

Accepted Manuscript

Title: Isolation of apigenin from subcritical water extracts:
Optimization of the process

Author: Aleksandra Cvetanović Jaroslava Švarc-Gajić Uroš
Gašić Živoslav Tešić Gökhan Zengin Zoran Zeković Saša
Đurović



PII: S0896-8446(16)30382-5
DOI: <http://dx.doi.org/doi:10.1016/j.supflu.2016.10.012>
Reference: SUPFLU 3781

To appear in: *J. of Supercritical Fluids*

Received date: 10-5-2016
Revised date: 23-10-2016
Accepted date: 24-10-2016

Please cite this article as: Aleksandra Cvetanović, Jaroslava Švarc-Gajić, Uroš Gašić, Živoslav Tešić, Gökhan Zengin, Zoran Zeković, Saša Đurović, Isolation of apigenin from subcritical water extracts: Optimization of the process, The Journal of Supercritical Fluids <http://dx.doi.org/10.1016/j.supflu.2016.10.012>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Isolation of apigenin from subcritical water extracts: optimization of the process

Aleksandra Cvetanović^{1*}, Jaroslava Švarc-Gajić¹, Uroš Gašić², Živoslav Tešić², Gökhan Zengin³,

Zoran Zeković¹, Saša Đurović¹

¹Faculty of Technology, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia

²Faculty of Chemistry, University of Belgrade, P.O. Box 51, 11158 Belgrade, Serbia

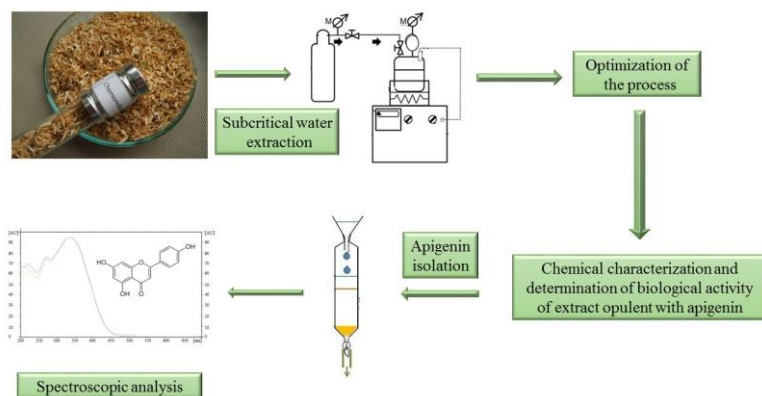
³Department of Biology, Faculty of Science, Selcuk University, Campus/Konya, Konya, Turkey

*To whom correspondence should be addressed.

Department of Biotechnology and Pharmaceutical Engineering, University of Novi Sad, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia

Tel: +381 64 1590019; Fax: +381 21 450413; E-mail: a.c.istrzivac@gmail.com

Graphical abstract



Highlights

- Simultaneous extraction and hydrolysis of apigenin bound forms from chamomile
- Hydrolytical potential of SCW for liberation of apigenin from its bound forms
- Isolation of apigenin using column chromatography
- Evaluation of biological activity of extract obtained under optimal conditions
- Identification of polyphenolic compounds using UHPLC-HESI-MS/MS analysis

Abstract

Apigenin is a phytochemical which has been associated with numerous beneficial health effects. However, due to its low concentration and poor bioavailability, isolation from natural sources is very difficult. Subcritical water (SCW) is an excellent solvent for the extraction of moderately polar and non-polar flavonoids. Carefully balanced moderate hydrolytical potential of SCW was used in this work to liberate apigenin from its bound forms. In this research simultaneous extraction and hydrolysis of apigenin bound forms from chamomile has been performed. Optimal operation parameters for maximum yields of apigenin have been investigated and defined (solvent-to-sample ratio 1:30; agitation rate 3 Hz; pressure 45 bar; temperature 115°C; time 30 min). Apigenin was further isolated in its pure form from water extracts obtained under optimal conditions using column chromatography. The purity of isolated compound was confirmed by spectroscopic analysis. Biological activity of extracts obtained under optimal conditions were determined as well. In order to identify polyphenolic compounds UHPLC-HESI-MS/MS analysis was performed.

Keywords: Subcritical water extraction, apigenin, phenols, UHPLC-HESI-MS/MS analysis, extraction parameters.

1. Introduction

Apigenin (4',5,7-Trihydroxyflavone) is common dietary flavonoid classified under the flavone group with a skeleton of 2-Phenylchromen-4-one [1–3]. Scientific findings indicate that apigenin is one of the most potent biologically and pharmacologically active agents. Various biological activities are attributed to this compound, in the first place anti-inflammatory, anti-cancer, antioxidant and anti-allergic activities [4–9]. Recent reports indicate its potential as antiviral agent against hepatitis C virus (HCV), herpes simplex virus type I (HSV-1), influenza virus and human immunodeficiency virus type I (HIV-1) [10–14]. Furthermore, apigenin has been screened for its antiparkinson's activity in transgenic *Drosophila melanogaster* and it has been reported to enhance locomotor ability [15]. *In-vitro* study on microglial cells proved that apigenin has inhibitory effect on inflammatory mediators, which suggests its neuroprotective properties important in neurodegenerative diseases [16]. This flavone is also associated with lower prevalence of cardiovascular diseases [17] and it has cardioprotective effects in myocardial ischemia/reperfusion injury [18]. Special focus has been put on its anticancer activities and numerous scientific investigations have focused on effects of apigenin on apoptosis, inhibition of cell growth and angiogenesis [19]. Its protective role against a wide variety of cancers with high selectivity for cancer cells has been confirmed in numerous studies [19–22].

Although apigenin is present in many herbs, fruits and vegetables, one of the most common sources of apigenin is chamomile (*Chamomilla matricaria* L.). Maximum concentrations of apigenin in chamomile infusion are ranging from 0.8 to 1.2% [22]. Depending on the plant source and used extraction technique chamomile extracts may contain significantly varying concentrations of apigenin, thus demonstrating different effects [23–25]. Bearing in mind the great pharmacological potential of this flavone, it is very important to find a safe “green” and efficient

extraction method for obtaining extracts with the highest biological potential that can be used as nutraceuticals or pharmaceuticals.

Traditional techniques for extracting phytochemicals involve the use of organic solvents, and usually require long extraction time [26,27]. Modern extraction techniques (ultrasound-assisted extraction, microwave-assisted extraction, subcritical fluid extraction, supercritical extraction with carbon-dioxide, etc.) are conveniently used for the extraction of biologically active compounds from natural sources. Among these, extraction with sub- or supercritical fluids are the most appealing due to the fact that those usually rely on safe solvents, allowing at the same time high extraction yields and shorter processing times [28–30]. Water in its supercritical state offers numerous advantages over carbon dioxide and it is inexpensive thus it represents suitable solvent for the industrial extraction of medical plants. Physical and chemical properties of superheated water can be manipulated by concurrently controlling the temperature and pressure. Under subcritical conditions, the intermolecular hydrogen bonds of water break down and the dielectric constant of water decreases. As temperature increases to 250 °C, water dielectric constant drops to value close to dielectric constant of ethanol [31,32]. In comparison to supercritical carbon-dioxide whose dielectric constant falls within the range 1-2, subcritical water having adjustable polarity within the range of dielectric constant 13-80 (350-25 °C) thus it offers advantageous selectivity, especially towards polar and moderately polar compounds which require mildest operational parameters [24]. Consequently, pressurized water under sub-critical conditions can easily solubilize organic polar (at lower temperatures) and nonpolar (at higher temperatures) compounds such as phytochemicals which are normally insoluble in ambient water [33]. A further advantage of sub-critical water extraction is that the high temperature and pressure produce high diffusion rates which promote very efficiently the extraction process [33–35].

In recent years, different studies have been published on the applicability of subcritical water for the extraction of biologically active compounds from natural sources [25]. Nevertheless, according to available literature there are no reports on feasibility of subcritical water for apigenin recovery from chamomile. Moreover, there is no published applications of subcritical water for simultaneous extraction and hydrolysis in controlled conditions for the recovery of natural biologically-active compounds. Thus, this work represents an overview of optimization of operational parameters where subcritical water is used for simultaneous extraction and release of apigenin-bound forms.

The influence of the most important operational parameters (solvent-to-sample ratio, agitation rate, pressure, temperature, time) on the yield of total phenols, flavonoids and apigenin was investigated and defined. Apigenin was further isolated from water extracts obtained under optimized conditions and its purity was confirmed by spectroscopic analysis. Polyphenolic profile of obtained subcritical water (SCW) extracts was done by using Ultra High Performance Liquid Chromatography coupled with mass spectrometry UHPLC-HESI-MS/MS analysis.

2. Material and methods

2.1. Chemicals and reagents

Folin-Ciocalteu reagent, trichloroacetic acid, 1,1-D-2-picrylhydrazyl (DPPH), α -amylase solution (ex-porcine pancreas, EC 3.2.1.1), α -glucosidase solution (from *Saccharomyces cerevisiae*, EC 3.2.1.20), L-glutathione, tyrosinase, 3,4-Dihydroxy-L-phenylalanine (L-DOPA), kojic acid, acarbose and polyphenolic standards (analytical grade; purity $\geq 99\%$) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Aluminium chloride hexahydrate, sodium carbonate, PNPG (4-N-trophenyl- α -D-glucopyranoside), silica-gel (GF₂₅₄) and sodium acetate trihydrate were purchased from Merck (Darmstadt, Germany). Potassium ferricyanide and ferric chloride, were

obtained from Zorka (Šabac, Serbia). Polyamide (MN-polyamid-SC6) was obtained from Macherey-Nagel (Düren, Germany). Acetonitrile and acetic acid (both of MS grade), methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Ultrapure water (ThermoFisher Scientific, Bremen, Germany) was used to prepare standard solutions and blanks. Syringe filters (13 mm, PTFE membrane 0.45 μm) were purchased from Supelco (Bellefonte, PA, USA). All other chemicals and reagents were analytical reagent grade.

2.2. Plant material

Chamomile ligulate flowers (CLF) used in this study were produced by the Institute of Field and Vegetable Crops, Bački Petrovac, Serbia. Plant material was collected in the end of April. At the time of chamomile harvest ligulate flowers stranded horizontally, and from the tubulare ones pollen released indicating optimal period for chamomile harvest. Chamomile flos were dried at the temperature of 40 °C in a solar dryer. The layer thickness of plant material was 5 cm. Drying was performed until the moisture content of 12%. After drying the CLF were separated from the tubular parts by sieving. Separated CLF were packed in paper bags and stored in the dark place until further use.

2.3. Subcritical water extraction

Subcritical water extraction was performed in a homemade subcritical water extractor/reactor presented in Fig. 1. Total capacity of high-pressure stainless steel vessel (6) was 1.7 L. Pressurization of the vessel was performed with 99.999% nitrogen (Messer, Germany) through valve (3). Nitrogen was used in order to prevent oxidation on high temperatures. The operating pressure in the vessel was monitored by in-built manometer (2) (Inol, Slovenia, model IM 811A12).

Extraction vessel was heated through a heating plate (1500 W) that allowed heating rate of approximately 10°C/min. The process temperature was measured by a thermocouple Pt100 (4) and regulated by a temperature controller (8) (Nigos, Serbia, model 1011P). Vibrating platform (7) was used in order to increase mass transfer and prevent local overheating in contact with heater (11). Agitation was assured at the frequency range of the vibration platform between 2 Hz and 6 Hz. Extraction duration in all experiments was 30 min, except for the experiments on time influence.

Figure 1.

After the extraction, the process vessel was immediately cooled in flow-through water-bath at 20 °C. Depressurization was done by valve opening and purging nitrogen through a valve. Obtained extracts were filtrated and stored in the refrigerator until analysis.

2.4. Determination of total phenols content

The Folin-Ciocalteu method [36,37] was used to determine the total phenolics content. The reaction mixture was prepared by mixing 0.1 mL of the extract, 7.9 mL of distilled water, 0.5 mL of Folin-Ciocalteu reagent and 1.5 mL of sodium carbonate (20%, w/w). After incubation at room temperature for 1 hour, absorbance was measured at 750 nm. The blank was prepared by replacing the extract with distilled water. Measurements were made in triplicate for each sample. Total phenols content in obtained extracts was calculated by interpolating the measured sample absorbance into calibration curve defined with standard solutions of chlorogenic acid, defined for the concentration range 0.02–0.1 mg/mL ($Y = 4.91 C - 0.00833$, $R^2 = 0.998$). The results were expressed as chlorogenic acid equivalents per gram of dry plant material (mg CAE/g).

2.5. Determination of total flavonoids content

Flavonoids in obtained extracts were determined using colorimetric assay based on the procedure described by Markham [38]. SCW extracts of chamomile (1 mL) were mixed with 5% NaNO₂ solution (0.3 mL). After 5 minutes aluminium chloride hexahydrate (10%, 0.3 mL) was added and allowed to stand for 6 minutes. Sodium-hydroxide (1 mol/dm³, 1 mL) was added to the mixture. Immediately, distilled water was added to bring the final volume to 10 mL. The blank was prepared by replacing the extract with distilled water. Immediately after mixing, absorbance was measured at 510 nm. Total flavonoid content in obtained extracts was calculated by interpolating the measured sample absorbance into calibration curve defined with standard solutions of rutin, defined for the concentration range 0.02–0.1 mg/mL ($A = 1.88727 C - 0.0096$, $R^2 = 0.997$). The results were expressed as rutin equivalents (RE) per gram of dry plant material (mg RE/g).

2.6. Determination of apigenin by thin layer chromatography (TLC)

Determination of apigenin was done according to the previously described procedure [39]. Thin layer chromatography (TLC) was performed on 20 x 20 cm glass plates (silica gel GF₂₅₄, 0.5 mm thickness). The aliquots of chamomile extracts were spotted on the plate as start points. Mixture of toluene:methylethylcetone:methanol (55:30:15, v/v/v) was used as a mobile phase. Development was performed at room temperature in a saturated glass chamber. Detection was done under UV light (254 and 365 nm). Ratio of front (R_f value) of apigenin standard was used to identify apigenin in analyzed extracts.

Spots of apigenin on silica gel were scraped and dissolved in 50% ethanol (5 mL). After 30 min of intensive mixing, the mixture was centrifuged at 3000 min⁻¹ for 10 min. Obtained supernatant, i.e.

apigenin solution, was evaporated using nitrogen and dissolved in 5 mL of 50% ethanol. Absorbance (A) of apigenin solution was measured at 340 nm. Apigenin content (C) in obtained extracts was calculated by interpolating the measured sample absorbance into calibration curve defined with standard solutions of apigenin, defined for the concentration range 0.02–0.1 mg/mL ($A = 6.91937 C + 0.03996$, $R^2 = 0.997$).

2.7. Isolation and identification of apigenin by column chromatography

Isolation of apigenin from subcritical water extract was done by column chromatography. The poly-amid chromatography column was packed as follows: the exit of the chromatographic column was plugged with a cotton wool to retain solids. Poly-amid (2 g, grain size < 0.07 mm) was suspended in water (60 mL), and then transferred to the column (30 cm length \times 1.5 cm i.d.). The column was rinsed with 50 mL of water. Prior to sample application, the level of water was lowered 0.5 cm above the stationary phase. Elution was performed with ethanol at a constant flow rate of 0.4 mL/min. Collected fractions (1 mL each) were analyzed by TLC. TLC analyses were performed on GF₂₅₄ silica gel plates at room temperature, using toluene:ethylmethylcetone:methanol (55:30:15, v/v/v) as developing reagent. Spots were visualized under an ultraviolet lamp at 254 and 365 nm. Fractions containing apigenin were pooled and concentrated under reduced pressure. Precrystallisation of apigenin was done from the absolute ethanol. Yellow apigenin crystals were stored at 4 °C for the subsequent HPTLC-UV/VIS analysis.

2.8. HPTLC-UV/VIS analysis

Samples (15 μ L of isolated apigenin and 5 μ L of commercially purchased apigenin standard) were applied as 5 mm bands at 35 mm distance from the lower edge of the plate with a rate of 30 nL/s using semi-automatic applicator device (Linomat 5 – Camag, Muttenz, Switzerland). The

chromatographic analysis was performed on silica gel GF₂₅₄ HPTLC plates (20 cm × 10 cm) using the mobile phase toluene:methylethylcetone:methanol (55:30:15, v/v/v). The plates were developed at room temperature in chromatographic chamber (Camag - ADC2) pre-saturated for 15 min with a mobile phase. After development and 3 min of automatic drying qualitative analyses of the plates was performed by TLC Scanner 4 (Camag) in absorption/reflectance mode at 340 nm, using slit dimensions 6 mm × 0.30 mm, scanning speed 20 mm/s and data resolution 100 µm/step. Plate was observed under UV lamp (254 and 365 nm).

2.9. UHPLC–DAD MS/MS analysis of polyphenolic compounds

The separation, determination and quantification of the polyphenolic compounds in SCW extract were performed using a Dionex Ultimate 3000 UHPLC system equipped with a diode array detector (DAD) that was connected to TSQ Quantum Access Max triple-quadrupole mass spectrometer (ThermoFisher Scientific, Basel, Switzerland). The elution was performed at 40 °C on a Synchronis C18 column (100 × 2.1 mm, 1.7 µm particle size) from ThermoFisher Scientific. The mobile phase consisted of water + 0.01% acetic acid (A) and acetonitrile (B), which were applied in the following gradient elution: 5% B in the first 2.0 min, 2.0–12.0 min 5–95% B, 12.0–13.0 min from 95% to 5% B, and 5 % B until the 20th min. The flow rate was set to 0.3 mL/min and the detection wavelengths to 254 and 280 nm. The injection volume was 5 µL.

1 mg/mL stock methanolic solutions of polyphenolics were prepared. The stock solutions were mixed and diluted with water yielding working solution of concentrations 0.01, 0.05, 0.10, 0.25, 0.50, 0.75, and 1.00 mg/L.

A TSQ Quantum Access Max triple-quadrupole mass spectrometer equipped with an heated electrospray ionization (HESI) source was used with the vaporizer temperature kept at 250 °C, and the ion source settings as follows: spray voltage 4500 V, sheet gas (N₂) pressure 27 AU, ion sweep

gas pressure 0 AU and auxiliary gas (N₂) pressure 7 AU, capillary temperature 275 °C, skimmer offset 0 V, and capillary offset -35 V. The mass spectrometry data were acquired in the negative ionization mode, in the m/z range from 100 to 1000. Multiple mass spectrometric scanning modes, including full scanning (FS), and product ion scanning (PIS), were conducted for the qualitative analysis of the targeted compounds. The collision-induced fragmentation experiments were performed using argon as the collision gas, and the collision energy was varied depending on the compound. The time-selected reaction monitoring (tSRM) experiments for quantitative analysis were performed using two MS² fragments for each compound that were previously defined as dominant in the PIS experiments [40]. Xcalibur software (version 2.2) was used for instrument control. Phenolics were identified and quantified according to the corresponding spectral characteristics: molecular ion, mass spectra, characteristic fragmentation, and characteristic retention time. The limits of detection (LOD) and quantification (LOQ) were calculated using standard deviations of the responses (SD) and the slopes of the calibration curves (S) according to the formulas: $LOD = 3(SD/S)$ and $LOQ = 10(SD/S)$. The values of standard deviations and slopes were obtained from the calibration curves created in MS Excel. The total amounts of each compound were evaluated by calculation of the peak areas and are expressed as mg kg⁻¹.

2.10. Biological characterization of extract

2.10.1. Antioxidant activity

The antioxidant activity of chamomile extracts was evaluated using DPPH and reducing power methods. For the DPPH radical-scavenging assay the procedure followed the method of Espin et al. (2000) [41] in which the extracts showed antioxidant activity by the reduction of purple colored DPPH to the yellow colored diphenylpicrylhydrazine derivatives. The extracts were mixed

with methanol (95 %) and 90 μM DPPH to give final concentrations of 0.01; 0.02; 0.05; 0.1 and 0.2 mg/mL. The mixture was incubated at room temperature for 60 minutes, and the absorbance was measured at wavelength of 515 nm. Methanol was used to set zero of transmittance. All tests were performed in triplicates. The results were expressed as inhibitory concentration at 50% (IC_{50}), which is the concentration of the test solution for achieving 50% of the radical scavenging capacity. The antioxidant activity was also determined by the reducing power ability following the procedure described by Oyaizu (1986) [42]. Various concentrations of the extracts (0.1; 0.2; 0.3; 0.4; 0.5, 0.6; 0.7; 0.8; 0.9 and 1 mg/mL) were mixed with phosphate buffer (1 mL, 0.2 M, pH 6.6) and 1 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C. After 20 min of incubation, the reaction mixture was acidified with 1 mL of trichloroacetic acid (10%). The mixture was further centrifuged at 3000 min^{-1} for 10 minutes. The obtained supernatant (2 mL) was mixed with double distilled water (2 mL) and 0.1% FeCl_3 solution (0.4 mL). Absorbance was measured at 700 nm. The blank was prepared by using water instead of an extract. All tests were performed in triplicates. The capacity of extracts to convert Fe^{3+} into Fe^{2+} was expressed as EC_{50} values, which represents the concentration of extract that gives half-maximal response.

2.10.2. Enzyme inhibitory activity

The enzyme inhibitory activity of chamomile extracts was evaluated by measuring α -amylase, α -glucosidase as well as tyrosinase inhibitory activity. α -Amylase inhibitory activity was performed using Caraway-Somogyi iodine/potassium iodide (IKI) method [43]. Sample solution (2 mg mL^{-1} ; 25 μL) was mixed with α -amylase solution (ex-porcine pancreas, EC 3.2.1.1, Sigma) (50 μL) in phosphate buffer (pH 6.9 with 6 mM sodium chloride) in a 96-well microplate and incubated for 10 min at 37 °C. After pre-incubation, the reaction was initiated with the addition of

starch solution (50 μL , 0.05%). Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme (α -amylase) solution. The reaction mixture was incubated for 10 min at 37 $^{\circ}\text{C}$. The reaction was then stopped with the addition of HCl (25 μL , 1 M). This was followed by addition of the iodine-potassium iodide solution (100 μL). The sample and blank absorbances were read at 630 nm. The absorbance of the blank was subtracted from that of the sample and the α -amylase inhibitory activity was expressed as acarbose equivalent (mg ACE/g extract).

α -Glucosidase inhibitory activity was performed by the previous method [43]. Sample solution (2 mg/mL; 50 μL) was mixed with glutathione (50 μL), α -glucosidase solution (from *Saccharomyces cerevisiae*, EC 3.2.1.20, Sigma) (50 μL) in phosphate buffer (pH 6.8) and PNPG (4-N-trophenyl- α -D-glucopyranoside) (50 μL) in a 96-well microplate and incubated for 15 min at 37 $^{\circ}\text{C}$. Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme (α -glucosidase) solution. The reaction was then stopped with the addition of sodium carbonate (50 μL , 0.2 M). The sample and blank absorbances were read at 400 nm. The absorbance of the blank was subtracted from that of the sample and the α -glucosidase inhibitory activity was expressed as acarbose equivalent (mg ACE/g extract).

Tyrosinase inhibitory activity was measured using the modified dopachrome method with 10 mM L-DOPA (3,4-Dihydroxy-L-phenylalanine) as substrate, as previously reported [44] with slight modification. Sample solution (2 mg/mL; 25 μL) was mixed with tyrosinase solution (40 μL) and phosphate buffer (100 μL , pH 6.8) in a 96-well microplate and incubated for 15 min at 25 $^{\circ}\text{C}$. The reaction was then initiated with the addition of L-DOPA (40 μL). Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme (tyrosinase) solution. The sample and blank absorbances were read at 492 nm after a 10 min incubation at 25 $^{\circ}\text{C}$. The

absorbance of the blank was subtracted from that of the sample and the tyrosinase inhibitory activity was expressed as kojic acid equivalent (mg KAE g⁻¹ extract).

2.11. Statistical analysis

All analysis were run in triplicate and were expressed as means \pm standard deviation (SD). Mean values were considered significantly different at $p < 0.05$ confidence level, after the performance of the ANOVA single/double factor statistical analysis followed by Tuckey test.

3. RESULTS AND DISSCUSSION

3.1. Optimization of the subcritical water extraction

3.1.1. Influence of the solvent-to-sample ratio

It is well known that the extraction conditions affect the efficiency of the extraction process and consequently the content of target compounds in obtained extracts. Extractability of phenolic compounds depends on many parameters, such as solvent, agitation, chemical structure, temperature, pressure, sample matrix, etc, and there is no universal procedure that is suitable for the extraction of all phenolics or a specific class of phenolics from plant samples. In comparison to other extraction techniques, SCW extraction shows competitive effectiveness for extraction of plant samples [25]. Nevertheless, it is necessary to optimize operational parameters in respect to desired components. Within this paper optimization was performed in terms of apigenin content, but content of total phenols and flavonoids was monitored as well. Despite the fact that used spectrophotometric methods for total phenols and flavonoids have some limitations they still represents the most common methods for their quantifications, and may serve for fast screening. From that reason those methods were used during the optimization process.

The first parameter which affects the concentration of the target compounds in the extracts is solvent-to-sample ratio (SSR). Influence of this parameter on the recovery of phenolic compounds from different plant matrices was frequently overlooked and it is very important to find optimal SSR for the maximum concentration.

In present study the influence of the SSR on the extraction yield was studied for four SSRs (1:30; 1:50 1:70; and 1:100) which were selected based on previously reported values for chamomile extraction [45]. Extraction was performed at 100 °C for 30 min. During the extraction the pressure was 20 bar and it was maintained constant during the entire extraction run. Influence of different SSR on the total phenols content is presented in Fig. 2.

Figure 2.

According to the results presented in Fig. 2, it is evident that the increase of SSR yielded in the increase of total phenols concentration. This is in correlation with mass transfer principles which suggest that the concentration gradient between the sample matrix and the bulk of the solvent is the driving force during extraction. With SSR increase the difference of the concentration between bulk solution and sample matrix increased releasing more solutes [46]. Obtained results for total phenols obtained in thus study were in agreement with the results of other studies suggesting that high SSRs were favorable for the extraction of phenolic compounds.

Bearing in mind great pharmacological potential of apigenin, special attention in this research was put on this flavone, and results for the effects of SSR on apigenin content in water extracts are presented in Fig 3. According to the presented results it could be concluded that unlike for phenols, the highest yields of flavonoids and apigenin were obtained for the ratio of 1:30. Further increase in SSR was followed by a decrease of the extraction yield (Fig. 3).

Figure 3.

Herodež et al. [47] stated that during extraction yield of solutes does not increase after equilibrium was reached, thus it could be assumed that equilibrium steady state for total flavonoids and apigenin was achieved at SSR of 1:30. SSR significantly affects the equilibrium constant, thus the maximum yields are achieved in a steady state [48]. The solubility of solutes is affected by changes in the activity coefficient, which varies with the temperature and composition of the solution [49]. Interactions between the compounds and the solvent are modify the activity coefficients and consequently the solubility of the compounds [50].

Overall, experimental results obtained in presented study showed that SSR had significant effect on total phenol and flavonoid yields, as well as on the yield of apigenin in chamomile extracts obtained by SCW. Bearing in mind that optimization was performed with the aim of apigenin isolation, SSR of 1:30 was chosen as optimal, and it was used in all further experiments. Using this ratio avoids the dilution effect while still ensuring the desired yield.

3.1.2. Influence of the agitation rate

Among other factors, agitation and mass transfer mechanisms are one of the most important factors influencing the recovery of key compounds from natural sources. In order to develop the optimal extraction protocol, agitation influence on extraction yields of phenols, flavonoids, as well as apigenin from chamomile flowers was investigated.

In the designed extractor agitation of the sample/solvent mixture was provided by vibrational movements of a platform that housed the extraction cell. Vibrational mode allowed efficient mixing and convective mass transfer. Agitation influence on the extraction yields of target compounds was

investigated in the range from 2 Hz to 6 Hz at the temperature of 100 °C, applying the extraction time of 30 minutes. The process was performed at a pressure of 20 bar maintaining previously defined optimal solvent-to-sample ratio 1:30. Total phenolic and flavonoid contents were determined from the calibration curves of chlorogenic acid ($Y = 4.91 X - 0.00833$; $R^2 = 0.998$) and rutin ($Y = 1.88727 X - 0.0096$; $R^2 = 0.997$), respectively. The total phenolic and flavonoid contents in obtained extracts are presented in table 1.

Table 1.

As can be seen from the Table 1, the highest concentrations of total extracted phenols and flavonoids was achieved by applying agitation frequency of 2 Hz. Greater agitation rates caused the decrease in the extraction efficiency of both phenols and flavonoids. In case of total flavonoids with increase of agitation rate from 3 to 4 and further from 4 to 5 Hz extraction efficiency changed insignificantly ($p = 0.99958$; $p = 0.128161$). However, in case of total phenols increase of agitation from 3 to 4 Hz resulted in significant changes in yield ($p = 0.00088$) while further increase from 4 to 5 Hz resulted in insignificant changes in yield of phenolic compounds ($p = 0.99784$). The lowest extraction efficiency was seen for the highest agitation frequency of 6 Hz. According to these results, it can be concluded that agitation frequency of 2 Hz provided maximum contents of total phenols and flavonoids in the extracts. As in the case of total phenols and flavonoids, the lowest extraction efficiency of apigenin was seen at the agitation rate of 6 Hz (1.30 mg/g). The maximum yield of the key compound (3.57 mg/mL) was obtained for the agitation rate of 3 Hz, thus, this optimal frequency was used in all further investigations.

3.1.3. Influence of the pressure

On oppose to supercritical carbon-dioxide extraction in which principal operational parameter influencing extraction efficiency is a density as a function of pressure and temperature, in SCW extraction principal operational parameters is a temperature, and pressures applied serve only to maintain water in its liquid state [51]. Although the effects of pressure on the recovery of most substances from plants is usually negligible when applying subcritical water [52–54], this factor must be carefully studied to fully optimize the extraction protocol. High pressures during the extraction helps solvent penetration into the pores of sample matrix and controls problems related to the formation of air bubbles within the matrix, which hinder the solvent from reaching the analyte [51,55].

In order to determine the influence of pressure on the recovery of target compounds investigation was carried out at previously defined optimal SSR (1:30) applying adopted agitation rate (3 Hz). Extraction temperature (100 °C) and time (30 min) were the same as in previous experiments. Obtained results are showed in the Fig. 4 and Fig. 5.

Figure 4.

Extracts obtained under the pressure of 10 bar demonstrated the lowest phenol contents (82.72 mg RE/g). Increase in operational pressure from 10 to 30 bar led to increase in phenols concentration with the pressure of 30 bar providing the highest phenols yield (181.51 mg CAE/g). Total phenols concentrations in extracts obtained under 45, 60 and 90 bar were 149.51, 139.69, and 154.54 mg CAE/g, respectively. Statistical analysis showed that increases of pressure from 45 to 60 bar and from 60 to 90 bar caused significant difference between yield of phenolic compounds ($p = 0.003109$ and $p = 0.000251$, respectively). However, results of performed statistical analyses

suggested that there were insignificant differences in yields obtained at 45 and 90 bar ($p = 0.13363$). This fact implies that efficient phenol extraction does not require high pressures. This fact goes along with practical convenience of the extraction protocol.

Figure 5.

Similar situation could be noticed for total flavonoids and apigenin. Interestingly, there was an identical pressure influence on the content of flavonoids and apigenin which is not so surprising taking into account that apigenin structurally belongs to a class of flavonoids. At pressures above 45 bar, there was a decrease in flavonoid and apigenin content. As Fig. 5 shows, the highest yields of total extracted flavonoids was achieved applying pressure of 45 bars, followed by pressures of 60 bar (31.62 mg RE/g), 30 bar (29.54 mg RE/g) and 90 bar (28.15 mg RE/g). Obtained results for flavonoid contents were in correlation with those obtained for apigenin content, i.e., increase in pressure above 45 bar yielded lower concentration. Within the examined range (10-90 bar) apigenin content differed from 2.78 mg/g (10 bar) to maximum content of 5.33 (45 bar). Taking into account the highest yields of target compound at the pressure of 45 bar this value was adopted as optimal and kept constant in all further extractions.

3.1.4. Influence of the temperature

Regardless on the applied extraction technique, temperature shows strong effects on process efficiency, due to its influence on analyte-sample matrix interaction, i.e. desorption kinetics, solubility of solutes, surface tension and viscosity, as well as mass transfer [51]. With temperature increase cohesive (molecule-molecule) and adhesive (analyte and the sample matrix) interactions can be overcome by decreasing the activation energy required for the desorption process [55].

Temperature effects on surface tension and viscosity reflect the efficiency of pore filling and the contact between the solvent and sample matrix. In subcritical water extraction temperature is a crucial factor that affects the extraction efficiency and selectivity of the process. By varying the extraction temperatures, dielectric constant of water can be modulated. At room temperature water is a very polar solvent with dielectric constant close to 80. However, its polarity can be significantly reduced to values close to 27 for working temperature of 250 °C while maintaining its liquid state by applying sufficient pressure [51]. Consequently, the polarity of water at elevated temperatures becomes equivalent to that of common organic solvents. This implies that more polar solutes soluble in ambient water are extracted efficiently at lower temperatures, whereas moderately polar and non-polar targets require a less polar medium induced by elevated temperatures [56]. Higher temperatures decrease hydrogen bonding strength in water leading to more efficient extraction of hydrophobic compounds. On the other hand, extraction at too high temperature may cause degradation of thermo-labile compounds. Thus, it is very important to define the optimal temperature for target compounds in a tested matrix.

In current study, extraction procedures were performed for 30 min at seven different temperatures (65-210 °C) applying the pressure of 45 bars and agitation frequency of 3 Hz. Color of obtained extracts changed from light yellow to dark brown. The darkest extracts were obtained at temperature of 130 °C. Further increase in temperature led to lightening of the extracts indicating possible hydrolytical process of sample matrix compounds.

Plot presented in Fig. 6 shows the dependence of total phenols content and the temperature. Significant effects of the extraction temperature on total phenolic content could be observed in the temperature range of 65-130 °C. Temperature increase resulted in the increase of total phenolic content, which is correlated with changes in extracts dye. By increasing the temperature above 65 °C, extracts become darker which is followed by increasing in yield of phenols content. At

temperature of 130 °C, the maximum yield of phenols was obtained (215 mg CAE/g), which match with color of extracts (darkest shade). At temperatures above 130 °C curve in the Fig. 6 decreases, and the color of extracts becomes brighter. In other words, by an increasing the temperature above 130 °C results in a decrease in phenols content, thus the lighter coloration of extracts could be observed.

At high temperatures different chemical reactions take place in organic medium. In superheated water, depending on various factors, including temperature, Maillard reactions are probable, especially under acidic conditions. Many of the Maillard reactions products are strongly colored causing browning of the reaction mixture [57,58]. The color of extracts probably resulted both from extracted compounds and formed Maillard reaction products. Both the yield and Maillard reactions increase with the temperature being responsible for the color of the extracts. Browning reactions increase exponentially with temperature [59] and the velocity of these reactions is the highest at temperatures between 140 and 160°C.

Figure 6.

The same situation could be observed in the case of total flavonoids and apigenin contents. The results of temperature influence on flavonoids and apigenin recovery are given in Table 2. With the temperature increasing the content of these compounds is rising. This effect has been observed in all points up to 115 °C. At this point amounts of flavonoids (36.96 mg RE/g) and apigenin (10.90 mg/g) achieved their maximum values. Further rising in temperature is followed by decreasing in yield of these compounds.

Table 2.

3.1.5. Influence of the extraction time

Optimal extraction time should be the shortest possible time upon equilibration of solutes concentration between sample matrix and bulk solvent. Longer extraction time should be avoided in order to prevent analyte degradation and due to practice consideration.

In order to determine the influence of the extraction time on phenols, flavonoids and apigenin contents in obtained extracts, extraction time was varied from 5 to 60 minutes. All others operational parameters relied on previously adopted values, temperature of 115 °C, agitation frequency of 3 Hz, pressure of 45 bar and solvent-to-sample ratio of 1:30. Fig. 7 illustrates the influence of the extraction time on the yields of the observed bioactive compounds.

Figure 7.

The extraction yields of all three observed outputs increased gradually during the first 30 minutes of the extraction process, after that a decline was observed. This declining tendency in the extraction yield may be related to degradation of the components. The extraction yields at peak extraction time (30 min) for total phenols, flavonoids and apigenin were 186.48 mg CAE/g, 38.89 mg RE/g and 11.50 mg/g, respectively. For total phenols content, the lowest concentration was achieved at the extraction time of 60 min (143.37 mg CAE/g), while minimal concentration of apigenin was seen after 5 minutes of the extraction (5.84 mg/g). For total flavonoids extraction duration did not exhibit profound effects as in the case of other two outputs, and within examined time window (5-60 min) total flavonoids content varied within the range from 29.15 to 38.89 mg RE/g. According to these results, extraction time of 30 min was adopted as optimal for the extraction of phenols, flavonoids and apigenin by SCW, under defined optimal conditions.

Obtained results were in collision with those one from the literature. Namely, according to Ko et al., [60] optimal conditions for apigenin recovery from plant material were temperature of 190°C and time of 15 min. However, in this work apigenin was extracted from parsleys and except the temperature and time no one parameter was monitored. For example, in above mentioned report pressure was constant (100 bar) and all other factors was not considered. Moreover, unlike that study in this manuscript extraction was performed with convective mass transfer which can be very influential. From economical point of view, optimal temperature of 115°C has advantages.

3.2.Isolation and identification of apigenin

Although numerous synthetic bioactive compounds have been developed, medicinal plants still remain one of the most important sources and models of new drugs [61,62]. Taking into account numerous advantages of natural compounds over synthesized, isolation of bioactive compounds from natural sources represents a great scientific challenge. In obtained extracts, usually great number of chemically similar components are present, thus isolation of particular compound can be very difficult.

Considering great pharmacological potential of apigenin, in this work SCW extraction was optimized to achieve its highest yields. Apigenin was further isolated by column chromatography from water extracts obtained under optimal conditions. Separation of apigenin was performed at polyamide column and the compound was precrystalised from absolute ethanol by joining apigenin-containing fractions. In order to confirm apigenin purity spectral analysis of precrystalised apigenin was performed in parallel with commercially purchased standard.

Figure 8.

Absorption spectra for apigenin and apigenin standard were presented in the Figure 8. Two main absorption band could be noticed and were measured at about 340 nm and 230 nm. Both bands were results of the two π - π^* HOMO-LUMO (H-L) transitions. Band at 340 nm derived from H \rightarrow L and H-1 \rightarrow L, while band at 230 nm derived from H \rightarrow L+1 and H-1 \rightarrow L+1 transitions. On the other hand, the dim signal measured at 270 nm was the result of the n- π^* transitions. This signal is composed of H-2 \rightarrow L transition [63,64]. Considering those transitions between HOMO and LUMO orbitales, it could be concluded that band at 340 nm was result of the absorption in the B-C rings (cinnamoyl segment of the structure) of apigenin molecule, while band at 230 nm was measured as the result of the absorption in the A-C rings (1,4-benzopyrone segment of the molecule). Such spectra suggested that isolated compound was apigenin. In addition, the purity and identity of isolated apigenin were confirmed by fragmentation spectrum by MS/MS analysis.

Although the chamomile has been considered as good source of apigenin, isolation process can be very difficult due to its low concentrations. According to the literature data [65] for apigenin isolation, purification of extracts, before column process, is obligatory because of the large number of compounds which hinder its isolation from the extracts. In this work high hydrolytic potential of SCW was carefully balanced and exploited to liberate apigenin from its bound forms, thus resulting in increased concentration of apigenin in obtained extracts. Simultaneously, SCW offers extracts without interfering compounds facilitating the isolation process. According to conducted investigations, the proposed technique shows to be promising in respect to extract purity and yields of key compound. Apigenin was isolated from subcritical water extract without using organic solvents, avoiding the need for further purification. Thus, this perspective approach provides obtaining economically-viable and safe functional ingredient which could be incorporated in numerous pharmaceuticals.

3.3. Biological characteristic of extracts obtained under optimal extraction conditions

In this study the biological activity of the extract obtained under the optimal extraction conditions was established by measuring its both antioxidant capacity, as well as the enzyme inhibition ability. The antioxidant power was estimated by using two different tests. The first one was DPPH assay which provide information through the reactivity of compounds with stabile radicals, while the second one, reducing power assay, served as an indicator of their antioxidant ability. The obtained results are presented as IC_{50} and EC_{50} values, which represent a concentration of extracts capable for inhibition of 50% of free radicals. The results are shown in Table 3.

Presented results reached by those two assays may be compared with previously published results [25]. According to Cvetanović et al. [25], the water extracts of chamomile obtained under subcritical conditions possess very strong antioxidant potential, more precisely, stronger than extracts obtained by using other extraction methods. However, the results presented by Cvetanović et al. [25] had been obtained under non-optimized conditions. In the interest of performing the extraction process with maximum apigenin yield, the experiments presented in this article were conducted under the optimal conditions. The results obtained by this group of author was as follows: IC_{50} value 0.0211 mg/mL and EC_{50} 0.578 mg/mL. These IC_{50} and EC_{50} values for chamomile SCW extracts obtained in the current study, under optimized conditions, were 0.0153 mg/mL and 0.339 mg/mL, respectively. It could be concluded that the antioxidant capacity of chamomile extracts become higher although the aim of this study was optimization for achievement the maximum apigenin yield, but not the optimization of antioxidant capacity.

Table 3.

Diabetes mellitus (DM) is considered as a global health problem due to its high world prevalence. The total number of people with DM is projected to rise from 415 million in 2015 to 642 million in 2040 [66]. In this sense, the exploration of new therapeutic approaches is very important for the management of DM. In recent years, key enzyme inhibitory theory is one of the most accepted theories for DM. α -amylase and α -glucosidase are key enzymes in the catabolism of carbohydrates. The enzymes catalyze the hydrolyzing of α -(1,4) bond in the carbohydrate and, therefore, the blood glucose level is increasing [67]. At this point, the inhibition of these enzymes could be considered as important tool for the management of blood glucose level in DM patients. For this purpose, several synthetic inhibitors are produced as oral anti-diabetic drugs, but they have several unfavorable health side effects including gastrointestinal disturbances [68]. Thus, there is a great interest in the discovery of natural and safer inhibitors from medicinal plants. α -amylase and α -glucosidase activities of the extract were tested by using micro-plate reader. The results were expressed as acarbose equivalents (mmol ACAE/g extract). The values were 0.45 mmol ACAE/g for α -amylase and 2.54 mmol ACAE/g for α -glucosidase (Table 3). Apparently, the extract was more active toward the α -glucosidase than α -amylase. Similar observations were realized by several researchers [69,70].

Tyrosinase is a key enzyme in the synthesis of melanin and thereby the inhibition of this enzyme could be valuable for the management of skin disorders especially in pigmentation disorders [71]. Similar to DM, several tyrosinase inhibitors are synthetically produced. However, they exhibited several side effects such as dermatitis and cytotoxicity [72]. In this sense, this phenomenon has encouraged researchers to seek natural and safe tyrosinase inhibitors for the management of skin disorders. Tyrosinase inhibitor effects was tested by using dopachrome method and the result was expressed as kojic acid equivalent. Anti-tyrosinase activity of the extract was determined as 11.38 mg KAE/g extract (Table 3). This value is comparable to the values

reported in the literature for other several plant species such as *Asphodeline* species (18.57-33.84 mg KAE/g extract in eight *Asphodeline* taxa, reported by Zengin et al. [73], *Macaranga* species (4.8-6.8 mg KAE/g in four *Macaranga* species, reported by Lim et al. [74] and *Phlomis* species (1.996-29.560 mg KAE/g in two *Phlomis* species, reported by Sarikurkcu et al. [75]). To the best of our knowledge, the present study is the first report on the enzyme inhibitory effects of the chamomile extract obtained by subcritical water extraction (P = 45 bar; t = 115°C).

Such strong biological activity implies that subcritical water extracts of chamomile obtained by above discussed conditions serve not only for apigenin isolation. Optimized SCW extraction offers obtaining extracts with improved biological activity. Taking into account that extracts were made by using water as a solvent, the step of their further purification can be avoided. Due to the safety of subcritical water such extracts are convenient for oral functional formulations.

3.4. UHPLC–HESI-MS/MS analysis of polyphenolic compounds in extract obtained under optimal extraction conditions

Quantification of polyphenolic compounds present in SCW extracts was performed using UHPLC-HESI-MS/MS analysis. A total of 18 polyphenolic compounds were identified using available standards. The list of identified compounds together with mass spectrometry data and limits of detection (LOD) and quantification (LOQ) is presented in Table 4.

Table 4.

Obtained results showed that five of eighteen polyphenols were phenolic acids (*p*-hydroxybenzoic, *p*-coumaric, chlorogenic, ellagic and sinapic acid), four were aglycones (luteolin, naringenin, apigenin and kaempferol). However, the majority of identified compounds were glucosides.

It is well known that the extraction of a phenols is greatly influenced by both the extraction conditions of the subcritical water solvent and the structure of the compound. Presence of glucosides in considerable level in comparison to other polyphenolic compounds can be explained by their structure. Namely, this group of compounds has a sugar portion of monosaccharide, and the presence of sugars (glucose or rhamnose) tends to make them more soluble in polar solvents, so they are extracted well at a lower temperature [60]. More preciously, molecules of glucosides can exhibit hydrogen bonding interactions with the water solvent due to their large charges. Therefore they could be well extracted using subcritical water at relatively lower temperature, whose high dielectric constant facilitates hydrogen bonding interactions than aglycones [60].

However, some of tested compounds were not detected in the extract. In the first place these were aglycones of flavonoids (quercetin, phloretin, chrysin, galangin). This could be related to the fact that aglycones possess stabile structure in subcritical water and most of them is well extracted at higher temperature. For example in the study Ko et al. [60] it was demonstrated that quercetin is well extracted at temperature of 170°C which represent more higher temperature than that one used for obtaining this extract. Another possibility of absence of these compounds is possible lack thereof in started plant material.

Obtained results (Table 4) showed that apigenin was the most present phenolics in investigated extract. It is present in an amount of 1501.25 mg/kg. A very high content of *p*-

hydroxybenzoic acid (496.83 mg/kg) was determined in SCW extract, while from the class of flavonoids, beside apigenin, luteolin (97.19 mg/kg) and kaempferol (60.37 mg/kg) were most abundant. As for glycosides, cynaroside (luteolin-7-*O*-glucoside) was found in highest concentration (278.37 mg/kg).

Conclusion

Taking into account poor solubility of apigenin in water, its low concentration in plant material and difficulties in its isolation, current research was designed to investigate optimal conditions for obtaining extracts with high apigenin concentration and to isolate it. By adjustment operational parameters which influence its content in chamomile extracts, samples with maximum apigenin content were obtained. Optimal operational conditions were found to be as follows: solvent-to-sample ratio of 1:30, agitation rate of 3 Hz, pressure of 30 bar, temperature of 130 °C and time of 30 min. Influence of each singular parameters was observed and explained in detail. Apigenin isolation was performed by column chromatography. Using HPTLC presence and purity of apigenin were confirmed. Extract obtained under optimal extraction conditions was characterized in terms of its biological activity. Qualitative and quantitative analysis of extract was done by using UHPLC-MS/MS analysis.

Acknowledgment

The present work was carried out within the projects of the Serbian Ministry of Education, Science and Technological Development, (Projects No. TR31013 and 172017). The authors are grateful to Msc Aleksandar Gajić for technical support and Dr Dušan Adamović, Institute of Field and Vegetable Crops, Bački Petrovac, Serbia, for his support in supplying plant material.

References

- [1] N. Haleagrahara, S. Chakravarthi, A. Bangra Kulur, T.M. Yee, Plant flavone apigenin protects against cyclosporine-induced histological and biochemical changes in the kidney in rats, *Biomed. Prev. Nutr.* 4 (2014) 589–593.
- [2] K.H. Miean, S. Mohamed, Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants, *J. Agric. Food Chem.* 49 (2001) 3106–3112.
- [3] K.N. Rithidech, M. Tungjai, E.B. Whorton, Protective effect of apigenin on radiation-induced chromosomal damage in human lymphocytes, *Mutat. Res. Toxicol. Environ. Mutagen.* 585 (2005) 96–104.
- [4] H. Ueda, C. Yamazaki, M. Yamazaki, Luteolin as an anti-inflammatory and anti-allergic constituent of *Perilla frutescens*, *Biol. Pharm. Bull.* 25 (2002) 1197–1202.
- [5] G. Seelinger, I. Merfort, U. Wölflle, C. Schempp, Anti-carcinogenic effects of the flavonoid luteolin, *Molecules.* 13 (2008) 2628–2651.
- [6] G. Seelinger, I. Merfort, C. Schempp, Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin, *Planta Med.* 74 (2008) 1667–1677.
- [7] W. Ren, Z. Qiao, H. Wang, L. Zhu, L. Zhang, Flavonoids: Promising anticancer agents, *Med. Res. Rev.* 23 (2003) 519–534.
- [8] H.P. Kim, K.H. Son, H.W. Chang, S.S. Kang, Anti-inflammatory plant flavonoids and cellular action mechanisms, *J. Pharmacol. Sci.* 96 (2004) 229–245.
- [9] M. Kawai, T. Hirano, S. Higa, J. Arimitsu, M. Maruta, Y. Kuwahara, T. Ohkawara, K. Hagihara, T. Yamadori, Y. Shima, A. Ogata, I. Kawase, T. Tanaka, Flavonoids and related compounds as anti-allergic substances, *Allergol. Int.* 56 (2007) 113–123.
- [10] M. Amoros, C.M.O. Simões, L. Girre, F. Sauvager, M. Cormier, Synergistic effect of

- flavones and flavonols against Herpes simplex virus type 1 in cell culture. Comparison with the antiviral activity of propolis, *J. Nat. Prod.* 55 (1992) 1732–1740.
- [11] J.S. Lee, H.J. Kim, Y.S. Lee, A new anti-HIV flavonoid glucuronide from *Chrysanthemum morifolium*, *Planta Med.* 69 (2003) 859–861.
- [12] A.-L. Liu, B. Liu, H.-L. Qin, S. Lee, Y.-T. Wang, G.-H. Du, Anti-influenza virus activities of flavonoids from the medicinal plant *Elsholtzia rugulosa*, *Planta Med.* 74 (2008) 847–851.
- [13] M.-M. Liu, L. Zhou, P.-L. He, Y.-N. Zhang, J.-Y. Zhou, Q. Shen, X.-W. Chen, J.-P. Zuo, W. Li, D.-Y. Ye, Discovery of flavonoid derivatives as anti-HCV agents via pharmacophore search combining molecular docking strategy, *Eur. J. Med. Chem.* 52 (2012) 33–43.
- [14] X.Lv, M. Qiu, D. Chen, N. Zheng, Y. Jin, Z. Wu, Apigenin inhibits enterovirus 71 replication through suppressing viral IRES activity and modulating cellular JNK pathway, *Antiviral Res.* 109 (2014) 30–41.
- [15] Y.H. Siddique, F. Naz, S. Jyoti, M. Afzal, Protective effect of apigenin in transgenic *Drosophila melanogaster* Model of Parkinson's disease, *Pharmacol. Online.* 3 (2011) 790–795.
- [16] K. Rezai-Zadeh, J. Ehrhart, Y. Bai, P.R. Sanberg, P. Bickford, J. Tan, R.D. Shytle, Apigenin and luteolin modulate microglial activation via inhibition of STAT1-induced CD40 expression, *J. Neuroinflammation.* 5 (2008) 41–51.
- [17] R. Estruch, E. Ros, J. Salas-Salvadó, M.-I. Covas, D. Corella, F. Arós, E. Gómez-Gracia, V. Ruiz-Gutiérrez, M. Fiol, J. Lapetra, R.M. Lamuela-Raventos, L. Serra-Majem, X. Pintó, J. Basora, M.A. Muñoz, J. V. Sorlí, J.A. Martínez, M.A. Martínez-González, Primary prevention of cardiovascular disease with a Mediterranean diet, *N. Engl. J. Med.* 368 (2013) 1279–1290.
- [18] J. Hu, Z. Li, L. Xu, A. Sun, X. Fu, L. Zhang, L. Jing, A. Lu, Y. Dong, Z. Jia, Protective

- effect of apigenin on ischemia/reperfusion injury of the isolated rat heart, *Cardiovasc. Toxicol.* 15 (2015) 241–249.
- [19] D. Patel, S. Shukla, S. Gupta, Apigenin and cancer chemoprevention: Progress, potential and promise (Review), *Int. J. Oncol.* 30 (2007) 233–245.
- [20] S. Gupta, F. Afaq, H. Mukhtar, Selective Growth-Inhibitory, Cell-Cycle Deregulatory and apoptotic response of apigenin in normal versus human prostate carcinoma cells, *Biochem. Biophys. Res. Commun.* 287 (2001) 914–920.
- [21] J. Lu, Z. Zhang, Z. Ni, H. Shen, Z. Tu, H. Liu, R. Lu, QM/MM–PB/SA scoring of the interaction strength between Akt kinase and apigenin analogues, *Comput. Biol. Chem.* 52 (2014) 25–33.
- [22] S. Shukla, S. Gupta, Apigenin: A promising molecule for cancer prevention, *Pharm. Res.* 27 (2010) 962–978.
- [23] M. Stanković, L. Stanojević, Tehnologija lekovitog i začinskog bilja, Faculty of Technology Leskovac, Leskovac, 2014.
- [24] J. Švarc-Gajić, A. Cvetanović, The influence of temperature on apigenin extraction from chamomile (*Matricaria recutita*) by superheated water, *WASET.* 2 (2015) 626.
- [25] A. Cvetanović, J. Švarc-Gajić, P. Mašković, S. Savić, L. Nikolić, Antioxidant and biological activity of chamomile extracts obtained by different techniques: perspective of using superheated water for isolation of biologically active compounds, *Ind. Crops Prod.* 65 (2015) 582–591.
- [26] M.D. Luque de Castro, L.E. García-Ayuso, Soxhlet extraction of solid materials: an outdated technique with a promising innovative future, *Anal. Chim. Acta.* 369 (1998) 1–10.
- [27] L. Wang, C.L. Weller, Recent advances in extraction of nutraceuticals from plants, *Trends Food Sci. Technol.* 17 (2006) 300–312.

- [28] I. Rodríguez-Meizoso, F.R. Marin, M. Herrero, F.J. Señorans, G. Reglero, A. Cifuentes, E. Ibáñez, Subcritical water extraction of nutraceuticals with antioxidant activity from oregano. Chemical and functional characterization, *J. Pharm. Biomed. Anal.* 41 (2006) 1560–1565.
- [29] J.W. King, Advances in critical fluid technology for food processing, *Food Science Technol. Today.* 14 (2000) 186–191.
- [30] M.S.Y. Kumar, R. Dutta, D. Prasad, K. Misra, Subcritical water extraction of antioxidant compounds from Seabuckthorn (*Hippophae rhamnoides*) leaves for the comparative evaluation of antioxidant activity, *Food Chem.* 127 (2011) 1309–1316.
- [31] L. Yang, H. Qu, G. Mao, T. Zhao, F. Li, B. Zhu, B. Zhang, X. Wu, Optimization of subcritical water extraction of polysaccharides from *Grifola frondosa* using response surface methodology, *Pharmacogn. Mag.* 9 (2013) 120–129.
- [32] P.P. Singh, M.D.A. Saldaña, Subcritical water extraction of phenolic compounds from potato peel, *Food Res. Int.* 44 (2011) 2452–2458.
- [33] X. Liang, Q. Fan, Application of sub-critical water extraction in pharmaceutical industry, *J. Mater. Sci. Chem. Eng.* 1 (2013) 1–6.
- [34] M.H. Eikani, F. Golmohammad, S. Rowshanzamir, Subcritical water extraction of essential oils from coriander seeds (*Coriandrum sativum* L.), *J. Food Eng.* 80 (2007) 735–740.
- [35] A. Basile, M.M. Jiménez-Carmona, A.A. Clifford, Extraction of rosemary by superheated water, *J. Agric. Food Chem.* 46 (1998) 5205–5209.
- [36] V.L. Singleton, J.A. Rossi, Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, *Am. J. Enol. Vitic.* 16 (1965) 144–158.
- [37] M.P. Kähkönen, A.I. Hopia, H.J. Vuorela, J.-P. Rauha, K. Pihlaja, T.S. Kujala, M. Heinonen, Antioxidant activity of plant extracts containing phenolic compounds, *J. Agric. Food Chem.* 47 (1999) 3954–3962.

- [38] K.R. Markham, Flavones, flavonoids, and their glycosides, in: J.B. Harborne, P.M. Dey (Eds.), *Methods in Plant Biochemistry*, Academic Press Ltd, London, 1989: pp. 197–235.
- [39] B. Pekić, Ž. Lepojević, B. Slavica, Determination of apigenin and apigenin-7-*O*- β -glucoside in the *Matricaria chamomilla* ligulate flowers, *Arh Farm.* 39 (1989) 169–174.
- [40] U.M. Gašić, M.M. Natić, D.M. Mišić, D. V. Lušić, D.M. Milojković-Opsenica, Ž.L. Tešić, D. Lušić, Chemical markers for the authentication of unifloral *Salvia officinalis* L. honey, *J. Food Compos. Anal.* 44 (2015) 128–138.
- [41] J.C. Espín, C. Soler-Rivas, H.J. Wichers, Characterization of the total free radical scavenger capacity of vegetable oils and oil fractions using 2,2-Diphenyl-1-picrylhydrazyl Radical, *J. Agric. Food Chem.* 48 (2000) 648–656.
- [42] M. Oyaizu, Studies on product of browning reaction prepared from glucose amine, *Japanese J. Nutr.* 44 (1986) 307–315.
- [43] G. Zengin, C. Sarikurkcu, A. Aktumsek, R. Ceylan, O. Ceylan, A comprehensive study on phytochemical characterization of *Haplophyllum myrtifolium* Boiss. endemic to Turkey and its inhibitory potential against key enzymes involved in Alzheimer, skin diseases and type II diabetes, *Ind. Crops Prod.* 53 (2014) 244–251.
- [44] I.E. Orhan, F.S. Senol, A.R. Gulpinar, N. Sekeroglu, M. Kartal, B. Sener, Neuroprotective potential of some terebinth coffee brands and the unprocessed fruits of *Pistacia terebinthus* L. and their fatty and essential oil analyses, *Food Chem.* 130 (2012) 882–888.
- [45] Z. Zeković, Determination, extraction and chemical transformations of Chamomile (*Matricaria chamomilla* L.) flavonoids, University of Novi Sad, Faculty of Technology, 1993.
- [46] M. Mohamad, M.W. Ali, A. Ripin, A. Ahmad, Effect of extraction process parameters on the yield of bioactive compounds from the roots of *Eurycoma longifolia*, *J. Teknol.*

- (Sciences Eng. 60 (2013) 51–57. d
- [47] Š.S. Herodež, M. Hadolin, M. Škerget, Ž. Knez, Solvent extraction study of antioxidants from Balm (*Melissa officinalis* L.) leaves, *Food Chem.* 80 (2003) 275–282.
- [48] S. Hamdan, H.G. Daood, M. Toth-Markus, V. Illés, Extraction of cardamom oil by supercritical carbon dioxide and sub-critical propane, *J. Supercrit. Fluids.* 44 (2008) 25–30.
- [49] T.C. Frank, J.R. Downey, S.K. Gupta, Quickly screen solvents for organic solids, *Chem. Eng. Prog.* 95 (1999) 41–61.
- [50] P.W. Tan, C.P. Tan, C.W. Ho, Antioxidant properties: Effects of solid-to-solvent ratio on antioxidant compounds and capacities of pegaga (*Centella asiatica*), *Int. Food Res. J.* 18 (2011) 557–562.
- [51] J. Švarc-Gajić, *Sampling and Sample Preparation in Analytical Chemistry*, Nova Science Publishers, New York, 2012.
- [52] M.P.K. Choi, K.K.C. Chan, H.W. Leung, C.W. Huie, Pressurized liquid extraction of active ingredients (ginsenosides) from medicinal plants using non-ionic surfactant solutions, *J. Chromatogr. A.* 983 (2003) 153–162.
- [53] C. Deng, J. Ji, X. Wang, X. Zhang, Development of pressurized hot water extraction followed by headspace solid-phase microextraction and gas chromatography-mass spectrometry for determination of ligustilides in *Ligusticum chuanxiong* and *Angelica sinensis*, *J. Sep. Sci.* 28 (2005) 1237–1243.
- [54] C. Deng, N. Li, X. Zhang, Rapid determination of essential oil in *Acorus tatarinowii* Schott. by pressurized hot water extraction followed by solid-phase microextraction and gas chromatography–mass spectrometry, *J. Chromatogr. A.* 1059 (2004) 149–155.
- [55] A. Mustafa, C. Turner, Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review, *Anal. Chim. Acta.* 703 (2011) 8–18.

- [56] R.M. Smith, Superheated water: the ultimate green solvent for separation science, *Anal. Bioanal. Chem.* 385 (2006) 419–421.
- [57] M. Plaza, M. Amigo-Benavent, M.D. del Castillo, E. Ibáñez, M. Herrero, Facts about the formation of new antioxidants in natural samples after subcritical water extraction, *Food Res. Int.* 43 (2010) 2341–2348.
- [58] F.J. Morales, M.-B. Babbel, Antiradical efficiency of Maillard reaction mixtures in a hydrophilic media, *J. Agric. Food Chem.* 50 (2002) 2788–92.
- [59] R.S. Shallenberger, G.G. Birch, *Sugar Chemistry*, Avi Publishing Co Inc., Westport, 1975.
- [60] M.-J. Ko, C.-I. Cheigh, M.-S. Chung, Relationship analysis between flavonoids structure and subcritical water extraction (SWE), *Food Chem.* 143 (2014) 147–155.
- [61] M.S. Butler, The role of natural product chemistry in drug discovery, *J. Nat. Prod.* 67 (2004) 2141–2153.
- [62] D.J. Newman, G.M. Cragg, K.M. Snader, The influence of natural products upon drug discovery, *Nat. Prod. Rep.* 17 (2000) 215–234.
- [63] A. Amat, C. Clementi, C. Miliani, A. Romani, A. Sgamellotti, S. Fantacci, Complexation of apigenin and luteolin in weld lake: a DFT/TDDFT investigation, *Phys. Chem. Chem. Phys.* 12 (2010) 6672–6684.
- [64] A. Amat, C. Dementia, F. De Angelis, A. Sgamellotti, S. Fantacci, Absorption and emission of the apigenin and luteolin flavonoids: A TDDFT investigation, *J. Phys. Chem. A.* 113 (2009) 15118–15126.
- [65] Ž. Lepojević, *Praktikum hemije i tehnologije farmaceutskih proizvoda*, University of Novi Sad, Novi Sad, 2000.
- [66] International Diabetes Federation, *IDF Diabetes Atlas*, 7th ed., International Diabetes Federation, Brussels, Belgium, 2015.

- [67] U. Etxeberria, A.L. de la Garza, J. Campión, J.A. Martínez, F.I. Milagro, Antidiabetic effects of natural plant extracts via inhibition of carbohydrate hydrolysis enzymes with emphasis on pancreatic alpha amylase, *Expert Opin. Ther. Targets*. 16 (2012) 269–297.
- [68] F.A. van de Laar, Alpha-glucosidase inhibitors in the early treatment of type 2 diabetes, *Vasc. Health Risk Manag.* 4 (2008) 1189–1195.
- [69] I. Lazarova, G. Zengin, O. Bender, D. Zheleva-Dimitrova, S. Uysal, R. Ceylan, R. Gevrenova, A. Aktumsek, M. Acar, M. Gunduz, A comparative study of Bulgarian and Turkish *Asphodeline* lutea root extracts: HPLC–UV profiles, enzyme inhibitory potentials and anti-proliferative activities against MCF-7 and MCF-10A cell lines, *J. Funct. Foods*. 15 (2015) 254–263.
- [70] D. Kumar, N. Gupta, R. Ghosh, R.H. Gaonkar, B.C. Pal, α -Glucosidase and α -amylase inhibitory constituent of *Carex baccans*: Bio-assay guided isolation and quantification by validated RP-HPLC–DAD, *J. Funct. Foods*. 5 (2013) 211–218.
- [71] M. Shiino, Y. Watanabe, K. Umezawa, Synthesis of *N*-substituted *N*-nitrosohydroxylamines as inhibitors of Mushroom Tyrosinase, *Bioorg. Med. Chem.* 9 (2001) 1233–1240.
- [72] M.E. Chiari, D.M.A. Vera, S.M. Palacios, M.C. Carpinella, Tyrosinase inhibitory activity of a 6-isoprenoid-substituted flavanone isolated from *Dalea elegans*, *Bioorg. Med. Chem.* 19 (2011) 3474–3482.
- [73] G. Zengin, M. Locatelli, R. Ceylan, A. Aktumsek, Anthraquinone profile, antioxidant and enzyme inhibitory effect of root extracts of eight *Asphodeline* taxa from Turkey: can *Asphodeline* roots be considered as a new source of natural compounds?, *J. Enzyme Inhib. Med. Chem.* (2015) 1–6. doi:10.3109/14756366.2015.1063623.
- [74] T. Lim, Y. Lim, C. Yule, Evaluation of antioxidant, antibacterial and anti-tyrosinase activities of four *Macaranga* species, *Food Chem.* 114 (2009) 594–599.

- [75] C. Sarikurkcu, M.C. Uren, B. Tepe, M. Cengiz, M.S. Kocak, Phenolic content, enzyme inhibitory and antioxidative activity potentials of *Phlomis nissolii* and *P. pungens* var. *pungens*, Ind. Crops Prod. 62 (2014) 333–340.

Figure Captions

Figure 1. Schematic diagram of subcritical water extraction system: (1) nitrogen cylinder; (2) manometer; (3) input gas valve; (4) thermocouple for temperature measurement; (5) coverlid of extraction vassel; (6) extraction vassel; (7) vibrating platform; (8) digital temperature controller; (9) main swich; (10) swich for the vibrating platform; (11) heating plate.

Figure 2. Extraction yield of phenols depending on the sample-to-solvent ratio.

Figure 3. Concentration of total flavonoids and apigenin in chamomile subcritical water extracts depending on the sample-to-solvent ratio.

Figure 4. The influence of the extraction pressure on the efficiency of total phenols content

Figure 5. The influence of the extraction pressure on the efficiency of total flavonoids and apigenin content.

Figure 6. Temperature influence on the total phenols content in subcritical water extraction.

Figure 7. Influence of the extraction time on total phenol, flavonoid and apigenin contents.

Figure 8. A) 3D-UV and B) UV spectra of apigenin isolated from SCW extract and standard apigenin.

Figure Captions

Figure 1. Schematic diagram of subcritical water extraction system: (1) nitrogen cylinder; (2) manometer; (3) input gas valve; (4) thermocouple for temperature measurement; (5) coverlid of extraction vassel; (6) extraction vassel; (7) vibrating platform; (8) digital temperature controller; (9) main swich; (10) swich for the vibrating platform; (11) heating plate.

Figure 2. Extraction yield of phenols depending on the sample-to-solvent ratio.

Figure 3. Concentration of total flavonoids and apigenin in chamomile subcritical water extracts depending on the sample-to-solvent ratio.

Figure 4. The influence of the extraction pressure on the efficiency of total phenols content

Figure 5. The influence of the extraction pressure on the efficiency of total flavonoids and apigenin content.

Figure 6. Temperature influence on the total phenols content in subcritical water extraction.

Figure 7. Influence of the extraction time on total phenol, flavonoid and apigenin contents.

Figure 8. A) 3D-UV and B) UV spectra of apigenin isolated from SCW extract and standard apigenin.

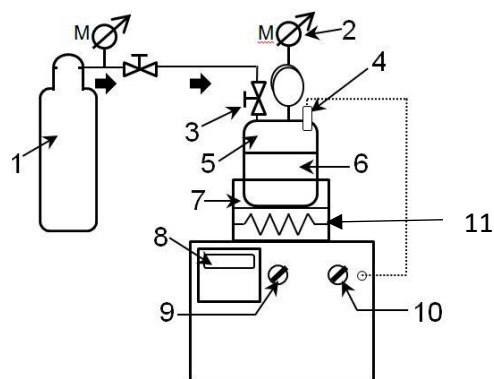


Fig. 1. Schematic diagram of subcritical water extraction system: (1) nitrogen cylinder; (2) manometer; (3) input gas valve; (4) thermocouple for temperature measurement; (5) coverlid of extraction vassel; (6) extraction vassel; (7) vibrating platform; (8) digital temperature controller; (9) main switch; (10) switch for the vibrating platform; (11) heating plate.

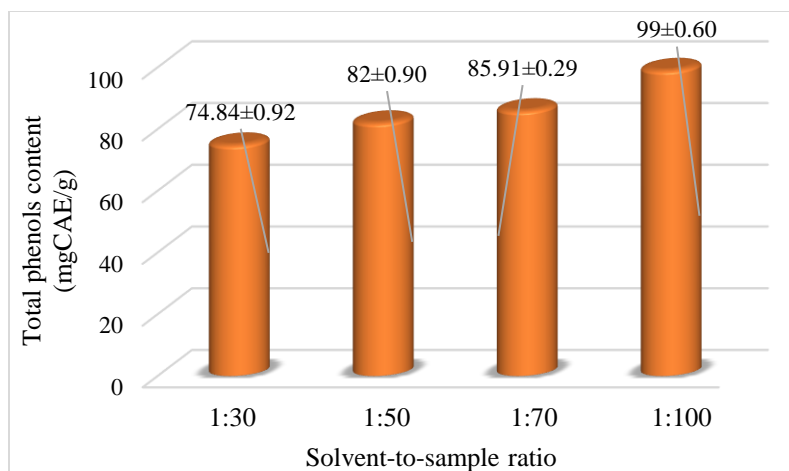


Fig. 2. Extraction yield of phenols depending on the solvent-to-sample ratio.

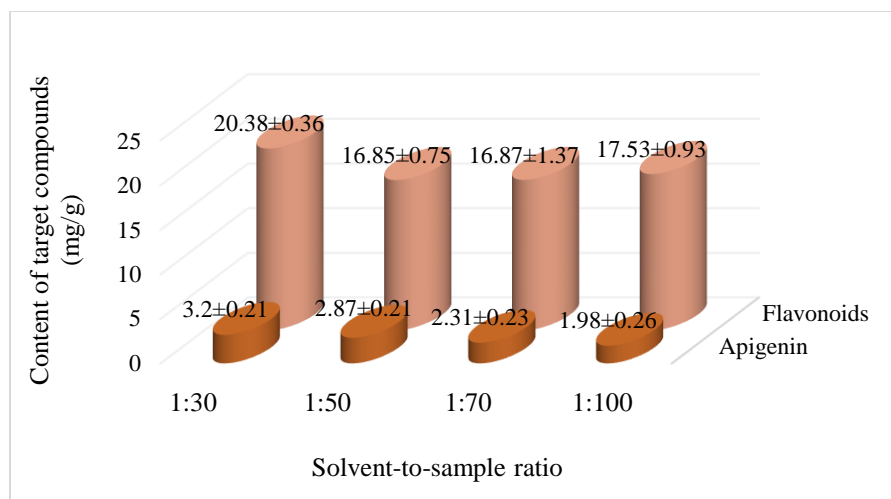


Fig. 3. Concentration of total flavonoids and apigenin in chamomile subcritical water extracts depending on the solvent-to-sample ratio.

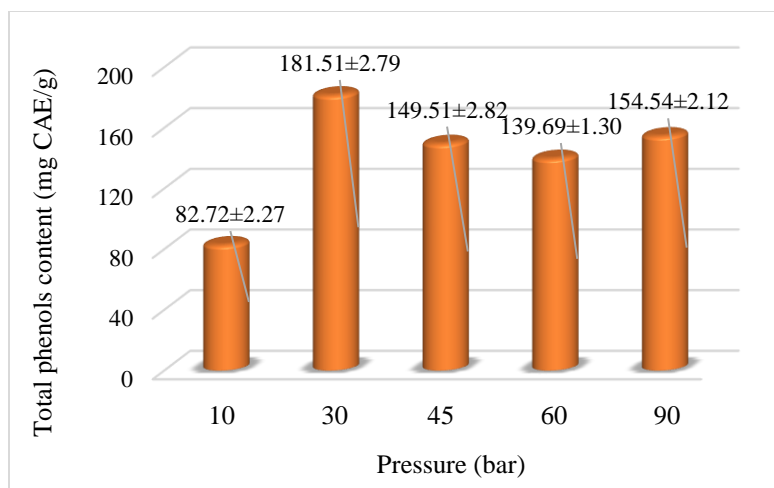


Fig. 4. The influence of the extraction pressure on the efficiency of total phenols content

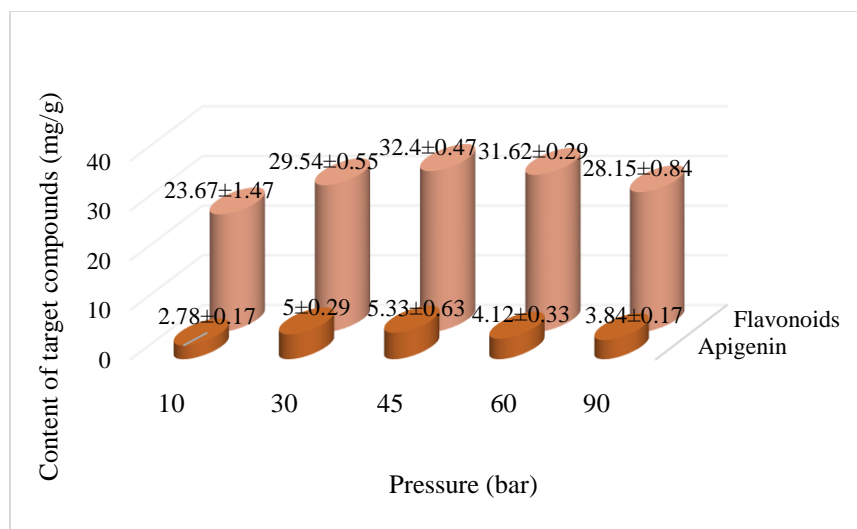


Fig. 5. The influence of the extraction pressure on the efficiency of total flavonoids and apigenin content.

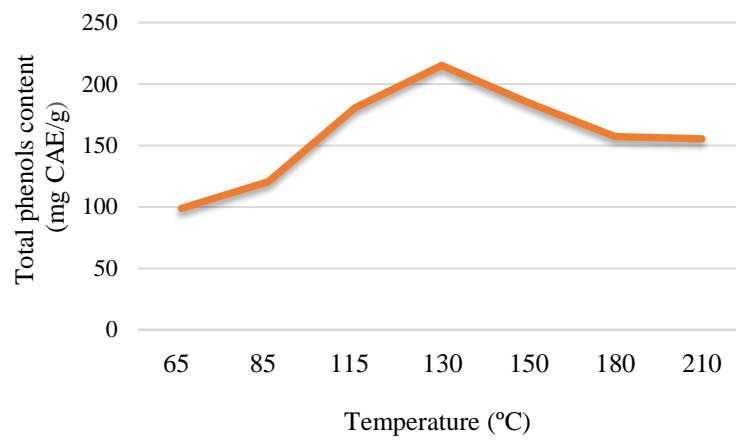


Fig.6. Temperature influence on the total phenols content in subcritical water extraction.

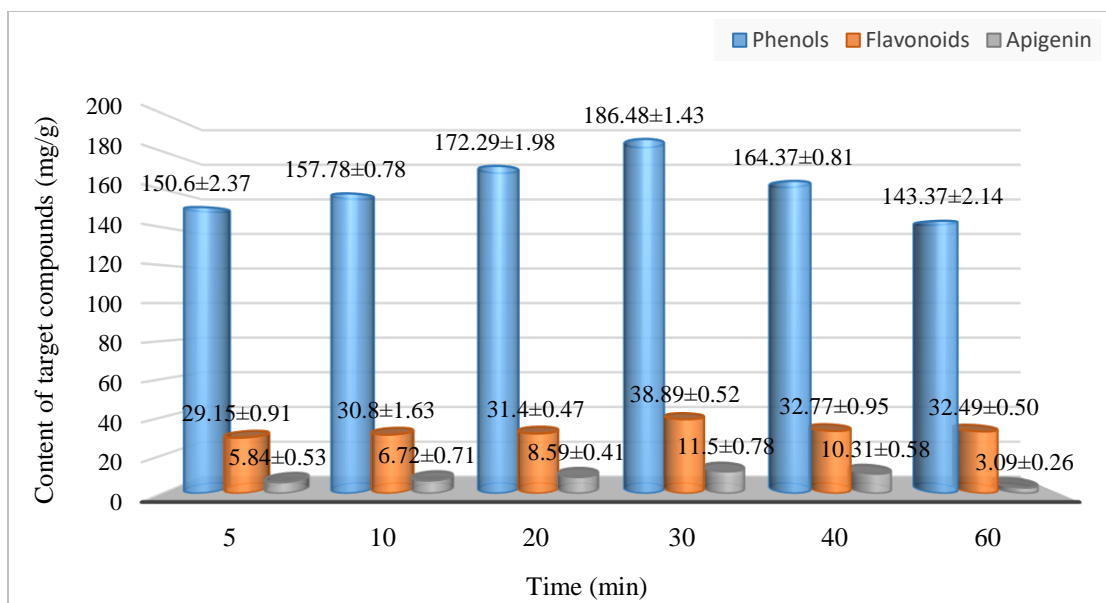


Fig. 7. Influence of the extraction time on total phenol, flavonoid and apigenin contents.

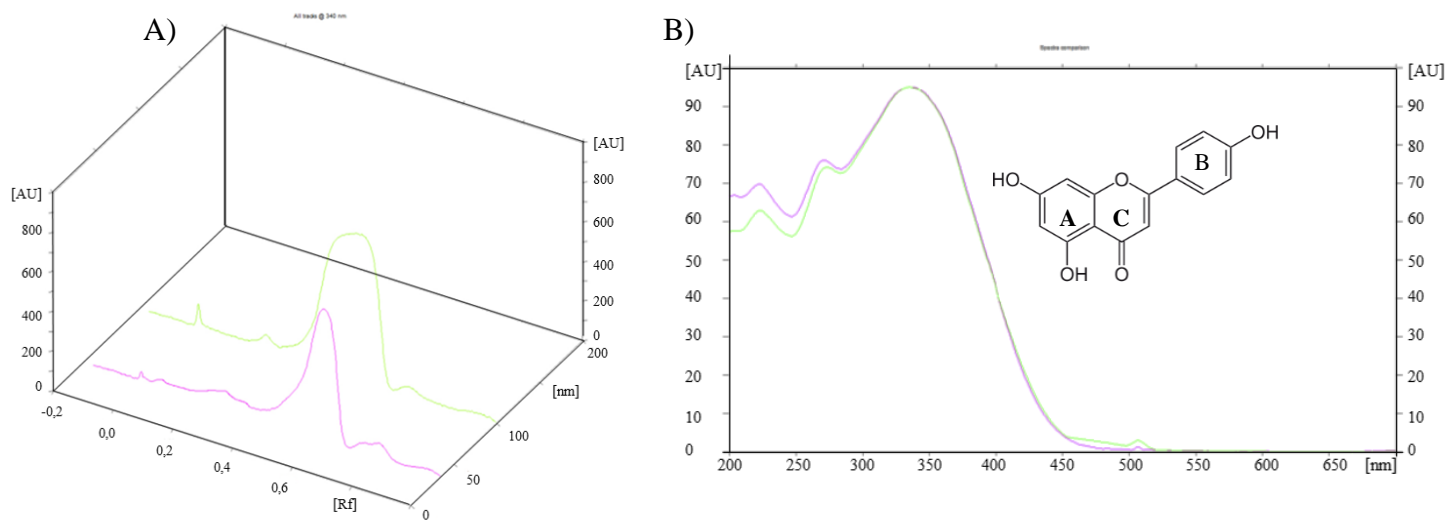


Fig. 8. A) 3D-UV and B) UV spectra of apigenin isolated from SCW extract and standard apigenin.

Table 1. The influence of the agitation rate on total phenol, flavonoid and apigenin contents

Vibration frequency (Hz)	Total phenols content (mg CAE/g)	Total flavonoids content (mg RE/g)	Apigenin content (mg/g)
2	96.66±1.55*	20.16±0.56	2.36±0.51
3	90.30±1.85	19.46±0.79	3.57±0.55
4	83.93±0.63	19.54±0.28	1.93±0.15
5	83.63±1.53	18.52±0.19	2.48±0.51
6	77.87±0.16	15.99±0.20	1.30±0.41

*±2SD

Table 2. The influence of the temperature on the yields of phenols, flavonoids and apigenin

Temperature (°C)	Total phenols content (mg CAE/g)	Total flavonoids content (mg RE/g)	Apigenin content (mg/g)
65	98.78±1.09*	14.72±0.4	3.33±0.32
85	120.60±1.76	20.96±0.09	3.18±0.23
115	180.60±1.58	36.96±0.77	10.90±0.62
130	215.15±0.84	36.66±0.45	8.03±0.15
150	184.84±4.83	34.48±0.33	5.60±0.31
180	157.27±5.88	34.36±0.26	3.48±0.4
210	155.45±2.43	33.93±0.87	3.03±0.06

*±2SD

Table 3. Biological activity of chamomile subcritical water extracts obtained under optimal extraction conditions

Antioxidant activity		
DPPH (mg/mL) ^a		Reducing power (mg/mL) ^a
0.0153±0.0005*		0.339±0.0062*
Enzyme activity		
α -amylase inhibition (mmol ACE/g) ^b	α -glucosidase inhibition (mmol ACE/g) ^b	tyrosinase inhibition (mg KAEs/g) ^c
0.45±0.03*	2.54±0.03*	11.38±0.54*

^amg of dry extract per mL;

^b mmol acarbose equivalent per g of dry extract;

^c kojic acid equivalent per g of dry extract.

* ± 2SD

Table 4. Phenolics identified in SCW extracts using UHPLC/(-) MS/MS analysis (tSRM experiment), mean expected retention times (t_R), mass of parent ions in a negative ion mode (m/z), masses of product ions (m/z) with specified collision energies (eV), and limits of detection (LOD) and quantification (LOQ).

Compound	t_R , <i>min</i>	Parent Ion, [M-H]⁻, m/z	Product Ions, [M-H]⁻, m/z (Collision Energy, eV)	LOD, mg/L	LOQ, mg/L	Yield (mg/kg)*
Aesculin	4.86	339.080	133.09 (44); 177.06 (25)	0.09	0.31	85.14
<i>p</i>-Hydroxybenzoic acid	5.20	137.057	93.19 (19); 108.33 (22)	0.16	0.54	496.83
Chlorogenic acid	5.31	353.103	191.28 (25)	0.06	0.21	24.33
Rutin	6.23	609.197	299.98 (42); 301.20 (32)	0.10	0.33	5.35
<i>p</i>-Coumaric acid	6.39	163.031	93.12 (39); 119.09 (16)	0.11	0.37	13.60
Hyperoside	6.40	463.002	271.01 (44); 300.02 (29)	0.10	0.32	4.69
Cynaroside	6.48	447.000	284.01 (40); 285.03 (27)	0.22	0.75	278.37
Ellagic acid	6.57	300.980	284.00 (32); 300.04 (30)	0.11	0.37	18.45
Apiin	6.73	563.056	267.98 (53); 269.01 (41)	0.04	0.12	4.67
Naringin	6.74	579.241	151.42 (43); 217.36 (33)	0.16	0.53	2.32
Sinapic acid	6.75	223.082	149.21 (36)	0.11	0.36	69.07
Astragalin	6.83	447.008	255.03 (43); 284.03 (29)	0.05	0.16	24.53
Apigetrin	6.92	431.004	239.11 (53); 268.03 (36)	0.01	0.03	69.90
Coniferyl aldehyde	7.50	177.060	97.00 (14); 162.00 (17)	0.06	0.21	2.30
Luteolin	8.17	285.035	133.05 (30); 150.95 (24)	0.06	0.21	97.19
Naringenin	8.92	271.036	119.10 (25); 151.07 (19)	0.14	0.46	7.87
Apigenin	8.92	269.032	117.24 (43); 149.00 (24)	0.10	0.32	1501.25
Kaempferol	9.02	285.074	211.00 (32); 227.00 (32)	0.25	0.84	60.37

*mg of observed compound per kg of dry extract