

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Processing of berries – Effects on functionality, stability and bioactivity of
anthocyanins

GABRIEL BARBOSA DE OLIVEIRA



Food and Nutrition Science
Department of Biology and Biological Engineering
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GABRIEL BARBOSA DE OLIVEIRA

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Department of Biology and Biological Engineering

Food and Nutrition Science

Chalmers University of Technology

SE-412 96 Gothenburg

Sweden

Telephone: + 46 (0) 31 772 1000

Fax: + 46 (0) 31 772 38 30

Cover: Strawberry with blueberry and kiwi fruit depicting a happy face in a culture plate containing YPD media and *Saccharomyces cerevisiae* spread with bilberries and fruit extracts. *Illustrated by Gabriel Barbosa de Oliveira.*

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GABRIEL BARBOSA DE OLIVEIRA

Department of Biology and Biological Engineering
Chalmers University of Technology, Gothenburg, Sweden

ABSTRACT

Berries are rich natural sources of anthocyanins, water-soluble pigments in plants that have been linked to beneficial effects against chronic diseases, such as cardiovascular diseases, type 2 diabetes and neurodegenerative diseases. However, anthocyanins as well as other polyphenolic compounds are often sensitive to degradation during processing, storage and digestion. The stability of anthocyanins is affected, for example, by exposure to high temperatures, oxygen and increasing pH. Non-thermal and mild processing approaches have been revealed as useful tools to extend the shelf-life of berries and preserve phenolic compounds during processing.

The overall aims of this thesis were first to evaluate different mild drying techniques and the fractionation of bilberry press cake from juice production toward obtaining phenolic-rich ingredients for incorporation into value-added food products. Second, to assess the recovery of anthocyanins in semi-dried berries and in coated whole berries, and for future analyses of compounds derived from breakdown or metabolism of anthocyanins, by developing a sensitive LC-MS/MS method. The recovery of anthocyanins was measured after non-thermal treatments and simulated gastrointestinal digestion of i) strawberry samples pre-treated with pulsed electric fields (PEF) prior to osmotic dehydration (OD) and ii) blueberry samples coated with chitosan and procyanidin. The third aim was to investigate the protective effect of digested anthocyanin extracts against H₂O₂-induced oxidative stress in yeast cells (*Saccharomyces cerevisiae*).

Hot air drying and microwave drying applied to bilberry press cake resulted in a small and similar reduction in the content of anthocyanins. Milling of bilberry press cake into powders with small particle size (< 500 µm) provided a powder with the highest apparent content of phenolic compounds. Considering the non-thermal treatments, the application of PEF-assisted OD of strawberries and the use of edible coatings to blueberries maintained or enhanced the stability of anthocyanins during *in vitro* gastrointestinal digestion. Digested strawberry samples osmotically dehydrated with trehalose and digested blueberries coated with chitosan and stored for 14 days, had the highest recovery of anthocyanins. A protective effect of anthocyanins from berry extracts was observed only when the yeast cells were pre-incubated with digested extracts. The results imply a biological effect, i.e. a changed phenotype during growth induced by digested anthocyanin-rich extracts, rather than a chemical effect.

In summary, berries can be tailored by mild processing to produce fortified ingredients and stabilise (poly)phenols during digestion. Also, the changes in their initial structures, occurring during digestion of berry extracts, are crucial to consider. Further studies on the bioactivities of anthocyanins and their transformed derivatives are needed to clarify the protective effects of digested anthocyanin-rich extracts.

Keywords: Berry fruits, anthocyanins, polyphenols, press cake, powder, hot air and microwave drying, gastrointestinal *in vitro* digestion, pulsed electric field, osmotic dehydration, edible coating, yeast, oxidative stress.

LIST OF PUBLICATIONS

This doctoral thesis is based on the work contained in the following papers:

- I. Lovisa Eliasson, **Gabriel Oliveira**, Maria Ehrnell, Evelina Höglund, Marie Alminger. Tailoring bilberry powder functionality by processing: effects of preprocessing. *Food Science and Nutrition*. 2019, 1-8.
- II. **Gabriel Oliveira**, Lovisa Eliasson, Maria Ehrnell, Evelina Höglund, Thomas Andlid, Marie Alminger. Tailoring bilberry powder functionality through processing: effects of drying and fractionation on the stability of total polyphenols and anthocyanins. *Food Science and Nutrition*. 2019, 1-10.
- III. **Gabriel Oliveira**, Urszula Tylewicz, Marco Dalla Rosa, Thomas Andlid, Marie Alminger. Effect of pulsed electric field-assisted osmotic dehydration and edible coating on the recovery of anthocyanins from gastrointestinal *in vitro* digested berries. *Foods*. 2019, 8, 505.
- IV. **Gabriel Oliveira**, Nataša Radovanovic, Marie Alminger and Thomas Andlid. Digested berry extracts increase survival of *Saccharomyces cerevisiae* during H₂O₂-induced oxidative stress. Submitted manuscript

Related publication not included in the thesis:

- V. Evelina Höglund, Lovisa Eliasson, **Gabriel Oliveira**, Valérie L. Almli, Nesli Sozer, Marie Alminger. Effect of drying and extrusion processing on physical and nutritional characteristics of bilberry press cake extrudates. *LWT – Food Science and Technology*. 2018, 92, 422 – 428.
- VI. Urszula Tylewicz, **Gabriel Oliveira**, Marie Alminger, Liisa Nohynek, Marco Dalla Rosa, Santina Romani. Antioxidant and antimicrobial properties of organic fruits subjected to PEF-assisted osmotic dehydration. Submitted manuscript

CONTRIBUTION REPORT

Paper I: The author, Gabriel Oliveira (GO), participated in the experimental work performing analyses of total phenolic content, interpreted data and writing of the manuscript.

Paper II: GO participated in a major part of the design of the study, performed the analyses of total phenolic content and anthocyanins and was responsible for writing the manuscript.

Paper III: GO performed the experimental work including the *in vitro* digestions analyses and the identification and quantification of anthocyanins, interpreted the data and was responsible for writing the manuscript.

Paper IV: GO participated in a major part of the design of the study, performed the experimental work, interpreted data and was responsible for writing the manuscript.

ABBREVIATIONS

CFU	Colony Forming Units
CID	Collision Induced Dissociation
CVDs	Cardiovascular Diseases
cy-glc	cyanindin-3- <i>O</i> -glucoside
DW	Dry weight
ESI	Electrospray Ionization
F-C	Folin-Ciocalteu
ff	flow factor index
GAE	Gallic Acid Equivalent
GI	Gastrointestinal
GIT	Gastrointestinal tract
Glut2	Glucose Transporter2
HAD	Hot Air Drying
HPLC	High Press Liquid Chromatography
LC	Liquid Chromatography
ma-glc	malvidin-3- <i>O</i> -glucoside
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MWHA	Microwave Hot Air
MWD	Microwave Drying
NND	New Nordic Diet
OD	Osmotic Dehydration
PEF	Pulsed Electric Field
ROS	Reactive Oxygen Species
SGLT1	Sodium Dependent Glucose Transporter1
SSF	Simulated Salivary Fluid
T2DM	Type-2 Diabetes Mellitus
TPC	Total Phenolic Content
UV-vis	Ultraviolet-visible
YNB	Yeast Nitrogen Base
YPD	Yeast Extract Peptone Dextrose

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1 INTRODUCTION

The consumption of berry fruits, which are rich sources of polyphenols, has been associated with health benefits in humans [1,2]. Several of these protective effects have been attributed to the intake of anthocyanins, particularly by protecting against chronic diseases, such as neurodegenerative diseases, diabetes and cardiovascular diseases [3–7] but little is still known about the mechanisms of action of anthocyanins.

Anthocyanins are found as glycosides in plant tissues, with glucose, galactose or arabinose being the most common sugar moieties linked to the aglycon structure (anthocyanidin) [8]. The glycosylation of anthocyanidins contributes to the stability and solubility of these pigments inside plant cells [9]. Anthocyanin-rich berries, such as blueberry and strawberry, are perishable seasonal products and their shelf-life is normally short. Substantial amounts of anthocyanins are enriched in peels and berry skins (press cakes), which are typically discarded by-products from juice production. These can be processed into more stable products used to add value and functionality to other food products. Considering that anthocyanins are unstable under certain conditions of temperature, light, oxygen, metallic ions and pH [8,10], the development of mild processing techniques, such as mild heat drying, pulsed electric field, osmotic dehydration and edible coating are interesting approaches to preserve the nutritional quality and extend the shelf-life of berries.

The choice of processing methods and conditions has a large impact on the characteristics of fruit and vegetable products including microstructure, flavour and micronutrient retention, textural properties, etc. [11]. In the hot air drying (HAD) process, which is a drying technique commonly used for berry preservation, the heated air meets the surface of the wet material that transfers heat from the air into the solid primarily by conduction [12]. On the other hand, microwave-assisted hot air (MWA) drying uses microwaves together with hot air to increase drying efficiency and decrease drying time. The MWA drying mechanism is different from HAD since the microwaves penetrate the product and increase the internal temperature. Drying processes using different methods have previously been reported to have significant effects on the degradation of bioactive compounds in berries [13]. Dried berry materials, such as powder and its fractions may be further used as food ingredients, for example, in extruded or dairy products.

Pulsed electric fields (PEF) is a promising emerging food preservation method that uses short electric pulses for microbial inactivation and cell tissue disintegration, to minimise detrimental effects on food quality [14]. PEF treatment applied to plant food processing is based on electrical stimulation pulses that increase the permeabilisation of the membrane of the cells leading to intracellular liquid release [15]. As shown in previous studies [16,17], a potential strategy to enhance the dehydration rate of berries could be the application of PEF as a pre-treatment of osmotic dehydration (OD), which is a useful tool to obtain semi-dried fruits. In the OD process, a counter-current mass transfer occurs, and water is eluted while the solute moves into the cell [18]. However, it is a slow process which depends on a number of factors, such as temperature, pressure, molecular weight and the type of osmotic substance [19].

Another mild processing approach to extend the shelf-life of fruits is the application of an edible coating which may create a semi-permeable barrier, improve textural quality, reduce microbial growth and retain the flavour and colour of the fruit [20]. Natural materials such as

polysaccharides, plant extracts, protein and lipids have been employed in the formulation of edible coatings [21]. For instance, chitosan combined with procyanidins from grape seeds has been successfully used as an edible coating ingredient to extend the shelf-life of fruits and vegetables [22,23]. Chitosan, an aminopolysaccharide derived from chitin, possesses antimicrobial properties [24] while procyanidins, polyphenols containing exclusively (epi)catechin, show antioxidant activities [25].

In this thesis, the stability of polyphenols, particularly anthocyanins, from blueberries and strawberries during processing was evaluated. Focus was on the effects of processing on the recovery of anthocyanins after *in vitro* gastrointestinal digestion as well as on the potential protective effects of digested anthocyanins and their derivatives against H₂O₂-induced oxidative stress in yeast cells. The objectives of this work were: i) to investigate whether different preprocessing applied to bilberry (a wild type of blueberry) might influence its powder quality ii) to evaluate whether bilberry press cake functionalities such as dispersibility and flowability could be tailored by applying different mild drying processes iii) to study the application of PEF-assisted OD or edible coating as non-thermal processing methods on the recovery of anthocyanins during processing and simulated GI-digestion and iv) to investigate the effects of *in vitro* digested berry extracts on the survival of yeast treated with hydrogen peroxide and whether these effects are due to chemical or biological activities.

The use of simulated gastrointestinal digestion is an interesting approach to investigate the impact of chemical transformation of anthocyanins and their effects on living systems such as *Saccharomyces cerevisiae*. *S. cerevisiae* is a well-characterised eukaryotic organism providing a relevant biological model for studying cellular pathologies in humans since its genome is significantly similar to higher eukaryotes, including humans [26]. Moreover, the use of *S. cerevisiae* as a cellular model is more feasible, less expensive and avoids ethical and regulatory considerations when compared to rodents and other mammal models. The yeast and the mammalian oxidative stress response mechanisms are similar and more than 25% of the genes related to ageing and human-degenerative pathologies, which pave the way to use yeast as a cell model for oxidative stress [27].

This PhD project was part of the European Union (ERA-NET Core Organic Plus) project “Innovative and eco-sustainable processing and packaging for safe and high quality organic berry products with enhanced nutritional value” with the overall aim to evaluate technologies to naturally extend the shelf-life of fresh organic berries and to process berries into a wide variety of value-added products with high nutritional quality and low environmental impact.

2 OVERALL AIM AND OBJECTIVES

The overall aim of this work was to evaluate the functionality of berry powders as well as the stability and biological activity of polyphenols, particularly anthocyanins, from berry fruits during processing and *in vitro* gastrointestinal digestion. The aim was also to study the protective effect of anthocyanins and their derivatives from simulated digestion on a cellular level using yeast cells stressed with hydrogen peroxide as a model.

The specific objectives of the studies included in this thesis were:

- To evaluate the effects of applying different preprocessing techniques (purée mixing and juice pressing vs. untreated whole berries) prior to drying, milling and fractionating on bilberry powder functionalities such as dispersibility and flowability.
- To assess the effects of hot air drying (HAD) and microwave drying (MWD) on total phenolic content (TPC) and anthocyanin composition of bilberry press cake powder fractionated into three different particle sizes. The goals were to identify the least destructive method that would preserve the quality of berry powders and their potential to be used as fortification ingredients.
- To study the effects of particle size on the dispersibility of hot air-dried bilberry press cake powders in water and dairy cream.
- To develop a sensitive LC-MS/MS method capable to detect and quantify anthocyanins and derivatives at very low concentration, as found in samples with digested berry extracts.
- To investigate the impact of non-thermal processing parameters on the stability of berry anthocyanins during simulated gastrointestinal digestion by assessing the effects of pulsed electric field-assisted osmotic dehydration treatment of strawberries to obtain semi-dried products and the use of edible coatings (made with chitosan from mushroom and procyanidins from grape seeds) on fresh, whole blueberries.
- To evaluate the bioactivity and potential protective effects of transformed anthocyanin derivatives, after *in vitro* digestion of berry extracts, against H₂O₂-induced oxidative stress using yeast cells (*Saccharomyces cerevisiae*) as a model.

3 BACKGROUND

3.1 Berries

Berries are fruits highly appreciated by the majority of consumers due to, for instance, their colours, texture, nutritional aspects and unique flavours. The major cultivated berry crops, excluding grapes, are strawberries, black currants, blueberries, red raspberries, gooseberries, cranberries and blackberries [28].

In common usage, the word berry refers to small, coloured and soft fleshy fruits that usually can be eaten whole such as strawberries, blueberries, blackberries and raspberries. However, botanical definitions would classify most of these as compound fruits while a "true" berry is a single fruit having seeds and edible pulp produced from a single ovary [29]. Therefore, by adopting the botanical concept, a banana and a tomato, for instance, are classified as berries while strawberry is not. In this thesis, we are referring to berries based on the common usage of this word.

3.2 Bilberry – a wild blueberry

The plant genus *Vaccinium* is a diverse genus in the Ericaceae family and includes over 200 described species, of which more than 100 are predominantly circumpolar. Blueberries are a group of small fruit-bearing plants belonging mainly to the species *Vaccinium corymbosum* (North-American highbush blueberry), *Vaccinium angustifolium* (Canadian lowbush blueberry) and *Vaccinium myrtillus* (European blueberry, also called bilberry). Differently from cultivated blueberries (*V. corymbosum* and *V. angustifolium*), bilberries (*V. myrtillus*) only grow in the wild. They are smaller in size and grow individually on the shrub making mechanical harvesting difficult compared to the cultivated varieties which are bigger and grow in clusters. In addition, another important difference between bilberries and domesticated blueberries is the anthocyanin content. The total content of anthocyanins in bilberries is over three-fold higher [30] compared to what is found in blueberries and the skin and flesh are both dark blue, compared to less anthocyanin-rich blueberries (Fig. 1).

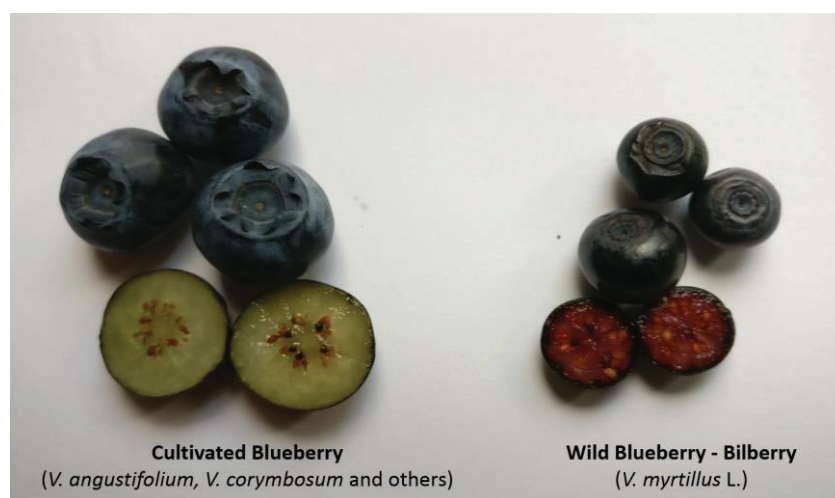


Figure 1. Main differences between bilberries and cultivated blueberries. Photo by Gabriel Oliveira, August 2019.

The content of anthocyanins in bilberries is not only significantly higher than cultivated blueberries but also higher than in many other berries and fruits, making bilberries one of the best natural sources of anthocyanins [31,32]. The chemical composition of bilberry can vary significantly depending on ripeness stage, climate conditions among other factors [33–36]. Table 1 shows the average chemical profile of bilberry fruits collected in Norway, Finland and Slovenia.

Table 1. Chemical profile of bilberry.

Chemical component	Average concentration (mg·Kg⁻¹ DW)	Reference
Glucose	25260	[35]
Fructose	25300	[35]
Total sugars	51900	[35]
Ascorbic acid	478	[33]
Citric acid	10640	[35]
Malic acid	4060	[35]
Quinic acid	6250	[35]
Shikimic acid	133	[35]
Fumaric acid	1.18	[35]
Total acids	21000	[35]
Total anthocyanins	35000	[36]
Total flavanols	1170	[37]
Total flavonols	1600	[37]
Total phenols	60300	[37]

Bilberry is a seasonal fruit and its production and consumption are higher during the summer in northern countries. For instance, in Sweden, around 20,000 tons of wild berries are picked annually making the country one of the biggest producers of wild berries in the world [38]. Unfortunately, less than 7% of the total yield of bilberries is collected [39]. Moreover, around 30% of the fruit in the form of press cakes is discarded as a residue by juice producers. Considering that the peel of the berries is the base of the press cake composition and contains high concentrations of anthocyanins, methods and technologies addressing the preservation and utilisation of bilberry press cakes are potentially of great value from both a nutritional and environmental perspective.

3.3 Processing of berries

3.3.1 Air drying and Microwave drying

A fast development of the market for food ingredients has been a consequence of the increasing consumer demand for diverse food products. Most of these products are supplied in a powdered form which requires the development of technologies capable of maintaining their quality and functionality. Powder forms of fruits can be used in many different products to enhance their colour, texture, flavour and nutritional quality [12,40]. The use of dried fruits and their application in powder form is an interesting subject to be developed since many of the fruits are highly perishable because of their high moisture content (around 80%) [21]. Drying a food product involves the evaporation of the loosely bound water and the free water from inside the solid material to the atmosphere. It is an intense and energy-consuming process responsible for 10-25% of the total energy used globally in the food processing industry [41].

Hot-air drying (HAD) also called convective or conventional drying is one of the most commonly adopted techniques in the food industry, even though long drying times and high air temperatures are required [12]. Compared with freeze-drying which is considered the gentlest food dehydration technique, costs of HAD have been quantified to be 4-8 times lower [42]. Basically, in the HAD process, heat is transferred by means of heated air by conduction to the solid food and, consequently, by air convection, liquid migrates to the surface of the material and evaporates. The whole process can be characterised in two different stages based on the drying rate. First, free water moves to the surface and is removed from the material by evaporation. Second, an increase in the viscosity of the solid material occurs, as the drying progresses, the liquid phase and consequently the drying rate declines since it takes more time for the internal moisture to move to the surface [43,44]. Temperature is one of the most important factors to be considered in the HAD process since it determines the quality of the end product [45,46]. Undesirable degradations such as colour deterioration, phytochemical depletion and shrinkage can be induced by high temperatures and have negative impacts on the quality [47–50].

In contrast to HAD, Microwave Drying (MWD) is a drying method where the mechanism is based on the electrical field's ability to rotate dipoles creating a fast heating rate (reducing drying time by up to 69% compared to HAD) due to a direct heating of the product [38,51]. In MWD food processing, a heating-up period occurs in which MW energy penetrates the food and creates internal temperature increase (electromagnetic energy is absorbed by water and converted into heat by molecular agitation) followed by a rapid drying period during which thermal energy is used for moisture vaporisation. Finally, a reduced drying rate period occurs during which the local moisture is reduced to a point that the energy needed for moisture vaporisation is lower than the thermal energy induced by MW [52,53]. In general, MWD technology has shown advantages in overall product quality such as colour and aroma, nutrient retention as well as considerable energy savings [50,54]. However, some studies reported drawbacks of MW drying such as textural damage and development of off-flavours [55–57]. Some disadvantages of MWD can be reduced by the combination of MWD with other drying methods such as HAD. Microwave-assisted hot air drying uses microwaves and hot air to increase drying efficiency and decrease drying time [58].

3.3.2 Pulsed electric field

The pursuit of environmentally friendly processing technologies connected with the increasing consumer interest in nutrient-rich fresh-like food products has boosted the development of non-thermal technologies such as pulsed electric fields (PEF). Regarding the study of alternative energy-efficient applications, PEF as a non-thermal food processing application has attracted much interest and developed into a major field of research in the last couple of decades [59]. This method has been shown to effectively extend the shelf-life of food products, enhancing extractability of phytochemicals, inactivating microorganisms and also retaining important aspects of freshness, including physical, nutritional and sensory qualities [14,60–63]. Compared with traditional thermal processes, PEF technology also exhibits several advantages, such as shorter processing time, lower treatment temperature, continuous flow and less degradation of nutritional and sensory characteristics of foods [64,65].

PEF is basically the application of an electric field in the form of short- or high-voltage pulses to food items, placed in a treatment chamber confined between two electrodes, for a short time, usually in the milli- or microsecond scale [65]. An electric potential is induced across the cell

membrane when an external electric field is applied to the food samples causing selective damage to cells: the membrane has been shown to be the only part affected with lipid portion being the site of the electric interaction [14]. This potential causes rapid electrical break-down and mechanical changes in the cell membrane. Consequently, membrane permeability drastically increases and pores are simultaneously formed in the membrane in a process called electroporation [59].

Pore formation is a dynamic process and can be reversible or irreversible depending on the treatment intensity [15]. In this process, the cell membrane acts as a capacitor with a low dielectric constant and free charges of opposite polarities present on both sides of a membrane, creating transmembrane potential. Accumulation of charges increases transmembrane potential when cells are exposed to an external electric field. Consequently, this additional potential raises compression pressure on the membrane with pore formation occurring in the sub-microsecond range at a given value of the applied field [66–68].

PEF has been demonstrated to be effective as a pre-treatment to increase water loss. For instance, Tylewicz et al. [16] have reported that PEF treatment prior to osmotic dehydration was found to positively affect the mass transfer, in terms of water loss from strawberry tissue. In another study, Nazari et al. [69] reported that the use of PEF as a pre-treatment increased water loss and solid gain by adjusting pulse number to an optimal condition in the osmotic dehydration of apple.

3.3.3 Osmotic dehydration

Osmotic dehydration (OD) of food products is a process which consists of the counter-current transfer of mass with the removal of moisture from the interior of the food to the hypertonic solution (water is eluted) while the solute flows into the matrix of biological tissues [18,70]. During the process, chemical, physical and biological activities, which deteriorate the foods, are lowered considerably; hence extending the shelf-life of food products [18]. The osmotic pressure difference between the food material and the hypertonic solution provides the necessary driving force for the removal of water from the food. The osmotic dehydration phenomenon continues until the water activity of both the solution and the sample attain the equilibrium state [70].

Many different factors can affect the osmotic dehydration process such as temperature and type/concentration of the osmotic agent. Temperature is the most important factor affecting the rate of osmotic mass transfer [71]. As the temperature rises, water loss accelerates [19] due to the plasticising effect of cell membranes and also to the lower viscosity of the osmotic medium. However, temperature greater than 60°C can damage plant tissues. Regarding the osmotic agent, the OD process is affected by its physicochemical properties, molecular weight, solubility and ionic state [72]. The most commonly used osmotic agents are glucose, sucrose, sodium chloride, glycerol and sorbitol [73]. It is important to mention that the use of sugar solutions reduces browning by preventing oxygen entry, providing stability to pigments and helping retain volatile compounds during drying of osmotically treated materials. Vicente et al. [74] have reported a significantly positive influence of the use of trehalose as an osmotic agent on apple tissue undergoing osmotic dehydration. Trehalose has a more protective effect on membranes than glucose. It is a disaccharide of glucose, which has more flexibility than other disaccharides. Colla et al. [75] suggested that the hydrogen bonding between trehalose and the polar headgroups of lipids in the cellular membranes contribute to preserving the integrity of biological structures.

The application of OD is a simple technique with low cost and low complexity reducing heat damage to the colour and flavour, as well as retarding enzymatic browning [73]. These partially dehydrated products can be used in products such as yoghurt, ice cream, desserts, confectionery products, snacks or components of cereals for direct usage [76]. On the other hand, it is a very slow process which makes the combination of OD with other processes an interesting strategy to reduce drying time and minimise energy costs.

3.3.4 Edible coatings

Edible coatings are defined as thin layers of edible material applied to the surface of food products to protect them and improve their quality. These primary packagings can play an important role in the conservation, distribution and marketing of fruits and vegetables [77]. Edible coatings act as hurdles between food and the surrounding environment being a barrier to moisture and gas and therefore slowing down the ripening rate of fruits [78]. They can protect the product from mechanical and physical damage as well as chemical and microbiological activities [79].

The increase in interest and research activity with regard to edible coatings is due to their ability to extend the shelf-life of foods and to rising consumer awareness concerning the harmful environmental effects of non-biodegradable waste resulting from packaging [78]. Edible coatings may be an interesting alternative because they are biodegradable and environmentally-friendly and can reduce the use of plastic packaging [80,81].

Edible coatings are applied in liquid form by methods that include brushing, spraying and dipping (usually by immersing the product in a solution formed with the structural matrix) [82]. The most common raw material used to form edible coating matrices are: A) Lipids – due to their hydrophobic nature, lipids possess good moisture barrier properties, which are important for preventing food products from physiologically deteriorating. On the other hand, lipids have poor mechanical properties and poor adhesion to food products with hydrophilic surfaces [83]. B) Proteins – which offer a high variety of physical and mechanical properties. On the other hand, proteins have a substantial risk of causing allergenic reactions that limit their use. C) Polysaccharides – such as cellulose derivatives, chitosan, starch, pectin, gums, algae-derived materials such as alginate and others are widely available, produce few allergic reactions and work as efficient oxygen blockers due to their well-ordered hydrogen bond network shape. Polysaccharides possess a defined chemical structure for each monomer unit which allows predicting and controlling of coating properties. However, they do not behave well as moisture barriers because they are hydrophilic in nature [84].

Chitosan coatings have been used successfully in the food industry, due to their structural properties that allow the formation of continuous layers. This polysaccharide is produced by deacetylation (in concentrated alkali solution) of chitin, which is a naturally occurring polysaccharide composed of N-acetyl-D-glucosamine units mainly found in arthropods and fungi. Chitosan is a safe, allergen-free and biocompatible polymer [85] with well-documented antimicrobial properties [86,87]. However, there are some reported challenges to the use of chitosan as an antioxidant and antifungal agent, such as identifying its optimum concentration to retard oxidation and fungal growth without undesirable effects on sensory properties of the final product [85].

A combination of two or more components, sometimes from different classes, can improve edible coating [88]. For example, polyphenols have been added to chitosan coating to enhance functional and antioxidant properties [22].

3.4 Phenolic compounds

Phenolic compounds are one of the biggest and widely distributed groups of aromatic substances produced as secondary metabolites in plants [89]. This group of substances is chemically heterogeneous comprising one (phenolic acids) or more (polyphenols) aromatic rings in their structures. The term (poly)phenols include mono and polyphenolic structures [90,91]. The main classes of dietary (poly)phenols are phenolic acids and flavonoids while the less common are stilbenes and lignans (Fig. 2).

Phenolic acids constitute near one-third of the phenolic compounds in the human diet. They are characterised by having a benzoic ring, a carboxylic group, and one or more hydroxyl and/or methoxy groups in the molecule [92]. Lignans are formed by two phenylpropane units while stilbenes are characterised by the presence of a 1,2-diphenylethylene nucleus [93,94]. Flavonoids are the most widely distributed and studied phenolic compounds in plant foods. They are classified according to the degree of hydroxylation and the presence of a C2-C3 double bond in the heterocyclic pyran ring [95]. Flavonoids are associated with a broad spectrum of health-promoting effects and among flavonoids, anthocyanins are considered one of the most biologically active compounds [96,97].

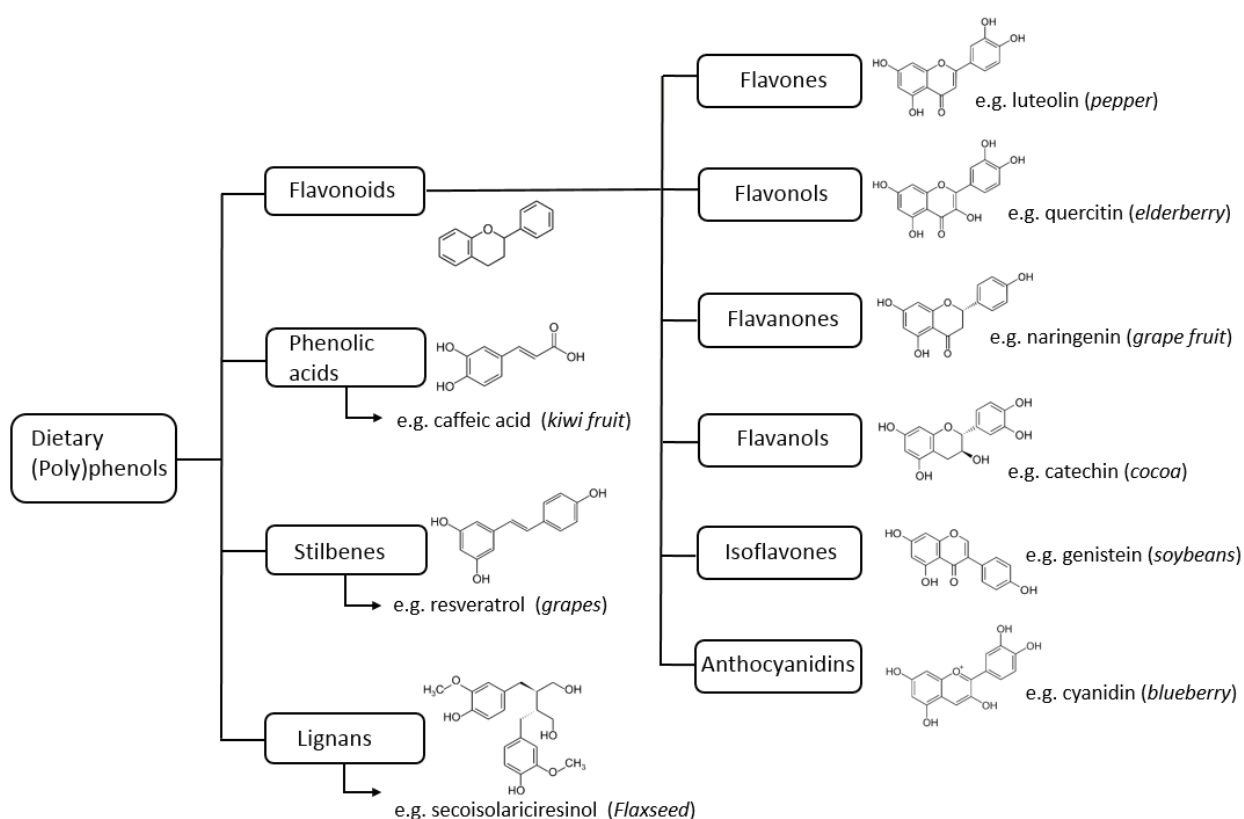


Figure 2. Schematic classification of (poly)phenols with their main chemical structures and examples of compounds and dietary sources [98–100].

3.5 Anthocyanins

3.5.1 Chemical structure and characteristics

Anthocyanins (of the Greek *anthos*, which means flower and *kianos*, which means blue) are the most important water-soluble pigments in plants that give the red, purple, and blue colouration of many fruits, flowers, and leaves [101]. The basic structure of anthocyanins (Fig. 3) is the flavylium cation, called anthocyanidin (2-phenylbenzopyrylium), a C6-C3-C6 structure which consists of an aromatic ring (A) bonded to a heterocyclic pyran ring (C) that is also bonded by a carbon-carbon bond to a second aromatic ring (B). Anthocyanidins are not present in nature owing to their instability. Anthocyanins are glycosides forms of anthocyanidins which vary in the number of methoxy and/or hydroxyl groups as well as in the type and number of the sugar moieties attached to the aglycon structure [8,102]. The most common sugar moieties (glucose, galactose, arabinose, rutinose, rhamnose and xylose) are bound to the anthocyanidins as mono-, di-, or trisaccharides, mainly at the C3-position of the C-ring (Fig. 3). The glycosylation of anthocyanidins contributes to maintain the stability and also to increase the solubility of these pigments inside the plant cells [9]. These sugars can also be acylated with aromatic acids, such as p-coumaric, ferulic, caffeic, and p-hydroxybenzoic acids and aliphatic acids, such as malonic, acetic, malic, and oxalic acids [103,104].

More than 700 types of anthocyanins have been identified and structurally elucidated from nature [105] and although more than 30 different types of anthocyanidins have been described only (delphinidin, cyanidin, petunidin, peonidin, malvidin, and pelargonidin) are the most common in the human diet (Fig. 3).

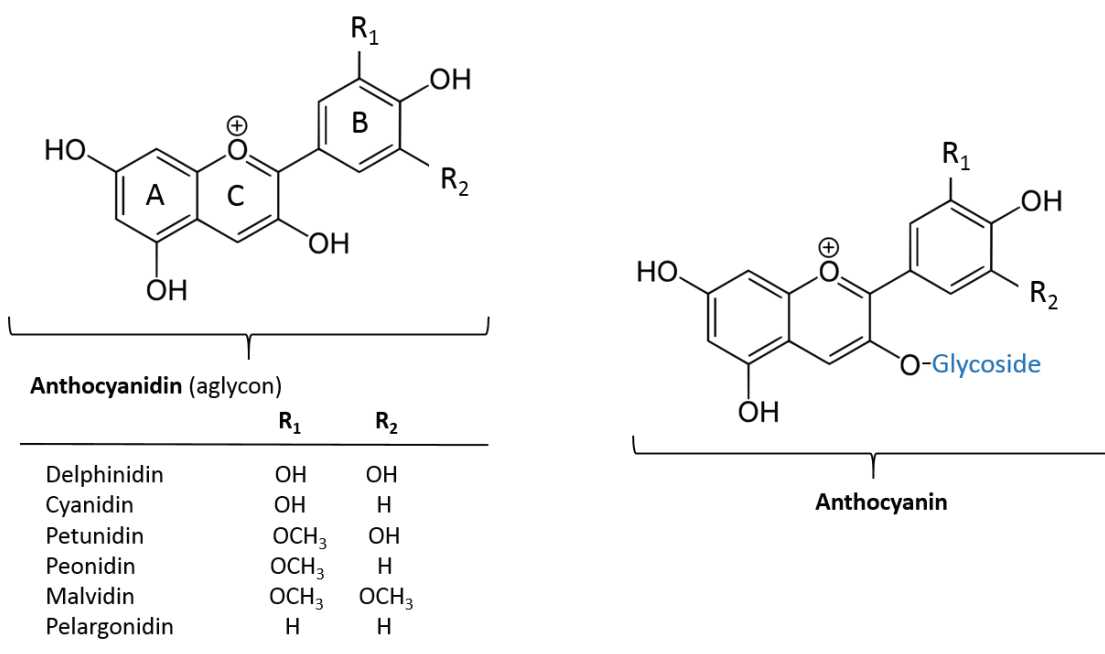


Figure 3. Basic chemical structures of the common anthocyanidins/anthocyanins [8,105–109].

3.5.2 Stability of anthocyanins

Anthocyanins, together with carotenoids, are among the most utilised vegetable colourants in the food industry [110]. However, the main challenge in the use of anthocyanins as natural colourants is the low stability of most of them to processing and storage conditions. The stability of anthocyanins depends on their chemical structure and also is determined by a combination of different factors such as temperature, enzymes, pH shifts, light, presence of other phenolic compounds, oxygen and metal ions [111–113]. Anthocyanins are among the least thermally stable flavonoids. For instance, Liu et al. [10] have reported that a preservation rate of total anthocyanin content in blueberry at pH 3.0 heated for 10 h dropped from 95% to 19% at 80°C. The same study has shown that the effect of pH on the total anthocyanin content of blueberry was significant and a low pH value (pH < 6) was beneficial to maintain the stability of blueberry anthocyanins.

The study of the effect of pH on the stability of anthocyanins is especially interesting since these pigments are exposed to different pH levels during transit through the digestive tract and the molecular transformation of anthocyanins from mouth to the intestine may interfere in their biological activity. Anthocyanins show higher stability at a pH of between 1 and 3, where they occur as flavylium cations (red-coloured). At pH between 2 and 4, the flavylium cation transforms, reversibly, either to the blue quinoidal base or to the colourless hemiketal, which further at pH between 5 and 6 undergoes ring-opening and produces the pale-yellow chalcone. At a pH higher than 6, chemical degradation of chalcone can occur, irreversibly, producing phenolic acids (Fig. 4) [102,105,109].

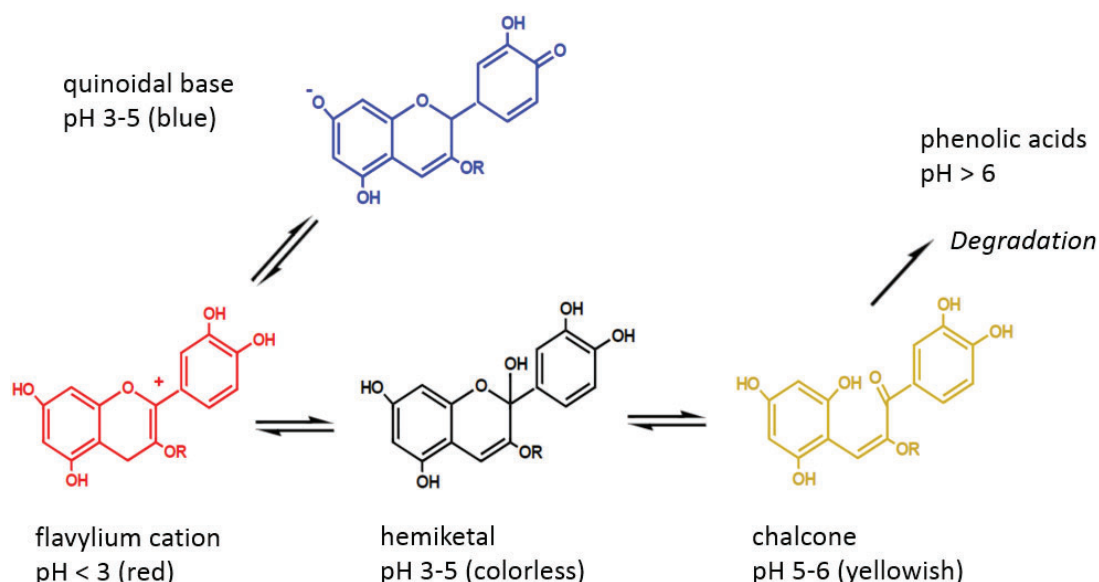


Figure 4. Chemical transformation of anthocyanins in different pH conditions [8,102,107].

3.5.3 Health effects of anthocyanins

Accumulating scientific evidence suggests protective effects of anthocyanin consumption against several chronic diseases, such as cardiovascular diseases (CVDs), type 2 diabetes mellitus (T2DM) and neurodegenerative diseases [43,44,53]. For many years, the proposed main mechanism of action behind the health effects provided by the consumption of anthocyanins were their antioxidant properties. It was proposed that by exerting a direct antioxidant effect against reactive oxygen species (ROS), anthocyanins could prevent inflammatory responses and consequently many diseases [115,116]. However, recent studies using cell models have shown that anthocyanins possess more complex mechanisms of action, such as cell signaling and modulation of gene expression [117,118]. It is also important to take into account that the products of the degradation of anthocyanins formed in the digestive tract are generally much more abundant than the residual anthocyanins and could mediate most of the potential effects of anthocyanins.

Cardiometabolic diseases such as cardiovascular diseases and diabetes mellitus are a major public health issue nowadays [119]. Only CVDs account for almost one-third of deaths worldwide [120]. Diet and lifestyle play an important role in the initiation and progression of these and other diseases. Recently, Yang et al. conducted a systematic review and meta-analysis of randomised controlled trials (RTC) assessing the effects of anthocyanins on glycemic regulation and lipid profiles in both healthy population and those with cardiometabolic diseases. From 33 eligible studies, 27 trials reported outcomes on lipid profiles and indicated that anthocyanin treatments were associated with decreased LDL and total cholesterol. The same meta-analysis study also showed a larger reduction of fasting glucose and LDL after treatments with purified anthocyanins or anthocyanin-rich extracts. Moreover, many other systematic reviews of animal and human studies have shown significant improvements in low-density lipoprotein oxidation after intakes of anthocyanins [121–124].

Type-2 diabetes mellitus (T2DM) is one of the most common non-communicable diseases all over the world that has been estimated to affect around 592 million people by 2035 [125]. This is a complex metabolic disease characterised by insulin resistance and progressive loss of β -cell insulin secretion leading to hyperglycemia. Different mechanisms of action are proposed for flavonoids with anti-diabetic effects. For instance, Su et al. [126] showed that pelargonidin-3-*O*-glucoside, an anthocyanin extracted from wild raspberry, attenuates hyperglycemia in hepatocyte cell models enhancing glucose uptake via autophagy induction.

3.5.4 Dietary intake and metabolism of anthocyanins

Anthocyanins are especially abundant in berries, among them bilberry (the low-bush blueberry species), blackcurrant, cowberry, chokeberry, cranberry and crowberry [127]. Also, a variety of berry products such as juice, wine, jam and food colourants (E163) contribute to the intake of these pigments. Other food plants rich in anthocyanins include eggplant, red cabbage and red onions [128]. The intake of anthocyanins differs depending on factors such as region, gender and culture (Fig. 5).

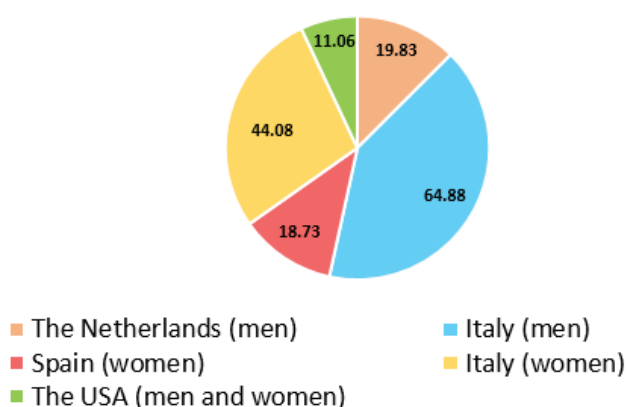


Figure 5. Average intake of anthocyanins (mg/day) for adults (age between 35 and 74) in Europe and the USA [129,130].

Differently from other flavonoids, the route of absorption and metabolism of anthocyanins after oral administration follows a unique pattern (Fig. 6). Considering the mouth, there is no specific evidence regarding the effects of saliva on anthocyanins, but the deglycosylation of flavonoids has been reported at this level of digestion [131]. At the low pH of the stomach, anthocyanins are significantly stable and absorption might begin in the gastric mucosa since, within 10 min to 30 min of intake of anthocyanin-rich fruits (before reaching the small intestine), high concentrations of anthocyanins have been reported to be detected in the plasma of rats and humans [132–134]. The gastric mechanism of absorption is not completely clear but studies suggest the involvement of the organic anion membrane carrier bilitranslocase [102,135] and that this absorption depends on the glycoside moiety present in the anthocyanin structure [134,136]. Continuing along the digestive tract, anthocyanins reach the small intestine where, in contrast to the stomach, the pH is close to neutral (around 6) and further in the colon, the pH can reach up to 8. Therefore, anthocyanins are converted from the stable flavylium cation to the unstable quinoidal/hemiketal base and to chalcones (Fig. 4) which can undergo degradation to phenolic acids and aldehydes.

The metabolism of anthocyanins in the intestine, their main site of absorption, seems to be complex but the main steps are suggested to be as follows. First, deglycosylation might occur by β -glucosidase in the lumen or/and by lactase phlorizin hydrolase at the brush border [137] releasing the lipophilic anthocyanidin form (aglycon), which could be able to passively enter the epithelial cells. An alternative way might be the absorption of intact anthocyanins by sodium-dependent glucose transporter 1 (SGLT1) [138] or glucose transporter 2 (GLUT2) [139], however, these mechanisms are still a matter of debate. Furthermore, the microbial population, particularly in the colon, has been shown to play an important role in the metabolism of anthocyanins [106,140].

Besides promoting the cleavage of glycosidic linkages (deglycosylation) due to the production of β -glucosidases, the microbiota is also responsible for the breaking of the heterocyclic C-ring of anthocyanins (Fig. 3) and degradation into phloroglucinol derivatives (from A-ring) and benzoic acids (from B-ring) [141]. For instance, Forester et al. [142] reported that anthocyanins from grapes, when incubated anaerobically in the contents of the large intestine of freshly slaughtered pigs, are completely degraded after 6 h and transformed into two phenolic acids (3-*O*-methylgallic acid and syringic acid) and one aldehyde (2,4,6-trihydroxybenzaldehyde). In another study using isotopically labeled cyanidin-3-glucoside (6,8,10,3',5'-¹³C₅-C3G) in humans, high ¹³C-dose recovery and maximal concentration of total metabolites were found in faeces after 24 h of intake, emphasising the importance of the microbiota in the metabolism of anthocyanins [143]. Likely, these new phenolic compounds, which may be absorbed and exhibit different bioactivity from the intact anthocyanins, are significantly important as promoters of health benefits.

The majority of anthocyanins are found in the circulation as glucuronidated, methylated and sulfated conjugates [144]. The enzymes responsible for these biotransformations (methylation, glucuronidation, and sulfation) are found in many tissues such as intestine, liver and kidney [104]. The route of elimination of anthocyanins depends on the type and the site of production of the conjugates. For instance, glucuronides formed in the intestine tend to enter the systemic circulation directly and are not readily available for biliary excretion unlike those newly formed in the liver, which are predominantly excreted into the bile [108].

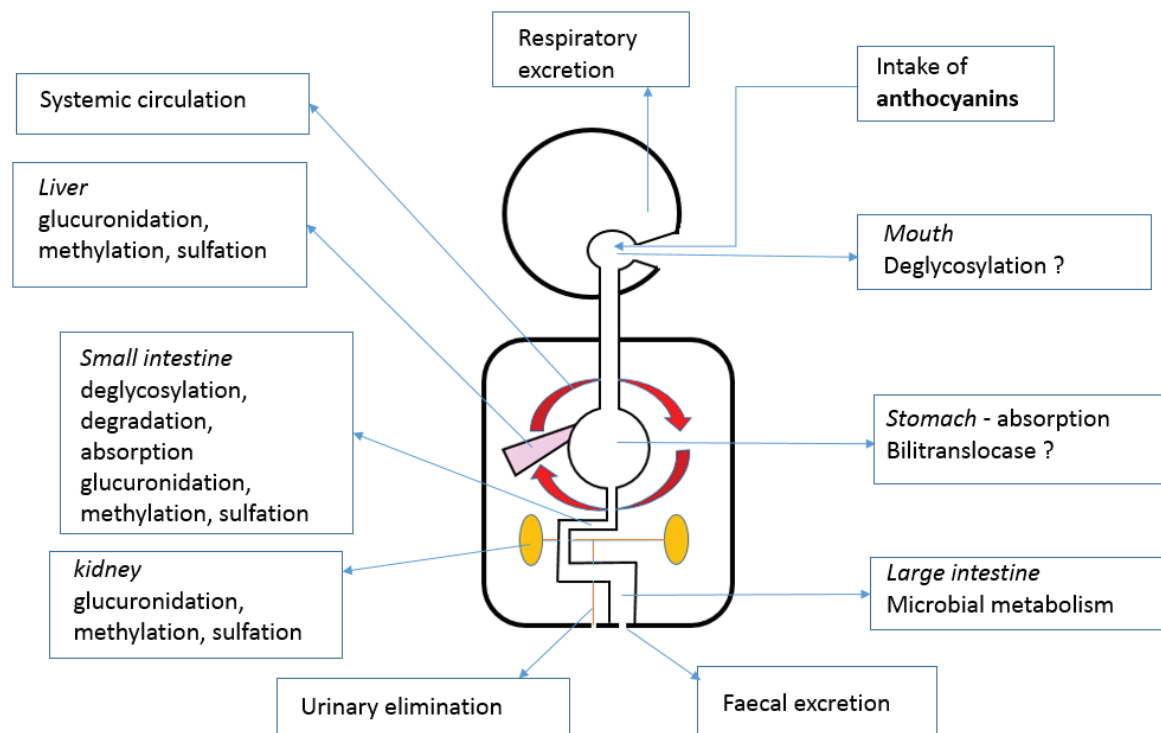


Figure 6. Schematic potential route of anthocyanin absorption, metabolism and elimination [105,107,108,145].

3.5.5 Analysis of polyphenols and anthocyanins

The Folin Ciocalteu (F-C) assay is the most commonly used colourimetric procedure for the determination of total phenolic content (TPC) in food extracts [146]. This method is based on electron transfer reactions in an alkaline medium between the F-C reagent (phosphomolybdic or phosphotungstic acid complexes) and the phenolic compounds which lead to a blue colour [147]. It is not a method specific to a particular group of phenolic compounds but serves to quantify the total concentration of phenolic hydroxyl groups. In the F-C assay, the TPC is expressed as “mg standard equivalent/g sample” with gallic acid use as a common standard in many different studies [148–150]. Gao et al. [147] have shown that among different commercial standards, gallic acid (which is a phenolic compound found in many plants) was comparably appropriate to be used in F-C assay based on its ionisation potential. The main drawback of the F-C assay occurs when determining the TPC of extracts obtained from fruits rich in ascorbic acid such as orange, kiwi fruit and strawberry. Ascorbic acid and also reducing sugars can interfere with the F-C reagent and result in overestimation of the TPC. There are some analytical strategies that can be applied to improve the selectivity of the F-C assay. For instance, partial purification of extracts based on solid-phase extraction; calculation of TPC by subtracting ascorbic acid or pre-treatment of extracts with oxidative agents at the levels that oxidise only the interfering compounds [146]. However, in fruits such as bilberry (*Vaccinium myrtillus*), which contain high levels of phenolic compounds compared with ascorbic acid and reducing sugars, these interferences may be negligible [30,33,35].

Conventionally, anthocyanins are identified and quantified using high-performance liquid chromatography (HPLC) coupled with ultraviolet/visible (UV-vis) spectrophotometry [151–154]. The mobile phase system is consistently binary, with an acidified aqueous phase and either methanol or acetonitrile. It is important to note that the mobile phase system has a low pH to keep the anthocyanins in their flavylum cation form, but caution must be taken to avoid degrading the column at such low pH values [155]. Anthocyanins show a maximum specific absorption at around 520 nm which allows separation of these compounds apart from other flavonoids in the plant extract. Moreover, the different anthocyanidin forms or the sugar moieties that form anthocyanins do not seem to have an effect on the observed spectra but do significantly change their retention times [155]. For instance, delphinidin has the greatest degree of polarity because it has the most hydroxyl groups, thus eluting first, on the other hand, malvidin has the most methoxy groups, giving it a more hydrophobic character and making it the last to elute from a reverse-phase column.

When anthocyanins need to be quantified at very low concentrations, such as in samples collected from the intestinal digestion or in plasma samples, HPLC-UV methods lack sensitivity and requires long run time to achieve optimal resolution. Therefore, sensitive analytical techniques such as high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) are needed to characterise and quantify these compounds accurately [101,103]. MS shows higher sensitivity compared with UV detectors and also higher selectivity can be achieved with the utility of multiple reaction monitoring (MRM) [156]. In the MRM approach, the use of collision-induced dissociation (CID) allows first the selection of a precursor ion in one quadrupole and this ion is further fragmented in a collision cell followed by a selection of a specific fragment ion in the second quadrupole. Among different types of ionisation methods, electrospray ionisation (ESI), is

useful to analyze anthocyanins because it is soft enough to avoid fragmentation of the molecular ion, providing the molecular mass of the analyte.

3.6 Static *in vitro* gastrointestinal digestion

Simulated gastrointestinal digestion methods are tools to estimate the effect of human digestion on food and specific compounds by mimicking physiological *in vivo* conditions. *In vitro* models allow screening of a large number of samples and enable prediction of changes during different stages of digestion. Although *in vivo* studies provide the most accurate results they are also more expensive, time-consuming and involve ethical issues [157], which makes *in vitro* models attractive alternatives.

These methods typically consist of sequential digestion steps in the oral, gastric and small intestinal parts of the gastrointestinal tract (GIT), taking into account, for example, the presence of digestive enzymes as well as their concentrations, pH, digestion time and salt concentration.

Dynamic models can simulate the continuous changes of the conditions in the GIT, including pH changes, altering enzyme concentration, peristaltic forces and transport of the digesta through the digestive tract [158]. However, static methods are the most common models described in the literature [159]. During static *in vitro* digestion, food is mixed with simulated fluids containing the appropriate electrolytes and enzymes corresponding to each step of digestion and then incubated at 37°C, with agitation [106,160–162].

The individual models described in the literature show significant variation in the use of *in vitro* digestion parameters. In studies III and IV in this thesis, a static digestion method is used (Fig. 7) based on the report from the COST Action INFOGEST, a consensus method derived from an international network joined by more than 200 scientists from 32 countries working in the field of digestion [163].

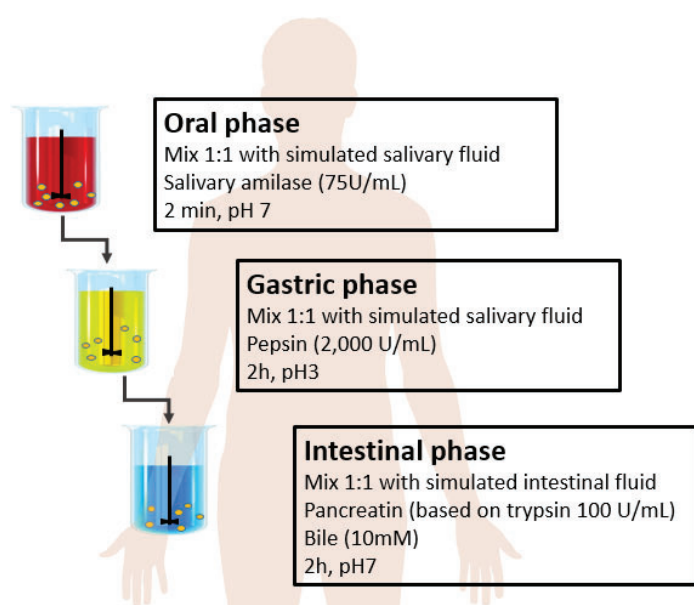


Figure 7. Flow diagram of the standardised INFOGEST *in vitro* digestion method.

3.7 *Saccharomyces cerevisiae* as a cell model for oxidative stress

Throughout the history of biological studies, the use of model organisms has been responsible for almost all knowledge accumulated regarding the fundamental properties of living cells. Among different model organisms used in science, baker's or brewer's unicellular yeast *Saccharomyces cerevisiae* is one of the most relevant ones [164]. It is the best-characterised eukaryotic organisms and the model system of choice for molecular and cellular biology studies. The genome of *S. cerevisiae* was the first eukaryote genome to be completely sequenced and was published in 1996. [165,166]. It revealed that a significant degree of homology and conserved pathways exist between yeast and higher eukaryotes, including human cells. Approximately 30% of human disease-associated genes significantly match yeast genes, which supports the relevance of using this model in the study of human disorders [167,168]. The use of yeast as a cellular model has many advantages over *in vivo* model as it is less expensive and avoids ethical and regulatory concerns when compared with the use of mouse, rat and other mammalian models.

S. cerevisiae has been successively used as a model to understand cellular oxidative stress damage and its relation to ageing and human neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease since its antioxidant response is similar to that of mammals [26,27,169–171]. Moreover, some studies have evaluated the antioxidant activity of dietary phytochemicals using *S. cerevisiae* as a model [172–174].

Yeast cells can grow on a wide variety of energy sources but consume glucose and fructose in preference to other sugars. In *S. cerevisiae*, glucose is able to repress the mitochondrial function (which is called Crabtree effect) and consequently this yeast ferments glucose to produce ethanol even under aerobic conditions [175]. A similar effect is observed in a variety of cancer cells and it has been proposed, as one of the possible explanations, that this aerobic fermentation serves as a means of accelerating growth by facilitation of mass accumulation [176].

4 METHODS AND METHODOLOGICAL CONSIDERATIONS

The experimental set-ups and methodologies included in the studies of this thesis are briefly described in this chapter. For a detailed description, the reader is referred to the corresponding papers.

4.1 Study overview

This thesis is based on four studies, I-IV, and an overview of their designs can be found in Figure 8. In studies I and II, the focus was on bilberry (*Vaccinium myrtillus*), which is one of the richest sources of anthocyanins among berry fruits. In study I, we evaluated the effects of applying different preprocessing techniques prior to hot air drying and milling on bilberry functionalities. Study II, as a follow-up of study I, focused on bilberry press cake powder to assess different drying techniques toward obtaining phenolic/anthocyanin-rich ingredients for incorporation into value-added food products and investigated the effects of drying and fractionation of bilberry press cake powders on the stability, dispersibility and solubility of phenolics/anthocyanins in water and dairy cream. In study III, the impact of non-thermal processing parameters on the stability of berry anthocyanins during simulated GI-digestion was evaluated by quantifying (using a sensitive LC-MS/MS method) the recovery of anthocyanins after digestion of i) strawberry samples, pre-treated with PEF, at 100 or 200 V·cm⁻¹, prior to osmotic dehydration and ii) blueberry samples coated with chitosan and procyanidin. In study IV we investigated the effect of anthocyanins extracts from berries and their derivatives after simulated GI-digestion on the survival of *Saccharomyces cerevisiae* exposed to H₂O₂.

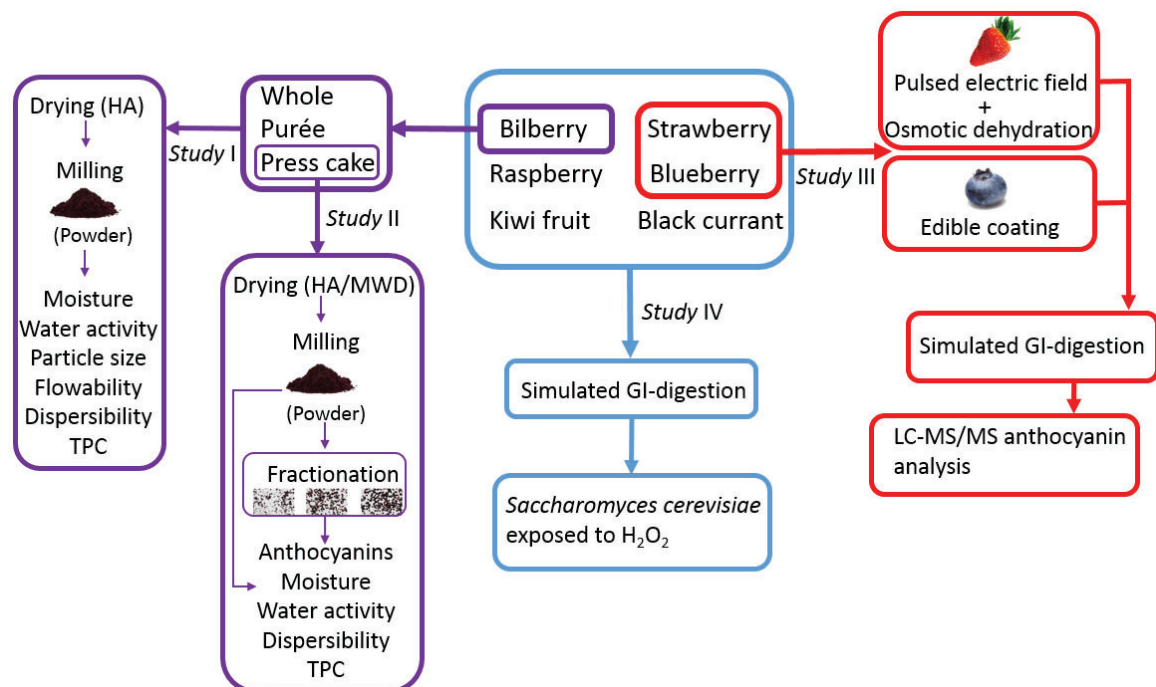


Figure 8. Schematic overview of the study designs. HA refers to hot air and MWD refers to microwave drying. TPC refers to total phenolic content and GI refers to gastrointestinal.

4.2 Preprocessing of bilberry (studies I and II)

4.2.1 Raw material and sample preparation

Bilberry samples (moisture content of $873 \pm 5.6\text{g/Kg}$) were supplied by Olle Svensson AB (Olofström, Sweden) and stored in the dark at -40°C prior to experiments. This raw material was used for studies I and II.

Before drying, the whole berries were thawed in the dark at room temperature for 2 h. The purée was prepared by mixing 280 g of whole berries in duplicate in a mini chopper (C3; Empire Sweden AB, Bromma, Sweden) for 40 s. The press cake was prepared by mechanical pressing (Hafico, Germany) of 900 g of berries that were thawed in the dark at 6°C for 16 h.

4.2.2 Drying and milling

In study I, HAD at 40°C was applied to the whole berries, puréed berries, and press cake until a water activity of around 0.5 was reached. Drying was carried out in a hot air oven (Garomat 142; Electrolux AB, Stockholm, Sweden) with an air velocity of 6.1 m/s.

In study II, where we focused only on bilberry press cake, besides HAD, MWD technique at 40°C was also applied. MWD was performed according to the procedure described by Kerbstadt et al. [38]. The microwave system consisted of a microwave cavity (Tivox AB, Tidaholm, Sweden) and an air-heating unit (Honeywell INU Control AB, Borås, Sweden). The microwaves were generated by a MagDrive-1400 (Tivox, Tidaholm, Sweden), and the microwave power was regulated automatically by the software MagDrive ver. 3.1 (Tivox Masik AB, Tidaholm, Sweden), depending on the temperature of the press cake material.

The dried material was stored from light at -40°C until milling. Each berry material was milled in triplicate (75 x 3) in a knife mill (Grindomix GM 200; Retsch GmbH, Haan, Germany) at 7,500 rpm for 30 s (study I). After milling, the bilberry powders were stored at -80°C until analysis. In study II, the milling time was 10 s instead of 30 s because we learned from study I that it was needed to produce larger particle sizes and consequently improve the flowability of the powder.

4.2.3 Moisture content, water activity and particle size distribution

In studies I and II, the moisture content of bilberry samples was determined gravimetrically in a vacuum oven (Sanyo Gallenkamp, Loughborough, UK). The water activity was measured in triplicate using the Aqua Lab 4TE (Decagon Devices, Pullman, WA, USA). Determination of the particle size distribution of the powder was achieved using a vibratory sieve shaker (Analysette 3; FRITSCH GmbH, Idar-Oberstein, Germany) with sieves of mesh sizes $250\ \mu\text{m}$, $500\ \mu\text{m}$, and $710\ \mu\text{m}$. Sieve shaking was performed for 10 min at an amplitude of 1.5 mm and an interval time of 10 s. In study II, the HAD press cake powders were separated into three different fractions: small ($< 500\ \mu\text{m}$); intermediated ($500\ \mu\text{m} - 710\ \mu\text{m}$) and large particle sizes ($> 710\ \mu\text{m}$).

4.2.4 Flowability

In study I, the flowability was evaluated and expressed as the powder flow function, which is the best-known indicator of powder flowability. The powder flow function, which represents a plot of the unconfined failure strength versus the major principal consolidation stress, gives a measure of the strength that the powder retains at a stress-free surface at a given stress level. A powder

flow tester (Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) was used to determine the flowability of the berry samples. The measurements were performed over five major principal consolidation stresses of approximately 1.6, 3.3, 6.9, 14.0, and 28.2 kPa. The bilberry powder was loaded into a volume shear cell of 38 cm³, and a vane lid was used to compress the sample during the analysis. The flowability of the powder was classified according to the flow factor index (ff): nonflowing, $ff < 1$; very cohesive, $1 < ff < 2$; cohesive, $2 < ff < 4$; easy-flowing, $4 < ff < 10$; and free-flowing, $10 < ff$ [177].

4.2.5 Dispersibility

In this thesis, in studies I and II, dispersibility was defined as the amount of particles, and the proportional number thereof, that remains suspended in the liquid phase after mixing of the powder with a liquid and subsequent resting time. The dispersibility levels of the bilberry powders were studied in water and dairy cream (15% fat, purchased in a local market), using a method adapted from those used previously [178,179]. Briefly, 5 g of dry powder was added to 100 g liquid (tap water or dairy cream) and stirred for 5 min using a magnetic stirrer (RTC basic; IKA-Werk GmbH, Staufen, Germany). After stirring, the suspension was left to rest for 12.5 min, to allow the suspended particle to settle, and thereafter, 7 g of the supernatant was carefully pipetted into a petri dish. The petri dishes with supernatants were placed in a vacuum oven (Fistreem International Ltd., Loughborough, UK) at 80°C and 90kPa, and dried until a constant weight was reached. A separate dispersibility experiment was performed to acquire samples for determination of the TPC of the supernatant.

4.2.6 Determination of total phenolic content

The total phenolic content was determined in studies I and II according to the methods described previously [180,181]. Freeze-dried bilberry powder and samples from the dispersibility experiments (0.2 ± 0.015 g) were extracted in triplicate with acidified methanol [MeOH:H₂O (70:30 mix) plus 1% trifluoroacetic acid]. After the addition of Folin-Ciocalteu reagent, the extracts were analysed spectrophotometrically against a standard curve of gallic acid measuring the absorbance at 765nm in a Safire² plate reader (TECAN) with the Magellan software. The total phenolic content (TPC) was presented as gallic acid equivalents (GAE) per kg of dry weight (g GAE/kg DW).

4.3 Analysis of anthocyanins in bilberry press cake powders (study II)

Bilberry press cake has the potential to be used as start material in the production of powder enriched in anthocyanins. Also, the use of this material has a sustainable approach and may contribute to adding value as an ingredient to other foodstuffs. Considering that anthocyanins are the most abundant polyphenols in bilberry, in study II, we decided to evaluate the stability of these compounds in the processed press cake. To do so, we extracted and analysed these pigments as described below.

4.3.1 Extraction of anthocyanins

Freeze-dried bilberry powder and samples from the dispersibility experiment were extracted using acidified methanol according to Bunea et al. [6] and You et al. [182]. Freeze-dried samples (0.200 ± 0.015 g) were mixed by vortexing for 30 s with 3 ml of methanol that contained 0.3 % HCl (v/v)

in a glass tube with a screw cap. Nitrogen gas was used to flush the air from the tube. The extraction mixture was placed in the dark at 4°C for 18 hr. After sonication for 15 min at 20°C, 37kHz (S15 Elma Sonicator, Elma Schmidbauer GmbH), the samples were centrifuged at 2,000 g for 10 min and the supernatant fluid was collected. Re-extraction was carried out by adding 3 ml of acidified methanol to the pellet, followed by vortexing, centrifugation, and finally pooling the supernatants from each extraction. The supernatants (6 ml) were centrifuged at 4,000 g for 15 min and stored at -20°C until analysis. It is important to mention that extraction based on Methanol:H₂O₂ (50:50) acidified using acetic acid 0.1 mL/L [182] was also performed, however, the method using acidified methanol (as described above) was found to be the best.

For the extraction of anthocyanins from bilberry powder samples dispersed in cream, an adaption of the method described by Jing et al [183] was used. The initial extraction steps were similar to those in the method described above but with the modification that 4 ml of acidified methanol was used and after shaking for 15 s, the mixture was stored in the dark at 4°C for 18 h. After sonication for 15 min and centrifugation, 3 ml of chloroform was mixed thoroughly with the supernatant in a test tube with a screw cap. After the addition of 3 ml of ultrapure water and mixing, the mixture was centrifuged at 4,500 g and 4°C. The upper aqueous phase containing the anthocyanins was transferred to a new tube, and the extraction was repeated again with the remaining chloroform phase by adding 3 ml of ultrapure water, followed by centrifugation.

4.3.2 HPLC-UV analysis of anthocyanins

The HPLC system consisted of a quaternary gradient pump (Jasco PU-2089 Plus; Jasco Inc., Easton, MD, USA), a cooled (8°C) auto-sampler (Jasco AS-2057 Plus), and a UV detector operating at 520 nm (Shimadzu SPD-10A UV-Vis detector; Shimadzu Corp., Kyoto, Japan). Separation of the individual anthocyanins was achieved with a Phenomenex Luna C₁₈ column (150 x 3.0 mm, 3 µm) at 40°C, with 5% aqueous formic acid (A) and methanol (B) as eluents. A linear gradient was applied at a flow rate of 0.5 ml/min with an increase from 13% to 25% of eluent B over 25 min, followed by an increase to 50% of eluent B over 2 min, and isocratic elution at 50% with eluent B for 5 min prior to injection of the next sample. The injection volume was 10 µL. Identification was based on retention time, UV spectra, comparison with commercial standards (cyanin chloride, malvin chloride, malvidin-3-*O*-glucoside chloride, peonidin chloride, petunidin-3-*O*-glucose chloride, cyanidin-3-*O*-glucoside chloride, malvidin chloride, cyanidin chloride, delphinidin chloride, cyanidin-3-*O*-arabinoside chloride, delphinidin-3-*O*-galactoside chloride, and peonidin-3-*O*-glucoside chloride from Extrasynthese, Genay, France), and the relative retention values from literature [127,184,185].

For quantification of the identified compounds, a five-point external standard calibration curve was prepared by dissolving the anthocyanin standards in aqueous methanol (50% v/v). The linearity of the standard curve was found to be acceptable ($R^2 > 0.998$). Internal standards were prepared by spiking some extracted samples with cyanin chloride, to evaluate any fluctuation in the response, and malvidin-3-*O*-glucoside was used to evaluate matrix effects.

4.4 Effects of non-thermal processing on the stability of anthocyanins during simulated GI-digestion (study III)

Strawberry and blueberry are among the most consumed and appreciated berry fruits. However, these berries are highly sensitive and perishable. Considering that strawberry is an interesting model to the development of semi-dried fruits and blueberry a model to study edible coatings of whole fruit, many studies have been conducted to extend the shelf-life of these fruits without reducing their nutritional value. In study III, we decided to investigate a further step on this approach: the effect of non-thermal processing, specifically PEF-assisted OD and edible coating on the stability of anthocyanin during simulated GI-digestion.

4.4.1 Raw materials

Organic strawberries and blueberries were purchased from the local market in Cesena, Italy. The fruits were kept at $4 \pm 1^\circ\text{C}$ until used (maximum storage time of one week). Fresh fruits with similar colour and size and without damage were selected. The strawberries were washed, hand de-stemmed and divided in half along the central axis of the fruit, while blueberries were used as whole berries.

4.4.2 PEF-assisted osmotic dehydration treatment on strawberry samples

Strawberry samples were pre-treated using a pulse generator (S-P7500 60A, 8 kV, Alintel srl., Bologna). A rectangular treatment chamber (5 x 5 x 5 cm) equipped with two stainless steel electrodes (5 x 5 cm). The conductivity of the water was adjusted using sodium chloride to achieve a value of 1.6 mS/cm (measured by EC-Meter basic 30+, Crison). The following parameters were used for PEF pre-treatment: electric field strength of 100 and 200 $\text{V}\cdot\text{cm}^{-1}$, rectangular pulses, pulse number of 1000, pulse width of 10 μs , frequency of 100 Hz, and treatment time of 10 s. The electric field strength 100 $\text{V}\cdot\text{cm}^{-1}$ was selected in order to obtain reversible pores and 200 $\text{V}\cdot\text{cm}^{-1}$ for irreversible pores, in accordance with previous reports [16,17,186]. Field strengths above 200 $\text{V}\cdot\text{cm}^{-1}$ were avoided since the application of field strengths of 250 and 400 $\text{V}\cdot\text{cm}^{-1}$ have been reported to damage cells in apples [187].

The OD treatment was carried out by immersing the strawberry samples in hypertonic sucrose or trehalose solutions (40%, w/w) with the addition of 1% (w/w) of calcium lactate (CaLac) as a structuring agent [16]. The samples were continuously stirred at 25°C during the whole treatment (120 min). The fruit to solution rate was 1:4 (w/w), to avoid concentration changes in the solution during the treatment.

4.4.3 Edible coating on blueberry samples

Two different coating solutions were prepared, the first one contained 1% (w/w) of chitosan from mushrooms provided by Agrovin (Alcazar de San Juan, Spain), while the second one was prepared by combining chitosan from mushroom (1% w/w) with 0.8% (w/w) procyanidins (extracted from grape seeds, Cesena, Italy). To each solution, 1.5% (w/w) of glycerol, 0.2% (w/w) of Tween® 20 and 1% of citric acid solution were added, as described by Mannozi et al. [20].

The coating solutions were applied by dipping in two steps (each step 30 s). The first dipping was followed by 60 min of drying at $25 \pm 1^\circ\text{C}$, and the second by 30 min at the same temperature.

Afterwards, coated berry samples were placed in plastic trays, in closed micro-perforated bags and stored at 4°C for 14 days.

4.4.4 *In vitro* GI-digestion of processed berry samples

To investigate the effects of processed berries on the stability of anthocyanins during digestion, human studies would be ideal because of the high complexity of all physiological aspects involved in the gastrointestinal tract. However, there are some advantages in using *in vitro* studies rather than *in vivo* studies such as low cost, no ethical issues and high reproducibility.

A static *in vitro* digestion method based on the standardised protocol published by Minekus et al. [163] was used in this study. The samples (in powder forms) were submitted to IVD method which consists basically of three steps: an initial step simulating the oral phase in the mouth with simulated salivary fluid (SSF) containing alfa-amylase, followed by digestion with pepsin/HCl to simulate conditions in the stomach, and a third step with bile salts/pancreatin to simulate conditions in the small intestine. The whole digesta for each sample was frozen (-80°C), freeze-dried and stored at -20°C prior to extraction and analysis of anthocyanins.

4.4.5 *Development of a sensitive LC-MS/MS method for analysis of anthocyanins*

In study III, anthocyanins were extracted using the same method described previously in study II. However, since the anthocyanin concentrations were low in some samples, due to degradation during digestion, a sensitive method was needed and a LC-MS/MS method was developed and used. The separation of anthocyanins was made at 40°C with a Zorbax Eclipse Plus C18 – 2.1x50mm – 1.8 m column (Agilent Technology) using a Shimadzu LC 30A system combined with a Shimadzu 8030+ MS detector with an electrospray ionisation (ESI) source. The mobile phase consisted of an aqueous 0.5% formic acid (A) and 0.5% formic acid in methanol (B). The flow rate was set at 0.5 mL/min. The samples were eluted using a linear gradient: 0-1 min, 15% B; 1-5 min, 15-50% B; 5-5.1 min, 50-100% B; 5.1-6.0 min, 100% B isocratic; 6.0-6.1 min, 100-15% B. The sample injection volume was set at 5 µL. All the MS/MS parameters were optimised by the infusion of each of the standards (cyanidin-3,5-di-*O*-glucoside chloride, malvidin-3,5-diglucoside, malvidin-3-*O*-glucoside chloride, petunidin-3-*O*-glucose chloride, cyanidin-3-*O*-glucoside chloride, cyanidin-3-*O*-arabinoside chloride, delphinidin-3-*O*-galactoside chloride, and peonidin-3-*O*-glucoside chloride) at 1µg/mL. The optimal ionisation conditions and fragmentation patterns were determined using instrument software (Labsolutions, Shimadzu). Multiple reaction monitoring (MRM) parameters for each anthocyanin standard were determined and is presented at paper III. In positive ion mode, the mass spectrometry conditions were as follows: nebuliser gas flow, 2 L/min; drying gas flow, 10L/min; heating block temperature 400°C; DL temperature 250°C; mass range from 50 to 1200 *m/z*. Standard calibration curves were constructed for all analytes and the linearity for all curves were evaluated. The coefficient of variation CV% (also called RSD) was calculated for each concentration of anthocyanin extract. The limit of quantification (LOQ) for each anthocyanin was based on the lowest concentration measured where the CV % was <20 %.

The recovery of anthocyanins in treated strawberry and blueberry samples was estimated by measuring the relative amount of anthocyanins left after *in vitro* digestion. The initial quantity of anthocyanins in the sample before *in vitro* digestion was considered 100%. The recovery of each

anthocyanin (%) was calculated by dividing the amount of anthocyanins after digestion by the amount of anthocyanins before digestion and multiplying this quotient by 100.

4.5 Protective effects of digested anthocyanins (Study IV)

In the previous studies, we were trying to identify treatments and processing methods that could enhance the stability of anthocyanins during processing and digestion. In study IV, we hypothesised that the gastrointestinal digestion process plays an important role in the beneficial effects associated with anthocyanins and that anthocyanins and/or their derivatives might have protective effects against oxidative stress. To the approach described below, using a combination of *in vitro* digestion to provide physiologically relevant samples, and *Saccharomyces cerevisiae* as a cellular model was used to evaluate the potential bioactivity of the berry samples.

4.5.1 Berry raw material

Strawberries (*Fragaria ananassa*), of the variety Malwina and black currant (*Ribes nigrum*) of the variety Ben Tron, were collected in Dingle and in Fagerfjäll, Sweden, respectively and bilberry (*Vaccinium myrtillus*), a wild variety of blueberry, was provided by Immun Skellefteå, Sweden. Raspberries (*Rubus idaeus*), of the variety Glen Ample, were harvested in Norway and kiwi fruit (*Actinidia deliciosa*), of the variety Hayward, was purchased from the local market in Cesena, Italy. All samples were freeze-dried, milled and stored at -80°C prior to analysis.

4.5.2 *In vitro* digestion of berry samples and extraction of anthocyanins

In study IV, the *in vitro* digestion method applied to the berry samples was the same as described previously in study III. The extraction of anthocyanins was also the same as described before in the studies II and III, with the difference that in this study (IV), the solvent (acidified methanol) was completely evaporated from the final solution and the remaining compounds in the tube were resolubilised in yeast nitrogen base media (YNB) and stored at -20°C until analysis.

4.5.3 Yeast strain and growth conditions

In all experiments of study IV, we used the diploid yeast strain *Saccharomyces cerevisiae* SKQ2n (ATCC 448227; a/alpha; ade1/+; ade2; +/-his1). This strain has previously been used as a reference/model in fundamental studies of osmotic stress response [188,189]. The cells cultured in sterilised medium containing 0.67% (wt/vol) yeast nitrogen base (YNB, Difco Laboratories) with 5% ammonium sulfate, supplemented with 2% of glucose. The yeast cells were grown at 30°C in 10 mL tubes in a LabRoller™ Rotator at 30 rpm and maintained on plates with YPD agar (per 1: 10g of yeast extract, 20 g of peptone, 20 g of D-glucose and 20 g of agar). Optical densities were measured at 600nm (OD₆₀₀) using a ULTROSPEC 10 cell density meter (Amersham Biosciences).

4.5.4 Evaluation of the optimal concentration of H₂O₂ for oxidative stress

A pre-culture was grown in YNB supplemented with 2% glucose, for approximately 9 h until an OD₆₀₀ of 6 was reached. The cells were harvested by centrifugation at 4000 g for 5 min. The pellet was resuspended in 0.9% NaCl and adjusted to an OD₆₀₀ of 1.0 in a 10 mL culture tube and subsequently diluted 10 times. Oxidative stress was induced by dividing the cellular suspension in 10 tubes and adding H₂O₂ to different concentrations ranging from 0.1 to 1.8 M to a final volume of 1 mL for each sample. The samples were incubated at room temperature with shaking at 180

rpm for 10 min and immediately diluted with saline in 10 mL culture tubes to stop the stress exposure. Analyses of viable cells were performed by making serial dilutions with 0.9 % NaCl in the range of 10^1 - 10^4 for each sample and spotting 10 μ L drops of the 10^2 - 10^4 dilutions onto YPD plates in pentaplicates. The plates were incubated at 30°C for 2 days and thereafter analysed for colonies. The relative survival rate of the yeast cells was calculated as percentage by dividing the number of colony-forming units (CFU/mL) after exposure to H₂O₂ by the number of CFU/mL untreated cells (control), which was considered as 100%.

4.5.5 Assessment of the protective effect of berry extracts against oxidative stress

A pellet from a pre-culture grown as described previously was suspended and adjusted to OD₆₀₀ of 0.2 in culture tubes containing YNB supplemented with 2% of glucose, chloramphenicol (200 mg.L⁻¹) and digested or non-digested berry extracts. The concentration of berry extracts was set at 10 mg.L⁻¹, which has been reported as non-toxic to *S. cerevisiae* [173,190]. The tubes were incubated at 30°C in a rotator at 30 rpm during 9 h. As controls, tubes containing the yeast cells without berry extracts or with the addition of reagents used during the *in vitro* digestion experiments were cultivated, using the same conditions as described above. The yeast cells were also cultivated in YNB with extracts from *in vitro* digested berry extract plus the addition of selected commercially purchased phenolic acids (Table 2). The choice of supplemented compounds was based on prior studies showing these to be the most commonly detected phenolic acids in human plasma after berry intake [191]. The oxidative stress assay with H₂O₂ was made in triplicates (three independent yeast suspensions) for each experimental mix as described above, at room temperature (180 rpm, 45 min). At intervals, samples (100 μ L) were removed from each cellular suspension every 5 min and immediately diluted in order to stop the stress exposure. Also in parallel, the cellular suspensions were diluted, spotted onto YPD plates and analysed for the number of colonies and survival was calculated according to the procedure described above.

Table 2. Phenolic acids used as supplements in the yeast cultures.

Phenolic acids	Concentration (nM)
homovanillic acid	600
protocatechuic acid	120
vanillic acid	5000
ferulic acid	60
syringic acid	100
p-coumaric acid	40
sinapinic acid	70
caffeic acid	70
gallic acid	16000
pyrogallol	500
p-hydroxybenzoic acid	200
3,4 dihydroxyphenylacetic acid	800

Two additional experiments were conducted to investigate whether the protective effects of berry extracts on yeast cells result mainly from a direct chemical neutralisation of H₂O₂ or from biological mechanisms: I) samples cultured with berry extracts were washed with 0.9% NaCl just before exposure to H₂O₂ and II) yeast cells were cultivated without berry extracts using the same growing

conditions described previously, with the berry extracts being added to the cultured tubes at 5 min prior to exposure to peroxide.

The yeast cells were also cultured with digested and non-digested kiwi fruit extracts to evaluate if a different fruit matrix without anthocyanins such as the kiwi fruit of the variety Hayward [192] could influence the survival rate of the cells during stress.

4.6 Statistical analysis

One-way analysis of variance (ANOVA) and *Tukey's* Honestly Significant Difference (HSD) test were applied to compare effects between treatments. Statistical Significance was defined as $p < 0.05$. Statistical analyses were performed using the IBM® SPSS® Statistics ver. 24 software.

5 RESULTS AND DISCUSSION

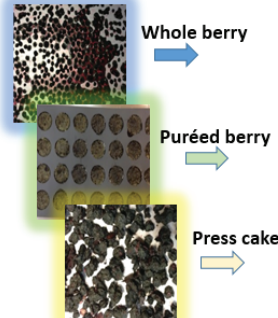
5.1 Effects of preprocessing of bilberry

5.1.1 Drying time and moisture content

In study I, the drying time required to attain a water activity of about 0.5 in bilberry samples was strongly influenced by the choice of preprocessing. Whole untreated berries showed the longest drying time (27 h), followed by 15 h for the puréed berry and 7.5 h for the press cake (Table 3). The waxy skin of whole berries acts as a barrier during drying, retarding moisture removal. By blending the berries to a purée, the drying time was reduced by 44%, due to extensive disruption of the bilberry skin. The press cake had a lower moisture content from the start (693 g/kg), compared to the whole berries (873 g/kg) and puréed berries (873 g/kg), which explains the more rapid drying of the press cake. In addition, mechanical pressing ruptures the bilberry skin, thereby facilitating moisture removal. Together with the juice, sugars and acids likely disappear from the press cake as described by Laaksonen et al. [36]. A lower sugar content of the press cake, reducing its hygroscopicity, could also facilitate moisture removal during drying [193].

The moisture contents of the whole berry and puréed berry powders were 40.9% - 45.1% higher than those of the press cake powder, although the water activity for all the powders lies within the range of 0.49 - 0.50 (Table 3). The higher moisture contents of the whole berry and puréed berry powders could possibly be connected to higher sugar contents in these samples compared to the press cake powder in which these components likely have been removed together with the juice [36]. Sugars bind water, thereby reducing the proportion of free water and lowering the water activity [194]. Pretreatment of raspberries in different sugar and acid solutions before drying has previously been shown to influence the equilibration between moisture content and water activity in dried raspberries [195].

Table 3. Moisture content (g/Kg), drying time (h), and water activity of powder from dried whole berries, puréed berries and press cake.



	Drying time (h)	Moisture content (g/Kg)	Water activity
Whole berry	27	132 ± 1.1	0.49 ± 0.001
Puréed berry	15	142 ± 0.7	0.50 ± 0.001
Press cake	7.5	78 ± 0.2	0.5 ± 0.003

5.1.2 Particle size distribution

The particle size distributions of food powders are of industrial importance because of their influences on various properties, such as flowability, bulk density, and compressibility [196]. A similar pattern of particle size distribution was noted for all the bilberry powders, with some differences. As shown in Figure 9, press cake powders, had the highest proportion of small particle

size (<250µm) fraction with 29.4% (294 g/kg) compared to 13.8% and 14.5% for whole berry and puréed berry powder, respectively. In contrast, the whole berry (48.5%) and puréed berry powders (42.8%) showed a higher proportion of the size fraction 250-500 µm compared to press cake powder (34.6%). After drying, the press cake was lower in moisture content than the whole berries and puréed berries, which likely rendered the material more friable and therefore easier to mill into smaller particles. Also, differences in sugar content, the shape and size of the dried materials before milling could have influenced the final particle size. To summarise, the use of different pre-processing material depending on the applicability was found to be a useful means to tailor bilberry powder.

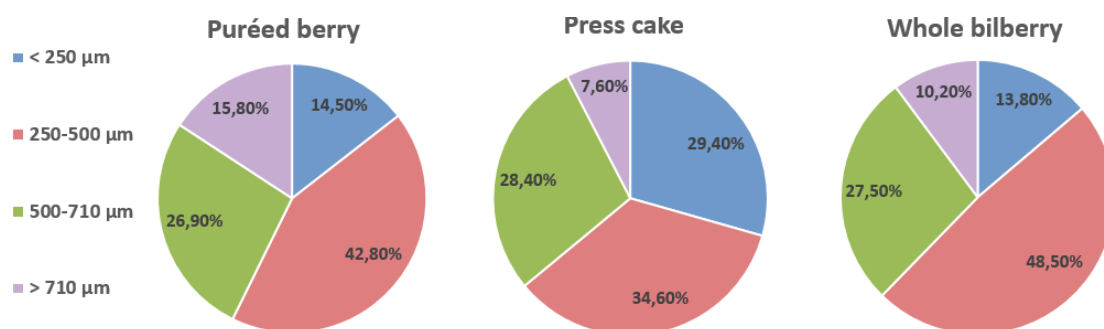


Figure 9. Particle size distribution (%) of bilberry powders from dried whole berries, puréed berries, and press cake.

5.1.3 Effects of preprocessing on flowability

The flow behaviours of food powders are crucial parameters for handling and processing operations such as storage, transportation, mixing, and packaging, and they are affected by, for example, moisture content, composition, particle size and particle structure [197]. The flow functions of both the whole berry and puréed berry powders showed a highly cohesive behaviour, while the press cake powder was cohesive on the cross-section to easy-flowing. A powder becomes more cohesive with increasing moisture content due to the formation of liquid bridges between the particles [197]. Therefore, the lower moisture content of the press cake powder (Table 3) makes it easier to flow than the other powders. Furthermore, sugars have likely been removed from the press cake with the juice as described by Laaksonen et al. [36]. A reduced sugar content in the press cake powder could have reduced its cohesiveness. The presence of sugars and acids increases the stickiness of the powder by reducing its glass-transition temperature, thereby resulting in a less-free-flowing powder [178,198].

The puréed berry powder showed a more cohesive behaviour than the whole berry powder, which may reflect its slightly higher moisture content (142 ± 0.7 g/kg vs. 132 ± 1.1 g/kg). That the puréed berry undergoes a higher degree of tissue disruption is another possible explanation. Excessive tissue disruption and leaching of sugars to the surface increase the stickiness when drying sugar-rich fruits [199].

From the industrial point of view, the highly cohesive flow behaviours of the whole bilberry and puréed berry powders could cause problems during handling and processing operations, and the addition of a high-molecular-weight drying aid might be needed. Kim and Kerr [198] produced dried powders from whole blueberry fruit and found that it was necessary to add maltodextrin to reduce stickiness and thereby improve flowability. To avoid the use of additives such as

maltodextrin, press cake is preferred as the starting material for bilberry powder production, as it naturally results in a more-easy-flowing powder. For this reason, we focused on press cake in study II.

The particle size of a powder strongly influences its flowability. In a finely milled powder, there is an increased risk of cohesion and the flowability is reduced. Teunou et al. [197] have reported that powders with particle sizes larger than 200 μm are, in general, free-flowing. Therefore, in study II of this work, we tried to improve the flowability characteristics of the bilberry press cake powder by applying a less-extensive milling regimen, resulting in larger particle sizes.

5.1.4 Effects of preprocessing on dispersibility

Bilberry powders are suitable as ingredients in liquid food products, such as beverages, milk drinks, yoghurt, and ice cream, as they provide texture, colour, nutrients, and flavour. Therefore, the dispersibility of the bilberry powders was evaluated in two different liquids, water and cream. As bilberry powders have a high content of insoluble dietary fiber, dispersibility was considered to be a more relevant quality index than solubility for these types of powders [160]. Powders with low dispersibility frequently cause problems related to high sedimentation volumes in the liquid, and this negatively influences the food quality [200].

The whole berry and puréed berry powders showed a similar degree of dispersibility in water and dairy cream, whereas the press cake powder showed lower dispersibility (Fig. 10). This result is likely connected to the removal of water-soluble components, such as low-molecular-weight sugar and organic acids, together with the juice in the pressing step [36]. That the press cake powder had lower contents of sugar and acids was confirmed by measuring the pH and %Brix values of the supernatants of the powders dispersed in water. The pH values of the berry powders dispersed in water were 3.0 for the whole berry and puréed, and 3.4 for the press cake. The %Brix values of the supernatants from the whole berry and puréed berry powders were about 10-fold higher (3.3 vs 0.3) than those obtained for the press cake. Sugars and acids may leak out from the powder particles and solubilise in the liquid. Particles that contain sugars are probably also more easily dispersed in the liquid due to the water-binding properties of sugar, thereby increasing the dispersibility of the whole berry and puréed berry powders, whereas particles with a low content of sugar readily sediment to the bottom of the liquid. Shittu and Lawal [179] have shown that sugar is one of the most important factors influencing the reconstitution properties, such as dispersibility, of cocoa powder.

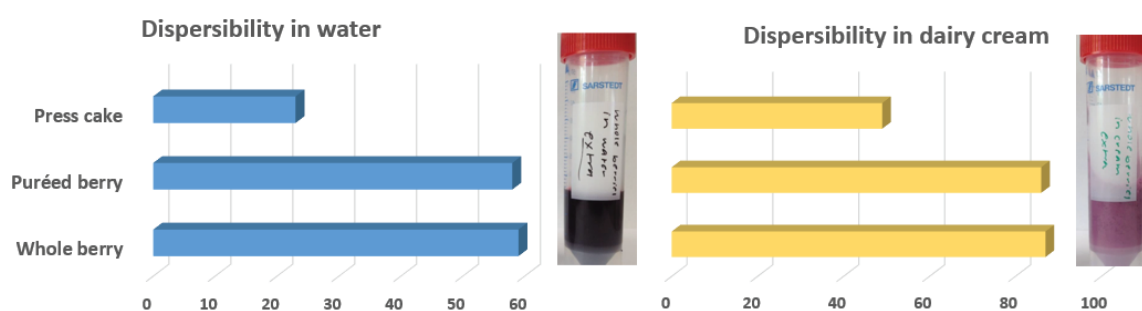


Figure 10. Dispersibility (% w/w) of bilberry powders from dried whole berries, puréed berries, and press cake in water and cream (150 g/kg fat).

For all types of bilberry powders, dispersibility was higher in cream than in water (Fig. 10). Cream is an emulsion of fat globules in water, and it contains proteins that are in a colloidal suspension in the aqueous phase [201]. It is likely that cream constituents (fat globules, proteins, and carbohydrates) entrap the bilberry particles, preventing their sedimentation, which explains the increased dispersibility of the particles in cream. These findings indicate that bilberry powders, in terms of dispersibility, have the potential to be used as ingredients in dairy products.

5.1.5 Total phenolic content

The press cake powder showed the highest TPC (Table 4), which is explained by the higher TPC of the starting material before drying, owing to a concentration effect when the sugar and acids were removed with the juice in the pressing step [36]. This is in agreement with Oszmiański et al. [193], who found that the use of pomace from honeysuckle berries increased the total polyphenol content of berry powders, as compared to the use of whole berries. In addition, mechanical disruption of cell walls during pressing may have improved the extraction efficiency during the TPC analysis.

Table 4. Total phenolic content (g GAE/kg DW) in bilberry powder from dried whole berries, puréed berries, and press cake, compared to whole berries and press cake before drying.

Sample	Total phenolic content (g GAE/kg DW)
Whole berries before drying	21.38 ^b ± 3.81
Press cake before drying	36.48 ^a ± 2.96
Whole berry powder	21.9 ^b ± 1.15
Puréed berry powder	26.3 ^b ± 2.53
Press cake powder	39.2 ^a ± 1.10

Notes. Values followed by the same letters were not significantly different ($p < 0.05$) based on Tukey's test.

DW: dry weight; GAE: Gallic acid equivalent.

No reduction in TPC was observed during drying for either the press cake or the whole berries, although the drying time was up to 27 h for the whole berries. Different studies have reported a reduction of TPC when drying at 40°C or higher temperatures [202,203]. However, comparison of the impacts of various processing methods on the stability levels of bioactive compounds is difficult due to the use of different processing conditions, including pretreatment and storage conditions, as well as the use of different berry varieties. Nevertheless, the air-drying temperature is one of the most important factors determining the quality (e.g., content of bioactive compounds and sensory attributes) of the end-product [46,204]. The lower initial TPC of the fresh material (21 g/kg DW) in the present study, as well as differences in drying time and temperature, may explain why the reduction in TPC reported in previous studies has been more pronounced. Skrede et al. [205] found that the stability of anthocyanins in blueberries was influenced to different extents during processing depending on the type of anthocyanin structure.

Due to the presence of other constituents (fat, protein, and carbohydrates) in cream, the dispersed TPC expressed per DW (Table 7) is lower in cream (0.95-2.81 g/kg DW) than in water (14.64-24.14 g/kg DW), where only dry matter from the bilberry powder is present. The dispersion of bilberry powders negatively influences the content of phenolic compounds (Table 5), as

compared to the initial powder (Table 4). After dispersion in water, the reduction was in the range of 33%-40% compared to the initial powder. It is possible that there is a degradation of phenolic compounds due to exposure to light and moisture. In addition, the TPC may be different for particles that sediment to the bottom and particles that are dispersed in solution. In summary, the dispersibility of bilberry powders in water and cream result in the reduction of TPC and this reduction varies depending on the matrix where the powder is dispersed.

Table 5. TPC of dispersed bilberry powders in water and cream expressed as g GAE/kg dry weight.

Sample	Total phenolic content(g GAE/kg DW)	
	Water	Cream
Whole berries powder	14.64 ^b ± 1.35	2.81 ^c ± 0.14
Puréed berry powder	15.93 ^b ± 1.57	2.81 ^c ± 0.10
Press cake powder	24.14 ^a ± 0.89	0.95 ^d ± 0.05

Notes. Values followed by the same letters were not significantly different ($p < 0.05$) based on Tukey's test.

DW: dry weight; GAE: Gallic acid equivalent.

5.2 Effects of drying and fractionation on anthocyanins from bilberry powder

5.2.1 HAD and MWD applied to bilberry press cake

The press cake was dried to constant weight, corresponding to a water activity of about 0.5, and a moisture content of 8% (Table 6). The drying time was slightly reduced (7%) by applying MWD instead of HAD and the difference in drying time longer. In a previous study, applying MWD and HAD to obtain bilberry press cake with a moisture content of 17%, MWD was reported to reduce the drying time by 40%, compared with HAD [58]. A possible explanation for the different results can be that the powders in the present study were dried to a lower moisture content (about 8%), and since microwaves primarily affect the water molecules in the samples, the heating caused by microwaves in the MWD process became less prominent as the moisture content decreased.

Table 6. Moisture content, drying time to reach constant weight, and water activity of the bilberry powders obtained by different drying techniques and after fractionation

Sample	Moisture (% w/w)	Drying time (h)	Water activity
Press cake before drying	71.2 ± 0.69	-	-
MWD, whole powder	8.4 ± 0.14	7	0.52 ± 0.006
HAD, whole powder	8.4 ± 0.18	7.5	0.52 ± 0.003
HAD < 500 µm	8.0 ± 0.19	7.5	0.47 ± 0.001
HAD 500-710 µm	7.3 ± 0.18	7.5	0.49 ± 0.004
HAD > 710 µm	8.1 ± 0.27	7.5	0.51 ± 0.003

Note: MWD, microwave-assisted hot air drying; HAD, hot air drying

A milling time of 10 s was applied to obtain an appropriate size distribution of particles (based on study I), enabling separation into three fractions of finely milled powder (<500 µm); seed fraction (500-710 µm); and peel fraction (>710 µm). No difference in particle size distribution was observed between the HAD and the MWD dried press cake samples (Fig. 11).

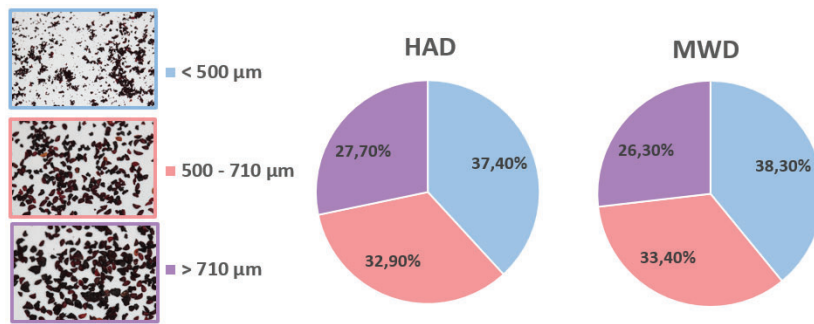


Figure 11. Particle size distribution (% w/w) of bilberry powders from press cake dried by hot air drying (HAD) and microwave-assisted hot air drying (MWD). The powders were fractionated into three particles sizes with: <500 μm ; 500-710 μm ; and >710 μm .

It is important to highlight that due to similar levels of anthocyanins found in the HAD and MWD dried powders and since that HAD technology is more likely to be suitable for commercial upscaling purposes and applicable to industry, a decision was taken to continue the work with only the HAD powder.

5.2.2 Dispersibility characteristics of bilberry powders in water and dairy cream

The dispersibility profiles of the MWD and HAD dried whole bilberry powders showed similar behaviours, as shown in Figure 12. Separation of the HAD into fractions with different particles sizes affected the dispersibility characteristics of the fractionated powders.

The dispersibility of the intermediate fraction (500-710 μm) in water was lower than the larger and the smaller fractions, probably due to the high content of seeds in this fraction, which sank to the bottom and was not well dispersed. In cream, however, the structure of the cream matrix supported the dispersion of the seeds. Similar behaviours were noted for the large and small fractions when comparing cream and water. In cream, a trend of higher dispersibility with decreasing particle size was observed, although differences were not significant.

In general, the dispersibility values of the different powders in cream were higher than those in water likely due to that the higher viscosity of the cream matrix better supports the dispersed particles. Therefore, the use of bilberry powders as ingredients for the fortification of dairy cream products seems to have the advantage of resulting in more homogenous products compared with water-based products.

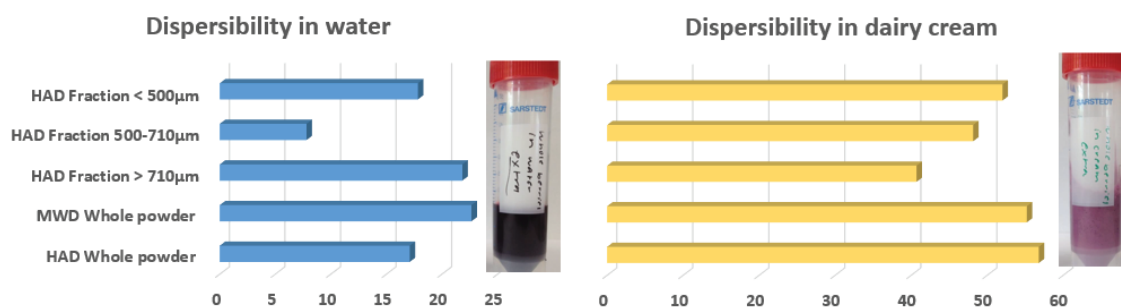


Figure 12. Dispersibility (% w/w) of powders from microwave-assisted hot air-dried (MWD), hot air-dried (HAD), and fractionated HAD powders from bilberry press cake.

5.2.3 Total phenolic content in fractionated powders

The total apparent phenolic contents of the dried and fractionated bilberry powders are shown in Table 7. The TPC values varied, with the highest level found for the fraction with the smallest particles (< 500 µm) and the lowest for the powder with intermediate particle sizes (500-700 µm). Although the fraction with the largest particles (>710 µm) also contained peel flakes, the TPC was similar to that of the whole dried bilberry powder. This may be explained by a reduced extraction efficiency due to the larger particle size. The presence of particles from the peel, in combination with the small particle size (contributing to enhance the apparent phenolic content), explains why the highest TPC was noted for the fraction with the smallest particle size. The low TPC in the intermediate size fraction (500-710 µm) is probably linked to the high proportion of intact seeds in this fraction [206].

Table 7. Total apparent phenolic content of whole berries, press cake, and powders from MWD, HAD, and fractionated HAD powders. Values followed by the same letter were not significantly different ($p < 0.05$) based on Tukey's test.

Sample	Total phenolic content (g GAE/kg DW)
Whole berries before drying	36.54 ^d ± 3.12
Press cake before drying	51.87 ^{ab} ± 0.38
MWD, whole powder	48.28 ^{bc} ± 0.61
HAD, whole powder	45.52 ^c ± 1.16
HAD, >710 µm	44.72 ^c ± 1.10
HAD, 500-710 µm	33.62 ^d ± 0.76
HAD, < 500 µm	55.33 ^a ± 0.06

Note: DW, dry weight; GAE, gallic acid equivalent; HAD, hot air drying; MWD, Microwave-assisted hot air drying.

5.2.4 TPC of bilberry powders dispersed and dissolved in water and cream

Although the lowest TPC was obtained for the intermediate size fraction (500-710 µm; Table 7), the phenolics dispersed well in water, given that there was no significant difference between the levels of phenolics in the whole bilberry press cake and the different fractions dispersed in water (Table 8). This is likely connected to that the intermediate fraction also had the lowest dispersibility (Fig. 12), as the intact seeds present in this fraction sedimented. Consequently, the fraction of the powder that was dispersed and dissolved in water was probably enriched in phenolics. This is in agreement with the results obtained for the dispersibility in cream, with the lowest TPC being seen for the intermediate fraction (Table 8). In contrast to the values obtained for water dispersibility, the dispersibility of the intermediate powder fraction was high in cream (Fig. 12), which might be due to that the cream matrix prevents the seeds from sinking to the bottom. Since anthocyanins are concentrated in the peel of bilberries [30,207], the presence of more seeds did not enhance the apparent total phenolic for this sample. The higher TPC detected in the sample dispersed in water (Table 8), despite its lower dispersibility compared with that in the cream, might be explained by the presence of water-soluble compounds in the press cake.

Table 8. Total apparent phenolic content of powders from MWD, HAD, and fractionated HAD powders from bilberry press cake dispersed in water and cream expressed as mg/g dry weight. Values followed by the same capital letter for water and small letter for cream were not significantly different ($p < 0.05$) based on Tukey's test.

Sample	Total phenolic content(g GAE/kg DW)	
	Water	Cream
MWD, whole powder	26.49 ^A ± 0.92	19.36 ^{ab} ± 2.25
HAD, whole powder	23.06 ^A ± 0.31	17.43 ^{ab} ± 3.25
HAD, >710 µm	26.62 ^A ± 2.39	7.26 ^{bc} ± 1.33
HAD, 500-710 µm	25.55 ^A ± 0.27	4.62 ^c ± 0.27
HAD, < 500 µm	26.80 ^A ± 1.69	25.48 ^a ± 5.12

Note: DW, dry weight; GAE, gallic acid equivalent; HAD, hot air drying; MWD, Microwave-assisted hot air drying.

In dairy cream, the TPC of the MWD powder was slightly higher than that of the HAD powder (Table 8). This could be due to particles of the microwave-dried powder having different structures than HAD particles, which may have promoted the release of polyphenols in the cream. The highest TPC in cream was observed in the fraction with the smallest particle size (< 500 µm), while the two fractions with larger particle sizes had significantly lower content of phenolic compounds. The lower TPCs of the intermediate fractions (500-710 µm) are likely related to their higher proportions of intact seeds, which contain lower levels of phenolics than the other parts of the press cake (Table 7). The fraction with the particles of the smallest size (< 500 µm) dispersed in cream had a higher TPC than the whole powder and the larger-sized particle fractions, probably due to more efficient extraction of phenolic compounds in the cream owing to the larger surface area and ruptured cell walls. In addition, this fraction might also contain broken seeds and might release polyphenols in the forms of ellagitannins and flavonols [208].

5.2.5 Anthocyanin composition

In the chromatogram obtained from the anthocyanin analyses (HPLC and UV detection) of freeze-dried bilberry press cake powder, a total of 14 peaks were identified (Fig. 13), using standard compounds and data from the literature [205,209,210].

The quantification of malvidin-3-*O*-glucoside, delphinidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, cyanidin-3-*O*-glucoside, and petunidin-3-*O*-glucoside (Fig. 13) was achieved using calibration curves prepared with an external standard. Evaluation of the method by the spiking of some sample with internal standards showed satisfactory performance, with no response fluctuations or matrix effect, as compared with the external calibration curve.

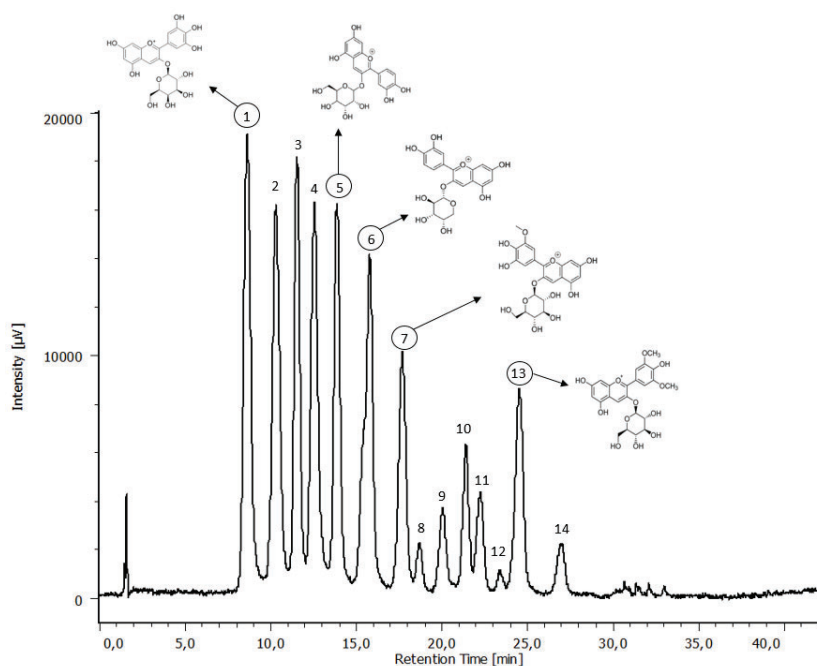


Figure 13. HPLC chromatogram of anthocyanins in freeze dried bilberry press cake powder. Peak identification: 1. delphinidin-3-*O*-galactoside, 2. delphinidin-3-*O*-glucoside, 3. cyanidin-3-*O*-galactoside, 4. delphinidin-3-*O*-arabinoside, 5. cyanidin-3-*O*-glucoside, 6. cyanidin-3-*O*-arabinoside, 7. petunidin-3-*O*-glucoside, 8. peonidin-3-*O*-galactoside, 9. petunidin-3-*O*-arabinoside, 10. peonidin-3-*O*-glucoside, 11. malvidin-3-*O*-galactoside, 12. peonidin-3-*O*-arabinoside, 13. malvidin-3-*O*-glucoside , 14. malvidin-3-*O*-arabinoside.

In figure 14, the total contents of the five most abundant anthocyanins in the HAD, MWD, and fractionated powders are shown. All the bilberry powders showed a similar anthocyanin profile. The most abundant anthocyanin was delphinidin-3-*O*-galactoside (7.50 ± 0.18 mg/g DW for untreated press cake), which is in agreement with previous studies reporting that delphinidin glycosides are among the most common anthocyanins in bilberries [211]. The different drying methods (MWD and HAD) did not induce any significant differences in terms of the total anthocyanins or the anthocyanin profile, and as with the TPC, the highest levels of anthocyanins were found in the press cake fraction with the smallest particle size. After dispersion of the different powder fractions in cream, markedly higher levels of anthocyanins and especially the relative amount of delphinidin-3-*O*-galactoside were quantified in samples from the small particle size fractions ($< 500 \mu\text{m}$) compared to the two fractions with larger particle size (Fig. 15). This suggests that the choice of the finest powder to be used as an ingredient may be a good option to boost the content of anthocyanins in the final product.

The total amount of the five anthocyanins in the untreated bilberry press cake powder was 26.3 mg/g (Fig. 14). The effect of the fractionation and dispersion on the anthocyanin profile was studied by comparing the total amount of the five identified anthocyanins in the different fractions. The ratio of the sum of all the quantified anthocyanins found in the HAD press cake fraction with the smallest particle size to the intermediate fraction was 1.8 (Fig. 14).

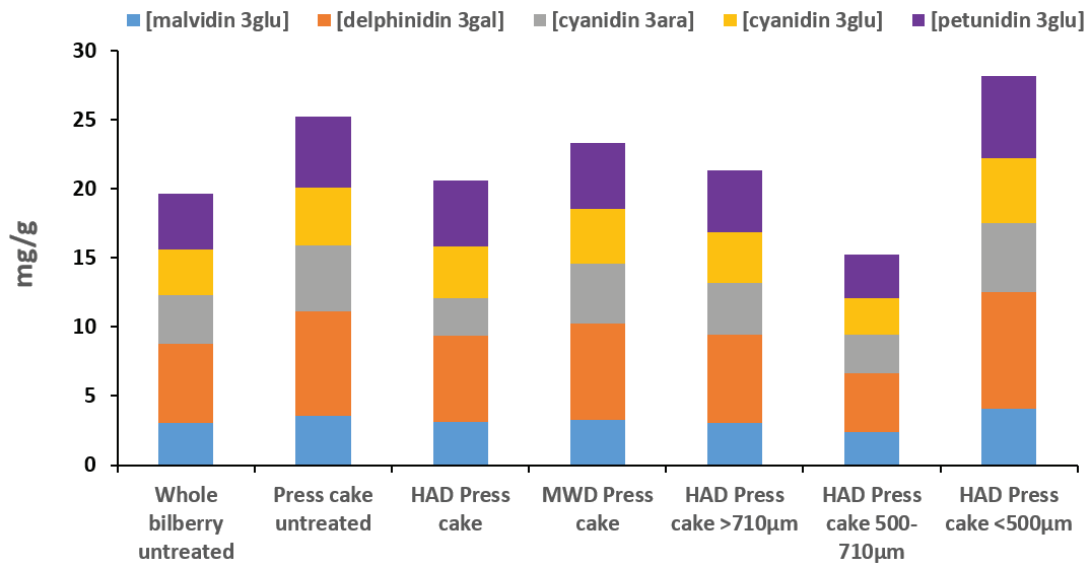


Figure 14. Concentration (mg/g dry weight) of anthocyanins in bilberry press cake powders after hot air drying (HAD) and microwave drying (MWD) treatments and fractionation of HAD powder.

After dispersion of these samples in cream, the corresponding ratio was 4 (Fig. 15). It is possible that the combination of small particle size and the cream matrix promoted interactions between the anthocyanins and other compounds, such as proteins [9], thereby enhancing the release of anthocyanins in the system.

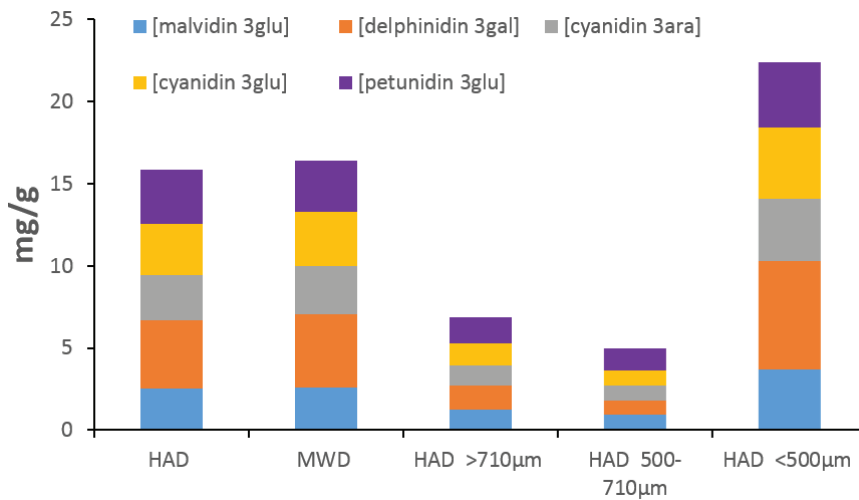


Figure 15. Concentration (mg /g dry weight) of anthocyanins in bilberry press cake powders after hot air drying (HAD) and microwave drying (MWD) treatments and fractionation of HAD powder dispersed in the dairy cream.

Before dispersion in cream, the anthocyanin content of the larger particle size fraction (>710 µm) was similar to that of the whole powder. However, after dispersion, both the content and profile changed, as compared to the whole powder. This probably reflects the lower dispersibility, as well as the large size of the flakes limiting the release of anthocyanins into the solution.

5.3 Effects of non-thermal processing prior to digestion on anthocyanins

5.3.1 Effect of PEF and OD on the recovery of anthocyanins in strawberry

Four different anthocyanins were identified and quantified in untreated and treated strawberry samples (Table 9), with cyanidin-3-*O*-glucoside (cy-glc) as the major anthocyanin, which is in agreement with other studies reporting this anthocyanin as one of the most abundant in strawberries [212,213]. The highest content of anthocyanins was found in samples treated with PEF at 200 V·cm⁻¹, without OD as pretreatment; while the application of PEF treatment at 100 V·cm⁻¹ seemed to have no positive effect on the detectability of anthocyanins, with the exception of peonidin-3-*O*-glucoside (Table 9).

Table 9. Anthocyanins quantified in untreated and treated strawberry samples before *in vitro* digestion. Values followed by the same letter in each column were not significantly different ($p < 0.05$) based on Tukey's test.

Sample	Anthocyanins mg/kg DW			
	cyanindin-3- <i>O</i> -glucoside	petunidin-3- <i>O</i> -glucoside	cyanidin-3- <i>O</i> -arabinoside	peonidin-3- <i>O</i> -glucoside
Untreated	91.75 ± 5.48 ^b	0.61 ± 0.01 ^a	0.037 ± 0.001 ^{bc}	0.36 ± 0.02 ^{bc}
PEF_100	90.50 ± 5.51 ^b	0.37 ± 0.02 ^d	0.026 ± 0.001 ^{cde}	0.41 ± 0.03 ^b
PEF_200	107.15 ± 4.16 ^a	0.56 ± 0.03 ^{ab}	0.054 ± 0.004 ^a	0.55 ± 0.01 ^a
OD_S	81.99 ± 4.33 ^{bc}	0.33 ± 0.01 ^d	0.037 ± 0.003 ^{bc}	0.34 ± 0.01 ^{bcd}
OD_T	66.56 ± 5.20 ^{de}	0.47 ± 0.01 ^c	0.057 ± 0.005 ^a	0.37 ± 0.01 ^b
PEF_100+OD_S	63.76 ± 0.67 ^e	0.35 ± 0.04 ^d	0.020 ± 0.003 ^{de}	0.37 ± 0.03 ^b
PEF_200+OD_S	73.51 ± 1.89 ^{cd}	0.38 ± 0.02 ^d	0.031 ± 0.002 ^{bcd}	0.27 ± 0.01 ^d
PEF_100+OD_T	79.14 ± 1.80 ^c	0.38 ± 0.01 ^d	0.039 ± 0.001 ^b	0.39 ± 0.02 ^b
PEF_200+OD_T	75.17 ± 4.54 ^{cd}	0.35 ± 0.02 ^d	0.016 ± 0.001 ^e	0.30 ± 0.01 ^{cd}

Note: PEF_100 and PEF_200 refer to a pulsed electric field, at 100 or 200 V·cm⁻¹ respectively, and OD refers to osmotic dehydration with sucrose (S) or trehalose (T).

It is well-known that when plant cells are exposed to an external low voltage electric field (< 0.5 kV), pores in the membrane may be formed (electroporation), which leads to increased permeability and mass transfer in the plant tissue [214]. The level of membrane alteration, including pore formation, depends on the specific characteristics of the membrane as well as the strength of the electric field. For instance, Dellarosa et al. [187] have shown that electric fields strength of 250 and 400 V·cm⁻¹ damaged apple cells. Likely, in the present study, the lower electric field strength (100 V·cm⁻¹) yielded only reversible electroporation of the cell membrane, whereas, at the higher voltage (200 V·cm⁻¹), our data suggest that larger and irreversible pores were formed. This may have facilitated the release of intracellular compounds and increased the extractability of the anthocyanins [215].

Table 9 also shows that all the combinations of PEF as a pre-treatment of OD resulted in lower levels of anthocyanins than in the untreated sample. A hyperosmotic solution causes efflux of water through the plasma-membranes of cells until the isosmotic condition has been reached. However, if the membrane integrity has been disrupted by pores, not only water will leave the cells but all dissolved compounds smaller than the pores, including anthocyanins. This will

continue until equilibrium has been reached for all solutes, which explains the consistently lower level of anthocyanins in the berries after the combination of PEF and OD (Table 9).

Table 10 shows the amount of anthocyanins (mg/kg, DW) quantified in uncoated blueberries (F) and in blueberries coated with chitosan (C) or chitosan and procyanidins from grape seeds (Cp). The most abundant anthocyanin found in blueberry was malvidin-3-*O*-glucoside (ma-glc), followed by delphinidin-3-*O*-galactoside and petunidin-3-*O*-glucoside. For all samples (coated and uncoated), storage for 14 days (T14) increased the concentration of anthocyanins in the fruit (Table 10). The observed increase is likely due to a combination of ripening-related accumulation, losses and extractability of anthocyanins[216,217].

Table 10. Anthocyanin quantified in uncoated and coated blueberry samples before *in vitro* digestion. Values followed by the same letter in each column were not significantly different ($p < 0.05$) based on Tukey's test.

Sample	Anthocyanins mg/kg DW		
	cyanidin-3- <i>O</i> -glucoside	malvidin-3- <i>O</i> -glucoside	petunidin-3- <i>O</i> -arabinoside
FT0	285.06 ± 14.41 ^c	3115.57 ± 6.72 ^b	1608.55 ± 103.11 ^d
FT14	345.98 ± 14.65 ^{ab}	3397.17 ± 97.75 ^a	1897.46 ± 24.70 ^{bc}
CT0	210.87 ± 10.78 ^d	2519.57 ± 99.88 ^c	1223.83 ± 46.11 ^e
CT14	355.21 ± 17.17 ^a	3529.05 ± 18.77 ^a	2121.66 ± 58.58 ^{ab}
CpT0	297.71 ± 9.56 ^c	2528.33 ± 112.29 ^c	1685.97 ± 77.91 ^{cd}
CpT14	310.04 ± 6.47 ^c	3547.34 ± 84.20 ^a	2237.28 ± 107.31 ^a
	cyanidin-3- <i>O</i> -arabinoside	peonidin-3- <i>O</i> -glucoside	delphinidin-3- <i>O</i> -galactoside
FT0	163.25 ± 4.62 ^b	144.27 ± 1.20 ^c	1715.19 ± 21.22 ^c
FT14	198.91 ± 1.70 ^a	171.49 ± 3.97 ^b	2134.96 ± 98.39 ^{ab}
CT0	167.62 ± 9.92 ^b	114.45 ± 3.36 ^d	1389.58 ± 1.96 ^d
CT14	194.50 ± 0.31 ^a	163.77 ± 1.12 ^b	2313.76 ± 118.86 ^a
CpT0	159.81 ± 11.08 ^b	122.31 ± 3.68 ^d	2020.33 ± 10.24 ^b
CpT14	197.04 ± 3.99 ^a	193.75 ± 9.61 ^a	2117.93 ± 54.36 ^{ab}

Note: F refers to uncoated sample, C to sample coated with chitosan and Cp to sample coated with chitosan and procyanidins. (T0) without storage and (T14) after storage for 14 days.

5.3.2 Recovery of anthocyanins from processed strawberry and coated blueberry after *in vitro* digestion using LC-MS/MS

The developed LC-MS/MS method showed to be suitable to analyse the lower recoveries of anthocyanins found after digestion of the berry extracts. The linearity for all curves was found to be acceptable ($0.95 < R^2 < 0.99$). The LOQ ranged from 0.008 to 0.08 ppm.

After digestion of the treated samples, significantly higher content of anthocyanins could be quantified in strawberry (cyanidin-3-*O*-glucoside) and in blueberry (malvidin-3-*O*-glucoside), compared with the non-treated samples, with recoveries ranging from 2.3% to 5% and 0.3% to 1.5%, respectively (Tables 11 and 12). Tagliacruzchi et al. [218] reported a recovery of anthocyanins ranging from 0.1% to 58% after *in vitro* gastrointestinal digestion of black carrot (*Daucus carota*) jams and marmalades. It is worthwhile to mention that in an aerobic *in vitro* digestion system

performed in open-air tubes, as applied in the present study, the degradation of polyphenols may be overestimated due to the considerable sensitivity of polyphenols to oxygen [219]. Therefore, it is important to take into account the losses of anthocyanins due to oxidative degradation, which could explain the low recoveries of malvidin and cyanidin glucosides found in our study.

The recovery of cy-glc in strawberry samples treated with PEF at 200 V·cm⁻¹ was 37% higher than the untreated or treated with PEF at 100 V·cm⁻¹ (Table 11). It is likely due to an enhanced extractability from the samples treated with PEF 200 V·cm⁻¹, as a consequence of larger and more stable pores created in the cell membranes during the treatment at higher electric field strength. Even though the pores formed after PEF at 200 V·cm⁻¹ may lead to losses and degradation of some anthocyanins during digestion, the processing may also promote the release of intracellular compounds and hence, enable a higher recovery of anthocyanins.

Table 11. Relative recovery of cyanidin-3-*O*-glucoside after *in vitro* digestion of untreated and treated strawberry samples. Different letters indicate significant differences ($p < 0.05$) between treatments, based on Tukey's test, (n=3).

Sample (Strawberry)	cyanidin-3- <i>O</i> -glucoside mg/kg DW	Recovery (%)
Untreated	2.20 ± 0.119 ^d	2.5
PEF_100	2.08 ± 0.068 ^d	2.3
PEF_200	4.28 ± 0.366 ^b	4.0
OD_S	2.45 ± 0.236 ^c	3.0
OD_T	3.32 ± 0.220 ^a	5.0
PEF_100+OD_S	1.91 ± 0.146 ^c	3.0
PEF_200+OD_S	2.05 ± 0.090 ^{cd}	2.8
PEF_100+OD_T	2.29 ± 0.007 ^{cd}	2.9
PEF_200+OD_T	2.17 ± 0.055 ^{cd}	2.9

Note: PEF refers to pulsed electric field, at 100 or 200 V·cm⁻¹ and OD refers to osmotic dehydration with sucrose (S) or trehalose (T).

Despite that osmotic dehydration with trehalose (OD_T) markedly reduce the concentration of cy-glc (Table 9) compared with the control (untreated), after digestion, this anthocyanin showed the highest recovery (Table 11). It is known that trehalose preserves lipid bilayers during dehydration and protect biomolecules [220,221]. According to Tanaka et al., [222] one of the possible explanation for this protective effect is that trehalose, by replacing water to form hydrogen bonds between its own OH groups and lipid headgroups, preserves lipid bilayers during dehydration. Anthocyanins are localised inside vacuoles in the plant cells where these pigments are protected [223,224]. Hence, trehalose may in our experiments have stabilized the cell and vacuole membranes, and consequently limited the degradation of cy-glc during digestion. On the other hand, using sucrose to raise the osmolarity in the surrounding solution resulted in a lower recovery of cy-glc compared with other molecules [225]. Further investigations are needed to understand the effects of different sugar-based matrices on the protection of anthocyanins during *in vitro* digestion.

Edible coating is a promising technique for the preservation of fruits and berries. This is especially interesting when natural compounds, such as chitosan, are used as a coating matrix. Chitosan is a polysaccharide formed by the deacetylation of chitin, commonly found as a component of the exoskeleton of insects and crustaceans, and in cell walls of most fungi. Chitosan cannot be

enzymatically cleaved in the human body but has been found safe and applicable as a food ingredient [226,227].

There was a notable difference between the recovery of ma-glc from *in vitro* digested blueberry samples coated with only chitosan and stored (FT14, Table 12), and uncoated and stored blueberries (CT14, Table 12). The use of chitosan as a single ingredient may contribute to a more stable coating than when combined with procyanidins (Cp14, Table 12), and hence enhance the protection of the anthocyanins during digestion. A protective effect of anthocyanins by chitosan in simulated gastrointestinal fluid has been reported by He et al. [228] who showed that, compared to preparations containing free anthocyanins, loading of anthocyanins on chitosan nanoparticles resulted in slower degradation and improved stability of anthocyanins in a model beverage system. Chitosan is a cationic molecule [229] and may by ionic interactions with anthocyanins increase the stability of some structures and prevent degradation.

Table 12. The combined recoveries of malvidin-3-*O*-glucoside from untreated and treated blueberry samples. Different letters indicate significant differences ($p < 0.05$) between treatments, based on Turkey's test ($n=3$).

Sample (blueberry)	malvidin-3- <i>O</i> -glucoside mg/kg DW	Recovery (%)
FT0	9.35 ± 1.2 ^e	0.3
FT14	33.97 ± 0.9 ^c	1.0
CT0	7.55 ± 0.8 ^e	0.3
CT14	49.41 ± 2.6 ^a	1.5
CPT0	12.64 ± 1.1 ^d	0.5
CpT4	42.57 ± 0.7 ^b	1.2

Note: F refers to uncoated sample, C to sample coated with chitosan and Cp to sample coated with chitosan and procyanidins. (T0) without storage and (T14) after storage for 14 days.

Blueberry samples coated with chitosan combined with procyanidins (Cp0, Table 12) had the higher recovery of ma-glc directly after coating than after storage for 14 days, suggesting that this coating combination may be unstable during storage.

It is important to take into account that even though procyanidins and anthocyanins belong to the same family of polyphenolic compounds (flavonoids), the former exists primarily in glucoside forms while procyanidins are usually present as aglycons [230]. Procyanidins are a subgroup of condensed tannins containing exclusively (epi)catechin [25,231]. According to previous HPLC-MS/MS data analysis, MS fragments of procyanidins and anthocyanins as well as their retention times are different, thus limiting the possibility of interference during analyses of these compounds [213,232].

5.4 Protective effects of digested berry extracts on oxidative stress

The pharmacologic value of anthocyanins is commonly suggested to originate from their antioxidant and anti-inflammatory activities related to their chemical structure [233]. However, also epigenetic activities have been found in flavonoids, leading to changed gene expression suggested to protect e.g. against cancer [234]. Mechanisms for cell protection are not fully understood and neither is the relative degree of protection by intact anthocyanins versus

derivatives resulting from GI-digestion. Our intention was to provide new knowledge regarding the protective effects of digested anthocyanin-rich berry extracts using the unicellular model-organism *S. cerevisiae*. In this study, we focused on comparing cellular protection from intact berry extracts with the same extracts exposed for GI-digestion.

5.4.1 Determination of respiratory state and optimal H_2O_2 concentration for oxidative stress

The yeast *Saccharomyces cerevisiae* SKQ2n displays two exponential phases with different catabolism and growth rates when cultured in synthetic medium with one single carbon energy source. These patterns are identified in figure 16 (respiro-fermentative phase, RF and respiratory phase, R). Since our intention was to assess oxidative stress resistance, we chose to collect cells in a state of oxidative catabolism (R) when the yeast defense system for reactive oxygen species is active. We, therefore, selected cells at time 9 h of growth, which is in the middle of the respiratory phase. All subsequent stress experiments (with berry extracts) in this article are performed with cells harvested at this time-point (9 h; mid-respiratory phase). According to the literature, production of ROS in this phase induces antioxidant defenses by triggering transcription factors such as Yap1p and Msn2/Msn4 [107].

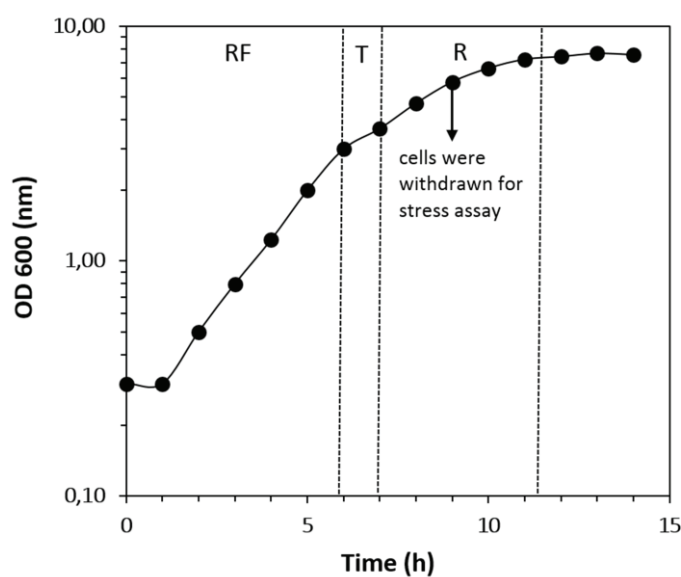


Figure 16. Growth curve of the *Saccharomyces cerevisiae* SKQ2n, cultured in synthetic medium YNB with 2% as the sole carbon and energy source and monitored by optical density (OD). Dotted lines represent transitions from one physiological state to another. RF: respiro-fermentative phase; T: transition phase and R: respiratory phase.

A H_2O_2 concentration of 0.45 M (yielding survival of around 25% after 10 min) was selected for all following studies since it allows for assessment of both increased and decreased survival in response to exposure for treatment (in our study: berry extracts).

5.4.2 Effect of *in vitro* digested berry extracts on yeast survival

S. cerevisiae SKQ2n was cultured in a synthetic medium, supplemented with different berry anthocyanin extracts (bilberry, black currant, strawberry and raspberry) to assess whether this would affect viability during stress. In addition to different berries, anthocyanin extracts with and without exposure to simulated GI-digestion were compared. The survival curves are displayed in Figure 17. All *non-digested* berry extracts (representing the anthocyanin mixture in forms as would be when entering the mouth) showed a *decrease* in the survival capacity of the yeast, suggesting toxicity. However, all *in vitro* GI-digested extracts (representing the anthocyanin mix in forms as would be present in the small intestine) enhanced yeast survival (Fig. 17A-D). These findings are in accordance with some studies, which have reported protective effects of digested (poly)phenols-rich food against oxidative injury in yeast and human cell models [161,174,235]. Likely, these effects were due to chemical alterations of anthocyanins caused by the digestion process, which may have generated modified compounds with different biological properties. Therefore, the derivatives from digested berry extracts or even the reduced concentrations of anthocyanins after digestion play an important role in the protection against oxidative stress in yeast. Different mechanisms have been suggested to explain the beneficial biological activity of polyphenols in *S. cerevisiae*, as for instance through the activation of the Yap1 and Msn2 stress-responsive regulators [236].

The cells cultured with digested strawberry extracts showed the highest increase in the survival capacity (Fig. 17C); approximately 70% increase in viability, compared with the control after 10 min of oxidative stress. Interestingly, strawberry does not possess the highest concentration of total anthocyanins among the studied samples. The concentration of anthocyanins in bilberry is 33 mg in 1 g of dried weight [185], while in strawberry, it is 8.16 mg in 1g [237]. Moreover, the anthocyanin profile of strawberries is markedly different compared with bilberry, black currant and raspberry. Pelargonidin-3-*O*-glucoside is the most abundant anthocyanin in strawberry, representing around 54% of the total anthocyanin content, followed by other types of pelargonidin glycosides [237]. Digested extracts from raspberry, black currant and bilberry, also resulted in strongly increased survival of approximately 62%, 53% and 47%, respectively, after 10 min of oxidative stress (Figures 17B, 17D and 17A). Simplified, this means that the GI-digested anthocyanins roughly doubled the resistance against severe oxidative stress. Bilberry possesses a higher diversity of anthocyanins with at least 16 different types being delphinidin and cyanidin glucosides the most abundant ones. Black currant is rich in delphinidin and cyanidin rutosides [238] while raspberry has cyanidin-3-*O*-sophoroside as the main anthocyanin [239]. It suggests that likely, either the concentration of pelargonidin glycosides or their derivatives were more effective compared with the other berry digested extracts.

To different degrees depending on berry type, the addition of phenolic acids to yeast cultured with digested berry extracts (table 4) yielded, added protective effect on the yeast cells. The 12 different phenolic acids used as supplements were chosen because they have been reported to be found as the main metabolites in human plasma after intake of bilberries. [191]. Therefore, we hypothesised that these phenolic acids could mimic to some extent the metabolites produced after intake of bilberry and promote a more realistic chemical environment to the cells in terms of phenolic protection during oxidative stress. Digested bilberry extracts supplemented with phenolic acids increased the survival rate from 47% to 93% (Fig. 17A). This increase was higher

than the other extracts at similar conditions. It may suggest that interactions between the supplements and other compounds present in the digested extract of bilberry may help to improve the survival of the cells. It is also important to take into account that many of these added phenolic acids are usually metabolite products from the gut microbiota after intake of berry fruits which highlight the important role of microorganisms in the metabolism of anthocyanins and its relevance to health benefits.

To verify that the observed protective effect of digested extracts came from the berry compounds and not from the physico-chemical background of the digestion mix, it was also essential to perform the experiment with the digestion mix and procedure *without* added berry extract (background). This resulted in a survival curve which closely followed the control in all experiments (no extract, no digestion chemicals) concluding that the observed cell protection came from the berry compounds (Fig. 17C, IVD blank. Same for the other samples but omitted in A, B and D).

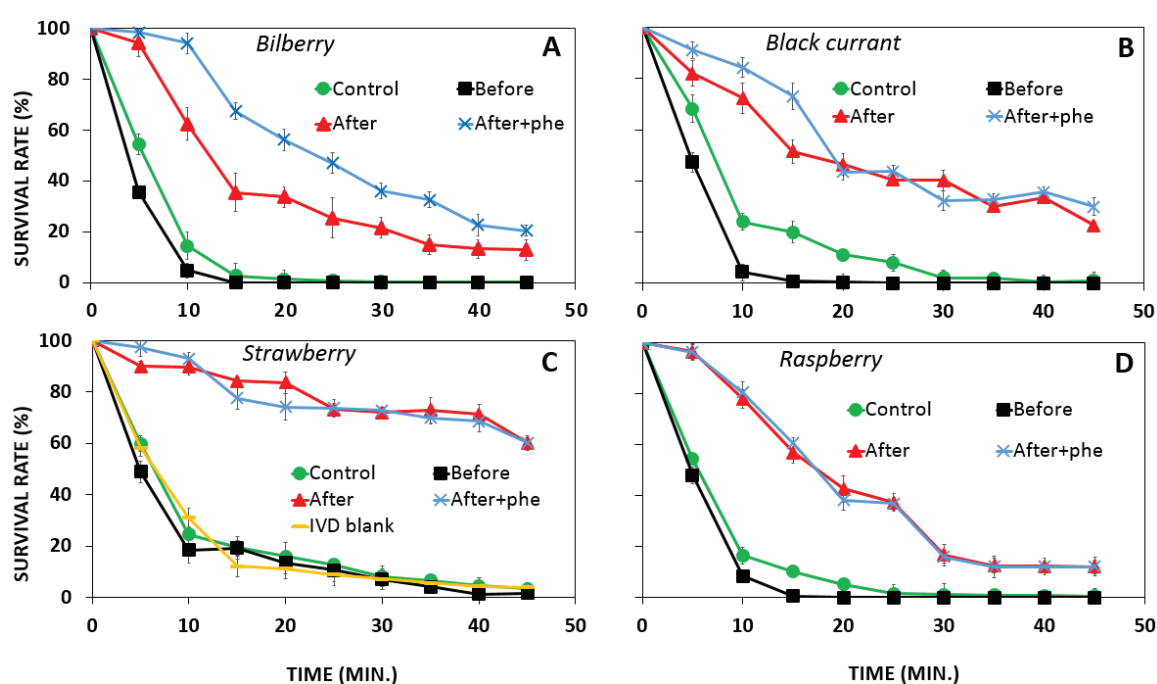


Figure 17. Survival curves of *S. cerevisiae* SKQ2n stressed by H₂O₂ (0.45 M) cultured in YNB glucose 2% without berry extracts (CONTROL), with berry extracts before *in vitro* digestion (BEFORE), after *in vitro* digestion without supplementation (AFTER) and with supplementation (AFTER+PHE) and with blank from the *in vitro* digestion experiment (IVD). A (bilberry), B (black currant), C (strawberry) and D (raspberry).

The negative effect observed when non-digested anthocyanin extracts were applied suggests that intact (poly)phenols in relatively high concentration may decrease the mechanisms of defense against oxidative stress in yeast. It is in accordance with the findings of Wei et al. [240], who showed that propyl gallate, a phenolic compound synthesised by the condensation of gallic acid and propanol in certain conditions, may increase the levels of ROS and/or promote depletion on protective enzymes levels, such as glutathione. Moreover, studies have reported that high levels of (poly)phenols can inhibit enzymes, such as peroxidases and catalases [241,242]. Also, it is well known from the literature that (poly)phenols extracts from berries can exhibit antimicrobial activity [243], however, in this study cells were cultured under non-toxic concentrations of undigested berry extracts [173,190] and reached similar density after 9 h of incubation.

5.4.3 Protective effect remains after removal of digested extracts

In order to investigate whether the observed protection was a chemical neutralisation of the H₂O₂ effect *outside* the cells, or the berry compounds induced a biological response *inside* the yeast cells leading to a phenotype with enhanced stress resistance, two more experiments were performed: 1) cultivation of yeasts in the presence of digested extract followed by removal of the extract before the stress assay (centrifugation plus washing of cells) and 2) cultivation of yeasts *without* extract (yeast medium only) followed by *addition* of the digested extract just before the stress assay.

The cells grown in yeast medium plus digested extract showed significantly higher stress resistance (Fig. 18A; as expected and shown also in Fig. 17A) compared with control. On the other hand, no increase in survival was observed when cultures were exposed to berry extracts only during H₂O₂ stress exposure (Fig. 18B). Our results demonstrate that the yeast cells somehow changed their phenotype to a more resistant state in the presence of the anthocyanins derivatives and the resistant cell state remained even after removing the compounds. The cells must have sensed and most likely were able to take up the digested anthocyanins and inside the cytoplasm, the anthocyanins derivatives somehow affected the defense state against oxidative stress. Either this happened by changed gene expression or by altering the activity of the existing pool of enzymes. However, this potential mechanism of alterations in expression or enzyme activities is unknown. This will be addressed in near coming studies. The intention is thereafter to use the yeast data to design corresponding experiments with human cell-lines.

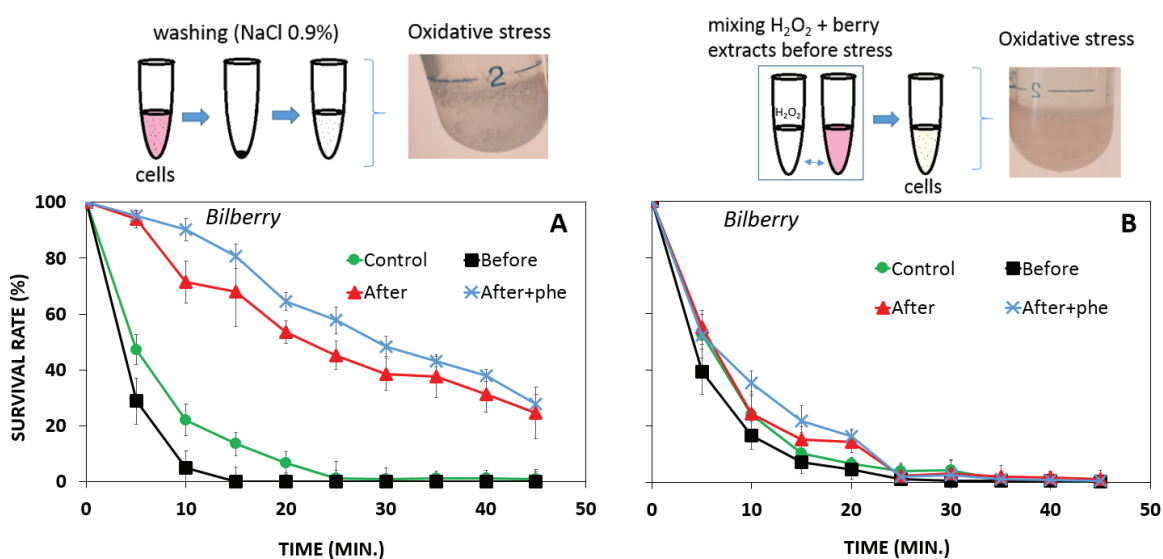


Figure 18. Survival curve of the yeast strain SKQ2n. Cells cultured with bilberry extracts but washed before stressing with H₂O₂ (A) and cultured without bilberry extracts but mixed with bilberry extracts 5 min before stress (B). Cultures without bilberry extract (CONTROL), with bilberry extracts non-digested (BEFORE), after *in vitro* digestion without supplementation (AFTER) and with supplementation (AFTER+PHE).

5.4.4 Effect of digested anthocyanin-rich extracts on yeast under oxidative stress

Considering that bilberry, strawberry, black currant and raspberry are rich natural sources of anthocyanins we hypothesised that these pigments in small concentrations and/or their derivatives produced after digestion were likely responsible for the protective effect described before. However, berry fruits are complex food matrices and other compounds rather than anthocyanins present in the fruit could be the effective ones. To address this hypothesis, we performed another experiment using an anthocyanin-free fruit (kiwi fruit of the variety Hayward) [192], which has a similar level of total (poly)phenolics as in strawberries [244] to grow yeast cells in the presence of its digested and non-digested extracts before being challenged with H₂O₂, similarly as was performed previously with anthocyanin-rich berries. Kiwifruit, with and without exposure to *in vitro* digestion did not improve yeast survival in the presence of oxidative stress, compared to the other digested berry extracts (Fig. 19).

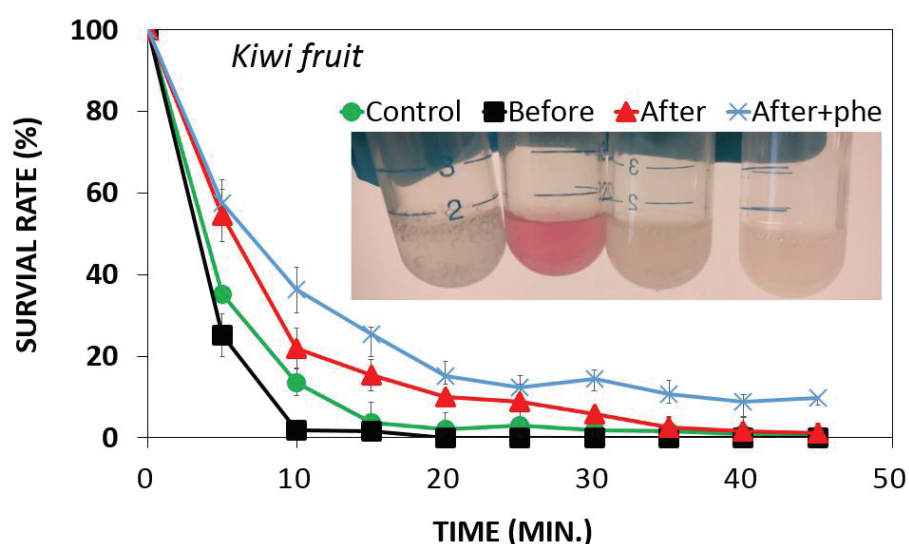


Figure 19. Survival curve of the yeast strain SKQ2n stressed by H₂O₂ (0.45 M) cultured in YNB glucose 2% without kiwi fruit extracts (CONTROL), with kiwi fruit extracts before *in vitro* digestion (BEFORE), after *in vitro* digestion without supplementation (AFTER) and with supplementation (AFTER+PHE).

These findings suggest that the protective effects are linked to the presence of anthocyanins and their derivatives. This study contributes with evidence that anthocyanins derivatives may exert biological activity increasing the oxidative stress defense of yeast. However, further studies need to be carried out in order to understand the specific mechanisms of protection. Also, the *in vitro* GI-digestion approach results in transformed phenolic compounds from berries, intended to mimic the derivative forms reaching the small intestine [245]. Naturally, the complexity in the human gut is larger due to e.g. the GI-biota, which is absent in our model.

6 CONCLUSIONS

The work presented in this thesis has been focused on the evaluation of the functionality and stability of phenolic compounds in processed berries and, to study the potential protective role of anthocyanins and their transformation obtained during digestion, against H₂O₂-induced stress in yeast cells. The main conclusions are:

- Bilberry powder can be tailored in a gentle way by applying different mechanical preprocessing techniques without using any additives, organic solvents, or high temperatures. The press cake from bilberry has the potential to be recycled from juice processing producing (poly)phenol-rich ingredients and sustainable use of raw materials.
- Hot air drying (HAD) and microwave drying (MWD), applied on bilberry press cake at 40°C yield similar and small decreases on the content of total phenolics and anthocyanins. Mild drying and fractionation of the powder were found to be promising approaches to transform bilberry press cake into stable and deliverable ingredients that can be used for fortification of food products with high levels of phenolic compounds.
- Milling of bilberry press cake into powders with small particle size (< 500 µm) provided a powder with the highest apparent content of phenolic compounds and anthocyanins indicating that this fraction shows promise to be used to as ingredients in value-added foods with a high content of phenolic compounds.
- Dispersibility of all dried powders samples was higher in dairy cream than in water suggesting that bilberry press cake powders are applicable to add nutritional value to dairy products such as yoghurt.
- A systematic approach was developed for optimising identification of anthocyanins in *in vitro* digested berry extracts using LC-MS/MS instrumentation. The method was found to be sensitive enabling detection and quantification of low concentrations of anthocyanins and providing adequate separation, response and high-performance characteristics.
- The application of PEF-assisted osmotic dehydration (OD) to strawberries and the use of edible coatings of blueberries maintained or enhanced the stability of anthocyanins during gastrointestinal *in vitro* digestion. The recovery of cyanidin-3-*O*-glucoside, the main anthocyanin detected in strawberry samples after digestion, was higher after PEF treatment at 200 V·cm⁻¹ compared with PEF 100 V·cm⁻¹, untreated samples and PEF-assisted OD.
- Strawberries osmotically dehydrated with trehalose had the highest recovery of cyanidin-3-*O*-glucoside while the recovery of malvidin-3-*O*-glucoside was the highest in blueberries coated with chitosan and stored for 14 days. Our results suggest that trehalose and chitosan work as protective compounds reducing the degradation of anthocyanins, during gastrointestinal *in vitro* digestion.

- *In vitro* digested extracts from anthocyanin-rich berry fruits are able to protect yeast cells stressed with hydrogen peroxide, while non-digested extracts, in contrast, increase cells sensitivity to oxidative stress. The results imply that the anthocyanins may have been structure-modified or transformed into other compounds during digestion, which may have a different biological effect than non-digested extracts.
- The digested anthocyanin extracts from strawberry, which is the richest source of pelargonidin glycosides among the studied berries, showed the highest protection of the stressed yeast cells. On the other hand, the combination of bilberry derivatives with supplemented phenolic acids significantly increased the protective effect suggesting a synergistic effect leading to enhance the ability to reduce damage from oxidative stress.

7 FUTURE PERSPECTIVES

This thesis work may contribute to more knowledge of the beneficial effects of applying the selected mild thermal and non-thermal processing techniques on the stability of anthocyanins. Also, these findings may increase knowledge regarding the effects of *in vitro* gastrointestinal digestion on the bioactivity of anthocyanins. However, further studies are needed and suggestions for continued work are:

- A more detailed investigation of the mechanisms behind the putative interactions between anthocyanins and other compounds such as proteins in the cream matrix.
- Further investigation is needed to elucidate the protective effects of trehalose used as hypertonic solute in osmotic dehydration and chitosan applied as an edible coating on anthocyanins during *in vitro* gastrointestinal simulated digestion. It would also be interesting to evaluate the non-thermal processing of different fruits and vegetables in order to compare the results with different food matrices.
- The protective effect of digested anthocyanins extracts on stressed yeast cells should be studied to elucidate the mechanisms of protection, for instance, the transcriptome and expression of genes related with oxidative stress when yeast is cultured in the presence/absence of anthocyanins and their derivatives.
- It is important to characterise (identify and quantify) the (poly)phenolic profile of the berry extracts before and after digestion using, for instance, Mass Spectrometry methods.
- More research is needed to understand in greater detail the potential interactions of anthocyanin derivatives and phenolic acids in improving the survival rate of cells under oxidative stress.
- The use of human cell models should also be used in future work addressing the protective effect of digested anthocyanins on oxidative stress. Also, the gut microbiota should be considered in order to take into account polyphenol microbial metabolism.

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