Species Differentiation by Multivariate Analysis of Phospholipids from Canned Atlantic Tuna

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Multivariate statistical analyses have been applied to different parameters regarding phospholipid classes with the aim of differentiating between three species of canned Atlantic tuna: albacore (*Thunnus alalunga*), bonito (*Sarda sarda*), and big eye tuna (*Thunnus obesus*). Using forward discriminant function analysis to generate classification functions, 14 variables were chosen, showing the content of the 20:4*n*−6 fatty acid in the phospholipid fraction, the highest value of Wilk's lambda. An excellent percentage of right classification in the three species groupings was obtained. The efficiency of the generated functions was tested using a set of canned tuna samples. Commercial albacore and bonito samples, samples caught during different years, and overprocessed samples were successfully identified employing this technique.

Keywords: *Tuna species; characterization; phospholipids; discriminant analysis*

INTRODUCTION

Tuna processing is an industry of well-known economic importance in many countries such as Spain, supporting a significant market demand and playing an important role in the field of human nutrition as components of the Spanish diet (Alimarket, 1992). One of the most important economic problems associated with these products is that regarding the authentication of the tuna species employed. Identification of fish species in marine foods is a prerequisite for complying with labeling regulations because some characteristics of food items have to be declared on the label, such as nutritional composition and a complete list of ingredients (Ashoor and Knox, 1985). In addition, since tuna species employed for canning can have different market prices, the need for characterizing which species the manufacturer or the consumer is buying is obvious.

Most raw tuna species can be easily recognized by their sarcoplasmic electrophoretic profiles. This technique has been employed for fish species identification since 1953 and is recognized by the AOAC as the official method for fish species inspection (Connel, 1953; AOAC, 1980). However, during a process, such as canning, most of these biochemical characteristics are removed or so damaged that these approaches become useless for identification purposes.

The first advance could be the use of biochemical components which are less damaged during processing. Some authors have suggested that selected lipid characteristics may be useful for species characterization purposes (Lichfield, 1972; Ackman, 1989). In particular, the analysis of the fatty acid distribution of triacylglycerols (TG) has been employed to distinguish fats and oils from animal or vegetable sources (Litchfield, 1972; Brockerhoff *et al.*, 1963). The method has been applied to detect the presence of pork meat in meat mixtures (Rugraff and Karleskind, 1983; Verbecke and De Brabander, 1985).

For fish lipids, however, the influence of both external and internal factors on lipid composition makes it difficult to apply that approach, since important variations in the TG pattern of these organisms have been reported (Dotson, 1976). Several studies have confirmed that the phospholipid (PL) pattern of marine organisms is mainly determined by their taxonomic position (De Koning, 1970; Thomas and Patton, 1972; Ackman, 1989). Variability of the acyl PL composition of different marine organisms due to seasonal and dietary changes has been the subject of several researches, but no definitive conclusions have been achieved (Ackman, 1989). Factors such as sex, age, or gonad development did not reveal remarkable differences in the content of total PLs and their fatty acid composition in an ample variety of fish and crustaceans (Ackman, 1989). Exogenous factors could have more influence, but the available information is contradictory. There are clear examples where the PLs and their fatty acid composition are dependent on temperature, salinity, or nutrition (Ackman, 1989; Hazel, 1989). Alternatively, other studies carried out did not find any change in these lipid classes (Ackman, 1989).

The aim of the present work was to differentiate between three of the most frequently consumed canned Atlantic tuna species, albacore (*Thunnus alalunga*), bonito (*Sarda sarda*) and big eye tuna (*Thunnus obesus*), using parameters concerning their PL composition. These species differ from a quality point of view, and consequently, they have different market prices with albacore having the highest economic value. Variables such as the PL classes distribution and the fatty acyl composition of the total PL fraction were determined employing several chromatographic techniques (column chromatography, high-performance liquid chromatography (HPLC), and gas chromatography (GC)). Multivariate statistical methods were then applied to data in order to investigate the potential of the method for classification of species of canned Atlantic tuna.

MATERIALS AND METHODS

Raw Material. The three commercial tuna species used are as follows: albacore (*Thunnus alalunga*) (15 fish), big eye tuna (*Thunnus obesus*) (8 fish) and bonito (*Sarda sarda*) (15 fish). The fish came from the Atlantic Ocean and were purchased at a recognized commercial market. All fish landed frozen. After arrival at our laboratory, all samples were stored at −20 °C prior to analysis.

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Cooking and Canning. Processing was performed in the pilot plant of the Instituto de Investigaciones Marinas del CSIC (Vigo, Spain). Whole eviscerated and beheaded fish were steamed (102−103 °C) until a final backbone temperature of 65 °C was achieved (90 min). They were then cooled at room temperature (14 °C) for about 5 h. The fish were cleaned, and the red muscle was removed. Portions of 90 g of cooked white muscle were placed in RO-100 cans (6.52 cm diameter, 3 cm height) and filled with oil (20 mL of Spanish soybean oil obtained at a local market and 2 g of sodium chloride). The cans were vacuum-sealed and sterilized in a retort at 110 °C (55 min). The cans were stored at room temperature until required for analysis (3 months).

Lipid Extraction. Lipids were extracted from the white muscle by the Bligh and Dyer method (1959), and the muscle content was determined gravimetrically as described by Herbes and Allen (1983). All organic solvents employed were reagent grade (E. Merck, Darmstadt, Germany).

Determination and Purification of Phospholipids. Organic phosphorus was determined on total lipid extracts according to the method of Raheja *et al.* (1973) based on a complex formation with ammonium molybdate (E. Merck). Purified PLs were obtained by means of column chromatography using an elution in chloroform to eliminate the TG and then in methanol to recover the PL as previously described by Christie (1982). During this process, possible TGs from the soybean oil were also separated from tuna PL.

Fractionation of Phospholipid Classes. HPLC was performed with a silica gel 60 column (4.6 mm i.d. × 25 cm; Supelco, Inc., Bellefonte), coupled with a Perkin Elmer solvent pump (Beaconsfield, Buckinghamshire, U.K.), a Perkin Elmer LC-65 UV variable wavelength detector set at 205 nm, and a Hewlett-Packard 8380A integrator (Wotingham, Berkshire, U.K.). The separation was achieved with an isocratic elution in a solvent mixture of $CH_3CN/CH_3OH/H_3PO_4$ (98:1:1, v/v/v). The flow rate was maintained at 1 mL/min. All solvents employed were of HPLC grade (E. Merck).

Polar lipids (150 μg) were injected into the HPLC system, and the phospholipid classes were separated under the conditions described above. The identity of peaks was verified using phospholipid standards (Matreya, Inc., Pleasant Gap, PA). Diacyl forms of phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and lysophosphatidylcholine (LPC) were separated completely, whereas other minor complex classes and plasmalogens (PLA) eluted with the eluent front.

Determination of Phospholipid Fatty Acid Composition. Total PL extract obtained from column chromatography and purified diacyl PL classes coming from the above HPLC separation were converted into the corresponding fatty acid methyl esters (FAME) by the method of Lepage and Roy (1986). To eliminate the excess of H_3PO_4, a washing step with an alkaline solution (6% K_2CO_3) (E. Merck) prior to the reaction was necessary.

FAME were analyzed by GC (Perkin Elmer 8700 chromatograph) employing a fused silica gel capillary column (SP-2330, 0.25 mm i.d. × 30 m; Supelco Inc., Bellefonte, PA), programmed from 145 to 190 °C at 1.0 °C/min and from 190 to 210 °C at 5.0 °C/min, followed by a hold for 13.5 min at 210 °C. Nitrogen at 10 psig was the carrier gas, and a flame ionization detector set at 250 °C was used. A programmed temperature injector was used in the split mode (150:1) and heated from 45 to 275 °C at 15 °C/min.

Peaks were identified by comparison of their retention times with standard FAME mixtures (Supelco, PUFA no. 1 and no. 2, Larodan, Qualmix Fish). For quantitation purposes, peak areas were automatically integrated and 19:0 fatty acid (Sigma) was used as an internal standard. Quantification of diacyl PL classes (PE, PC, PS, PI) and the monoacyl LPC was done knowing their respective fatty acid composition and the structure of the non-acyl moiety.

Statistical Analysis. Data obtained were subjected to analysis of variance (ANOVA) one-way method according to Sokal and Rohlf (1981). Kolmogorov−Smirnov and Cochran tests for normality and homogeneity of variance and a forward stepwise discriminant analysis were carried out using a Statistica package (Statsoft, 1994).

Table 1. Lipid and Phospholipid Contents[a] in White Muscle of Six Tuna Species

tuna species	lipid[b]	phospholipid[b]
albacore	2.43 ± 0.68^{a}	0.39 ± 0.06^{a}
big eye	1.13 ± 0.33^{b}	0.28 ± 0.04^{ab}
bonito	3.71 ± 1.40^{a}	0.23 ± 0.04^{b}

[a] Results are expressed as mean ± standard deviation for different samples. Values with different following letters in the same column are significantly different. Significance was declared at $p < 0.05$. [b] Expressed as g/100 g of wet muscle.

Samples Employed for Validation. To test the ability of the statistical functions generated, several canned tuna samples from different sources were employed. Commercial tuna cans came from a commercial market, labeled as albacore and bonito from Atlantic Ocean and packed in soybean oil. Overprocessed Atlantic albacore samples were prepared in our pilot plant. Sterilization was carried out using temperatures (120 °C) and times (2 h) higher than usual conditions (115 °C during 55 min). Tuna samples caught in the same seasonal periods and geographical zones as those of raw data but during different years were used. Finally, albacore canned samples from Pacific Ocean packed in soybean oil and purchased from a commercial market were analyzed. All these samples were subjected to the same analysis described above to calculate the values of PL parameters. They were then classified using the discriminant functions generated.

RESULTS AND DISCUSSION

Table 1 shows the values obtained for lipid and PL content for the white muscle of canned tuna species. Albacore and bonito samples showed the highest levels of lipid according to data previously described for fresh muscle (Medina *et al.*, 1993, 1995a). Differences in the lipid content between raw white muscle of tuna species have been already observed depending on both environmental and endogenous factors (Dotson, 1976). For each species, the lipid content was rather constant, as can be inferred from the low deviations calculated for all individual samples. Albacore and big eye tuna showed the highest levels of PL content expressed as g/100 g of wet muscle (Table 1). These species showed high percentages of PL related to total lipids (Table 1).

A small amount of filling oil remains in the fish muscle in spite of the draining operation, increasing the real value of the lipid content in canned muscle (Sacchi *et al.*, 1993; Medina *et al.*, 1995b). This additive effect concerns the quantities of total TG or diacylglycerides found in lipids extracted from muscle but does not affect the absolute values of PL or their fatty acid composition since soybean oils lack PL. The intrinsic location of tuna PL at cell membranes avoids a possible extraction into the filling soybean oil (Aubourg *et al.*, 1990). Variables regarding the PL composition appeared to be independent of the oil employed as packing medium.

The three tuna species analyzed showed similar qualitative and quantitative patterns of PL classes (Table 2). In all cases, PC was the major class followed by PE and LPC. The main source of LPC in fresh fish muscle is related to the storage at relatively high freezing temperatures before processing (Ohshima *et al.*, 1985). Therefore, in addition to the PLA degradations occurring during thermal treatments (Medina *et al.*, 1993), a possible hydrolysis of PC to result in LPC has been also described during sterilization of tuna muscle (Aubourg *et al.*, 1990).

The more remarkable differences among the species can be found in the content of the minor PL classes such

Table 2. Percentage Distribution[a] of Phospholipid Classes in White Muscle of Canned Tuna Species

PL class	tuna species		
	albacore	big eye	bonito
PE	19.12 ± 1.88[a]	19.25 ± 3.16[a]	21.71 ± 2.50[a]
PI	5.00 ± 0.64[a]	11.39 ± 1.43[b]	5.54 ± 2.04[a]
PS	7.44 ± 0.92[a]	4.99 ± 0.91[a]	5.85 ± 2.84[a]
PC	52.54 ± 2.31[a]	40.94 ± 3.83[b]	52.90 ± 3.03[a]
SPH	3.13 ± 0.59[a]	3.38 ± 0.91[a]	1.36 ± 0.33[b]
LPC	13.08 ± 1.80[a]	15.85 ± 1.84[a]	9.95 ± 3.47[a]

[a] Results are expressed as mean ± standard deviation for different samples. Values with different following letters in the same row are significantly different. Significance was declared at $p < 0.05$.

Table 3. Fatty Acid Composition[a] of Phospholipids from White Muscle of Canned Tuna Species

acid	tuna species		
	albacore	big eye	bonito
14:0	0.57 ± 0.23[a]	0.50 ± 0.24[a]	0.99 ± 0.23[a]
15:0	0.35 ± 0.19[a]	0.28 ± 0.08[a]	0.39 ± 0.11[a]
16:0	15.14 ± 5.16[a]	13.64 ± 1.46[a]	19.37 ± 2.61[a]
17:0	0.60 ± 0.26[a]	0.47 ± 0.10[a]	1.34 ± 0.33[b]
18:0	0.95 ± 0.14[a]	0.94 ± 0.27[a]	0.85 ± 0.24[a]
16:1*n*−7	11.83 ± 1.05[a]	11.84 ± 2.20[a]	12.08 ± 2.02[a]
18:1*n*−9	12.03 ± 2.14[a]	11.58 ± 1.12[a]	10.88 ± 3.28[a]
18:1*n*−7	2.15 ± 0.25[a]	1.65 ± 0.20[b]	3.49 ± 0.60[c]
20:1*n*−9	1.56 ± 0.60[a]	0.51 ± 0.15[b]	1.56 ± 0.57[a]
18:2*n*−6	0.86 ± 0.23[a]	0.51 ± 0.08[a]	0.88 ± 0.45[a]
20:4*n*−6	3.09 ± 0.49[a]	6.84 ± 0.30[b]	2.83 ± 0.39[a]
20:5*n*−3	5.31 ± 1.05[ab]	3.82 ± 0.47[a]	8.60 ± 2.65[b]
22:6*n*−3	45.55 ± 5.54[ab]	47.42 ± 1.69[a]	38.42 ± 3.33[b]

[a] Data are given in mol % as means and standard deviations for different samples. Values with different following letters in the same row are significantly different. Significance was declared at $p < 0.05$.

as PI, PS, and SPH. These PLs have been found in several marine samples, although their concentrations vary depending on the tissue and organ considered (Vaskowski, 1989).

Table 3 shows the fatty acid composition of total PL extracted of white canned muscle of each tuna species. Fish PLs are remarkable for their high content in polyunsaturated fatty acids (PUFA), and substantial differences in their fatty acid composition can be found depending on the muscle tissue or organism (Ackman, 1989). As can be inferred from Table 3, the fatty acid composition exhibits certain differences between species. An ANOVA test was performed to single out the significant differences for each fatty acid. All species showed major saturated and monounsaturated fatty acids, 16:0 and 18:1*n*−9, respectively. The preponderance of 16:0 over 18:0 is a consequence of the major proportion of PC found in tuna PL and the enrichment of this PL class in palmitic acid in marine tissues (Ackman, 1989). More differences are found in minor fatty acids, such as 17:0 and 20:1*n*−9. PUFA showed the major differences between species, with important variations in the magnitudes of 20:4*n*−6, 20:5*n*−3, and 22:6*n*−3.

To provide a method for differentiating the three canned tuna species, a multivariate statistical analysis was carried out. Before proceeding with this, homogeneity of variance and normality were confirmed (Cochran and Kolmogorow−Smirnow tests, respectively). Forward stepwise discriminant analysis, using as variables the PL content, the proportions of each PL class, and the content of the total PL fatty acids, was employed as the classification method.

Table 4 shows the discriminant function analysis

Table 4. Stepwise Analysis Summary[a]

variable	Wilk's lambda	partial lambda	*F*-remove	*p*-level	toler
20:4*n*−6	0.002 848	0.162 906	35.969 44	0.000 003	0.220 142
PL	0.000 514	0.901 881	0.761 56	0.485 338	0.447 900
18:1*n*−7	0.000 893	0.519 559	6.472 97	0.010 220	0.125 090
LPC	0.000 584	0.794 355	1.812 18	0.199 574	0.130 430
20:1*n*−9	0.000 692	0.670 141	3.445 56	0.060 696	0.282 023
PI	0.000 957	0.484 689	7.442 26	0.006 284	0.231 082
PS	0.000 693	0.669 216	3.460 00	0.060 112	0.222 751
20:5*n*−3	0.000 575	0.807 026	1.673 82	0.222 953	0.110 570
16:1*n*−7	0.000 655	0.708 346	2.882 17	0.089 479	0.082 056
14:0	0.000 863	0.537 213	6.030 22	0.012 913	0.136 416
PE	0.000 659	0.704 099	2.941 79	0.085 790	0.165 861
SPH	0.000 619	0.749 640	2.337 81	0.133 037	0.144 922
18:2*n*−6	0.000 585	0.792 657	1.831 06	0.196 606	0.148 039
17:0	0.000 559	0.830 286	1.430 83	0.272 016	0.364 445

[a] Variables currently in the model with their corresponding parameters.

Table 5. Discrimination Functions[a]

variable	discrimination functions	
	function 1	function 2
20:4*n*−6	1.9477	−0.2211
PL	0.2481	−0.4073
18:1*n*−7	1.3492	1.4625
LPC	1.2212	0.3242
20:1*n*−9	−1.0826	−0.0977
PI	1.3243	0.7196
PS	−0.2451	−1.2230
20:5*n*−3	−1.2689	−0.3982
16:1*n*−7	1.4544	1.2376
14:0	−1.7593	−0.5865
PE	1.2985	0.3467
SPH	0.8269	−1.0499
18:2*n*−6	0.3307	−1.1644
17:0	0.6429	−0.2435

[a] Standardized coefficients of each variable.

summary. The model included 14 variables, with the last one being the content of the 17:0 fatty acid. The Wilk's lambda value obtained was 0.000 46. The content of the 20:4*n*−6 fatty acid was the first variable entered with the highest value of Wilk's lambda calculated if this variable is eliminated in the model (0.002 848), and a partial lambda of 0.1629 that indicates the contribution of this variable to the total discrimination ability. Parameters such as the *F* value associated with partial lambda coefficient, tolerance, and the *p*-level associated with the respective *F* were computed. Variables with tolerance values lower than 0.01 resulted as more than 99% redundant with the other variables already in the model; therefore, they were not considered in the analysis.

Table 5 shows the two discriminant functions with the standardized coefficients for each variable. In the first function, the content of the 20:4*n*−6 fatty acid showed the largest coefficient, since the role of this variable to discrimination between groups was the most relevant. With respect to the second discriminant function, the content of 18:1*n*−7 showed the highest coefficient value. The nature of the discrimination for each discriminant function was identified by plotting the individual scores for the two functions (Figure 1). The second discriminant function discriminated between albacore and bonito canned samples and the first function between these two groups and big eye canned samples.

All the cases were classified by using the classification functions of each group (Table 6). Each case was classified as belonging to the group for which it showed

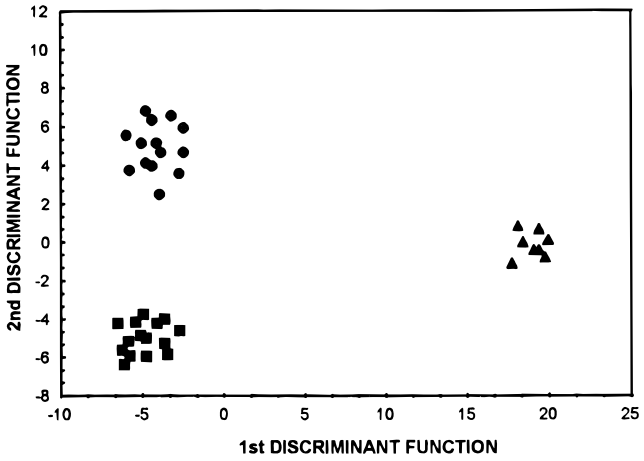

Figure 1. Plot of the two discriminant function scores for the canned tuna species: (■) albacore, (●) bonito, and (▲) big eye.

Table 6. Classification Functions of Each Tuna Group[a]

	classification functions		
variable	albacore	bonito	big eye
20:4*n*−6	188.13	186.21	297.43
PL	446.70	369.45	532.99
18:1*n*−7	176.16	211.21	267.96
LPC	29.16	30.65	40.93
20:1*n*−9	−107.32	−112.44	−188.97
PI	38.47	43.66	61.89
PS	4.85	−1.24	−0.97
20:5*n*−3	−34.98	−37.52	−52.63
16:1*n*−7	250.34	297.17	398.51
14:0	−412.43	−441.78	−606.91
PE	41.40	43.13	54.95
SPH	23.10	17.13	32.70
18:2*n*−6	17.37	−3.23	22.63
17:0	174.23	167.11	228.24
constant	−1219.54	−1273.55	−2478.56

[a] Constant and coefficients of each variable.

Table 7. Classification of Tuna Cans from Commercial Sources, Overprocessed Albacore Canned Samples, Atlantic Tuna Canned Samples Caught during Different Years, and Albacore Samples from Pacific Ocean, According to the Discriminant Functions Generated

tuna samples (*n*)[a]	percent of correct classification
commercial albacore (4)	100
commercial bonito (2)	100
overprocessed albacore (4)	100
albacore caught during different years (3)	100
bonito caught during different years (3)	100
big eye caught during different years (3)	100
albacore from Pacific Ocean (2)	0

[a] *n*, number of canned samples.

the highest classification score. Results obtained showed an excellent classification percent of tuna canned samples (100% for each group), with a clear differentiation between species according to results visualized in Figure 1.

The efficiency of the generated functions was tested using a set of canned samples from different sources. Results are summarized in Table 7. Atlantic albacore and bonito commercial samples were successfully classified. Big eye is commercially labeled as light tuna. With that denomination, other tuna species such as skipjack (*Katsuwomus pelamis*) or bluefin (*Thunnus thynus*) can be also processed and labeled. To distinguish these species, a detailed description of their PL parameters would be necessary to compute the corresponding discriminant and classification functions.

A high percent of right characterization was obtained for all overprocessed samples. This result seems to indicate that the classification PL model resulted independent of the thermal treatment employed during canning.

With respect to Atlantic tuna samples caught during different years, all cans were successfully classified. However a misclassification was obtained for albacore samples from Pacific Ocean. This result may be useful for identifying different origins for a given fish species.

CONCLUSIONS

The results show the discriminant analysis application using the PL composition of canned tuna as a potential approach in the identification of canned Atlantic tuna species. The content of the 20:4*n*−6 fatty acid in the PL fraction was identified as one of the most important variables in aiding discrimination. Using this methodology, Atlantic albacore was easily differentiated and identified. This fact has noteworthy importance since this species is the most costly tuna species in Spain due to its quality as marine food and, subsequently, its commercial acceptance. The method could be also useful to discriminate different geographical origins in a given fish species. Research with the aim to distinguish between big eye and other Atlantic tuna species commercially labeled as light tuna may also be useful. A detailed description of the PL content in these species should be done.

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