Analysis of Lipid Classes by Solid-Phase Extraction and High-Performance Size-Exclusion Chromatography

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An improved method to analyze lipid classes of edible oils and fats by solid-phase extraction (SPE) and high-performance size-exclusion chromatography (HPSEC) is presented. A mixture of lipid standards was fractionated by the solid-phase extraction procedure (NH_2 phase) into polar and nonpolar fractions; these were then submitted to analysis by HPSEC. The size-exclusion chromatographic columns were three styrene/divinylbenzene columns with pore sizes of 100 Å and 50 Å. Light-scattering was used for the detection system, and the parameters of the detector were optimized to minimize the difference between the responses of the compounds studied. With this procedure it was possible to separate the following lipid classes: triacylglycerols, diacylglycerols, monoacylglycerols and free fatty acids, sterols, sterol esters, tocopherols and carotenoids. Quantitative analysis was studied for a light-scattering detector with several lipid standards of different molecular weights and unsaturation levels.

KEY WORDS: High-performance size-exclusion chromatography, light-scattering detector, lipid class analysis, solid-phase extraction.

High-performance size-exclusion chromatography (HPSEC) has been used to analyze triacylglycerols, diacylglycerols, monoacylglycerols and free fatty acids in edible oils (1). It has also proven to be a useful method to evaluate the alteration profiles of oils. With HPSEC, a heterogeneous material such as autoxidized oil can be fractionated into a few main groups of compounds that have equal hydrodynamic volumes. A combination of adsorption and size-exclusion chromatography permits analysis of different alteration products such as polymers, polar triacylglycerol monomers, diacylglycerols, monoacylglycerols and free fatty acids. Compounds deriving from autoxidative, thermooxidative and hydrolytic alteration can be distinguished by HPSEC (2,3). Several HPSEC methods have been presented that are particularly useful for analyzing the polymerization level of frying oils (2–5) or fish oils (6,7).

If these lipid classes are to be analyzed with size-exclusion chromatography, other lipids that are present in the oils and fats must be considered. Free and bonded sterols are present in most edible oils. The sterol content of many vegetable oils is reported to be in the range of 60–900 mg/100 g of oil (8). Also many other minor lipid classes are present in edible oils and fats and might in some instances interfere with this analysis. The total tocopherol content of edible oils and fats is reported to be 3–250 mg/100 g (9), but in some fish oils the total tocopherol content is as high as 800 mg/100 g (5). In contrast, the contents of alteration products analyzed by HPSEC can be as low as 50 mg/100 g (4).

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The refractive index detector is most commonly used for HPLC analyses of lipids. The ultraviolet (UV) detector is less widely used, owing to the weak ultraviolet absorbance of many triacylglycerols. The light-scattering detector (LSD) has become increasingly popular for lipid analysis (10–15) and has also been used for quantitation of the autoxidation products in marine lipids (7). The principle and the theory of the light-scattering detector have been described in several papers (10,16,17). In the light-scattering detector the eluent is nebulized by a gas stream and the vapor goes into a heated pipe where the solvent molecules evaporate. The solute molecules, as fine particles, pass through a narrow light beam, and the scattered light is collected by a photomultiplier. The light-scattering detector is considered universal because the photomultiplier signal is proportional to sample concentration. However, response factors for different compounds are reported to depend slightly on the detector parameters, *e.g.*, temperature and gas flow, as well as on the melting point, weight and shape of the molecules (17).

This study presents a method to analyze the different lipid classes of edible oils by HPSEC. Solid-phase extraction (SPE) separation into polar and nonpolar fractions is needed because the large amount of triacylglycerols present in edible oils and fats would interfere with the HPSEC separation of other lipid classes with elution volumes near that of triacylglycerols. The lipid classes of interest were triacylglycerols, diacylglycerols, monoacylglycerols, free fatty acids, sterols, sterol esters, tocopherols (α-tocopherol) and carotenoids (β-carotene). A light-scattering detector was used for the quantitation of triacylglycerols, diacylglycerols, monoacylglycerols and free fatty acids. The effect of the detector parameters on the response factors of different lipid standards was studied.

EXPERIMENTAL PROCEDURES

Solid-phase extraction. The solid-phase extraction columns tested were silica and NH_2 columns (Bond Elut 500 mg, Analytichem International, Harbor City, CA). Their separation efficiency was tested with triolein, diolein, monoolein and oleic acid (NuChek Prep, Inc., Elysian, MN), a sterol mixture (ultrasitosterol, Kaukas Oy, Lappeenranta, Finland) and a synthetized sterol ester mixture as standards. The sterol ester mixture was obtained from Raisio Group, Edible Oils and Fats Division, Research Laboratory (Raisio, Finland). It had been synthetized from ultrasitosterol and rapeseed oil to achieve a sterol and fatty acid composition comparable to that of rapeseed oil. Two different standard mixtures were used for SPE optimization: i) a mixture of 80% triolein, 4% diolein, 5% sterol ester, 3% monoolein, 2% free oleic acid and 6% sterols; and ii) a mixture of 92.7% triolein, 4.3% diolein, 2.3% monoolein and 0.2% free oleic acid. Standard mixtures were prepared to correspond to an edible oil

sample that has 80–90% triglycerides and 1–5% of each of the minor lipid classes.

The amount of the sample was 20–60 mg/SPE column. The nonpolar fraction was eluted with 20 mL hexane-diethylether (9:1), and the polar fraction with 10 mL chloroform: methanol (2:1) and 10 mL methanol:acetic acid (98:2). All the solvents were high-performance liquid chromatography (HPLC) grade (Rathburn Chemicals Limited, Walkerburn, Scotland, United Kingdom). The solvent mixtures were allowed to flow under gravity at an approximate solvent flow rate of 0.5–1 mL/min.

The recovery of lipid classes was calculated from an HPSEC chromatogram based on refractive index detector response. The efficiency of the SPE separation was also checked by thin-layer chromatography (TLC), which was carried out on plates coated with Kieselgel 60 (Merck, Darmstadt, Germany). The eluent system was hexane: diethylether:acetic acid (80:20:2), and the fractions were detected by spraying with 10% sulphuric acid in methanol and heating.

High-performance size-exclusion chromatography. The HPLC system used was a Waters 6000A HPLC pump equipped with a Rheodyne injector (Model 7125) with a 50 μL loop (Waters, Milford, MA). A refractive index detector (Waters 410) was used for testing the SPE separations; otherwise the light-scattering detector (Cunow DDL21, Cunow Department DMS, Gergy, St. Christophe, France) was used. The gas used was compressed air filtered before the detector through a 0.45-μm filter. The pressure used was 1.0 bar giving a flow rate of 7 L/min. The size-exclusion column series included one 100 Å and two 50 Å columns (PLGEL, 30 × 0.8 cm i.d., Polymer Laboratories Inc., Amherst, MA). The stationary phase is a highly crosslinked styrenedivinylbenzene copolymer, particle size 5 microns. The mobile phase was HPLC-grade tetrahydrofuran (Rathburn Chemicals Limited). Butylated hydroxytoluene (BHT) (0.025%) was used as a stabilizer, and the solvent was kept under helium atmosphere to avoid peroxide formation. The flow rate was 0.6 mL/min at ambient temperature. The sample concentration was approximately 0.1 mg/mL and the injection volume was 10–50 μL. The integrator used was a Hewlett-Packard model 3390A (Palo Alto, CA).

Light-scattering detector (LSD) optimization. The optimized LSD parameters were the working pressure of the gas, make-up gas flow and evaporation temperature. Detector responses were obtained for the following lipid standards (NuChek Prep Inc.): triacylglycerols (18:2, 18:1, 18:0 and 16:0), diacylglycerols (18:2, 18:1, 18:0 and 16:0), monoacylglycerols (18:2, 18:1, 18:0 and 16:0) and free fatty acids (18:2, 18:1, 18:0 and 16:0). The response of each compound was analyzed in duplicate.

The fatty acids selected are the most common in vegetable oils. The standards were selected to study the effects of melting point and unsaturation level, as well as molecular shape and weight of the lipid molecules on the response of the detector.

The relative responses of the compounds were calculated as peak area units/μg. All response factors were normalized to that of triolein at a detector temperature of 54°C.

RESULTS AND DISCUSSION

Solid-phase extraction. SPE fractionation of the standard mixtures with silica columns was not reproducible, possibly because water content had too great an effect on the cartridge efficiency. This problem was eliminated when amino columns were used. As an adsorbent the amino phase is slightly less polar than silica and moisture has less effect on its lipid adsorption capacity.

The recovery of the lipid classes from the two standard mixtures is given in Table 1. The nonpolar elution system of hexane:diethylether (9:1) permitted 98% and 92% of the triacylglycerols and sterol esters, respectively, to be eluted from the amino columns (Table 1). Less than 1% of the triacylglycerols and no sterol esters were detected in the polar fraction. Quantitative elution of the polar compounds was more difficult to accomplish. When the polar compounds were eluted with 10 mL of methanol, recovery was unsatisfactory (60–80%). To elute all the test compounds quantitatively, chloroform-methanol (2:1, 10 mL) and methanol-acetic acid (98:2, 10 mL) had to be used as eluents. The recovery of the compounds then was 91–110%. The SPE separation of lipid classes after HPSEC analysis is shown in Figure 1.

The amount of polar material in the test mixture affected fractionation. Small amounts (0.2%) of free fatty acids eluted into the nonpolar fraction, but larger amounts (2%) required methanol: acetic acid (98:2) for quantitative elution.

Separation of lipid classes by HPSEC. One column of 100 Å and two 50 Å columns were used in series. This configuration allowed separation of triacylglycerols, diacylglycerols, sterol esters, monoacylglycerols, free fatty acids and free sterols (Fig. 2). β-Carotene (elution volume V_E = 19.88 mL) eluted near monoolein (V_E = 20.10 mL), and α-tocopherol (V_E = 20.45 mL) eluted between monoolein and oleic acid (V_E = 20.83 mL). A flow rate of 0.6 mL/min was considered optimal because resolution was good while the effect of band broadening was minimal. With the three columns, analysis time was 35 min. This type of separation was not possible with the shorter column

TABLE 1

Recovery (% mean ± SD) of Lipid Classes in Solid-Phase Extraction of Standard Mixtures[a]

	Standard mixture	
	1 (n = 4)	2 (n = 2)
Nonpolar fraction		
Triolein	98 ± 1	99 ± 2
Sterol ester	92 ± 4	*b*
Diolein	<0.1	<0.1
Monoolein	<0.1	<0.1
Oleic acid[c]	<0.1	80 ± 20
Sterol mixture[c]		*b*
Polar fraction		
Triolein	<1	<0.1
Sterol ester	<0.1	*b*
Diolein	95 ± 2	95 ± 2
Monoolein	96 ± 5	103 ± 2
Oleic acid[c]	96 ± 3	
Sterol mixture[c]		

[a] Mixture 1 and 2: see Materials and Methods.

[b] Not in the standard mixture.

[c] Free fatty acid and sterol quantitated as one peak.

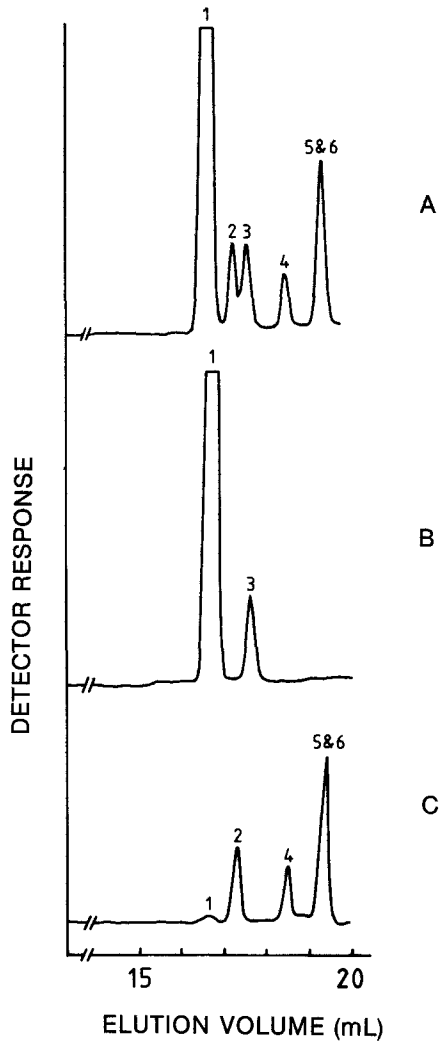

FIG. 1. Separation of lipid classes by solid-phase extraction. HPSEC chromatograms of standard mixtures: 1. triolein, 2. diolein, 3. sterol ester, 4. monoolein, 5. oleic acid and 6. sterol mixture. (A) Standard mixture before solid-phase extraction; (B) nonpolar fraction; and (C) polar fraction. Refractive index detector.

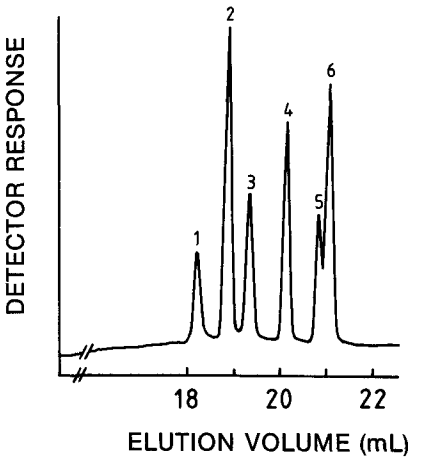

FIG. 2. HPSEC chromatogram of lipid standard mixture: 1. triolein, 2. diolein, 3. sterol ester, 4. monoolein, 5. oleic acid and 6. free sterol. Light-scattering detector. Conditions: See Experimental Procedures.

series studied (18). Diacylglycerols or free fatty acids could not be analyzed, if bonded or free sterols were present in the oil, without pretreatment of the sample or better separation efficiency of the HPSEC columns. The elution volume of tocopherols is equal to that of monoacylglycerols if a shorter column series is used.

Earlier papers dealing with HPSEC analysis of triacylglycerols, diacylglycerols, monoacylglycerols and free fatty acids provide no information on the interfering lipid classes.

Quantitation with LSD. The effect of detector parameters on the response factors of different types of lipids was studied. The detector parameters studied were evaporation temperature, total gas pressure and make-up gas flow.

The evaporation temperature was found to be the most critical parameter for the detector. It had an especially marked effect on the response of saturated monoacylglycerols, as seen in Figure 3. The response/temperature curves of these compounds differed significantly from those of other compounds tested. At a lower temperature (35°C), the response of saturated compounds was 3.5–4 times higher than that of triolein (relative response at 54°C = 1.00). Being more readily volatile in the evaporation tube, lighter molecules such as free fatty acids lost 90% of the response compared to that of triolein. At too high a temperature (58°C), the loss of lighter molecules was even greater.

The high responses at low temperatures are apparently related to the melting points of the compounds. At lower temperatures, the saturated monoacylglycerols (m.p. 71–81°C) condense as solid particles, which apparently scatter light by a different mechanism than liquid particles (17). This does not explain why saturated diacylglycerols (m.p. 72–80°C) do not have high response factors at lower temperatures as do the monoacylglycerols. It may be because the diacylglycerols used were all mixtures of 1,2- and 1,3-isomers, which do not form crystals as easily as the individual isomers. The monoacylglycerols tested were all pure α-isomers. The relationship between high response factors and melting points has previously been reported (17,19,20). Coulombe (21), on the other hand, reported that the response factors of different compounds differed radically at higher temperature settings of the detector but less markedly at lower temperature settings.

The optimum detector temperature in this study was considered to be 54°C. At this temperature, the differences between the response factors of the compounds studied were at minimum, and the free fatty acids were not lost. The average relative response of triacylglycerols was 0.93 ± 0.09, and those of diacylglycerols, monoacylglycerols and free fatty acids were 0.70 ± 0.13, 0.94 ± 0.22 and 0.15 ± 0.12, respectively. The relative responses of each compound tested at 54°C are listed in Table 2.

The relative responses of unsaturated tri- and diacylglycerols are slightly larger than those of the saturated compounds. The relative standard deviation (SD%) of the triacylglycerols was 10% and that of diacylglycerols 20%. The high SD% of free fatty acids is due to the low response of these compounds. The amounts tested were near the detection limit of free fatty acids.

The gas pressure was adjusted to 1.0 psi. This parameter affected the noise/response ratio and the total response of the compounds, but the relative responses did not

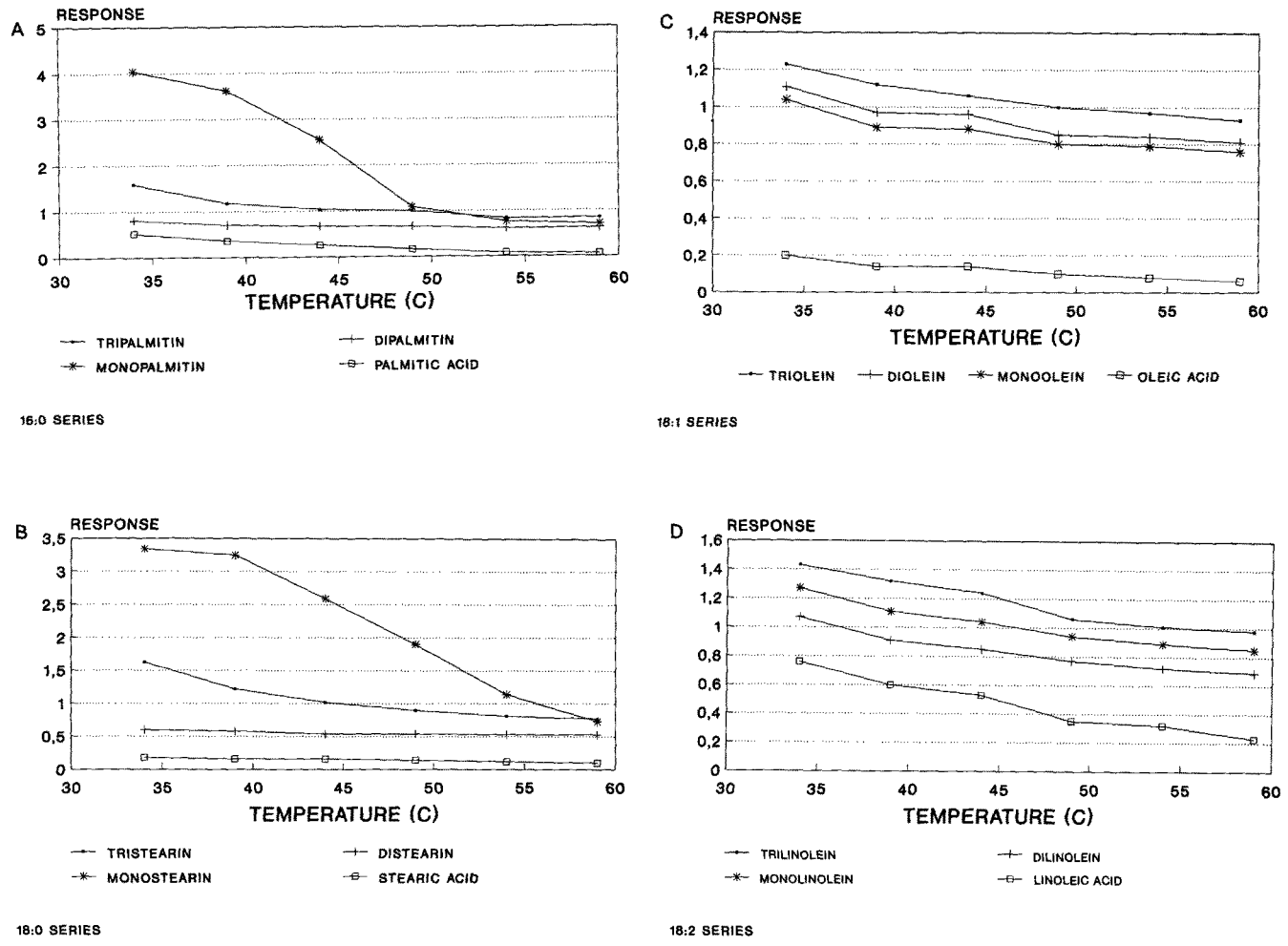

FIG. 3. Optimization of the light-scattering detector: relative detector response as a function of temperature (°C). The relative responses are calculated as area units/μg (see Experimental Procedures). (A) 16:0 standards; (B) 18:0 standards; (C) 18:1 standards; and (D) 18:2 standards.

TABLE 2

Response of Lipid Standards with Light-Scattering Detector (temperature 54°C)[a]

Standard	Relative response	Standard	Relative response	Standard	Relative response	Standard	Relative response
Triacylglycerols		Diacylglycerols		Monoacylglycerols		Free fatty acids	
18:2	1.01	18:2	0.72	18:2	0.89	18:2	0.32
18:1	1.00	18:1	0.88	18:1	0.84	18:1	0.13
18:0	0.87	18:0	0.59	18:0	1.25	18:0	0.07
16:0	0.83	16:0	0.61	16:0	0.76	16:0	0.08
Mean ± SD	0.93 ± 0.09	Mean ± SD	0.70 ± 0.13	Mean ± SD	0.94 ± 0.22	Mean ± SD	0.15 ± 0.12

[a]The relative responses are calculated as area units/μg (see Experimental Procedures).

change. The effect of make-up gas flow is seen in Figure 4. The effect of this parameter on most of the compounds tested was marginal but the response of monostearin changed significantly at excessively high gas flows.

The calibration curves for triolein, diolein, monoolein and oleic acid are seen in Figure 5. The correlation factors calculated for each of the calibration curves were 0.96–0.97 and the response was only approximately linear over the weight range studied. For quantitative work, calibration curves near the area of interest should be used.

The method discussed in this study permits better separation of lipid classes present in edible oils than methods presented in earlier papers. The fractionation of polar triacylglycerol monomers and the polymeric material of autoxidized oils by this method are now being studied and will be reported later. The sensitivity of

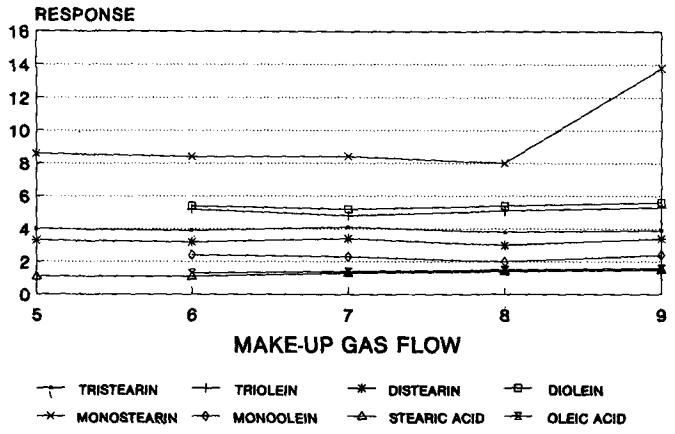

FIG. 4. Optimization of the light-scattering detector: relative detector response as a function of make-up gas flow expressed as an arbitrary valve setting.

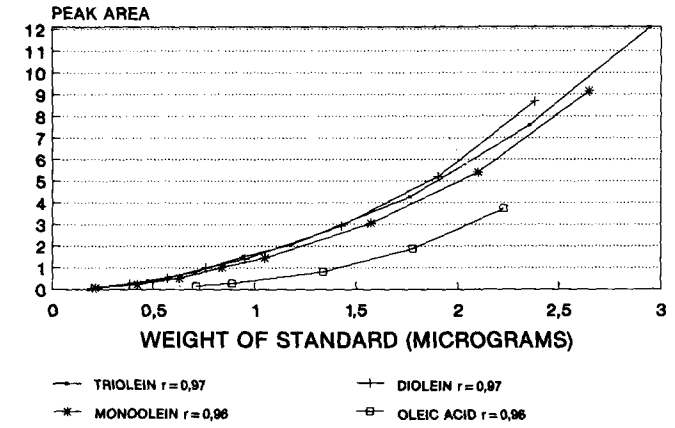

FIG. 5. Calibration curves of each lipid standard injected: weight injected *versus* detector response. Each point represents the mean of two injections. SD% between the injections <5%.

the analysis is better with an LSD than with a refractive index detector, but a careful optimization of the detector parameters is required.

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