Identification and Quantification of Feruloylated Mono-, Di-, and Triacylglycerols from Vegetable Oils

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ABSTRACT: The use of HPLC–MS to separate and identify the feruloylated acylglycerols formed during the transesterification of ethyl ferulate with TAG was examined. Novozym 435 (*Candida antarctica* lipase B)-catalyzed transesterifications of ethyl ferulate and soybean oil resulted in a mixture of feruloylated MAG, DAG, and TAG and diferuloylated DAG and TAG. These feruloylated acylglycerols have recently garnered much interest as cosmeceutical ingredients. The ratio of the various feruloylated acylglycerol species in the resultant oils is presumed to affect the oil's cosmetic efficacy as well as its physical (formulation) properties. Thus, it was desirable to develop an analytical method to separate, identify, and quantify the individual feruloylated acylglycerols to determine their relative ratios. The feruloylated acylglycerols were successfully separated and identified by HPLC–MS using a phenyl-hexyl reversed-phase column developed with a water/methanol/1-butanol gradient. The chromatograms of the feruloylated acylglycerols from soybean oil were convoluted by myriad fatty acids; therefore, feruloylated acylglycerols from triolein were studied as a model reaction. Hydrolysis of the feruloylated acylglycerols from triolein catalyzed by Lipase PS-C "Amano" I (*Burkholderia cepacia*), which showed no hydrolysis reactivity toward ethyl ferulate, allowed for the chromatographic assignment of the feruloyl acylglycerol positional isomers.

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KEY WORDS: Ferulic acid, feruloylated lipids, HPLC–MS, lipase, lipids, transesterification.

Vegetable oils have been biocatalytically and chemically converted to commercially successful structured lipids for food and skin-care applications (1). Vegetable oils have also been used as feedstocks for large-scale industrial products such as surfactants, paints, lubricants, and biodiesel (2–4). These biocatalytic and chemical processes are rarely quantitative and often result in MAG and DAG intermediates and by-products as well as free glycerol, FFA, and unreacted TAG. These intermediates and by-products are often unwanted in the final product; thus, methods have been developed to identify and monitor these contaminants during synthesis and purification.

A convenient, versatile method for separation and identification of vegetable lipids and their intermediate glycerols is HPLC incorporating UV detection, ELSD, and MS. A variety of HPLC methods have been developed to optimize the resolution of fats and oils, including regular-phase (5), reversed-phase (6,7), argentation (silver ion) (8,9), 2-D silver-ion reversed-phase (10), and size-exclusion (11) chromatography. An examination of various HPLC methods for resolving acyl lipids recently has been published (12). These HPLC methods are used to resolve TAG species based on fatty acid composition, positional isomers, and degree of desaturation. The biocatalytic and chemical conversion of TAG to structured lipids and fatty acid esters results in intermediate DAG and MAG as well as FFA. HPLC methods have been developed to resolve the DAG and MAG and elucidate the positional isomers of these intermediate glycerols (13–15). A few of these HPLC methods have been reviewed in detail elsewhere (16).

Partial substitution of fatty acid groups in vegetable oils by aryl groups presents new analytical challenges. HPLC methods that have previously been developed for the resolution of phenolic lipids have focused on catecholic (e.g., urushiol, anacardic, ginkgolic), resorcinolic, and hydroquinonic lipids, which are more structurally similar to FFA (17,18). The present work establishes an HPLC method to separate and identify various feruloyl (phenolic)-substituted MAG, DAG, and TAG.

A reversed-phase HPLC method using a water/methanol/1-butanol gradient and a phenyl-hexyl column was used to elucidate the molecular species derived from enzymatically feruloylated vegetable oils. The lipase-catalyzed transesterification of ethyl ferulate (EF) and soybean oil (SBO) resulted in a mixture of feruloylated acylglycerol species (19,20). The UV absorption and antioxidant properties of the feruloylated acylglycerols have recently led to interest in them as all-natural ingredients for use in daily-wear cosmetics and skin-care products. Each of the feruloylated acylglycerol molecular species is presumed to have intrinsic chemical and physical properties that will affect the overall efficacy and formulation capabilities of the bulk oil. Thus, it was desirable to develop an HPLC method to separate, identify, and quantify the various feruloylated acylglycerol species. This method will guide the optimization of reaction parameters such as reactant ratio and reaction time to control the relative ratios of the feruloylated species in the resultant oil, which influence the optimization of the ingredient's efficacy and formulation properties.

EXPERIMENTAL PROCEDURES

Materials. EF (ethyl 4-hydroxy-3-methoxy cinnamate) was purchased from Shanghai OSD (Shanghai, China). Novozym

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435 (*Candida antarctica* lipase B immobilized on acrylic beads) was purchased from Novozymes North America (Franklinton, NC). Lipase PS-C "Amano" I (*Burkholderia cepacia* immobilized on ceramic particles) was graciously donated by Amano Enzyme USA Co. (Elgin, IL). Triolein was purchased from Nu-Chek-Prep (Elysian, MN). SBO was purchased at a local grocery. All other reagents were from Sigma-Aldrich (St. Louis, MO). Solvents and glycerol (<0.1% w/w water) were spectroscopic grade.

Synthesis of feruloylated mono- and dioleoylglycerols. Varying amounts of glycerol (0.0 to 0.368 g, 0.0 to 4.2 mmol) were blended with 200 mg of silica gel in 50-mL Schlenk flasks. Triolein (3.54 g, 4.0 mmol) and Novozym 435 (0.221 g) were added to the Schlenk flasks and the mixtures were degassed under vacuum for 30 min. The evacuated flasks were shaken on an orbital shaker (200 rpm) at 60°C for 24 h. EF (0.888g, 4.0 mmol) was added to the flasks, and the flasks were evacuated for 5 min. The reaction mixtures were allowed to shake (200 rpm) at 60°C for 120 h. Aliquots (100 mL) were removed at timed intervals for analytical HPLC and UV spectroscopy (325 nm). The flasks were evacuated for 5 min after each sampling. The feruloylated reaction products (Fig. 1) were identified by HPLC-MS (see below).

Deacylation of feruloylated mono- and dioleoylglycerols. After reaching equilibrium (120 h), the feruloylated oleoylglycerol reaction mixtures were extracted with 10 mL of 2-methyl-2-propanol. The solutions were filtered to remove the Novozym 435 and silica gel. The solids were rinsed with two 2-mL portions of 2-methyl-2-propanol (*t*-BuOH). The combined filtrates (14 mL) were transferred to 50-mL Schlenk flasks. Water (1.0 g, 55.0 mmol) and Lipase PS-C "Amano" I (0.100 g) were added to the flasks, and the flasks were shaken (175 rpm) at 37°C. Aliquots (20 mL) were removed at timed intervals for analytical HPLC and UV spectroscopy (325 nm).

Synthesis of feruloylated SBO. The packed-bed bioreactor synthesis of feruloylated SBO has been detailed previously (20). A solution of EF (40 g) dissolved in SBO (160 g) at 60°C was circulated over a bed of Novozym 435 (34 g) at 60°C for 144 h. The reaction progress was monitored by analytical HPLC (see below). By-products and unreacted starting material were removed from the reaction mixtures by molecular distillation at 120°C.

Deacylation of feruloylated SBO. Feruloylated SBO (240 g) and water (14.5 g) were dissolved in 250 mL of 2-methyl-2-propanol in a 1-L Fernbach flask. Lipase PS-C "Amano" I (2.5 g) was added and the mixture was shaken (125 rpm) at 37°C for 144 h. Aliquots (100 mL) were removed at timed intervals for analytical HPLC and UV spectroscopy (325 nm). The solvent was removed *in vacuo* at 60°C for 24 h, and the resultant oily residue was dissolved in 100 mL of acetone containing 1.0% acetic acid. The feruloylated monoacylglycerol (F-MAG) and diferuloylated diacylglycerol (F_2-DAG) were isolated by preparative HPLC (see below). The effluents containing the F-MAG collected during the preparative HPLC separations were combined and the solvent removed under vacuum at 40°C to yield a cloudy, colorless viscous oil. The isolated F-MAG was

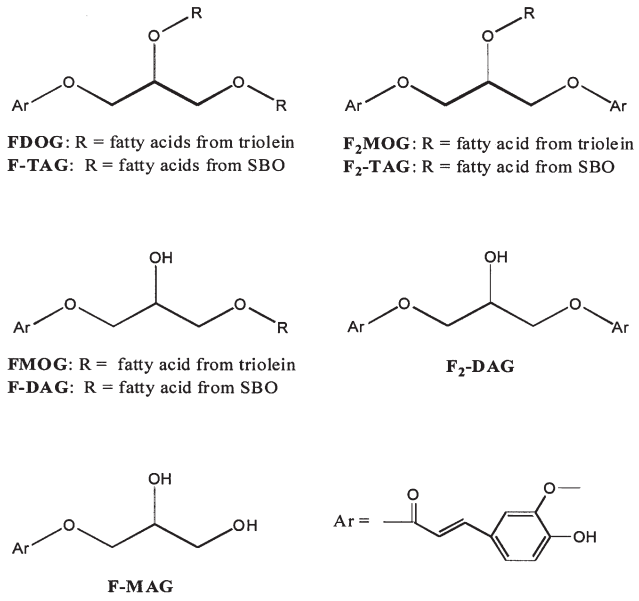

FIG. 1. The feruloylated species obtained from Novozym 435-catalyzed transesterification of triolein or soybean oil (SBO) with ethyl ferulate (EF). The structures shown do not denote all possible *sn*-positional isomers. F-MAG, feruloylated MAG; F-DAG, feruloylated DAG; F-TAG, feruloylated TAG; FMOG, feruloyl monooleoylglycerol; FDOG, feruloyl dioleoylglycerol; F_2-DAG, diferuloylated DAG; F_2-TAG, diferuloylated TAG; F_2MOG, diferuloyl monooleoylglycerol.

analyzed by analytical HPLC and was found to be pure. Yield: 3.69 g. ^{1}H NMR (d_6-acetone): δ (ppm) 7.60 (1 H, *d*, *J* = 16.1 Hz, H-8), 7.31 (1 H, *s*, H-2), 7.12 (1 H, *d*, *J* = 8.2 Hz, H-5), 6.85 (1 H, *d*, *J* = 8.1 Hz, H-6), 6.37 (1 H, *d*, *J* = 16.1 Hz), 4.19 (2 H, *dm*, *J* = 24.3 Hz, H-11), 3.90 (3 H, *s*, H-7), 3.87 (1 H, *m*, H-12), 3.58 (2 H, *m*, H-13). ^{13}C NMR (d_6-acetone): δ (ppm) 167.5 (C-10), 150.1 (C-4), 148.8 (C-3), 145.8 (C-8), 127.5 (C-1), 123.9 (C-6), 116.1 (C-5), 115.8 (C-9), 111.3 (C-3), 71.0 (C-12), 66.3 (C-13), 64.1 (C-11), 56.3 (C-7). Atmospheric pressure CI (APCI)-MS: 266.9 $[M - H]^-$ (calcd. $C_{13}H_{16}O_6$: 268.1).

The effluents containing the F_2-DAG collected during the preparative HPLC separations were combined and the solvent removed under vacuum at 40°C to yield a white solid. The isolated F_2-DAG was analyzed by analytical HPLC and was found to be pure. Yield: 2.3 g. ^{1}H NMR (d_6-acetone): δ (ppm) 7.62 (2 H, *d*, *J* = 16.1 Hz, H-8), 7.30 (2 H, *s*, H-2), 7.10 (2 H, *d*, *J* = 6.9 Hz, H-5), 6.85 (2 H, *d*, *J* = 6.5 Hz, H-6), 6.40 (2 H, *d*, *J* = 16.1 Hz), 4.27 (4 H, *m*, H-11), 4.18 (1 H, *m*, H-12), 3.88 (3 H, *s*, H-7). ^{13}C NMR (d_6-acetone): δ (ppm) 167.4 (C-10), 150.1 (C-4), 148.8 (C-3), 146.1 (C-8), 127.4 (C-1), 124.0 (C-6), 116.1 (C-5), 115.6 (C-9), 111.4 (C-3), 68.35 (C-12), 66.0 (C-11), 56.4 (C-7). APCI-MS: 464.9 $[M - H + Na]$, 443.0 $[M - H]^-$ (calcd. $C_{23}H_{24}O_9$: 444.14).

Analytical HPLC. Analyses were performed using a Thermo Separation Products (San Jose, CA) HPLC system consisting of a Spectra System AS3000 autosampler, a Spectra System P4000 pump, a Spectra System UV6000LP detector, an Alltech (Deerfield, IL) 500 ELSD, and a Luna Phenyl-Hexyl column (5 μm, 250 × 4.6 mm; Phenomenex, Torrance, CA). Solvents were filtered using Whatman 0.45 mm nylon mem-

brane filters (Sigma-Aldrich) and degassed using a Thermo Separation Products SCM 1000 Membrane Degasser. The feruloylated lipid species were determined using a three-solvent gradient. Solvent A was water (268 mL), methanol (70 mL), 1-butanol (11 mL), and glacial acetic acid (1 mL). Solvent B was water (93 mL), methanol (245 mL), 1-butanol (33 mL), and glacial acetic acid (1 mL). Solvent C was methanol. The column was developed at 1.0 mL/min with a 5 min isocratic flow of 3:1 A/B, a 2-min linear gradient to 100% B, a 5-min isocratic flow of 100% B, a 2-min linear gradient to 100% C, a 13-min isocratic flow of 100% C, followed by a 3-min linear gradient to 3:1 A/B. The UV detector response (325 nm, 7 nm bandpass) was calibrated with EF. Injection volumes were 10 μL.

HPLC–MS. HPLC–APCI–MS was conducted with a Finnigan-Thermoquest (San Jose, CA) LCQ LC–MS system (AS3000 autoinjector, P4000 HPLC pump, UV6000 PDA detector, LCQ ion-trap mass spectrometer, and a nitrogen generator) all running under the Xcaliber 1.3 software system. The MS was run with the electrospray ionization (ESI) interface operating in the negative ion mode. The source inlet temperature was set at 220°C and the sheath gas rate was set at 90 arbitrary units. The MS was optimized by using the autotune feature of the software while infusing a solution of F2-DAG in with the effluent of the column and tuning on an atomic mass unit of 531 $[M - H]^-$. The software package was set to collect mass data between 100 and 1500 amu. Generally the most significant sample ions generated under these conditions were $[M - 1]^-$. The LC conditions were the same as those described above for the analytical system, with the liquid flow exiting the diode array detector split 1:10 and the low-flow split being directed into the ESI–MS interface.

Preparative HPLC. Preparative HPLC was used to isolate F-MAG and F_2-DAG. A Shimadzu (Columbia, MD) preparative HPLC system was used with dual 8A pumps, SIL 10vp autoinjector, SPD M10Avp photodiode array detector, and a SCL 10Avp system controller, all operating under the Shimadzu Class VP operating system. Sample aliquots (2 mL) in 1% acetic acid in acetone (vol/vol) were injected on a Waters (Milford, MA) Bondapak C18 PrepPak column (15–20 mm, 125 Å, 47 × 300 mm) in a radial compression module. The column was preequilibrated with 1% acetic acid, 10% acetonitrile, and 89% acetone at a flow rate of 50 mL/min, and the effluent was monitored at 360 nm. The column was developed to 23% acetonitrile over 10 min. Peaks were collected by hand. The procedure was repeated 40 times to obtain sufficient quantities of purified F-MAG and F_2-DAG.

RESULTS AND DISCUSSION

Synthesis of feruloyl and diferuloyl acylglycerols. SBO was biocatalytically transesterified with EF to form UV-absorbing oils (19,20). The Novozym 435-catalyzed transesterifications were typically conducted by passing solutions of EF in SBO over a bed of Novozym 435 at 60°C for 144 h. It was previously reported that the transesterifications resulted in an equilibrium in which 65% of the EF was converted to a mixture of feruloylated TAG (F-TAG), feruloylated DAG (F-DAG), and feruloylated MAG (F-MAG) as well as trace amounts of ferulic acid (FA), which resulted from the hydrolysis of EF (Fig. 1). Herein, an improved, reversed-phase HPLC–MS method is discussed that reveals that the lipase-catalyzed transesterifications also produce diferuloylated TAG (F_2-TAG) and diferuloylated DAG (F_2-DAG), which had not been previously identified.

Recent collaborative interests in the use of F-MAG as a carbon source and selection substrate for the directed evolution of feruloyl esterases led to efforts to deacylate the F-DAG and F-TAG mixtures to form F-MAG. The feruloylated acylglycerols were enzymatically hydrolyzed with an excess of water in 2-methyl-2-propanol solutions at 37°C using a *sn*-1,3-specific enzyme, Lipase PS-C "Amano" I (Fig. 2), which showed no transesterification or hydrolysis activity towards ethyl ferulate (19). Fatty acid hydrolysis was facile and reached equilibrium after 48 h. Unexpectedly, the deacylation of the feruloylated acylglycerols resulted in two feruloylated glycerol species, F-MAG and F_2-DAG; the latter was incorrectly identified as F-MAG using our original water/methanol

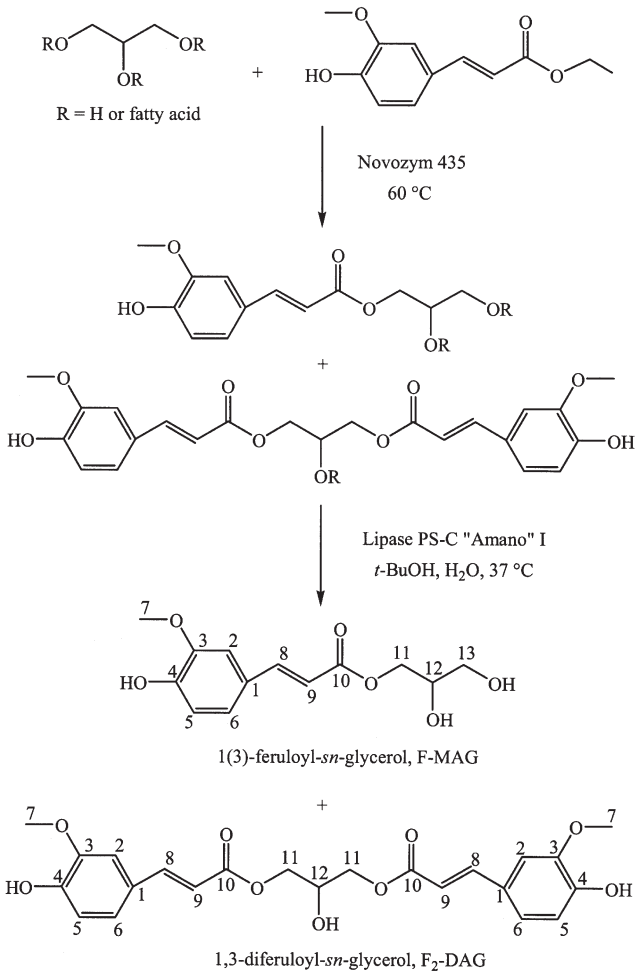

FIG. 2. The lipase-catalyzed transesterification of triolein or SBO with EF followed by lipase-catalyzed hydrolysis. For abbreviations see Figure 1.

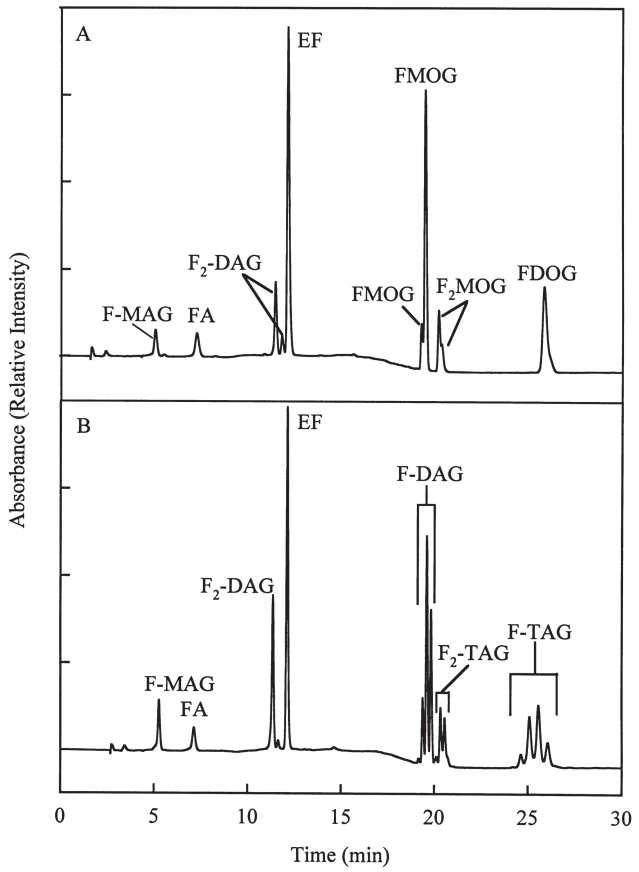

FIG. 3. Analytical reversed-phase HPLC chromatograms of the Novozym 435-catalyzed transesterification of (A) triolein and (B) SBO with EF. The chromatograms were obtained using a Luna phenyl-hexyl column developed at 1.0 mL/min with a gradient of a tertiary solvent system consisting of water, methanol and *n*-butanol monitored at 325 nm. See Figure 1 for acronym definitions.

and acetone/acetonitrile, reversed-phase, C8 column, HPLC methods (19). The isolation of the two feruloylated glycerol species by preparative HPLC was achieved using an isocratic development of a C18 column with an amended acetone/acetonitrile solvent system. ^{1}H NMR analysis of the isolated solids allowed for the unambiguous identification of 1(3)-feruloyl-*sn*-glycerol (F-MAG) and 1,3-diferuloyl-*sn*-glycerol (F_2-DAG). The F-MAG spectrum consisted of three unique sets of glycerol protons in a 2:1:2 ratio (H-11/H-12/H-13), indicating an *sn*-1(3) substitution of the glycerol backbone. The ratio of the feruloyl methyl peak (H-7) to the glycerol protons confirmed a single feruloyl moiety per glycerol. The F_2-DAG spectrum showed two sets of glycerol protons (H-11/H-12) in a 4:1 ratio, consistent with an *sn*-1,3 substitution pattern. The ratio of the feruloyl methyl peak (H-7) to the glycerol protons confirmed two feruloyl moieties per glycerol. Identification of the F-MAG and F_2-DAG was confirmed by HPLC-MS (discussed shortly).

The unexpected discovery of the F_2-DAG species indicated that a significant quantity of the F_2-TAG must be produced during the transesterification of SBO with EF. However, previous HPLC–MS methods had not revealed the presence of any diferuloylated acylglycerol species (19). Therefore, a new HPLC–MS method was developed to better separate and identify the feruloylated and diferuloylated acylglycerol species.

HPLC–MS analysis of feruloyl and diferuloyl acylglycerols. The transesterification of SBO with EF resulted in a mixture of feruloylated and diferuloylated acylglycerol species. A reversed-phase HPLC method using a water/methanol/1-butanol solvent gradient developed on a phenyl-hexyl column was investigated for the separation of the feruloylated and diferuloylated acylglycerols. As shown in Figure 3B, the HPLC method was effective in separating the F-MAG and F_2-DAG from the FA and residual EF. These species were chromatographically identified using FA and EF standards and the F-MAG and F_2-DAG isolated and purified by preparative HPLC (discussed earlier). The F-DAG through F-TAG regions [retention time (R_t) = 18 to 27 min], however, were convoluted with peaks due to the multiple fatty acids in SBO, making the assignment of the diferuloylated species ambiguous.

To deconvolute the HPLC–MS chromatogram and simplify the analysis of the feruloylated species, the lipase-catalyzed transesterification of triolein with EF was investigated. During the study of this reaction, it was observed that using glycerol as a co-reactant enhanced production of the feruloylated species. Thus, the transesterification of triolein with EF detailed herein was performed in the presence of glycerol. The chromatogram of the reaction of a 2:2:1 ratio of EF/triolein/glycerol after reaching equilibrium (120 h) is shown in Figure 3A. The F-MAG, F_2-DAG, EF, and FA were identified by using standards as described above. The remaining peaks were identified by HPLC–APCI–MS in the negative ion mode.

Table 1 lists the peaks obtain by HPLC–MS chromatography and their corresponding R_t and major ions. The identification of the F-MAG and F_2-DAG isolated by preparative HPLC and characterized by ^{1}H and ^{13}C NMR spectroscopy was confirmed by HPLC–MS. The major ions of the F_2-DAG species were 23 mu greater than their calculated exact mass, which corresponds to the addition of a Na^+ atom to the negative F_2-DAG ion. Ion contaminants from salts (e.g., Na^+ and Li^+) often form adducts with the major ion (21).

The presence of the previously undetected diferuloyl monooleoylglycerol (F_2MOG) species was confirmed by HPLC–MS using the new reversed-phase pheny-hexyl column method. The F_2MOG eluted as a single peak (R_t = 20.2 min; Fig. 3A) with the expected mass (Table 1) followed by a small shoulder. The major ion detected for the shoulder also corresponded to the mass for F_2MOG. Because Novoym 435 has an *sn*-1(3)-positional acylation preference, it was assumed that the major F_2MOG peak corresponded to the 1,3-diferuloyl-2-oleoyl-*sn*-glycerol isomer and the shoulder was the less abundant 1,2-diferuloyl-3-oleoyl-*sn*-glycerol. Similarly, the other diferuloylated species, F_2-DAG, eluted as two peaks, each possessing the same mass. The larger peak (R_t = 11.1 min; Fig. 3A) was identified by the F_2-DAG standard isolated by preparative HPLC and was confirmed by ^{1}H NMR to be the 1,3-diferuloyl-*sn*-glycerol (discussed earlier). Thus, the less abundant peak was assumed

TABLE 1
Feruloylated Species Obtained from the Novozym 435-Catalyzed Transesterification of Triolein with Ethyl Ferulate (EF)

Species[a]	R_t (min)[b]	Exact mass	Major ion (*m/z*)[c]	Peak area (%)[d]
F-MAG	6.7	268.1	266.9 $[M - H]^-$	8.2
FMOG	18.5	532.3	531.1 $[M - H]^-$	29.6
FDOG	25.3	796.6	795.3 $[M - H]^-$	11.7
F_2-DAG	11.1	444.1	464.9 [M – H + Na]	13.8
F_2-DAG[e]	11.4	444.1	465.0 [M – H + Na]	0.6
F_2MOG	20.2	708.1	714.3 [M – H + Li]	1.1
F_2MOG[e]	20.2 sh[f]	708.1	707.1 $[M - H]^-$	0.9

[a]Feruloylated species obtained after the enzyme-catalyzed reaction of 2:2:1 mole ratio of EF/triolein/glycerol at 60°C (120 h). FA, ferulic acid; F-MAG, feruloylated MAG; FMOG, feruloyl monooleoylglycerol; FDOG, feruloyl dioleoylglycerol; F_2-DAG, diferuloylated DAG; F_2MOG, diferuloyl monooleoylglycerol.
[b]Retention times were determined using the UV6000 detector (325 nm) during HPLC–MS.
[c]Major ions detected by HPLC–MS in negative ion mode.
[d]The percent peak area of the species related to the total peak area of all feruloylated species, including FA and EF, as detected by HPLC using the UV detector (325 nm).
[e]*sn*-1,2-Diferuloyl positional isomers.
[f]Shoulder.

to be 1,2-diferuloyl-*sn*-glycerol. The elution of the 1,3-F_2-DAG slightly before the 1,2-F_2DAG is consistent with the reversed-phase HPLC elution sequence of 1,3-dipalmitoyl glycerol before 1,2-dipalmitoyl glycerol and 1,3-dilinoleoyl glycerol before 1,2-dilinoleoyl glycerol (6). Therefore, it can be concluded that the *sn*-1,3-diferuloyl isomers eluted before the *sn*-1,2-diferuloyl isomers, further suggesting that the larger F_2MOG peak was the 1,3-diferuloyl-2-oleoyl-*sn*-glycerol.

Feruloyl monooleoylglycerol (FMOG) species eluted as a small shoulder preceding a single peak (R_t = 18.5 min; Fig. 3A). The main peak possessed a major ion of the expected mass, but the MS data for the shoulder were inconclusive. It was likely, however, that the shoulder was also an FMOG isomer. Based on the *sn*-1(3) positional acylation preference of Novozym 435, it was concluded that the feruloyl group of the various monoferuloyl acylglycerol species occupies the *sn*-1(3)-position. Hydrolysis of the feruloylated oleoylglycerols offered evidence as to which FMOG positional isomer, the 1-feruloyl-3-monooleoyl-*sn*-glycerol or 1-feruloyl-2-oleoyl-*sn*-glycerol, corresponded to the shoulder and which corresponded to the main peak. Consider the Novozym 435-catalyzed transesterification of triolein with EF without glycerol, which produced the highest feruloyl dioleoylglycerol (FDOG) to FMOG ratio (Table 2). The FDOG, 1-feruloyl-dioleoyl-*sn*-glyerol, when hydrolyzed by the predominantly *sn*-1,3-specific *B. cepacia* lipase, which showed no hydrolysis activity toward EF (19), would be expected to yield the 1-feruloyl-2-oleoyl-*sn*-glycerol FMOG isomer. The HPLC chromatogram of the hydrolyzed reaction mixture after 24 h (data not shown) showed a large increase in the FMOG shoulder (R_t = 18.5 min) and complete consumption of the FDOG. This strongly suggested that the FMOG shoulder was the 1-feruloyl-2-oleoyl-*sn*-glycerol isomer. Thus, the FMOG main peak was designated as the 1-feruloyl-3-oleoyl-*sn*-glycerol isomer. The FMOG main peak was essentially consumed within 24 h under the hydrolysis conditions and would be expected to be converted to F-MAG. Indeed, the F-MAG peak increased relative to the consumption of the FMOG main peak, further supporting that the FMOG main peak was the1-feruloyl-3-oleoyl-*sn*-glycerol.

The results just presented show that the reversed-phase, phenyl-hexyl column, HPLC–MS method allowed for the separation and unambiguous identification of the various feruloylated and diferuloylated oleoylglycerols and their positional isomers formed during the lipase-catalyzed transesterification of triolein with EF. Although the many feruloylated acylglycerols formed during the transesterification of SBO with EF (R_t

TABLE 2
Ratio of Reactants and the Corresponding Distribution of Feruloylated Mono- and Dioleoyl Glycerol Products

		Product species distribution (%)[a]							
Trial	Triolein/EF/glycerol (mole ratio)	F-MAG (6.7)[b]	F_2-DAG (11.1)[b]	F_2-DAG (11.4)[b]	FMOG[c] (18.5)[b]	FMOG (18.5)[b]	F_2MOG (19.2)[b]	F_2MOG[c] (19.2)[b]	FDOG (25.3)[b]
A	1:1:1	18.6	12.2	0.3	4.0	32.5	0.9	0.6	7.5
B	2:2:1	8.2	13.8	0.6	3.9	29.6	1.1	0.9	11.7
C	4:4:1	3.0	15.7	0.6	3.0	21.7	2.3	1.1	16.0
D	1:1:0	0.4	6.2	0.0	1.1	11.0	8.2	0.0	19.7

[a]The product species distributions at reaction equilibrium were determined as the percent peak area of the species related to the total peak area of all feruloylated species, including FA and EF, as detected by HPLC using the UV detector (325 nm). See Table 1 for acronym definitions.
[b]The R_t (min) corresponding to the peaks in Figure 2A.
[c]Ratio of shoulder to the main peak (see Fig. 2A).

= 18.0–28.0 min, Fig. 3B) could not be as cleanly separated using the phenyl-hexyl HPLC–MS column method because of SBO's multiple fatty acids, the results from the triolein esterifications were used to assign the peaks to classes of feruloylated SBO glycerols. Figure 3B shows that the F-DAG, F_2-TAG, and F-TAG eluted at times similar to those of their corresponding feruloylated oleoylglycerols. This allowed for quantification of the three groups of feruloylated acylglycerols, which was not possible using previous HPLC methods (19,20).

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