Phenolic Compounds and Ascorbic Acid in Black Currant (*Ribes nigrum* L.)

Variation due to Genotype, Ontogenetic Stage, Harvest Date and Location

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Phenolic compounds and ascorbic acid in black currant (*Ribes nigrum* L.) – Variation due to genotype, ontogenetic stage, harvest date and location

Abstract

Black currant is an edible soft fruit crop that is now attracting increased scientific attention due to its high content of potentially beneficial phenolic compounds and ascorbic acid. Using HPLC and spectrophotometry, this thesis examined the content of phenolic compounds in buds, leaves and fruits of black currant plants grown in southern and northern Sweden. In addition, the content of ascorbic acid, soluble solids, titratable acidity and total anthocyanins were studied in the fruits. Differences due to genotype, ontogenetic stage, harvest date and location were determined.

The genotypes 'Ben Finlay', 'Poesia' and 'JHI 8944-13' had the highest content of several compounds in both buds and fruits. Among the different bud ontogenetic stages, dormant buds had the highest content of total phenols. In the leaves, the content of phenolic compounds generally varied depending on the position of the leaf on the shoot and on harvest date. A higher content of total phenols was recorded late in the season, except in the basal leaves.

Black currant fruits grown in the south had higher contents of most phenolic compounds, ascorbic acid and soluble solids than those grown in northern Sweden. Buds picked from plants grown in the north had higher content of flavan-3-ols, phenolic acids and several flavonols than buds from the south.

In conclusion, proper selection of genotype and location for cultivation is essential for promoting the food and health attributes of black currant. Moreover, knowledge related to influence of ontogenetic stage and harvest time on content of specific bioactive compounds in black currant could help tailor functional foods or pharmaceutical products. Black currant production could thereby be carefully planned to enhance the content of specific compounds for product optimisation.

Keywords: anthocyanins, antioxidants, cultivar, extraction, flavonoids, growth stage, HPLC, polyphenols, vitamin C.

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Dedication

To my family

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **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.

II **Vagiri, M**., Ekholm, A., Öberg, E., Johansson, E., Andersson, S.C. & Rumpunen, K. (2013). Phenols and ascorbic acid in black currants (*Ribes nigrum* L.): variation due to genotype, location, and year. *Journal of Agricultural and Food Chemistry 61*(39), 9298-9306.

III **Vagiri, M**., Conner, S., Stewart, D., Andersson, S.C., Verrall, S., Johansson, E. & Rumpunen, K. (2014). Phenolic compounds in black currant (*Ribes nigrum* L.) leaves relative to leaf position and harvest date. *Food Chemistry 172*(2015), 135-142.

IV **Vagiri, M**., Ekholm, A., Johansson, E., Andersson, S.C. & Rumpunen, K. Major phenolic compounds in black currant (*Ribes nigrum* L.) buds. Variation due to genotype, ontogenetic stage and location. (Manuscript)

The contribution of Michael Rajeev Vagiri to the papers included in this thesis was as follows:

I Planned the experiment with supervisors, performed all field, experimental and practical work, data analysis and wrote the manuscript together with co-authors.

II Performed all experimental and practical work, data analysis and wrote the manuscript together with co-authors.

III Planned the experiment together with supervisors, performed all field and experimental work and data analysis and wrote the manuscript together with co-authors.

IV Planned the experiment together with supervisors, performed all field, experimental and practical work and data analysis and wrote the manuscript together with co-authors.

1 Introduction

Due to their nutritional composition and health benefits associated with their consumption, crops in the genus *Ribes* have been the subject of increasing scientific investigation in recent years. The genus includes more than 150 diploid species (Brennan, 1996). At present, 10-12 species of *Ribes* are propagated for fruit production, the majority of which are black (*Ribes nigrum* L.), red and white currant (*R. rubrum* L., synonyms *R. vulgare* Jancz. and *R. sativum* Syme.) and gooseberry (*e.g.* European gooseberry: *R. uva-crispa* L., synonym *R. grossularia* L., and American hairystem gooseberry: *Ribes hirtellum* Michx.) (Brennan, 2008). Black currant has the greatest economic importance in this genus and is cultivated in temperate Europe, North America, Asia and mountainous regions of South America, New Zealand and North Africa (Brennan, 1996; 2008). Total production of black currant fruit worldwide during 2013 was 192,405 tonnes, with 183,405 tonnes in the EU[1].

Black currant is mainly cultivated for juice and beverage production and for processing into jams, jellies, yoghurts, pureés, teas and functional food products, but to some extent is sold as fresh fruit (Hummer & Barney, 2002). In the UK, about 75% of the crop is contracted for juice production for a brand named 'Ribena™ '. The growing trend for consumer interest in black currant is attributed to the high content of ascorbic acid (vitamin C) and beneficial phenolic compounds such as anthocyanins, flavonols, flavan-3-ols and phenolic acids that can be found in the fruit, but also in buds and leaves (Tabart *et al*., 2011, 2006; Karjalainen *et al*., 2008; Anttonen & Karjalainen, 2006; Lister *et al*., 2002). Therefore black currant is regarded as a health-promoting, high value raw food material worldwide.

1. IBA, 2013 http:// www.internationalblackcurrantassociation.com accessed July 2014.

1.1 Taxonomy

The genus *Ribes* was originally considered to be a member of the *Saxifragaceae* family, but taxonomic studies have placed the genus in the *Grossulariaceae* family due to morphological characteristics such as inferior ovaries, syncarpous gymnosperm and fleshy fruit (Brennan, 1996; Cronquist, 1981).

Black currant is thus classified botanically as:
Kingdom − Plantae
Division − Magnoliophyta
Class – Magnoliopsida
Order – Saxifragales
Family – Grossulariaceae
Genus – *Ribes*
Sub-genus – Coreosma
Species – *Ribes nigrum* L.

1.2 Origin, history and cultivar development

The word 'currant' is derived from the ancient Greek word for the city Corinth, and was first used to describe grapes cultivated in that region. Earlier English references described the cultivated species of *Ribes* with word variations such as 'corinthes', 'corans', 'currants' and 'bastarde corinthes' (Hedrick, 1925). Black currants have been cultivated in northern Europe for about 400 years, and records were first found in the 17th century herbals, with *e.g.* Gerarde (1636) describing the use of black currant leaves and berries in tea and medical concoctions (Barney & Hummer, 2005). John Tradescant originally collected black currant plants from the wild in Holland and it was introduced into the UK in 1611. By 1800, the use of black currant as a hedge or home garden shrub had become common in the UK (Brennan, 1996).

The first cultivar descriptions date from 1826 and were made by the Royal Horticultural Society in the UK. The society recognised five black currant cultivars: 'Black Naples', 'Black Grape', 'Common Black', 'Wild Black' and 'Russian Grape'. By 1920, the number of cultivars of *Ribes nigrum* descent had increased to 26, solely due to the efforts of gardeners and common people (Hatton, 1920). During World War II, cultivation of black currant was promoted throughout Europe, where citrus fruits could not be grown or were difficult to import (Hummer & Barney, 2002). From that time on the number of cultivars steadily increased due to various breeding programmes by

professional plant breeders at government-funded institutes and commercial companies (Brennan, 2008).

In the UK, the cultivar 'Baldwin', which is of unknown origin but known to be 150 years old, dominated crop production from the late 19th century until the late 20th century. Valued for its rich flavour, 'Baldwin' is still grown today, albeit on a very small scale. With the development of the 'Ben' series of late-flowering frost-tolerant cultivars (*e.g.* cv. 'Ben Lomond') at the James Hutton Institute (formerly Scottish Crop Research Institute), the problems with frost injury at flowering were overcome. The `Ben' series has produced further releases of commercial significance, for instance 'Ben Gairn' and 'Ben Finlay', which are considered to be resistant to black currant reversion virus (reversion disease) and black currant gall mite (*Cecidophyopsis ribis* Westw.), respectively. The 'Ben' series of cultivars now occupies over 95% of the cropping area in the UK and around 50% worldwide (Brennan, 2008).

In Russia, black currant breeders used European subspecies with the goal of developing cultivars with winter hardiness, high yield, large fruit size and high content of ascorbic acid. Later, with increasing incidence of gall mite, reversion disease and anthracnose (*Drepanopeziza ribis* (Kleb.) Höhnel), wild germplasm of *R. nigrum* var. *sibiricum* (gall mite resistant), *R. pauciflorum* and *R. dikuscha* was used in the crosses, leading to cultivar releases such as 'Primoskij Cempion' (Brennan, 2008).

In the USA, black currant was probably introduced along with red currant in the 17th century, but initially received little attention in terms of domestication and cultivation (Barney & Hummer, 2005). Subsequent breeding programmes in Canada led to the development of rust-resistant cultivars, namely 'Consort', 'Crusader' and 'Coronet', by crossing wild germplasm of *R. ussuriense* with the cultivar 'Kerry' (Hummer & Barney, 2002).

In Sweden, the first cultivar trial started in 1940, at 12 different locations. The most prominent local varieties developed at the Öjebyn research station in northern Sweden were 'Haparanda', 'Sunderbyn', 'Öjebyn' and 'Östersund' (Hjalmarsson & Wallace, 2007). Breeding programmes at Balsgård in southern Sweden during the 1960s resulted in cultivars such as 'Stella I', 'Stella II', 'Stellina', 'Stor-klas', 'Polar' and 'Intercontinental', with the focus on resistance to fungal disease, hardiness, yield and berry size (Trajkovski, 1992, 1986). Other cultivars of commercial importance, such as 'Titania' and 'Triton', were developed at Tollarp in southern Sweden.

1.3 Biology

Black currant, like all other *Ribes* species, is normally diploid and has a chromosome number of 16 (2n=2x=16), and natural polyploids are rarely seen (Brennan, 1996). The chromosomes are relatively small (1.5-2.5 µm), with the mitotic and meiotic process being regular (Zielinski, 1953). The plant is an unarmed, aromatic, deciduous shrub that can grow up to 2 m tall (Figure 1). It starts yielding fruit after about 2-3 years.

Figure 1. (Left) Black currant bush and (right) black currant fruit. Photo: M. Vagiri.

The growth habit varies, with upright, semi-upright or spreading forms (Figure 2). Shoot type varies depending on genotype. In some cultivars, shoots are straight, firm, long and thick at the base, a little pubescent and prominently brown in colour, while others are straight or rather short, firm and grey or buff-brown in colour (Wassenaar & Hofman, 1966). Buds are strongly aromatic and can vary from short and thick to long and slender, fusiform, ovoid or conical in shape with a few brown scales, greenish, reddish or yellowish in colour. The buds turn intensely reddish as the winter progresses. The leaves are strongly perfumed, pale green, simple, alternately paired, broad, 3-5 cm long, palmate with five lobes and glabrous upper, slightly pubescent with many sessile and aromatic glands on the lower leaf surface.

Figure 2. (Left) Black currant buds and (right) a spreading black currant bush. Photo: M. Vagiri.

The flowers are pinkish green to reddish in colour, with curved sepals and white petals, and the racemes (strigs) hang down and bear about 10 flowers. Flowering takes place during the spring. In extreme northern latitudes with long day length, flowering commences in late June/July, and can last up to 3-4 weeks depending on the cultivar, location and climate conditions. Floral initiation in black currant is induced by external factors such as photoperiod and temperature (Wright, 1985). For floral initiation, a day length of approx. 16 hours is prescribed for the crop, but is known to vary between different cultivars (Heide & Sønsteby, 2011). Cross-pollination can play an important role in achieving optimum yield, because some black currant cultivars are partly self-sterile (Denisow, 2003). Insects such as bumble bees are important agents for pollination. The fruit (Figure 1) is an edible berry, about 1 cm or more in diameter, purple or shiny black in colour when ripe and containing a large number of seeds. Green and yellow fruiting cultivars also exist (Hummer & Barney, 2002). The fruits begin to ripen 70-100 days after bloom (Brennan, 1996).

1.4 Breeding objectives

In general, the objectives of early black currant breeding programmes were to develop cultivars with good physical characteristics, such as upright and vigorous growth habit and long strigs, high yield, ease of mechanical harvesting and strong pest and disease resistance. However, in recent years there have been significant changes in black currant breeding. These changes are closely aligned to the needs of the processing sector, mainly for juice production, and encompass traits relating to flavour and colour of the fruit and the content of compounds providing potential health benefits (Brennan & Graham, 2009). Modern breeding is therefore focusing on satisfying the demands of the processing industry, leading to the development of cultivars

bearing fruits rich in ascorbic acid, balanced sugar to acid ratio, good flavour and improved sensory characteristics (Brennan & Gordon, 2001). In addition, there is an increasing preference for cultivars with enhanced levels of phenolic compounds, including flavonoids such as anthocyanins and flavonols, due to the high antioxidant activity of these compounds (Brennan & Graham, 2009; Lister *et al*., 2002). The content of phenolic compounds and of sugars, organic acids and ascorbic acid is influenced by genotype, latitude, environment and agronomic practices (Krüger *et al*., 2012; Zheng *et al*., 2012, 2009; Tabart *et al*., 2006; Anttonen & Karjalainen, 2006). Since black currant is commonly grown under contract for juice production, it is essential that the quality and sensory components of the fruits remain predictable and, if possible, constant. Hence, breeding efforts now focus on testing different cultivars/genotypes over different locations for more than one year to minimise the influence of environmental factors on the phytochemical content.

However, the above-mentioned quality traits do not supersede agronomic traits such as spring frost resistance, late flowering, high yield, pest and disease resistance, which have long formed the basis of breeding objectives (Brennan & Gordon, 2001). All traits are important in light of *e.g*. increasing concerns and preferences among consumers and processors about residue-free fruits and stringent legislation (Mitchell *et al*., 2011). Therefore there is an important move toward integrated crop management that limits the use of chemical control in black currant (Brennan & Graham, 2009). In organic cultivation of black currant in particular, the need for resistant cultivars to pests and diseases is paramount and the main objective of the Swedish breeding programme for black currant is to develop better cultivars for organic growing. Besides the most serious pests of black currant, *i.e*. gall mite and reversion virus, resistance to foliar pathogens such as mildew (*Sphaerotheca mors-uvae* (Schwein.) Berk & Curtis), septoria leaf spot (*Mycosphaerella ribis* (Fuckel) Lindau), anthracnose and rust fungus *(Cronatium ribicola* Fisch.) is still the focus of many breeding programmes. In recent years, production of 'dessert' quality black currants for the fresh market has been steadily increasing, so breeding programmes are also focusing on producing varieties with large, firm fruit with dry pick characteristics and a palatable and sweet taste.

At present, breeding programmes are established in a few northern European countries, *e.g*. Scotland, Poland, Lithuania, Latvia, Estonia, Sweden. New Zealand also has a large breeding programme. Breeding of black currant is also being undertaken in *e.g*. Ukraine, Russia and China, but little information is available on that work.

1.5 Crop uses

1.5.1 Buds

Black currant buds contain remarkable amounts of essential oils rich in aroma volatile compounds. These volatile compounds are mainly hydrocarbons and oxygenated fractions of terpenes (Dvaranauskaite *et al*., 2009). The essential oils isolated from the dormant buds release a strong terpenic aroma, which is overwhelmed by a 'catty' note (Piry *et al*., 1995; Le Quere & Latrasse, 1990). Therefore, black currant buds are used as aroma enhancers in cosmetics, as well as ingredients for fragrance (Castillo del Ruiz & Dobson, 2002; Piry *et al*., 1995). The buds can be processed and used as extracts and polyphenol supplements. Glycerinate extract from buds is commercially available as a food supplement (Tabart *et al*., 2011). Phytochemical studies have identified black currant buds as a possible source of total phenols and as possessing higher antioxidant capacity than any other part of the black currant plant (Tabart *et al*., 2011, 2007, 2006; Dvaranauskaite *et al*., 2008). The major phenolic compounds contained in the buds are myricetin, quercetin and kaempferol, and also flavan-3-ols and phenolic acids (Liu *et al*., 2014; Tabart *et al*., 2011, 2006).

Although the composition of phenolic compounds in black currant buds has been determined, there is limited information regarding the composition and variation in phenolic compounds among different genotypes and how cultivation location and ontogenetic stage influence the variation in these compounds. If buds are to be used for functional food products or extracts, choice of optimum stage for harvesting and a suitable location for cultivation is important in optimising the plant material for content of specific phenolic compounds.

1.5.2 Leaves

Black currant leaves have been used in herbal medicines since ancient times to treat rheumatism and inflammatory problems and for their diuretic properties (Declume, 1989). The anti-inflammatory and antioxidant effects of black currant leaves have been confirmed using *in vitro* and *in vivo* models (Garbacki *et al*., 2005). Recent research has also demonstrated that extracts of black currant leaves have significant anti-influenza activity for *in vitro* and *in vivo* cell cultures (Ehrhardt *et al*., 2013). The leaves are used for products such as tea and the '*louhisaari*' drink prepared from young leaves picked during early summer[2]. Black currant leaves when added to sweetened vodka make a

2. Food:http://www.food.com/recipe/louhisaari-black-currant-drink-120759 accessed July 2014.

greenish beverage with a sharp flavour and tangy taste[3]. Studies on black currant leaves show a five-fold higher content of total phenols than in fruits or other black currant parts (Tabart *et al*., 2006). The phenolic profile of black currant leaves includes flavonoids such as kaempferol, quercetin, myricetin and phenolic acids (Liu *et al*., 2014; Raudsepp *et al*., 2010). The leaves could therefore be of interest for industrial applications in health and functional foods, especially if plants could be bred for high levels of phenolic compounds.

Therefore, information regarding the accumulation of phenolic compounds in black currant leaves is required. Furthermore, if black currant leaves are to be used in diverse food supplements, knowledge about the variation and composition of phenolic compounds in leaves harvested from different positions of the shoot on different occasions is essential for optimisation of plant material.

1.5.3 Fruits

Industrially, the fruits of black currant are considered a natural high value raw material and a source of many essential nutritional components. The fruits are characterised by good organoleptic properties, *i.e*. rich flavour, taste and intense colour, and are therefore of use in diverse food applications. The processed products include jams, jellies, purees, pie fillings, ice creams, flavoured waters, sweets, toppings for desserts and perfumes (Hummer & Dale, 2010). In various countries, black currant fruits are used for the production of *liqueur de cassis* or *crème de cassis* and for converting white wine to rosé (Brennan, 1996), as well as for strong alcoholic drinks (40%) such as 'Absolut' vodka and black currant schnapps. The fruits are also eaten fresh and this market is steadily increasing with the development of dessert-quality fruits with high soluble solid content (Pluta *et al*., 2012).

Furthermore, the seed oils from black currant fruits are widely used as a health supplement due to their high content of γ-linoleic acid (GLA) together with other nutritionally important fatty acids (Dobson *et al*., 2012). Pomace (press residue), a by-product during juice production, can also be used as a raw material for functional food because of its nutritional properties (Sójka & Król, 2009). The extracts prepared from black currant pomace have been found to contain high levels of phenolic compounds, especially anthocyanins (Sójka *et al*., 2009).

Scientifically, the fruits of black currant are well known to be a good source of ascorbic acid, which is one of the major driving forces in marketing of black currants (Brennan & Graham, 2009). They also contain high levels of phenolic

3. Incredible edibles: http://www.edible.co.nz/fruits.php?fruitid=22 accessed July 2014.

compounds, including flavonoids such as anthocyanins, flavonols, phenolic acids, flavan-3-ols, and tannins, with health-promoting properties (Gopalan *et al*., 2012; Borges *et al*., 2009; Karjalainen *et al*., 2008; Anttonen & Karjalainen, 2006). In addition, the aroma profile of black currant juice contains more than 120 volatile compounds such as terpenes, esters and alcohols (Varming *et al*., 2004). Compared with other fruits, black currents are low in calories and sodium and have moderate levels of vitamin A (carotenoids), vitamin B1 (thiamin), vitamin B3 (niacin), vitamin E (tocochromanols), iron, potassium and calcium (Hummer & Barney, 2002).

1.6 Bioactive compounds

1.6.1 Vitamin C (ascorbic acid)

Vitamin C is an important essential nutrient for normal metabolic functions in humans. Vitamin C can be found in its reduced form, ascorbic acid, as well as its oxidised form, dehydroascorbic acid (DHA) (Linster & Van Schaftingen, 2007). In many fruit crops, DHA is of minor importance and normally comprises 5-10% of the total vitamin C content (Lee & Kader, 2000). However, the content of DHA tends to increase during storage and other post-harvest conditions (Buescher *et al*., 1999).

Ascorbic acid, also known as ascorbate, is the most abundant water-soluble antioxidant in plant tissues (Walker *et al*., 2006). Humans cannot synthesise vitamin C due to lack of enzyme L-gluconolactone oxidase, but the vitamin is easily absorbed by the body (Davey *et al*., 2000). The deficiency of vitamin C in humans is associated with the clinical syndrome scurvy, a disease characterised by dryness of the skin, bleeding and swollen gums, fatigue and impairment of wound healing capacity (Naidu, 2003).

In plants, biosynthesis of ascorbic acid takes place via L-galactose (Wheeler *et al*., 1998) and D-galacturonic acid (Agius *et al*., 2003). The ascorbic acid content in commonly grown black currant cultivars varies from between 130-200 mg/100mL juice to over 350-450 mg/100mL juice in some breeding lines (Brennan & Graham, 2009). Studies on biosynthesis of ascorbic acid in black currants have demonstrated that accumulation occurs during early stages of fruit development via the L-galactose pathway (Hancock *et al*., 2007).

For extraction of ascorbic acid from plant tissues, pure water or acid solutions such as metaphosphoric acid (MPA) or oxalic acid, alone or in combination with other acids/organic solvents, can be used (Campos *et al*., 2009). However, in many laboratories MPA is used due to its capacity to

rapidly precipitate proteins and reduce the pH of the matrix, promoting the stability of AsA (Walker *et al*., 2006).

1.6.2 Phenolic compounds

Phenolic compounds are secondary plant metabolites and constitute one of the most widely distributed groups of natural products in plants. Phenolic compounds possess an aromatic ring having one or more hydroxyl substituents (Pereira *et al*., 2009). They are needed for normal growth and development of the plant and also protect it against adverse factors such as drought, UV radiation, infection or physical damage. Scientific research has shown that phenolic compounds are bioactive components with health-promoting benefits against degenerative diseases such as cancer, cardiovascular disease, immune system disease and brain function (Rodriguez-Mateos *et al*., 2014; Ferreyra *et al*., 2012; Karjalainen *et al*., 2008; Scalbert *et al*., 2005). Phenolic compounds are formed from a common intermediate, phenylalanine (PAL), or its precursor shikimic acid (Tsao, 2010). In berry fruits, the main classes of phenolic compounds are phenolic acids, flavonoids, stilbenes, tannins and lignans (Paredes-López *et al*., 2010). The classification is illustrated in Figure 3.

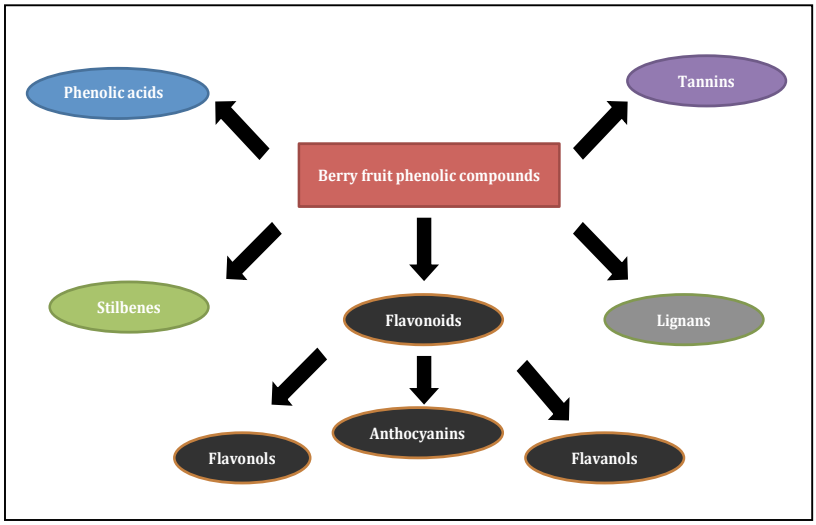


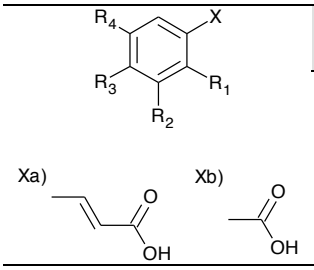
Figure 3. Classification of phenolic compounds in berry fruits (image modified from Paredes-López *et al*., 2010).

The most commonly used solvents for extraction of phenolic compounds from plant material are alcohols (methanol, ethanol), acetone, diethyl ether and

ethyl acetate (Khoddami *et al*., 2013; Stalikas, 2007). The most convenient and frequently used technique for separation, identification and quantification of phenolic compounds is high performance liquid chromatography (HPLC) coupled to a diode array detector (DAD). Electrospray ionisation (ESI) and tandem mass spectrometry (MS^n) provide molecular mass information and structural details of the phenolic compounds (Gavrilova *et al*., 2011). Based on polarity, various classes of phenolic compounds can generally be determined by reverse phase (RP) columns (Khoddami *et al*., 2013).

Phenolic acids

Phenolic acids are non-flavonoid phenols that are saturated to carboxylic acid. They contain two distinguishing carbon frameworks: the hydroxycinnamic acid (Xa) and the hydroxybenzoic acid (Xb) structures (Robbins, 2003) (Figure 4).



Compound	R_1	R_2	R_3	R_4	X
p-coumaric acid	H	H	OH	H	a
Ferulic acid	H	OCH_3	OH	H	a
Caffeic acid	H	OH	OH	H	a
Vanillic acid	H	OCH_3	OH	H	b
Protocatechuic acid	H	OH	OH	H	b

Figure 4. Structure of some of the prominently occurring phenolic acids (images modified from Robbins, 2003).

The common hydroxybenzoic acids are p-hydroxybenzoic, protocatechuic, vanillic, gallic, ellagic and syringic acids, and these acids are present in fruits such as strawberries, raspberries and blackberries (Häkkinen *et al*., 1999). The corresponding hydroxycinnamic acids are p-coumaric, caffeic, ferulic and sinapic acids, which are present in nearly all plants (Tsao, 2010). The highest content of hydroxycinnamic acids, predominantly caffeic acid, is found in fruits such as blueberries, cherries, apples and plums (Mattila *et al*., 2006). The most common esters of hydroxycinnamic acids are chlorogenic acid (5-*O*-caffeoylquinic acid) derivatives (esters of caffeic and quinic acids) (Robbins, 2003) (Figure 5).

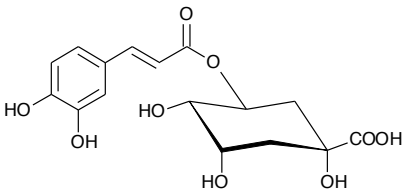


Figure 5. Structure of chlorogenic acid (image modified from Robbins, 2003).

The other forms include neo chlorogenic acid (3-*O*-caffeoylquinic acid) and cryptochlorogenic acid (4-*O*-caffeoylquinic acid). In black currants, the average phenolic acid content is reported to 23 mg/100 g fresh fruit, consisting predominantly of p-coumaric acid and caffeic acid (Mattila *et al*., 2006; Häkkinen *et al*.,1999).

Flavonoids

Flavonoids constitute one of the most abundant and diverse groups of phenolic compounds in plants, with over 4000 compounds. They are a class of phenylpropanoids and a group of low molecular weight compounds (Heim *et al*., 2002). Flavonoids are synthesised from PAL and are products of the shikimic acid pathway (Ferreyra *et al*., 2012). Their biosynthesis involves the formation of PAL from phenylpyruvate. The PAL is transformed to an activated form of cinnamic acid (4-coumarol-CoA), which is then hydrolysed to p-coumaric acid. The p-coumaric acid condenses with malonyl-coA units to form chalcone. Subsequent hydration and ring exposure give rise to compounds such as catechins (3-hydroxyflavonoids) and flavonols (3,4-diolflavonoids) (Aherne & O'Brien, 2002).

The basic flavonoid skeleton is made from two aromatic rings (A- and B-rings) connected by a three-carbon (C-ring) bridge (1,3-diphenylpropanone), usually in the configuration of a heterocyclic C ring (Figure 6). Flavonoids are classified according to substitution patterns of ring C into different subclasses, *e.g*. anthocyanins, flavonols and flavan-3-ols (Heim *et al*., 2002). Due to their different substitutions to ring A and B, there are different compounds within each subclass (Ignat *et al*., 2011).

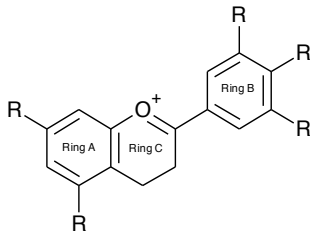


Figure 6. Flavonoid skeleton (image modified from Heim *et al*., 2002).

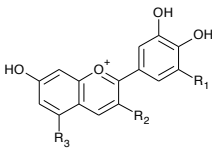
Flavonoids are referred to as glycosides when they are bound to one or more sugar groups (or glucosides in the case of a glucose moiety), and as aglycone when no sugar group is present (de Rijke *et al*., 2006). Flavonoids are localised in outer and aerial tissues of the plant (*e.g*. leaves) and their biosynthesis is known to be stimulated by light (Jaakola *et al*., 2004).

Flavonoid subclasses

Anthocyanins

Anthocyanins are the most commonly occurring natural water-soluble pigments. They are responsible for imparting colour to flower petals, fruits, vegetables and other plant parts. In addition, anthocyanins play an important role in attracting animals to carry out pollination and dispersal of seeds in plants. In fruits, anthocyanins are mainly found in the external layers of the hypodermis (peel) (Szajdek & Borowska, 2008).

The colour of anthocyanins is dependent on the pH of the solution, *i.e*. red in acidic conditions and blue in basic conditions (Giusti & Wrolstad, 2001). However, other factors, *e.g*. storage temperature, concentration, light, oxygen, presence of enzymes and metallic ions, can also affect the colour of anthocyanins (Castañeda-Ovando *et al*., 2009). In recent years, the stability of anthocyanins has been the main focus of analysis, mainly due to their potential applications as physiological function foods, their health benefits and use as natural colourants (Bakowska-Barczak & Kolodziejczyk, 2011; Castañeda-Ovando *et al*., 2009).



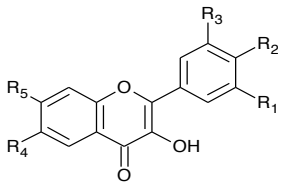
Compound	R_1	R_2	R_3
Cyanidin-3-*O*-glucoside	H	*O*-glucose	OH
Cyanidin-3-*O*-rutinoside	H	*O*-rutinose	OH
Delphinidin-3-*O*-glucoside	OH	*O*-glucose	OH
Delphinidin-3-*O*-rutinoside	OH	*O*-rutinose	OH

Figure 7. Structure of four major anthocyanins in black currants (image modified from Nielsen *et al*., 2003).

The four major anthocyanins in black currants are cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside and delphinidin-3-*O*-rutinoside (Figure 7). The relative proportions of these compounds vary between different genotypes (Rumpunen *et al*., 2011; Anttonen & Karjalainen, 2006; Nielsen *et al*., 2003; Slimestad & Solheim, 2002). Anthocyanins are the most important phenolic compounds in black currants, constituting 80% of all flavonoids present and with an average content of approximately 250 mg/100 g in fresh fruits (Sójka *et al*., 2009). Cultivars with enhanced levels of anthocyanins are in demand from the processing sector due to high antioxidant activity and potential health benefits (Gopalan *et al*., 2012; Brennan & Graham, 2009; Lister *et al*., 2002).

Flavonols

Another main class of flavonoids present in black currants is the flavonols. Flavonols derive from the simple flavonol 3-hydroxyflavone (Perez-Vizcaino & Duarte, 2010). The most common flavonols found in plant foods, including black currants, are quercetin, myricetin, kaempferol and isorhamnetin (Figure 8), and their glycosides. Flavonols occur as glycosides with mono-, di-, tri- and tetrasaccharides of glucose, galactose, rhamnose, arabinose, xylose and glucuronic acid (Hollman & Arts, 2000).



Compound	R_1	R_2	R_3	R_4	R_5
Quercetin	OH	OH	H	OH	OH
Kaempferol	H	OH	H	OH	OH
Myricetin	OH	OH	OH	OH	OH
Isorhamnetin	OCH_3	OH	H	OH	OH

Figure 8. Four major flavonols in black currants (image modified from Perez-Vizcaino & Duarte, 2010).

The main sources of flavonols in are onions, curly kale, broccoli, teas, berries and apples (Scalbert *et al*., 2005). Berries such as bilberries, blueberries and black currants are good sources of flavonols, especially quercetin and myricetin (Borges *et al*., 2009; Määttä-Riihinen *et al*., 2004; Häkkinen *et al*., 1999).

Flavanols

Flavanols or flavan-3-ols occur in monomeric form as catechins or in polymerised form as proanthocyanidins. Flavan-3-ols have a C6-C3-C6 flavonoid skeleton. Flavanols are generally found in their non-glycosylated form, unlike anthocyanidins or flavonols (Aron & Kennedy, 2008).

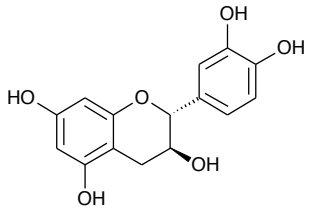


Figure 9. Structure of catechin (image modified from Hollman & Arts, 2000).

The most abundant flavan-3-ols are catechin (Figure 9), epicatechin, epigallocatechin and their galloyl-substituted derivatives (Tsao, 2010). Flavan-3-ols are present in many types of foods, *e.g*. grapes and red wine, but chocolates and green teas are considered to be the richest sources (Gu *et al*., 2004). Proanthocyanidins, traditionally known as condensed tannins, are oligomers or polymers of flavanols, the prodelphinidins and procyanidins. The common flava-3-ols present in different parts of black currant plants are epigallocatechin, gallocatechin, catechin, epicatechin and epigallocatechin gallate (Tabart *et al*., 2011). Substantial amounts of proanthocyanidins are also found in black currants (Wu *et al*., 2004).

1.7 Fruit quality

Fruit quality includes a range of physiological traits, *e.g*. berry size, colour, firmness, shelf life in the case of fresh fruit and characters associated with chemical composition, such as sweetness, sourness and content of nutritionally important compounds (Brennan & Graham, 2009). Besides bioactive compounds, the contents of soluble solids, sugars and organic acids are equally important. The composition and content of sugars and acids is known to affect the organoleptic characteristics of the fruit (Mikulic−Petkovsek *et al*., 2012).

The ratio of sugars and organic acids is an important indicator of perceived taste, maturity/ripeness and general quality, which may serve as an index of consumer acceptance (Bordonaba & Terry, 2008). Fruits with a pleasant taste are normally considered to have a high content of sugars and relatively low levels of organic acids (Zheng *et al*., 2009). Organic acids are important drivers for sensory properties of black currants such as flavour, mouth feel and tart taste. Organic acids are present in high levels in black currants and maintain the quality during processing and regulate pH within the fruit. The major organic acids in black currants include citric acid, with minor contents of succinic, malic, oxalic and quinic acids. Sugars impart sweetness to the juice, thereby affecting its palatability. In black currants, glucose and fructose are the dominant sugars, along with small traces of sucrose (Mikulic-Petkovsek *et al*., 2012). In addition to sugars and organic acids, certain flavonols and phenolic acids have been reported to contribute to the astringency and puckering taste in black currants (Laaksonen *et al*., 2013). Black currants can be prone to consumer rejection if they are too acidic and astringent in taste. Therefore selection of genotypes with enhanced levels of sugars and low content of organic acids is essential.

1.8 Health-promoting properties of black currants

A growing body of epidemiological and scientific studies suggests that increased intake of fruits, berries and vegetables is associated with a lower risk of cardiovascular events and other degenerative symptoms (Soto-Vaca *et al*., 2012; Perez-Vizcaino & Duarte, 2010; Battino *et al*., 2009). Moreover, recent studies suggest that consumption of berry fruits rich in phytochemicals (*i.e*. phenolic compounds) helps prevent certain cancers and other chronic diseases (Folmer *et al*., 2014; McDougall & Stewart, 2012; Paredes-López *et al*., 2010). Phytochemicals such as flavonoids and other phenolics possessing antioxidant activity are considered to account for the beneficial effects of a diet containing berries (Nile & Park, 2014; Del Rio *et al*., 2013; Scalbert *et al*., 2005). Similarly, flavonoids and other phenolic compounds are considered to be the major bioactive compounds contributing to the health-promoting effects of black currants (Gopalan *et al*., 2012).

Some of the key beneficial effects of black currants are presented in this thesis. In studies using different *in vitro* and animal models, black currant fruit phenolic extracts are reported to have *e.g*. antioxidant, anti-inflammatory, vasomodulatory, anti-haemostatic and muscle-relaxing effects, improvement of visual function and neuroprotective and anticancer activities (Jia *et al*., 2012;

Karjalainen *et al*., 2008; Ghosh *et al*., 2006; Matsumoto *et al*., 2005a; Lister *et al*., 2002). Furthermore, research has demonstrated that extracts of black currant leaves have significant antioxidant and anti-inflammatory activities and may possess anti-influenza virus activity (Ehrhardt *et al*., 2013; Tabart *et al*., 2012; Garbacki *et al*., 2005). In addition, essential oils of black currant buds have been shown to exert anti-microbial activity against infectious bacteria and therefore can be used in the prevention and as an alternative remedy for treatment of infections (Oprea *et al*., 2008). In a recent study, a supplement of polyphenol-rich black currant extract significantly decreased obesity-induced inflammation in rats (Benn *et al*., 2014).

In human studies, oral administration of black currant anthocyanins has been found to bring about a reduction in dark adaptation threshold and subjective symptoms of visual fatigue in healthy subjects (Nakaishi *et al*., 2000). In other studies, intake of black currant extract improved blood flow to shoulder muscles during typing work, thereby reducing shoulder stiffness and muscle fatigue (Matsumoto *et al*., 2005b). In another study, intake of black currant juice in healthy women subjects induced peripheral vasodilatation and led to increased blood flow and decreased blood pressure (Yonei *et al*., 2009). Supplementation with black currant anthocyanins in 120 dyslipedmic subjects increased high-density lipoprotein-cholesterol (HDL-C) by 14% and decreased low-density lipoprotein cholesterol (LDL-C) by 14%, with no changes in total serum cholesterol levels (Qin *et al*., 2009). Thus it is not just the fruits, but also other plant tissues of black currant, *e.g*. buds and leaves, that possess health-promoting properties.

Several clinical studies have shown considerable improvement in cardiovascular risks such as endothelia function, arterial stiffness and blood lipids after consumption of blueberries, strawberries, cranberries or phenolic extracts from other fruits or vegetables in healthy or diseased individuals (Macready *et al*., 2014; Rodriguez-Mateos *et al*., 2014, 2013). However, these effects have not yet been proven for black currants.

1.9 Variation in content of bioactive compounds

The content and composition of different bioactive compounds in plants are certainly not constant and are influenced by several factors such as genotype, environmental conditions and ontogenetic stage, but also by year, harvest date and growing season. Furthermore, the degree of ripeness, post-harvest handling (*e.g*. storage conditions), extraction procedure and analytical method affect the content of bioactive compounds.

1.9.1 Genetic variation

The content and composition of bioactive compounds in plants vary between species and cultivars, with some compounds being found specifically in certain species and genotypes. The content of bioactive compounds in black currant is reported to be more or less influenced by genotype (Kaldmaee *et al*., 2013; Krüger *et al*., 2012; Zheng *et al*., 2012; Brennan & Graham, 2009; Mikkonen *et al*., 2001). For instance, the content of ascorbic acid in ripe fruits is highly variable among commonly grown cultivars, ranging from 130-200 mg/100 mL juice to even higher levels (400 mg/100 mL juice) in some breeding lines (Brennan & Graham, 2009). It has been shown that the accumulation of ascorbic acid is highest during the fruit expansion phase and that genotypic differences persist in later ripening stages (Hancock *et al*., 2007). Furthermore, a strong maternal influence on the heritability of ascorbic acid has been reported, but there are also significant variations caused by environmental factors (Brennan, 2008). It has been shown that although the content of ascorbic acid may vary from year to year due to environmental influences, the cultivar rankings remain stable (Walker *et al*., 2010). The contents of individual sugars and organic acids, along with soluble solids and titratable acidity, largely depend on genotype, but a strong correlation with temperature has also been observed during ripening (Kaldmaee *et al*., 2013; Zheng *et al*., 2009).

There is also a rich genetic diversity in content of phenolic compounds. For example, a 2.1-fold and 3.4-fold variation in content of total phenols and anthocyanins, respectively, and even a 4.4-fold variation in ascorbic acid have been reported among black currant genotypes (Krüger *et al*., 2012; Moyer *et al*., 2002). Similarly, the flavonol content varies and a three-fold variation has been found in the content of myricetin and kaempferol among black currant germplasm (Mikkonen *et al*., 2001). The composition and content of monomeric anthocyanins and other major phenolic compounds is reported to be significantly affected by genotype (Zheng *et al*., 2012; Bordonaba & Terry, 2008). In terms of monomeric anthocyanins, the Western European black currant cultivars contain higher levels of cyanidin derivatives, while delphinidin derivatives have been found to be high in Scandinavian cultivars (Karjalainen *et al*., 2008). Genetic variation in the content of flavonol glycosides and other phenolic compounds in buds and leaves of black currant during the growing season has also been reported recently (Liu *et al*., 2014).

Thus, the high variability in content of different bioactive compounds (*e.g*. ascorbic acid and polyphenols) and sensory characters (*e.g*. sugars and acids) in different genotypes offers possible avenues for selection of suitable cultivars

for production of various black currant products with health-promoting properties.

1.9.2 Environmental conditions

Environmental conditions generally have a strong influence on the content of bioactive compounds in plants (Hewett, 2006). Cultivation location (latitude) is often associated with changes in environmental conditions such as day length, light intensity, water stress, chilling requirements during winter and temperature (Tiwari & Cummins, 2013; Jaakola *et al*., 2004; Lee & Kader, 2000). In addition, a wide range of other factors change with location/latitude, *e.g*. precipitation, humidity, solar radiation, snow cover and soil type (Jaakola & Hohtola, 2010). Similarly, the reported effect of growing season as well as monthly and between-year differences on the content of bioactive compounds is most likely due to environmental conditions (Remberg *et al*., 2012; Hewett, 2006). Other possible within-year activities such as cultivation practices (*e.g*. pruning), relative amount of soil nutrients and degree of pests and diseases can vary markedly, affecting the content of bioactive compounds (Anttonen & Karjalainen, 2008; Wang, 2006). Growing season temperature and light are known to influence the photochemical content in plants (Wang, 2006). Jaakola *et al*. (2004) reported a higher content and increased expression of flavonoid pathway genes in apical leaves exposed to direct sunlight than basal leaves on the same branch in blueberries. This could be an effect of light, temperature or a combination of both. In a study on black currant, plants growing on south-facing slopes receiving much sunlight contained 20% more ascorbic acid than plants grown on north-facing slopes at the same location (Walker *et al*., 2010). Although the content of bioactive compounds is genetically determined, it can be affected by environmental conditions (*e.g*. light, photoperiod, temperature *etc*.) and genotype x environment interactions (Tiwari & Cummins, 2013). In a study conducted on blueberries, much greater variation in total phenols, anthocyanins, flavonols and hydroxycinnamic acid content was reported between genotypes than between years (Howard *et al*., 2003). In the same study, however, significant genotype x environment interactions demonstrated that certain genotypes vary in their capacity to synthesise phenolic compounds under different growing conditions.

For black currant, the influence of location, environmental condition and yearly variation on the content of ascorbic acid, phenols and other compounds has been described previously (Kaldmaee *et al*., 2013; Krüger *et al*., 2012; Walker *et al*., 2010; Zheng *et al*., 2009). Compositional differences due to seasonal variation have also been investigated for different parts (buds, leaves and fruits) of the black currant plant (Liu *et al*., 2014; Tabart *et al*., 2006). The

results indicate a significant influence of environmental conditions on the levels of bioactive compounds. However, further research on the effect of location and year on bioactive compounds is needed. Such research could identify potential growing sites promoting high levels of a desired bioactive compound that are least influenced by environmental conditions.

1.9.3 Ontogenetic and harvesting factors

Physiological maturity of both fruits and vegetables can greatly influence the content of bioactive compounds in different plant parts. The variation in content of bioactive compounds at physiological maturity depends on the biosynthesis of the compounds during plant growth and development during maturity (Pantelidis *et al*., 2007). This variation is evident when flavonoids or carotenoids provide colour to the fruit during the ripening process (Kalt, 2005). Maturity stage of the fruit has been reported to influence the content of total phenols and anthocyanins in blackberry, raspberry and strawberry cultivars (Wang & Lin, 2000). According to Wang & Lin (2000), the content of total phenols increases in black and red raspberry from the pink to the ripe stage, but for strawberry and blackberry, less ripe fruit contains higher contents of total phenols than fully ripe fruit. Those authors also showed that maturity of leaves has an effect on phenol content, with young leaves containing higher levels of total phenols than mature leaves. Furthermore, decreasing levels of phenolic compounds during leaf ontogeny have been reported in *e.g*. tea and pear (Andreotti *et al*., 2006; Lin *et al*., 1996). In black currant, Tabart *et al*. (2006) reported that buds harvested in March (fully opened) and leaves harvested in June had a higher content of total phenols than fully ripe fruits (July). Similarly, in black currant leaves, Nour *et al*. (2014) reported a high total phenol content in mid-June, followed by a considerable decrease until early August. Cosmulescu & Trandafir (2011) have shown that in walnut leaves, the content of total phenols increases during the month of June and July and then decreases in August, whereas in a study conducted on apple leaves, the highest content of total phenols was found in August (Mikulic-Petkovsek *et al*., 2009). Therefore pre-harvest factors such as ontogeny and harvest date may play an important role for the content of bioactive compounds. This underlines the need to investigate the content of a specific compound and determine the most suitable harvest occasion, thereby optimising plant material aimed at functional food and pharmaceutical products.

2 Objectives

The main aim of this thesis was to determine the major phenolic compounds in buds, leaves and fruits of black currant (*Ribes nigrum* L.) and the content of ascorbic acid, titratable acidity, soluble solids and total anthocyanins in the fruit. A further aim was to optimise extraction methods for phenolic compounds and their chromatographic separation and identification.

Specific objectives were to:

- Develop an optimised method for extraction, identification and analysis of phenolic compounds in buds, leaves and fruits by HPLC-DAD and HPLC-ESI-MS^n (Paper I)
- Investigate the role of genotype, location and year on the content of ascorbic acid, single phenolic compounds, total phenols, total anthocyanins, soluble solids and titratable acidity in black currant fruits (Paper II)
- Investigate the content of phenolic compounds in black currant leaves collected from different positions of the shoot during the growing season (Paper III)
- Investigate the role of genotype, ontogenetic stage, cultivation location and season on the content of phenolic compounds in black currant buds (Paper IV).

The hypotheses tested in Papers II-IV were as follows:

- The content of phenolic compounds, ascorbic acid and other compounds in fruits is higher in black currant plants grown at northerly latitudes than in plants grown further south (Paper II).

- The content of single and total phenolic compounds in leaves varies with harvest date and leaf position on the shoot (Paper III).

- Buds have a higher content of phenolic compounds when dormant and the content declines during onset of burst. In addition, buds picked from plants grown further south have a lower content than buds picked from plants grown at northerly latitudes (Paper IV).

3 Materials and methods

This section of the thesis gives an overview of the plant material and methodological details used for the chemical analyses of bioactive compounds (Papers I-IV).

3.1 Plant material

Black currant genotypes 'BRi 9508-3A' and 'BRi 9508-3B' were used for Papers I and III, respectively. For Papers II and IV, the black currant genotypes used in the experiments were two advanced selections 'JHI 8944-13', 'BRi 9504-2-227' and the three cultivars 'Ben Finlay', 'Poesia', and 'Titania' (Figures 10 and 11). A description of all genotypes is given in Table 1.

Table 1. *Selected black currant genotypes used in Papers I-IV with their parentage and description*

Genotypes	Parentage	Description
'BRi 9508-3A'	'Gagatai' x 'Intercontinental'	Selection made at SLU Balsgård from a joint Swedish-Latvian-Lithuanian breeding programme. Early ripening, large berries, rich flavour, low acidity, low content of ascorbic acid, low content of anthocyanins, healthy foliage but with minor susceptibility to powdery mildew, low susceptibility to gall mite. Suitable for hand harvesting.
'BRi 9508-3B'	'Gagatai' x 'Intercontinental'	Selection made at SLU Balsgård Sweden from a joint Swedish-Latvian-Lithuanian breeding programme. Early ripening, large berries, low content of ascorbic acid, high content of anthocyanins, healthy foliage. Suitable for hand harvesting and possibly also mechanical harvesting.
'BRi 9504-2-227'	65-59-5 x 'Storklas'	Selection made at SLU Balsgård Sweden from a joint Swedish-Latvian-Lithuanian breeding programme. High yielding, large berries, distinct flavour, high acid content, average ascorbic acid, healthy looking leaves, Suitable for hand harvesting and possibly also mechanical harvesting. Low susceptibility to gall mite.
'JHI 8944-13'	'S13-2-97' x 'S10-2-78'	Selection from JHI. Early-mid season ripening, high yielding, dense foliage, small berries, high ascorbic acid, medium-low chilling requirement, gall mite resistant.
'Ben Finlay'	'S13-14-9' x 'B1834-67'	Scottish cultivar. Early-mid season ripening, high yielding, vigorous growth habitat, dense foliage, small berries, very high ascorbic acid, medium-low chilling requirement, gall mite resistant.
'Poesia' ('Poezia')	'Minaj Shmyriov' x 'Öjebyn'	Russian cultivar. Medium-sized berries, mid-season ripening, good taste, high anthocyanins and average ascorbic acid, gall mite resistant, leaf spot susceptible.
'Titania'	'Altajskaja Desertnaja' x ('Consort' x 'Kajaanin Musta')	Swedish cultivar. Mid-season ripening, high yielding, large berries, below average ascorbic acid, resistant to mildew, leaf spot and rust, susceptible to gall mite.

‘BRi 9058-3A’

‘BRi 9504-2-227’

‘JHI 8944-13’

‘Poesia’

‘Ben Finlay’

‘Titania’

Figure 10. Black currant genotypes used for the experiments in Papers II and IV. Photo: M. Vagiri.

Figure 11. Representative fruits of (left to right) the genotypes 'Ben Finlay', 'JHI 8944-13', 'BRi 9504-22-7', 'Poesia' and 'Titania'. Photo: M. Vagiri.

3.2 Growing location

Two comparative organic trials were established in 2006 at Öjebyn (Piteå) in northern Sweden (65°21'N, 21°23'E) and Balsgård (Kristianstad) in southern Sweden (56°06'N, 14°10'E) (Figure 12). The distance between the two sites is 1,102 km.

Figure 12. (Left) Map of Sweden showing the two cultivation locations (image from Google maps, accessed July 2014) and (right) the black currant plantations at Öjebyn (upper image) and Balsgård (lower image). Photo: M. Vagiri.

In general, the Öjebyn site is characterised by a subarctic climate, with severe winters, no dry season and cool, short summers with very long day length during the growing season (Figure 13). In contrast, the Balsgård site has a mild climate with a dry spring season, usually warm summers, winter precipitation falling as rain and a maximum day length during the growing season of 17 hours (Figure 13).

At both sites, one-year-old black currant plants were planted in the field in a randomised block design consisting of five blocks (rows) with one plant per genotype (replicates) planted in these rows. The planting distance was 4 m between rows and 2 m between plants. The general fertilisation and cultivation regimes are described in detail in Paper II.

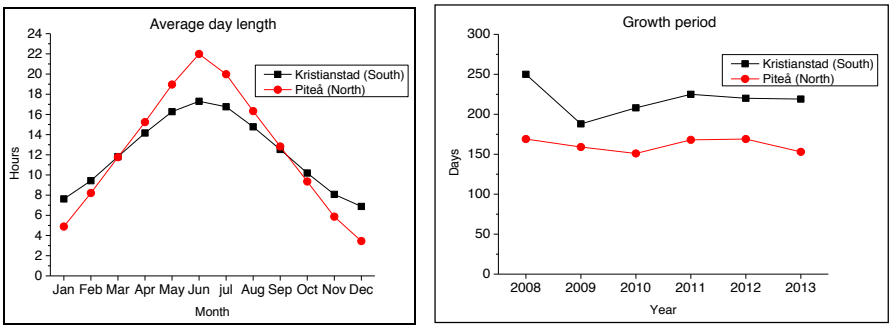


Figure 13. (Left) Average day length in different months and (right) growing period length for the period 2008-2013 at the Öjebyn site in northern Sweden (red circles) and the Balsgård site in southern Sweden (black squares). (Data from Swedish Meteorological and Hydrological Institute, SMHI.)

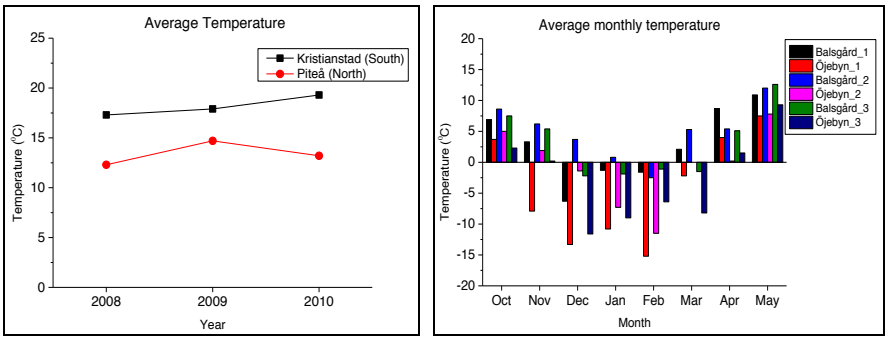


Figure 14. (Left) Mean temperature during the month of fruit harvesting (August in north, red circles; July in south, black squares) and (right) mean monthly temperature during bud harvesting in the north and south of Sweden (1 = 2010-2011; 2 = 2011-2012; 3 = 2012-2013). (Data from Swedish Meteorological and Hydrological Institute, SMHI.)

3.3 Harvesting

For the method optimisation study (Paper I), fruits, leaves and buds were harvested in July, September and October 2010, respectively, at Balsgård. For the ascorbic acid and phenolic compounds analyses (Paper II), fruits were sampled at full maturity based on organoleptic properties such as sweetness and mouth feel, in July at Balsgård and in August at Öjebyn, during the period 2008-2010 (Figure 15).

Figure 15. Fully ripe black currant fruits harvested from different genotypes at Balsgård. Similar representative fruit sampling was carried out for genotypes grown at Öjebyn. Photo: M. Vagiri.

For the analyses of phenolic compounds in leaves (Paper III), five leaves each were taken from the apical (young), middle and basal (old) shoot positions. The leaves were harvested only at Balsgård, at five different dates during the growing season (6 June, 23 June, 26 July, 6 August and 26 August) in 2011.

For the analyses of phenolic compounds in buds (Paper IV), samples were picked when the buds were dormant, swollen and during onset of burst during three seasons (2010-2011 = Season 1; 2011-2012 = Season 2; 2012-2013 = Season 3) at both Balsgård and Öjebyn.

Sample collection (Papers I-IV) was carried out during daytime and the samples were stored at -20 °C. At the end of harvest, all samples were freeze-

dried for 1 week and then stored again at -20 °C until sample preparation and analyses.

3.4 Chemical analyses

Phenolic compounds in different parts of black currant plants were identified by reverse phase HPLC along with either a diode array detector (DAD) or electrospray ionisation mass spectrometry (ESI-MS). For identification of novel compounds, HPLC-ESI-MS^n was used (Paper I). Major phenolic compounds in black currant fruits and buds were analysed by HPLC-DAD in Papers II and IV, respectively. HPLC with ESI-MS^n detection was used to analyse phenolic compounds in leaves, with slight modifications to the HPLC method used in Paper III. All single phenolic compounds were quantified using external standards and results expressed as μg/g dry weight (DW) (Papers II-IV). Total phenols were measured by the Folin Ciocalteau method (Singleton *et al*., 1999) and all results in Papers I-IV were corrected for ascorbic acid interference. Total anthocyanin content was measured by the pH differential method (Giusti & Wrolstad, 2001) (Papers I-II). Soluble solids content in fruit samples was measured using a digital refractometer and titratable acidity using an automatic titrator (Paper II). Ascorbic acid was analysed using an isocratic HPLC method with an UV-Vis detector (Papers I-IV). The extraction procedures for each plant part are described in detail in Papers I-IV.

3.5 Statistics

General linear model (GLM) analysis, followed by Duncan's post hoc test, analysis of variance (ANOVA), regression analysis, mixed procedure and Pearson correlation coefficients, were performed using SAS (SAS Institute Inc., Cary, NC). One-way analysis of variance and principal component analysis (PCA) on the data were carried out using Minitab (State College, PA, USA) software. MS Excel and Origin (OriginLab, Northampton, MA) were used for bar and line charts.

4 Results and discussion

4.1 Optimisation of extraction procedure and HPLC method

One of the aims of this thesis was to identify the optimum extraction procedure for analyses of phenolic compounds, estimated as total phenols in black currant buds and leaves and as total anthocyanins in fruits. The optimum extraction procedure was determined through testing different concentrations of ethanol-water (30, 50, 70% v/v) (see Materials and Methods section in Paper I), a simple extraction method for analyses of phenolic compounds. High yields of phenols were obtained with solvent containing 50% ethanol-water mixture (v/v) with 0.05M H_3PO_4 in extracts of both buds and leaves following an extraction time of 15 min using an ultrasonic bath.

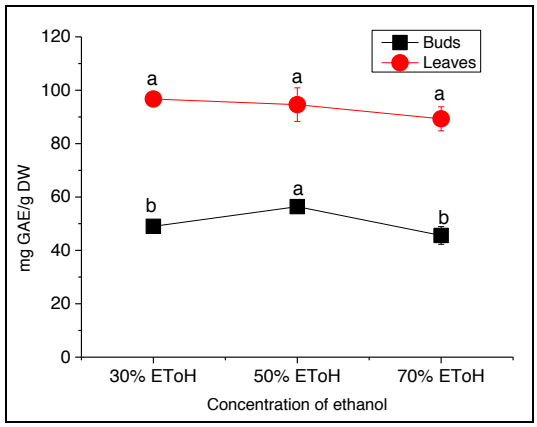


Figure 16. Total phenols (mg GAE/g DW) in leaves (red circles) and buds (black squares) of black currant obtained using different concentrations of EtOH/water (30, 50, 70% v/v) with 0.05 M H_3PO_4 as the solvent. Different letters (a,b) for the respective plant part indicate significant differences ($p<0.05$) .

It was observed that total phenols content was higher in the leaves than the buds, regardless of the concentration of ethanol used. The total phenols content of leaves ranged from 89-97 mg GAE/g DW, whereas that of buds ranged from 45-56 mg GAE/g DW (Figure 16). Similarly, Nour *et al*. (2014) obtained high levels of total phenols using 40% ethanol as the extraction solvent, whereas the use of 80% ethanol led to significantly lower yield of total phenols in black currant leaves. In another study, aqueous acetone was found to be more effective than methanol and water in extracting phenols from black currant buds and leaves (Tabart *et al*., 2006). However, as there is a risk associated with methanol or acetone toxicity, the use of water or ethanol is preferred in the functional food and health industry. Moreover, for extraction of phenolic compounds from foods, ethanol and water mixtures are commonly preferred, sometimes in combination with weak or strong acids (Rusak *et al*., 2008; Naczk & Shahidi, 2004). This is due to the wide array of phenolic compounds that an ethanol-water mixture (moderate polar solvent) can dissolve and extract. Similarly, in our studies, 50% ethanol in water (v/v) was preferred as an extraction solvent as it was able to extract both polar (water-soluble) and less polar phenolic compounds at the same time.

Since the anthocyanins are highly unstable and susceptible to degradation depending on *e.g*. pH and storage temperature, their stability during storage at room temperature was tested for a period of 6 days using different extraction solvents (Figure 17). As can be seen from the results, major degradation of total anthocyanins occurred over time (Figure 17). It was found that 50% ethanol (EtOH) was the most unstable extraction solution, with a decrease of 15.7% and 33.7% at day 3 and 6, respectively. When acidified methanol (MeOH) was used, there was significant decrease of 13.0% on day 3 and 22.3% on day 6, although significantly more total anthocyanins could be extracted compared with the other solvents. Total anthocyanins extracted with acetonitrile (ACN)/formic acid (5:10 v/v in water) seemed to be slightly more stable than those in other extraction solutions, with a decrease of 8.1% and 15.7% on day 3 and 6, respectively.

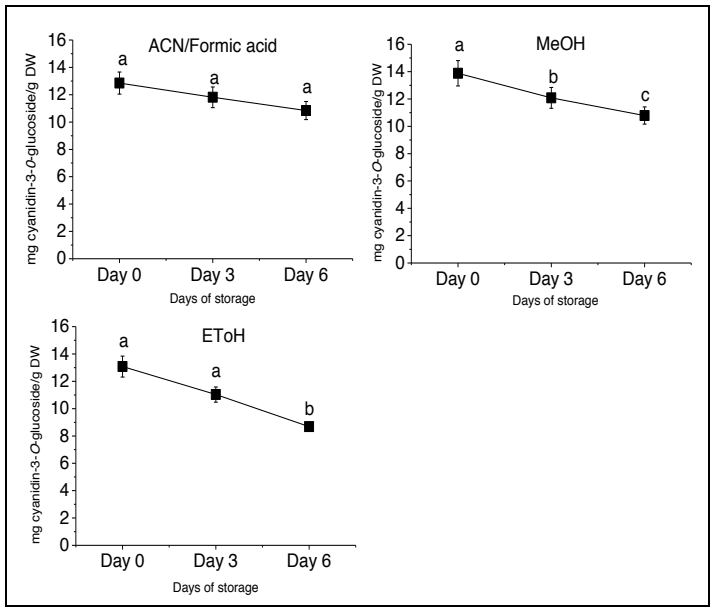


Figure 17. Content of total anthocyanins (mg cyanidin-3-*O*-glucoside/g DW) analysed after 0, 3 and 6 days in different fruit extracts stored at room temperature. Letters (a-c) indicate significant differences ($p<0.05$) for the specific extraction solvents within storage period.

The results showed that a 50% ethanol-water (v/v) mixture resulted in the highest yields among the different ethanol concentrations tested for extracting total phenols from buds and leaves. However, an acetonitrile/formic acid (5:10 v/v in water) mixture was the best in terms of stability for extraction of anthocyanins from fruits. Therefore, 50% ethanol-water (v/v) with 15 minutes extraction time using an ultrasonic bath was chosen for extraction of phenolic compounds from leaves (Papers I, III) and buds (Papers I, IV), whereas for fruits (Papers I, II) a solution of formic acid and acetonitrile (10:5 v/v) in water was used following an extraction time of 20 minutes.

A Synergie Hydro RP-80A column using a gradient of 7% formic acid in water (v/v) as mobile phase A and a mixture of acetonitrile-methanol-water (90:5:5 v/v) as mobile phase B was chosen for the HPLC analyses in Paper I. All compounds identified were successfully separated with good resolution (Figures 5-7 in Paper I). The HPLC-DAD method developed in Paper I was chosen for use in Papers II and IV. A Thermo Scientific Hypersil gold column with mobile phases 0.4% formic acid in water (A) and 90:5:5 (v/v) acetonitrile-methanol-water (B) was chosen for HPLC-ESI-MS^n analyses in Paper III, because it was a shorter method than that used in Papers II and IV, while still providing reliable results.

4.2 Bioactive compounds in buds, leaves and fruits

A total of 23 kinds of phenolic compounds were identified in buds and 22 in leaves and fruits in Paper I. Only major phenolic compounds were selected for quantification using HPLC-DAD in Papers II and IV, because some of the compounds were below the limit of reliable detection and hence could not be quantified. However, for Paper III, since the analysis of leaves was performed on an HPLC-ESI-MS^n instrument, all 13 phenolic compounds identified (Figure 2 in Paper III) were quantified. A summary of all the phenolic compounds within each class identified in buds, leaves and fruits is given in Table 1 in Paper I.

In brief, two phenolic acids, neo chlorogenic acid and chlorogenic acid, were identified in the buds, leaves and fruits of black currant. Both these compounds have been reported previously in black currant fruits and leaves (Oszmiański *et al*., 2011; Raudsepp *et al*., 2010; Anttonen & Karjalainen, 2006), but Paper I is the first study to identify these compounds in black currant buds (at 320 nm; Figure 5 in Paper I). In the case of flavan-3-ols, epigallocatechin, catechin and epicatechin have been reported previously in black currant (Rumpunen *et al*., 2011; Tabart *et al*., 2011; Määttä *et al*., 2003). In the present work, all these three flavan-3-ols were detected in the buds, leaves and fruits of black currant, at 280 nm (Figures 5, 6 and 7, respectively, in Paper I). Four major anthocyanins, delphindin-3-*O*-glucoside, delphindin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside, were detected in buds, leaves and fruits, at 520 nm (Figures 5, 6, and 7, respectively, in Paper I). All anthocyanins identified are in agreement with earlier findings (Gavrilova *et al*., 2011; Anttonen & Karjalainen, 2006; Slimestad & Solheim, 2002). Twelve flavonol glycosides (*i.e*. myricetin, quercetin, kaempferol and isorhamnetin conjugates) were identified in the buds, leaves and fruits at 360 nm (Figures 5, 6 and 7, respectively, in Paper I). Of these, kaempferol rutinoside was identified for the first time in black currant leaves. However, this compound has been identified previously in the leaves of *e.g*. strawberry and raspberry (Oszmiański *et al*., 2011; Gudej & Tomczyk, 2004). In addition, quercetin was detected only in buds. One peak (Peak 10) remained unidentified but was found in all three plant parts analysed. The flavonols detected in this study confirm previous findings (Oszmiański *et al*., 2011; Raudsepp *et al*., 2010; Anttonen & Karjalainen, 2006; Määttä *et al*., 2003).

All phenolic compounds were identified based on their UV-vis spectra (260-550 nm) using available literature and retention times relative to available external standards, as well as through peak spiking whenever possible to support the identification.

In fruits (Paper II), delphindin-3-*O*-rutinoside and cyanidin-3-*O*-rutinoside dominated among the anthocyanins studied. Among the flavonols, quercetin glucoside was the most abundant, along with quercetin rutinoside, whereas isorhamnetin glucoside was present in the lowest amounts. Rubinskiene *et al*. (2006) observed a similar difference in terms of anthocyanin composition, with cyanidin-3-*O*-rutinoside and delphinidin-3-*O*-rutinoside being present in the highest concentrations and cyanidin-3-*O*-glucoside in the lowest concentrations among the genotypes studied.

In leaves (Paper III) and buds (Paper IV), quercetin malonylglucoside was the most abundant flavonol glycoside. Interestingly, epigallocatechin was among the compounds present in the smallest amount in leaves, but was present at the highest concentrations of all the investigated compounds in buds. Among the phenolic acids detected, chlorogenic acid was the most abundant in both fruits and buds, while neo chlorogenic acid was the most abundant in leaves. There were also significant correlations between certain compounds (Papers II and III).

4.3 Influence of various sources on variation in bioactive compounds

This thesis represents a first attempt to evaluate the relative influence of different sources (genotype, ontogenetic stage, location and season) on the content of bioactive compounds in the fruits (Paper II) and buds of black currant (Paper IV), using R^2 values from regression analyses.

In fruits, a combination of genotype, location and years explained 7.0->70% of the variation (Figure 18). Genotype was the most important source of variation in several of the phenolic compounds analysed (Figure 18), whereas year was the most important source explaining variation in delphindin-3-*O*-glucoside (18.2%), delphindin-3-*O*-rutinoside (4.5%) and total anthocyanins (13.5%). Location was the most influential source of variation for myricetin malonylglucoside (9.0%), quercetin glucoside (5.0%) and quercetin malonyl glucoside (18.5%). The sources studied explained >65% of the variation in total phenol content, with genotype explained the highest proportion of variation (60.4%). Similarly, a high proportion of variation from the sources studied was observed for ascorbic acid (71.2%), soluble solids (57.5%) and titratable acidity (9.8%), with genotype being the most important contributing factor (Figure 18).

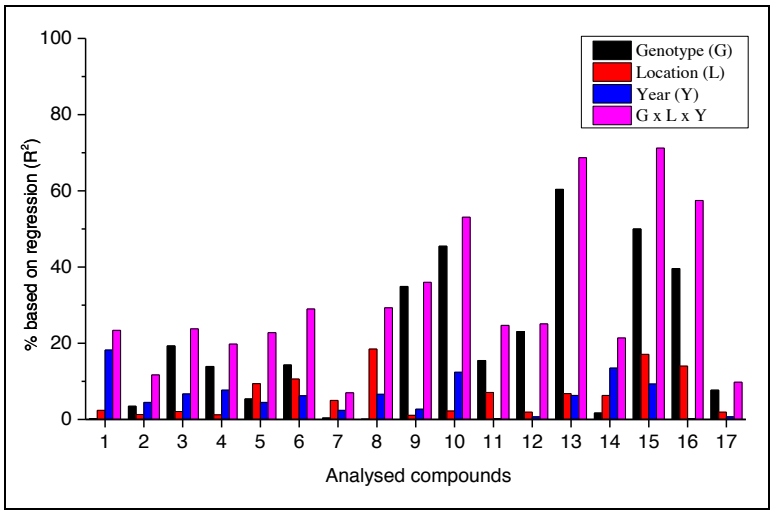


Figure 18. Relative amounts of variance (%, based on R^2 values from regression analyses) explained by the different sources studied for compounds analysed in fruits. Codes: 1 = delphindin-3-*O*-glucoside; 2 = delphinidin-3-*O*-rutinoside; 3 = cyanidin-3-*O*-glucoside; 4 = cyanidin-3-*O*-rutinoside; 5 = myricetin malonylglucoside; 6 = quercetin rutinoside; 7 = quercetin glucoside; 8 = quercetin malonylglucoside; 9 = qaempferol glucoside; 10 = isorhamnetin glucoside; 11 = neo chlorogenic acid; 12 = chlorogenic acid; 13 = total phenols; 14 = total anthocyanins; 15 = ascorbic acid; 16 = soluble solids; 17 = titratable acidity.

For the buds, a combination of different sources (genotype, ontogenetic stage, location and season) in analyses performed for two locations and two seasons explained 0.0-50.9% of the variation for the phenolic compounds analysed (Figure 19). Although the degree of variation for quercetin rutinoside (9.34%) and isorhamnetin glucoside (8.29%) was low, genotype was the most influencing factor (Figure 19). Location was the most influencing source for catechin (33.1%), chlorogenic acid (23.0%) and kaempferol malonylglucoside (20.8%). The variation in epigallocatechin was influenced by both ontogenetic stage and season to the highest extent (>26%), whereas ontogenetic stage was the source of highest influence for content of epicatechin (28.7%) and total phenols (19.4%). Season was the most important factor for myricetin malonylglucoside, quercetin galactoside, quercetin glucoside, quercetin malonylglucoside and kaempferol glucoside, explaining 12.2-50.9% of the variation in concentrations (Figure 19).

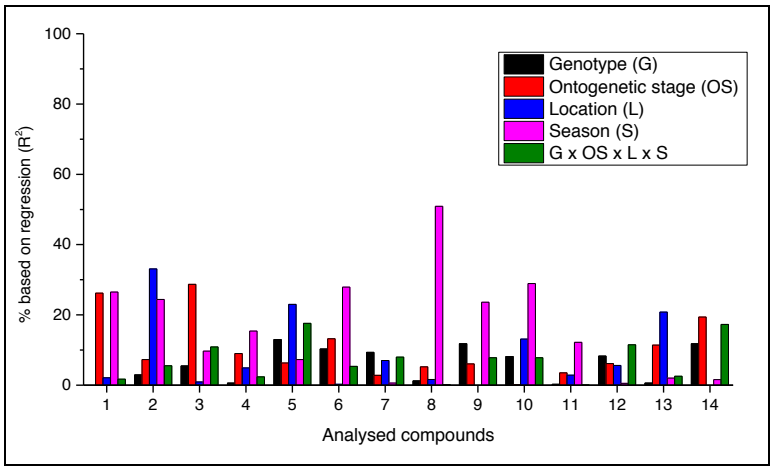


Figure 15. Relative amounts of variance (%, based on R^2 values from regression analyses) explained by the different sources studied for the compounds analysed in buds over two seasons. Codes: 1 = epigallo catechin; 2 = catechin; 3 = epicatechin; 4 = neo chlorogenic acid; 5 = chlorogenic acid; 6 = myricetin malonylglucoside; 7 = quercetin rutinoside; 8 = quercetin galactoside; 9 = quercetin glucoside; 10 = quercetin malonylglucoside; 11 = kaempferol glucoside; 12 = isorhamnetin glucoside; 13 = kaempferol malonylglucoside; 14 = total phenols.

In leaves (Appendix 1 in Paper III), the influence of leaf position and harvest date was calculated using F values from mixed model analyses. The position of leaves on the shoots influenced the content of a number of compounds, such as myricetin malonylglucoside, myricetin malonylglucoside isomer, quercetin rutinoside, quercetin glucoside, quercetin malonylglucoside, kaempferol glucoside, kaempferol malonylglucoside and total phenols. Harvest date was also found to significantly influence the content of all compounds analysed.

4.4 Genotypic variation

The content of various compounds varied significantly between the genotypes. In Paper II (see Table 2 in that paper), high content of ascorbic acid, total phenols, total anthocyanins and titratable acidity, along with low levels of soluble solids in fruits, differentiated 'Ben Finlay' from the other genotypes. A two-fold difference was observed between the genotypes with the highest and lowest content of ascorbic acid. Previous studies have shown even four- and six-fold differences in ascorbic acid content (Viola *et al*., 2010). The content of

total phenols and total anthocyanins is partly linked to berry size and thickness of peel (Moyer *et al*., 2002). Large berry size implies less accumulation of total phenols and the thicker the peel, the more anthocyanins. Since berry size is a genotype-specific trait, the abundant amounts of total anthocyanins and total phenols observed in 'Ben Finlay' might be due to the small berry size and thicker peel seen in this cultivar. Moderate levels of total phenols and total anthocyanins were found in selection 'BRi 9054-2-227', which might be due to this genotype having large berries (Figure 11) and thin peel. The genotypes 'Poesia' and 'JHI 8944-13' contained abundant amounts of several of the compounds investigated and thereby differed from the other genotypes. The cultivar 'Titania' had lower levels of several compounds, including total phenol and ascorbic acid content, than the other four genotypes studied.

Similarly, in buds a high content of total phenols distinguished 'Ben Finlay' from the other four genotypes, and the lowest content was observed for 'Titania' (Table 3 in Paper IV). The cultivar 'Poesia' contained abundant levels of all flavonols analysed except isorhamnetin glucoside. Intriguingly, 'Poesia' was characterised by absence of quercetin galactoside. Liu *et al*. (2014) also reported the absence of this compound in some cultivars ('Morti' and 'Jalaste no. 15'). This implies that the presence/absence of the compound quercetin galactoside is genotype-specific.

For fruits and buds, principal component analysis (PCA) was applied to visualise the underlying source of genetic variation in Paper II and IV, respectively. Three components explained 69.1% of the total variation for the fruit (Paper II), with PC1 and PC2 explaining 34.8% and 20.7% of the variation, respectively (Figure 2a in Paper II). From a score plot, it was evident that biochemical content of the samples depended on genotype, location and year (Figure 2a in Paper II). Furthermore, the genotypes were grouped in more or less compact clusters, which were distinct from one another. For the cultivar 'Ben Finlay', a clear separation in the samples collected from north and south in 2009 and 2010 was observed. Samples of 'BRi 9504-2-227' clustered tightly irrespective of year and location, indicating a strong genotypic effect on the content of metabolites. The selection 'JHI 8944-13' clustered with a yearly effect when grown in the north in 2008 and 2010. A north-south location effect was also observed for 'Poesia', while the cultivar 'Titania' exhibited a complex pattern driven by different years regardless of the location.

For the buds (Paper IV), PCA was performed for samples from north and south separately, as location effects were obvious. Three components explaining 65.1% of the total variation were obtained from PCA of bud samples grown in three successive seasons in the south. PC1 and PC2 explained 33.2% and 18.6% of the data, respectively (Figure 2a in Paper IV).

Two major clusters were observed, with the first cluster consisting of the genotypes 'Ben Finlay', 'Titania', 'JHI 8944-13' and 'BRi 9504-2-227', and the second cluster consisting solely of 'Poesia'. Surprisingly, the same clustering was observed for buds of 'Poesia' for both seasons and ontogenetic stages, which could be attributed to a higher content of multiple phenolic compounds (Figure 2a and 2b in Paper IV). A strong ontogenetic effect regardless of genotype and season, with a clear separation between dormancy and bud burst stages, was observed for the remaining genotypes. Furthermore, an effect of season was observed at the swollen bud stage across all genotypes.

In PCA analysis of bud samples from the north, three components explained 79.3% of the total variation in samples harvested over two seasons. PC1 and PC2 explained 47.0 and 18.5% of the variation, respectively (Figure 3a in Paper IV). It was observed that the samples from the north differed in clustering pattern to those from the south. From the score plot (Figure 3a in Paper IV), it was evident that genotype did not have a particularly strong effect on the clustering of different genotypes based on the content of different phenolic compounds. However, a strong ontogenetic and seasonal effect on the content of phenolic compounds was observed across all genotypes.

4.5 Variation due to cultivation location

Black currant fruits (Table 3 in Paper II) grown in the south had higher contents of sum of monomeric anthocyanins, sum of flavonols, total phenols, total anthocyanins, ascorbic acid and soluble solids compared with those grown in the north of Sweden. Thus our hypothesis of higher content of these compounds in samples collected from plants grown in the north was rejected. However, in the case of phenolic acids, a higher content was found in fruits grown in the north. The opposite was found in a study in Finland, where fruits grown at higher latitudes contained significantly less anthocyanins and flavonols and a higher content of phenolic acids, ascorbic acid and soluble solids (Zheng *et al*., 2012, 2009). An explanation might be differences in the climate conditions in southern Sweden and southern Finland, but the genetic background of the plant material, the maturity of fruits at harvest and soil nutrients might also be part of the explanation.

This thesis presents the first study to analyse phenolic compounds in black currant buds sampled at different growing locations (Paper IV). The sum of flavan-3-ols, sum of phenolic acids and sum of flavonols were higher in the samples collected from the north than in those from the south, corroborating our initial hypothesis. However, some individual compounds differed in content between locations, *e.g*. epigallocatechin, myricetin malonylglucoside

etc. were higher in buds from the south than in those from the north (Table 5 in Paper IV).

In black currant fruits, weather variables such as temperature and radiation have previously been reported to influence the content of specific compounds such as delpinidin-3-*O*-glucoside, delphindin-3-*O*-rutinoside and myricetin glucoside (Zheng *et al*., 2012). Those authors also reported that the content of delphindin glycosides increased as temperature and radiation increased, whereas the cyanidin-3-*O*-glucoside did not show a consistent change. Likewise, in this thesis the content of delphindin-3-*O*-glucoside was higher in the south, which had higher temperatures during the month of harvest (Figure 14). In strawberries, higher temperatures have been shown to increase the content of flavonols and anthocyanins (Wang & Zheng, 2001). Solar radiation during the growing season was found to increase the content of ascorbic acid in black currant fruits (Walker *et al*., 2010). Similarly, warm weather conditions are reported to increase the content of soluble solids and accumulate less acid in black currant fruits (Kaldmaee *et al*., 2013).

In this thesis, the difference between locations in terms of anthocyanin sum, total anthocyanins, total phenols, ascorbic acid and soluble solids content in fruits could tentatively be explained by higher average temperatures recorded in the south during the month of harvest (Figure 14). In addition, it was observed for fruits that the composition of certain compounds, such as cyanidin-3-*O*-rutinoside and isorhamnetin glucoside, and the titratable acidity did not differ much between the locations. Likewise, in buds the content of total phenols did not vary between the locations. Therefore, the composition of these compounds seems to be rather stable regardless of growing location. Furthermore, it was observed from the PCA plots (Figures 2 and 3 in Paper IV) that the seasonal effect was more pronounced in the north, whereas genotypic and ontogenetic effects were more pronounced in the south for the phenolic compounds analysed.

The results suggest that southern Sweden is the best location for growing black currant fruits with a higher content of most phenolic compounds, ascorbic acid and soluble solids, whereas if a higher content of sum of flavan-3-ols, phenolic acids and flavonols in buds if desired, the north of Sweden could be chosen.

4.6 Ontogenetic variation

The content of different phenolic compounds in black currant buds (Table 4 in Paper IV) depended on the ontogenetic stage of bud development, confirming our initial hypothesis.

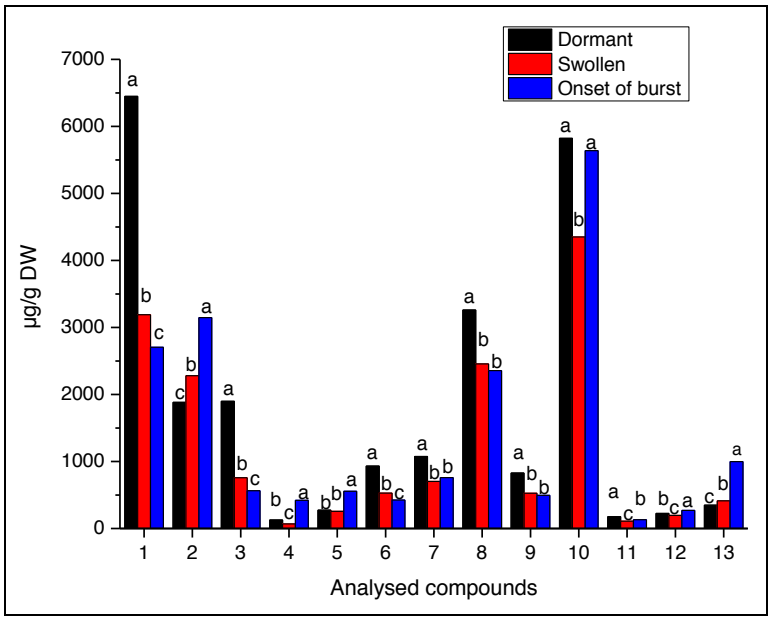


Figure 20. Means (averages of genotype, location and season) for the phenolic compounds analysed at different ontogenetic stages. Different letter/letters indicate significant differences ($p<0.05$) between the ontogenetic stages for each compound. Codes: 1 = epigallocatechin; 2 = catechin; 3 = epicatechin; 4 = neo chlorogenic acid; 5 = chlorogenic acid; 6 = myricetin malonylglucoside; 7 = quercetin rutinoside; 8 = quercetin galactoside; 9 = quercetin glucoside; 10 = quercetin malonylglucoside; 11 = kaempferol glucoside; 12 = isorhamnetin glucoside; 13 = kaempferol malonylglucoside.

The flavan-3-ols (epigallocatechin and epicatechin) decreased during bud development (Figure 20), and hence harvesting at bud dormancy could be recommended if high levels of these compounds are desired. In contrast to epigallocatechin and epicatechin, the content of catechin increased during bud development. Both phenolic acids (neo chlorogenic acid and chlorogenic acid) showed their highest content at bud burst, although the content of neo chlorogenic acid dropped at bud swell. The content of the flavonols myricetin malonylglucoside, quercetin rutinoside, quercetin galactoside, quercetin glucoside and kaempferol glucoside and total phenols decreased from bud dormancy to bud burst (Figure 20). On the other hand, quercetin

malonylglucoside remained at similar levels, with a decline at bud swell, and quercetin malonylglucoside, isorhamnetin glucoside and kaempferol malonylglucoside increased from dormancy to bud burst (Figure 20).

4.7 Variation in relation to leaf position and harvest date

The content of specific phenolic compounds in black currant leaves of the genotype 'BRi 9508-3B' (Paper III) was rather stable at different harvest dates and leaf positions for some compounds, but varied among harvest dates and leaf positions for other compounds, with some significant interactions (Figure 21).

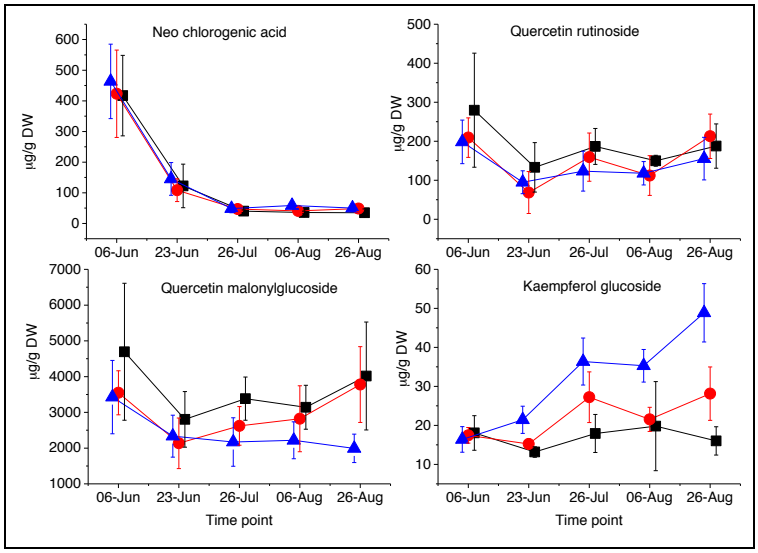


Figure 21. Content (µg/g DW) of neo chlorogenic acid, quercetin rutinoside, quercetin malonyl glucoside and kaempferol glucoside analysed from apical (■), middle (●) and basal (▲) leaves of the black currant genotype 'BRi 9508-3B' at different dates of harvest in 2011.

Two compounds, neo chlorogenic acid and epigallocatechin, exhibited their highest levels at the first harvest date (Figure 21), and therefore early harvest is recommended to obtain high contents of these contents. Many of the other compounds (*e.g.* quercetin rutinoside, quercetin malonylglucoside) were rather stable over harvest seasons and leaf position (except for basal leaves in quercetin malonylglucoside) (Figure 3 in Paper III) (Figure 21). Thus, harvest can be carried out with no specific attention to timing to obtain high levels of these compounds. Variation in environmental conditions, *e.g.* day and night

temperature and, intensity of radiation, could have influenced the results. Chlorogenic acid showed higher amounts during the first and last harvest dates. For kaempferol rutinoside, kaempferol malonylglucoside kaempferol malonylglucoside isomer, quercetin glucoside and kaempferol glucoside (Figure 21), leaf position in particular is worth considering, as the content was highest in the apical leaves for kaempferol rutinoside, kaempferol malonylglucoside and kaempferol malonylglucoside isomer and in the basal leaves for quercetin glucoside and kaempferol glucoside (Figure 3 in Paper III).

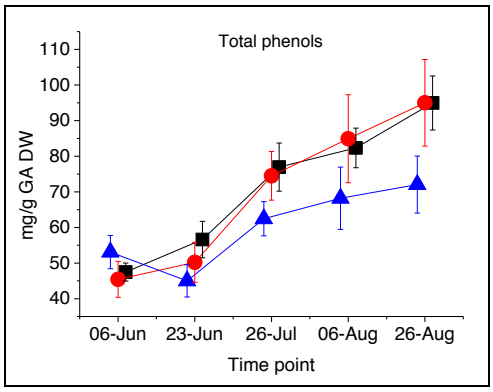


Figure 22. Total phenol content (mg/g GA DW) in apical (■), middle (●) and basal (▲) leaves of the black currant genotype 'BRi 9508-3B' at different dates of harvest in 2011.

Total phenols (except for basal leaves) exhibited a higher content late in the season (Figure 22). This contradicts results obtained by Tabart *et al*. (2006) and Nouir *et al*. (2014), who reported higher total phenol content in leaves picked during June. This difference in results may be explained by different genetic material being used and possibly the age of the plant material sampled.

4.8 Annual variation

The variation between years can be assumed to be an environmental effect if field management practices remain constant. The environmental factors responsible for annual variation include solar radiation, temperature, chilling requirements, day length, precipitation, snow cover and humidity. Other parameters besides annual variation that affect the content of bioactive compounds include soil fertility and irrigation, pruning activities and degree of foliar diseases. During this study, no extraordinary events occurred that could explain the variation between years, with the exception that cultivar 'Ben

Finlay' did not set sufficient fruits during 2008 (Paper II). In addition, bushes were not pruned during 2011, but pruning activities were performed in the remaining years (2012, 2013) for analyses of phenolic compounds in buds (Paper IV).

The fruits had the highest amounts of monomeric anthocyanins and flavonols in 2010 (Table 4 in Paper II). In 2009 and 2010, the contents of total phenols, total anthocyanins, soluble solids and ascorbic acid were significantly higher than in 2008. Sum of phenolic acids and neo chlorogenic acid were highest in 2009, whereas chlorogenic acid was higher in 2008 and 2010. The content of kaempferol glucoside was high in both 2008 and 2009. In 2008, all the above compounds except chlorogenic acid, quercetin glucoside and kaempferol glucoside displayed their lowest values. For titratable acidity, the yearly influences were not so pronounced, although a slight increase in content was observed in 2008.

Similarly, in buds there was a difference in the content of phenolic compounds obtained between years/seasons (Table 6 in Paper IV). In season 1 (2010-2011), the buds showed their highest content of flavan-3-ols and flavonols. Season 2 (2011-2012) yielded the highest content of total phenols and neo chlorogenic acid, but the content of chlorogenic acid was higher in season 1.

These annual variations are in agreement with previous studies on the content of bioactive compounds in black currants. For instance, in a three-year study (2003-2005), Krüger *et al*. (2012) found that the content of total phenols was higher in 2004 and 2005 and anthocyanin levels were lower in 2005. Kaldmaee *et al*. (2013) reported irregular variation in content of ascorbic acid, soluble solids and titratable acidity between 2006-2010 within each cultivar studied. Given that there was no major variation in environmental factors, this suggests that the content of bioactive compounds in black currant responds to changes in microclimate. Hence the plant material should be tested for a number of successive years to establish whether any annual variations observed are consistent. Additional studies on adaptability or stability assessments could be carried out to ascertain the levels of consistency in compound concentrations.

5 Conclusions and perspectives

The key findings of this thesis were that:

- Choice of extraction solvent is an important factor determining the degree of extraction and stability of phenolic compounds in black currants. The stability of total anthocyanins degrades during storage at room temperature and is an important factor to consider when analysing large numbers of samples.

- The fruits, buds and leaves of the black currant plant all contain beneficial phenolic compounds. Neo chlorogenic and chlorogenic acids were identified for the first time in black currant buds, while kaempferol rutinoside was identified for the first time in black currant leaves.

- Genetic background is an important factor determining the composition and content of bioactive compounds in black currant fruits and buds. There are differences between genotypes in terms of content of different compounds and adaptability to different locations, indicating that it is possible by breeding to maximise the content of bioactive compounds, leading to more health-promoting black currant material.

- The genotypes ‘Ben Finlay’, ‘JHI 8944-13’ and ‘Poesia’ have high concentrations of many beneficial phytochemicals in both buds and fruits. Although these genotypes are not sufficiently high-performing, they could be ideal for use as parents in breeding programmes for the development of cultivars with enhanced

phytochemical content suitable for Scandinavian growing conditions.

- Variation between years/seasons, leaf age and cultivation location can have an impact on the content of different phenolic compounds in black currant buds, leaves and fruits. Thus, better knowledge of the impact of these factors and of other cultivation parameters on content of different bioactive compounds is essential in breeding programmes, to minimise the influences of external factors such as weather conditions. Based on such knowledge, it would be possible to optimise the plant material in relation to different compounds for different purposes in the food and health industry.

- Black currant fruits grown at lower latitudes (southern Sweden) may have a higher content of ascorbic acid, soluble solids and certain bioactive phenolic compounds than those grown at higher latitudes (northern Sweden).

- Harvesting during the dormancy stage of buds is recommended in order to obtain a high content of total phenols.

- Black currant leaves should be harvested as young leaves if high contents of neo chlorogenic acid and epigallocatechin are desired. For chlorogenic acid, harvesting should preferably be carried out in the beginning and end of the growing season. Late harvesting is recommended if a high content of total phenols (with choice of apical and middle leaves) and kaempferol glucoside and quercetin glucoside (with choice of basal leaves) is desired. Differences between harvest dates are relatively small for quercetin malonyl glucoside, with apical leaves containing a higher content.

- There is a positive correlation between titratable acidity and soluble solids, implying that it could be difficult to lower the level of acids and still have a high content of sugars. However, the positive correlation between total anthocyanins, total phenols and ascorbic acid indicates that a high content of all these three traits is an achievable goal.

Scientific perspective

It would be interesting to:

- Study variation in fruit maturity stages, including gene expression associated with bioactive compounds. Such studies would provide insights into the developmental cues in black currant.

- Augment the findings of this thesis by investigating gene expression and biochemical pathways in buds, leaves and fruits to elucidate the reason behind the variations in different compounds.

- Examine how the content of different phenolic compounds, individual sugars and organic acids varies over different time spans, for example by harvesting fruits at different scales of ripeness during the harvest season.

- Determine a suitable marker for maturity of black currant fruits, for instance any phenolic compound, carotenoid or chlorophyll, to obtain the optimum content of desired compounds.

- Evaluate the effects of different fertilisation regimes and stress conditions on different quality parameters.

- Investigate the health effects of different fractions of specific phenolic compounds from buds, leaves and fruits of black currant on human health. This could involve administering extracts containing different concentrations of known compounds to human subjects and investigating the effects on *e.g.* arterial stiffness, blood coagulation, cholesterol levels, muscle relaxation effects, type-2 diabetes levels *etc*.

Applied perspectives

The results obtained in this thesis are of relevance for black currant growers and industry. The finding that not only black currant fruits, but also buds and leaves, contain beneficial phenolic compounds could be further exploited for the production of health-related products from the whole plant. The results show that it is relevant to consider the genotype, cultivation location, harvest date, bud development stage and leaf position in order to obtain the optimum content of desired bioactive compounds. In practice, cultivar choice will be

especially important, and it should be considered carefully whether early or late harvest is best to obtain high contents of desired phytochemicals. In addition, harvest technology and practice may determine whether it is possible to differentiate and thus harvest only leaves and buds with a high content of beneficial compounds. Moreover, higher yield at a late harvesting date may compensate for/counterbalance the lower content of specific phenolic compounds at the beginning of the growing season (if the compounds are to be extracted).

The results presented in this thesis are also of relevance for black currant breeders. The content of different compounds was shown to vary among cultivars, cultivation location and year/season with respect to fruits and the ontogenetic stage in buds. In addition, harvest date/season at different stages of leaf development influenced the content of phenolic compounds in leaves. Therefore, it may be relevant for breeders to test breeding material and new varieties for phenolic and ascorbic acid content together with key sensory traits at several geographical sites and during at least a few seasons before commercial release of new cultivars.

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