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Improved high-performance liquid chromatographic method for the separation and quantification of lipid classes: application to fish lipids

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Abstract

An improved straight-phase HPLC method for the separation and quantification of lipid classes is described. Two binary gradient solvent systems were used, one for polar and one for neutral lipids, and detection was performed with a light-scattering detector. The developed HPLC methods were highly reproducible and allowed base-line separation of all investigated polar lipid classes (phosphatidic acid, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, phosphatidylserine, phosphatidylinositol and lysophosphatidylcholine) and neutral lipid classes (triacylglycerol, free fatty acid, diacylglycerol, cholesterol and monoacylglycerol) except of cholesterol ester and wax ester. Application of the chromatographic systems demonstrated that the methods are suitable for quantitative analysis of the major lipid classes present in lipid extracts from livers and eggs of Atlantic salmon (*Salmo salar*). © 1997 Elsevier Science B.V.

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1. Introduction

Detailed investigations of lipid metabolism require accurate methods for the separation and quantification of lipid classes. One of the problems in lipid class analysis is the lack of a satisfactory detection method that enables the quantification of all the major lipid classes present in animals [1]. In recent years, the application of HPLC for lipid analysis has become important, and HPLC together with light-scattering detection can be a very useful technique for lipid class analysis. This technique, as well as the principles and properties of the light-scattering detector, has been thoroughly described [1–5]. Quantitative analysis with light-scattering detectors does not require a positive identification of the fatty acid composition of the analyte since, unlike UV detectors, the chain-length and degree of unsaturation of the fatty acid compound have little effect on the detector response [1,4–7].

Numerous applications of HPLC combined with light-scattering detection for the separation and quantification of lipid classes have been reported [2,3,5,7–19]. Some of these methods involve complex ternary gradient programs, or the authors have not presented separation of all the major lipid classes present in animal tissue. In addition, the vast majority of the earlier studies describe procedures for the separation and quantification of phospholipids [2,3,5,7–19] and far less information is available on

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the analysis of neutral lipid classes by HPLC and light-scattering detection [2,3,12,14,15].

The aim of the present study was to develop binary gradient solvent systems for HPLC that are able to separate and quantify the major classes present in lipid extracts from fish. Light-scattering detection was employed and the responses of various lipid classes and the reproducibility of the methods were evaluated.

2. Experimental

2.1. Chemicals

Phosphatidylcholine (PC, dipalmitoyl), phosphatidylethanolamine (PE, dipalmitoyl), lysophosphatidylcholine (LPC, 1-palmitoyl), sphingomyelin (SM, principally stearic and nervonic acids), triacylglycerol (TAG, triolein), diacylglycerol (DAG, 1,2-diolein), monoacylglycerol (MAG, 1-monoolein), cholesterol ester (CE, oleate), free fatty acid (FFA, oleic acid), cholesterol (C) and wax ester (WE, oleyl linoleate) were obtained from Larodan Fine Chemicals (Malmö, Sweden). Phosphatidic acid (PA, dipalmitoyl, free acid), diphosphatidylglycerol or cardiolipin (DPG, sodium salt from bovine heart), phosphatidylinositol (PI, sodium salt from soya bean) and phosphatidylserine (PS, from bovine brain) were obtained from Sigma (St. Louis, MO, USA).

Acetic acid, butylated hydroxytoluene (BHT), chloroform, 2′,7′-dichlorofluorescein, *n*-hexane, isopropanol, KCl, methanol, NaCl, thin-layer chromatography (TLC) plates (20×20 cm×0.25 mm, coated with silica gel 60, H) and triethylamine were obtained from Merck (Darmstadt, Germany). The hexane and isopropanol used for chromatography were of HPLC grade (LiChrosolv) and all other chemicals were of analytical grade. The water used was deionized and filtered through a millipore system (Milli-Q, UF Plus, Millipore, Bedford, MA, USA).

2.2. Animal samples

Atlantic female salmon (*Salmo salar*) with an average weight of 8 kg were caught in the river Lule älv in northern Sweden during July to September on their spawning migration from the Baltic Sea and kept in basins at a fish farm (Hedens laxodling, Boden, Sweden). Eggs were obtained in November by stripping ovulated females. After stripping, each female was killed by a blow on the head and the liver was dissected. Egg and liver samples were rapidly frozen in liquid nitrogen, transported to the laboratory and stored at −80°C until lipid extraction.

2.3. Lipid extraction

Total lipid of livers and eggs were extracted by the method of Bligh and Dyer [20] as modified by Silversand and Haux [21]. All samples were extracted and analysed in duplicate and all solvents used for lipid extraction contained 50 mg/l BHT as an antioxidant.

2.4. HPLC system

Lipid separation was accomplished by straight-phase HPLC, using two solvent delivery pumps (Kontron Instruments, model 322, Milan, Italy). The original solvent mixer of model 322 was bypassed and an external solvent mixer (high pressure mixer, M 800, Kontron Instruments) with a mixing volume of 1.5 ml was connected between the pumps and the column. The original high pressure solvent mixer of model 322 has a mixing volume of 20 μl which was too small for the applications in the present study. A 20-μl volume of sample was injected into the column using a 20-μl sample loop. The chromatographic column was a LiChrosphere 100 Diol (5 μm), 250×4 mm (Merck). The flow-rate was 1.0 ml/min and the column temperature was kept at 45°C during all runs. A light-scattering detector was used for the detection (Sedere, Sedex 45, Vitry-sur-Seine, France) and the gas connected was compressed air. The detector temperature was 45°C and the air pressure was 2.0 bar. The photomultiplier sensitivity was adjusted to the mean value of the total photomultiplier range (gain 7). The detector signal was recorded and integrated by a personal computer and a software program (GynkoSoft Chromatography Data System, version 4.22, Gynkotek, Munich, Germany).

2.5. Chromatographic conditions

The polar and neutral lipid classes were separated by two different solvent mixtures and gradient systems.

2.5.1. Polar lipids

The two solvents in the binary gradient used for the separation of polar lipid classes were as follows: (A) hexane–isopropanol–acetic acid (82:17:1.0, v/v/v); (B) isopropanol–water–acetic acid (85:14:1.0, v/v/v). Triethylamine (0.08%, v/v) was added to the solvents. The samples were injected at time 0 and the gradient profile started at 5% for solvent mixture B and was increased to 40% B in 23 min. The gradient then continued from 40 to 100% B in 5 min, after which it was kept at 100% B for 1 min. Finally, the gradient was reduced from 100 to 5% B in 10 min and then maintained at 5% B for 5 min. In total, the solvent program for the separation of polar lipids took 44 min. All samples analysed for polar lipid class composition were dissolved in hexane–isopropanol–acetic acid (50:50:1.0, v/v/v) plus triethylamine (0.08%, v/v) prior to chromatography. However, the lipids were kept dissolved in chloroform or chloroform–methanol (1:1, v/v) while stored and just before HPLC analysis evaporated to dryness and dissolved in hexane–isopropanol–acetic acid (50:50:1.0, v/v/v) containing triethylamine (0.08%, v/v). This handling procedure was required because it was observed that the polar lipids did not stay in solution for more than 24 h in hexane–isopropanol–acetic acid (50:50:1.0, v/v/v). The total lipid extracts were used for analysis of polar lipid classes without further purification.

2.5.2. Neutral lipids

The two solvents in the binary gradient for neutral lipids were as follows: (A) hexane–acetic acid (99:1.0, v/v); (B) hexane–isopropanol–acetic acid (84:15:1.0, v/v/v). The samples were injected at time 0 and the gradient profile started at 0% for solvent mixture B and was changed to 100% B in 25 min. The gradient was kept at 100% B for 1 min and was then reduced to 0% B in 3 min and kept at 0% B for another 2 min. The solvent program for the separation of neutral lipid classes took 31 min in total. All samples analysed for neutral lipid class composition were dissolved in hexane–acetic acid (99:1.0, v/v) prior to chromatography. Neutral lipids were isolated from polar lipids before analysis. This was achieved by HPLC and the solvent program described above for polar lipids. The column was disconnected between the outlet of the column and the detector, and the eluted solvent collected between 1 and 4.5 min. The collected fraction, containing the neutral lipids, was evaporated at 40°C for 30 min and the neutral lipids were redissolved in hexane–acetic acid (99:1.0, v/v) and analysed.

2.6. Identification and quantification

Pure lipid standards were chromatographed individually to confirm retention times and purity. Lipid standards were also co-eluted together with samples in order to identify peaks in unknown samples. Furthermore, different lipid class fractions obtained by HPLC were collected and analysed by TLC to establish their purity. Lipid classes were isolated by TLC using a solvent system of hexane–diethyl ether–acetic acid (80:20:2.0, v/v/v) for neutral lipids and chloroform–methanol–acetic acid–water (25:15:4.0:2.0, v/v/v/v) for polar lipids. Lipids on TLC were stained with 2′,7′-dichlorofluorescein and visualised under ultraviolet light.

Solutions of known concentrations of different lipid classes were mixed and lipid standard curves were generated to study the linearity of the detection method and to quantify lipid classes in unknown samples. Standard lipids (15–25 μg) were dried at 40°C under a stream of N_2 for 16 h and the amount was determined gravimetrically. Calibration curves were prepared for 2.0–20 μg of PA, DPG, PE, PC, SM, PS, PI and LPC. Calibration curves were also prepared for 0.8–5.0 μg of TAG, FFA, C, DAG, MAG and either CE or WE. CE and WE were chromatographed separately because they co-eluted. Each concentration of the standard solutions was injected twice and the average peak area for each lipid was plotted against the absolute amount of lipid. Correlation (r^2) was determined for all curves by linear regression analysis.

3. Results

A typical chromatogram of standard polar lipid classes is shown in Fig. 1. All lipids eluted within

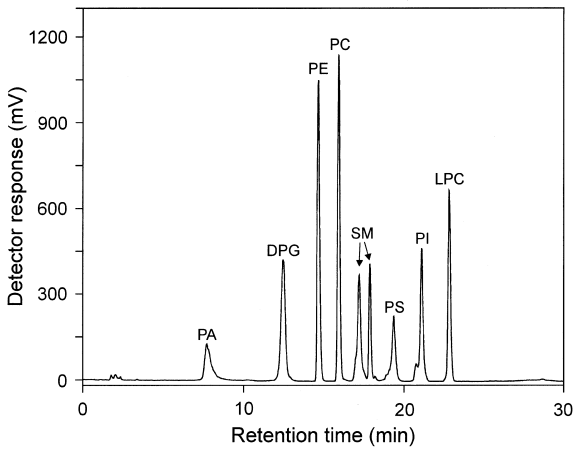

Fig. 1. HPLC of standard polar lipid classes. The chromatographed sample contained 20±2 μg each of phosphatidic acid (PA), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylserine (PS), phosphatidylinositol (PI) and lysophosphatidylcholine (LPC). Details of elution conditions are described in Section 2.5.

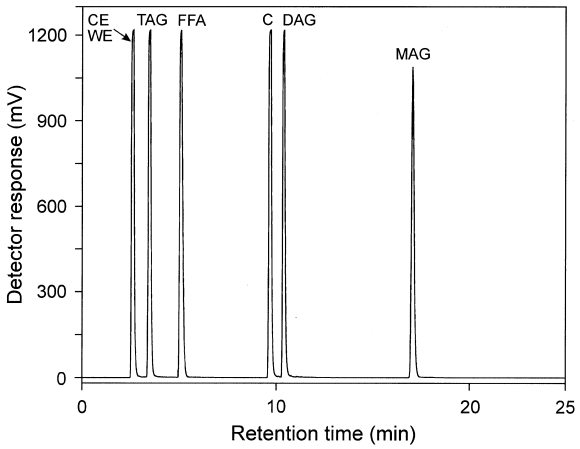

Fig. 2. HPLC of standard neutral lipid classes. The chromatographed sample contained 4.0 μg each of cholesterol ester (CE) and wax ester (WE) and μg each of triacylglycerol (TAG), free fatty acid (FFA), cholesterol (C), diacylglycerol (DAG) and monoacylglycerol (MAG). Details of elution conditions are described in Section 2.5.

the first 25 min and the remaining solvent program was needed for the equilibration of the column. All polar lipid classes were baseline separated and nearly all eluted as single peaks, except SM and PI. The peak appearance and the elution position upon HPLC were highly reproducible for most polar lipid classes, although PA and DPG exhibited some variation in the elution position and detector response. Triethylamine was added to the solvents for HPLC of polar lipids in order to improve peak shape and resolution. Furthermore, triethylamine also improved the reconditioning of the column and made sure that no lipids remained adsorbed in the column after each analysis.

Fig. 2 shows the HPLC elution profile of a mixture of standard neutral lipid classes. All standard neutral lipid classes emerged as single peaks which were baseline separated from each other, except for the CE and WE, which had identical retention times. Only the elution profile for the first 25 min is shown because no compounds eluted later. As for polar lipids, the peak appearances and the retention times for neutral lipid classes were highly reproducible.

The calibration curves for polar lipid classes are shown in Fig. 3A and B. Approximately linear relationships between peak areas and absolute amounts of lipids were found for all polar lipid classes in the range of 2.0–20 μg. The r^2 for a linear curve fitting varied between 0.98 and 1.00 except for PA for which r^2 was 0.92. The SM content was calculated from the sum of the two peaks. At sample loads above 20 μg of polar lipids the peaks reached the upper limit for detection, while at loads below 4.0 μg the response declined rapidly. In order to investigate the reproducibility of the calibration curves, a dilution series containing PE, PC and PI was prepared and five calibration curves for each lipid class were generated. The average and standard deviation of the five calibration curves for each lipid class is shown in Fig. 3C.

Fig. 3D and E shows the calibration curves for neutral lipids. The calibration curves were approximately linear for all neutral lipids in the range 0.8–5.0 μg with r^2 of 0.98–0.99. Neutral lipids exhibited greater detector responses than polar lipids. The concentration range for the quantification of neutral lipid classes was small due to the sharp peak form of neutral lipids. At neutral lipid amounts above 3.8–5.0 μg, depending on class, the peak reached the upper limit of the detector and for amounts below 1.0 μg the response decreased. The reproducibility of the calibration curves for neutral lipids was evaluated by preparing a dilution series

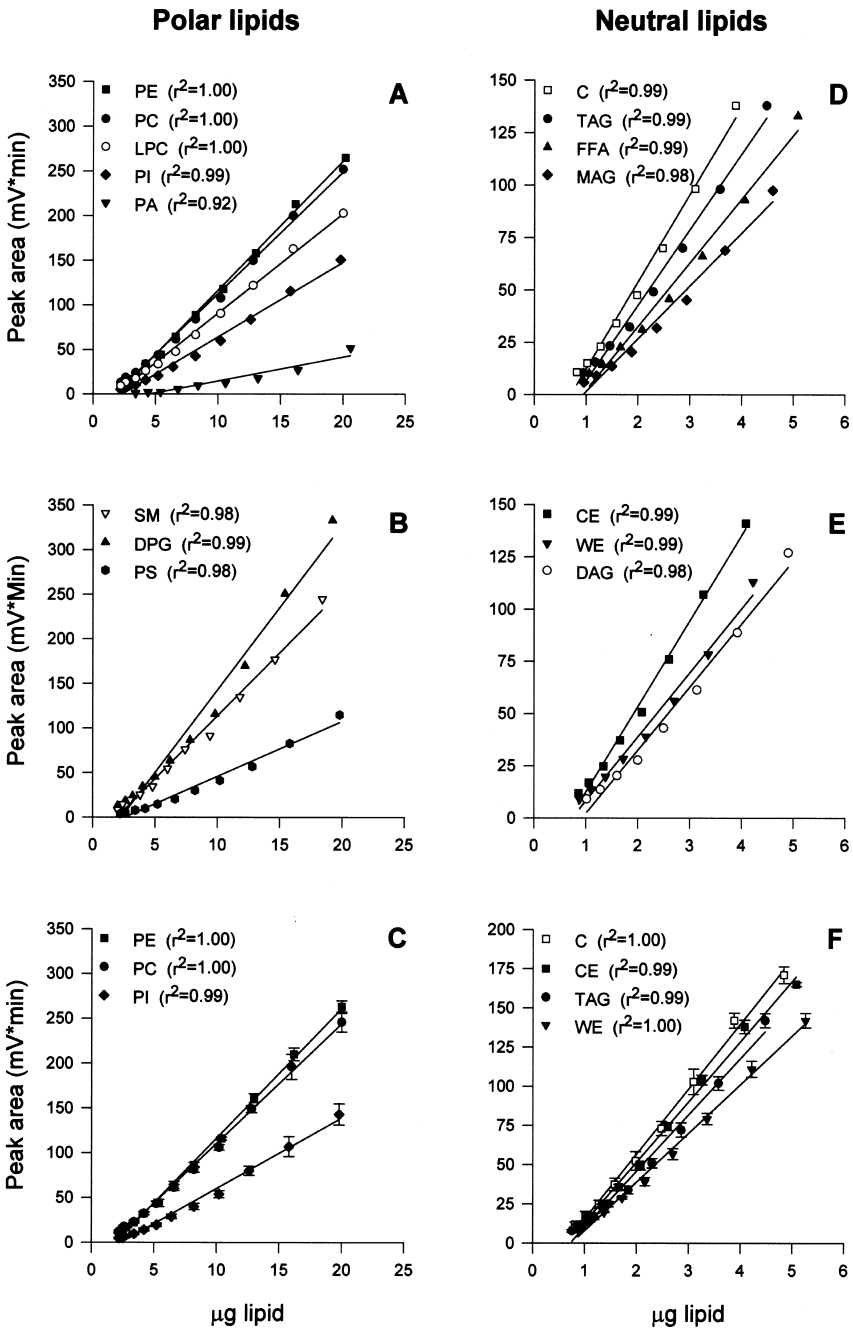

Fig. 3. Calibration curves for lipid classes analysed by HPLC and light-scattering detection. The peak area for each lipid class is plotted against absolute injected amount and the correlation was determined by linear regression analysis. (A,B) Calibration curves for polar lipid classes. Each point represents the average of two analyses. (C) The average of five calibration curves for PE, PC and PI. Error bars indicate the standard deviation of the mean. (D,E) Calibration curves for neutral lipid classes. Each point represents the average of two analysis. (F) The average of five calibration curves for C, CE, TAG and WE. Error bars indicate standard deviation of the mean. DPG, diphosphatidylglycerol; LPC, lysophosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; C, cholesterol; CE, cholesterol ester; DAG, diacylglycerol; FFA, free fatty acid; MAG, monoacylglycerol; TAG, triacylglycerol; WE, wax ester.

containing C, CE, TAG and WE and generating five calibration curves for each lipid class. Fig. 3F shows the average and standard deviation of five calibrations for each lipid class.

The separation obtained when lipids from liver and eggs of Atlantic salmon were subjected to the HPLC procedures are shown in Figs. 4 and 5. All lipid classes present in liver and eggs were well separated with minor molecular species separation. Chromatography of polar lipids revealed that liver and eggs contained PE, PC, SM and PI (Fig. 4A and B). The liver also contained minor amounts of DPG and PS (Fig. 4A). Fig. 5 shows the elution profiles when neutral lipids from liver and eggs were subjected to HPLC. Prior to analysis of neutral lipids, these were isolated from polar lipids by HPLC as described. The recovery of neutral lipids was high (>98%) when isolated with the solvent program used for polar lipids. This was evaluated by injections of standard neutral lipids of known concentrations. Between 300 and 500 μg of total lipid was applied onto the column for the isolation of the neutral lipids from the polar lipids. HPLC of neutral lipids from Atlantic salmon livers showed the pres-

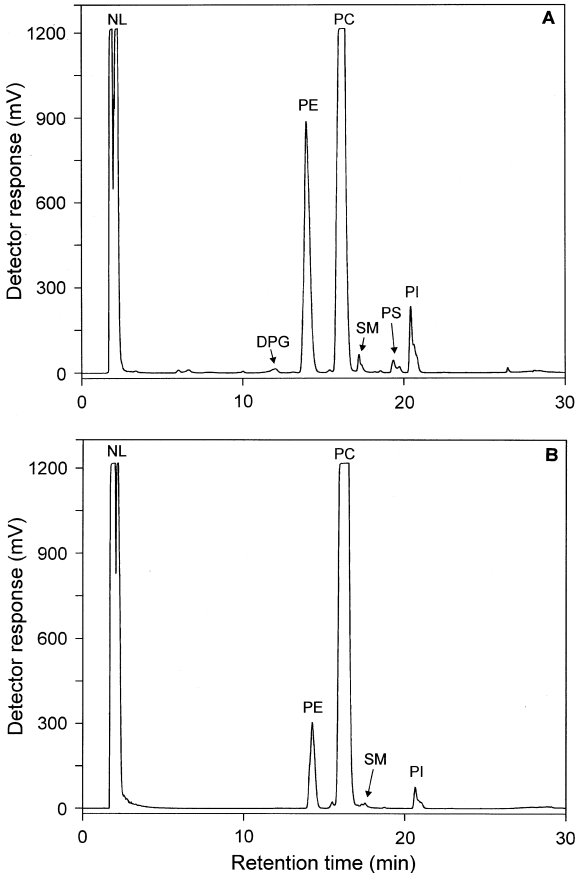

Fig. 4. HPLC of polar lipid classes extracted from liver and eggs of Atlantic salmon. (A) Shows the elution profile for 200 μg of total lipid extracted from liver and (B) represents 150 μg of total lipid from eggs. NL, neutral lipids; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol.

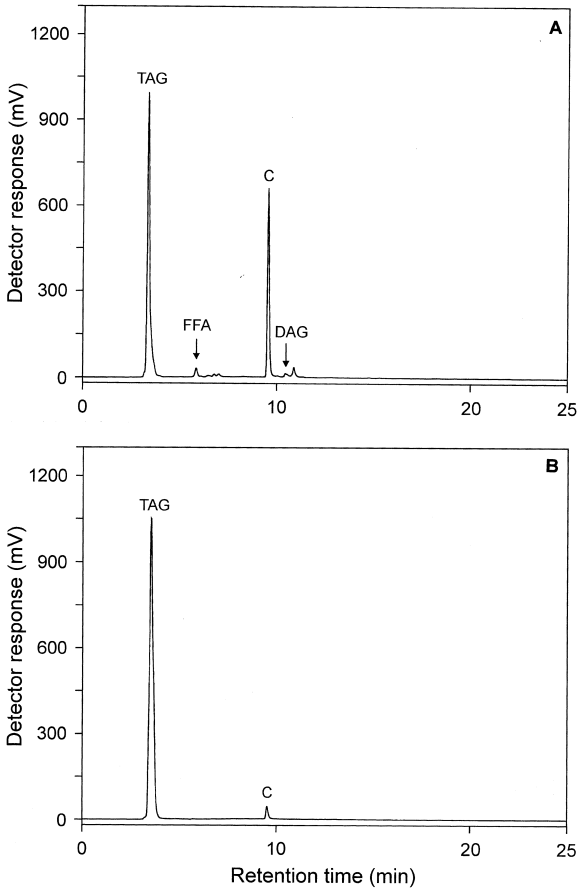

Fig. 5. HPLC of neutral lipid classes extracted from liver and eggs of Atlantic salmon. (A) Shows the elution profile for 20 μg of neutral lipids from livers and (B) represents 10 μg of neutral lipids from eggs. TAG, triacylglycerols; FFA, free fatty acids; C, cholesterol; DAG, diacylglycerols.

ence of TAG, FFA, C and DAG (Fig. 5A). Neutral lipids of salmon eggs consisted of TAG and small amounts of C (Fig. 5B). All lipid class peaks, both of polar and neutral lipid classes, were pure as measured by TLC.

The peak areas for the lipid classes in livers and eggs were converted into absolute amounts using the linear regression analysis of the calibration curves generated from standard lipids. The percentage lipid composition of livers and eggs of Atlantic salmon is shown in Table 1. The most abundant polar lipid class was PC followed by PE. TAG was the major neutral lipid in both liver and eggs. Each sample was analysed twice and the average peak area was used for correlation. Lipid classes present at various levels in the samples were determined by injecting the lipid extracts at different concentrations. For quantitative analysis of polar lipids from liver and eggs, 50–500 μg of total lipid was sufficient to obtain accurate analysis of the major polar lipid classes. For reliable analysis of liver and egg neutral lipids, 5–50 μg of total lipid was needed.

Table 1
Lipid class composition of liver and eggs from Atlantic salmon

	Liver	Eggs
Total polar lipid classes (%)	81±5.0	51±4.6
Total neutral lipid classes (%)	19±5.0	49±4.6
Polar lipid classes (% of total lipid)		
Phosphatidic acid (PA)	–	–
Phosphatidylcholine (PC)	47±5.0	44±4.4
Phosphatidylethanolamine (PE)	16±2.0	4.3±0.7
Phosphatidylinositol (PI)	11±0.6	2.6±0.2
Phosphatidylserine (PS)	4.5±0.6	–
Lysophosphatidylcholine (LPC)	–	–
Diphosphatidylglycerol (DPG)	–	–
Sphingomyelin (SM)	2.6±0.4	–
Neutral lipid classes (% of total lipid)		
Cholesterol (C)	8.8±2.0	5.2±0.7
Cholesterol-wax ester (CE-WE	–	–
Free fatty acid (FFA)	–	–
Triacylglycerol (TAG)	11±4.1	44±4.8
Diacylglycerol (DAG)	–	–
Monoacylglycerol (MAG)	–	–

Lipid class compositions are expressed as percentage by wt. of total detected lipid classes and values represents means±S.D. of six fish.
–=Not present or in minor amounts (<1%).

4. Discussion

This paper describes modified HPLC procedures for the separation and quantification of lipid classes using two binary-solvent systems, one for polar and one for neutral lipids. The procedures yield high resolution between most lipid classes in a relatively short processing time, exhibit a linear dose–response curve within certain concentration intervals and are highly reproducible. Analysis of lipids from Atlantic salmon liver and eggs reveals that the method is suitable for natural lipid extracts. This study demonstrates that HPLC with light-scattering detection is an attractive alternative to TLC methods for the analysis of fish lipids.

The solvent system used for polar lipids in the present study was originally designed to separate plant phospholipids on a diol column [11]. In order to apply the chromatographic method for the analysis of fish lipids, and since several lipid classes were poorly resolved in the previous study, the method was further developed. Taken together, the HPLC procedures used in the present study (both for neutral and polar lipids) show better lipid class resolution than earlier studies, and separation of more lipid classes was also achieved. The resolution of 15 lipid classes was investigated and all of the classes showed baseline separation except CE and WE. The peaks of both polar and neutral lipid classes were relatively homogenous with little molecular species separation except for SM. Several other authors have described a double peak elution of SM, presumably because of partial separation of molecular species [2,3,7,10,12,18,19].

The peak area increased linearly with increasing sample concentrations for all lipid classes in the present study. However, the linearity was limited to certain concentration intervals. Approximately linear response curves for light-scattering detectors during lipid class analysis have been reported by others [2,7,9,17]. The finding that the detector response is different for various lipid classes means that an accurate quantitative analysis of lipids requires individual response factors for each lipid class to be studied. However, the fact that the detector response for compounds belonging to the same lipid class varies very little with the acyl group of the molecule [1,4–7], makes it possible to quantify lipid classes

directly in unknown samples by the use of standard calibration curves.

To quantify the lipid classes present in a sample, it may either be concentrated or diluted to obtain concentrations giving peak areas within the detection interval. Two or three different concentrations of each sample have to be injected in order to quantify all classes present which is a limitation with the method. Approximately 10 μg of each polar lipid class and 2 μg of each neutral lipid class are more than sufficient for analysis. A reservation for PA has to be made due to the much lower relative response of this lipid class. Alternatively, lipids present at different concentrations can also be quantified by changing the sensitivity of the detector. However, in that case, new calibration curves have to be generated for each detector sensitivity. The sensitivity of the detector was kept constant (gain 7) during the whole study, but if necessary the sensitivity can be increased substantially.

Polar lipid classes were the major constituents of lipids from the liver of Atlantic salmon, whereas eggs of the same species contained approximately equal amounts of polar and neutral lipids. Livers from Atlantic salmon in the present study contained considerably more polar lipids (80% wt of total lipid) than were found in an earlier study on farmed Atlantic salmon (54% wt) [22]. This difference is probably explained by the dietary variations of the fish. In teleost fish, the liver is the main organ for lipid metabolism and its lipid content and composition therefore depends on the diet [23]. The observed lipid class composition of Atlantic salmon eggs was similar to that analysed previously by TLC and flame ionisation detection (Iatroscan TH 10, Iatron Laboratories, Japan) [24].

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