Waste animal fats represent a very attractive feedstock for biodiesel production. Primary fatty acid amides (PFAA) are a class of bioactive signaling lipids found in mammalian organisms, as well as several plant families. Waste animal fat coming from rendering plants can obtain up to 2.0% PFAA. After the conversion process they pose a risk in the final biodiesel as they can lead to deposits in storage tanks or plug fuel filters. In this paper, a method for efficient separation and quantification of PFAAs in waste animal fat is presented. The method consists of separation of PFAAs via solid phase extraction (SPE) using cartridges with 60 Å silica as stationary phase. The most effective eluents are determined to be hexane: ethyl acetate followed by chloroform: 2-propanol. The isolated PFAAs are identified and quantified via gas chromatography-flame ionization detector (GC-FID) using nonadecanoic acid amide as an internal standard. The recovery of PFAAs in lard as matrix is determined to be around 100%. Six real samples are analyzed for the content of PFAAs leading to concentrations between 0.04% and 1%. Additionally, the limits of detection and quantification are determined to be 0.002% and 0.005%, respectively.

Practical applications: The developed method is an efficient tool for characterization and determination of primary fatty acid amides in animal fat used as starting material for biodiesel production. The developed simple and efficient separation of PFAA via SPE and GC-FID analysis without derivatization can also be applied to other biological fat samples, such as fat tissue, and plasma samples or microbial oils.

1. Introduction

Waste animal fat, as a by-product of the meat processing industries, has become a material that can be converted into high-quality biodiesel that meets the international specifications.\textsuperscript{1,2} Biodiesel produced from vegetable oils and animal fat can contain insoluble contaminants which can cause filter clogging or precipitation at longer storage time at lower temperatures. A series of compounds in fat and oil feedstocks were identified as reason for these problems. In pure vegetable oils, it could be sterol glucosides or waxes, during incomplete transesterification also saturated monoglycerides could lead to insoluble precipitates.\textsuperscript{3} In waste animal fat, especially plastic material from packages like polyethylene could lead to similar problems. Primary fatty acid amides (PFAA) were identified by Madl and Mittelbach\textsuperscript{4} as one of the impurities in biodiesel produced from animal fat. Two examples of PFAA are depicted in Figure 1. Rendered animal fat contains up to 1% of PFAA\textsuperscript{4} which do not result from naturally occurring amides. Most probably these amides are formed during the rendering process when free fatty acids or triglycerides react with ammonia originating from proteins, which, unfortunately, until now has not yet been confirmed.

Fatty acid amides are structurally diverse molecules found in mammalian organisms as well as several plant families. They belong to the class of bioactive signaling lipids that perform various functions in physiological and pathological processes.\textsuperscript{5} Because of their chemical structure, they are soluble in polar organic solvents, such as chloroform and 2-propanol but also in moderately polar tetrahydrofuran (THF) or in nonpolar toluene.\textsuperscript{6}

Some previous reports on different techniques for the separation of fat components include separation and determination of PFAA. Bilyk et al.\textsuperscript{7} developed a method for separation of neutral lipids using thin layer chromatography (TLC). In addition, a separation of amides from other components was reported. Gee et al.\textsuperscript{8} reported a method for determination of PFAA by derivatization using gas chromatography-mass spectrometry (GC-MS). A first method for separation and quantification of PFAA in animal fats was reported by Madl and Mittelbach.\textsuperscript{9} This method is based on separation of amides by use of a C18 solid phase extraction (SPE) column. The triacylglycerols are eluted with 120 mL of hexane, followed by elution of primary fatty acid amides with 120 mL of THF. Isolated amides are then quantified using high-performance liquid chromatography-mass spectrometry (HPLC-MS). Sultana and Johnson\textsuperscript{10} reported separation of amides on an aminopropyl bonded SPE column with quantification using GC-MS. Palardy et al.\textsuperscript{11} developed a method for identification and quantification of fatty amides in crude oil obtained from algal biomass by fractionation of lipid class groups.
by SPE followed by quantification by GC-FID (flame ionization detector).

However, all of these methods have some drawbacks. For instance, Bilyk et al.\textsuperscript{[7]} focused their work only on separation and identification of PFAs, without quantification. The method of Gee et al.\textsuperscript{[8]} included an additional step of derivatization by trimethyl-silylation before quantification. Madl and Mittelbach\textsuperscript{[4]} separated amides with considerable amounts of eluents (240 mL in total), which is time-consuming in routine analysis. Additionally, HPLC-MS is not a very precise method for quantification. Separation of amides in the method from Sultana and Johnson\textsuperscript{[9]} was performed in two steps, on two different SPE columns. Palardy et al.\textsuperscript{[10]} reported the separation of amides in the first eluting fraction. However, this method was not found suitable enough, because the amide fraction still contained interfering triacylglycerols.

The method described in this paper is a combination or a very fast SPE separation between neutral lipids and PFAs using small amounts of solvents followed by quantification with GC-FID. Nonadecanoic acid amide was used as an internal standard (ISTD). For development and optimization of the method, lard was used as a fatty matrix. Individual fatty acid amides were synthesized from their corresponding fatty acids. After establishment of the method, different waste samples which were acquired from industrial biodiesel producers were analyzed for the content of PFAs.

2. Experimental Section

2.1. Materials

Edible grade lard was purchased from a local food store. Oleamide (99%) was purchased from Sigma Aldrich Chemie (Germany), hexadecanamide (95%) was from TCI GmbH (Germany). Thionyl chloride and ammonia solution (25%) were purchased from Merck (Darmstadt, Germany). Nonadecanoic acid (98%) was purchased from Sigma Aldrich Chemie (Germany), heptadecanamide acid from Acros Organics (USA), and stearic acid (n.a.) from Fluka Chemie (Switzerland). Chloroform (HPLC grade), ethyl ether (technical grade), ethanol (99%), ethyl acetate (99.8%), acetic acid (99.9%), and 2-propanol (>99.9%) were purchased from VWR Chemicals (France). Hexane (HPLC grade), tetrahydrofuran (HPLC grade), and methanol (99.8%) were obtained from Fischer Chemicals (UK). Petrol ether (boiling range 40–60 °C) was purchased from Carl Roth (Germany).

For SPE, silica Strata SI-1 cartridges were purchased from Phenomenex (USA) with following properties: 1 g per 6 mL volume, 56 μm average particle size, 60 Å pore size, 0.89 mL g\textsuperscript{-1} pore volume, and 474 m\textsuperscript{2} g\textsuperscript{-1} surface area.

An Agilent Technologies 6890 GC/FID system equipped with HP-5MS column (30 m × 250 μm × 0.1 μm) was used for GC/FID analysis. For GC/MS analysis, an Agilent Technologies 5973 GC System equipped with DB 5MS column (30 m × 250 μm × 0.25 μm) was used. For measuring the melting point of amides, Gallenkamp AF3 melting point apparatus was used. Six animal fat samples from rendering processes were obtained from Argent Energy Ltd. (UK) and BDI-BioEnergy International GmbH (Austria).

2.2. Synthesis

Synthesis of stearamide and nonadecanoic acid amide was performed according to a literature method in two steps.\textsuperscript{[11]} First, stearic acid chloride and nonadecanoic acid chloride were synthesized from their corresponding fatty acids. 20 mmol of each fatty acid was mixed with an excess of thionyl chloride (21.2 g, 180 mmol) and refluxed for 1 h at 85 °C. Then excess thionyl chloride was distilled off and the remaining mixture was mixed with 20 mL THF and 5 mL ammonium hydroxide (25% in water) under ice cooling to yield the corresponding amides. The product was mixed with water, filtered, washed with water, and dried. The yield of amides was ≈70%. The purity of synthesized amides was confirmed by GC-FID. Additionally, melting points were determined and compared to literature results; \(T_m\) of C18:0 amide was 108.5 °C (lit.: 107–109 °C\textsuperscript{[12]}), \(T_m\) of C19:0 amide was 124 °C (no available literature data).

To correctly identify and quantify the amides which were expected to be found in animal fat as well as of amide that was used as ISTD, a sample containing a mixture of C16:0, C18:0, C18:1, and C19:0 amide was analyzed with GC-FID using the temperature program established for this method, and the structures were confirmed by GC-MS equipped with the same column and the same temperature program.

2.3. TLC

To determine which solvents are the most efficient in separating different components from fatty samples, four solvents with different polarities were tested: hexane and hexane:ethyl acetate in three different ratios (90:10, 80:20, and 70:30, v/v). For determining the retention index of triacylglycerols on TLC plates, lard was used as a reference standard.

For TLC analysis, aluminum sheets coated with silica gel (60 F\textsubscript{254}) were used as stationary phase and petrol ether:diethyl acetic acid (70:30:1, v/v) as mobile phase. For detection, 10% iodine impregnated on silica gel H was used. Small aliquots from each eluted fraction were applied on the plate and developed in the glass chamber with mobile phase. Reaction of iodine with double bonds in triacylglycerols and other components revealed their relative quantities in the eluents. TLC experiments provided fast input on which solvent was the most efficient in removing triacylglycerols from the samples.

2.4. SPE

In these experiments, lard was used as a fatty matrix. Lard was spiked with 0.15% (m/m) of C18:0 amide by dissolving the amide
Table 1. Instrumental conditions for GC-FID analysis of fatty acid amides.

| Parameter      | Condition                                                                 |
|----------------|---------------------------------------------------------------------------|
| Column         | HP-5MS (Agilent Technologies, USA), 30 m × 250 µm × 0.1 µm                |
| Carrier gas    | Helium 5.0                                                                |
| Oven           | Initial temperature 50 °C                                                  |
| Initial time   | 3 min                                                                     |
| Rate           | 15–300 °C held for 10 min                                                 |
| Volume         | 1 µL                                                                      |
| Mode           | Split                                                                    |
| Split ratio    | 40:1                                                                      |
| Split flow     | 1.2 mL min⁻¹                                                              |
| Temperature    | 250 °C                                                                    |
| Detector       | Temperature 310 °C                                                        |
| H₂ flow        | 40 mL min⁻¹                                                               |
| Air flow       | 400 mL min⁻¹                                                              |
| Makeup flow (N₂) | 40 mL min⁻¹                                                            |

in THF (c = 1 mg mL⁻¹) and adding the appropriate amount of this solution to the previously melted lard, mixing it and removing the solvent by evaporation until the constant mass of the sample was obtained. 0.5 g of the sample dissolved in 10 mL of hexane was loaded on the Si SPE column previously conditioned with hexane. The sample was first eluted with 20 mL of hexane:ethyl acetate (70:30, v/v) which separated triacylglycerols, vitamins, pigments, and other minor components, followed by elution with 10 mL of chloroform:2-propanol (2:1, v/v). The SPE extraction was performed under a slight vacuum. The second eluting fraction was evaporated, re-dissolved in 4 mL of THF, and 1 mL of C19 amide solution in THF (1 mg mL) was added as an ISTD.

2.5. Quantification

The second eluting fraction from SPE separation, with C19:0 amide as ISTD, was analyzed via GC-FID equipped with HP-5MS 5% phenyl methyl siloxane column with conditions described in Table 1.

2.6. Method Validation

Recovery of amides from lard was determined by performing five consecutive experiments with the developed method. For these experiments, lard spiked with 0.15% (m/m) of C18:0 amide was used as a sample, and C19:0 amide was used as an ISTD. All of the experiments were performed with the same batch of sample (5 g of lard mixed with 7.5 mg of C18:0 amide, as described in the previous section), on the same day, under the same conditions. The recovery was determined by integrating the peaks representing the C18:0 and C19 amides and calculated using following formula (Equation (1))

\[ R = \frac{m_{\text{ref}} \times A_{\text{amide}}}{A_{\text{ref}} \times m_{\text{amide}}} \times 100 \]  

where \( R \) represents the recovery, \( m_{\text{ref}} \) and \( m_{\text{amide}} \) refer to the mass of the reference amide (ISTD) and the mass of the analyzed amide, respectively, \( A_{\text{amide}} \) and \( A_{\text{ref}} \) refer to the peak areas of analyzed amide and reference amide (ISTD), respectively.

In order to determine the detection and quantification limit, lard with 11 different concentrations of C18:0 amide was prepared: 0.2%, 0.1%, 0.05%, 0.025%, 0.015%, 0.0075%, 0.005%, 0.0035%, 0.002%, 0.001%, and 0.0005% (m/m). A sample from each concentration was analyzed, using C19 amide as ISTD. For each concentration, two experiments were performed, with two injections for each experiment.

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

These values were calculated manually from the individual chromatograms, by measuring the height of the analyte signal peak and noise magnitude around the analyte retention time and dividing the two values.

2.7. Method Performance

To determine the amount of PFAA from real samples, six different samples originating from rendering processes were analyzed with the developed method. For these experiments, C19 amide was used as ISTD. 1 mL of C19 amide solution in THF (c = 1 mg mL) was added to 0.5 g of each sample, THF was removed by evaporation, and the samples were analyzed as described previously.

3. Results and Discussion

3.1. TLC

The primary goal was to separate amides from triacylglycerols, mainly because triacylglycerols would be retained in the GC column and cause contaminations in the succeeding measurements. For this purpose, four different eluents were investigated: hexane and hexane:ethyl acetate in three different ratios.

For each of the eluents tested, the sample was eluted on SPE column multiple times with 5 mL aliquots and each eluted fraction was analyzed with TLC. TLC of eluates from SPE column eluted with hexane showed that for complete removal of triacylglycerols more than 50 mL of hexane is necessary. On the other hand, hexane:ethyl acetate (70:30) was much more efficient, with 20 mL eluting all the triacylglycerols from the sample. The absence of triacylglycerols was determined by lack of spots resulting from triacylglycerols on TLC plate.

3.2. GC-FID

Prior to quantification, the retention times of most common amides were determined with the developed GC-FID method by
analysing the mixture containing the most important amides. Additionally, the identity of each of the amides was unambiguously confirmed by GC-MS.

Figure 2 shows the chromatogram of a sample mixture of different PFAA. A very good separation of the three relevant FAA palmitamide, stearamide, and oleamide found in animal fat as well as the ISTD can be seen.

The recovery of the SPE separation was done with commercially available edible grade lard, after confirmation that no traces of PFAA were found in the original sample. 0.5 g of the sample was spiked with C 18:0 amide (0.75 mg), separated with SPE, additivated with ISTD (1 mg), and analyzed. The recovery of C18:0 amide was calculated according to Equation (1). The results of five independent measurements are shown in Table 2.

As it can be seen in Table 2, the average of the recovery could be determined as 101.3%. Theoretically this value cannot be higher than 100%; one reason for that might be some nonvolatile impurities in the ISTD, which could not be found in the gas chromatogram. Furthermore, the standard deviation of the values is quite high, so these results should be seen as preliminary, more detailed work has to be done to get a better performance. Nevertheless, the recovery is higher than similar methods described in literature.

### 3.3. Determination of Detection and Quantification Limits

For each concentration of analyte in the sample, signal-to-noise ratio was calculated from the individual chromatograms. Signal-to-noise ratio was found to be 3 at the concentration of analyte of 0.002%, so 20 ppm was accepted as a limit of detection. Similarly, the signal-to-noise ratio at a concentration of the analyte of 0.005% was 10.3, so 50 ppm was accepted as a limit of quantification.

### 3.4. Determination of Amides in Real Samples

All the samples were analyzed following the scheme of developed method shown in Figure 3.

A representative chromatogram from one sample is shown in Figure 4. The calculated amounts of amides in real samples ranged from 0.04% to 1%.

The most represented amides in the analyzed samples were palmitamide, stearamide, and oleamide. The overview of fatty acid amides found in the samples, as well as the total amount of amides is given in Table 3. These results were expected, considering the types of fatty acids which are most common in animal fats.[14]

With all of the samples analyzed, the method proved to be repeatable. Analyzing the chromatograms from all the samples, it was evident that there are very few peaks which do not represent amides, which leads to the conclusion that the SPE method presented in this paper is much more efficient in separating amides from other compounds than reported previously.[4,10] For example, with the method by Madl and Mittelbach,[4] free fatty acids were co-eluted with the amides, while with this method no free fatty acids were detected in the amide eluting fraction. Additionally, the recovery of amides is improved compared to 93% and 80% stated in the previously mentioned articles. Furthermore, this method is much more time efficient, primarily because the elution is performed with small amounts of only two eluents, making it much more suitable for routine analysis. The advantages of the method are summarized in Table 4.
Table 3. Amide distribution and average content in real samples.

| Sample | C16:0 amide [%] | C18:0 amide [%] | C18:1 amide [%] | Total amide content (average) [%] | Standard deviation |
|--------|-----------------|-----------------|-----------------|----------------------------------|-------------------|
| A      | 6               | 94              | n.d.            | 0.18                             | 0.01              |
| B      | 15              | 85              | n.d.            | 0.75                             | 0.01              |
| C      | 50              | 35              | 15              | 0.99                             | 0.15              |
| D      | 68              | 11              | 21              | 0.04                             | <0.01             |
| E      | 76              | 9               | 15              | 0.05                             | <0.01             |
| F      | 81              | 6               | 13              | 0.94                             | <0.01             |

a) Calculated according to peak areas of amines; b) Calculated according to peak area of internal standard.

Table 4. Summarized method advantages over literature.

| New Literature | Old Literature |
|----------------|----------------|
| Total solvent consumption | 30 mL | (4) 120 + 120 mL |
| Sample pretreatment | not necessary | (8) derivatization |
| Instrumentation | GC/FID | (8) GC/MS |
| Solid phase extraction | 1 SPE column | (9) 2 SPE columns |
| Quality of separation | very efficient | (4,10) no quantitative separation of triacylglycerols and free fatty acids |
| Recovery | almost 100% | 80–93% |

4. Conclusion

Analysis of the real samples acquired from industrial processes revealed the presence of three different major primary amides (palmitamide, oleamide, and stearamide). These results correspond to types of fatty acids which are the most represented in animal fat. The developed method enables the reliable and efficient determination of minor components PFAA in waste animal fat used as starting material for biodiesel production. This is an important quality control of the feedstocks in order to prevent from unwanted contamination in the final product. Further research has to be carried out to prove the applicability of the method for similar biological samples.

Acknowledgements

The authors acknowledge BDI-BioEnergy International GmbH (Austria, Raaba-Grambach) for providing samples for analysis and also for their financial support.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

M.J.: Writing-original draft. S.S.: Supervision. M.M.: Supervision.

Data Availability Statement

Data sharing not applicable & no new data generated.

Keywords

animal fat, biodiesel, fatty acid amides, GC-FID, solid phase extraction
[1] T. M. Mata, N. Cardoso, M. Ornelas, S. Neves, N. S. Caetano, Energy Fuels 2011, 25, 4756.
[2] I. M. Atadashi, M. K. Aroua, A. A. Aziz, Renewable Sustainable Energy Rev. 2010, 14, 1999.
[3] R. Dunn, Prog. Energy Combust. Sci. 2009, 35, 481.
[4] T. Madl, M. Mittelbach, Analyst 2005, 130, 565.
[5] B. F. Cravatt, A. H. Lichtman, Chem. Phys. Lipids 2002, 121, 135.
[6] J. Richmond, Cationic Surfactants: Organic Chemistry, Taylor & Francis, London 1990.
[7] A. Bilyk, G. J. Piazza, R. G. Bistline, M. J. Haas, Lipids 1991, 26, 405.
[8] A. J. Gee, L. A. Groen, M. E. Johnson, J. Chromatogr. A 1999, 849, 541.
[9] T. Sultana, M. E. Johnson, J. Chromatogr. A 2006, 1101, 278.
[10] O. Palardy, C. Behnke, L. M. L. Laurens, Energy Fuels 2017, 31, 8275.
[11] J. D. Roberts, M. C. B. Caserio, Basic Principles of Organic Chemistry, Benjamin, Menlo Park, CA 1977.
[12] W. M. Haynes, CRC Handbook of Chemistry and Physics, 95th ed., CRC Press, Hoboken, NJ 2015.
[13] A. Shrivastava, V. Gupta, Chron. Young Sci. 2011, 2, 21.
[14] Fat Content and Composition of Animal Products: Proceedings of a Symposium, Washington, D.C., December 12–13, 1974, National Academy of Sciences, Washington, D.C. 2010.