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Doctoral dissertation

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Department of Clinical Nutrition
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ABSTRACT

The purpose of this thesis was to identify and quantify the non-anthocyanin phenolic compounds in Finnish berries. The compounds of interest were kaempferol, quercetin and myricetin (flavonols); p-coumaric, caffeic and ferulic acids (hydroxycinnamic acids); p-hydroxybenzoic, gallic and ellagic acids (hydroxybenzoic acids). These compounds were selected because of their proposed health-promoting effects as antioxidants and anticarcinogens. High-performance liquid chromatographic methods for the screening of these phenolic compounds and for the quantification of flavonols and ellagic acid in berries were optimised and validated.

Phenolic profiles were determined in 19, flavonols in 25 and ellagic acid in eight berries. Marked differences were observed in the phenolic profiles among the berries, with certain similarities within families and genera. Total contents of flavonols (100–263 mg/kg) in cranberry (*Vaccinium oxycoccos*), bog whortleberry (*Vaccinium uliginosum*), lingonberry (*Vaccinium vitis-idaea*), black currant (*Ribes nigrum*) and crowberry (*Empetrum nigrum* and *Empetrum hermaphroditum*) were higher than those reported for the commonly consumed fruits or vegetables, except for onion, kale and some lettuces. Ellagic acid content varied from 350 to 700 mg/kg, being highest in arctic bramble (*Rubus arcticus*). Varietal differences were observed in the contents of flavonols and phenolic acids among the six strawberry and four blueberry cultivars studied. Some regional differences were detected in strawberries grown in Finland or in Poland. No consistent differences between conventional and organic cultivation of strawberries were detected.

The effects of juicing, cooking or crushing on flavonols were studied in five berries commonly consumed in Finland. Juicing or crushing of the berries by common domestic methods resulted in marked losses of flavonols (40–85%), whereas jam-cooking caused only a small loss (<20%) of flavonols and ellagic acid. Compared to steam-extraction, cold-pressing was a superior juicing method in extracting flavonols from black currants. The contents of flavonols and ellagic acid in the frozen berries and berry products were analysed after 3, 6 and 9 months of storage in a domestic refrigerator or freezer. Effects of freezing on quercetin varied in different berries. Myricetin and kaempferol were more susceptible than quercetin to losses during processing and long-term storage of berries. Ellagic acid content decreased during jam-making and storage of berries.

In 1998, the average daily intakes of flavonols and ellagic acid from berries by the Finnish population were 3.4 and 8.7 mg, respectively. Berries accounted for 30% of the total dietary intake of flavonols, and they probably represented the most important source of ellagic acid. In conclusion, the present results demonstrate that Finnish berries are excellent sources of flavonols and ellagic acid. They also show that consumption of berries plays a significant role in the dietary intake of these phenolics by the Finnish population.

National Library of Medicine Classification: QU 220, QU 145.5, WB 430
Medical Subject Headings: anticarcinogenic agents; antioxidants; bioflavonoids; chromatography, high pressure, liquid; coumaric acids; eating; ellagic acid; Finland; food/analysis; food handling; fruit; hydroxybenzoic acids; quercetin

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Vantaa, September 2000

Sari Häkkinen

ABBREVIATIONS

API-MS	atmospheric pressure ionisation – mass spectrometry
CA	caffeic acid
CA4H	cinnamic acid 4-hydroxylase
CID	collision-induced dissociation
4CL	4-coumarate: coenzyme A ligase
CO	p-coumaric acid
CV	coefficient of variation
d	day
DAD	diode array detection
E	ellagic acid
EC	electrochemical
ESI-MS	electrospray ionisation – mass spectrometry
f.w.	fresh weight
GC-MS	gas chromatography – mass spectrometry
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
K	kaempferol
LC-MS	liquid chromatography – mass spectrometry
M	myricetin
MS	mass spectrometry, mass spectrometer
m/z	mass – charge ratio
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NaOH	sodium hydroxide
ODS	octadecylsilane
PAL	phenylalanine ammonialyase
PCA	principal component analysis
PDA	photo-diode array detection
Q	quercetin
RP	reversed-phase
SID	source-induced dissociation
TBHQ	*tert*-butylhydroquinone
TIC	total ion chromatogram
TLC	thin-layer chromatography
UV	ultra violet
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–VII**:

I Häkkinen SH, Kärenlampi SO, Heinonen IM, Mykkänen HM, Törrönen AR. HPLC method for screening of flavonoids and phenolic acids in berries. J Sci Food Agric 1998; 77: 543–551.

II Häkkinen S, Heinonen M, Kärenlampi S, Mykkänen H, Ruuskanen J, Törrönen R. Screening of selected flavonoids and phenolic acids in 19 berries. Food Res Int 1999; 32: 345–353.

III Häkkinen SH, Kärenlampi SO, Heinonen IM, Mykkänen HM, Törrönen AR. Content of the flavonols quercetin, myricetin, and kaempferol in 25 edible berries. J Agric Food Chem 1999; 47: 2274–2279.

IV Häkkinen S, Auriola S. High-performance liquid chromatography with electrospray ionisation mass spectrometry and diode array ultra violet detection in the identification of flavonol aglycones and glycosides in berries. J Chromatogr A 1998; 829: 91–100.

V Häkkinen SH, Törrönen AR. Content of flavonols and selected phenolic acids in strawberries and Vaccinium species: Influence of cultivar, cultivation site and technique. Food Res Int 2000; 33: 517–524.

VI Häkkinen SH, Kärenlampi SO, Mykkänen HM, Heinonen IM, Törrönen AR. Ellagic acid content in berries: Influence of domestic processing and storage. Eur Food Res Technol, in press.

VII Häkkinen SH, Kärenlampi SO, Mykkänen HM, Törrönen AR. Influence of domestic processing and storage on flavonol contents in berries. J Agric Food Chem 2000; 48: 2960-2965.

In addition, some previously unpublished results are presented.

CONTENTS

1 INTRODUCTION

In addition to many essential nutritional components, plants contain phenolic substances, a large and heterogeneous group of biologically active non-nutrients (Schahidi and Naczk 1995). Flavonoids are divided into many categories, including flavonols, flavones, catechins, proanthocyanidins, anthocyanidins and isoflavonoids (Havsteen 1983, Shahidi and Naczk 1995). Phenolic acids present in plants are hydroxylated derivatives of benzoic and cinnamic acids (Herrmann 1989, Shahidi and Naczk 1995). Flavonoids and phenolic acids have many functions in plants. They act as cell wall support materials (Wallace and Fry 1994) and as colourful attractants for birds and insects helping seed dispersal and pollination (Harborne 1994). Phenolic compounds are also important in the defence mechanisms of plants under different environmental stress conditions such as wounding, infection, and excessive light or UV irradiation (Bennet and Wallsgrove 1994, Dixon and Paiva 1995).

The biological potency of secondary plant phenolics was found empirically already by our ancestors; phenolics are not only unsavoury or poisonous, but also of possible pharmacological value (Strack 1997). Flavonoids have long been recognised to possess antiallergenic, anti-inflammatory, antiviral and antiproliferative activities (Kühnau 1976, Harborne 1994). Flavonoids and phenolic acids also have antioxidative (Osawa et al. 1987, Frankel et al. 1993, Rice-Evans et al. 1996, Robards et al. 1999) and anticarcinogenic effects (Hayatsu et al. 1988, Strube et al. 1993, Sharma et al. 1994, Stavric 1994). Inverse relationships between the intake of flavonoids (flavonols and flavones) and the risk of coronary heart disease (Hertog et al. 1993a, 1995, Knekt et al. 1996), stroke (Keli et al. 1996), lung cancer (Knekt et al. 1997, Le Marchand et al. 2000), and stomach cancer (Garcia-Closas et al. 1999) have been shown in epidemiological studies. In other epidemiological studies, however, no association was found between the intake of flavonoids and the risk of heart disease (Hertog et al.1994, Rimm et al. 1996) or cancer (Hertog et al. 1995, 1997). Although the role of flavonoids and phenolic acids in the maintenance of health and prevention of diseases seems positive, the evidence is still limited and conflicting. Moreover, the bioavailability of flavonoids and phenolic acids from various foods, and the extent and mechanism of absorption in the human body are poorly known.

Berries belong traditionally to the Nordic diet. A multitude of phenolic compounds has been detected in berries (e.g. Wildanger and Herrmann 1973, Schuster and Herrmann 1985, Hertog et al.

1992b, Justesen et al. 1998), their content being highly variable in different berries. Recent studies have shown that extracts of berries, in particular strawberries and berries of the genus *Vaccinium*, have antioxidative (Costantino et al. 1992, Wang et al. 1996, Prior et al. 1998, Kalt et al. 1999, Kähkönen et al. 1999) and anticarcinogenic (Bomser et al. 1996) effects *in vitro*, which are partly proposed to be due to phenolic compounds. Diets supplemented with blueberry or strawberry extracts were beneficial in reversing the course of neuronal and behavioral ageing in rats (Joseph et al. 1998, 1999). A freeze-dried strawberry preparation was found to be an effective inhibitor of esophageal cancer in rats (Stoner et al. 1999). Furthermore, interesting data on the effects of berries in humans have been reported. In elderly women, serum and urine antioxidant capacity was increased following consumption of strawberries (Cao et al. 1998).

In Finland, the season during which fresh berries are available is short, lasting from late June (strawberry) to October (cranberry). Therefore, only a small proportion of berries is consumed fresh and most of the harvest is preserved by freezing or by processing to juices, jams, jellies, etc. Influences of processing and storage on the quality and quantity of flavonol glycosides have been reported in vegetables (Price et al. 1997, 1998a, b, Hirota et al. 1998) and apples (Burda et al. 1990, Price et al. 1999). Effect of juice- and wine-making on ellagic acid content has been studied in red raspberries (Rommel and Wrolstad 1993c) and grapes (Auw et al. 1996). There are, however, no other previous reports on the effects of processing and storage either on flavonol or on ellagic acid contents of berries.

The compounds of interest in the present study were: kaempferol, quercetin and myricetin (flavonols); p-coumaric, caffeic and ferulic acids (hydroxycinnamic acids); p-hydroxybenzoic, gallic and ellagic acid (hydroxybenzoic acids). These compounds were selected because of their proposed health-promoting effects. The aim of this thesis was to determine the main non-anthocyanin phenolics in berries grown or cultivated in Finland and to quantify flavonols and ellagic acid in berries. Since the comparison of the contents of phenolic compounds in different berries has been difficult because of the varying analytical methods used, one of the aims of this thesis was the optimisation and validation of the methods. The influences of cultivar, cultivation site and cultivation technique on the content of phenolic compounds were studied in strawberry and blueberry. In addition, influences of domestic processing (e.g. jam-cooking and juicing) and storage methods on the contents of flavonols and ellagic acid in berries were assessed. Finally, the contribution of berries to the dietary intake of flavonols and ellagic acid in Finland was estimated.

2 REVIEW OF THE LITERATURE

2.1 Structures of flavonols and phenolic acids

2.1.1 Flavonols

Flavonoids are compounds which all posses the same C_{15} (C_6 - C_3 - C_6) flavone nucleus (Harborne 1988, Macheix et al. 1990): two benzene rings (A and B) linked through an oxygen-containing pyran or pyrone ring (C) (Figure 1). This structure is common to 3-deoxyflavonoids (flavones, flavanones, isoflavones and neoflavones) and 3-hydroxyflavonoids (flavonols, anthocyanins, flavan-3,4-diols and flavan-3-ols), i.e. whether a hydroxyl group is present at the C3 position or not (Kühnau 1976).

Flavonols (kaempferol, quercetin and myricetin) (Figure 1) are pale yellow, poorly soluble substances present in flowers and leaves of at least 80% of higher plants and also in fruits and berries (Kühnau 1976). Flavonols occur in foods usually as *O*-glycosides, D-glucose being the most common sugar residue. Other sugar residues are D-galactose, L-rhamnose, L-arabinose, D-xylose and D-glucuronic acid. The preferred binding site for the sugar residue is C3 and less frequently the C7 position (Herrmann 1976, 1988).

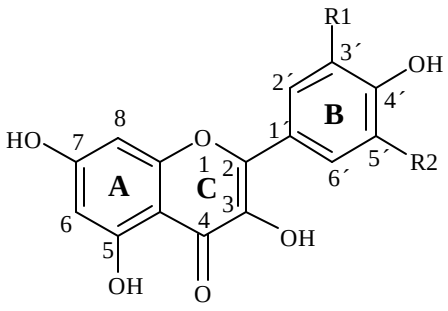


Figure 1. Chemical structure of flavonols: kaempferol, R1=H, R2=H; quercetin, R1=OH, R2=H; myricetin, R1=OH, R2=OH.

2.1.2 Phenolic acids

Hydroxybenzoic acids

Hydroxybenzoic acids have a general structure of C_6-C_1 derived directly from benzoic acid (Figure 2a). Variations in the structures of individual hydroxybenzoic acids lie in the hydroxylations and methylations of the aromatic ring (Macheix et al. 1990). Four acids occur commonly: p-hydroxybenzoic, vanillic, syringic, and protocatechuic acid. They may be present in soluble form conjugated with sugars or organic acids as well as bound to cell wall fractions, e.g. lignin (Schuster and Herrmann 1985, Strack 1997). A common hydroxybenzoic acid is also salicylic acid (2-hydroxybenzoate). Gallic acid (Figure 2a) is a trihydroxyl derivative which participates in the formation of hydrolysable gallotannins (Haslam 1982, Haddock et al. 1982, Strack 1997). Its dimeric condensation product (hexahydroxydiphenic acid) and related dilactone, ellagic acid (Figure 2b), are common plant metabolites. Ellagic acid is usually present in ellagitannins as esters of diphenic acid analogue with glucose (Haslam 1982, Haddock et al. 1982, Maas et al. 1992).

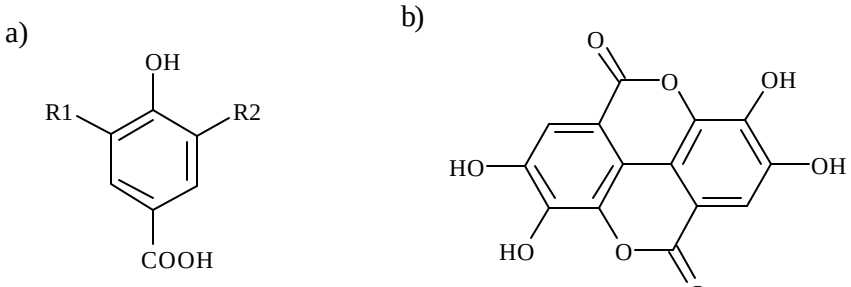


Figure 2. Chemical structures of (a) hydroxybenzoic acids: p-hydroxybenzoic acid, R1=H, R2=H; gallic acid, R1=OH, R2=OH, and (b) ellagic acid.

Hydroxycinnamic acids

The four most widely distributed hydroxycinnamic acids in fruits are p-coumaric, caffeic, ferulic and sinapic acids (Figure 3) (Macheix et al. 1990). Hydroxycinnamic acids usually occur in various conjugated forms, the free forms being artefacts from chemical or enzymatic hydrolysis during tissue extraction. The conjugated forms are esters of hydroxyacids such as quinic, shikimic and tartaric acid, as well as their sugar derivatives (Schuster and Herrmann 1985, Macheix et al. 1990, Shahidi and Naczk 1995).

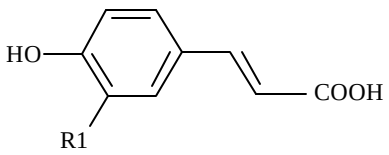


Figure 3. Chemical structure of three common hydroxycinnamic acids: p-coumaric acid, R1=H; caffeic acid, R1=OH; ferulic acid, R1=OCH_3.

2.2 Flavonols and phenolic acids in plants

2.2.1 Biosynthesis of phenolic compounds in plants

Phenolics display a wide variety of structures, ranging from simple moieties containing a single hydroxylated aromatic ring to highly complex polymeric substances (Strube et al. 1993, Harborne 1994). The biosynthetic pathways of phenolic compounds in plants are quite well known (Haddock et al. 1982, Harborne 1988, Macheix et al. 1990, Dixon and Paiva 1995, Strack 1997). The biosynthetic pathways of some flavonols and phenolic acids are shown in Figure 4. The biosynthesis and accumulation of secondary compounds can be an endogenously controlled process during developmental differentiation (Macheix et al. 1990, Strack 1997) or it can be regulated by exogenous factors such as light, temperature and wounding (Bennet and Wallsgrove 1994, Dixon and Paiva 1995). Phenylalanine, produced in plants via the shikimate pathway, is a common precursor for most phenolic compounds in higher plants (Macheix et al. 1990, Strack et al. 1997) (Figure 4). Similarly, hydroxycinnamic acids, and particularly their coenzyme A esters, are common structural elements of phenolic compounds, such as cinnamate esters and amides, lignin, flavonoids and condensed tannins (Macheix et al. 1990) (Figure 4). The phenylalanine/hydroxycinnamate pathway is defined as 'general phenylpropanoid metabolism'. It includes reactions leading from L-phenylalanine to the hydroxycinnamates and their activated forms (Strack 1997). The enzymes catalysing the individual steps in general phenylpropanoid metabolism are phenylalanine ammonialyase (PAL), cinnamic acid 4-hydroxylase (CA4H), and hydroxycinnamate: coenzyme A ligase (C4L). These three steps are necessary for the biosynthesis of phenolic compounds (Macheix et al. 1990, Strack 1997). A growing body of evidence indicates that phenylpropanoid and flavonoid pathways are catalysed by several membrane-associated multienzyme complexes (Dixon and Paiva 1995, Winkel-Shirley 1999).

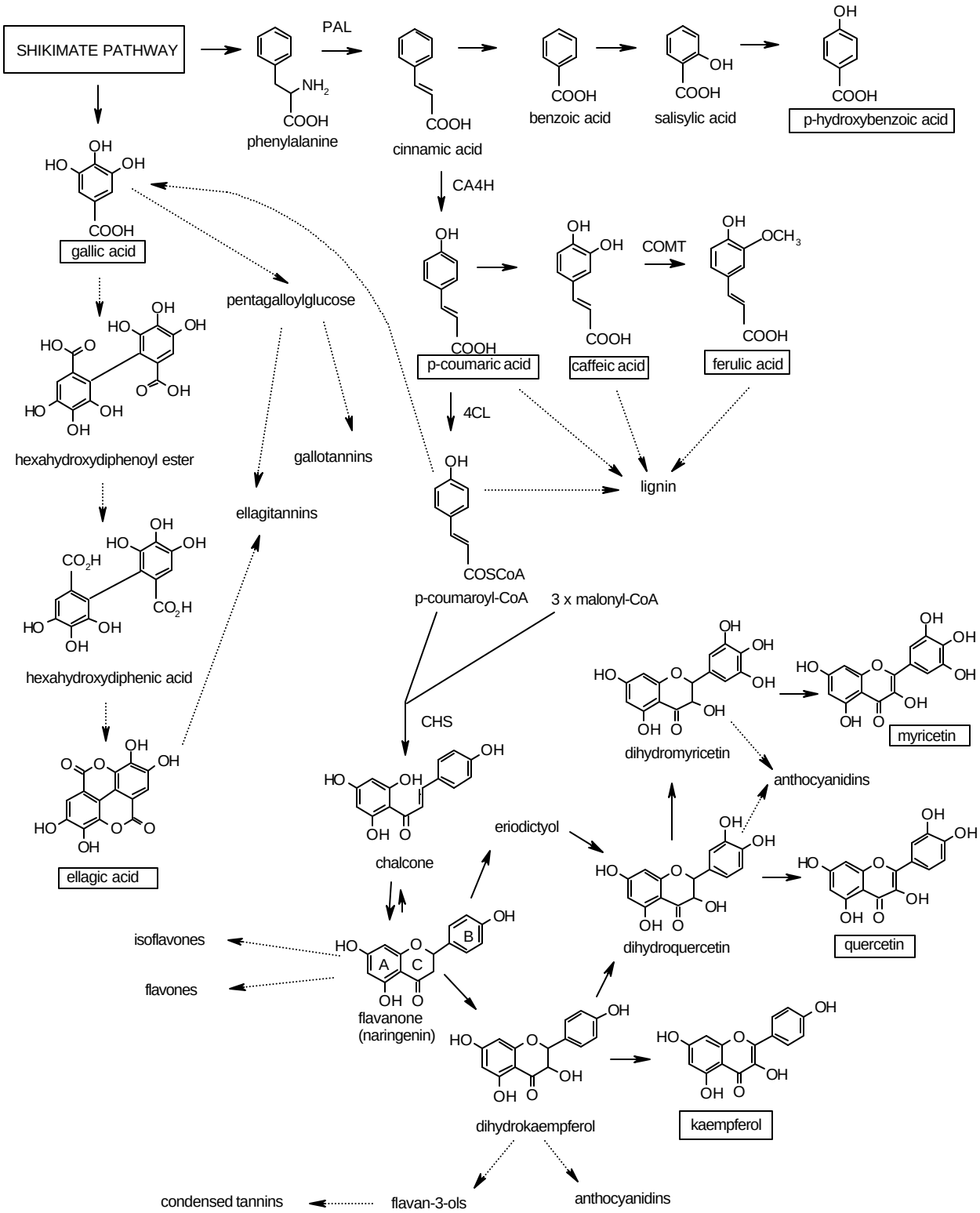


Figure 4. Biosynthesis of hydroxycinnamic acids, hydroxybenzoic acids and flavonoids (Haddock et al. 1982, Harborne 1988, Hahlbrock and Scheel 1989, Maas et al. 1992, Bennet and Wallsgrove 1994, Dixon and Paiva 1995, Strack 1997). Solid arrows represent well-characterised reactions catalysed by single enzymes. Dashed lines represent transformations that require multiple enzymes, that are less characterised, or vary among plant species. Enzymes: CA4H, cinnamic acid 4-hydroxylase; CHS, chalcone synthase; 4CL, 4-coumarate:coenzyme A ligase; PAL, phenylalanine ammonialyase.

Biosynthesis of hydroxycinnamic acids

The formation of hydroxycinnamic acids (caffeic, ferulic, 5-hydroxyferulic and sinapic acids) from p-coumaric acid requires two types of reactions: hydroxylatio n and methylation. The introduction of a second hydroxyl group into p-coumaric acid to give caffeic acid (Figure 4) is catalysed by monophenol mono-oxygenases, a well-known group of plant enzymes (Macheix et al. 1990, Strack 1997). Methylation of caffeic acid leads to the formation of ferulic acid which, together with p-coumaric acid, are the precursors of lignins (Figure 4). The methylation is catalysed by an O-methyltransferase (Macheix et al. 1990, Strack 1997). Caffeic acid is the substrate for rare 5-hydroxyferulic acid, which yields sinapic acid as a result of O-methylation.

The formation of hydroxycinnamic acid derivatives requires the formation of hydroxycinnamate-CoAs (e.g. p-coumaroyl-CoA) catalysed by hydroxycinnamoyl-CoA ligases or by the action of O-glycosyl transferases. The hydroxycinnamate-CoAs enter various specific phenylpropanoid reactions (Figure 4), such as condensations with malonyl-CoA leading to flavonoids or NADPH-dependent reductions leading to lignins. Moreover, hydroxycinnamate-CoAs can conjugate with organic acids (Macheix 1990, Strack 1997). In the biosynthesis of sugar derivatives of hydroxycinnamic acids, the transfer of glucose from uridine diphosphoglucose to hydroxycinnamic acid is catalysed by glucosyl transferase (Macheix et al. 1990, Strack 1997).

Biosynthesis of hydroxybenzoic acids

It is likely that there are several pathways for the biosynthesis of individual hydroxybenzoic acids, depending on the plant. They can be derived directly from the shikimate pathway (Figure 4), especially from dehydroshikimic acid; this reaction is the main route to gallic acid (Haddock et al. 1982, Strack 1997). However, they can also be produced by the degradation of hydroxycinnamic acids, in a similar manner to the β-oxidation of fatty acids; the main intermediates are cinnamoyl-CoA esters (Macheix et al. 1990, Strack 1997) (Figure 4). Hydroxybenzoates are also produced occasionally by the degradation of flavonoids (Strack 1997). Moreover, hydroxylations and methylations of hydroxybenzoic acids are known to occur in an analogous way to the phenylalanine/ hydroxycinnamate pathway (Gross 1981, Strack 1997). Knowledge of the mechanisms and, particularly, the enzymes involved in the biosynthesis of hydroxybenzoic acids and their derivatives is rather limited, especially regarding fruits, although gallic acid and its derivatives play an important role in the formation of hydrolysable tannins (Macheix et al. 1990).

The biogenesis of hexahydroxydiphenoyl esters and their hydrolysis to give ellagic acid (Haddock et al. 1982) is presented in Figure 4. The existence of isozymes in the biosynthesis of ellagic acid have not been determined (Maas et al. 1992). Ellagic acid is formed by oxidation and dimerization of gallic acid (Maas et al. 1991a). Oxidation is hastened by alkaline conditions, whereas hydrolysis and lactonization are favored by acidic conditions (Tulyathan et al. 1989). Gallic acid and its dimeric form ellagic acid can react with hydroxyl-containing compounds to form esters. Gallic acid and ellagic acid are the main components of hydrolysable tannins. Tannins which yield only gallic acid are defined as gallotannins, and those which give hexahydroxydiphenic acid - normally as ellagic acid, the dilactone form of hexahydroxydiphenic acid - are called ellagitannins (Haslam 1981).

Biosynthesis of flavonols

A key step of flavonoid biosynthesis is the condensation of three molecules of malonyl-CoA with p-coumaroyl-CoA to form the C_{15} intermediate 4,2',4',6'-tetrahydroxychalcone (Figure 4) (Harborne 1988, Strack 1997). The enzyme catalysing this step is chalcone synthase. For all chalcone synthases tested so far, 4-coumaroyl-CoA is the best substrate and, in general, it appears that the second B-ring hydroxyl is inserted at a later stage to give flavonoids with 3',4'-di-OH substitution (Harborne 1988).

The next step after chalcone synthesis is its stereospecific isomerization to a (2S)-flavanone, naringenin (Figure 4), catalysed by chalcone isomerase. Flavanones represent a branch point in the biosynthesis since they may be converted to either flavones (e.g. apigenin) or to isoflavones (e.g. genistein). The next enzyme along the pathway, flavanone-3-hydroxylase catalyses the conversion of (2S)-naringenin to (2R,3R)-dihydrokaempferol and also (2S)-eriodictyol to (2R,3R)-dihydroquercetin (Britch and Grisebach 1986) (Figure 4). The enzyme flavonol synthase converts dihydrokaempferol to kaempferol (Spribille and Forkmann 1984). The enzymatic hydroxylation of the flavonol ring B at C-3' and C-5' has been demonstrated although the possibility that, in some cases, p-coumaric acid is hydroxylated to caffeic acid before being incorporated into the flavonoid molecule has not been ruled out (Britton 1983). Additional hydroxylations can apparently occur at virtually all levels of oxidation of the flavonoid skeleton. Dihydroflavonol may enter another pathway leading to anthocyanins. An NADPH-dependent dihydroflavonol 4-reductase catalyses the formation of leucoanthocyanidin structure (Grisebach 1982, Strack 1997). The enzymatic steps catalysing the conversion of leucoanthocyanidins to coloured anthocyanidins are not well

characterised but involve an oxidation and dehydration step (Heller and Forkmann 1988, Strack 1997). It is likely that the enzymes involved in these reactions are anthocyanidin synthases (Holton and Cornish 1995).

Most of the flavonoids occur as glycosides in actively metabolising plant tissues. There are hundreds of different glycosides, with glucose, galactose, rhamnose, xylose and arabinose as the most frequently found sugar moieties (Strack 1997). The two major types of linkages are O- and C-glycosides (Harborne 1994). Glycosyl transferase catalyses the glycosylation of flavonoids. Flavonoid conjugation is not restricted to glycosylation. Many flavonoids contain acylated sugars. The acyl groups are either hydroxycinnamates or aliphatic acids such as malonate (Strack 1997). In the acylation reaction, the sugar hydroxyl and acid groups undergo esterification reaction (Markham 1989).

2.2.2 Functions of flavonoids and phenolic acids in plants

Phenolics are of great importance as cell-wall support materials (Wallace and Fry 1994, Strack 1997). They form an integral part of the cell-wall structure, mainly in the form of polymeric materials such as lignins, serving as mechanical support and barrier against microbial invasion. Lignins are, after cellulose, the second most abundant organic structures on earth (Wallace and Fry 1994, Strack 1997).

A most significant function of the phenolic flavonoids, especially the anthocyanins, together with flavones and flavonols as co-pigments, is their contribution to flower and fruit colours (Harborne 1994, Strack 1997). This is important for attracting insects and birds to the plant for pollination and seed dispersal. Phenolics may influence the competition among plants, a phenomenon called 'allelopathy'. Besides the well-known volatile terpenoids, toxic water-soluble phenolics, such as simple phenols, hydroxybenzoic acids and hydroxycinnamic acids may serve as allelopathic compounds (Strack 1997). A recent finding concerning the function of phenolics, especially flavonoids, is that they can act as signal molecules (host-recognition substances) in the interaction between the plant and the nitrogen-fixing bacteria in certain leguminuos plants (Strack 1997).

An important function of flavonoids and phenolic acids is their action in plant defence mechanisms (Britton 1983, Bennet and Wallsgrove 1994, Dixon and Paiva 1995). Stress conditions such as excessive UV light, wounding or infection induces the biosynthesis of phenolic compounds. Thus,

environmental factors may have a significant contribution to the content of flavonoids and phenolic acids in plants, e.g. in berries. Phenolic compounds contribute to the disease resistance mechanisms in plants. Two modes of action appear to operate in plant defence mechanisms, direct toxic effects (e.g. free radicals formed from lignin precursors) and the active and rapid deposition of barriers such as lignin (Bennet and Wallsgrove 1994, Strack 1997). Phenolics may accumulate as inducible low-molecular-weight compounds, called 'phytoalexins', as a result of microbial attack. Phytoalexins are post-infectional, i.e. although they might already be present at low concentrations in the plant, they rapidly accumulate upon attack (Strack 1997). In contrast, the pre-infectional toxins are constitutive compounds. They are present in healthy tissues at concentrations high enough for defence, either as free toxins or in conjugated forms from which they are released after attack (Strack 1997). Among the phenolic phytoalexins and toxins, hydroxycoumarins and hydroxycinnamic acids are of major importance (Strack 1997) but also flavonols play a role in defence.

Influence of light and UV irradiation

Light is one of the most extensively studied environmental factors in the phenolic metabolism (Macheix et al. 1990). In general, light stimulates the synthesis of flavonoids, especially anthocyanins and flavones, phenylalanine ammonialyase being the major inducible enzyme (Britton 1983, Macheix et al. 1990, Dixon and Paiva 1995). It is thought that phenolic compounds help to attenuate the amount of light reaching the photosynthetic cells (Beggs et al. 1987). Numerous data are available on the relationship between light and phenolic compounds, mostly anthocyanins and, to a lesser extent, flavonol glycosides and hydroxycinnamic derivatives (Macheix et al. 1990). Anthocyanin contents of crowberries vary from year to year according to the overall radiation and the number of hours of sunshine (Linko et al. 1983).

UV irradiation induces synthesis of flavonoids, particularly kaempferol derivatives, in *Arabidopsis* (Li et al. 1993, Lois 1994). By accumulating primarily in the epidermal layers or leaves and stems, these UV-absorbing compounds are thought to provide a means of protection against UV-B damage and subsequent cell death by protecting DNA from dimerization and breakage (Strack 1997).

Influence of injury or infection

The synthesis of flavonoids, phenolic acids or other phenylpropanoids is increased in plant tissues following wounding or infection by pathogenic organisms or feeding by herbivores (Britton 1983,

Bennet and Wallsgrove 1994, Dixon and Paiva 1995, Strack 1997). The accumulation of flavonols such as kaempferol and its glycosides is induced by wounding in the stigma of *Petunia* (Mo et al.1992, Vogt et al. 1994); these flavonoids may also serve to prevent microbial infection in an otherwise nutrient-rich environment. Excessive anthocyanin production can be observed in infected plant tissues (Britton 1983). Chlorogenic acid, alkyl ferulate esters and cell-wall -bound phenolic esters may act directly as defence compounds or may serve as precursors for the synthesis of lignin and other wound-induced polyphenolic barriers (Halbrock and Scheel 1989, Bernards and Lewis 1992). Simple phenolic acids, as well as complex tannins on the surface of the plant, are effective deterrents e.g. in plant-bird interactions where they interfere with the digestion through interaction with the microbial flora of the cecum (Strack 1997). Moreover, it is thought that the astringency of high-tannin plants makes them less appealing to birds (Bennet and Wallsgrove 1994).

Influences of other stresses

Levels of anthocyanins increase e.g. in grapes and apples following cold stress and nutritional stress (phosphate limitation), but the reasons for this are unclear (Macheix et al. 1990, Christie 1994). Low iron levels can cause increased release of phenolic acids (Dixon and Paiva 1995). Additionally, low nitrogen levels induce flavonoids and isoflavonoids serving as *nod* gene inducers and chemoattractants for nitrogen-fixing symbionts (Graham 1991). In cranberries, anthocyanin formation was found to be inversely related to increasing applications of nitrogen and phosphorus (Francis and Atwood 1961). Also, in grape, treatment with large amounts of nitrogen reduced the anthocyanin content of fruits and delayed the maturation (Kliewer 1977).

2.2.3 Content of flavonoids and phenolic acids in berries

The contents of flavonoids and phenolic acids in berries vary widely according to the literature (Table 1). Variation in the content of phenolic compounds within one species is mainly due to differences in the berry varieties (Schuster and Herrmann 1985, Bilyk and Sapers 1986, Maas et al. 1991b, Amiot et al. 1995, Prior et al. 1998) or in growth conditions (Macheix et al. 1990, Bennet and Wallsgrove 1994, Dixon and Paiva 1995). Also, methodological differences (Hertog et al. 1992a, Hollman and Venema 1993, Heinonen et al. 1998) may contribute to the variability in the reported flavonoid and phenolic acid concentrations. Discrepancies found may also partly be due to

differences in the maturity stage of the fruits (Stöhr and Herrmann 1975b, Starke and Herrmann 1976, Amiot et al. 1995, Prior et al. 1998).

Anthocyanins constitute the main group of phenolic compounds in berries (Table 1). The contents of flavonols (especially quercetin) and flavan-3-ols [(+)-catechin and (-)-epicatechin] have been studied quite extensively in blueberry, currants, cranberry, strawberry and red raspberry (Table 1). Generally, the content of flavonols is higher than that of flavan-3-ols in berries. However, in gooseberry, red raspberry and strawberry, the content of flavan-3-ols has been reported to be higher than that of flavonols (Herrmann 1992, Heinonen et al. 1998, Arts et al. 2000). In many of the above studies on flavonols (Wildanger and Herrmann 1973, Starke and Herrmann 1976) and flavan-3-ols (Mosel and Herrmann 1974, Stöhr and Herrmann 1975a,b), a combination of thin-layer chromatographic (TLC) and spectrophotometric methods have been used without optimisation of the extraction and hydrolysis steps, most probably leading to an underestimation of the levels of these flavonoids (Hertog 1994).

Only a few studies are available on the content of phenolic acids in berries, although it is higher than that of flavonols or flavan-3-ols in blueberry, black currant, gooseberry, red raspberry and strawberry (Table 1). For many berries (e.g. bilberry, cranberry, lingonberry, rowanberry), no data on the contents of hydroxycinnamic or hydroxybenzoic acids are available. In red raspberry and strawberry, the content of ellagic acid is reported to be high (Daniel et al. 1989, Hollman and Venema 1993), but not higher than that of anthocyanins (Table 1).

Table 1. Flavonoid and phenolic acid contents in berries.

	Flavonoids (mg/kg fresh weight)					Phenolic acids (mg/kg fresh weight)					
	Kaempferol	Quercetin	Myricetin	Anthocyanins (total)	Flavan-3-ols (total)	p-Coumaric acid	Caffeic acid	Ferulic acid	p-Hydroxy-benzoic acid	Gallic acid	Ellagic acid
Blueberry	0 [a]	24–160 [a, b, c]	9–69 [a]	626–4840 [d, e, f]	11–70 [d, g, y]	6–20 [h]	1860–2090 [h]	13–14 [h]	5-6 [h]	3–7 [h]	–
Bilberry	0 [i]	32–37 [i, j]	0–37 [i, j]	2996 [e]	–*	–	–	–	–	–	–
Currant, black	<0.1-10 [a, b]	33–68 [a, b, j]	41–55 [a, i, j]	2350 [f]	3–12 [g, k, y]	20–44 [h]	68–84 [h]	18–24 [h]		4–13 [h]	4–11 [h]
	–										
Currant, red	0.1-2 [a, i, v]	2–27 [a, b, i, v]	<0.1 [i]	119–186 [f, l]	4–36 [g, k, y]	5–15 [h]	3–8 [h]	1–3 [h]	9–13 [h]	3–08 [h]	–
Currant, white	0.2-2 [a, i]	3–28 [a, i]	<1 [i]	–	4–13 [g, y]	–	–	–	–	–	–
Cranberry	0-3 [c]	104–250 [b, c, j]	11–249 [b, c, j, x]		577–1720 [m, n]		42 [y]	–	–	–	– –
	120 [o,**]										
Crowberry	–	–	–	3200–5600 [p]	–	–	–	–	–	–	–
Gooseberry	0 [i]	<0.1 [i]	0 [i]	–	15–36 [k, y]	12–15 [h]	10–19 [h]	2–11 [h]	2 [h]	9–14 [h]	–
Lingonberry	<1-5 [g, i]	34–210 [b, i, j]	0 [i]	322 [q]	–	–	–	–	–	–	–
Raspberry, red	<0.1 [a]	8–29 [a, b]	0 [a]	230–9950 [d, f, r, s]	32–480 [d, t, y]	6–25 [h, t]	4–10 [h, t]	3–17 [h, t]		15–59 [b, t]	19–38 [t]
	284–1240 [o, u]										
Rowanberry	–	106 [j]	10 [j]	–	–	–	–	–	–	–	–
Strawberry	5–12 [b, v]	6–8.6 [b, s]	–	786–3851 [d, s]	6–126 [d, w, y]	7–27 [h, e]	<0.5–7 [h, w]	2 [h]	10–36 [h, w]	1–44 [h, w]	90–402 [o, u]

[a] Starke and Herrmann 1976, [b] Justesen et al. 1998, [c] Bilyk and Sapers 1986, [d] Heinonen et al. 1998, [e] Prior et al. 1998, [f] Costantino et al. 1992, [g] Stöhr and Herrmann 1975a, [h] Schuster and Herrmann 1985, [i] Wildanger and Herrmann 1973, [j] Kumpulainen et al. 1999, [k] Herrmann 1992, [l] Øydvin 1974, [m] Lees and Francis 1972, [n] Sapers et al. 1983b, [o] Daniel et al. 1989, [p] Linko et al. 1983, [q] Kähkönen et al. (personal communication), [r] Torre and Barrit 1977, [s] Wang and Lin 2000, [t] Mosel and Herrmann 1974, [u] Hollman and Venema 1993, [v] Hertog et al. 1992b, [w] Stöhr and Herrmann 1975b, [x] Hertog et al. 1992a, [y] Arts et al. 2000, * no data available, ** mg/kg dry weight.

2.2.4 Compartmentation of flavonoids and phenolic acids in fruits

Phenolic compounds are not evenly distributed in fruits either at the subcellular level or in the tissues (Macheix et al. 1990). Knowledge of the compartmentation is thus of importance in order to optimise the yield of phenolics in the processed products of berries, fruits and vegetables (Sapers et al. 1983a, McRae et al. 1990, Hirota et al. 1998). These compounds are mainly deposited in the cell-wall where lignin and the more simple molecules (flavonoids and ferulic acid esters) accumulate, and in the vacuoles where soluble phenolic compounds and their derivatives are stored (Guern et al. 1987, Monties 1989, Ibrahim and Barron 1989). In grape, flavonol glycosides, anthocyanins and hydroxycinnamic esters accumulate in the vacuoles of subepidermal cells (Moskowitz and Hrazdina 1981).

Accumulation of soluble phenolic compounds is greater in the external tissues of fleshy fruits (epidermal and subepidermal layers) than in the internal tissue (mesocarp and pulp) (Macheix et al. 1990, Wollenweber 1994). Since the formation of phenolic compounds depends on light, they are mainly found in the skins of berries and fruits. In many fruits (e.g. black currant, grape, apple, peach), flavonol glycosides are mainly, or even solely, located in the outer part of fruits or in the epicarp (Hawker et al. 1972, Wildanger and Herrmann 1973, Pérez-Ilzarbe et al. 1991, Price et al. 1999). Anthocyanins may be distributed throughout the fruit, as in strawberry, red raspberry and currants or, as in other fruits, located mainly in the skin (Macheix et al. 1990). Also catechins and tannins are frequently more abundant in the external than in the internal tissues of fruits. In pear and apple, (+)-catechin and (-)-epicatechin contents are greater in the skin than in the rest of the fruit (Risch and Herrmann 1988, Pérez-Ilzarbe et al. 1991, Bengoechea et al. 1997). Also, the highest levels of hydroxycinnamic acids and especially caffeic acid derivatives are often found in the external parts of the ripe fruit (Risch and Herrmann 1988, Herrmann 1989, Macheix et al. 1990). However, in apple, distribution of hydroxycinnamic acids appears to vary with the cultivar (Macheix et al. 1990, Price et al. 1999).

Little is known about the localisation of hydroxybenzoic acids in fruits (Macheix et al. 1990). They are either present in skin and pulp like in tomato and melon (Schmidtlein and Herrmann 1975) or found only in skin as in grape (Singleton and Trousdale 1983). According to Daniel et al. (1989), 96% of ellagic acid in strawberries is present in the pulp and 4% in seeds. On the other hand, Maas et al. (1991b) reported that ellagic acid content was higher in the seeds (8.5 or 9 mg/g) than in the

pulp (1.5 or 1.6 mg/g) of ripe strawberries. Similarly, the seeds of red raspberry contained 88% of ellagic acid, and 12% was present in the pulp (Daniel et al. 1989).

2.2.5 Changes during growth and maturation

Accumulation of phenolic compounds varies strongly in relation to the physiological state of the fruit, being a result of an equilibrium between biosynthesis and further metabolism including turnover and catabolism. The most important control mechanisms in the phenolic metabolism include the amount of enzymes, regulation of enzyme activities, compartmentation of enzymes, availability of precursors and intermediates, and integration in the differentation and development programs (Macheix et al. 1990, Harborne 1994).

Numerous investigations have confirmed that concentrations of phenolic compounds are generally higher in young fruits and tissues (Britton 1983, Macheix et al. 1990). In particular, anthocyanins are often produced in large amounts in young shoots and leaves (Britton 1983). In fruits, the total phenol content (mg/g f. w.) falls during growth, but two distinct phenomena can be observed. Either the level continues to fall steadily, as in the case of white-coloured species and varieties, e.g. white grape cultivars, mango and banana, or it rises at the end of maturation as in the case of red fruits in which anthocyanins or flavonoids accumulate (Macheix et al. 1990).

Phenolic acids

Caffeic acid, p-coumaric acid and ferulic acid concentrations are generally high in young fruits of red raspberries, black currants and strawberries, falling first rapidly and then more slowly during maturation and postharvest storage (Mosel and Herrmann 1974, Stöhr and Herrmann 1975a, b). Data on hydroxybenzoic acids in fruits are fragmentary and it is difficult to draw any conclusions about the general features of the variations. In strawberry, p-hydroxybenzoic acid appears at a relatively late stage of the fruit development (Stöhr and Herrmann 1975b). The ellagic acid content of strawberries is higher in green than in red fruit pulp (Maas et al. 1991b).

Flavonoids

The contents of anthocyanins and total phenolics increase with maturity in red and black raspberry (Wang and Lin 2000). Also in blueberry and bilberry, more mature fruits at harvest have

increased anthocyanin and total phenolic concentrations (Prior et al. 1998). Similarly, the amount of flavonols, i.e. glycosides of quercetin and particularly myricetin, increases markedly during ripening of black currants (Stöhr and Herrmann 1975a). In cultivated blueberries, myricetin content in ripe fruit is also higher than that in unripe fruit. On the other hand, in most fruits (red and white currants, sour cherries, cultivated blueberries, elderberries) the concentrations of kaempferol and quercetin glycosides are lower in ripe than in unripe fruits (Stöhr and Herrmann 1975a). Also, the content of total phenolics decreases significantly in blackberry and strawberry as the fruit matures (Wang and Lin 2000). Moreover, the content of flavan-3-ols decreases during growth and maturation of strawberries and red raspberries (Mosel and Herrmann 1974, Stöhr and Herrmann 1975b).

2.3 Dietary sources and intake

The content of the flavonols quercetin, kaempferol and myricetin was determined by Hertog et al. (1992a, b, 1993b) from vegetables, fruits and beverages commonly consumed in the Netherlands. Quercetin levels in the edible parts of most vegetables were generally below 10 mg/kg, except for onions (284–486 mg/kg), kale (110 mg/kg), broccoli (30 mg/kg), French beans (32–45 mg/kg), and slicing beans (28–30 mg/kg) (Hertog et al. 1992b). Similar levels of quercetin for these vegetables were reported by Justesen et al. (1998) in foods on the Danish market. Kaempferol was found in kale (211 mg/kg), endive (15–91 mg/kg), leek (11–56 mg/kg), and turnip-tops (31–64 mg/kg) (Hertog et al. 1992b). Justesen et al. (1998) found kaempferol also in broccoli (60 mg/kg), parsley (11 mg/kg), brussels sprouts (9 mg/kg) and spring onion (6 mg/kg). In most fruits, the quercetin content was 15 mg/kg, on average, except for different apple varieties in which the quercetin content was 21–72 mg/kg (Hertog et al. 1992b). Justesen et al. (1998) reported quercetin levels higher than 20 mg/kg in several berries and fruits (cowberry, lingonberry, cranberry, blueberry, black currant, blue grapes, rosebud, apple and apricot). According to Hertog et al. (1992b), the content of myricetin was below the limit of detection (<1 mg/kg) except for fresh broad beans (26 mg/kg). Justesen et al. (1998) detected a high level of myricetin in cranberry (230 mg/kg).

Red wines and grape juice had quercetin levels of 4 to 16 mg/l and of 7 to 9 mg/l, respectively (Hertog et al. 1993b). According to Justesen at al. (1998), the mean quercetin and myricetin levels in 21 red wines were 8 and 10 mg/kg, respectively. McDonald et al. (1998) reported the content of flavonols (quercetin and myricetin) in 65 red wines to vary from below 6 to 40 mg/l. Quercetin

levels in fruit juices were generally below 5 mg/l, except for lemon juice (7 mg/l) and tomato juice (13 mg/l) (Hertog et al. 1993b). In black tea infusions, quercetin (10–25 mg/l), kaempferol (7–17 mg/l), and myricetin (2–5 mg/l) have been detected (Hertog et al. 1993b, Justesen et al. 1998).

Kühnau (1976) estimated the average intake of total flavonoids in the USA in 1971 to be 1 g/d. Flavonols would contribute 115 mg/d. However, this estimate was not very accurate because only limited data on flavonoid contents in foods was available. Hertog et al. (1993a) used the data of the Dutch National Food Consumption Survey 1987–1988 to report the intake of flavonols (quercetin, kaempferol, myricetin) and flavones (apigenin, luteolin) among 4112 adults in the Netherlands. The average intake of these flavonoids was 23 mg/d based on HPLC analysis of fruits, vegetables and beverages (Hertog et al. 1992a, b, 1993b). The most important flavonoid was quercetin (70% of the total intake) followed by kaempferol (17%) and myricetin (6%). The most important sources of flavonoids in the Netherlands were tea (48% of total intake), onions (29%) and apples (7%).

Hertog et al. (1995) compared the flavonoid (flavonols and flavones) intake of men (40–59 years of age) in 16 cohorts in 7 countries (Seven Countries Study 1958–1964). The mean intake of flavonoids was lowest in Finland, 2.6 and 9.6 mg/d in western and eastern Finland, respectively, and highest in Japan (68.2 mg/d). Tea was the main dietary source of flavonoids in Japan, in the Netherlands and in the United Kingdom (Hertog et al. 1995). In Finland, in the former Yugoslavia, in Greece and in USA, vegetables and fruits, particularly onions and apples, represented the main dietary sources of flavonoids. According to Knekt et al. (1996, 1997) the intake of flavonols in Finland was as low as 3–4 mg/d, based on food consumption data from appr. 40 years ago. The dietary intake of flavonols was 20 mg/d in USA (Rimm et al. 1996) and 26 mg/d in the United Kingdom (Hertog et al. 1997). Hollman and Katan (1998) estimated the daily intake of total flavonoids to be a few hundred milligrams per day expressed as aglycones. Flavonols comprise only a small fraction. However, reliable quantitative data on the intake of other flavonoids such as catechins or anthocyanidins are not yet available (Hollman et al. 1999b).

The intake of hydroxycinnamic and hydroxybenzoic acids by adults was studied in a Bavarian subgroup of the National Food Consumption Survey in Germany (Radtke et al. 1998). A database containing the phenolic acid contents of foods (data from literature) was built and 7-day dietary protocols of 63 women and 56 men (age 19–49 years) of the German National Food Consumption Survey were evaluated. The average phenolic acid intake of men and women was 222 mg/d within a large range. The most important phenolic acid was caffeic acid (206 mg/d) followed by ellagic acid

(5.2 mg/d). Significant differences among sexes were found for some of the phenolic acids. Particularly, the average intake of caffeic acid by women (229 mg/d) was higher than that of men (179 mg/d), being caused by the high coffee consumption. The major sources of phenolic acids were coffee with 92% of the caffeic acid intake, and fruits (including fruit products and juices) with 75% of the salicylic acid and 59% of the p-coumaric acid intake.

2.4 Analytical methods

Several reviews have been published on the analysis of phenolic compounds in plants (van Sumere et al. 1978, Harborne 1989, Waterman and Mole 1994, Harborne 1998) and in plant-based foods (Macheix et al. 1990, Lee and Widmer 1996). Herrmann (1989) and Waksmundzka-Hajnos (1998) have reviewed the analysis of hydroxycinnamic and hydroxybenzoic acids in plants and plant-based foods. The analysis of flavonoids has been reviewed extensively by Markham (1982, 1989), by Harborne (1988, 1994) and by Robards and Antolovich (1997).

The analysis of phenolics in raw or processed food matrix begins with extraction. The extraction procedure depends on the type of food to be analysed, the phenolic compound in question, and the analytical procedure to be used (Lee and Widmer 1996). The first step is to crush, mill, macerate, or grind the sample to increase the surface area, allowing a better contact of the extracting solvent with the sample (Waterman and Mole 1994). This also helps in mixing the sample to ensure that the extracted portion is representative of the entire sample. Since many phenolic compounds occur as glycosides or esters, the sample preparation may include alkaline, acid or enzymatic hydrolysis to free the bound phenolics. The hydrolysis step is omitted if the phenolics are to be analysed as derivatives.

2.4.1 Flavonols

2.4.1.1 Extraction and hydrolysis techniques

Flavonoids are generally stable compounds and may be extracted from fresh or dried, ground plant material with cold or hot solvents. Suitable solvents are aqueous mixtures containing ethanol, methanol, acetone or dimethylformamide (Robards and Antolovich 1997). Extraction of flavonols

has been performed by maceration of the fresh, undried fruit or plant sample in the extracting solvent (Wildanger und Herrmann 1973, Bilyk and Sapers 1986, Treutter et al. 1988, Dick et al. 1987, Price et al. 1999), by extracting an aliquot of homogenised fresh fruit sample (Amiot et al. 1995, Heinonen et al. 1998) or by extracting a freeze-dried (lyophilised) sample (Hertog et al. 1992a, b, Crozier et al. 1997, Justesen et al. 1998, Price et al. 1998, Ewald et al. 1999).

Quantitative analysis of individual flavonol glycosides in berries and fruits is difficult because most reference compounds are not commercially available. Furthermore, more than 30 different flavonol glycosides have been identified in fruits (Macheix et al. 1990). Hydrolysis of flavonol glycosides to their corresponding aglycones offers a practical method for the quantification of flavonols in foods (Hertog 1994, Robards and Antolovich 1997). Hydrolysis of flavonols with hydrochloric acid (HCl) has been described by Harborne (1965). Wildanger and Herrmann (1973) and Bilyk and Sapers (1986) studied the flavonol contents in berries using acid hydrolysis but without optimisation of the extraction and hydrolysis procedure. Hertog et al. (1992a) optimised the extraction and acid hydrolysis conditions (in aqueous methanol with HCl) for the analysis of flavonols and flavones in freeze-dried vegetables and fruits (Hertog et al. 1992a, 1993b). Extraction and hydrolysis in aqueous methanol with HCl has also been applied for studies of flavonoid aglycones in fruits and vegetables by Justesen et al. (1998) and Ewald et al. (1999). Rommel et al. (1993a, b) used alkaline hydrolysis (2 N NaOH) to study flavonols, hydroxycinnamic and hydroxybenzoic acids in red raspberry juices. The rate of acid/base hydrolysis of glycosides depends on the acid/base strength, nature of the sugar moiety and its position in the flavonoid nucleus. Glucuronides resist acid hydrolysis better than glucosides which are rapidly cleaved (Hertog 1994, Robards and Antolowich 1997).

Enzymatic hydrolysis offers a rapid method for the cleavage of specific monosaccharides from flavonoid O-glycosides. Enzymatic hydrolysis with β-gluronidase and sulfatase has been used to study flavonols in human plasma by Manach et al. (1998) and Erlund et al. (1999).

2.4.1.2 Chromatographic techniques

Paper chromatographic methods were developed for flavonoids in the 1950s and 1960s (Markham 1982, Robards and Antolowich 1997). These techniques were replaced by thin-layer chromatography (TLC) in the 1970s providing an inexpensive and useful technique for the

simultaneous analysis of several samples (Robards and Antolowich 1997, Harborne 1998). Selection of a suitable stationary phase and solvent depends on the class(es) of flavonoids to be examined. Hydrophilic flavonoids, such as flavonols, can be readily separated by TLC on polyamide or microcrystalline cellulose (Wildanger and Herrmann 1973, Robards and Antolowich 1997). TLC is still in common use for preparative separations (Lee and Widmer 1996) and as a rapid low-cost screening method for determining the flavonoid classes present in fruits (Fernández de Simón et al. 1992) and honey (Sabatier et al. 1992).

Gas chromatography (GC) has only a limited applicability in the analysis of flavonoids and other phenolics due to their limited volatility; the main disadvantage is an extra step required to ensure the volatility of the phenolics (Lee and Widmer 1996, Robards and Antolowich 1997). However, GC analysis with mass spectrometric (MS) detection has been applied for the analysis of flavonols in black tea (Finger et al. 1991) and cabbage (Nielsen et al. 1993). Advantages of GC analysis include an improved separation of closely related isomers and simple coupling to MS detectors for identification through the fragmentation pattern (Mouly et al. 1993, Schmidt et al. 1994).

High-performance liquid chromatography (HPLC) has been the most widely employed chromatographic technique in flavonoid analysis during the past 20 years (Harborne 1988, Robards and Antolowich 1997, Merken and Beecher 2000). It has added a new dimension to the investigation of flavonoids in plant and food extracts. Particular advantages are the improved resolution of flavonoid mixtures compared to other chromatographic techniques, the ability to obtain both qualitative and accurate quantitative data in one operation, and the great speed of analysis (Harborne 1988, Markham 1989). Normal-phase chromatography has been used for the separation of flavonoids (flavone, flavonol and flavanone aglycones) in orange juice (Galensa and Herrmann 1980a, b). Flavonoid acetates were separated isocratically on LiChrosorb Si60 using benzene-acetonitrile, benzene-ethanol or iso-octane-ethanol-acetonitrile solvent systems and detected at 312 or 270 nm. However, for normal-phase systems, there is a concern that highly polar materials may be irreversibly retained in the column (vande Casteele et al. 1983), with the result that the separation characteristics could be gradually altered. Thus, reversed-phase (RP) chromatography has invariably been the method of choice for the separation of flavonols and other flavonoid groups in fruits (Table 2). The normal way of separation uses a C_{18} -column (particle size 3–5 μm) together with aqueous mobile phases and methanol or acetonitrile as an organic modifier. Small amounts of acetic acid, formic acid or phosphate buffers incorporated in the mobile phase tend to markedly improve

separations of flavonoids and other phenolic compounds (Lamuela-Raventós and Waterhouse 1994, Merken and Beecher 2000).

Other modern separation systems such as capillary zone electrophoresis have been applied only to a limited extent mainly for the analysis of anthocyanins in berries (Bridle and Garciá-Viguera 1997, Costa et al. 1998) and of polyphenolics in wine (Andrade et al. 1998, Arce et al. 1998, Prasongsidh and Skurray 1998).

T*able 2*. High-performance liquid chromatography of flavonols and other flavonoid groups in fruits.

Compounds	Fruit sample	Column	Solvent system	Detection	Reference
Flavonols	cranberry, blackberry, blueberry	300 x 3.9 mm µBondapak C18	water/acetic acid/methanol (42:8:50)	UV 280 nm	Bilyk and Sapers (1986)
Flavonols, flavan-3-ols	apple	Novapak C18	A: aqueous trifluoroacetic acid B: acetonitrile	UV 270 nm	McRae et al. (1990)
Flavonols, flavan-3-ols	juices: apple, apricot, grape, orange, peach, pear, pineapple	300 x 3.9 mm Novapak C18	A: aqueous 2% acetic acid B: water/ methanol/ acetic acid (68:30:2), 0–85% B in 65 min	PDA [a] 254, 280, 340, 365 nm	Fernández de Simón et al. (1992)
Flavonols, flavan-3-ols	apricot, peach, plum, strawberry, sour orange, pear (jams)	125 x 4 mm LiChroCA RT C18	A: aqueous formic acid B: methanol 20–80% B in 35 min	PDA [a] 280, 350 nm	Tomás-Lorente et al. (1992)
Flavonols, flavones	apple, apple sauce, apricot, grape, peach, pear, plum, red currant, strawberry, sweet cherry	Novapak C18	acetonitrile/phosphate buffer (25:75, v/v, pH 2.4)	PDA [a] 370 nm	Hertog et al. (1992b)
Flavonols	red raspberry (juice)	250 x 4.6 mm Spherisorb ODS-1	A: aqueous 1% acetic acid B: acetonitrile 16–100% B in 77 min	PDA [a] 360 nm	Rommel and Wrolstad (1993b)
Flavonols, flavan-3-ols	apple	220 x 4.6 mm Aquapore C18	A: aqueous acetic acid B: acetonitrile	UV 350 nm	Lister et al. (1994)
Flavonols, flavan-3-ols	pear	150 x 4.6 mm Adsorbosphere C18	A: water, pH 2.6 with phosphate buffer B: acetonitrile/methanol/water (1:3:1), 0–50% B in 70 min	PDA [a] 280, 360 nm	Amiot et al. (1995)

Flavonols, flavan-3-ols	apple, peach (concentrates and purées)	300 x 3.9 mm Novapak C18	A: water/acetic acid (78:2) B: water/acetonitrile/acetic acid (78:20:2), 0–90% B in 70 min	PDA [a]	Bencoechea et al. (1997)
Flavonols, flavones, flavan-3-ols	apple, apricot, black currant, blueberry, cherry, cowberry, cranberry, grapefruit, grape, lemon, lime, orange, red currant, red raspberry, rosebud, strawberry	250 x 4.6 mm Phenomenex C18	A: methanol-water (30:70, v/v) with 1% formic acid B: methanol 25–86% B in 50 min	PDA [a] 290, 365 nm MS (API) [b]	Justesen et al. (1998)
Flavonols, flavan-3-ols, anthocyanins	blackberry, blueberry, red raspberry, strawberry, sweet cherry	150 x 3.6 mm Novapak C18	A: 50 mM dihydrogen ammonium phosphate (pH 2.6) B: 20% A with 80% acetonitrile C: 0.2 M orthophosphoric acid (pH 1.5) (100:0:0) to (0:80:20) in 60 min	PDA [a] 365, 520 nm	Heinonen et al. (1998)
Flavonols	apple	250 x 4.6 mm Phenomenex C18	A: water/tetrahydrofuran/ trifluoroacetic acid (98:2:0.1) B: acetonitrile	PDA [a] 370 nm	Price et al. (1999)

[a] PDA=photo-diode array detection [b] MS (API)=mass spectrometry (atmospheric pressure ionisation)

2.4.2 Phenolic acids

2.4.2.1 Extraction and hydrolysis techniques

The most common solvents used for the extraction of phenolic acids from plant matrices are ethyl acetate (Azar et al. 1987, Fernández de Simón et al. 1990, 1992), diethyl ether (Fernández de Simón et al. 1990, 1992), methanol or aqueous methanol (Kuninori and Nishiyama 1986, Torres et al. 1987, McRae et al. 1990, Tomás-Lorente 1992). Enzymatic hydrolysis with β-glucosidase (Kanes et al. 1993) or hydrocinnamoyl-quinate esterase (Goupy et al. 1990) has been applied for the analysis of phenolic acids. However, acid and alkaline hydrolysis are more commonly used for the determination of phenolic acids in plants (Lee and Widmer 1996).

Acid hydrolysis has been done by heating the sample with HCl for 2 h or more (Kuninori and Nishiyama 1986). Hydrolysis of benzoic acid and cinnamic acid esters with alkali can be performed with NaOH at room temperature for 4–24 h (Seo and Morr 1984, Torres et al. 1987, Peleg et al. 1991, Roussef et al. 1992a,b, Rommel and Wrolstad 1993a) or for 90 min at 60 °C under N_2 (Kuninori and Nishiyama 1986). Rommel and Wrolstad (1993a) tested acid (HCl) and base (NaOH) hydrolysis in the analysis of phenolic composition (ellagic acid, hydroxybenzoic acids, hydroxycinnamic acids, flavonols and flavan-3-ols) of red raspberry juice. The phenolic pattern of the alkaline-hydrolysed sample was very similar to that of the acid-hydrolysed sample of the same juice. Only one ellagic acid compound was hydrolysed more effectively in alkaline than in acidic conditions (Rommel and Wrolstad 1993a). Recoveries were quite poor (57–67%) for hydroxycinnamic acids extracted from fruit juices after alkaline hydrolysis (Peleg et al. 1991). However, Seo and Morr (1984) found that hydrolysis with NaOH leads to better recovery of ferulic acid from soybean protein products than hydrolysis with HCl. Hollman and Venema (1993) tested extraction and acid hydrolysis of ellagic acid from walnuts and berries (strawberry, blackberry, red raspberry) using different HCl and aqueous methanol concentrations together with a variation of hydrolysis period. The hydrolysis characteristics of ellagitannins of walnuts (optimum 5 M HCl in 57% aqueous methanol for 1 h) differed from those of berries (optimum 3.5 M HCl in 72% aqueous methanol for 4–8 h). In general, optimisation of extraction and hydrolysis conditions is always needed when phenolic acids are quantified from fruits or other plant materials (Lee and Widmer 1996).

2.4.2.2 Chromatographic techniques

TLC applications for quantitative analysis of phenolic acids (Azar et al. 1987, Regnault-Roger et al. 1987, Srisuma et al. 1989, Agbor-Egbe and Rickard 1990) are usually carried out using normal-phase chromatography on cellulose or silica layers and separating the compounds with a mixture of hydrocarbon carriers (toluene, dioxane or benzene) and polar organic modifiers (acetone, butanol, ethanol or acetic acid). The advantages in screening the sample extract by TLC prior to HPLC are the detection of contaminants that may absorb to the stationary phase in the HPLC column, or the determination of solvent conditions necessary for a successful separation of phenolic compounds (Fernández de Simón et al. 1992, Lee and Widmer 1996).

In the 1980s, GC was applied for the analysis of phenolic acids in fruits (Möller and Herrmann 1983, Schuster and Herrmann 1985) and vegetables (Huang et al. 1986). However, like for flavonols, HPLC and, particularly, RP-HPLC is the method of choice in the chromatographic analysis of phenolic acids (Table 3). The solvent systems used in the analytical HPLC usually include binary gradient elutions using solvents of aqueous acetic, formic or phosphoric acids with methanol or acetonitrile as an organic modifier. The pH and ionic strength of the mobile phase are known to influence the retention of phenolics in the column depending on protonation, dissociation, or a partial dissociation (Marko-Varga and Barcelo 1992). A change in pH which increases the ionisation of a sample could reduce the retention in a reversed-phase separation. Thus, small amounts of acetic (2–5%), formic, phosphoric or trifluoroacetic acid (0.1%) are included in the solvent system to suppress ionisation of phenolic and carboxylic groups and hence to improve resolution and reproducibility of chromatographic runs.

Table 3. High-performance liquid chromatography of hydroxycinnamic acids, hydroxybenzoic acids and ellagic acid in fruits.

Compounds	Fruit sample	Column	Solvent system	Detection	Reference
Hydroxycinnamic acids	apple, apricot, peach, pear, plum, sour and sweet cherry	250 x 4 mm LiChrosorb RP-18	A: aqueous 2% acetic acid B: methanol 7–35% B in 50 min	UV 320 nm	Möller and Herrmann (1983)
Hydroxycinnamic and hydroxybenzoic acids	bilberry juice	250 x 4 mm LiChrosorb C18	A: 2% acetic acid in water B: 2% acetic acid/water/ acetonitrile (2:68:309) 7–100% B in 50 min	UV 280 nm	Azar et al. (1987)
Ellagic acid	cranberry, blackberry, blueberry, raspberry, strawberry	250 x 4.6 mm Alltech C18 RP	A: 30 mM ammonium dihydrogen phosphate/ methanol (50:50) B: 10 mM ammonium dihydrogen phosphate 30–100% A in 40 min	UV 254 nm	Daniel et al. (1989)
Hydroxycinnamic acids	orange, grapefruit	LiChrospher 100-RP-18	aqueous 1.5% acetic acid/ methanol (77:23)	UV 300 nm	Peleg et al. (1991)
Hydroxycinnamic acids	apple (and juice)	250 x 4.6 mm Spherisorb ODS2	A:0.2% 1 M HCl in water B: 0.2% 1 M HCl in methanol 0–50% B in 85 min	UV 260, 310 nm	Delage et al. (1991)
Hydroxycinnamic acids	apricot, peach, plum, strawberry (jams)	LiChroCART 100-RP-18	A: water/formic acid (95:5) B: methanol 5–80% B in 35 min	UV 280, 350 nm	Tomás-Lorente et al. (1992)
Hydroxycinnamic and hydroxybenzoic acids	juices: apple, apricot, grape, orange, peach, pear, pineapple	300 x 3.9 mm Novapak C18	A: aqueous 2% acetic acid B: water/methanol/acetic acid (68:30:2), 0–85% B in 45 min	PDA[a] 280, 240 nm	Fernández de Simón et al. (1992)

Hydroxycinnamic acids	orange juice	Bondasil C18	tetrahydrofuran/acetonitrile/ aqueous 2% acetic acid (12:5:83) to tetrahydrofuran/ aqueous 2% acetic acid (35:65) in 28 min	UV 280, 324 nm Fluorescence	Roussef et al. (1992a)
Hydroxycinnamic acids	apple juice	LiChrospher 100-RP-18	A: aqueous 0.5% acetic acid B: methanol 10–100% B in 50 min	PDA[a]	Cliffe et al. (1994)
Hydroxybenzoic, hydroxycinnamic and ellagic acids	red raspberry juice	250 x 4.6 mm Spherisorb ODS-1	A: aqueous 1% acetic acid B: acetonitrile 5–100% B in 77 min	PDA[a] 260, 280, 320, 360 nm	Rommel and Wrolstad (1993a)
Ellagic acid	blackberry, red raspberry, strawberry	Novapak C18	methanol/ 0.025 M potassium-hydrogen phosphate, pH 2.4 (40:60) (post column derivatisation with $Na_2B_4O_7$)	Fluorescence	Hollman and Venema (1993)
Hydroxybenzoic and hydroxycinnamic acids	blackberry, blueberry, red raspberry, strawberry, sweet cherry	150 x 3.6 mm Novapak C18	A: 50 mM dihydrogen ammonium phosphate (pH 2.6) B: 20% A with 80% acetonitrile C: 0.2 M orthophosphoric acid (pH 1.5) (100:0:0) to (0:80:20) in 60 min	PDA[a] 280, 316 nm	Heinonen et al. (1998)

[a] PDA=photo-diode array detection

2.4.3 Detection and identification of flavonols and phenolic acids

Phenolic compounds absorb in the UV region and the most commonly used detector for HPLC is a variable-wavelength UV or UV-vis detector (Lee and Widmer 1996, Robards and Antolowich 1997) (Tables 2 and 3). No single wavelength is ideal for monitoring all classes of phenolics since they display absorbance maxima at different wavelengths (Delage et al. 1991). Most benzoic acid derivatives display their maxima at 246–262 nm, except for gallic acid and syringic acid which have absorption maxima at 271 and 275 nm, respectively (Torres et al. 1987). Hydroxycinnamic acids absorb in two UV regions, one maximum being in the range of 225–235 nm and the other in the range of 290–330 nm (Ribereau-Gayon 1972). At 320 nm, cinnamic acid derivatives can be detected without any interference from benzoic acid derivatives, which have a higher absorptivity at 254 nm. However, detection at 280 nm is the best alternative for the determination of both classes of phenolic compounds (Pussayanawin and Wetzel 1987). The absorption range 350–370 nm has been widely used for flavonol aglycones and 280 nm for flavan-3-ol and flavonol glycosides (Robards and Antolowich 1997).

The extensive use of photo-diode array detection (PDA) in the analysis of flavonoids and phenolic acids can be attributed to the ability to collect on-line spectra (Hertog et al. 1992a,b, Rommel and Wrolstad 1993a,b, Justesen et al. 1998) without using stopped-flow techniques. This has led to a considerable improvement in the HPLC analysis for identification purposes and demonstrated the usefulness of qualitative information in phenolic analysis that is based on the absorption spectrum (Jaworski and Lee 1987, Mazza and Velioglu 1992, Fernández de Simón et al. 1992). PDA has three major advantages: multiple wavelength detection, peak identification, and peak purity determination (Lee and Widmer 1996). For the peak purity check, the on-line spectra of the eluting peak from up-slope, down-slope and peak apex are compared.

The UV detection has the disadvantage of not being as sensitive or selective as fluorescence detection, and interfering peaks are more common. However, fluorescence detection has not been applied widely to phenolics (Lee and Widmer 1996). In the analysis of phenolics in orange juice, fluorescence detection offers major advantages over UV detection in terms of enhanced selectivity and sensitivity (Roussef et al. 1992b). Fluorescence detection has also been applied for the analysis of phenolic acids in persimmons (Gorinstein 1994), ellagic acid in berries and nuts (Hollman and

Venema 1993), and flavonols (Hollman et al. 1996) and resveratrol isomers in plasma (Giachetti et al. 1999).

Electrochemical detection (EC) is very sensitive for compounds that can be oxidised or reduced at low-voltage potentials. EC detection is becoming increasingly important for the determination of very small amounts of phenolics, as it shows enhanced sensitivity and selectivity compared to UV detection (van Sumere 1989, Akasbi et al. 1993). EC detection has been applied for the detection of flavonols and phenolic acids in vegetables (Chiavari et al. 1988), beverages (Lunte 1987, 1988a, b), and plasma (Erlund et al. 1999).

HPLC-MS is a fast and reliable method for structural analyses of non-volatile phenolic compounds, since better techniques (interfacing systems) have been developed for the removal of the liquid mobile phase before ionisation (Careri et al. 1998). Pietta et al. (1994) showed that thermospray liquid chromatography (LC) MS is an excellent technique for the analysis of flavonol glycosides from medicinal plants. Flavan-3-ols (Lin et al. 1993) and various groups of polyphenols including flavonol glycosides (Kiehne et al. 1993) in tea have been studied using thermospray LC-MS. Positive ion fast atom bombardment MS and tandem MS have been used to study the glycosidic linkages in diglycosyl flavonoids (Li and Claeys 1994). Electrospray and its several variations are more recent developments in atmospheric pressure ionisation mass spectrometers (Robards and Antolowich 1997). HPLC electrospray ionisation (ESI)-MS offers advantages in terms of sensitivity and capacity for the analysis of large, thermally labile and highly polar compounds (Robards and Antolowich 1997, Careri et al. 1998). HPLC-ESI-MS has been used to study flavonoids in tea (Poon 1998) and in tomato and plasma (Mauri et al. 1999). Atmospheric pressure ionisation (API)-MS technique has been applied for the analysis of flavonoids in fruits and vegetables (Justesen et al. 1998).

3 AIMS OF THE STUDY

The general aim of this series of studies was to determine the content of selected flavonoids and phenolic acids in wild and cultivated berries in Finland. Special emphasis was given to the optimisation of the extraction and hydrolysis methods of phenolic compounds, as well as to their chromatographic separation and identification techniques.

The aims of the individual studies were:

- to determine the main non-anthocyanin flavonoids and phenolic acids (phenolic profiles) in berries by using an optimised, semi-quantitative HPLC method (**I, II**);
- to assess the content of the flavonols quercetin, myricetin, kaempferol, and ellagic acid in berries by using optimised, quantitative HPLC methods (**III, IV, VI**);
- to investigate the influence of cultivar, cultivation site and cultivation technique on the flavonol and phenolic acid contents of strawberries and blueberries (**V**);
- to study the effects of domestic processing and storage on flavonols and ellagic acid in selected berries and berry products (**VI, VII**);
- to estimate the contribution of berries to the dietary intake of flavonols and ellagic acid in Finland (**III, VI**).

4 MATERIALS AND METHODS

4.1 Berry samples

The berries investigated in the individual studies are described in Table 4. The samples were of Finnish origin (except for two strawberry samples from Poland in Study **V**) and were collected mainly from eastern Finland in 1994–1998. Sample information about the berries investigated in individual studies is presented in Table 5. The berries in Studies **I** and **II** were stored for 6 months at –20 °C until crushed (while still frozen) and concentrated by lyophilisation. The freeze-dried berries were ground to pass a sieve and the seedless powders were stored at –20 °C. In Studies **III**–**VII**, fresh berries were frozen in 100-g batches and stored at –20 °C until analysed.

Table 4. Berries investigated in the individual studies **I**–**VII**.

Berry	Wild/ cultivated	Family	Genus	Studies
Cranberry	wild	Ericaceae	*Vaccinium oxycoccos*	**II–IV**
Lingonberry	wild	Ericaceae	*Vaccinium vitis-idaea*	**II–IV, VII**
Blueberry	cultivated	Ericaceae	*Vaccinium corymbosum* 'Northblue'	**II, III, V**
Blueberry	cultivated	Ericaceae	*Vaccinium corymbosum* 'Northcountry'	**II, III, V**
Bilberry	wild	Ericaceae	*Vaccinium myrtillus*	**II–V, VII**
Bog whortleberry	wild	Ericaceae	*Vaccinium uliginosum*	**III -V**
Gooseberry	cultivated	Grossulariaceae	*Ribes uva-crispa*	**II, III**
Black currant	cultivated	Grossulariaceae	*Ribes nigrum* 'Öjebyn'	**I–IV, VII**
Red currant	cultivated	Grossulariaceae	*Ribes* x*pallidum* 'Red Dutch'	**II, III**
White currant	cultivated	Grossulariaceae	*Ribes* x*pallidum* 'White Dutch'	**II, III**
Green currant	cultivated	Grossulariaceae	*Ribes nigrum* 'Vertti'	**II, III**
Chokeberry	cultivated	Rosaceae	*Aronia mitschurinii* 'Viking'	**II, III**
Rowanberry	wild	Rosaceae	*Sorbus aucuparia*	**II - IV**
Sweet rowan	cultivated	Rosaceae	*Grataegosorbus mitschurinii* 'Granatnaja'	**II, III**
Strawberry	cultivated	Rosaceae	*Fragaria* x *ananassa*	**I–VII**
Cloudberry	wild	Rosaceae	*Rubus chamaemorus*	**II, III, VI**
Red raspberry	wild/cultivated	Rosaceae	*Rubus idaeus*	**II–IV, VI**
Arctic bramble	cultivated	Rosaceae	*Rubus arcticus*	**II, III, VI**
Sea buckthorn berry	cultivated	Elaeagnaceae	*Hippóphaë rhamnoides*	**II–IV**
Crowberry	wild	Empetraceae	*Empetrum nigrum*	**II, III**
Crowberry	wild	Empetraceae	*Empetrum hermaphroditum*	**III, IV, VII**

Berries analysed in Studies **VI** and **VII** were from the same batches as those used in Study **III**. After harvesting, the berries were stored at +5 °C or, for comparison, at room temperature (+22 °C), and analysed within 24 h. The berries were frozen, stored at –20 °C and analysed after 3, 6 and 9 months. Black currants and crowberries used for the preparation of cold-pressed juice in Study **VII** were analysed after 1 and 4 months of storage at –20 °C, respectively. Three sets of strawberry jam, bilberry soup, crushed lingonberries, unpasteurised lingonberry juice, and steam-extracted black currant juice were prepared in the laboratory as described in Study **VII**, and the content of flavonols (and ellagic acid in strawberry jam) was analysed within 24 h and after 3, 6 and 9 months of storage at +5 or –20 °C.

Table 5. Sample information about the berries investigated in individual studies.

Study	Number of samples (berries/products)	Year of sample collection	Origin [a] of sample	Storage time at –20 °C (months)	Form of samples analysed
I	2	1994	EF	6	lyophilised, powder
II	19	1994, 1995	EF, WF, NF	1 or 6	lyophilised, powder
III	25	1997	EF, WF, NF	3	thawed (frosty)
IV	11	1997	EF, WF, NF	4–6	thawed (frosty)
V	21	1997	EF, SWF, P	8–9	thawed (frosty)
VI	8/1	1997	EF, WF	0, 3, 6 and 9	fresh/ thawed (frosty)
VII	6/7	1997, 1998	EF, NF	0, 3, 6 and 9	fresh/ thawed (frosty)

[a] EF= eastern Finland, WF= western Finland, NF= northern Finland, SWF= south-western Finland, P= Poland

4.2 Analytical methods

Flavonoids and phenolic acids were determined by HPLC after extraction and hydrolysis to the corresponding aglycones or unconjugated acids. For the identification of the phenolic compounds, diode-array detection (DAD) was used in all studies. Electrospray ionisation mass spectrometry (ESI-MS) was used to identify the flavonol aglycones and glycosides in Studies **III** and **IV**. The analyses were carried out in duplicate (Studies **I, II**, **IV**–**VI**) or in triplicate (Studies **III** and **VII**).

4.2.1 Extraction and hydrolysis

Extraction and hydrolysis conditions for the berry samples in individual studies are described in Table 6. Flavonoid glycosides in Studies **III**–**V** and **VII** were extracted and hydrolysed to their corresponding aglycones in 50% (v/v) aqueous methanol containing hydrochloric acid (1.2 M) with a method modified from that of Hertog et al. (1992b).

For the extraction of flavonoid glycosides in Study **IV**, the thawed and homogenised berries (5 g) were extracted in 50% (v/v) aqueous methanol without added HCl. The mixture was shaken at room temperature (+21°C) for 2 h.

Table 6. Extraction and hydrolysis conditions for flavonoids and phenolic acids in individual studies.

Study	Amount of sample (g)	Compounds analysed [a]	Time (h)	Temperature (°C)	Antioxidant
I-II	0.5 [b]	FLA, HCA, E	16	35	ascorbic acid
V	5	HCA	16	35	ascorbic acid
III-V, **VII**	5	FLA	2	85	TBHQ [c]
V-VI	5	E	20	85	-

[a] FLA = flavonols (kaempferol, quercetin and myricetin), HCA = hydroxycinnamic acids (*p*-coumaric, caffeic and ferulic acids), E = ellagic acid. [b] Freeze-dried sample. [c] TBHQ = *tert*-butylhydroquinone.

4.2.2 Chromatographic conditions in semi-quantitative HPLC analyses (**I**–**II**)

In the testing of various chromatographic systems (Study **I**), an HPLC apparatus consisting of a Hewlett-Packard (Waldbronn Analytical Division, Germany) 1050 Series pump, an auto-sampler and variable-wavelength detector was used. In the testing and evaluation of the method chosen as well as in the analysis of phenolic profiles (Studies **I** and **II**), an HPLC apparatus consisting of a Hewlett-Packard 1050 Series pumping system, an injector and a 1040M Series II photodiode array UV-vis detector was used. Separations were performed with an ODS-Hypersil column (Hewlett-Packard, Germany) protected with guard column RP-18. Three solvents were used for the gradient elution: (A) 50 mM ammonium dihydrogen phosphate; (B) 0.2 mM ortho-phosphoric acid; and (C) 20% solvent A in 80% acetonitrile. The solvent gradient elution program is described in Study **I**. A

diode-array detection was used for the identification of the compounds. Retention times and UV-vis spectra of the peaks were compared with those of the standards.

4.2.3 Chromatographic conditions in quantitative HPLC analyses (**III-VII**)

Chromatographic systems

The HPLC system used for UV quantification of flavonols and ellagic acid in Studies **III, VI** and **VII** was a Hewlett-Packard 1050 Series pump, an autosampler and a variable-wavelength detector. For the identification of flavonols and ellagic acid in berries (Studies **III**, **IV**, **VI**, **VII**), an HPLC apparatus consisting of a Hewlett Packard 1050 Series pump, an injector and a 1040M Series II diode array UV-vis detector was used. The system used for HPLC-MS analyses (Study **IV**) was a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with a Rheos 400 HPLC pump (Danderyd, Sweden). The HPLC system used for UV-vis quantification and identification in Study **V** was a Hewlett-Packard 1100 Series instrument with a diode array UV-vis detector. In all analyses, a LiChroCART column (Merck, Darmstadt, Germany) protected with a LiChroCART guard column was used. Solvent A was 1% formic acid and solvent B acetonitrile; flow rate was 0.5 ml/min.

The gradient elution system for flavonol analysis in Studies **III**–**IV** and **VII** is described in Study **III**. The gradient used for flavonol analysis in study **V** was slightly modified from that of Study **III**; the amount of 1% formic acid was decreased (pH and hydrophobicity was increased) in the elution solvent more quickly in study **V** than in Study **III**.

The gradient used for the analysis of ellagic acid is described in Study **VI**. In Study **V**, the gradient used in the analysis of ellagic acid and hydroxycinnamic acids was modified from that of Study **VI**; the amount of 1% formic acid was decreased in the elution solvent more slowly in Study **V** than in Study **VI**.

Identification procedures

Ellagic acid was detected at 260 nm, hydroxycinnamic acids at 320 nm and flavonols at 360 nm. To identify the phenolic compounds in berries, the retention times and UV-vis spectra of the flavonols and phenolic acids in berry samples were compared with those of standards. Additionally, spectra were recorded up-slope, apex and down-slope (220–450, 2 nm steps). Peaks were considered to be pure when there was a correspondence of >900 (flavonols, hydroxycinnamic

acids) or >950 (ellagic acid) among the spectra. Moreover, HPLC-ESI-MS was used for the identification of flavonol aglycones and flavonol glycosides in berries (Study **IV**). All mass spectrometric data were acquired in the positive ionisation mode. Total ion chromatograms (TIC) were measured and, in addition, the instrument was set to alternatively measure four events: (1) full scan source induced dissociation (SID) was used to screen the samples for kaempferol, quercetin or myricetin containing glycosides in the berry samples; (2) MS (full scan) was used to measure the $[M+H]^+$ ions revealing the molecular weights of the components; (3) MS-MS was used to break down the most abundant $[M+H]^+$ ion from MS with dependent collision-induced dissociation (CID); (4) MS3 was used to break down the most abundant fragment ion from MS-MS with CID.

4.2.4 Validation of the methods

Optimisation procedures

In Study **I**, extraction and hydrolysis with 50% methanol was tested with two different acid concentrations (0.6 or 1.2 M HCl) and at three different temperatures, i.e., room temperature (16 h), 35°C (16 h), and 85°C (2 h). To optimise the HPLC analysis, three reversed-phase columns were tested: Spherisorb ODS1 (Phase Separations Inc., Norwalk, CT, USA); LiChrospher 100RP-18 (Hewlett-Packard, Germany); ODS-Hypersil (Hewlett-Packard). In addition, three solvent systems were tested: acetonitrile/1% acetic acid in water, 5% acetic acid in water/methanol, 50 mM ammonium dihydrogen phosphate (A)/0.2 mM ortho-phosphoric acid (B)/20% solvent A in 80% acetonitrile.

In Study **III**, extraction solutions with methanol concentrations of 25, 50 and 64% in 1.2 M HCl were tested in the analysis of flavonols. Also, the influence of TBHQ antioxidant on flavonol concentrations was investigated. Moreover, the influence of thawing method was tested. The berries were thawed in a refrigerator (7 °C, 16 h), at room temperature (21 °C, 1.5 h), or in a microwave oven (2–3 min) in plastic containers. In all cases, the berries were cold (5–10 °C) when homogenised.

To optimise ellagic acid analysis in berries (Study **VI**), extraction and hydrolysis times of 2, 3, 4, 6, 16, 20, 40 and 46 h were tested at 85ºC in 50% aqueous methanol and 1.2 M HCl. The extraction and hydrolysis procedure modified from that of Daniel et al. (1989) for strawberry was also tested.

Analytical quality control

The recoveries (Studies **I**, **III**, **VI**–**VII**) from berry and berry product samples were measured by adding pure standards to the extraction solutions prior to extraction and hydrolysis. The amount of each standard added was chosen to be between 50 and 100% of the content of the corresponding compound previously analysed from the authentic berry samples. To study the linearity of the methods, new standard curves with freshly prepared standards from stock solutions were determined every week. The standards used for the quantitative analysis of flavonols (Studies **III, V, VII**), ellagic acid (Studies **V** and **VI**) and hydroxycinnamic acids (Study **V**) were prepared from the stock solutions of the individual standards in methanol (1000 ì g/ml). In studies **I** and **II**, quercetin and p-coumaric acid were used as secondary standards for flavonols and phenolic acids, respectively. To study the repeatability of the HPLC analysis, six injections of the same hydrolysed berry sample were analysed (Study **I**). To study the within-laboratory repeatability (within-day precision), flavonol content of a frozen berry sample was analysed six times within one day (Studies **III** and **VI**). Within-laboratory reproducibility of the whole method was studied by duplicate analyses of a freeze-dried sample during a period of six weeks (Study **I**), seven months (Study **III**) or eight months (Study **VI**).

4.3 Calculation of the intake

The dietary intake of flavonols (Study **III**) and ellagic acid (Study **VI**) was calculated by using food consumption data obtained from the Finnish Household Survey 1990 (Statistics Finland 1993). The survey recorded the purchase of foods (250–300 food items) of households. In 1990, the final sample size was 8258 households. Additionally, the intake of flavonols and ellagic acid was calculated by using food consumption data from the Finnish Household Survey 1998 (Statistics Finland, unpublished data). In 1998, the final sample size was 4359 households.

Fresh berries recorded in the Finnish Household Survey were black currant, red and white currants, strawberry, other garden berries, bilberry, lingonberry, cranberry, cloudberry and other wild berries. For other flavonol-rich foods, the flavonol contents reported by Hertog et al. (1992b, 1993b) were used.

4.4 Statistical analyses

The phenolic profile data (Study **II**) were analysed with principal component analysis (PCA). For PCA calculations, program Statistica, version 5.0 (Statsoft Inc., USA) was used.
In Studies **V** and **VI**, statistical analysis was done by one-way analysis of variance (Minitab for Windows), and in Study **VII** by non-parametric Friedman's one-way analysis of variance (SPSS 8.0 for Windows). Values of $P < 0.05$ were considered statistically significant.

5 RESULTS

5.1 Validation of the methods

5.1.1 Optimisation of the procedures

Semi-quantitative analysis (**I**)

The best conditions for extraction and hydrolysis of strawberry and black currant were obtained by shaking the samples in a water bath at 35 °C for 16 h in 50% (v/v) aqueous methanol with 1.2 M HCl (**I:** Table 4). When using this system, the mean relative peak areas of all phenolic compounds were highest compared to other methods tested. Moreover, no compound was completely destroyed by this method, in contrast to the other methods evaluated. Of the chromatographic conditions tested, the ODS-Hypersil column with the solvents 50 mM ammonium dihydrogen phosphate (A), 0.2 mM ortho-phosphoric acid (B), and 20% solvent A in 80% acetonitrile gave the best separation for the phenolics of interest (**I:** Figure 1). This gradient elution program was modified from the method of Lamuela-Raventós and Waterhouse (1994), described originally for the measurement of wine phenolics.

Quantitative analysis of flavonols and ellagic acid (**III, VI**)

Thawing of the berries at room temperature resulted in lower quercetin contents than thawing in the refrigerator or microwave oven. However, variability in the apparent quercetin content was higher when the berries were thawed in the refrigerator (17%) compared to thawing in the microwave oven (12%). Therefore, thawing in the microwave oven was chosen. This was also the most practical method for routine analysis.

When extracted with 50% aqueous methanol (the final method), the apparent flavonol contents in the berries were 20 or 70% higher than those recovered with 64 or 25% methanol, respectively (Study **III**). In three berries studied, the apparent quercetin content increased by 8-30% when TBHQ was present in the extraction and hydrolysis solution. Therefore, TBHQ was included as an antioxidant in the flavonol analyses.

Optimal recoveries of hydrolysed ellagic acid were achieved by 20-h extraction and hydrolysis

time at 85 °C in 50% aqueous methanol and 1.2 M HCl (**VI:** Figure 3). When using 20-h extraction and hydrolysis time, 50% more ellagic acid was measured from strawberry sample compared to that extracted and hydrolysed for 2 h. With the extraction and hydrolysis procedure modified from that of Daniel et al. (1989) for strawberry, the amount of ellagic acid obtained was less than 15% of that obtained with the method chosen here.

5.1.2 Reliability of the methods

Detector responses were linear over the concentration ranges used in all studies (**I-III, V-VII**). In Study **I**, the recoveries were good or tolerable (70–96%) for most compounds studied in strawberry and black currant (**I:** Table 5). In the calculation of the final results, the recoveries were not taken into account. The coefficient of variation (CV) for the repeatability of the HPLC injections was good being less than10% (**I:** Table 6). The CVs for the long-term variation (reproducibility) were between 7.7 and 56.9% (**I:** Table 6). The highest CVs were detected for the compounds present at the lowest amounts in the sample. The CVs for the reproducibility of the phenolic profile for a black currant sample varied between 7.9 and 62.2%, being highest for ellagic acid present at a very low concentration (**I:** Table 7). In general, the precision was better for flavonoids than for phenolic acids. For the identification of the phenolic compounds in Studies **I** and **II**, the retention times and UV-vis spectra of the peaks were compared with those of the standards; the purity of the peaks was not confirmed using the diode-array purity test.

In Study **III**, the recovery of pure quercetin from the berries ranged from 50 to 106%, that of myricetin from 52 to 99% and that of kaempferol from 67 to 88% (**III:** Table 1). Because of the differences in the recoveries of the flavonols among the berries, the results were corrected for the recoveries in Studies **III** and **VII**. The CVs of selected berries (Study **III**) for the within-day repeatability and within-laboratory reproducibility of the HPLC method were good (<10%) for quercetin and myricetin.

Using HPLC-DAD and HPLC-ESI-MS techniques it was possible to confirm the identity and purity of the flavonol aglycones in berries (**IV:** Table 1). These identification techniques were used in the analysis of flavonol contents in Studies **III** and **VII**. An HPLC-DAD chromatogram of the peak (purity factor >900) and an extracted ion chromatogram for the ion m/z 303 (quercetin) in a sea buckthorn berry sample are shown in Figure 2 in Study **IV**. An MS spectrum of m/z 303.3 ion

resulted in MS-MS fragmentation spectrum in which the main ions matched with the fragmentation spectrum of quercetin. Using these techniques, quercetin aglycone was identified and its purity confirmed with both ESI-MS and DAD in all berries studied. Myricetin was identified with both techniques in cranberry, crowberry and black currant, and kaempferol in strawberry.

Precision of the method used in Study **VI** was good. Recoveries of the ellagic acid standard from strawberry and strawberry jam were 80 and 85%, respectively. In the calculation of the final results, the recoveries were not taken into account. CVs for the repeatability of ellagic acid analysis were <10%. The CV for the within-laboratory reproducibility was 10%. Monitoring of the ellagic acid peak from the samples using diode-array detection confirmed its identity and purity (peak purity factor >950).

5.2 Phenolic profiles in berries (II)

The relative contents of flavonoids and phenolic acids among the berries studied varied considerably (Figure 5). Certain similarities were observed within families and genera (**II:** Tables 3 to 6). In the members of the family *Ericaceae*, genus *Vaccinium*, the main phenolics found were flavonols (lingonberry and cranberry) and hydroxycinnamic acids (blueberries and bilberry). Flavonols dominated also in gooseberry, black currant and red currant (family *Grossulariaceae*, genus *Ribes*), in sea buckthorn berry (family *Elaeagnaceae*) and in crowberry (family *Empetraceae*). Ellagic acid was the main phenolic compound in the family *Rosaceae* genus *Fragaria* (strawberry) and genus *Rubus* (cloudberry, red raspberry, arctic bramble).

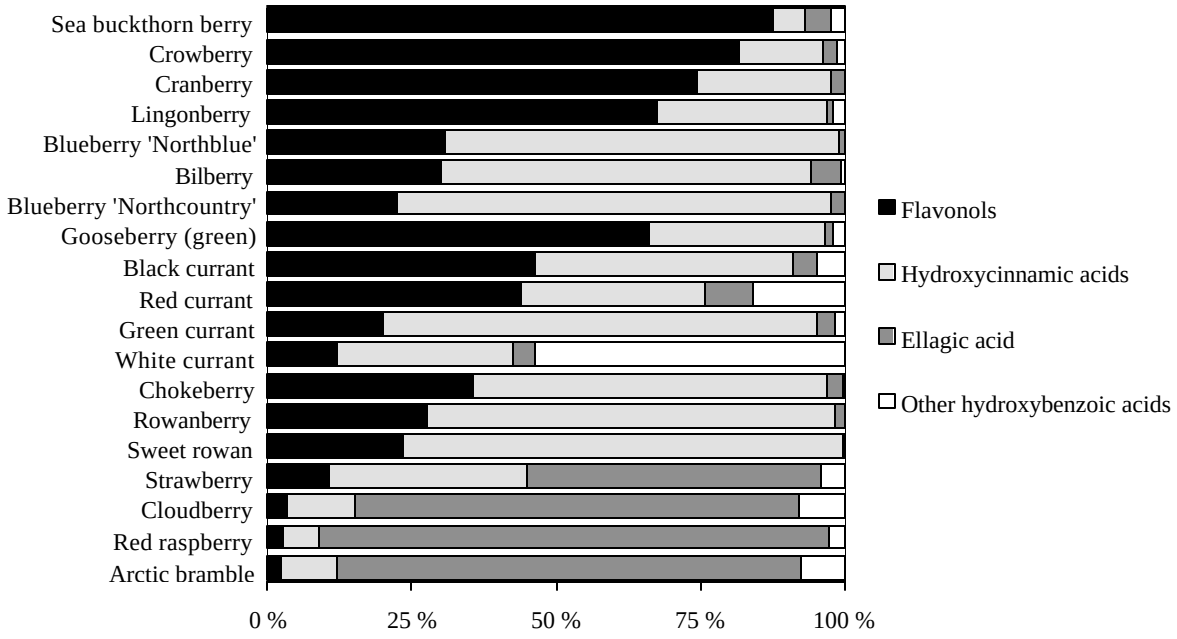


Figure 5. Percentage distributions of groups of phenolic compounds analysed in berries (Study **II:** Figure 1).

5.3 Flavonol contents in berries and berry products

Flavonol aglycones in berries (**III**)

Large differences among the berries were found in their mean flavonol contents (from 6 to 210 mg/kg, f. w.) (Figure 6). Quercetin was found in all berries, the contents being highest (> 50 mg/kg f. w.) in bog whortleberry, lingonberry, cranberry, chokeberry, sweet rowan, rowanberry, sea buckthorn berry and crowberry (**III**: Table 2). Myricetin was detected in cranberry, black currant, crowberry, bog whortleberry, blueberries and bilberry. Kaempferol was detected only in gooseberry and strawberry.

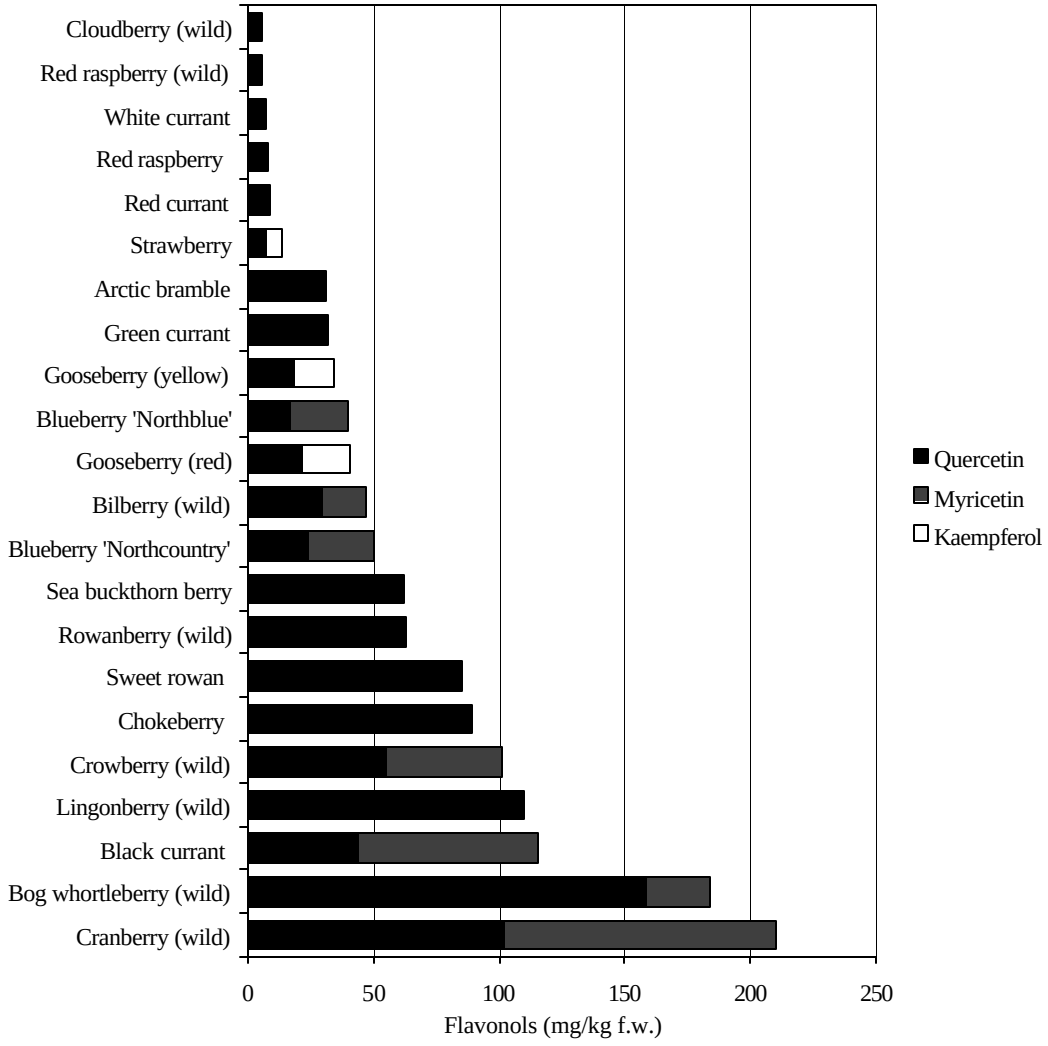


Figure 6. Concentrations of flavonols in berries.

Flavonol glycosides in berries (**IV**)

The TIC and SID chromatograms, as well as MS, MS-MS and MS3 spectra were used to identify a diglycoside, as an example, in the unhydrolysed chokeberry sample in Study **IV**. SID-generated ion chromatograms of aglycone ions were used for screening flavonols (e.g. quercetin m/z 303) in the HPLC-MS chromatogram (**IV:** Figure 3b-e). The molecular weight of the diglycoside compound ($[M+H]^+$ ion at 611) was determined from the MS. The MS-MS spectrum of the $[M+H]^+$ ion of the diglycoside shows an ion at 465 indicating a loss of a 146 mass unit (u) ion (probably deoxyhexose sugar) and also exhibits an ion of m/z 303.3. The difference (162 u) between the two masses 465 and 303 suggests a further loss of a hexose sugar. The MS3 spectrum of the m/z 303

ion resulted in a fragmentation spectrum in which the main ions matched with the fragmentation spectrum of quercetin. Using these techniques we identified several glycosides in berries (**IV:** Table 2). Hexoses, deoxyhexose-hexoses and pentoses of quercetin (Q) were the most abundant glycosides identified.

Effects of processing and storage on flavonol aglycones of berries and berry products (**VII**)

Effects of processing methods on flavonol contents in berry products are presented in Fig. 7. Cooking strawberries with sugar to make jam resulted in minor losses in quercetin and kaempferol concentrations. In contrast, less than 30% of myricetin and 15% of quercetin originally present in berries was extracted in juices by common domestic methods (steam-extracted black currant juice, unpasteurised lingonberry juice) (Figure 7). Cold-pressing was superior to steam-extraction in extracting flavonols from black currants.

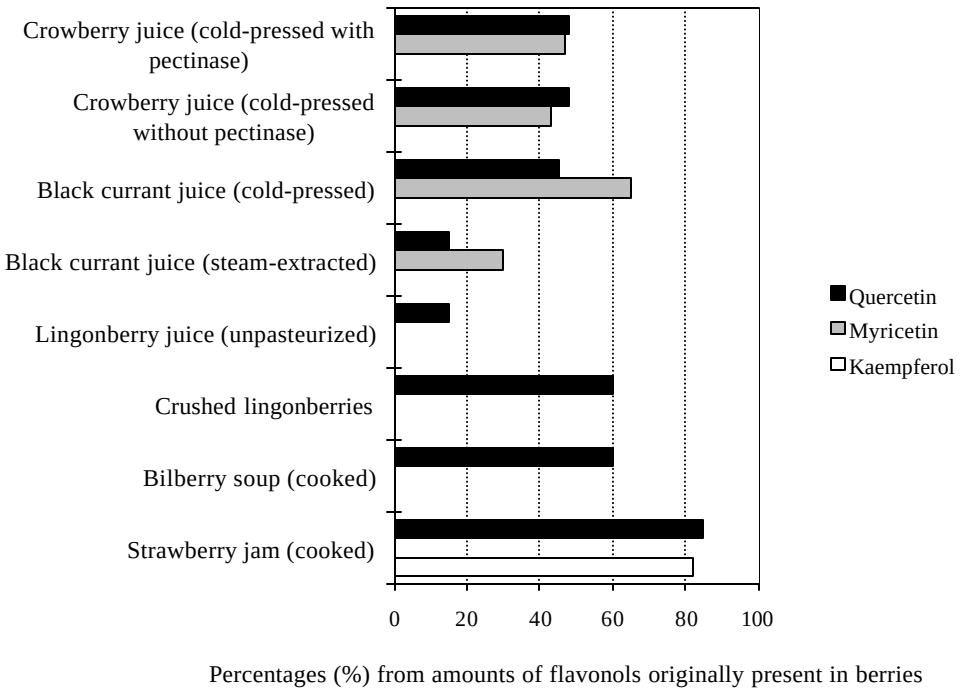


Figure 7. Effects of different processing methods on quercetin, myricetin and kaempferol contents in berries as percentages from the amounts originally present in unprocessed berries.

The post-harvest temperature had an effect on quercetin and kaempferol contents in strawberry, and on myricetin content in black currant (**VII:** Table 1). Approximately 20% lower levels were measured in the berries stored for 24 h at room temperature compared to those stored in a refrigerator. During 9 months of storage in a freezer, quercetin content decreased markedly in bilberries and lingonberries, but not in black currants or red raspberries (**VII:** Table 1) (Figure 8). Higher apparent quercetin levels were measured from strawberries after 9 months of storage than from fresh berries (Figure 8).

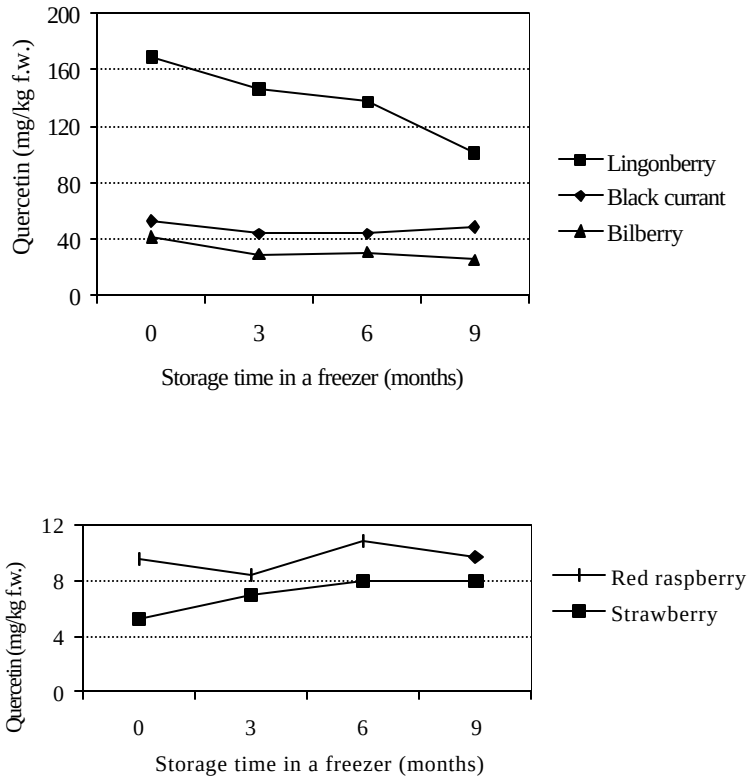


Figure 8. Effects of storage on quercetin contents in frozen berries.

On the other hand, although kaempferol was detected in fresh strawberries and strawberry jam, it could not be quantified after 3 or 6 months of storage, respectively (**VII**: Tables 1 and 2). Also, the level of myricetin in fresh black currants and in the freshly-prepared black currant juice was high, but decreased during storage and was not detectable (peak purity < 900) after 9 months (Figure 9).

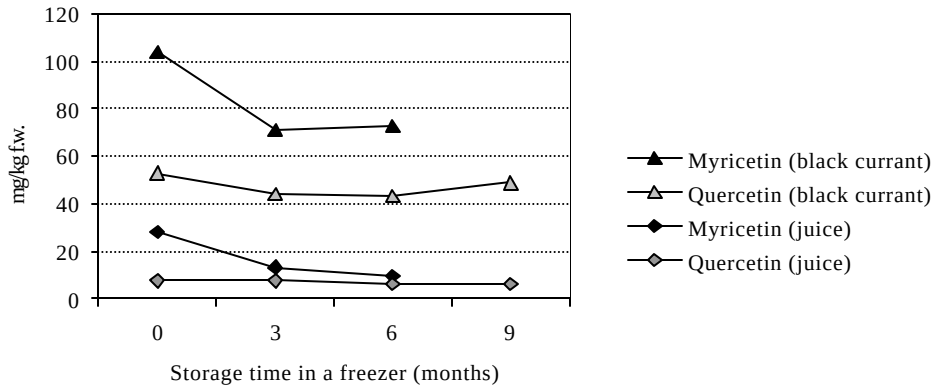


Figure 9. Effect of storage in a freezer on the content of quercetin and myricetin in black currants and black currant juice.

5.4 Influence of cultivar, cultivation site and technique on phenolics in berries (V)

In various strawberry cultivars, the sum of phenolics analysed [quercetin (Q), kaempferol (K), ellagic acid (E), p-coumaric acid (CO)] varied from 421 to 544 mg/kg f. w., being lowest in 'Senga Sengana' and highest in 'Jonsok' (**V:** Table 1). The varietal differences were largest in p-coumaric acid contents.

In the blueberry cultivars studied, the sum of phenolics analysed [Q, myricetin (M), caffeic acid (CA)] was highest in 'Tumma' (92 mg/kg) and lowest in 'Northcountry' cultivated in Kuopio (44 mg/kg) (**V:** Table 3). Thus, the variation was larger among the blueberry than the strawberry cultivars. The varietal differences among blueberry cultivars were highest in the contents of caffeic acid and quercetin.

Some regional differences were observed between strawberries grown in Finland and in Poland. Two strawberry samples cultivated in Finland had a significantly higher sum of phenolics analysed (Q, K, E, CO) compared to those cultivated in Poland (**V:** Table 2). This difference was due to the higher concentrations of p-coumaric and ellagic acids in the berries grown in Finland. However, in another 'Senga Sengana' sample cultivated in Finland, only the content of p-coumaric acid was significantly higher than in those cultivated in Poland. Significant regional differences were found also in the contents of quercetin and caffeic acid in the blueberry cultivars 'Northblue' and 'Northcountry', respectively, grown in two locations in Finland (**V:** Table 3). However, apparent differences in the sums of phenolic compounds (Q, K, E, CO) were neither

observed in strawberries cultivated in two locations in Finland nor in two quality classes of strawberries cultivated in Poland.

Similar levels of phenolic compounds were observed in two strawberry cultivars whether conventional or organic cultivation techniques were used (**V:** Table 1). Only in the cultivar 'Jonsok', the organically cultivated berries had a significantly higher sum of all analysed compounds (Q, K, E, CO), ellagic acid and kaempferol content compared to those cultivated by conventional techniques.

5.5 Ellagic acid in berries: content and effects of jam-making and storage (VI)

The ellagic acid contents after 3 months of storage at –20 °C varied between 315 (strawberry 'Senga Sengana') and 686 mg/kg f. w. (arctic bramble) (**VI:** Table 1). We found relatively small but statistically significant differences between two cultivars of strawberries ('Jonsok', 'Senga Sengana') and red raspberries ('Ottawa', 'Muskoka') (**VI:** Table 1). The location where the berries were picked appeared to have no significant effect on ellagic acid levels in cloudberries. The ellagic acid content in strawberry jam was 238 mg/kg f. w., being 80% of that in unprocessed strawberries. The post-harvest temperature (5 °C or 22 °C for 24 h) had no apparent effect on ellagic acid content in fresh strawberries (hydrolysed samples). During the 9 months of storage at –20 °C, a statistically significant reduction of 30 and 40% in ellagic acid content was observed in red raspberries and strawberries, respectively (**VI:** Table 1).

5.6 Intake of flavonols and ellagic acid from berries (III, VI)

The total annual consumption of unprocessed berries in 1990 in Finland was 10 kg/person providing, on average, an intake of 1.2 mg flavonols/d (**III:** Table 3). Assuming that one third of the meals were eaten outside of home, the total intake of flavonols would have been approximately 11 mg/d. The total annual consumption of unprocessed berries in 1998 was 23.1 kg/person, providing 3.4 mg flavonols/d (Table 6) (Statistics Finland, unpublished data). The total intake of flavonols in 1998 was 11.3 mg/d (Table 6) (Statistics Finland, unpublished data). Assuming that one third of the meals was eaten outside of home, the total intake of flavonols would have been 17 mg/d.

Table 6. Intake of flavonols in Finland in 1998.

Foodstuff	Annual onsumption [a] (kg or l [b])	Flavonol content (mg/kg or mg/l [b])	Intake (mg/d)
Unprocessed berries			
Black currants	3.32	115	1.05
Red and white currants	3.22	9	0.08
Strawberries	5.68	15	0.23
Bilberries	3.23	47	0.42
Lingonberries and cranberries [c]	4.51	130	1.61
Cloudberries (and other wild berries)	2.09	6	0.03
Other cultivated berries	0.94	- [d]	
Other berries	0.05	- [d]	
Other foodstuffs and beverages			
Onions	4.3	347 [e]	4.09
Tomatoes	7.5	8 [e]	0.16
Apples	13.0	36 [e]	1.28
Grapes	1.3	13 [e]	0.05
Juice drinks (as orange juice)	24.9	5 [e]	0.34
Tea [f]	20	35 [e]	1.92
Red wine [g]	0.8	16 [e]	0.04
Total			11.30

[a] Statistics Finland (unpublished data). [b] Juices, tea, wine. [c] Consumption ratios of lingonberries and cranberries are estimated to be 80 and 20%, respectively. [d] Flavonol content is not known. [e] Contents of flavonols from Hertog et al. (1992b, 1993b). [f] Tea prepared from 0.2 kg of tea leaves. [g] Twenty percent of the consumption of wines (alcohol 9-15 %, v/v) (3.9 l).

The total annual consumption of unprocessed strawberries and cloudberries in 1990 in Finland was 4.00 and 1.24 kg/person, respectively (Statistics Finland 1993). The mean ellagic acid content (**VI**: Table 1) in strawberries and cloudberries is 350 and 583 mg/kg, respectively; these two berries alone provided thus 5.8 mg ellagic acid/d. In 1998, the total annual consumption of strawberries and cloudberries was 5.68 and 2.09 kg/person, respectively (Statistics Finland, unpublished data), providing 8.7 mg ellagic acid/d.

6 DISCUSSION

6.1 Evaluation of the methods used

Optimisation of the procedures

A simple, semi-quantitative screening method for routine analysis of the major phenolics in berries was developed in Study **I**. Since the phenolics were analysed as aglycones, a hydrolysis step was needed. Flavonols occur in plants usually as O-glycosides with sugars bound at the C3 position (Herrmann 1976). Ellagic acid is usually present in plant vacuoles as hydrolysable, water-soluble ellagitannins, i. e. esters of glucose with a diphenic acid analogue (Bate-Smith 1972, Wilson and Hagerman 1990, Maas et al. 1991b). Hydroxycinnamic acids may be bound to the cell-wall polymers, or they occur as simple esters with glucose, quinic acid or other carboxylic acids (Herrmann 1989). Hydroxybenzoic acids occur as glycosides in berries (Schuster and Herrmann 1985). The method chosen in the present study was a compromise and aimed at efficient hydrolysis of all various derivatives while not destroying the compounds. The method did not give the best yields for flavonols or ellagic acid but it gave information about those compounds which would have been lost under more rigorous conditions (85 °C), e.g. those used by Hertog et al. (1992a,b) for flavonoids only.

The ODS-Hypersil column combined with a gradient elution system of increasing hydrophobicity and changing pH (Lamuela-Raventós and Waterhouse 1994) was chosen for the final method in Studies **I** and **II** because all compounds of interest were successfully separated. We also tested Spherisorb ODS1 column using a gradient of acetonitrile and 1% acetic acid (Rommel and Wrolstad 1993a), but the peaks were broad and ellagic acid and myricetin, in particular, did not separate properly. Moreover, when testing LiChrospher 100RP-18 column (Treutter 1988), the separation of hydroxycinnamic acids was poor when butanol was used in the solvent system; this was also recognised by Treutter (1988).

In the quantitative analysis of flavonols and ellagic acid (Studies **III-VII**), a higher extraction and hydrolysis temperature (85 °C) was used compared to that of the semi-quantitative method. In the analysis of flavonols, the extraction with 50% aqueous methanol resulted in higher apparent flavonol contents in the berries than did extraction with 64 or 25% methanol. These results are in accordance with the studies of Hertog et al. (1992a) showing that the apparent flavonoid contents in vegetables

were highest when 50% aqueous methanol was used. TBHQ was used as an antioxidant in flavonol analyses of berries and berry products because the apparent quercetin and myricetin contents were increased in berries when TBHQ was present in the extraction and hydrolysis solution. TBHQ was also used in the flavonol analysis of vegetables, fruits and beverages by Hertog et al. (1992a,b, 1993b).

Optimal recoveries of hydrolysed ellagic acid were achieved by ten times longer extraction and hydrolysis time compared to that for flavonol analysis at the same temperature and similar concentrations of methanol and HCl. Thus, under these conditions, flavonol aglycones were more easily extracted and hydrolysed from the corresponding glycosides than ellagic acid was released from ellagitannins in the berry samples.

Reliability of the methods

Information about the analytical quality is lacking or poor in many previous studies on the phenolic compounds in berries or fruits. Herrmann and co-workers (e.g. Wildanger and Herrmann 1973, Schuster and Herrmann 1985) did not provide information about the analytical quality control, nor did Bilyk and Sapers (1986). However, Rommel and Wrolstad (1993a) reported recoveries of flavonol aglycones and ellagic acid approaching 100%, but the recovery of benzoic acid standards from C18 cartridges was poor and variable. Hertog et al. (1992a) determined the CVs for within-laboratory repeatability (<5%) and within-laboratory reproducibility (<9%) of flavonol analysis. The recoveries of flavonols varied from 77 to 110%. Justesen et al. (1998) reported CVs for reproducibility for flavonol analysis (<13%). The recoveries of the flavonols were good, except for the myricetin standard (30%).

In Study **I**, the within-day repeatabilities of the HPLC injections for different phenolics in black currant were good or tolerable. However, the within-laboratory reproducibility of the semi-quantitative HPLC method was poor, especially for the compounds present at low concentrations. When compared to Hertog et al. (1992a), the variation in our results was higher, probably due to the long extraction and hydrolysis time (16 h) and long chromatographic run (75 min). Also the fact that the hydrolysis conditions applied were not optimal for these compounds could result in poor reproducibility (Hertog et al. 1992a). The relatively poor precision limits the use of this method only to qualitative or semi-quantitative studies. The recoveries of the phenolic compounds were good or reasonable (>70%), except for p-coumaric acid in strawberry.

In the analysis of flavonols in Study **III**, CVs both for the within-day repeatability and for the within-laboratory reproducibility of the HPLC method were good for quercetin and myricetin, and comparable to those of Hertog et al. (1992a). The recoveries of added pure flavonol aglycones from berries and berry products varied widely in Studies **III** and **VII**. Therefore, the results for the flavonol contents were corrected for the recovery in these studies. The influence of some berry matrices (black currant, gooseberry, lingonberry, rowanberry, sea buckthorn berry) was detrimental for the analysis of flavonol aglycones (recoveries < 70%). This may be due to their chemical reactions as metal chelators or to co-pigmentation reactions with other phenolics, e.g. anthocyanidins (Britton 1983). Most likely, enzymatic reactions did not cause the poor recoveries of flavonols in some berries, because methanol and HCl in the extraction medium denaturate plant enzymes such as polyphenol oxidase (Markham and Bloor 1998). Furthermore, too severe hydrolysis conditions could have resulted in degradation and low recovery of flavonols (especially myricetin) as was reported by Hertog et al. (1992a) and Justesen et al. (1998).

In the analysis of ellagic acid from berries (Study **VI**), repeatability, reproducibility and recoveries of the method were good. Rommel et al. (1993c) reported a high variation in ellagic acid measurements, most likely due to low solubility of the external standard (ellagic acid) in ethanol under neutral or acidic conditions. In the present study, the ellagic acid standard was therefore first dissolved in dimethyl sulfoxide and then in methanol.

6.2 Phenolic profiles in berries

Family Ericaceae, genus Vaccinium

In accordance with earlier reports (Wildanger and Herrmann 1973, Bilyk and Sapers 1986, Hertog et al. 1992a), quercetin was shown to be the main flavonol in lingonberry and cranberry, and the content of myricetin was relatively high in cranberry (Study **II**). p-Coumaric acid was the main phenolic acid in lingonberry, and hydroxycinnamic acids represented the main phenolic acids analysed in cranberry (Study **II**). No other published data on phenolic acids in these berries are available except for ellagic acid in cranberry (Daniel et al. 1989).

In blueberries and bilberry, the relative content of quercetin was higher than that of myricetin or kaempferol (Study **II**). Similar findings have been reported by Starke and Herrmann (1976) in several European bilberry varieties. Ferulic acid was the main phenolic compound in blueberries

'Northcountry' and 'Northblue' and the content of caffeic acid was low (Study **II**). These results are not in accordance with those of Stöhr and Herrmann (1975a), who found caffeic acid to be the main phenolic acid in blueberries. Neither was ferulic acid detected in Study **V** in blueberry varieties. Most probably, in Study **II**, an unknown hydroxycinnamate compound (e.g. isoferulic acid or sinapic acid) eluted together with ferulic acid in blueberry samples, resulting in its misinterpretation as ferulic acid (peak purity was not confirmed in Study **II** as was done in Study **V**). However, this misinterpretation did not affect the percentage distributions of different groups of the phenolic compounds reported for blueberries in Study **II**. In bilberry, the main phenolic acid was p-coumaric acid (Studies **II** and **V**).

Family Grossulariaceae, genus Ribes

Quercetin was the main flavonol in black, red, white and green currants as well as in gooseberries (Study **II**). Results of this study agree well with the literature (Wildanger and Herrmann 1973, Starke and Herrmann 1976, Hertog et al. 1992b). In addition, the relative content of myricetin was high in black currant (Study **II**). For green currants, no previous results on phenolic compounds have been published.

In accordance with the earlier report by Stöhr and Herrmann (1975a), caffeic acid was the main phenolic acid in green gooseberry and p-coumaric acid in black currant (Study **II**). Contrary to the study of Stöhr and Herrmann (1975a), the relative content of p-hydroxybenzoic acid in white currant was found to be exceptionally high (Study **II**). One explanation might be that the white currants were not ripe in our study. In red currant, the amounts of ferulic, p-coumaric and caffeic acids have been reported to be almost similar (Stöhr and Herrmann 1975a), but in Study **II** the relative contents of caffeic and p-coumaric acids were higher than that of ferulic acid.

Families Rosaceae, Elaeagnaceae and Empetraceae

In cloudberry and arctic bramble, ellagic acid was the main phenolic compound and the percentage distribution of flavonols was very low (Study **II**). In rowans and chokeberry, ferulic acid and quercetin were the most dominant phenolics. No data on phenolic acids or flavonols in these berries of the family *Rosaceae* have been previously published.

In line with the earlier studies (Daniel et al. 1989, Maas et al. 1991a, b), ellagic acid was the main phenolic compound in red raspberry and strawberry. Quercetin was the main flavonol in red

raspberry as was also reported by Wildanger and Herrmann (1973). The relative content of quercetin in strawberry was higher than that of kaempferol (Study **II**), which is not in agreement with the study of Hertog et al. (1992b), nor with Studies **III** and **V**, where kaempferol was detected as the main flavonol in strawberries. The results in Study **VII** suggested that kaempferol and myricetin were more susceptible to losses than was quercetin during processing and storage. Similarly, the lower content of kaempferol compared to quercetin could be due to the long (over 20 h) semi-quantitative analysis procedure used in Study **II**.

Quercetin was the main phenolic compound in sea buckthorn berry and crowberry (Study **II**). In addition, the relative content of myricetin was high in crowberry. There are no previous data published on phenolics in these berries of the families *Elaeagnaceae* and *Empetraceae.*

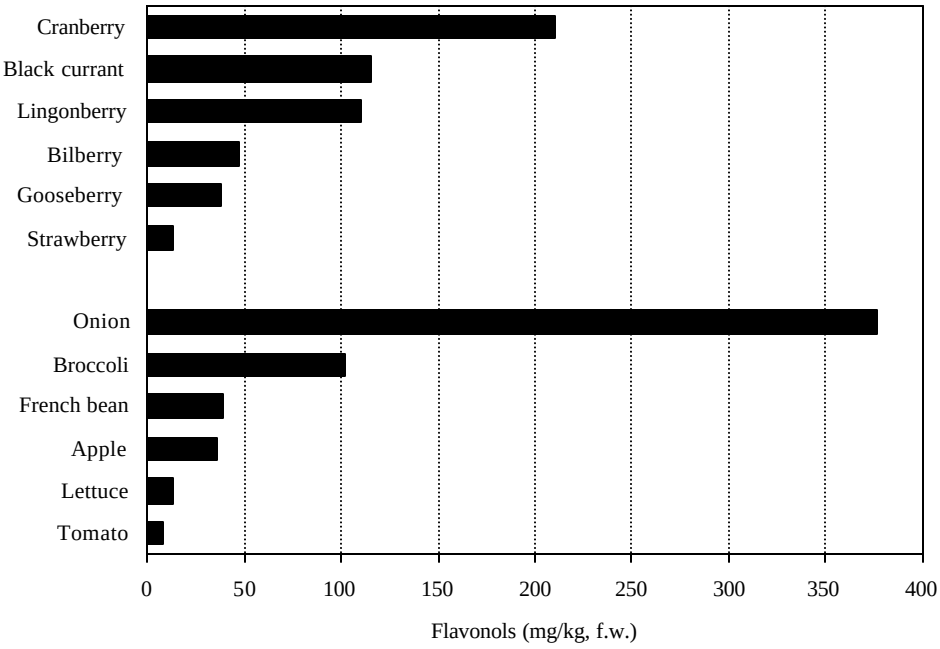
6.3 Flavonol and ellagic acid contents of berries

In general, the flavonol contents obtained in Study **III** were in accordance with those published in earlier reports (e.g. Wildanger and Herrmann 1973, Hertog et al. 1992b, Justesen et al. 1998). However, in contrast with the results of Wildanger and Herrmann (1973) and Bilyk and Sapers (1986), we detected myricetin in blueberries and bilberries (Study **III**). Also in wild cranberry (*Vaccinium oxycoccos*), higher levels of myricetin were reported in the present study compared to those reported in many different cranberry varieties by Bilyk and Sapers (1986) or in *Vaccinium macrocarpon* Ait. by Hertog et al. (1992b). These discrepancies in myricetin content may be due to differences in varieties or cultivars; according to Bilyk and Sapers (1986), the variation in flavonol contents among six cranberry varieties was large (50-70%).

Compared to Study **III**, Herrmann and co-workers (Wildanger and Herrmann 1973, Starke and Herrmann 1976) reported lower flavonol levels for lingonberries, gooseberries and black currants. On the other hand, Justesen et al. (1998) reported somewhat higher flavonol contents e.g. in lingonberries, and Heinonen et al. (1998) in several Californian berries, than was reported in Study **III**. Differences in flavonol contents may partly be due to different cultivars or varieties in different countries, as discussed earlier, or result from the development of the methodology from the early seventies to late nineties. Also, environmental factors (e.g. light, temperature, soil nutrients) may influence phenylpropanoid metabolism and flavonol concentrations in plants (Dixon and Paiva 1995). Discrepancies may also be due to differences in fruit ripeness (Amiot et al. 1995, Prior et al. 1998).

No data on flavonol content in cloudberry, arctic bramble, sea buckthorn berry, bog whortleberry or crowberry have been previously reported. Several berries commonly consumed in Finland (Study **III**) had higher total flavonol contents than have been reported for other fruits or most vegetables (Hertog et al. 1992b, Crozier et al. 1997) (Figure 10). Only onions, kale and broccoli contain similar or higher amounts of flavonols. In addition, many berries had similar or higher flavonol levels (Study **III**) than reported for apples (Hertog et al. 1992b) (Figure 10). Based on Hertog et al. (1995), a flavonol content > 50 mg/kg in food can be considered high. Accordingly, the flavonol content was high in 12 berries (Study **III**). Only cloudberries, red raspberries, and red and white currants contain low levels of flavonols (<10 mg/kg).

Figure 10. Content of flavonols in berries (Study **III**), vegetables and fruits (Hertog et al. 1992b) commonly consumed in Finland.



Study **VI** gave new information about ellagic acid contents in berries per f. w.; they were considerably higher than the flavonol contents in any of the Finnish berries in Study **III**. The ellagic acid contents in strawberry cultivars 'Senga Sengana' and 'Jonsok' were lower (20 and 25%, respectively) in Study **VI** compared to Study **V**, although the year of sample collection was the same in these studies. The ellagic acid contents in Study **VI** were similar to or higher than those reported by Daniel et al. (1989) and Radtke et al. (1998) for strawberries and red raspberries. However,

Hollman and Venema (1993) reported higher ellagic acid contents for strawberries and red raspberries (360-480 and 1200 mg/kg, respectively) compared to those in Study **VI**.

6.4 Flavonol glycosides in berries

According to Hollman (1997), Paganga and Rice-Evans (1997) and Hollman et al. (1999a), quercetin could be absorbed as glycosides into human plasma. Manach et al. (1998) and Erlund et al. (1999) reported that quercetin is probably conjugated with glucuronide and sulphate groups in the liver and no free quercetin or significant amounts of quercetin glycosides can be found in plasma. More research is needed to determine flavonol glycosides from various dietary sources, including berries, and to understand the mechanisms of intestinal absorption of flavonols and the biological activity of the possible conjugates of flavonols.

In Study **IV**, HPLC-ESI-MS was used to identify flavonol glycosides in berries. To confirm the data, the sugars were also analysed using GC-MS. From lingonberry, Q-hexose, Q-pentose and Q-deoxyhexose were identified with ESI-MS (Study **IV**). According to Kühnau (1976), the sugars are galactose, arabinose and rhamnose, respectively. In the GC-MS analysis of sugars both Q-glycoside and Q-galactoside were identified from lingonberry extract (Study **IV**). In accordance with the present study, M-3-rutinoside, M-3-glucoside, Q-3-rutinoside and K-3-rutinoside have been identified in black currant (Siewek et al.1984). In agreement with the study of Kühnau (1976), two deoxyhexose-hexoses of quercetin were identified from sea buckthorn berry with HPLC-ESI-MS, and in the GC-MS analysis of both fractions, glucose and rhamnose were identified. In line with the findings of Kühnau (1976) in cranberry, Q-hexose, Q-pentose, Q-deoxyhexose and Q-di-pentose were identified with ESI-MS technique.

The flavonol glycosides of chokeberry, crowberry and bog whortleberry have not been previously studied. Q-hexose and myricetin aglycone were identified in crowberry whereas in bog whortleberry, two Q-pentoses, Q-hexose and quercetin aglycone were identified with ESI-MS technique (Study **IV**). In chokeberry, e.g. Q-deoxyhexose-hexose and Q-hexose-pentose were identified with ESI-MS technique, and these structures were tentatively confirmed with GC-MS as Q-rhamnose-glucose and Q-glucose-arabinose, respectively.

6.5 Influence of cultivar, cultivation site and cultivation technique on phenolics in berries

Effect of cultivar

Compared to the variation in the contents of phenolic compounds among six strawberry cultivars (Study **V**), Maas et al. (1991b) reported a higher variation in ellagic acid content of the red fruit pulp of strawberry for 35 cultivars and selections. On the other hand, Stöhr and Herrmann (1975b) reported a lower variation in the p-coumaric acid content for 16 strawberry varieties. No studies on the variation in flavonol concentrations of strawberry cultivars are available, except for our study on two cultivars 'Senga Sengana' and 'Jonsok' in which similar levels of quercetin and kaempferol were found (Study **III**). The apparent levels of quercetin and kaempferol in these two cultivars were higher in Study **III** than in Study **V**, partly because the recoveries were not taken into account in Study **V**. During the storage in a freezer, the apparent quercetin content increased in 'Jonsok' (Study **VII**) but kaempferol could not be detected after 9 months of storage. In Study **V**, kaempferol could be detected in all strawberry cultivars after 8 months of storage at –20 °C; this might be due to its initially higher content in fresh strawberries. Moreover, the gradient elution program in Study **V** was slightly modified from the one used in Study **VII**, probably resulting in a better chromatographic separation of kaempferol from the impurities. Among the strawberry cultivars studied (Study **V**), the flavonol content was highest in cultivar 'Honeoye', a potential cultivar for industrial use in the future. The content of both flavonols (14 mg/kg) and total phenolics (Q, K, E, CO) (522 mg/kg) in cultivar 'Honeoye' was significantly higher than in 'Senga Sengana' (9 and 421 mg/kg, respectively) which is conventionally used as the main strawberry by the Finnish food industry.

Variation in quercetin contents of four blueberry cultivars studied was larger than that reported by Bilyk and Sapers (1986) for four highbush blueberry varieties. In general, quercetin and myricetin contents in 'Northblue' and 'Northcountry' samples in Study **V** and **III** were quite similar, except for the higher quercetin content measured in 'Northblue' in Study **V** compared to that in Study **III**. Variation in caffeic acid contents of the blueberry cultivars (Study **V**) was smaller than that reported by Stöhr and Herrmann (1975a) in three blueberry cultivars. Moreover, the caffeic acid concentrations were higher in the study of Stöhr and Herrmann (1975a) compared to those in Study **V**. Differences may be due to genetic and environmental factors or methodological differences. p-Coumaric acid was detected only in bilberry, and ferulic acid could not be detected in any of the samples in Study **V**.

Effect of cultivation site

Only a few studies are available on the influence of cultivation site on the content of phenolic compounds in food or beverages. Maas et al. (1991b) reported ellagic acid concentrations of strawberry cultivars and selections in two different locations in the US (north and east). Regional differences up to 70% have been reported for the flavonol and catechin contents of wines made from the same grape (Cabernet Sauvignon) around the world (Goldberg et al. 1998, McDonald et al. 1998). However, these differences may be partly due to different methods of vinification. In Study **III**, the location where the berries were collected had no influence on quercetin levels in wild bilberries and cloudberries. However, in wild lingonberries collected from two different parts of Finland, quercetin contents differed markedly (50%). Also, wild cranberries collected from western Finland had less flavonols than those collected from eastern Finland. The location effect may partly explain this difference. Cranberries with the lower flavonol content had also been exposed to night frost, and the maturity of the berries may have been different although both berries were ripe. In the study of Starke and Herrmann (1976), the contents of quercetin and myricetin glycosides varied considerably in ripe and unripe black currants and blueberries.

In Study **V**, two 'Senga Sengana' strawberries cultivated in Finland had a significantly higher sum of phenolic compounds analysed (Q, K, E, CO) compared to those cultivated in Poland. However, one sample harvested from eastern Finland and analysed in order to study the varietal differences contained the sum of phenolics not significantly higher than those cultivated in Poland. Regional differences in phenolic contents in strawberries between these two countries were thus not obvious.

Significant differences in the sum of phenolic compounds analysed (Q, K, E, CO) among the 'Senga Sengana' strawberries were neither observed in two quality classes cultivated in Poland nor in two locations in Finland. Small differences were observed in blueberry cultivars grown in eastern Finland compared to those cultivated in south-western Finland. Significant regional differences (40 and 30%) were found in the contents of quercetin and caffeic acid in blueberries, respectively. Because the data were obtained from one growing season only, our results about the influence of cultivation site on the phenolics should be considered tentative.

Effect of cultivation technique

Despite the growing interest of consumers towards organic food, no studies on the influence of organic farming on the contents of flavonoids and phenolic acids in fruits, vegetables or berries are available. Phenolic compounds are induced in plants by various biotic and abiotic stresses (Bennet and Wallsgrove 1994, Dixon and Paiva 1995). Thus, it was expected that the synthesis of phenolic compounds might be different in organic berry production where herbicides, pesticides and insecticides are not used and nutrients are applied without using fertilisers (Kivijärvi 1999). However, organic farming had no consistent effect on the levels of phenolic compounds in strawberries.

6.6 Influence of processing and storage on flavonol and ellagic acid contents of berries

6.6.1 Effect of processing

Cooked berries

Cooking of strawberries with sugar for 30 min caused a smaller loss of flavonols than did the other processing methods studied in any of the berries (Study **VII**). This is probably due to the fact that whole berries were cooked without crushing. In addition, polyphenol oxidase is inactivated by the high temperature (Waterman and Mole 1994). In ellagic acid content, a decrease similar to that of flavonols was observed during strawberry jam preparation. Differing from our procedure, Zafrilla et al. (1999) analysed the content of free ellagic acid (without hydrolysis) in strawberry and raspberry jams. The content of free ellagic acid increased by 150% during jam-cooking. This increase was related either to a release of hexahydroxydiphenic acid from ellagitannins, which is transformed to ellagic acid, or to an easier extractability of this compound from processed products due to the degradation of the cell structures.

Cooking for 10 min with water resulted in a 40% loss of quercetin in bilberries (Study **VII**). Cooking of tomatoes and onions by boiling for 15 min resulted in losses of conjugated quercetin twice as great as in our bilberry study (Crozier et al. 1997). Similarly, only 14–28% of the individual flavonol glycosides of broccoli florets were retained in the cooked tissue, the remainder being largely leached into the cooking water (Price et al. 1998a). Also during cooking of bilberries, quercetin is probably leached into the water fraction that is not, however, discarded. This may explain the much smaller loss of quercetin compared to cooking tomatoes, onions, and broccoli.

Crushed berries

In lingonberries preserved in the traditional way - partially crushed and stored in their own juice in a refrigerator - the apparent level of quercetin was decreased by 40% within a day. This might be due to enzymatic reactions that start when the sub-cellular compartmentation breaks down and enzymes come into contact with potential substrates to which they are normally not exposed (Waterman and Mole 1994). Moreover, the loss of membrane integrity increases the potential of oxidation of phenolic compounds (Waterman and Mole 1994). The level of quercetin remained quite stable during 6 months of storage in a refrigerator, most probably due to the slowing down of the enzymatic and oxidative reactions.

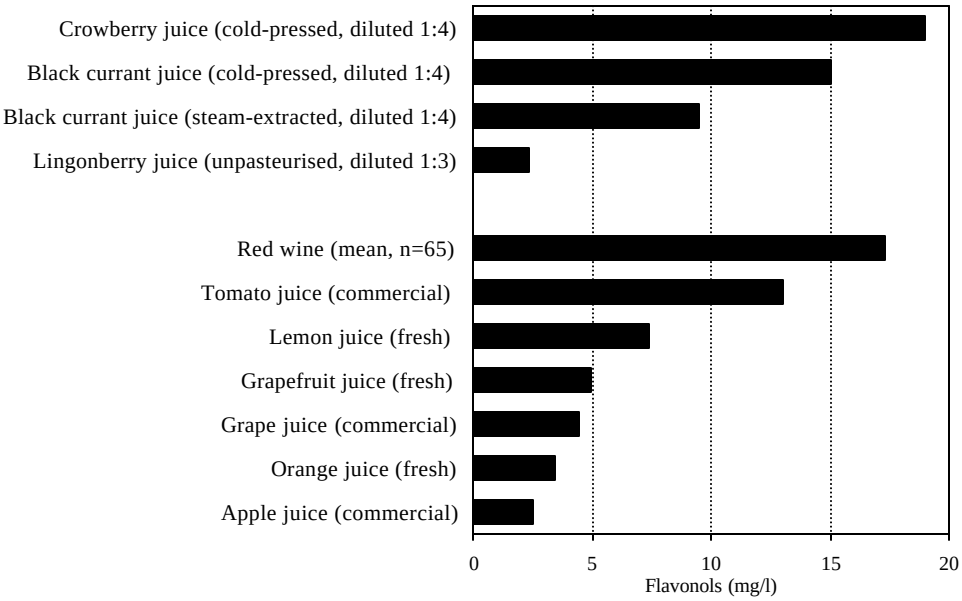
Juices

When juices were made using common domestic processing methods, considerable reduction in flavonol contents were observed (Study **VII**). This is due to the fact that berry skins were removed, and flavonols are known to be concentrated mainly in the skins of fruits (Hawker et al. 1972, Wildanger and Herrmann 1973, Price et al. 1999). According to van der Sluis et al. (1997), only 10% of the original quercetin was found in raw apple juice produced by enzymatic (pectinase) pulping. Also, commercial scale pressing of cider apple varieties resulted in juice that contained 9.9 to 12.7% of the flavonols, the rest remaining in the pomace (Price et al. 1999). Peach-based products are completely devoid of flavonol derivatives due to the removal of the skin in the manufacturing process (Bengoechea et al. 1997).

The cold-press method was superior to the traditional steam-extraction method in extracting quercetin and myricetin from black currant (Study **VII**). This could be due to a more effective extraction of flavonols from berry material (mainly the skins) by mechanical cold-pressing compared to steam-extraction. One reason for the differences in the extractability of flavonols might be that black currants were not subjected to freeze-thaw treatment prior to the steam-extraction process as was done with the berries used in the cold-pressing process. According to Sapers et al. (1983a), freeze-thaw treatment increases the apparent anthocyanin content of cranberry juice. The treatment facilitates the migration of anthocyanins from the exocarp into the mesocarp and endocarp during thawing of cranberries and thus enhances pigment extraction during processing. This might also occur for flavonols in black currants.

It was of interest that the myricetin/quercetin ratio was higher (3.8) in steam-extracted black currant juice than in intact berries (2.1) or cold-pressed juice (1.2). One explanation might be that myricetin is more effectively extracted by hot water than is the less polar quercetin. Differences in these ratios might also reflect the different localisation of myricetin and quercetin within the berries. According to Price et al. (1999), the individual flavonol conjugates in different apple varieties are not necessarily distributed similarly between the flesh and peel of the fruit. This could also partly explain the observation that the extractability of myricetin to cold-pressed juices varied depending on the berry. A higher percentage of myricetin was extracted to black currant juice than to crowberry juice. Also, differences in the structures of myricetin glycosides in these berries (Study **IV**) might affect the extractability.

The black currant and crowberry juices are consumed after dilution with water. Despite the reduction during the juicing, the flavonol content in diluted (1:4 v/v) steam-extracted black currant juice compares well with flavonol levels found in other fruit juices (Hertog et al. 1993b) (Figure 11). In the cold-pressed black currant and crowberry juices, the flavonol concentrations after dilution are higher than those reported for fruit juices (Hertog et al. 1993b) and compare well with the levels found in red wines (McDonald et al. 1998)



(Figure 11).

Figure 11. Content of flavonols in berry juices (Study **VII**), red wine (McDonald et al. 1998) and other fruit juices (Hertog et al. 1993b).

6.6.2 Effect of storage

Post-harvest storage

The effects of post-harvest temperature were studied in strawberry and black currant (Study **VII**). One day at room temperature resulted in lower total flavonol (Q, M, K) contents compared to storage in a refrigerator. However, the post-harvest temperature had no apparent effect on ellagic acid content (bound in ellagitannins) in strawberries. However, Gil et al. (1997) reported that the apparent content of free ellagic acid (not bound in ellagitannins) significantly increased in strawberries during 10 days of storage in a refrigerator. This increase was explained by the degradation of ellagitannins. The conclusion is that the practice of keeping berries at low temperature during post-harvest storage and transportation is advantageous also from the point of view of saving the flavonols, not to mention other obvious advantages.

Storage of the berries and berry products in a refrigerator or freezer

To our knowledge, no previous studies on the effects of storage by freezing on flavonol or ellagic acid contents in foods are available. The results obtained with five berries show that the effects of storage in a freezer or in a refrigerator on flavonols vary among berries and berry products (Study **VII**).

In strawberries and strawberry jam, the apparent amount of quercetin increased markedly during the 9 months of storage in a freezer and in a refrigerator (jam) (Study **VII**). The most probable explanation for these unexpected observations could be that quercetin in frozen strawberries and jam becomes more easily extractable and hydrolysable during storage. This might be due to degradation of cell structures during storage. Previously, an increase in flavonol contents during storage in a refrigerator has been reported for strawberries (Gil et al. 1997), pears (Amiot et al. 1995), and freeze-dried onion bulbs (Price et al. 1997).

Quercetin was well preserved in frozen red raspberries and black currants and in black currant juice, since almost no changes were observed during 9 months (Study **VII**). In contrast to quercetin, myricetin contents were significantly reduced in frozen black currants and black currant juice during 6 months of storage. According to our results, myricetin is more stable in intact berries than in juice during the storage in a freezer (Study **VII**).

Quercetin content decreased markedly in bilberries and lingonberries during 9 months of storage in a freezer, although in lingonberries the reduction was not statistically significant (Study **VII**). One explanation for this loss might be the low content of vitamin C in these two berries (Study **III**). The high content of vitamin C in black currant, strawberry and red raspberry (Study **III**) might protect quercetin during the storage in a freezer. In crushed lingonberries, the level of quercetin remained quite stable during 6 months of storage in a refrigerator, most probably due to the slowing down of the enzymatic reactions. However, quercetin was less stable in crushed lingonberries (50% loss) and in intact frozen lingonberries (40% loss) than in unpasteurised lingonberry juice (no losses) stored in a refrigerator for 9 months.

The content of ellagic acid in strawberries and red raspberries was significantly reduced during the 9 months of storage in a freezer (Study **VI**). Hexahydroxydiphenic acid may be released from ellagitannins during the storage and/or thawing leading to spontaneous formation of ellagic acid (Zafrilla et al. 1999). Thus, free ellagic acid may act as an antioxidant in berries due to its metal chelating capacity and ability to react with free radicals (Osawa et al. 1987) resulting in a reduction in

its total amount during storage and/or thawing. In strawberry jams, a decrease in ellagic acid content during the storage was not as apparent as in berries, possibly due to the fact that most of the (antioxidative) reactions of ellagic acid had already occurred during jam-making (Study **VI**).

6.7 Intake of flavonols and ellagic acid from berries

According to the annual consumption data in 1990, the main sources of flavonols in the average Finnish diet were onions (41%), tea (21%), berries (17%) and apples (15%) (Study **III**). However, the amount consumed in 1990 was much lower (10 kg/person) than that in 1998 (23 kg/person). It should be recognised that this might be partly due to a more accurate way of collecting the berry consumption data from households in 1998 (Statistics Finland, unpublished data) compared to that in 1990 (Statistics Finland 1993). Thus, according to the annual consumption data in 1998 (Statistics Finland, unpublished), the main sources of flavonols in the average Finnish diet are onions (36%) and berries (30%), followed by tea (17%) and apples (11%).

The estimated flavonol intake in Finland in 1998 was 17 mg/d which is almost 3 times the intake (6 mg/d) 50 years ago as reported by Hertog et al. (1995), and 4 times the flavonoid intake (3–4 mg/d) ca. 40 years ago as reported by Knekt et al. (1996, 1997). In the studies of Hertog et al. (1995) and Knekt et al. (1996, 1997), methods different from that in the present study were used for the collection of food consumption data. Moreover, data of the flavonol contents in most of the Finnish berries were not available in the studies of Hertog et al. (1995) and Knekt et al. (1996, 1997).

Strawberries and cloudberries alone provided 5.8 mg ellagic acid/d per person in Finland in 1990 (Study **VI**) and 8.7 mg/d in 1998 (Statistics Finland, unpublished). These are higher dietary intakes of ellagic acid (from berries and nuts) than that reported for adults (5.2 mg/d) in a Bavarian subgroup of the German National Food Consumption Survey (Radtke et al. 1998). In that study, berries (strawberry, red raspberry) and nuts (walnut) provided 38 and 54%, respectively, of the ellagic acid intake. The ellagic acid contents in walnuts, red raspberries and strawberries were 7400, 650 and 223 mg/kg, respectively (Radtke et al. 1998). In Finland, the total annual consumption of nuts (mainly peanuts) in 1997 was 0.92 kg/person (Balance Sheet for Food Commodities 1999). Thus, the unprocessed berries studied, together with nuts, apparently make the main contribution to the total dietary intake of ellagic acid in Finland. The dietary intake of ellagic acid calculated in Study **VI**

was over 4 times that of flavonols from berries (Study **III**). However, according to the consumption data in 1998 (Statistics Finland, unpublished data), the dietary intake of ellagic acid (8.7 mg/d) was over 2 times that of flavonols (3.4 mg/d) from berries. Because the ellagic acid and flavonol data were obtained from berries collected during one growing season only, our estimations must be considered tentative.

7 SUMMARY AND CONCLUSIONS

The aim of this study was to determine the content of flavonols and selected phenolic acids in wild and cultivated berries in Finland. The specific aims were to examine the main phenolic compounds in berries by using a semi-quantitative HPLC method (Studies **I**, **II**), the amounts of flavonols (quercetin, myricetin, kaempferol) and ellagic acid in berries by using quantitative HPLC methods (Studies **III**, **IV**, **VI**), the influence of cultivar, cultivation site and cultivation technique on flavonol and phenolic acid contents in selected berries (Study **V**), the effects of domestic processing and storage on flavonols and ellagic acid in selected berries (Studies **VI**, **VII**), and to estimate the contribution of berries to the dietary intake of flavonols and ellagic acid in Finland (**III**, **VI**).

The results of this series of studies can be summarised as follows:

1. The methods developed and validated were well suited for screening the main non-anthocyanin phenolic compounds in berries as well as for determining the contents of flavonols and ellagic acid in berries and berry products. HPLC-ESI-MS technique was valuable in the identification of flavonol aglycones and glycosides from berry extracts.

2. There were marked differences in the phenolic profiles of berries commonly consumed in Finland. Similarities could be seen within plant families and genera.

3. The flavonol content was high (>50 mg/kg) in 12 out of 25 berries, e.g. in cranberry, lingonberry, black currant and bilberry commonly consumed in Finland.

4. Varietal differences in the contents of phenolic compounds were observed among the six strawberry and four blueberry cultivars studied. Some regional differences in phenolic concentrations were observed in strawberries grown in Finland or in Poland and in blueberry cultivars grown in two different parts of Finland. No consistent differences between conventional and organic cultivation were detected in strawberries.

5. The ellagic acid content was high in arctic bramble, red raspberry, cloudberry and strawberry. Its content was reduced in the berries during jam-making and storage in a freezer.

6. Juicing or crushing the berries by common domestic methods resulted in considerable losses of flavonols, whereas jam-making caused a small loss of flavonols. Effects of freezing on quercetin varied in different berries. Myricetin and kaempferol were more susceptible than quercetin to losses during processing and storage of berries.

7. The unprocessed berries make a marked contribution (30%) to the total dietary intake of flavonols in Finland. Strawberries, cloudberries and red raspberries, together with nuts, make the main contribution to the total dietary intake of ellagic acid in Finland.

This study shows that berries commonly consumed in Finland are excellent sources of dietary flavonols and ellagic acid. The results also indicate that juicing or crushing the berries results in significant losses of flavonols. Despite this, diluted (1:4) cold-pressed berry juices have flavonol contents similar to that of red wine. The effects of storage in a freezer on flavonol contents of berries are variable. These results are important in assessing the contribution of flavonols and phenolic acids to the beneficial health effects of berries in the diet.

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