Berry press-residue
- a valuable source of polyphenols with potential health effects

Philosophiae Doctor (PhD) Thesis

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Ås, March 2014 Linda Holtung

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Abbreviations

ACE	angiotensin I-converting enzyme
ALAT	alanine transaminase
BP	blood pressure
BPV	blood pressure variance
BPEC	black currant press-residue extract concentrate
COX-2	cyclooxygenase-2
CVD	cardiovascular disease
DBP	diastolic blood pressure
DHAA	dehydroascorbic acid
FPG	formamidopyrimidine DNA glycosylase
GAE	gallic acid equivalents
H_2O_2	hydrogen peroxide
HCA	hydroxycinnamic
HDL	high-density lipoprotein
HPLC	high performance liquid chromatography
IFN	interferon

IL	interleukin
iNOS	inducible nitric oxide synthases
L-AA	L- ascorbic acid
LDL	low-density lipoprotein
LPS	lipopolysaccharide
NF-κB	nuclear factor kappa B
ROS	reactive oxygen species
SB	strand breaks
SBP	systolic blood pressure
TMA	total monomeric anthocyanin
TNF	tumor necrosis factor
TP	total phenolics
WHO	world health organization

1 Introduction

In recent years, the health benefits of fruit and vegetables have gained increased interest worldwide. Several epidemiological studies have shown an inverse association between the intake of fruit and vegetables and the risk of cancer,[1, 2] cardiovascular disease (CVD) [3-5], and diabetes [6, 7]. According to the world health organization (WHO) report 2002, nearly 5 % of deaths (2.7 million) were attributable to low fruit and vegetable intake.[8] Thus, higher intake of fruit and vegetables offers an effective tool for preventing serious diseases, and may reduce the need for medication.

WHO recommends eating ≥400 g per day of fruits and vegetables for the prevention of chronic diseases [9]. Nevertheless, in more than half of the countries of the WHO European Region the consumption of fruit and vegetables is lower than 400 g per day.[10] Although the epidemiological evidence for the health benefit of consuming fruit and vegetables is very strong, the evidence for a health benefit of a specific fruit and vegetable is less convincing. Thus, the dietary advice to the general population is still a generic "eat a large variety of plant-based foods".

Berries are traditionally an important part of the daily diet for many people and are among the most widely consumed fruits in the human diet.[11] Strawberries (*Fragaria* x *ananassa*), raspberries (*Rubus idaeus*), bilberries (*Vaccinium myrtillus L.*), and black currants (*Ribes*

nigrum L.) are popular berries consumed in the Norwegian diet, both in fresh, frozen and processed forms such as beverages and jams. The consumption of berry fruits and their contribution to improving health is a subject of considerable interest. However, epidemiological evidence on the health effects of berries alone is scarce. A report from the Iowa Women's Health Study showed a significant reduction in heart disease mortality in woman reporting consumption of strawberries or blueberries (*Vaccinium* sect. *Cyanococcus)*.[12] In Women's Health study in US, association between higher strawberry intake and decreasing trend for CVD was found.[13]

1.1 Health promoting compounds in fruits and berries

There has been a growing interest in understanding the reason for the health benefits of berries and to identify the components with health protective effects. The health benefits of berries have mostly been linked to their high polyphenol content, but berries contain also essential dietary components such as vitamins, minerals, fibre, and energy (mainly in the form of sugar) that may be important components of a healthy diet.[14-16]

1.1.1 Polyphenols

Polyphenols are products of the secondary metabolism inside the plants and are chemically defined as substances which possess an aromatic ring bearing one (phenol) or more (polyphenol) hydroxyl substituents, including functional derivatives (esters, methyl esters, glycosides etc.).[17] Several thousand molecules having a polyphenol structure have been identified in plants.[18] In the plant, polyphenols have roles in defense mechanism against herbivore animals, insects attach or competing plants. Further, they function as signals in the

form of color, aroma, and flavor to attract pollinating or seed dispersing animals, and protect the plant against free radicals generated during photosynthesis.[19]

The most important polyphenol sources are food or drink products that are both rich in polyphenols and consumed in large quantities, such as fruit and berries.[20, 21] Berries contain a wide range of polyphenols such as flavonoids (e.g. anthocyanins, flavonols, and flavanols), tannins (proanthocyanidins and ellagitannins), stilbenoids (e.g. resveratrol), phenolic acids (e.g. hydroxycinnamic acids), and lignans (**Figure 1**). [22-24] Genotype, the degree of maturity at harvest, preharvest conditions, processing, and subsequent storage, do all affect the quality, content and bioactivity of polyphenols.[25-27]

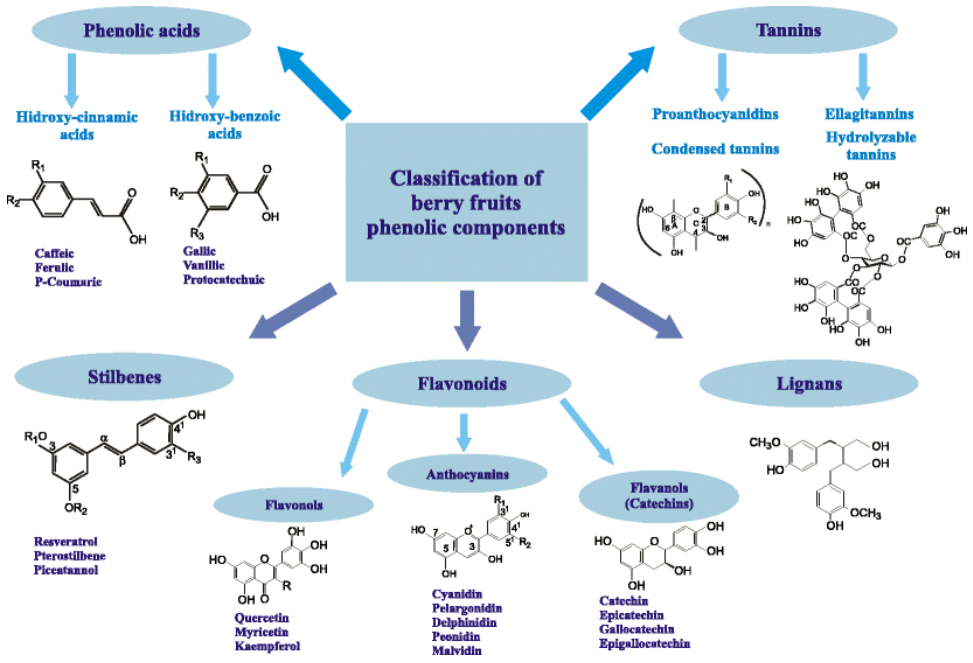

Figure 1. Phenolic components found in berry fruits with examples of each class. Reprinted from Paredes-López 2010.[24]

Flavonoids (particularly anthocyanins) and phenolic acids are the most abundant polyphenols in the fruits and berries studied in this thesis, and will be discussed further in the following text.

1.1.1.1 Flavonoids

Flavonoids consist of 2 aromatic rings that are bound together by 3 carbon atoms that form an oxygenated heterocycle (**Figure 1**). There are six subclasses of flavonoids: flavonols, flavones, isoflavones, flavanones, anthocyanins, and flavanols, based on the type of heterocycle involved.[18, 28] Within each subclass, individual compounds are characterized by specific hydroxylation and conjugation patterns.[29] Flavonoids are found in fruits, berries, vegetables, and other plant food and beverage products.[18] Several epidemiological studies and meta-analyses have shown an inverse association between the intake of flavonoid-rich diets and development of many aging-associated diseases including cancers, cardiovascular disease, diabetes, osteoporosis, and neurodegenerative disorders.[15, 30-34]

1.1.1.1.1 Anthocyanins

Anthocyanins, that are glycosidically bound anthocyanidins, are one of the most ubiquitous polyphenols in berries and are pigments responsible for the orange, pink, red, violet, and blue colors.[35-37] The most common anthocyanidins (aglycones) are cyanidin, delphinidin, malvidin, peonidin, pelargonidin and petunidin.[30] The aglycones are bound to one or more saccharide residues such as glucose, galactose, rhamnose or arabinose.[37] Anthocyanins are found in berries, fruits, and vegetables.[18] The total amounts of anthocyanins vary between bilberries (300-627 mg/100 g), elderberries (170-1000 mg/100 g), chokeberries (398-542 mg/100 g), black currants (110-430 mg/100 g), blueberries (73-430 mg/100 g) and raspberries (32-68

mg/100 g) and other berries.[38] Furthermore, the anthocyanin compositions of the berries differ.[39] Anthocyanins undergo rearrangements in response to pH. At low pH (pH 1-3), the anthocyanins are colored while at higher pH, a colorless form is dominant.[22, 36] Degradation of anthocyanins also occur in presence of oxygen, light, ascorbic acid and/or metal ions and various enzymes.[40] There is a research interest in exploring the broad spectrum of the health-giving properties of anthocyanins.

1.1.1.2 Phenolic acids

There are two types of phenolic acids: hydroxybenzoic acids and hydroxycinnamic acids. Phenolic acids are mostly found in bound insoluble form in some berries, fruits and vegetables.[18] Caffeic acid and ferulic acids, two types of hydroxcinnamic acids, are the most common phenolic acid in berries.[24] Chokeberries (*Aronia melanocarpa*) is characterized by high amounts of phenolic acids (96 mg/100 g) as well as blueberries (85 mg/100 g), while bilberries (40-51 mg/100 g), raspberries (26-29 mg/100 g), lingonberries (*Vaccinium vitis-idaea*) (24 mg/100 g), black currants (100 mg/100 g) contain lower concentrations.[41] Phenolic acids may have protective effects against oxidative stress, inflammation and cancer.[42, 43]

1.1.1.3 Metabolism and bioavailability

A typical plant-based meal contains many different polyphenols. These polyphenols may differ in their metabolism and bioavailability. Bioavailability is usually defined as; "the proportion of the nutrient that is digested, absorbed and metabolized through normal pathways".[44] The polyphenols differ greatly in bioavailability, and the understanding of all mechanisms related to absorption and distributions are far from complete. Chemical structures of the polyphenols determine their rate and extent of intestinal absorption and the nature of

the metabolites circulating in the plasma, and are thus one of the main factors that influence the bioavailability of the polyphenols.[21] When polyphenols exist as polymers or in glycosylated forms, which is the case for most of the polyphenols in food, the polyphenols, except for anthocyanins which may be detected as intact glycosides in the circulation, cannot be absorbed and are hydrolyzed by intestinal enzymes or by the colonic microflora before absorption.[18, 21-24, 40, 45-48] After absorption the polyphenols undergo even more structural modifications, in the small intestine and later in the liver, due to conjugation processes, such as methylation, sulfation, and glucuronidation, which affect the bioavailability of the polyphenols.[18, 40, 44, 45, 47] The urinary excretion of metabolites ranges from 0.3% to 43% of the ingested dose, depending on the phenolic compound. Gallic acid and isoflavones are the most well-absorbed polyphenols, while proantocyanidines and anthocyanins are the least well-absorbed polyphenols.[49]

Since the polyphenols are extensively modified, the polyphenols absorbed in cells and tissues are often chemically, biologically, and possibly functionally different from the original dietary form.[46] Many of the metabolites are still largely unknown, and the knowledge of their tissue disposition is scarce.[50]

1.1.2 Vitamin C

Berries, especially black currants (200 mg/100 g) and strawberries (59-60 mg/100 g), contain high amounts of vitamin C.[51, 52] Vitamin C is essential for humans who must satisfy their vitamin C requirements through the diet due to lack of the last enzyme, L -gulono-lactone oxidase, on the vitamin C synthesis pathway.[53, 54] The function of vitamin C is primarily as an electron donor in redox reactions. The principal compound with vitamin C activity is L-

ascorbic acid (L-AA), which is easily and reversibly oxidized to dehydroascorbic acid (DHAA), which also exhibits vitamin C activity. L-AA is very susceptible to chemical and enzymatic oxidation, and decrease during processing and storage. [51]

There is a strong evidence to link dietary L-AA with protective effects against various oxidative stress-related diseases such as cancers and cardiovascular diseases.[52] L-AA is known to play a role in the synthesis of collagen, enhancing the activity of leucocytes and other aspect of the immune system, scavenging reactive oxygen species, protecting protein and DNA from oxidative damage among others.[52, 54]

1.2 Health effects of berries

A wide number of *in vitro* and *in vivo* studies, discussed in the sections below, have been performed in order to get an understanding of the health effects of berries and their constituents, mainly polyphenols, on lifestyle diseases such as cancer, CVD, and diabetes, as well as the underlying mechanisms. *In vitro* experiments are valuable tools and have provided much information on the biological effects and mechanism of action of polyphenols from berries.[46] *In vitro* means "in glass", and is used conventionally about measurements on cell and organ cultures outside the host, but is also used about biochemical and molecular reactions carried out in a test tube. Although the model systems used in *in vitro* studies may be helpful in describing the molecular mechanism behind any health effect, use of *in vivo* experiments are required to study the contribution of degraded polyphenols and colonic metabolites in human body for the health effect. *In vivo* means "in the living plant or animal",[55] and can be performed both as animal and human studies, and may be an important

link between nutritional epidemiological studies and *in vitro* studies. The next sections will discuss health effect of berries found in *in vitro* and *in vivo* studies.

1.2.1 Antioxidative properties

The antioxidant properties of the polyphenols have been suggested as a part of the explanation of the health effect of eating berries. To understand the mechanism of the antioxidant effect of polyphenols, terms such as free radicals and oxidative stress must be explained. A free radical is a molecule or fragment of a molecule that contains one or more unpaired electrons, and is thus highly reactive.[56] There are many types of radical, but those of most concern in biological systems are derived from oxygen, and known as reactive oxygen species (ROS). The term ROS includes superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical, peroxynitrite, and others.[57] In biological systems, ROS are generated as a natural byproduct of the normal cellular metabolism, are involved in energy production, synthesis of biologically important compounds, regulation of cell growth, intracellular signalling, and are an important part of the immune defence by killing pathogens. In cases of environmental stress, such as cigarette smoke, air pollutants, UV radiations, drugs, inflammation and ischemia-reperfusion, ROS levels can increase dramatically, known as oxidative stress.[58, 59] Oxidative stress may result in significant damage to DNA, proteins and lipids and is associated with different human diseases such as cancer, heart diseases, and chronic inflammation.[58-61] Normally, cells defend themselves against ROS damage by special enzymes (e.g. superoxide dismutase, glutathione peroxidase, catalase)[27] or by non-enzymatic antioxidants (e.g. ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, caretonoids, flavonoids).[62] The antioxidant effect of polyphenols may assist in preventing oxidative stress by chelating redox active metals or scavenging free radicals, and thus prevent further radical formation and associated diseases.[21, 59, 60, 63, 64]. Figure 2 shows how phenols scavenges free radicals by donate hydrogen

atoms and form a stable antioxidant radical by delocalize unpaired electrons and/or intramolecular hydrogen binding or by further oxidation (**Figure 2**)

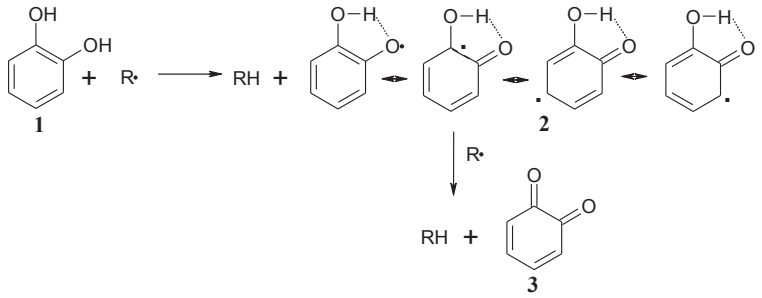

Figure 2. Radical scavenging of free radicals (*R˙)* by a phenol (1). The phenoxyl radical (2) is stabilized by electron delocalization and intramolecular hydrogen bond. The initially formed phenoxyl radical may react with a second radical and be further oxidized to a stable quione (3).

Although the antioxidant effect of polyphenols is probably a contributor to lower the chronic disease risk by scavenging oxygen species and free radicals, it has gradually become clear that the mechanisms of action go beyond their activity as antioxidants. There is increasing evidence towards a role of polyphenols having other properties, such as inhibiting enzymes or specific receptor interactions, affecting the cell cycle and modulating signaling pathways thereby regulating cell activities.[35, 65-68] The polyphenols impact on epigenetic mechanisms (including change in DNA methylation pattern, regulation of histone modifications and changes in the expression of some miRNAs) seem to play a major role in their effects on gene expression.[69] The chemical structure of polyphenols will affect their antioxidant activity, specific interactions with receptors and enzymes and other properties,[21] and as mentioned previously (section 1.1.1.3), the metabolism of the dietary polyphenols possibly change the function from the original dietary form. Although the potential effect of polyphenols is

currently receiving ample attention, most of the literature data are still obtained from *in vitro* studies.

Although individual compounds may affect a pathway one way, the presence of several polyphenols together may change these effects. Some polyphenols exhibit additive and synergistic effects on antioxidant activity.[27, 70-73] Additive effects occur when two or more polyphenols in a mixture interact to provide a combined effect that is equal to the sum of the effects of the individual polyphenols, while synergistic effect refers to cases when combinations of polyphenols exert effects that are greater than the sum of the individual polyphenols.[71] Thus, studying a single polyphenol alone may not reflect the actual health effect. For example, no single polyphenol seems to be able to scavenge all types of radicals or perform optimally for all the mechanisms in the cells; thus combinations of a large number of polyphenols can be more beneficial and prevent free radical-induced diseases than just a high concentration of certain types of polyphenols. Studying whole food will thus give a better understanding of the health effect of polyphenols in the diet.

1.2.2 Anti-inflammatory effects

Inflammation is a complex series of responses to stimuli or agents that are perceived as harmful to the organism. The mechanisms involved in the inflammation response are extremely complex and provide numerous molecular targets.[69, 74] If prolonged, inflammation may progress into a chronic inflammation, which may contribute to the pathogenesis of chronic diseases such as CVD, diabetes, neurodegenerative diseases and several cancers.[75-77]

Berry extracts have been shown in various *in vitro* and *in vivo* studies to impact specific steps in cell signaling pathways that are known to be involved in the inflammation process.[74, 78, 79] The transcription factor NF-κB (nuclear factor kappa B) is found to play a critical role in cellular stress-, immune- and inflammatory responses, and is activated by several agents including cytokines, free radicals, inhaled particles, ultraviolet irradiation, and bacterial or viral products.[80, 81] Once NF-κB is activated, it stimulates the expression of a number of genes including those responsible for the production of cytokines and chemokines.[74] Although NF-κB is essential in normal physiology, inappropriate activation of NF-κB has been linked to chronic inflammation, atherosclerosis and cancer.[80, 82-84] Several berry extracts, such as crowberries (*Empetrum nigrum*), wild strawberries (*Fragaria vesca*), blueberries, and black berries (*Rubus sp.,)* have been found to modulate NF-κB activity after a simulated bacterial infection in human monocytic cell line *in vitro*, indicating that the berry extracts' ability to contribute to the inflammation process go through modulation of NF-κB.[85] In another study, black berries, raspberries, black currants and blueberries have been found to inhibit cyclooxygenase-2 (COX-2) *in vitro.*[86] COX-2 is an enzyme induced by proinflammtory stimuli and data indicate that COX-2 is involved in many inflammatory process and various carcinomas.[87] In the same study black currants and raspberries inhibited activation of NF-κB.[86]

Furthermore, anthocyanins seem to have anti-inflammatory activity, both *in vitro* and *in vivo*, including modulation of NF-κB activity and NF-κB related pro-inflammatory cytokines and chemokines (Interleucin (IL)-4, IL-13, IL-8 and interferon (IFN)-α).[74, 88-90] Studies have also shown that polyphenols have positive effect on expression of inducible nitric oxide synthases (iNOS), COX2, IL-1β, tumor necrosis factor (TNF), IL-6, and other markers.[90-92]

1.2.3 Anticancer effect

The most studied mechanisms of the anticancer effect of berries are their ability to modify proliferation and apoptosis *in vitro*. Black currants, blackberries, bilberries, lingonberries, strawberries and raspberries are some of the berries that have been shown to control cancer growth by inhibiting the proliferation of cancer cells *in vitro*.[50, 93-96] Furthermore, the berry extracts stimulated apoptosis of a human colon cancer cell line, HT-29.[94, 96] Studies indicate that, in addition to their effects on cancer cell proliferation and apoptosis, the anticancer effects of berries may also be partially mediated through their abilities to counteract, reduce, and also repair damage resulting from oxidative stress and inflammation, and involvement in regulation of carcinogen and xenobiotic metabolizing enzymes, various transcription and growth factors, and inflammatory cytokines.[50]

It is well established that free radicals react with all components of DNA, thus damaging its bases and the deoxyribose backbone and causing mutations in genes which may lead to cancer.[60] In animal studies, berries are found to have protective effect against cancer and the effect seems to be the berries ability to influence carcinogen metabolism and reduce levels of DNA damage.[47, 97, 98] Results from the animal studies confirm the possible health benefit of bioactive compounds *in vivo* against oxidative stress. Furthermore, berries or berry juices have also been found to decrease oxidative damage to DNA, a biomarker of oxidative stress, in a number of intervention studies.[98-104]

Several studies have shown that anthocyanin extracts have strong antioxidants activity.[40, 63, 105] *In vitro* tests have suggested that the anticancer effects of anthocyanins may be due to their

anti-proliferative, apoptotic effects, and their effect on capability to regulate gene expression.[91, 106 40, 107, 108]

1.2.4 Anti-atherosclerosis and cardioprotection

CVD is a complex age-related disease that occurs after a long time period and involves oxidative stress, inflammation and tissue damage. Development of atherosclerosis is a key process in the development of CVD and involves the build-up of cholesterol and formation of fatty lesions in the arterial and may results in narrow vessels and constriction of normal blood flow.[109] Berries may play an important role in inhibiting events that occur in the progression of CVD, and their effect on platelet function, hypertension, and lipid metabolism, which all are risk marks associated with CVD, have been widely studied.[110]

The major biological function of platelets is to stop blood loss at sites of injury by forming a haemostatic plug or thrombus.[111] Increased platelet activity and therefore platelet aggregation may contribute to the initiating and progression of atherosclerosis and the occurrence of thrombotic events.[112, 113] Hypertension is a risk factor for many cardiovascular diseases, and is also associated with diabetes as well as oxidative stress.[114-117] Hypertension is defined as systolic blood pressure (SBP) greater than 140 mm Hg or diastolic blood pressure (DBP) greater than 90 mm Hg.[110] Elevated blood pressure (BP) can lead to organ damage, including endothelial dysfunction and renal impairment.[111] Elevated plasma low-density lipoprotein (LDL) cholesterol and low plasma high-density lipoprotein (HDL) cholesterol are associated with increased CVD risk.[118] ROS is known to lead to oxidation of low density lipoprotein (OxLDL) which may result in endothelial dysfunction. [60]

An intervention study has shown that bilberries, lingonberries, black currants, chokeberries, raspberries, and strawberries significantly improved HDL-cholesterol, BP and platelet function.[119] Other studies that include intake of chokeberries, strawberries and mix of bilberries, lingonberries, and black currants have all shown a decrease in LDL oxidation.[13, 120, 121] Also chokeberry extracts lowered the BP and showed a decrease in angiotensin I-converting enzyme (ACE) in myocardial infarction survivors taking statin.[122] ACE is an important enzyme involved in maintaining vascular tension. The two primary functions of the enzyme are to catalyze the conversion of the inactive angiotensin I into a powerful vasoconstrictor and salt retaining compound angiotensin II, and to inactivate bradykinin, a potent vasodilator, which is conductive to lowering BP.[123] These two actions make inhibition of this enzyme a goal in the treatment of conditions such as high BP. *In vitro* studies suggest that black currants are able to control BP by inhibiting angiotensin I-converting enzyme (ACE) involved in maintaining vascular tension.[124] Anthocyanin extracts are also found to inhibit angiotensin-converting enzyme (ACE).[125, 126] Upregulated endohelia nitric oxidase synthase, decreased activities of carbohydrate digestive enzymes, decreased oxidative stress, and inhibited inflammatory gene expression and foam cell formation are some other mechanism thought to be involved in improving cardiovascular health on eating berries.[13]

1.2.5 Anti-diabetic effect

Hyperglycemia is a condition characterized by an excess of glucose in the blood and has been linked to the onset of type II insulin-independent diabetes mellitus and associated cardiovascular complications including hypertension. [127, 128] One therapeutic approach for treating diabetes II is retarding the intestinal absorption of glucose through the inhibition of the key α-glucosidase involved in intestinal glucose absorption and to reduce starch hydrolysis by inhibition α-amylase.[129, 130] α-glucosidase and α-amylase inhibitors, such as

acarbose, are recommended for the treatment of obesity and diabetes.[129, 131] Several extracts from berries have been shown to control glucose level in blood by inhibiting α-glucosidase and α-amylase *in vitro*.[124, 132-136] Natural α-glucosidase and α-amylase inhibitors from berries can offer an attractive strategy to control the glucose level in the blood and thereby have the potential for management of hyperglycemia linked to type II diabetes.

Some berry extracts, such as lingonberries, strawberries, and raspberries have also been found to be effective inhibitors of pancreatic lipases, enzymes that split triglycerides into absorbable glycerol and fatty acids, *in vitro*.[137]

Higher consumption of anthocyanins and anthocyanin-rich fruits is associated with a lower risk of type 2 diabetes.[138] Anthocyanins from different sources have been shown to affect glucose absorption and insulin level and lipid metabolism *in vitro* and *in vivo*.[139-141] Anthocyanins have also been reported to have anti-obese and anti-diabetic effects in various animal models.[138, 142-144] Recently, high fat fed mice supplemented with lingonberries, black currants, raspberries or bilberries gained less weight and had lower fasting insulin levels than the control group receiving high-fat diet without berries.[145] Mouse studies have also shown that blueberries have a protective effect against insulin resistance and hyperglycemia.[146, 147]

In a Finnish epidemiological study, intake of quercetin and myricetin through mainly apples and berries was shown to be inverse associated with risk of type 2 diabetes.[148] Human intervention studies have shown that berries significantly improve insulin sensitivity,[149] reduce fasting plasma glucose,[150] and reduce the postprandial glucose response to a sucrose

load.[151] In these studies blueberry powder, blueberry leaf extracts, and purée of bilberries, black currants, cranberries (*Vaccinium macrocarpon*), and strawberries were evaluated. Interestingly, studies using freeze dried strawberry powder were not able to correct glycemia.[120, 121]

1.3 Juice processing

As a consequence of the growing awareness among consumers of the beneficial effects of fruits and berries, fruit and berry juices experience an increasing popularity and represent an alternative way of consuming fresh fruits. This has led to an increased production of fruit and berry juices. Today, berries are important raw material for the berry processing industry. During the juice processing, the berry mash is heated prior to maceration and pectinase treatment to inactivate endogenous enzymes and increase release of juice. [152] The mash is pressed, and the raw juice is clarified using enzymes, before filtration to remove fiber or pulp (**Figure 3**). Processing affects the polyphenol and vitamin contents of present berries, and thereby their bioactivities and probably also effects on factors involved in health effects.[25, 26, 153-155]

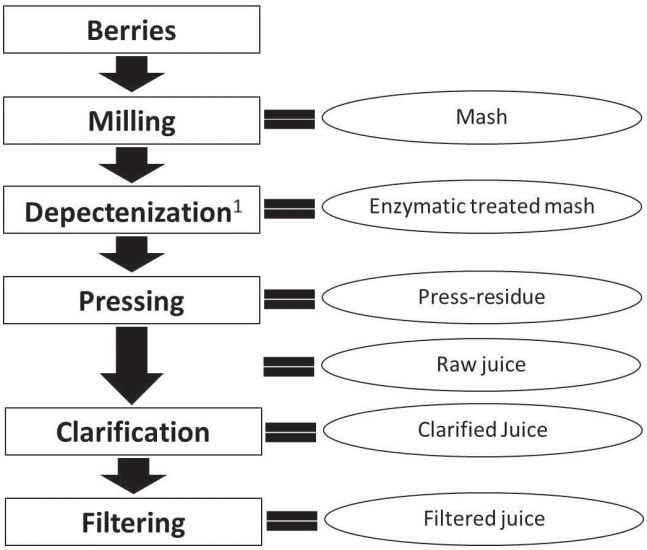

Figure 3. Flow sheet for the juice processing of berries. [1] The temperatures during enzymatic treatment are between 40 and 50°C in 2 h. Some berries are also exposed to a "hot break" (90 °C in 2 min) to deactivate berry enzymes before enzymatic treatment.

1.3.1 Press-residue

Today's production procedures of berry juices are not able to extract all the polyphenols present in fruit and berries. In the literature, the number of studied press-residues has increased as a result of the interest of the agro- and food industry in the added value of recycling, and also of the increased awareness of health promoting effect of berry polyphenols. During the juice-processing, seeds and peels end up in the press-residue. Since seeds and peels contain high concentrations of polyphenols,[156-158] a great part of the polyphenols from berries ends up in the press-residue, resulting in considerable lower concentrations of polyphenols in the juice.[25, 26, 155, 156, 159-161] Knowing that a great part of the polyphenols remain in the press-residue indicates that processing may have a major impact on the bioactivity of the juice compared to berries. Furthermore, utilization of berry press-residue as a source of polyphenols is of interest.

Extraction of polyphenols from press-residue by use of different extraction solvents such as methanol, ethanol and acetic acid, has been reported.[162-165] Enzyme-aided pressing has been tried to increase the yield of polyphenols in the juice, but the studies showed that considerable amounts of polyphenols were still not released from the press-residue.[25, 161] Another way to utilize the polyphenols from the press-residue is to extract the polyphenols in a separate step. Use of ultrasound-assisted extraction, pectinolytic enzymes, different extraction solvents, reduction of particle size, and different extraction times, temperatures, and solid to ratio are factors that have been shown to influence the extraction of polyphenols extracted from press-residues.[166-168]

1.4 Background for the study

The interest in utilization of fruit processing press-residues as a source of polyphenols is growing. Furthermore, the focus on processing waste has increased world-wide. The challenge in the current project was to develop procedures that would extract more of the polyphenols with beneficial biological effects from berry press-residues and thereby increase the value of the press-residue and reduce waste. This thesis has been part of a larger project where the aim was to investigate press-residue after juice processing of Norwegian apples, raspberries, black currants, and bilberries. Furthermore, the aim was also to develop a high-value, natural polyphenol rich food based on one or more of the mentioned commodities. The four chosen commodities have different structures and polyphenol compositions. Since the extraction of press-residue can affect the polyphenol composition, the knowledge of the chemical structures and composition of the compounds together with their biological activity are important.

2 Aims of the study

The objective of the study was to better utilize plant resources by increased release of polyphenols from press-residues after juice processing of black currants and bilberries and to develop high-value, natural polyphenol rich food products with characterized beneficial biological effects from industrial press-residue from black currant. Specifically, the aims were to:

1. Optimize extraction of polyphenols from black currant and bilberry press-residue with regard to phenolic composition and biological effects (*in vitro*) for better utilization of raw materials.
2. Develop a drink product enriched with extracted polyphenols from press-residue of black currant, by taking into consideration phenolic composition, biological effects (*in vitro*), and sensory quality.
3. Determine the effects of the polyphenol enriched drink product on BP and DNA damage using a human intervention study.

3 Results

3.1 Effect of processing of black currant press-residue on polyphenol composition and cell proliferation (Paper I)

In this study, extraction of phenolic compounds from black currant press-residue, which extraction conditions and solvents compatible with food use, was optimized. Measurements of total phenolics (TP) and total monomeric anthocyanins (TMA) in all stages of black currant juice processing showed that the concentrations of TP and TMA in the press-residue, that is 2004 mg gallic acid equivalents (GAE)/100 g of sample and 686 mg/100 g of sample, respectively, were approximately 3- fold higher than in the berries and approximately 4- fold higher than in the raw juice. Calculated on a dry weight basis, the concentration in the press-residue was still 1.7 fold higher than in the berries. Further, 57% of the polyphenols originally present in the berries ended up in the raw juice, while the remaining 43% was mainly retrieved from the press-residue or lost during the processing. The effects of temperature (20-90 °C), time (4-30 min) and sonication on extraction yield and amount of TP and TMA were found using a multiple regression model. Preliminary studies showed that enzymatic treatment of the press-residue did not increase yields of TMA. Within the factors tested in the experimental design, the optimal conditions for aqueous extraction of phenolic compounds were 15 min extraction at 90 °C, while ultrasound-assisted extraction had no effect. The percentage contribution of the anthocyanins delphinidin- and cyanidin-3-glucoside to TP had a positive linear correlation with temperature, while delphinidin- and cyanidin-3-rutinoside had a negative correlation with temperature. Furthermore, extracts obtained at higher extraction temperatures showed a stronger inhibition of proliferation of colon cancer cell lines (Caco-2, HT-29 and HCT 116) than extracts obtained at lower temperature.

3.2 Extraction of phenolic compounds from bilberry (*Vaccinium myrtillus* L.) press-residue: Effects on phenolic composition and cell proliferation (Paper II)

The influence of extraction conditions of bilberry press-residue on phenolic composition and cell proliferation was investigated. Analyzing the phenolic content in the different stages during the juice processing showed that TP and TMA in filtered juice were 46% and 52% of the concentration in the berries, respectively. Furthermore, the TP concentration was 2.5-fold higher in press-residue (1447 mg GAE/100 g of sample) than in the berries. The effect of temperature (22-100 °C) and time (4-45 min) on aqueous extraction of TP and TMA were determined using a multiple regression model. Based on the results of the black currants experiment, sonication was excluded from the experimental design. Both temperature and extraction time had a significant positive effect on the yield of TP. Furthermore, the concentration of TP was curvilinearly related to both temperature and extraction time, meaning that TP increased with increasing temperature and extraction time up to a point before decreasing or leveling out. The yield of TMA in the extracts increased with temperature, while different extraction time did not significantly affect the yield of TMA. The effect of extraction time on the extraction yield of TMA, however, showed significant interaction with extraction temperature. Extraction at 80 °C for 15 min and 100 °C for 4 min gave the highest retrieval of both TP and TMA that is, 37% and 67-68%, respectively. The individual phenolic compounds were differently affected by the extraction conditions. As for black currant press-residue, bilberry press-residue extracts obtained at high temperature gave a stronger inhibition of cell proliferation of colon cancer cell lines (Caco-2, HT-29, and HCT 116) than extracts obtained at lower temperature.

3.3 Potential health effects of fruit juice enriched with polyphenols from black currant press-residue analyzed using *in vitro* model systems (Paper III)

The optimized extraction procedure found in paper I was transferred to the industrial scale. The resulting black currant press-residue concentrate (BPEC) was added to a commercial fruit juice (MANA) containing red grape, aronia, cherry, and bilberry to increase the diversity of polyphenol composition. *In vitro* health effects of the new complex drinks were screened in various model systems. Several combinations of MANA and BPEC were tested in order to make an optimized healthy drink product. Increasing amounts of BPEC in MANA, standardized to the same concentration of TP (μg GAE/ml sample), gave stronger inhibition of cell proliferation of HCT 116 and Caco-2 cells, angiotensin I-converting enzyme (ACE) activity, and α-glucosidase activity than MANA alone. BPEC gave a significant increase of the basal NF-κB activity compared to the control, but was not significant difference from MANA, while MANA, and not BPEC, had an inhibitory effect on the lipopolysaccharide-induced (LPS) NF-κB activity.

3.4 Polyphenol-rich juice reduce blood pressure measures in a randomized trial in high normal and hypertensive volunteers (Paper IV)

The objective of this study was to investigate if a long-term intake of polyphenol-rich fruit could lower the BP *in vivo*. Based on the *in vitro* results from paper III and sensory judgment evaluation, the optimized juice (Optijuice) was made of MANA containing 15% black currant press-residue concentrate (BPEC). Randomly selected men and women (n=134), between 50 and 70 years, were randomized into three groups consuming 500 mL of placebo, MANA, or Optijuice daily for 12 weeks. The BP was measured before intervention and after 6 and 12 weeks. The BP was measured three times at 1-minute intervals. Both the first measure (BP1), the mean of measure number two and three (BPmean), and the standard deviation of all three measurements (BPV) were analyzed. Analyses where MANA and Optijuice groups were pooled were also performed to increase statistical power. The systolic BP1 (SBP1) was significantly reduced over time compared to the placebo group in the pooled juice group (6.9 and 3.4 mm Hg after 6 and 12 weeks, respectively p=0.01). Similarly, SBP1 was significantly reduced in hypertensive participants in the pooled juice group after 12 weeks compared to placebo (6.8 mm Hg, p=0.04), but not in normotensive participants (+1.0 mm Hg, p=0.71). There was also a significant reduction in BPV in the pooled juice group after 6 and 12 weeks compared to placebo (1.4 and 1.7 mm Hg, respectively, p=0.03). There was not found any significant differences between the groups when time curves for BPmean were investigated. There was a significant difference only in the liver enzyme activity of alanine transaminase (ALAT) between the three study groups. No significant differences were found for other biomarkers related to cardiovascular diseases or other diseases.

3.5 Consumption of polyphenol-rich juice causes a decrease in oxidative damage in DNA in healthy participants with elevated blood pressure (Paper V)

In paper V, blood samples collected from the intervention study described in paper IV were analyzed to study the effect of polyphenol-rich juice on background DNA strand breaks (SBs) and DNA oxidization damage, measured by comet assay. There were no significant differences between the three study groups during the study, either in change in SBs or DNA oxidative damage. The Optijuice group was, however, the only group having a significant decrease of DNA oxidation damage during the intervention study, and the response was more pronounced in participants starting with lower DNA oxidation damage than participants starting with higher damage by baseline.

4 Discussion

4.1 Optimized extraction of polyphenols from berry press-residue (Papers I, II)

During the juice processing, only 46% of the phenolic compounds in bilberries and 50% of the phenolic compounds in black currants ended up in the final juice. The concentrations of TP and TMA were higher in black currant press-residue (2004 mg GAE/100 g of sample and 686 mg/100 g of sample, respectively) than in bilberry press-residue (1447 mg GAE/100 g of sample and 458 mg/100 g of sample, respectively). Both the press-residues from after black currant and bilberry juice production are rich in phenolic compounds and appear to be good sources for further extraction.

To utilize the high amounts of polyphenols in the press-residue, the polyphenols have to be released from the press-residue. Earlier studies have shown that use of the extraction solvents, such as ethanol and methanol, enhance the extraction of polyphenols from plant press-residues compared to water.[163, 164] Reduction of particle size of the sample, thereby increasing surface area, is known to enhance the yield of polyphenols and anthocyanins in black currant press-residue extracts.[167] In the same study, Landbo et al. have shown that water can be as good an extraction solvent as methanol when used on fine milled black currant press-residue. Thus, the present studies did not elucidate the effect of particle size on the yield of extracted polyphenols. Rather, drawing up the knowledge from earlier studies, the press-residues from black currants and bilberries used in the optimizing experiments in lab scale and in the industrial process were homogenized and reduced in particle size.

In order to optimize the extraction of polyphenols from press-residue of black currants and bilberries, several factors compatible with food use were tested. The effect of different temperatures (20-90 °C for black currant press-residue and 22-100 °C for bilberry press-residue) and extraction times (4-30 min for black currant press-residue and 4-45 min for bilberry press-residue) for aqueous extraction of polyphenols from the press-residue was tested. The yields of extracted polyphenols from black currant press-residue (5-34% of TP and 5-43% of TMA originally present in the berries) were lower than the yields of polyphenols from the bilberry press-residue extracts (9-40% of TP and 10-68% of TMA originally present in the berries). The thicker cell walls of black currants containing more pectin may explain the lower extractability of polyphenols from black currant press-residue than from the bilberry press-residue. [25, 169]

Although treatment of berries with cell wall degrading enzymes, such as pectinases, is known to significantly increase the juice yield and the amount of polyphenols in juices,[25, 161, 170-172] no significant difference in the yields of extracted TMA from black currant press-residue was found in response to enzymatic treatment with pectinases. The black currants have, however, already been exposed to pectinase treatment during juice processing and this may be the reason for why the enzyme had no effect in the present study. Moreover, enzyme-catalyzed anthocyanin pigment degradation is a recognized adverse effect of pectinases used in fruit juice processing.[173, 174] Findings in this study are in accordance with Landbo and Meyer where enzymes had negative or no effects on the yield of anthocyanins obtained from black currant press-residue, depending on types of enzymes. Several of the enzymes by Landbo and Meyer, however, significantly increased the yield of total phenols.[167] Use of enzymes have also been found to enhance the extraction of polyphenols from grape press-residue.[175]

Since the use of ulatrasound-assisted extraction has been shown to increase the yield of polyphenols from citrus peels,[168] ultrasound-assisted extraction was tested for the ability to enhance the extraction of polyphenols from black currant press-residue. During UAE the propagation of an ultrasound wave (from 20 to 800 kHz) through a liquid initiates formation and activity of bubbles.[176]. The collapse of bubbles can produce physical, chemical, and mechanical effects [177] resulting in swelling and hydration of the plant cell and thereby improve the diffusion process of polyphenols. An increase in the swelling of the cell can result in cell wall disruption improving the washing out process.[178] However ultrasound did not affect the extraction yield, TMA or TP. Enzymatic treatment and sonication of bilberry press-residue was not performed in view of the lack of effect were obtained by enzymatic and sonication treatment of black currant press-residue.

The temperatures and extraction times tested in the experiments gave different effects on extraction yield and contents of TMA and TP in black currant press-residue extracts. Increasing temperature had a positive effect on the extraction yield from black currant press-residue up to about 55°C before the yield decreased or leveled out, while extraction time had no significant effect on the extraction yield. Several studies have shown that higher solvent to solid ratio increase the extraction yield during fruit and berry extraction.[166, 179] It is, however, advantageous to have lower solvent to solid ratio in the industrial juice process due to the work required to remove water to obtain higher concentrations of polyphenols in the extracts.

Temperature had a positive significant effect on the extraction yield of TMA from the black currant and bilberry press-residues. However, there was a significant interaction between

temperature and extraction time, meaning that the effect of extraction time on extraction yield of TMA was dependent on extraction temperature. Although temperature had no effect on TP during extraction from black currant press-residue, a significant interaction between temperature and extraction time was documented. Furthermore, the concentrations of TP were curvilinearly related to temperature in the extracts from black currant press-residue, meaning that TP increased up to a point before decreasing or leveling out. The concentration of TP in bilberry extracts also had a curvilinear relationship to temperature. According to other reports, extraction temperature affects the compound stability due to thermal decomposition and may be the main mechanism causing decrease in polyphenol content.[72]

Extraction time alone had positive effect, but had curvilinear effect on the concentration of TMA in black currant press-residue and concentration of TP in bilberry press-residue extracts. According to another study, the yields of anthocyanins in black and red currant press-residue extracts decreased with time of extraction at room temperature.[163]

4.2 Polyphenols in press-residue extracts (Papers I, II, III)

The anthocyanin compositions of the berries used for juice processing in Paper I and Paper II, analyzed by high performance liquid chromatography (HPLC), were mainly in accordance with previous findings in black currants[25, 180] and bilberries.[25, 181-183]. The anthocyanin composition found in black currant press-residue was also in accordance with other literature,[162] but with a slightly higher content of cyanidin-3-rutinoside.

The results in Paper I and Paper II also showed that temperature and extraction time affected the composition of anthocyanins and other phenolic compounds. In extracts from black currant press-residue obtained at higher temperature (90 °C), the percentage contribution to TP of flavonols, hydroxycinnamic acids (HCA), cyanidin-3-rutinoside, cyanidin-3-glucoside, delphinidin-3-rutinoside, and delphinidin-3-glucoside decreased compared to extracts obtained at lower temperature (40-80 °C), which means that new not quantified compounds appeared with higher extraction temperature and made a considerable contribution to TP. The same trend was seen in bilberry press-residue extracts, where the contribution of anthocyanins to TP in extracts obtained at 30 min extraction time increased with increasing temperature to 74% to TP at 60 °C, before the percentage contribution of anthocyanins decreased to 41% in extracts obtained at 100 °C.

Also individual anthocyanins in black currant press-residue extracts were affected by temperature and extraction time. The negative correlation between cyanidin-3- and delphinidin-3-rutinsoide and extraction temperature and the positive correlation between cyanidin-3- and delphinidin-3-glucoside and extraction temperature, in extracts obtained after 30 min, may indicate that the rutinosides (dissaccharides) were decomposed to glucosides (monosaccharides) at higher temperatures. Temperatures beyond 35°C have been shown to degrade anthocyanins in extracts from black currant in another study,[179] while the decrease in percentage contribution of anthocyanins in the present study did not occurred before the temperature was 90 °C. Also anthocyanin composition in bilberry press-residue extracts obtained after 30 min changed with increasing extraction temperatures. The higher percental contents of delphinidin-3-galactoside and delphinidin-3-glucoside at higher extraction temperatures may be due to enhanced extractability of delphinidin hexosides at higher temperatures, and the lower contents of cyanidin-3 and malvidin-3-arabinoside may be due to

lower stability of anthocyanin arabinosides compared to glucosides and galactosides.[184, 185] Furthermore, aglycones detected in bilberry press-residue extracts obtained at increased extraction time (30 and 45 min) and higher temperature (100 °C) shows that there was an extensive degradation of anthocyanins in these samples.

In black currant press-residue extracts, percentage contributions of both flavonols and HCA to TP had a negative correlation with the extraction temperatures. The concentrations of both HCA and flavonols, however, increased in response to higher temperatures. Total yield of flavonols and HCA increased twice in extracts obtained at 90 °C (16.0 mg/100 g and 15.9 mg/100 g, resepcetively) compared to extracts obtained at 40 °C (7.9 mg/100 g and 8.2 mg/ 100 g, respectively). Also the concentrations of flavonols and HCA in bilberry press-residue extracts increased with increasing temperature, and the yield of flavonols, except for aglycons, and HCA reached 100 and 63%, repectively, at 100 °C. This means that HCA and flavonols were effectively extracted from the press-residues by hot water.

Since TP is measured by colorimetric assay and calculated as gallic acid equivalents and individual polyphenols were measured by HPLC and use of appropriate standards, the percentage contribution is not numerically correct, but illustrates differences between samples. Further, also other compounds such as sugars and organic acids including ascorbic acid may contribute to TP in the samples.[186]

4.3 *In vitro* effects of press-residue extracts (Papers I, II, III)

Since the extracts produced by different extraction conditions differed in polyphenol composition (Papers 1 and II), the extracts were screened for *in vitro* bioactivities to study any differences in their biological effects (Papers I, II and III).

Unregulated cell proliferation together with suppressed apoptosis is the basis for cancer evolution and progression.[187] Earlier studies have shown that berry extracts can inhibit cell proliferation *in vitro*,[93-95, 188, 189] and phenolic compounds have been suggested to be at least partly responsible for the observed antiproliferative effects of the berries.[50, 93, 188, 190] The black currant and bilberry press-residue extracts showed antiproliferative effects in a dose-dependent manner, which is in agreement with earlier findings showing that black currant and bilberry extracts are able to inhibit cancer cell proliferation. [93, 188, 189] Interestingly, extracts of black currant and bilberry press-residue obtained at higher temperatures had increased antiproliferative effects. The extracts were diluted to the same concentration of TP, indicating that the compounds in extracts obtained at higher temperatures had a higher antiproliferative effect than compounds extracted at lower temperatures. Based on the polyphenol composition of the press-residue extracts (Papers I and II), it is reasonable to suggest that the decomposed polyphenols obtained at high temperatures increased the antiproliferative effects of the press-residue extracts by being more available to the cells. Other studies have shown that cyanidin and delphindin inhibit the growth of human cancer cells to a higher degree than the corresponding glycosylated anthocyanins.[188, 191] Furthermore, it has been shown that glycosylation of phenolic compounds restrict cellular antioxidant activity,[192] aglycones have higher cell permeability than the glucosides,[193] and that more nonpolar compounds have a higher permeability coefficient.[194] This supports the theory that decomposed compounds may be more active or more accessible to the cells. The positive effects of black currant and

bilberry press-residue extracts on induction of apoptosis, in the present study (Papers I and II), indicate that the inhibition of cell proliferation is due to increased apoptosis.

Based on the results of the laboratory scale experiments, the most promising processing procedure of black currant press-residue with regard to beneficial effects was transferred to an industrial scale. Although the optimal extraction temperature found in Paper I was 90°C, only 80 °C was achieved after 30 min heating of homogenized black currant press-residue in the industrial-scale experiment. The industrial made BPEC contained 7682 mg GAE/L and 2091 mg TMA/L. As seen in Paper III, the industrial processed BPEC significantly inhibited the cell proliferation of HCT 116 and Caco-2, like the black currant press-residue extracted at 90 °C for 30 min in laboratory scale (Paper I). Neither the laboratory made black currant press-residue extract nor the BPEC had significant effect on HT-29, which may be due to a different regulation of this cell line compared to Caco-2 and HCT 116.

Due to the bitter taste of black currant press-residue extract, the BPEC was added to MANA, a commercial polyphenol-rich juice, to make a drink with good taste and to increase the content and diversity of possible health beneficial phenolic compounds. The aim of the experiments performed in Paper III was to screen the *in vitro* health effects of different mixtures of MANA and BPEC in various model systems in order to optimize a new complex drink to be tested in an intervention study. The mixtures of MANA and BPEC were tested in model systems for their inhibitory effect on cell proliferation, ACE activity and α-glucosidase activity and their ability to modulate NF-κB activity. The significant effects of MANA to inhibit cell proliferation of Caco-2, compared to the control, found in Paper III, are in accordance with resulted found in Paper II and other studies that have shown that bilberry,

aronia, and red grape (some of the fruits found in MANA) have antiproliferative effects. MANA did not, however, significantly affect the cell proliferation of HT-29 and HCT 116, which indicates that the combination and/or concentration of the fruits and berries in MANA were insufficient to reduce cell proliferation of these cell lines. In spite of equal concentrations of TP, BPEC had a stronger inhibitory effect on the cell proliferation of HCT 116 and Caco-2 than MANA, indicating that there is no correlation between antiproliferative effects and total phenolics concentration. Increasing the percentage of BPEC in MANA gave stronger inhibition of cell proliferation.

The ability of BPEC to inhibit the activity of ACE activity and α-glucosidase indicates that BPEC is a good source of polyphenols with potential protective effects against hypertension and diabetes mellitus. Earlier studies have also shown that black currant has an inhibitory effect on ACE and α-glucosidase activity.[124, 135] BPEC adjusted to equal TP concentration as MANA gave no significant difference in inhibitory effect of ACE compared to MANA. However, compared to control, BPEC adjusted to equal concentration as MANA had a significant effect, while no significant effect of MANA was found. This indicates that the polyphenols found in BPEC may have a higher inhibitory effect on ACE activity than the polyphenols found in MANA. Adding BPEC to MANA also significantly increased the inhibition of α-glucosidase compared to MANA. As found in Paper I and Paper III, black currant press-residue is distinguished by high concentrations of anthocyanins. Cyanidin-3-rutinoside, delphindin-3-glucoside, and cyanidin-3-glucoside found in BPEC, but not or in low concentrations in MANA, are known to inhibit α-glucosidase,[139] ACE activity,[125] and growth of colon cancer cell lines.[108] These compounds may contribute to the stronger effects of BPEC than MANA in several of the *in vitro* assays performed in the present study.

Monocytes stably transfected with a NF-κB-luciferase reporter construct were used to screen ability of the different mixtures of MANA and BPEC to modulate NF-κB.[195] NF-κB is central in inflammatory responses, and modulation of NF-κB activity can be a potentially effective prevention strategy for controlling certain diseases. MANA containing 10-50 % BPEC and BPEC alone gave a significant increase in the basal NF-κB activity compared to the control. The mechanisms behind the NF-κB modulation of berry extracts can be thought to act at several levels in the complex signaling pathway. Inhibitors of NF-κB may for example act upon signals that activate the cascades, translocates NF-κB into the nucleus, DNA binding of dimers and interaction with the transcription machinery.[196] Although increasing amount of BPEC in MANA gave stronger inhibition of α-glucosidase, ACE activity, cell proliferation and basal NF-κB acitivty *in vitro*, only MANA and MANA enriched with 5-50% BPEC had a significant inhibitory effect on LPS-induced NF-κB activity. This shows that MANA, and not BPEC, has an effect on acute inflammation in this model system. This is in agreement with another study showing that black currants have no significant effect on LPS-induced NF-κB activity.[85] On the other hand, due to the content of anthocyanins in BPEC, an anti-inflamamtory effect were expected as anthocyanidins are generally found to have anti-inflammatory effects and exhibit this largely through the NF-κB pathway.[91] Interesting, unpublished results from this project have shown that black currant press-residue extracts obtained at lower temperature (40 °C) have stronger inhibitory effect on LPS induced NF- κB activity than extracts obtained at higher temperatures (90 °C), indicating that the process temperature affects the inhibiting effect of black currant press-residue on LPS induced NF-κB activity. Furthermore, black currants are found to repress the TNF-α induced expression of NF-κB regulating alkaline phosphatase in prostate cancer cells, so it seems likely that the ability of black currants to significantly modulate NF-κB activity may also depend on the cell

type. Interestingly, adding 10-30% BPEC to MANA resulted in an increased (although not significant) inhibitory effect of LPS-induced NF-κB activity, indicating that the new combination of polyphenols may act synergistically.

The effectiveness of the samples tested on the *in vitro* assays studied in Paper I, II and III were compared on the basis of equivalent amounts of total phenolics as measured by the Folin-Ciocalteau's method. However, also other reducing substances (including vitamin C, organic acids, sugars etc.) than polyphenols will reduce the reagent in the Folin-Ciocalteau's method. Therefore, it has to be kept in mind that also other compounds may contribute to the effect of the samples on the *in vitro* assays. In addition, compounds that do not affect the Folin-Ciocalteau's method, i.e. compounds that do not have reducing properties, may also contribute to the effect of *in vitro* assays.

In this project we have studied health effect of extracts from fruit and berries *in vitro* and *in vivo*, and not the effect of individual polyphenols. Use of extracts is more likely to mimic the effect of the particular food items; although information on the individual components and their potential effects is lost. It is expected, however, that the health effects of foods may arise from combinations of compounds and/or from concurrent effects on multiple targets.

Cell cultures have been used as model systems in several of the analyses performed in this study. There are several advantages of working with cell cultures, such as the control of the environment, that is physiochemical environment and physiological conditions are stable, facilitation of the study of the molecular mechanism behind an effect, inter-laboratory

repeatability, relatively low cost, and time efficiency.[55, 197] Cell studies may give mechanistic knowledge related to results found in clinical trials. The disadvantages of cell studies, however, are the lack of human complexity and the likelihood that the *in vitro* results are directly transferrable to *in vivo* conditions. Cancer cell lines do not represent a typical population since they are originally from one patient, so the results should be generalized with care.

In conclusion, the most beneficial combination of BPEC and MANA, optimized with regard to polyphenol composition, *in vitro* biological effects and sensory qualities (results not shown), was MANA containing 15-20% polyphenol-rich BPEC. The new product, called Optijuice, contains an even more diverse composition of polyphenols than MANA, which may account for the stronger effect in several of the *in vitro* model system tested.

4.4 *In vivo* effects of berry juice (Papers IV, V)

The effect on BP and health-related biomarkers of such as CVD, diabetes, and oxidative stress (DNA damage) of drinking the optimized product, Optijuice, was tested in a human intervention study.

4.4.1 Effect on blood pressure

High BP and BP variability are significant CVD risk factors,[115, 116, 198, 199] and reducing these factors by natural means may have an impact on public health. Other studies have suggested that the polyphenols in fruit and berries can reduce BP.[119, 122, 200, 201] The results from the intervention study conducted in this project, support these finding by strongly indicating that

polyphenol rich juice can reduce BP and short term BP variability (BPV). Thus, polyphenol-rich berry juices may offer an attractive therapeutic option for decreasing BP.

How to measure BP is debated.[202] There are several guidelines for BP measurement procedures varying from at least two measurements spaced by 1-2 min after resting for several minutes,[203] to more specific procedure such as resting 5 min, taking three measures and averaging the two latter.[204] In research, the measurement procedures are more standardized both for resting conditions and number of measurements taken, but still there are different traditions as to how many and which measurements to use. In this study, the mean of second and third measurements was used, to follow the latest guideline.[204] In addition, the first recorded blood pressure (BP1) and BP variance (BPV) were analyzed separately.

The observation in this study, that BP1 was higher than the two next, is in accordance with other literature.[205] BP1 is probably more sensitive to stress and sympathetic activation, similar to the elevated BP observed during mental or acute stress tests.[206-208] The reduction in systolic BP1 (SBP1) in the groups drinking MANA or Optijuice suggests that the mechanism behind the beneficial effects on CVD of fruits and berries may be due to their ability to reduce the elevated BP during stressful situations and not necessarily on the resting BP (BPmean), which was not significantly changed during the intervention period compared to the control group. No significant change in BPmean was in line with an intervention study where effects of anthocyanins had no effect on BP.[209] However, the results are in contrast with other intervention studies where berries had positive effect on BP.[119, 122, 210]

The polyphenol content in the intervention beverages was determined to relate the obtained health effects to the phenolic composition in the beverages. The main differences between the juices were the high content of anthocyanins, the major phenolic compounds in the juices, where Optijuice had about 3-fold higher concentration than MANA. In addition, the composition of anthocyanins differed. Since no difference was observed in the effect of BP between MANA and Optijuice, it was not possible to reveal any effect of dose- or content of polyphenols. Therefore, the participants drinking MANA and Optijuice were pooled to increase the statistical power in the analyses.

The strongest decrease in SBP1 was found after 6 weeks (6.3 mm Hg in the pooled group), while the change between week 6 and week 12 was only 0.9 mm Hg. This time course indicates that this intervention was not able to decrease the averaged BP in the pooled group more than the averaged BP obtained after 6 weeks. Analysis of the polyphenol content in the polyphenol-rich juices showed that the contents of TP, anthocyanins, flavonols, and HCA in Optijuice and MANA decreased during the intervention study, by 5%, 51%, 9%, and 4%, respectively, in Optijuice and 2%, 52%, 13%, and 1%, respectively, in MANA. The decrease in polyphenol content, especially the anthocyanins, in the juices during the intervention study may be one of the factors contributing to the lack of further decrease in BP after 6 weeks. Interestingly, the time curve for SBP1 in the placebo group had a different shape. Only small changes were observed after 6 weeks (+0.6), while an evident reduction occurred between week 6 and 12 (-4.4). The intervention study performed in this study started at the end of winter/beginning of spring (March) and ended in beginning of summer (June). It is known that the BP varies by season,[211] and this may partly explain the SB1 time curve of the placebo group. These observations underline the great importance of having a placebo group in intervention studies to obtain reliable results.

Visit-to-visit variability is shown to be a strong predictor of stroke, independent of mean of the systolic BP.[199] Furthermore, short term BPV within 24 h is found to be closely associated with the development, progression and severity of cardiac and renal organ damage independently of mean BP.[198] A possible mechanism behind these findings is that high BPV leads to stress on the vessel wall which again may result in damage and initiation of CVD. Thus, since both visit-to-visit and ambulatory BPV are predictors of cardiovascular incidents,[212] we suggest that the BPV over a time period of 3-4 minutes also may reflect relevant pathophysiological conditions, similar to BPV determined by visit-to-visit and ambulatory measurements. Another polyphenol intervention study has shown a reduction in BPV,[213] but this is (to our knowledge) the first placebo controlled clinical trial showing a reduction in BPV. The BPV decrease observed in the pooled juice group in the intervention study in this project supports the suggestion that the positive health effect of berries on CVD may be due to reduction in BPV.

It is of particular interest to decrease the BP in participants that are hypertensive (SBP/DBP≥ 140/90 mm Hg) at baseline. The results from this study showed that there was a significant reduction in SBP1 in hypertensive participants in the pooled group compared to the placebo (7.3 and 6.8 mm Hg after 6 and 12 weeks, respectively), which is in accordance with other findings where intervention with fruit and berries had stronger effect on reduction of BP of hypertensive participants.[119, 201]

Although other intervention studies have reported a decrease in BP after intakes of berries,[119, 122, 210, 214] the mechanisms behind the effects of polyphenol-rich food have not been identified.

Analysis of blood samples taken during the intervention study may help us to understand the mechanisms behind the BP reduction. As mentioned before, ACE act as a major regulator of BP and thus ACE activity is a major therapeutic target in treatment of hypertension. Although MANA enriched with BPEC resulted in an inhibition of ACE *in vitro* (Paper III), the significant inhibition was not found *in vivo* (Paper IV). In this study, only alanine transaminase (ALAT), a liver enzyme, showed a significant change during the study. If the liver is damaged or not functioning properly, the plasma level of ALAT increases. Thus, measurement of serum ALAT level is used to find liver diseases.[215] None significant change was found in total antioxidant status in plasma during the intervention study. It is known, however, that anthocyanins are absorbed rapidly and reach the circulatory system within 0.25-2 h.[216] Since the last intake of the polyphenol-rich juices occurred 10-14 hours before blood sample was collected, the anthocyanins may no longer be present in the blood. Generally, all the baseline blood sample biomarkers were within the normal range, which make it more difficult to find significant changes during an intervention.

Hypertension remains an important public health challenge, and this and other studies indicate that berries may control the BP. Integrating nonpharmacological approaches such as increased berry intake may help to improve clinical outcomes as well as decrease the economic burden of hypertension.

4.4.2 Effect on DNA damage

Since oxidative stress is associated with different human diseases such as cancer, heart diseases, and chronic inflammation,[58, 59, 61] it was of interest to study the effect of polyphenol rich juices on DNA oxidation damage by using blood samples from the intervention study

(Paper V). Oxidative damage to DNA is one of the most widely measured biomarkers of oxidative stress.[217, 218] Thus, reducing the frequency of DNA damage or enhancing the repair mechanism may be a feasible explanation for the protective effects of berries against cancer and other chronic diseases. DNA damage were evaluated by the comet assay, which is one of the standard methods to assess DNA damage in form of strand breaks (SB) as well as DNA oxidation damage, when used in combination with formamidopyrimidine DNA glycosylase (FPG) that converts altered purines, including 8-oxoguanine, to breaks.[219]

Previously, intervention studies with fruit and berry juices have given divergent effects on DNA damage. Several berry and fruit juices, containing blueberry, red grape, blackberry, sour cherry, black currant, elderberry and/or chokeberry significantly decreased oxidative DNA damage.[100, 101, 104, 220, 221] Black currant juice and kiwi juice, however, had no effect on endogenous DNA damage.[222, 223] The polyphenol rich juices in this study had no effect on DNA strand breaks (SB), which means that the polyphenol-rich juices had no effect on the strand breaks resulting from a variety of sources of damage. The decrease in DNA oxidation damage in the group drinking Optijuice indicates that the Optijuice may inhibit formation of 8-hydroxydeoguanosine (8-OHdG) which is the most abundant DNA damage formed during oxidative stress.[224] The lower levels of DNA oxidation damage may not result just from antioxidant effects, but also from an enhancement of DNA repair.[225] Despite of a significant decrease in DNA oxidation damage in the Optijuice group, no significant differences were found between the three drink groups.

The significant correlation between DNA oxidation damage before and after the intervention indicates that participants with initial high DNA oxidation damage have higher DNA

oxidation damage also after the intervention compared to the participants with initial lower DNA oxidation damage, implying that the estimates of oxidative damage to DNA bases, as measured on the comet assay, reflect biological reality. Our study also showed that there were no correlations between SBs and DNA oxidation damage, meaning that those with high SBs did not necessarily have high DNA oxidation damage.

Since a high frequency of DNA oxidation lesions may be a result of oxidative stress in cells, and DNA oxidation damage may be linked to cancer and other diseases, it is of particular interest to decrease DNA damage in participants starting with high DNA oxidation damage at baseline (over 10% in tail DNA after FPG treatment). The results from this study, however, indicated that Optijuice was more effective at decreasing the levels of DNA oxidation damage in participants with initial lower DNA oxidation damage (a 50% drop of the level of DNA oxidation damage) than participants with initial higher level of FPG sites (a 14% drop of the level of DNA oxidation damage).

The analysis of damage in frozen blood is a relative new approach, and it is therefore difficult to compare the levels of SBs and DNA oxidation level in the participants in this intervention study with other studies. Older studies have performed the comet assay on fresh blood, and often on isolated lymphocytes. Also other scoring methods, including visual scoring, have been used. Although Al-Salamani et al.[226] have shown successful storage of whole blood at -80°C for up to 1 month with no indication of elevated DNA strand breakage, the blood samples from the present study had been frozen for a longer period before analysis. Analysis of 10 blood samples from this intervention study, however, showed that the levels of FPG-

dependent breaks are comparable with levels seen in other human trials.[227] This indicates that the DNA oxidation damage seems to be unaffected of long time freeze storage.

Pooling participants drinking MANA and Optijuice to increase the statistical power gave neither significant difference in SBs nor DNA oxidation damage between the pooled group and placebo (results not shown). The baseline values of SBs were significantly different in the three study groups when the dataset was separated into participants starting with high or low DNA oxidation damage, making it difficult to do further statistical analysis. There was, however, no significant difference between the groups in DNA oxidation damage at baseline, and the participants starting with low DNA oxidation damage drinking polyphenol-rich juice had a significantly stronger decrease in DNA oxidation damage (-1.3% tail DNA) than the placebo group (+0.67% tail DNA) (independent t-test, p=0.037).

Several studies have tested antioxidant effects of fruits and berries by exposing isolated lymphocytes from the blood samples taken during the intervention study to H_2O_2 (*ex vivo)* to induce SBs.[222, 228] This method has been used as a semiquantitive measurement of the donor's antioxidative status.[224] Intake of kiwi, kiwi juice, blueberry, blueberry, blueberry/apple juice and grape juice have been shown to decrease H_2O_2 sensitivity, while intake of 600 grams fruit and vegetables per day had no effect.[100, 220, 224, 229-232] The suggested mechanisms responsible for this antigenotoxic activity for some fruits and berries have been described as increased antioxidant activity.[98] The ability of H_2O_2 to induce DNA breaks is severely attenuated in whole blood compared with freshly isolated lymphocytes, and may be explained by the breakdown of H_2O_2 by catalase or by the catalytic action of Fe-ions from haem released from lysed erythrocytes.[226, 227]

Oxidative stress causes DNA damage, which, when left unrepaired, can lead to base mutation, single- and double strand breaks, DNA cross-linking, and chromosomal breakage and rearrangement.[73] Although results from this study, as well as other studies, indicate that berries may have the ability to decrease DNA damage, DNA damage is only an intermediate biomarker that is plausibly linked to cancer and other chronic diseases because DNA damage likely plays a role in mutation rate and cancer risk. The blood cells are not a target tissue for cancer and it is not clear that the damage detected in blood cells reflects the damage in actual target tissue.[233] In the absence of clinical trials using definitive end points such as cancer, these results represent only weak evidence for anticancer or other effects related to diseases. However, the results indicate that berries' ability to decrease DNA damage may be one of the mechanisms behind their protective effect found in epidemiological studies.

The results from the papers in this thesis are not suited to confirm or reject the "antioxidant hypotheses": since oxidative stress causes diseases and polyphenols are antioxidants, polyphenols reduce the risk of oxidative stress related diseases through their antioxidant function *in vivo*. However, since fruit juices also contain other substances, it has to be kept in mind, that their preventing effect may not be attributed alone to the presence of polyphenols. These results contribute to the discussion of how fruit and berries can reduce the risk of chronic diseases by showing that fruit and berry juice can affect biomarkers for chronic diseases *in vivo*.

5 Concluding remarks and future perspectives

5.1 Conclusion

The results obtained in the present work demonstrate that the press-residue from juice production of black currants and bilberries provides a good source of polyphenols that may have health benefits. The bilberry and black currant press-residue extracts, especially extracts obtained at high extraction temperatures had promising antiproliferative effects on different human colon cancer cell lines *in vitro*. Furthermore, increasing percentage of black currant press-residue extract concentrate (BPEC) in an already existing fruit juice (MANA) gave increasing inhibition of ACE activity, α-glucosidase and cell proliferation of colon cancer cell lines and increased the basal NF-κB activity, *in vitro*.

Optijuice (15% BPEC and 85% MANA) and MANA significantly reduced SBP1 in a group of middle-aged individuals with elevated BP. The reduction was more pronounced in hypertensive than in normotensive subjects. Furthermore, Optijuice and MANA significantly reduced the variation of BP measurements during the intervention study. In the same intervention study we found that drinking Optijuice for 12 weeks resulted in significant reduction in DNA oxidation damage, and this response was more pronounced in participants with initial lower DNA oxidation damage than participants with initial higher damage at baseline.

Although this study design did not permit elaboration of causal relationships between specific compounds and health outcomes, the extracts/juices tested differed in polyphenol composition. The extracts and juices were rich in anthocyanins, and several of the detected anthocyanins, such as cyanidin-3-rutinoside, delphinidin-3-glucoside, and cyanidin-3-glucoside, are previously shown to have relevant bioactivities.

This thesis describes optimization of extract of polyphenols from black currant and bilberry press-residues which can be used in the food industry. The project has provided increased knowledge and upgrading of skills for all partners involved. The project has offered more knowledge about components and biological effects in press-residue extracts. This knowledge may provide a basis for new and healthier products in the future.

5.2 Future perspectives

An overwhelming and rapidly growing number of studies suggest that berries may have a potential for prevention of several diseases, but there are still gaps of the knowledge of the potential mechanisms behind the effects of berries in chronic disease prevention. Although it is rare to find rigorous evidence of the dietary benefits associated with the consumption of specific polyphenols, or even classes of compounds, there is a general belief that the polyphenols present in plant foods contribute positively to long–term human health. To reach conclusive evidence of the effectiveness of polyphenols in disease prevention and human health improvement, it is essential to determine the distribution of these compounds in our diet, estimate their content in each food item, have a better knowledge of their bioavailability and fate of their metabolites, and evaluate their biological activity and mechanism of action within the whole body. Since the stability and bioavailability of most of the polyphenols in

berries are affected by cooking, processing and storage, additional studies are needed to further understand their optimal preparation.

Overall, *in vitro* studies provide details on mechanistics and enhance the understanding of the potential health effects of berries and their compounds. The concentrations used in many *in vitro* experiments are, however, higher than the physiological concentrations that are possible through dietary intake.[234, 235] After eating polyphenol-rich foods or taking dietary supplements, the plasma polyphenol concentrations may be in the nanomolar range, but the polyphenol concentration used in *in vitro* studies are often in the micromolar range .[35] Elevated *in vitro* doses can be used to reinforce positive outcomes, but the results obtained with such high doses must be translated to *in vivo* situations with great care. On the other hand, one may argue that ingesting polyphenols in the human body may mean consistent exposure of cells to these polyphenols, and that prolonged exposure can produce significant effects even when tissue concentrations are low.[46] Further, since the polyphenols are quickly modified and metabolized in the human body, there is a challenge to find the physiological relevance from the *in vitro* experiments using plant polyphenols to the situation *in vivo*. Investigation of undigested polyphenols in model systems may not reflect the effects of *in vivo* metabolites that may be responsible for at least part of the health effects of polyphenols. During the recent years, scientists have started to focus on more physiologically relevant concentrations and it seems that the future research will pay special attention on the bioavailability of polyphenols. Better knowledge of the concentrations and bioavailability of dietary polyphenols will be essential in the future to properly evaluate their role in the prevention of diseases.

Whereas *in vitro* studies shed light on the mechanism of action of dietary polyphenols; these findings need support from *in vivo* experiments. It is important to actively investigate the potential biological activity of the metabolites of dietary polyphenols. Further *in vivo* investigations are required to translate the evidence obtained *in vitro* into actual outcomes and to understand the factors and mechanisms regulating the biological effects of berries and polyphenols.

While limited epidemiological data inversely associate consumption of berries with cancer, CVD and diabetes, these conclusions need to be strengthened in future prospective cohort studies investigating the long-term health benefits of berries in specific populations. Because extrapolations cannot be made between *in vitro* and *in vivo* systems, further studies should be designed to investigate the disease-preventive potential of berries in animal models and human subjects. In conclusion, it is strongly recommended that research of berries continues, as this may lay the foundation for the development of diet-based strategies for the prevention and therapy of several types of human cancers and other diseases. Research focus on nutrigenomics (effects of nutrients on the genome, proteome, and metabolome) and nutrigenetics (effects of genetic variation on the interaction between diet and disease) will be essential, as well as individual differences in metabolism and activity. Research efforts should continue to focus on elucidation of mechanisms of action at the cellular and molecular levels.

Finally, continued attention to increase fruit and vegetable consumption is a practical and important means for optimizing nutrition intake to reduce disease risk and maximize good health.

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Paper I

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Paper V