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Separation of glyceride positional isomers by silver ion chromatography

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Abstract

Separation of triglyceride and diglyceride positional isomers by silver ion high-performance liquid chromatography coupled with an evaporative light-scattering detector is described. The triglyceride isomers had a fatty acid composition of CLC and CCL, where C and L were caprylic acid and linoleic acid, respectively. Diglyceride isomers, 1,2(2,3)-diglyceride and 1,3-diglyceride, which contained caprylic acid were separated too. A solvent system based on *n*-hexane, 2-propanol, ethyl acetate, and acetonitrile with a flow-rate of 0.8 ml/min was developed. Calibration curves of CLC and CCL were achieved with triolein as internal standard. Using this method, the incorporation of linoleic acid onto specific a position of glycerol backbone can be monitored.

Keywords: Positional isomers; Silver ion chromatography; Glycerides; Diglycerides; Triglycerides; Dicaprylins; Fatty acids

1. Introduction

In recent years with increasing knowledge and understanding about the effects of fatty acid chain length, unsaturation, and positional distribution on metabolism and health, studies have been attempted on the modification of natural fats and oils or for the production of structured triglycerides (TGs). These contain different fatty acids esterified at a specific position of glycerol backbone and may provide the most effective means of delivering desired fatty acids for nutritive or therapeutic purposes, targeting specific diseases and metabolic conditions [1].

TGs with medium-chain fatty acids in the *sn*1- and *sn*3-positions of glycerol and unsaturated long-chain fatty acids in the *sn*2-positions, have been reported as alternatives to medium-chain triglycerides for treatment of patients with lipid malabsorption, e.g., pancreas insufficiency [2]. Enzymatic methods for the synthesis of such TGs are becoming increasingly attractive with regard to chemical methods.

With the aim of an enzymatic synthesis of CLC (C: caprylic acid, $C_{8:0}$ and L: linoleic acid, $C_{18:2}$ Fig. 1), the positional distribution of acyl groups in TGs during the enzymatic reaction must be known, and in particular the number of linoleic acids which are incorporated at a specific glycerol position. In

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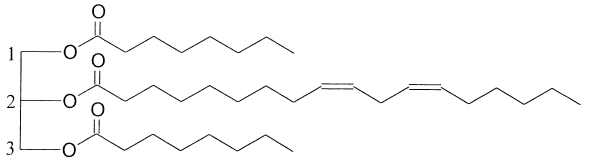

Fig. 1. Structured triglyceride CLC. C is caprylic acid (octanoic acid, $C_{8:0}$) and L is linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid, $C_{18:2}$).

order to monitor the esterification reaction between 1,3-dicaprylin (1,3-dC) and linoleic acid catalysed by lipase, a simple and rapid analysis method is needed. Silver ion high-performance liquid chromatography (Ag-HPLC) allows the simultaneous determination of both the molecular species composition of TGs and the positional distribution of fatty acids [3]. Han et al. [4] reported a separation of TGs containing eicosapentaenoic acid (EPA) for the monitoring of the transesterification of tricaprylin with EPA ethyl ester. However, no separation of isomeric 1,2(2,3)-diglyceride and 1,3-diglyceride was obtained. Adlof [5] had reported a separation of palmitic acid diglyceride positional isomers but as acetate forms. In this work, we report a method based on Ag-HPLC to separate the different isomers of TGs which contain linoleic acid incorporated onto the glycerol molecule. Furthermore the two positional isomer diglycerides 1,3-dicaprylin and 1,2 (2,3)-dicaprylin are also separated under these conditions.

2. Experimental

2.1. Reagents

n-Hexane was obtained from SDS (Peypin, France). Acetonitrile was purchased from Carlo Erba (Val de Reuil, France). 2-Propanol and dichloromethane were supplied by Merck (Nogent-sur-Marne, France). Ethyl acetate, dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (4-DMAP) and glycerol were provided by Acros (Noisy le Grand, France). Tricaprylin and 1,2-dicaprylin were purchased from Sigma (St. Quentin Fallavier, France), linoleic acid and caprylic acid from Aldrich (St. Quentin Fallavier, France). Triolein was purchased from Fluka (St. Quentin Fallavier, France). Immobilized lipase IM60 from *Mucor miehei* was supplied by Novo Nordisk (Bagsvaerd, Denmark). All solvents were of HPLC grade and used as received. 1,3-dC and the different TGs were synthesised according to Section 2.4 and were characterised by ^{1}H and ^{13}C nuclear magnetic resonance (NMR). The specific fatty acid in the 1(3)-, 2-, and 3(1)-positions are designated by the letters C (caprylic; octanoic acid, $C_{8:0}$), and L (linoleic; *cis*-9,*cis*-12-octadecadienoic acid, $C_{18:2}$). The 1- and 3-positions are not differentiated.

2.2. Chromatographic instrumentation

The chromatographic separation was performed with a Kontron HPLC apparatus (St. Quentin Yvelines, France). A Rheodyne 7725 injector (Rheodyne, Cotati, CA, USA) with a 20-μl injection loop was used. Samples were detected with evaporative light-scattering detection (ELSD; Sedex 35 detector from Sedere, France). Temperature and pressure of detector were 40°C and $2.5 \cdot 10^{5}$ Pa, respectively. A Chrompack silver ion chromatography column (250×4.6 mm, Varian-Chrompack, Middelburg, The Netherlands) was used. The data processing was performed with a Kontron data system 450-PCIP MT2.

2.3. Operating conditions

Mobile phases were: (A) *n*-hexane–2-propanol–ethyl acetate (820:40:140) and (B) *n*-hexane–2-propanol–acetonitrile (956:40:4). The flow-rate was standardised at 0.8 ml/min. The solvent gradient program used is shown in Table 1. Triolein was used as internal standard.

Table 1
The mobile phase gradient program

Time (min)	A (%)	B (%)	Flow-rate (ml/min)
0	100	0	0.8
8	100	0	0.8
15	0	100	0.8
18	100	0	0.8
40	100	0	0.8

Solvent A: *n*-hexane–2-propanol–ethyl acetate (820:40:140). Solvent B: *n*-hexane–2-propanol–acetonitrile (956:40:4).

2.4. Preparation of standards

2.4.1. Preparation of 1,3-dicaprylin (1,3-dC)

A solution of glycerol (2.30 g, 25 mmol), and caprylic acid (7.21 g, 50 mmol) was prepared to which immobilized *Mucor miehei* lipase (IM 60, 375 mg) was added. Vacuum was applied after 1 h for water removal. The mixture was stirred at 40°C for 24 h. The lipase was separated off by filtration and the diglyceride was purified by silica gel column chromatography using light petroleum–diethyl ether (80:20) as eluent (35% yield).

2.4.2. Preparation of 1,3-dicapryloyl-2-linolein (CLC)

A solution of 4-DMAP (3 mg, 0.024 mmol) and DCC (180.2 μl of 1 *M* solution in dichloromethane, 0.180 mmol) with 180 μl dichloromethane was prepared. Next, linoleic acid (45.91 mg, 0.164 mmol) and 1,3-dicaprylin (84.50 mg, 0.246 mmol) were added. The resulting mixture solution was stirred at room temperature for 4 h. The liberated urea was filtered and the filtrate was concentrated. The crude product was purified by silica gel column chromatography with light petroleum–diethyl ether (95:5) as eluent (95% yield).

2.4.3. Preparation of 1,2-dicapryloyl-3-linolein (CCL)

The procedure was identical to CLC preparation, except for the use of 1,2-dicaprylin as starting material (70% yield).

2.4.4. Preparation of 2,3-dilinoleoyl-1-caprylin (CLL)

The procedure was identical to CLC preparation using 4-DMAP (6 mg, 0.048 mmol), DCC (360.4 μl of 1 *M* solution in dichloromethane, 0.360 mmol), linoleic acid (91.82 mg, 0.327 mmol), 1-monocaprylin (53.53 mg, 0.245 mmol), (50% yield). In this case, ^{1}H- and ^{13}C-NMR showed small amounts of LCL.

3. Results and discussion

The enzymatic catalysis on 1,3-dC and L mixture led to different products which are listed in Fig. 2. Their separation was obtained with the solvent program described in Table 1. This chromatogram (Fig. 3) was performed with commercial or synthesised standards (see Section 2.4). Under these conditions, the elution order of TGs containing linoleic acid are related to the number of linoleic acid molecules and their isomeric position. Indeed, silver ion chromatography is based on the property of silver ions to form reversible polar complexes with π-electrons of carbon–carbon double bond in organic molecules [3]. Unsaturated compounds are also fractionated according to the number, configuration and position of the double bond. Interactions of silver ion with the free electron pair of the carbonyl-group oxygens have also been implicated in the separation of substrates [3]. Tricaprylin appeared at 3.96 min, CLC and its positional isomer, CCL, had retention times of 8.02 and 8.50 min, respectively. This observed elution order was in agreement with

Compounds		Abbreviations
Starting compounds		
1,3-dicaprylin	$C_{8:0}$–OH–$C_{8:0}$	1,3-dC
Linoleic acid	$C_{18:2}$	L
Expected products		
1,3-dicapryloyl-2-linolein	$C_{8:0}$–$C_{18:2}$–$C_{8:0}$	CLC
1,2-dicapryloyl-3-linolein	$C_{8:0}$–$C_{8:0}$–$C_{18:2}$	CCL
1,2-dilinoleoyl-3-caprylin	$C_{18:2}$–$C_{18:2}$–$C_{8:0}$	CLL
1,3-dilinoleoyl-2-caprylin	$C_{18:2}$–$C_{8:0}$–$C_{18:2}$	LCL
Tricaprylin	$C_{8:0}$–$C_{8:0}$–$C_{8:0}$	CCC
1,2-dicaprylin	$C_{8:0}$–$C_{8:0}$–OH	1,2-dC
Internal standard		
Triolein	$C_{18:1}$–$C_{18:1}$–$C_{18:1}$	OOO

Fig. 2. Starting compounds of the esterification reaction and expected products.

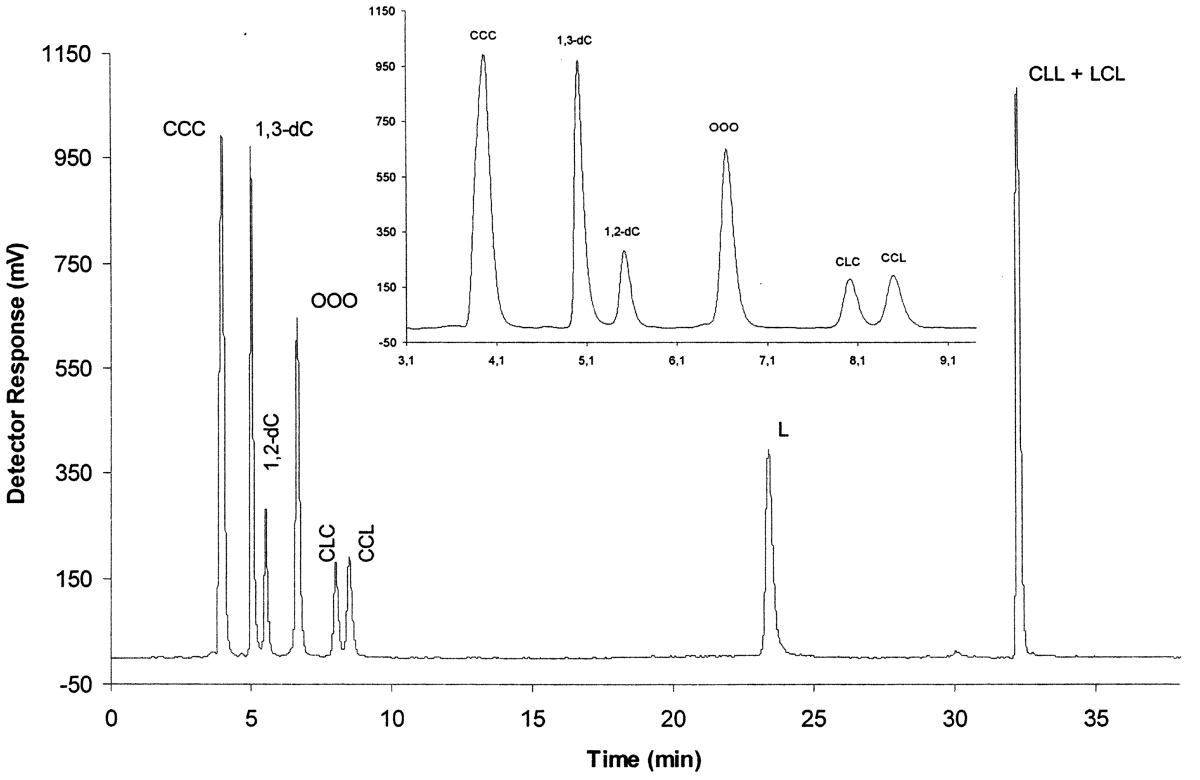

Fig. 3. Analysis of product mixture containing CCC; 1,3-dC; 1,2-dC; OOO; CLC; CCL; L; CLL by silver ion high-performance liquid chromatography (Ag-HPLC) and a magnification from 3.1 to 9.1 min. For abbreviations see Fig. 2.

the TG elution order noted by Han et al. [4] and Adlof [6]. Quantification of the targeted CLC was therefore possible taking triolein as an internal standard (retention time: 6.65 min). Calibration curves of CLC and CCL were achieved (Fig. 4). Because the ELSD response was not linear, a linear log–log calibration curve was established with good correlation coefficients of $R^2=0.997$ and 0.993. It is interesting to note that triolein which contains three double bonds in the whole molecule was less retained than CLC and CCL which contain only two double bonds but on the same fatty acid. The normal-phase contributions of the remaining unreacted silanol groups of the column may influence the separation. These effects have been used by Adlof [7] to explain the retention characteristics of homogeneous TGs containing saturated fatty acids which were found to be inversely related to the number of carbon atoms. In our case, the normal effects tended override the silver ions contributions resulting in a shorter retention time of triolein compared with CLC and CCL.

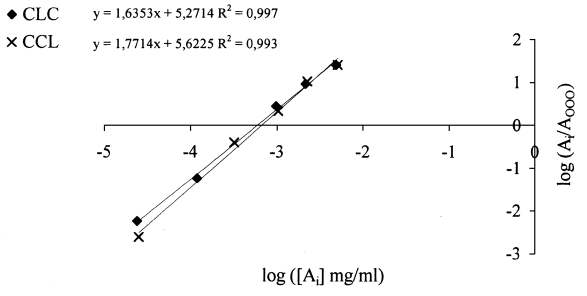

Fig. 4. Calibration curves of CLC and CCL. A_i represents the peak area of CLC and CCL. A_{OOO} represents peak area of internal standard OOO, triolein.

The two positional isomer diglycerides, 1,3-dicaprylin and 1,2-dicaprylin were also separated. Their retention times were 5.01 and 5.52 min, respectively. This elution pattern is in accordance with Adlof [5] who studied the separation of posi-

tional isomer diglycerides, notably of palmitic acids as acetate forms. These substrates do not contain carbon–carbon double bonds. The interactions of the acetate constituent with the original silanol groups of the column (normal-phase effects) have been suggested in the observed differences retention time. Chang et al. [8] separated 1,2- and 1,3-distearylin by reversed-phase chromatography and steric effects have been proposed as explanation.

The separation of 1,3-dicaprylin and 1,2-dicaprylin allows one to quantify the apparition of 1,2-dicaprylin, which could result from acyl migration during enzymatic catalysis.

Use of acetonitrile had been necessary in order to elute TGs containing two linoleic acids. However, in this case CLL and LCL are eluted together. Adlof [6] separated TG positional isomers formed by two unsaturated fatty acids and one saturated fatty acid using two columns connected in series and 0.7 to 0.9% acetonitrile in hexane as solvent.

4. Conclusion

The described technique demonstrates a satisfactory separation of TG positional isomers according to the number and the position of linoleic acid. It is a rapid alternative to currently used analytical and chemical methods, since it allows the simultaneous determination of both the molecular species composition of TGs and the positional distribution of linoleic acid in less than 40 min. Furthermore, a good separation of the two positional isomer diglycerides, 1,3-dicaprylin and 1,2-dicaprylin was obtained. The technique represents a good tool to optimise the enzymatic production of the targeted structured CLC.

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