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Supercritical carbon dioxide extraction of okra (*Hibiscus esculentus* L) seeds

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Abstract: Pilot-scale supercritical fluid extraction of okra seeds was carried out, using carbon dioxide as solvent, at temperatures of 40, 50 and 60 °C and pressures of 150, 300 and 450 bar. Laboratory-scale Soxhlet extraction of the ground seeds was carried out with ethanol and n-hexane. The yields of supercritical fluid extraction and n-hexane Soxhlet extractions were similar. The ethanol Soxhlet extraction gave the highest yield, but the concentrations of β-sitosterol and tocopherols in this extract were lower than in the supercritical fluid extraction product. The fatty acid profiles of the extracts were determined, and a high unsaturated/saturated ratio was observed. The fatty acid compositions were only slightly different for oils obtained by the different extraction methods.

Keywords: supercritical fluid extraction; okra; β-sitosterol; tocopherol; fatty acids

INTRODUCTION

The supercritical fluid extraction (SFE) of natural compounds from plant raw materials is an excellent method for both industrial and analytical applications, especially for use in the food and pharmaceutical industries. The solvent is tunable; by changing the parameters of the extraction process (pressure and temperature), the selectivity of the solvent is changed and two operations, extraction and fractionation, can be achieved in a single process. The extract, after depressurization in the separators, remains solvent-free. The most utilized supercritical solvent is carbon dioxide. Its advantage over organic solvents is that it is non-flammable, non-toxic and environmentally benign, and also available in high (food-grade) purity. Owing to the low critical temperature of carbon dioxide (31.1 °C), it is ideal for extraction of thermally labile, non-polar natural compounds from plant raw materials.[1–3]

SFE technology is widely used in the food, cosmetic and pharmaceutical industries. Coffee and tea decaffeination, hop extraction, extraction of spices and herbs and production of flavours and fragrances are the major applications. The commercial plants are mainly located in the developed areas of Europe, North America and Asia. There are many applications in development in the fields of herbal medicines, nutraceuticals and natural antioxidants and colorants.[1–4] It has been demonstrated that large capacity plants, with optimized design and operation, have costs that are very often comparable with those of classical processes submitted to similar constraints in terms of environmental and consumer protection.[5] Although SFE can perform many types of separation, case-by-case evaluations are always required, and many factors should be evaluated.

The okra plant (*Hibiscus esculentus* L, member of Malvaceae) is found in many parts of Africa, the Mediterranean region and throughout the cotton belt of the USA. Plant parts, such as flowers and green tissues, may be consumed as specialty foods, while the seed oil may be used for industrial or food purposes. The reported composition of okra seed oil is approximately ($g\,kg^{-1}$): 255–297 linoleic acid, 415–419 oleic acid and 288–297 saturated acids. The extracted meal is comparable with other meals in commercial use for feeding livestock.[6,7]

Recently, a comparative study on oils of 22 seed genomes of perennial and woody species and hybrids was completed.[8] Neutral lipids were extracted with

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carbon dioxide at 80 °C and 537 bar for 45 min. Polar lipids were subsequently extracted with a mixture of carbon dioxide and 150 g kg^{-1} ethanol at the same temperature and pressure. The average oil yield across the seeds examined was 122.7 g kg^{-1}. Fatty acid composition and polar phospholipid profiles were determined.

In this study, SFE of oil components from okra seeds, with respect to the total yield, the fatty acid composition of the oils obtained and the yields of minor components, was investigated.

MATERIALS AND METHODS

Materials

The raw material (commercially available okra seeds) was purchased from Atena Belissaride Co (Thessaloniki, Greece) and used for extraction. The Soxhlet extraction solvents, ethanol (food grade, 960 g kg^{-1}), n-hexane (reagent grade) and other analytical-grade reagents were purchased from Reanal Ltd (Budapest, Hungary). The carbon dioxide used in supercritical fluid extraction (food grade, 995 g kg^{-1} purity) and helium for GC carrier gas were supplied by Messer Griesheim Hungary Ltd (Budapest, Hungary). The fatty acids RM-5 standard (rape seed oil methyl ester) and β-sitosterol standard were purchased from Supelco (Bellefonte, PA, USA). The α- and γ-tocopherol standards were supplied by Aldrich (Steinheim, Germany).

Methods

Preparation of the raw material for extraction

The raw material was ground with a hammer mill before extraction. After the SFE the particle size distribution was determined by sieving the extracted meal with a set of standardized sieves. The parameters of the Rosin–Rammler–Bennett (RRB) distribution model used, which is suitable for the representation of many fine grinding products, were determined with Statistica for Windows® 5.0 (StatSoft, Tulsa, OK, USA).

Solvent extraction

Laboratory Soxhlet extractions of ground seeds were carried out in triplicate according to the *Hungarian Pharmacopoeia*, edition VII, using n-hexane and ethanol.

Supercritical fluid extraction

The raw material was extracted with carbon dioxide in a high-pressure pilot plant equipped with 5 l volume extractor vessel (delivered by NATEX, Ternitz, Austria). A more detailed description of the equipment and extraction is given elsewhere.[9]

The extraction vessel was filled with about 2.0–2.2 kg of ground seeds. The desired temperature and pressure were adjusted and the CO_2 feed was started. The operating parameters in the separator were 40 bar and 20–25 °C. The accumulated product samples were collected and weighed at certain time intervals. The CO_2 flow rate, measured with a Mikro Motion RFT 9729 type mass flow meter (Micro Motion Europe, Veenendaal, The Netherlands), was about 6 kg CO_2 h^{-1} kg^{-1} raw material. The extraction was carried on until the amount of the product sample collected during 1 h decreased to under 1 g kg^{-1} raw material. The extraction time was in the range of 240–800 min, depending on the extraction conditions.

Determination of β-sitosterol by TLC and densitometry evaluation

The standard solution was made by dissolving 200 mg β-sitosterol in 10 cm^3 of diethyl ether. The samples were prepared by dissolving 100 mg extract in 10 cm^3 trichloromethane.

Thin layer chromatography (TLC) was effected on POLIGRAM® SIL G pre-coated plastic sheets (Macherey-Nagel, Düren, Germany). For the developing agent a mixture of n-hexane–diethyl ether (1:1, v:v) was applied. The sterol spots were visualized using iodine vapour (quick identification) and sulphuric acid (0.6 M in methanol) for the purpose of qualitative analysis. The intensities of the spots were evaluated using a Chromscan (Yoce Loebl, Gateshead, UK) densitometer. The measurements were made at $\lambda = 400$ nm; the slit width was 0.2 mm. The peaks were evaluated at $0.2R_f$ (for β-sitosterol) and $0.85R_f$ (for β-sitosterol-esters). For calibration the quantity of standard samples was plotted against peak height.

Determination of total fatty acid composition by gas chromatography

To a vial containing a precisely measured quantity of extracted oil (100–200 mg) were added 3 mg diethyl ether and 0.2 mg of 200 g l^{-1} tetramethylammonium hydroxide (TMAH) in methanol. The vial was shaken for about 2 min, and then the contents were allowed to separate into two phases. Three drops of thymol blue indicator plus enough methanolic HCl solution (0.5 M) to change the blue colour of the solution to yellow were added dropwise. To produce a single phase solution, 0.5 cm^3 of methanol was added. Fifty microlitres of this solution were injected into the gas chromatographic apparatus.[10]

The RM-5 standard (rape seed oil methyl ester) was used for determination of fatty acid composition of the extracts. The gas chromatographic (GC) apparatus utilized for the analysis of fatty acid composition was a Varian Star 3400 CX (Palo Alto, CA, USA), with a capillary column OmegaWax 320 (length 25 m, id 0.25 mm, film layer thickness 0.25 μm) and flame ionization detector (FID). As carrier gas, high-purity helium was used (flow rate 5 ml min^{-1}). The injection temperature was 220 °C, the detector temperature was 270 °C and a temperature gradient method was used, with the following programme for the column temperature: 100 °C (5 min), 10 °C min^{-1} to 220 °C, held at 220 °C for 3 min.

Determination of tocopherols by HPLC

Tocopherols were separated by high-performance liquid chromatography (HPLC; Waters 2690 separation module; Waters, Milford, MA, USA). Samples of 250 mg of extract were dissolved in 25 ml n-hexane. Ten microlitres of the solution were injected onto a Nova-Pak silica column (150 × 3.9 mm, 4 μm; Waters). Elution was performed with n-hexane–tetrahydrofuran (99:1, v:v) mobile phase at a flow rate of 1.0 ml min^{-1} under isocratic conditions. The column was tempered at 30 °C, and the sample temperature was 20 °C. Detection was performed by fluorescence detector (Waters 474 FLD) with excitation wavelength 295 nm and emission wavelength 330 nm. Identification was made by comparing with external standards.

RESULTS AND DISCUSSION

Characterization of the raw material

The moisture content of the ground okra seeds was 92.8(±1.2)g kg^{-1}. The particle size distribution of the solid material was determined by passing the ground seeds through sieves of various mesh size and weighing the fraction taken from each tray. The RRB equation[11] was used to characterize the size distribution: $R = 1000\exp[-(x/x_0)^n]$ where R is the proportion by mass of particles greater than screen size x, and x_0 and n are parameters related to the characteristic size (the particle size corresponding to 36.78% of the cumulative probability distribution), and the shape spread of the distribution function, respectively. A small n denotes a wide spread of particle sizes. A typical particle size distribution is shown in Fig 1. The RRB equation gave a good fit (variance 0.9766). The mean and standard deviation (in parentheses) of 11 replicates were $x_0 = 0.519$ mm(±0.057 mm) and $n = 1.66(\pm 0.28)$

Extraction yield

The effect of pressure and temperature of the supercritical fluid in the extractor was studied using a 3^2 full factorial design. Three experiments were performed at the centre of the design. The upper pressure level was set to 450 bar by considering the maximum allowed pressure in the extractor; the lower level (150 bar) was determined by the appropriate density (thus solubility). As the maximum temperature 60 °C was used in order to avoid thermal decomposition of sensitive components, while the lower temperature level (40 °C) was set slightly above the critical temperature of carbon dioxide. The yield was used as the characteristic variable for the efficiency. From the Pareto chart [Fig 2(a)] we found that the linear and quadratic terms of pressure and the interaction between pressure and temperature were the most important effects; the latter is in fact related to the density. Other terms have been found to be statistically significant but less important. Figure 2(b) represents the response surface which shows both the sensitivity and the optimal operating conditions.

Recovery of sterols

The total sterol content of the oil samples was determined by TLC densitometry. The Pareto chart for the standardized effects and the estimated response surface are given in Fig 3(a) and 3(b), respectively. Only the linear term of temperature and the interaction between the quadratic terms were statistically non-significant at the 95% confidence level ($p = 0.05$). The interaction between the pressure and temperature and the pressure had the main effects on the recovery of sterols. The best extraction conditions for sterols in the investigated range were 450 bar and 60 °C. A comparison of response surfaces for yield [Fig 2(b)] and recovery of sterols [Fig 3(b)] shows that the surfaces are very similar. Thus, higher oil yields result in higher recoveries of the sterol components. About 100 g kg^{-1} of the total sterols were free β-sitosterol.

Comparison of the products obtained by different extraction methods

Table 1 shows the comparison of the yields achieved by traditional solvent extraction (Soxhlet procedure

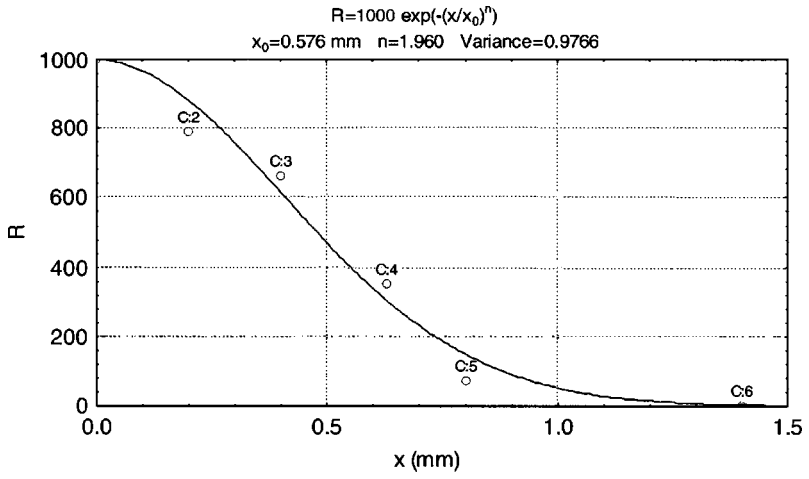

Figure 1. Particle size distribution of ground okra seeds: R is the proportion (g kg^{-1}) by mass of particles greater than screen size x; O, measured proportion retained on sieves C:2–C:6, respectively.

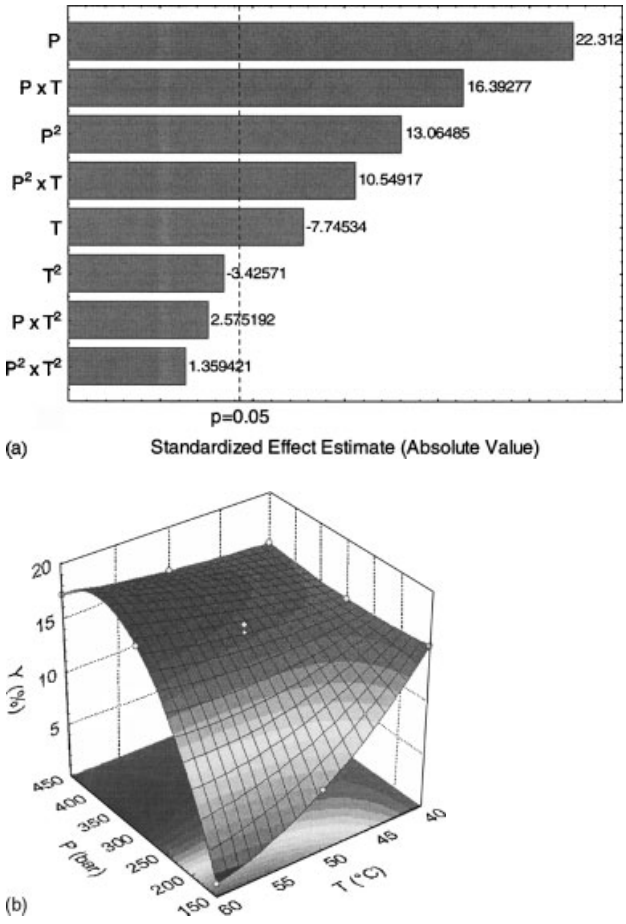

Figure 2. Effects of extraction pressure (*P*) and temperature (*T*) on oil yield (*Y*): (a) Pareto chart; (b) response surface estimated for the extraction yield.

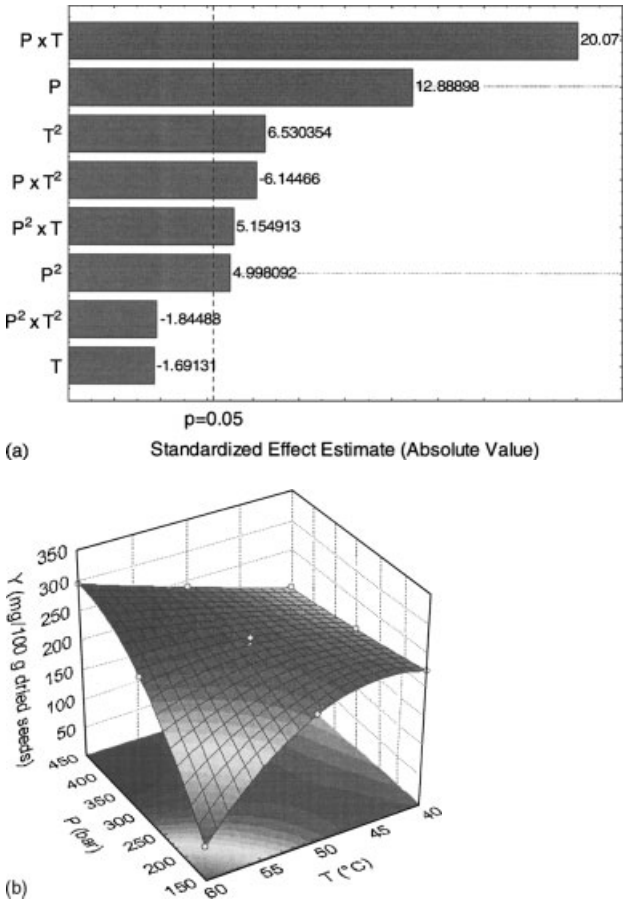

Figure 3. Effects of extraction parameters on recovery of β-sitosterol: (a) Pareto chart; (b) response surface estimated for the recovery of β-sitosterol.

using ethanol and n-hexane, respectively) with that obtained by SFE. SFE resulted in almost the same yield as hexane extraction and remarkably lower yield than ethanol extraction.

The fatty acid compositions of the extracts were measured by gas chromatography and the results are summarized in Table 2. The compositions are given as percentage chromatographic area. No significant differences in the compositions of the different solvent-extracted oils can be observed. All oils show a high degree of unsaturation, with linoleic acid as major constituent. The highest sterol concentration was obtained in the CO_2-extracted oil (see Table 1). However, the recovery of sterols was higher by alcoholic extraction due to the higher oil yield. Similar results were obtained by isolation of γ-tocopherol. Remarkably, α-tocopherol was recovered by SFE. While the α-tocopherol yields were considerably lower in oils obtained by Soxhlet extraction, the β- and δ-tocopherols were under the detection level in the oils.

CONCLUSIONS

SFE with carbon dioxide is a suitable technique for concentration of biologically active compounds (β-sitosterol, sitosterol esters and tocopherols) from okra

Table 1. Comparison of different extraction methods: extraction yields and yields of the active components

	SFE (450 bar, 50 °C)	n-Hexane extraction	Ethanolic extraction
Yield ($g\,kg^{-1}$)[a]	159.6 (4.8)	163.1 (5.6)	207.4 (9.4)
β-Sitosterol content of oil ($g\,kg^{-1}$)[b]	15.0	12.3	12.9
β-Sitosterol yield ($g\,kg^{-1}$)[b]	2.39	2.01	2.68
α-Tocopherol content of oil ($mg\,kg^{-1}$)[b]	930	780	620
α-Tocopherol yield ($mg\,kg^{-1}$)[b]	148	127	129
γ-Tocopherol content of oil ($g\,kg^{-1}$)[b]	2.55	2.33	2.38
γ-Tocopherol yield ($mg\,kg^{-1}$)[b]	407	380	494

[a] Mean of tree replicates; numbers in parentheses are the standard deviations.
[b] Mean of two replicates.

Table 2. Fatty acid composition of okra seed oil (percentage chromatographic area; the values represent the means of two GC runs)

	Extraction method		
Fatty acid components	SFE	*n*-Hexane	Ethanol
Palmitic acid (C16)	31.6	32.5	29.9
Stearic acid (C18)	3.4	2.8	3.2
Oleic acid (C18:1)	17.2	16.1	17.4
Linoleic acid (C18:2)	46.0	47.4	47.5
Linolenic acid (C18:3)	1.3	1.2	1.3
Arachidic acid (C20)	0.5	—	0.2
Behenic acid (C22)	—	—	0.2
Erucic acid (C22:1)	—	—	0.3
Unsaturated/saturated ratio	1.82	1.83	1.98

seeds. The overall yields of oil and sterols by SFE are very similar to those obtained using n-hexane as solvent, but the extraction temperature in the case of SFE is lower and the resulting cake and extract do not contain any residual solvent. The best process parameters were determined with the response surface method as $P = 450$ bar and $T = 60\,^{\circ}\mathrm{C}$, but, to avoid the possibility of thermal degradation of valuable components, it is recommended to use the intermediate temperature $T = 50\,^{\circ}\mathrm{C}$, because the yield decrease in this case is unimportant. The grinding of the seeds is essential, because internal diffusion is the rate-determining process in SFE of oleaginous seeds.

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