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Analysis of Fatty Acid Amides in Waste Animal Fat

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AFFIDAVIT

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Dad, I always believed that my greatest capabilities I learned from you. Without them, I could not accomplish what I have so far…

Abstract

The aim of this thesis is development and optimization of an efficient separation and quantification method for primary fatty acid amides (PFAMs) in waste animal fat. The method consists of separation of PFAMs via solid phase extraction. The most effective eluents were determined to be hexane: ethyl acetate (70:30) (for triglycerides, vitamins, pigments and other minor components) and chloroform: 2-propanol (2:1) (for PFAMs).

The isolated PFAMs are identified and quantified via GC-FID using nonadecanoic acid amide as an internal standard. The recovery of PFAMs in rapeseed oil and lard as matrices was determined to be around 100%.

After establishment of the method, six real samples were analyzed for the content of PFAMs. The quantification of PFAMs by GC-FID yielded concentrations 0.04% to 2%.

Additionally, the limits of detection and quantification were determined to be 0.002% and 0.005%, respectively.

Zusammenfassung

Das Ziel dieser Arbeit ist die Entwicklung und Optimierung einer effizienten Trennungs- und Quantifizierungsmethode für primäre Fettsäureamide (PFAMs) in tierischem Fett. Das Verfahren besteht aus der Trennung von PFAMs mittels Festphasenextraktion. Als wirksamste Eluenten wurde Hexan: Ethylacetat (70:30) (für Triglyceride, Vitamine, Pigmente und andere Nebenkomponenten) und Chloroform: 2-Propanol (2:1) (für PFAMs) bestimmt.

Die isolierten PFAMs werden mittels GC-FID unter Verwendung von Nonadecansäureamid als internem Standard identifiziert und quantifiziert. Die Rückgewinnung von PFAMs in Rapsöl und Schmalz als Matrix lag bei rund 100%.

Nach Etablierung der Methode wurden sechs reale Proben auf den Gehalt an PFAMs untersucht. Die Quantifizierung dieser mittels GC-FID ergab Konzentrationen von 0,04% bis 2%.

Zusätzlich wurden die Nachweis- und Quantifizierungsgrenzen der Methode mit 0,002% und 0,005% bestimmt.

Table of content

1. Introduction

Over the last few decades, the humane society has become more and more dependent on fossil fuels to produce enough energy to satisfy fast growing population and rapid technological developments. However, burning of fossil fuels has caused huge environmental issues such as rising of carbon dioxide levels in the atmosphere, increase of earth's average surface temperature. Furthermore, fossil fuels are non-renewable, taking them millions of years to create while humans are harvesting them in non-comparable rate. As a consequence, a global movement towards substituting fossil fuels with renewable resources for energy production has yielded many alternatives in each energy sector.

Biodiesel has been established as a very important alternative for Diesel fuel in the transport sector. It is ecologically acceptable substitute for the conventional fuel, because it is renewable, readily available, cheap, has a better emission profile and higher Cetane number [1]. One of the main advantages of biodiesel is its role in reducing global greenhouse gas emissions, as well as reducing global dependence on the petroleum reserves.

Usually it is produced from wide range of edible vegetable oils, such as rapeseed oil, soybean oil, palm oil or sunflower oil. However, there are some limitations of using biodiesel, such as compatibility with some rubber material parts, as well as corrosion and wear of engine parts of the fuel supply system components [2]. The use of edible oils for the production of biodiesel has also raised many controversies, mainly because the land that was primarily used for agriculture is used for energy crops. Furthermore, the prices of edible vegetable oils are expected to increase in the future. This has caused considerable efforts during the last years to find the cheaper and more acceptable alternative for biodiesel production.

In this regard, waste animal fat, as a by-product of the meat processing industries, has become a material of great interest for biodiesel production. Animal fat can be converted into high-quality biodiesel that meets the international specifications. However, there are some drawbacks and challenges, such as a high cloud point due

to a relatively high melting temperature of animal fats [3]. Additionally, waste animal fat contains impurities that can present interference with the biodiesel process, resulting in lower biodiesel quality.

This thesis focuses on fatty acid amides (FAA), as one of the occurring impurities in waste animal fat. FAAs, being insoluble in animal fat, as well as in the corresponding products, such as biodiesel, pose a risk during biodiesel production as they can plug filters [4].

The aim of this work is the development and optimization of a method for determination and quantification of FAAs in waste animal fat.

2. Theoretical part

2.1. Biodiesel from waste animal fat

In the past decade, many efforts have been focused into the development of alternative fuels that could replace fossil fuels. Numerous studies revealed that the combustion of biodiesel significantly reduced harmful emissions, which launched biodiesel to be the most used renewable energy source after ethanol. [5]

Normally, biodiesel is produced from high quality vegetable oils. However, the price of feedstock contributes to 85% of biodiesel production expenses [6], and these prices are expected to rise in the future. On the other hand, most of the animal fats are not considered suitable for human consumption. Instead, they are used for pet food, animal feed or for soap production, which makes their cost considerably lower than the cost of vegetable oil, making them a very attractive feedstock for biodiesel production.

Numerous studies were carried out on biodiesel production from low cost feedstocks, such as frying oils, non-edible vegetable species, new vegetable species and animal fats [3] [7].

Because of the negative physical properties, animal fats cannot be directly combusted in diesel engines. Therefore, they are converted into fatty acid methyl esters (FAME) in a two stage production process: initial esterification of free fatty acids using acid catalyst and transesterification of triglycerides using alkaline catalysts [8]. Waste animal fat (WAF) biodiesel has lower or equal NO_x emissions compared to vegetable oil biodiesel [9, 10]. Furthermore, biodiesel produced from animal fat has a higher Cetane number resulting from higher ratio of saturated fatty acids, leading to better performance of engine and reduced emissions [11]. Similarly, the saturated fatty acids contribute to a better oxidative stability of biodiesel [12]. However, animal fat derived biodiesel has some negative properties, such as higher cloud point and cold filter plugging point, thus, it is not possible to use them during cold periods in pure form [13]. The simplest and least expensive way to overcome this drawback is mixing of methyl esters of animal origin with vegetable methyl esters and/or with fossil diesel fuel [14].

Currently, there are two biodiesel production techniques, namely chemical and biocatalytic techniques. In chemical synthesis, the fat is converted to FAME using acidic or basic catalysts. Biocatalytic techniques employ enzymes for this conversion, mostly lipases. Although chemical production techniques are currently the most used techniques, they have several drawbacks, like deterioration of fats with strong acids and complex downstream purification process, especially for the raw glycerol. Enzyme-based production can overcome some of these limitations. For instance, enzyme catalyzed production limits the risk of soap formation and the need to use high molar ratio of alcohol. However, main limitations of enzyme-catalyzed technology are high cost and easy inactivation of enzyme with methanol, which is the leading cause that this process is still not commercialized. Methods, which involve enzyme immobilization, allow simple recovery and reuse of enzyme are still under investigation. [12]

2.2. Waste animal fat

Main sources of animal fat for production of biodiesel are beef tallow, lard, poultry fat and fish oils. Due to their high content of saturated fatty acids, animal fats are highly viscous and generally in solid form at ambient temperature.

Fatty wastes that come from the meat processing industries are usually not suitable for human consumption, either because of aesthetic or sanitary reasons. Products of rendering industry usually have lower market value making them a very attractive feedstock for biodiesel production. Waste animal fats (WAFs) are gathered from slaughterhouses, butcher houses and supermarkets and transported to rendering plants. [15] However, contaminated animals could present a biosafety issue, therefore special attention has to be given.

Animal fats can be divided into two general categories: edible and inedible. Edible animal fats are produced from healthy slaughtered animals, and are declared as fit for human consumption. Inedible fats are usually slaughter by-products, which are not intended for human consumption, and may include blood, bones, feet and other parts of the animal. [16]

Following the BSE crisis in the UK, in 2000, Animal-By-Product-Regulation (ABPR) was published with the intention to regulate safe collection, treatment and use of animal by-products. The ABPR divides animal by-products into three categories. Category 1 materials have the highest risk of contamination, where contaminants cannot be destroyed by normal rendering process. Category 2 products pose known and manageable risk of diseases that can be reduced by a sterilization step. These products are usually used like fertilizers or as oleo chemical products. Category 3 products are the lowest risk products that are suitable for animal feed, but not for human consumption. [16]

Although majority of biodiesel is still produced from vegetable oils, mostly palm oil and soybean oil, around 7% is made from animal fats (1.4 Mt in average), and that share has stayed constant even though biodiesel production has increased [17]. From 2014 to 2017 tallow and lard contributed about 3% to 6% of total feedstock for biodiesel. [18] By year 2016, around 11 Mt of category 3 fat was produced annually, while category 1 and 2 were produced in average 5 Mt annually. Approximately 10% of that was used as feedstock for biodiesel, while rest was used for combustion of meat and bone meal, fat combustion and fertilizer. In 2016, around 500.000 t of category 1 and 2 fat and close to 450.000 t of category 3 was used for production of biodiesel [19].

2.2.1. Fat rendering

Rendering is a process that converts waste animal tissue into stable, usable materials. All rendering processes use the application of heat, extraction of moisture and separation of fat. [20] Depending on whether the fat is obtained from an already dried material, or from a wet phase, wet and dry rendering processes are distinguished, respectively. Generally, wet rendering is preferred for sensitive, valuable products, like fish oils or edible fats. [16]

Wet rendering: The raw material is minced and melted in a cooker at 70 – 90^{o}C either with steam or with heat. Three phases are separated: solid phase, aqueous phase and melted fat. The liquid phase is decanted from the solids and sent to the dryer. [16]

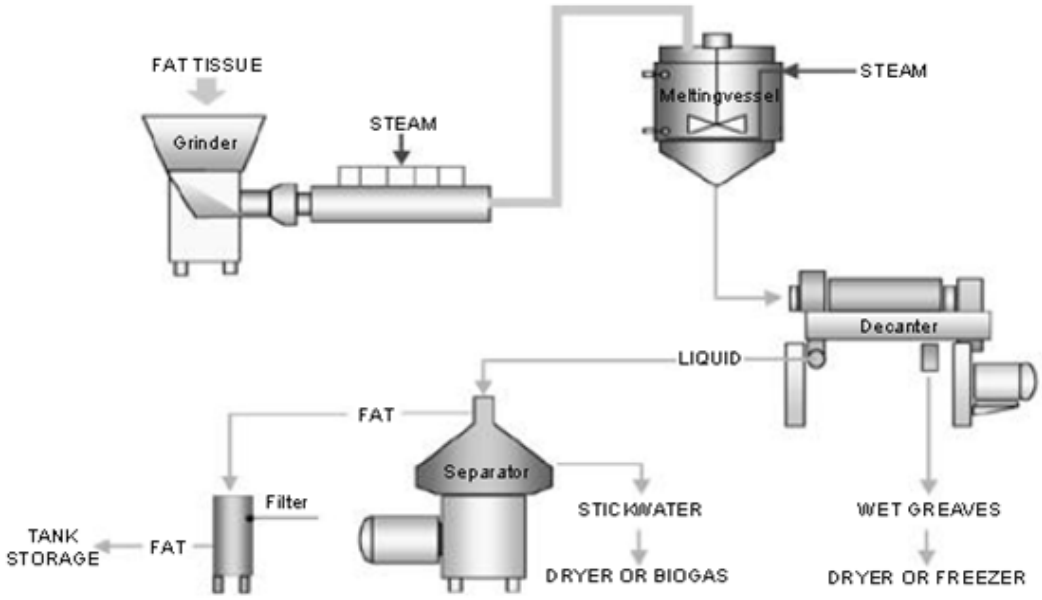


Figure 1. Wet rendering process [16]

Dry rendering: The material is crushed, cooked and dried. The dried material is then pressed to separate fat and solids. [16]

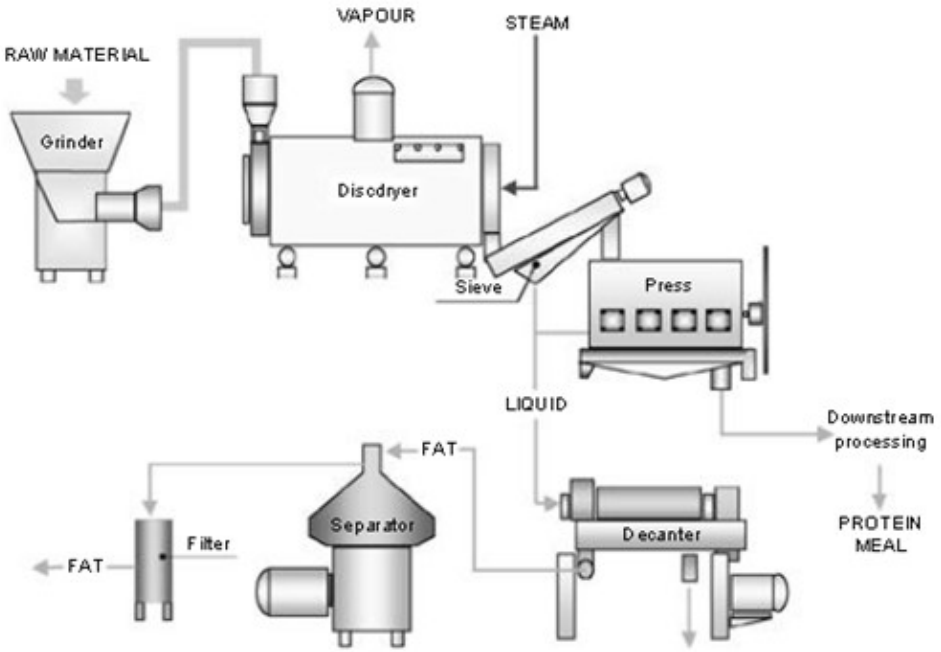


Figure 2. Dry rendering process [16]

The main components of animal fat are triglycerides, fatty acids, phospholipids and sterols. The triglyceride molecule is an ester derived from glycerol and three fatty acids. During transesterification reaction, fatty acids react with alcohol to produce fatty acid alkyl ester (biodiesel). The degree of saturation in the carbon chain determines the physical properties of fats and oils. Oils are in liquid state because of unsaturated fatty acids whereas animal fats, because of the high amount of saturated fatty acids, exist in solid state. When fats are exposed to wet environment for longer time, they are easily hydrolyzed, which increases the content of free fatty acids. As a consequence, the quality of the fat for biodiesel production is reduced [21]. Table 1. represents the chemical composition (% by weight) of fatty acids present in various animal fats.

Table 1. Chemical composition of fatty acids in different animal fats [22]

Fatty acid	*Beef tallow*	*Pork lard*	*Poultry fat*	*Fish oil [23]*
Myristic acid C 14:0 [%]	1 - 1.50	1 - 1.50	/	5.77
Palmitic acid C16:0 [%]	24 - 28	24 - 28	20 - 24	16.94
Palmitoleic acid C 16:1 [%]	2 - 3	2 - 3	5 - 9	5.42
Stearic acid C 18:0 [%]	20 - 24	13 - 14	4 - 6	4.31
Oleic acid C 18:1 [%]	40 - 43	43 - 47	33 - 44	19.20
Linoleic acid C18:2 [%]	2 - 4	8 - 11	18 - 20	16.05
Linolenic acid C 18:3 [%]	<1	<1	1 - 2	2.82
Arachidic acid C 20:0 [%]	<1	<1	/	/
Eicosenoic acid C 20:1 [%]	1.80	1.34	/	/
Eicosapentaenoic acid C 20:5 [%]	/	/	/	15.55
Docosapentaenioc acid C 22:5 [%]	/	/	/	2.45
Docosahexaenoic acid C 22:6 [%]	/	/	/	11.36

2.2.2. Tallow

Tallow is extracted from animal tissues containing fat either by rendering or melting processes. Mainly it is based on beef, mutton or sheep by-products. It is odorless, tasteless and waxy white fat, consisting of suet (raw, hard fat found around the loins and kidneys). Tallow was mainly used in the manufacture of soaps, cosmetic industry and as fuel for smaller boilers, until the development of synthetic surfactants made it available as a base for chemicals and lubricants.

Tallow consists mainly of triglycerides, whose major constituents are stearic, palmitic and oleic acids. Approximately 50% of total fatty acids in tallow are saturated, resulting in a high melting point and high viscosity of the fat. [24]

Tallow can contain up to 10% of free fatty acids, and for the production of biodiesel, the content of FFA has to be reduced to below 2%. Therefore, the production of biodiesel from tallow is usually done in two stages: acid catalyzed esterification of free fatty acids followed by transesterification. [11]

2.2.3. Lard

Lard is fat obtained from any part of the pig with high amount of fatty tissue. The highest-grade lard is gained from visceral fat deposit around kidneys and inside the loin. The next highest grade lard is obtained from fatback, the hard subcutaneous fat between the pig's back skin and muscle. The lowest grade is obtained from the soft caul fat surrounding digestive organs. [20]

The main fatty acids found in lard are palmitic, stearic, oleic and linoleic acids. Compared to other animal fats, lard is characterized by higher levels of unsaturated fatty acids (approximately 60%), comprising mostly of oleic (~ 39%) and linoleic (~19%) fatty acid.

2.2.4. Chicken fat

Chicken fat is obtained from chicken rendering and processing. Most often, it is discarded as waste because it is not suitable for human consumption, which makes it a valuable feedstock for biodiesel production. Compared to other animal fats, chicken fat was found to be the most promising choice for high quality biodiesel production because of its low price, availability and easy processing [25]. Total body fat content of chicken is approximately 11% [26], with high levels of linoleic acid (between 17.9% and 22.8%) [27]. However, several disadvantages of using chicken fat as feedstock have been reported, such as high content of free fatty acids, high viscosity, high pour point and flashpoint [28].

2.3. Solid Phase Extraction

Solid phase extraction is one of the most prevalent sample preparation techniques by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties. Most often, it is used to isolate and extract semi volatile or nonvolatile analytes of interest from a wide variety of matrices like biological fluids such as plasma, urine, blood; environmental samples such as water, air, soil; food products like grains, meat; pharmaceuticals; beverages or industrial products. [29] [30]

Solutes that are dissolved or suspended in a liquid (known as the mobile phase) are passed through a solid (known as the stationary phase or sorbent) are separated according to their affinity into desired and undesired components. Either the desired analytes of interest or undesired impurities are retained on the stationary phase. If the analytes are recovered from the sorbent in a final volume that is only a fraction of the sample volume, then besides isolation, the concentration is achieved.

There are three major benefits of SPE:

1. Simplification of complex sample matrix including compound purification; interfering substances are separated from sample matrix, which allows much simpler identification and quantification of compounds of interest.

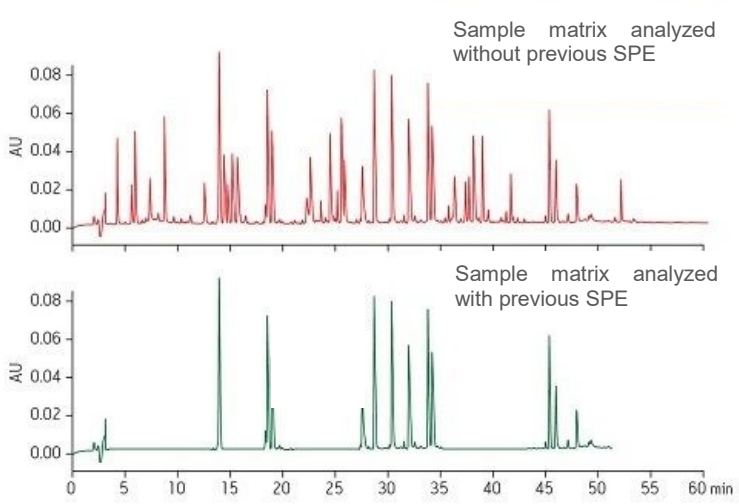


Figure 3. Chromatogram of sample matrix before and after SPE [30]

Another benefit of simplifying the sample matrix is improved quantitation accuracy, because the peak of interest may contain some contamination from the matrix with the same retention time resulting in larger peak area. [30]

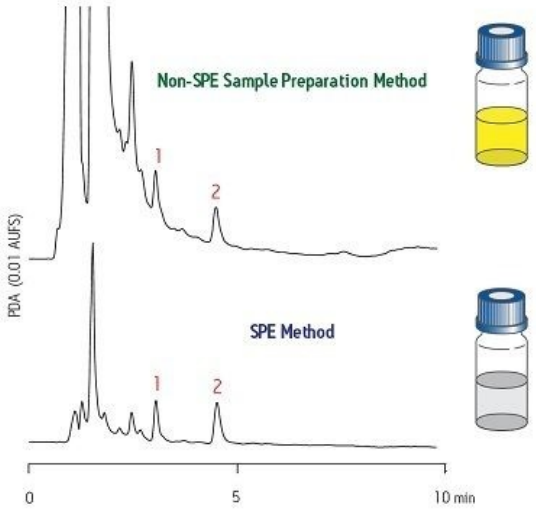


Figure 4. Chromatogram of sample matrix before and after SPE [30]

2. Capability to fractionate sample matrix to analyze compounds by class; an SPE method can be developed to separate different classes of compounds. For example, polar compounds can be separated from the more non-polar, which allows a much more efficient analysis. [30]

3. Enrichment of very low level compounds; for compounds which concentration in the original sample is lower than the sensitivity of the analytical instrument, SPE gives a possibility to concentrate the desired analyte resulting in increased signal strength for this compound [30]

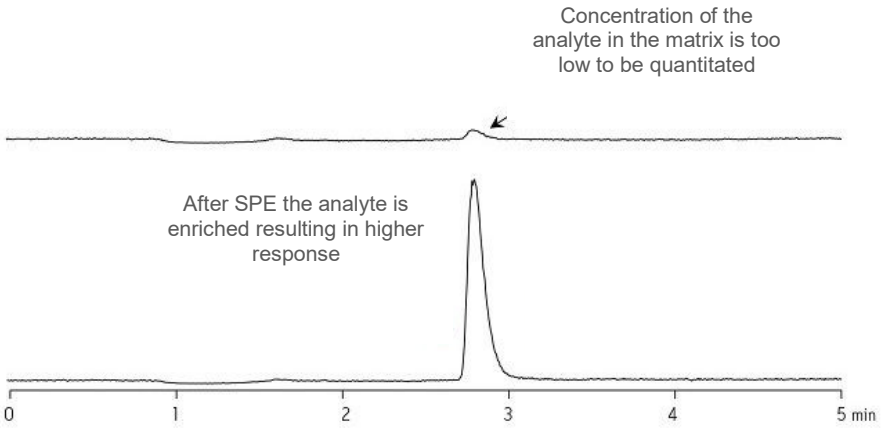


Figure 5. Enrichment of compounds as a result of SPE [30]

SPE consists of four steps; conditioning, sample loading, washing and elution.

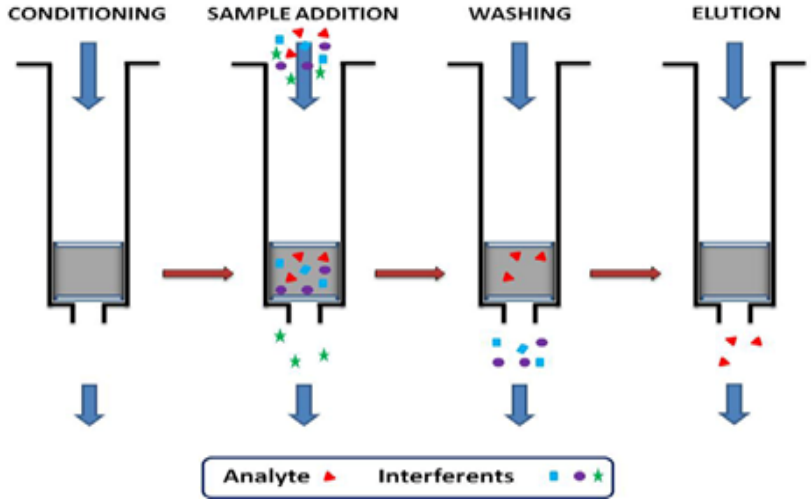


Figure 6. Four steps of SPE [31]

Conditioning of the column is done with an organic solvent that acts as a wetting agent and solvates the functional groups of the sorbents. The sample is loaded on the column by means of gravity or by applying a vacuum. In the washing stage, interferences are removed from the matrix while the analyte is retained on the column. Elution of the analyte is completed by disruption of analyte - sorbent interaction with an appropriate

solvent. The retained analytes are often eluted using small aliquots of an organic solvent concentrating them enough for analysis without further evaporation and concentration procedures. [32]

3.3.1. Normal phase SPE

Normal phase SPE involves a polar analyte, a mid- to non-polar matrix (e.g. hexane, acetone or chlorinated solvents) and a polar stationary phase. Retention of an analyte is primarily due to interactions between polar functional groups of the analyte and polar groups on the sorbent surface. A stationary phase of polar functionally bonded silicas with short carbons chains frequently makes up the solid phase. A solvent that is more polar than the original matrix disrupts the binding mechanism between analyte and the solid phase eluting adsorbed compounds. [33]

3.3.2. Reversed phase SPE

The stationary phase is non-polar due to derivatization with hydrocarbon chains, which retains compounds of mid to low polarity due to the hydrophobic effect. A typical stationary phase contains hydrophilic silanol groups at the surface of silica packing, which have been chemically modified with hydrophobic alkyl or aryl functional groups. Attractive forces between the carbon-hydrogen bonds in the analyte and the functional groups on the silica surface are responsible for the retention of the analyte. The analyte can be eluted by washing the cartridge with a non-polar solvent, which disrupts the interaction of the analyte and the stationary phase.

A stationary phase of silicon with carbon chains is commonly used. Relying on mainly non-polar, hydrophobic interactions, only non-polar or very weakly polar compounds will adsorb to the surface. [33]

3.3.3. Ion exchange SPE

Ion exchange sorbents separate analytes based on electrostatic interactions between charged functional group of the analyte of interest and the positively or negatively

charged groups that is bonded to the silica surface. For ion exchange to occur, both the stationary phase and sample must be at a pH where both are charged.

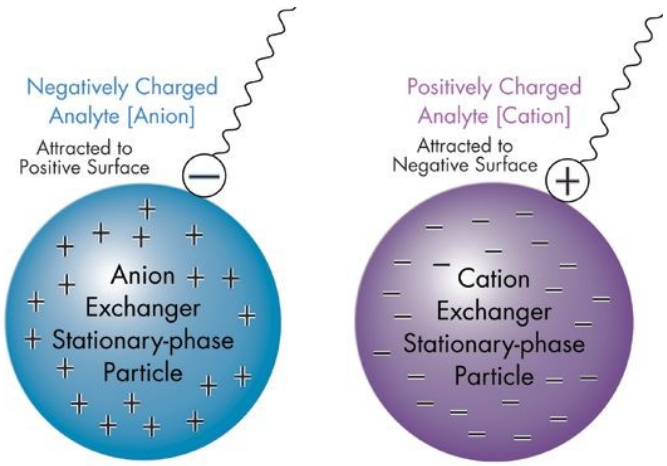


Figure 7. Ion exchange SPE [34]

For elution of the analyte, a solution that neutralizes either the functional group of the analyte or on the sorbent surface is used.

Anion exchange sorbents are derivatized with positively charged functional groups that retain negatively charged anions, such as acids. Strong anion exchange sorbents contain quaternary ammonium groups that have a permanent positive charge in aqueous solutions. These sorbents bind acidic impurities in the sample separating them from the analyte of interest. Weak anion exchange sorbents use amine groups that are charged when the pH is below 9. [33]

Cation exchange sorbents are derivatized with functional groups that retain positively charged cations, such as bases. The sorbent material contains silica with aliphatic sulfonic acid groups bonded on the surface. These groups attract or exchange cationic species in the sample solution, and are charged over the whole pH range allowing them to separate both strong and weak cationic compounds. [33]

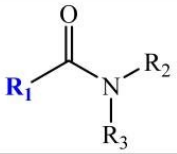
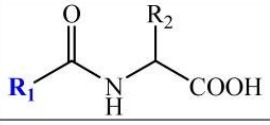
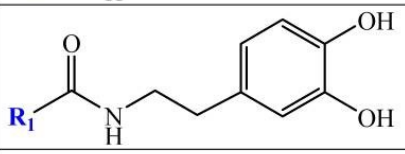
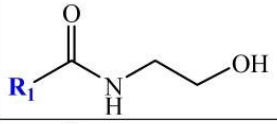
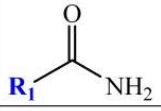
2.4. Fatty acid amides

Fatty acid amides (FAA) are structurally diverse molecules found in mammalian organisms as well as several plant families. As many other nitrogen derivatives of fatty

acids, they have a considerable economic importance and have, therefore, been an object of many research activities [35]. Because of their chemical features resulting in high surface activities, today they are produced in large scale. They are used as detergents, lubricants, textile softeners, antistatic and floatation agents, wax additives and plasticizers.

They are an emerging class of bioactive signaling lipids that perform various functions in physiological and pathological processes [36]. Five general classes of endogenous FAAs have been identified – N-acylamides (NAMs), *N*-acylethanolamines (NAEs), *N*-acylamino acids (NAAs), *N*-acyldopamine (NAD) and primary fatty acid amides (PFAM). [37]

Table 2. Five classes of fatty acid amides [37]

Fatty Acid Amide	Structure
N-Acylamide (NAM)	
N-Acylamino acid (NAA)	
N-Acyldopamine (NDA)	
N-Acylethanolamines (NAE)	
Primary fatty acid amides (PFAM)	

Primary fatty acid amides are a subclass of lipids that have been identified in the last decade. They consist of carboxamide headgroup and an acyl tail of various length and saturation. [38]

PFAMs are waxy solids with melting temperatures from 68 – 108°C, depending on their chain length, and have low solubility in water. They are soluble in non-polar organic

solvents, such as chloroform, isopropyl alcohol and toluene, and their solubility increases with temperature. When completely saturated, amides are stable to air oxidation, heat and diluted acids or bases. Unsaturated primary amides are susceptible to oxidation leading to darker colored products. [39]

Endogenous FAAM were first isolated from human plasma [40]. However, the interest in these compounds and their anabolic pathways was modest because their metabolism and function *in vivo* was unknown, until the discovery that oleamide and (9(*Z*)-octadecenamide) erucamide accumulate in the cerebrospinal fluid of sleep deprived cats [41] increased an interest in the structure and functions of PFAMs. Since then, it has been revealed that oleamide has the ability to affect memory processes, increase food intake, reduce anxiety and pain, stimulate release of Ca(II) ion and relax blood vessels, among other functions [42].

The main path for PFAM *in vivo* degradation is the hydrolysis to fatty acids and ammonia, a reaction catalyzed by fatty acid amide hydrolase (FAAH), an integral membrane protein [36].

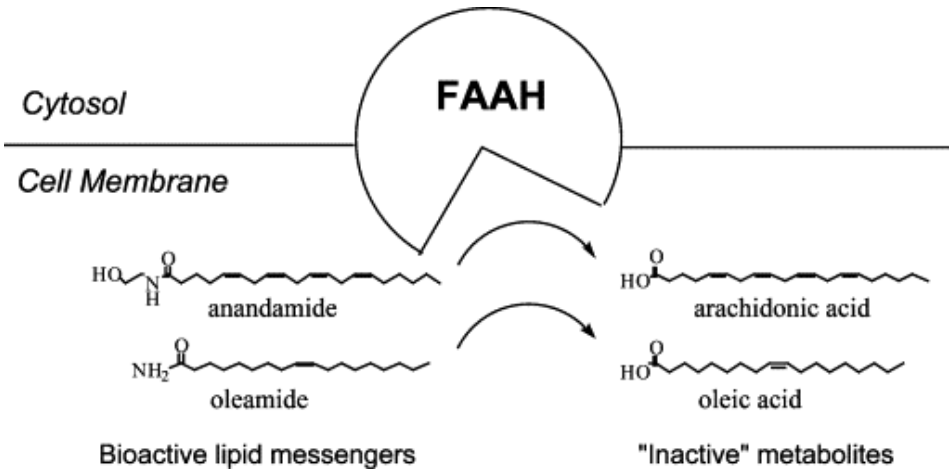


Figure 8. Hydrolysis of PFAMs [36]

The biosynthetic route for PFAMs biosynthesis is still not fully understood. There are several suggested reactions for production of PFAMs. One is the direct amidation of fatty acyl-CoA thioesters with ammonia, however, it is still not clear which enzyme catalyzes this reaction in vivo [37]. A second proposed pathway for PFAM biosynthesis involves the PAM-mediated cleavage of *N*-fatty acylglycines [43, 44].

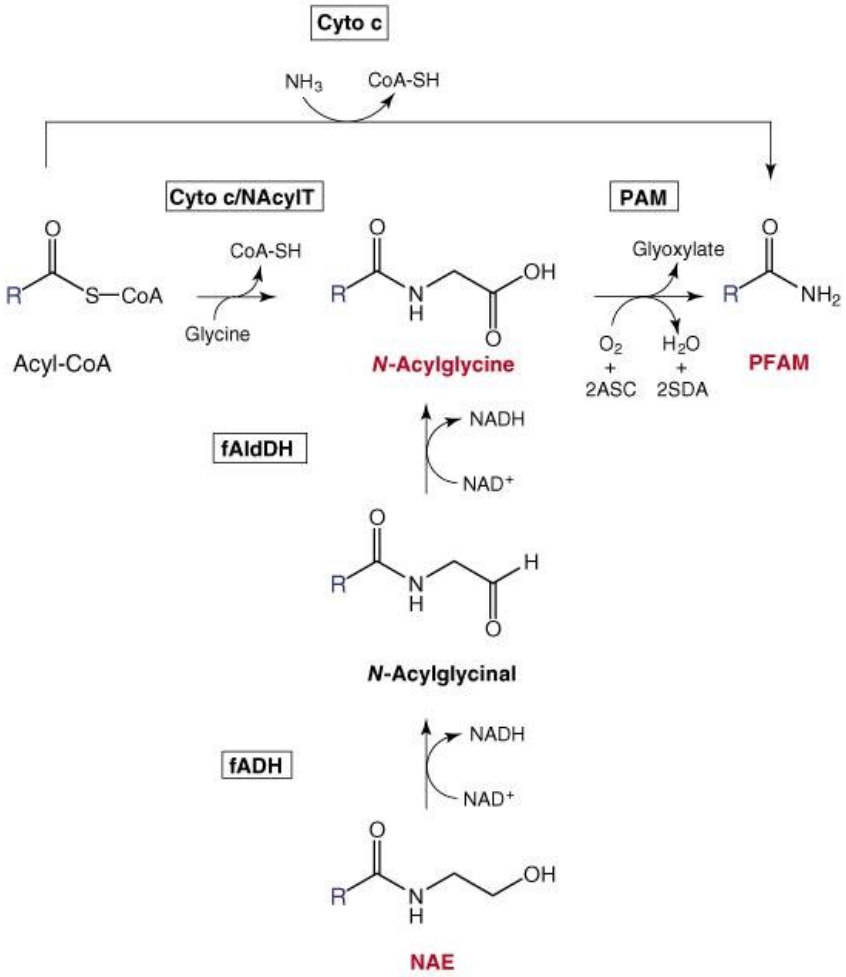


Figure 9. Biosynthesis of fatty acid amides via amidation of Acyl-CoA [42]

The enzymes responsible for conversion of the fatty-CoA to the primary amide are still a matter of debate. It was reported that an enzyme peptidylglycine α-amidating monooxigenase (PAM) converts N-myristoylglycine (C14:0) to the primary enzyme, myristamide [45], which led scientists to assume that PAM could also amidate other fatty glycines to their corresponding amides. Subsequent NMR studies confirmed these assumptions to be true. [38]

Another enzyme that was reported to convert both oleoyl-CoA and oleoyl glycine to oleamide is cytochrome c [46]. Bile acid CoA:amino acid N-acyl transferase (BAAT) has also been found to catalyze conversion of fatty – CoA substrates to fatty glycines [47].

2.4.1. Chemical synthesis of fatty acid amides

2.4.1.1. Chlorinating agents

Synthesis of amides is most often carried out via acid derivatives with a good leaving group, which are converted into the amide upon aminolysis.

The formation of amides from acid chlorides and amines, known as Schotten – Baumann reaction, is performed in a two-phase system. The acid chloride is usually synthesized by reacting a carboxylic acid with a chlorinating agent, such as thionyl chloride or phosphorous trichloride followed by aminolysis. [48]

Coupling of halogenating agents with PPh3 has been reported as an effective way for conversion of fatty acid esters and their derivatives into fatty acid amides. [49]

Xu et al [50] reported a synthesis of 9(*Z*)-octadecenamide and 9(*Z*),12(*Z*)-octadecadienamide from solution of corresponding free fatty acids in CH_2Cl_2 with oxalyl chloride at room temperature for four hours. The obtained acid chlorides are treated with saturated aqueous NH_4OH at 0ºC and amides are extracted with EtOAc.

Cheng et al [51] used the same procedure as previous for the synthesis of oleamide, but instead of using aqueous NH_4OH, they reported using gaseous ammonia.

2.4.1.2. Synthesis using catalysts

Heterogeneous catalysts offer many advantages such as selectivity, easy workup, nonhazardous nature and usage in catalytic amounts. Additionally, they present an environmentally friendly approach to chemical synthesis, as they can be easily separated and reused. [52]

Nano sulfated titanium – direct amidation of fatty acids in the absence of coupling agents and solvents. Stearic acid was treated with aniline for three hours, at 115ºC with catalytic amount of nano sulfated TiO_2. The yield of stearamide was reported to be 98%. [52]

Several metal-based homogeneous catalysts are known to catalyze the hydration of nitriles to primary amides, such catalysts based on ruthenium [53], iridium [53], rhodium [54] and gold [55]. These reactions are usually done at elevated temperatures. [48]

2.4.1.3. Enzyme catalyzed amide synthesis

Lipase SP525 immobilized on Accurel EP 100 (lipase from *Candida antarctica* B immobilized on a macroporous acrylic resin) was reported to convert oleic acid to oleamide in the presence of n-butyl alcohol and t-amyl alcohol. The reaction is performed in two stages; first stage is esterification at 60°C under vacuum for 24 hours followed by saturation with ammonia for 96 hours at 60°C. After the reaction, the enzyme is filtered off and solvent evaporated. Oleamide is re-crystallized from hexane, with a total isolated yield of 90% (84% purity). [56]

2.4.2. Identification and quantification of fatty acid amides

Several published papers investigated different techniques for the separation of different fractions of fat. Some of them also reported separation and determination of fatty acid amides from various samples. Bilyk et al [57] developed a method for separation of neutral lipids using thin-layer chromatography. In addition, a separation of amides from other components was reported. Samples applied on TLC plates are developed in two phases; in the first phase, a solvent consisting of toluene: diethyl ether: ethyl acetate: acetic acid (75:10:13:1.2 v/v) is used, followed by development of plates with a second solvent consisting of hexane: diethyl ether: formic acid (80:20:2 v/v). Palardy et al [58] developed a method for identification and quantification of fatty amides in crude oil obtained from algal biomass. The method consists of fractionation of lipid class groups by solid phase extraction (SPE). The amides are isolated with the neutral fraction of the oils, which is separated from the sample using chloroform: isopropanol (2:1 v/v), followed by the elution of fatty acids with diethyl ether: acetic acid (98:2 v/v) and elution of polar lipids with methanol: chloroform (6:1 v/v) and methanol: ethyl acetate (6:1 v/v). Amides are identified via GC-MS and quantified via GC-FID.

Separation and quantification of fatty acid amides so far has been based on paper by Madl and Mittelbach “Quantification of primary fatty acid amides in commercial tallow

and tallow fatty acid methyl esters by HPLC-APCI-MS" [4]. In this method, the sample is first separated on C18 SPE column. The triglycerides are eluted with 120 ml of hexane, followed by elution of primary fatty acid amides with 120 ml of tetrahydrofuran. Isolated amides are then quantified using HPLC.

In praxis, this method was modified. Previous to separation, esterification of free fatty acids using methanolic boron trifluoride solution is performed. With the addition of heptane, the solution is divided in organic and inorganic phase. The organic phase is applied on Silica column and eluted with previously reported eluents. Isolated amides are quantified using GC-FID.

The basis of this work was a published paper from Sultana and Johnson [59] "Sample preparation and gas chromatography of primary fatty acid amides". This method is based on the isolation of primary fatty acid amides using SPE and quantification by GC-MS. The lipid extract is first separated on Silica SPE column with following elution solutions:

Table 3. Elution solutions for Si SPE column

Eluent	*Quantity (ml)*
Hexane	5
Hexane: acetic acid (99:1)	5
Hexane: ethyl acetate (90:10)	5
Hexane: ethyl acetate (80:20)	5
Hexane: ethyl acetate (70:30)	5
Chloroform: 2-propanol (2:1)	7
Methanol	2

The amides are contained in the fraction six (chloroform: 2-propanol (2:1)). This fraction is then separated on NH_2 column using following eluents:

Table 4. Eluting solutions for NH_2 SPE column

Eluent	*Quantity (ml)*
Chloroform	2
2% 2-propanol in chloroform	2
2% 2-propanol in chloroform	1
3% 2-propanol in chloroform	3
Methanol	2

Fraction three was found to contain only amides.

3. Experimental part

3.1. Chemical, raw materials and instrumentation

3.1.1. Technical instrumentation

GC-MS:

Device	Agilent 5973 GC System
Inlet	Split inlet (split ratio 40:1)
Column	DB 5 MS
	30 m X 250 µm x 0.25 µm

GC-FID:

Device	Agilent 6850 GC System
Inlet	Manual injection
	Split inlet (split ratio 40:1)
Column	HP-5MS 5% phenyl methyl siloxane
	30 m X 250 µm x 0.1 µm

SPE Column:

Phenomenex Strata SI-1 Silica
1g/ 6 ml tubes
Pore size (Å): 60
Average particle size (µm): 56

Isolute C18 (EC)
2 g/ 6 ml tubes
Pore size (Å): 68
Average particle size (µm): 51

Rotary evaporator: VWR IKA RV 10 digital; VWR IKA HB10 digital heating bath; Büchi Rotavapor R-114

Oven: Heraeus electronic T 5042 5

Vacuum pump: Vacuubrand MD 4C; Vacuubrand MZ 2C

Ultrasonic bath: Elma Transsonic T460/H

3.1.2. Solvents, chemicals and gasses

Table 5. Used solvents

Solvent	*Producer*	*Purity*
Acetic acid	VWR Chemicals; France	99.90%
Chloroform	VWR Chemicals; France	99.20%
Diethyl ether	VWR Chemicals; France	Technical
Ethanol	VWR Chemicals; France	99.00%
Ethyl acetate	VWR Chemicals; France	99.80%
Hexane	Fischer Chemicals; UK	HPLC
Isopropanol	VWR Chemicals; France	100%
Methanol	Fischer Chemicals; UK	99.80%
Petrolether	Carl Roth Gmbh; Germany	n.a.
Tetrahydrofuran	Fischer Chemicals; UK	HPLC

Table 6. Used chemicals

Chemicals	*Producer*	*Purity*
Ammonia solution	E. Merck; Germany	25%
Heptadecanoic acid	Acros Organics; USA	n.a.
Iodine	Institute of Chemistry	n.a.
Ion exchange resin (strong basic, Cl^- - type)	E. Merck, Lewatit M 5020; Germany	n.a.

Nonadecanoic acid	Sigma - Aldrich Chemie Gmbh; Germany	98%
Oleic acid	Sigma - Aldrich Chemie Gmbh; Germany	90%
Palmitic acid	Fluka AG; Switzerland	n.a.
Phenolphthalein	Sigma - Aldrich Chemie Gmbh; Germany	
Potassium hydroxide	VWR Chemicals; France	99%
Stearic acid	Fluka AG; Switzerland	n.a.
Thionyl chloride	E. Merck; Germany	99%

Table 7. Used gasses

Gas	*Producer*	*Purity*
Ammonia	Messer; Austria	5.0
Compressed air	Messer; Austria	5.0
Helium	Messer; Austria	5.0
Hydrogen	Messer; Austria	5.0
Nitrogen	Messer; Austria	5.0

3.1.3. Raw materials

Table 8. Used raw materials

Sample name	*Company*	*Collection date*
December	Argent Energy; Austria	08.03.2018
January	Argent Energy; Austria	08.03.2018
February	Argent Energy; Austria	08.03.2018
Fraction I	BDI; Austria	26.03.2018
Feed	BDI; Austria	26.03.2018
CAT2	Rendering plant	2017

Lard	Schachinger; Austria	2017
Rapeseed oil	Spar; Austria	04.2018

3.2. Synthesis of amides

Chemical synthesis of fatty acid amides was performed according to synthetic method reported in a paper by Madl and Mittelbach [4].

Prior to synthesis, thionyl chloride was distilled to remove any possible impurities. The first step is the synthesis of acid chloride by refluxing the corresponding fatty acid with tenfold excess of thionyl chloride as halogenating agent, at 85°C for one hour. After the reaction, any excess of thionyl chloride was distilled on a rotavapor. THF was added to fatty acid chloride and the solution was treated with a 25% solution of ammonium hydroxide in THF under ice cooling. After addition of water, the obtained amide was filtered, washed with water and dried. The purity of the amides was confirmed by GC-FID.

3.3. Sample preparation

All the analyzed samples consisted of some fatty matrix (rapeseed oil, lard…) were spiked with fatty acid amides. First, the amide is dissolved in THF [c = 1mg/ml]. An appropriate amount of this solution is added to the matrix that is being analyzed to obtain the desired concentration of amide in the matrix. THF is then completely removed from the sample by evaporation on rotavapor until the constant mass of the sample is obtained.

All the samples were kept in the oven at 60°C between measurements to prevent the amides from precipitating.

3.4. Sample: Rapeseed oil with 0.1% of C19 amide.

3.4.1. Solid Phase Extraction

1 g of sample was dissolved in 10 ml of hexane, loaded on the SPE column and eluted with the following eluents:

Table 9. Starting eluting solutions

Fraction	*Eluent*	*Quantity [ml]*
Zero	Hexane (preconditioning)	10
I	Hexane + sample (loading)	10
II	Hexane (washing)	5
III	Hexane: acetic acid (99:1)	5
IV	Hexane: ethyl acetate (90:10)	5
V	Hexane: ethyl acetate (80:20)	5
VI	Hexane: ethyl acetate (70:30)	5
VII	Chloroform: 2-propanol (2:1)	7

3.4.2. Thin layer chromatography

Thin layer chromatography (TLC) is used to separate mixtures into their components. In this experimental work, TLC was a method of choice for evaluating the influence of different solvents on the material that is being analyzed, because it is simple, fast, and many different solvents can be analyzed at the same time.

To compare the efficiency of different eluents on removal of different components from sample, a series of TLCs were performed with eluates collected from SPE separation.

In the first experiment, the sample was eluted with hexane, and in the second, with hexane: ethyl acetate in different proportions.

- Stationary phase: sheets coated with silica gel

- Mobile phase: petrol ether: diethyl ether: acetic acid (70:30:1)
- Detection: Iodine

The sample was eluted with 5 ml aliquots of eluents. Each aliquot was applied on the silica plate in the same amount using glass capillary, dried and developed in a chamber containing the mobile phase.

Additionally, the necessity of using hexane: acetic acid (99:1) as an eluent was evaluated by comparing chromatograms from experiments performed with and without this eluent.

In the first experiment, the sample was eluted with the following eluents:

Table 10. Eluting solutions

Fraction	*Eluent*	*Quantity [ml]*
I	Hexane	5
II	Hexane: acetic acid (99:1)	5
III	Hexane: ethyl acetate (70:30)	20
IV	Hexane: ethyl acetate (70:30)	5
V	Chloroform: 2-propanol (2:1)	7

Subsequently, the same experiment was performed two times with the following eluents:

Table 11. Eluting solutions

Fraction	*Eluent*	*Quantity [ml]*
I	Hexane: ethyl acetate (70:30)	20
II	Hexane: ethyl acetate (70:30)	5
III	Chloroform: 2-propanol (2:1)	7

3.4.3. Gas Chromatography

The sample and all the fractions collected from SPE were analyzed on GC-FID and/or GC-MS.

Operating conditions:

- Injection volume: 1µl
- Column temperature program: 50°C hold for 3 minutes; programmed at 15°C up to 310°C; final temperature hold for 10 minutes
- Carrier gas flow: 1.2 ml/min

3.5. Recovery of amides

As a sample, rapeseed oil with 0.15% of C18 amide was prepared, as described in 4.3. 1 g of sample was dissolved in 10 ml of hexane, applied to the preconditioned column and eluted with the following eluents:

Table 12. Final eluting solutions

	Eluents	*Quantity [ml]*
1.	Hexane: ethyl acetate (70:30)	20
2.	Chloroform: 2-propanol (2:1)	7

The second eluting fraction was evaporated and the residue dissolved in 10 ml of THF. C17 amide was added as a reference and the samples were analyzed using GC-FID.

The experiment was conducted two times.

3.6. Sample: Lard

To investigate the influence of eluting solutions on separation of amides from the animal fat, lard was used as a matrix. Initially, to make sure that there are no amides in the lard, the content of amides in the pure lard was determined using the previously

described method. 1 g of lard was diluted in 10 ml of hexane, loaded on the SPE column and eluted with previously established eluting solutions. The second fraction was evaporated, dissolved in THF and analyzed via GC-FID and GC-MS.

Additionally, the content of free fatty acids in lard was determined by titration. 0.5 g of sample was dissolved in 100 ml of ethanol: diethyl ether (1:1) and titrated with 0.1M KOH in ethanol. To ensure that all the amount of titrant was used to neutralize the free fatty acids in the sample, the solvent was previously neutralized with KOH/EtOH with phenolphthalein as an indicator.

Table 13. Amounts of sample and titrant used for titration

Experiment	*m (sample) [g]*	*V (KOH/EtOH) [ml]*
1	0.5182	0.20
2	0.5131	0.18

3.6.1. Recovery of amides from lard

Lard spiked with 0.15% of C18 amide was prepared the same way as it is described in 4.3. 1g of the sample was dissolved in 10 ml of hexane and eluted on Si SPE column. The second fraction from SPE column was evaporated and C19 amide was added as a reference. The samples were analyzed with GC-FID.

To ensure that reproducible results are obtained, five consecutive experiments were performed with the same sample.

Table 14. Weights of sample and C19 amide for calculation of recovery of amides from lard

Experiment	*m (sample) [g]*	*m (C19 amide) [g]*
1	1.0177	0.0018
2	1.0080	0.0019
3	1.0101	0.0021

4	1.0076	0.0020
5	1.0086	0.0022

3.6.2. Sample: Lard with high amount of free fatty acids

To assess the behavior and potential interference of free fatty acids during separation and measurement of amides from the sample, lard spiked with 0.15% of C18 amide was infused with different amounts of oleic acid and eluted on a SPE column as previously described.

Table 15. Samples with different concentrations of oleic acid

Experiment	*Lard [g]*	*C18a (1mg/ml) [ml]*	*m (oleic acid) [g]*	*c (oleic acid in sample) [%]*
1	5.01	10	1.00	20.0
2	3.01	6	0.52	17.0
3	3.02	6	0.46	15.0
4	3.04	6	0.31	10.0
5	3.11	6	0.15	5.0
6	3.04	6	0.08	3.0
7	2.39	4	0.00	1.5

3.6.3. Residual amides on SPE column

After elution of amides from the sample with 20% of oleic acid with 7 ml of chloroform: 2-propanol, two samples were further eluted with additional 5 ml of chloroform: 2-propanol, to verify whether some amides were still left on the column.

The additional fraction was evaporated, the residue was dissolved in THF, and after the addition of C19 amide as a reference, analyzed on GC-FID.

3.6.4. Reduction of sample amount and anion exchange resin

Because the recovery of amides from the sample with high amount of oleic acid was evidently much lower than from sample without added oleic acid, two different approaches were carried out:

- Reduction of the amount of sample that was loaded on the SPE column (from 1 g to 0.5 g). In this case, the sample was eluted with reduced amounts of eluents (10 ml of hexane: ethyl acetate and 5 ml of chloroform: 2-propanol), and the evaporated second fraction was dissolved in 5 ml of THF.

- Addition of ion exchange resin on the top of the SPE column before the extraction (approximately 1.5 g of anion exchange was placed on top of the column, the column was equilibrated with hexane, loaded with sample and eluted with eluents)

3.7. Sample: Rapeseed oil methyl ester with 0.2% C18 amide

Rapeseed oil methyl ester was mixed with 0.2% of C18 amide, as described in chapter 2.3. 0.5 g of the sample was dissolved in 10 ml of hexane and applied on Silica and C18 SPE columns. The sample applied on Silica column was eluted with hexane: ethyl acetate (70:30), followed by chloroform: 2-propanol (2:1). The sample applied on C18 column was eluted with 120 ml of hexane, followed by 120 ml of THF.

The second fractions from both columns were evaporated, dissolved in 5 ml of THF, and C19 amide was added as a reference. The samples were analyzed on GC-FID.

3.8. Determination of amides in real samples

Six different samples, which were acquired from companies and already tested for content of amides with the existing method described in the introduction (in further content referred to as “old method”), were analyzed again for content of amides using the method developed in this research work (in further content referred to as “new method”).

All the samples were prepared with C19 amide as a reference, as described in section 2.1.

Table 16. Weights of reals samples for determination of amides

Sample	*Experiment*	*m (sample) [g]*	*C19a (1mg/ml) [ml]*
Fraction 1	I	1.01	0.0018
	II	1.00	0.0027
Feed	I	1.00	0.0025
	II	1.01	0.0019
December	I	1.03	0.0020
	II	1.02	0.0020
January	I	1.06	0.0020
	II	1.01	0.0020
February	I	1.00	0.0020
	II	1.01	0.0020
CAT2	I	1.02	0.0020
	II	1.01	0.0020

3.9. Elution of amides from the reduced sample amount

All six samples were analyzed again using 0.5 g of sample, with regular amount of elution solutions (20 ml of hexane: ethyl acetate (70:30) and 10 ml of chloroform: 2-propanol (2:1)). For each sample, three experiments were performed, with three injections for each experiment.

Table 17. Reduced weights of samples for determination of amides

Sample	*Experiment*	*m (sample) [g]*	*C19a (1mg/ml) [ml]*
Fraction 1	I	0.50	0.001
	II	0.50	0.001
	III	0.51	0.001
Feed	I	0.50	0.001
	II	0.50	0.001
	III	0.50	0.001
December	I	0.50	0.001
	II	0.51	0.001
	III	0.50	0.001
January	I	0.51	0.001
	II	0.51	0.001
	III	0.51	0.001
February	I	0.50	0.001
	II	0.50	0.001
	III	0.51	0.001
CAT2	I	0.50	0.001
	II	0.51	0.001
	III	0.50	0.001

3.10. Determination of the detection and quantification limit

A series of samples of tallow with different concentrations of C18 amide was prepared. From each sample, 0.5 g was loaded on the Si SPE column and eluted with following eluents:

Table 18. Elution solutions

Fraction	*Eluent*	*Quantity [ml]*
I	Hexane: ethyl acetate (70:30)	20
II	Chloroform: 2-propanol (2:1)	10

Table 19. Tallow with different concentration of C18 amide

Experiment	*m (tallow) [g]*	*m (C18a (1mg/ml) [ml]*	*c (C18a) [%]*
1	2.06	0.004	0.2
2	2.10	0.002	0.1
3	2.10	0.001	0.05
4	0.50	0.0005	0.025
5	0.52	0.0003	0.015
6	0.51	0.00015	0.0075
7	0.51	0.00007	0.0035
8	0.51	0.00002	0.0010
9	0.51	0.00001	0.0005

The second fraction was evaporated, dissolved in THF and analyzed on GC-FID with C19 amide as a reference.

For each concentration, two experiments were performed, with two injections for each experiment.

The detection and quantification limit were calculated from the signal-to-noise ratio from the obtained chromatograms. Detection limit corresponds to three time the noise level, while quantification limit corresponds to 10 times the noise level.

These values were calculated manually on the chromatogram printouts, by measuring the height of the analyte signal peak and noise magnitude around the analyte retention time and dividing the two values.

4. Results and Discussion

4.1. Synthesis of fatty acid amides

Fatty acid amides were synthesized from the corresponding fatty acids, as described in 4.2. These amides were used as standards in all the following experiments, therefore their purity was analyzed with GC-FID.

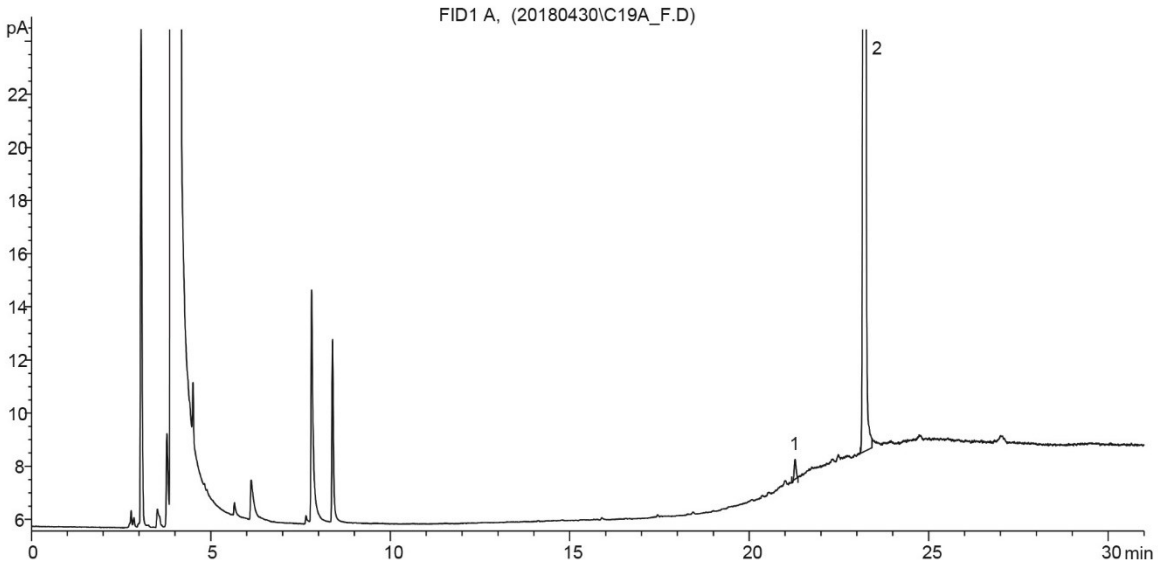


Figure 10. GC-FID of synthesized C19 amide

Table 20. GC-FID results from C19 amide

#	*Time*	*Area*	*Area%*	*Compound*
1	21.284	2.6	0.7	C 19 fatty acid
2	23.208	354.8	99.3	C 19 amide

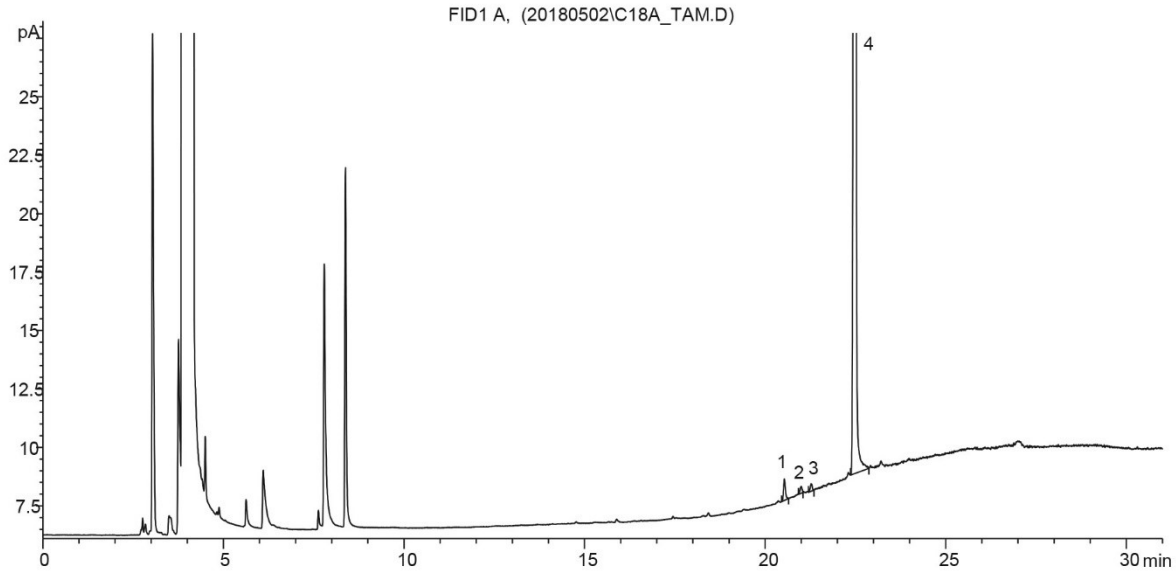


Figure 11. GC-FID of synthesized C18 amide

Table 21. GC-FID results of C18 amide

#	Time	Area	Area%	Compound
1	20.531	3.3	0.7	C18 fatty acid
2	20.999	1.1	0.2	C18 nitrile
3	21.271	1.2	0.3	unknown
4	22.475	445.1	98.8	C18 amide

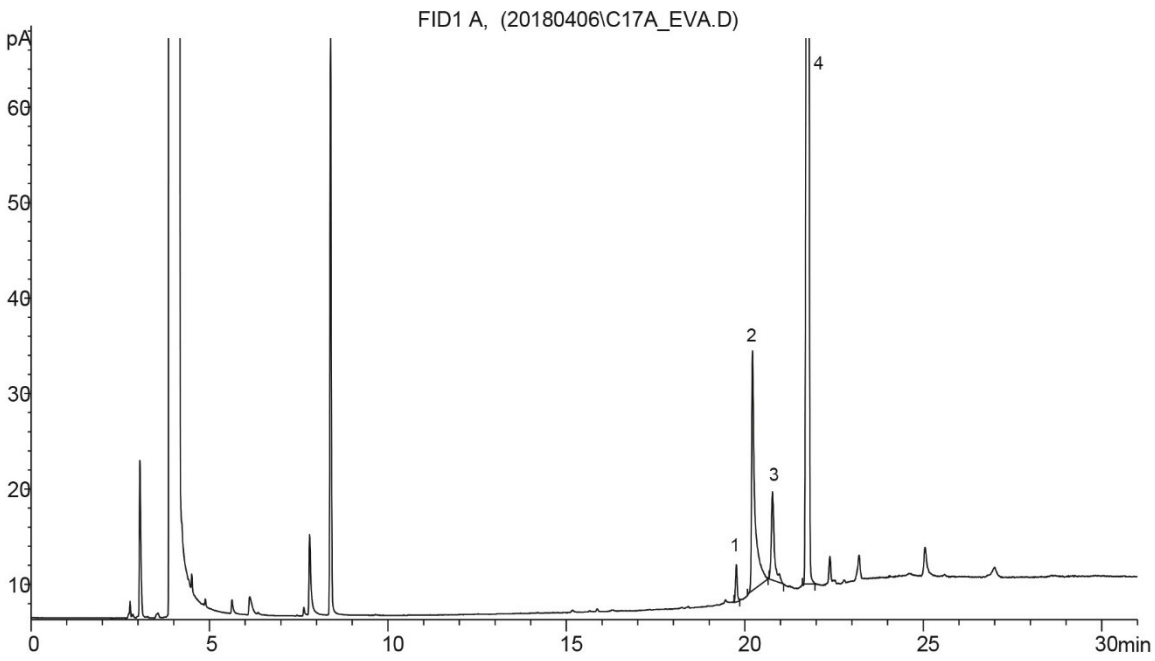


Figure 12. GC-FID of synthesized C17 amide

Table 22. GC-FID results of C17 amide

#	Time	Area	Area%	Compound
1	19.76	13.0	0.8	C17 nitrile
2	20.216	155.7	9.9	C17 fatty acid
3	20.776	54.4	3.5	unknown
4	21.761	1344.2	85.8	C17 amide

The main problem with the synthetized amides was that during time it was observed that they were hydrolyzed to free fatty acids, resulting in lower purity of the standards. Several approaches were made to purify them, all based on the fact that free fatty acids are soluble in hexane, while fatty acid amides are insoluble. Amides were mixed with hexane (both cold and warm) and filtered; amides mixed with hexane (cold and warm) were kept in ultrasonic bath, and then filtered; amides mixed with hexane were filtered with the addition of ion exchange on the filter. Additionally, during amide synthesis, gaseous ammonia was used instead of ammonium hydroxide. However, none of these methods gave satisfactory results. More experimental work is yet to be done regarding the purification of amides.

4.2. Gas Chromatography

The sample (rapeseed oil infused with 0.1% C19 amide) was eluted on SPE with six different eluents, as described in 4.4.1. The sample, as well as all six fractions were analyzed on GC-FID and/or GC-MS.

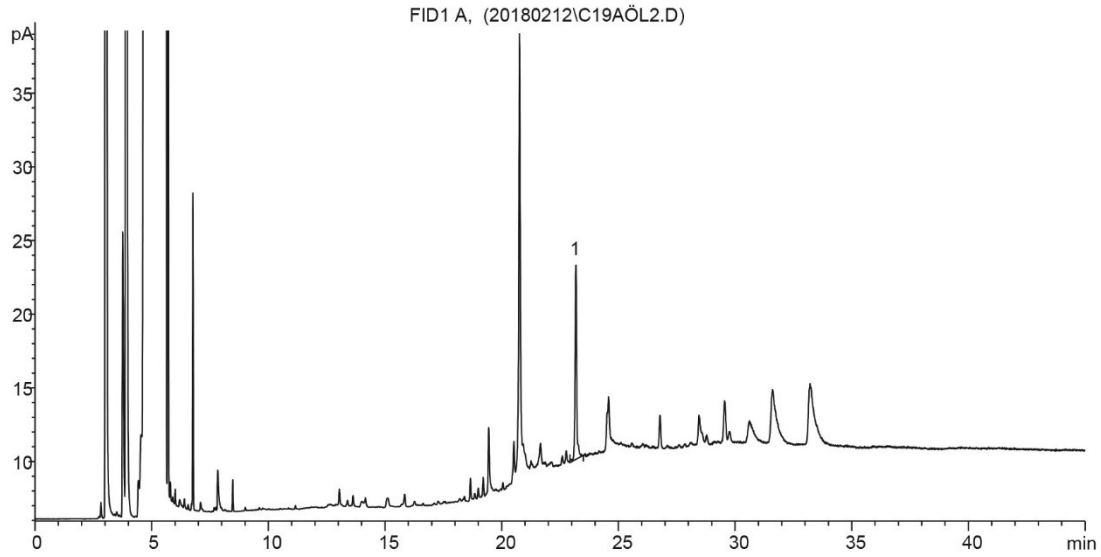


Figure 13. GC-FID of the Rapeseed oil with 0.1% of C19 amide; 1 - C19 amide

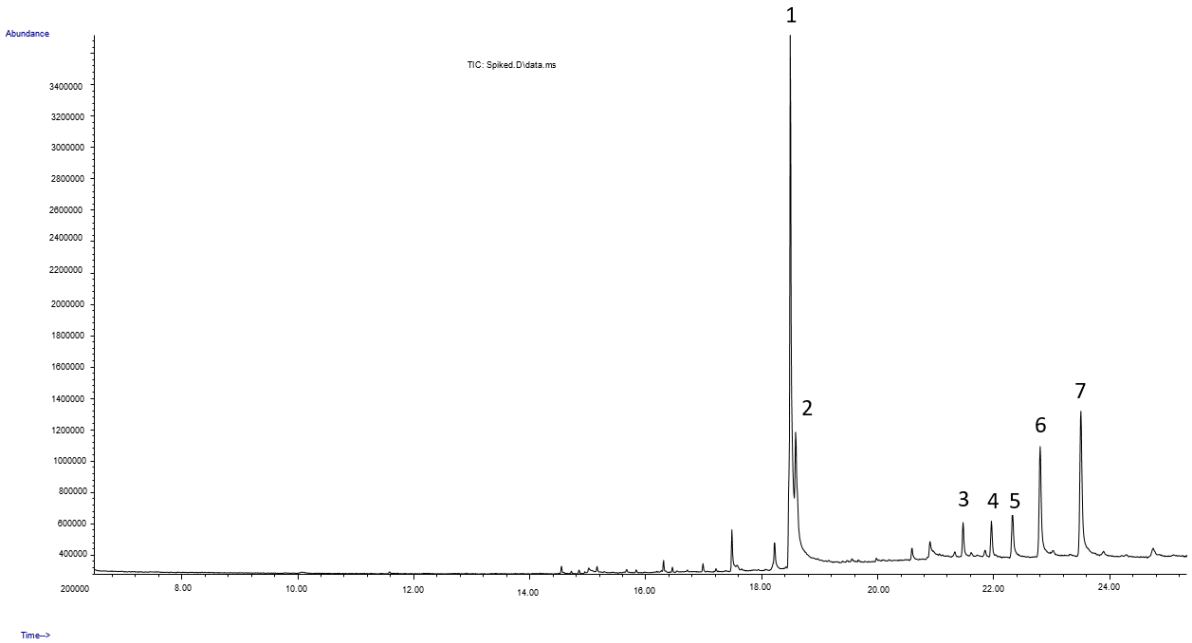


Figure 14. GC-MS of the sample: 1 – 9-octadecenal; 2 – C19 amide; 3 – α-tocopherol; 4 – γ-tocopherol; 5 – brassicasterol; 6 – campesterol; 7 – sitosterol [60]

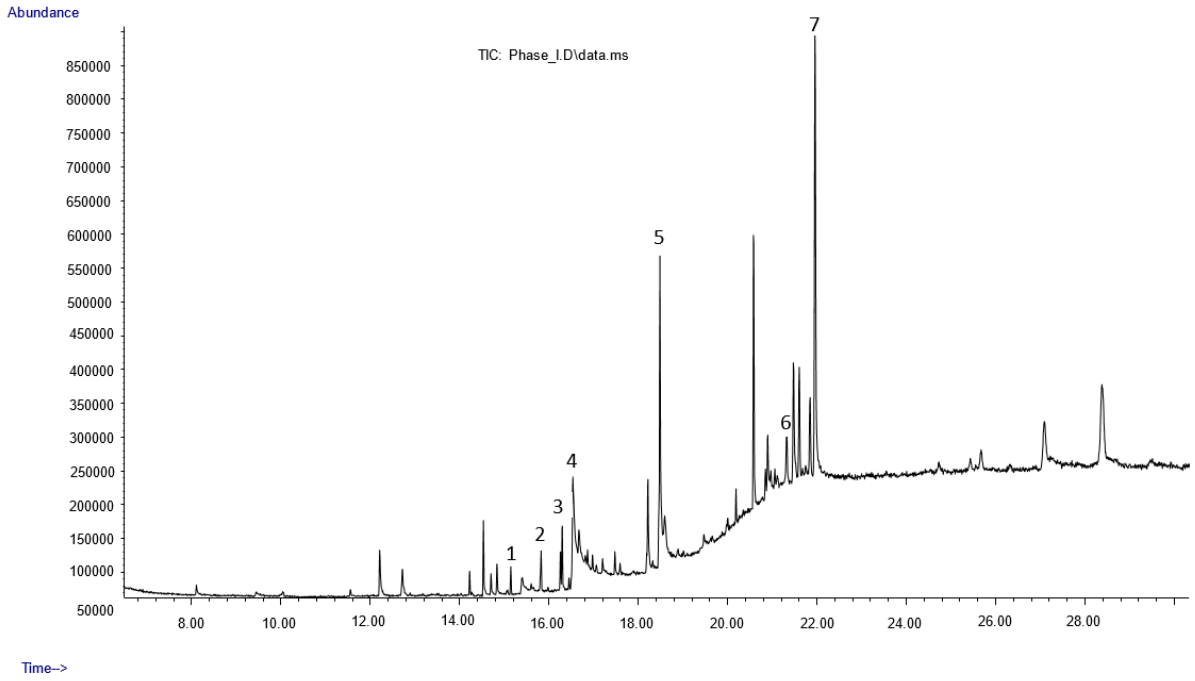


Figure 15. GC-MS of Fraction I (loading the sample on the column): 1 – palmitic acid methyl ester, 2 – E-15-heptadecenal (1-octadecene); 3 – stearic acid methyl ester; 4 – oleic acid; 5 – 9-octadecenal; 6 – α-tocopherol; 7 – γ-tocopherol

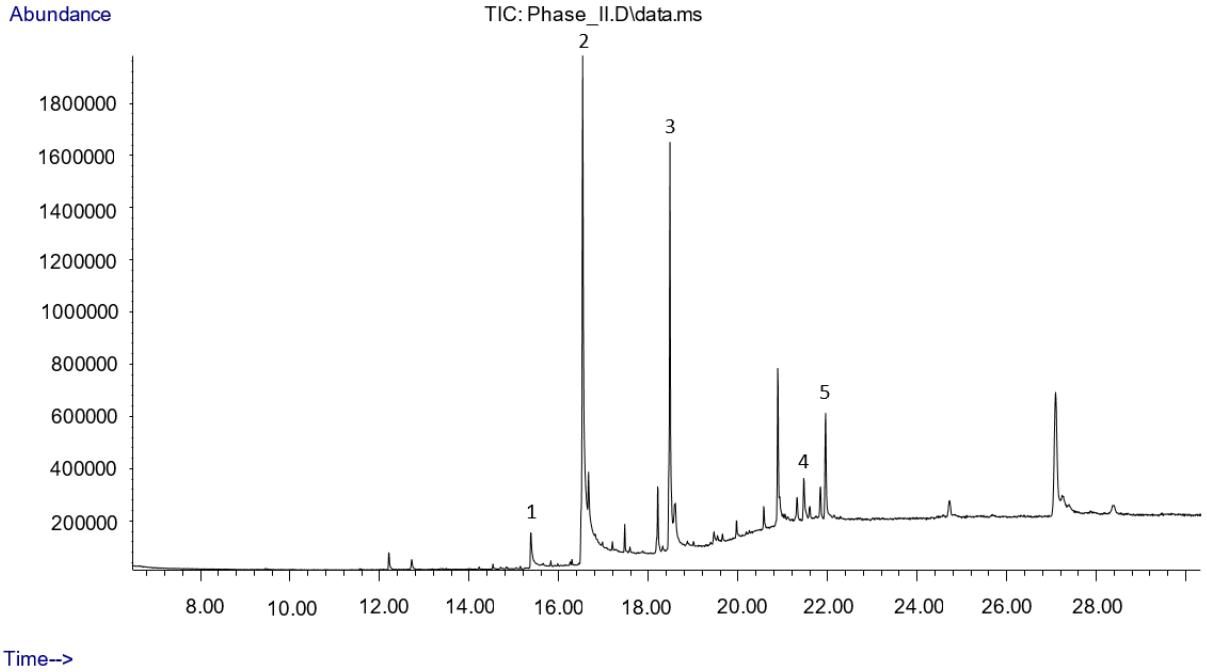


Figure 16. GC-MS of Fraction II (washing of sample with hexane): 1 – palmitic acid; 2 – oleic acid; 3 – 9-octadecenal; 4 – α-tocopherol; 5 – γ-tocopherol

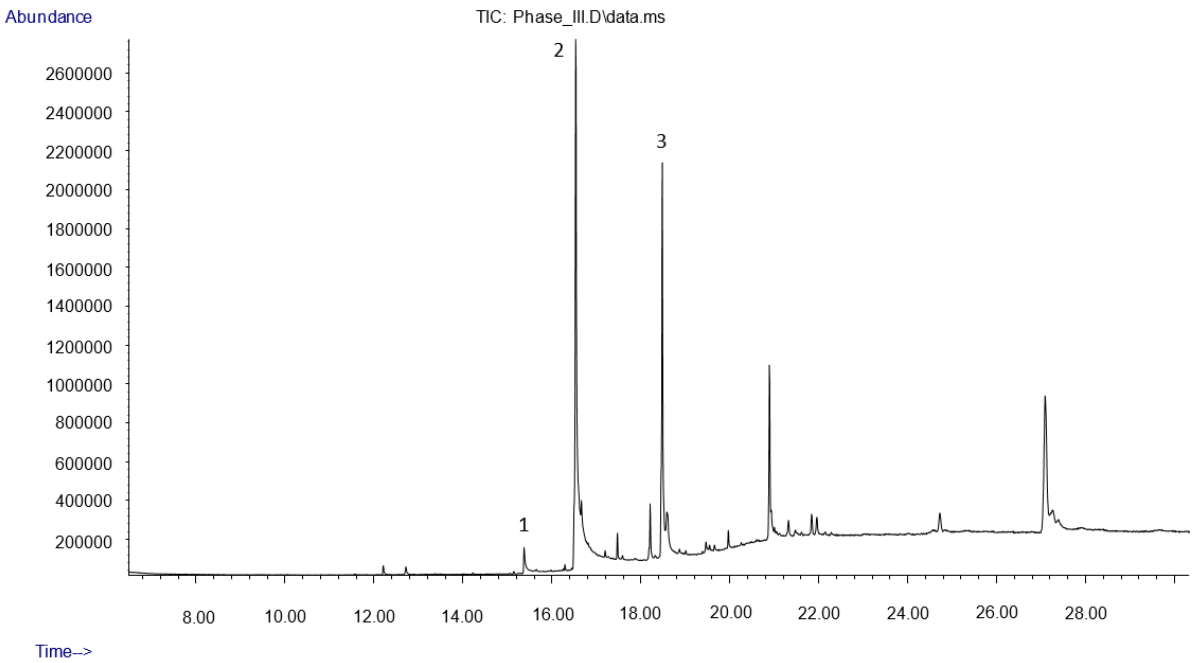


Figure 17. GC-MS of Fraction III (elution of sample with hexane: acetic acid (99:1)): 1 – palmitic acid; 2 – oleic acid; 3 – 9-octadecenal

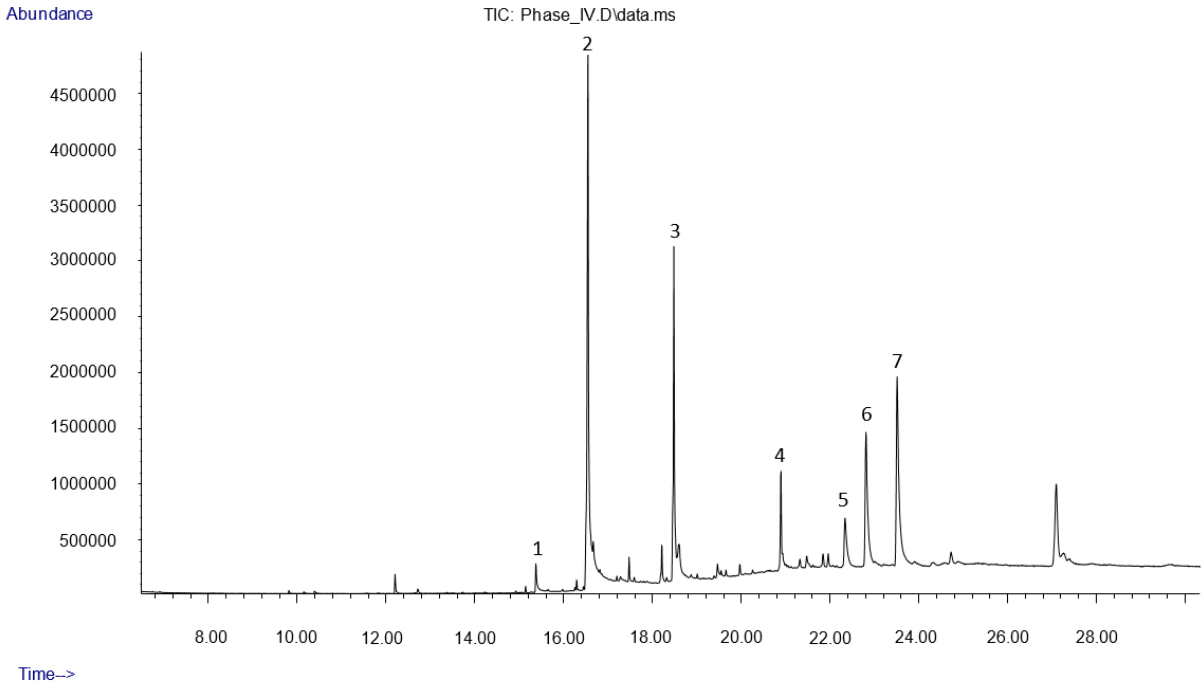


Figure 18. GC-MS of Fraction IV (elution of sample with hexane: ethyl acetate (90:10)): 1 – palmitic acid; 2 – oleic acid; 3 – 9-octadecenal; 4 – monoglyceride; 5 – brassicasterol; 6 – campesterol; 7 – sitosterole

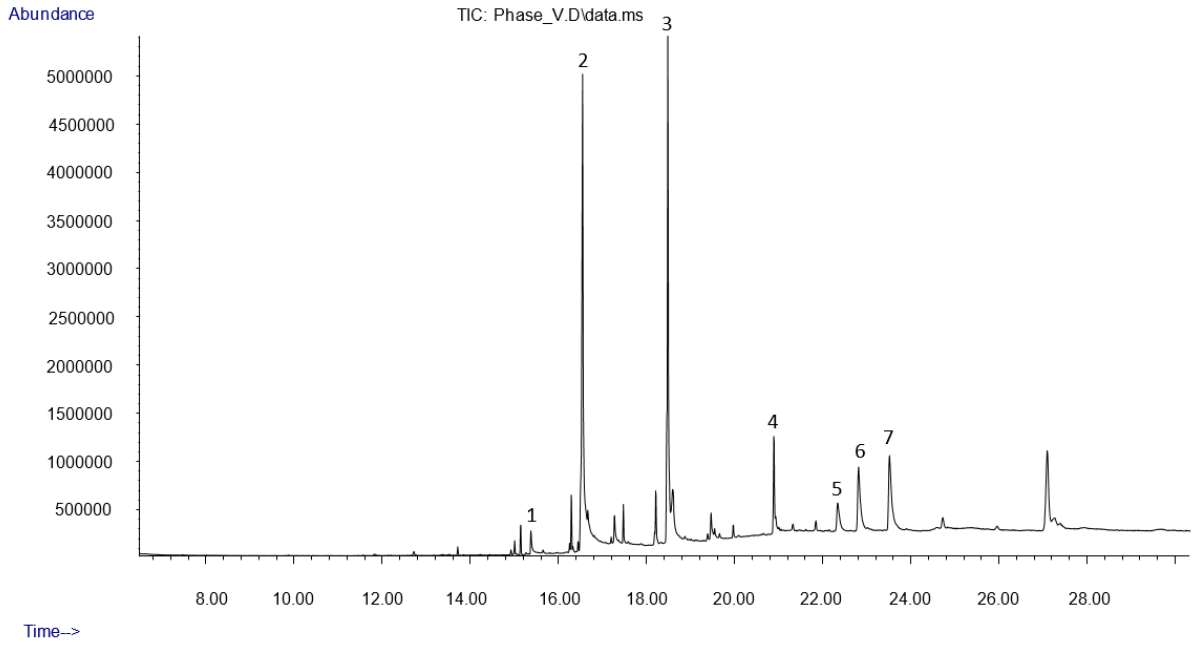


Figure 19. GC-MS of Fraction V (elution of sample with hexane: ethyl acetate (80:20)): 1 – palmitic acid; 2 – oleic acid; 3 – 9-octadecenal; 4 – monoglyceride; 5 – brassicasterol; 6 – campesterol; 7 – sitosterole

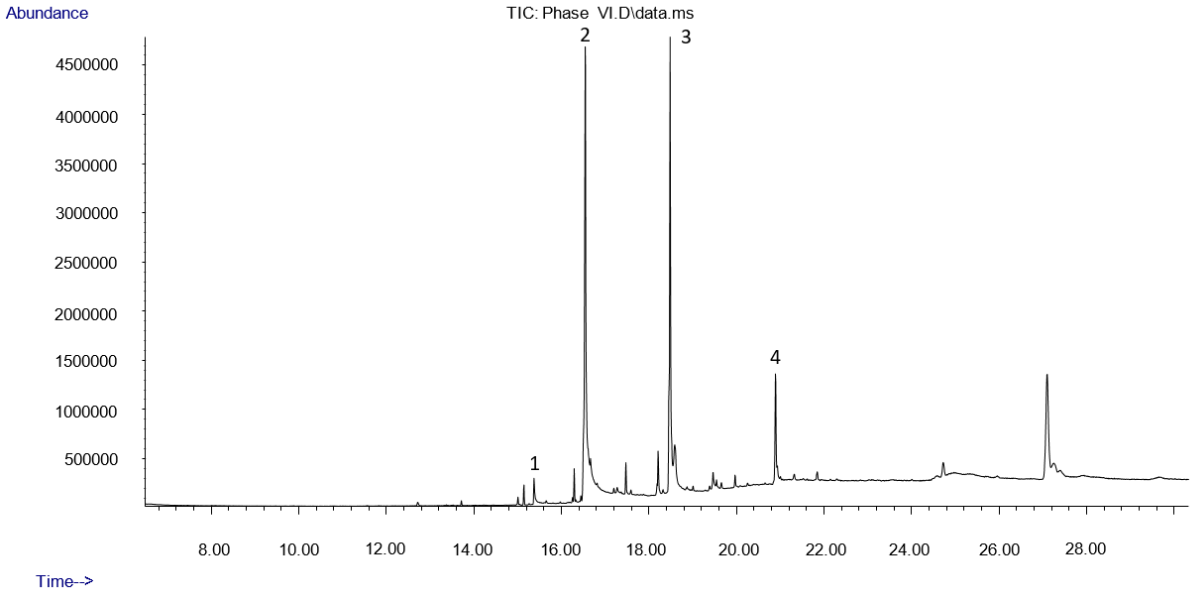


Figure 20. GC-MS of Fraction VI (elution of sample with hexane: ethyl acetate (70:30)): 1 – palmitic acid; 2 – oleic acid; 3 – 9 - octadecenal; 4 – monoglyceride

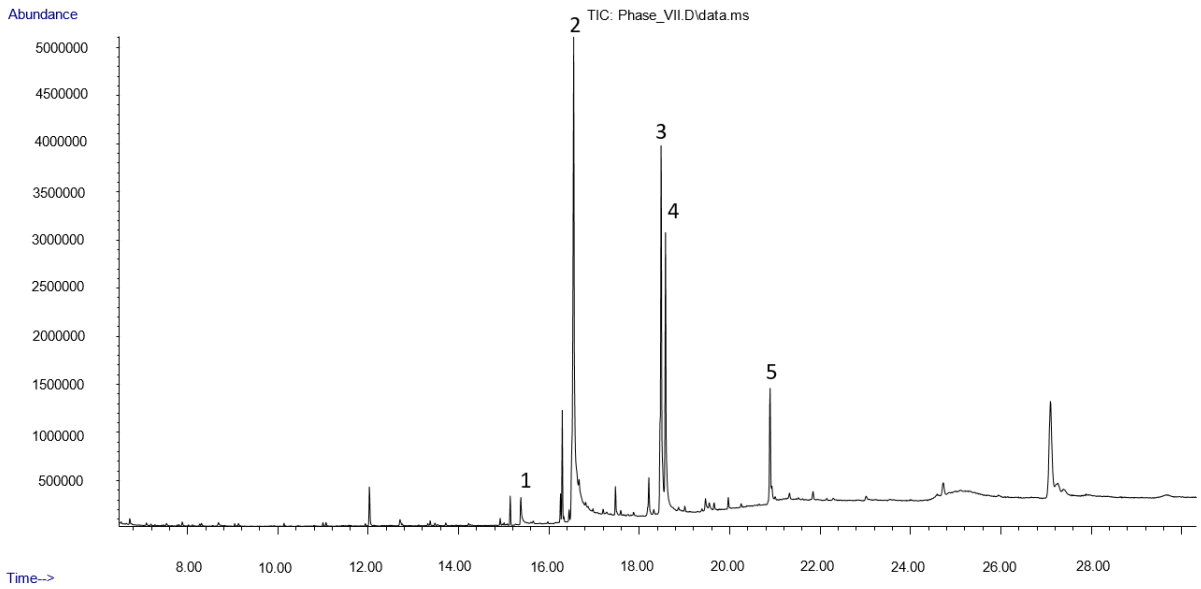


Figure 21. GC-MS of Fraction VII (elution of sample with chloroform: 2-propanol (2:1)): 1 – palmitic acid; 2 – oleic acid; 3 – 9 - octadecenal; 4 – C19 amide; 5 – monoglyceride

Analyzing the chromatograms from GC-MS of the fractions, it can be concluded that most of the components from the rapeseed oil are eluted with the eluent hexane: ethyl acetate. The predominant component in all the fractions, as it was expected, is oleic acid. C19 amide was eluted with chloroform: 2-propanol (2:1), and it is not present in any of the previous fractions.

4.3. Thin Layer Chromatography

The efficiency of different eluents on separation of different components from the sample was examined using TLC. In the first experiment, rapeseed oil infused with 0.1% C19 amide was eluted with 5 ml aliquots of hexane using SPE.

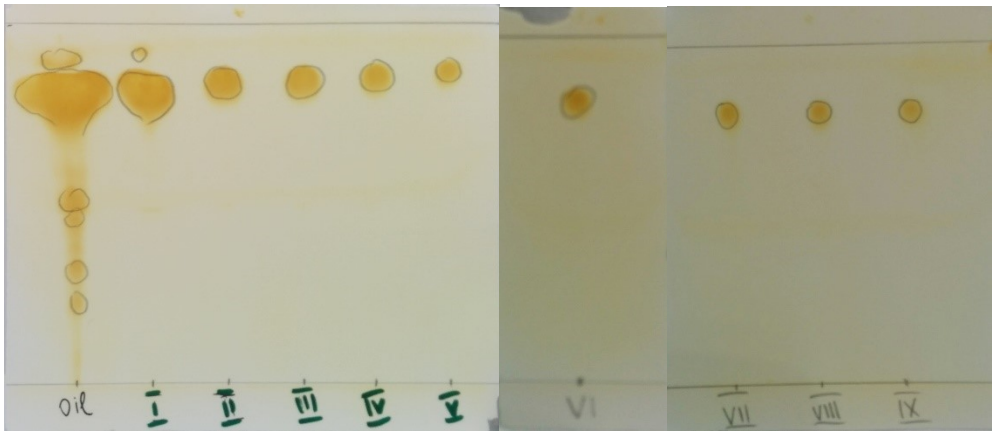
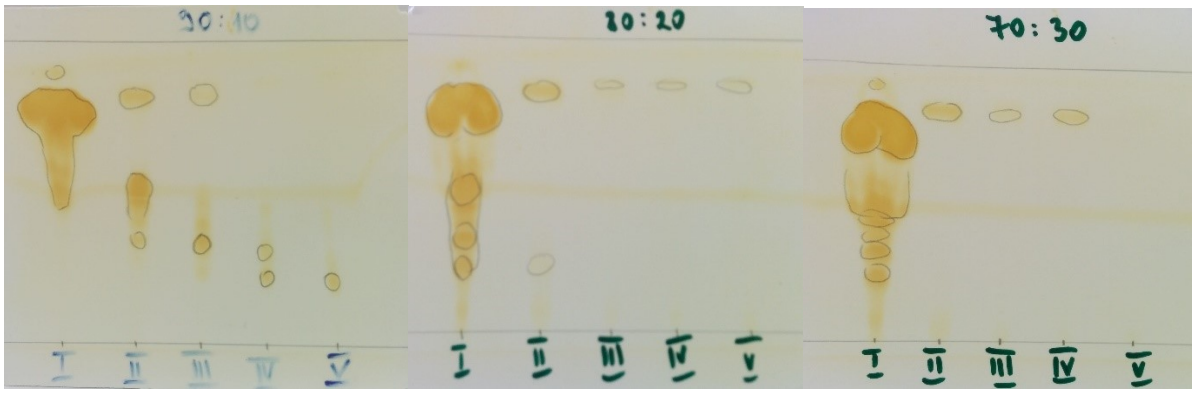


Figure 22. Elution of sample with hexane

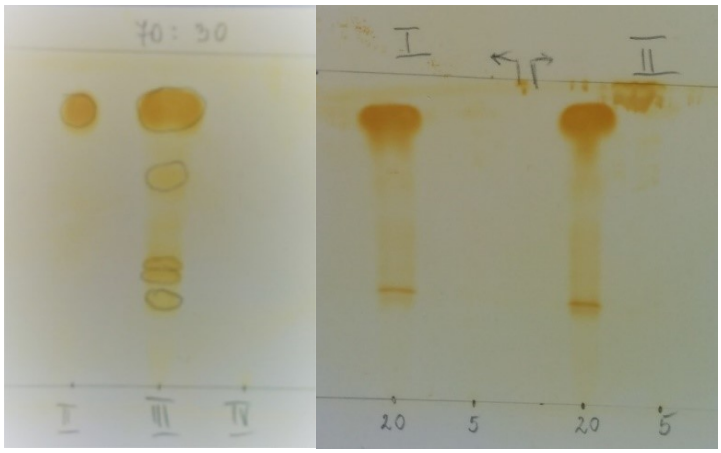
In the second experiment, the same sample was eluted with hexane: ethyl acetate in different proportions (90:10, 80:20, 70:30). Again, 5 ml aliquots from the SPE from each eluting solution were developed on TLC plate.



a) *b)* *c)*

Figure 23. Elution of sample with hexane: ethyl acetate; a) Hexane: ethyl acetate (90:10); b) Hexane: ethyl acetate (80:20); c) Hexane: ethyl acetate (70:30)

The two experiments above undeniably show that elution of different components from the sample is much more effective with hexane: ethyl acetate (70:30) compared to elution with just hexane. After eluting the sample with 45 ml of hexane (Figure 11.), there were still triglycerides present in the sample. Comparing different proportions of hexane: ethyl acetate, it is evident that 20 ml of hexane: ethyl acetate (70:30) completely removed the triglycerides and other minor components (vitamins, pigments…), while first two eluents were not that effective (Figure 12.).



a) b)

Figure 24. Elution of sample with hexane: acetic acid (99:1) a) and without b)

The eluent hexane: acetic acid (99:1) does remove some of the triglycerides, as it is observed in Figure 13. a). However, the Figure 13. b) shows that the same effect is obtained without this eluent; after elution with 20 ml of hexane: ethyl acetate (70:30) all triglycerides and other components were completely removed from the sample. From this, it can be concluded that eluting solution hexane: acetic acid (99:1) is unnecessary. Therefore, the final eluting solutions are as followed:

Table 23. Eluting solutions

	Eluents	*Quantity [ml]*
1.	Hexane: ethyl acetate (70:30)	20
2.	Chloroform: 2-propanol (2:1)	10

4.4. Recovery of amides

Rapeseed oil infused with 0.15% C18 amide was eluted with 20 ml of hexane: ethyl acetate (70:30) and 7 ml of chloroform: 2-propanol (2:1). The second eluting fraction was evaporated, dissolved in THF, and after the addition of C17 amide as reference, analyzed with GC-FID. Two experiments were performed with the same sample.

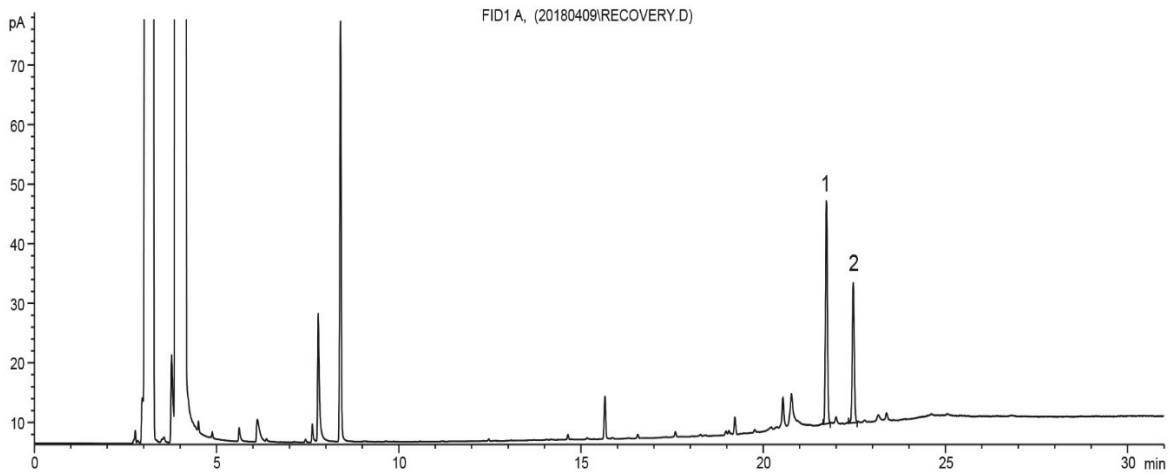


Figure 25. GC-FID of the second eluting fraction of rapeseed oil with 0.15% of C18 amide; C17 amide was used as a reference; 1 – C17 amide; 2 – C18 amide

Table 24. Summarized results obtained with GC-FID of amide recovery from rapeseed oil

Experiment	*m (sample) [g]*	*m (C18 amide) [g]*	*m (C17 amide) [g]*	*Area of C17 amide*	*Area of C18 amide*	***Recovery of C18a [%]***
1	1.01	0.0014	0.0025	199.1	144.6	**114**
2	1.00	0.0014	0.0023	192.5	141.5	**107**

In both experiments, the recovery of C18 amide was above 100%, which indicates that the entire amount of amides that was loaded on the column with the sample was eluted with the second eluting fraction.

4.5. Experiments with lard

4.5.1. Content of free fatty acids and amides

The content of free fatty acids in lard was determined by titration with 0.1 M ethanolic KOH. The experiment was conducted two times.

Table 25. Content of free fatty acids in lard

Experiment	*Free fatty acid content [%]*
Experiment I	1.06
Experiment II	0.96

Lard used for further experiments had approximately 1% of free fatty acids. Additionally, GC-FID and GC-MS showed no amides in lard.

4.5.2. Recovery of amides

Lard infused with 0.15% C18 amide was eluted on a Si SPE column, with C19 amide added as a reference after the elution. The amide-containing fraction was analyzed with GC-FID.

To ensure reliable results, the experiment was repeated five times with the same sample.

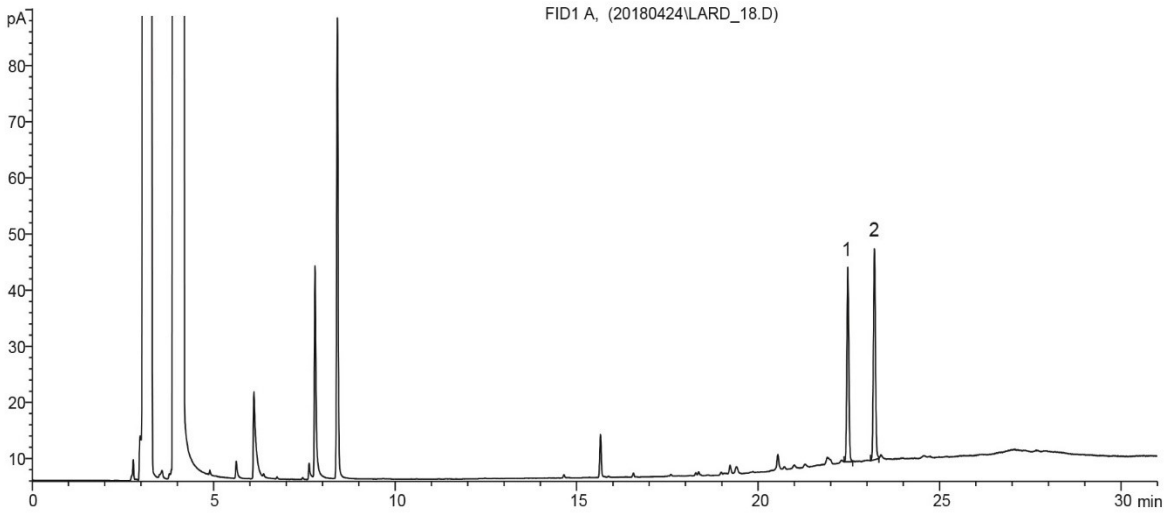


Figure 26. GC-FID of the second eluting fraction of lard spiked with 0.15% C18 amide, with C19 amide as a reference

Table 26. Summarized results from GC-FID of amide recovery from lard

Experiment	*m (sample) [g]*	*m (C19 amide) [g]*	*Area of C18 amide*	*Area of C19 amide*	***Recovery of C18a [%]***
1	1.02	0.0018	130.2	148.4	**108.3**
2	1.01	0.0019	121.1	162.8	**95.9**
3	1.01	0.0021	127.0	158.2	**113.0**
4	1.01	0.0020	129.9	170.3	**103.4**
5	1.01	0.0022	126.5	207.2	**91.2**

The average recovery of amides was 102.35%. Standard deviation was calculated to be 8.9%.

4.5.3. Lard with high amount of free fatty acids

Lard spiked with 0.15% C18 amide was mixed with different amounts of oleic acid, and after the elution on SPE column, the recovery of C19 amide from each sample was calculated from chromatograms obtained with GC-FID.

Table 27. Summarized results from GC-FID of lard with different amounts of oleic acid

Oleic acid [%]	*Area C18 amide*	*Area C19 amide*	***Recovery of C18 amide [%] (average)***
20	17.00	*16.00	
17	95.50	197.60	**48.40**
15	101.25	189.15	**53.00**
10	122.85	195.05	**62.75**
5	100.50	201.60	**49.76**
3	121.40	197.90	**61.20**
1.5			**84.75
0			**≈100**

* *C19 amide was added before the elution as an internal standard*
** *Lard + 0.15% C19 amide + 1.5% C19 free fatty acid*

The results summarized in the table above indicate that with increasing amount of oleic acid in the sample the recovery of amides is obstructed more.

A chromatogram of a sample containing 20% of oleic acid is shown as an example. All the chromatograms look the same, the only difference being in height of the peaks.

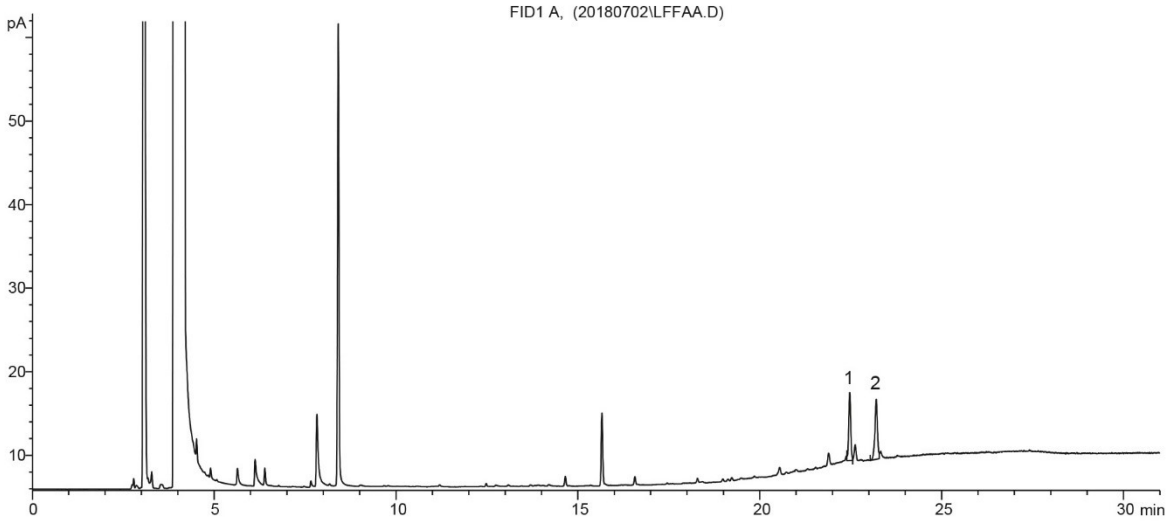


Figure 27. GC-FID of second eluting fraction of Lard with 0.15% C18 amide and 0.20% C19 amide infused with 20% of Oleic acid; 1 – C18 amide; 2 – C19 amide

Comparing the chromatograms obtained from the samples with and without oleic acid, it is evident that the presence of free fatty acids in the sample hinders the recovery of amides:

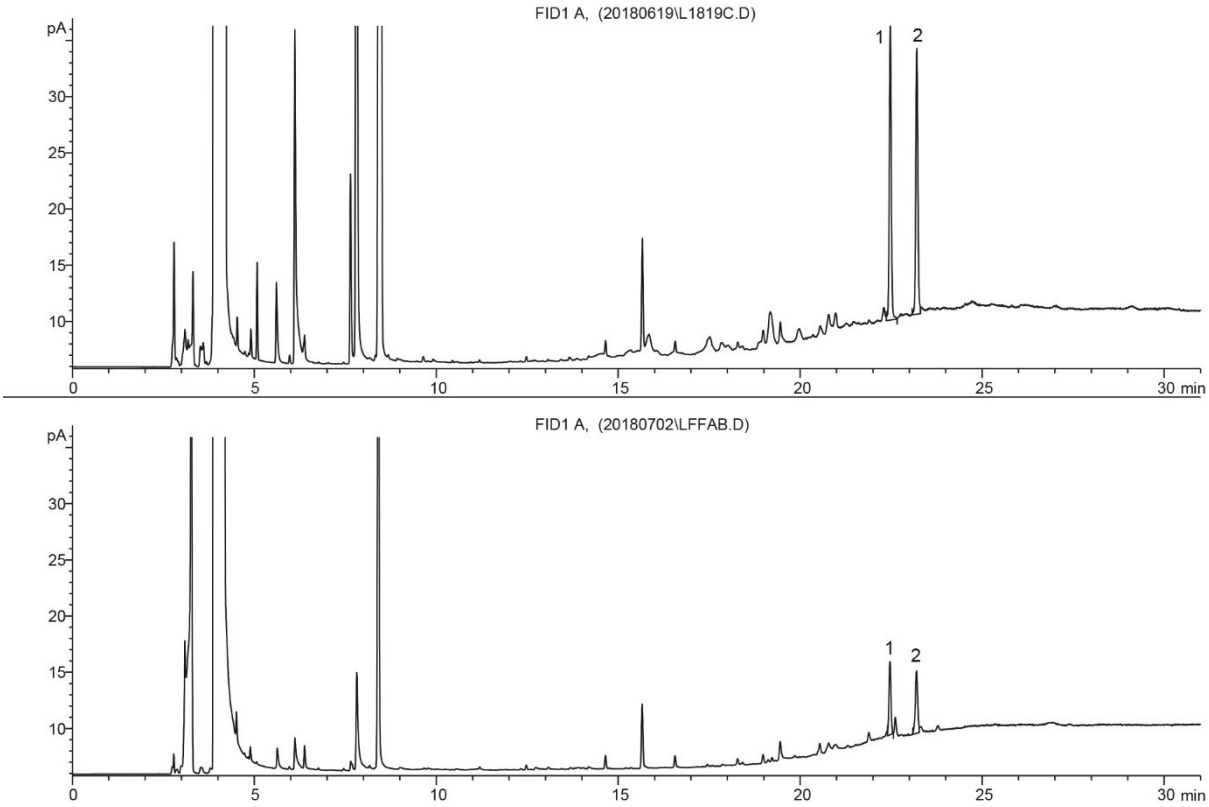


Figure 28. GC-FID of second eluting fraction of lard without oleic acid (upper chromatogram) and lard with 20% of oleic acid (lower chromatogram)

4.5.4. Residual amides on the SPE column

The very poor recovery of amides in the sample containing higher amount of free fatty acids, led to the assumption that in some way free fatty acids are omitting the elution of amides, and because of that they are being left behind on the column. To check this assumption, two samples were eluted with additional 5 ml of chloroform: 2-propanol and analyzed on GC-FID:

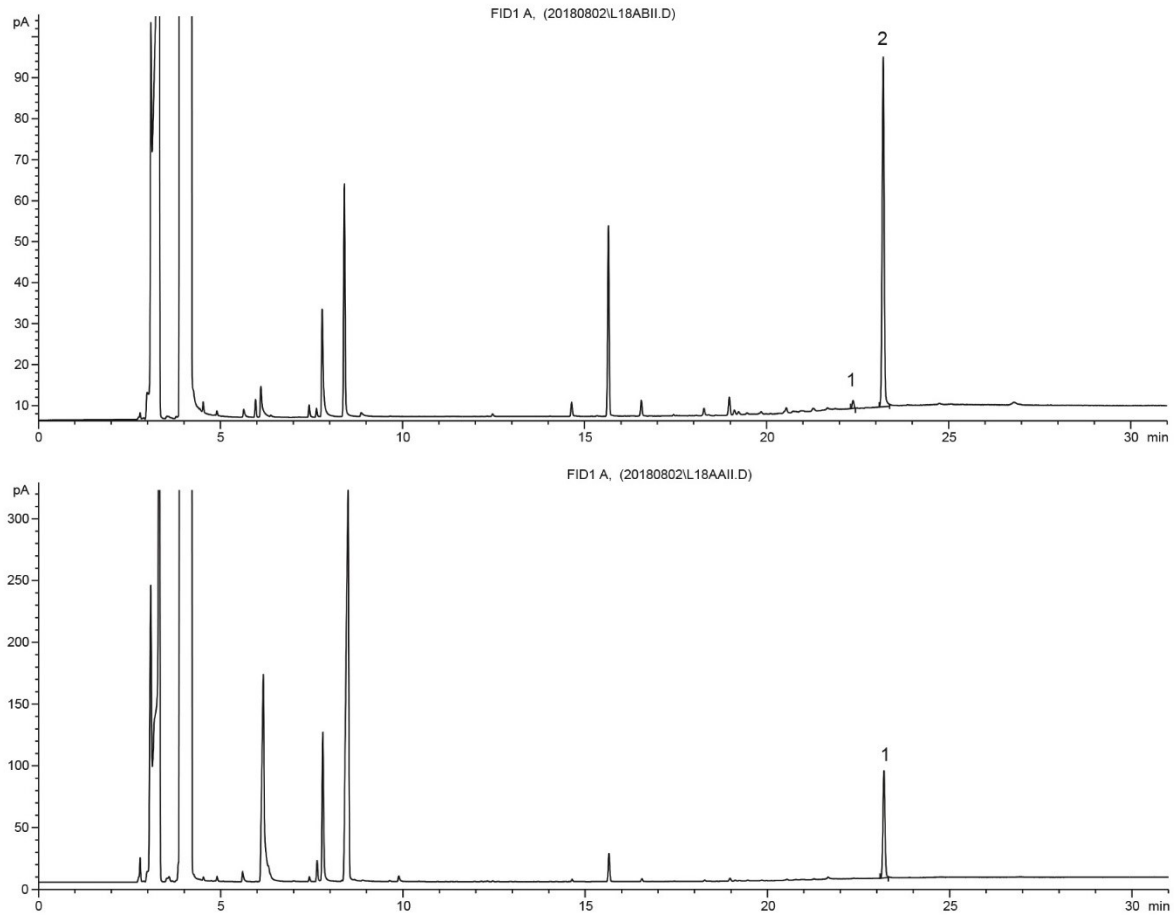


Figure 29. GC-FID of additional 5 ml of second eluting fraction from two samples; 1 – C18 amide; 2 – C19 amide

In the first sample, a small peak was detected at the retention time of C18 amide, however, the peak was too small to be integrated.

In the second sample, the peak belonging to C18 amide was big enough to be integrated. The calculated amount of C18 amide in this case was approximately 0.025 mg, which corresponds to 1.6% of the total amount of C18 amide that was loaded on the SPE column with the sample. This additional eluted amount of amide does not add up to the total amount that was loaded on the column. Nevertheless, the volume of second eluting fraction was increased to 10 ml.

4.5.5. Reduction of sample amount and use of an anion exchange resin

To overcome the low recovery of amides, two approaches were investigated: reduction of the sample amount loaded on the column and the use of anion exchange resin.

In the first approach, the assumption was that the column is overloaded with the sample, and free fatty acids are occupying all the binding spots on the sorbent, instead of amides, resulting in elution of some amides with the first fraction. By reducing the sample amount, possibly, enough binding spots will still be available for amides to interact.

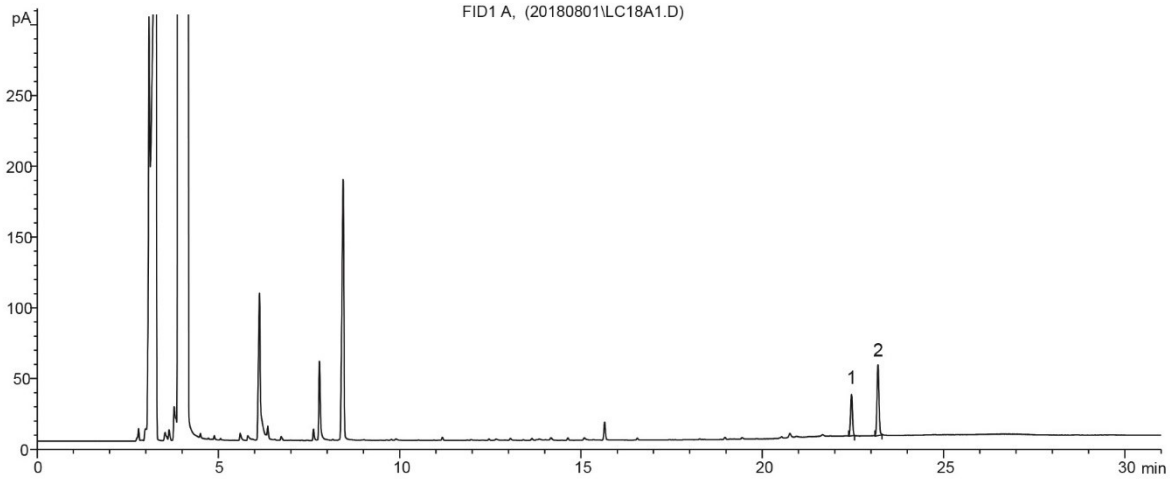


Figure 30. GC-FID of the second eluting fraction of 0.5 g of lard with 0.20% of C18 amide and 20% of oleic acid; 1 – C18 amide; 2 – C19 amide

Table 28. Comparison of recovery of C18 amide from 1 g of sample and 0.5 g of sample

Sample amount [g]	*Area C18 amide*	***Recovery of C18 amide [%]***
1	17.0	**9.00**
0.5	110.8	**28.05**

The aim of the second experiment was to separate free fatty acids from the sample before the SPE column. By placing the anion exchange on top of the SPE column, free fatty acids would be detained in the anion exchange, and the sample without free fatty acids would pass through to SPE column.

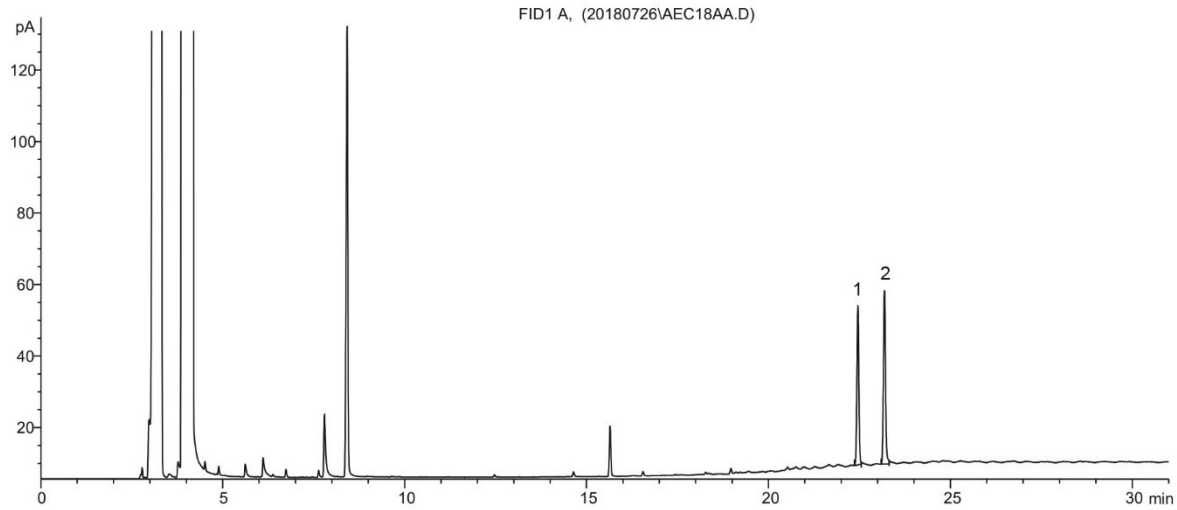


Figure 31. GC-FID of the second eluting fraction of 1 g of lard with 0.20% of C18 amide and 20% of Oleic acid eluted with the anion exchange; 1 – C18 amide; 2 – C19 amide

Table 29. Summary of the results of recovery of C18 amide with the use of anion exchange

Experiment	*Area C18 amide*	*Area C19 amide*	***Recovery of C18 amide [%]***
1	166.8	194.6	**85.5**
2	141.5	180.1	**78.5**
3	134.8	189.2	**71.0**
4	128.9	191.0	**67.5**
			Average 75

As two previous experiments show, by reducing the sample amount loaded on the SPE column by half, the recovery of amides increases but only up to ~28%. The use of anion exchange increases the recovery of amides up to 75%.

4.5.6. Rapeseed oil methyl ester

Rapeseed oil methyl ester spiked with 0.2% C18 amide was analyzed on C18 column according to paper published by Madl and Mittelbach [4]. Eluents in this experiment were hexane (120 ml) and THF (120 ml). After elution, the THF fraction was analyzed on GC-FID using C19 amide as a reference.

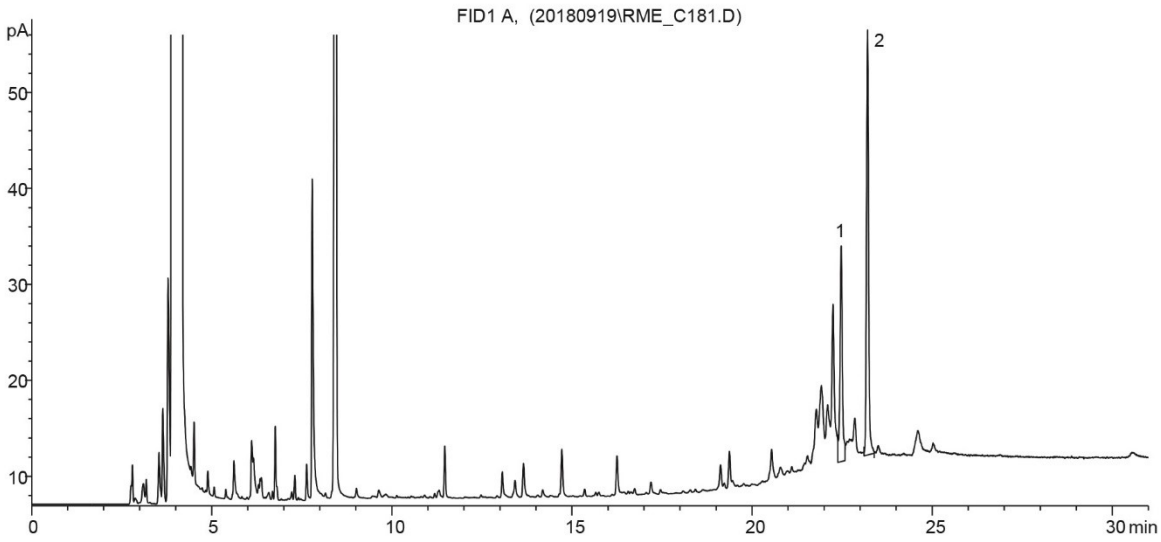


Figure 32. GC-FID of the second eluting fraction from the sample eluted on C18 column

Table 30. Results of the recovery of C18 amide from the C18 column

Experiment	*Area C18 amide*	*Area C19 amide*	***Recovery of C18 amide [%]***
1	101.8	181.6	**131**
2	95.7	180.3	**131**

In the second experiment, the same sample was analyzed on a Si SPE column with 20 ml of hexane: ethyl acetate (70:30) and 10 ml of chloroform: 2-propanol as eluents. The second fraction was evaporated, dissolved in THF and analyzed on GC-FID using C19 amide as a reference.

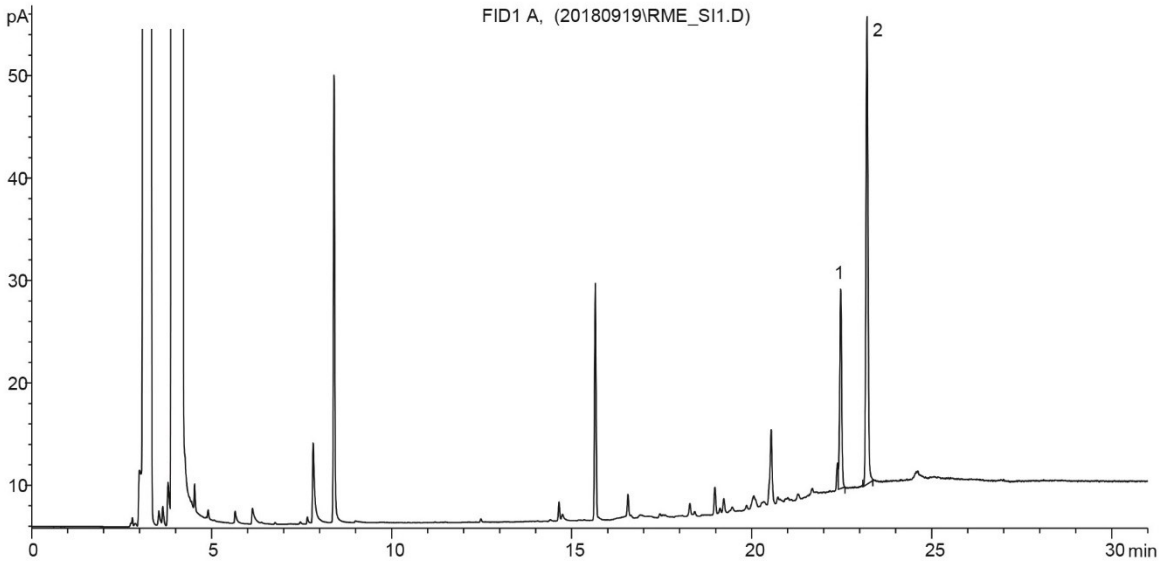


Figure 33. GC-FID of the second eluting fraction from the sample eluted on Silica column

Table 31. Results of the recovery of C18 amide from the silica column

Experiment	*Area C18 amide*	*Area C19 amide*	***Recovery of C18 amide [%]***
1	74.3	182.7	**50**
2	61.1	175.3	**35**

As the results above show that the recovery of amides in the first experiment is incomparably better than in the second experiment, meaning that the combination of Si SPE column and solvents that were used are not suitable for separation of amides from biodiesel. Because the recovery of C18 amide was much worse from Silica column compared to recovery from C18 column, the first eluting fraction from the Silica column was also analyzed on GC-FID:

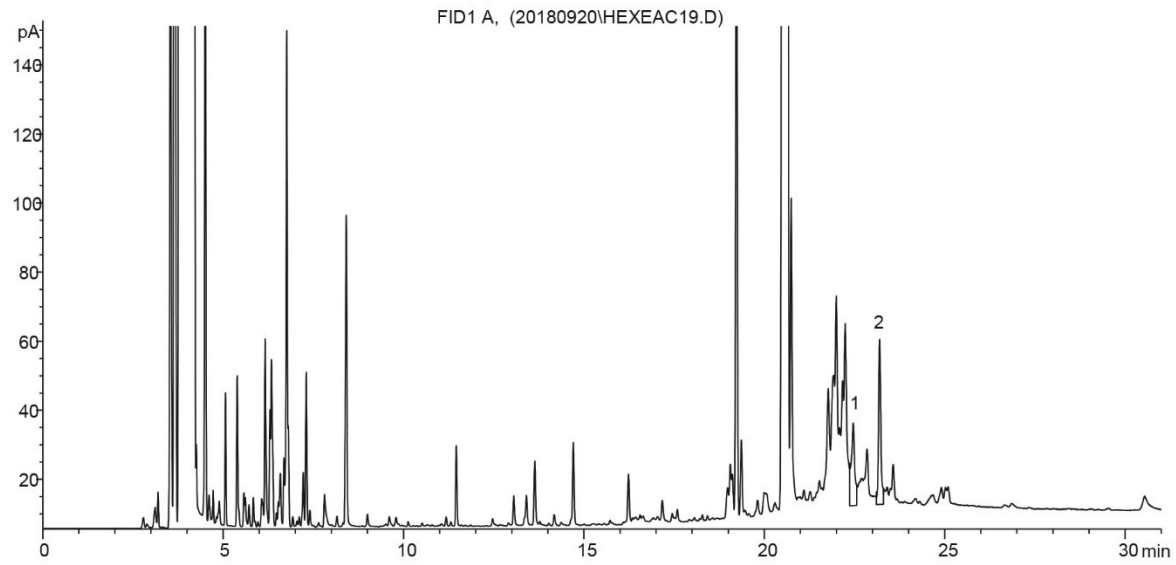


Figure 34. GC-FID of the first eluting fraction from the sample eluted on Silica column

As the previous chromatogram shows, recovery of amides from the silica column is very low because part of the amides are eluted with the first eluting fraction.

Although the recovery of amides from biodiesel is above 100% when the experiment is performed according to the paper from Madl and Mittelbach [4], the use of eluents is excessive. These were the preliminary experiments done with biodiesel. More research has to be done in the future on this subject.

4.6. Determination of amides in real samples

4.6.1. Sample "Feed 18-18"

The content of free fatty acids in the sample was determined to be 0.55%.

Table 32. Content of free fatty acids in sample "Feed 18-18"

Experiment	*m (sample) [g]*	*V (KOH/EtOH) [ml]*	*Content of FFA [%]*
1	0.50	0.1	0.54
2	0.50	0.1	0.55

The sample with C19 amide as internal standard (added before the elution) was eluted on the SPE column and analyzed on GC-FID:

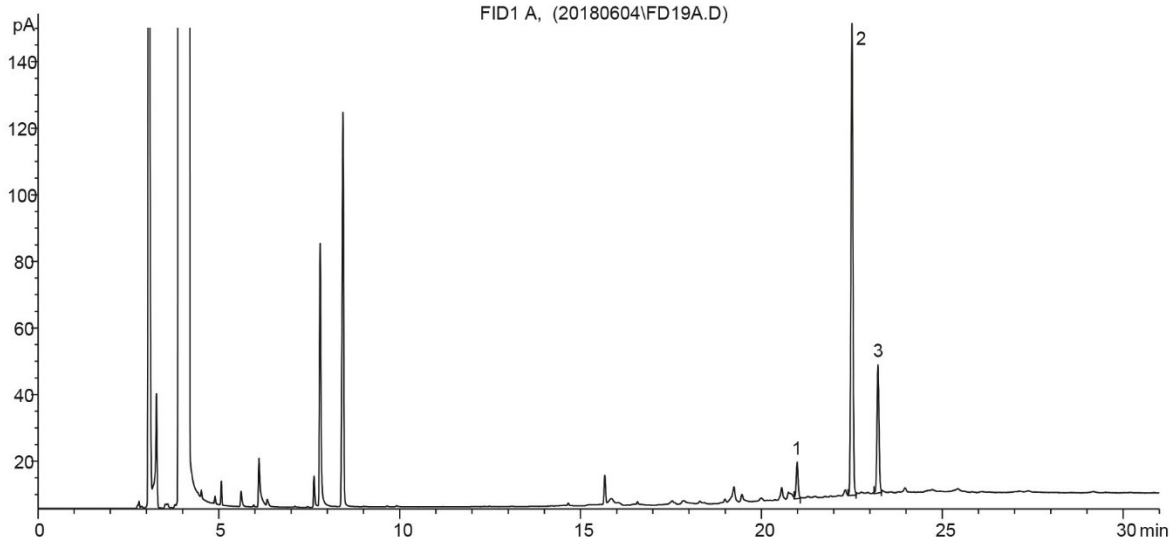


Figure 35. GC-FID of the second eluting fraction of the sample "Feed 18-18"; 1 – C16 amide; 2 – C18:1 amide; 3 – C19 amide

Table 33. Summarized results of GC-FID of the sample "Feed 18-18"

Experiment	*m (sample) [g]*	*Area C16 amide*	*Area C18 amide*	*Area C19 amide*	*Total amount of amides [%]*
1	1.01	35.5	548.9	145.4	0.80
2	1.01	28	451.8	117.5	0.80
Old method	1.00	165.4	645.2	210.2	0.77

With the old method, the content of amides was determined to be 0.77%. The chromatograms obtained with new and old method are compared:

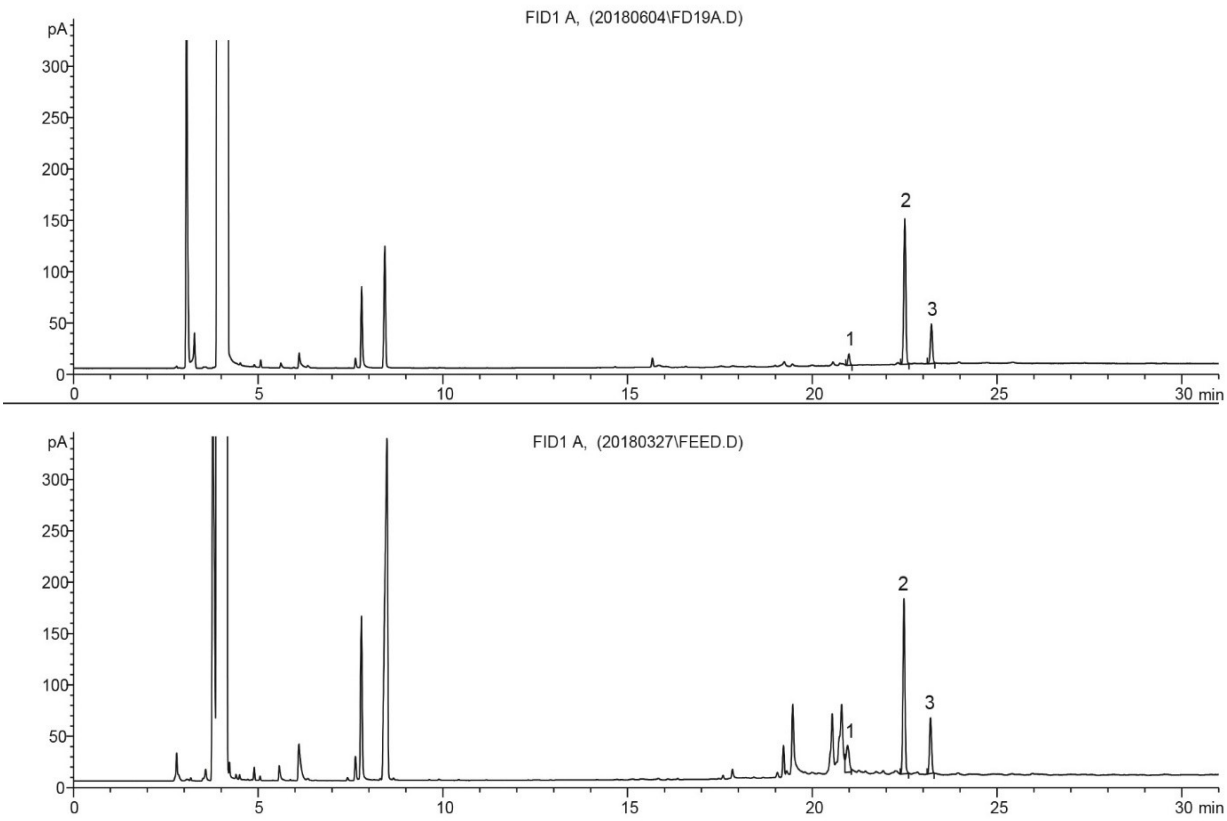


Figure 36. Comparison of chromatograms obtained with the new (up) and old (down) method

The results obtained with new and old method are comparable. However, it is evident that with the new method more interfering substances were removed compared with the old method.

4.6.2. Sample Fraction I

The content of free fatty acids in the sample was determined to be 0.52%.

Table 34. Content of free fatty acids in sample "Fraction I"

Experiment	*m (sample) [g]*	*V (KOH/EtOH) [ml]*	*Content of FFA [%]*
1	0.51	0.10	0.55
2	0.50	0.09	0.50

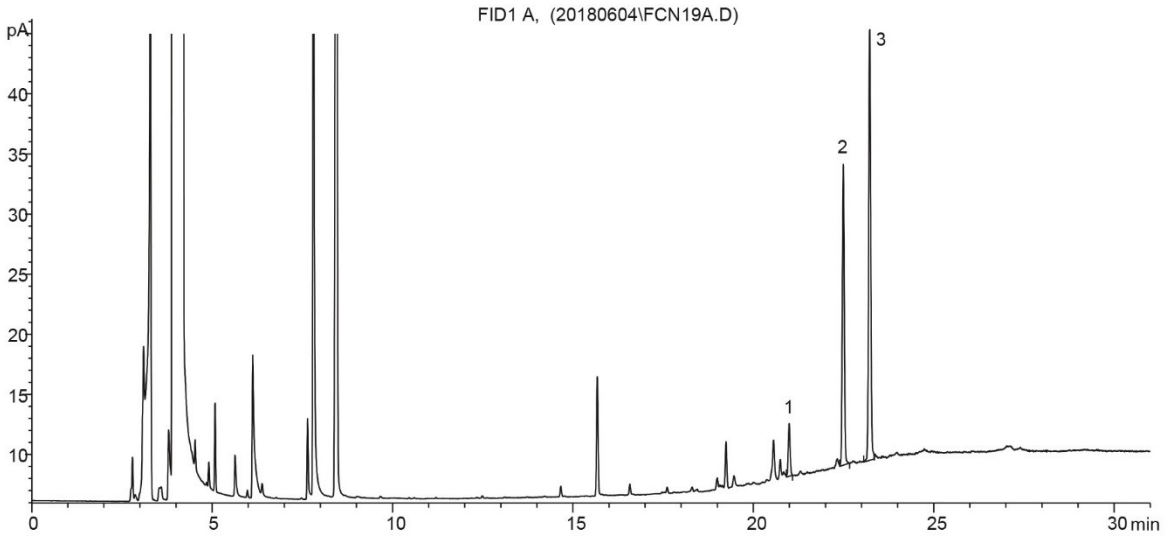


Figure 37. GC-FID of the second eluting fraction of the sample "Fraction I"; 1 – C16 amide; 2 – C18:1 amide; 3 – C19 amide

Table 35. Summarized results of GC-FID of the sample "Fraction I"

Experiment	*m (sample) [g]*	*Area C16 amide*	*Area C18 amide*	*Area C19 amide*	*Total amount of amides [%]*
1	1.00	16.7	95.2	142.6	0.16
2	1.01	16.2	100.8	163.0	0.15
Old method	1.00	113.5	129.3	222.2	0.22

With the old method, the content of amides was calculated to be 0.22%. The chromatograms obtained with new and old method are compared:

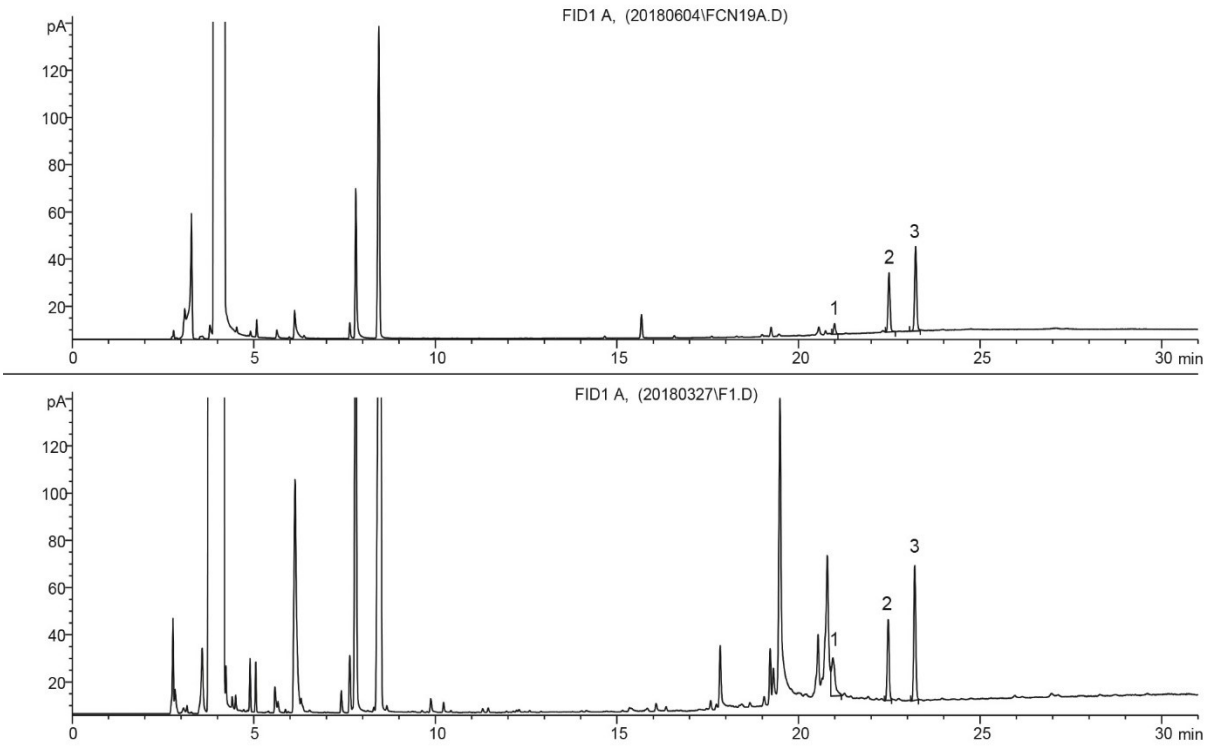


Figure 38. Comparison of chromatograms obtained with the new (up) and old (down) method

The biggest difference in results obtained with the new and the old method is in the area of the C16 amide peak. It appears that the area of C16 amide peak is much bigger when measured with the old method, resulting in higher content of amides. However, with the old method, there are additional peaks around this retention time, so it is possible that some other component comprised the peak of C16 amide, giving apparently a higher result.

4.6.3. Sample CAT2

The content of free fatty acids in the sample was determined to be 39%.

Table 36. Content of free fatty acids in sample "CAT2"

Experiment	*m (sample) [g]*	*V (KOH/EtOH) [ml]*	*Content of FFA [%]*
1	0.51	21	38.27
2	0.50	22	39.01

1 g of sample with C19 amide as an internal standard was separated on SPE column and analyzed on GC-FID:

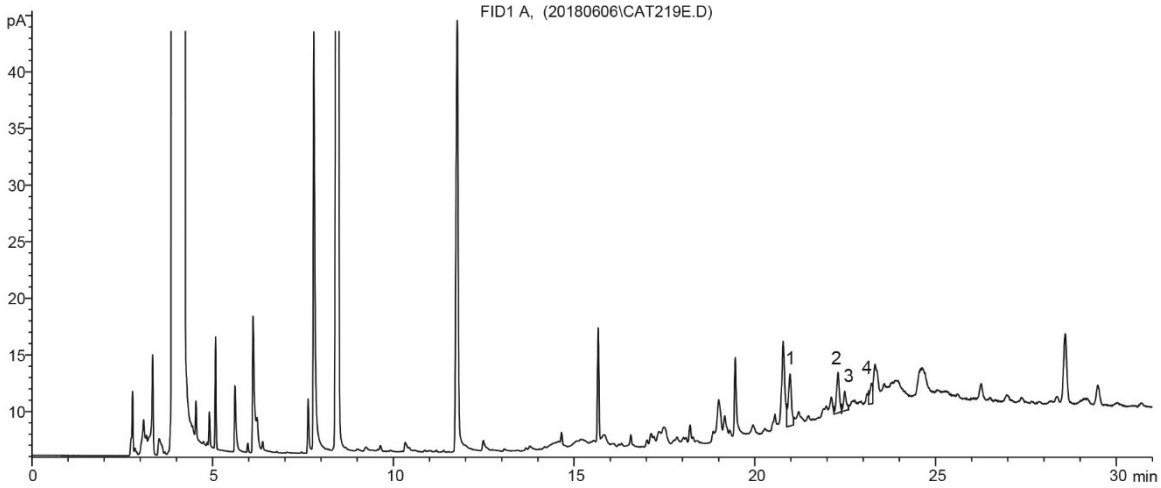


Figure 39. GC-FID of the second eluting fraction of the sample "Fraction I"; 1 – C16 amide; 2 – C18:1 amide; 3 – C18 amide; 4 - C19 amide

Table 37. Summarized results of GC-FID of the sample "Fraction I"

	m (sample) [g]	*Area C16 amide*	*Area C18:1 amide*	*Area C18 amide*	*Area C19 amide*	*Total amount of amides [%]*
Experiment 1	1.02	33.2	23.7	11.6	10.5	1.29
Experiment 2	1.01	28.7	20.5	9.9	10.7	1.10
Experiment 3	1.01	26.2	18.8	7.9	11.0	0.95
Experiment 4	1.03	28.3	18.0	7.5	8.8	1.18
Old method	1.00	382.2	640.8	234.7	271.0	0.99

With the old method, the content of amides was calculated to be 0.99%. The chromatograms obtained with the new and the old method were compared:

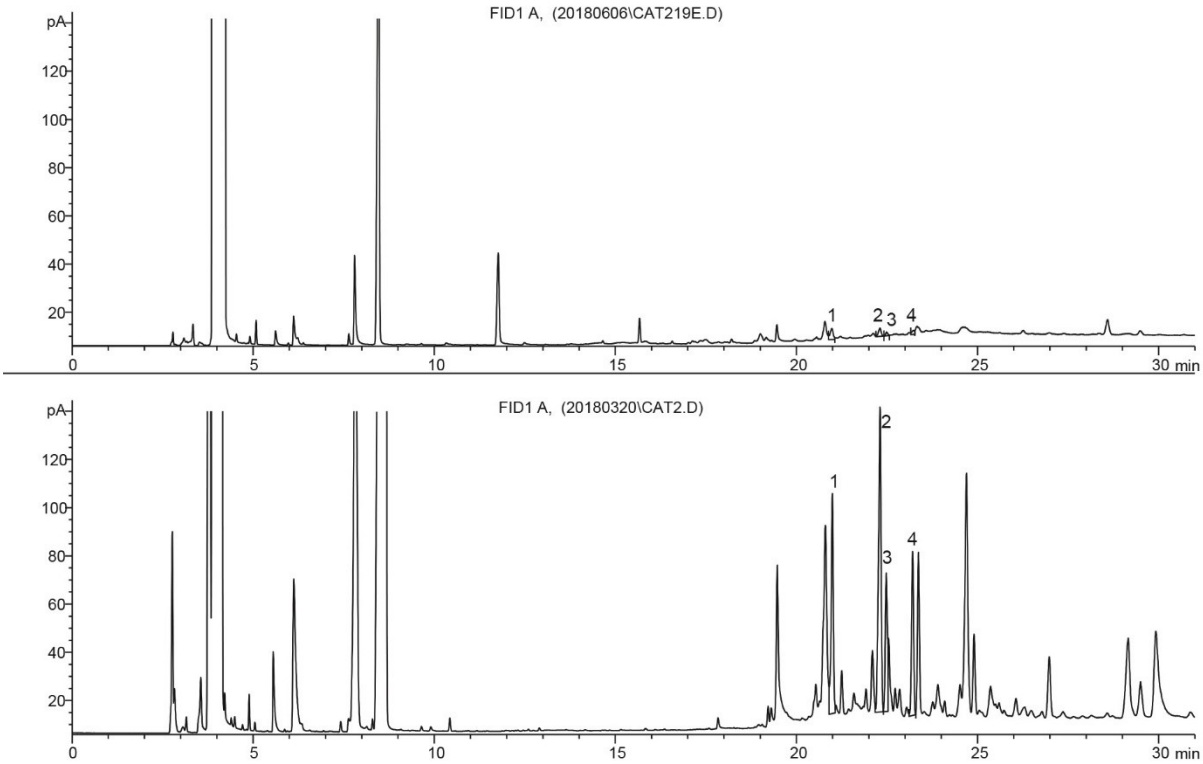


Figure 40. Comparison of chromatograms obtained with the new (up) and old (down) method

The new method gave comparable results as the old one; however, it was again obvious that the recovery of amides in the sample with high amount of free fatty acids (CAT2) was much lower with new method. Additionally, the peaks on the chromatogram obtained with the new method are not as expressed as with the old method. Therefore, the sample CAT2 was analyzed again, this time with the reduction of the sample amount loaded on the SPE column (0.5 g of sample) and with the use of the anion exchange resin (with 0.5 g of sample):

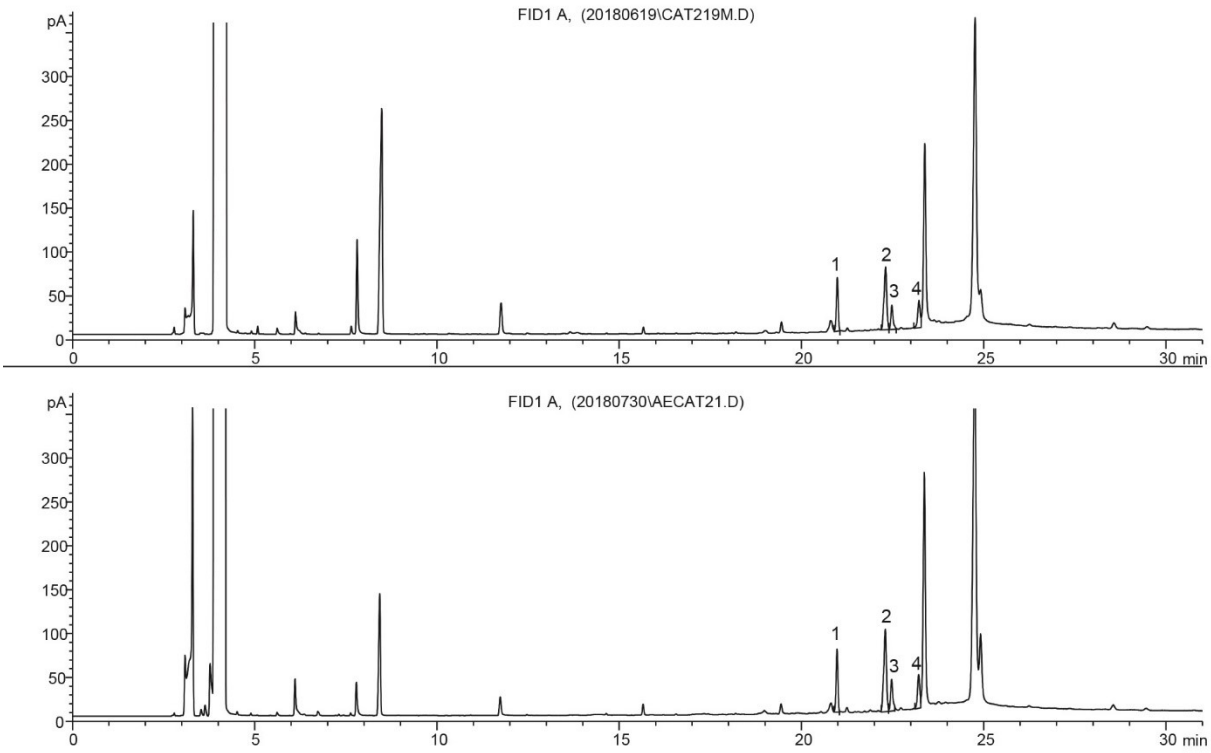


Figure 41. Comparison of chromatograms obtained with the reduced amount of sample (up) and reduced amount of sample + anion exchange (down); 1 – C16 amide; 2 – C18:1 amide; 3 – C18 amide; 4 – C19 amide

Table 38. Comparison of amides peak areas recovered in experiments with and without the anion exchange

	Peak area					
	Without Ion Exchange			With Ion Exchange		
C16 amide	189.6	233.5	220.0	202.5	251.0	217.1
C18:1 amide	277.7	363.0	380.5	304.6	488.1	386.3
C18 amide	81.6	120.3	125.7	92.6	166.9	123.8
C19 amide	121.5	146.3	150.2	139.1	179.7	175.9

The results with the use of the anion exchange resin with sample CAT2 were not as conclusive as with lard. As the table above indicates, the peaks from amides are not conclusively more expressed with the use of anion exchange. More influence on the results in this case seemed to have reduced the amount of sample that was loaded on the SPE column. Comparing chromatograms from experiments with 1 g and 0.5 g of sample also indicates that better recovery of amides is obtained with 0.5 g of sample:

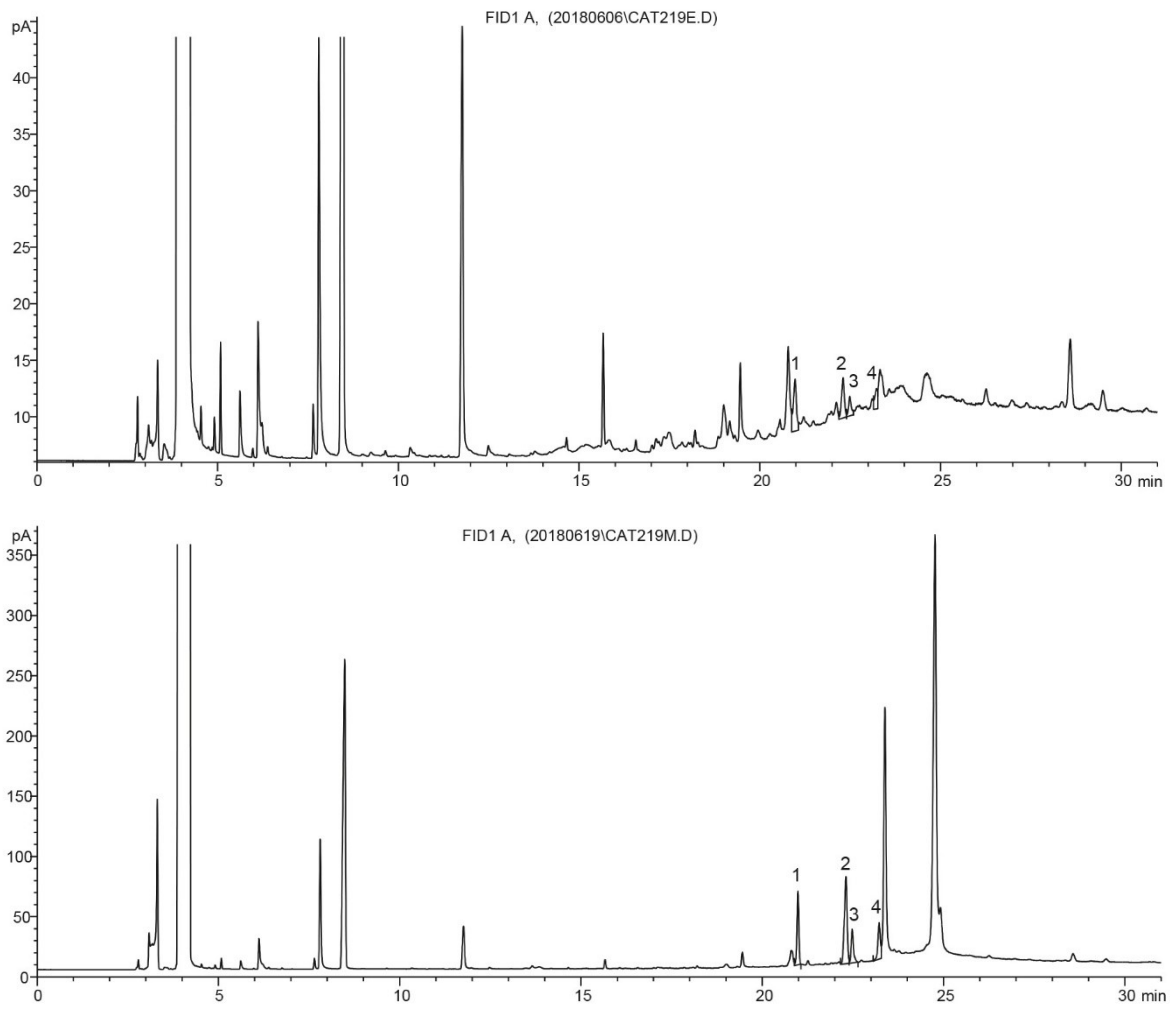


Figure 42. Comparison of chromatograms obtained with 1 g of sample (up) and 0.5 g of sample (down)

4.6.4. Elution of amides from reduced sample amount

After the previous experiments showed that the best and most reliable results are obtained when, instead of 1 g, 0.5 g of sample is analyzed, the real samples were analyzed again using a reduced amount of sample and regular amount of eluting solutions (20 ml of hexane: ethyl acetate (70:30) and 10 ml of chloroform: 2-propanol (2:1)). For the reproducibility of the results, each sample was analyzed three times, with three injections for each experiment.

Table 39. Summarized results of experiments with sample "Fraction I"

Fraction 1

Experiment	I			II			III		
Injection	a)	b)	c)	a)	b)	c)	a)	b)	c)
Area C16a	5.9	6.1	6.3	5.6	5.8	5.5	5.3	5.2	5.4
Area C18a	54.3	49.4	47.6	52.7	53.4	52.5	51.6	50.6	51.1
Area C19a	61.5	54.6	51.8	62.4	62.8	62.5	63.7	62.4	63.4
Amides [%]	**0.19**	**0.2**	**0.2**	**0.18**	**0.18**	**0.18**	**0.17**	**0.17**	**0.17**

Table 40. Summarized results of experiments with sample "Feed 18-18""

Feed 18-18

Experiment	I			II			III		
Injection	a)	b)	c)	a)	b)	c)	a)	b)	c)
Area C16a	11.6	12.0	11.8	12.0	12.2	12.5	11.3	11.0	10.8
Area C18a	220.2	227.6	227.9	227.6	231.6	233.4	212.5	212.9	212.9
Area C19a	59.3	62.5	63.0	62.5	62.5	63.3	59.6	59.4	60.0
Amides [%]	**0.77**	**0.76**	**0.75**	**0.77**	**0.76**	**0.76**	**0.74**	**0.74**	**0.74**

Table 41. Summarized results of experiments with sample "January"

January

Experiment	I			II			III		
Injection	a)	b)	c)	a)	b)	c)	a)	b)	c)
Area C16a	12.2	11.9	11.9	13.2	13.4	13.4	12.4	12.1	12.1
Area C18a	1.5	1.4	1.4	1.4	1.7	1.7	1.8	1.7	1.6
Area C19a	55.6	56.4	54.8	57.5	58.5	57.2	58.6	57.5	58.6
Amides [%]	**0.05**	**0.05**	**0.05**	**0.05**	**0.05**	**0.05**	**0.05**	**0.05**	**0.05**

Table 42. Summarized results of experiments with sample "February"

February

Experiment	I			II			III		
Injection	a)	b)	c)	a)	b)	c)	a)	b)	c)
Area C16a	9.1	9.7	9.6	9.1	9.1	9.1	9.2	9.5	9.1
Area C18a	2.2	2.2	2.2	1.6	1.6	1.9	1.7	1.6	1.7
Area C19a	53.6	54.9	55.0	54.0	56.7	55.2	60.3	58.7	59.0
Amides [%]	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**

Table 43. Summarized results of experiments with sample "December"

December

Experiment	I			II			III		
Injection	a)	b)	c)	a)	b)	c)	a)	b)	c)
Area C16a	9.9	10.1	10.0	10.0	9.8	10.1	9.9	9.7	10.0
Area C18a	2.0	2.0	2.1	1.7	1.7	1.5	1.5	1.5	1.4
Area C19a	59.6	56.7	57.3	57.3	56.4	59.3	59.2	60.8	60.1
Amides [%]	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**

Table 44. Summarized results of experiments with sample "CAT2"

CAT2

Experiment	I			II			III		
Injection	a)	b)	c)	a)	b)	c)	a)	b)	c)
Area C16a	37.4	38.8	39.3	25.5	27.6	28.3	37.0	37.3	38.2
Area C18:1a	61.6	70.1	73.0	45.1	49.6	51.1	66.1	67.3	68.5
Area C18a	19.0	20.1	20.3	13.2	14.2	14.1	18.8	19.5	20.0
Area C19a	13.3	14.2	14.6	8.2	8.8	9.0	10.7	11.6	11.5
Amides [%]	**1.75**	**1.78**	**1.78**	**1.90**	**1.90**	**1.90**	**2.16**	**1.96**	**2.16**

4.7. Determination of the limits of detection and quantification

In order to determine the limits of detection and quantification tallow was spiked with different amounts of C18 amide. C19 amide was added to the sample after the elution as a reference. All the samples were analyzed on GC-FID.

Table 45. Summarized results of experiments with lard with 0.2% of C18 amide

0.2% C18 amide				
Experiment	I		II	
Injection	a)	b)	a)	b)
C18a	95.1	92.4	184.2	182.4
C19a	188.2	184.1	180.6	179.9
Recovery [%]	**100**	**100**	**99.03**	**99.03**

Table 46. Summarized results of experiments with lard with 0.1% of C18 amide

0.1% C18 amide				
Experiment	I		II	
Injection	a)	b)	a)	b)
C18a	106.3	112.1	103.0	98.7
C19a	191.4	199.2	199.7	188.4
Recovery [%]	**102.8**	**104.6**	**101.9**	**102.6**

Table 47. Summarized results of experiments with lard with 0.05% of C18 amide

0.05% C18 amide				
Experiment	I		II	
Injection	a)	b)	a)	c)
C18a	52.5	49.5	53.8	53.0
C19a	191.7	182.4	191.4	193.1
Recovery [%]	**108.0**	**108.0**	**112.4**	**108.0**

Table 48. Summarized results of experiments with lard with 0.025% of C18 amide

0.025% C18 amide				
Experiment	I		II	
Injection	a)	b)	a)	b)
C18a	26.90	27.13	26.6	27.0
C19a	193.2	196.9	194.0	201.0
Recovery [%]	**111.4**	**111.4**	**107.7**	**104.0**

Table 49. Summarized results of experiments with lard with 0.015% of C18 amide

0.015% C18 amide				
Experiment	I		II	
Injection	a)	c)	b)	c)
C18a	15.5	14.5	17.3	14.5
C19a	197.5	189.5	197.2	189.5
Recovery [%]	**101.3**	**99.3**	**110**	**99.3**

Table 50. Summarized results of experiments with lard with 0.0075% of C18 amide

0.0075% C18 amide				
Experiment	I		II	
Injection	a)	b)	a)	b)
C18a	7.6	8.2	8.7	9.2
C19a	196.8	197.0	208.1	213.1
Recovery [%]	**102.9**	**110.1**	**110.5**	**113.1**

Table 51. Summarized results of experiments with lard with 0.0035% of C18 amide

0.0035% C18 amide				
Experiment	I		II	
Injection	a)	b)	a)	b)
C18a	3.7	3.9	4.1	4.2
C19a	206.2	205.8	203.7	202.9
Recovery [%]	**102.5**	**108.3**	**111.1**	**111.1**

Table 52. Summarized results of experiments with lard with 0.001% of C18 amide

0.001% C18 amide				
Experiment	I		II	
Injection	a)	b)	a)	b)
C18a	1.4	1.2	1.1	1.1
C19a	202.7	201.6	209.7	204.2
Recovery [%]	**119.0**	**102.6**	**104.0**	**108.0**

Table 53. Summarized results of experiments with lard with 0.0005% of C18 amide

0.0005% C18 amide				
Experiment	I		II	
Injection	a)	b)	a)	b)
C18a	n.d	n.d	n.d	n.d
C19a	194.6	194.3	201.8	199.4
Recovery [%]	-	-	-	-

The lowest concentration at which the peak of the analyte was possible to integrate is 0.001%. However, the signal-to-noise ratio at this concentration is 2.5, so this value cannot be taken as a detection limit. At the concentration of analyte of 0.0035%, the signal-to-noise ratio is 7.5. Therefore, it can be estimated that the detection limit is

closer to the concentration of 0.001% than to 0.0035%, so the value of 0.002% is accepted as a detection limit.

Similarly, for the limit of quantification, the signal-to-noise ratio at a concentration of the analyte of 0.0035% is 7.5; at the concentration of analyte of 0.0075% is 18. Therefore, it is estimated that the limit of quantification is closer to the concentration of 0.0035% then to 0.0075%, and the value of 0.005% of analyte is accepted as a quantification limit.

Method detection limit:	0.002%
Method quantification limit:	0.005%

6. Conclusions

The previously used method for the determination of FAA involved esterification and transesterification of animal fat samples before SPE. This procedure was time consuming, and huge amounts of eluents were used for SPE (120 ml of hexane and 120 ml of THF). The aim of this work was to develop a method with simpler sample preparation, and using less chemicals that would yield reproducible results relatively fast.

Initial experiments with TLC showed that an optimal combination of solvents to separate amides from other components of animal fat was found to be: hexane: ethyl acetate (70:30) and chloroform: 2-propanol. Additionally, this combination of eluents allows the direct separation of amides from the sample, without previous esterification and transesterification.

Experiments with rapeseed oil, and then later with lard spiked with amides, showed that the recovery of amides from SPE column is approximately 100%, meaning that all the amides that were present in the sample were eluted from the SPE column. However, further experiments proved that FFA in the sample omit the recovery of amides. Even smaller amounts of FFA (1.5%) decrease the recovery to approx. 85%. The easiest way to overcome this problem was the reduction of sample amount that is loaded on the SPE column. With experiments with lard with 20% of oleic acid, by reducing the sample amount from 1 g to 0.5 g, the recovery increased from 9% to 28%. However, the major advantage of sample reduction was that the obtained chromatograms were much favorable, meaning that amides peaks were much higher, more distinct and more easy to integrate. For instance, the sum peak of all amides in sample CAT2 (C16 amide, C18:1 amide and C18 amide) increased from 60 (mean value) to 650 (mean value).

After the method was developed using rapeseed oil and lard as matrixes, real samples obtained from companies were analyzed, and the results were compared with those obtained with the previous method. With all of the samples analyzed, the method proved to be repeatable, and the results were comparable to those of the previous

method. Analyzing the results obtained with the new and the old method, it is evident that chromatograms obtained from the new method are “cleaner”, i.e. there are fewer interfering peaks, which additionally validates the conclusion that the eluents in the new method are much more efficient in separating amides from other compounds.

By analyzing a series of samples of lard with different concentrations of amide, it was calculated that the limit of detection is 0.002%, while the limit of quantification is 0.005%.

7. List of Abbreviations

ABPR - Animal By-Products Regulations

BAAT - bile acid CoA:amino acid N-acyl transferase

BSE - Bovine spongiform encephalopathy

FAA – fatty acid amides

FAAH – fatty acid amide hydrolase

FAME – fatty acid methyl esters

FFA – free fatty acids

GC-FID – gas chromatography – flame ionization detection

GC-MS – gas chromatography – mass spectrometry

HPLC-APCI-MS – high-performance liquid chromatography – atmospheric pressure chemical ionization – mass spectrometry

n.a – not available

n.d – not detected

NAA - *N*-acylamino acid

NAD - *N*-acyldopamine

NAE - *N*-acylethanolamine

NAM - N-acylamide

NMR – nuclear magnetic resonance

PAM - peptidylglycine α-amidating monooxigenase

PFAM – primary fatty acid amides

PPh3 – triphenylphosphine

SPE – solid phase extraction

THF - tetrahydrofuran

TLC – thin layer chromatography

WAF – waste animal fat

8. List of tables

9. List of figures

10. References

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