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SVETOLIK MAKSIMOVIC[1]
VANJA TADIC[2]
JASNA IVANOVIC[1]
TANJA RADMANOVIC[1]
STOJA MILOVANOVIC[1]
MILICA STANKOVIC[3]
IRENA ZIZOVIC[1]

[1]University of Belgrade, Faculty of Technology and Metallurgy, Belgrade, Serbia
[2]Institute for Medical Plant Research "Dr Josif Pančić", Belgrade, Serbia
[3]University of Niš, Faculty of Medicine, Niš, Serbia

SCIENTIFIC PAPER

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UTILIZATION OF THE INTEGRATED PROCESS OF SUPERCRITICAL EXTRACTION AND IMPREGNATION FOR INCORPORATION OF *Helichrysum italicum* EXTRACT INTO CORN STARCH XEROGEL

Article Highlights

- Starch xerogels were impregnated *with H. italicum* extract by $scCO_2$ extraction-impregnation process
- Influence of different process parameters on impregnation yield was studied
- Influence of ethanol on the extract's chemical profile and impregnation yield was determined
- Selected process conditions enabled sufficient quantity of impregnated extract

Abstract

Supercritical CO_2 extraction of Helichrysum italicum and impregnation of starch xerogels with the extract by using an integrated $scCO_2$ extraction and impregnation process were performed at 350 bar and 40 °C in order to produce biomaterials for possible oral intake of the extract. Xerogels produced by air-drying of acetogels and alcogels were used as carriers in the supercritical impregnation process. The effect of ethanol as a co-solvent, contact time, plant material/carrier mass ratio and xerogel preparation on the impregnation loading was studied. The highest impregnation loading (1.26±0.22%) was achieved after 5 h impregnation of the xerogel obtained from alcogel using pure $scCO_2$ and plant material/carrier mass ratio of 10. Chemical analysis of the extracts showed that the addition of ethanol as co-solvent had a positive effect on $scCO_2$ selectivity to terpene fraction and total flavonoids, while it lowered the total phenolic content. Despite the difference in chemical composition, both extracts expressed similar antioxidant activity according to the DPPH and FRAP methods. The integrated process was shown to be a feasible method for isolation and incorporation of bioactive components of H. italicum into starch xerogels.

Keywords: antioxidant activity, Helichrysum italicum, supercritical extraction, supercritical impregnation, starch xerogels.

The genus *Helichrysum* consists of an estimated 600 species, in the sunflower family (*Asteraceae*). *Helichrysum italicum* is commonly known as curry plant or everlasting. It is a small aromatic shrub with yellow flowers. The stems are woody at the base and can reach 30-70 cm in height. It grows on dry, rocky or sandy ground around the Mediterranean [1].

Traditional use of this plant includes the application for treatment of allergies, colds, cough, skin, liver and gallbladder disorders, inflammation, infections and sleeplessness [1]. Essential oils and extracts of *H. italicum* are reported for antimicrobial, anti-inflammatory, anti-viral, antioxidant and anti-larvicidal activities which is mainly due to the presence of terpenes and phenolic compounds [2].

Although *H. italicum* is traditionally used for treatment of digestive system disorders, only a few articles in the open literature reported use of *H. itali-*

Correspondence: S. Maksimovic, University of Belgrade, Faculty of Technology and Metallurgy, Karnegijeva 4, 11120 Belgrade, Serbia.
E-mail: smaksimovic@tmf.bg.ac.rs
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cum isolates in this area. Recently Rosa *et al.* [3], performing extraction from *H. italicum* by acetone, reported the protective effect of the extract's component arzanol on oxidative modification of lipid components induced by Cu^{2+} in human low-density lipoprotein (LDL) and the reduction of polyunsaturated fatty acids and cholesterol levels, inhibiting the increase of oxidative products. Previously, Facino *et al.* [4] tested free-radical scavenger properties of the single glycosyl-flavonoids, isolated from the ethanolic extract of *H. italicum*, and *in toto* glycosidic fraction with *in vitro* systems where different reactive oxygen species were generated and on lipid peroxidation in rat liver microsomes. It was shown that *in toto* fraction inhibited superoxide ions and hydroxyl radicals to a lesser extent than the lipid peroxidation in microsomes. Finally, Rigano *et al.* [5], performing extraction of *H. italicum* by ethanol, showed that this extract elicited antispasmodic actions in the isolated mouse ileum and inhibited transit preferentially in the inflamed gut. Biological assays on human colonic epithelial cells indicated the extract's component 12-acetoxytremetone antioxidant effects, specifically by reducing reactive oxygen species [6].

Supercritical fluid technology, including the application of carbon dioxide as the main solvent, has gained wide acceptance during the past decades as an alternative to conventional processes [7]. Carbon dioxide is the most used solvent in supercritical processes because it is safe, readily available and low-cost [8]. Also, it has low critical pressure and temperature values (73.86 bar and 31.06 °C, respectively). The main advantage of supercritical CO_2 ($scCO_2$) extraction compared to conventional extraction processes is the production of solvent-free and highly valued plant extracts, after removing the solvent by decompression. On the other hand, $scCO_2$ can induce swelling of polymers and reduce viscosity of the polymer melt by up to an order of magnitude [9]. This and the near-zero surface tension of $scCO_2$ facilitates incorporation of compounds soluble in $scCO_2$ into the polymer matrix [10]. The main advantages of supercritical impregnation over the conventional impregnation processes are the production of high-purity and solvent-free impregnated materials, better distribution of solute inside the carrier matrix during the short time compared to conventional processes and avoidance of toxic reagents and additional treatment of the impregnated products.

Data on extraction from *H. italicum* using $scCO_2$ were reported in few articles found in the literature [11-18]. In these studies, extraction pressure and temperature were varied in the range of 79.3-350 bar and 35.86-64.14 °C respectively, with the extraction times ranging from 1.5 h to 4 h. Obtained yields were in the range of 0.35-6.31%. It can be noted that there is a lack of data regarding the extract composition and co-solvent effects at higher pressures [19].

Starch is a natural biodegradable polymer suitable to be a carrier for oral intake of bioactive components. Impregnation of starch-based materials with bioactive components soluble in $scCO_2$ can be performed using supercritical solvent impregnation (SSI). A literature survey on SSI of starch materials for different applications is given in Table 1.

The articles listed in Table 1 included starch of various origins: potato and Eurylon7 amylomaize [20], maize [21], corn [22-24], sorghum and rice [25], pea

Table 1. Literature survey of supercritical CO_2 impregnation of starch with different active components

Form of starch	Active component	Pressure bar	Temperature °C	Impregnation time h	Loading, %	Application	Reference
Aerogel	Ibuprofen, paracetamol	180	40	70	10-22,10-25	Controlled drug delivery	[20]
Microparticles, modified with *n*-octenil succinate (OSA)	Lavandin essential oil	100-120	40-50	2	2.5-14.7	Biocide formulation	[21]
Pregelatinized starch prepared by spray drying	Oleic acid, flax oil	150-300	40-80	8	4.89-11.86, 0.51-6.60	Food packaging	[22]
Aerogel	Ketoprofen	180	40	1-8	2.1-11.53	Controlled drug delivery	[23]
Aerogel	Ketoprofen, Benzoic acid	180	40-55	24	12.84,21.54	Controlled drug delivery	[24]
Spherules and particles	Oregano essential oil	80-150	40-50	3-24	2.5-15	Food preservation	[25]
Biocomposite films	Cinnamaldehyde	150-250	35	3-15	0.1-0.25	Food packaging	[26]
Xero- and aerogel	Thymol	155	35	24	0.58-4.01	Pharmaceuticals and nutraceuticals	[27]

[23], cassava [26] and tapioca [27]. Starch used in the mentioned articles showed considerable potential as a carrier for the production of controlled drug delivery systems. Furthermore, the highest impregnation loadings were obtained when starch aerogels were impregnated due to their outstanding surface area and porosity achieved by supercritical drying [28]. On the other side, production of polymer aerogels requires a substantial flow of $scCO_2$ during the drying process [29]. Starch in the form of xero- and aerogel was used as carrier for SSI with thymol [27]. The study showed that xerogels expressed high thymol loading capacities at given experimental conditions (Table 1), whereby the xerogels were easy to produce in a low-cost process. Finally, it can be noted that in all the presented articles SSI of starch with different active components was performed in a batch mode – the carrier and active component were exposed to $scCO_2$ in the same vessel as a batch and after a certain time, decompression was performed.

This research was aimed to investigate the simultaneous $scCO_2$ extraction from *H. italicum* and impregnation of corn starch xerogels with the extract in order to produce a suitable system for oral intake of *H. italicum* bioactive components. As previously mentioned, *H. italicum* extract was shown to be effective in treatment of disorders in the digestive system. The main objective of this research was feasibility testing of the application of the recently introduced [30] integrated supercritical fluid extraction (SFE) and SSI (SFE-SSI) process in isolation of *H. italicum* extract and its incorporation into corn starch xerogels using $scCO_2$. Influence of the xerogel preparation method as well as the SFE-SSI process parameters (impregnation time, mass of plant material to carrier mass ratio and addition of ethanol as co-solvent) on the impregnation loading was analyzed. Furthermore, the influence of ethanol as a co-solvent on the composition and antioxidant activity of *H. italicum* extracts was studied.

EXPERIMENTAL

Materials

H. italicum flowers were collected in August 2013 in the region Konavle (the southern part of Croatia) and kept away from direct sunlight at room temperature during the drying process. Corn starch (amylose content 28%) was obtained from Starch Industry Jabuka (Serbia) in the form of white to pale yellow powder. The density of starch given by the manufacturer was 1.5 g/cm^3. Commercial CO_2 (purity 99%) was purchased from Messer-Tehnogas, (Serbia). Commercial acetone (purity 99.7%) and 96% ethanol (purity 99.8%) were purchased from Zorka Pharma-Hemija (Serbia). 2,2-diphenyl-1-picrylhydrazyl (DPPH, purity ≥99%), 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ, purity ≥99%) and L-ascorbic acid (purity ≥99%) were purchased from Sigma-Aldrich GmbH (Germany). Folin-Ciocalteu reagent was purchased from Merck (Germany). Gallic acid (purity >98%) was obtained from TCI Europe (Belgium).

Starch xerogels preparation

Starch (10 g) was mixed with distillated water in the mass ratio of 1:10. The suspension was heated in a silicon oil bath and stirred with a magnetic stirrer (500 rpm) for 20 min at 100 °C to obtain hydrogels [27]. Hydrogels were immersed into the acetone for 5 days in the fridge at 8 °C to replace the water in the hydrogel pores. The volume of the acetone was the same as the volume of water used for the preparation of hydrogels (100 ml). The obtained acetogel films (thickness of 4.0±0.5 mm) were cut into small discs (diameter of 1 cm). The acetone was removed from the acetogels by air-drying at ambient conditions for 10 days to obtain xerogels (XG-Ac).

Xerogels were also produced from alcogels by gradual replacement of water in starch hydrogels. Hydrogels were poured into Petri dishes and warm 10% ethanol solution in water (temperature of 40 °C) was added. After cooling at ambient conditions, the hydrogel was left in the fridge at 8 °C during the night. Next day, the solvent for water replacement was removed and replaced with 20% ethanol solution and the hydrogel was returned into the fridge where it remained overnight. This procedure was repeated during the following days by using solvents with successively higher content of ethanol (30-96%) to prevent gel shrinkage (pore collapse). The obtained alcogel films (thickness of 4.0±0.5 mm) were cut into small discs (diameter of 1 cm). The ethanol was removed from the alcogels by air-drying at ambient conditions for 10 days to obtain xerogels (XG-Alc).

$ScCO_2$ extraction and impregnation

In order to investigate the influence of ethanol as a co-solvent on chemical composition and antioxidant activity of the obtained extract, $scCO_2$ extraction of *H. italicum* was performed first, apart from the integrated SFE-SSI process. The SFE was performed at the pressure of 350 bar and temperature of 40 °C. Moderately low temperature (40 °C) is suggested to prevent thermal degradation of terpenes [31]. Higher CO_2 pressure (density) is suggested for it favors extraction of phenolic compounds [32]. Due to the high polarity of phenolic compounds, especially flavonoids,

an addition of small amounts of organic co-solvents like ethanol and methanol in order to change the solvent polarity and increase the solvating power towards the desired compounds is required [32,33]. Ethanol in quantity of 10 mass% of CO_2 consumed in experiment without co-solvent was added to the milled plant material. Extraction was carried out in the extraction part of the experimental unit for the combined SFE-SSI process described in details below. The average extraction time was 5.5 h, while the average CO_2 flow rate was 0.19 kg/h. The extraction yield (y) was calculated using the following equation:

$$y(\%) = \frac{100 m_e}{m_s} \quad (1)$$

where m_e is the mass of obtained extract, while m_s is the mass of plant material at beginning of the process.

The integrated SFE-SSI process was carried out in the previously described [34] high pressure extraction adsorption (HPEA) 500 unit (Eurotechnica GmbH, Germany), Figure 1.

Starch xerogel was placed in the 100 mL adsorption column, made of stainless steel and designed to be operated at maximum pressure of 690 bar and temperature of 250 °C, while milled *H. italicum* flowers (26.6±0.3 g) were put in the 280 mL stainless steel extractor, designed to be operated at maximum pressure of 534 bar and temperature of 120 °C. In experiments with co-solvent, ethanol in a quantity of 10 mass% of CO_2 in a system at operating conditions in the experiment without co-solvent was placed in the extractor as well. Plant material to carrier mass ratio of 10 and 20 was used. Liquid CO_2 was supplied from a CO_2 cylinder with a siphon tube, then cooled in a cryostat to prevent vaporization and finally pumped into the system by a liquid metering pump (Milton Roy, France). After the filling of vessels with CO_2 and reaching the operational conditions (350 bar and 40 °C) circulation of the solution (CO_2 + extract) through both vessels (extractor and adsorption column) during the 5 or 8 h followed. A high pressure gear pump designed to be operated at maximum pressure of 500 bar and temperature of 100 °C was used for circulation of the supercritical solution. At the end of each experiment the system was depressurized at the rate of 35 bar/min. The impregnation loading (I) was calculated using the following equation:

$$I(\%) = 100 \frac{m_i - m}{m_i} \quad (2)$$

where m_i is the mass of impregnated carrier, while m is the mass of carrier before the impregnation. All experiments were performed in duplicates.

Chromatographic analysis

Samples of *H. italicum* extract obtained with and without use of ethanol as a co-solvent were used to analyze the chemical composition and antioxidant activity thereof. Prior to the analysis, ethanol was removed from the *H. italicum* extract by rotary vacuum evaporation. Furthermore, a sample of *H.*

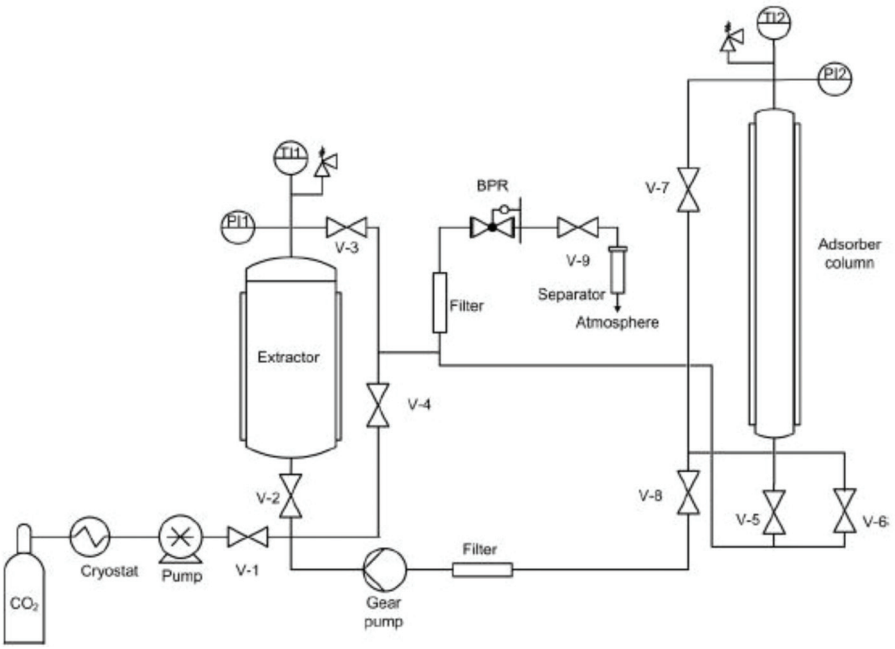

Figure 1. Schematic view of the HPEA 500 unit.

italicum extract incorporated in XG-Alc without co-solvent was also analyzed after its dissolving in a chloroform/methanol mixture (7:3).

Gas chromatography analysis of the extracts was carried out on a HP-5890 Series II GC apparatus (Hewlett-Packard, Waldbronn, Germany), equipped with split-splitless injector and automatic liquid sampler, attached to HP-5 column (25 m×0.32 mm, 0.52 μm fil thickness) and fitted to flame ionization detector (FID). Carrier gas flow rate (H_2) was 1 mL/min, split ratio 1:30, injector temperature was 250 °C, detector temperature 300 °C, while column temperature was linearly programmed from 40 °C to 260 °C (at rate of 4 °C/min), and then kept isothermally at 260 °C for 10 min. Solutions of samples dissolved in chloroform/methanol mixture (7:3) were consecutively injected in amount of 1 μL. Area percent reports, obtained as result of standard processing of chromatograms, were used as the base for the quantification analysis.

The same analytical conditions as those mentioned for GC/FID were employed for GC/MS analysis, along with column HP-5MS (30 m×0.25 mm, 0.25μm film thickness), using HP G 1800C Series II GCD system (Hewlett-Packard, Palo Alto, CA, USA). Helium was used as a carrier gas. Transfer line was heated at 260 °C. Mass spectra were acquired in EI mode (70 eV), in m/z range 40-450. The amount of 0.2 μL of the sample solution in chloroform/methanol mixture (7:3) was injected. The components of the oil were identified by comparison of their spectra to those from Wiley 275 and NIST/NBS libraries, using different search engines. The experimental values for retention indices were determined by the use of calibrated Automated Mass Spectral Deconvolution and Identification System Software (Amdis, ver. 2.1), compared to those from the available literature (Adams) [35] and used as additional tool to approve MS findings.

Determination of total phenolic content

The content of total phenolics in the extracts was determined by a modified Folin-Ciocalteu method [36]. The extracts were diluted in methanol to a final concentration of 1 mg/mL. 0.1 mL of extracts was shaken for 1 min with 0.5 mL of Folin-Ciocalteu reagent and 6 mL of distilled water. After the mixture was shaken, 1.5 mL of 20% Na_2CO_3 was added and the mixture was shaken once again for 0.5 min. Finally, the solution was brought up to 10 mL by adding distilled water. After incubation for 2 h at room temperature, the absorbance was measured at 750 nm using glass cuvettes against a blank (100 μL of methanol instead of test samples). The total phenolic content was calculated using the standard calibration curve of gallic acid (from 1 to 1.500 μg/mL). Spectrophotometric measurements were performed by using UV-Vis spectrophotometer HP 8453 (Agilent Technologies, USA) in order to determine total phenolic and total flavonoid contents. The analysis was performed in triplicate and the results were expressed as milligrams of gallic-acid equivalents (GAE) per g of dried extract.

Determination of total flavonoid content

Total flavonoid content was measured by means of the aluminum chloride colorimetric assay [37]. An aliquot (1 mL) of 0.02, 0.04, 0.06, 0.08, 0.10 mg/mL methanolic catechin solutions or methanolic plant extracts (1 mg/mL) was added into a 10 mL volumetric flask containing 4 mL of water. Then 0.3 mL of 5% $NaNO_2$ was added and after 5 min, 0.3 mL of 10% $AlCl_3$, was added. After 6 min, 2 mL of 1 M NaOH was added and the total volume was made up to 10 mL with water. The solution was well mixed and the absorbance was measured against the prepared blank at 510 nm. Total flavonoids were expressed as g of catechin equivalents (CE) per g of the dry extract.

Determination of antioxidant activity of the extracts

Antioxidant activity of the extracts was measured on the basis of scavenging activities of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [38]. 80 μL of extract previously diluted in methanol to obtain at least four different concentrations (0.2 to 1 mg plant material/mL), 2.92 mL of methanol and 1.0 mL of freshly prepared DPPH methanol solution (90 μmol/L) were shaken vigorously and left in the dark for 30 min at room temperature. The absorbance was measured against a blank (methanol) at 517 nm. Inhibition of the DPPH radical was calculated as a percentage (PE, %) using the following equation:

$$PE\,(\%) = 100\,\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \tag{3}$$

where A_{control} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Synthetic antioxidant L-ascorbic acid was used as a positive control and all tests were carried out in triplicates.

The FRAP (the ferric reducing ability of plasma) assay is based on the reduction, at low pH, of a yellow ferric complex (Fe^{3+}-2,4,6-tri(2-pyridyl)-*s*-triazine, TPTZ) to a blue-colored ferrous complex (Fe^{2+}-TPTZ) by the action of electron-donating antioxidants. The

reduction is monitored by measuring the change of absorbance at 593 nm. The measurement was carried out according to the method reported by Benzie and Strain [39]. The working FRAP reagent was prepared daily by mixing 10 volumes of 300 mM acetate buffer pH 3.6 (containing 6.4 mL 2 M sodium acetate solution and 93.6 mL 2 M acetic acid solution diluted in a volumetric flask (1 L)) with 1 volume of 10 Mm TPTZ (in 40 mM HCl) and with 1 volume of 20 mM ferric chloride. A standard calibration curve was constructed using aqueous solutions of $FeSO_4 \cdot 7H_2O$ at different concentrations (0.1–1 mM). For antioxidant activity determination, 100 μL of methanol plant extract (in concentration of 1mg of dry extract/mL) were mixed with 3 mL of the FRAP reagent. The absorbance readings were started after 10 min and they were performed at 593 nm against a blank (100 μL of methanol instead of test samples). The FRAP value was calculated and expressed as mmol Fe (II) equivalents per gram of extract (mmol Fe (II)/g) based on the standard calibration curve.

Characterization of xerogels

Morphology of the starch xerogels was investigated by the field emission scanning electron microscopy (FE-SEM, Mira 3 XMU TESCAN a.s., Czech Republic) operated at the accelerating voltage of 10 kV. The samples of the starch xerogels were coated with a thin layer of Au/Pd (85/15), using a sputter coater (Polaron SC502, Fisons Instruments, UK) prior to the analysis.

Density and porosity of the xerogel samples were determined by the pycnometer method. The density of the samples (ρ_x) was calculated using Eq. (4) according to the previously described procedure [40]:

$$\rho_x = \frac{\rho_{H_2O} m_1}{m_1 + m_2 - m_3} \tag{4}$$

where m_1 is the mass of sample, m_2 is the mass of pycnometer and water, m_3 is the mass of glass with water and the sample. The density of water used for determination of the same for XG-Ac at 28.9±0.2 °C was 996.0±0.1 kg/m^3, while the density of water used for determination of the same for XG-Alc at 27.8±0.2 °C was 996.3±0.1 kg/m^3. The measurements were performed in triplicates. Porosity of the xerogels (ε) was calculated using the following equation [41]:

$$\varepsilon(\%) = 100\left(1 - \frac{\rho_x}{\rho_{starch}}\right) \tag{5}$$

whereby ρ_{starch} is the density of raw starch given by the manufacturer.

RESULTS AND DISCUSSION

$ScCO_2$ extraction and characterization of extracts

The extraction of *H. italicum* with pure $scCO_2$ resulted in an extraction yield of 2.7±0.5%. The addition of ethanol in quantity of 10 mass% of the CO_2 consumed in experiment without the co-solvent caused the increase of extraction yield to 5.9±0.7%. The extractions were performed in duplicate. Obtained results indicated strong ethanol's influence on changing $scCO_2$ solubility power.

Compounds identified in *H. italicum* extracts obtained with pure $scCO_2$ (97.1%) and using co-solvent (93.9%), according to the GC/MS analysis can be classified in 5 groups of compounds (Table 2). Based on presented data, the addition of ethanol led to a 1.5 times increase of the overall content of terpenes (total terpenic content) in *H. italicum* extract and to the reduction of the content of fatty acid esters, aldehydes and alcohols (4 times). The presence of the co-solvent resulted in a significant increase of content of coumarin and amorphene derivatives and hydrocarbons, including waxes. Main components of the *H. italicum* extract obtained with pure $scCO_2$ were terpenes – neryl acetate (1.5%), ar-curcumene (4.1%), β-selinene (4.6%) and xanthorrhyzol (2.1%), ester methyl caprylate (9.2%), hydrocarbons nonacosane (3.0%) and untriacontane (2.7%) and 3,4-dihydro--4,4,5,7,8-penthamethyl coumarin-6-ol (5.9%). Main components of the *H. italicum* extract obtained using co-solvent were terpenes neryl acetate (2.0%), ar-curcumene (6.4%), β-selinene (4.9%) and α-selin-11-en--4-ol (1.8%), 2-methyl-3-oxo-valeric acid methyl ester (5.2%), hydrocarbons nonacosane (5.9 %) and untriacontane (7.4%) and 2α-acetoxy-11-metoxy amorpha--4,7-diene (7.0%) and 3,4-dihydro-4,4,5,7,8-penthamethyl coumarin-6-ol (6.4%).

The Folin-Ciocalteu method and aluminum chloride colorimetric assay were used to quantify fractions of higher molecular weight compounds in *H. italicum* supercritical extracts. Obtained results (Table 3) indicated significantly higher total phenolic content in the extract obtained with pure $scCO_2$, compared to values reported for *H. italicum* essential oil (74±1.642 mg GAE/g sample [42]), ethanolic extract (31.97 1.42 mg GAE/g dry extract [43]) and aqueous extract (15.69±0.17 mg GAE/g dry extract [44]). On the other hand, according to the obtained results (Table 3), addition of ethanol caused an increase of total flavonoid content by 20%. Kladar *et al.* [43] determined the

Table 2. Results of GC/MS analysis of H. italicum extracts obtained at 350 bar and 40 °C

Compound group	Content, %		
	Extract obtained with pure $scCO_2$	Extract obtained with addition of co-solvent	Extract obtained after reextraction from impregnated XG-Alc
Terpenes	28.3	40.8	14.3
Fatty acids, esters, aldehydes and alcohols	50.3	13.3	56.6
Hydrocarbons	8.9	18.9	18.1
Coumarin and amorphene derivatives	8.1	20.9	4.5
Others	1.5	0.0	0.2
Total	97.1	93.9	93.7

Table 3. Total phenolics, flavonoids and antioxidant activities of H. italicum extracts obtained at 350 bar and 40 °C; the experimental results were expressed as mean ± standard deviation (SD) of three replicates

Sample	TP[a], mg GAE/g dry extract*	TF[b], mg CE/g dry extract	DPPH[c] PE[d], % (1 mg/mL)	FRAP[e], mmol Fe^{2+}/g dry extract
Extract obtained with pure supercritical CO_2	130.27±1.14	40.67±0.53	11.50±0.10	0.32±0.04
Extract obtained with addition of co-solvent	57.77±1.01	48.02±0.31	12.84±0.76	0.34±0.02

[a]Total phenolics (TP) was measured by Folin-Ciocalteu method and expressed as gallic-acid equivalents (GAE); [b]total flavonoids (TF) was measured by the aluminium chloride colorimetric assay expressed as catechin equivalents (CE); [c]DPPH radical scavenging activity; [d]percentage inhibition (PE); [e]ferric reducing ability (FRAP)

total flavonoid content in *H. italicum* ethanolic extract, being 20.68±0.66 mg QE/g dry extract, expressed as quercetin equivalents, while in this study we obtained 130.27±1.14 and 57.77±1.01 mg CE/g dry extract, expressed as catechin equivalents, for the extract obtained with pure $scCO_2$ and extract obtained using co-solvent, respectively. Results presented in Tables 2 and 3 indicated the significant role of ethanol as a co-solvent in modification of $scCO_2$ selectivity.

Antioxidant activity of *H. italicum* $scCO_2$ extracts obtained at 350 bar and 40 °C was tested by DPPH free-radical and FRAP assays. Results given in Table 3 indicated moderate antioxidant potential of *H. italicum* extracts, which is in accordance with previously reported data [15,16]. Costa *et al.* [15] showed that the DPPH radical scavenging ability of the $scCO_2$ extracts of *H. italicum* isolated at 120 bar and 40 °C increased in a dose-dependent manner (approximately 15–60% at concentration of 0.625–5.0 mg/mL), while the extracts isolated at 90 bar and 40 °C were not able to reduce the DPPH radical at the studied concentrations. On the other side, Poli *et al.* [16] reported scavenging effect of *H. italicum* extracts obtained at 260 bar and 50 °C of 10.79±0.3–95.2±1.5% at concentration of 5–200 μg/mL, tested by DPPH method, 0.5±0.026–0.857±0.04% after 28–56 h, assessed by the β-carotene bleaching test and none to full superoxide radical scavenging activity at concentration of 25–200 μg/mL. Addition of ethanol as a co-solvent resulted in a slight increase of antioxidant activity in both tests. Since the extract obtained with the addition of ethanol contained 40.8% terpenes (1.5 times higher content than in the extract obtained with pure CO_2), whereas the extract obtained with pure $scCO_2$ contained 50.3% fatty acids, esters, aldehydes and alcohols and 2 times higher total phenolic content than the extract obtained with addition of ethanol, it could be concluded that both types of compounds equally contribute to the antioxidant activity of *H. italicum* extracts.

Characterization of xerogels

The SEM images of dried XG-Ac and XG-Alc were given in Figure 2. Both types of xerogel possessed compact and porous structures which could be due to the completed gelatinization in the applied temperature range.

The determined density of the XG-Ac and XG-Alc samples was 1285.3±3.2 kg/m^3 and 1213.9±6.8 kg/m^3, respectively. Corresponding porosity of the XG-Ac and XG-Alc samples was 14.3±0.2% and 19.1±0.5%, respectively.

Integrated SFE-SSI process

Obtained impregnation loadings of *H. italicum* extract in the xerogels at selected conditions (350 bar and 40 °C) for different process parameters (plant to carrier mass ratio, contact time, use of co-solvent) are listed in Table 4.

In the case of XG-Ac impregnation, the influence of all mentioned parameters was tested. Addition of the co-solvent in the case of XG-Ac resulted in almost two times lower impregnation loading (decrease from 0.99±0.06 to 0.54±0.13%) despite the fact that the

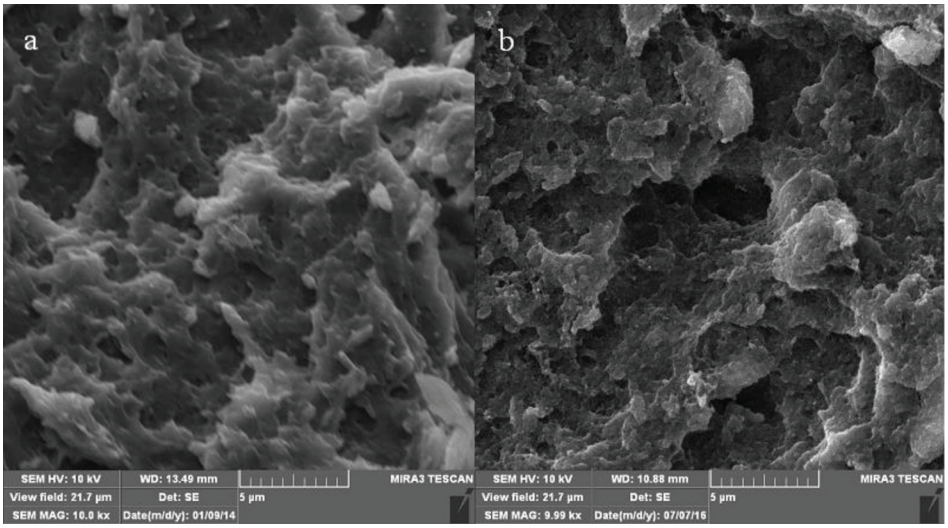

Figure 2. FE-SEM images of starch xerogels obtained by drying of: a - acetogels; b - alcogels.

solubility of *H. italicum* extract was higher in the $scCO_2$+EtOH system, than in pure $scCO_2$ (according to the obtained extraction yields). This could be due to the higher affinity of *H. italicum* extract to the $scCO_2$+EtOH mixture than to the starch xerogel. Increase of the process time from 5 to 8 h resulted in the decrease of the impregnation loading from 0.99±0.06 to 0.83±0.05%. It could be assumed that impregnation of the xerogel with *H. italicum* extract in a particular moment reaches its maximum, after which desorption of the extract takes place [34]. Increase of the plant material/carrier mass ratio from 10 to 20, in the case of XG-Ac, resulted in the decrease of the impregnation loading from 0.99±0.06 to 0.88±0.01%. Therefore, the process time of 5 h, plant material/carrier mass ratio of 10 and impregnation without co-solvent were found to be the optimal conditions with respect to the obtained loading in the case of XG-Ac. Based on these results, impregnation of XG-Alc was performed without the co-solvent during 5 h, whereby the plant material/carrier mass ratio was varied. Increasing of the plant material/carrier mass ratio had a negative effect on the impregnation loading. As presented in Table 4, the highest impregnation loading of 1.26±0.22% was achieved for the SFE-SSI of xerogel obtained from alcogel (XG-Alc) with pure $scCO_2$ during 5 h and with the plant material/carrier mass ratio of 10. Significantly higher impregnation loading for the SFE-SSI of XG-Alc under the same process conditions compared to XG-Ac (Table 4) indicated a positive influence of gradual water replacement during the preparation of XG-Alc. SEM analysis indicated that gradual water replacement positively affected porosity of the xerogels (Figure 2) which was confirmed by the determined porosity values (higher porosity was determined for the XG-Alc sample). Therefore, this could be the reason for 27% higher impregnation loading of the extract in XG-Alc (1.26± ±0.22%) in comparison to XG-Ac (0.99±0.06%) at the same SFE-SSI conditions (Table 4).

Finally, xerogels obtained in the SFE-SSI process with the co-solvent had a more intensive yellow color compared to the one obtained in the SFE-SSI process with pure $scCO_2$. This was assumed to be due to the change of $scCO_2$ selectivity in the presence of ethanol (Table 2).

GC/MS analysis of the *H. italicum* extract dissolved from the impregnated XG-Alc resulted in identification of 93.7% compounds. Content of the compound groups is listed in Table 2. As can be seen, the

Table 4. Experimental data for integrated SFE-SSI process; m_{cos} - mass of co-solvent, m_c - mass of carrier, m_s/m_c - plant material to carrier mass ratio, I - impregnation loading

Xerogel type	Time, h	m_{CO2}, g	m_{cos}, g	m_c, g	m_s/m_c	I, %
XG-Ac	5	450	-	2.66	10	0.99±0.06
	5	400	45	2.69	10	0.54±0.13
	8	450	-	2.66	10	0.83±0.05
	5	450	-	1.34	20	0.88±0.01
XG-Alc	5	450	-	2.69	10	1.26±0.22
	5	450	-	1.34	20	0.71±0.12

carrier showed affinity towards fatty acids, esters, aldehydes and alcohols and hydrocarbons at the expense of terpenes, coumarin and amorphene derivatives. Main components of the extract were terpenes ar-curcumene (5.6%), *β*-selinene (1.8%) and *δ*-cadinene (2.2%), esters methyl isomyristate (6.9%) and methyl myristate (4.0%), oleic acid (6.1%), hydrocarbons pentacosane (4.3%), nonacosane (3.5%) and untriacontane (4.0%) and 3,4-dihydro-4,4,5,7,8-penthamethyl coumarin-6-ol (4.1%).

CONCLUSION

$ScCO_2$ extraction from *H. italicum* and SSI of starch xerogels with the extract were performed by the integrated SFE-SSI process.

It was shown that the addition of ethanol as a co-solvent changed selectivity of $scCO_2$ increasing total terpenic content as well as the content of coumarin and amorphene derivatives and flavonoids. Both $scCO_2$ extracts showed moderate antioxidant activity.

In the case of XG-Ac ethanol as a co-solvent significantly lowered the impregnation loading, which led to the conclusion that the extract possesses higher affinity to $scCO_2$+ethanol mixture than to the xerogel. The increase of impregnation time from 5 to 8 h, as well as the plant material to carrier mass ratio caused decrease of the impregnation loading. The highest impregnation loadings were obtained in the processes with pure $scCO_2$, during 5 h and with plant material to carrier mass ratio of 10. Significantly higher impregnation loading for XG-Alc could be attributed to the different porosity and morphology of the xerogels which was visible on the SEM images. Obtained results indicated feasibility of the combined SFE-SSI process application in extraction and posterior impregnation of *H. italicum* extract into the starch xerogels. In further research a more thorough analysis of the influence of contact time and impregnation mode on the impregnation loading and chemical composition of the loaded extract is needed.

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SVETOLIK MAKSIMOVIĆ[1]
VANJA TADIĆ[2]
JASNA IVANOVIC[1]
TANJA RADMANOVIĆ[1]
STOJA MILOVANOVIC[1]
MILICA STANKOVIC[3]
IRENA ZIZOVIC[1]

[1]Univerzitet u Beogradu, Tehnološko--metalurški fakultet, Karnegijeva 4, 11120 Beograd, Srbija
[2]Institut za proučavanje lekovitog bilja "Dr Josif Pančić", Tadeuša Košćuška 1, 11000 Beograd, Srbija
[3]Univerzitet u Nišu, Medicinski fakultet, Bulevar Zorana Đinđića 81, 18 000 Niš, Srbija

NAUČNI RAD

PRIMENA INTEGRISANOG PROCESA NATKRITIČNE EKSTRAKCIJE I IMPREGNACIJE ZA INKORPORACIJU EKSTRAKTA *Helichrysum italicum* U KSEROGEL KUKURUZNOG SKROBA

Ekstrakcija iz smilja (Helichrysum italicum) pomoću natkritičnog CO_2 i impregnacija kserogelova skroba ekstraktom primenom integrisanog procesa ekstrakcije i impregnacije pomoću natkritičnog CO_2 izvođeni su na 350 bar i 40 °C u cilju proizvodnje biomaterijala za potencijalni oralni unos ekstrakta. Kserogelovi dobijeni sušenjem na vazduhu acetogelova i alkogelova upotrebljeni su kao nosač u procesu natkritične impregnacije. Ispitani su uticaji etanola kao kosolventa, vremena kontakta, odnosa masa biljnog materijala i nosača i načina pripreme kserogela na prinos impregnacije. Najveći prinos impregnacije (1,26±0,22%) postignut je nakon 5 h impregnacije kserogelova dobijenih od alkogelova, upotrebom čistog natkritičnog CO_2 i pri odnosu masa biljnog materijala i nosača od 10. Hemijska analiza ekstrakata pokazala je da je dodatak etanola kao kosolventa pozitivno uticao na selektivnost natkritičnog CO_2 prema terpenima i ukupnom sadržaju flavonoida, dok je ukupan sadržaj fenola smanjen. Bez obzira na razliku u hemijskom sastavu, oba tipa ekstrakta su pokazala slično antioksidantno dejstvo, na osnovu primene DPPH i FRAP testova. Integrisani proces se pokazao kao pogodan metod za izolaciju i inkorporaciju bioaktivnih komponenata smilja (H. Italicum) u kserogelove skroba.

Ključne reči: antioksidantno dejstvo, Helichrysum italicum, natkritična ekstrakcija, natkritična impregnacija, kserogelovi skroba.