Behavior of Diglycerides and Conjugated Fatty Acid Triglycerides in Reverse-Phase Chromatography

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The behavior of conjugated fatty acid triglycerides and diglycerides on reverse-phase chromatography was studied. Trieleostearin is a geometric isomer of trilinolenin. The conjugated double bond arrangement in trieleostearin enhances its hydrophobic interaction with the stationary phase and causes it to be eluted later than trilinolenin. In separation of "critical pairs" of tri- and diglycerides, diglycerides elute later than triglycerides due to the longer fatty acid constituent. Position isomers of 1,2- and 1,3-diglycerides can be separated by reverse-phase high-performance liquid chromatography.

KEY WORDS: Diglycerides, reverse-phase HPLC, triglycerides.

Separation of triglycerides by reverse-phase high-performance liquid chromatography (HPLC) with various high-efficiency packed columns and mobile phases was well documented in the late 1970s and early 1980s (1-9). These columns allowed separation of triglycerides not only by their acyl chainlength but also by the degree of unsaturation within the same chainlength. Rules for the chromatographic behavior of triglyceride molecular species have been developed based on equivalent carbon number (ECN) (9), theoretical carbon number (TCN) (10) and matrix model (11).

In general, ECN and TCN methods are used in predicting the elution order of triglycerides on reverse-phase HPLC. El-Hamdy and Perkins (9) gave the definition of ECN as:

$$ECN = CN - 2n \quad [1]$$

where CN = actual number of carbon and n = number of double bonds per molecule.

A lower ECN triglyceride elutes prior to those with higher ECN. Within each ECN triglyceride category, elution begins with the triglyceride having the greatest number of double bonds and terminates with those that have the least number of double bonds. *Cis-trans* isomers of a triglyceride can also be separated on a reverse-phase column (9). However, separation of position isomers has not been reported.

During studies of formation of eleostearic acid (9*c*,11*t*,13*t*-octadecatrienoic acid) in tung nut and isolation of its corresponding synthetase, we have established the elution position of trieleostearin on reverse-phase HPLC. In the present report, the behavior of a triglyceride with conjugated double bonds during reverse-phase HPLC and its elution order in relation to triglycerides and diglycerides of the same ECN are elucidated. A mechanism for separation of diglyceride positional isomers by reverse-phase HPLC is postulated.

EXPERIMENTAL PROCEDURES

Materials. Triglyceride and diglyceride standards were obtained from Sigma (St Louis, MO). Acetone, acetonitrile and tetrahydrofuran (THF) (HPLC-grade; Burdick & Jackson Brand) were purchased from Baxter International Inc. (McGaw, IL). Triglyceride and diglyceride standards were solubilized in THF at 100 mg/mL.

Oil extraction. The oil was extracted from tung kernels with petroleum ether according to the procedure of Conkerton *et al.* (12). A typical extraction was as follows: Dry kernels (2.5 g) were placed in a Stomacher bag with 100 mL of petroleum ether, then extracted in a Stomacher Lab-Blender 400 (Tekmar Co., Cincinnati, OH) for 2.5 min. The oil was collected by filtration, and petroleum ether was removed by evaporation under nitrogen. The oil was solubilized in THF at 100 mg/mL.

Triglycerides analyses. Triglycerides and diglycerides were analyzed by reverse-phase HPLC according to the procedure of Chang *et al.* (13). Analyses are performed in triplicate. Ten μL of solution were injected onto two in-series Supelco C-18 columns (150 × 4.6 mm; Supelco Inc., Bellefonte, PA). Samples were separated by isocratic elution at a flow rate of 1.0 mL/min; a mixture of acetone and acetonitrile (3:1) was used as the mobile phase. Peaks were detected by a refractive index detector, and individual peaks were identified by comparing the retention time of the peak with that of reference triglycerides or diglycerides. Peaks for which commercial standards were not available were collected and derivatized to fatty acid methyl esters (FAMEs). The FAMEs were identified by gas chromatography and mass spectrometry.

RESULTS AND DISCUSSION

Complex mixtures of triglycerides containing varied acyl components are difficult to resolve during HPLC analysis and separation procedures. One of these difficulties is the formation of "critical pairs," i.e., structures of identical ECN. Figure 1a shows the triglyceride profile of oil extracted from tung nuts. Tung oil contains five triglycerides in two basic forms, trieleostearin and substituted dieleostearins. Eleostearic acid has three conjugated double bonds. Eleostearic and linolenic acid have the same ECN, and, therefore, they represent a critical pair. Similarly, trieleostearin (ECN = 36) and trilinolenin (ECN = 36) are critical pairs. Figure 1b shows the separation of trieleostearin and trilinolenin. Trieleostearin eluted 0.4 min after trilinolenin. Trieleostearin and trilinolenin are also geometrical isomers that differ in double bond arrangement. Therefore, reverse-phase HPLC has the capability to separate geometric isomers of triglyceride as well as *cis* and *trans* isomers (9). It also appears, from retention time data, that double bonds arranged in a conjugated manner increase the hydrophobic interaction of a triglyceride with the stationary phase of the column.

Rules for separation of diglycerides on reverse-phase HPLC are the same as the rules for triglycerides, i.e., separation is based on ECN. Figure 2 shows the elution profiles of triglyceride and diglyceride standards. With ECN as the criterion for defining critical pairs, distearin (ECN = 36) and trilaurin (ECN = 36) form a critical pair. Distearin eluted at 12.0 min, 2.5 min after trilaurin. The acyl chainlength in the diglyceride (C = 18) is longer than that in the triglyceride (C = 12). These results indicate

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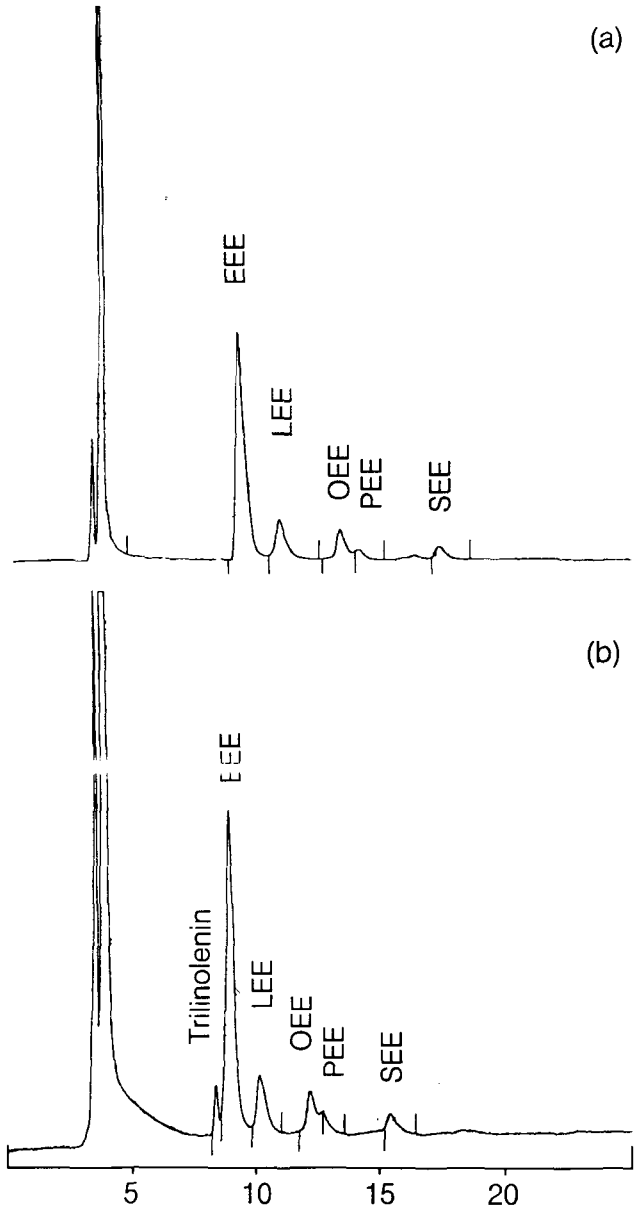

FIG. 1. Reverse-phase high-performance liquid chromatography (HPLC) separation of tung oil. (a), tung oil; (b) trilinolenin plus tung oil. EEE, Trieleostearin, LEE, linoleoyl–dieleostearin; OEE, oleoyl–dieleostearin; PEE, palmitoyl–dieleostearin; SEE, stearoyl–dieleostearin. See text for HPLC conditions.

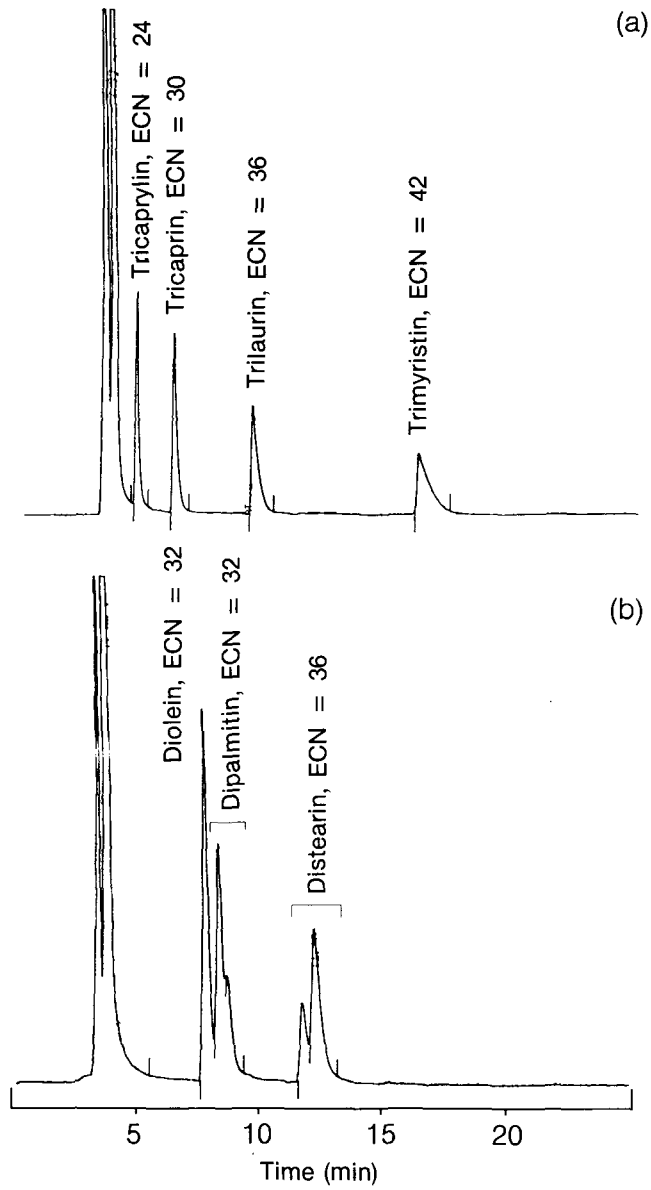

FIG. 2. HPLC separation of triglyceride and diglyceride standards; (a), triglyceride; (b), diglyceride. See text for HPLC conditions. ECN, equivalent carbon number. See Figure 1 for other abbreviation.

that the length of the fatty acid chain provides a basis for separation of critical pairs of diglycerides and triglycerides.

The separation of triglyceride positional isomers cannot be achieved by reverse-phase HPLC (14). 1,2-Dioleoyl-3-stearoyl-*rac*-glycerol and 1,3-dioleoyl-2-stearoyl-*rac*-glycerol are coeluted as a single peak during reverse-phase HPLC. However, the positional isomers of a diglyceride are separable by reverse-phase HPLC. Figure 3 shows the reverse-phase HPLC separation of 1,2- and 1,3-distearin, which are positional isomers of the diglyceride. The 1,3-isomer eluted 0.7 min ahead of the 1,2-isomer. Fatty acids attached at the 1,2-positions of the glycerol moiety are arranged on opposite sides of the carbon axis of glycerol, whereas those of the 1,3-position are on the same side (Fig. 4). It is proposed that the former arrangement of fatty acids enhances the affinity of the diglyceride for the stationary phase. In other words, fatty acid arrangements in the 1,3-position of diglyceride create a stearic effect that decreases hydrophobic interaction of the diglyceride and stationary phase. Therefore, the retention time of the 1,2-isomer is longer than that of the 1,3-isomer.

In summary, the conjugated arrangement of double bonds in a triglyceride increases hydrophobic interaction with the stationary phase. Separation of critical pairs consisting of a diglyceride and a triglyceride is dependent

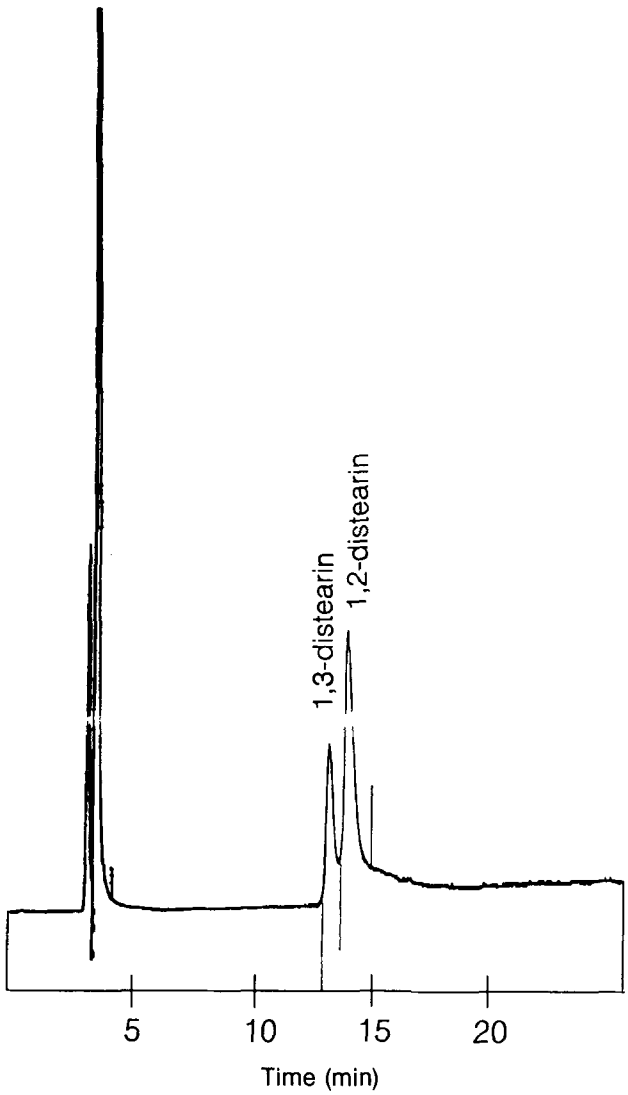

FIG. 3. HPLC separation of 1,3- and 1,2-diglyceride isomers. See text for HPLC conditions. See Figure 1 for abbreviation.

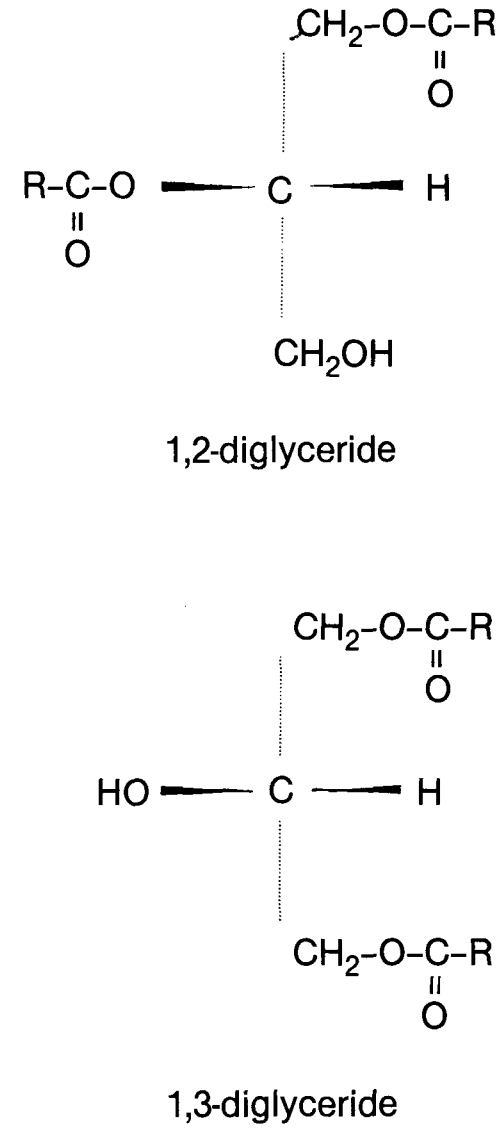

FIG. 4. General structure of diglycerides.

on the fatty acid constituent, i.e., diglycerides containing longer chain fatty acids elute later than the triglyceride with the same ECN but containing shorter-chain fatty acids. Position isomers of diglycerides (1,2- and 1,3-), can be separated by reverse-phase HPLC because of the arrangement of the fatty acids on the glycerol axis.

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