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High-performance liquid chromatography separation and light-scattering detection of phospholipids from cooked beef

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Abstract

A sensitive high-performance liquid chromatography (HPLC) method for the separation and quantitative analysis of major phospholipids (PLs) in biological systems is described. PLs were purified by solid-phase extraction with an amino (NH_2) phase. Separation of PLs was carried out on an HPLC silica gel column, with a mobile phase consisting of chloroform, methanol and ammonium hydroxide, and detection was performed with a light-scattering evaporative detector. HPLC analysis of PLs extracted from ground beef cooked under different conditions and capillary gas chromatography of the fatty acid methyl esters showed that cooking treatments did not have a significant effect on the PL composition and fatty acid contents of the single PLs in ground beef.

1. Introduction

Phospholipids (PLs) are ubiquitous constituents of all living tissues, since they are one of the main structural and functional components of cell membranes. PLs are, therefore, present in all foodstuffs and, because of their emulsifying properties, they can exert profound effects during food processing. PLs play an important role in governing the quality of meat during cooking and processing [1–3] and they are important flavour precursors because of their high content of long-chain polyunsaturated fatty acids. Fatty acids are important precursors of beef flavor since they are the primary source of carbonyl compounds upon heating [4,5]. In general, the more unsaturated fatty acids are the most susceptible to oxidation, giving a greater rate of oxidation [6]. Hornstein et al. [7] observed that upon exposure to atmosphere, PLs extracted from pork and beef muscles developed rancid off flavours much faster than neutral fats. This high susceptibility of PLs to oxidation is attributed to their high concentration of polyunsaturated fatty acids [8].

Numerous HPLC methods have been described for the separation of PLs. With a few exceptions [9–12], silica gel has been frequently used as the stationary phase. With respect to the mobile phase, different mixtures have been utilized; *n*-hexane–2-propanol–water [13–15], acetonitrile–methanol–water [16,17] or chloroform–methanol–ammonium hydroxide [18–20].

However, detection of PLs has been a major problem. Due to the absence of a specific absorption peak for lipids, UV detection does not allow a quantitative estimation of these compounds and refractive index detection is not compatible with gradient elution. The light-scattering (LS) evaporative detector, on the other

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hand, allows detection of non-volatile substances with a very good sensitivity level and, moreover, it is compatible with all gradient elution. In fact, PL quantities ranging from 0.25 and 4 μg have been detected by LS detection [20].

The objective of this work is to develop an HPLC method for separation and quantitative analysis of PLs in biological systems. An application of this method was performed on ground beef subjected to different cooking treatments, in order to determine the PL composition and the fatty acid composition of the major PLs.

2. Experimental

2.1. Reagents and standards

All chemicals were purchased from Carlo Erba (Milan, Italy). Methanol and chloroform were HPLC grade; ammonium hydroxyde (30%), *n*-hexane, diethyl ether and acetic acid were analytical grade. Freshly deionized and distilled water was used. Fatty acid methyl ester standards (FAMEs) were obtained from Sigma (St. Louis, MO, USA).

PL standards were from Sigma: L-α-phosphatidylethanolamine (PE) from bovine brain, L-α-phosphatidylcholine (PC) from bovine liver, L-α-phosphatidyl-L-serine (PS) from bovine brain, sphingomyelin (Sph) from bovine brain, L-α-phosphatidylinositol (PI) from soybean, L-α-phosphatidyl D,L-glycerol (PG) from egg yolk lecitin.

Silica column regeneration solution was purchased from Supelco (Bellefonte, PA, USA).

2.2. Sample preparation

Patties (120 g) were prepared from ground beef purchased in a local supermarket (Bologna, Italy). These patties were then subjected to the following treatments: boiling, roasting in an oven, microwave heating, barbecue cooking style, roasting over a metal plate without oil (MP), and a combination of roasting and microwave heating. The microwave oven was a Model Sfornatutto Combi 7 plus, Dé Longhi (Treviso, Italy). Cooking was performed on both sides of the patties (half time per side). Cooking times and conditions are given in Table 1; temperatures were measured at the surface of the patties. All samples were compared against a raw patty (control).

2.3. Lipid extraction and separation

Total lipids were extracted from 30 g of patty, using the procedure described by Folch et al. [21]. A 250-mg amount of lipids was then dissolved in 250 μl of chloroform and applied to a Bond Elut (500 mg size) column with amino (NH_2) bonded phase (Varian, Harbor City, CA, USA), which was previously conditioned with hexane. The elution was carried out by adding 2.5 ml of a chloroform–isopropanol mixture (2:1, v/v) (two times), 2.5 ml of a 2% (v/v) solution of acetic acid–diethyl ether (two times) and 1 ml of methanol (four times) [22]. The

Table 1
Cooking times and temperatures for six cooking treatments

Cooking treatments	Cooking time (min)	Cooking conditions
BO	20	100°C
R	30	225°C
M	6	1000 W[a]
BA	18	200°C
MP	15	180°C
COMB	10	225°C + 1000 W[a]

Abbreviations: BO = boiling; R = roasting in an oven; M = microwave heating; BA = barbecue cooking style; MP = roasting over a metal plate without oil; COMB = combination roasting–microwave heating.
[a] Power of microwave heating.

methanolic fraction, which contained the PLs, was then diluted 10 times and analyzed by HPLC.

2.4. Fatty acid methyl ester preparation

The major phospholipids in the beef samples, i.e. PE and PC, were recovered by the HPLC three-way side vent valve from Scientific System (State College, PA, USA) and were transmethylated directly with 2 *M* KOH, according to Christopherson and Glass [23]. FAMEs were then analyzed by capillary gas chromatography (cGC).

2.5. Calibration of standard curves

Standard curves for HPLC analysis were run with the commercial standards of PC, PE, PS, PI, PG and Sph. Solutions contained 0.25–4 μg of PL and were injected in an increasing concentration order, in each run. Three replicates were run for each concentration. Regression analyses were done using the quadratic function $y = (a + bx)^2$.

2.6. High-performance liquid chromatography

The HPLC system comprised the following components: ERC degasser, Erma (Tokyo, Japan); Rheodyne injector Model 7125 (Cotati, CA, USA); Knauer pump Model 64 (Berlin, Germany); Autochrom gradient controller M-300 (Milford, MO, USA); Sedere light-scattering evaporative detector Sedex Model 45 (Vitry sur Seine, France). The HPLC system was equipped with an on-line filter (Rheodyne). The HPLC columns were LiChrosorb 60, 10 μm (25 cm × 4.6 μm I.D.) Wellington House (Macclesfield, UK) and Spherisorb Si 10 μm (25 cm × 4.6 mm I.D.) Phase Separations (Deeside, UK). The chromatograms were recorded with a Spectra-Physics 4290 integrator (San Jose, CA, USA). The LS detector was set at 60°C of evaporation temperature and at 2 atm of pressure of nebulization gas (compressed air) (1 atm = 101 325 Pa).

A methanolic ammonium hydroxide gradient in chloroform was chosen as eluent system because of its low viscosity and good solvent properties [18,19]. After optimization, the following binary gradient was utilized: (A) chloroform–methanol–ammonium hydroxide 30% (80:19.5:0.5, v/v) and (B) chloroform–methanol–water–ammonium hydroxide 30% (60:34:5.5:0.5, v/v). The flow-rate was 1.5 ml/min. The solvent program is shown in Table 2.

Table 2
HPLC solvent program for a binary gradient

Time (min)	A (%)	B (%)
0	100	0
8	45	55
15	40	60
20	40	60
35	100	0

A = chloroform–methanol–ammonium hydroxide 30% (80:19.5:0.5, v/v); B = chloroform–methanol–water–ammonium hydroxide 30% (60:34:5.5:0.5, v/v).

The time required for returning to the starting conditions and for column equilibration (15 min) was observed, in order to avoid significant variations in the retention volumes.

2.7. Capillary gas chromatography

cGC analysis were performed using a Carlo Erba 4260 gas chromatograph (Rodano, Milan, Italy) equipped with split injector and flame ionization detector. The cGC column was a 25 m fused-silica capillary column (0.25 mm I.D. and 0.25 μm film thickness) coated with cyanopropyl methyl silicone from Quadrex (New Haven, CT, USA). The oven temperature program was from 150 to 240°C at a rate of 3°C/min. Injector and detector temperatures were both set at 260°C. The helium carrier gas flow-rate was 2 ml/min, with a split ratio of 1:30. cGC chromatograms were recorded with a Spectra-Physics 4290 integrator.

3. Results and discussion

Calibration curves for each of the PL standards were run with concentrations ranging from

0.25 to 4 μg of PLs and exponential curves were obtained according to the equation $y = (a + bx)^2$. The quadratic regression constants (a, b) and the correlation coefficients (r^2) for each PL calibration curve are given in Table 3, for which the statistic curve fit F was found to be significant at a 0.01% level. Malton [24] has found a linear response for the calibration curves, but using considerably larger amounts of PLs. The minimum quantifiable amount of PL was 100 ng, except for the case of Sph in which 250 ng was the minimum quantitated. In fact, Sph gives two bands as reported by some authors [20,25,26]. For quantitative purposes, the Sph content was calculated from the sum of two peaks. The method described here, therefore, is sensitive enough to quantify the PL levels present in biological systems.

Fig. 1 shows a HPLC trace of a 15-min analysis of a mixture of PL standards. Once the adequacy of this method was established, some considerations were made with respect to the HPLC column, which was packed with a silica phase and used with an aqueous eluent. Silica phase columns are able to give optimum separation of PL mixtures [27,28], besides being relatively inexpensive. Moreover, according to Vanderdeelen et al. [29], the irregular phase seems to be more suitable for this type of analysis than the spheric configuration; thereby, an irregular phase column was used in this work. However, a strong decay of the column properties was observed after 2 months of continuous use. It was possible to delay the column removal for a week, by immersing the column in a commercial regenerating liquid overnight. Despite these difficulties, the total cost of the analysis was still affordable due to both the low cost of the column and the relatively high number of analyses that could be run. On the other hand, solid-phase extraction (SPE) columns with a NH_2 bonded phase gave an optimal purification of the PL fraction with a 99% recovery.

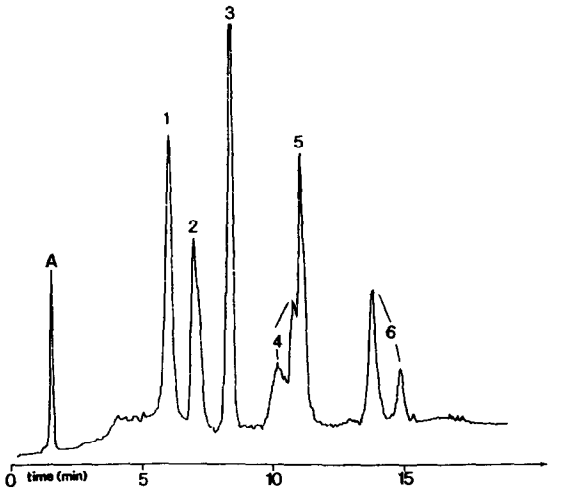

Fig. 1. HPLC trace of phospholipid standards. Peaks: A = free fatty acids; 1 = PG; 2 = PE; 3 = PI; 4 = PS; 5 = PC; 6 = Sph.

Table 3
Quadratic regression constants (a, b) and correlation coefficients (r^2) for phosopholipid calibration curves

PL	a	b	r^2
PC	501.514	554.701	0.9737
PE	444.424	570.524	0.9811
PS	628.386	428.922	0.9333
PI	478.605	727.641	0.9879
PG	329.380	562.413	0.9748
Sph	459.484	565.489	0.9764

Abbreviations: PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PG = phosphatidylglycerol; Sph = sphingomyelin

This methodology was then applied to study the effect of different cooking treatments in the PL composition of ground beef, from a qualitative and quantitative point of view. Many authors have already reported the PL composition of bovine muscle from a biological standpoint [24,30], rather than from a food technology aspect. Since this work analyses lipids in foods, instead of lipids in an specific muscle or organ, a direct comparison with those data would not be valid.

Fig. 2 shows the HPLC trace of roasted ground beef, where a good peak separation of the main PLs was obtained.

Table 4 reports the PL concentrations in ground beef samples (expressed in mg/g of fat). Traces of PS and PG were also found (data not

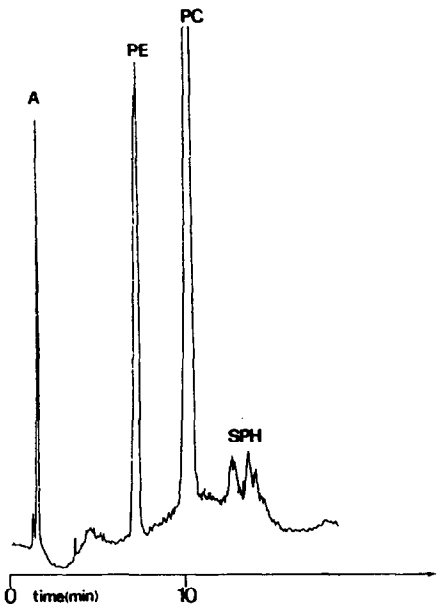

Fig. 2. HPLC trace of phospholipids from roasted ground beef. A = Free fatty acids.

shown). In general, cooking treatments produced small variations in the PL content. On the other hand, Sph decreased uniformly in all cooked samples; this might be due to a weaker interaction between Sph and the cellular membrane so that part of the Sph is lost with some fat during cooking.

With respect to the FAME preparation, transmethylation was carried out using the method by Christopherson and Glass [23], since its application to PLs has been also confirmed by other authors [31,32]. Although other methodologies have been suggested for this purpose [33], the method followed in this study was used because of the fast sample preparation at room temperature and the quantitative validity of the results.

Table 4
Effect of cooking treatments on the phospholipid contents in ground beef (expressed in mg/g of fat)

Treatment	PC	PE	Sph	Total
RM	15.36	11.36	9.36	36.08
BO	15.84	16.8	7.76	40.4
R	17.36	15.44	8.16	40.96
M	17.44	15.2	7.92	40.56
BA	17.68	18.8	7.2	43.68
MP	14.24	11.52	6.48	32.24
COMB	16.96	13.28	6.64	36.88

Abbreviations as in Tables 1 and 3.

FAMEs were identified by comparison with a cGC trace of a standard mixture of FAMEs and confirmation was with a fractionation by silver ion TLC [34], which separated the different methyl esters by degree of unsaturation, and with a further cGC analysis of the fractions. Identification was in agreement with previous papers [35,36]. Results from Table 5 and 6 show little variations in the FAME content of the PLs of cooked beef samples. No increase was observed in the amount of *trans* fatty acids, except in the case of C16:1. Changes in the polyunsaturated fatty acids content could have been expected due to an oxidation process which takes place during cooking; however, no differences were noticed in the PLs of raw and the cooked patties in this respect.

The analytical procedures given in this work allow rapid (total run of 35 min, included restoring of the initial conditions), accurate and sensitive analysis from animal tissues. The SPE method allows the separation of PLs from other types of lipid and a quantitative concentration of minor amounts of PLs for further HPLC analysis.

The type of detector used in this investigation was a light-scattering one, which is based on the nebulization of the eluate of the HPLC column by evaporation of the mobile phase. Detection is carried out by measuring the amount of light scattered by the solid particles that are left after evaporation of the mobile phase.

The high sensitivity of the LS detector used in this investigation permits detection and quantification of small amounts of PLs. This innovative model of detector selects by split the more homogeneous and minute particles of the nebulized solute, so that interaction is avoided among the larger droplets that have not been completely evaporated. Moreover, this detector has a more accurate control of the temperature and of the evaporation pressure than the previous models. These parameters along with the cell geometry have a direct influence on both the nebulization

Table 5
Effect of cooking treatments on FAME content (%) in ground phosphatidycholine

Treatment	Fatty acids %																		
	14:0	15.0	16.0	16:1t	16:1	17:0	17:1	18:0	18:1t	18:1	18:2	18:3	20:1	20:3	20:4	20:5	22:4	22:5	22:6	others
RM	0.7	0.3	24.0	0.2	2.1	0.7	0.7	10.0	0.6	30.9	22.6	1.2	0.5	0.7	3.1	0.3	0.2	0.6	0.1	0.5
BO	0.1	0.3	20.5	0.7	1.3	0.5	0.8	7.1	0.6	27.4	29.4	1.0	0.2	1.6	5.3	0.5	0.4	1.4	0.4	0.6
R	0.1	0.2	21.9	0.6	1.3	0.5	0.5	7.3	0.8	27.7	28.9	0.9	tr	1.5	4.5	0.8	0.3	0.9	0.2	0.5
M	0.1	0.2	23.0	0.7	1.3	0.5	0.5	7.7	0.8	29.3	25.4	1.0	tr	1.6	4.8	0.9	0.3	1.0	0.2	0.5
BA	0.2	0.3	22.9	0.7	1.5	0.5	0.7	7.1	0.4	27.4	28.5	0.2	tr	1.4	4.6	0.4	0.7	1.3	0.2	0.6
MP	0.2	0.3	23.2	0.6	1.3	0.5	0.6	7.2	0.5	27.3	28.3	0.8	0.2	1.1	4.4	0.3	0.4	0.8	tr	0.5
COMB	0.2	0.3	23.1	0.7	1.5	0.6	0.9	7.3	0.6	26.6	28.0	1.0	0.2	1.5	4.8	0.4	0.5	1.1	0.2	0.6

t = *trans*; tr = trace; RM = raw; other abbreviations as in Table 1.

Table 6
Effect of cooking treatments on FAME content (%) in ground beef phosphatidylethanolamine

Treatment	Fatty acids %																
	16:0	16:1	17:0	17:1	18:0	18:1t	18:1	18:2	18:3*n*6	18:3*n*3	20:1	20:3	20:4	20:5	22:4	22:5	22:6
RM	3.2	0.6	0.2	0.2	18.9	0.2	15.8	26.9	tr	0.5	0.2	3.2	22.5	1.2	2.0	3.8	0.8
BO	1.7	0.6	0.3	0.3	18.7	0.8	15.1	28.2	0.1	0.7	0.5	3.3	22.7	1.4	1.8	3.3	0.6
R	2.0	0.8	0.2	0.2	20.4	0.5	15.7	31.1	0.2	0.7	0.4	3.5	19.5	1.0	1.1	2.3	0.4
M	5.4	1.0	0.3	3.2	20.7	0.9	14.5	29.5	0.2	0.8	0.2	3.4	24.3	1.6	2.0	4.3	0.4
BA	2.0	0.7	0.3	0.6	24.0	0.3	12.4	21.9	0.2	1.0	0.4	2.5	25.0	1.4	1.8	3.5	2.0
MP	3.2	1.1	0.6	1.3	17.6	0.1	13.5	26.7	0.1	1.2	0.3	2.2	25.1	0.8	1.3	3.7	1.0
COMB	2.9	0.7	0.4	0.2	19.5	0.1	14.5	27.1	0.1	0.7	0.2	4.4	22.2	1.3	2.0	3.2	0.5

Abbreviations as in Table 5.

homogeneity and the evaporation velocity, therefore affecting directly the sensitivity and repeatability of the instrument. Due to these improvements, more precise quantifications are achieved, thus reducing respectively the effects of underestimation and overestimation of the amounts of major and minor component of the mixture, respectively.

On the other hand, all types of solvent gradient can be utilized with the LS detector. However, the solvent mixture should be prepared only with very volatile acid or bases in the absence of buffers.

A good recovery of the single PLs is achieved by using the split valve, allowing further analysis of the FAMEs by cGC.

With respect to the analysis of the ground beef samples, it can be concluded that no differences were observed in the FAME contents of the PLs, despite the different cooking methods used. Moreover, it seems that lipids in foods are much more protected than those from model systems, which are generally used to simplify complex events that occur in nature. As demonstrated by previous investigations, other nutrients present in foods have a protective effect on fatty substances [37] and when lipids are constituents of an organized biological structure, this "protection" phenomenon is more evident.

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