Supercritical Fluid Chromatographic Analysis of New Crop Seed Oils and Their Reactions[1]

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ABSTRACT: Supercritical fluid chromatography (SFC) with an open tubular column of nonpolar stationary phase separated triglycerides from crambe, meadowfoam, *Euphorbia lagascae*, and vernonia oils based on their molecular weight. The triglyceride compositions were consistent with the literature. SFC proved also to be a valuable tool in analyzing lipase-catalyzed transesterification reactions where lesquerella oil and estolides were among the substrates employed. Analyte molecular weights could be estimated from a retention time- (or elution density-) molecular weight calibration curve. An increase in isothermal column temperature during SFC pressure or density programming improved the resolution of high-molecular-weight (>600 Da) analytes but yielded poorer resolution for analytes of molecular weight < 200. A simultaneous pressure and temperature ramping program proved superior in enhancing resolution in several instances.

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KEY WORDS: Esters, *Euphorbia lagascae* oil, lesquerella oil, meadowfoam oil, supercritical fluid chromatography, triglycerides, vernonia oil.

Supercritical fluid chromatography (SFC) is becoming a valuable tool in the analysis of lipids as it is replacing high-performance liquid chromatography (HPLC) and gas chromatography (GC) as the analytical tool of choice for several applications. SFC can be considered a hybrid of GC and HPLC because supercritical fluids possess properties (e.g., density, viscosity, and self-diffusion coefficient) intermediate between gases and liquids. For example, separation of analytes by relative volatility, as encountered in GC, can also be achieved with SFC but at much lower elution temperatures and without derivatization. The use of lower temperatures protects analytes that are susceptible to thermal degradation, such as polyunsaturated and oxygenated lipids. With the onset of equipment that allows addition of modifiers to the supercritical fluid mobile phase, HPLC-like separations can be achieved by SFC, but a larger range of detectors—GC-style [e.g., flame-ionization (FID), mass spectrum, and electron capture] and HPLC-style (e.g., ultraviolet and evaporative light-scattering)—can be employed with SFC. Furthermore, SFC possesses the versatility to allow use of both packed (HPLC-like) and capillary (GC-like) columns that contain a variety of stationary phases. However, resolution can be reduced in SFC compared to GC (1).

SFC of lipids has been recently reviewed (2–4). When open tubular columns with nonpolar stationary phases are employed, separation of glycerides occurs *via* molecular weight (MW), or equivalently, carbon number, but not by degree of unsaturation (1,4–8). Determination of an analyte's MW can be determined simply by creation of a retention time–MW calibration curve (5,6). A recent example demonstrated the ability of SFC to elute triglycerides (TG) with MW over 1500 Da. (6). SFC with open tubular columns of more polar stationary phase (1,4,7,8, Hayes, D.G., and R. Kleiman, unpublished data) or packed columns and carrier fluid that contain modifiers (4,7,9) have been successful in separating glycerides by MW and degree of polarity. However, the large number of eluted peaks makes analysis difficult. For this reason, we applied SFC with open tubular columns of nonpolar stationary phase for the analysis of several new crop seed oils from *Crambe abyssinica*, *Vernonia galamensis*, *Euphorbia lagascae*, *Lesquerella fendleri*, and *Limnanthes alba* (meadowfoam). These oils have triglycerides of high MW and/or contain polyunsaturation or oxygenation that makes them susceptible to thermal degradation. Resolution was enhanced by raising or programming column temperature.

Our research group (6,10), and others (11,12) have found SFC a valuable tool for analyzing reactions catalyzed by the enzyme lipase (EC 3.1.1.3). For example, Berg *et al.* (12) analyzed lipase-catalyzed alcoholysis of edible fat hosted in supercritical carbon dioxide by on-line SFC. Examples of SFC analysis of lipase-catalyzed reactions are given here that demonstrate the utility, simplicity, and limitations of SFC.

MATERIALS AND METHODS

Refined vernonia, lesquerella, and crambe oils were donated by Dr. K.D. Carlson at our research center. Two batches of refined meadowfoam oil (Batch 1 supplied in 1991, Batch 2 in 1993) were obtained from the Oregon Meadowfoam Growers Association (Salem, OR). *Euphorbia lagascae* oil was ob-

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[3]Retired

tained from the NCAUR's oilseed collection (Inventory No. 44776). Estolide derived from oleic acid was donated by Dr. T.A. Isbell at our research center. Lipozyme IM, *Rhizomucor miehei* lipase immobilized on anion exchange resin, was donated by Novo-Nordisk (Danbury, CT). All solvents and other reagents were of high purity.

Lipase-catalyzed reactions were carried out in batch mode with Lipozyme being dispersed by magnetic stirring. Further details of enzymatic reaction are included elsewhere (10,13). Vernonia oil was fractionated *via* column chromatography on silica gel (SG 60, 6.0 nm., 230–400 mesh) from Aldrich (Milwaukee, WI) with hexane/ethyl acetate in increasing polarity as eluate. The oil was separated into four fractions, which differed in the number of epoxy fatty acids (0–3) per triglyceride.

The fatty acid content of vernonia and *E. lagascae* oils was determined by GC with a BPX70 (25 m × 0.25 mm × 0.21 μm) open tubular column from SGE (Austin, TX). The carrier gas was helium at a flow rate of 0.39 mL/min through the column. The FID and injector temperatures were held at 220 and 250°C, respectively, while the oven temperature was programmed as follows: 150°C initially, raised to 220°C at 2°C/min, then held at 220°C until all analytes were eluted. The oil was transesterified with sodium methoxide to create fatty acid methyl esters (FAME) prior to GC injection.

SFC was performed on a model 600 chromatograph, equipped with a syringe pump, (nonpolar) SB-Methyl-100 (10 m × 50 μm i.d) column and an FID. The latter two were connected through a frit restrictor. All SFC equipment was purchased from Dionex (Salt Lake City, UT). The carrier fluid was SFC/SFE-grade CO_2 from Air Products (Tamaqua, PA). All runs employed an FID temperature of 350°C. Analytes were dissolved in either methylene chloride, hexane, or acetone at concentrations of *ca.* 0.1–10 mg/mL. The amount of analyte placed in the column was controlled also by setting the duration of the time-split injection. Four different temperature-pressure (or -density) programs were employed: Method A—The temperature was held isothermally at a value in the range 100–200°C. The pressure was initially held for 9 min at 125 atm, then increased at 5 atm/min up to 400 atm or until all analytes were eluted. Method B—The temperature was held isothermally at a value between 100–200°C. The density was initially held for 9 min at 0.15 g/mL, then ramped at 0.01 g/mL/min until all analytes were eluted. Method C—The temperature was held at 100°C for 9 min, then increased at 2.2°C/min up to 200°C, then held for 1 min. The pressure was programmed as in Method A, with the final pressure being 355 atm. Method D—The temperature was held isothermally at 200°C for 25 min, then decreased at 1.6°C/min to 150°C. The pressure was programmed as in Method A, with the final pressure being 355 atm.

RESULTS AND DISCUSSION

SFC triglyceride (TG) analysis of new crop oils by using both pressure (density) and temperature programming. Figure 1 contains SFC chromatograms of meadowfoam and crambe oils with pressure programming (Method A) at either 100 or 200°C. The former oil contains mostly long-chain fatty acids (FA), i.e., of chain length ≥20, with unusual unsaturation: *cis*-double bonds at the C_5 position, while the latter is rich (57–59%) in erucic ($22:1^{13}$) acid (14,15). Crambe oil is resolved fairly well at a column temperature of 100°C, with separation based on carbon number, but the resolution is equivalent to that obtained by GC (16). The distribution of crambe oil TG, based on carbon number obtained by SFC, compares favorably with that reported for GC (Table 1). The TG analysis of crambe oil by either Method A or C was identical, suggesting that thermal degradation of analyte at 200°C did not occur. However, meadowfoam oil TG, of a similar MW distribution as crambe oil TG, were poorly resolved at 100°C. An increase of column temperature greatly improved the resolution, and temperature programming (Method C) provided even more improvement. The distribution of meadowfoam TG, based on carbon number by SFC (Fig. 1d), compares favorably with a recent study (17) that employed reverse-phase and silver-ion HPLC and with GC (Table 1). The *T*62, *T*64, and *T*66 species of meadowfoam oil separate into two SFC peaks each. The promoter of these separations is probably either the amount of di-unsaturated acyl groups or acyl groups that contain Δ5 unsaturation per TG. Peaks *T*62B and *T*64B of meadowfoam oil elute at similar retention times (densities) as *T*62 and *T*64 of crambe oil, which contain acyl groups with "normal" unsaturation positions, at the n-9 posi-

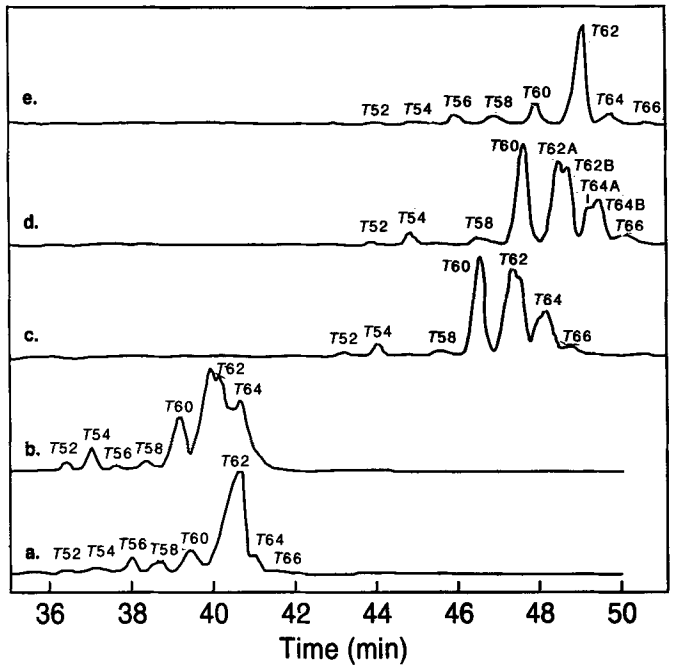


FIG. 1. Supercritical fluid chromatography chromatograms of meadowfoam (Batch 1) and crambe oils by pressure programming. (a) crambe oil; isothermal hold at 100°C (Method A), (b), meadowfoam oil; isothermal at 100°C (Method A), (c) meadowfoam oil; isothermal hold at 200°C (Method A), (d) meadowfoam oil; positive temperature ramp (Method C), (e) crambe oil; positive temperature ramp (Method C). Carbon numbers of the triglyceride species are contained in the figure.

TABLE 1
SFC Analysis of TG from Meadowfoam and Crambe Oils

	Meadowfoam oil		Crambe oil			
TG species	SFC[a,b]	SFC[a,c]	HPLC[d]	GC[a,e]	SFC[a]	GC[e]
*T*52	0.6	0.4	0.1	1.1	0.5	0.9
*T*54	2.8	0.6	0.7	3.5	1.3	2.0
*T*56	0.7	0.5	0.2	0.6	4.6	5.3
*T*58	2.3	2.2		1.8	5.6	4.9
*T*60	28.8	28.2	31.2	29.4	11.8	12.2
*T*62	40.4[f]	40.8[f]	42.6	40.0	67.3	65.3
*T*64	20.6[f]	21.7[f]	16.5	19.5	7.7	6.9
*T*66	3.9	4.4	0.9	3.5	1.1	0.7
Unknown			7.3			

[a]Method C employed; SFC, supercritical fluid chromatography. TG, triglycerides; HPLC, high-performance liquid chromatography; GC, gas chromatography.
[b]Meadowfoam oil Batch 1.
[c]Meadowfoam oil Batch 2.
[d]From Reference 17.
[e]From Carlson, K.D., unpublished data employing the methodology described in Reference 16, but using a 15°C/min temperature ramp.
[f]Peaks *T*62A and *T*62B added; *T*64A and *T*64B added.

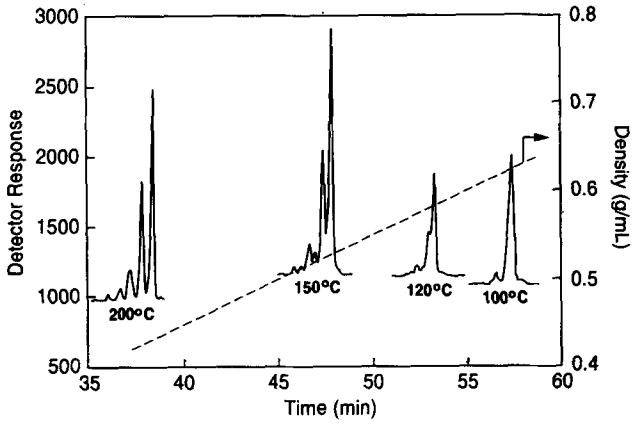


FIG. 2. Separation of vernonia oil triglyceride by supercritical fluid chromatography with density programming (Method B) at different isothermal temperatures.

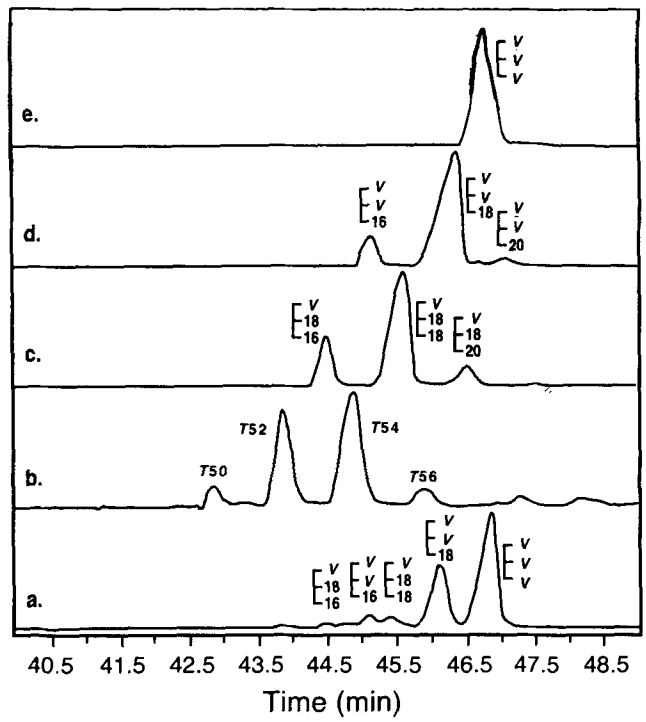


FIG. 3. Supercritical fluid chromatography chromatograms of vernonia oil and its fractions by using simultaneous pressure and temperature programming (Method C). (a) vernonia oil, (b–e) fractions of vernonia oil that contain 0–3 epoxy acyl groups per triglycerides (TG), respectively. Positional assignments of TG shown in figure do not necessarily represent actual positional assignments. *V* represents vernolic acyl groups.

tion (Fig. 1d and e). This would suggest that meadowfoam oil peaks *T*62B and *T*64B contain mostly C_{18}-Δ9 and erucic acyl groups. *T*54 peaks for both meadowfoam and crambe oils elute at the same time. This supports the above statement because the *T*54 species in meadowfoam do not contain Δ5 unsaturation (17). Further experimentation will be required to identify the structure of *T*62A, *T*62B, *T*64A, and *T*64B.

Vernonia oil contains a large portion (77–81%) of vernolic (*cis*-12,13-epoxy-*cis*-9-octadecenoic) acid, an epoxy acid with application in paints, coatings, and plastics (14,18,19). Other acyl groups present are C_{16}, C_{18}, and C_{20} saturates and unsaturates that are commonly found in other seed oils (18,19). As exhibited above for meadowfoam oil, an increase in isothermal column temperature dramatically improved the resolution of vernonia oil TG (Fig. 2). The resolution at a given temperature was not significantly altered when pressure programming (Method A) was employed instead of density programming (Method B) (data not shown). Others (8,20) also obtained improved resolution of TG when the isothermal column temperature was raised to 150°C, but temperatures above 150°C did not improve resolution further.

We examined simultaneous pressure and temperature programming by using each a positive (Method C) and a negative (Method D) temperature program. We found that both types of temperature programs improved resolution of vernonia oil TG, and that the positive temperature program performed slightly better (Fig. 3). Others also have encountered improved resolution of high-MW compounds by using simultaneous pressure and temperature programming (21,22). Peaks were identified from fractions of vernonia oil separated by degree of polarity (i.e., number of epoxy acyl groups per TG) *via* liquid chromatography on silica gel. The analysis indicated that SFC separated TG based on MW, with trivernolin being the most abundant TG species. The TG compositions of vernonia oil and *E. lagascae* oil, another oil rich in vernolic acid, are contained in Table 2. The percentage of triepoxy TG (54.4%) we obtained is slightly higher than that measured by HPLC (18,24) and than that predicted theoretically with use of a random distribution: 41.9%, based on the fatty acid composition measured by us by GC, or 44.3%, cal-

TABLE 2
SFC Analysis of Vernonia Oil and *Euphorbia lagascae* Oil TG

TG species[a]	*E. lagascae* oil (SFC)[b]	Vernonia oil (SFC)[b]	Vernonia oil (Theoretical)[c]	Vernonia oil (LC)[d]
[16,18,18]	3.1	0.8		
[16,18,*V*]	2.1	1.2	2.0	
[18,18,18]	3.4	1.3		
[16,*V*,*V*]	6.2	4.8	4.4	
[18,18,*V*]	12.3	5.0	9.7	
[18,*V*,*V*]	45.3	29.6	37.1	
[18,*V*,20]		0.8[e]	0.3	
[*V*,*V*,*V*]	23.2	54.4	44.3	
[*V*,*V*,20]	1.2	1.3[d]	0.9	
Triepoxy TG	23.2	54.4	44.3	50.3
Diepoxy TG	52.7	35.7	42.4	20.6
Monoepoxy TG	14.4	7.0	12.0	18.5

[a]V = vernolic acid; 16, 18, 20 = C_{16}, C_{18}, and C_{20} fatty acids.
[b]SFC operated by Method C.
[c]From Reference 23.
[d]From Reference 18 by liquid chromatography (LC). See Table 1 for the abbreviations.
[e]Peaks buried within trivernolin peak; determined based on SFC analysis of silica gel column chromatography fractions of di- and monoepoxy TG.

culated by Anderson *et al.* (23). In agreement, Neff *et al.* (18) found vernolic acid to occur more frequently at the 1- (3-) TG position. In contrast, the percentage of trivernolin in *E. lagascae* oil (23.2%) is nearly identical to the percentage based on random distribution (23.5%), but slightly larger than that measured previously, 18–20% (24,25). The accuracy of the TG assignments are examined in Table 3, where the fatty acid compositions calculated from SFC TG assignments are compared to those measured by GC. The FA compositions for *E. lagascae* oil are nearly identical, while the calculated vernolic acid percentage based on SFC is slightly higher. An explanation for this difference is unknown. Our own results, as well as those of others (20), indicate that FID detector response per unit mass of analyte is invariant.

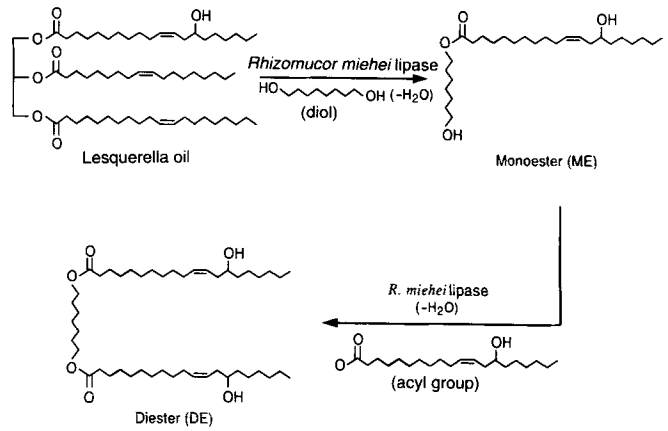
Use of SFC in analysis of lipase-catalyzed reactions. SFC with nonpolar capillary columns has been particularly useful in the analysis of lipase-catalyzed reactions that involve new crop seed oils and estolides. As a first example, SFC analysis (Method A) of the reaction between α,ω-diol and *L. fendleri* oil, catalyzed by immobilized *R. miehei* lipase (Scheme 1), is displayed in Figure 4. Lesquerella oil is a rich source of C_{20} hydroxy fatty acids, which are under investigation for a variety of industrial applications (26). Alcoholysis of lesquerella oil with *R. miehei* lipase, a 1,3-specific lipase, yields esters that contain mostly C_{20}-hydroxy acyl groups because these groups comprise over 80% of the acyl groups present at the 1- and 3-glycerol positions (27). Initially, the reaction medium contains a stoichiometric excess of TG relative to diol (Fig. 4a). During the initial stages of the reaction, monoester (ME) and diglyceride (DG) are synthesized (Fig. 4b); then, as the diol supply becomes depleted, the ME is converted into diester (DE), hence the decrease in ME and increase in DE amount (Fig. 4c). At that stage of the reaction, diol was added to the medium (Fig. 4d), which caused the further decrease in TG and DG amounts and the increase in ME concentration relative to DE (Fig. 4e). The presence of several reaction substrates, products, and intermediates presents difficulties in analysis. However, the technique presented here allows separation of analytes by MW only, i.e., no separation by degree of unsaturation exists, which greatly reduces the number of peaks, and simplifies the analysis. However, the SB-Methyl 100 column allowed partial separation between saturated and unsaturated free fatty acid (FFA) or fatty acid (mono) esters, as exhibited in Figure 4e between 18:0 and 18-unsaturated monoesters.

Analyte MW can be estimated from near-universal calibration plots, such as the one illustrated in Figure 5, for analytes present during α,ω-diolysis and lipolysis of lesquerella

TABLE 3
A Check of Fatty Acid (FA) Composition Based on SFC Analysis of *Euphorbia lagascae* and Vernonia Oil Against Literature Values

FA species	*E. lagascae* oil[a]	*E. lagascae* oil[b]	Vernonia oil[a]	Vernonia oil[b]
C_{16}	4.1	4.0	2.3	3.2
C_{18}	29.8	31.8	15.6	20.7
C_{20}	0.4	0.7	0.7	0.7
Vernolic	63.4	62.1	80.5	75.1

[a]Calculated based on TG assignments (Table 2) as determined by SFC.
[b]Measured by gas chromatography analysis of fatty acid methyl esters. See Table 1 for other abbreviations.



SCHEME 1

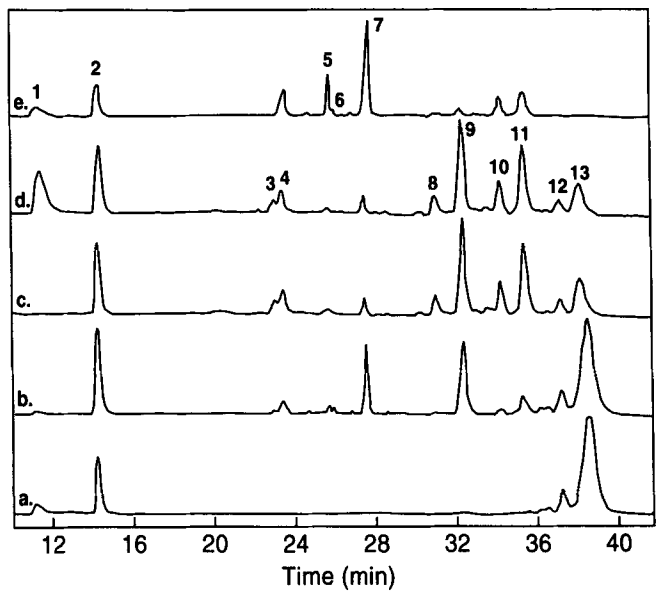


FIG. 4. Supercritical fluid chromatography (SFC) chromatograms of the immobilized *Rhizomucor miehei* lipase-catalyzed transesterification between 1,8-octanediol and lesquerella oil by pressure programming (Method A) at 100°C. Reaction times: (a) 0.0 h, (b) 1.1 h, (c) 24.1 h (d) 24.1 h (after addition of further diol), (e) 69.8 h. SFC peaks: (1) diol, (2) $C_{16}H_{34}$ internal standard, (3) C_{20}-hydroxy acid (occurs from hydrolysis—a side-reaction), (4) C_{18} monoglyceride, (5) C_{18} unsaturate monoester (ME), (6) 18:0 ME, (7) 20-OH ME, (8)—diglyceride (DG)36, (9) DG(18,20-OH), (10) diester(18,20-OH), (11) DG(20-OH,20-OH), (12) triglyceride (TG)(18,18,20-OH), (13) TG(20-OH,18,20-OH).

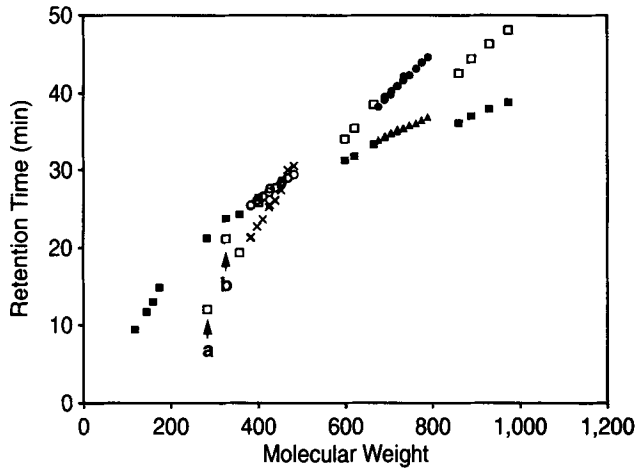


FIG. 5. Supercritical fluid chromatography retention time–molecular weight calibration with pressure programming (Method A) at either 100 or 200°C. (100°C, 200°C)—(■,□) lesquerella oil free fatty acid (FFA), monoglycerides, diglycerides, and triglycerides; (○,X) diol-lesquerella monoester; (▲,●) diester. Peaks a and b refer to C_{18} and 20-OH FFA, respectively.

oil. Similar SFC calibration curves also have been displayed elsewhere (5,28,29). The slope of the calibration curve is initially steep; then it gradually decreases. The initial steepness occurs because separation in the low MW region is based on the relative volatility, or equivalently, MW, of the analytes, as occurs in GC. In the higher-MW portion of the curve, where a higher, hence more liquid-like, carrier fluid density is required, separation is based less on relative volatility and more on interactions between solute, solvent, and stationary phase, as occurs in HPLC, hence the smaller slope. By increasing column temperature to 200°C, both resolution (proportional to the slope of the calibration curve) and retention times decreased for low-MW (<200 Da) analytes and increased for higher-MW analytes. The low-MW analytes, in fact, were poorly resolved from the solvent peak. These trends with respect to column temperature were exhibited during analysis of high-MW TG of vernonia, crambe, and meadowfoam oils (see above) and for surfactant analysis with a similar nonpolar stationary phase (28). When elution density replaces retention time as the ordinate, two separate but nearly parallel curves result, with the curve for the analysis at 200°C being below the 100°C curve (Fig. 6). The use of density (Method B) rather than pressure programming (Method A) had little effect on the calibration curve (Fig. 6) except for low-MW analytes at 100°C, where density programming yielded better resolution. This probably occurred because the initial density was lower with Method B (density programming) rather than Method A (pressure programming).

Data points a and b in Figures 5 and 6 refer to C_{18} and C_{20}-OH FFA from lesquerella oil lipolysis. The retention behavior of these two species is not described by the calibration curves because their peak widths at 200°C are quite large and the peak shapes are non-Gaussian. Others also found that the resolution of FFA decreases at higher temperatures (28). As a compromise between improved resolution of FFA at low temperatures and of DG and TG at higher temperatures, the simultaneous positive pressure and temperature ramping program (Method C) has proven to be valuable in the analysis of lesquerella oil lipolysis (data not shown).

A second example is the lipase-catalyzed esterification of estolides and alcohols. Estolides, oligomers of hydroxy fatty acids, are under investigation by our research group. We found that esterification of estolides by 1,3-specific lipase produced esters at high yields without degradation or hydrolysis of the estolide, with the esters having a lower viscosity

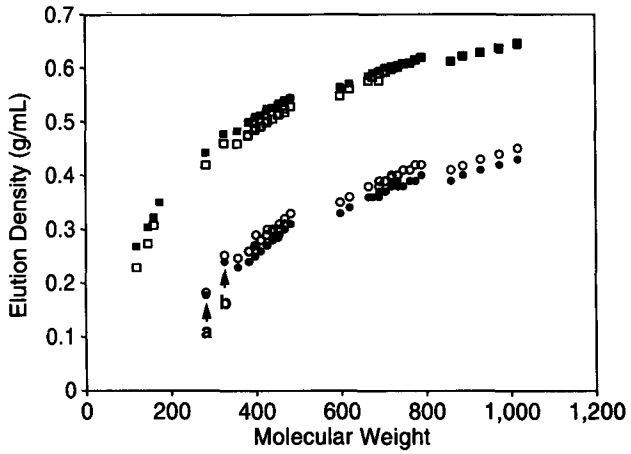


FIG. 6. Supercritical fluid chromatography elution density–molecular weight calibration of lesquerella oil glycerides and diol esters by using Method A (filled symbols) and Method B (outlined symbols). (■,□) 100°C, (●,○) 200°C. Assignments for peaks a and b equal those of Figure 5.

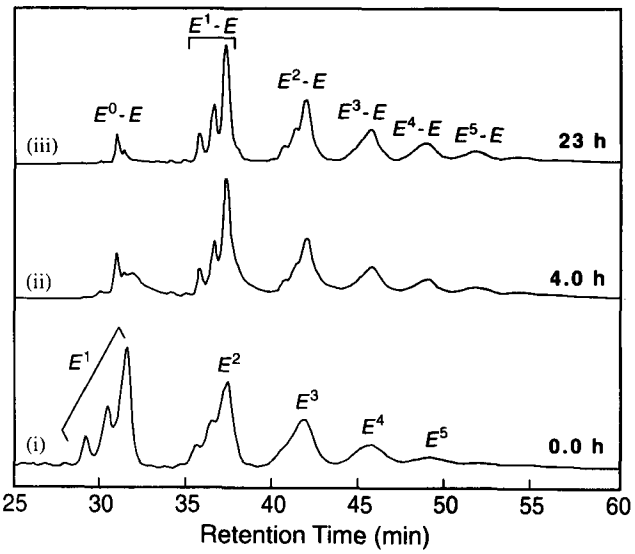
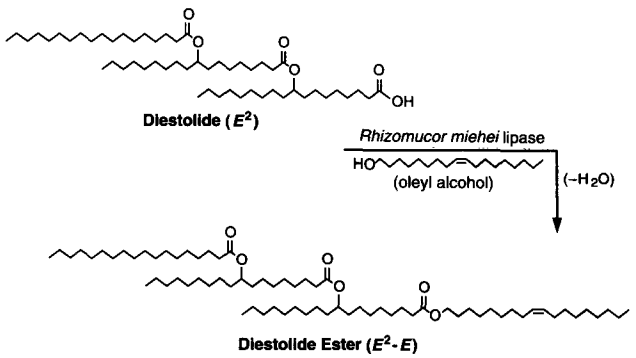


FIG. 7. Supercritical fluid chromatography chromatograms of lipase-catalyzed esterification of oleyl alcohol and polyestolide derived from oleic acid. Reaction times are listed in the figure. E^i refers to estolide containing i estolide bonds (i + 1 acyl groups); E^i-E refers to estolide oleyl esters.



SCHEME 2

and higher viscosity index than the corresponding estolides (10). SFC analysis of the synthesis of estolide-oleyl alcohol esters (Scheme 2) is displayed in Figure 7. The low-MW free estolides are resolved much better than the larger oligomers, with pentaestolide, E^5 (5 estolide ester bonds, 6 acyl groups), at a MW of 1683 being the largest analyte resolved (Fig. 7, curve i). A major reason for the poor resolution of high-MW analytes is the high velocities that occur at the high pressures (near 400 atm) required for their elution (30). After four hours of reaction time, new peaks have formed, which according to the retention time–MW calibration are estolide–oleyl esters (Fig. 7, curve ii). After 23 h of reaction time, the free estolide peaks have nearly completely disappeared, with only estolide ester peaks being present. Thus, at least for the smaller-MW estolides, esterification occurred at nearly 100%. Estolide ester peaks are better resolved compared to the free estolide peaks. For example, the E^5-E peak can be clearly detected, even though its MW is quite high (1933 Da). This indicates that the absence of free COOH groups improves the resolution of estolide analytes. An increase of column temperature to 200°C improved the resolution of free mono- and diestolides, but shortened the elution range (data not shown).

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