DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU

Effects of Genetic and Environmental Factors on Proanthocyanidins in Sea Buckthorn (*Hippophaë rhamnoides*) and Flavonol Glycosides in Leaves of Currants (*Ribes* spp.)

WEI YANG

Food Chemistry and Food Development
Department of Biochemistry

DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU
Food Chemistry

Effects of Genetic and Environmental Factors on Proanthocyanidins in Sea Buckthorn (*Hippophaë rhamnoides*) and Flavonol Glycosides in Leaves of Currants (*Ribes* spp.)

WEI YANG

Food Chemistry and Food Development
Department of Biochemistry

TURKU, FINLAND – 2017

Food Chemistry and Food Development
Department of Biochemistry
University of Turku, Finland

Supervised by

Professor Baoru Yang, Ph.D.
Department of Biochemistry
University of Turku
Turku, Finland

Professor emeritus Heikki Kallio, Ph.D.
Department of Biochemistry
University of Turku
Turku, Finland

Reviewed by

Professor Encarna Gómez-Plaza, Ph.D.
Food Science and Technology Department
University of Murcia
Murcia, Spain

Professor Laura Jaakola, Ph.D.
Department of Arctic and Marine Biology
UiT The Arctic University of Norway
Tromsø, Norway

Opponent

Professor Marina Heinonen, Ph.D.
Department of Food and Environmental Sciences
University of Helsinki
Helsinki, Finland

Research director

Professor Baoru Yang, Ph.D.
Department of Biochemistry
University of Turku
Turku, Finland

The originality of this dissertation has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service

ISBN 978-951-29-6780-3 (print)
ISBN 978-951-29-6781-0 (pdf)
ISSN 2323-9395 (print)
ISSN 2323-9409 (pdf)
Painosalama Oy – Turku, Finland 2017

TABLE OF CONTENTS

ABSTRACT

Sea buckthorn (*Hippophaë rhamnoides*) and currants (*Ribes* spp.) are rich sources of flavonoids and other polyphenols. Both proanthocyanidins (PAs) in sea buckthorn and flavonol glycosides (FGs) in currant leaves have biological activity and may be effective in maintaining health.

The aim of this thesis was to establish methods for qualitative and quantitative analysis of complex PAs in sea buckthorn berries and FGs in currant leaves, and to investigate the effects of genetic background and growth environment on the content and composition of these compounds.

A rapid and sensitive method based on hydrophilic interaction liquid chromatography combined with electrospray ionization mass spectrometry (HILIC-ESI-MS) was established for profiling of PAs of sea buckthorn berries. B-type PAs up to degree of polymerization 11 were detected. Among them, the main monomeric units were (epi)gallocatechins. Three PA dimers, four PA trimers and five PA tetramers were quantified by HILIC-ESI-MS in selected ion recording mode (SIR) using reference compound.

Sea buckthorn berries of three subspecies (*H. rhamnoides* ssp. *rhamnoides*, ssp. *mongolica* and ssp. *sinensis*) including nine varieties grown in Finland, Canada and China were collected and the contents of PA oligomers and total PAs were detected using HILIC-ESI-MS-SIR method and the Brunswick Laboratories 4-dimethylaminocinnamaldehyde (BL-DMAC) method, respectively. The three subspecies were separated by three validated factors in the partial least squares discriminant analysis model (PLS-DA). The content of PA oligomers ranged from 0.11 to 0.54 mg/100 g FW. The content of total PAs in ssp. *rhamnoides* was significant higher than that in ssp. *mongolica* ($p < 0.05$). Five varieties 'Prevoshodnaya', 'Prozcharachnaya', 'Chuiskaya', 'Oranzhevaya' and 'Vitaminaya' of ssp. *mongolica* were separated in principal component analysis (PCA) model.

Sea buckthorn berries of ssp. *rhamnoides* ('Tytti', 'Terhi' and one of wild origin) harvested during 2007–2013 in Finland and of ssp. *sinensis* (wild) harvested in 2008 in China were investigated by the contents of PAs based on growth environments. In ssp. *rhamnoides*, the levels of total PAs in the northern samples (high latitude) (610-970 mg/100g DW) were significantly higher than that in the southern samples (low latitude) (340-450 mg/100g DW) ($p < 0.05$). The three varieties ('Tytti', 'Terhi' and one of wild origin) were all well discriminated by latitude in the PLS-DA model (four factors, R^2 0.75, Q^2 0.70). Negative correlation was found between total PAs and most of the temperature and radiation-related variables ($p < 0.01$), and positive correlation with most of precipitation and humidity variables. In the northern ssp.

rhamnoides samples, clearly decreasing trends were found in the contents of total PAs during the growth season until harvest ($p < 0.01$) when length of the growth season (r = -0.805), temperature sum (r = -0.829), and total radiation (r = -0.874) were increasing. No significant correlation was found in southern samples. Within ssp. *sinensis*, latitude correlated negatively to total PAs and positively to PA oligomers. In comparison to the latitude, altitude showed less impact on PAs.

The leaves of three black currant (*Ribes nigrum* L.) cultivars, 'Mortti', 'Mikael' and 'Jaloste n:o 15', a green currant (*R. nigrum*) 'Vertti', a red currant (*R. rubrum*) 'Red Dutch' and a white currant (*R. rubrum*) 'White Dutch' were collected at four time points during growth season in southern and northern Finland, 2012. Twenty-seven FGs were identified in the leaf extracts by HPLC-DAD combined with electrospray ionization tandem mass spectrometry, NMR analysis and reference compounds. Quercetin and kaempferol were the major aglycones of the glycosides. Quercetin-3-*O*-(2,6-*α*-dirhamnopyranosyl-*β*-glucopyranoside), quercetin-3-*O*-(2-*β*-xylopyranosyl-6-α-rhamnopyranosyl-*β*-glucopyranoside), kaempferol-3-*O*-(3,6-*α*-dirhamnopyranosyl-*β*-glucopyranoside) and kaempferol-3-*O*-*β*-(6'-malonyl)glucopyranoside were identified by NMR for the first time in currant leaves. The leaves of 'Mortti', 'Jaloste n:o 15', green currant 'Vertti' and red currant 'Red Dutch' had similar profiles of FGs, which differed from those of 'Mikael' and 'White Dutch' had different profiles. During the growth season, the highest contents of FGs in the southern samples occurred a few weeks earlier than in the northern samples. The levels of malonylated FGs decreased regularly during the growth season. Currant leaves collected randomly and from the middle positions had different profiles of FGs.

SUOMENKIELINEN ABSTRAKTI

Tyrni (*Hippophaë rhamnoides*) ja herukat (*Ribes* spp.) ovat saaneet yhä enenevää huomiota osakseen sisältämiensä flavonoidien ja muiden fenolisten yhdisteiden vuoksi. Sekä tyrnimarjojen proantosyanidiinit (PA) että herukan lehtien flavonoliglykosidit (FG) ovat biologisesti aktiivisia ja terveyttä edistäviä yhdistitä.

Työn tavoitteena oli kehittää ja soveltaa menetelmiä tyrnimarjan kompleksisten PA-yhdisteiden ja herukanlehtien FG-yhdisteiden kvalitatiiviseen ja kvantitatiiviseen analyysiin sekä selvittää perimän ja kasvuympäristön vaikutuksia fenolisten yhdisteiden määrään ja koostumukseen.

Optimoitu, nopea ja herkkä menetelmä tyrnimarjojen proantosyanidiinien profilointiin perustui hydrofiilisten vaikutusten nestekromatografiaan yhdistettynä sähkösumutusionisaatiomassaspektrometriaan (HILIC-ESI-MS). B-tyypin PA-yhdisteitä tunnistettiin aina polymeraatioasteeseen 11asti, ja tärkeimmät monomeeriset rakenneyksiköt olivat (epi)gallokatekiineja. Kolme PA-dimeeriä, neljä PA-trimeeriä ja viisi PA-tetrameeriä kvantitoitiin HILIC-ESI-MS-analyysin valitun ionien seurantamenetelmää (SIR) käyttäen vertailuyhdisteen avulla.

Tutkimuksen kohteina olevat kolmen alalajin tyrnimarjat (ssp. *rhamnoides*, ssp. *mongolica* and ssp. *sinensis*) koostuivat yhdeksän lajikkeen marjoista, jotka olivat peräisin Suomesta, Kanadasta ja Kiinasta. PA-oligomeerit määritettiin HILIC-ESI-MS-SIR-menetelmällä ja PA-yhdisteiden kokonaismäärät 4-dimetyylikanelialdehydimenetelmällä (BL-DMCA, Brunswick Laboratories). Kolme alalajia erottuivat toisistaan PLS-diskriminanttianalyysimallin (PLS-DA, Partial Least Squares Discriminant Analysis) kolmella validoidulla faktorilla. PA-oligomeerien pitoisuudet vaihtelivat välillä 0,11 – 0,54 mg/100 g tuorepainoa. PA-yhdisteiden kokonaismäärä alalajilla *rhamnoides* oli merkittävästi suurempi kuin alaljilla *mongolica* ($p < 0.05$). Viisi lajiketta, ‘Prevoshodnaya’, ‘Prozcharachnaya’, ‘Chuiskaya’, ‘Oranzhevaya’ and ‘Vitaminaya’ (ssp. *mongolica*) erottuivat toisistaan pääkomponenttianalyysissa (PCA).

Alalajin *rhamnoides* marjat (‘Tytti’, ‘Terhi’ ja luonnonkanta) korjattiin vuosina 2007–2013 (Suomessa) ja alalajin *sinensis* (luonnonkanta) vuonna 2008 (Kiinassa), ja työssä tutkittiin kasvuolosuhteiden vaikutusta PA-pitoisuuksiin. Alalajilla *rhamnoides* PA-yhdisteiden kokonaismäärät pohjois-suomalaisissa marjoissa (610–970 mg/100g kuivapainoa) olivat merkitsevästi suuremmat kuin eteläsuomalaisissa näytteissä (340–450 mg/100g) ($p < 0.05$). Suomalaiset marjat erottuivat hyvin latitudin perusteella PLS-DA mallissa (neljä faktoria, R^2 0.75, Q^2 0.70). PA:n kokonaismäärän ja useimpien

lämpötilaan tai valaistukseen vaikuttavien tekijöiden välillä oli negatiivinen korrelaatio ($p < 0.01$), kun taas korrelaatio sademäärään ja kosteuteen oli positiivinen. Pohjoisissa *rhamnoides*-alalajin marjoissa oli havaittavissa selvä PA-yhdisteiden aleneva suuntaus kasvukauden aikana aina sadonkorjuuseen asti ($p < 0.01$) kun kasvukauden pituus (r = -0.805), lämpösumma (r = -0.829) ja säteilyn konaismäärä (r = -0.874) lisääntyivät. Eteläisissä näytteissä ei havaittu merkitsevää riippuvuutta. Kasvupaikan leveyspiiri korreloi negatiivisesti alalajin sinensis proantosyanidiinien kokonaismäärään, mutta positiivisesti PA-oligomeereihin. Kasvupaikan korkeudella oli pohjoisuutta vähäisempi vaikutus proantosyanidiineihin.

Kolmen mustaherukkalajikkeen (*Ribes nigrum* L., cv.'Mortti', cv. 'Mikael' ja cv. 'Jaloste n:o 15') yhden viherherukkalajikkeen (*R. nigrum*, cv. 'Vertti') yhden punaherukkalajikkeen (*R. rubrum*, 'Red Dutch') ja yhden valkoherukkalajikkeen (*R. rubrum*, 'White Dutch') lehtiä kerättiin neljänä ajankohtana kasvukauden aikana Etelä- ja Pohjois-Suomessa vuonna 2012. Lehtiuutteista tunnistettiin 27 flavonoliglykosidia HPLC-DAD-ESI-MS/MS-menetelmän, NMR-analyysin ja vertailuyhdisteiden avulla. Kversetiini ja kemferoli olivat glykosidien tärkeimmät aglykonit. Kversetiini-3-*O*-(2,6-*α*-diramnopyranosyyli-*β*-glukopyranosidi), kversetiini-3-*O*-(2-*β*-ksylopyranosyyli-6-α-ramnopyranosyyli-*β*-glukopyranosidi), kemferoli-3-*O*-(3,6-*α*-diramnopyranosyyli-*β*-glukopyranosidi) ja kemferoli-3-*O*-*β*-(6'-malonyyli)glukopyranosidi tunnistettiin NMR-analyysin avulla ensimmäistä kertaa herukan lehdistä. 'Mortin', 'Jaloste n:o 15:n', 'Vertin' and red 'Red Dutch'-lajikkeen flavonoliglykosideilla oli samanlaiset profiilit, jotka erosivat muista jalosteista. Kasvukauden aikana eteläiset näytteet saavuttivat korkeimmat FG-pitoisuutensa muutamaa viikkoa aikaisemmin kuin pohjoiset näytteet. Malonyloidut flavonoliglykosidit vähenivät säännöllisesti kasvukauden edetessä. Lehden kasvupaikka pensaassa vaikutti FG-yhdisteiden profiiliin.

LIST OF ABBREVIATIONS

ANR	anthocyanidin reductase
ANS	anthocyanidin synthase
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
BFF	benzofuran-forming
*b*HLH	basic helix–loop–helix
BL-DMAC	Brunswick Laboratories 4-dimethylaminocinnamaldehyde
C4H	cinnamate 4-hydroxylase
CBG	cytosolic *β*-glucosidase
CHI	chalcone isomerase
CHS	chalcone synthase
CK2	casein kinase II
COSY	correlation spectroscopy
CVD	cardiovascular disease
DAD	diode array detector
DFR	dihydroflavonol reductase
DP	degree of polymerization
DW	dry weight
ESI	electrospray ionization
F3'5'H	flavonoid 3', 5'-hydroxylase
F3H	flavanone 3-hydroxylase
F3'H	flavonoid 3'-hydroxylase
FGs	flavonol glycosides
FL	fluorescence
FLS	flavonol synthase
FW	fresh weight
Gal	galactose
Glu	glucose
GST	glutathione *S*-transferase
GT	glucosyltransferase
GTFs	glycosyltransferases
HILIC	hydrophilic interaction liquid chromatography
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum correlation
HRF	heterocyclic ring fission
HSQC	heteronuclear single quantum coherence
IC	ion chromatography
IL-1, IL-6	interleukin 1 and 6
LAR	leucoanthocyanidin reductase
LD_{50}	lethal dose, 50%
LPH	lactase phloridzin hydrolase
MRM	multiple reactions monitoring
Nrf2-ARE	nuclear erythroid 2-related factor 2-antioxidant responsive element

PAL	phenylalanine ammonia lyase
PA	proanthocyanidin
PDA	photo diode array
QM	quinone methide fission
RDA	retro-Diels-Alder reaction
Rha	rhamnose
SIM	selected ion monitoring
SIR	selected ion recording
SRM	selected reaction monitoring
TAS2Rs	taste receptors type 2
TNFα,	tumor necrosis factor alpha
TT19	transparent testa 19
Xyl	xylose

LIST OF ORIGINAL PUBLICATIONS

I. Kallio, H., Yang, W., Liu, P. & Yang, B. (2014). Proanthocyanidins in wild sea buckthorn (*Hippophaë rhamnoides*) berries analyzed by reversed-phase, normal-phase, and hydrophilic interaction liquid chromatography with UV and MS detection. *Journal of Agricultural and Food Chemistry*, *62*(31), 7721-7729.

II. Yang, W., Alanne, A. L., Liu, P., Kallio, H. & Yang, B. (2015). Flavonol glycosides in currant leaves and variation with growth season, growth location, and leaf position. *Journal of Agricultural and Food Chemistry*, *63*(42), 9269-9276.

III. Yang, W., Laaksonen, O., Kallio, H. & Yang, B. (2016). Proanthocyanidins in sea buckthorn (*Hippophaë rhamnoides* L.) berries of different origins with special reference to the influence of genetic background and growth location. *Journal of Agricultural and Food Chemistry*, *64*(6), 1274-1282.

IV. Yang, W., Laaksonen, O., Kallio, H., & Yang, B. (2017). Effects of latitude and weather conditions on proanthocyanidins in berries of Finnish wild and cultivated sea buckthorn (*Hippophaë rhamnoides* L. ssp. *rhamnoides*). *Food Chemistry*, *216*, 87-96.

1 INTRODUCTION

Sea buckthorn (*Hippophaë rhamnoides* L.) is a species of berry-bearing, thorny, deciduous shrubs with nitrogen-fixing root nodules in the family Eleagnaceae (Rousi A, 1971). It is divided into eight subspecies which are native to Asia and Europe and also introduced to North America and South Africa (Sun et al., 2002). The plant can withstand extreme temperatures from – 43°C to 40°C, and it is also drought-resistant. It grows typically in dry sand areas and is useful for soil erosion control, land reclamation, wildlife habitat enhancement and farmstead protection (T. S. Li, 2002; Rongsen, 1992). In ancient Greece, the leaves of sea buckthorn have been used as horse feed for weight gain and shiny hair. The Latin name '*Hippophae*' has the meaning of a shiny horse in Greek words (Rongsen, 1992). In Asia, the nutritional and medicinal effects of sea buckthorn have been recorded in traditional Chinese medicine as early as during the Tang Dynasty (Suryakumar & Gupta, 2011). The berries of sea buckthorn contain health beneficial compounds such as vitamins, minerals, carotenoids, fatty acids and flavonoids (Gao, Ohlander, Jeppsson, Bjork, & Trajkovski, 2000; Kallio, Yang, Tahvonen, & Hakala, 2003). For the past 60 years, sea buckthorn berries have been used in clinical treatment of burns, skin diseases, and gastric ulcers in China and Russia (Li & Schroeder, 1996; Singh et al., 2006). Recent research further has increased understanding of the biological activity and health benefits of sea buckthorn including anti-oxidative (Geetha, Sai Ram, Singh, Ilavazhagan, & Sawhney, 2002; Suryakumar & Gupta, 2011), hepatoprotective (Geetha et al., 2008), radioprotective (Chawla et al., 2007), tissue repairing (Upadhyay et al., 2009) and cardiovascular effects (Xu, Kaur, Dhillon, Tappia, & Dhalla, 2011).

Sea buckthorn berries are typically described as sour, bitter and astringent. The sour taste is elicited by abundant fruit acids mainly malic acid and quinic acid (Tiitinen, Hakala, & Kallio, 2005). The bitterness and astringency are primarily due to flavonols, flavanols and proanthocyanidins (PAs) (Lesschaeve & Noble, 2005). Moreover, the degree of polymerization of PAs also affects the profile of bitterness and astringency. In addition, PAs have been reported to possess various biological effects including antioxidative (Hsieh, Shen, Kuo, & Hwang, 2008), anti-inflammatory (Terra et al., 2011), anticancer (Nandakumar, Singh, & Katiyar, 2008) and antimicrobial activities (Zang et al., 2013) *in vivo* and *in vitro*. However, the identification and quantification of PAs is problematic due to the highly complex mixtures in sea buckthorn. These restrictions hinder the understanding of the compounds, and new analytical method needs to be established for identification of PAs in sea buckthorn berries.

Currants (*Ribes* spp. L.) are small, perennial, fruit-bearing and deciduous shrubs of the genus *Ribes* of the family Grossulariaceae. They are mainly distributed in temperate regions of Europe and the North America. The species *R. nigrum* (black currants and green currants) and *R. rubrum* (red currants and white currants) are commonly cultivated in home gardens for fruits and as ornamental shrubs (Geils, Hummer, & Hunt, 2010; Hummer & Dale, 2010). The less common unpigmented green and white currants are variants of black and red currant, respectively (Määttä, Kamal-Eldin, & Törrönen, 2001). In Europe, black currants are widely cultivated for industrial processing.

The currant berries have a number of health-promoting effects due to the high content of bioactive compounds (Cassidy et al., 2012; Gopalan et al., 2012; McCullough et al., 2012; C. Wang, Mehendale, Calway, & Yuan, 2011). Also the leaves of currants have received considerable attention as rich sources of phenolic compounds (He et al., 2010; Tabart et al., 2007; Vagiri, Ekholm, Andersson, Johansson, & Rumpunen, 2012). In traditional folk medicines, black currant leaves have been used to treat rheumatism and arthritis (Declume, 1989). Recent research has shown anti-inflammatory properties of black currant leaves (Garbacki, Kinet, Nusgens, Desmecht, & Damas, 2005). It has been shown that black currant leaves harvested in June have higher antioxidant activity and a higher content of total phenolics than fully ripened berries (Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2006). The main group of phenolics in currant leaves are flavonoids, especially derivatives of kaempferol, quercetin and myricetin (Liu, Kallio, & Yang, 2014; Vagiri et al., 2012). These flavonol glycosides (FGs) are of particular interest, because they have shown anti-inflammatory, antiviral and antioxidant properties, and may reduce the risk of coronary heart disease (Haasbach et al., 2014; Middleton, Kandaswami, & Theoharides, 2000; Moon, Tsushida, Nakahara, & Terao, 2001; Tabart et al., 2012).

As an important berry, the world harvest of currants amounted to approximately 770,000 tonnes in 2013 (data from www.helgilibrary.com), mainly for the juice processing industry. Meanwhile, a large number of leaves are produced every year. Although black currant leaves are used as raw materials to produce tea and beverages in some European countries, the majority of the leaves are still left unused as agricultural waste. In order to provide better exploitation of currant leaves, isolation, identification and quantitative determination of their FGs are of special importance.

Biosynthesis and accumulation of plant secondary metabolites, such as PAs and FGs, are regulated especially by genetic background but also by the growth environment. The growth locations at different latitudes and altitudes have their own environmental profiles, such as growth season, day length, temperature, radiation, precipitation and humidity. Accumulation of secondary metabolites

in plants is a result of complex gene-environment interactions. Investigation on the impact of different genetic backgrounds and environmental factors are essential for a better understanding of plant physiology, and for berry breeding and cultivation as well as for optimization of use of raw materials.

In this thesis, sea buckthorn of three subspecies (ssp. *rhamnoides*, ssp. *mongolica* and ssp. *sinensis*) were compared based on the content and composition of PAs. Moreover, the impact of latitudes, altitudes and weather conditions at the growth sites were also studied. In the study related to currants, the FGs in leaves of three cultivars of black currant (*R. nigrum*), one of green currant *(R. nigrum*), one of red currant (*R. rubrum*) and one of white currant (*R. rubrum*) were compared, taking growth seasons, growth locations, and leaf positions into account.

In the literature review, the recent publications are summarized on chemical structures, sensory characteristics and biological activities of PAs and FGs in food. Moreover, the review also covers the effects of growth environment on biosynthesis of PAs and FGs, as well as the most recent methods for analysis of these two groups of compounds.

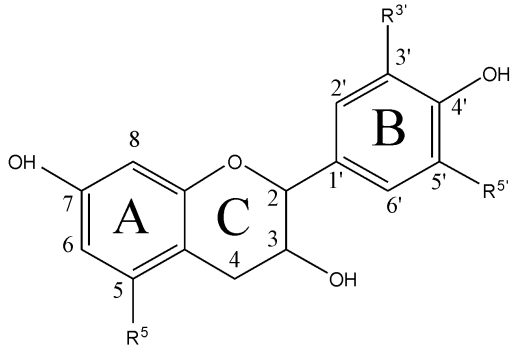
2 REVIEW OF THE LITERATURE

2.1 Proanthocyanidins

Proanthocyanidins (PAs), also known as condensed tannins, are oligomers and polymers consisting of flavan-3-ol units produced *via* the biosynthetic pathway of flavonoids. PAs are widely distributed in seeds, bark, flowers, leaves, roots and even fruit skin in woody and some herbaceous plants, providing specific physiological functions. Research on PAs has attracted high interest over the past decades due to their potentially beneficial effects on human health and due to their contribution to astringency and bitterness in food and beverages such as wine, tea and juices.

2.1.1 Structures and existence of proanthocyanidins

As the monomeric unit of PAs, flavan-3-ols have the typical C6–C3–C6 flavonoid skeleton. The structures of PAs vary depending on the hydroxylation pattern and stereochemistry of the flavan-3-ol units, as well as on the type of linkages between monomeric units and on the degree of polymerization (DP).



Proanthocyanidins (PAs)	Flavan-3-ol	$R^{3'}$	$R^{5'}$	R^{5}
Proguibourtinidins (PGs)	(epi)guibourtinidol	H	H	H
Propelargonidins (PPs)	(epi)afzelechin	H	H	OH
Profisetinidins (PFs)	(epi)fisetinidol	OH	H	H
Procyanidins (PCs)	(epi)catechin	OH	H	OH
Prorobinetinidins (PRs)	(epi)robinetinidol	OH	OH	H
Prodelphinidins (PDs)	(epi)gallocatechin	OH	OH	OH

Fig. 1 Structures of flavan-3-ol units

The names of flavan-3-ols and classes of PAs are shown in **Figure 1**. The most common PA oligomers and polymers consisting of only (epi)catechin, (epi)gallocatechin or (epi)afzelechin subunits are called procyanidins (PCs), prodelphinidins (PDs) or propelargonidins (PPs), respectively. Oligomers or polymers of the other three flavan 3-ols listed in **Figure 1** are less common in nature (Porter, 1988). Flavan-3-ols differ by the presence of hydroxyl groups at positions C3', C5' and C5 (substituents $R^{3'}$, $R^{5'}$ and R^{5}) on the aromatic rings A and B, as well as on the stereochemistry of substituents at C2 and C3 in the heterocyclic ring C. There are chiral centers at C2 and C3 forming four diastereoisomers, two of them are in *cis* configuration and the other two in *trans* configuration. In order to distinguish the *cis*/*trans* isomers, the prefix 'epi' is used for *cis* stereochemistry. *R* and *S* configurations at C2 and C3 are used to distinguish the stereoisomers, such as (–)-epicatechin (2*R*,3*R*) (**Figure 2A**), (+)-catechin (2*R*,3*S*) (**Figure 2B**) (Santos-Buelga & Scalbert, 2000).

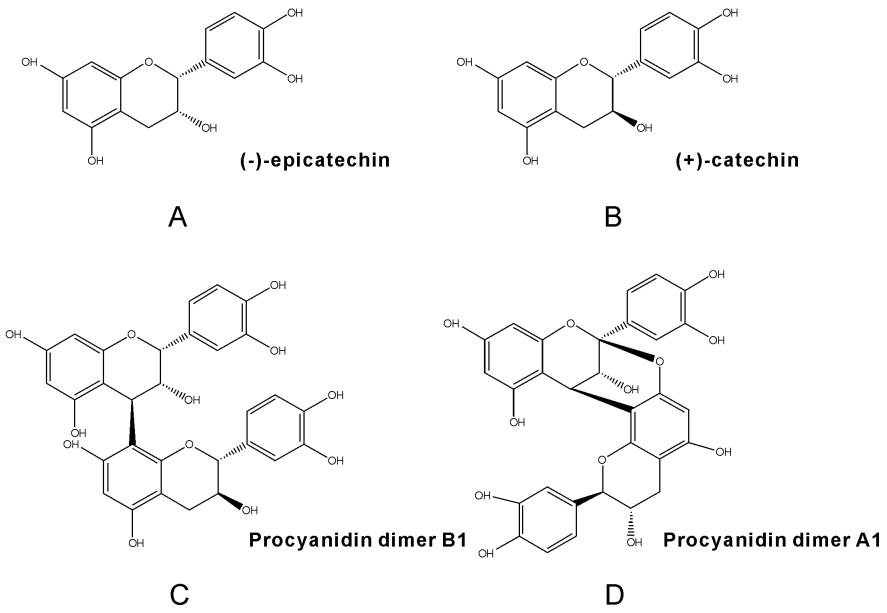


Fig. 2 Structures of (–)-epicatechin (2*R*,3*R*) (A), (+)-catechin (2*R*,3*S*) (B), B-type procyanidin dimer B1 (C) and A-type procyanidin dimer A1 (D)

Two types (B-type and A-type) of linkages between these monomeric units occur in PAs. B-type PAs consist of flavan-3-ol units linked *via* C4–C8 and/or C4–C6 carbon-carbon bonds (**Figure 2C**). A-type PAs contain an additional ether bond C2–O–C7 between the monomeric units (**Figure 2D**) (Porter, 1988). Most of the common PAs are of B-type, whereas the A-type PAs are present in a few specific foods only (Hellström, Törrönen, & Mattila, 2009). As shown in

Table 1, A-type PCs are found exclusively in cranberries, lingonberries, plums, avocados and peanuts. Mixed PCs and PPs coexist in raspberries, strawberries, almonds and some beans. Cinnamon contains even highly heterogeneous mixture of PCs, PDs, and PPs.

Table 1. Proanthocyanidins in common foods (Hellström et al., 2009)

Linkage	Type	Food
A B	PC	cranberries, lingonberries, plums, avocados, peanuts
A B	PC PD PP	cinnamon
B	PC	apple, apricots, bananas, barley, blueberries, blackberries, black eye peas, black beans, cashews, chocolate, choke berries, cherries, cates, grape seed , indian squash, mangos, nectarines, marion berries, peaches, pears, kiwis, sorghum, walnuts
B	PC PD	beer, blackcurrants, green grapes, red grapes, grape juice, red wine, hazelnuts, pecans, pistachios, sea buckthorn berries
B	PC PP	raspberries, strawberries, pinto beans, small red beans, red kidney beans, almonds

In addition, PA esters and PA glycosides are also commonly found in food. Gallic acid can be esterified with the hydroxyl groups of flavan-3-ol units at C3 position, such as epigallocatechin gallate, epicatechin gallate and epiafzelechin gallate present in green tea. Esterification with hydroxyl groups at positions C5 or C7 is rare. PA esters can be e.g. monogalloylated as dimeric epicatechin-(4$\beta \rightarrow$ 8)-epicatechin-3-*O*-gallate (Phansalkar et al., 2015), or highly galloylated as trigalloylated procyanidin trimers (Russo et al., 2013). The PA glycosides are generally formed through the hydroxyl group at C3 position, but also at C5, C6 and C7 positions are possible. Complex structures of PAs bring many difficulties in naming the individual compounds. The formal names are listed according to the names of flavan-3-ol units, interflavanoid linkages and the terminal flavan-3-ol units. The interflavanoid linkages give the connection direction of C4 (B-type) or/and C2 (A-type), and their stereochemistry bonds distinguished by α and β. In addition, common names are also widely used for individual PAs (**Table 2**).

Table 2. Common names and structures of PA dimers. Adapted from Karonen, 2007.

Name	Extension unit	Interflavan Linkage	Terminal Unit
Procyanidin B1	Epicatechin	(4β →8)	Catechin
Procyanidin B2	Epicatechin	(4β →8)	Epicatechin
Procyanidin B3	Catechin	(4α →8)	Catechin
Procyanidin B4	Catechin	(4α →8)	Epicatechin
Procyanidin B5	Epicatechin	(4β →6)	Epicatechin
Procyanidin B6	Catechin	(4α →6)	Catechin
Procyanidin B7	Epicatechin	(4β →6)	Catechin
Procyanidin B8	Catechin	(4α →6)	Epicatechin
Procyanidin A1	Epicatechin	(2β→7,4β→8)	Catechin
Procyanidin A2	Epicatechin	(2β→7,4β→8)	Epicatechin
Prodelphinidin B1	Epigallocatechin	(4β →8)	Gallocatechin
Prodelphinidin B2	Epigallocatechin	(4β →8)	Epigallocatechin
Prodelphinidin B3	Gallocatechin	(4α →8)	Catechin
Prodelphinidin B4	Gallocatechin	(4α →8)	Epigallocatechin
Prodelphinidin B5	Epigallocatechin	(4β →6)	Epigallocatechin
Prodelphinidin A1	Epigallocatechin	(2β→7,4β→8)	Gallocatechin
Prodelphinidin A2	Epigallocatechin	(2β→7,4β→8)	Epigallocatechin

2.1.2 Functions of proanthocyanidins in plants

PAs and their derivatives are widely distributed in plant tissues, such as bark, leaves, fruits, seeds and roots, where the metabolites exhibit several biological and physiological properties. PAs have properties related to protein-binding and metal-binding. The former may result in aversion to the plant and protect the plant from predation by herbivores. PAs also contribute to the astringency and bitterness in plants (Aziz, Paiva, May, & Dixon, 2005; Dixon, Xie, & Sharma, 2005). The compounds may be even poisons to insects and other herbivores. PAs in glandular hair of plant cuticles further illustrate this fact. However, for specific species of insects, PAs can act as feeding and oviposition stimulants (Barbehenn & Constabel, 2011; Sato, Islam, Awata, & Yamasaki, 1999). Plants may also accumulate PAs in the endothelium cell layers or subepidermal layers of fruit and seed coat to fight against bacteria or other

pathogens (Barbehenn & Constabel, 2011). The mechanism for the activity may be derived from the competition between PAs and bacteria to bind iron (Scalbert, 1991). In most plants, PAs are involved in dormancy and germination during the seed development (Debeaujon, Leon-Kloosterziel, & Koornneef, 2000; Lepiniec et al., 2006). In the case of the invasive species *Centaurea maculosa* (spotted knapweed) in western America, PAs are released from the root as phytotoxin which may influences growth of the neighbouring plants (Bais, Vepachedu, Gilroy, Callaway, & Vivanco, 2003).

2.1.3 Role of proanthocyanidins in sensory properties of food

The astringency and bitterness are important oral sensations. Astringency is generally defined as "the complex of sensations due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums or tannins" (ASTM International, 2004). One characteristic of PAs is the ability to complex or precipitate salivary proteins, thus producing tactile sensation by friction in the oral cavity (Breslin, Gilmore, Beauchamp, & Green, 1993; De Freitas & Mateus, 2001). Bitterness is a taste perception perceived through the bitter taste receptor cells (TRC) in taste buds, then transmitted to the brain through the nerves. Taste buds are distributed on the tongue and each of them contains 50–150 different TRCs. In humans, about 25 taste receptors type 2 (TAS2Rs) are responsible for bitter taste (Conte, Ebeling, Marcuz, Nef, & Andres-Barquin, 2002).

PAs play an essential role in sensory properties of astringency and bitterness in food, especially in fruits, wine, tea and cider (Peleg, Gacon, Schlich, & Noble, 1999). In fruits and berries, the content of total PAs ranged from 20 to 2200 mg/100g (FW) (Hellström et al., 2009). In water solution, the threshold value of astringency with polymeric PAs, procyanidin B1, procyanidin B2 and procyanidin B3 is 22, 139, 110 and 116 mg/L, respectively. The threshold concentrations of B-type PAs for bitterness are 230–280 mg/L (Hufnagel & Hofmann, 2008; Schwarz & Hofmann, 2007). It is believed that the intensity of astringency and bitterness sensation depends on the stereochemistry and linkage of PAs, degree of polymerization, galloylation and esterification.

As the subunits and monomers of PAs, (–)-epicatechin and (+)-catechin are stereoisomers, but the former is more bitter and astringent than the latter. The composition of the monomeric subunits affect the intensity of astringency and bitterness of PAs. Cala et al. compared four PC dimers and one PC trimer by their ability to to bind peptides (14 amino acid residues) as a mechanism for simulating astringency. The order of binding capacity was PC C2 > PC B2 > PC B4 > PC B1 > PC B3 (Cala et al., 2010). In addition, the ratio of PC: PD

may influence the profiles of astringency (Laaksonen, Salminen, Mäkilä, Kallio, & Yang, 2015). In the time-intensity evaluation of astringency and bitterness, PC B6 with interflavan linkage (4α→6) is more astringent and bitter than PC B3 and B4 with the linkage (4α→8) (Peleg et al., 1999). The degree of polymerization (DP) also affects astringency and bitterness of PAs. PAs with higher DP values contain more hydrophobic groups, which may increase the ability to bind proteins (Soares, Mateus, & De Freitas, 2007). In contrast to astringency, bitterness has a negative correlation with DP values of PAs (Chira, Schmauch, Saucier, Fabre, & Teissedre, 2008; Peleg et al., 1999). Esterification of PAs with gallic acid can also affect the ability to bind proteins. Esterified PAs in grape seeds are more astringent than the non-esterified PAs. (−)-Epicatechin simultaneously responding to TAS2R4, TAS2R5, and TAS2R39 receptors, whereas PC trimer (PC C2) can only activate TAS2R5 receptor. The bitterness of PAs experienced by consumers may be related to the type and quantity of TAS2Rs (Soares et al., 2013).

2.1.4 Heath effects of proanthocyanidins

PAs are the most common flavonoids in the human diet, and they have received attention in the past decades because of their antioxidative properties and other potential benefits to human health. As natural antioxidants, PAs can eliminate the free radicals that cause oxidative damage to DNA, lipids, and proteins in human body (Es-Safi, Guyot, & Ducrot, 2006; Oki et al., 2002). PAs have also anti-inflammatory properties. The mechanism of action is related to inhibiting the formation of inflammatory cytokines (such as TNFα, IL-1, IL-6) and to limiting the endothelial expression of the adhesion molecules (Giovinazzo & Grieco, 2015; Zhang & Tsao, 2016). According to studies with tumor models created *in vitro* and *in vivo*, PAs can activate the immune system, inhibit cell proliferation and malignant transformation of benign tumors to carcinoma, induce apoptosis of cancer cells and arrest the cancer cell cycle in Gap 2 phase (Katiyar, 2016; Kaur, Singh, Gu, Agarwal, & Agarwal, 2006). A variety of molecular targets modulated by PAs confirmed that PAs can play a potential role in anti-cancer therapy (Nandakumar et al., 2008). In epidemiological surveys, dietary PA consumption is significantly associated with reduced risk of chronic diseases, such as cardiovascular disease (CVD) and type 2 diabetes, which are major causes of morbidity and mortality in Western countries (Kruger, Davies, Myburgh, & Lecour, 2014; X. Wang, Ouyang, Liu, & Zhao, 2014; Zamora-Ros et al., 2014). Recently, Ribas et al. reported that PAs from grape seed extract can modulate the body circadian rhythm and have chronobiological properties (Ribas‐Latre et al., 2015). Moreover, the grape seed PAs can relieve postweaning diarrhea in rats (Song et al., 2011) and

enhance the biological stability of dentin collagen in clinical trials (Y. Liu et al., 2013). Sea buckthorn seed PA extract showed protective effects against light-induced retinal degeneration (Wang et al., 2016).

The antioxidative and anti-inflammatory effects are influenced by the structure of PAs. PDs with trihydroxyphenyl structures show stronger free radical scavenging activity than PCs with dihydroxyphenyl structures (Santos ‐ Buelga & Scalbert, 2000). The antioxidant activity increases with the increasing DP values and with increasing number of esterified groups in ring B (Da Silva Porto, Patrıcia Andréia Leite, Laranjinha, & de Freitas, Victor Armando Pereira, 2003; Osakabe et al., 2002). In a study of anti-inflammatory properties of PAs from peanut skin, the dimers and trimers showed stronger activities against melanogenesis and cytokine production than monomers or tetramers of PAs (Tatsuno et al., 2012).

2.1.5 Absorption and metabolism of proanthocyanidins

Absorption of PAs depends largely on their degree of polymerization (DP). Only PA monomers, dimers, trimers and tetramers are absorbed through the wall of the small intestine by passive diffusion and the rest of PA polymers with DP > 4 are not absorbed as such in the small intestine (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). Compared with absorption rates of PA dimers, trimers and tetramers, the PA monomer has a higher rate of absorption. The metabolites of (–)-epicatechin are present as methylated, glucuronidated and sulphated conjugates in blood and urine (Romanov-Michailidis et al., 2012). PC dimers are absorbed without conjugation or methylation and are directly detected in plasma. The absorption rate of A-type PC dimers (A1 and A2) is higher than that of B-type dimers (B2) (Appeldoorn, Vincken, Gruppen, & Hollman, 2009; Holt et al., 2002; Sano et al., 2003; Serra et al., 2010). However, these absorbable oligomers are not degraded and they do not affect the concentration of (–)-epicatechin (monomeric flavanol) in blood or urine (Ottaviani, Kwik-Uribe, Keen, & Schroeter, 2012). Also food matrix affects the absorption of PAs. Carbohydrate-rich and protein-rich diets may have a negative effect on the absorption of PAs (Serafini et al., 2003; Serra et al., 2010).

The bioavailability of PAs is limited by DP, only a small amount of absorbable PAs are absorbed in the small intestine. The remaining majority of PAs pass from the small intestine into the colon, where these non-absorbable PAs with DP > 4 are catabolized by gut microflora, and still have potential health benefits due to prebiotic activities and microbial metabolites (Ou & Gu, 2014). In the early stage of microbial catabolism of B-type PAs (procyanidin B2), the C-ring and A-ring are cleaved and oxidized. In addition, also the

interflavan bond can be cleaved by bacteria to form monomeric PAs (Stoupi, Williamson, Drynan, Barron, & Clifford, 2010). Compared to B-type PAs, A-type PAs are more stable due to their additional C2–O–C7 bond and only C-ring is cleaved during degradation (Engemann, Hübner, Rzeppa, & Humpf, 2012). In the model of fermentation with human colonic fecal bacteria, major catabolites of PAs are benzoic acid derivatives, phenylacetic acids, and phenylpropionic acids, which may play a role in inhibiting inflammatory factors and preventing atherosclerosis (Cueva et al., 2013; Engemann et al., 2012; Karlsson et al., 2005). PAs also offer potential health benefits by modulation of intestinal flora. Recent studies have shown that grape seed PAs promote the growth of *Bifidobacterium, Lactobacillus* and *Enterococcus* species, and decrease the population of *Clostridium histolyticum* and other clostridia (Cueva et al., 2013). Moreover, A-type PAs from cranberries combined with probiotics as a synergistic functional food inhibit *in vitro* invasion of gut epithelial cells by extra-intestinal pathogenic *Escherichia coli* (Polewski, Krueger, Reed, & Leyer, 2016).

2.2 Flavonol glycosides

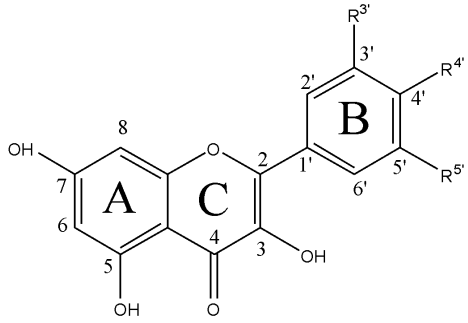
Flavonols are a class of flavonoids which widely occur in fruits, vegetables, and beverages. Though some flavonols exist as aglycones, most of them are typically conjugated with sugars as FGs (Del Rio et al., 2013). Increasing evidences suggest that dietary flavonols and FGs may have protective effects against human chronic diseases, and may reduce the risk of certain cancers (Del Rio et al., 2013; Y. Xie, Huang, & Su, 2016)

2.2.1 Structure of flavonol glycosides

The structure of flavonols also consists of two aromatic rings and one heterocyclic pyran ring (C6–C3–C6) (**Figure 3**). C-ring of flavonols has a double bond between C2 and C3, a keto group at C4 and a hydroxyl group in position C3.

The positions C3′, C4′ and C5′ in B-ring may be substituted with methoxy groups or hydroxyl groups, thus forming different flavonol aglycones (Aherne & O'Brien, 2002). Practically, the common aglycones are galangin, kaempferol, quercetin, myricetin, and isorhamnetin (**Figure 3**). The aglycones can be glycosylated by even up to four or more saccharides. The most common monosaccharides are glucose (Glu), galactose (Gal), rhamnose (Rha) and xylose (Xyl), whereas the common disaccharides are sophorose (Glu-β-(1→2)-Glu), gentiobiose (Glu-β-(1→6)-Glu), and rutinose (Rha-α (1→6)-Glu) (Veitch & Grayer, 2008). The preferred site of glycosylation is C3, and less frequently

at position C7 (Fossen, Pedersen, & Andersen, 1998). The presence of various saccharides and complex linkages results in diverse FGs. For example, more than 200 kaempferol glycosides are found in plants (Del Rio et al., 2013). With the rapid development of MS and NMR analysis, more complex FGs can be identified.



Name	$R^{3'}$	$R^{4'}$	$R^{5'}$
Galangin	H	H	H
Kaempferol	H	OH	H
Quercetin	OH	OH	H
Myricetin	OH	OH	OH
Isorhamnetin	OCH_3	OH	H

Fig. 3 The structure of flavonols

2.2.2 Flavonols and flavonol glycosides in food

Dietary flavonols are widely distributed in vegetables and fruits where FGs are the main forms of existence. FGs are mainly accumulated in leaves and peels, but rare in the rhizome with the exception of onions (Aherne & O'Brien, 2002). Among the dietary flavonols, glycosides of kaempferol, quercetin, myricetin and isorhamnetin are most widespread. Kaempferol is abundantly found in leafy vegetables and some spices, such as spinach, kale, endive and fennel, with concentrations between 0.1 and 27 mg/100 g FW. Capers, cocoa, onions and berries are good sources of quercetin glycosides (Tan et al., 2003). The richest sources of myricetin are some spices and medicinal herbs such as parsley and dill weed, as well as some berries such as currants and cranberries (Ong & Khoo, 1997). Isorhamnetin is present in some spices such as fennel, and in fruits like almonds and sea buckthorn berries at levels ranging from 1 to 10 mg/100 g FW (De la Rosa, Alvarez-Parrilla, & Gonzalez-Aguilar, 2009; Ma et al., 2016). Moreover, beverages such as tea and wine are also good sources of flavonols, together with PAs contributing to the astringent taste (Hufnagel & Hofmann, 2008).

2.2.3 Health effects of flavonols

Flavonols are bioactive compounds directly associated with human health. As the active ingredients of some traditional Chinese medicine, such as white

mulberry leaf (*Morus alba* L., Sang ye), sophorae flower (*Sophora japonica* L., Huai hua), and polygoni avicularis herba (*Polygonum aviculare* L., Bian xu), flavonols and their glycosides have been used as drugs for centuries (Matkowski, Jamiolkowska-Kozlowska, & Nawrot, 2013). Flavonols are non-essential nutrients, but long-term moderate dietary intake may play a role in promoting health. In the past decades, evidences of epidemiological studies have suggested that dietary flavonols can significantly reduce the incidence of some chronic diseases such as type 2 diabetes, CVD and even cancers (X. Wang et al., 2014; Y. Xie et al., 2016). Flavonols have the common properties of flavonoids, of which the antioxidant and anti-inflammatory activities are the most representative. Recent research suggestes that quercetin induces autophagy, nuclear erythroid 2-related factor 2-antioxidant responsive element (Nrf2-ARE), and antioxidant/anti-inflammatory enzyme paraoxonase 2 against oxidative stress (Costa, Garrick, Roquè, & Pellacani, 2016). Flavonols and FGs have antibacterial and antiviral activity, they can inhibit bacterial DNA synthesis and various enzymes related to the life cycle of viruses (Cushnie & Lamb, 2005; Kumar & Pandey, 2013). Quercetin has been shown to inhibit *Escherichia coli* DNA gyrase, and to prevent infection and replication cycle of herpes simplex virus type-1 and dengue virus type-2 (Kumar & Pandey, 2013). Among the flavonols, myricetin can affect glucose metabolism by increasing hepatic glycogen synthase I activity and stimulating the transport of glucose alleviating the symptoms of diabetes (Ong & Khoo, 2000). Moreover, a recent study showed that oral flavonols may be used as inhibitors of serine-threonine kinase CK2, which is over-expressed in various cancers (McCarty, 2015).

Structures of aglycones and the total number of sugar moieties affect the properties and biological activity. Comparing the antioxidant capacity and toxicology of four aglycones, myricetin, quercetin, kaempferol and galangin, Kim et al. reported that the intracellular antioxidant activity and LD_{50} values enhanced with the increasing number of hydroxyl groups on B-ring (Kim, Liu, Guo, & Meydani, 2006). Usually, the antioxidant activity of aglycones is stronger than their corresponding glycosides (Kumar & Pandey, 2013). In aqueous phase trolox equivalent antioxidant capacity assay, the effect of quercetin is almost twice as high as that of quercetin monoglycosides and diglycosides. The effect of kaempferol is about 3–10 times higher than that of kaempferol glycosides. (Plumb, Price & Williamson, 2013)

2.2.4 Absorption and metabolism of flavonols

Glycosides are the main forms of dietary flavonol compounds. Particularly, the type and number of glycosidic moieties affect the bioavailability and absorption of flavonols. Free flavonol aglycones are relatively hydrophobic and

can be transported by passive diffusion (Day, Gee, DuPont, Johnson & Williamson, 2003). With the addition of a glycosidic substituent, hydrophilicity is enhanced, which reduces the capability of passive diffusion, and the glucose transporter can transport the FGs (Aherne & O'Brien, 2002; Hollman, de Vries, van Leeuwen, Mengelers & Katan, 1995). Nelson et al. detected FGs in urine after consumption of cranberry juice. Among the five FGs studied, myricetin-3-galactoside was the earliest postprandial peak of FGs in plasma, whereas appearance of quercetin-3-arabinoside took the longest time. Quercetin-3-galactoside was the most abundant FG in urine after consumption of cranberry juice (Nelson et al., 2016). Quercetin glucoside is more efficiently absorbed than the aglycone itself, whereas rutin with disaccharide rutinose is less efficiently absorbed than quercetin aglycone (Hollman, de Vries, van Leeuwen, Mengelers & Katan, 1995).

The first step of metabolism of FGs is deglycosylation. There are two possible ways of hydrolysis of flavonoid glycosides. One is the hydrolysis by the lactase phloridzin hydrolase (LPH) at the brush border membrane of epithelial cell. LPH can deglycosylate FGs by the specificity for flavonoid-*O*-*β*-D-glucosides, after which the aglycone is absorbed by passive diffusion (Day et al., 2000). Another is the transport mediated by cytosolic *β*-glucosidase (CBG) in epithelial cells. Hydrolysis by CBG requires active transport of the FGs into epithelial cell as entirety (Gee, DuPont, Day, Plumb, Williamson & Johnson, 2000). After absorption, flavonols undergo hydroxylations, methylations and sulfation in multiple organs such as liver, small intestine, colon and kidney (Aherne & O'Brien, 2002). Liver is considered the major metabolic organ, but a research suggests that sulfation of quercetin occurs only in the small intestine instead of liver or colon (Del Rio, Rodriguez-Mateos, Spencer, Tognolini, Borges & Crozier, 2013). In a metabolic study with rats, radiolabeled quercetin-4'-glucoside (4 mg/kg body weight) was rapidly degraded to phenolic acids, principally to 3-hydroxyphenylacetic acid and benzoic acid in gastrointestinal tract, and then excreted with urine in 72 hours (Mullen et al., 2008).

Increasing evidence shows that FGs are not only absorbed in epithelial cells, but also can be degraded by gut microflora into aglycones and phenolic acids. Rutin and robinin (kaempferol-3-*O*-robinoside-7-*O*-rhamnoside) are bacterially converted into quercetin and kaempferol in human gut, respectively. These mechanisms are associated with cleavage of glycosidic bonds by bacterial glycosidases and/or C-ring cleaving enzyme produced by intestinal bacteria (Aherne & O'Brien, 2002).

2.3 Biosynthesis of proanthocyanidins and flavonol glycosides

2.3.1 General biosynthetic pathway of proanthocyanidins and flavonol glycosides

Both PAs and FGs are synthesized as end products in different branches of the flavonoid pathway. They share the same upstream pathways, the acetate/malonate pathway and the shikimate pathway. The main steps of the biosynthesis pathways of PAs and FGs are shown in **Figure 4**.

In the first step, *p*-coumaroyl-CoA produced from the shikimate pathway and three malonyl-CoAs from the acetate/malonate pathway are combined to form chalcone by chalcone synthase (CHS). *P*-coumaroyl-CoA is considered to form the aromatic B-ring and the heterocyclic C-ring, and three malonyl-CoAs are added through sequential decarboxylation and condensed to A-ring (Tsao, 2010). The second step of the pathway is the isomerization reaction from chalcone to naringenin. This reaction can occur spontaneously but it can also occur more efficiently by the action of chalcone isomerase (CHI) (Cain, Saslowsky, Walker & Shirley, 1997). Naringenin is further transformed into dihydrokaempferol by flavanone 3-hydroxylase (F3H) (Holton & Cornish, 1995). Dihydrokaempferol is converted to dihydroquercetin and dihydromyricetin by the catalysis of flavonoid 3'-hydroxylase (F3'H) and flavonoid 3', 5'-hydroxylase (F3'5'H), respectively. These compounds can also be converted from eridictyol and pentahydroxyflavanone derived from naringenin in the previous step. From dihydroflavonols, PAs and FGs will have their own branches of pathway.

Dihydroflavonols (dihydrokaempferol, dihydroquercetin and dihydromyricetin) are converted to kaempferol, quercetin and myricetinis mediated by flavonol synthase (FLS) (Tsao, 2010). These flavonols are glycosylated, thereby formed FGs by glycosyltransferases (GTFs) (De la Rosa, Alvarez-Parrilla & Gonzalez-Aguilar, 2009). The dihydroflavonols are reduced by dihydroflavonol reductase (DFR) to (2*R*, 3*S*, 4*S*)-leucoanthocyanidins and further to (2*R*, 3*S*)-flavan-3-ols by leucoanthocyanidin reductase (LAR), which are subunits of PAs. (2*R*, 3*S*, 4*S*)-leucoanthocyanidins can also be oxidized by anthocyanidin synthase (ANS) to anthocyanidins, which are converted to (2*R*,3*R*)-flavan-3-ols by anthocyanidin reductase (ANR) (He, Pan, Shi & Duan, 2008, Tsao, 2010). All the (2R, 3S, 4S)-leucoanthocyanidins, (2*R*, 3*S*)-flavan-3-ols and (2*R*, 3*R*)-flavan-3-ols are potential precursors for PAs (Dixon, Xie & Sharma, 2005; Tian, Pang & Dixon, 2008).

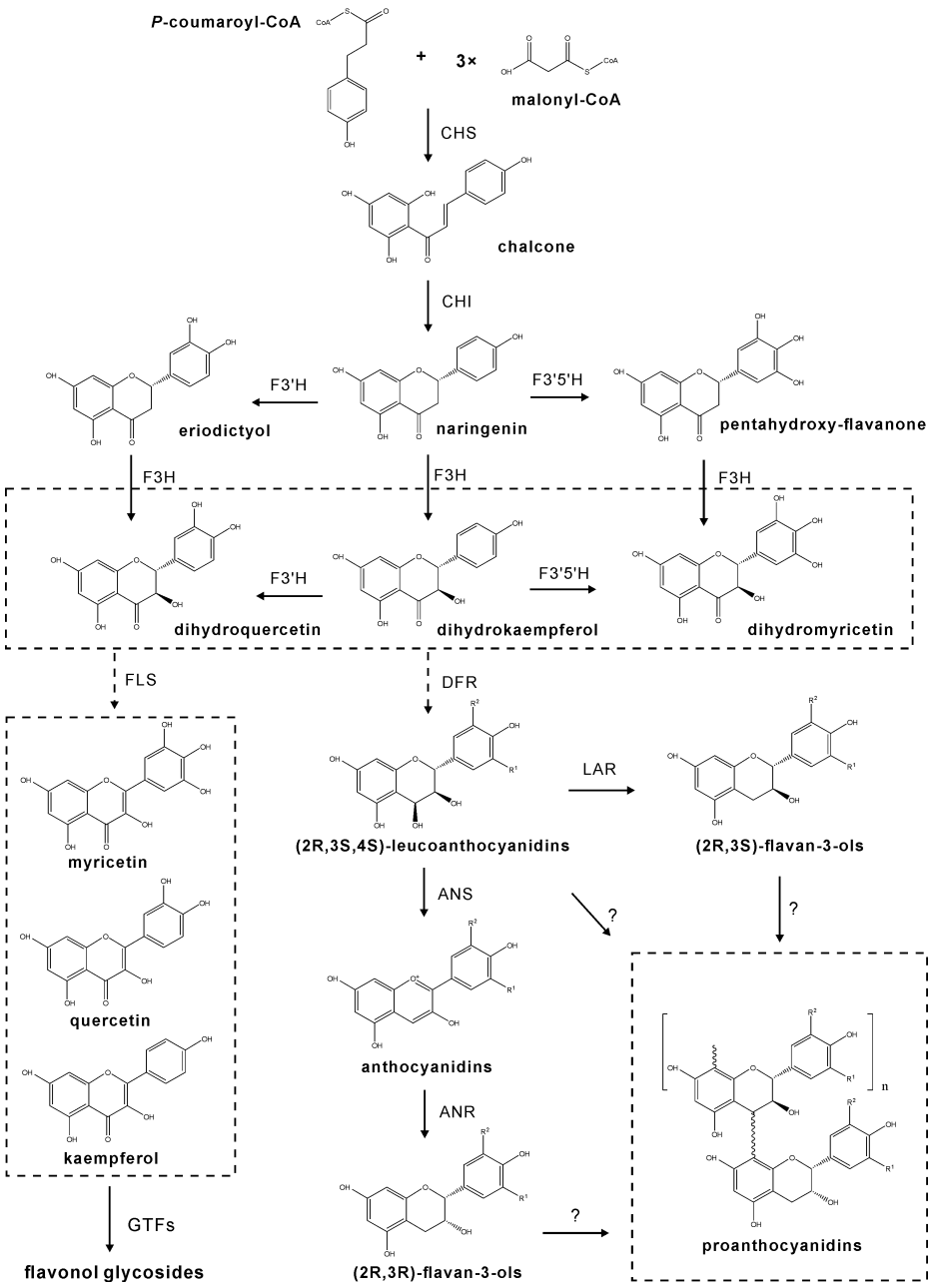


Fig. 4 The biosynthetic pathway of proanthocyanidins and flavonol glycosides. Adapted from He et al., 2008. CHS, chalcone synthase; CHI, chalcone isomerase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3', 5' -hydroxylase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; GTFs, glycosyltransferases ; DFR, dihydroflavonol reductase; LAR,

leucoanthocyanidin reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase.

2.3.2 Transportation and polymerization of proanthocyanidins

Proanthocyanidins generally accumulate in the vacuoles, whereas their precursors are mostly located in endoplasmic reticulum or cytoplasm (Saslowsky & Winkel-Shirley, 2001). Up to now, the mechanisms of transport are still controversial. More and more attention has been paid to the role of transparent testa 19 (TT19), a glutathione *S*-transferase (GST), in accumulation and transformation of anthocyanidins in a variety of plants (Zhao, Pang & Dixon, 2010). TT19 can be combined to flavonoids and prevent oxidation of anthocyanins, and then transfer anthocyanins into vacuoles (Zhao, Pang & Dixon, 2010). However, the role of TT19 in transformation of PAs is unclear. A possible process is that PAs are bound to TT19, which contributes to mediating intracellular membrane trafficking (Ichino et al., 2014). Polymerization mechanisms of PAs are still poorly understood. Although several potential mechanisms have been studied, such as catalysis by polyphenol oxidase, regulation of transparent testa 10 gene and non-enzymatic polymerization, none of them have been proven by enough evidence and direct experimental support (He, Pan, Shi & Duan, 2008; Zhao, Pang & Dixon, 2010). This unresolved question deserves further investigation.

2.4 Analysis of proanthocyanidins and flavonol glycosides

As natural flavonoids, the structure, composition and content of PAs and FGs significantly affect their biological activities, absorption and metabolism as reviewed in the previous section. Qualitative and quantitative analysis aims to increase the overall understanding and exploitation of these flavonoids. Although instrumentation and techniques of analysis are rapidly developing, the complex glycosylation and polymerization still present many challenges. This section is intended to review the preparation, extraction, purification and analysis of PAs and FGs from plant foods.

2.4.1 Sample preparation

Fruits, berries, vegetables and beverages (wine, tea, juice) are the major sources of flavonoids in the human diet. Sample preparation for analysis usually depends on composition and matrix of the materials to be studied. In the process of collection, transportation and preservation, plant samples have to be handled with care. In order to avoid deterioration of the samples and degradation of the target compounds by enzymes or oxidazion, samples are

usually dried or frozen after collection (Stalikas, 2007). Samples are milled or ground to obtain a certain particle size for higher extraction yields (Gião, Pereira, Fonseca, Pintado & Malcata, 2009). In this case, liquid nitrogen is usually the best choice, which reduces damage of phenolic compounds compared to high-temperature drying.

2.4.2 Extraction and purification

Liquid–liquid and solid–liquid extractions are the most common technique used to isolate flavonoids from food samples. As classes of flavonoids, PAs and FGs in their natural forms are relatively hydrophilic. Thus, water, polar organic solvents such as methanol, ethanol, acetone, and mixtures of water and organic solvents are employed in extraction (Tsao, 2010). Also the weak lipophilic solvent ethyl acetate is used to extract FGs. For instance, 70 % aqueous acetone has been applied for extracting FGs from currant leaves (Liu, Kallio & Yang, 2014; Yang, Alanne, Liu, Kallio & Yang, 2015). PAs are generally more stable at low pH conditions and weak or strong acids at low concentrations may be added in the extraction solvent. E.g. 0.5 % acetic acid in the solvent has been used for PA extractions (Kallio, Yang, Liu & Yang, 2014). When extracting PAs from grape seeds, 15 % aqueous ethanol combined with maceration can give the highest extraction yields (Hernández-Jiménez, Kennedy, Bautista-Ortín & Gómez-Plaza, 2011). In recent years, other techniques have arisen, such as supercritical fluid extraction, ionic liquids extraction and ultrasound-assisted extraction. Compared to the conventional extraction procedures, the solvents used in the new methods are easily recycled, non-flammable and non-toxic (Khoddami, Wilkes & Roberts, 2013; Yang, Sun, Yang, Zhao, Zhang & Zu, 2012) but their applicability and efficiency are highly case-specific.

PAs and FGs have similar solubilities as many other flavonoids, which may make the analyses complicated. Therefore, from samples rich in various groups of flavonoids, isolation of the target compounds is necessary. Molecular imprinting can be applied in solid-phase extraction of flavonols and FGs. Molecularly imprinted polymer is prepared using target compound (template), functional monomer and crosslinker. The porogen is used to remove the template, leaving behind specific recognition sites for target compounds. As the sorbent material, rutin, kaempferol and quercetin may be selected as a specific class of compounds by specific recognition sites (Theodoridis, Lasakova, Škeříková, Tegou, Giantsiou & Jandera, 2006; Xie, Zhu, Luo, Zhou, Li & Xu, 2001).

Sephadex LH-20 is commonly used to fractionate PAs (Naczk & Shahidi, 2004). Carbohydrates and low-molecular weight phenolics can be removed by

20 % – 50 % aqueous methanol in Sephadex LH-20 column chromatography. PA oligomers and polymers are eluted by 70 % aqueous acetone (Kallio, Yang, Liu & Yang, 2014). In addition, other solid phases such as C18 Sep-Pak, Sephadex G-25 and MCI gels are also used for purification of PAs (Karonen, 2007).

2.4.3 High performance liquid chromatography

In order to accurately characterize PAs and FGs, individual target compounds must be well separated. High performance liquid chromatography (HPLC) is the most widely used tool for separation of flavonoids. Reversed phase liquid chromatography (RP-HPLC) and normal phase liquid chromatography (NP-HPLC) are both used for separation of flavnoids (Karonen, 2007). Hydrophilic interaction liquid chromatography (HILIC) is an alternative LC method, a variant of NP-HPLC, which is used for separating polar compounds. Moreover, a combination of two of the methods above forms two-dimensional liquid chromatography (2D-HPLC), which is a powerful technique for the separation and analysis of complex samples, especially of PAs.

In RP-HPLC, the mobile phase is more polar than the stationary phase and elution of the compounds takes place in the order of decreasing polarity. In the late 1970s, RP-HPLC was applied to the analysis of PAs, and the method separated successfully PA oligomers. A typical elution order of the PA monomers is (+)-gallocatethin, (–)-gallocatethin, (+)-catethin, (–)-catethin and the order of PC oligomers is C2, B3, B1, B6, B4, B2, B7, C1 and B5 (Karonen, 2007, Koupai-Abyazani, McCallum & Bohm, 1992). PAs with *cis* stereochemistry and linkage through C4–C8 are eluted earlier than other PAs in RP-HPLC. However, PA polymers are not well separated by RP-HPLC and they overlap with oligomers due to similar polarity, which gives challenges to mass spectrometric analysis.

RP-HPLC is used to separate also flavonols. The more polar compounds are generally eluted first and the elution order is: flavonol diglycosides, flavonol monoglycosides, and flavonol aglycones (Harborne & Mabry, 2013, Watson, 2014). Also the structures of the sugar moieties affect the elution order. In case of same aglycone and same position of glycosylation, the elution order is: galactoside, glucoside, pentoside, rhamnoside, and glucurunoside (Cuyckens & Claeys, 2004, Ma et al., 2016).

In NP-HPLC analysis, combination of a polar stationary phase (silica) and a mobile phase of low polarity (organic solvent) is used. In 1993, NP-HPLC was for the first time used in separation of PCs up to tetramers from cacao beans and grape seeds (Rigaud, Escribano-Bailon, Prieur, Souquet & Cheynier, 1993). Then, the methods were improved to further separate the PAs up to decamers

(Gu et al., 2002), and successfully applied to separate PAs in 39 different foods including fruits, vegetables, cereals, nuts, beverages and spices (Gu et al., 2003a). In NP-HPLC, PAs are usually eluted in the order of increasing DP. In addition, also galloylation of PAs influence the order of elution. The non-galloylated PAs are eluted earlier than the galloylated ones, and increasing the number of galloyl substituents increases elution time (Rigaud, Escribano-Bailon, Prieur, Souquet & Cheynier, 1993).

In 1990, HILIC was the first time proposed for analysis of peptides and nucleic acids (Alpert, 1990). Although a variant of NP-HPLC, the separation mechanism of HILIC is different and more complex than in NP-HPLC. HILIC shares the same stationary phases with NP-HPLC (silica, amino) and uses similar mobile phase as in RP-HPLC (water and acetonitrile). Further, it is also compatible with ion chromatography (IC) for separating charged substances (**Figure 5**) (Buszewski & Noga, 2012). Compared with traditional LC methods, HILIC has its advantages. Aqueous mobile phase is more suitable for polar samples and high concentrations of organic solvents make samples more conductive to ionization in mass spectrometry (MS). HILIC has been successfully applied for separation of PAs (Buszewski & Noga, 2012; Karonen, Liimatainen & Sinkkonen, 2011; Kelm, Johnson, Robbins, Hammerstone & Schmitz, 2006; Yanagida et al., 2007). PAs are eluted according to DP in HILIC, which is similar with NP-HPLC, but HILIC has better stability and higher sensitivity (Kallio, Yang, Liu & Yang, 2014).

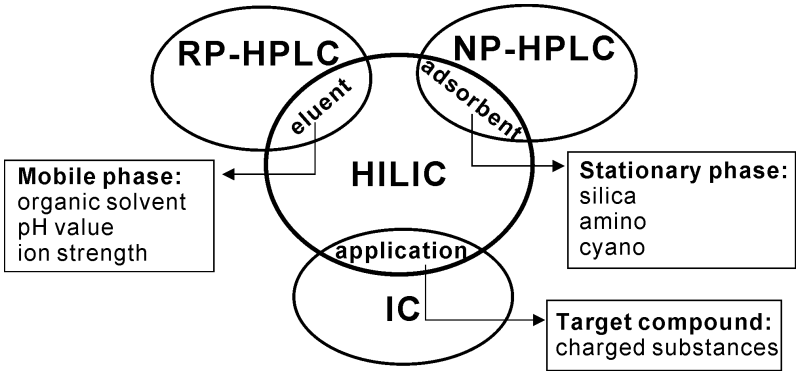


Fig. 5 The characteristics of HILIC overlap with those of RP-HPLC, NP-HPLC and IC.

Polymeric PAs with the same DP values generally include a number of isomers due to multiple linkages of subunits. Separation of individual compounds from these complex mixtures of PAs cannot be implemented by any single method of RP-HPLC, NP-HPLC or HILIC. With two-dimensional

HPLC technique, separation of complex mixtures of PAs became possible in a single run. In this method, the first-dimension and second-dimension columns have different separation mechanisms. Compounds with poor separation in the first dimension may be well separated in the second dimension. Two-dimensional HPLC has been successfully applied to the analysis of PAs (Kalili & de Villiers, 2009; Montero, Herrero, Prodanov, Ibáñez & Cifuentes, 2012; Montero, Herrero, Ibáñez & Cifuentes, 2013). Usually, NP-HPLC or HILIC is used as the first dimension, which can separate PAs by DP, and RP-HPLC is employed in the second dimension for eluting PAs isomers of the same DP by polarity.

The individual compounds separated by HPLC needs to be identified based on spectral properties. Ultraviolet-visible (UV-Vis), photo diode array (PDA) / diode array detector (DAD) and fluorescence detector (FLD) are the most common detectors for HPLC. PAs and FGs have characteristic absorbance peaks at 280 nm and 360 nm, respectively. The two channels are usually recorded simultaneously to exclude interference from other compounds. Fluorescence detection is more suitable and selective for PAs and it is often conducted with an excitation wavelength of 230 nm and an emission wavelength of 321 nm (Huemmer & Schreier, 2008)

2.4.4 Mass spectrometry

Mass spectrometry is the primary tool in identification of specific compounds. It provides indication of chemical structures with excellent sensitivity and selectivity. Atmospheric pressure ionization (API) interface is often coupled with HPLC, mainly in electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) modes (Thomson, 1998). Compared to APCI, ESI is more suitable for analysis of phenolic compounds due to better sensitivity and lower background noise (Pérez-Magariño, Revilla, González-SanJosé & Beltrán, 1999). ESI is a kind of soft ionization method, in which the analytes in the samples are atomized into charged droplets (Diameter 20 nm) by high voltage capillary and reach the mass analyzer (Greaves & Roboz, 2013). Commonly used analyzers for flavonoid analyses include quadrupoles, quadrupole ion traps, and time of flight analyzers. Moreover, multi-analyzers can also be used in conjunction, called tandem mass spectrometry (MS/MS), which can examine selectively the fragmentation of particular ions in a mixture of ions (Greaves & Roboz, 2013).

ESI-MS and ESI-MS/MS have been widely applied in the analysis of PAs (Callemien & Collin, 2008, Gu et al., 2002, Karonen, Liimatainen & Sinkkonen, 2011, Karonen, Loponen, Ossipov & Pihlaja, 2004, Montero, Herrero, Prodanov, Ibáñez & Cifuentes, 2012; Montero, Herrero, Ibáñez &

Cifuentes, 2013). The fragmentation pathway of PAs includes retro-Diels−Alder reaction (RDA), heterocyclic ring fission (HRF), benzofuran-forming (BFF), and quinone methide (QM) fissions (Li & Deinzer, 2007). The ESI source operated in negative ion mode is commonly used for PAs, where PA oligomers and polymers are detected as $[M-H]^-$ ions, or multiply charged $[M-2H]^{2-}$ and $[M-3H]^{3-}$ ions in full scan mode (Gu et al., 2003b; Hayasaka, Waters, Cheynier, Herderich & Vidal, 2003; Kallio, Yang, Liu & Yang, 2014). In selected ion recording (SIR)/selected ion monitoring (SIM) mode of MS and in selected reaction monitoring (SRM) or multiple reactions monitoring (MRM) mode of MS/MS, PAs with specific *m/z* values based on full scan mode are detected thus increasing the separation capacity (Kallio, Yang, Liu & Yang, 2014; Prasain et al., 2009). Recent research showed that ESI-MS/MS with optimized cone voltages can be used for analyses of PAs of a wide range of DP values (Engström, Pälijärvi, Fryganas, Grabber, Mueller-Harvey & Salminen, 2014).

FGs are the most common forms of flavonols in nature. ESI-MS and ESI-MS/MS analyses in positive ion mode produce fragments providing significant amount of information on the structures of the compounds. Loss of sugar residues, glucose as the most common one in FGs, was revealed. In addition, typical existence of galactose, rhamnose, pentose and disaccharide substituents were found in fruits and leaves of various plants (Liimatainen, 2013). Aglycone ions are formed by the cleavage of sugar moities. However, identification of FGs is still problematic even though the type and position of the sugar substitution of the FGs affect to some extent the fragmentation of aglycone (Hvattum & Ekeberg, 2003). SRM/MRM methods with the appropriate precursor ion improve the selectivity (Wang, Sha, Yu & Liang, 2015). In addition, a method for differentiating between sugar residues with the same molecular weight is proposed by identifying radical and even-electron flavone ions (March, Lewars, Stadey, Miao, Zhao & Metcalfe, 2006).

2.4.5 NMR

NMR has an irreplaceable role in analyses when accurate configurational and conformational information on molecular structures is required. Briefly, the principle of NMR is based on the energy transition of certain atomic nuclei in an external magnetic field. The nuclei are excited with a radio frequency pulse. The frenquency of the signals emitted by the nuclei is measured and processed for analysis. The most commonly used one-dimensional and two-dimensional (2D) NMR techniques are ^{1}H, ^{13}C, correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple quantum correlation (HMQC), and heteronuclear multiple bond correlation

(HMBC). These techniques are widely used to identify PAs and FGs. The ratio of 2,3-*cis* to 2,3-*trans* stereochemistries in PAs can be obtained from the difference in C2 position chemical shifts through ^{13}C NMR (Behrens, Maie, Knicker & Kögel-Knabner, 2003). In sea buckthorn containing mixtures of PCs and PDs, most subunits of PAs possessed a 2, 3-*trans* configuration (Rösch, Mugge, Fogliano & Kroh, 2004). ^{1}H NMR was employed to estimate the DP of PAs by integrating the A-ring proton signals (Guyot, Le Guernevé, Marnet & Drilleau, 1999). 2D-NMR measurements proved the existence of four rotameric forms in two native PC trimers (Tarascou et al., 2007). Moreover, recent research demonstrated that quantum-mechanics driven ^{1}H iterative Full Spin Analysis (QM-HiFSA) of ^{1}H NMR spectra can distinguish spectral detail and rapidly provide information of the 3D structure and bonding activity of PA oligmers (Nam et al., 2015). For FGs, ^{1}H NMR is particularly useful in identifying the aglycone type and the anomeric configuration of the glycoside moieties, while ^{13}C NMR is more suitable for identification of the type of glycoside moieties. (Pinheiro & Justino, 2012). HMBC helps to distinguish anomeric resonances from aromatic resonances in high resolution (Liimatainen, 2013).

2.5 Effects of growth conditions

Environmental factors such as latitude, altitude, light, temperature, precipitation and soil have influence on biosynthesis and accumulation of the secondary metabolites in plants. As a part of the plant defense mechanism, secondary metabolites are self-regulated for adaption to various environmental conditions. Flavonoids have important physiological functions in plants and the compounds also exert biological activities in humans. Thus, it is necessary to understand the impact of environmental factors on the biosynthesis of flavonoids.

2.5.1 Effects of latitude and altitude

Changes in the latitude and the altitude of the growth place are usually accompanied by changes in environmental factors, such as temperature, radiation, photoperiod, light quality and light intensity. The further north the growth site, the higher is the impact of latitude on flavonoids. At high latitudes like in Scandinavia, latitude typically correlates negatively with temperature, radiation and length of the growth season whereas positive correlation is seen with day-length during the growth season.

Impact of latitude on the flavonoid contents in various plant species has been reviewed by Jaakola and Hohtola (Jaakola & Hohtola, 2010). Lätti et al.

analyzed anthocyanins in bilberry (*Vaccinium myrtillus*) and both anthocyanins and flavonols in bog bilberry (*Vaccinium uliginosum*) samples collected from northern and southern Finland. The contents of anthocyanins and flavonols in the northern samples were higher than those in the southern samples (Latti, Jaakola, Riihinen & Kainulainen, 2010, Lätti, Riihinen & Kainulainen, 2008). Similar results have been obtained in bilberries grown in Sweden and Denmark (Åkerström, Jaakola, Bång & Jäderlund, 2010). In studies of pomegranate and red currants, the total contents of anthocyanins were significantly higher in samples grown at higher latitude (Borochov-Neori et al., 2011; Schwartz et al., 2009; Yang, Zheng, Laaksonen, Tahvonen & Kallio, 2013). The concentration of phenolic compounds and terpenoids in juniper (*Juniperus communis*) needles have been shown to increase with latitude and altitude in Finland. (Martz, Peltola, Fontanay, Duval, Julkunen-Tiitto & Stark, 2009). A recent study showed that berries of most varieties of sea buckthorn (*Hippophaë rhamnoides* ssp. *mongolica*) grown in northern Finland (high latitude) had higher levels of total FGs compared to the same varieties grown in southern Finland or in Québec, Canada (low latitude) (Ma et al., 2016). In this thesis research, sea buckthorn berries (*H. rhamnoides* ssp. *rhamnoides*) grown further north contained more than twice the amount of total PAs compared with the berries grown in southern Finland (Yang, Laaksonen, Kallio & Yang, 2016).

The effects of altitude on flavonoids have been observed in a variety of plants. In a study on grape fruits (*Vitis vinifera* L. cv. Malbec) grown at three altitudes (500 m, 1000 m, 1500 m), the total anthocyanins and total polyphenols in the skin showed a significantly increasing trend as the altitude increased from 500 m to 1000 m (Berli, D'Angelo, Cavagnaro, Bottini, Wuilloud & Silva, 2008). Camas et al. investigated St. John's Wort (*Hypericum orientale* L. and *Hypericum pallens* Banks & Sol.) grown at different altitudes (from 400 m to 3250 m) and found that both species produced significantly higher amount of flavonols at higher altitudes (Camas, Radusiene, Ivanauskas, Jakstas & Cirak, 2014). Leaves of Du-Zhong (*Eucommia ulmoides* Oliv.) are a known raw material for traditional Chinese medicine. Leaves of four selected superior clones grown at different altitudes (from 550 m to 1180 m) were investigated, and the results showed that the contents of chlorogenic acid and flavonoids correlated positively with altitude ($p < 0.05$) (Dong, Ma, Wei, Peng & Zhang, 2011). Similarly, the contents of quercetin-3-*O*-rutinoside in elderberries (*Sambucus nigra* L.) and of phenolic compounds in juniper needles clearly increased with increasing altitude (Martz, Peltola, Fontanay, Duval, Julkunen-Tiitto & Stark, 2009; Rieger, Müller, Guttenberger & Bucar, 2008). In a study on wild sea buckthorn berries (*H. rhamnoides* ssp. *sinensis*), increasing trends were found in the contents of most flavonols as the altitude increased from 200 m to 3000 m (Ma et al., 2016). In

the same subspecies grown in China, the content of total PAs slightly increased as the altitude increased ($p < 0.05$) (Yang, Laaksonen, Kallio & Yang, 2016).

Multiple environmental factors are overall reflections of latitude and altitude, among which temperature and light conditions are considered as the most important factors affecting flavonoid biosynthesis in plants (Azuma, Yakushiji, Koshita & Kobayashi, 2012; Jaakola & Hohtola, 2010; Zoratti, Karppinen, Escobar, Häggman & Jaakola, 2014; Yang, Laaksonen, Kallio & Yang, 2016).

2.5.2 Effects of temperature

As an expression of self-defense mechanism of plants, secondary metabolites are produced by temperature stress. Typically, when grown at low temperatures plants have higher photosynthetic rates compared to those at higher temperatures, providing more carbon source for synthesis of secondary metabolites (Jaakola & Hohtola, 2010, Zobayed, Afreen & Kozai, 2005). High latitude and high altitude are usually accompanied by low temperature, which partially explains the increased content of flavonoids at higher latitudes and altitudes, as described in many studies in previous literature. For instance in grape skin, reduction of phenolic compounds was found with increasing temperature. Accumulation of anthocyanins at 20 ℃ was significantly higher than at 30 ℃ (Goto-Yamamoto, Mori, Numata, Koyama & Kitayama, 2010; Tarara, Lee, Spayd & Scagel, 2008, Yamane, Jeong, Goto-Yamamoto, Koshita & Kobayashi, 2006).

Christie et al. and Mori et al. have analyzed the transcript abundance of the flavonoid pathway genes and anthocyanin degradation at high or low temperature stress. They found that inhibition of mRNA transcription and degradation occurred at a high temperature, whereas transcript levels of phenylpropanoid pathway increased several fold at a lower temperature (Christie, Alfenito & Walbot, 1994; Mori, Goto-Yamamoto, Kitayama & Hashizume, 2007).

Flavonoid biosynthesis of the pathways from phenylalanine to the final products as reviewed in section 2.3.1 requires coordinated expression of enzymes. The key enzymes are PAL, C4H, CHS, CHI, F3H, FLS, ANS and ANR. Several studies have shown increased expression of PAL, C4H and CHS at low temperature in leaves of tomato (*Solanum lycopersicum*, cv. Suzanne) and ginkgo (*Ginkgo biloba* L.) (Løvdal, Olsen, Slimestad, Verheul & Lillo, 2010; Wang, Cao, Wang & El-Kassaby, 2015). Similar results have been obtained in expression of PAL in leaves of *Arabidopsis thaliana* L., and of C4H and CHS in grape skin (‘Pione’, *V. vinifera* × *V. labruscana*) (Azuma, Yakushiji, Koshita & Kobayashi, 2012, Leyva, Jarillo, Salinas & Martinez-Zapater, 1995). Again, F3H, the key enzyme of flavonoids biosynthesis and

FLS, the essential enzyme of flavonols biosynthesis, showed increased expression in ginkgo leaves at low temperature (Wang, Cao, Wang & El-Kassaby, 2015).

2.5.3 Effect of light

A prerequisite for plants to survive is light, which can be classified according to photoperiods, intensity, direction and quality. All these affect also the biosynthesis and accumulation of secondary metabolites in plants (Zoratti, Karppinen, Escobar, Häggman & Jaakola, 2014). Changes in latitude and altitude are accompanied by variation in light effects. The short growth season of plants at high latitudes is characterized by long day-time, low total solar irradiance but high level of UV radiation. Photoperiod affects biosynthesis of secondary metabolites by regulating the circadian clock (Zoratti, Karppinen, Escobar, Häggman & Jaakola, 2014). In a previous study, accumulation of anthocyanins and flavonols in black currant (*Ribes nigrum* L.) increased under long photoperiod (Woznicki, Aaby, Sønsteby, Heide, Wold & Remberg, 2016). A long-day photoperiod upregulated synthesis of flavanols and catechins in sweet potato leaf and tea leaf (Carvalho, Cavaco, Carvalho & Duque, 2010; Pavarini, Pavarini, Niehues & Lopes, 2012). Similar results in purple and red-flesh potatoes were obtained by Reyes et al. who observed increase in anthocyanin content with longer photoperiod (Reyes, Miller & Cisneros-Zevallos, 2004). In a period of six years of research in Finnish black currants ('Mortti', 'Ola' and 'Melalahti'), the content of anthocyanins (delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside) and content of FG (myricetin-3-*O*-glucoside) correlated positively with radiation in all three cultivars (Zheng et al., 2012).

As primary regulators in flavonoid biosynthesis, the R2R3 MYB transcription factors can control the accumulation of flavonoids (Czemmel, Heppel & Bogs, 2012; Jaakola, 2013). Recent studies have shown that some of R2R3 MYB transcription factors are induced by light and are thus indirectly regulating the synthesis of flavonoids in several fruit species (Zoratti, Karppinen, Escobar, Häggman & Jaakola, 2014). Zoratti et al. summarized the functions of these light-inducible R2R3 MYB transcription factors. Five factors were identified in apple, eight in grape, and one was identified in Chinese bayberry, litchi, nectarine, pear, and strawberry (Zoratti, Karppinen, Escobar, Häggman & Jaakola, 2014). These light-induced factors regulate flavonoid biosynthesis through interaction with *b*HLH (basic helix–loop–helix) (Ravaglia et al., 2013, Vimolmangkang, Zheng, Han, Khan, Soria-Guerra & Korban, 2014). Moreover, these factors also bring about interaction with the MYB recognition element, which is necessary for activation of promoter of CHS, and

is thereby a step in flavonoid biosynthesis (Feldbrügge, Sprenger, Hahlbrock & Weisshaar, 1997; Hartmann, Sagasser, Mehrtens, Stracke & Weisshaar, 2005).

Generally, plants exposed to high levels of UV radiation are susceptible to DNA damages, and abiotic stress stimulates flavonoid accumulation for protection (Woznicki, Aaby, Sønsteby, Heide, Wold & Remberg, 2016). Flavonols generally protect plants from UV radiation, and a significant increase in flavonols was demonstrated in plants exposured to elevated UV-B light, which even generated an epigenetic memory of UV stress (Muller-Xing, Xing & Goodrich, 2014). With this feature, UV radiation has been applied as a novel technique to increase the content of flavonols to elongate the shelf life of lettuce, and to enhance health-promoting properties of grape (Allende & Artés, 2003; Cantos, Espín & Tomás-Barberán, 2001). Liu et al. investigated the UV-B induced pathways in *Vitis vinifera* L. var. Sauvignon blanc grapes, expression of *vvFLS*4 (flavonol synthase genes) and the transcription factor *VvMYB12* responded to UV-B, the regulation and transcription factors of both genes are involved in regulating FLS, thus affecting the synthesis of flavonols.

2.5.4 Genome × environment interaction

The biosynthesis of flavonoids is affected by interaction of genetic background and the environmental factors, which is essential for adaptation of plants to distinct environments. The interaction was investigated in six cultivated strawberry (*Fragaria* × *ananassa* Duch.) cultivars grown at two locations in Italy. Traits associated with levels of flavonols and PAs showed significant dependence on environmental factors, whereas the level of anthocyanins was more dependent on the genetic background (Carbone et al., 2009). In strawberry, flavonols and PAs accumulated at early stages of development to protect from feeding by animals and from attack of pathogens, whereas anthocyanin are mainly visual attractants, and their synthesis begun at later stages (Fait et al., 2008). In another research, clones of bilberries (*Vaccinium myrtillus* L.) originating from northern and southern Finland were studied in a controlled experiment at 12 °C and 18 °C. Northern clones showed significantly higher contents of total anthocyanins and shorter ripening times at 12 °C (Uleberg et al., 2012). Northern clones of bilberries, even in changed growth environment, maintained their northern genetic behavior such as shorter ripening time at lower temperature and higher level of total anthocyanins. These results suggest latitude–related genetic adaptation. In a research period of seven years of Finnish wild sea buckthorn berries (ssp. *rhamnoides*), native bushes from southern Finland were transplanted to northern Finland. In the berries of northern bushes, the content of total PAs was significantly higher

than in those of the southern bushes ($p < 0.05$) (Yang, Laaksonen, Kallio & Yang, 2017).

More studies related to gene–environment interaction have been reviewed by Jaakola and Hohtola (Jaakola & Hohtola, 2010). Some results suggest the existence of environment-related genetic adaptation. At the same time, there is also maintenance of gene expression. The genetic adaptation to environment may also be understood as "plant memory". Plants can remember the external stresses from environmental changes through epigenetic mechanisms. Several studies indicate the existence of epigenetic memory caused by radiation in some plant species (Muller-Xing, Xing & Goodrich, 2014). However, the conclusion of gene–environment interaction is still not clear. A better understanding of secondary metabolism in various plant species requires long-term studies.

3 AIMS OF THE STUDY

PAs in sea buckthorn berries and FGs in currant leaves have benefits on human health. A better understanding of these compounds may contribute to a better use of these crops. In this thesis, PAs and FGs have been investigated in sea buckthorn berries and currant leaves, respectively. The main aim was to characterize these compounds and understand the impact of genetic background and environmental factors on their contents.

The main objectives of the current investigation were:

1. To establish an HPLC method combined with UV and MS detection for qualitative and quantitative analysis of the key PA oligomers in sea buckthorn berries (Paper **I**).
2. To characterize the FGs from leaves of six currant cultivars with HPLC, MS and NMR methods (Paper **II**).
3. To investigate the effects of growth environment and leaf positions on the content and composition of FGs in currant leaves (Paper **II**).
4. To investigate the effects of genetic background and growth environment on the content and composition of PAs in sea buckthorn berries (Papers **III** and **IV**).

4 MATERIALS AND METHODS

4.1 Plant samples

The sea buckthorn berries and currant leaves used in this thesis are summarized in **Table 3** and **Table 4**, respectively. Berries of three subspecies of sea buckthorn and leaves of two species (six cultivars) of currant were investigated.

PAs of berries of three subspecies of *Hippophaë rhamnoides* L., i.e. ssp. *rhamnoides*, ssp. *sinensis* and ssp. *mongolica* were investigated. Two varieties and one wild origin of ssp. *rhamnoides* were cultivated in southern and northern Finland (harvests 2007-2013). The two varieties 'Tytti' and 'Terhi' were cultivated in Turku (south) and Kittilä (north). The wild bushes from Uusikaupunki (south) were transplanted also to Kolari (north). Wild berries of ssp. *sinensis* (ten locations in six provinces in China) and five varieties of ssp. *mongolica* (one location in Canada) were harvested in 2008 (**Table 3**). In each growth place, the bushes were divided into two to four field blocks, and berries were picked randomly from different blocks when optimally ripe. In addition, overripe berries of 'Terhi' and 'Tytti' grown in Turku were collected at four time points during September–December, 2011. All the berries were frozen immediately after picking and stored at -18 °C until analysis. Wild berries of ssp. *rhamnoides* from Uusikaupunki (2008) were investigated in Paper **I**, berries from Finland, Canada and China were studied in paper **III** and ssp. *rhamnoides* berries from four locations in Finland were analyzed in paper **IV.**

The currant study includes leaves of three black currant (*Ribes nigrum* L.) cultivars, 'Mortti', 'Mikael' and 'Jaloste n:o 15', one green currant (*R. nigrum*) cultivar 'Vertti', one red currant (*R. rubrum*) cultivar 'Red Dutch' and one white currant (*R. rubrum*) cultivar 'White Dutch' (paper **II**). All the leaves were collected from the test fields of LUKE (Natural Resources Institute Finland) located in Piikkiö (south Finland) and Rovaniemi (north Finland) at four time points during July–September in 2012 and 2013 (**Table 4**). Each cultivar was planted in four blocks with 3–4 bushes each. Leaf samples were divided into two groups by position. Leaves in the middle position of new, berry bearing branches are referred as "middle leaves" (**Figure 6**), which were analyzed to investigate the changes in the content and composition of FGs during the growth season (2012 and 2013) in the two growth locations. A separate set of samples referred as "random leaves", were randomly collected from the bushes grown in Piikkiö at four time points in 2012. These samples were compared with the "middle leaves" in terms of the content and profile of the FGs. All the leaves were mixed well after collection, frozen immediately and stored at -18 °C until analysis.

Fig. 6 The position of "middle leaves" in bushes of currant (Photo by Baoru Yang)

Table 3. Sea buckthorn berries investigated.

Subspecies	Variety	Growth site (abbreviation)	Longitude	Latitude	Altitude (m)	Harvest Time
rhamnoides	Terhi	Turku, Finland (TU)	22°09′ E	60°23′ N	1	Aug 30, 2007; Aug 28, 2008; Aug 31, 2009; Aug 26, 2010; Sep 23, 2011; Sep 4, 2012; Aug 21, 2013 Oct 14, 2011*; Nov 4, 2011*; Nov 25, 2011*; Dec.16, 2011*
		Kittilä, Finland (KI)	24°37′ E	68°02′ N	210	Oct 10, 2008; Sep 29, 2009; Oct 2, 2010; Sep 26, 2011; Sep 22, 2013
	Tytti	Turku, Finland (TU)	22°09′ E	60°23′ N	1	Aug 30, 2007; Aug 28, 2008; Aug 31, 2009; Aug 26, 2010; Sep 23, 2011; Sep 4, 2012; Aug 21, 2013 Oct 14, 2011*; Nov 4, 2011*; Nov 25, 2011*; Dec.16, 2011*
		Kittilä, Finland (KI)	24°37′ E	68°02′ N	210	Oct 10, 2008; Sep 26, 2011; Sep 22, 2013
	Finnish (wild)	Uusikaupunki, Finland (UU)	21°15′ E	60°54′ N	1	Sep 12, 2007; Sep 29, 2008; Sep 30, 2009; Oct 1, 2010; Sep 28, 2011; Sep 13, 2012; Sep 5,2013
		Kolari, Finland (KO)	24°42′ E	67°07′ N	163	Sep 22, 2007; Oct 4, 2008; Sep 21, 2009; Sep 30, 2010; Sep 14, 2011; Oct 3,2012; Sep 10, 2013
mongolica	Chuiskaya	Québec, QC, Canada	71°17′ W	46°47′ N	100	Sep 3, 2008
	Oranzhevaya	Québec, QC, Canada	71°17′ W	46°47′ N	100	Sep 3, 2008
	Vitaminaya	Québec, QC, Canada	71°17′ W	46°47′ N	100	Sep 3, 2008
	Prevoshodnaya	Québec, QC, Canada	71°17′ W	46°47′ N	100	Sep 3, 2008
	Prozcharachnaya	Québec, QC, Canada	71°17′ W	46°47′ N	100	Sep 3, 2008
sisensis	Chinese (wild)	Heilongjiang, China (HL)	127°06′ E	47°14′ N	210	Dec 15, 2008
		Heibei, China (HB)	116°34′E	41°17′ N	832, 818	Oct 16, 2008
		Shanxi, China (SX)	113°52′E	37°05′ N	1515, 2182	Oct 16, 2008
		Sichuan, China (SC)	106°54′E	31°01′ N	3000, 2500, 2000	Oct 15, 2008
		Qinghai, China (QH)	101°23′E	36°45′ N	3115	Oct 25, 2008
		Inner Mongolia, China (IM)	109°48′E	39°47′ N	1480	Oct 25, 2008

*overripe samples

Table 4. Currant leaves investigated.

Species	Cultivar	Growth site (abbreviation)	Longitude	Latitude	Altitude (m)	Harvest Time
Ribes nigrum	Mortti (Black Currant)	Piikkiö, Finland	22°33´E	60°23′ N	10	Jul 3, 2012; Jul 24, 2012; Aug 14,2012; Sep 4,2012; Jul 3, 2013; Jul 23, 2013; Aug 14,2013; Sep 3,2013
		Apukka, Finland	26°00´E	66°34′ N	102	Jul 3, 2012; Jul 24, 2012; Aug 14,2012; Sep 4,2012; Jul 3, 2013; Jul 23, 2013; Aug 14,2013; Sep 3,2013
	Mikael (Black Currant)	Piikkiö, Finland	22°33´E	60°23′ N	10	Jul 3, 2012; Jul 24, 2012; Aug 14,2012; Sep 4,2012; Jul 3, 2013; Jul 23, 2013; Aug 14,2013; Sep 3,2013
		Apukka, Finland	26°00´E	66°34′ N	102	Jul 3, 2012; Jul 24, 2012; Aug 14,2012; Sep 4,2012; Jul 3, 2013; Jul 23, 2013; Aug 14,2013; Sep 3,2013
	Jaloste n:o 15 (Black Currant)	Piikkiö, Finland	22°33´E	60°23′ N	10	Jul 3, 2012; Jul 24, 2012; Aug 14,2012; Sep 4,2012; Jul 3, 2013; Jul 23, 2013; Aug 14,2013; Sep 3,2013
		Apukka, Finland	26°00´E	66°34′ N	102	Jul 3, 2012; Jul 24, 2012; Aug 14,2012; Sep 4,2012; Jul 3, 2013; Jul 23, 2013; Aug 14,2013; Sep 3,2013
	Vertti (Green Currant)	Piikkiö, Finland	22°33´E	60°23′ N	10	Jul 3, 2012; Jul 24, 2012; Aug 14,2012; Sep 4,2012; Jul 3, 2013; Jul 23, 2013; Aug 14,2013; Sep 3,2013
		Apukka, Finland	26°00´E	66°34′ N	102	Jul 3, 2012; Jul 24, 2012; Aug 14,2012; Sep 4,2012; Jul 3, 2013; Jul 23, 2013; Aug 14,2013; Sep 3,2013
Ribes rubrum	Red Dutch (Red Currant)	Piikkiö, Finland	22°33´E	60°23′ N	10	Jul 3, 2012; Jul 24, 2012; Aug 14,2012; Sep 4,2012; Jul 3, 2013; Jul 23, 2013; Aug 14,2013; Sep 3,2013
		Apukka, Finland	26°00´E	66°34′ N	102	Jul 3, 2012; Jul 24, 2012; Aug 14,2012; Sep 4,2012; Jul 3, 2013; Jul 23, 2013; Aug 14,2013; Sep 3,2013
	White Dutch (White Currant)	Piikkiö, Finland	22°33´E	60°23′ N	10	Jul 3, 2012; Jul 24, 2012; Aug 14,2012; Sep 4,2012; Jul 3, 2013; Jul 23, 2013; Aug 14,2013; Sep 3,2013
		Apukka, Finland	26°00´E	66°34′ N	102	Jul 3, 2012; Jul 24, 2012; Aug 14,2012; Sep 4,2012; Jul 3, 2013; Jul 23, 2013; Aug 14,2013; Sep 3,2013

4.2 Sample extraction

4.2.1 Extraction of proanthocyanidins from sea buckthorn berries

In paper **I,** about 50 g of sea buckthorn berries were accurately weighed. The whole berries were crushed with a disperser at 7000 rpm and extracted three times with 200 mL of solvent consisting of acetone, water and acetic acid (80:19.5:0.5, v/v) by sonicating for 15 min for each time of extraction. The extracts were centrifuged and combined, and acetone was evaporated. The remaining aqueous extract was defatted with petroleum ether and filtered. In papers **III** and **IV**, about 10 g sea buckthorn berries were used in the analysis. Extraction of PAs was accomplished according to the method in paper **I**, except for the changes in the amount of extract solvent. The amount was 30 mL for each time.

4.2.2 Extraction of flavonol glycosides from currant leaves

In the study on currant leaves (paper **II**), about 5 g of frozen leaves of each cultivar were powdered in liquid nitrogen. One gram of leaf powders was weighed in triplicate. The samples were extracted three times with a mixture of acetone and water (70:30, v/v) by sonicating for 20 min for each time of extraction. After centrifugation, the combined supernatants were evaporated to dry extract under vacuum, re-dissolved in 1 mL methanol and clarified through a polytetrafluoroethylene (PTFE) filter. For preparative chromatography, about 10 g leaves of ‘Mikael’ and ‘White Dutch’ were milled and extracted using the same method as described above.

4.3 Purification of proanthocyanidins

Further purification of the sea buckthorn berry samples was required and carried out with activated Sephadex LH-20 column chromatography. In paper **I**, the final extract (about 100 mL) was loaded to the column and eluted with 250 mL of methanol and water (20:80, v/v) (Fraction I), 200 mL of acetone and water (70:30, v/v) (Fraction II), and 250 mL of acetone and water (70:30, v/v) (Fraction III) in sequence. The three fractions were collected for the traces of PAs. The fractions were dried by vacuum rotary evaporator, re-dissolved in methanol and filtered (PTFE) before analysis. In papers **III** and **IV**, the final extract (about 25 mL) was loaded to the column. The order of eluent was 150 mL of water, 100 mL of methanol and water (20:80, v/v), 150 mL of acetone and water (70:30, v/v) and 100 mL of methanol. The fraction eluted by acetone and water (70:30, v/v) was collected.

4.4 LC and MS analysis

4.4.1 HPLC/UPLC-DAD-MS analysis of proanthocyanidins

Analysis of samples in paper **I** were carried out with a Waters Acquity Ultra High Performance LC system coupled with Waters Quattro Premier triple quadrupole mass spectrometer with an electrospray-ionization (ESI) source (Waters Corp., Milford, MA). The UPLC system consisted of of a sample manager, a binary solvent delivery system and a Waters 2996 PDA Detector. The instrument was operated using the MassLynx 4.1 software. RP-HPLC, NP-UPLC, and HILIC analyses were used to identify PAs in sea buckthorn berries (paper **I**). In papers **III** and **IV**, only HILIC-MS analysis was applied.

In RP-HPLC, a Phenomenex Luna RP-C18 column (5 µm, 250 × 4.60 mm, Torrance, CA) combined with a Phenomenex Prodigy guard column (5 µm, 30 × 4.60 mm) was employed. Formic acid/water (0.5:99.5, v/v) was used as solvent A and acetonitrile/methanol (80:20, v/v) as solvent B for the gradient elution. The gradient program was 0−5 min, 10 % B; 5−15 min, 10–18 % B; 15−25 min, 18 % B; 25−30 min, 18−25 % B; 30−35 min, 25 % B; 35−40 min, 25−35 % B; 40−45 min, 35−60 % B; 45−50 min, 60−10 % B; and 50−55 min, 10% B. The injection volume was 20 µL. Chromatograms were recorded at 280 nm and 360 nm. The total flow was 1 mL/min, which was split after UV detector into the mass spectrometer with 0.3 mL/min, and the rest into a waste bottle.

In NP-UPLC analysis, the column was Restek Pinnacle DB Silica UPLC column (1.9 µm, 100 × 2.1 mm, Bellefonte, PA). The mobile phase was run as gradient elution consisting of dichloromethane, methanol, water, and acetic acid (82:14:2:2 v/v) as solvent A and methanol, water, and acetic acid (96:2:2 v/v) as solvent B. The gradient program was 0−15 min, 0−15 % B; 15−20 min, 15–30 % B; 20−25 min, 30−80 % B; 25−30 min, 80−0 % B; and 30−35 min, 0 % B. The flow rate was 0.4 mL/min. The injection volume was 10 µL. Chromatograms were recorded at 280 nm and 360 nm. The total flow of 0.4 mL/min was all transferred to the mass spectrometer after the UV detector.

In HILIC analysis, a Phenomenex Luna HILIC 200A column (3 µm, 150 × 3.00 mm, Torrance, CA) combined with a Phenomenex Security Guard Cartridge Kit was used. A binary solvent system was employed consisting of formic acid/water (0.5:99.5, v/v) as solvent A and acetonitrile as solvent B. The gradient program was 0−5 min, 5−20 % B; 5−10 min, 20–30 % B; 10−12 min, 30−40 % B; 12−15 min, 40−65 % B; 15−17 min, 65−5 % B; and 17−30 min, 5 % B. The injection volume was 10 µL. The peaks were monitored at 280 nm and 360 nm. The total flow of 0.5 mL/min was directed to the ion source of the MS.

The mass spectrometer was operated in negative ion ESI ion mode. Full scan (*m/z* 500−3000) and selected ion recording (SIR) modes were employed. The most abundant PA ions were chosen for recording SIR chromatograms.

4.4.2 HPLC-DAD-ESI-MS/MS analysis of flavonol glycosides

For flavonol glycosides (paper **II)**, the HPLC and MS systems were the same as used in proanthocyanidins analysis. A Phenomenex Aeris peptide XB-C18 column (3.6 μm, 150 × 4.60 mm, Torrance, CA) combined with a Phenomenex Security Guard Cartridge Kit was used. The mobile phase consisting of two solvents, were formic acid/water (0.1:99.9, v/v) as solvent A and formic acid/acetonitrile (0.1:99.9, v/v) as solvent B. The gradient program was 0−15 min, 15−20 % B; 15−20 min, 20–25 % B; 20−25 min, 25 % B; 25−30 min, 25−60 % B; 30−35 min, 60−15 % B; 35−40 min, 15 % B. The injection volume was 10 μL and the flow rate was 0.5 mL/min. The chromatograms were monitored at 360 nm. Positive ion mode ESI was used for MS analysis. The MS analysis was first carried out in full scan mode by scanning ions between *m/z* 100 and 1000. Moreover, tandem MS (daughter scan and parent scan) was also applied for identification of unknown FGs.

4.4.3 Semi-Preparative HPLC isolation of unknown flavonol compounds

The unknown FGs were isolated for NMR analyses (paper **II**) with a semi-preparative HPLC system by using UV/VIS detection and a fraction collector (Shimadzu, Kyoto, Japan). The mobile phase was run as gradient elution of formic acid/water (0.1:99.9, v/v) as solvent A and formic acid/acetonitrile (0.1:99.9, v/v) as solvent B. The total flow rate was 5 mL/min. Two gradient programs were optimized to achieve sufficient separation of FGs from currant leaves of different cultivars. The gradient program for cultivar ‘Mikael’ was 0−15 min, 15−30 % B; 15−20 min, 30–60 % B; 20−25 min, 60–15 % B; and 25−30 min, 15 % B. For ‘White Dutch’, the gradient program was 0−17 min, 15 % B; 17−25 min, 15–60 % B; 25−30 min, 60−15 % B; and 30−35 min, 15 % B. The injection volumes for both gradient programs were 300 μL. The chromatograms were monitored at 360 nm.

4.5 NMR analysis of purified flavonol compounds

The unknown compounds collected by Semi-Preparative HPLC were further analyzed with NMR (paper **II**). NMR spectra were acquired using a Bruker Avance 500 spectrometer operating at 500.13 and 125.77 MHz for ^{1}H and ^{13}C, respectively. The spectra were recorded at 25 °C using CD_3OD as a solvent

with a non-spinning sample in a 5 mm NMR-tube. The spectra were calibrated on the solvent residual signal of CD_3OD at 3.31 ppm for ^{1}H and 49.15 for ^{13}C. The spectra were processed with the TopSpin 3.2 software.

4.6 Quantitative analysis of proanthocyanidins and flavonol glycosides

4.6.1 Quantitative analysis of proanthocyanidins by HPLC-MS

Quantitative analysis of PA oligomers (dimers, trimers and tetramers) of sea buckthorn berries was carried out using HILIC-ESI-SIR and procyanidin B2 as an external standard (papers **I**, **III** and **IV**). The peak areas in full scan and SIR modes were corrected by isotopic abundances (^{13}C, ^{14}C, ^{2}H, ^{3}H, ^{17}O and ^{18}O). The calibration curves were constructed by analysis of standard solutions of procyanidin B2 in methanol with the concentration range of 0.01–10 mg/100 mL. The contents of PA oligomers were calculated with the calibration curves and correction factors (isotopic abundances and ionization efficiency).

4.6.2 Quantitative analysis of flavonol glycosides in currant leaves by HPLC-DAD

The quantitative analysis of FGs in currant leaves was carried out using HPLC-DAD method and external standards (six commercial reference compounds and four compounds isolated from currant leaves in our lab) (paper **II**). The equipments and parameters of gradient elution were the same as described above in section 4.4.2. The concentrations of the external standard solutions ranged from 0.01 to 0.15 mg/mL. The injection volume was 10 μL for each standard solution. The contents of FGs were calculated by calibration curves of reference compounds based on peak areas at 360 nm.

4.6.3 Determination of total proanthocyanidins content by DMAC

In papers **III** and **IV**, the contents of total PAs were determined using BL-DMAC assay and procyanidin B2 as an external standard. Preparation of the DMAC reagent was performed according to the method described previously (Prior et al., 2010). A 96-well plate was used as the reaction vessel. 100 μL of DMAC solution, 60 μL of extraction solvent and 10 μL of sea buckthorn extract solution were added sequentially to each well and mixed thoroughly. The OD values at 640 nm were detected at 30 min by a plate reader (Hidex, Finland) against blank, which consisted of 100 μL of DMAC solution and 70 μL of extraction solvent. The concentrations of procyanidin B2 were ranged 1–

20 mg/mL and the calibration curves were constructed by plotting the OD values.

4.7 Determination of dry matters of sea buckthorn berries and currant leaves

For dry matter measurement in papers **I**, **III** and **IV**, about 5 g of sea buckthorn berries were weighed accurately in triplicate. The berries were thawed at room temperaturein, and cut by a scalpel. The residue on the knife was rinsed by distilled water. Samples were dried 103-105 °C, cooled in a desiccator and weighed. The drying and weighing was continued till a constant weight was reached. For samples of currant leaves (paper **II**), about 5 g fresh leaves of each cultivar were milled into fine powder in liquid nitrogen. About one gram of powder was weighed accurately in triplicate, dried to a constant weight at 103–105 °C, cooled in a desiccator and weighed. In all the results of this thesis, the contents of PAs and FGs were expressed as mg/100 mg (DW) or mg/g (DW).

4.8 Weather conditions

The meteorological data from Finland used in paper **IV** were provided by the Finnish Meteorological Institute (Helsinki, Finland). The meteorological data for Turku and Uusikaupunki were recorded at the weather stations in Kaarina (Yltöinen, 60°23′N, 22°33′E, 6 m) during 2007–2008 and in Turku (Artukainen, 60°27′ N, 22°10′ E, 8 m) during 2009–2013. Data for Kittilä and Kolari were recorded at the weather station in Kittilä (Pokka, 68°10′ N, 25°47′ E, 275 m) during 2007–2013. The weather variables and abbreviations are listed in **Table 5**.

4.9 Statistical analyses

Statistical analyses and multivariate models were performed using SPSS 16.0.1 (SPSS Inc., Chicago, IL) and Unscrambler X, version 10.3 (CAMO Software, Oslo, Norway). A one-way analysis of variance (ANOVA) and independent-sample t test were performed to compare the content and composition of PAs in different subspecies, varieties, latitudes, altitudes and harvest time points (papers **III** and **IV**), and the FGs of currant leaves at different growth locations (paper **II**). Bivariate (Pearson's Correlation Coefficients) and partial correlation analyses was applied to investigate the correlation coefficients among content of PAs, latitude and altitude in sea buckthorn berries (paper **III**). Partial least squares regression discrimination analysis (PLS-DA) was used to explain the

difference among subspecies, varieties and growth locations according to the PA contents in sea buckthorn berries (papers **III** and **IV**). Principal component analysis (PCA) was applied to explain the difference of PAs among varieties of sea buckthorn berries (paper **III**) and to study the effects of weather condition on the content and composition of PAs in sea buckthorn (paper **IV**).

Table 5. The abbreviations of weather variables. Reprinted from the original publication **IV** (Yang, Laaksonen, Kallio & Yang, 2017) (supplementary) with permission from Elsevier.

Abbreviations	Weather variables
Dgs	Length of the growth season (day)
ΣTgs	Temperature sum of growth season (℃)
ΣTgh	Temperature sum from start of growth season until harvest (℃)
ΣTmon	Temperature sum over 5 ℃ of the last month before harvest (℃)
HDgh	Hot days (>25℃) during growth season until harvest (day)
HDmon	Hot days (>25℃) during last month before harvest (day)
TJan TFeb...TSep	Average temperature in January, February...September (℃)
MaTJan MaTFeb...MaTSep	Extreme maximum temperature of January, February...September (℃)
MiTJan MiTFeb...MiTSep	Extreme minimum temperature of January, February...September (℃)
Tmon	Average temperature during the last month before harvest (℃)
Tw	Average temperature during the last week before harvest (℃)
ΔTmon	Mean temperature difference between the highest and lowest temp in the day during last month before harvest (℃)
MiTmon	Extreme lowest temperature during the last month before harvest (℃)
LoTmon	Average of daily lowest temperature during last month before harvest (℃)
MaTmon	Highest temperature during the last month before harvest (℃)
HiTmon	Average of highest daily temperature during last month before harvest (℃)
ΣRgh	Radiation sum during growth season until harvest (kJ/m2)
ΣRmon	Radiation sum during the last month before harvest (kJ/m2)
ΣRw	Radiation sum during last week before harvest (kJ/m2)
ΣRJan, ΣRFeb...ΣRSep	Radiation sum of January, February...September (kJ/m2)
Prgh	Precipitation in growth season until harvest (mm)
PrM	Precipitation in the last month before harvest (mm)
PrW	Precipitation in the last week before harvest (mm)

PrJan, PrFeb... PrSep	Precipitation of January, February...September (mm)
Hgh	Average humidity during growth season until harvest (mm)
Hmon	Average humidity during last month before harvest (mm)
Hweek	Average humidity during last week before harvest (mm)
HJan, HFeb... HSep	Relative humidity of January, February...September (mm)
DH20to30g	Percent of humidity 20-30% days during growth season until harvest
DH30to40g	Percent of humidity 30-40% days during growth season until harvest
DH40to50g	Percent of humidity 40-50% days during growth season until harvest
DH50to60g	Percent of humidity 50-60% days during growth season until harvest
DH60to70g	Percent of humidity 60-70% days during growth season until harvest
DH70to80g	Percent of humidity 70-80% days during growth season until harvest
DH80to90g	Percent of humidity 80-90% days during growth season until harvest
DH90to100g	Percent of humidity 90-100% days during growth season until harvest
DH50to60m	Percent of humidity 50-60% days during last month until harvest
DH60to70m	Percent of humidity 60-70% days during last month until harvest
DH70to80m	Percent of humidity 70-80% days during last month until harvest
DH80to90m	Percent of humidity 80-90% days during last month until harvest
DH90to100m	Percent of humidity 90-100% days during last month until harvest

5 RESULTS AND DISCUSSION

5.1 Proanthocyanidins in sea buckthorn berries

5.1.1 Purification of proanthocyanidins by column chromatography

Sephadex LH-20 column chromatography has been successfully used for purification of PAs in number of studies (Naczk & Shahidi, 2004). Due to hydrophilicity, carbohydrates, low-molecular weight phenolic compounds and flavonols were also present in the crude extracts. In order to reduce these disturbances in chromatographic and mass spectrometric analyses, purification of PAs was needed. Typically, PAs have a maximum absorption at 280 nm without a band beyond 300 nm. Some other substances in the crude extract, such as flavonols and their glycosides had absorption maxima around both 280 nm 360 nm. The crude extract and three fractions were analyzed by RP-HPLC at 280 nm and 360 nm (paper **I**). In the fraction eluted by 250 mL methanol and water (20:80, v/v) (Fraction I) only non-tannin substances were found. In the fraction eluted by 200 mL acetone and water (70:30, v/v) (Fraction II, PA fraction), PAs were detected according to UV Spectra. Additional eluent of 250 mL acetone and water (70:30, v/v) (Fraction III) was collected and used to confirm that no PAs remained in the column after fraction II. The RP-HPLC chromatograms of the crude extract and of the PAs fraction recorded at 280 nm and 360 nm are presented in **Figure 7**.

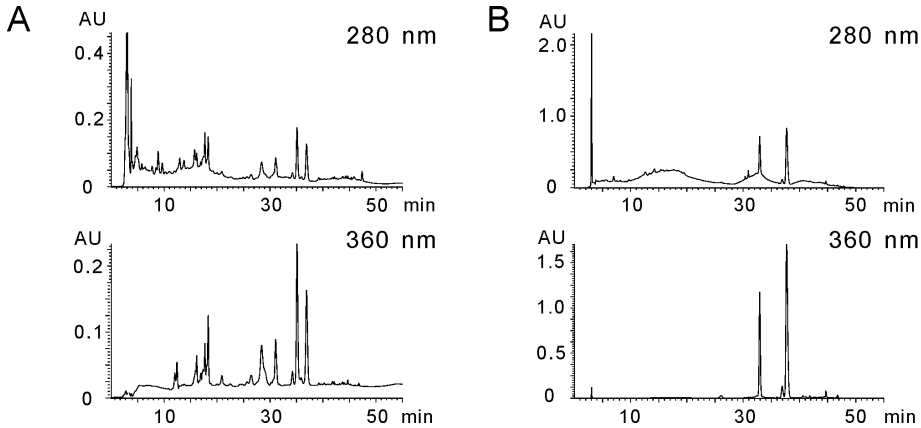


Fig. 7 RP-HPLC-DAD chromatograms measured at 280 nm and 360 nm of the crude extract of sea buckthorn berries (A) and of the Sephadex-purified fraction of PAs (B).

5.1.2 Identification of proanthocyanidins

RP-HPLC, NP-UPLC, and HILIC combined with DAD and ESI-MS were employed to identify the PAs in sea buckthorn berries in paper **I** . Among the chromatograms recorded at 280 nm (Kallio, Yang, Liu & Yang, 2014), the peaks overlapped seriously with each other in both RP-HPLC and NP-UPLC and no legible spectra of individual PAs were obtained. In the HILIC-DAD chromatogram at 280 nm, 14 peaks were recorded between 3.5 min and 9 min. All these peaks were tentatively identified as PAs based on the UV spectra. In full-scan mode, scanning from *m/z* 500 to 3000 was applied to obtain the profiles of PAs. The total ion spectra were recorded in 14 min (from 3.6 to 17.6 min) in HILIC-ESI-MS and in 22 min (from 3 to 25 min) in NP-UPLC-ESI-MS. The recording times were obtained from duration of PAs in chromatograms recorded by UV at 280 nm. As shown in the publication (Kallio, Yang, Liu & Yang, 2014), the HILIC-ESI-MS method was the best and most sensitive among the three types of LC analyses, and B-type oligomeric PAs up to DP11 were detected. Among the PA oligomers, PAs with DP from 2 to 6 and with DP from 8 to 11 were detected in form of $[M-H]^-$ ions and $[M-2H]^{2-}$ ions, respectively. PAs with DP 7 were detected in both $[M-H]^-$ ions and $[M-2H]^{2-}$ ions (**Figure 8**).

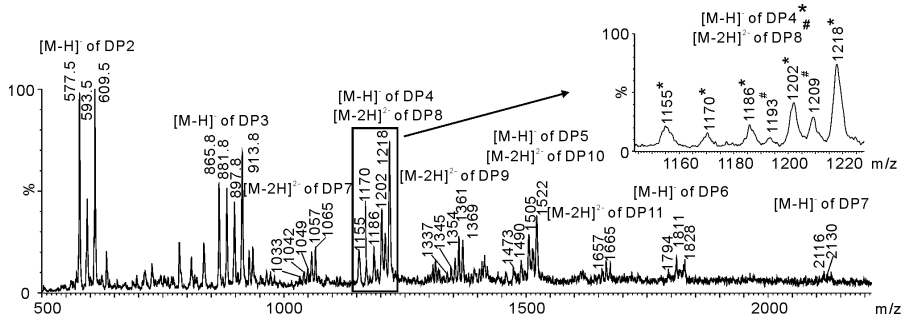


Fig. 8 The total ion spectrum of PA fraction from 3.6 to 17.6 min analyzed by HILIC-ESI-MS. Reprinted from the original publication **I** (Kallio, Yang, Liu & Yang, 2014), with permission from American Chemical Society.

Due to the high complexity of PAs in sea buckthorn, neither the UV spectra nor the total ion spectra could give more information about retention times and isomers. In this case, SIR mode of ESI-MS was applied to selectively analyze these PAs in 12 channels (PA dimers, *m/z* 577, 593, and 609; PA trimers, *m/z* 865, 881, 897, and 913; PA tetramers, *m/z* 1153, 1169, 1185, 1201 and 1217). The NP-UPLC-ESI-MS-SIR and HILIC-ESI-MS-SIR analyses provided separation methods based on molecular weight, and the RP-HPLC-MS-SIR

method complemented the separation of PAs by polarity. In the chromatograms of both NP-UPLC and HILIC-ESI analyses, PA oligomers were well separated according to molecular weight within each PD, but the HILIC method had more resolution and resulted in sharper peak shapes. In the chromatogram of RP-HPLC-ESI-MS-SIR, at least 28 PA dimers (five isomers with $[M-H]^-$ *m/z* 577, eleven isomers with $[M-H]^-$ *m/z* 593, twelve isomers with $[M-H]^-$ *m/z* 609), and at least 32 PA trimers (eight isomer with $[M-H]^-$ *m/z* 865, eight isomer with $[M-H]^-$ *m/z* 881, eight isomer with $[M-H]^-$ *m/z* 897, eight isomer with $[M-H]^-$ *m/z* 913), and numerous PA tetramers were detected. All these oligomers with their molecular formulas, molecular weights, and number of subunits are summarized in **Table 6**. However, the exact structures of PA oligomers, such as interflavanoid linkages of monomeric units, the stereoisomerism of the B-ring and hydroxyl group at position 3 are still unknown.

Table 6. Composition of oligomeric proanthocyanidins estimated from the total ion spectra obtained by HILIC-ESI-MS. Reprinted from the original publication **I** (Kallio, Yang, Liu & Yang, 2014) with permission from American Chemical Society.

DP [a]	Molecular formula	Number of subunits [b]		Exact mass	Detected mass	
		(E)C	(E)GC		$[M-H]^-$	$[M-2H]^{2-}$
2	$C_{30}H_{26}O_{12}$	2	0	578.14	577.52	
2	$C_{30}H_{26}O_{13}$	1	1	594.14	593.52	
2	$C_{30}H_{26}O_{14}$	0	2	610.13	609.52	
3	$C_{45}H_{38}O_{18}$	3	0	866.21	865.77	
3	$C_{45}H_{38}O_{19}$	2	1	882.20	881.77	
3	$C_{45}H_{38}O_{20}$	1	2	898.20	897.77	
3	$C_{45}H_{38}O_{21}$	0	3	914.19	913.77	
4	$C_{60}H_{50}O_{24}$	4	0	1154.27	1154.97	
4	$C_{60}H_{50}O_{25}$	3	1	1170.26	1170.34	
4	$C_{60}H_{50}O_{26}$	2	2	1186.25	1186.02	
4	$C_{60}H_{50}O_{27}$	1	3	1202.25	1202.06	
4	$C_{60}H_{50}O_{28}$	0	4	1218.25	1217.88	
5	$C_{75}H_{62}O_{32}$	3	2	1474.32	1473.89	
5	$C_{75}H_{62}O_{33}$	2	3	1490.32	1490.04	
5	$C_{75}H_{62}O_{34}$	1	4	1505.31	1505.34	
5	$C_{75}H_{62}O_{35}$	0	5	1522.31	1522.16	
6	$C_{90}H_{74}O_{40}$	2	4	1794.38	1794.07	
6	$C_{90}H_{74}O_{41}$	1	5	1810.37	1811.01	
6	$C_{90}H_{74}O_{42}$	0	6	1826.37	1828.03	
7	$C_{105}H_{86}O_{45}$	4	3	2066.44		1032.84
7	$C_{105}H_{86}O_{46}$	3	4	2082.43		1041.91
7	$C_{105}H_{86}O_{47}$	2	5	2098.43		1049.18
7	$C_{105}H_{86}O_{48}$	1	6	2114.43	2115.64	1057.13
7	$C_{105}H_{86}O_{49}$	0	7	2130.42	2130.49	1065.00
8	$C_{120}H_{98}O_{50}$	6	2	2337.51		1168.25[c]
8	$C_{120}H_{98}O_{52}$	4	4	2369.49		1184.24[c]
8	$C_{120}H_{98}O_{53}$	3	5	2386.50		1193.29
8	$C_{120}H_{98}O_{54}$	2	6	2402.49		1200.24[c]
8	$C_{120}H_{98}O_{55}$	1	7	2418.49		1209.18
8	$C_{120}H_{98}O_{56}$	0	8	2434.48		1216.23[c]
9	$C_{135}H_{110}O_{56}$	7	2	2626.58		1312.95
9	$C_{135}H_{110}O_{57}$	6	3	2642.57		1319.95
9	$C_{135}H_{110}O_{58}$	5	4	2658.57		1328.61
9	$C_{135}H_{110}O_{59}$	4	5	2674.56		1336.64
9	$C_{135}H_{110}O_{60}$	3	6	2690.56		1344.59
9	$C_{135}H_{110}O_{61}$	2	7	2706.55		1353.51
9	$C_{135}H_{110}O_{62}$	1	8	2722.54		1361.31
9	$C_{135}H_{110}O_{63}$	0	9	2738.54		1369.33
10	$C_{150}H_{122}O_{64}$	6	4	2945.62		1472.31[d]
10	$C_{150}H_{122}O_{66}$	4	6	2978.62		1488.30[d]
10	$C_{150}H_{122}O_{67}$	3	7	2994.61		1496.57
10	$C_{150}H_{122}O_{68}$	2	8	3010.61		1504.30[d]
10	$C_{150}H_{122}O_{69}$	1	9	3026.60		1513.96
10	$C_{150}H_{122}O_{70}$	0	10	3042.60		1520.29[d]
11	$C_{165}H_{134}O_{75}$	2	9	3314.67		1657.09
11	$C_{165}H_{134}O_{76}$	0	11	3330.66		1665.49

[a]DP=degree of polymerization; [b] (E)C=(epi)catechin, (E)GC=(epi)gallocatechin; [c] $[M-2H]^{2-}$ ions of DP8 overlapped with the $[M-H]^-$ ions of DP4; [d] $[M-2H]^{2-}$ ions of DP10 overlapped with the $[M-H]^-$ ions of DP5.

5.1.3 Quantitative profiling of oligomeric proanthocyanidins

The HILIC-ESI-MS-SIR was used to study the quantitative profiles of oligomeric PAs in sea buckthorn in papers **I**, **III** and **IV**. The proportions of the peak areas in SIR mode corrected by isotopic abundances were quite close to the results in the total ion spectra (full scan mode). In wild sea buckthorn berries, (epi)catechin was the main subunit of PA dimers, whereas (epi)gallocatechin was the most abundant in both PA trimers and tetramers. The peak areas of PAs with DP > 4 were quite small both in ESI-MS-SIR analyses and in the total ion spectra. More details are shown in Table 2 in publication **I**.

Our results showed that the peak areas of PC dimers and trimers in SIR mode were very close to their molar proportions. The content of PC dimer with $[M-H]^-$ *m/z* 577 was calculated based on Procyanidin B2. Thus, the contents of PA dimers ($[M-H]^-$ *m/z* 593, $[M-H]^-$ *m/z* 609, PA trimers ($[M-H]^-$ *m/z* 865, $[M-H]^-$ *m/z* 881, $[M-H]^-$ *m/z* 897, $[M-H]^-$ *m/z* 913), PA tetramers ($[M-H]^-$ *m/z* 1153, $[M-H]^-$ *m/z* 1169, $[M-H]^-$ *m/z* 1185, $[M-H]^-$ *m/z* 1201, $[M-H]^-$ *m/z* 1217) were all quantified by a known concentration of PC dimer with $[M-H]^-$ *m/z* 577. The contents of PA dimers, PA trimers and PA tetramers ranged between 1.4–8.9 mg/100 g, 1.3–10.7 mg/100 g, and 1.0–8.3 mg/100 g DW, respectively.

5.1.4 Comparison of subspecies and varieties

The contents of total PAs and PA oligomers in each sample were determined by BL-DMAC method and HILIC-ESI-MS-SIR method, respectively. The content and composition of PAs were compared among the three subspecies (ssp. *rhamnoides*, ssp. *mongolica* and ssp. *sinensis*) (papers **III** and **IV**). Significant differences in contents of total PAs, dimers, Dim-2, Dim-3, Tri-1, Tet-1 and Tet-3 were found between ssp. *rhamnoides* and ssp. *mongolica* ($p < 0.05$). Ssp. *sinensis* had significantly lower content of dimers, trimers, tetramers, Dim-1, Dim-3, Tri-2, Tri-4, Tet-2 and Tet-5 than the other subspecies ($p < 0.05$). The three subspecies were relatively well classified with three validated factors (R^2 0.724, Q^2 0.677) in the PLS-DA model (**Figure 9**). In this model, the ssp. *rhamnoides* berries were characterized by more trimers and tetramers, the ssp. *mongolica* berries were characterized to contain more dimers, whereas the ssp. *sinensis* berries contained less all PAs (excluding total PAs) than other two subspecies.

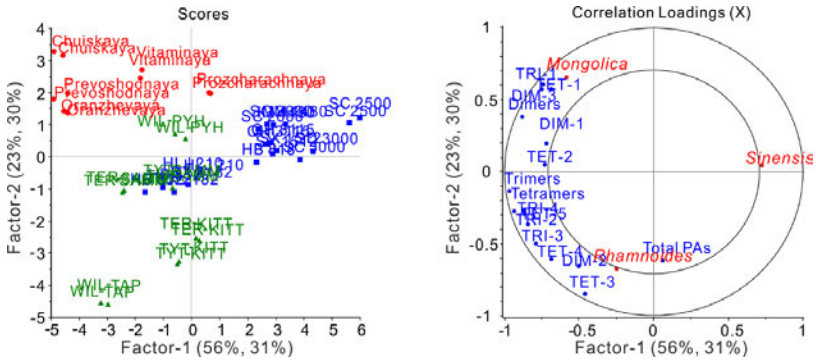


Fig. 9 PLS-DA model for sea buckthorn samples (n = 20 × 2) classified according to subspecies (ssp. *rhamnoides*, green triangles; ssp. *mongolica*, red circles; and ssp. *sinensis*, blue squares) with the PA contents (variables; n = 16). Reprinted from the original publication **III** (Yang, Laaksonen, Kallio & Yang, 2016) with permission from American Chemical Society.

Within ssp. *rhamnoides*, the berries of 'Terhi', 'Tytti' and the wild origin harvested during 2007–2013 were compared according to the content and composition of PAs. There were no significant differences in the content of total PAs among the three sea buckthorn origins. The PA composition of 'Terhi' and 'Tytti' were similar to each other, except that the content of Tri-3 was significantly higher in 'Terhi' ($p < 0.05$). In the wild berries, the contents of some PA oligomers (Tri-1, Tri-2, Tri-3, Tet-1, Tet-2 and Tet-3) were significantly lower than those in 'Terhi' ($p < 0.05$). In PLS-DA model, the three origins were poorly separated. Most berries of 'Tytti' located on the central axis were mixed with 'Terhi' and wild berries. 'Terhi' contained more PA oligomers, and the wild berries were characterized by lower content of PA oligomers. The overlapping distributions in PLS-DA model may be due to the extreme variation of the PA contents in different growth places. Further analyses based on the growth places will be described later.

Within ssp. *mongolica*, five varieties 'Prevoshodnaya', 'Prozcharachnaya', 'Chuiskaya', 'Oranzhevaya' and 'Vitaminaya' cultivated in Quebec, Canada were compared with each other based on the content and composition of PAs. 'Prevoshodnaya' had higher content of total PAs than the other varieties, whereas the lowest level of total PAs was found in 'Prozcharachnaya' ($p < 0.05$). In PCA model, the five varieties were well separated. 'Prozcharachnaya' was characterized by lower levels of all PAs, 'Chuiskaya' was characterized by high contents of Dim-1 and Dim-2, whereas 'Prevoshodnaya' samples were characterized by high levels of total PAs, Dim-3, Tet-2 and Tet-3. 'Oranzhevaya' was located between 'Chuiskaya' and 'Prevoshodnaya',

correlated with PA dimers, trimers and tetramers. It is important to remember that the results are based on one year samples only.

5.1.5 Comparison of latitudes

Three origins of ssp. *rhamnoides* berries, 'Terhi', 'Tytti' and wild, were used to investigate the effects of growth latitude on the content and composition of PAs in sea buckthorn (papers **III** and **IV**). The effects of altitude (within around 200 m) were ignored in this section. The samples were collected during 2007–2013 in both southern (60°–61°N) and northern Finland (67°–68°N) separated by a distance of around 800 km. An independent-sample t test was applied to study the differences between samples from the south and the north. In all the three sample groups, the contents of total PAs in the berries from the north were significantly higher than in the berries from the south; the difference was 2.0 times in 'Terhi', 2.3 times in 'Tytti', and 1.7 times in the wild sea buckthorn ($p < 0.05$). Most of the PA oligomers in both 'Terhi' and 'Tytti' had the same trend, whereas, in wild samples, an opposite trend was found in the content of Tri-1, Tet-1 and Tet-2. PLS-DA models were created to study the impact of altitude on the content and composition of PAs. In PLS-DA model with all the samples, berries from higher latitudes were well separated from those at lower latitudes by higher levels of PAs (**Figure 10**). In PLS-DA models within each variety, the loading plots showed similar trends and more detailed information. In 'Terhi' and 'Tytti', the PA content variables (total PAs, Tri-4, Tet-4 and Tet-5) were located close to high latitude samples, whereas, variables Tet-1, Tet-2 and Tri-1 were close to low latitude samples. Tet-1, Tet-2 and Tri-1 located closer to the low latitude samples of wild berries than to the samples of 'Terhi' and 'Tytti'. This was in agreement with the statistical results in the independent-sample t test. Among PA oligomers, PDs were the main components (Tri-4, Tet-4 and Tet-5) correlating positively to latitude, whereas, PCs of the major oligomers (Tet-1, Tet-2 and Tri-1) correlated negatively to latitude.

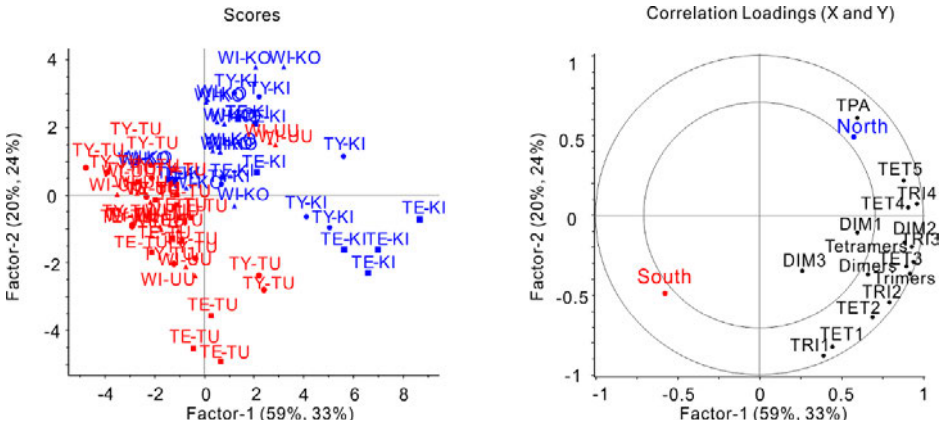


Fig. 10 PLS-DA models for all the three origins ('Tytti', 'Terhi' and one of wild origin) of sea buckthorn as proanthocyanidins as variables (n = 16) (South samples, Blue icon).

5.1.6 The impact of latitude and altitude on proanthocyanidins in Chinese berries of ssp. *sinensis*

To study the effect of latitude and altitude on the content and composition of PAs, wild sea buckthorn berries of ssp. *sinensis* were collected from ten different locations in six provinces in China (paper **III**). The spans of latitude and altitude were from 31°N to 47°N and from 210 m to 3000 m, respectively. Among the samples, the content of total PAs increased as the latitude decreased and as the altitude increased (R^2=0.1608), whereas PA oligomers (dimers, trimers and tetramers) decreased as the latitude decreased and as the altitude increased (R^2=0.2345, R^2=0.4254, R^2=0.3914, respectively). Bivariate correlation analysis (Spearman's correlation) showed that most of the PA oligomers correlated positively to latitude, whereas total PAs correlated negatively ($p < 0.01$). Altitude correlated negatively to all PAs, three (Tri-3, Tri-4 and Tet-5) of which significantly ($p < 0.01$). The main components of Tri-3, Tri-4 and Tet-5 were PDs, analogously to the results of our previous studies related to latitude. Partial correlation analysis showed similar results as in bivariate correlation analysis, which indicated most of the PA oligomers to correlate positively with latitude and negatively with altitude ($p < 0.01$). An exception was total PA with negative correlation to latitude ($p < 0.01$). It is worth mentioning that the effect of latitude in China does not have the same influence as in Finland. Due to the complex topography in China, the effect of latitude on radiation and temperature may be negligible. Moreover, the samples collected at different altitudes from Shanxi (SX) (1515 m and 2182 m) and Sichuan (SC) (2000 m, 2500 m, 3000 m) were investigated ignoring the impact

of latitude. The levels of total PAs decreased as the altitude increased in both SC and SX samples ($p < 0.01$). In SX berries, the contents of PA oligomers increased as the altitude increased, whereas SC samples showed opposite results. The changes in contents were not significant at altitudes above 2500 m.

5.1.7 Effects of weather conditions on proanthocyanidins

Changes in latitude and altitude are usually accompanied by changes in the weather conditions. In order to understand the impact of climatic and weather conditions on the content and composition of PAs in sea buckthorn, berries of varieties 'Terhi' and 'Tytti' as well as wild ssp. *rhamnoides* were collected from southern and northern Finland during 2007–2013 (paper **IV**). Bivariate correlation analysis (Pearson's correlation coefficient) showed positive correlation between total PAs and most of the precipitation and humidity-related variables, whereas the correlation was negative between total PAs and most of the variables of temperature and radiation in 'Terhi' and 'Tytti' ($p < 0.01$).

PCA models were employed to investigate correlations among specific weather conditions and PA compositions in 'Terhi' and 'Tytti'. All PA variables were located around the northern growth sites, correlating negatively with variables of temperature, radiation, precipitation around time of harvest and relatively low humidity.

Separated PCA models of Kittilä (northern Finland) and Turku (southern Finland) were created to evaluate respective correlations. In Kittilä, total PAs were positively associated with low temperature and radiation, high precipitation and humidity (weather conditions in 2008). High radiation, low precipitation and low humidity (weather conditions in 2011) contributed the accumulation of PA oligomers. In Turku, weather conditions in 2012 with low temperature, low radiation, low humidity and high precipitation before ripening were likely favorable for PA accumulation. In 'Terhi', 'Tytti' and wild sea buckthorn, correlations between the contents of PAs and Dgs (length of the growth season), between PAs and ΣTgh (temperature sum during growth season until harvest) as well as between PAs and ΣRgh (radiation sum during growth season until harvest) were analyzed. The level of total PAs decreased as the Dgs, ΣTgh and ΣRgh increased ($p < 0.01$), whereas no significant correlation between PA oligomers and these variables was observed.

5.1.8 Effects of over-ripening on proanthocyanidins in sea buckthorn berries

Over-ripe sea buckthorn berries of 'Terhi' and 'Tytti' were collected at four different time points in Turku in 2011 (paper **IV**). Significant differences in the

contents of total PAs were found between optimally ripe and over-ripe stages in both 'Terhi' and 'Tytti' ($p < 0.05$). Most of the PA oligomers in 'Tytti' showed significant differences between the two stages, whereas only Dim-1 in 'Terhi' was found to be significantly influenced. 'Terhi' and 'Tytti' had the same changing trend of total PAs and PA oligomers during the stage from optimally ripe to overripe.

5.2 Flavonol glycosides in currant leaves (paper II)

5.2.1 Identification of flavonol glycosides

Twenty-seven FGs were identified/tentatively identified in the extracts of currant leaves. Among them, seven FGs were identified using reference compounds, four FGs were purified and identified using NMR (**Figure 11**) and the remaining ones were tentatively identified by the UV spectra and the mass spectra. The FGs are summarized in **Table 7**.

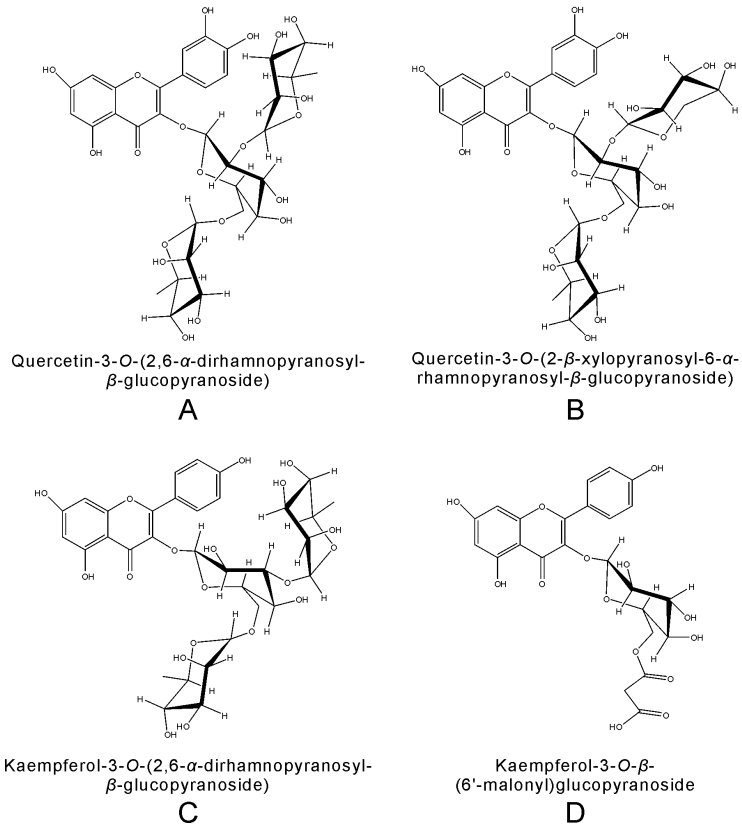


Fig. 11 Structures of four flavonol glycosides (A-D) identified by NMR

Table 7. Flavonol glycosides determined by HPLC-DAD and positive ion ESI-MS in the extracts from the leaves of black, green, red and white currants. Reprinted from the original publication **II** (Yang, Alanne, Liu, Kallio & Yang, 2015) (supplementary), with permission from American Chemical Society.

Compounds	RT [a]	λ_{max}	$[M+H]^+$	Other ions in MS	MS^2	Preliminary identification [b]	Cultivars [c]
	(min)	(nm)	(*m/z*)	(*m/z*)	(*m/z*)		
1	5.67	266, 348	611	287, 449, 633	287, 449, 611	Kaempferol-di-hexoside	BC1,BC2, BC3, GC, WC
2	8.42	280, 320	433	287		Kaempferol-rhamnoside	BC1,BC2, BC3, GC, RC
3	9.87	256,354	757	303, 465, 611	303, 465, 611, 757	Quercetin-3-*O*-(2,6-*α*-dirhamnopyranosyl-*β*-glucopyranoside) #	WC
4	10.25	256, 354	743	303, 465, 611	303, 465, 611, 743	Quercetin-3-*O*-(2-*β*-xylopyranosyl-6-*α*-rhamnopyranosyl-*β*-glucopyranoside) #	WC
5	11.13	264, 340	481	319	319, 481	Myricetin-3-*O*-glucoside *	BC1, BC2, BC3, GC, RC, WC
6	11.87	262, 348	627	319, 481, 627	319, 481, 627	Myricetin-rhamnosylhexoside	BC1, RC
7	12.27	268, 356	481	319	319, 481	Myricetin-hexoside	BC1, BC2, BC3, GC, RC
8	12.35	265, 354	597	303, 465	303, 465, 597	Quercetin–hexosylpentoside	WC
9	12.55	266, 348	741	287, 449, 595	287, 449, 595, 741	Kaempferol-3-*O*-(2,6-*α*-dirhamnopyranosyl-*β*-glucopyranoside) #	WC
10	13.88	267, 346	595	287, 449	287, 595	Kaempferol-rhamnosylglucoside I	WC
11	14.72	267, 356	567	319	319, 567	Myricetin-malonylhexoside	BC1, BC2, BC3, GC, RC, WC
12	14.97	256, 355	611	303, 465	303, 465, 611	Quercetin-rhamnosylhexoside	BC1
13	15.28	256, 354	611	303, 465	303, 458, 611	Rutin *	BC1,BC2, BC3, GC, RC, WC

14	15.98	267, 351	595	287, 449	287	Kaempferol-rhamnosylglucoside II	BC1,BC2, BC3, WC
15	16.63	256, 356	465	303	303, 465	Hyperoside *	BC1
16	17.28	256, 354	465	303	303, 465	Isoquercitrin *	BC1,BC2, BC3, GC, RC,WC
17	19.73	266, 350	595	287, 449, 595	287, 449, 595	Kaempferol-3-*O*-rutinoside *	BC1,BC2, BC3, GC, RC,WC
18	20.02	266, 348	449	287, 471	287, 449	Kaempferol-hexoside I	BC1
19	20.28	256, 355	551	303, 551	303, 551	Quercetin-3-*O*-*(*6"-malonyl*)*-glucoside *	BC1,BC2, BC3, GC, RC
20	21.30	256, 355	449	287, 449	287, 449	Kaempferol-3-*O*-glucoside *	BC1,BC2, BC3, GC, RC, WC
21	21.73	266, 316	551	303	303, 551	Quercetin-malonylhexoside I	BC1,BC2, BC3, GC, RC
22	22.60	265, 344	419	287	287, 419	Kaempferol-pentoside	BC1
23	22.97	267, 346	535	287	287, 535	Kaempferol-malonylhexoside I	BC1
24	23.53	267, 345	535	287	287, 535	Kaempferol-malonylhexoside II	BC1
25	24.00	266, 348	535	287	287, 535	Kaempferol-3-*O*-*β*-(6'-malonyl)glucopyranoside [#]	BC1,BC2, BC3, GC, RC
26	24.90	267, 345	535	287	287, 535	Kaempferol-malonylhexoside IV	BC1,BC2, BC3, GC, RC
27	25.52	267, 345	535	287	287, 535	Kaempferol-malonylhexoside V	BC1

[a] The retention times are according to the HPLC-DAD chromatograms. [b] Compounds with * were identified with commercial reference compounds, with [#] were identified by NMR, the others were identified based on UV and mass spectra. [C] BC1, 'Mikael'; BC2, 'Mortti'; BC3, 'Jaloste n:o 15'; GC, green currant 'Vertti'; RC, red currant 'Red Dutch'; WC, white currant 'White Dutch'.

Interestingly, quercetin and kaempferol with the same sugar moieties were eluted in pairs, as seen with quercetin-3-*O*-(2,6-*α*-dirhamnopyranosyl-*β*-glucopyranoside) and kaempferol-3-*O*-(2,6-*α*-dirhamnopyranosyl-*β*-glucopyranoside), quercetin-3-*O*-rutinoside (rutin) and kaempferol-3-*O*-rutinoside, quercetin-3-*O*-glucoside (isoquercitrin) and kaempferol-3-*O*-glucoside, quercetin-3-*O*-(6''-malonyl)-glucoside and kaempferol-3-*O*-*β*-(6'-malonyl)-glucoside, and quercetin-malonylhexoside I and kaempferol-malonylhexoside V. The flavonol glycosides with aglycone of quercetin eluted faster than those of kaempferol.

5.2.2 Comparison of cultivars

Quercetin and kaempferol were the most important flavonol aglycones in the FGs of all the currant cultivars. The profiles of FGs were quite similar in leaf extracts of black currants 'Mortti' and 'Jaloste n:o 15', green currant 'Vertti' and red currant 'Red Dutch'. Black currant 'Mikael' and white currant 'White Dutch' had their own profiles of FGs. The black currant cultivar 'Mikael' was characterized by quercetin-rhamnosylhexoside, hyperoside, kaempferol-hexoside, kaempferol-pentoside, kaempferol-malonylhexoside I, kaempferol-malonylhexoside II, kaempferol-malonylhexoside V, whereas the white currant 'White Dutch' was characterized by the presence of quercetin-3-*O*-(2,6-*α*-dirhamnopyranosyl-*β*-glucopyranoside), quercetin-3-*O*-(2-*β*-xylopyranosyl-6-*α*-rhamnopyranosyl-*β*-glucopyranoside), quercetin-hexosylpentoside, kaempferol-3-*O*-(2,6-*α*-dirhamnopyranosyl-*β*-glucopyranoside), kaempferol-rhamnosylglucoside I.

5.2.3 Comparison of different time points within growth seasons

The twelve most abundant identified FGs accounted for approximately 90 % proportion of the total peak area of the twenty-seven FGs detected. The contents of FGs in leaves collected from the middle position at four different time points were compared. The contents of FGs changed significantly during the growth season. In Piikkiö (south Finland), the contents reached the highest levels around late July to mid–August, followed often by a decrease. The peaks were typically postponed by three to four weeks in the samples from Apukka (north Finland). The ratios of malonylated and non-malonylated FGs decreased during the growth season from early July to early September. Such compounds were e.g. quercetin-3-*O*-(6''-malonyl)-glucoside and isoquercitrin, kaempferol-3-*O*-*β*-(6'-malonyl)-glucoside and kaempferol-3-*O*-glucoside. Moreover, the contents of FGs were compared in 2012 and 2013 and all the cultivars displayed similar changing trends in these two years.

5.2.4 Comparison of growth places

The average contents of FGs at the four time points were compared in currant leaves from the two orchards (Piikkiö in the north and Apukka in the south). No significant differences were found between the two locations based on the average contents of flavonol glycosides ($p > 0.05$), and the contents were thus not affected by latitude. Only the peak in the content of FGs was postponed in Apukka.

5.2.5 Comparison by leaf position

The leaves of green currant 'Vertti', red currant 'Red Dutch' and white currant 'White Dutch' were collected at random and middle positions in Piikkiö in 2012. The contents of total FGs and of individual FGs were compared between the samples harvested with the two methods. Some changing trends were noticed. In random leaves, the increasing trends of total FGs were due to the special increase of rutin, isoquercitrin, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside at the end of growth season. Compared with the randomly collected leaves, the leaves collected from the middle position contained higher levels of malonylated FGs i.e. (quercetin-3-*O*-*(*6"-malonyl*)*-glucoside and kaempferol-3-*O*-*β*-(6'-malonyl)glucopyranoside) as well as quercetin-3-*O*-(2,6-*α*-dirhamnopyranosyl-*β*-glucopyranoside), quercetin-3-*O*-(2-*β*-xylopyranosyl-6-*α*-rhamnopyranosyl-*β*-glucopyranoside) and kaempferol-3-*O*-(2,6-*α*-dirhamnopyranosyl-*β*-glucopyranoside) were higher than that in random leaves.

6 SUMMARY AND CONCLUSION

A rapid and sensitive method for profiling of proanthocyanidins (PAs) in sea buckthorn (*Hippophaë rhamnoides* ssp. *rhamnoides* L.) berries was established based on HILIC-ESI-MS analysis. PA oligomers with degree of polymerization from 2 to 11 were detected. All of them were B-type PAs with (epi)gallocatechins as the main monomeric units. PA oligomers (dimers, trimers and tetramers) were quantified with HILIC-ESI-MS method.

The three sea buckthorn subspecies (ssp. *rhamnoides*, ssp. *mongolica* and ssp. *sinensis*) were well classified with three validated factors (R^2 0.724, Q^2 0.677) in PLS-DA model. Ssp. *rhamnoides* contained more PA trimers and tetramers, ssp. *mongolica* contained more PA dimers, whereas berries of ssp. *sinensis* had lower contents of all PAs compared with the other two subspecies. Within ssp. *rhamnoides*, berries of 'Terhi' and 'Tytti' had similar compositions of PAs, which were different from the wild ones in most PA oligomers ($p < 0.05$). Within ssp. *mongolica*, varieties 'Prevoshodnaya' and 'Prozcharachnaya' had the highest and lowest contents of total PAs among the five varieties, respectively ($p < 0.05$), and 'Oranzhevaya' was characterized by high contents of PA dimers, trimers and tetramers.

Growth environments, such as latitude, altitude and weather conditions showed significant influence on the content and composition of PAs in sea buckthorn. 'Terhi', 'Tytti' and wild sea buckthorn of ssp. *rhamnoides* grown at higher latitude in northern Finland contained higher levels of PAs. The length of the growth season, temperature sum and total radiation during the growth season till harvest correlated negatively with total PAs in the berries from the north. No respective correlations were found in the samples grown at the lower latitude in southern Finland. In ssp. *sinensis* from China, the content of total PAs correlated negatively with latitude, whereas PA oligomers showed positive correlation. In comparison to latitude, altitude showed less impact on PAs in ssp. *sinensis* berries.

Flavonols were analyzed in leaf extracts of four *Ribes nigrum* cultivars, i.e. three black currants, 'Mortti', 'Mikael' and 'Jaloste n:o15' and a green currant 'Vertti', as well as of two *Ribes rubrum* cultivars, i.e. one red currant 'Red Dutch', and one white currant 'White Dutch'. Twenty-seven flavonol glycosides (FGs) were identified/tentatively identified using reference compounds, UV spectra, mass spectra and NMR analysis. Among them, four major previously unknown compounds quercetin-3-*O*-(2,6-*α*-dirhamnopyranosyl-*β*-glucopyranoside), quercetin-3-*O*-(2-*β*-xylopyranosyl-6-α-rhamnopyranosyl-*β*-glucopyranoside), kaempferol-3-*O*-(3,6-*α*-dirhamnopyranosyl-*β*-glucopyranoside) and kaempferol-3-*O*-*β*-(6'-

malonyl)glucopyranoside) were identified by NMR for the first time in currant leaves.

The profiles of FGs were highly affected by the genetic background. 'White Dutch' differed clearly from all the other cultivars, and black currant 'Mikael' showed a different profile compared to black currants 'Mortti', 'Jaloste n:o15' and green currant 'Vertti'. The ratios between malonylated and non-malonylated FGs reduced regularly during the growth season. The highest contents of FGs in leaves of currants grown at the higher latitude were typically postponed by a few weeks compared to the corresponding cultivars grown at lower latitudes. The position of leaves had an effect on profiles of FGs.

This study investigated systematically the influence of genetic background and growth environments on PAs in berries of three subspecies of sea buckthorn and FGs of six cultivars of currants leaves. The results contribute to understanding of the accumulation of PAs and FGs in plants in different environments. The research provides guidance for the cultivation of sea buckthorn berries and the exploitation of currant leaves as potential raw material of nutraceuticals.

ACKNOWLEDGEMENTS

The work of this thesis was carried out in the Food Chemistry and Food Development unit at the Department of Biochemistry, University of Turku, Finland under the supervision of Professor Baoru Yang and Professor emeritus Heikki Kallio.

I am grateful to the China Scholarship Council (CSC) and the Magnus Ehrnrooth Foundation for the financial support. I would also like to thank the Doctoral Programme in Molecular Life Sciences (DPMLS) of the University of Turku Graduate School (UTUGS), the Turku University Foundation and the Magnus Ehrnrooth foundation for travel grants.

I would like to express my warm regards and deep sense of gratitude to my supervisors, Professor Baoru Yang and Professor emeritus Heikki Kallio for their patience and supervision. Professor Baoru Yang provided me an excellent opportunity to pursue this PhD education and first introduced me to the field of berries and phenolic compounds. I am grateful to Professor emeritus Heikki Kallio for his trust and encouragement as well as effort and interest in supervising the thesis despite his retirement.

I would like to thank Professor Encarna Gómez-Plaza and Professor Laura Jaakola for reviewing the thesis and for the valuable comments and suggestions.

I wish to express my thanks to my co-authors for their contributions. Especially, I would like to thank Oskar Laaksonen for always timely comments and fruitful discussions. Oskar Laaksonen and Maaria Kortesniemi also have helped me a lot in the thesis writing and printing stage.

I want to thank all my colleagues in Food Science Unit for creating such an excellent working environment. I am grateful for Marko Tarvainen, Pengzhan Liu, Jukka-Pekka Suomela, Mika Kaimainen and Marika Kalpio for their help in the instrumental analysis in the lab. Jani Sointusalo and Anu Hirvensalo deserve special thanks for their technical support. Also, I wish to thank to Mirva Jalo and Minna Lintala for their administrative support. I warmly thank Maisa Lintala for her contribution to samples preparation.

I am grateful to Mr Hannu Lappalainen, Ms Inkeri Peltomaa, Mr Markku Peltomaa, Mr Seppo Lappalainen, Mr Bärtil Lappalainen, Ms Elli Ravelin and Ms Elvi Kaija for providing the sea buckthorn berries. I also would like to acknowledge Professor Risto Tahvonen, Professor Saila Karhu, Alpo Heinonen, Hilma Kinnanen, Jarkko Mäkinen, Jorma Hellstén, Kaisa Soppela from Natural Resources Institute Finland (Luke) for providing the currant leaves, and Anni Lindstedt, Anssi Vuorinen, Maaria Kortesniemi for sample collection. Especially, many thanks to Jie Zheng for the sample management.

I warmly thank my close friends Qing Miao, Shiyu Miao, Lan Yu for sharing extracurricular time and delicious food.

Finally, I would like express my sincere gratitude to my family. I am thankful to my parents and parents-in-law for their support and encouragement. My heartfelt thanks to my dear wife Xueying for her love and understanding from beginning to end. Thank you for everything.

Turku, March 2017

REFERENCES

Aherne, S. A., & O'Brien, N. M. (2002). Dietary flavonols: Chemistry, food content, and metabolism. *Nutrition, 18*(1), 75-81.

Åkerström, A., Jaakola, L., Bång, U., & Jäderlund, A. (2010). Effects of latitude-related factors and geographical origin on anthocyanidin concentrations in fruits of *vaccinium myrtillus* L.(bilberries). *Journal of Agricultural and Food Chemistry, 58*(22), 11939-11945.

Allende, A., & Artés, F. (2003). UV-C radiation as a novel technique for keeping quality of fresh processed 'Lollo rosso'lettuce. *Food Research International, 36*(7), 739-746.

Alpert, A. J. (1990). Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. *Journal of Chromatography A, 499*, 177-196.

Appeldoorn, M. M., Vincken, J. P., Gruppen, H., & Hollman, P. C. (2009). Procyanidin dimers A1, A2, and B2 are absorbed without conjugation or methylation from the small intestine of rats. *The Journal of Nutrition, 139*(8), 1469-1473.

ASTM International. (2004). *Annual book of ASTM standards* ASTM International.

Aziz, N., Paiva, N. L., May, G. D., & Dixon, R. A. (2005). Transcriptome analysis of alfalfa glandular trichomes. *Planta, 221*(1), 28-38.

Azuma, A., Yakushiji, H., Koshita, Y., & Kobayashi, S. (2012). Flavonoid biosynthesis-related genes in grape skin are differentially regulated by temperature and light conditions. *Planta, 236*(4), 1067-1080.

Bais, H. P., Vepachedu, R., Gilroy, S., Callaway, R. M., & Vivanco, J. M. (2003). Allelopathy and exotic plant invasion: From molecules and genes to species interactions. *Science, 301*(5638), 1377-1380.

Barbehenn, R. V., & Constabel, C. P. (2011). Tannins in plant–herbivore interactions. *Phytochemistry, 72*(13), 1551-1565.

Behrens, A., Maie, N., Knicker, H., & Kögel-Knabner, I. (2003). MALDI-TOF mass spectrometry and PSD fragmentation as means for the analysis of condensed tannins in plant leaves and needles. *Phytochemistry, 62*(7), 1159-1170.

Berli, F., D'Angelo, J., Cavagnaro, B., Bottini, R., Wuilloud, R., & Silva, M. F. (2008). Phenolic composition in grape (*vitis vinifera* L. cv. malbec) ripened with different solar UV-B radiation levels by capillary zone electrophoresis. *Journal of Agricultural and Food Chemistry, 56*(9), 2892-2898.

Borochov-Neori, H., Judeinstein, S., Harari, M., Bar-Ya'akov, I., Patil, B. S., Lurie, S., & Holland, D. (2011). Climate effects on anthocyanin accumulation and composition in the pomegranate (*punica granatum* L.) fruit arils. *Journal of Agricultural and Food Chemistry, 59*(10), 5325-5334.

Breslin, P., Gilmore, M., Beauchamp, G., & Green, B. (1993). Psychophysical evidence that oral astringency is a tactile sensation. *Chemical Senses, 18*(4), 405-417.

Buszewski, B., & Noga, S. (2012). Hydrophilic interaction liquid chromatography (HILIC)—a powerful separation technique. *Analytical and Bioanalytical Chemistry, 402*(1), 231-247.

Cain, C. C., Saslowsky, D. E., Walker, R. A., & Shirley, B. W. (1997). Expression of chalcone synthase and chalcone isomerase proteins in arabidopsis seedlings. *Plant Molecular Biology, 35*(3), 377-381.

Cala, O., Pinaud, N., Simon, C., Fouquet, E., Laguerre, M., Dufourc, E. J., & Pianet, I. (2010). NMR and molecular modeling of wine tannins binding to saliva proteins: Revisiting astringency from molecular and colloidal prospects. *Official Publication of the Federation of American Societies for Experimental Biology, 24*(11), 4281-4290.

Callemien, D., & Collin, S. (2008). Use of RP-HPLC-ESI(−)-MS/MS to differentiate various proanthocyanidin isomers in lager beer extracts. *Journal of the American*

Society of Brewing Chemists, 66(2), 109-115.

Camas, N., Radusiene, J., Ivanauskas, L., Jakstas, V., & Cirak, C. (2014). Altitudinal changes in the content of bioactive substances in *hypericum orientale* and *hypericum pallens*. *Acta Physiologiae Plantarum, 36*(3), 675-686.

Cantos, E., Espín, J. C., & Tomás-Barberán, F. A. (2001). Postharvest induction modeling method using UV irradiation pulses for obtaining resveratrol-enriched table grapes: A new "functional" fruit?. *Journal of Agricultural and Food Chemistry, 49*(10), 5052-5058.

Carbone, F., Preuss, A., De Vos, R. C., D'AMICO, E., Perrotta, G., Bovy, A. G., & Rosati, C. (2009). Developmental, genetic and environmental factors affect the expression of flavonoid genes, enzymes and metabolites in strawberry fruits. *Plant, Cell and Environment, 32*(8), 1117-1131.

Carvalho, I. S., Cavaco, T., Carvalho, L. M., & Duque, P. (2010). Effect of photoperiod on flavonoid pathway activity in sweet potato (*ipomoea batatas* (L.) lam.) leaves. *Food Chemistry, 118*(2), 384-390.

Cassidy, A., Rimm, E. B., O'Reilly, E. J., Logroscino, G., Kay, C., Chiuve, S. E., & Rexrode, K. M. (2012). Dietary flavonoids and risk of stroke in women. *Stroke, 43*(4), 946-951.

Chawla, R., Arora, R., Singh, S., Sagar, R. K., Sharma, R. K., Kumar, R., & Prasad, J. (2007). Radioprotective and antioxidant activity of fractionated extracts of berries of *hippophae rhamnoides*. *Journal of Medicinal Food, 10*(1), 101-109.

Chira, K., Schmauch, G., Saucier, C., Fabre, S., & Teissedre, P. (2008). Grape variety effect on proanthocyanidin composition and sensory perception of skin and seed tannin extracts from bordeaux wine grapes (cabernet sauvignon and merlot) for two consecutive vintages (2006 and 2007). *Journal of Agricultural and Food Chemistry, 57*(2), 545-553.

Christie, P. J., Alfenito, M. R., & Walbot, V. (1994). Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: Enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta, 194*(4), 541-549.

Conte, C., Ebeling, M., Marcuz, A., Nef, P., & Andres-Barquin, P. J. (2002). Identification and characterization of human taste receptor genes belonging to the TAS2R family. *Cytogenetic and Genome Research, 98*(1), 45-53.

Costa, L. G., Garrick, J. M., Roquè, P. J., & Pellacani, C. (2016). Mechanisms of neuroprotection by quercetin: Counteracting oxidative stress and more. *Oxidative Medicine and Cellular Longevity, 2016: 2986796.*

Cueva, C., Sanchez-Patan, F., Monagas, M., Walton, G. E., Gibson, G. R., & Martin-Alvarez, P. J., Moreno-Arribas, M. V. (2013). In vitro fermentation of grape seed flavan-3-ol fractions by human faecal microbiota: Changes in microbial groups and phenolic metabolites. *FEMS Microbiology Ecology, 83*(3), 792-805.

Cushnie, T. T., & Lamb, A. J. (2005). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents, 26*(5), 343-356.

Cuyckens, F., & Claeys, M. (2004). Mass spectrometry in the structural analysis of flavonoids. *Journal of Mass Spectrometry, 39*(1), 1-15.

Czemmel, S., Heppel, S. C., & Bogs, J. (2012). R2R3 MYB transcription factors: Key regulators of the flavonoid biosynthetic pathway in grapevine. *Protoplasma, 249*(2), 109-118.

Da Silva Porto, Patrıcia Andréia Leite, Laranjinha, J. A. N., & de Freitas, Victor Armando Pereira. (2003). Antioxidant protection of low density lipoprotein by procyanidins: Structure/activity relationships. *Biochemical Pharmacology, 66*(6), 947-954.

Day, A. J., Cañada, F. J., Díaz, J. C., Kroon, P. A., Mclauchlan, R., Faulds, C. B., & Williamson, G. (2000). Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin

hydrolase. *FEBS Letters, 468*(2-3), 166-170.

Day, A. J., Gee, J. M., DuPont, M. S., Johnson, I. T., & Williamson, G. (2003). Absorption of quercetin-3-glucoside and quercetin-4′-glucoside in the rat small intestine: The role of lactase phlorizin hydrolase and the sodium-dependent glucose transporter. *Biochemical Pharmacology, 65*(7), 1199-1206.

De Freitas, V., & Mateus, N. (2001). Structural features of procyanidin interactions with salivary proteins. *Journal of Agricultural and Food Chemistry, 49*(2), 940-945.

Debeaujon, I., Leon-Kloosterziel, K. M., & Koornneef, M. (2000). Influence of the testa on seed dormancy, germination, and longevity in arabidopsis. *Plant Physiology, 122*(2), 403-414.

Declume, C. (1989). Anti-inflammatory evaluation op a hydroalcoholic extract op black currant leaves (*ribes nigrum*). *Journal of Ethnopharmacology, 27*(1), 91-98.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P., Tognolini, M., Borges, G., & Crozier, A. (2013). Dietary (poly) phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxidants & Redox Signaling, 18*(14), 1818-1892.

Dixon, R. A., Xie, D., & Sharma, S. B. (2005). Proanthocyanidins–a final frontier in flavonoid research?. *New Phytologist, 165*(1), 9-28.

Dong, J., Ma, X., Wei, Q., Peng, S., & Zhang, S. (2011). Effects of growing location on the contents of secondary metabolites in the leaves of four selected superior clones of *Eucommia ulmoides*. *Industrial Crops and Products, 34*(3), 1607-1614.

Engemann, A., Hübner, F., Rzeppa, S., & Humpf, H. (2012). Intestinal metabolism of two A-type procyanidins using the pig cecum model: Detailed structure elucidation of unknown catabolites with fourier transform mass spectrometry (FTMS). *Journal of Agricultural and Food Chemistry, 60*(3), 749-757.

Engström, M. T., Pälijärvi, M., Fryganas, C., Grabber, J. H., Mueller-Harvey, I., & Salminen, J. (2014). Rapid qualitative and quantitative analysis of proanthocyanidin oligomers and polymers by UPLC-MS/MS. *Journal of Agricultural and Food Chemistry, 62*(15), 3390–3399.

Es-Safi, N., Guyot, S., & Ducrot, P. (2006). NMR, ESI/MS, and MALDI-TOF/MS analysis of pear juice polymeric proanthocyanidins with potent free radical scavenging activity. *Journal of Agricultural and Food Chemistry, 54*(19), 6969-6977.

Fait, A., Hanhineva, K., Beleggia, R., Dai, N., Rogachev, I., Nikiforova, V. J., & Aharoni, A. (2008). Reconfiguration of the achene and receptacle metabolic networks during strawberry fruit development. *Plant Physiology, 148*(2), 730-750.

Feldbrügge, M., Sprenger, M., Hahlbrock, K., & Weisshaar, B. (1997). PcMYB1, a novel plant protein containing a DNA-binding domain with one MYB repeat, interacts in vivo with a light-regulatory promoter unit. *The Plant Journal, 11*(5), 1079-1093.

Fossen, T., Pedersen, A. T., & Andersen, Ø M. (1998). Flavonoids from red onion (*allium cepa*). *Phytochemistry, 47*(2), 281-285.

Gao, X., Ohlander, M., Jeppsson, N., Bjork, L., & Trajkovski, V. (2000). Changes in antioxidant effects and their relationship to phytonutrients in fruits of sea buckthorn (*hippophae rhamnoides* L.) during maturation. *Journal of Agricultural and Food Chemistry, 48*(5), 1485-1490.

Garbacki, N., Kinet, M., Nusgens, B., Desmecht, D., & Damas, J. (2005). Proanthocyanidins, from *ribes nigrum* leaves, reduce endothelial adhesion molecules ICAM-1 and VCAM-1. *Journal of Inflammation, 2*, 9.

Gee, J. M., DuPont, M. S., Day, A. J., Plumb, G. W., Williamson, G., & Johnson, I. T. (2000). Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway. *The Journal of Nutrition, 130*(11), 2765-2771.

Geetha, S., Jayamurthy, P., Pal, K., Pandey, S., Kumar, R., & Sawhney, R. (2008). Hepatoprotective effects of sea buckthorn (*hippophae rhamnoides* L.) against carbon tetrachloride induced liver injury in rats. *Journal of the Science of Food and Agriculture, 88*(9), 1592-1597.

Geetha, S., Sai Ram, M., Singh, V., Ilavazhagan, G., & Sawhney, R. C. (2002). Anti-oxidant and immunomodulatory properties of seabuckthorn (*hippophae rhamnoides*)-an in vitro study. *Journal of Ethnopharmacology, 79*(3), 373-378.

Geils, B. W., Hummer, K. E., & Hunt, R. S. (2010). White pines, *ribes*, and blister rust: A review and synthesis. *Forest Pathology, 40*(3-4), 147-185.

Gião, M. S., Pereira, C. I., Fonseca, S. C., Pintado, M. E., & Malcata, F. X. (2009). Effect of particle size upon the extent of extraction of antioxidant power from the plants *agrimonia eupatoria*, *salvia* sp. and *satureja montana*. *Food Chemistry, 117*(3), 412-416.

Giovinazzo, G., & Grieco, F. (2015). Functional properties of grape and wine polyphenols. *Plant Foods for Human Nutrition, 70*(4), 454-462.

Gopalan, A., Reuben, S. C., Ahmed, S., Darvesh, A. S., Hohmann, J., & Bishayee, A. (2012). The health benefits of blackcurrants. *Food & Function, 3*(8), 795-809.

Goto-Yamamoto, N., Mori, K., Numata, M., Koyama, K., & Kitayama, M. (2009). Effects of temperature and water regimes on flavonoid contents and composition in the skin of red-wine grapes. *Journal International des Sciences de la Vigne et du Vin,43*, 75-80.

Greaves, J., & Roboz, J. (2013). *Mass spectrometry for the novice* CRC Press.

Gu, L., Kelm, M. A., Hammerstone, J. F., Beecher, G., Holden, J., Haytowitz, D., & Prior, R. L. (2003). Screening of foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation. *Journal of Agricultural and Food Chemistry, 51*(25), 7513-7521.

Gu, L., Kelm, M. A., Hammerstone, J. F., Zhang, Z., Beecher, G., Holden, J., & Prior, R. L. (2003). Liquid chromatographic/electrospray ionization mass spectrometric studies of proanthocyanidins in foods. *Journal of Mass Spectrometry, 38*(12), 1272-1280.

Gu, L., Kelm, M., Hammerstone, J. F., Beecher, G., Cunningham, D., Vannozzi, S., & Prior, R. L. (2002). Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC-MS fluorescent detection method. *Journal of Agricultural and Food Chemistry, 50*(17), 4852-4860.

Guyot, S., Le Guernevé, C., Marnet, N., & Drilleau, J. (1999). Methods for determining the degree of polymerization of condensed tannins: A new ^{1}H-NMR procedure applied to cider apple procyanidins. *Plant polyphenols 2* (pp. 211-222) Springer.

Haasbach, E., Hartmayer, C., Hettler, A., Sarnecka, A., Wulle, U., Ehrhardt, C., & Planz, O. (2014). Antiviral activity of Ladania067, an extract from wild black currant leaves against influenza A virus in vitro and in vivo. *Frontiers in Microbiology, 5*

Harborne, J. B., & Mabry, T. J. (2013). *The flavonoids: Advances in research* Springer.

Hartmann, U., Sagasser, M., Mehrtens, F., Stracke, R., & Weisshaar, B. (2005). Differential combinatorial interactions of cis-acting elements recognized by R_2R_3-MYB, BZIP, and BHLH factors control light-responsive and tissue-specific activation of phenylpropanoid biosynthesis genes. *Plant Molecular Biology, 57*(2), 155-171.

Hayasaka, Y., Waters, E. J., Cheynier, V., Herderich, M. J., & Vidal, S. (2003). Characterization of proanthocyanidins in grape seeds using electrospray mass spectrometry. *Rapid Communications in Mass Spectrometry, 17*(1), 9-16.

He, D., Huang, Y., Ayupbek, A., Gu, D., Yang, Y., Aisa, H. A., & Ito, Y. (2010). Separation and purification of flavonoids from black currant leaves by high-speed countercurrent chromatography and

preparative HPLC. *Journal of Liquid Chromatography & Related Technologies, 33*(5), 615-628.

He, F., Pan, Q., Shi, Y., & Duan, C. (2008). Biosynthesis and genetic regulation of proanthocyanidins in plants. *Molecules, 13*(10), 2674-2703.

Hellström, J. K., Törrönen, A. R., & Mattila, P. H. (2009). Proanthocyanidins in common food products of plant origin. *Journal of Agricultural and Food Chemistry, 57*(17), 7899-7906.

Hernández-Jiménez, A., Kennedy, J. A., Bautista-Ortín, A. B., & Gómez-Plaza, E. (2011). Effect of ethanol on grape seed proanthocyanidin extraction. *American Journal of Enology and Viticulture, 63*(1), 57-61.

Hollman, P. C., de Vries, J. H., van Leeuwen, S. D., Mengelers, M. J., & Katan, M. B. (1995). Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *The American Journal of Clinical Nutrition, 62*(6), 1276-1282.

Holt, R. R., Lazarus, S. A., Sullards, M. C., Zhu, Q. Y., Schramm, D. D., Hammerstone, J. F., & Keen, C. L. (2002). Procyanidin dimer B2 [epicatechin-(4*β*-8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa. *The American Journal of Clinical Nutrition, 76*(4), 798-804.

Holton, T. A., & Cornish, E. C. (1995). Genetics and biochemistry of anthocyanin biosynthesis. *The Plant Cell, 7*(7), 1071-1083.

Hsieh, M. C., Shen, Y. J., Kuo, Y. H., & Hwang, L. S. (2008). Antioxidative activity and active components of longan (*dimocarpus longan* lour.) flower extracts. *Journal of Agricultural and Food Chemistry, 56*(16), 7010-7016.

Huemmer, W., & Schreier, P. (2008). Analysis of proanthocyanidins. *Molecular Nutrition & Food Research, 52*(12), 1381-1398.

Hufnagel, J. C., & Hofmann, T. (2008). Orosensory-directed identification of astringent mouthfeel and bitter-tasting compounds in red wine. *Journal of Agricultural and Food Chemistry, 56*(4), 1376-1386.

Hummer, K., & Dale, A. (2010). Horticulture of *ribes*. *Forest Pathology, 40*(3-4), 251-263.

Hvattum, E., & Ekeberg, D. (2003). Study of the collision-induced radical cleavage of flavonoid glycosides using negative electrospray ionization tandem quadrupole mass spectrometry. *Journal of Mass Spectrometry, 38*(1), 43-49.

Ichino, T., Fuji, K., Ueda, H., Takahashi, H., Koumoto, Y., Takagi, J., & Shimada, T. (2014). GFS9/TT9 contributes to intracellular membrane trafficking and flavonoid accumulation in *arabidopsis thaliana*. *The Plant Journal, 80*(3), 410-423.

Jaakola, L. (2013). New insights into the regulation of anthocyanin biosynthesis in fruits. *Trends in Plant Science, 18*(9), 477-483.

Jaakola, L., & Hohtola, A. (2010). Effect of latitude on flavonoid biosynthesis in plants. *Plant, Cell & Environment, 33*(8), 1239-1247.

Kalili, K. M., & de Villiers, A. (2009). Off-line comprehensive 2-dimensional hydrophilic interaction× reversed phase liquid chromatography analysis of procyanidins. *Journal of Chromatography.A, 1216*(35), 6274-6284.

Kallio, H., Yang, B., Tahvonen, R., & Hakala, M. (2003). Composition of seabuckthorn berries of various origins. *The Global Seabuckthorn Research and Development, 1*(1), 34-39.

Kallio, H., Yang, W., Liu, P., & Yang, B. (2014). Proanthocyanidins in wild sea buckthorn (*hippophaë rhamnoides*) berries analyzed by reversed-phase, normal-phase, and hydrophilic interaction liquid chromatography with UV and MS detection. *Journal of Agricultural and Food Chemistry, 62*(31), 7721-7729.

Karlsson, P. C., Huss, U., Jenner, A., Halliwell, B., Bohlin, L., & Rafter, J. J. (2005). Human fecal water inhibits COX-2 in colonic HT-29 cells: Role of phenolic

compounds. *The Journal of Nutrition, 135*(10), 2343-2349.

Karonen, M. (2007). *Plant proanthocyanidins: Characterization and quantification by degradation methods, HPLC-DAD/ESI-MS and NMR* University of Turku.

Karonen, M., Liimatainen, J., & Sinkkonen, J. (2011). Birch inner bark procyanidins can be resolved with enhanced sensitivity by hydrophilic interaction HPLC-MS. *Journal of Separation Science, 34*(22), 3158-3165.

Karonen, M., Loponen, J., Ossipov, V., & Pihlaja, K. (2004). Analysis of procyanidins in pine bark with reversed-phase and normal-phase high-performance liquid chromatography-electrospray ionization mass spectrometry. *Analytica Chimica Acta, 522*(1), 105-112.

Katiyar, S. K. (2016). Dietary proanthocyanidins inhibit UV radiation-induced skin tumor development through functional activation of the immune system. *Molecular Nutrition & Food Research, 60*(6), 1374-1382.

Kaur, M., Singh, R. P., Gu, M., Agarwal, R., & Agarwal, C. (2006). Grape seed extract inhibits in vitro and in vivo growth of human colorectal carcinoma cells. *Clinical Cancer Research, 12*(20), 6194-6202.

Kelm, M. A., Johnson, J. C., Robbins, R. J., Hammerstone, J. F., & Schmitz, H. H. (2006). High-performance liquid chromatography separation and purification of cacao (*theobroma cacao* L.) procyanidins according to degree of polymerization using a diol stationary phase. *Journal of Agricultural and Food Chemistry, 54*(5), 1571-1576.

Khoddami, A., Wilkes, M. A., & Roberts, T. H. (2013). Techniques for analysis of plant phenolic compounds. *Molecules, 18*(2), 2328-2375.

Kim, J., Liu, L., Guo, W., & Meydani, M. (2006). Chemical structure of flavonols in relation to modulation of angiogenesis and immune-endothelial cell adhesion. *The Journal of Nutritional Biochemistry, 17*(3), 165-176.

Koupai-Abyazani, M. R., McCallum, J., & Bohm, B. A. (1992). Identification of the constituent flavanoid units in sainfoin proanthocyanidins by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A, 594*(1), 117-123.

Kruger, M. J., Davies, N., Myburgh, K. H., & Lecour, S. (2014). Proanthocyanidins, anthocyanins and cardiovascular diseases. *Food Research International, 59*, 41-52.

Kumar, S., & Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: An overview. *The Scientific World Journal, 2013*, 162750.

Laaksonen, O., Salminen, J., Mäkilä, L., Kallio, H., & Yang, B. (2015). Proanthocyanidins and their contribution to sensory attributes of blackcurrant juices. *Journal of Agricultural and Food Chemistry, 63*(22), 5373-5380.

Latti, A. K., Jaakola, L., Riihinen, K. R., & Kainulainen, P. S. (2010). Anthocyanin and flavonol variation in bog bilberries (*vaccinium uliginosum* L.) in Finland. *Journal of Agricultural and Food Chemistry, 58*(1), 427-433.

Lätti, A. K., Riihinen, K. R., & Kainulainen, P. S. (2008). Analysis of anthocyanin variation in wild populations of bilberry (*vaccinium myrtillus* L.) in Finland. *Journal of Agricultural and Food Chemistry, 56*(1), 190-196.

De la Rosa, A., Alvarez-Parrilla, E., & Gonzalez-Aguilar, G. A. (2009). *Fruit and vegetable phytochemicals: Chemistry, nutritional value and stability* John Wiley & Sons.

Lepiniec, L., Debeaujon, I., Routaboul, J., Baudry, A., Pourcel, L., Nesi, N., & Caboche, M. (2006). Genetics and biochemistry of seed flavonoids. *Annual Review of Plant Biology, 57*, 405-430.

Lesschaeve, I., & Noble, A. C. (2005). Polyphenols: Factors influencing their sensory properties and their effects on food and beverage preferences. *The American Journal of Clinical Nutrition, 81*(1S), 330S-335S.

Leyva, A., Jarillo, J. A., Salinas, J., & Martinez-Zapater, J. M. (1995). Low temperature induces the accumulation of phenylalanine ammonia-lyase and chalcone synthase mRNAs of arabidopsis thaliana in a light-dependent manner. *Plant Physiology, 108*(1), 39-46.

Li, H., & Deinzer, M. L. (2007). Tandem mass spectrometry for sequencing proanthocyanidins. *Analytical Chemistry, 79*(4), 1739-1748.

Li, T. S. (2002). Product development of sea buckthorn. *Trends in New Crops and New Uses. ASHS Press, Alexandria*, 393-398.

Li, T. S., & Schroeder, W. (1996). Sea buckthorn (*hippophae rhamnoides* L.): A multipurpose plant. *HortTechnology, 6*(4), 370-380.

Liimatainen, J. (2013). *Phenolics of the inner bark of silver birch: Characterization and intraspecific variation* University of Turku.

Liu, P., Kallio, H., & Yang, B. (2014). Flavonol glycosides and other phenolic compounds in buds and leaves of different varieties of black currant (*ribes nigrum* L.) and changes during growing season. *Food Chemistry, 160*, 180-189.

Liu, Y., Chen, M., Yao, X., Xu, C., Zhang, Y., & Wang, Y. (2013). Enhancement in dentin collagen's biological stability after proanthocyanidins treatment in clinically relevant time periods. *Dental Materials, 29*(4), 485-492.

Løvdal, T., Olsen, K. M., Slimestad, R., Verheul, M., & Lillo, C. (2010). Synergetic effects of nitrogen depletion, temperature, and light on the content of phenolic compounds and gene expression in leaves of tomato. *Phytochemistry, 71*(5), 605-613.

Ma, X., Laaksonen, O., Zheng, J., Yang, W., Trépanier, M., Kallio, H., & Yang, B. (2016). Flavonol glycosides in berries of two major subspecies of sea buckthorn (*hippophaë rhamnoides* L.) and influence of growth sites. *Food Chemistry, 200*, 189-198.

Ma, Y., Mani, A., Cai, Y., Thomson, J., Ma, J., Peudru, F., & Chapman, R. G. (2016). An effective identification and quantification method for *ginkgo biloba* flavonol glycosides with targeted evaluation of adulterated products. *Phytomedicine, 23*(4), 377-387.

Määttä, K., Kamal-Eldin, A., & Törrönen, R. (2001). Phenolic compounds in berries of black, red, green, and white currants (*ribes* sp.). *Antioxidants and Redox Signaling, 3*(6), 981-993.

Manach, C., Williamson, G., Morand, C., Scalbert, A., & Rémésy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. review of 97 bioavailability studies. *The American Journal of Clinical Nutrition, 81*(1), 230S-242S.

March, R. E., Lewars, E. G., Stadey, C. J., Miao, X., Zhao, X., & Metcalfe, C. D. (2006). A comparison of flavonoid glycosides by electrospray tandem mass spectrometry. *International Journal of Mass Spectrometry, 248*(1), 61-85.

Martz, F., Peltola, R., Fontanay, S., Duval, R. E., Julkunen-Tiitto, R., & Stark, S. (2009). Effect of latitude and altitude on the terpenoid and soluble phenolic composition of juniper (*juniperus communis*) needles and evaluation of their antibacterial activity in the boreal zone. *Journal of Agricultural and Food Chemistry, 57*(20), 9575-9584.

Matkowski, A., Jamiolkowska-Kozlowska, W., & Nawrot, I. (2013). Chinese medicinal herbs as source of antioxidant compounds–where tradition meets the future. *Current Medicinal Chemistry, 20*(8), 984-1004.

McCarty, M. (2015). Flavones and flavonols may have clinical potential as CK2 inhibitors in cancer therapy. *Cancer Studies and Molecular Medicine, 2*(1), 39-51.

McCullough, M. L., Peterson, J. J., Patel, R., Jacques, P. F., Shah, R., & Dwyer, J. T. (2012). Flavonoid intake and cardiovascular disease mortality in a prospective cohort of US adults. *The American Journal of Clinical Nutrition, 95*(2), 454-464.

Middleton, E., Kandaswami, C., & Theoharides, T. C. (2000). The effects of plant

flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. *Pharmacological Reviews, 52*(4), 673-751.

Montero, L., Herrero, M., Ibáñez, E., & Cifuentes, A. (2013). Profiling of phenolic compounds from different apple varieties using comprehensive two-dimensional liquid chromatography. *Journal of Chromatography A, 1313,* 275-283.

Montero, L., Herrero, M., Prodanov, M., Ibáñez, E., & Cifuentes, A. (2012). Characterization of grape seed procyanidins by comprehensive two-dimensional hydrophilic interaction× reversed phase liquid chromatography coupled to diode array detection and tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, *405*(13), 4627-4638.

Moon, J., Tsushida, T., Nakahara, K., & Terao, J. (2001). Identification of quercetin 3-*O*-*β*-D-glucuronide as an antioxidative metabolite in rat plasma after oral administration of quercetin. *Free Radical Biology and Medicine, 30*(11), 1274-1285.

Mori, K., Goto-Yamamoto, N., Kitayama, M., & Hashizume, K. (2007). Loss of anthocyanins in red-wine grape under high temperature. *Journal of Experimental Botany, 58*(8), 1935-1945.

Mullen, W., Rouanet, J., Auger, C., Teissèdre, P., Caldwell, S. T., Hartley, R. C., & Crozier, A. (2008). Bioavailability of [2-^{14}C] quercetin-4′-glucoside in rats. *Journal of Agricultural and Food Chemistry, 56*(24), 12127-12137.

Muller-Xing, R., Xing, Q., & Goodrich, J. (2014). Footprints of the sun: Memory of UV and light stress in plants. *Frontiers in Plant Science, 5*, 474.

Naczk, M., & Shahidi, F. (2004). Extraction and analysis of phenolics in food. *Journal of Chromatography A, 1054*(1), 95-111.

Nam, J., Phansalkar, R., Lankin, D., Bisson, J., McAlpine, J., Leme, A., & Pauli, G. (2015). Chemical nano shifts explain the NMR fingerprints of dentin-enhancing oligomeric proanthocyanidins. *Planta Medica, 81*(11), PY8.

Nandakumar, V., Singh, T., & Katiyar, S. K. (2008). Multi-targeted prevention and therapy of cancer by proanthocyanidins. *Cancer Letters, 269*(2), 378-387.

Nelson, H. N., Kaiser, A. J., Reker, N. C., Wang, Y., Singh, A. P., Vorsa, N., & Wilson, T. (2016). Postprandial cranberry flavonol glycoside bioavailability in human urine. *Official Publication of the Federation of American Societies for Experimental Biology 30*(1 Supplement), 690.19.

Oki, T., Masuda, M., Kobayashi, M., Nishiba, Y., Furuta, S., Suda, I., & Sato, T. (2002). Polymeric procyanidins as radical-scavenging components in red-hulled rice. *Journal of Agricultural and Food Chemistry, 50*(26), 7524-7529.

Ong, K. C., & Khoo, H. (1997). Biological effects of myricetin. *General Pharmacology: The Vascular System, 29*(2), 121-126.

Ong, K. C., & Khoo, H. (2000). Effects of myricetin on glycemia and glycogen metabolism in diabetic rats. *Life Sciences, 67*(14), 1695-1705.

Osakabe, N., Yasuda, A., Natsume, M., Takizawa, T., Terao, J., & Kondo, K. (2002). Catechins and their oligomers linked by C4→C8 bonds are major cacao polyphenols and protect low-density lipoprotein from oxidation in vitro. *Experimental Biology and Medicine, 227*(1), 51-56.

Ottaviani, J. I., Kwik-Uribe, C., Keen, C. L., & Schroeter, H. (2012). Intake of dietary procyanidins does not contribute to the pool of circulating flavanols in humans. *The American Journal of Clinical Nutrition, 95*(4), 851-858.

Ou, K., & Gu, L. (2014). Absorption and metabolism of proanthocyanidins. *Journal of Functional Foods, 7*, 43-53.

Pavarini, D. P., Pavarini, S. P., Niehues, M., & Lopes, N. P. (2012). Exogenous influences on plant secondary metabolite levels. *Animal Feed Science and Technology, 176*(1), 5-16.

Peleg, H., Gacon, K., Schlich, P., & Noble, A. C. (1999). Bitterness and astringency of

flavan-3-ol monomers, dimers and trimers. *Journal of the Science of Food and Agriculture, 79*(8), 1123-1128.

Pérez-Magariño, S., Revilla, I., González-SanJosé, M., & Beltrán, S. (1999). Various applications of liquid chromatography–mass spectrometry to the analysis of phenolic compounds. *Journal of Chromatography A, 847*(1), 75-81.

Phansalkar, R. S., Nam, J., Chen, S., McAlpine, J. B., Napolitano, J. G., Leme, A., & Pauli, G. F. (2015). A galloylated dimeric proanthocyanidin from grape seed exhibits dentin biomodification potential. *Fitoterapia, 101*, 169-178.

Pinheiro, P. F., & Justino, G. C. (2012). *Structural analysis of flavonoids and related compounds-A review of spectroscopic applications* INTECH Open Access Publisher.

Plumb, G., Price, K., & Williamson, G. (2013). Antioxidant properties of flavonol glycosides from tea. *Redox Report,*

Polewski, M., Krueger, C., Reed, J., & Leyer, G. (2016). Ability of cranberry proanthocyanidins in combination with a probiotic formulation to inhibit in vitro invasion of gut epithelial cells by extra-intestinal pathogenic *E. coli*. *Journal of Functional Foods, 25*, 123-134.

Porter, L. J. (1988). Flavans and proanthocyanidins. *The flavonoids* (pp. 21-62) Springer.

Prasain, J. K., Peng, N., Dai, Y., Moore, R., Arabshahi, A., Wilson, L., & Watts, R. L. (2009). Liquid chromatography tandem mass spectrometry identification of proanthocyanidins in rat plasma after oral administration of grape seed extract. *Phytomedicine, 16*(2), 233-243.

Prior, R. L., Fan, E., Ji, H., Howell, A., Nio, C., Payne, M. J., & Reed, J. (2010). Multi-laboratory validation of a standard method for quantifying proanthocyanidins in cranberry powders. *Journal of the Science of Food and Agriculture, 90*(9), 1473-1478.

Ravaglia, D., Espley, R. V., Henry-Kirk, R. A., Andreotti, C., Ziosi, V., Hellens, R. P., & Allan, A. C. (2013). Transcriptional regulation of flavonoid biosynthesis in nectarine (*prunus persica*) by a set of R_2R_3 MYB transcription factors. *BMC Plant Biology, 13*(1), 1.

Reyes, L., Miller, J., & Cisneros-Zevallos, L. (2004). Environmental conditions influence the content and yield of anthocyanins and total phenolics in purple- and red-flesh potatoes during tuber development. *American Journal of Potato Research, 81*(3), 187-193.

Ribas-Latre, A., Del Bas, J. M., Baselga-Escudero, L., Casanova, E., Arola-Arnal, A., Salvadó, M., & Bladé, C. (2015). Dietary proanthocyanidins modulate melatonin levels in plasma and the expression pattern of clock genes in the hypothalamus of rats. *Molecular Nutrition & Food Research, 59*(5), 865-878.

Rieger, G., Müller, M., Guttenberger, H., & Bucar, F. (2008). Influence of altitudinal variation on the content of phenolic compounds in wild populations of *calluna vulgaris*, *sambucus nigra*, and *vaccinium myrtillus*. *Journal of Agricultural and Food Chemistry, 56*(19), 9080-9086.

Rigaud, J., Escribano-Bailon, M., Prieur, C., Souquet, J., & Cheynier, V. (1993). Normal-phase high-performance liquid chromatographic separation of procyanidins from cacao beans and grape seeds. *Journal of Chromatography A, 654*(2), 255-260.

Romanov-Michailidis, F., Viton, F., Fumeaux, R., Léveques, A., Actis-Goretta, L., Rein, M., & Barron, D. (2012). Epicatechin B-ring conjugates: First enantioselective synthesis and evidence for their occurrence in human biological fluids. *Organic Letters, 14*(15), 3902-3905.

Rongsen, L. (1992). Seabuckthorn: A multipurpose plant species for fragile mountains. *Kathmandu: International Centre for Integrated Mountain Development 62p.*

Rösch, D., Mugge, C., Fogliano, V., & Kroh, L. W. (2004). Antioxidant oligomeric proanthocyanidins from sea buckthorn (*hippophae rhamnoides*) pomace. *Journal of Agricultural and Food Chemistry, 52*(22), 6712-6718.

Rousi A. (1971). The genus hippophae L. A axonomic study. *Annales Botanici Fennici, 8*, 177-227.

Russo, D., Kenny, O., Smyth, T. J., Milella, L., Hossain, M. B., Diop, M. S., & Brunton, N. P. (2013). Profiling of phytochemicals in tissues from *sclerocarya birrea* by HPLC-MS and their link with antioxidant activity. *ISRN Chromatography, 2013*

Sano, A., Yamakoshi, J., Tokutake, S., Tobe, K., Kubota, Y., & Kikuchi, M. (2003). Procyanidin B1 is detected in human serum after intake of proanthocyanidin-rich grape seed extract. *Bioscience, Biotechnology, and Biochemistry, 67*(5), 1140-1143.

Santos-Buelga, C., & Scalbert, A. (2000). Proanthocyanidins and tannin-like compounds–nature, occurrence, dietary intake and effects on nutrition and health. *Journal of the Science of Food and Agriculture, 80*(7), 1094-1117.

Saslowsky, D., & Winkel-Shirley, B. (2001). Localization of flavonoid enzymes in arabidopsis roots. *The Plant Journal, 27*(1), 37-48.

Sato, M., Islam, S. Q., Awata, S., & Yamasaki, T. (1999). Flavanonol glucoside and proanthocyanidins: Oviposition stimulants for the cerambycid beetle, monochamus alternatus. *Journal of Pesticide Science, 24*(2), 123-129.

Scalbert, A. (1991). Antimicrobial properties of tannins. *Phytochemistry, 30*(12), 3875-3883.

Schwartz, E., Tzulker, R., Glazer, I., Bar-Ya'akov, I., Wiesman, Z., Tripler, E., & Holland, D. (2009). Environmental conditions affect the color, taste, and antioxidant capacity of 11 pomegranate accessions' fruits. *Journal of Agricultural and Food Chemistry, 57*(19), 9197-9209.

Schwarz, B., & Hofmann, T. (2007). Sensory-guided decomposition of red currant juice (*ribes rubrum*) and structure determination of key astringent compounds. *Journal of Agricultural and Food Chemistry, 55*(4), 1394-1404.

Serafini, M., Bugianesi, R., Maiani, G., Valtuena, S., De Santis, S., & Crozier, A. (2003). Plasma antioxidants from chocolate. *Nature, 424*(6952), 1013-1013.

Serra, A., Macia, A., Romero, M., Valls, J., Bladé, C., Arola, L., & Motilva, M. (2010). Bioavailability of procyanidin dimers and trimers and matrix food effects in *in vitro* and *in vivo* models. *British Journal of Nutrition, 103*(07), 944-952.

Singh, V., Singh, V., Yang, B., Kallio, H., Bala, M., Sawhney, R., & Tolkachev, O. (2006). Seabuckthorn (*hippophae* L.) in traditional medicines. *Seabuckthorn (Hippophae L.).A Multipurpose Wonder Plant.Vol II: Biochemistry and Pharmacology,* Daya Publishing House.

Soares, S., Kohl, S., Thalmann, S., Mateus, N., Meyerhof, W., & De Freitas, V. (2013). Different phenolic compounds activate distinct human bitter taste receptors. *Journal of Agricultural and Food Chemistry, 61*(7), 1525-1533.

Soares, S., Mateus, N., & De Freitas, V. (2007). Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary α-amylase (HSA) by fluorescence quenching. *Journal of Agricultural and Food Chemistry, 55*(16), 6726-6735.

Song, P., Zhang, R., Wang, X., He, P., Tan, L., & Ma, X. (2011). Dietary grape-seed procyanidins decreased postweaning diarrhea by modulating intestinal permeability and suppressing oxidative stress in rats. *Journal of Agricultural and Food Chemistry, 59*(11), 6227-6232.

Stalikas, C. D. (2007). Extraction, separation, and detection methods for phenolic acids and flavonoids. *Journal of Separation Science, 30*(18), 3268-3295.

Stoupi, S., Williamson, G., Drynan, J. W., Barron, D., & Clifford, M. N. (2010). A comparison of the in vitro biotransformation of (–)-epicatechin and procyanidin B2 by human faecal microbiota. *Molecular Nutrition & Food Research, 54*(6), 747-759.

Sun, K., Chen, X., Ma, R., Li, C., Wang, Q., & Ge, S. (2002). Molecular phylogenetics of *hippophae* L.(elaeagnaceae) based on the

internal transcribed spacer (ITS) sequences of nrDNA. *Plant Systematics and Evolution, 235*(1-4), 121-134.

Suryakumar, G., & Gupta, A. (2011). Medicinal and therapeutic potential of sea buckthorn (*hippophae rhamnoides* L.). *Journal of Ethnopharmacology, 138*(2), 268-278.

Tabart, J., Franck, T., Kevers, C., Pincemail, J., Serteyn, D., Defraigne, J., & Dommes, J. (2012). Antioxidant and anti-inflammatory activities of *ribes nigrum* extracts. *Food Chemistry, 131*(4), 1116-1122.

Tabart, J., Kevers, C., Pincemail, J., Defraigne, J., & Dommes, J. (2006). Antioxidant capacity of black currant varies with organ, season, and cultivar. *Journal of Agricultural and Food Chemistry, 54*(17), 6271-6276.

Tabart, J., Kevers, C., Sipel, A., Pincemail, J., Defraigne, J., & Dommes, J. (2007). Optimisation of extraction of phenolics and antioxidants from black currant leaves and buds and of stability during storage. *Food Chemistry, 105*(3), 1268-1275.

Tan, W., Lin, L., Li, M., Zhang, Y., Tong, Y., Xiao, D., & Ding, J. (2003). Quercetin, a dietary-derived flavonoid, possesses antiangiogenic potential. *European Journal of Pharmacology, 459*(2), 255-262.

Tarara, J. M., Lee, J., Spayd, S. E., & Scagel, C. F. (2008). Berry temperature and solar radiation alter acylation, proportion, and concentration of anthocyanin in merlot grapes. *American Journal of Enology and Viticulture, 59*(3), 235-247.

Tarascou, I., Ducasse, M., Dufourc, E. J., Moskau, D., Fouquet, E., Laguerre, M., & Pianet, I. (2007). Structural and conformational analysis of two native procyanidin trimers. *Magnetic Resonance in Chemistry, 45*(2), 157-166.

Tatsuno, T., Jinno, M., Arima, Y., Kawabata, T., Hasegawa, T., Yahagi, N., & Ohta, T. (2012). Anti-inflammatory and anti-melanogenic proanthocyanidin oligomers from peanut skin. *Biological and Pharmaceutical Bulletin, 35*(6), 909-916.

Terra, X., Pallarés, V., Ardèvol, A., Bladé, C., Fernández-Larrea, J., Pujadas, G., & Blay, M. (2011). Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. *The Journal of Nutritional Biochemistry, 22*(4), 380-387.

Theodoridis, G., Lasakova, M., Škeříková, V., Tegou, A., Giantsiou, N., & Jandera, P. (2006). Molecular imprinting of natural flavonoid antioxidants: Application in solid-phase extraction for the sample pretreatment of natural products prior to HPLC analysis. *Journal of Separation Science, 29*(15), 2310-2321.

Thomson, B. A. (1998). Atmospheric pressure ionization and liquid chromatography/mass spectrometry—together at last. *Journal of the American Society for Mass Spectrometry, 9*(3), 187-193.

Tian, L., Pang, Y., & Dixon, R. A. (2008). Biosynthesis and genetic engineering of proanthocyanidins and (iso) flavonoids. *Phytochemistry Reviews, 7*(3), 445-465.

Tiitinen, K. M., Hakala, M. A., & Kallio, H. P. (2005). Quality components of sea buckthorn (*hippophae rhamnoides*) varieties. *Journal of Agricultural and Food Chemistry, 53*(5), 1692-1699.

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. *Nutrients, 2*(12), 1231-1246.

Uleberg, E., Rohloff, J., Jaakola, L., Trôst, K., Junttila, O., Häggman, H., & Martinussen, I. (2012). Effects of temperature and photoperiod on yield and chemical composition of northern and southern clones of bilberry (*vaccinium myrtillus* L.). *Journal of Agricultural and Food Chemistry, 60*(42), 10406-10414.

Upadhyay, N., Kumar, R., Mandotra, S., Meena, R., Siddiqui, M., Sawhney, R., & Gupta, A. (2009). Safety and healing efficacy of sea buckthorn (*hippophae rhamnoides* L.) seed oil on burn wounds in rats. *Food and Chemical Toxicology, 47*(6), 1146-1153.

Vagiri, M., Ekholm, A., Andersson, S. C., Johansson, E., & Rumpunen, K. (2012). An optimized method for analysis of phenolic compounds in buds, leaves, and fruits of black currant (*ribes nigrum* L.).

Journal of Agricultural and Food Chemistry, 60(42), 10501-10510.

Veitch, N. C., & Grayer, R. J. (2008). Flavonoids and their glycosides, including anthocyanins. *Natural Product Reports, 25*(3), 555-611.

Vimolmangkang, S., Zheng, D., Han, Y., Khan, M. A., Soria-Guerra, R. E., & Korban, S. S. (2014). Transcriptome analysis of the exocarp of apple fruit identifies light-induced genes involved in red color pigmentation. *Gene, 534*(1), 78-87.

Wang, C., Mehendale, S. R., Calway, T., & Yuan, C. (2011). Botanical flavonoids on coronary heart disease. *The American Journal of Chinese Medicine, 39*(04), 661-671.

Wang, G., Cao, F., Wang, G., & El-Kassaby, Y. A. (2015). Role of temperature and soil moisture conditions on flavonoid production and biosynthesis-related genes in ginkgo (*ginkgo biloba* L.) leaves. *Natural Products Chemistry & Research, 3*(1), 1000162.

Wang, X., Ouyang, Y. Y., Liu, J., & Zhao, G. (2014). Flavonoid intake and risk of CVD: A systematic review and meta-analysis of prospective cohort studies. *The British Journal of Nutrition, 111*(1), 1-11.

Wang, Y., Zhao, L., Huo, Y., Zhou, F., Wu, W., Lu, F., & Deng, Q. (2016). Protective effect of proanthocyanidins from sea buckthorn (*hippophae rhamnoides* L.) seed against visible light-induced retinal degeneration *in vivo*. *Nutrients, 8*(5), 245.

Wang, Z., Sha, Y., Yu, X., & Liang, Y. (2015). Determination of flavonol glycosides in tea samples by ultra-high performance liquid chromatography-photodiode array detection-tandem mass spectrometry. *Chinese Journal of Chromatography, 33*(9), 974-980.

Watson, R. R. (2014). *Polyphenols in plants: Isolation, purification and extract preparation* Academic Press.

Woznicki, T. L., Aaby, K., Sønsteby, A., Heide, O. M., Wold, A., & Remberg, S. F. (2016). Influence of controlled post-flowering temperature and daylength on individual phenolic compounds in four black currant cultivars. *Journal of Agricultural and Food Chemistry, 64*(4), 752-761.

Xie, J., Zhu, L., Luo, H., Zhou, L., Li, C., & Xu, X. (2001). Direct extraction of specific pharmacophoric flavonoids from gingko leaves using a molecularly imprinted polymer for quercetin. *Journal of Chromatography A, 934*(1), 1-11.

Xie, Y., Huang, S., & Su, Y. (2016). Dietary flavonols intake and risk of esophageal and gastric cancer: A meta-analysis of epidemiological studies. *Nutrients, 8*(2), 91.

Xu, Y., Kaur, M., Dhillon, R. S., Tappia, P. S., & Dhalla, N. S. (2011). Health benefits of sea buckthorn for the prevention of cardiovascular diseases. *Journal of Functional Foods, 3*(1), 2-12.

Yamane, T., Jeong, S. T., Goto-Yamamoto, N., Koshita, Y., & Kobayashi, S. (2006). Effects of temperature on anthocyanin biosynthesis in grape berry skins. *American Journal of Enology and Viticulture, 57*(1), 54-59.

Yanagida, A., Murao, H., Ohnishi-Kameyama, M., Yamakawa, Y., Shoji, A., Tagashira, M., & Shibusawa, Y. (2007). Retention behavior of oligomeric proanthocyanidins in hydrophilic interaction chromatography. *Journal of Chromatography A, 1143*(1), 153-161.

Yang, B., Zheng, J., Laaksonen, O., Tahvonen, R., & Kallio, H. (2013). Effects of latitude and weather conditions on phenolic compounds in currant (*ribes* spp.) cultivars. *Journal of Agricultural and Food Chemistry, 61*(14), 3517-3532.

Yang, L., Sun, X., Yang, F., Zhao, C., Zhang, L., & Zu, Y. (2012). Application of ionic liquids in the microwave-assisted extraction of proanthocyanidins from *larix gmelini* bark. *International Journal of Molecular Sciences, 13*(4), 5163-5178.

Yang, W., Alanne, A., Liu, P., Kallio, H., & Yang, B. (2015). Flavonol glycosides in currant leaves and variation with growth season, growth location, and leaf position. *Journal of Agricultural and Food Chemistry, 63*(42), 9269-9276.

Yang, W., Laaksonen, O., Kallio, H., & Yang, B. (2016). Proanthocyanidins in sea buckthorn (*hippophaë rhamnoides* L.) berries of different origins with special reference to influence of genetic background and growth location. *Journal of Agricultural and Food Chemistry, 64*(6), 1274-1282.

Yang, W., Laaksonen, O., Kallio, H., & Yang, B. (2017). Effects of latitude and weather conditions on proanthocyanidins in berries of Finnish wild and cultivated sea buckthorn (*Hippophaë rhamnoides* L. ssp. *rhamnoides*). *Food Chemistry*, *216*, 87-96.

Zamora-Ros, R., Forouhi, N. G., Sharp, S. J., Gonzalez, C. A., Buijsse, B., Guevara, M., & Wareham, N. J. (2014). Dietary intakes of individual flavanols and flavonols are inversely associated with incident type 2 diabetes in european populations. *The Journal of Nutrition, 144*(3), 335-343.

Zang, X., Shang, M., Xu, F., Liang, J., Wang, X., Mikage, M., & Cai, S. (2013). A-type proanthocyanidins from the stems of *ephedra sinica* (ephedraceae) and their antimicrobial activities. *Molecules, 18*(5), 5172-5189.

Zhang, H., & Tsao, R. (2016). Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Current Opinion in Food Science, 8*, 33-42.

Zhao, J., Pang, Y., & Dixon, R. A. (2010). The mysteries of proanthocyanidin transport and polymerization. *Plant Physiology, 153*(2), 437-443.

Zheng, J., Yang, B., Ruusunen, V., Laaksonen, O., Tahvonen, R., Hellsten, J., & Kallio, H. (2012). Compositional differences of phenolic compounds between black currant (*ribes nigrum* L.) cultivars and their response to latitude and weather conditions. *Journal of Agricultural and Food Chemistry, 60*(26), 6581-6593.

Zobayed, S., Afreen, F., & Kozai, T. (2005). Temperature stress can alter the photosynthetic efficiency and secondary metabolite concentrations in st. john's wort. *Plant Physiology and Biochemistry, 43*(10), 977-984.

Zoratti, L., Karppinen, K., Escobar, A. L., Häggman, H., & Jaakola, L. (2014). Light-controlled flavonoid biosynthesis in fruits. *Frontiers in Plant Science, 5, 534.*

Turun yliopisto
University of Turku