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Silver ion solid-phase extraction for the analysis of trans fatty acids in human adipose

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Silver ion solid-phase extraction is suggested for the analysis of trans fatty acids in human adipose. Acid catalyzed esterification of the fatty acids protocol using boron trifluoride–methanol complex as a catalyst was used. Silica based Ag^+-SPE conditions are presented. A mixture of dichloromethane and hexane with the content of dichloromethane increasing from 5 to 100% was used as an eluent. The presented method allows eliminating from the trans chromatographic zone about 75% of cis isomers of fatty acid methyl esters, thus enabling to determine trace contents of trans fatty acids in the samples containing big amounts of dominant saturated, monounsaturated and polyunsaturated fatty acids.

Keywords: Ag^+-SPE, trans fatty acids, human adipose

INTRODUCTION

The obesity is growing dramatically in many developed countries including those of the European Union [1]. Obesity is clearly associated with pathophysiologic processes and comorbidities, such as metabolic syndrome, type 2 diabetes mellitus and cardiovascular disease, which is the most common cause of death in the Western World [2]. Those diseases are strongly associated with the dietary fatty acids and especially their trans isomers [3–11]. For example, low concentrations of trans-C16:ln 7-palmitoleate in the blood serum indicate a risk of metabolic disease [12]. Some fatty acids (polyunsaturated fatty acids (PUFAs), trans fatty acids, and odd-numbered and branched-chain fatty acids) can play a role as biomarkers to assess dietary intake [13–15]. Because of this, it is very important to have sensitive and easy methods for determination of fatty acids in human adipose samples.

60 years have passed after Morris concluded that for the analysis of the lipids silver ion (or argentation) chromatography was third in importance to gas chromatography (GC) and thin layer chromatography (TLC) [16]. Silver ion chromatography is based on the ability of Ag^+ to form weak reversible charge transfer complexes with π electrons of the double bonds of unsaturated fatty acids. The retention depends on the number of double bonds, on their configuration and on the distance between double bonds [17–18].

The separation achieved by using silver ion TLC (Ag^+-TLC) for fatty acid methyl esters (FAMEs) with different numbers and geometric configuration of double bounds was a great complement to the GC separation of FAMEs on packed columns [19]. Lately developed very long and highly polar GC capillarity columns made possible to resolve most of geometrical and position isomers. However, the improvement of GC resolution only exacerbated the need for the reliable method to help identify many resolved and sometimes overlapping monounsaturated fatty acids (MUFA) and PUFA isomers. For this reason not only Ag^+-TLC but also silver ion solid phase extraction (Ag^+-SPE) and silver ion high performance liquid chromatography (Ag^+-HPLC) are involved now. Ag^+-SPE offers the same separation

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potential as would be obtained by either Ag^+-TLC or Ag^+-HPLC, but Ag^+-SPE fractionation is easier and faster than that of Ag^+-TLC. In addition, since Ag^+-SPE fractions are isolated in a solution, the PUFAs are protected from oxidation and eluates are suitable for a direct GC analysis [5]. Ag^+-HPLC provides a higher chromatographic resolution, more reproducible separations and allows real time monitoring of the separation [20–23]. However, it may require to use several columns connected in series to achieve a good separation of trans isomers of fatty acids [24]. High resolution can be provided by combination of Ag^+-HPLC and GC techniques [25], but it requires an expensive instrumentation just for preparative purposes.

In this paper a fractionation technique of trans esterified adipose tissue on a home-made silica based Ag^+-SPE column for the gas chromatographic determination of trans fatty acids in human adipose is suggested.

EXPERIMENTAL

Reagents and solutions

All the reagents were of analytical grade. Trans-9-elaidic methyl ester (10 mg ml^{-1}) solution in heptanes was obtained from Sigma-Aldrich (Germany). Mixture ME 100 (10 mg ml^{-1}) solution in dichloromethane (methyl butyrate 4%; methyl hexanoate 4%; methyl octanoate 4%; methyl decanoate 4%; methyl undecanoate 2%; methyl dodecanoate 4%; methyl tridecanoate 2%; methyl tetradecanoate 4%; methyl myristoleate (9c) 2%; methyl pentadecanoate 2%; methyl 10c-pentadecenoate 2%; methyl hexadecanoate 6%; methyl palmitoleate (9c) 2%; methyl heptadecanoate 2%; methyl 10c-heptadecenoate 2%; methyl octadecanoate 4%; methyl oleate (9c) 4%; methyl elaidate (9tr) 2%; methyl linoleate (9c, 12c) 2%; methyl linoelaidate (9tr, 12tr) 2%; methyl linolenate (9c, 12c, 15c) 2%; methyl gamma linolenate (6c, 9c, 12c) 2%; methyl eicosanoate 4%; methyl 11c-eicosenoate 2%; methyl 11c, 14c-eicosadienoate 2%; methyl eicosatrienoate (11c, 14c, 17c) 2%; methyl 8c, 11c, 14c-eicosatrienoate 2%; methyl arachidonate (5c, 8c, 11c, 14c) 2%; methyl eicosapentaenoate (5c, 8c, 11c, 14c, 17c) 2%; methyl heneicosanoate 2%; methyl docosanoate 4%; methyl erucate (13c) 2%; methyl docosadienoate (13c, 16c) 2%; methyl docosahexaenoate (4c, 7c, 10c, 13c, 16c, 19c) 2%; methyl tricosanoate 2%; methyl tetracosanoate 4%; methyl nervonate (15c) 2%.) was obtained from Larodan (Sweden). Dichloromethane (99.8%), methanol (99.9%), n-hexane (98.0%), isooctane (99.0%), sodium sulfate (99.0%), boron trifluoride-methanol complex (20% solution in methanol) were purchased from Merk (Germany). Sodium hydroxide was obtained from Eurochemicals (EU). Silica gel 60 (particle size 0.063–0.2 mm) was purchased from Sigma-Aldrich (Germany). Silver nitrate was obtained from Girochem (Slovenija).

Instrumentation

The chromatographic analysis was performed on a Shimadzu GC-2010 plus a gas chromatograph equipped with an AOC-20i auto injector and a flame ionization detector. The GC system was equipped with an Agilent CP-Select-CB for a FAME capillary column (100 m × 0.25 mm fused silica WCOT)

GC conditions

Helium was employed as a carrier gas with a linear velocity of 32.0 cm s^{-1}. The injector temperature was held at 225 °C. Injection was performed in a split mode. The split ratio was 10:1.

The oven temperature was programmed as follows: 120 °C for 1 min, from 120 to 250 °C at 2 °C min^{-1}, and held at 250 °C for 1 min.

The flame ionization detector temperature was held at 260 °C. Nitrogen gas was used as make up gas at a flow rate of 30 ml min^{-1}. The hydrogen flow rate was 40 ml min^{-1}, the air flow rate was 400 ml min^{-1}. The signal acquisition rate was 40 msec.

Derivatization

Boron trifluoride in methanol, a catalyst, was used for derivatization. 50 mg of lipids were transferred into a 100 ml flask, 5 ml of a methanolic sodium hydroxide solution (0.5 mol l^{-1}) was added and the mixture was refluxed in a water bath for 30 min. Then 5 ml of borontrifluoride in methanol (20% w/v) was added through the top of a condenser, and the mixture was refluxed for 3 min. The flask was removed from the water bath, and 5 ml of isooctane and 20 ml of saturated sodium chloride were added. The flask was closed and shaken for 30 s, 50 ml of distillated water was added. After separation of two phases, the upper layer was removed and dried with sodium sulphate. The extract was filtered through glass wool and kept in a dark glass tube until analysis.

Preparation of a silica based Ag^+-SPE tube

1 g of silica was placed in a 0.9 × 6 cm tube, between glass frits. The tube was covered with aluminum foil. 3 ml of a 1% silver nitrate solution were passed through the column. The tube was washed with 10 ml of methanol, 10 ml of dichlormethane and 10 ml of n-hexane in turn.

RESULTS AND DISCUSSION

Silica based silver ion solid-phase extraction

Adipose tissue consists of fatty acids triglycerides that are low volatility compounds and cannot be directly analyzed using gas chromatography. Therefore, transesterification was applied prior to the Ag^+-SPE and consequent GC analysis. Methylation procedures have been reviewed in detail by

Christie [26]. There are two most common transesterification protocols: catalyzed by acid or base. In this work one of the most popular catalysts – boron trifluoride in methanol – was used. The Lewis or Brønstedt acid-catalyzed esterification of carboxylic acids with alcohols to give esters is a typical reaction in which the products and reactants are in equilibrium (Fig. 1). The equilibrium may be influenced by either removing one product from the reaction mixture (for example, removal of water by azeotropic distillation or absorption by molecular sieves) or by employing an excess of one reactant. In this case high excess of methanol is used.

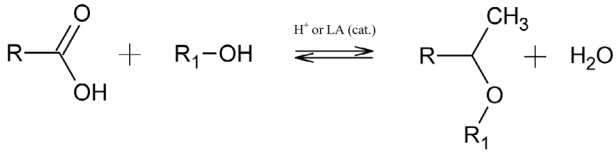

Fig. 1. Typical scheme of Fischer esterification

A chromatogram of the adipose tissue sample derivatized using boron trifluoride in methanol is presented in Fig. 2. A good resolution of FAMEs that differ by a chain length and a number of double bonds was achieved, but geometric isomers were not resolved properly. The main reason for that is a high amount of a dominant geometric oleic isomer C18:1 9-cis that makes about 50% of the total fatty acid content.

The huge peak of C18:1 9-cis overlaps with the peaks of different C18:1 isomers. As it can be seen from the standard mixture chromatogram, one of the isomers in the overlapping zone is C:18:1 9-trans (Fig. 3). In order to completely separate geometric isomers with the retention times of 32–34 min, prefractionation of the sample prior to the GC analysis using Ag^+-SPE was employed.

For this, a home-made silica based Ag^+ functionalized SPE system was elaborated. Silica based TLC plates

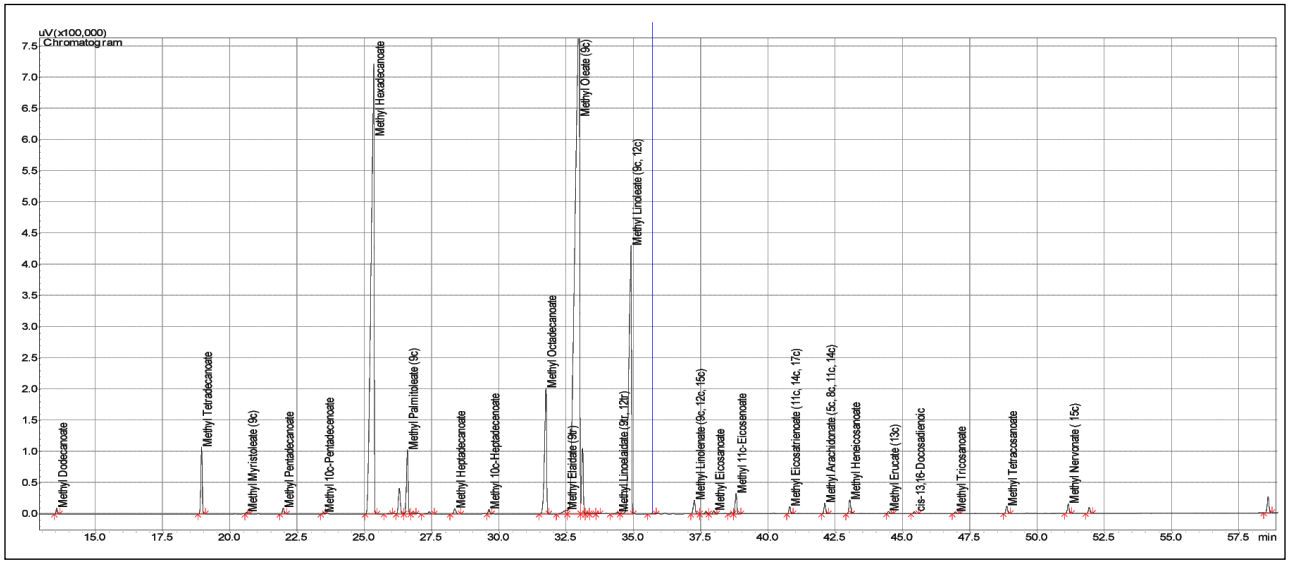

Fig. 2. Chromatogram of the human adipose tissue sample derivatized using boron trifluoride in methanol

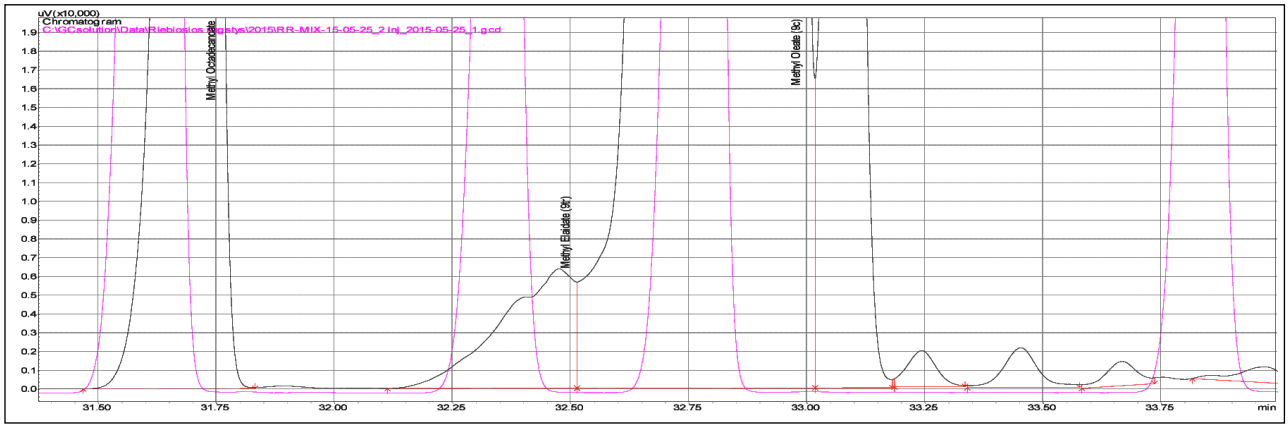

Fig. 3. A fragment of the chromatogram of derivatized human adipose tissue sample (black) and of the chromatogram of standard FAME mixture (pink online)

were used widely in silver ion chromatography. For this reason silica was chosen as a promising sorbent in the Ag-SPE system. High excess of a 0.1 mol l^{-1} silver nitrate solution (3 portions, 1 ml each) was used to fully cover a silica sorbent with Ag^+ ions. As a primary test subject, rape seed oil derivatized using boron trifluoride in methanol as described above was used. Rape seed oil was selected as it contains a high amount of oleic acid, similarly to human adipose.

Selection of an appropriate eluent plays the main role in Ag^+-SPE. It was noticed that even high volumes of n-hexane do not elute FAMEs from the column. In order to achieve a more efficient elution, a mixture of n-hexane and dichloromethane (DCM) was used as an eluent. Initially, a linearly increasing amount of DCM (from 10 to 55%) was applied (Fig. 4). The results were promising, but the separation conditions needed to be optimized as C18:0 and C18:1 were not separated completely. Moreover, cis and trans isomers of C18:1 were eluted in the same fraction.

The results of the first trial suggested that in order to separate geometric isomers the first portions of the eluent should be weaker, i. e. should contain less DCM. Thus, after the sample was loaded, the column was washed with 10 ml of 5% DCM solution in hexane and then with 10 ml of 10% DCM solution in hexane. Those fractions did not contain fatty acids (results not shown). Then the elution was proceeded using a stepped gradient of DCM in hexane. A profile of the gradient was optimized and full separation of C18:0 and C18:1 was achieved (Fig. 5). Due to the use of weaker elution conditions as presented in Fig. 5, PUFAs fractions were not eluted and collected.

Geometric isomers were not completely separated. However, elimination of the major portion of cis isomers from the trans isomers fraction (38–47 ml of eluent) totally removes overlapping of the peaks. Base line separation of the most problematic geometric isomers C18:1 cis and C18:1 trans is achieved with a retention factor of 1.84 (Fig. 6).

Application

The proposed method was applied for the separation and determination of fatty acids trans isomers in human adipose. The sample of adipose tissue was obtained from the Department of General Surgery, Vilnius University Hospital. The derivatization and Ag-SPE procedures were as described above.

After the Ag^+-SPE clean-up, only C18:1 isomers were present in the cis configuration in the 47–50 ml fraction. As can be seen from the chromatogram of this fraction (Fig. 7), C18:1 cis isomers with a different position of a double bound are easy detectable and can be identified.

A full separation of 6-cis and 9-cis isomers is not achieved, but the resolution is much better than in the extract before fractionation. Moreover, a comparison of the chromatograms presented in Figs. 3 and 7 demonstrates that in the chromatogram before fractionation (Fig. 3) the peak with the retention time presents two different substances – C18:1 6-cis and C18:1 11-trans.

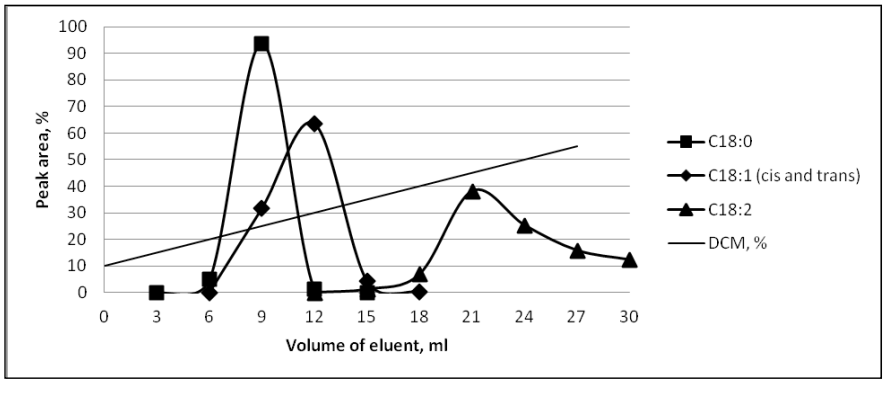

Fig. 4. Separation of C18 FAMEs on the silica based Ag^+-SPE column using a mixture of *n*-hexane and DMC as eluent

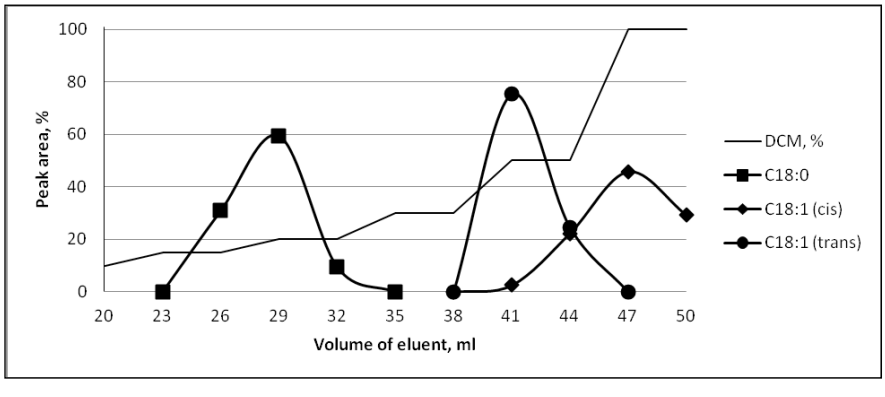

Fig. 5. Separation of C18 FAMEs on the silica based Ag^+-SPE column under optimized conditions

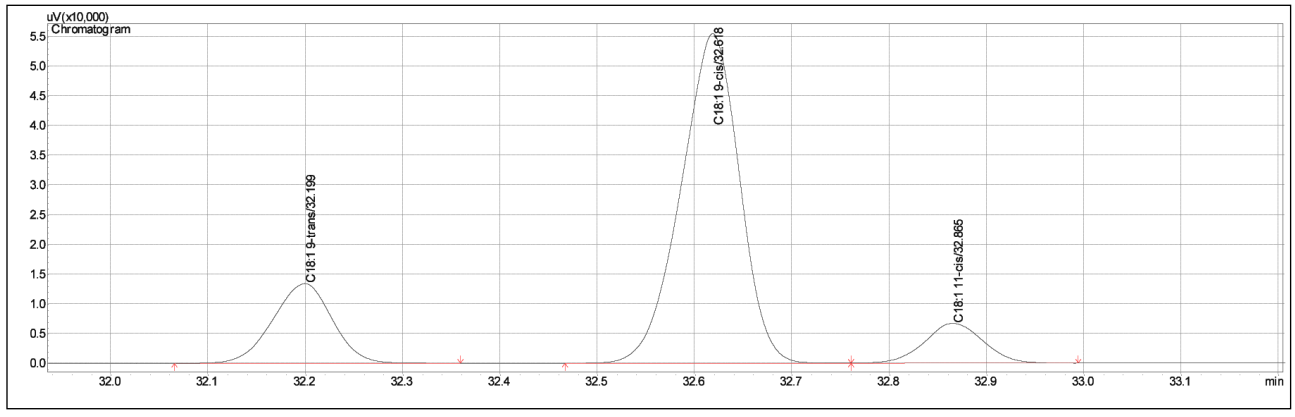

Fig. 6. A fragment of the chromatogram of rape seed oil spiked with elaidic acid fraction (38–47 ml of eluent) eluted from the Ag^+-SPE column

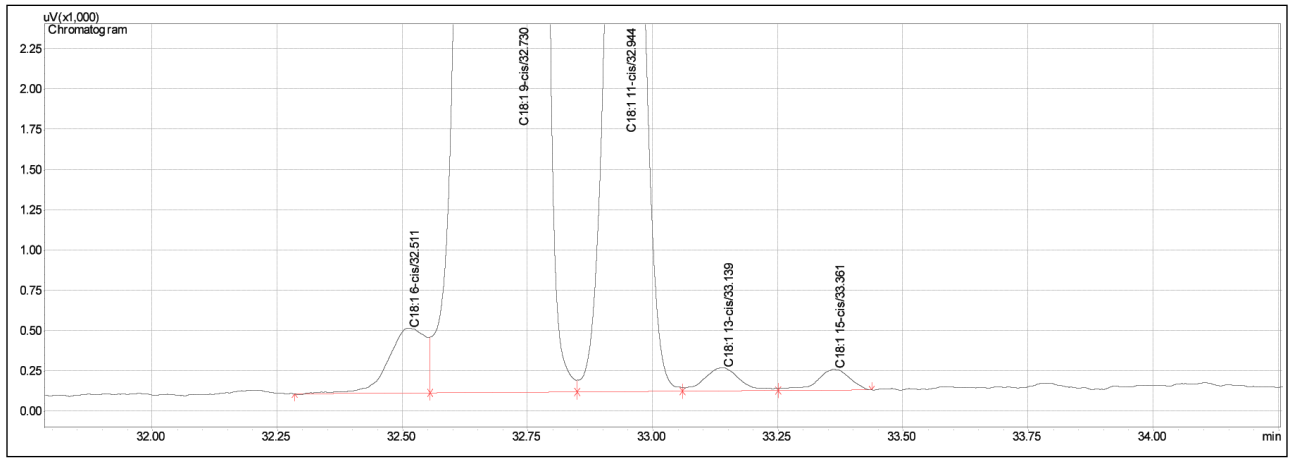

Fig. 7. A fragment of the chromatogram of C18:1 cis fraction (47–50 ml) of human adipose extract eluted from the Ag^+-SPE column

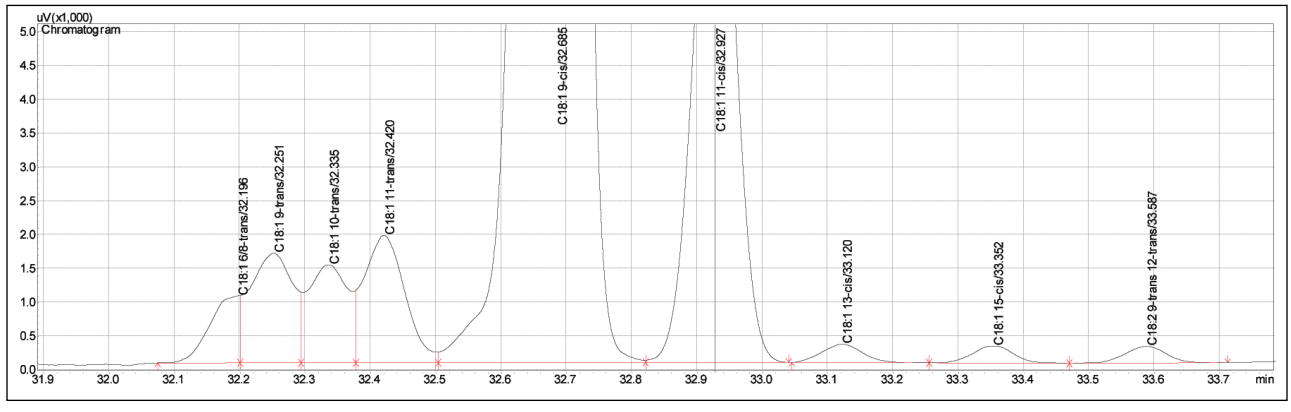

Fig. 8. A fragment of the chromatogram of 38–47 ml fraction of human adipose extract eluted from the Ag^+-SPE column

The peaks of cis isomers are observed also in the chromatogram of trans fraction (41–47 ml). However, Ag^+-SPE clean-up removed from the trans fraction about 75% of dominating C18:1 6-cis, C18:1 9-cis and C18:1 11-cisisomers. A significant decrease of the dominating isomers eliminated overlapping of the peaks and improved the separation of trans fatty acids. In Fig. 8 five trans isomers of C18:1 (C18:1 6 and 8-trans, C18:1 9-trans, C18:1 10-trans and C18:1 11-trans) are observed, that were under one peak before fractionation. This enables one to determine trace contents of trans fatty acids in the samples containing big amounts of dominant saturated fatty acids, MUFAs and PUFAs.

Quantification of trans fatty acids was accomplished. The FID response to methyl esters of different C18:1 isomers is identical. Considering this, the calculation of total trans fatty acid content was accomplished using a calibration curve constructed for C18:1 9-trans ME.

The sum of C18:1 trans fatty acids in human adipose after the silver ion solid phase extractions procedure was 0.19% of total FAMEs.

CONCLUSIONS

This work demonstrated that self-made Ag^+-SPE columns can be successfully applied for the preparative cis and trans fatty acids separation. The presented method allows eliminating about 75% of cis isomers of FAMEs from the trans fraction. Although a complete separation of all C18:1 trans fatty acids methyl esters was not achieved, the fractionation enabled us to identify most of trans isomers and to assess the percentage of trans isomers in human adipose tissue. Since a direct separation of geometric FAMEs isomers cannot be fully achieved even on 200 m highly polar ionic liquid columns [27], Ag^+-SPE is a powerful tool to supplement GC analyses.

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SIDABRO JONŲ KIETAFAZĖ EKSTRAKCIJA TRANSRIEBALŲ RŪGŠTIMS ŽMOGAUS RIEBALINIAME AUDINYJE NUSTATYTI

S a n t r a u k a

Transriebalų rūgštims žmogaus riebaliniame audinyje nustatyti pasiūlytas kietafazės ekstrakcijos, naudojant sidabro jonais padengtą silikagelio sorbentą, metodas. Riebalų rūgščių metilo esteriams formuoti pasirinktas rūgštinės katalizės variantas, katalizatoriumi naudojant boro trifluorido metanolinį kompleksą. Tinkamu eliuentu kietafazei ekstrakcijai parinktas dichlormetano ir heksano mišinys, kuriame dichlormetano dalis kinta nuo 5 iki 100 %. Pasiūlytas metodas leidžia eliminuoti iš transfrakcijos 75 % dominuojančių riebalų rūgščių cis izomerų ir nustatyti pėdsakinius riebalų rūgščių transizomerų kiekius mėginiuose, kurių sudėtyje yra daug dominuojančių sočiųjų, mononesočiųjų ir polinesočiųjų riebalų rūgščių.