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ORIGINAL ARTICLE

Head Space Single Drop Micro Extraction Gas Chromatography Flame Ionization Detection (HS-SDME-GC-FID) Method for the Analysis of Common Fatty Acids

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ABSTRACT

Objectives: Post-marketing/surveillance studies show that most of the many vegetable oils that are sold with health-promoting claims or statements with high nutritional values and are beneficial against diseases are off-limits of related monographs/criteria. Defining the oil with a fast, cheap, and efficient analytical method is needed to express fatty acids in any herbal product to authenticate, trace, specify, and classify the content.

Materials and Methods: Here, we define a new simple tool with a headspace single drop microextraction (HS-SDME) method coupled with a gas chromatography-flame ionization detector (GC-FID) for the analysis of common fatty acids (FAs) in oils. Linolenic acid, γ-linolenic acid, and linoleic acid in olive oil, thyme oil, and fish oil were determined. Derivatization was performed with 0.2 mL of 2 mol/L KOH in methanol to transfer the FAs of oils into their methyl esters (FAMEs). Then, FAMEs were extracted using a head space single drop, which is 2.0 µL of sodium dodecyl sulfate:1-butanol (1:3, *v/v*) mixture.

Results: The most suitable extraction condition was that 360 µL of the FAMEs, 2.0 mL vial, 0.07 g NaCl as a salting-out effect, 45 °C extraction temperature, and 35 min extraction time. The precision of the method was below 12%, with accuracy validated by the GC-FID reference method.

Conclusion: The HS-SDME can be used effectively for extracting FAs from oils for improved analysis of other FAs. The method is of direct importance and relevance for the herbal, pharmaceutical, and cosmetics industries.

Key words: Head space, microextraction, gas chromatography flame ionization detection, HD-SDME-GC-FID, fatty acids

INTRODUCTION

Fatty acids are monocarboxylic acids with double carbon numbers, cis structures, unbranched or straight chains, or acyclic structures. Vegetable-derived oils usually contain fatty acids with one or more double bonds in their structure. The melting points of fatty acids vary depending on the length of the chain and the degree of unsaturation. The melting points of short-chain fatty acids are lower than those of long-chain fatty acids. Thus, the melting point decreases with an increase in the number of double bonds.[1,2]

The most common fatty acid in nature is oleic acid (18:1, n-9 or omega-9); however, there is also linoleic acid (18:2, n-6 or omega 6), linolenic acid (18:3, n-3 or omega-3), and arachidonic acid (20:4, n-6 or omega-6). Although animal organisms can synthesize only a single pair of double-bond fatty acids, fatty acids with multiple double bonds (*i.e.* linoleic acid, α-linolenic acid) are also essential and should be taken externally.[3,4] Omega-3 and omega-6 fatty acids, which cannot be synthesized by the body, are converted into longer-chain fatty acids when taken into the body. Linoleic acid is metabolized to arachidonic acid in the

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body, while α-linolenic acid is metabolized to docosahexaenoic acid (22:6, DHA) and eicosapentaenoic acid (20:5, EPA), which play roles in cell membrane formation, blood clotting, wound healing, and inflammation.[3,5]

Oily fish are rich in omega-3 and omega-6, which are the fatty acids required for growth and development. Insufficient digestion of these fatty acids may result in dermatitis in children, which is characterized by a slowdown in brain development, kidney failure, and hematuria. Olive oil, which is a vegetable oil, is quite rich in terms of oleic acid (56-85%), linoleic acid (3.5-20%), and linolenic acid (1.2%).[6] Digestion of linoleic acid-rich food may have prophylactic and therapeutic effects in diseases such as cystic fibrosis, diabetes, and dermatitis.[5] There is a linear relationship between the appropriate consumption of fat (especially conjugated linoleic acid, CLA) and weight loss.[4,7]

The fatty acid content of vegetable oils depends on the plant seed because fatty acids are partially hydrolyzed by lipase enzymes because of bacterial contamination in the presence of high temperature and humidity.[8] Thus, fatty alcohols are formed through ketones, aldehydes, and short-chain fatty acids, which are produced during the contamination of certain fungi such as *Aspergillus niger*.[9] Auto-oxidation of oils may result in free radicals that cause food to decay, loss of taste quality, damage tissues, cause cancer, atherosclerosis, inflammatory events, and accelerate aging. Several factors affecting lipid auto-oxidation are the type and amount of fatty acids in the composition of fat, the presence of oxygen in the environment, metals (Cu and Fe), light, temperature, moisture, and storage conditions.[10]

Fatty acids are widely analyzed by different analytical methods such as high-performance liquid chromatography (HPLC),[11] gas chromatography (GC),[12] and capillaries electrophoresis.[13] Derivatization, which is a chemical process that increases chromatographic selectivity and sensitivity, is required to transform compounds into volatile states to prepare them for GC analysis. Compounds containing polar functional groups such as -COOH, -OH, -NH, and -SH are less volatile for gas chromatographic analysis because they form hydrogen bonds by themselves, thereby decreasing their volatility or interaction with the column. In contrast, gas chromatographic analyses can be performed by making these compounds volatile through derivatization because of the polarization of the compounds. The carboxyl group of fatty acid reacts with alcohols in the presence of the catalyst (acid, or base) to give the ester species, which is more volatile.[14-16]

Lipids are extracted from matrices using non-polar solvents and saponified with base (NaOH or KOH) to produce free fatty acid salts. The fatty acid salts are further derivatized to fatty acid methyl esters by refluxing with methanolic sodium or potassium hydroxide to improve peak symmetry, increase volatility, and decrease sample activity.[6,15] Finally, the FAMEs were extracted with a non-polar solvent (*e.g.*, heptane) for GC analysis.

The preliminary process plays a major role in the separation and enrichment of analytes in a complex matrix environment for precise, accurate, and fast analysis. Most sample preparation methods include purification steps that are time-consuming and expensive because of the chemicals used during the process.[14] In recent years, techniques such as flow injection, liquid-phase extraction, and solid-phase extraction methods have been developed to simplify and reduce the amount of solvents used in purification.[17] Although the liquid-liquid extraction (LLE) method is one of the most preferred classical sample preparation methods, it is still time-consuming and requires a large amount of organic solvents. On the other hand, the solid-phase microextraction method requires less solvent than the LLE method but contains complex extraction steps. Due to these challenges, interest has increased in micro-extraction methods compatible with gas chromatography, capillary electrophoresis, and high-performance liquid chromatography, which can be applied more easily, simplify the extraction steps, provide automation, reduce the use of organic solvents to microliter (μL) levels, and provide better enrichment. Single drop microextraction (SDME) is the most preferred method of liquid phase microextraction techniques because it is cheap, easy to apply, reduces solvent usage, and can be applied in analytical systems in the form of direct pre-concentration. It can be easily applied to GC, inductively coupled plasma mass spectrometry (ICP-MS), capillary electrophoresis (CE), and electrothermal atomic absorption spectrometry.[18] In the SDME method, the extraction solvent droplet (1-10 μL), which does not mix with the gas or liquid sample, hangs on the nozzle. Following extraction, the substances are resuspended from the aqueous sample by passive diffusion and analyzed *via* GC, HPLC or CE.[19]

Volatile and semi-volatile compounds can be determined using headspace SDME (HS-SDME).[20-23] The HS is used for extracting volatile compounds from gaseous and aqueous phases using a stir bar[24] or a single liquid collecting droplet.[25] The HS-SDME method is based on placing the microdrop of the appropriate solvent in the tip of a microsyringe needle into the space at the top of the vial (HS), which contains the sample solution, and the extraction of volatile analytes to the microdrop. This method, which has three phases: aqueous phase, gas phase, and organic drop, is used for the enrichment of volatile organic compounds and allows easy removal of analytes from the complex matrix.[20,26,27]

In our study, we aimed to develop a high-precision analytical method with a simple preliminary process for fast and accurate analysis of linoleic acid, linolenic acid and, γ-linolenic acid in fish oil, olive oil, and thyme oil. For this purpose, an esterification reaction with methanol and an alkaline catalyst was performed to transform the essential fatty acids in the samples into their methyl esters, which were extracted from the matrix using the HS-SDME method and analyzed *via* GC-FID.

MATERIALS AND METHODS

Reagents and chemicals

All chemicals used were of gas chromatographic quality. Linolenic acid, γ-linolenic acid, linoleic acid, potassium hydroxide (KOH), heptane (C_7H_{16}, 99%), and sodium dodecyl

sulfate were purchased from Sigma-Aldrich (Germany). The standards of FAs were stored at -20 ˚C. Methanol, (CH_3OH, containing less than 0.5% water) and 1-butanol were obtained from Merck (Darmstadt, Germany). NaCl, purchased from J.T. Baker, Deventer, Holland, was used to adjust the ionic strength of the solution. Helium, hydrogen, and dried air gases (for gas chromatography) were purchased from Oksan Co., Ltd. (Ankara, Türkiye). Deionized water was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). The screw-top vial used in single-drop microextraction was 2 mL of amber (Agilent Technologies Inc., US). The three commercial oil samples (olive oil, thyme oil, and fish oil) were purchased in 2018 from a local store in Ankara (Türkiye) and stored in the absence of light at ambient temperature until analysis. All solutions containing FAs were prepared in an ice bath.

Chromatographic conditions

GC-FID analysis was performed on an Agilent Technologies 7890A GC System (Santa Clara) equipped with an autosampler (Agilent 7693A Automatic Liquid Sampler, China), and J&W HP-5 (5%-phenyl)-methylpolysiloxane non-polar capillary column (30 m x 0.32 µm ID x 0.25 µm film thickness, Agilent Technologies, US). The oven temperature program was as follows: hold at 80 ˚C for 2 min, increase from 4 ˚C/min to 210 ˚C, then hold for 5 min, increase from 15 ˚C/min to 300 ˚C, and hold for 5 min. The column carrier gas was high-purity helium (≥ 99.999%) at a constant flow rate of 1 mL/min. The injection and detector temperatures were 270 ˚C and 280 ˚C, respectively. The injection volume was 1 µL with a split ratio of 100:1. Flame gases were hydrogen and dried air gases. Identification of the fatty acid methyl esters of oil samples was performed on the basis of the comparison of their retention times (t_R) with those of pure standards under the same chromatographic conditions. Peak areas were used to evaluate the number of FAMEs as a percentage.

Derivatization of the standards

Linolenic acid, γ-linolenic acid, and linoleic acid (10 mg of each) standards were dissolved in 200 µL of heptane, separately, and incubated in an ice bath. 0.2 mL of 2 mol/L potassium hydroxide in methanol was added to each solution, and caps were closed immediately, mixed with vortexing for approximately 30 s, and waited until the upper phase became clear. The FAME-containing upper phase was analyzed *via* GC-FID for 12 h.[28]

Derivatization of the samples

Each sample (olive oil, thyme oil, and fish oil) was heated in a closed system at 100 ˚C for about 3 h. One gram of oil sample was weighed, 2 mL of heptane was added, shaken, and then 0.2 mL of 2 mol/L KOH in methanol solution was added. The caps were closed and mixed with vortexing for 30 s. The clear upper phase, which contains fatty acid methyl esters, was taken for enrichment with HS single drop microextraction.

The intraday precision of FAMEs was evaluated by GC-FID analysis of the olive oil sample on the same day at four different times (0^{th}, 6^{th}, 12^{th} and 24^{th} hour) and the relative standard deviation (RSD %) was found to be 2.1-18.3% at 12 h and 2.1-25.0% for 24 h. FAMEs were used on the HS-SDME-GC-FID method for 12 h, and all analyses were performed in triplicate.

Headspace single-drop microextraction

After the derivatization of samples, microextraction and injection procedures were performed using a Hamilton gastight syringe (1700 series, SL syringe, 50 µL, needle size 22s ga, Germany). 360 µL of fatty acids methyl esters sample and 0.07 g of NaCl were placed in a 2 mL glass vial (Agilent Technologies Inc., US) equipped with a screw cap and silicone septum. Hamilton gastight syringe containing 2.0 µL of sodium dodecyl sulfate: 1-butanol mixture (1:3, *v/v*) was immersed from the septum up to 0.5 cm of the solution. The temperature was adjusted to 45 ˚C for 35 min. Following microextraction, the solvent was retracted into the gastight syringe, and the syringe's valve was turned off until it was transferred to the heated injection port of the GC-FID.

RESULTS AND DISCUSSION

All microextraction parameters were determined in the derivatized olive oil sample according to the peak area of the linolenic acid methyl ester. The optimization assay used a successive optimization approach, in which one parameter was changed at a time, and the others were kept constant. The most important step for the HS-SDME method is selecting the appropriate extraction solvent. Thus, the boiling point of the solvent and its viscosity are important parameters in the selection criteria for the solvent in the micro-extraction method. Hence, the solvent's boiling point must be low enough to conform to gas chromatographic analysis, but higher than the boiling point of the analytes to prevent evaporation during the extraction process. Moreover, the solvent must have a high viscosity sufficient to adhere to the tip of the syringe needle, as well as a viscosity low enough to allow rapid diffusion of the analyte to the drop that significantly affects the extraction time. Various types of extraction solvents (1-butanol, 1-octanol, acetonitrile, diethyl acetate, toluene, hexane, heptane, dichloromethane: hexane: toluene, sodium dodecyl sulfate: 1-butanol) were attempted to select the best solvent for the microextraction of FAMEs. Sodium dodecyl sulfate:1-butanol mixture (1:3, *v/v*) was the most suitable extraction solvent for HS-SDME. The extraction efficiency of the analyte depends on the microdrop volume; however, as the drop volume increases, the drop on the needle of the syringe becomes unstable. Therefore, 2 µL of extraction solvent was used in further steps of the experiment.

Optimization of other variables in the HS-SDME was the quantity of the salting-out reagent (NaCl), the micro-extraction time, and temperature. The total vial volume was tested with 10 mL and 2 mL of vial, where 2 mL of vial was selected without stirring in further studies.

In conventional extraction methods, the addition of salt to the aqueous sample solution is an accepted approach to increase the extraction efficiency and ensure that the polar analytes in the organic phase remain intact. As the ionic strength of the medium increases with the addition of electrolytes to the

samples prepared in the aqueous medium, the water solubility of the polar analytes and the organic compounds decreases. Thus, the rate of analyte passing to the organic phase and the extraction efficiency of the method increases. The reduction of the solubility of analytes in water occurs when the ionic salt molecules (salting out effect) in the aqueous medium surround the hydration layer. In addition, salt molecules may interact with polar molecules electrostatically, leading to reduced mass transfer of analytes. Depending on the solubility of analytes, the amount of extraction may increase with the salting out effect at high salt concentrations or the extraction of analytes may decrease due to the attraction forces between charged particles, electrolytes, and analytes dispersed in the solution. Although adding salt to the sample solution generally increases the extraction efficiency, the presence of salt in high concentrations may change the physical properties of the extraction film and decrease the diffusion rate of the analyte in the organic phase.[29,30] In the present study, the extraction efficiency of FAs from the aqueous phase to a single drop was determined using NaCl as the inorganic salt. The effect of increasing the ionic strength of the solution was evaluated by adding NaCl with the highest peak area of 0.07 g NaCl.

The extraction time was tested between 15 and 45 min with the optimum extraction time being 35 min (Figure 1). The extraction temperature was determined between 25 and 45 °C , and the maximum peak area was obtained at 45 °C (Figure 2).

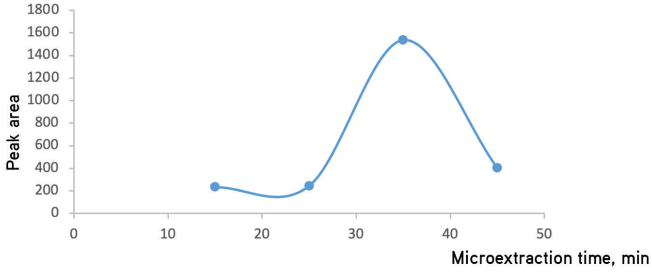

Figure 1. Effect of time on the extraction efficiency for HS-SDME-GC-FID method (360 µL sample, 2 µL microdrop of sodium dodecyl sulfate: 1-butanol (1:3, *v/v*), extraction temperature 45 °C, 0.07 g NaCl)

HS-SDME-GC-FID: Headspace single-drop microextraction- gas chromatography-flame ionization detector

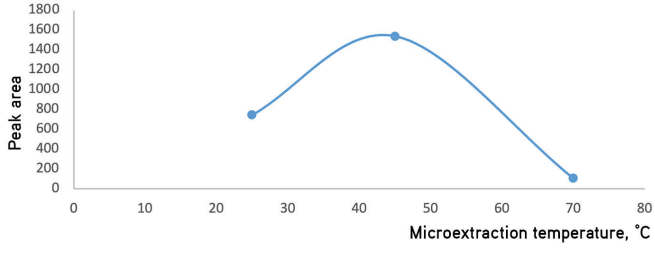

Figure 2. Effect of temperature on the extraction efficiency for HS-SDME-GC-FID method (360 µL sample, 2 µL microdrop of sodium dodecyl sulfate: 1-butanol (1:3, *v/v*), extraction time 35 min, 0.07 g NaCl)

HS-SDME-GC-FID: Headspace single-drop microextraction-gas chromatography-flame ionization detector

Further microextraction studies were conducted by 360 µL FAMEs, 0.07 g NaCl in 2.0 mL vials, and applying microextractions for 35 min at 45 °C. The applied extraction conditions and obtained highest efficiency values are summarized in Table 1.

The GC-FID chromatograms of the linolenic acid, γ-linolenic acid, and linoleic acid methyl ester standards are presented in Figure 3. The retention times (t_R) of linolenic acid methyl ester, γ-linolenic acid methyl ester, and linoleic acid methyl ester were found to be 35.82, 35.04, and 34.32 min, respectively.

Identification of the fatty acid methyl esters in oil samples was performed on the basis of the comparison of their retention times (t_R) with those of pure standards. The peak areas were used to evaluate the number of FAMEs as a percentage under the optimum HS-GC-FID chromatographic conditions. Chromatograms of thyme oil, olive oil, and fish oil fatty acid methyl esters with GC-FID and HS-SDME-GS-FID at optimum conditions are shown in Figures 4-6, respectively.

The accuracy was determined by comparing the HS-SDME-GC-FID quantitative results with the GC-FID reference[6,31] and methods (Table 2). Olive oil, thyme oil, and fish oil fatty acid methyl esters were calculated on the basis of the total fatty acid methyl ester amounts according to the normalization method received from the GC-FID and HS-SDME-GS-FID analysis results. The results were found to be compatible with the European Pharmacopoeia criteria.[6] Statistical analysis was performed at 95% confidence limit (p = 0.05). HS-SDME-GC-FID and GC-FID reference method values were compared using Student's t-test at 95% confidence limit. "t" values were calculated from equation 1,[32,33] and s values were calculated from equation 2.[32,33] Because the calculated "t" values were smaller than t_{table}, they are statistically acceptable and the proposed method is significant for accurate use in further studies.

$$t = [(|x_{ort} - y_{ort}|)/s\ ] \times \sqrt{[(m \times n) / (m+n)]}$$

Equation 1

$$s = \sqrt{[(\sum x_i^2 - (\sum x_i)^2/m + \sum y_i^2 - (\sum y_i^2)/n) / (m+n-2)]}$$

Equation 2

s: standard deviation of two series

x_{mean}: Mean value of Serie 1 (HS-SDME-GC-FID results)

y_{mean}: Mean value of Serie 2 (GC-FID results)

m: Number of data points of Serie 1

n: Number of data points of Serie 2

The precision of the HS-SDME-GC-FID method was determined

Table 1. Factors and experimental condition applied for optimization of the HS-SDME

Factors	Experimental conditions			
NaCl (g)	0.02	0.04	0.07*	
Extraction time, min	15	25	35*	45
Extraction temperature, °C	25	45*	70	
Sodium dodecyl sulfate:1-butanol (*v/v*)	1:0	1:1	1:2	1:3*

*Optimum value

Table 2. Percentage of FAMEs obtained with GC-FID (n: 3) and HS-SDME-GC-FID (n: 3). % (*w/w*) and *t*-value (p = 0.05)

Sample	γ-Linolenic acid methyl esther (mean % ± SD*)	Linolenic acid methyl esther (mean % ± SD*)	Linoleic acid methyl esther (mean % ± SD*)
Fish oil (HS-SDME-GC-FID)	1.0 ± 0.2	2.9 ± 0.4	1.2 ± 0.3
Fish oil (GC-FID)	1.5 ± 0.1	2.8 ± 0.2	1.0 ± 0.2
$t_{calculated} < t_{table}$	1.78 < 2.78	0.61 < 2.78	1.1 < 2.78
Reference value of fish oil (EU 2011)		0.5-3.0	0.5-3.0
Thyme oil (HS-SDME-GC-FID)	23.7 ± 1.3	1.0 ± 0.3	2.5 ± 0.1
Thyme oil (GC-FID)	25.4 ± 1.5	1.4 ± 0.2	2.7 ± 0.2
$t_{calculated} < t_{table}$	2.55 < 2.78	1.00 < 2.78	2.08 < 2.78
Reference value of thyme oil (EU 2014)		max. 1.2	3.5-20.0
Olive oil (HS-SDME-GC-FID)	5.2 ± 0.5	1.7 ± 0.2	2.2 ± 0.1
Olive oil (GC-FID)	5.9 ± 0.2	1.9 ± 0.1	2.1 ± 0.2
$t_{calculated} < t_{table}$	1.21 < 2.78	0.51 < 2.78	0.78 < 2.78
Reference value of olive oil (EU 2011)		Max 1.2	3.5-20

*Standard deviation
HS-SDME-GC-FID: Headspace single-drop microextraction-gas chromatography-flame ionization detector

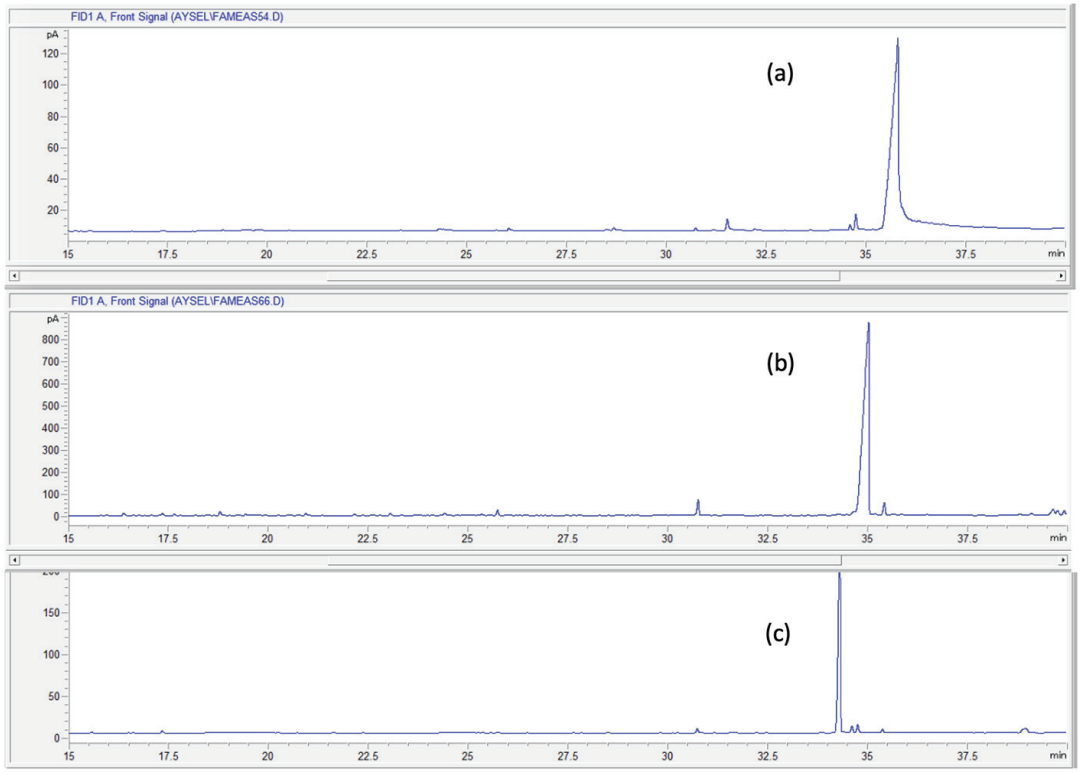

Figure 3. GC-FID chromatogram of (a) linolenic acid methyl esther (t_R: 35.82 min), (b) γ-linolenic acid methyl esther (t_R: 35.04 min), (c) linoleic acid methyl esther (t_R: 34.32 min)
GC-FID: Gas chromatography-flame ionization detector

as RSD % (n: 5) as 11.7% for linoleic acid methyl ester, 12.4% for γ-linolenic acid methyl ester, and 11.2% for linolenic acid methyl ester.

The extraction efficiency of the method was calculated to be 41.5 ± 3.7% according to the GC-FID and HS-SD-GC-FID peak areas.

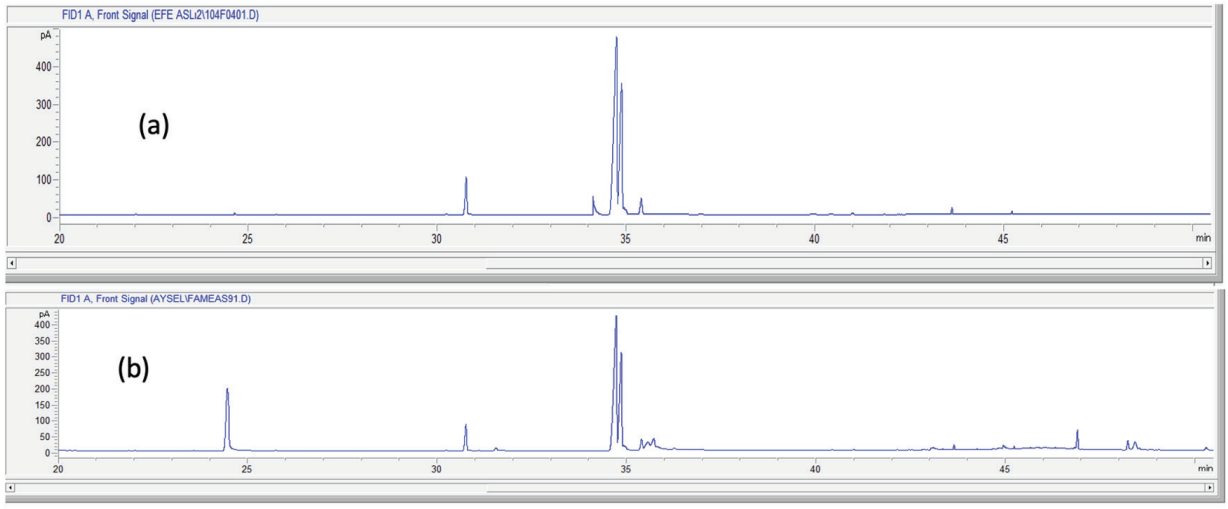

Figure 4. Chromatogram of thyme oil methyl esters obtained with a) GC-FID b) HS-SDME-GC-FID.
HS-SDME-GC-FID: Headspace single-drop microextraction- gas chromatography-flame ionization detector

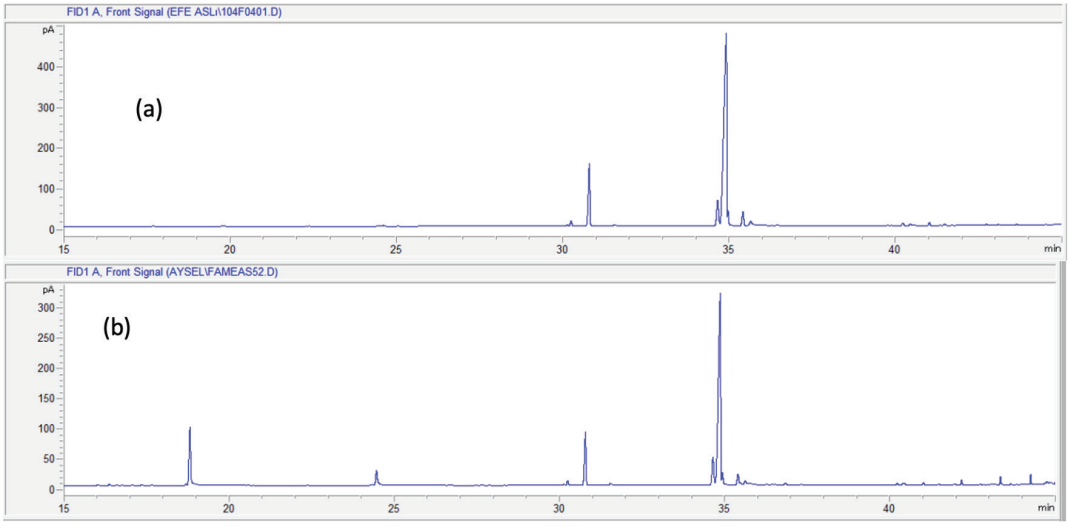

Figure 5. Chromatogram of olive oil methyl esters obtained with a) GC-FID b) HS-SDME-GC-FID.
HS-SDME-GC-FID: Headspace single-drop microextraction- gas chromatography-flame ionization detector

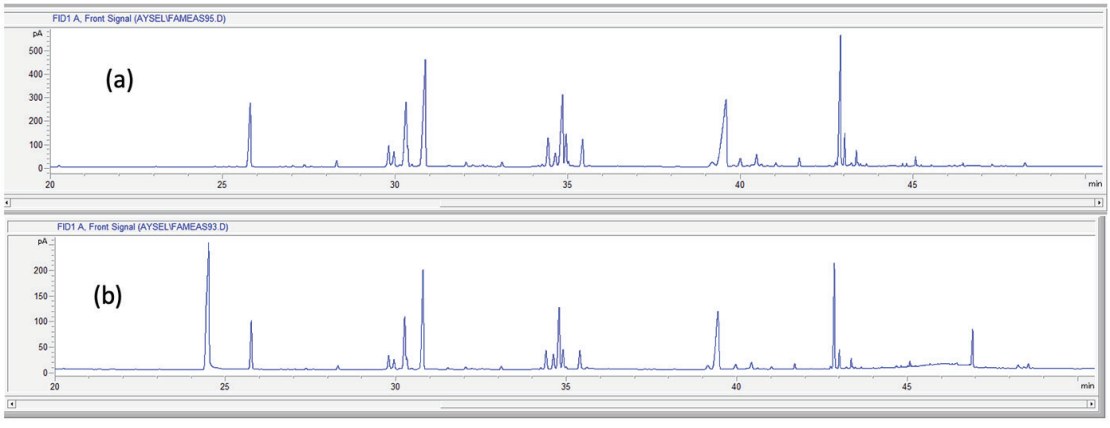

Figure 6. Chromatogram of fish oil fatty acid methyl esters obtained with a) GC-FID b) HS-SDME-GC-FID.
HS-SDME-GC-FID: HS-SDME-GC-FID: Headspace single-drop microextraction- gas chromatography-flame ionization detector

CONCLUSION

HS-SDME-GC-FID is a rapid, reproducible, and accurate method for the analysis of FAMEs (γ-linolenic acid, linolenic acid, and linoleic acid methyl esters). This method reduces the amount of extraction solvent (green chemistry) and thereby the cost. High precision (below 12.4%), simple sample preparation, enrichment of the analyte, and removal of the matrix from the analyte may allow the use of this proposed method for routine analysis in industry and research laboratories.

It is also possible to determine fatty acids in samples using an external calibration method using the standards of γ-linolenic acid, linolenic acid, and linoleic acid methyl esters. However, because of the cost of standard fatty acids, studies are often designed on percentage quantities. However, fatty acid methyl esters can also be easily analyzed using a GC mass spectrometry (GC-MS), its existing library without using costly standards, although libraries must contain accurate all *m/z* spectra of oil, which type of library may cost high as standards' itself. Here, the method developed was analyzed using carbon-sensitive FID detector due to the long carbon chain structures of fatty acids. The method we developed with easy sample preparation and repeatable results could also be used successfully in the analysis of different types of oils and fatty acids using standards containing methyl esters of all fatty acids. Because it is defined using a fast, cheap, and efficient analytical method, the new technique can also be used to express fatty acids in any food or product, to authenticate, trace, specify, and classify the content, whatever or wherever such technique is needed.

Ethics

Ethics Committee Approval: There is no requirement for ethical approval.

Peer-review: Externally peer reviewed.

Authorship Contributions

Concept: A.E., O.K.U., A.B., Design: A.E., O.K.U., A.B., Data Collection or Processing: A.E., O.K.U., A.B., Analysis or Interpretation: A.E., O.K.U., A.B., Literature Search: A.E., O.K.U., A.B., Writing: A.E., O.K.U., A.B.

Conflict of Interest: No conflict of interest was declared by the authors.

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