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ARTICLES

Influence of Extraction Parameters on the Phytochemical Characteristics of Extracts from Buckwheat (*Fagopyrum esculentum*) Herb

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In recent years, the interest in herbal medicinal products, especially in the field of dermatology and cosmetics, has risen enormously. Many plant-derived substances show photoprotective properties in terms of absorption of UV radiation and preventing photodamage to molecular structures of human skin. Modern phytopharmaceutics as well as phytocosmetics require standardized, defined extracts from the herbal matrix. Buckwheat herb is rich in flavonoids, which have been identified as potent antioxidants. Up to now, there have been no systematic investigations available concerning the extraction conditions for phenolic substances from buckwheat herb. In this paper, we report the influence of three extraction parameters, ethanol concentration, temperature, and extraction time, on the response variables extractable matter, antioxidant activity, and content of fagopyrin, rutin, and chlorogenic acid. Our results suggest that an extract with good antioxidant activity, a high content of phenolics, and a low content of the phototoxic fagopyrin can be yielded by agitated maceration with 30% ethanol at 60 °C for 2 h. Furthermore, there is good correlation between the antioxidant activity and the rutin content, whereas the extractable matter is not an appropriate parameter for extract quality. Huge differences in the content of rutin and chlorogenic acid when using herbal drugs from different suppliers confirm the demand of standardized procedures for the production of herbal drugs.

KEYWORDS: Buckwheat; *Fagopyrum esculentum*; flavonoids; extraction conditions

INTRODUCTION

In recent years, the interest in herbal medicinal products, especially in the field of dermatology, has risen enormously (*1*). Many plant-derived substances show photoprotective properties in terms of absorption of UV radiation and preventing photodamage to molecular structures of human skin (*2*). Buckwheat (*Fagopyrum esculentum* Moench) is traditionally used in venous diseases (*3*). It has a high content of phenolic substances such as flavonoids and phenolic acids (*4*). The main phenolics are rutin, chlorogenic acid, and hyperoside (**Figure 1**). Rutin, whose content in buckwheat herb is about 5−8%, has been shown to absorb UV radiation and to scavenge free radicals as superoxide anions, hydroxyl radicals, and peroxyl radicals (*5*), so an use in suncare products seems to be interesting. Hyperoside and chlorogenic acid were shown to have antioxidant activity (AA), too (*6*).

Modern phytopharmaceutics require standardized, defined extracts from the herbal matrix. The extraction procedure is important because the extraction conditions determine the quality and the yield of the individual constituents. Although a lot of innovative extraction techniques such as subcritical fluid extraction, microwave-assisted extraction, pressurized liquid extraction, and ultrasonic extraction have been developed during the past decade (*7*), classical agitated maceration at an elevated temperature is still prevalent in the pharmaceutical industry. There are only a few systematic investigations about the influence of solvent extraction parameters for drugs containing phenolic substances (*8*−*13*). The AA has been reported before for extracts from buckwheat hulls and flour (*14*−*16*) and buckwheat honey (*17*). Niesel could show that rutin is released completely from buckwheat herb by maceration with boiling water (herbal infusion) (*18*), but no publications are available that study the influence of extraction variables on the recovery of different constituents from buckwheat herb methodically.

The aim of this paper was to evaluate the influence of extraction parameter on the extract quality in a systematic way.

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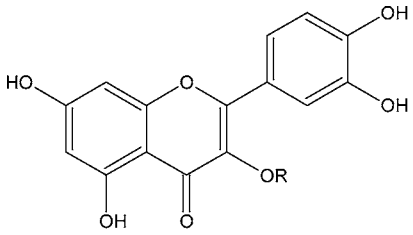
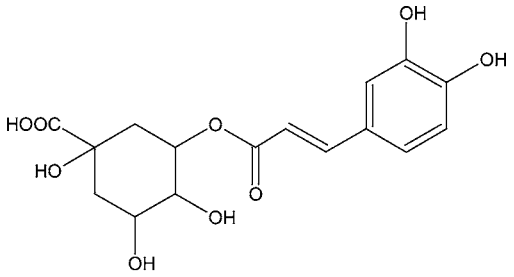


R = H quercetin
R = Glc-Rha rutin
R = Gal hyperoside
R = Rha quercitrine



chlorogenic acid

Figure 1. Most important phenolics occurring in buckwheat herb.

Table 1. Experimental Design for Three Variables

run	concn (%)	time (h)	temp (°C)	run	concn (%)	time (h)	temp (°C)
1	70	24	60	5	70	24	25
2	30	24	60	6	30	24	25
3	70	2	60	7	70	2	25
4	30	2	60	8	30	2	25

Therefore, a 2^3 factorial design was used. Temperature, solvent concentration, and extraction time are the most important factors that influence the extraction efficacy in terms of quality and yield. Mixtures of ethanol and water were chosen as nontoxic and environmentally friendly solvents, which have been shown to be effective in the extraction of quercetin glycosides (*10*). The determined response variables are representatives of the phytochemical characteristics of the prepared extracts. The extractable matter (EM) is the yield of the extraction process. Rutin and chlorogenic acid are the desirable components of the extract. They can be quantified by means of high-performance liquid chromatography (HPLC) analysis as has been demonstrated by Kreft and Krawczyk (*19*, *20*). In contrast, the fagopyrin content in the extract should be limited due to its phototoxic potential (*21*). In plant extracts, there are often more compounds than can be quantified individually by HPLC analysis. The AA of the extracts with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined to take into account the contribution of minor compounds to the overall AA of the extracts.

MATERIALS AND METHODS

Materials. Chemicals and reagents were obtained from the following commercial sources: rutin (Acros Organics, Geel, Belgium), DPPH and chlorogenic acid (Sigma, Deisenhofen, Germany), and acetic acid (Roth, Karlsruhe, Germany). Methanol (HPLC grade) was purchased from J. T. Baker (Deventer, Netherlands). Hypericin was a kind gift from W. Schwabe (Karlsruhe, Germany). All samples, solutions, and buffers were prepared from bidistilled water. The dried buckwheat herb was obtained from Agrargenossenschaft Calbe (Calbe, Germany) and from Caelo (Hilden, Germany). The respective drugs are further referred to as "Calbe drug" and "Caelo drug".

Preparation of Extracts. Five grams of the respective drug was macerated in a wather bad shaker with 100 mL of the respective solvent. The combinations of the extraction parameters can be found in **Table 1**. After the given time, the drug and the solvent were separated by filtration to obtain the fluid extract. The fluid extract was used to determine the EM. For freeze drying, ethanol was removed from the fluid extracts under vacuum and the remaining aqueous phase was submitted to the freeze dryer (Alpha 2-4, Christ, Osterode, Gemany) for 24 h at −30 °C and a pressure of 0.370 mbar. The freeze-dried extracts were stored in airtight bottles in the refrigerator.

Determination of Extractable Matter. An exactly weighed amount of fluid extract (approximately 2 g) in a Petri dish was kept in a drying oven for 3 h at 100−105 °C. After it was cooled in a desiccator for 1 h, the residue was weighed and the EM (%) was calculated as mass of residue/mass of fluid extract·100. The experiments were performed with eight aliquots of the fluid extract.

Determination of Fagopyrin Content. The freeze-dried extracts were solved in methanol at 2 mg/mL. After filtration, the absorbance at 590 nm was determined (UV/vis photometer Shimadzu 1202, Shimadzu Europa GmbH, Duisburg, Germany) in comparison to hypericine, the structure and UV spectrum of which is very similar to fagopyrin. The experiments were performed in triplicate.

DPPH Assay. The freeze-dried extract was solved in methanol at a concentration of 1 mg/mL. The DPPH concentration in methanol was 100 μg/mL. The measurement was done in microtiter plates (96 wells, BMG Labtechnologies, Offenburg, Germany) according to Fukumoto et al. (*22*). One hundred microliters of extract solution was mixed with 100 μL of DPPH, and the absorption was recorded after 10 min at 540 nm (microtiter plate reader Polar Star Galaxy, BMG Labtechnologies). The blank was a mixture of 100 μL of extract solution and 100 μL of methanol; the control was a mixture of 100 μL of DPPH and 100 μL of methanol. AA was calculated according to the following equation:

$$\text{AA}\ (\%) = 100 \cdot (A_c - A_s)/A_c \qquad (1)$$

where A_s is the absorbance of the sample and A_c is the absorbance of the control. The measurements were performed in triplicate.

HPLC Method. The content of rutin and chlorogenic acid in the freeze-dried extracts was determined by HPLC analysis with a Merck-Hitachi apparatus equipped with an autosampler AS 4000, interface D-6000A, pump L6200A, UV−vis detector L4250, and column oven Jetstream 2 plus. Twenty microliters of extract solved in methanol: water 50 + 50 (v/v) was injected onto a Eurospher-100 RP8 column (250 mm × 4 mm, 5 μm, Knauer, Berlin, Germany), which was held at 25 °C. Detection was at 324 and 350 nm. The mobile phase consisted of two components, (A) water−methanol−acetic acid (90:10:0.5, v/v) and (B) methanol−acetic acid (100:0.5, v/v), and followed the gradient program in **Table 2**. The mobile phase flow rate was 1.1 mL/min. The following phenolic substances were identified by comparing the retention time to that of reference substances: chlorogenic acid, rutin, hyperoside, and quercitrin. In this paper, only the data for rutin and chlorogenic acid are presented. The validation parameters can be found in **Table 3**.

Statistics. A 2^3 factorial design was chosen for investigating the effects of extraction conditions [ethanol concentration (X_1), extraction time (X_2), and temperature (X_3)] on dependent variables of the extract. All data are given as means ± standard deviations of two independent batches. Effects of the independent variables and their interactions were calculated as the differences between the means on the high and the low levels, respectively. The significance of the effects was evaluated by comparing their values to the confidence intervals based on the mean

Table 2. Gradient Profile Used in HPLC for Analysis of Buckwheat Extracts

program time (min)	mobile phase A (%)	mobile phase B (%)	program time (min)	mobile phase A (%)	mobile phase B (%)
0	90	10	19	0	100
6	60	40	32	0	100
9	60	40	33	90	10
10	65	35	40	90	10
18	65	35			

Table 3. Validation Parameters for the HPLC Analysis of Buckwheat Extracts

rutin	LOD (in μg/mL)[a]	0.5
	precision (in %)	2.59
	linearity	0.5–600 μg/mL, $r^2 = 0.999$
chlorogenic acid	LOD (in μg/mL)[a]	1.4
	precision (in %)	1.32
	linearity	1–100 μg/mL, $r^2 = 0.998$

[a] Limit of detection.

standard deviation for the respective response variables (*23*). Linear regression was performed with Microsoft Excel. For the comparison of drugs, statistical significance was determined by analysis of variance after logarithmic transformation of the data and Newman−Keuls post test. *P* values equal or less than 0.05 were considered significant (Graph Pad Prism 2.0, GraphPad Software Inc., San Diego, CA).

RESULTS AND DISCUSSION

Phenomena that may influence extract quality during the maceration process are solvent saturation or drug exhaustion (which both lead to a steady state), solvent selectivity due to composition and temperature, degradation of components, and many others. The results for the response variables are given in **Table 4**. The effect of the investigated parameters may be either independent or interactive for every single response variable. In the latter case, the respective parameters have to be considered in combination.

EM. For the EM, a general parameter measuring the yield of the extraction process, the effect of the three-factor interaction (TFI), is significant ($p < 0.05$). Looking to the results in more detail, it becomes clear that the extraction yield is higher at 60 °C. Increasing the extraction time is only relevant for the extraction at 25 °C, independent of the concentration used. This means that the steady state between drug and solvent is reached more quickly at the higher temperature because a longer extraction time does not influence the yield at 60 °C but only at 25 °C.

AA. For the AA, the three factor interaction is significant as well ($p < 0.01$). So at 60 °C, neither concentration nor extraction time influence the AA whereas at the lower temperature, the use of the lower concentration leads to a decrease with a longer extraction time. The AA against the DPPH radical is affected by the amount of reduceable substances. In buckwheat herb, these are flavonoids and phenolcarbonic acids as well as their degradation products, which are mainly hydroxybenzoic acids (*24*). As there is a decrease in AA with longer extraction time when using 30% ethanol, major degradation processes may occur as minor degradation should not have influenced the amount of reduceable substances to such an extent.

Fagopyrin. Both concentration and temperature as well as their interaction have a significant positive effect on fagopyrin content ($p < 0.001$). Further consideration of the results reveals that raising the temperature increases fagopyrin content when using 70% ethanol but not at the lower solvent concentration. This can be attributed to the lipophilicity of fagopyrin (*25*). Theurer et al. yielded similar results as these reported here when comparing the fagopyrin level in extracts from buckwheat herb prepared by extraction with water or 50% ethanol (*21*). Ethanol 30% seems to have such a poor selectivity for fagopyrin that the fagopyrin level cannot be increased by higher temperature. This result can be used for obtaining extracts with a low level of fagopyrin by using 30% ethanol as the extraction solvent.

Content of Rutin and Chlorogenic Acid. For rutin content, there are two significant interactions between concentration and extraction time ($p < 0.05$) and between concentration and temperature ($p < 0.001$). Raising the temperature to 60 °C leads to an increase of rutin content, which is higher for the lower solvent concentration. For the extraction with 30% ethanol, a longer extraction time yields a loss of rutin content. In the case of chlorogenic acid, there is only a negative significant effect for concentration ($p < 0.001$).

The results found for rutin content are consistent with findings of Baumgertel et al. They found a flavonol-3-O-β-heterodisaccharidase in buckwheat herb, which has a high activity up to a temperature of 55 °C and a methanol content of 33%. A higher temperature as well as a higher alcohol concentration decrease the enzyme activity, which leads to a better stability of rutin in solution (*26*). Liu et al. performed similar extraction experiments with St. John's wort (*Hypericum perforatum*), which contains partially the same flavonoids as buckwheat. They found that raising the temperature from 23 to 55 °C has a positive effect on the EM as well as on the recovery of quercetin glycosides (*8*). Interestingly, while extracting with a 50% ethanol−acetone combination at room temperature, they observed an increase of recovery up to an extraction time of approximately 480 min whereas at longer extraction times degradation of constitutents took place. In the results presented here, increasing the extraction time to more than 2 h did not lead to a higher yield. At room temperature, it even provoked degradation of phenolic substances. So a high level of rutin can be yielded by using 30% ethanol at 60 °C. Using the lower concentration solvent is also favorable for obtaining higher levels of the more water soluble

Table 4. Influence of Extraction Parameters on Phytochemical Characteristics[a]

run	EM (%)	AA (%)[b]	fagopyrin (%)	rutin (%)	chlorogenic acid (%)
1	1.48 ± 0.01	99.74 ± 0.36	0.16 ± 0.01	7.91 ± 0.34	0.29 ± 0.08
2	1.33 ± 0.01	99.99 ± 0.02	0.02 ± 0.003	7.77 ± 0.52	0.43 ± 0.04
3	1.47 ± 0.07	99.47 ± 0.25	0.19 ± 0.04	7.05 ± 0.52	0.28 ± 0.01
4	1.33 ± 0.00	100.00 ± 0.00	0.03 ± 0.001	8.07 ± 0.45	0.47 ± 0.06
5	1.30 ± 0.02	99.98 ± 0.03	0.11 ± 0.001	6.77 ± 1.30	0.37 ± 0.05
6	1.09 ± 0.01	86.89 ± 0.73	0.03 ± 0.001	1.03 ± 0.31	0.43 ± 0.01
7	1.16 ± 0.02	97.88 ± 2.89	0.07 ± 0.01	5.98 ± 1.18	0.38 ± 0.09
8	1.04 ± 0.01	94.42 ± 1.06	0.04 ± 0.05	2.42 ± 0.21	0.50 ± 0.02

[a] Data are given as means ± standard deviations of two independent batches. [b] AA against DPPH.

Table 5. Overview on Effects of the Independent Variables and Their Interactions[a]

	concn (X_1)	time (X_2)	temp (X_3)	$X_1 \times X_2$[b]	$X_2 \times X_3$[c]	$X_1 \times X_3$[d]	TFI
EM	**+27.15**	**+9.27**	**+45.94**	**+4.36**	**–8.16**	–1.90	**–3.24**
AA	**+18.96**	–6.21	**+24.07**	**+11.90**	**+6.82**	**–20.81**	**–11.23**
fagopyrin	**+44.35**	+0.25	**+17.59**	+3.05	–7.27	**+20.50**	–6.99
rutin	**+21.81**	+0.66	**+38.39**	**+8.23**	+2.37	**–26.55**	–1.99
chlorogenic acid	**–45.54**	–9.82	–18.75	+8.93	+3.57	–12.50	–0.89

[a] Data are given as a percentage of the total sum of effects. + and – indicate the direction of the effects. Significant effects are printed in bold ($p < 0.05$). [b] Interaction of concentration and time. [c] Interaction of time and temperature. [d] Interaction of concentration and temperature.

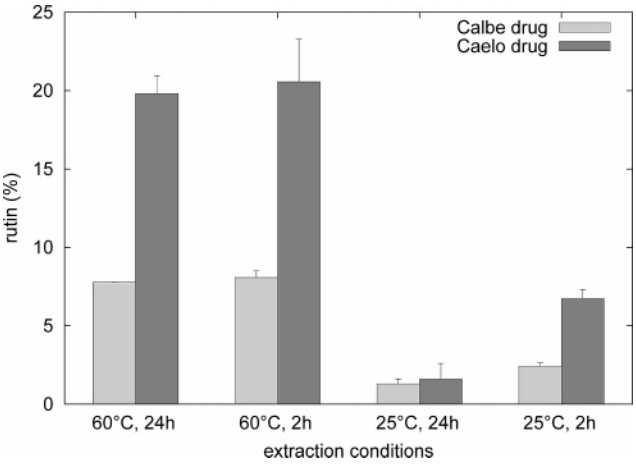


Figure 2. Influence of the drug used on the content of rutin. Extracts were prepared with 30% ethanol. Data are given as means ± standard deviations of two independent batches.

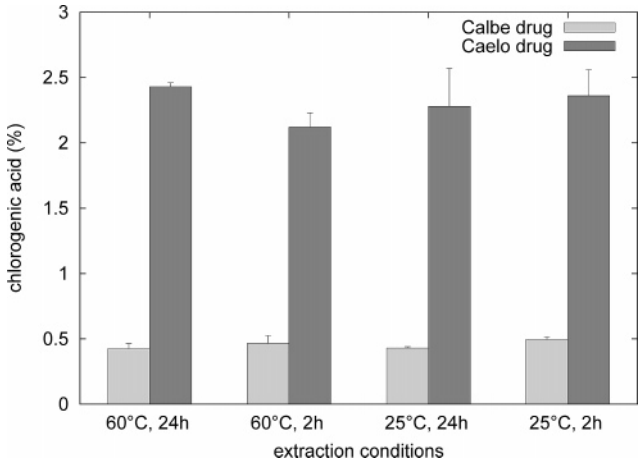


Figure 3. Influence of the drug used on the content of chlorogenic acid. Extracts were prepared with 30% ethanol. Data are given as means ± standard deviations of two independent batches.

chlorogenic acid. Quercetin glycosides seem to be more stable toward higher temperatures than anthocyanins for which a degradation has been reported by Cacace et al. when raising the temperature from 30 to 35 °C (*11*).

In **Table 5**, the percentages of individual effects on the total sum of effects are summarized. It is shown that only for the EM and the AA, TFIs are relevant. The interaction of the factors concentrations and temperature is significant for three of five response variables, and the respective percentage is in the same order of magniture as for the main factors. Overall, the experimental design revealed that an extract with a high content of phenolics, a low fagopyrin content, and good AA can be obtained by adjusting the extraction parameters as follows: ethanol concentration, 30%; temperature, 60 °C; and extraction time, 2 h.

The rutin content can be correlated well with AA of the extracts ($r^2 = 0.8682$) in contrast to chlorogenic acid content ($r^2 = 0.1522$). This probably can be attributed to the fact that rutin has a higher AA than chlorogenic acid (*5*). The amount of EM can be correlated neither with AA ($r^2 = 0.5109$) nor with rutin ($r^2 = 0.7154$) or chlorogenic acid content ($r^2 = 0.5516$). Consequently, the EM cannot be considered as a surrogate parameter for the extract quality. In contrast, a high content of rutin provides good AA of the extract.

Effect of Raw Material Used. To investigate the influence of the starting material, the experimental design was repeated partially (the respective runs with 30% ethanol) with a drug from another supplier. Rutin and chlorogenic acid content were chosen as response variables. The results (**Figures 2** and **3**) show that for chlorogenic acid, the yield is 5-fold higher when using the Caelo drug instead of the Calbe drug ($p < 0.001$). Regarding rutin content, the results are more complex. At 60 °C, the difference between the drugs is significant ($p < 0.05$) and the rutin content is higher for the Caelo drug. This difference is also present for the extraction conditions 25 °C/2 h, whereas for 25 °C/24 h no difference can be found between the drugs. This may be due to rutin degradation as suggested above, which affects the results more than the rutin content of the starting materials.

The surprising results for the differences between the starting materials used were further investigated by determining the rutin content in both drugs according to Hagels (*4*). Indeed, the results showed that the Calbe drug contained 6.35% rutin, whereas in the Caelo drug, 6.66% of rutin could be found. Further inquiries revealed that the supplier of the Calbe drug harvested the buckwheat plants too late, which can lead to a deterioration of rutin content (*27*). This confirms the necessity of standardization of plant material already in the state of seeding and harvesting as it is required by the Herbal Medicinal Products Working Party (HMPWP) (*28*). Although the difference in rutin content seems to be small, the extraction process leads to diverging quality of the respective extracts.

ABBREVIATIONS USED

AA, antioxidant activity; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EM, extractable matter; HMPWP, Herbal Medicinal Products Working Party; TFI, three-factor interaction.

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