Determination of essential oil content in caraway (*Carum carvi* L.) species by means of supercritical fluid extraction

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ABSTRACT

Dependently on planting conditions caraway fruits contain 1–9% of essential oils consisting of about 30 compounds. Carvone and limonene account for the main portion, about 95%. To evaluate the quality of various registered caraway (*Carum carvi* L.) cultivars (Kepron, Prochan and Rekord) planted during 1998–2000, regarding the effect of sample grinding and preparation, plant treatment and time of harvest, the amounts of essential oil and the carvone/limonene ratio were determined. Both whole and ground caraway seeds were extracted. As obvious from the results, SFE is not suitable for the determination of essential oils in whole seeds since the results are lower in comparison with those of ground caraway. The way of grinding was also examined. Of the three mills used, a splintery mill VIPO seemed to be the most suitable. Further, the amount of essential oil was studied in caraway gathered at the beginning of maturation (sample A) and at full ripeness (sample B) of caraway seeds. It was found that the samples gathered at full ripeness (samples B) had more essential oil. It was also concluded that the use of the regulator Roundup Bioaktiv during caraway maturation to unite the ripening of achenes in the main umbel and the first-order umbels, and the use of fungicides (Alert S and Prelude 10) affected positively the amount of essential oil in caraway. Possibilities of SFE application for the essential oil determination in small samples gathered during breeding were investigated. The results were used as a one of the criteria during breeding. Classical way of the essential oil determination does not allow this option. An alternative method for the isolation and determination of essential oils – supercritical fluid extraction (SFE) – was investigated in this study.

Keywords: caraway; cultivar; carvone; limonene; gas chromatography (GC); supercritical fluid extraction (SFE)

The evaluation of caraway fruits according to the current regulation brings large differences between individual analyses as the prescription of sample preparation can be explicated in different ways. That is why we started to search for an alternative method, enabling faster analyses with more precise and accurate results.

Nowadays, the instrumentation and automation of analytical methods is obviously on a higher level than those of sample preparation and treatment. Some samples can be analysed directly, but most of them has to be transferred into a liquid medium. Traditional liquid/liquid extraction methods have many disadvantages. A long extraction time (several hours or days), the need of preconcentration that can cause analyte losses or contamination of samples, and very often also the high toxicity and ecological noxiousness of used organic solvents belong to the most important. The method of supercritical fluid extraction (SFE) is able to minimise these deficiencies in part.

Currently, SFE represents a dynamically evolving separation technique for the determination of essential oils. It is an efficient method for isolation of volatile compounds from complex natural matrices, comparable with older field-proven methods, such as steam distillation, liquid/liquid (solvent) extraction or hydrodistillation. Physicochemical properties of supercritical fluids represent the transition between the properties of gases and liquids, which is the main reason for current increasing interest in the application of supercritical fluids (SFE, SFC etc.). In comparison with a liquid, the diffusivity of supercritical fluid is approximately one order of magnitude higher and the viscosity is one order of magnitude lower while the high solvent power is maintained. Thus, from the viewpoint of mass transfer, the properties of gases with the solvation properties of liquids are combined in supercritical fluids.

In SFE, carbon dioxide is used predominantly (Engelhardt and Gross 1988, Bounoshita et al. 1993, Kallio et al. 1994) because of its low critical temperature (31°C) and low critical pressure (7.38 MPa), negligible toxicity, incombustibility and low reactivity. Its polarity and extraction power are close to *n*-hexane. The extraction efficiency decreases with increasing analyte polarity. In the determination of volatile, reactive and thermosensitive terpenes, analysed in this work, the low critical temperature and the nonpolar character of supercritical fluid are preferred.

The content of essential oils, the amount of carvone and limonene in the oil and the ratio of both substances are the main quality criteria determined in caraway pro-

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duction. Since 1998, Regulation No. 330/97, an implementing rule of Food Act No. 110/97, has been compulsory for the control of caraway quality. The Czech codices from 1997 require the essential oil content higher than 3% and at least 50% content of carvone in the essential oil.

At present, steam distillation according to the standard ČSN 58 0110 and ČSN 6571 (Sedláková et al. 1998) and *n*-hexane distillation (Bouwmeester et al. 1995) or a combination of both methods are used for the isolation and determination of essential oil content. The monoterpenes can also be isolated from the gaseous phase by Solid Phase Microextraction (SPME) followed by thermal desorption with on-line injection into a GC capillary column (Mani and Wholley 1995). SFE is used as a new separation method.

To determine carvone and limonene contents, mostly gas chromatography with flame ionisation (GC/FID) or mass spectrometry (GC/MS) detection are used. High-performance liquid chromatography (HPLC) with UV (Kovar and Bock 1983, Seif el Din et al. 1983, Thies 1984) or polarimetric detection (Kovar and Bock 1983), derivative spectrophotometry (Seif el Din et al. 1983) and proton magnetic resonation (Mossa et al. 1989) can also be applied.

MATERIAL AND METHODS

Plant materials

Kepron, Prochan and Record, caraway (*Carum carvi* L.) varieties, planted in Agritec Šumperk (1998, 2000) were used to compare these varieties and to find out how the treatment affects the amount of essential oil in these species, and to determine the amount of essential oil in seeds harvested in two different periods (A, B).

New plant breeding selections of caraway from an experimental plot Huštěnovice (1999) were used to determine the essential oil content by the SFE method. The materials were newly-bred varieties with shortened vegetation period. The aim of the study was to verify the possibilities of using SFE for the treatment of the ground for further plant breeding work where the essential oil content in the caraway is one of the crucial selection criteria.

The variety Kepron, obtained from a field of the plant breeding company ROLS Lešany (2000), was used for the selection of the best SFE modifier. Prior to the extraction and analysis, the caraway seed samples were stored and preserved at a room temperature and protected from light.

Chemicals

l-Carvone and *d*-limonene (Fluka, Switzerland) were used as standards to test the extraction efficiency. *n*-Hexane (for HPLC, Merck, Germany) was used for the trapping of extracted substances. Liquid carbon dioxide (for the food industry), nitrogen (4.0) and/or nitrogen (4.6), helium (5.0) and medicinal oxygen (all AGA Ltd., Brno) were used for SFE and GC. Methanol (for HPLC), ethanol (for UV spectrometry), acetonitrile (for HPLC, all Merck, Germany), acetone (p.a., ONEX, Czech Republic), dichloromethane (p.a.), chloroform (p.a.), toluene (p.a., all Lachema Brno, Czech Republic) were used as SFE modifiers. Highly pure water (Millipore) was used for sample preparation and dilution.

The investigated varieties were treated with a ripeness regulator Roundup Bioaktiv (Monsanto Europe S.A., Belgium). The dose of this compound was 3 l/ha. The formulations Alert S and Prelude 10 (Dupont, France) were tested to prevent the varieties against fungal diseases during the experiments.

Supercritical fluid extraction

A supercritical fluid extractor SE-1 (SEKO-K, s.r.o., Brno) was used for SPE extractions. The instrument was controlled from a control station. Extraction programmes developed by the users could be stored in the extractor memory and all values were shown on the display. The pressure values from 7 to 40 MPa were adjusted by a pneumatically controlled piston micro-pump. The whole system worked with three gas cylinders (extraction and cooling CO_2 and N_2 as a pressure gas). A minimum N_2 pressure of 1.5 MPa was necessary to achieve the CO_2 working pressure of 40 MPa. The volume of the piston micro-pump (10 ml) was not large enough for a long extraction time (which is influenced by the restrictor volume – length and i.d.).

Before the pump filling started, the pump head was cooled with a stream of liquid CO_2 to 3°C, and then the filling time started to be counted. In case that the extraction medium stored in the pump was depleted during the extraction, the Valco valve switched automatically and the pump filling procedure started again. The time of pump filling could be preset to 1–9 min. When the pump was filled, the Valco valve was switched again and the pressure was increased up to the required value. After the preset extraction time expired, the extraction was terminated automatically.

Approximately 400–500 mg (±0.01 mg) of a ground sample garbled through a 1mm mesh, was treated in a splintery VIPO grinder, and weighed into an extraction cartridge. The cartridge was inserted into a stainless steel extraction cell of the inner volume 0.7–7.0 ml according to the sample amount and closed with frit on both sides. The extraction cell, supplied with a depressurisation screw, was fastened with extraction cap. The restrictor heating was adjusted to 120°C to prevent the restrictor plugging. Carbon dioxide, the extraction medium, came out from the extraction cell through a restrictor (of CO_2 flow rate), leading to a trapping vial with *n*-hexane. A fused silica capillary of 30 μm i.d. was used as a restrictor. Heating and regulation of the trapping vial were off during these procedures. Trapping was carried out at

a room temperature. Carvone and limonene trapped in *n*-hexane were analysed by GC.

For the determination of essential oil in ground caraway, the pressure of 40 MPa, extraction cell temperature 80°C and restrictor temperature 120°C were used. The extracts were kept in a refrigerator until the time of GC analysis. Three different types of mills – ETA 0067 with millstones (ETA, a.s., Czech Republic), splintery VIPO mill (Mechanical Workshop, Litomyšl, Czech Republic) and single ball cryogenic grinder Vibrom 2S (Jebavý Ltd., Czech Republic) using liquid nitrogen – were used for sample grinding.

Gas chromatography (GC)

A gas chromatograph HP 4890 D was used to determine the carvone to limonene ratio in SFE extracts. The separation was carried out on an HP-5 column (cross-linked 5% PH ME siloxane, 15 m × 0.53 mm i.d. × 1.5 μm film thickness, all Hewlett Packard) at the helium flow rate 2 ml/min, injector temperature 220°C, detector temperature 240°C. The temperature programme 60°C, 40°C/min to 220°C, 2 min at 220°C was applied. Portions of *n*-hexane solution (2 μl) of essential oil were injected into the GC column. Final chromatograms were processed by means of CSW station (ver. 1.7, Data Apex, Prague, Czech Republic).

RESULTS AND DISCUSSION

To determine the essential oil content in caraway species, steam distillation according to the standard ČSN 58 0110, Pharmaceutical Code of Practice and Czech Pharmacopoeia is used as a standard method. The essential oil determination by steam distillation brings large differences in results. That is why the aim of this study was to find an alternative method. SFE (supercritical fluid extraction) seems to be suitable. The best SFE conditions were explored in the first experiments. The influence of sample pre-treatment was investigated – entire vs. ground caraway seeds. In the second part of this study, the impact of various grinding methods was examined. Various types of mills were compared because the grinder type is not specified in the ČSN 58 0110 standard. In the fourth part the effect of harvest time on the amount of essential oils was investigated. Caraway seeds were harvested before maturation and at full ripeness. Further, the influence of treatment on the amount of essential oil in individual varieties was investigated. The formulation Roundup Bioaktiv was used as a ripening modifier, Alert S and Prelude 10 as fungicides. The use of SFE for the essential oil determination in small samples, obtained during the plant breeding process, was also investigated. The analyses of 45 plants that differed from the remaining plants were carried out, and the results were used in further plant breeding. Finally the effect of modifiers that can enhance the extraction efficiency of the essential oil recovery obtained by SFE was investigated.

Effect of sample grinding and different ways of grinding

The effect of sample grinding on the essential oil recovery was investigated. As for the SF extraction of whole caraway seeds, 0.09 weight % of essential oil was found, with relative standard deviation (*RSD*) = 1.56%, and in the case of ground caraway, 2.55 weight % of essential oil was found with *RSD* = 2.00%. It is obvious from these values that the SFE method is not suitable for the essential oil determination in caraway seeds that were not ground. The extraction yield is one order of magnitude lower in comparison with the results of SFE extraction of ground caraway fruits. The low yields are probably caused by the restricted analyte diffusion from the inner part of caraway fruits.

The ratio of carvone to limonene content is also different in the case of SFE extractions from the whole and ground caraway fruits. In the extracts from the whole caraway fruits, the amount of carvone is 81.5%, while in the extracts from the ground caraway the content of carvone declined to 66.4%. That is why further experiments were focused only on the extraction of essential oil from ground caraway seeds.

The comparability of the results of some sample grinding methods using three different types of mills (ETA 0067 with millstones, splintery VIPO mill and cryogenic mill Vibrom 2) was also investigated. The type of mill significantly influenced the amount of extracted essential oil. The best extraction yield (2.55 weight % with *RSD* = 2.00%) was achieved using the splintery mill VIPO. The respective values of 1.79 weight % with *RSD* = 1.36% and 1.72 weight % with *RSD* = 1.50% were achieved using the ETA and the cryogenic mill. Both the type of mill and the grinding refinement can affect the amount of essential extracted oil.

Effect of harvest time

The essential oil contents in caraway fruits collected before maturation (samples A) and at ripeness (samples B) are given in Table 1. The samples (A) had lower contents of essential oil than the samples (B) harvested at full ripeness. The marked morphological differences be-

Table 1. Qualitative parameters of caraway (Šumperk 1998)

	Kepron		Prochan	
	A	B	A	B
Essential oil (%)	1.26	3.36	1.50	3.94
RSD (%)	2.01	3.01	2.00	1.98
Limonene (%)	26.77	18.24	35.13	27.45
Carvone (%)	73.23	81.76	64.87	72.55

A – before maturation, B – after ripeness
RSD – relative standard deviation

Table 2. Qualitative parameters of variety Kepron (Šumperk 2000)

	Untreated	Roundup Bioaktiv	*MS**
Essential oil (%)	3.41	3.66	0.063**
RSD (%)	0.92	0.42	–
Limonene (%)	43.57	42.34	1.44
Carvone (%)	56.43	57.66	1.54

* *MS* – mean square calculated by statistical program UNISTAT
RSD – relative standard deviation

Table 3. Essential oil content (%) in caraway after the treatment of fungicides (Šumperk 2000)

Treatment	Kepron	Prochan	Rekord
Untreated	3.41	3.22	3.32
Alert S	6.75	5.56	5.39
Prelude 10	5.41	4.42	4.03

tween the samples were also visually apparent. The samples collected before harvest had fruits of elongate, narrow shape while samples gathered after maturation had round-shaped fruits. The amount of carvone was enhanced during maturation by about 12% (Table 1).

Effect of caraway treatment on the essential oil amount

Samples of the caraway varieties Kepron, Prochan and Rekord, planted in Šumperk (in 2000) were used for a comparison of plant treatment procedures. The amount of essential oil increased after the Roundup Bioaktiv treatment (see comprehensive Table 2). The fact is also proved by a highly significant difference of the analysis of variance performed under the programme UNISTAT.

Further, after the treatment of all varieties Kepron, Prochan and Rekord with fungicides (fungicide 1 – Alert S and fungicide 2 – Prelude 10), the amount of essential oil increased significantly (Table 3). The highest increase was achieved after the treatment of plants with the Alert S fungicide, as proved by the highly significant *MS* value for the treatment in the analysis of variance (*MS* – 10.026***). No significant differences were found between the varieties (*MS* – 1.52), and the interaction and treatment of the varieties did not show any significant differences either (*MS* – 0.297).

SFE use for the determination of essential oil in small samples

The essential oil content is a significant selection factor for plant breeders. The use of SFE is favourable for

Table 4. Qualitative parameters of variety Prochan in the small samples (Huštěnovice 1999)

Sample	*x*	*RSD* (%)	Limonene	Carvone	Sample	*x*	*RSD* (%)	Limonene	Carvone
1	1.09	6.14	48.11	51.89	24	0.90	8.57	36.19	63.81
2	1.24	3.98	44.37	55.63	25	0.71	6.04	51.95	48.05
3	1.15	4.75	46.85	53.15	26	1.05	10.50	46.83	53.17
4	0.71	0.18	43.83	56.17	27	1.19	9.83	48.05	51.95
5	0.36	–	30.08	69.92	28	0.59	2.66	29.05	70.95
6	1.00	1.10	43.56	56.44	29	1.43	8.23	50.78	49.22
7	0.95	6.38	46.09	53.91	30	1.45	7.49	50.64	49.36
8	0.95	5.43	57.58	42.42	31	1.08	3.59	40.14	59.86
9	0.98	3.33	38.95	61.05	32	0.80	6.67	25.81	74.19
10	1.19	4.75	55.29	44.71	33	1.44	8.38	53.75	46.25
11	0.92	4.61	49.75	50.25	34	**1.83**	**3.84**	**44.87**	**55.13**
12	0.88	7.90	48.50	51.50	35	1.10	4.85	47.19	52.81
13	1.00	1.72	46.47	53.53	36	0.91	8.73	40.77	59.23
14	1.28	2.77	45.63	54.37	37	1.06	8.11	44.37	55.63
15	0.98	5.08	44.61	55.39	38	**1.80**	**9.06**	**38.29**	**61.71**
16	1.32	4.84	52.81	47.19	39	1.03	1.26	41.64	58.36
17	1.37	1.24	61.73	38.27	40	1.09	5.52	44.54	55.46
18	0.74	8.66	60.32	39.68	41	1.06	8.78	36.24	63.76
19	1.04	–	45.30	54.70	42	**1.56**	**8.87**	**34.49**	**65.51**
20	0.64	3.20	73.66	26.34	43	**1.59**	**8.00**	**16.79**	**83.21**
21	0.64	8.84	58.29	41.71	44	1.47	2.09	48.54	51.46
22	0.99	3.20	62.99	37.01	45	1.48	–	55.92	44.08
23	0.60	5.91	40.77	59.23					

– not repeated, low amount of the sample, *RSD* – relative standard deviation

the isolation of essential oil from small samples gathered during breeding. The analyses of 45 plants that differed from the remaining plants (positively – taller height, higher number of umbels etc.) were carried out. The analysis of essential oil content was performed in a half of sample seeds from the main umbel, while the second half of sample seeds was re-sown again.

The highest amounts of essential oil (Table 4) were found in samples 34, 38, 42 and 43, while the lowest essential oil contents were found in samples 5, 20 and 21. The plants from which the seed samples were obtained underwent the morphological analysis, but no correlation was found between the plant height, the number of the 1st and 2nd order umbels and the content of essential oils.

Table 5. Qualitative parameters of variety Kepron after the addition of modifiers (Lešany 2000)

Modifier	*x* (%)	Limonene (%)	Carvone (%)
Untreated	2.43	42.03	57.97
Methanol	2.95	42.23	57.77
Ethanol	2.85	42.11	57.89
Acetone	3.30	42.11	57.89
Acetonitrile	2.91	42.89	57.11
Hexane	3.23	42.47	57.53
Dichloromethane	3.24	42.84	57.16
Chloroform	3.44	41.89	58.11
Toluene	3.25	42.78	57.22

Use of modifiers

In SFE, carbon dioxide is a widely used supercritical fluid because of its low critical pressure and temperature. However, low polarity is a prevailing problem of carbon dioxide because the solubility of analytes decreases with their higher polarity. To avoid this problem, a small amount (up to 10% of volume) of a polar solvent (modifier) is added to the stream of supercritical CO_2. The effect of modifier addition on the extracted essential oil amount and on the carvone to limonene ratio was investigated.

Methanol, ethanol, acetone, acetonitrile, *n*-hexane, dichloromethane, chloroform and toluene were tested as modifiers. About 100 µl of modifier was added directly into the extraction cell (*in situ*). SFE was performed under these parameters: 40 MPa, 80°C, and extraction time 30 min.

As shown in Table 5, the use of modifiers can enhance the amount of extracted essential oil. The amount of essential oil extracted without modifier was found to be about 85% of the amount obtained by steam distillation. Chloroform approved itself to be the most efficient modifier. With the use of a modifier (chloroform), the amount of extracted essential oil increases up to about 91% of the amount obtained by steam distillation. Only carvone and limonene were determined, and as their proportion in the essential oil is 95%, the total amount of essential oil in the case of determination of all compounds is very close to the results of steam distillation. The carvone/limonene ratios hardly change as a result of the use of different modifiers.

CONCLUSIONS

Due to various breeding interventions and plant selection within the framework of further breeding caraway seeds contain various amounts of essential oil in the range of 1–9%.

The supercritical fluid extraction (SFE) method at pressure 40 MPa, temperature 80°C and extraction time 30 min was used to separate the essential oil from caraway seeds in different experimental conditions. SFE seems to be a very useful method of sample preparation suitable for the isolation of essential oil from complex real matrices followed by gas chromatographic (GC) determination. It is comparable to state-of-the-art methods such as steam distillation, solvent extraction or hydrodistillation, but it is faster and provides more accurate results due to the experiment performer cutoff. However, it requires a GC-equipped laboratory.

Full and ground caraway fruits were extracted. The results proved that SFE is not suitable for the extraction of essential oil from whole caraway seeds. The results are lower than those of ground seeds probably due to the poor analyte diffusion from the inner parts of fruits. The carvone to limonene ratio also differs in the case of extraction of whole and ground caraway seeds. In the ground seed extract, the value of carvone portion decreased distinctly by about 15% in comparison with whole seeds. Thus, SFE is suitable only for the essential oil determination from ground seeds.

Further, the various grinding methods were compared because the ČSN 58 0110 standard does not specify either the way of grinding or the mill type. The splintery mill VIPO seemed to be the most suitable out of all the three ones. The applicability of SFE was verified on the samples obtained from experiments where various breeding interventions were monitored.

The content of essential oil in caraway fruits harvested at the beginning of maturation (sample A) and at full ripeness (sample B) was investigated. The samples (B), harvested at full ripeness, had more essential oils. The difference between samples A and B was also visually evident. The samples harvested at the beginning of maturation had elongate, narrow seeds, while the samples obtained at full ripeness had seeds of rounded shape. The amount of carvone was higher in the sample harvested at full ripeness.

The content and quality of essential oil were also monitored in plants treated in a different way. The effect of Roundup Bioaktiv regulator applied to unite the maturation of caraway seeds in the main umbel and in the first-order umbels on caraway maturation was examined. The effect of fungicides (Alert S and Prelude 10) applied to control the content and quality of essential oil was also

investigated. The use of both Roundup Bioaktiv and fungicides was found to have a positive influence on the amount of essential oil in caraway.

Likewise, the possibilities of SFE use for the determination of essential oil content in small samples gathered during breeding were tested. Essential oil determination was performed in 45 plants obtained by positive or negative selection among the plants of new varieties. The results were applied as new breeding criteria. The classical methods for the determination of essential oil do not allow anything of this kind.

Further, the effect of modifiers that can enhance the extraction efficiency on the amount of SFE extracted essential oil was investigated. Methanol, ethanol, acetone, acetonitrile, hexane, dichloromethane, chloroform and toluene were used as modifiers. The carvone/limonene ratio hardly changed with individual modifiers. Chloroform was found to be the most efficient. The amount of essential oil extracted without modifier was found to be approximately 85% of the value obtained by steam distillation. The use of modifier (chloroform) enhances the amount of extracted essential oil up to 91% of the steam distillation recovery.

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ABSTRAKT

Stanovení obsahu silic v kmínu (*Carum carvi* L.) pomocí superkritické fluidní extrakce

Nažky kmínu obsahují v závislosti na podmínkách pěstování 1–9 % silic, v nichž je přítomno asi 30 látek. Karvon a limonen tvoří asi 95 % celkového obsahu. U registrovaných odrůd Kepron, Prochan a Rekord kmínu kořenného (*Carum carvi* L.), pěstovaných v letech 1998–2000, byl sledován vliv pomletí a přípravy vzorku, ošetření a doby sklizně na jejich kvalitu. Jako ukazatel jakosti sloužily celkové množství silice a vzájemný poměr karvonu a limonenu u jednotlivých odrůd. Kmín kořenný byl extrahován celý a mletý. Ukázalo se, že pro stanovení silic z nemletého kmínu není SFE vhodná, neboť získané hodnoty jsou nižší oproti SFE z mletého kmínu. Byl rovněž ověřován vliv způsobu mletí. Ze tří použitých mlýnků se jako nejvhodnější ukázal tříštivý mlýnek VIPO. Dále byl sledován obsah silic v kmínu sklizeném na začátku zrání (vzorek A) a v plné zralosti (vzorek B) plodů kmínu. Více silic měly vzorky B, které byly sklizeny v plné zralosti. Použití přípravku Roundup Bioaktiv pro sjednocení dozrávání nažek na hlavním okolíku a okolících prvního řádu a fungicidů Alert S a Prelude 10 ovlivnilo pozitivně množství silice v kmínu. Byly zkoumány možnosti využití SFE pro stanovení silic u malých vzorků odebíraných v průběhu šlechtění. Výsledky byly využity jako další kritérium pro šlechtění, což klasický způsob stanovení obsahu silic neumožňuje. Byla testována též alternativní metoda superkritické fluidní extrakce (SFE).

Klíčová slova: kmín; odrůda; karvon; limonen; plynová chromatografie (GC); superkritická fluidní extrakce (SFE)

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