HPLC Analysis of Phospholipids by Evaporative Laser Light-Scattering Detection

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High-performance liquid chromatography (HPLC) for analysis of phospholipids has traditionally employed ultraviolet detection of the eluted compounds. The evaporative laser light-scattering detector (ELSD) offers new opportunities for quantitative analysis of lipids. Phospholipids were isolated from crude and degummed oils prepared from soybeans subjected to storage at high moisture content. Analytical and preparative separations of phospholipids by normal-phase HPLC were accomplished. Major class fractions were analyzed by transmethylation and capillary column chromatography for fatty acid composition, and by reverse-phase C-18 HPLC (RP-HPLC) for molecular species composition. The RP-HPLC-ELSD system was limited to the analysis of phosphatidylcholine and phosphatidylethanolamine.

KEY WORDS: Capillary gas chromatography, crude soybean oil, damaged soybeans, degummed soybean oil, high performance liquid chromatography, phospholipids.

High-performance liquid chromatography (HPLC) for analysis of phospholipids has traditionally employed ultraviolet detection of the eluted compounds (1–6). Several researchers have only reported on the qualitative analysis of phospholipids (1–3), while quantitative analysis requires the development of calibration curves (5) or the use of phosphorimetry (4,6). We previously reported the analysis of phospholipids (PL) isolated from damaged crude oils by HPLC with ultraviolet detection at 206 nm (7).

Mass detectors have been utilized for quantitative analysis of phospholipids (8–11). Both flame ionization (8,9) and light-scattering detection systems (10,11) reportedly also give good quantitation. Recently, Breton *et al.* (11) separated and quantitated the main classes of animal membrane phospholipids by a procedure in which binary-solvent HPLC was combined with a light-scattering detector.

Crude oils from soybeans that have been damaged in the field, during storage or during handling and transportation contain significant amounts of nonhydratable phospholipids (NHP) (12,13). NHP are those phosphatides that during the degumming of crude soybean oil with water do not hydrate, swell, form gels or precipitate from the oil and that are not removed by centrifugation (14). Neilsen (14) reported that the NHP are composed of phosphatidic (PA) and lysophosphatidic acids. Other researchers have hypothesized that the NHP are products of the action of phospholipase D on the base group of phosphatidylcholine (PC) and/or phosphatidylethanolamine (PE) (15,16). Nakayama *et al.* (15) stored soybeans at 13% moisture for six months and determined the changes in lipid composition of crude oil extracted from ground soybeans by two-dimensional thin-layer chromatography. They found that PA was preferentially retained in degummed oil.

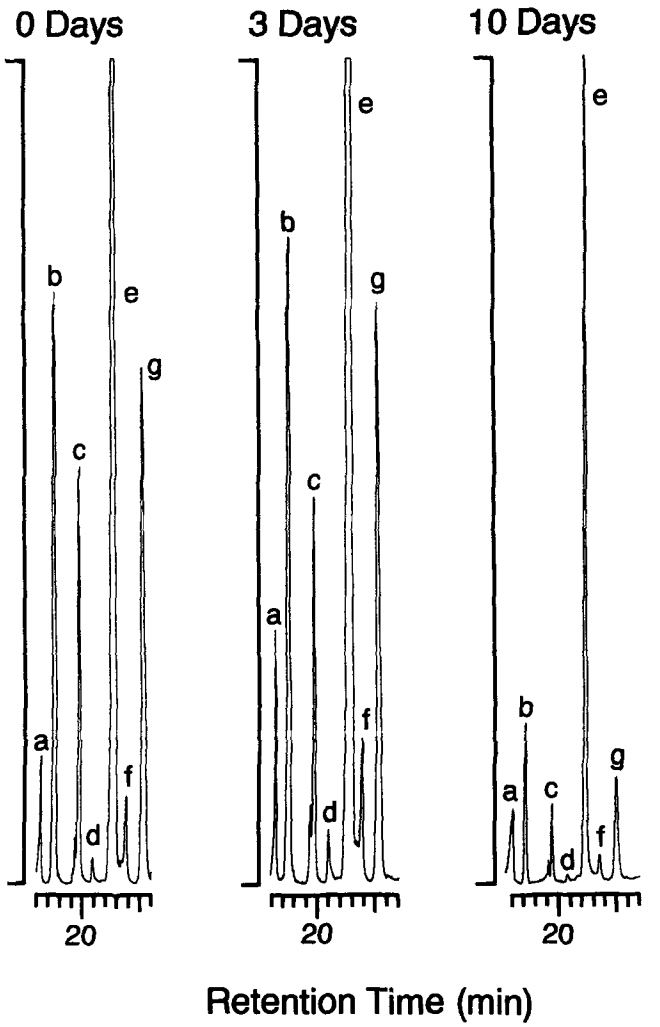

FIG. 1. HPLC of phospholipids isolated from crude oils extracted from soybean flakes stored at high moisture (14%). Column, Lichrosorb Si-60, 10μ; solvents, A. $CHCl_3$:THF (1:1), B. CH_3OH:NH_4OH:$CHCl_3$ (92:7:1); linear gradient elution 0% to 100% (B) in 30 min, 15 min at 100% (B) and 0% to 100% (A) in 10 min; flow rate, 1 mL/min; evaporative light scattering detection; peaks: a, unidentified component; b, phosphatidylethanolamine; c, phosphatidylinositol; d, unidentified component; e, phosphatidic acid; f, unidentified component; g, phosphatidylcholine.

List *et al.* (17), employing laboratory simulations of commercial processing procedures, showed that formation of NHP in soybeans and soy flakes was influenced by moisture, heat and enzymatic activity. Formation of NHP was particularly rapid in soy flakes stored at 14% moisture and 40°C. Inactivation of phospholipase D by treatment of soy flakes with live steam or microwave treatment of whole soybeans inhibited the formation of NHP.

Results obtained in our previous study (7) indicated that the susceptibility of PL to attack by phospholipase D was PC > phosphatidylinositol (PI) > PE. PA accumulated as the deterioration of the oil progressed.

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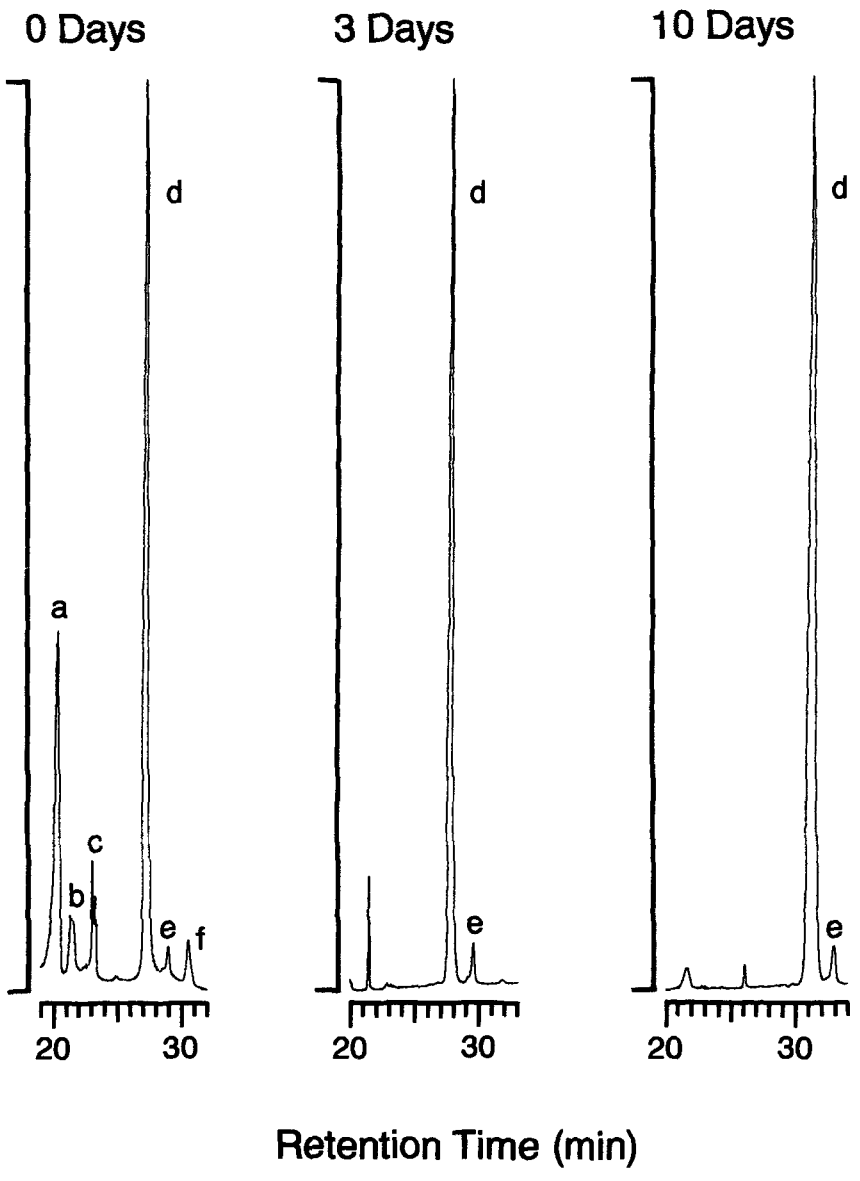

FIG. 2. HPLC of phospholipids isolated from degummed oils from soybean flakes stored at high moisture (14%). Conditions as in Figure 1. Peaks: a, phosphatidylethanolamine; b, unidentified component; c, phosphatidylinositol; d, phosphatidic acid; e, unidentified component; f, phosphatidylcholine.

To better understand the formation of NHP during soybean deterioration, we have modified Breton and colleagues' (11) procedure to characterize the PL fraction isolated from crude soybean oils extracted from soy flakes stored at high-moisture content and their degummed oils prepared by laboratory simulations of commercial degumming procedures.

EXPERIMENTAL PROCEDURES

Materials. Williams-certified seed-grade soybeans purchased locally were used. All solvents were reagent-grade suitable for HPLC. Phospholipid standards (PA, PC, PE and PI) were all derived from soybeans (Avanti Polar Lipids, Pelham, AL). Silica Gel (60–200 mesh) was purchased from Baker Analytical Co. (Newark, NJ).

Sample preparation. Whole beans (8000 g) were tempered to 14% moisture content by placing them in plastic bags with the required amount of water and allowing them to equilibrate at ambient temperature until all moisture had been adsorbed, *i.e.*, overnight. Soybeans were then cracked, dehulled and flaked as described previously (14). Duplicate samples of soy flakes, 1000 g each, were aged for 3, 5 and 10 days and, along with duplicate unaged samples, were hexane-extracted; crude soybean oil (SBO) was recovered and subsequently degummed according to laboratory procedures previously described (17,18). Soybeans at 10% moisture were processed as an unaged control sample.

Phosphorus contents of the crude and degummed oils were determined by AOCS standard method Ca 12-55 (19). PL were isolated from the crude and degummed oils prior

TABLE 1

Phosphorus Content and Phospholipid Composition (wt% ± S.D.) of Crude and Degummed Oil from Soybean Flakes Stored at High Moisture (14%)[a]

Days of storage	Phosphorus content (ppm)	Phospholipid classes (%)			
		PE	PI	PA	PC
Crude					
0	900 ± 3.8[b]	20.2 ± 0.8[b]	11.8 ± 0.5[b]	46.6 ± 1.8[b]	21.4 ± 0.8[b]
3	773 ± 3.1[c]	19.4 ± 0.6[b]	9.4 ± 0.3[c]	51.7 ± 1.7[b]	19.5 ± 0.6[b]
5	728 ± 3.1[c]	17.8 ± 0.5[b]	7.1 ± 0.2[c]	61.6 ± 1.9[c]	13.5 ± 0.4[c]
10	617 ± 2.7[d]	12.9 ± 0.4[c]	11.7 ± 0.3[b]	63.2 ± 1.7[c]	12.3 ± 0.3[c]
Degummed					
0	304 ± 1.3[b]	16.0 ± 0.2[b]	5.0 ± 0.1[b]	73.0 ± 0.9[b]	6.0 ± 0.1[b]
3	196 ± 0.8[c]	8.9 ± 0.1[c]	n.d.	91.1 ± 0.8[c]	n.d.
5	212 ± 0.8[c]	4.5 ± 0.1[d]	0.8 ± 0.0[c]	94.3 ± 0.8[c]	0.4 ± 0.0[c]
10	242 ± 1.0[c]	5.5 ± 0.1[d]	n.d.	94.3 ± 0.9[c]	0.2 ± 0.0[c]
10% Control	1000 ± 4.2	41.1 ± 1.7	19.5 ± 0.8	7.5 ± 0.3	31.9 ± 1.4

[a]Abbreviations: PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; n.d., not detected. Values in the same column (within the same subgroup) with different superscript letters (b–d) are significantly different ($P<0.05$) as measured by Duncan's Test.

to analysis by HPLC. Approximately 5 g of oil was fractionated on a 5-g column of silica gel (60–200 mesh), by sequential elution with 200 mL chloroform, 100 mL acetone, 100 mL methanol and 100 mL 0.1% phosphoric acid in methanol. The methanol fractions were combined for recovery of the total PL. Solvent was removed in a rotary evaporator at room temperature, the sample residue was dissolved in chloroform, washed three times with 1 mL of saturated salt solution, then sodium bicarbonate was added until neutral. The sample was dried with sodium sulfate and filtered, and the solvent was removed by rotary evaporation at room temperature. The sample was then frozen until analyzed. Samples were diluted with chloroform to give a 1 mg/μL solution for analysis.

Methods. Analytical and preparative PL separations were performed with a Spectra-Physics Model 8800 solvent delivery system, a Rheodyne 7125 injector (10 μL and 100 μL sample loops for analytical and preparative separations, respectively) (Spectra-Physics, Inc., San Jose, CA) and a Varex Evaporative Light Scattering Detector (ELSD) (model ELSD II, Varex Corp., Rockville, MD). The analog signal from the ELSD was interfaced with a real-time computer (ModComp Inc., Fort Lauderdale, FL) programmed to calculate peak areas and component relative percentage composition. PL (3–5 mg) were analyzed with a normal-phase column, Lichrosorb Si-60, 10μ (250 × 4.6 mm i.d.) (Alltech, Deerfield, IL), and a linear-gradient elution from chloroform/tetrahydrofuran, 1:1 (v/v) to methanol/ammonium hydroxide/water, 92:7:1 (v/v/v) in 30 min, a hold for 15 min, and a reverse linear gradient to the starting solvent in 10 min. Flow rate was 1 mL/min.

PL classes were separated and collected from a normal-phase preparative column, Lichrosorb Si-60, 10μ (250 × 10 mm i.d.) (Alltech), and the elution gradient was identical with that used in the analytical column; however, the flow rate was 2 mL/min. A micrometric sample splitter was set at a 40/60 ratio to deliver 0.8 mL/min to the evaporative laser light-scattering detector (ELSD) and 1.2 mL/min for peak collection. Multiple samples of 25–50 mg each were separated to accumulate adequate amounts of each peak for subsequent reverse-phase HPLC separations of molecular species.

Reverse-phase HPLC separations were perfomed with a RSIL-C18, HL, 10μ (250 × 4.6 mm i.d.) column (Alltech). The eluting solvent was methanol/water/chloroform at 20:1:1 (v/v/v) for PE and 30:1:1 (v/v/v) for PC. Flow rate was 2 mL/min. The column flow was split at a 50/50 ratio to deliver 1.0 mL/min to the ELSD and 1.0 mL/min to collect peaks for subsequent analysis of fatty acid composition.

PL fractions were converted to fatty acid methyl esters (FAME) by HCl-methanol (20). Analysis of FAME was performed in a Varian model 3400 gas chromatograph equipped with a 30 m × 0.25 mm (0.2 μm coating) SP 2330 fused-silica capillary column (Supelco, Bellefonte, PA). Helium carrier gas and a flame-ionization detector were used. The oven temperature was programmed from 170–220°C at 3°C/min, after an initial 10-min holding period.

The data were analyzed by Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (Duncan's Test) (21). Statistical significance was accepted at a level of $P < 0.05$.

RESULTS AND DISCUSSION

Breton *et al.* (11) reported that the ELSD gives a linear response for amounts of 5–40 μg of different PL and reproducible results in quantitative analysis. We confirmed his findings in preliminary analyses of PL standards. Representative chromatograms of the analysis of isolated PL from crude and degummed oils initially and after storage of high-moisture flakes are presented in Figures 1 and 2, respectively. Peak identification was accomplished by comparison with the retention times of standards. The peak shapes and phospholipid elution profiles obtained with the current HPLC mobile phase and ELSD are noticeably improved relative to those previously reported with ultraviolet (UV) detection (7). As reported by previous investigators (11), the baseline was stable, and reproducible retention times and quantitative analysis were achieved.

The phosphorus contents and PL distributions deter-

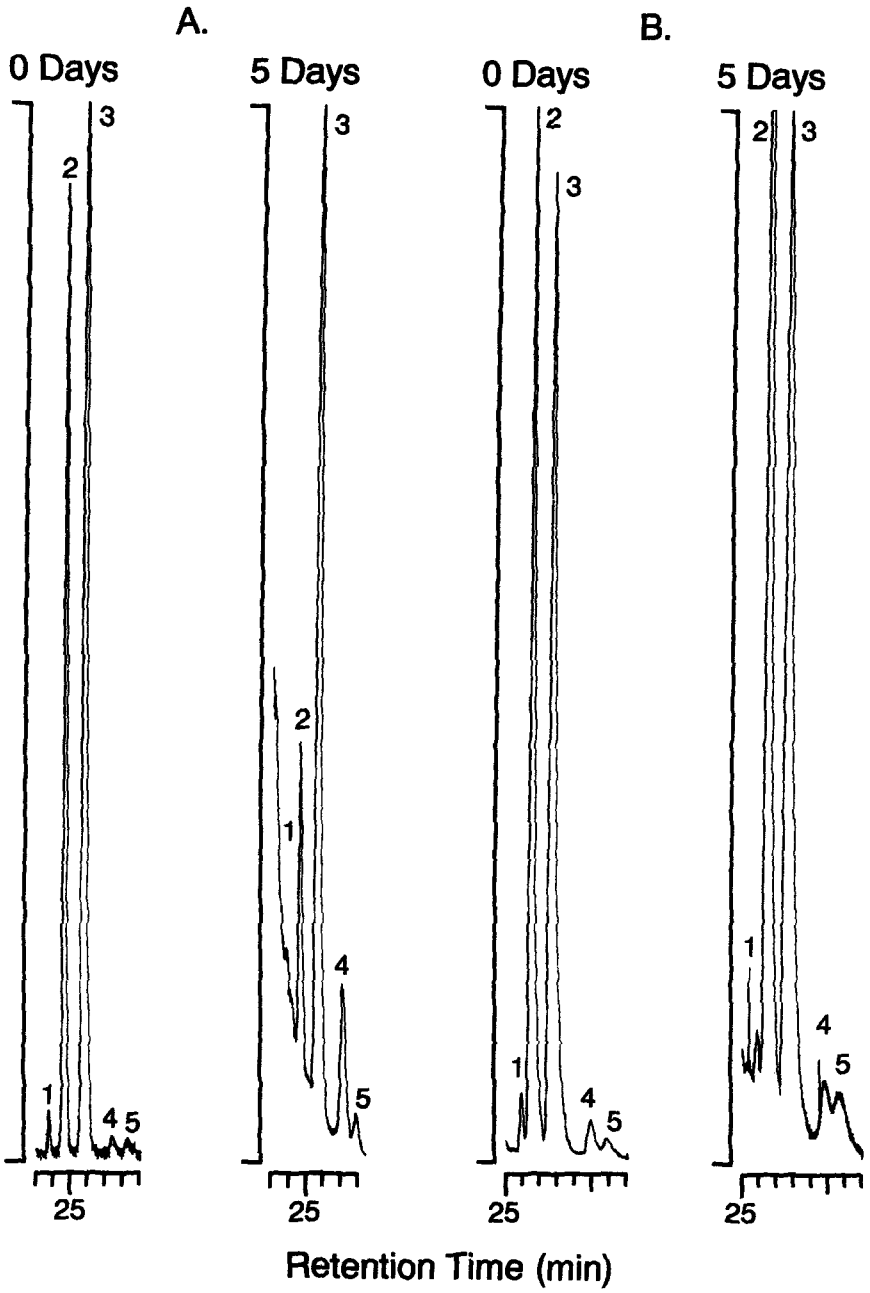

FIG. 3. RP-HPLC analysis of A. phosphatidylethanolamine and B. phosphatidylcholine. Column, RSIL-C18 HL, 10μ; solvent, methanol/water/chloroform, for A. (20:1:1), for B. (30:1:1); flow rate, 2 mL/min (split 1.0 mL/min to detector; 1.0 mL/min, collected); evaporative laser light-scattering detector; peaks identified by capillary GC, Tables 2 and 3.

mined by HPLC-ELSD for crude and degummed oil are presented in Table 1. Duplicate samples showed a coefficient of variation (CV) of less than 5%. A decrease in the total phosphorus content of crude oil is indicative of PL destruction through the action of phospholipases, *i.e.*, cleavage of phosphorus-containing moieties (15,16). PL deterioration has been reported to be particularly rapid in soybeans and soy flakes at 14% moisture (22).

In the present studies, comparing analyses of the PL from unaged 10%- and 14%-moisture soy flakes indicated that 10% of the PL was decomposed during the initial extraction process. After 10 days storage, nearly 40% of the PL was destroyed. Analysis of the phospholipid classes indicated that PE and PC decreased from 41.1 and 31.9% to 12.9 and 12.3%, respectively, while PA increased significantly, both initially and during storage. The NHP content of the crude oil, estimated from the phosphorus content of the degummed oil, ranged from 30 to 40%. Unaged soyflakes at 10% moisture content gave crude oil containing only 7% NHP. The main PL component of degummed oil is PA. This is in agreement with earlier findings reported by Nakayama *et al.* (15).

TABLE 2

Molecular Species Composition (%) of PE from Crude Oil from Soybean Flakes Stored at High Moisture (14%)[a]

Peak number	Fatty acid class[b]	0 Days	5 Days
1	C16:0/C18:0	1.2	tr
2	C18:2/C18:2	37.2[c]	14.6[d]
3	C16:0/C18:2	59.0[c]	69.4[d]
4	C16:0/C18:1	1.1[c]	13.3[d]
5	C18:0/C18:1	1.5[c]	2.8[c]

[a]Reversed-phase HPLC as described in the Experimental Procedures section. Values in the same row with different superscript letters (c-d) are significantly different (P>0.05) as measured by Duncan's Test.

[b]Identification by capillary-GC analysis of methyl esters of collected peak.

TABLE 3

Molecular Species Composition (%) of PC from Crude Oil from Soybean Flakes Stored at High Moisture (14%)[a]

Peak number	Fatty acid class[b]	0 Days	5 Days
1	C16:0/C18:0	tr	3.1
2	C18:2/C18:2	64.4[c]	47.7[d]
3	C16:0/C18:2	35.6[c]	49.2[d]
4	C16:0/C18:1	tr	—
5	C18:0/C18:1	tr	—

[a]Reversed-phase HPLC as described in the Experimental Procedures section. Values in the same row with different superscript letters (c-d) are significantly different (P>0.05) as measured by Duncan's Test.

[b]Identification by capillary-GC analysis of methyl esters of collected peak.

Chromatograms for the reversed-phase HPLC analysis of PE and PC that were isolated from crude oils extracted from 0-time and 5-day-storage soy flakes are shown in Figure 3. Each peak was collected and FAME were prepared and analyzed by capillary gas chromatography (GC) to identify the molecular species. The quantitative analysis of molecular species is presented in Tables 2 (PE) and 3 (PC). The contents of the di-C18:2 species in both PE and PC were selectively reduced by phospholipid destruction during high-moisture storage. This may indicate that phospholipid deterioration under these conditions occurs enzymatically both by action of lipoxygenases and peroxidases (23,24), *i.e.* oxidatively, and by action of phospholipases (15,16), *i.e.* cleavage of phosphorus-containing moieties or end groups.

Separations of the molecular species of PI and PA were not achieved by use of the reverse phase high-performance liquid chromatography-evaporative light-scattering detector (RP-HPLC-ELSD) system. RP-HPLC systems with UV detection have been developed to analyze the molecular species of standard samples of PI and PA (25,26). Application of these systems to the PI and PA fractions isolated in this study will be reported in a later publication.

ACKNOWLEDGMENT

R.K. Holloway provided assistance in the preparation and high-moisture aging of soybean flakes.

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[Received September 19, 1991; accepted February 12, 1992]