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Anja Primetta

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## *The Potential for Authenticity Analyses*

Publications of the University of Eastern Finland
*Dissertations in Forestry and Natural Sciences*

UNIVERSITY OF EASTERN FINLAND

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**ABSTRACT**

Part of the interest in health promoting phenolic compounds centres around their feasibility for use in authentication studies. The phenolic profiles of berries and fruits are mainly genetically determined and thus characteristic for each genus, species, even cultivars. The establishment of a botanical authentication specification demands a thorough knowledge of the chemical composition of the initial raw material and the natural ranges of variation. Furthermore, latitudinal differences in the phenolic profiles may be applicable in the determination of geographical authenticity. The objectives of this study were to use HPLC-DAD/ESI-MS in the analysis of phenolic compounds to characterize berries of some *Vaccinium* species (*Vaccinium* × *intermedium* Ruthe, *Vaccinium arctostaphylos* L.), to study the geographical variation (*Vaccinium myrtillus* L., *Vaccinium uliginosum* L.), and to evaluate the variation in phenolic profiles in *Vaccinium* genus and subsequently, the potential use of phenolic compounds in the authenticity studies of berry raw material and products.

Hybridization is a common phenomenon in *Vaccinium* populations, playing a role in the evolution of secondary metabolites. These include the anthocyanins which are phenolic compounds visible as the red and blue colours of berries. Lingonberries (*Vaccinium vitis-idaea* L.) contained only three cyanidin glycosides of anthocyanins. The anthocyanin profile of bilberries (*V. myrtillus*) was characterized by fifteen anthocyanidin glycosides. Hybrid berries (*V.* × *intermedium*) contained the same anthocyanins as bilberries, but the inheritance from lingonberry was showed in the predominance of cyanidin. The inheritance of proanthocyanidins was qualitatively closer to lingonberry but quantitatively to bilberry.

Bilberries contained the highest amount of anthocyanins with delphinidin and cyanidin being the major anthocyanidins detected. Bog bilberries (*V. uliginosum*) had qualitatively similar anthocyanin profile to bilberries but the total content was about 50% lower. The dominating anthocyanidins were delphinidin and malvidin. The berries of *V. uliginosum* contained an abundance of flavonol glycosides. The total anthocyanin content of Caucasian blueberries (*V. arctostaphylos*) was at the same level as that in bog bilberries. The most predominant anthocyanidin was delphinidin, followed by almost equal relative proportions of petunidin and malvidin. Anthocyanidin sambubiosides were typical for Caucasian blueberries and not detected in bog bilberries.

The geographical variation displayed similar latitudinal differences in the anthocyanins of bilberries and bog bilberries in Finland. The total anthocyanin, delphinidin and petunidin con-

tents in the berries of both species from the northern regions were significantly higher compared with those from the south. The relative proportion of malvidin was 12% higher in the southern bog bilberries than in their northern counterparts. The northern bog bilberries had significantly higher contents of the total flavonols, myricetin and quercetin glycosides compared with their southern counterparts. No geographical related effects could be detected in the profiles of anthocyanidins in Turkish bilberries.

The Finnish and Turkish bilberries did not significantly differ in the profile of their anthocyanidins, which may be considered as a consistent feature for botanical authentication. However, Finnish and Turkish origins were distinguished in the analysis of sugar moieties of anthocyanins. A logistic regression model based on glucoside proportions classified 96.7% of the samples correctly into their geographical region of origin. This may be used as a novel discriminating criterion for distinguishing bilberries among different geographical origins. To conclude, despite observed natural variation, the phenolic profiles were distinctive between *Vaccinium* species thus being applicable as a part for authenticity analyses in conjunction with the determination of other chemical compounds.

*Universal Decimal Classification: 547.972, 547.973, 581.19, 582.688.3, 634.73*

*CAB Thesaurus: secondary metabolites; phenolic compounds; phenylpropanoids; flavonoids; anthocyanins; flavonols; bilberries; blueberries; Vaccinium; Vaccinium myrtillus; Vaccinium uliginosum; Vaccinium vitis-idaea; hybrids; hybridization; geographical variation; cultivar authenticity; adulteration; identification; HPLC; mass spectrometry; regression analysis; Finland; Turkey*

*Yleinen suomalainen asiasanasto: fenoliset yhdisteet; flavonoidit; flavonolit; antosyaanit; marjat; mustikka; puolukka; hybridit; alkuperä; todentaminen; verifiointi; väärennökset; nestekromatografia; massaspektrometria; regressioanalyysi; Suomi; Turkki*

# *Preface*

This work was mainly carried out at the Department of Environmental Science, University of Eastern Finland (before 1.1.2010 University of Kuopio). I wish to express my sincere thanks and deep gratitude to my supervisors Docent Kaisu Riihinen, Ph.D. and Docent Laura Jaakola, Ph.D., for their guidance, support, confidence and endless patience throughout this work. I am also grateful to Docent Pirjo Kainulainen, Ph.D. for her contribution and supervision of a great part of this work. I also wish to thank the co-authors Professor Faik A. Ayaz Ph.D., Sema Hayirlioglu-Ayaz Ph.D. and Associate Professor Huseyin Inceer Ph.D.

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Finally, I am the most grateful to my friends, especially to Terttu, and to my family, my mother Mirja, my dear daughter and son, Anni and Antti for all the support and patience. I dedicate this thesis to my father who passed away in March 2012.

Kuopio, December 2013

Anja Primetta

**LIST OF ABBREVIATIONS**

| | |
|---|---|
| ANOVA | analysis of variance |
| C | carbon, e.g. in numbering C-1 |
| CHS | chalcone synthase |
| cv. | cultivar |
| DAD | diode array detector |
| DW | dry weight |
| ESI | electrospray ionization |
| FW | fresh weight |
| HCl | hydrochloric acid |
| HCOOH | formic acid |
| HPLC | high-performance liquid chromatography |
| MeCN/ACN | acetonitrile |
| mDP | mean degree of polymerization |
| MS | mass spectrometry |
| NaOH | sodium hydroxide |
| OH | hydroxyl |
| PAL | phenylalanine ammonialyase |
| PA(s) | proanthocyanidin(s) |
| RP | reversed-phase |
| SD | standard deviation |
| SM | secondary metabolite |
| sp. | species (singular) |
| spp. | species (plural) |
| ssp. | subspecies |
| UV | ultraviolet |
| Vis | visible |

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the data presented in the following articles, referred to by the Roman numerals (**I–V**).

**I** Lätti AK, Riihinen KR and Jaakola L. Phenolic compounds in berries and flowers of a natural hybrid between bilberry and lingonberry (V*accinium* × *intermedium* Ruthe). *Phytochemistry 72: 810–815, 2011*

**II** Lätti AK, Riihinen KR and Kainulainen PS. Analysis of anthocyanin variation in wild populations of bilberry (*Vaccinium myrtillus* L.) in Finland. *Journal of Agricultural and Food Chemistry 56: 190–196, 2008*.

**III** Lätti AK, Kainulainen PS, Hayirlioglu-Ayaz S, Ayaz FA and Riihinen KR. Characterization of anthocyanins in Caucasian blueberries (*Vaccinium arctostaphylos* L.) native to Turkey. *Journal of Agricultural and Food Chemistry 57: 5244–5249, 2009.*

**IV** Lätti AK, Jaakola L, Riihinen KR and Kainulainen PS. Anthocyanin and flavonol variation in bog bilberries (*Vaccinium uliginosum* L.) in Finland. *Journal of Agricultural and Food Chemistry 58, 427–433, 2010.*

**V** Primetta AK, Jaakola L, Ayaz FA, Inceer H and Riihinen, KR. Anthocyanin fingerprinting for authenticity studies of bilberry (*Vaccinium myrtillus* L.). *Food Control 30: 662–667, 2013.*



## AUTHOR'S CONTRIBUTION

The author has planned the major part of the study design of all of the studies. She was the main author in writing the manuscripts except for the article **I**. The author has the main responsibility of the laboratory work. The author has conducted all of the statistical evaluations.

# *Contents*

# 1 Introduction

The genus *Vaccinium* L. includes a wide range of berry species of economic importance. *Vaccinium* berries are consumed as fresh, dried or frozen food. The products include jams, pies, syrups, juices, soft drinks, wines and liqueurs. Berries are also commercially utilized in food supplements (e.g. capsules, extracts) or cosmetic formulations (e.g. soaps, shampoos, lotions, make-ups).

*Vaccinium* contains three major cultivated crops, blueberry, large cranberry, and lingonberry. Many wild *Vaccinium* species are used in their local geographical region or have potential as new crops. Cultivated or semicultivated blueberries consist of various species, e.g. *Vaccinium corymbosum* L., *Vaccinium angustifolium* Ait., *Vaccinium virgatum* Ait./*Vaccinium ashei* Reade, and their interspecific hybrids (Brevis et al. 2008). The highbush blueberry (*V. corymbosum*) is the most important cultivated berry crop produced in the United States, Canada, Europe, Australia, New Zealand, Chile and Argentina (Hancock et al. 2003, Boches et al. 2006). Wild bilberries (*Vaccinium myrtillus* L.) are valued in Europe and Asia. North American wild blueberries (*Vaccinium deliciosum* Piper, *Vaccinium membranaceum* Douglas *ex* Torr.) from the same section are in commercial terms the most extensively utilized (Barney 2003). Lingonberries (*Vaccinium vitis-idaea* L.) are collected predominantly from the forests, although the cultivated species has been domesticated recently. Lingonberry is important in northern Europe and Newfoundland (Vander Kloet & Dickinson 2009). Bog bilberries (*Vaccinium uliginosum* L.) have been traditionally collected for domestic use in Finland, Canada, and Japan (Fediuk et al. 2002, Hirai et al. 2010).

*Vaccinium* berries are known for their high levels and wide diversity of phenolic compounds (Johnson et al. 2010) which are classified as the plant secondary metabolites. The most predominant secondary metabolites in *Vaccinium* berries are phenolic acids and flavonoids (e.g. anthocyanins, flavonols). The distribution of the phenolic compounds varies within and between berry genera. These variations may confer the qualitative properties (the presence/absence of certain compound(s)) and/or quantitative features (contents and relative proportions of each of the compounds present) (Macheix et al. 1990).

The content of phenolic compounds is mainly under genetic control although they can be influenced by environmental factors. The long days and cool night temperatures in the northern latitudes appear to favour, for example, anthocyanin biosynthesis of strawberries in the north compared with the south (Hårdh &

Hårdh 1977). Recently, bilberries from seven Slovenian growing locations were differentiated by conducting a linear discriminant analysis with 27 parameters, including their anthocyanin content (Može et al. 2011).

Due to intense competition in today's marketplace, there are considerable economic incentives to sell or manufacture adulterated raw material or products. Adulteration is a serious global problem. A database (Food Chemical Codex, United States Pharmacopeia 2012) of food ingredient fraud issues was recently compiled. It includes 1305 records from publicly available references (Moore et al. 2012). Fruit juices, concentrates, jams, purees and preserves constituted one of the largest categories of recorded frauds. In addition, there were some reports of wine and liquor frauds. Previously, studies were published on mislabelled and adulterated bilberry extracts and capsules on the market (e.g. Penman et al. 2006, Cassinese et al. 2007).

Thus, there is a need to identify species along the entire production process, from the level of the starting material up to the verification of labelling claims in order to protect the consumer and to avoid unfair competition. Common adulteration modes include adulteration with substitutes such as synthetic chemicals or dilution and mislabelling about the botanical or geographical origin (Cordella et al. 2002). Due to the distinctive differences, the phenolic profiles of berries have been proposed as representing tools to be applied in authenticity studies.

Over the last ten years, consumers have shown a renewed interest in foods strongly identified with a particular place of origin (Luykx & van Ruth 2008). This has meant that more and more products with a distinct geographical identity are being marketed. Therefore, the determination of geographical origin has become increasingly important. For authentication analyses, the use of comprehensive data banks and a combination of composition (e.g. anthocyanin, volatile profiles) and isotopic data are generally recommended in order to obtain a characteristic fingerprint related to the geographical origin of the sample (Cordella et al. 2002, Peres et al. 2007, Luykx & van Ruth 2008).

This thesis examines phenolic compounds in the berries of selected *Vaccinium* species. The phenolic profiles of *V.* × *intermedium*, *V. myrtillus*, *V. vitis-idaea*, *V. uliginosum* and *V. arctostaphylos* were analysed with the emphasis on the anthocyanidin and flavonol glycosides. The aim was to evaluate genetic, geographical and some technique-analytical factors as sources of the variations in the profiles of phenolic compounds. The qualitative and quantitative phenolic marker compounds were compared between the berries of *Vaccinium* species and berries and fruits of other genera. The comparisons were made between the published data and with the results obtained in the present thesis. Finally, the potential of

using the phenolic (e.g. anthocyanin) profile as an authentication tool for *Vaccinium* berries is discussed.

# 2 Literature review

## 2.1 *Vaccinium* L.

*Vaccinium* L. is a morphologically diverse genus of about 450–500 species. The number of estimated species varies according to the classification philosophy being used by the taxonomists. *Vaccinium* plants are mainly terrestrial, less frequently epiphytic or epipetric, varying in form from lianas to trailing vines, shrubs and small trees (Vander Kloet 2004, Pedraza-Peñalosa & Luteyn 2011). New *Vaccinium* species are still recognized (Pedraza-Peñalosa & Luteyn 2011). The Vaccinieae tribe includes all the Ericaceae with epigynous berries that develop from inferior ovaries (Ballington 2001). Thus, in the botanical sense, they are called false or accessory fruits (Mauseth 1998).

The taxonomical relationships between selected *Vaccinium* species and some related berry species are represented in ***Figure 1***. However, the relationships of *Vaccinium* to the other members of the Vaccinieae, or the sectional relationships are not always clear (Kron et al. 1999, Powell & Kron 2002, Pedraza-Peñalosa & Luteyn 2011).

### 2.1.1 Geographical distribution

Wild *Vaccinium* species are widespread. *Vaccinium* is the only genus in the Vaccinieae tribe that occurs both in cold, temperate and tropical climates (Kron et al. 2002b, Hancock et al. 2003, Vander Kloet & Dickinson 2009). About 90 species of *Vaccinium* can be found in America (Pedraza-Peñalosa & Luteyn 2011). The greatest species diversity exists in south-eastern Asia with about 300 species (Powell & Vander Kloet 1997). *Vaccinium* populations are absent from Australia, New Zealand, Antarctica and most of Africa, but they do grow in Madagascar (Kron et al. 2002b, Vander Kloet & Dickinson 2009).

The distribution of individual wild species is narrow in many cases. A wild lowbush blueberry (*V. angustifolium*), for example, is localized to the extreme north-eastern United States and Maritime Provinces of Canada (Smith 2002). Certain species are endemic to islands, such as *Vaccinium padifolium* Smith which only grows in Madeira (Portugal) (Powell & Kron 2002). Five *Vaccinium* species naturally occur in northern Europe: *V. myrtillus*, *V. vitis-idaea*, *V. uliginosum*, *Vaccinium oxycoccus* L. and *Vaccinium microcarpum*

Turcz. *ex* Rupr. (Hämet-Ahti et al. 1992). A deciduous shrub, bilberry (*V. myrtillus*), is a common species in coniferous forests preferring shady places. It occurs also in the pine and oak woods and upland heaths extending from Europe to the Caucasus and northern Asia (Ritchie 1956). In western North America and central Japan there are a few disjunct populations (Vander Kloet & Dickinson 1999). Two subspecies (ssp.) has been delineated on the basis of the morphological differences: *Vaccinium myrtillus* ssp. *myrtillus* and *Vaccinium myrtillus* ssp. *oreophilum* (Rydb.) Love, Love and Kapoor (Tirmenstein 1990), but no scientific consensus has been reached on this issue.

A circumboreal lingonberry (*V. vitis-idaea*) is one the most widely distributed *Vaccinium* species. This evergreen dwarf shrub reaches the greatest abundance in pinewoods (Ritchie 1955a). It can naturally hybridize with *V. myrtillus*, forming fertile V. × *intermedium* Ruthe (Ritchie 1955b). *Vaccinium vitis-idaea* ssp. *minor* (Lodd.) Hultén, called partridgeberry, has been described as the only form of the species which occurs in North America (Ritchie 1955a, Kalt et al. 2008). Lingonberry and partridgeberry have been considered to be two subspecies (*V. vitis-idaea* ssp. *minor* and *minus*, respectively) or ecotypes of *V. vitis-idaea* (Vander Kloet, according to Kalt et al. 2008). A circumboreal bog bilberry (*V. uliginosum*) is native to North America, Europe and Asia. This deciduous plant grows in waterlogged upland heaths, bogs and in tundra (Jacquemart 1996, Brochmann et al. 2004). A southern related species with a disjunct distribution is a Caucasian blueberry (*V. arctostaphylos*) occurring mainly along the Black Sea from south-western Bulgaria through European and Asiatic Turkey to the Caucasus. It is a deciduous understory shrub growing in open beech or oak woods as well as in a variety of coniferous stands (Vander Kloet & Dickinson 1992).

Species distinction has been based e.g. on morphological, cytological, ecological, molecular and/or chemical data (Powell & Vander Kloet 1997, Vander Kloet & Dickinson 1999, Kron et al. 2002a, Powell & Kron 2002, Vander Kloet & Dickinson 2009). The polyploidy changes and overlapping morphologies have caused species distinction problems in the sections *Myrtillus, Cyanococcus* and *Oxycoccus* (Lyrene et al. 2003). Misidentifications have happened in the studies of wild species (Vander Kloet & Dickinson 1999). In the case of cultivated species, the genetic background of cultivars (cv.) is not always entirely known (Boches et al. 2006, Brevis et al. 2008). For example, the group of cultivars loosely referred to as "southern highbush blueberries" have a genetic base derived from seven *Vaccinium* species. Hybridization is one of the factors complicating species distinctions (Govindaraghavan et al. 2012).

### 2.1.2 Hybridization and introgression

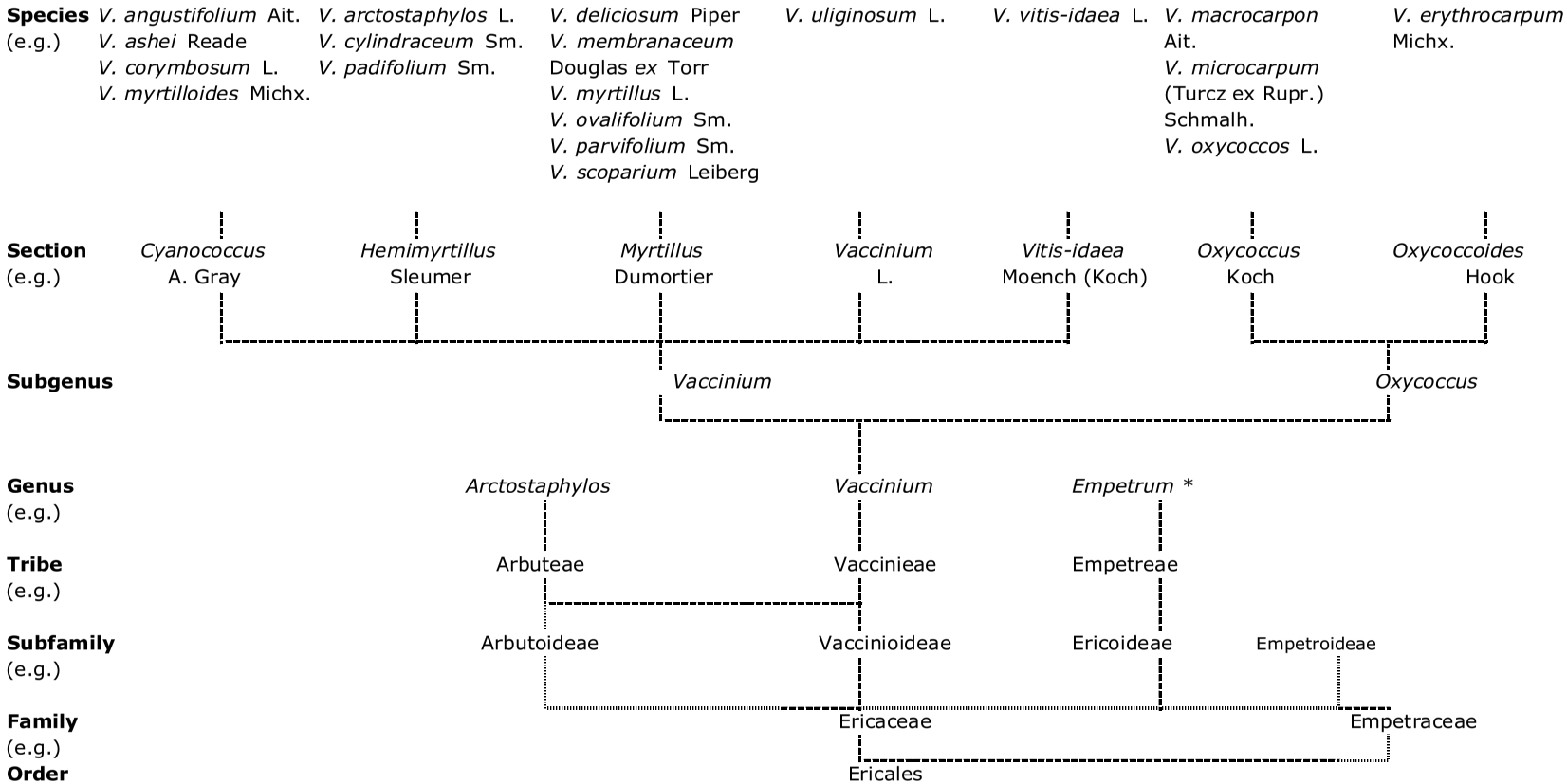
Hybridization and introgression occur among wild and cultivated *Vaccinium* species (Lyrene et al. 2003, Hancock et al. 2008). Hybridization may sometimes lead to introgression which can be defined as the stable integration of genetic material from one species into another through repeated backcrossing (Baack & Rieseberg 2007). They are frequent phenomena in wild plant populations (Orians 2000) playing an important role in the evolution of the taxonomic groups. As a result, new species with the same ploidy level or different ploidy levels may arise (Whitney et al. 2010).

There are closely related diploid and tetraploid species and a widespread ability of the diploid species to form fertile $F_1$ hybrids in the section Myrtillus (Lyrene et al. 2003). *V. corymbosum* cultivars (section Cyanococcus) have escaped, became feral and interbred with bog bilberries (*V. uliginosum*; section *Vaccinium*) (Vander Kloet & Dickinson 2009). *V. uliginosum* has produced fertile progeny after hybridization with *V. angustifolium* (Jacquemart 1996). All the polyploid *Cyanococcus* seem to be of multiple origins and active introgression between species is still ongoing (Hancock et al. 2008). Both *V. uliginosum* and *V. oxycoccus* consist of morphologically different diploid to hexaploid races. The tetraploids of both species are regarded to be among the most widespread (Jacquemart 1996, Brochmann et al. 2004, Vorsa & Polaschok 2005). The tetraploid race of *V. oxycoccus* likely carries genes from *V. macrocarpon* (Hancock et al. 2008).

Hybridization has been used in *Vaccinium* to develop new cultivars (Lyrene & Ballington 1986, Lyrene et al. 2003). The most important commercial cultivated fruit crops have been developed from the species and hybrid species of *Vaccinium* (Ballington 2001). These include the cultivated and semi-cultivated blueberries, derived from species in *Vaccinium* the section *Cyanococcus* and the large cranberry, developed from *V. macrocarpon* in the section *Oxycoccus*.

Hybridization will produce progeny that differs from the parents in many traits (e.g. morphological, chemical). The patterns of introgression may vary across the genome. Alleles at some loci do not introgress while others do so readily (Baack & Rieseberg 2007). The traits are not always just an intermediate between the parental taxa. An important consequence of hybridization is the formation of qualitative and quantitative variation in secondary compounds (Orians 2000). For example, a natural hybrid (*V.* × *intermedium*) possessed a richer phenolic composition in its leaves than either of the parental taxa (Hokkanen et al. 2009).

***Figure 1.*** *The taxonomic ranks and groups between some wild or cultivated Vaccinium berry species (Anderberg 1993, Ballington 2001, Kron et al. 2002a, USDA Grin 2013). Taxonomic relationships to some other common northern wild berry genera are presented as well. * Also placed in Empetraceae.*



## 2.2 Structures of phenolic acids and flavonoids

Phenolic compounds are a large group of plant secondary metabolites (SMs) including many tens of thousands identified structures (Cheynier et al. 2013). In this section, the structures and some general features of phenolic acids and flavonoids [anthocyanins, flavonol glycosides, flavan-3-ols, proanthocyanidins (PAs)] will be presented. The focus is on the structural forms found in berries and fruits (Macheix et al. 1990).

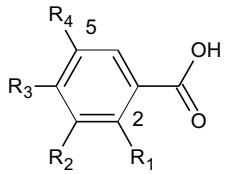
### 2.2.1 Phenolic acids

Soluble forms of phenolic acids are localized within the plant cell vacuoles (Naczk & Shahidi 2006) as a variety of conjugates. However, they are generally found mainly in insoluble (nonextractable, unextractable) forms linked through ester, ether or acetal bonds to structural components (e.g. lignin, polysaccharides) (Stalikas 2007, Pérez-Jiménez & Torres 2011).

*Hydroxybenzoic and hydroxycinnamic acids*

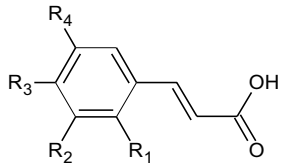
Phenolic acids are non-flavonoid polyphenolic compounds which can be further divided into two main types, hydroxybenzoic acid and hydroxycinnamic acid derivatives based on C1–C6 and C3–C6 backbones (Tsao 2010). The common hydroxybenzoic acids are *p*-hydroxybenzoic (4-hydroxybenzoic), protocatechuic (3,4-dihydroxybenzoic), vanillic (3-methoxy-4-hydroxybenzoic) and syringic (3,5-dimethoxy-4-hydroxybenzoic) acids. The corresponding hydroxycinnamic acids are *p*-coumaric (4-hydroxycinnamic), caffeic (3,4-dihydroxycinnamic), ferulic (3-methoxy-4-hydroxycinnamic) and sinapic (3,5-dimethoxy-4-hydroxycinnamic) acids, respectively. These derivatives differ in the patterns of hydroxylations and methoxylations in their aromatic rings (***Figure* 2**).

**a)**




| Common name | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| Gallic acid | H | OH | OH | OH |
| Syringic acid | H | $OCH_3$ | OH | $OCH_3$ |
| Vanillic acid | H | $OCH_3$ | OH | H |
| Protocatechuic acid | H | OH | OH | H |
| *p*-Hydroxybenzoic acid | H | H | OH | H |
| Gentisic acid | OH | H | H | OH |
| Benzoic acid | H | H | H | H |

**b)**




| Common name | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| Sinapic acid | H | $OCH_3$ | OH | $OCH_3$ |
| Ferulic acid | H | $OCH_3$ | OH | H |
| Caffeic acid | H | OH | OH | H |
| *p*-Coumaric acid | H | H | OH | H |
| Cinnamic acid | H | H | H | H |

***Figure 2.*** *Chemical structures of benzoic* ***(a)*** *and cinnamic acids* ***(b)****, and their common phenolic derivatives.*

Hydroxybenzoic acids are most commonly present in the conjugates with glucose (Herrmann 1989). Gallic acid may be conjugated as such, or as its dimer (ellagic), trimer (tergallic) and tetramer (gallagic) (Tomás-Barberán & Clifford 2000). It participates in the formation of hydrolysable gallo- and ellagitannins (Arapitsas 2012). Gallotannins consist of a polyol core (e.g. glucose) esterified with gallic acid(s) (Dai & Mumper 2010). Ellagitannins are characterized by one or more hexahydroxydi-

phenoyl (HHDP) moieties esterified to sugar, usually glucose (Landete 2011).

Soluble hydroxycinnamic acids occur mainly as two types of derivatives. First, the carboxyl group may be esterified with mono-/disaccharides or organic acids, for example, to yield caffeoylglucose or caffeoylquinic acid (El-Seedi et al. 2012). Caffeic acid esterified with hydroxyl (OH)-groups at C-5, C-3 and C-4 of quinic acid are called chlorogenic acid (5-*O*-caffeoylquinic acid), neochlorogenic acid (3-*O*-caffeoylquinic acid) and cryptochlorogenic acid (4-*O*-caffeoylquinic acid). Second, the phenolic oxygen may be glycosylated with sugar, for example as in a *p*-coumaric acid *O*-glucoside (Murkovic 2003). Insoluble hydroxycinnamic acids in the cell wall are generally covalently bound to cellulose, proteins and polysaccharides (e.g. pectin) via ester linkages (Kroon & Williamson 1999, El-Saadi et al. 2012).

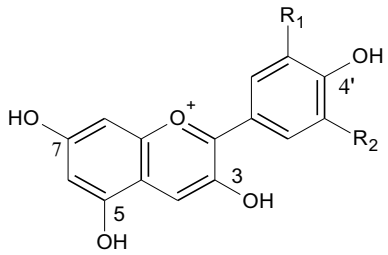
### 2.2.2 Flavonoids

Flavonoids, a class of phenylpropanoids, presents from low to high molecular weight compounds with an amount of 10 000 molecules known today (Cheynier et al. 2013). They all possess a basic $C_{15}$ ($C_6—C_3—C_6$) skeleton structure. In the narrow sense, the skeleton consists of two aromatic rings (A and B) connected by a three carbon bridge i.e. 1,3-diphenylpropane.

Variations in substitution patterns to the central heterocyclic ring C result in the major flavonoid subclasses (e.g. anthocyanidins, flavonols, flavan-3-ols). Substitutions to ring A and B give rise to different compounds within each subclass (Ignat et al. 2011). The differences in individual compounds result from the variations both in number and arrangement of the OH-groups (Rice-Evans et al. 1996). Further flavonoid modifications can include methoxylation and OH-group *O*-glycosylation (Aron & Kennedy 2008). Tannins, the relatively high molecular weight compounds are subdivided into hydrolysable and condensed tannins (PAs). The former are esters of gallic acid (See ***Chapter 2.2.1***) whereas the latter are polymers of polyhydroxyflavan-3-ols (Porter 1989).

Most flavonoids occur as glycosides. They can be present as *O*-glycoside or *C*-glycosyl compound with the former being the most common (de Rijke et al. 2006). One exception is the flavan-3-ols since their glycosides are less common (Aron & Kennedy 2008).

*Anthocyanins (=anthocyanidin glycosides)*




| Common name | $R_1$ | $R_2$ |
|---|---|---|
| Delphinidin | OH | OH |
| Cyanidin | OH | H |
| Petunidin | $OCH_3$ | OH |
| Peonidin | $OCH_3$ | H |
| Malvidin | $OCH_3$ | $OCH_3$ |
| Pelargonidin | H | H |

***Figure 3**. Chemical structures of anthocyanidins.*

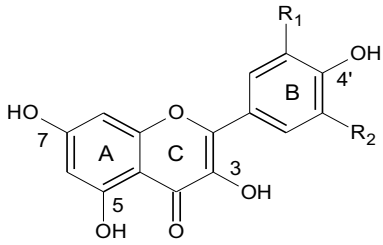
The basic structure of an anthocyanidin is that of the 3,5,7,4'-tetrahydroxyflavylium cation (***Figure 3***) (Brouillard 1982). Anthocyanidins contain a conjugated system of double bonds throughout the molecule giving rise to the orange, red, purple or blue colors. Anthocyanins are water soluble anthocyanidin glycosides i.e. consisting of the glycone and the aglycone moieties. A total of 19 types of anthocyanidins are known (Tanaka et al. 2008), but only six anthocyanidins are common in fruits (***Figure 3***) (Macheix et al. 1990). Since anthocyanidins are unstable in water and much less soluble than anthocyanins, glycosylation is assumed to confer solubility and stability onto the pigment (Brouillard 1982). The sugar acylation further improves anthocyanin stability (Brouillard 1982, Grotewold 2006).

In flowers and fruits, most anthocyanidins are linked to one or more sugars in a family- or species-specific manner (Brouillard 1982, Tanaka et al. 2008, Castañeda-Ovando et al. 2009). The sugar group is usually attached to the OH of C3-position of the ring C, followed by the OH of C5-position (de la Rosa et al. 2010). The attached sugar is mostly glucose, whereas other monosaccharides are galactose, arabinose, rhamnose and xylose (Miller et al. 2011). In the diglycosidic forms, the sugars may be linked either to positions 3 and 5 of the aglycone or as disaccharide to OH of C3-position (Macheix et al. 1990). The most common disaccharides en-

countered are rutinose (*O*-rhamnosyl-glucose), sambubiose (*O*-xylosyl-glucose), and sophorose (*O*-glucosyl-glucose) (Bueno et al. 2012). Occasionally, an extra sugar molecule of 3-diglycosides is bound to OH at position C5, or three sugar molecules are linked to the OH at position C3 to form anthocyanidin triglycoside. Nonetheless, triglycosides are not widely distributed in fruits (Macheix et al. 1990).

Acylated anthocyanins are found quite often in fruits. Glycosyl moieties of anthocyanins are modified by aromatic (hydroxycinnamic or hydroxybenzoic) and/or aliphatic (malonic, acetic, or succinic) acyl moieties (Tanaka et al. 2008).

*Flavonols*

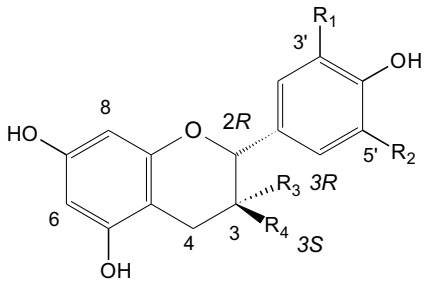



| Common name | $R_1$ | $R_2$ |
|---|---|---|
| Myricetin | OH | OH |
| Quercetin | OH | H |
| Laricitrin | $OCH_3$ | OH |
| Isorhamnetin | $OCH_3$ | H |
| Syringetin | $OCH_3$ | $OCH_3$ |
| Kaempferol | H | H |

***Figure 4.*** *Chemical structures of flavonols*

Flavonols (i.e. 3-hydroxyflavones) are characterized by their double bond between C2 and C3 and by the presence of an OH-group in the C3-position (Murkovic 2003) (***Figure 4***). The preferred glycosylation site is the OH at the C3-position, and less frequently, at the C7-position (Aherne & O'Brian 2002). The most common sugar moieties are galactose, glucose, arabinose, rhamnose and xylose (Macheix et al. 1990). There may also be glucuronides and in that respect they differ from the anthocyanins.

There are two types of diglycosides. Two sugars may be attached to the OH of the same carbon (always in C3) or to the OHs of two different carbons. Of these, 3,5-diglycosides are more common in fruits than 3,7-diglycosides. Branched triglycosides seem to be less typical for flavonols than for anthocyanins (Macheix et al. 1990).




| Common name | Stereochemistry | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Pro-anthocyanidin |
|---|---|---|---|---|---|---|
| (+)-Gallocatechin | 2,3-*trans*-2R,3S | OH | OH | H | OH | Prodelphinidin |
| (-)-Epigallocatechin | 2,3-*cis*-2R,3R | OH | OH | OH | H | Prodelphinidin |
| (+)-Catechin | 2,3-*trans*-2R,3S | OH | H | H | OH | Procyanidin |
| (-)-Epicatechin | 2,3-*cis*-2R,3R | OH | H | OH | H | Procyanidin |
| (+)-Afzelechin | 2,3-*trans*-2R,3S | H | H | H | OH | Propelargonidin |
| (-)-Epiafzelechin | 2,3-*cis*-2R,3R | H | H | OH | H | Propelargonidin |

***Figure 5.*** *Chemical structures of flavan-3-ols. Most natural flavan-3-ols are 2R- isomers.*

There is no double bond between C2 and C3, and no C4 carbonyl in the C-ring of flavanols unlike in most flavonoids (***Figure 5***). This and the hydroxylation at C3 allows flavanols to have two chiral centers in the molecule (on C2 and C3) i.e. four possible diastereoisomers. As an example, catechin is the isomer with a *trans*-configuration and epicatechin is the form with *cis*-configuration. Each of these two configurations contains two stereoisomers, i.e., (+)-catechin, (−)-catechin, (+)-epicatechin and (−)-epicatechin. The most common structural units are two epimers, (−)-epicatechin and (+)-catechin (Dixon et al. 2005).

Flavan-3-ols (i.e. flavanols) occur as monomers and as structural units in oligomeric or polymeric PA chains (condensed tannins) (Porter 1989, Aron & Kennedy 2008). In the B-type PA chains, flavan-3-ol units are linked through carbon-carbon C4→C8 or C4→C6 bonds (Dixon et al. 2005). In other words, the C4-position of the upper unit (extension unit) is connected to the lower units (terminal unit) C8 or C6. The less common A-type of PAs have a more rigid conformation due to presence of two interflavan linkages. One of these is the same as is found in the B-type (C4→C8 or C4→6). The second bond is the linkage between the position C2 of the upper unit and ether C5 or C7 of the lower unit (Aron & Kennedy 2008).

## 2.3 Biosynthesis of phenolic compounds

Secondary metabolism has been regarded as having developed early in the evolution of land plants (Boudet 2007, Wink 2008). Some of the genes involved have been postulated to have been inherited from their bacterial symbionts or as a result of duplication of the essential genes of primary metabolism (Stafford 1991, Boudet 2007, Wink 2008, Sing et al. 2010, Vogt 2010). Their biosynthesis has evolved in response to changes in the environment (Cooper-Driver & Bhattacharya 1998, Pichersky & Gang 2000) such as in use as sunscreens when being adapting to land from an aquatic environment (Boudet 2007, Cheynier et al. 2013). The phenolic compounds have become progressively enriched to provide specific adaptations for different plant families (Boudet 2007).

***Figure 6*** shows some branch points in the biosynthesis of phenolic compounds. Phosphoenolpyruvate (PEP), from glycolysis, and erythrose 4-phosphate (E 4-P), from the pentose phosphate pathway, are converted into chorismic acid (Herrmann & Weaver 1999, Tzin & Galili 2010). The plant shikimate pathway is the entry point in the biosynthesis of phenyl propanoids the intermediates of which also serve as starting points for a wide variety of compounds. For example, the dehydroquinic acid (DHQ) can be converted to quinic acid, the precursor to the chlorogenic acid (Herrmann & Weaver 1999). Gallic acid is most likely formed via dehydrogenation of 5-dihydroshikimate (Vogt 2010).

Phenylalanine ammonialyase (PAL) catalyzes the deamination of phenylalanine to *trans*-cinnamic acid. Tyrosine is similarly deaminated by tyrosine ammonialyase (TAL), or at lower efficiency by PAL, to form *p*-coumaric acid (de la Rosa et al. 2010). It is these deaminations that initiate the general phenylpropanoid pathway.

Phenolic acids are produced in plants via shikimic acid through the phenylpropanoid pathway and as breakdown products of lignin and cell wall polymers (Mandal et al. 2010). Hydroxylation of the aromatic ring at C4-position by cinnamate 4-hydroxylase (C4H) generates *p*-coumaric acid. The addition of a second OH to the C3-position yields a caffeic acid, whereas *O*-methylation of OH produces ferulic acid (***Figures 2*** and ***6***). The biosynthesis of hydroxycinnamic acid derivatives is based on two main pathways: the formation of CoA esters and then transesterification with hydroxyacids (e.g. quinic, malic, shikimic), or directly from hydroxycinnamic acid for glucose derivatives (Macheix et al. 1990).

The flavonoid biosynthesis pathway (***Figure 7***) starts with catalysis of the synthesis of tetrahydroxychalcone (THC) (i.e. naringenin chalcone or chalcone) from one molecule of 4-coumaroyl-

CoA and three molecules of malonyl-CoA (Sing et al. 2010). This reaction is catalysed by chalcone synthase (CHS). THC is rapidly and stereospecifically isomerized into the colorless (2S)-naringenin by chalcone isomerase (CHI) (Tanaka et al. 2008).

Ring B and the central three-carbon bridge forming the C-ring originate from phenylalanine. The A-ring is derived from three units of malonyl CoA (de la Rosa et al. 2010). Naringenin is hydroxylated at the 3-position by flavanone 3-hydroxylase (F3H) to yield dihydrokaempferol. Flavonoid 3′-hydroxylase (F3′H) catalyzes the hydroxylation of dihydrokaempferol to form dihydroquercetin. Dihydromyricetin may be formed from both dihydrokaempferol and dihydroquercetin via the action of flavonoid 3′5′-hydroxylase (F3′5′H). The dihydroflavonol 4-reductase (DFR) catalyzes the reduction of 3-OH flavanones (dihydroflavonols) to colorless leucoanthocyanidins (flavan-3,4-diols). They are converted to anthocyanidins by anthocyanidin synthase (ANS), an enzyme which is also called leucoanthocyanidin dioxygenase (LDOX) (Tanaka et al. 2008). 3′-*O*-methyltransferase (3′-OMT) converts quercetin into isorhamnetin and myricetin to laricitrin, respectively. Laricitrin is converted to syringetin by methylation 5′-*O*-methyltransferase (5′-OMT). Similarly, delphinidin is converted into petunidin and cyanidin into peonidin by 3′-OMT. Finally, 5′-*O*-methylation of petunidin produces malvidin.

Anthocyanidins are stabilized by UFGT (UDP glucose-flavonoid 3-*O*-glucosyltransferase) (Vogt 2010, Routray & Orsat 2011). Glycosylation is thought to be the last step in anthocyanin biosynthesis although the possibility of conversion of dihydroflavonol 3-glucoside to anthocyanidin 3-glucoside cannot be excluded (Macheix et al. 1990).

In all, the various transcription factors control different sets of structural genes (Grotewold 2005). The transcription factors that directly regulate the expression of the structural flavonoid pathway genes have been isolated from many species. The regulation occurs through interactions with R2R3 MYB transcription factors, MYC like basic helix-loop-helix (bHLH) and WD40-repeat proteins (Allan et al. 2008). Thus, the chemical diversity in the plant kingdom is closely linked to the evolution of the transcription factors that control the corresponding pathways.

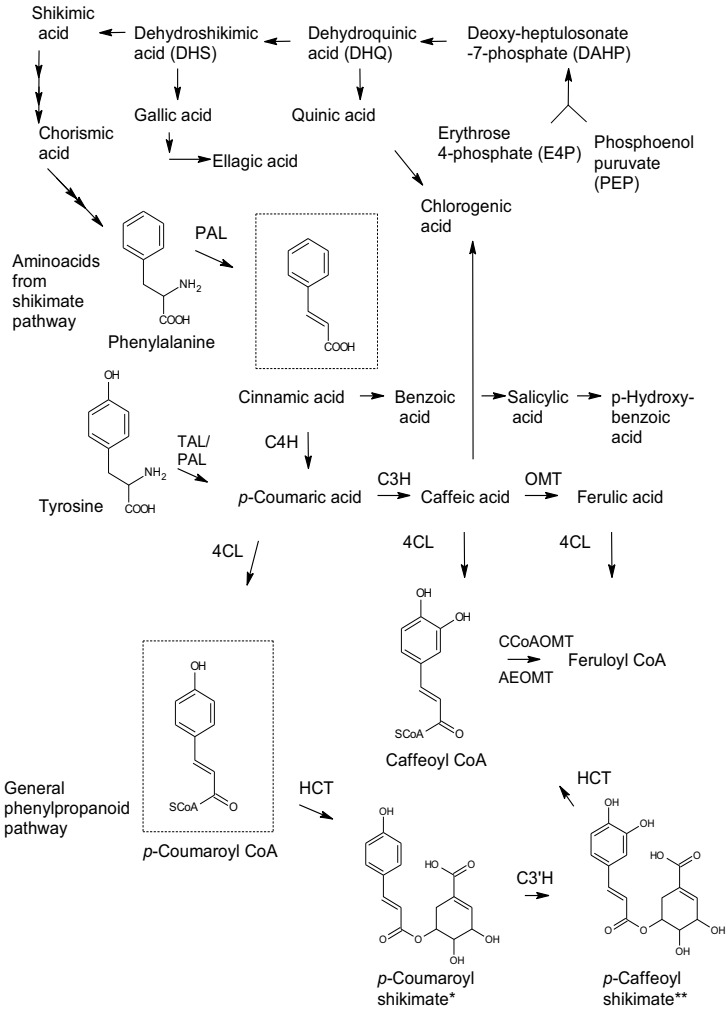



***Figure 6.*** *The general view of shikimic acid and phenylpropanoid pathways leading to phenolic acids and their derivatives in plants.*

* *p*-Coumaroyl quinate and **caffeoyl quinate may be also formed. Modified from: Herrmann & Weaver 1999, de la Rosa et al. 2010, Mandal et al. 2010, Umezawa 2010. Enzyme abbreviations: AEOMT (hydroxycinnamic acid/hydroxycinnamoyl CoA esters *O*-methyltransferase), CCoAOMT (caffeoyl CoA 3-*O*-methyltransferase), C4H (cinnamate 4-hydroxylase), C3H (*p*-coumarate 3-hydroxylase), C3'H (*p*-coumaroyl shikimate/quinate 3-hydroxylase), 4CL (4-coumarate CoA ligase), HCT (hydroxycinnamoyl CoA: shikimate/quinate hydroxycinnamoyl transferase). OMT (*O*-methyltransferase), PAL (phenylalanine ammonia lyase), TAL (tyrosine ammonia lyase).

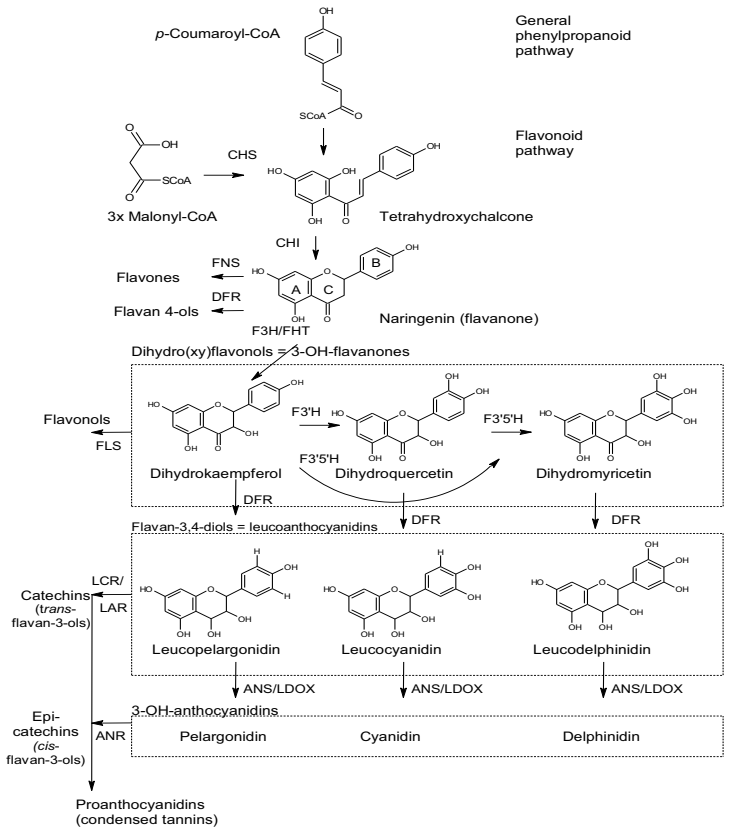



***Figure 7.*** *Simplified representation of the main flavonoid pathway.*

Enzyme abbreviations: ANR (anthocyanidin reductase), ANS/LDOX (anthocyanidin synthase/leucoanthocyanidin dioxygenase), CHI (chalcone isomerase), CHS (chalcone synthase), DFR (dihydroflavonol 4-reductase), FNS (flavone synthase), F3H/FHT (flavanone 3-hydroxylase/flavanone hydroxylase), F3′H (flavonoid 3′′-hydroxylase), F3′5′H (flavonoid 3′5′ hydroxylase), LCR/LAR (leucoanthocyanidin reductase).

## 2.4 Roles of phenolic compounds in flowers and berries

Phenolic compounds have a variety of functions in plants such as protection from UV-radiation, pathogen invasion, cold temperature, pollinator attraction, pigmentation, and playing an essential role in reproduction (Dixon & Paiva 1995, Winkel-Shirley 2002, Grotewold 2006, Mandal et al. 2010, Singh et al. 2010).

The biosynthesis of phenolic compounds is tissue specific and developmentally regulated in different plant organs (Pichersky & Gang 2000, Vogt 2010, Miller et al. 2011). Glycosylation affects

plant signalling. For example, the flavonol aglycones kaempferol and quercetin are required for pollen germination whilst the corresponding glycosides are inactive (Jones & Vogt 2001).

European *Vaccinium* species display some variation in floral traits related to their pollination ecology (Jacquemart 2003). They are still rather similarly colored. Bee-pollinated flowers in temperate latitudes are generally blue or yellow, rarely red (Miller et al. 2011). Light colored bilberry (*V. myrtillus*) flowers with its green to pink–purple hue contains a small amount of cyanidin glycosides (Ritchie 1956, Riihinen et al. 2008) similarly to blueberry (*V. corymbosum* × *V. angustifolium*) flowers (Riihinen et al. 2008). In the tropics, blueberry (*Vaccinium* spp.) flowers that hummingbirds visit tend to be brightly colored (red to orange). The flowers of *V. floribundum* represent an exception. This common blueberry species in South America has white-pinkish corollas and is bee pollinated (Luteyn 2002).

The hydroxycinnamic acid content in the blueberry flower is over three times higher than in the bilberry flower (Riihinen et al. 2008). The flower of bilberry contains much higher amounts of anthocyanins than that of blueberry cultivar whereas the quercetin content is clearly lower. Myricetin has not been found in the flowers of these species, but kaempferol can be detected in the flower of blueberry (Riihinen et al. 2008).

Bilberry and blueberry flowers contain considerably higher levels of hydroxycinnamic acids than the berries (Riihinen et al. 2008). Phenolic acids serve as precursors for a wide array of secondary metabolites such as salicylates, coumarins, lignin and flavonoids. The next most abundant molecular species are flavonols, followed by PAs, in both bilberry and blueberry flowers. The major flavonol of bilberry, quercetin, has its highest levels in the flower with about one third of that concentration present in small-sized green fruits (Jaakola et al. 2002).

The content of the PAs decreases at the same time that the anthocyanins begin to accumulate at the beginning of the ripening period. Similar to the situation with other berries from the section *Myrtillus*, the flesh of bilberry is fully pigmented. The pigments are missing in the pulp of berries from the sections *Cyanococcus* (e.g. *V. corymbosum* × *V. angustifolium)* and *Vaccinium* (*V. uliginosum*) (Riihinen et al. 2008). The levels of flavonols in bilberries and cranberries were more constant over the period of fruit development (Jaakola et al. 2002, Vvedenskaya & Vorsa 2004) displaying significant differences only in the early phases of coloration (Castrejón et al. 2008). PAs, the end products of the flavonoid pathway, confer an astringent taste. They are believed to provide protection from too early feeding or possibly combat against fun-

gal or bacterial pathogens (Cooper-Driver & Bhattacharya 1998, Dixon et al. 2005, Singh et al. 2010).

## 2.5 Analyses of phenolic compounds

High-performance liquid chromatography (HPLC), coupled with UV/Vis and mass spectrometric (MS) detectors, is the most widely used analytical technique in the quantification and tentative identification of phenolic compounds (Merken & Beecher 2000, Dai & Mumper 2010). NMR-analysis provides an accurate structural identification.

In the following section, the stages of the phenolic analyses have been reviewed especially from the point of view of authenticity studies i.e., the effects of some technique-analytical factors on the qualitative and quantitative phenolic profiles. There can be different ways to perform authenticity studies e.g. from the analysis of the reference samples for the databank establishment to the quality control analyses at the various stages of the production process. The procedure appropriate needs to be selected according to the authentication case and its objectives. The detection of specific phenolic markers may be sufficient in certain cases, if they are unique to some plant species (See ***Table 1***), thus revealing adulterant. However, in most cases the quantitative analysis (See **Chapter 2.6.3**) is needed (Cordella et al. 2002, Carcea et al. 2009, Smillie & Khan 2010).

### 2.5.1 Collection, preparation and extraction of samples

A well planned and performed sample preparation (e.g. collection, storage, pretreatment) is an essential part of all chemical analyses. The first part involves the collection of samples. *Vaccinium* populations can contain high levels of genetic diversity (Kreher et al. 2000). In sampling, it should be taken account that wild *Vaccinium* stands are composed of the mixtures of genotypes (i.e. genetic individuals, genets, clones), where one genet consists of a collection of ramets forming a variable sized patch in a field (Albert et al. 2005).

The correct identification of species should be verified. The species distinction problems have occurred especially in the sections *Myrtillus, Cyanococcus* and *Oxycoccus* (***Figure 1***) (Lyrene et al. 2003). The cooling (with liquid nitrogen/ice) of samples should be conducted as soon as possible to slow or stop the degradation processes. Samples are often dehydrated to a constant weight in order to ensure a reliable assay. Dehydration reduces also enzyme activities thus minimizing degradation of phenolic compounds (Tsao 2010). The accurate documentation about the plant material

and the location of the collection site is vital [e.g. date, the location of the collection site, i.e. global positioning satellite position (GPS-coordinates), habitat information, organoleptic characteristics and processing steps used (e.g. drying method, time, temperature)] (Smillie & Khan 2010).

Solvent extraction is the most commonly used technique as a sample pre-treatment for phenolic analysis (de Rijke et al. 2006). The yield depends on the type of solvents which have varying polarities, extraction times, temperatures, sample-to-solvent ratios, particle sizes and the sample matrices (Khoddami et al. 2013). Ultrasonic radiation (frequencies > 20 kHz) or microwaves (frequencies 300 MHz–300 GHz) may be used with an appropriate solvent to facilitate the extraction (Khoddami et al. 2013). Pre-concentration may require the use of a solid-phase extraction (SPE) technique (Santos-Buelga & Williamson 2003).

Aqueous alcoholic (MeOH/EtOH) solvents are generally used for the extraction of low molecular weight phenolic glycosides. MeOH/$H_2O$-water tends to be better for the extraction of low molecular weight PAs whereas acetone/$H_2O$ is preferred for higher molecular weight PAs (Arapitsas 2012).

Acetone/ $H_2O$ (in ratios of 60–70:30–40) has been found to be the optimal solvent for the extraction of PAs from blueberries (Naczk & Shahidi 2004). Wu et al. (2004) detected six artificial anthocyanins with acetone/$H_2O$/acetic acid (70:29.5:0.5) extraction of black currants. Although the compounds only occurred in minor quantities, acetone was not recommended for anthocyanin extraction.

The anthocyanins are stable only in acid media at room temperature (Brouillard 1982). Weak organic acids (e.g. HCOOH) or low concentrations of strong acids (<1% HCl) are recommended if one wishes to obtain the best yield during anthocyanin extraction (Dai & Mumper 2010). Anthocyanins may be dissolved in an eluent used in reversed-phase (RP) separation such as in a mixture of 86:14 (5% aqueous HCOOH:MeOH) (Wang et al. 2000); or 87% MeCN/3% $H_2O$/10% HCOOH (Müller et al. 2012) to enhance resolution in the subsequent HPLC-analysis.

High concentrations of strong acids may be deleterious for acylated anthocyanins (Tura & Robards 2002). Moreover, the acidic conditions in extraction may lead to an underestimation of the oligomeric and polymeric species of PAs due to their conversion to anthocyanidins (Tura & Robards 2002).

Antioxidants (e.g. BHT, *tert*-butylhydroquinone, ascorbic acid) or EDTA have been added to the extraction medium to prevent oxidation (Häkkinen et al. 1999a, Häkkinen et al. 1999b, Sellappan et al. 2002, Mattila et al. 2006, Naczk & Shahidi 2006, Arapitsas 2012). However, the use of ascorbic acid during flavanol extrac-

tion may produce a certain degree of degradation of PAs (Santos-Buelga & Williamson 2003). It is advisable to use temperatures below 30°C to prevent breakdown of heat-sensitive anthocyanins (Mazza et al. 2004).

*Hydrolysis*

Methods, based on either pure extraction or extraction with hydrolysis (e.g. hydroxycinnamic acid analysis), have been used in authenticity analyses (e.g. Rapisarda et al. 1998, Cassinese et al. 2007). Hydrolysis means breaking the bonds by chemical or enzymatic means. Soluble phenolic compounds may be directly quantified from the supernatants of aqueous–organic extractions or after hydrolysis (Häkkinen et al. 1998, Sellappan et al 2002, Mazza et al. 2004). Esterified soluble phenolic acids may be measured in the supernatants after performing alkaline hydrolysis (Pérez-Jiménez & Torres 2011). Acid hydrolysis targets glycosidic bonds. Insoluble (nonextractable, bound) phenolic acids, which remain in the residues from the extraction and hydrolysis, require the hydrolysis treatment of the extraction residue in order to release phenolic acids that are bound to the cell walls (either to polysaccharides, lignins, or proteins) (Pérez-Jiménez & Torres 2011).

The recoveries have been variable after hydrolysis. Structures may partly degrade and some information will be lost. Thus, hydrolysis is not generally recommended e.g. for anthocyanin fingerprinting analyses (e.g. Cassinese et al. 2007). Mattila & Kumpulainen (2002) found that phenolic acids were more stable during alkaline as compared to acid hydrolysis. The loss under acid conditions varied from 15% to 95% for *O*-coumaric (Robbins 2003). The recovery for *p*-coumaric acid varied from 42% (strawberry) to 70% (black currant) (Häkkinen et al. 1998). Gallic acid may be unstable under alkaline conditions (Mattila & Kumpulainen 2002). Chlorogenic and other caffeoylquinic acids may be hydrolyzed to caffeic acid. A combination of acid and alkaline hydrolysis may be necessary in order to achieve the analysis of all forms of nonextractable phenolic acids present (Pérez-Jiménez & Torres 2011). Delphinidins were found to be the least stable of the bilberry anthocyanidins under acid hydrolysis conditions (Zhang et al. 2004). It has been reported that hydrolysis conditions may destroy flavonols to some extent (Häkkinen et al. 1999b, Merken & Beecher 2000).

### 2.5.2 Analyses by spectrophotometric and HPLC–chromatographic techniques

Spectrophotometric methods have been developed for the estimation of the content of the phenolic compounds. The most commonly used method is pH-differential method for the estimation of total anthocyanins based on the characteristic behaviour under acidic conditions (Naczk & Shahidi 2004). However, it lacks specificity and is not suitable for identification berry and fruit extracts of different species (Cassinese et al. 2007).

Analysis of phenolic compounds in HPLC is usually carried out in the RP columns, most commonly on C18–bonded silica (4.6 × 100–300mm) (de Rijke et al. 2006, Dai & Mumper 2010). The ideal resolution of phenolic compounds is achieved by adjusting the ratio of aqueous acid/organic solvent, the acid strength, the choice of organic solvent and the column, temperature and flow rate (Snyder et al. 1997, Stalikas 2007). Aqueous mixtures of ACN and MeOH are the most common mobile phases utilized (Khoddami et al. 2013). An acid modifier (e.g. HCOOH, acetic acid) is necessary to suppress the ionization of the phenolic OH-groups. This means that sharper peaks will be obtained with peak tailing minimized in the chromatograms (Santos-Buelga & Williamson 2003, Dai & Mumper 2010). Normal phased (NP) columns can be useful for the separation of non-polar or weakly polar phenolics such as polymethoxylated flavones, flavanones and flavanols, according to their degree of polymerization (Santos-Buelga & Williamson 2003, Hellström et al. 2009).

The outflow from HPLC is connected to a detector for the identification and quantification of the separated compounds. The quantification of phenolic compounds is usually performed by UV/Vis spectrophotometry, using the appropriate absorption wavelengths depending on which class of flavonoids the analysis is focussing. Other detectors (e.g. fluorometric, electrochemical coulometric) are less commonly utilized in flavonoid analyses (Naczk & Shahidi 2006). Phenolic acids, flavonols and anthocyanins are generally quantified at 280, 360 and 520 nm, respectively (Mazza et al. 2004, Corradini et al. 2011). The concentrations of anthocyanins and flavonols, or their aglycones after hydrolysis, have been determined by constructing a calibration plot using external and internal standards (Sellappan et al. 2002, Taruscio et al. 2004, Koponen et al. 2007, Müller et al. 2012, Uleberg et al. 2012).

### 2.5.3 Structural characterization by RP-HPLC-DAD-ESI-MS

HPLC with DAD detection is a satisfactory tool in distinguishing various flavonoid sub-classes from each other (de Rijke et al. 2006). Generally, phenolic acids are eluted from RP columns

according to decreasing polarities i.e. the loss of polar OH-groups and the presence of $CH_3$-groups increase the retention time (Stalikas 2007). Retention times together with the UV/Vis spectral data can assist in the recognition of aglycones and help identify the types of glycosylation or acylation (Hong & Wrolstad 1990). MS detectors coupled to HPLC (HPLC-MS/MS) have been currently employed in tentative structural characterizations.

*UV/Vis detection*

The obtained on line-UV/Vis-spectra can provide information on the nature of the aglycone, glycosylation and possibile acylation. Phenolic acids with the benzoic acid carbon framework have their absorption maxima between 200–290 nm. The only exception is gentisic acid, which has an absorbance that extends to 355 nm. Caffeic acid derivatives have maxima in the regions 328–332nm and 240–245nm, with a shoulder in the range 290–300nm (Santos-Buelga & Williamson 2003). *p*-Coumaric acid derivatives have their primary maximum in the range 310–315nm (Santos-Buelga & Williamson 2003). The absorption spectrum of both *trans*-ferulic acid and sinapic acid is very similar to that of *trans*-caffeic acid. *O*-glycosidic bond caused a hypsochromic effect (shift to shorter wavelength i.e. blue shift) in the absorption maxima compared to the aglycone (Williams & Fleming 1987, Määttä et al. 2003). Bathochromic effect (shift to longer wavelength i.e. red shift) of 4 nm was found in the case of esterification of the carboxylic functions (Williams & Fleming 1987, Määttä et al. 2003). If hydroxycinnamic acids are conjugated to anthocyanins, it can be recognized by a third absorption band at about 310–330 nm (Strack & Wray 1989).

Spectral characteristics of anthocyanins provide very useful qualitative information (Castañeda-Ovando et al. 2009). The wavelength maximum in the visible range is related to the hydroxylation pattern of each anthocyanidin: derivatives of delphinidin can be distinguished from derivatives of cyanidin, which in turn can be distinguished from pelargonidin derivatives (Hong & Wrolstad 1990, Mazza et al. 2004). Anthocyanins with 3-glycosides and 3,5-diglycosides each have unique UV/Vis spectra. The former compounds absorb stronger around 400–460nm as compared to the 3,5-diglycosides (Dossett et al. 2008).

Flavonols exhibit two major absorption bands in the UV/Vis region. Band I in the 320–385 nm range represents the B ring absorption, whereas Band II in the 250–285 nm range is attributable to the A ring absorption (Rice-Evans et al. 1996). An increase in the numbers of OH-groups induces a shift to a longer wavelength. *O*-methylation and glycosylation evoke hypsochromic effects (Rice-Evans et al. 1996). The composition of mobile phase slightly affects

the on line-spectral characteristics. Thus, direct comparisons between the absorption maxima values published in the literature may be inappropriate (Hong & Wrolstad 1990). For example, increasing solvent polarity influences the spectral characteristics of anthocyanins by decreasing the visible $\lambda_{max}$.

*MS-detection*

HPLC combined with DAD and MS are commonly used for structural characterization of phenolics together with the help of standards and reference data (Naczk & Shahidi 2004, de Rijke et al. 2006). Mass spectrometer can detect charged molecular ions and fragments separated according to their molecular weights (Andersen & Markham 2006).

ESI (electrospray ionization) and APCI (atmospheric pressure chemical ionisation) seem to be the most useful ionisation techniques for the characterization of flavonoids. They are soft ionization techniques that cause moderate fragmentation of the molecule (Andersen & Markham 2006). ESI technique permits the detection of the molecular ion, either as a protonated molecule $[M + H]^+$, adduct $[M + Na]^+$, or as a deprotonated molecule, $[M - H]^-$ (Andersen & Markham 2006). In a more detailed structural characterization, fragmentation is induced by using collision induced dissociation (CID). During a CID MS-MS analysis, precursor ions extracted in the first analyser collide with atoms of an inert gas (e.g. helium). Then the ionized fragments created are separated in the second analyzer (Andersen & Markham 2006).

The ESI-MS/MS achieves very low limits of detection (ng/ml) (Valls et al. 2009). Ion-trap mass spectrometers (ITMS), with their high sensitivity in the scanning mode and the ability to perform MS/MS experiments, are well suited for identification purposes. They can carry out sequential fragmentations first of the parent molecular ion and second of the daughter ions (Valls et al. 2009). The fragmentation pathways are still largely independent of the ionization mode and the type of mass analyzer (triple quadrupole or ion trap) used (de Rijke et al. 2006). Compared with ESI, APCI produces more fragment ions due to the harsher processes (Andersen & Markham 2006).

Co-eluting flavonoids can be scanned separately using the MS detector. HCOOH is one of the recommendable modifiers (Valls et al. 2009). The elution conditions have been found to be critical in order to achieve good sensitivity (Cuyckens & Claeys 2004). The recommended percentages of organic/weak acids for ESI are 0.3–1% HCOOH (Cuyckens & Claeys 2002). The anthocyanins are commonly detected in the positive mode with the [M + H]+ ion subjected to further fragmentation to allow identification of the anthocyanidin (aglycone) core. The MS/MS approach permits the

characterization of molecular weight of anthocyanidin and sugar moiety (Ignat et al. 2011). Flavonol and flavanone glycosides have produced responses in both the positive and negative ionization modes (Häkkinen & Auriola 1998, Lin & Harnly 2007). The best response for PAs and hydrolysable tannins is usually obtained in the negative ionization mode (Valls et al. 2009, Arapitsas 2012). Ek et al. (2006) reported that *V. vitis-idaea* catechins could be easily detected with both positive and negative electrospray polarities in LC-MS chromatograms.

There is a possibility of misidentifications if one relies exclusively on HPLC-MS data, since some anthocyanins share the same mother and fragmentation ions (Lee et al. 2012). One example is the misidentification of cyanidin 3-xylosylrutinoside as cyanidin 3-sambubioside-5-rhamnoside. In this instance, both anthocyanins share the same molecular (*m/z* 727) and fragmentation (*m/z* 581 and 287) ions. Hence, they can be differentiated on the basis of their distinct UV/Vis spectra (Lee et al. 2012).

## 2.6 Phenolic compounds of berries and fruits from the view of authenticity analyses

### 2.6.1 Chemotaxonomy

Phenolic compounds have been used in chemotaxonomy studies of many plant genera (*Leontodon*, *Rubus*, *Vaccinium, Vitis*) (Jennings & Carmichael 1980, Andersen 1987a, Ballington et al. 1988a, Zidorn & Stuppner 2001, Picariello et al. 2014). Chemotaxonomy has been traditionally distinguished plants and other organisms according to differences and similarities in their biochemical compositions (Vander Kloet & Bohm 1991, Iwashina 2000, Reynolds 2007).

### 2.6.2 Qualitative profile – the marker compounds untypical for *Vaccinium* berries

Each species (or even a cultivar) displays a distinct, qualitative phenolic profile (presence/absence of certain compound(s)) (Macheix et al. 1990). The detection of phenolic compounds which are not characteristic for the berry or fruit indicated on the label may be evidence of adulteration. The specific markers bring some information about the adulterant(s). In this chapter, untypical compounds for *Vaccinium* berries and their potential botanical sources are reviewed. In ***Table 1***, there are some examples of phenolic compounds that have not been detected by LC-MS (in most cases) in *Vaccinium* berries. In addition, the examples of the berry and fruit species with their typical characteristic compounds are presented.

*Phenolic acids*

There are some interspecific differences in the hydroxybenzoic and hydroxycinnamic acids present in fruits with regard to qualitative profile. Generally, tartaric acids (***Table 1***) esterified with caffeic, coumaric and/or ferulic acids are regarded as indicators for grape (*V. vinifera*) (Clifford 1999, Hollecker et al. 2009, Zhang et al. 2009, Tamborra & Esti 2010). However, recently low amounts (< 0,34 g/100g) of soluble tartaric acids were detected in the berries of *V. myrtillus*, *V. macrocarpon* and *V. vitis-idaea*, but not in blueberries (*V. corymbosum*) (Mikulic-Petkovsek et al. 2012a). The rather rare glucaric (feruloyl, *p*-coumaroyl and diferuloyl) and galactaric acid (feruloyl, *p*-coumaroyl) derivatives can be considered as indicators for citrus fruits (Clifford 1999). *Vaccinium* berries are qualitatively quite rich in phenolic acids; their phenolic acid profiles are presented in ***Table 2***.

*Anthocyanins*

Each fruit can be characterized by its anthocyanidin glycosides. Blue-black colored *Vaccinium* berries contain at least five anthocyanidins (aglycones) (e.g. Baj et al. 1983, Määttä-Riihinen et al. 2004, Rieger et al. 2008). Many of them are absent in similarly colored berries of most other genera (Lee & Hong 1992, Määttä-Riihinen et al. 2004). The taxonomically related (***Figure 1***) crowberries (*Empetrum nigrum* L.) have been reported to contain five anthocyanidins similarly to berries of *V. myrtillus*, *V. uliginosum* and *V. corymbosum* (Baj et al. 1983, Martinelli et al. 1986, Koskela et al. 2010, Määttä-Riihinen et al. 2004).

Pelargonidin is rarely found in *Vaccinium* berries. It has been reported to occur in one endemic species of *V. japonicum* (Andersen 1987a). The presence of pelargonidin is typical for certain berries. The red color of strawberries (*Fragaria* spp.) is mainly attributable to pelargonidin 3-glucoside (Määttä-Riihinen et al. 2004, Bakowska-Barczak et al. 2007). Similarly, the fruits of *Prunus tomentosa* Thunb were rich in pelargonidin, especially as 3-rutinoside derivatives (Bakowska-Barczak et al. 2007).

Certain conjugated forms of anthocyanins are absent, or very rare, occurring in low amounts in *Vaccinium* berries. Those include 3-sambubioside-5-glucosides, 3,5-glucosides, rutinosides and sophorosides (See ***Table 1***). Malvidin 3-rutinoside has been found only in the berries of *V. padifolium* (Cabrita & Andersen 1999). Rare anthocyanidin triglycosides were found in the berries of this species (Cabrita et al. 2000). Some other rare triglycosides are characteristic for other species too (***Table 1***). For example, the cyanidin 3-(2-glucosylrutinoside) content was high in red currants (*Ribes* × *pallidum* Otto & Dietr.) (Määttä et al. 2003, Wu et al. 2004). Cya-

nidin 3-dioxalylglucoside was specific for blackberries (*Rubus armeniacus* Focke) (Grant & Helleur 2008).

*Flavonols*

All the six aglycones (myricetin, quercetin, laricitrin, isorhamnetin, syringetin, kaempferol) have been found in *Vaccinium* berries (Vvedenskaya et al. 2004, Koponen et al. 2008, Vrhovsek et al. 2012). Hence, most of the studies have dealt only with myricetin, quercetin and kaempferol (e.g. Sellappan et al. 2002, Taruscio et al. 2004, Riihinen et al. 2008).

Myricetin, isorhamnetin and kaempferol have not been detected in the berries of *V. vitis-idaea* and *V. macrocarpon* (Ek et al. 2006, Grant & Helleur 2008, Mikulic-Petkovsek et al. 2012b). However, in the berries of some *V. ashei* cultivars and *V. uliginosum*, myricetin was the most abundant flavonol (Sellappan et al. 2002, Taruscio et al. 2004). Quercetin was the major flavonol detected in the berries of blueberry species from the sections *Cyanococcus* and *Pyxothamnus* (*V. angustifolium* × *V. corymbosum*, *V. corymbosum*, *V. deliciosum*, *V. membranaceum*, *V. ovalifolium*, and *V. ovatum*) and bilberries from the section *Myrtillus* (Sellappan et al. 2002, Taruscio et al. 2004, Riihinen et al. 2008, Vrhovsek et al. 2012).

Isorhamnetin occurred as a minor compound in the berries of *V. myrtillus* and *V. macrocarpon* (Vvedenskaya et al. 2004, Koponen et al. 2008, Mikulic-Petkovsek et al. 2012b) and in some *V. corymbosum* hybrid varieties (Vrhovsek et al. 2012). Minor amounts of laricitrin and syringetin have been found in the berries of *V. corymbosum* and its hybrids and *V. myrtillus* (Koponen et al. 2008, Mikulic-Petkovsek et al. 2012b, Vrhovsek et al. 2012).

Flavonol 3-rutinosides were not detected in the berries of *V. myrtillus*, *V. vitis-idaea* and *V. uliginosum* by LC-MS/MS (Ek et al. 2006, Koponen et al.2008, Kusznierewicz et al. 2012); except quercetin 3-rutinosides in blueberries (*V. angustifolium*, *V. corymbosum*) (Grant & Helleur 2008, Mikulic-Petkovsek et al. 2012b). Myricetin 3-rutinoside, a flavonol which occurs abundantly in *R. nigrum* berries (***Table 1***), was not found in *Vaccinium* berries. Similarly, many quercetin diglycosides that occur in the berries of *Amelanchier*, *Fragaria* and *Ribes* genera have not been found in *Vaccinium* berries (See ***Table 1***).

*Tannins*

Ellagitannins occur abundantly in the berries and fruits from *Punica*, *Rosa*, *Rubus* and *Fragaria* genera (Koponen et al. 2007, Zhang et al. 2009, Landete et al. 2011, Lee et al. 2012). The most abundant ellagitannin, agrimoniin, in the berries of *Fragaria* is unique for this genus (Aaby et al. 2012). Ellagitannins have not

been found in the berries of *V. myrtillus, V. oxycoccus, V. uliginosum* and *V. vitis-idaea* (Koponen et al. 2007).

On type of PAs, propelargonidins (i.e. (epi)afzelchin units), have not been detected in *Vaccinium* berries. They have been found in the berries of *Rubus* and *Fragaria* genera (Gu et al. 2003, Hellström et al. 2009). Gallolyated PAs are invariably present in grapes (*Vitis* spp.) but absent in *Vaccinium* berries (Hellström et al. 2009).

There are no reports about the existence of flavones in *Vaccinium* berries. Dihydrochalcones and flavanone glycosides have a rather restricted distribution among fruits and berries, being absent in *Vaccinium*. Dihydrochalcones are characteristic especially to *Malus* and flavanones to *Citrus* fruits (See ***Table 1***).

***Table 1.*** *Phenolic marker compounds untypical for Vaccinium berries.*

| | **Not found in the selected *Vaccinium* berries[a] Phenolic group/Compound** | **Fruit and berry source(s) [b]** | **Examples of Refs.** |
|---|---|---|---|
| | Phenolic acids | | |
| 1 | Caftaric acid (=caffeoyltartaric acid) | ***Vitis vinifera*** L. | 1, 2, 3 |
| 2 | Coutaric acid (=coumaroyltartaric acid) | *V. vinifera,* common grapevine | 1 |
| 3 | Fertaric acid (=feruloyltartaric acid) | *V. vinifera* | 1 |
| | Anthocyanins | | |
| 4 | Delphinidin 3-sambubioside-5-glucoside | ***Aristotelia chilensis*** (Molina) Stuntz, Chilean wineberry | 4, 5 |
| 5 | Delphinidin 3,5-diglucoside | *A. chilensis* | 4 |
| | | *Punica granatum* L., pomegranate | 6 |
| | | *Vitis rotundifolia* L., muscadine grape | 7 |
| 6 | Delphinidin 3-rutinoside (=3-(6-rhamnosyl-glucoside) | ***Hyeronima macrocarpa*** Müll. Arg., motilón | 8 |
| | | *Lonicera uthensis* S. Wats., red twinberry | 9 |
| | | ***Ribes nigrum*** **L**., black currant | 10, 11, 12, 13, 14 |
| | | (*Sambucus nigra* L., European elderberry) | 15 |
| 7 | Cyanidin 3-(2-glucosylrutinoside) | *Rubus* hybrids*; R. idaeus* | 16, 17, 18 |
| | | *R. loganbaccus* × *baileyanus* | 19 |
| | | ***R. × pallidum*** **;** ***R. rubrum*** | 11, 12 |
| 8 | Cyanidin 3-sophoroside-5-rhamnoside | *R. idaeus* | 14 |
| 9 | Cyanidin 3-sambubioside-5-glucoside | *A. chilensis* | 4 |
| | | *S. canadensis* , American elderberry; ***S. nigra*** | 15 |
| 10 | Cyanidin 3-(2-xylosylrutinoside) | *R. × pallidum;* ***R. rubrum*** *;* ***R. occidentalis*** | 11, 12, 13, 14, 20 |
| 11 | Cyanidin 3,5,-diglucoside | *Lonicera caerulea* L. subspp. edulis, *kamtschatica* and *pallassi* , blue honeysuckle | 21 |
| | | ***L. uthensis*** *;* ***P. granatum*** | 6, 9 |
| | | *Rubus idaeus* L. | 17 |
| | | *Sambucus canadensis* L.*, American elderberry* | 15 |
| | | *S. nigra* | 15 |
| | | *Vitis labrusca* L.*, fox grape; V. rotundifolia* | 7, 22 |
| 12 | Cyanidin 3-sophoroside | *M. nigra* | 23 |
| | | ***R. idaeus*** | 14, 16, 17 |
| | | *Rubus* hybrids***; R. loganbaccus × baileyanus*** | 18, 19 |
| | | *R. × pallidum; R. rubrum* | 11, 12 |
| 13 | Cyanidin 3-rutinoside | *L. caerulea subspp. edulis, kamtschatica* and *pallassi* | 21 |
| | | *Morus nigra L.,* mulberry | 14 |
| | | ***Prunus avium*** **L**., sweet cherry | 24 |
| | | ***Prunus cerasus*** **L.,** tart cherry | 24 |
| | | *Ribes grossularia* L., gooseberry; ***R. nigrum*** | 10, 11, 12, 13, 14 |
| | | *Ribes × pallidum* , red currant | 11 |
| | | *Ribes rubrum* , red currant | 12, 13 |
| | | ***Ribes sanguineum*** Pursh, red flower currant | 25 |
| | | *Rubus glaucus* Benth., Andean blackberry | 26 |
| | | *Rubus* hybrids, blackberry cultivars | 27, 28 |
| | | *Rubus idaeus* L., raspberry | 16, 17 |
| | | *Rubus loganbaccus* × *baileyanus* Nutt., boysenberry | 19 |
| 14 | Cyanidin 3-coumaroylsambubioside-5-glucoside | ***S. canadensis*** | 15 |
| 15 | Cyanidin 3-dioxalylglucoside | *Rubus fruticosus; Rubus* hybrids | 14, 26, 29 |
| 16 | Petunidin 3,5-diglucoside | *V. rotundifolia* | 7 |
| 17 | Petunidin 3-rutinoside | *R. nigrum* | 10, 12 |
| 18 | Peonidin 3,5-diglucoside | *V. rotundifolia* | 7 |
| 19 | Peonidin 3-rutinoside | *L. caerulea subspp. edulis, kamtschatica* and *pallassi* | 21 |
| | | *R. grossularia; R. nigrum* | 10, 12 |
| 20 | Pelargonidin 3,5-diglucoside | *P. granatum* | 6 |
| | | *V. rotundifolia* | 7 |

***Table 1*** *continues.*

| | | | |
|---|---|---|---|
| 21 | Pelargonidin 3-rutinoside | *Fragaria* × *ananassa* Dusch., strawberry | 16, 30 |
| | | *M. nigra;* ***Prunus tomentosa*** Thunb., red nanking cherries; *(R. nigrum)* | 9, 10, 12, 14, 23 |
| | | *R. glaucus; R. idaeus* | 16, 28 |
| 22 | Pelargonidin 3-glucoside | ***F. × ananassa*** | 14, 16, 30 |
| | | *L. caerulea; M. nigra* | 21, 23 |
| | | *R. idaeus* | 16, 30 |
| | | *(R. nigrum);* (*S. nigra*) | 10, 12, 15 |
| 23 | Pelargonidin 3-malonylglucoside | *F.* × *ananassa* | 16, 30 |
| | Flavonol glycosides | | |
| 24 | Myricetin 3-rutinoside | ***R. nigrum*** | 11, 31, 32 |
| 25 | Myricetin 3-malonylglucoside | *R. nigrum* | 11, 31, 32 |
| 26 | Quercetin 3-rhamnosylgalactoside (=quercetin 3-robinobioside) | *Amelanchier alnifolia Nutt.,* saskatoon fruit; *(Aronia melanocarpa* Michx., black chokeberry) | 10, 33 |
| 27 | Quercetin 3-arabinosylglucoside (=quercetin 3-vicianoside) | *A. alnifolia;* (*A. melanocarpa*) | 10, 33 |
| 28 | Quercetin 3-glucosylxyloside | *Rubus* hybrids | 26 |
| 29 | Quercetin 3-xylosylglucuronide | *Rubus* hybrids | 26 |
| 30 | Quercetin 3-malonylglucoside | *F.* × *ananassa; R. nigrum; R.* × *pallidum* | 11, 30, 31, 32 |
| 31 | Isorhamnetin 3-rutinoside | *R. nigrum; S. canadensis, S. nigra* | 15, 31, 32 |
| 32 | Kaempferol 3-rutinoside | *R. nigrum; S. canadensis, S. nigra* | 15, 31, 32 |
| 33 | Kaempferol 3-malonylglucoside | *F.* × *ananassa; R. nigrum* | 11, 30 |
| | Dihydrochalcones | | |
| 34 | Coumaroylphloretin 2-glucoside | *Malus* sp., apple | 33 |
| 35 | Phloretin 2-xylogalactoside | *Malus* sp. | 33 |
| 36 | Phloretin 2-xyloglucoside | *Malus* sp. | 33 |
| 37 | Phloridzin | *Malus* sp. | 33 |
| | | (*F.* × *ananassa*)(V. macrocarpon L., cranberi | 33, 34, 35 |
| | Flavanone glycosides | | |
| 38 | 4'5,7,-trihydroxyflavanone-7-rutinosic (=naringin) | ***Citrus* grandis**, pomelo; ***Citrus limon***, lemon fruit; **Citrus paradisi**, grapefruit | 36, 37 |
| 39 | hesperetin 7-rutinoside | *Citrus sinensis*, sweet orange | 37 |
| 40 | naringetin 7-rutinoside | *C. sinensis* | 37 |
| | Ellagitannins | | |
| 41 | Lambertianin C | *F.* × *ananassa* | 38 |
| | | ***Rubus chamaemorus*** L., cloudberry | 39 |
| | | *R. fruticosus;* ***R. idaeus*** | 17, 39, 40 |
| 42 | Punicalagin isomers | ***P. granatum*** | 6 |
| 43 | Sanguiins (=HHDP-galloylglucoses) | *F.* × *ananassa* | 38 |
| | | ***R. chamaemorus*** *;* ***R. idaeus*** | 39, 40 |
| | | *R. fruticosus, V. rotundifolia* | 40, 41 |

[a] According to the literature about the following species (see below). The superscripts: * the identification is made by HPLC-MS (tentative) and/or NMR studies of the phenolic acids ([ph]), anthocyanins ([an]), flavonols ([fl]) and/or ellagitannins ([et]).

*V. angustifolium* (Gao & Mazza 1994[an], Grant & Helleur 2008[an,fl*], Grace et al. 2009[an*,] Nicoué et al. 2007[an*]), *V. corymbosum* (Gao & Mazza 1994[an], Cho et al. 2004[an], [fl*], Gavrilova et al. 2011[an,fl,ph*]), *V. deliciosum* (Lee et al.

2004a[an]), *V. floribundum* (Vasco et al. 2009[an,fl,ph*], Schreckinger et al. 2010[an*]), *V. japonicum* (Andersen 1987[an]), *V. macrocarpon* (Vvedenskaya et al. 2004[fl*], Pappas & Schaich 2009[an,fl,ph]), *V. membranaceum* (Lee et al. 2004b[an, ph*]), *V. meridionale* (Garzón et al. 2010[an, fl*]), *V. myrtillus* (Dugo et al. 2001[an*], Koponen et al. 2007[et], Koponen et al. 2008[fl*], Gavrilova et al. 2011[an, fl,ph*]), *V. myrtilloides* (Bakowska-Barczak et al. 2007 [an*], Nicoué et al. 2007[an*]), *V. ovatum* (Lee et al. 2004[an,ph*]), *V. oxycoccus* (Andersen 1989[an], Koponen et al. 2007[et], Česonienė et al. 2011[an*]), *V. padifolium* (Cabrita & Andersen 1999[an*], Cabrita et al. 2000[an*]), *V. uliginosum* (Koponen et al. 2007[et*]), *V. vitis-idaea* (Ek et al. 2006[an,fl*], Koponen et al. 2007[et]).

[b] Bold letters: The compound occurs typically in high amounts in the berries or fruits of that species, cultivar or hybrid; or if they are written in the parentheses: the compound is a minor one and usually below HPLC-DAD quantification level.

References: [1] Naczk & Shahidi 2006, [2] Hollecker et al. 2009, [3] Liang et al. 2012, [4] Schreckinger et al. 2010, [5] Escribano-Bailón et al. 2006, [6] Fischer et al. 2010, [7] Sandhu & Gu 2010, [8] Santacruz et al. 2012, [9] Bakowska-Barczak et al. 2007, [10] Slimestad & Solheim 2002, [11] Määttä et al. 2003, [12] Wu et al. 2004, [13] Gavrilova et al. 2011, [14] Ogawa et al. 2008, [15] Lee & Finn 2007, [16] Määttä-Riihinen et al. 2004, [17] Remberg et al. 2010, [18] Kaume et al. 2012, [19] Cooney et al. 2004, [20] Lee et al. 2012; [21] Chaovanalakit et al. 2004; [22] Lago-Vanzela et al. 2011, [23] Dugo et al. 2001, [24] Liu et al. 2011, [25] Jordheim et al. 2007, [26] Vasco et al. 2009, [27] Cho et al. 2004, [28] Siriwoharn et al. 2004, [29] Grant & Helleur 2008, [30] Aaby et al. 2007, [31] Anttonen & Karjalainen 2006, [32] Koponen et al. 2008, [33] Ozga et al. 2007, [34] Gosch et al. 2010, [35] Hilt et al. 2003, [36] Turner et al. 2005, [37] Zhang et al. 2011, [38] Medina-Remón et al. 2011, [39] Aaby et al. 2012, [40] Buendia et al. 2010, [41] Kähkönen et al. 2012, [42] Gasperotti et al. 2010, [43] Lee et al. 2005.

### 2.6.3 Quantitative comparisons

The contents and relative proportions of phenolic compounds in fruit families and genera vary greatly. Quantitative variation involves variations of amounts of individual compounds and their relative proportions (Macheix et al. 1990).

In this section, the quantitative comparisons have been made mainly between *Vaccinium* species but also between *Vaccinium* species and species from other genera. Especially, in those cases when the amounts of individual compounds, or their relative proportions, in *Vaccinium* berries are very low, some examples about the species of berries in which they are prevalent are presented. Multiple markers have been shown to be useful especially in distinguishing closely related species (Sudberg et al. 2010). In some cases, the analysis of minor compounds is also advisable e.g. since it is not economically feasible for adulterators to adjust the levels of all components, many of which are expensive (Smillie & Khan 2010, Sudberg et al. 2010).

*Phenolic acids*

When reviewing the literature on phenolic acid contents, there are several difficulties related to the methodology in use. The extraction methods differ markedly (See ***Chapter 2.5.1***). Most reports about the contents after the hydrolysis of extracts with varying recoveries. Some have included also the nonhydrolyzed portion in the quantitative results (Mattila et al. 2006). ***Table 2*** shows the results from the methods using hydrolysis and without hydrolysis separately.

The hydroxybenzoic acid content in berries and fruits including *Vaccinium* berries is generally low. They were below quantification level (by DAD), or about 10mg/100g FW at the highest (Häkkinen et al. 1999a, Taruscio et al. 2004, Ayaz et al. 2005, Mattila et al. 2006). The berries of *V. parvifolium* are an exception (See ***Table 2***). Certain fruits of the Rosaceae family (blackberry, raspberry, strawberry) consist of high amounts of hydroxybenzoic acids (Tomás-Barberán & Clifford 2000). They are rich in ellagic acid which is present mainly in the form of ellagitannins (Tomás-Barberán & Clifford 2000, Määttä-Riihinen et al. 2004, Kaume et al. 2012, Lee et al. 2012). Low amounts of ellagic acid were found (≤7 mg/100g FW) in the berries of some *Vaccinium* species (*V. ashei*, *V. corymbosum*, *V. myrtillus*) (Može et al. 2011, Sellappan et al. 2002).

In general, *Vaccinium* berries contain high levels hydroxycinnamic acids**.** The most common phenolic acids include the isomers of caffeoylquinic acids which differ in their abundance in *Vaccinium* species (See ***Chapter 5.4.4***). Neochlorogenic acid (3-*O*-caffeoylquinic acid) has been reported to occur as a minor phenolic component in the berries of *V. floribundum*, *V. myrtillus*, *V. ovatum* and *V. uliginosum* (Lee et al. 2004b, Kusznierevicz et al. 2012, Prencipe et al. 2014). In contrast, it was the dominant phenolic acid (123 mg/100g FW) in the berries of *Aronia melanocarpa* (Slimestad et al. 2005). Furthermore, a predominance of neochlorogenic acid with lesser amounts of its 5-isomer has been considered as being a typical characteristic of the berries of *Empetrum nigrum*, *Prunus avium*, *Prunus spinosa* and some *Sorbus aucuparia* cultivars (Määttä-Riihinen et al. 2004, Hukkanen et al. 2006, Liu et al. 2011).

The berries of *V. angustifolium* × *V. corymbosum* and *V. corymbosum* contained high levels of chlorogenic acid (~130–140 mg/100g FW) (Taruscio et al. 2004). Low levels (~20 mg/100g FW) of chlorogenic acid were found in *V. arctostaphylos* berries (Ayaz et al. 2005). The berries of *V. ovalifolium* and *V. uliginosum* did not display quantifiable levels of this phenolic acid (UV/Vis detection) (Taruscio et al. 2004). Caffeic acid was the predominant phenolic acid present in the berries of *V. uliginosum* and *V. arctostaphylos* (Taruscio et al. 2004, Ayaz et al. 2005). In lingonberries, *p*-coumaric

acid was reported to be the major phenolic acid (Määttä-Riihinen et al. 2004).

*Anthocyanins*

The dark colored blue-black berries contain an abundance of anthocyanins. The highest contents (FW as cyanidin 3-glucoside equivalents) of anthocyanins have been analysed in the berries of *Aronia melanocarpa* (1300–1500 mg/100g), *V. myrtillus* (500–1600mg/100g) and *E. nigrum* (400–1100 mg/100g). They are followed by the berries of *R. nigrum* (300–600mg/100g), *S. nigra* (500–1400 mg/100g) and *V. uliginosum* (400–600mg/100g) (Nyman & Kumpulainen 2001, Määttä-Riihinen et al. 2004, Wu et al. 2004, Koskela et al. 2010, Može et al. 2011).

The occurrence of aglycone moieties (anthocyanidins) in the berries of *Vaccinium* species may vary from the prevalence of one anthocyanidin to the occurrence of at least five different anthocyanidins (e.g.Andersen 1985, Andersen 1987b, Määttä-Riihinen et al. 2004, Rieger et al. 2008). Delphinidin and cyanidin were the major aglycone moieties in the berries of *V. myrtillus* and *R. nigrum*. Furthermore, the berries of *V. myrtillus* contain also petunidin, peonidin and malvidin (Määttä-Riihinen et al. 2004, Buchert et al. 2005, Cassinese et al. 2007, Koponen et al. 2007). These aglycones have been occasionally detected in very low contents in the black currants (*R. nigrum*) (Slimestad & Solheim 2002, Ogawa et al. 2008). Bog bilberries (*V. uliginosum*) and crowberries (*Empetrum* spp.) have five different aglycone moieties (Koponen et al. 2007). Two major anthocyanidins of bog bilberries have been either delphinidin and malvidin or delphinidin and cyanidin (Andersen 1987b, Taruscio et al. 2004, Koponen et al. 2007, Xiao-yan et al. 2010). The anthocyanin rich berries of *A. melanocarpa* and *S. nigra* contain cyanidin as major anthocyanidin (Slimestad et al. 2005, Lee & Finn 2007). Red currants (*R.* × *pallidum*/*R. rubrum*) and red gooseberries (*R. uva-crispa*) have similarly only cyanidin glycosides but in lower contents (Määttä-Riihinen et al. 2004, Koponen et al. 2007).

*Flavonols*

Myricetin was the most abundant flavonol in the berries of some *V. ashei* cultivars and *V. uliginosum* (Sellappan et al. 2002; Taruscio et al. 2004). Quercetin was reported to be the most abundant flavonol in the berries of *V. ashei*, *V. corymbosum*, *V. floribundum*, *V. myrtillus*, *V. oxycoccus*, *V. uliginosum* and *V. vitis-idaea* (Häkkinen et al. 1999a, Häkkinen & Törrönen 2000, Vasco et al. 2009, Može et al. 2011, Gavrilova et al. 2011, Wang et al. 2012, Vrhovsek et al. 2012).

*Vaccinium* berries seem to be low in kaempferol. It has been detected as a minor flavonol in the berries of *V. ashei*, *V. corymbosum*,

*V. macrocarpon*, *V. oxycoccus*, *V. uliginosum*, *V. vitis-idaea*, *V. darrowii* and its hybrid cultivars) (Häkkinen et al. 1999a, Sellappan et al. 2002, Zheng & Wang 2003, Määttä-Riihinen et al. 2004, Vrhovsek et al. 2012). The berries of *Amelanchier canadensis* L., *R. nigrum* and *Ribes grossularia* L. had clearly higher amounts of kaempferol (Mikulic-Petkovsek et al. 2012b). Kaempferol sophoroside was a characteristic compound found in the berries of *R. grossularia*.

Minor contents of laricitrin, syringetin or isorhamnetin in *Vaccinium* berries have been reported (Yan et al. 2002, Vvedenskaya & Vorsa 2004, Koponen et al. 2008, Gavrilova et al. 2011, Mikulic-Petkovsek et al. 2012b, Vrhovsek et al. 2012). The higher content of isorhamnetin have been quantified in the berries of *Hippophaë rhamnoides* L. (17mg/100g FW), *R. uva-grispa*, or *F.* × *ananassa* (2–8mg/100g FW) (Määttä-Riihinen et al. 2004, Aaby et al. 2012, Mikulic-Petkovsek et al. 2012b).

*Tannins*

The levels of a particular class of tannin molecule, that is, either condensed (PAs) or hydrolyzable (ellagitannins) tannins, vary considerably between the different berries. *Vaccinium* berries contain predominantly PAs (Hellström et al. 2009). A-type linkages among PAs dominate in *Vaccinium* and *Empetrum* berries (Määttä-Riihinen et al. 2005, Hellström et al. 2009). Their proportions are especially high in lingonberries (Hellström et al. 2009).

Prodelphinidins were absent in the berries of *V. uliginosum* and *V. vitis-idaea*. Previously, they were detected in the berries of *V. myrtillus* and *V. oxycoccus* (Määttä-Riihinen et al. 2004, Hellström et al. 2009). A recent study examined differences in the procyanidin profiles and concentrations between *V. macrocarpon*, *V. oxycoccus* and *V. vitis-idaea*. The berries of V. *oxycoccus* lacked A-type trimers. Instead, they occurred in the berries of *V. macrocarpon* and *V. vitis-idaea* (Jungfer et al. 2012). The smallest oligomers (from monomers to trimers) predominated in the berries of *V. uliginosum*, whereas highly polymerized PAs (DP >10) dominated in the berries of *V. oxycoccus* and *V. myrtillus* (Hellström et al. 2009).

***Table 2.*** *The contents of phenolic acids in the berries and fruits from the families of Ericaceae, Grossulariaceae, Rosaceae, Rutaceae and Vitaceae.*

| Family / Genus / Species | | Contents [a] 1) without hydrolysis 2) with hydrolysis — Hydroxycinnamic: Chloro-genic | *p*-Coumaric | Caffeic | Ferulic | Sinapic | Hydroxybenzoic: *p*-OH-benzoic | Proto-catechuic | Vanillic | Syringic | Gallic | Ellagic [b] | Refs. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ericaceae | | | | | | | | | | | | | |
| *Vaccinium* | | | | | | | | | | | | | |
| *myrtillus* | 1) | **23** | <0.5/7 | <1 | <1 | | | | | | 5 | 1 | 1,2 |
| | 2) | | 2–8 | <1/**10–11** | 1 | <1 | $nd^{DAD}$ | $nd^{DAD}$/7 | 6–7 | **14–15** | 2–3 | | 3,4 |
| *parvifolium* | 2) | 5–8 | 8–11 | **14–16** | 4 | | **41–69** | | | | | | 5 |
| *oxycoccos* | 2) | 5 | 1/**10–11** | 1–2 | <1/5–7 | <1 | <1 | 2 | 3 | 1 | $nd^{DAD}$ | | 4,5 |
| *ovatum* | 2) | **47** | 3–4 | <1 | **10–12** | | 1 | | | | | | 5 |
| *uliginosum* | 2) | <0.1 | <0.5/7–8 | **2–16** | 1–8 | $nd^{DAD}$ | $nd^{DAD}$/1 | $nd^{DAD}$ | 1 | **15** | 2 | | 4,5 |
| *vitis-idaea* | 2) | | 4 | 5 | 2 | <0.5 | 1 | 4 | <1 | $nd^{DAD}$ | 4 | | 4 |
| *ashei* | 1) | **22** | | | | | | | | | | | 6 |
| cv. Tifblue | 2) | | $nd^{DAD}$ | $nd^{DAD}$ | **17** | | $nd^{DAD}$ | | | | **259** | $nd^{DAD}$ | 7 |
| *corymbosum* | 1) | **129** | | <0.5 | 2 | | | | | | | | 8 |
| cv. Bluecrop [c] | 2) | **158** | <1 | **22** | 5 | | <0.1 | | | | | | 5 |
| Grossulariaceae | | | | | | | | | | | | | |
| *Ribes* | | | | | | | | | | | | | |
| *nigrum* | | | | | | | | | | | | | |
| cv. Öjebyn/ Rosenthal | 1) | <1 | 2–4 | 2–5 | 1–2 | 1 | | | | | | | 8,9 |
| cv. Ben Sarek | 1) | | <0.5 | <0.1 | $nd^{DAD}$ | $nd^{DAD}$ | <0.1 | <0.5 | <0.5 | $nd^{DAD}$ | <0.1 | | 10 |
| | 2) | | **12** | **11** | 3 | <1 | 5 | 8 | 2 | <0.5 | **21** | | 10 |
| cv. Mortti | 1) | | 3 | 1 | 2 | | | | | | | | 11 |
| Rosaceae | | | | | | | | | | | | | |
| *Amelanchier* | | | | | | | | | | | | | |
| *alnifolia* | 1) | **44–72**/ 10–13[N] | | | | | | <0.5 | | | | | 12,13 |
| cv. Honeywood | | | | | | | | | | | | | |
| cv. unknown | 2) | | 3 | **43** | 2 | <1 | <1 | 8 | <1 | $nd^{DAD}$ | $nd^{DAD}$ | | 4 |

***Table 2*** *continues.*

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Fragaria* | | | | | | | | | | | | | |
| × *ananassa* | | | | | | | | | | | | | |
| cv. Elsanta | 1) | | nd[MS] | nd[MS] | 7 | 5 | 2 | <0.5 | 1 | nd[MS] | <0.1 | | 10 |
| | 2) | | **13** | 2 | 2 | nd[MS] | 5 | <1 | <1 | nd[MS] | **14** | | 10 |
| cv. Polka | 2) | | 2–5 | <0.5 | nd[DAD]/<0.5 | nd[DAD] | 4–5 | nd[DAD] | <0.5 | nd[DAD] | 2–5 | **51–85** | 3,4,14 |
| cv. Jonsok | 2) | | 2–4 | <0.5 | <0.5 | nd[DAD] | 4–6 | nd[DAD] | nd[DAD] | nd[DAD] | 3–4 | **52–80** | 3,4,14 |
| *Prunus* | | | | | | | | | | | | | |
| *avium* [d] | 1) | 2–4/**20–65**[N] | | | | | | | | | | | 15 |
| cv. *Ambrunés* | 1) | 2/**39**[N] | **21** | | | | | | | | | | 16 |
| *cerasus*, cv. CAB | 1) | **15/15**[N] | | | | | | | | | | | 15 |
| sp. (cherry) | 2) | | 5 | 17 | <0.5 | nd[DAD] | <1 | 3 | 1 | nd[DAD] | nd[DAD] | | 4 |
| *Rubus* | | | | | | | | | | | | | |
| *glaucus* | 2) | | 0.4–1 | <0.5 | <1 | | | | | | 5–6 | **393–503** | 17, 18 |
| *idaeus* | 1) | 2 | 2 | | | | | | | | nd[MS] | 4–19 | 2, 14, 19 |
| | 2) | | 1–2 | 1 | 1 | <0.5 | 2 | nd[DAD] | ≤1* | nd[DAD] | **21–22** | **264–331** | 4, 14 |
| cv. Autumn | 1) | | nd[MS] | <0.05 | 1 | 1 | 1 | <0.1 | <0.5 | 2 | <0.1 | | 10 |
| Bliss | 2) | | 4 | 1 | 3 | 1 | 11 | 4 | 2 | 2 | **26** | | 10 |
| Rutaceae | | | | | | | | | | | | | |
| *Citrus* | | | | | | | | | | | | | |
| *paradisi* | 2) | | 1–4 | 3–6 | **11–12** | 1–2 | nd[DAD]/<0.5 | nd[DAD] | 1–2 | nd[DAD]/<0.1 | nd[DAD] | | 4 |
| *sinensis* | 2) | | 2 | 3–6 | 9–10 | 1–2 | <1 | nd[DAD] | <2 | nd[DAD] | nd[DAD] | | 4 |
| Vitaceae | | | | | | | | | | | | | |
| *Vitis* | | | | | | | | | | | | | |
| *vinifera (green)* | 2) | | 1 | 3 | nd[DAD] | nd[DAD] | nd[DAD] | nd[DAD] | nd[DAD] | nd[DAD] | 3 | | 4 |

[a] Free and conjugated phenolic acids mg/100g FW as the weight of the aglycone, [b] The contents of ellagic acid equivalents after acid hydrolysis of ellagitannins are included (*Fragaria* sp., *Rubus* sp.), [c] cv. Bluecrop: The genetic background consists of *V. corymbosum* and *V. angustifolium* (6.3%) (Wang et al. 2012), [d] 10 cultivars, [N] = neochlorogenic acid, nd[DAD] = monitored by DAD; and not detected (nd), [MS] = monitored by MS. The major compounds within a species or cultivar are indicated with bold letters.

References: [1] Može et al. 2011, [2] Määttä-Riihinen et al. 2004, [3] Häkkinen & Törrönen 2000, [4] Mattila et al. 2006, [5] Taruscio et al. 2004, [6] Wang et al. 2012, [7] Sellappan et al. 2002, [8] Gavrilova et al. 2011, [9] Anttonen & Karjalainen. 2006, [10] Russell et al. 2009, [11] Zheng et al. 2012, [12]Ozga et al. 2007, [13] Lavola et al. 2012, [14] Koponen et al. 2007, [15] Liu et al. 2011, [16] Serradilla et al. 2011, [17]Mertz et al. 2007, [18] Vasco et al. 2009, [19] Mattila & Kumpulainen 2002.

### 2.6.4 Geographical variation in phenolic compounds

In this Chapter some general background and major factors that lie behind the geographical variation (e.g. genetic diversity, phenotypic response) of the phenolic compounds in wild species are reviewed to better understand the phenomenon although any individual effects are not studied in this thesis work. Some examples are included about differences caused by genetic and environmental factors in the phenolic compounds of cultivated berries and fruits. Even though in the case of cultivated species, the genetic background have evolved in different way (natural selection vs. breeding selection) compared to wild species.

The environmental conditions in different latitudes vary with season, day length, light quality and temperature. A wide range of factors also change with the altitude e.g. precipitation, temperature and its extremes, snow-cover and radiation intensities. Enhanced UV-B radiation has been regarded as the most important factor affecting the secondary metabolism of plants growing at high altitudes (Zidorn 2010). The content and composition of phenolic compounds are mainly under genetic control although they can be affected by a number of environmental factors (e.g. light, photoperiod, temperature, soil fertility) and their interactions (Macheix et al. 1990, Dixon & Paiva 1995). The environmental factors have shaped the genetic patterns of natural populations in a long term (Linhart & Grant 1996). The phenotypic responses in natural populations are driven by genetic diversity and phenotypic plasticity (Linhart & Grant 1996). The plastic responses are both trait and resource specific, and represent evolved characteristics that vary among genotypes, populations and species (Sultan 2000). Plasticity can influence patterns of evolutionary diversification – if individual genotypes are sufficiently plastic to produce phenotypes appropriate to different local environments, natural selection will not occur for genetically distinct, locally specialized ecotypes (Sultan 2000).

The degree of anthocyanin accumulation in berries is primarily dependent on the light conditions. In addition, a favorable impact of low temperature and a limiting effect of high temperatures have been reported (Ortega-Regules et al. 2006, Azuma et al. 2012). Shading resulted in an increase in anthocyanins with B-ring disubstitution (cyanidin, peonidin) compared to trisubstitution (delphinidin, petunidin, malvidin) in grape berries (*V. vinifera*) over three years (Cohen & Kennedy 2010). The response of different anthocyanins on increased temperature may vary. For example, exposure to direct solar radiation promoted dihydroxylation of the B-ring of the anthocyanidin in grape berries (cv. Merlot) (Tarara et al. 2008). Authors concluded that anthocyanin accumulation appear to be determined by a synergistic combina-

tion of solar radiation and berry temperature, which is complex and needs further investigation.

In the study of Koyama et al. (2012), visible light primarily induced biosynthesis of PAs and affected their composition, whereas UV-light specifically induced biosynthesis of flavonols in grape berries. The use of UV barriers significantly reduced the accumulation of flavonols in grape berries in a 2-year study described by Cohen & Kennedy (2010).

Various hypotheses have been postulated to explain the type, distribution and abundance of plant SMs e.g. carbon-nutrient balance (CNB) (Bryant et al. 1983, Koricheva et al. 1998). The theory of Close & Arthur (2002) highlights the role of plant phenolics as protectors from photodamage. Accordingly, plants increase phenolic production directly in response to the oxidative pressure exerted by the abundance of light. The indirect response emerges from low temperature, which in turn limits photosynthesis, thus increasing oxidative stress and consequently, phenolic levels. Thus, the continuous light during the most of the growing season combined with low night temperatures, have been claimed to increase the production of phenolic compounds in the northern latitudes.

The concept that the concentration of carbon-based secondary compounds (CBSCs) in plant tissues is controlled by the availability of carbon and nitrogen in the environment emerges from the CNB hypothesis (Bryant et al. 1983). The hypothesis states that more carbon is left for CBSCs only when plant growth is restricted through a lack of mineral nutrients (e.g. nitrogen). Conversely, plants growing slowly because of low nutrient availability, but with sufficient light for normal photosynthetic rates, will have 'extra', 'cheap', or 'cost-free' carbon to be allocated to CBSCs.

Altitude and latitude related features in phenolic compounds in wild plants have been reported. The contents of caffeic acid derivatives and the ratio of flavonoids with 3',4'-dihydroxylated ring B to the flavonoids without this substitution pattern correlated positively with altitude in the flowering heads of *Arnica montana* L. (Spitaler et al. 2006). The flavonoid contents of flowering heads of *Leontodon autumnalis* L. correlated positively with altitude but there was no connection with the phenolic acid contents (Zidorn & Stuppner 2001). The concentrations of the soluble phenolics, PAs and flavonols in juniper needles (*Juniperus communis* L.) increased with latitude (Martz et al. 2009). The leaves of *V. myrtillus* from higher latitudes and higher altitudes had greater soluble phenolic and flavonol levels and lower contents of chlorogenic acid derivatives (Martz et al. 2010). The ratio between di- and monohydroxylated flavonoids increased from south to north in

the leaves of the alpine plant (*Oxyria digyna* L. (Hill)) (Nybakken et al. 2004).

Site-specific variation may occur in different growth locations at the same latitude (Jaakola & Hohtola 2010). Site-specific differences have been reported in flavonoids of *Betula nana* leaves from dwarf shrub tundra in Abisko (Sweden 68°21" N) and Toolik Lake (Alaska 68°38" N) (Graglia et al. 2001). The content of the more hydroxylated myricetin derivatives was significantly higher in Abisko, but quercetin derivatives were higher at Toolik Lake. The authors assumed that the Toolik Lake population had much higher flexibility in its carbon allocation which may be caused by differences in genetic control, environmental conditions or a differential evolutionary response to stress.

Among cultivated species, the berries of six strawberry (*F.* × *ananassa*) cultivars from northern Italy (44°09"N) contained significantly higher content of the total flavonols (quercetin and kaempferol glycosides) than the berries from the south (40° 23"N) (Carborne et al. 2009). Moreover, epicatechin/catechin -ratio and PA mDP values were significantly higher in strawberries grown at the northern locations compared to those grown in the south. The berries of white, green and red currants (*Ribes* cvs. Red Dutch, White Dutch and Vertti) growing in the northern Finland (66°34"N) had at least 10% higher total content of phenolic compounds than those growing in the south (60°23"N) (Yang et al. 2013). In the same study, it was found that the contents of hydroxycinnamic acid conjugates in white and green currants from the north were 30% higher than in the berries of the same species from the south.

Opposite was found in another study as the berries of *R. nigrum* (cv. Melalahti, cv. Mortti, cv. Ola) grown at higher latitude (66°34"N) contained significantly less anthocyanins, flavonols and total phenolic compounds than those grown at lower latitude (60°23"N) (Zheng et al. 2012). Similarly, in the study of Vagiri et al. (2013) black currants grown in the southern Sweden had higher content of anthocyanins and phenolic compounds compared to those grown in the north. In the case of phenolic acids, they were more abundant in the berries grown in the north. Vagiri & coauthors (2013) showed that the effect of genotype (cvs. Ben Finlay, Poesia and Titania) on the content of phenolic compounds in black currants (*R. nigrum*) was more significant than that of location and year. The variation in the contents of phenolic compounds between the cultivated blueberry genotypes (*V. corymbosum*) was found greater than that found between years, and the interaction between the genotypes and environment was significant (Howard et al. 2003).

### 2.6.5 Authenticity assessments using phenolic compounds

Phenolic compounds have been used for authentication of berry and fruit cultivars, such as grapes (*Vitis* spp. and *Vitis* hybrids) and their corresponding wines (Pomar et al. 2005, Radovanović et al. 2010, de la Gruz et al. 2012, Rosso et al. 2012, Garrido & Borges 2013, Guo et al. 2013, Fraige et al. 2014), botanical supplements, extracts (Cassinese et al. 2007, Madrigal-Carballo et al. 2009), fruit juices (Cautela et al. 2008, Borges et al. 2010), and geographical origin of wines (Anastasiadi et al. 2009, Di Paola-Naranjo et al. 2011, de Andrade et al. 2013) and juices (Guo et al. 2013).

Phenolic compounds of wines are mostly originated from grape berries and some of them from chemical and biochemical reactions during the winemaking process (Ali et al. 2010). The concentrations of catechin and quercetin were proposed to be used as markers for the authentication of Cabernet Sauvignon wines (Radovanović et al. 2010). Borges & coauthors (2010) proposed that the concentration of anthocyanins together along with the ellagitannin profile can be used as indicator of authenticity of pomegranate juices. Flavanone glycosides charasteristic for the bergamot (*Citrus bergamia* Risso and Poit.) juice and absent in the lemon [*Citrus limon* (L.) Burm. f.] juice was used to determine and quantify the fraudulent addition of bergamot juice to lemon juice above an adulteration level of 1% (Cautela et al. 2008).

The chemometric classification of wines according to their phenolic profile analysed by HPLC and $^{1}$H NMR allowed discrimination between wines from different wineries of the same wine-producing zone (Anastasiadi et al. 2009). Hydroxycinnamic acids and flavan-3-ols were mainly used for differentiation (100% correctly) of the geographical origin of 35 commercial Riesling white wines produced from 9 terroirs in Czech Republic over the years 2006–2008 (Kumšta et al. 2012). Rastija & co-workers (2009) established flavonols and *trans*-resveratrol patterns as the basis for classification of wines according to their geographical origin. The combined use of elemental, isotopic and phenolic (flavanols, flavonols, *trans*-resveratrol) data with chemometric techniques enabled the differentiation of Argentinean red wines of various geographical origins (Di Paola-Naranjo et al. 2011).

Anthocyanin, flavanone or other phenolic profiles are used in some commercial laboratories to detect adulteration of red colored juices e.g. cranberry, pomegranate, raspberry and strawberry juices and citrus juices (e.g. Eurofins Scientific, Krueger Food Laboratoires Inc., SGF Internationale e. V.).

# 3 Aims of the study

The objectives of this study were to characterize the phenolic compounds in less known *Vaccinium* berries (*Vaccinium* × *intermedium* Ruthe, *Vaccinium arctostaphylos* L.), to study the geographical variation (*Vaccinium myrtillus* L., *Vaccinium uliginosum* L.), and to evaluate the variation in phenolic profiles in *Vaccinium* genus and subsequently, the potential of using phenolic compounds in the authenticity studies of berries and fruits.

The specific aims of the individual Studies (**I–V**) were:

- To analyze the content of anthocyanins and PAs in the flowers and berries of the *V.* × *intermedium* in comparison with the parent species, bilberry (*V. myrtillus*) and lingonberry (*V. vitis-idaea*) (**I**).
- To identify tentatively the phenolic compounds in the flowers and berries of the *V.* × *intermedium* by HPLC-DAD and HPLC-ESI-MS (**I**).
- To evaluate the anthocyanin variation in the berries of *V. myrtillus* growing in 20 populations in Finland (**II**).
- To examine the content and composition of anthocyanins in *V. arctostaphylos* berries native to Turkey by combining data obtained from DAD and ESI-MS after separation by RP-HPLC (**III**).
- To evaluate the anthocyanin and flavonol variation in the berries of *V. uliginosum* from 15 populations in Finland (**IV**).
- To tentatively identify the flavonoids by combining data obtained from DAD and ESI-MS after separation by RP-HPLC (**IV**).
- To compare the anthocyanin profiles in the berries of *V. myrtillus* from Turkish populations to their Finnish counterparts using the consistent, optimized, repeatable RP-HPLC-DAD method (**V**).
- To evaluate the potential of anthocyanin fingerprinting as an authentication tool (**V**).

# 4 Materials and methods

## 4.1 Flower and berry samples

The flower and berry samples of *V.* × *intermedium* were collected from the Botanical Garden of University of Oulu (***Table 3***). The berries of *V. myrtillus* and *V. vitis-idaea*, used as references, were collected as bulk population samples during summer 2009 (**I**). The berries of *V. myrtillus* (179 plant individuals) (**II**, **V**) and *V. uliginosum* (137 plant individuals) (**IV**) from wild populations in Finland were handpicked during the summer of 2005. The bushes were randomly selected within the populations, with the preconditions that should be a minimum distance between the studied plants of 10 m in order to ensure that samples would be collected from different genets (Albert et al. 2005). The berries (*V. arctostaphylos*, *V. myrtillus*) from Turkey were collected as bulk population samples during the summers of 2007 and 2008 (**III**, **V**). All the samples (**I**–**V**) were cooled immediately and stored at –20–25 °C. They were freeze-dried to a constant weight in a random order within the next 3 months. The lyophilized samples were stored in a desiccator in a freezer room (**I**, **II**, **IV**, **V**) or in nylon boxes in a freezer containing silica gel (**III**, **V**) until being sent to Finland.

***Table 3.*** *The berries of the selected Vaccinium species investigated in Studies **I**–**V**.*

| Botanical name | Sample | Number of populations | Location/ Latitude N | Altitude m (a. s. l.)[a] | Country | Study |
|---|---|---|---|---|---|---|
| *V. myrtillus* | Berries | 1 | Kuusamo/68°05" | 300 | Finland | **I** |
| *V. vitis-idaea* | Berries | 1 | Hankasalmi/62°2 | ND[b] | Finland | **I** |
| *V.* × *intermedium* | Flowers | 1 | Oulu/65°01" | ND | Finland | **I** |
| | Berries | 1 | Oulu/65°01" | ND | Finland | **I** |
| *V. myrtillus* | Berries | 20 | 60°21"–68°34" | 12–308 | Finland | **II,V** |
| *V. arctostaphylos* | Berries | 5 | 40°35"–41°40" | 600–1250 | Turkey | **III** |
| *V. uliginosum* | Berries | 15 | 60°23"–68°34" | 20–300 | Finland | **IV** |
| *V. myrtillus* | Berries | 10 | 39°41"–41°52" | 1300–2290 | Turkey | **V** |

[a] a. s. l. = above sea level
[b] ND = Not Determined

## 4.2 Methods

*Extraction*

The extractions were performed in duplicate. The extraction solution consisted of 10% solvent A and 90% solvent B. The solvents used were MeCN:MeOH (85:15 v/v) (A) and 8.5% aqueous HCOOH (B). Studies **II–V**: Freeze-dried berries were ground into a powder, weighed (270mg), and 4 mL of extraction solution was added. The sample was vortexed (1 min), sonicated (10 min), vortexed again, and centrifuged (4500 rpm, 5 min, 4 °C). The filter cake was re-extracted three times (3 × 2 mL). The supernatants were combined. The volume was adjusted to 10 mL.

Study **I**: Due to a lower amount of material, smaller extraction volumes were used than in the other studies, but with the same mass/volume ratio (27mg/mL). The flowers and berries of *V.* × *intermedium* were extracted with 2 mL. The extractions of the berries of *V. myrtillus* and *V. vitis-idaea* were performed in 5 mL. All the extracts were filtered through a regenerated cellulose filter equipped with a glass prefilter prior to HPLC-analysis. Semi-quantification of PAs was employed according to the procedure published by Toivanen et al. (2009). Aliquots of extracts were incubated at +70 °C for three hours to depolymerize PAs to anthocyanidins. The absorbances were read at 520nm before and after incubation for estimation the content of proanthocyanidins as an increase of absorption.

*HPLC-DAD -analyses*

The used RP-HPLC-DAD method was originally optimised for the separation and quantification of anthocyanins in all studied berries. It was also found to be suitable for the quantification of flavonol glycosides in the berries of *V. uliginosum*. In the case of the berries of *V. myrtillus* and *V. arctostaphylos*, the levels of flavonols were too low for quantification. Anthocyanins were detected at 520nm, flavonols at 360nm and phenolic acids and PAs at 280 nm, respectively. The characterization of the studied compounds was performed using the spectroscopic and/or retention properties of the aglycone and/or sugar(s) moieties (Goiffon et al. 1991, Mazza et al. 2004, Koponen et al. 2008).

The chromatographic system consisted of a Hewlett-Packard (Waldbronn Analytical Division, Germany) instrument with a quaternary pump, an autosampler (HP 1050), and a DAD (HP 1040M). Analytical separation of the studied compounds was carried out using a 150 × 4.6 mm i.d., 5 μm, Phenomenex Gemini C-18 column equipped with a 4 × 3 mm C-18 precolumn. The gradient program was composed of two mobile phases, MeCN/MeOH (85:15) and aqueous 8.5% HCOOH. It is described in detail in

Study **II**. A low pH (below 2) was used in order to stabilize anthocyanins in the form of flavylium cations. The injection volume was 20 μL. The equilibration time between runs was 3 min. Two anthocyanin quality control standards were analyzed in the beginning and at the end of every run series to control for any possible fluctuations in the response of anthocyanins.

The stability of the anthocyanin standard solutions was tested at the pH value 1.6 and in the concentrations of 2, 5, 10, 70, 100 and 200 μg/mL. Standards proved to be stable for at least 2 months at 9 °C in the dark (**II**). The coefficients of variation of peak areas of six standards between four measurements during 2 months were 2.4–5.4%, on average 3.4%. These values were considered to be acceptable.

The repeatability of retention times was tested (within-day precision) by analyzing the standard solution (100 μg/mL) 7 times within 1 day (**II**). The coefficient of variation was 0.3%. The repeatability of the peak area (within-day precision) was studied by seven injections (20 μL, 100μg/mL). It was found to be 0.3%. The between day precisions of the standards (70, 100 μg/mL) were also studied during 3 weeks in 20 runs. The repeatabilities (CV%) of peak areas were 2.1 and 3.2, respectively. The determined values were considered to be acceptable.

*HPLC-ESI-MS -analyses*

Studies **I**, **III**, **IV**: Hydroxycinnamic acids, anthocyanins, flavonols and PAs were tentatively identified by their UV/Vis spectra, elution order and ESI-MS fragmentation patterns (Koponen et al. 2008, Hokkanen et al. 2009). In study **I**, NMR-identified (Riihinen et al. 2013) quercetin glycosides isolated from the berries of *V. vitis-idaea* were used as standards. The commercial standards were cyanidin 3-glucoside, quercetin 3-glucoside, syringetin 3-galactoside and syringetin 3-glucoside. Tandem MS (MS/MS) was used to characterize individual compounds in the separate ionization and fragmentation steps. $MS^3$ spectra were acquired by fragmenting the two major target ions observed in the $MS^2$ spectra.

The HPLC-ESI-MS system consisted of a Finnigan Survey HPLC and a Finnigan LTQ ion trap mass spectrometer (Thermo, San Jose, CA, USA). The column and organic mobile phase were the same as those in HPLC-DAD analyses. In the case of the aqueous phase, 8.5% HCOOH was replaced with 1% HCOOH to achieve the lowest possible detection limit in the positive ion mode (Valls et al. 2009). The gradient program was slightly modified in order to have the first peaks to elute earlier. According to our studies, the retention order of anthocyanins is constant and not dependent on the strength of HCOOH in the mobile phase

(Jaakola et al. 2002, Määttä et al. 2003, Määttä-Riihinen et al. 2004). The gradient program is described in detail in Study **III**.

Conditions for the initial ionization in the positive ionization mode included capillary voltages at +4.5 kV and a temperature of 225 °C. Full scan mass spectra were measured from *m*/*z* 250 to 700. Tandem MS (MS/MS) was performed using helium as the collision gas. The collision energy was set at 35%. MS revealed the positive molecular ions, and MS/MS broke down the most abundant ions with dependent collision-induced dissociation (CID). The data was analyzed with Finnigan Xcalibur 1.4 SR1 software (Thermo Fischer Scientific Inc., Waltham, MA, USA).

*Quantification*

The purity of the standards were checked by HPLC monitoring the chromatogram at 520nm (cyanidin 3-glucoside) and at 360nm (quercetin 3-glucoside). A six-point anthocyanin calibration curve was generated from duplicate standards of cyanidin 3-glucoside (2, 5, 10, 70, 100 and 200 μg/mL), the linearity of which was found to be acceptable ($R^2 > 0.998$). Flavonols were quantified by dissolving duplicate standards of quercetin 3-glucoside in solvent A (10%) and solvent B (90%) similarly to cyanidin 3-glucoside. The linearity of a five-point calibration curve (4, 15, 50, 90 and 120 μg/mL) was found to be acceptable ($R^2 > 0.9996$).

*Statistical analyses*

The variations in anthocyanin (**I**, **IV**) and flavonol (**IV**) levels between Finnish populations and regions were evaluated using one-way analysis of variance (ANOVA) and the Tukey HSD procedure. The evaluation and comparisons of the anthocyanins between Finnish and Turkish populations were carried out using the non-parametric Mann-Whitney U test (**V**). A logistic regression model was chosen to estimate the probability that the bilberry sample would fall into the correct origin according to the anthocyanidin glycoside content and/or glycone moiety (**V**). All analyses were performed with SPSS for Windows 14.0, (**I**) or 16.0 (**IV**) or 17.0 (**V**), respectively. Differences reaching a confidence level of 95% were considered as statistically significant.

# 5 Results and discussion

## *5.1* The distinctive features of phenolic profiles in the berries *of V. × intermedium, V. myrtillus, V. vitis-idaea, V. uliginosum* and *V. arctostaphylos (I–V)*

### 5.1.1 Handling and analyses of samples

To minimize degradation of compounds to be analyzed in the large set of samples they were freeze-dried. All the samples were analyzed in random order. The large sets of samples (**II**, **IV**) were analyzed within a year, the other samples within two months. The RP-HPLC-DAD method was developed and optimized to quantify the major anthocyanins in bilberries (*V. myrtillus*) in Studies **II** and **V**. Moreover, the good separation of bilberry anthocyanins (**II**: ***Figure 3***) permitted their quantification in Caucasian blueberries (*V. arctostaphylos*) (**III**), bog bilberries (*V. uliginosum*) (**IV**), lingonberries (*V. vitis-idaea*) (**I**), hybrid berries (*V. × intermedium*) and flowers (**I**).

The method proved to be valid for the quantification of flavonol glycosides in the berries of *V. uliginosum* (**IV**). It was suitable for the tentative identification of the flavonol glycosides and hydroxycinnamic acid derivatives in the berries and flowers of *V. × intermedium*, berries of *V. myrtillus* and *V. vitis-idaea* in LC-ESI-MS analysis, respectively.

The responses of the two quality control standards (cyanidin 3-glucoside) at the beginning and at the end of run series (n=20) varied by 8% (70 μg/mL) and 9% (100 μg/mL), respectively. External calibration method was chosen for anthocyanins (all studies) and flavonols (**IV**), since 15 closely eluting *V. myrtillus* anthocyanin peaks did not allow using internal standard.

*Methodological considerations about anthocyanin analyses*

The blue-black *Vaccinium* berries contain generally at least 15 anthocyanins to be quantified, such as bilberries (*V. myrtillus*). A challenge in the quantification of anthocyanins by HPLC is the unavailability of most standards on a commercial basis. Furthermore, some of those that are commercialized are sold in small quantities and are very expensive. In addition, if one aims to isolate the standards, the isolation of each anthocyanin is considered problematic mainly because of the difficulties of preparing crystalline anthocyanins with high purity grade and in sufficient amounts to allow reliable weighing under optimal conditions.

Each anthocyanin has unique color which is related to its molecular structure and the size of energy transitions associated with

absorption of visible light. Furthermore, anthocyanins exist in various structural forms (e.g. quinoidal base, the carbinol pseudobase, the chalcone pseudobase), the relative amount of which vary with both pH and the structure of anthocyanin (Brouillard 1982, Heredia et al. 1998). At pH below 2 (in this study) anthocyanins exist mainly in basic cationic form in eqilibrum with those mesomeric forms (Brouillard 1982).

Due to these difficulties and large sets of samples to be analysed, the external standard method was chosen for quantification, i.e. separated anthocyanins were individually calculated against on commercially available external anthocyanin standard (cyanidin 3-glucoside). It did not give a real amount of individual anthocyanins because of the differences in their molar absorptivities due to differences in their molecular structure and mobile phase composition during a gradient HPLC-run.

The amount of the variation caused by using one external calibration standard can be evaluated to some extent. Kähkönen et al. (2003) found that the relative proportions of 3-glucosides of delphinidin, petunidin and malvidin were 0.3–0.9% lower compared with quantification using cyanidin 3-glucoside as a standard instead of the corresponding standard. Due to the lower relative proportions of these standards, the relative proportion of cyanidin 3-glucoside was in this case 2.2% higher. These methodological aspects would be discussed, especially if the possible differences are small, and cyanidin contents are higher than those of delphinidin ones among studied anthocyanins.

### 5.1.2 Anthocyanins

Despite the variation caused by both analytical and environmental factors, the influence of the genetic background on the anthocyanin profiles is still strong i.e. the phenolic profiles of the berries were species-specific. Though the variation is found high within a species in the total anthocyanin contents, the relative proportion of different anthocyanidins is quite constant.

A summary of the relative proportions of anthocyanidins and the total anthocyanin content of *Vaccinium* berries from populations of various origins analysed in this thesis work and in other studies is given in **Table 5** to help in the comparisons and to show distinct traits for anthocyanin profiles of berries of *Vaccinium* species.

*Total anthocyanins*

The berries of *V. myrtillus* (section *Myrtillus*; ***Figure 1***) from Finland analyzed in Studies **I** and **II** contained higher amounts of anthocyanins than the berries of *V. ovalifolium* from the same section (Taruscio et al. 2004). Previously, the berries of *V. ovalifolium* has been reported to be the best source of anthocyanins

among the related species from the section *Myrtillus* (*V. deliciosum*, *V. membranaceum*) and the section *Pyxothamnus* (*V. ovatum*) (Lee et al. 2004a,b, Taruscio et al. 2004). Moreover, the blueberries in the section *Cyanococcus* (*V. angustifolium*, *V. ashei*, *V. corymbosum*, *V. myrtilloides*) seem to have distinctly lower anthocyanin contents compared with the Finnish bilberries (*V. myrtillus*) (Gao & Mazza 1994, Prior et al. 1998, Kalt et al. 1999, Katsube et al. 2003, Määttä-Riihinen et al. 2004 ). The results of this thesis work were similar as reported by Määttä-Riihinen & co-workers (2004), as the berries of *V. vitis-idaea* and *V. uliginosum* were found to have clearly lower anthocyanin content (**I**, **III**, **IV**) than the berries of *V. myrtillus* (**II**).

One factor that contributes to the high anthocyanin content of bilberries is the fact that the fruit pulp of *V. myrtillus* in addition to the skin is intensely colored as opposed to green or white pulp in bog bilberries (section *Vaccinium*) studied in this work, or blueberries (section *Cyanococcus*). For a given volume of fruit, the amount of skin (surface) area increases as berry size decreases. This has lead to the general observation that smaller-sized berries of the same species contain more anthocyanins per unit volume (Prior et al. 1998). However, berry size-anthocyanin content relationship was not found among lowbush (*V. angustifolium*) or southern highbush (*V. corymbosum*) clones (Kalt et al. 2001) but Prior et al (1998) did found it in some *V. corymbosym* cultivars. Anyway, the relationship was shown to break down when compared the anthocyanin contents between the berries of early and late harvest dates: the total anthocyanin content increased, but the surface/volume estimate did not change. Moyer et al. (2002) did observe a high positive correlation for highbush blueberries, but not for eight other *Vaccinium* species and hybrids: they found that larger berries of one *Vaccinium* species may still contain more anthocyanins as compared to smaller berries of other species. For example, the berries of *V. ashei* were relatively large, but still contained the highest amount of anthocyanins within 31 *Vaccinium* samples (Moyer et al. 2002). Environmental factors such as water availability or the differences in the distribution of anthocyanin pigments in the layers of the peel, and the thickness of the peel, may influence on this relationship.

The berries of *V.* × *intermedium* had higher total anthocyanin content compared to the berries of *V. deliciosum*, *V. membranaceum* and *V. parvifolium* from the section *Myrtillus* analysed in the previous studies (***Table 4***). Similarly, the red colored berries of *V. parvifolium*, *V. oxycoccus* and *V. vitis-idaea* exhibited lower anthocyanin contents (***Table 4***) than *V.* × *intermedium*. This is most probably due to its other parent species with berries known to be anthocyanin rich, i.e. *V. myrtillus*. In some cases, the berries of *V. uliginosum* of some Finnish and Chinese origins have been re-

ported to contain more anthocyanins than the berries of *V.* × *intermedium* (***Table 4***).

On the basis of the results of this work, the decreasing order of the anthocyanin contents analysed by the same method was *V. myrtillus* > *V.* x *intermedium* > *V. arctostaphylos* > *V. uliginosum* > *V. vitis-idaea*. However, the difference between the berries of *V. arctostaphylos* and *V. uliginosum* is not very high and the results are only from the one season. Excessive generalisation should avoid, since the number of the samples (genotypes, clones) within a population and the number of the studied populations is low in many reported studies (***Table 4***). There may be differences in the phenotypic plasticity of the production of anthocyanins between genotypes and populations, and the environmental factors vary between seasons. Therefore, further studies are needed.

*Qualitative comparisons of anthocyanin profiles*

The characterization of anthocyanins was performed by using spectroscopic and retention properties of the aglycone and sugar moities. At first, peaks were grouped by studying their spectroscopic properties at 510–540 nm. The difference of 8nm in the absorption maxima between the glycosides of delphinidin/petunidin/malvidin (526 nm) and those of cyanidin/peonidin (518 nm) enabled their differentiation of the resolved peaks into two groups. The tentative identification of the RP-HPLC-DAD peaks was further confirmed according to ESI-MS and literature.

Blue-black colored *Vaccinium* berries contain at least five anthocyanidins (***Table 4***) which are attached to sugars. The peaks of 15 major anthocyanidin glycosides of bilberries (*V. myrtillus*) were well separated in acid RP-HPLC conditions (**II**: ***Figure 3***). The profile (**I**, **II**) was in accordance with the previously published studies (e.g. Jaakola et al. 2002, Koponen et al. 2007).

Additionally, minor anthocyanins, cyanidin hexose-hexoside and coumaroylated anthocyanidin hexosides were tentatively identified (**I: *Table 1*)**. Previously, acylated anthocyanins have not been found in the berries of *V. myrtillus*, but for example, the berries of *V. angustifolium* from the section *Cyanococcus* have contained a remarkable portion of acylated anthocyanins (Kalt et al. 1999).

No anthocyanidin sambubiosides were detected in the present study, even though sambubioside conjugates have been previously found from bilberry fruit (Du et al. 2004). With respect to the other *Vaccinium* berries, anthocyanidin 3-*O*-sambubiosides have been found in the berries of *V. padifolium* (Cabrita & Andersen 1999) from the section of Hemimyrtillus (***Figure 1***).

The berries of *V. vitis-idaea* consisted of three major anthocyanins (**I: *Table 2***) in accordance with the other studies

(Andersen 1985, Ek et al. 2006, Grant & Helleur 2008). The berries of *V.* × *intermedium* contained all the same 15 major anthocyanins (**I:** ***Table 2***) as the berries of the other parent species, *V. myrtillus*.

The anthocyanins in the berries of *V. arctostaphylos* were separated and tentatively identified by HPLC-DAD and HPLC-ESI-MS systems for the first time (**III**). The characteristic HPLC-DAD chromatogram at 520 nm was obtained with 19 anthocyanin peaks (**III**: ***Figure 3***), whereas HPLC-ESI-MS revealed 26 anthocyanins (**III**: ***Table 1***). Furthermore, tentatively identified anthocyanidin sambubiosides were typical for these berries similarly to the berries of *V. padifolium* (Cabrita & Andersen 1999) from the same section of *Hemimyrtillus*.

The berries of *V. uliginosum* were found to be devoid of anthocyanidin sambubiosides. Individual anthocyanins were detected at 520 nm and quantified by means of HPLC-DAD. They were designated with numbers (1–11, 13–15, 17, 18, 23) as shown in **Figure 8**. The new minor ones were subsequently identified via HPLC-ESI-MS detection and coded with letters (A, C) (**IV**: ***Table 3***).

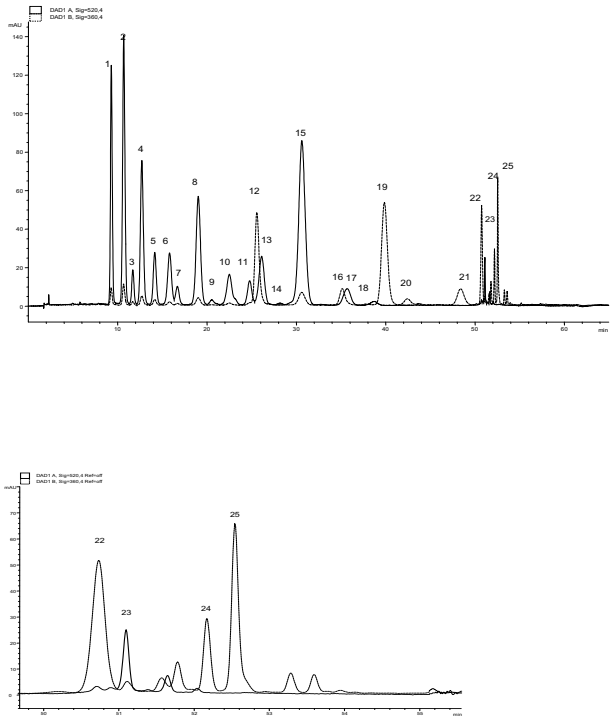


***Figure 8***. HPLC-DAD profile of the anthocyanins and flavonols in bog bilberries (*V. uliginosum*) monitored at 520 and 360 nm.

*Quantitative comparisons of anthocyanin profiles*

The major anthocyanidins in the berries of *V. myrtillus* were delphinidin and cyanidin. The minor anthocyanidin was peonidin (**II**: ***Figure 3***; ***Table 4***). On the basis of anthocyanidin profile, the berries of *V. myrtillus* might be distinguished from blueberries from the section of *Cyanococcus* (***Figure 1***). In general, the greatest difference seems to be in the relative proportion of malvidin, which was over 10% greater in *V. corymbosum* (Kalt et al. 1999, Taruscio et al. 2004). The berries of *V. ovatum* from the section of *Pyxothamnus* (***Figure 1***) had at least 22% greater (Ballington et al. 1988b, Taruscio et al. 2004) relative proportion of cyanidin compared to the berries of *V. myrtillus* (n=20; **II**).

Differently to bilberries, the major anthocyanidins in the berries of *V. uliginosum* (section *Vaccinium*; ***Figure 1***) were found to be delphinidin and malvidin (**IV**). The minor compounds were peonidin and cyanidin. However, a clearly higher (~20%) relative propor-

tion of delphinidin and a lower (26%) proportion of malvidin have been reported for the *V. uliginosum* berries from two populations of the northwestern United States (Taruscio et al. 2004). It is known that *V. uliginosum* is a genetically and phenotypically very variable species (Jacquemart 1996, Alsos et al. 2005) which may be reflected in its anthocyanidin profile.

The berries of *V. arctostaphylos* from the section of *Hemimyrtillus* (***Figure 1***) had three major anthocyanidins with the dominance of delphinidin (***Table 4***). It was followed by almost equal shares of petunidin and malvidin, respectively (**III**). The proportion of delphinidin was about 9% greater than that found in the berries of *V. padifolium* from the same section (Cabrita & Andersen 1999). It was up to 28% greater than that in the berries of *V. ovatum* from the section *Pyxothamnus* (Ballington et al. 1988b, Lee et al. 2004b) enabling their differentiation.

The rather low relative proportion (≤15%) of peonidin seems to be a common feature for most bilberries and blueberries in the sections *Hemimyrtillus*, *Myrtillus*, *Cyanococcus*, and *Vaccinium* (Ballington et al. 1988a, Cabrita & Andersen 1999, Lee et al. 2004b, Taruscio et al. 2004). Conversely, a high relative proportion peonidin (>45%) is a typical characteristic of northern cranberries (*V. oxycoccus*) from the section Oxycoccus (***Figure 1***) (Andersen 1989, Määttä-Riihinen et al. 2004). The similarly red colored berries of *V. vitis-idaea* from the section *Vitis-idaea* (***Figure 1***) contained mainly (≥ 99%) cyanidin derivatives (**I**).

There are considerable differences between the relative proportions of different glycone moieties of anthocyanins in berries of *Vaccinium* species. The anthocyanidins in the berries of *V. myrtillus* (average ± SD; n = 179) were mainly combined to glucosides (39 ± 10%) followed by galactosides (32 ± 6%) and arabinosides (29 ± 5%) (**II**). Similarly, the glucoside conjugates formed the major part of the anthocyanins in the berries of *V. uliginosum* (n = 137), but in the higher relative proportion (59 ± 9%) (**IV**). Galactosides (19 ± 7%) and arabinoside derivatives (20 ± 4%) of *V. uliginosum* anthocyanins occurred in nearly equal proportions. Xylosides were the less extensively represented (2 ± 1%).

Instead, anthocyanidin glucosides formed the lowest relative proportion (~ 4%) in the berries of *V. vitis-idaea* (**I: *Table 2***). For the galactosides and arabinosides, the respective values were ≈78% and ≈17%. The highest proportion of anthocyanidin glucosides was detected in the berries of *V. arctostaphylos*, 61 ± 7% (**III**). For the other sugar moieties the relative proportions were 11 ± 2%, 22 ± 4%, 5 ± 1% and 1% for galactosides, arabinosides, xylosides and sambubiosides, respectively. The relative proportion of glucosides in the berries of *V. arctostaphylos* was over 50% higher than those of reported in the berries of *V. ovatum* (section *Pyxothamnus*) and

V. *membranaceum* (section *Myrtillus*) (Ballington et al. 1988a,b, Lee et al. 2004b).

Despite the finding that berries both of the species of *V. myrtillus* and *V. uliginosum* of some plant individuals (n=7 for bilberries; n= 2 for bog bilberries) had exceptionally low relative proportions of anthocyanidin glucosides, < 4% and <10% (**II**, **IV**), in general, characteristic differences between the species could still be found.

### 5.1.3 Flavonols

Flavonol profiles were found to be distinctive among the berries of *V. myrtillus*, *V. uliginosum*, *V. arctostaphylos* and *V. vitis-idaea* both qualitatively and quantitatively in this thesis work. The average (n = 137) content of the total flavonols in the berries of *V. uliginosum* was 1133mg/100 g of DW (154 mg/100g FW) (**IV**). Bog bilberries were rich in flavonol glycosides and approximately one-quarter (22%) of them contained greater amounts of flavonols than anthocyanins.

*Qualitative comparisons*

In this study, the anthocyanins and flavonol glycosides in the berries of *V. uliginosum* were simultaneously extracted for analysis via RP-HPLC-DAD. The quantified flavonol glycosides monitored at 360nm were designated with numbers (12, 16, 19–22, 24, 25) and shown in ***Figure 8***. The peaks assigned as flavonol glycosides exhibited typical UV/Vis spectra with shapes and characteristic absorption maxima in the region of 354–358 nm (**IV**: ***Table 3***).

Fourteen flavonol glycosides (i.e. myricetin, quercetin, laricitrin, isorhamnetin, syringetin, kaempferol conjugates) were tentative identified in the berries of *V. uliginosum* on the basis of ESI-MS data, elution order, literature and available standards (**IV**: ***Table 3)***. Of them, the kaempferol and isorhamnetin aglycons were tentatively identified for the first time. All the same aglycone conjugates were detected in the berries of *V. myrtillus*, except kaempferol (**I:** ***Table 4***).

Laricitrin, isorhamnetin, and syringetin are less frequently found aglycones in *Vaccinium* berries. All these three aglycone moieties have been recently found in the berries of blueberry hybrid cultivars (e.g. cv. Legacy) from the section Cyanococcus (Brevis et al. 2008, Vrhovsek et al. 2012, USDA Grin 2013). Both laricitrin and isorhamnetin have been previously detected in the berries of *V. myrtillus* (Koponen et al. 2008). The latter also occurred in the berries of *V. macrocarpon* (cv. Stevens) (Yan et al. 2002).

Quercetin 4″-(3-hydroxy-3-methylglutaroyl)rhamnoside (HMG-rhamnoside) was present in the berries of *V. vitis-idaea* (**I**: ***Table 4***).

It was not detected in the berries of *V. myrtillus* and *V. uliginosum* (**I**, **IV**). In contrast to acylated anthocyanins, acylated flavonol glycosides have been more rarely reported. Recently, Vrhovsek et al. (2012) reported the presence of quercetin 3-glucoside acetate in the berries of blueberry hybrid cultivars (e.g. cv. Legacy).

Quercetin galactoside, glucoside, glucuronide and pentoside were detected in both berries and flowers of *V.* × *intermedium* (**I: *Table 4***). Furthermore, galactosides and glucosides of myricetin were found. They were absent in the berries of *V. vitis-idaea*. Laricitrin hexoside and glucuronide were found only in the berries of *V. myrtillus*. Kaempferol hexoside and deoxyhexoside were detected in hybrid berries and lingonberries but not in bilberries (**I**).

A quercetin 3-HMG-rhamnoside was not found in the berries of *V.* × *intermedium* (**I**). Syringetin hexoside was the only flavonol derivative detected in both parent species but lacking in the hybrid. The biosynthesis of syringetins occurs via the methylation of OH-group in the position 5′ of laricitrins. In the berries of *V.* × *intermedium*, the occurrence of the minor relative proportion of malvidin (4%) that also require methylation of OH-group in the position 5′ of delphinidins suggests low activity of *O*-methyltransferase (5'–OMT). This could explain the lack, or at least very low levels of syringetin in hybrid berries.

*Quantitative comparisons*

Study **IV** was the first quantitative study of five flavonol aglycone conjugates in the berries of *V. uliginosum*. Quercetin has been reported to be more abundant than myricetin in the Nordic berries of *V. uliginosum* (Häkkinen et al. 1999b, Häkkinen & Törrönen 2000, Määttä-Riihinen et al. 2004). This was found to be true for most of the individuals in Study **IV**, though myricetin dominated in 17 plant individuals (of the total of 137). The average relative proportions of the major flavonols were 35% for myricetin, and 53% for quercetin in bog bilberries. The minor flavonols, laricitrin, isorhamnetin, and syringetin occurred below relative proportions of 10%. Quercetin has been reported to be the major flavonol in blueberries (*V. angustifolium* × *V. corymbosum*, *V. corymbosum*, *V. deliciosum*, *V. membranaceum*, *V. ovalifolium*, and *V. ovatum*) and bilberries (*V. myrtillus*) from the sections *Cyanococcus*, *Myrtillus*, and *Pyxothamnus* (Sellappan et al. 2002, Zheng & Wang 2003, Taruscio et al. 2004, Riihinen et al. 2008).

### 5.1.4 Other phenolic compounds

The number of the tentatively identified flavan-3-ols and PA dimers was about two times higher in the berries of *V. vitis-idaea* than in those of *V. myrtillus* (**I: *Table 3***). Previously, both prodel-

phinidins and procyanidins have been detected in the berries of *V. myrtillus* and *V. oxycoccus*, whereas only procyanidins have been observed in the berries of *V. uliginosum* and *V. vitis-idaea* (Määttä-Riihinen et al. 2004, Hellström et al. 2009). Accordingly, both (-)-(epi)gallocatechin and (–)-epicatechin were detected in the berries of *V. myrtillus* in this study and (–)-epicatechin and (+)-catechin in the berries of *V. vitis-idaea* (**I**). The number of tentatively identified PAs was in hybrid berries two times higher than in bilberries but at about the same levels as in lingonberries.

PAs are the predominant phenolic compounds in the berries of *V. vitis-idaea* and *V. microcarpon*. They have constituted a portion of over 60% of the phenolic compounds in the berries of both these species (Kylli et al. 2011). Correspondingly, the level of PAs in lingonberries was found to be eight times higher (8000mg/100g DW) than in bilberries as estimated by the semiquantitative spectrophotometric method (**I)**.

The distribution of hydroxycinnamic conjugates was quite similar in the berries of *V. myrtillus* and *V. vitis-idaea*, except that caffeoyl hexose was not detected in the latter berries (**I: *Table 4***). Two caffeoylquinic acid isomers (See Chapter **2.2.1** Phenolic acids) were detected in the berries of both of these species. The one is probably chlorogenic acid (5-*O*-caffeoylquinic acid) which has been quantified as the dominant phenolic acid in the berries of *V. myrtillus*, whereas another might be neochlorogenic acid, which has been quantified as minor one (HPLC-DAD) (Može et al. 2011, Kusznierewicz et al. 2012, Prencipe et al. 2014). However, there are no published data about caffeoylquinic acid isomers in the lingonberries.

*Flowers vs. berries*

The flowers of *V.* × *intermedium* contained only cyanidin glycosides (**I: *Table 2***) similarly to the flowers of *V. myrtillus* reported by Jaakola et al. (2002). The content of anthocyanins was forty times higher in hybrid berries compared with the flowers. The opposite was in the case of PAs**.**

The flowers of *V.* × *intermedium* showed a more diverse profile of hydroxycinnamic acids than the berries. They contained twice the number of hydroxycinnamic acid conjugates than the berries (**I**: ***Table 4***). Previously, bilberry and blueberry flowers have been showed to contain considerably higher levels of hydroxycinnamic acids than the respective berries (Riihinen et al. 2008).

### 5.2 Heritability of phenolic compounds in hybridization (I)

The berries and flowers of the natural hybrid (*V.* × *intermedium*) between *V. myrtillus* and *V. vitis-idaea* exhibited characteristics in-

herited from both parent species in the distribution and contents of phenolic compounds. In comparison with the parent species, hybrid berries possessed a more diverse profile of phenolic compounds (**I:** ***Tables 1, 3, 4***)) similarly to previously studied hybrid leaves (Hokkanen et al. 2009). This may be explained in the following manner: qualitatively the mode of inheritance of most phenolic compounds is Mendelian with dominance. If both parents produce a chemical, then the hybrids will almost always produce it; furthermore when only one parent synthesizes a chemical, the hybrid usually produce it as well (Orians 2000).

The berries of *V.* × *intermedium* contained all the same 15 major anthocyanins and 3 minor acylated compounds as those of *V. myrtillus* (**I**: ***Table 1***). The inheritance from *V. vitis-idaea* was showed in the predominance of cyanidin. The proportions of delphinidin, petunidin and malvidin were lower in hybrid berries than in bilberries (**I**: ***Table 2***).

The effect of hybridisation between species on the glycone moieties of anthocyanins is difficult to evaluate. In the thesis study, the relative proportions of galactosides, glucosides and arabinosides in the berries of *V.* × *intermedium* were between those of the parent species. In a study of interspecific hybrids between *V. macrocarpon* and *V. oxycoccus*, the proportions of the glucosides and galactosides were intermediate between those of the parental species (Vorsa & Polashock 2005). However, the proportion of arabinosides was lower than that present in either of the two hybrids. The authors suggested that the glycosylation was more likely to be controlled by more than one locus.

A-type PAs have been shown to be typical for the berries of both species, *V. vitis-idaea* and *V. myrtillus* (Määttä-Riihinen et al. 2004). It was found in this study that they were inherited by the hybrid. The inheritance of PAs in hybrid berry was qualitatively closer to lingonberry (**I**: ***Table 3***) but quantitatively to bilberry. Overall, if one considers the anthocyanin and PA profiles, it can be concluded that hybrid berries had inherited many of the special characteristics of both parents, i.e. the diverse anthocyanin profile from bilberry and the PA profile from lingonberry. The levels of PAs were lower in the hybrid berries than in lingonberry but the anthocyanin content of hybrid berries was clearly closer to bilberry than lingonberry.

***Table 4.****The anthocyanidin profiles of berries of Vaccinium species from 9 sections showing their characteristic relative proportions of aglycone moieties and variable total anthocyanin contents. The distant geographical origins and methods used in the studies are also summarized.*

| ***Vaccinium*** **Section** ***species*** | **Country, region** | **n**[a] | **year** | **Relative proportions (%) (lower bound–upper bound)** **Delphinidin** | **Cyanidin** | **Petunidin** | **Peonidin** | **Malvidin** | **Other** | **Acyl-ated** | **Total** **DW** | **FW** | **Method**[b] | **Ref.** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *V. × intermedium* Ruthe | Finland, N | 1 | 2009 | 17 | 66 | 5 | 8 | 4 | | | 2131 | 320 | FD, MS | [1] |
| *Myrtillus* Dumort. | | | | | | | | | | | | | | |
| *myrtillus* L. | Finland, S, C, N[c] | 20 | 2005 | 33 ± 5 (25–43) | 33 ± 4 (26–40) | 14 ± 1 (13–16) | 7 ± 2 (4–11) | 13 ± 2 (9–15) | | | 2894 ± 607 (1934–3877) | 412 ± 52 (350–525) | FD | [2] |
| myrtillus | Finland, N | 1 | 2007 | 38 | 31 | 14 | 5 | 12 | | tr[d] | 3067 | 429 | FD, MS | [1] |
| *myrtillus* | Finland | 1 | 2000 | 35 | 36 | 14 | 5 | 10 | | | NA | 536 | FD, H | [3] |
| *myrtillus* | Finland, S | 1 | 2002 | 34 | 38 | 10 | 2 | 15 | 1 (u.i.)[e] | | NA | 1201 | | [4] |
| *myrtillus* | Finland | 1 | 2003 | 36 | 31 | 12 | 6 | 15 | | | NA | 904 | | [5] |
| *myrtillus* | Finland | 1 | 2005 | 35 | 35 | 12 | 6 | 12 | | | NA | 767 | | [5] |
| *myrtillus* | Finland | 1 | 2002 | 39 | 33 | 12 | 5 | 11 | | | NA | 922 | | [6] |
| *myrtillus* | Sweden, S, C N; Denmark, C | 4 | 2007 | 38 ± 4 (32–42) | 34 ± 6 (28–42) | 15 ± 1 (13–16) | 5 ± 1 (4–6) | 9 ± 2 (7–10) | | | 3985 ± 1290 (3135–5889) | NA | FD, H | [7] |
| *myrtillus* | Sweden, S, C, N; Denmark, C | 5 | 2008 | 41 ± 4 (36–45) | 33 ± 3 (30–37) | 14 ± 1 (13–16) | 4 ± 1 (3–5) | 8 ± 1 (7–9) | | | 3034 ± 572 (2343–3782) | NA | FD, H | [7] |
| *myrtillus* | Slovenia. NW, N, C, NE | 7 | 2007 | 41 ± 4 (37–49) | 30 ± 2 (27–34) | 14 ± 1 (13–16) | 6 ± 2 (3–8) | 8 ± 3 (4–12) | | | NA | 1188 ± 209 (982–1593) | RD, SPE | [8] |
| *myrtillus* | Austria, C | 3 | 2004 | 41 ± 4 (37–44) | 26 ± 1 (25–26) | 17 ± 1 (17–18) | 16 ± 4 (pn + mv) (13–20) | | | | 2050 ± 566 (1670–2700) | NA | AD, MS | [9] |
| *myrtillus* | Austria, C | 3 | 2005 | 42 ± 1 (42–43) | 25 ± 1 (25–26) | 18 ± 1 (17–18) | 15 ± 1 (pn + mv) (14–15) | | | | 1777 ± 533 (1370–2380) | NA | AD, MS | [9] |
| *myrtillus* | Montenegro N, NE | 11 | 2009 | 37 ± 4 (32–42) | 32 ± 3 (28–37) | 9 ± 1 (8–9) | 6 ± 1 (5–8) | 16 ± 3 (11–21) | | | NA | 246 ± 67 (127–337) | H | [10] |

***Table 4*** *continues.*

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *myrtillus* | Turkey, N, NW, NE, MW | 10 | 2008 | 34 ± 6 (22–40) | 34 ± 8 (23–51) | 14 ± 3 (9–18) | 7 ± 2 (5–12) | 11 ± 2 (7–15) | | | 2651 ± 781 (1708–3647) | 475 ± 187 (241–795) | FD | [11] |
| *deliciosum* | USA, NW | 1 | 1985 | 28 | 35 | 13 | 9 | 15 | | | NA | NA | pa | [12] |
| Piper *deliciosum* | USA, NW | 2 | 2001 | 41 (40–42) | 31 (27–34) | 12 12 | 5 (4–6) | 12 (8–15) | | | NA | 152 ± 2 (151–154) | H | [13] |
| *membranaceum* | USA, NW | 1 | 1985 | 13 | 62 | 5 | 13 | 7 | | | NA | NA | pa | [12] |
| Douglas *ex* Torr. *membranaceum* | USA, NW | 3 | 2001 | 31 ± 3 (29–34) | 45 ± 3 (43–48) | 9 ± 0,4 (9–10) | 7 ± 2 (5–9) | 8 ± 2 (6–10) | | | NA | 199 ± 18 (179–215) | H | [13] |
| *ovalifolium* | USA, NW | 2 | 1984 | 31 | 28 | 15 | 8 | 17 | | | NA | NA | pa | [12] |
| Sm. *ovalifolium* | USA, NW | 3 | 1985 2001 | (26–26) 33 (27–37) | (24–31) 45 (41–51) | (13–17) 8 (7–9) | (7–9) 7 (6–9) | (15–18) 7 (6–7) | | | NA | 335 ± 76 (271–419) | H | [13] |
| *parvifolium* | USA, NW | 3 | 2001 | 9 ± 1 (8–9) | 63 ± 3 (61–67) | 9 ± 1 (8–9) | 10 ± 1 (9–11) | 9 ± 1 (8–10) | | | NA | 18 ± 2 (17–19) | H | [13] |
| Sm. *parvifolium* | USA, NW | 1 | 1985 | ND | 100 | ND | ND | ND | | | NA | NA | pa | [12] |
| *Oxycoccus* Koch | | | | | | | | | | | | | | |
| *oxycoccus* L. | USA, NW | 1 | 2001 | 16 | 56 | 3 | 21 | 3 | | | NA | 49 | H | [13] |
| oxycoccus | Norway, S | 1 | 1985 | 2 | 46 | 1 | 46 | 4 | 1 | | NA | 78 [g] | pa | [14] |
| oxycoccus | Finland, E | 1 | 2002 | ND | 45 | ND | 55 | ND | | | NA | 131 | | [4] |
| Oxycoccoides Hook | | | | | | | | | | | | | | |
| *erythrocarpum* Michx. | USA, NW | 2 | 1984 | *ND* | *93* | *5* | *1* | *1* | | | NA | NA | pa | [15] |
| *japonicum* | Norway, SW | 1 | 1985 | ND | 59 | ND | ND | ND | 41 (Pg)[f] | | NA | 113 | | [16] |
| *Vitis-idaea* Koch | | | | | | | | | | | | | | |
| *vitis-idaea* L. | Norway | 1 | 1983 | <0.1 | 100 | ND | ND | ND | | | ND | 174 [g] | pa | [17] |
| *vitis-idaea* | Finland, C | 1 | 2002 | ND | 99 | ND | 1 | ND | | | NA | 204 | | [4] |
| vitis-idaea | Finland, C | 1 | 2009 | ND | 100 | ND | ND | ND | < 1 (u.i.) | ND | 343 | 65 | FD, MS | [1] |

***Table 4*** *continues.*

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Vaccinium* L. | | | | | | | | | | | | | |
| *uliginosum L.* | Finland, S, C, N | 15 | 2005 | 28 ± 4 (22–36) | 7 ± 2 (4–10) | 19 ± 1 (17–21) | 4 ± 1 (3–5) | 41 ± 6 (28–49) | | 1425 ± 398 (1077–1676) | 194 ± 56 (153–245) | FD, MS | [18] |
| *uliginosum* | Finland | 1 | | 32 | 6 | 15 | 4 | 43 | | NA | 219 | | [5] |
| *uliginosum* | Finland, N | 1 | 2002 | 28 | 19 | 14 | 2 | 31 | 6 | NA | 379 | | [4] |
| | Finland, C, N | 2 | 2002 | 35 | 4 | 20 | ND | 38 | 3 | NA | 566 | | [4] |
| *uliginosum* | Norway | 1 | | 31 | 7 | 14 | 5 | 43 | | NA | 233[g] | [i] | [19] |
| *uliginosum* | USA, NW | 2 | 2002 | 48 (48–49) | 22 (22–23) | 12 (11–12) | 3 (2–4) | 15 (15–16) | | NA | 113 (69–157) | H | [13] |
| *uliginosum* | China, NW | 1 | | 41 | 3 | 18 | 3 | 36 | | NA | 560[g] | pa, SPE | [20] |
| *Cyanococcus angustifolium* Ait. A. Gray | Canada, NE | 3 [h] | | 23 ± 1 (22–23) | 12 ± 1 (10–13) | 14 ± 1 (14–15) | 5 (5 – 6) | 31 ± 6 (26–37) | | 15 ± 4 (11–19) | NA | NA | pa | [21] |
| *Conchophyllum* Sleumer *meridionale Sw.* | Colombia | 1 | | 23 | 77 | ND | ND | ND | | | NA | 329 | pa | [22] |
| *Hemimyrtillus* Sleumer *arctostaphylos* L. | Turkey, N | 5 | 2007 2008 | 41 ± 4 (37–45) | 16 ± 5 (11–23) | 19 ± 1 (18–20) | 4 ± 1 (3–5) | 20 ± 3 (16–23) | 1 (u.1.) (0,5–1) | 1420 ± 111 (1299–1562) | 261 ± 21 (239–288) | FD, MS | [23] |
| *padifolium* Sm. | Portugal, Madeira | 1 | 1995 | 32 | 8 | 22 | 2 | 31 | 5 | NA | NA | pa | [24] |
| *Pyxothamnus* (Nutt.) Sleumer *ovatum* Pursh | USA, NW | 1 | 1984 | *14* | *55* | *5* | *15* | *11* | | NA | NA | pa | [15] |
| *ovatum* | USA, NW | 2 | 2001 | 18 (17–19) | 69 (68–71) | 4 4 | 5 5 | 4 (3–4) | | NA | 287 (283–290) | H | [13] |

[a] n = number of populations (or batch); [b] Appreviations used for method features: AD = air drying, FD = freeze drying, H = hydrolysis, SPE = solid phase extraction, MS = identification based also on mass spectrometry detection, RD = drying using rotavapor, pa = comparison based only on peak areas (%); [c] Appreviations used for regions: S = south, C = central, N = north, NE = northeast, NW = northwest, MW = midwest; [d] tr = traces; [e] u. i. = unidentified; [f] Pg = pelargonidin; [g] Total content only using spectrophotometry; [h] Samples were harvested from a wild stand where also the bushes of *V. myrtilloides* were growing; [i] The pulp was removed before the skin was macerated with the extraction solvent..

References used were as follows: [1] Lätti et al. 2011, [2] Lätti et al. 2008, [3] Nyman & Kumpulainen 2001, [4] Määttä-Riihinen et al. 2004, [5] Koponen et al. 2007, [6] Buchert et al. 2005, [7] Åkerström et al. 2010, [8] Mozé et al. 2011, [9] Rieger et al. 2008, [10] Jovancevic et al. 2011, [11] Primetta et al. 2013, [12] Ballington et al. 1988a, [13] Taruscio et al. 2004, [14] Andersen 1989, [15] Ballington et al. 1988b, [16] Andersen 1987a, [17] Andersen 1985, [18] Lätti et al. 2010, [19] Andersen 1987b, [20] Xiao-yan et al. 2010, [21] Kalt et al. 1999, [22] Garzón et al. 2010, [23] Lätti et al. 2009, [24] Cabrita & Andersen 1999.

### 5.3 Geographical variation of flavonoids in the berries of *V. myrtillus* and *V. uliginosum* (II, IV)

#### 5.3.1 Anthocyanins of Finnish bilberries (*V. myrtillus*)

Variations in the anthocyanins of the Finnish bilberries (*V. myrtillus*) of many plant individuals from multiple populations in the different regions (south, central, north) of the same country have not been previously systematically investigated. In Study **II**, significant differences in the contents of the total anthocyanins of bilberries were found between 20 populations (consisting of 179 plant individuals) (**II**: ***Table 1***). Despite these local differences, the total anthocyanin, delphinidin and petunidin contents in the bilberries from the southern regions (60°21"–61°17"N) was significantly lower compared to the central (north of 62°56"N) and the northern regions (**II**: ***Figure 4***). Later, a similar trend with increasing anthocyanidin levels toward north has been reported in the berries of *V. myrtillus* growing in Sweden (Åkerström et al. 2010).

The differences of the 76 Finnish bilberry genotypes of southern and northern origins have been presented by both the phenological and morphological characteristics growing at the same field which has supported the division of the clones into southern and northern genetically different climatic ecotypes. Although the border of climatic ecotypes is not generally distinct, they estimated the transition zone separating these ecotypes lying roughly along the latitude of 64° (Vänninen et al. 1988). These findings suggest that even if *V. myrtillus* is considered a very plastic species; the southern and northern origins in northern Europe have evolved genetically different which has been found in many chacteristics (e.g. Vänninen et al. 1988).

The strong genetic influence on anthocyanin production has been shown by a recent trial (Åkerström et al. 2010) in which the berries of *V. myrtillus* clones of northern origins had the highest anthocyanidin contents even when growing in the same location as the southern clones. Recently, the bilberries of northern clones have been reported to contain more total anthocyanins when grown under controlled temperature and light conditions (Uleberg et al. 2012).

The environmental heterogeneity generates genetic heterogeneity (Linhart & Grant 1996). The maximum difference in altitude between bilberry collection locations was only about 296m. Higher altitudinal differences might have generated more variability as the bilberry clones originating from along an altitudinal gradient (200–1100m) in Scotland have maintained many distinctive morphological and physiological characters when grown in a controlled chamber trial (Woodward 1986). The anthocyanin con-

tents in bilberries from Austria have been found to decrease with altitude (800–1200m) (Rieger et al. 2008).

In the berries of *V. myrtillus*, differences were observed in the relative proportions of anthocyanins (**II**). The delphinidin glycosides dominated (38% for north vs. 29% for south) in the northern bilberries whereas the cyanidin glycosides (31% for north vs. 36% for south) were most common in their southern counterparts (**II**). A higher delphinidin to cyanidin ratio has been detected in more northern clones growing at the same experimental field (Åkerström et al. 2010). The recent results of Uleberg et al. (2012) have indicated a positive effect of low temperature on levels of delphinidin glycosides in bilberries. Similarly, delphinidin accumulation has been enhanced in the pomegranates (*Punica granatum* L.) growing during a cooler season (Borochov-Neori et al. 2011).

In Study **II,** the relative proportion (n = 179) of cyanidin was 17% higher than those reported in the berries of *V. myrtillus* of Italian origin (Martinelli et al. 1986). Nonetheless, they have detected higher contents of cyanidin glycosides in bilberries from more northern countries (Norway, Sweden) whereas delphinidin glycosides dominated in bilberries from Italy and Romania. The geographical coordinates and the number of the samples within the country and from how many populations they were collected within the country, were not informed, which complicates the comparison. The contradictory results compared to Study **II** may be due to geographical trends in the studied countries similar those to found in Finland in Study **II** and/or site-specific or seasonal differences.

A divergent anthocyanin profile was found in the berries of *V. myrtillus* of seven individuals mainly originating from eastern Finland with very low amounts of anthocyanidin glucosides (**II**). The reason for this variation can be traced to genetic origins, but will need to be confirmed in further studies. In the study of Vorsa & Polaschock (2005) tetraploid cranberry (*V. oxycoccus*) had very low relative proportion of cyanidin and peonidin glucosides, 1.5% and 4.7%, respectively. Instead, diploid *V. oxycoccus* had the respective proportions of 19.5% and 54.5%. It is possible that the reduced biosynthesis of anthocyanidin glucosides is a result of hybridization, or that of introgression, which are important sources of genetic variation in wild populations resulting in a complex mixture of parental genes. It has been estimated that hybrids comprise about 6% of the populations in section *Myrtillus* (Vander Kloet & Dickinson 1999).

### 5.3.2 Anthocyanins of Turkish bilberries (*V. myrtillus*)

As far as I am aware, the anthocyanin profiles in the berries of *V. myrtillus* from Turkish populations (**V**: ***Table 1***) representing climatically diverse regions were compared for the first time. The total anthocyanin content was the lowest in the bilberries from midwestern (Balikesir) Turkey (**V**: ***Table 2***), whereas the highest anthocyanin content (3647 mg/100g DW) was observed in bilberries originating from northeastern Turkey (Artvin). In contrast to the situation in Finnish bilberries (**II:** ***Figure 4***)) no latitudinal related effects were found in the contents or in the proportions of anthocyanidins or the total anthocyanins in Turkish bilberries. Although the difference between the highest and the lowest altitude (990m) was clearly higher than in Finland (296m) there were no altitude related effects. One reason for this might be that locations in Turkey were situated in geographically large areas with high altitudinal variations. In the study of Rieger et al. (2008) anthocyanin contents in bilberries have decreased with altitude, where the altitude samples have been collected from the same geographical area (Upper Styria, Austria).

The greater variation in the anthocyanin contents in Turkish bilberries might be due to more significant genetic and environmental heterogeneity. In natural populations, different environments generate different selection pressures, and these, in turn, lead to genetic heterogeneity. Environmental heterogeneity, such as elevation, quite often generates significant barriers to gene flow, e.g. via an effect on phenology. In this way genetic differentiation is promoted among isolated populations (Linhart & Grant 1996).

### 5.3.3 Anthocyanins and flavonols in Finnish bog bilberries (*V. uliginosum*)

The total anthocyanin content in the berries of *V. uliginosum* from the southern region was significantly lower as compared to that in the berries from the central and northern regions (**IV:** ***Figure 2***), in accordance with Study **II**. Still, exceptionally low anthocyanin contents were found in the berries from the most northern population (Ivalo) which was mainly due to the reduced amounts of malvidin in berries from that location. The reason for this phenomenon need to be studied with cloned material of southern and northern origins. The contents of the two nonmethylated anthocyanidins, delphinidin and cyanidin, were significantly higher in the north than in other parts of the country. The content of the methylated anthocyanidin, malvidin, was significantly lower in northern than in central Finland, as in the berries of *V. myrtillus* (**II:** ***Figure 2***).

The flavonol contents in the berries of *V. uliginosum* from the southern and central regions were significantly lower than those

in the berries collected in the north. **(IV**: ***Figure* 2**) The flavonol contents at the population level rose as one moved north, being the highest in the most northern location (Ivalo) (**IV**: ***Table* 2**). There were also significant regional differences in the contents of flavonol glycosides; i.e. there were more myricetin and quercetin glycosides in the berries of *V. uliginosum* originated from the north than from the other regions (**IV**: ***Figure* 2**). More studies from several years will need to be performed to find out if latitudinal differences are a general trend or a curiosity found in this study.

In addition to the variation in the responses of anthocyanidin and flavonol contents in the berries from the northern populations (Ivalo), the variation in the response between studied flavonoid groups was found in the relative proportions (**IV**). The differences in the relative proportions of flavonol aglycons between regions were not as high as in the case of anthocyanidins. The greatest difference (5%) between the south and the north was found in the proportion of quercetin which dominated in the northern origins.

Despite the significant variation between the wild populations and plant individuals, the evaluation of the results of this study suggests that northern climatic conditions favor the biosynthesis of phenolic compounds in wild *Vaccinium* berries, especially those with more OH-groups. Other studies performed later with cloned material of wild *V. myrtillus* of various origins support the major effect of genetic background (Åkerström et al. 2010, Uleberg et al. 2012). Latitude related genetic adaptation of plants to varying environmental conditions in a long-term was seen for example, in the bilberries of northern origins with higher anthocyanin contents and delphinidin/cyanidin ratios than those of southern origins even they were grown at the same experimental field (Åkerström et al. 2010).

Even though the genetic background of cultivated berries has evolved in a different way than those of wild berries analysed in this study, similar kind of results in the latitudinal differences have been obtained in some of the studies with cultivated berries. The strawberries (*F.* × *ananassa*) growing in the northern Italy have been contained significantly more flavonols (quercetin, myricetin) compared to those grown in the south (Carborne et al. 2009). The white and green currants (*Ribes* spp.) growing in the north have had significantly higher total phenolic and hydroxycinnamic acid contents than those growing in the southern Finland (Yang et al. 2013). Hydroxycinnamic acids have been more abundant in the northern black currants (*R. nigrum*) compared to those ones in the south (Vagiri et al. 2013). Furthermore, the berries of *R. rubrum* cultivated in the northern conditions have been higher in the total phenolic and anthocyanin contents (Yang et al. 2013).

However, opposite results have been obtained in the anthocyanins and total phenolic compounds of black currants (Zheng et al. 2012, Vagiri et al. 2013) as well as in the flavonols (Zheng et al. 2012) growing in Finland and Sweden, since the berries growing in the south contained more of those compounds than the berries growing in the north.

When comparing to the latitudinal differences in other plant parts (leaves, needles) of wild species, total flavonols (*J. communis*, *V. myrtillus*), quercetin glycosides (*Betula pubescens* Ehrh.) and the ratio of dihydroxylated/monohydroxylated flavonoids [*Oxyria digyna* (L.) Hill] have increased with latitude (Nybakken et al. 2004, Stark et al. 2008, Martz et al. 2009, Martz et al. 2010), similarly to this study. However, there were differences in contents among studied compound groups, since hydroxycinnamic acids of leaves of *V. myrtillus* and flavones of leaves and needles of *B. pubescens* and *J. communis* have been decreased with latitude (Stark et al. 2008, Martz et al. 2009, Martz et al. 2010).

In all, there is variation in the response between species and within phenolic groups the amount of which would be important to know in authenticity analyses. As the genetic background is the major determinant of phenolic profiles, the adaptation through evolution might contribute the main differences in the responses between species. The phenolic compounds have various functions in plants such as protection from cold temperature and UV-radiation (See **Chapter 2.4**.). The genetic background of wild populations (e.g. *V. myrtillus*, *V. uliginosum*) evolve through natural selection (**Chapter 2.6.4**). In the case of cultivated species, genetic background has been changed through breeding strategies. Thus, their response of phenolic compounds to environmental factors may vary compared to wild species. Some species, such as *Ribes* spp. has undergone breeding selection as early as in the 1400s (Hummer & Dale 2010) whereas blueberry (e.g. *V. corymbosum*; section *Cyanococcus*) is a more recent major fruit crop to be brought under cultivation; breeding did not begin until 1909 (Brevis et al. 2008).

### 5.4 Anthocyanin profile together with other phenolic markers as an authentication tool for *Vaccinium* berries and products derived from them

The following three basic concepts have mainly guided this work: The prequisites for quantitative phytochemical data for differentiation between taxa (botanical origin) are as follows (1) the degree of variation between populations of a given taxon (**II**, **IV**); (2) the variation between different individuals within a single population (**II**, **IV**); (3) the variation between populations growing in different ecological environments (**II**, **III**, **IV**, **V**)(Zidorn & Stuppner 2001).

Furthermore, the data may be exploited in the authentication of geographical origin (**V**) together in conjunction with multivariate statistical methods (Luykx et al. 2008, Carcea et al. 2009).

### 5.4.1 Botanical origin – a case of bilberries (*V. myrtillus*)

Anthocyanin profiles have been proposed for authentication analyses as berries and fruits in each genus have their own typical anthocyanin profile (Hong & Wrolstad 1990, Macheix et al. 1990). In Study **V,** the anthocyanin fingerprints of the berries of *V. myrtillus* originating from Asian populations (**V**: ***Table 1*)** were compared to the previously studied northern European bilberries (**II:** ***Figure 2*, *Table 1***).

There were no statistically significant differences in the contents or relative proportions of anthocyanidins or in the contents of the total anthocyanins between Turkey and Finland (**V**: ***Table 2***). The study revealed that the proportions of aglycones are consistent, regardless of the distinct geographical origin. Consequently, they do not appear to be suitable for the authenticity analyses of geographical origin for bilberries (*V. myrtillus*), although latitude and altitude related features have been reported in **Study II** as well as in other studies about bilberries (Rieger et al. 2008, Åkerström et al. 2010) or berries of other species (*Ribes* cvs.) (Vagiri et al. 2013, Yang et al. 2013). The recent study (Fraige et al. 2014) supports the result (**V**) although the studied geographical origins were not as distinct. The discrimination of 11 grape varieties (*V. vinifera*) and one hybrid variety was based only on their anthocyanidin profiles but the geographical origin (southern, southeastern and northeastern Brazil) of grapes could not be discriminated. The use of higher number of variables, in addition to anthocyanins gives more discriminating power, for example, bilberries from seven Slovenian locations were differentiated in this way (Može et al. 2011).

Therefore, the profile of anthocyanidin relative proportions and the total anthocyanin contents represent a promising authentication tool for distinguishing *V. myrtillus* from other berry or fruit species and V*accinium* species. ***Table 4*** summarises the anthocyanidin profiles of berries of *Vaccinium* species from 9 sections showing the characteristic relative proportions of aglycone moieties.

### 5.4.2 Geographical origin of bilberries (*V. myrtillus*)

Clear differences were found in the contents and relative proportions of sugar moieties of anthocyanins between Finland and Turkey (**V**). The contents of galactosides and arabinosides were significantly lower in the Turkish bilberries than in their Finnish counterparts. In contrast, the content of glucosides was higher in

Turkish bilberries, although the difference was not statistically significant.

There are only a few previous studies on the sugar profiles of anthocyanins in the berries of *V. myrtillus*. The sugar proportions of bilberries in Study **II** and those reported by Buchert et al. (2005) were more close to each other but different from Turkish ones. Furthermore, the relative proportion of glucosides (42%) has dominated in Slovenian bilberries (Mozˆe et al. 2011) similarly as in Turkish bilberries (52%) examined in the present study.

A logistic regression was calculated to predict the origin of the berries of *V. myrtillus* (Finland versus Turkey). A preliminary model based on glucoside proportions classified 96.7% of samples correctly into their geographical origin (**V**). The significant differences found in the relative proportions of the sugar moieties may be used as a novel discriminating criterion for distinguishing the berries of *V. myrtillus* from different geographical origins.

Future challenges in the determination of geographical authenticity of *V. myrtillus* and related species might involve the collection of isotope ratio mass spectrometry (IRMS) data in addition to confirming the anthocyanin, and other phenolic, profiles. It is necessary to use of multiple techniques if one wishes to achieve the highest possible certainty level.

### 5.4.3 Markers of adulteration of *Vaccinium* berry raw material and its products with berries from other genera

As an example, the relatively high price of bilberry (*V. myrtillus*) raw material and subsequently, the products derived from it, has made it a target for sophisticated adulteration (Cassinese et al. 2007, Foster & Blumenthal 2012). Bilberry extracts have been adulterated with berries of European elderberries (*S. nigra*) and Chinese mulberries (*Morus australis*; *M.* spp.). Recently, Yamamoto & coauthors (2013) have reported the adulteration of bilberry tablets with black currants (*R. nigrum*).

If the authenticity of *V. myrtillus* raw material or its products (e.g. extracts, juices) are to be confirmed, it is practical to conduct an HPLC-DAD analysis. The mixture of the anthocyanins in the sample is separated as they pass through a column based on their properties (e.g. hydrophilicity) (See ***Chapters 2.5.2*** and ***2.5.3***). Next, the obtained chromatogram at 520nm should be compared to the chromatograms established for the genuine sample material. It is advisable to record the chromatogram at 360nm in the same run to check the number and level of flavonols (**Figure 8**). Possible extra peaks, i.e. markers, in the chromatogram provide information about the presence of an adulterant.

There are many potential anthocyanidin diglycosides which may be observed as atypical peaks in the HPLC-chromatogram at

520nm obtained from the adulterated *V. myrtillus* sample (***Table 1***). Thus, they may be evidence of the addition of berries of fruits from the genera of *Fragaria*, *Lonicera*, *Prunus*, *Ribes*, *Rubus* and *Sambucus* genera. In addition, specific compounds among phenolic acids, flavonols, dihydrochalcones, flavanone glycosides and ellagitannin may be used as markers for genera such as *Vitis*, *Ribes*, *Malus*, *Citrus* and *Rubus* too (***Table 1***).

In general, the typical relative proportions of anthocyanidins of a given *Vaccinium* species (***Table 4***) should be checked. An untypically high proportions (> 50%) of cyanidin found in bilberry (*V. myrtillus*) juices or dietary supplements (e.g. extracts or tablets) point to adulteration with cyanidin-rich berry material. For example, the berries of *Aronia mitchurinii* have contained only cyanidin glycosides and their levels have been about 2.9–fold higher than those in bilberries (Määttä-Riihinen et al. 2004).

Cheaper apple (*Malus* sp.) raw material is a common adulterant in berry products (e.g. Downey & Kelly 2004, Vaclavik et al. 2012). Ten phloretin derivatives among the group of compounds called dihydrochalcones have been described to occur in the *Malus* species (Gosch et al.2010). The addition of apple can be proved, if quantifiable amounts of several phloretin glycosides are detected. Generally, these compounds have not been found in *Vaccinium* berries, except phloretin 2′-*O*-glucoside, which have been fractionated and concentrated from juice concentrate of *V. macrocarpon* (Turner et al. 2005).

Of the reported adulterants, the fruits of *Morus australis*, are rich in cyanidin 3-rutinoside (Song et al. 2009). However, these compounds are absent in *Vaccinium* berries. Rutinosides and glucosides of pelargonidin can be used as additional markers, because they have been found in many *Morus* species (*Morus atropurpurea* Roxb., *M. nigra*) (Isabelle et al. 2008, Pawlowska et al. 2008). The best markers for the berries of *R. nigrum* are its most abundant compounds i.e. 3- rutinosides of delphinidin, cyanidin and myricetin. Indicators for red currants (*R.* × *pallidum*, *R. rubrum*) include 3-(2-glucosylrutinoside), 3-(2-xylosylrutinosides and rutinosides of cyanidin (***Table 1***).

Adulteration with *S. nigra* berries can be suspected if there are high contents of cyanidin 3-sambubioside-5-glucoside, a compound not found in *Vaccinium* berries. Similarly, cyanidin 3,5-diglucoside and 3-rutinosides of isorhamnetin and kaempferol are characteristic for the berries of *S. nigra* (***Table 1***). Moreover, bilberries are low in flavonols (11mg/100g FW) whereas in *S. nigra* berries their concentrations are 4–5-fold (45–57mg/100g FW) higher (Määttä-Riihinen et al. 2004, Mikulic-Petkovsek et al. 2012b). Adulteration with American elderberry (*S. canadensis*)

could be detected by the presence of high amounts of cyanidin 3-coumaroylsambubioside-5-glucoside (***Table 1***).

Tartaric acids (caffeoyl-, coumaroyl- and feruloyltartaric) are usually considered an indicator that there has been *V. vinifera* addition to a more expensive raw material. The existence of 3,5-diglucosides of delphinidin, cyanidin, petunidin, peonidin and pelargonidin is the evidence of the addition of muscadine grape (*V. rotundifolia*). (***Table 1***)

In conjunction with the phenolic compounds, it is additional specific chemical markers that one relies on. These include sorbitol, which is absent in *Vaccinium* berries but have been quantified in chokeberries (*Aronia melanocarpa*), eastern shadbush (*Amelanchier canadensis*), apple (*Malus* sp.) and pears (Mikulic-Petkovsek et al. 2012a). With respect to the organic acids, quinic/citric, quinic/malic, and citric/malic ratios have been found to be important in proving the authenticity of cranberry juice (Ehling & Cole 2011).

### 5.4.4 Markers of adulteration of *V. myrtillus* raw material and its products with berries from the same genus

*V. myrtillus* extracts have also been adulterated from related berry sources. For example, the berries of *V. uliginosum* and *V. vitis-idaea* have been offered to world markets as "Chinese domestic bilberry" extracts (Foster & Blumenthal 2012). The addition of the berries of *V. parvifolium* could be suspected by unusual high contents of hydroxybenzoic acids (e.g. *p*-hydroxybenzoic acid) since their content are low in other *Vaccinium* berries, also in those of *V. myrtillus* (***Table 2***).

There are major differences in the contents of caffeoylquinic isomers between *Vaccinium* species. The most abundant molecular species seems to be chlorogenic acid (i. e. 5-caffeoylquinic acid) (***Table 2***). The berries of *V. angustifolium* × *V. corymbosum* and *V. corymbosum*, were the richest sources of chlorogenic acid (Taruscio et al. 2004) (**Table 2**). An exceptionally high content of this phenolic acid in *V. myrtillus* raw material or products derived from it could indicate from the addition of the berries of these species. They are cultivated in mass production and are subsequently cheaper. Neochlorogenic acid (i. e. 3-caffeoylquinic acid) has been occurred as a minor phenolic acid in the berries of *V. myrtillus* (Kusznierevicz et al. 2012) whereas in the berries of *V. membranaceum* it has been found to be the major phenolic compound (Lee et al. 2004b). Subsequently, it might be a potential indicator for this species in the authenticity studies of *V. myrtillus*.

A rather low (≤ 14%) relative proportion of peonidin is a common feature typically present in the berries of *V. myrtillus* as well as in the berries from the sections *Hemimyrtillus*, *Myrtillus*,

*Cyanococcus*, and *Vaccinium* (***Table 4***). The berries of *V. vitis*-idaea (section *Vitis-idaea*) and *V. erythrocarpum* (section *Oxycoccoides*) have mainly cyanidin (>90%). The berries of *V. japonicum* (section *Oxycoccoides*) have almost equal shares of cyanidin and pelargonidin. As an example, an unusual high content of cyanidin or pelargonidin, the presence of typical sugars (galactoside, glucoside, arabinoside) and the absence of untypical ones (e.g. rutinoside, diglucoside or sophoroside as indicators of berries from other genera) in bilberry products might indicate of addition of one of these *Vaccinium* berries.

In the berries of *V. myrtillus* (section *Myrtillus*) there are five anthocyanidins present but in the different relative proportions (***Table 4***). The distinguishable difference between the berries of *V. myrtillus* and blueberries (*V. corymbosum*) from the section *Cyanococcus* occurs in the relative proportion of malvidin. This has been clearly higher in blueberries (Kalt et al. 1999, Taruscio et al. 2004). The range of the relative proportions of cyanidin and malvidin in the berries of *V. myrtillus* (n=20; 26–40% and 9–15%, respectively) compared with the berries of *V. uliginosum* (n=15; 4–10% and 28–49%, respectively), found in this study, would make it possible to differentiate them. The berries of *V. uliginosum* are among the richest sources of flavonols (30–100 mg/100g FW) in comparison to the berries of *V. myrtillus* and *V. corymbosum* cultivars (1–11mg/100g) (Häkkinen & Törrönen 2000, Määttä-Riihinen et al. 2004, Može et al. 2011) which may be used as an adulteration indicator for this species.

Quercetin HMG-rhamnoside was a typical lingonberry flavonol glycoside. Since it was absent in the berries of *V.* × *intermedium*, *V. myrtillus* and *V. uliginosum*, it could be considered to be a suitable marker for *V. vitis-idaea*.

If the berries of the related *Vaccinium* species are mixed in *V. myrtillus* raw material, according to the quantitative differences of multiple phenolic markers it might be possible to assess if it is authentic or not. However, in most cases, complementary markers are needed to confirm about what is an adulterant species.

### 5.4.5 Effects of processing on the anthocyanin profiles in *Vaccinium* berries

The phenolic profiles are distinctive between the berries of *Vaccinium* species. The main aim of this work was to evaluate the natural variation of anthocyanins and other phenolic compounds. The obtained results may also be applicable being combined with data from other chemical compounds in authenticity studies of processed products. However, in that case, one needs to consider how processing may affect phenolic profiles.

Processing effects on the anthocyanin profiles follow the same trends depending on the technology. The anthocyanin content of berries diminishes to some extent in processing. The short pasteurization time (60–90s at 90°C) used for blueberry juices has generally resulted in only minor (<10%) losses of the anthocyanins (Howard et al. 2012). The qualitative pattern which was characteristic and unique to berry species and cultivars still remained. In other words, the same major and minor compounds could be found although their relative proportions change, amount of which depends on technology treatment(s). In cranberry (*V. macrocarpon*) products (freeze-dried berries, extract powder, juice, juice cocktail) the galactosides of peonidin and cyanidin were most abundant, with cyanidin glucoside being the least one and the arabinosides of cyanidin and peonidin showing intermediate values (Brown & Shipley 2011). Similarly, the same major and minor anthocyanins were quantified in frozen cranberries, blanched mash, pectinase treated mash, unclarified juice, clarified juice and pasteurized juice (White et al. 2011)

Delphinidin glycosides appear to be the most unstable of this class whereas malvidin glycosides tend to be the most stable (Howard et al. 2012). Delphinidin derivatives decreased their content by about 6% in favour of malvidin and cyanidin derivatives when berries were converted into steam-blanched blueberry juice (Brambilla et al. 2008). However, the percentage fluctuations were found to follow the same trend as previously reported for blueberry juices traditionally processed. The anthocyanin profiles of the bilberry and blueberry juices were found to correspond to those of the respective fruits (Müller et al. 2012). It was found that bilberry anthocyanins were stable during capsule and tablet manufacturing processes (Cassinese et al. 2007).

### *5.4.6 Phenolic compounds as authentication tools*

Phenolic compounds are recommendable tools for authenticity analyses of berry or fruit raw material and products which are not manufactured under very harsh production conditions. Phenolic compounds are mainly genetically determined. Thus, certain limitations of their applicability are based especially on genetic factors. The authenticity assessment of *Vaccinium* cultivar using phenolic compounds is simpler because the genetic background is generally better known than that of a wild relative species. Wild *Vaccinium* stands are composed of a mixture of genotypes where, for example, hybridization is a common phenomenon. It is one of the factors which may complicate species distinctions (Govindaraghavan et al. 2012). Secondary metabolite profiling, including phenolic profiles, complements the taxonomic and genomic authentication of plant species (Waterman 2007,

Govindaraghavan et al. 2012). In the determination of botanical authenticity the use of marker compounds and the profiles of the relative proportions of anthocyanidin and flavonol glycosides might be most useful way to utilize the phenolic compounds. The flavonol glycosides were only quantified in the berries of *V. uliginosum* in this thesis work, but it was noticed that already their contents vary remarkable between *Vaccinium* species, such as between the berries of *V. myrtillus* and *V. uliginosum*. For example, the studies about the identification of grape (*V. vinifera*) cultivars suggested that the anthocyanin (e.g. Pomar et al. 2005) and flavonol (e.g. Ledda et al. 2010) profiles based on relative proportions are promising tools to discriminate cultivars.

Phenolic compounds have similar limitations such as other phenotypic features (e.g. morphological) in the botanical identification due to overlapping characteristics, especially in closely related species. The variation in phenolic profiles may be a result of heritable differences (genotypic variation); or be due to environmental differences (phenotypic variation;) or a combination of both factors. The qualitative phenolic profile (i.e. the phenolic compounds present in a given genotype) is only marginally affected by environmental factors. The phenotypic plasticity is the amount by which the expressions of individual characteristics of a genotype are changed by different environments. Some traits are more constant than others. The determination of geographical authenticity is much more complex. The use of many variables, preferably different types of ones [e.g. organic (anthocyanin, flavonol, hydroxycinnamic), inorganic, isotopic), is often necessary for reliable authentication. Statistical multivariate methods can be used to facilitate data interpretation when a large set of variables are to be analyzed.

Overall, authentication tools may vary widely, depending on the plant and processes being investigated, i.e. from a botanical or morphological evaluation to very elaborate chemical or genetic approaches (Smillie & Khan 2010). The authenticity specification may e.g. consist of analyses of anthocyanins, tannins, sugars and their ratios, sugar alcohols, organic and amino acids (Zhang et al. 2009). The use of multiple techniques is necessary in order to achieve the highest possible certainty level. The use of multiple markers in the authentication adds another layer of complexity and further cost to combating fraudulent practises.

# 6 Summary and conclusions

*Hybrid berries inherited the special characteristics of both parents* **(I)**
The berries and flowers of the natural hybrid (*V.* × *intermedium*) between *V. myrtillus* and *V. vitis-idaea* showed characteristics inherited from both parent species in the distribution and contents of phenolic compounds. As compared with the parent species, hybrid berries possessed a more diverse profile of phenolic compounds.

Inheritance from lingonberry was revealed in the predominance of cyanidin. The proportions of delphinidin, petunidin and malvidin were lower in the berries of *V.* × *intermedium* than in *V. myrtillus*. The inheritance of PAs in hybrid berry was qualitatively closer to lingonberry but quantitatively to bilberry. Hybrid berries had inherited a diverse anthocyanin profile from *V. myrtillus* and the PA profile from *V. vitis-idaea*. The levels of PAs were lower in the hybrid berries than in lingonberry but the anthocyanin content of hybrid berries was clearly closer to bilberry than lingonberry.

*The distinctive phenolic profiles of the berries of V.* × *intermedium, V. myrtillus, V. vitis-idaea, V. uliginosum and V. arctostaphylos have potential for authenticity purposes* **(I–V)**
The berries of *V. myrtillus* contained the highest amount of anthocyanins followed by the berries of *V.* × *intermedium*, *V. arctostaphylos*, *V. uliginosum* and *V. vitis-idaea*. The berries of *V. arctostaphylos* had a diverse anthocyanin profile consisting of 26 anthocyanin peaks detected by HPLC-ESI-MS. Of these, anthocyanidin sambubiosides were characteristic for these berries being absent in the berries of *V. uliginosum*. The berries of *V. uliginosum* contained 15 major anthocyanidin glycosides found in both the berries of *V. myrtillus* and *V. arctostaphylos* but in the different relative proportions.

The major anthocyanidins in the berries of *V. myrtillus* were delphinidin and cyanidin. The most minor anthocyanidin was peonidin. The berries of *V.* × *intermedium* displayed one major anthocyanidin, cyanidin, similarly to the berries of the other of the parent species, *V. vitis-idaea*. The major anthocyanidins in the berries of *V. uliginosum* were delphinidin and malvidin. The berries of *V. arctostaphylos* contained three major anthocyanidins. The delphinidin predominated being followed by almost equal shares of petunidin and malvidin, respectively. In all, the studied berries of *V.* × *intermedium*, *V. myrtillus*, *V. uliginosum*, *V. arctostaphylos* and *V. vitis-idaea* displayed distinctive anthocyanidin aglycone profiles which are summarised in ***Table 4***.

Flavonol profiles were distinctive between the berries of *V. myrtillus*, *V. uliginosum*, *V. arctostaphylos* and *V. vitis-idaea* qualitatively. Quercetin HMG-rhamnoside was a typical lingonberry flavonol glycoside being absent in the berries of *V.* × *intermedium*, *V. myrtillus* and *V. uliginosum*. In Study **IV**, the presences of kaempferol and isorhamnetin aglycons, as well as the tentative identification of 14 flavonol glycosides (i.e. myricetin, quercetin, laricitrin, isorhamnetin, syringetin, kaempferol conjugates) in the berries of *V. uliginosum* were reported.

Study **IV** was the first quantitative study of flavonol conjugates in *V. uliginosum* berries. The berries of *V. uliginosum* contained high amounts of flavonols, 1133 mg/100 g of DW (154 mg/100g FW). The flavonol contents were too low for accurate quantification in the berries of *V. myrtillus*, *V. vitis-idaea* and *V. arctostaphylos*.

*Northern climatic conditions favour the biosynthesis of anthocyanidin and flavonol glycosides, especially the more hydroxylated derivatives* (**II**, **IV, V**)

Statistically significant differences in the total anthocyanins of Finnish bilberries were found between the populations. Despite these local differences, the total anthocyanins content in the berries of *V. myrtillus* from the southern regions was significantly lower in comparison to the central and the northern regions. There might be some latitude related genetic differences in anthocyanin production of berries similarly to other phenotypic characters. A divergent anthocyanin profile in bilberries of seven individuals was found mainly originating from eastern Finland with very low amounts of anthocyanidin glucosides. In contrast to Finnish bilberries no latitudinal (or longitudinal) related effects were found in the contents or in the proportions of anthocyanidins or the total anthocyanins in the Turkish bilberries.

The total anthocyanin content in the berries of *V. uliginosum* from the southern region was significantly lower compared to that in the berries from the central and northern regions. The contents of the two nonmethylated anthocyanidins, delphinidin and cyanidin, were significantly higher in the north than in other parts of the country. The content of the methylated anthocyanidin, malvidin, was significantly lower in northern than in central Finland, as in *V. myrtillus*.

The flavonol contents in the berries of *V. uliginosum* from the southern and central regions were significantly lower than those in berries gathered in the north. Furthermore, there were more myricetin and quercetin glycosides in the berries of *V. uliginosum* from the north than from the other regions. Similarly to *V. myrtillus*, plants with a very low proportion (<10%) of anthocyanidin glucosides were found.

*Anthocyanin profile provides a promising authentication tool for bilberries (V. myrtillus)* (**V**)

There were no statistically significant differences in the contents or proportions of anthocyanidins or in the contents of the total anthocyanins in the berries of *V. myrtillus* between Turkey and Finland. The study revealed that the proportions of aglycones are consistent, regardless of the distinct geographical origin. Consequently, they do not appear to be suitable for use in the authenticity analyses of the geographical origin. Therefore, the profile of anthocyanidin proportions represents a promising authentication tool for distinguishing *V. myrtillus* from other berry species and V*accinium* species.

Clear differences were found in the contents and proportions of sugar moieties of anthocyanins between Finland and Turkey. A logistic regression was utilized to predict the origin of bilberries (Finland versus Turkey). A preliminary model based on glucoside proportions classified 96.7% of samples correctly according their geographical origin. The significant differences found in the proportions of the sugar moieties may be used as novel discriminating criteria for distinguishing bilberries from different geographical origins.

Future challenges in the determination of geographical authenticity of *V. myrtillus* and related species might involve the collection of isotope ratio mass spectrometry (IRMS) data in addition to confirming the anthocyanin, and other phenolic, profiles. The use of multiple techniques is necessary if one wishes to achieve the highest possible certainty level. One analytical technique is usually insufficient to detect all kinds of adulteration ways commonly practised. Therefore, in most cases only combined analyses can ensure reliable authenticity assessment of a product.

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# *Phenolic Compounds in the Berries of the Selected Vaccinium Species*

*The Potential for Authenticity Analyses*

Due to intense competition in today's marketplace, food fraud is a growing problem. Mislabelled and adulterated berry and fruit products constitute one of the largest groups of recorded frauds. In this work, the botany and biology of *Vaccinium* L. as well as the occurrence and the geographical variation of phenolic compounds in the berries of the selected *Vaccinium* species are reviewed from the view of authenticity analyses. This thesis brings new information about the natural variation of phenolic compounds in *Vaccinium* berries providing examples of how to detect adulterated *Vaccinium* products.

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