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Retention properties of triacylglycerols on silver ion high-performance liquid chromatography

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Abstract

The retention properties of series of synthetic disaturated–monounsaturated (1 to 6 double bonds) triacylglycerols and the common seed oil triacylglycerols on a silver ion high-performance liquid chromatography column have been studied in quantitative terms. Retention factors were found to increase stepwise, with a saturated dimonoenoic species being held ten times and trilinolenin ten thousand times more strongly than a disaturated monoenoic species, for example. Formation of chelate-type of pi-complexes between silver ions and double bonds, with participation of the carbonyl oxygen in the complexation, have been assumed in order to explain the high separation power and specificity of the silver ion column.

1. Introduction

In recent years, high-performance liquid chromatography (HPLC) has become one of the most widely applied separation techniques in lipid analysis [1]. Until recently, separation of triacylglycerols by reversed-phase HPLC (RP-HPLC) had no rival, but with the introduction of a stable silver-loaded column [2] silver ion HPLC (Ag-HPLC) has become an established technique, which has been reviewed recently [3]. The advantage of Ag-HPLC is that unlike RP-HPLC it separates species according to a single property: the number (with the configuration) of their double bonds. This simplifies the analysis of natural triacylglycerol mixtures to a great extent as they are resolved into groups with equal unsaturation [4]. It is evident that Ag-HPLC and RP-HPLC applied in a complementary way are to date the most powerful tools in the isolation and analysis of triacylglycerols [5–11].

The general elution order of triacylglycerols obtained with the silver-loaded column described by Christie [5] is:

$$\begin{aligned} &SSS > SSM > SSD > MMM > SMD > MMD \\ &> SDD = SST > SMT = MDD > MMT \\ &> SDT = DDD > MDT \geq STT > DDT \\ &> MTT > DTT > TTT \end{aligned}$$

where S = saturated, M = monoenoic, D = dienoic and T = trienoic residues. One dienoyl residue is retained more strongly then two monoenoyls in one triacylglycerol molecule and one trienoyl (e.g. α-linolenoyl) residue is the equivalent of two dienoyls [5], but there are no quan-

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titative data on the retention characteristics. The aim of the present work was to acquire such data for the most common constituents of natural seed oils as an aid to the understanding of the underlying separation mechanism. Also, for the latter purpose, a series of disaturated monounsaturated (1 to 6 double bonds) triacylglycerols was synthesised and the chromatographic behaviour was examined.

2. Experimental

2.1. Materials

All solvents were HPLC grade and were used without further purification. Oleic, linoleic, α-linolenic, arachidonic, eicosapentaenoic, docosahexaenoic acids, tripalmitin and 1,2-dipalmitin were purchased from Sigma (Poole, UK). Olive oil and sunflower were supplied by LipidTeknik AB (Sweden); linseed oil was from a local shop. The pure triacylglycerols fractions were isolated from each oil by eluting the crude sample through an Isolute silica cartridge (Crawford Scientific, Strathaven, UK) with hexane–acetone (99:1, v/v; 10 ml).

2.2. Triacylglycerol synthesis

Dipalmitoyl monounsaturated triacylglycerols were synthesised via 1,2-dipalmitin and the appropriate acid chlorides [12]. The latter were produced by reacting the free fatty acid (20 mg) with oxalyl chloride (0.5 ml) (Aldrich, Gillingham, UK) at room temperature for 36 h in a test tube [12]. The excess reagents were then removed in a stream of nitrogen and finally by a vacuum pump. The acid chloride was redissolved in 0.5 ml of toluene and was immediately added to dipalmitin (10 mg) in toluene (1 ml)/pyridine (0.2 ml). The mixture was left overnight at 50°C. The excess reagents were removed in a stream of nitrogen and the residue was dried under vacuum. It was then redissolved in hexane (5 ml), washed twice with water (5 ml) and cleaned by elution through a Florisil mini-column (Pasteur pipette) with hexane–acetone (99:1, v/v; 10 ml). The purity was checked by TLC on silica gel with a mobile phase of hexane–acetone (100:8, v/v), and eventually by silver ion HPLC.

2.3. Silver ion high-performance liquid chromatography

A Hitachi L-6200A HPLC pump was used with a Varex Model IIA evaporative light-scattering detector (P.S. Instruments, Sevenoaks, UK). A Nucleosil 5SA column (25 cm × 4.6 mm I.D.) (Hichrom Ltd, Reading, UK) was converted to the silver form as described earlier [2]. The integrator response was regulated by using tripalmitin as test substance. The temperature of the drift tube in the detector was 90°C.

The temperature of the column was maintained at 20.0 ± 1.0°C by fitting it into a water jacket through which water was pumped from a temperature control unit. Dichloroethane–dichloromethane (1:1, v/v) and either acetone or acetonitrile were used in the mobile phases, the compositions of which are listed in Tables 1 and 2.

Table 1
Mobile phase composition (Volume %) for elution of the synthetic disaturated triacylglycerols

Mobile phase composition[a]	Triacylglycerols resolved	
DCE–DCM–AcN, 1:1:0.01	16:0 16:0 18:1	16:0 16:0 18:2
DCE–DCM–AcN, 1:1:0.02	16:0 16:0 18:2	16:0 16:0 18:3
DCE–DCM–AcN, 1:1:0.03	16:0 16:0 18:3	16:0 16:0 20:4
DCE–DCM–AcN, 1:1:0.04	16:0 16:0 20:4	16:0 16:0 20:5
DCE–DCM–AcN, 1:1:0.05	16:0 16:0 20:5	16:0 16:0 22:6

[a] Abbreviations: DCE, dichloroethane; DCM, dichloromethane; AcN, acetonitrile.

Table 2
Experimental conditions for resolution of natural triacylglycerols mixtures on a silver ion loaded ion-exchange column

Oil	Mobile phase composition	Triacylglycerols resolved[a]
Olive oil	DCE–DCM–Acetone (1:1:0.1)	SSM, SMM
	DCE–DCM–Acetone (1:1:1)	SSM, SMM, MMM, SMD
	Acetone (100)	SMM, MMM, SMD, MMD
Sunflower	Acetone (100)	SMM, MMM, SMD, MMD
	Acetone–AcN (100:1)	SMD, MDD, SDD, MDD
	Acetone–AcN (100:2)	SDD, MDD, DDD
Linseed	Acetone–AcN (100:2)	MMM, SMD, MMD, SDD + SST, MDD, SMT
	Acetone–AcN (100:3)	SMT + MDD, MMT, DDD + SMT
	Acetone–AcN (100:4)	MMT, DDD + SDT, MDT
	Acetone–AcN (100:6)	MDT, STT, DDT, MTT
	Acetone–AcN (100:7)	MDT, STT, DDT, MTT, DTT
	Acetone–AcN (100:8)	MDT, STT, DDT, MTT, DTT, TTT

[a] Abbreviations: S, saturated; M, monoenoic; D, dienoic; T, trienoic fatty acid moieties; see also footnote to Table 1.

3. Results and discussion

Triacylglycerols are resolved by silver ion chromatography according to the total number of double bonds in the fatty acid moieties. The position of double bonds in a fatty acyl chain, the chain length of the fatty acids and the position of an unsaturated acyl group on the glycerol moiety have relatively small or negligible effects and are not considered. The principle question addressed here is whether a triacylglycerol molecule participates in complexation as a single entity or whether the general effect is a sum of the separate retention properties of the different fatty acid moieties. It has been established from crystallographic studies that a single silver ion can form a complex with two double bonds in the same or different molecules [13], and the specificity of this type of Ag-HPLC column has been interpreted as due to a dual interaction between a silver ion and either two double bonds or one double bond and an electron-rich centre [5].

Saturated acyl moieties have no significant participation in the retention. PPP (P = palmitic acid) had a k' value of 0.193 with a mobile phase of dichloroethane–dichloromethane (1:1, v/v), compared to PPO (O = oleic acid) with one double bond which eluted with a retention factor of 10.1.

When the polarity of the mobile phase was increased, the k' value of PPP equalled that of a non-retained substance, docosane. If palmitoyl moieties are neglected, triacylglycerols of the SSU type (U = unsaturated acyl moiety) can be treated as derivatives of fatty acids with 1 to 6 double bonds and their chromatographic behaviour can be compared with that of the methyl esters of the same fatty acids. There was no simple relationship between the fatty acid component of a triacylglycerol and its retention on the column, and the results for the k' values of the synthetic PPU triacylglycerols in Table 3 clearly reveal this. The ratio k'_{PPU}/k'_{PPO} is also listed since it expresses the increase in retention with an increasing number of double bonds in the molecule. If a fatty acid moiety were the only factor to affect the retention of a fatty acid derivative in the column, through the interaction of its double bond(s) with silver ion(s), the retention of the different types of derivative would be similar. The general patterns were comparable. As has been found with the methyl esters [5], the greatest relative increase in retention of the PPU triacylglycerols was observed when the number of double bonds in the mole-

Table 3
Calculated k' values of the synthetic dipalmitoyl monounsaturated triacylglycerols for a mobile phase of dichloroethane–dichloromethane–acetonitrile (1:1:0.1 by volume)

Triacylglycerols	k'	k'_{PPL}/k'_{PPO}	$k'_{UFA}/k'^{a}_{18:1}$
16:0–16:0–18:1	0.95	1.0	1.0
16:0–16:0–18:2	20.1	21.2	2.9
16:0–16:0–18:3	35.9	37.8	7.1
16:0–16:0–20:4	53.2	56.4	10.3
16:0–16:0–20:5	134.4	141.8	16.7
16:0–16:0–22:6	196.9	207.7	21.5

[a] For the corresponding fatty acid methyl ester [5].

cule increased from one to two. This could be ascribed to the appearance of a second complexing site in the molecule which enables the formation of a chelate type of complex, i.e. there is a simultaneous interaction of two double bonds and one silver ion. While methyl esters and triacylglycerols are similar in qualitative terms, there are quantitative differences. Methyl linoleate, for example, is held three times as strongly as methyl oleate, but dipalmitoyllinolein is held twenty times more strongly than dipalmitoylolein. Generally, the retention factor of a triacylglycerol was about 5 to 10 times greater than that of a comparable methyl ester.

The higher retention factors of triacylglycerols can be explained through two possible interactions: formation of a stronger silver ion complex because the triacylglycerol molecule is more rigid so that the double bond is approached more easily, and simultaneous interaction of a silver ion with the free electron pair of any of the three carbonyl oxygens. Both effects may play a part. Adsorption effects with the silica gel would not be expected to differ appreciable between methyl esters and triacylglycerols, and with the mobile phases used, hydrophobic interactions with the propylphenyl spacer should be minimal.

Table 4 presents the k' values of the most abundant natural triacylglycerols from olive, sunflower and linseed oil, i.e. the full series of triacylglycerols with zero to 9 double bonds. It is clear from the results that the retention of a monoacid or mixed acid triacylglycerol is not a simple sum of the k' values of the respective monounsaturated triacylglycerols. The data reveal that much more complex interactions occur when the number of double bonds increases. From an SSM triacylglycerol (1 double bond) to TTT (9 double bonds), the relative k' values increased from 1.0 to 10 000. As has been already observed, between triacylglycerols with an equal number of double bonds, one in which the double bonds are concentrated in one fatty

Table 4
k' values for natural triacylglycerols from olive oil, sunflower oil and linseed oil

Triacylglycerols[a]	k'	k'_{TG}/k'_{PPO}
SSM	0.95	1.0
SMM	10.6	11.2
SSD	19.5	20.6
MMM	30.2	31.9
SMD	61.4	64.8
MMD	139.6	147.2
MDD	403.3	425.4
SDD + SST	209.9	221.5
SMT	425.4	448.7
MMT	619.1	653.0
DDD + SDT	796.7	840.4
MDT	1154	1217
STT	1764	1861
DDT	1913	2018
MTT	2543	2683
DTT	4301	4538
TTT	9681	10212

[a] For the abbreviations see the footnote to Table 2.

acid moiety is held more strongly. Thus, SSD is held more than twice as strongly as SMM, and SMD more than twice as strongly as MMM, while the k' of SST is about seven times greater than that of MMM.

In the light of earlier findings [5], it seems probable that one silver ion can interact with two double bonds in different acyl moieties of one triacylglycerol at the same time, although the extent of this interaction may be limited by steric constraints. Whether double bonds in different acyl residues of one triacylglycerol molecule can interact with more than one silver atom simultaneously is not known. Neither is anything known of the nature of the surface of the stationary phase, the topology of silver ions and the conformation of the triacylglycerol molecule, so it is not possible to estimate what type of interaction is more probable. It seems likely that by increasing the number of double bonds leading to a different spatial position of the molecule, the number of single and separate complexation interactions with silver ions increases. The result is that the molecule spends a longer time on the stationary phase.

The greatest relative increase in k' value was between PPO and POO where the replacement of one saturated moiety with one monoenoic increased the k' value 10 times. This may indicate that chelate-type complexes are formed within the molecule. With an increasing number of double bonds, however, the relative rate of increase in the k' values tended to decrease, perhaps because the formation of chelate-type complexes is hindered by stereochemical factors. For example, the spatial arrangement of carbon atoms in different acyl chains may be such that double bonds are held too far apart to interact with a single silver ion simultaneously.

It is of interest to compare the data in Tables 3 and 4 from a practical point of view. The retention factor, k', is related to the selectivity of the separation. It was not possible to find conditions for isocratic elution that would provide reliable results in a reasonable time, and gradient elution seems essential. Critical pairs of triacylglycerols occur and these always have more than four double bonds. Of course, the selectivity of resolution is governed by other factors, and it may yet prove possible to select a mobile phase and gradient to solve these problems. Isocratic elution with acetone–acetonitrile (100:0.2, v/v) enabled successful resolution of the critical pair MDD–SMT. With increasing unsaturation, the difficulties become greater, although some useful separations have been achieved even with fish oils [7,14,15]. Acetonitrile is an essential component of the system, as it complexes strongly with silver ions displacing even polyunsaturated eluents. A more drastic change in the nature of the mobile phase will have profound influences on the order of elution as was observed, for example, in silver ion supercritical fluid chromatography [16].

The silver ion HPLC column has a rather low content of silver (less than 80 mg silver nitrate) [2], and this must be held as a mono-molecular layer at some distance from the silica surface (by the propylbenzenesulphonate spacer). The layer may not be uniform because of repulsion effects of the sulphonate residues. When operated isocratically, peak widths and shapes for unsaturated molecules are far from ideal and measurements of column efficiency indicated that there were only about 170 theoretical plates, although this is rather dependent on sample load. That the column works so well in practise, especially under gradient elution conditions, is undoubtedly due to the strength of the interaction between the silver ion and double bonds and the remarkable differences in the strengths of the interactions with molecules of different degrees of unsaturation, shown in Table 4.

4. Conclusion

The results therefore provide further evidence for the formation of chelate-type pi-complexes between silver ions and double bonds, with participation of the carbonyl oxygen in the complexation as a secondary effect. It is the strengths of these complexes, rather than column efficiency, which explain the high separating power of such columns.

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