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# Multi-stage recovery of phytochemicals from buckwheat (*Fagopyrum esculentum* Moench) flowers by supercritical fluid and pressurized liquid extraction methods

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ABSTRACT

Supercritical fluid extraction with carbon dioxide and co-solvent ethanol (SFE–$CO_2$/ET) and pressurized liquid extraction (PLE) with the increasing polarity solvents, hexane (HX), acetone (AC), ethanol/water (ET/W) and water (W) were applied for recovery of bioactive substances from buckwheat (*Fagopyrum esculentum*) flowers (BWF). The products obtained were evaluated for their antioxidant capacity and phytochemical composition. Extract yields varied from 1.78 to 35.6% with the highest values for ET/W (21.7–35.6%), while the total yields were in the range of 37.02–64.05%. Comparing total phenolic content (TPC) and radical scavenging capacity in ABTS assay W extracts were weaker antioxidants than AC and ET/W extracts, while ORAC was in the range of 672–2114 μmol Trolox equivalents/g. The highest total TPC and TEAC was obtained using PLE at 140 °C, while the highest ORAC value was obtained using SFE–$CO_2$/PLE combination. Antioxidant activity indicators of solid plant substances were followed during extraction by using QUENCHER method. Lipophilic fractions were weaker antioxidants although they contained high concentrations of tocopherols, up to 392 μg/g dry weight of extract (DWE). Seven phytochemicals were quantified in BWF, rutin being the major constituent (70–110 mg/g DWE) followed by quercitrin (6.4–88.0 mg/g DWE) and citric acid (0.31–31.3 mg/g DWE). Extracts increased the oxidative stability of rapeseed oil and emulsion in Oxipres and Rancimat assays. High pressure fractionation schemes have never been applied to BWF previously: as a result new promising fractions have been obtained, which may serve as a good platform for the development of new industrial products.

## 1. Introduction

The search, evaluation and application of natural antioxidants and other phytochemicals has become one of the most popular topics among agricultural, nutritional, pharmaceutical and food scientists due to the following reasons: (i) increasing evidence of health effects of numerous plant origin natural compounds; (ii) rapid developments in functional foods, nutraceuticals, natural pharmaceuticals and cosmetics; (iii) vast diversity of various under investigated species in the plant kingdom; (iv) increasing consumer preference for natural ingredients and additives in foods and other products; (v) development of analytical techniques enabling fast and effective isolation and characterisation of natural compounds. The discovery of a very potent anti-malaria drug artemisinin, which was isolated from *Artemisia annua*, is one of the most exciting examples in the great success of natural product research (Tu, 2011).

Buckwheat is a Polygonaceae family gluten-free pseudocereal, which has been grown and used for food and medicine since ancient times. Common buckwheat (*Fagopyrum esculentum* Moench.) and tartary buckwheat (*F. tataricum* Gaertn.) are nowadays the most important commercially species. The cultivation of buckwheat declined sharply in the 20th century; however, nowadays it regains the popularity as a raw material for healthy foods. Bioactive compounds and biofunctional properties of buckwheat grain have been comprehensively reviewed: high quality proteins, resistant starch, dietary fiber, lignans, flavonoids, phytosterols, fagopyrins, fagopyritols, phenolic compounds, vitamins, minerals and antioxidants were reported as the vital constituents in buckwheat making it a highly valuable pseudocereal (Ahmed et al.,

*Abbreviations:* AA, antioxidant activity; AAPH, 2,2′–azobis (2–amidinopropane) dihydrochloride; ABTS, 2,2′–azino–bis(3–ethylbenzothiazoline–6–sulphonic acid; AC, acetone; BWF, buckwheat flowers; $CO_2$–ET, carbon dioxide ethanol; DWE, dry weight of extract; DWP, dry weight of plant material; EM, emulsion; ET/W, ethanol/water; GA, gallic acid; HX, hexane; IP, induction period; ORAC, oxygen radical absorbance capacity; PF, protectionfactor; PLE, pressurised liquid extraction; RMCD, randomly methylated β–cyclodextrin; RSC, radical scavenging capacity; RO, rapeseed oil; SET/HAT, single electron transfer/hydrogen atom transfer; SFE, supercritical fluid extraction; TEAC, trolox equivalents antioxidant capacity; TPC, total phenolic content; UPLC/ESI–QTOF–MS, ultra performance liquid chromatography–electrospray ionization–quadrupole time of flight–mass spectrometry; W, water

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2014; Giménez-Bastida, 2015; Zhang et al., 2012). Although grain-like seeds are the main edible part of buckwheat, phytochemical composition and antioxidant properties of its leaves, stems, flowers, roots, hulls and sprouts have also been studied, mainly from the beginning of the 21st century (Acar et al., 2011; Bystrickà et al., 2011; Dadáková and Kalinová, 2010; Hinneburg and Neubert, 2005; Holasova et al., 2002; Kim et al., 2008; Lee et al., 2014; Li et al., 2013; Liu et al., 2008; Peng et al., 2004; Quettier-Deleu et al., 2000; Sytar, 2014; Suzuki et al., 2009; Uddin et al., 2013; Zielińska et al., 2012). These studies resulted in the identification of various (poly)phenolics, mainly belonging to the classes of phenolic acids (ferulic, vanillic, chlorogenic, *p*-coumaric, *p*-anisic, salicylic, methoxycinnamic, vanillic, salicylic, *p*-anisic, 4-hydroxybenzoic, 4-hydroxy-3-methoxy benzoic), flavonoids (catechin, epicatechin, quercetin, kaempferol) and their glycosides (rutin, quercitrin) (Sytar, 2014; Sytar et al., 2014; Uddin et al., 2013). Squalene and α-tocopherol were reported in buckwheat leaves (Kalinova et al., 2006). However, rutin was reported as the most abundant flavonoid in buckwheat; its content in leaves and flowers was remarkably higher than in seeds (Zielińska et al., 2012). Antioxidant properties of buckwheat inflorescences has also been reported in several articles (Acar et al., 2011; Bystrickà et al., 2011; Hinneburg and Neubert, 2005; Holasova et al., 2001, 2002; Kim et al., 2008; Li et al., 2013; Liu et al., 2008; Quettier-Deleu et al., 2000; Sytar, 2014; Suzuki et al., 2009; Zielińska et al., 2012). For instance, Hinneburg and Neubert (2005) showed that the extract with good antioxidant activity, high content of phenolics, and low content of the phototoxic fagopyrin can be obtained by agitated maceration with 30% ethanol at 60 °C for 2 h.

However, previously performed studies applied conventional solvent extraction methods, using mainly highly toxic solvent methanol and were performed mainly for analytical purposes, while more comprehensive fractionation schemes of buckwheat flowering parts for recovery of valuable fractions, which could be used for upscaling to pilot and industrial production, have not been investigated until now. Moreover, to the best of our knowledge, high pressure methods such as supercritical fluid extraction with carbon dioxide (SFE–$CO_2$) and pressurized liquid extraction (PLE) have not been applied for the recovery of antioxidants and phytochemicals from buckwheat flowers previously, while the most recent study of *Bergenia crassifolia* roots and leaves indicates that the multistep processing approach provides promising results (Kraujalienė et al., 2016). So far as these methods possess several advantages, systematic studies of their application for other botanicals (in our case buckwheat flowers) in obtaining high value fractions with antioxidant and other bioactivities may be of great interest in the development of natural functional ingredients suitable for various applications including cosmetics, foods and pharmaceuticals.

The aim of the present work was to investigate various schemes of processing buckwheat flowering parts using high pressure extraction methods (SFE–$CO_2$ and PLE) and to evaluate antioxidant properties and phytochemical composition of the fractions obtained. Such a systematic approach is expected to provide more comprehensive data on antioxidant properties and the possibilities of obtaining new phytochemical and antioxidatively active fractions from BWF. The data obtained may be used for upscaling the production of new functional ingredients from buckwheat to industrial levels and for the preliminary prognosis of their possible health benefits.

## 2. Materials and methods

### 2.1. Chemicals and plant material

Buckwheat flowering parts (BWF) were collected in September 2013 in an organic herb farm located in Dzūkija National Park, Panara village (54°06′22″N, 24°06′40″E), Varėna district (Lithuania). The plants were dried at 30 ± 2 °C during 48 h by active ventilation using a solar collector for air heating and stored in the dark. Agricultural origin ethanol was from Stumbras (Kaunas, Lithuania), acetone (pure p. a., 99.5%), hexane (pure p. a., 99.0%) and methanol (pure p. a., 99.8%) from Chempur (Piekary Śląskie, Poland). Reference compounds, citric (ACS reagent, ≥99.5%) and chlorogenic (≥95%) acids, isoorientin (≥98%), myricitrin (≥99%), quercetin (≥98%), quercetin 3-D-galactoside (≥97.5%), quercetin 3-O-α-L arabinopyranoside (≥95%), quercitrin hydrate (≥95%), rutin (≥94%), ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid, ≥98%) and HPLC grade solvents used for chromatographic analyses were purchased from Sigma-Aldrich (Steinheim, Germany). Commercial refined, deodorised rapeseed oil (RO) "Tyras" without any added by the producer antioxidants was from Obelių Aliejus (Lithuania).

### 2.2. Sample preparation and extraction

Dried BWF were ground in an ultra-centrifugal mill ZM 200 (Retsch, Haan, Germany) using 0.2 mm hole size sieve. SFE–$CO_2$ was performed in a Helix extraction system (Applied Separation, Allentown, PA, USA) with 99.9% $CO_2$ (Gaschema, Jonava, Lithuania) from 10 g of ground material placed in a 50 $cm^3$ cylindrical extractor, 14 mm inner diameter and 320 mm length. Cotton wool was placed on the top and in the bottom of the extraction vessel. In all extractions $CO_2$ flow rate was kept constant, 2 L/min (standard conditions), static extraction time was 10 min. Extraction pressure and temperature (45 MPa, 60 °C) giving high extract yields were selected based on previously published data for various botanicals (Kemzūraitė et al., 2014; Šulniūtė et al., 2016; Mackėla et al., 2015) and set automatically by PC control, while extraction time was 30, 60 and 90 min. After completing the static extraction phase, the flow for dynamic extraction was set by the lever according to the flow meter reading. The $CO_2$ extracts were collected in glass vials and when the extraction was completed the vials were kept until constant weight to avoid $CO_2$ residues. The extracts were weighed and transferred to opaque bottles. In addition, SFE–$CO_2$ with 10% ethanol as a co–solvent was performed at 45 MPa pressure, 60 °C during 60 min, which was established as sufficient for exhaustive extraction. The extracts were kept at approx. 4 °C until further analysis.

PLE was performed as described previously (Kraujalis et al., 2013; Kraujalienė et al., 2016) in a Dionex ASE 350 system (Dionex, Sunnyvale, CA) both from the initial material and from the residues remaining after SFE–$CO_2$. Five g of material were mixed with diatomaceous earth (1/1) and placed in a 34 mL stainless–steel cells. The extraction was performed consecutively using the solvents of increasing polarity, namely hexane (used only for initial material for removing lipophilic substances), acetone, a mixture of ethanol/water (80/20, v/v) and water; which are further abbreviated as HX, AC, ET/W and W, respectively. It should be noted that hydroethanolic solvents have been traditionally used in the preparation of herbal medicines. Extraction time was 15 min, pressure 10.3 MPa, temperature 70 °C and 140 °C (further referred as $PLE_{70}$ and $PLE_{140}$). Organic solvents were removed in a rotary vacuum evaporator at 40 °C, while the residual water was removed in a freeze dryer. The extracts after solvent evaporation were kept under nitrogen flow for 20 min and stored in dark glass bottles at −18 °C.

Extract yields were determined both for the residues (DWR) and for the initial plant material (DWP). For the 1st extraction step the yields are similar because the process was applied directly to the initial material, while after further extraction steps, the yields were directly measured for the DWR by weighing, and afterwards the results obtained were recalculated for the DWP taking into account the reduction of plant mass after each extraction step.

### 2.3. Antioxidant capacity assays

#### 2.3.1. $ABTS^{\bullet+}$ scavenging

Trolox equivalent antioxidant capacity (TEAC) was measured by using $ABTS^{\bullet+}$ scavenging assay (Re et al., 1999). Firstly, phosphate buffered saline (PBS) solution (75 mmol/L; 7.4 pH) was prepared by