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Simultaneous determination of flavonols and phenolic acids by HPLC-CoulArray in berries common in the Nordic diet

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Chemical compounds studied in this work:
Gallic acid (PubChem CID: 370)
Vanillic acid (PubChem CID: 8468)
Caffeic acid (PubChem CID: 689043)
p-coumaric acid (PubChem CID: 637542)
Ferulic acid (PubChem CID: 445858)
Myricetin (PubChem CID: 5281672)
Quercetin (PubChem CID: 5280343)
Isorhamnetin (PubChem CID: 5281654)
Kaempferol (PubChem CID: 5280863)

ABSTRACT

In this study we evaluated and applied an HPLC coulometric array (HPLC-CoulArray) method for simultaneous determination of flavonols and phenolic acids in bilberry, lingonberry, cloudberry and sea-buckthorn berry. Berry samples purchased from local supermarkets were freeze-dried, hydrolysed and extracted with 50% aqueous methanol containing TBHQ antioxidant and 1.2 M HCL, and analysed with HPLC-CoulArray. We found that phenolic profiles differed between berries. Quercetin was found in almost all berries, with the highest concentrations observed in lingonberry. Myricetin and isorhamnetin were only detected in bilberry and sea-buckthorn, respectively. Hydroxycinnamic acids (caffeic, ferulic, *p*-coumaric) were also detected in all berries, but hydroxybenzoic acids (gallic, vanillic) were not found in sea-buckthorn. Total concentration of phenolic compounds was highest in sea-buckthorn berry (270.5 mg/100 g DW), followed by bilberry (253.6 mg/100 g DW), lingonberry (219.7 mg/100 g DW) and cloudberry (121.7 mg/100 g DW).

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1. Introduction

The traditional Nordic diet includes local seasonal food items with high nutritional value and potential beneficial effects on human health (Bere & Brug, 2009; Flint et al., 2009; Higdon, Delage, Williams, & Dashwood, 2007). A number of recent studies have investigated the beneficial effects of different healthy Nordic diets (Adamsson et al., 2012; Mithril et al., 2013; Olsen et al., 2011; Uusitupa et al., 2013). The diets tested have typically included fibre-rich foods such as whole grain oat and rye, nuts, cabbage, pears, apples, root vegetables, vegetable oils, fish, low-fat dairy products and berries (Nordic Council of Ministers, 2008; Bere & Brug, 2009; Olsen et al., 2011).

Berries (wild or cultivated) are an essential part of the traditional Nordic diet and an important component of the healthy Nordic diets investigated recently. Bilberries, lingonberries, cloudberries and sea buckthorn berries are abundant wild fruits in the Nordic region and easily available for consumption.

Consumption of sufficient amounts of berries is recommended as part of a healthy diet for prevention of chronic disease (Expert Consultation on Diet, Nutrition, and the Prevention of Chronic Diseases, Weltgesundheitsorganisation, & FAO, 2003), due to their high content of dietary fibre, vitamins C, E, K, folate and polyphenols, carotenoids, phytosterols and other bioactive compounds (Nordic Council of Ministers, 2008). The naturally occurring flavonoids are the most abundant and common group of polyphenols and secondary metabolites in berries (Yao et al., 2004) and derive from phenylalanine and tyrosine. The carbon ring skeleton of all flavonoids is C6–C3–C6, but based on degree of hydroxylation, substitution and

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conjugation they are allocated to different subclasses, namely anthocyanins, flavonols, flavones, isoflavones, flavanones and monomeric, oligomeric and polymeric flavanols (Santos-Buelga & Williamson, 2003; Yao et al., 2004). Phenolic acids are the other important class of phenolic compounds in berries and include two fundamental structures of hydroxycinnamic acids (HCAs) and hydroxybenzoic acids (HBAs) with a carbon ring skeleton of C6–C3 and C6–C1, respectively. Phenolic acids exhibit strong antioxidant activity, which is related to the number and position of hydroxyl groups in the carbon ring (Khoddami, Wilkes, & Roberts, 2013; Pereira, Valentão, Pereira, & Andrade, 2009).

There is growing evidence that fruit (including berries) and vegetable consumption is related to lowered incidence and mortality from cardiovascular disease and, less consistently, from cancer (Hollman & Katan, 1999; Wang et al., 2014; Ivey, Hodgson, Croft, Lewis, & Prince, 2015).

Flavonols and phenolic acids occur in bound form with different sugars, free acids and plant cell walls (Azar, Verette, & Brun, 1987). Therefore extraction and hydrolysis procedures play an important role in release and separation of these compounds from matrices. Temperature, acid concentration and appropriate solvents are among the most important factors determining the effectiveness of flavonoid extraction yield and hydrolysis (Aaby, Grimmer, & Holtung, 2013).

In order to evaluate the impact of berries and their associated bioactive compounds on human health, determination of their chemical content is essential. Hence, simple and reliable extraction and separation methods for identification and quantification of phenolic compounds in different berries are required and food composition data are needed. In recent years, various HPLC-based methods using different detectors such as UV, DAD and MS have been used to analyse flavonoids and other phenolic compounds in different types of berries (Mikulic-Petkovsek, Slatnar, Stampar, & Veberic, 2012; Veberic, Slatnar, Bizjak, Stampar, & Mikulic-Petkovsek, 2015). HPLC combined with coulometric array detection (HPLC-CoulArray) has not yet been fully exploited for such analysis, but possesses great potential due to its high sensitivity, selectivity and production of suitable chromatograms for fingerprinting (Guo, Cao, Sofic, & Prior, 1997; Roy et al., 2002; Jandera et al., 2005; Beňová & Hájek, 2010). Nearly all previous studies of phenolic compounds in berries undertaken in the Nordic countries have examined Finnish berries, while studies on the content and composition of phenolic compounds in berries consumed in Sweden are still lacking. Therefore, the aim of the present study was to optimise extraction and hydrolysis methods and evaluate and apply HPLC-CoulArray for simultaneous determination of flavonol (quercetin, myricetin, kaempferol, isorhamnetin) and phenolic acid (gallic, vanillic, ferulic, *p*-coumaric, caffeic) aglycones in four berry species commonly consumed in Sweden.

2. Materials and methods

2.1. Materials

Frozen berry products comprising bilberry (*Vaccinium myrtillus*), lingonberry (*Vaccinium vitis-idaea*), cloudberry (*Rubus chamaemorus*) and sea-buckthorn (*Hippophae rhamnoides* L.) were purchased from major supermarket chains in Uppsala, Sweden. The use of store-purchased berries instead of obtaining berries from the cultivars grown under known geographic and meteorological conditions would best reflect what will be used by consumers in Nordic countries. In order to estimate intakes, such data is needed. For each berry type, three product brands were selected and for each product brand, three packages from different supermarkets were purchased. Berries (100 g) from each package were lyophilised at −110 °C for 24–96 h (Scanvac Coolsafe, Labogene, Denmark) and the freeze-dried samples were then powdered using liquid nitrogen and kept at −20 °C until analysis. Organic freeze-dried bilberry powder was received from Immun (Sweden) and kept at −20 °C before use in method optimisation.

2.2. Chemicals and standards

Hydrochloric acid 37%, *ortho*-phosphoric acid 85%, HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Milli-Q water was purchased from Millipore, sodium dehydrate phosphate (NaH_2PO_4 98%) from Prolabo, VWR, ascorbic acid ≥99%, *tert*-butylhydroquinone (TBHQ) 97%, quercetin dehydrate 98%, resveratrol, ferulic acid, monohydrate gallic acid, caffeic acid, vanillic acid and *p*-coumaric acid were purchased from Sigma-Alderich (Steinheim, Germany). Kaempferol, isorhamnetin and myricetin were purchased from Fluka, France. All chemicals were of analytical grade.

2.3. Preparation of standards and calibrations

Stock solutions were prepared by dissolving compounds in methanol at a concentration of 1 mg/mL and stored at −80 °C before use. Working standard solutions were prepared in the concentration range 100–7000 ng/mL for seven-point calibration curve and method validation assays. External standard calibration curves were set up for bilberry and cloudberry and a standard additional calibration technique for lingonberry and sea-buckthorn was conducted based on a pooled sample of each of these two berry types from all brands and supermarkets.

2.4. Extraction and hydrolysis

Phenolic acids (hydroxybenzoic, hydroxycinnamic) and flavonols were extracted and hydrolysed to their aglycones as described previously (Hertog, Hollman, & Venema, 1992; Häkkinen, Kärenlampi, Heinonen, Mykkänen, & Törrönen, 1999) with slight modifications. In brief, lyophilised berry samples (0.5 g) were dissolved in aqueous methanol (40 mL, 62.5%) containing *tert*-butylhydroquinone (TBHQ) (2 g/L). Hydrochloric acid (HCL) (10 mL, 6 M) was added to the solution and bubbled with nitrogen for 30 s. Two extraction conditions were evaluated using freeze-dried bilberry powder. In the first, the bilberry powder was heated at 85 ± 2 °C for 2 h in a shaking water bath (Memmert, Germany), while in the second it was heated with reflux at 85 ± 5 °C for 2 h. The extracts were then allowed to cool in a dark box (1 h), methanol (50 mL) was added and the samples were sonicated (5 min). Extract (2 mL) was filtered with a 0.45-μm filter specifically designed for organic solvents (GHP membrane, Pall Corporation, USA) and analysed by HPLC-CoulArray detection as described below.

In addition, two concentrations of HCl (0.6 M, 1.2 M) and two different antioxidants (ascorbic acid, TBHQ) were tested in a hydrolysis and extraction method in a shaking water bath to evaluate their influence on yield of bilberry extract. In all steps, the samples were kept in darkness to avoid the risk of oxidation. All samples were prepared and analysed in triplicate.

2.5. Determination of flavonols and phenolic acids by HPLC-CoulArray

Sample extract (15 μL) was injected using a Midas auto-sampler (Holand Spark, Switzerland) into two LC-10 AD Shimadzu pumps coupled to an ESA 5600A coulometric detector (ESA Inc., Chelmsford, MA, USA) with eight porous graphite electrodes. The separation was conducted at room temperature on an ODS-3 Intersil

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