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## Efficient extraction of flavonoids of sea buckthorn berries

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### ABSTRACT

Six different extraction methods were tested to establish an efficient method for extracting flavonoids from sea buckthorn (*H. rhamnoides* L. ssp. *rhamnoides*) berries. Twelve flavonoids were studied and HPLC was used to quantitate recovery. The content of total HPLC flavonoids was higher in acetone/petroleum ether extracts compared to other extraction methods. Acetone/petroleum ether extraction optimised recovery of quercetin derivative (1), isorhamnetin 3,7-diglucoside, isorhamnetin rhamnodi-glucoside, isorhamnetin 3-glucoside 7-rhamnoside, isorhamnetin-glucoside (3), quercetin derivative (3) and dicoumaroyl isoquercitrin compared to the other extraction methods. Acetone/petroleum ether, hexane, methanol and ethanol as extraction solvents gave similar concentration for five other compounds, while propanol/hexane and chloroform/methanol were not found to be useful extraction solvents due to very low extraction efficiency. These results indicate that the extraction of flavonoid compounds of sea buckthorn berries is maximized using acetone/petroleum ether extraction method. © 2011 Trade Science Inc. - INDIA

### KEYWORDS

HPLC;  
Flavonoids;  
Phenolic extraction;  
*Sea buckthorn*;  
Berries;  
Lipid-rich tissue.

### INTRODUCTION

Fruit of sea buckthorn (*Hippophae rhamnoides* L.) is a storehouse of anti-oxidants and other potentially bioactive substances. The berries contain ascorbic acid (30-2750 mg 100 g<sup>-1</sup>), tocopherols (50-250 mg 100 g<sup>-1</sup> of oil) and carotenoids (1-120 mg 100 g<sup>-1</sup>)<sup>[1]</sup>. Sea buckthorn also produces a wide variety of flavonoids (168-859 mg 100g<sup>-1</sup>) particularly flavonol glycosides of isorhamnetin, quercetin and kaempferol, together with high concentrations of condensed tannins<sup>[1-4]</sup>.

Accurate determination of the levels of flavonoids is limited by their extractability, which is determined by

the structure and solubility of the compounds of interest and by the chemical and physical characteristics of the plant tissue. It is estimated that approximately 30 % of the errors in analytical measurements are caused by the sample preparation<sup>[5]</sup>. When analysing sea buckthorn berries, the berry oils must be removed from the sample. If the berry oils and other lipophilic compounds are not removed, they are co-extracted with more polar compounds such as phenolics, but the extract forms a two phase systems which limits recovery of the analytes of interest. Several solvents, such as acetone, chloroform, ethanol, ethyl acetate, hexane, methanol, propanol, water and their combinations have been used

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to extract phenolics from sea buckthorn berries, but the efficiency of extraction has not been compared<sup>[2,6-10]</sup>. Because of large variability in methods and results reported, this study was established to optimize phenolic extraction from sea buckthorn berries.

### EXPERIMENTAL

#### Study field

The Finnish sea buckthorn cultivars Terhi, Tytti and Tarmo used in this study descended from wild sea buckthorns originating in the Baltic Sea region in Finland<sup>[11]</sup>. The saplings were grown at the study field in a coastal area in Merikarvia, western Finland (61°52'N, 21°30'E), see experimental design of the field in<sup>[12]</sup>. Berry samples were collected from sea buckthorn bushes at the end of the growing season, in 2006. Fresh berries were stored at -20°C.

#### Sample preparation

Seeds were separated from the icy berries by small forceps. Berries (80 berries) were then freeze-dried and crushed with liquid nitrogen in a mortar. On average 36 mg of powder from freeze-dried powder was extracted using each extraction solvents and protocol.

#### Chemical analyses

Six different solvent systems were tested for phenolic extraction from sea buckthorn berries. Four replicate analyses were carried out on each extract. In addition, a recovery test was carried out for the acetone/petroleum ether extraction method to evaluate whether the concentration of phenolic compounds decreased during the extraction.

#### Preparation of plant extracts

The solvents systems were: I) hexane II) methanol III) ethanol IV) aqueous acetone/petroleum ether V) propanol/hexane (2:1; v/v) VI) chloroform/methanol (2:1; v/v).

Solvent systems I, II, III, V, and VI. The berries were homogenized with the glass rod (using it as a pestle) for 5 minutes in 600 µl of solvent. Samples were then centrifuged at 11 500 g for 3 minutes. The extract was collected, and the residues were re-extracted two more times (1×5 min and 1×5 s) in 600 µl of solvent.

#### Removal of berry oil

Solvent systems I, V, and VI. The entire extract, a homogenous solution, was used for HPLC to determine whether nonpolar solvents extract flavonoids in addition to lipids.

#### Flavonoid extraction

Solvent systems I, V, and VI. The residues were air dried and homogenized with the glass rod for 5 minutes in 600 µl of methanol and centrifuged at 11 500 g for 3 minutes. The extract was collected, and the residues were re-extracted two more times (1×5 min and 1×5 s) with 600 µl of methanol.

Solvent systems I, II, III, V, and VI: all extracts were combined, and solvent was evaporated off under nitrogen. For analyses with high performance liquid chromatography (HPLC), the residue was re-dissolved in 600 µl of methanol.

#### Aqueous acetone/petroleum ether extraction

Solvent system IV. The berries were homogenized with glass rod for 5 minutes in 600 µl of acetone/water (3:1; v/v). Samples were then centrifuged as mentioned earlier. The extract was collected, and the residues were re-extracted two more times (1×5 min and 2×5 s) 600 µl of acetone/water. All extracts were combined, and acetone was evaporated under nitrogen. The sea buckthorn oil was removed from the extract by washing the sample three times with 600 µl of petroleum ether (it was removed and used for HPLC to determine whether nonpolar solvents extract flavonoids in addition to lipids). For analyses with high-performance liquid chromatography (HPLC), the samples were dissolved in 500 µl of methanol and water up to 1.5 ml.

#### Recovery test for aqueous acetone / petroleum ether extraction method

The berries, (freeze-dried and powdered, four replicates, 30 mg each) were homogenized with glass rod for 5 minutes in 600 µl of acetone/water (3:1; v/v). The sample was then centrifuged at 11 500 g for 3 minutes. The extract was collected, and the residues were re-extracted three more times (1×5 min and 2×5 s) 600 µl of acetone/water. All extracts were combined, and acetone was evaporated off under nitrogen. The sea buckthorn oil was removed from the residue by

washing three times with 600  $\mu$ l of petroleum ether, which was removed by pipette and the rest was evaporated under nitrogen. The extract was dissolved in water up to 1.0 ml.

- (1) For the (HPLC) control, 180  $\mu$ l of extract was re-dissolved in 500  $\mu$ l of methanol and water up to 1.5 ml.
- (2) For recovery tests another 180  $\mu$ l of extract was re-dissolved in 450  $\mu$ l of acetone and homogenized with glass rod for 5 minutes. Sample was then centrifuged at 11 500 g for 3 minutes. The extract was collected, and the residues were re-extracted three more times (1 $\times$ 5 min and 2 $\times$ 5 s) 600  $\mu$ l of acetone/water (3:1; v/v). All extracts were combined, and acetone was evaporated off under nitrogen. The sea buckthorn berry oil was removed from the extract by washing the sample three times with 600  $\mu$ l of petroleum ether, which was removed by pipette and the rest was evaporated under nitrogen. For analyses with high-performance liquid chromatography (HPLC), the samples were dissolved in 500  $\mu$ l of methanol and water up to 1.5 ml.

The concentrations of flavonoids in recovery tests (2) were compared to flavonoids in (HPLC) control (1).

### HPLC analyses

The flavonoids were analyzed by HPLC. The system used was an Hewlett-Packard (Avondale, Pennsylvania) instrument with a quaternary pump (HP 1050), an autosampler (HP 1050), and a photo diode array detector (HP 1040A) controlled by HP Chem Station Software. A 3- $\mu$ m HP Hypersil ODS column (60 $\times$ 4.6 mm ID) was used. The gradient elution systems consisted of aqueous 1.5 % tetrahydrofuran and 0.25% o-phosphoric acid (=A) and 100% methanol (=B). The samples were eluted as follows: 0-5 min 100% A; 5-10 min 85% A, 15% B; 10-20 min 70% A, 30% B; 20-30 min 65% A, 35% B; 30-50 min 50% A, 50% B; 50-55 min 100% B; 55-60 min 100% A. The flow rate was 2 ml/min. The injection volume was 20  $\mu$ l. The injector and column temperature were 22 and 30 $^{\circ}$ C, respectively. The flavonoids were identified using their retention times and the UV-spectra and HPLC-MS (see Heinaaho et al., in press). For HPLC-MS eluent A contained 0.25% of acetic acid instead of o-phosphoric

acid, and a 3- $\mu$ m HP Hypersil ODS column (100 x 2.1 mm ID) was used. Other conditions were as reported in Julkunen-Tiitto and Sorsa<sup>[13]</sup>.

The HPLC runs were monitored at 320 nm. Analyzed secondary metabolites were quantified against commercial standards: quercetin 3-galactoside (Roth, Karlsruhe, Germany) for isorhamnetin derivatives and quercetin derivatives.

### Statistical analyses

The data from this experiment was analyzed using appropriate models of analysis of variance (ANOVA). To meet the requirements of ANOVA, if the chemical data were not normally distributed, they were log- or square root-transformed. The multiple comparisons with extraction methods were made using Bonferroni correction test. The ANOVAs were analyzed with SPSS 16.0.1 for Windows (SPSS Inc., Chicago, IL, USA, 2007).

## RESULTS AND DISCUSSION

In this study, the berries of sea buckthorn cultivar Tytti were used to compare six methods for extraction of berry flavonoids. The concentrations of twelve compounds were compared using HPLC with UV detection.

The concentration of total HPLC flavonoids was highest in acetone extracts that were delipidified with petroleum ether. This method of extraction was more effective than extraction with hexane ( $P < 0.01$ ), propanol/hexane or chloroform/methanol extraction solvents ( $P < 0.001$ ) (Figure 1). The concentrations of six out of twelve individual compounds [such as quercetin derivative (1), isorhamnetin 3,7-diglucoside,

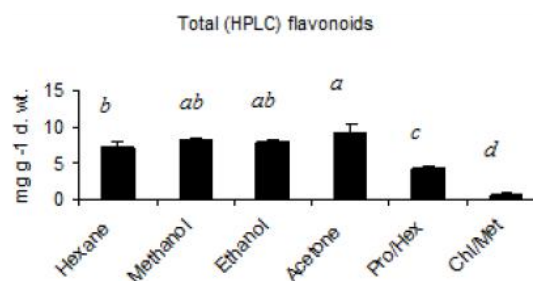


Figure 1 : The sum of total (HPLC) flavonoids with six different extraction methods. The bars denote means of  $\pm$  standard deviation (SD). The bars with same letters has no statistical difference between each other

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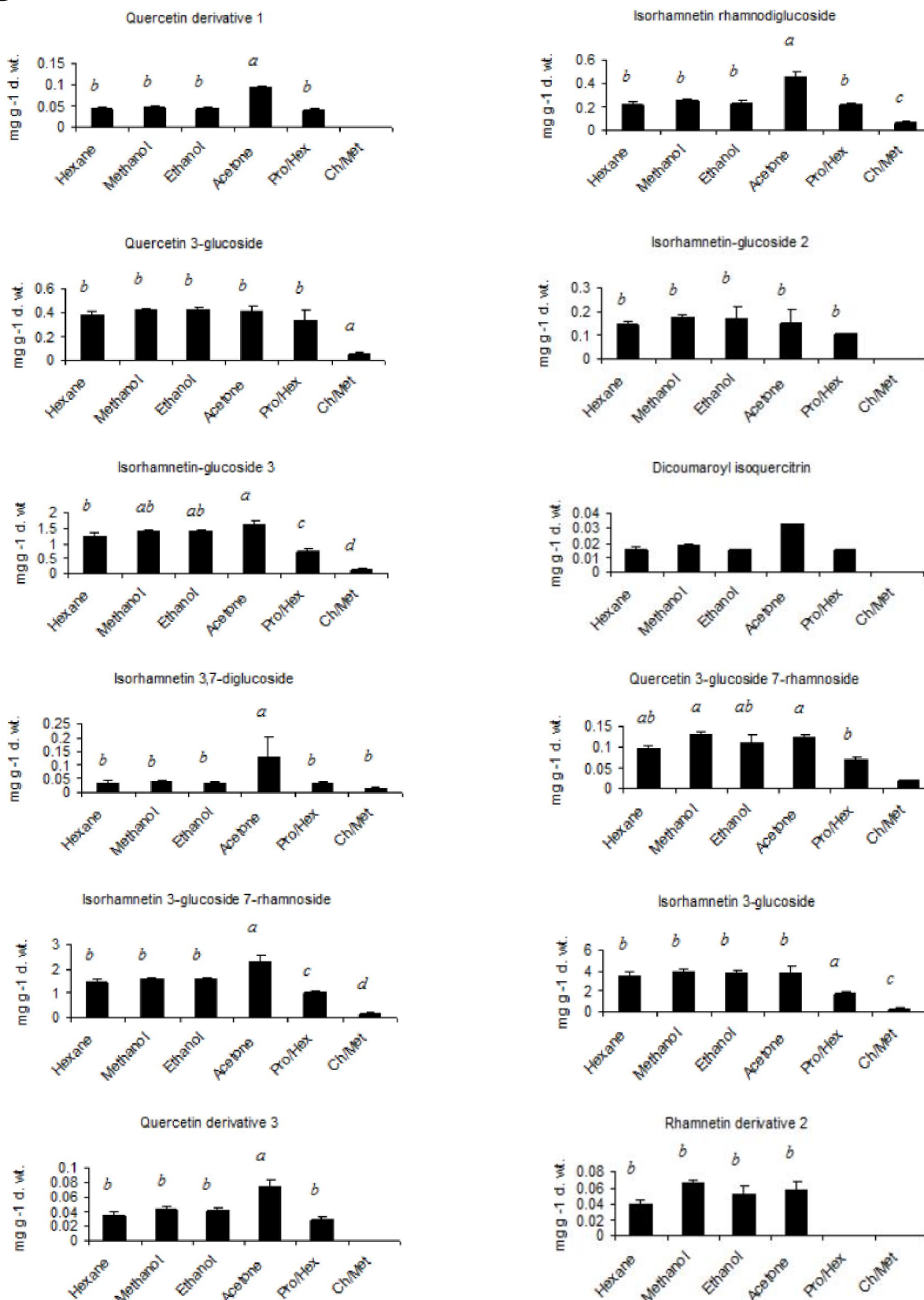


Figure 2 : The concentrations of flavonoids with six different extraction methods. The bars denote means of  $\pm$  standard deviation (SD). The bars with same letters have no statistical difference between each other. In cases of quercetin 3-glucoside 7-rhamnoside and dicoumaroyl isoquercitrin, the bars without letters have had too few results for bonferroni correction test



isorhamnetin rhamnoglucoside, isorhamnetin 3-glucoside 7-rhamnoside, quercetin derivative 3, ( $P < 0.001$ ) and dicoumaroyl isoquercitrin ( $P = 0.001$ ) were higher in the acetone/petroleum ether extracts compared to the other extraction solvents (Figure 2). For isorhamnetin-glucoside 3 methanol and ethanol were as effective as was acetone/petroleum ether. However, its concentration was higher in these solvents compared to propanol/hexane and chloroform/methanol extraction solvents ( $P < 0.001$ ). For other five, quercetin 3-glucoside 7-rhamnoside, quercetin 3-glucoside, isorhamnetin-glucoside 2, isorhamnetin 3-glucoside or rhamnetin derivative 2, hexane, methanol and ethanol were as effective as acetone/petroleum ether ( $P > 0.05$ ).

The other extraction methods, such as chloroform/methanol or propanol/hexane, removed high concentrations of flavonoids into an oil-rich fraction. For example, the concentration of isorhamnetin 3-glucoside 7-rhamnoside in propanol/hexane berry oil fraction was  $21 \pm 2\%$ , and in chloroform/methanol berry oil fraction  $60 \pm 1\%$  of the concentration of same flavonoids in acetone/petroleum ether extract. On the other hand petroleum ether berry oil fraction had only  $0.6 \pm 0.04\%$  of isorhamnetin 3-glucoside 7-rhamnoside, and  $0.8 \pm 0.18\%$  of isorhamnetin 3-glucoside, and hexane oil fraction  $0.5\%$ , of the concentration of acetone/petroleum ether extract. It has been earlier found that oil extraction with hexane decreased the concentrations of phenolic compounds by 30-40%<sup>[14]</sup>. It has been isolated sea buckthorn oils also successfully using chloroform/methanol, but in this study did not analyze phenolic compounds<sup>[15]</sup>. Our results indicate that in addition to removing sea buckthorn lipids, chloroform/methanol and propanol/hexane extract significant amounts of flavonoids, too. Petroleum ether removes only a small amount of flavonoids and is found to be suitable solvent for oil removal<sup>[16]</sup>.

Hakkinen<sup>[17]</sup> extracted flavonoid glycosides from sea buckthorn berries in 50% methanol/water (v/v). We found methanol, hexane or ethanol extracted phenolics, but the sea buckthorn berry oil was difficult to remove from the sample. After evaporation under nitrogen there was a small drop of berry oil left in the sample vial, making it difficult to prepare the sample for

**TABLE 1 : Recovery test for acetone/petroleum ether extraction method**

Compound group	Recovery (%)
Quercetin derivative 1	$92 \pm 6$
Isorhamnetin 3,7-diglucoside	$93 \pm 9$
Isorhamnetin rhamnoglucoside	$90 \pm 5$
Quercetin 3-glucoside 7-rhamnoside	$81 \pm 4$
Quercetin 3-glucoside	$91 \pm 4$
Isorhamnetin 3-glucoside 7-rhamnoside	$92 \pm 5$
Isorhamnetin-glucoside 2	$101 \pm 3$
Isorhamnetin 3-glucoside	$91 \pm 5$
Isorhamnetin-glucoside 3	$89 \pm 5$

#### HPLC.

Recovery of phenolic compounds should be more than 70%, to ensure reasonable estimation of phenolic content<sup>[16,17]</sup>. In our experiment, the recovery of compounds was determined using the oil-purified flavonoid extracts of the berries and measures recovery of individual flavonoids after acetone/petroleum ether extraction and HPLC-analyses. There was marked variation in recovery-% among different compounds (TABLE 1). Quercetin 3-glucoside 7-rhamnoside and isorhamnetin-glucoside 3 had the lowest recoveries ( $81 \pm 4\%$  and  $89 \pm 5\%$ ), respectively. Other compounds had more than 90% recoveries indicating that the concentration of phenolics do not reduce significantly during the acetone/petroleum ether extract.

In conclusion, we tested six extraction methods to find an accurate method to analyse the concentrations of flavonoids in sea buckthorn berries. The concentration of total HPLC flavonoids was highest when acetone was used to extract flavonoids, and the extracted material was then defatted by extraction with petroleum ether. With individual compounds, seven out of twelve compounds gave the highest concentrations of phenolics in the acetone/petroleum ether extract. With five compounds the concentrations did not differ from hexane, methanol or ethanol extracts. It was difficult to remove sea buckthorn berry oil from the sample without losing the flavonoids. The oil was left in the sample with hexane, methanol and ethanol extracts, which made HPLC analysis difficult. However, this problem was not faced using acetone with petroleum ether to defat the samples as the extraction solvent.

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