Optimized Separation of Nonpolar and Polar Lipid Classes from Wheat Flour by Solid-Phase Extraction

Jose A. Prieto*, Andrés Ebri and **Concepción Collar**
Cereals Laboratory, Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Jaime Roig, 11, 46010 Valencia, Spain

A method combining solid-phase extraction on prepacked silica and aminopropyl bonded-phase (Bond-Elut) columns has been developed for the separation of neutral lipids, glycolipids and phospholipids from wheat flour into individual classes in high yield and homogeneity. Chromatography on a single silica column (500 mg) with solvent combinations of increasing polarity resulted in complete separation of steryl esters, triglycerides, free fatty acids, 1,2-diglycerides, 1,3-diglycerides, monoglycerides, monogalactosylglycerides, digalactosylglycerides, phosphatidylcholine and lysophosphatidylcholine. Chromatography on an aminopropyl bonded-phase column (500 mg) with ternary mixtures of chloroform/methanol/ammonium hydroxide led to the proper separation of N-acyl-phosphatidylethanolamine and N-acyl-lysophosphatidylethanolamine, previously coeluted on the prepacked silica column. Cross contamination among phospholipid classes tested by normal-phase high-performance liquid chromatography was always less than 14%.

KEY WORDS: Chromatography, glycolipids, phospholipids.

Traditional methods to separate and isolate neutral and polar lipids from wheat flour, bread dough and gluten involve adsorption and partition chromatography on preparative plates (1–3) and low-pressure columns (4–7). Recently, solid-phase extraction (SPE) has been reported as a rapid, efficient and high-recovery method for the isolation and fractionation of lipids (8–10), especially with small and medium sample sizes, which overcomes significant problems associated with classical procedures and high-performance liquid chromatography (HPLC) (11). SPE columns on silica (12) and aminopropyl (11) stationary phases have been used for proper separation of nonpolar lipid classes with recoveries greater than 98%. Separation of polar lipid classes appears to be harder to achieve (12,13). Nevertheless, the combination of stationary and mobile phases available offers the possibility to optimize the methodology.

The work detailed in this report outlines optimized SPE procedures involving the use of disposable silica and aminopropyl prepacked columns, under pressure, to isolate and purify individual phospholipid (PL), glycolipid (GL) and neutral lipid (NL) classes from wheat flour with high percent of recovery and homogeneity.

MATERIALS AND METHODS

Materials. Standards of NL [steryl ester (SE), triglyceride (TG), 1,3-diglyceride (1,3-DG), 1,2-diglyceride (1,2-DG), free fatty acids (FA), α-monoglyceride (α-MG) and β-monoglyceride (β-MG)], GL [monogalactosyl diglyceride (MGDG) and digalactosyl glyceride (DGDG)] and PL [phosphatidylcholine (PC) and lysophosphatidylcholine (LPC)] were purchased from Sigma Chemical Co. (St. Louis, MO). Bond Elut silica and aminopropyl disposable prepacked columns (500 mg) were supplied by Analitichem International (Harbor City, CA). A Supelco vacuum elution apparatus (Supelco, Bellefonte, PA) with adaptors was used for all experiments. Thin-layer chromatography (TLC) plates (Silica Gel 60) were from Merck (Darmstadt, Germany). Solvents used were analytical (SPE, TLC) and HPLC grade, and were obtained from Merck and SDS (Peypin, France), respectively. Chromatography-quality water for aqueous solvent mixtures was supplied by Merck. Standard lipid solutions were made to a concentration of 5–10 mg/mL.

Lipid extraction. Bonded lipids from wheat flour (1 g) were extracted with water-saturated n-butanol (6 mL) at room temperature by intermittent mechanic stirring of the slurry during 10 min. After centrifugation (2500 rpm, 10 min) extracts were evaporated to dryness under nitrogen atmosphere, and redissolved (8–9 mg/mL) in hexane/diethyl ether (200:3) for chromatography.

NL and GL elution. Lipid separation was performed by SPE on Bond-Elut (500 mg) prepacked columns (stainless steel frits). Mobile phases used for NL separation were based on those reported earlier by Hamilton and Comai (12) and Kaluzny *et al.* (11). GL elution was carried out by solvent combinations commonly used in low-pressure chromatography (14). Silica and aminopropyl columns were conditioned before use by washing with 12 mL of hexane and 12 mL of initial mobile phase, with care to prevent the columns from becoming completely dry. A lipid sample (1 mL) was applied to each column (760 mm Hg), and 2 mL of the mobile phase was pulled through. This left the entire lipid mixture on the column. Next, the columns were eluted under vacuum (5–10 mm Hg) with mixtures of solvents with increasing polarity as detailed in Table 1. Columns were dried after pulling through each mobile phase. The eluates were collected, evaporated and redissolved in chloroform/methanol (2:1, 0.5 mL) for further analysis of purity.

PL elution. Separation of the main PL from wheat flour was checked on both silica and aminopropyl prepacked columns, both separately and in conjunction by means of several mixtures of solvents with increasing polarity (tetrahydrofuran/acetonitrile/propan-2-ol, tetrahydrofuran/acetonitrile/methanol, acetonitrile/methanol, methanol), and increasing ionic strength (Chloroform/methanol/ammonium hydroxide) as mobile phases, prior to the optimized utilization of both columns with elution of PC and LPC (silica) under increasing polarity, and of N-acyl-phosphatidylethanolamine (NAPE) and N-acyl-lysoPE (NALPE) (aminopropyl) under ionic suppression elution conditions (Table 2). Eluates were collected, evaporated and redissolved in chloroform/methanol (2:1, 0.5 mL) before analyses.

TLC. Routine visual checks of the chromatographic purity of eluate fractions was done by TLC on Silica Gel 60 plates. Samples (20 μL) were spotted with a 10 μL syringe supplied by Hamilton (Reno, NV). Plates were previously conditioned by i) A vertical development with chloroform/methanol (2:1) up to 0.5 cm from the top;

*To whom correspondence should be addressed.

TABLE 1

Mobile Phases Used in the Prepacked Silica Column for the Fractionation of Lipid Classes from Wheat Flour[a]

Name	Solvents, volume ratio	Volume (mL)	Lipid eluted[b]
A	Hexane/diethyl ether (200:3)	15	SE
B	Hexane/diethyl ether (96:4)	20	TG
C	Hexane/acetic acid (100:0.2)	20	—
D	Hexane/diethyl ether/acetic acid (100:2:0.2)	20	FA
E	Hexane/ethyl acetate (95:5)	15	—
F	Hexane/ethyl acetate (85:15)	15	1,2-DG, 1,3-DG
G	Diethyl ether/acetic acid (100:0.2)	15	α-MG, β-MG
H	Diethyl ether/acetone (50:50)	20	MGDG + MGMG
I	Acetone	20	DGDG + DGMG
J	Tetrahydrofuran/acetonitrile/ propan-2-ol (40:35:25)	5	trace amount GL
K	(30:35:35)	5	—
L	(20:35:45)	5	NAPE
M	Tetrahydrofuran/acetonitrile/ methanol (15:45:40)	5	NAPE + NALPE
N	(15:35:50)	5	—
O	(10:35:55)	5	PC
P	(5:35:60)	5	—
Q	Acetonitrile/methanol (35:65)	5	LPC

[a]Lipid extracts were processed by the procedures described in Methods. The volumes represent the amounts of each to elute the particular lipid, and are dependent upon the amount of stationary phase.

[b]Lipids (8–9 mg/mL A) loaded onto silica column (1000 mg). If Bond-Elut (500 mg) is used, some lipid classes change elution solvent—1,3-DG (E, 20 mL), 1,2-DG (E, 40 mL or F, 20 mL), PC (N) and LPC (P).

TABLE 2

Mobile Phases Used for Optimized Separation of Main Phospholipids Classes from Wheat Flour by Solid-Phase Extraction on Combined Silica and Aminopropyl Prepacked Columns

Name	Solvent, volume ratio	Volume (mL)	PL eluted[a,b]
N	Tetrahydrofuran/acetonitrile/ methanol (15:35:50)	10	PC[a]
Q	Acetonitrile/methanol (35:65)	10	LPC[a]
α	Chloroform/methanol/ ammonium hydroxide (85:15:0.1)	25	—[b]
β	(80:20:0.1)	20	NAPE[b]
γ	(75:25:0.1)	20	—[b]
δ	(50:50:0.1)	20	NALPE[b]
ω	(0:100:0.1)	20	—[b]

[a]Eluted on Bond-Elut prepacked silica column (500 mg).

[b]Eluted on Bond-Elut aminopropyl column (500 mg).

ii) thermal activation at 130°C for 1–2 hr; and iii) cooling at room temperature. At least 5 μg of lipid was spotted *vs.* known standards and, in some cases, the plates were deliberately overloaded (>50 μg per spot) to assess contamination of each fraction with other lipids. NL, GL and PL were chromatographed following the procedures described by Morrison *et al.* (3).

For NL and GL, plates were developed vertically in two solvent systems: i) Ethyl ether/toluene/ethanol/acetic acid (10:50:2:0.2); ii) ethyl ether/hexane (6:94), up to 12 cm and 18 cm, respectively, from the bottom (NL); and iii) of chloroform/acetone/acetic acid/water (10:90:2:3); iv) ethyl ether/acetic acid (99:1), up to 15 cm and 18 cm from the bottom, respectively (GL).

For PL separation, a single vertical development with chloroform/methanol/33% ammonium hydroxide (65:35:2.5) was performed up to 18 cm from the bottom. Visualization was accomplished, respectively, by charring with 50% sulfuric acid (NL, GL) and with the modified Dittmer-Lester reagent (PL) described by Stillway and Harmond (15).

HPLC. A Hewlett-Packard HPLC (Palo Alto, CA), composed of a 1050 pumping system, a rheodyne injector, a 1040A diode array detector set at 206 nm and a 9000 Pascal Chem Station, was used. Purity of eluate fractions and percent recovery of PL classes fractionated by SPE were assessed on a Silica Gel Hypersil column (3 μm, 60 × 4.6 mm i.d.) in line with a low-dispersion column-inlet filter, both from Hewlett-Packard. Lipids were eluted by a gradient system from an initial mobile phase of hexane/2-ol-propanol/water (60:38.5:1.5 to 52.6:42:5.4 in 9 min, then 38.7:51.6:9.7 at 15 min, and finally isocratically to 20 min). The columns were operated at a flow rate of 1 mL/min at room temperature.

RESULTS AND DISCUSSION

Separation of wheat flour lipids on prepacked silica columns. SPE of lipid classes from wheat flour on a single silica column with solvent mixtures of increasing polarity led to the fractionation of NL, GL and PL classes (Table 1). SE, TG, FA, 1,3-DG, 1,2-DG, MG, monogalactosyl glyceride (MGG), digalactosyl glyceride (DGG), PC and LPC were separated properly. A visual verification of the purity of each lipid fraction is shown in Figure 1. Elution of some lipid classes is closely dependent on the amount of stationary phase. On a Supelco silica column (1000 mg), 1,2-DG and 1,3-DG are coeluted by solvent F, and PC and LPC are eluted with solvents O and Q, respectively. If a Bond Elut silica column (500 mg) is used, 1,3-DG and 1,2-DG are completely separated and eluted with solvents E and F, respectively, as well as with increasing volumes of solvent E—20 mL (1,3-DG), 40 mL (1,2-DG). In addition, PC and LPC are less retained on the column, and emerged in solvent N (PC) and solvent P (LPC), respectively. Under the above conditions, no separation of NAPE and NALPE could be achieved, and they were found mainly in fraction M (Table 1).

Separation of wheat flour lipids on aminopropyl bonded-phase column. Separation of NL, GL and PL on aminopropyl bonded-phase column (Bond-Elut, 500 mg) under the elution scheme displayed in Table 1, differed from silica column fractionation. FA were eluted in the D fraction, 1,2-DG and 1,3-DG emerged from solvent E, MGDG and MGMG (H) were contaminated with α-MG and O-glucosides, PC and LPC emerged together in solvent M, contaminated by minority lipids and proteins, and NAPE and NALPE were strongly retained and could not be eluted from the column. As a result of the better separation of NL and GL on silica columns, PL fractionation was checked on aminopropyl columns by an elution scheme with increasing polarity and ionic strength (Table 3). Under these conditions, N-acyl-phospholipids could be separated properly by increasing ionic strength of binary mixtures methanol/water; but overlapping of PC and LPC, coeluted in fraction M, took place again.

Separation of main PL classes on combined prepacked silica and aminopropyl columns. On the basis of the

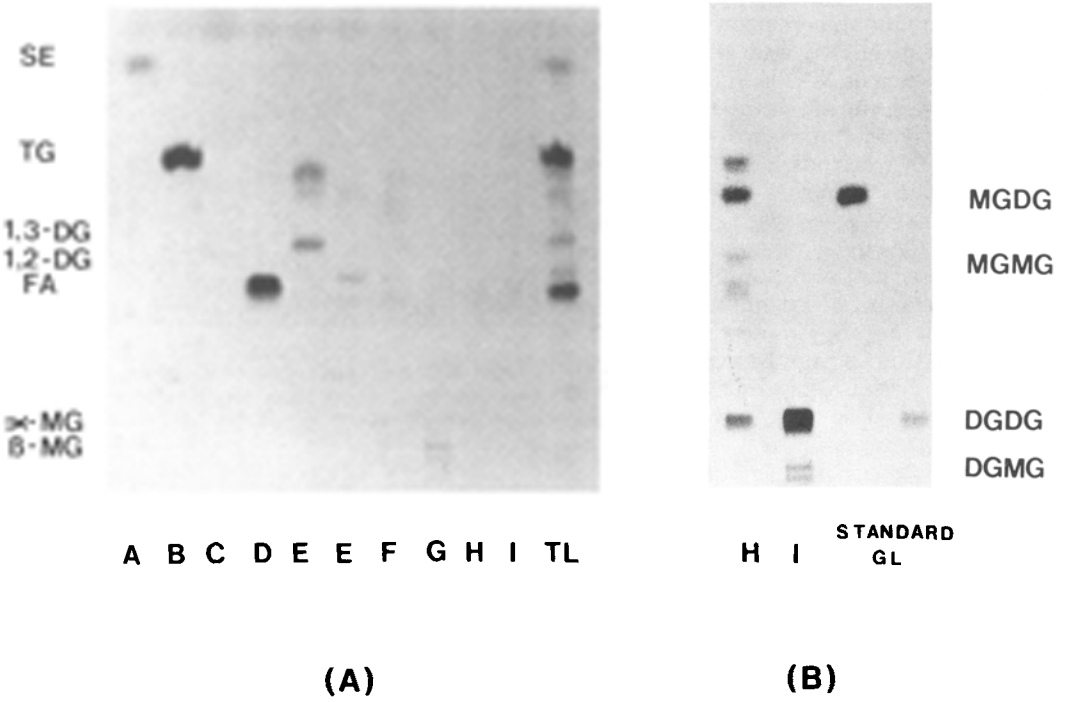

FIG. 1. Visual verification of the purity of neutral lipids (A) and glycolipid (B) classes separated by Bond-Elut from wheat flour. Lipid classes were fractionated on a silica prepacked column (500 mg) as described in Methods. Aliquots of each fraction were chromatographed and the plate was visualized as given in Methods. The letters under each lane represent the solvent used to elute the fraction chromatographed in that lane.

TABLE 3

Mobile Phases Used in the Aminopropyl Bonded-Phase Column for the Fractionation of Main Phospholipid Classes from Wheat Flour

Name	Solvent, volume ratio	Volume (mL)	PL eluted[a,b]
J	Tetrahydrofuran/acetonitrile/ propan-2-ol (40:35:25)	20	—
K	(30:35:35)	20	—
L	(20:35:45)	20	—
M	Tetrahydrofuran/acetonitrile/ methanol (15:45:40)	20	PC + LPC
N	(15:35:50)	20	—
R	Methanol	20	—
S	Methanol/0.3 mM ammonium hydroxide in 1 mM acetic acid (pH 4.0) (9:1)	20	NAPE
T	Methanol/3 mM ammonium hydroxide in 10 mM acetic acid (pH 4.0) (9:1)	20	—
U	Methanol/33 mM ammonium hydroxide in 0.1 M acetic acid (pH 4.0) (9:1)	20	NALPE
V	Methanol/33 mM ammonium hydroxide in 0.1 M acetic acid (pH 4.0) (5:5)	20	—

[a]Eight to nine mg of total lipids loaded onto aminopropyl column (500 mg).
[b]NL and GL fractionated as described in Table 1.

previous results obtained for separation of the main PL classes on silica and aminopropyl columns, the combination of both stationary phases and respective elution systems, successfully used for PC and LPC (silica), and NAPE and NALPE (aminopropyl), was checked for optimal separation.

Thus, PL were loaded onto a silica column and eluted with solvents J–Q (Table 1). Fractions L and M were pooled together, and NAPE and NALPE were fractionated as earlier reported (solvents R–V, Table 3). Results showed good separation and homogeneity of PC (N) and LPC (P); but coelution of NAPE and NALPE (U), indicating a close dependence of NAPE, retained more on the column than expected, on the number and polarity of the solvents previously used for the elution. For that reason, a large number of fractions would be needed for the separation of the four PL classes. To avoid this, the separation of N-acyl-phospholipids was performed under ionic suppression conditions by using an increasing polarity gradient of chloroform/methanol/ammonium hydroxide (Table 2) with good results. Optimum separation of NAPE and NALPE (aminopropyl) and of PC and LPC (silica) could be achieved (Fig. 2) from 10 mg TL maximum sample loading without loss of resolution. Cross contamination of PC and LPC was less than 14%, and recovery

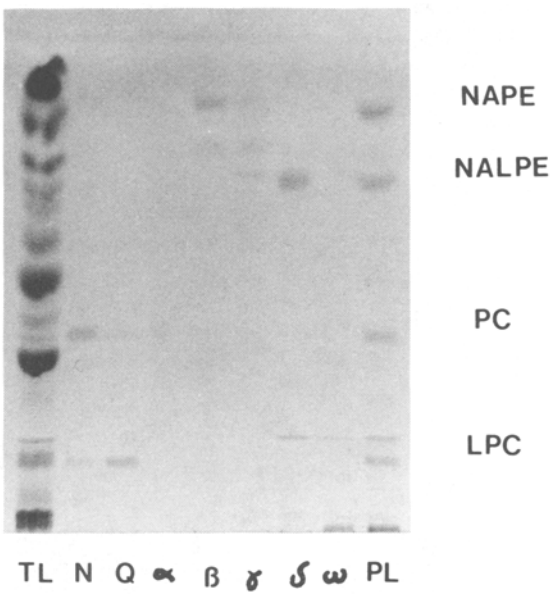

FIG. 2. Visual verification of the purity of the main phospholipid classes from wheat flour, fractionated on silica and aminopropyl prepacked columns. See Table 2 for mobile phases.

TABLE 4

Recovery of Phospholipids (% ± σ) from Bond-Elut Silica and Aminopropyl Prepacked Columns[a]

PL class	Bond-Elut column	Fraction N	Fraction Q	% Recovery[b] Fraction α	Fraction β	Fraction γ	Fraction δ	Fraction ω
PC	Silica	85.9 ± 0.9	12.8 ± 2.5	—	—	—	—	—
LPC	Silica	14.1 ± 0.9	87.2 ± 2.5	—	—	—	—	—
NAPE	Aminopropyl	—	—	—	87.6 ± 0.7	12.4 ± 0.7	—	—
NALPE	Aminopropyl	—	—	—	—	12.9 ± 0.7	81.0 ± 2.0	6.1 ± 1.3

[a]Lipids from wheat flour were extracted and fractionated as described in Materials and Methods.
[b]Percent of recovery of each PL class was assessed by normal-phase HPLC (see Methods) and calculated by assuming the total content of each class as the sum of the respective amounts in the eluted fractions.

of NAPE and NALPE was better than 87% and 81%, respectively (Table 4).

SPE is a rapid, efficient and versatile technique to perform the isolation and fractionation, on a preparative scale, of the main lipid classes from wheat flour. Chromatography on a single Silica Gel column allows the separation of NL into eight classes, as well as monogalactosyl and digalactosyl glycerides, in high purity and homogeneity. PL resolution can be achieved by properly combining aminopropyl and Silica Gel stationary phases. This method can probably be extended and adapted to the separation of complex lipid mixtures from sources other than wheat flour.

ACKNOWLEDGMENTS

This work was financed in part by the Comisión Interministerial de Ciencia y Tecnología (CICYT), Spain.

REFERENCES

1. Daniels, N.W.R., *J. Sci. Fd. Agric. 17*:20 (1966).
2. Chiu, C.-M., and Y. Pomeranz, *J. Food Sci. 31*:753 (1966).
3. Morrison, W.R., S. Leng Tan and K.D. Hargin, *J. Sci. Fd. Agric. 31*:329 (1980).
4. Mann, D.L., and W.R. Morrison, *Ibid. 25*:1109 (1974).
5. Chung, O.K., Y. Pomeranz, K.F. Finney and M.D. Shogren, *Cereal Chem. 54*:484 (1977).
6. Békés, F., U. Zawistowska, R.R. Zillman and W. Bushuk, *Ibid. 63*:327 (1986).
7. Dieffenbacher, A., B. Durieux and C. Jordan, *Rev. Franç. Corps Gras 35*:495 (1988).
8. Hamilton, J.G., and K. Comai, *J. Lipid Res. 25*:1142 (1984).
9. Juaneda, P., and G. Rocquelin, *Lipids 20*:40 (1985).
10. Figlewicz, D.A., C.E. Nolan, I.N. Singh and F.B. Jungalwala, *J. Lipid Res. 26*:140 (1985).
11. Kaluzny, M.A., L.A. Duncan, M.V. Merritt and D.E. Epps, *Ibid. 26*:135 (1985).
12. Hamilton, J.G., and K. Comai, *Lipids 23*:1146 (1988).
13. Janero, D.R., and C. Burghardt, *J. Chromatogr. 526*:11 (1990).
14. Christie, W.W., *Gas Chromatography and Lipids*, edited by W.W. Christie, The Oily Press, Ayr, Scotland, 1989, p. 86.
15. Stillway, L.W., and S.J. Harmond, *J. Lipid Res. 21*:1141 (1980).

[Received September 13, 1991; accepted January 9, 1991]