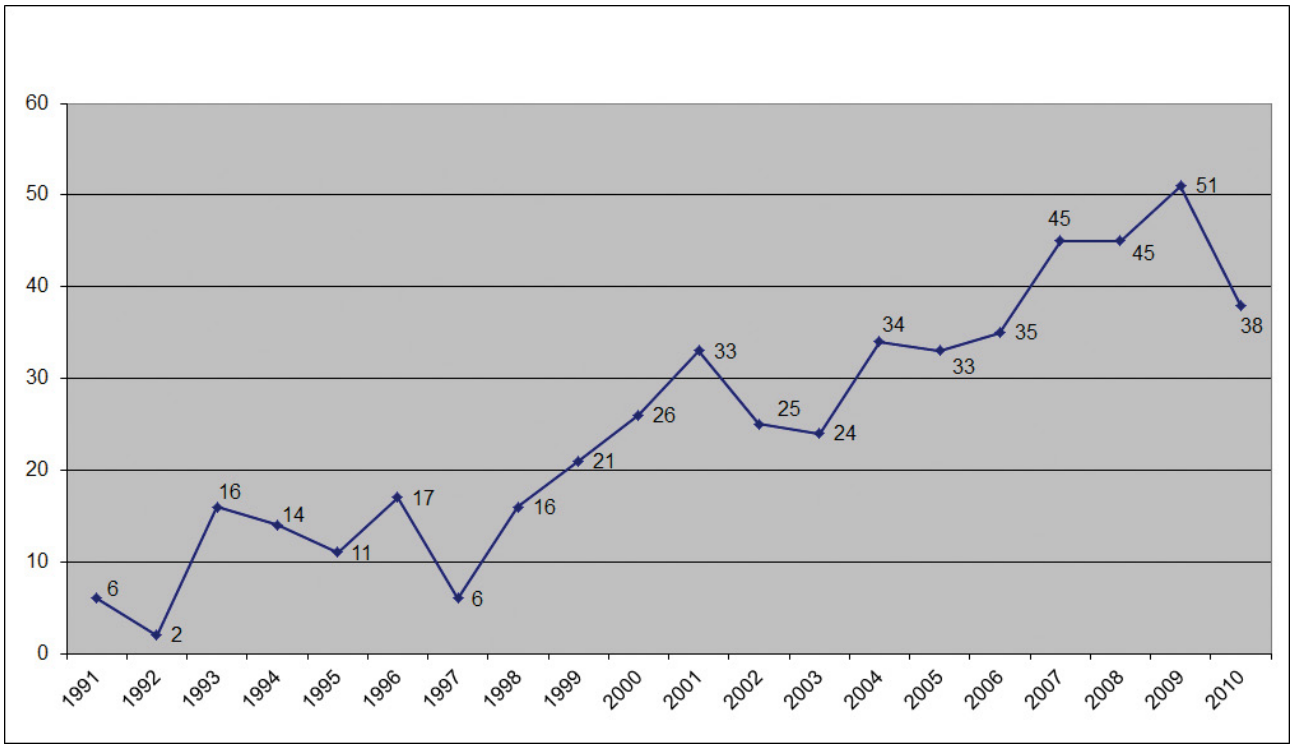


Fig. 1 - Evolution of publication output from 1991 to 2010.

Table 1 - Journals with 4 o more published papers.

Journal title	1991-1995	1996-2000	2001-2005	2006-2010	Total	IF 2010*	Journal Country
American Journal of Enology and Viticulture	13	9	13	15	50	1.667	United States
Journal of Agricultural and Food Chemistry	2	10	11	10	33	2.816	United States
Vitis	8	8	5	8	29	0.662	Germany
Plant Disease	3	5	2	4	14	2.387	United States
Phytopathology	6	2	2	4	14	2.428	United States
Applied and Environmental Microbiology	1	1	5	4	11	3.778	United States
Theoretical and Applied Genetics	-	-	3	4	7	3.264	Germany
Plant Pathology	-	2	4	-	6	2.237	United Kingdom
Proceedings of the National Academy of Sciences of the United States of America	-	1	-	4	5	9.771	United States
Food Chemistry	-	1	3	1	5	3.458	United Kingdom
Annals of Applied Biology	-		2	3	5	1.681	United States
Journal of Applied Microbiology	-	2	1	2	5	2.365	United Kingdom
Journal of Cardiovascular Pharmacology	-	1	2	2	5	2.406	United States
European Journal of Plant Pathology	-	2	1	2	5	1.575	Netherlands
Journal of Chromatography A	1	2	1	1	5	4.194	Netherlands
Journal of Phytopathology	1	1	-	2	4	0.937	Germany
BMC Genomics	-	-	-	4	4	4.206	United Kingdom
Plant Cell Reports	2	1	-	1	4	2.279	Germany
Journal of Plant Pathology	-	-	1	3	4	1.054	Italy
14 journals with 3	-	-	-	-	42		
37 journals with 2	-	-	-	-	74		
168 journals with 1	-	-	-	-	168		
Total	49	86	149	214	498		

3. Institutional productivity and citations

The published papers reflected involvement by 691 institutions, with the University of California Davis contributing the most (149 publications). Other American institutions that collaborated with the European Union were Cornell University (New York) through the departments of Food Science and Technology, Plant Pathology and Horticulture Sciences and the Agricultural Research Service of the United States Department of Agriculture (n=27) (Table 3).

Among European Union institutions, the most collaborative institutions with the US were Institut Nationale de la Recherche Agronomique (France), the Italian universities of Milan, Bologna and Udine, and the Spanish University of Barcelona.

The literature search was limited to articles that were signed by European Union and US institutions. Therefore, institutions that did not belong to these Countries included foreign institutions who had published articles in conjunction with these Countries, such as the University of Stellenbosch (South Africa; n=6), the Australian Wine Research Institute (Australia; n=3), and the University of Basel (Switzerland; n=2), among others.

In terms of the citations received, the most frequently cited institution was also the University of California (n=5,695), followed by the University of Barcelona (Spain; n=1,931), the Institut Nationale de la Recherche Agronomique (France; n=342), the Australian Res Ctr GmbH (n=1,135) and the Agricultural Research Service-United States Department of Agriculture (US, n=1,029). Eighty institutions received 100 or more citations (11.6%), 454 (65.7%) received 10 or more citations and only 28 received no citations. It should be stressed the fact some institutions such as the Agricultural Research Service-United States Department of Agriculture (Usa), the Consejo Superior de Investigaciones Científicas (Spain) and the University of Udine (Italy) have a remarkable growth in the rate of citations per document in the 2006-2010 period.

Table 2- Papers published and citations by Countries.

Country	Documents (D)					Cites (C)						Rate C/D
	1991-1995	1996-2000	2001-2005	2006-2010	Total	1991-1995	Rate C/D	1996-2000	Rate C/D	2001-2005	Rate C/D	2006-2010	Rate C/D	Total	
USA	49	86	149	214	498	1,822	37.18	5,264	61.21	3,960	26.58	2584	12.07	13,630	27.4
Italy	23	21	36	66	146	929	40.39	866	41.24	1,183	32.86	874	13.24	3,852	26.4
France	11	20	29	57	117	207	18.82	1217	60.85	1,256	43.31	628	11.02	3,308	28.3
Spain	11	10	21	31	73	635	57.73	1745	174.5	745	35.48	276	8.90	3,401	46.6
Germany	3	8	24	15	50	69	23	173	21.63	441	18.38	65	4.33	748	15
UK	-	10	9	28	47	-	-	213	21.3	186	20.67	956	34.14	1,355	28.8
Hungary	5	6	13	17	41	71	14.2	94	15.67	157	12.08	146	8.59	468	11.4
Denmark	-	6	7	5	18	-	-	574	95.67	92	13.14	52	10.4	718	39.9
Netherlands	-	1	6	9	16	-	-	315	315	199	33.17	116	12.89	630	39.4
Austria	-	4	6	5	15	-	-	1199	299.75	290	48.33	45	9	1,534	102.3
Australia	1	1	5	7	14	4	4	48	48	75	15	57	8.14	184	13.1
Portugal	-	6	5	2	13	-	-	121	20.17	157	31.4	12	6	290	22.3
Switzerland	2		4	7	13	49	24.5	-	-	186	46.5	113	16.14	348	26.8
Canada	-	4	1	6	11	-	-	162	40.5	13	13	79	13.17	254	23.1
Greece	1	-	2	7	10	1	1	-	-	11	5.5	88	12.57	100	10
Other 40	9	12	30	71	122	185	-	742	9	435	6.75	1426	29.89	2,788	21.8
Countries with less than 10 documents															
Total	115	195	347	547	1,204	3,972		12,733		9,386		7,517		33,608	

3. Authors' productivity and citations

The 498 articles were published by 2028 different authors, of whom 1629 (80.3%) published a single article. Table 4 presents authors with five or more published papers, the number of citations received and the evolution of these variables from 1991 to 2010. The most productive author was Dipak K Das (n=35), followed by Andrew L Waterhouse (n=25) and two authors with 20 articles respectively, Carole P Meredith and Marc Fuchs.

Regarding the citations (Table 2), five authors received more than one thousand citations. The most frequently cited authors were Rosa M Lamuela-Raventos (n=1,847), followed by Andrew L Waterhouse (n=1,751), Dipak K Das (n=1,299), VL Singleton (n=1,140) and R Orthofer (n=1,123). Singleton and Orthofer are not included in Table 3 because they published only two and one articles, respectively; 95% of the authors were cited at least once, and the remaining 5% received no citations.

4. Indicators of collaboration and networks

The collaboration index (average number of authors per paper) was 6.2 for the entire period, increasing from 4.7 in the first five years to 7.4 in the latter five-year period (results not shown). Regarding collaboration between the US and European Countries, the Country with the most papers was Italy (n=146), followed by France (n=117), Spain (n=73) and Germany (n=50). Other Countries that published articles in collaboration with the US and at least one EU Country (that is, a triple collaboration) were Australia (n=14), Switzerland (n=13), Canada (n=11), South Africa (n=9), Israel (n=6) and the People's Republic of China (n=5), among others.

For the representation of networks of collaboration, a threshold of 5 co-authored articles was used. Eighteen groups were created with different components or numbers of authors in each group (Fig. 2). The largest group had 19 components and two nuclei: in the first one (with a stellar structure), the central author was Meredith, whereas in the second one (with a spherical structure due to the high degree of collaboration between members), the central authors were Velasco and Traggio. The authors that linked both nuclei were This and Grando. In the second group, with 13

Table 3 - Institutions with more than 5 papers.

Institution	Documents (D)					Cites (C)									Rate C/D
	1991-1995	1996-2000	2001-2005	2006-2010	Total	1991-1995	Rate C/D	1996-2000	Rate C/D	2001-2005	Rate C/D	2006-2010	Rate C/D	Total	
Univ. Calif Davis (USA)	20	37	48	44	149	796	39.8	3,083	83.32	1104	23	712	16.18	5,695	38.22
Cornell Univ. (USA)	12	12	11	33	68	227	18.92	314	26.17	90	8.18	137	4.15	768	11.29
Institut Nationale de la Recherche Agronomique (France)	7	8	12	23	50	115	16.43	433	54.13	462	38.5	263	11.43	1273	25.46
Agricultural Research Service-United States Department of Agriculture(USA)	5	3	10	9	27	416	83.2	63	21	231	23.1	319	35.44	1029	38.11
Univ. Connecticut (USA)	-	2	8	14	24	-	-	288	144	284	35.5	220	15.71	792	33
Univ. Milan (Italy)	-	6	8	9	23	-	-	347	57.83	255	31.88	144	16	746	32.43
Univ. Bologna (Italy)	12	2	1	6	21	600	50	45	22.5	14	14	22	3.67	681	32.43
Consiglio Nazionale delle Ricerche (Italy)	4	3	6	5	18	146	36.5	61	20.33	85	14.17	41	8.2	333	18.50
Univ. Udine (Italy)	5	1	5	7	18	108	21.6	36	36	243	48.6	156	22.29	543	30.17
Univ. Barcelona (Spain)	5	6	4	1	16	528	105.6	1,266	211	121	30.25	16	16	1,931	120.69
Oregon State Univ. (USA)	-	3	5	8	16	-	-	68	22.67	84	16.8	92	11.5	244	15.25
Univ. Calif Berkeley (USA)	2	5	1	8	16	131	65.5	185	37	13	13	52	6.5	381	23.81
Harvard Univ. (USA)	-	5	1	9	15	-	-	711	142.2	11	11	101	11.22	823	54.87
Univ. Florence (Italy)	2	5	4	3	14	131	65.5	260	52	29	7.25	37	12.33	457	32.64
Univ. Bari (Italy)	4	2	4	4	14	122	30.5	40	20	79	19.75	12	3	253	18.07
Consejo Superior de Investigacion. Científicas (Spain)	-	3	5	5	13	-	-	412	137.33	268	53.6	116	23.2	796	61.23
Univ. Bordeaux 2 (France)	1	1	4	7	13	25	25	63	63	160	40	55	7.86	303	23.31
Univ. Strasbourg 1 (France)	-	-	1	11	12	-	-	-	-	4	4	41	3.73	45	3.75
Washington State Univ. (USA)	1	3	2	4	10	25	25	64	21.33	22	11	38	9.5	149	14.9
Univ. Reims (France)	-	1	5	4	10	-	-	76	76	280	56	32	8	388	38.8
Univ. Hohenheim (Germany)	-	3	5	1	9	-	-	44	14.67	30	6	2	2	76	8.44
Res. Inst. Viticulture & Enol. Kecskemet (Hungary)	1	1	1	5	8	13	13	8	8	4	4	20	4	45	5.63
Szent Istvan Univ. (Hungary)	-	-	4	4	8	-	-	-	-	11	2.75	16	4	27	3.38
Virginia Polytech Inst. & State Univ. (USA)	-	1	4	2	7	-	-	67	67	268	67	27	13.5	362	51.71
Univ. Georgia (USA)	-	3	2	2	7	-	-	169	56.33	64	32	25	12.5	258	36.86
Natl. Univ. Ireland Univ. Coll Cork (Ireland)	-	2	3	2	7	-	-	26	13	13	4.33	2	1	41	5.86
Univ. Padua (Italy)	-	1	3	3	7	-	-	36	36	207	69	174	58	417	59.57
Hungarian Acad. Sci. (Hungary)	2	3	1	1	7	20	10	39	13	4	4	1	1	64	9.14
Debrecen Univ. Med. (Hungary)	-	-	3	4	7	-	-	-	-	32	10.67	48	12	80	11.43
Univ. Turin (Italy)	1	2	1	3	7	37	37	84	42	113	113	27	9	261	37.29
Inst. Valenciano Invest. Agr. (Spain)	1	-		5	6	8	8	-	-	-	-	13	2.6	21	3.5
IASMA Res. Ctr. Istituto Agrario San Michele all'Adige (Italy)	-	-		6	6	-	-	-	-	-	-	246	41	246	41
Univ. Florida (USA)	-	-	4	2	6	-	-	-	-	34	8.50	11	5.5	45	7.5
Univ. Católica Portuguesa (Portugal)	-	4	2	-	6	-	-	37	9.25	11	5.50	-	-	48	8
Missouri State Univ (USA)	-	-		6	6	-	-	-	-	-	-	18	3	18	3
Univ. Basilicata (Italy)	1	2	2	1	6	16	16	46	23	67	33.50	9	9	138	23
Univ. Calif Riverside (USA)	2	1	2	1	6	11	5.5	9	9	12	6	1	1	33	5.50
Univ. Bourgogne (France)	-	2	1	3	6	-	-	140	70	8	8	7	-	155	25.83
Centre National de la Recherche Scientifique (France)	-	1	1	4	6	-	-	24	24	5	5.00	38	9.5	67	11.17
Univ. Naples Federico 2 (Italy)	-	-		6	6	-	-	-	-		-	60	10	60	10
Univ. Stellenbosch (South Africa)	-	1	1	4	6	-	-	63	63	18	18.00	27	6.75	108	18
Tech. Univ. Denmark (Denmark)	-	5	1	-	6	-	-	523	104.6	11	11.00	-	-	534	89

Table 4 - Authors with more than 5 papers.

Author	Documents (D)					Cites (C)				
	1991-1995	1996-2000	2001-2005	2006-2010	Total	1991-1995	1996-2000	2001-2005	2006-2010	Total
Das, Dipak K.	-	4	12	19	35	-	576	391	332	1,299
Waterhouse, Andrew L.	6	9	6	4	25	596	936	187	32	1,751
Meredith, Carole P.	-	4	13	3	20	-	325	553	73	951
Fuchs, Marc	-	-	4	16	20	-	-	36	64	100
Burr, Thomas J.	3	8	3	4	18	32	176	10	5	223
Walker, M Andrew	4	4	7	1	16	35	59	136	2	232
Lamuela-Raventos, Rosa M.	4	7	3	1	15	502	1280	49	16	1847
Martelli, Giovanni P.	2	2	5	4	13	63	32	53	113	261
Davis, R.E.	3	4	5	1	13	185	77	77	7	346
Boulton, Roger B.	2	3	5	2	12	22	34	92	4	152
Kovacs, Laszlo G.	3	-	1	7	11	58	-	1	21	80
Edwards, Charles G.	-	6	2	2	10	-	128	14	15	157
This, Patrice	-	1	3	6	10	-	138	290	180	608
Vigne, Emmanuelle	-	-	1	9	10	-		14	37	51
Noble, A.C.	3	4	3	-	10	83	101	48	-	232
Gonsalves, D.	3	2	4	1	10	71	46	42	6	165
Bertelli, Alberto A.E.	-	2	4	4	10	-	288	151	80	519
Demangeat, Gerard	-	-	1	8	9	-	-	14	15	29
Intrigliolo, Diego S.	-	-		9	9	-	-	-	38	38
Sule, Sandor	3	4	1	1	9	38	51	4	1	94
Frankel, E.N.	-	8	1	-	9	-	809	11	-	820
Credi, R.	7	1	1	-	9	218	9	14	-	241
Barka, Essaid Ait	-	-	6	3	9	-	-	518	42	560
Bertelli, A.	-	2	4	3	9	-	288	223	55	566
Fidelibus, Matthew W.	-	-	2	6	8	-	-	10	13	23
Rowhani, Adib	2	4	1	1	8	23	74	2	5	104
Maulik, Nilanjana	-	1	4	3	8	-	73	112	70	255
Velasco, Riccardo	-	-	-	8	8	-	-	-	425	425
Tosaki, Arpad	-	-	5	3	8	-	-	166	47	213
Di Gaspero, Gabriele	-	-	-	7	7	-	-	-	128	128
Lemaire, Olivier	-	-	-	7	7	-	-	-	15	15
Reisch, Bruce I.	2	3	-	2	7	44	155	-	28	227
Minafra, Angelantonio	3	2	-	2	7	84	40	-	9	133
Mortimer, R.K.	2	5	-	-	7	131	185	-	-	316
Cortesi, P.	-	4	2	1	7	-	60	28	2	90
Mills, David A.	-	-	4	3	7	-	-	128	252	380
Smart, David R.	-	-	6	1	7	-	-	66	8	74
Szegedi, Erno	-	2	1	4	7	-	16	4	9	29
Spencer, Robert G.M.	-	-	-	7	7	-	-	-	117	117
Troggio, Michela	-	-	-	7	7	-	-	-	286	286
Kikkert, Julie R.	2	2	-	2	6	44	78	-	28	150
Komar, Veronique	-	-	1	5	6	-	-	14	16	30
Dangl, G.S.	-	-	5	1	6	-	-	322	1	323
Boursiquot, J.M.	1	1	3	1	6	15	138	132	3	288
Forneck, A.	-	3	3	-	6	-	44	25	-	69
Vezzulli, Silvia	-	-	-	6	6	-	-	-	219	219
Das, Samarjit	-	-	1	5	6	-	-	46	102	148
Kocsis, L.	-	2	4	-	6	-	19	43	-	62
Ebeler, Susan E.	-	1	4	1	6	-	14	182	10	206
Lakso, Alan N.	2	-	-	4	6	57	-	-	13	70
Polsinelli, M.	2	4	-	-	6	131	156	-	-	287
Pindo, Massimo	-	-	-	6	6	-	-	-	245	245
Jones, G.V.	-	-	2	4	6	-	-	54	52	106
Meyer, A.S.	-	5	1	-	6	-	523	11	-	534
Balestrieri, Maria Luisa	-	-	-	6	6	-	-	-	47	47
Bagchi, D.	-	-	5	1	6	-	-	218	41	259
Napoli, Claudio	-	-	-	6	6	-	-	-	68	68

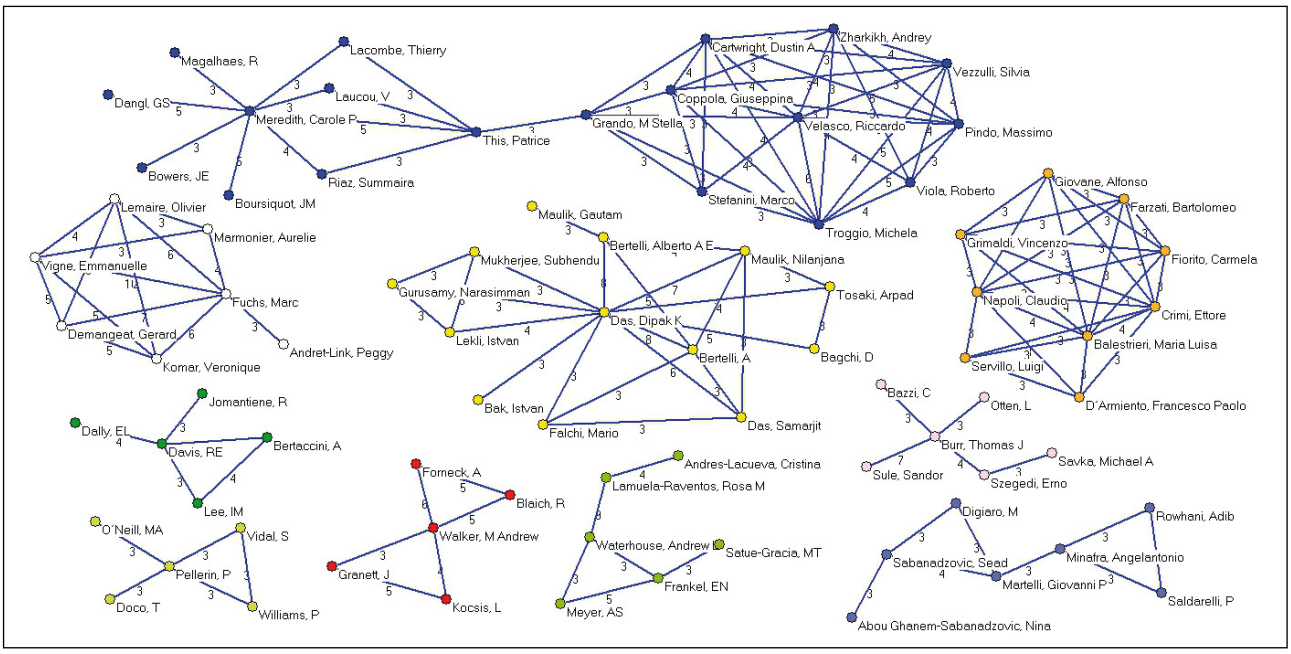


Fig. 2 - Author's network (1).

members, Das was the central author and was linked to 11 other authors. Fig. 2 also presents a group of 9 components, for which the central authors were Balestrieri and Crimi, and other smaller groups: two with seven members, two with six members and three with five members.

Fig. 3 represents the collaborative relationships between institutions. A threshold of three co-authored signed articles was established. The University of California Davis is situated in a central position in the network, and its most intense collaborations have been with the Institute National de la Recherche Agronomique (France), with 16 articles signed jointly, and the University of Barcelona (Spain), with 13 articles. Other centres that occupy a central, although less intense, position are Cornell University (US), with 16 articles signed jointly with the Institute Nationale de la Recherche Agronomique and 10 with the University of Strasbourg; the University of Connecticut, with 12 papers signed in collaboration with the University of Milan; the Agricultural Research Service-United States Department of Agriculture (US); the University of Bologna (Italy); Consiglio

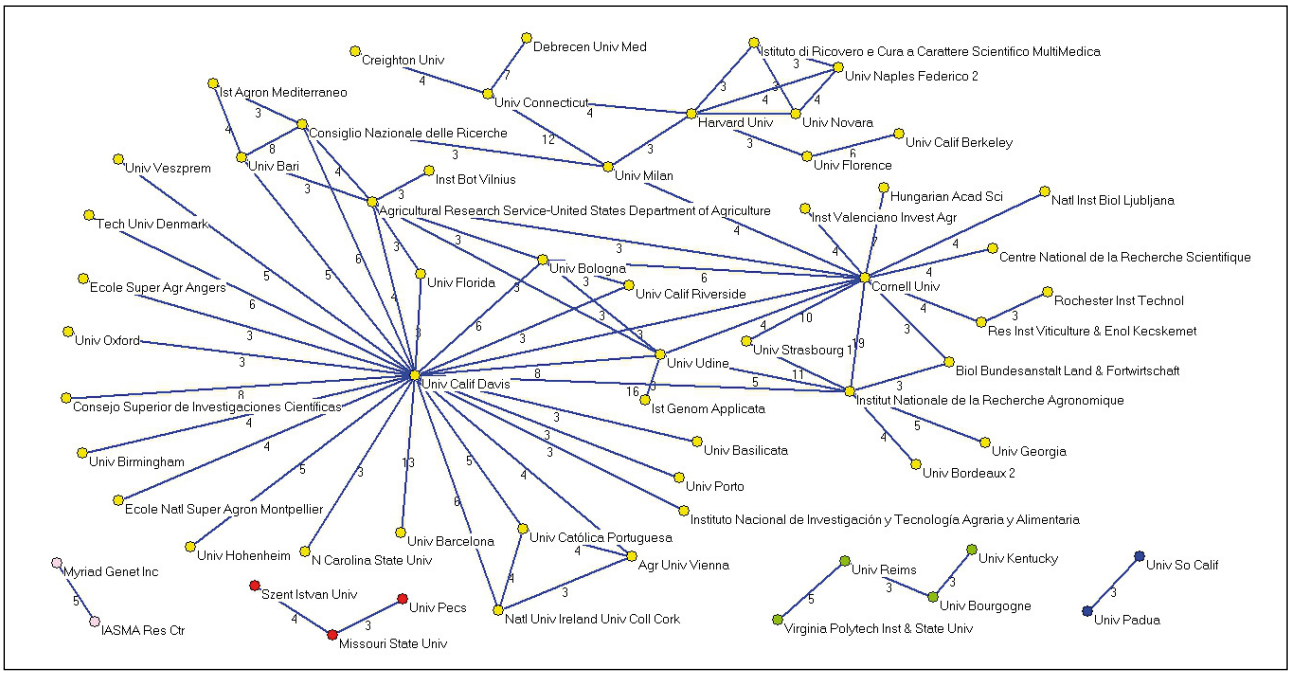


Fig. 3 - Institutions network.

Nazionale delle Ricerche (Italy); and University of Udine. Fig. 4 presents the collaborative network among Countries with the USA in a central position, as well as United Kingdom, Italy, France, Spain, Germany, and Australia.

5. Most cited papers

The 28 papers that received 100 or more citations (hot papers) are presented in Table 5. The most frequently cited paper was by Singleton, Orthofer and Lamuela-Raventos, published in 1999 in the journal Flavonoids and other Polyphenolsse Methods in Enzymology, which has received 1,123 citations. This is the only paper with more than one thousand citations. Two other papers received more than three hundred citations and were published by Gil and colleagues in the Journal of Agricultural and Food Chemistry in 2000 and by Rimm and colleagues in the journal Annals of Internal Medicine in 1996.

DISCUSSION

This paper analysed collaboration in scientific journals between the United States and the European Union in the field of viticulture and oenology and identified the most productive journals and the institutions and authors with the greatest impact. Furthermore, this paper examined the composition of research groups that have significant collaboration and, according to NEWMAN (2004), can be considered the elite or the leaders of research on this topic.

In the annual distribution of published articles, an increasing trend can be seen during the 20 years studied, especially from the second half of the 1990s, with a peak of 51 articles in 2009. Two distinct periods in the evolution of the annual number of published articles can be observed, one extending to the end of the first decade, with moderate production (27% of the papers published during this period) and an average of 13 articles per year, and another that runs from the beginning of the second decade, with an average of 36 papers per year (73%).

Reasons for this increase may include the growing importance of trade relations between the US and the EU-27 (FERNÁNDEZ *et al.*, 2006; GLANZEL and VEUGELERS, 2006). Other reasons may include the overall growth of the SCIE database, where an increasing number of journals have been added during last decades, which implies a rising number of retrievable relevant literature, and the increasing number of oenology and viticulture journals included in SCIE. In 2000, there was only one specific journal, the American Journal of Enology and Viticulture, whereas in 2010, there were 5 more: Australian Journal of Grape and Wine Research (Australia), Ciencia e Tecnica Vitivinicola (Portugal), Journal International des Sciences de la Vigne et du Vin (France), South African Journal of Enology and Viticulture (South Africa) and VITIS (Germany).

The analysis of the subjects of journals can indicate the areas covered in articles in the current multidisciplinary viticulture and oenology field. It has been observed that researchers publish not only in journals that are specific to the area but also in other areas, such as food science and technology (Journal of Agricultural and Food Chemistry and Food Chemistry), plant sciences (Plant Disease, Plant Pathology and Jour-

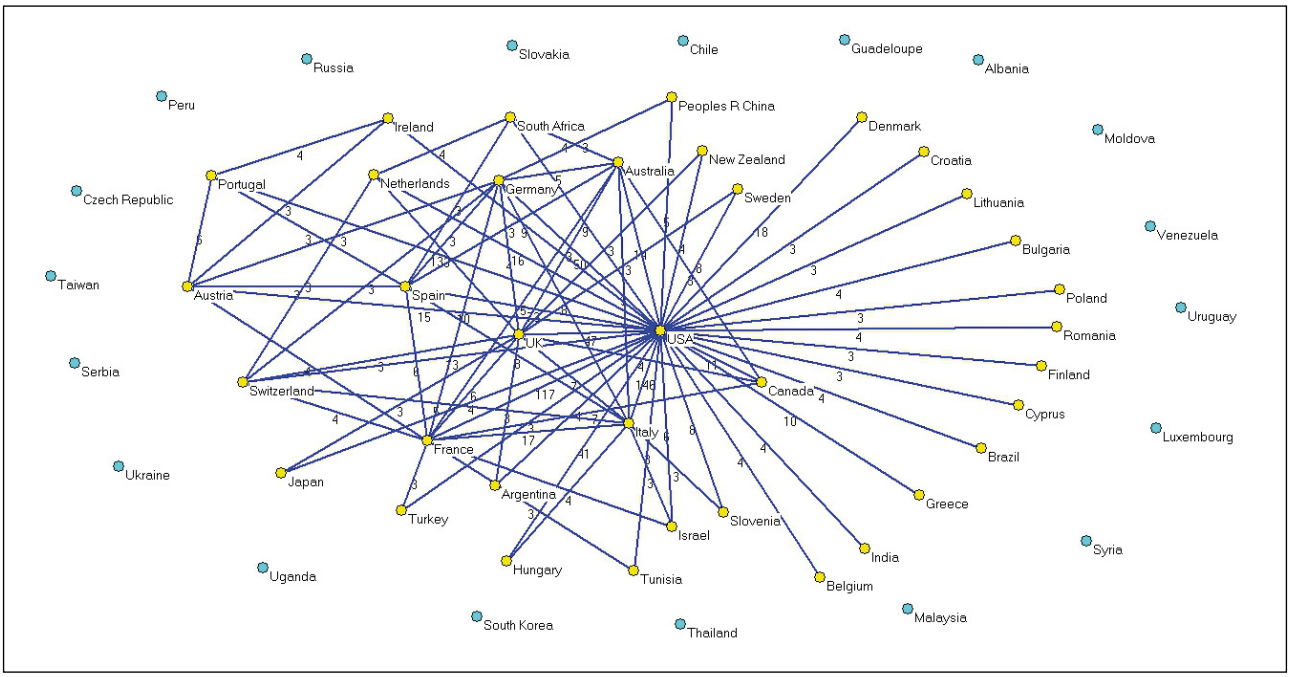


Fig. 4 - Countries network.

Table 5 - Hot papers (more than 100 citations).

Authors	Title	Journal	Year	Vol.	Iss.	First Page	Last Page	No. Cites
Singleton V.L.; Orthofer R.; Lamuela-Raventos R.M.	Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent in Enzymology	Flavonoids and other Polyphenolsse Methods	1999	299		152	178	1123
Gil M.I.; Tomas-Barberan F.A.; Hess-Pierce B.; Holcroft D.M.; Kader A.A.	Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing	Journal of Agricultural and Food Chemistry	2000	48	10	4581	4589	340
Rimm E.B.; Katan M.B.; Ascherio A.; Stampfer, M.J.; Willett W.C.	Relation between intake of flavonoids and risk for coronary heart disease in male health professionals	Annals of Internal Medicine	1996	125	5	384	389	315
Teissedre P.L.; Frankel E.N.; Waterhouse A.L.; Peleg H.; German J.B.	Inhibition of in vitro human LDL oxidation by phenolic antioxidants from grapes and wines	Journal of the Science of Food and Agriculture	1996	70	1	55	61	287
Makarova K.; Slesarev A.; Wolf Y.; Sorokin A.; Mirkin B.; Koonin E.; *et al*.	Comparative genomics of the lactic acid bacteria	Proceedings of The National Academy of Sciences of the United States of America	2006	103	42	15611	15616	250
Ray P.S.; Maulik G.; Cordis G.A.; Bertelli .AA.E.; Bertelli A.; Das D.K.	The red wine antioxidant resveratrol protects isolated rat hearts from ischemia reperfusion injury	Free Radical Biology and Medicine	1999	27	1-2	160	169	215
Rahman I.; Biswas S.K.; Kirkham P.A.	Regulation of inflammation and redox signaling by dietary polyphenols	Biochemical Pharmacology	2006	72	11	1439	1452	198
Lee I.M.; Bertaccini A.; Vibio M.; Gundersen D.E.	Detection of multiple phytoplasmas in perennial fruit-trees with decline symptoms in Italy	Phytopathology	1995	85	6	728	735	186
Velasco R.; Zharkikh A.; Troggio M.; Cartwright D.A.; Cestaro A.; Pruss D.; *et al*.	A high quality draft consensus sequence of the genome of a heterozygous grapevine variety	Plos One	2007	2	12	0	0	173
Tapiero H.; Tew K.D.; Ba G.N.; Mathe G.	Polyphenols: do they play a role in the prevention of human pathologies?	Biomedicine & Pharmacotherapy	2002	56	4	200	207	165
Compant S.; Duffy B.; Nowak J.; Clement C.; Barka E.A.	Use of plant growth-promoting bacteria for biocontrol of plant diseases: Principles, mechanisms of action, and future prospects	Applied and Environmental Microbiology	2005	71	9	4951	4959	163
Lamuela Raventos R.M.; Romero Perez A.I.; Waterhouse A.I.; De La Torre Boronat M.C.	Direct hplc analysis of cis-resveratrol and trans-resveratrol and piceid isomers in Spanish red vitis-vinifera wines	Journal of Agricultural and Food Chemistry	1995	43	2	281	283	162
Kris-Etherton P.M.; Hecker K.D.; Bonanome A.; Coval S.M.;Binkoski A.E.; Hilpert K.F.;*et al*.	Bioactive compounds in foods: Their role in the prevention of cardiovascular disease and cancer	American Journal of Medicine	2002	113		71	88	158

Table 5 - Continues.

Authors	Title	Journal	Year	Vol.	Iss.	First Page	Last Page	No. Cites
Lamuela Raventos R.M.; Waterhouse A.L.	A direct hplc separation of wine phenolics	American Journal of Enology and Viticulture	1994	45	1	1	5	156
Meyer A.S.; Yi O.S.; Pearson D.A.; Waterhouse A.L.; Frankel E.N.	Inhibition of human low-density lipoprotein oxidation in relation to composition of phenolic antioxidants in grapes (Vitis vinifera)	Journal of Agricultural and Food Chemistry	1997	45	5	1638	1643	146
Meyer A.S.; Heinonen M.; Frankel E.N.	Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation	Food Chemistry	1998	61	1-2	71	75	142
Bowers J.; Boursiquot J.M.; This P.; Chu K.; Johansson H.; Meredith C.	Historical genetics: The parentage of chardonnay, gamay, and other wine grapes of Northeastern France	Science	1999	285	5433	1562	1565	138
Manach C.; Donovan J.L.	Pharmacokinetics and metabolism of dietary flavonoids in humans	Free Radical Research	2004	38	8	771	785	133
M.W.; Xu S.Q.; Maitland-Toolan K.A.; Zuccollo A.; Hou X.Y.; Jiang B.B.; et al.	Polyphenols stimulate AMP-activated protein kinase, lower lipids, and inhibit accelerated atherosclerosis in diabetic LDL receptor-deficient mice	Diabetes	2006	55	8	2180	2191	129
Prince J.P.; Davis R.E.; Wolf T.K.; Lee I.M.; Mogen B.D.; Dally E.L.; et al.	Molecular-detection of diverse mycoplasmalike organisms (mlos) associated with grapevine yellows and their classification with aster yellows, x-disease, and elm yellows mlos	Phytopathology	1993	83	10	1130	1137	123
Tomas-Barberan F.A.; Gil M.I.; Cremin P.; Waterhouse A.L.; Hess-Pierce B.; Kader A.A.	HPLC-DAD-ESIMS analysis of phenolic compounds in nectarines, peaches, and plums	Journal of Agricultural and Food Chemistry	2001	49	10	4748	4760	122
Meyer A.S.; Donovan J.L.; Pearson D.A.; Waterhouse A.L.; Frankel E.N.	Fruit hydroxycinnamic acids inhibit human low-density lipoprotein oxidation in vitro	Journal of Agricultural and Food Chemistry	1998	46	5	1783	1787	117
Mortimer R.K.; Romano P.; Suzzi G.; Polsinelli M.	Genome renewal - a new phenomenon revealed from a genetic-study of 43 strains of saccharomyces-cerevisiae derived from natural fermentation of grape musts	Yeast	1994	10	12	1543	1552	115
This P.; Jung A.; Boccacci P.; Borrego J.; Botta R.; Costantini L.; et al.	Development of a standard set of microsatellite reference alleles for identification of grape cultivars	Theoretical And Applied Genetics	2004	109	7	1448	1458	113
Cavalieri D.; Townsend J.P.; Hartl D.L.	Manifold anomalies in gene expression in a vineyard isolate of Saccharomyces cerevisiae revealed by DNA microarray analysis	Proceedings of the National Academy of Sciences of the United States of America	2000	97	22	12369	12374	104
Pellerin P.; Doco T.; Vidal S.; Williams P.; Brillouet J.M.; ONeill M.A.	Structural characterization of red wine rhamnogalacturonan II	Carbohydrate Research	1996	290	2	183	197	104
Donovan J.L.; Meyer A.S.; Waterhouse A.L.	Phenolic composition and antioxidant activity of prunes and prune juice (Prunus domestica)	Journal of Agricultural and Food Chemistry	1998	46	4	1247	1252	101

nal of Plant Pathology), microbiology and biotechnology (Applied and Environmental Microbiology, Journal of Applied Microbiology), and health sciences (Journal of Cardiovascular Pharmacology), among others. This diversity of subject areas should alert researchers seeking information on viticulture and oenology to not only investigate the specific journals in this area but to expand their search to other related journals, such as those identified in this study (KLEIN, 1996).

The University of California Davis takes a leadership role in the collaboration with European Union Countries in this area, mainly through the departments of Viticulture and Enology, Plant Sciences, Plant Pathology and Microbiology. The goal of the UC Davis Viticulture and Enology Department is to develop a knowledgeable workforce to form the basis of the state's wine industry and to make California wine more approachable to consumers. Although the program dates back 120 years, the current UC Davis Department of Viticulture and Enology was established in 1935. Many disciplines are studied in the department's complex curriculum. One of the department's innovations was emphasising communication between the vineyard and the winery. This communication effectively bridged the gap that had developed in California between grape growing and winemaking. At Cornell University, several departments have participated, mainly the Department of Plant Pathology & Plant Microbe Biology, the Department of Food Science & Technology and the Department of Ecology & Evolutionary Biology.

The existence of many Italian, French and Spanish institutions that collaborate with the US is not surprising, for two reasons. The first reason is that these Countries are the most important producers of wine in Europe and, consequently, have developed an important research structure in this area. The second reason is that it is very common among European researchers to maintain contact and active research collaborations with many national and international universities and research organisations from the United States. This collaboration involves agreements related to the exchange of students and foreign researchers as well as many collaborative research projects. The promotion of residency in foreign research centres is considered crucial for promoting the internationalisation of science in any Country (PONDS, 2009), and the United States is one of the preferred destination Countries for European researchers. In this work, we have seen a steady increase in collaboration within this area, consistent with the data provided by GLÄNZEL and VEUGELELRS (2006). This study identified other non-American and European institutions that collaborated in the retrieved papers, usually from Countries that also have a tradition of wine production, mainly Australia and South Africa (ALEIXANDRE-BENAVENT *et al.*, 2012). With regard to indicators, particularly citation impact indicators, they should be carefully interpreted because may fail to compare large and small institutions or nations (COSTAS *et al.*, 2009; KATZ, 2000).

Regarding the authors of published articles, the top producer of collaborative articles was Das, who is currently a researcher at the University of Connecticut (Cardiovascular Research Center, School of Medicine, Farmington, Usa). This researcher specialises in wine and health and focuses on the beneficial effects of resveratrol, a red wine- and grape-derived polyphenolic antioxidant that prevents cardiovascular diseases. Waterhouse is a professor in the Department of Viticulture & Enology of the University of California Davis, specialising in research on the health and taste effects of phenolics and other components of wine and their antioxidant activity. The main line of research of Meredith, who also belongs to the Department of Viticulture & Enology at the University of California Davis, is genetics in grape species.

This study identified the research front in the collaboration between the US and the EU in this area by applying social network analysis because this method allowed us to detect the main groups of researchers with scientific collaborations (GEORGHIOU, 1998; GLÄNZEL and SCHUBERT, 2004). Knowledge of these groups has several advantages for researchers. First, it provides researchers with trustworthy information about existing teams that have high research activity. This information provides the opportunity to expand researchers' networks and to participate in discussion forums and exchanges of ideas on significant topics (GONZÁLEZ-ALCAIDE *et al.*, 2008; SCOTT, 2001; WAGNER and LEYDESDORFF, 2005). Second, this knowledge allows researchers to determine which organisations work together and which ones occupy central places in the network, which is helpful in choosing which institutions may be suitable for collaborative activities and multicentre studies. Some authors argue that collaborative networks with many links are preferentially selected by new research groups to begin scientific relationships, and a group rich in connections will increase its influence more quickly due to its leading role in this area (WAGNER and LEYDESDORFF, 2005). As an additional advantage, collaboration between teams with analogous interests and research topics prevents repetition of experiments, increasing research effectiveness (BARABÁSI, 1999; FIGG *et al.*, 2006; GEORGHIOU, 1998; TEASLEY and WOLINSKY, 2001). Smaller groups consisting of a small number of authors with no links to other groups may reflect teams at the beginning of their research activity in this area (BEAVER, 2001; MERRILL and HRIPCSAK, 2008; SAEGUSA, 1999).

Some of the following limitations of this study should be discussed:

1. The SCIE database does not incorporate all published scientific papers on viticulture and oenology, and other complementary sources of in-

formation could have been used. Nevertheless, the use of SCIE has several advantages:

a) SCIE is extensively used in studies investigating scientific activity in science and technology because it includes the highest-impact international journals. Consequently, the use of SCIE allows us to focus on the most important researchers and organisations. b) SCIE provides all of the authors who signed the papers and all of their institutional affiliations, allowing us to obtain indicators of collaboration between authors and institutions. c) SCIE provides the number of citations received by the articles, allowing us to determine the number of citations for their authors and institutions. d) The additional resource JCR provides the impact factor of journals, information not usually present in other databases.

2. The social network analysis based on coauthorship of scientific papers has only recently been employed. For this reason, we have not found similar papers in the scientific literature that analyse the social networks for viticulture and oenology to explain these results.

3. The papers, authors and groups identified in this study did not allow us to examine their contributions to the development and dissemination of knowledge beyond their citations in later publications (GARFIELD, 2006).

CONCLUSIONS

This study offers indicators to measure the state and evolution over the past 20 years of collaborative research in viticulture and oenology between United States of America and the European Union based on articles published in the high-impact international scientific journals included in the SCIE database. This study identified the journals, subject areas, scientific productivity and impact of researchers and organisations. Collaboration patterns between these researchers and organisations were also established, identifying those that were most significant, central and connected to one another. We note a considerable increase in collaboration in recent years, with American, French, Italian and Spanish institutions leading this collaboration. Future lines of research could analyse the behaviour and changes of the identified groups, the increase or decrease in the number of researchers in each group, the quality of published papers based on the number of citations received, and emerging groups and their evolution.

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PAPER

FATE OF *LISTERIA MONOCYTOGENES* DURING PRODUCTION AND STORAGE OF ARTISAN WATER BUFFALO MOZZARELLA CHEESE

A. SERRAINO[1]*, G. FINAZZI[2], G. MARCHETTI[1], P. DAMINELLI[2], R. RIU[1], F. GIACOMETTI[1], M.N. LOSIO[2] and R. ROSMINI[1]

[1]University of Bologna, Faculty of Veterinary Medicine, Department of Veterinary Medical Sciences, Via Tolara di sopra 50, 40064 Ozzano Emilia, Bologna, Italy

[2]Experimental Institute for Zooprophylaxis in Lombardy and Emilia Romagna, Department of Microbiology, Via Bianchi 7/9, 25124 Brescia, Italy

*Corresponding author: andrea.serraino@unibo.it

ABSTRACT

The study aim was to assess the behaviour of *Listeria monocytogenes* during the production and shelf life of artisan water buffalo mozzarella cheese (WBMC) under different storage conditions. Raw milk was deliberately contaminated by *L. monocytogenes* and the evolution of *L. monocytogenes* count during production and shelf life was monitored. In traditional WBMC production technology *L. monocytogenes* can multiply in the curd during ripening, but its growth rate expressed in log CFU/g/h is lower than the growth rate reported by theoretical predictions. Stretching proved to be a process with good repeatability and able to reduce *L. monocytogenes* contamination by about 2 Log CFU/g. The intrinsic characteristics of traditional WBMC proved to be able to obstacolate the growth of *L. monocytogenes* during storage even in the case of severe thermal abuse.

- Keywords: *Listeria monocytogenes*, water buffalo mozzarella cheese, stretching, shelf life, food safety -

INTRODUCTION

Water buffalo mozzarella cheese (WBMC) is a fresh "pasta filata" cheese produced from whole chilled buffalo milk moulded into various shapes, most commonly oval-spherical. Although pasteurization of milk and the use of defined starter cultures are recommended, traditional technology involving the use of unpasteurized milk and natural whey cultures is still employed for WBMC production in Italy. The production process was described by ADDEO and COPPOLA (1983) and VILLANI *et al.* (1996). In the artisan mozzarella cheese factory, mozzarellas are stored at room temperature in a conditioning liquid commonly composed of water resulting from stretching, acidified with whey from the previous manufacture (VILLANI *et al.* 1996) or, more recently, by tap water salted and acidified with lactic or citric acid.

Foodborne diseases associated with cheese consumption are reported worldwide but diseases associated with the consumption of cheeses produced by pasteurized milk are less common. The main concerns related to the use of unpasteurized milk for making WBMC are the pathogenic bacteria *Salmonella* spp. and *Listeria monocytogenes*, which may contaminate raw milk and, consequently, be harboured in the cheese. *Listeria monocytogenes* has been implicated in a number of food poisoning outbreaks involving milk, dairy products, meat products and vegetables. Between 1988 and 2007 dairy products accounted for 41.5% of 53 foodborne outbreaks of listeriosis reported internationally and *L. monocytogenes* was involved in 6.6% of foodborne outbreaks caused by the consumption of dairy products (GREIG and RAVEL, 2009). The notification rate in the EU in 2008, independently by the food involved, was 0.3 cases per 100,000 population (ANON, 2010). Several listeriosis outbreaks involving cheese as a vehicle have been reported in the EU in recent years (KOCH *et al.*, 2010). The incidence of listeriosis in Europe appears to increase among persons over 60 years of age (GOULET *et al.*, 2008).

Potential sources of contamination of WBMC by *L. monocytogenes* are raw milk (LOVETT *et al.*, 1987; RYSER, 1999), natural whey starter cultures, whey used as an ingredient of the conditioning liquid (VILLANI *et al.*, 1996), not sanitized surfaces or dairy tools or equipment, and handlers (D'AMICO and DONNELLY, 2009).

According to Regulation (EC) 2073/2005, ready to eat food in which *L. monocytogenes* can grow must not contain the bacterium in 25 g at the time when the food leaves the production plant and *L. monocytogenes* must not be present in levels above 100 Colony Forming Units (CFU)/g during shelf life. In addition, producers should be able to make the decision on the shelf life assigned to food, and the indication on duration and storage temperature should be based on a product-specific risk analysis, taking into account reasonable storage conditions and use by consumers. The Regulation suggests specific challenge tests to be carried out on experimentally contaminated food. Producers' instructions on the storage conditions of artisanal WBMC during shelf life differ widely: some producers claim a shelf life of five days keeping the product at room temperature, others claim storage at refrigerator temperature for up to three weeks, and yet others a shelf life of five to ten days storing the product at room temperature for one to three days and in a refrigerator thereafter.

The purpose of this study was therefore to assess the behaviour of *L. monocytogenes* during production and shelf life of WBMC under four different storage conditions assuming raw milk as the source of contamination.

MATERIALS AND METHODS

All experiments were carried out for four batches: three inoculated batches and one non-inoculated batch for control.

Bacterial strains

The following five strains of *L. monocytogenes* were evaluated: *L. monocytogenes*: ATCC strain n. 19115 (Id. Riboprinter DUP1042, ECORI 189-11-S-1); field strain IZSLER n. 2007/34985/2 isolated from cheese (Id. Riboprinter DUP 1042 ECORI 189-11-S-1); field strain IZSLER n. 2007/32929/2 isolated from cheese (Id. Riboprinter DUP 1060, ECORI 189-15-S-4); field strain IZSLER n. 2008/323272/2 isolated from raw milk (DUP 1046, ECORI 189-554-S-3); field strain IZSLER n. 2008/148454/2 isolated from raw milk (DUP 1044, ECORI 189-939-S-1). Strains were grown separately on blood agar base with 5% defibrinated sheep blood (Oxoid, Basingstoke, United Kingdom) incubated at 37°C for 24 h; bacterial colonies were collected by washing with saline (NaCl 0.85%, VWR International, Milan, Italy) and 5 mL of suspensions obtained were used to inoculate 1 L of brain heart infusion broth (Oxoid, Basingstoke, United Kingdom). The broth inoculated was incubated under stirring conditions at 37°C for 24 h. Cells were collected by centrifugation (3,000 g for 1 h) and the pellet was resuspended in 100 mL of saline (NaCl 0.85% VWR International, Milan, Italy). Milk was contaminated by adding equal parts of each bacterial suspension to obtain a final density of about 10^6 CFU/mL of milk of each bacterial strain. The contamination was performed after heating the milk to 38°-40°C and before the natural whey starter and rennet addition.

Water buffalo mozzarella cheese production

To avoid unknown contamination during processing tools used for the experimental productions were sterilized by autoclaving; the surfaces

in contact with milk, curd or cheese were sanitized by 94° ethanol and by steaming; all workers wore sterile gloves during production. Mozzarella was produced according to the traditional technology using 50 litres of unpasteurized milk and natural whey culture as starter for each batch. Briefly the raw milk was heated to 38°-40°C; the natural whey starter and rennet were added and the curd was left to ripen at 35°-38°C for about 4 hours. The curd was extracted from the whey and stretched in hot water (85°-90°C) for about 2 min. The stretched curd was then molded in the traditional round shape. Each mozzarella weighed 250 g. The conditioning liquid was prepared with tap water, salt up to 2° Bé and lactic acid 80% to a final pH of 2.79 and a titratable acidity of 5.5° SH/50 mL. Single 250 g WBMC were packaged in trays completely covered by the conditioning liquid. The temperature of the curd during production and stretching was measured by a Hobo H08-002-02 data logger.

Storage test

For each batch 60 packaged WBMC were divided into four groups (15 WBMC for each group) for the storage tests at four different temperatures (5°, 10°, 15°, and 20°C) for 12 days. The storage conditions were chosen to simulate optimal storage conditions (5°C), domestic storage (10°C, BEAUFORT *et al.*, 2008) and thermal abuse (15° and 20°C).

L. monocytogenes count

Before inoculating *L. monocytogenes*, each batch of raw milk and natural whey starter was tested as described by ISO 11290-2:1998 in order to exclude the presence of unknown strains of *L. monocytogenes*.

L. monocytogenes count for each batch was performed in duplicate for inoculated raw milk, curd at the end of ripening, curd after stretching and WBMC after 60 minutes of packaging and during shelf life at 0, 1, 3, 5, 7 and 12 days. Curd and WBMC were homogenized by a stomacher, then serially diluted and plated on Agar Listeria Ottaviani Agosti (ALOA) (Biolife, Milan, Italy). Plates were incubated aerobically at 30°C for 48 h.

Determination of cell density of lactic acid bacteria, pH and a_w

The following samples were collected in duplicate from each batch: natural whey starter, raw milk after *L. monocytogenes* inoculation, milk after natural whey starter addition, curd at the end of ripening; curd after stretching, WBMC 60 min after packaging in the conditioning liquid and packed WBMC at 0, 1, 3, 5, 7 and 12 days at each storage temperature (5°, 10°, 15°, and 20°C). The following analyses were made on each sample: count of mesophilic and thermophilic lactococci by decimal dilution and inclusion in M17 agar plates (Oxoid, Basingstoke, United Kingdom) incubated aerobically at 30° and 42°C respectively for 48 hours; count of mesophilic and thermophilic lactobacilli by decimal dilution and inclusion in MRS agar plates (Oxoid, Basingstoke, United Kingdom) incubated under microaerophilic conditions at 30° and 42°C respectively for 48 hours; pH was measured by an instrument with automatic temperature compensation (Hanna Instruments HI 223); a_w was determined by AquaLab model series 3 (Decagon Devices Inc. Pullmann, Usa).

Data, including calculation of *L. monocytogenes* generationtime were calculated using programs available on www.combase.cc. Statistical analysis was performed by T-test using SPSS software 12.0.

RESULTS

L. monocytogenes behaviour

L. monocytogenes was not detected in non-inoculated raw milk or natural whey starter.

L. monocytogenes count showed a moderate but significant increase ($p < 0.01$) during curd ripening (from 7.13 to 7.39 Log CFU/g in about 4 h) (Table 1); the combase theoretical predicted growth rate was 0.45 Log/CFU/h at pH 5 and 0.79 log CFU/g/h at pH 6. A decrease (about 1 Log CFU/g) of *L. monocytogenes* count was observed at the end of curd stretching. Sixty min after packaging in the conditioning liquid a further, but not significant 1 Log CFU/g decrease of *L. monocytogenes* count was observed.

During storage test, after a moderate increase for one day at all storage temperatures considered, *L. monocytogenes* count at 5°C storage decreased by about 0.5 Log CFU/g; increasing the storage temperature enhanced the decrease of *L. monocytogenes* count (up to 1.58 Log CFU/g at 20°C storage) (Table 2). By contrast, Combase prediction reported a theoretical growth of *L. monocytogenes* at all storage temperatures ranging from a generation time of 3 days and 20 h at 5°C storage to 9 h at 20°C storage.

pH, a_w and temperature profile

From inoculation of raw milk to the end of ripening pH decreased from 6.89 to 5.18; a further decrease to 5.08 was observed in WBMC at 60 min after packaging in the conditioning liquid (Table 1). During the first days of storage test pH dropped to about 5.0, 4.8, 4.7 and 4.4 during storage at 5°, 10°, 15°, and 20°C respectively and then remained unchanged till the end of the storage test (Table 2). a_w values remained substantially unchanged during storage test at all temperatures ranging from an initial value of 0.979 to a final value of 0.974. No significant differences were observed in the pH and a_w values of the inoculated and non-inoculated batches (data not shown).

Table 1 - Evolution of pH and of *Listeria monocytogenes* and lactic acid bacteria populations count (Log CFU/g) during water buffalo mozzarella cheese traditional production process (Mean of 3 batches ± standard deviation).

Sample	*L. monocytogenes*	pH	Mesophilic lactobacilli	Thermophilic lactobacilli	Mesophilic lactococci	Thermophilic lactococci
Raw milk	n.d.[1]	6.89±0.00	n.a.[2]	n.a.	n.a.	n.a.
Natural whey starter	n.d.	4.00±0.17	8.21±0.59	8.52±0.25	9.16±0.06	9.18±0.08
Inoculated raw milk	7.13±0.06[a]	6.89±0.00	4.25±0.54	3.11±0.45	4.86±0.38	4.32±0.57
Raw milk after natural whey starter addition	n.a.	6.40±0.01	5.98±0.46 [a]	6.98±0.06 [a]	7.76±0.08 [a]	7.73±0.05 [a]
Curd at the end of ripening	7.39±0.04[b]	5.18±0.36	7.91±0.62 [b]	8.38±0.36 [b]	8.64±0.34 [b]	8.67±0.29 [b]
Curd after stretching	6.21±0.27 [c]	5.21±0.14	5.92±0.65 [c]	5.26±0.23 [c]	4.11±0.53 [c]	4.33±0.16 [c]
WBMC 60 min after packing in conditioning liquid	5.14±1.45 [c]	5.08±0.09	5.56±1.18 [c]	1.55±0.29 [d]	3.33±1.46 [d]	2.85±1.06 [d]

[1]: not detected;
[2]: not analysed.
Different letters in a column show significant differences ($p < 0.01$).

Table 2 - Evolution of pH and of *Listeria monocytogenes* and mesophilic lactobacilli count (Log CFU/g) during water buffalo mozzarella cheese storage test at 5°, 10°, 15°, and 20°C (Mean of 3 batches ± standard deviation).

	Storage temperature 5°C			Storage temperature 10°C		
Days after packaging	*L. monocytogenes*[a]	pH	Mesophilic lactobacilli[b]	*L. monocytogenes*[c]	pH	Mesophilic lactobacilli[c]
0	5.14±1.45	5.08±0.14	5.56±1.18	5.14±1.45	5.08±0.14	5.56±1.18
1	5.51±0.41	5.14±0.08	6.95±0.34	5.53±0.76	5.07±0.11	6.89±0.40
3	5.04±0.42	5.09±0.11	7.40±0.28	5.22±1.36	4.88±0.15	6.76±1.05
5	5.02±0.92	4.99±0.11	6.63±0.37	5.19±1.06	4.83±0.16	7.14±0.13
7	5.01±1.49	4.97±0.13	6.60±1.25	4.33±0.93	4.83±0.15	7.73±0.11
12	4.97±1.46	4.83±0.12	6.56±0.60	3.46±1.61	4.82±0.15	7.66±0.13
	Storage temperature 15°C			**Storage temperature 20°C**		
Days after packaging	*L. monocytogenes*[c]	pH	Mesophilic lactobacilli[c]	*L. monocytogenes*[c]	pH	Mesophilic lactobacilli[c]
0	5.14±1.45	5.08±0.14	5.56±1.18	5.14±1.45	5.08±0.14	5.56±1.18
1	5.29±1.13	4.93±0.11	6.87±0.96	5.95±0.47	4.81±0.09	7.41±0.31
3	5.19±1.00	4.72±0.09	6.86±0.66	5.22±0.95	4.42±0.11	7.53±0.40
5	4.34±0.89	4.71±0.13	7.76±0.24	3.48±1.42	4.41±0.17	7.91±0.47
7	5.26±0.26	4.70±0.11	7.67±0.38	3.23±3.21	4.40±0.18	8.06±0.22
12	3.33±2.14	4.71±0.12	7.87±0.23	3.56±1.89	4.41±0.17	8.18±0.09

[a]: a non significant reduction in *L. monocytogenes* count was shown by T test ($p > 0.01$);
[b]: a non significant increase in mesophilic lactobacilli count was shown by T test ($p > 0.01$);
[c]: a significant increase was shown by T test ($p < 0.01$).

The temperature reached by the curd during stretching of inoculated curds is reported in Fig. 1. The maximum temperature reached during curd stretching was 71.6°C±1.6. Curd temperature during stretching remained over 65°C for 3 min in all four batches (data not shown).

Evolution of lactic acid population

During curd ripening an increase of 4-5 Log CFU/g was observed in all lactic acid bacterial populations. The heat treatment of stretching reduced the counts of the different lactic acid bacteria populations by about 1.99 to 4.5 Log CFU/g (Table 1). During the storage test thermophilic lactobacilli and thermophilic lactococci counts remained substantially unchanged at regardless of the temperature (data not shown). Mesophilic lactococci count decreased by 0.96 Log CFU/g when WBMC was stored at 5°C but was unaffected by higher storage temperatures (data not shown). Mesophilic lactobacilli count showed a not significant increase ($p > 0.01$) during storage at 5°C, but a significant increase ($p < 0.01$) was observed at 10°, 15°, and 20°C (Table 2). No significant differences were observed in the evolution of lactic acid bacteria populations of the inoculated and non-inoculated batches (data not shown).

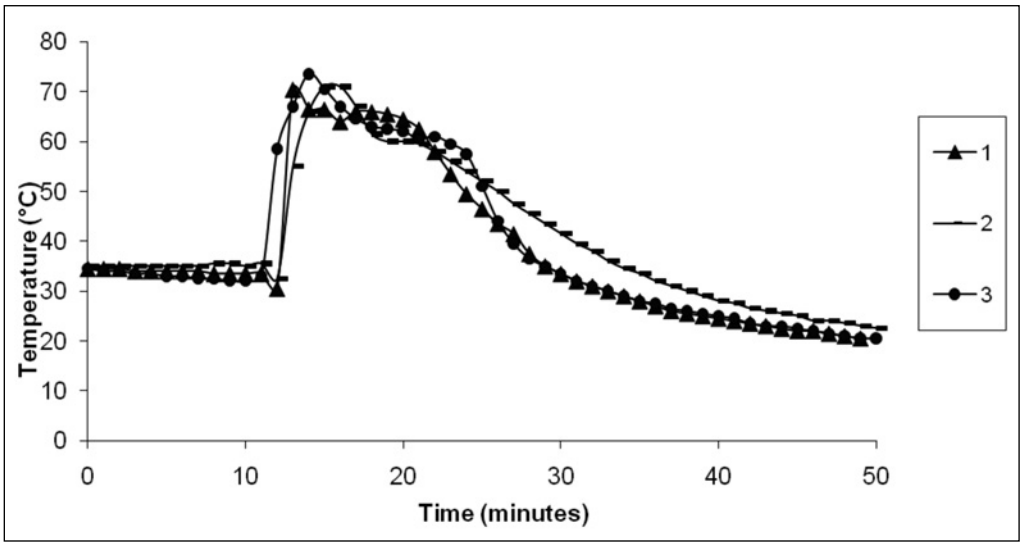


Fig. 1 - Evolution of the temperature during stretching of experimentally contaminated water buffalo mozzarella cheese (3 batches).

DISCUSSION

The results of this study show that if contamination levels are high, stretching cannot ensure the complete destruction of *L. monocytogenes* in traditional WBMC. Many authors have argued that although stretching is capable of destroying pathogenic bacteria, the variability of factors such as temperature, time and initial level of milk contamination do not allow to consider the stretching process as a substitute of the pasteurization (ADDEO and COPPOLA, 1983; KIM *et al.*, 1998; MURRU *et al.*, 1999a). Stretching, however, is a very important stress factor for *L. monocytogenes*: a study conducted by BUAZZI *et al.* (1992) proved that the stretching phase in mozzarella cheese is able to eliminate *L. monocytogenes* contamination if the curd retains a temperature of 77°C for three to four minutes. MURRU *et al.* (1999a) claim that a stretching phase run at a temperature between 65°C and 69°C for five minutes cannot eliminate *L. monocytogenes* contamination in WBMC if the initial contamination level of the pathogen is higher than 4 Log CFU/g. The contamination of raw milk, if present, is assumed to be low because the few available studies on water buffalo raw milk contamination reported the absence of *L. monocytogenes* (HAN *et al.* 2007; MURRU *et al.* 2009b); nevertheless it must be evaluated that *Listeria* spp., including outbreak strains of *L. monocytogenes*, are regularly isolated from dairy processing and cheese-making environments (D'AMICO and DONNELLY, 2009) and the ability of *L. monocytogenes* to adhere to many materials found in food-processing environments (BERESFORD *et al.* 2001) may represent a significant source of contamination increasing the natural contamination of milk or of the curd in non-experimental conditions. In our study the temperature of the curd reached a maximum of 71.6°C and the temperature remained above 65°C for three minutes. The decrease of 2 Log CFU/g of *L. monocytogenes* (1 Log CFU/mL during stretching and 1 Log in the following 60 min) is in agreement with the data reported by VILLANI *et al.* (1998). Although according to Regulation (EC) 2073/2005, WBMC could be considered a ready to eat food able to support the growth of *L. monocytogenes* (pH < 4.4 or a_w < 0.92 or pH <5.0 and a_w < 0.94), under our experimental conditions *Listeria monocytogenes* was not able to grow during storage of WBMC, even in the case of severe thermal abuse. *L. monocytogenes* is known to grow over a wide range of temperatures (-1.5° to 45°C) and pH (4.3 to 9.4) (TE GIEFFEL and ZWIETERING, 1999). MILLET *et al.* (2006) reported a pH growth/no growth limit in Saint-Nectaire-type cheese of 5.2. Other authors reported a pH growth limit of 4.6 in cottage cheese and in feta cheese (PEARSON and MARTH, 1990). The ability to grow at different temperatures was strictly correlated with the pH of the cheeses. A decrease of pH value coincides with an inhibition of *L. monocytogenes* growth as reported by ROGGA *et al.* (2005) and UHLICH *et al.* (2006) in Queso Blanco, whereas an increase in pH leads to an increase of *L. monocytogenes* cell density (BACK *et al.*, 1993; MENDOZA-YEPES *et al.*, 1999; GENIGEORGIS *et al.*, 1991); RAMSARAN *et al.* (1998) observed an increased number of *L. monocytogenes* during initial ripening of feta cheese followed by a growth arrest after 24 h when the pH value dropped down.

During WBMC storage we observed a growth of mesophylic lactobacilli accompanied by a drop in pH value more enhanced at higher storage temperatures. The growth/no growth interfaces of *Listeria* reported by LE MARC *et al.* (2002) are

5.50, 4.6-4.7, 4.50 and 4.4-4.5 respectively at 5°, 10°, 15°, and 20°C. In our study these no growth pH limits were reached after 3 days of storage at 20°C, whereas they were never reached at 10° and 15°C. Therefore an increase in *L. monocytogenes* count rather than a decrease should be observed, as calculated by Combase prediction. SCHVARTZMAN *et al.* (2010) demonstrated that the growth limits of *L. monocytogenes* are matrix-dependent and that solid foods may limit the diffusion rate of organisms throughout space in food and this can be an additional factor limiting the growth of *L. monocytogenes* in WBMC. The literature reports additional factors influencing the growth of *L. monocytogenes* in milk and dairy products. The inhibitory effect of natural microbiota, namely lactic acid bacteria, lead to a longer lag phase and higher generation time of *L. monocytogenes* in pasteurized milk with respect to UHT milk or autoclaved milk (ROSENOW and MARTH, 1987; WALKER *et al.*, 1990; BOVILL *et al.*, 2000). The addition of organic acids or starter cultures in cheeses increased the minimal growth temperature of *L. monocytogenes* or negatively affected its survival (EL-SHENAWY and MARTH, 1990; GLASS *et al.*, 1995; MENDOZA-YEPES *et al.*, 1999). Additional hurdles, due to the effect of lactic acid added to the conditioning liquid and to the growth of lactic acid bacteria (competition for carbohydrates, acids and bacteriocins production) may be hypothesized, but are difficult to evaluate in a product made with raw milk and natural whey cultures. Lactic acid bacteria are known to inhibit different pathogens (NUÑEZ *et al.*, 1997; RODRIGUEZ *et al.*, 2005) and a study conducted by GAY and AMGAR (2005) found that lactic acid bacteria have a twofold higher inhibitory activity towards pathogenic microorganisms in products derived from raw milk (like artisan WBMC) than in products derived from pasteurized milk. However, because the inhibitory activity of lactic acid bacteria in dairy products depends on the biotype and cell density of lactic acid bacteria, this feature should be further investigated in WBMC.

CONCLUSIONS

In traditional WBMC production technology *L. monocytogenes* can multiply during curd ripening, but its growth rate is lower than the growth rate reported by the theoretical predictions. Therefore, it is expected that, if the contamination level of milk is low, during curd ripening *L. monocytogenes* will not reach values of cell density so high to represent a risk for consumers' health. Under our experimental conditions, curd stretching proved to be a process with good repeatability and able to decrease *L. monocytogenes* cell density of curd by about 2 Log CFU/g. Because the intrinsic characteristics of traditional WBMC were able to limit the growth of *L. monocytogenes* during storage (even in the case of severe thermal abuse), it may be issued that the key factor for reducing the risk for consumers' health is the implementation of appropriate hygiene measures to minimize the contamination of raw milk and post processing contamination.

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PAPER

EVALUATION OF A SENSORY SCORECARD FOR GRATED PARMIGIANO-REGGIANO CHEESE

M. ZANNONI[1]* and E.A. HUNTER[2]
[1]Organismo Controllo Qualità Produzioni Regolamentate,
Via Ferruccio Ferrari 6, 42122 Reggio Emilia, Italy
[2]Independent Chartered Statistician and Chartered Scientist, 23 St. Ninians Terrace, Edinburgh, EH10 5PW, United Kingdom
*Corresponding author: mariozan@ocqpr.it

ABSTRACT

The Parmigiano-Reggiano cheese is sold more and more as grated cheese and in 2002 the official certification body of Parmigiano-Reggiano added the sensory analysis to its array of analytical tools. The scorecard is quantitative-descriptive with 21 descriptors. The scorecard has also 4 qualitative evaluation (appearance, smell, texture, and taste) for the compliance with the regulations. The scorecard was evaluated tasting twice 24 samples of grated Parmigiano-Reggiano. Analysis of variance showed that all the descriptors discriminate well among the samples. The results measured with GPA showed a good agreement between assessors for every modality and only some problems for the "odour".

- Keywords: Cheese, Parmigiano-Reggiano, quality control, sensory analysis -

INTRODUCTION

The Parmigiano-Reggiano (original Parmesan) cheese traditionally was sold in wheels by the producer to the retailer which were then cut at the counter. In the '70s pre - (vacuum) packed slices became popular with consumers and in the '90s the grated form packed in modified atmosphere.

Currently grated Parmigiano-Reggiano is the market segment growing fastest, in fact the quantity has risen from 4.323 tonnes in 2002 to 10.996 tonnes in 2010, an increase of 154% in 8 years. Commercial firms are increasingly interested in this product which has a high service element. The interest in the grated form of the cheese by the consumer can stimulate production of counterfeit Parmigiano-Reggiano cheese or the production of a poor quality product by less scrupulous processors.

Some research about grated Parmigiano-Reggiano cheese was carried out for chemical (free aminoacids) (RESMINI and PELLEGRINO, 1986) (PELLEGRINO and HOGENBOOM, 2007) and microbiological (BOTTAZZI *et al.*, 2001) characteristics.

Sensory features of grated Parmigiano-Reggiano cheese were nevertheless dealt only with a paper introducing the method for sensory quality control of this kind of cheese (ZANNONI, 2006). Bibliographic research shows that papers about grated cheese are rare, also sensory analysis is used together other analytical methods as a tool to evaluate packaging conditions (TROBETAS *et al.*, 2008; MEXIS *et al.*, 2011).

Grated Parmigiano-Reggiano is under the control of Protected Designation of Origin (PDO) regulations (OFFICIAL JOURNAL OF THE EUROPEAN UNION, 2006). The official regulation for this PDO cheese provides regulations for milk production, cheese making and features of the cheese at point of sale.

The control body of the PDO Parmigiano-Reggiano the "Organismo Controllo Qualità Produzioni Regolamentate" (OCQ PR) is in charge of implementing the official PDO regulations and this is done by control of documentation, inspections of the production sites and testing of the cheese prior to marketing.

According to the regulations, the grated cheese shall have the typical features of Parmigiano-Reggiano. Clearly the grating process changes the sensory characteristics of the cheese, for instance the odours are more rapidly lost due to the large surface area in contact with air. The texture is different and the solubility is increased due to the very small particles which "melt" in the mouth.

Sensory analysis of Parmigiano-Reggiano in blocks (i.e. not grated) is today carried out using a specific scorecard developed in 90s (LAVANCHY *et al.*, 1994; BERODIER *et al.*, 1997; NIELSEN and ZANNONI, 1998; ZANNONI *et al.*, 2006; GARAVALDI *et al.*, 2010).

In 1993, the Consorzio Parmigiano-Reggiano defined another sensory scorecard specifically for evaluating the grated form of the cheese (ZANNONI, 2006). In 2002 the OCQ PR adapted the scorecard for regulatory purposes by adding compliance with the standards. The quantitative descriptive scorecard now has 21 descriptors but also 4 evaluations of compliance (appearance, smell, texture and taste) with a 7 points scale. These latter are useful for determining the quality of the product and the minimum level acceptable for the PDO. This addition was introduced because of the requirements of the processors to directly assess the quality of their product rather than indirectly with a spider plot using an array of descriptors whose interpretation is not clear to them. The boundaries between the product acceptable for PDO and those not acceptable were set using the experience of the Consorzio, the organization of producers, and that of the OCQ PR, the control body, both having many years of experience with the product including the grated form.

Grated Parmigiano-Reggiano cheese has been found to be a delicate product subject to rapid change and thus the grating and packaging processes have to be carried out with great care. The quality of the raw material also has a major influence on the quality of the final product. The aim of this paper is to first test the validity of the scorecard for the grated form of Parmigiano-Reggiano whilst also evaluating the performance of the sensory panel.

MATERIAL AND METHODS

A) Cheese samples

According to the regulations only certified PDO Parmigiano-Reggiano can be used for grating purposes, so the minimum age of the product is 12 months but a maximum age is not defined. The grated cheese can be produced only in plants authorized by the control body, the OCQ PR. The processor's grating plant requires to be located in the production area of Parmigiano-Reggiano and is required to follow the production regulations. The processing plants use normal industrial graters and modified atmosphere packaging equipment. The most common size of pack is 100 g of grated cheese in plastic bags. There are 29 such plants active in the production area. For this research 24 samples of 400 g of freshly grated Parmigiano-Reggiano were collected from 24 different plants.

B) Sample preparation and testing

Each of the 24 products was divided into two parts for sensory analysis, products were coded A-X. Thus there were 48 samples to be evaluat-

ed each labelled with a unique accession number. Four of these samples were evaluated in each of 12 sessions by the assessors, samples were randomly split. Unknown to the assessors two of the samples in a session were from the same product thus testing the assessors ability to rate the cheeses consistently. A set of three mutually orthogonal latin squares of 4 rows and 4 columns were used to order the samples so that each assessor rated the samples in a different order. The samples were refrigerated to 2°-8°C and stored for about 25 days before sensory analysis, the expiry date of the products being between 2 and 4 months hence. The evening before tasting, the samples for tasting that day were transferred to a cabinet at 13°C to allow them to slowly reach the temperature of tasting. Half an hour before the test, the samples were put at room temperature in order to reach the tasting temperature of about 16°C. The tasting room was prepared according to the norm ISO/8598, in the booths the neutralizing agent was low salt mineral water and un-salted crackers. When the bags were opened for the tasting session 200 g of sample was put on a plastic dish and mixed, then quantities of 20 g were put in plastic Petri dishes and submitted to the tasters.

C) Scorecard and references

The modalities of evaluation were appearance, odour, texture and taste (as basic taste plus aroma by way of back of the nose). The descriptors were chosen using previous work (BÉRODIER *et al.*, 1997; LAVANCHY *et al.*, 1994) (ZANNONI and PIGONI, 2006). The grated form of the cheese suggested the introduction of three new descriptors for appearance viz. particle size, large grains and long threads. For texture the descriptors rind particles and sandy were introduced.

The 20 descriptor scorecard for Parmigiano-Reggiano (Table 1) was tested extensively with samples of PDO and non PDO grated cheese and this experience confirmed it's suitability. Four descriptors for compliance with the regulations were then added at the request of the control body of Parmigiano-Reggiano who needed to have a score to define the compliance of the sample with the PDO sensory description. For some descriptors the 1-7 scale, used also for the scorecards of Parmigiano-Reggiano in blocks, was retained but for descriptors connected to defects a 1-4 scale was better accepted by the panellist.

The training of the panel was done with references taken by the two "guides" (BÉRODIER *et al.*, 1997; LAVANCHY *et al.*, 1994). For the appearance, photographs were used as references. Digital photographs proved to be an effective way to show colour tonalities and particle sizes. To assess the presence of rind, panellist were trained to taste a mixture of grated rind coming from 2 different 24-months Parmigiano-Reggiano wheels. To set the standard for the panel for the 4 evaluations of compliance (appearance, odour, texture, taste) a mixture of two 24 months good quality Parmigiano-Reggiano grated cheese was used.

Two sessions of panel training were organized before starting to assess the 24 products. In each of these a sample of grated Parmigiano-Reggiano, another of Parmigiano-Reggiano plus about 10% rind scraps and another of imitation Parmigiano-Reggiano were assessed. These sessions were held to familiarize with the product of good quality i.e. D.O. and products of inferior quality.

D) Sensory panel

The 9 panellists were aged between 34 and 69 years with an experience of sensory analysis of cheese from 5 to 18 years. According to the internal procedures of the OCQ PR the panel was periodically assessed with reference substances for cheese; 7 tests a year were done covering appearance, odour/aroma, taste and texture.

E) Statistical data analysis

The data were analysed one variable at a time (univariate analysis) and also by groups of variables (multivariate analysis) e.g. Compliance. These are different ways of looking at the data giving different information about the samples and about the performance of the panel.

For Colour, Appearance, Odour, Texture, Aroma/Taste essentially there is an intensity measurement followed by measurements of defects. The data are in the form of a small number of points on a scale which is then converted to the numbers 1, 2, 3..... (half points being allowed in intensity attributes).

In order to make the most of the complicated structure of the data, and bearing in mind that this is a research project, a linear mixed model was fitted to each variable in turn using the technique of Residual Maximum Likelihood (REML) to estimate the parameters of the model. The main purpose of REML is to estimate sample means (i.e. sample x replicate means) free of Assessor and Order (of presentation) effects. This technique is an advance on analysis of variance and regression and yet also provides tests of significance and estimates of (corrected) means. It thus allows more complex models to be fitted to large data sets. The technique is implemented in Genstat (VSN International), and in other statistical packages (GALWEY, 2006).

For multivariate analysis Generalised Procrustes Analysis (GPA) was used (DIJKSTERHUIS and GOWER, 1991-2; GOWER and DIJKSTERHUIS, 2004).

Using the GPA scores for the samples an analysis of variance gives the 95% confidence estimate for the products means. The usual way

Table 1a* - Scorecard for grated Parmigiano-Reggiano cheese. Visual assessment.

1) VISUAL ASSESSMENT	**Score Compliance ..... (1-7 scale)**			
A) Colour (yellow):	*Pale*	*Medium*	***Dark***	
➢ Intensity	●.....●.....●.....●.....●.....●.....●			
➢ Notes:	1 2 3 4 5 6 7			
----------	*Not perc.*	*Low*	***Medium***	***High***
Brown	○	○	○	○
Lemon Yellow	○	○	○	○
Other colours:	○	○	○	○
B) Appearance:	*Fine*	*Medium*	***Large***	
➢ Particles size	●.....●.....●.....●.....●.....●.....●			
➢Notes:	1 2 3 4 5 6 7			
----------	*Not perc.*	*Low*	***Medium***	***High***
Large Grains	○	○	○	○
Long Threads	○	○	○	○

*Note: in bold underscored the intensities considered as defects.

Table 1b* - Scorecard for grated Parmigiano-Reggiano cheese. Tasting assessment.

2) TASTING ASSESSMENT- Odour:	**Score Compliance ..... (1-7 scale)**			
	Low	*Medium*	*High*	
➢ Intensity	●.....●.....●.....●.....●.....●.....●			
➢ Notes:	1 2 3 4 5 6 7			
	Not perc.	*Low*	***Medium***	***High***
Rancid	○	○	○	○
Cheese Rind	○	○	○	○
Sour	○	○	○	○
3) Texture	**Score Compliance ..... (1-7 scale)**			
	Low	*Medium*	*High*	
➢ Solubility	●.....●.....●.....●.....●.....●.....●			
➢ Notes:	1 2 3 4 5 6 7			
	Not perc.	*Low*	***Medium***	***High***
Dryness	○	○	○	○
Rind Particles	○	○	○	○
Sandy	○	○	○	○
4) Aroma (retronasal) and Taste:	**Score Compliance ..... (1-7 scale)**			
	Low	*Medium*	*High*	
➢ Intensity	●.....●.....●.....●.....●.....●.....●			
➢ Notes:	1 2 3 4 5 6 7			
	Not perc.	*Low*	***Medium***	***High***
Salty	○	○	○	○
Pungent	○	○	○	○
Sour	○	○	○	○
Rancid	○	○	○	○
Cheese Rind	○	○	○	○

*Note: in bold underscored the intensities considered as defects.

of displaying the results for each pair of dimensions, is to plot a map of the Products. Adding a 95% confidence ellipse for the products to the bottom right corner of these diagrams (Fig. 1) is a way of dealing with the problem of the variability being very different for each dimension of the diagram, and thus it facilitates interpretation of the results in the way that confidence intervals for means helps the interpretation of tables.

In order to study differences between Assessors the entirely different multivariate technique of Principal Co-ordinate Analysis (PCO) was used. A matrix of dis-similarities between each pair of Assessors was formed (rather like a road atlas which gives a table of the distances between all pairs of cities). PCO allows the table to be used to plot (map) co-ordinates of each assessor, thus presenting all assessors in a single map. On the assumption that the co-ordinates are normally distributed possibly with outliers, using a robust estimates of variance, a 95% confidence ellipse was super imposed on the map of assessors. It then becomes clear which assessors (if any) are alike and which have a different view of the samples.

RESULTS AND DISCUSSION

Results

UNIVARIATE

A mixed model with fixed effects for Assessor, Order (of presentation), Sample and Session and with random effects for Assessor by Session (a measure of the extent to which assessors vary from session to session) and for Sample (the variation between replicate Samples) was fitted to each variate in turn.

The discriminating abilities of each descriptor are given in Table 2a, Statistical tests of Sample (Product) effects. All attributes differentiate between the products except *Other colours*. Overall, Appearance and Texture differences were perceived to be larger than Odour and Aroma/Taste. In Appearance differences between products in *Colour intensity* was perceived to be large but differences in *Brown, Lemon yellow* and *Other colours* are much smaller though the first two are significant at $p<0.001$. For modality Compliance the differences between Products for *Compliance texture* ($F=30.45$) and for *Compliance visual* ($F=20.96$) are greater than *Compliance aroma/ taste* ($F=12.29$) which is greater than for *Compliance odour* ($F=6.40$).

The full results of F-tests of the fixed effects and t-tests of the random effects are given in Table 2b.

Assessor effects, the tendency of assessors to consistently score high or low for a variate are often statistically important. The difference between the maximum and minimum mean can be as great as one scale point but is generally much lower. Only for Compliance variables are there relatively small consistent Order of testing effects. This emphasises the importance of building in procedures to control order of tasting in the sensory laboratory.

The effects of Session are partly confounded (5 df) with product but 6 df remain to test this effect. The results can be interpreted as meaning the panel do not drift from session to session. Alongside the other results for Assessors and Session this is evidence that the assessors fix a personal scale and adhere to it.

The Assessor by Session effect, the amount by which Assessors vary from session to session is not significant.

The absence of a Sample effect within products is evidence that the products were homogeneous.

The relatively high variation of F values related to product effect across some variables can be explained considering that there are variables influenced by process choices (i.e. P*articles size-visual, Large grains*, *Long threads*, *Rind odour*, *Rind particles*) or quality problems of raw matter (i.e. *Rancid*).

Table 2a - Statistical Tests of Sample (Product) Effects. Discrimination level of descriptors.

Modality	Attribute	Sample (Product) (23 df)	
		F	prob
Colour	Intensity	17.77	<0.001
	Brown	3.9	<0.001
	Lemon yellow	2.38	<0.001
	Other colours	1.84	ns
Appearance	Particles size	15.03	<0.001
	Large grains	29.01	<0.001
	Long threads	23.46	<0.001
Odour	Intensity	3.64	<0.001
	Rancid	5.59	<0.001
	Rind	9.04	<0.001
	Sour	2.32	<0.001
Texture	Solubility	19.87	<0.001
	Dryness	14.07	<0.001
	Rind particles	16.68	<0.001
	Sandy	10.93	<0.001
Aroma/Taste	Intensity	6.80	<0.001
	Salty	3.17	<0.001
	Pungent	3.95	<0.001
	Sour	3.07	0.010
	Rancid	8.21	<0.001
	Rind	13.84	<0.001
Compliance	Visual	20.96	<0.001
	Odour	6.40	<0.001
	Texture	30.45	<0.001
	Aroma/ taste	12.29	<0.001

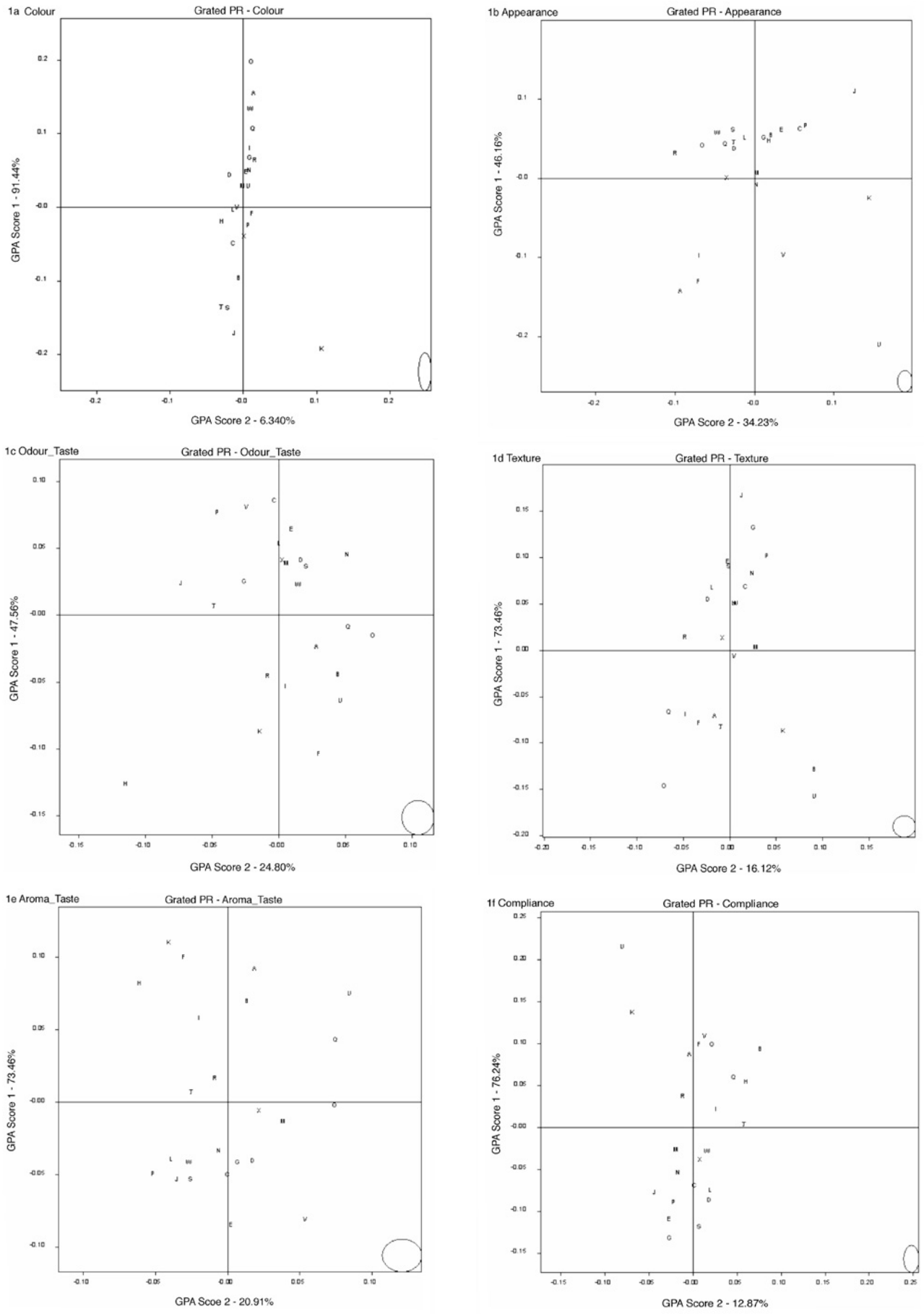


Fig. 1 a,b,c,d,e,f - GPA scores, sample plots for: a) Colour, b) Appearance, c) Odour, d) Texture, e) Aroma/taste, f) Compliance. Letters are the samples and standard error of each cheese is given by the ellipse.

Table 2b - Results from univariate (REML) analysis of sensory data.

a. Colour

		Fixed effects								Random effects			
		Assessor (8 df)		Order (3 df)		Sample (Product) (23 df)		Session (6 df)		Assessor. Session		Sample (Replicate)	
Variable	Mean	F	prob	F	prob	F	prob	F	prob	t	prob	t	prob
Intensity	3.19	25.91	<0.001	1.57	ns	17.77	<0.001	2.85	0.030	2.270	0.05	0.540	ns
Brown	0.07	1.93	0.067	1.88	ns	3.90	<0.001	0.33	ns	1.160	ns	0.000	ns
Lemon yellow	0.09	4.38	<0.001	2.67	0.048	2.38	<0.001	2.01	0.066	1.370	ns	0.000	ns
Other colours	0.08	3.22	0.003	0.76	ns	1.84	ns	0.18	ns	0.080	ns	0.000	ns

b. Appearance

		Fixed effects								Random effects			
		Assessor (8 df)		Order (3 df)		Sample (Product) (23 df)		Session (6 df)		Assessor. Session		Sample (Replicate)	
Variable	Mean	F	prob	F	prob	F	prob	F	prob	t	prob	t	prob
Particles size	3.29	26.01	<0.001	2.47	0.062	15.03	<0.001	0.65	ns	3.56	<0.001	1.46	ns
Large grains	0.31	1.54	ns	0.08	ns	29.01	<0.001	1.05	ns	1.40	ns	0	ns
Long threads	0.49	2.68	0.012	2.09	ns	23.46	<0.001	0.60	ns	2.12	ns	0.86	ns

MULTIVARIATE

The data were divided into the modalities identified in Table 1. A GPA analysis was carried out for each modality. The results are summarised in tabular form in Table 3 Generalised Procrustes Analysis of data (Table 3a: percentage of variation for each modality accounted for by dimensions of the GPA consensus, Table 3b: scaling factor for each assessor for each modality, Table 3c: correlation of each assessors data with GPA consensus) The information from these table, divided by every modality, is summarized as follows:

Colour

From Table 3a, it is clear that Colour is essentially one dimensional, this dimension is mostly defined by *Intensity*.

Appearance

In contrast for Appearance three dimensions are needed to characterise the products (Table 3a) and so all three descriptors are required, thus showing a more complex modality.

Odour

The fact that three (4 descriptors) sensory dimensions are required to characterise 88.8% of the variation between products suggests that the Assessors have found difficulty with this modality or alternatively that it is intrinsically complex. The correlations of Table 3c (0.46-0.76) show that the consensus was only loosely correlated with the individual Assessors data so there may be a case for more training and/or more (or different) sensory descriptors.

Texture

Differences between products can be characterised by two Sensory Dimensions. *Solubility* and *Rind particles* effectively define these dimensions; *Dry* and *Sandy* appear to be closely related.

Aroma-taste

The fact that three (6 descriptors) sensory dimensions are required to characterise 77.6% of the differences between products points to the complexity of this modality. Although the correlations for each individual Assessors data with the consensus are low they are marginally higher (0.52-0.72) than for Odour.

Compliance

Three dimensions (4 descriptors) are required to characterise 98.0% of the variation in the compliance data. With the exception of Assessor 2 (0.53), Assessors largely agreed with the consensus (0.71-0.84). All 4 descriptors appeared to play a part in defining the sensory dimensions.

The (linear) correlations of descriptors scores with compliance calculated by REML data showed (Table 4) that some descriptors are significantly correlated with compliance. The only positive significant correlation is that of *Solubility* and negative correlations were found, obviously, in descriptors (with the 4 points scale)

Table 2b - Continues.

c. Odour

		Fixed effects								Random effects			
		Assessor (8 df)		Order (3 df)		Sample (Product) (23 df)		Session (6 df)		Assessor. Session		Sample (Replicate)	
Variable	Mean	F	prob	F	prob	F	prob	F	prob	t	prob	t	prob
Intensity	3.56	9.20	<0.001	2.82	0.039	3.64	<0.001	1.13	ns	2.48	0.05	0.00	ns
Rancid	0.53	4.92	<0.001	0.76	ns	5.59	<0.001	0.55	ns	2.55	0.05	0.00	ns
Rind	0.76	10.91	<0.001	0.41	ns	9.04	<0.001	0.81	ns	1.68	ns	0.00	ns
Sour	0.23	0.60	ns	2.23	ns	2.32	<0.001	0.62	ns	2.23	0.05	0.00	ns

d. Texture

		Fixed effects								Random effects			
		Assessor (8 df)		Order (3 df)		Sample (Product) (23 df)		Session (6 df)		Assessor. Session		Sample (Replicate)	
Variable	Mean	F	prob	F	prob	F	prob	F	prob	t	prob	t	prob
Solubility	3.90	8.02	<0.001	0.70	ns	19.87	<0.001	0.49	ns	2.79	0.050	0.00	ns
Dryness	0.83	12.59	<0.001	1.75	ns	14.07	<0.001	0.63	ns	1.38	ns	0.00	ns
Rind particles	0.62	4.85	<0.001	0.26	ns	16.68	<0.001	0.77	ns	2.25	0.050	0.00	ns
Sandy	0.63	7.21	<0.001	0.38	ns	10.93	<0.001	0.33	ns	3.39	0.050	0.00	ns

e. Aroma/Taste

		Fixed effects								Random effects			
		Assessor (8 df)		Order (3 df)		Sample (Product) (23 df)		Session (6 df)		Assessor. Session		Sample (Replicate)	
Variable	Mean	F	prob	F	prob	F	prob	F	prob	t	prob	t	prob
Intensity	4.31	6.75	<0.001	0.62	ns	6.80	<0.001	0.88	ns	3.07	0.001	0.00	ns
Salty	0.60	2.54	0.016	0.37	ns	3.17	<0.001	1.25	ns	3.31	0.001	0.00	ns
Pungent	0.77	9.71	<0.001	4.56	0.00	3.95	<0.001	2.14	0.051	2.41	0.050	0.00	ns
Sour	0.70	16.70	<0.001	1.93	ns	3.07	0.010	0.70	ns	0.69	0.000	0.21	ns
Rancid	0.89	8.58	<0.001	0.55	ns	8.21	<0.001	0.27	ns	3.21	0.001	0.00	ns
Rind	1.11	1.95	0.059	0.74	ns	13.84	<0.001	0.51	ns	2.73	0.050	0.05	ns

f. Compliance

		Fixed effects								Random effects			
		Assessor (8 df)		Order (3 df)		Sample (Product) (23 df)		Session (6 df)		Assessor. Session		Sample (Replicate)	
Variable	Mean	F	prob	F	prob	F	prob	F	prob	t	prob	t	prob
Visual	4.96	7.79	<0.001	6.66	<0.001	20.96	<0.001	1.06	ns	1.50	ns	0.000	ns
Odour	4.55	11.19	<0.001	5.49	0.001	6.40	<0.001	1.36	ns	1.75	ns	0.000	ns
Texture	4.68	5.90	<0.001	5.62	<0.001	30.45	<0.001	0.77	ns	3.35	0.001	0.000	ns
Aroma/Taste	4.38	6.62	<0.001	3.38	0.019	12.29	<0.001	1.61	ns	3.25	0.001	0.550	ns

Table 3 - Generalised Procrustes Analysis of data.

a. Percentage (%) variation accounted for by Dimensions of the GPA consensus.

Modality	No. of descriptors	1st Dimension	2nd Dimension	3rd Dimension	Sum Dimensions 1-3
Colour	4	91.4	6.3	1.9	99.6
Appearance	3	46.2	34.2	19.6	100.0
Odour	4	47.6	24.8	16.4	88.8
Texture	4	73.5	16.1	6.0	95.6
Aroma/Taste	6	43.5	20.9	13.2	77.6
Compliance	4	76.2	12.9	8.9	98.0

b. Scaling Factor for each assessor for each modality.

	Assessor								
	2	**3**	**5**	**6**	**7**	**10**	**15**	**16**	**17**
Colour	1.06	0.87	1.02	1.51	0.88	1.06	0.96	1.12	0.87
Appearance	0.98	1.00	0.91	1.35	0.96	1.08	1.03	0.93	0.93
Odour	0.99	1.14	1.00	1.00	0.91	1.16	1.01	1.09	0.84
Texture	1.32	1.08	1.06	0.73	1.01	1.07	0.97	1.08	1.03
Aroma/ Taste	0.97	1.21	1.01	0.74	1.01	1.28	1.18	0.93	1.01
Compliance	1.41	0.98	0.93	1.00	0.77	1.14	1.21	1.09	0.87

c. Correlation of each assessors data with GPA consensus.

	Assessor								
	2	**3**	**5**	**6**	**7**	**10**	**15**	**16**	**17**
Colour	0.68	0.63	0.79	0.68	0.86	0.78	0.72	0.70	0.77
Appearance	0.74	0.86	0.77	0.63	0.86	0.87	0.88	0.85	0.86
Odour	0.49	0.48	0.72	0.58	0.66	0.65	0.46	0.56	0.76
Texture	0.58	0.73	0.80	0.76	0.75	0.73	0.72	0.72	0.79
Aroma/Taste	0.63	0.56	0.70	0.72	0.70	0.52	0.58	0.60	0.69
Compliance	0.53	0.71	0.76	0.75	0.81	0.72	0.72	0.77	0.84

considered defects: *Large grains*, *Long threads*, *Rancid odour*, *Rind odour, Dry, Rind particles*, *Sandy, Rancid aroma*, *Rind aroma*.

GPA samples plots

The plots for each modality (Figs. 1 a,b,c,d,e,f) show different levels of discrimination between Products. The modality Compliance gives the clearest discrimination between products, the panel is able to discriminate among the most pairs of samples. The results for appearance, odour and texture shows that many Products are different. As expected the graphic that shows least discrimination is that of aroma/taste. However, this graphic does show that 10 Products are distinctly different from the other 14 Products.

Assessors plots

One of the great advantages of using multivariate techniques is that it provides techniques to monitor the Assessors (Reference to Figs. 2 a,b,c,d,e,f, PCO assessors plots).

Four plots calculated with Principal Co-ordinate Analysis (PCO) (colour, texture, aroma-taste and compliance) show a good agreement among tasters, only one of them being clearly out of the ellipse area where the 95% of the panel should lie. For appearance we have two tasters outside the area and for odour four. This last results confirms those of tab 3 c about the necessity to work with the tasters to improve the situation.

DISCUSSION

The assessors showed some limits during their activity in the use of scale, they had also some problems with odour evaluation both in finding a consensus and in discrimination among products. On the contrary the good results of compliance give confidence in the competence of the panel in identifying the sensory characteristics which could drift from the traditional standard provided for by the official requirements of PDO Parmigiano-Reggiano cheese.

The scorecard was developed bearing in mind such requirements. It has to be considered that in the process of grating the wheels of Parmi-

Table 4 - Correlations of Component data with Compliance data.

a. Colour with Compliance. Visual.			
Variable	**Mean**	**Corr**	**Prob**
Intensity	3.19	0.04	ns
Brown	0.07	-0.44	0.014
Lemon yellow	0.09	0.19	ns
Other colours	0.08	-0.45	0.013
b. Appearance with Compliance. Visual.			
Variable	**Mean**	**Corr**	**Prob**
Particles size	3.29	-0.05	ns
Large grains	0.31	-0.84	<0.001
Long threads	0.49	-0.47	0.009
c. Odour with Compliance. Odour.			
Variable	**Mean**	**Corr**	**Prob**
Intensity	3.56	0.06	ns
Rancid	0.53	-0.57	0.001
Rind	0.76	-0.39	0.028
Sour	0.23	-0.16	ns
d. Texture with Compliance. Texture.			
Variable	**Mean**	**Corr**	**Prob**
Solubility	3.90	0.92	<0.001
Dryness	0.83	-0.81	<0.001
Rind particles	0.62	-0.72	<0.001
Sandy	0.63	-0.61	0.001
e. Aroma/taste with Compliance. Aroma/Taste.			
Variable	**Mean**	**Corr**	**Prob**
Intensity	4.31	0.37	0.036
Salty	0.60	0.11	ns
Pungent	0.77	-0.04	ns
Sour	0.70	-0.07	ns
Rancid	0.89	-0.63	<0.001
Rind	1.11	-0.66	<0.001
f. Compliance means.			
Variable	**Mean**		
Visual	4.96		
Odour	4.55		
Texture	4.68		
Aroma/Taste	4.38		

giano-Reggiano cheese, the differences among the samples could be originated by: a) raw matter mostly in terms of odour, aroma/taste and less in terms of texture; b) processing technology mostly in terms of texture than in terms of odour or aroma/taste.

In the first case we could have differences due to different aromatic composition of the original cheese (for aromas of quality products but also for off flavours), but the texture of the wheel before grating could also affect the sensory evaluation, for instance by moisture content or proteolysis. On the other hand the technology could influence the percentage of rind presented in the grated cheese that, according to regulations, should not exceed the 18% of the weight, but could be also inferior. Different percentage of rind in grated cheese affect dramatically the quality of the products. Other technological decisions , for instance waiting too long between the opening of the wheels and the grating affect the quality of the grated cheese. Too fine grating affect also the quality.

Raw matter and technology conditions thus affect specific sensory quality characteristics of the cheese and the descriptors chosen in the scorecard were found useful in describing the important features and defects of grated Parmigiano-Reggiano.

Some descriptors nevertheless which are not so useful in product discrimination (*Other colours*) or difficult to assess (*Salty, Sour*) or redundant (*Sandy)* could be removed bearing in mind the utility of reducing the length of the scorecard.

CONCLUSIONS

This research demonstrates that this scorecard can be used for the sensory analysis of grated Parmigiano-Reggiano. It is shown to be fully "fit for the purpose" of differentiating between different samples of grated Parmigiano-Reggiano and identifying the differences. As always the assessors showed modest differences in personal performance, but in general excellent agreement was found. Overall, assessors clearly have more problems with the complex modalities i.e. Odour and Aroma/Taste. This emphasizes the need for frequent training exercises and constant monitoring for this most demanding job.

The discrimination among products was particularly good for Texture and Compliance.

The results for Compliance showed that the assessors have understood the proper meaning and use of this modality. These excellent results confirm the use of this scorecard by the official control body of the PDO Parmigiano-Reggiano to test samples of grated cheese for compliance with PDO requirements. We suggest also that this scorecard could be used by the producers as useful tool to monitor their production in sensory quality control.

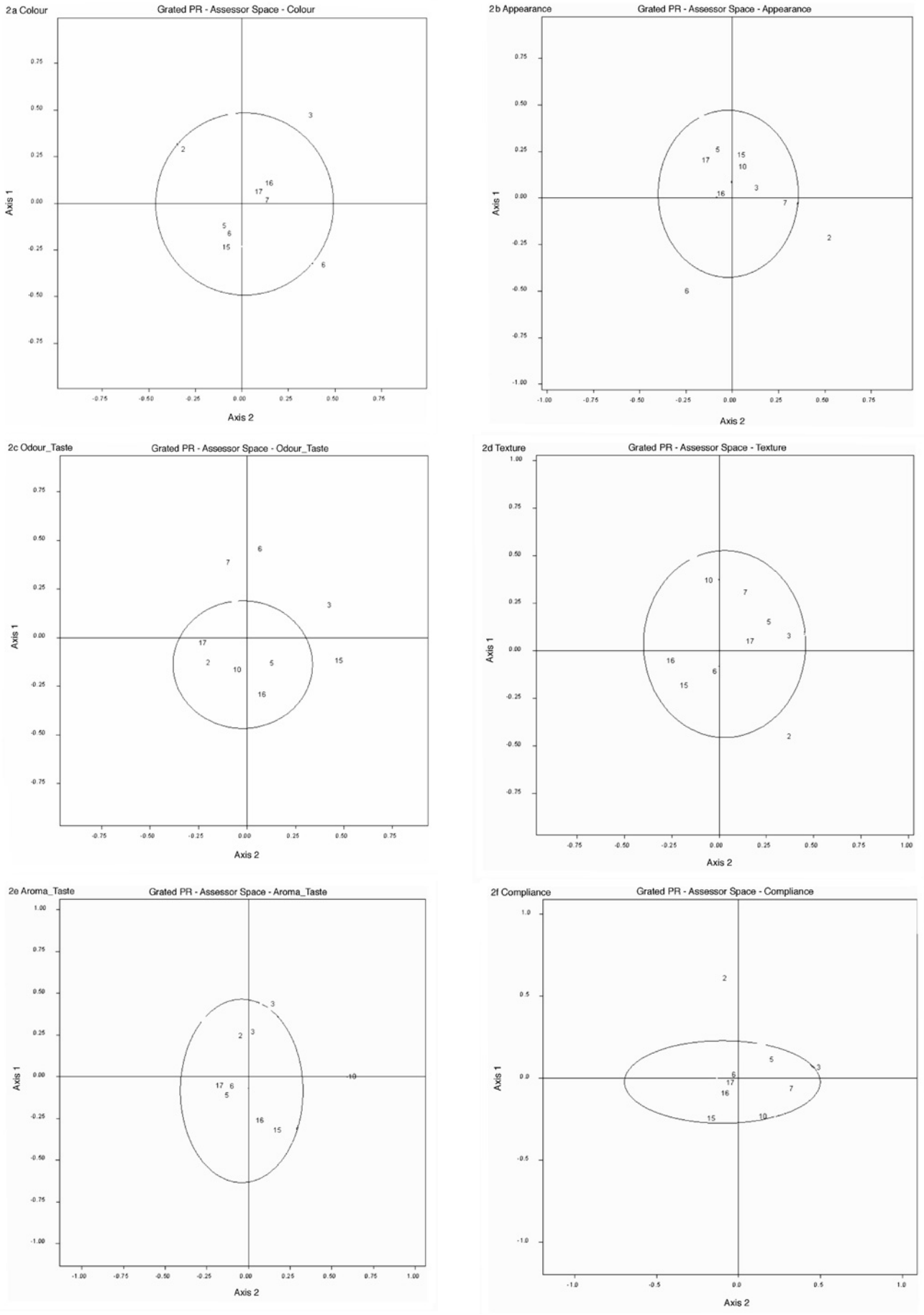


Fig. 2 a,b,c,d,e,f - PCO assessors plots for: a) Colour, b) Appearance, c) Odour, d) Texture, e) Aroma/taste, f) Compliance. Numbers represent assessor and the ellipse encloses the area where 95% of the panel is expected to lie.

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PAPER

INVESTIGATION OF CHEESE-MAKING FACTORS EFFECT ON RIPENING OF A SEMI-HARD CHEESE FOR PRODUCT QUALITY ENHANCEMENT

V. GIANOTTI[1]*, E. ROBOTTI[1], D. PATTONO[2], M. BENZI[1] and E. MARENGO[1]
[1]University of Piemonte Orientale, DISIT Department of Scienze e Innovazione Tecnologica,
Viale T. Michel 11, 15121 Alessandria, Italy
[2]University of Torino, Department of Animal Pathology,
Via Leonardo da Vinci 44, 10097 Grugliasco (TO), Italy
*Corresponding author: Tel. +39 0131 360271, Fax +39 0131 360250,
email: gianotti@mfn.unipmn.it

ABSTRACT

The work is devoted to understand how cheese making factors can influence the quality of cheese along ripening. The study is focused on a semi-hard cheese (Toma). Three parameters (type of milk, of curd and of starter bacteria) were studied by a full factorial experimental design, providing a set of eight cheese samples characterised, after one and two months of ripening respectively, by compositional parameters and the content of biogenic amines. Principal Component Analysis and the regression models built for one and two months relating cheese making factors to quality parameters, allowed to point out important relationships existing between cheese making conditions, ripening time and quality and led to suggest best practices in the cheese making procedure of Toma cheese, according to the improvement in both food safety and quality.

The approach is intended as a guideline for product optimization taking into account the technological features of the product, food safety issues and the receipt constraints.

- Keywords: Experimental Design, food quality, food safety, biogenic amines determination, cheese ripening, Principal Component Analysis -

INTRODUCTION

The ripening process is responsible for the positive features of cheese involving flavour, texture and aroma; on the other hand, during ripening the proteolytic process can lead to the formation of compounds that are potentially toxic for humans (KALIT *et al.*, 2005). The entire process leads both to changes in the chemical-physical properties of cheese (pH, moisture, etc.) and to the formation of biogenic amines (BAs) due to the decarboxylation of free amino acids.

BAs are organic bases characterised by a biological activity as they play psycho- and vasoactive effects on humans: the consumption of large amounts of BAs can in fact cause troubles of different entity, from light illnesses as headache and nausea to more dangerous diseases as hyper- and hypo-tension, cardiac palpitation and anaphylactic shock syndrome (NOVELLA-RODRIGUEZ, 2000).

Many different factors have been studied to characterise cheese and also investigate variations during cheese ripening processes: moisture, salt, proteins, D+L lactic acid, nitrogen fractions soluble in water and acid, free amino acids content, BAs profile and fatty acid methyl ester content, protein fractions (ATTAIE, 2005; BARILE *et al.*, 2006; GENNARO *et al.*, 2003; KEBARY *et al.*, 1999; KUCHROO and FOX, 1982; PINTADO *et al.*, 2008; PUERTO *et al.*, 2004; WATKINSON *et al.*, 2001).

During ripening, changes are evident in moisture, amount of proteins, salt, pH, nitrogen soluble fractions and amount of BAs; all these parameters are influenced by cheese making procedures and storage conditions (GENNARO *et al.*, 2003; KEBARY *et al.*, 1999; KUCHROO and FOX, 1982; MICHAELIDOU *et al.*, 2003; NIKOLAOU *et al.*, 2002; NOVELLA-RODRIGUEZ *et al.*, 2004; SKEIE and ARDO, 2000; VARLIK and UGUR, 2002; WALLACE and FOX, 1997; WATKINSON *et al.*, 2001).

In terms of food safety, a previous work (GENNARO *et al.*, 2003) carried out by our research group showed the correlation between amount of BAs and production conditions: this kind of knowledge can suggest optimal production and storage conditions to provide the minimization (and/or decrease) of the BAs content in cheese.

The aim of this work is deepening the knowledge of the effect played by cheese making parameters on the quality parameters of the final product along with ripening, providing in the meantime information about the best practices in cheese production according to the improvement of food quality and safety. The study is focused on a semi-hard cheese, namely "Toma" by full factorial experimental design and Principal Component Analysis in order to point out relationships existing between cheese making conditions, ripening time and quality of the final products.

The approach can be intended as a guideline for product optimization that allows to contemporarily consider the technological features of the product, food safety issues and the existence of receipt constraints.

MATERIALS AND METHODS

Sample preparation

The cheese samples were prepared by varying three cheese making conditions according to a two-level full factorial design (BOX and HUNTER, 1978; CARLSON, 1992; DEMING and MORGAN, 1993): a) the milk pre-treatment (raw or pasteurised), MILK; b) the starter bacteria (thermophile or mesophile), STRAIN; c) the treatment of the curd (raw or heated curd), CURD. The experiments required are 8 and three replicates of the overall experimental plan were produced, thus providing a total of 24 cheese samples. Factorial design theory is not further deepened here since it is widely described elsewhere (BOX *et al.*, 1978; CARLSON, 1992; DEMING and MORGAN, 1993).

Chemical and physico-chemical parameters were determined on each independent replication after one and two months ripening. Each set of cheese samples was obtained from separate batches of 50 litres of bovine whole milk, coagulation was obtained at 35°C by means of liquid bovine rennet (1:10000). In the case of "heated curd", a temperature of 44°C was applied for 12 min. In all cases, curd was put in cylindrical moulds (diameter 25 cm) then salted in a 21% NaCl w/w brine for 12 h and ripened at 10°C and 85% R.H. up to two months.

Bacterial cultures were two different commercial starters, with different composition:

- *Mesophilic strains: Lactococcus lactis* (subspecies *lactis*), *Lactococcus lactis* (subsp. *cremoris), Lactococcus lactis* (subsp. *biovar), Lactococcus lactis* (subsp. *diacetylactis)* and *Leuconostoc mesenterioides* (subsp. *cremoris)*;
- *Thermophilic strains: Streptococcus thermophilus* and *Lactobacillus delbreuckii (*subsp. *Bulgaricus).*

Reagents

Ultrapure water from Milli-Q system (Millipore), acetonitrile, HCl 0.1 M and acetone HPLC grade were purchased from Merck. Histamine dihydrochloride, tyramine hydrochloride, putrescine dihydrochloride, cadaverine dihydrochloride, sodium hydrogen carbonate and dansylchloride were Sigma-Aldrich reagents.

Instrumentation

An HPLC Lachrom, Merck-Hitachi (Tokyo, Japan), equipped with a quaternary pump L-7100, an UV detector L-7400 and an interface L-7000

were used; the chromatographic column was a Merck LiChrospher 100 RP-18 250 x 4 mm (5 μm) coupled with a guard column of the same material. A homogeneiser UltraTurrax T25 (IKA, Warke, Germany) and a centrifuge 5804 (Eppendorf, Hamburg, Germany) were used. A Crison pH2001 pH-meter equipped with a combined glass-calomel electrode was employed for the pH measurements.

Methods

Amine identification and quantification were made by HPLC, after extraction and dansyl-chloride derivatization, performed according to the protocol from MORET and CONTE (1996). The mobile phase was a water/ACN mixture in the following gradient elution: 0-5 min water/ACN 35/65, 5-20 min water/ACN 25/75. Flow-rate was 0.8 mL/min and UV detection was performed at 254 nm. pH and moisture determinations were performed according to FENELON and GUINEE (1999); water and acid soluble nitrogen were measured by the method reported by KUCHROO and FOX (1982), while total nitrogen content was estimated by the AOAC standard method 2001.14. D+L Lactic acid was determined by a Boehringer-Mannheim enzymatic kit and the total aminoacids quantity was evaluated as in POLYCHRONIADOU (1988).

Principal Component Analysis, regression models and all graphical representations were performed by Statistica 7.1 (Statsoft Inc., USA) and Excel 2003 (Microsoft Corporation, USA).

Data analysis

Principal Component Analysis (PCA) (MASSART *et al.*, 1988; VANDEGINSTE *et al.*, 1998) was carried out here to provide a dimensionality reduction; PCA was applied with a Varimax rotation of the relevant PCs (i.e. the maximisation of the variances of all the original variables on each PC), to obtain a clearer insight of the investigated phenomena (MASSART *et al.*, 1988; VANDEGINSTE *et al.*, 1998). The Varimax rotation is particularly suitable for this application to identify the macro-variables hidden in the data structure, e.g. the ripening time or the content of biogenic amines.

The effect played by the factors investigated (MILK, CURD and STRAIN) was studied by a two-level full factorial design that allows the evaluation of the effect of the principal factors and of their interactions (two- and three-way interactions) on the investigated responses. Eight cheese samples corresponding to the experiments deriving from a 2^3 full factorial design were performed (replicated three times to provide 24 final products). The samples obtained were ripened for two months and analyzed after one and two months respectively by the compositional parameters (moisture, pH, lactic acid, aminoacids, nitrogen fraction soluble in water and in trichloroacetic acid (TCA)) and the BAs content (cadaverine, histamine, putrescine and tyramine).

The Ordinary Least Squares (OLS) regression model that can be investigated by the 2^3 full factorial design applied in this case is:

$$y = b_0 + b_1 \cdot MILK + b_2 \cdot STRAIN + b_3 \cdot CURD + \\ + b_{12} \cdot MILK \cdot STRAIN + b_{13} \cdot MILK \cdot CURD + \\ + b_{23} \cdot STRAIN \cdot CURD \\ + b_{123} \cdot MILK \cdot STRAIN \cdot CURD$$

where the coefficients b_i measure the effect of the corresponding variable and of their interactions on the final response y. The variable y can be either a direct experimental measurement (e.g. the concentration of investigated analytes) or a variable taking into account the correlation structure of the data (score on a significant PC). Here, the regression models were built for each PC calculated by PCA after Varimax rotation.

Data analysis consisted therefore in a multistep procedure:

1) Principal Component Analysis.

PCA was first applied to the overall dataset, provided in Supplementary Information 1, in which values lower than the LOD were set to 0. After autoscaling, the Varimax rotation was applied to the first four PCs calculated, explaining about 85% of the total variance. This first step provides a reduced set of macro-variables.

2) Evaluation of the experimental error.

For each regression model, the experimental error was obtained calculating the pooled variance as:

$$s^2_{pe} = \frac{\sum_{i=1,N} \left(\nu_i \cdot s_i^2 \right)}{\sum_{i=1,N} \nu_i}$$

where:

$\upsilon_i = n_i - 1$ is the number of degrees of freedom of each estimate of the experimental error s^2_i of the *i-th* replicated experiment (n is the number of replications; here $n_i=3$ for each s^2_i). The sum runs on the $N=8$ experiments of the experimental design.

3) Calculation of regression models

Regression models were then built relating each rotated principal component to the investigated cheese making factors (MILK, STRAIN, CURD) and their interactions. Statistically relevant coefficients of factors and/or interactions to be included in the model were identified as in (BOX and HUNTER, 1978), comparing the coefficients to the corresponding standard error:

$$StdError = \frac{S_{pe}}{\sqrt{N}}$$

where N is the number of experiments (here, N=24).

Thank to the availability of several replicated experiments, each model containing only the relevant factors and interactions could be tested for lack-of-fit. The test was carried out according to ANOVA (ANalysis Of VAriance), by a Fisher F-test (BOX and HUNTER, 1978) comparing the residual information due to lack-of-fit, MSS_{lof} (not accounted for by the), model to the residual information due to pure experimental error (MSS_{pe}):

$$F_{lof,calc} = \frac{MSS_{lof}}{MSS_{pe}}$$

If the $F_{lof,calc}$ is smaller than the F-Fisher tabulated for the corresponding degrees of freedom of MSS_{lof} and MSS_{pe}, at a significance level =0.05, no lack-of-fit is detected.

The analysis of the regression models provides general information about the effect played by each factor on the general characteristics of cheese along ripening and allows the identification of best practices in cheese making procedures. Independent regression models for one and two months ripening times were built, in order to identify possible changes in the effect played by the cheese making conditions.

RESULTS AND DISCUSSION

Principal Component Analysis

PCA was applied to the overall dataset after autoscaling and Varimax rotation of the first 4 factors, explaining about 85% of the total variance.

PC_1 accounts for the largest amount of total variance (about 31%) and the corresponding loading plot (Fig. 1a) shows large positive weights of the variables related to the BAs content and at a lower extent, to the fraction of N_2 extracted in water. This PC therefore mainly accounts for the overall BAs content (more positive scores on PC_1 correspond to a larger BAs content).

PC_2 (about 28% explained variance) shows a positive contribution (positive weight; Fig. 2a) of the amount of nitrogen extracted by TCA, amino acids and pH; a lower but still positive contribution is also present for nitrogen extracted in water. The amount of nitrogen extracted by TCA is usually related to non protein nitrogen, while N extracted in water is commonly considered to have a protein origin (KALIT *et al.*, 2005; KUCHROO and FOX, 1982) [1, 7]. The amount of variance accounted for by water soluble nitrogen is thus separated into two contributions: one related to proteolysis and one related to the BAs content. From this point of view, the generation of BAs can be looked at as a secondary product of proteolysis and an increase of pH and of the amount of N in TCA can be related to proteolysis working-out (WATKINSON *et al.*, 2001) [10], i.e. to a correct ripening from the technological point of view. An increase of the scores on PC_2 can therefore be related to the presence of a proteolyc process.

PC_3 (13% explained variance) accounts for the information on moisture, showing a large negative weight (loading plot in Fig. 3a); more negative scores on this component are therefore related to a larger moisture.

The fourth PC, explaining about 12% of total variance, accounts for the contribution of lactic acid (large negative weights). Since PC_4 does not show systematic variations among the samples, it is not discussed further here since no relevant models could be built.

Regression models

Table 1 reports the regression models calculated for each PC after 1 and 2 months ripening: coefficients in parenthesis were not included in the final model (p-level > 0.05). R^2 and F_{lof} values refer therefore to the final model excluding not significant parameters and/or interactions. As it can be noticed good fitting abilities for all models were obtained (R^2 values always > 0.92), with no evidence of lack-of-fit ($F_{lof, calc} < F_{lof, tab}$).

Hereafter, the models obtained are discussed in more detail for each PC independently to extrapolate the effect played by each cheese making factor on the evolution of the physical-chemical characteristics of the samples along ripening. Models are discussed on the basis of surface response graphics showing the corresponding PC score along the z-axis and the variation of two parameters at a time on the x and y axes respectively. All response surfaces are represented on a colour scale from red (large positive scores) towards blue (large negative scores).

- **PC_1: content of biogenic amines**

For what regards the 1st month ripening, the effect played by the cheese making conditions must be discussed on the basis of the three-way interaction, that appears relevant in the model (Table 1). Fig. 1b represents the response surfaces illustrating the three way interaction. Passing from raw to pasteurised milk, BAs decrease (more negative scores) independently from the type of strain and curd. However, with heated curd, the effect of the type of milk is larger (steeper curve) with thermophile than for mesophile strains.

Therefore, the use of pasteurised milk, after one-month ripening, smoothes the effects of the other two variables on the BAs content. Either

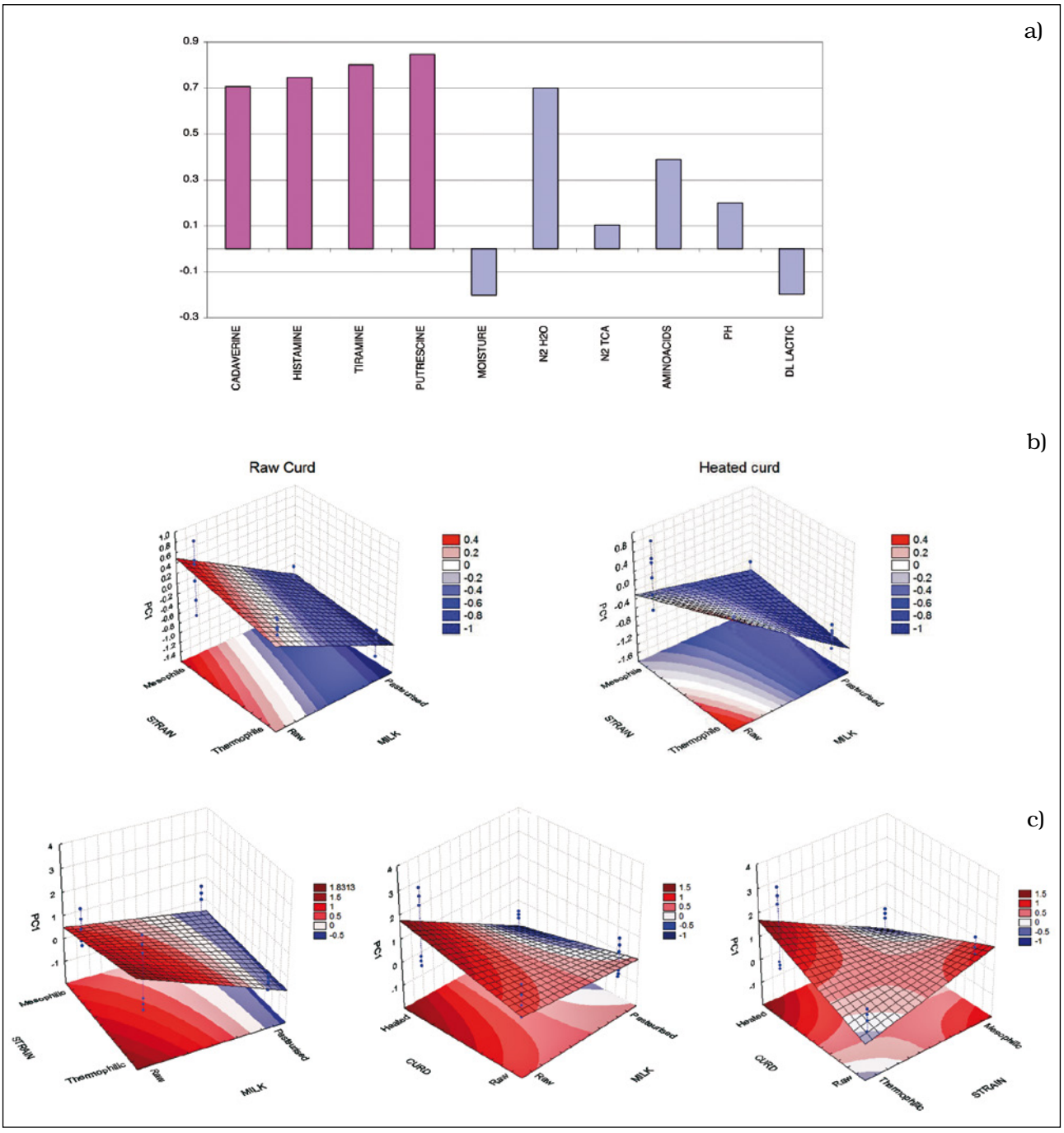


Fig. 1 - Results obtained for PC_1 (Biogenic Amines): loading plot (a); surface plots for the three-way interaction for raw and heated curd separately for the model after one month ripening (b); surface plots for the two-way interactions for the model after two months ripening (c).

heating or not heating the curd, it is useful to adopt pasteurised milk to obtain a minor content of BAs: this is true for both types of strain. The smallest possible concentration of BAs is obtained with pasteurised milk, heated curd and mesophile strain.

If raw milk has to be used, instead, the interaction between curd and strain becomes relevant: thermophile strains have to be used with raw curd and mesophile strains if curd is heated.

For what regards the 2nd month regression model, the effects of the factors are discussed on the basis of the surface plots of all the relevant two-way interactions (Table 1 and Fig. 1c):

- *MILK * STRAIN* Interaction. With pasteurised milk, the change of the type of strain does not produce effects on the BAs content (they are in general at low levels), while the starter bacteria plays an effect with raw milk: passing from thermophile to mesophile strains, BA decrease. Passing from raw to pasteurised milk produces a decrease of the BAs content with both thermophile and meso-

phile strains but the effect is larger with the first ones. The smallest amount of amines is detected with pasteurised milk and thermophile strains.

- *MILK * CURD* Interaction. Passing from raw to heated curd, BAs decrease with pasteurised milk and increase with raw milk. With both raw and heated curd, milk pasteurisation decreases amines but the effect is more evident if the curd is heated. The most different situations can be detected with heated curd: with pasteurised milk the content of amines is the smallest, with raw milk their content is the largest.
- *STRAIN * CURD* Interaction. Passing from raw to heated curd, BAs decrease with mes-

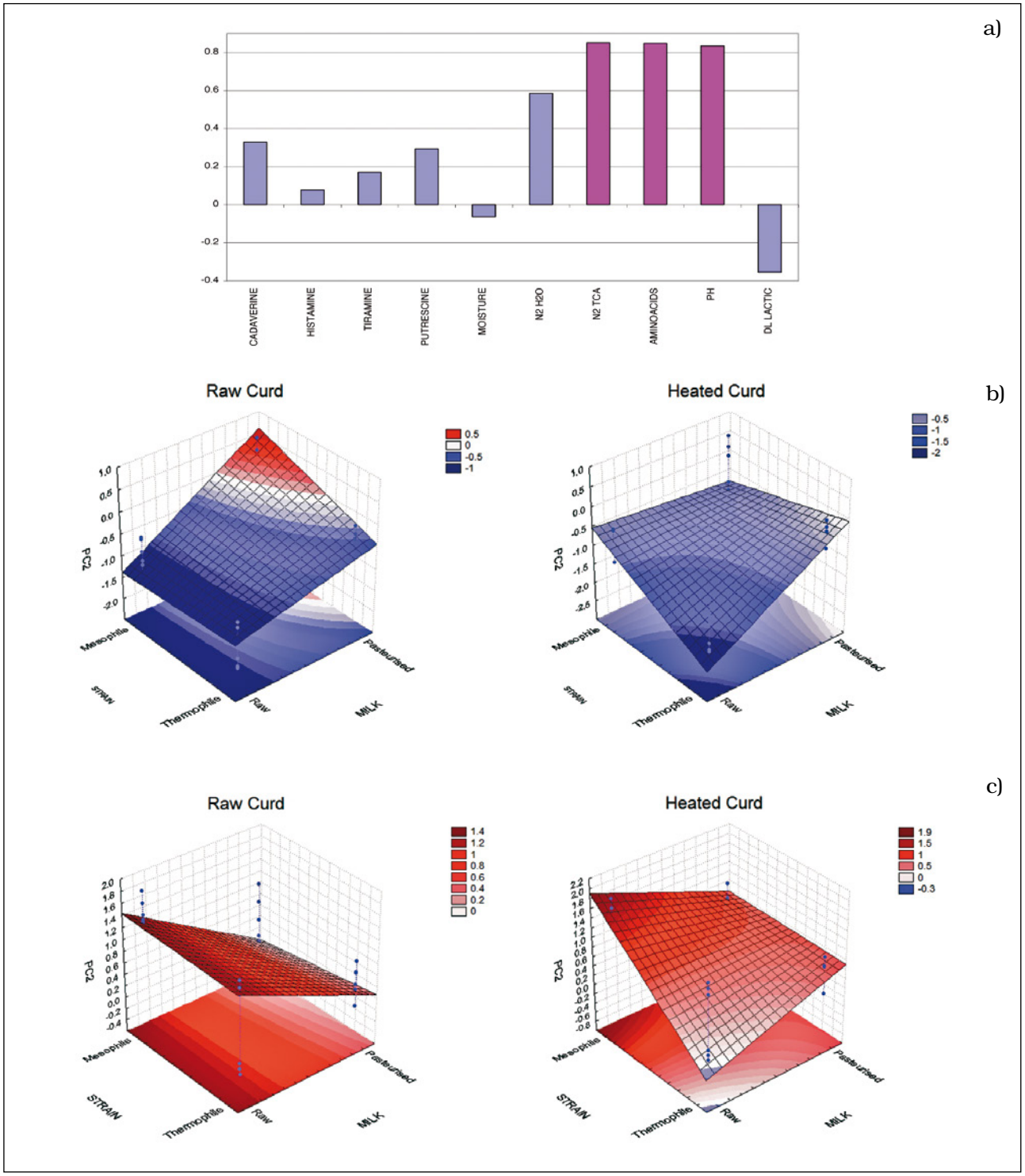


Fig. 2 - Results obtained for PC_2 (Proteolysis): loading plot (a); surface plots for the three-way interaction for raw and heated curd separately for the model after one month ripening (b); surface plots for the three-way interaction for raw and heated curd separately for the model after two months ripening (c).

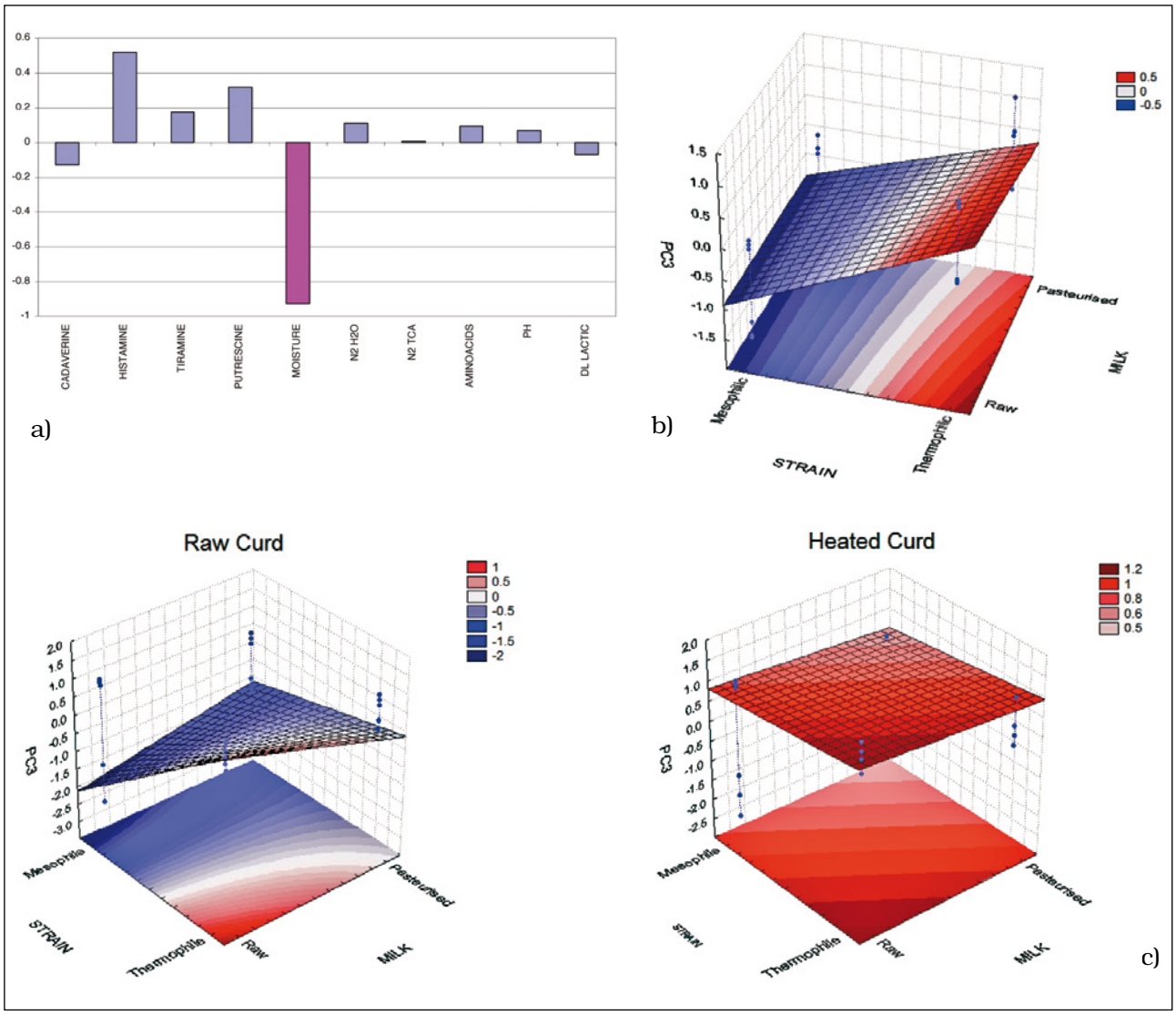


Fig. 3 - Results obtained for PC_3 (Moisture): loading plot (a); surface plots for the two-way MILK*STRAIN interaction for the model after one month ripening (b); surface plots for the three-way interaction for raw and heated curd separately for the model after two months ripening (c).

ophile strains and increase with thermophile strains. Passing from thermophile to mesophile strains, amines increase with raw curd and decrease if the curd is heated. The smallest amounts of amines are detected in correspondence to raw curd/thermophile strain and heated curd/mesophile strain and the largest amount in the other two extreme experimental points.

If the three two-way interactions are considered simultaneously, it appears clear that also after two months ripening the smallest amount of amines is achieved with pasteurised milk, heated curd and mesophile strains. For what regards the achievement of low levels of BAs, potentially dangerous for health, it is better to heat the curd with mesophile strains or using raw curd with thermophile strains: in such cases fermentation is not pushed to extremes. The heating of the curd does not produce always smaller levels of amines: this is true only if pasteurised milk is used.

Comparing the two models (Table 1) the only parameter showing a change of its sign is the intercept (negative value for the first model and positive value for the second model): the average BAs content after one month ripening is low (negative score), while it is larger after two months (positive score), showing that a general increase is recorded during ripening.

In order to maintain low concentrations of BAs, it seems to be sufficient to pasteurise milk with a ripening of one month; for a ripening time of two months it is necessary, in addition, to heat the curd. If the procedure of cheese making requires the use of raw milk, the use of mesophile strains and heated curd assures a low concentration of BAs.

Table 1 - OLS regression models for PC_1 (biogenic amines), PC_2 (proteolysis) and PC_3 (moisture) for the first and second months: regression coefficients, corresponding calculated t-Student and p-level, experimental error (S_{pe}), standard error calculated on the coefficients (Std Error). Coefficients in parenthesis were not included in the final model (p-level > 0.05); R^2, F-Fisher calculated and tabulated for the evaluation of lack-of-fit are indicated for the model excluding not significant coefficients.

PC_1: Biogenic amines	**1st Month**			**2nd Month**		
	Coeff.	**t calc**	**p-level**	**Coeff.**	**t calc**	**p-level**
Intercept	-0.3086	8.38	0.0000	0.3086	5.69	0.0000
MILK	-0.5061	13.74	0.0000	-0.7110	13.10	0.0000
STRAIN	(0.0332)	0.90	0.3802	-0.2623	4.83	0.0002
CURD	-0.1594	4.33	0.0005	(-0.0398)	0.73	0.4739
MILK*STRAIN	0.0893	2.42	0.0276	0.2463	4.54	0.0003
MILK*CURD	(0.0180)	0.49	0.6317	-0.4177	7.69	0.0000
STRAIN*CURD	-0.1270	3.45	0.0033	-0.7437	13.70	0.0000
MILK*STRAIN*CURD	0.1276	3.47	0.0032	(0.1072)	1.98	0.0657
	S_{pe}	**Std Error**	**R^2**	**S_{pe}**	**Std Error**	**R^2**
	0.1804	0.0368	0.9330	0.2660	0.0543	0.9577
	$F_{lof,calc}$	**$F_{lof,tab}$**		**$F_{lof,calc}$**	**$F_{lof,tab}$**	
	0.53	4.69		2.22	4.69	
PC_2: Proteolysis	**1st Month**			**2nd Month**		
	Coeff.	**t calc**	**p-level**	**Coeff.**	**t calc**	**p-level**
Intercept	-0.7603	18.44	0.0000	0.7603	19.18	0.0000
MILK	0.4863	11.80	0.0000	-0.2800	7.06	0.0000
STRAIN	0.1869	4.53	0.0003	0.2150	5.43	0.0001
CURD	-0.1572	3.81	0.0015	(-0.0093)	0.24	0.8171
MILK*STRAIN	-0.1053	2.56	0.0212	-0.2193	5.53	0.0000
MILK*CURD	-0.0945	2.29	0.0359	0.2553	6.44	0.0000
STRAIN*CURD	(0.0234)	0.57	0.5782	0.2610	6.59	0.0000
MILK*STRAIN*CURD	-0.3209	7.79	0.0000	-0.1548	3.91	0.0013
	S_{pe}	**Std Error**	**R^2**	**S_{pe}**	**Std Error**	**R^2**
	0.2019	0.0412	0.9379	0.0377	0.0396	0.9290
	$F_{lof,calc}$	**$F_{lof,tab}$**		**$F_{lof,calc}$**	**$F_{lof,tab}$**	
	0.32	6.11		0.06	6.11	
PC_3: Moisture	**1st Month**			**2nd Month**		
	Coeff.	**t calc**	**p-level**	**Coeff.**	**t calc**	**p-level**
Intercept	-0.2002	6.42	0.0000	0.2002	3.68	0.0020
MILK	-0.0774	2.48	0.0245	-0.1359	2.49	0.0239
STRAIN	-0.5839	18.73	0.0000	-0.5756	10.57	0.0000
CURD	0.6183	19.83	0.0000	0.6770	12.43	0.0000
MILK*STRAIN	0.0791	2.54	0.0219	0.2269	4.17	0.0007
MILK*CURD	(-0.0446)	1.43	0.1714	(-0.1137)	2.09	0.0532
STRAIN*CURD	(0.0537)	1.72	0.1041	0.3889	7.14	0.0000
MILK*STRAIN*CURD	(-0.0040)	0.13	0.9000	-0.2256	4.14	0.0008
	S_{pe}	**Std Error**	**R^2**	**S_{pe}**	**Std Error**	**R^2**
	0.1527	0.0312	0.9729	0.2668	0.0545	0.9462
	$F_{lof,calc}$	**$F_{lof,tab}$**		**$F_{lof,calc}$**	**$F_{lof,tab}$**	
	1.68	4.08		4.36	6.11	

- **PC_2: Proteolysis**

The models built after one and two months ripening show that the three-way interaction is relevant (Table 1); therefore, both models must be discussed considering the surface plots representing the corresponding three-way interactions (Figg. 2b-c).

- *First month ripening* (Fig. 2b). With raw curd, the largest proteolysis (largest positive score) is observed with mesophile strains and pasteurised milk. If raw milk is used, proteolysis is always low. With raw curd, the strain plays an effect only if pasteurised milk is used. Milk pasteurisation always plays the same effect: it increases proteolysis, but the effect is more relevant (steeper curve) if mesophile strains are used. When the curd is heated, instead, proteolysis is always low (negative scores), particularly with thermophile strains and raw milk. With heated curd, the effect of the type of strain is the most relevant (steepest curve) with raw milk (opposite to the behaviour with

raw curd). The effect of the type of milk is relevant only with thermophile strains (less negative scores).

- *Second month ripening* (Fig. 2c). One of the most evident differences between 1st and 2nd month models (Table 1) is the sign of the intercept, changing from negative (first model) to positive (second model): after one month ripening the average proteolysis is low, while it increases on the average after two months ripening. In general, we can state that the effects played by the factors remain almost unvaried with heated curd even if proteolysis in general increases after the second month: the smallest proteolysis is always obtained with raw milk and thermophile strains. However, proteolysis is large above all with raw milk and mesophile strains. With heated curd the effect of milk is similar to the first month but in this case it is relevant even with mesophile strains (opposite to that recorded for thermophile strains). With raw curd, the behaviour after two months ripening is quite different from one month ripening: proteolysis is large with raw milk and low with pasteurised milk, while the strain does not play a relevant effect. With raw curd, the type of milk plays the same effect with both strains but the effect is opposite to that recorded after one month ripening (proteolysis increases with raw milk). The type of strain plays no relevant effect nor with raw or pasteurised milk. The effect of the use of raw milk, when using raw curd, becomes visible only at the second month, increasing proteolysis, but shows no effect on proteolysis if the ripening is stopped at the first month.

- **PC_3: Moisture**

In the 1st month model only the three principal factors and the interaction *MILK - STRAIN* are relevant (Table 1). The regression coefficient of *CURD* shows that passing from raw to heated curd, moisture of the final product decreases, since draining increases. The effect of *MILK* and *STRAIN* instead must be discussed on the basis of the two-way interaction (Fig. 3b). With both pasteurised and raw milk, passing from mesophile to thermophile strains decreases moisture (more positive scores). In particular with mesophile strains, pasteurising milk does not produce relevant effects (moisture is always large), while it reduces moisture with thermophile strains.

For what regards the 2nd month model, the effects must be discussed on the basis of the three-way response surface (Table 1), reported in Fig. 3(c). With raw curd, the interaction between MILK and STRAIN seems similar to the first month: the lowest moisture is recorded with raw milk and thermophile strains. On the contrary, the largest values are recorded with mesophile strains for both types of milk. In general, the effect of the type of milk and strain on the final moisture is more significant with raw curd.

With heated curd, moisture is always low (positive scores) but the lowest possible values are recorded when raw milk and thermophile strains are adopted. Heating of the curd therefore levels moisture after two months ripening.

Again, if 1st and 2nd month models are compared, only the intercept changes its sign: a negative value for the first model corresponds to an average large value of moisture after one month, while the positive value for the second model corresponds to a general decrease of the moisture along ripening, as expected.

In moisture control, the most significant parameter is the heating of curd, as expected; if cheese has to be produced from raw curd, the use of thermophile strains, in association with both pasteurised and raw milk, assures its minimum value.

CONCLUSIONS

The conclusions that can be drawn from the joint analysis of the regression models built for each PC independently, can lead to the establishment of optimal cheese making practices aimed to obtain products characterised by high quality features and high food safety standards.

The approach here presented can be used as a guideline for product optimization: the optimization step has to take into account the technological features of the product and food safety issues but also receipt constraints. In facts, the optimal cheese-making conditions will not probably correspond to the absolute optimal conditions but will be the best conditions considering all receipt constraints, e.g. the use of raw milk, the use of raw curd etc.

Since the use of raw or heated curd leads to fundamentally different final products, final considerations are drawn according to a preliminary choice of a desirable ripening time (one or two months) and of the procedure used for curd preparation. For example, if the making conditions state a maximum ripening of one month and the use of raw curd (the worst conditions for BAs production), it emerges that pasteurised milk has to be preferred since the final product shows anyway a small BAs amount and very advanced proteolysis (especially when mesophile strains are used); the use of mesophile strains leads to a very moist product, but if thermophile strains are adopted, intermediate moisture levels can be obtained.

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PAPER

EFFECT OF STARCHY COATING FILMS ON THE REDUCTION OF FAT UPTAKE IN DEEP-FAT FRIED POTATO PELLET CHIPS

MALAK M. ANGOR[a], RADWAN AJO [a], WALID AL-ROUSAN[a] and BASEM AL-ABDULLAH[b]*

[a]Al-Huson University College, Al-Balqa Applied University, Al-Huson, Jordan

[b]Department of Nutrition and Food Technology, Faculty of Agriculture, University of Jordan, Amman, Jordan

*Corresponding author: basem@ju.edu.jo

ABSTRACT

The aim of this work was to investigate the influence of starch and bread crumbs as coating films at different levels (1, 2, 3, 4, and 5%) on fat reduction and product quality in deep fried potato pellet chips. The results obtained have shown that all coated samples increased fat reduction percent at all levels of starch and bread crumbs ranged from 12.89 to 44.37% and from 14.98 to 27.15%, respectively. The most effective level of starch was 4% for fat reduction, while the best level of bread crumbs was 3%. There were no significant ($P > 0.05$) differences between all samples in specific volume for fried potato pellet chips. The sensory attributes were improved as a result of using the coating materials at different levels and this is may be attributed to the reduction of fat absorption.

- Keywords: coating films, deep fat frying, fat reduction, potato pellet chips -

INTRODUCTION

Deep fat frying plays an important role in food preparation, and it is one of the oldest cooking methods widely used to prepare tasty, distinctive flavor, aroma and crispy foods. It makes a unique contribution to sensory characteristics (RIMAC-BRNČIĆ *et al.,* 2004). Despite its considerable fat content and intensified consumer's awareness of the relationships between food, nutrition and health, frying remains a principal cooking method (SAGUY and DANA, 2003).

Some of the fried foods may contain up to 50% fat. For example, lipid content of French Fries increases from 0.2 to 14% and may reach 40% in fried potato chips (PINTHUS *et al.*, 1993; AKDENIZ *et al.*, 2006). Thus, fried foods have become a health concern. High consumption of lipids has been related to obesity and other diseases like coronary heart diseases. Furthermore, lipid oxidation during frying generates different oxidation byproducts, some of which are also associated with many diseases such as premature aging, membrane damage, heart disease and cancer (FALGUERA *et al.,* 2011).

Fried foods contribute a significant proportion of the total fat consumed in the world. Yet, aside from their high caloric value, fried foods can be nutritious and favorably compared with other cooking methods such as baking and boiling (PEDRESCHI and MOYANO, 2005).

Properties of fried food surfaces are significant in influencing fat absorption. Application of coating is therefore a promising route to reduce oil content (NGADI *et al.*, 2007). Coatings are reported to form barriers to moisture loss and subsequently fat uptake during frying. Deep fat frying involves the transfer of heat from the surrounding oil to the food interior together with the transfer of oil to the food and the transfer of water in the form of vapor from the food to the oil (SALVADOR *et al.*, 2008).

It has been well documented that edible coatings applied to food before frying aid in limiting moisture and oil transfer during frying (ALBERT and MITTAL, 2002). The surface modification by the hydrocolloid coatings can contribute to the reduction of the oil uptake during frying (KIM, 2011).

Various surface coating materials such as isolated whey protein, powdered bread crumbs, starch, carrageenan, and their combinations were used to reduce moisture loss and fat uptake of chicken meat balls during deep fat frying (AL-ABDULLAH *et al.*, 2011). However, there is little published information about the use of starch and bread crumbs as coating materials to reduce moisture loss and fat uptake of potato pellet chips during deep-fat frying. Therefore, the aims of this study are to study the effect of different percentages of starch and bread crumbs as coating material films for potato pellet chips during deep-fat frying on the level of fat reduction, and to study some important chemical, physical and sensory properties.

MATERIALS AND METHODS

Materials

Potato pellet chips were purchased from a local market (JOPELLETS, Amman, Jordan). The coating materials used were commercial cornstarch (packed by Al- Tahuona Al- Fakhera Est. Amman) and bread crumbs that were prepared from pan bread by removing the crust of the pan bread (bread crumbs), then dried at 40°C in an air oven (Memmert, 854 Schwapach, West Germany) for 8 hours.

After that the dried bread crumbs was ground by using a mixer (Type 241, Moulinex, France), and then the ground crumbs were sieved to 150 μm particle size using a sieve (Type ASTME 11-70, Mauer, Germany) and were placed in a glass jar until use.

Coating solution preparation

Coating solutions were prepared. For each coating material, five solutions of 1, 2, 3, 4, and 5% w/v were prepared. Each solution was heated to 90°C for 5 minutes then cooled to room temperature. After that glycerol was added at a level of 3% w/v to these solutions as plasticizer. Potato pellet chips were dipped for 10 seconds in the coating solutions followed by air drying using an air blower; the dipping and drying procedures were repeated again to ensure uniform coating of the film on the products. Potato pellet chips without coating were used as control.

The coated and uncoated samples were fried in an electrical deep-fat fryer (Emjoi power, Model UEDL- 206, China) filled with corn oil (Yara oil, Kafak Trading Company, Jordan) at 180° ± 2°C for 10 seconds (controlled temperature and time) during which a golden color surface was observed and the temperature of frying oil monitored by a digital thermocouple (K- type, APPA-51, Taiwan). The oil was replaced after four frying batches. In each batch, twenty potato pellet chips were placed inside the frying basket. Frying was carried out by immersing the basket in the oil. After frying, samples were put on absorbing tissue paper for 5 minutes prior to testing. This was done to minimize the absorption of oil due to the cooling down period (SOUTHERN, 2000). The fried samples were left to cool down to ambient conditions for 1 hour. One part of the freshly fried samples was taken immediately for sensory evaluation, while the other parts were placed in plastic bags until chemical and physical analysis.

Samples analysis:

Chemical methods

Samples were analysed for moisture and fat content according to Association Official Analytical Chemists (AOAC) 2000 and each analysis was carried out in duplicate.

Moisture content (MC) was determined by an oven-drying method in which potato pellet chips samples were placed in dishes and held in an oven (Memmert, 854, Schwapach, Germany) at 105°C until constant weight was reached.

Fat content (FC) was determined by the Soxhlet method, which involve continuous vaporisation and condensation of petroleum ether during passage through a 5 g, moisture-free sample placed in the extraction thimble and covered with cotton wool. After 16 h, the extract was collected and subjected to distillation in order to collect the crude fat, which was then dried and weighed.

The fat reduction capabilities of the edible coating in relative to control (uncoated samples) were estimated as the percent of fat content differences between uncoated and coated samples.

Acidity percent (as oleic acid) and peroxide value were analyzed for frying oil at each frying batch according to AOAC (2000). The acidity and peroxide value for the frying oil were ranged from 0.22 to 0.25 and 9.9 to 9.96, respectively.

Physical methods

The volume of each sample was determined by immersion in a graduated cylinder filled with paraffin oil. Sample specific volume was calculated by dividing the sample weight by the volume of paraffin oil displaced (AKDENZ *et al.*, 2006).

Samples from each treatment were evaluated for sensory evaluation by an 11-member trained panel, chosen from the employees and students of the Department of Nutrition and Food Technology, Al-Balqa University. The panellists were of both sexes and different ages (18-50 years). Before evaluating the samples, they were familiarised with the test procedure and use of the scoring system.

Each sample was evaluated for general appearance, colour, flavor, taste, crispiness and overall acceptability, using a descriptive nine-point hedonic scale on which 9 was 'like extremely' and 1 was 'dislike extremely'. Small amounts of bread and water were used between samples to neutralise any lingering taste from the previous sample. All treatments were evaluated in duplicate on separate occasions.

Statistical analysis

Statistical analysis of data was carried out using Statistical Analysis System package (SAS Inc., 1997). The data obtained were analyzed using a Completely Randomized Design (CRD) to study the effect of treatments on the fat%, moisture%, specific volume, and sensory scores. The significant difference of the mean was determined using Least Significant Difference (LSD) method.

RESULTS AND DISCUSSION

Table 1 shows the fat and moisture content of the deep fried potato pellet chips coated with different levels of starch. The data shows that all levels of starch coating films used were effective in reducing the fat percent when compared to those of the uncoated one (control). However, the level of 4% of starch coating film sample had significantly ($P \leq 0.01$) the lowest fat 22.23%, and the level of 1% starch coating film sample had significantly ($P \leq 0.01$) the highest fat content 34.80% when compared with other coated samples, while the fat percent for uncoated sample (control) had 39.95 % fat. The moisture percent of different levels of starch as coating films for deep fried potato pellet chips ranged from 0.82 to 2.2% for all samples. The lower fat content of these samples was associated with higher moisture content. This result was as expected, since oil absorption occurs as moisture is removed from food during the frying process. The finding results confirmed by (SALVADOR *et al.*, 2008; GARCÍA *et al.*, 2004).

The results of the analysis of fat and moisture content of the coated samples of potato pellet chips with different levels of bread crumbs and uncoated samples after deep-frying, are shown in Table 2. The data shows that the uncoated potato pellet chips sample had significantly ($P \leq 0.01$) higher fat content 39.95% than those of the coated samples. Among the coated potato pellet chips samples, the sample coated with 3% bread

Table 1 - Fat and moisture content of the deep fried potato pellets chips at different levels of starch as coating films.

Treatments[1] Starch %	Fat %	Moisture %
1%	34.80^{b}	1.00^{bc}
2%	31.55^{c}	1.34^{b}
3%	26.42^{d}	2.02^{a}
4%	22.23^{e}	2.20^{a}
5%	26.00^{d}	2.04^{a}
control	39.95^{a}	0.82^{c}
P[2]	**	**

[1]Values are the means of two replicates, means with different matching letters within the same column are significantly (*; $p \leq 0.05$, **; $p \leq 0.01$) different according to LSD.
[2]*; $p \leq 0.05$, **; $p \leq 0.01$.

Table 2 - Fat and moisture content of the deep fried potato pellets chips at different levels of bread crumb as coating films.

Treatments[1] (Bread crump)	Fat %	Moisture %
1%	32.98[b]	1.29[c]
2%	33.08[b]	1.54[c]
3%	29.11[c]	2.09[b]
4%	32.92[b]	2.18[b]
5%	33.97[b]	2.49[a]
control	39.95[a]	0.82[d]
P[2]	**	**

[1]Values are the means of two replicates, means with different matching letters within the same column are significantly (*; $p \leq 0.05$, **; $p \leq 0.01$) different according to LSD.
[2]*; $p \leq 0.05$, **; $p \leq 0.01$.

crumbs had significantly ($P \leq 0.01$) the lowest fat content 29.11% while that sample coated with 5% bread crumbs had the highest fat 33.97%. These results agreed with (Al-ABDULLAH *et al.*, 2011). They found that the level of 3% of bread crumbs as coating films for chicken meat balls during deep-frying reduced fat uptake significantly when compared with the uncoated one (control). The moisture percent of the product at different levels of bread crumbs as coating films for deep fried potato pellet chips ranged from 0.82 to 2.49 for all samples.

Fat reduction percent of control and coated samples with different levels of starch and bread crumbs as coating films for the deep fried potato pellet chips was shown in Fig. 1 and Fig. 2, respectively. All samples coated showed fat reduction at different levels of starch and bread crumbs. These levels ranged from 12.89 to 44.37% and from 14.98 to 27.15%, respectively. The most effective level of starch for fat reduction was at 4%. While the best level of bread crumbs was at 3%. These results were confirmed by (Al-ABDULLAH *et al.*, 2011) who concluded that using starch as coating films for minced chicken meat balls reduced fat uptake. Also agreed with (ALTUNAKAR *et al.*, 2006; AKDENIZ *et al.*, 2006), who concluded that fried foods coated with batters containing hydroxypropyl methylcellulose, xanthan, or guar gum had reduced oil content. KIM *et al.* (2011) were also obtained that the use of hydrocolloid as coating films of potato strips reduced the oil content up to 41% compared to control.

The specific volume for all samples coated (starch and bread crumbs) and uncoated ranged from 2.49 to 2.61 g/mL. There were no significant ($P > 0.05$) differences in specific volume of the fried potato pellet chips between all samples. This reflects that the coated films did not affect the specific volume of the treatments. These results confirmed by (Al-ABDULLAH *et al.*, 2011). They reported that minced chicken meat balls coated with different coating films showed no significant differences among all treatments, indicating a lack of any coating effect, on the product specific volume.

Sensorial characteristics of the fried potato pellet chips are attractive to the consumers. Mean hedonic scores for appearance, color, flavor, taste, crispness and overall acceptability of the fried potato pellet chips coated with different levels of starch and bread crumbs are presented in Table 3 and Table 4, respectively. With the exception of the uncoated treatment the overall acceptability ratings for all treatments showed no significant differences ($P > 0.05$) between them for all the samples coated with different levels of starch and bread crumbs. The coated samples with different levels of starch and bread crumbs enhanced the overall acceptability. These results agreed with (AL-ABDULLAH *et al.*, 2011) they concluded that the starch coating films for minced chicken meat balls yielded the highest overall-acceptability rating. Also (KILINCCEKER *et al.*, 2009) concluded that the sen-

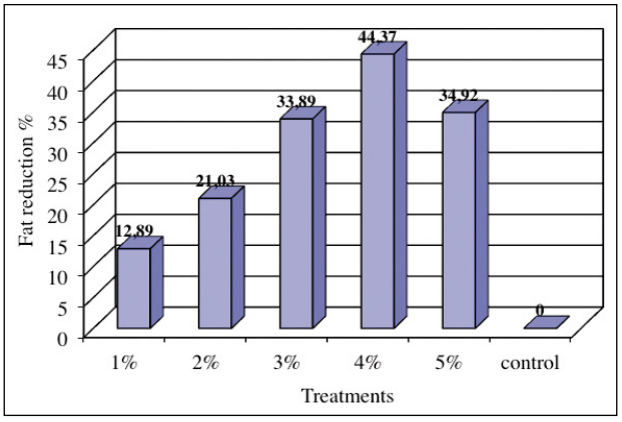


Fig. 1 - Fat reduction of deep fried potato pellet chips coated with different levels of starch.

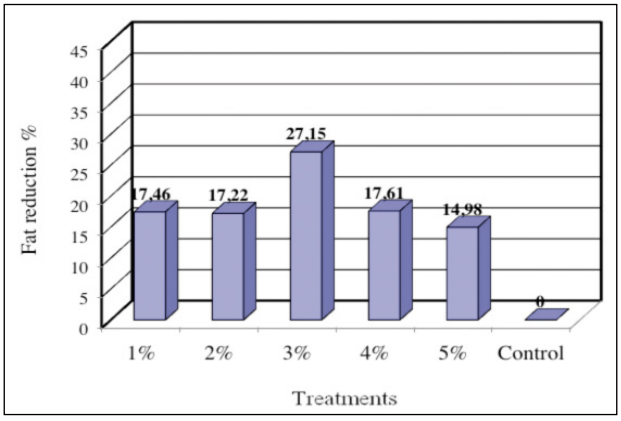


Fig. 2 - Fat reduction of deep fried potato pellet chips coated with different levels of bread crumb.

Table 3 - Means of sensory evaluation scores of deep fried potato pellets chips coated with starch at different levels[1].

Treatments	Appearance	Color	Flavor	Taste	Crispy	Overall acceptability
1%	7.70	7.80	7.40	7.30	7.40	7.80[a]
2%	7.20	7.60	7.40	7.30	7.40	7.90[a]
3%	7.30	7.40	7.60	7.40	7.50	7.90[a]
4%	8.00	7.90	7.60	7.50	7.90	8.20[a]
5%	7.00	7.40	7.85	7.70	8.00	8.50[a]
control	7.00	7.00	6.70	6.50	6.70	6.90[b]
P[2]	ns	ns	ns	ns	ns	**

[1]Each mean is the average of 11 readings, where 9 refer to like extremely, 5 neither like or dislike and 1 refers to dislike extremely. Values within the same column followed by different letter are significantly (*; $p \leq 0.05$, **; $p \leq 0.01$) different according to LSD.
[2]ns; $p > 0.05$, *; $p \leq 0.05$, **; $p \leq 0.01$.

Table 4 - Means of sensory evaluation scores of deep fried potato pellets chips coated with bread crumbs at different levels[1].

Treatments	Appearance	Color	Flavor	Taste	Crispy	Overall acceptability
1%	7.70	7.90	7.80[ab]	7.70[a]	7.70[a]	7.90[a]
2%	7.30	7.80	8.00[a]	7.70[a]	7.80[a]	8.30[a]
3%	8.10	7.60	8.1[a]	7.40[a]	8.00[a]	8.30[a]
4%	8.00	8.00	7.4[abc]	7.40[a]	8.30[a]	8.30[a]
5%	7.20	7.80	7.10[bc]	7.70[a]	8.10[a]	8.10[a]
control	7.10	7.40	6.70[c]	6.5[b]	6.70[b]	6.9[b]
P[2]	ns	ns	**	*	**	**

[1]Each means is the average of 11 readings, where 9 refers to like extremely, 5 neither like or dislike and 1 refers to dislike extremely. Values within the same column followed by different letter are significantly (*; $p \leq 0.05$, **; $p \leq 0.01$) different according to LSD.
[2]ns; $p > 0.05$, *; $p \leq 0.05$, **; $p \leq 0.01$.

sorial features of the product were improved and that the sensorial properties of the coated fillets were more desirable than those of the uncoated fillets. While (MALLIKARJUNAN *et al.*, 1997) reported that hydroxypropyl methylcellulose (HPMC) coatings improved the sensory attributes of the fried chicken nuggets than the uncoated samples.

The other attributes (appearance, color, flavor, taste, and crispness) scores for all treatments of the fried potato pellet chips coated with different levels of starch and bread crumbs were found between the ranges of "like" very much to "like slightly", included the uncoated (control) sample. There were no significant ($P > 0.05$) differences between different levels of starch coating samples for these attributes. Also bread crumb coating samples and control had no significant ($P > 0.05$) differences between different levels for only appearance and color, While these samples had significant differences for flavor, taste and crispy. The differences rating between all the treatments for all attributes were small, but significant in some cases. These results agreed with (AL-ABDULLAH *et al.*, 2011) who obtained that minced chicken meat ball samples were coated with starch, bread crumb films and non-coated acceptable by the sensory panel and any differences in sensory rating between products were generally small.

Also these results were supported by (SALVADOR *et al.*, 2008), who concluded that the differences between the coated and uncoated samples were generally small but significant in some cases. In any case, all attributes were acceptable to the sensory panels.

CONCLUSION

Using starch and bread crumbs as edible coating film for potato pellet chips prior deep fat-frying at different levels 1, 2, 3, 4, and 5% reduced fat absorption. The starch and bread crumbs coating at 4 and 3% levels were the most effective levels for fat reduction 44.27 and 27.15%, respectively. The coating films of starch and bread crumbs did not affects the specific volume of the potato pellet chips. Regarding, the sensory evaluation of the fried potato pellet chips, the sensory attributes were improved as a result of using the coating materials at different levels and this is may be due to the reduction of fat absorption.

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PAPER

QUALITY AND ANTIOXIDANT ACTIVITY OF BREAD FORTIFIED WITH FLAXSEED

R. MERAL* and I. SAIT DOGAN
Yüzüncü Yıl University, Faculty of Engineering-Architecture, Department of Food Engineering, 65080 Van, Turkey
*Corresponding author: Tel. +90 432 2251024, Fax +90 432 2251104,
email: raciyemeral@yyu.edu.tr

ABSTRACT

The objective of this study was to determine the effects of flaxseed on the rheological and bread-making properties and antioxidant activity of bread. Wheat flour was replaced with flaxseed at the level of 2, 4, and 8%. The water absorption, dough development time, stability and mixing tolerance index of the dough did not significantly change with the addition of flaxseed. The test baking results showed that the addition of %2 flaxseed yielded the highest loaf volume. The incorporation of flaxseed increased the antioxidant properties of the bread when compared to the control bread.

- Keywords: antioxidant activity, bread, DPPH, dough rheologhy, flaxseed, phenolic compounds, TEAC -

INTRODUCTION

Bread is one of the major components of the human diet in most parts of the world. For several thousands of years, people have used wheat to produce bread. In the past few decades, researchers have worked on fortifying bread with natural compounds because of demands to natural and healthier food. Thus, whole grains and other seeds have become popular in the production of bread. Many researchers have studied the fortification of bread, and looked at, for example dietary fibre (WANG *et al.*, 2002; GOMEZ *et al.*, 2003; KAACK *et al.*, 2006) and antioxidants (HOLTEKJOLEN *et al.*, 2008; PENG *et al.*, 2010). Although the impact of various ingredients on the antioxidant properties of bread has been widely studied, the impact of flaxseed on the antioxidant activity of bread is still fairly unknown.

Flaxseed (*Linum usitatissimum*) is a readily available oilseed and a major dietary source of α-linolenic acid, dietary fibres and lignans (UDENIGWE and ALUKO, 2010). Flaxseed contains 30-40% fat, 20-25% protein, 20-28% dietary fibre, 4-8% ash and the oils contains vitamins A, B, D and E, minerals and amino acids (MENTES *et al.*, 2008). Flaxseed is also a rich source of an oligomeric complex containing the lignan secoisolariciresiniol (SDG). SDG is a precursor of mammalian lignans which are recognized as protecting against hormonal-dependent breast cancer. Other phenolic compounds in the complex are two hydroxycinnamic acid derivates, *p*-coumaric acid-4-*O*-glucoside and ferulic acid-4-*O*-glucoside (STRANDAS *et al.*, 2008). The mentioned flaxseed components have been reported to possess various physiological activities relevant to human health sustenance, especially in the prevention of cardiovascular disease, cancer and diabetes. Flaxseed consumption has been demonstrated to exhibit potential health benefits, including decreased tumor growth, reduced serum cholesterol levels, and a decreased in the formation of breast, prostate, and colon cancers (HOSSEINIAN *et al.*, 2010).

The objectives of this research were to observe the influence of flaxseed on bread-making quality and the antioxidant properties of breads.

MATERIALS AND METHOD

Materials

The experimental wheat flour was obtained from a local miller (Van, Turkey). The salt and instant active dry yeast were purchased from a commercial local food ingredient company. The flour treatment agent (Pantera) which included emulsifier, alpha amylase, ascorbic acid and citric acid, was obtained from Puratos, Istanbul, Turkey. The flaxseed was also purchased from a local market. The flaxseed was ground with a laboratory mill (Perten LM 120, Sweden) just before use. After the flaxseed was ground (particle size<0.5 mm) wheat flour was replaced with ground flaxseed at the levels of 0, 2, 4, and 8%.

Chemicals

Gallic acid, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium persulfate ($K_2S_2O_8$), Folin-Ciocalteu reagent, sodium carbonate (Na_2SO_3), sodium chloride (NaCl) were obtained from Sigma-Aldrich Company (St. Lois, MO, USA). Methanol for extraction was analytical grade and purchased from Merck (Darmastadt, Germany).

The rheological characteristics of dough

The effects of the flaxseed on dough rheology during mixing were determined by using Dough Test Instrument (Yücebaş Machine Company, İzmir, Turkey), that followed the AACC method (1983). The parameters obtained were water absorption (WA), dough development time (DDT), dough stability and mixing tolerance index (MTI).

The viscoelastic characteristics of dough

The viscoelastic behaviour of the dough was determined by Texture Analyser, using SMS/Kieffer dough and gluten extensibility rig. The dough samples prepared as above were placed in a Teflon mould. The whole mould was placed in a chamber at 30° and 85% RH for 90 min to allow stress relaxation. The extension test of a strip of dough was evaluated using SMS/Kieffer Dough and Gluten Extensibility Rig with a 5 kg load cell. For each kind of dough sample, four measurements were performed (WANG *et al.*, 2003).

Bread-making and evaluation

Bread samples were prepared using a straight dough method with slight modifications. The basic dough formula for 500 g flour consisted of salt (7.5 g), instant active dry yeast (5 g), a flour treatment agent (4 g) and water, which needed to have a consistency of 500 BUE. After all the ingredients were added the dough was kneaded for the DDT. After mixing, the dough was placed in the fermentation cabin (Özköseoğlu, İstanbul, Turkey) at a temperature of 30°C and 85% RH for 45 min. The fermented dough was divided into three 200 g pieces, moulded using a dough forming device (Şimşek Laborteknik, Ankara, Turkey) and put

into pans (Teksan Machine Company, Bursa, Turkey) for the final proofing. The dough samples were fermented until they reached 2 cm above the rim and then baked at 218°C for 21 min. Following baking, the bread loaves were cooled at room temperature. The loaf weights and volumes were measured 1 h after removal from the oven. The loaf volumes were measured using the rapeseed displacement method (PLESSAS *et al.*, 2003) and specific volumes were calculated. Bread firmness was determined using TA.XT plus Texture analyser (TA.TX2. Stable Micro Systems Ltd. Godalming Surrey, UK) equipped with 5 kg load cell and 36 mm cylinder aluminum probe (P36/R) after 3 and 72 h of baking. The firmness was expressed as the force required to compress 25% of the bread.

Extraction of phenolics from bread

The bread (control and flaxseed-enhanced bread) was dried at 40°C. The bread was ground into fine powder using a laboratory mill (Perten LM 120, Sweden). Samples from each type of bread of approximately 10 g were mixed with 30 mL methanol and stirred for 22 h in the dark and at 35°C using a shaking incubator (Heidolph Unimax 1010, Germany). After this, the homogenates in the tube were centrifuged at 12,000 *g* 15 min at 20°C and the supernatant was transferred into an amber volumetric flask. The precipitate was extracted again using the same solvent and in the same conditions and made up to a final volume of 100 mL. The extracts were stored at -20°C for four weeks (IACOPINI *et al.*, 2008; YEMIS *et al.*, 2008).

Determination of total phenolic content

The concentration of total phenolics (TPC) in the methanol extract of bread samples was determined using the Folin-Ciocalteau colorimetric method (YEMIS *et al.*, 2008). Briefly, a 150 µL sample extract and 3.0 mL of 2% sodium carbonate (w/v in water) were transferred into a test tube. After about two minutes 150 µL Folin-Ciocalteu's reagent (1:1, v/v in water) was added and mixed throughly. The mixture was left to stand for 45 min at room temperature in the dark. The absorbance of the reaction mixture was measured at 765 nm in a spectrophotometer (PG Instrument T80 UV/VIS Spectrophotometer, UK). All the spectrometric measurements were carried out in triplicate**.** The calibration curve was performed using gallic acid and the results were expressed as mg of gallic acid equivalents 100 g of sample (mg GAE/100 g of sample).

Determination of total flavonoid content

The concentration of total flavonoids (TF) in the methanol extract of the bread samples was determined using the colorimetric method (RAMOMOORTHY and BONO, 2007). 1 mL of each bread extract was mixed with 1 mL aluminium trichloride in methanol. The mixture was left to stand for 10 min at room temperature in the dark. The absorbance of the reaction mixture was measured at 510 nm in the spectrophotometer. The calibration curve was performed using quercetin, and the results were expressed as mg of quercetin equivalents for 100 g of the samples (mg QE/100 g of sample).

Free radical scavenging activity

The free radical scavenging activity of the extract was measured using DPPH· free radical scavenging method (BRAND-WILLIAMS *et al.*, 1995) with some modifications. One mL of the sample extract was added to 3 mL DPPH· solution (4 mg L^{-1} in methanol). The mixture was shaken vigorously and allowed to stand for 30 min at room temperature in the dark. Then the absorbance was measured at 517 nm. Methanol was used instead of the sample for the control measurements. The inhibition of DPPH· was determined according to the following equation.

$$\% \, Inhibition = \left[\frac{(A \; control - A \; sample)}{A \; control} \right] * 100$$

A control: Absorbance of control at 517 nm
A sample: Absorbance of sample at 517 nm

Trolox equivalent antioxidant capacity (TEAC)

The TEAC value was determined using YEMIS *et al.* (2008) with slight modifications. 7 mM ABTS salt solution was reacted with 2.45 mM potassium persulphate solution and the reaction mixture was allowed to stand in the dark for 16 h at room temperature and used in 2 days. Then the radical solution was diluted with a potassium phosphate-saline buffer (pH 7.4) to an absorbance of 0.7±0.005 at 734 nm. 50 µL of the sample extract or standard (different concentrations of trolox) and 3 mL of diluted ABTS· solution were added to the macrocuvvete and absorbance was taken as being at 734 nm on a spectrophotometer after 6-min incubation. The results were expressed as TEAC values (µmol trolox/g sample). Triplicate analyses were performed.

Statistical methods

The data were analysed using the StatGraphics Centrium 15.1 (STATGRAPHICS, 2006) for one-way ANOVA. The Student Newman Keuls (SNK) procedure was used to identify any significant differences (p<0.05) between the samples.

Table 1 - Influence of flaxseed on dough properties.

Substitute level (%)	Water absorption (%)	DDT (min)	Stability (min)	MTI (BUE*)
0	57.7±0.42^{a}	5.2±0.14^{ab}	6.4±0.14^{b}	41.1±9.54^{b}
2	58.6±0.35^{a}	6.5±0.42^{a}	5.9±0.28^{b}	63.7±0.07^{a}
4	58.7±0.35^{a}	5.8±0.07^{ab}	6.1±0.07^{b}	54.6±0.14^{b}
8	58.9±0.14^{a}	5.7±0.21^{ab}	7.5±0.42^{a}	38.6±3.25^{b}

*BUE: brabender units equivalent. The values are given as mean±SD (n=2). Values followed by different superscripts within the same column are significantly different (p<0.05).

RESULTS AND DISCUSSION

Influence of flaxseed on dough properties

The effects of the flaxseed on dough rheology are given in Table 1. The addition of flaxseed did not alter the WA and DDT. The stability value and MTI are indicators of the flour strength, with higher stability and lower MTI values suggesting a stronger dough. Flaxseed did not change the stability up to the addition level of 8%. While the MTI increased at the level of 2%, further increases did not significantly change it. A possible reason is the dilution of gluten by the added non-gluten proteins. Similar findings were observed with the addition of triticale flour to wheat flour (COSKUNER and KARABABA, 2005).

Influence of flaxseed on the viscoelastic characteristics of dough

Two parameters were used to characterize the extensigram: extensibility at maximum resistance to extension (E at R_{max}) and maximum resistance to extension (R_{max}). Extensibility represents the deformation of the dough before it ruptures. The larger the extensibility, the further the dough can be extended before it ruptures (WANG *et al.* 2003). The effects of flaxseed on viscoelastic characteristics of dough are shown in Table 2. The extensibility of the dough including flaxseed was significantly higher (p<0.05) than that of the control dough. The addition of flaxseed led to a decrease in the R_{max} value. High extensibility and low R_{max} indicates lower elasticity. A possible reason for this is the dilution of gluten by the added non-gluten proteins. Kieffer extensibility analysis gives information about the viscoelastic behaviour of a dough and measures dough extensibility and resistance to extension (WANG *et al.*, 2003). A combination of good resistance and good extensibility results in desirable dough properties (KARAOGLU, 2006).

Table 2 - Influence of flaxseed on viscoelastic properties of dough.

Substitute level (%)	E at R_{max} (mm)	R_{max} (g)
0	43.2±1.3^{d}	30.5±0.7^{a}
2	70.2±0.9^{a}	26.8±0.3^{b}
4	67.6±3.0^{b}	19.2±0.4^{c}
8	63.8±2.8^{c}	14.2±1.0^{d}

The values are given as mean±SD (n=4). Values followed by different superscripts within the same column are significantly different (p<0.05).

Influence of flaxseed on bread quality evaluation

The effect of flaxseed substitution on bread quality characteristics are summarized in Table 3.

The specific volume value of bread containing 0, 2, 4 and 8% flaxseed were 3.9, 5.5, 5.4 and 5.0 cm^3/g, respectively. The addition of flaxseed increased the specific volume. The baking test results showed that the addition of flaxseed at the 2% level gave the highest bread volume. The specific volume increased by 30, 28 and 23%, respectively. MENTES *et al.* (2008), who observed the effects of flaxseed on bread properties, determined that there was a significant increase in the specific volume value of bread with the addition of flaxseed. The effect of flaxseed on the spe-

Table 3 - Influence of flaxseed on bread quality.

Substitute level (%)	Specific volume (cm^3/g)	Crumb firmness (g) (3 h)	Crumb firmness (g) (72 h)
0	3.9±0.07^{c}	426±14.14^{a}	1423±11.31^{a}
2	5.5±0.14^{a}	227±18.38^{b}	747±4.24^{b}
4	5.4±0.21^{a}	192±4.24^{c}	730±0.70^{c}
8	5.0±0.00^{b}	153±9.19^{d}	600±13.43^{d}

The values are given as mean±SD (n=3). Values followed by different superscripts within the same column are significantly different (p<0.05).

Table 4 - TPC, TF content and antioxidant activity of breads containing flaxseed.

Substitute level (%)	TPC (mg GAE/100 g)	TF (mg QE/100 g)	DPPH inhibition (%)	TEAC (µmol Trolox)
0	0.37±0.14^{d}	0.12±0.05^{d}	29±0.0^{b}	1.10±0.21^{d}
2	0.49±0.70^{c}	0.50±0.02^{c}	35±0.0^{a}	1.21±0.17^{c}
4	0.53±0.14^{b}	0.58±0.01^{b}	35±0.7^{a}	1.41±0.11^{b}
8	0.55±0.01^{a}	0.67±0.00^{a}	37±2.8^{a}	1.93±0.16^{a}

The values are given as mean±SD (n=3). Values followed by different superscripts within the same column are significantly different (p<0.05).

cific volume value might be due to fat and protein existing in the flaxseed. When fat is used in the bread formula, it contributes to a homogeneous mix. Fat wraps around the gas cells and prevents gas release from the dough. Thus, a higher volume is obtained. Fat also protects the freshness of the bread and retards staling (ELGUN and ERTUGAY, 1997).

The texture analysis revealed that the addition of flaxseed decreased the initial crumb firmness. The firmness values of bread including 2, 4 and 8% flaxseed were lower than that of the control bread (p<0.05). Similar findings were obtained by MENTES *et al.* (2008). Flaxseed also reduced the firmness of bread during storage. The firmness of the bread containing flaxseed after 72 h of storage was lower than that of the control bread. Among the evaluated breads, the lowest firmness value was observed in bread containing 8% flaxseed. SABANIS and TZIA (2009) reported that a larger loaf size produced softer bread. Another reason might be the higher protein content of flaxseed, which contributes to the reduction of the firming rate during staling (KIM and D'APPOLONIA, 1977) and its higher water-binding capacity.

Influence of flaxseed on the TPC, TF and antioxidant activity of breads

The TPC, TF and antioxidant activities of breads are given in Table 4. The TPC values of bread ranged from 0.37-0.55 mg GAE/100 g. The TPC increased significantly with increasing flaxseed levels in the bread and the highest TPC was found in bread containing 8% flaxseed. Many studies have shown that phenolic compounds have antioxidant activity. There is a positive correlation between the TPC and antioxidant activity. The TF value of bread made with flaxseed ranged from 0.12-0.67 mg QE/100 g. The TF increased significantly with increasing flaxseed levels in bread. There is a positive correlation between the TF and antioxidant activity (SANTAS *et al.*, 2010).

The antioxidant activities of bread prepared with different levels of flaxseed substituted for wheat flour were analysed using the DPPH radical scavenging activity and TEAC assays and the results are shown in Table 4. The antioxidant activities of the bread increased significantly as the level of flaxseed substitution increased. Bread containing flaxseed (0, 2, 4, and 8%) showed 29-37% DPPH inhibition. DPPH inhibition increased by 17-22% with the addition of flaxseed. The TEAC values of the bread containing flaxseed ranged from 1.10-1.93 µmol Trolox. The TEAC values of bread significantly increased. The results showed that the addition of flaxseed greatly enhanced the antioxidant activity of bread. AMAROWICZ *et al.* (1997) found that the hydrophobic parts of flaxseed contain a higher proportion of phenolic compounds and are a more powerful antioxidant than the hydrophilic parts. Compared to other oilseeds, flaxseed contains a low amount of phenolic acids. Therefore, the antioxidant activity of flaxseed flours and extracts might be due to the presence of other types of phenolic compounds (PHILIPH, 1974).

CONCLUSION

Flaxseed improved the rheological properties and extensibility of dough. The specific volume value increased by 30, 28, and 23%. Flaxseed also reduced the firmness of the bread crust and the bread stayed fresh during storage. The addition of flaxseed improved the antioxidant activity of the bread. Overall, flaxseed could be included in the formula to improve the functionality of bread.

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PAPER

POSTHARVEST QUALITY OF 'HACHIYA' ASTRINGENT PERSIMMONS (*DIOSPYROS KAKI* L.) AS AFFECTED BY HOT WATER TREATMENT AND MODIFIED ATMOSPHERE PACKAGING

M.H. OZER, B. AKBUDAK* and I. ALTIOGLU
Department of Horticulture, Faculty of Agriculture, University of Uludag, 16059 Bursa, Turkey
*Corresponding author: Tel. +90 224 2941476, Fax +90 224 4429098,
email: bakbudak@uludag.edu.tr

ABSTRACT

This study was conducted with the aim of determining the effects of hot water and modified atmosphere packaging (MAP) on some quality changes in the astringent persimmon *cv* 'Hachiya'. The harvested fruits were immersed in 48°C water for 10 minutes, and different thickness polyethylene plastic packaging materials were used for the MAP. Treated and untreated fruits were stored at 0°±1°C and 90±5% relative humidity for 90 days. After the storage period, they were kept at 20°±1°C and 60±5% RH for 7 days to determine their shelf life. The HW+50 μ PE treatment was better compared with the other treatments.

- Keywords: persimmon, postharvest treatment, quality, storage -

INTRODUCTION

The cultivation of persimmons, which originated in China, has become common worldwide in recent years. Although when persimmons were introduced to Turkey is not exactly known, a limited amount of persimmon cultivation has been reported since ancient times (ONUR, 1990; ANONYMOUS, 2010). However, the production of persimmons has been increased by 2-2.5 fold compared with the past (ANONYMOUS, 2009). In addition to astringent cultivars, some non-astringent cultivars have been recommended for commercial planting in recent years. Extra artificial removal of astringency is not necessary because the non-astringent cultivars have a firm, edible fruit at harvest. This advantage reduces the cost of the postharvest stage. Hence, these fruits are less sensitive to handling and transportation injuries than astringent persimmon fruits. However, long-term storage may result in chilling injury, especially in the non-astringent cultivars. 'Fuyu' is chill-sensitive, but 'Hachiya' is not (CRISOSTO, 2004). The results of the research that was conducted on the storage of persimmon fruit in response to 1-methyl cyclopropene (1-MCP) treatment indicate that the chilling injury symptoms may be attributed to an altered oxidative status (ZHANG *et al.*, 2010). Despite the increase in persimmon production, the persimmon has important problems after harvest, including standardisation, storage, marketing and transportation. The shelf life of persimmons is short, and losses in quality occur due to a variety of reasons, such as weight loss, fruit softening, changes in fruit skin and changes in flesh colour, decreasing its market value and storage duration.

In the last decade, research on the persimmon has been conducted to evaluate the combined effects of 1-MCP, hot water treatments, and MAP storage. 1-MCP, a gaseous ethylene activity inhibitor, is an effective compound to delay poststorage flesh softening of 'Fuyu' persimmon fruits (AHN and CHOI, 2010). Non-astringent 'Fuyu' persimmon fruits have an excellent crispy texture due to their firm flesh. The flesh, however, softens rapidly if the fruits are exposed to an ambient temperature after low temperature storage. Softening is the most ethylene-sensitive ripening response. Therefore, CHOI (2010) investigated the ethylene production of the fruits with respect to the flesh softening and the effect of aminoethoxyvinylglycine (AVG) and 1-MCP on ethylene production and flesh firmness. They found that the AVG treatment could not only inhibit the transitional ethylene production but also shortened the time to the initiation of climacteric ethylene production. Unlike AVG, 1-MCP reduced the respiration burst and effectively retarded the softening rate at ambient temperature. In another study, 'Fuyu' persimmon fruit were treated with 500 μL/L 1-MCP for 24 h at 20°C and then stored at 4°C for 45 days to investigate the effects of 1-MCP on chilling injury (CI) during storage at 4°C. The results suggested that the reduction in the CI symptoms in persimmon fruit in response to 1-MCP treatment may be attributed to an altered oxidative status (ZHANG *et al.*, 2010). In a study, 'Fuyu' sweet persimmon fruits treated with 1-MCP (1,000 μL/L) followed by cold storage (2 months) had a higher firmness at 60, 75 and 90 days following the application of cold storage than fruits with a 1-month cold storage followed by postload 1-MCP with additional cold storage (1 month). The repeated application of 1-MCP, pre- and post-load, did not increase fruit firmness compared with a single application of preload 1-MCP. 1-MCP-treated fruits had a greater fruit diameter than the controls at 60, 75 and 90 days following the application of cold storage, but the flesh weight, soluble solid content, and electrical conductivity was not affected by 1-MCP treatment at 30, 45, 60, 75 and 90 days following the application of cold storage (CHOI *et al.*, 2012). LEE *et al.* (2010) performed a study to establish an effective method of hot-water treatment and MAP storage for disinfesting insects that disturb quarantine and the quality preservation of sweet persimmon fruits during postharvest storage and shipping. They determined that the best treatment to control insects and, at the same time, suppress fruit quality deterioration was hot-water dipping at 48°C for 10 min, which can be applied practically in the industry. In this study, the packaging treatments for persimmons of hot water (HW) and modified atmosphere (MAP) following harvest were compared singly or in combination, and the storage duration of these treatments and their effects on fruit quality were examined.

MATERIALS AND METHODS

This research was conducted at the Cold Storage Research and Practice Unit and the Postharvest Physiology Laboratory of the Department of Horticulture, Faculty of Agriculture, Uludag University. In this study, the astringent persimmon *cv* 'Hachiya' (from 10-year-old trees, grafted onto *Diospyros lotus* rootstock and planted at a spacing of 5x5 m) obtained from Yalova Ataturk Garden Cultures Central Research Institute Research and Practice Garden were used. By examining at the type-specific size, shape and colour of the fruits, the most appropriate harvest time was determined (CRISOSTO, 2004). Fruits were brought into the Postharvest Physiology Laboratory within a few hours after harvest, and the initial analyses and pre-storage treatments were completed. Then, the persimmons were stored as specified below:

a) Fruits stored at normal atmosphere (NA): The fruits in this group were stored at NA (0.03:21 [CO_2:O_2%] at 0°±1°C with 90±5% relative humidity [RH]) without any treatments and served as the control.

b) Fruits stored at MAP: The fruits in this group were stored in 35 or 50 μ PE plastic wrapping material under passive MAP conditions.

c) Fruits treated with HW+NA: The fruits in this group were dipped into HW at 48°C for 10 minutes and then stored at NA.

d) Fruits treated with HW+MAP: The fruits in this group were dipped into hot-water at 48°C for 10 minutes and then stored in 35 or 50 μ PE plastic wrapping material under MAP conditions.

After air-drying, three persimmon fruits from the treated and non-treated lots were enclosed in plastic film packages that were sealed using a Petra FS 500 plastic covering machine. The MAP studies were performed using 35 and 50 μ PE. All NA- and MAP-treated fruits were stored at 0°±1°C and 90±5% relative humidity (RH) (KITAGAWA and GLUCINA, 1984; CRISOSTO, 2004; CANDIR *et al.*, 2010). Moreover, to determine the shelf life, in addition to the storage time, the fruits were kept under room conditions at 20°±1°C with 60±5% RH for 7 days.

To determine the differences between the treatments, the quality parameters explained below were examined in the fruits at 1-month intervals during the storage.

In the study, to determine the weight loss, the fruits were numbered and individually weighed at each analysis period using a Radwag PS 3600/C1 scale (0.01 g precision, Radwag Balances and Scales, Radom, Poland), and the weight losses were calculated as the percentage loss of the initial weight. The total soluble solids (TSS) in the juice, obtained separately from the fruits in each replication, were measured using a NOW (0-32%) (Tech-Jam International Inc., Tokyo, Japan) hand refractometer. The fruit flesh firmness (FFF) was determined in kg using a Sundoo 50 SH (Digital Push Pull Gauge, Wenshou, China) penetrometer after removing the skin at the equator level in two opposing areas with a diameter of 1 cm each. During each analysis period, the titratable acidity (TA) changes in the fruits were obtained by the neutralisation of a 10 mL sample with NaOH and expressed as percentages in terms of malic acid. The pH values of the samples were measured using an Inolab (Level 1, Weilheim, Germany) digital pH meter. The skin and flesh colours of the fruits in each replication were established with a Minolta CR-300 (Konica-Minolta, Osaka, Japan) colour reading apparatus. Moreover, in the fruits, at the beginning and at 1-month intervals during the storage period, sensorial evaluations (overall appearance and taste) were performed by considering the external view, structure and flavour of the fruits by 5 panellists. In these evaluations, each fruit was given a score ranging from 1 to 5 (1: very bad, 2: bad, 3: neither bad nor good, 4: good, 5: very good), and the analyses were conducted so that there would be 3 parallels in each replication. The tests were conducted in the Postharvest Physiology Laboratory of Horticulture Department. Eighteen samples were evaluated for each session, and the samples were arranged randomly. The panellists performing the sensory analyses were academic personnel of the Horticulture Department, and they were trained in the evaluation criteria of persimmons.

A randomised plot factorial experimental design with 3 replications, consisting of 3 fruits per replicate, was used in the trial. The results were analysed using ANOVA, and the means were compared using the least significant difference (LSD) test (P<0.05).

RESULTS AND DISCUSSION

In the persimmons, there were weight losses that depended on the storage time. These losses were larger under the NA conditions compared with MAP. Moreover, there were significant differences between the treatments in terms of weight loss (Table 1). In the control group, the HW treatment had a negative effect on weight loss. Additionally, film thickness did not have any effect on the weight loss. Compared with the 50 μ PE treatment, the changes in weight loss supported the observation that the HW+50 μ PE packaging had a positive effect on the storage time of the fruit. In a study on the effects of PVC plastic wrapping material on the physical and chemical structure of *cv* 'Taubate', the fruits covered with plastic wrapping material and kept at 0°C for 72 days had a reduction in the weight loss (DEMOURA *et al.*, 1997). However, SALVADOR *et al.* (2004) tested some concentrations of 1-MCP on the persimmon *cv* 'Rojo Brillante' and found that the 1-MCP concentration did not affect the weight loss.

The increase in TSS associated with ripening in persimmons is an important parameter determining the edibility characteristics of the fruit (WRIGHT and KADER, 1996). The TSS levels fluctuate around an almost constant value. Changes in the TSS amount were also significant during storage (Table 1). In 'Hachiya', the TSS values varied from 6.00 to 8.00%, but no linear increase or decrease was observed during storage. This finding can be explained as a result of varying weight loss and respiratory rate depending on the storage treatment. In our study, when HW-treated and -untreated fruits were evaluated, the 50 μ PE treatment had the lowest increase in TSS. These fluctuations occurring in the TSS levels during storage are in agreement with the results of the study by KUZUCU and KAYNAS (2002). Similarly, CHOI *et al.* (2012) reported that 1-MCP and MAP applications did not significantly affect the TSS ratios. However, DEMOURA *et al.* (1997) found a sharp decrease in the TSS values of the fruits in their study on the persimmon *cv* 'Taubate'.

Fruit softening during the storage period is a serious problem that limits the success of shelf life as FFF values below 1.02 kg can be considered unmarketable (CRISOSTO, 2004). In our

Table 1 - Changes in weight loss, TSS, FFF, TA, pH, overall appearance and taste occurred during NA and MAP storage of persimmons.

Storage period (day)	Treatment 1	Treatment 2	Weight loss (%)	TSS[c] (%)	FFF[d] (kg)	TA[e] (%)	pH	Overall appearance	Taste
0	Control	NA	0.00 a[f]	14.00 a	5.38 a	0.69 a	5.79 a	5.00 a	1.00 a
		35 µ PE[b]	0.00 a	14.00 a	5.38 a	0.69 a	5.79 a	5.00 a	1.00 a
		50 µ PE	0.00 a	14.00 a	5.38 a	0.69 a	5.79 a	5.00 a	1.00 a
	HW[a]	NA	0.00 a	14.00 a	5.38 a	0.69 a	5.79 a	5.00 a	1.00 a
		35 µ PE	0.00 a	14.00 a	5.38 a	0.69 a	5.79 a	5.00 a	1.00 a
		50 µ PE	0.00 a	14.00 a	5.38 a	0.69 a	5.79 a	5.00 a	1.00 a
	LSD		0.00	1.78	0.14	0.56	0.09	0.06	0.06
30	Control	NA	2.13 bc	14.25 b	3.06 bc	0.72 a	5.97 a	5.00 a	1.00 c
		35 µ PE	0.00 d	16.33 a	4.18 b	0.54 a	5.67 b	4.00 b	1.50 b
		50 µ PE	2.83 b	15.50 ab	5.44 a	0.70 a	5.64 b	4.00 b	1.50 b
	HW	NA	5.39 a	16.50 a	2.16 c	0.67 a	5.53 b	5.00 a	2.00 a
		35 µ PE	0.56 cd	16.50 a	5.28 a	0.75 a	5.58 b	5.00 a	2.00 a
		50 µ PE	1.14 bcd	14.33 b	5.65 a	0.52 a	5.90 a	5.00 a	2.00 a
	LSD		1.79	1.92	1.07	0.32	0.22	0.06	0.06
60	Control	NA	6.81 b	11.50 b	1.53 c	0.35 b	6.16 bc	4.00 b	1.50 b
		35 µ PE	0.25 c	7.33 c	3.20 b	0.33 b	6.29 ab	4.00 b	1.50 b
		50 µ PE	7.98 b	6.33 c	3.69 ab	0.38 b	6.33 ab	4.00 b	1.50 b
	HW	NA	18.43 a	14.00 a	0.84 c	0.37 b	6.49 a	3.00 c	2.00 a
		35 µ PE	0.56 c	9.67 b	4.33 a	0.64 a	6.04 c	5.00 a	2.00 a
		50 µ PE	1.41 c	11.33 b	3.98 ab	0.48 ab	5.71 d	5.00 a	2.00 a
	LSD		1.87	2.00	1.07	0.23	0.21	0.06	0.06
90	Control	NA	14.61 b	14.00 a	2.20 abc	0.14 ab	6.06 d	4.00 c	2.00 c
		35 µ PE	0.76 d	12.33 b	1.20 c	0.15 ab	6.35 bc	4.00 c	2.50 b
		50 µ PE	7.98 c	15.00 a	3.23 ab	0.26 ab	6.35 bc	3.50 d	2.50 b
	HW	NA	27.57 a	14.50 a	1.32 bc	0.20 ab	6.20 c	3.00 e	4.00 a
		35 µ PE	0.56 d	11.67 b	2.75 abc	0.30 a	6.40 ab	4.50 b	4.00 a
		50 µ PE	1.72 d	14.00 a	3.69 a	0.11 b	6.51 a	5.00 a	4.00 a
	LSD		1.99	1.44	1.97	0.17	0.15	0.06	0.06
90+7	Control	NA	26.60 b	15.50 b	0.75 cd	0.14 a	5.95 b	1.50 e	3.00 c
		35 µ PE	0.76 d	14.67 bc	2.02 a	0.27 a	4.61 d	3.00 b	3.50 b
		50 µ PE	7.98 c	13.67 cd	1.42 ab	0.17 a	5.72 c	2.00 d	3.50 b
	HW	NA	42.25 a	16.83 a	0.17 d	0.22 a	6.04 ab	2.50 c	5.00 a
		35 µ PE	0.56 d	13.33 d	0.18 d	0.13 a	6.16 a	3.00 b	5.00 a
		50 µ PE	1.72 d	12.67 d	1.10 bc	0.23 a	6.15 a	4.00 a	5.00 a
	LSD		1.80	1.19	0.64	0.19	0.20	0.06	0.06

[a]HW: Hot water; [b]PE: Polyethylene; [c]TSS: Total soluble solids; [d]FFF: Fruit flesh firmness; [e]TA: Titratable acidity; [f]Different letters in the same column indicate significant differences, $P<0.05$.
LSD, least significant difference.

study, the FFF evaluations indicated that the HW and packaging material treatments had an important effect during storage. During storage, significant differences with a continuous decrease were observed between the FFF values. Compared with the control samples, an examination of the values obtained at the beginning and during storage revealed that the fruits covered with the 50 µ PE wrapping material yielded more successful results in terms of FFF (Table 1). Generally, the HW treatment caused a decrease in the FFF. Moreover, OZDEMIR *et al.* (2009) reported similar results, showing that a significant decrease occurred in the FFF values in correlation with the length of the storage time. Similarly, in a different study with *cv* 'Fuyu', after the fruits were kept for 3 months under MA at 2°C, the fruit flesh browning was higher in the control treatment, and softening in the control group fruits occurred faster (CHAE *et al.*, 2004). In another study conducted with the same cultivar, 1-MCP, an ethylene action blocker, reduced the respiration burst and effectively retarded the softening rate at ambient temperature (CHOI, 2010).

The changes in the TA ratios during storage are given in Table 1. Reductions, which were not very significant, occurred during the NA and MAP storage. The TA values varied from 0.11 to 0.75% during storage. According to our findings, there was a linear decrease in the TA values. For all samples, the TA decreased with time, and there were no treatment effects (HW and MAP) or film thickness effects (35 and 50 µ) (Table 1). Howev-

er, at the end of the 60th day, there was a sudden decrease in the TA values of the fruits kept under the NA and MAP conditions. During this period, the TA values varied from 0.33 to 0.64%. When the values obtained at the beginning and during storage were examined, the fruits in the control group covered with the 35 μ PE packaging material yielded successful results with respect to the TA values compared with those treated with HW and covered with 50 μ PE packaging material. Moreover, our results are in agreement with those of USTUN *et al.* (1997), who reported similar physical and chemical structures and sensorial characteristics of the persimmon *cvs*.

The postharvest pH value of *cv* 'Hachiya' was 5.79. The pH increased slightly in all samples during storage (Table 1). Moreover, the statistical analysis indicated that the varying means of the treatments were significantly different (Table 1). The highest pH value was obtained in the HW-treated fruits packaged with 35 and 50 μ PE. In the HW-treated fruit, the best results in terms of pH were obtained with the HW+50 μ PE treatment. Moreover, KUZUCU and KAYNAS (2002) reported that the pH values showed a continuous declining trend in a similar study.

The evaluation of the sensorial quality criteria of the fruits revealed that the overall appearance and taste changed. Overall, at the end of storage and at the shelf life of 90+7 days, the fruits subjected to the HW+MAP treatment had the highest values. The flavour test findings indicated that the packaging materials used in the study had an important effect on the eating quality of the fruit (Ta-

Table 2 - Changes in fruit skin and flesh colour occurred during NA and MAP storage of persimmons.

Storage period (day)	Treatment 1	Treatment 2	Fruit skin colour			Fruit flesh colour		
			L	a	b	L	a	b
0	Control	NA	47.32 a[c]	4.18 a	25.49 a	58.46 a	7.17 a	27.03 a
		35 μ PE[b]	47.32 a	4.18 a	25.49 a	58.46 a	7.17 a	27.03 a
		50 μ PE	47.32 a	4.18 a	25.49 a	58.46 a	7.17 a	27.03 a
	HW[a]	NA	47.32 a	4.18 a	25.49 a	58.46 a	7.17 a	27.03 a
		35 μ PE	47.32 a	4.18 a	25.49 a	58.46 a	7.17 a	27.03 a
		50 μ PE	47.32 a	4.18 a	25.49 a	58.46 a	7.17 a	27.03 a
	LSD		0.99	4.44	0.66	2.98	1.78	2.25
30	Control	NA	47.84 ab	14.62 bc	28.24 ab	52.67 a	7.47 c	22.85 b
		35 μ PE	49.70 ab	22.74 ab	29.47 a	56.20 a	14.62 a	28.93 a
		50 μ PE	49.93 a	14.10 bc	28.53 a	55.43 a	12.27 ab	28.68 a
	HW	NA	47.52 ab	24.37 a	27.37 ab	57.31 a	14.94 a	29.74 a
		35 μ PE	48.37 ab	19.88 abc	28.30 ab	57.70 a	11.41 ab	28.63 a
		50 μ PE	46.07 b	11.94 c	25.65 b	58.74 a	8.58 bc	27.28 a
	LSD		3.65	9.20	2.85	6.48	3.86	3.43
60	Control	NA	44.98 b	24.77 a	26.81 ab	21.58 c	2.34 c	6.00 c
		35 μ PE	30.90 c	5.78 c	12.73 c	26.30 c	2.62 c	9.03 c
		50 μ PE	25.48 d	1.97 c	8.40 d	25.10 c	2.27 c	8.08 c
	HW	NA	46.00 b	21.60 a	26.82 ab	42.40 b	12.06 ab	22.23 b
		35 μ PE	44.93 b	13.80 b	24.80 b	55.87 a	8.18 b	24.16 ab
		50 μ PE	50.55 a	20.52 a	29.54 a	57.70 a	12.89 a	27.74 a
	LSD		4.41	5.65	3.34	7.45	4.71	3.96
90	Control	NA	62.01 a	19.73 ab	59.89 ab	42.52 b	1.90 b	15.86 d
		35 μ PE	50.16 b	12.18 b	42.79 b	40.07 b	4.23 b	21.07 cd
		50 μ PE	61.88 a	14.12 b	60.23 a	64.30 a	17.29 a	60.03 a
	HW	NA	55.34 ab	22.66 ab	46.98 ab	54.25 ab	6.91 b	35.86 bc
		35 μ PE	63.49 a	29.82 a	63.91 a	58.96 a	6.47 b	42.28 b
		50 μ PE	57.08 ab	15.05 b	52.24 ab	65.21 a	7.11 b	38.65 b
	LSD		9.77	13.64	17.39	15.31	7.90	15.10
90+7	Control	NA	43.02 b	12.56 ab	22.09 b	33.42 cd	3.44 b	13.58 b
		35 μ PE	41.60 b	12.97 ab	27.09 b	31.16 d	0.54 c	13.18 b
		50 μ PE	45.71 ab	12.59 ab	36.08 ab	36.62 bcd	3.16 bc	16.35 b
	HW	NA	46.57 ab	17.07 a	31.01 b	40.18 bc	6.12 a	24.04 b
		35 μ PE	55.49 a	18.00 a	49.49 a	51.14 a	5.74 ab	43.90 a
		50 μ PE	57.44 a	11.31 b	37.38 ab	42.12 b	3.24 b	25.68 b
	LSD		10.69	8.92	17.59	7.36	2.65	13.47

[a]HW: Hot water; [b]PE: Polyethylene; [c]Different letters in the same column indicate significant differences, $P<0.05$.
LSD, least significant difference.

ble 1). KOBILER *et al.* (2011) and LEE *et al.* (2010) doused the *cv* 'Triumph' with chlorinated water and HW and stored them under MA conditions. In agreement with our findings, the investigators found that those treatments removed the effects of fungal and insect damage on the fruits, maintaining the general view of the fruits and extending their storage time. In another study that was conducted on insect disinfestation and the quality changes of 'Fuyu' persimmon fruit influenced by hot-water treatment methods and MAP storage, a higher visual fruit quality was observed after hot-water brushing compared with hot-water dipping (LEE *et al.*, 2010).

The skin colour measurements of the 'L' and 'a' values of the fruits showed fluctuation, both increasing and decreasing. During the storage of the fruits, the colour changed from green-orange to orange. When the colour measurements of the 'L' and 'a' values at the beginning and end of storage were compared, the fruits packaged with 50 µ PE gave the closest results to beginning (Table 2). The fruit flesh colour values (L, a, b) are given in Table 2. At the beginning of storage, no significant changes occurred in the fruit flesh colour. Generally, the fruit flesh colour in the control treatment changed in monthly intervals, but no significant change was observed in the fruit flesh colour in the HW+MAP treatment during storage. However, toward the end of the storage time, colour changes to dark orange were also observed in the fruit flesh colour. Our results are in agreement with those of KUZUCU and KAYNAS (2002), who found increases and decreases in the fruit skin colour during storage associated with the effects of the different packaging types on the shelf life, ripening and quality of the persimmons.

In conclusion, the present study investigating the effects of HW and different packaging materials on the quality criteria of persimmon fruits were examined singly and in combination revealed that these treatments were more successful in terms of the examined compared with the controls. Moreover, among the treatments, covering the fruits with 50 µ PE packaging material was determined to be more promising with respect to storage time and fruit quality.

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PAPER

ANTIOXIDANT ACTIVITY OF PROBIOTIC YOGHURT FORTIFIED WITH MILK PROTEIN BASED INGREDIENTS

G. UNAL[1,*], S.N. EL[2], A.S. AKALIN[1] and N. DINKCI[1]
[1]Ege University, Faculty of Agriculture, Department of Dairy Technology, 35100 Izmir, Turkey
[2]Ege University, Faculty of Engineering, Department of Food Engineering, 35100 Izmir, Turkey
*Corresponding author: Tel. +90 232 3112732, Fax +90 232 3425713,
email: gulfem.unal@ege.edu.tr

ABSTRACT

The effect of the fortification with sodium calcium caseinate (SCC) and whey protein concentrate (WPC) on antioxidant activity of probiotic yoghurt, containing *Bifidobacterium lactis* Bb12, was investigated. Probiotic yoghurt fortified with WPC had a higher DPPH$^{\bullet}$ (2,2,-diphenyl-1-picrylhydrazyl) radical-scavenging activity when compared to that fortified with SCC. In Fe^{2+} chelating activity method, probiotic yoghurts fortified with SCC and a blend of SCC-WPC showed greatest chelating activity by 30 and 60 min. There were no significant differences in the H_2O_2 scavenging activity among samples except control yoghurt, which showed the lowest activity, at a concentration of 0.2 g/mL.

- Keywords: antioxidant activity, probiotic yoghurt, sodium calcium caseinate, whey protein concentrate -

INTRODUCTION

Oxidation is essential to many living organisms for the production of energy to fuel biological processes (UNAL and AKALIN, 2006). Free radicals and active oxygen species are physiological metabolites formed as a result of respiration in aerobic organisms. Any excessive amount of reactive radicals can damage all types of cellular macromolecules, including proteins, carbohydrates, lipids, and nucleic acids, leading to cell death and tissue damage (UNAL and AKALIN, 2006; FURST, 2009). This cellular damage can initiate the risk of several diseases including atherosclerosis, arthritis, diabetes, and cancer (SARMADI and ISMAIL, 2010; ANDRÉ *et al.*, 2010). It is also well-known that lipid oxidation occurring in food products causes deteriorations in food quality, like rancid flavor, unacceptable taste, and shortening of shelf life (PIHLANTO, 2006).

Milk proteins are regarded as a source of energy and essential amino acids, which are needed for growth and maintenance of physiological functions (SARMADI and ISMAIL, 2010). Bioactive peptides are considered to be specific protein fragments that are inactive within the sequence of the parent protein. They liberate from milk proteins by the enzymatic hydrolysis with digestive enzymes or by the fermentation of milk by proteolytic starter cultures and action of enzymes derived from these microorganisms. After they are released during gastrointestinal digestion or food processing they may exert various physiological functions (SARMADI and ISMAIL, 2010). One of these functions of bioactive peptides derived from milk proteins is antioxidant activity. Studies have shown that casein and whey proteins have antioxidant properties, presumably based on their ability to bind transition metals and scavenge free radicals (RIVAL *et al.*, 2001).

In recent years, the consumption of yoghurt has increased due to the fact that it fulfils many dietary needs. In addition to its health effects, yoghurt has proved to be an excellent vehicle for the production of functional foods, especially those containing probiotic bacteria and/or milk protein based ingredients. As there is no difference in gross composition between yoghurt and milk, which could explain the high antioxidant capacity, it is assumed that peptides and amino acids liberated during fermentation play a significant role (FARVIN *et al.*, 2010b). Antioxidant activity has been reported for milk proteins (WOO *et al.*, 2009), sodium caseinate and whey protein concentrate (SUGIARTO *et al.*, 2009) and yoghurt (MCCUE and SHETTY, 2005; FARVIN *et al.*, 2010a; 2010b). The antioxidative ability of lactic acid bacteria has been shown by many researchers (KAIZU *et al.*, 1993; SANDERS *et al.*, 1995; LIN and YEN, 1999; KUDOH *et al.*, 2001; LIU *et al.*, 2005; SAIDE and GILLILAND, 2005; KIM *et al.*, 2005; VIRTANEN *et al.*, 2007; LIU and PAN, 2010; WANG *et al.*, 2011; MONAJJEMI *et al.*, 2012). The antioxidant activity of probiotic bacteria, especially Bifidobacteria, has also been investigated by both *in vitro* and *in vivo* studies (KANSAL, 2009; SHEN *et al.*, 2010; 2011; HUANG *et al.*, 2011; XU *et al.*, 2011; RAJPAL and EJTAHED *et al.*, 2012). Most of these researchers concluded that the development of antioxidant activity was strain-specific characteristic. Furthermore, WANG *et al.* (2006) investigated the antioxidant activity of fermented soymilk and concluded that soymilk fermented with lactic acid bacteria and bifidobacteria exhibited a significantly higher antioxidant activity that did the soymilk fermented lactic acid bacteria or bifidobacteria alone. To the best of our knowledge, the antioxidant activity of probiotic yoghurt and the influence of the fortification with milk protein based ingredients on this activity have not yet been studied. Furthermore, hydrogen peroxide scavenging activity of yoghurt, as an indicator of antioxidant capacity, has not been previously investigated.

The objective of this work was to evaluate the antioxidant activity of probiotic yoghurt, containing *B. animalis* subsp. *lactis*, and the influence of the fortification with sodium-calcium caseinate and whey protein concentrate on this activity. To perform this study, a set of *in vitro* antioxidant assays, including the 2,2,-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging effect, inhibition of hydrogen peroxide (H_2O_2), and Fe^{2+} chelating activity, were used.

MATERIALS AND METHODS

Strains and ingredients

The commercial yoghurt starter culture, containing *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (YC X-11), and *Bifidobacterium animalis* subsp. *lactis* (Bb-12) were in a freeze-dried direct vat set (DVS) form containing 10^{11} and 10^{10} cfu g^{-1}, respectively, and were obtained from Chr. Hansen A/S, Hørsholm, Denmark. Cultures were stored after procurement according to the manufacturer's recommendation.

Skim milk powder (SMP) and whole milk were kindly provided by Pinar Dairy Products, Izmir, Turkey. Other dairy ingredients, whey protein concentrate (WPC, Oragel DY 101 XP) and sodium calcium caseinate (SCC) were obtained from Armor Proteinés, Saint Brice en Coglés, France. The concentrations of protein in the supplements were 33.7 (±0.79), 80 (±2.7), and 88.0 (±2.8) g/100 g for SMP, WPC, and SCC, respectively.

Yoghurt preparation

Set-type probiotic yoghurt was prepared by using whole milk containing 3.65 % (w/v) milk fat that was standardized with SMP to obtain

100 g L^{-1} of non-fat milk solids. The milk was divided into four lots and supplemented with 2 % SMP, SCC, WPC, and the blend of (1:1) SCC-WPC. After they were mixed properly, each milk base was heated at 85°C for 30 min, by circulation in a hot water bath and cooled to 43°C in an ice bath. At this point, they were inoculated with both yoghurt starter culture and *B. lactis*. All cultures were used according to the manufacturer's instructions. Yoghurt starter culture was poured into 1 L sterilized milk at 40°C and mixed thoroughly, and then 4 mL of the mix was added for each 1 L of mixture. *B. lactis* was also added as 0.01%. The mixtures were then put into 200 mL plastic containers and incubated at 40°C for about 4.5 h until a pH 4.70 was reached. After fermentation, the yoghurt samples were cooled and transferred to a refrigerator and stored at 4°C for analysis. Analyses to determine the antioxidant activity of the samples were run within the first week of yoghurt production.

Chemicals and equipment

Trolox (6-Hydroxy-2, 5, 7, 8- tetramethylchroman-2- carboxylic acid, Cat: 23,881-3), DPPH• (2,2,-diphenyl-1-picrylhydrazyl, D-9132), and ferrozine [3-(2-Pyridyl)-5,6-diphenyl-1, 2, 4-triazine-4", 4"-disulfonic acid sodium salt, P-5338] were purchased from Sigma-Aldrich (Steinheim, Germany). All other reagents and solvents commercially obtained were of analytical grade. All spectrophotometric data were acquired using a Cary 50 Scan UV-Visible spectrophotometer (UK).

Determination of the Scavenging Activity on the DPPH· Radical

The DPPH• scavenging activity of the probiotic yoghurt samples was estimated according to the procedure described by MCCUE and SHETTY (2005) with minor modifications. A 0.1 mM DPPH• radical solution in 95% ethanol was prepared. Eight milliliters of ethanolic DPPH• solution was placed in a 50 mL centrifuge tube and mixed with 2 mL of sample or 95% ethanol (as control), vortexed well, and then incubated for 30 min at room temperature. The samples were then centrifuged for 5 min at 13,500 rpm at room temperature. Supernatants were filtered using Whatman No 40 filter paper. Absorbance of each sample at 517 nm was measured. Trolox was used as a reference antioxidant at a concentration of 0.25 mg/mL.

DPPH• scavenging activity percent was calculated as follows:

DPPH• scavenging activity (%) =
= [(control absorbance-extract absorbance) /
/ (control absorbance)] x 100

Determination of the Fe^{2+} chelating activity

The chelating activity of the samples on Fe^{2+} was measured according to the method described by YEN and WU (1999) and EL and KARAKAYA (2004) with minor modifications. One milliliter of sample (1 g/mL) was mixed with 3.7 mL deionized water. Each sample was incubated with 0.1 mL $FeCl_2 4H_2O$ (2.0 mM) for 0, 10, 30 and 60 min. After incubation, the reaction was initiated by addition of 0.2 mL ferrozine (5.0 mM). The mixture was then shaken vigorously and left at room temperature for 10 min. The absorbance of the mixture (formation of the ferrous iron-ferrozine complex) was measured at 562 nm. The control was performed in the same way using water instead of sample. EDTA (0.1 mg/mL) was also run in the same way for comparison. The chelating activity was calculated as follows:

Fe^{2+} chelating activity (%) = [1-(absorbance of sample / absorbance of control)] x 100

Determination of the Scavenging Activity of H_2O_2

Five and 10 g yoghurt samples were dissolved in 50 mL distilled water (at 0.1 and 0.2 g/mL concentrations), centrifuged at 9,500 rpm for 10 min at room temperature. The supernatants are then filtered using Whatman No 40 filter paper.

The ability of the samples to scavenge H_2O_2 was determined spectrophotometrically (EL and KARAKAYA, 2004). One milliliter (0.1 and 0.2 g/mL) of sample was mixed with 3.4 mL of 0.1 M phosphate buffer (pH 7.4), and then 0.6 mL of a 43 mM solution of H_2O_2 in the same buffer added. Absorbance values were measured at 0 and 40 min at 230 nm. The values of the reaction mixtures were recorded against a blank solution without H_2O_2 for each sample. The concentration (mM) of H_2O_2 in the assay medium was determined using a standard curve. For the standard curve, 3.4 mL phosphate buffer was added to 0.6 mL of 10, 15, 25, 43, and 50 mM solutions of H_2O_2. The equation was determined by linear regression as follows:

$$A_{(230nm)} = (9.4x10^{-3}) \times C\ (H_2O_2, mM) + 1.13x10^{-2}\ (r^2=0.9993)$$

Trolox was used as the reference antioxidant at a concentration of 50 ppm. H_2O_2–scavenging activity of the samples was calculated using the following equation:

Scavenging activity (%) = 100 -
+ (H_2O_2 concentration of medium /
/ H_2O_2 concentration of control) x 100

Statistical analysis

The experiments, including yoghurt manufacture, were performed in triplicate and parallel.

Six values for each sample were averaged (n=6). The data obtained were processed by one-way ANOVA using the general linear model (GLM) procedure of the SPSS version 11.05 (SPSS Inc., Chicago, IL, USA). The means were compared with the Duncan test at $p < 0.05$ level.

RESULTS AND DISCUSSION

Several methods have been developed to evaluate the antioxidant capacity of food compounds or biological systems. However, none of them can be used as a universal standardized method. For different reasons linked to the mechanisms, endpoint, quantification method, analysis conditions, and substrate used, there is often a lack of correlation between results obtained through different assays. This is the reason why, in this study, three *in vitro* tests were used to get a better understanding on the antioxidant activity of the samples.

Antiradical activity of samples against to DPPH•

The antioxidative mechanism of antioxidants can result from metal chelation, free radical scavenging, or cooperative effects of these properties (RIVAL *et al.*, 2001). The DPPH• radical is long-lived organic nitrogen radical and DPPH• method is based on the ability of a sample to scavenge the free stable radical DPPH• by hydrogen donation (SMET *et al.*, 2008; KARADAG *et al.*, 2009).

The DPPH• scavenging activity of the probiotic yoghurt samples are shown in Fig. 1. The probiotic yoghurt extracts really possessed high DPPH• scavenging activity that ranged from 90 to 93%. Trolox at a concentration of 0.25 mg/mL showed a DPPH• scavenging activity of 81.02%. The probiotic yoghurt fortified with WPC had a higher scavenging activity when compared to that fortified with SCC ($p<0.05$). This higher scavenging effect of the whey proteins can be attributed to lactoferrin which has been reported as a key component for high scavenging activity (SHINMOTO *et al.*, 1992; CHIANG and CHANG, 2005). In addition, α-lactalbumin (SADAT *et al.*, 2011) and β-lactoglobulin (HERNÁNDEZ-LEDESMA *et al.*, 2005; DEL MAR CONTRERAS *et al.*, 2011) may also contribute to enhancing the scavenging activity of yoghurt. The higher DPPH scavenging activity of yoghurt fortified with WPC can be also attributed to the higher viable counts of *L. bulgaricus* when compared to the viable counts of *L. bulgaricus* in yoghurt containing SCC (data not shown). This is supported by the results of KUDOH *et al.* (2001) who determined a peptide with DPPH scavenging activity found in milk fermented with *L. bulgaricus.* The sample fortified with the blend of (1:1) SCC-WPC showed DPPH• scavenging activity as high as the sample fortified with WPC. The antioxidant activities of some fermented dairy products were found similar to our results. FARVIN *et al.* (2010a) studied the antioxidant activity of different fractions of yoghurt and found the DPPH• radical scavenging activity of crude yoghurt (0.2 mg/mL) to be 94.47%. The authors suggested that the oxidative stability of yoghurt might be due to antioxidant peptides released during the fermentation of milk by lactic acid bacteria. They also concluded that these peptides act as electron donors and could react with free radicals to convert them to more stable products. MCCUE and SHETTY (2005) also investigated the DPPH• scavenging activity of soy yoghurt produced by Kefir cultures and reported the activity as 92.3% after 48 h of production.

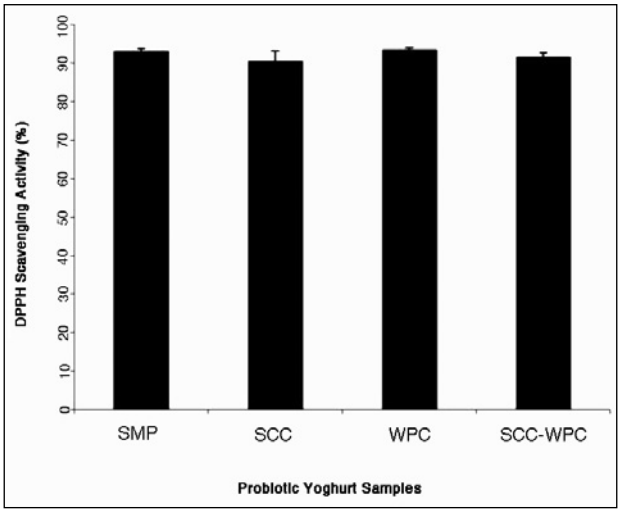


Fig. 1 - DPPH scavenging activity (%) of probiotic yoghurt samples.
SMP: control probiotic yoghurt containing skim milk powder; SCC: probiotic yoghurt containing sodium calcium caseinate; WPC: probiotic yoghurt containing whey protein concentrate; SCC-WPC: probiotic yoghurt containing the blend of (1:1) sodium calcium caseinate and whey protein concentrate.

Chelating effect of samples on Fe^{2+}

Iron is essential for life, because it is required for oxygen transport, respiration, and the activity of many enzymes (DUH *et al.*, 2001). However, it may work as a catalyst for the generation of reactive oxygen species in pathological conditions. The reduced form of iron potentates oxygen toxicity by converting, via the Fenton reaction, the less reactive hydrogen peroxide to the more reactive oxygen species, the hydroxyl radical (OH•) and the ferryl ion (LIU *et al.*, 2005). Therefore, minimization of the Fe^{2+} concentration in the Fenton reaction affords protection against oxidative damage (RIVAL *et al.*, 2001).

All probiotic yoghurts, except the control sample showed more than 60% chelating effect on ferrous ions for an incubation time of 60 min (Table 1). The increase in incubation time sig-

Table 1 - Iron chelating activity of probiotic yoghurt samples.

Sample	0 min	10 min	30 min	60 min
SMP	18,13±8,23^{cA}	36,62±15,58^{bA}	46,85±10,44^{abB}	54,69±7,23^{aC}
SCC	22,46±5,95^{cA}	45,17±8,72^{bA}	52,84±7,94^{bAB}	77,11±6,61^{aA}
WPC	22,02±7,53^{dA}	35,84±7,82^{cA}	49,38±7,05^{bB}	64,62±5,56^{aB}
SCC-WPC	32,38±11,85^{cA}	50,81±10,40^{bA}	66,18±9,69^{aA}	80,15±3,51^{aA}

SMP: control probiotic yoghurt containing skim milk powder; SCC: probiotic yoghurt containing sodium calcium caseinate; WPC: probiotic yoghurt containing whey protein concentrate; SCC-WPC: probiotic yoghurt containing the blend of (1:1) sodium calcium caseinate and whey protein concentrate.
$^{A-C}$: Means in the same column with different superscripts are significantly different ($p<0.05$);
$^{a-d}$: Means in the same row with different superscripts are significantly different ($p<0.05$).

nificantly improved the iron-chelating activities of all probiotic yoghurt types. When the sample was not incubated with $FeCl_2$, and the reaction was initiated at zero time by the addition of ferrozine. The capability to chelate the ferrous ion of samples was recorded with the increasing incubation times with $FeCl_2$. The iron-chelating activity of all samples varied 18.13-32.38% at zero time. The chelating activity of yoghurt fraction (10-30 kDa) was determined as 28.15% in the study of FARVIN *et al.* (2010a) at an incubation time of 3 min.

No significant differences were determined among samples in 10 min of incubation time whereas, probiotic yoghurts fortified with SCC and the blend of SCC-WPC showed greatest iron-chelating activities by 30 and 60 min. The binding of iron (ferrous sulfate) to two commercial milk protein products, sodium caseinate and whey protein isolate (WPI), was examined by SUGIARTO *et al.* (2009). Sodium caseinate showed more iron binding sites than WPI, and a higher affinity for iron attributed to the casein's phosphoserine clusters, known to strongly bind divalent cations. The metal ion chelating actions of casein peptides and the inhibition of lipid oxidation in different model systems have also been reported by several researchers (RIVAL *et al.*, 2001; DIAZ *et al.*, 2003; KANSCI *et al.*, 2004). Furthermore, CERVATO *et al.* (1999) demonstrated that casein exhibited inhibitory action against Fe-induced peroxidation.

The iron-chelating activity of probiotic yoghurt samples ranged from 46.85 to 66.18% at 30 min of incubation time. However, EDTA showed more effective chelation capacity (99.54%) than samples at a lower concentration (0.1 mg/mL) at an incubation time of 30 min.

Several researchers have investigated the ability of milk proteins to bind ferric or ferrous ions, e.g., lactoferrin, serum albumin, casein, and a high molecular weight fraction of whey, all of which have been reported to demonstrate some iron-chelating activity (MEUCCI *et al.*, 1991; TONG *et al.*, 2000). In general, milk fractions containing a greater number of phosphoseryl serine groups reveal a greater affinity for iron, although the carboxyl group of the amino acids asparagines and glutamine can bind iron as well (WONG and KITTS, 2003). To the best of our knowledge, there is no study that has previously focused on the iron-chelating activity of probiotic yoghurt fortified with milk protein based ingredients. According to our results, it can be concluded that fortification of yoghurt with SCC or the blend of SCC-WPC increases iron-chelating activity more than fortification with only WPC.

H_2O_2 scavenging activity of samples

Hydrogen peroxide is regarded as poorly reactive because of its weak oxidizing capability but it can form a highly reactive hydroxyl radical through the Fenton reaction (YEN and DUH, 1994; LEE *et al.*, 2004). As shown in Fig. 2, the samples are capable of scavenging H_2O_2 in concentration-dependent fashion after 40 min of incubation. None of the concentrations of samples assayed could completely remove H_2O_2 from the assay medium. The highest ($p < 0.05$) inhibition

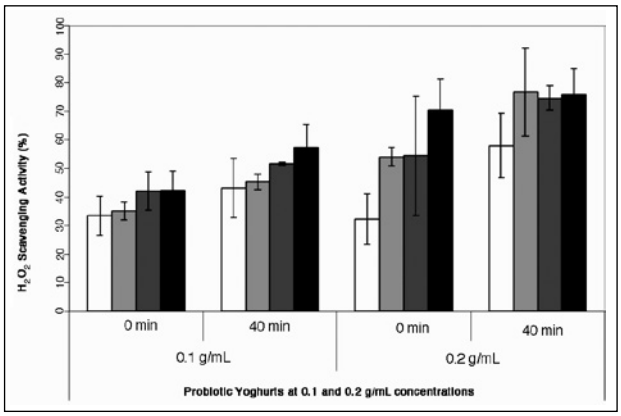


Fig. 2 - H_2O_2 scavenging activity (%) of probiotic yoghurt samples.
□ (SMP): control probiotic yoghurt containing skim milk powder;
▒ (SCC): probiotic yoghurt containing sodium calcium caseinate;
▒ (WPC): probiotic yoghurt containing whey protein concentrate;
■ (SCC-WPC): probiotic yoghurt containing the blend of (1:1) sodium calcium caseinate and whey protein concentrate.

values were obtained for probiotic yoghurts fortified with WPC and the blend of SCC-WPC at the concentration of 0.1 g/mL. The higher H_2O_2 scavenging activity of yoghurt fortified with WPC when compared to that of fortified with only SCC can be due to the higher viable counts of *B. lactis* (data not shown). This result can be supported by the study of LIN and YEN (1999) who detected the highest H_2O_2 scavenging ability in Bifidobacteria among lactic acid bacteria. KIM *et al.* (2005) investigated the antioxidant activity of several Lactobacillus species and concluded that *L. bulgaricus* LB207 showed a strong hydroxyl radical scavenging activity. So, as WPC added yoghurt had higher viable counts of *L. bulgaricus* (data not shown) than that of fortified with only SCC, this could have caused a higher H_2O_2 scavenging activity. At 0.2 g/mL concentration there were no significant differences ($p > 0.05$) in the scavenging activity among the samples, except control yoghurt which showed lower values ($p < 0.05$). Some researchers have reported the role of milk protein fractions in the scavenging activity of H_2O_2 (LINDMARK-MÅNSON and ÅKESSON, 2000; KITTS, 2005). KITTS (2005) informed that the sequestering of ferrous ion by casein phosphopeptides corresponded to reducing the Fenton reaction generated hydroxyl radical ($OH^{\bullet}$) formation. In addition, lactoferrin has been said to have an influence on the conversion of hydrogen peroxide into the hydroxyl radical which can be controlled by the availability of iron ions (LINDMARK-MÅNSON and ÅKESSON, 2000). In our study, Trolox, at a concentration of 50 ppm, showed 28 and 29% H_2O_2 scavenging activity in 0 and 40 min, respectively. No previous study has investigated the H_2O_2 scavenging activity of probiotic yoghurt and also the influence of fortification with milk based ingredients on this activity. Our results indicated the effective use of SCC and WPC in the fortification of probiotic yoghurt in terms of H_2O_2 scavenging activity.

CONCLUSIONS

In conclusion, the present study demonstrated that fortification of probiotic yoghurt, containing *B. animalis* subsp. *lactis*, with milk protein based ingredients increases the antioxidant activity in comparison to the fortification with skim milk powder. Determination of a free radical and H_2O_2 scavenging activity and iron-chelating effect are important parameters in evaluating the *in vitro* antioxidant capacity. The antioxidant activity of the samples determined with different methods cannot be compared. Because these methods differ from each other in terms of reaction mechanisms, oxidant, reaction conditions, comparisons may thus not be appropriate (EL and KARAKAYA, 2004; KARADAG *et al.*, 2009). Using $DPPH^{\bullet}$ radical-scavenging method, fortification of probiotic yoghurt with whey protein concentrate was more advantageous than fortification with sodium-calcium caseinate. On the other hand, probiotic yoghurt fortified with sodium-calcium caseinate had higher Fe^{2+} chelating activity than fortified with whey protein concentrate. Fortification with sodium-calcium caseinate or whey protein concentrate caused similar effects on H_2O_2 scavenging activity. As antioxidant activity occurs by different mechanisms, employing a method depending on one mechanism may not reflect the true antioxidant capacity. Therefore, in order to gain a good understanding on the antioxidant properties of dairy products, the use of several different methods is necessary (CHEN *et al.*, 2003). In all methods, yoghurt fortified with the blend of sodium-calcium caseinate and whey protein concentrate showed antioxidant activity as high as that fortified with only one ingredient. Therefore, using a blend of these two ingredients can be recommended in the fortification of yoghurt in order to obtain an increase in the antioxidant activity.

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APPENDIX

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PAPER

CRUDE FIBRE, WATER EXTRACTS, TOTAL ASH, CAFFEINE AND MOISTURE CONTENTS AS DIAGNOSTIC FACTORS IN EVALUATING GREEN TEA QUALITY

S.O. AROYEUN
Cocoa Research Institute of Nigeria, PMB, 5244 Ibadan, Oyo State, Nigeria
Tel. +234 8059158795,
email: aroyeun2000@yahoo.co.uk

ABSTRACT

This experiment was carried out to diagnose the qualities of green tea from Nigeria using some quality markers like crude fibre, water extracts, total ash, caffeine and moisture contents respectively.

Green Tea was processed from seven different tea clones from Mambilla Highland located on 1840m above mean sea level. The clonal materials used were processed into green tea using Chinese methods viz: Plucking, Fixing (Pan firing), Rolling and Drying. The green tea produced were analyzed for moisture contents, (MC); Crude Fibre, (CF).; Water Extract, (WE).; Total Ash, (TA).; Water Soluble Ash, (WSA).; Alkaline Insoluble Ash, (ALIA) and Acid Insoluble Ash, (AIA). The quality of the green tea produced was compared to the recommended ISO standard for Tea.

Our findings showed that there were significant differences ($p<0.05$) in the chemical components studied as a result of clonal variations which made the quality characteristics of the green tea to vary accordingly. The WE, of all the green tea examined except sample made from clone 236 were within values recommended for good quality green tea as speculated by ISO 3720, % MC was between 4.11-12.5% with average value being 10.2%. The High MC recorded was due to the high humidity of the processing environment where sunlight was inadequate The percentage CF ranged between 4.37-20.8%, the ALIA, 0.90-1.30, TA, 4.2-6.5%, WSA, 50-67% and WE 21.7-43.6% and the caffeine contents were within 1.29-2.56%.

In conclusion, quality of green tea produced in Nigeria has comparable quality characteristics with other tea producing countries of the world and conformed to international standard for green tea.

- Keywords: tea clones, green, black, crude fibres, water extracts, caffeine -

INTRODUCTION

Crude fibre in plants originates from certain structural units, cellular walls, skelrenchyma, collenchymas and transporting tissues. Young cells have thin cellular walls that becomes hard as the plant grows and provide the plants with protection from excess transpiration and influence of other undesirable factors (STRASBERGER, 1962). Fibre is composed of many different compounds in particular cellulose, hemicelluloses and wood-wool. Most of them are polysaccharides with the exception of wood-wool. The content of crude fibre in young tea leaves is much less than in older ones. The fundamental (and one of the older) definition of crude fibre states that it is the residue after treating with boiling 0.255N sulfuric acid and 0.313N sodium hydroxide (BARTMIKOWSKA, 1997). Crude fibre is the main part of the insoluble matter of black tea. The fibre content can vary and ranges between 7 and 20% in tea products. In general, when the fibre content of tea increases, most of the quality characteristics component decreases (FERMUZ *et al.*, 1993). Coarse plucking and aged leaves result in higher levels of Fibre. Coarse leaves and stalks are mixed with the finer parts of the shoots during the different rolling processes and it is very difficult to remove all of them from tea in later processes. Tea is one of the oldest beverages in the world. It is second most popular beverage after water (SMIECHOWSKA and DMOWSKA, 2006). Its quality is determined among other things by aroma and taste properties. Much attention has been drawn to the fact that crude fibre is an important parameter in the quality evaluation of tea and influences its sensory properties. Another very important parameter that determines tea quality according to FERMUZ *et al.* (1993) is water extract (WE). A tea shoot of two leaves and a bud contain about 25% solid matter. About half of the solid matter is soluble in water and this is termed extract. Many components of the liquor influence tea quality. The amount of these components generally increases in proportion to the amount of Tea extract. Thus, the percentage of extract is limited by the International Standard Organization (ISO). The amount of extracts decreases as plucking intervals lengthens and also decreases due to coarse plucking and aging of the leaf (SMIECHOWSKA and DMOWSKA, 2006).

Caffeine is an alkaloid that can constitute approximately 5% of the Dry Matter in fine tea shoots. Tea is widely consumed all over the world and caffeine plays a very important role in explaining why Tea is used as a beverage because it has a stimulating effect on the human body. A high content of caffeine contributes to briskness, which is one of the factors indicating Quality Tea. The Caffeine content of green tea increases after harvesting particularly during the withering process. Some authors have reported that different rolling process do not affect the caffeine content of processed black tea Green tea (unfermented tea) is a major beverage in Asian Countries such as China and Japan whereas, black tea (fermented tea) is more popular in other parts of the world. Although green tea and black tea are made from the leaves of the same plant, *Camellia sinensis*, differences in the processing of the leaves result in differing chemical components. Since this study is the first report on green tea production in Nigeria, with no previous reports in the literature. This work was, however, designed with the objective of diagnosing the quality of green tea processed from Nigerian tea clonal materials using quality markers like crude fibre, Caffeine, Moisture contents, water extracts and total Ash according to the International quality standards for tea.

MATERIALS AND METHODS

Green tea samples used in this study were processed on the Mambilla Highland from seven different tea clones 35, 68, 228, 318, 363, 61 and 236, respectively The fresh tea leaves used in the green tea production were plucked from vegetatively propagated (VP) tea fields at the Cocoa Research Institute of Nigeria (CRIN) substation, Kusuku, Mambilla located around 1,840 m above mean sea level, latitude 6° 24´ N and longitude 10° 35´E. The plants were grown under recommended agronomic conditions. The tea clones were plucked at the tea plantation and processed by the miniature green tea processing facilities. The plucked tea leaves were withered shortly followed by fixing to inactivate the activities of the polyphenol oxidase enzyme using the Chinese methods of Pan fixing. The pan-fixed leaves were later rolled and dried using oven. The green tea produced were later packaged in paper board boxes and reserved for Analysis.

Preparation of the tea solution

The tea preparations consisted of addition of boiling water (200 mL) to leaf tea (2 g), then the tea solution was filtered through cotton wool and the residue was washed with distilled water (30x10 mL). The tea solution was cooled to room temperature and washings were diluted 250 mL with distilled water. The samples were analyzed in triplicate.

Caffeine and water extracts

The methods used for the analyses of caffeine and water extracts of the tea solution were based on international standards (ISO 1839), 1980; (ISO 9768, 1994); YAO *et al.* (1992) as stated below.

Caffeine

Lead Acetate solution $(CH_3COO)_2Pb$.
100 g was dissolved and diluted to 200 mL with distilled water.
Hydrochloric acid solution, HCl.
36%, specific gravity, 1.18, 0.9 mL was diluted to 100 mL with distilled water.

Sulphuric acid solution

Sulphuric acid (98%) H_2SO_4, specific gravity, 1.84, 16.7 mL) was diluted to 100 mL with distilled water.

Measurements: Tea solution (10 mL), HCl (5 mL) and Lead acetate solution (1 mL) were mixed in a 100 mL volumetric flask and diluted with distilled water. The solution was then filtered through Whatman No. 1 quantitative filter paper. The filtrate (25 mL) and sulphuric acid solution (0.3 mL) were placed in a volumetric flask and diluted to 50 mL with distilled water. The solution was filtered using the same type of filter paper. The absorbance of the filtrate was measured using Ultraspec III UV /visible spectrophotometer at 274 nm. The measurement was performed in triplicates.

Standard curve

Caffeine stock solution (10 mL), 1 mg/mL, w/v in distilled water was diluted to 200 mL with distilled water. Next, 0, 10, 20, 30, 40, or 50 mL of the diluted caffeine solution were separately mixed, each with Hydrochloric acid solution (4 mL) in a volumetric flask are diluted to 100 mL with distilled water. Thereafter, the remaining steps were repeated as described earlier. The readings of the standard solution against the concentrations were used to prepare the standard curve.

Calculation

$$\text{Caffeine (\%)} = E/1{,}000 \times V_0 \times (100/V_1) \times \times (50/25)/W = 0.2EV_0/V_1/W$$

Where E is 'mg' of caffeine from the standard curve against the reading of the spectrophotometer and E/1,000 is to convert 'mg' into 'g'. V_0 is the total volume of the tea solution (250 mL); V_1 is the volume used for the measurements (10 mL), and 100/V1 indicates 10 mL tea solutions that were diluted to 100 mL, while 50/25 shows that another dilution from 25 mL tea filtrate made to 50 mL in the measurement. W is the dry weight of the tea sample.

Water extracts

Measurements
Tea solution (50 mL) was placed in a weighed evaporating dish and was then evaporated to dryness over a water bath. The residue (tea extracts) in the dish was fully dried in a vacuum oven at 75°C with a negative pressure of 65kPa for 4h until the weight of the dish with extract was constant.

Calculation

$$\text{Water extract (\%)} = (D_1 - D_0) \times V_0 \times 100/V_1 W$$

Where D_1 is the weight of the dry tea extracts with the dish; D_0, is the weight of the dish, V_0 is the total volume of the tea solution (250 mL), V_1 is the volume used for the analysis (50 mL); W is the dry weight of the tea sample Moisture Content.

The tea moisture was measured using a vacuum oven based on an international standard method ISO 1573, BS 6049-2, 1981.

Table 1 - Chemical composition of green tea processed from seven tea clones*.

	Tea clonal varieties							
Chemical parameters	**228**	**318**	**68**	**35**	**61**	**363**	**236**	**ISO standard**
% Water extracts	42.0	42.0	42.0	43.0	42.0	21.7	42.0	Minimum 32.0
% Total ash	6.5	6.2	6.3	4.3	4.2	6.3	6.3	Maximum 8.0 Minimum 4.0
% Water soluble ash	66.6	67.0	66.6	50.0	50.0	67.0	67.0	Minimum 45.0
% Acid insoluble ash	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Maximum 1.0
Alkaline insoluble ash	1.10	1.10	1.00	0.90	0.70	1.30	0.90	Maximum 3.0 Minimum 1.0
% Crude Fibre	8.35	8.35	12.50	8.60	20.80	20.80	4.37	Maximum 16.50
% Moisture content	8.94	6.72	7.11	5.64	6.22	7.20	4.11	Maximum 6.5
% Caffeine	2.18	2.56	1.29	1.68	1.37	1.96	2.39	-

*Data presented are means of triplicates.

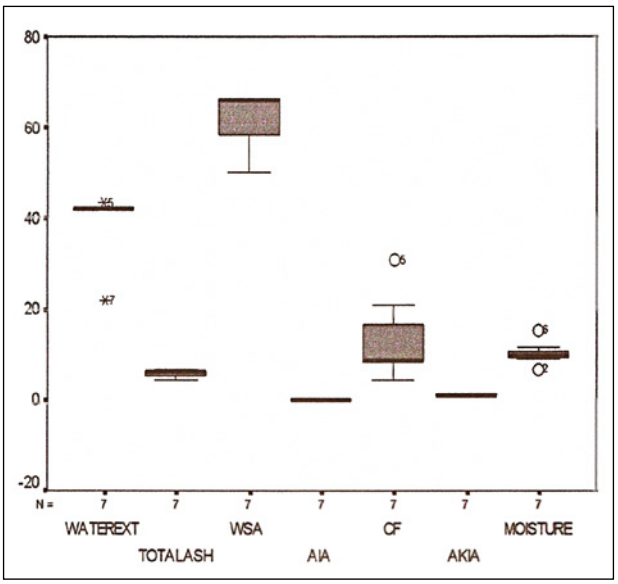


Fig. 1 - Boxplot showing the variations in some chemical properties of green tea of some selected tea clones.

Statistical analysis

One way Analysis of Variance (ANOVA) was performed to show the general trend of the experimental data. SPSS version 10.0 (SPSS Inc. Chicago, IL, USA) statistical Package was used. The mean and standard errors of means (SEM) in terms of the chemical compositions of the tea clones were expressed by Box plot using SPSS 10.0. version.

RESULTS AND DISCUSSION

Some samples of the results are shown in Table 1 while the summary has been displayed in the box plots (Fig. 1). To avoid error of interpretation, it should be noted that certain variables, such as water soluble ash (WSA), water extracts and crude fibre (CF) of the tea samples, have higher numerical values compared to other chemical components like the ash content, alkaline insoluble ash and moisture contents respectively (Fig. 1).

One of the parameters described in quality norms is crude fibre content. According to Table 1, the averages of the crude fibre contents ranged between 4.30-20.80% dry matter for the tea clones from Nigeria. The crude fibre contents were generally similar in values to the values determined in Turkish black tea samples as reported by FERMUZ *et al.* (1993). Crude fibre of tea has been linked to shooting period. Black tea from the first shooting period has been reported to have the lowest amount of fibres whereas the fibre contents were found to be higher in the tea of the third shooting period. This result indicated that the seven tea clones used in this study differed significantly in crude fibres, suggesting large generic variations (MAGOMA *et al.*, 2000). Indeed, the significant differences ($p<0.05$) in the levels of the crude fibres suggested that the quality potential of these clones could be different. It is noteworthy that the quality requirements for black tea states that crude fibre contents should not exceed 16.5% (ISO 3720, 1986). From the data collected in our own studies, it appears that on average, the least crude fibre was found in clone 236, whereas the highest crude fibre was recorded only for both clones 61 and 363. In all the seven tea clones analyzed, only these two clones deviated from the ISO standard 3720-1986 for crude fibre. From our data, it was obvious that minimum crude fibre from clone 236 was lower than the minimum from China and the maximum was recorded for samples from, Malawi. (Table 2). Moreover, the extent of crude fibre in tea from Malawi ranged very drastically from 5.83 to 43.27%. SMIECHOWSKA and DMOWSKA (2006) reported that the crude fibre can be accurate parameters to describe tea age. The younger the tea leaves used to process the green tea, the lower is the fibre content. In the so called leafy teas, a significant content of fibre in the products may show that lower quality material has been used in the production (5^{th} to 6^{th} leaf) due to high contents of careless mechanical harvest of tea when the yield contained not only leaves but also stems. This factor may not be significant in this study as leaves plucked were strictly 1 leaf + a bud for green tea. It could be suspected that the maturity of the tea used in the experiment might not have been the same. The genetic nature of the tea clones might also be responsible. The knowledge of water extracts, caffeine, moisture contents, crude fibre are very important physical and chemical parameters' as well as economic in screening tea clones for green tea and black tea processing. The water extracts found in this study ranged between 21.7-43.4% giving a mean of 39.3%. This is in agreement with previous studies (FERMUZ *et al.*, 1993). According to the international standard, water extracts of a tea is the soluble matter extracted from a test portion by boiling water, under the condition specified in this international standard

Table 2 - Crude Fibre (g/100 g) of tea from different Countries.

Countries	n.	Range	x±SD
China*	10	10.96-12.92%	11.49±0.89
India*	12	9.56-27.89%	15.24±7.91
Malawi*	12	5.83-43.27%	21.08±15.70
Nigeria	27	4.30-20.80%	10.3±0.12

n., number of samples; x, mean value; SD, standard deviation.
*SMIECHOWSKA and DMOWSKA, 2006.

expressed by mass on a dry basis (ISO 9768, 1994). In this study, the water extracts of all the tea clones examined except clone 236 conformed to the ISO 9768, 1994 standard which stipulated that the water extract in tea should be more than or equal to 32% of the dry mass. Compared to the water extracts of green tea produced from other Countries like Chinese green tea, 36.79%; India, 36.89-41.95%, but lower than water extracts of tea from Sri-Lanka, 36.72-46.9% and Kenya, 44.12%. It is however established that the mean contents of water extracts in green tea from Nigerian tea clones as evidenced in this study is relatively high and compared favourably well with the water extracts from leading tea producing Countries of the world. The mean content of water extracts in all the tea clones were within those reported by LIU *et al.*, 2006, who reported 39.18% in six green tea bags from Australian supermarkets. The similarity in the values obtained in our experiment is not unexpected as water extracts from black and green tea cannot be the same, green being reportedly higher than black tea due to differences in manufacturing conditions leading to decomposition of tea components to different degrees (HARA *et al.*, 1995; HARBOWY and BALENTINE, 1997).

Moisture content of green tea

Moisture content is an important quality parameter of tea (ROBERTS and SMITH, 1963) and is usually neglected by researchers but not by the industries or tea traders. Tea Researchers (OTHIENO and OWUOR, 1984; ROBINSON and OWUOR, 1993) suggested that the moisture content of the teas should be controlled to lie below 6.5% for marketing teas, whereas Millin, 1987 noted that teas had a moisture content of 7.0-8.5 during retailing. In this study, the mean moisture content obtained was 10.17% which was above 6.5% and deviated from the recommended standard for good quality green tea. The high level of moisture in our study samples was due to the high humidity of the processing environment as dried tea picked up moisture during the holding time between production and Analysis In this study, the variation in tea clones did not have any significant effects on the moisture contents. The main important factor for the moisture recorded was due to the high humidity of the processing environments. The main implications of this finding is that high moisture content will produce negative effects on shelf life of the green tea produced. There is however, a need for an improved quality control for the drying and Packaging of green tea produced in Nigeria. The dried tea leaves must be packaged in a moisture barrier material to prevent moisture pick up after drying. As for Water extracts (WE), ISO 9768 standard, min = 32. All the tea clones have more than this level except clone 363 having only 21.7% WE. The Total Ash of the clones varied according to variations in clones and ranged between 4.2-6.65 and fell within the recommended ISO 1575 standard for total ash of good quality green tea (min, 4, maximum, 8).The water soluble ash (WSA) of the clones were all within the recommended ISO 1576 standard, minimum = 45%. Acid insoluble Ash (AIA) of all the tea clones were also appropriate when compared to ISO 5998, apart from clone 61 and clone 363 with high values of 20.8% each than the recommended.

Caffeine

The caffeine contents varied significantly according to the tea clones. The green tea caffeine for clone 228, 318 and 236 were within the standard level of 2-5%. Other clonal varieties like clone 61, 35, 68 and 363 were relatively low in caffeine. Caffeine is an important component of tea. Green tea contains abundant caffeine, which is an alkaloid that can have negative effects on human body depending on the level of intake. Less than 300 mg intake per day is not harmful for adults, but an intake of more than 500 mg has been shown to cause excessive excitation in the central nervous system and cause arrhythmia and vertigo (PASPA and VASSAILA, 1984; SEALE *et al.,* 1984). Since not all the body can withstand the strong caffeine in the system, green tea from clones 61, 35, 68 and 363 can be good material for producing green tea, with clone 61 being the best. It is noteworthy therefore that close attention should be paid to caffeine intake by children and pregnant women because it is slowly metabolized and can remain in the body for a prolonged period (GIANNELLI *et al.* 2003; RASCH, 2003). Accordingly the removal or marked reduction of the caffeine content of green tea has been actively attempted by establishing a new technique called decaffeination in the manufacturing process. Methods using organic solvents, supercritical carbon IV oxide and hot water have been reported for the decaffeination of green tea (LEE *et al.,* 2007; LIANG *et al.*, 2007; PARK *et al.*, 2008; PARK *et al.*, 2007).

In conclusion, the combination of good yields coupled with processing potential of the clone should be used as a base for plant breeders to develop a clone that can be recommended for use in the processing of green tea with the minimum caffeine contents.

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PAPER

THE EFFECTS OF TOBACCO SMOKE EXPOSURE ON CAFFEINE METABOLISM

A. GRELA[1], M. KULZA[2], W. PIEKOSZEWSKI[1,3]*, M. SENCZUK-PRZYBYLOWSKA[2], E. GOMOŁKA[4] and E. FLOREK[2]

[1]Department of Analytical Chemistry, Faculty of Chemistry, Jagiellonian University, 30-060 Krakow, Poland

[2]Laboratory of Environmental Research, Department of Toxicology, University of Medical Sciences, 60-631 Poznan, Poland

[3]Laboratory of High Resolution Mass Spectrometry, Regional Laboratory of Physicochemical Analysis and Structural Research, Faculty of Chemistry, Jagiellonian University, 30-060 Krakow, Poland

[4]Laboratry of Analytical Toxicology and Drug Monitoring, Jagiellonian University, 31-501 Krakow, Poland

*Corresponding author: Tel. +48 12 663 6500, Fax +48 12 663 6501, email: wojciech.piekoszewski@uj.edu.pl

ABSTRACT

Caffeine and nicotine are some of the most often self-administered substances worldwide. Very often they are taken simultaneously and it seems that this fact is correlated with the amount of caffeine and nicotine administered. The aim of this study is to determine, whether tobacco smoke influences the metabolism of caffeine. The secondary task is to establish whether caffeine has an effect on elimination of cotinine, nicotine's main metabolite. The results showed that tobacco smoke influences the metabolism of caffeine by accelerating its elimination, by the means of induced CYP1A2 activity. As far as cotinine is concerned, no influence of caffeine on its elimination was observed.

- Keywords: caffeine, cotinine, metabolism, tobacco smoke -

INTRODUCTION

Caffeine and nicotine are some of the most commonly self-administered drugs worldwide. Caffeine is the vital compound found in coffee and energizing beverages, whereas nicotine is the main psychoactive substance in tobacco and tobacco smoke. The daily intake of caffeine varies worldwide, as it depends on the type of beverage that is consumed. According to a projection made by the Food and Agriculture Organization (FAO), consumption of caffeinated beverages in 2010 is forecasted to increase (FAO, 2010).

Caffeine affects the respiratory, cardiovascular and, most notably, central nervous system (CNS). As far as the cardiovascular effect is concerned, caffeine both decreases heart rate and increases blood pressure (KOURTIDOU-PAPADELI *et al.*, 2002).

Caffeine's most important metabolites are: theobromine (3,7-dimethylxanthine), paraxanthine (1,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine). Enzymes responsible for caffeine metabolism are cytochrome P450 isoforms (Fig. 1).

Cytochrome CYP1A2 is mainly involved in transformation of caffeine to paraxanthine, but also to theophylline and theobromine, to some extent. CYP2E1 metabolizes caffeine to theobromine and theophylline (ARNAUD, 2011).

Tobacco smoke is the product of tobacco combustion. There are two kinds of tobacco smoke: mainstream smoke, inhaled by the smoker, and side-stream smoke, emitted by the cigarette between puffs (MAURER and SYRIGOS, 2006). The smoke that remains suspended in the air is called Environmental Tobacco Smoke (ETS). The most frequently used biomarker of tobacco smoking and exposure to ETS is cotinine, the metabolite of nicotine, which is also a biomarker of ETS (PRIGNOT, 2011).

Tobacco smoke contains over 4,300 different compounds, among them many established carcinogens, which results in increased risk of developing various forms of cancer, e.g. lung cancer (MAURER and SYRIGOS, 2006). WHO study reports that the highest prevalence of tobacco use is in Europe, North America and Australia (WHO, 2011).

As mentioned before, tobacco smoke contains various substances that can interact with administered drugs, among others with caffeine and its metabolism (ZEVIN and BENOWITZ, 1999). Polycyclic aromatic hydrocarbons induce cytochrome P450 activity which may result in rapid caffeine metabolism. Furthermore, nicotine was believed to induce CYP1A2 activity (ZEVIN and BENOWITZ, 1999), recent studies shown relation between nicotine induction of the cytochrome P450 isoform and caffeine metabolism (HUKKANEN *et al.*, 2011). However, other components of tobacco smoke, such as heavy metals and carbon monoxide are known inhibitors of the cytochrome P450 (ZEVIN and BENOWITZ, 1999).

According to findings of a study concerning the interaction between caffeine and tobacco smoke published in 1989 (BROWN and BENOWITZ, 1989), the cigarette consumption is greater during caffeine intake and the concentration of nicotine is higher during low-dose of caffeine compared with no-caffeine intake. It also seems that smokers drink more coffee, however, there are also reports stating that caffeine consumption had practically no influence on nicotine intake (TANDA and GOLDBERG, 2000). According to another paper, although coffee-drinking smokers smoked more than non-coffee drinkers, the number of cigarettes smoked was uncorrelated to the type of beverage drank (caffeinated or non-caffeinated) (BLANK *et al.*, 2007). Moreover, smokers drinking non-caffeinated beverages smoked significantly more than non-drinking smokers.

The aim of this study was to describe the effect of exposure to tobacco smoke on caffeine metabolism in animal model.

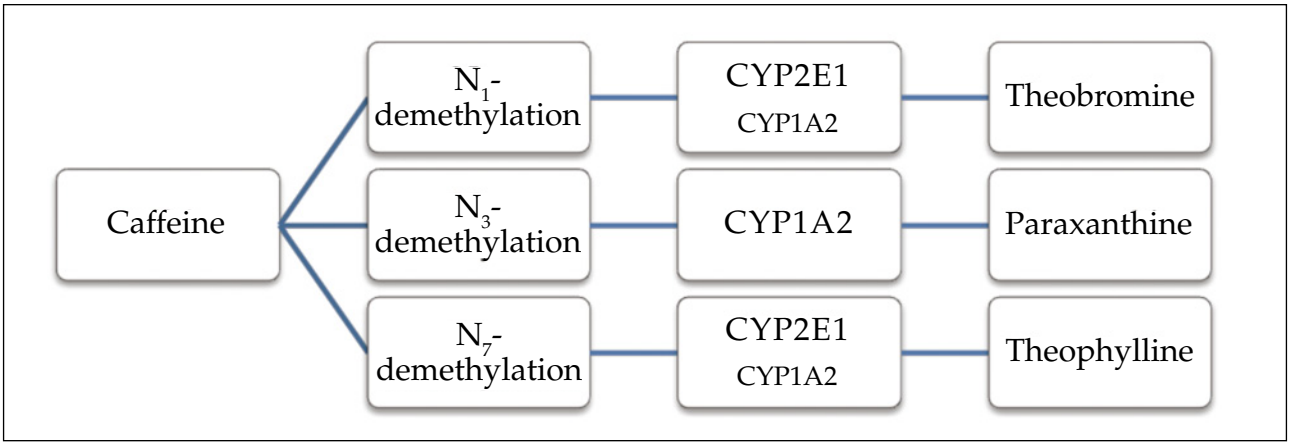


Fig. 1 - Schematic workflow of caffeine biotransformation to its methylxanthine metabolites.

MATERIALS AND METHODS

Reagents

Caffeine, theophylline, theobromine and paraxanthine, as well as cotinine in the form of crystal powder were obtained from Sigma-Aldrich, NJ, USA, and 8-bromotheophylline also in the form of crystal powder was synthesized in the Department of Organic Chemistry, Faculty of Chemistry of the Jagiellonian University, Krakow, Poland. Stock solutions (1 mg/mL) of the analytes were prepared in water. Working solutions at following concentrations: 0, 10 (only for cotinine), 50, 100, 200, 500, 1,000, 2,000 and 5,000 ng/mL were prepared in water by appropriate dilution of the stock solution. Concentration of internal standard (8-bromotheophylline) in water was 50 μL/mL.

Sample preparation

Firstly, acidic extraction was performed: 200 μL of a phosphate buffer (pH 3) and 20 μL of internal standard (8-bromotheophylline) were added to 500 μL of rat serum or to 500 μL of standard solution (when preparing calibration curve) and mixed on a Vortex. To each test tube 3 mL of chloroform (Sigma, HPLC Grade) was added and solutions were shaked for 15 min and centrifuged for 20 min at 3,000 rpm. The organic phase was separated (2 mL) and evaporated at 40°C in nitrogen stream. The dry residue was dissolved in 300 μL of mobile phase.

The process of cotinine extraction was carried out using the same method as caffeine and its metabolites (theobromine, theophylline and paraxanthine).

Chromatographic condition

100 μL of solution was injected into the chromatographic column (Supelcosil LC-8, 25 cm x 4.6 mm, 5 μm). A diode-array detector (Merck Hitachi L-3000 Photo Diode Array Detector) and a UV-spectrophotometer (275 nm) (Spectra 100 Therma Seperation Products) were used simultaneously. The DAD-detector was used for higher concentrations and the UV-detector for lower ones. Cotinine samples were measured at 254 nm.

For the analysis the isocratic elution was applied. The flow of the mobile phase (1.19 g of sodium octanesulfonate and 4.54 g of potassium hydrogen phosphate in 880 mL of deionised water and 120 mL of acetonitrile (Sigma, HPLC Gradient Grade)) was 1.1 mL/min and constant.

The retention times of the analytes were as follows: theobromine 7,6 min, paraxanthine 12.1 min, theophylline 12.5 min, caffeine 25.6 min and that of 8-bromotheophylline was 28.8 min. Cotinine's retention time was 9.1 min.

Animal experiment

One hundred and five Wistar rats (250-300 g) bred at the Department of Toxicology of the Poznan University of Medical Sciences were housed in polycarbonate cages containing sawdust bedding. A standard pellet diet and water were available ad libitum. The 12/12 h light/dark cycle, temperature 20°-22°C and humidity 50-60% were maintained throughout the study. After 14 days of acclimatization, the rats were randomized and divided into three groups of 45 animals each. The first group of rats was given caffeine intraperitoneally in a dose of 10 mg/kg of body weight. Animals from the second group animals were exposed to tobacco smoke for 6 h per day, for 5 days. CO concentration was taken as an index of tobacco smoke concentration and was maintained at 1,500 mg CO/m^3. Rats were exposed in a dynamic toxicological chamber (FLOREK and MARSZAŁEK, 1999) to tobacco smoke generated from a Polish brand of cigarettes without a filter tip. Animals from the third group were exposed to tobacco smoke at a concentration of 1,500 mg CO/m^3 of air during 5 days, 6 hours a day and after the last day of exposure, caffeine was injected intraperitoneally in the dose of 10 mg/kg body weight. After exposure to tobacco smoke (second group) or administration of caffeine (first and third group), animals were anesthetized (xylocaine 40 mg/kg and ketamine 5 mg/kg). Blood samples were collected at nine time-points (15 and 30 min, and 1, 2, 3, 6, 9, 12 and 18 h) with three rats per point.

After blood clotting, the samples were centrifuged and the obtained serum was kept at a temperature of -18°C until analysis was performed (no longer than two weeks). The protocol for this animal experiment was approved by the Local Ethics Commission for Animal Studies in Poznan.

Statistic and pharmacokinetic calculation

In our study analysis of variances was applied for statistic data. Furthermore, all pharmacokinetic parameters, that is: area under the curve (AUC), area under the first moment curve (AUMC), clearance (Cl; calculated only for caffeine), volume of distribution (V; calculated only for caffeine), mean residence time (MRT), elimination rate constant (k; calculated for caffeine and cotinine), biological half-time ($t_{1/2}$) were calculated by the means of the statistical moment analysis. To calculate the parameters we used the non-compartmental model.

RESULTS

In our studies paraxanthine, theophylline and theobromine were used as markers for influence of tobacco smoke on caffeine metabolism

Table 1 - Analytical parameters for caffeine and its metabolites.

Parameter	Caffeine	Paraxanthine	Theophylline	Theobromine	Cotinine
Linearity [ng/mL]	0-500 ng/mL	0-1,000 ng/mL	0-1,000 ng/mL	0-1,000 ng/mL	0-5000 ng/mL
Slope of calibration curve a	0.0038	0.0008	0.0007	0.0014	1.8022
Intercept b	0.0403	0.0068	0.0385	0.0558	-32.277
Correlation coefficient R^2	0.9952	0.9964	0.9898	0.9954	0.9953
LOD [ng/mL]	17	17	17	17	3
LOQ [ng/mL]	50	50	50	50	10
Intra-day precision	16	9	37	10	32
at 100 ng/mL RSD %	(n = 5)	(n = 4)	(n = 5)	(n = 3)	(n = 5)
Intra-day precision	6	9	3	18	12
at 500 ng/mL RSD %	(n = 4)	(n = 4)	(n = 3)	(n = 3)	(n = 5)

Table 2 - Pharmacokinetic parameters for caffeine and its metabolites (value±standard deviation).

	Caffeine		Paraxanthine		Theophylline		Theobromine	
Parameter	Rats exposed to caffeine	Rats exposed to caffeine and tobacco smoke	Rats exposed to caffeine	Rats exposed to caffeine and tobacco smoke	Rats exposed to caffeine	Rats exposed to caffeine and tobacco smoke	Rats exposed to caffeine and	Rats exposed to caffeine tobacco smoke
AUC [h×mg/L]	29.3±7.4	14.6±4.4 *	13.7±2.8	10.2±3.3	13.5±3.0	6.5±1.7 *	15.6±2.1	20.2±9.2
AUMC [mg×h^2/h]	86.77±36.69	21.8±10.6 *	111.2±30.9	53.6±17.8 *	98.4±18.0	33.4±5.8 *	93.3±13.1	91.1±19.0
Cl [L/kg/h]	0.36±0.10	0.73±0.22 *	-	-	-	-	-	-
V [L/kg]	1.00±0.07	0.99±0.09	-	-	-	-	-	-
MRT [h]	2.87±0.54	1.43±0.32 *	8.02±0.66	5.24±0.07 *	7.32±0.27	5.14±0.94 *	5.96±0.06	8.90±4.07
k [h^{-1}]	0.42±0.07	0.73±0.04 *	0.16±0.01	0.24±0.04 *	0.27±0.02	0.46±0.16 *	0.32±0.03	0.31±0.17
$t_{1/2}$ [h]	1.69±0.26	0.96±0.05 *	4.48±0.41	2.91±0.40 *	2.55±0.22	1.65±0.65 *	2.20±0.21	2.70±1.15

*Statistically significant differences (Analysis of variances).

and cotinine as an indicator of exposure to tobacco smoke.

Due to the analytical aspect of the paper, Table 1 contains analytical parameters of our study.

The lowest concentration on the calibration curve was taken as the limit of quantitation, whereas the limit of detection was calculated as three times lower as the limit of quantitation. Linearity is defined as the concentration range was the concentration is directly proportional to the analytical signal – in the case of our study the area under the peak.

Tables 2 and 3 display the values of pharmacokinetic parameters of caffeine and its metabolites (Table 2), as well as of cotinine (Table 3).

Table 3 - Pharmacokinetic parameters for cotinine (value±standard deviation).

	Cotinine	
Parameter	Rats exposed to caffeine	Rats exposed to caffeine and tobacco smoke
AUC [h×mg/L]	914.4±255.8	818.3±271.9
AUMC [mg×h^2/h]	7256.9±3034.9	6354.2±3086.3
MRT [h]	7.72±1.16	7.60±1.11
k [h^{-1}]	0.13±0.02	0.13±0.02
$t_{1/2}$[h]	5.40±0.71	5.27±0.91

The pharmacokinetic parameter describing exposure to tobacco smoke (area under curve of caffeine) has a lower value in the group exposed to tobacco smoke and caffeine comparing to animals that had only caffeine administered.

Furthermore, mean residence time and biological half-time (twofold lower) as well as clearance and the elimination rate constant (two-times higher), depict a faster elimination after exposure to tobacco smoke. No change in volume of distribution indicates lack of influence of tobacco smoke on caffeine distribution throughout the organism. The faster elimination of caffeine due to tobacco smoke exposure is also depicted in Fig. 2.

Caffeine metabolism is catalyzed by cytochrome P450 isoforms: CYP2E1 and CYP2A1 (ARNAUD, 2011). Acceleration of caffeine elimination after tobacco smoke exposure is mainly caused by polycyclic aromatic hydrocarbons which are known to induce cytochrome P450 isoforms (VILLARD *et al.*, 1998; ZEVIN and BENOWITZ, 1999).

Paraxanthine is caffeine's main metabolite in man (ARNAUD, 2011). According to data collected in Table 2, area under concentration-time curve is the only pharmacokinetic parameter for paraxanthine that is not statistically different for tobacco and non-tobacco smoke exposed groups. Mean resident time and biological half-

time decreased 1.5-time after exposure to tobacco smoke, whereas elimination rate constant increased 1.5-time. The faster elimination of paraxanthine is shown in Fig. 3. The highest concentrations were in the third hour for both with and without tobacco smoke exposure.

The metabolite which is of high medical importance, as it is used for asthma treatment, is theophylline. All its pharmacokinetic parameters were statistically different after tobacco smoke exposure. Theophylline highest concentrations were in the sixth hour and second hour for rats exposed only to caffeine and for rats exposed to tobacco smoke, respectively. Exposure to tobacco smoke accelerates the process of caffeine's biotransformation to theophylline (Fig. 3). Moreover, its elimination is more rapid, which is confirmed by the two times higher elimination rate constant as well as statistically significantly lower values of MRT and biological half-time. Area under curve decreased in half (Table 2).

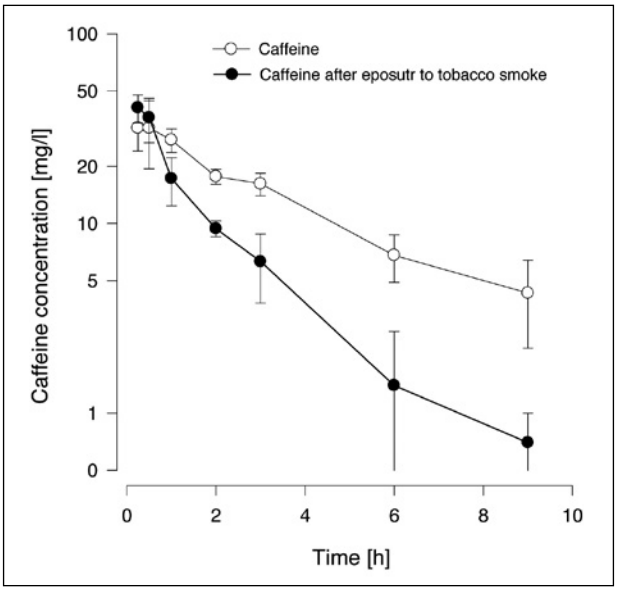


Fig. 2 - Time course of the effect of tobacco smoke on caffeine concentration in rat serum.

For theobromine the concentration-time profile (Fig. 3) and all pharmacokinetic parameters (Table 2) were similar.

In both experimental groups the maximum concentration of cotinine was achieved 30 min after end of exposure. Calculated pharmacokinetic parameters of cotinine in group exposed to tobacco smoke and in the group exposed to smoke and caffeine indicated that caffeine cotreatment does not influence cotinine elimination (Table 3). None of the parameters describing rate of elimination (biological half-lives, elimination rate constants and mean resident times) differ statistically.

DISCUSSION

According to our research, caffeine has no influence on nicotine's metabolism, since there were no changes in pharmacokinetic parameters describing a xenobiotic's elimination rate after co-administering of caffeine and nicotine. This is supported by a paper published by Kozlows-

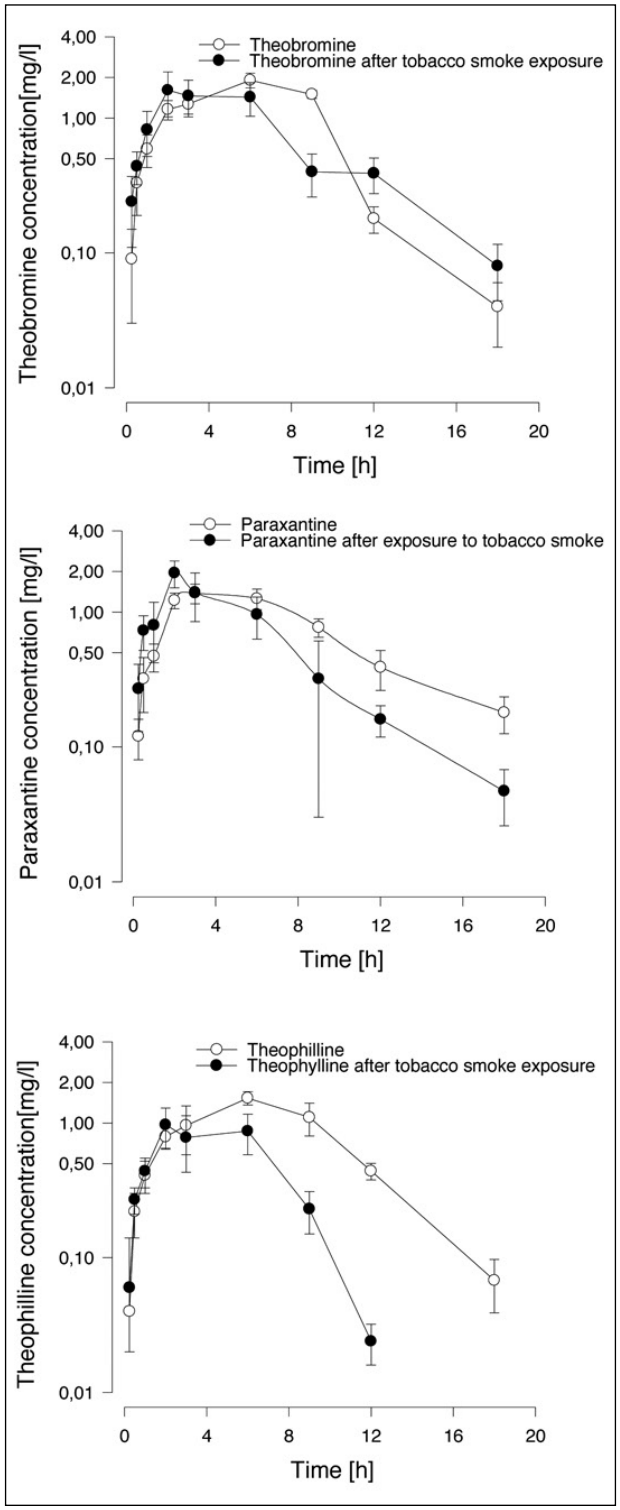


Fig. 3 - Time course of the effect of tobacco smoke on theobromine (up), paraxanthine (middle) and theophylline (down) concentration in rat serum.

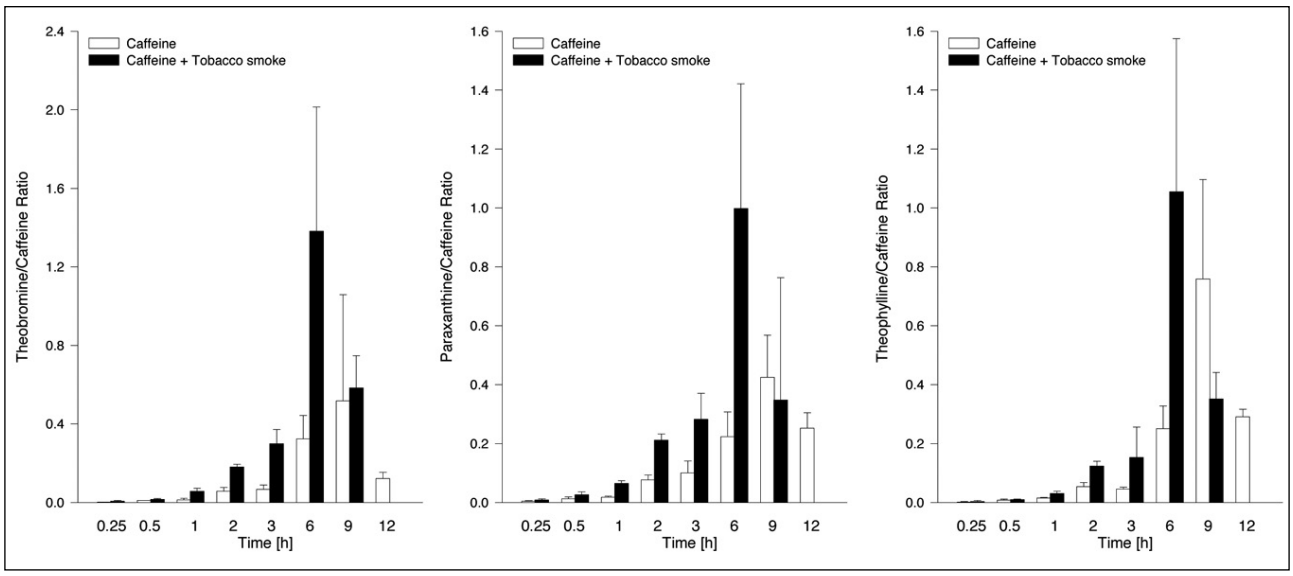


Fig. 4 - The change of theobromine/caffeine (left), paraxanthine/caffeine (middle) and theophylline/caffeine (right) ratio in time.

ki (1976), who found that smokers absorb more nicotine while not drinking coffee. Similarly, in a different paper can be found that caffeine had no influence on nicotine intake (CHAIT and GRIFFITHS, 1983). However, a different study (MARSHALL *et al.*, 1980) reports that coffee drinking did increase the number of smoked cigarettes, although they observed this effect also for water and a non-caffeinated drink which may suggest that the increase of nicotine consumption is not solely connected to caffeine's pharmacologic effects.

Furthermore, our study revealed that tobacco smoke does increase caffeine's elimination rate as well as its two metabolites: theophylline and paraxanthine. Pharmacokinetic parameters for theobromine (Table 2) did not differ after exposure to tobacco smoke and this suggests that tobacco smoke does not alter its elimination rate

Changes in caffeine's metabolism due to tobacco smoke exposure is also described in MARSHALL *et al.* (1980). According to their findings coffee drinkers smoke more than non-drinkers. In a different study (DE LEON *et al.*, 2003) it is reported that smokers had lower plasma caffeine concentration than non-smokers which also supports our conclusion that tobacco smoke increases caffeine metabolism.

Paraxanthine, theophylline and theobromine are caffeine's metabolites, all catalysed by cytochrome P450 isoforms. The first one, only by CYP1A2, and the rest mainly by CYP2E1 and partially CYP2A1 (ARNAUD, 2011). One of the tobacco smoke constituents is polycyclic aromatic hydrocarbons which induces cytochrome P450 isoforms, mainly CYP1A2 (KROON, 2007).

According to data collected during the experiment, tobacco smoke increases elimination of caffeine (Table 2 and Fig. 2), as that of paraxanthine and theophylline (Table 2 and Fig. 3).

In the sixth hour, paraxanthine/caffeine ratios as well as the theophylline/caffeine ratio for the tobacco-smoke exposed group are over four-times higher than in the group exposed only to caffeine (Fig. 4). In the case of theobromine, the ratio comparison shows tobacco-smoke exposed group ratio to be over 5.5-times higher than for the caffeine-exposed one (Fig. 4).

The comparison of the group exposed to tobacco smoke and both tobacco smoke and caffeine, after nine hours, reveals that theobromine/caffeine and paraxanthine/caffeine ratios are similar. Theophylline/caffeine ratio of the group exposed to both factors (i.e. tobacco smoke and caffeine) is twofold lower than the ratio of the group exposed only to caffeine.

In our study we observed that paraxanthine and theophylline are metabolized faster than theobromine. A similar study that investigated the effect of rutaecarpine, which is also a CYP450 inducer, shows an increase of elimination rate of all caffeine metabolites after exposure to this drug (NOH *et al.*, 2011). However, according to our findings, tobacco smoke, which contains CYP450 inducer, such as polycyclic aromatic hydrocarbons, has no such effect on theobromine (Table 2). The tobacco smoke related cytochrome induction can serve as an explanation. CYP450 isoforms involved in caffeine, as well as paraxanthine and theophylline, metabolism are induced by polycyclic aromatic hydrocarbons present in tobacco smoke, whereas those connected to theobromine biotransformation seem not to be altered. Caffeine metabolism is accelerated, theobromine metabolism does not change, therefore, theobromine/caffeine ratio is high.

As mentioned before, the reason for this is the

presence of polycyclic aromatic hydrocarbons in tobacco smoke. These compounds of tobacco smoke induce several of CYP450 isoforms, including CYP1A2, which is not only responsible for caffeine metabolism but also the metabolites' (paraxanthine and theophylline) biotransformation to their respective metabolites (ARNAUD, 2011). CYP1A2 catalyses theophylline transformation to both 1-methylxanthine and 3-methylxanthine. Therefore, higher activity of this enzyme causes faster elimination, via biotransformation, of caffeine as well as paraxanthine and theophylline.

CONCLUSION

The aim of the study was to determine if tobacco smoke influences caffeine's metabolism and caffeine's influence on nicotine metabolism using cotinine as a marker. No changes in pharmacokinetic parameters describing rate of elimination of cotinine indicate lack of caffeine's influence on the metabolism of nicotine. However, exposure to tobacco smoke does accelerate caffeine elimination. Our study demonstrates that tobacco smoke significantly increases caffeine elimination, hence its lower concentration in the organism. Tobacco smoke also influences elimination of two caffeine metabolites – theophylline and paraxanthine, whereas pharmacokinetic parameters for theobromine are unchanged after tobacco smoke exposure.

These data can serve as an explanation for the observed phenomenon of smokers drinking more coffee than non-smokers.

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PAPER

FILLET FATTY ACID COMPOSITION, ESTIMATED INDEXES OF LIPID METABOLISM AND OXIDATIVE STATUS OF WILD AND FARMED BROWN TROUT (*SALMO TRUTTA* L.)

A. DAL BOSCO[1]*, C. MUGNAI[2], V. ROSCINI[1] and C. CASTELLINI[1]
[1]Department of Applied Biology, University of Perugia, Borgo XX Giugno 74, 06100 Perugia, Italy
[2]Department of Food Science, University of Teramo, Viale F. Crispi 212, 64100 Teramo, Italy
*Corresponding author: Tel. +39 075 5857110, Fax +39 075 5857122,
email: dalbosco@unipg.it

ABSTRACT

The aim of the present study was to compare the fillet fatty acid composition and the oxidative stability of wild and cultivated brown trout (*Salmo trutta* L.). The study was carried out in Valnerina (Perugia, Italy), where 20 two-year-old farmed brown trout (250 ± 15 g) were utilized; another 20 trout were chosen from a sample caught during the same period from the Nera River (Perugia, Italy) based on their weight (± 10% of weight of farmed ones). The fatty acid profile, fatty acid index and oxidative stability of the fish fillets were assessed. The farmed trout had a significantly higher fat (3.16 *vs* 2.80%, $P<0.05$) and energy content than the wild ones, whereas no significant differences were observed with respect to protein and moisture. The linoleic acid content was higher than the linolenic content in farmed trout, while in the wild fish, the inverse situation was observed. The docosahexaenoic and eicosapentaenoic acid contents in farmed trout were higher than those in wild trout. The wild fish showed higher thioesterase and Δ^9-desaturase (18) and lower Δ^5- Δ^6-desaturase activity. Regarding oxidative stability, the wild fish showed a significantly higher development of oxidative processes associated with a lower peroxidability index.

- Keywords: *Salmo trutta*, farmed, wild, fatty acid, oxidative stability -

INTRODUCTION

The total consumption of Brown trout (*Salmo trutta* L.) in Italy amounts to approximately 5,000 tons per year (ISMEA, 2006). In Italian rivers fishing for this species is highly developed and thus there is also a food consumption of wild trout. The demand cannot be satisfied by catching wild fish, essentially because the world fish stocks are limited; thus, the number of aquaculture products is increasing.

Many nutritional and organoleptic characteristics of fish are correlated with the quantity and quality of lipids and are affected by intrinsic (genotype, age, sex, physiological stage) and extrinsic (environment, water temperature, feeding) factors and differences in the lipid composition of various farmed or wild-caught fish species have been described in a number of studies (CAHU *et al.*, 2004). Concerning feeding, the wild brown trout has a typically carnivorous diet and frequently preys on larval and adult insects as well as crustaceans, annelids, and gastropods; as the fish grows, it may also prey on other fish. To our knowledge, only a few studies (BOCCIGNONE *et al.*, 1987; BOCCIGNONE *et al.*, 1988; ZIINO *et al.*, 1991; AKPINAR *et al.*, 2009; BAYIR *et al.*, 2010) have analyzed the fatty acids of different wild *Salmo trutta* sub-species; thus, the fatty acid dynamics of wild fishes is not well known. Accordingly, in this present study, the fatty acid compositions and the oxidative stability of the flesh of wild and farmed brown trout were compared.

MATERIAL AND METHODS

Rearing conditions, diets and fishing conditions

The study was carried out on March 2011 at the Ichthyic Genetic Centre of the Province of Perugia located in Borgo Cerreto in Valnerina (Italy), where brown trout are reared to produce the fish necessary to restock different rivers of Central Italy.

Twenty two-year-old brown trout (approximately 20 cm long and weighing approximately 250 ± 15 g) provided by the Center were utilized, and another 20 were chosen from a sample caught from the Nera River based on their weight (±10% of weight of farmed fish). This river originates in the Sibillini Mountains (Central Italy) and flows for approximately 125 km before joining the Tiber River. The water temperature in the Ichthyic Genetic Centre of the Province of Perugia ranges from 6° to 15°C. The farmed fish fed a commercial diet (Veronesi Verona spa, Italy; 40% of crude protein, 24% lipids, 6.5% Ash, 2% fiber; 7.9% linoleic acid, C18:2n-6 LA, 2.9% linolenic acid, C18:3n-3 ALA and 15.5% total n-3 fatty acid) at approximately 1.5% of their live weight for the last year of life. The main ingredients of the diet were soybean meal, fish meal, fish oil, wheat meal, pea protein concentrate, wheat gluten, minerals, amino acids and antioxidants. After capture, all fish (wild and farmed) were slaughtered in water and ice and immediately transferred (packed into an insulated polystyrene box with ice) to the laboratory of the Department of Applied Biology of Perugia (transport time equal to one hour) for the chemical analyses.

Fillets and samples preparation

After washing with running water, the fish were manually eviscerated, the tails and heads were removed, and eighty fillets (mean weight 65 ± 2 g) were dissected for analysis.

Chemical composition

Analyses were immediately carried out in duplicate to determine the proximate composition. Moisture, ash and total nitrogen contents were determined using AOAC methods (N. 950.46B, 920.153, and 928.08, respectively). The total protein content was calculated using the Kjeldahl nitrogen method and a conversion factor of 6.25. The total lipids content was extracted (FOLCH *et al.*, 1957) from 5 g of each homogenized sample and determined gravimetrically. Energy content of fillets was determined by an adiabatic bomb calorimeter (model IKA Calorimeter C400, Adiabatic 2800, Bremen, Germany).

Fatty acid profile and indexes

The fatty acids content was determined by gas chromatography after lipid extraction according to the method developed by FOLCH *et al.* (1957). One milliliter of lipid extract (from 0.2 to 0.3 mg lipids) was evaporated under a stream of nitrogen, and the residue was derivatized by adding 3 mL of sulfuric acid (3% in methanol). After incubating at 80°C for one hour, methyl esters were extracted with petroleum ether, and 1 μL was injected into a gas chromatograph (Fisions Mega 2 Carlo Erba Gas Chromatograph, model HRGC Milan, Italy) equipped with a flame ionization detector.

The separation of fatty acid methyl esters (FAME) was carried out on an Agilent (J W) capillary column (30 m x 0.25 mm I.D, CPS Analitica, Milan, Italy) coated with a DB-Wax stationary phase (film thickness of 0.25 μm). The operating conditions of the column injection were as follows: the temperatures of the injector and detector were 270° and 280°C, respectively and the detector gas flows were H_2 50 mL/min and air 100 mL/min. The oven temperature was programmed to give good peak separation; the initial temperature was set to 130°C and then increased at a rate of 4.0°C/min until reaching a temperature of 180°C, which was held for 5 min;

the temperature was subsequently increased at a rate of 5.0°C/min until it reached 230°C, which was held for 5 min. Helium was used as a carrier gas at a constant flow rate of 1.1 mL/min. Individual fatty acid methyl esters were identified with reference to the retention time of tridecanoic acid (C13:0) methyl ester added before extraction as an internal standard. The concentration of each fatty acid (mg/100 g of fish) was calculated from the lipid content of the fish and a conversion factor of 0.91 according to JOHANSSON *et al.* (2000). The mean value of each fatty acid was used to calculate the sum of the saturated (SFA), monounsaturated (MUFA) and PUFA, and several indexes were used to estimate the desaturase and elongase activity of the muscle tissue (VESSBY *et al.*, 2002). The activities of Δ^9-desaturase and elongase were estimated by relating the percentage of product to the percentage of the precursor (OKADA *et al.*, 2005). Specifically, the Δ^9-desaturase index for the C18:1 was calculated as 100 times the ratio of oleic acid (C18:1) to the sum of C18:1 and stearic acid (C18:0). The total Δ^9-desaturase index (both 16 and 18) was calculated as 100 times the ratio of the sum of C16:1 and C18:1 to the sum of C16:1, C16:0, C18:1 and C18:0.

The elongase index was calculated as the ratio of C18:0 to C16:0, whereas the thioesterase index was calculated as the ratio of C16:0 to myristic acid (C14:0) (ZANG *et al.*, 2006).

To evaluate the activity of both Δ^5-desaturase and Δ^6-desaturase, the enzymes catalyzing the formation of long-chain n-6 and n-3 polyunsaturated fatty acids (PUFAs) starting from the precursors C18:2n-6 and C18:3n-3, the following equation was used (SIRRI *et al.*, 2010):

$$\Delta^5\text{-desaturase} + \Delta^6\text{-desaturase} = [(C20{:}2n\text{-}6 + C20{:}4n\text{-}6 + C20{:}5n\text{-}3 + C22{:}5n\text{-}3 + C22{:}6n\text{-}3)/(C18{:}2n\text{-}6 + C18{:}3n\text{-}3 + C20{:}2n\text{-}6 + C20{:}4n\text{-}6 + C20{:}5n\text{-}3 + C22{:}5n\text{-}3 + C22{:}6n\text{-}3)] \times 100$$

The index of nutritional quality (INQ) was calculated based on the eicosapentaenoic (EPA) + docosahexaenoic (DHA) acid level using the formula suggested by GODBE (1994) on 100 g of fillets used to calculate a ratio of the achieved percentage of the requirement of the sum of these fatty acids to the percentage of a 2000 kcal intake (TESTI *et al.*, 2006).

Peroxidability index (PI) was calculated according to the equation proposed by ARAKAWA and SAGAI (1986):

$$PI = (\%\ \text{monoenoic} \times 0.025) + (\%\ \text{dienoic} \times 1) + (\%\ \text{trienoic} \times 2) + (\%\ \text{tetraenoic} \times 4) + (\%\ \text{pentaenoic} \times 6) + (\%\ \text{hexaenoic} \times 8)$$

The amount of each fatty acid was used to calculate the indexes of Atherogenicity (AI) and Thrombogenicity (TI), as proposed by ULBRICHT and SOUTHGATE (1991), and the hypocholesterolaemic/hypercholesterolaemic ratio (HH), as suggested by SANTOS-SILVA *et al.* (2002):

$$AI = (C12{:}0 + 4 \times C14{:}0 + C16{:}0)/[(\Sigma MUFA + \Sigma (n\text{-}6) + \Sigma (n\text{-}3)];$$

$$TI = (C14{:}0 + C16{:}0 + C18{:}0)/ [(0.5 \times \Sigma MUFA + 0.5 \times (n\text{-}6) + 3 \times (n\text{-}3) + (n\text{-}3)/(n\text{-}6)];$$

$$HH = [(C18{:}1n\text{-}9 + C18{:}2n\text{-}6 + C20{:}4n\text{-}6 + C18{:}3n\text{-}3 + C20{:}5n\text{-}3 + C22{:}5n\text{-}3 + C22{:}6n\text{-}3)/(C14{:}0 + C16{:}0)]$$

Assessment of oxidative stability

The extent of lipid oxidation was evaluated in fillets as thiobarbituric acid reactive substances (TBARs) by a spectrophotometer (set at 532 nm, Hitachi U-2000, Theodor - Heuss - Anlage 12, Mannheim, F.R. Germany), which measured the absorbance of thio-barbituric acid-reactive substances, and a tetraethoxypropane calibration curve in sodium acetate buffer (pH=3.5), according to the modified method of KE *et al.* (1977). Oxidation products were quantified as malondialdehyde (MDA) equivalents (mg MDA/kg muscle).

Statistical analysis

The qualitative traits were analyzed using a one-way ANOVA (STATA, 2005) for the comparison of wild *vs* farmed trout. Least squares means and planned comparisons were used for mean separation when the differences were significant ($P < 0.05$).

RESULTS AND DISCUSSION

In Table 1, the proximate composition of the trout fillets of this study is reported. The wild trout had a significantly lower fat than the farmed ones, most likely due to the high dietary fat level in the feed (24%) and lower activity of the latter. Protein and moisture did not show significant differences.

The observed differences between wild and farmed are in agreement with the data obtained by other authors (RENON *et al.*, 1994a; RENON *et al.*, 1994b; REA *et al.*, 2000), who observed a higher fat content in cultivated fish. Within species, genotype, age, sex and feeding (REA *et al.*, 2000) affect the lipids content, with relevant repercussions on the nutritional characteristics of the meat.

Concerning the fatty acid content, palmitic acid (C16:0) was the major SFA, and myristic (C14:0) and stearic (C18:0) acids followed. Oleic acid (C18:1n-9) was the major MUFA, whereas palmitoleic acid (C16:1n-7) was found only in small quantities. Rainbow trout reared in the same habitat show similar levels of MUFA and oleic acid (DAL BOSCO *et al.*, 2007).

Table 1 - Fillet chemical composition (%) and fatty acid profile (mg/100 g fresh fillet) of cultivated and wild *Salmo trutta*.

		Cultivated	Wild	SED
Moisture	%	73.86	73.93	4.84
Protein	"	21.54	21.92	2.41
Lipids	"	3.16^{b}	2.80^{a}	0.46
Energy	Kcal/100 g fresh fillet	133.3	132.0	4.84
Fatty acids				
C14:0		182.3^{B}	85.17^{A}	25.3
C16:0		547.8	569.5	89.5
C18:0		98.3^{a}	133.3^{b}	10.8
Others		44.6	39.5	8.4
SFA		761.5^{a}	862.7^{b}	73.5
C16:1n-7		2.9^{A}	9.9^{B}	4.1
C18:1n-9		294.2^{A}	394.9^{B}	68.8
Others		136.9	118.7	23.1
MUFA		433.9^{A}	555.2^{B}	92.1
C18:2n-6	LA	123.9	118.7	18.9
C20:4n-6		33.9	40.5	8.2
C18:3n-3	ALA	34.8^{A}	179.9^{B}	26.8
C20:5n-3	EPA	310.8^{B}	179.9^{A}	97.3
C21:5n-3		109.2	100.5	26.5
C22:5n-3		104.9^{a}	59.6^{b}	40.1
C22:6n-3	DHA	675.2^{B}	281.0^{A}	185.3
Others		110.5	100.9	21.6
PUFA		1560.6^{B}	1118.8^{A}	256.3

N=20 per group.
Least square means in the same row with different superscript letters are significantly different A..B: P<0.01; a..b: P<0.05.
SED: Standard Error Deviation.
SFA: Saturated Fatty Acids.
MUFA: Monounsaturated Fatty Acids.
PUFA: Polyunsaturated Fatty Acids.

In the PUFA fraction, EPA and DHA were the major fatty acids detected.

Concerning the comparison between wild and reared fish, the former had a lower LA content than ALA content, while in cultivated fish, the inverse situation was observed.

Wild trout showed lower levels of myristic acid (C14:0) and higher levels of stearic acid (C18:0), SFA and MUFA. These results are in accordance with those observed by REA *et al.* (2000) in fillets of farmed and wild sea bream.

The results regarding ALA are in agreement with those of BOCCIGNONE *et al.* (1988), who reported higher values in wild than in farmed brown trout (2.09 *vs* 1.36%; 7.70 *vs* 1.26% in our trial; data not shown).

The absolute contents of DHA and EPA in wild trout were lower than those in farmed trout. DE FRANCESCO *et al.* (2004) found lower values (479 and 182 mg/100 g, respectively) in rainbow trout than in farmed brown trout of our study.

The literature regarding the EPA and DHA content in wild and farmed fishes shows some conflicting results. Many authors, in agreement with our findings, reported lower levels of EPA and DHA in wild fish with respect to farmed ones (BOCCIGNONE *et al.*, 1988; REA *et al.*, 2000; SAGLIK *et al.*, 2003). In contrast, RENON *et al.* (1994a) and RENON *et al.* (1994b) found higher EPA and DHA contents in wild sea bream, as did KRAJNOVIC-OZRETIC *et al.* (1994) and REA *et al.* (2000) in sea bass fillets.

The assessment of standard fatty acid profiles is more difficult for wild fish for larger variations among samples due to size/age, reproductive status, geographic location and season (NETTLETON and EXLER, 1985; ACKMAN, 1989; SAITO *et al.*, 1999; DAL BOSCO *et al.*, 2012).

Concerning the indexes of fatty acid metabolism, wild subjects showed higher thioesterase and Δ^9-desaturase (18) and lower Δ^5-Δ^6-desaturase activities (Table 2), which are mainly due to the feeding regimen.

Also BAYIR *et al.* (2010) affirmed that the fatty compositions of neutral and phospholipids of wild *Salmo trutta caspius*, *Salmo trutta labrax* and *Salmo trutta macrostigma* were strongly affected by many factors, especially reproduction and diet.

FOCHETTI *et al.* (2003), studying the eating habits of brown trout of the Nera River, observed selective feeding behavior, with a negative selection for some species of Ephemeroptera and Diptera and a positive selection for species of Trichoptera. In another study on the same river, FOCHETTI *et al.* (2008) observed that Plecopteran nymphs were more preyed upon by older subjects, while ephemeropteran nymphs were also preyed upon by young fish. Trichopteran larvae were the most abundant prey in trout younger than two years, while their percentage decreased considerably in older fish. The remaining aquatic prey (except Dipteran larvae) was scarce, and terrestrial prey was consumed more by older individuals.

Table 2 - Indexes of lipid metabolism, oxidative status and nutritional traits of in fillets of cultivated and wild *Salmo trutta*.

		Cultivated	Wild	SED
Elongase		0.18	0.23	0.08
Thioesterase		3.00^{A}	6.69^{B}	1.01
Δ^9-desaturase (16+18)		74.95	74.77	3.24
Δ^9-desaturase (18)		0.52^{A}	1.70^{B}	0.42
Δ^5/Δ^6-desaturase		88.71^{b}	70.65^{a}	3.81
INQ		22.82^{b}	10.07^{a}	4.15
H/H		2.16^{b}	1.88^{a}	0.21
n-6/n-3		0.18^{A}	0.27^{B}	0.04
Peroxidability index		301.05^{B}	189.79^{A}	54.19
Atherogenic index		0.72^{b}	0.64^{a}	0.11
Thrombogenic index		0.21	0.30	0.10
TBARs	mg MDA/kg	0.65^{A}	2.06^{B}	0.99

N=20 per group.
Least square means in the same row with different superscript letters are significantly different A..B: P<0.01; a..b: P<0.05.
IQN: Index of Nutritional Quality.
H/H: Hypocholesterolaemic/Hypercholesterolaemic acids ratio.
TBARs: Thio-Barbituric Acid Reactive substances.

Additionally, SALAVATIAN *et al.* (2011) observed in brown trout a diet that consisted mainly of benthic animals, chiefly represented by Oligocheta, Gastropoda, Diptera, Coelifera, Trichoptera, Lepidopetra, Hemiptera, Coleoptera, Odonata, Homoptera and Archnida, and the feeding behavior of these fish was directly related to the abundance of prey in the river.

GHIONI *et al.* (1996), in analyzing the fatty acid composition of neutral lipids and phospholipids of some freshwater insects, observed that in the above-mentioned classes the major fatty acid present was EPA.

Considering that the feeding behavior of young wild trout (< 2 years of age) is based on insects with a high content of n-3 long-chain fatty acids, it is probable that these fish developed a low elongase and desaturase capacity, which could explain the lower DHA content compared to that of farmed trout. Paradoxically, the diet of wild fish, rich in EPA, renders DHA biosynthesis less efficient when compared with the feed administered to the farmed trout, which is partially composed of vegetal components and contain 7.9% of LA and 2.9% of ALA. According to BELL and DICK (2004), to optimize the synthesis of DHA from ALA, in farmed trout, it would be appropriate to avoid supplementation with fish oil, especially at an early age, and the presence of the long-chain n-3 PUFA in fish oil and meal reduces the synthesis of DHA from ALA by approximately 10 fold. The ability to convert LA and ALA, abundant in vegetable oils, into C20 and C22 PUFAs is well established in some species, such as rainbow trout (KANAZAWA *et al.*, 1978; BUZZI *et al.*, 1996; MOURENTE and TOCHER, 1998). These statements are reinforced by the study of TURCHINI and FRANCIS (2009), who observed that when a diet is characterized by high EPA levels, a large proportion of this fatty acid is used for energy production and only a small quantity is further bioconverted in DHA.

The nutritional properties of brown trout fillets are reported in Table 2.

The INQ index of wild trout was significantly lower respect that of the farmed ones (22.82 *vs* 10.07), that considering the equal energy value, indicates a better achievement of dietary recommendations for EPA + DHA for fillets of farmed trout.

Also the HH ratio was significantly lower in wild brown trout, and in general lower than that found by TESTI *et al.* (2006) in rainbow trout fillets and by us (DAL BOSCO *et al.*, 2007). The atherogenic indexes were both higher than those reported by TURCHINI *et al.* (2003) for brown trout (0.45), whereas the thrombogenic index was similar.

The n-6/n-3 ratio of the fillets was 0.27 and 0.18, respectively for wild and farmed trout, similar to the results of another study in brow trout (TURCHINI *et al.*, 2003).

Regarding the differences in n-6/n-3 ratio between wild and farmed fish, the results of this experiment are in disagreement with most of those found in the literature because in this trial, the wild subjects showed higher values than the farmed, contrary to what was observed in other fish specimen by RENON *et al.* (1994a), KRAJNOVIC-OZRETIC *et al.* (1994) and REA *et al.* (2000).

Concerning oxidative stability, wild subjects showed a lower oxidative stability accompanied by a lower PI and this situation could be related to different factors:

- the different stresses that fish have suffered at the time of capture, which were certainly lower in farmed fishes with respect to wild ones because the wild ones had to fight at the moment of being fished (DIGGLES and ERNST, 1997);
- farmed trout fed a diet fortified in antioxidants, specifically, 11,500 IU vit. A, 1.800 IU vitamin D3, 150 mg Vitamin E, 100 mg vit. C per kg;
- some studies (BASTROP *et al.*, 2002; PASCUAL *et al.*, 2003) have reported that dietary restriction can increase oxidative stress in fish; ELLIOTT and HURLEY (1998; 2000) observed that in severe environments, such as streams at high altitude, where temperatures frequently reach below 10°C and feed resources are scarce, brown trout eat with lower frequencies and are satiated with lower quantities of food compared with farmed fish.

CONCLUSIONS

Wild and farmed brown trout analysed in this study differ mainly in their total lipid content and fatty acid profile. These results, to be confirmed by further investigations on a larger number of fish during different seasons, can be explained mainly by considering the availability and composition of feed, particularly regarding the fatty acid profile and antioxidants, which are the main factors affecting the lipid metabolism and consequently the fatty acid profile of fillets and their nutritional properties.

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PAPER

CHARACTERIZATION AND TRIALS OF A JERTE VALLEY CHERRY PRODUCT AS A NATURAL ANTIOXIDANT-ENRICHED SUPPLEMENT

M. GARRIDO[a], D. GONZÁLEZ-GÓMEZ[b], M. LOZANO[b], C. BARRIGA[a], S.D. PAREDES[c] and A.B. RODRÍGUEZ[a]*

[a]Department of Physiology (Neuroimmunophysiology and Chrononutrition Research Group), Faculty of Science, University of Extremadura, 06006 Badajoz, Spain

[b]Department of Horticulture and Pomology, Technological Institute of Food and Agriculture of Extremadura (INTAEX), 06071 Badajoz, Spain

[c]Department of Physiology, School of Medicine, Complutense University of Madrid, 28040 Madrid, Spain

*Corresponding author: Tel. +34 924 289 300 Ext.89035, Fax +34 924 289 388, email: moratino@unex.es

ABSTRACT

As a food, the cherry fruit is nutrient dense, with a relatively low caloric content and major amounts of important phytochemicals. The nutritional and functional properties of a Jerte Valley (Cáceres, Extremadura, Spain) cherry product were characterized with the aim of assessing its potential use in improving the antioxidant status in humans. The results showed the product to have high levels not only of nutritional compounds but also of bioactive compounds. In a subsequent placebo-controlled trial with human volunteers, the ingestion of this product improved their antioxidant status. This cherry-based product may therefore reliably be categorized as a natural antioxidant-enriched supplement.

- Keywords: antioxidant status, melatonin, phenolics, serotonin, sweet cherry, tryptophan -

INTRODUCTION

Since phytochemicals present in fruits and vegetables have known actions as antioxidants (PRIOR *et al.*, 2007), it is assumed worldwide that the consumption of fruits and vegetables exerts a protective effect on health (PITSAVOS *et al.*, 2005). In fact, since the 1990s, several international organizations have recommended increasing the consumption of fruits and vegetables to five or more daily servings in order to provide a desirable intake of antioxidants, which exert health-promoting effects (WHO, 1990).

The cherry fruit is considered a nutrient-dense food with a relatively low caloric content and a significant amount of important bioactive components including anthocyanins, quercitin, and carotenoids (McCUNE *et al.*, 2011). Several studies have reported the *in vitro* antioxidant effects of the aforementioned compounds. Thus, anthocyanins, which are referred to as the prevailing phenolics in sweet and sour cherries, inhibit lipid oxidation and play an important role as scavengers of free radicals (WANG *et al.*, 1997; CHUN *et al.*, 2003). Likewise, clinical studies demonstrate that the consumption of sweet cherries, in particular, Bing and Jerte Valley cherries, increases the plasma lipophilic and urinary antioxidant capacities, respectively (JACOB *et al.*, 2003; GARRIDO *et al.*, 2010). In this respect, the vast majority of works related to the anti-inflammatory and antioxidant effects produced by the ingestion of cherries or cherry-derived products attribute these benefits to the amount of phenols present in both sweet and sour cherries (FERRETTI *et al.*, 2010). Moreover, anthocyanins are able to inhibit nitric oxide (NO) production and other pro-inflammatory factors, such as tumour necrosis factor-alpha (TNF-α) (WANG and MAZZA, 2002a,b), thereby highlighting the anti-inflammatory functions of these molecules. The inhibitory effect of inflammatory pathways has been hypothesized to be the main molecular mechanism to explain the relationship between the consumption of cherries or cherry-derived products and the alleviation of several chronic diseases such as arthritis and gout (JACOB *et al.*, 2003; HE *et al.*, 2006). The beneficial properties of fruits and vegetables have also been proposed to come from the additive and synergistic effects of their phytonutrients (MILDE *et al.*, 2007). In this regard, it has recently been reported that Jerte Valley sweet cherries contain not only high concentrations of anthocyanin pigments and other phenolic compounds (GONZÁLEZ-GÓMEZ *et al.*, 2010), but also high levels of tryptophan (CUBERO *et al.*, 2010), serotonin, and melatonin (GONZÁLEZ-GÓMEZ *et al.*, 2009), which are molecules with well-known antioxidant effects (PAREDES *et al.*, 2007a, b, c).

Since *in vitro* measurements of high antioxidant capacities of foodstuffs may or may not be an indication of their potential for altering the *in vivo* antioxidant status (PRIOR *et al.*, 2007), the main objective of this work was to determine the nutritional and functional characteristics of a Jerte Valley (Cáceres, Extremadura, Spain) cherry product, and subsequently to evaluate its potential use to improve the antioxidant status in humans.

MATERIAL AND METHODS

Nutritional analysis

Moisture was determined by the loss of weight of 1 g of freeze-dried cherry product in a Selecta Vaciotem vacuum oven (JP Selecta, Barcelona, Spain) at 100°C. Ash content was measured by calcination of 1 g of freeze-dried cherry product in a Selecta muffle furnace (Andaluza de Instrumentación, Mairena del Aljarafe, Seville, Spain) at 550°C for 24 h. Protein analysis was carried out by the Kjeldahl method, using a Gerhardt Kjeldatherm digestor and a Gerhardt Vapodest distillation system (Izasa, Seville, Spain). Dietary fibre was measured by enzymatic digestion of 1 g of freeze-dried cherry product, according to the enzymatic-gravimetric method established by the AOAC, 1980.

Functional analysis

Total antioxidant activity

Determination of total antioxidant activity (TAA) was carried out using the end-point method established by CANO *et al.* (2000). Briefly, 0.03 g of freeze-dried cherry product was diluted in 10 mL of phosphate buffer, and then 20 μL of the diluted sample was put into a spectrometer cuvette containing 1 mL of the radical cation 2,20-azino-*bis*(3-ethylbenzothiazoline 6-sulfonate) (ABTS). The initial absorbance at 730 nm was compared with the absorbance obtained after 20 min of reaction. The measurements were carried out in a UV-2401PC Shimadzu spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD, USA). Results are expressed as mg of Trolox per 100 g of freeze-dried cherry product.

Total polyphenols content

Phenols were extracted from 0.15 g of freeze-dried cherry product diluted with 90 mL of a solution of 80% EtOH (1% hydrochloric acid), following the method described by LIMA *et al.* (2005). They were then determined using the Folin-Ciocalteu reagent (FOLIN and CIOCALTEU, 1927) in a UV-2401PC Shimadzu spectrophotometer. Gallic acid was used as standard, and the results are expressed as mg of gallic acid per 100 g of freeze-dried cherry product.

Total anthocyanins content

Anthocyanins were extracted from 0.3 g of freeze-dried cherry product in 50 mL of methanol solution (0.1% hydrochloric acid) for 24 h at -10°C. The absorbance of the resulting extracts was measured at 515 nm in a UV-2401PC Shimadzu spectrophotometer. Quantification was carried out using malvidin as standard. The results were expressed as mg of malvidin per 100 g of freeze-dried cherry product.

Quantification of the amino acid tryptophan

To prepare the freeze-dried cherry product for analysis, 1 g of freeze-dried cherry product was accurately weighed, and 0.5 g of $Ba(OH)_2$ (ALEGRÍA *et al.*, 1996) plus 0.5 mL of distilled H_2O were added in a cryotube. This solution was mixed by rocking shaker for 10 min. Hydrolysates were heated in the oven at 100°C for 12 h and centrifuged for 15 min at 13 200×*g*. The supernatant was collected and filtered through 0.45 μm pore filters, and finally frozen at -28°C.

Upon analysis, the samples were adjusted to a working pH of 5.5 by means of sodium acetate/acetic acid buffer. Each assay was initiated with the injection of 20 μL of sample into a high performance liquid chromatography device equipped with a fluorescence detector (HPLC/FL) (Thermo FL 3000, San José, USA). The HPLC/FL system was controlled by ChromQuest™ 4.2 software, and the chromatographic analysis was performed using a ZORBAX Eclipse XDB-C_{18} column (150 mm × 4.6 mm, 5 μm; Agilent Technologies, Madrid, Spain). The binary gradient system consisted of eluent A (methanol:acetic acid:water, 10:2:88, v:v:v) and eluent B (methanol). The excitation and emission wavelengths were 284 and 340 nm, respectively, and the mean retention time was 7.9 ± 0.2 min. Peak areas were correlated with the concentrations in accordance with the calibration curve. The results correspond to the assay of each sample carried out in triplicate.

Quantification of the indoles serotonin and melatonin

To extract melatonin and serotonin from the freeze-dried cherry product, 15 mL of phosphate buffer (1 M; pH 8.0) and 3 mL of KOH (1 M; pH > 8.0) were added to 6 g of freeze-dried product. Once homogenized, the sample was filtered and rinsed with 3 mL of chloroform, and then the mixture was shaken for 10 min. After that, the mixture was centrifuged for 5 min at 15 320×*g*, and then the organic phase was separated and evaporated using a nitrogen gas stream. Then 200 μL of formic acid (0.45%) solution was used for sample reconstitution, and finally the sample was injected into an HPLC system with an electrospray ionization mass spectrometry (MS-ESI)-quadrupole detector (Agilent 1100). The separation of melatonin and serotonin was accomplished with an Xterra C_{18} HPLC column (100 mm × 2.1 mm, 3.5 μm; Waters Cromatografía, S.A., Barcelona, Spain) heated at 30°C. The mobile phase used for the separation was 0.45% formic acid and acetonitrile (95:5, v:v) in gradient mode. From minute 0 to minute 5, isocratic elution was applied (95% of 0.45% formic acid and 5% of acetonitrile).

Following the serotonin elution, a linear gradient was used for melatonin elution, passing from minute 5 to minute 8 the acetonitrile portion of mobile phase from 5 to 100%, and then maintaining it in this proportion until minute 16.

Once the peaks had been characterized, the determination of melatonin and serotonin was performed by selected ion monitoring (SIM) of the generated ions (m/z 233 for melatonin and m/z 177 for serotonin). Quantitative calculations were made with the peak areas of melatonin and serotonin. ACOC software was used for the calibration and statistics calculations (ESPINOSA-MANSILLA *et al.*, 2005), and Agilent ChemStation software for data acquisition and peak area integration.

Determination of antioxidant status in humans

Participants

The study was carried out on young (20-30 years old, n = 10; 5 men and 5 women), middle-aged (35-55 years old, n = 10; 5 men and 5 women), and elderly (65-85 years old, n = 10; 5 men and 5 women) volunteers whose weight, height, and body mass index (BMI) values are presented in Table 1. The study was approved by the Ethics Committee of the University of Extremadura (Badajoz, Spain) in accordance with

Table 1 - Weight, height, and body mass index (BMI) of young, middle-aged, and elderly participants.

	Young		Middle-aged		Elderly	
	Men	Women	Men	Women	Men	Women
Weight	81.00±2.70	60.5±8.22	76.00±3.91	65.2±4.54	77±9.62	64.33±9.13
Height	1.80±0.04	1.61±0.02	1.74±0.06	1.65±0.03	1.75±0.01	1.61±0.05
BMI	24.86±0.68	23.07±2.57	24.89±0.78	23.84±2.54	24.97±2.84	24.76±2.88

Each value represents the mean ± SD of five participants.

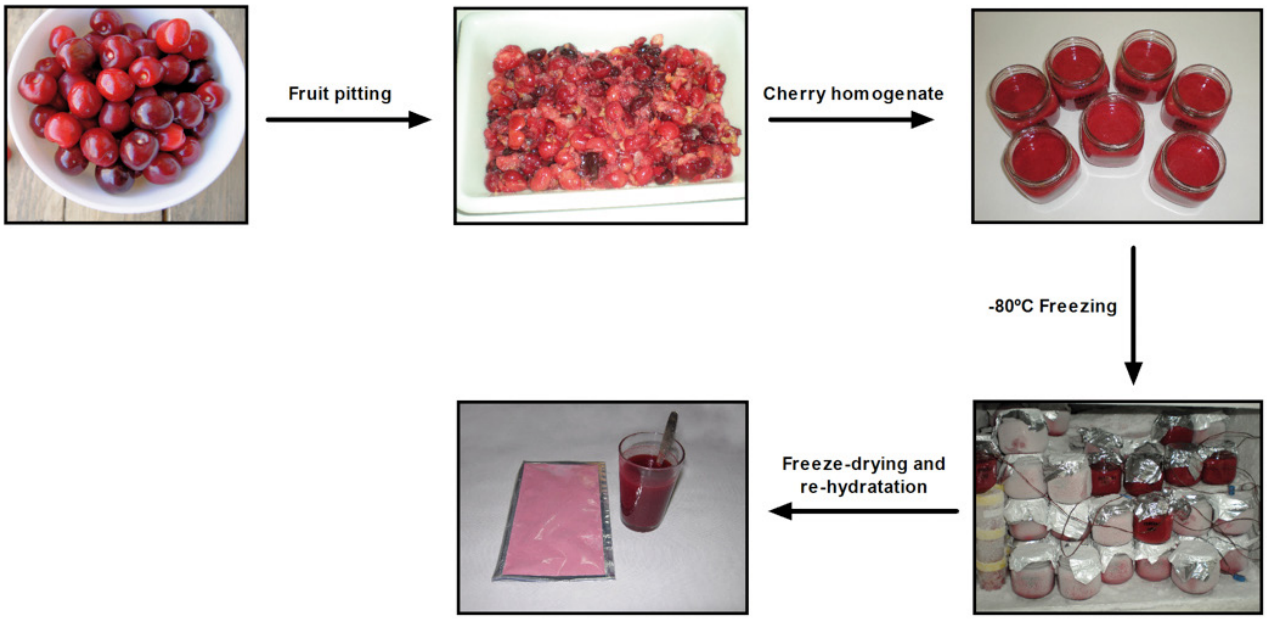


Diagram 1 - The cherry product was made from 141 g of fresh cherries in equal parts of four Jerte Valley cultivars: Bourlat, Navalinda, Pico Negro, and Pico Colorado. Once pitted, 7.5 g of maltodextrin and 1.5 g of ascorbic acid were added to fresh fruit to obtain a cherry homogenate. Subsequently, the cherry homogenate was frozen at -80°C, freeze-dried, and then ground in order to get a powder that can be easily dissolved in water.

the Declaration of Helsinki, the Council of Europe, and the Universal Declaration of UNESCO on human rights, biomedicine, and human genome. All participants were of Caucasian ethnicity. Each participant was ascertained to be in good health from their medical history and a clinical examination including routine laboratory tests and screening. The participants were non-smokers, were not using any medication, and abstained from alcohol. Informed consent was obtained from all participants.

Experimental design

The study was a blind, placebo-controlled, randomized, crossover design with two treatment periods of five days each, separated by a washout period of one week. Either the placebo or the Jerte Valley (Cáceres, Extremadura, Spain) cherry product was consumed twice a day, as lunch and dinner desserts. One dose of the Jerte Valley cherry product (27.85 g) consisted of 18.85 g of pitted, freeze-dried cherries (equivalent to 141 g fresh cherries) consisting in equal parts of four Jerte Valley cherry cultivars (Bourlat, Navalinda, Pico Negro, and Pico Colorado), plus 7.5 g maltodextrin and 1.5 g ascorbic acid (Spanish patent no. ES 2342141 B1). Once it was freeze-dried, the cherry-based product was ground in order to get a powder that was then packed in plastic containers (Diagram 1). Before its consumption, the Jerte Valley cherry product was diluted in 125 mL of water. The placebo was a commercial unsweetened cherry-flavoured soft drink (Kool-Aid®, Kraft Foods, USA; ingredients listed: citric acid, salt, calcium phosphate, red 40, artificial flavour, ascorbic acid, artificial colour, blue 1) which was prepared by mixing it with water in the proportion recommended by the manufacturer (Fig. 1).

Urinary antioxidant capacity assay

First-void morning urines were collected before the trial (basal values), after a 5-day intake of the Jerte Valley cherry-based product or the placebo (trial values), and one day following the termination of the treatment (post-trial values). The samples were stored at -20°C until biochemical assay.

Total antioxidant capacity was measured in the urines using a colorimetric assay kit (Cayman, MI, USA) according to the manufacturer's instructions (MILLER *et al.*, 1993; RICE-EVANS and MILLER, 1994).

Determination of blood glucose levels

Blood glucose levels were determined in all participants using a blood glucose level testing kit (Medisense, Abbott Laboratories, Ab-



Fig. 1 - Jerte Valley cherry product (left panel) and placebo (right panel).

bott Park, IL, USA). Glucose levels were measured at 08:00 h, before the trial (basal values), during the 5-day intake of the Jerte Valley cherry-based product or the placebo (trial values), and one day following the termination of the treatment (post-trial values). Results are expressed in mg/dL.

Statistical analysis

The Kolmogorov-Smirnov test was applied to examine the normality of the distribution of the results. Once confirmed that the data did not fit a normal distribution, the Friedman non-parametric test followed by Dunns' multiple comparison tests were used to analyse the results. Each value represents the mean ± SD of the number of determinations, unless otherwise indicated. The degree of significance was set at $P < 0.05$. All analyses were performed using GraphPad Prism (version 5.0, 2007; GraphPad Software Inc., San Diego, CA, USA).

RESULTS AND CONCLUSIONS

Prevention of health disorders is commonly associated with lifestyle, and particularly with diet. Phytochemicals present in fruits and vegetables, e.g., phenols, anthocyanins, and carotenoids, possess a great potential to avoid or delay the development of such disorders as cancer, obesity, or cardiovascular diseases. In the present study, several nutritional and functional parameters were analysed in the Jerte Valley cherry product (Tables 2 and 3, respectively). With respect to the proximate analysis, one observes in Table 2 that the Jerte Valley cherry freeze-dried product contains comparatively high levels of fibre (7.83 ± 0.26%) and proteins (2.99 ± 0.05%), since the content of fibre and proteins in other commonly consumed cherry products is roughly 2% and 1%, respectively (GEBHARDT *et al.*, 2006). Both fibre and proteins may contribute to food intake and body weight regulation, being strong determinants of short-term satiety (ANDERSON and MOORE, 2004; SUTER, 2005). Indeed, high fibre diets have been associated with improved blood glucose control and reduced cholesterol levels, thus further highlighting the potential beneficial effects of this nutrient (SUTER, 2005; KEENAN *et al.*, 2006).

Table 2 - Nutritional characterization of the Jerte Valley cherry product (values expressed per 100 grams of freeze-dried product and per dose, i.e., 27.85 g freeze-dried product).

Parameter	Per 100 g product	Per dose (27.85 g)
Moisture (%)	5.09±0.07	1.41±0.02
Ash (%)	6.55±0.20	1.82±0.05
Protein (%)	2.99±0.05	0.83±0.01
Total dietary fibre (%)	7.83±0.26	2.18±0.07

All the data are the mean ± SD of four replicates.

With regard to the functional analysis (Table 3), the Jerte Valley cherry product contains 5669.61 ± 127.75 mg total phenolics in 100 g of freeze-dried product, which is equivalent to 1578.98 ± 35.57 mg total phenolics per dose (roughly 28 g of freeze-dried product). Interestingly, the content of polyphenols in a 28 g serving of the Jerte Valley cherry product is 10-fold higher than that found in Bing sweet cherries, which present 160-170 mg total polyphenols in a 100 g serving (CHAOVANALIKIT and WROLSTAD, 2004). Similarly, the total polyphenols in a 100 g serving of Montmorency tart cherries (301 mg and 558 mg in flesh and skins, respectively) (CHAOVANALIKIT and WROLSTAD, 2004) are much lower than those in the Jerte Valley cherry product. These bioactive compounds have long-term human health benefits due to their wide range of biological activities, including antioxidant, antiallergic, anticarcinogenic, antimicrobial, antimutagenic, and anti-inflammatory properties (BENAVENTE-GARCÍA *et al.*, 1997). In particular, cherry phenolics protect neuronal cells against oxidative stress-mediated damage, one of the main factors contribut-

Table 3 - Functional characterization of the Jerte Valley cherry product (values expressed per 100 grams of freeze-dried product and per dose, i.e., 27.85 g freeze-dried product).

Parameter	Per 100 g product	Per dose (27.85 g)
Total antioxidant activity (mg) [a]	2478.65±113.68	690.30±38.06
Total phenolics (mg) [b]	5669.61±127.75	1578.98±35.57
Total anthocyanins (mg) [c]	110.31±8.53	30.72±2.37
Tryptophan (mg)	6.95±1.06	1.93±0.29
Serotonin (ng)	95.73±6.21	26.66±1.73
Melatonin (ng)	58.38±5.31	16.25±1.48

All the data are the mean ± SD of four replicates, except for tryptophan content (n = 3).
[a]mg Trolox equivalents.
[b]mg gallic acid equivalents.
[c]mg malvidin equivalents.

ing to the development of neurodegerative diseases such as Alzheimer's disease (SMITH *et al.*, 2000). Cherries also show antioxidant effects *in vivo*, since an increased activity of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) as well as a decrease of lipid peroxidation have been reported in mice fed sour cherry juice (SARIC *et al.*, 2009). In the present study, the total anthocyanins measured in the Jerte Valley cherry product were 110.31 ± 8.53 mg per 100 g of freeze-dried product, which is equivalent to 30.72 ± 2.37 mg total anthocyanins per dose. Anthocyanins present one of the strongest antioxidant activities among phenolic compounds. In fact, the anthocyanin content of these cherries is comparable to that of other plant foods in which evidence has suggested health-promoting effects related to their anthocyanin content (STEVENSON and HURST, 2007).

In recent years, the amino acid tryptophan, the neurotransmitter serotonin, and the indole melatonin have been identified as important bioactive compounds in plants. This may have major implications for animal and human health given the potent antioxidant activity of these molecules in a variety of disorders and diseases in which enhanced production of free radicals has been reported (REITER *et al.*, 2009; RICHARD *et al.*, 2009). In the cherry product of the present study, tryptophan reached a concentration of 6.95 ± 1.06 mg per 100 g of freeze-dried product, equivalent to 1.93 ± 0.29 mg per dose. Serotonin reached 95.73 ± 6.21 ng per 100 g of freeze-dried product, equivalent to 26.66 ± 1.73 ng per dose. And melatonin reached 58.38 ± 5.31 ng per 100 g of freeze-dried product, equivalent to 16.25 ± 1.48 ng per dose.

The total antioxidant capacity of the product was assessed. The results were that it contained 2478.65 ± 113.68 mg Trolox per 100 g of freeze-dried product, equivalent to 690.30 ± 38.06 mg per dose. Interestingly, a 28 g serving of the Jerte Valley cherry product possesses a greater antioxidant capacity than a 100 g serving of fresh Jerte Valley sweet cherries (GONZÁLEZ-GÓMEZ *et al.*, 2010).

This cherry product was administered to young, middle-aged, and elderly volunteers in an attempt to elucidate whether the consumption of this natural product is beneficial for health. Fig. 2 shows that the consumption of a placebo did not modify the urinary antioxidant capacity in any of the age groups studied, whereas the ingestion of the Jerte Valley cherry product raised ($P < 0.05$) the urinary antioxidant capacity with respect to their corresponding basal values in all three groups: young (2.01 ± 0.95, Fig. 2A), middle-aged (1.47 ± 0.37, Fig. 2B), and elderly (3.23 ± 0.78, Fig. 2C). These values returned to basal values ($P < 0.05$) one day following the trial's termination (post-trial values) in the young (1.26 ± 0.56) and middle-aged (1.07 ± 0.50) volunteers. It is noteworthy that the greatest urinary antioxidant increase after the ingestion of the cherry product (more than three-fold) was found in the elderly age group, with the value remaining elevated (2.74 ± 0.42; $P < 0.05$) with respect to its corresponding basal level one day following the trial's termination. These findings agree with previous reports that the antioxidant status after the ingestion of several Jerte Valley cherries tends to be higher in the elderly (GARRIDO *et al.*, 2010). Despite the fact that total energy intake declines with age, the requirements for many nutrients increase in order to maintain organ systems with declining functionality (MARTYN *et al.*, 1996). For this reason, dietary components with high antioxidant activity have

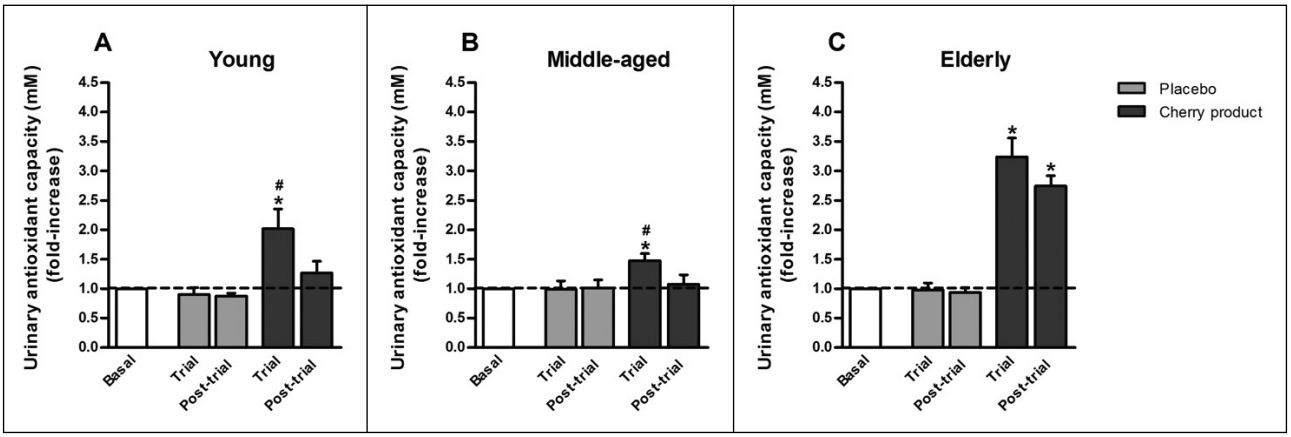


Fig. 2 - Effect of a Jerte Valley cherry product and a placebo on urinary antioxidant levels obtained in first-void morning urines, in young (A), middle-aged (B), and elderly (C) participants. Basal values are urine samples obtained before the intake of the cherry product or the placebo, assay values are urine samples taken on the last day of a 5-day cherry product intake or placebo intake, and post-trial values are urine samples obtained after the termination of the assay. Data are expressed as fold increase over the basal levels. Each value represents the mean ± SEM from ten participants.
*$P < 0.05$ with respect to their corresponding basal values.
#$P < 0.05$ with respect to their corresponding post-trial values.

Table 4 - Blood glucose (mg/dL) levels measured in young, middle-aged and elderly subjects before the beginning of the trial (basal), during the 5-day intake of the placebo or the cherry-product (trial), and one day following its termination (post-trial).

		Placebo		Cherry product	
	Basal	Trial	Post-trial	Trial	Post-trial
Young	91.50±2.02	92.00±3.16	91.25±1.76	91.50±1.63	91.87±1.86
Middle-aged	102.20±12.75	99.10±9.21	97.60±8.70	99.80±11.74	100.60±11.89
Elderly	101.25±5.74	102.12±4.69	97.75±5.25	98.62±4.48	99.37±4.29

Results are expressed in mg/dL. Each value represents the mean ± SEM of ten participants.

to be the object of particular attention because of their potential role in modulating the oxidative stress associated with ageing and chronic conditions (MEYDANI, 2001). In this respect, the indole melatonin, which declines gradually from young adulthood onwards, may play an important role in the regulation of the ageing process, since it is reportedly a potent antioxidant and free radical scavenger (PAREDES *et al.*, 2007b). In fact, the rate of ageing and the time of onset of age-related disorders can be retarded by melatonin supplementation or treatments that preserve the high amplitude of the endogenous rhythm of melatonin formation (BONDY *et al.*, 2004).

The measurements of blood glucose levels (Table 4) showed no significant effects after the consumption of either the placebo or the cherry-based product in any age group. In this respect, cherry fruit has an estimated glycæmic index of 22, generally lower than most other fruits such as apricot (57), grapes (46), peach (42), blueberry (40), or plum (39). Furthermore, numerous studies have shown that anthocyanins are effective in improving insulin secretion in response to varying glucose levels (GHOSH and KNONISHYI, 2007). Consequently, it has been suggested that cherries may aid in diabetes control (LACHIN and REZA, 2012), being a potentially particularly appropriate fruit-based snack food (as compared with many other fruits) for diabetic individuals due to their lower glycæmic index (McCUNE *et al.*, 2011).

The current development of functional products with antioxidant effects is mainly focused on the production of phenolic-enriched products (RAMÍREZ-TORTOSA *et al.*, 2004; CERDA *et al.*, 2006). However, in advanced age, an exogenous supplementation of melatonin and its precursors, tryptophan and serotonin, may exert beneficial effects as potent antioxidants. Therefore, the consumption of the Jerte Valley cherry product, which contains not only high concentrations of total phenolics, including anthocyanin pigments, but also high levels of tryptophan, serotonin, and melatonin, may be used as an antioxidant-enriched supplement to fight against disorders in which oxidative stress plays a deteriorating role.

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PAPER

GOVERNANCE MECHANISMS IN FOOD COMMUNITY NETWORKS

S. PASCUCCI[1], A. LOMBARDI[2], L. CEMBALO[2]* and D. DENTONI[1]
[1]Dipartimento di Economia e Politica Agraria, Università degli Studi di Napoli Federico II, Via Università 96, 80055 Portici, NA, Italy
[2]Management Studies Group, Wageningen University, Hollandseweg 1, Building No. 201, 6706 KN Wageningen, the Netherlands
*Corresponding author: cembalo@unina.it, lcembalo@gmail.com

ABSTRACT

This paper discusses the concept of the food community network (FCN) and how consumers and farmers organize credence food transactions. The FCN is based on pooling specific resources and using membership-based contracts to assign decision and property rights. It implies an organization based on a combination of several *democratic* and *communitarian* elements, with few *market-like* and *bureaucratic* elements. By applying arguments from new institutional economics and organizational science, case studies on community-supported agriculture reported elsewhere were used to describe how FCN governance works. The results indicate a great variety of FCN organizational forms.

- Keyword: food community network, community-supported agriculture, credence food transactions, governance mechanisms -

INTRODUCTION

The agro-food industry, farmers and consumers are in the process of setting up new organizations to exchange food worldwide (PASCUCCI, 2010). This is especially evident when food has the features of a credence good. Hence the way it is produced matters more than other intrinsic attributes. In previous studies such organizations have been defined as alternative supply chains, civic food networks or food community networks (FCNs) (RENTING *et al.*, 2003; AUGLIA *et al.*, 2008; BOUGHERARA *et al.*, 2009; PASCUCCI, 2010; CARACCIOLO and LOMBARDI, 2012). In these organizations, transactions of credence foods are carried out through direct interaction of consumers and farmers, who share resources and stakes. In a more recent contribution a more formalized approach was proposed to define this organization, mainly through a new institutional economics oriented approach, and using Grounded Theory as a methodological tool (PASCUCCI *et al.*, 2011). However, a crystalline definition of FCNs is still some way off. In this paper we propose a further step in the direction of identifying the elements constituting FCNs.

Several approaches to describe and analyse organizational design can be used when dealing with network organizations. HATCHUEL (2001), for example, proposed to combine organization and design theories to describe collective actions, specifically to understand the interplay between learning, innovating and imposing routines. NOOTEBOOM (2004) also highlights the interplay between governance and competence to describe network organizations and forms of cooperation among economic actors. Though the literature on network organizations is far-reaching and extensive we mainly build our analysis on organizational design arguments, comparing the new institutional economics (NIE) approach (WILLIAMSON, 1991; MÉNARD, 2004) with a more recent approach in organizational science proposed by GRANDORI and FURNARI (2008)[1]. While design theories and competence studies are very helpful in describing "creative" and innovative network formation, NIE has been extensively used to describe network organizations in the food sector (Menard, 2004). However, while the "standard" NIE approach to organizations emphasises alternative governance structures (e.g. markets, hybrids or hierarchies) looking at networks as alternative to both markets and hierarchies, the GRANDORI and FURNARI approach (GFA) proposes a way to analyse alternative organizations by considering more basic elements which can be combined, and allow for complementarities (GRANDORI and FURNARI, 2008). Therefore, unlike the NIE approach, the GFA is not limited to explaining differences within the same "cluster/category" of governance structures. This is particularly handy when it comes to analysing emerging governance structures, such as FCNs. More specifically, in this paper we use the GFA to investigate different FCN case studies, mainly using internet-based sources and literature reviews. Even though the FCN case studies analysed are based on North American CSAs, we believe that our research findings can be generalized to any FCN organization operating in various parts of the world.

MATERIALS AND METHODS

The FCN is an emerging type of organization which often challenges more traditional, "mainstream" types of organizations dealing with food production and distribution (e.g. supermarkets and "hard discounts") (HENDRICKSON and HEFFERNAN, 2002; SCHIFANI and MIGLIORE, 2011; CEMBALO *et al.*, 2012). More specifically, the FCN seems to be a competitive organization when it comes to exchanging foods with a higher degree of credence attributes. For such foods "the way" they are produced and distributed is more important than "what" constitutes them (i.e. chemo-physical elements). Traditional organizations of credence foods are affected by the presence of several legally independent actors, like food companies, farms, public agencies and consumers (or households), which coordinate quantity and quality issues by means of standards, logos, brands and labels (RAYNAUD *et al.*, 2005; CEMBALO *et al.*, 2008). All these actors have to deal with issues of asymmetric information and moral hazard problems, therefore often experiencing high transaction costs. Typical solutions are the use of arrangements such as *bilateral contracts*, *strategic networks and alliances*, and *vertical integration* (MÉNARD and VALCESCHINI, 2005; PASCUCCI, 2010). Such solutions often rely on public monitoring (VETTER and KARANTININIS, 2002). However, FCNs are organized in a different way. But how?

According to preliminary empirical evidence, FCNs have been described as based on a group of highly motivated consumers and a (group of) producer(s) that decide to vertically coordinate, and partially integrate, on the basis of a long-term relationship to produce and transact credence foods (PASCUCCI, 2010). In this section we further investigate the organization of FCNs by looking at two theoretical approaches: new institutional economics and organizational science.

A new institutional economics perspective

A new institutional economics approach can be used to characterize FCNs, examining different attributes of *governance mechanisms*, namely the degree of resource *pooling*, and types of *contracting mechanisms*, the latter being divided

[1] GRANDORI and FURNARI (2008) provide an extended discussion on this topic which we recommend to those interested.

into *coordination*, and internal-external *competition* (WILLIAMSON, 1991; MÉNARD 2004; MÉNARD and VALCESCHINI, 2005; KARANTININIS, 2007). The governance mechanisms used in the FCN are based on intense resource pooling, while coordination is based on limited authority and relational contracts (PASCUCCI, 2010). Moreover, there is limited competition among members while external competition with other organizations (such as supermarkets) is very high. Following this approach we can view the FCN as a *form of vertical integration* between consumers and farmers, leading to the constitution of a hybrid such as a formalized network. The integration process is based on using *membership* to assign decision and property rights, and driven by the need to share and pool resources which are specific to the members. When membership is strongly formalized, the network assumes the form of a club which, in some cases, can lead to the legal form of a producer-consumer cooperative (CEMBALO *et al.*, 2013). Resource pooling and sharing is slightly different between consumer and producer members: (1) consumers provide time, information, knowledge and financial resources by participating directly in the organization of the production process; (2) farmers provide land and capital assets but also specific skills and knowledge (PASCUCCI, 2010). They also transfer decision rights to consumers. On the one hand, consumers receive as pay-offs leisure and credence foods while reducing transaction costs (i.e. the costs of monitoring). The time spent by consumers in the production process is assumed to be leisure time (PASCUCCI *et al.*, 2011). The time allocated to the production process is either related to manual working and to its organization (managerial tasks). The time allocated by consumers is also used to monitor the process, and hence to reduce the risk of producers' moral hazard. Since consumers can coordinate participation and their time spent on participation is not a cost but a utility-enhancing activity, we can assume that the overall monitoring costs of the process can be considerably reduced by this mechanism. On the other hand, farmers reduce part of their production and transaction costs (i.e. labour costs, certification costs, etc.), investment uncertainty and income instability (PASCUCCI, 2010). Producers reduce their production or transaction costs by allowing consumers to directly participate in the organization of the production process. They also limit uncertainty and can reduce lock-in problems of investing in specific assets related to credence food production.

An organizational science approach

NIE is limited in addressing the "micro-elements" of organizations (GRANDORI and FURNARI, 2008). The main limitation of NIE is the challenge in addressing specific and basic elements of organizational design, while being more focused on the (quantitative) analysis of discrete structural alternatives (WILLIAMSON, 1991). MILGROM and ROBERTS (1995) introduced an approach where several complementarities among different organizational practices could be considered. However, the list of different practices remains extensive and not sufficiently systematic in nature. For example, it is not clear which practices (governance elements) are complementary and under what circumstances (GRANDORI and FURNARI, 2008). This constitutes a major shortcoming when seeking to analyse "new types" of organizations, for which empirical evidence is still limited, as is the case of FCNs. GRANDORI and FURNARI (2008) proposed to overcome this problem by using a "chemistry of organization" type of approach. Building on three theoretical frameworks in the tradition of organizational science, namely complementary and configurational approaches, transaction cost economics and contingency theory approaches, they identify a "combinative" approach to analyse multiple effective organizational combinations under different contingencies (GRANDORI and FURNARI, 2008).

Their approach is based on identifying basic elements, or "building blocks", which constitute an organization. More precisely, they distinguish between four categories of elements, namely: (i) *market-like elements*, which include price-like and control-by-exit devices; (ii) *bureaucratic elements*, including formal rules and plans, and articulation of the division of labour; (iii) *communitarian elements*, including knowledge and value sharing, and common culture; (iv) *democratic elements*, including the allocation of ownership, decision and representation rights. Market-like elements mainly concern the capacity of coordinating action with minimal communication through high-powered incentives. Bureaucratic elements are linked to formal elements of governance mechanisms such as formal rules, procedures and evaluation systems. Community elements are related to organizational practices, infusing cohesion and aligning interests, for example through value and knowledge sharing. Finally democratic elements refer to the diffusion of ownership, decision and representation rights which enable the organization to integrate different interests among and between their members (GRANDORI and FURNARI, 2008).

In this approach it is assumed that a combination of different elements is not only possible but constitutes the potential advantage of an organization. This is particularly useful in the case of FCNs where a clear definition is still lacking and contrasting elements persist. For example, in market-like practices organization members base their decisions on the economic incentives they receive. In FCNs consumers often pay farmers a "market" price for the (box of) products delivered. Specific forms of "pay-for-

performance" are also used, for example when the farmer receives a "premium" for "extra-quality" products, such as for niche or fresh products. However, discounts can also be arranged, even for high-quality products, as in the case when greater quantities of products are bought, or "share-based" payment is organized at the beginning of each season. In this case a lower price per product is not linked to lower quality, and it does not work as a disincentive for the farmer. On the contrary, it might positively affect trustworthiness between farmers and consumers, reinforcing the willingness to buy and produce high-quality products. Therefore it can be said that also the exchange activities of FCNs are coordinated through a type of price mechanism.

Although FCNs are far from being hierarchical organizations, some form of authority, for example in strategic decisions, is often adopted. For example, authority is used to decide on investments, to control quality issues, to arrange delivery service and solve disputes between farmers and members. As indicated by GRANDORI and FURNARI (2008), the main advantage in using bureaucratic power is basically to have a higher capacity to control opportunism, especially where transaction costs are high or when specific investments were made by FCN members. However, authority and bureaucratic elements represent a challenge for FCNs because hierarchical mechanisms are often in contrast with cooperative behaviour and trust. FCNs are often characterized by participatory decision-making, open access/open exit membership. This can lead to the problem of free-riding of some members (as in many cooperative-type organizations). To address the issue of free-riding, authority (and formalized rules) can be used to mitigate the tension between group-based interests and those of individual members.

Communitarian elements constitute a fundamental component of FCNs. They are formed by shared norms, which are mainly based on informal rules within each community. They are built to facilitate, motivate and coordinate types of collective actions led by community members because they can prevent opportunism by limiting the expectation that other members will behave opportunistically. In this way they can create trust and trustworthiness, which can also reduce transaction costs (NOOTEBOOM, 2007). In the FCN context, trust is an important feature, for example, to reduce the cost of safeguarding against opportunism. This is mainly due to information and knowledge exchange, which leads to control over members' reputations.

In general, communitarian elements can be effective at building trust if shared norms can be created within the community. This process is also leading to greater member involvement and commitment, thus reducing the need for economic-based "punishment" for opportunistic behaviour, while supporting non-economic rewards for group loyalty. It is important to stress that in communitarian practices participants are often trustworthy not due to control mechanisms (punishment) or economic incentives (rewards), but because they choose on the basis of intrinsic motivation. In general, as with other collective organizations, the capacity of FCNs to build trust, and hence reduce transaction costs, is conditional upon the size and homogeneity of the group of participants\members. Especially in their early stages FCNs tend to select highly motivated members, while excluding (indirectly) less aligned and motivated participants.

Distinct from issues of trust and trustworthiness, democratic elements refer to the component of FCNs based on sharing decision rights and fairness of decision-making. More participatory decision making and shared ownership of strategic resources are seen as enforcing commitment in the group interests, also leading to transaction costs reduction. Many FCNs adopt democratic procedures, for example, to check the quality of processes and products, and to enhance investments. The possibility to control but also to deliberate on strategic issues is a fundamental aspect of the potential competitive advantage of FCNs (NOOTEBOOM, 2007).

Classification of FCNs: evidence from CSA in North America

Using data available on-line, through websites and blogs, we analysed and classified a sample of 95 community-supported agriculture organizations operating in North America (US and Canada) by implementing K-means cluster analysis. The information was gathered in the second half of 2011. Using this information we derived variables related to both the new institutional economics approach, such as pooling and contracting, and the organizational science approach, such as market-like, bureaucratic, communitarian and democratic elements. Of the various types of FCN, CSA is the most widespread in the English-speaking world, especially in urban and peri-urban areas of US and Canada. CSA schemes are often established from an innovative dynamic strategy of farmers, who seek to establish relations with consumers in the same area. FCNs are based on local food supply and maintain a high sense of community. CSA schemes are often led by educated, highly skilled farmers, who work in a mosaic of small-scale farms. CSA prospers where many small farms can satisfy consumer needs with a wide range of food products, for a sizable urban population living in proximity of the farms in question (ADAM, 2006). The concept of CSA originated in the 1960s, when Japanese women, concerned with the increase in imported food and the loss of farmers and farmland, asked lo-

cal farmers to grow vegetables and fruit directly for their families. Starting from that, a number of families committed themselves to supporting their region's agriculture. In this way, the *teikei* concept was born which, literally translated, means "food with the farmer's face on it" (VAN EN, 1995). This model, first implemented in the United States in the mid 1980s, became known as CSA. As defined by GRADWELL *et al.* (1999), CSA is a partnership between farmers and community members working together to create a local food system. CSA farmers may produce vegetables, fruit, meat, dairy products, fibres, etc., directly for local community members. CSA differs from direct marketing because its members commit to a full-season price in the spring, sharing the risks of production (CICIA *et al.*, 2011). With this up-front support, farmers can concentrate on growing quality food and caring for the land. In return, members know where their food comes from and how it is grown; they share a connection to the land and farmers who produce for them, establishing a direct economic and social link between farmers and community members (WELLS and GRADWELL, 2001). Table 1 describes the variables used to identify pooling and contracting elements; Table 2 describes the organizational elements we used according to the organizational science approach.

We use these variables to build indexes of intensity for the six elements of governance we considered: pooling, contracting, market-like, bureaucratic, communitarian and democratic. We decided not to assign a weight to different variables, nor to different elements. Therefore each variable equally contributes to determine the intensity of the relevant element. If the specific variable was found we assigned a score of 1, otherwise we gave a score of zero. In table 3 we report the descriptive statistics of the six governance elements we measured in the sample.

Table 2 - Organizational Science elements.

Element	Variables
Market-like	Presence of incentive for subscribing annual shares Presence of private quality certification system
Bureaucratic	Participation in the community is formalized Subscription of annual shares is formalized Formal public and\or private quality certification system Formalized membership Formalized default conditions Scheduled visit in the farm Participation in the production process is formalized
Communitarian	Members are stimulate dot share knowledge Participation in the community is informal Participation in the production process is informal Free-access to the farm Informal certification system Quality is checked through blogs, discussions and\ or debates
Democratic	Participation in the community is open Quantity of products per box is jointly decided Portfolio composition of products is jointly decided Quantity of each product is jointly decided Quality of each product is jointly decided (i.e. organic non organic) Type of inputs to be used is jointly decided Production decisions are shared Visiting the farm is possible Information are shared

RESULTS

Table 1 - Pooling and contracting elements.

Elements	Variable	Meaning
Pooling	Knowledge	Presence of practices to share knowledge between members and producers
	Labour time	Members provide time to work in the community
	Financial capital	Members provide financial capital, for example through subscribing membership shares
	Physical capital	Members share their assets, such as machineries or facilities for supporting the activities of the community
	Decision right on product portfolio quantity	Members can decide how much products to receive from the CSA
	Decision right on product portfolio composition	Members can decide the type of products to receive from the CSA
	Decision right on specific product quantity	Members can decide the quantity of each product to receive from the CSA
	Decision right on specific product quality	Members can decide whether receive organic or non- organic products
	Input	Members provide inputs for the production process (other than labour and capital)
Contracting	Production process checking	Members directly participate and check farming processes
	Direct visit	Members can visit the farm (either in a flexible or a scheduled way)
	Quality checking	Members have the opportunity to comment, complain and discuss about the quality of the products (i.e. through a blog, website, etc.)
	Certification system	Private and/or public formalized certification system is in place
	Contracting membership	Membership contract is formalized
	Default conditions	Contract is specified default conditions (i.e. default in deliveries, breakdown in quality levels, etc.)

Table 3 - Descriptive statistics for the 6 organizational elements.

Elements	Min.	Max.	Mean	Std. Deviation
Market-like	0	2	1,22	0,47
Bureaucratic	0	5	3,24	1,00
Communitarian	0	6	3,27	1,82
Democratic	0	7	1,81	1,44
Pooling	1	9	4,06	1,62
Contracting	0	11	5,62	2,49

The results of K-means cluster analysis indicate the presence of five types of CSA. Table 4 shows the average indexes of governance intensity for each type:

Group 1 is characterized by a high level of formalization and contracting. We use the term "bureaucrats" for this group to indicate that governance mechanisms are mainly based on formalized rules. Decision-making is "centralized" and still remains within the area of the farmers' power. The "hard participants" are those belonging to group 2. In this group all indexes have high values and indicate extensive participation of members in all activities and governance issues of the CSA. Group 3, called Democratic shares, is more based on democratic mechanisms than group 2 and more based on sharing resources. Group 4 consists of soft participants, indicating that they are not so much involved in the CSA. Finally group 5, named Relational shares, is mainly based on communitarian elements with a strong combination of both pooling and contracting issues.

CONCLUSIONS

In this paper we discussed and analysed CSA within the framework of food community networks. In this type of organization, consumers and farmers closely integrate their functions by using combinations of different organizational elements such as market-like elements, bureaucratic, communitarian, democratic, contracting and pooling. More specifically we have studied several CSAs operating in North America. We identified five main distinct types but also confirmed some common features. For example, CSA often makes use of very formalized membership, to define not only the type of "delivery" service consumers wish to receive but also to share risks and transfer relevant decision rights. However, we found that consumers' decision rights on the production phase are sometimes limited, especially as regards the allocation of land to different uses.

The presence of different types of organizations indicates that within the framework of FCNs internal organizational dynamics have to be further investigated and linked to FCN different performance. In the current debate on alternative or short supply chains such links between different organizational structures and performance are under-investigated. Despite the broad consensus that FCNs can contribute, for example, to local sustainable development, it is still unclear which types of FCNs may be most suited to that purpose. Another issue requiring in-depth understanding is participation and whether a specific organizational structure leads the FCN to attract a target group of members. Understanding the relation between the type of FCN and type of participation mechanism may be important to better implement development-oriented strategies. For example, FCNs may be suitable to preserve local products, or to increase healthy food diets in a target group. Understanding whether a more or less formalized membership facilitates participation can be considered a key factor.

The analysis provided in this paper should be seen in the light of a descriptive contribution. Stricter research hypotheses on factors contributing to adoption of different governance mechanisms could not be tested, given the type and nature of information available. Analysis of FCN performance or member participation also has to be implemented. These are points to be developed in a future research agenda.

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Table 4 - Typologies of CSA.

Element	Typologies				
	Bureaucrats	Hard participants	Democratic sharers	Soft participants	Relational sharers
Market-like	1,3	1,4	1,4	0,9	1,1
Bureaucratic	3,7	3,9	2,9	2,1	3,1
Communitarian	1,9	5,4	2,7	0,9	4,2
Democratic	0,5	2,8	4,6	0,9	1,9
Pooling	2,1	5,0	6,9	2,9	4,9
Contracting	5,3	8,9	4,7	2,1	5,6
N. of cases	22,0	22,0	7,0	16,0	28,0

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SHORT COMMUNICATION

CHEMICAL COMPOSITION AND SELECTED PARAMETERS OF TECHNOLOGICAL SUITABILITY OF CAPRINE MILK PRODUCED IN ORGANIC AND CONVENTIONAL FARMS

J. BARŁOWSKA[1]*, Z. LITWIŃCZUK[2], A. WOLANCIUK[1] and T. SZMATOŁA[1]

[1]Department of Commodity Science and Processing of Raw Animal Materials, University of Life Sciences, Akademicka 13, 20-950 Lublin, Poland

[2]Laboratory of Organic Production of Animal Derived Food, University of Life Sciences, Akademicka 13, 20-950 Lublin, Poland

*Corresponding author: joanna.barlowska@up.lublin.pl

ABSTRACT

Despite the lower productivity (approximately 0.2 kg daily), goats maintained on an organic farm *vs* a conventional farm produced milk of a more favorable chemical composition while benefiting from parameters of technological suitability. The production season associated with the lactation stage had a significant influence on milk yield, its basic chemical composition, acidity, heat resistance and cytological quality.

- Keywords: chemical composition, goat milk, organic farms, production season, somatic cell count, technological parameters -

INTRODUCTION

Goat milk, in addition to its obvious nutritional features, has a therapeutic value. Unique characteristics exclusive to caprine milk as compared to bovine milk include: lower allergenicity, increased digestibility, buffer capacity, higher proportion of short-chain fatty acids, and favorable immunological and antibacterial properties (MICHAELIDOU, 2008). Despite the fact that goat milk has a similar basic chemical composition as that of a cow, it has slightly worse technological properties. It is less resistant to heat treatment and has different parameters for coagulation. Clotting time is shorter and therefore the curd obtained is weaker and more susceptible to tearing (BARŁOWSKA *et al.*, 2011).

Dairy products manufactured from goat milk are usually characterized by a regional denomination. Products can be described as having specific sensory properties (such as taste, aroma and flavor) as well as nutritional properties (including the composition of protein fraction and fatty acid profile) due to the diverse botanical composition of organic pastures and regional microclimate. Those products can meet the requirements and expectation of the consumers with the most sophisticated taste (SCINTU and PIREDDA, 2007).

Almost all farms in Poland that keep goats are considered to employ the extensive farming system. Utilization of natural pastures without fertilization is very common according to NARDONE *et al.* (2004), which gives an existing opportunity to accelerate the transition of such farms to the organic production system.

The aim of this study was the evaluation of the chemical composition and selected parameters of technological suitability of milk obtained from goats maintained in two types of farming systems, namely organic and conventional.

MATERIAL AND METHODS

The examination included 238 samples of goat milk, obtained within 3 production seasons; namely, spring (97), summer (70) and autumn (71). The production season was predominantly associated with the lactation phase of goats. Milk was collected from animals maintained in two farms of different type, i.e. certified organic (131) and conventional (107). In both herds approximately 100 animals were kept and the production season corresponded with the phase of lactation. Conventional farm maintained goats of Polish Color-Improved breed, whereas organic farm maintained Non-Improved goats of diverse color. In the spring and summer season (from May to September), all animals were grazing on the pastures. In the autumn season (October and November) goats were fed mainly with hay silage. Goats maintained on the organic farm received crushed oat-barley (1:1) meal obtained from own crops, and in the conventional farm – industrial breeder. Goats were milked twice a day and the milk was measured individually. Representative samples (350 mL) for analysis were collected from two entire milking procedures and the following values were established: basic chemical composition, namely the content of fat, protein, lactose and dry matter was determined using an Infrared Milk Analyzer apparatus manufactured by Bentley; content of casein by Wolker's method according to PN-68/A-86122; acidity (pH value) with Pioneer 65 pH-meter manufactured by Radiometer Analytical; heat stability by White and Davies' method in 140°C in oil bath manufactured by TEWES-BIS; rennet coagulation time with Shern's method (the time value indicated by the moment of first appearance of the casein flakes); somatic cell count (SCC) with Somacount apparatus manufactured by Bentley.

The results were analyzed statistically with StatSoft Inc. STATISTICA 6 software with the implementation of two-factor analysis of variations with the interaction. The significance of differences between means for analyzed groups was calculated by Fisher's LSD test.

RESULTS AND DISCUSSION

It has been determined that goats maintained in the conventional farm produced slightly more milk (by 0.2 kg) daily ($p \leq 0.01$) than goats from organic farm (Table 1). The higher milk yield specific to Polish Color-Improved goats from conventional farm might be the result of higher genetic potential and more intensive feeding system of these animals (mainly in the peak of lactation, which is in the summer months).

The analysis of daily performance of goats between seasons (corresponding to the phase of lactation) has indicated that animals in both types of farms produced milk at a similar level (approximately 1.5 kg) in the spring season (early lactation). The lactation peak occurred in the summer season; however, the goats on the conventional farm produced more than 1 kg of milk as compared to the organic ones. The end of lactation, in the autumn season, was marked by a more rapid decline in milk production (by 0.35 kg less of milk) for animals maintained on the conventional farm in comparison to goats from the organic farm (Fig. 1). From an economic point of view, lactation characterized by a balanced distribution of the quantity of milk produced during the whole period of lactation duration is the most beneficial, as explained by PULINA *et al.* (2007).

Caprine milk from organic farms was characterized by a more favorable chemical composition. It contained significantly ($p \leq 0.01$) more

Table 1. Daily milk yield, basic chemical composition and selected parameters of technological suitability of milk from goats maintained on the organic and conventional farms in regard to the production season.

Item		Farm type		Production season			Interaction farm type × production season
n		organic	conventional	spring	summer	autumn	
		131	107	97	70	71	
Daily milk yield		1.40^{A}	1.60^{B}	1.47^{Y}	2.12^{Z}	0.89^{X}	**
(kg)	SD	0.56	0.99	0.59	0.86	0.36	
Fat		4.03^{B}	3.58^{A}	3.66^{X}	3.72^{X}	4.13^{Y}	**
(%)	SD	0.67	0.54	0.56	0.48	0.84	
Protein		3.43^{B}	2.95^{A}	3.23^{Y}	3.10^{X}	3.32^{Y}	**
(%)	SD	0.40	0.38	0.57	0.34	0.45	
Casein		2.79^{B}	2.41^{A}	2.64^{Y}	2.52^{X}	2.64^{Y}	**
(%)	SD	0.30	0.36	0.39	0.33	0.41	
Protein-to-fat ratio		0.87	0.84	0.89^{Y}	0.84^{X}	0.82^{X}	**
	SD	0.13	0.14	0.16	0.10	0.15	
Lactose		4.51	4.46	4.53^{Y}	4.53^{Y}	4.39^{X}	ns
(%)	SD	0.20	0.24	0.19	0.19	0.5	
Dry matter		12.71^{B}	11.59^{A}	12.10^{X}	11.96^{X}	12.58^{Y}	**
(%)	SD	1.06	0.92	1.09	1.00	1.28	
pH		6.73^{B}	6.47^{A}	6.63^{y}	6.52^{x}	6.67^{y}	*
	SD	0.09	0.15	0.16	0.19	0.14	
Heat stability		100^{B}	50^{A}	104^{Y}	58^{X}	60^{X}	**
(s)	SD	40	22	43	35	20	
Rennet coagulation time		176^{B}	135^{A}	190^{Y}	138^{X}	132^{X}	ns
(s)	SD	90	61	98	54	58	
SCC		937^{A}	$1,822^{B}$	990^{X}	$1,199^{X}$	$1,941^{Y}$	ns
(thousands/mL)	SD	0.73	1.58	0.88	0.89	1.74	

A, B: Differences between farming systems significant at p≤0.01; X, Y, x, y: Differences between production seasons; X, Y: significant at p≤0.01; x, y: significant at p≤0.05.
**significant at p≤0.01; ns: not significant.

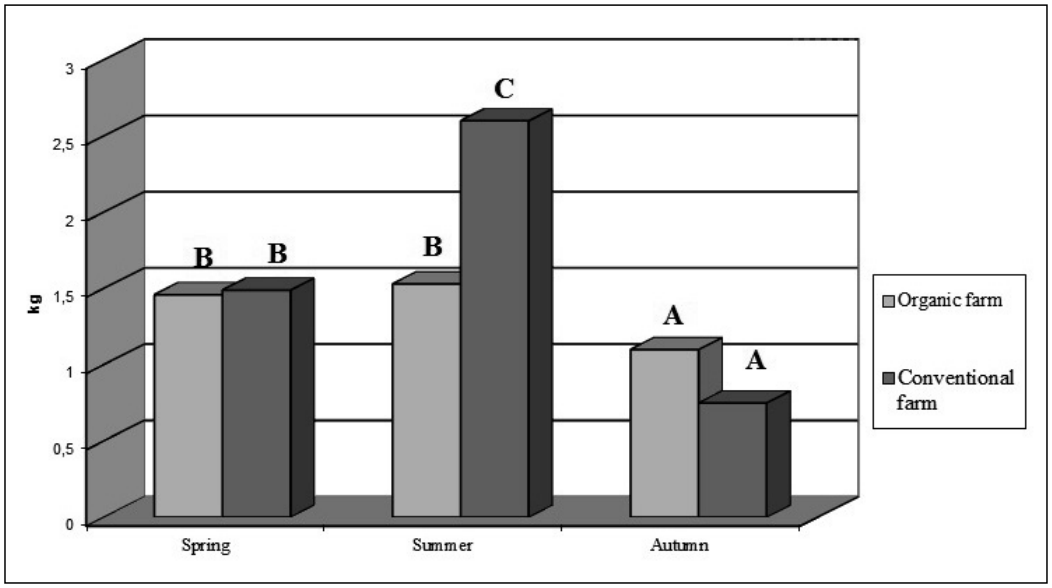


Fig.1 - Daily milk yield. A, B, C: The difference significant within the farm at p≤0.01.

fat (by 0.45 %), protein (by 0.48 %), including casein (by 0.38 %) and dry matter (by 1.12 %). TUDISCO *et al.* (2010) confirm the higher production of milk fat by goats of the same breed (Cilentana) maintained in the organic system (65.9 g/day) as compared to the conventional (54.3 g/day). This study demonstrated significantly ($p \leq 0.01$) higher concentration of protein, including casein in milk of Non-Improved goats maintained on the organic farm. Numerous authors attribute this fact to a specific genotype of these animals. Local (indigenous) goat breeds are characterized by a higher frequency of so-called "strong" genes of αs_1-casein, i.e. A, B and C (MARTIN 1993; BARŁOWSKA *et al.* 2007; STRZAŁKOWSKA *et al.* 2004). These genes determine a higher synthesis of αs_1-casein fraction, and thus the concentration of casein and consequently amount of total protein.

It was stated that, regardless of the farm type, goats produced milk with the highest concentration of fat, protein, including casein and dry matter, and low lactose in the autumn season (late lactation) (Table 1). Significantly higher pH in goat milk from the organic farm also had impact on its higher heat stability. Short time of enzymatic coagulation of goat milk causes formed clot to be less firm, which entails a greater loss of casein (BARŁOWSKA *et al.*, 2011). Longer clotting time of goat milk from the organic farm obtained in our study (which should be treated as beneficial) could be associated with the higher content of dry matter, especially protein (CLARK and SHERBON, 2000).

It was determined that goats maintained on the organic farm produced milk with almost twice as lower somatic cell count (937 thousands/mL on organic farm in comparison to 1,822 thousands/mL in conventional farm). Somatic cell count has significantly ($p \leq 0.01$) increased with the progress of lactation in both types of farms (Table 1). The deterioration of milk cytological quality along with the lactation progress is confirmed by VACCA *et al.* (2010).

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SURVEY OF 11 TRACE ELEMENTS OF TOXIC AND NUTRITIONAL SIGNIFICANCE IN CHINESE RICE

L.L. YAN[1], Y.M. HE[1], Q.Q. FAN[1], J. HU[1] and Y. ZHOU[1,2]*
[1]College of Chemical Engineering and Materials Science, Zhejiang University of Technology,
18 Chaowang Road, Hangzhou 310032, Zhejiang Province, China
[2]Research Center of Analysis and Measurement, Zhejiang University of Technology,
18 Chaowang Road, Hangzhou 310032, Zhejiang Province, China
*Corresponding author: Tel. +86 571 88320568,
email: yingzhou@zjut.edu.cn

ABSTRACT

11 trace elements (Cr, Mn, Co, Ni, Cu, As, Se, Cd, Pb, Sn, Hg) of 21 different kinds of rice samples from main production regions in China were analyzed by ICP-MS (inductively coupled plasma mass spectrometry). The recoveries were in the range of 80.0-113.7%. The detection limits ranged from 0.010 to 1.904 ng g^{-1}. The results revealed that the concentrations of Hg of 4 rice samples, Pb of 3, Cd of 2 and Cr of 1, exceeded the Chinese permitted levels in foods. And the estimated daily intake was 28.1-46.8 μg for As and 9.4-15.6 μg for Se. This study demonstrates that the potential risk to people's health which is attributed to the daily consumption of these rice samples is probably being existing.

- Keywords: ICP-MS, nutritional, rice, toxic, trace elements -

INTRODUCTION

Rice is a staple food in China and even Asia, which is treated as the major crop for 65% of China's population. And the production and consumption of rice account for 30% in the world. Therefore, trace element analysis in rice is rather important from nutritional and toxicological levels. As we all know, humans require adequate intakes of essential elements through food ingestion, such as chromium (III), manganese, cobalt, nickel, copper, selenium, tin and so on. On the other hand, toxic elements such as arsenic, lead, cadmium and mercury severely upset the functions of humans' organism (TSAI *et al.*, 2010). Thus it is essential to determine the contents of the above mentioned trace elements in rice, which is an important dietary component for many people around the world.

However, with the accelerated process of industrialization, heavy metal pollution has become a global problem. Due to the absorption of crops in the soil affected by human activities strongly, heavy metals are transferred into the crops, which results in the human health problems. The survey conducted by Hang showed that the contents of Pb, Hg and Cd in rice samples from Changshu in China exceeded their national maximum levels in foods (HANG *et al.*, 2009). Therefore, it is worthwhile to examine the contents of trace elements in rice from different regions in China.

To determine the trace elements in cereal samples, various atomic spectrometry techniques such as atomic absorption spectrometry (AAS) (MOHAMED and TAHA, 2003), flame (FAAS) (SHAR *et al.*, 2004; DICKSON, 2010) or electrothermal atomic absorption spectrometry (ETAAS) (MANJUSHA *et al.*, 2008), inductively coupled plasma atomic emission spectrometry (ICP-AES) (ZENG *et al.*, 2008) or optical emission spectrometry (ICP-OES) (HORITA *et al.*, 2009) have been employed. Nowadays, inductively coupled plasma mass spectrometry (ICP-MS) is regarded as the most promising technique in the routine analysis. ICP-MS has the ability for sequential determination of macro, trace and ultra-trace elements with low detection limits, compared with the methods mentioned above. It has been widely applied for the quantitative determination of multiple elements in beverage (STOZHKO *et al.*, 2007), various food samples (DAWCZYNSKI *et al.*, 2007; NARDI *et al.*, 2009) and botanicals and dietary supplements (AVULA *et al.*, 2010).

The aims of this study were to make a survey of trace elements in rice from the main production regions (Zhejiang, Jiangsu, Heilongjiang, Jilin, Hubei, Sichuan, Anhui and Jiangxi provinces) in China, to evaluate its quality according to the maximum levels of trace elements set by various organizations and to estimate people's health effects in terms of the daily consumption of these kinds of rice.

MATERIALS AND METHODS

Apparatus

An ICP-MS (Elan DRC-e, Perkin Elmer, USA) equipped with a dynamic reaction cell was used for analysis. The sample introduction system consists of a Ryton spray chamber and a cross-flow nebulizer connected by tubes to the ICP-MS's peristaltic pump. The ICP-MS was operated with nickel sampler and skimmer cones obtained from Perkin Elmer. Samples are digested by a Multiwave 3000 XF100 (Anton Parr, Graz, Austria) microwave reaction system. An Ultra Centrifugal Mill ZM200 (Retsch, Germany) was used for grinding the rice. A Milli-Q Element A10 water purification system (Millipore Corporation, Bedford, MA, USA) was used in ultrapure water preparation.

Reagents

HNO3 (69.0-71.0%) MOS grade and H_2O_2 (30%) guaranteed reagent from Sinopharm Chemical Reagent Limited Corporation were used for digesting the rice powder. Setup solution provided by Perkin Elmer was introduced for optimizing the instrument. Multi-element ICP-MS standard solution (10 mg L^{-1}), rare earth element standard (10 mg L^{-1}) and mercury standard (10 mg $^{L-1}$) from Perkin Elmer were used for preparing the stock standard solutions (500 μg L^{-1}), nine elements solution (Cr, Mn, Co, Ni, Cu, As, Se, Cd and Pb), Sn standard and Hg standard, respectively. High-purity argon (99.999%, Hangzhou Jingong Specialty Gases Co., Ltd., Hangzhou, China) was used as the carrier gas. Ultrapure water (resistivity 18.2 MΩ cm-1) was used in sample and solution preparation throughout this work. The certified reference material-corn (GBW10012) was purchased from the National Research Centre for Certified Reference Materials (China).

Sample collection and treatment

21 rice samples were purchased from the local markets in eight main regions including Zhejiang, Jiangsu, Jiangxi, Hubei, Heilongjiang, Anhui, Jilin and Sichuan provinces in China. Besides, Thai rice was as a control, because it is very popular with the people all over the world. The dried rice samples were ground by the ZM200 Mill. The powder was collected and stored in refrigerator at below 10°C for the following experiments. Approximately 0.2 g rice samples were weighed directly into the PTFE vessels and digested with a mixture of 4 mL of HNO_3 and 2 mL of H_2O_2. After half an hour at room temperature, the vessels were screwed into position on the microwave equipment according to the following operating program (Table 1). The resulting solutions were cooled, transferred to a 25 mL

Table 1 - Microwave digestion program.

Step	Power (W)	Ramp (min)	Hold (min)	Fan (n)
1	600	10:00	10:00	1
2	800	10:00	10:00	1
3	0	0	15:00	3
P-Rate: 0.3 bar s^{-1}; IR: 200℃; P: 50 bar; Drive: Rot.				

volumetric flask, and diluted to the final volume with ultrapure water. The final solutions were used for determination by ICP-MS. One sample with reagent blank was digested in triplicate.

Determination of trace elements in rice samples

The rice samples were determined by ICP-MS using the external standard method. The optimum operating conditions for determining trace elements in rice samples were presented in Table 2.

RESULTS AND DISCUSSION

Analytical performance

Calibration curves for all 11 elements were made. Calibration data indicated linearity (r>0.9997) for all standard elements (0.04-1 μg L^{-1} for Hg, 1-20 μg L^{-1} for Sn, and 1.0-80 μg L^{-1} for Cr, Mn, Co, Ni, Cu, As, Se, Cd, Pb). The detection limits obtained for Cr, Mn, Co, Ni, Cu, As, Se, Cd, Sn, Pb and Hg were 0.399, 0.814, 0.010, 0.357, 0.403, 0.066, 0.447, 0.029, 0.396, 1.904 and 0.039 ng g^{-1}, respectively, which were determined as 3 standard deviation (SD) of the 11 consecutive measurements of the reagent blanks multiplied by the dilution factor used for sample preparation. All standards and samples were injected in triplicate. The RSD for the samples ranged from 1.08 to 2.86% for all elements tested. The recovery experiment was carried out with one rice sample selected randomly. And two kinds of concentrations between 1.5 and 25,000 ng g^{-1} were added for different contents of each element. The recoveries between 80.0 and 113.7% were as follows: Cr (105.1, 113.7%), Mn (100.4, 99.5%), Co (95.0, 94.6%), Ni (101.1, 95.3%), Cu (103.3, 89.5%), As (97.5, 102.7%), Se (88.4, 104.3%), Cd (80.0, 83.3%), Sn (94.3, 95.9%), Pb (93.1, 90.0%), and Hg (96.0, 108.0%).

In order to verify the precision and accuracy of the proposed method, GBW10012 corn from the National Research Centre for Certified Reference Materials (China) was digested and analyzed. Except for Sn and Cd, all other elements (Cr, Mn, Co, Ni, Cu, As, Se, Pb and Hg) were determined. The certified reference material was treated in triplicate. As can be seen from Table 3 data measured were in good agreement with certified values.

Toxic elements (As, Cd, Pb, Hg) in the rice samples

Table 4 listed the levels of trace elements in all the determined rice samples. The levels of toxic elements (As, Cd, Pb, Hg) were described in this table. Arsenic has been widely recognized as a highly dangerous element. Exposure to As can cause an accumulation of As in tissues, affecting liver, kidney and gastrointestine. Arsenic levels in the Chinese rice samples varied from 36.7±1.7 to 132.8±0.7 ng g^{-1}. Among Zhejiang and Jiangsu rice samples, the As content was more than 60 ng g^{-1}. The lowest level occurred in Sichuan-1 rice sample.

Cadmium is a very toxic element which accumulates in the liver and kidney via air, food and water. It influences central nervous, immune system, and even speeds up cancer development. The Cd content in these rice samples varied from ND to 431.7±13.2 ng g^{-1}. Three rice samples from Sichuan presented high levels from 155 to 432 ng g^{-1}.

Lead is also a harmful heavy metal which causes clinical symptoms such as colic, anemia, myopathy and so forth. It accumulates in human body through food and breath, impairing diges-

Table 2 - Instrument settings for ICP-MS.

ICP-MS operation parameters	
RF power (W)	1100
Gas flow rates (L min^{-1})	Plasma 15; Auxiliary 1.2; Nebulizer 0.75
Sample uptake rate (μL min^{-1})	400
Nebulizer	Crossflow
Spray chamber	Ryton
Sweeps	20
Readings	1
Replicates	3

Table 3 - Analytical results of element contents in GBW10012 (n=3).

Element	Measured value[a] (μg g^{-1})	Certified or reference value (μg g^{-1})
Cr	0.11	0.11
Mn	1.51±0.04	1.55±0.08
Co	0.012±0.001	0.012
Ni	0.100±0.006	0.097±0.014
Cu	0.66±0.09	0.66±0.08
As	0.028±0.001	0.028±0.006
Se	0.019±0.001	0.021±0.008
Pb	0.07±0.01	0.07±0.02
Hg	0.0016±0.0001	0.0016

[a]Values are expressed as mean±standard deviation(SD).

Table 4 - Concentrations of trace elements in rice samples.

Rice samples	Cr* (ng g^{-1})	Mn (ng g^{-1})	Co (ng g^{-1})	Ni (ng g^{-1})	Cu (ng g^{-1})	As (ng g^{-1})	Se (ng g^{-1})	Cd (ng g^{-1})	Pb (ng g^{-1})	Sn (ng g^{-1})	Hg (ng g^{-1})
Zhejiang Hangzhou-1	211.4±13.5[e]	6654±83	ND	94.0±7.0	1158±12	85.1±1.2	13.3±2.6	ND	ND	86.4±1.3	8.6±0.4
Zhejiang Hangzhou-2	214.8±8.3	10355±32	ND	544.7±9.3	4515±71	132.8±0.7	37.5±1.4	13.5±0.3	142.9±6.1	121.0±10.8	8.6±1.5
Zhejiang Huzhou-1[b]	98.3±0.7	13922±71	ND	154.1±6.4	3904±35	130.3±1.4	50.2±1.3	19.8±0.2	264.5±8.9	24.9±1.2	11.2±0.7
Zhejiang Huzhou-2[a]	102.0±8.2	10990±125	1.4±0.2	195.0±6.0	2170±9	99.3±8.1	22.4±4.2	35.0±0.9	102.6±1.2	387.1±6.8	42.2±1.1
Zhejiang Xiangshan	ND	6173±415	ND	5.2±1.9	1077±179	95.6±12.3	19.3±0.9	10.2±0.4	ND	39.3±1.4	1.0±0.6
Zhejiang Jiaxing	99.7±2.0	9511±3	ND	81.0±4.0	1748±12	65.0±0.7	12.1±1.8	14.3±0.4	60.5±2.7	356.2±6.0	1.1±0.3
Zhejiang-1	170.4±5.4	7254±18	1.4±0.1	143.2±9.7	1332±7	132.3±4.7	8.4±1.3	ND	2.6±0.8	101.2±8.0	4.1±0.3
Jiangsu-1	148.2±5.6	10041±38	ND	187.8±9.5	2395±38	111.6±0.1	35.3±2.0	11.8±0.9	111.6±7.2	117.8±11.6	9.2±0.3
Jiangsu-2	ND	6713±30	ND	138.9±1.8	2301±19	87.0±1.9	16.8±1.7	26.1±0.7	107.2±4.7	87.3±2.6	1.9±0.2
Jiangsu-3	ND	4264±39	ND	25.6±2.6	1915±21	62.2±2.0	2.5±0.6	17.0±0.3	ND	50.4±4.8	2.0±0.2
Sichuan-1[b,c]	141.5±3.6	12269±90	6.5±0.1	401.5±20.4	2407±46	36.7±1.7	44.8±0.4	431.7±13.2	239.6±12.8	203.7±16.1	9.5±0.4
Sichuan-2[a,b,d]	2702±2	10545±62	26.0±4.9	2652±64	2499±20	56.3±2.5	44.4±0.9	155.8±7.6	464.4±12.4	749.7±15.8	31.6±0.4
Sichuan-3[a]	407.3±4.4	11752±9	12.4±0.5	503.6±6.2	1144±14	97.8±1.9	58.0±2.3	62.7±0.5	149.7±2.1	444.9±8.0	40.0±0.9
Sichuan-4[a,c]	335.7±3.5	12169±15	7.4±0.2	1232±3	1246±45	93.0±0.9	80.1±0.8	262.2±9.5	169.4±7.2	2021±12	38.8±1.3
Jiangxi	523.4±15.4	10559±180	12.2±0.1	397.4±13.8	2425±61	112.8±5.1	41.1±1.5	182.4±2.9	ND	ND	7.7±0.7
Heilong jiang-1	188.4±10.4	10905±27	1.8±0.6	349.2±26.3	2519±111	128.5±2.4	26.7±0.1	1.7±0.2	1.2±0.3	27.1±0.6	3.2±0.2
Heilong jiang-2	255.7±5.3	38336±64	ND	1437±4	6403±9	57.3±1.0	37.1±0.9	106.4±0.3	ND	51.9±1.7	1.3±0.1
Anhui	110.9±2.5	6033±94	ND	42.4±0.6	1115±34	102.8±5.6	28.1±1.7	2.5±0.5	ND	77.7±4.6	1.6±0.1
Hubei-1	652.0±5.5	9376±40	18.5±3.2	414.6±6.6	2742±12	95.1±0.5	43.6±2.3	122.5±1.1	ND	ND	3.7±0.4
Hubei-2	258.0±6.6	12065±48	8.2±0.7	63.1±9.7	5505±14	70.9±2.3	11.5±0.4	29.9±2.7	ND	150.4±3.4	4.6±0.6
Jilin	146.4±7.0	8951±31	ND	195.9±3.2	1427±3	112.3±5.0	22.9±1.1	ND	52.5±0.7	142.6±9.4	ND
Thailand	80.5±2.9	5278±28	ND	22.3±2.7	671.5±5.5	58.1±3.6	18.1±0.7	24.2±0.1	ND	43.1±0.7	1.9±0.5

[a, b, c, d] represent excessive samples for Hg, Pb, Cd and Cr, respectively; [e]Values are expressed as mean±standard deviation(SD); *n=3; ND: not detected.

tive, nervous, respiratory and immune system. In these rice samples, the lead content ranged from ND to 464.4±12.4 ng g^{-1}. Nine rice samples from Zhejiang, Jiangsu and Sichuan presented high levels higher than 100 ng g^{-1}.

Mercury is considered as one of the most toxic elements to humans. Low concentration exposure to mercury shows a potential hazard to human health and produces adverse effects on cardiovascular, gastrointestinal and reproductive systems, resulting in the clinical symptoms including tremor, gingivitis and neural conduction disorder. Hg levels varied from ND to 42.2±1.1 ng g^{-1}. The Hg content in four rice samples was more than 30 ng g^{-1}. Others were all below 10 ng g^{-1}.

Essential elements (Cr, Mn, Cu, Co, Ni, Se, Sn) in rice samples

Chromium (III) is beneficial to human. It plays an important role in physiological function coacting with other materials such as hormone, insulin, enzymes and genetic substances. But chromium (VI) has a carcinogenic effect on humans. Its content ranged from ND to 2,702±2 ng g^{-1}. Except for the highest value in Sichuan-2 rice sample, the values of all the other rice samples were lower than 700 ng g^{-1}.

Manganese is regarded as the most close element to psychiatric science. It can keep normal brain function and sustain glucose and lipid metabolism. Manganese levels in the Chinese rice samples varied from 4,264±39 to 38,336±64 ng g^{-1}.

Copper is also an essential trace element, which can activate the formation of hemoglobin and promote absorption and use of iron. Just like manganese, the copper levels in these rice samples were very high, ranging from 1,077±179 to 6,403±9 ng g^{-1}. The contents of both elements in the Brazilian rice were also very high (BATISTA *et al.*, 2010).

Cobalt is the component of vitamin B12 complex, stimulating hematopoietic system of medulla in human. Cobalt levels in the Chinese rice samples varied from ND to 26.0±4.9 ng g^{-1}. Cobalt was not detected in half of these samples.

Nickel is an essential element which could be penetrated into skin, causing the dermatitis and eczema. The levels in the Chinese rice samples varied from 5.2±1.9 to 2,652±64 ng g^{-1}. Rice samples from Zhejiang, Jiangsu, Heilongjiang and Jiangxi provinces presented high levels of Ni except Xiangshan rice and Jiangsu-3 rice sample. All the Sichuan rice samples presented much higher Ni levels of more than 400 ng g^{-1}.

Selenium is also essential for human health. It is an active component of GSH-Px involved in protection against oxidation action, further preventing diabetes. Adequate Se can strengthen immunity by removing toxins and clearing cholesterol. We found that Se levels in rice samples varied from 2.5±0.6 to 80.1±0.8 ng g^{-1}. Sichuan rice samples showed slightly higher levels.

Tin is not yet considered as an essential element for humans. But Sn deficiency has been associated with metabolic disease of protein and nucleic acid, preventing growth and development, especially for the children, while dizziness, diarrhea and nausea are attributed to excessive Sn. Tin levels in the Chinese rice samples varied from ND to 2,021±12 ng g^{-1}. Sichuan-4 rice presented the highest level which was about 80 times higher than the Huzhou-1 rice that could be detected.

The contents of trace elements in the Thai rice sample were also summarized in Table 4.

According to these data, the trace elements contents in the Chinese rice samples were a little higher.

Comparison of concentrations of trace elements with different standards

The different levels of trace elements in rice have beneficial or adverse effects on human health. So the concentrations in rice samples compared with maximum levels of Cr, As (inorganic), Se, Cd, Pb, Hg in GB 2762-2005 (China) were shown in Fig. 1. Other elements are not established in this regulation. The details were as follows: Cr concentration in Sichuan-2 rice exceeded the maximum level of 1,000 ng g^{-1} stipulated for crop by China, and a little higher than the mean value of 1,287 ng g^{-1} in brown rice (ZENG *et al.*, 2008); As and Se concentrations of all the samples were both below the maximum levels of 150 ng g^{-1} and 300 ng g^{-1} stipulated for rice and crop, respectively; Cd concentrations in Sichuan-1 and Sichuan-4 rice exceeded the Chinese permitted level of 200 ng g^{-1} stipulated for rice, but still below the value of 0.67±0.02 μg g^{-1} in 1 rice sample (YE *et al.*, 2003); Pb concentrations in Huzhou-1, Sichuan-1 and Sichuan-2 rice exceeded the maximum level of 200 ng g^{-1} stipulated for cereal, close to the concentrations in late xian rice and polished rice (NI *et al.*, 2008), but much lower than the highest level of 957 ng g-1 in Changshu rice (HANG *et al.*, 2009); Hg concentrations in Huzhou-2, Sichuan-2, Sichuan-3 and Sichuan-4 rice exceeded the Chinese permitted level of 20 ng g^{-1} stipulated for crop, and similar to the mean value of 37.4 ng g^{-1} in the early xian rice (NI *et al.*, 2008), but lower than the highest level of 60.2 ng g^{-1} in Changshu rice (HANG *et al.*, 2009). The results showed that the concentrations of trace elements in rice from the cities in Yangtze River Delta Region were normal, except Pb excess of Huzhou-1 rice and Hg excess of Huzhou-2 rice, respectively. However, Cd and Pb in Sichuan-1 rice, Cr, Pb and Hg in Sichuan-2 rice, Hg in Sichuan-3 rice and Cd and Hg in Sichuan-4 rice exceeded the maximum levels, respectively. This may result from a large number of coal mines

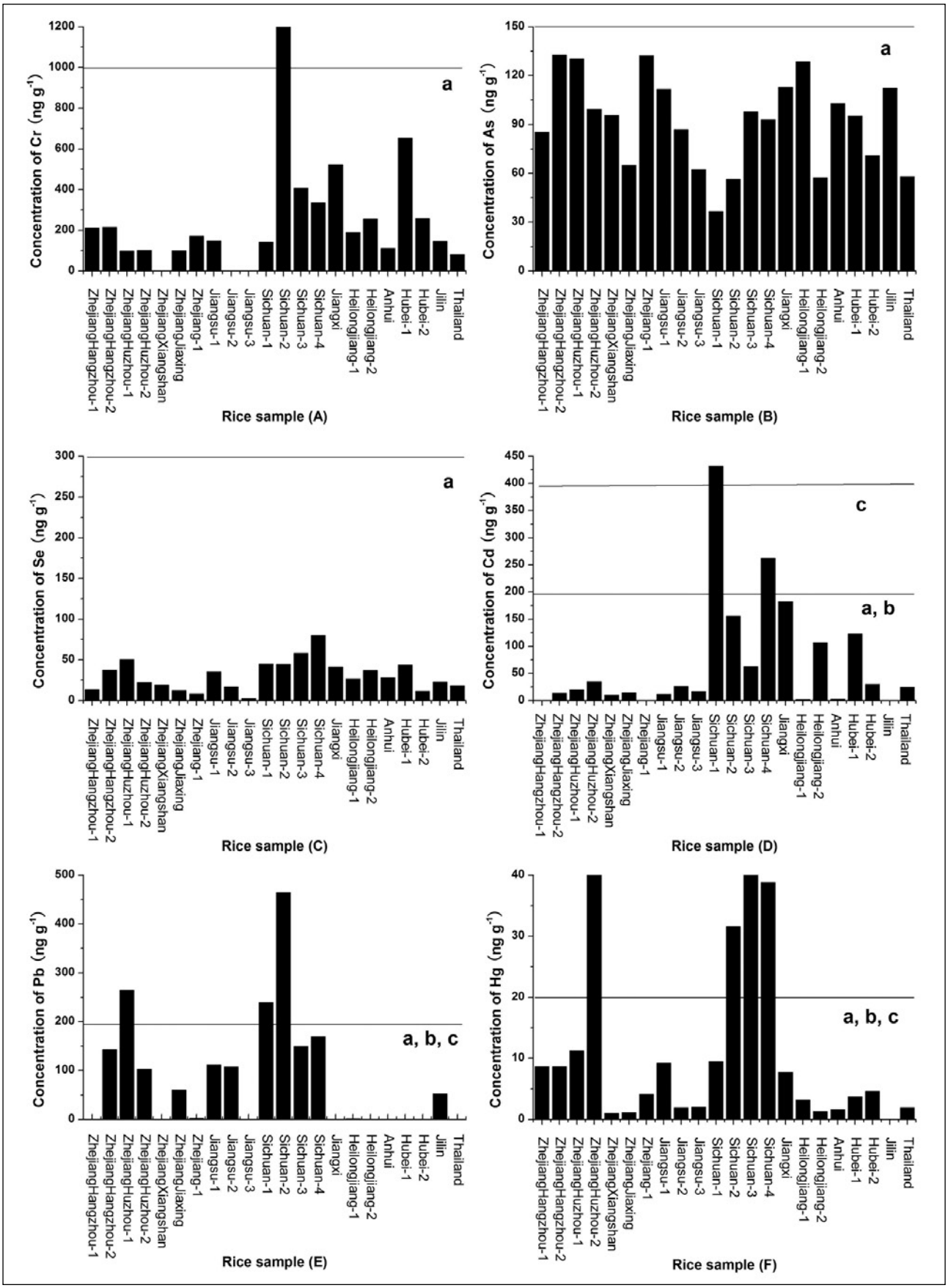


Fig. 1 - The concentrations of Cr, As, Se, Cd, Pb, Hg in rice samples. A: The concentration of Cr in rice; B: The concentration of As (inorganic) in rice; C: The concentration of Se in rice; D: The concentration of Cd in rice; E: The concentration of Pb in rice; F: The concentration of Hg in rice. Line represents maximum levels of elements in foods set by different organisations. a: GB 2762-2005; b: European Union 2006; c: CODEX 2007.

and environmental pollution nearby in Sichuan. Also, comparison with the standards set by European Union and Codex Alimentarius Commission (CAC) was presented in Fig. 1. The only difference was that Cd level in Sichuan-1 rice exceeded the maximum level set by CAC.

Daily intakes via rice consumption

In addition, although As and Se concentrations were within the maximum allowable levels, the estimation of daily intake of them was conducted through rice consumption. The average levels of them in all the Chinese rice samples analyzed were used. Since daily dietary rice intake in China is about 300-500 g/day for an adult (provided by China Nutrition Society), the estimated daily intake is 28.1-46.8 μg for As and 9.4-15.6 μg for Se. Estimated daily intake was calculated as:

$$EDI = C_e \times M_{rcd}$$

Where EDI is the estimated daily intake of chemical elements (mg/day/person or μg/day/person); C_e is the average concentration of element (μg g^{-1}); and Mrcd is the amount of rice consumed daily (g day^{-1}). Estimated daily intake was compared with the provisional tolerable daily intake (PTDI) for elements (Joint FAO/WHO Expert Committee on Food Additives (JECFA)/ WHO guidelines 2000; World Health Organization (WHO) 2004). PTDI was considered as provisional tolerable weekly intake (PTWI) divided by 7. Considering the average weight of an adult as 70 kg, the PTDI values for As (inorganic) and Se are 150 and 55 μg/day (BATISTA *et al.*, 2010), respectively. As a result, rice contributes to 18.7-31.2 and 17.0-28.4% of the PTDI for As and Se, respectively. According to the levels of 6.3 and 5.4% in Brazilian rice (BATISTA *et al.*, 2010), Chinese rice contributes much higher to the PTDI of the elements for Chinese. And estimated daily intakes of Cr, Mn, Co, Ni, Cu, Cd, Sn, Pb and Hg are 127.0-211.6 μg, 3.3-5.4 mg, 2.9-4.8 μg, 132.3-220.4 μg, 0.7-1.2 mg, 25.1-41.8 μg, 82.7-137.9 μg, 43.1-71.9 μg and 3.5-5.8 μg, respectively. In comparison with the values (1.9 mg for Mn, 3.1 μg for Co, 0.2 mg for Cu and 2.4 μg for Cd) in Brazilian rice (BATISTA *et al.*, 2010), intake of Co only is quite close to each other, and intakes of Mn, Cu, Cd are considerably higher. It is due to that rice consumption in China is almost 5 times larger than the consumption (86 g/day) in Brazil.

CONCLUSION

Rice samples obtained from markets in China were analyzed and evaluated for their trace elements contents by comparing the concentrations of elements with the maximum levels based on different standards. This study suggests that significant differences exist in the trace elements levels for rice samples from different regions. Compared with Thai rice, all the trace elements concentrations were slightly higher. The results could serve as a basis on comparison with other rice samples in China and even the World. Four samples for Hg, three samples for Pb, two samples for Cd and one sample for Cr were found to contain elevated contents above the maximum levels, respectively. If these rice samples are consumed daily, there will be up to 18.7-31.2 and 17.0-28.4% of the PTDI for As and Se, posing potential risk for people, which should be paid great attention to.

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PREVIEW ON VOLUME XXV No. 2, 2013

OPINION

Health Quality and Nutritional Value of Rye Bread Produced on a Small and Large Scale in Poland
M. Radzyminska, B. Garbowska and D. Jakubowska

PAPERS

Anthocyanin Esterification in Sangiovese Grapes
L. Rustioni, M. Rossoni, O. Failla and A. Scienza

Effect of Technological Factors on Glycoalkaloids and Nitrates Content in Dehydrated Potato
E. Rytel

Influence of Chemical Composition and Structure of Strawberry Gels on the Choosen Physical Properties of Freeze-Dried Final Product
A. Ciurzyńska, A. Lenart and W. Traczyk

Ultrasounds Application in Winemaking: Grape Maceration and Yeast Lysis
P. Ferraretto, V. Cacciola, I.F. Batlló and E. Celotti

Antioxidative Peptides Derived from Denaturated Egg White Protein
A. Zambrowicz, M. Pokora, E. Eckert, J. Chrzanowska, M. Szołtysik, A. Dąbrowska and T. Trziszka

Preliminary Characterization of a Bacteriocin-Like Substances Produced by a *Bacillus Subtilis* Isolated from Argentinean Vegetable Food
M.F. Fangio and R. Fritz

Quality of Bread Containing *Potentilla Anserine* L. Cultivated in China
Ying Ji, Jie Gang and Wenzhong Hu

Modification of the Rheological Behaviour of Sodium Alginate by Chitosan and Multivalent Electrolytes
B. Abu-Jdayil and D. Abu Fara

Environmental Assessment of the Citrus Fruit Production in Sicily Using LCA
A. Lo Giudice, C. Mbohwa, M.T. Clasadonte and C. Ingrao

Effect of UV-C Light in the Preservation of Raw Fermented Beverages
M. Borcakli, J. Lucas, L. Caputo, T. Ozturk, F. Baruzzi, V. Fusco, G.M. Quero, L. Quintieri and M. Houghton

Different Stabilization Treatments Of Rice Bran Added to Wheat Flour Determine Different Properties in Partially Baked Wheat Bread
J. Quilez, M. Zator, J. Salas-Salvadò and L. Alvarez

Prediction of the Major Fatty Acids in the Backfat From In- and Outdoor Iberian Pigs at Slaughter
A. Daza, M.A. Latorre and C.J. Lòpez-Bote

SHORT COMMUNICATIONS

Mineral Content of a Moss Tea for Hypertension
B. Pejin, D. Belic, G. Vuckovic, Y. Kien-Thai and B. Stanimirovic

First Data on Antimicrobial Activity of *Yucca Filamentosa L.* Bark Extracts
M. Bononi, G. Guglielmi, P. Rocchi and F. Tateo

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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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(3) Figures must be prepared and saved separately in **TIF, JPEG, EPS (300 dpi resolution).** They should be prepared so that on 50% reduction, lines, figures and symbols will be clearly legible and not overcrowded. All figures must be given Arabic numbers, e.g. Fig. 3. Legends for figures must be self-explanatory and should be typed on a separate sheet under "Legends to Figures".

(4) Standard Usage, Abbreviations and Units. The Concise Oxford and Webster's English Dictionaries are the references for spelling and hyphenation. Statistics and measurements should always be given in figures, e.g. 10 min, except when the number begins a sentence. When the number does not refer to a unit of measurement it should be spelled out unless it is 100 or greater. Abbreviations should be used sparingly, only when long or unwieldy names occur frequently, and never in the title; they should be given at the first mention of the name. International Standard abbreviations should generally be used except where they conflict with current practice or are confusing. For example, 3 mm rather than $3x10^{-3}$ m. Abbreviations should be defined the first time they are used in the text and they should be used consistently thereafter. Temperatures should be expressed in the Celsius (centigrade) scale. Chemical formulae and solutions must specify the form used, e.g. anhydrous or hydrated, and the concentration must be in c1early defined units.

Common species names should be followed by the Latin binomial (italics) at the first mention. For subsequent use, the generic name should be contracted to a single letter if it is unambiguous.

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RESEARCH INSTITUTES

Dipartimento di Scienze e Tecnologie Agroalimentari e
Microbiologiche (DI.S.T.A.A.M.),
Università del Molise, Campobasso Fax +39-0874-404652

DeFENS, Department of Food, Environmental
and Nutritional Sciences, Università di Milano, Milano Fax +39-02-50316601

Dipartimento di Valorizzazione e Protezione delle Risorse
Agroforestali (DI.VA.P.R.A.), Sezione Microbiologia
ed Industrie Agrarie, Università di Torino, Grugliasco Fax +39-011-6708549

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VOLUME XXV IJFS No. 1, 2013

CONTENTS

PAPERS

Analysis of the Collaboration Between the US and the EU in Viticulture and Oenology
R. Aleixandre-Benavent, J.L. Aleixandre Tudó, G. González Alcaide and J.L. Aleixandre 3

Fate of *Listeria Monocytogenes* During Production and Storage of Artisan Water Buffalo Mozzarella Cheese
A. Serraino, G. Finazzi, G. Marchetti, P. Daminelli, R. Riu, F. Giacometti, M.N. Losio and R. Rosmini 16

Evaluation of a Sensory Scorecard for Grated Parmigiano-Reggiano Cheese
M. Zannoni and E.A. Hunter 23

Investigation of Cheese-Making Factors Effect on Ripening of a Semi-Hard Cheese for Product Quality Enhancement
V. Gianotti, E. Robotti, D. Pattono, M. Benzi and E. Marengo 35

Effect of Starchy Coating Films on the Reduction of Fat Uptake in Deep-Fat Fried Potato Pellet Chips
Malak M. Angor, Radwan Ajo, Walid Al-Rousan and Basem Al-Abdullah 45

Quality and Antioxidant Activity of Bread Fortified with Flaxseed
R. Meral and I. Sait Dogan 51

Postharvest Quality of 'Hachiya' Astringent Persimmons (*Diospyros Kaki* L.) as Affected by Hot Water Treatment and Modified Atmosphere Packaging
M.H. Ozer, B. Akbudak and I. Altioglu 57

Antioxidant Activity of Probiotic Yoghurt Fortified with Milk Protein Based Ingredients
G. Unal, S.N. El, A.S. Akalin and N. Dinkci 63

Crude Fibre, Water Extracts, Total Ash, Caffeine and Moisture Contents as Diagnostic Factors in Evaluating Green Tea Quality
S.O. Aroyeun 70

The Effects of Tobacco Smoke Exposure on Caffeine Metabolism
A. Grela, M. Kulza, W. Piekoszewski, M. Senczuk-Przybylowska, E. Gomołka and E. Florek 76

Fillet Fatty Acid Composition, Estimated Indexes of Lipid Metabolism and Oxidative Status of Wild and Farmed Brown Trout (*Salmo Trutta* L.)
A. Dal Bosco, C. Mugnai, V. Roscini and C. Castellini 83

Characterization and Trials of a Jerte Valley Cherry Product as a Natural Antioxidant-Enriched Supplement
M. Garrido, D. González-Gòmez, M. Lozano, C. Barriga, S.D. Paredes and A.B. Rodrìguez 90

Governance Mechanisms in Food Community Networks
S. Pascucci, A. Lombardi, L. Cembalo and D. Dentoni 98

SHORT COMMUNICATION

Chemical Composition and Selected Parameters of Technological Suitability of Caprine Milk Produced in Organic and Conventional Farms
J. Barłowska, Z. Litwińczuk, A. Wolanciuk and T. Szmatoła 105

SURVEY

Survey of 11 Trace Elements of Toxic and Nutritional Significance in Chinese Rice
L.L. Yan, Y.M. He, Q.Q. Fan, J. Hu and Y. Zhou 109

PREVIEW ON VOLUME XXIV No. 2, 2013 117
GUIDE FOR AUTHORS 118
CONTRIBUTORS 121