



Selective extraction of phospholipids from soybeans with supercritical carbon dioxide and ethanol

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Abstract

Supercritical carbon dioxide (SC-CO₂) is very effective in removing oils from a variety of seed matrices, devoid of any appreciable amount of phospholipid (PL) content. However, the limited solubility of PLs in SC-CO₂ leaves behind a potentially valuable by-product in the spent seed matrix. Any PL extraction process from the spent matrix must maintain the structure and the quality of PLs and must be compatible with the end use of the seed protein meal as animal feed or for human consumption. An initial SC-CO₂ extraction of soybean flakes was performed at 32 MPa and 80°C to extract the oil, leaving the PLs in the defatted soybean flakes (DSF). A second step was performed on the DSF using $X_{\text{eth}}=0.10$, varying the pressure from 16.6 to 68.9 MPa and the temperature from 60 to 80°C. For all SC-CO₂/ethanol extractions, a fraction rich in PLs was obtained. The fractions extracted from defatted soybean flakes were dried and then re-dissolved in chloroform before HPLC-ELSD analysis. Quantitative and qualitative analysis of PLs on soybean seeds, DSF, and different extracted phospholipid fractions was carried out, to ascertain the effort of combinations of extraction pressure and temperature. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Phospholipids (PLs) are polar lipids [1,2]. The terms lecithin and phosphatidylcholine (PC) are often used interchangeably [2]. However, the term lecithin refers to a complex, naturally occurring mixture of PLs, traditionally obtained by water-washing crude vegetable oil and separating and drying the hydrated gums [3]. Therefore, the term lecithin is often used to describe a diverse group

of commercially available PL mixtures, including fractions containing one or more PLs, triglycerides, pigments, carbohydrates, sterols, cerebrosides, in different proportions [3,4].

Previous studies conducted at the National Center for Agricultural Research in Peoria, IL by Friedrich and co-workers [5,6] showed that supercritical carbon dioxide (SC-CO₂) was very effective in removing oils from a variety of seed matrices, devoid of any appreciable PL content. This property has recently been exploited by List et al. [7] to continuously degum pre-extracted soybean oil using SC-CO₂. However, the limited solubility of

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PLs in SC-CO₂ leaves behind a potentially valuable by-product in the spent seed matrix that could be recovered to economic advantage. In addition, any PL recovery process must also be compatible with the end use of the seed protein meal as an animal feed, or for use in human consumption. Since neat CO₂ will not effectively dissolve PL moieties, the choice of a suitable co-solvent to enhance their solubility must be made not only on a thermodynamic basis, but also with regard to its food safety status, i.e. 'Generally Recognized As Safe' (GRAS). A logical choice for a co-solvent is ethanol, which enjoys GRAS status in the US. Ethanol has been used previously to fractionate PLs [8], although not in an SFE process; however, it has been utilized by Temelli [9] to remove the phospholipids from canola seed using SC-CO₂. Since high pressure phase equilibrium data are available for ethanol/CO₂ mixtures [10], we focused on the use of this co-solvent in fractionating the PL mixtures. The potential use of SC-CO₂/ethanol mixture for extraction and fractionation of PLs from defatted flaked soybean seeds has been investigated previously [11,12]. Initial studies indicated that a small amount of ethanol (5%) in the SC-CO₂ was not enough to extract the PLs, but by using a molar fraction of ethanol (X_{eth}) corresponding to 10%, the total recovery of PLs can be increased considerably. Otherwise, the relative amount of phosphatidylcholine (PC) and phosphatidyl-ethanolamine (PE) in the resulting extract can be varied, using amounts greater than 10%. Since SC-CO₂ extraction allows different kinds of products to be obtained by changing the operating parameters (pressure and temperature), the influence of these processing parameters on the extraction and fractionation was studied.

The individual properties of the constituents of lecithin are not well known, due to the difficulties in isolating large quantities of these compounds for study. For this reason it is highly desirable to obtain phospholipid-enriched fractions, and considerable effort is being put into developing schemes for phospholipid purification, particularly those based on the relative alcohol solubility of the individual phospholipids [13]. Moreover, the demand for high PC containing lecithin has been

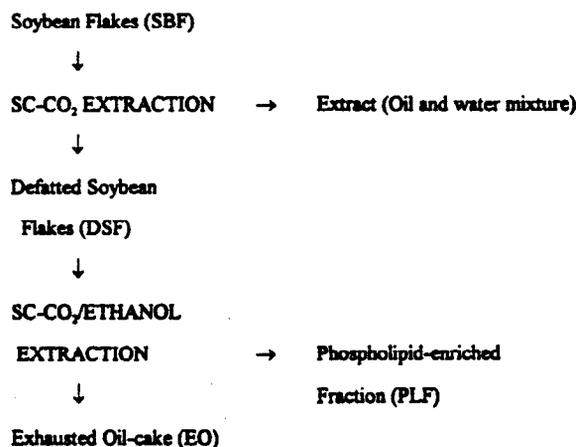
increasing in the cosmetic, pharmaceutical, food and other industries [14].

The objectives of this research were: (a) to evaluate the possibility of completely extracting the PLs present in the DSF by using a small amount of ethanol in SC-CO₂ (10% molar fraction), thereby confirming the possibility of carrying out a dual procedure for the removal of oil and phospholipid fractions from seed matrices using supercritical fluids; (b) to fractionate PLs by changing the pressure and the temperature of SC-CO₂/ethanol mixture. These objectives should be achievable considering that the solubility data for PC and PE in net ethanol (in subcritical status) show that PC is easier to dissolve than PE. Therefore, a first PC-enriched phospholipid fraction should be obtained; then, by changing the operating parameters, a second PC-depleted phospholipid fraction should be obtained.

2. Experimental procedure

The soybean seeds were provided by Mignini S.p.A. (Petignano d'Assisi, PG, Italy). They had an oil content of 20.6% by weight, and their moisture content was 12.7% by weight.

The following two SFE steps were carried out:



The first SC-CO₂ extraction was performed at 32 MPa and 80°C by using a Muller SFE pilot plant (Muller Extract Company GMBH, Coburg, Germany), on 600 g of soybean flakes placed in a

Table 1
Mass balance flow sheet (g) of CO₂ net extraction

	In				Out			
	Wet M.	Dry M.	Water	Oil	Wet M.	Dry M.	Water	Oil
SBF	600	523.8	76.2	123.6				
Extract					138.5	119.5	19.0	119.5
DSF					445.1	408.6	36.5	8.9
Total	600	523.8	76.2	123.6	583.6	528.1	55.5	128.4
Δ (out – in)					–16.4	+4.3	–20.7	+4.8

Table 2
Extraction conditions

<i>P</i> (MPa)	68.9	68.9	68.9	52.4	40.7	40.7	40.7	30.1	23.9	23.9	23.9	19.4	16.6	16.6	16.6
<i>T</i> (°C)	80	70	60	80	80	70	60	80	80	70	60	80	80	70	60
ρ^a (g/ml)	0.95	0.97	0.99	0.90	0.85	0.88	0.91	0.80	0.75	0.80	0.84	0.70	0.65	0.72	0.78

^a ρ = SC-CO₂/ethanol mixture density, calculated by using 'SF-Solver'® [15].

1 l nominal vessel. The extract (138.5 g containing 13.7% moisture) was separated by reducing the pressure at the pressure let down valve to 10 MPa, leaving 445.1 g of DSF in the extractor with a total fat content of 2.0% by weight and a moisture content of 8.2% by weight. Table 1 reports the mass balance flow sheet of this first extraction. Subsequent extractions were performed with an Isco SFX® 3560 extractor equipped with two 100DX syringe pumps (Isco, Inc., Lincoln, NE, USA). These extractions were performed using $X_{\text{eth}}=0.10$, varying the pressure from 16.6 to 68.9 MPa, and varying the temperature from 60 to 80°C. The *P*–*T* combinations that were utilized are given in Table 2. Density values (ρ) of the SC-CO₂/ethanol mixture were calculated by using 'SF-Solver'® software [15] and are in quite good accord with the experimental data found by Pöhler and Kiran [16].

These extractions were conducted for 30 min on samples of 4.5 g DSF. Each extraction was replicated three times. Both the CO₂ and ethanol syringe pumps were thermostated at 4°C, their flows were chosen to obtain a flow of 2 ml/min for the SC-CO₂/ethanol mixture ($X_{\text{eth}}=10\%$). PLs were also extracted from SBF and DSF by five-fold extraction using the extraction solvent mixture of Bollmann (1:1:1 benzene:ethanol:petroleum

ether) [17,18], then dried, and re-dissolved in chloroform.

Standard phospholipids, L- α -phosphatidylethanolamine (PE), L- α -phosphatidylcholine (PC) and L- α -phosphatidic acid sodium salt (PA) from egg yolk, L- α -phosphatidylinositol sodium salt (PI) from soybean, were from Sigma (Sigma-Aldrich, Milano, Italy). All the HPLC grade and RPE grade solvents were from BDH (Milano, Italy).

Dry matter and crude fat were determined on the SBF, extract, and DSF according to AOCS methods [19,20]. PL analyses were performed using a Varian 2020 gradient programmer, and two Varian 2010 pumps (Varian, USA) were used. The procedure of Becart et al. [21], slightly modified, was used as follows. A normal phase silica column μ Porasil (3.9 mm i.d. \times 300 mm) (Waters, Milford, MA, USA) utilizing a Hamilton silica precolumn (Hamilton, Reno, NV, USA) was used. The mobile phase was the following binary gradient: (A) chloroform:methanol:water:30% ammonium hydroxide at the v/v ratio 60:34:5.5:0.5, respectively; (B) chloroform:methanol:30% ammonium hydroxide in the v/v ratio 80:19.5:0.5, respectively.

The HPLC flow rate was 1 ml/min and the gradient was from 0 to 100% A in 14 min, then 100% A isocratic to 23 min and again to 0% A in

29 min. There was a 5 min isocratic equilibration time before each injection. A 10 μ l sample containing 10 to 25 μ g of PLs was injected into the column. Quantitative analyses were carried out for phospholipids where high purity standards were available. Calibration curves for each of the PL standards were run by injected amounts ranging from 1 to 10 μ g. An Alltech Varex MKIII ELSD (Alltech Associates, Inc., Deerfield, IL, USA) was used to detect the HPLC eluate, nebulized with an air flow of 3.5 ml/min to a drift tube set at 80°C. The signal output from all the detectors was analyzed using a Varian DMS 654 integration station. Statistical analysis was done using Statgraphics® software (Statgraphics Manual, 1992). Because this study is part of an international bilateral project, the HPLC analysis was replicated on the extracted PLFs in the laboratories of the National Center for Agricultural Utilization Research, United States Department of Agriculture, Peoria, IL. There were no consistent discrepancies.

3. Results

Table 3 reports the amount and the percentage repartitioning of PLs extracted with the Bollmann reagent from SBF and DSF, and Table 4 reports the amount and the percentage repartitioning of PLs extracted via SFE at 80°C at different pressures, using $X_{eth} = 10\%$. All data were normalized to 1 g DSF. From these results, the following considerations can be drawn. (a) Comparing the

PLs present in SBF and in DSF, it is possible to see that no PLs were extracted in the first SFE with net CO₂. (b) The highest amount of PLs was extracted at 68.9 MPa, and they are 74.8% of the total present in the DSF according to the extraction performed using the Bollmann reagent. At this pressure no enrichment of any PL was obtained, i.e. the relative percentages of PE, PC, PI, and PA were the same as obtained using the Bollmann reagent. (c) Decreasing the pressure decreased the amount of PLs extracted, but the relative percentage of each PL changed. In particular, at 19.4 MPa, PC represented 80.1% of the total PLs extracted, while with the Bollmann reagent only 64.7%. (d) More PI was extracted with the Bollmann reagent than with SC-CO₂/ethanol 10% mixtures. Its maximum extraction yield was obtained at 68.9 MPa (0.26 mg PI/g DSF in comparison with 0.90 mg PI/g DSF extracted with the Bollmann reagent). (e) PA was well extracted from 68.9 to 40.7 MPa (from 0.18 to 0.16 mg PA/g DSF), while only 0.12 mg PA/g DSF was extracted at 30.1 MPa. Lower pressures did not allow a significant amount of PA to be extracted. (f) At 16.6 MPa no significant amount of PLs was extracted.

Table 5 reports the amount of PLs extracted from DSF at 68.9, 40.7, and 23.9 MPa at different temperatures (80, 70, and 60°C). Since no significant amount of PLs was extracted at 16.6 MPa at any temperature, these data are not reported. All data were normalized to 1 g of DSF. From these results, the following considerations can be drawn.

Table 3
PLs extracted with the Bollmann reagent from SBF and DSF

	Phospholipid	mg/g DSF	%
SBF treated by five-fold extraction with Bollmann reagent	PE	2.51	26.6
	PC	6.20	65.6
	PI	0.69	7.2
	PA	0.15	0.6
	Total	9.54	100
DSF treated by five-fold extraction with Bollmann reagent	PE	2.34	24.5
	PC	6.19	64.7
	PI	0.90	9.4
	PA	0.22	1.5
	Total	9.65	100

Table 4
PLs extracted from DSF at 80°C at different pressures, using $X_{\text{eth}} = 10\%$

P and ρ^a	PLs	mg PLs/g DSF	%	P and ρ^a	PLs	mg PLs/g DSF	%
68.9 MPa 0.95 g/ml	PE	1.81	25.1	23.9 MPa 0.75 g/ml	PE	0.60	18.5
	PC	4.97	68.8		PC	2.51	77.7
	PI	0.26	3.6		PI	0.09	2.8
	PA	0.18	2.5		PA	0.03	1.0
	Total	7.22	100		Total	3.23	100
52.4 MPa 0.90 g/ml	PE	1.44	24.0	19.4 MPa 0.70 g/ml	PE	0.30	14.5
	PC	4.28	71.4		PC	1.67	80.1
	PI	0.11	1.8		PI	0.07	3.4
	PA	0.17	2.8		PA	0.04	2.0
	Total	6.00	100		Total	2.09	100
40.7 MPa 0.85 g/ml	PE	0.84	20.8	16.6 MPa 0.65 g/ml	PE	0.04	44.1
	PC	2.95	73.4		PC	0.02	20.7
	PI	0.07	1.8		PI	0.04	35.2
	PA	0.16	4.0		PA	0.00	0.0
	Total	4.02	100		Total	0.10	100
30.1 MPa 0.80 g/ml	PE	0.54	19.0				
	PC	2.12	73.9				
	PI	0.08	2.9				
	PA	0.12	4.2				
	Total	2.87	100				

^a $\rho = \text{SC-CO}_2/\text{ethanol}$ mixture density, calculated by using 'SF-Solver' [15].

Table 5
PLs extracted from DSF at different pressures and temperatures, using $X_{\text{eth}} = 10\%$

T and ρ^a at 68.9 MPa	PLs	mg PL/g DSF	%	T and ρ^a at 40.7 MPa	PLs	mg PL/g DSF	%	T and ρ^a at 23.9 MPa	PLs	mg PL/g DSF	%
80°C 0.95 g/ml	PE	1.81	25.1	80°C 0.85 g/ml	PE	0.84	20.8	80°C 0.75 g/ml	PE	0.60	18.5
	PC	4.97	68.8		PC	2.95	73.4		PC	2.51	77.7
	PI	0.26	3.6		PI	0.07	1.8		PI	0.09	2.8
	PA	0.18	2.5		PA	0.16	4.0		PA	0.03	1.0
	Total	7.22	100		Total	4.02	100		Total	3.22	100
70°C 0.97 g/ml	PE	2.15	20.0	70°C 0.88 g/ml	PE	0.55	17.4	70°C 0.80 g/ml	PE	0.17	18.1
	PC	8.20	76.2		PC	2.53	80.5		PC	0.78	81.9
	PI	0.19	1.8		PI	0.04	1.2		PI	0.00	0.0
	PA	0.23	2.0		PA	0.03	0.9		PA	0.00	0.0
	Total	10.77	100		Total	3.14	100		Total	0.95	100
60°C 0.99 g/ml	PE	1.80	22.0	60°C 0.91 g/ml	PE	0.67	19.9	60°C 0.84 g/ml	PE	0.14	20.5
	PC	6.22	75.9		PC	2.60	77.7		PC	0.56	79.5
	PI	0.14	1.7		PI	0.06	1.7		PI	0.00	0.0
	PA	0.03	0.4		PA	0.02	0.7		PA	0.00	0.0
	Total	8.20	100		Total	3.35	100		Total	0.70	100

^a $\rho = \text{SC-CO}_2/\text{ethanol}$ mixture density, calculated by using 'SF-Solver' [15].

(a) At 68.9 MPa, decreasing the temperature increased the amount of extracted PLs. At 70°C more PLs were extracted than when using the Bollmann reagent, i.e. 10.77 mg PLs/g DSF in comparison to 9.65 mg PLs/g DSF (see Table 3). At 60°C 8.20 mg PLs/g DSF were extracted. An enrichment of PC was obtained at 70 and 60°C. (b) At the lower pressures, reducing the temperature gave an overall reduction of the total extractable PLs. (c) The most interesting enrichment in PC was achieved at 40.7 MPa and 70°C. In fact, at this P and T 3.14 mg PLs/g DSF were extracted, of which 2.53 mg were PC (80.5% as relative percentage) equal to 40.9% of the total extractable PC using the Bollmann reagent (6.19 mg PC/g DSF, see Table 3). Other phospholipid fractions with more than 80% PC (as relative percentage) were obtained. They correspond to a lower amount of the total extractable PC: at 19.4 MPa and 80°C (see Table 4) 1.67 mg PC/g DSF (equal to 27.0% of the total extractable PC using the Bollmann reagent); at 23.9 MPa and 70°C only 0.78 mg PC/g DSF (equal to 12.6% of the total extractable PC using the Bollmann reagent).

4. Conclusions

The above results show the possibility of completely extracting the PLs present in DSF by using a 10% SC-CO₂/ethanol mixture, thereby confirming the possibility of carrying out a dual procedure for the removal of oil and the phospholipid fractions from soybean seeds using supercritical fluids. This has remarkable interest because the traditional extraction with hexane leaves about 50% of the total PLs in the spent seed matrix, while the other 50% are extracted into the hexane. Hence, only this portion of the PLs is recovered from the extraction process using hexane to extract the raw oil. Moreover, by changing the pressure and temperature of a SC-CO₂/ethanol mixture, some PC-enriched PLFs were obtained. These results, and those shown in previous papers where PL extractions were performed by changing the X_{eth} always at 68.2 MPa and 80°C [11,12], confirm the possibility of obtaining a PC-enriched PLF and yield a fractionated extract of PLs from DSF.

New trials are presently in progress to evaluate the influence of P , T , and X_{eth} simultaneously. These studies are based on a new statistical design, on solubility data determination of PLs, and on some results determined by using model systems.

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