A Preferred Solvent System for High-Performance Liquid Chromatographic Analysis of Soybean Phospholipids with Evaporative Light-Scattering Detection

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ABSTRACT: Normal-phase high-performance liquid chromatography separations of phospholipids (PL) depend heavily on the variation in mobile phases. Incorporation of pure tetrahydrofuran to a mobile phase of chloroform, methanol, and ammonium hydroxide improved the separation of polar phospholipids. However, impurities were often found to be present in tetrahydrofuran that caused severe peak interferences with PL analytes. The use of a common chromatographic solvent *tert.*-butyl methyl ether, in lieu of tetrahydrofuran in mobile phases, eliminated problems associated with solvent contamination and yielded reproducible results. The modified method has been applied for the quantitative analysis of phospholipids in crude soybean oils.

JAOCS 73, 535–536 (1996).

KEY WORDS: Analysis, HPLC, mobile phase solvent, phospholipids, separations, soybean oil, *tert.*-butyl methyl ether, tetrahydrofuran.

Recently, we reported on the normal-phase high-performance liquid chromatographic (HPLC) analysis of major soybean phospholipids (PL) with mobile phases that contained tetrahydrofuran (THF) as a cosolvent (1,2). Adding THF to a chloroform/methanol/ammonium hydroxide mobile phase enhances the separation of phosphatidylcholine (PC) from phosphatidic acid (PA) (2). In these earlier studies, excellent separations of the PL components were achieved in all experiments with no background band overlaps, as pure THF was fortuitously employed in the mobile phase (1,2). However, during subsequent routine analyses, it was found that HPLC with different batches of HPLC-grade THF posed problems for PL analyses. We report the observation of variable degrees of peak interferences from impurities present in the commercial THF solvent. As an alternative, another common HPLC solvent, *tert.*-butyl methyl ether (BME), was used in place of THF for HPLC assays of soybean PL.

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EXPERIMENTAL PROCEDURES

Materials. PL standards PA, PC, phosphatidylethanolamine (PE), and phosphatidylinositol (PI) were obtained from (Avanti, Pelham, AL). A PL sample from a crude Hardin soybean oil was prepared according to procedures previously described (1). HPLC solvents and ammonium hydroxide were obtained from Fisher (Fair Lawn, NJ). BME was obtained from Aldrich (Milwaukee, WI). Several batches of THF with and without stabilizers from various industrial sources (Fisher, EM Separations, and Aldrich) were used as received.

HPLC. HPLC analyses with THF mobile phases were carried out according to previously published procedures (1). Aliquots (1–5 mg) of fresh PL samples in chloroform were injected onto an EM Separations (Gibbstown, NJ) Lichrospher Si 60/II column (3 μm, 250 × 4 mm i.d.) *via* a Rheodyne (Cotati, CA) injector (1). A modified mobile phase, consisting of solvent (A), chloroform/BME (75:15), and solvent (B) chloroform/methanol/ammonium hydroxide (1:92:7), was pumped at 0.5 mL/min. The elution cycle was initiated with a linear gradient of 100% to 0% (A) in 30 min, then held at 100% (B) for 10 min, and ended with a linear gradient of 0 to 100% (A) in 10 min.

RESULTS AND DISCUSSION

As stated earlier, HPLC of PL with suitable mobile phases of chloroform, reagent-grade THF, methanol, and ammonium hydroxide led to complete separations of PE, PI, PA, and PC with no additional peaks (1,2). However, clean HPLC peak profiles of PL were not always obtained because of frequent interfering ghost bands near PE peaks on HPLC chromatograms (Fig. 1A and B).

Consequently, a series of diagnostic experiments were conducted to determine the origin of the problems. An HPLC experiment with impure THF in a mobile phase system (1,2) and without sample injection produced a peak, which also appeared in another HPLC run without ammonium hydroxide in the mobile phase. Changes in gradient profiles of additional HPLC runs (no samples injected) resulted in the reappearance

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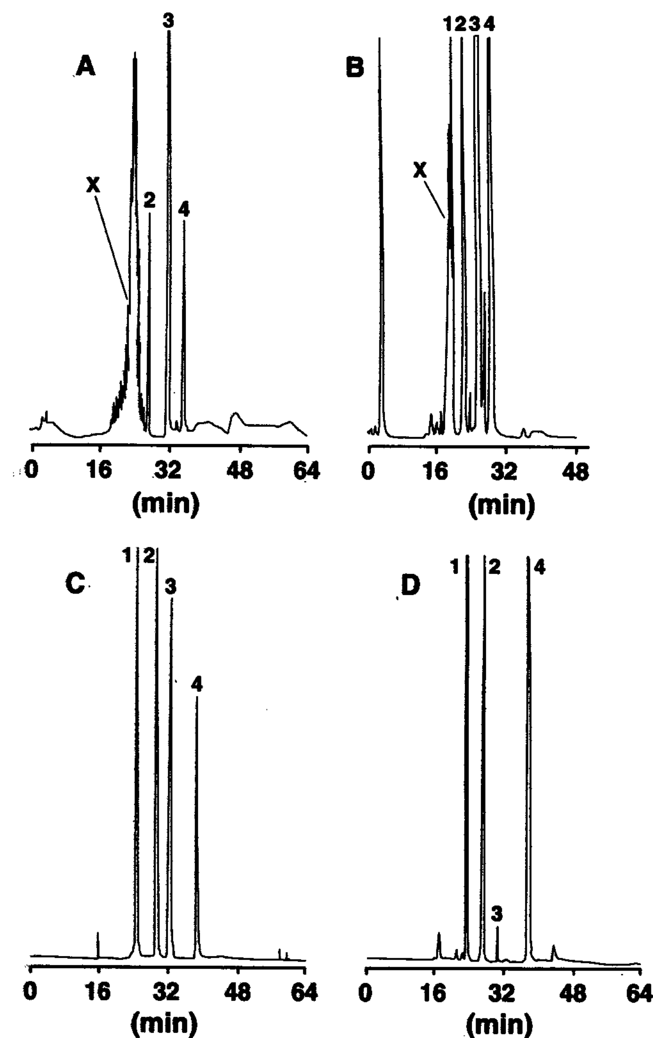

FIG. 1. A. Separations of phospholipid (PL) standards with moderately impure tetrahydrofuran (THF)/$CHCl_3$ (1:1) in a mobile phase. Peak identification: X = THF-derived band, 1 = phosphatidylethanolamine, 2 = phosphatidylinositol, 3 = phosphatidic acid, 4 = phosphatidylcholine. For high-performance liquid chromatographic (HPLC) conditions, see References 1 and 2. B. Separations of PL components in a degummed soybean oil with 14% moisture. Mobile phase contained impure THF/$CHCl_3$ (1:4). For peak identification and HPLC conditions, see A. C. Separations of PL standards with *tert.*-butyl methyl ether in a mobile phase. For peak identification and HPLC conditions, see respective A and the Experimental Procedures section. D. Separations of PL components in a genetically modified soybean oil. Mobile phase contained *tert.*-butyl methyl ether. For peak identification and HPLC conditions, see C.

of similar unknown peaks with different retention times. The experiments were later repeated with the same solvent systems, but with the column removed. No peak was observed in each instance. Upon addition of 25% methanol into solvent (A), the unidentified peaks were significantly diminished and shifted. They eventually disappeared from chromatograms

TABLE 1
Normal-Phase High-Performance Liquid Chromatography (HPLC)–Evaporative Light-Scattering Detection of Crude Soybean Oils[a]

Sample	Composition (%)[b]			
	PE	PI	PC	PA
Hardin-1	27.4	22.0	48.5	2.1
	(3.4)[c]	(6.1)	(1.9)	(1.2)
Hardin-2	26.1	24.7	43.2	6.0
	(7.2)	(5.6)	(5.3)	(1.0)

[a]See the Experimental Procedures section for HPLC conditions.
[b]Compositions are mean values of triplicate determinations based on calibration with individual phospholipid standards. PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine; PA, phosphatidic acid.
[c]Coefficients of variation are given in parentheses.

obtained with solvent (A) devoid of THF. Further, the peak interferences were not seen at all in isocratic HPLC experiments with solvent (B) alone. The results appeared to indicate that some intrinsic impurities in commercial THF were presumably retained, concentrated, and eluted from the silica column as peaks or bands that often interfered with the PL peaks of interest. The exact nature of the THF-derived contaminants was not further investigated.

To solve the HPLC problems associated with impure THF as described here, we replaced THF with BME for the analysis of PL in soybean oil. With BME in the mobile phase, the four major soybean PL were well separated on a 3-μm Lichrospher silica column (3), and separations were at least comparable and often superior to those in previous reports (1). The chromatograms obtained with the modified mobile phase system invariably exhibited neat PL peaks with no interfering foreign peaks (Fig. 1, C, and D). Moreover, the HPLC results were highly reproducible, regardless of which batches of commercial-grade BME were used in the analysis. Table 1 shows examples of the HPLC analysis of PL in two crude oils derived from two different bags of identical Hardin soybeans. This modified HPLC method has been routinely used in the analysis of PL in vegetable oil samples.

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[Received July 27, 1995; accepted January 4, 1996]