

VILNIUS UNIVERSITY
CENTER FOR PHYSICAL SCIENCES AND TECHNOLOGY

Vilius
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Development and assay of solid phase extraction systems for separation of trans fatty acids

SUMMARY OF DOCTORAL DISSERTATION

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VILNIAUS UNIVERSITETAS
FIZINIŲ IR TECHNOLOGIJŲ MOKSLŲ CENTRAS

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Kietafazės ekstrakcijos sistemų skirtų trans riebalų rūgščių atskyrimui kūrimas ir tyrimas

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INTRODUCTION

There are two major sources of trans fatty acids (TFA): TFA are produced industrially through partial hydrogenation of liquid plant oils in the presence of a metal catalyst, vacuum, and high heat or can occur naturally in meat and dairy products, where ruminant animals biohydrogenate unsaturated fatty acids via bacterial enzymes. Recent research suggests that TFA from different sources, as well as individual isomers, elicit differential biological effects. The industrially produced TFA clearly have adverse health effects in humans, are associated with cardiovascular disease, cancer, inflammation and oxidative stress, metabolic syndrome, type 2 diabetes mellitus. On the other hand, there are data of health benefits of ruminal TFA isomers. For example, the major ruminant derived trans fatty acid, vaccenic acid, is a precursor for conjugated linoleic acid (CLA). It was determined that CLA supplementation for 24 months in healthy, overweight adults was well tolerated, induced a decrease of body fat, and may help maintain initial reductions in body fat mass and weight in the long term. However, a database on beneficial health effect of ruminal TFAs or certain TFA isomers is lacking. Fatty acids are well absorbed by a human body and long-term intake is reflected in the fatty acid composition of adipose tissue. Thus, sensitive and easy methods for determination of TFA in food and biological samples should help to reveal a correlation between individual TFA content and certain diseases and are therefore important.

Fatty acids contained in fat sample are most commonly determined by gas chromatography after their extraction and conversion into fatty acid methyl ester (FAME) derivatives. However, high amounts of cis and low amounts of trans fatty acids are common for real samples in which oleic acid composes up to 60% of total FAs meanwhile the quantity of trans isomers usually does not exceed 0.5%. Because of this even with the use of long and

efficient capillary columns cis/trans isomers of fatty acids are hardly separated and their peaks often overlap (**Fig. 1**). Also, it is not possible to concentrate samples to get better sensitivity for trans isomers without overloading column with dominating FAMES. Thus, pre-separation of trans FA before GC analysis is required. To separate FAME cis/trans isomers, silica based silver ion solid phase extraction (Ag^+ -SPE) can be employed. Ag^+ -SPE is based on the ability of silver ions 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.

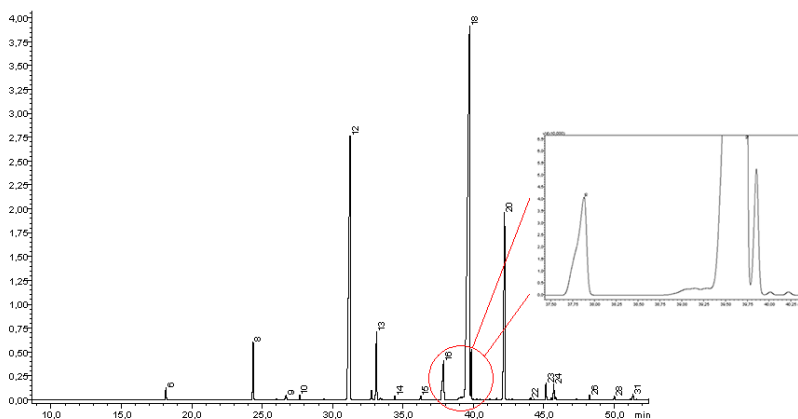


Fig. 1. Chromatogram of the fatty acid methyl esters of human adipose and the area of trans isomers of oleic acid.

Monolithic sorbents could be an attractive alternative to conventional sorbents. They possess some unique features such as ease of fabrication, high surface area that enhances interactions with the analytes and thus separation efficiency, greater (through-pore size)/(skeleton size) ratios than conventional particle-packed columns (1–2 versus 0.25–0.4), resulting in higher permeability in comparison with particulate columns with a similar number of theoretical plates.

For the separation of cis/trans fatty acids, monolithic sorbents should be modified in order to tune their surface properties. As it was mentioned above, at present silver ions are applied in the separation of cis/trans fatty acids. However, in its ionic state, silver is not stable for a long period of time, especially under the exposure of light.

Because of the delocalization of charge in silver nanoparticles (Ag NPs), the particles tend to have affinity toward electron-rich unsaturated bonds on their surface. Therefore, for the separation of cis/trans fatty acids instead of ionic silver, silver nanoparticles could be used.

The main goal of present work was to develop and investigate silver ion and silver nanoparticle modified solid phase extraction systems and apply them for the separation of geometric isomers of fatty acid methyl esters.

The tasks for achievement of the aims

1. To create a silver ion modified solid phase extraction system using silica gel as a sorbent and to select appropriate fatty acids fractionation conditions.
2. To compare the characteristics of home-made and commercial ionic silver ion solid phase extraction systems.
3. To synthesize and characterize monolithic silica sorbent modified with aminopropyl groups.
4. To select appropriate conditions for the synthesis of silver nanoparticles and to perform this synthesis in monolithic sorbent.
5. To investigate the interaction of silver nanoparticle-coated monolithic sorbent with methyl esters of cis and trans fatty acids.

Main statements for the defence

1. Fatty acid trans isomers can be identified and quantified in complex matrices using silver ion or silver nanoparticle modified solid phase extraction systems and gas chromatographic analysis.
2. An efficient and inexpensive silver ion-modified solid phase extraction system for the separation of trans fatty acids can be prepared using silica gel as a sorbent and silver nitrate as silver ion source.
3. Aminopropyl modified monolithic silica sorbent has a large surface area and pore volume.
4. The interaction of silver nanoparticles with fatty acids depends on the degree of unsaturation of fatty acid. The formation of silver nanoparticle aggregates weakens and can completely eliminate the interaction of metal silver with unsaturated compounds.
5. Aminopropyl modified monolithic silica sorbent can be coated with silver nanoparticles using precursor silver nitrate and reductor formaldehyde.
6. Silver nanoparticles modified solid phase extraction system is characterized by high stability and reusability. Effective separation of compounds is achieved by using lower amounts of sorbent and organic solvents than for silver ion-based solid phase extraction systems.

Structure of dissertation

The doctoral dissertation is written in Lithuanian language and consists of eight chapters: abbreviations, introduction, literature review, experiment methodology, results and discussion, conclusions, list of publications and list of references.

Introduction

The introduction describes the relevance of the work. The importance of fatty acids for the body and harmful effects of trans fatty acids is discussed. The possibility of using fatty acid isomers as disease markers is described, but the problem of determining these isomers is also emphasized. It is noted that the most efficient way to simplify the analysis of trans fatty acids is to fractionate a sample using a sorbent that can separate cis and trans isomers.

1. Literature review

The literature review consists of five parts. The first part describes the properties of trans fatty acids, their prevalence and the possibilities of gas chromatographic analysis. The second part provides an overview of the possibilities of using silver for chromatographic analysis. Part three reviews polymer, silica and hybrid monolithic sorbents. Possible modifications of these sorbents are described in the fourth section. The last section summarizes the possibilities of using modified monolithic sorbents in the analysis.

2. Experiment methodology

This section consists of sixteen chapters to describe the experimental part of the dissertation. It describes the equipment,

reagents and solutions used during the study. There are also included methods of formation of fatty acid methyl esters, conditions of gas chromatographic analysis and silver elemental analysis. Eight chapters are designed to describe the conditions for the preparation and use of silver solid phase extraction systems. The last section in detail describes the of evaluation results.

3. Results and discussion

This chapter consists of two sections. The first section describes the selection of conditions for the formation of methyl esters of fatty acids. The second section describes preparation, optimization and application of silver ions and silver nanoparticles solid phase extraction systems for the fractionation of methyl esters of fatty acids.

3.1 Derivatization of fatty acids

This section describes the selection of the optimal method for the formation of methyl esters of fatty acids using an acid catalyst. Two-step fat transesterification scheme using a strong base for fatty acid saponification and an acid catalyst BF_3 for esters formation was tested. An esterification reaction was also performed *in situ* using acetyl chloride in methanol. The dependence of methyl ester formation on time was investigated. Samples were heated 10, 20, 30, and 40 minutes using tricosanoic acid as an internal standard. After gas chromatographic analysis of the samples, it was determined that methyl esters were fully formed after 10 minutes, and the time extension did not change the results. However, this method of fatty acids methyl esters formation resulted in a fairly high distribution of results (3.5% - 6.7%).

Using a two-step esterification scheme, the derivatization lasted longer (35 min.) and more sophisticated hardware was required.

Nonetheless, this method produced an extremely low spread of the results (0.14% - 1.65%). For this reason, it was decided to use it for further work.

3.2 Silver modified solid phase extraction systems

This section is composed of six sub-sections. The first sub-section describes the use of a commercial silver ion solid phase extraction system for analysis of trans fatty acids in human adipose. The second sub-section describes the preparation of home made silica based silver ion solid phase extraction system, optimization of elution conditions and application. The results obtained using home made silver ion solid phase extraction column were compared with the results obtained with a commercial column. Sub-chapters 3-5 are dedicated to a nano silver solid phase extraction system based on modified silica monolith. They describe the synthesis, modification, coating with nanoparticles and application of the system for the fractionation of fatty acids. In the last sub-chapter commercial silver ion, home made silver ion and silver nanoparticle coated monolithic solid phase extraction systems are compared.

3.2.1 Supelco Discovery Ag-Ion Solid Phase Extraction System

Fractionation of the fatty acid methyl esters of human adipose was successfully carried out under the conditions provided by the column manufacturer (**Fig. 2**). This solid phase extraction system allowed the elimination of all polyunsaturated fatty acids from the fraction containing trans fatty acid methyl esters, so compounds of this class could be added to the test fraction as an internal standard. Using an internal standard allows to concentrate the sample to small volumes (100 μ l or less) and strongly increase the sensitivity of the method.

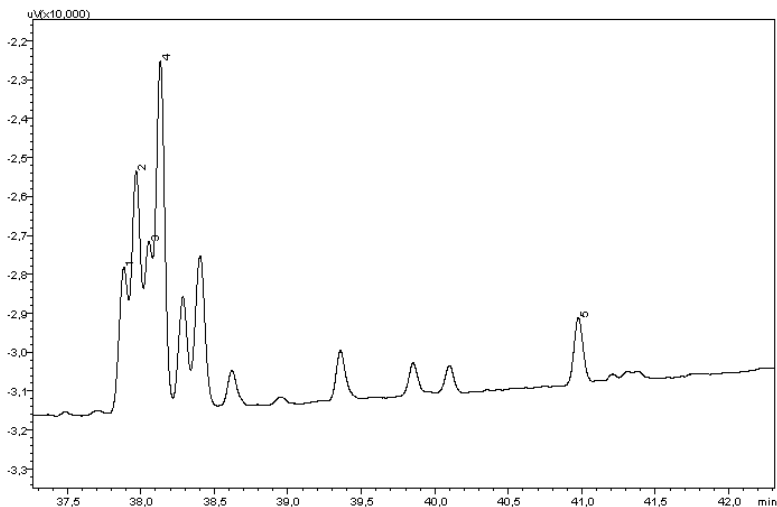


Fig. 2. Fragment of trans fraction of C18: 1 isomers of esterified human adipose tissue. 1 – C18:1-6tr; 2 – C18:1-9tr; 3 – C18:1-10tr; 4 – C18:1-11tr; 5 – C18:2-9,12c (internal standart).

3.2.2 Silver ion coated silica gel solid phase extraction system

Attempts were made to produce a simple, reliable and inexpensive solid phase extraction column based on a silica gel. 1 g of silica gel was placed in a plastic tube between glass filters. 0.1 mol l⁻¹ silver nitrate solution was used as a precursor to coat the sorbent with silver ions. It was determined that 3 ml of this solution is enough to fully cover 1 g of silica with silver ions. First try of such column demonstrated retention of fatty acids methyl esters based on a number of double bounds, so futher optimization of elution conditions and sample load was performed. It was determined that optimum amount of analytes for such system is 1 mg and 95% of cis isomers can be eliminated by using n-hexane – dichlormethane mixture as eluent (**Fig.3**).

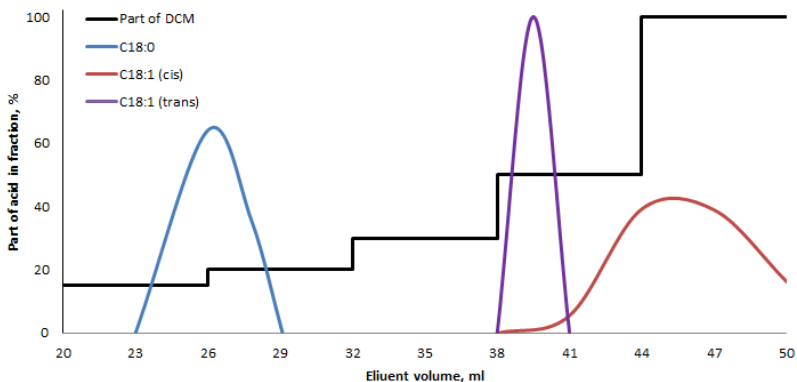


Fig. 3. Separation of C18 fatty acids methyl esters under optimized conditions.

The results obtained using home made silver ion solid phase extraction column were compared with the results obtained with a commercial column (table 1).

Table 1.

Reproducibility of silver ion solid phase extraction methods

Isomer	Silica Ag ⁺ - SPE				Commercial Discovery Ag ⁺ - SPE			
	Internal standart		External calibration		Internal standart		External calibration	
	Amount*	s _r *	Amount	s _r	Amount	s _r	Amount	s _r
C18:1-6tr	0,124	9,3	0,123	13,0	0,115	10,0	0,108	9,8
C18:1-9tr	0,192	4,5	0,199	6,5	0,190	6,6	0,187	7,0
C18:1-10tr	0,119	6,0	0,116	7,4	0,116	9,0	0,109	9,0
C18:1-11tr	0,255	9,6	0,268	6,7	0,277	6,1	0,281	6,7

* Amount of trans fatty acid is expressed as % from all fatty acids. Relative standard deviation is expressed in %.

3.2.3 Synthesis of silver nanoparticles

The operating principles of monolithic sorbents modified by silver nanoparticles are similar to those of silver-ion modified

sorbents, however, silver nanoparticles incorporated in the sorbent are much more stable. Four methods of synthesis of silver nanoparticles were investigated. Silver ions were reduced by trisodium citrate, ascorbic acid, alcohols and formaldehyde. The results of silver nanoparticle synthesis in solutions are summarized in table 2.

Table 2.

Synthesis of silver nanoparticles in solutions.

Reductor	Synthesis media / conditions	Size of synthesized particles, nm
Trisodium citrate	–	50 – 120
Ascorbic acid	pH = 6,6	90 – 100
	pH = 7	70 – 80
	pH = 8,7	40 – 50
Formaldehyde	Ag/PEI = 1	10 – 20
	Ag/PEI = 2	40 – 50
	Ag/PEI = 3	60 – 70
	Ag/PEI = 4	≥ 200
Methanol	Ag/PEI = 1	70 – 90
Ethanol	Ag/PEI = 1	70 – 150
1-Propanol	Ag/PEI = 1	100 – 150
1-Butanol	Ag/PEI = 1	100 – 200

The smallest and most uniform nanoparticles are formed from silver amine complexes using formaldehyde as a reducing agent.

3.2.4 Synthesis of monolithic sorbent coated with silver nano particles

Monolithic silica has been synthesized by employing one-pot acid–base two-step reaction using tetramethoxysilane (TMOS) as a precursor and catalyzed by acetic acid (the first step) and hydrolysis product of urea (the second step). Slow hydrolysis of urea into

carbon dioxide and ammonia at 60 °C leads to the base-catalyzed polycondensation of TMOS and results in a silica monolith formation. The synthesized monolith was calcified at 800 °C. After calcification the monolith kept its cylinder shape, however its diameter strongly decreased (from 20 to 16 mm). **Fig. 4** shows micrometer-scale morphology of the calcified silica monolith. It consists of 1.5 μm macropores and 8.2 nm mesopores. Rod-like skeletons can be recognized from the SEM image and nitrogen isotherm with type-IV hysteresis. The specific surface area of the silica monolith is 443 m² g⁻¹ and total pore volume is 0.902696 cm³ g⁻¹.

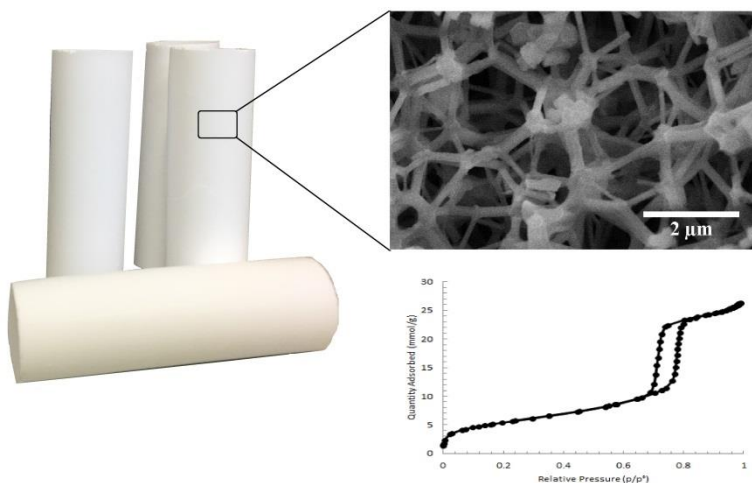


Fig. 4. Photo, SEM image and N₂ adsorption–desorption isotherm of silica monolith calcified at 800 °C.

Modification of the monolith with aminopropyl groups was achieved by the reaction of (3-Aminopropyl)triethoxysilane (APTES) and silanol groups of the monolith. A successful modification of the monolith with aminopropyl groups was proved by thermogravimetric analysis. A resolved exothermic peak between

270 and 330 °C together with the weight lost in same temperature region was observed due to the pyrolysis of aminopropyl groups grafted on the surface of silica, meanwhile no weight lost at this temperature region was observed in unmodified sorbent. Elemental analysis was performed to confirm successful monolith modification and to determine nitrogen content. It showed that nitrogen composed 2.75% by weight of the monolith.

The reduction potential for $\text{Ag}(\text{NH}_3)^{+2}/\text{Ag}$ is lower (+0.373) than that for the redox pair Ag^+/Ag (+0.799). Thus, a reducing agent able to reduce complexed silver but too weak to reduce free silver ions had to be applied. Based on this, we have applied formaldehyde to reduce silver ions bonded to aminopropyl groups modified monolithic sorbent. Successful modification was confirmed by scanning electron microscopy and the distribution and morphology of Ag NPs embedded in the monolith was observed by transmission electron microscopy (*Fig. 5*). The average size of Ag NPs was about 20 nm.

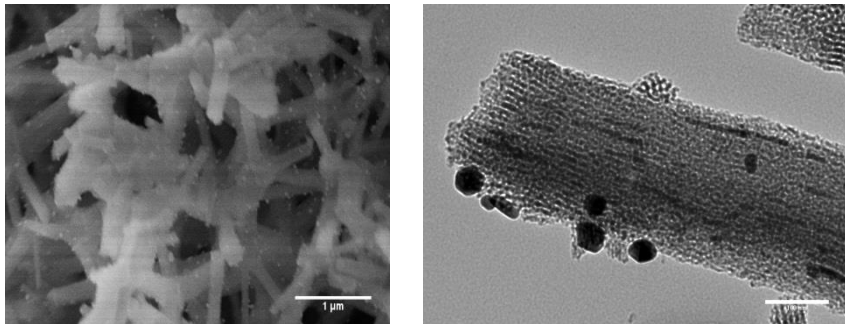


Fig. 5. SEM and TEM images of silver nanoparticles on aminopropyl groups modified sorbent.

3.2.5 Investigation of silver nanoparticle modified silica monolith solid phase extraction system

Ag NPs modified monolithic sorbent was tested for fractionation of rape seed oil fatty acids methyl esters spiked with elaidic acid methyl ester ($60 \mu\text{g mL}^{-1}$) in order to make a ratio of C18:1 cis and C18:1 trans approximately 100:1. High amounts of C18:1 cis and low amounts of C18:1 trans fatty acids are common for real samples. For the fractionation, 0.2 g of grinded sorbent was packed into a 2 mL syringe between glass frits. The sorbent was washed with 3 mL of hexane, then 50 μl of esterified rape seed oil (10 mg mL^{-1}) spiked with methyl elaidate was loaded onto the sorbent and six fractions (1 ml each) were collected and analyzed on GC-FID. Stepped gradient of hexane : dichlormethane was used as eluent (**Fig.6**).

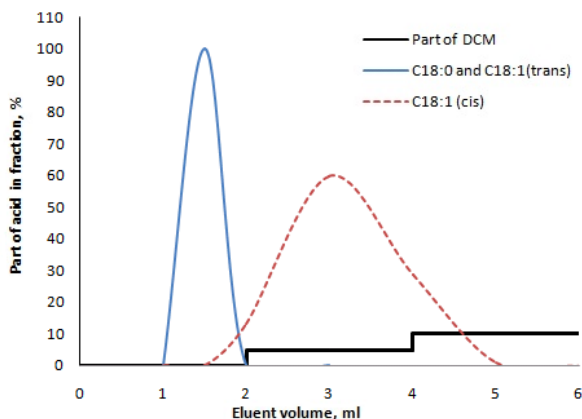


Fig. 6. Separation of fatty acids on the Ag NPs coated monolithic silica column.

After the separation, saturated FAMES, monounsaturated trans FAMES and only a small part of cis FAMES were eluted in the second fraction, thus the ratio of C18:1 cis and C18:1 trans strongly decrease (**Fig 7**). To increase sensitivity of the method pre-concentration step can be used for collected trans fraction. Moreover, only 0.2 g of the sorbent was enough to achieve sufficient separation of FAMES with 50 μg sample load.

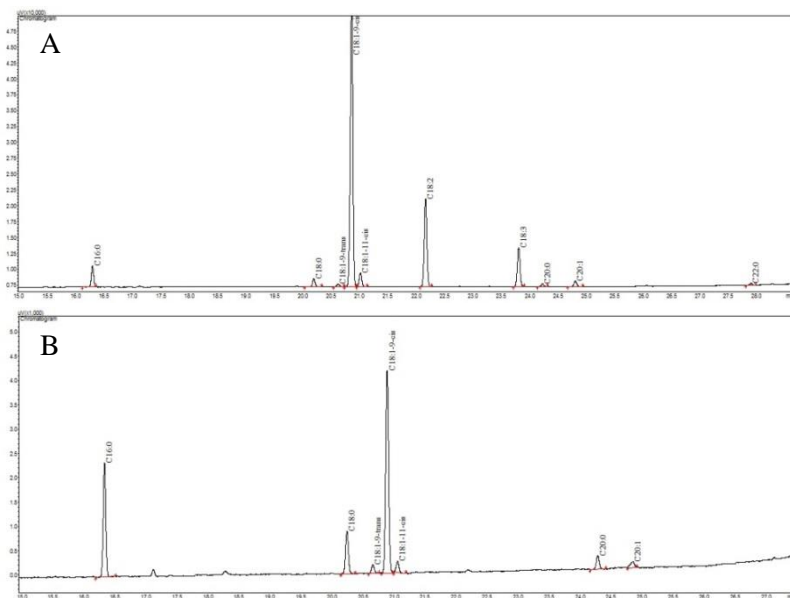


Fig. 7. Chromatograms of a fatty acid methyl ester mixture before fractionation (A) and trans fraction (B).

3.2.6 Comparison of silver solid phase extraction systems

Summarizing the results of the research of silver ion and silver nanoparticle solid phase extraction systems, it can be stated that all the systems used can be successfully applied to the separation of trans fatty acids. The main advantages and disadvantages of each system are presented in table 3.

Table 3.

3.2.6 Comparison of silver solid phase extraction systems

	Discovery Ag-Ion	Silica Ag⁺	Silica monolithic Ag⁰
Commercially available	✓		
Possibility to use internal standard	✓	✓	✓
Possibility of reuse			✓
Fast and efficient separation of trans isomers	✓	✓	✓
Low price		✓	
Small amounts of organic solvents used	✓		✓

The biggest drawback of silver nanoparticle-modified silica monolithic sorbent is the synthesis that requires long and specific laboratory equipment, but, unlike ionic silver systems, it is extremely stable and reusable. Also, using this solid phase extraction system, the separation of trans isomers takes place very quickly, and the target fraction is collected in just 1 ml of solvent.

CONCLUSIONS

1. Silver ion modified solid phase extraction system with silica gel sorbent has been developed. The conditions for solid phase extraction of this system have been investigated and optimized. The system is designed to quantify trans fatty acids in human adipose tissue.
2. Commercial Discovery Ag-Ion solid phase extraction system for quantitative determination of trans fatty acids in human adipose tissue has been investigated. Comparison of the analytical characteristics of the home made and commercial Discovery Ag-Ion SPE column revealed that both columns were suitable for trans fatty acid separation.
3. Synthesis and modification of silicon monolithic sorbent with aminopropyl groups has been performed. The sorbent had a very large surface area of $443 \text{ m}^2 \text{ g}^{-1}$.
4. The synthesis conditions of silver nanoparticles in solutions have been investigated and optimized. It has been found that the smallest and the most uniform size particles were obtained by reducing the silver amine complexes with formaldehyde. Formed particle size is 10-20 nm.
5. Optimized conditions for the synthesis of silver nanoparticles have been successfully applied to their formation in silica monolithic sorbent. Average particle size was 20 nm. Created nanoparticle-modified solid phase extraction system was applied for the separation of trans fatty acids.
6. Silver nanoparticles modified solid phase extraction system for fatty acid separation is as effective as ionic silver based solid phase extraction system. The retention of compounds depends on the number of double bounds and their configuration.
7. It has been determined that only single silver nanoparticles interact with fatty acids depending on their degree of unsaturation. The fformation of silver nanoparticle aggregates

weakens and can completely eliminate the interaction of metallic silver with unsaturated compounds.

8. The silver nanoparticle modified solid phase extraction system is highly stable and reusable. Effective separation of compounds is achieved by using lower amounts of sorbent and organic solvents than for silver ion-based solid phase extraction systems.

SUMMARY IN LITHUANIAN

Riebalų rūgščių izomerų analizė dažniausiai atliekama dujų chromatografijos metodu, tačiau tai yra ganėtinai sudėtinga, nes tiriamuose objektuose paprastai yra didelis kiekis cis konfigūracijos nesočiųjų riebalų rūgščių, kurios lemia chromatografinių smalių persiklojimą. Efektyviausias būdas supaprastinti analizę – mėginį frakcionuoti naudojant sorbentą, kuris geba atskirti nesočiųjų rūgščių cis ir trans izomerus. Šioje daktaro disertacijoje apibendrintų mokslinių tyrimų tikslas – sukurti ir ištirti sidabro jonais ir nanodalelėmis modifikuotus kietafazės ekstrakcijos sorbentus ir pritaikyti juos riebalų rūgščių metilo esterių geometrinių izomerų frakcionavimui.

Darbo metu riebalų rūgščių geometrinių izomerų atskyrimui buvo pritaikytos trys kietafazės ekstrakcijos sistemos. Naudojant silikagelio sorbentą buvo sukurta ir optimizuota sidabro jonų kietafazės ekstrakcijos sistema. Ši sistema sėkmingai pritaikyta trans riebalų rūgščių atskyrimui žmogaus riebaliniame audinyje, o gauti rezultatai palyginti su komercine sidabro jonų kietafazės ekstrakcijos kolonėle gautais rezultatais. Pagrindinis sidabro jonų veikimu paremtų sorbentų trūkumas yra joninio sidabro nestabilumas, kuris riboja tokio tipo sorbentų pakartotinio naudojimo galimybes.

Sidabro nanodalelėmis modifikuoti monolitiniai sorbentai nesenai tapo daug žadančia medžiagų klase. Jie gali būti sėkmingai panaudoti cheminėje analizėje, o tokių sorbentų veikimo principai yra panašūs kaip sidabro jonų veikimu paremtų sorbentų. Disertaciniame darbe ištirtos ir optimizuotos sidabro nanodalelių sintezės sąlygos tirpaluose. Nustatyta, kad mažiausios ir vienodžiausio dydžio dalelės yra gaunamos redukuojant sidabro amininius kompleksus formaldehidu. Susidariusių nanodalelių dydis 10-20 nm. Optimizuotos sidabro nanodalelių sintezės sąlygos sėkmingai pritaikytos jų formavimui laboratorijoje susintetintame ir aminopropilo grupėmis modifikuotame silikagelio monolitiniame

sorbente, kuris pasižymis itin dideliu paviršiaus plotu ($442 \text{ m}^2 \text{ g}^{-1}$) ir porų tūriu ($0,9 \text{ cm}^3 \text{ g}^{-1}$). Vidutinis sidabro nanodalelių dydis 20 nm. Sukurta nanodalelėmis modifikuota kietafazės ekstrakcijos sistema trans riebalų rūgštims atskirti. Parodyta, kad ši sistema riebalų rūgštis atskiria taip pat efektyviai kaip joninio sidabro sistemos. Junginių sulaikymas priklauso nuo dvigubųjų ryšių skaičiaus ir jų konfigūracijos. Didžiausias sidabro nanodalelėmis modifikuoto silikagelio monolitinio sorbento trūkumas yra ilga ir specifinės laboratorinės įrangos reikalaujanti sintezė, tačiau priešingai nei joninio sidabro sistemos, ši pasižymi itin dideliu stabilumu ir pakartotinio naudojimo galimybe. Naudojant šią kietafazės ekstrakcijos sistemą trans izomerų atskyrimas vyksta labai greitai, o tikslinė frakcija surenkama vos 1 ml tirpiklio tūryje.

LIST OF PUBLICATIONS

Publications in journals:

1. V. Poskus, V. Vickackaite, G. Brimas. Silver ion solid-phase extraction for the analysis of trans fatty acids in human adipose. *Chemija* **27** (3), 2016, p. 179.
2. V. Poskus, V. Vickackaite, J. Dargyte, G. Brimas. Home-made silver ion solid-phase extraction system for the analysis of trans fatty acids: comparison with commercial Discovery Ag-Ion SPE. *Chemija* **29** (1), 2018, p. 49.
3. V. Poskus, V. Vickackaite. Silver-coated monolithic silica column for separation of trans fatty acids. *Separatios science plus* **1** (11), 2018, p. 738.

Publications in international conferences proceedings:

1. V. Poskus, V. Vickackaite, G. Brimas. Trans fatty acid separation by home-made silica based Ag ion modified solid phase extraction tube. International Conference „There is no future without the past”. Vilnius, Lithuania, (2017) p. 74
2. V. Poskus, V. Vickackaite, G. Brimas. Silver ion solid-phase extraction for the analysis of trans fatty acids in human adipose. Europe's Analytical Chemistry Meeting „XIX Euroanalysis 2017”, Stockholm, Sweden, (2017) p. 257.
3. V. Poskus, V. Vickackaite. Silver-coated monolithic silica column for separation of trans fatty acids. 61st international conference for students of physics and natural sciences „Open readings 2018”, Vilnius, Lithuania, (2018) p. 283.
4. V. Poskus, V. Vickackaite. Trans fatty acids separation by silver nanoparticles modified monolithic silica column. International Scientific Conference „EcoBalt”, Vilnius, Lithuania, (2018) p. 29.

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Work experience:

2011 till now	„Eurofins Labtarna Lietuva“ JSC Chemist.
2017 till now	Vilnius University department of Chemistry and Geosciences. Junior assistant.

Project activities:

2013	Student scientific activity promotion. „Synthesis of pyrolo[2,3d]pyrimidine derivates and Pd(II) catalysed reactions study“
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