JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Chemical Characterization and DNA Tracking of Sardinian Botargo by *Mugil cephalus* from Different Geographical Origins

ANDREA BARRA,*,† VINCENZO LUIGI GARAU,† SANDRO DESSI,‡ GIORGIA SARAIS,† ELISABETTA CERETI,§ MARCO ARLORIO,§ JEAN DANIEL COISSON,§ AND PAOLO CABRAS†

Department of Toxicology, University of Cagliari, Via Ospedale 72, 09124 Cagliari, Italy, Department of Public Safety, Laboratorio di Igiene degli Alimenti, University of Cagliari, Via Porcell 4, 09124 Cagliari, Italy, and Dipartimento di Scienze Chimiche, Alimentari, Farmaceutiche e Farmacologiche (DISCAFF), Universita' degli Studi del Piemonte Orientale, via Bovio 6, 28100 Novara, Italy

The chemical composition of the Sardinian botargo by *Mugil cephalus* from different geographical origins was investigated. Fat (~20%), proteins (~50%), moisture (~22%), and salt (~7%) were measured in ground (G) and whole (W) commercial products. Among the nutritional compounds, ω-3 fatty acids were ~8%, squalene was ~15 mg/100 g, vitamin E was ~8.5 mg/kg, and cholesterol was ~300 mg/100 g, on average in both products. Antioxidant properties, assessed by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) test and expressed as Trolox equivalent antioxidant capacity (TEAC), showed quite good activity in extracted oil (0.8−1.1 mmol of Tolox/L). Major constituents in the samples varied noticeably, but only few statistical differences were evidenced between G or W products or between samples from different origins. Principal component analysis (PCA) of random amplified polymorphic DNA (RAPD) and proteins, coupled with both, did not differentiate samples from different origins. On the basis of our results, chemical and molecular data exclude the differentiation of samples from diverse origins.

KEYWORDS: Botargo by *Mugil cephalus*; ω-3 fatty acids; traceability; RAPD; PCA

INTRODUCTION

Botargo, from the Arabian word *botarikh*, is a typical fish-based food obtained from the eggs of *Mugil cephalus*, known by Egyptian and other Mediterranean people since 5000 B.C. Sardinia has a strong tradition of producing and using botargo by *M. cephalus* caught locally. In the past few years, this product became successful and now is exported abroad. Production of Sardinian botargo exceeds 350 tons/year, and this expansion necessitated using eggs from different geographical origins such as the U.S.A., Brazil, Mauritania, and Centre-West Atlantic (FAO 31 area). In the market are now available products made following the traditional method but using eggs from different geographical origins. Briefly, the industrial production of botargo starts from crude eggs extracted from *M. cephalus* that are cleaned, salted, dried, and then selected to be marketed whole (W) or ground (G).

European Union (EU) International commercial policy is oriented toward preserving the European gastronomic world from indiscriminate reproduction to guarantee typical food products (*1*). In this context, the IGP designation of origin has been recently requested for "botargo of Sardinia".

To our knowledge, no study has been carried out describing the chemical composition of botargo by *M. cephalus*.

The random amplified polymorphic DNA (RAPD) technique is used widely for DNA tracking and allows for the examination of genomic variation without prior knowledge of DNA sequences. The technique has been applied in fish to evaluate the degree of inter/intraspecific polymorphism (*2*–*4*). Although the genomic characterization of *M. cephalus* using RAPD was never investigated, Murgia et al. (*5*) used DNA mitochondrial sequences and specific primers to identify the origin of botargo from *M. cephalus*. In another study, 43 sequence-tagged microsatellites were isolated from *M. cephalus* of Mediterranean origin (*6*). The protein pattern of the *M. cephalus* eggs has also been previously characterized (*7*), but protein patterns have not yet been investigated as discriminators of sample origin.

The aims of the present study were (i) to determine the chemical and nutritional compositions of botargo, including the more relevant health-related compounds, such as ω-3 fatty acids, vitamin E, squalene, and cholesterol, and (ii) to assess the difference between botargo obtained with eggs from Sardinia

* To whom correspondence should be addressed. E-mail: abarra@unica.it.

† Department of Toxicology, University of Cagliari.

‡ Department of Public Safety, Laboratorio di Igiene degli Alimenti, University of Cagliari.

§ Universita' degli Studi del Piemonte Orientale.

10.1021/jf802363k CCC: $40.75
Published on Web 10/23/2008

and eggs from different geographical origins using chemical, genetic, and protein markers.

MATERIALS AND METHODS

Sampling. A total of 71 commercial samples of W ($n = 42$) and G ($n = 29$) botargo by *M. cephalus* were purchased from a local market and kept at −18 °C until analysis. Samples came from different origins: Brazil (W = 15; G = 10), Mauritania (W = 11; G = 6), U.S.A. (W = 6; G = 5), Centre-West Atlantic (FAO 31 area) (W = 6; G = 8), and Sardinia (W = 5).

Chemical. Heptadecanoic acid methyl ester, squalene, squalane, cholesterol, stigmasterol, α- and δ-tocopherol, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), all analytical standard grade, were purchased from Sigma-Aldrich (Milan, Italy). The marine oil FAME mix was purchased from Restek (Bellefonte, PA). *n*-Hexane, methanol, chloroform, and Na_2SO_4, all analytical grade, were purchased from Carlo Erba (Milan, Italy). Water was distilled and filtered through a Milli-Q apparatus (Millipore, Milan, Italy).

Analytical Methods. *Extraction of Lipids for Determination of Fatty Acids, Squalene, Vitamin E, Cholesterol, and the Antioxidant Activity.* Lipids were extracted according to the method of Bligh and Dyer (*8*). The Bligh and Dyer extracts were then stored at −20 °C before analysis.

Fatty Acid Composition and Squalene. Fatty acid methyl esters (FAMEs) from the extracts were prepared according to Association of Official Analytical Chemists (AOAC) (*9*) official method Ce 1b-89 and expressed as a percentage after peak normalization. Squalene content was determined in the same extracts using squalane as an internal standard and expressed as mg/100 g of botargo (wt/dry wt). The different FAMEs and squalene were separated with a Carlo Erba HRGC 5160 Mega series gas chromatograph equipped with an autosampler (AS-800 CE instruments, Thermoquest, Milan, Italy) and flame ionization detector (FID). A fused silica DB-23 (23% phenylmethylpolysyloxane, 60 m, 0.25 mm inner diameter; 0.25 μm film thickness) (J & W Scientific, Folsom, CA) column was used. The injector and detector were operated at 240 °C. One microliter sample was injected in split mode (1:100). The oven was programmed as follows: 160 °C, raised to 240 °C at 3 °C/min, and held isothermally for 40 min. Nitrogen at 220 and 75 kPa, was used as a carrier and make up gas, respectively. H_2 and air (zero grade) were used at 150 and 100 kPa, respectively.

Cholesterol. Cholesterol was determined in the lipid extracts according to the method of Botsoglu et al. (*10*) but using stigmasterol as an internal standard. The results are expressed as mg/100 g of botargo (wt/dry wt).

Vitamin E. An high-performance liquid chromatograph, LaChrom-Merk-Hitachi (Tokyo, Japan), D7000, equipped with a L-7100 pump, L-7200 autosampler, and L-7485 fluorescence detector (excitation at 290 nm; emission at 330 nm), was employed to quantify different tocopherols according to a slightly modified version of the method described by Brenes et al. (*11*). The tocopherols were separated on a Spherisorb 5 μm ODS2 (460 × 25 mm Waters, Milan, Italy) column, eluted isocratically with water/acetonitrile (50:50, v/v). The injection volume was 50 μL, and the flow rate was 1 mL/min. External calibration curves for the tocopherols using this method have been reported to exhibit regression coefficients between 0.995 and 0.999. Results are expressed as mg/kg of botargo (wt/dry wt).

Antioxidant Activity. Antioxidant activity was determined by the DPPH spectrophotometric test on the lipid extracts. DPPH assays were carried out according to Brand-Williams et al. (*12*). Briefly, stock standard solutions of DPPH (0.4 mM) and Trolox (20 mM) were prepared 24 h before the experiment. Diluted standard solution of DPPH in ethyl acetate (0.04 mM) was prepared just before the experiment. Standard solutions of Trolox at different concentrations (0.1, 0.4, 1.0, 2.0, and 4.0 mmol of Trolox/L) were prepared in ethyl acetate to construct the Trolox calibration curve. A total of 10 μL of Trolox standard dilutions or oil was dissolved in 3 mL of diluted DPPH standard solution (0.04 mM) and hand-shaken. Blank samples was prepared by adding 10 μL of ethyl acetate instead of standard dilutions of Trolox or samples. After 1 h of incubation in the dark at room temperature, absorbance was read versus the blank at $\lambda_{517\ nm}$ using a Cary 50 spectrophotometer (Varian, Milan, Italy). Results were expressed as Trolox equivalent antioxidant capacity (TEAC) in mmol of Trolox/L.

Table 1. Random Primers (10-mer) Used in This Study

primer code	sequences (5′ → 3′)
A1	CAGGCCCTTC
A2	TGCCGAGCTG
A3	AGTCAGCCAC
A4	AATCGGGCTG
A5	AGGGGTCTTG
A7	GAAACGGGTG
A8	GTGACGTAGG
A9	GGGTAACGCC
A12	TCGGCGATAG
B1	GTTTCGCTCC
B2	TGATCCCTGC
C1	TTCGAGCCAG
C4	GATGACCGCC

Lipid Content. Total lipid content in the samples was determined according to the AOAC method (*9*) with a Soxtherm-rapid extraction unit (Gerhardt, Konigswinter, Germany), using ethyl ether/petroleum ether (50:50) as the extraction solvent. Results are expressed as a percentage of dry weight.

Moisture. Moisture content of the samples was determined according to the AOAC method (*9*) by weighing samples until constant weight was achieved after drying in an oven at 60 °C. Results are expressed as a percentage of dry weight.

Protein. The total nitrogen in the samples was determined using the Kjeldahl digestion method with a Buchi K-424 digestor coupled with a Buchi K314 distillation unit. The protein content was calculated as N × 6.25. Results are expressed as a percentage of dry weight.

Salt. Sodium chlorine content in all samples was titrated according to Volhard's method as described in AOAC official method 937.09 (*9*). Results are expressed as a percentage of dry weight.

Ash. Ash content was determined by liming at 550 °C for 18 h.

pH. A total of 100 μg of each sample was dispersed in 20 mL of distilled water, and the pH was measured with a 420 A Orion pH-meter (Milano).

All of the analyses were performed in duplicate.

Molecular Tecniques. *Samples.* Five whole samples from each of the following areas were selected to perform the genetic analysis: U.S.A., Sardinia, Brazil, and Mauritania. Samples from FAO 31 were of very low amount for these analyses.

Genomic DNA Extraction. Total DNA was extracted according to the following sodium dodecyl sulfate (SDS)-based protocol. In a test tube, about 200 mg of each sample was frozen in liquid nitrogen and crushed in a mortar. A volume of 500 μL of extraction buffer [200 mM Tris-HCl at pH 8.5, 200 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% SDS] was added, and the tubes were left in the Thermomixer at 65 °C for 1 h. After centrifugation for 10 min at 14000*g*, the supernatant was transferred to another tube and the DNA was purified by one extraction with phenol/chloroform/isoamyl alcohol (25:24:1, v:v:v), followed by one extraction with chloroform, and precipitated with ice-cold absolute ethanol. The DNA was pelleted by centrifugation at 14000*g* for 10 min and, after two washes with 70% ethanol in pure water, was dried and resuspended in 100 μL of TE buffer (100 mM Tris-HCl and 1 mM EDTA at pH 8.0).

DNA Amplification. A total of 13 decameric primers were chosen arbitrarily and used during this study, all purchased from Invitrogen (Milan, Italy) (**Table 1**). To consider genetic markers able to distinguish different populations, two replicated samples from each population were screened with all of the random primers. RAPD assays were run using a PE 9700 thermocycler (Applied Biosystems, Monza, Italy) according to the following standardized conditions. For each sample was prepared a 25 μL reaction mixture containing about 25 ng of template DNA (quantified employing the Smart-Spec 3000 spectrophotometer, Bio-Rad, Milan, Italy), 1× reaction buffer (500 mM KCl and 100 mM Tris at pH 9.0, provided by the enzyme supplier), 0.8 mM of dNTPs mixture,

Table 2. Constituent Composition of Commercial Botargo by *M. cephalus*[a]

	pH		moisture[b]		ash[b]		salt[b]		lipids[b]		proteins[b]	
origin	G	W	G	W	G	W	G	W	G	W	G	W
FAO 31 mean	5.5 a	5.6 a	24.6 a	31.4 b	9.3 a	9.5 a	8.1 a	6.8 a	25.7 a	21.3 a	49.6 a	43.0 a
Brazil mean	5.7 a	5.6 a	21.4 a	22.4 a[c]	7.2 a	6.8 a	7.7 a	6.8 a	18.8 a	14.2 a	50.1 a	48.9 a
Mauritania mean	5.3 a	5.6 a	16.8 a[c]	28.1 b	8.2 a	8.7 a	6.7 a	8.0 a	28.6 a	20.8 a	56.9 a	49.6 a
U.S.A. mean	5.3 a	5.3 a	19.1 a	23.2 a	7.2 a	6.7 a	6.2 a	5.8 a	26.7 a	16.9 a	49.5 a	48.2 a
Sardinia mean		5.3		29.5[d]		5.6		4.3[e]		13.3[c,e]		47.7
total mean ± SD	5.5 ± 0.4	5.6 ± 0.4	21.1 ± 4.1	23.7 ± 5.6	8.0 ± 2.9	7.6 ± 1.1	6.9 ± 1.7	6.9 ± 1.1	23.5 ± 7.1	16,2 ± 7.3	51.8 ± 7.7	49.5 ± 7.5

[a] SD, standard deviation; G, ground; W, whole. Lowercase letters indicate a comparison in the column between G and E products. Data marked with different letters are significantly different ($p \leq 0.01$) by LSD test. Data marked with "*c*", "*d*", or "*e*" are significantly different from that corresponding to FAO 31, Brazil, or Mauritania, respectively. [b] Expressed as a percentage of dried weight.

Table 3. Fatty Acids, Squalene, and Cholesterol in Commercial Botargo by *M. cephalus*[a]

	origin											
	FAO 31		U.S.A.		Brazil		Mauritania		Sardinia		mean ± SD	
fatty acid[b]	G	W	G	W	G	W	G	W	G	W	G	W
C14:0	1.0 a	1.0 a	0.9 a	0.8 a	0.9 a	1.1 a	0.8 a	1.4 a		1.7	0.9 ± 0.1	1.0 ± 0.2
C16:0	12.1 a	13.5 a	16.4[d]	19.4 a[d]	15.1 a	15.8 a	11.0 a	15.1 b		16.5	13.7 ± 2.5	15.9 ± 2.5
C17:0	9.8 a	11.2 a	5.8 a[d]	6.8 a[d]	11.8 a[e]	7.9 b[d]	14.6 a[d,e]	6.1 b[d]		10.8[f]	10.5 ± 3.7	8.1 ± 2.3
C18:0	6.5 a	5.8 a	7.4 a	7.7 a	7.1 a	7.2 a	5.3 a	8.0 b		5.5	6.5 ± 0.9	7.2 ± 1.0
C20:0	0.9[e,f,g]	t	t	t	t	0.8[d,e]	t	0.4[d,e]		0.6[d,e]	0.6 ± 0.4	0.6 ± 0.3
∑SAFA	30.3 a	31.5 a	30.5 a	34.7 a	34.9 a	32.8 a	31.7 a	30.8 a		35.2	32.2 ± 1.5	32.8 ± 0.3
C14:1	1.6 a	0.4 b	0.7 a	0.6 a	0.7 a	0.8 a	0.9 a	0.3 b		0.4[g]	1.0 ± 0.4	0.5 ± 0.2
C16:1	3.2 a	2.9 a	4.1 a	3.3 a	3.4 a	4.1 a	4.1 a	4.0 a		5.0	3.7 ± 0.5	3.5 ± 0.5
C18:1 *n*-9	4.9 a	4.5 a	5.1 a	4.9 a	4.8 a	5.3 a	5.2 a	4.7 a		6.2	5.0 ± 0.2	4.8 ± 0.3
C18:1 *n*-7	4.2 a	4.4 a	4.5 a	3.0 a	4.2 a	4.0 a	4.2 a	4.0 a		5.1	4.2 ± 0.2	3.8 ± 0.6
C20:1	t	t	t	t	t	0.3[d,e]	t	0.3[d,e]		0.6[d,e]		0.3 ± 0.3
C24:1	7.7 a	6.1 b	5.8 a	3.8 b[d,f,g]	5.7 a	6.2 a	5.4 a	7.0 b		5.2	6.1 ± 1.1	5.7 ± 1.4
∑MUFA	21.6 a	18.3 a	20.2 a	15.6 a	18.8 a	20.7 a	19.8 a	20.3 a		22.4	20.3 ± 1.1	18.6 ± 1.2
C18:2	0.7 a	0.7 a	0.9 a	0.6 b	0.7 a	0.7 a	1.3 a	0.5 b		1.1[f]	0.9 ± 0.3	0.7 ± 0.3
C18:3 ω-3	0.9 a	0.7 a	1.0	0.6 a	0.8 a	0.8 a	0.5 a	0.4 a		0.7	0.8 ± 0.2	0.6 ± 0.2
C20:4 ω-3	3.2 a	4.5 a	4.2 a	3.0 b	3.5 a	4.0 a	4.2 a	2.6 b[d,g]		3.2	3.8 ± 0.5	3.7 ± 1.2
C20:3	1.9	0.8 a	1.4 a	0.2 b	0.8 a	0.6 b	0.7 a	0.3 b		0.1[d,g]	1.1 ± 0.7	0.4 ± 0.2
C20:5 ω-3	11.0 a	8.0 a	9.2 a	8.9 a	9.7 a	9.4 a	9.7 a	10.5 a		8.0	9.9 ± 0.8	9.2 ± 1.0
C22:6D ω-3	24.8 a	26.3 a	25.2 a	27.9 a	22.0 a	24.2 a	22.6 a	22.2 a[e]		22.0[e]	23.7 ± 1.5	25.1 ± 2.5
∑PUFA	40.3 a	40.6 a	40.8 a	41.0 a	36.4 a	38.8 a	37.5 a	36.1 a		35.2	38.8 ± 2.1	39.1 ± 2.2
∑ω-3	39.5 a	40.0 a	39.6 a	39.6 a	35.8 a	38.1 a	36.5 a	35.6 a		33.9	37.9 ± 1.9	38.3 ± 2.0
∑unidentified	8.8 a	9.6 a	7.6 a	9.0 a	8.9 a	9.2 a	11.6 a	7.6 a		8.1	9.3 ± 1.7	8.9 ± 1.0
cholesterol[c]	307 a	302 a	298 a	273 a	268 a	200 b	304 a	184 b[d]		277[f]	314 ± 109	212 ± 61
squalene[c]	19 a	15 a	18 a	19 a[g]	14 a	10 a	15 a	17 a		12	17 ± 2	13 ± 5

[a] SD, standard deviation; t, trace (<0.1%); G, ground; W, whole. Lowercase letters indicate a comparison in the column between G and E products. Data marked with different letters are significantly different ($p \leq 0.01$) by LSD test. Data marked with "*d*", "*e*", "*f*", or "*g*" are significantly different from that corresponding to FAO 31, U.S.A., Mauritania, or Brazil, respectively. [b] Expressed as a mean percentage of dried weight. [c] Expressed as mg/100 g of dried weight.

0.2 μM of primer, 2.5 mM $MgCl_2$, and 1 unit of Taq polymerase (Labogen, Milan, Italy). All amplifications were performed by a thermal program that included a total of 40 cycles of 40 s at 94 °C (denaturation), 1 min at 32 °C (primer annealing), and 1 min at 72 °C (extension), using the fastest available transitions between each temperature.

DNA Detection. Aliquots (10 μL) of amplification products were separated in a 2% agarose gel, stained with Sybr Safe (Invitrogen, Milan). Bands were visualized by UV fluorescence and analyzed using a Fluor-S MultImager detector equipped with Quantity One software (Bio-Rad, Milan, Italy).

Analysis of the Protein Pattern. Samples (100 mg) were extracted in a Thermomixer at 40 °C for 2 h in 1 mL of a urea-containing buffer (0.1 M Tris-glycine and 6 M urea). After centrifugation for 20 min at 14000*g* and filtering (0.22 μm filter Millipore), 25 μL of supernatant was added to 225 μL of absolute ethanol and the precipitated proteins were obtained at 4 °C for 18 h. After centrifugation for 10 min at 14000*g*, the ethanol was discarded and the pellet was dissolved in 100 μL in Laemmli's sample buffer (4% SDS, 20% glycerol, 0.004% bromphenol blue, and 0.125 M Tris-HCl at pH 6.8). Electrophoresis was performed using SDS−polyacrylamide gel electrophoresis (PAGE) precast gels (ExCelgel 2D homogeneous, 12.5 acrylamide from Pharmacia) in the Multiphor II system (Pharmacia). The electrophoresis was run at 300 V/50 mA/30 W for 15 min and then at 600 V/50 mA/30 W until bromophenol blue reach the anode strip. The gel was fixed in 10% acetic acid and 40% ethanol solution for 30 min and then stained with Coomassie Blue R-250 for 30 min to reveal bands. Fluor-S Multimager and Quantity One software (Bio-Rad) were used for gel analysis. Gel bands are expressed as a relative percentage for each sample and used as variables for statistical analysis.

Statistical Analysis. Analysis of variance (ANOVA) of the chemical data was performed by GenStat, version 7.1 software (VSN International Ltd., Herts, U.K.), an using unbalanced treatment structure design when appropriate ($p \leq 0.01$), followed by the LSD test. Principal component analysis (PCA) of the molecular data was performed using the free statistical software R (R Development Core Team, 2008).

RESULTS AND DISCUSSION

Compositional Data. *(i) Crude Composition.* **Table 2** reports the pH, moisture, salt, ash, lipid, and protein contents for W and G products. Mean pH across all samples was 5.5. The moisture content was on average 21.1 and 23.7% for G and W products, respectively. Normally, crude *M. cephalus* eggs possessed 50% moisture on average (data not reported). Mean ash was 8.0 and 7.6% for G and W products, respectively, while salt was 6.9% for both products. The amount of salt and ash in

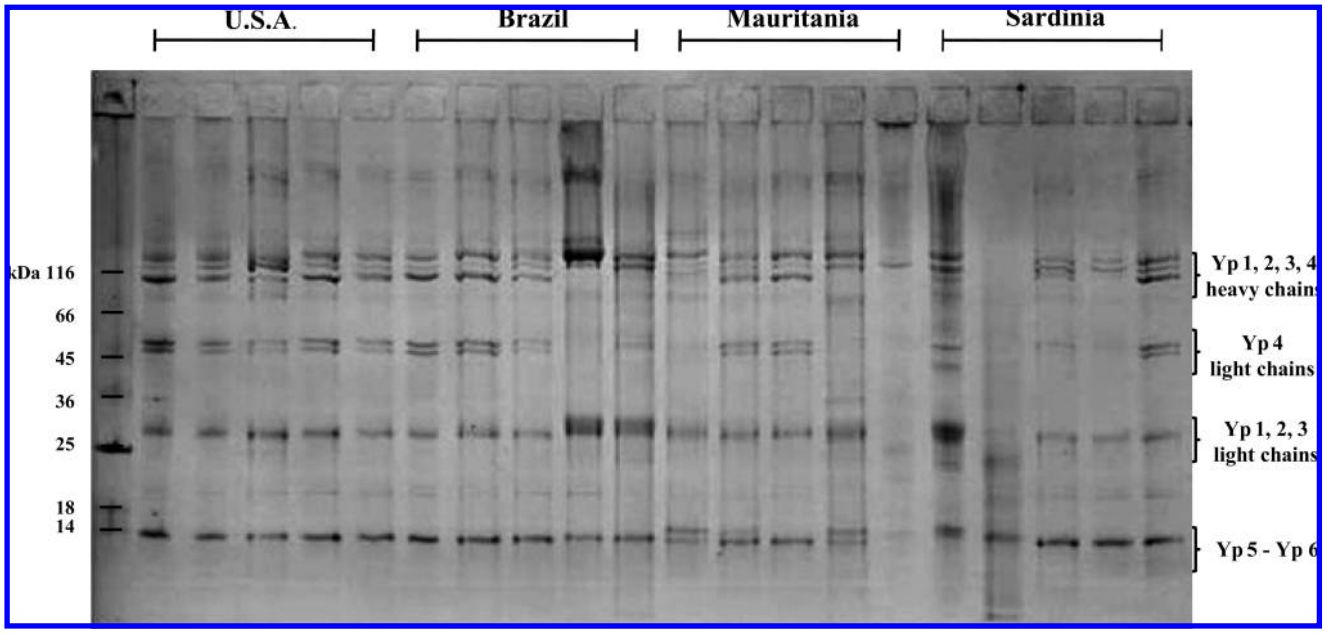

Figure 1. SDS−PAGE fingerprints of proteins of commercial botargo samples from different geographic origins.

crude eggs was ∼2.5% (data not shown), indicating that the salting process contributes more than 90% of the total ash. Lipid contents were 23.5 and 16.2% for G and W products, respectively. Protein content was 50% on average for all samples analyzed, ranging from 35 to 65%. Moisture content was usually higher for the W products, as expected, because G products could lose water easily. Sardinian samples were different from those of Mauritania for salt and lipid contents and from those of FAO 31 and Brazil for lipid and moisture, respectively.

(ii) Fatty Acid Composition. **Table 3** shows the fatty acid composition of the commercial products from different geographical origins. Given are the general averages of saturated fatty acids (SAFAs) (∼32%), mono-unsaturated fatty acids (MUFAs) (∼20%), and poly-unsaturated fatty acids (PUFAs) (∼38%). While the non-identified compounds represent ∼10% of total fatty acids, nevertheless, each unidentified compound was of very low abundance.

The most abundant SAFAs were C16:0 (∼15%) and C17:0 (∼9%). C17:0 has been identified previously as a typical chemical marker of *M. cephalus* oil (*13*).

Among the MUFA, C18:1 (*n*-9) (∼5%) was slightly more abundant than C18:1 (*n*-7) (∼4%) but slightly less abundant than C24:1 (∼6%). A total of 38% of the total fatty acids were *ω*-3. This percentage was about the same as that for PUFAs, with the majority of those being C22:6 [4,7,10,13,16,19-docosahexaenoic acid (DHA)] and C20:5 [5,8,11,14,17-eicosapentaenoic acid (EPA)]. The fatty acids *ω*-3 content could be expressed as ∼8% (wt/dry wt) of commercial products on average. This value is higher than the percentage reported for caviar (4.8%) (*14*) or *Morone saxatilis* eggs (*15*) (1.3%). High PUFA content is important because they are known for their significant nutritional role (*13*). Although a few differences of single fatty acid were evidenced between W and G products or between samples having different geographical origins, no differences were found comparing the sums of the major classes of fatty acids.

(iii) Squalene. Squalene is a hydrocarbon known for its antioxidant activity (*13*). The amount of squalene detected in the samples was 15 mg/100 g in the two commercial products on average, but this low amount is unable to exert good antioxidant activity. Nevertheless, no relevant differences were evidenced between G or W products, and only W samples of the U.S.A. were distinguished from the corresponding ones of FAO 31 (**Table 3**).

Table 4. α- and δ-Tocopherol in Commercial Botargo by *M. cephalus* from Different Origins and Antioxidant Activity of Its Lipid Extract[a]

	δ-tocopherol[b]		α-tocopherol[b]		TEAC[c]	
origin	G	W	G	W	G	W
FAO 31	0.1 a	0.1 a	9.5 a	12.6 a	0.8 a	1.2 a
Brazil	0.2 a	0.2 a	5.3 a	5.2 a	1.3 a	0.6 b
Mauritania	0.04 a	0.07 a	3.3 a	12.2 b	1.2 a	0.7 a
U.S.A.	0.1 a	0.3 b	7.9 a	6.4 a	0.9 a	0.6 a
Sardinia		0.1		7.8		0.7
total mean ± SD	0.2 ± 0.2	0.1 ± 0.1	8.8 ± 5.9	8.3 ± 4.9	1.1 ± 0.4	0.8 ± 0.3

[a] SD, standard deviation; G, ground; W, whole. Lowercase letters indicate a comparison in the column between G and W products. Data marked with different letters are significantly different ($p \leq 0.01$) by LSD test. [b] Means are reported and expressed as mg/kg on dried weight. [c] Expressed as mmol/L of Trolox referred to the extracted oil.

(iv) Cholesterol. Cholesterol is one of the sterols characterizing animal fats. Cholesterol amount was 212 and 314 mg/100 g for the W and G samples, respectively (**Table 3**). The content of cholesterol in W samples from Brazil and Mauritania was lower than that in the corresponding G samples. The W samples of Mauritania contained significantly less cholesterol than in the corresponding FAO 31 samples, and samples of Sardinia were different only from those of Mauritania.

(v) Vitamin E. Vitamin E, generally expressed as the sum of tocopherols, exerts its antioxidant activity as a radical scavenger in the lipid peroxidation process (*16*). **Table 4** reports the vitamin E content of the W and G products. Both α- and δ-tocopherols were identified in the samples, but the amount of δ-tocopherol was 100-fold lower than that of α-tocopherol, while γ-tocopherol was not detected. The amount of vitamin E was ∼8.5 ppm in W and G samples on average, and no relevant statistical differences were found, except for the W samples of Mauritania. Aro et al. (*17*) reported a low content of vitamin E in salted herring submitted through similar technological processes as botargo. Preliminary experiments showed that a loss of vitamin E (5−6-fold reduction) occurs during processing

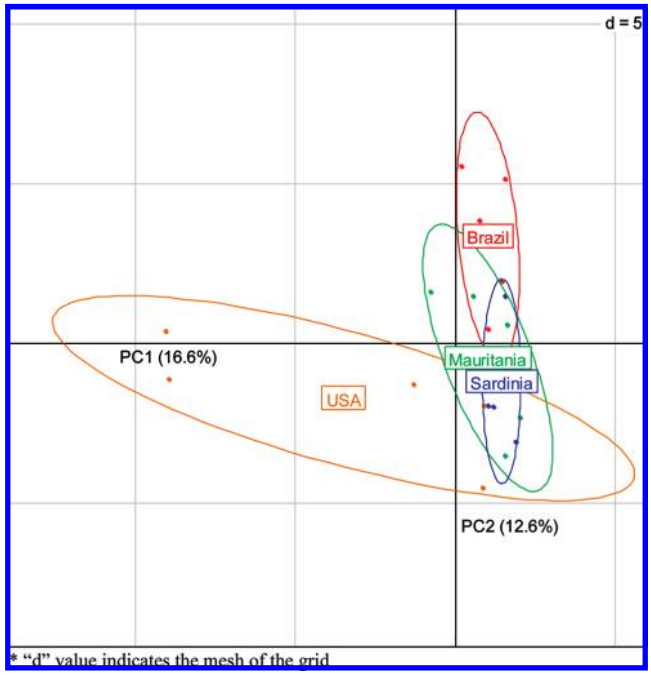

Figure 2. PCA scores plot of the information obtained by amplification using five primers RAPD (29.2% of the total variation).*

of the eggs to obtain botargo (data not shown). This led to the hypothesis that processing significantly reduces the content of vitamin E.

(vi) TEAC. The results of the DPPH tests are reported in **Table 4**. The extracts exhibited antioxidant activity ranging between 0.8 and 1.1 mmol/L for G and W products, respectively. Only one statistically significant difference between G and W samples from Brazil was found, but no difference among samples from different geographical origins was evidenced. These values agree with the literature data on arachid (0.61 mmol/L) and olive oils (1.79 mmol/L) (*18*). The investigated products were manipulated differentially (washed, salted, dried, and/or ground), explaining the high loss of the antioxidants during processing. Regardless, some antioxidant activity was preserved in all samples, but it could not be related to the low amount of tocopherols or squalene.

Molecular Data. *(i) Protein Pattern Analysis.* The protein pattern of botargo was analyzed by SDS−PAGE (12% acrylamide) after extraction in urea buffer. The patterns obtained are presented in **Figure 1**. Bands can be identified according to the results by Amano et al. (*7*). The bands at 90−110 kDa are the heavy chains of lipovitellins (yolk proteins: Yp1, Yp2, and Yp3) derived from vitellogenin. The light chains of the same proteins are at 30 kDa. The Yp4 protein, a proteolytic variant consisting of lipovitellin and phosvitin of vitellogenin A, exhibits mainly a heavy chain (around 110 kDa) and another band at 54 kDa. The band at 15 kDa is attributed to Yp5 and Yp6, identified as the β' component derived from vitellogenins A and B (*7*). Given the similar protein patterns for all of the samples, PCA of those data (expressed as relative percentages of each band), does not enable sample differentiation (data not shown).

(ii) Genetic Analysis. It is known that reproducibility of the RAPD technique depends upon the effects of different reaction parameters on band profiles (*19*). Thus, many experiments were performed to test the effect of template DNA, primer ratio, Taq polymerase, Mg^{2+} concentration, and annealing temperature to standardize the reaction conditions. Moreover, the quality of the template DNA and the method of extraction could affect the profiles. Thus, DNA was always extracted by the same operator using an efficient SDS-based protocol and then purified, employing a phenol/chloroform/isoamyl alcohol (25:24:1, v:v:v) mixture. The quantification of DNA yield was made using a spectrophotometer at a 260 nm absorbance value, checking the DNA purity parameter 260/280 absorbance ratio. The values obtained were reproducible.

Initial experiments using some specific primers and simple sequence repeat (SSR) mugil-specific primer pairs according to the literature data (*5, 6*) did not reveal genetic polymorphisms (data not shown). Thus, RAPD molecular markers were applied to the fingerprint of the samples. All random primers yielded satisfactory amplification products with all samples tested. The reaction protocol was optimized, and each primer produced a unique band pattern of amplified DNA.

Only five primers (A3, A4, A7, A8, and A9) led to polymorphic products and the number of RAPD bands per primer varied from 7 to 24. A total of 58 amplification polymorphic products were scored and used in statistical analyses.

Before PCA, arbitrary values were assigned to all generated bands. RAPD bands were recorded in a binary form, giving "1" value for the presence of the band and "0" value for the absence of the band. Bands that were unique to a single sample or did not show reliability within the replicated samples were eliminated and not used in the computation. The bands were noted and collected in an apposite data matrix. A lot of statistical analysis using PCA of the RAPD data were performed. **Figure 2** shows the results of the bidimensional principal components (2 PC) that explain 29.2% of the total variance. The three-dimensional principal components (3 PC) explain only 40.1% of the total variance. Higher principal components (maximum of 10 PC) and their bidimensional combination (e.g., 2 PC versus 4 PC) does not cluster the samples. Overall, the results do not reveal genetic polymorphism of the samples related to their geographic origin.

The results presented in this study give a chemical evaluation of both the principal nutrients (lipids, ∼20%; proteins, ∼50%; moisture, ∼22%; and salt, ∼7%) and the major health-related compounds, such as ω-3 fatty acids, squalene, vitamin E, and cholesterol, of botargo by *M. cephalus*. The ω-3 fatty acids contents were higher compared to the amount reported in other fish eggs. Cholesterol was abundant, but this was expected because eggs are a cholesterol-rich food. The antioxidant compounds studied (squalene and vitamin E) were in low amounts, probably explained by the industrial processing of botargo. The major constituents of the samples varied noticeably, but only few statistical significant differences were evidenced between G and W products or between samples from different origins. This finding prevents one from differentiating W from G samples or samples from different geographical origins based on the constituents.

The molecular techniques evidenced similar genetic and protein patterns that also prevent discrimination of samples of different origins. Our results indicate that botargo obtained with eggs of *M. cephalus* from Sardinia cannot be distinguished from those obtained with eggs derived from different geographical origins.

ACKNOWLEDGMENT

The authors thank Dr. Gavin Mountjoy, University of Kent, U.K., for helpful discussion.

LITERATURE CITED

(1) Schirone, M.; Vergara, A.; D'Annunzio, T.; Ianieri, A. *Ind. Aliment.* **2007**, *46*, 1125–1133.

(2) Bardakci, F.; Skibinski, D. O. F. Application of the RAPD technique in Tilapia fish—Species and subspecies identification. *Heredity* **1994**, *73*, 117–123.
(3) Borowsky, R. L.; McClelland, M.; Cheng, R; Welsh, J. Arbitrarily primed DNA fingerprinting for phylogenetic reconstruction in vertebrates—The *Xiphophorus* model. *Mol. Biol. Evol.* **1995**, *12*, 1022–1032.
(4) Mamuris, Z.; Apostolidis, A. P.; Theodorou, A. J.; Triantaphyllidis, C. Application of random amplified polymorphic DNA (RAPD) markers to evaluate intraspecific genetic variation in red mullet (*Mullus barbatus*). *Mar. Biol.* **1998**, *132*, 171–178.
(5) Murgia, R.; Tola, G.; Archer, S. N.; Vallerga, S.; Hirano, J. Genetic identification of grey mullet species (Mugilidae) by analysis of mitochondrial DNA sequence: Application to identify the origin of processed ovary products (bottarga). *Mar. Biotechnol.* **2002**, *4*, 119–126.
(6) Miggiano, E.; Lyons, R. E.; Li, Y.; Dierens, L. M.; Crosetti, D.; Sola, L. Isolation and characterization of microsatellite loci in the striped mullet *Mugil cephalus*. *Mol. Ecol. Notes* **2005**, *5*, 323–326.
(7) Amano, H.; Fujita, T.; Hiramatsu, N.; Shimizu, M.; Sawaguchi, S.; Matsubara, T.; Kagawa, H.; Nagae, M.; Sullivan, C. V.; Hara, A. Egg yolk protein in gray mullet (*Mugil cephalus*): Purification and classification of multiple lipovitellins and other vitellogenin-derived yolk proteins and molecular cloning of the parent vitellogenin genes. *J. Exp. Zool.* **2007**, *307*, 324–341.
(8) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Phys.* **1959**, *37*, 911–917.
(9) Association of Official Analytical Chemists (AOAC). Official Methods of Analysis of the Association of Official Analytical Chemists. AOAC, Arlington, VA, 1996.
(10) Botsoglu, N.; Fletouris, D.; Psomas, I.; Mantis, A. Rapid gas chromatographic method for simultaneous determination of cholesterol and α-tocopherol in eggs. *J. AOAC Int.* **1998**, *81*, 1177–1183.
(11) Brenes, M.; Gracia, A.; Dobarganes, M. C.; Velasco, J.; Romero, C. Influence of thermal treatments simulating cooking processes on the polyphenol content in virgin olive oil. *J. Agric. Food Chem.* **2002**, *50*, 5962–5967.
(12) Brand-Williams, W.; Culier, M. E.; Berset, C. Use of free radical method to evaluate antioxidant activity. *Lebensm.-Wiss. Technol.* **1995**, *28*, 25–30.
(13) Pigott, G. M.; Tuker, B. W. Fish oils. In *Encyclopaedia of Food Sciences and Nutrition*, 2nd ed.; Elsevier: Amsterdam, The Netherlands, 2003; Vol. 4, pp 2491−2501.
(14) Gessnes, J.; Wirth, M.; Kirschbaum, F.; Kruger, A.; Patriche, N. Caviar composition in wild and cultured sturgeons-impact of food sources on fatty acid composition and contaminant load. *J. Appl. Ichthyol.* **2002**, *18*, 665–672.
(15) Harrel, M. R.; Curry Woods, L., III. Comparative fatty acid composition of eggs from domesticated and wild stripped bass (*Morone saxatilis*). *Aquaculture* **1995**, *133*, 225–233.
(16) Visoli, F.; Galli, C. The effect of minor constituents of olive oil on cardiovascular disease: New findings. *Nutr. Rev.* **1998**, *56*, 142–147.
(17) Aro, T. L.; Larmo, P. S.; Backman, C. H.; Kallio, H.; Tahvonen, R. L. Fatty acids and fat-soluble vitamins in salted herring (*Clupea harengus*) products. *J. Agric. Food Chem.* **2005**, *53*, 1482–1488.
(18) Pellegrini, N.; Serafini, M.; Colombi, B.; Del Rio, D.; Salvatore, S.; Bianchi, M.; Brighenti, F. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays. *J. Nutr.* **2003**, *133*, 2812–2819.
(19) Meunier, J. R.; Grimont, P. A. Factors affecting reproducibility of random amplified polymorphic DNA-fingerprinting. *Res. Microbiol.* **1993**, *144*, 373–379.

Received for review July 30, 2008. Revised manuscript received September 16, 2008. Accepted September 17, 2008. The authors thank "Fondazione Banco di Sardegna" for financial support.

JF802363K