Berry Phenolics and Their Antioxidant Activity

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Phenolic profiles of a total of 26 berry samples, together with 2 apple samples, were analyzed without hydrolysis of glycosides with HPLC. The phenolic contents among different berry genera varied considerably. Anthocyanins were the main phenolic constituents in bilberry, bog-whortleberry, and cranberry, but in cowberries, belonging also to the family Ericaceae genus *Vaccinium*, flavanols and procyanidins predominated. In the family Rosaceae genus *Rubus* (cloudberry and red raspberry), the main phenolics found were ellagitannins, and in genus *Fragaria* (strawberry), ellagitannins were the second largest group after anthocyanins. However, phenolic acids were dominant in rowanberries (genus *Sorbus*) and anthocyanins in chokeberry (genus *Aronia*). In the family Grossulariaceae genus *Ribes* (currants and gooseberry), anthocyanins predominated, as well as in crowberries (family Empetraceae genus *Empetrum*). In apples, hydroxycinnamic acids were the main phenolic subgroup. Extraction methods for berries and apples were studied to produce phenolic extracts with high antioxidant activity. Evaluation of antioxidant activity was performed by autoxidazing methyl linoleate (40 °C, in the dark). The extraction method affected remarkably both the phenolic composition and the antioxidant activity, but with statistical analysis the observed activity could not be well explained with the contents of individual phenolic subgroups.

Keywords: *antioxidant; berry; fruit; phenolics; extraction*

INTRODUCTION

Fruits, including berries, are one of the most important sources of phenolic compounds in our diets. Especially hydroxybenzoic and hydroxycinnamic acid derivatives, anthocyanins, flavonols, catechins, and tannins, hydrolyzable or condensed, are frequently present (*1*). Many of these compounds exhibit a wide range of biological effects, including antioxidant, antimicrobial, anti-inflammatory, and vasodilatory actions (*2*, *3*). Phenolic compounds have numerous defense functions in plants, and thus several environmental factors, such as light, temperature, humidity, and internal factors, including genetic differences, nutrients, hormones, etc., contribute to their synthesis (*4*).

Fruit extracts have shown high antioxidant potential in several studies. Phenolic crowberry, rowanberry, cloudberry, cranberry, whortleberry, gooseberry, chokeberry, bilberry, cowberry, raspberry, and black currant extracts were effective in inhibiting the formation of hydroperoxides in bulk methyl linoleate (*5*). In an earlier study, phenolic extracts of berries (blackberries, red raspberries, sweet cherries, blueberries, and strawberries) inhibited human low-density lipoprotein (LDL) and liposome oxidation (*6*). Berries have also shown a remarkably high scavenging activity toward chemically generated active oxygen species (*7*−*10*). However, scarce information is available on the contribution of different phenolic subgroups or single compounds in this observed activity. In the studies published on the antioxidant activity of berry phenolics, different extraction procedures, oxidation models, conditions, and measurements have been applied, and therefore interpretation of results is complicated. Reliable analytical methods for the extraction of phenolics from fruits, as well as simple, controlled methods for the evaluation of phenolic composition and antioxidant activity of these extracts are necessary tools when the aim is to produce berry extracts with high antioxidant potential. It is obvious that the specificity and sensitivity of one method cannot lead to excellent recovery for all phenolic subclasses. Therefore, knowledge of the phenolic constituents, that is, their quality and contribution to the studied activity, is essential in the choice of an extraction procedure for a given fruit.

Solvent extraction has been the most common method in fruit sample preparation. Phenolic compounds have been extracted from ground, dried, or freeze-dried berry and fruit samples or by macerating the fresh sample with the extracting solvent (*11*). Most common solvents are aqueous mixtures with ethanol, methanol, and acetone (*1*). The raw extracts produced contain also nonphenolic substances such as sugars, organic acids, proteins, and pigments (*1*), which can interfere during antioxidant evaluation. Therefore, solid-phase extraction (SPE) has been used to remove free sugars from fruit extracts or fruit wines prior to antioxidant testing (*5*, *12*).

Although a number of studies have been made on the content of phenolics in berries and fruits, they are generally restricted to their total phenolic and/or total tannin, total anthocyanin, and phenolic acid contents. Furthermore, hydrolysis of glycoside bonds is often used in the extraction procedure, and thereby essential information on the native forms of phenolics is lost. As a consequence, knowledge on the relationship between antioxidant activity and phenolic composition of berries and fruits is limited as well.

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10.1021/jf010152t CCC: $20.00
Published on Web 07/26/2001

Domestic berries, both wild and cultivated species, and apples are consumed in abundance in Finland, as other fruits are not as successfully grown in the northern climate. In this study we included 26 berry and 2 apple samples, all of Finnish origin. The aim was to characterize the phenolic profiles, to seek possible statistical correlation between phenolic composition and antioxidant activity, and to develop an extraction and purification method for berries and apples to produce phenolic extracts with high antioxidant activity.

MATERIALS AND METHODS

Chemicals. Cyanidin 3-glucoside was purchased from Extrasynthese (Genay, France), and (+)-catechin, cholorogenic acid, ellagic acid, gallic acid, rutin, and α-tocopherol were obtained from Sigma Chemical Co. (St. Louis, MO). Methyl linoleate (MeLo) was purchased from Nu-Check-Prep, Inc. (Elysian, MN). All organic solvents used were of HPLC grade.

Sampling. Berry samples were either purchased from a market place or collected from nature. The materials included chokeberry, crowberry, gooseberry, black and red currants, cloudberry, raspberry, rowanberry, bilberry, cranberry, bog-whortleberry, cowberry, strawberry, and apple. Varieties (apples and cultivated berries) and growing locations (wild berries) are listed in Table 1. The leaves and branches were picked from berry samples; apples were cored and cut into slices with peels. Samples were packed immediately into a vacuum and stored in a freezer at −18 °C. Frozen samples were lyophilized before analysis. Maximum time between freezing and analysis of the samples was 8 weeks.

Sample Extraction. To study the effect of extraction method and sugar removal on the phenolic yields and antioxidant action of the extracts, three berries, bilberry, cowberry, and raspberry, and apple were chosen to represent different types of materials. Bilberry is rich in anthocyanins, cowberry in procyanidins and flavonols, and raspberry in ellagitannins, whereas apple contains high amounts of hydroxycinnamic acids. The solvents tested were 70% aqueous acetone, 60% aqueous methanol, hexane, H_2O at room temperature, and boiling H_2O (refluxing). On the basis of the test results, acetone extraction was chosen to be used in preparing phenolic extracts from the entire set of 28 samples.

Extraction was carried out as follows: 500 mg of lyophilized material was weighed into a centrifuge tube, 10 mL of solvent (70% aqueous acetone, 60% aqueous methanol, hexane, or H_2O in room temperature) was added, and the sample was homogenized (Ultra-Turrax) for 1 min. Tubes were centrifuged (3000 rpm, 15 min), and the clear supernatant was collected. The procedure was repeated with another 10 mL of solvent. One more repetition was carried out when H_2O was used as the solvent. Supernatants were combined and taken to dryness.

Refluxion was carried out as follows: 0.5 g of lyophilized material was weighed into a centrifuge tube, 10 mL of H_2O was added, and the sample was homogenized (Ultra-Turrax) for 1 min. The tube was sealed, placed into a hot bath filled with sand, and cooked for 5 min. After cooking, the tube was allowed to cool to room temperature and then centrifuged (3000 rpm, 15 min). The supernatant was collected and the procedure repeated with another 10 mL of H_2O. Supernatants were combined and taken to dryness.

The solid residues were dissolved in methanol and stored in a freezer for a maximum of 1 week before analysis. To determine dry weights, a part of the extract was lyophilized and the solid residue weighed.

Purification. Apple, bilberry, cowberry, and raspberry extracts were purified according to the method described before (*12*). BondElut C_{18} SPE tubes (500 mg; Varian, Middelburg, The Netherlands) were preconditioned with methanol and H_2O. The sample, dissolved in H_2O (0.5 mL), was transferred to the tube, and the tube was washed with 2 mL of H_2O (pH 2.0). The phenolic fraction was eluted with 2.5 mL of methanol and eventually taken to the volume of 1 mL under N_2. The effect of sugar removal on the amount of phenolics in the extracts was monitored colorimetrically using the Folin−Ciocalteau method and HPLC determination.

Determination of Total Phenolics. The amount of total phenolics in extracts was determined according to the Folin−Ciocalteu method (*13*) and expressed as gallic acid equivalents (GAE), milligrams per gram of dry matter.

Determination of Phenolic Profiles. Phenolic profiles were determined using an analytical HPLC method described by Lamuela-Raventós and Waterhouse (*14*). The HPLC system (Waters, Milford, MA) consisted of a WISP 712 autosampler, three 501 pumps with a pump control module, a column oven with a temperature control module, a PDA996 diode array detector, and a Millennium 2020C/S software data module. Analytical separation of phenolic compounds was carried out on a Nova-Pak C18 column (150 mm × 3.9 mm, 4 μm; Waters) equipped with a C18 quard column. The mobile phase consisted of 50 mM dihydrogen ammonium phosphate adjusted to pH 2.6 with orthophosphoric acid (solvent A), 20% A with 80% acetonitrile (solvent B), and 0.2 M orthophosphoric acid adjusted with ammonia to pH 1.5 (solvent C). The temperature of the column oven was set at 40 °C. The elution conditions were as follows: isocratic elution 100% A, 0−5 min; linear gradient from 100% A to 96% A/4% B, 5−15 min; to 92% A/8% B, 15−25 min; stepwise to 8% B/92% C 25−25.01 min; linear gradient to 20% B/80% C, 25.01−45 min; to 40% B/60% C, 45−55 min; to 80% B/20% C, 55−65 min; isocratic elution 80% B/20% C, 65−70 min; linear gradient to 100% A, 70−75 min; post-time 15 min before next injection; flow rate = 0.5 mL/min. On the basis of spectral identification, phenolics were quantified in six subclasses: flavanols and procyanidins [expressed as (+)-catechin equivalents; detection wavelength = 280 nm], hydroxybenzoic acids (as gallic acid equivalents, 280 nm), ellagitannins (as ellagic acid equivalents, 280 nm), hydroxycinnamic acids (as chlorogenic acid equivalents, 320 nm), flavonols (as rutin equivalents, 365 nm), and anthocyanins (as cyanidn 3-glucoside equivalents, 520 nm), and expressed as milligrams per 100 g of dry material.

Oxidation of MeLo. Antioxidant testing was carried out by autoxidazing MeLo. The method has been used in a previous antioxidant activity study of plant extracts (*5*). Both raw and SPE-purified extracts were tested at the concentration of 500 ppm according to the dry weight of the extract. Methanolic extracts were added to MeLo (0.2 g), and methanol was evaporated under nitrogen. Oxidation of MeLo was carried out in the dark at 40 °C. Sample aliquots (10 mg) were taken at the starting point and after 72 h of oxidation. Aliquots were dissolved in 5 mL of 2,2,4-trimethylpentane (isooctane), and the conjugated diene absorption at 234 nm was measured (Perkin-Elmer lambda 15 UV−vis spectrophotometer, Norwalk, CT). The amount of hydroperoxides was calculated using absorptivity of 26000 (*15*). The antioxidant activity was expressed as percentual (%) inhibition of formation of MeLo hydroperoxides after 72 h of oxidation. α-Tocopherol (20 ppm) was used in each experiment as a reference antioxidant.

Statistical Analysis. HPLC and antioxidant test results were processed by using one-way variance analysis (ANOVA). Differences at $P < 0.05$ were considered to be significant. In addition, Pearson product moment correlation and multiple regression analysis were performed for the extraction test data. The computer program employed was Statgraphics Plus for Windows 3.0.

RESULTS

Phenolic Profiles of Berry and Apple Samples. Table 1 shows the contents of phenolic compounds subgroups (anthocyanins, flavonols, hydroxycinnamic acids, benzoic acids, ellagitannins, and flavanols and procyanidins) in the berry and apple extracts produced using 70% acetone as extraction solvent. Anthocyanins predominated in bilberry, bog-whortleberry, cranberry, chokeberry, black and red currants, gooseberry, crowberry, raspberry, and strawberry extracts, with concentrations varying from 83 (gooseberry) to 3090 (bilberry)

Table 1. Anthocyanin, Flavonol, Hydroxycinnamic Acid (HCA), Hydroxybenzoic Acid (HBA), Ellagitannin, Flavanol and Procyanidin (FL+P), and Total Phenolic Contents of Raw Extracts Produced Using 70% Acetone as Extraction Solvent and Antioxidant Activity (Percentual Inhibition) of SPE-Purified Berry Extracts[a]

fruit sample	genus, cultivar	growing location	growing year	antho-cyanin[b]	flavonol[c]	HCA[d]	HBA[e]	ellagi-tannin[f]	FL+P[g]	total phenolics[h]	inh % after SPE[i]
apple	*Malus pumila* Punakaneli	Sipoo	1997	3.4 ± 0.5a	14 ± 0.5a	219 ± 2a	1.1 ± 0.1a	ND	48 ± 1a	1300 ± 20a	88
apple	*Malus pumila* Valkea kuulas	Laajasalo	1997	4.2 ± 0.1a	58 ± 1b	257 ± 2b	NDb	ND	64 ± 1b	1310 ± 50a	95
chokeberry	*Aronia mitschurinii* Viking	Mäntsälä	1997	1041 ± 11	79 ± 3	422 ± 7	1.8 ± 0.1	ND	57 ± 2	4210 ± 100	93
bilberry	*Vaccinium myrtillus*	Orimattila	1997	2298 ± 88a	54 ± 2a	231 ± 8a	5.8 ± 0.1a	ND	9.0 ± 0.2a	3300 ± 50a	92
bilberry	*Vaccinium myrtillus*	Mäntyharju	1998	2657 ± 117a	88 ± 3b	113 ± 1b	3.3 ± 0.1b	ND	13 ± 1a	3480 ± 80ab	
bilberry	*Vaccinium myrtillus*	Nurmes	1998	3090 ± 56b	130 ± 4c	222 ± 4a	7.2 ± 0.3c	ND	19 ± 1b	3820 ± 100b	
black currant	*Ribes nigrum* Öjebyn	Vesanto	1997	756 ± 12a	74 ± 1a	58 ± 2a	6.0 ± 0.1a	ND	114 ± 1a	2230 ± 10a	83
black currant	*Ribes nigrum* Öjebyn	Nurmijärvi	1998	1064 ± 8b	87 ± 1b	93 ± 3b	12 ± 1b	ND	208 ± 7b	2790 ± 20b	
black currant	*Ribes nigrum* Titan	Keuruu	1998	955 ± 35c	72 ± 3a	79 ± 3c	9.7 ± 0.5b	ND	159 ± 1c	2580 ± 0c	
bog-whortleberry	*Vaccinium uligonosum*	Keminmaa	1997	1297 ± 66	790 ± 13	54 ± 1	4.3 ± 0.3	ND	24 ± 1	2910 ± 80	95
cloudberry	*Rubus chamaemorus*	Kemijärvi	1997	6.0 ± 0.2a	56 ± 3a	68 ± 1a	44 ± 2a	1423 ± 17a	2.9 ± 0.1a	1710 ± 20ab	97
cloudberry	*Rubus chamaemorus*	Kilpisjärvi	1998	5.7 ± 0.1a	34 ± 2b	80 ± 2b	29 ± 1b	1110 ± 37b	1.4 ± 0.1b	1690 ± 40bc	
cloudberry	*Rubus chamaemorus*	Pyhäjärvi	1998	9.2 ± 0.2b	28 ± 1bc	77 ± 2b	43 ± 1a	1630 ± 3c	1.6 ± 0.1b	1840 ± 50c	
cloudberry	*Rubus chamaemorus*	Nurmes	1998	4.4 ± 0.1c	21 ± 1c	56 ± 3c	23 ± 1c	1090 ± 8b	1.4 ± 0.0b	1510 ± 40a	
cowberry	*Vaccinium vitis-idaea*	Puumala	1997	230 ± 5a	102 ± 1a	64 ± 2a	10 ± 1a	ND	636 ± 9a	2600 ± 50a	91
cowberry	*Vaccinium vitis-idaea*	Petäjävesi	1998	340 ± 15b	153 ± 1b	46 ± 1b	13 ± 1ab	ND	876 ± 10b	2820 ± 70b	
cowberry	*Vaccinium vitis-idaea*	Alavus	1998	350 ± 5b	131 ± 2c	70 ± 4a	16 ± 1c	ND	1170 ± 18c	2780 ± 60b	
cranberry	*Vaccinium oxycoccus*	Kemijärvi	1997	397 ± 2	200 ± 4	147 ± 2	3.8 ± 0.2	ND	219 ± 10	2200 ± 100	96
crowberry	*Empetrum nigrum*	Keminmaa	1997	2473 ± 94	163 ± 2	169 ± 3	38 ± 1	ND	423 ± 6	5240 ± 140	98
gooseberry	*Ribes grossularia* Lepaan punainen	Perniö	1997	83 ± 2	51 ± 3	39 ± 2	6.1 ± 0.1	ND	65 ± 1	1320 ± 10	93
raspberry	*Rubus idaeus* Ottawa	Kerimäki	1997	172 ± 1a	15 ± 1a	26 ± 1a	25 ± 1a	1692 ± 30a	4 ± 0.1a	2730 ± 50a	88
raspberry	*Rubus idaeus* Ottawa	Kesälahti	1998	219 ± 2b	25 ± 1b	23 ± 1a	30 ± 1b	1754 ± 9a	4 ± 0.2a	2860 ± 90a	
raspberry	*Rubus idaeus* Muskoka	Savitaipale	1998	298 ± 4	30 ± 1c	27 ± 1a	16 ± 1c	1706 ± 7a	3 ± 0.1b	2990 ± 70a	
red currant	*Ribes rubrum* Red dutch	Lappeenranta	1997	113 ± 5	9.5 ± 0.1	9.4 ± 0.1	14 ± 1	ND	68 ± 2	1400 ± 40	60
rowanberry	*Sorbus aucuparia*	Valkeakoski	1997	26 ± 1	165 ± 6	679 ± 30	13 ± 1	ND	18 ± 2	2090 ± 30	98
strawberry	*Fragaria ananassa* Bounty	Pyhäsalmi	1997	184 ± 1a	10 ± 1a	47 ± 1a	18 ± 1a	81 ± 7a	9.2 ± 0.7a	2410 ± 10a	60
strawberry	*Fragaria ananassa* Jonsok	Pielavesi	1997	195 ± 4b	6.3 ± 0.2b	63 ± 1b	11 ± 0.0b	115 ± 7b	10 ± 1a	1840 ± 40b	52
strawberry	*Fragaria ananassa* Senga sengana	Suonenjoki	1997	232 ± 3c	20 ± 0.5c	58 ± 1c	55 ± 1c	184 ± 3c	8.1 ± 0.1a	1600 ± 20c	57

[a] Composition data expressed as mg/100 g of dw (mean ± SD of duplicate assays). Values in the same column for each fruit having the same letter are not significantly different at $P < 0.05$. ND, not detected. [b] Concentration based upon cyanidin 3-glucoside as standard. [c] Concentration based upon rutin as standard. [d] Concentration based upon chlorogenic acid as standard. [e] Concentration based upon gallic acid as standard. [f] Concentration based as ellagic acid as standard. [g] Concentration based as (+)-catechin as standard. [h] Concentration based upon gallic acid as standard. [i] Kähkönen et al. 1999 (*5*).

mg/100 g of dry weight (dw). Hydroxycinnamic acids prevailed in rowanberries (679), apples (>250), and cloudberries (56−80 mg/100 g of dw). However, in cloudberries, as well as in raspberries, ellagitannins were the predominant phenolic constituents. Lower levels of ellagitannins were found in strawberries. In these three berries, all belonging to the family Rosaceae, the content of hydroxybenzoic acids was relatively high compared to that of berries from other families. Flavanols together with their oligo- and polymeric forms, procyanidins, constituted the main subgroup in cowberries, with concentrations ranging from 636 to 1170 mg/100 g of dw. This phenolic group was abundant also in crowberries, cranberries, and currants. Apples were rich in phenolic acids.

Influence of Cultivar or Growing Site and Growing Season on Phenolic Profiles. The total phenolic contents of the two black currant cultivars, Öjebyn and Titan, showed relatively small but statistically significant difference [2790 and 2580 mg/100 g of dw, respectively; relative standard deviation (RSD) = 6%] when the samples were collected during the same growing season (Table 1). However, among the two Öjebyn samples collected in two different growing seasons, greater variation was observed (RSD = 19%). For the phenolic subgroups, the greatest differences among the two cultivars and betweem the two Öjebyn samples grown in different years were found in flavanol and procyanidin contents (RSD = 19 and 41%, respectively). The two raspberry cultivars studied, Ottawa and Muskoka, showed also a quite small, insignificant variation in the concentration of total phenolics (2860 and 2990 mg/100 g of dw, respectively; RSD = 3%), but yet significant differences could be detected in anthocyanin (RSD = 22%) and hydroxybenzoic acid (RSD = 43%) contents. The variation in Ottawa from year to year was not as clear as the variation in the black currant cultivar, but significant differences were found in anthocyanin, flavonol, and hydroxybenzoic acid contents. Greater varietal differences were observed in various strawberry cultivars, the total phenolic content ranging from 1600 (cv. Senga Sengana) to 2410 (cv. Bounty) mg/100 g of dw (RSD = 21%). The divergence was especially great in hydroxybenzoic acid contents (RSD = 84%). In apple, differences in the total phenolic contents between the two cultivars were insignificant (1100 and 1210 mg/100 g of dw), but in cv. Punakaneli, flavonol content was 76% smaller than in cv. Valkea Kuulas.

Some differences were found between wild berries grown in different locations in Finland. Two bilberry samples collected in 1998 were relatively similar with respect to their total phenolic (3480 and 3820 mg/100 g of dw; RSD = 7%) and anthocyanin (RSD = 11%) contents, but great differences were found in other phenolic subclasses. All of these differences were statistically significant. Significant differences were also detected among bilberry samples from different harvest years. In the two cowberry samples studied, the variation was statistically insignificant in total phenolic content (RSD = 1%), but still, quite large significant differences were found in hydroxycinnamic acid (RSD = 29%) and flavanol and procyanidin contents (RSD = 20%). The cowberry sample collected in 1997 had significantly lower total phenolics, anthocyanin, flavonol, and flavanol and procyanidin contents. In cloudberry, amounts of total phenolics in the three different samples from 1998 were relatively close to each other (RSD = 10%). The sample from the previous year was also quite similar with respect to the total phenolic content. However, variation was great within the phenolic subgroups.

Effect of Phenolic Composition on Antioxidant Activity. The phenolic composition data (Table 1) showed no remarkable correlation between antioxidant activity and total phenolics ($R^2 = 0.30$, $P = 0.09$, $n = 34$). Similarly, antioxidant activity correlated weakly with total phenolics in the extraction method data in Table 2 ($R^2 = 0.42$, $P = 0.02$, $n = 32$; hexane extracts excluded). Statistically significant correlation was observed between flavonol content and antioxidant activity ($R^2 = 0.78$, $P = 0.00$, $n = 34$) and between hydroxycinnamic acid content and antioxidant activity ($R^2 = 0.54$, $P = 0.00$, $n = 34$) in the phenolic composition data. However, in the extraction test data, no statistically significant correlation could be seen between antioxidant activity and single studied phenolic subgroups.

To study more closely the relationships between phenolic compositions and antioxidant activities, the data were analyzed using multiple regression analysis. The aim in this method is to choose from a large group of independent variables those variables which explain best the variation of the dependent variable, that is, which phenolic components are most important to the formation of antioxidant response, and to calculate a multiple linear regression model to describe the relationship between the dependent and independent variables. In this analysis, independent variables were the anthocyanin, flavonol, hydroxycinnamic acid, hydroxybenzoic acid, ellagitannin, and flavanol and procyanidin contents after SPE, and the dependent variable was the antioxidant activity after SPE (inhibition). Previous to the statistical analysis, the data points below the detection limits were substituted by 0.5 times the detection limit. However, with the six independent variables from all of the fruit samples included in the analysis, it was not possible to build a reasonable regression model; that is, the inhibition of hydroperoxide formation could not be well explained by any combination of the variables. For example, flavonol and hydroxycinnamic acid contents, which showed positive correlation with antioxidant activity, explained together only 31% of the variability in the antioxidant response ($P < 0.05$). A similar result was obtained from the extraction method survey data.

Effect of Extraction Method on Phenolic Profiles. Changing the extraction solvent from aqueous methanol to aqueous acetone altered the phenolic composition of the samples (Table 2). With acetone extraction, the yields of hydroxycinnamic acids and anthocyanins were higher, but the differences were statistically significant only in the apple and bilberry anthocyanins and in the cowberry and raspberry hydroxycinnamic acids. Ellagitannins, the main phenolic subgroup in raspberry, were also extracted more efficiently with acetone extraction, the difference being statistically significant. The highest yield of flavanols and procyanidins was achieved with 60% methanol except in apple, in which acetone extraction yielded the highest levels. Extraction with 100% H_2O at room temperature resulted in quite low yields of phenolic compounds compared to mixtures of water and organic solvents, whereas refluxing yielded nearly as high or higher total phenolic amounts in apple, bilberry, and cowberry.

Table 2. Anthocyanin, Flavonol, Hydroxycinnamic Acid (HCA), Hydroxybenzoic Acid (HBA), Ellagitannin, Flavanol and Procyanidin, and Total Phenolic Contents (Data Expressed as Milligrams per 100 g of Weight), and Antioxidant Activity (Data Expressed as Inhibition Percentage) of Apple, Bilberry, Cowberry, and Raspberry Extracts Produced Using Different Extraction Methods[a]

fruit sample, cultivar	extraction method	anthocyanin[b]	flavonol[c]	HCA[d]	HBA[e]	ellagitannin[f]	flavanol[g]	total phenolics[h]	inh % raw[i]	inh % after SPE[j]
apple, Punakaneli	methanol, 60%	1.4 ± 0.1a	13 ± 0.1a	190 ± 5a	0.2 ± 0.0a	ND	79 ± 1a	948 ± 5a	16 ± 1a	81 ± 3a
	acetone, 70%	2.3 ± 0.4b	10 ± 2a	207 ± 6a	0.4 ± 0.0b	ND	68 ± 2b	1148 ± 20b	23 ± 2b	88 ± 2b
	H_2O	0.8 ± 0.0c	7.1 ± 0.4a	70 ± 2b	0.2 ± 0.0ab	ND	14± 1c	391 ± 6c	13 ± 3ad	23 ± 4c
	refluxing	ND	8.9 ± 0.2a	120 ± 2c	3.0 ± 0.1d	ND	14 ± 1c	857 ± 10d	70 ± 5c	96 ± 1d
	hexane	ND	ND	trace	ND	ND	ND	ND	10 ± 6d	NA
bilberry	methanol, 60%	2023 ± 6a	62 ± 4a	203 ± 4ab	3.1 ± 0.2a	ND	13 ± 1a	3057 ± 55a	46 ± 10ab	93 ± 2ab
	acetone, 70%	2387 ± 30b	54 ± 2a	228 ± 10a	3.9 ± 0.1a	ND	7.1 ± 0.2b	3343 ± 84a	55 ± 4a	96 ± 2b
	H_2O	944 ± 30c	18 ± 1b	133± 4c	1.0 ± 0.0b	ND	8.0 ± 0.1b	1110 ± 21b	38 ± 1c	92 ± 4a
	refluxing	721 ± 20d	30 ± 1b	168 ± 1bc	5.8 ± 0.1c	ND	10 ± 1c	2925 ± 40a	48 ± 8bc	92 ± 4
	hexane	6.0 ± 0.4e	ND	0.2 ± 0.0d	ND	ND	ND	ND	6 ± 5d	NA
cowberry	methanol, 60%	228 ± 6a	80 ± 1a	49 ± 1a	16 ± 2a	ND	524 ± 15a	2241 ± 30a	82 ± 7a	93 ± 1a
	acetone, 70%	234 ± 6a	98 ± 1b	64 ± 2b	12 ± 1a	ND	476 ± 14a	2403 ± 47a	82 ± 8ab	97 ± 1a
	H_2O	144 ± 1b	65 ± 3c	22 ± 1b	0.2 ± 0.0b	ND	430 ± 21a	1510 ± 19b	88 ± 1b	94 ± 5a
	refluxing	129 ± 3b	65 ± 1c	37 ± 1d	10 ± 0a	ND	800 ± 25b	2462 ± 51a	27 ± 7c	62 ± 6b
	hexane	0.1 ± 0.0c	ND	ND	ND	ND	16 ± 3c	ND	86 ± 2ab	NA
raspberry, Ottawa	methanol, 60%	160 ± 7ab	25 ± 3a	19 ± 1a	23 ± 1a	1400 ± 50a	7.9 ± 0.3a	2488 ± 73a	20 ± 3a	92 ± 2a
	acetone, 70%	170 ± 1a	15 ± 1b	30 ± 2b	27 ± 1a	1642 ± 17b	6.9 ± 0.5a	2702 ± 100a	31 ± 5b	93 ± 1a
	H_2O	132 ± 5b	7.0 ± 1c	14 ± 1a	9.0 ± 0.4b	378 ± 7c	6.0 ± 0.1a	1574 ± 30b	38 ± 4b	92 ± 1a
	refluxing	71 ± 2c	14 ± 1b	19 ± 1a	16 ± 1c	312 ± 3c	7.2 ± 0.5a	1516 ± 41b	67 ± 4c	97 ± 0b
	hexane	0.2 ± 0.0d	ND	ND	ND	ND	ND	ND	15 ± 7d	NA

[a] Means ± SD of duplicate assays. Values in the same column for each berry having the same letter are not significantly different at $P < 0.05$. ND, not detected. NA, not analyzed. [b] Concentration based upon cyanidin-3-glucoside as standard. [c] Concentration based upon rutin as standard. [d] Concentration based upon chlorogenic acid as standard. [e] Concentration based upon gallic acid as standard. [f] Concentration based as ellagic acid as standard. [g] Concentration based as (+)-catechin as standard. [h] Concentration based upon gallic acid as standard. [i] Inhibition of methyl linoleate hydroperoxide formation after 72 h of incubation at the concentration of 500 ppm of dry raw extract. [j] Inhibition of methyl linoleate hydroperoxide formation after 72 h of incubation at the concentration of 500 ppm of dry extract after sugar removal with SPE.

Table 3. Effect of Sugar Removal on Anthocyanin, Flavonol, Hydroxycinnamic Acid (HCA), Hydroxybenzoic Acid (HBA), Flavanol and Procyanidin (FL+P), and Total Phenolic Contents of Apple, Bilberry, Cowberry, and Raspberry Extracts Produced Using 70% Acetone[a]

fruit sample	cultivar	anthocyanin[b] loss (%)	flavonol[c] loss (%)	HCA[d] loss (%)	HBA[e] loss (%)	ellagitannin loss (%)	FL+P[g] loss (%)	total phenolics[h] loss (%)
apple	Punakaneli	6.3 ± 0.2	7.7 ± 0.2	8.3 ± 0.3	40 ± 1		18 ± 1	11 ± 0.4
bilberry		4.8 ± 0.2	2.0 ± 0.03	7.3 ± 0.2	45 ± 2		26 ± 1	6.3 ± 0.2
cowberry		5.8 ± 0.1	5.7 ± 0.05	6.2 ± 0.1	37 ± 1		16 ± 1	7.5 ± 0.2
raspberry	Ottawa	6.1 ± 0.1	5.8 ± 0.1	7.5 ± 0.2	32 ± 2	8.9 ± 0.3	22 ± 1	5.2 ± 0.1

[a] Data are expressed as loss of phenolics (%) after SPE treatment (means ± SD of triplicate assays). [b] Concentration based upon cyanidin 3-glucoside as standard. [c] Concentration based upon rutin as standard. [d] Concentration based upon chlorogenic acid as standard. [e] Concentration based upon gallic acid as standard. [f] Concentration based upon ellagic acid as standard. [g] Concentration based as (+)-catechin as standard. [h] Concentration based upon gallic acid as standard.

Effect of SPE Purification on Phenolic Profiles. Sugar removal with SPE reduced the amount of total phenolics by 5−11% (Table 3). The treatment altered the phenolic profiles: yields of anthocyanins, flavonols, and hydroxycinnamic acids remained nearly constant, whereas the amount of benzoic acids, flavanols, and procyanidins decreased remarkably.

Effect of Extraction Method and SPE Purification on Antioxidant Activity in MeLo. All raw extracts tested before sugar removal were less active than purified extracts (Table 2). Especially the activity of apple and raspberry extracts increased significantly after SPE treatment. For example, the raw acetone/water extract of raspberry inhibited the formation of MeLo hydroperoxides only by 31%, whereas the purified extract gave an inhibition value as high as 93%. Fruit extracts produced using different extraction methods showed variation in antioxidant response. Overall, extraction with acetone/water gave more active extracts than methanol/water or pure water extraction. Refluxing yielded active apple, bilberry, and raspberry extracts; inhibitions of 96, 92, and 97% were measured, respectively, at the concentration of 500 ppm. All hexane extracts showed low activity except cowberry extract, which showed high inhibition of MeLo oxidation (86%). The reason for the high activity of hexane-extracted cowberry remained unclear; possibly the extract contained fat-soluble antioxidants such as tocopherols, which were not analyzed in this study.

DISCUSSION

The phenolic contents among the studied berries varied greatly, but some consistencies were observed within families and/or genera. In *Vaccinium* species, that is, bilberry, bog-whortleberry, and cranberry, anthocyanins were the major phenolic subgroug except in cowberries, in which the flavanol and procyanidin concentration exceeded the anthocyanin concentration. In family Rosaceae genus *Rubus* (cloudberry and red raspberry) and in genus *Fragaria* (strawberry), ellagitannins predominated. Rowan and chokeberry are members of the same Rosaceae family, but ellagitannins were detected in neither one of them; hydroxycinnamates

were dominant in rowanberries (genus *Sorbus*) and anthocyanins in chokeberry (genus *Aronia*). In black and red currants and gooseberry, belonging to family Grossulariaceae genus *Ribes*, anthocyanins predominated, as well as in crowberries (family Empetraceae genus *Empetrum*). In apples, the phenolic content was fairly low, hydroxycinnamates being the main phenolic subgroup. In general, our qualitative and quantitative data for the phenolic contents in berries are in accordance with other recent studies on Finnish berries (*16*−*19*). However, in these studies acid hydrolysis was performed prior to analysis and only the flavonol and/or phenolic acid contents were measured. There are very scarce previously published data on anthocyanin, flavanol, procyanidin, and hydrolyzable tannin contents of wild berries, yet knowledge of the phenolic composition is essential to evaluate the importance of berries in the human diet as a source of bioactive compounds, as well as in utilization of berries and berry extracts in the food or medicine industry.

Even though the data showed significant variations in the phenolic profiles between different berry and apple cultivars, between wild berries harvested from different locations in Finland, and between berries grown during different growing seasons, it is difficult to distinguish between the numerous factors causing these differences. In a recent study of Häkkinen and Törrönen (*17*), both varietal and regional differences in the flavonol and phenolic acid content were found in strawberries and blueberries, whereas cultivation technique (conventional or organic) had no consistent effect on the phenolic levels. Growing location had no effect on antioxidant (ORAC) activity or anthocyanin and total phenolic contents of different varieties of blueberries, whereas varietal differences were remarkable (*8*). The effect of light on the phenolic metabolism has been studied extensively. Increase in solar radiation generally yields higher contents of phenolics, especially anthocyanins, in fruits (*1*). In crowberries, the anthocyanin content varied from one year to the next in relation to overall radiation and the number of hours of sunshine (*20*). Low temperatures may increase anthocyanin accumulation in fruits (*1*). In grapes, a favorable effect of cool climate and a short growing season on phenolic content has been reported (*21*). Low nitrogen and phosphorus levels have been shown to increase the formation of anthocyanins in cranberries (*22*), and in grapes, treatment with large amounts of nitrogen reduced the anthocyanin formation (*23*). In addition, wounding or infection increases the flavonoid synthesis in plant tissue (*4*). Finally, differences in the ripeness of the fruit may have had an effect on the phenolic profiles, as concentrations of phenolic compounds are usually higher in young fruits than in mature fruits with the exception of anthocyanins, which generally accumulate during the maturation of red fruits (*1*). On the basis of all these different factors affecting phenolic metabolism in fruits, it is probable that wild berries grown in the cool northern climate, under a short growing season, and without fertilizers, pesticides, or herbicides have high phenolic contents compared to cultivated berries that grow in a warm climate, in fertilized soil, and protected against plant diseases and insects with pesticides and herbicides. To study this hypothesis, controlled research on the phenolic composition of wild and cultivated berries of the same genera grown in different climates would be necessary.

The relationship between the antioxidant activity of berry and apple extracts and their phenolic composition is complex, and thus it is very difficult to describe it with statistical tools. Antioxidant activity correlated at a statistically significant level with flavonols and hydroxycinnamic acids, but together they explained only a relatively small part (31%) of the antioxidant activity of the extracts. One reason for this is the divergence within the phenolic subgroups; antioxidant properties of single compounds within the group can vary remarkably, and thereby equal levels of, for example, total hydroxycinnamates do not necessarily mean equal antioxidant response. Another important aspect is the antioxidant methodology used. Heinonen et al. (*6*) found earlier that the antioxidant activity of a berry extract in the LDL oxidation was related to the presence of anthocyanins, but the activity in the liposome oxidation was correlated with the amount of hydroxycinnamates. Positive correlation has been found also between oxygen radical absorbance capability (ORAC) and anthocyanin content (*8*). In complex lipid systems, where several different antioxidant and prooxidant actions occur simultaneously, it is obviously more difficult to observe the effect of a single factor than in simplified radical scavenging models. In lipid oxidation models, peroxyl radical scavenging and metal inactivation properties are very important mechanistic factors, but the polarity of the compound and the physical state of the lipid system also affect the behavior of antioxidants. In addition, synergism, that is, the ability of antioxidant compounds to reinforce each other, can have a significant effect on the antioxidant response (*24*). Ascorbic acid is a well-known synergist, and obviously the tested fruit extracts contain variable amounts of ascorbic acid. It did show a peak in the HPLC chromatograms, but as it eluted nearly in the dead volume of the column, it was not quantified. However, the concentrations in the purified extracts were presumably quite low, as the peak nearly disappeared after SPE treatment. Ascorbic acid does not act as an antioxidant in bulk MeLo alone (*25*), but it is possible that it had some synergistic effects with phenolic components.

The evaluation of different extraction methods produced valuable information considering the use of berry phenolics in foods or medicines, as the data show clearly that the extraction and purification methods used to produce fruit extracts affect significantly both the phenolic composition and the antioxidant activity of the extracts. Aqueous acetone extraction solvent was shown to be slightly superior to aqueous methanol in extracting phenolics in berries and apples. This result is in accordance with a previous report of Heinonen et al. (*6*). To gain the best possible yield of flavanols, procyanidins, and flavonols, methanol/water may be a more appropriate choice for the solvent, but in most berries the significance of these phenolic subgroups in the total content of phenolics is fairly low. Addition of a small amount (0.1−1%) of organic acid, for example, trifluoroacetic acid or formic acid, could increase the yield of anthocyanins without causing any changes in acylated anthocyanins (*1*, *26*). SPE purification strengthens the activity remarkably, as nonphenolic components, mainly free sugars and to a lesser extent organic acids, are removed and thereby the relative concentration of phenolics in the extracts increases. Berry and apple extracts produced using the conventional acetone/water or methanol/water mixtures as solvents showed high

antioxidant activity, but, surprisingly, the berry extracts produced using only H_2O as solvent were also very active, despite the lower levels of detected phenolics. The hydroxylation activity of polyphenol oxidase probably remains during the extraction treatment with H_2O at room temperature, which may decrease the phenolic content in the extracts. Refluxing is quite a rude treatment for many labile phenolics, but it inactivates the enzymes and thus yielded highly active extracts. The results on berry and apple extracts produced using hot water show that it is possible to prepare highly active extracts with high phenolic contents without organic solvents, which may be problematic in the food industry or medicine production.

ACKNOWLEDGMENT

The technical assistance of Mia Suovaniemi and Marja Vainionpää is gratefully acknowledged.

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Received for review February 6, 2001. Revised manuscript received June 12, 2001. Accepted June 12, 2001. This work was supported by the Finnish Graduate School on Applied Bioscience, the Finnish Ministry of Agriculture and Forestry, and Tekes Technology Development Center.

JF010152T