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Fatty Acids Analysis Using Gas Chromatography-Mass Spectrometer Detector (GC/MSD) - Method Validation Based on Berry Seed Extract Samples

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Abstract Oily seed extracts were used as research samples in Analytical Laboratory of Raw Materials and Plant Products. Supercritical carbon dioxide extraction on an industrial scale was used to prepare extracts from raw plant materials, usually berry seeds: blackcurrant, raspberry, strawberry, and chokeberry. Gas chromatography analysis was performed on Agilent equipment with single quadrupole mass spectrometer detector and split/splitless injector. Fatty acids (FAs) were determined using indirect method, where they were converted to corresponding methyl esters (FAMEs) during trimethylsulfonium hydroxide solution reaction. Comparing the mass spectrum and retention time peak, more than 30 compounds in different oily extracts were qualitatively identified. Quantification of individual fatty acids was based on two different methods. Firstly, it was based on the received peak area, and the results were normalized without correction factor. Secondly, the quantification was based on external calibration curve, for 34 identified fatty acids. The method was validated, and the results, e.g., linearity, precision, limit of detection (LOD), and limit of quantification (LOQ), were presented.

Keywords Fatty acids · Oily plant extracts · GC/MSD · Method validation

Introduction

Plants are not easy to research. They have complicated matrix, which is problematic during analysis (*matrix effect*) (Yaroshenko and Kartsova 2014); however, they also contain many valuable, healthy substances known as bioactive compounds including flavonoids (polyphenols), phenolic and hydroxycinnamic acids, lignans, vitamins, carotenoids, monoterpenes, and lipids (phytosterols, tocopherols, and saturated and unsaturated fatty acids).

Analysts usually use all parts of plants (roots, stalks, leaves, flowers, fruits, and seeds), as well as juices, concentrates, and extracts which are the final products of the technological processes. Agricultural production and the berry fruit industry have a significant position in Poland (Nawirska et al. 2007; Agricultural Market Agency 2014; Kraciński 2014). In 2013, the fruit share in the value of commodity crop production was more than 15%. In 2004–2013, berry fruit collection, in Poland (Fig. 1), were 5–11% of the fruit products in EU. In 2013, they were at the record level of 4.13 million tons, about 29% higher than average yields in 2004–2012 (Agricultural Market Agency 2014; Kraciński 2014).

What fuelled the growth of fruit production were farmlands in quite good conditions and a large group of well-educated and experienced young fruit growers, especially in the berry fruit sector, which in 2013 was at the level of 607,000 t.

A proper processing base is one of the key factors in the development of berry fruit production. Among the processed fruits, frozen ones dominate (40%), but concentrated juices (30%) and pomaces (16%) also have a significant share.

Extracts can be obtained from pomaces, which are the wastes or by-products in plant production processes and contain up to 50% (*w/w*) of seeds, rich in nutrients and biological active substances. Dry seeds from blackcurrant, raspberry, or strawberry may contain up to 25% of fat and 20% of proteins,

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Fig. 1 The structure of berry fruit crops in Poland in 2013 (Agricultural Market Agency 2014)
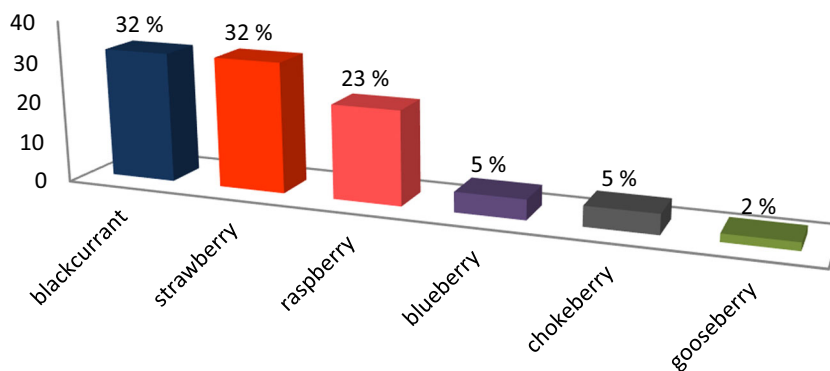
and in this way, they can be an interesting material to receive oily extracts rich in unsaturated and polyunsaturated fatty acids (Nawirska 2007; Rój et al. 2009; Nowak 2005; Dobrzyńska et al. 2014).

There are different methods for obtaining extracts from solid samples like plants and plant seeds. The most popular in laboratories and manufactories were conventional simple solvent extractions (solid–liquid extraction, Soxhlet extraction). Nowadays, liquid–liquid or solid–liquid extractions are usually assisted by external factors (e.g., mechanical agitation, pressing and/or heating system) giving more rapid and automated methods (e.g., pressurized liquid extraction (PLE), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), accelerated solvent extraction (ASE)). They have an advantage over conventional methods because they are time saving and solvent reducing and can be carried out with no oxygen or light which prevents the degradation of desired substances (Nayak et al. 2015; Rombaut et al. 2014; Chemat et al. 2015; Da Porto et al. 2009). Another extraction method, old but reactivated now because of their similarity to "the Green Analytical Chemistry," is the cold-pressed extraction (Armenta et al. 2008; Chemat et al. 2012; Tobiszewski et al. 2009; Płotka et al. 2013; Tobiszewski and Namieśnik 2012; Van Hoed et al. 2011). As we can read in the Codex Alimentarius, "*cold pressed fats and oils are edible vegetable fats and oils obtained by mechanical procedures e.g. expelling or pressing, without the application of heat*" (Codex Alimmentarius Commission; Obiedzińska and Waszkiewicz-Robak 2012). The obtained extract can only be purified by washing with water, precipitating, filtrating, or centrifuging.

In general, the food industry prefers "green extraction and processing" to ensure safe and high-quality extracts (Nayak et al. 2015; Chemat et al. 2012).

These assumptions fully comply with another type of extraction: supercritical fluid extraction (SFE). This method has been used in the New Chemical Syntheses Institute in Puławy, Poland, since 2000, and was the first one of this type used in Central and Eastern Europe (Skowroński 2005; Skowroński

and Mordecka 2001; Rój 2009; Rój and Skowroński 2006a, b). In the middle of 2011, the Institute launched another research and production of a supercritical CO_2 (scCO_2) extraction plant to extract oils from raw plant materials. It has 2000 t/ year processing ability and works in a pressure range 20– 53 MPa (Rój et al. 2009; Rój et al. 2013). SFE is solvent and waste-free, is faster than conventional liquid–liquid methods, and with easy parameter control, provides a certain selectivity (Meyer et al. 2012; Aladić et al. 2015).

In a significant number of publications about scCO_2 extraction of raw plants or their parts, we can find that several authors described matrix effects (Aladić et al. 2015; Araus et al. 2009; Azmir et al. 2013), and others discuss optimized reaction parameters in the production of plant oils using, for example, response surface methodology (Rój and Skowroński 2006a, b; Azmir et al. 2013; Stamenic et al. 2010; Watros et al. 2013; Da Porto et al. 2012a, b; Ara et al. 2015). Most of them used scCO_2 laboratory-scale extraction installation (up to 2 L extraction vessel) to obtain oils from the most popular fruits, vegetables, or herbs, e.g., grapes, olives, and sunflowers (Yang et al. 2011; Rai et al. 2016; Aladić et al. 2015; Milić et al. 2015; Duba and Fiori 2015; Del Valle 2015; Da Porto et al. 2012a, b), but only a few of them used supercritical CO_2 industry-scale extraction plant to obtain oils, especially from berry seeds (Rój et al. 2013).

In this study, scCO_2 extracts from blackcurrant, strawberry, raspberry, and chokeberry seeds from an industry-scale plant (extraction vessel 2200 L) were used as samples in order to determine fatty acid content. The composition of oils obtained by the described method can be compared with the oils extracted by other techniques and can be used in the future study on the characteristics of berry seed oils according to various contents of unsaturated and polyunsaturated fatty acids. Also, the achieved value of fatty acid (FA) content can help to choose appropriate oils for, e.g., pharmacy, medicine, or food industry.

Most regulations and quality standards in laboratories required validation of analytical methods. The results from method validation can be used to determine the quality,

reliability, and consistency of analytical results, which is an integral part of any good analytical practice. To obtain consistent, reliable, and accurate data from analytical measurement, the method of fatty acid analysis in berry seed oils was validated.

Materials and Methods

Chemicals, Standards, and Reference Materials

All chemicals and reagents were of analytical reagent grade. Analytical standards of FAMEs (methyl butyrate; methyl hexanoate, methyl octanoate; methyl decanoate; methyl undecanoate; methyl laurate; methyl tridecanoate; methyl myristate; methyl pentadecanoate; methyl palmitate; methyl palmitoleate; methyl heptadecanoate; methyl cis-10-heptadecenoate; methyl stearate; methyl elaidate; methyl oleate; methyl linolelaidate; methyl linoleate; methyl γ-linolenate; methyl arachidate; methyl linolenate; methyl cis-11-eicosenoate; methyl heneicosanoate; cis-11,14-eicosadienoic acid methyl ester; cis-8,11,14-eicosatrienoic acid methyl ester; methyl behenate; methyl arachidonate; cis-11,14-17-eicosatrienoic acid methyl ester; methyl tricosanoate; methyl all-cis-5,8,11,14,17-eicosapentaenoate; cis-13,16-docosadienoic acid methyl ester; methyl tetracosanoate; methyl cis-15-tetracosenoate; all-cis-4,7,10,13,16,19-mocosahexaenoic acid methyl ester) were from Sigma-Aldrich Co. (St. Louis, MO, USA). Trimethylsulfonium hydroxide (TMSH) solution (~0.25 M in methanol) for GC derivatization was delivered by Fluka (Sigma-Aldrich Co., St. Louis, MO, USA). Tert-butyl methyl ether (MTBE) 99.8%, sodium chloride, and isooctane were purchased from POCH S.A. (Gliwice, Poland). Boron trifluoride (~1.3 M in methanol) was from Fluka (Sigma-Aldrich Chemie GmbH, Steinheim, Switzerland). Standard Reference Material 3251 *Serenoa repens* Extract was from NIST; Extract Reference Material (XRM) *Serenoa serrulata* (Saw Palmetto) Fruit CDXA-XRM-001 was from ChromaDex.

All standards and materials were stored in appropriate conditions (a fridge or a freezer).

Plant Material

Seeds of different species of blackcurrant, strawberry, raspberry, and chokeberry were purchased from external suppliers, as a by-product in plant production processes. All obtained scCO$_2$ berry seed extract samples were stored in a fridge, under nitrogen atmosphere, in 25–50-mL orange glass bottles.

Fatty Acid Extraction

scCO$_2$ extraction allows extraction of plant oils at temperature above 35 °C and pressure above 20 MPa. Extracts from blackcurrant, strawberry, raspberry, and chokeberry seeds obtained from scCO$_2$ industry-scale installation, using extraction vessel 2200 L, were prepared and delivered to the laboratory by internal suppliers. According to Rój and collaborators (Rój et al. 2009), during the processes some of scCO$_2$ extraction conditions were changed (extraction time and pressure from 28 to 36 MPa), and others were constant (T = 50 °C; CO$_2$ flow = 80 kg/h), so that the mixture of extracts were analyzed. Some samples were collected separately every 15–20 min and were used to define the FA profile during the cycle time process. Seeds were prepared as described in earlier publications (Rój et al. 2013).

FAME Preparation

In the first part of the experiment, two different methods to obtain FAMEs were used. Both of them are normalized and described in the Polish version of the European Standard EN ISO 5509:2000.

A first few extract samples were prepared as follows: About 150 mg of extract from berry seeds and 4 mL of sodium hydroxide methanol solution (0.5 M) were placed into a 50-mL round-bottom flask. The reflux condenser was placed, and the flask was heated for 25 min. Next, 5 mL of boron trifluoride (BF$_3$ ~ 1.3 M in methanol) was added via the upper end of a reflux condenser and heated for 5 min. Then, 3 mL of isooctane was added into a boiling mixture, the reflux condenser was disconnected, and the mixture was cooled in an ice water bath, as fast as possible. From 20 to 40 mL saturated NaCl solution was added to the mixture, shaked vigorously, and left for phase separation. The isooctane phase (1–2 mL) was placed into an autosampler vial and directed for GC analysis.

The described method was appropriate for the analyzed type of samples. BF$_3$ as acylation reagent for GC derivatization required temperature about 100 °C, so that the method was solvent- and time-consuming. The high number of samples received from industry plant and necessity of online analysis of FA content requires faster method application.

The second method described in EN ISO 5509 was used and validated. Samples (without internal standards) were prepared at room temperature as follows: About 10 mg ± 3 mg of analytical sample was placed into a 1.5-mL autosampler vial. Five hundred microliters of tert-butyl methyl ether was added and mixed for 5 min to dissolve the sample. Next, 250 μL of TMSH solution (~0.25 M in methanol) was added and, without heating, all mixture was shaken for 25 min. Samples were usually diluted 1:10 or 1:20 with MTBE/CH$_3$OH (9:1 *v/v*).

After that, the sample vial was placed into the autosampler and analyzed.

GC-MSD Analysis

Gas chromatography analysis were performed on Agilent equipment (GC 6890N) with single quadrupole mass spectrometer detector (MSD 5975) and split/splitless injector. Both systems were controlled by MSD ChemStation, version E.02.02.1431 (Agilent Technologies, Inc.). An Agilent J&W GC capillary column, type HP-88, with 88% cyanopropylaryl polysiloxane phase (60 m, 0.25 mm i.d., 0.20 μm film thickness) was used to separate FAMEs. Some notes or applications (EN-ISO 5509 2000; Sigma-Aldrich Brochure 2007) do not recommend this type of column phase with TMSH-prepared samples, especially during cold on-column injection or when lipids with hydroxy groups occur. Any undesirable effects were not observed in our analysis. The oven temperature program was started at 90 °C held for 2 min, increased to 152 °C at a rate of 4 °C/min, held for 1 min, and increased to 218 °C at the rate of 2 °C/min, held for 1 min. Total analysis time was 53 min. The split/splitless injector was used with injector temperature 250 °C and split ratios 6:1 and 120:1. Helium was used as a carrier gas, with a flow rate of 1.5 mL/min. Inject volume was 1 μL, solvent delay 3.5 min, and MSD ionization voltage 70 eV. Data were collected in SCAN and SIM mode. FAMEs were identified by comparing their mass spectrum (Fig. 2) and fragmentation patterns in the NIST library and by comparing retention time peak with appropriate standards. The FA contents were expressed as weight percentages, % w/w (g FA/100 g of sample). Samples were prepared separately in duplicates, and the average values were presented as final results. The obtained data were analyzed statistically during method validation using internally prepared spreadsheets in Microsoft Office Excel 2007 and online available authorized computer program e-Stat (available in Polish version).
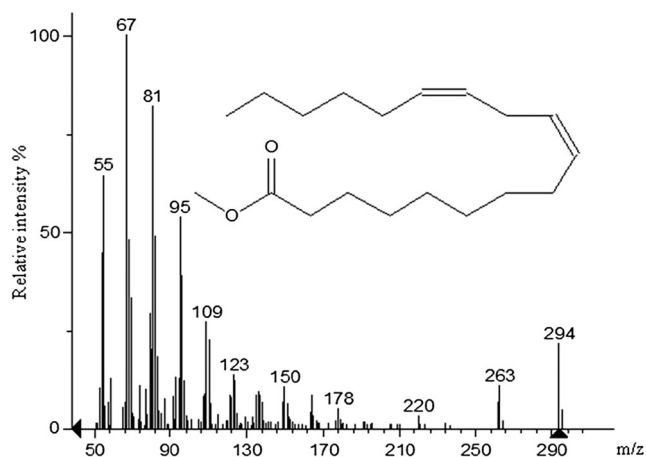

Fig. 2 Mass spectrum of the linoleic acid methyl ester

Results and Discussion

Chromatography Results

The pro-health approach to fatty acids forced the producers of oils and food, as well as companies that deal with this subject, to check the level and content determination of individual fatty acids and their isomers. It is not easy, especially for little-known matrix, and the extracts of oil obtained by scCO₂ extraction are this kind of matrices.

All unsaturated lipids are oxidized under the influence of many external factors, and spontaneous oxidation is observed with regard to mainly unsaturated hydrocarbon chains of fatty acids. The rate of this reaction increases with the increase in the degree of unsaturation. According to the literature, linoleic acid is oxidized 10–40 times faster than oleic acid and linolenic acid 2–4 times faster than linoleic acid (Drozdowski 2007).

The developed method allows the preparation of a draft specification of oil extracts and indicates the quantity and nature of present acids (cis/trans isomers and saturated and unsaturated acids), from which you can determine the durability of the extract and the rate in the oxidation process.

A typical chromatogram of the analysis of the chosen 34-compound FAME standard, obtained on the HP-88 column, is shown in Fig. 3.

A very good separation is obtained, except the following compounds: C20:4 (n6) coelute at 39.5 min with C20:3 (n3). Also very close, but separated, are C20:5 (n3) at 42.1 min and C22:2 at 42.2 min. However, this separation is sufficient for the analyzed extract from the chosen berry seeds.

Using this method, all FAs can by determined in scCO₂-obtained extracts. This is demonstrated in Figs. 4 and 5, where the analysis of blackcurrant and raspberry seed oil samples is shown. Peaks are identified by giving retention time presented in Table 1.

Free FAs can be analyzed directly on polar stationary phases, but more robust and reproducible chromatographic data are obtained if the fatty acids are derivatized to the corresponding methyl esters. Different methods and reagents are available for the derivatization, selective response, and detection in complex matrices (Sigma-Aldrich Brochure 2007). The TMSH method, mentioned in this research paper, is easy to use and does not require expensive equipment and reagents. Also, during reaction with TMSH, there is no isomerization of polyunsaturated fatty acids and removing excess of reagent is not required, because in the injector at a temperature of 250 °C pyrolysis occurs (methanol and $(CH_3)_2S$ evaporate).

Using an HP-88 column and the presented gradient temperature program, all compounds in the standard mixture and in the berry seed extracts are well separated. What is important is the separation of cis/trans isomers like C18:1 (n9) cis/trans and C18:2 (n6) cis/trans and the separation of polyunsaturated
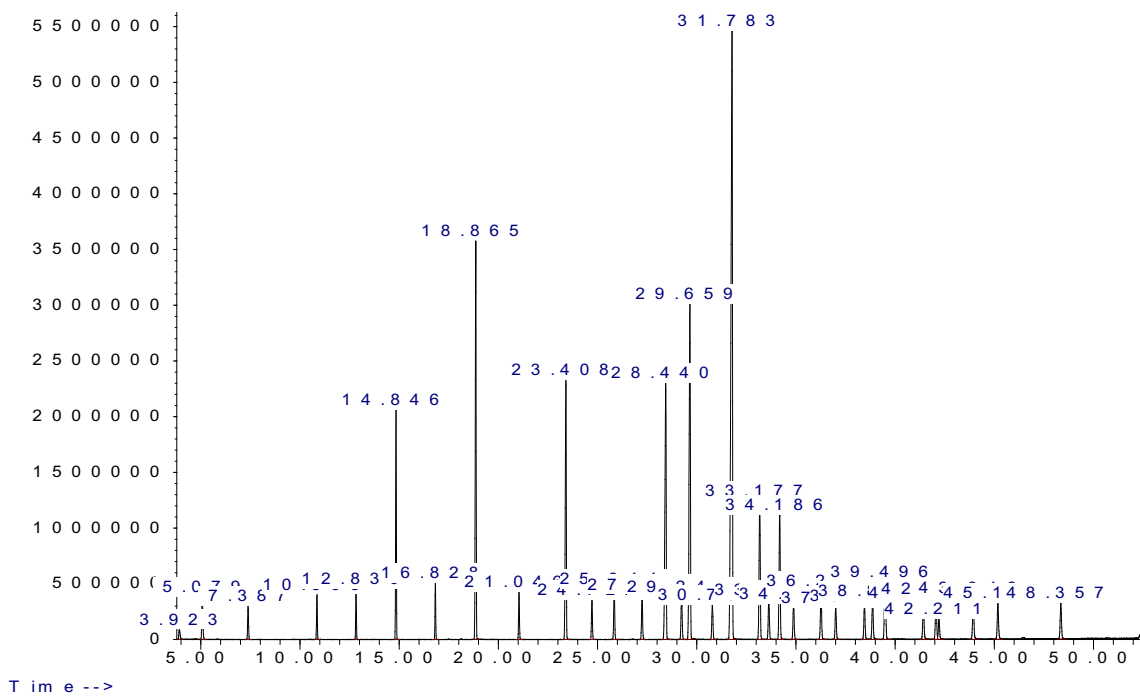
Fig. 3 GC/MSD analysis of 34-component FAME mixture on HP-88 column

components like C20:5 (n3) EPA (42.1 min) and C22:6 (n3) DHA (48.4 min). This method is useful for the determination of omega-3 fatty acids (such as EPA and DHA) and also omega-6 fatty acids, such as C18:2 cis (n6), C18:3 (n6), C20:2 (n6), C20:3 (n6), and C20:4 (n6) presented at retention times 31.8, 33.2, 37.0, 38.5, and 39.5 min, respectively. Figure 4 or 5 demonstrates that for real samples containing

several acids and different isomers, separation with the HP-88 column is a good choice.

Examining the qualitative composition of selected samples presented in Table 1 and changes to the composition, during the extraction process (Table 2, Fig. 6), it can be concluded that these extracts obtained a good source of unsaturated fatty acids.
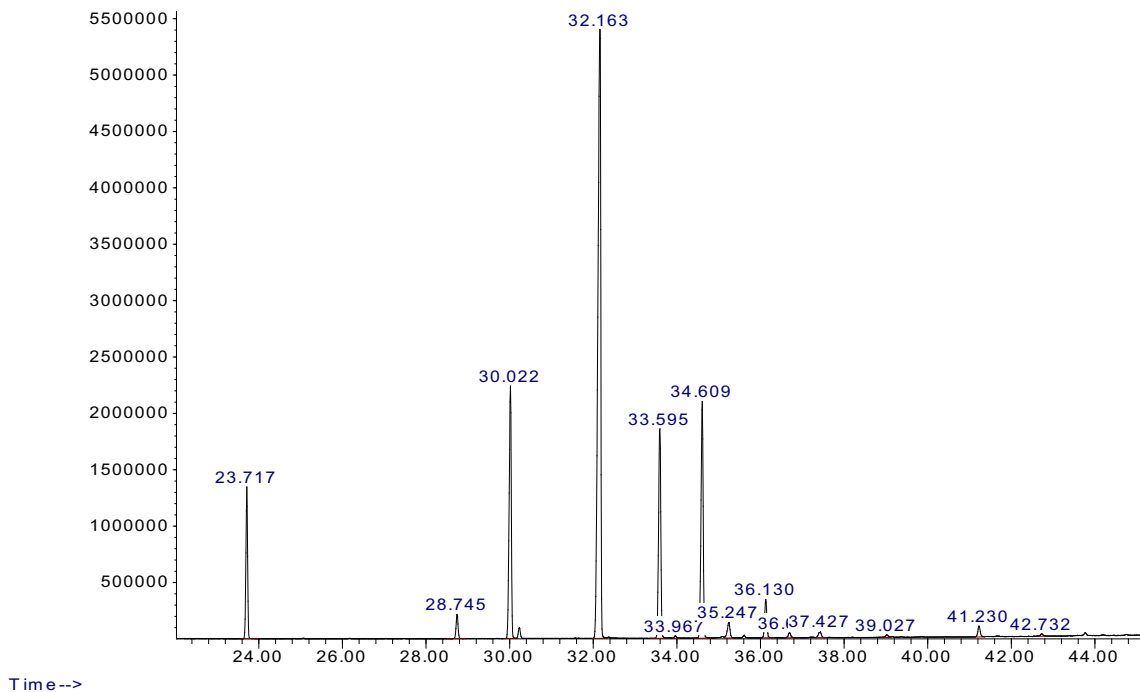

Fig. 4 GC/MSD analysis of FAMEs from scCO$_2$-extracted blackcurrant seeds on HP-88 column
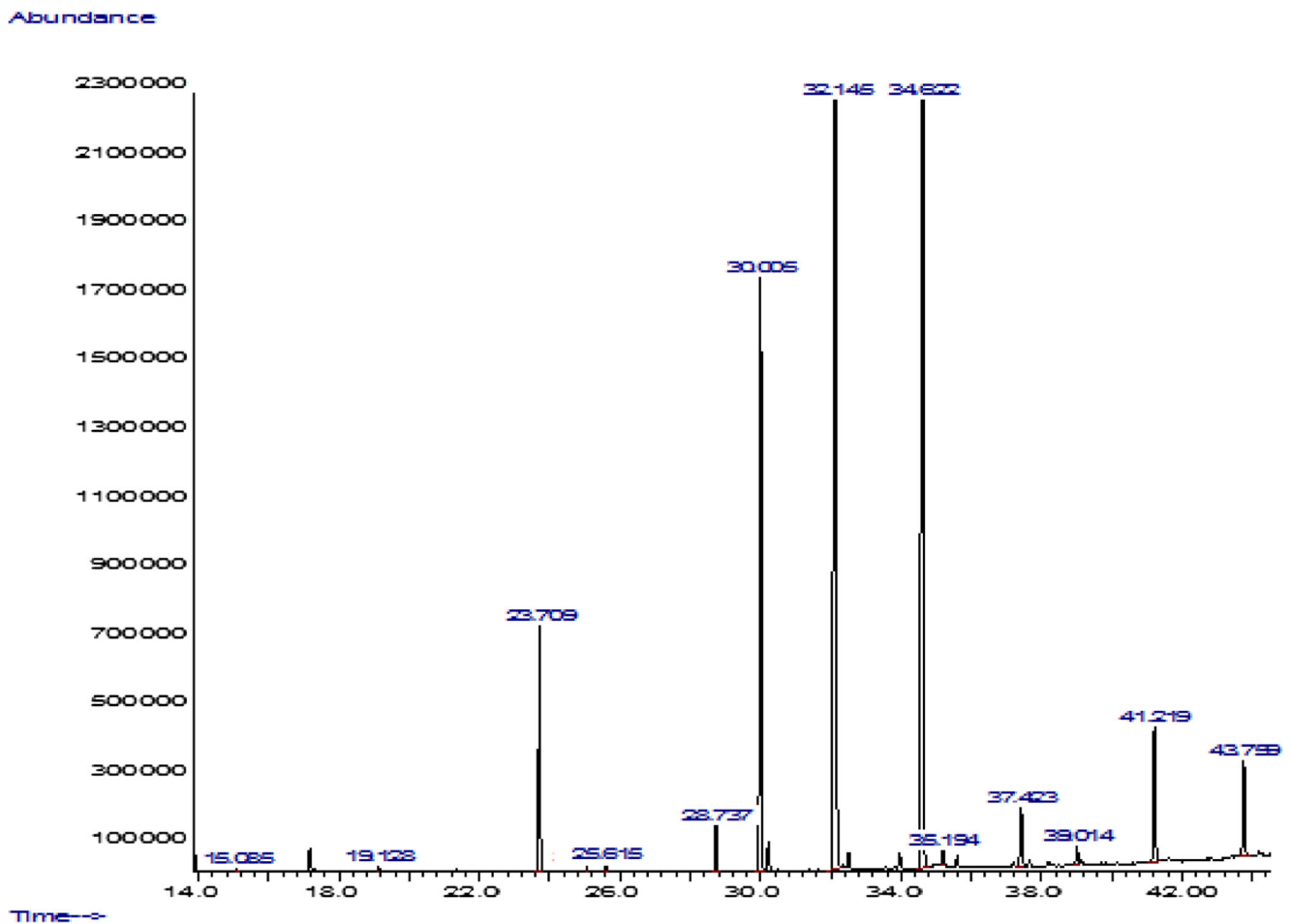
Fig. 5 GC/MSD analysis of FAMEs from scCO$_2$-extracted raspberry seeds on HP-88 column

For the analyzed samples of oily extracts, more than 30 fatty acids were shown. The basic ten are C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C18:1 cis (n9), C18:2 cis (n6), C18:3 (n6), and C18:3 (n3). The others are present in the amounts of less than 0.2% or below specified values of detection limits of the analytical method.

The highest total content of fatty acids was obtained during the analysis of the most easily accessible material of blackcurrant seeds. Total content of FAs was 63–78%, and in one sample even 88%. Next comes a strawberry extract with values of the total content of fatty acids from 50 to 69%, the raspberry interval 44–66%, and chokeberry 25–58%. As shown in Table 1, one raspberry sample had a content of total FAs of 98%. The biggest change in the composition of the extract relates to changes in extraction time. Changes in pressure did not affect significantly the profile change of fatty acids and their concentrations (Dobrzyńska et al. 2014).

Among the saturated fatty acids, palmitic acid dominates, whose value ranges from 3.2% w/w to 9.2% w/w for blackcurrant extract (average concentration about 5.2% w/w in analyzed samples), 0.9–4,5% w/w for raspberry (average

conc. 3.5% w/w), 1.4–3.5% w/w for chokeberry (average conc. 3.3% w/w), and 1.7–3.2% w/w for the extract of strawberry seeds (average conc. 2.8% w/w). Also, stearic acid is present and berry seed oils contain it in the amount of 1.3% w/w for blackcurrant and 0.7–0.9% w/w for raspberry and strawberry oils, and 0.6% w/w average content was found in the samples of chokeberry.

In the studied samples, there is high content of unsaturated fatty acids (UFA). They accounted for over 90% of the total FAs in received extracts (UFA/total FAs: 94.6% for raspberry, 93.5% for strawberry, 92.6% for chokeberry, 91.3% for blackcurrant). From UFA, three of the fatty acids (C18:2 cis (n6), C18:3 (n3), C18:3 (n6)) are the measures of good-quality test plant materials. Blackcurrant seed oil contains more than 9.8% w/w of C18:3 (n3). Significantly, higher levels of alfa-linolenic acid (ALA) were obtained for the analyzed raspberry oil (average conc. 22.7% w/w) and strawberry oil (average conc. 18.9% w/w). This high level of ALA, especially in the raspberry, strawberry, and blackcurrant oils, makes them interesting products with a positive n-6/n-3 ratio. It is also interesting when we think that those extracts are obtained from the seeds separated from the pomaces—

Table 1 Fatty acid composition of selected scCO₂-extracted berry seed oils (quantitation based on scan method; ions are given for information only)

No.	Abbreviation	Name	MW (g/mol) FA	MW (g/mol) FAME	RT ± 0.5 (min)	Ion FAME	Fatty acid concentration (% w/w) Blackcurrant	Raspberry	Chokeberry	Strawberry
1	C4:0	Butyric acid	88	102	3.92	74.59.87.102	0.039	n.d.	n.d.	0.009
2	C6:0	Caproic acid	116	130	5.08	74.87.95	0.010	0.008	0.009	n.d.
3	C8:0	Caprylic acid	144	158	7.39	74.87.127.158	n.d.	0.009	0.005	0.003
4	C10:0	Capric acid	172	186	10.86	74.87.143.186	n.d.	n.d.	n.d.	n.d.
5	C11:0	Undecanoic acid	186	200	12.83	74.87.55.200	0.010	0.056	0.009	0.053
6	C12:0	Lauric acid	200	214	14.85	74.87.143.214	0.074	n.d.	n.d.	n.d.
7	C13:0	Tridecanoic acid	214	228	16.83	74.87.143.228	0.042	0.103	0.085	0.188
8	C14:0	Myristic acid	228	242	18.87	74.87.199.242	n.d.	n.d.	n.d.	n.d.
9	C15:0	Pentadecanoic acid	242	256	21.05	74.87.213.256	n.d.	n.d.	n.d.	n.d.
10	C16:0	Palmitic acid	256	270	23.41	74.87.227.270	5.694	3.379	3.281	2.835
11	C16:1	Palmitoleic acid	254	268	24.72	55.69.83.268	0.096	0.080	0.104	0.082
12	C17:0	Heptadecanoic acid	270	284	25.84	74.87.143.284	0.053	0.066	0.048	0.105
13	C17:1	cis-10 Heptadecanoic acid	268	282	27.25	55.69.83.282	0.043	0.075	0.029	n.d.
14	C18:0	Stearic acid	284	298	28.44	74.87.255.298	1.202	0.755	0.562	0.791
15	C18:1 trans (n9)	Elaidic acid	282	296	29.24	55.97.264.296	n.d.	n.d.	n.d.	n.d.
16	C18:1 cis (n9)	Oleic acid	282	296	29.66	55.69.83.296	11.598	8.866	11.061	10.718
17	C18:2 trans (n6)	Linolelaidic acid	280	294	30.79	67.55.81.294	0.021	0.028	0.014	0.019
18	C18:2 cis (n6)	Linoleic acid	280	294	31.78	67.81.95.294	41.467	47.143	43.105	32.543
19	C18:3 (n6)	Linolenic acid	278	292	33.18	67.79.194.292	11.615	0.207	n.d.	n.d.
20	C20:0	Arachidic acid	312	326	33.64	74.87.143.326	0.182	0.540	0.163	0.191
21	C18:3 (n3)	alfa-Linolenic acid	278	292	34.19	79.67.95.292	13.596	35.816	0.619	19.898
22	C20:1 (n9)	cis-11 Eicosenoic acid	310	324	34.89	55.69.292.324	1.118	0.407	0.163	0.287
23	C21:0	Heneicosenoic acid	326	340	36.27	74.87.143.340	0.097	0.038	0.086	0.029
24	C20:2 (n6)	cis-11,14 Eicosadienoic acid	308	322	37.01	67.81.178.322	0.889	0.275	0.105	0.124
25	C20:3 (n6)	cis-8,11,14 Eicoastrienoic acid	306	320	38.46	67.93.150.320	n.d.	n.d.	n.d.	n.d.
26	C22:0	Behenic acid	340	354	38.87	74.87.143.354	0.086	0.228	0.096	0.163
27	C20:4 (n6)	Arachidonic acid	304	318	39.49	79.67.133.332	0.032	n.d.	n.d.	n.d.
28	C20:3 (n3)	cis-11,14,17 Eicosatrienoic acid	306	320	39.49	79.95.108.135	0.032	0.019	0.007	0.010
29	C23:0	Tricosanoic	354	368	41.43	74.87.143.368	0.032	0.048	0.010	0.019
30	C20:5 (n3)	cis-5,8,11,14,17 Eicosapentanoic acid (EPA)	302	316	42.06	79.67.55.201	0.050	n.d.	n.d.	n.d.
31	C22:2	Cis-13,16 Docosadienoic acid	336	350	42.21	67.81.95.319	n.d.	n.d.	n.d.	n.d.
32	C24:0	Lignoceric acid	369	383	43.95	74.87.143.382	0.076	0.114	0.054	0.067
33	C24:1	Nervonic acid	366	380	45.19	348.55.69.380	0.032	n.d.	n.d.	n.d.
34	C22:6 (n3)	cis-4,7,10,13,16,19 Docosahexaenoic acid (DHA)	328	342	48.36	79.67.105.119	0.021	n.d.	n.d.	n.d.
		Others (unidentified compounds)					11.75	1.74	40.39	31.87
		Total FAs					88.25	98.26	59.61	68.13
		Total saturated FAs (SFA)					7.64	5.34	4.41	4.45
		Total unsaturated FAs (UFA)					80.61	92.92	55.21	63.68
		SFA:UFA					0.095	0.058	0.08	0.07

n.d. not detected

Table 2 FAs content in one scCO₂ extraction series of blackcurrant seeds

Sample number	Stearic acid % (w/w)	Palmitic acid % (w/w)	Oleic acid % (w/w)	alfa-Linolenic acid % (w/w)	gamma-Linolenic acid % (w/w)	Linoleic acid % (w/w)	Total FA %
1	2.90	9.16	13.13	1.12	1.22	50.79	78.32
2	1.44	6.24	8.77	7.23	7.86	33.40	64.94
3	1.31	5.81	8.42	8.12	8.84	32.10	64.59
4	1.27	5.65	8.35	8.24	8.90	31.65	64.06
5	1.18	5.52	8.52	8.77	9.61	33.03	66.63
6	1.24	5.13	8.55	8.95	9.72	32.84	66.44
7	1.24	4.95	8.62	8.98	9.88	32.97	66.64
8	1.19	5.01	8.88	9.48	10.38	34.21	69.14
9	1.18	5.22	9.24	10.09	10.94	35.97	72.63
10	1.18	5.30	9.54	10.27	11.22	36.73	74.23
11	1.17	5.14	9.32	10.05	11.12	36.09	72.88
12	1.17	5.04	9.26	10.09	11.06	36.08	72.71
13	1.13	4.93	9.28	10.00	10.94	35.48	71.77
14	1.13	4.86	9.16	10.09	10.90	35.55	71.69
15	1.16	5.02	9.72	10.87	11.78	37.86	76.42
16	1.21	4.86	9.75	10.75	11.48	37.28	75.32
17	1.24	4.58	9.93	10.85	11.44	37.73	75.75
18	1.34	4.09	10.15	10.53	10.53	37.41	74.06
19	1.48	3.82	10.63	10.83	10.34	38.14	75.24
20	1.50	3.47	10.34	10.23	9.61	36.28	71.44
21	1.59	3.29	10.33	9.85	8.89	35.28	69.23
22	1.68	3.17	10.30	9.50	8.50	34.21	67.37
23	1.63	3.20	9.72	8.83	7.73	31.96	63.07
24	1.49	5.57	9.54	7.75	8.08	36.14	68.56

waste or by-products in plant production processes. Furthermore, blackcurrant seed extracts contain more than 10.9% w/w of C18:3 (n6) gamma-linolenic acid, which is the active component in the n-6 fatty acid group.

Method Validation

A method was validated for the quantitation of fatty acids in scCO₂-obtained berry seed extracts using GC/ MSD Agilent equipment. As a representative sample of the berry seed oils, blackcurrant oil was selected. The concentration range of target FAs used in this validation was chosen to fit the commonly encountered range of analyte concentration in previously tested samples. Therefore, the standard concentrations of the basic eight fatty acids were at a higher level than the rest of them.

Matrix samples, blanks, standards, and reference materials were used during the validation process. Berry
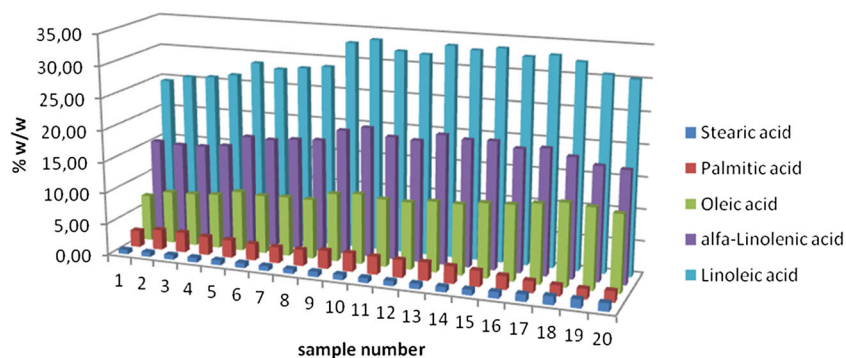
Fig. 6 FA profile changing during one scCO₂ extraction series of strawberry seeds

Table 3 Validation parameters of berry seed extracts (based on blackcurrant seed extract samples, matrix sample, and calibration curve)

No.	Abbr.	Fatty acid name	sd % w/w	rsd	CV %	r repeatability % w/w	LOD % w/w	LOQ % w/w	Linearity r^2	Working range mg/mL	Working range % w/w
1	C4:0	Butyric acid	n.d.	n.d.	n.d.	n.d.	0.0103	0.0171	0.998	0.02–0.004	0.15–0.03
2	C6:0	Caproic acid	0.005	0.3573	35.73	0.01	0.0187	0.0311	0.998	0.02–0.004	0.15–0.03
3	C8:0	Caprylic acid	n.d.	n.d.	n.d.	n.d.	0.0071	0.0119	0.999	0.02–0.004	0.15–0.03
4	C10:0	Capric acid	0.0113	0.1009	10.09	0.03	0.0068	0.0102	0.999	0.02–0.004	0.15–0.03
5	C11:0	Undecanoic acid	n.d.	n.d.	n.d.	n.d.	0.0086	0.0143	0.999	0.02–0.004	0.15–0.03
6	C12:0	Lauric acid	0.005	0.3573	35.73	0.01	0.0245	0.0408	0.999	0.10–0.01	0.75–0.075
7	C13:0	Tridecanoic acid	0.0112	0.1603	16.03	0.03	0.0103	0.0172	0.998	0.02–0.004	0.15–0.03
8	C14:0	Myristic acid	0.0112	0.2933	29.33	0.03	0.027	0.0451	0.999	0.10–0.01	0.75–0.075
9	C15:0	Pentadecanoic acid	0.0112	0.2933	29.33	0.03	0.0159	0.0265	0.996	0.02–0.004	0.15–0.03
10	C16:0	Palmitic acid	0.421	0.0701	7.01	1.19	0.0435	0.0522	0.997	0.10–0.01	0.75–0.075
11	C16:1	Palmitoleic acid	0.0112	0.1235	12.35	0.03	0.0176	0.0293	0.995	0.02–0.004	0.15–0.03
12	C17:0	Heptadecanoic acid	0.0112	0.2295	22.95	0.03	0.0204	0.034	0.995	0.02–0.004	0.15–0.03
13	C17:1	cis-10 Heptadecanoic acid	0.0112	0.2933	29.33	0.03	0.0255	0.0425	0.995	0.02–0.004	0.15–0.03
14	C18:0	Stearic acid	0.0695	0.0551	5.51	0.2	0.0265	0.0318	0.999	0.10–0.01	0.75–0.075
15	C18:1 trans (n9)	Elaidic acid	n.d.	n.d.	n.d.	n.d.	0.0027	0.00445	0.998	0.02–0.004	0.15–0.03
16	C18:1 cis (n9)	Oleic acid	0.7606	0.0625	6.25	2.15	0.0372	0.0446	0.999	0.10–0.01	0.75–0.075
17	C18:2 trans (n6)	Linolelaidic acid	0.0077	0.3626	36.26	0.02	0.0237	0.0396	0.998	0.02–0.004	0.15–0.03
18	C18:2 cis (n6)	Linoleic acid	3.0857	0.0709	7.09	8.73	0.0128	0.0256	0.998	0.40–0.01	3.00–0.075
19	C18:3 (n6)	gamma-Linolenic acid	1.1166	0.0915	9.15	3.16	0.0125	0.0375	1.000	0.10–0.01	0.75–0.075
20	C20:0	Arachidic acid	0.0117	0.0665	6.65	0.03	0.0157	0.0262	0.997	0.02–0.004	0.15–0.03
21	C18:3 (n3)	alfa-Linolenic acid	1.273	0.0891	8.91	3.6	0.015	0.0432	1.000	0.10–0.01	0.75–0.075
22	C20:1 (n9)	cis-11 Eicosenoic acid	0.1175	0.1006	10.06	0.33	0.015	0.0249	0.996	0.02–0.004	0.15–0.03
23	C21:0	Heneicosenoic acid	0.0112	0.1235	12.35	0.03	0.0192	0.032	0.996	0.02–0.004	0.15–0.03
24	C20:2 (n6)	cis-11,14 Eicosadienoic acid	0.0407	0.0465	4.65	0.12	0.0242	0.0404	0.998	0.02–0.004	0.15–0.03
25	C20:3 (n6)	cis-8,11,14 Eicoastrienoic acid	0.0295	0.1181	11.81	0.08	0.0207	0.0345	0.995	0.02–0.004	0.15–0.03
26	C22:0	Behenic acid	0.0112	0.1395	13.95	0.03	0.0206	0.0343	0.995	0.02–0.004	0.15–0.03
27	C20:4 (n6)	Arachidonic acid	0.0112	0.4064	40.64	0.03	0.0188	0.0313	0.996	0.02–0.004	0.15–0.03
28	C20:3 (n3)	cis-11,14,17 Eicosatrienoic acid	0.0112	0.4064	40.64	0.03	0.0174	0.0289	0.996	0.02–0.004	0.15–0.03
29	C23:0	Tricosanoic acid	0.0112	0.4064	40.64	0.03	0.0278	0.0463	0.995	0.02–0.004	0.15–0.03
30	C20:5 (n3)	cis-5,8,11,14,17 Eicosapentanoic acid (EPA)	0.0119	0.0602	6.02	0.03	0.0305	0.0509	0.997	0.02–0.004	0.15–0.03
31	C22:2	cis-13,16 Docosadienoic acid	0.0202	0.0652	6.52	0.06	0.0161	0.0269	0.997	0.02–0.004	0.15–0.03
32	C24:0	Lignoceric acid	0.0112	0.1603	16.03	0.03	0.0229	0.0383	0.999	0.02–0.004	0.15–0.03
33	C24:1	Nervonic acid	0.0112	0.4064	40.64	0.03	0.0166	0.0277	0.998	0.02–0.004	0.15–0.03
34	C22:6 (n3)	cis-4,7,10,13,16,19 Docosahexaenoic acid (DHA)	0.0077	0.3626	36.26	0.02	0.0188	0.0313	0.996	0.02–0.004	0.15–0.03

n.d. not detected during blackcurrant seed oil analysis, *r* range of repeatability

seed oils were tested without internal standards. Samples were analyzed in duplicates. All 34 available standards were prepared separately in volume flasks by dissolving an appropriate weight in 25 mL of MTBE. For linoleic acid methyl ester (C18:2 n6), the weight was 0.50 g giving a concentration of 20.0 mg/mL; for methyl laurate (C12:0), methyl myristate (C14:0), methyl palmitate (C16:0), methyl stearate (C18:0), methyl oleate (C18:1), methyl γ-linolenate (C18:3 n6), and methyl linolenate (C18:3 n3), it was 0.125 g into 25 mL of MTBE giving a concentration of 5.0 mg/mL FAME, and for the rest of the 26 FAMEs, it was 0.025 g/25 mL of MTBE giving the final concentration of 1.0 mg/mL. Next, standards on these base concentrations were diluted 50 times, in one flask, to obtain a mix of FAMEs in concentrations of 0.4, 0.1, and 0.02 mg/mL, respectively. All these concentrations could be converted into weight percentage of FAMEs or FAs and all standard solutions diluted again, if needed.

The analytical method was validated according to English and Polish versions of European standards (PN-ISO 3534-1 2009; PN-ISO 3534-2 2010; PN-ISO 5725-1-6 2002; ISO/TS 21748 2004; PN-EN ISO/IEC 17025 2005), internal test procedures, and standard protocols (IB03 research procedure, the research methods validation; IB05 research procedure, the estimation of measurement uncertainty). It was not validated for robustness, carryover, dilution integrity, or mass spectrometer parameter changes (e.g., ion source and quadruple temperatures, ionization voltage). Validation studies included sensitivity measured by the limit of detection (LOD) and limit of quantitation (LOQ), working range, linearity and calibration model fits (correlation), precision (repeatability expressed by standard deviation and relatively standard deviation), accuracy (recovery pattern, certificated value of CRM), coefficient of variation, and uncertainty. All calculations were performed using the ChemStation software Excel 2007 and online available authorized computer program e-Stat.

## Limit of Detection and Limit of Quantitation

Limit of detection (LOD) is the lowest concentration of analyte in the sample that can be detected but not necessarily quantified. The limit of quantification (LOQ) is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

In this paper, a standard mixture, dissolved in MTBE, was used as the sample because it contained all 34 FAs. The sample extract from berry seeds that contains all those selected to identify acids was not found.

Also, a calibration curve equation was used to assess the LOD and LOQ values.

Samples with decreasing amounts of the analyte were injected. They were prepared by 2-, 5-, 8-, and 10-fold diluting standard mixtures of FAMEs at the concentrations of 0.4, 0.1, and 0.02 mg/mL to final concentrations of 0.04, 0.01, and 0.002 mg/mL. This prepared sample was analyzed 10 times. Average concentration values and standard deviations were calculated. LOD was equal to the sum of the total blank value and three times the value of the standard deviation. LOQ was equal to three times the value of LOD. The identical results of LOD and LOQ for all 34 fatty acids were achieved by performing the calculation based on the equation of the linear calibration curve. LOD was then equal to six times the value of the residual standard deviation ($s_{y/x}$) divided by the value of the slope (a). LOQ was equal to 10 times the $s_{y/x}$ divided by the value of the slope. All individual values for LOD and LOQ are presented in Table 3. Generally, LOD was 0.03% w/w and LOQ was 0.05% w/w.

Fig. 7 Oleic acid calibration curve. Calibration range 0.075–0.75% w/w

y = 1E+07x - 331524
R² = 0,999
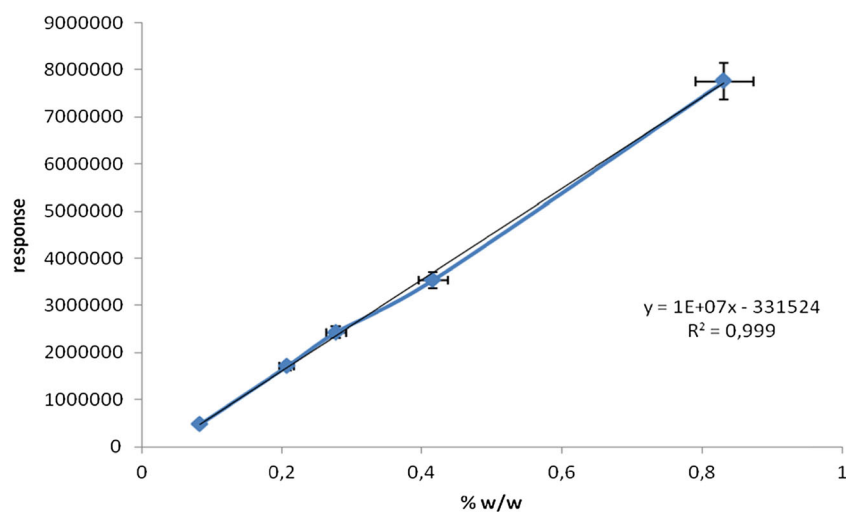
Range, Linearity, and Calibration Model Fits

The identification criteria for working range determination (calibration curve range) were LOQ, linearity, and calibration model fits (correlation). The working range was set as the range of concentrations from the LOQ to the maximum of the calibration curve, maintaining the correlation coefficient (r^2) above 0.995.

The calibration curves were constructed over the range of 0.4–0.004 mg/mL by replicate injections ($n = 3$) of standard mixtures. The calibration curves, determined by the least squares regression method, were linear over the range, with r^2 above 0.995 (see Table 3). It was found that the linear fit was an appropriate calibration model for all 34 fatty acids in the analyzed samples.

Figure 7 shows the example calibration curve for oleic acid (C18:1 cis n9).

Precision

The precision of the samples was measured and evaluated as the repeatability expressed by standard deviation (sd) and relative standard deviation (rsd) and as the coefficient of variance (% CV) for the inter-run analysis. The standard acceptance criteria for inter-run precision were ±30% at each concentration. The inter-run precision, as shown in Table 3, ranged from 5 to 29% CV and was within the acceptance criteria for most of FAs in the blackcurrant extract. Only for eight fatty acids was % CV higher than 30% (values marked in red), but it is worth noticing that seven of them were at the LOQ-level concentration (Table 1).

Accuracy

In accordance with current metrological nomenclature, the accuracy is defined as the closeness of agreement between the true value or an accepted reference value and the value found. The true value can be obtained from an established reference method or by sample analysis with known concentrations, for example certified reference material (CRM), standard reference material (SRM), or extract reference material (XRM).

To assess the accuracy and recovery in our method, materials (NIST SRM 3251 *Serenoa repens* Extract, ChromaDex Extract Reference Material (XRM) *Serenoa serrulata* (*Saw Palmetto*) Fruit CDXA-XRM-001) and spiked extract samples of blackcurrant seeds were used. The sample matrix was spiked with the known standard amount, by volume. The concentration should cover the range of concern and should include the amount close to the LOQ, in the middle of the range and at the high end of the calibration curve. Acceptance criteria for recovery were 100 ± 30%. During this analysis, samples were

Table 4 Validation parameters for linear regression, statistical significance factors, accuracy, and uncertainty

No.	Abbr.	a	b	s_a	s_b	$s_{y/x}$	s_m	CV %	r^2	t_{crit}	t_a	t_b	t_r	Accuracy SRM %	Accuracy XRM %	Recovery %
1	C8:0	2.98E+07	−7.54E+04	4.42E+05	3.54E+04	45,770	0.00154	2.357	0.9993	3.182	67.34	2.133	65.45	97.2	–	124.1
2	C10:0	3.42E+07	−1.29E+05	5.12E+05	3.56E+04	46,070	0.00135	2.374	0.9993	3.182	66.82	3.625	65.45	95.6	109.3	109
3	C12:0	3.09E+07	−1.20E+05	5.30E+05	1.66E+05	214,500	0.00695	2.725	0.9991	3.182	58.25	0.7214	57.71	101.9	102.5	91.5
4	C14:0	2.98E+07	−2.10E+06	5.47E+05	2.33E+05	301,200	0.01012	2.915	0.999	3.182	54.42	0.9037	54.74	99.4	104.1	117.3
5	C16:0	3.35E+07	−1.38E+06	1.03E+06	4.25E+05	549,500	0.01639	4.868	0.9972	3.182	32.59	3.239	32.69	98.9	102.8	102
6	C18:0	3.57E+07	−6.49E+05	6.67E+05	2.35E+05	379,900	0.01065	4.024	0.9986	2.776	53.5	2.761	53.41	71.4	75.5	113.2
7	C18:1 cis (n9)	9.68E+06	−3.31E+05	1.80E+05	8.01E+04	103,700	0.01071	2.949	0.999	3.182	53.79	4.135	54.74	101.2	94	103.3
8	C18:2 cis (n6)	1.08E+07	−3.31E+04	2.72E+05	3.95E+05	511,000	0.04751	4.019	0.9981	3.182	39.49	0.0839	39.7	67.2	–	78.1
9	C18:3 (n6)	8.81E+06	−2.26E+05	6.96E+04	1.84E+04	23,810	0.0027	1.253	0.9998	3.182	126.6	12.3	122.5	–	–	107.1
10	C18:3 (n3)	1.26E+07	−3.88E+05	1.14E+05	3.16E+04	40,830	0.00324	1.438	0.9998	3.182	110.4	12.28	122.5	100.5	–	105.3
11	C20:1 (n9)	8.36E+06	−8.81E+04	2.91E+05	2.08E+04	26,960	0.00323	5.52	0.9964	3.182	28.74	4.706	28.82	–	–	125.1
12	C24:0	1.95E+07	−3.13E+05	8.63E+05	7.46E+04	96,540	0.00496	7.036	0.9991	3.182	22.56	4.2	22.48	–	–	93.6

spiked with concentrations in the middle of the range. Accuracy results are shown in Table 4. The recovery ranges from 78 to 125% for selected analytes. The SRM/XRM accuracy ranged from 71 to 109%.

Table 4 shows all the following validation parameters (for selected FAs):

1. Linear regression: a—slope, b—intercept, s_a—standard uncertainty (standard deviation) of slope, s_b—standard uncertainty (standard deviation) of intercept, $s_{y/x}$—residual standard deviation, s_m—method standard error, CV—coefficient of variance, r^2—correlation coefficient
2. Statistical significance factors: t_{crit}—Student t critical value in a two-sided test, t_a—slope significance factor, t_b—intercept significance factor, tr—correlation significance factor
3. Accuracy (recovery pattern, certified value of SRM/XRM)
4. Expanded uncertainty

Uncertainty

The experimental approach was used to define uncertainty. Expanded uncertainty $U(y)$ was calculated by Eq. (1), where k is the coverage factor (equal 2 for 95% confidence level) and $u(y)$ is the combined uncertainty.

$$U(y) = k \times u(y) \qquad (1)$$

The values of the expanded uncertainty were determined for certain levels of concentration. The highest value (among selected seven FAs) was 33% for C24:0 (conc. range 0.05–0.10% w/w). For C18:3 (n3) and (n6), the value was 19% (conc. range 0.50–0.75% w/w), 15% for C16:0, C18:2 (n6) (conc. range 0.25–0.36% w/w, 1.82–2.36% w/w), 14% for C18:1 (n9) (conc. range 0.51–0.73% w/w), and 13% for C18:0 (conc. range 0.05–0.09% w/w). Taking into account the value of the expanded uncertainty, the results can be represented by Eq. (2), where c_{FA} is the concentration of fatty acid.

$$c_{FA} \pm U(c_{FA}) \qquad (2)$$

Conclusion

Validation shows that the GC/MSD method provides reliable results for the quantitation of FAs. The method displays good accuracy and precision, as well as recovery and uncertainty.

The developed method, based on existing standards and regulations, allows the initial characterization of selected berry seed $scCO_2$-obtained extracts. Chemical composition characteristics, including the content of fatty acids, which could be carried out using the gas chromatography technique, are an important element connecting the chemical industry with research and allow the optimization of $scCO_2$ extraction processes. The whole procedure allows better quality control of the final product (high-quality polyunsaturated oils), evaluates its suitability for a specific market (medical, pharmaceutical, cosmetic, food), and provides the ability for monitoring and modifying the process parameters.

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