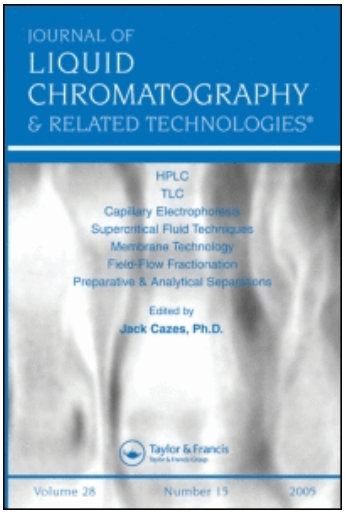

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SILVER ION HPLC FOR THE ANALYSIS OF POSITIONALLY ISOMERIC FATTY ACIDS

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SILVER ION HPLC FOR THE ANALYSIS OF POSITIONALLY ISOMERIC FATTY ACIDS

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ABSTRACT

The utilization of silver ion HPLC is evaluated for the resolution of positionally isomeric monoenoic and polyenoic fatty acids. The impact of the fatty acid derivative on the resolution is discussed and the advantage of using specified aromatic derivatives is demonstrated. Emphasized, are the achievements in the separation of octadecenoates in partially hydrogenated oils and of octadecadienoates with conjugated double bonds in tissues and pharmaceutical products.

Key Words: Silver ion HPLC; Unsaturated fatty acids; Fatty acid derivatives; Configurational isomers; Positional isomers; Mobile phases

INTRODUCTION

With few exceptions, positionally isomeric *cis* and *trans* fatty acids do not occur in nature, but are products of natural oils and fats processing in the food

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industry. Their determination is, therefore, of substantial importance in food control. Due to the close similarity in their chemical properties, the analysis of the isomers has always been a challenge. Chromatography is the method of choice and, undoubtedly, gas chromatography (GC) has the leading role (see, for example, the recent review by Ratnayake,[1] although overlapping between *cis* and *trans* positionally isomers cannot be fully avoided when GC is used as a single separation method, even when employing 100 m cyanosilicone columns. Silver ion chromatography is supposed, by many authors, to have a supporting role only, since it is used as a preliminary step for the fractionation of complex fatty acid mixtures into groups according to the number and geometry of double bonds. These fractions are isolated and subjected to GC for resolution of the positional isomers.[1] Indeed, this combination of methods ensures the best results in the determination of isomeric fatty acids achieved so far and finds wide application.

On the other hand, efforts to utilize silver ion chromatography as a stand-alone approach for the resolution of positionally isomeric fatty acids have been made since the introduction of this technique. In 1967, Gunstone and co-workers[2] reported the migration properties of the complete series of *cis* and *trans* octadecenoates in silver ion thin-layer chromatography (Ag-TLC). In spite of the constant efforts of later work by others, the differences in *Rf* values were too small to be of practical value. Analytical procedures were developed only for the three naturally occurring octadecenoic isomers: petroselinic acid (*cis*-6-18 : 1), oleic acid (*cis*-9-18 : 1) and *cis*-vaccenic acid (*cis*-11-18 : 1) after appropriate derivatization into methyl esters[3–5] and phenacyl esters.[6,7]

A major step forward was made when silver ion high-performance liquid chromatography (Ag-HPLC) was introduced into practice and, especially, when an effective procedure for loading the column with silver ions was offered by Christie in 1987.[8] Manufacturers employed the principle of this procedure and a commercial silver loaded column is now available. Since the utilization of these columns, the resolution of positionally isomeric fatty acids again attracted serious attention and promising results were obtained. Presented here are some of the achievements in resolution of positionally isomeric fatty acids by using Ag-HPLC reported in the literature.

GENERAL NOTES

The main problem to solve in performing HPLC in the silver ion mode has been the introduction of silver ions into the system. Two approaches have been tested so far, and both have been employed in the separation of isomers. The first attempts were performed on columns, laboratory-packed with slurry of the stationary phase (silica, mostly), impregnated with silver nitrate of defined

concentration in the 5% to 30% range, by weight. Although these columns were capable of providing acceptable resolution of some isomers, they were not stable and separations were not reproducible. Much better results were obtained with the silica-based cation-exchange column (Nucleosil™ 5SA, for example) converted into the silver ion form, as proposed by Christie.[8] The loading procedure is simple and easy to perform. The column can be used for a long period (12 to 18 months, for example, depending on the intensity of use) without losing resolution. Interfering leakage of silver ions is not detectable and the separations are reproducible. Using the same principles, Chrompack (now part of Varian) produces a silver loaded column, ChromSpher™ 5Lipids (Varian), which is recently widely and successfully employed.

Fatty acids are usually analyzed as methyl or aromatic esters. The derivatization procedures have not been reported to cause any changes in the case of monounsaturated fatty acid isomers. There is some controversy of whether derivatization causes intraisomerization and losses of certain geometrical isomers in the case of conjugated linoleic acids (CLA). While some authors claim that such processes occur,[9,10] others,[11–20] including the present authors,[21] have not detected changes in the composition due to isomerization. It is probably true that, in order to choose an appropriate derivatization method for CLA, one should take into account the nature of the sample.[22,23] Recently, successful resolution of underivatized octadecadienoic fatty acids with conjugated double bonds has been demonstrated, which undoubtedly simplifies the analysis and eliminates the occurrence of side reactions.[9,24] On the other hand, the type of the ester moiety was found to substantially affect the selectivity of resolution and much better separations were achieved when converting fatty acids into aromatic esters.[25]

Isocratic elution with mobile phases based on hexane[26] or dichloromethane[27] as major components and acetonitrile (mostly), methanol, iso-propanol, or tetrahydrofurane, as a modifier, has been employed with good results.

In principle, all detectors used in Ag-HPLC[28,29] are suitable for this particular application. The reported procedures utilize either UV-detectors or evaporative light-scattering detectors (ELSD). UV detection is by far the most common in HPLC. It is, however, more suitable for fatty acids tagged with a strong chromophore (in the ester moiety). With the exception of species with conjugated double bonds, methyl esters are poorly detected in the UV. ELSD does not put any such limitations and has been used with good results for the resolution of methyl and aromatic esters, as well as of underivatized fatty acids.

RESOLUTION OF MONOENOIC FATTY ACID ISOMERS

The monounsaturated geometrical and positional isomers are typical components of partially hydrogenated vegetable and fish oils and all derived

dietary products. Monoenes of chain length C_{18} dominate in products based on vegetable oils, while isomers of all chain lengths, ranging from C_{16} to C_{22}, are possible in products containing partially hydrogenated fish oils. So far, Ag-HPLC was applied only for the separation of 18 : 1 isomers in reference mixtures and real samples, as well as for some *cis*-20 : 1 isomers in reference mixtures only. The aim of Ag-HPLC is to achieve clear separation between *cis* and *trans* isomers while ensuring reasonable resolution of the positional isomers in each of these groups.

One of the first promising results was reported by Battaglia and Frohlich in 1980.[30] Isomeric fatty acids (as methyl esters) in margarine samples were separated in about 30 min on a column packed with Spherisorb S5W impregnated with 5% $AgNO_3$. A mobile phase of hexane–tetrahydrofurane (99.4 : 0.6, v/v), at a flow rate of 2.2 mL/min and detection at 205 nm, was used. *Trans*- and *cis* isomers were clearly separated, while the resolution of the positional isomers was only partial. The elution order was found to be: *trans* 13-, 12-, 11-, 10-, 9-, 8-, 7-18 : 1, and *cis* 12-, 11-, 10-, 9-, 8–18 : 1 (in the order of increasing retention). Increasing the silver ion content substantially shortened the analysis time, but did not improve the resolution.

Practically the same elution order: *trans* 14-, 13-, 12-, 11-, 10-, 8- + 9–18 : 1 and *cis* 13-, 12-, 11-, 10-, 8- + 9–18 : 1 methyl esters (in order of increasing retention) was observed for a sample of partially hydrogenated vegetable oil when using ChromSpher™5Lipids column (detection at 206 nm).[26] The mobile phase was 0.08% acetonitrile in hexane at a flow rate of 1.0 mL/min. Sample size was small: 0.4 μg; nevertheless, the resolution of positional isomers remained partial (Fig. 1). Significant resolution losses of all positional isomers occurred when sample size was increased to 0.1 mg. The utilization of two ChromSpher 5 Lipids columns, connected in series, led to some, but not to substantial, improvement in resolution, according to the authors.

The sensitivity of the analysis could be considerably increased using strongly UV-absorbing fatty acid derivatives instead of methyl esters, as is the practice in reversed-phase HPLC of fatty acids.[31] Thus, when introducing aromatic esters of fatty acids in Ag-HPLC, the aim was only to facilitate the detection and quantification. *p*-Bromophenacyl esters were the first employed and a standard mixture of *cis*- and *trans*-11- and 9–18 : 1 esters was separated on a column packed with aluminosilicate impregnated with 4% $AgNO_3$.[32] The mobile phase was 0.01% acetonitrile in hexane–chloroform (13 : 1, v/v). The *cis* positional isomers were completely resolved, while the resolution of *trans* 11- and 9–18 : 1 was only partial. Later, the same isomers were fully resolved by Christie and Breckenridge on a Nucleosil™5SA column loaded with silver ions, after conversion in phenacyl esters.[27] The separation was performed with a mobile phase of 1,2-dichloroethane–dichloromethane (1 : 1, v/v), at a flow rate of 1.5 mL/min and UV-detection at 242 nm. The same chromatographic conditions

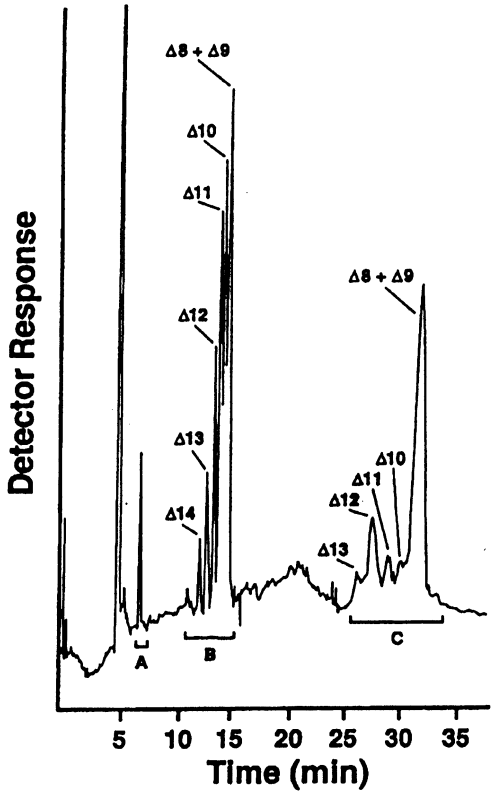

Figure 1. Ag-HPLC separation of isomeric fatty acid methyl esters in partially hydrogenated vegetable oil on ChromSpher 5 lipids column; mobile phase 0.08% acetonitrile in hexane; flow rate 1.0 mL/min; UV-detection at 206 nm; sample size: 0.4 μg. Fractions: A, saturates; B, *trans*-18 : 1; C, *cis*-18 : 1.[26] (Reproduced by kind permission of the authors and of Journal of American Oil Chemist Society).

were applied for the separation of *trans* and *cis* positional isomers of 18 : 1 fatty acids in partially hydrogenated soybean oil.[27] The elution order was practically the same as that reported for methyl esters, but the resolution was much better. Thus, while *trans* 8- and 9–18 : 1 methyl esters gave a single peak in the chromatogram,[26,30] they were fully resolved as phenacyl esters. The separation of *cis* isomers was also improved although, probably because of the long elution time (about 50 min), the peaks were quite broad.[27]

Having once attracted attention, the utilization of aromatic esters in Ag-HPLC was extensively studied.[25,33–36] First, the retention characteristics of

methyl and phenacyl esters of series of positionally isomeric *cis* octadecenoic, octadecadienoic and some polyunsaturated fatty acids with methylene-interrupted double bonds were studied.[25] Two different columns, Nucleosil™5SA and ChromSpher™5Lipids, were tested at fixed temperatures (10°C and 15°C), with evaporative light-scattering detection. A mobile phase of 1,2-dichloroethane-dichloromethane, (1 : 1, v/v) with various proportions of acetonitrile, was used, at a flow rate of 1.5 mL/min. Octadecene was employed as an internal standard to accurately measure the respective retention factors (k''). When plotting the k'' values against the position of the double bond(s), a pattern was obtained which was similar to the sinusoidal curves reported earlier in Ag-TLC by Gunstone and co-workers for methyl octadecenoates[2] and by Christie for methyl octadecadienoates,[37] i.e., the retention order of phenacyl esters was similar to that of methyl esters. The differences in k'' values between some of the isomers were large enough to be of practical interest and, indeed, a base-line resolution of the three naturally occurring cis octadecenoic isomers (*cis* 6-, 9- and 11–18 : 1) as phenacyl esters was achieved by isocratic elution with mobile phase 1,2-dichloroethane–dichloromethane–acetonitrile, 50 : 50 : 0.025 (by volume) over 25 min (Fig. 2) and it was the best reported so far for these isomers, irrespective of the separation techniques employed. In order to test whether and how the nature of the ester moiety affects the resolution, the investigation was extended to studying the retention and resolution of various esters of positionally isomeric fatty acids. Examined were allyl-, butenyl-, pentenyl- and hexenyl-esters,[33] benzyl- and phenacyl-esters bearing functional groups in the benzene ring; normal- and branched-chain alkyl esters,[34] phenethyl-, phenacyl- and *p*-methoxyphenacyl esters,[35] 2-naphthacyl-, 9-anthrylmethyl- and 2-naphthylmethyl esters.[36] All experiments were performed on a Nucleosil 5SA column loaded in silver ion form, with solvent mixtures of hexane, 1,2-dichloroethane, dichloromethane and acetonitrile in various proportions as mobile phases. Detection was carried out either by evaporative light-scattering detector,[33,34] or by UV-detector[35,36] at appropriate wavelengths (245 nm for phenacyl- and *p*-methoxyphenacyl esters, 250 nm for 2-naphthacyl- and 9-anthrylmethyl esters, 254 nm for phenethyl esters, 275 nm for 2-naphthylmethyl esters). Reference mixtures of commercially available isomeric fatty acids were employed in most cases.[33,34,36] It was found that, in the series of alkyl esters examined, the resolution of *cis*-6- and *cis*-9–18 : 1 improves in the order 2-methyl-1-butyl-, 3-methyl-1butyl-, methyl-, ethyl-∼, propyl-, *n*-butyl-, 2-methyl-2-butyl-, isopropyl ∼ 2-methyl-propyl esters, while 9- and 11–18 : 1 were fully resolved irrespective of the type of the ester moiety.[34] Also, *cis*-6-, 9- and 11–18 : 1 were not resolved when converted into *p*-bromobenzyl-, *p*-methoxybenzyl- and pentafluorobenzyl- derivatives, and were fully resolved as phenacyl-, *p*-bromophenacyl- and *p*-methoxyphenacyl esters (the resolution increasing in this order).[34] The effect of other ester moieties, such as: phenethyl-[35]

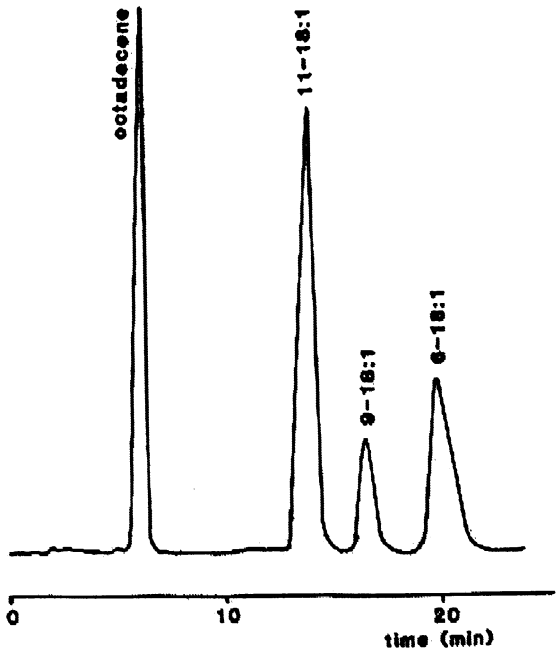

Figure 2. Ag-HPLC separation of *cis*-11-, *cis*-9- and *cis*-6–18 : 1 fatty acid phenacyl esters and octadec-1-ene (internal standard) on a silver-loaded Nucleosil 5SA column; mobile phase 1,2-dichloroethane–dichloromethane–acetonitrile (50 : 50 : 0.025, v/v/v); flow rate 1.5 mL/min; evaporative light-scattering detection; sample size: 0.1 mg of each component.[25] (Reproduced by kind permission of the authors and of Journal of Chromatography).

2-naphthylmethyl-, 2-naphtacyl- and 9-anthrylmethyl-[36] was also studied using the limited number of commercially available *cis*- and *trans* isomeric octadecenoic fatty acids. While the elution order remained the same irrespective of the ester moiety, the selectivity of resolution substantially increased in the order phenethyl-, 2-naphthylmethyl-, phenacyl- ∼, 2-naphthacyl-, 9-anthrylmethyl-, *p*-methoxyphenacyl-octadecenoates.[35,36] Thus, the most selective separation was achieved with *p*-methoxyphenacyl derivatives; this is demonstrated in Fig. 3. Presented in the figure is the fatty acid composition of a partially hydrogenated sunflower oil, as determined by Ag-HPLC with a mobile phase consisting of hexane–dichloromethane–acetonitrile (60 : 40 : 0.2, v/v/v).[35] The peaks were identified by spiking the sample with the respective reference fatty acids. The separation was performed over 22 min (compare to the 35 min analysis time reported in Ref. [26] and the 50 min required in Ref. [27]).

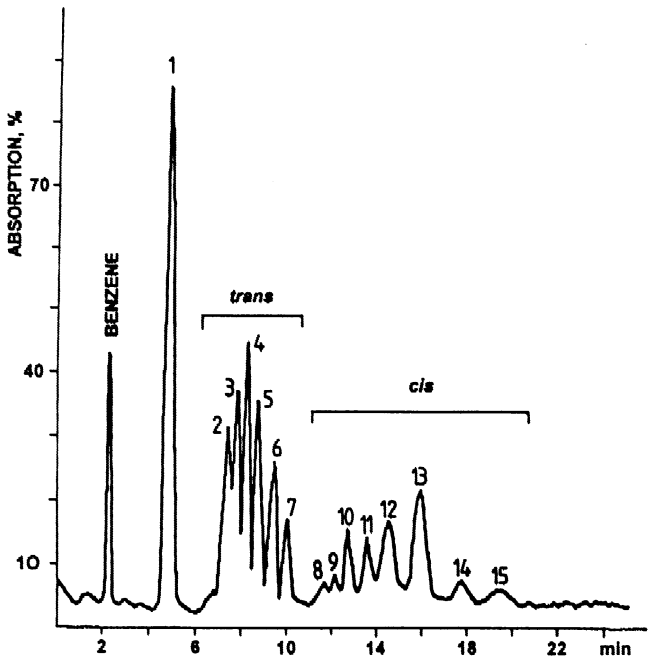

Figure 3. Ag-HPLC separation of isomeric fatty acids in partially hydrogenated sunflower oil after conversion in *p*-methoxyphenacyl esters on a silver-loaded column Nucleosil 100-5SA; mobile phase hexane–dichloromethane–acetonitrile (60 : 40 : 0.2, v/v/v); flow rate 1.5 mL/min; UV-detection at 245 nm; sample size: 20 µg. 1, saturated; 2, unknown; 3, *trans* 11–18 : 1; 4, unknown; 5, *trans* 9–18 : 1; 6, unknown; 7, *trans* 6–18 : 1; 8, *cis* 13–18 : 1; 9, *cis* 12–18 : 1; 10, *cis* 15–18 : 1; 11, *cis* 11–18 : 1; 12, unknown; 13, *cis* 9–18 : 1; 14, unknown; 15, *cis* 6- and *cis* 7–18 : 1 fatty acids.[35] (Reproduced by kind permission of Journal of Chromatography A).

p-Methoxyphenacyl esters were found to be effective in the separation of eicosenoic (20 : 1) fatty acids as well.[38,39] Complete resolution of the four naturally occurring eicosenoic isomers *cis* 5-, 8-, 11-, and 13–20 : 1 was achieved on the Nucleosil^{TM}5SA column loaded with silver ions, with mobile phases of either hexane–dichloromethane–acetonitrile (70 : 30 : 0.2, v/v/v) or hexane–dichloromethane–isopropanol (60 : 40 : 0.2, v/v/v) at a flow rate of 1.5 mL/min; detection at 270 nm. Using a mobile phase of hexane–dichloromethane–isopropanol (60 : 40 : 0.2, v/v/v), it was possible to clearly separate the mixture of all naturally occurring 18 : 1 and 20 : 1 isomers, which eluted, under these conditions, in the order: 13–20 : 1, 11–20 : 1, 11–18 : 1, 9–18 : 1, 8–20 : 1, 5–20 : 1, and 6–18 : 1 (in increasing retention order)[39] (Fig. 4).

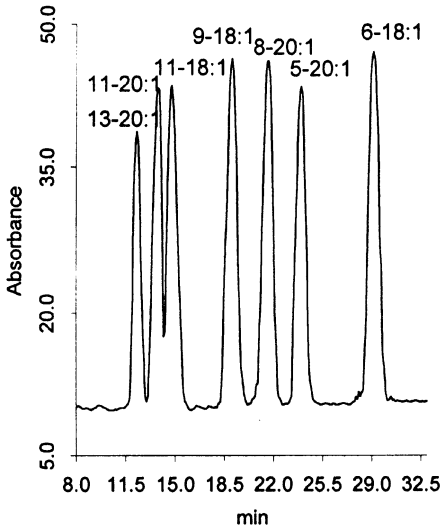

Figure 4. Ag-HPLC separation of isomeric *cis*-monoenoic fatty acids after conversion into *p*-methoxyphenacyl esters on a silver-loaded column Nucleosil 100-5SA, mobile phase hexane–dichloromethane–isopropanol (60 : 40 : 0.2, v/v/v) at flow rate 1.5 mL/min, UV-detection at 270 nm, sample size: 1–2 μg of each component.[39] (Reproduced by kind permission of Journal of Liquid Chromatography & Related Technologies).

The effect of the mobile phase composition on the selectivity of resolution of aromatic esters is also of interest and series of solvent systems based on either hexane or dichloromethane and modified with acetonitrile, methanol or isopropanol, have been examined on some *cis*-monoenoic isomers.[38–40] Hexane and dichloromethane, admixed in proportions 70 : 30 to 60 : 40 (by volume) and modified with acetonitrile or isopropanol, provide the most selective resolutions. Equally efficient is the solvent system of dichloromethane–acetonitrile. Expectedly, both retention and resolution rapidly decrease with increasing proportion of the modifier in the mobile phase. In addition, isopropanol has been found to reverse the elution order of 5–20 : 1 and 8–20 : 1 which, as seen from Fig. 4, are difficult to resolve and give two adjacent chromatographic peaks. 5–20 : 1 elutes ahead of 8–20 : 1 in the presence of acetonitrile or methanol and the order reverses when isopropanol was the modifier.[38,39]

The effect of the mobile phase composition on the retention and resolution of *trans* positional isomers is limited so far to the commercially available 6-, 9-, and 11–18 : 1.[40] The three *trans* isomers were completely resolved on Nucleosil 5SA column in silver ion form with all the above mobile phases.

The elution order is 11-, 9-, 6–18 : 1 (in increasing retention order) and remains constant irrespective of the mobile phase composition.

RESOLUTION OF POLYENOIC FATTY ACID ISOMERS

Polyenoic Fatty Acids with Methylene-Interrupted Double Bonds

To the best of our knowledge, examples of separation by Ag-HPLC of dienoic positionally isomeric fatty acids with methylene-interrupted double bonds have not been published yet. The retention characteristics (i.e., capacity factor values) are only reported for a series of positionally isomeric methyl- and phenacyl-derivatives, including fatty acids with more than one methylene group between the double bonds.[25] It should be noted that the fatty acids with separated double bonds are retained more strongly than are the methylene-interrupted octadecadienoates. The k'' values of the respective methyl esters depend on the number of methylene groups that separate the two double bonds. The highest value is measured for the *cis*,*cis*-6,10–18 : 2 (two methylene groups between the double bonds) and it is about four times higher than that of *cis*,*cis*-9,12–18 : 2, for example. The prospect for the resolution of isomeric octadecadienoates has not been commented, however.[25]

Complete resolution of the *p*-methoxyphenacyl esters of *cis*,*cis*,*cis* 9,12,15–18 : 3 and 6,9,12–18 : 3, and of 11,14,17–20 : 3 and 5,8,11–20 : 3 (in increasing retention order) on Nucleosil 5SA in silver ion form, using a mobile phase of dichloromethane–acetonitrile (100 : 0.7, v/v) was reported.[38] The elution order reversed when the mobile phase was modified with isopropanol.[38] By isocratic elution with dichloromethane–acetonitrile (100 : 0.4, v/v), it was possible to separate all four trienoic isomers (Fig. 5).[39]

Octadecadienoic Fatty Acids with Conjugated Double Bonds

Recently, due to the reported beneficial health effects, the analysis of natural and commercial samples containing isomeric octadecadienoic fatty acids with conjugated double bonds, usually denoted as CLA (conjugated linoleic acids), has been attracting increasing attention. The chromatographic procedures have been reviewed, recently, and the great potential of Ag-HPLC was especially emphasized.[11–13]

CLA isomers comprise a very complex mixture of three groups of geometrical isomers: *trans*,*trans*-, *trans*/*cis*-, *cis*,*cis*-(eluting in this order in Ag-HPLC) each being, in its turn, a mixture of positional isomers. As has been

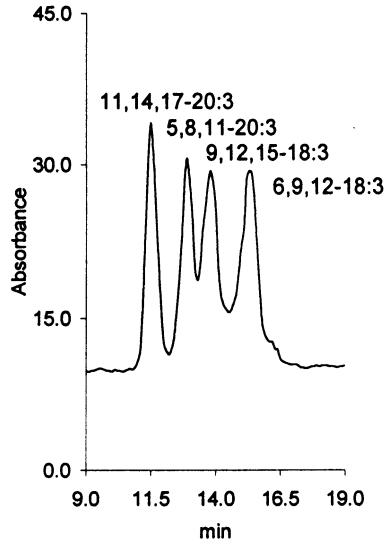

Figure 5. Ag-HPLC separation of isomeric *cis*,*cis*,*cis*-trienoic fatty acids after conversion into *p*-methoxyphenacyl esters on a silver-loaded Nucleosil 100-5SA column; mobile phase dichloromethane–acetonitrile (100 : 0.4, v/v); flow rate 1.5 mL/min; UV-detection at 270 nm; sample size: 1–2 μg of each component.[39] (Reproduced by kind permission of Journal of Liquid Chromatography and Related Technologies).

shown,[25,41,42] in silver ion chromatography, conjugated octadecadienoates are held more strongly than are *trans*-octadecenoates, but less strongly than are the *cis*-octadecenoates. While the separation by Ag-HPLC of the geometrical CLA isomers is easily achieved, the resolution of the positional isomers in each of the three groups causes problems. In spite the difficulties, efforts were made to achieve acceptable resolution of CLA isomers by Ag-HPLC. Advantageously, the conjugated double bonds system is a chromophore with reasonably high absorption at 233–234 nm, thus allowing for UV detection.[11–20] In most procedures, CLA are converted in methyl esters and subjected to Ag-HPLC on ChromSpher™5 Lipids column. Employing a mobile phase of 0.1% acetonitrile in hexane complete separation of the *trans*,*trans*-, *trans*/*cis*- and *cis*,*cis*-isomers in a standard mixture[14–16] and in cheese products[16,17] was achieved. Since the positional isomers were resolved only partially on a single column, up to six columns were connected in series.[18–20] The resolution of the isomers progressively improved with increasing numbers of columns, but the analysis time was increased to 120 min. Therefore, a series of three columns was assumed to be the optimal compromise, allowing for satisfactory resolution of most CLA isomers in natural products (Fig. 6).[18–20]

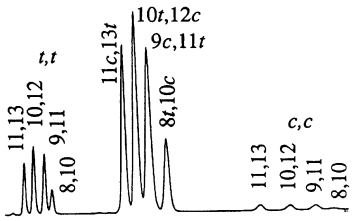

Figure 6. Ag-HPLC separation of a commercial conjugated linoleic acid (CLA) methyl esters on three ChromSpher 5 Lipids columns connected in series; mobile phase: 0.1% acetonitrile in hexane; flow rate 1.0 mL/min, UV-detection at 234 nm.[19] (Reproduced from the reference by kind permission of the authors and of Lipids).

A separation comparable to that discussed above was achieved by subjecting the free fatty acids to Ag-HPLC.[9,24] The separation was performed again on ChromSpher™5 Lipids column with a solvent system of 2.5% acetic acid and 0.025% acetonitrile in hexane and detection at 234 nm. This approach has the advantage that the intermediate steps of producing and purifying derivatives were eliminated, thus eliminating the probable sample losses, the formation of artifacts, or the possible danger of double bond migration related to these procedures.[9,24]

As with the monoenes, the ester moiety was found to influence the resolution of positional CLA isomers (Fig. 7).[21] Shown in the figure is the resolution of a standard mixture of CLA isomers when subjected to Ag-HPLC (Nucleosil™5SA column in silver ion form) after conversion to either methyl-, phenacyl- or *p*-methoxyphenacyl esters. The mobile phases used were hexane–acetonitrile, 100 : 0.1, v/v (methyl esters), hexane–dichloromethane–acetonitrile, 70 : 30 : 0.2, v/v/v (phenacyl esters), and hexane–dichloromethane–acetonitrile 50 : 50 : 0.2, v/v/v (*p*-methoxyphenacyl esters), with UV-detection at 233, 245, or 270 nm, respectively. Evident is the substantial improvement in the resolution when replacing the methoxy-moiety in the ester molecule with phenacyl- and, especially, with the *p*-methoxyphenacyl group. The resolution of the CLA isomers as *p*-methoxyphenacyl esters is the best achieved on a single Ag-HPLC column.

When dealing with samples of natural origin, the presence and eventual interference of other fatty acids has to be taken into account. This is not a problem when using UV detection and fatty acid methyl esters, since CLA isomers are the only components detected. All components are UV-detectable in a mixture of *p*-methoxyphenacyl esters. Whether this is an advantage or a disadvantage depends on the purpose, on the complexity of the sample, and on the quality of resolution. Chromatographic conditions for single-column

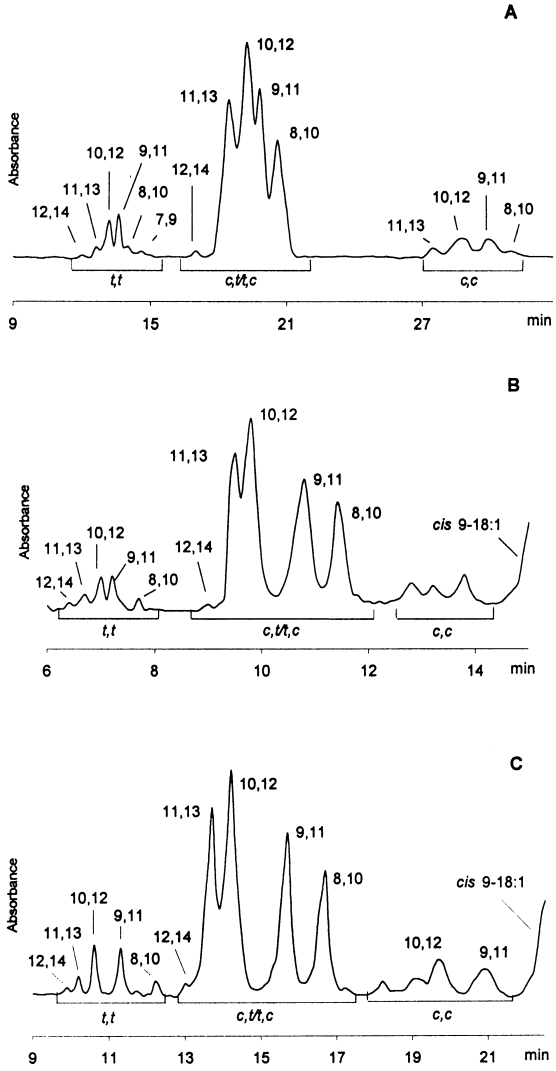

Figure 7. Ag-HPLC separation of a commercial conjugated linoleic acid (CLA) fatty acid mixture on a silver-loaded Nucleosil 100-5SA column after conversion in: (A) methyl esters; (B) phenacyl esters; (C) *p*-methoxyphenacyl esters; mobile phases: (A) hexane–acetonitrile (100 : 0.1, v/v); (B) hexane–dichloromethane–acetonitrile (70 : 30 : 0.2, v/v/v); (C) hexane–dichloromethane–acetonitrile (50 : 50 : 0.2, v/v/v); flow rate 1.0 mL/min; UV-detection at: (A) 233 nm; (B) 245 nm; (C) 270 nm, sample size: 10–100 µg of the respective esters.[21] (Reproduced by kind permission of Journal of High Resolution Chromatography).

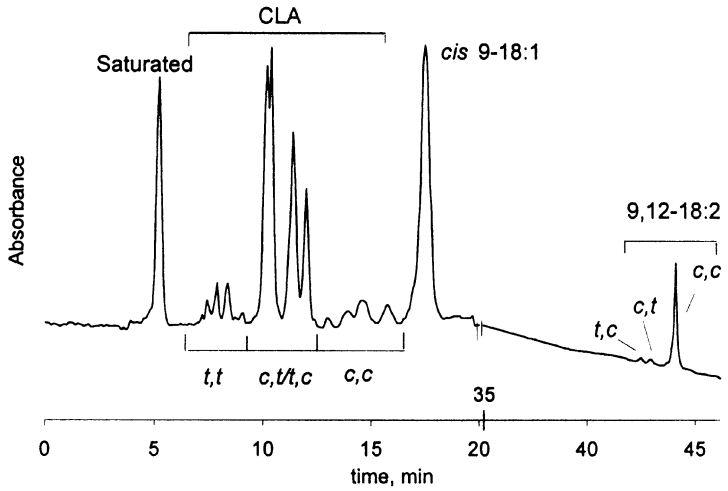

Figure 8. Ag-HPLC separation of the fatty acids in the commercial sample CLA 60 after conversion in *p*-methoxyphenacyl esters on a silver-loaded Nucleosil 100-5SA column, mobile phase: stepwise gradient from 100% hexane–dichloromethane–acetonitrile (40 : 60 : 0.2, v/v/v) over 30 min changed to 100% dichloromethane–acetonitrile (100 : 1, v/v) over 10 min and held over further 20 min; flow rate 1.0 mL/min; UV-detection at 270 nm, sample size: 50 µg.[21] (Reproduced from reference by kind permission of Journal of High Resolution Chromatography).

resolution of saturated, *trans*,*trans*-, *trans*/*cis*- and *cis*,*cis*-CLA positional isomers, *cis*-monoenoic and methylene-interrupted dienoic fatty acids as *p*-methoxyphenacyl esters were established using a stepwise gradient from hexane–dichloromethane–acetonitrile (40 : 60 : 0.2, v/v/v) to dichloromethane–acetonitrile (100 : 1, v/v) (Fig. 8). Since, in many cases, the CLA content is very low, a preliminary pre-concentration step is advisable.[21]

QUANTIFICATION

Quantification of positionally isomeric fatty acids is performed, at present, indirectly by gas chromatography, after preliminary fractionation by silver ion chromatography. Silver-ion thin-layer chromatography (Ag-TLC) is preferable for fractionation because it is simpler, versatile, and does not require expensive instrumentation.[42,43] Having in mind the potential of Ag-HPLC in the resolution of isomeric fatty acids, it seems important to utilize this technique for direct

quantification of fatty acid isomers; moreover, some of the detectors employed are suitable for direct measurements.[28,29]

Flame-ionization (FID), refractive index (RI), and UV-detectors have been employed for direct quantification of isomeric fatty acid methyl esters in partially hydrogenated vegetable oil by Ag-HPLC.[26] However, correction factors are required when using RI- and UV-detectors. A large sample size is needed when employing FID, thus reducing the selectivity of resolution.[26]

Aromatic esters were supposed to have a serious potential in performing direct quantification of fatty acids in Ag-HPLC with UV detection.[27] The detector response was supposed to be rectilinearly related to the molar proportion of phenacyl esters in the range 0–200 μg at 242 nm.[27] Calibration curves to confirm this statement have not been shown, however; instead, the relative proportions of saturated, *trans*- and *cis*-monoenoic, dienoic-, and trienoic fatty acid phenacyl esters were determined in two standard mixtures and in samples of some oils and fats. The results of the Ag-HPLC quantification of the standard mixtures were compared with those of gravimetric analysis and excellent agreement was found.[27]

On the other hand, Ag-HPLC determination of conjugated linoleic acids as methyl esters[9,11,14–17,19,20] or as underivatized fatty acids[9] has been performed using the strong UV-absorption of the conjugated double bond system at 234 nm. The quantitative proportions reported were not confirmed by independent quantitative analysis.

The authors consider, therefore, that additional work is required in order to validate the quantification of fatty acids in general, and of positional isomers in particular, by Ag-HPLC, in order to accept this method as reliable from an analytical point of view.

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REFERENCES

1. Ratnayake, W.M.N. Analysis of Dietary Trans Fatty Acids. J. Oleo Sci. **2001**, *50*, 339–352.
2. Gunstone, F.D.; Ismail, I.A.; Lie Ken Jie, M. Fatty Acids, Part 16. Thin Layer and Gas-Liquid Chromatographic Properties of the Cis and Trans Methyl Octadecenoates and of some Acetylenic Esters. Chem. Phys. Lipids **1967**, *1*, 376–385.

3. Morris, L.J.; Wharry, D.M.; Hammond, E.W. Chromatographic Behaviour of Isomeric Long-Chain Aliphatic Compounds. II. Argentation Thin-Layer Chromatography of Isomeric Octadecenoates. J. Chromatogr. **1967**, *31*, 69–76.
4. Nikolova, B.M.; Tarandjiiska, R.B.; Chobanov, D.G. Determination of Petroselinic Acid, Oleic Acid and the Major Triglyceride Groups in some *Umbelliferae* Seed Oils. Comt. Rend. Acad. Bulg. Sci. **1985**, *38*, 1231–1234.
5. Breuer, B.S.; Fock, H.P. Separation of Fatty Acids or Methyl Esters Including Positional and Geometrical Isomers by Alumina Argentation Thin-Layer Chromatography. J. Chromatogr. Sci. **1987**, *25*, 302–306.
6. Nikolova-Damyanova, B.; Christie, W.W.; Herslof, B. Improved Separation of some Positional Isomers of Monounsaturated Fatty Acids, as Their Phenacyl Derivatives by Silver Ion Thin-Layer Chromatography. J. Planar Chromatogr. **1994**, *7*, 382–385.
7. Nikolova-Damyanova, B.; Momchilova, Sv.; Christie, W.W. Determination of Petroselinic, *cis*-Vaccenic, and Oleic Acids in some Seed Oils of *Umbelliferae* by Silver Ion Thin-Layer Chromatography of Their Phenacyl Esters. Phytochem. Anal. **1995**, *7*, 136–139.
8. Christie, W.W. A Stable Silver-Loaded Column For the Separation of Lipids by High Performance Liquid Chromatography. J. High Resol. Chromatogr. & Chromatogr. Commun. **1987**, *10*, 148–150.
9. Ostrowska, E.; Dunshea, F.; Muralitharam, M.; Cross, R. Comparison of Silver-Ion High-Performance Liquid Chromatographic Quantification of Free and Methylated Conjugated Linoleic Acids. Lipids **2000**, *35* (10), 1147–1153.
10. Yamasaki, M.; Kishihara, K.; Ikeda, I.; Sugano, M.; Yamada, K. A Recommended Esterification Method for Gas Chromatographic Measurement of Conjugated Linoleic Acid. J. Am. Oil Chem. Soc. **1999**, *76*, 933–938.
11. Mossoba, M.M.; Kramer, J.K.G.; Yurawecz, M.P.; Sehat, N.; Roach, J.A.G.; Eulitz, K.; Fritsche, J.; Dugan, M.E.R.; Ku, Y. Impact of Novel Methodologies on the Analysis of Conjugated Linoleic Acid (CLA). Implications of CLA Feeding Studies. Fett/Lipid **1999**, *101* (6), 235–243.
12. Banni, S.; Martin, J.-C. Conjugated Linoleic Acid and Metabolites. In *Trans Fatty Acids in Human Nutrition*; Sebedio, J.-L., Christie, W.W., Eds.; The Oily Press: Dundee, Scotland, 1998; 261–302.
13. Yurawecz, M.P.; Morehouse, K.M. Silver-Ion HPLC of Conjugated Linoleic Acid Isomers. Eur. J. Lipid Sci. Technol. **2001**, *103*, 609–613.
14. Sehat, N.; Yurawecz, M.P.; Roach, J.A.G.; Mossoba, M.M.; Kramer, J.K.G.; Ku, Y. Silver-Ion High-Performance Liquid Chromatographic Separation and Identification of Conjugated Linoleic Acid Isomers. Lipids **1998**, *33* (2), 217–221.

15. Adlof, R.O.; Copes, L.C.; Walter, E.L. Changes in Conjugated Linoleic Acid Composition Within Samples Obtained From a Single Source. Lipids **2001**, *36* (3), 315–317.
16. Rickert, R.; Steinhart, H.; Fritsche, J.; Sehat, N.; Yurawecz, M.; Mossoba, M.; Roach, J.A.G.; Eulitz, K.; Ku, Y.; Kramer, J.K.G. Enhanced Resolution of Conjugated Linoleic Acid Isomers by Tandem-Column Silver-Ion High Performance Liquid Chromatography. J. High Resol. Chromatogr. **1999**, *22*, 144–148.
17. Sehat, N.; Kramer, J.K.G.; Mossoba, M.M.; Yurawecz, M.P.; Roach, J.A.G.; Eulitz, K.; Morehouse, K.M.; Ku, Y. Identification of Conjugated Linoleic Acid Isomers in Cheese by Gas Chromatography and Mass Spectral Reconstructed Ion Profiles. Comparison of Chromatographic Elution Sequences. Lipids **1998**, *33* (10), 963–971.
18. Sehat, N.; Rickert, R.; Mossoba, M.M.; Kramer, J.K.G.; Yurawecz, M.P.; Roach, J.A.G.; Adlof, R.O.; Morehouse, K.M.; Fritsche, J.; Eulitz, K.D.; Steinhart, H.; Ku, Y. Improved Separation of Conjugated Fatty Acid Methyl Esters by Silver Ion-High Performance Liquid Chromatography. Lipids **1999**, *34* (4), 407–413.
19. Eulitz, K.; Yurawecz, M.P.; Sehat, N.; Fritsche, J.; Roach, J.A.G.; Mossoba, M.M.; Kramer, J.K.G.; Adlof, R.O.; Ku, Y. Preparation, Separation, and Confirmation of the Eight Geometrical Cis/Trans Conjugated Linoleic Acid Isomers 8,10-through 11,13–18 : 2. Lipids **1999**, *34* (8), 873–877.
20. Fritsche, J.; Fritsche, S.; Solomon, M.; Mossoba, M.M.; Yurawecz, M.P.; Morehouse, K.; Ku, Y. Quantitative Determination of Conjugated Linoleic Acid Isomers in Beef Fat. Eur. J. Lipid Sci. Technol. **2000**, *102*, 667–672.
21. Nikolova-Damyanova, B.; Momchilova, Sv.; Christie, W.W. Silver Ion High-Performance Liquid Chromatographic Separation of Conjugated Linoleic Acid Isomers, and Other Fatty Acids, After Conversion to *p*-Methoxyphenacyl Derivatives. J. High Resolut. Chromatogr. **2000**, *23* (4), 348–352.
22. Kramer, J.K.G.; Fellner, V.; Dugan, M.E.R.; Sauer, F.D.; Mossoba, M.M.; Yurawecz, M.P. Evaluating Acid and Base Catalysts in the Methylation of Milk and Rumen Fatty Acids with Special Emphasis on Conjugated Dienes and Total *Trans* Fatty Acids. Lipids **1997**, *32*, 1219–1228.
23. Park, S.J.; Park, C.W.; Kim, S.J.; Kim, J.K.; Kim, Y.R.; Park, K.A.; Kim, J.O.; Ha, Y.L. Methylation Methods for the Quantitative Analysis of Conjugated Linoleic Acid (CLA) Isomers in Various Lipid Samples. J. Agric. Food Chem. **2002**, *50*, 989–996.
24. Cross, R.F.; Ostrowska, E.; Muralitharam, M.; Dunshea, F.D. Mixed Mode Retention and the Use of Competing Acid for the Ag-HPLC Analysis of Underivatized Conjugated Linoleic Acids. J. High Resolut. Chromatogr. **2000**, *23* (4), 317–323.

25. Nikolova-Damyanova, B.; Herslof, B.; Christie, W.W. Silver Ion High-Performance Liquid Chromatography of Derivatives of Isomeric Fatty Acids. J. Chromatogr. **1992**, *609*, 133–140.
26. Adlof, R.O.; Copes, L.C.; Emken, E.A. Analysis of the Monoenoic Fatty Acid Distribution in Hydrogenated Vegetable Oils by Silver-Ion High-Performance Liquid Chromatography JAOCS **1995**, *72* (5), 571–574.
27. Christie, W.W.; Breckenridge, G.H.McG. Separation of Cis and Trans Isomers of Unsaturated Fatty Acids by High-Performance Liquid Chromatography in the Silver Ion Mode. J. Chromatogr. **1989**, *469*, 261–269.
28. Christie, W.W. Detectors for HPLC. In *High-Performance Liquid Chromatography and Lipids*; Pergamon Press: Oxford, England, 1987; 18–30.
29. Christie, W.W. Detectors for High-Performance Liquid Chromatography of Lipids with Special Reference to Evaporative Light-Scattering Detection. In *Advances in Lipid Methodology-One*; Christie, W.W., Ed.; The Oily Press: Ayr, Scotland, 1992; 239–271.
30. Battaglia, R.; Frohlich, D. HPLC Separation of Cis and Trans Monounsaturated Fatty Acids. Chromatographia **1980**, *13*, 428–431.
31. Nikolova-Damyanova, B. Reversed-Phase High-Performance Liquid Chromatography: General Principles and Application to the Analysis of Fatty Acids and Triacylglycerols. In *Advances of Lipid Methodology—Four*; Christie, W.W., Ed.; The Oily Press: Dundee, Scotland, 1997; 193–251.
32. Lam, S.; Grushka, B. Silver Loaded Aluminosilicate as a Stationary Phase for the Liquid Chromatographic Separation of Unsaturated Compounds. J. Chromatogr. Sci. **1977**, *15*, 234–238.
33. Nikolova-Damyanova, B.; Christie, W.W.; Herslof, B. Silver-Ion High-Performance Liquid Chromatography of Esters of Isomeric Octadecenoic Fatty Acids with Short-Chain Monounsaturated Alcohols. J. Chromatogr. A **1995**, *693*, 235–239.
34. Nikolova-Damyanova, B.; Christie, W.W.; Herslof, B. Mechanistic Aspects of Fatty Acid Retention in Silver Ion Chromatography. J. Chromatogr. A **1996**, *749*, 47–54.
35. Momchilova, Sv.; Nikolova-Damyanova, B.; Christie, W.W. Silver Ion High-Performance Liquid Chromatography of Isomeric *cis*- and *trans*-Octadecenoic Acids. Effect of the Ester Moiety and Mobile Phase Composition. J. Chromatogr. A **1998**, *793*, 275–282.
36. Momchilova, Sv.; Nikolova-Damyanova, B. Silver Ion High Performance Liquid Chromatography of Polynuclear Aromatic Derivatives of Positionally Isomeric Octadecenoic Fatty Acids. J. Liq. Chrom. & Rel. Technol. **2000**, *23* (9), 1319–1330.
37. Christie, W.W. Chromatography of Isomeric Methylene Interrupted Methyl Cis, Cis-Octadecadienoates. I. Argentation Thin Layer Chromatography. J. Chromatogr. **1968**, *34*, 405–406.

38. Momchilova, Sv.; Nikolova-Damyanova, B. Silver Ion HPLC of *p*-Methoxyphenacyl Derivatives of Unsaturated Fatty Acids. I. Mobile Phase Effects. J. Liq. Chrom. & Rel. Technol. **2000**, *23* (15), 2303–2316.
39. Momchilova, Sv.; Nikolova-Damyanova, B. Silver Ion HPLC of *p*-Methoxyphenacyl Derivatives of Unsaturated Fatty Acids. II. Chain Length vs. Double Bond Position. J. Liq. Chrom. & Rel. Technol. **2000**, *23* (15), 2317–2325.
40. Momchilova, Sv.; Nikolova-Damyanova, B. Silver Ion HPLC of *p*-Methoxyphenacyl Derivatives of Unsaturated Fatty Acids. III. Mobile Phase Effects on Trans 6-, 9-, and 11–18 : 1. J. Liq. Chrom. & Rel. Technol., **2002**, *25* (4), 615–625.
41. Chobanov, D.; Tarandjiiska, R.; Nikolova-Damyanova, B. Quantitation of Isomeric Unsaturated Fatty Acids by Argentation TLC. J. Planar Chromatogr. **1992**, *5*, 157–163.
42. Nikolova-Damyanova, B. Silver Ion Chromatography and Lipids. In *Advances in Lipid Methodology-One*; Christie, W.W., Ed.; The Oily Press: Ayr, Scotland, 1992; 181–237.
43. Nikolova-Damyanova, B.; Momchilova, Sv. Silver-Ion Thin-Layer Chromatography of Fatty Acids. A Survey. J. Liq. Chrom. & Rel. Technol. **2001**, *24* (10), 1447–1466.

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