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KAISU RIIHINEN

Phenolic Compounds in Berries

Doctoral dissertation

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Institute of Applied Biotechnology
Food and Health Research Centre,
Department of Clinical Nutrition
University of Kuopio

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ABSTRACT

Phenolic compounds are secondary metabolites of plants, and are adundant constituents of plant-based foods and beverages. Current scientific evidence indicates that they contribute to the health benefits of diets rich in fruits and vegetables. The primary aim of this work was to analyse the distribution and contents of native forms of phenolic compounds in twenty-two berry species of six families. Phenolic compounds were identified and quantified by reversed-phase high-performance liquid chromatography combined with diode array detection. Mass spectrometric detection and literature data were used for further identification of the phenolic compounds.

A sequential procedure was developed for the simultaneous extraction of the major classes of phenolic compounds present in berries. Adequate quantitative data was obtained on the contents of hydroxycinnamic acid conjugates, ellagitannins, flavonol glycosides and anthocyanins, but determination of flavan-3-ol and proanthocyanidin contents remains tentative. Two strategies used in the quantification of ellagitannins, either as native forms (in gallic acid equivalents) or as acid hydrolysis products (in ellagic acid equivalents), produced comparable values for most berries, except for strawberries.

Quercetin glucoside and cyanidin glucoside were the most widespread phenolic compounds in the studied berries. Esters of *p*-coumaric and caffeic acid were the most typical conjugates of hydroxycinnamic acids. Rare A-type proanthocyanidins were tentatively identified in lingonberry, cranberry, bilberry and bog whortleberry. Distinctive similarities were found among berry species of the same family in the distribution of their conjugated forms of the phenolic compounds. On the other hand, there were differences in chromatographic profiles of conjugates and composition of aglycons especially in the case of anthocyanins. The lack of anthocyanins in colour variants of berries was linked to the lower contents of myricetin (flavonol) and changes in the composition of hydroxycinnamic acids.

The highest contents of hydroxycinnamic acids were detected in chokeberry, sweet rowanberry and half-highbush blueberry (340-890 mg/kg FW). The level of ellagic acid in various forms ranged from 650-850 mg/kg in strawberry to 1900-3900 mg/kg in raspberry, arctic bramble and cloudberry. The content of flavonols was highest in bog whortleberry (820-1020 mg/kg), followed by sea buckthorn, chokeberry, elderberry and cranberry (270-420 mg/kg). The highest contents of anthocyanins were detected in chokeberry, bilberry and northern crowberry (7680-8420 mg/kg), followed by black currant, southern crowberry, bog whortleberry and elderberry (3320-4320 mg/kg). Lingonberry showed ten times higher levels of flavan-3-ols and low molecular weight procyanidins than the other berries.

This thesis shows that berries are rich sources of a diverse spectrum of phenolic compounds. Compared to other foods, they provide high or moderate amounts of ellagitannins, anthocyanins and flavonols.

Universal Decimal Classification: 581.19, 547.97, 634.7, 577.19
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Kuopio, September 2005

Kaisu Riihinen

ABBREVIATIONS

C	carbon, e.g. in numbering C-3
DAD	diode array detection
ESI-MS	electrospray ionization mass spectrometry
FW	fresh weight
HCl	hydrochloric acid
HHDP	hexahydroxydiphenic acid
HPLC	high-performance liquid chromatography
LC-MS	liquid chromatography mass spectrometry
NaOH	sodium hydroxide
RP	reversed-phase
UV	ultraviolet
Vis	visible

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals **I-VI**.

I **Määttä K**, Kamal-Eldin A, Törrönen R. Phenolic compounds in berries of black, red, green, and white currants (*Ribes* sp.). Antioxid Redox Signal 2001; 3: 981-993.

II **Määttä KR**, Kamal-Eldin A, Törrönen AR. High-performance liquid chromatography (HPLC) analysis of phenolic compounds in berries with diode array and electrospray ionization mass spectrometric (MS) detection: *Ribes* species. J Agric Food Chem 2003; 51: 6736-6744.

III **Määttä-Riihinen KR**, Kamal-Eldin A, Törrönen AR. Identification and quantification of phenolic compounds in berries of *Fragaria* and *Rubus* species (family Rosaceae). J Agric Food Chem 2004; 52: 6178-6187.

IV **Määttä-Riihinen KR**, Kamal-Eldin A, Mattila PH, González-Paramás AM, Törrönen AR. Distribution and contents of phenolic compounds in eighteen Scandinavian berry species. J Agric Food Chem 2004; 52: 4477-4486.

V Jaakola L, **Määttä K**, Pirttilä AM, Törrönen R, Kärenlampi S, Hohtola A. Expression of genes involved in anthocyanin biosynthesis in relation to anthocyanin, proanthocyanidin, and flavonol levels during bilberry fruit development. Plant Physiol 2002; 130: 729-739. (Only the studies on phenolic content of this collaborative work are included in the thesis)

VI **Määttä-Riihinen KR**, Kähkönen MP, Törrönen AR, Heinonen IM. Catechins and procyanidins in berries of *Vaccinium* species and their antioxidant activity. J Agric Food Chem 2005; in press. (The studies on antioxidant activity of the fractions in this collaborative work are not included in the thesis)

CONTENTS

1 INTRODUCTION

Berries are small globular or ovate juicy fruits, which may be consumed fresh as such or in jams, jellies, juices, wines, and liquors. Due to their excellent nutritional properties, berries are appreciated as part of a healthy diet. Phenolic compounds are involved in colour and organoleptic properties of edible berries, and may also be responsible for some of their health effects.

The abundant phenolic classes in berries include hydroxybenzoic acids, hydroxycinnamic acids and hydrolysable tannins as well as anthocyanins, flavonols, flavan-3-ols and condensed tannins (Häkkinen et al. 1999a, Häkkinen et al. 1999b, de Pascual-Teresa et al. 2000, Häkkinen et al. 2000a, Kähkönen et al. 2001, Moyer et al. 2002, Gu et al. 2004). Other phenolic compound classes such as lignans, lignins, suberins, cutins, flavanones and isoflavonoids, occasionally found in berries (Macheix et al. 1990), will not be discussed in this thesis.

Phenolic compounds have functional roles in berry plants as colourful attractants for birds helping in seed dispersal, as cellular support materials (Faulds and Williamson 1999), and as a part of the plant stress defence mechanism (Strack 1997). The content of phenolic compounds may respond to environmental stress factors such as pathogen attack, excess UV-light, low temperature and low availability of nitrogen and phosphorus (Dixon and Paiva 1995, Winkel-Shirley 2002).

Numerous studies have been performed to try to better understand the occurrence, distribution, biosynthesis, metabolism and functions of phenolic compounds in plants (Harborne 1989, Macheix et al. 1990, Winkel-Shirley 2001, Winkel-Shirley 2002). Food and health scientists are interested in these substances since they have been reported to have multiple biological properties including antioxidant, antimutagenic, anticarcinogenic, anti-inflammatory, antiproliferative and antimicrobial activities (Haslam 1996, Lairon and Amiot 1999, Morton et al. 2000, Parr and Bolwell 2000). Their antioxidant activities are of interest not only in the light of their possible health effects, but also for their ability to prevent enzymatic browning and oxidation of food lipids, i.e. thus they have potential as food preservatives (Kaack and Austed 1998, Bonilla et al. 1999, Tapiero et al. 2002, Mihalev et al. 2004). Phenolic compounds are the most abundant antioxidants present in the diet.

The relationship between the intake of phenolic compounds from fruits and vegetables and the reduced risk of cardiovascular diseases and lung cancer has been demonstrated in a number of epidemiological studies (Arts and Hollman 2005). The antioxidant activity of phenolic compounds may be responsible for these health effects by protecting the body tissues against oxidative stress (Prior 2003). The cancer-preventive properties of phenolic compounds can be attributed to other parallel mechanisms, including cell cycle arrest, apoptosis, altered cellular signaling, and induction of detoxifying enzymes (Chen and Kong

2004). However, in the case of high-dose supplementation, there is a risk that the phenols may induce harmful effects in the human body, such as pro-oxidant activity, mitochondrial toxicity, as well as interactions with drug-metabolizing enzymes (Galati and O'Brien 2004).

The health effects of phenolic compounds do not only depend on the amount consumed but also on their bioavailability. The matrix of plant food and the covalent conjugates influence their release and absorption in the human intestine (Manach et al. 2004). Insoluble phenolic compounds are highly polymerized forms or components of cell walls, while soluble forms are compartmentalized within plant cell vacuoles (Ibrahim and Barron 1989). Soluble phenolic compounds are found in the free form or combined with sugars or other polyols *via* *O*-glycosidic or ester bonds in fruits (Macheix et al. 1990). Polymeric phenolic compounds are resistant to solubilization especially in the classes of lignins and tannins. As an analytical question, the solubility of compounds depends on the plant matrix and on the solvent used in the extraction (Merken and Beecher 2000b, Robbins 2003). In this dissertation, the term "soluble" is used for compounds extractable in aqueous-alcoholic solvents and "insoluble" for those ones which remain in the extraction residue. The earlier literature provides only scattered data on contents of different kinds of conjugated forms and the insoluble forms of phenolic compounds in berries and other plant foods.

When considering berries as a source of phenolic compounds, a simultaneous determination of all the major phenolic classes is needed. The main objective of the present studies was to determine the distribution and contents of soluble and insoluble phenolic compounds in selected wild and cultivated berries grown in Scandinavia. In addition, the levels of anthocyanins, proanthocyanidins and flavonols were analysed during bilberry fruit development. Simultaneous extraction of all phenolic classes with one extraction solvent is impracticable due to their different solubility and stability characteristics (Tura and Robards, 2002). Therefore, a novel procedure was developed for the sequantial extraction of various phenolic compounds for the qualitative and the quantitative analyses. The obtained data were subjected to an evaluation of the distribution in berries and to an assessment of berries as a source of possible bioavailable forms.

2 REVIEW OF THE LITERATURE

2.1 Berries

2.1.1 Relationships of berry plants

Berries – wild or cultivated – are small fruits of herbs, shrubs or trees. Berries with the other woody plants may be organized into a hierarchy based on taxonomy relationships of families (**Figure 1**). The basic taxonomic unit is the species (sp.), which is recognized by the two-part Latin name (e.g. *Ribes nigrum* L.). The first part of the name is the genus, the second part the species, and the letter refers to the classification system developed by Carl von Linné (1707-1778). The closely related plants within a family, which share some general or generic features, are grouped in the same genus (Stace 1984). A cultivar is a cultivated variety of plant species, which may be propagated either vegetatively (cloned) or from seeds. Some cultivars are hybrids between species, which is indicated with the multiplication symbol (e.g. *Fragaria* x *ananassa*). Wild berry species represent many individual plant clones adapted to survive, grow and develop in their own environment.

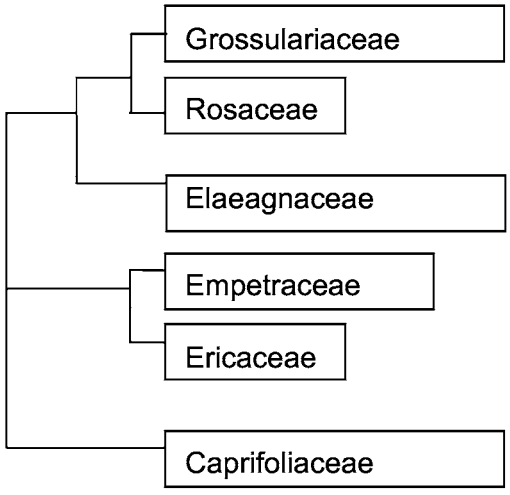


Figure 1. Taxonomic relationships of berry families.

Chemotaxonomy uses compounds produced within the plant to identify their evolutionary relationships (Stace 1984). Phenolic compounds, especially anthocyanins, have been widely used for purposes of chemotaxonomy particularly on the genus *Ribes* (e.g. black currant, gooseberry) and *Vaccinium* (e.g. lingonberry) (Harborne and Hall 1964, Øydvin 1974, Macheix et al. 1990). Moreover, the chemotaxonomy of anthocyanins has been applied to the authentication of fruit products (Robards and Antolovich 1997).

2.1.2 Berries as a natural resource

Berries are a traditional part of the diet and an important natural resource in Finland. In recent years, consumption of berries was 75 Mkg, which means 15 kg for every Finn (Anonym 2004a). The most widely cultivated berries are black currant and strawberry, accounting for 91% of total berry production in Finland (12 Mkg in 2004) (Anonym 2004b). The estimated harvest of wild berries is 40 Mkg on an annual basis (Salo 1997). Commercially the most valuable wild berries are lingonberry, bilberry, and cloudberry, accounting for 98% of the market (Salo 1997).

2.2 Structures of phenolic compounds

2.2.1 Biosynthetic relationships of phenolic compounds

All phenolic compounds share the same intermediate, phenylalanine, or its precursor, shikimic acid (Harborne 1989). They can be divided into different phenolic classes based on their general chemical structures. In the present thesis, seven phenolic classes are presented, *i.e.* hydroxybenzoic acids, hydroxycinnamic acids, hydrolysable tannins, flavonols, flavan-3-ols, proanthocyanidins (*syn.* condensed tannins) and anthocyanidins which are commonly found in plant based foods. **Figure 2** shows the biosynthetic relationships of these phenolic classes. Details of the biosynthesis pathway have been described in detail in the recent reviews and articles (Strack 1997, Winkel-Shirley 2001, Winkel-Shirley 2002, Marles et al. 2003, Schijlen et al. 2004, Xie et al. 2004). The simple structure of cinnamic acid (C_6-C_3) is converted by a cleavage of acetate to hydroxybenzoic acid or by hydroxylation and methoxylation to hydroxycinnamic acid molecules. Phenolic acids are structural units in the biosynthesis of lignans and polymeric lignins, suberins and cutins (Strack 1997). The basic skeleton of flavonoid (C_6-C_3-C_6) is first encountered in chalcone, which is derived from *p*-coumaric acid and three malonic acids (Winkel-Shirley 2002). Chalcone is converted to flavanones, dihydroflavonols and flavan-3,4-diols, which are precursors of other flavonoids named flavonols, flavan-3-ols, proanthocyanidins and anthocyanidins. Flavanones are also the precursor molecules to isoflavones and flavones, which are common in *Citrus* fruits (Macheix

et al. 1990). Recently, enzymes that can convert anthocyanidins into the corresponding flavan-3-ols in the biosynthesis of condensed tannins were discovered (Marles et al. 2003, Xie et al. 2004). Gallic acid may arise through the same biosynthetic pathways as the other hydroxybenzoic acids, although a direct aromatisation of dehydroshikimic acid is considered to represent the main pathway (Haddock et al. 1982). Gallic acid is the biosynthetic precursor structure to hydrolysable tannins and ellagic acid (Haddock et al. 1982).

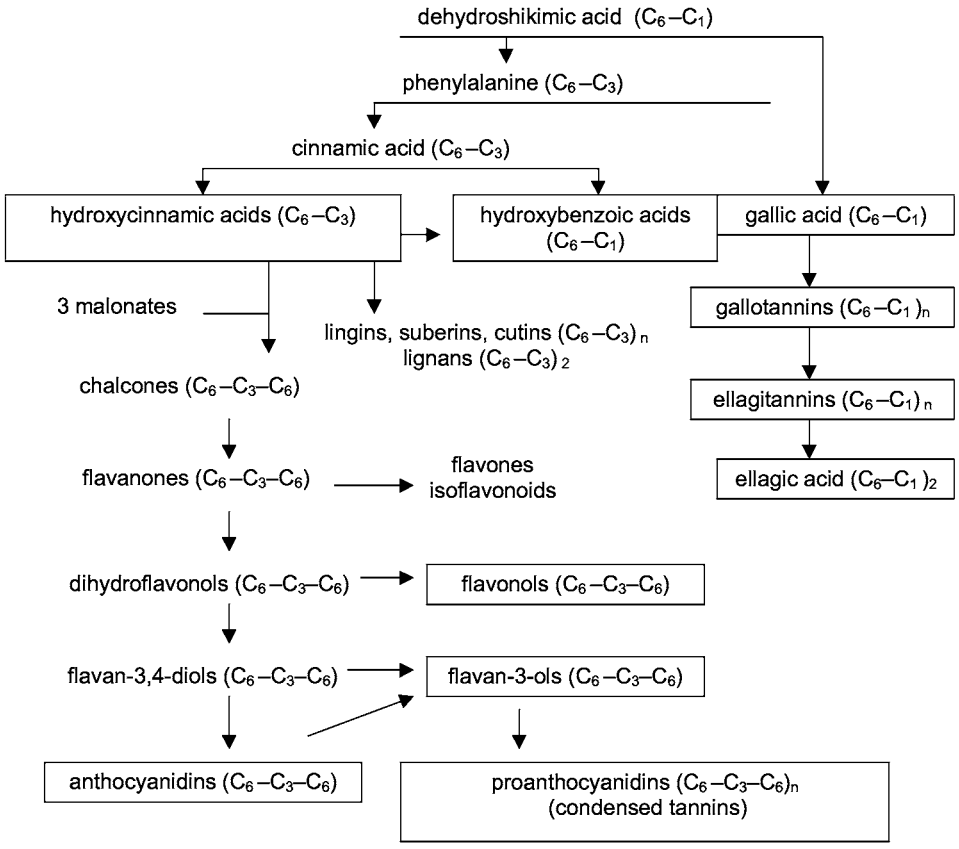
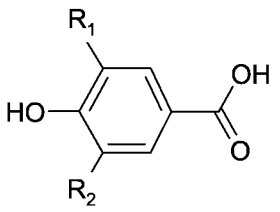


Figure 2. Biosynthetic relationships of phenolic compounds (Haddock et al. 1982, Winkel-Shirley 2001, Winkel-Shirley 2002, Marles et al. 2003, Schijlen et al. 2004, Xie et al. 2004).

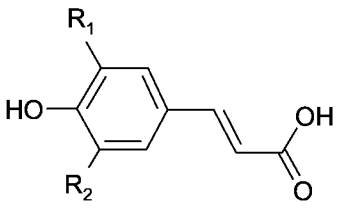
2.2.2 Phenolic acids

Hydroxybenzoic acids and hydroxycinnamic acids

The structures of hydroxybenzoic and hydroxycinnamic acids have a similar backbone of an aromatic ring with variations in the hydroxylation and methoxylation patterns (**Figure 3**). The four common hydroxybenzoic acids are *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids, and the corresponding hydroxycinnamic acids with the three carbon side chain are *p*-coumaric, caffeic, ferulic and sinapic acids, respectively (Macheix et al. 1990). The double bond in the side chain of hydroxycinnamic acids is most frequently in the *trans* form (Strack 1997). Insoluble hydroxybenzoic and hydroxycinnamic acids are constituents of complex polymers in lignins, suberins and cutins (Macheix et al. 1990). Moreover, ferulic acid has been found to be ester- or ether-linked to polysaccharides and other cell wall components (Faulds and Williamson 1999). The soluble forms of hydroxybenzoic and hydroxycinnamic acids occur in various conjugates and quite seldom in free forms in the cytoplasm of the plant cell (Ibrahim and Barron 1989). The carboxyl group may be esterified with sugars or quinic acid, shikimic acid or other organic acids, or the phenolic oxygen may be glycosylated with sugar (Herrmann 1989). The most frequently encountered sugar in the conjugates is glucose (Strack 1997). Trivial nomenclature is commonly used for caffeic acid esterified with hydroxyl groups at C-3, C-4 and C-5 of quinic acid. Chlorogenic acid is 5-ester (5-caffeoylquinic acid), neochorogenic acid is 3-ester (3-caffeoylquinic acid) and cryptochlorogenic acid is 4-ester (4-caffeoylquinic acid).



Hydroxybenzoic acids:
p-Hydroxybenzoic acid $R_1 = R_2 = H$
Protocatechuic acid $R_1 = OH$, $R_2 = H$
Vanillic acid $R_1 = OCH_3$, $R_2 = H$
Syringic acid $R_1 = R_2 = OCH_3$,



Hydroxycinnamic acids:
p-Coumaric acid $R_1 = R_2 = H$
Caffeic acid $R_1 = OH$, $R_2 = H$
Ferulic acid $R_1 = OCH_3$, $R_2 = H$
Sinapic acid $R_1 = R_2 = OCH_3$,

Figure 3. Chemical structures of hydroxybenzoic and hydroxycinnamic acids.

Gallic acid, ellagic acid and hydrolysable tannins

Gallic acid is a trihydroxylated benzoic acid which participates in the formation of hydrolysable gallo- and ellagitannins. The simple soluble forms of gallic acid (**Figure 4**) occur as a free acid, as conjugates with glucose and quinic acid, or combined with flavan-3-ols (Macheix et al. 1990). Ellagic acid (**Figure 4**) is found free and in methoxylated and glycosylated forms, and as a part of uncharacterized polymers (Zafrilla et al. 2001, Mullen et al. 2003). Hydrolysable tannins are complex polymeric phenolic compounds which may be degraded under hydrolytic conditions into sugars, gallic acid and ellagic acid (Bate-Smith 1972, Lei et al. 2001). Hydrolysable tannins consist of a polyol core (e.g. glucose or quinic acid) esterified with either gallic acid(s) in gallotannins or/and hexahydroxydiphenic acid(s) (HHDP) in ellagitannins (**Figure 4**). Additional galloyl residues may be attached to glucose *via* the so-called *meta*-depside bonds (Haddock et al. 1982). Mono- and digalloyl esters of glucose are not classified as gallotannins, since they lack the typical property of tannins to complex strongly with carbohydrates and proteins (Porter 1989). After acid hydrolysis of ellagitannins, HHDP is released and spontaneously lactonized to ellagic acid (Bate-Smith 1972). Gallo- and ellagitannin monomers can be further oxidized in plants to form dimers, trimers and tetramers with molecular weights up to 4000 (Clifford and Scalbert 2000). Simple conjugates are soluble forms, but the highly polymerized ellagitannins may be considered as insoluble forms of phenolic compounds.

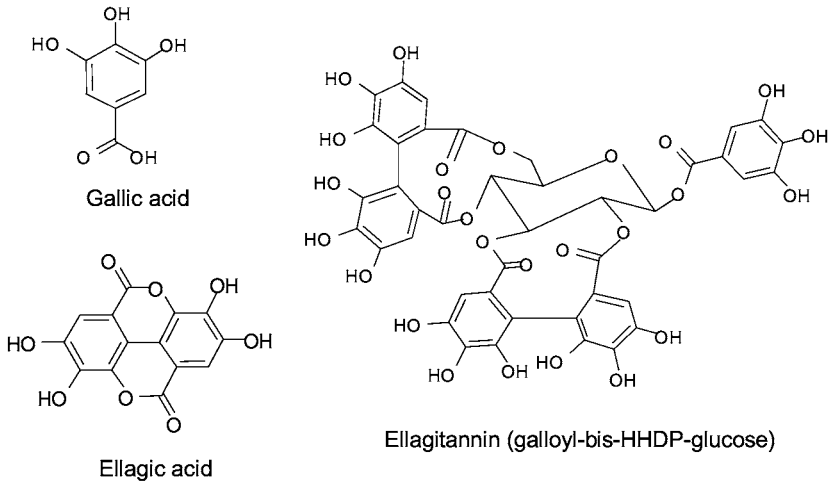


Figure 4. Chemical structures of gallic acid, ellagic acid and ellagitannin.

2.2.3 Flavonoids

Flavonols

Flavonols are flavonoids characterized by a double bond between C-2 and C-3 and by the presence of a hydroxyl group at C-3. The most typical flavonols are quercetin, kaempferol, myricetin and isorhamnetin (**Figure 5**) (Hollman and Arts 2000). Flavonols occur usually as *O*-glycosides, with the sugar moiety preferentially on the hydroxyl group at the 3-position. Other possible glycosylation sites at positions 7 and 4' are more rare in plant foods (Aherne and O'Brien 2002). The possible sugar residues are D-glucose, D-galactose, L-rhamnose, L-arabinose, D-xylose, and D-glucuronic acid (Aherne and O'Brien 2002). All these sugars are found in monoglycosides, but also in diglycosides and occasionally in triglycosides (Macheix et al. 1990). The most widespread diglycosides are 3-rutinosides in which rhamnose and glucose residues are joined by a 1→6 bond (Robards and Antolovich 1997). Flavonol glycosides have been found acylated with simple organic acids like acetic acid and malonic acid as well as hydroxybenzoic and hydroxycinnamic acids, but they have only rarely been reported in edible plants (Macheix et al. 1990, Nielsen et al. 1993, DuPont et al. 2000). Flavonol glycosides are considered soluble according to their molecular structure (Markham 1989).

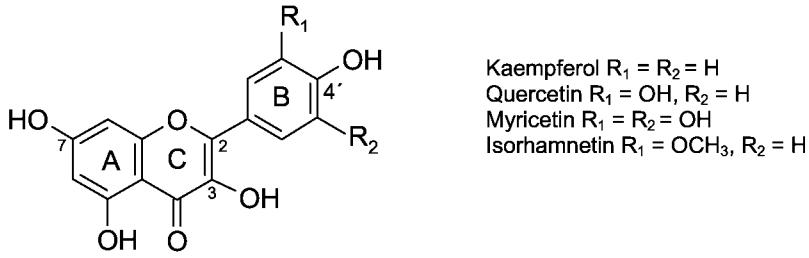


Figure 5. Chemical structures of flavonols.

Anthocyanins

Anthocyanins are pigments which give fruits and berries their variable colours from light red to bluish-black or reddish-black. Anthocyanin is a glycoside composed of an aglycon named anthocyanidin and a sugar residue. The basic structure of anthocyanidin, flavylium phenyl-3-benzopyrylium cation, exists in equilibrium with other mesomeric forms such as the quinoidal base, the carbinol pseudobase and the chalcone pseudobase in acidic aqueous solutions (Strack and Wray 1989). The six most widespread anthocyanidins are cyanidin, delphinidin, peonidin, pelargonidin, petunidin and malvidin (**Figure 6**) (Strack and Wray

1989). Anthocyanidins occur most commonly as *O*-glycosides. The possible sugar residues are D-glucose, D-galactose, L-rhamnose, L-arabinose and D-xylose (Strack and Wray 1989). These five sugars are involved in the formation of monoglycosides, in which a sugar residue almost always is located at the hydroxyl group of C-3. Diglycosides may be formed when another sugar is linked to monoglycoside at C-3 or to another hydroxyl group at C-5. More rare triglycosides may be formed similarly from diglycosides. Three linked sugars in a side chain may form linear or branched structures. Acylated anthocyanins are typically found in some plant foods such as blueberry, red onion and potato (Gao and Mazza 1995a, Ferreres et al. 1996, Clifford 2000a). Acylation by phenolic acid appears to be related to the stabilization of anthocyanins in the acid environment of the cell sap (Strack and Wray 1989). Anthocyanins are considered soluble because they are charged molecules.

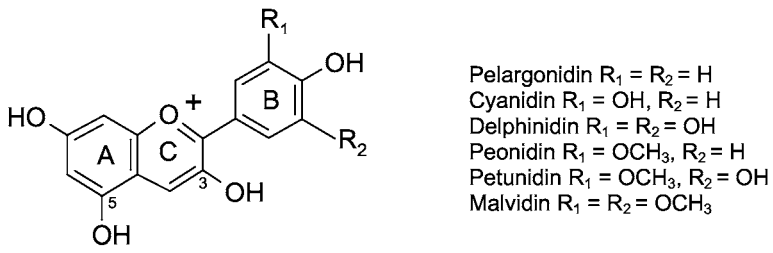
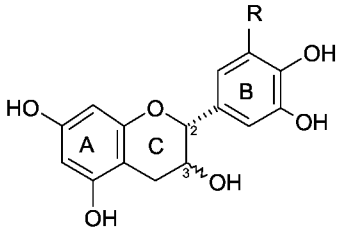


Figure 6. Chemical structures of anthocyanidins.

Flavan-3-ols and proanthocyanidins

Flavans are characterized by a saturated ring C (**Figure 7**). Flavan-3-ols are found as monomers as well as structural units in proanthocyanidin chains, also known as condensed tannins (Porter 1989). The four commonly occurring flavan-3-ols are (+)-catechin, (-)-epicatechin, (+)-gallocatechin and (-)-epigallocatechin (**Figure 7**) (Rohr et al. 2000). This common nomenclature with the sign of the optical rotation of flavan-3-ols at the sodium D line is still used despite contravening I.U.P.A.C rules (Porter 1989). These monomeric flavan-3-ols are generally found free, but there are also some reports on glycosylated or esterified forms in plant food (Gu et al. 2003a).



(+)-Catechin (2R, 3S) R = H
(-)-Epicatechin (2R, 3R) R = H
(+)-Gallocatechin (2R, 3S) R = OH
(-)-Epigallocatechin (2R, 3R) R =OH

Figure 7. Chemical structures of flavan-3-ols.

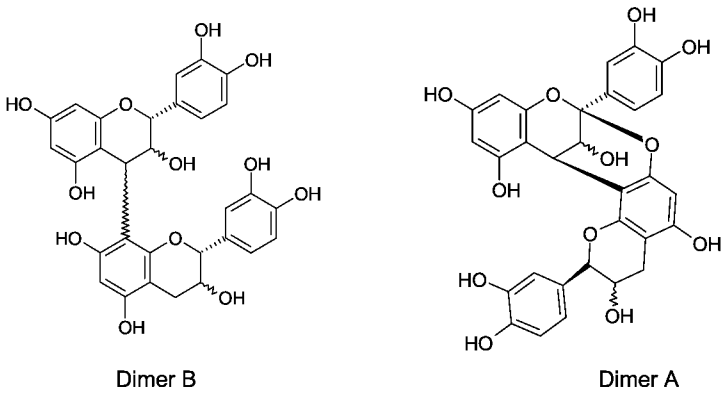


Figure 8. Chemical structures of B- and A- type proanthocyanidins.

Proanthocyanidins are named by their characteristic hydrolysis in acidic medium which yields anthocyanidins. The trivial name, condensed tannin, is based on the traditional use of proanthocyanidins as tanning agents to complex with proteins and on their condensation reactions in acidic and basic media. The structural diversity of proanthocyanidins is based on the monomer units of (epi)catechins, (epi)gallocatechins and (epi)afzelechins, on the different types of interflavonoid bonds and on the different length of chains (Foo and Porter 1981, Gu et al. 2002). Anthocyanidins, released by acid hydrolysis, are used in the classification of proanthocyanidins as procyanidins, prodelphinidins and propelargonidins. Carbon-carbon bonds C4→C8 or C4→C6 link the flavan-3-ol units in the B-type proanthocyanidin chain. In the respective A-type proanthocyanidins, a C4→C8 bond and an ether bond O7→C2 are found (**Figure 8**). **Table 1** shows the trivial and official nomenclature of major A- and B-type dimeric proanthocyanidins found in plant foods. The length of the proanthocyanidin chains may vary from dimers to high molecular weight polymers (up to 150 000) (Santos-Buelga and Scalbert 2000). Polymeric proanthocyanidins form helical structures in which the central core

is composed of rings A and C of the flavan repeat unit and ring B projects laterally from this core.

Table 1. Nomenclature of the most typical procyanidins in plant food (Rohr et al. 2000).

Trivial name	Official nomenclature
B1	Epicatechin-(4β→8)-catechin
B2	Epicatechin-(4β→8)-epicatechin
B3	Catechin-(4α→8)-catechin
B4	Catechin-(4α→8)-epicatechin
B5	Epicatechin-(4β→6)-epicatechin
B7	Epicatechin-(4β→6)-catechin
A1	Epicatechin-(2β→ 7, 4β→8)-catechin
A2	Epicatechin-(2β→ 7, 4β→8)-epicatechin
C1	Epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin
C2	Catechin-(4β→8)-catechin-(4β→8)-catechin

2.3 Methods for analysis of phenolic compounds

The development of analytical methods for phenolic compounds has a long and eventful history since year 1957 (Harborne 1989). Analytical problems and choices for methods have been previously reviewed (Merken and Beecher 2000b, Tura and Robards 2002, Robbins 2003, Mazza et al. 2004). The choice of method and sample handling strategy depends partly on how the results are to be applied since the requirements are different, e.g. systematic studies in botany may require different techniques than those aiming at the compilation of food composition databases.

2.3.1 Sample handling strategies

Storage and homogenization

The first challenges are in the sampling and in the storage of plant material prior to analysis. Analyses can rarely be conducted directly after harvest and, therefore, the storage conditions need to be carefully chosen to maintain the original distribution and contents of the unstable phenolic compounds. Freezing in liquid nitrogen rapidly disrupts the biosynthetic reactions in the living plant cells and these conditions prevail at –70 °C. Freezing at –20 °C for a shorter period may be considered adequate for food composition analysis (Häkkinen et al. 1999b, Häkkinen et al. 2000b). Samples are frequently freeze-dried for the storage and for sample pre-treatment (Hertog et al. 1992b, Justesen et al. 1998, Arts et al. 2000b, Kähkönen et al.

2001, Goncalves et al. 2004, Gu et al. 2004). The next step in the sample handling is solubilizition of phenolic compounds in solvents. The sample matrix is milled or crushed to a fine powder to increase the surface area and to facilitate maximun release of phenolic compounds during solvent extraction and chemical hydrolysis of the sample.

Solvent extraction

The extractability of soluble phenolic compounds is influenced by their chemical nature and the extraction method employed. The ultimate goal is the preparation of sample extract, where all components of interest are quantitatively enriched and which are free from interfering matrix components. There are no completely satisfactory extraction conditions for all the soluble phenolic compounds, due to the variable degrees of polarity of both solvents and compounds (Tura and Robards 2002, Robbins 2003, Mazza et al. 2004). Moreover, the choice of extraction solvent depends on the food matrix.

Methanol, ethanol, acetone, water, ethyl acetate and their combinations are the most frequently used solvents (Merken and Beecher 2000b, Bloor 2001, Tura and Robards 2002, Robbins 2003, Mazza et al. 2004). Alcoholic-aqueous solvents are the predominant means of extraction of the various forms of phenolic compounds (Winter and Herrmann 1986, Sakakibara et al. 2003, Goncalves et al. 2004, Schütz et al. 2004). Ethyl acetate is immiscible with aqueous solvents and this can be utilized in the enrichment and purification of phenolic compounds (Bomser et al. 1996, Foo et al. 2000a, Alasalvar et al. 2001, Slimestad and Solheim 2002) as well as in the extraction of phenolic acids from hydrolysates and from coffee beverages (Andreasen et al. 2000, Mikkonen et al. 2001, Nardini et al. 2002). Aqueous acetone is considered to be the most effective solvent to dissolve polymeric condensed and hydrolysable tannins, especially from fresh tissue, due to its ability to break hydrogen bonds (Porter 1989, Rohr et al. 2000, Tura and Robards 2002). The new extraction techniques, e.g. supercritical fluid and microwave assisted extractions, have enabled reduced solvent consumption and analysis time (Tura and Robards 2002).

Complete extraction may be the most frequent goal in choosing the extraction solvent, but sometimes it is preferable to avoid chemical modification such as destruction and formation of artifacts during the extraction process (Tura and Robards 2002). An alternative goal is to macerate the fresh plant matrix in the extraction solvent for uniform plant-solvent suspension (Tura and Robards 2002). High temperatures and long extraction times should be avoided especially in the case of reactive proanthocyanidins (Rohr et al. 2000). Extraction of anthocyanins should be carried out under acidic conditions to obtain the highest yields of flavylium forms which are coloured red and stable in a high acid medium (Strack and Wray 1989). Mineral acid (e.g. HCl) is commonly used, but in the case of acylated anthocyanins, an organic acid (e.g. formic acid) is favoured (Strack and Wray 1989, Mazza et al. 2004). Acidification is recommended in extraction of condensed and hydrolysable tannins, since the

increased polarity of the acidified solvents shifts partition equilibrium of polar components towards solvent medium (Hagerman and Klucher 1986, Clifford and Ramirez-Martinez 1991). Flavan-3-ols may be destroyed in acidified solvents (Porter 1989), whereas flavonol glycosides are generally considered as stable compounds (Markham 1989). In some cases, plant extracts need to be purified to remove chlorophyll, excessive sugars or other disturbing compounds prior to analysis (Tura and Robards 2002). Simple techniques for purification include liquid-liquid extraction and solid phase extraction e.g. on a reversed-phase cartridge (Bloor 2001, Tura and Robards 2002, Mazza et al. 2004).

Hydrolysis

Ester or glycosidic bonds combine phenolic compounds to the conjugates or to the plant cell structures. These bonds may be broken by chemical or enzymatic hydrolysis. Enzymatic hydrolysis optimally cleaves specific bonds without additional destruction of compounds, and is favoured in the analysis of low concentrations of phenolic compounds in the human body fluids and in the isolated plant tissues (Kanes et al. 1993, Manach et al. 2004). However, it may not be used for the analysis of wide ranges of conjugates due to specificity reasons. Traditional chemical hydrolysis conducted with acid (e.g. hydrochloric acid) or base (e.g. sodium hydroxide) has been used to release phenolic compounds in the plant extracts, juices or whole plant samples (Peleg et al. 1991, Hertog et al. 1992b, Rommel and Wrolstad 1993, Häkkinen et al. 1999b, Vuorinen et al. 2000, Hernanz et al. 2001, Sun et al. 2001, Mattila and Kumpulainen 2002, Sellappan et al. 2002). Cell wall bound phenolic compounds are thought to be released in the hydrolysis of the whole sample. Optimal release was achieved for esters in alkaline conditions and for glycosides in acidic conditions (Häkkinen et al. 1999b, Andreasen et al. 2000, Hernanz et al. 2001, Mattila and Kumpulainen 2002, Nardini et al. 2002). However, both types of chemical hydrolyses may break both ester and ether linkages depending on the prevailing conditions such as temperature and concentration of acid or base (Rommel and Wrolstad 1993, Sun et al. 2001). The release of aglycons allows their practical quantitative analysis, since fruits contain multiple conjugates for which reference compounds are not available. Most frequently hydrolysis for quantitative purposes is made separately for one phenolic class, which may be due to variations in the optimal hydrolysis conditions between the different classes (Häkkinen et al. 1999a). Hydrolysis may also be used as part of the structural analysis of phenolic conjugates to identify the aglycon or to release the acyl group attached to the sugar residue (Markham 1982, Bloor 2001).

Phenolic acids have been released from the conjugates and the plant matrix using acid hydrolysis, base hydrolysis (Peleg et al. 1991, Rommel and Wrolstad 1993, Häkkinen et al. 1999a, Andreasen et al. 2000, Hernanz et al. 2001, Nardini et al. 2002), or their combination (Sun et al. 2001, Mattila and Kumpulainen 2002). Method optimization revealed that the traditional mild alkaline hydrolysis and acid hydrolysis released only part of the ester- and

ether-linked phenolic acids (Sun et al. 2001). Therefore, Sun et al. (2001) recommended using hot alkaline hydrolysis for the cleavage of both types of bonds. Moreover, phenolic acids were more stable during alkaline than acid hydrolysis (Mattila and Kumpulainen 2002). According to Robbins (2003), there are no truly robust sample preparation techniques available for total phenolic acids.

Flavonols and anthocyanidins occur mostly as soluble glycosides. Validated methods based on acid hydrolysis have been developed for the analysis of flavonol aglycons (Hertog et al. 1992b, Häkkinen et al. 1999b, Sellappan et al. 2002). Anthocyanins have also been analysed as anthocyanidin aglycons (Nyman and Kumpulainen 2001, Zhang et al. 2004), but less frequently since there is often destruction of the aglycons (Mazza et al. 2004) and the formation of the same depolymerization products of proanthocyanidins. Flavonoids are considered to be quite stable during acid hydrolysis since the recoveries of respective standard compounds have been adequate (Häkkinen et al. 1999b, Nyman and Kumpulainen 2001). Recoveries of standards from various plant matrices may be used for correction of the quantified concentrations of flavonols (Häkkinen et al. 1999b).

Condensed and hydrolysable tannins are found both in soluble and insoluble forms. Therefore, the methods based on hydrolysis may be considered essential for their analyses. Condensed tannins (*syn.* proanthocyanidins) are depolymerized to anthocyanidins under acid treatment (Bate-Smith 1954). This conversion reaction has been studied for kinetics and yields (Rohr et al. 2000) and has been optimized for higher yields using metal ions to accelerate oxidation (Porter et al. 1986). However, the yields have remained low, due to other products formed in side reactions (Powell et al. 1995). The best possible standard would be a proanthocyanidin fraction purified from the plant matrix under analysis, but there may still remain problems such as the biphasic calibration curve and a decrease of color yield during storage of the isolate (Rohr et al. 2000). Depolymerization of condensed tannins in the presence of benzylmercaptan (thiolysis) or phloroglucinol is used in the studies on the content of oligo- and polymeric proanthocyanidins, the nature of the constitutive flavanol units and degree of polymerization (Santos-Buelga and Scalbert 2000, Gu et al. 2003a, Guyot et al. 2003).

Hydrolysable tannins (gallotannins and ellagitannins) are depolymerized by hydrolysis to gallic acid and ellagic acid, respectively (Bate-Smith 1972, Lei et al. 2001). Maximum yields of deconjugation products have been evaluated and found to be achieved in acidic (Daniel et al. 1989, Maas et al. 1991, Wang et al. 1994, Häkkinen et al. 2000a, Lei et al. 2001) rather than in alkaline conditions (Rommel and Wrolstad 1993). It is recommended that hydrolysis should be performed in alcoholic-aqueous solutions, because of the poor solubility of released ellagic acid in water (Daniel et al. 1989). The most frequently used acids are hydrochloric acid (Häkkinen et al. 2000a, Wada and Ou 2002) and trifluoroacetic acid (Daniel et al. 1989, Maas et al. 1991, Wang et al. 1994). Acid hydrolysis has been performed for macerated plant-

solvent suspensions (Häkkinen et al. 2000a, Lei et al. 2001, Mattila and Kumpulainen 2002) or for plant extracts (Maas et al. 1991, de Ancos et al. 2000, Wada and Ou 2002). Higher yields of ellagic acid from berry-solvent suspension were obtained using HCl instead of trifluoroacetic acid and a hydrolysis time of 20 hours instead of 2 hours (Häkkinen et al. 2000a). Ellagic acid has been found to be stable in severe and long acid hydrolysis conditions (Häkkinen et al. 2000a, Lei et al. 2001).

2.3.2 Methods for separation, identification and quantification

Separation of phenolic compounds

The classical paper and thin-layer chromatographic procedures have been replaced by the modern instrumental techniques and are nowadays used to a variable extent in screening and preparative separations (Harborne 1989, Bloor 2001, Mazza et al. 2004). Phenolic acids may be analysed as volatile derivatives using gas chromatography (Zuo et al. 2002, Robbins 2003), and flavonoids, especially anthocyanins, may be separated by capillary zone electrophoresis (Bridle and García-Viguera 1997, da Costa et al. 2000, Mazza et al. 2004). However, the most frequently used analytical technique for the separation of phenolic compounds is high-performance liquid chromatography (HPLC) (Hyound and Hong 1992, Merken and Beecher 2000a). Reversed-phase (RP) columns are routinely used for separation of all classes of phenolic compounds by HPLC (Hyound and Hong 1992, Robards and Antolovich 1997, Robbins 2003, Mazza et al. 2004), except for proanthocyanidins which may be separated according to their sizes in normal-phase columns (Hammerstone et al. 1999, Saucier et al. 2001, Gu et al. 2003b). The solvent systems used in RP-separation include gradient elution using aqueous phase and organic modifier such as methanol or acetonitrile (Merken and Beecher 2000b, Robbins 2003). The resolution of phenolic compounds is achieved by the choice of pH and the ionic strength of mobile phase (Hyound and Hong 1992, Merken and Beecher 2000a, Rohr et al. 2000, Bloor 2001). Surprisingly reproducible elution orders have been reported for low molecular weight proanthocyanidins (Rohr et al. 2000) and anthocyanins (Goiffon et al. 1991) and other flavonol glycosides except rutinosides (Pietta et al. 1994, Bengoechea et al. 1997, Schieber et al. 2001, Tomás-Barberán et al. 2001, Mullen et al. 2002a). Therefore, flavonoids may be partly characterized by the retention order or peak profiles in commonly used gradients in RP-HPLC systems. The diversity of phenolic compounds in plant extracts complicates their simultaneous separation in HPLC analysis (Merken and Beecher 2000b), but these problems can be overcome by developing novel solvent systems and more selective columns (Sakakibara et al. 2003). The remaining challenge is the limited number of standards available for quantification of native conjugated forms of phenolic compounds.

Identification and quantification of phenolic compounds

Traditionally, identification of isolated phenolic compounds has been based on spectral procedures using ultraviolet-visible (UV-Vis), infra-red (IR), mass (MS) and nuclear magnetic resonance (NMR) spectrometry and fluorometry (Markham 1982, Harborne 1989). Nowadays, these procedures are more frequently used as detection techniques after the separation of phenolic compounds in HPLC (Harborne 1989). Electrochemical detectors are available for phenolic compounds with oxidative or reducing properties (Milbury 2001). UV-Vis absorption has become the most popular detection for HPLC systems (Merken and Beecher 2000b, Robbins 2003, Mazza et al. 2004). Variable wavelength detectors monitor the absorption of phenolic compounds simultaneously and separately at selected wavelengths. However, the collection of on-line UV-Vis absorption spectra by the use of diode-array detection (DAD) is currently the most commonly used technique for the routine qualitative and quantitative analysis of phenolic compounds (Escarpa and Gonzalez 1999, He et al. 2000, Schieber et al. 2001). The UV-Vis absorption spectra enable identification and classification of chromatographic peaks into phenolic classes according to the respective spectra of authentic standards (Harborne 1989).

Identification of the structural units of isolated compounds has traditionally been made after controlled enzymatic or chemical cleavages (Markham 1982, Harborne 1989). Some structural features such as substitution patterns can be identified by interpretation of changes in UV-Vis spectrum after adding shift reagents (Markham 1982). The development of soft ionization techniques in mass spectrometry (MS) combined to HPLC has partly replaced these classical procedures. They provide the molecular masses in MS, and when tandem MS is used, some restricted structural data on the thermally labile, non-volatile polar phenolic compounds may be obtained (Cuyckens and Claeys 2004). Ionization may be performed in the positive and/or the negative ion mode, depending on the chemical nature of the compounds. In the negative ionization mode, the acidic structure of hydroxybenzoic and hydroxycinnamic acids means that they deprotonate readily (Tomás-Barberán et al. 2001), and in the positive ionization mode, they form adducts with the cations in the sample or mobile phase, mostly with sodium and ammonium ions (Swatsitang et al. 2000, Baderschneider and Winterhalter 2001). Depending on the chromatographic conditions, monomeric flavan-3-ols and dimeric and trimeric proanthocyanidins favour both protonation to positive ions (Lin et al. 1993) and deprotonation to negative ions (Poon 1998, Pérez-Magariño et al. 1999, Friedrich et al. 2000, Tomás-Barberán et al. 2001), the latter taking place more easily with the longer proanthocyanidin chains (Hammerstone et al. 1999). Flavonol glycosides have also shown responses in both positive and negative ionization modes (Lin et al. 1993, Häkkinen and Auriola 1998, Andlauer et al. 1999), and anthocyanins have been mainly identified as their most common native forms, i.e. positive flavylium cations (Giusti et al. 1999, da Costa et al. 2000). The combination of these ionization modes

of MS may reveal more details on the structures of phenolic compounds (He et al. 2000, Justesen et al. 2000). The common tandem technique of DAD and MS is becoming more popular in the tentative identification of phenolic compounds in plant extracts. The latest technological approach is the use of HPLC-NMR in the identification of phenolic compounds but its use is still restricted because of cost and required operation expertise.

2.3.3 Simple methods for analysis of total contents

Traditional quantification of total anthocyanin content is based on light absorbance in alcoholic-aqueous solution at the visible wavelengths of 500-550 nm (Strack and Wray 1989). The same simple strategy has been applied for other phenolic compounds, such as flavonols in onion which can be measured at their absorption maxima 340-360 nm (Lombard et al. 2002). In the case of phenolic compounds other than anthocyanins, the contribution of interfering compounds in a mixture should be accounted for (Bloor 2001). Another spectrophotometric method for total anthocyanin content is the pH differential method frequently used in recent publications (Moyer et al. 2002, Zheng and Wang 2003). Absorbance is measured at two different pH levels to differentiate the true content of anthocyanins from the interfering compounds, e.g. decomposition products of anthocyanins.

Even in the latest studies, the widely used method for the estimation of total phenolic compounds in plant extracts, foods, and beverages is the Folin-Ciocalteu assay (Harborne 1989, Goncalves et al. 2004). This method is based on redox reactions, which means that non-phenolic compounds with this chemical property interfere with the assay. Chemical assays are often used for the simple analysis of variable sizes and forms of proanthocyanidins. Vanillin and dimethylaminocinnamaldehyde assays are the most common methods based on chemical reactions with the functional groups of proanthocyanidins, even though there are some problems with specificity and sensitivity (Rohr et al. 2000). Nowadays, the former method has occasionally been used as a support for instrumental analyses (Papagiannopoulos et al. 2004) and the latter has been used as a post-column derivatization reaction in liquid chromatographic analysis of proanthocyanidins (Treutter et al. 1994, de Pascual-Teresa et al. 1998).

2.4 Phenolic compounds in plant foods

In this section, beverages, cereals, vegetables and fruits most rich in particular phenolic compounds are briefly reviewed with the special emphasis on berries. However, the best dietary sources of phenolic compounds depend on the amount of each food item consumed. This means that the relevant comparisons of contents are made by serving based calculations or by comparing to the food consumption databases, as has been done in recent review articles

(Clifford 2000b, Clifford 2000a, Hollman and Arts 2000, Manach et al. 2004). Destruction of phenolic compounds, especially phenolic acids and flavan-3-ols, may be remarkable under boiling, oven baking, microwaving and other processing conditions (Clifford 2000b, Clifford 2000a, Hollman and Arts 2000, Tomás-Barberán and Clifford 2000). In the following section, literature data on the contents have been collected for unprocessed plant foods and beverages on a fresh weight (FW) basis. Scientific names are shown when there are results for two or more species from the same family. The contents have been recalculated for the phenolic residues using conversion factors based on molecular weights and fresh weights using the reported estimated water contents of foods (Belitz and Grosch 1999). The composition of phenolic compounds is further evaluated for the conjugated soluble and insoluble forms, since these forms may contribute to the bioavailability of phenolic compounds.

2.4.1 Hydroxybenzoic acids

p-Hydroxybenzoic acid, vanillic acid, syringic acid and protocatechuic acid are widely distributed in plant foods (Robbins 2003). The occurrence of gallic acid is discussed in connection of hydrolysable tannins. Estimation of the total hydroxybenzoic acid content is complicated. Firstly, phenolic acids are found both as soluble conjugates and insoluble constituents in lignins and fibre (Macheix et al. 1990). Most studies report the hydroxybenzoic acid contents only for soluble forms (Tomás-Barberán and Clifford 2000). Secondly, large differences are found in hydroxybenzoic acid contents between varieties of the same species (Herrmann 1989).

Glucosides of *p*-hydroxybenzoic, vanillic and protocatechuic acids have been the most frequently encountered conjugates of hydroxybenzoic acids in berries and fruits, but their contents remain at low levels (Herrmann 1989). The analysis of hydrolysed extracts revealed that the content of *p*-hydroxybenzoic and protocatechuic acids in blackberry (70-200 mg/kg), black currant (10-60 mg/kg) and raspberry (40-60 mg/kg) (Mosel and Herrmann 1974, Stöhr and Herrmann 1975b) were higher than in other plant foods (Tomás-Barberán and Clifford 2000). However, potatoes with 50-250 mg/kg of hydroxybenzoic acids (Lewis et al. 1998) can be considered as a more relevant food source for these compounds due to the higher consumption of potatoes than berries (Tomás-Barberán and Clifford 2000).

2.4.2 Hydroxycinnamic acids

One or more of the common hydroxycinnamic acids, *p*-coumaric acid, caffeic acid, ferulic acid or sinapic acid, are present in nearly all plants (Ibrahim and Barron 1989). These acids are found in insoluble forms as structural components and in soluble forms as simple conjugates (Ibrahim and Barron 1989). The diversity of these forms makes the comparison of

quantitative results complex, especially due to the variable analytical methods used (Robbins 2003). Phenolic acids are concentrated into the outer parts of plants (Herrmann 1989). Therefore, hydroxycinnamic acids are mainly found in brans and less in sieved flours in grain products. According to this literature review, blueberry was one of the best dietary sources of hydroxycinnamic acids (**Table 2**). However, it should be noted that other berries such as rowanberries may be relevant sources (Pyysalo and Kuusi, 1974) but adequate quantitative data is lacking.

Spinach and cereal brans were defined as the main dietary sources of *p*-coumaric acid (Clifford 2000b). The soluble forms of *p*-coumaric acid were tartaric acid esters present in spinach and quinic acid esters in sweet cherry, savoy cabbage and apple (Herrmann 1989, Gao and Mazza 1995b, Clifford 1999, Goncalves et al. 2004). Insoluble *p*-coumaric acid was a part of cell walls in barley and rye (Andreasen et al. 2000, Hernanz et al. 2001)

Coffee beverages, blueberry and apple were defined as the major dietary sources of caffeic acid (Clifford 2000b). Caffeic acid was found as a part of chlorogenic acid (5-*O*-caffeoylquinic acid) in coffee, apple, pear, peach, eggplant, corn-salad, potato, carrot, and blueberry (Herrmann 1989, Lewis et al. 1998, Clifford 1999, Alasalvar et al. 2001, Tomás-Barberán et al. 2001, Nardini et al. 2002, Whitaker and Stommel 2003, Taruscio et al. 2004). Moreover, coffee contained other conjugates of caffeic acid released by alkaline hydrolysis (Nardini et al. 2002). Caffeic acid occurred mainly as a part of neochlorogenic acid in sweet cherry and plum (Gao and Mazza 1995b, Tomás-Barberán et al. 2001, Goncalves et al. 2004). Green artichoke head contained caffeic acid in various mono- and diester forms with quinic acid (Schütz et al. 2004). A very high caffeic acid content was reported in lettuce after hydrolysis (800-1600 mg/kg) (Schmidtlein and Herrmann 1975), but much lower as native tartaric acid conjugates (40 mg/kg) (Baur et al. 2004). Similarly, recent results indicated lower chlorogenic acid contents in kiwifruit compared to earlier findings (Macheix et al. 1990, Dawes and Keene 1999).

Coffee, citrus juices, sugar beet fibre and cereal brans were defined as the major dietary sources of ferulic acid (Clifford 2000b). Synytsya et al. (2003) made a rough estimation of the high ferulic acid content directly as a part of the cell walls in sugar beet pulps using spectroscopic methods. The peel structures of orange and grapefruit could be potential sources of ferulic acid, but the contents were lower in juice sacs (Peleg et al. 1991). The ferulic acid content in coffee was substantial but lower than that of caffeic acid (Nardini et al. 2002). The bran layers of cereals such as barley, brown rice, oat groat and rye were rich in ferulic acid dehydrodimers cross-linked with cell wall polymers (Andreasen et al. 2000, Hernanz et al. 2001, Tian et al. 2004).

Broccoli, kale, other leafy brassicas and citrus juices have been described as being the major dietary sources of sinapic acid (Clifford 2000b). However, the content of sinapic acid in savoy cabbage, orange and grapefruit was lower compared to other hydroxycinnamic acids

(Herrmann 1989, Peleg et al. 1991). The typical conjugated form was sinapoylglucose (Herrmann 1989). The content of insoluble sinapic acid was substantial in rye (Andreasen et al. 2000).

Table 2. Dietary sources with high contents of hydroxycinnamic acids (mg/kg FW)[a].

	p-coumaric acid	caffeic acid	ferulic acid	sinapic acid	Ref.
Apple	10	30-200	≤5	NR[b]	1
Artichoke head	NR	830	NR	NR	2
Barley	70-230	5-20	320-530	NR	3
Blueberry	≤5	330-790	30-50	NR	4, 5 6
Brown rice	20	≤5	160	≤5	7
Carrot[c]	≤5	20-270	5-40	NR	1, 8
Coffee	10	830	140	NR	9
Eggplant	NR	260-750	10	NR	1, 10
Grapefruit juice	≤5	10	30	≤5	11
Lettuce	NR	800-1600	NR	NR	12
Oat groat	40-60	20	140-150	≤5	13
Orange juice	≤5	≤5	30	10	11
Peach	≤5	40-200	≤5	NR	1
Plum	20	50-440	10	NR	1
Potato[c]	5-40	20-450	5	NR	1, 14, 15
Rye	30-60	110-970	780-1110	60-120	16
Savoy cabbage	10-60	5-40	≤5	10-20	1
Spinach	100-130	10	10	20-30	1
Sugar beet pulp	NR	NR	1000-4000	NR	17
Sweet cherry	100-650	110-970	<5	NR	1, 18, 19

[a] Contents are expressed as the weight of the phenolic residue. [b]NR, not reported. [c] Range from unpigmented to coloured cultivars. [1]Herrmann 1989, [2]Schütz et al. 2004, [3]Hernanz et al. 2001, [4]Taruscio et al. 2004, [5]Zheng and Wang 2003, [6]Gao and Mazza 1994, [7]Tian et al. 2004, [8]Alasalvar et al. 2001, [9]Nardini et al. 2002, [10]Whitaker and Stommel 2003, [11]Peleg et al. 1991, [12]Schmidtlein and Herrmann 1975, [13]Clifford 2000, [14]Lewis et al. 1998, [15]Mattila and Kumpulainen 2002, [16]Andreasen et al. 2000, [17]Synytsya et al. 2003, [18]Goncalves et al. 2004, [19]Gao and Mazza 1995.

2.4.3 Gallic acid and ellagic acid

Hydrolysable tannins, gallotannins and ellagitannins, and other free and conjugated forms of gallic acid and ellagic acid occur preferentially in woody plants, but are frequently found also in some plant foods (Haddock et al. 1982, Lei et al. 2001). The contents of ellagitannin in different studies have been variable, since the conditions present in the extraction and acid hydrolysis were found to significantly affect the yields of ellagic acid (Clifford and Scalbert 2000, Häkkinen et al. 2000a, Kähkönen et al. 2001, Lei et al. 2001). Therefore, in the following section, the results for ellagitannins are considered according to the principles of sample preparation.

Tea may be considered as the most relevant source of free gallic acid (40-6700 mg/kg in various dry tea leaves) and (gallo)catechin gallates (1600-150 000 mg/kg) (Cabrera et al. 2003). In fermented teas, 38-41% of dry matter was soluble in hot water after brewing for 3 minutes (Belitz and Grosch 1999), which leads to a high gallic acid contents also per litre of tea beverage (recalculated value ~1200 mg/l in black tea) (Cabrera et al. 2003). However, the gallic acid content in tea beverage brewed from a tea bag indicated a much lower content (20 mg/l), which was close to the content of free form reported for berries of American *Rubus* species (3-14 mg/kg) (Tomás-Barberán and Clifford 2000, Wada and Ou 2002) and acid released forms in black currant (30-60 mg/kg) and strawberry (10-40 mg/kg) (Stöhr and Herrmann 1975a, Stöhr and Herrmann 1975b, Tomás-Barberán and Clifford 2000). Hydrolysis may release gallic acid from the simple conjugates or gallotannins. Both methylgallate and gallotannins (tri- and pentagalloylglucose) have been detected in strawberry (Stöhr and Herrmann 1975a, Haddock et al. 1982). Content of gallic acid in common grapevines (*Vitis vinifera*) and spirits may reach 30-40 mg/l during maturation in wood barrels (Tomás-Barberán and Clifford 2000).

Berries of *Rubus* species have been reported as the best dietary sources of ellagic acid (Daniel et al. 1989). The free form of ellagic acid was found in muscadine grape juices (*Vitis rotundifolia,* 4-20 mg/l), pomegranate juice (140 mg/l), strawberry (10 mg/kg) and berries of *Rubus* species such as red raspberry (*R. idaeus,* 30 mg/kg), marionberry (*R. ursinus,* 60 mg/kg) and boysenberry (*R. ursinus x idaeus,* 100 mg/kg) (Tomás-Barberán and Clifford 2000, Wada and Ou 2002, Lee and Talcott 2004, Seeram et al. 2004). Ellagic acid glycosides have been quantified in muscadine grape juices (3-30 mg/l) and red raspberry jam (30 mg/kg) (Zafrilla et al. 2001, Lee and Talcott 2004). However, major amounts of ellagic acid are present as variable sizes of ellagitannins in plant foods (Clifford and Scalbert 2000). The total content of native ellagitannins and other sources of ellagic acid in pomegranate juice was 1770 mg/l (Seeram et al. 2004). In the acetone extracts of cloudberry, raspberry and strawberry, the native ellagitannin contents were 1600-2400, 2500-2600 and 80-180 mg/kg, respectively (Kähkönen et al. 2001). These amounts were different from those achieved by acid hydrolysis of ellagitannin to ellagic acid, since acid released higher amounts from strawberry extracts (40-460 mg/kg) and strawberry-solvent suspensions (310-380 mg/kg) but lower amounts from red raspberry extracts (210-285 mg/kg) and raspberry-solvent suspensions (510-1600 mg/kg) (Daniel et al. 1989, Maas et al. 1991, de Ancos et al. 2000, Häkkinen et al. 2000a, Mattila and Kumpulainen 2002). For other *Rubus* species, the following contents of free and released ellagic acid were obtained: cloudberry 560-600 mg/kg, arctic bramble 690 mg/kg, blackberry (*R. fruticosus)* 1600 mg/kg, black raspberry 135 mg/kg, and marionberry 100 mg/kg (Häkkinen et al. 2000a, Lei et al. 2001, Wada and Ou 2002). The respective total ellagic acid content was 310 mg/kg in pecans, 570 mg/kg in walnuts (Daniel et al. 1989) and 105-320 mg/l in muscadine grape juices (Lee and Talcott

2004). The content of ellagitannins in common grapevine (*Vitis vinifera*) was low, but increased over the maturation period in wood barrels (Tomás-Barberán and Clifford 2000). The major ellagitannins were sanguiin H-6 and lambertianin C in raspberry (Mullen et al. 2002b), pedunculagin in walnut (Cerdá et al. 2005) and punicalagin in pomegranate (Gil et al. 2000, Seeram et al. 2004).

2.4.4 Flavonols

Quercetin, myricetin, kaempferol and isorhamnetin are the most widespread flavonols in plant foods (Markham 1989). The main sources of flavonols in average diets were onions, tea, berries and apples (Hollman and Arts 2000, Häkkinen 2000). The contents of quercetin were below 50 mg/kg in most of the beverages, vegetables and cereals, except in yellow onions, red onions and kale (**Table 3**) (Ferreres et al. 1996, Price and Rhodes 1997, Hollman and Arts 2000). Quercetin is glycosylated mainly with glucose and sophorose (glucose-glucose) in onions (Ferreres et al. 1996, Price and Rhodes 1997, Bonaccorsi et al. 2005). Due to the high consumption, black tea infusion is a substantial source of quercetin (Hertog et al. 1993, Crozier et al. 1997, Justesen et al. 1998, Price et al. 1998). Quercetin rutinoside (glucose-rhamnose) was quite common in fermented black teas whereas triglycosides predominated in green teas (Plumb et al. 1999). With respect to the berries and fruits, the quercetin contents were substantial in apple, bog whortleberry, lingonberry, cranberry, chokeberry, rowanberry, sweet rowanberry, and elderberry (**Table 3**) (Hertog et al. 1992a, Justesen et al. 1998, Kaack and Austed 1998, Häkkinen et al. 1999b, Häkkinen et al. 2000b, Häkkinen and Törrönen 2000). Quercetin is conjugated with various sugars such as glucose, galactose, rhamnose, arabinose (in monoglycosides), rutinose and sophorose (in diglycosides) in berries and apple (Macheix et al. 1990, Escarpa and Gonzalez 1998, Häkkinen and Auriola 1998, Paganga et al. 1999, Zheng and Wang 2003).

A high kaempferol content has been found in kale, endive, and broccoli (Hertog et al. 1992a, Justesen et al. 1998, DuPont et al. 2000). The major kaempferol glycosides were glucoside, glucuronide, and malonylglucoside in endive (DuPont et al. 2000), and sophoroside in broccoli (Vallejo et al. 2004). The myricetin content was substantial in cranberry and black currant (Häkkinen and Auriola 1998, Häkkinen et al. 2000b). The contribution of isorhamnetin to the total flavonol content was mostly low in plant foods, except for red onions (Ferreres et al. 1996).

Table 3. Dietary sources with high contents of flavonols (mg/kg FW)[a].

	myricetin	quercetin	kaempferol	isorhamnetin	Ref.
Apple	NR[b]	20-40	NR	NR	1, 2
Black currant	70-100	40-50	1	NR	2-4
Bog whortleberry	30-120	160-200	NR	NR	3, 5
Broccoli	NR	30-40	60-70	NR	1, 2
Chokeberry	NR	90	NR	NR	3
Cranberry	70-140	80-160	NR	NR	2, 3
Elderberry	NR	30-60	NR	NR	6
Endive	NR	≤5	20-50	NR	1, 7
Kale	NR	110-120	210-470	NR	1, 2
Lingonberry	NR	70-210	5	NR	2-4
Onion, red	NR	200-450	NR	40-230	2, 8-10
Onion, yellow	NR	40-480	NR	NR	2, 9, 11, 12
Rowanberry	NR	60	NR	NR	3
Sweet rowanberry	NR	90	NR	NR	3
Black tea infusion	≤5	5-40	10-20	NR	2, 13, 14

[a] Contents are expressed as the weight of the phenolic residue.[b] NR, not reported. [1]Hertog et al. 1992, [2]Justesen et al. 1998, [3]Häkkinen et al. 1999, [4]Häkkinen et al. 2000, [5]Häkkinen and Törrönen 2000, [6]Kaack and Austed 1998, [7]DuPont et al. 2000, [8]Crozier et al. 1997, [9]Ferreres et al. 1996, [10]Bonaccorsi et al. 2005, [11]Hollman and Arts 2000, [12]Price and Rhodes 1997, [13]Hertog et al. 1993, [14]Price et al. 1998.

2.4.5 Anthocyanins

Anthocyanins are water-soluble pigments. The six most widespread anthocyanidins are cyanidin, delphinidin, peonidin, pelargonidin, petunidin and malvidin in decreasing order of occurrence (Robards and Antolovich 1997). As a simple rule for content: The strongly coloured vegetables, fruits and berries contain high levels of anthocyanins (Macheix et al. 1990, Espín et al. 2000, Kähkönen et al. 2001) and are also considered as the best sources. Due to their high consumption, red wines (100-200 mg/l) and the anthocyanin-based food colorant (E163) are considered as abundant sources of anthocyanins (Manach et al. 2005b). Those dietary sources, with a high amount of one or more anthocyanidins, are presented in **Table 4**.

Cyanidin glycosides are the most typical anthocyanins, these being found in almost all (90%) coloured fruit species (Macheix et al. 1990). A high cyanidin content was quantified as a part of monoglycosides in chokeberry (Zheng and Wang 2003, Wu et al. 2004), blueberry (Gao and Mazza 1994, Zheng and Wang 2003) and cultivated lingonberry (Zheng and Wang 2003). In addition, high total anthocyanins levels with substantial amounts of cyanidin monoglycosides were detected in bilberry, wild lingonberry, crowberry, and rowanberry (the fruit of the mountain ash tree, *Sorbus aucuparia*) (Pyysalo and Kuusi 1974, Kärppä et al. 1984, Andersen 1985, Kalt et al. 1999). Diglycosides co-occurred with monoglycosides in sweet cherry (500-1450 mg/kg) (Gao and Mazza 1995b, Goncalves et al. 2004), black currant and elderberry (Wu et al. 2004). Red raspberry cultivars contained variable composition of

cyanidin mono-, di- and triglycosides (**Table 4**) (de Ancos et al. 1999). Other *Rubus* species (evergreen blackberry, boysenberry, marionberry and black raspberry) contained a higher total anthocyanin content than red raspberry and there was a distinctive composition of acylated cyanidin mono-, di- and triglycosides (Wada and Ou 2002, Cho et al. 2004). Red onions contained both monoglycosides and malonylmonoglycosides (Ferreres et al. 1996).

Delphinidin contents were dominant in blueberry (Gao and Mazza 1994, Zheng and Wang 2003) and black currant (Wu et al. 2004) as well as in bilberry and crowberry, according to the substantial peak areas and the high total anthocyanin content (Kärppä et al. 1984, Kalt et al. 1999). Delphinidin was in form of monoglycoside in berries other than black currant, in which delphinidin rutinoside co-occurred with the glucoside (Macheix et al. 1990). The high amount of delphnidin in eggplants (Paganga et al. 1999, Sakakibara et al. 2003) is accounted for delphinidin-3-(*p*-coumaroylrutinoside)-5-glucoside isolated from peels (Noda et al. 2000) and other delphinidin glycosides (Macheix et al. 1990).

Bilberries and crowberries could be potential sources for petunidin, peonidin and malvidin (Kärppä et al. 1984, Kalt et al. 1999). Other appreciable anthocyanidins were malvidin in blueberry (Gao and Mazza 1994, Zheng and Wang 2003) and red grape (Mazza 1995, Cho et al. 2004) as well as peonidin in cultivated cranberry (*Vaccinium macrocarpon*) (Zheng and Wang 2003) and blackthorn (Espín et al. 2000). Strawberry cultivars contained pelargonidin as mono-, di- and acylmonoglycosides (Wang and Zheng 2001).

Table 4. Dietary sources with high contents of anthocyanidins (mg/kg FW)[a].

	cyanidin	delphinidin	peonidin	pelargonidin	petunidin	malvidin	Ref.
Black currant	530-1040	590-2720	NR[b]	NR	NR	NR	[1]
Blackberry	720-1520	NR	NR	NR	NR	NR	[2]
Blueberry	50-290	210-470	10-190	NR	230	330-690	[3, 4]
Chokeberry	2020-9450	NR	NR	NR	NR	NR	[1, 4]
Cranberry	100	NR	240	NR	NR	NR	[4]
Grape, red	10-240	20-810	20-530	NR	10-960	120-2500	[2]
Eggplant	NR	690-1100	NR	NR	NR	NR	[5, 6]
Elderberry	8750	NR	NR	NR	NR	NR	[1]
Lingonberry	360	NR	30	NR	NR	NR	[4]
Onion, red	130	NR	NR	NR	NR	NR	[7]
Raspberry, red	210-710	NR	NR	NR	NR	NR	[8]
Strawberry	10-40	NR	NR	220-770	NR	NR	[9]
Sweet cherry	130-1450	NR	5-110	5-20	NR	NR	[10, 11]

[a] Contents are expressed as the weight of the phenolic residue. [b] NR, not reported. [1]Wu et al. 2004, [2]Cho et al. 2004, [3]Gao and Mazza 1994, [4]Zheng and Wang 2003, [5]Paganga et al. 1999, [6]Sakakibara et al. 2003, [7]Ferreres et al. 1996, [8]de Ancos et al. 1999, [9]Wang and Zheng 2001, [10]Gao and Mazza 1995, [11]Goncalves et al. 2004.

2.4.6 Flavan-3-ols and proanthocyanidins

Detailed quantitative information has been obtained on the contents of flavan-3-ols and proanthocyanidins only in recent years (Arts et al. 2000a, Arts et al. 2000b, de Pascual-Teresa et al. 2000, Gu et al. 2004). The main sources of flavan-3-ols and dimeric and trimeric proanthocyanidins are tea, chocolate, apples, pears, grapes, and red wine (Manach et al. 2005a). Recently, procyanidin contents ranging from dimers to high molecular weight forms were reported by Gu et al. (2004). Among the beverages, the highest contents of flavan-3-ols were found in black tea infusions (100-420 mg/l) and red wine (20-100 mg/l) (Arts et al. 2000a, Gu et al. 2004). Red wine and grape juice contained also high amounts of proanthocyanidins (290 mg/l and 500 mg/l, respectively) (Gu et al. 2004). Among the plant foods, the highest content of flavan-3-ols and proanthocyanidins was in beans (50-490 mg/kg and 4450-7810 mg/kg, respectively) (Arts et al. 2000b, de Pascual-Teresa et al. 2000, Gu et al. 2004). Substantial amounts of flavan-3-ols have been detected in some fruits, such as cherry (40-120 mg/kg), plum (60-110 mg/kg) and apple (Arts et al. 2000b, de Pascual-Teresa et al. 2000, Gu et al. 2004). The flavan-3-ol content varied greatly among the apple varieties (20-150 mg/kg) with the highest average level being present in Red Delicious (70-150 mg/kg) (de Pascual-Teresa et al. 2000, Hammerstone et al. 2000, Gu et al. 2004). These fruits were also good sources of proanthocyanidins, especially plums (2150-2470 mg/kg) and the apple cultivars, Granny Smith and Red Delicious (870-1400 mg/kg) (Hammerstone et al. 2000, Gu et al. 2004). In a recent study on selected berries, high amounts of proanthocyanidins were quantified in chokeberry (6590 mg/kg), black currant (1200-1650 mg/kg) and gooseberry (450-1330 mg/kg), even though the contents of flavan-3-ols were 50 mg/kg in chokeberry and below 10 mg/kg in black currant and gooseberry (Wu et al. 2004). With respect to the other common berry species, a high flavan-3-ol content has been reported in blackberry (40-190 mg/kg) (Arts et al. 2000b, de Pascual-Teresa et al. 2000). As one of the food sources, chocolate contained a substantial amount of flavan-3-ols and procyanidins (total 3790-4900 mg/kg) (Hammerstone et al. 2000).

2.5 Absorption of phenolic compounds

Absorption of phenolic compounds from the intestine is an important contributor to their bioavailability. Ingested dietary phenolic compounds are absorbed in the gastrointestinal tract after metabolism such as release by hydrolysis and/or conjugation. This section focuses on the absorption of soluble and insoluble forms of phenolic compounds except for the hydroxybenzoic acids. However, it should be noted that these simple phenolic acids are some of the major decomposition products of other larger-mass dietary phenolic compounds formed by the gut flora fermentation processes (Scalbert et al. 2002). The processes involved in the

intestinal and hepatic metabolism (phase 1 and phase 2 transformations), plasma transport and elimination in bile and urine as well as bioavailability have been extensively described for diverse phenolic compounds in recent reviews (Clifford 2000b, Manach et al. 2004, Manach et al. 2005b, Williamson and Manach 2005).

2.5.1 Hydroxycinnamic acids

Soluble portions of free and esterified hydroxycinnamic acids can be metabolized and absorbed in the human gastrointestinal tract (Bourne and Rice-Evans 1998, Kern et al. 2003a, Kern et al. 2003b, Manach et al. 2004). There are no studies which have assessed the absorption of hydroxycinnamic acid glycosides. The absorption efficiency of free caffeic acid was greater than that of chlorogenic acid from coffee, suggesting that esterification reduces the absorption of hydroxycinnamic acids (Olthof et al. 2001, Konishi and Kobayashi 2004b). Chlorogenic acid is probably hydrolysed to caffeic acid prior to absorption in the gastrointestinal tract (Nardini et al. 2002). De-esterification reactions have been shown to occur all along the length of the small intestine (Kern et al. 2003a, Kern et al. 2003b) and also by the microflora of the large intestine (Andreasen et al. 2001). However, there are conflicting opinions on the ability of the tissues to hydrolyse ester bonds (Manach et al. 2004). Chlorogenic acid metabolites of microbial origin have been detected in the urine and plasma of rats, indicating that the ingested chlorogenic acid is metabolized by the colonic microflora instead of being absorbed in the small intestine (Azuma et al. 2000, Gonthier et al. 2003b). Traces detected in plasma samples have been used as evidence for the absorption of hydroxycinnamic acids from tomatoes (Bourne and Rice-Evans 1998), beer (Bourne et al. 2000) and prunes (Cremin et al. 2001). The fibre-bound forms of ferulic acids in cereals must be released by microbial esterases in the colon prior to absorption (Andreasen et al. 2001), otherwise these insoluble forms may be considered as unavailable forms of hydroxycinnamic acids. The presence of cereal matrix has been shown to limit the bioavailability of free ferulic acid (Adam et al. 2002).

The intestinal transport system for hydroxycinnamates is assumed to be the same or similar to that responsible for the active uptake of glucose (Clifford 2000b). The monocarboxylic acid transporter may also be responsible for the absorption of hydroxycinnamic acids and their colonic metabolites (Konishi et al. 2004, Konishi and Kobayashi 2004a, Konishi and Kobayashi 2004b).

2.5.2 Gallic acid and ellagic acid

There are very few reports on the absorption and metabolism of different sources of gallic acid and ellagic acid. 4-*O*-Methylgallic acid, the main metabolite of gallic acid, was detected rapidly in relevant concentrations in human plasma and urine after the ingestion of pure free gallic acid and wine (Shahrzad and Bitsch 1998, Caccetta et al. 2000). It was concluded that free gallic acid is extremely well absorbed, but there is no respective data on its simple conjugates (Manach et al. 2005b). Ellagic acid showed strong affinity for proteins and poor absorption in small animals (Maas et al. 1991). *In vitro* studies with rat intestinal contents showed that ellagitannins can be hydrolysed to ellagic acid at the pH found in the small intestine and cecum but not in the stomach (Daniel et al. 1991). Free ellagic acid was present in human plasma after ingestion of both ellagic acid and ellagitannin from pomegranate juice (Seeram et al. 2004). However, free ellagic acid was not detected in biological fluids in another study. Instead, a new biomarker for ingested ellagitannins, the 3,8-dihydroxydibenzopyran-6-one (urolithin B) derivative, was found in plasma of rats and humans (Cerdá et al. 2003, Cerdá et al. 2005). This biomarker was concluded to be a metabolite produced by gut microflora, since there was extensive variation in the urinary excretion of metabolites between individuals (Cerdá et al. 2005). Urolithin B derivatives represented the excretion products of all forms of ellagitannins as well as ellagic acid glycosides consumed. In a comparison of the sources, urinary excretion was lower for ingested strawberries (2.8%) and red raspberry (3.4%) than for walnuts (16.6%).

2.5.3 Flavonol glycosides and anthocyanins

Flavonols and anthocyanidins are found as soluble glycosylated forms, which influences their absorption from the intestine. There may be some hydrolysis of phenolic glycosides to aglycons in the stomach, but it is thought that most of the glycosides arrive intact in the duodenum (Manach et al. 2004). In the latest studies, interest has been focused on the impact of other food components on the rate of absorption of flavonols and anthocyanins (Bitsch et al. 2004b, Lesser et al. 2004).

Most of the studies have been based on absorption of quercetin as aglycon, glucoside and rutinoside (Hollman and Katan 1998). Aglycon and glucoside of quercetin can be absorbed from the small intestine, whereas at least diglycosides and rhamnoside must reach the colon where they will be hydrolysed by the microflora prior to absorption (Hollman and Katan 1999). Quercetin pentosides such as arabinoside and xyloside probably behave similarly to rhamnoside (Manach et al. 2004), but the fate of galactoside is obscure. As a general rule, glucoside and aglycon are the best absorbable forms of quercetin (Manach et al. 2004). This was also revealed in the higher rates of absorption of quercetin from glucoside in onions

compared to rutinoside and other conjugates in apples and tea (Hollman and Katan 1998, Crozier et al. 2000). Results obtained with quercetin may not be directly applicable to the other flavonols, since the differences in hydroxylation pattern of the B-ring markedly affected their absorption in rats (Hou et al. 2003). However, unabsorbed flavonol glycosides may be responsible for the beneficial health effects of phenolic compounds via their metabolites formed by colon flora (Olthof et al. 2003).

Absorption of anthocyanins appears to be much lower than that of quercetin (Prior 2003, Mullen et al. 2004), and the mechanisms might be different. The coloured flavylium cation form of anthocyanins is thought to exist in the acidic pH of the stomach, but the other base forms will predominate in lower portions of the gastrointestinal tract (Clifford 2000a). Anthocyanidin arabinoside, glucoside and galactoside from bilberry origin were all detected in plasma and urine samples in a rat study (Clifford 2000a). The stomach was shown to be a site of anthocyanin absorption in animal studies and the rapid absorption of anthocyanins from various berry extracts indicated that the same mechanism exists in humans (Manach et al. 2005b). Therefore, the effect of the sugar moiety on absorption is not as clear in the case of anthocyanins as it is in the case of quercetin. The urinary excretion of anthocyanins was reported to be low for all berry extracts and juices studied (Manach et al. 2005b), indicating a similar low absorption for mono- di- and triglycosides. Anthocyanins were more extensively absorbed from elderberry extract than from blackcurrant juice (Bitsch et al. 2004a). The effect of sample handling has not been adequately taken into account in bioavailability studies and the role played by microflora in the absorption of anthocyanins is unclear (Manach et al. 2005b).

Hollman et al. (1995) suggested that quercetin glucoside could be transported into enterocytes by the sodium-dependent glucose transporter. In support of this proposal, it was noted that quercetin glucoside inhibited the sodium-dependent glucose uptake, whereas quercetin galactoside, glucorhamnoside (rutinoside) and the aglycon as well as cyanidin diglucoside were ineffective (Cermak et al. 2004). Anthocyanins were considered to be transported by bilitranslocase in the stomach (Manach et al. 2004).

2.5.4 Flavan-3-ols and proanthocyanidins

Absorption from the intestine differs markedly between different forms of flavan-3-ols and proanthocyanidins. Monomeric flavan-3-ols as well as dimeric and trimeric proanthocyanidins are absorbed at least to some extent (Manach et al. 2005a). Galloylation of flavan-3-ols reduces their absorption (Van Amelsvoort et al. 2001). Proanthocyanidin dimers are absorbed, but at about one hundred fold lower rates than the flavan-3-ol monomers (Holt et al. 2002). In addition, trimeric forms were transported across monolayers of human intestinal epithelial cells in an *in vitro* model (Deprez et al. 2001), but the overall evidence for

the absorption of the dimeric and trimeric proanthocyanidins is scarce (Manach et al. 2005b). Tetrameric and higher polymeric proanthocyanidins are not absorbed as such (Manach et al. 2004), neither are they deconjugated to shorter chain forms in acidic conditions of the stomach *in vivo* (Rios et al. 2002). Microbial metabolites formed in colon are considered to be the major forms, via which the unabsorbed flavan-3-ols and proanthocyanidins can be ultimately be absorbed (Manach et al. 2005b). However, in a rat study the amount of the formed metabolites was found to be reduced by increasing the degree of polymerization from catechin to procyanidin polymers (Gonthier et al. 2003a). It should be noted that the beneficial effects of proanthocyanidins may be due to direct effects on the intestinal mucosa by protecting it against oxidative stress or the actions of carcinogens (Manach et al. 2005b).

3 AIMS OF THE STUDY

The main objective of the present studies was to determine the contents and composition of soluble and insoluble phenolic compounds in selected wild and cultivated berries commonly consumed in Scandinavia. Special emphasis was given to method development for the simultaneous extraction of various phenolic compounds in their native forms.

The specific aims of the individual studies were:

- To study the contents of flavonoids and phenolic acids in berries of *Ribes nigrum* (black and green currants) and *Ribes x pallidum* (red and white currants). (**I**)
- To identify and quantify the individual conjugated forms of phenolic compounds in currants. (**II**)
- To identify and quantify individual conjugated forms of phenolic compounds in four berry species of the Rosaceae family, namely strawberry, red, yellow and wild (red) raspberry, cloudberry and arctic bramble. (**III**)
- To analyse the distribution and contents of the free and conjugated forms of hydroxycinnamic acids, flavonols, anthocyanidins as well as flavan-3-ols and low molecular weight and insoluble proanthocyanidins in various Finnish and Swedish berries. (**IV**)
- To analyse the contents of flavonols, proanthocyanidins and anthocyanins during the development of bilberry from the flower to ripe fruit. (**V**)
- To tentatively identify flavan-3-ols and proanthocyanidins in lingonberry, cranberry, bilberry and bog whortleberry. (**VI**)

4 MATERIALS AND METHODS

4.1 Berry samples

The berries investigated in the present studies are described in **Table 5**. The berries were harvested at maturity in Finland at 62-63°, 66° and 69° N, and in Sweden at 60° N (Studies **I, II, III** and **IV**). Cultivated berries were obtained from the Research Garden of the University of Kuopio and from local farmers. Berries of the family *Vaccinium* were purchased from a Finnish supplier (Study **VI**).

Table 5. Berries investigated in Studies **I-VI**.

Latin name	Trivial name and cultivar (cv.)	Color of the berry	Studies
Family Grossulariaceae			
Ribes nigrum L.	black currant, cv. Öjebyn	shiny black	**I, II, IV**
Ribes nigrum L.	green currant, cv. Vertti and unnamed	yellowish-green	**I, II, IV**
Ribes × *pallidum* Otto & F. Dietr	red currant, cv. Red Dutch	shiny scarlet	**I, II, IV**
Ribes × *pallidum* Otto & F. Dietr	white currant, cv. White Dutch	shiny white	**I, II, IV**
Ribes uva-crispa L.	gooseberry, red, cv. Hinnonmaki's red	deep red	**IV**
Ribes uva-crispa L.	gooseberry, yellow, cv. Hinnonmaki's yellow	yellow	**IV**
Family Ericaceae			
Vaccinium uliginosum L.	bog whortleberry (or northern bilberry), wild	blue	**IV, VI**
Vaccinium myrtillus L.	bilberry (or whortleberry), wild	bluish-black	**IV, V, VI**
Vaccinium corymbosum L.	half-highbush blueberry, cv. Aino	blue	**IV, VI**
Vaccinium vitis-idaea L.	lingonberry (or cowberry), wild	scarlet	**IV, VI**
Vaccinium oxycoccos L.	cranberry, wild	dark red	**IV, VI**
Family Rosaceae			
Fragaria x ananassa Duch.	strawberry, cv. Honeoye, Jonsok and Polka	light scarlet	**III**
Rubus idaeus L.	red raspberry, cv. Maurin makea and Muskoka	red	**III**
Rubus idaeus L.	yellow raspberry, cv. unknown	yellow	**III**
Rubus idaeus L.	red raspberry, wild	red	**III**
Rubus arcticus L.	arctic bramble, cv. Mespi and Pima	red	**IV**
Rubus chamaemorus L.	cloudberry, wild	orange	**IV**
Prunus spinosa L.	blackthorn (or sloe), wild	bluish-black	**IV**
Aronia mitschurinii (or *Aronia melanocarpa* var. *grandifolia)*	chokeberry, cv. Viking	reddish-black	**IV**
x Crataegosorbus mitschurinii[a]	sweet rowanberry, cv. Granatnaja	red	**IV**
Family Empetraceae			**IV**
Empetrum hermaphroditum L.	northern crowberry, wild	shiny black	**IV**
Empetrum nigrum L.	southern crowberry, wild	shiny black	**IV**
Family Elaeagnaceae			
Hippophaë rhamnoides L.	sea buckthorn, cv. mixture[b]	orange	**IV**
Family Caprifoliaceae			
Sambucus nigra L.	elderberry, cv. unknown	shiny black	**IV**

[a] Crossbreed of *Crataegus sanguinea* Pall. and *Sorbus aucuparia* L. [b] Mixture of cultivars Obilnaja, Zheltaja Rannaja, Kaliningradskaja, Finskaja, and Vorobjovskaja

Bilberry samples (Oulu, Finland) in Study **V** were at six different ripening stages. The berries were frozen at –24±2 °C until being analysed within three to six months. A portion of raspberry (a mixture of cultivars) was freeze-dried for the analysis with the present and alternative methods in Study **IV**.

4.2 Extraction methods

The overall scheme of the extraction procedure for soluble and insoluble phenolic compounds is shown in **Figure 9**. This procedure was applied in Studies **I** and **II** without the sodium acetate liquid-liquid extraction, which was introduced in Studies **III** and **IV**. Ellagitannins (Study **III**) were analysed only in those berries which were known to contain major amounts of these compounds (Häkkinen et al. 2000a).

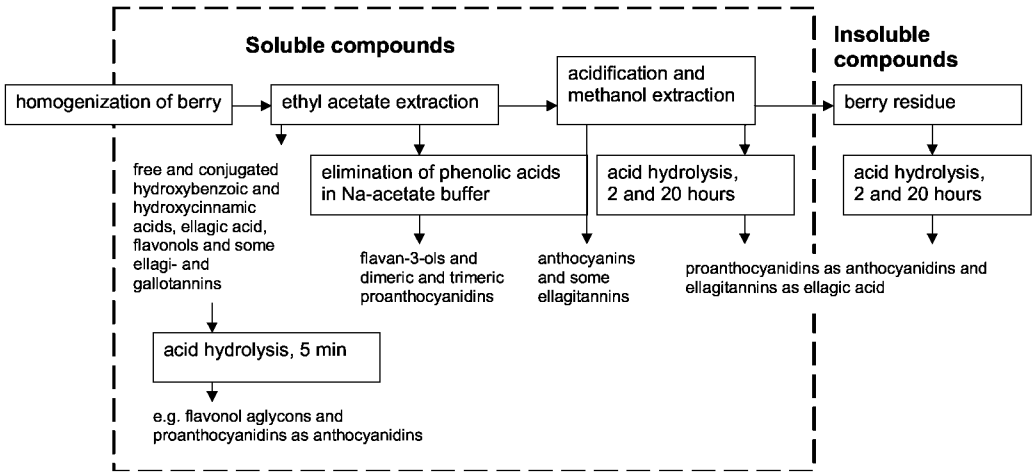


Figure 9. Sequential extraction of soluble and insoluble phenolic compounds from berry material.

4.2.1 Sequential extraction procedure

The edible parts of frozen berries (100 g) were homogenized and samples (5 g) were weighed in centrifuge tubes for extraction. In Studies **III** and **IV,** rare flavonol, morin (1 mg/ml methanol, 0.1 ml) was added as an internal standard, as it exhibited similar solubility in extraction solvents as flavan-3-ols. Extractions were performed by repeated vigorous vortexing of samples with ethyl acetate (4 x 10 ml) followed by centrifugation. The combined

ethyl acetate extracts were divided into two portions (20 ml each) containing soluble free and conjugated forms of hydroxybenzoic acids, hydroxycinnamic acids, gallic acid, ellagic acid and flavonols as well as flavan-3-ols, low molecular weight proanthocyanidins and some ellagitannins.

The whole 40-ml portion (Studies **I** and **II**) or one 20-ml portion (Studies **III** and **IV**) of the ethyl acetate extract was evaporated to dryness with a rotary evaporator, dissolved in 1 ml of methanol and analysed with HPLC-DAD in Studies **I-IV** and HPLC-ESI-MS in Studies **II** and **III**. In Studies **III** and **IV**, the other 20-ml portion was extracted with 2 x 10 ml of sodium acetate buffer (0.1 M, pH 7.0) and then with 10 ml of water to remove ionizable phenolic acids into the aqueous phase. After this purification step, the extract was evaporated to dryness and dissolved in 1 ml of methanol for the analysis of flavan-3-ols and dimeric proanthocyanidins with HPLC-DAD. After ethyl acetate extraction, the berry residue was acidified with HCl (2 M, 2 ml), and ellagitannins and anthocyanins were extracted with methanol four times to a total volume of ~50 ml. An aliquot of the methanol extract (10 ml) was evaporated to dryness, dissolved in 1 ml of methanol and analysed with HPLC-DAD in Studies **I-IV** and HPLC-ESI-MS in Studies **II** and **III**.

4.2.2 Berry-solvent suspension

A simple suspension method was developed for the small amounts of bilberry samples available in Study **V**. Frozen bilberry samples (1-2 g) were crushed with a mortar and pestle and macerated in 10 ml of acidified (0.6 M HCl) methanol. After removing 1 ml of the berry-solvent suspension for the analysis of anthocyanins with HPLC-DAD and HPLC-ESI-MS, the rest of the samples were acid hydrolysed for analysis of insoluble proanthocyanidins and flavonol glycosides as described below.

4.2.3 Acid hydrolysis

After analysis with HPLC-DAD, the ethyl acetate extracts were acidified to 0.6 M with conc. HCl and heated for 5 min in a boiling water bath (70-80 °C) to release the aglycons from flavonol glycosides in Studies **I** and **IV**. During this procedure, low molecular weight proanthocyanidins were depolymerized to red anthocyanidins, and they were quantified in this form in Study **I**. Upon acid hydrolysis, the conversion of proanthocyanidins to anthocyanidins has been shown to occur by oxidation following acid-catalysed cleavage of the interflavanoid bond (Porter et al. 1986).

The conditions of acid hydrolysis of proanthocyanidins were optimized using berry-methanol suspension of green currant, to provide the highest possible anthocyanidin yield. In the optimized method, aliquots of the methanol extracts (10 ml) and the methanol suspended

extraction residues obtained from sequential extraction (10 ml) were acidified (to 0.6 M with conc. HCl) and refluxed for 2 hours (60 °C) for analysis of the soluble and insoluble proanthocyanidins, respectively. In addition, phenolic glycosides were deconjugated to aglycons, which were utilized in the analysis of flavonol aglycons in the berry-solvent suspension in Study **V** and in the analysis of insoluble glycosides in Studies **I, III** and **IV**. For the analysis of soluble and insoluble ellagitannins in Study **III**, acid hydrolysis was carried out for 20 hours, since these conditions were previously found optimal for the conversion of ellagitannins to ellagic acid (Häkkinen et al. 2000a, Lei et al. 2001). Acid hydrolysates were analysed with HPLC-DAD.

4.2.4 Extraction and fractionation of flavan-3-ols and proanthocyanidins

Ethyl acetate extraction and liquid-liquid extraction with sodium acetate buffer were made for *Vaccinium* berries as described in 4.2.1 but on a larger scale (Study **VI**). The purified ethyl acetate extract was reconstituted into 10 ml with methanol for fractionation in an open Sephadex LH-20 (Amersham Pharmacia, Sweden) column (30 x 2 cm) equilibrated with sodium acetate buffer. The extract was mixed with an equal volume of sodium acetate buffer, the precipitating components were eliminated by centrifugation, and the supernatant was applied to the column. Residues of phenolic acids were removed in elution with 40 ml of sodium acetate buffer and 50 ml of 30% methanol in water. Flavan-3-ols were eluted with 50 ml of 60% methanol in water, and dimeric and trimeric proanthocyanidins were retrieved with 50 ml of pure methanol. Flavonol glycosides co-occurred in these fractions. Fractions were concentrated by vacuum evaporation and further purified with the preparative HPLC method described by Kähkönen et al. (2003). No phenolic compounds other than catechins and proanthocyanidins were detected in the purified fractions by means of HPLC-DAD and HPLC-ESI-MS.

4.3 Analytical methods

4.3.1 Chromatographic systems

All samples were filtered through a 0.45-μm syringe filter before injection into the HPLC. The HPLC-DAD apparatus consisted of a Hewlett-Packard instrument with a 1100 series quaternary pump, an autosampler, and a diode array detector linked to an HP-ChemStation data handling system (Waldbronn Analytical Division, Germany). The system used for HPLC-ESI-MS analysis was a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with a Rheos 400 HPLC pump (Danderyd, Sweden). HPLC separation of the phenolic compounds was achieved on a (125 x 3 mm i.d., 5 μm) LiChroCART Purospher

RP-18e column (Merck, Darmstadt, Germany) protected with a guard column of the same material (4 x 4 mm). A 20-min linear gradient of acetonitrile in 1% formic acid was used to separate the mixture of phenolic compounds in the ethyl acetate extracts. A step gradient of acetonitrile in 5% formic acid was used to separate anthocyanins as follows: 5-10% acetonitrile (0-5 min), 10% acetonitrile (5-10 min), 10-40% acetonitrile (10-25 min), and finally 40-90% acetonitrile (25-35 min). For a better separation of flavan-3-ols and dimeric and trimeric proanthocyanidins, 5% acetonitrile was used isocratically (0-5 min) and then increased to 20% in a linear gradient (5-35 min). All three gradients were followed by raising acetonitrile to 90% in 10 min, a return to initial conditions in 5 min and then re-equilibration of the column for 10 min. The flow rate for all separations was 0.5 ml/min. The chromatographic performance was followed by analysis of a standard mixture (*p*-hydroxybenzoic acid, caffeic acid and rutin) at the beginning of every sample series.

4.3.2 Identification

HPLC combined with diode array detection was used for UV-Vis spectral analysis and quantification. Identification of the conjugated and free forms of phenolic compounds in the chromatograms was based on retention times and on comparison of their UV-Vis spectral shape, wavelengths of maximum absorption and wavelengths of shoulders (sh) with those of representative standards (**Table 6**). Classifications of free and conjugated forms of gallic acid and ellagic acid as well as ellagitannins were based on typical spectral characteristics (**III**: Figure 3). Low molecular weight proanthocyanidins showed similar UV-spectra to flavan-3-ols (Bartolomé et al. 1996). Further identification was based on literature data of known conjugated hydroxycinnamic acids (Pyysalo and Kuusi 1974, Azar et al. 1987, Herrmann 1989), flavonol glycosides (Pyysalo and Kuusi 1974, Azar et al. 1987, Häkkinen and Auriola 1998, Yan et al. 2002, Mullen et al. 2003, Zheng and Wang 2003) and anthocyanins (Kärppä et al. 1984, Andersen 1985, Goiffon et al. 1991, Espín et al. 2000, Huopalahti et al. 2000, Chandra et al. 2001, Zheng and Wang 2003) in the respective berries. Identification of flavonol glycosides was confirmed by releasing the aglycons by acid hydrolysis of ethyl acetate extracts. Insoluble proanthocyanidins were depolymerized to cyanidin, delphinidin and pelargonidin by acid hydrolysis.

The native phenolic compounds were tentatively identified by HPLC with electrospray ionization mass spectrometric (ESI-MS) detection. The parameters for positive ionization were adapted from Häkkinen and Auriola (1998) and those for negative ionization from Mämmelä et al. (2000). The conditions for the initial ionization in the positive and negative ionization modes included capillary voltages at +4.5 kV and -3 kV, respectively, and temperature at 225 °C. The MS data were acquired as full scan mass spectra at *m/z* 150-1500 by using 200 ms for collection of the ions in the trap. Tandem MS was performed using

helium as the collision gas, and the collision energy was set at 30%. MS revealed the positive or negative molecular ions and tandem MS broke down the most abundant ones with dependent collision-induced dissociation.

4.3.3 Quantification

Identified individual compounds were quantified within the linear range using standard curves of representative standards. The response factors were determined from freshly prepared solutions in the following concentration ranges of aglycons: anthocyanins 1.5-85 mg/l and other phenolic compounds 2-250 mg/l. The response factors of anthocyanidins and anthocyanins were determined in acidified methanol (0.6 M HCl). The representative standards (vanillic acid, *p*-hydroxybenzoic acid, gallic acid, rutin, anthocyanidin glucosides, hydroxycinnamic acids, flavonol aglycons, flavan-3-ols and anthocyanidins) were analysed near their wavelengths of maximum absorption: hydroxybenzoic acids at 260 nm, hydroxycinnamic acids at 320 nm, ellagic acid and flavonol glycosides at 360 nm, anthocyanins at 520 nm, and flavan-3-ols at 280 nm. Dimeric proanthocyanidins were quantified using the response factor of (-)-epicatechin at 280 nm. Native ellagitannins were quantified using the response factor of gallic acid at 280 nm. Morin was used as an internal standard in the quantification of flavan-3-ols and low molecular weight proanthocyanidins to correct losses due to the purification step of the ethyl acetate extract. The contents of phenolic compounds were the sum of the soluble forms in the ethyl acetate and methanols extracts plus the insoluble forms in the extraction residue, and are expressed for the weight of the phenolic aglycon. Ellagitannins were depolymerized to ellagic acid (Rt = 16.1 min) and an unknown, less polar, ellagic acid derivative (Rt = 18.0 min) in the 20-hours acid hydrolysis of the methanol extract and the extraction residue. Since ellagic acid glycosides deconjugate to ellagic acid in acid hydrolysis, the contents of free and conjugated forms of ellagic acid were subtracted from the ellagitannin contents in the acid hydrolysates.

Table 6. UV-Vis spectral characteristics of phenolic compounds in alcohol [1-4] and on-line in HPLC-DAD [5] (Studies **II** and **IV**) .

	Wavelengths (nm) of maximun absorption and shoulders (sh)		
	aglycon	glycoside (with glucose)	esters
Hydroxycinnamic acids			
p-Coumaric acid	234, 300sh, 310 (**std**); 226, 292sh, 309 [1, 5]	234, 296 (**II**)	with glucose 236, 300sh, 312-314 (**II**); 225, 316 [2]
Caffeic acid	240, 300sh, 324-326 (**std**); 238, 293sh, 323 [1]		with glucose 244, 300sh, 326-330 (**II**); with quinic acid 244, 300sh, 326 nm (**std**)
Ferulic acid	240, 300sh, 324-326 (**std**)	211, 284 [2]	with glucose 244, 300sh, 326-330 (**II**); 240, 325 [5] with quinic acid 300sh, 325 [3]
Sinapic acid	236, 326 (**std**); 302sh, 327 [3]		with glucose 305sh, 328 [3]
Flavonoids			
Myricetin	254-260, 300sh, 373 (**std**)	254, 300sh, 354 (**II**)	
Quercetin	254, 300sh, 371 (**std**)	254, 300sh, 354 (**II**)	
Kaempferol	265, 367 (**std**)	264, 290, 348 (**II**); 263, 344 [5]	
Isorhamnetin	254, 371 (**std**)	254, 300sh, 354 (**IV**)	
Delphinidin	274, 528 (**std**)	278, 524 (**std**)	
Cyanidin	274, 524 (**std**)	278, 516 (**std**)	
Petunidin		278, 528 (**std**)	
Peonidin		278, 516 (**std**)	
Malvidin		278, 528 (**std**)	
Pelargonidin		276, 504 (**std**)	
Gallocatechin	271 [4]		
Catechin	278 (**std**) [4]		

[1]Moran et al. 1998, [2]Baderschneider and Winterhalter 2001, [3]Ibrahim and Barron 1989, [4]Bartolomé et al. 1996, [5]Escarpa et al. 2002.

5 RESULTS AND DISCUSSION

5.1 Evaluation of the methods

The requirements of sample preparation techniques are demanding when one wishes to analyse both soluble and insoluble phenolic compounds in the plant matrix. Soluble phenolic compounds are compartmentalized within the cell vacuoles as free aglycons or as conjugated forms of glycosides and esters. Hydroxybenzoic and hydroxycinnamic acid conjugates, ellagic acid glycosides, flavonol glycosides, anthocyanins, and flavan-3-ols, as well as the lower molecular weight forms of proanthocyanidins and ellagitannins are considered to be soluble compounds according to their structures. Insoluble phenolic compounds are components of cell walls or high molecular weight forms of proanthocyanidins (condensed tannins) and ellagitannins. These high molecular weight tannins are also strongly bound to polar fibrous matrices in cell walls (Rohr et al. 2000). The first step in sample preparation is to homogenize the sample matrix to a fine powder, thereby increasing the surface area for extraction. In the present studies, berries were crushed while still frozen so that the skins and bulb were in a fine form (Studies **I-VI**). The following evaluates the methods developed for the extraction of soluble phenolic compounds and for acid hydrolysis of soluble and insoluble phenolic compounds (**Figure 9**).

5.1.1 Extraction procedures

Berries contain a wide range of phenolic compounds in different conjugated forms with variable solubility and stability characteristics, a fact that makes their simultaneous extraction and analysis a difficult task (Macheix et al. 1990). Therefore, a sample preparation technique based on a sequential extraction procedure was developed (**Figure 9**) (Studies **I-IV**). Ethyl acetate was chosen to the first extraction solvent, since it is easily evaporated in low temperatures and it selectively extracts other phenolic compounds but not anthocyanins, which were extracted subsequently in acidified methanol. Available standards may be spiked in samples for the evaluation of method performance in terms of their recovery (Escarpa and Gonzalez 1998, Sato et al. 2001). Spiked standards show the possible destruction of compounds and the interactions with sample matrix and solvents during extraction, but they do not give information on the extraction efficiency of the variably compartmentalized and conjugated forms of phenolic compounds. The release and extractability in the solvents were determined for hydroxycinnamic acids, flavonols and anthocyanins in Studies **III** and **IV** by their quantification in the ethyl acetate extract, in the subsequent methanol extract, and in the extraction residue (**Table 7**). Information on extraction efficiency can only be obtained by comparing results from, at least two, independent methods. Therefore, in Study **IV,** freeze-

dried raspberry was analysed with the present method and with alternative methods, where the types of sample pre-treatment and quantification were different (Treutter et al. 1994, de Pascual-Teresa et al. 1998, Häkkinen and Auriola 1998, Mattila and Kumpulainen 2002).

Extraction of hydroxycinnamic acids and flavonols

The free and conjugated forms of hydroxycinnamic acids and flavonols were mainly detected in the ethyl acetate extracts, with some residual amounts present in the subsequent methanol extracts and extraction residues (**Table 7**). The exceptions were those berries which contain high amounts of phenolic compounds and/or diglycosides of flavonols that are more polar and less soluble in ethyl acetate than monoglycosides. However, the total content was expressed as a sum of the compounds present in the two solvents and the extraction residue. The total hydroxycinnamic acid content in the freeze-dried raspberry was comparable to that obtained with the alternative method (Mattila and Kumpulainen 2002), where aglycons were released by acid and alkali hydrolysis (Study **IV**). The analysis of flavonol glycosides by the present method gave higher contents than quantification of their aglycons after acid hydrolysis in the alternative method (Häkkinen and Auriola 1998) (Study **IV**). Previously, the contents of flavonols were corrected with recoveries to balance the losses in acid hydrolysis (Häkkinen et al. 1999b). The sequantial extraction procedure was found to be adequate to extract the major part of both hydroxycinnamic acid conjugates and flavonol glycosides. Moreover, ethyl acetate extraction was found to be necessary for the analysis of the easily desctructable structures of the flavonol malonylglucosides (Study **II**).

Extraction of flavan-3-ols and proanthocyanidins

Flavan-3-ols and dimeric and trimeric proanthocyanidins were extracted in ethyl acetate (**Figure 9**). Studies **I** and **II** with black, green, red, and white currants revealed that conjugated hydroxycinnamic acids co-eluted with peaks of flavan-3-ols and proanthocyanidins. To solve this problem, liquid-liquid extraction of one half of the ethyl acetate extract with sodium acetate buffer was incorporated into the experimental procedure in Studies **III** and **IV**. A comparison with the alternative method (Treutter et al. 1994, de Pascual-Teresa et al. 1998) revealed that the initial method had underestimated the contents of flavan-3-ols and dimeric and trimeric proanthocyanidins (Study **IV**). This difference may be due to the lower extractability of flavan-3-ols and low molecular weight proanthocyanidins from the homogenized berry in ethyl acetate than in the aqueous methanol used in the alternative method (Rohr et al. 2000). Aqueous acetone is considered to be the most effective solvent to dissolve proanthocyanidins (Rohr et al. 2000, Tura and Robards 2002) and was recently used for the extraction of variable sizes of proanthocyanidins (Gu et al. 2002). However, the developed sample extraction and purification was found to be a quick and

inexpensive sample pretreatment procedure prior to the estimation of levels of catechins and low molecular weight proanthocyanidins in the comparison of the different berry samples.

Table 7. Release and extractability of hydroxycinnamic acids, flavonols and anthocyanins, as quantified proportions (%) in ethyl acetate (EtAc) and subsequent methanol extracts (MeOH) and extraction residue (Res)[a] (Studies **III** and **IV**).

	Hydroxycinnamic acids			Flavonols			Anthocyanins		
	EtAc	MeOH	Res[b]	EtAc	MeOH	Res[b]	EtAc	MeOH	Res[b]
Black currant	100	ND[c]	ND	50	47	3	1	99	tr
Green currant	77	23	ND	75	25	ND	ND	ND	ND
Red currant	88	12	ND	100	ND	ND	ND	100	ND
White currant	95	5	ND	86	14	ND	ND	ND	ND
Gooseberry, red	90	10	ND	54	46	ND	33	67	ND
Gooseberry, yellow	58	42	ND	67	33	ND	ND	ND	ND
Bog whortleberry[d]	100	ND	ND	73-76	27-24	ND	1-2	98-99	tr
Lingonberry	86	7	7	94	6	ND	2	98	ND
Cranberry	97	3	tr	86	11	3	4	96	ND
Bilberry	100	ND	ND	84	16	ND	2	98	tr
Blueberry	95	3	2	89	11	ND	9	91	tr
Chokeberry	49	51	1	32	65	2	1	97	2
Sweet rowanberry	71	28	1	42	58	ND	3	97	ND
Blackthorn	53	47	ND	74	26	ND	1	99	tr
Strawberry[e]	100	ND	ND	56-77	ND	23-44	21-24	76-79	ND
Red raspberry	100	ND	ND	100	ND	ND	1	99	tr
Yellow raspberry	100	ND	ND	100	ND	ND	ND	ND	ND
Red raspberry	100	ND	ND	100	ND	ND	1	99	tr
Arctic bramble	83	17	ND	86	14	83	2	98	tr
Cloudberry	100	ND	ND	100	ND	100	ND	100	ND
Northern crowberry	100	ND	ND	83	17	ND	2	98	tr
Southern crowberry	67	9	24	88	12	ND	2	98	tr
Sea buckthorn	100	ND	ND	40	29	31	ND	ND	ND
Elderberry	67	14	19	48	52	ND	1	99	tr

[a]The scheme for the extraction procedure is shown in **Figure 9**. [b] Flavonols and hydroxycinnamic acids were quantified as aglycons after acid hydrolysis of the extraction residue. Traces (tr) of anthocyanins were shown as a light red colour in the extraction residue after suspension with acidified methanol. [c] ND, not detected.
[d] Variation for berries collected from three different locations. [e] Variation for three berry cultivars.

Extraction of anthocyanins, ellagitannins and insoluble proanthocyanidins

Subsequent to ethyl acetate extraction, the berry residue was acidified with hydrochloric acid to favour extractability, equilibrium and stability of anthocyanins as flavylium cations in methanol. Extractability of anthocyanins into the acidified methanol was found to be adequate, since only traces were detected in the extraction residue (**Table 7**). Soluble forms of ellagitannins were extracted in ethyl acetate and methanol, whereas insoluble forms were released by acid hydrolysis from the solid extraction residue (**III:** Table 3; **IV:** Table 2). The

total content of ellagitannins in raspberry (in the extracts and extraction residue) was comparable to earlier results on the content of soluble forms extracted with 70% acetone (Kähkönen et al. 2001). Aqueous acetone would be the recommended extraction solvent in studies on the native forms of ellagitannins.

Berry-solvent suspension method

The maceration of sample-solvent suspension was used to study the contents of flavonol glycosides, anthocyanins and proanthocyanidins in bilberry samples available in small portions (Study **V**). Fresh samples were crushed and macerated in acidified methanol, to ensure that the compounds were uniformly distributed in the solvent and sample matrix. However, double the amount of anthocyanins was quantified in bilberry using the sequential extraction procedure (8000 mg/kg in Study **IV**) compared to the suspension method (4000 mg/kg in Study **V**). The most probable explanation for the difference is the particle size of samples. In a later study, where the same maceration method was used for frozen tissue crushed in liquid nitrogen to a more fine powder, the anthocyanin content of bilberry samples was 8000 mg/kg (Table 2 in Jaakola 2003). The berry-solvent suspension method is a quick and convenient sample handling procedure for the quantification of non-acylated flavonol glycosides and anthocyanins.

5.1.2 Acid hydrolysis of extracts, extraction residues and berry-solvent suspensions

Acid hydrolysis prior idenfication

Acid hydrolysis was used in the identification of flavonol aglycons in the ethyl acetate extracts in Studies **I** and **IV** (**Figure 9**). Short (5 min) acid treatment of the ethyl acetate extract was optimal to release myricetin, quercetin, kaempferol or isorhamnetin from glycosides (Studies **I**).

Acid hydrolysis prior quantification

Acid hydrolysis was used prior to quantification of phenolic glycosides (possibly cell wall bound), proanthocyanidins and ellagitannins in extracts, extraction residues and berry-solvent suspensions (Studies **I, III, IV** and **V**). Hydrolysis times of 2 and 20 hours for flavonol glycosides and ellagitannins, respectively, were adapted from the literature (Häkkinen et al. 1999b, Häkkinen et al. 2000a). The highest yield of anthocyanidins from proanthocyanidins (Study **I**) was achieved in the same conditions as in the method developed for the quantitative analysis of flavonols. High temperatures (~80 °C) used in acid hydrolysis have been shown to destroy hydroxycinnamic acids (Häkkinen and Auriola 1998). Therefore, the conditions (~60 °C in methanol under refluxion) employed in the acid hydrolysis might not have been optimal for conjugated or cell wall bound phenolic acids.

Analysis of proanthocyanidins using acid depolymerization has long been used to identify and tentatively quantify proanthocyanidins (Bate-Smith 1954). In the conventional method, pigment yield was measured spectrophotometrically, but in the present method cyanidin, delphinidin and pelargonidin were analysed with HPLC-DAD (Studies **I, III, IV** and **V**), providing compositional data on proanthocyanidins present in the berries. One problem encountered was the deconjugation of anthocyanins to anthocyanidins. Moreover, the content of anthocyanins decreased to 80% of the original content due to their destruction during acid hydrolysis, which was shown in a preliminary test in Study **V** and has been noted in the literature (Merken and Beecher 2000b). Therefore, 80% of the content of cyanidin (anthocyanin origin) were subtracted from the total acid released cyanidin content for estimation of proanthocyanidin contents in the slightly red coloured bilberry samples (Study **V**). In other studies, acid hydrolysis was applied for anthocyanin free berry extracts and extraction residues to estimate the levels and composition of proanthocyanidins in different berry species (Studies **I, III** and **IV**). Two hours of acid hydrolysis was found to be a quick and convenient sample handling strategy prior to the estimation of the composition and content of proanthocyanidins.

Analysis of ellagitannins after acid treatment was introduced by Bate-Smith (Bate-Smith 1972). The method has been applied mainly for wood samples but also for plant food samples (Daniel et al. 1989, Maas et al. 1991, de Ancos et al. 2000, Häkkinen et al. 2000a, Lei et al. 2001, Mattila and Kumpulainen 2002). Ellagitannins were depolymerized by acid hydrolysis to ellagic acid and a less polar derivative (20-26% of the content) which is assumed to be the methyl ether (methoxyl group) or methyl ester of ellagic acid (Study **III**). Both conversion products were quantified, even though previously the content was presented only for ellagic acid (Häkkinen et al. 2000a, Lei et al. 2001, Mattila and Kumpulainen 2002). In the methanol extracts, ellagitannins were both acid hydrolysed to ellagic acid equivalents and analysed as their native forms in gallic acid equivalents (Study **III**). In the comparison of these two strategies, the ellagitannin contents were comparable in the berries of *Rubus* species, but not in strawberries (**III:** Table 3). In the literature, the total content of ellagitannins in strawberry were higher using the acid hydrolysis method (Daniel et al. 1989, Maas et al. 1991, Häkkinen et al. 2000a, Mattila and Kumpulainen 2002) than was shown for acetone extract (Kähkönen et al. 2001). The possible explanation is that the fibrous berry matrix of strawberry, which was partly solubilized in methanol or aqueous acetone, may contain complex insoluble ellagitannins. Therefore, acid hydrolysis would be more recommendable for strawberry samples than solvent extraction of native ellagitannins. The method based on acid hydrolysis of ellagitannins to ellagic acid equivalents may not cause any major destruction of the acid released products. Moreover, acid hydrolysis can be used for various plant materials and for simultaneous analysis of all forms of ellagitannins. Therefore, 20 hours of acid hydrolysis is the most practical sample handling procedure prior to the quantification ellagitannins as

ellagic acid equivalents. The most authentic results would be obtained by quantification of both ellagic acid and the other acid released conversion product of the ellagitannins.

5.1.3 Remarks on quantification of native forms of phenolic compounds

The chromatographic performance was followed by analysis of a standard mixture prior to every sample series. The retention times of the standards increased slowly by 0.2-0.4 min during the lifetime of the column (7 months, *ca* 1000 analyses). Day-to-day variation in the peak areas of standards was acceptable (CV <3%, n=40) for the valid use of response factors of the representative standards.

Quantification of the native forms of phenolic compounds is challenging, since standards are not available for all of the compounds. In the present method, representative standards were selected for quantification. The spectral maxima of free hydroxycinnamic acids, *p*-coumaric acid, caffeic acid and ferulic acid, are close to their ester conjugates (**Table 6**) (Ibrahim and Barron 1989, Moran et al. 1998, Baderschneider and Winterhalter 2001, Escarpa et al. 2002). The maximum of quercetin rutinoside is almost the same as that of myricetin, quercetin and isorhamnetin glycosides and close to the maxima of kaempferol glycosides (**Table 6**) (Escarpa et al. 2002). The acid and water concentrations are important factors in the quantification of anthocyanins, since they influence the balance of coloured and colourless forms in solvents (Clifford 2000a, Mazza et al. 2004). In the present studies, anthocyanin standards were analysed at 0.6 M HCl in methanol, and the acid concentrations in the methanol extracts (water content <5%) were 0.08 M for strongly coloured berries, 0.8 M for slightly coloured berries and 0.6 M for berry-solvent suspension. In the case of flavonol glycosides and anthocyanins, the intensity of absorption is affected by the linked sugar moiety (Pietta et al. 1994, Mazza et al. 2004), which is neglected in the use of representative standards. However, the present strategy is adequate for quantification purposes in the analysis of a variety of berries, otherwise the isolation of all native conjugated phenolic compounds would be a very laborious assignment.

5.2 Distribution of phenolic compounds in berries

5.2.1 Identification of the native forms of phenolic compounds

This study presents and compares the distribution and contents of the soluble and insoluble phenolic compounds in the edible berry species. Soluble phenolic compounds in the ethyl acetate and methanol extracts were primarily identified using HPLC-DAD. Peaks in the chromatograms were classified into hydroxybenzoic acids, hydroxycinnamic acids, ellagic acid, flavonols, ellagitannins, anthocyanins, flavan-3-ols, and proanthocyanidins by the

comparison of their UV-Vis spectra with those of the available standards. Special information on the type of the bond was obtained from the UV-Vis absorption spectra of the compounds. Bathochromic shifts (shifts to longer wavelength) in the UV-Vis absorption spectra indicated esterification of the aglycons, and the respective hypsochromic shifts (shifts to shorter wavelength) indicated glycosidation (**Table 6**). The existing literature on berries presented in **Table 8** was a useful tool, since the order of retention of conjugated phenolic compounds in the RP-column is known to be reproducible in certain solvent systems (Goiffon et al. 1991, Pietta et al. 1994, Bengoechea et al. 1997, Schieber et al. 2001, Tomás-Barberán et al. 2001, Mullen et al. 2002a).

The detailed description and interpretation of HPLC-ESI-MS identification results are provided for currants, raspberry, arctic bramble, and cloudberry phenolic compounds in Studies **II** and **III**, for bilberry anthocyanins in Study **V** and for bog whortleberry, bilberry, lingonberry and cranberry proanthocyanidins in Study **VI**. Phenolic acids displayed sodium adducts as the most abundant positive ions, which fragmented to sodium adduct ions of the hexose moiety (Study **II**: Fig. 4B). Systematic identification of flavonol glycosides, ellagic acid glycosides and anthocyanins was feasible, because they fragmented to the aglycons in tandem MS (Lin et al. 1993, Häkkinen and Auriola 1998, Andlauer et al. 1999, Giusti et al. 1999, da Costa et al. 2000, Mullen et al. 2003). The bound sugar moieties consisted of hexoses (glucose or galactose), deoxyhexoses (rhamnose), pentoses (xylose or arabinose), glucuronic acid, acetylpentose, malonylglucose, and succinylglucose. Flavan-3-ols and dimeric and trimeric proanthocyanidins were identified according to the previously reported fragmentation pattern of proanthocyanidins (Poon 1998, Pérez-Magariño et al. 1999, Friedrich et al. 2000, Tomás-Barberán et al. 2001). Ellagitannins were identified according to the masses known to originate from HHDP group and galloyl-HHDP-glucose (Barry et al. 2001).

Table 8. Distribution of hydroxycinnamic acids, flavan-3-ols, flavonol glycosides and anthocyanins in berries of six families.

Phenolic compound[a]	t_R[b] min	Grossulariaceae	Ericaceae	Rosaceae	Empetraceae	Elaeagnaceae Caprifoliaceae	References
		bc, black currant gc, green currant rc, red currant wc, white currant rgb, red gooseberry ygb, yellow gooseberry	bw, bog whortleberry bib, bilberry blb, blueberry lb, lingonberry cb, cranberry	swb, strawberry rb, raspberry arb, arctic bramble cb, cloudberry ch, chokeberry sr, sweet rowanberry bt, blackthorn	ncr, northern crowberry scr, southern crowberry	sb, sea buckthorn eb, elderberry	Studies **(II, III, V)** Literature [1-20]
Hydroxycinnamic acid conjugates							
Neochlorogenic acid	7.2	rgb, ygb	NR[c]	ch, sr, bt, rb	ncr, scr	eb	[1-3]
p-Coumaric acid 4-glucoside	7.6	bc, gc, rc, wc,	bw, bib, blb, lb, cb	sr	NR	NR	**(II)** [2]
Caffeoylglucose	7.9	bc, gc, rc, wc, rgb, ygb	NR	rb, arb	NR	NR	**(II, III)** [2]
Ferulic acid glucoside	8.9	NR	blb	NR	NR	NR	[2]
p-Coumaric acid ester	9.4	bc, gc, rgb, ygb	bw, bib	swb, rb, arb, ch, sr, bt	ncr, scr	eb	**(II)**
p-Coumaroylglucose	10	bc, gc, rc, wc, rgb, ygb	bw, lb	NR	NR	NR	**(II)** [2]
Chlorogenic acid	9.9	NR	bib, blb, cb	ch, sr, bt	ncr, scr	eb	[1, 3, 4]
Feruloylglucose	11.0	bc, gc, wc	lb, cb	rb	NR	NR	**(II)**
Caffeic acid	11.4	NR	bw	cb	NR	NR	**(III)**
p-Coumaric acid ester	11.7	NR	blb, cb	NR	NR	NR	
Feruloylquinic acid	11.7	NR	NR	sr	NR	NR	[1]
p-Coumaric acid ester	12.5	NR	NR	ch, sr	ncr	NR	
Caffeic/ferulic acid ester	12.8	NR	blb	NR	NR	NR	
p-Coumaric acid	14.7	NR	lb	cb	NR	NR	
p-Coumaric acid ester	15.7	NR	lb	NR	NR	NR	
p-Coumaric acid ester	15.9	NR	bib, lb	ch	NR	NR	
Ferulic acid	16.0	NR	NR	cb	NR	NR	
Caffeic acid hexose derivative	15.8	bc, gc	NR	NR	NR	NR	**(II)**
Caffeic/ferulic acid ester	18.1	NR	NR	ch, sr	NR	NR	

p-Coumaric acid hexose derivative	18.4	bc, gc, rgb, wc	lb	ch	NR	NR	**(II)**
Caffeic/ferulic acid ester	19.9	NR	lb	NR	NR	NR	
Ferulic acid hexose derivative	19.1	bc, gc	NR	NR	NR	NR	**(II)**
Flavan-3-ols							
(+)-Catechin	9.2	bc, gc, rc, wc, rgb, ygb	bib, blb, lb, cb	swb, rb, arb, cb, bt	ncr, scr	sb	**(II,III)**
(-)-Epicatechin	12.0	bc, gc, rc, rgb, ygb	bw, bib, blb, lb, cb	swb, rb, arb, cb, ch, cr	ncr, scr	sb	**(II,III)**
Flavonol glycosides							
Quercetin diglycoside	13.7	NR	NR	ch, sr	NR	eb	[3, 5]
Myricetin 3-rutinoside	14.0	bc, gc	NR	NR	NR	NR	**(II)**
Myricetin 3-galactoside	14.2	NR	bw, bib, cb	NR	ncr, scr	NR	[6]
Myricetin 3-glucoside	14.4	bc, gc	bib	NR	ncr, scr	NR	**(II)** [5]
Quercetin diglycoside	14.7	NR	NR	ch, sr	NR	sb eb	[3, 5]
Myricetin 3-arabinose	14.7	NR	NR	NR	NR	NR	[4]
Quercetin 3-glucosylrhamnoside	15.1	NR	NR	NR	NR	sb	[5]
Myricetin 3-malonylglucoside	15.4	bc, gc	NR	NR	NR	NR	**(II)**
Quercetin 3-rutinoside	15.7	bc, gc, rc, wc, rgb, ygb	NR	ch, sr, bt	NR	sb eb	**(II)** [1, 5, 7, 8]
Quercetin glycoside	15.6	NR	bw	NR	NR	NR	
Quercetin glucurone-deoxyhexoside	15.8	NR	NR	arb	NR	NR	**(III)**
Quercetin glycoside	15.9	NR	bib, blb, lb, cb	NR	ncr, scr	NR	
Quercetin 3-galactoside	16.3	NR	bw, bib, blb lb, cb	ch, sr, bt	ncr, scr	NR	**(II)** [1, 4, 5, 9, 10]
Quercetin 3-glucoside	16.5	bc, gc, rc, wc, rgb, ygb	bw, bib, blb	rb, ch, sr, bt	ncr, scr	sb eb	**(III)** [4, 8, 9, 11]
Quercetin 3-glucuronide	16.7	NR	NR	swb, rb, arb, cb	NR	NR	[11, 12] **(III)**
Quercetin 3-xyloside	17.3	NR	cb, lb	bt	NR	NR	[6]
Quercetin 3-malonylglucoside	17.6	bc, gc, rc, wc	NR	NR	NR	NR	**(II)**
Isorhamnetin 3-glucosylrhamnoside	17.7	NR	NR	NR	NR	sb	[8]
Isorhamnetin glycoside	17.7	rgb, ygb	NR	NR	NR	NR	
Quercetin 3-arabinoside	17.7	NR	bw, blb, lb, cb	ch, sr, bt	ncr, scr	eb	[4-6]

Quercetin 3-rhamnoside	18.1	NR	bw, bib, blb, lb, cb	bt	ncr, scr	NR	[4-6]
Quercetin glycoside	18.4	NR	lb, cb	NR	NR	NR	**(II)** [4]
Kaempferol 3-glucoside	18.3	bc, gc	NR	NR	NR	NR	**(II)**
Kaempferol 3-glucuronide	18.5	NR	NR	NR	arb	NR	**(III)**
Isorhamnetin 3-rutinoside&3-glucoside	18.5	NR	NR	NR	NR	sb	[8]
Isorhamnetin 3-glucuronide	18.9	NR	NR	arb	NR	NR	**(III)**
Kaempferol 3-malonylglucoside	19.7	bc, gc	NR	NR	NR	NR	**(II)**
Quercetin glycoside	21.3	NR	lb	NR	NR	NR	
Myricetin	20.1	NR	bib, blb	NR	ncr, scr	NR	
Quercetin	24.1	NR	bib, lb, cb	NR	ncr, scr	NR	
Kaempferol	26.0	NR	bib	NR	NR	NR	
Isorhamnetin	26.2	NR	NR	NR	NR	sb	
Anthocyanins							
Cyanidin 3-sambubioside-5-glucoside	8.4	NR	NR	NR	NR	eb	[13]
Delphinidin 3-galactoside	8.7	NR	bw, bib, blb	NR	ncr, scr	NR	**(V)** [14]
Delphinidin 3-glucoside	9.6	bc	bw, bib, blb	NR	NR	NR	**(II)** [15]
Cyanidin 3-sophoroside	9.9	rc	NR	rb, cb	NR	NR	**(II)**
Cyanidin 3,5-diglucoside	10.0	NR	NR	NR	NR	eb	[13]
Delphinidin 3-rutinoside	10.4	NR	NR	NR	NR	NR	**(II)** [15]
Cyanidin 3-galactoside	10.5	NR	bw, bib, blb, lb, cb	ch, sr	ncr, scr	NR	**(V)** [4, 14, 16-18]
Cyanidin 3-(2^G-glucosylrutinoside)	10.6	rc	NR	rb, cb	NR	NR	**(II, III)** [19]
Delphinidin 3-arabinoside	10.8	NR	bw, bib, blb	NR	NR	NR	**(V)**
Cyanidin 3-sambubioside	11.9	rc	NR	NR	NR	eb	**(V)** [20]
Cyanidin 3-glucoside	12.1	bc, rc, rgb	bw, bib, blb, lb, cb	swb, rb, arb, cb, ch, sr	NR	eb	**(II, III, V)** [4, 14, 18, 20]
Cyanidin 3-(2^G-xylosylrutinoside)	12.2	rc	NR	NR	NR	NR	**(II)**
Petunidin 3-galactoside	12.6	NR	bw, bib, blb, cb	NR	ncr, scr	NR	**(V)** [14]
Cyanidin 3-rutinoside	13.9	bc, rc, rgb	NR	rb, arb, cb, bt	NR	NR	**(II, III)** [15, 17, 19]

Cyanidin 3-arabinoside	14.1	NR	bw, bib, blb, lb, cb	ch, sr	ncr, scr	NR	**(V)** [4, 14, 16-18]
Petunidin 3-glucoside	14.4	NR	bw, bib, blb	NR	NR	NR	**(V)**
Pelargonidin 3-glucoside	15.0	NR	NR	swb, rb, arb	NR	NR	**(III)** [19]
Peonidin 3-galactoside	15.3	NR	bw, bib, blb, cb	NR	ncr, scr	NR	**(V)**[14, 18]
Petunidin 3-arabinoside	15.6	NR	bw, bib, blb,	NR	NR	NR	**(V)**
Pelargonidin 3-rutinoside	15.8	NR	NR	swb, rb, arb,	NR	NR	**(III)** [19]
Peonidin 3-glucoside	16.2	NR	bw, bib, blb, cb	bt	NR	NR	[17, 18]
Malvidin 3-galactoside	16.2	NR	bw, bib, blb	NR	ncr, scr	NR	**(V)** [14]
Peonidin 3-rutinoside	16.6	NR	NR	bt	NR	NR	[17]
Cyanidin 3-xyloside	16.7	NR	NR	ch	NR	NR	[4]
Peonidin 3-arabinoside	16.8	NR	bw, bib, blb, cb	NR	ncr, scr	NR	**(V)** [18]
Malvidin 3-glucoside	16.8	NR	bw, bib, blb	NR	NR	NR	**(V)**
Malvidin 3-arabinoside	17.4	NR	bw, bib, blb	NR	ncr, scr	NR	**(V)**
Pelargonidin 3-malonylglucoside	18.1	NR	NR	swb	NR	NR	
Pelargonidin 3-succinylglucoside	18.7	NR	NR	swb	NR	NR	

[a]Characterization of phenolic compounds is based on standards, UV-Vis spectral characteristics, aglycons of flavonol glycosides released in acid hydrolysis and retention times of conjugated forms of phenolic compounds, and further identification in references. [b]HPLC separation was achieved on a LiChroCART Purospher RP-18e column (125 x 3 mm i.d., 5 μm) using a gradient of acetonitrile in 1% formic acid for the hydroxycinnamic acid conjugates and flavonol glycosides, and a gradient of acetonitrile in 5% formic acid for anthocyanins (**I-V**). [c] NR, not reported. [1]Pyysalo and Kuusi 1974, [2]Herrmann 1989, [3]Gil-Izquierdo and Mellenthin 2001, [4]Zheng and Wang 2003, [5]Häkkinen and Auriola 1998, [6]Yan et al. 2002, [7]Macheix et al. 1990, [8]Rösch et al. 2003, [9]Azar et al. 1987, [10]Vasilets et al. 1988, [11]Mullen et al. 2003, [12]Wang and Zheng 2001,[13]Goiffon et al. 1999, [14]Kärppä et al. 1984, [15]Slimestad and Solheim 2002, [16]Andersen 1985, [17]Espín et al. 2000, [18]Huopalahti et al. 2000, [19]Goiffon et al. 1991, [20]Chandra et al. 2001.

5.2.2 Hydroxybenzoic acids

The occurrence of hydroxybenzoic acids was studied in currants (Studies **I** and **II**), but not in the other berries. Esters of *p*-hydroxybenzoic acid and vanillic acid were tentatively identified in red and white currants (**II:** Table 1). Glycosides were not detected as the native conjugates in currants even though they have been identified earlier in these berries (Schuster et al. 1986).

5.2.3 Hydroxycinnamic acids

Composition of aglycons

p-Coumaric acid was the most abundant hydroxycinnamic acid in currants, red gooseberry, strawberry, raspberry, cloudberry, bog whortleberry, lingonberry, and crowberry (**III:** Table 2 and **IV:** Table 2) and caffeic acid (including minor amounts of ferulic acid) in the rest of the studied berries. There is a possibility for variation within species, since previously *p*-coumaric acid has been shown to dominate in some but not all raspberry cultivars (Schuster and Herrmann 1985). Sinapic acid was not detected in the studied berries. The composition of hydroxycinnamic acids was consistent with that reported in similar kinds of studies on the native conjugates in these berries (Pyysalo and Kuusi 1974, Azar et al. 1987, Herrmann 1989, Zheng and Wang 2003), but somewhat inconsistent with the studies on aglycons of acid hydrolysates (Häkkinen et al. 1999a, Taruscio et al. 2004). This inconsistency was highlighted in the relatively higher amounts of ferulic acid in acid hydrolysates, which may have been released from cell walls (Faulds and Williamson 1999, Häkkinen et al. 1999a, Taruscio et al. 2004).

Composition of soluble forms

Soluble hydroxycinnamic acids are usually found as conjugated rather than free forms in fruits (Macheix et al. 1990). Exceptionally, free hydroxycinnamic acids were detected as a dominant soluble form in cloudberry and were present also in bog whortleberry and lingonberry (**Table 8**). It is possible that free hydroxycinnamic acids are released at the late stages of ripening due to the environmental stress factors or that the unbound acids are typical for wild berries. More studies on wild berries grown in different environmental circumstances are needed before any final conclusion can be drawn. *p*-Coumaric acid glucoside was detected in currants, bog whortleberry, bilberry, half-highbush blueberry, cranberry, lingonberry and sweet rowanberry (**Table 8**), even though glycosides of hydroxycinnamic acids have been reported as being rare conjugates in berries and fruits (Herrmann 1989). Caffeic acid glucoside was not detected in the studied berries, and ferulic acid glucoside was only detected

in blueberry in agreement with reports in the literature (**Table 8**) (Schuster and Herrmann 1985, Macheix et al. 1990).

Esters were the most typical hydroxycinnamic acid conjugates in the studied berries. Esters consisted of quinic acid in all other families, except for glucose esters in family Grossulariaceae (**Table 8**). Previously, neochlorogenic acid was found in both black currant and gooseberry (Schuster and Herrmann 1985), but in the present study it was detected only in the latter berry (**Table 8**). Chlorogenic acid was the major hydroxycinnamic acid conjugate in bilberry, half-highbush blueberry, cranberry, and elderberry and dominated with neochlorogenic acid in chokeberry and sweet rowanberry in agreement with literature (**IV**: Figures 2-6) (Pyysalo and Kuusi 1974, Azar et al. 1987, Herrmann 1989, Zheng and Wang 2003). A predominance of neochlorogenic acid with lesser amounts of chlorogenic acid was typical for crowberry and blackthorn (**IV:** Figures 4 and 5) similarly to other stone fruits like cherries and plums (Möller and Herrmann 1983, Piga et al. 2003, Goncalves et al. 2004).

Less polar hydroxycinnamic acid esters were tentatively identified in currants (**II:** Table 1). These phenolic acids contained sugar and an unknown structure with a mass unit 96 or 98. Other hard-coated berries, lingonberry, chokeberry and sweet rowanberry, contained the same and other unpolar hydroxycinnamic acid conjugates (**Table 8**). Since these compounds were easily extracted in ethyl acetate, they may be part of the waxy surface of berries (Macheix et al. 1990).

Insoluble hydroxycinnamic acids

Insoluble hydroxycinnamic acids were quantified in the extraction residues of lingonberry, southern crowberry and elderberry (**Table 7**). All these berries are hard-coated, and these acids may be released from surface structures. It should be noted that acid hydrolysis was not optimized for quantitative analysis of hydroxycinnamic acids in the present study.

5.2.4 Gallic acid, ellagic acid and hydrolysable tannins

Free and conjugated forms of gallic acid and ellagic acid

Gallic acid, ellagic acid as well as their conjugated forms were quantified in *Fragaria* and *Rubus* species. Free gallic acid was found in cloudberry and soluble galloylesters in strawberry, yellow raspberry and arctic bramble (**III:** Table 2). Previously, free gallic acid was detected in the American *Rubus* species (Wada and Ou 2002) and methylgallate and gallotannins (tri- and pentagalloylglucose) in strawberry (Stöhr and Herrmann 1975a, Haddock et al. 1982). Monogalloyl ester has been found in mango fruit (Berardini et al. 2004), but otherwise gallotannins are rare in plant foods. Free ellagic acid was detected in all *Fragaria* and *Rubus* species in Study **III**. It is present in these berries, but was partly released from ellagitannins or ellagic acid glycosides due to the acidified conditions in sample

handling. The various forms of ellagic acid glycosides were typical to raspberry, especially wild raspberry, in accordance with the reports in the literature (Zafrilla et al. 2001, Mullen et al. 2003). Recently, ellagic acid glycosides were reported also in muscadine grape juices (Lee and Talcott 2004), but otherwise they are rare and have been less extensively studied than ellagitannins in plant foods.

Ellagitannins

The presence of ellagitannins in both ethyl acetate and methanol extracts of strawberry, raspberry, arctic bramble and cloudberry as well as in the extraction residues suggests variable molecular sizes and structures (**III**: Table 3). The distribution of two major ellagitannins was similar in all *Fragaria* and *Rubus* species. The two major ellagitannins were assumed to be sanguiin H-6 and lambertianin C, having two and three 1-galloyl-2,3:4,6-bis-HHDP-glucose units, respectively, in their structure. This structural unit was tentatively identified in HPLC-ESI-MS, and these ellagitannins have been previously found in raspberry (Mullen et al. 2002b, Mullen et al. 2003).

5.2.5 Flavonols

Composition of aglycons

Quercetin was the major flavonol in the studied berry species except black currant, crowberry, gooseberry and sea buckthorn (**II:** Table 3; **II:** Table 2; **IV:** Table 2). Myricetin was the major aglycon in black currant (cv. Öjebyn) in Study **IV**, in accordance with reports in the literature (Häkkinen et al. 1999b, Vuorinen et al. 2000, Mikkonen et al. 2001), but in Study **II** myricetin was present at the same level as quercetin. This inconsistency may be explained by the level of ripeness of the berries, since the amount of myricetin increased by 100% and that of quercetin by 50% during a two week follow-up period (Vuorinen et al. 2000). Study **V** on bilberry fruit development revealed that synthesis of myricetin begins in the half-coloured red berry, while the quercetin content decreased from the green berry to the early stage of colouring and was almost constant in the latest stages of ripening. Overall, the composition of flavonol aglycons was similar as previously reported and reviewed by Häkkinen (2000), except in the case isorhamnetin. Deconjugation of flavonol glycosides to aglycons by acid hydrolysis revealed the precence of isorhamnetin in gooseberry and sea buckthorn at the same level as quercetin (**IV:** Table 2). According to the recent study of Rörsch et al. (2003), isorhamnetin was the most important flavonol in sea buckthorn. A minor isorhamnetin glycoside was tentatively identified in arctic bramble in Study **III** and previously in raspberry (Mullen et al. 2002b).

Distribution of glycosides

Glucose was the most typical sugar moiety of quercetin glycosides (**Table 8**). The dominant pairs of flavonol glycosides were myricetin and quercetin galactosides in bog whortleberry, cranberry and crowberry as well as quercetin glucoside and galactoside in chokeberry and sweet rowanberry. In bilberry, equal amounts of quercetin and myricetin were conjugated with glucose and galactose. These findings on the distribution of the glycosides are supported by earlier reports on chokeberry, cranberry (Zheng and Wang 2003), bilberry (Azar et al. 1987), rowanberry (Gil-Izquierdo and Mellenthin 2001) and southern crowberry (Kärppä et al. 1984). Quercetin arabinoside and rhamnoside were typical to blackthorn berry, and were also detected in its flower (Olszewska and Wolbis 2002). Glucuronic acid was the typical and predominant conjugate in strawberries, raspberries, arctic bramble and cloudberry. The distribution of flavonol glycosides in strawberry and raspberry was consistent with the earlier results (Wang and Zheng 2001, Mullen et al. 2003).

In black, green and white currants and elderberry, quercetin was found mainly as both rutinoside and glucoside (**II:** Table 3, **IV:** Figure 6). Diglycosides other than rutinoside were detected in chokeberry, sweet rowanberry, blackthorn, crowberry, and elderberry (**Table 8**). A rare flavonol triglycoside was found in white and red currants in agreement with literature (Siewek et al. 1984). Flavonol glucoside-malonates were tentatively identified and quantified in the berries of currants for the first time (Study **II**).

Insoluble forms

Insoluble flavonols were released by acid hydrolysis of the extraction residues. Substantial amounts of flavonols were released from the berry matrices of strawberry (cv. Honeoye, Polka and Jonsok) and sea buckthorn (**Table 7**). Insoluble flavonols (kaempferol) in strawberry are considered to represent the cell wall bound phenolic compounds. Insoluble myricetin and quercetin in sea buckthorn are believed to be released from seeds, which were partly broken down during homogenization and were resistant to solubilization during solvent extraction.

5.2.6 Anthocyanins

Historically, studies on the distribution of anthocyanins have been used for purposes of chemotaxonomy (Harborne and Hall 1964, Øydvin 1974). Moroever, characteristic anthocyanin compositions are found to be useful in detecting adulteration or authenticity of fruit juices, jams and wines (Clifford 2000a). Scattered data on the distribution of anthocyanins were available for all of the berry species analysed in Studies **I-V** and were used to support the identifications (Macheix et al. 1990, Goiffon et al. 1991, Hyound and Hong 1992). Cyanidin was detected in all pigmented berry species (**III:** Table 2, **IV:** Table 2) and it

was the single dominant aglycon in red currant, red gooseberry, chokeberry, sweet rowanberry, elderberry, and cloudberry. Actually, there are few fruits which do not contain cyanidin (Macheix et al. 1990).

Tools to distinguish berries with two to five anthocyanidins

The composition of two to five anthocyanidins was evaluated to differentiate the species of berries from each other. The amount of delphinidin was clearly (Study **IV**) or slightly (Study **I**) higher compared to cyanidin in black currant (cv. Öjebyn), consistent with the composition in various cultivars (Iversen 1999, Kähkönen et al. 2003, Wu et al. 2004). Peonidin was found at higher content than that of cyanidin in blackthorn (**IV:** Table 2), whereas other *Prunus* species, such as cherry and plum, have been reported to contain only traces of peonidin compared to cyanidin (Harborne and Hall 1964). Cyanidin and peonidin occurred at the same level in cranberry, but cyanidin dominated clearly in lingonberry. Bog whortleberry, bilberry, blueberry and crowberry contained five aglycons, cyanidin, delphinidin, peonidin, petunidin and malvidin. The major aglycons were delphinidin and malvidin in bog whortleberry, delphinidin and cyanidin in bilberry, but mainly delphinidin in half-highbush blueberry consistent with European bilberry (France) and a mixture of wild clones of low-bush blueberry (Canada) (Kalt et al. 1999). The environmental, geographical or other factors in the northernmost latitudes of Finland (Ivalojoki) were correlated with higher relative content of cyanidin in bog whortleberry (Study **IV**). Cyanidin was the predominant anthocyanidin in northern crowberry and malvidin in southern crowberry. In the study on bilberry fruits of various origin, it was shown that cyanidin glycosides were present in slightly higher amounts in berries from the northern latitudes (Norway and Sweden) and delphinidin glycosides from the southern latitudes (Italy, Poland and Romania) (Martinelli et al. 1986). Bog whortleberry, bilberry, blueberry, cranberry and lingonberry showed galactose, glucose and arabinose as the sugar residues (Andersen 1985, Kalt et al. 1999, Huopalahti et al. 2000, Kähkönen et al. 2003, Zheng and Wang 2003), whereas only galactosides were found in crowberry (**Table 8**) (Kärppä et al. 1984).

Tools to distinguish berries with one major anthocyanidin

Other berry species, which contained only or mainly cyanidin, can be differentiated by their dominant glycosides. Red currant (cv. Red Dutch) was distinguished by its composition of six cyanidin glycosides including glucoside, three diglycosides and two triglycosides (2^G-glucosylrutinoside and 2^G-xylosylrutinoside) (Øydvin 1974). Glucose and rutinose were the typical sugar residues of red gooseberry (cv. Hinnonmaki's red), as reported recently for cultivars Whinham and Lancastine (Wu et al. 2004). The predominance of two major anthocyanins, cyanidin sambubioside and glucoside, in elderberry is well characterized (Goiffon et al. 1999, Chandra et al. 2001, Wu et al. 2004). Cyanidin sophoroside was the

major anthocyanin in cultivated red raspberry in Study **III** and it is known to be typical for European raspberry cultivars (de Ancos et al. 1999). However, a distinguishable anthocyanin profile was found in wild raspberry with equal amounts of cyanidin sophoroside and cyanidin glucoside (**III:** Table 2). Cyanidin rutinoside was the characteristic anthocyanin in arctic bramble. Cyanidin triglycoside was found in raspberry and cloudberry but not in arctic bramble. Pelargonidin glucoside was the major anthocyanin in the studied strawberry cultivars (Honeoye, Jonsok, Polka), similarly to various other cultivars in literature (Bakker et al. 1994, Bridle and García-Viguera 1997, Espín et al. 2000, Wang and Zheng 2001, Lopes-da-Silva et al. 2002). Chokeberry and sweet rowanberry showed almost identical chromatographic profiles for their anthocyanins with cyanidin galactoside, glucoside, arabinoside and xyloside (Study **IV:** Figure 4) in accordance with previous studies (Pyysalo and Kuusi 1974, Espín et al. 2000, Zheng and Wang 2003, Wu et al. 2004).

5.2.7 Flavan-3-ols

(+)-Catechin and (-)-epicatechin were identified and quantified in the berries (**III**: Table 2; **IV**: Table 2), but other forms of flavan-3-ols remained without quantification. (-)-Epigallocatechin was detected in all currants in HPLC-ESI-MS analysis (II: Table 1), but it was overlapped by other compounds in HPLC-DAD analysis. According to literature, (+)-gallocatechin and (-)-epigallocatechin are found in black currant, red currant, gooseberry and strawberry (Arts et al. 2000b, de Pascual-Teresa et al. 2000).

(+)-Catechin dominated in green, red and white currants, lingonberry and cranberry, while (-)-epicatechin dominated in black currant, red gooseberry, bog whortleberry and bilberry. These compositions of currants, gooseberry and cranberry exhibited some inconsistencies compared to berries (unknown cultivars) investigated in previous studies (Macheix et al. 1990, Arts et al. 2000a, de Pascual-Teresa et al. 2000). In general, there is marked variation in the composition flavan-3-ols between cultivars of the same species (Macheix et al. 1990). .(+)-Catechin was dominant in strawberry, arctic bramble, blackthorn, and sea buckthorn, while (-)-epicatechin dominated in red raspberry, chokeberry, sweet rowanberry, and crowberry in agreement with earlier studies (Macheix et al. 1990, Arts et al. 2000b, de Pascual-Teresa et al. 2000, Rösch et al. 2003).

5.2.8 Proanthocyanidins

Composition of low molecular weight proanthocyanidins was analysed in the ethyl acetate extracts of *Ribes*, *Fragaria*, *Rubus* and *Vaccinium* species. Dimeric and trimeric proanthocyanidins were tentatively identified as GC-(4,8)-GC, C-(4,8)-GC and dimer B3 (**II:** Table 2) in currants and dimer B2 in raspberry, arctic bramble and cloudberry (**III**: Table 1),

in accordance with previous results for red currant and raspberry (de Pascual-Teresa et al. 2000). It should be noted that some proanthocyanidins overlapped in the HPLC-ESI-MS detection. Our later study (Puupponen-Pimiä et al. 2005) on isolated fractions of proanthocyanidins revealed the occurrence of dimers B3, B1, and B4 in cloudberry and dimers B4 and B2 in raspberry as well as one B-type trimer (C2 according to the retention order) in both of these berries. Similarly, the fractions of proanthocyanidins were isolated from bog whortleberry, bilberry, cranberry and lingonberry to investigate their antioxidant activity in Study **VI**. Both A- and B-type dimers and trimers were tentatively identified in these fractions (**VI:** Table 1). According to the retention order of proanthocyanidins (Rohr et al. 2000), dimer B2 and B7 were found in all studied wild *Vaccinium* species and dimer B3 and B5 in lingonberry and cranberry, in agreement with studies on related species (Morimoto et al. 1988, de Pascual-Teresa et al. 2000, Foo et al. 2000b). Two A-type dimers, according to the literature, A1 and A2, and five A-type trimers were found in cranberry and lingonberry (Morimoto et al. 1988, Foo et al. 2000b). In addition, bilberry and bog whortleberry contained A-type proanthocyanidins, even though primary B-type forms have been reported in other bluish-black coloured *Vaccinium* species (Prior et al. 2001, Schmidt et al. 2004). Among the common plant foods, A-type linkages were found in plums, avocados and peanuts (Gu et al. 2004). This unique doubly linked chain structure of flavonoids has aroused special interest, because it is suspected to contribute to antiadhesion activity against bacteria (Milner 2002) and to their antiviral effects (de Bruyne et al. 1999).

The extraction residues were acid hydrolysed to liberate insoluble proanthocyanidins as anthocyanidins (Studies **I, III** and **IV**). According to the spectral characteristics, the released anthocyanidins were cyanidin, delphinidin and pelargonidin, which represent the constituent units (epi)catechin (procyanidin), (epi)gallocatechin (prodelphinidin) and (epi)afzelechin (propelargonidin), respectively. Proanthocyanidins can be characterized by their procyanidin/prodelphinidin or procyanidin/propelargonidin ratios (Foo and Porter 1981, Gu et al. 2003a, Wu et al. 2004). Only procyanidins were detected in bog whortleberry, lingonberry, sweet rowanberry, chokeberry and elderberry in accordance with the recent report for these latter two species (Wu et al. 2004). Procyanidin dominated (>55%) in bilberry, half-highbush blueberry, cranberry, blackthorn and sea buckthorn, whereas prodelphinidin (>55%) dominated in currants and red gooseberry. These results support the earlier finding that prodelphinidin units dominate in the Grossulariaceae family and procyanidin in the Ericaceae and Rosaceae families (Foo and Porter 1981, Wu et al. 2004). As was recently noted (Gu et al. 2003a), propelargonidin was another proanthocyanidin in *Fragaria* and *Rubus* species of Rosaceae family. In strawberry, cloudberry and arctic bramble procyanidin units dominated (approx. 80%), but in raspberry, propelargonidin units were detected in approximately equal amounts.

5.3 Phenolic compounds in colour variants and fruit development stages

5.3.1 Coloured species compared to their uncoloured variants

Pigmented and unpigmented variants of currants, gooseberry and bilberry were investigated in Studies **I**, **II** and **V**. The clear difference between these berries is the lack of anthocyanins in the unpigmented berries. Interest was focused on changes in the composition of hydroxycinnamic acids, since they are precursors of other phenolic compounds in the biosynthetic pathway (**Figure 2**). The content of *p*-coumaric acid was almost doubled by the mutation from black to green currant. In white currant, the contents of both *p*-coumaric acid and caffeic/ferulic acid were higher than in red currant. Yellow gooseberry contained double the amount of *p*-coumaric acid, but lower amounts of caffeic and ferulic acids than red gooseberry. These results suggest that the lack of anthocyanins is associated with changes in the levels of hydroxycinnamic acids. In a similar study with grapes, the relative proportions of caffeic and ferulic acid derivatives increased with the change from coloured to white forms (Macheix et al. 1990). However, there is no trend to indicate that reduced synthesis of anthocyanins would be replaced by enhanced synthesis of phenolic acids. From the results obtained in Study **IV**, the opposite may be suggested, since increased production of other phenolic compounds was associated with high amounts of anthocyanins. Similarly, coloured potato tuber cultivars contained twice as much phenolic acids as white tubers (Lewis et al. 1998).

There was also an interesting difference in the composition of flavonols. Black currant contained both quercetin and myricetin, but only quercetin was detected in the respective unpigmented variant, green currant. There was similar assocation between bilberry and its natural colour variants (Study **V**). The possible explanation is the organization of flavonoid pathway genes as suggested by Jaakola (2003).

5.3.2 Bilberry fruit development

The composition and contents of flavonols, proanthocyanidins and anthocyanins in bilberry was determined from flower to ripe fruit (Study **V**). The content of quercetin was highest in flower and at the beginning of berry development, and decreased during the later stages of ripening (**V**: Fig. 5 a). Myricetin was detected and quantified only during the later stages of ripening, and reached the level of quercetin in ripe bilberry. Procyanidins were the major flavonoids in early developmental stages, but were not quantified in the strongly pigmented bilberries due to analytical problems (5.1.2). However, the results indicated that there is a reduction in the proanthocyanidin content during ripening of white coloured bilberry variants. The astringent taste of proanthocyanidins may protect plants against early feeding (Harborne

and Williams 2000). The anthocyanins in the flower consisted of cyanidin glycosides only (**V**: Fig. 6), whereas other bilberry anthocyanins appeared later with increasing contents during the progress of berry ripening. The role of biosynthetic genes in the accumulation of bilberry flavonoids was discussed by Jaakola (2003).

5.4 Berries as a source of phenolic compounds

The best dietary sources of phenolic compounds are frequently estimated using food consumption databases (Clifford 2000b, Clifford 2000a, Hollman and Arts 2000, Häkkinen 2000, Manach et al. 2004). In the following section, the emphasis is focused on the composition of the conjugated phenolic compounds, since these forms may in some extent contribute to the bioavailability of the phenolic compounds.

5.4.1 Hydroxycinnamic acids

Hydroxycinnamic acids are found in plants as soluble conjugates or insoluble structural components (Ibrahim and Barron 1989). In the studied berries they were available mostly in soluble forms (**Table 7**). The hydroxycinnamic acid contents varied from low (< 20 mg/kg) in red currant, raspberries, bog whortleberry and sea buckthorn berry to very high (700-900 mg/kg) in chokeberry and sweet rowanberry, in accordance with previously published results (Azar et al. 1987, Herrmann 1989, Gil-Izquierdo and Mellenthin 2001, Kähkönen et al. 2001, Mattila and Kumpulainen 2002, Zheng and Wang 2003).

The fate of hydroxycinnamic acids in the gastrointestinal tract depends on their native forms in plant sources. Extensive studies have been made for evaluation of metabolism and absorption of hydroxycinnamic acids in soluble free and conjugated forms (Olthof et al. 2001, Nardini et al. 2002, Kern et al. 2003a, Kern et al. 2003b, Konishi et al. 2004, Konishi and Kobayashi 2004a, Konishi and Kobayashi 2004b) and in insoluble fibre-bound forms (Azuma et al. 2000, Andreasen et al. 2001, Gonthier et al. 2003b). These studies suggest that the soluble hydroxycinnamic acids have better bioavailability than the insoluble forms. Moreover, soluble free forms are absorbed from the small intestine more readily than conjugated forms. Cloudberry was a good source of hydroxycinnamic acids providing 50-60 mg/kg of free hydroxycinnamic acids. Chokeberry, sweet rowanberry, blueberry, elderberry, bilberry, northern crowberry and lingonberry were good sources of hydroxycinnamic acids due to their high contents (100-900 mg/kg). These berries are comparable to other food items with high contents of hydroxycinnamic acids, as shown in **Figure 10**. Hydroxycinnamic acids are mainly present as insoluble forms in rye and barley, which reduces their value as sources of bioavailable phenolic compounds (Sun et al. 2001). Coffee, similarly to fruits and vegetables in **Figure 10**, contains mainly caffeic acid esterified with quinic acid (Herrmann 1989, Lewis

et al. 1998, Andreasen et al. 2000, Hernanz et al. 2001, Nardini et al. 2002, Goncalves et al. 2004, Schütz et al. 2004).

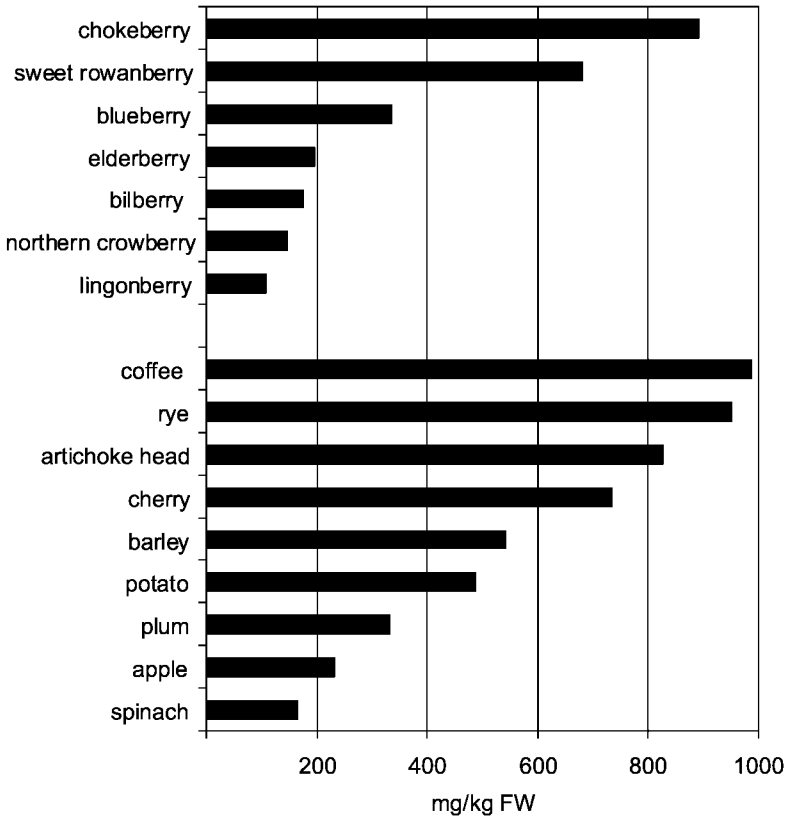


Figure 10. The highest contents of hydroxycinnamic acids in berries (Studies **III** and **IV**) compared to other plant foods (Herrmann 1989, Lewis et al. 1998, Andreasen et al. 2000, Hernanz et al. 2001, Nardini et al. 2002, Goncalves et al. 2004, Schütz et al. 2004).

5.4.2 Ellagic acid, ellagi- and gallotannins

Soluble forms of gallic acid and gallotannins were found in the extracts of *Fragaria* and *Rubus* species (**III:** Table 3). Strawberry was found to be a good source of galloylesters (120 mg/kg), ten times better than reported earlier (Stöhr and Herrmann 1975a). Free gallic acid is well absorbed, but there is no respective data on its conjugates (Manach et al. 2005b). Only cloudberry contained free gallic acid among the studied berries, with a content (40 mg/kg)

equal to a tea infusion (20 mg/l) made using a tea bag (Tomás-Barberán and Clifford 2000). The contents of gallic acid and galloylesters were low or negligible in the other *Rubus* species, consistent with the contents of free gallic acid reported for the American *Rubus* species (Wada and Ou 2002).

Ellagic acid was present as free and glycosylated forms and as ellagitannins subjected to varying degrees of polymerization. Previously, berries of *Fragaria* and *Rubus* species have been reported as the best sources of ellagic acid (Tomás-Barberán and Clifford 2000) compared to other fruits and nuts (Daniel et al. 1989). According to Study **III,** the contents in these berries may be even higher than those previously reported (Daniel et al. 1989, Maas et al. 1991, de Ancos et al. 2000, Häkkinen et al. 2000a, Mattila and Kumpulainen 2002). The estimated intake of ellagic acid should include free and conjugated forms of ellagic acid as well as soluble and insoluble ellagitannins. These contents (mg/kg FW) would be approximately 650-850 in strawberry, 1900 in cultivated raspberry, 2700 in wild raspberry, 3900 in arctic bramble and 3600 in cloudberry. Ellagitannins contributed to 94-99% of ellagic acid equivalents. Gut microflora was recently found to have an important role in the metabolism and absorption of ellagitannins and ellagic acid glycosides from the intestine (Cerdá et al. 2003, Cerdá et al. 2005). Considerable inter-individual differences were noted identifying "high and low metabolite excreters" depending on the health status of the gut flora (Cerdá et al. 2005). Therefore, bioavailability of ellagic acid from various sources is less dependent on food matrix, solubility and conjugation factors.

5.4.3 Flavonols

The contents of total flavonols in berries provided in Studies **III** and **IV** are compared to those of other food items, with high flavonol contents (**Figure 11**). Bog whortleberry, sea buckthorn, chokeberry, elderberry and cranberry contained higher or equal contents with those present in onions and kale. In general, the content represents soluble flavonol glycosides, except in the case of sea buckthorn. Insoluble flavonols (30%) in sea buckthorn may be considered as unabsorbable forms. As a general rule for the soluble flavonol glycosides, the glucosides and aglycons are the most absorbable forms of flavonols, whereas all other glycosides must reach the colon where they need to be hydrolysed by the microflora prior to possible absorption (Hollman and Katan 1999). These results have been obtained for quercetin and the hydroxylation pattern of the B-ring of flavonol may affect absorption (Hou et al. 2003). Therefore, quercetin glucoside is the only form of flavonol glycosides, which has been proven to be absorbed in the small intestine according to current knowledge.

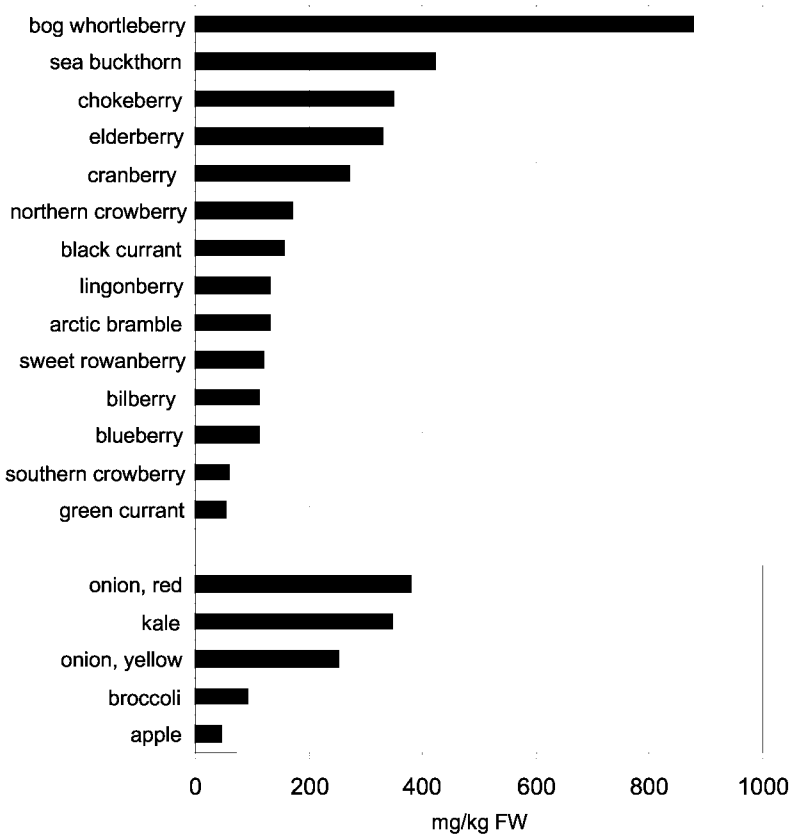


Figure 11. The highest contents of flavonols in berries (Studies **III** and **IV**) compared to other plant foods (Hertog et al. 1992, Ferreres et al. 1996, Price and Rhodes 1997, Hollman and Arts 2000).

The original quantitative data presented in Study **IV** was reanalysed for the contents of quercetin glucosides. Quercetin glucoside was a minor flavonol glycoside in bog whortleberry, cranberry, lingonberry and crowberry. The amount of quercetin provided from glucoside was 83 mg/kg in elderberry, 58 mg/kg in chokeberry, 31 mg/kg in bilberry, 23 mg/kg in green currant, 20 mg/kg in sea buckthorn, 18 mg/kg in sweet rowanberry, 18 mg/kg in half-highbush blueberry and 17 mg/kg in black currant. The total quercetin content was 40-450 mg/kg in onions (Ferreres et al. 1996, Crozier et al. 1997, Price and Rhodes 1997, Justesen et al. 1998, Hollman and Arts 2000, Bonaccorsi et al. 2005), of which 20-200 mg/kg was in form of glucoside (Price and Rhodes 1997). Onion is a more relevant source of quercetin glucoside than the berries studied. However, other flavonol glycosides and insoluble flavonols ingested from berries may be bioavailable as the aglycon or other forms after fermentation by gut flora (Hollman and Katan 1999, Olthof et al. 2003). In the study of

Erlund et al. (2003), black currant, lingonberry and bilberry were all good sources of bioavailable quercetin, but no conclusions were available for comparison of these berries. Quercetin galactoside, rhamnoside and unknown glycosides were present in lingonberry instead of quercetin glucoside (Study **IV**), suggesting that also these glycosides may be relevant sources of bioavailable quercetin.

5.4.4 Anthocyanins

The food items with the highest contents of anthocyanidins are easily recognized, since the deep red or even bluish-black colour is a good indicator of the presence of these pigments. However, there are few studies to show the contents on an aglycon basis or in values which could be recalculated. Analytical differences also influence the true comparability of the available data on anthocyanin contents (Clifford 2000a). In Studies **I-V**, the contents of anthocyanins are expressed as the weight of the phenolic aglycon.

The highest contents of anthocyanins (7680-8420 mg/kg) were detected in chokeberry, bilberry and northern crowberry. Black currant, southern crowberry, bog whortleberry and elderberry contained about half of that content (3320-4320 mg/kg). Moderate contents of anthocyanins in half-highbush blueberry and lingonberry (130-150 mg/kg) make them as good sources as the other coloured plant foods, such as cherries, red onions and eggplants (Gao and Mazza 1995b, Paganga et al. 1999, Sakakibara et al. 2003). Raspberry, cranberry, arctic bramble and sweet rowanberry contained anthocyanins 70-90 mg/kg. The present anthocyanin contents are comparable to earlier results in the case of chokeberry, black currant, raspberry, and blueberry (Gao and Mazza 1994, de Ancos et al. 1999, Zheng and Wang 2003, Wu et al. 2004).

The effect of the sugar moiety on absorption of anthocyanin in humans is not as clear as in the case of quercetin. The urinary excretion of anthocyanins was reported to be low for all berry extracts and juices studied (Manach et al. 2005b), indicating that all anthocyanins have similar low absorption efficiency. Further studies are needed to show the possible role of the sugar moiety and the role of microflora in the absorption of anthocyanins (Manach et al. 2005b).

5.4.5 Flavan-3-ols and proanthocyanidins

The present extraction method was not optimal for flavan-3-ols and proanthocyanidins. Therefore, the quantitative results obtained are generally lower than those reported in literature (Arts et al. 2000b, de Pascual-Teresa et al. 2000, Hammerstone et al. 2000, Gu et al. 2004, Manach et al. 2005a). The main sources of flavan-3-ols and proanthocyanidins are tea, chocolate, apples, pears, grapes, red wine and beans (Hammerstone et al. 2000, Gu et al.

2004, Manach et al. 2005a). According to the contents of flavan-3-ols in the studied berry species, lingonberry was the best source of flavan-3-ols with ten times higher contents in than in other berries. Among the other common berry species, high flavan-3-ol content has been reported for blackberry (Arts et al. 2000b, de Pascual-Teresa et al. 2000). High levels of insoluble proanthocyanidins were detected in chokeberry, sweet rowanberry and blackthorn, consistent with the astringent taste of these berries and the recent study of Wu et al. (2004).

6 SUMMARY AND CONCLUSIONS

Performance of the methods

The developed extraction methods were based on either sequential extraction or berry-solvent suspension. Phenolic compounds were identified and quantified by reversed-phase high-performance liquid chromatography combined with diode array detection. Mass spectrometric detection and literature data were used for the further identification.

Adequate quantitative data was obtained on the contents of hydroxycinnamic acid conjugates, ellagitannins, flavonol glycosides and anthocyanins. The contents of flavan-3-ols and proanthocyanidins provided by the sequential extraction method remain tentative since determination of these classes would demand their own separate extraction and purification procedure. The sequential extraction was necessary for those berry samples containing both easily destructible structures of flavonol malonylglucosides and anthocyanins, which prefer acidified extraction conditions. The berry-solvent suspension method is a convenient sample handling procedure if one wishes to to quantify non-acylated flavonol glycosides and anthocyanins. The two strategies used for the quantification of ellagitannins, either as native forms (in gallic acid equivalents) or as acid hydrolysis products (in ellagic acid equivalents), were comparable in the analysis of the *Rubus* berries, but not in the case strawberry. However, the method based on acid hydrolysis was found to be the most practical for the quantification of ellagitannins.

Characteristics of the distribution of phenolic conjugates in berries

Special attention was paid to the distribution and composition of phenolic compounds in the studied berries. This data is informative for studies on chemotaxonomy and authenticity of berry raw materials.

1. Distinctive similarities were found among berry species of the same family in the distribution of conjugated forms of phenolic compounds. However, differences in the chromatographic profiles of conjugates and the composition of aglycons were observed especially in the case of anthocyanins. Notably, the chromatographic profiles of chokeberry and sweet rowanberry (family Rosaceae) were similar. (Studies **I**, **II**, **III**, and **IV**)
2. *p*-Coumaric acid was the major hydroxycinnamic acid present in currants, red gooseberry, strawberry, raspberry, cloudberry, bog whortleberry, lingonberry and crowberry, and caffeic acid dominated in bilberry, half-highbush blueberry, cranberry, chokeberry, sweet rowanberry and elderberry. Esters were the most typical conjugates of hydroxycinnamic acids. (Studies **I**, **II**, **III**, and **IV**)
3. The composition of two major ellagitannins was similar in strawberry, raspberry, arctic bramble and cloudberry. (Study **III**)

4. Quercetin was the major flavonol in green, red and white currants, bog whortleberry, bilberry, blueberry, lingonberry, cranberry, chokeberry, sweet rowanberry, blackthorn, strawberry, raspberry, arctic bramble, cloudberry and elderberry, and dominated with myricetin in black currant and crowberry and with isorhamnetin in gooseberry and sea buckthorn. Glucose was the most widespread sugar moiety of the quercetin glycosides. Glucoside-malonates were tentatively identified and quantified in the berries of currants for the first time. (Studies **I**, **II**, **III**, and **IV**)
5. Cyanidin was detected in all pigmented berry species and it was the single dominant aglycon in red currant, red gooseberry, chokeberry, sweet rowanberry, elderberry and cloudberry. (Studies **I**, **II**, **III**, and **IV**)
6. Both A- and B-type proanthocyanidin dimers and trimers were tentatively identified in lingonberry, cranberry, bilberry and bog whortleberry. (Study **VI**)
7. The lack of anthocyanins in colour variants of berries and in the early stages of bilberry fruit development was connected to their lower content of myricetin and to the changes in the composition of hydroxycinnamic acids. (Studies **I**, **II**, **III**, **IV** and **V**)

Berries as a source of dietary phenolic compounds

The composition of native forms was evaluated for berries with the highest contents of phenolic compounds, since these forms may contribute to the bioavailability of phenolic compounds.

1. The highest hydroxycinnamic acid contents were observed in chokeberry (890 mg/kg), followed by sweet rowanberry (680 mg/kg), half-highbush blueberry (340 mg/kg), elderberry, bilberry, northern crowberry and lingonberry (110-200 mg/kg). The major chlorogenic acid and neochlorogenic acid are soluble and according to recent knowledge may be more bioavailable than fibre-bound forms in cereals. (Studies **III** and **IV**)
2. The estimated amounts of ellagic acid in various forms are approximately 650-850 mg/kg in strawberry, 1900 mg/kg in cultivated raspberry, 2700 mg/kg in wild raspberry, 3900 mg/kg in arctic bramble and 3600 mg/kg in cloudberry. (Study **III**)
3. The highest flavonol contents were detected in bog whortleberry (820-1020 mg/kg) followed by sea buckthorn, chokeberry, elderberry and cranberry (270-420 mg/kg). According to current knowledge, quercetin glucoside is the form of flavonol glycosides which can be absorbed from the small intestine. The amount of quercetin provided as glucoside was 83 mg/kg in elderberry, 58 mg/kg in chokeberry, and 31 mg/kg in bilberry. (Study **IV**)
4. The highest contents of anthocyanins were determined in chokeberry, bilberry and northern crowberry (7680-8420 mg/kg), followed by black currant, southern crowberry, bog whortleberry and elderberry (3320-4120 mg/kg). (Studies **I**, **II**, **III**, and **IV**)

5. Lingonberry contained ten times higher levels of flavan-3-ols and low molecular weight proanthocyanidins than the other berries. (Study **VI**)

This thesis shows that berries are rich sources of a diverse spectrum of phenolic compounds. Compared to other foods, they provide high or moderate amounts of ellagitannins, anthocyanins and flavonols. In quantitative terms, anthocyanins and ellagitannins are the predominant phenolic classes encountered in berries.

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