HPLC Separation and Determination of 12 Cholesterol Oxidation Products in Fish: Comparative Study of RI, UV, and APCI-MS Detectors

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A simple, fast, and sensitive method for the extraction through direct saponification, separation, quantification, and identification of 12 cholesterol oxidation products (COPs) and cholesterol in a single isocratic, normal-phase, high-performance liquid chromatography (HPLC) was developed. Three detectors were compared for determination of COPs and cholesterol in fish samples: refractive index (RI), ultraviolet (UV), and atmospheric pressure chemical ionization mass spectrometry (APCI-MS). The results did not show significant differences ($p > 0.05$) between the concentration of the cholesterol oxides and cholesterol obtained with these detectors. The present study demonstrated the presence of 19-hydroxycholesterol, 22"R"-hydroxycholesterol, 22"S"-hydroxycholesterol, 24"S"-hydroxycholesterol, and 25"R"-hydroxycholesterol for the first time in fish samples.

KEYWORDS: Cholesterol oxides; cholesterol; HPLC-UV-RI; HPLC-APCI-MS; fish

INTRODUCTION

Cholesterol (5-cholesten-3β-ol) is found in substantial amounts in most foodstuffs of animal origin. Because it is an unsaturated alcohol, it can generate numerous oxidation products commonly known as cholesterol oxidation products (COPs) (*1*). COPs found in living organisms are derived both from diet and from endogenous enzymatic processes (*2*). Several of theses compounds have been shown to possess undesiderable biological effects such as sterol metabolism interference, cytoxicity, atherogenicity, mutagenicity, carcinogenicity, and changes in cellular membrane properties (*3*).

Processed animal foodstuffs such as milk powder, egg powder, freeze-dried meat and fish, heated animal fats, and meat are the main sources of cholesterol oxides in the human diet (*4*). Absorption of these compounds has been clearly demonstrated; therefore nutritionists as well as food scientists are interested in detecting sources of COPs and quantifying them accurately (*5*). The analysis of COPs is strongly influenced by their chemical structure because these compounds have different functional groups that provide diverse polarity and chemical properties to the molecules. On the other hand, some COPs are isomers and thus have similar chemical, spectrophotometric, and fragmentation characteristics (*6*). Since COPs occur mainly at low levels, purification is necessary before final quantification (*7*). Extraction and purification of the COP fraction involves saponification and/or chromatographic fractionation of lipid extracts (*7*). This is the most critical step for the analytical procedure due the complexity of the lipid fraction of many foods (*8*). Usually the lipid fraction is saponified, although some authors attempted to saponify the whole food sample directly (*6, 7*). Dionisi et al. (*9*) recommended the direct saponification method, for extraction and purification of COPs from milk powder samples, based on recovery, precision, minimal artifacts formation, and relative simplicity.

COPs are usually analyzed by gas chromatography (GC) or GC coupled to mass spectrometry (GC-MS) (*10*). GC, although efficient, can produce artifacts from thermal degradation of cholesterol and its oxides. It requires derivatization, making recovery difficult (*6, 11*). As a consequence of the limitations of GC, high-performance liquid chromatography (HPLC) has become the technique of choice (*12−16*) mainly because the analyses are carried out at ambient temperature. Numerous detection systems have been used to determine COPs by HPLC such as spectophotometric (UV), refractive index (RI), mass spectrometric (MS), fluorimetric, evaporative light scattering, flame ionization, and chemiluminescence's detectors (*6*). The UV detector displays high sensitivity and specificity and is very popular in HPLC determination of COPs. However, some oxysterols of biological importance, such as 5,6α-epoxy-cholesterol, 5,6β-epoxy-cholesterol, and cholestane-3β,5α,6β-triol do not absorb at UV wavelengths, and other detection methods must be used (*6, 15*). RI is a universal detector which can be used with any type of solvent under isocratic conditions

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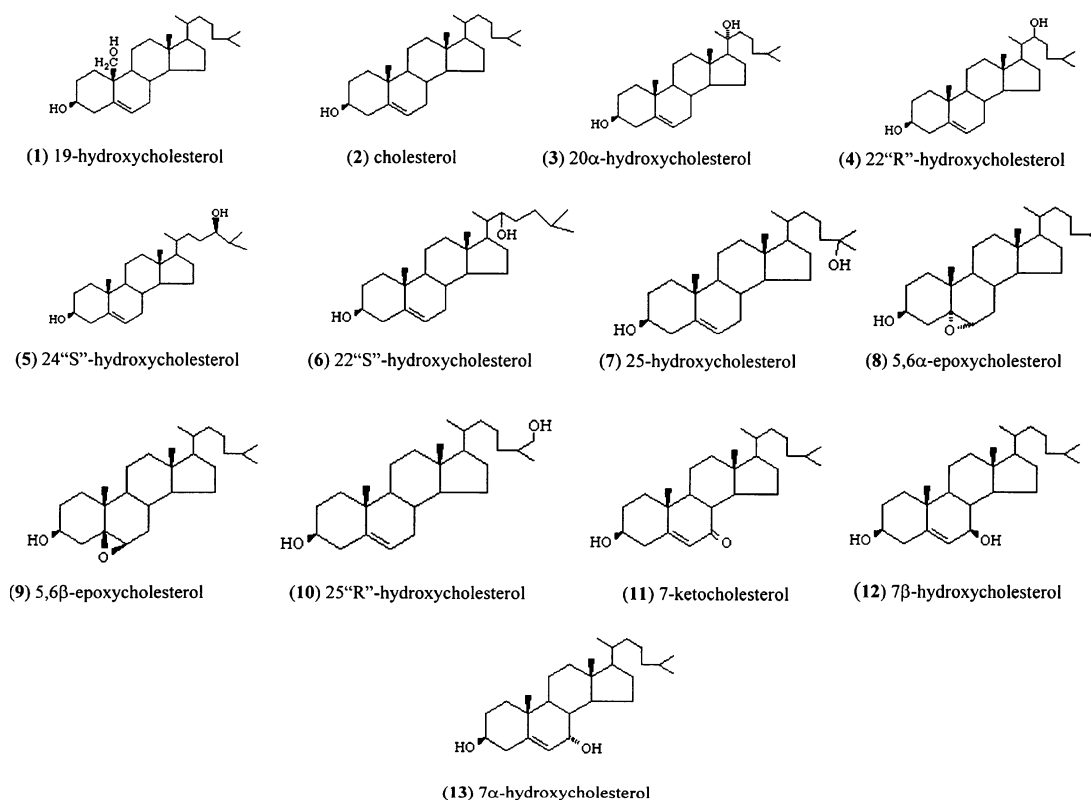† Faculty of Food Engineering.
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Figure 1. Chemical structures of cholesterol (**2**) and cholesterol oxides (**1**, **3**–**13**) numbered according to the elution order on the CN column with *n*-hexane–2-propanol (97:3).

and allows the simultaneous detection of all COPs, although it displays a low sensitivity and cannot be used with solvent gradient (*6*).

Recent studies have described the use of HPLC coupled to mass spectrometry to determine cholesterol oxides, since this sensitive technique can detect polar and labile trace compounds (*12*). Careri et al. (*12*) applied HPLC coupled to particle beam mass spectrometry and separated cholesterol and five of its oxides. Using HPLC with atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS), Manini et al. (*17*) separated cholesterol and five COPs, Razzazi-Fazeli et al. (*18*) determined seven COPs in butter, Raith et al. (*19*) quantified cholesterol and five COPs in processed foods such as pork, beef, chicken, and egg, and Al-Saghir et al. (*20*) determined seven COPs in salmon.

The goals of this study were (i) the development of a simple method of cholesterol and COPs extraction by direct saponifica-tion, (ii) the validation of an analytical method using an isocratic, normal-phase HPLC system using a cyano-bonded HPLC column, and (iii) the investigation of the applicability of RI, UV, and APCI-MS detectors in the simultaneous separation and quantification of cholesterol and 12 cholesterol oxides. This paper describes the determination of the following isomeric cholesterol oxides in food matrixes for the first time: 22"*R*"-hydroxycholesterol, 24"*S*"-hydroxycholesterol, 22"*S*"-hydroxy-cholesterol, and 25"*R*"-hydroxycholesterol.

MATERIALS AND METHODS

Chemicals and Reagents. All chemicals and reagents came from Sigma (Milford, MA), Steraloids (Newport, RI), Mscience (Darmstadt, Germany), and Merck (Darmstadt, Germany). The purities of the standards varied from 95 to 98%. The trivial names and chemical structures of the compounds, in elution order, are presented in **Figure 1**.

Fish Samples. Samples of 1 kg each of salted and dried Pacific codfish (*Gadus macrocephalus*) and fresh merluza (*Merluccius cap-ensis*) were used for the development of the extraction method. A 2 kg amount of salted and dried Pacific codfish was used for the development and validation of the methodology for the simultaneous determination of cholesterol and cholesterol oxides. Samples of 1 kg each of salted and dried Northern codfish (*Gadus morhua*), fresh sardine (*Sardinella spp*), and Atlantic hake (*Merluccius hubbsi*) were used for the application of the method. The fishes were purchased at a local store in Sao Paulo, Brazil. The salted and dried codfish were left in water for 24 h to remove the salt. The samples were completely ground and homogenized in a food processor prior to extraction.

Extraction Method. Several methods found in the literature to determine cholesterol oxides in fish use the lipid extract to obtain these products (*21*–*24*); however, in other samples direct saponification was used (*6*, *7*, *9*, *25*). In the present study sample preparation consists of two steps: direct saponification and extraction of the nonsaponifiable material (*26*). For the saponification step the following conditions were tested through experimental planning: (i) sample weights (0.5–2.0 g), (ii) percentage of KOH in water (20–50%), (iii) volume of ethyl alcohol (2–5 mL), and (iiii) time of saponification (12–24 h). To extract the nonsaponifiable fraction, *n*-hexane and diethyl ether were tested, with three, four, and five extractions. All the conditions were evaluated through chromatographic injections and statistically compared. Recovery of the cholesterol and cholesterol oxides was made by the addition of 100 μg of all standards, stablishing the accuracy of the extraction method. The precision of the extraction procedure was calculated by repeating the complete analysis of pieces of the same codfish sample 20 times.

Method Validation. Reliability of the method was tested for linearity, precision, sensitivity, and recovery. Linearity was observed through correlation coefficients (r^2), and detection limits (DL) were calculated using a signal-to-noise ratio of 3. The intraassay precision, expressed as a relative standard deviations (RSD%), was determined by the extraction and analysis of 10 pieces of the same codfish sample on the same day. The recovery was determined in the salted and dried codfish sample at two different fortification levels, using 10 extractions

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for each level of addition, to assess the extraction efficiency of the proposed method.

HPLC Analysis. A Shimadzu (Tokyo, Japan) liquid chromatograph equipped with UV (SPD-10 AVVP) and RI (RID-10 A) detectors was used. The chromatographic conditions used in the present study was as follow: analytical column, 300×3.9 mm i.d. $\times 4$ μm; Nova Pack CN HP (Waters, Milford, MA) with a 7.5×4.6 mm i.d. $\times 5$ μm; BDS CN precolumn (Alltech, Deerfield, IL); oven temperature, 32 °C. The eluent was *n*-hexane−2-propanol (97:3, v/v) at a flow rate of 1 mL/min and an analysis time of 30 min (*14*, *15*). In addition, a reverse-phase HPLC column C18, 300×3.9 mm, 4 μm, (Nova Pak, Waters, Ireland), was tested. Cholesterol and the epimeric 5,6-epoxides were quantified using the RI detector, because these oxides do not absorb in the UV wavelengths and cholesterol is better separated. The other oxides were quantified using the UV detector at 210 nm. Cholesterol and cholesterol oxides were identified by comparison of retention times of peaks in samples with those of reference standards and quantified by external calibration. The standards were diluted in ethyl acetate, the final concentration of cholesterol being 1.006 mg/mL and that of the oxides 1 μg/mL. The calibration curves were made with six points, with concentrations that varied from 0.5 to 70 μg/mL for the oxides and from 0.1 to 2.0 mg/mL for cholesterol. Internal standards could not be used for quantification since the ones tested (6-ketocholesterol and 7-pregnenolone) failed to respond adequately, and 19-hydroxycholesterol was found in the samples studied.

HPLC-APCI-MS was used to quantify and confirm the identity of cholesterol and cholesterol oxides in the Pacific codfish samples. Isocratic HPLC was carried out using a Waters Alliance 2695 pump (Milford, MA) and the same chromatographic conditions as above. The mass spectrometer used was a Qtrap (Applied Biosystems, Concord, Ontario, Canada) with a QqQ (linear ion trap) configuration. The injection of cholesterol and cholesterol oxide standards was used to optimize the MS conditions. Optimum conditions were as follows: positive ion mode APCI; scan range, m/z 250−500; temperature, 375 °C; nitrogen as carrier gas (70 L/min), sheath gas (60 L/min), and curtain gas (30 L/min); nebulizer current set at 3000 V; declustering potential at 30 V; entrance potential at 9 V. Chromatograms were obtained in the selective ion monitoring (SIM) mode for the ions m/z 367, 369, 385, 401, and 403.

Statistical Analysis. One-way analysis of variance (ANOVA) was applied to the data. The results obtained of the quantification of three different detectors were compared using Tukey multiple comparisons test, significance being based on a 0.05 probability level.

RESULTS AND DISCUSSION

Evaluation of Extraction Procedure. In the saponification conditions evaluated, 2 g of the samples were employed since in the lower concentrations of **3**, **4**, and **5** oxides were not detected. Total dissolution of the sample without emulsion formation was achieved using a solution of 4 mL of 50% KOH in water added to 6 mL of ethyl alcohol. The samples were tested at 12, 18, 20, 22, and 24 h, being that only at 22 and 24 h the samples were totally dissolved. No significant difference ($p > 0.05$) was observed between 22 and 24 h, so for the experiments the time of 22 h was used.

In the nonsaponifiable fraction higher recoveries were found using *n*-hexane compared with diethyl ether. Analyzingly in the samples extracted with *n*-hexane, no significant differences ($p > 0.05$) were observed between four and five extractions for evaluation of the oxides; thus, four extractions were used.

The ideal extraction procedure is described as follows. A sample weighing of 2 g was treated with 4 mL of a 50% aqueous solution of KOH plus 6 mL of ethanol, to perform a saponification at room temperature for 22 h in the dark. For the extraction of the unsaponifiable matter, 5 mL of distilled water and 10 mL of hexane were added to the samples, which were then shaken and the hexane fraction was then separated. The extraction with 10 mL of hexane was repeated three times (total

of four extractions). Subsequently, the solution was dried in a rotatory evaporator and the residue dissolved in 5 mL of hexane, transferred to a screw top flask, dried under N$_2$, diluted with 1 mL of the mobile phase, filtered through a 22 μm Millipore filter, and injected into the HPLC system. The present methodology was also tested in fresh merluza samples, and, in this case, not all the COPs were found, clearly showing that the extraction method does not produce artifacts.

Separation of Cholesterol and Cholesterol Oxides. Initially both normal-phase and reverse-phase HPLC were tested with a variety of eluents, but only the normal-phase HPLC with *n*-hexane/2-propanol (97:3, v/v) was capable of separating isomers **3**, **4**, **5**, and **6** as well as **8** and **9**. In addition, under the chromatographic conditions used, it was possible to separate within 30 min cholesterol (**2**) and 12 cholesterol oxides: **1**, **3**, **4**, **5**, **6**, **7**, **8**, **9**, **10**, **11**, **12**, and **13**. **Figures 2−4** show the chromatograms of the standards and a codfish sample using HPLC-UV-RI and HPLC-APCI-MS. The peaks **8** and **9** were only determined by RI and APCI-MS, since they do not absorb in the UV region. The other oxides were determined using the UV detector at 210 nm as well as APCI-MS.

The driving force of the separation is most probably the interaction of the CN phase with the hydroxyl groups of the COPs rather than that with the double bonds. Isomers **4** and **6** showed good separation with the CN column although, according to literature (*27*), these compounds were only separated previously using a chiral column. Although C18 columns are widely used in the determination of COPs, the baseline separation of all the isomers could not be achieved with reverse-phase HPLC.

Table 1 presents the main molecular and/or fragment ions of cholesterol and its oxides produced in positive ion mode APCI-MS. Results from in-source dissociation leading to the loss of one or two molecules of water, and the fragment ions, were similar to those found in other studies using APCI-MS (*17*, *18*). The fragments observed for cholesterol and oxides **1**, **7**, **10**, and **11** showed distinctive ion distributions; however, **3**, **4**, **5**, and **6** are structurally very similar isomers, resulting in small differences in their ion distributions. Nevertheless, these differences, coupled to their characteristic retention times, were sufficient to confirm the identity of these oxides. Oxides **8** and **9** displayed similar fragmentation patterns, but, with well-resolved chromatographic peaks with good resolution, they could be distinguished by their retention times. The same occurred with isomers **12** and **13**. When analyzing the codfish samples, despite the high cholesterol concentration, the peaks of the oxides could be clearly observed and quantified. Considering these results, for an unequivocal identification of cholesterol and COPs by HPLC-APCI-MS, a complete HPLC separation of the compounds is mandatory. On the other hand, since tandem mass spectrometry of these compounds results in nonspecific fragmentation (*19*), the method was not useful for structure elucidation.

In the present study good ionization and precision were observed using a nonpolar mobile-phase and normal-phase HPLC. Reverse-phase HPLC is preferred for HPLC-MS, since it displays higher reproducibility than normal phase and the polar mobile phase favors positive ionization (*6*). However, methanol adducts were observed for several COPs with reverse-phase HPLC-MS (*18*) which were not observed with normal-phase HPLC-MS (*17*).

Analytical Performance. Excellent linearity was observed for all tested compounds with correlation coefficients (r^2) of >0.996 between the three detectors evaluated. All curves were
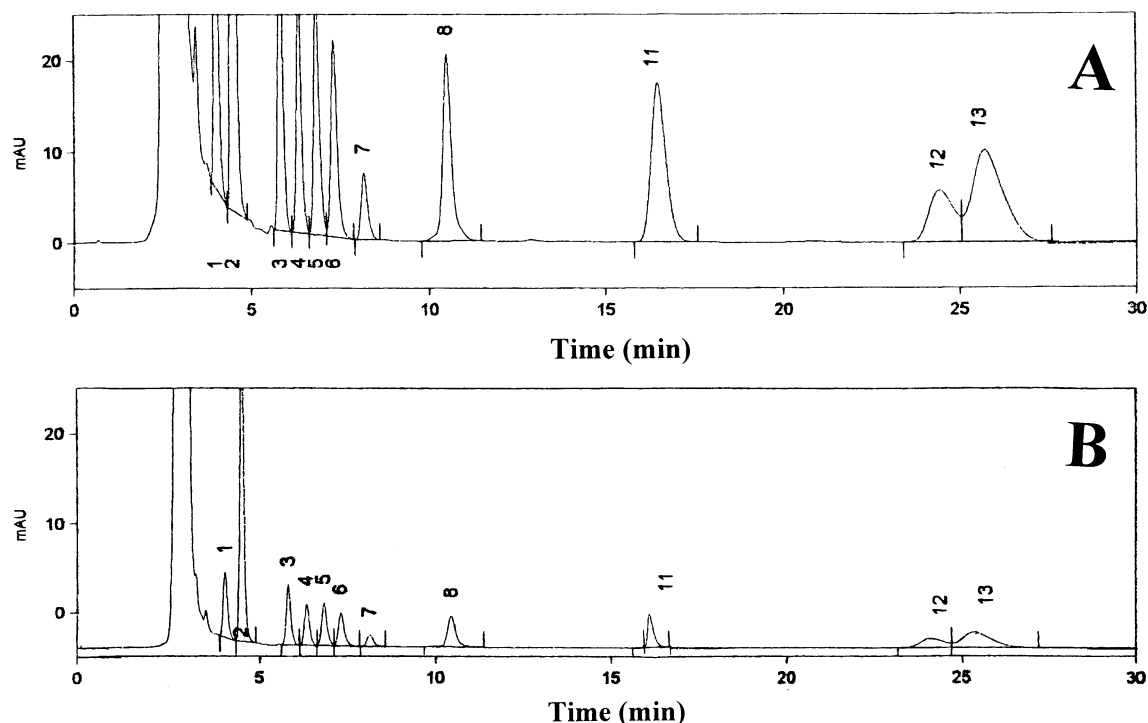
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Figure 2. Chromatogram of cholesterol and cholesterol oxide standards (**A**) and the codfish sample (**B**) determined using the UV detector.
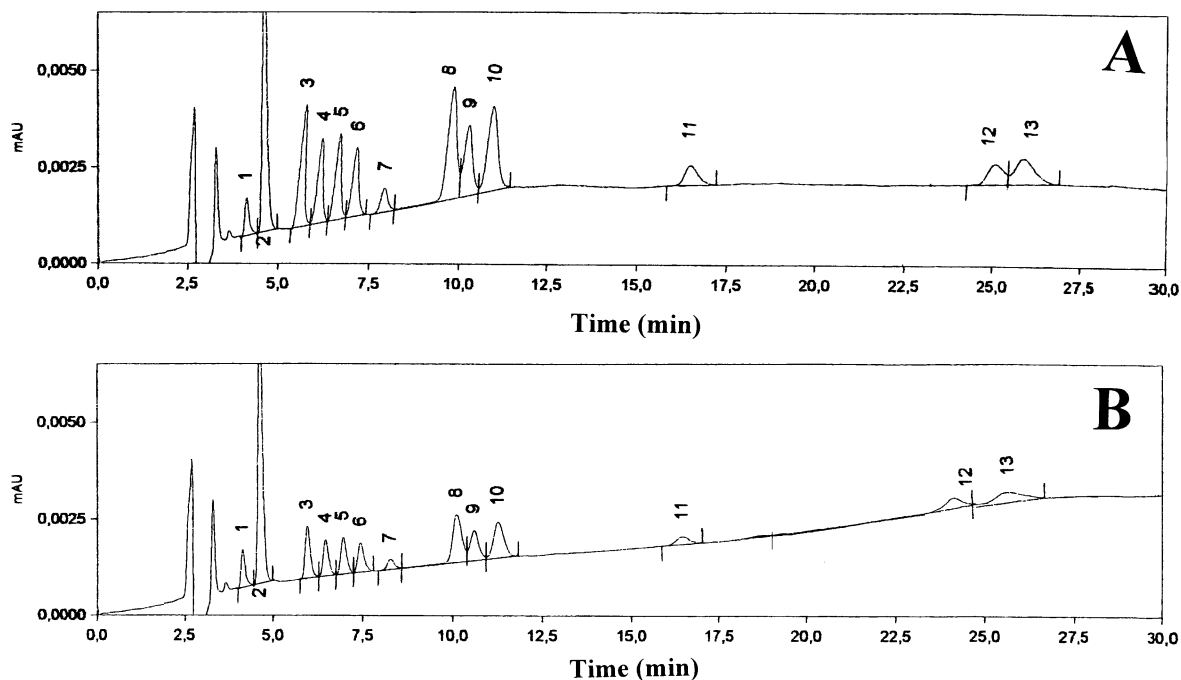

Figure 3. Chromatogram of cholesterol and cholesterol oxide standards (**A**) and the codfish sample (**B**) determined using the RI detector.

linear in the range of 0.5−70 μg/mL for cholesterol oxides and 0.1−2.0 mg/mL for cholesterol. Similar results were obtained with use of the CN column and UV detection (*15*) and with APCI-MS (*18*).

The detection limits (DL) of the method varied according to the detector used. In the UV detector the DL for cholesterol and for cholesterol oxides varied from 6 to 70 ng/mL; in the RI detector the DL for cholesterol and for cholesterol oxides varied from 10 to 180 ng/mL; and in the APCI-MS detector the DL for cholesterol and for cholesterol oxides varied from 1 to 5 ng/mL, respectively. As expected, analysis with the APCI-MS detector was more sensitive than with the UV and RI detectors, which showed comparable DL. However, good DL

for the RI detector was obtained. In contrast, UV detection of COPs has been found to be 1000 times more sensitive than RI detection (*13*). The LD obtained for the APCI-MS detector was lower than those found by Raith et al. (*19*) (15−30 ng/mL) and higher than those determined by Razzazi-Fazeli et al. (*18*) (0.1−0.75 ng/mL).

The differences in the results obtained for the different COPs and cholesterol were not significant ($p > 0.05$) between the three detectors used (**Table 2**). The intraassay precision, expressed as a relative standard deviation (RSD%), varied between 0.03 and 3.2% for cholesterol and the cholesterol oxides, which demonstrates the good precision of the methodology and excellent response for all detectors.
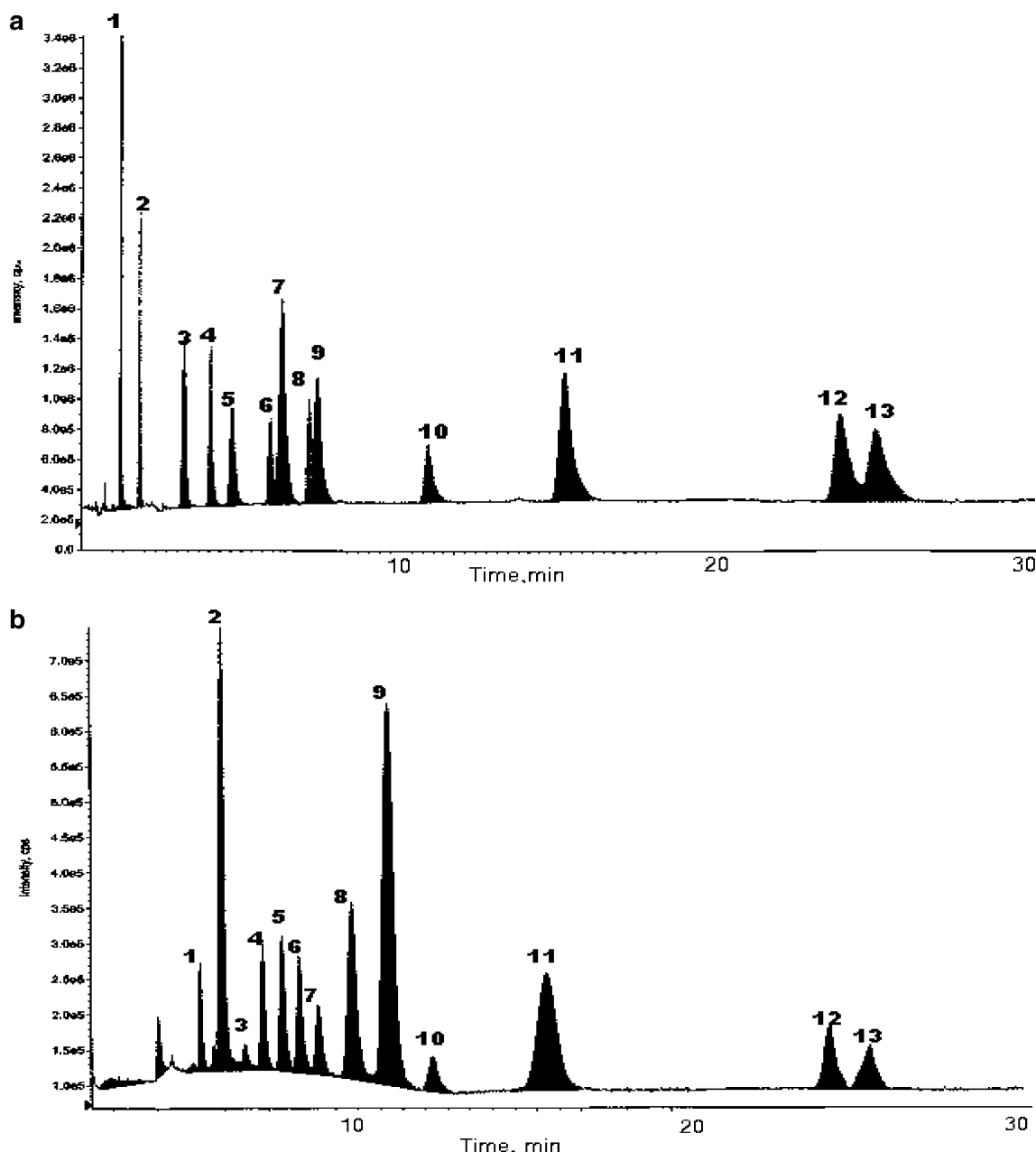
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Figure 4. Chromatogram of cholesterol and cholesterol oxide standards (**A**) and the codfish sample (**B**) determined using HPLC-APCI-MS.

One of the objectives of the present study was to compare the performance of different detectors for the quantification of cholesterol oxides and cholesterol. Because no significant differences ($p > 0.05$) were found in the concentrations of the evaluated products among the detectors studied, the recovery of the cholesterol and COPs was determined only using the UV and RI detectors (**Table 3**).

Good results were found for both moderately polar (epoxides) and polar oxysterol, with recoveries between 95 and 104%. Compared with the results reported by Baggio et al. (*14*) in smoked processed meat products, using HPLC-DAD-RI, a higher recovery for oxides **9** and **11** was obtained. Using HPLC-UV lower values for **11** (88%), **12** (85%), **13** (87%), and **7** (93%) were determined in pig muscle (*28*).

Application of the Method in Fish Samples. The level of individual cholesterol and cholesterol oxides in the fish samples assessed by HPLC-UV-RI are reported in **Table 4**.

In cod samples, all the cholesterol oxides separated by the method employed were detected, while for hake and sardine

only six COPs were found. This is probably due to differences in the type of sample, since hake and sardine were raw and fresh while the cod was salted and dried. The cholesterol oxides most commonly reported in foods are those derived from the B-ring of the main cholesterol chain, such as **8**, **9**, **11**, **12**, and **13**, and, in smaller concentrations, derivatives of the side chain, such as, for example, oxides **3** and **7** (*29*). In this study, some oxides not normally found in food matrixes were determined, such as **1**, **5**, and **10**, in addition to the stereoisomers **4** and **6**. In fact, the origin of these compounds should be enzymatic, whereas the COPs having the oxygenated group on the ring are generated by chemical oxidation (auto, photo, and thermo). 19-Hydroxycholesterol is generally not present in food, and this is the reason it is usually employed as an internal standard. The presence of these compounds in fish has to be explained, and it is probably due to the fish metabolism. Several papers have been published on COPs in fish, but the only compound oxidized on the lateral chain reported was 25-hydroxycholesterol (*20–24, 29–32*). However, side chain oxidation products other than

Table 1. Relative Intensity of the Main Ions of Cholesterol and Cholesterol Oxides in HPLC-APCI-MS

			relative intensity (*m/z*)				
peak no.[a]	molecular mass	[M − H$_2$O][b]	367	369	385	401	403
1	402.65	384.65	50	10	100		
2	386.65	350.65	5	100	5		
3	402.65	384.65	100	10	95		
4	402.65	384.65	100	10	100		
5	402.65	384.65	100	10	100		
6	402.65	384.65	85	10	100		
7	402.65	384.65	100	10	30		
8	402.65	384.65	50	10	100		20
9	402.65	384.65	25	10	100		10
10	402.65	384.65			100		
11	400.64	364.64				100	10
12	402.65	384.65	100	10	30		
13	402.65	384.65	100	10	30		

[a] Peak numbered according to elution order. [b] Loss of one or two molecules of water.

Table 2. Cholesterol and COP Contents in Pacific Codfish Samples Quantified by HPLC-UV-RI and HPLC-APCI-MS

	UV		RI		APCI-MS	
peak no.[a]	mean[b] (µg/g)	RSD (%)	mean[b] (µg/g)	RSD (%)	mean[b] (µg/g)	RSD (%)
1	3.45	0.86	3.36	1.19	3.44	1.45
2	6917	0.05	6981	0.06	6857	0.03
3	0.76	1.31	0.72	1.38	0.72	1.38
4	1.19	1.68	1.15	1.73	1.20	2.50
5	0.31	3.22	0.32	3.10	0.33	3.00
6	0.49	2.00	0.50	2.00	0.51	1.96
7	8.96	1.22	8.84	1.35	8.90	0.89
8			2.00	0.90	2.18	0.45
9			3.79	1.80	3.66	1.93
10	1.03	0.97	1.12	1.78	1.05	0.95
11	6.30	2.53	6.40	2.50	6.48	1.54
12	3.50	0.85	3.42	2.30	3.37	0.89
13	5.30	0.37	4.95	2.00	5.34	1.49

[a] Peak numbered according to elution order. [b] Values are means of 10 pieces of the same raw codfish sample (dry basis), and there are no significant differences (*p* > 0.05) between detectors.

25-hydroxycholesterol could be included in the seafood products because many unidentified peaks normally appear on the gas chromatograms (*29*).

In the evaluated samples, the amounts of some oxysterol derivatives of the side chain were high in comparison with those derived from the B-ring. Similar values for **7** and higher values for **8**, **9**, **11**, and **12** than in the present results were obtained by Shozen et al. (*22*) in fish. Only four cholesterol oxides were determined in cod pastes (*32*), and the levels for 7α-OH, 7β-OH, and 7-keto were much higher than the ones formed in the present work. According to the authors, the technological conditions applied during the elaboration of the pastes, especially the heating intensity, would explain these lower values. Cholesterol oxides were not detected in fresh sardine, only in the dried product (*24*), and their results were similar to the ones formed in the present work.

In summary, a fast, simple and sensitive method for simultaneous determination of cholesterol and 12 cholesterol oxides products by direct saponification and the accurate quantification using normal-phase HPLC and UV−RI detection is presented. The main achievements of this work include the following: no significant differences (*p* > 0.05) in the quantification of all

Table 3. Recovery (%) of Cholesterol and Cholesterol Oxides in Pacific Codfish Samples[a]

		UV		RI	
peak no.[b]	spike (µg/g)	mean	RSD (%)	mean	RSD (%)
1	25	99	3.23	95	1.68
	50	98	2.00	98	1.86
2	50	102	2.83	98	0.81
	100	100	2.01	100	0.65
3	25	101	1.48	97	1.88
	50	98	1.32	97	1.70
4	10	97	2.12	95	1.18
	25	99	2.65	99	1.40
5	10	102	1.13	103	1.65
	25	101	1.47	99	1.30
6	25	99	0.86	95	1.05
	50	100	0.47	97	1.44
7	10	98	0.91	95	2.84
	25	100	0.85	98	2.33
8[c]	50			97	2.06
	75			100	1.45
9[c]	50			98	3.07
	75			98	2.93
10	50	100	1.35	98	3.30
	75	104	1.62	95	3.02
11	50	102	0.98	102	3.00
	75	103	0.34	99	2.69
12	10	99	0.39	96	2.54
	25	98	0.28	97	2.75
13	35	99	1.12	101	1.92
	50	97	2.25	99	2.30

[a] Means and relative standard deviation (RSD) of 10 pieces of the same raw codfish sample. [b] Peak numbered according to elution order. [c] Peaks 8 and 9 only detected by RI.

Table 4. Cholesterol and COP Contents in Northern Codfish, Hake, and Sardine Samples by HPLC-RI-UV[a]

	codfish		hake		sardine	
peak no.[b]	mean (µg/g)	RSD (%)	mean (µg/g)	RSD (%)	mean (µg/g)	RSD (%)
1	4.29	1.16	12.37	0.80	14.00	0.85
2	5857	0.10	3894	0.08	3420	2
3	1.50	2.60				
4	0.89	1.12	1.89	2.11		
5	5.14	1.50			1.39	1.40
6	10.65	1.59	2.30	1.30	1.26	0.80
7	3.27	2.14	5.33	1.68	0.30	0.10
8	3.34	0.89	4.14	2.41		
9	4.85	2.00	8.93	2.68		
10	1.58	2.53			1.20	0.35
11	5.23	2.67			1.20	0.50
12	12.61	1.42				
13	5.60	0.95				

[a] Mean and relative standard deviation (RSD) of the 10 analysis (dry basis). [b] Peak numbered according to elution order.

compounds using UV, RI, and APCI-MS detectors; unambiguous identification and confirmation of a large number of COPs by HPLC-APCI-MS; and, more importantly, the detection and structure elucidation of oxides **1**, **4**, **5**, **6**, and **10** not previously reported in fish.

NOTE ADDED AFTER ASAP PUBLICATION

The original posting of May 11, 2006, contained an incorrect version of Figure 1. Figure 1 has been corrected with the posting of May 19, 2006.

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