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Note

Prediction of retention times of hydroxylated triacylglycerols in reversed-phase high-performance liquid chromatography

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Enzymic modification of lipids appears to be a promising area of industrial lipase technology, in which substrate- or regiospecificities of these enzymes are utilized to produce ester lipids for tailor-made applications[1–4]. Castor oil, for example, containing up to 90% ricinoleic acid (12-hydroxyoctadec-9-enoic acid), is an excellent natural source of hydroxylated fatty acids that are used to modify fats and oils via interesterification with lipase. Although no single analytical technique can resolve all triacylglycerol species produced, reversed-phase high-performance liquid chromatography (RP-HPLC) nevertheless provides a powerful tool for the rapid routine analysis of such complex mixtures. One problem in RP-HPLC analysis, however, is the formation of "critical pairs", *i.e.*, triacylglycerol species having the same equivalent carbon number (*ECN*) in spite of differences in chain length and number of double bonds[5,6]. The problem of hydroxylated species in such analyses has not been addressed so far, only one group having reported the separation of castor oil triacylglycerols by RP-HPLC[7,8]. In this paper we report a new equation based on equivalent carbon numbers (*ECN*), which allows relative retention times of hydroxylated triacylglycerols to be predicted and such species to be identified in complex mixtures with straight-chain triacylglycerols.

EXPERIMENTAL

Materials

Castor oil was purchased from Alberdingk and Boley (Krefeld, F.R.G.) and beef tallow from Fischermanns (Duisburg, F.R.G.). Crude lipase from *Chromobacterium viscosum* was obtained from Toyo Jozo (Shizuoka, Japan). Triricinolein was isolated from castor oil by flash chromatography[9], 12-hydroxystearic acid was purchased from Serva (Heidelberg, F.R.G.) and juniperic (16-hydroxypalmitic) acid, tripalmitin, tristearin, triolein and trilinolein were purchased from Sigma (Munich, F.R.G.). Mixtures of standard triacylglycerols containing hydroxylated fatty acids were obtained by lipase-catalysed interesterification of the standard straight-chain triacylglycerols (25 μmol) with triricinolein (25 μmol), juniperic acid (75 μmol) and 12-hydroxystearic acid (75 μmol), respectively. The reactants were dissolved in 4 ml of light petroleum (b.p. 60–80°C) and incubated with 7.7 mg of crude lipase for 48 h at 50°C after addition of 5 μl of 0.1 *M* phosphate buffer (pH 7.0).

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Analytical techniques

The RP-HPLC system consisted of an HPLC pump (Kontron Model 420) coupled with a differential refractometer (Erma ERC-7510) and a Rheodyne Model 7125 loop (20-μl) injector. An integrator (Shimadzu Chromatopac C-R6A) was used to monitor accurate retention times at a chart speed of 0.2 cm/min. The columns used for separations were arranged in series and consisted of a 30 × 4.0 mm I.D. guard column and two 120 × 4.0 mm I.D. Spherosil S3 ODS-2 main columns, 3-μm particles (Knauer, Berlin, F.R.G.). The columns were maintained at 35°C by means of a column oven (Microlab, Aarhus, Denmark). RP-HPLC analysis was performed isocratically using acetonitrile–tetrahydrofuran (74:26, v/v) as the mobile phase at a flow-rate of 1 ml/min; the solvents were degassed with helium for 5 min before use.

The sample size was 20 μl of a 10–20% solution of triacylglycerol in acetone–dichloromethane (50:50, v/v).

RESULTS AND DISCUSSION

It is well known that the retention times of triacylglycerols with constituent saturated and unsaturated fatty acids (including the polyunsaturated type with a divinylmethane system of double bonds) can be predicted from their equivalent carbon umber (*ECN*) using the formula $ECN = CN - 2n$, where *CN* is the sum of the carbon numbers of the acyl groups and n the sum of double bonds in a triacylglycerol molecule[5,6]. This concept was very helpful when the resolving power of RP-HPLC was limited. Modern HPLC stationary phases, solvent systems based on acetonitrile[10] or propionitrile[11] and lower temperatures[12] allow, however, the resolution of triacylglycerols that earlier were considered "critical pairs".

A mixture of two natural fats alone leads to an enormous number of different triacylglycerol species, and after interestification of these oils additional species are obtained. The situation is even more complex when a hydroxyl group, such as in 12-hydroxyoctadec-9-enoic (ricinoleic) acid, becomes a third parameter determining retention times. However, when product formation of triacylglycerols with hydroxylated compounds is monitored by RP-HPLC, rapid interpretation of the chromatograms of the complex mixtures formed is necessary for further optimization of the reaction conditions and product control.

As standards containing hydroxylated triacylglycerols were not available, we prepared such mixtures by interesterification of triricinolein with, *e.g.*, triolein, catalysed by nonspecific lipase from *Chromobacterium viscosum*. The resulting mixture, composed of RRR, RRO, ROO and OOO, was analysed by RP-HPLC (for abbreviations, see legend to Fig. 1). All other standard mixtures containing RRP, RRS, RRL, ROL, RSP, etc., were synthesized in the same way and identified by their retention times in RP-HPLC in comparison with those of the starting triacylglycerol mixture. The number of individual standards thus prepared equalled the number of triacylglycerol species expected from interesterification of castor oil with beef tallow. On combining two or more standard mixtures of triacylglycerols that contain ricinoleic acid, several critical pairs, *e.g.*, RRS and RLL or RSP and LLO, are seen in RP-HPLC analysis. By comparing the retention times of such critical pairs, we found that the *ECN* concept can be applied to triacylglycerols containing hydroxylated fatty acids, based on the observation that one hydroxyl group reduced the retention time by the equivalent of six carbon atoms.

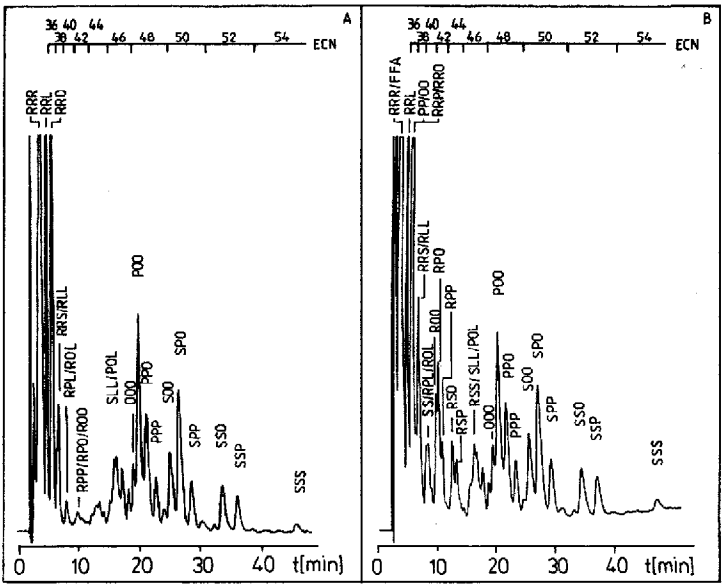

Fig. 1. RP-HPLC of a 2:1 (w/w) mixture of castor oil and beef tallow. (A) Analysis of triacylglycerols of the initial blend; (B) analysis of triacylglycerols after lipase-catalysed interesterification of the initial blend. Analytical conditions as described under Experimental. Abbreviations: OOO, POO, etc., denote triacylglycerol species containing the fatty acyl groups designated P, S, O, L, R, H, J (palmitoyl, stearoyl, oleoyl, linoleoyl, ricinoleoyl, hydroxystearoyl and juniperoyl, respectively) regardless of their position in the glycerol moiety. FFA, free fatty acids. The numbers, *e.g.*, 46, denote equivalent carbon numbers (*ECN*), where $ECN = CN - 2n - 6m$. All critical pairs having the same *ECN*, *e.g.*, 48 for OOO, POO, etc., are denoted as fraction *ECN* 48 in the text. Peaks and shoulders not denoted are triacylglycerols containing the minor fatty acids of castor oil and beef tallow.

The *ECN* equation can now be extended to

$$ECN = CN - 2n - 6m$$

where m is the sum of hydroxyl groups in a triacylglycerol molecule. To compromise between sufficient resolution and an acceptable analysis time and with omission of time-consuming derivatization procedures, we used acetonitrile–tetrahydrofuran (74–26, v/v) as the mobile phase. More polar solvent systems lead to the precipitation of saturated compounds. The RP-HPLC analysis was performed at 35°C to avoid further broadening of peaks of high-melting triacylglycerols. In this instance the resolving power is limited and cannot be improved by inclusion of additional columns in series owing to the pressure drop.

One important application was the expeditious identification of newly synthesized triacylglycerols that were obtained by interesterification of castor oil and beef tallow, catalysed by lipase from *Chromobacterium viscosum*. Fig. 1A shows the RP-HPLC trace for an initial blend of castor oil and beef tallow (2:1, w/w) that was analysed with sufficient separation within 45 min. After incubation of this mixture with the lipase a complex pattern of old and newly synthesized triacylglycerols was obtained with new peaks arising from *ECN* 36 to 46 (Fig. 1B). The new equation

TABLE I

EQUIVALENT CARBON NUMBERS (*ECN*) AND RELATIVE RETENTION TIMES OF TRIACYLGLYCEROLS CONTAINING HYDROXYLATED FATTY ACIDS

Abbreviations as in the legend to Fig. 1.

ECN	*Triacylglycerols containing ricinoleic acid*	*Retention time[a] in RP-HPLC*	*Triacylglycerols containing juniperic acid or 12-hydroxystearic acid*	*Retention time[a] in RP-HPLC*
36	RRP/RRO	0.29	JJO	0.28
38	RRS/RLL	0.34	JJS	0.34
40	RPL/ROL	0.41	HHO	0.39
42	RPP/RPO/ROO/RSL	0.52	JOO/HHS	0.54
44	RSP/RSO	0.65	HOO	0.61
46	RSS	0.84	JSS	0.87
48	–	–	HSS	1.00

[a] Relative to triolein (OOO = 1).

allowed the precise identification of all triacylglycerols containing ricinoleic acid by their predicted retention times (Table I).

Further investigations showed that the extended equation is applicable not only to the RP-HPLC analysis of triacylglycerols containing ricinoleic acid, but also to those containing other hydroxylated fatty acids, such as juniperic acid or 12-hydroxystearic acid. As demonstrated in Table I, good correlations between calculated *ECN*s of hydroxylated triacylglycerols and their retention times relative to triolein were observed.

The *ECN* concept as reported here may be transferred to other hydroxylated compounds. As a rule of thumb, this concept is very helpful for the identification of complex reaction mixtures in routine RP-HPLC analyses, where a complete separation of hydroxylated compounds in the presence of unsaturated triacylglycerols is tedious and time consuming.

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