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Positional and configurational separation of fatty acid isomers by micro reversed-phase liquid chromatography with an Ag^+-containing mobile phase

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Abstract

Different positional and configurational fatty acids, derivatised into the phenacylesters, were analyzed by reversed-phase liquid chromatography (RPLC) on micropacked fused-silica columns with an Ag^+-containing mobile phase. The π-complexation strength was evaluated by plotting the selectivity coefficients (αAg^+) as a function of $[Ag^+]$. The equilibrium constants (K^*) were determined by means of the hyperbolic dependence of the capacity factors on $[Ag^+]$. Different fat hydrolysates and margarines taken at different stages of palm oil hydrogenation were analyzed. Data obtained with micropacked RPLC and capillary GC for the determination of the *cis*/*trans* ratio of monoenoic acids are compared.

Keywords: Argentation; Fats; Isomers, configurational; Isomers, positional; Margarines; Silver ion complexation; Fatty acids

1. Introduction

Argentation chromatography (AgC) has been extensively applied in different chromatographic techniques to improve the separation of solutes containing olefinic bonds [1–6]. Electrophilic transition metal cations interact with π-orbitals to form reversible π-complexes (Lewis acid–base pairs). The stability of these complexes is low and their formation depends on the number, position, geometry and steric hindrance of the double bonds.

Argentation in liquid chromatography (LC) can be achieved in two different ways namely in the normal-phase mode with Ag^+-loaded stationary phases or in the reversed-phase (RP) mode by adding silver ions to the mobile phase.

Since its introduction, AgC applying thin layer chromatography (TLC) or LC, has been widely used

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for the analysis of lipids. The reader is referred to the reviews of Nikolova-Damyanova [7], Firestone and Sheppard [8], Christie [9] and Ratnayaka [10] for more detailed information. Triglycerides (TGs) are separated according to the number of double bonds and fatty acids (FAs) are separated according to the number, position and geometry of the double bonds. Special attention has been paid to the determination of *trans* unsaturation in fats and oils of animal and vegetable origin (dairy products, shortenings, vegetables, etc.). *trans* Unsaturation can arise from commercial and/or biological (intestinal tract of ruminants) hydrogenation. *trans*-FAs are non essential FAs that, along with lauric, myristic and palmitic acids, have been classified as cholesterol raising compounds by increasing low density lipoproteins and serum lipoprotein Lp(a) while decreasing high density lipoproteins [11]. Determination of *trans* unsaturation has been achieved on silver nitrate-loaded LC columns [6,12] or using a stable ion-exchange column in the Ag^+ form [13–15]. Separations of some unsaturated and heterocyclic solutes with considerably increased selectivity have been carried out by RPLC using mobile phases containing silver salts [3,16–19]. Complexation of a solute with a complexing ion in the mobile phase is not only useful to increase selectivity but also to measure complex stability constants from chromatographic data [20–22]. The stationary phase properties are, in principle, independent of the complexing ion concentration in the mobile phase.

We recently described the separation of different fatty acids, derivatized into their phenacylesters (FAPEs) to permit UV detection at 242 nm, by micropacked RPLC [23]. Good resolution of oleic and elaidic phenacyl esters and of three out of four geometrical isomers of 9,12-octadecadienoic phenacyl esters was obtained but separation of the positional isomers of monoenoic FAPEs could not be realized. The effect of adding Ag^+ ions to the mobile phase on the separation of geometrical and positional unsaturated FAPE isomers was therefore studied. The separation principle was applied to investigate fat hydrolysates and margarines at different stages of hydrogenation on their *cis*/*trans* C18 monoenoic acid ratio. Data of micropacked RPLC are compared to those obtained with capillary gas chromatography (cGC) on a highly polar cyanosilicone stationary phase.

2. Experimental

2.1. Materials and reagents

Fatty acid standards and the derivatization reagents were purchased from Sigma (Bornem, Belgium). All solvents were HPLC grade and supplied by Lab-Scan (Dublin, Ireland). Silver nitrate 99.8% was from Panreac (Barcelona, Spain). Fat and margarine samples were kindly donated by the Slovenian Institute of Hygiene and by Unilever (Vlaardingen, The Netherlands), respectively.

2.2. Sample preparation

Samples of collected daily meals (Slovenian Institute of Hygiene) were homogenized and dried at 105°C to constant mass prior to fat extraction. Soxhlet extraction was performed on 20 g of homogenized meals with 100 ml of petroleum ether at 80°C for 4 h. After extraction, the solvent was removed by distillation, followed by complete evaporation under a stream of nitrogen.

Fatty acid methyl ester derivatives (FAMEs) were prepared by reacting 10 mg of fat in 5 ml of hexane in the presence of methanolic KOH (2 *M*). After vortex mixing for 30 s, the hexane fraction was analyzed by cGC. FAPEs were prepared as described in the literature [24].

2.3. Capillary gas chromatography

FAME analysis according to the AOCS Official Method [25] was performed on a Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detection system and a 7673 Hewlett-Packard autosampler. A fused-silica capillary column 50 m×0.32 mm I.D., coated with a 0.25 μm film of BPX70 (70% cyanopropyl–polysilphenylene–siloxane) phase (SGE, Austin, TX, USA) was employed. Operating conditions were as follows: hydrogen carrier gas at 0.6 ml/min; injector at 255°C; detector at 300°C; oven temperature programmed from 50°C at 25°C/min to 150°C, and then at 5°C/min to 240°C; volume injected 1 μl with a split ratio of 1/50.

2.4. Micropacked LC

Micropacked LC was performed on a Varian 5000 liquid chromatograph operated in the split-flow mode. Detection was done by means of a Wescan variable-wavelength UV detector (Model WEJ55B4) equipped with a 0.32 mm I.D. fused-silica cell and operated at 242 nm. Samples were injected by means of a Valco valve (Valco, Houston, TX, USA) having a 60 nl internal loop. The micropacked columns were prepared by the slurry packing method using constant pressure (560 bar, 3 h), as described in the literature [26]. The stationary phase (RoSil C_{18} HLD 3 μm) was obtained from Bio-Rad (Eke, Belgium). The fused-silica column dimensions were 25 cm× 0.32 mm I.D. The quality of the columns was determined by injecting phenetol (D_M $1.45 \cdot 10^{-9}$ m^2/s) with acetonitrile–water (65:35) as mobile phase. Columns exhibiting a reduced plate height of 2.5 or smaller were used. The mobile phase was methanol–water (95:5) at a flow-rate of 2 μl/min to which concentrations of 10 to 100 m*M* Ag^+ ions were added. The reduced plate height significantly dropped to ca. 10 in AgC.

2.5. Fatty acid names and symbols

The fatty acids addressed in the present work are indicated in Table 1 by peak number, name and symbol.

3. Results and discussion

3.1. Micropacked LC

Micropacked fused-silica columns were used in this work because, besides of their well-known advantages over conventional LC like lower solvent consumption and increased detectability, they offered a more robust approach for AgC in which silver ions are added to the mobile phase. Similar experiments with stainless steel columns with I.D.s of 2 and 4.6 mm were not very successful in this respect. Blocking of the metal frits regularly occurred by precipitation of metal ions as already observed by Vonach and Schomburg [17] who eliminated blocking by using paper frits and thorough filtration of the silver containing mobile phase or by substituting $AgNO_3$ by $AgClO_4$. On the other hand, on conventional columns higher silver ion concentrations were required to obtain the same effects. Nevertheless, daily and thorough rinsing of micropacked columns with pure methanol and water after silver ion experiments was a prerequisite for a long column lifetime. Similar precautions were taken by Vonach and Schomburg [17].

3.2. Effect of silver ion concentration on retention

In accordance with Ref. [17], the silver ion concentration in the mobile phase was varied from 10 to 100 m*M*. Fig. 1 shows the relevant part (10 to

Table 1
Peak number, name and symbol for the fatty acids

No.	Name	Symbol
1	Capric acid or decanoic acid	10:0
2	Myristic acid or tetradecanoic acid	14:0
3	Palmitic acid or hexadecanoic acid	16:0
4	Stearic acid or octadecanoic acid	18:0
5	Petroselinic acid or 6-*cis*-octadecenoic acid	6c-18:1
6	Petroselaidic acid or 6-*trans*-octadecenoic acid	6t-18:1
7	Oleic acid or 9-*cis*-octadecenoic acid	9c-18:1
8	Elaidic acid or 9-*trans*-octadecenoic acid	9t-18:1
9	*cis*-Vaccenic acid or 11-*cis*-octadecenoic acid	11c-18:1
10	*trans*-Vaccenic acid or 11-*trans*-octadecenoic acid	11t-18:1
11	Linoleic acid or 9-*cis*-12-*cis*-octadecadienoic acid	9,12c,c-18:2
12	Linolelaidic acid or 9-*trans*-12-*trans*-octadecadienoic	9,12t,t-18:2
13	Linolenic acid or 9-*cis*-12-*cis*-15-*cis*-octadecatrienoic acid	9,12,15c,c,c-18:3

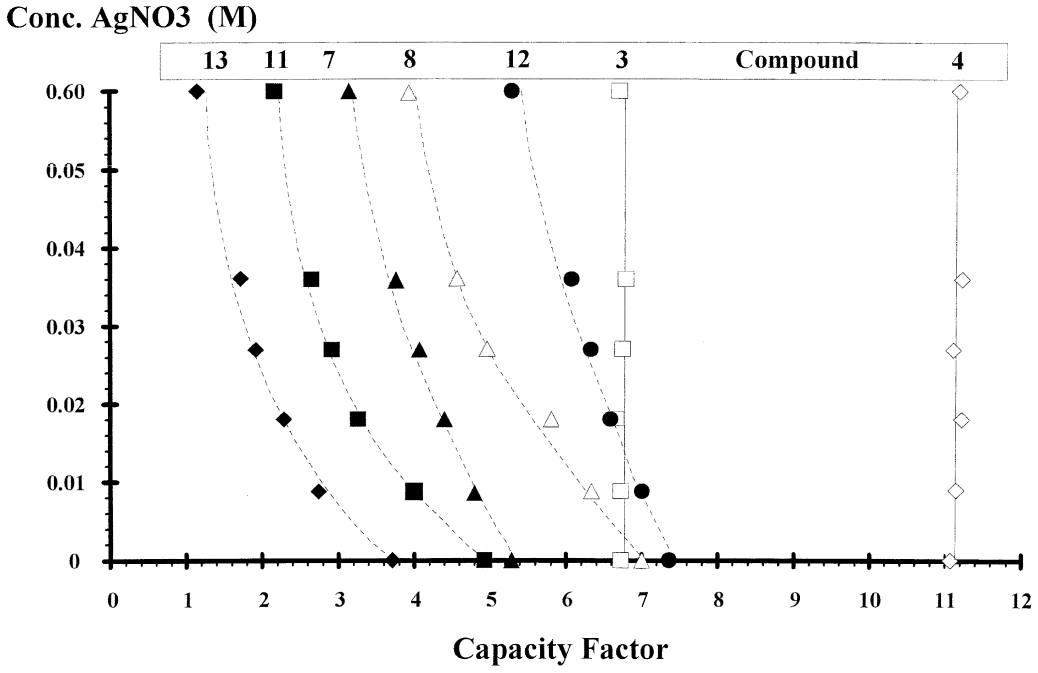

Fig. 1. Capacity factors as function of the silver ion concentration in the mobile phase for some FAPEs (Table 1). For conditions see Experimental.

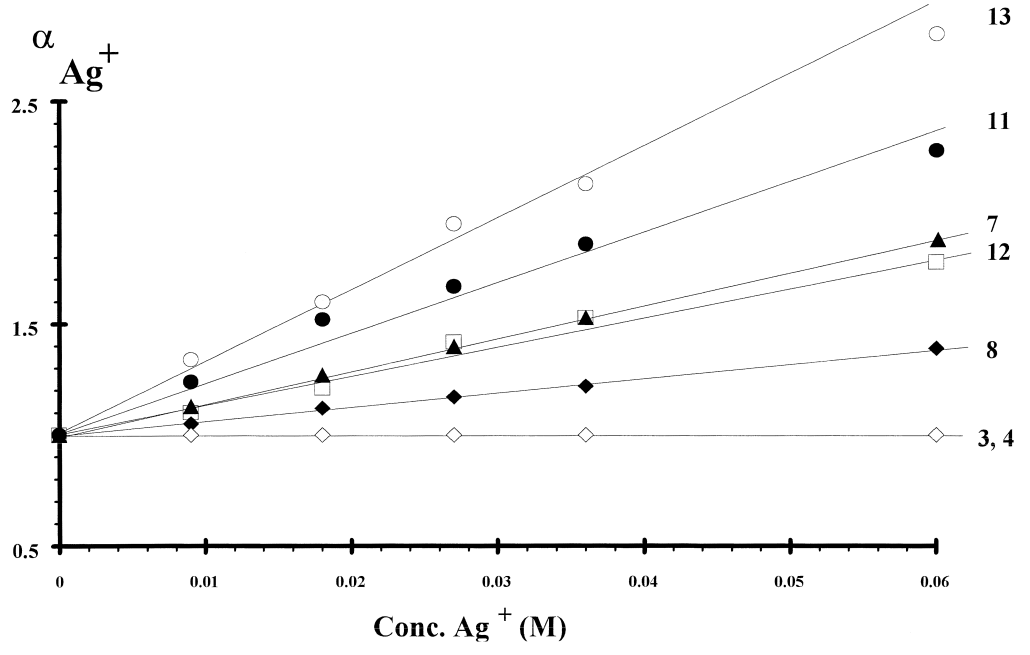

Fig. 2. The selectivity coefficients (α_{Ag^+}) as function of the silver ion concentration for some FAPEs.

Table 2
Strength of Ag^+ complexation for some unsaturated FAPEs

	FAPE				
	9,12,15c,c,c-18:3	9,12c,c-18:2	9,12t,t-18:2	9c-18:1	9t-18:1
Slope	27.4	22.1	11.3	13.6	6.5
r^2	0.981	0.985	0.999	0.983	0.998
Complexation ratio	–	1.9	2.1		

60 m*M*) of the capacity factors versus silver ion concentration plots for a mixture consisting of the saturated FAPEs 16:0 and 18:0, the monoenoic FAPEs 9-c-18:1 and 9-t-18:1, the dienoic FAPEs 9,12-c,c-18:2 and 9,12-t,t-18:2, and the trienoic FAPE 9,12,15-c,c,c-18:3. The addition of $AgNO_3$ generates no effects in the mobile phase and stationary phase polarity since no changes are observed in the retention time (t_R) and capacity factors of the saturated FAPEs. The systematic decrease in elution time of unsaturated FAPEs can only be attributed to π-complexation. The Ag^+-complex having a greater polarity elutes faster than the non-complexed solute. The *cis*-FAPE–Ag^+ complexes, being more stable than the *trans*-ones, show more affinity for the mobile phase and consequently elute faster.

The π-complexation strength is different for each unsaturated species and depends on the number and geometry of the double bonds. The strength of the complexation can be conveniently evaluated by the characteristic selectivity coefficients [17] (also known as retention modulus [20]) plotted as a function of the silver ion concentration. The selectivity coefficient (α_{Ag+}) is defined by Eq. (1), where k and k_0 are the capacity factors measured under identical conditions of the complexed and uncomplexed solutes, respectively.

$$\alpha_{Ag^+} = \frac{k_0}{k} \quad (1)$$

By plotting α_{Ag^+} as function of $[Ag^+]$ (Fig. 2), a linear correlation is observed and the slopes of the curves are proportional to the strength of complex formation between Ag^+ and the double bonds. The slope and the linear correlation coefficient (r^2) values, as well as the complexation ratio between *cis*/*trans* pairs are given in Table 2. As expected, *cis*-isomers show a stronger π-complexation than *trans*-isomers because of their favorable steric configuration. The fact that the complexation ratio remains independent against the number of double bonds (pairs 11/12 and 7/8, respectively) can only be explained by a 1:1 complexation stoichiometry.

According to the theory developed by Horvath et al. [20–22] which has been applied for fatty acids by e.g., Nikolova-Damyanova et al. [19], for measuring stability constants in ion-pair chromatography, the simplest case occurs when one solute molecule forms a complex with one metal cation (1:1 complex). In this situation, the plot capacity factors versus complexing ion concentration follows a hyperbolic function described by Eq. (2), in which k_c is the capacity factor of the complex formed with an infinite high concentration of metallic ion in the mobile phase and K^* is the corresponding stability constant.

$$k = \frac{k_0 + k_c K^*[Ag^+]}{1 + K^*[Ag^+]} \quad (2)$$

In order to calculate stability constants using this

Table 3
Stability constants for Ag^+–FAPE complexes

	FAPEs from compounds				
	9,12,15c,c,c-18:3	9,12c,c-18:2	9,12t,t-18:2	9c-18:1	9t-18:1
K^* (M^{-1})	30.78	21.77	8.99	15.81	6.28
r^2	0.9999	0.9998	0.9992	0.9998	0.9921

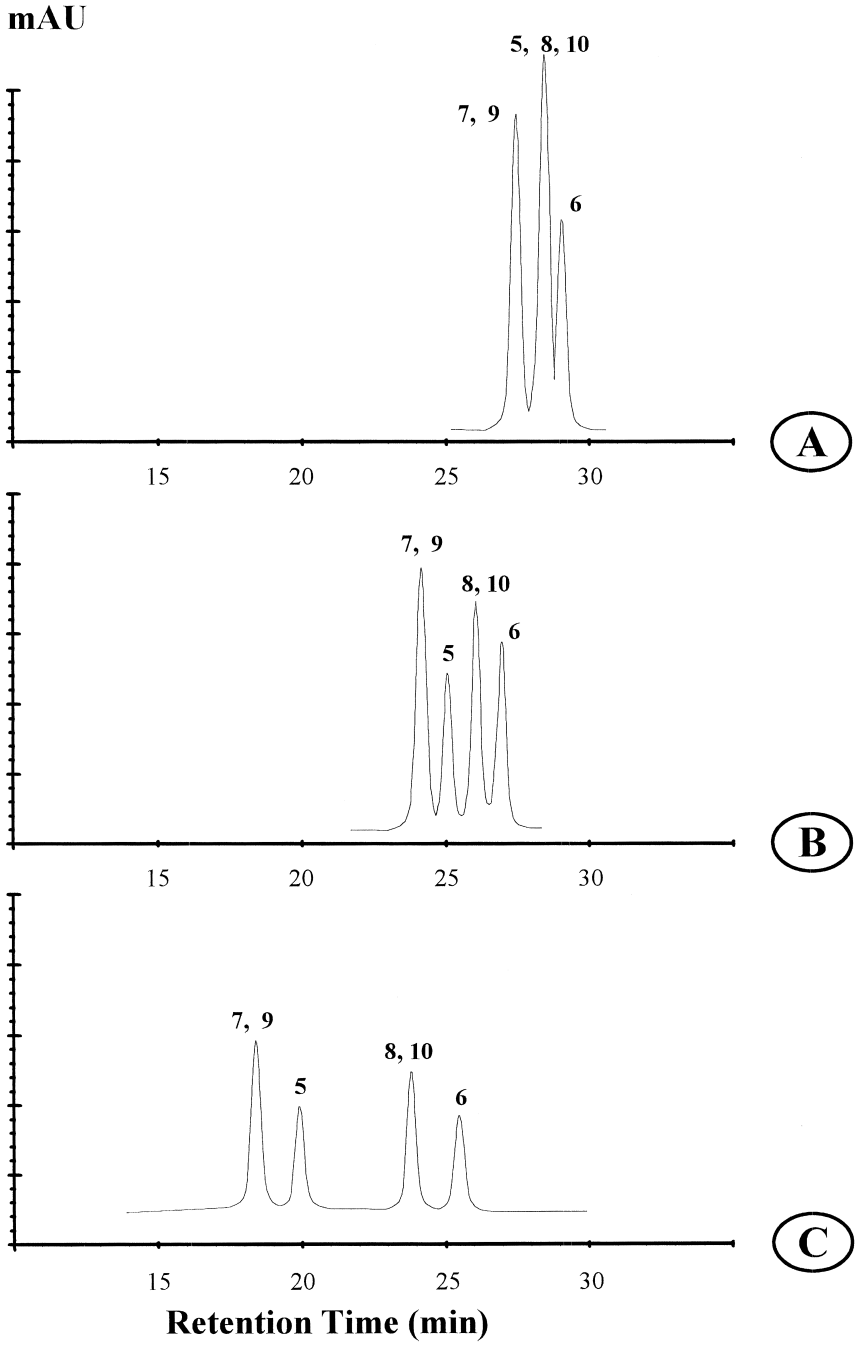

Fig. 3. Separation of positional and geometrical 18:1 FAPE isomers as a function of silver ion concentration. (A = 0; B = 10 m*M*; C = 60 m*M*).

approach, the following assumptions are made: (a) only formation of a 1:1 complex is considered, formation of other complexes (e.g., 2:1 complexes) is negligible; (b) complex formation in the mobile phase is fast compared to the chromatographic run time; (c) sample size is so small that the retention values are independent of the sample amount even at low concentration of complexing ion in the mobile phase.

Linearization of Eq. (2) can be done by changing the variables [20] in order to evaluate graphically the stability constants for complexes formed in the mobile phase, starting from experimental retention data. One of such algorithms, given by Eq. (3), fits better the experimental data.

$$\frac{[Ag^+]}{k-k_0} = \frac{1}{k_c - k_0}[Ag^+] + \frac{1}{(k_c - k_0 K^*)} \quad (3)$$

Eq. (3) can be rewritten in the form $y = bx + a$, where:

$$y = \frac{[Ag^+]}{k-k_0}; x = [Ag^+]; K^* = \frac{b}{a}$$

The results of the calculations are shown in Table 3. It is observed that not only the complexation of monounsaturated, but also of di- and triunsaturated FAPEs fit Eq. (3) with a very good linear correlation coefficients (r^2). This behavior led us to conclude that 1:1 complexes are formed preferentially and this independent of whether or not the solute contains two or more double bonds. The same observation has been made before in a GC study of Ag^+–olefin complexes [27]. Contrary to that observation, it has been reported that Ag^+ preferentially forms a complex with two ethylene molecules (2:1 complex) [28]. That experiment, however, was performed in the gas phase and in the presence of an excess of ethylene. This does not apply to this study in which an excess of silver ions is used. The phenacyl group introduced by the derivatization does not impede the Ag complexation which is in accordance with Vonach and Schomburg who, moreover, observed no change in the capacity factor of benzene [17]. Some experiments, however, carried out in our laboratory for separating polyaromatic hydrocarbons on a silver loaded stationary phase demonstrated the contrary. Nevertheless, for FAPEs, the influence of the

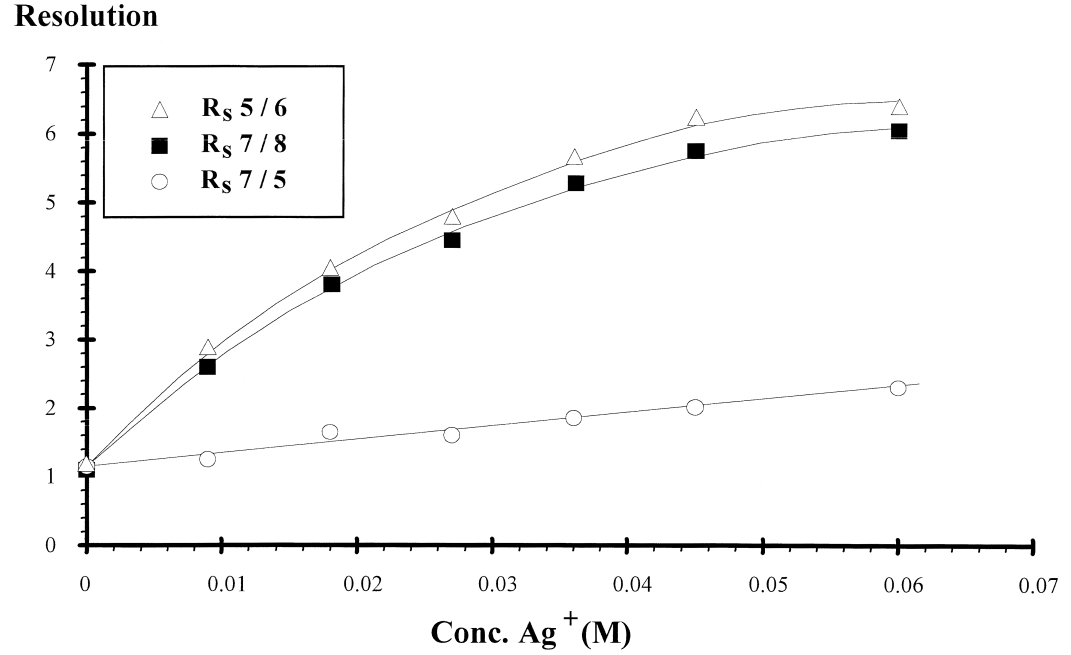

Fig. 4. Plot of the resolution of positional and geometrical 18:1 FAPE isomers versus silver ion concentration in the mobile phase.

phenacyl group is constant. The equilibrium constant for oleic phenacyl ester–Ag^+ complex reported in the literature ($16\pm1\ M^{-1}$) [19] is in agreement with the formation constant of 15.81 M^{-1} ($K_{dissociation} = 0.063\ M$) calculated in this work.

3.3. Effect of silver ion concentration on the selectivity for positional and configurational monoenoic isomers

Silver ions have a tremendous effect on the selectivity for the separation of monoenoic isomers. Fig. 3 shows some chromatograms for the separation of six positional and geometrical isomers of octadecenoic phenacyl esters obtained by varying the silver ion concentration in the mobile phase. Without $AgNO_3$ (Fig. 3A) 7/9, both *cis*, and 5/8/10 *cis*, *trans*, *trans*, respectively, are coeluting while the last eluting *trans* isomer peak 6 is nicely separated. Addition of only 10 m*M* $AgNO_3$ gives faster elution of the *cis* isomers compared to the *trans* isomers which results in the separation of the *cis* peak 5 out of 8 and 10. Further addition of $AgNO_3$ up to 60 m*M* do not achieve separation of the 7/9 and 8/10 pairs of positional isomers but the *cis*/*trans* resolution increases. Differentiation between *cis*/*trans* monoenoic acids in fats and oils become possible. The lack of resolution between the pairs 7/9 and 8/10 needs further explanation. When the double bond is positioned around the middle part of the hydrocarbon chain, π-interaction with silver ions is stronger and this as well for *cis* as for *trans* configurations but positions 9 and 11 cannot be differentiated. In the case of 5 and 6, the double bond is closer to the

Table 4
Ratio of *cis*/*trans* monoenoic acids in fat daily intakes and margarine

Sample	AgC–micro RPLC *cis*:*trans* ratio	GC *cis*:*trans* ratio
Margarine	1.2	1.5
Sample 1	2.7	3.4
Sample 2	2.7	3.1
Sample 3	3.1	3.8
Sample 4	3.2	3.2
Sample 5	3.1	4.0
Sample 6	21.00	26.7
Sample 7	4.2	4.2

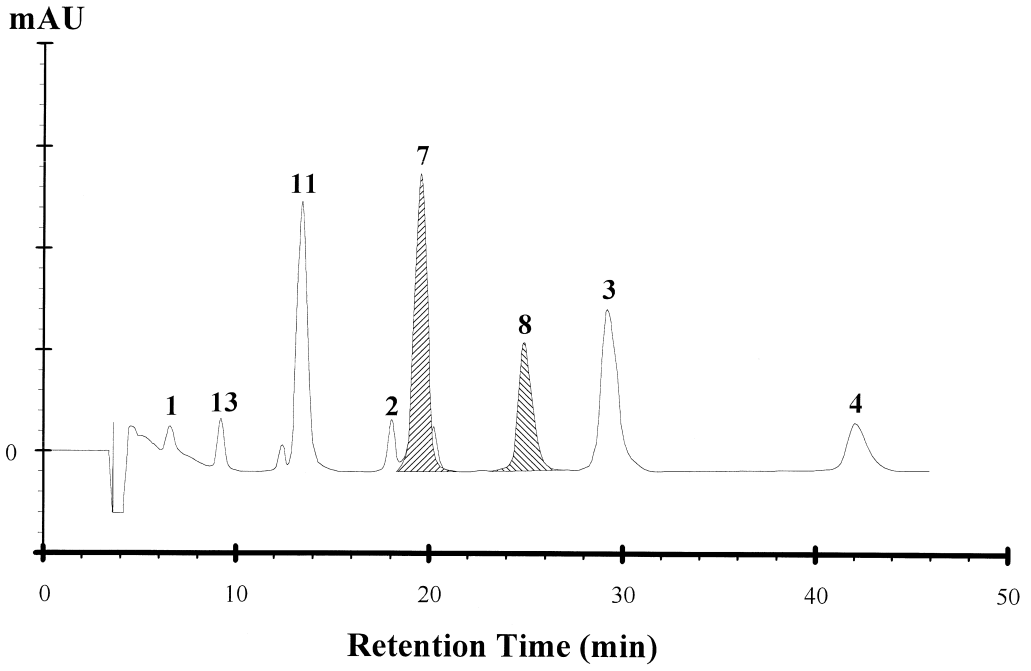

Fig. 5. FAPE separation of a fat daily intake. 60 m*M* silver ion concentration. For conditions see Experimental.

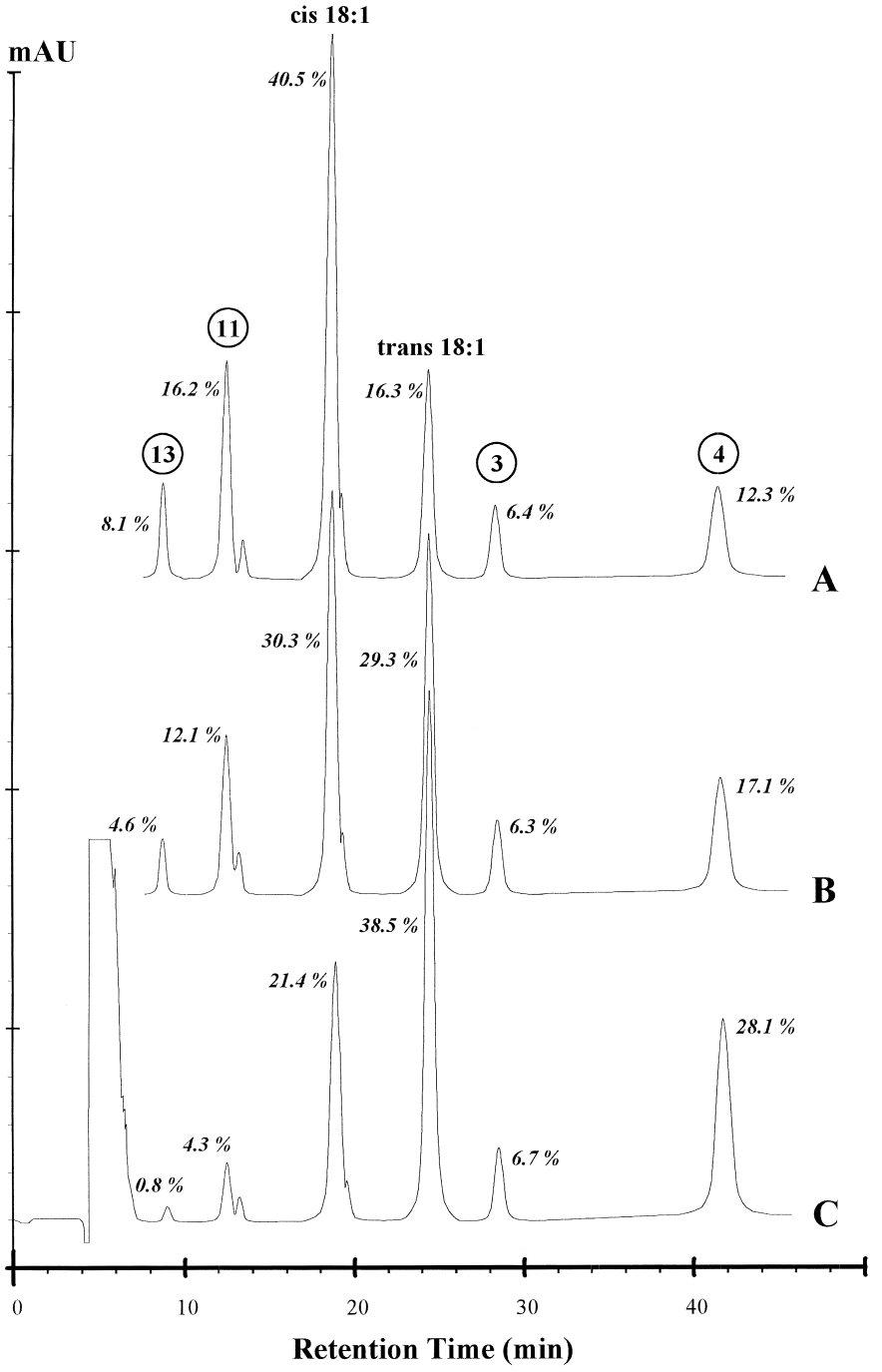

Fig. 6. Separation of FAPEs from samples taken at different stages of palm oil hydrogenation. 60 m*M* silver ion concentration. For conditions see Experimental.

carboxyl moiety, and steric effects most probably hinders interaction with the silver ions, resulting in increased retention.

Variation of the chromatographic resolution of the configurational pairs seems to be logarithmic related to the silver ion concentration in the mobile phase while a linear relationship is observed for positional pairs as illustrated in Fig. 4. It should be noted that for resolution of positional isomers only, a separation system in which the silver ions are bound to ion-exchange material [29], and papers cited therein, [30] is far superior compared to the described separation system. This approach, however, is less practical for real sample analysis because of overlap of positional isomers with fatty acids with different carbon numbers or degree of unsaturation.

3.4. Determination of the cis/trans monoenoic ratio in real world samples

From Fig. 4 it can be deduced that with AgC, conformational isomers can be differentiated and the method was therefore applied to elucidate the ratio of *cis*/*trans* monounsaturated fatty acids in different samples of fat daily intake and experimental margarines. Fig. 5 shows a representative chromatogram of a fat daily intake. Monoenoic acids with double bonds close to the carboxyl moiety e.g., the 6 position (peaks 5 and 6 in figures) seem not to occur neither as *cis* nor as *trans* isomers. Those isomers would be separated and should elute between *cis* 18:1 and *trans* 18:1 and between *trans* 18:1 and 16:0 (peak 3), respectively. The origin of the *trans* monoenoic acids can therefore only be by intake of a partially hydrogenated vegetable oil i.e., a low quality margarine [10].

The same samples were converted into their FAMEs and analyzed by cGC. A good correlation between the two techniques can be observed (Table 4). The differences can be attributed to the fact that in GC co-elution can lead to underestimation of the *trans* isomers. Repetitive analyses ($n=3$) of FAPEs by micropacked RPLC shows a relative standard deviation (RSD) below 2.5%. The chromatograms of three experimental margarine samples of a palm oil hydrogenation process are shown in Fig. 6. During hydrogenation, with the exception of palmitic acid (peak 3), all fatty acid quantities modify. The *trans* 18:1 isomers and 18:0 increase at the expense of the mono-, di- and trienoic species.

4. Conclusion

AgC in which the silver ions are introduced in the mobile phase of micropacked RPLC allows one to elucidate configurational isomers of monoenoic fatty acids in fat and oil samples, whereas only some geometrical isomers can be separated.

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