Betalains and Phenolics in Red Beetroot (*Beta vulgaris*) Peel Extracts: Extraction and Characterisation

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The extraction of red beetroot (*Beta vulgaris*) peel betalains and phenolics was compared with two extraction methods and solvents. The content of total phenolics in the extracts was determined according to a modification of the Folin-Ciocalteu method and expressed as gallic acid equivalents (GAE). The profiles of extracts were analysed by high-performance liquid chromatography (HPLC). The compounds of beetroot peel extracted with 80% aqueous methanol were characterised from separated fractions using HPLC- diode array detection (HPLC-DAD) and HPLC- electrospray ionisation-mass spectrometry (HPLC-ESI-MS) techniques. The extraction methods and the choice of solvent affected noticeably the content of individual compounds in the extract. The betalains found in beetroot peel extract were vulgaxanthin I, vulgaxanthin II, indicaxanthin, betanin, prebetanin, isobetanin and neobetanin. Also cyclodopa glucoside, *N*-formylcyclodopa glucoside, glucoside of dihydroxyindolcarboxylic acid, betalamic acid, L-tryptophan, *p*-coumaric acid, ferulic acid and traces of unidentified flavonoids were detected.

Introduction

Phenolic compounds are ubiquitous in the plant kingdom and they have been reported to possess many biological effects. Recently plant phenolics have received interest due to their supposed properties in promoting human health and the possibility to use them as natural food additives, since phenolic compounds influence the quality, acceptability and stability of foods by acting as flavorants, colorants and antioxidants (Decker, 1995).

The red beetroot (*Beta vulgaris*) has been cultivated for many hundreds of years in all temperate climates. Beetroot is used as a vegetable, and its juice and extracts also as traditional medicine, food colorant and additive to cosmetics (Henry, 1996; Stuppner and Egger, 1996). The use of beetroot as source of colour has focused the beetroot investigations on betalains (red-violet betacyanins and yellow betaxanthins), which are a class of N-containing water-soluble plant pigments. Betalains are characteristic for the plant order Caryophyllales (Centrospermae), and there is a mutual exclusion of betalains and anthocyanins. However, other flavonoids coexist with the betalains (Steglich and Strack, 1990). Many betalain-bearing species have relatively large concentrations of ferulic acid in their cell walls (Jackman and Smith, 1996). Except ferulic acid, also other phenolic acids and phenolic acid conjugates have been reported in different beetroot materials (Winter and Herrmann, 1986; Bokern *et al.*, 1991; Waldron *et al.*, 1997; Harborne *et al.*, 1999). The antioxidant activity of beet extracts, especially beet peel extracts, reported recently increase the interest in beetroot compounds (Vinson *et al.*, 1998; Kähkönen *et al.*, 1999).

In this paper the effect of extraction method and solvent on the content of red beetroot peel extract are described. The characterisation of chemical compounds in detail is given for beetroot peel extract (80% aqueous methanol) by a high-performance liquid chromatography coupled with UV-detector, DAD-detector and a mass spectrometer equipped with electrospray ion source.

Materials and Methods

Chemicals

Folin-Ciocalteu's reagent was purchased from Fluka (Biochemica, Buchs, Switzerland), sodium carbonate and L-tryptophan were from Merck (Darmstadt, Germany) and gallic acid, ferulic acid

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and *p*-coumaric acid were from Sigma (St. Louis, MO, USA). All organic solvents used were of HPLC-grade.

Plant material

The red beetroots were obtained from a local farmer (*Beta vulgaris*, cultivar "Little Ball"). The beetroots were washed and hand-peeled, and the collected peels were cut into small pieces and stored at −25° C until lyophilisation. Lyophilised plant material was ground with a mortar and pestle.

Extraction (analytical)

Three replicates were prepared with both extraction methods and solvents.

Method A: 500 mg of lyophilised material was extracted with 25 ml of solvent (80% aqueous methanol or water) in planar shaker (Promax 2020, Heidolp, Germany) for 45 min. The homogenate was centrifuged for 10 min (1500×*g*) and the clear supernatant was collected. The insoluble part was re-extracted twice with 25 ml of solvent for 45 and 20 min. The combined extracts were taken to dryness by a rotary evaporator and the residue was dissolved in 20 ml of water.

Method B: 500 mg of lyophilised material was homogenised (Ultra-Turrax T 25, Janke & Kunkel, IKA-Labortechnik, Germany) for one min with 10 ml of solvent (80% aqueous methanol or water). The homogenate was centrifuged for 10 min (1500×*g*) and the clear supernatant was collected. The insoluble part was re-extracted with 10 ml of solvent. The combined extracts were taken to dryness by a rotary evaporator and the residue was dissolved in 20 ml of water.

Extraction (preparative) and fractionation

Prior to the HPLC-ESI-MS analysis a concentrated beetroot peel extract was prepared. The extract was prepared by a modification of the analytical method B, the initial amount of dry beetroot peel being 20 g and the volume of used solvent, 80% aqueous methanol, 800 ml. The combined extracts were taken to dryness and the residue was dissolved in 40 ml of water.

The concentrated extract was centrifuged for 10 min (1500×*g*) and the pH of the extract was adjusted to 2.0 using HCl. The preparative column (400 × 25 mm I.D., Pharmacia, Uppsala, Sweden) filled with Sephadex LH-20 (Pharmacia, Umeå, Sweden) gel was wrapped in aluminum foil to prevent exposure to light during the fractionation. The column was washed in advance with water (pH 2.0) and the extract was transferred on to the column. The beetroot peel compounds were eluted with acidic water (pH 2.0), neutral water and different strengths (20–100%) of aqueous methanol. The first fraction contained sugars and was discarded. The elution was continued and fractions (about 100 ml per fraction) were collected. The fractions were taken almost to dryness by a rotary evaporator and the residues were diluted to water in prior to HPLC and HPLC-ESI-MS analyses. Portion of the fractions were lyophilised for further analyses.

Determination of total phenolics

The content of total phenolics in the extracts was determined according to a modification of the Folin-Ciocalteu method (Nurmi *et al.*, 1996) on a Perkin-Elmer Lambda 12 UV/VIS Spectrometer (Norwalk, CT, USA). The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram of dry plant material.

HPLC analysis

Analytical chromatographic measurements were performed on an HPLC system consisting of a Merck-Hitachi L-7200 autosampler, Merck-Hitachi L-7100 HPLC pump, Merck-Hitachi L-7400 UV/VIS detector and Merck-Hitachi D-7000 interface (all from Hitachi, Tokyo, Japan). The absorption spectra (240–370 nm) of individual compounds of extracts and fractions were recorded on a system consisting the pump and LC-235 diode array detector (Perkin-Elmer, Norwalk, CT, USA) connected to a Graphic Printer GP-100 (Perkin-Elmer, Beaconsfield, UK). The compounds were detected by an HPLC method developed earlier in our laboratory (Ossipov *et al.*, 1995). The column used was a LiChrocart RP-18 (250 × 4.0 mm I.D., Purospher, 5-μm, Merck, Darmstadt, Germany) equipped with a precolumn. The injection volume was 20 μl. A constant flow rate was 1.0 ml min^{-1}. The eluents were acetonitrile (A) and formic acid – water (5:95, v/v) (B). The elution pro-

file was: 0–5 min, 100% B (isocratic); 5–60 min, 0–30% A in B (linear); 60–70 min, 30–60% A in B (linear). In order to define the composition of beetroot peel extract, both betalains and phenolics, 280 nm was chosen as detection wavelength, after also wavelenghts 320, 365 and 520 nm were tried.

HPLC-ESI-MS analysis

A high-performance liquid chromatographic-mass spectrometric analysis of red beetroot peel compounds were carried out with a Perkin-Elmer SCIEX API 365 triple-quadrupole mass spectrometer (Sciex, Toronto, Canada) incorporated to a Perkin-Elmer Series 200 HPLC system and Apple Macintosh 8.1 data system. The ion source was an ionspray (pneumatically assisted electrospray ionisation option; Sciex, Toronto, Canada). The HPLC system was comprised of two Perkin-Elmer Series 200 micro pumps (Perkin-Elmer, Norwalk, CT, USA) and a 785A UV/VIS detector (Perkin-Elmer, Norwalk, CT, USA). Samples were introduced into the system by a Perkin-Elmer Series 200 Autosampler (Perkin-Elmer, Norwalk, CT, USA). The separation of individual compounds was achieved on a column mentioned earlier.

The HPLC solvent system consisted of acetonitrile (A) and formic acid – water (0.4:99.6, v/v) (B). The elution profile was: 0–5 min, 100% B (isocratic); 5–50 min, 0–20% A in B (linear); 50–60 min, 20–70% A in B (linear). The injection volume was 20 µl. The flow rate was 1.0 ml min^{-1} before the ESI. The main part of the flow was split and only minor part (200 µl min^{-1}) was introduced into the ion source.

The mass spectrometer was operated in the negative and positive ion modes. Mass data were acquired by the scan mode, consisted of scanning from *m/z* 100 to 1100 with 0.30 amu steps. The spray needle potential for the negative ion experiments was −4000 V. Orifice plate voltage was set at − 35 V. Ring voltage was set at −220 V. Nebulizer gas (purified air) value was set to value 8 and curtain gas (N_2) to value 10. Settings for the positive ion experiments were: the spray needle potential, 5200 V; orifice plate voltage, 45 V; ring voltage, 220 V; nebulizer gas, value 8 and curtain gas, value 10. Heated nitrogen gas temperature was 300 °C.

Results and Discussion

The total phenolic contents of the red beetroot peel extracts prepared by different extraction methods and solvents decreased in the order 80% aqueous methanol extract prepared by method A (24.1 ± 0.3 mg/g GAE), water extract prepared by method A (20.5 ± 0.4 mg/g GAE), 80% aqueous methanol extract prepared by method B (18.8 ± 0.3 mg/g GAE) and water extract prepared by method B (17.4 ± 0.4 mg/g GAE). The extraction method and solvent were found to affect noticeably the content of individual compounds in beetroot peel extract on the basis of HPLC analyses. The area of neobetanin peak in the 80% aqueous methanol extracts made by method A and the area of cyclodopa glucoside peak in the 80% aqueous methanol extracts made by method B, were multiple compared with the other extracts. The area of isobetanin peak in the water extracts made

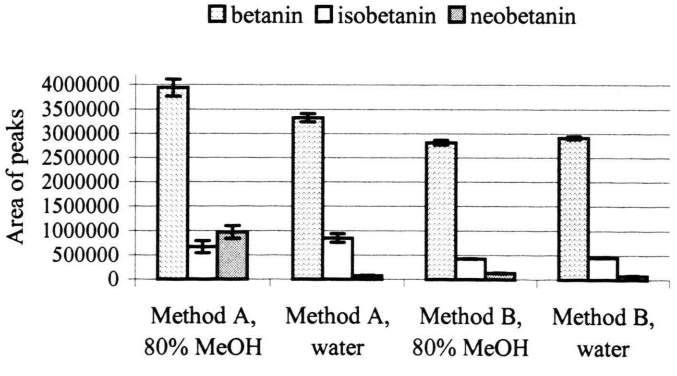

Fig. 1. Areas of betanin, isobetanin and neobetanin peaks in the chromatograms of red beetroot peel extracts prepared with two different extraction methods and solvents.

by method A was greater than in other extracts in regard to the area of betanin peak (Fig. 1).

Four betacyanins (betanin, prebetanin, isobetanin, neobetanin), three betaxanthins (vulgaxanthin I, vulgaxanthin II and indicaxanthin), cyclodopa glucoside, *N*-formylcyclodopa glucoside, betalamic acid, L-tryptophan, *p*-coumaric acid and ferulic acid were found in the water fractions of crude extract of beetroot peel. Glucoside of dihydroxyindolcarboxylic acid was found in the first methanolic fraction of crude extract. The identification of these compounds was based on their molecular weights defined from HPLC-ESI-MS analyses and the comparisons of the UV-spectral characteristics of the corresponding peaks with standards or literature data. The UV-spectral characteristics of L-tryptophan, *p*-coumaric acid and ferulic acid were compared with standards, and those of other compounds with literature data (betanin, isobetanin, vulgaxanthin I, vulgaxanthin II and betalamic acid (Musso, 1979); indicaxanthin (Piattelli *et al.*, 1964); prebetanin (Wyler *et al.*, 1967); neobetanin (Alard *et al.*, 1985); cyclodopa glucoside, *N*-formylcyclodopa glucoside and glucoside of dihydroxyindol carboxylic acid (Wyler *et al.*, 1984)). Vulgaxanthin I and vulgaxanthin II are known to cooccur in beetroot (Steglich and Strack, 1990). The vulgaxanthins coeluted in the HPLC analyses, but on the basis of the HPLC-ESI-MS analyses vulgaxanthin I was found to be predominant. On the basis of UV-spectral characteristics also flavonoids were found in the methanolic fractions.

The mass spectral data of red beetroot peel constituents are listed according to the retention order (analytical column) in Table I. Negative- and positive ion mass spectra of components of the extract allowed the definition of molecular masses. Except indicaxanthin and glucoside of dihydroxyindolcarboxylic acid, each compound gave clear $[M-H]^-$ ions and $[M+H]^+$ ions in the negative and positive modes, respectively, with relatively high intensity. Glucoside of dihydroxyindolcarboxylic acid and prebetanin were the only compounds in this study, which gave detectable fragment ions. Glucoside of dihydroxyindolcarboxylic acid (M.W. 355) showed fragment ion at *m/z* 192 with negative ion mode indicating the loss of glucose residue. Prebetanin (M.W. 630) showed fragment ion [betanidin + H]$^+$ at *m/z* 389 with positive ion mode. Moreover, in most spectra of the components, additional information in the forms of $[2M-H]^-$ or $[2M+H]^+$ ions were detected.

Table I. Mass spectral data (negative and positive detection) of beetroot peel constituents.

Structure attribution	*m/z*	Ions
Cyclodopa glucoside	356	$[M-H]^-$
	713	[2M-H]
	358	$[M+H]^+$
	715	$[2M+H]^+$
N-Formylcyclodopa glucoside	383	$[M-H]^-$
	767	$[2M-H]^-$
	385	$[M+H]^+$
Vulgaxanthin I	338	$[M-H]^-$
	677	$[2M-H]^-$
	340	$[M+H]^+$
Vulgaxanthin II	339	$[M-H]^-$
	341	$[M+H]^+$
Indicaxanthin	307	$[M-H]^-$
Betalamic acid	210	$[M-H]^-$
	421	$[2M-H]^-$
	212	$[M+H]^+$
Betanin	549	$[M-H]^-$
	551	$[M+H]^+$
Glucoside of dihydroxyindolcarboxylic acid	192	$[M-H\text{-glucose}]^-$
	354	$[M-H]^-$
	709	$[2M-H]^-$
L-Tryptophan	203	$[M-H]^-$
	407	$[2M-H]^-$
	205	$[M+H]^+$
	409	$[2M+H]^+$
Prebetanin	629	$[M-H]^-$
	389	[betanidin +H]$^+$
	631	$[M+H]^+$
Isobetanin	549	$[M-H]^-$
	551	$[M+H]^+$
Neobetanin	547	$[M-H]^-$
	549	$[M+H]^+$
p-Coumaric acid	163	$[M-H]^-$
	327	$[2M-H]^-$
	165	$[M+H]^+$
Ferulic acid	193	$[M-H]^-$
	195	$[M+H]^+$

Betanin is found as the predominant colouring in most varieties of beetroot (Henry, 1996). Other betacyanins reported in red beetroot materials are

betanidin, isobetanidin, isobetanin, prebetanin, neobetanin, amaranthin, lampranthin I and lampranthin II (Alard *et al.*, 1985; Pourrat *et al.*, 1988; Bokern *et al.*, 1991; Jackman and Smith, 1996). The solvents used to extract betalains are mainly water and aqueous methanol (50–80%). Degradation products of betanin are isobetanin, decarboxylated betanin, betalamic acid and cyclodopa glucoside (Jackman and Smith, 1996). Except decarboxylated betanin, all these compounds were detected in the fractions of crude extract. The natural occurrence of betalamic acid and cyclodopa glucoside has been reported (Wyler *et al.*, 1984; Steglich and Strack, 1990). The occurrence of free cyclodopa glucoside in red beetroot has also been reported to support its role as intermediate of betanin biosynthesis. The detected *N*-formylcyclodopa glucoside and glucoside of dihydroxyindolcarboxylic acid have been earlier reported in air exposed solutions of cyclodopa glucoside (Wyler *et al.*, 1984). 14,15-dehydroxybetanin, neobetanin, has been attributed to be an artefact formed during the isolation process (Wyler, 1986), but also its natural occurrence has been proved (Strack *et al.*, 1987).

Although red beetroot has been reported to contain many phenolic acids, only *p*-coumaric acid and ferulic acid were detected in the fractions of peel extract. Waldron *et al.* (1997) found ferulic acid, ferulic acid dehydrodimers, *p*-hydroxybenzoic acid, vanillic acid and *trans-p*-coumaric acid in cell walls of beetroot. Bokern *et al.* (1991) found five ferulic acid conjugates from cell cultures of beetroot, when freeze-dried cells were extracted with 50% aqueous methanol. Ferulic acid has also been found in the leaves of *Beta vulgaris* (Harborne *et al.*, 1999). Winter and Herrmann (1986) reported several phenolic acids from different parts of beetroot extracted with 80% aqueous methanol (3′-caffeoylquinic acid, 3′-*p*-coumaroylquinic acid, 3′-feruloylquinic acid, 1-*O*-*p*-coumaroyl-β-D-glucose, 1-*O*-feruloyl-β-D-glucose, 1-*O*-sinapoyl-β-D-glucose). Also dihydrocaffeic acid has been detected in beetroot (Harborne *et al.*, 1999).

L-Tryptophan is found as a protein amino acid in all plants, but it is often present in such low amounts that it can not be detected readily (Harborne *et al.*, 1999). The results of Kato-Noguchi *et al.* (1994) suggest that L-tryptophan may be an allelochemical, which affects the growth or germination of different plant species.

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