ELSEVIER

Journal of Chromatography B, 770 (2002) 71–75

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Separation of phospholipid classes in sea urchin, *Paracentrotus lividus* by high-performance liquid chromatography

A. Rodríguez-Bernaldo de Quiros, J. López-Hernández*, J. Simal-Lozano

Nutrition Bromatology Group, Analytical Chemistry, Nutrition and Bromatology Department, Pharmacy Faculty, University of Santiago de Compostela, E-15782 Santiago de Compostela, La Coruña, Spain

Abstract

A simple high-performance liquid chromatography (HPLC) method for determination of major phospholipid classes in sea urchin *Paracentrotus lividus* is described. The separation was performed on a Tracer Extrasil SI 5 μm 25×0.4 cm column and an isocratic mobile phase of acetonitrile–methanol 85%–phosphoric acid (50:50:1.8, v/v). The HPLC method utilizes UV detection at 205 nm. Five phospholipids were identified and quantified: phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyelin (SM). Fresh and canned samples were analyzed. Student's *t*-test showed no significant difference ($P \leq 0.05$) between the mean phospholipid contents of raw and canned sea urchin.

Keywords: *Paracentrotus lividus*; Phospholipids

1. Introduction

Sea urchins are marine invertebrates of the phylum Echinodermata. *Paracentrotus lividus* is an edible species with a globose shell (the test) covered with numerous sharp spines [1].

More than 750 species of sea urchins have been identified, of which *Paracentrotus lividus* is the most appreciated sea urchin. The edible portions are five gonads and constitute only 10% by weight [2].

Lipids are important constituents of food, whether they are added or endogenous to the components. They can have large effects on structure, texture and flavour, and they impart nutritional qualities [3].

Fish lipids have received much attention owing to their positive effects on cardiovascular diseases [4].

Lipids occur in the fish tissue in a wide variety of physical forms. Neutral lipids or triglycerides are parts of large aggregates in storage tissues and are extracted with relative ease. In contrast phospholipids are more difficult to extract since they are constituents of membranes, where they occur in close association with such compounds as proteins and polysaccharides [5].

Traditional techniques for separation of lipid classes included preparative thin-layer chromatography (TLC), solvent partitioning and high-performance liquid chromatography (HPLC). The HPLC detection of phospholipids is accomplished commonly by UV, refractive index or flame ionization detection. The last two detection methods lack sensitivity; UV detection has higher sensitivity but absorption by phospholipids in the range 200–210 nm make it difficult to use common chromatographic solvents, which are not transparent in this region.

Recently, numerous applications of HPLC combined with light-scattering detection for the sepa-

*Corresponding author.

E-mail address: qnlhjul@usc.es (J. López-Hernández).

1570-0232/02/$ – see front matter
PII: S1570-0232(01)00628-6

ration and quantification of lipid classes have been reported [6,7]. Some of these methods involve complex ternary gradient programs.

And more recently solid-phase extraction on either silica gel or bonded phase columns [8–10] has been explored for separation of lipid classes.

The aim of the present study was to develop an isocratic system for HPLC that is able to separate and quantify the major phospholipid classes present in sea urchin.

2. Experimental

2.1. Chemicals

L-α-Phosphatidylinositol (PI) from soy bean (P-0639), DL-α-phosphatidylethanolamine (dipalmitoyl) (PE) (P-3275), L-α-phosphatidylcholine (PC) from egg yolk (P-7318), sphingomyelin (SM) from bovine brain (S-7004) and L-α-phosphatidyl-L-serine from bovine brain (P-7769) all were obtained from Sigma (Madrid, Spain).

Chloroform, methanol, *n*-hexane, 2-propanol, acetonitrile and *ortho*-phosphoric acid 85% were obtained from Merck (Darmstadt, Germany).

Water used for all solutions was obtained from a Milli-Q water purification system (Millipore).

2.2. Equipment

Spectra Physics liquid chromatograph equipped with an SP8800 ternary pump (San Jose, CA, USA), a 20-μl Rheodyne injection loop (Cotati, CA, USA), and a UV–Vis forward optical scanning detector controlled with PC-1000 software.

2.3. Animal samples

Sea urchin (*Paracentrotus lividus*) were caught of the Atlantic coastal region in Galicia (NW Spain). Fresh and canned samples were obtained from a local canning factory (‘Conservas y Ahumados Lou’ Ribeira La Coruña, Spain).

The canning process involves heating to 112 °C for 50 min in an autoclave. Fresh samples were transported to the laboratory on ice. At the laboratory all samples were analyzed immediately.

2.4. Sample preparation

Lipids of sea urchin gonads were extracted by the method of Bligh and Dyer [11]. The extracts were evaporated to dryness under N_2 and the residue was redissolved in 1 ml of *n*-hexane–2-propanol (3:1, v/v), membrane-filtered (Millipore, 0.22 μm) to remove insoluble particles, then injected into the chromatograph.

All samples were analysed in duplicate.

2.5. Chromatographic conditions

The separation was achieved on a Tracer Extrasil SI 5 μm 25×0.4 cm TR 416069 N20 column (Teknokroma, Barcelona, Spain).

The mobile phase was acetonitrile–methanol: 85%–phosphoric acid (50:50:1.8, v/v). It was delivered to the column at a flow-rate of 1.5 ml/min, the column was thermostated at 28 °C.

The working wavelength was 205 nm.

3. Results and discussion

First, to obtain a better resolution we assayed different gradient program such as A=hexane/THF, B=iso-propanol, C=water [3]; and A=isoctane–tetrahidrofuran (99:1, v/v), B=acetone–methylenechloride (2:1, v/v), C=2-propanol–water (7.5 m*M* acetic acid, 7.5 m*M* ethanolamine) (85:15, v/v) [12]. But we did not obtain a good resolution and the time analysis was too long (40 or 44 min). We employ an isocratic method.

The chromatography procedure is a modification of the method of Ref. [13].

In developing the method, several changes in the proportions of the mobile phase components were assayed (100:10:1.8, v/v; 60:40:1.8, v/v; 50:50:1.8, v/v; 50:50:2.5, v/v) (acetonitrile–methanol 85%–phosphoric acid) and several flow-rates were tried (1, 1.5, 1.7 ml/min).

The column temperature was varied between 25 and 30 °C.

When the amount of phosphoric acid in the mobile phase was increased the analysis time is reduced but the resolution was not optimal.

We found that better resolution was obtained with

50:50:1.8 (v/v) acetonitrile–methanol–phosphoric acid; a flow-rate of 1.5 ml/min and a column temperature of 28 °C.

Identification of the different compounds was made by comparison of their retention times (t_R) and UV absorbance spectra with those of pure standards of known concentrations.

Five phospholipids were identified and quantified: PI (t_R=3.34), PS (t_R=3.52), PE (t_R=3.77), PC (t_R=4.20) and SM (t_R=4.97).

In order to choose the best wavelength, UV scanning was carried out between 200 and 210 nm, using phospholipids standards. The highest absorbance was found to be 205 nm [9]. For this reason 205 nm was selected as the working wavelength.

The method was calibrated using a series of phospholipid standards of known concentration. Calibration lines are given in Table 1.

Fig. 1 shows the chromatogram of a sea urchin sample (*Paracentrotus lividus*) sample.

The limit of detection of each component was calculated in accordance with ACS guidelines [14]. The results are given in Table 2.

Precision of measurement, was estimated as RSD% for eight determinations of a single aliquot of extract. The results are presented in Table 2.

Repeatability, was estimated as RSD% for determinations of eight extracts (each prepared separately from the same homogenized sample). The results are shown in Table 2.

Recoveries, estimated on the basis of determinations after spiking samples with known amounts of standards were 92.15% for PI, 93.82% for PS, 93.44% for PE, 103.13% for PC and 95.47% for SM.

Table 3 shows the mean concentrations of phospholipid in fresh and canned *Paracentrotus lividus* gonads.

The predominant phospholipids in both types of samples were PE and PC.

The *t*-test comparing the mean phospholipid contents of raw gonads and gonads sterilized at 112 °C for 50 min indicated that differences were not significant (P=0.05).

The total lipids were 11.12±1.02 (mean±SD) g per100 g of dry weight of canned samples and 12.71±3.16 g per 100 g of dry weight of fresh samples. Use of the *t*-test to compare the mean total lipid contents of raw and canned sea urchin gonads indicated that differences were not significant ($P \leq$ 0.05).

The phospholipid, PE and PC were the most predominant as reported previously for *Hemicentrotus pulcherrimus* [15].

Neutral lipid classes were the major constituents of lipids from the gonads of sea urchin *Paracentrotus lividus* [16].

Comparison of phospholipids content of sea urchin and other aquatic organisms reported in the literature showed that the phospholipids content of sea urchin was lower than in catfish *Ictalurus punctatus* [17].

Acknowledgements

This work was financed under project no. XUGA 20301B98 from the Xunta de Galicia. The authors thank D. Manuel Loureiro (director of Conservas y Ahumados LOU, Ribeira, La Coruña) for supplying the sea urchins.

Table 1
Parameters of calibration lines ($y = a + bx$) for the compounds studied

Phospholipids	*a*	*b*	Correlation coefficient
L-α-Phosphatidylinositol	60 447	3×10^6	0.9937
L-α-Phosphatidyl-L-serine	26 171	2×10^6	0.9997
DL-α-Phosphatidylethanolamine	−1086.7	1×10^6	0.9999
L-α-Phosphatidylcholine	1514.7	1×10^6	0.9999
Sphingomyelin	351.48	580 518	0.9999

Each line is based on four concentrations of standard.

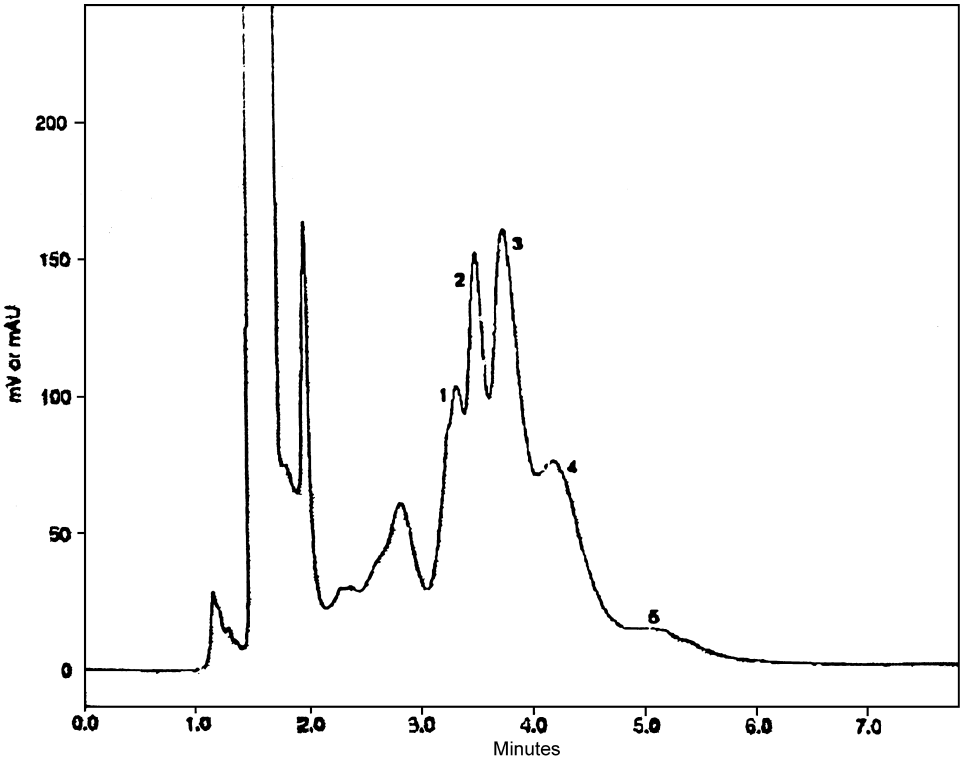

Fig. 1. Chromatogram of a sea urchin *Paracentrotus lividus* sample. Peaks: (1) L-α-phosphatidylinositol; (2) L-α-phosphatidyl-L-serine; (3) DL-α-phosphatidylethanolamine; (4) L-α-phosphatidylcholine; and (5) sphingomyelin.

Table 2
Repeatability, reproducibility and limits of detection for phospholipid classes in sea urchin, *Paracentrotus lividus*, by HPLC

Phospholipid	PI	PS	PE	PC	SM
Precision of measurement (RSD%)	1.07	1.29	1.00	2.62	2.50
Repeatability (RSD%)	2.41	2.42	1.32	2.67	2.75
Limit of detection (μg/ml)	1.30	3.40	1.75	1.77	2.35

Table 3
Phospholipid contents (mg per 100 g of dry weight) in fresh and canned samples of sea urchin *Paracentrotus lividus*

Compound	Fresh material	Canned material
L-α-Phosphatidylinositol	50.65±3.22	51.52±3.02
L-α-Phosphatidyl-L-serine	117.49±8.88	121.32±7.77
DL-α-Phosphatidylethanolamine	272.77±23.08	276.29±37.13
L-α-Phosphatidylcholine	211.32±19.92	228.47±14.78
Sphingomyelin	5.53±0.15	5.12±0.33

Values shown are means (±SD) for eight different samples. The value for each sample was the midpoint of two replicate determinations of a single extract.

References

[1] C. Gabin-Sánchez, F. Lorenzo de Dios, El erizo de mar un recurso con futuro. Aula del Mar, Fundación Caixa Galicia. Spain, 1993, p. 8.
[2] A.C. Campbell (Ed.), Guía de la flora y fauna de las costas de España y Europa, Omega, Barcelona, 1989, p. 248.
[3] E.W. Hammond, Trends Anal. Chem. 8 (1989) 308.
[4] I. Undeland, M. Härröd, H. Lingnert, Food Chem. 61 (1998) 355.
[5] W.W. Christie, in: Lipid Analysis, Pergamon, New York, 1982.
[6] W.W. Christie, J. Chromatogr. 361 (1986) 396.
[7] H. Bünger, U. Pison, J. Chromatogr. B 672 (1995) 25.
[8] M.A. Kaluzny, L.A. Duncan, M.V. Merritt, D.E. Epps, J. Lipid Res 26 (1985) 135.
[9] H. Kim, N. Salem, J Lipid Res. 31 (1990) 2285.
[10] J.A. Prieto, A. Ebri, C. Collar, J. Am. Oil Chem. Soc. 69 (1992) 387.
[11] E.G. Bligh, W.J. Dyer, Can. J. Biochem. Phys. 37 (1959) 911.
[12] R. Homan, M. Anderson, J. Chromatogr. B 708 (1998) 21.
[13] Shafiq-Ur-Rheman, J. Chromatogr. B 567 (1991) 29.
[14] American Chemical Society Subcommittee of Environmental Analytical Chemistry, Anal. Chem. 52 (1980) 2242.
[15] K. Shimada, N. Ogura, J. Food Sci. 55 (1990) 967.
[16] C. De la Cruz-Garcia, J. Lopez-Hernandez, M.J. Gonzalez-Castro, A.I. Rodriguez-Bernaldo de Quiros, J. Simal-Lozano, J. Sci. Food Agric. 80 (2000) 1189.
[17] M.C. Erickson, J. Food Sci. 58 (1993) 84.