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Supercritical fluid extraction of *Vitex agnus castus* fruit

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ABSTRACT

The effects of supercritical fluid extraction (SFE) conditions (pressure and temperature) on extraction yield and recoveries of biologically active components were studied using a 3^2 full factorial design. Pilot plant SFE experiments were performed in a 5×10^{-3} m^3 volume high-pressure vessel. The pressure and temperature were varied over the ranges of 100–450 bar and 40–60 °C, respectively. The yield and recoveries were compared to those obtained with *n*-hexane and ethanol (96%, v/v) extractions. The extract samples were analysed by TLC, TLC-densitometry, GC and HPLC methods.

The obtained yields changed between 1.9 and 65.7 g kg^{-1}, according to the solvent power of the supercritical fluid. The recoveries of the different minor components were (g minor components kg^{-1} dried raw material): 0.06–1.06 rotundifurane, 0.02–1.08 β-sitosterol, 0.04–0.63 β-amyrin, 0.87–2.71 casticin.

By evaluation the designed experiments 450 bar and 45 °C were chosen as the best conditions within the ranges investigated.

1. Introduction

Supercritical fluid extraction (SFE) is an outstanding method for the extraction of natural agents and valuable components from plant materials, for the heat-sensitive compounds can be extracted without any degradation and, in addition it is an environmentally accepted technology. Supercritical carbon dioxide (sc-CO_2) is the most useful supercritical fluid in the food industry, because it is non-toxic, non-explosive, available in high (food-grade) purity and can be removed from the extracted products without leaving any residue [1].

The chaste tree (*Vitex agnus castus* L., Verbenaceae family) is a deciduous, fragrant small tree or shrub native to Europe and Asia, that grows wild in the Middle East and the Mediterranean regions to a height of 2–3 m. It is now cultivated all over the world. Leaves have an aromatic flavour, which repels mosquitoes. *Vitex* has violet or light violet coloured flowers, which blossom in 10–15 cm spike from July till the end of September. Flowers are followed by fleshy fruit that contains four seeds that are similar to black pepper. The common names of the fruit of *Vitex agnus castus* are chasteberry or monk's pepper [2,3]. Its ripened fruits (*Agni casti fructus*) have been used as a popular folk phytomedicine in Europe for the treatment of the hormone imbalance syndromes in women for a long time. For more than 2500 years ago, in ancient Egypt, Greece and Rome, *Agni casti fructus* was used for a variety of gynecologic conditions [4–6]. Since the 1950s, many medicinal preparations of chasteberry from both wild and cultivated plants have been widely used in the treatment of premenstrual syndrome (PMS), cyclical breast discomfort, menstrual cycle irregularities, and dysfunctional uterine bleeding [5]. The berry of the chaste tree contains a number of bioactive compounds: flavonoids (i.e. casticin, penduletin and kaempferol), iridoid glycosides (i.e. aucubin and agnuside), terpenoids (i.e. vitexlactam and rotundifurane), essential oils (i.e. limonene, α- and β-pinene), and ketosteroids [5,7–14]. The last decade has provided several successful clinical trials supporting the use of *Vitex agnus castus* L. for treatment of premenstrual syndrome [15–18].

It was recently reported that the lipophilic fraction of *Vitex agnus castus* L. extracts contain diterpenes with pharmacological, dopaminergic activity. The lipophilic fraction can be extracted with *n*-hexane, which was shown to contain the active principle. Hoberg et al. [10] extracted 80 g ground fruit with *n*-hexane by turbo extraction and the yield (Y) was 7.25 g dry extract (Y = 9.1%). In another study [9] 4 kg fruit was extracted with *n*-hexane (3 × 8 L) and the yield was 200 g (Y = 5%). For some bioassay experiments the *Vitex agnus castus* L. fruit samples were first defatted with petroleum ether and then extracted with 90% methanol [6,19]. In a PMS treatment study the administered *Vitex agnus castus* L. extract was utilized in film-coated tablets. One tablet contained 4.0 mg dried ethanolic (70% EtOH) extract (E) which was equal to 40 mg herbal

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drug (D) (ratio of D/E = 10). Ohyama et al. [7] extracted 1 g ground *Agni casti fructus* with 10 mL EtOH (reflux, 2 h) and the obtained yield was 0.08–0.1 g from 1 g dried fruit (Y = 8–10%).

The main aim of present study was to investigate the effects of SFE conditions on the yield and recoveries of minor components from the fruit of *Vitex agnus castus* L. A further aim was to set up an environmentally friendly technology to obtain high-value biologically active extracts from the fruit of chaste tree.

2. Materials and methods

2.1. Materials

The chasteberry was obtained as dried raw material from Gradiens Ltd. (Budapest, Hungary). Food grade carbon dioxide (995 g kg^{-1} purity) was purchased from Linde Gas Co. (Budapest, Hungary). The solvents (food grade) used for the extractions and the analytical-grade reagents were purchased from Reanal Ltd. (Budapest, Hungary). The analytical standards β-sitosterol, β-amyrin, α-pinene, limonene, β-caryophyllene, α-humulene were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA) and the standard of rotundifurane and casticin were purchased from University of Szeged (Szeged, Hungary).

2.2. Methods

2.2.1. Preparation of the raw material for extraction

The dried fruit was ground with a hammer mill using a 1 mm mesh size screen before extraction. The particle size distribution was determined by sieving. Statistica for Windows® 8 software (StatSoft, Tusla, OK, USA) was used to determine the parameters of the Rosin–Rammler–Bennett (RRB) particle size distribution model, which is suitable for the representation of several crushed products [20].

The dry residue of ground samples were measured by method 2.8.16 described in *European Pharmacopoeia* (5th ed. 2005). About 2.00 g of the ground samples to be examined were dried in an oven at 105 °C for 3 h and allowed to cool in a desiccator over anhydrous silica gel and then weighed. Three repeated experiments were carried out.

2.2.2. Determination of essential oil content

Standard method 2.8.12 described in *European Pharmacopoeia* (5th ed. 2005) was applied for determination of the essential oil content of *Agni casti fructus* samples. The apparatus is for the purpose of steam distillation in a modified Clevenger vessel. About 100 g of raw material was measured into the round-bottomed flask with 1000–1300 mL distilled water. It was boiled for at least 3 h. In the top of the Clevenger vessel the essential oil was collected and measured in volume. Three repeated experiments were carried out.

2.2.3. Soxhlet extraction

About 20 g of dried ground fruit was weighted and appropriate volume (200 mL) of organic solvent (*n*-hexane, ethanol, 96%, v/v) was poured into the Soxhlet apparatus. The extraction process was continued until the extraction time was reached, while the colour of the extract seemed to fade. After the extraction the solvent was removed in a vacuum-distillation apparatus ($p \sim 0.5$ bar) and then the extracts were weighed.

2.2.4. Supercritical fluid extraction

The ground plant material was extracted in a high-pressure pilot plant with 5 L volume extractor vessel, which was designed and delivered by NATEX (Ternitz, Austria). The extraction vessel was filled with about 0.8 kg ground fruit (bulk density: 508 kg m^{-3}). The desired extraction conditions (temperature and pressure) were set and the CO_2 feed was started. The operational parameters in the separator were 40 bar and 20–25 °C during the process. The CO_2 flow rate was measured with a Micro Motion RFT 9729 type mass flow meter (Micro Motion Europe, Veenedaal, The Netherlands). The specific flow rate was about 7 kg CO_2 h^{-1} kg^{-1} raw material. Extract samples were collected and weighed every hour. Total extraction times were between 4 and 7 h. At 450 bar, where the extraction was the most effective in the studied range, the required extraction time was 4 h, while at 100 bar the process time was 7 h because of the low solubility of the compounds at this condition. Each extraction was continued until the amount of sample collected within 1 h period was less than 1 g kg^{-1} raw material.

The effects of extraction parameters (pressure and temperature) on yield and recoveries of active principles were studied using a 3^2 full factorial design. The results obtained with the above described methods were subjected to analysis of variance (ANOVA). Statistica for Windows® 8 software (StatSoft Inc., Tusla, OK, USA) was used for the statistical evaluation. The effects of the operational parameters were evaluated at 5% significance level (p = 0.05).

2.2.5. Determination of the content of the essential oil by TLC and GC

The essential oil obtained with hydro-distillation and the essential oil rich SFE product were investigated by TLC and GC analytical method.

The TLC was employed for quick comparison of the samples. The used TLC system was the next: sorbent: Kieselgel 60 F_{254} Merck, solvent system: *n*-hexane/ethyl acetate 80:20 (v/v) as mobile phase, detection: 3% vanillin in ethanol, 3% sulphuric acid in ethanol reagent by heating for 3 min on 100 °C and normal light after derivatization.

For the GC analysis the samples were prepared as follows: from the essential oil obtained with hydro-distillation 3 μL was dissolved in 2 mL chloroform and 0.4 μL was injected; 20 mg from the essential oil rich product of SFE was dissolved in 2 mL chloroform, and 0.4 μL was injected. The gas chromatograph equipment was a Fisons GC-8000 with FID detector (Fisons Plc., Ipswich, UK) and DB 1701 column (30 m, 0.32 mm i.d., with 0.25 μm film) (Agilent Technologies, Santa Clara, CA, USA). The column temperature program was 2 min at 60 °C isotherm, followed by ramp rate 8 °C/min up to 230 °C, then 5 min isotherm at 230 °C. The temperature of the injector was 200 °C, the carrier gas was nitrogen with a flow rate of 6–8 mL/min. The compounds were identified by their retention times compared to standards.

2.2.6. Determination of diterpenes by thin layer chromatography (TLC) and densitometry

The diterpenes content of the chaste tree fruit was investigated by TLC densitometry. The studied samples were obtained with sc-CO_2, *n*-hexane and ethanol (96%, v/v). Samples were prepared by dissolving 50 mg of extract in 10 mL of cyclohexane. The applied quantities of the samples were 5–5 μL, respectively. The reference material was 1, 3, 5, 7 and 10 μL of 880 mg L^{-1} rotundifurane in trichlorometane. During the analysis the mobile phase was benzene/ethyl acetate (95:5, v/v) and the stationary phase was silica gel (DC-Alufolien Kieselgel 60 GF_{254}, Merck & Co., Inc., Darmstadt, Germany). Concentrated H_2SO_4 was used for the evaluation. The concentrated H_2SO_4 was blown onto the surface of the plate, then dried at 105 °C for 2–3 min. The intensities of the spots were evaluated with a CHR-SCAN OE 540 densitometer (Chemotron Inc., Budapest, Hungary) at λ = 548 nm wavelength.

2.2.7. Determination of triterpenes by thin layer chromatography and densitometry

Due to eliminate the numerous disturbing components from the samples the non-saponifyable fraction had to be prepared. Exactly 1 g from the SFE extract, 0.1 g from the *n*-hexane extract and 0.4 g from the ethanolic (96% EtOH) extract was dissolved in 10 mL chloroform. Each of these dissolved extracts were refluxed for 2 h with 5% ethanolic KOH solution then cooled and mixed with 1% ammonium sulphate, after that the dissolved and refluxed samples were extracted with 3×30 mL and 2×20 mL diethyl ether. The combined diethyl etheric fractions were washed with distilled water then deflegmated with sodium sulphate (Na_2SO_4). The ether was evaporated after the samples diluted in 10 mL chloroform. Two triterpenes, β-amyrin and β-sitosterol were used as standards, from each of them 10 mg was diluted in 10 mL chloroform. The equipment was a Shimadzu CS-930 densitometer, $\lambda = 600$ nm, zigzag scan.

2.2.8. Determination of casticin by high-performance liquid chromatography (HPLC)

A HPLC method was developed to determine the casticin content in the extracts. For the sample preparation nearly 1.0 g sample was dissolved in 50 mL methanol in ultrasonic bath. This mixture was filtrated into a separating-flask then 50 mL distilled water was added. After mixing 2×50 mL chloroform was added for extraction the casticin. The lower, chloroformic fractions were combined and filtrated through sodium sulphate (Na_2SO_4). The solvent was evaporated fully in vacuum distillation. For the determination of casticin content, the dry residue was dissolved in 10 mL methanol. Precisely 20 μL of the solution was injected onto a Nucleosil 100 RP-C18 column (125 mm × 4 mm, 10 μm, Tracer C17935). The HPLC system included Merck Hitachi L-6000 A Pump, D-7500 Integrator (Merck & Co., Inc.). Elution was performed with mobile phase acetonitrile/water (40/60, v/v) with a flow rate of 0.8 mL min^{-1}. The column temperature was maintained at 25 °C. Detection was performed by UV detector (Merck Hitachi L-4250 UV–vis Detector, Merck & Co., Inc.) at 258 nm. Identification was made by using external standard.

3. Results and discussion

3.1. Characterization of raw material

The dry matter content of the ground chasteberry samples were 911.9(±3.7) g kg^{-1}.

The RRB equation was used to characterise the distribution of particle size

$$R = 100 \exp\left[-\left(\frac{d}{d_0}\right)^n\right],$$

where R is the proportion by mass of particles greater than screen size d, d_0 and n are the 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. The average size distribution parameters were: $d_0 = 0.47(\pm 0.04)$ mm and $n = 4.99(\pm 2.81)$.

Table 1
Comparison of extraction yields (Y) of chaste tree fruit obtained with ethanol (96%, v/v), *n*-hexane and supercritical carbon dioxide (sc-CO_2)

Solvents	Solubility parameter δ [$MPa^{1/2}$]	Yields Y [g kg^{-1}]
Ethanol	26.2	181.07 ± 9.55
n-Hexane	14.9	63.73 ± 0.75
sc-CO_2[a]	15.0	63.77 ± 2.06

[a] The operational parameters of the SFE: 275 bar and 50 °C.

3.2. Soxhlet extraction yields

Table 1 shows that the yields of the ethanolic extracts were 2.8 times higher than those provided by *n*-hexane. Table 1 contains the Hildebrand solubility parameters [21] of *n*-hexane and ethanol (96%, v/v) which represent their solvent power.

The physical attributes of the extracts like flavour, colour and viscosity changed depending on the used solvent. The sample had strong smell, brownish-yellow colour and easy spread consistency extracted with non-polar *n*-hexane. The ethanol (96%, v/v) provided dark-green-black coloured, highly viscous extract, as the most polar used solvent.

3.3. Supercritical fluid extraction yields

The designed extraction pressures were 100, 275 and 450 bar (100 bar is above the critical pressure of the solvent CO_2 (73 bar) and it is often suggested for extraction of essential oils. The upper working limit of the used equipment was 500 bar. Therefore the highest applied extraction pressure was 450 bar). The operational temperatures were 40, 50 and 60 °C. (The 40 °C is just above the critical temperature of the solvent CO_2 (31.06 °C) and this temperature is generally used in the extraction of plant materials by SFE. The selected upper limit of the temperature (60 °C) was low enough to avoid the damage of the heat sensitive compounds.). There were three repeated experiments in the center of the design (275 bar and 50 °C). The extraction yield (Y mass of extract/mass of dry matter) was used as an indicator of the effects of the extraction conditions (Table 2). Table 2 contains the solubility parameters of sc-CO_2 in case of different operation parameter sets. These values were calculated on the basis of the extraction pressure and temperature by using SF-Solver Software (ISCO Inc., Lincoln, NE, USA). From the Pareto chart (Fig. 1) we can see that the linear and qudratic terms of the extraction pressure and the extraction temperature

Table 2
The SFE yields of chaste tree fruit at different operation parameters

Experiments	Extraction pressure p [bar]	Extraction temperature T [°C]	Solubility parameter δ [$MPa^{1/2}$]	Extraction yield Y [g $kg^{-}1$]
SFE 1	100	40	10.6	36.0
SFE 2	100	50	6.7	8.5
SFE 3	100	60	5.1	1.9
SFE 4	275	40	15.8	62.3
SFE 5	275	50	15.0	65.7
SFE 6	275	50	15.0	64.0
SFE 7	275	50	15.0	61.6
SFE 8	275	60	14.2	64.2
SFE 9	450	40	17.1	63.6
SFE 10	450	50	16.5	63.8
SFE 11	450	60	16.0	62.0

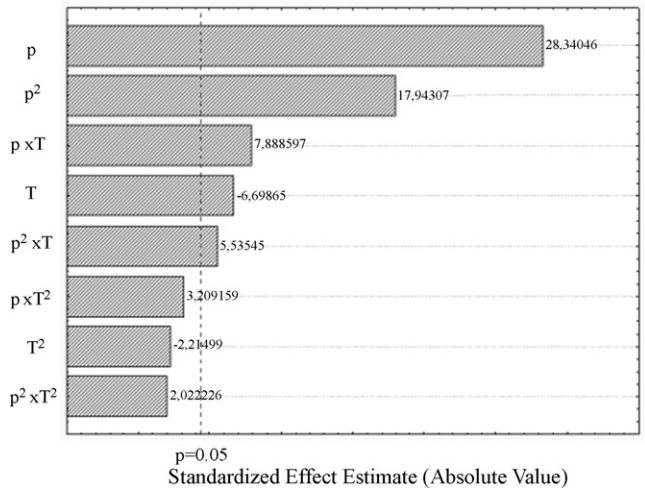

Fig. 1. Pareto chart for the effects of pressure (p) and temperature (T) on total extraction yield (Y).

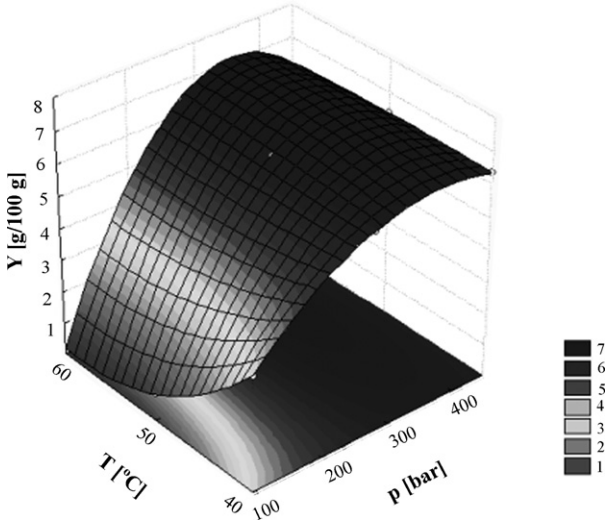

Fig. 2. Fitted response surface for SFE yield of the fruit of chaste tree. T: extractor temperature, p: extraction pressure, Y: extraction yield.

and in addition the interaction terms of linear and quadratic pressure and temperature have significant effects on the yield at the studied 5% significance level (for visual comparison the limit is shown at $p = 0.05$ with a broken line). Fig. 2 represents the fitted response surface for the measured points of extraction yield, which shows that yields reach a plateau above 300 bar with no regards to temperature. High yields can be achieved at pressures above 300 bar and temperatures 45 ± 5 °C within the ranges investigated. The solubility parameters show that the solvent power of the sc-CO_2 increases significantly in range 100–275 bar which phenomena was also represented in Fig. 2 (plateau above 300 bar). At 100 bar as the temperature increases the solubility parameters decrease significantly and the obtained yields decrease as well.

The optimal yield of the SFE was similar to the *n*-hexane extraction (Table 1), because of the similarity of the dissolving capacity of the supercritical carbon dioxide (sc-CO_2) and *n*-hexane. The yield of alcoholic extract was almost threefold higher than the SFE or *n*-hexane extracts. However the ethanol dissolved the whole unwanted soluble polar compounds, while the sc-CO_2 and *n*-hexane, as non-polar solvents, dissolved the non-polar components only.

Below 150 bar and above 45 °C is the range of the extraction of essential oils. The essential oils were fractionated with two serial connected separators ($p_{s1} = 80$ bar, $T_{s1} = 25$ °C, $p_{s2} = 20$ bar, $T_{s2} = 35$ °C, extraction pressure 450 bar, extraction temperature 50 °C) which were carried out separately from the 3^2 full factorial design. The volatile compounds (essential oils) were condensed in the second separator. The obtained amounts of the extracts in 100 g dried fruit in the first and second separators were 5.80 and 0.55 g, respectively. The amount of essential oil obtained by hydrodistillation in 100 g dried fruit were 0.11 g. The identified main volatile compounds of chasteberry by GC analytical method were α-pinene; limonene; 1,8-cineol; β-caryophyllene; *trans*-β-farnesene; α-humulene; spathulenol. The ratio of the identified components are nearly the same in the samples provided by different extraction processes.

3.4. Recovery of active components

3.4.1. Diterpene, triterpene and casticin contents

Table 3 shows the recoveries of biologically active compounds of chaste tree fruit.

The effects of SFE parameters (p, T) on the diterpene content of the supercritical extracts were investigated, namely the rotundifurane yield were evaluated. The Pareto chart (Fig. 3a) shows that the linear and quadratic terms of the extraction pressure and the interaction terms of pressure and temperature have significant effects at the 5% significance level ($p = 0.05$). Fig. 3b shows the response surface estimated for the rotundifurane recovery. It is well demonstrated in Fig. 3b that the temperature has significant effect on the recovery of rotundifurane at around 100 bar. It shows

Table 3
The recoveries of biologically active compounds [mg active compound 100 g^{-1} dry matter] of chaste tree fruit

Solvents	Experiments	Rotundifurane	β-Sitosterol	β-Amyrin	Casticin
Ethanol (96%, v/v)		85.00	88.00	60.50	225.00
n-Hexane		90.00	100.00	58.80	247.50
	SFE 1	85.90	38.56	40.31	87.50
	SFE 2	42.10	5.06	18.44	101.57
	SFE 3	5.91	1.63	3.55	n.d.[a]
	SFE 4	101.56	72.61	51.82	188.27
	SFE 5	86.54	96.57	58.05	227.30
sc-CO_2	SFE 6	96.61	87.19	46.80	204.38
	SFE 7	103.17	85.98	62.58	242.76
	SFE 8	102.66	91.29	51.86	233.93
	SFE 9	101.18	108.36	61.41	257.70
	SFE 10	105.90	102.90	55.45	245.09
	SFE 11	101.19	94.40	50.45	271.30

[a] n.d.: Non-detectable.

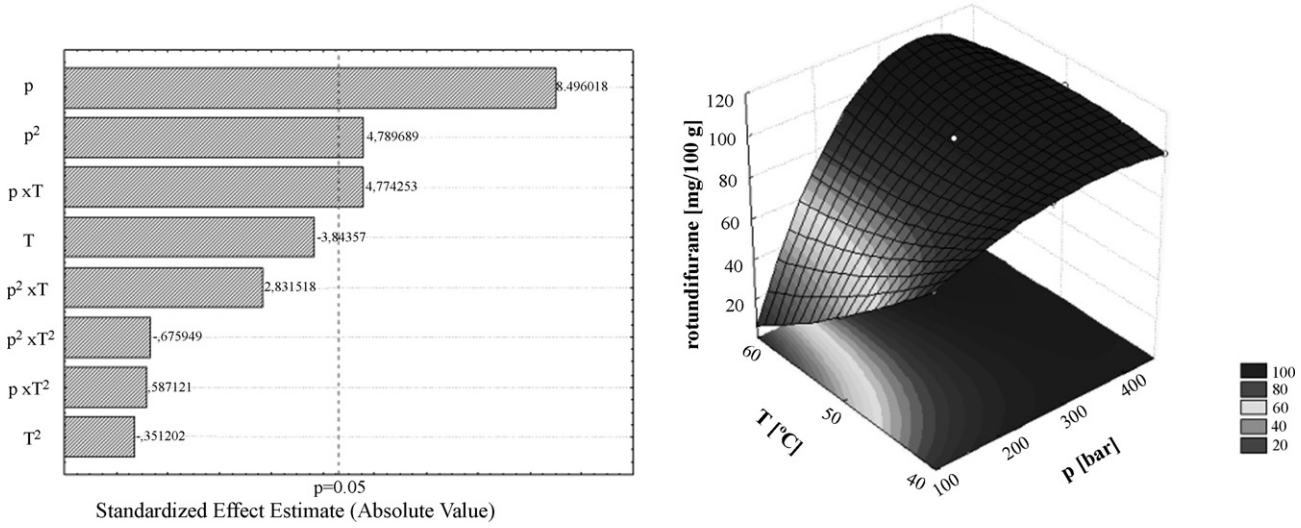

Fig. 3. (a) Pareto chart for the effects of pressure (p) and temperature (T) on the recovery of rotundifurane. (b) Fitted response surface for rotundifurane recovery of the fruit of chaste tree. T: Extractor temperature, p: extraction pressure.

the rotundifurane recovery changed between 5.91 and 85.90 mg $100\,g^{-1}$ at 100 bar. As the temperature increased, the yield of rotundifurane decreased. In the studied pressure range the effect of the temperature was less and less at higher pressure and finally (above 300 bar) it was negligible. This phenomena proves the temperature dependence of solubility parameters of sc-CO_2 at different conditions. The highest yield of the rotundifurane was obtained at 450 bar and 50 °C (1.06 mg g^{-1}).

The rotundifurane yields obtained by *n*-hexane and ethanol were 0.90 and 0.85 mg g^{-1}, respectively.

The triterpene content was measured at each point of the 3^2 full factorial design to determine the influence of the operational parameters (pressure and temperature) on the recovery of the triterpenes and in addition the *n*-hexane and alcoholic extract were investigated too. The studied triterpenes were β-sitosterol and β-amyrin. In case of β-sitosterol the linear and quadratic terms of the extraction pressure and the interaction terms of quadratic pressure and temperature have significant effects at the 5% significance level ($p = 0.05$) showed by Pareto chart (Fig. 4a). The fitted response surface for the β-sitosterol recovery (Fig. 4b) shows that the increase of the pressure slightly increases the yield of the β-sitosterol. The highest yield of the β-sitosterol was found at 450 bar and 40 °C. The Pareto chart (Fig. 5a) for the β-amyrin recovery shows that only the pressure has significant effect on the β-amyrin yield. Fig. 5b shows the response surface estimated for the β-amyrin recovery. It indicates that β-amyrin recovery is negligibly low at low pressures. Extracts with relatively high β-amyrin content can be obtained at medium temperature (45 °C) and high pressures (above 300 bar). Comparing Figs. 4b and 5b, it can well be seen that the yield of these two examined triterpenes were changed almost in the same amount as function of the temperature at 100 bar. In case of β-sitosterol the yield changed between 1.63 and 38.56 and in case of β-amyrin 3.55–40.31 mg $100\,g^{-1}$. But at higher pressure the maximum yield of β-sitosterol (108.36 mg $100\,g^{-1}$) was almost 2 times higher than the yield of β-amyrin (62.58 mg $100\,g^{-1}$) as Figs. 4b and 5b show.

The most effective isolation method of β-sitosterol was the SFE (1.08 mg g^{-1}). Nearly the same β-sitosterol yield was obtained by

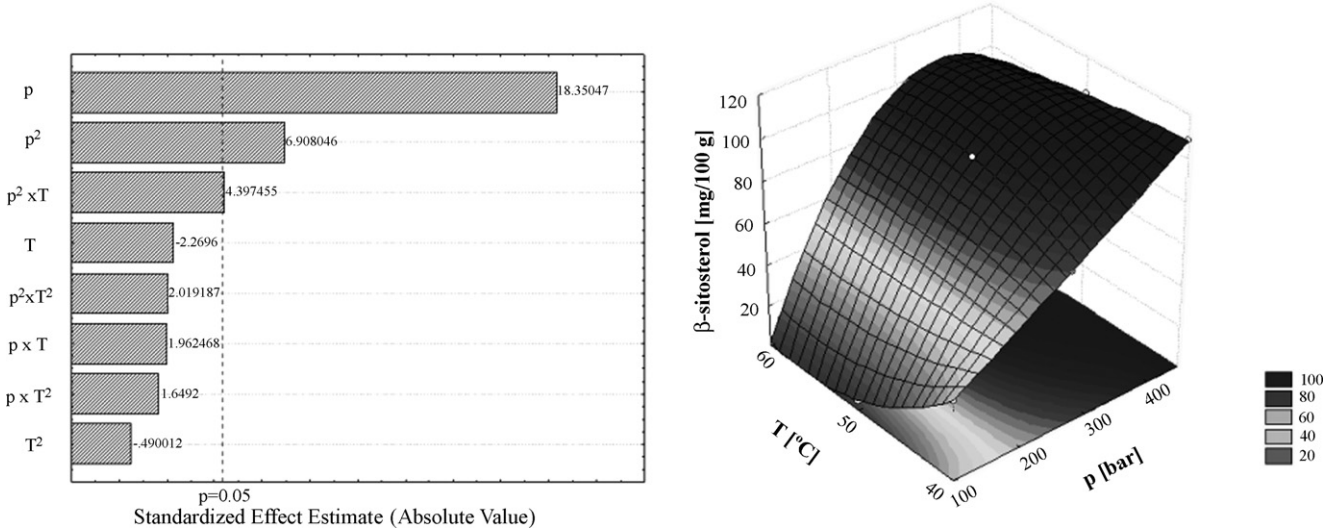

Fig. 4. (a) Pareto chart for the effects of pressure (p) and temperature (T) on the recovery of β-sitosterol. (b) Fitted response surface for β-sitosterol recovery of the fruit of chaste tree. T: Extractor temperature, p: extraction pressure.

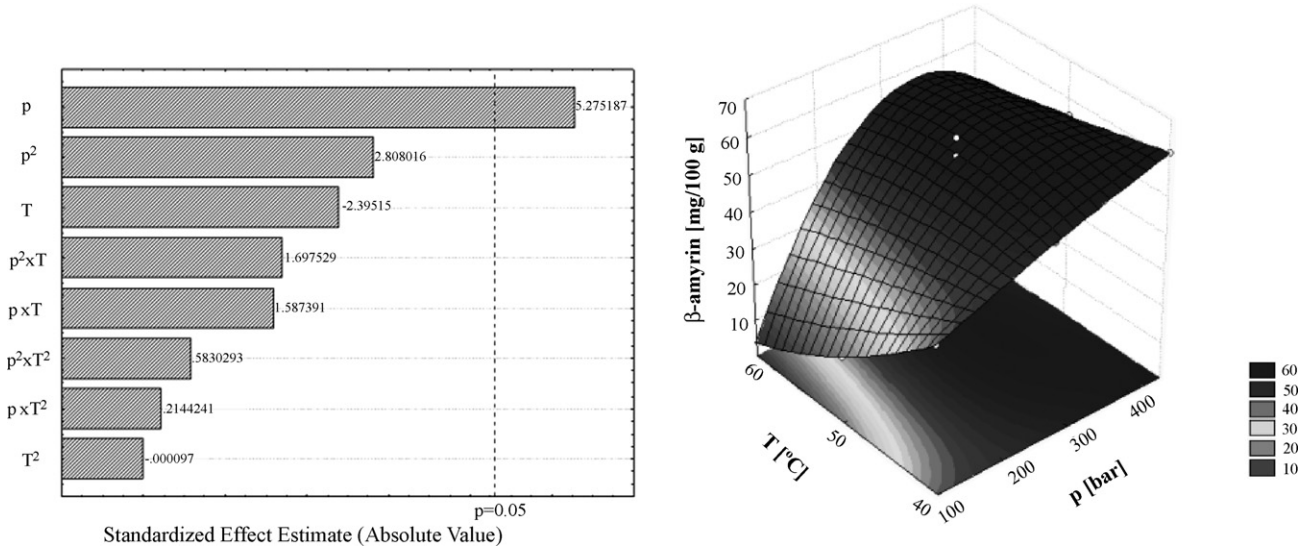

Fig. 5. (a) Pareto chart for the effects of pressure (p) and temperature (T) on the recovery of β-amyrin. (b) Fitted response surface for β-amyrin recovery of the fruit of chaste tree. T: Extractor temperature, p: extraction pressure.

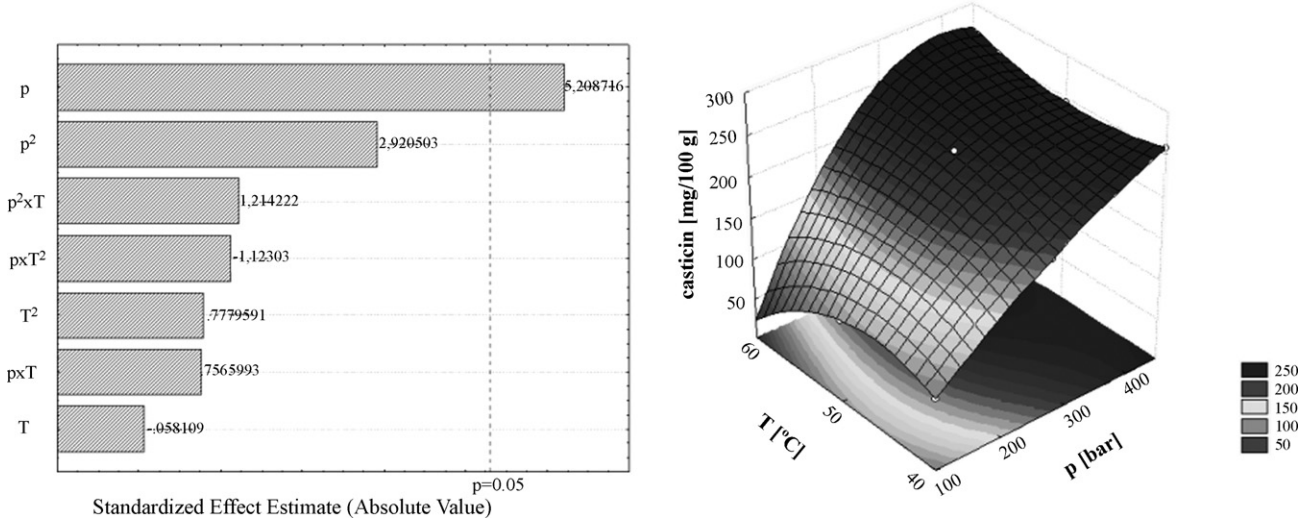

Fig. 6. (a) Pareto chart for the effects of pressure (p) and temperature (T) on the recovery of casticin. (b) Fitted response surface for casticin recovery of the fruit of chaste tree. T: Extractor temperature, p: extraction pressure.

n-hexane (1.00 mg g^{-1}). In the recovery of triterpenes the efficiency of the ethanol was lower (0.88 mg g^{-1}) than the two other studied solvents.

The casticin contents of the supercritical extracts were investigated in each sample of the experimental design and in addition the *n*-hexane and ethanolic extracts were analyzed too. The Pareto chart (Fig. 6a) for the effects of pressure and temperature on the recovery of casticin shows that the pressure has significant effect at the 5% significance level ($p = 0.05$). The highest casticin content (Fig. 6b) was found at 450 bar and 55 ± 5 °C. In Fig. 6b the significant effect of the pressure on the yield of casticin can be traced. The recoveries of casticin obtained by *n*-hexane (2.47 mg g^{-1}) was nearly the same and obtained by ethanol (2.25 mg g^{-1}) was lower than the recovery obtained by sc-CO_2.

4. Conclusion

The supercritical fluid extraction is a capable method for the valorization of the chaste tree fruit. The statistical evaluation of the experimental results showed the pressure has significant effect on the extraction. The suggested operational parameters of the SFE are 450 bar and $T = 45 \pm 5$ °C. At these conditions the rotundifurane, β-sitosterol, β-amyrin and casticin were obtained together in the highest amount.

In cases of extraction yield, recovery of diterpenes, triterpenes and casticin, the fitted response surfaces reached a plateau above 300 bar.

The obtained concentration (g minor components kg^{-1} extract) of rotundifurane, β-sitosterol, β-amyrin and casticin were the following: 4.7, 4.9, 3.4 and 12.4 in case of ethanol; 14.1, 15.7, 9.3 and 38.9 using *n*-hexane; 16.6, 17.3, 9.9 and 42.4 applying sc-CO_2, respectively. These data represent that 3–4 times higher concentration of the minor components in the extracts can be obtained by using sc-CO_2 than applying ethanol.

The extract obtained with sc-CO_2 contains valuable components like essential oil (β-caryophyllene, 1,8-cineol, etc.), diterpenes (rotundifurane), triterpenes (β-sitosterol, β-amyrin), flavonoids (casticin). The essential oil of *Vitex agnus castus* L. useful for the

self-care treatment of meanopausal balance [22]. Diterpenoids from the fruit of *Vitex agnus castus* L. showed dopamine D_2 receptor affinity. Via this attribute diterpenes normalize the output of oestrogens and progesterone and the correct prolactin level [10,13]. The β-sitosterol and β-amyrin as phytosterols are effective to decrease the cholesterol level without any side effects [23–25]. The casticin has antioxidant and antitumor activities [26].

These compounds can be extracted in higher amounts with sc-CO_2 than with traditional organic solvents, in addition the supercritical carbon dioxide extract does not contain any dangerous solvent after the extraction.

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