Recovery of added value compounds from beetroot juice industry wastes

A dissertation submitted

by

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To my parents, Dimitris and Athina, and my sister Ellie for their continuous support.

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Abstract

The aim of this research work is the recovery of added-value compounds from beetroot juice industry wastes. The recovery of added-value compounds from wastes is a waste valorisation strategy, which can lead to the reduction of the environmental footprint and the creation of additional revenue for companies.

In this work, the beetroot pomace is extracted mainly for the recovery of betalain pigments and phenolic compounds, using enzymatic-aided extraction. The extractions were performed in lab- and semi-pilot scales, using aq. ethanol solvents, and were compared to conventional extraction. The semi-pilot extracts were further subjected to UF and NF membrane cascade processing, for their clarification and the fractionation, concentration and purification of the potentially extracted compounds.

The initial analysis of the raw beetroot extracts and the beetroot juice, from the production of which the beetroot pomace derives as a waste, led to the detection of several betalain pigments. In contrast, the lab scale extraction of the beetroot pomace did not unveil any beetroot compounds, even after using different enzymatic treatment/extraction conditions and pomace batches.

The semi-pilot conventional and enzymatic extraction of the beetroot pomace did not lead to the detection of any beetroot compound, using HPLC-DAD and LC-ESI-MS chromatography, apart from a single compound, which could not be qualitatively determined. A colour change from green to blue was interestingly observed among the various retentate NF streams, but could not be correlated to individual pigments. Finally, the conventional extraction of the beetroot pomace using pure organic solvents demonstrated the presence of only low betanin amounts, solely in the methanol extracts.

These findings suggest that the beetroot pomace is depleted in most pigments; also, methanol cannot be combined with the commercial enzymes and the membranes in use. It is, therefore, concluded that the initial investigation has been completed and there are no further objectives.

Chapter 1

Introduction

1.1. Scope

The current work focuses on the valorisation of the beetroot juice industry wastes (pomaces), particularly through the recovery of potentially contained added-value compounds. Among the main target compounds expected to be found are betalain pigments and phenolic antioxidant compounds (Ninfali and Angelino, 2013; Chhikara *et al.*, 2019).

The processing strategy for the recovery of such compounds in this work involves the application of the enzymatic-aided extraction, which, although appears to be a promising technique for the extraction of plant-based materials such as most agri-food wastes (Gómez-García, Martínez-Ávila and Aguilar, 2012; Ruviaro, Barbosa and Macedo, 2019), it has not yet been applied for the extraction of the beetroot pomace.

The parameters of the extraction are intended to be optimised in lab scale, aiming at providing maximum recovery yields with minimal processing costs. The optimisation of the extraction's parameters is planned to be implemented based on a Central Composite Design (CCD) of design of experiments (DoE) approach, by also using Response Surface Methodology (RSM), with the benefit of additionally providing information on the process factors significance and interactions.

The enzymatic-aided extraction is intended, once optimised, to be up-scaled with the performance of semi-pilot enzymatic-aided extractions, following a subsequent cross-flow membrane cascade ultrafiltration (UF) and nanofiltration (NF) processing stage. The membrane processing is aiming at the clarification and purification of the target compounds inside a theoretically expected complex extract mixture containing various components, such oligosaccharides and enzymes. The membrane processing is lastly intended to be optimised through a suitable selection of membranes modules and membrane processing parameters, in favour of a better purification performance in combination with high permeate fluxes and low operational time and costs.

The investigation of this processing strategy is aiming to propose an approach for the exploitation of the beetroot juice industry wastes, by exploring its technological and economic feasibility, with the final aim of this finding practical application in the juice industry.

1.2. The waste problem and valorisation strategies

1.2.1. Fruit & vegetable industry: Market size and value

The fruit and vegetable sector is an important sector of the food industry. Although a large portion of fruits and vegetables is consumed directly as raw commodity, a significant amount is processed to a plethora of food products. According to Eurostat (2019), the processed fruits/vegetables industry was worth 51.5 billion EUR in 2017, corresponding to 6.5 % of the EU's food industry total market value. The vast majority of the processed fruits/vegetables is sold in the form of frozen/preserved products (e.g.

canned fruits/vegetables, jams, dried fruits), consisting of 72.5% of the total processed fruits/vegetables, while second in percentage is the processing to produce juices, accounting for 19.6 % (Antonella De Cicco, 2019).

Fruits and vegetable juice industry demonstrates sizable figures in output and market value, in EU-level and globally. The global fruit juice production was estimated at 38.9 billion litres with a total value of 94.3 billion EUR in 2015, but it declined to 36.2 billion litres and 85.4 billion EUR in 2017 (AIJN, 2016 & 2018). The world's fruit juice consumption was measured at 38.5, 32.2 and 35.9 billion litres in the years 2015, 2017 and 2018, respectively (AIJN, 2016, 2018 & 2019). Although Europe accumulated the 34% of the global fruit juice production and consumption, this share corresponded to only 30% of the global fruit juice market value, in 2017. On the contrary, the contribution of North America in the global fruit juice production and consumption was 24%, but this interestingly corresponded to a much larger global market value share of 37% in the same year. According to a survey, the global fruits and vegetable juice market size is expected to grow by a CAGR of 5.93% (annual growth rate), in the years 2018-2025 (Grand View Research, 2018).

The top five European fruit juice consumption markets, in declining order, are Germany (25.6%), France (15%), U.K. (11.7%), Poland (9.2%) and Spain (8.8%) (AIJN, 2019). In the UK, the total fruit juice production and consumption remained relatively steady between 1.060-1.192 million litres during the last 5-years, corresponding to 11.9% of EU's and 3% of the world's fruit juice production and consumption volume (AIJN 2015-2019). The UK's fruits and vegetable juice market was estimated at 2.8 million USD in 2020 and is expected to grow by a CAGR of 0.8% in the period 2020-2023 (Statista, 2020).

1.2.2. The problem of wastes

The fruit and vegetable sector discards annually significant amounts of biomass in the forms of wastes and by-products. A clear distinction should be drawn between the various terms of the food industry streams.

According to the High Level Panel of Experts on Food Security and Nutrition (HLPE, 2014) and the Food and Agriculture Organisation (FAO) (FAO 2013 & 2019) of the United Nations (UN), "food loss" (FL) is defined as the edible food mass, which was originally intended for human consumption, and is instead being discarded at all stages of the food supply chain prior to consumer level (i.e. production, processing, storage and distribution of the food), regardless of the cause. As "food waste" (FW) is defined the edible food mass which is discarded or is left to spoil at the retail and consumer stages of the food supply chain, also regardless of the cause. The term "Food Waste and Loss" (FWL) is often used to describe the total food mass which is being discarded at all levels of the food supply chain; this also refers to the edible food parts. Those definitions are primarily used to serve as indexes or tools for measuring and controlling global hunger, natural resources depletion and also for promoting food industry sustainability.

An alternative definition for the wastes of the food sector has been established and is described in the Waste Framework Directive of the EU (Directive 2008/98/EC). According to this Directive, the various streams of the food sector are mainly characterised either as (i) wastes or as (ii) by-products. The term "waste" describes any substance or object (edible or not) that the holder discards or intends or is required to discard (e.g. deteriorated or spoiled food, the peels and the core of fruits and vegetables, the bones and the skin of animals etc.); for the edible parts of food products which are discarded as wastes, the term "food waste" is also used. In contrast, the term "by-product" is used to define any secondary substance or object which is a result of the production process of another primary product, and which does not constitute a waste, but its further use is a certainty, and not a mere possibility. Finally, the term "bio-waste" is also adopted and defines those wastes that are biodegradable and derive from gardens, parks, households, restaurants, retail stores or food processing plants (e.g. spent food, peels, stems or leaves of plants etc.). Therefore, the definition of the EU Directive for "food waste" has a broader meaning in comparison to FAO's definition, as it includes edible parts that are discarded in all stages of the food supply chain; this is equivalent to the FAO's "Food Waste and Loss" term. Another key difference is that, the definitions of the EU directive, in contrast to FAO's definitions, have a rather legal context.

According to FAO (2011), the fruit and vegetable sector is producing approximately 25-30% of food losses and 10-20% of food waste in industrialised regions, while 45-50% of losses and 1-5% of wastes is generated out of the total production in developing regions. In the juice industry, yields and by-products depend significantly upon the extraction or pressing technique and the fruit and/or vegetable type and variety used for juice production. Depending on the type of fruit or vegetable, typical by-product generation levels can vary from as little as 20% to up to 60% of the total fruit/vegetable initial mass (Sagar et al., 2018).

The industrial food wastes, such as the wastes of the juice production, create severe environmental problems which are commonly associated with their disposal. Most problems can be attributed to the general characteristics of food wastes, which are their high biological and chemical oxygen demand (BOD/COD) values and organic load, the presence of pollutants/contaminants and, finally, their high microbial load and susceptibility to microbial growth (Lin *et al.*, 2013). Some common environmental problems caused by food wastes are:

- the direct contamination of the natural resources, through the transfer of contaminants and pollutants from the waste to the environment (air, water and soil – for example via leaching etc.; (Torres-León *et al.*, 2018)

- the indirect contamination of the natural environment and its resources. An example is the generation of methane and carbon dioxide gasses (Greenhouse gasses – GHGs) through the anaerobic digestion of the wastes, due to lack of oxygen, following their uncontrolled disposal in landfills. This is enhancing the green-house effect and global warming (Papargyropoulou *et al.*, 2014). Another example is the eutrophication phenomenon caused by the waste disposal in natural waters. This leads to the contamination of the aqueous habitat and to intense incidences of fish kills.

The food industry is in a constant search of sustainable waste management strategies in order to eliminate the environmental and economic repercussions of wastes. Such repercussions are:

A. Economic

- Cost of handling and/or environmental processing of the wastes (sewage and wastewater treatment), in order to reduce their environmental impact (Torres-León *et al.*, 2018)
- Penalty costs for not meeting the environmental targets/criteria as established by regulations of local/national/international authorities (Directive 2008/98/EC of the European Parliament and European Council)

B. Environmental

- Elevated environmental footprint of companies (carbon footprint, GHG emissions, natural resources pollution – water, soil, air etc.) (Anselm Eneh and Stephen Oluigbo, 2012; Kusch *et al.*, 2014; Matharu, de Melo and Houghton, 2016)
- Depletion of natural resources (feedstock, land, water, energy etc.) (Anselm Eneh and Stephen Oluigbo, 2012)

1.2.3. Waste management & waste valorisation

Waste management and valorisation has become a significantly popular research topic in the recent years. "Waste management" is termed as the application of techniques for the collection, recovery (including sorting), storage, transport, processing/treatment/handling and final disposal of wastes, in such way in order to minimise their negative impact to human health, wildlife and environmental systems, before their final disposal (Park and Allaby, 2007; Directive 2008/98/EC). Therefore, the primary focus of a successful waste management strategy is the safe disposal of the waste.

The common management practices of wastes for many years used to be: (a) the disposal to landfills and (b) the incineration of the wastes. However, these practises have adverse effects to the environment; landfilling is responsible for high GHG (greenhouse gas) emissions and for the contamination of soil and water (e.g. leaching,

odours), while incineration is responsible for the generation of hazardous gasses of combustion (dioxins, furans etc.) that are polluting the atmosphere (Wise and Trantolo, 1996; WHO, 2016). These waste management strategies are also contributing to the depletion of natural resources, as they do not involve waste recycling. Finally, they are posing significant costs to food companies, too; landfilling requires considerable transport and labour costs, whereas incineration is associated with high energy demands. There was clearly a need for the development of more sustainable novel waste management approaches.

In search of sustainable novel solutions, research is now focused towards new waste management strategies, other than their simple disposal. These strategies are aiming not only at improving waste management from an environmental perspective (i.e. in terms of reducing the wastes' environmental impact), but, also, at creating additional commercial revenue for the companies that generate these wastes. For this reason, these are referred to as "waste valorisation strategies". Waste valorisation is generally defined as the process of reusing, recycling or composting waste materials and converting them into more useful products including chemicals, materials, fuels and other sources of energy (Arancon *et al.*, 2017; AIChE, 2019; Peters and Seay, 2019).

Figure 1.1 *The waste hierarch of the Waste Framework Directive (Directive 2008/98/EC), established by the EU (Source: https://ec.europa.eu/environment/green-growth/waste-prevention-and-management/index_en.htm)*

Waste valorisation strategies are in line with the "waste hierarchy" concept, which is part of the Waste Framework Directive initiated and enacted by the EU (Directive 2008/98/EC). According to waste hierarchy, the actions that should be taken for a sustainable waste management of a waste should be directed, in an order of priority, towards; (a) the prevention (or minimisation) of the waste generation, (b) the reuse, (c) the recycling, (d) the recovery (e.g. energy recovery) and, finally, as the least favoured option, (e) the disposal of the waste (Figure 1.1).

Waste valorisation strategies have been categorised, in literature, into two groups: the "conventional" or 1st generation techniques and the "novel" or 2nd generation techniques (Arancon *et al.*, 2013; Lin *et al.*, 2013; Kusch *et al.*, 2014). The former refer to the strategies which are mainly currently in use, yet still under development, and often with relatively low value. Several examples of these strategies are: (a) the use of wastes as animal feed, (b) composting to produce compost which is a soil conditioner/fertilizer, (c) anaerobic digestion (AD) to produce biogas (a mixture of mostly CH_4 and CO2) which is used as a fuel, (d) incineration to generate thermal energy (in this case thermal energy is recovered), (e) fermentation to produce bioethanol, which is used as a biofuel, (f) gasification to produce syngas (mixture of H_2, CO and CO_2), which is used as a fuel gas and (g) pyrolysis to produce biochar, which is used as solid biofuel and soil amendment. Anaerobic digestion, incineration, pyrolysis, gasification and fermentation to bioethanol are categorised as "waste-to-energy" approaches.

The 2nd generation waste valorisation strategies are emerging strategies which focus on using waste as a feedstock for the production of higher value chemicals and marketable products. A common example of 2nd generation valorisation strategy is the fermentation of food waste by microorganisms to produce organic chemicals, such as lactic and succinic acid, which can be subsequently used to synthesise bio-based polymers (e.g. succinic-based polyester, polyamides, PLA etc.). Mainly, the forementioned strategies involve the recovery of compounds from wastes and their further conversion into different classes of compounds with greater marketable value. Once the valuable constituents of a waste mass have been extracted, the waste can be then processed using 1st generation valorisation strategies (animal feed, compost, anaerobic digestion etc.).

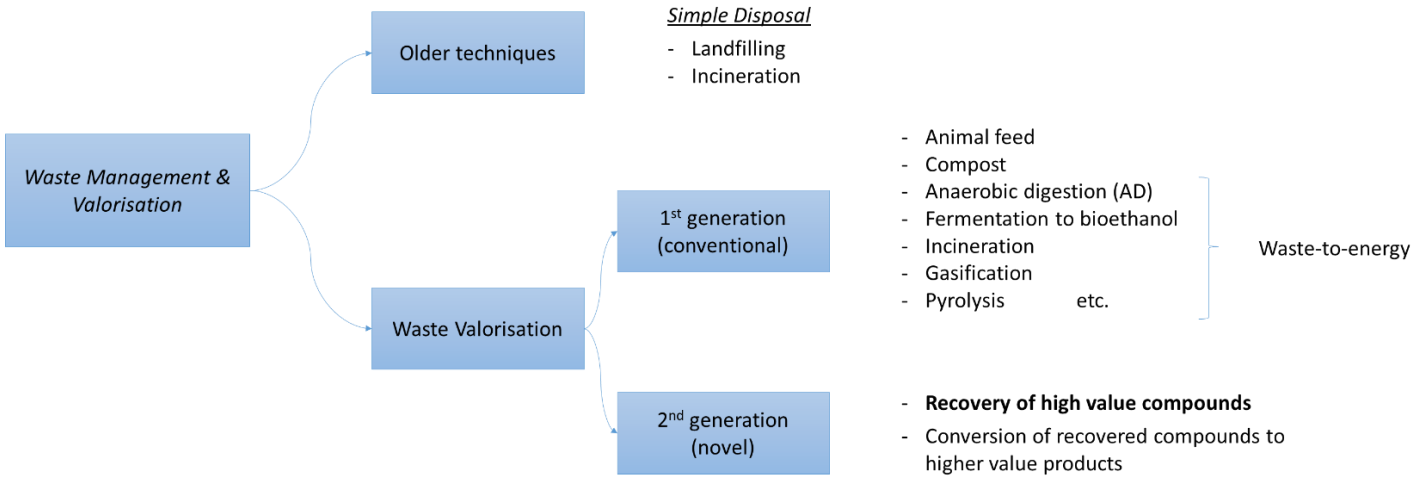


Figure 1.2 *A synopsis of the waste management and valorisation strategies*

The "biorefinery concept" is commonly used as a term to define a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals ideally from waste biomass, as analogous to today's petroleum refineries (Ángel Siles López, Li and Thompson, 2010).

An optimum biorefinery concept, for each specific type of waste, involves the selection, design and combination of a series of processes in a way that ensures maximum profitability and sustainability. All waste management and valorisation strategies should be described by a close-loop supply chain design, i.e. be designed in a way that all waste residues are fed back into the value chain (Lin et al., 2013). This close-loop design ensures that the goal of circular economy has been achieved.

This thesis focuses on the recovery of high value compounds from wastes of the beetroot juice industry.

1.2.4. Recovery of added value compounds

Wastes, especially those derived from the agri-food industry, could be cheap sources of high-value compounds which could remain inside the bulk waste at the end of a

processing procedure. These compounds, however, could find potentially significant application in several industrial sectors, predominately as ingredients in food, pharmaceutical or cosmetic products.

The recovery of high-value compounds consists the first stage of a waste valorisation design strategy; all high-value compounds should be isolated in order to capitalise on their value, prior to any further processing of the waste using different valorisation approaches (anaerobic digestion, fermentation etc.) (Lin *et al.*, 2013). The first step in a recovery procedure is the simple qualitative determination of compounds present in a waste. The next step is the optimisation of the compounds' recovery, with minimal cost, processing time and health and environmental impact, by choosing the optimal extraction technique and its processing parameters for each type of compound. Finally, every recovered compound is purified by separating it from all the other compounds contained in the extract and by removing any excess extraction solvent, and is eventually processed to its final marketable form (powder, syrup, tablet etc.).

In the recent years, the recovery of added-value compounds from agri-food wastes has become a cutting-edge topic in the literature. Typical examples are: (a) the isolation of pectin from the orange peels deriving as by-products of the orange juice production (Ma et al., 1993; Donaghy and McKay, 1994), (b) the recovery of dietary fibres from citrus wastes (Marín et al., 2007), (c) the recovery of anthocyanins from grape pomaces (Trikas et al., 2016), betalains from beetroot pomaces (Vulić et al., 2014) and carotenoids from carrot pomaces (Tiwari et al., 2019) and (d) the recovery of polyphenols from grape pomaces (Sagdic et al., 2011).

1.3. Beetroot

A synopsis of beetroot's main attributes and characteristics is given in this section, as this research work focuses on the recovery of added-value compounds from beetroot pomaces. Beetroot is a vegetable with a growing consumption trend in recent years, as it has been characterised as a superfood, due to late scientific claims on its potential benefits to human health. It is the main source of betalains, which are natural colourants

with significant marketable value, and contains relatively high amounts of phenolic compounds, known for their strong antioxidant effect.

1.3.1. Beetroot's taxonomy

Beetroot (*Beta vulgaris spp. vulgaris var. rubra L.)* is classified as a vegetable plant in the order of *Caryophyllales*, the family of *Chenopodiaceae*, the genus of *Beta*, the species of *Beta vulgaris* and the subspecies of *Beta vulgaris spp. vulgaris* (Ninfali and Angelino, 2013; Romeiras *et al.*, 2016). The subspecies of *Beta vulgaris spp. vulgaris* includes four different variations: (a) beetroot (or red beet or table beet or garden beet) (*Beta v. spp. vulgaris var. rubra; Abbr. BVr*), (b) Swiss chard (or leaf beets) (*Beta v. spp. vulgaris var. cicla; Abbr. BVc*), (c) Sugar beet (*Beta v. spp. vulgaris var. saccharifera*) and (d) mangel-wurzel (or fodder beets) (*Beta v. spp. vulgaris var. crassa*) (Figure 1.3 A-D) (Ninfali and Angelino, 2013; Encyclopeadia Britannica, 2019)

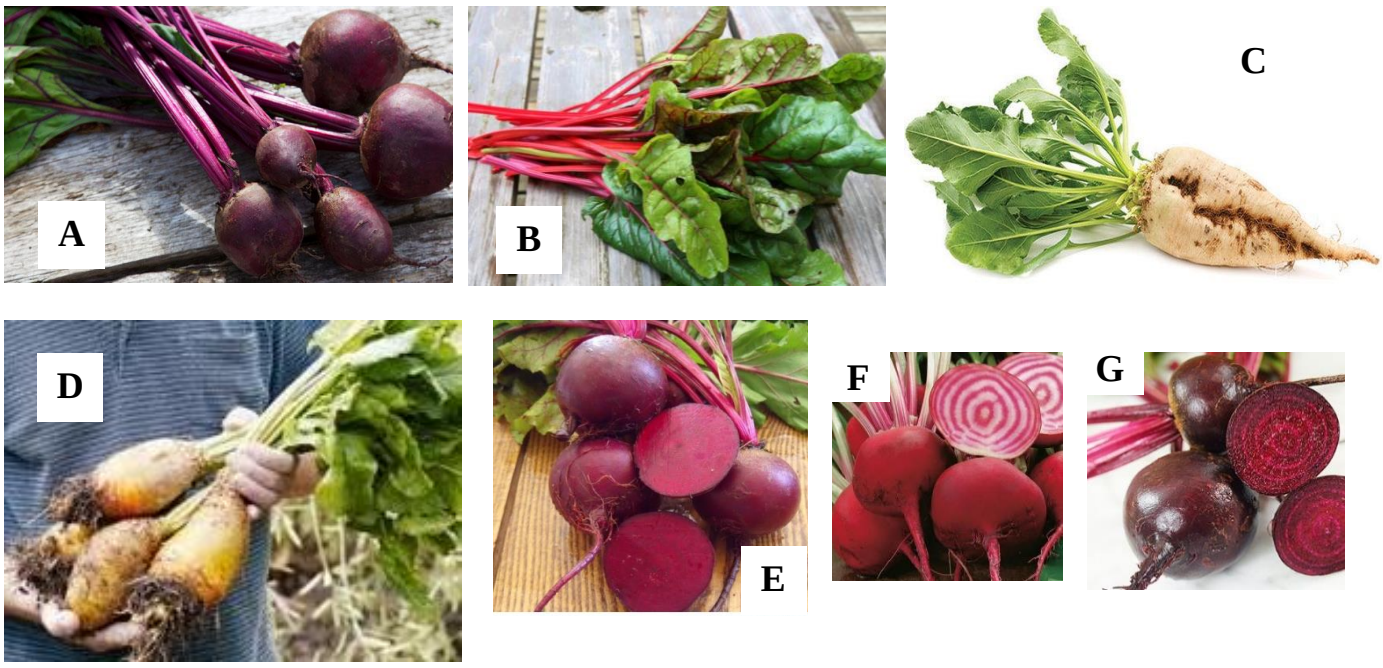


Figure 1.3 *The variations of Beta vulgaris spp. vulgaris: (A) Red beet or beetroot, (B) Swiss chard, (C) Sugar beet and (D) Mangel-wurzel, and the most common variations of red beet (BV rubra): (E) Detroit dark red, (F) Chioggia and (G) Ruby Queen.*

The *Beta vulgaris var. rubra* (i.e. the red beet) includes several varieties, with the more popular being: the Detroit dark red, the Chioggia and the Ruby Queen (Figure 1.3 E-G) (Ninfali and Angelino, 2013).

1.3.2. Plant Structure & Cultivation

The beetroot's plant consists of three major parts, which are: the taproot, the stems and the leaves (Figure 1.4) (Encyclopaedia Britannica, 2016). The taproot is the main edible portion of beetroot and grows underground. It has generally spherical or cylindrical shape, and colours ranging from purple-red to purple-pink, although it can also have golden-yellow to orange, red-white (in stripes) and plain white colour, depending on the variety of the beetroot (Chhikara *et al.*, 2019). The skin of the taproots is thin with a smooth texture.

The stems of beetroot grow on the top part of the root, which is named as hypocotyl. These stems are normally elongated, tapered and stout, and they can reach heights of 1-2 m above the ground (Chhikara *et al.*, 2019). At the top end of the stems, the leaves are growing. These generally have green to yellow colour with red tones, however their size, shape and colour depend on the variety. The seed of beetroot normally contains two to five germs, thus beetroot is classified as a multi-germ plant (Chhikara *et al.*, 2019).

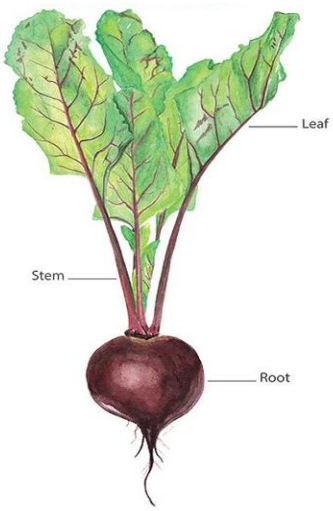


Figure 1.4 *The parts of beetroot's plant.*

Beetroot is mostly biennial and rarely perennial plant; the stems of beetroot develop during the second year of cultivation. The beetroot plant can grow throughout the year; its harvest times are between 75 to 90 days in summer and 100 to 120 days in the winter. Optimum growing temperatures are between 15 to 19^{o}C (Chhikara *et al.*, 2019). Although beetroot is tolerant to higher temperatures (up to 35^{o}C) (Starke Ayres, 2014), the lower temperatures favour the development of a more intense deep red pigmentation in the root (Chhikara *et al.*, 2019).

1.3.3. Chemical composition

Beetroot is a rich source of various macronutrients, micronutrients and other phytochemicals. The main types of compounds which can be found in beetroot are: carbohydrates, proteins, lipids, minerals, trace elements, vitamins, phenolic compounds, pigments, organic and inorganic acids, saponins and the compound geosmin. A detailed review of all these types of compounds found in beetroot according to the literature, is given in this section.

The macronutrients of beetroot are: carbohydrates, proteins and lipids. The main macronutrient which corresponds to higher amounts in beetroot is carbohydrates. The beetroot contains a total carbohydrate amount of 9.56 g/100g of edible part, of which 6.76 g consists of sugars and 2.8 g of dietary fibres (USDA, 2018). The most abundant sugar is sucrose (2.73 g/100g edible part), while fructose and glucose are contained in lower amounts (0.13 g and 0.08 g/100g edible part, respectively) (Nemzer et al., 2011; Jovanovic-Malinovska, Kuzmanova and Winkelhausen, 2014). Several oligosaccharides have also been detected in beetroot; those are two fructoligosaccharides (FOS), in particular 1-kestose (GF2) and nystose (GF3) (0.15g and 0.21g/100 g of edible part, respectively / Total FOS: 0.36g/100g edible part), and also stachyose (0.12g/100g edible part) (Jovanovic-Malinovska, Kuzmanova and Winkelhausen, 2014).

Proteins and lipids are contained in lower quantities in beetroot. The total protein amount of beetroot is 1.61 g/100g edible part (USDA, 2018). A study showed that beetroot contains nine (9) essential amino acids (histidine, isoleucine, leucine, lysine,

methionine, phelynalanine, threonine, tryptophan, valine) and nine (9) non-essential amino acids (aspartic acids, serine, glutaminic acid, proline, glycine, alanine, tyrosine, arginine, cycstine), however, in trace amounts (Nemzer *et al.*, 2011). Finally, the total lipid content of beetroot is estimated at 0.17 g/100g (USDA, 2018). Fatty acids have been analysed only in the *Beta v. spp. vulgaris cicla* (Swiss Chard) seed; linoleic, oleic and palmitic acids were found to be the most abundant (Ninfali and Angelino, 2013).

Beetroot is also a source of many minerals and vitamins. Minerals and trace elements that have been reported in literature to be present in the edible parts of beetroot (100g) include: potassium (325 mg), sodium (78 mg), phosphorus (40 mg), magnesium (23 mg), calcium (16 mg), iron (0.80 mg) and zinc (0.35 mg). In the beetroot juice, Wruss *et al.*, 2015 have reported the presence of chlorine (219 mg), manganese (833 μg), copper (210 μg) and nickel (27.4 μg), per 1 L of beetroot juice (mean values of 7 varieties). The vitamins which have been reported to be contained inside beetroot per 100g of the edible parts are: vitamin C (4.9 mg), vitamin A (2 mg), niacin (0.334 mg), vitamin B6 (0.064 mg), riboflavin (0.270 mg), thiamine (0.310 mg), folate (109 μg) and pantothenic acid (0.145 mg) (USDA, 2018;Chhikara *et al.*, 2019).

Two of the most important types of compounds that can be found in beetroot are phenolic compounds and betalains. Phenolic compounds are compounds which contain one or multiple phenols, i.e. one phenyl group (-C6H5) bonded to a hydroxy group (-OH). Those compounds exhibit strong antioxidant activity and are linked to various health benefits to humans. The beetroot plant is considered to be a great source of polyphenols. Most studies conclude that beetroot juice contains a total phenolic content (TPC) of 1000-1500 mg GAE/L (GAE: Gallic Acid Equivalents). Beetroot pomaces have also been reported to contain TPC values of 1-50 mg GAE/g. A map of all the individual phenolic compounds reported to have been detected inside raw beetroot, beetroot juice or beetroot pomace is given in Figure 1.5.

Beetroot is a rich source of certain pigments, called betalains. Betalains are categorised into two groups: (i) betacyanins and (ii) betaxanthins. Betacyanins have a characteristic red-violet colour (540 nm), whereas betaxanthins have a yellow-orange colour (480 nm). The most common betacyanins found in beetroot are betanin (and its aglycone structure, called betanidin), isobetanin, probetanin and neobetanin, whereas the most

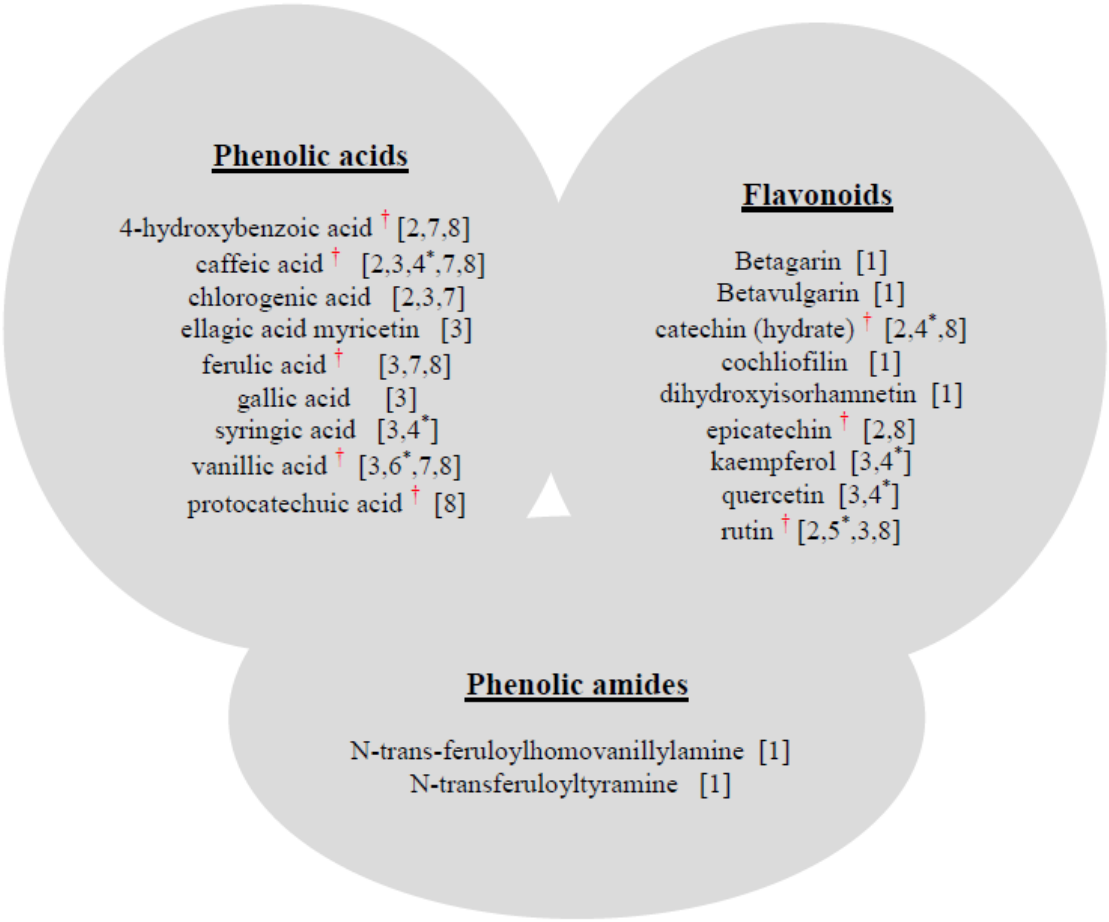


Figure 1.5 *Individual compounds which have been detected in beetroot in the literature (own compilation). [1] Kujala et al. (2002), [2] Georgiev et al. (2010), [3] Koubaier et al. (2014), [4] Pyo et al. (2004)*, [5] Ninfali et al. (2007)*, [6] Gennari et al. (2011)*, [7] Ravichandran et al. (2012), [8] Vulić et al. (2014) [† indicates the presence of the compound in beetroot pomace, * indicates compounds identified in Beta vulgaris cicla (Swiss chard)]*

common betaxanthins are vulgaxanthin (types I-V), miraxanthin, portulaxanthin and indicaxanthin (Nemzer et al., 2011; Gonalves et al., 2012). The total betalain content in beetroot juices was measured at 1103 mg/L, by Wruss *et al.* (2015). According to Vulić *et al.* (2012), the beetroot pomace contains approximately a total betalain content of 0.75-3.75 mg/g dry mass. Most studies on the analysis of individual betalain compounds in beetroot agree that betanin is the most abundant pigment (Kujala *et al.*, 2002; Vulić *et al.*, 2012, 2014).

Finally, three types of compounds that are contained in beetroot are organic and inorganic acids, saponins and geosmin. The main inorganic acids of beetroot are

nitrates, phosphates and sulfates. Beetroot is particularly one of the richest food sources of nitrates. According to studies, the nitrates amount ranges between 1275-1970 mg/L in beetroot juices, although it has been found to be highly variable. The amount of sulfates and phosphates has been measured at 257 mg/mL and 867 mg/L, respectively. Furthermore, the most predominant organic acid in beetroot is oxalic acid. It is regarded as an anti-nutritional factor, because it subtracts micronutrients during the digestive process in humans and participates in the formation of kidney stones (Ninfali and Angelino, 2013). Oxalic acid is estimated to be present at concentrations of 300-550 mg/L in beetroot juices. Saponins are a group of compounds that can be used as surfactants and insect pest repellents and insecticides (Liu *et al.*, 2017; Singh and Kaur, 2018). Beetroot contains 44 different triterpene saponins (Mroczek *et al.*, 2012; Mikołajczyk-Bator *et al.*, 2016).

Geosmin is the compound that gives beetroot its characteristic "earthy" taste and aroma. The removal of geosmin is often applied in order to diminish its unpleasant flavour (Ninfali and Angelino, 2013). The concentration of geosmin has been measured in two different cultivars, Chioggia (135 ± 30.6 μg/kg plant tissue) and Detroit Dark Red (65.2 ± 25.9 μg/kg plant tissue) by Lu *et al.* (2003).

1.3.4. Marketable Products and Health Benefits

One of the most significant application of beetroot in terms of marketable value is undoubtedly as natural food colorant. In particular, the beetroot's powdered extract is a natural colorant, commonly named as "beetroot red"; this is very popular and widely used in several food, pharmaceutical and cosmetic products. The beetroots' colouring ability comes from the presence of betalain pigments. It is a FDA- and EU- approved food additive, registered by the number E162 (Neelwarne, 2013). This pigment provides a dark red colour, without generally altering the flavour and taste of foods, and is stable in a relatively wide pH range of 3-7 (Ciriminna et al., 2018). Red beet is the only allowed source of betalains for the use as food additive in the European Union (Commission Directive 95/45/EC) and the USA (Title 21 CFR Part 73), and is exempt from certification (Neelwarne, 2013).

In the food industry, the total global demand of food colorants was covered at a 10% level from beetroot red in 2019 (nearly 45,000-50,000 tonnes of beetroot red) (Ciriminna et al., 2019). Beetroot's red pigment is currently sold at 130 €/kg by an Italian food colorant manufacturer (Ciriminna et al., 2019; New Decors Art srl., 2020). In recent years, there is a growing demand for natural instead of synthetic pigments, as consumers perceive them as being harmless or even healthy (Azeredo, 2009). This, in combination with the fact that beetroot's pigments, i.e. betalains, have been associated with significant health benefits to humans (as will be discussed in the next paragraph), is expected to soar the production of beetroots powders, which has been estimated to grow with an annual rate of 5% between 2017-2027, from 90,000 tonnes ($15 million value) in 2016 to 1.1 million tonnes in 2027 (Ciriminna et al., 2018).

An interesting aspect of beetroot is, from an economic perspective, its potential health benefits. Recently, many studies have been claiming that beetroot products demonstrate significant anti-cancer, antiradical and anti-inflammatory activities. Regarding the anticancer activity, betanin has been shown to have inhibitory role in the proliferation of several cancer cell lines *in vitro*, such as against liver, prostate, breast, colon, stomach, central nervous system and lung cancer cell lines (Reddy, Alexander-Lindo and Nair, 2005; J. Kapadia *et al.*, 2011; Lee *et al.*, 2014), and also is able to promote apoptosis of certain cancer cells *in vitro*, e.g. of human chronic myeloid leukemia cancer cell lines K-562 (Sreekanth *et al.*, 2007). Saponins have been further reported to have cytotoxic effect *in vitro* and *in vivo* against cancer cell lines (Podolak, Galanty and Sobolewska, 2010). I*n vivo* animals studies have confirmed the anticancer activity of beetroot pomace extract against Ehlirch carcinoma (EAC) and of beetroot extract against skin and lung tumours (Kapadia *et al.*, 1996; Vulić *et al.*, 2013). Betanin has been found to have both inhibitory and apoptotic activity against xenobiotic-induced esofagous, liver and skin cancer (Kapadia *et al.*, 2003; Lechner *et al.*, 2010)

Among other health effects, the beetroot's products and constituents appear to have a strong antioxidant activity. Numerous studies have depicted this high antioxidant activity of beetroot juice (Wootton-Beard and Ryan, 2011), raw beetroot extracts (Zitnanova *et al.*, 2006) and beetroot pomace extracts (Vulić *et al.*, 2013), using common *in vitro* antioxidant assays, such as DPPH and FRAP assays. Interestingly, according to two comparative studies, beetroot was classified as the second most potent

antioxidant among various fruits & vegetables (Zitnanova *et al.*, 2006; Clifford *et al.*, 2015). Individual compounds of beetroot have also been tested; betalains (Escribano, 1998; Kanner, Harel and Granit, 2001) and phenolic compounds (Manach *et al.*, 2005; Georgiev *et al.*, 2010; Shahidi and Ambigaipalan, 2015) present a strong antioxidant activity, which can be expressed synergistically (Georgiev *et al.*, 2010). Especially, betalains appear to have stronger antioxidant capacity than betaxanthins (Escribano, 1998). Nitrates and nitric oxide have been, additionally, described to exhibit significant antioxidant activities (Wink *et al.*, 2001, 2011).

The antioxidant capacity of beetroot and its constituents is responsible for their protective role against the oxidative stress of cellular components, such as against protein oxidation, lipid peroxidation and DNA damage. This role has been demonstrated in both *in vitro* (Kanner, Harel and Granit, 2001; Esatbeyoglu *et al.*, 2014) and *in vivo* (Kujawska *et al.*, 2009; Krajka-Kuźniak *et al.*, 2012; Szaefer *et al.*, 2014) studies. Finally, many studies have reported the ability of beetroot juice and betanin to reinforce and trigger the endogenous antioxidant mechanisms of organisms, *in vitro* (Krajka-Kuźniak *et al.*, 2013; Esatbeyoglu *et al.*, 2014) and *in vivo* (Szaefer *et al.*, 2014).

The anti-inflammatory activity is among other health benefits of beetroot and its constituents, which is supported by multiple studies. The majority of these studies attribute this anti-inflammatory activity to the ability of beetroot's constituents, mainly of betalains, to counteract the pro-inflammatory signalling cascade, through the inactivation of the cyclooxygenase (COX) and lipoxigenase enzymes, during the early stages of inflammation (Reddy, Alexander-Lindo and Nair, 2005; Vidal *et al.*, 2014). A study showed that betanin's anti-inflammatory activity was comparable *in vitro* with the activity of commercial anti-inflammatory drugs, such as Ibuprofen (Reddy, Alexander-Lindo and Nair, 2005). The anti-inflammatory capabilities of beetroot and betalains have been confirmed also by *in vivo* studies in animals (Lechner *et al.*, 2010; Krajka-Kuźniak *et al.*, 2012; El Gamal *et al.*, 2014; Tan *et al.*, 2015) and in humans (Pietrzkowski *et al.*, 2010).

Other health benefits of beetroot reported in the literature are: (a) the lowering of blood pressure (Coles and Clifton, 2012; Aamand *et al.*, 2013; Siervo *et al.*, 2013; Jajja *et al.*, 2014), (b) the anti-microbial properties (Vulić *et al.*, 2013), (c) the anti-depressant

properties (Pietrzkowski *et al.*, 2010; Sulakhiya *et al.*, 2016), (d) the beneficial effect on the endothelial function (Webb *et al.*, 2008; Hobbs, George and Lovegrove, 2013), (e) the healing effect against Acne skin disease (Maraie *et al.*, 2014) and (f) the improvement of the cognitive function of diabetes type 2 patients (Gilchrist *et al.*, 2014).

The beneficial role of beetroot products has finally been depicted in regard to the improvement of the endurance and performance of athletes. According to several studies, the beetroot's juice appears to be contributing to the lowering of the oxygen demand of athletes during exercise (Bailey *et al.*, 2009), leading to an increase in their endurance; this effect has been mainly attributed to the nitrate content in beetroot (Larsen *et al.*, 2007; Katherine E. Lansley *et al.*, 2011). Another study has eventually reported a significant improvement in the performance of cyclists, who consumed 0.5 L of beetroot juice, 2.5 hours prior to exercise (K E Lansley *et al.*, 2011).

Despite these multiple health effects of beetroot, there is still no use of beetroot or its components in the commercial production of pharmaceuticals (at least to the best of our knowledge). However, beetroot is widely manipulated for the production of health and sports supplements; due to its anti-oxidant,/anti-inflammatory/anti-cancer/cardiovascular-promoting role and its positive effect on the endurance and performance of athletes, beetroot has gained an increasing popularity as superfood. These supplements of beetroot are provided in several marketable forms, most often as capsules, powders or syrups. Indicatively, beetroot supplements are now being sold as capsules at £ 4-15 / 100 g, and as powders at £ 5-21 / 500 g (Holland & Barrett Retail Limited, 2020; Vivadeo Superfoods, 2020).

The betalain pigments are nowadays the beetroot's compounds with the highest commercial and marketable value.

1.3.5. Juice and waste production

According to FAO (2014), the world beetroot production was measured at 269,714 million tonnes in 2014 (Chhikara *et al.*, 2019). The largest producer of beetroot was France with 37.8 million tonnes, whereas UK was 9th in the list, producing 9.43 million

tonnes (Chhikara *et al.*, 2019). In general, 1 kg of beetroot yields approximately 0.5 L of juice (Wruss *et al.*, 2015). Nevertheless, data on the production of raw beetroot and beetroot juice, as well as the generation of beetroot wastes and by-products is scarce.

1.4. Extraction

The recovery of valuable compounds from waste materials requires the employment of certain separation processes, in particular of extraction processes. The extraction processes are mainly categorised into three groups: (1) solid-liquid extraction (SLE), (2) liquid-liquid extraction (LLE) and (3) solid phase extraction (SPE) (Patel, Panchal and Ingle, 2019). In the case of solid wastes, the use of solid-liquid extraction methods is employed in order to facilitate the transfer of the valuable compounds from the solid sample to a liquid extraction solvent (Tzia and Liadakis, 2003).

1.4.1. Solid-Liquid Extraction

Solid-liquid extraction involves traditionally the exposure of a solid waste to a liquid solvent under certain conditions. The aim of such process is the transfer of a target compound from the sample material to the bulk of the liquid solvent (Tzia and Liadakis, 2003; Patel, Panchal and Ingle, 2019). In a typical solid-liquid extraction process, the main mechanisms involved are: (i) the entrance of the solvent into the solid sample, (ii) the solubilisation and/or breakdown of components, (iii) the transport of the solute to the exterior of the solid sample and (iii) the migration of the extracted solute from the surface of the sample to the bulk of the extraction solvent (Tzia and Liadakis, 2003).

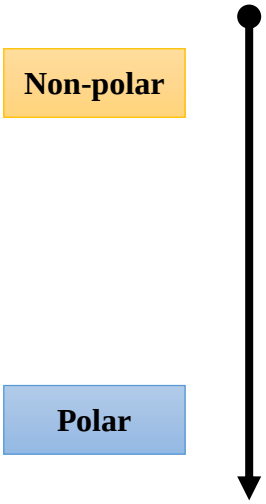
The transport phenomena have, therefore, a significant role in the optimal design of the process. An optimal extraction design aims at the recovery of the maximum amount of targeted compounds from a sample, in combination with the minimum cost, processing time and health and environmental impact (Tzia and Liadakis, 2003); in order to achieve this, the process parameters of the extraction must be manipulated in a way that facilitates these transport phenomena.

The main parameters of the extraction are: (a) the surface area of the sample (Tzia and Liadakis, 2003; Andres *et al.*, 2020), (b) the solid-to-solvent ratio (i.e. mass of the sample compared to the volume of the solvent) (Hernández-Carranza *et al.*, 2016; Andres *et al.*, 2020), (c) the solvent type (Hernández-Carranza *et al.*, 2016; Putnik *et al.*, 2016), (d) the temperature (Hernández-Carranza *et al.*, 2016; Putnik *et al.*, 2016; Andres *et al.*, 2020), (e) the pH of the solvent (Putnik *et al.*, 2016; Andres *et al.*, 2020), (f) the mixing type and speed/intensity (Mohamad *et al.*, 2013; Andres *et al.*, 2020) and (g) the extraction time (Hernández-Carranza *et al.*, 2016; Putnik *et al.*, 2016; Andres *et al.*, 2020).

In specific, key role regarding the efficiency of the process holds the type of solvent. The selection is primarily based upon (a) the solubility of the compound in the solvent and (b) the affinity, mainly in terms of polarity, between the compound and the solvent, while other important selection criteria are the solvent's safety in use (e.g. toxicity, flammability), eco-friendliness, wettability, stability and cost (Tzia and Liadakis, 2003; Aires, 2017). In regard to polarity, the solvents are generally better classified according to the Snyder's polarity index (P') (Snyder et al., 1988); water is regarded as the most polar solvent (P'=10.2), methanol and ethanol as moderately polar (P'=4.3-5.1), and hexane as a non-polar solvent (P'=0.1). Polarity index values for several extraction solvents are given in Table 1.1.

In the case that the extraction is intended for food applications (e.g. for the production of foodstuffs or food ingredients), the solvent type must be in compliance with the national legislation in-force. In the EU area, the extraction solvents for food applications are categorised, according to Directive 2016/1855/EC of the European Commission, to those that are generally safe for all uses, in compliance with good manufacturing practice (i.e. presence of solvent residues in the foodstuff or food ingredient only up to technically unavoidable quantities which present no danger to human health; e.g. ethanol, propanol, ethyl acetate etc.), and to those which are not regarded as safe and are only allowed for specific applications and whose maximum residue levels in the foodstuff or food ingredient is defined in the law and must be controlled (e.g. hexane, dimethyl ether, dichloromethane etc.). Methanol and propan-2-ol are permitted to be used for all uses, however with a maximum residue limit of 10 mg/kg in the final foodstuff or food ingredient .

Table 1.1 *The polarity index of various extraction solvents (Source: Harvey, 2000)*



Mobile Phase	Polarity Index (*P'*)
cyclohexane	0.04
n-hexane	0.1
carbon tetrachloride	1.6
i-propyl ether	2.4
toluene	2.4
diethyl ether	2.8
tetrahydrofuran	4.0
ethanol	4.3
ethyl acetate	4.4
dioxane	4.8
methanol	5.1
acetonitrile	5.8
water	10.2

The conventional solid-liquid extraction techniques involved, traditionally, either the simple exposure of a sample in a liquid extraction solvent under certain conditions of mixing and temperature (also referred to as maceration), or the use of the continuous Soxhlet extraction method (Aires, 2017). However, novel solid-liquid extraction techniques have recently emerged, that are able to achieve improved extraction yields; examples are the pressurised liquid extraction (PLE), the supercritical fluid extraction (SFE), the ionic liquid (IL) mediated extraction, the microwave-assisted extraction (MWAE), the ultrasound-assisted extraction (UAE), the Pulsed Electric Field (PEF)-assisted extraction, the high pressure extraction (HPE) and the enzymatic-assisted extraction (EAE) (Renard, 2018).

In the following section, a more detailed analysis of the enzymatic-assisted extraction (EAE) is given, as this technique is the core extraction methodology used in this project, for the recovery of high-value compounds from beetroot pomace wastes.

1.4.2. Enzymatic-aided extraction

The enzymatic-aided extraction is a novel extraction technique, which involves the employment of enzymes in order to improve the recovery yields of the target compounds (Shamraja S Nadar, Rao and Rathod, 2018).

In particular, plant-derived products, such as many agri-food wastes, comprise of a complex lignocellulosic structure, which is formed mainly of cellulose, hemicellulose, lignin and pectin. This complex lignocellulosic structure is organised in three distinctive layers; (a) the primary cell wall, (b) the secondary cell wall and (c) the middle lamella (Figure 1.6). The primary cell wall is formed of cellulose, hemicellulose and pectin, as well as of relatively low amounts of proteins. The secondary cell wall comprise mainly of cellulose, hemicellulose and lignin. The primary cell wall is the outer layer of the structure, whereas the secondary cell wall is the inner layer. The middle lamella is found between the primary and secondary cell walls; this layer consists mainly of pectin and has an adhesive role.

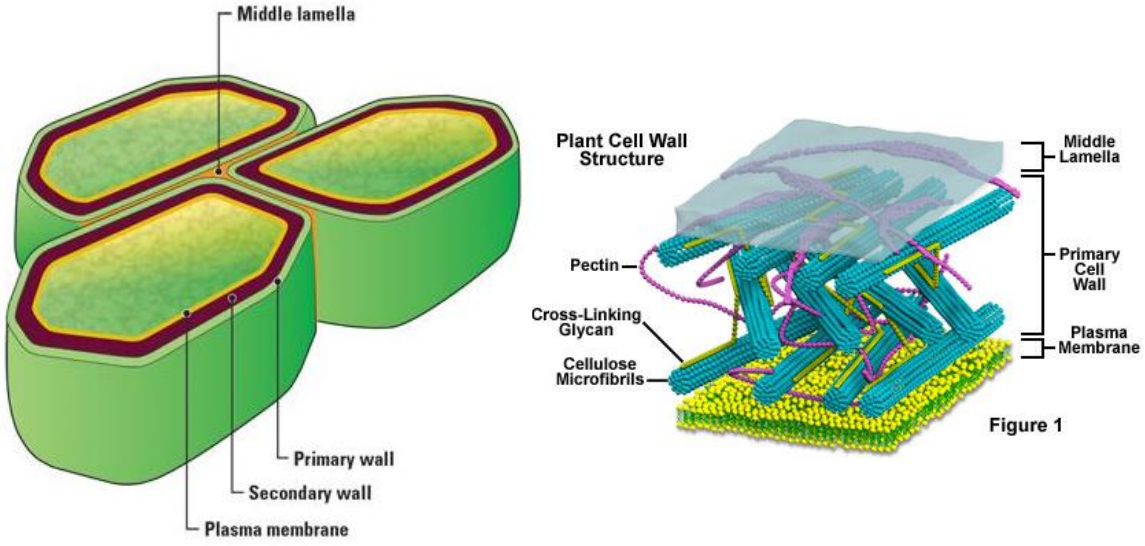


Figure 1.6 *The typical lignocellulosic structure of plant-derived materials, comprising of the primary cell wall, middle lamella and the secondary cell wall.*

Cellulose is an unbranched polysaccharide of glucose. Hemicellulose is a polysaccharide that consists of glucose and other types of sugars, such as xylose, mannose, galactose, rhamnose and arabinose; this is a branched and relatively shorter chain biopolymer compared to cellulose. Pectin is also a branched heteropolysaccharide, which mainly comprise of galacturonic acid. Finally, lignin is not a polysaccharide, but a polymer formed by cross-linked phenolic precursors.

The enzymatic-aided extraction is generally applied in the case of plant-derived products as the majority of the target compounds of the extraction are mostly found

inside the complex lignocellulosic structure, in which they remain either entrapped or even bound (Shamraja S Nadar, Rao and Rathod, 2018). A typical example is phenolic compounds; phenolic acids often form ether linkages with lignin through hydroxyl groups of their aromatic rings or ester linkages with the polysaccharides and proteins of the plant cell walls, flavonoids are often covalently linked to sugar moieties via glycosidic bonds through an hydroxyl group or through carbon-carbon bonds, and tannins commonly form strong complexes with proteins (Shamraja S Nadar, Rao and Rathod, 2018).

The use of specific degrading enzymes can decompose the plant's complex lignocellulosic structure, leading to the liberation of those potentially entrapped or bound compounds. As a consequence, this may enhance their availability during extraction and improve their extraction yields (Shamraja S Nadar, Rao and Rathod, 2018).

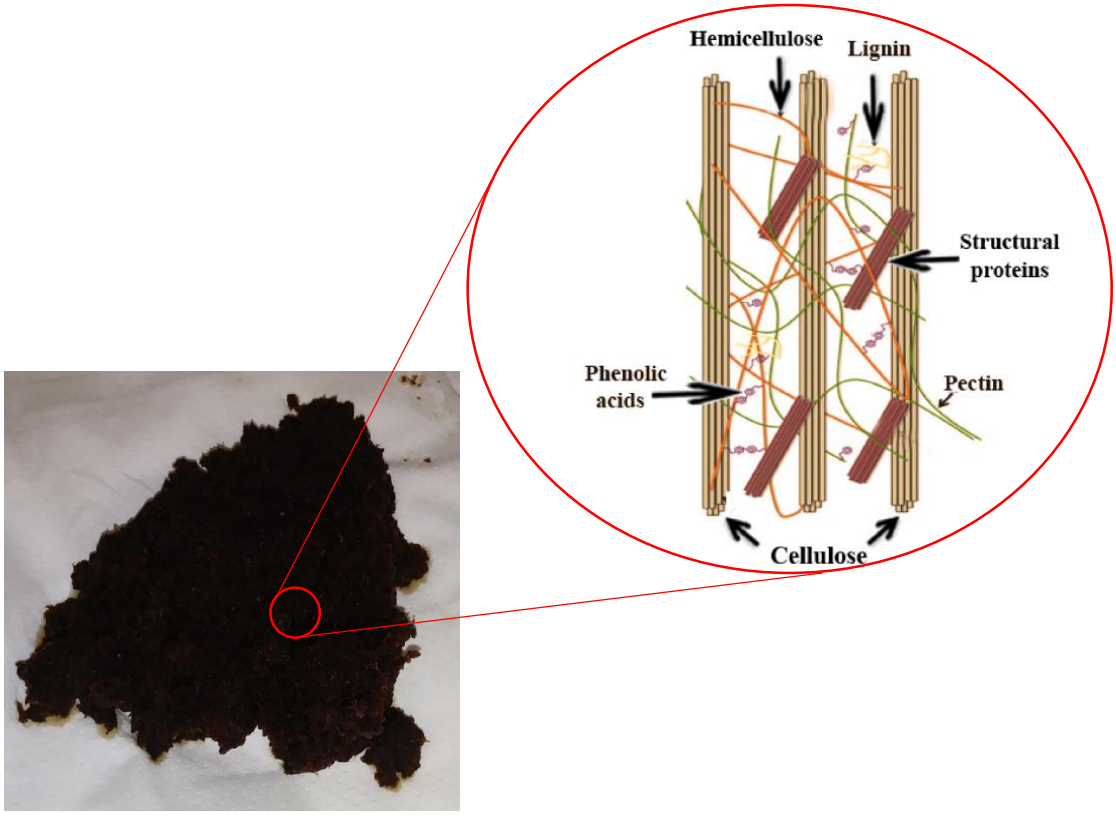


Figure 1.7 *The internal structure of a typical agri-food waste product, in particular of the beetroot pomace. Several compounds, such as phenolic compounds, remain entrapped inside the waste's complex lignocellulosic structure (Source: Nadar, Rao and Rathod, 2018).*

Each plant cell wall component is decomposed by specific enzymes; (a) cellulases are a group of enzymes which decompose cellulose into its glucose monomers or into shorter polysaccharides and oligosaccharides (Cheng and Timilsina, 2010), (b) hemicellulases are a group of enzymes that decompose hemicellulose into its monomer components (glucose, xylose, mannose, galactose, arabinose etc.) (Cheng and Timilsina, 2010), (c) pectinase is an enzyme which decomposes pectin and (d) Lignin-modifying enzymes (LME) and Lignin-degrading auxiliary enzymes (LDA) are groups of enzymes that decompose lignin (Janusz *et al.*, 2017).

These enzymes, except for the lignin degrading enzymes, decompose the plant's cell wall components through a reaction called enzymatic hydrolysis (Figure 1.8). In this type of reaction, a glycosidic linkage of a polysaccharide chain is cleaved via the addition of one molecule of water; the reaction is catalysed by the enzyme (Cheng and Timilsina, 2010; Shamraja S. Nadar, Rao and Rathod, 2018; Muhammad et al., 2019). Lignin degrading enzymes are not hydrolytic, but they decompose lignin via an oxidation mechanism (Chukwuma *et al.*, 2020).

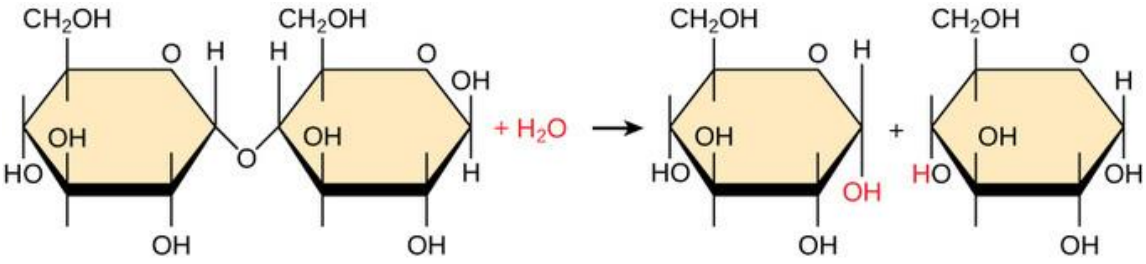


Figure 1.8 *A typical equation of an enzymatic hydrolysis reaction. The glycosidic bond between two monosaccharide molecule in a polysaccharide chain is cleaved via the addition of one molecule of water; the reaction is catalysed by the enzyme.*

The enzymatic-aided extraction can be typically performed in three possible configurations: (a) the performance of the enzymatic hydrolysis and the extraction simultaneously, where the enzymatic solution is also the extraction solvent (Ghandahari Yazdi *et al.*, 2019), (b) the performance of the enzymatic hydrolysis and the extraction in two subsequent stages, with a simple addition of the complementary extraction solvent components in the enzymatic mixture, at the end of the hydrolysis (e.g. ethanol

in the enzyme solution) (Li *et al.*, 2015; Fu *et al.*, 2019), and, finally, (c) the performance of the enzymatic hydrolysis and the extraction in two separate stages, involving the removal of the enzyme mixture after the hydrolysis and the addition of the extraction solvent into the solid sample, prior to extraction (Obama *et al.*, 2012).

Among the main parameters of the enzymatic-aided extraction are: (1) the type of the employed enzymes, (2) their concentration and (3) their relative concentration ratio in the mixture, (4) the solid-to-liquid ratio of the hydrolysis, and, lastly, several conditions of the treatment, mainly (5) the temperature, (6) the pH and (7) the treatment time of the hydrolysis.

Most of the commercial enzyme preparations, which are typically employed in the enzymatic-aided extraction of plant materials, such as cellulases and pectinases, are classified as food-grade and GRAS (Generally Recognised as Safe), by the US Food and Drug Administration (FDA) (FDA GRAS notices online inventory[1]; FDA, 2014 & 2015) and by FAO/WHO (Food and Agriculture Organization and World Health Organization) (FAO, 1992, 2000a and b).

In the EU, the regulatory framework for "food enzymes", i.e. those to be used in order to serve a technological function in the manufacture, production, preparation, transport or storage of foods, has been established with the Regulation (EC) 1332/2008. However, this regulation is not yet in force until the on-going assessment of the enzymes' safety is completed, which is due to take place with the publication of the Community list; this will include all the approved enzymes.

At present, the use of food enzymes is regulated by the national legislation of each Member State; for example, in France, most of the enzymes used in the enzymatic-aided extraction of plant materials has been authorised according to "Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires", while many other countries may require individual authorisation procedures. There is currently no EU legal framework established about the use of enzymes as "processing aids" for the production of food additives or ingredients.

[1] Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices. Searches include the following keywords: "cellulase", "pectin esterase", "pectin lyase" and "polygalacturonase".

The enzymatic-aided extraction may offer several advantages in comparison to conventional extraction techniques, such as higher extraction yields, lower extraction times, lower energy consumption and efficient recoveries with the use of simpler procedures, without, for instance, the use of toxic or hazardous solvents (Shamraja S Nadar, Rao and Rathod, 2018). In contrast, the enzymatic extraction displays certain limitations, which are mainly the relatively high cost of the enzymes and the difficulty of up-scaling to an industrial level process (Puri, Sharma and Barrow, 2012). However, these limitations may often be offset by the aforementioned benefits of the enzymatic-aided extraction, depending on each individual application.

1.5. Membrane processing

An extraction process is an essential procedure in an attempt to recover desired valuable compounds from solid materials; however this process leaves often extracts containing, in addition, several undesired products, too. For example, these could be the solid sample itself, an excess amount of extraction solvent or the presence of other extracted undesirable compounds. Downstream processes are, as a consequence, usually applied after the extraction, in order to separate and purify the target compounds from any undesired extract constituent.

Membrane processing is considered to be one of the most fundamental downstream processes and, in practice, it involves the subjection of a liquid sample to passing through a semi-permeable membrane, with pores of specific size on its surface. The driving force of the sample through the membrane depends on the type of membrane processing; this chapter focuses on pressure-driven membrane processes. In this type of processes, the driving force of a sample through the membrane is the pressure difference between its two sides (i.e. the feed and the permeate side), which is known as transmembrane pressure (TMP). Furthermore, the main mechanism of the membrane processing is size sieving (Visakh and Nazarenko, 2016). In specific, larger molecules that are generally able to be retained by a particular membrane, are separated from smaller molecules of a sample, which are able to pass through its pores. The selectivity of the retention is highly dependent on the size of the compounds and the pore size distribution of the membrane.

Membrane processes are divided into two modes of operation: (a) the dead-end filtration and (b) the cross-flow filtration. In dead-end filtration, the liquid feed sample is flowing vertically to the membrane surface, whereas in cross-flow filtration the liquid feed flows in parallel to the membrane's surface (tangential flow; Figure 1.9).

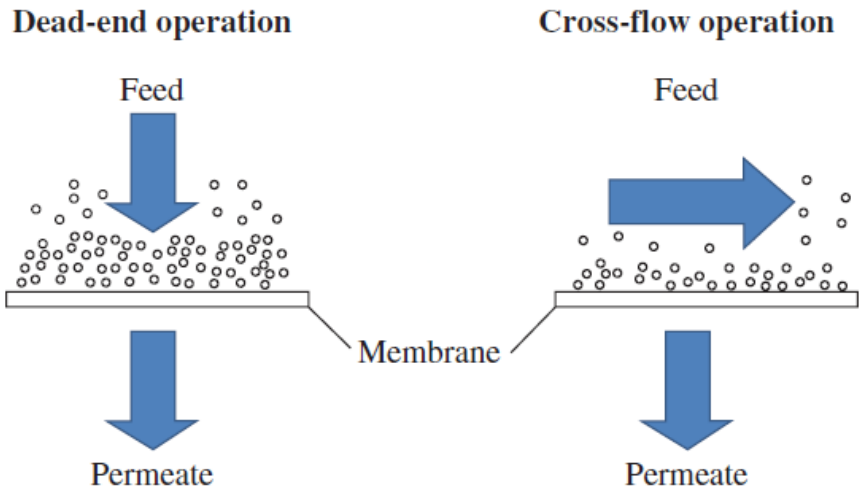


Figure 1.9 *A schematic diagram of dead-end and cross-flow filtration. (Source: Tamine, 2013)*

Table 1.2 *The four classes of the membranes according to their pore size, along with their nominal MWCO and their operating transmembrane pressures.*

Type of filtration	Pore size[1]	MWCO	Operating Pressures
Microfiltration (MF)	> 0.1 μm	Particle size	0.1 – 3 bar
Ultrafiltration (UF)	2 - 100 nm	1 – 500 kDa	1 – 10 bar
Nanofiltration (NF)	< 2 nm	100 – 1000 Da	5 – 35 bar
Reversed Osmosis (RO)	Typically < 1 nm	<200 Da	10 – 150 bar

[1] According to the "Terminology for membranes and membrane processes" of the International Union of Pure and Applied Chemistry (IUPAC, 1996) (Koros, Ma and Shimidzu, 1996)

The membranes are also divided into four classes according to their pore size: (i) Microfiltration (MF; > 0.1 μm), (ii) Ultrafiltration (UF; 2-100 nm), (iii) Nanofiltration (NF; 0.5-2 nm) and (iv) Reverse Osmosis (RO; < 0.5 nm) (Koros, Ma and Shimidzu, 1996; Table 1.2). The term nominal molecular-weight cut-off (MWCO) is oftentimes used, instead of the pore size, to indicate the lowest molecular weight of compounds that will be retained at a rejection level of 90%, by a membrane.

As cross-flow filtration is employed in this project, a brief description of this membrane process category is shown below.

1.5.1. Cross-flow filtration

Cross-flow filtration is a type of membrane processing in which, as mentioned previously, the liquid feed stream is flowing in parallel to the membrane surface. An advantage of cross-flow filtration is that, as a process, it generates two streams: a permeate and a retentate, while dead-end filtration generates only a permeate (Figure 1.9 & 1.10). A typical cross-flow system consists of the following components: (i) a feed tank, (ii) a pump, (iii) the filtration module (membrane), (iv) a recorder of the permeate mass, (v) several pressure, temperature and flow meters and (vi) a heat exchanger (optionally) (Figure 1.10 - B).

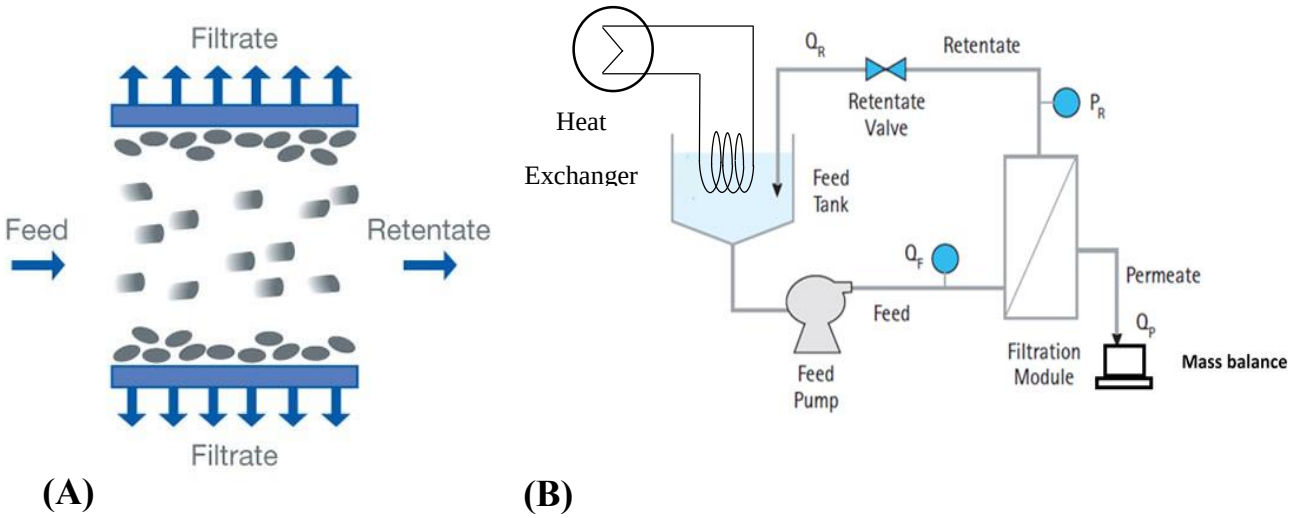


Figure 1.10 *Schematic diagrams of: (A) cross-flow filtration and (B) a typical cross-flow filtration system*

The generation of two streams, instead of only one, allows the operation of cross-flow filtration in several configurations. Among them, the most common are: (a) the total recycle mode (both permeate and retentate streams are recycled to the feed tank), (b) the batch concentration mode (retentate is fed back to the feed tank and permeate is taken out), and the single-pass mode (both permeate and retentate are taken out).

By using cross-flow filtration, the following objectives can be accomplished:

- Clarification (removal of suspended solids from the feed stream)
- Concentration (of the retained substances, due to the removal of solvent from the feed to the permeate)
- Fractionation (of molecules based on their MW in the permeate/retentate streams)
- Purification (of targeted compounds, attributed to the selective removal of other compounds in a sample, due to their different MW)

The main operational parameters of cross-flow filtration, which can be manipulated, are: (a) the transmembrane pressure (TMP), (b) the cross-flow velocity, (c) the temperature and (d) Volume Concentration factor (VCF) or time of operation (Tamine, 2013). In particular, the transmembrane pressure is defined as the average pressure applied to the membrane and is calculated as: $TMP\ [bar] = [(P_F + P_R)/2] - P_P$, where $P_{F, R, P}$ are the pressures in the feed, retentate and permeate side of the filtration module, respectively. Furthermore, the cross-flow velocity is the linear velocity of the feed flow tangential to the membrane surface, and is given in [m/s]. Finally, the Volume Concentration factor is number of times the volume of the feed has been reduced at a certain moment and is given as: $VCF = V_{Feed}/V_{Retentate}$; this parameter can be used instead of time of operation.

Other significant process factors can be divided to those related to the sample (e.g. feed concentration, pH, viscosity, charge of molecules, size distribution and shape of molecules, stickiness) and those related to the membrane (pore size distribution, surface charge, membrane material). Important factors that affect the retention of membranes are (i) the solute-membrane interactions (e.g. electrostatic interaction) and (ii) the

solute-solute interactions (e.g. tendency to form coagulants). As a consequence, a membrane separation process is dependent on a multitude of complex factors.

1.5.2. Concentration polarisation & Membrane fouling

A common problem of the membrane processing is the decrease of the permeate flux, during the filtration run. This is a frequently occurring undesirable phenomenon (Tamine, 2013), which can reduce the quality of the separation, increase the processing time and shorten membranes life. This reduction is caused by two factors: (i) the concentration polarisation and (ii) the fouling of pores (Tamine, 2013). Concentration polarisation is the accumulation of rejected solutes on the membrane surface, as the feed passes through the membrane. As a consequence, an increase of the rejected solutes concentration is created on the membrane's boundary layer, compared to the concentration of the feed bulk, which increases the osmotic pressure and reduces the net driving force through the membrane, thus decreasing the permeate flux (Cui and Muralidhara, 2010; Tamine, 2013).

Fouling is defined as the deposition of solutes on the membrane surface or inside the membrane pores, in a way that embeds the normal flow of the liquid stream through membrane. There are three main types of fouling: (a) cake or gel layer formation, (b) pore blocking and (c) adsorption (Cassano, Marchio and Drioli, 2007; Tamine, 2013). Cake or gel layer is formed from larger particles than the membrane pores, which tend to accumulate and form a deposition layer on the membrane surface, thus blocking its pores (Tamine, 2013). Pore blocking is the deposition of molecules of similar size to the membrane pore size, either internally or externally to the pore (Tamine, 2013). Finally, adsorption is the attachment of particles on the membrane surface (either externally or internally to the pore), due to hydrophobic, polar or electrostatic interaction, but not due to their size (Cuperus and Smolders, 1991; El Rayess *et al.*, 2011).

1.5.3. Membrane cascades

Membrane cascades consists of a series of membrane runs of an initial liquid sample, each by employing membrane modules of declining MWCO (Figure 1.11). In this type

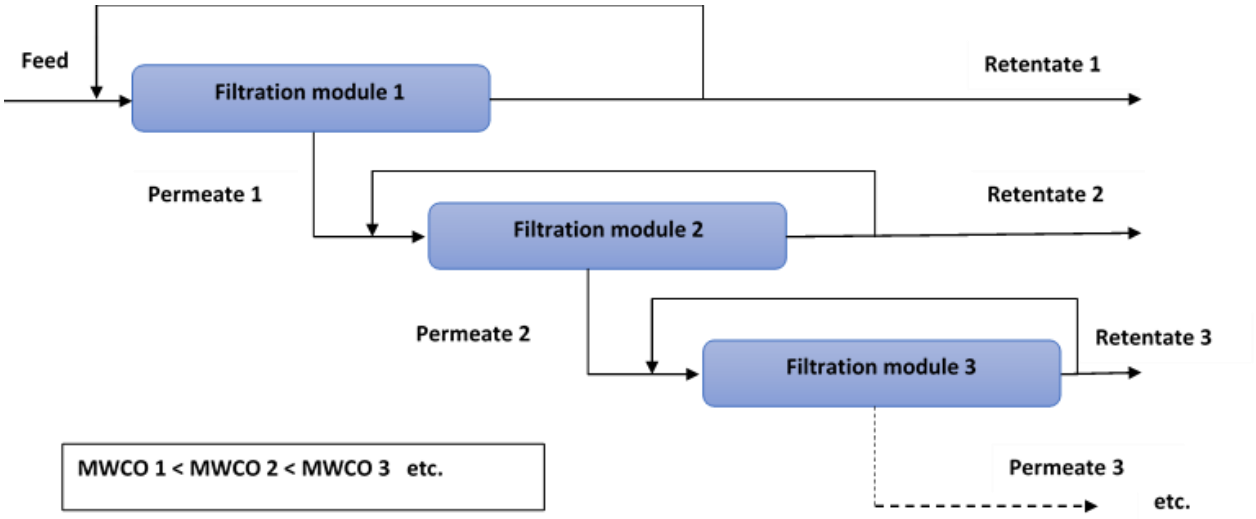


Figure 1.11 *A schematic diagram of a membrane cascade.*

of processes, the permeate of each membrane run is used as feed for the subsequent membrane run using a membrane module of a lower MWCO (Lin and Livingston, 2007; Cui and Muralidhara, 2010). During this procedure, molecules with size low enough to pass through the first module may be blocked by the second module. Membrane cascades allow the fractionation of molecules of an initial liquid into the various retentate and permeate streams, based on specific MW ranges (Siew *et al.*, 2013; Rizki *et al.*, 2020).

1.6. Optimisation methodology

A process can be visualised as a series of actions or operations combined for the accomplishment of a particular task. In general, most processes involve a number of independent (input) variables, which may be controllable or uncontrollable, and a number of output responses (Figure 1.12; Montgomery, 2012).

An experiment consists of a series of tests (runs), in which changes are made in input variables in order primarily (a) to observe the effects that those have on the output responses, (b) to explain the reasons behind those effects and (c) to determine the particular input setting that can lead to a desired output. The first two objectives assist

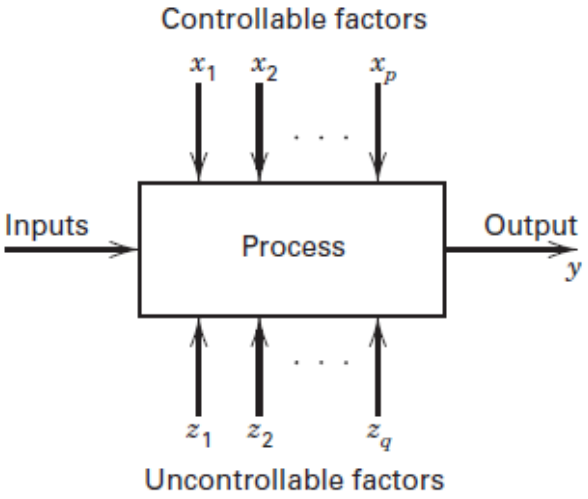


Figure 1.12 *A visual representation of a process, involving a number of controllable and uncontrollable input factors and output responses (Montgomery, 2012).*

mainly in understanding the underlying mechanism of a process. The last objective involves the optimisation of this process.

The experimental procedure consists mainly of (a) the design of the experiments and (b) the statistical analysis of the data. The design of experiments (DoE) involves the selection and planning of the input variables, in such way that would lead to the collection of data which, after appropriate statistical and mathematical analysis, will provide the desired conclusions about the process (Montgomery, 2012; Dadashzadeh, Duzgun and Yesiloglu-Gultekin, 2017). In multi-variable experiments, the experimental designs traditionally involved the use of an one-factor-at-a-time (OFAT) approach (DeLoach and Ulbrich, 2007), in which one factor is varied each time with all the other factors being kept constant at a specific level. However, factorial approaches, in which factors are combined together, so that each factor is examined at various levels of the other factors, have been proposed as more suitable and effective. The main reason is that factorial designs can give additional information on possible interactions between factors, something that the OFAT approaches fail to consider. Common factorial designs are (1) the Central Composite Design (CCD), (ii) the full factorial design and (iii) the Box-Behnken design (Figure 1.13; Montgomery, 2012).

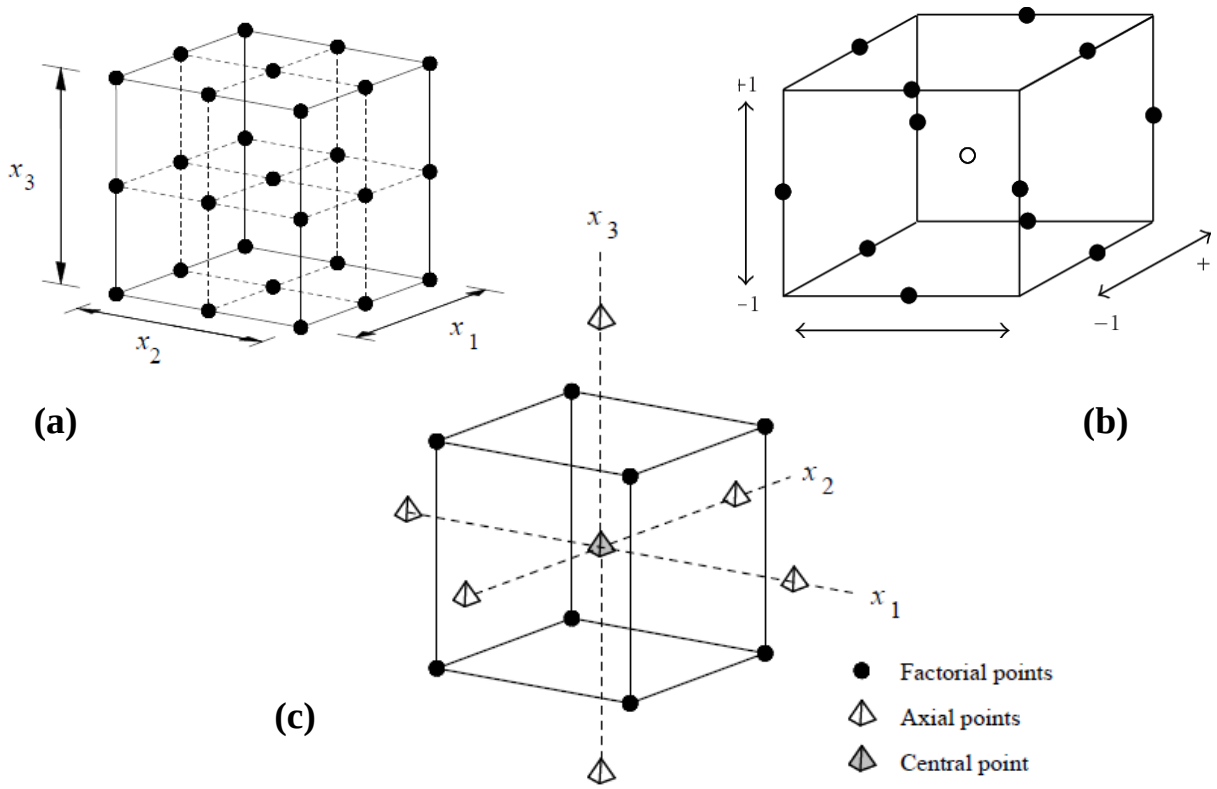


Figure 1.13 *Typical factorial designs of Design of Experiments (DoE); (a) full factorial design, (b) Box-Behnken design and (c) Central Composite Design (CCD)*

The mathematical and statistical analysis of the experimental data is usually performed using the Response Surface Methodology (RSM). In RSM problems, the data are represented in a surface plot, to understand the topography of the response surface including local maximums, minimums and ridge lines and to find areas where the desired response occurs (Figure 1.14; Aydar, 2018). The data are also fitted in a second-order polynomial empirical equation, as follows:

$$y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i<j} \sum \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \varepsilon$$

where β_o, β_i, β_{ij}, β_{ii} are the regression co-efficients of the constant term, the independent variable terms (x_i), the quadratic terms (x_i^2) and the interaction terms ($x_i \cdot x_j$), respectively, and ε is the experimental error. The sign of the coefficient indicates the

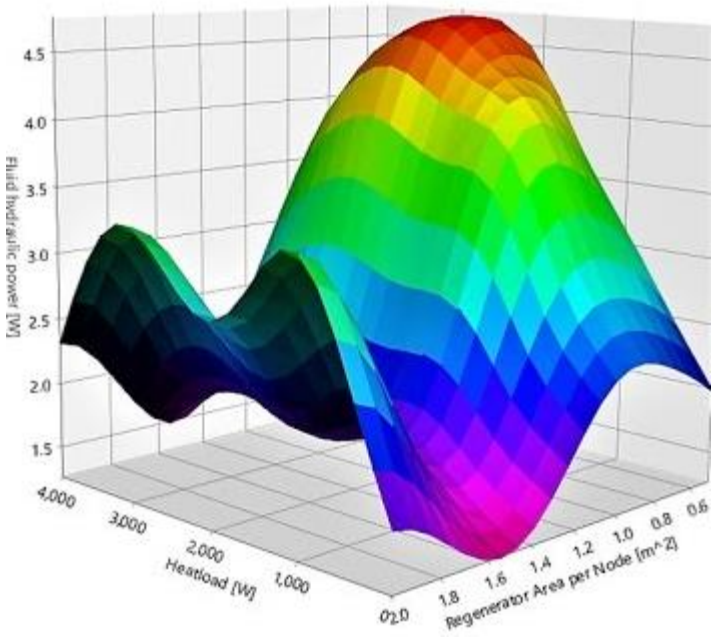


Figure 1.14 *A typical response surface plot of RSM. The plot allows to understand the topography of the response surface including local maximums, minimums and ridge lines, and to find those areas and factor value combinations in which the desired response occurs*

direction of the relationship, while the level of the value indicates the extend of this relationship. The regression and ANOVA analyses of the RSM give information regarding the statistical significance of the dependency of the response from each variable, the lack-of-fit or not of the model and its ability to significantly predict the response based on the variable values, and the statistical significance of the interactions between the variables. The terms that are found to be statistically insignificant are usually omitted and this typically increases the accuracy of the model. In case that the model is found to have adequate accuracy, it can be used to determine the optimum responses. At the end of the optimisation process, confirmatory experiments are performed at the model-predicted optimum conditions and a comparison is made between the actual and predicted optimum values.

Examples of software that performs DoE and RSM optimisation analysis are the Minitab Statistical software (Minitab LLC, USA), the Design-Expert® software (Stat-Ease Inc., USA) and the Statgraphics Centurion software (Statgraphics Technologies Inc., USA) etc.

1.7. Beetroot and agri-food waste processing

This section provides an extended literature review of the processing strategies employed for the recovery of valuable compounds from raw beetroot, the beetroot juice and the beetroot pomace. In particular, the aspects which are elucidated are the extraction methods, the type of target compounds, the analytical techniques and the recovery yields. Furthermore, the processing of other agri-food wastes is also presented with emphasis on the recovery of similar types of compounds and extraction methodologies, employed in this project.

1.7.1. Processing of raw beetroot, beetroot juice & beetroot pomace

This subchapter focusses on the extraction methods and the membrane processing of raw beetroot, the beetroot juice and beetroot wastes.

Conventional extraction

One of the earliest studies on the recovery of compounds from raw beetroot has been performed by Kujala *et al.* (2000). By using conventional aqueous extraction (solid to solvent ratio 1:20, two consecutive extractions of 1 min each, using an homogeniser, extracts were combined), it was found that the total polyphenol content (TPC) was 15.5 mg GAE/g dry weight in the peel and 4.2 mg GAE/g dry weight in the flesh of beetroot. Furthermore, Kujala, Loponen and Pihlaja (2001) performed the conventional extraction of beetroot, this time comparing two different solvents; 80% aq. methanol and pure water (solid to solvent ratio 1:50, three consecutive extraction 45/45/20 min, extracts were combined). The use of 80% aq. methanol as solvent led to higher polyphenol (24.1 mg GAE/g) and betalain (given in terms of peak area of the HPLC analysis) recoveries, compared to pure water (polyphenols measured at 20.5 mg GAE/g). Kujala *et al.* (2000, 2001, 2002) also performed the qualitative determination of individual phenolic and betalain compounds inside the beetroot extracts, using HPLC-ESI-MS, HPLC-DAD and NMR, from 4 cultivars. The authors confirmed the presence of phenolic compounds, given in Figure 1.5 and of betacyanins (betanin, isobetanin) and betaxanthins (vulgaxanthin I & II).

In another study, Georgiev *et al.* (2010) measured the amount of phenolic compounds at 12.27 mg FAE/g (FAE: Ferulic Acid Equivalent) and 0.55 mg FAE/g (with Folin assay), and of betalains at 0.61 mg FAE/g and 0.47 mg FAE/g (with UV-Vis), using conventional extraction (solid to solvent ratio 1:1, 70% aq. ethanol as solvent), in beetroot hairy roots and intact beetroot plants, respectively. In the same study, individual phenolic compounds were identified with HPLC-DAD.

In a more recent study, Neagu and Barbu (2014) demonstrated that the optimal solvent for the extraction of betanin is water with added 0.5% citric acid and 0.1% ascorbic acid, in comparison to 50% and 70% aq. ethanol with various concentrations of citric acid/ascorbic acid, by yielding 20 mg betanin/g of beetroot. The performance of further extractions at various conditions (T: 20^{o}C and 70^{o}C, S/L: 1:5 and 1:10, pH: 2.5 and 8, using water as solvent) showed that the optimal conditions for betanin were T=70^{o}C, regardless of the other two parameters (although graphs shows that pH 2.5 leads to higher recoveries). Finally, Kavalcová et al. (2015) measured the total phenolic content of extracts of red beetroot originating from various regions of Slovakia (S/L 1:2, 80% aq. ethanol, 16 hours), and found that the values ranged between 820-1280 mg GAE/kg beetroot.

The beetroot pomace has also been used, with conventional extraction methods, for the recovery of compounds. In a recent study, Kushwaha *et al.* (2017) attempted to optimise the conditions of the conventional aqueous extraction of beetroot pomace for the maximum recovery of phenolic compounds and betalains using Response Surface Methodology (RSM), by manipulating the following parameters: solid-solvent ratio (1:15 - 1:45), pH (1.5-5.5), temperature (30-70^{o}C), time (2.5-12.5 min). Based on the RSM model, the maximum predicted amounts calculated were: 160.67 mg/100 mL for total phenolic compounds (actual 156.54 mg/100 mL) (with Folin assay), 19.36 mg/100 mL for betacyanins (actual 17.07 mg/100 mL) and 17.40 mg/100 mL for betaxanthins (actual 15.04 mg/100 mL) (with UV-Vis), all under the same conditions: solid-solvent ratio 1:15, 50.04^{o}C, 10 min, pH 2.5. However, there were some discrepancies between the process variables reported in the abstract and the RSM table. These RSM table variable values may have been incorrectly assigned and taken into consideration in the RSM model equation, hence raising questions about its reliability. Finally, the results

were expressed per 100 mL of extract, instead of per mass of beetroot pomace, something which impedes the proper evaluation of the extraction.

Novel extraction

One of the earliest novel extraction techniques applied in the case of **raw beetroot** was the **Pulsed Electric Fields (PEF)-assisted extraction**, studied by Fincan, DeVito and Dejmek (2004). The authors assessed PEF-assisted extraction as an effective method with low-energy consumption, for the recovery of beetroot pigments, although the recoveries appeared to be higher for the conventional mechanical pressing. Nayak et al. (2006), furthermore, employed **gamma-irradiation**, as an extraction pre-treatment; betanin showed increasing recovery yields as a function of the gamma-irradiation dosage. The highest recovery of betanin was reported to be 7.11 mg/100 mL (corresponding to 1.78 mg/g beetroot, based on the S/L of 1:25), using the maximum tested dosage of 10 Gy, as compared to the blank's (no-irradiation) recovery of 5.58 mg/100 mL (1.36 mg/g beetroot), corresponding to an increase of 27%.

The two most studied novel extraction techniques involving raw beetroot are (i) the ultrasound-assisted extraction and (ii) the microwave-assisted extraction. In regard to the former technique, Sivakumar *et al.* (2009) optimised the **ultrasound-assisted extraction** of pigments from beetroot; the experiments demonstrated that the optimal conditions are 80 W for 3 h, using water-ethanol mixture of 1:1, yielding 0.13 g beetroot dye/g beetroot (using UV-Vis). Moreover, Guldiken *et al.* (2016) performed the ultrasound-assisted extractions of beetroot powders, and measured total phenolic contents of 255 mg/100 g, using Folin assay. In the same study, betalain pigments were determined with HPLC-DAD, but recoveries were not translated in concentration units (e.g. mg/mL).

The **microwave-assisted extraction** of raw beetroot has been initially studied by Ravichandran et al. (2012, 2013). In their first study, the authors reported an 1.5-fold increase of the antioxidant capacity of microwave-assisted extracts compared to conventional extracts, as measured with ABTS assay, while a similar increase was found also for roasting (ABTS assay) and boiling (DPPH assay) pre-treatments. However, there was not a good correlation among the antioxidant capacities measured

with DPPH and ABTS assays, for each sample. Furthermore, the amounts of the individual phenolic acids were lower in the microwave-assisted extracts compared to the conventional, as measured with HPLC-DAD (Ravichandran *et al.*, 2012). In their second study, the use of microwave-assisted extraction led to the increase of betaxanthins, with a maximum increase of 19% at the conditions of 1800 W for 3 sec (as measured with UV-Vis), and to the increase of betanin, with a maximum increase of 30% at 450 W (as measured with HPLC-DAD), in comparison to conventional extracts, whereas betacyanins were found to be even (as measured with UV-Vis) (Ravichandran *et al.*, 2013).

The optimisation of the microwave-assisted extraction has been further investigated involving raw beetroot, by applying the Response Surface Methodology (RSM). In particular, Cardoso-Ugarte et al. (2014) optimised the parameters of the microwave-assisted extraction, which was performed in both single and two-step modes, for the recovery of betanines and betaxanthins. The two-step processing mode, which involved two microwave extraction cycles with a cooling step in between, was proved more efficient in the recovery of betanines and betaxanthins, yielding 187 mg/g dry mass and 125 mg/g dry mass, respectively (as measured with UV-Vis), at the optimum conditions of 400 W, duty cycle 100% and 130 s – 100 s with added 40 mM ascorbic acid. In contrast, the single-step microwave extraction optimally recovered 128 mg/g dry mass of betanines (at conditions 400W/100%/100s) and 101 mg/g dry mass of betaxanthins (at conditions 400W/100%/140s). Furthermore, it was found that long microwave processing times lead to lower recoveries, due to the thermal degradation of the beetroot pigments. The addition of ascorbic acid was additionally proved as very efficient in protecting pigments against this thermal degradation. The microwave-assisted extraction achieved 2-fold increase of beetroot pigments in comparison to conventional extraction. Finally, Singh, Ganesapillai and Gnanasundaram (2017) optimised the microwave-assisted extraction of beetroot peels by using two different solvents, i.e. ethanol and citric acid aq. solution, and determined an optimal betanin recovery of 472 mg/L (as measured with UV-Vis), using ethanol, at optimum conditions of pH 4.74, 384.25 W, 74.91 s.

The novel extraction of **beetroot pomace** has only been investigated using **ultrasound-assisted extraction**. In their study, Vulić *et al.* (2012) estimated the TPC values of

pomace extracts from 4 beetroot varieties at 1,87-11,98 mg GAE/g dry mass (using Folin assay) and betalain content of 0,75-3,75 mg betalains/g dry mass (using UV-Vis). The HPLC-DAD analysis showed that the most predominant phenolic compounds are ferulic acid (0.850-1.200 mg/g dry mass) and caffeic acid (0.018-0.074 mg/g dry mass), while the most predominant pigment is betanin (0.038-0.368 mg/g dry mass). In another study, the same authors similarly reported that strong antiradical activity and reducing power of the ultrasound pomace extracts of 5 beetroot cultivars, and the presence of individual phenolic compounds, mainly ferulic acid and catechin, and of betalain pigments (betanin, isobetanin, vulgaxanthin I) (Vulić *et al.*, 2014). Both studies have not performed, however, a comparison of the ultrasound-assisted extraction to the conventional extraction.

Membrane processing

Three recent studies on the membrane processing of beetroot products have been published in literature; two involve the filtration of beetroot juice and one the filtration of red beet stalks extracts. Firstly, Thakur and Kumar Das Gupta (2005) investigated the effect of enzyme treatment (ET; using Pectinex Ultra SPL, Novozymes, Denmark, 0.07 and 0.15% w/v concentration, 45ºC, for 2.5 h) and a fining agent (FA; bentonite, 0.08% and 0.2% w/v concentration, for 1 h) on the ultrafiltration (UF) of beetroot juice. They reported that FA and ET increases the permeate flux of the UF, with FA giving the highest fluxes. They, also, reported that ET prior to UF leads to better clarity and colour values (CIELAB colour scale) in the processed juice.

A more recent study, by Mereddy *et al.* (2017), investigated the use of both laboratory and pilot scale membrane processing systems on the purification of betalains and the removal of dissolved solids and salts in beetroot juice. The lab-scale experiments showed that betalains are mainly separated in the retentate stream of the 1 kDa MWCO membrane (Millipore membrane disks, 63 mm diameter, for 200 mL Amicon bench-top module, at 4.5 bar and 25ºC) and that this separation is also obvious upon visual examination. The pilot scale experiments managed to retain 99% of betalains and only 6% of salts, 50% of dissolved solids and 40% of phenols, when using MF (0.1 μm pore size, Graver Technologies, USA) followed by 1 kDa MWCO (Koch-spiral wound) or

Loose RO (300 Da MWCO; Dow spiral wound) membranes, leading to the effective purification of betalains.

Finally, dos Santos et al. (2016) performed the extraction of red beet stalks and the subsequent MF (0.05 μm pore size) and UF (20 kDa MWCO; both from Andritz Separation) processing of the extracts. The study demonstrated a 99.5% reduction in peroxidase activity, a 99.9% reduction in turbidity and an increase in the redness and colour intensity of the final permeate, in comparison to the feed.

1.7.2. Processing of other agri-food wastes

Enzyme-assisted extraction

The enzymatic-aided extraction has been investigated as a potential novel extraction technique using various agri-food products and wastes, in order to improve the recovery of added-value compounds. More specifically, Choudhari and Ananthanarayan (2007) have reported a lycopene recovery of 108 μg/g from the whole tomato and 1104 μg/g from the tomato peels by using pectinase-assisted extraction (measured with UV-Vis), corresponding to increases of 224% and 206% in comparison to conventional extraction, respectively. Similarly, Neagu *et al.* (2014) managed to recover 26.59 mg/kg of lycopene, 3.11 mg/kg of β-carotene (as measured with HPLC-DAD), 164 mg GAE/kg of total phenols (as measured with Folin) from tomato tissues, corresponding to increases of 55%, 145% and 135% in comparison to conventional extraction, respectively.

In another study, the enzymatic-aided extraction of grape pomace increased the amounts of the total phenolic content from 4615 to 6055 mg/L (measured with Folin), using aq. acetone as solvent and Grindamyl pectinase (Danisko Ingredients, Denmark), corresponding to an increase of 31.2% as compared to conventional extraction (Meyer, Jepsen and Sørensen, 1998). Moreover, Gómez-García, Martínez-Ávila and Aguilar (2012) have further performed the enzymatic-assisted extraction of grape pomaces and measured optimum total phenolic contents of 40 mg/100g (as measured with Folin), corresponding to 25% increase as compared to conventional extraction, using the

commercial enzyme Novoferm (Novozymes). In the study of Ruviaro, Barbosa and Macedo (2019), the extraction of citrus juice by-products led to 2.5-fold increase of hesperidin when using cellulase, 4.7-fold increase of narirutin when using cellulase, and 111-fold increase of hesperetin when using β-glucosidase, while tangeritin remained unchanged, as compared to conventional extraction (measured with HPLC-DAD).

The study of Zheng and Chung (2013) optimised the enzymatic-assisted extraction of phenolic compounds from unripe apples, using the commercial enzyme Viscozyme L (Novozymes, Denmark), by applying the RSM methodology. The optimal recoveries were reported to be 110.52 mg GAE/100g in terms of total phenol content (as measured with Folin) and 43.13 mg/kg of caffeic acid content (as measured with HPLC-DAD), corresponding to 2.2-fold and 13.4-fold increase, respectively, compared to conventional extraction. Furthermore, Ghandahari Yazdi *et al.* (2019) reported increase of 112% in TPC (as measured with Folin), 71% in antioxidant capacity (as measured with DPPH assay), 3.8-fold increase in phloroglucinol (35,71 mg/g dry mass, as measured with HPLC-DAD) and 4.6-fold increase in gallic acid (121.1 mg/g dry mass, as measured with HPLC-DAD) recovered from pistachio green hull, using enzymatic-assisted extraction with a mixture of three enzymes; (i) pectinase BE color (Novozymes Ferment, Denmark), (ii) cellulase (Sigma, USA) and (iii) tannase (Kikkoman Biochemifa, Japan), as compared to conventional extraction.

The recent study of Saad et al. (2019) demonstrated that the enzymatic-assisted extraction of the raspberry pomace can increase the total phenolic content by 48% (measured with Folin assay) and the antioxidant activity by 25% (measured with DPPH assay), compared to conventional extraction. On the contrary, Roda-Serrat *et al.* (2019) have interestingly reported that the conventional extraction of sour cherry wine pomace managed to recover higher amounts of anthocyanins (48.6 mg/kg) and phenols (82.1 mg/kg), compared to the enzymatic-assisted extraction. However, the study demonstrated that the enzymes were able to increase the permeate flux of the extract's ultrafiltration, thus improving that process. Finally, the authors concluded that the low amounts which were recovered from the pomace are limiting its exploitation as a biosource of high-value products.

Membrane processing

The membrane processing of agri-food products in literature has been mainly focused towards the processing of juices. The ultrafiltration is generally aiming at the clarification of the juices by removing suspended solids, microorganisms, proteins and other macromolecules (Cassano, Marchio and Drioli, 2007; Cassano *et al.*, 2013; Conidi *et al.*, 2017). Furthermore, it is used as a pre-treatment step in order to reduce the fouling phenomena in the subsequent nanofiltration processing (Cassano *et al.*, 2013).

The nanofiltration is generally employed for the purification of the recovered compounds, mainly of phenolic compounds and pigments, by separating and removing other substances present in juices. It is also used for the concentration of those desired compounds in the retentate streams by removing the excess water to the permeate stream (Cassano *et al.*, 2013; Bazzarelli *et al.*, 2016; Conidi *et al.*, 2017). Another aim of nanofiltration is the fractionation of those desired compounds based on their MW (Syed *et al.*, 2017). Finally, the liquid phase of a sample, for example water in the case of juices, can be recovered in the permeate stream of a nanofiltration or reverse osmosis process, in a purified form and free of compounds (Bazzarelli *et al.*, 2016), leading to the reduction of their environmental impact, due to the removal of potential contaminants. A practical application of this is the nanofiltration or reverse osmosis of an extract to obtain the extraction solvent in the permeate side, in a purified form, which can then be re-used in subsequent extraction cycles anew (Syed *et al.*, 2017).

The enzymatic pre-treatment is often employed to improve the membrane processing of agri-food products. In specific, those agri-food products contain long-chained insoluble polysaccharides, such as cellulose, hemicellulose, lignin and pectin, which can create fouling problems, when the membrane processing is applied. The degradation of these compounds prior to the membrane processing, by using hydrolysing enzymes, e.g. cellulases and pectinases, has been shown to reduce the fouling phenomena, increase the permeate fluxes and improve the overall process (Doko *et al.*, 1991; Vaillant *et al.*, 2001; Tamine, 2013).

A study of Conidi et al. (2017) has reported that the membrane processing of the pomegranate juice, using two subsequent cross-flow UF processing runs (150 kDa

MWCO FUC 1582 membrane module, Microdyn Nadir, Germany, and 2 kDa MWCO Desal GK membrane module, GE Water & Process Technologies, USA) with a diafiltration step at the second membrane run, was able to retain 84.8% and 90.7% of the polyphenols and anthocyanins, respectively. At the same time, the process was able to remove both glucose and fructose to the permeate side, up to levels of 90% and 93%, respectively. In another study involving the UF processing of blood orange juice, Cassano, Marchio and Drioli (2007) achieved the clarification of the juice by effectively removing the suspended solids entirely, while maintaining the 80% of the total antioxidant capacity and of the content of compounds, such as ascorbic acid, total anthocyanins, narirutin and hesperidin (this 80% was commensurate to the permeate/feed volume ratio).

There are significantly fewer studies which have been published on the membrane processing of liquid waste streams and pomace extracts. In one of the studies, Bazzarelli *et al.* (2016) investigated the membrane processing of olive mill wastewaters (OMWWs) using a series of microfiltration (MF), nanofiltration (NF) and osmotic distillation (OD). The membrane process was able to recover an amount of 1463 g of polyphenols, corresponding to the 85% of the initial amount contained in the OMWWs, based on the processing of 1000 L of OMWWs, and at the same time to recover 800 L (80% of the initial volume) of purified water as permeate. Syed *et al.* (2017) performed the extraction of the grape pomace and the subsequent fractionation of the recovered flavon-3-ols, using nanofiltration with diafiltration of the extract, and reported that the process was able to fractionate 40% of the monomeric flavon-3-ols, while achieving the recovery of 90% of the extraction solvent with an additional reverse osmosis processing step.

In another study, Díaz-Reinoso *et al.* (2009) investigated the aqueous extraction of the grape pomace and the membrane processing of the extract using five different nanofiltration membranes. The authors concluded that all membranes demonstrate similar rejections to phenols and sugars, thus limiting their use to solely their concentration, without allowing the fractionation and purification of those compounds. Interestingly, the results suggested, however, that the best membrane might be “Inside Ceram” (Tami Industries, France), with a nominal MWCO of 1000 kDa, as it was able to achieve higher rejections of phenols (R = 80%), in combination with higher

concentration factors (VRF=6.5) and permeate fluxes (15-25 L/m^{2}h; at TMP = 2 bar, feed flow = 2-3 m/s, 20^{o}C).

The sub-critical water extraction of grape pomace and the membrane processing of the extract was finally studied using nine nanofiltration membranes by Yammine *et al.* (2019). As the results suggested, the membranes with MWCO of 500-1000 kDa were able to quantitatively recover the polymeric pro-anthocyanins in the retentate streams, while separating them from phenols passing to the permeate streams. On the other hand, membranes with MWCO of 300-600 kDa were able to fractionate the monomeric phenolic groups. All membranes employed managed to successfully remove the anthocyanins and flavonols in their retentate streams. In view of those findings, it was concluded that nanofiltration can effectively be used for the fractionation and concentration of the grape pomace extract.

1.8. Research gaps

The field of waste valorisation of the agri-food industry wastes through the recovery of added-value compounds has emerged as a very popular research topic, especially during the last decade.

However, there are still research gaps and questions which are yet to be answered in the published literature. A few of the most important research gaps and questions in this research area, are identified below:

1. the enzymatic-aided extraction of the beetroot pomace has not yet been studied. Some of the research questions which still remain unanswered are:

    (a) Is the enzymatic-aided extraction suitable for the extraction of beetroot pomace?
    (b) If yes, which are the optimum enzymatic-aided extraction parameters?
    (c) Is enzymatic-aided extraction a better approach to conventional extraction? Which of the two extraction techniques leads to higher yields and at what processing cost?

(d) Can the enzymatic-aided extraction be combined with membrane processing, which is a commonly used downstream process for the purification and concentration of extracts? Can the extraction process be easily up-scaled and can it have practical application in the beetroot juice industry from an economic and technical feasibility standpoint?

2. The investigation of membrane processing, and especially of membrane cascades, after the extraction of agri-food wastes is limited and has not been applied for beetroot pomace extracts. The combination of the enzymatic-aided extraction with membrane processes is also very limited in the literature. The research questions which consequently arise and are yet to be answered, are:

   (a) Is membrane processing suitable for the purification of the added-value compounds, which are expected to be extracted from the beetroot pomace? Can the membrane processing help in the clarification of the extracts and the concentration of those compounds?
   (b) Can the enzymatic-aided extraction be combined with membrane processing? Can the use of this extraction technique improve the overall performance of the membrane processing?
   (c) Which are the optimal parameters of the membrane cascade processing?
   (d) Is membrane cascade processing technically feasible and economically viable for the processing of beetroot pomace extracts and can it have practical application in the beetroot juice industry?

1.9. Aims of the project

The main aim of this research work is to propose a processing strategy and examine its technical and economic feasibility for the recovery of added-value compounds from beetroot juice industry wastes (beetroot pomaces), which consists the primary stage for the valorisation strategy of such wastes.

The suggested processing methodology initially involves the enzymatic-aided extraction of the beetroot pomace and subsequently the membrane cascade processing of the extracts, using ultrafiltration (UF) and nanofiltration (NF).

The specific research objectives of this work can be categorised and synopsised as follows:

For the enzymatic-aided extraction

1. The determination of already identified and potentially unidentified or unreported in the literature compounds, preferably of high marketable value, contained inside the beetroot pomace.

2. The optimisation of the extraction process of the most valuable compounds which will be found in the beetroot pomace, by determining their optimum extraction conditions.

3. The comparison of the enzymatic-aided to conventional extraction.

4. The modelling of the extraction yields as a function of the process parameters by using Response Surface Methodology (RSM) (i.e. “mapping” of the extraction conditions and yields).

5. The analysis of the significance of the extraction parameters and of potential interactions between the processing factors, by applying Response Surface Methodology (RSM).

For the membrane cascade processing

1. The evaluation of the UF processing of the beetroot pomace extracts, on the basis of its efficiency in removing the total suspended solids (TSS), for the clarification of the extracts.

2. The evaluation of the NF processing of the clarified beetroot pomace extracts, in terms of its efficacy to fractionate, purify and concentrate the recovered compounds.

3. The optimisation of the membrane cascade processing, through the selection of the most suitable membrane modules and processing parameters, in order to obtain the optimum rejection levels and purification efficiency for the

compounds, in combination with minimum fouling phenomena, processing times and operational costs.

4. The evaluation of the effect of the UF processing and the enzymatic-aided extraction on improving the efficacy of the NF processing of the beetroot pomace extracts.

A final research objective for this project would be to perform a techno-economic analysis for the suggested processing strategy, based on data to be obtained, so as to evaluate its feasibility for industrial application. In this case, the optimum processing parameters will be proposed for maximum profitability (i.e. by considering the extraction yields and the market value of the recovered compounds against the processing cost of their recovery).

Chapter 2

Materials & Methods

2.1. Beetroot pomace

The beetroot pomace in use was provided by a UK juice company, named "James White Ltd." (Ashbocking, U.K.), as the waste deriving from its beetroot juice production. The pomace was delivered in a frozen state. It had a solid and soft texture when it was unfrozen, and consisted mainly of the peels and the root tissue of the beetroot plant (Figure 2.1). Upon collection, the beetroot pomace was stored at frozen condition and was only thawed overnight in the fridge at the specific quantity to be used on a given day, to avoid any deterioration.

Figure 2.1 *The beetroot pomace which was used for the experiments, given by "James White Ltd." Juice company (UK)*

2.2. Chemicals

The enzymatic hydrolysis of the beetroot pomace was performed using the commercial enzymes Celluclast® 1.5L (Novozymes A/S, Denmark) and Pectinex® Ultra Mash (Novozymes A/S, Denmark).

The HPLC analysis was performed with mobile phases which were prepared using Acetonitrile, HPLC grade (Fisher Scientific UK Ltd., UK) and Methanol Chromasolv™, ≥99% HPLC grade (Honeywell Riedel-de Haën™ International Inc., USA), and using ultra-pure water obtained from a Milli-Q® Direct system (Millipore Merck KGaA, Darmstadt, Germany). The mobile phase pH was adjusted using formic acid (Honeywell Fluka™ International Inc., USA).

The standard compounds used in the experiments were the sodium carboxymethyl cellulose (CMC, average M_w ~ 250,000, degree of substitution 0.7; Sigma-Aldrich®, St. Louis, USA) and D-(+)-glucose (≥99.5% HPLC grade, Sigma-Aldrich®, St. Louis, USA). Furthermore, the phenolic compounds used were the gallic acid monohydrate (≥99% HPLC grade; Sigma-Aldrich®, St. Louis, USA), caffeic acid (≥98.0% HPLC grade; Sigma-Aldrich®, St. Louis, USA), vanillic acid (purum ≥97.0% HPLC grade; Sigma-Aldrich®, St. Louis, USA). Finally, betanin (red beet extract diluted with dextrin; Sigma-Aldrich®, St. Louis, USA) was used as standard.

The extractions were performed using the following as solvents: Ethanol Absolute for HPLC (Fisher Chemical, Fisher Scientific UK Ltd., UK), Methanol for HPLC (Fisher Chemical, Fisher Scientific UK Ltd., UK) and Acetone (HPLC Grade, ≥99.5%, Alfa Aesar, Fisher Scientific UK Ltd., UK). The pH of the extraction was adjusted using Hydrochloric acid (ACS Reagent, 37%, Honeywell Fluka™ International Inc., USA) and Sodium hydroxide pellets (ACS reagent, ≥97.0%; Sigma-Aldrich®, St. Louis, USA), diluted in water.

The Folin assay was performed using Folin-Ciocalteu's phenol reagent 2 M (with respect to acid; Sigma-Aldrich®, St. Louis, USA) and Sodium carbonate powder (≥99.5%, ACS reagent; Sigma-Aldrich®, St. Louis, USA).

The membrane modules were cleaned using Sodium hydroxide pellets (ACS reagent, ≥97.0%; Sigma-Aldrich®, St. Louis, USA), diluted in water (0.5% w/v). The membrane modules were stored using sodium metasulfide (ACS reagent, ≥97.0%; Sigma-Aldrich®, St. Louis, USA) diluted in water (0.15% w/v).

2.3. Processing methods

The processing methodology of this work involved initially the enzymatic-aided extraction of the beetroot pomace for the recovery of added-value compounds, and, subsequently, the membrane cascade processing of the extracts for the purposes of their clarification and, also, the concentration, fractionation and purification of the recovered compounds.

The experimental procedure was designed by initially performing conventional extractions of the raw beetroot, from which the beetroot pomace derived as waste, and also the analysis of the beetroot juice. This was expected to serve as an indication for the presence of compounds in the beetroot pomace in use and also to provide useful information about their extraction (Section 2.3.1.).

After examining both the raw beetroot and the beetroot juice, the experimental procedure subsequently involved the recovery of added-value compounds from the beetroot pomace, in two separate stages. In the first stage, the enzymatic-aided extraction of the beetroot pomace was performed in lab-scale, at few indicative screening conditions with the aim of simply determining compounds, in order to finally proceed in performing a larger number of experimental conditions for the optimisation of the process, according to a CCD design of DoE and the RSM approach. The

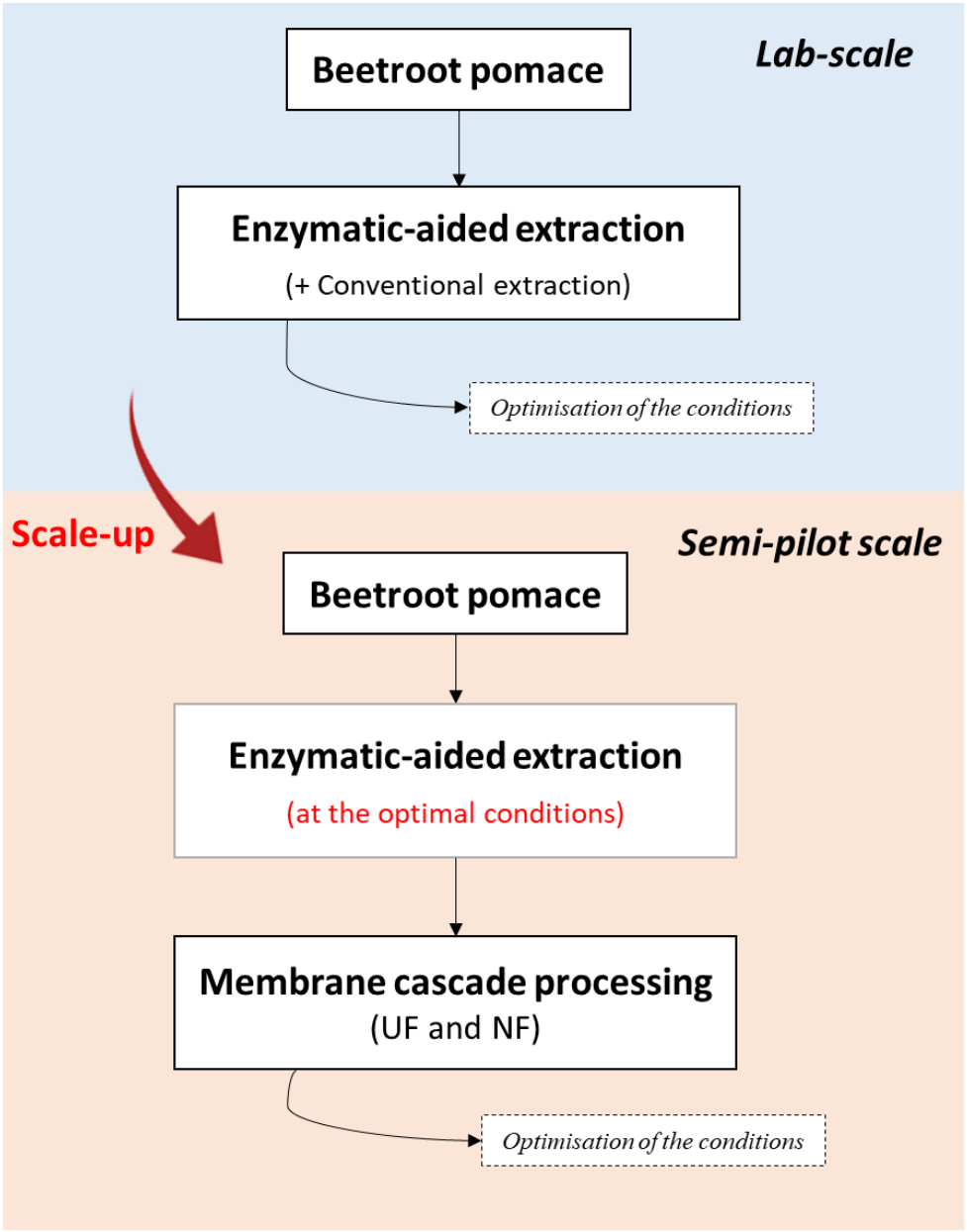


Figure 2.2 *A schematic diagram of the two experimental stages of beetroot pomace extraction; the first stage involving lab-scale extractions and the second stage involving semi-pilot scale extractions in combination with the membrane cascade UF and NF processing.*

extractions were performed in lab scale, as this can offer several advantages, such as testing a large number of experimental conditions in minimum time and cost. The conventional extraction of the beetroot pomace was also performed to be compared to the enzymatic extraction.

The second stage of the experimental procedure involved the scale-up of the process. In particular, the enzymatic-aided extraction was designed to be performed at the specific optimal conditions, as determined during the first stage, this time at a semi-pilot level, followed by the UF and NF membrane cascade processing of the extracts. At this stage, the membrane processing parameters were aimed to be optimised.

In the next paragraphs, the details of each experimental procedure are given, in the chronological order in which the experiments were performed. Deviations from the initial experimental design had to be made, mainly in regard to suspending the optimisation part of the process, and additional experiments were required to be introduced, due to the nature of the results and conclusions drawn from early experiments, described in Chapter 3.

This section is intended to give details that describe how each experimental procedure was executed, while the rationale, aims and reasoning for performing these experiments are primarily included in the results & discussions chapter (Chapter 3).

2.3.1. Raw beetroot extracts and beetroot juice

The conventional extraction of the raw beetroot, from the processing of which the beetroot pomace in use derives as waste, has been performed to investigate the presence of compounds inside this initial sample, as this could serve as an indication for the existence of compounds inside the beetroot pomace.

Those conventional extractions were performed by using three different solvents: (i) 15% v/v aq. ethanol, (ii) 100% ethanol and (iii) 100% methanol. In particular, raw beetroot was initially peeled to remove the external skin and was ground into a puree mass. Then, 30 g of the mixed ground beetroot and 300 mL of solvent were transferred

into a beaker (solid-to-solvent ratio of 1:10). The extraction took place on a heating magnetic stirrer, adjusted to a temperature of 25ºC, for an extraction time of 5 h and under constant mixing. The total volume of the liquid extract was then decanted to remove the solid beetroot mass and was subsequently evaporated using a rotary evaporator (as described in section 2.3.10), in order to be concentrated by removing the excess solvent. At the end of the evaporation, the extracts had been concentrated by a factor of 2.5-3.5 and were finally analysed using HPLC-DAD and LC-MS methods, as described in sections 2.4.2. and 2.4.3., respectively.

The analysis of the beetroot juice, from the processing of which beetroot pomace comes as a waste, was also implemented using HPLC-DAD and LC-MS methods, according to sections 2.4.2. and 2.4.3., respectively.

A. Lab-scale extractions

2.3.2. Sample preparation

In order to ensure the maximum uniformity of the sample and the minimum possible deterioration of compounds in the beetroot pomace, each beetroot pomace batch was initially thawed, mixed well (by hand) and divided into aliquots, each corresponding to the amount needed for the extraction experiments on a single experimental day. Those beetroot pomace aliquots were immediately frozen and were only thawed in the fridge, overnight prior to their use. This procedure was followed in order to ensure the most optimal and repeatable extraction outcome.

2.3.3. Enzyme mixture preparation

The Celluclast® 1.5L/Pectinex® Ultra Mash enzyme mixture was prepared at a fixed 1:1 ratio of enzyme activities. In particular, the mixtures were prepared by diluting the suitable mass of the enzymes in DI water, so as to achieve the desired enzyme activities. The calculations were based on the reported enzyme activities per gram, as given by the manufacturer of the commercial enzymes (700 EGU/g for Celluclast® 1.5L and 9500

PECTU/g for Pectinex® Ultra Mash)[2]. For example, the preparation of 100 mL of 250 U/mL•g enzyme mixture (i.e. a mixture of 250 EGU/mL•g Celluclast® 1.5L and 250 PECTU/mL•g Pectinex® Ultra Mash) was made by mixing 37.7143 g Celluclast® 1.5L, 2.6316 g Pectinex® Ultra Mash and 68.44 g DI water. The preparations were made according to the masses, and not based on the volumes of the commercial enzymes (given from density), using a four-digit analytical mass balance, with the commercial enzymes equilibrated at ambient temperature, to ensure maximum accuracy and repeatability.

In order to further achieve the maximum uniformity of the enzyme mixture in each vial and, therefore, the repeatability of the experiments, the total volume of the enzyme mixture required for one repetition of a set of extraction experiments (that included several hydrolysis/extraction conditions and the pure enzyme mixture blanks) was prepared in a single large stock solution. This large solution was mixed well after preparation and was distributed into containers, in aliquots needed for the performance of extraction experiments on a single experimental day. The containers were immediately frozen and were only thawed in the fridge, overnight prior to their use.

2.3.4. Enzymatic hydrolysis & subsequent extraction without intermediate separation of the hydrolysis medium from the pulp

This section describes the experimental procedure which was followed for the performance of the lab-scale enzymatic-aided and conventional extractions of the beetroot pomace.

These enzymatic-aided and conventional extractions took place inside small glassy vials (Figure 2.3). Initially, 1 g of beetroot pomace was added in each vial. Then, 10 mL of

[2] The catalytic activity of enzymes, as well as the enzyme amount, is typically measured in Units (U); in specific, 1 U is defined as the amount of the enzyme that catalyses the conversion of 1 μmol of substrate per minute under the specified conditions of the assay method (Labuda *et al.*, 2018). The terms EGU and PECTU stand for endoglucanase unit and pectinase unit, respectively; this relates to the type of the enzyme activity and, hence, the substrate used. The SI unit of the enzyme activity is katal (kat), which is defined as the amount of the enzyme that catalyses the conversion of 1 mol of substrate per second (1U = 16.67 nkatal; Labuda *et al.*, 2018). In this work, the enzyme activity was expressed in Units (U), as the manufacturer of the employed enzymes, as well the majority of the studies in the literature, specify enzyme activity in terms of Units.

the already prepared enzymatic mixture (according to paragraph 2.3.3), consisting of Celluclast 1.5L and Pectinex Ultra Mash at an enzyme activity of 250 U/mL·g, were added for the enzymatic-aided extraction. For the conventional extraction, 10 mL of DI water were added instead. In each vial, a magnetic bar was finally added.

The vials were subsequently placed on a heating magnetic stirrer, which was pre-adjusted to the tested temperature, according to the experimental conditions under examination (given in table). After the samples had equilibrated to the set temperature, the pH was also adjusted to the tested value (given in table), by adding drops of accordingly diluted H_2SO_4 or NaOH, and also considering the temperature correction of the pH measurement.

The vials were finally left at the set temperature and pH conditions, under constant mixing, for a specific time duration of 360 min, so as for the enzymatic hydrolysis to take place. After the end of the hydrolysis, 3 mL of ethanol were added in each of the vials. This water-ethanol mixture served as the solvent of the extraction step. Finally, the samples were re-adjusted to the same temperature and pH as during the enzymatic hydrolysis step and were left at these set conditions for a time duration of 2 hours, to allow the extraction to take place.

At the end of this final extraction step, the extracts were centrifuged at 4000 rpm for 10 min (at ambient temperature) to remove the pulp. All the liquid supernatants were collected, measured in terms of their volumes and kept refrigerated until the following day, for analysis using the Folin-Ciocalteu assay, according to section 2.4.1. Finally, aliquots of the samples were kept frozen and were analysed with HPLC-DAD, as described in section 2.4.2., collectively. The enzymatic-aided extractions were performed at the following fixed conditions:

- Enzyme ratio: Celluclast® 1.5L/Pectinex® Ultra Mash (1:1) [in terms of activity]
- Enzyme activity: 250 U/mL·g
- Solid to Solvent ratio: 1:10
- Hydrolysis medium volume: 10 mL
- Extraction duration: 2 hours

The different enzymatic hydrolysis conditions which were investigated are given in the table below (Table 2.1):

Table 2.1 *The enzymatic hydrolysis conditions which were investigated during the initial screening lab-scale extraction experiments of the beetroot pomace.*

Conditions	Temperature (°C)	pH	Enzymatic treatment time (min)	Enzyme activity (U/mL g)
1	55	4.5	360	250
2	55	6	360	250
3	45	4.5	360	250

The aforementioned enzymatic and conventional extractions of the beetroot pomace of each condition were performed in triplicates. In addition, three large enzyme mixture bulks were prepared, one for each repetition of a series of tested conditions. This experimental procedure was repeated using two different beetroot pomace batches.

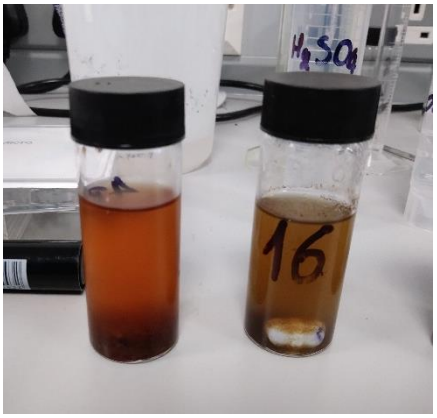


Figure 2.3 *The vials in which the lab-scale extractions took place*

The parameters of the enzymatic-aided extraction were selected with consideration to reports and findings in the already published literature.

In particular, the enzymatic-aided extractions were implemented by performing the enzymatic hydrolysis and the extraction in two subsequent stages, with a simple addition of the complementary extraction solvent component inside the enzymatic

mixture at the end of the hydrolysis, similarly to the procedures followed by Li *et al.* (2015) and Fu *et al.* (2019). This approach was chosen as more a suitable and effective, since it allows the enzymatic hydrolysis to be performed at a pure aqueous environment, in which the enzymes show higher activity, are more stable and are able to penetrate more easily into the substrate, while it simultaneously allows the extraction to be performed with a different solvent type, by simply adding the complementary solvent component.

The temperature range of the enzymatic-aided extractions was chosen to be 45-55^{o}C. According to the manufacturer of the commercial enzymes, Celluclast 1.5L mixture has an optimal activity at 65^{o}C (exhibits activity in the range of 45-65^{o}C) and shows stability at temperatures below 68^{o}C. Pectinex Ultra Mash enzyme mixture has an optimal activity at 50^{o}C and is stable at temperatures below 68^{o}C. In addition, phenolic compounds start to degrade at significant levels at temperatures above 70^{o}C (Mojzer *et al.*, 2016); degradation levels of 15-20% have been reported after heat treatment at 60^{o}C for 4 h (Volf *et al.*, 2014). Therefore, temperatures above 65^{o}C, especially for prolonged time periods, should generally be avoided. Betalains are also regarded to be relatively stable at temperatures below 60-70^{o}C (Slimen, Najar and Abderrabba, 2017). Considering the above, the temperature range of the extractions was chosen with a lower limit of 45^{o}C, in order for Celluclast 1.5L enzyme mixture to exhibit a substantial enzyme activity, and with an upper limit of 55^{o}C in order to avoid any degradation of phenolic or betalain compounds during the extraction process.

In regard to pH, Celluclast 1.5L mixture has an optimal activity at pH 5, and shows stability at a range of pH 3.5-6.5, while Pectinex Ultra Mash enzyme mixture has an optimal activity at pH 4 and is stable at a range of pH 2.8-6. Most phenolic compounds are generally stable in the range of pH 3-11, but few of those (caffeic, chlorogenic and gallic acids) which have been reported to have been found in beetroot (Georgiev *et al.*, 2010; Koubaier *et al.*, 2014), appear to be unstable at pH above 8 (Friedman and Jürgens, 2000). Finally, betalain pigments are relatively stable at a pH range of 3-7, but exhibit maximum stability at pH of 5-6 (Havlikova, Mikova and Kyzlink, 1983; Azeredo, 2009; Slimen, Najar and Abderrabba, 2017). In view of the above, the pH range of the screening extractions was chosen to be pH 4.5-6; the upper limit of pH 6 was chosen based on the activity level of the enzymes, while the lower limit of pH 4.5

according to the stability of betalains and the activity of Celluclast 1.5L enzyme mixture. The pH was adjusted using HCl and NaOH solutions, similarly to the study of Roselló-Soto *et al.* (2019).

The enzymatic dosage and treatment time of the extraction was chosen to be 250 U/g mL and 6 h, respectively. In regard to the dosage, there is no specific recommended value reported neither by the enzymes' manufacturer, nor in scientific papers. The suitable enzyme dosage level will be, generally, greatly affected by the type of the substrate, hence by the particular application (Shamraja S Nadar, Rao and Rathod, 2018). In general, the higher the enzyme dosage added in the hydrolysis medium, the greater the effect on degrading the substrate, thus leading to a better extraction of compounds (Laroze, Soto and Elvira, 2010; Shamraja S Nadar, Rao and Rathod, 2018), however the greater is also the cost of the enzymes and of the overall process. Enzyme dosages applied in the literature range from as low as 0.2 U/g mL (Ruviaro, Barbosa and Macedo, 2019) up to 3800 U/g mL (Catalkaya and Kahveci, 2019), but most typically are between 1-80 U/g mL (Catalkaya and Kahveci, 2019; Ghandahari Yazdi *et al.*, 2019; Ruviaro, Barbosa and Macedo, 2019). For the screening experiments, a relatively high enzyme dosage of 250 U/g mL was used, since there is no specific enzyme dosage recommendation given by their manufacturer and because the main aim of these experiments was not yet to specify the minimum effective enzyme dosage, but to use a dosage sufficient enough to safely degrade the pomace, in order to initially detect potentially present beetroot compounds.

Furthermore, there is not an agreement among scientific papers about the units in which the dosage is expressed; most papers report the enzyme dosage either in v/w (Catalkaya and Kahveci, 2019; Roda-Serrat *et al.*, 2019), in w/w (Choudhari and Ananthanarayan, 2007; Laroze, Soto and Elvira, 2010), in U/mL (Ghandahari Yazdi *et al.*, 2019), in U/g (Ruviaro, Barbosa and Macedo, 2019) or just as a percentage (%) (Li, Smith and Hossain, 2006).

In these experiments, the enzyme dosage was expressed in Units (U) per gram of substrate and volume of hydrolysis medium. The enzyme activity (given in units) is generally a more accurate term in expressing the amount of an enzyme inside a commercial enzyme mixture, instead of the mass or the volume of the commercial

mixture, since most of these mixtures are not pure enzyme solutions, but crude products containing several other components (Liu, 2017). Thus, the enzyme activity describes more accurately the amount of the enzyme that is added in a system, and also better allows the comparison among the various commercial products of the same enzyme that are made by different manufacturers, which usually contain diverse amounts of that enzyme per gram/volume of solution. The enzyme activity is also expressed as per mL of solution (this gives the concentration of the enzymes) and per gram of substrate, since the effective dosage is always analogous to the amount of the substrate.

The enzymatic treatment time is generally dependent on the substrate type and the specific application in use. In many studies, the optimum treatment time had been found to be 4-6 h (Li, Smith and Hossain, 2006; Catalkaya and Kahveci, 2019; Ghandahari Yazdi *et al.*, 2019). By considering these studies and since the aim of the screening experiments was not yet to specify the optimum treatment time, but to initially detect potential compounds in the beetroot pomace, the treatment time was decided to be 6 hours. It was also decided that longer enzymatic treatment times should be avoided, as this could potentially lead to partial degradation of extracted compounds. The enzyme dosage ratio between Celluclast 1.5L and Pectinex Ultra Mash was chosen to be 1:1 (in terms of their enzyme activity); this parameter was not examined during these screening experiments.

The solvent of the extraction step was chosen to be a mixture of 23% aqueous ethanol. In general, there is not a consensus about the solvent that is optimal for the extraction of phenols (Aires, 2017). However, many studies conclude that the most effective solvents are hydro-alcoholic mixtures; the aqueous component swells the plant material and helps the alcoholic component to penetrate and extract the phenolic compounds (Galanakis *et al.*, 2013). In many papers, the optimal concentration of the alcoholic component in water has been found to be approximately 50-80% v/v (Apak *et al.*, 2007; Silva, Rogez and Larondelle, 2007; Galanakis *et al.*, 2013; Sun *et al.*, 2015). Nevertheless, the concentration of the organic component in applications involving enzymes must be kept relatively low (Bisswanger, 2014), e.g. generally below 30% v/v when ethanol or methanol is used (Mozhaev *et al.*, 1989), as higher concentrations typically lead to enzyme denaturation. Lastly, the analysis of undiluted samples, which contain high concentrations of organic component, using Folin-Ciocalteu assay, can

lead to the formation of white salt precipitate (Singleton and Rossi, 1965) of Na_2CO_3, due to its low solubility, which impedes the analysis.

In many studies, methanol has been reported to be better solvent in extracting phenolic compounds than ethanol (Adil *et al.*, 2007; Zheng, Hwang and Chung, 2009; Mojzer *et al.*, 2016; Iglesias-Carres *et al.*, 2019). Moreover, methanol also appears to be a better solvent for the extraction of betalains than water (Kujala, Loponen and Pihlaja, 2001). Nevertheless, ethanol is often preferred as a solvent over methanol for food applications, due to its low toxicity (Adil *et al.*, 2007; Zheng, Hwang and Chung, 2009); according to the Directive 2016/1855/EC of the European Commission, methanol should be present at a maximum residue level of 10 mg/kg in the final foodstuff or food ingredient, therefore its use requires long additional purification steps, which are costly and time-consuming (Mojzer *et al.*, 2016). For all these reasons, ethanol was employed at a concentration of 23% for the final extraction step.

As the extraction time in most of the scientific papers is in the range of 0.5-2 hours (Kujala, Loponen and Pihlaja, 2001; Lee and Wrolstad, 2004; Kammerer *et al.*, 2005; Righi Pessoa da Silva, da Silva and Bolanho, 2018; Catalkaya and Kahveci, 2019) and also in order to avoid long extraction times which could lead to potential degradation of the extracted compounds, an extraction time of 2 hours was applied.

2.3.5. Investigation of the decreased Folin values

2.3.5.1. Phenol-protein and carbohydrate-protein interactions

The occurrence of potential protein-phenol and carbohydrate-phenol interactions was investigated as the cause for the observation of decreased Folin values in the enzymatic extracts in comparison to their pure enzyme mixture blanks, between the employed commercial enzymes and potentially present phenolic compounds and carbohydrates in the extracts.

The investigation of those interactions was performed by using model systems of the enzyme mixture in use, i.e. Celluclast 1.5 L and Pectinex Ultra Mash (250 U/mL), in combination with the carbohydrates glucose (sugar; 1% w/v) and carboxyl methyl cellulose (CMC; polysaccharide; 0.25% w/v), and with various phenolic compounds commonly reported to be present in beetroot products, according to the literature (Figure 1.4); more specifically, these compounds were: gallic acid (200 μg/mL), vanillic acid (120 μg/mL), caffeic acid (50 μg/mL) and rutin (saturated). The model systems were prepared both in pure solutions and binary protein-phenol and carbohydrate-protein mixtures, using standard compounds. The same concentrations, as in the mixtures, were used for the pure solutions of each compound. In regard to the saturated mixture of rutin, this was formulated by initially preparing a supersaturated solution of 100 μg/mL, and subsequently thoroughly mixing and filtering the solution using Corning® syringe filters 0.45 μm PES (Corning Ltd., UK), to obtain the saturated rutin solution. Each of the mixture and pure solutions was prepared in duplicates.

The prepared pure and mixture standard solutions were analysed and compared using the Folin-Ciocalteu assay, according to paragraph 2.4.1., with two measurements taken for each repetition of each solution, so as to simulate similar sample conditions in the enzymatic beetroot pomace extracts with the use of the same analytical method, but this time using standard solutions. The aim was to observe a potentially similar decrease of the Folin response in any of the mixtures as compared to the sum Folin response of their pure solution, which would suggest the occurrence of the interactions.

The UV-Vis spectrometric analysis of pure and mixture solutions of gallic acid and the enzyme mixture was further applied. In general, the UV-Vis absorbance of a two-compound mixture is known to be equal to the sum of the absorbance of their single component solutions at their same concentrations, when these compounds do not interact with each other (Inscoe et al., 1958). A deviation from this law would indicate that interactions between those compounds are taking place.

These experiments were performed with mixtures at the following concentrations: 12.5 U/mL enzymes – 150 μg/mL gallic acid, 12.5 U/mL enzymes – 30 μg/mL gallic acid, 12.5 U/mL enzymes – 15 μg/mL gallic acid, 12.5 U/mL enzymes – 3 μg/mL gallic acid. In addition, the pure solutions of the enzymes and of gallic acid were also prepared at

the same exact concentrations. The pure and mixture solutions were diluted using 0.05 M citrate buffer (pH 4.8), as solvent, prepared according to Wood and Bhat (1988). All the samples were prepared in triplicates. Finally, the pure and mixture solutions were mixed well and left standing at ambient temperature for the durations of 2, 4 and 6 hours, prior to the UV-Vis spectral analysis.

The UV-Vis spectrums were measured with the use of the Thermo Scientific UV-Visible Spectrophotometer Evolution 220 (Thermo Fisher Scientific UK Ltd., UK), set in the scanning range of 190-800 nm. The measurements were made using a UV quartz cuvette (3.5 mL, Alpha Nanotech, Canada). The blank sample used for the baseline correction of the measurements was the 0.05 M citrate buffer solvent.

2.3.5.2. Enzyme denaturation

The denaturation of the enzymes was investigated as a potential reason for the observation of decreased Folin values in the enzymatic beetroot pomace extracts in comparison to the pure enzyme mixture blanks. The investigation involved the preparation of Celluclast® 1.5L/Pectinex® Ultra Mash enzyme mixtures at the concentration of 250 U/mL, according to section 2.3.3. The mixtures were subjected to heat treatment at three different temperatures: (i) 55^{o}C, (ii) 65^{o}C and (iii) 90^{o}C, for a maximum exposure time of 6 h. The thermally treated samples, along with the untreated enzyme mixtures, were finally measured with the Folin-Ciocalteu assay, according to paragraph 2.4.1.

The experiments were performed in triplicates. In particular, the enzyme mixtures were prepared in three large stock solutions, each corresponding to one of the three repetition series (untreated, 55^{o}C, 65^{o}C, 90^{o}C).

A potential reduction in the Folin response of the thermally treated enzyme mixtures in comparison to the untreated mixtures would indicate that the denaturation of the enzymes occurs and that this denaturation is the main reason for the loss of Folin response in the enzymatic extracts.

2.3.5.3. Enzymatic hydrolysis & subsequent extraction with intermediate separation of the enzymes from the pulp using washings with DI water

A potential cause for the decreased values of the enzymatic extracts in comparison to the pure enzyme mixture was assumed to be the adsorption of the enzymes on the pulp. This hypothesis was investigated by performing experiments involving the washing of the pulp with DI water, immediately after the enzymatic hydrolysis step, so as to remove potentially adsorbed enzymes on the pulp.

Washing procedure

The experimental procedure was the same as in paragraph 2.3.2. and 2.3.3., in regard to the sample and enzyme preparation. The initial enzymatic hydrolysis step was performed also similarly as in paragraph 2.3.4, at the conditions of 55^{o}C, pH 4.5, treatment time of 240 min and enzyme activity of 250 U/mL·g. However, the extraction step was not performed after the end of the enzymatic hydrolysis. Instead, the samples were collected and centrifuged at 4000 rpm for 10 min, at ambient temperature. Then, the liquid supernatant (i.e. the hydrolysis medium) of the centrifugation of each sample was collected, while the precipitated pulp was re-diluted in 10 mL of DI water and mixed thoroughly for 3 minutes, in order to wash out and remove the potentially adsorbed enzymes on the pulp.

This pulp/DI water mixture was then centrifuged at 4000 rpm for 10 min, at ambient temperature, and subsequently both the liquid supernatant (1st washing) and the precipitated pulp were collected. This washing step procedure was repeated ten times in total, until the Folin response of the samples reached a plateau. For comparison purposes, the same procedure was followed without the use of enzymes, by adding 10 mL of DI water in the initial hydrolysis step, instead of the enzyme mixture. The washing procedure was performed in triplicates.

All the supernatants of the centrifugation (i.e. the hydrolysis medium and the washings) were collected and analysed using the Folin-Ciocalteu assay, according to section 2.4.1. Each repetition was measured twice. The sum of TPCs value of the hydrolysis medium

and the washings was calculated and compared to the TPC value of the pure enzyme mixture. A surplus value would indicate the presence of phenolic compounds recovered from beetroot pomace, while a lower value would suggest that the enzymes are strongly adsorbed on the pulp's surface and that their retrieval would not be practically feasible by performing the washing of the pulp with DI water.

Finally, the samples were analysed using the HPLC-DAD method, as described in section 2.4.2., aiming at detecting enzyme compounds inside the washing samples of the pulp, through the comparison to the respective chromatograms of the pure enzyme mixture blanks.

Enzymatic extraction at varied conditions using different pomace batches

The enzymatic and conventional extraction experiments with an initial hydrolysis step, two subsequent washing steps, as described in the previous paragraph, and a final extraction step of the beetroot pomace were performed at three different conditions and using two different pomace batches. These experiments focused on investigating the effect of the different conditions and of the pomace batch on the extraction of beetroot compounds, however this time in combination with the washing methodology procedure. It was hoped that the use of different conditions and pomace batches, after the performance of two washing steps to remove a major part of the adsorbed enzyme, could potentially lead to the measurement of a sum of TPCs responses in the supernatants (hydrolysis medium, 2 washings and extract), high enough to surpass the respective responses of the pure enzyme mixture.

The experimental procedure was the same as in paragraph 2.3.2. and 2.3.3., in regard to the sample and enzyme preparation. The conventional and enzymatic extractions were initially implemented by performing the enzymatic hydrolysis step and two washing steps, as described in the previous paragraph; for the conventional extractions, the pulp was mixed with 10 mL of DI water, instead of 10 mL of enzyme mixture, at the hydrolysis step. The hydrolysis steps were performed at the same fixed and variable conditions, as in paragraph 2.3.4. (see below). At the end of the second washing, the precipitated pulp was collected and mixed with 13 mL of extraction solvent (consisting

of 10 mL of DI water and 3 mL of ethanol). The samples were subsequently adjusted to the same temperature and pH as during the hydrolysis step and were left at these set conditions for a time duration of 2 hours, to allow the extraction to take place.

At the end of the final extraction step, the extracts were centrifuged at 4000 rpm for 10 min at ambient temperature, to remove the pulp. All the liquid supernatants were collected, measured in terms of their volumes and kept refrigerated until the following day, for analysis using the Folin-Ciocalteu assay, according to section 2.4.1. Finally, aliquots of the samples were kept frozen and were analysed with HPLC-DAD, as described in section 2.4.2., collectively.

The aforementioned enzymatic and conventional extractions of the beetroot pomace were performed in triplicate. Three enzyme mixture stock solutions were prepared, one for each repetition series. The experimental procedure was repeated using a second beetroot pomace batch.

The enzymatic extractions were performed at the following fixed conditions:

- Enzyme ratio: Celluclast® 1.5L/Pectinex® Ultra Mash (1:1) [in terms of activity]
- Enzyme activity: 250 U/mL·g
- Solid to Solvent ratio: 1:10
- Hydrolysis medium volume: 10 mL
- Extraction duration: 2 hours

The different enzymatic hydrolysis conditions which were investigated are given in the table below (Table 2.2):

Table 2.2 *The enzymatic hydrolysis conditions which were investigated during the initial screening lab-scale extraction experiments of the beetroot pomace.*

Conditions	Temperature (°C)	pH	Enzymatic treatment time (min)	Enzyme activity (U/mL g)
1	55	4.5	360	250
2	55	6	360	250
3	45	4.5	360	250

2.3.6. Investigation of the enzyme removal using UF filtration

The removal of the enzymes after the enzymatic hydrolysis of the beetroot pomace was investigated using UF filtration. This filtration was performed by employing suitable Vivapsin® UF filter tubes (Vivaspin® 20 Ultrafiltration unit PES 30 kDa MWCO, Sartorius AG, Germany), with a nominal MWCO of 30 kDa, as illustrated in Figure 2.4. The filters consist of two parts, which are divided by the membrane; the feed is loaded on the upper part of the tube, while the permeate is collected from the bottom part. The filtration using this filters takes place inside a centrifuge, with the centrifugal force acting as the driving force for the permeation of the sample through the membrane.

In regard to the procedure, the UF filter tubes were initially rinsed with DI water, in order to remove any trace elements of glycerine and sodium azide, according to the suggested procedure given by the product manufacturer. In particular, the rinsing was performed by loading 20 mL DI water in the upper part of the UF filter tube. The centrifugation was then performed at 7000 rpm for 10 min, at ambient temperature. At the end of the filtration, the total water volume had permeated the membrane. This water volume was collected from the bottom part of the UF filter tube and was discarded. The rising procedure was repeated for two additional times. Samples of the rinsing waters

Figure 2.4 The UF filter tube used for the filtration of the enzyme mixture (Vivaspin® 20 Ultrafiltration unit PES 30 kDa MWCO, Sartorius AG, Germany).

were kept as blanks and were analysed with HPLC-DAD for the presence of trace elements or other membrane impurities.

The UF filtrations of enzymatic and conventional extract supernatants, prepared and collected according to the procedure described in paragraph 2.3.4., and of the pure enzyme mixture blanks were then tested by loading each of the sample on the upper part of the a rinsed UF filter tube. The centrifugation was subsequently performed at 7000 rpm for 2 hours and at 6^{o}C, to avoid any deterioration of the sample. The filtration was terminated at the point that the majority of the sample had passed through the membrane, while leaving a small portion of retentate, so as to perform the mass balances of the feed, permeate and retentate. Finally, the retentates and the permeates of the UF filtrations were collected, and along with the feed samples which were kept before the filtration, were recorded in terms of their volume and were analysed with the Folin-Ciocalteu assay and the HPLC-DAD method, as described in sections 2.4.1. and 2.4.2., respectively.

B. Semi-pilot scale

2.3.7. Pre-Conditioning

The pre-conditioning of the membranes is a standard procedure, which is typically performed only prior to the first use of a membrane with a sample. This procedure involves operating the membrane using DI water as the feed, at an equal or higher pressure to the one to be used for the subsequent sample run (within the upper pressure limits of the membrane). During this procedure, the membrane pores are wetted and the membrane structure may go through compaction or swelling. The pre-conditioning ensures that the membranes will perform similarly throughout the process and according to the specifications of the manufacturer, in terms of the permeate flux and the rejection values (Sterlitech Co., 2016)

2.3.8. Characterisation

The characterisation of the membranes is a standard procedure, which is typically performed each time prior to the use of a membrane module with a sample. It involves

measuring the permeate flux of the membrane at several pressures, within the acceptable tolerable pressure range of the membrane according to its specifications, using DI water as the feed. The measured permeate flux corresponds to the pure water permeability of the membrane at a given pressure and is compared to the respective permeabilities given in the membrane specification sheets by their manufacturer. The characterisation procedure ensures that the membrane exhibits no apparent defects (e.g. non-reversible fouling or rip) and performs as expected, prior to its use.

2.3.9. Semi-pilot scale extractions of the beetroot pomace and subsequent membrane cascade processing of the extract

Enzymatic hydrolysis and extraction

The semi-pilot scale enzymatic-aided extraction of the beetroot pomace was performed by adding 750 g of beetroot pomace, 75 g of Celluclast 1.5L, 1 g of Pectinex Ultra Mash and 12 L of DI water in a 20 L vessel. The mixture was subjected to constant mixing of 600 rpm with the use of medium-sized propeller mixer (SciQuip Pro 40 homogeniser, SciQuip Ltd., UK). The temperature was adjusted to 50^{o}C with the use of a spiral coil heat exchanger connected to a water bath. The pH was also adjusted to 4.5 by adding drops of H_2SO_4 and NaOH solutions. After those adjustments, the mixture was left at these conditions for a time duration of 4 hours, so as for the hydrolysis to take place.

After the enzymatic hydrolysis, 2.7 L of ethanol were added into the mixture in order for the extraction to take place. The pH was re-adjusted to 4.5 and the mixture was left for an extraction time of 2 hours.

The conventional extraction was performed with a similar procedure, in which the enzymatic mixture was replaced with 62 mL of DI water; this was equivalent to the volume of the enzymes added in the enzymatic extraction.

At the end of the extraction, the extract was sieved to remove the solid beetroot pomace mass. The mass was further squeezed in order to obtain the maximum possible amount

of absorbed extract. Finally, the obtained extract was subjected to UF and NF cross-flow membrane cascade processing, in a batch concentration mode.

Membrane cascade processing

The configuration of the cross-flow membrane processing system in use consisted of the following parts, given in order: (i) a feed tank, (ii) a feed pump, (iii) a flow meter and a thermometer, (iv) the filtration module, which was placed inside a suitable stainless steel holder, (v) a retentate manometer (v) a retentate valve, (vi) a mass balance for measuring the permeate mass, (vii) a mass recorder integrated to a computer, (viii) a monitoring and recording instrument of the flow, pressure and temperature measurements, which was also a control panel for the flow (i.e. pump speed), and finally (ix) a spiral coil heat-exchanger connected to a water bath, which was immersed inside the feed tank (Figure 2.5).

The membrane cascade processing was applied by performing a series of membrane runs, each using a different membrane module. In the membrane cascade, the permeate of a membrane run was used as the feed for the subsequent run (Figure 2.6). These membrane runs were performed one-by-one, successively, using the same membrane rig, by placing the suitable membrane module and feed, each time.

The membrane modules which were employed, were the following, in the order of their use in the cascade: (i) Pentair UF Helix 5mm (UF 150 kDa MWCO), (ii) NXF Mexfil MP025 UF075 (UF, 75 kDa MWCO), (iii) HFS (UF, 10 kDa MWCO), (iv) NXF Mexfil MP025 dNF80 (NF, 800 Da MWCO), (v) NXF Mexfil MP025 dNF40 (NF, 400 Da MWCO) and (vi) NXF Mexfil MP025 dNF20 (NF, 200 Da MWCO). The main characteristics and specifications of the membrane modules (MWCO, maximum pressure and temperature, membrane material etc.) are synopsised in Table 2.3. In addition, the operating membrane parameters (TMP, temperature, cross-flow velocity, VCF) varied for each membrane module's run, according to the specification of the particular membrane module in use, and these are synopsised in Table 2.4.

The performance of each membrane run involved initially transferring the feed (i.e. the extract for the first Helix module or the permeate of the previous membrane run for the

subsequent modules) inside the feed tank and placing the membrane module into the suitable holder. As described in section 2.3.7 and 2.3.8., each membrane module was pre-conditioned prior to its first use and was characterised before each run by measuring its pure water permeability. The water bath was then adjusted to the temperature of the

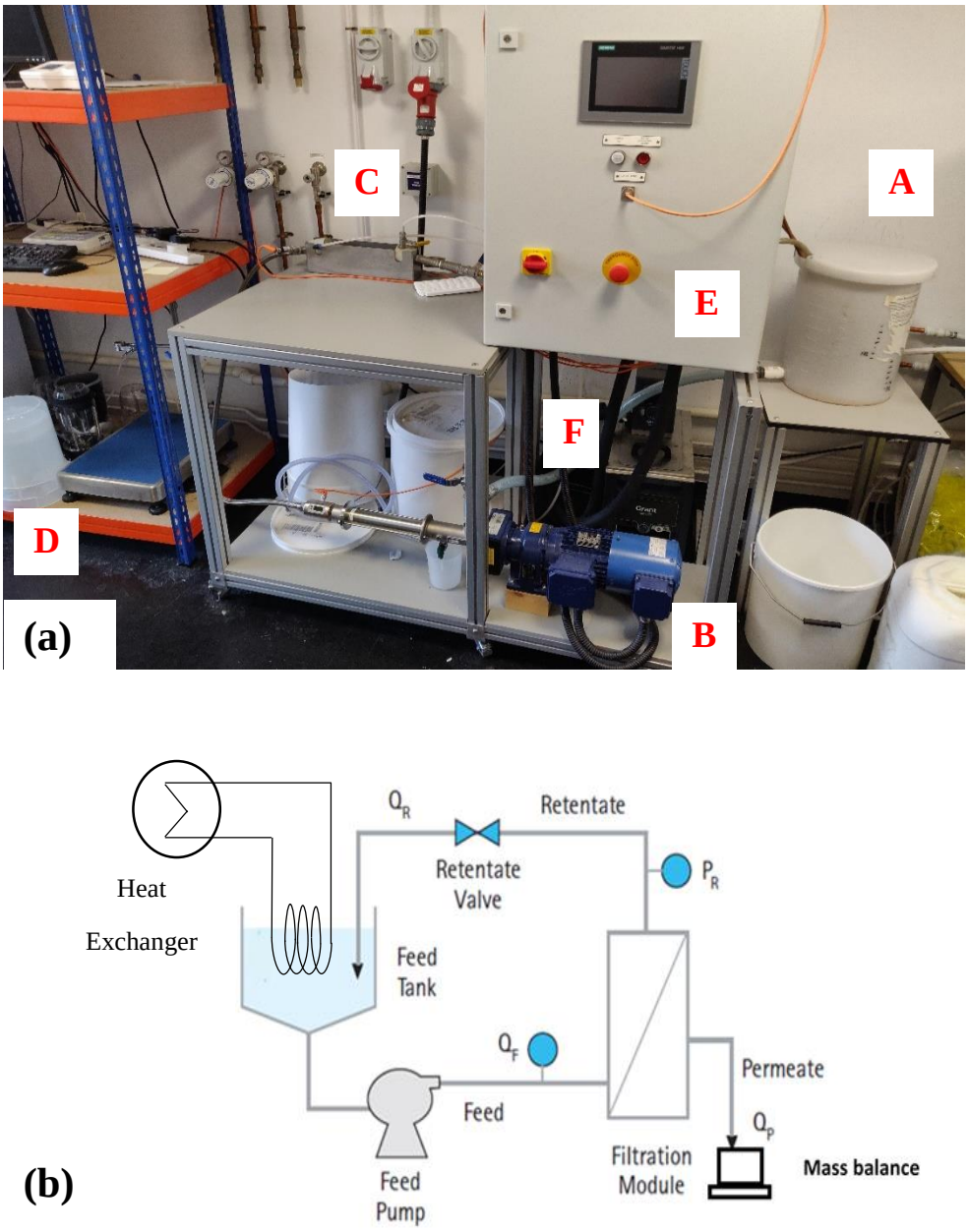


Figure 2.5 *The semi-pilot scale cross-flow membrane processing system used for the experiments. (a) In particular, the main parts of the system were: (A) a feed tank, (B) a feed pump, (C) the filtration module, (D) a mass balance and (E) a monitoring, recording and control system of the membrane parameters and (F) a water bath connected to a spiral coiled heat-exchanger immersed inside the feed tank. (b) A schematic depiction of the membrane system*

membrane run, and the coiled heat exchanger was placed inside the tank. The extract was subsequently left to equilibrate to the set temperature. Finally, the membrane run was initiated, after setting accordingly the pressure and the pump speed parameters of the run and, also, the recording of the membrane processing data (permeate mass, temperature, pressure, feed's flow rate).

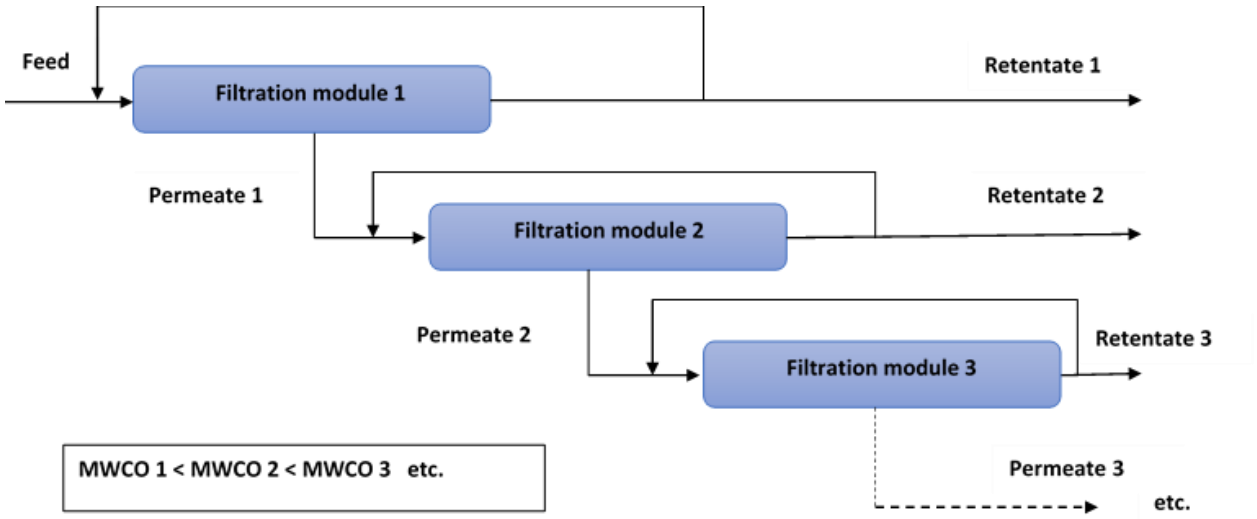


Figure 2.6 *A schematic diagram of a membrane cascade.*

Table 2.3 *The main characteristics of the filtration modules, used in the membrane cascade processing of the beetroot pomace extract.*

Order	Name of membrane	Ultrafiltration/ Nanofiltration	Manufacturer	Membrane Material	MWCO (kDa)	Max Temperature (°C)	Max Pressure (bar)
1	Pentair UF Helix 5mm	Ultrafiltration	Pentair Ltd.	PVDF/PVP	150	-	2,5
2	NXF Mexfil MP025 UF075	Ultrafiltration	Pentair Ltd.	Modified PES	75	50	3
3	HFS	Ultrafiltration	Pentair Ltd.	PES/SPES	10	-	5
4	NXF Mexfil MP025 dNF80	Nanofiltration	NXFiltration BV.	Modified PES	0,8 (25°C)	50	10
5	NXF Mexfil MP025 dNF40	Nanofiltration	NXFiltration BV.	Modified PES	0,4 (25°C)	50	10
6	NXF Mexfil MP025 dNF20	Nanofiltration	NXFiltration BV.	Modified PES	0,2 (25°C)	50	10

Table 2.4 *The main processing conditions of the membrane cascade processing of the beetroot pomace extract.*

Module		Mass (g)	Other parameters	
Helix 5mm	Feed	12990,5	Pressure	1 bar
	Retentate	3305,5	Temperature	40 °C
	Permeate	8468,5	$V_{cross\text{-}flow}$	0,23 m/s
	VCF	3,9	Duration	3 days
UF075	Feed	14945*	Pressure	1 bar
	Retentate	1231	Temperature	40 °C
	Permeate	14740	$V_{cross\text{-}flow}$	0,45 m/s
	VCF	12,1	Duration	1h
HFS	Feed	14441,5	Pressure	1 bar
	Retentate	1774,5	Temperature	40 °C
	Permeate	12677	$V_{cross\text{-}flow}$	0,25 m/s
	VCF	8,1	Duration	1h 40min
dNF80	Feed	12424	Pressure	5 bar
	Retentate	2354,5	Temperature	40 °C
	Permeate	9970	$V_{cross\text{-}flow}$	0,48 m/s
	VCF	5,3	Duration	5h 15min
dNF40	Feed	9609	Pressure	5 bar
	Retentate	1397,5	Temperature	40 °C
	Permeate	7936	$V_{cross\text{-}flow}$	0,48 m/s
	VCF	6,9	Duration	3h 7min
dNF20	Feed	7561	Pressure	5 bar
	Retentate	2080	Temperature	40 °C
	Permeate	5429	$V_{cross\text{-}flow}$	0,48 m/s
	VCF	3,6	Duration	3h 45min

* Addition of 7000 g of DI water, in order to obtain enough sample volume for the membrane cascade and achieve high VCF (concentration factors in the retentate) and high permeate fluxes (due to reduction in feed's concentration).

The membrane runs were completed until achieving the maximum possible VCF. This corresponded to different operating times for each module, depending on the level of the permeate flux reached. At the end of the run, the retentate was collected and stored for further concentration, using a rotary evaporator (as described in section 2.3.10.), and analysis, while the permeate was used as the feed for the subsequent membrane run. A sample of the permeate was collected, concentrated and analysed similarly to the

retentate. This procedure above was employed for each membrane module used in the membrane cascade design.

2.3.10. Rotary evaporation

The rotary evaporation of the membrane streams was performed using the Rotavapor® R-300 evaporator (Büchi Labortechnik AG, Switzerland), connected to a vacuum pump V-300 (Büchi Labortechnik AG, Switzerland) and a recirculating chiller F-308 (Büchi Labortechnik AG, Switzerland) (Figure 2.7).

The evaporation was performed by placing the sample in the evaporating flasks, with a maximum volume of 2/3 of the flask (approx. 250 mL). Generally, the evaporations were performed according to the 10/30/50 rule, i.e. with the chiller adjusted to a temperature of 10ºC and the water bath to a temperature of 50ºC. The low temperature of the water bath ensures the least possible deterioration of compounds during the evaporation.

Figure 2.7 *The rotary evaporator used for the concentration of the membrane processing streams; (A) the removed solvent and (B) the concentrate of a membrane stream.*

2.3.11. Conventional extraction of the beetroot pomace with organic solvents

The conventional extractions of the beetroot pomace were performed using three pure organic solvents: (i) pure ethanol, (ii) pure methanol and (iii) pure acetone. Initially, the beetroot pomace was thoroughly mixed and, then, 30 g of the mixed pomace were added in 300 mL of solvent, inside a beaker (solid-to-solvent ratio of 1:10). The extraction took place on a heating magnetic stirrer, adjusted to a temperature of 25^{o}C, for an extraction duration of 5 h and under constant mixing. The total volume of the liquid extract was then decanted to remove the solid pomace mass and was subsequently evaporated using a rotary evaporator (as described in section 2.3.10), in order to be concentrated by removing the excess solvent. At the end of the evaporation, the extracts had been concentrated by a factor of 2.5-3.5 and were finally analysed using HPLC-DAD and LC-MS methods, as described in sections 2.4.2. and 2.4.3., respectively.

2.4. Analytical techniques

In this section, the analytical techniques which were applied in this project are synopsised. These include methods primarily used for the determination of the recovered compounds in the beetroot extracts; these methods, briefly, are (a) the Folin-Ciocalteu assay (for the determination of the total phenolic content), (b) the HPLC-DAD method (for the detection mainly of individual phenolic compounds and betalain pigments, as well as of other potentially unknown compounds) and (iii) the LC-ESI-IT-TOF-MS method (for the detection and the qualitative determination of both expected and unknown extracted beetroot compounds).

The Gel Filtration Chromatography technique was also employed for the evaluation of the UF filter tubes, as described in section 2.3.6., in terms of retaining, and thus separating, the hydrolytic enzymes in use from the extracts.

2.4.1. Folin-Ciocalteu assay

The Folin-Ciocalteu assay was employed for measuring the Total Phenolic Content (TPC) of the extracts. In particular, the Folin-Ciocalteu procedure of Box (1983) was applied, with some slight modifications, as given below:

Briefly, 150 μL of 20% w/v Na_2CO_3 solution were added into 1 mL of sample and the mixture was mixed thoroughly. Then, 50 μL of the Folin reagent were added, the mixture was mixed again and left to stand in the dark for 60 min. The absorbance was finally measured at 750 nm, with the use of a UV-Vis spectrophotometer (Thermo Scientific UV-Visible Spectrophotometer Evolution 220, Thermo Fisher Scientific UK Ltd., UK). The samples were diluted appropriately prior to analysis, so that the readings of the spectrophotometer would fall within the range of 0.100-1.000 AU (absorbance units). The measurements were made using disposable "Brand® UV-cuvettes semi-macro" cuvettes (Brand GmbH, Germany). A reference sample (blank) was prepared by adding 150 μL of 20% w/v Na_2CO_3 and 50 μL of the Folin reagent into 1 mL of DI water, as described previously. The results were expressed as μg GAE/mL (GAE: Gallic Acid Equivalent), based on a calibration curve made with the use of gallic acid standards (1-30 μg/mL) (Figure 2.8).

In general, this method belongs to the category of the Electron-transfer (ET) antioxidant assays (Huang, Ou and Prior, 2005; Apak *et al.*, 2007; Everette *et al.*, 2010). Regarding the mechanism of the Folin assay, the Folin reagent consists of a mixture molybdotungstophosphoric heteropolyanion reagent (Prior, Wu and Schaich, 2005; A Agbor, Vinson and Donnelly, 2014; Sigma Aldrich, 2018). In alkaline conditions of approximately pH 10, the phenolic compounds are converted into phenolate ions, through the dissociation of a phenolic hydrogen (-OH of phenyl groups, which has a pK_a of 10). The alkaline conditions are achieved with the addition of sodium carbonate. The phenolate ions are capable of reducing the Folin reagent, mainly through reducing the molybdenum and tungsten species from Mo(VI) to M(V) and W(VI) to W(V), and the formation of $[Phenol\text{-}MoW_{11}O_{40}]^{-4}$ complexes, which all have characteristic blue colour (Apak *et al.*, 2007; Sánchez-Rangel *et al.*, 2013; Agbor, Vinson and Donnelly, 2014; Ford *et al.*, 2019). The alkaline environment of approximately pH 10 facilitates the fast reduction of the Folin reagent, while the reaction is much slower in acidic

environments (Singleton, Orthofer and Lamuela-Raventos, 1999; Medina, 2011; Agbor, Vinson and Donnelly, 2014). The formation of the blue colour is measured spectrophotometrically at 725-765 nm (Medina, 2011)

In essence, the Folin-Ciocalteu assay measures both the phenolic compounds and non-phenolic reducing compounds in a sample. Several reducing substances have been shown to react with the Folin reagent and give strong absorbances, by many papers (Robbins, 2003; Ozdal, Capanoglu and Altay, 2013; Ford *et al.*, 2019; Sigma Aldrich, 2018). Certain examples of such reducing compounds are: (i) vitamins (Prior, Wu and Schaich, 2005; Everette *et al.*, 2010; Berker *et al.*, 2013; Granato *et al.*, 2016), (ii) proteins and amino acids (Everette *et al.*, 2010; Berker *et al.*, 2013; Bastola *et al.*, 2017), (iii) reducing sugars (Prior, Wu and Schaich, 2005; Bastola *et al.*, 2017), (iv) organic acids (Prior, Wu and Schaich, 2005; Bastola *et al.*, 2017), (v) metal ions (Prior, Wu and Schaich, 2005; Everette *et al.*, 2010; Granato *et al.*, 2016), (vi) nucleic acids (Prior, Wu and Schaich, 2005; Everette *et al.*, 2010) and (vii) thiols (Everette *et al.*, 2010).

In view of the above, a correction of the value for the interfering substances must be performed during the analysis of as sample, in order for the determination to be accurate (Prior, Wu and Schaich, 2005; Sánchez-Rangel *et al.*, 2013; Ford *et al.*, 2019). Since the enzymatic extracts of our experiments contain lignocellulose degrading enzymes, which according to the literature are able to interfere with the Folin method, a correction of value is performed using pure enzyme standards at the same concentrations found in the extracts, during the Folin analysis of the samples.

In many papers, it is supported that the Folin assay is not determining the "Total Phenolic Content (TPC)" of samples, but instead the "Folin-Ciocalteu reducing capacity" (Huang, Ou and Prior, 2005; Granato *et al.*, 2016; Costa *et al.*, 2017). Nevertheless, the assay can give an idea and an approximation of the phenolic content in a sample (Granato *et al.*, 2018; Ford *et al.*, 2019).

The Folin assay is a simple, sensitive, fast and economic method (Prior, Wu and Schaich, 2005; Bastola *et al.*, 2017), but it lacks selectivity (Prior, Wu and Schaich, 2005; Ozdal, Capanoglu and Altay, 2013; Sánchez-Rangel *et al.*, 2013). Therefore, the Folin-Ciocalteu method is employed in this work, yet with the aim of having a

supportive role to more established and widely accepted analytical techniques (e.g. HPLC, LC-MS), in accordance with the literature (Chomchan *et al.*, 2016; Granato *et al.*, 2018; Ford *et al.*, 2019).

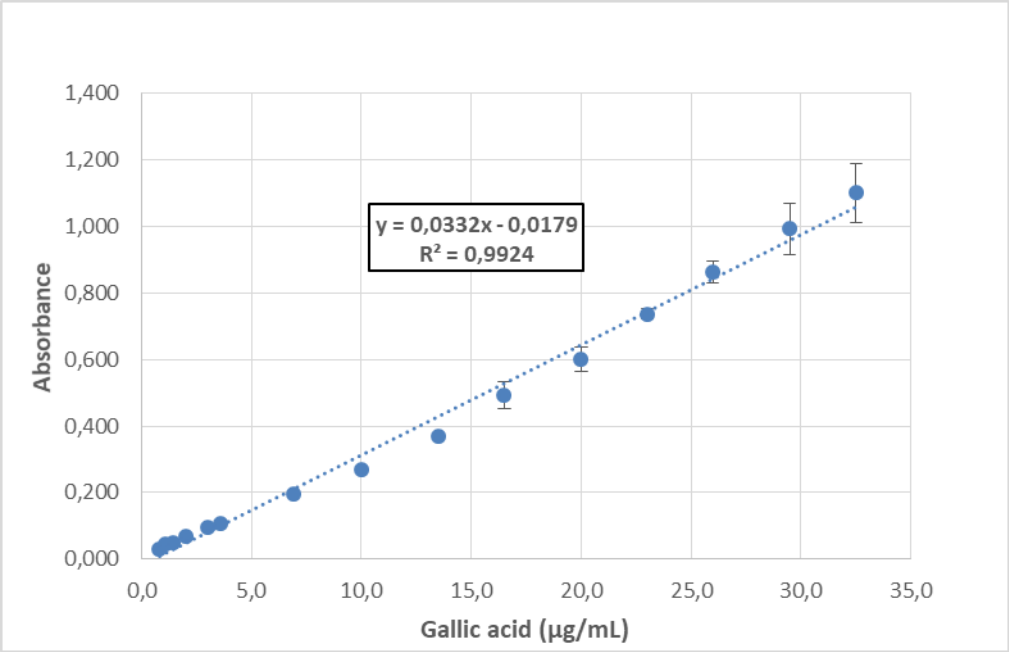


Figure 2.8 *The calibration curve of the Folin assay, using gallic acid (1-30 µg/mL)*

2.4.2. HPLC-DAD analysis

The High Performance Liquid Chromatography coupled with a Diode Array Detector (HPLC-DAD) is considered as one of the most established and accurate analytical techniques for the detection of phenolic and pigment compounds. In the majority of the cases, the use of reversed-phase chromatography is employed with C18 columns and mobile phases consisting of a combination of acidic aqueous and organic solvents (Robbins, 2003).

The HPLC-DAD analysis is based on the separation of the compounds contained inside a sample prior to their detection, hence it is suitable for detecting individual phenolic compounds, instead of the total amounts which are measured with the Folin-Ciocalteu

assay. For this reason, the HPLC-DAD method offers improved selectivity against the Folin-Ciocalteu assay.

The HPLC-DAD analysis of the extracts was performed using a Shimadzu NexeraX2 UHPLC system (Shimadzu Europa GmbH, UK). The chromatographic separation was implemented with the use of a Shim-pack XR-ODS (C18) column (L. 50 x I.D. 3 mm, particle size 2.2 μm; Shimadzu Europa GmbH, UK). In regard to the method, two mobile phases were used; (A) water with 0.1% v/v formic acid (pH of 2.7) and (B) a mixture of acetonitrile/methanol/water 80:10:10 v/v/v with 0.1% v/v formic acid. The samples were eluted according to the following method: (i) 0-10 min: 98% for solvent A (isocratic), (ii) 10-20 min: from 98% to 80% for solvent A (gradient), (iii) 20-29 min: from 80% to 50% for solvent A (gradient), (iv) 29-35 min: from 50% to 30% for solvent A (gradient), (v) 35-39 min: from 30% to 90% for solvent A (gradient), (vi) 39-40 min: from 90% to 98% for solvent A (gradient), (vii) 40-50 min: 98% for solvent A (isocratic; re-equilibration step). In addition, the flow rate was set at 0.3 mL/min and the column temperature at 45^{o}C. The injection volume of the samples was 10 μL. Finally, each sample was filtered with Corning® syringe filters 0.45 μm PES (Corning Ltd., UK), prior to analysis.

The determination of compounds was made based on their retention times and UV-Vis spectrums, in comparison to corresponding data found in the literature or acquired using standards (Robbins, 2003). In particular, the standards which were used were the following: betanin pigment (red beet extract diluted with dextrin), gallic acid, vanillic acid, caffeic acid and rutin (Figure 2.9 & 2.10). The determination was also based on LC-IT-TOF-MS data, which were acquired by using the same elution/separation conditions with the HPLC-DAD analysis; this was not, however, an integrated HPLC-DAD-MS analysis. In general, the HPLC-DAD analysis offers very low detection limits in the range of μg/mL (i.e. ppm), for phenolic compounds (Nour, Trandafir and Cosmulescu, 2013; Alberti *et al.*, 2014; Carvalho, Curto and Guido, 2015).

The calibration curve of betanin was determined using commercial standards of betanin (red beet extract in dextrin), at concentrations in the range of 0-30 mg/mL. In the chromatograms of the betanin standards, two characteristic peaks could be observed (Figure 2.9), which were closely eluting at rt. 16.8 (UV_{max} = 534 nm) and 18.1 min

(UV_{max} = 528 nm), and are attributed to betanin and isobetanin, respectively (Naderi et al., 2012; da Silva et al., 2019). The calibration curve was calculated based on the sum area of both peaks at their UV maxima, and is given in Figure 2.11.

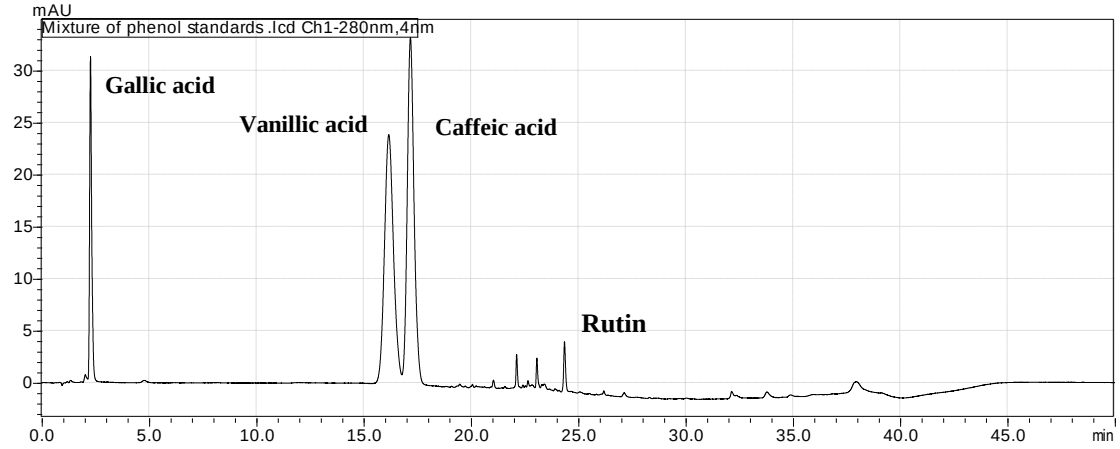


Figure 2.9 *The HPLC-DAD chromatogram of a mixture of standard phenolic compounds at 280 nm, with each phenolic compound assigned to its peak.*

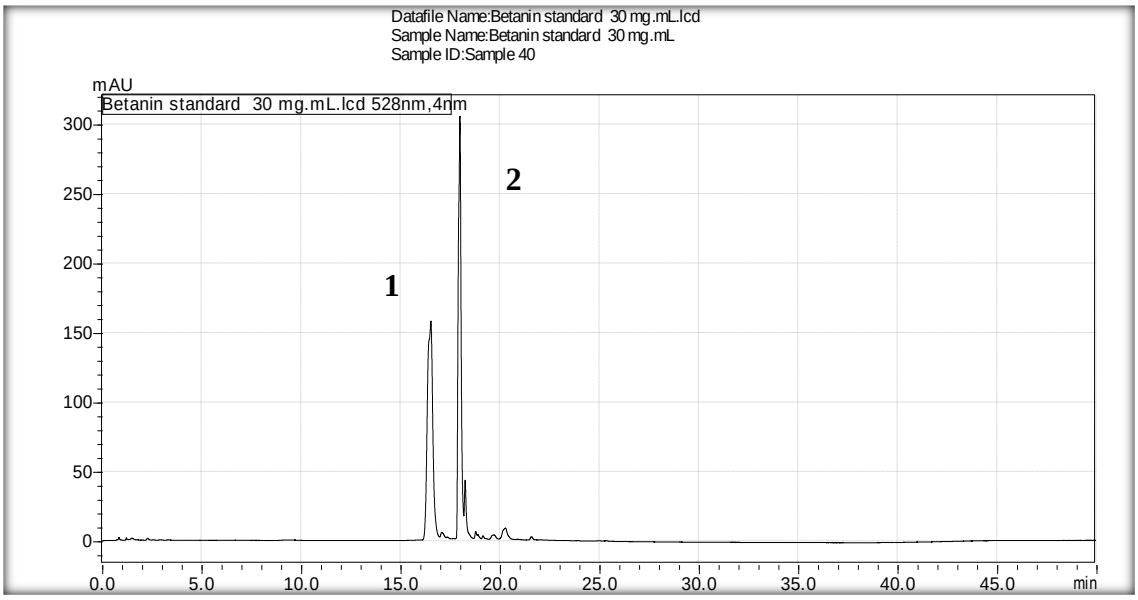


Figure 2.10 *The HPLC-DAD chromatogram of the commercial betanin standard (red beet extract with dextrin), at 538 nm. Two peaks can be spotted, attributed to: (1) betanin (rt. 16.8 min, UV_{max} = 534 nm) and (2) isobetanin (rt. 18.1 min, UV_{max} = 528 nm).*

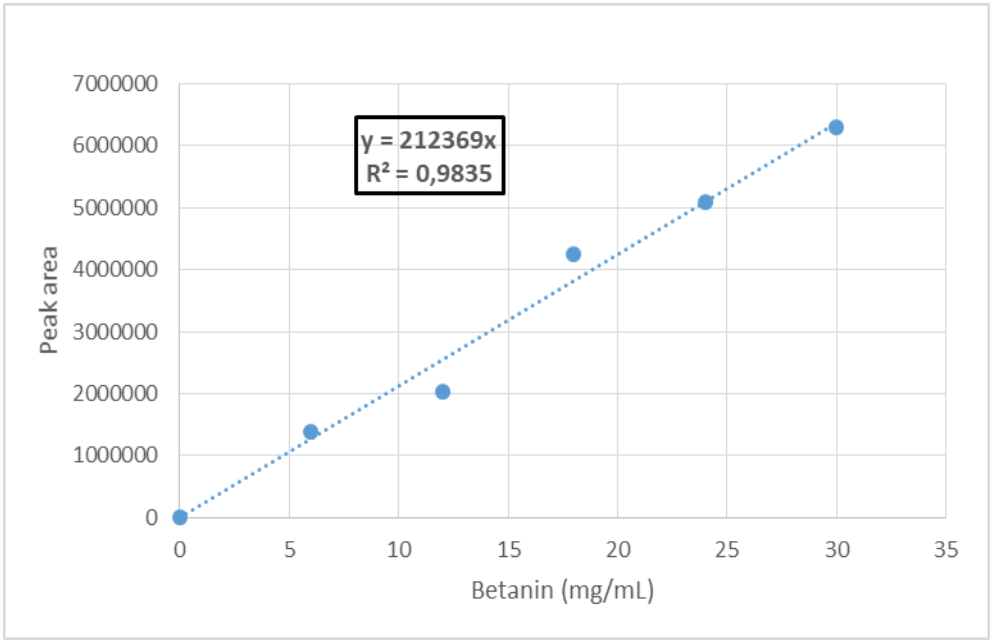


Figure 2.11 *The calibration curve of the commercial betanin standard (red beet extract with dextrin) using HPLC-DAD, in the concentration range of 0-30 mg/mL, calculated based on the sum area of both betanin and isobetanin peaks at their* UV_{max}.

2.4.3. LC-ESI-IT-TOF-MS analysis

The Liquid Chromatography coupled with a Mass Spectrometer was employed, also combined with an Electrospray Ionisation (ESI), a Quadrupole Ion Trap (QIT) and a Time-of-Flight (TOF) Mass analyser system. The system used was the Shimadzu NexeraX2 UHPLC system (Shimadzu Europa GmbH, UK) in combination with a Shimadzu LCMS-IT-TOF™ mass analyser (Shimadzu Europa GmbH, UK).

The LC-ESI-IT-TOF-MS is considered to be an established analytical technique for the detection of phenolic compounds (Robbins, 2003; Seraglio *et al.*, 2016), betalain pigments (Sawicki, Juśkiewicz and Wiczkowski, 2017) and saponins (Mroczek *et al.*, 2012; Mikołajczyk-Bator *et al.*, 2016). The LC-MS analytical technique offers low detection limits, as it is able to detect amounts in the range of ng/mL (i.e. ppb) for phenolic compounds (Seraglio *et al.*, 2016) and betalain pigments (Sawicki, Juśkiewicz

and Wiczkowski, 2017), and in the range of μg/mL (i.e. ppm) for saponins (Ha *et al.*, 2006; Zhang *et al.*, 2014).

The chromatographic conditions of the LC-ESI-MS analysis were chosen to be the same with the respective conditions of the HPLC-DAD analysis, in order to be able to correlate the peaks which would be detected in both instruments. More specifically, the elution method, the column selection, the column temperature, the flow rate and the injection volumes of the samples were chosen to be the same as in the HPLC-DAD analysis. The mass spectrometer scan range was set at 150-800 m/z. The combined information acquired from both chromatographic techniques for each peak was expected to facilitate primarily their qualitative determination.

2.4.4. Gel Filtration Chromatography

The Gel Filtration Chromatography (GFC) was performed combined with a Refractive Index Detector (RID), mainly for the detection of the lignocellulosic enzymes. In particular, the analysis was performed using a Shimadzu Prominence GPC system (Shimadzu Europa GmbH, UK). The separation was achieved using the Phenomenex Phenogel™ size-exclusion (SEC) column (5 μm Linear(2), LC Column 300 x 7.8 mm, Phenomenex Inc., USA), with a nominal fractionation range of MW 100 Da-10,000 kDa. Furthermore, the GFC analysis was performed using isocratic elution of a phosphate buffer aqueous solution (100 mM sodium phosphate buffer/0.025 % NaN_3) adjusted to pH of 6.8. The flow rate and the column's temperature were set at 1 mL/min and ambient temperature, respectively. Finally, the calibration curve was designed based on the following standards: (1) thyroglobulin bovine (670 kDa), immunoglobulin G dimer (300 kDa), γ-globulins from bovine blood (150 kDa), albumin chicken egg grade VI (44 kDa), myoglobin (horse) (17 kDa) and uridine (244 Da).

Chapter 3

Results & Discussions

3.1. Introduction

In this chapter, the beetroot juice and conventional extracts of the raw beetroot are initially analysed to determine and validate the presence of added-value beetroot compounds.

The lab-scale enzymatic and conventional extractions of the beetroot pomace are subsequently employed to determine the presence of added-value compounds in the waste material, with the final aim of optimising the extraction process parameters, in favour of achieving maximum recoveries in the most time- and cost- efficient manner.

The semi-pilot scale enzymatic and conventional extractions are finally employed, combined with the subsequent UF and NF membrane cascade processing of the extracts and the evaporation of the obtained streams, for the clarification of the extracts and the fractionation, concentration and purification of potentially recovered compounds. The concentration, during the membrane processing and evaporation stages, is expected to improve the detection of some of the added-value compounds, in comparison to the lab-scale experiments, due to their potential increase in concentration up to levels that will surpass the detection limits of the applied analytical methods. The final aim of the semi-pilot scale experiments is the optimisation of the membrane processing parameters, by achieving the aforementioned process objectives, in the most time- and cost- efficient manner.

3.2. Raw beetroot extraction & beetroot juice

This section focuses on the analysis of the beetroot juice and of extracts of raw beetroot from the processing of which the tested beetroot pomace derives as waste. The aim of the analysis is to validate the presence of compounds inside these samples, mainly using HPLC-DAD and LC-MS techniques. This may serve as an indication for the presence of compounds inside the beetroot pomace.

3.2.1. Raw beetroot extraction

Raw beetroot was firstly subjected to conventional extraction, using three different solvents: (i) 15% v/v aq. ethanol, (ii) 100% ethanol and (iii) 100% methanol. The extractions were performed at a solid-to-solvent ratio of 1:10 (30 g of beetroot in 300 mL solvent), at a temperature of 25^{o}C and for 5 h, under constant mixing. The extracts were then decanted from the solid material and were evaporated using a rotary evaporator, to concentrate the samples by removing the excess solvent. After evaporation, the extracts were concentrated by a factor of 2.5-3.5 and were finally analysed with HPLC-DAD and LC-MS.

The chromatograms of the HPLC-DAD analysis of these extracts are given in Figure 3.1. The analysis unveiled the presence of certain compounds inside beetroot. The retention times of the peaks found in each extract are synopsised in Table 3.1, along with their qualitative determination, based on the acquired HPLC-DAD and LC-MS data and the published literature.

In the methanol extract, the presence of a compound with retention time at 1.7 min was observed, with UV_{max}=471 nm and $[M+H]^+$ value of 340. This compound is attributed to Vulgaxanthin I, based on its early elution in RP-HPLC (Nemzer *et al.*, 2011; Koubaier *et al.*, 2014; Račkauskiene *et al.*, 2015; Slatnar *et al.*, 2015), its m/z value (Nemzer *et al.*, 2011; Koubaier *et al.*, 2014; Račkauskiene *et al.*, 2015; Slatnar *et al.*, 2015) and its UV_{max} value (Nemzer *et al.*, 2011; Slatnar *et al.*, 2015).

Two additional compounds were found at relatively similar retention times of 17 and 18 min, both having a UV_{max}=543 nm, corresponding to betanin and isobetanin, respectively. This was based on their perfect correlation with the betanin standard, their similar medium retention in RP-HPLC (Nemzer *et al.*, 2011; Koubaier *et al.*, 2014; Sawicki, Juśkiewicz and Wiczkowski, 2017) and their UV_{max} values (Nemzer *et al.*, 2011; Koubaier *et al.*, 2014; Slatnar *et al.*, 2015; Sawicki, Juśkiewicz and Wiczkowski, 2017). Finally, a compound was found with a retention time at 20.5 min, having a UV_{max}=464 nm. According to its elution, which was slightly later than betanin and isobetanin in RP-HPLC (Nemzer *et al.*, 2011; Račkauskiene *et al.*, 2015; Slatnar *et al.*, 2015; Sawicki, Juśkiewicz and Wiczkowski, 2017), and to its UV_{max} value (Nemzer *et al.*, 2011; Slatnar *et al.*, 2015), this peak can be attributed to neobetanin.

In the 15% v/v ethanol extract, the presence of vulgaxanthin I, betanin and isobetanin was determined. Apart from those compounds, a peak with a retention time at 6 min was further observed, having UV_{max}=274 nm and $[M+H]^+$=341 (MW=340 g/mol). However, this compound remained qualitatively unknown, as its matching with common phenolic compounds, pigments or saponins, reported to be present in beetroot in the literature, was unsuccessful. Another compound was finally found at a retention time of 23 min, having a UV_{max}=419 nm. Due to the late elution of this compound, which was later than neobetanin (Nemzer *et al.*, 2011; Račkauskiene *et al.*, 2015; Slatnar *et al.*, 2015), and to its UV_{max} (Nemzer *et al.*, 2011; Slatnar *et al.*, 2015), it can

be concluded that this peak corresponds to 2-Decarboxy-2,3-dehydro-neobetanin. In the 100% ethanol extract, no peaks were determined, thus suggesting that this solvent is not efficient for the extraction of beetroot.

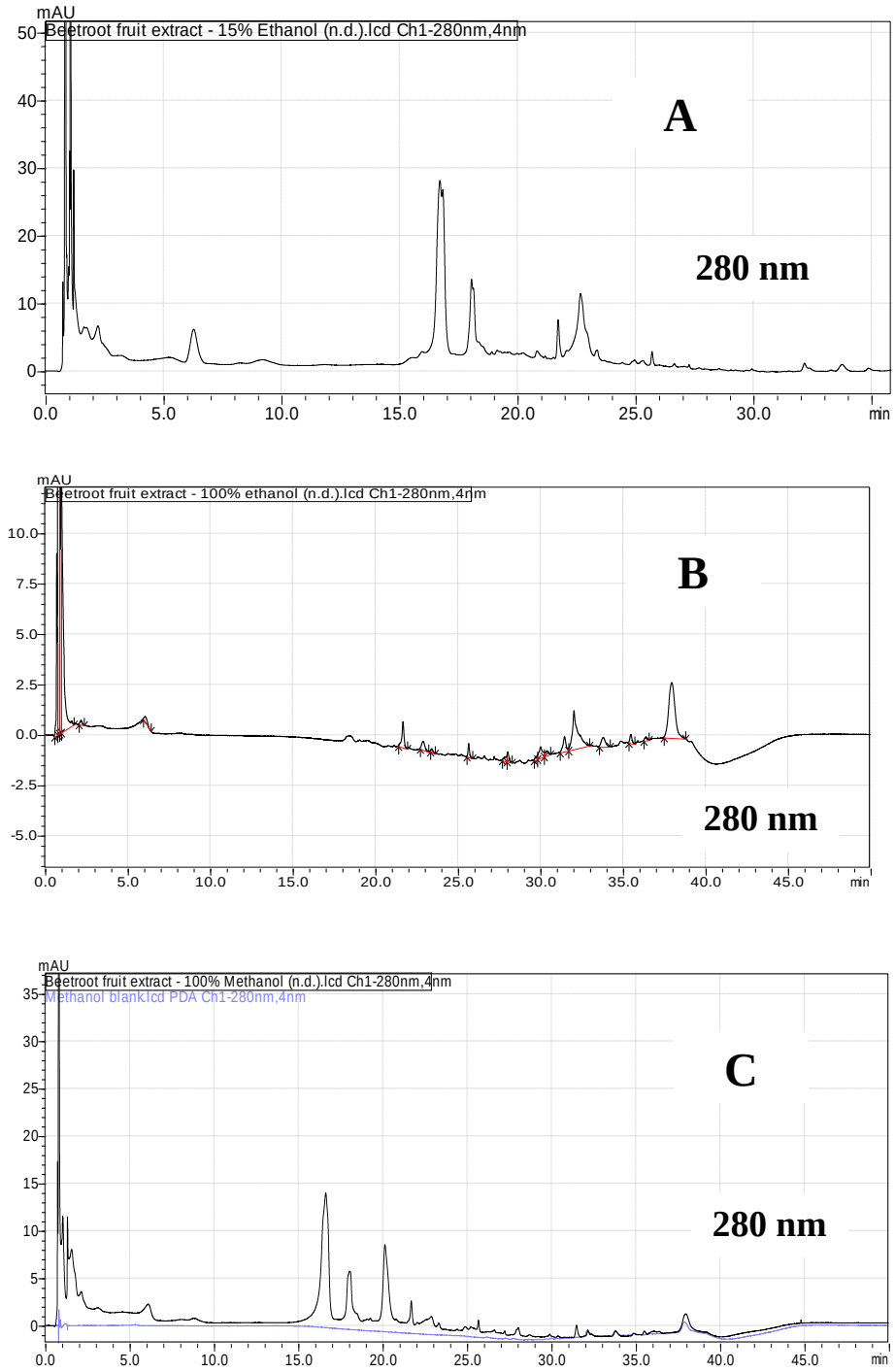


Figure 3.1 *The HPLC-DAD chromatogram of the conventional extraction of raw beetroot, using (A) 15% ethanol, (B) 100% ethanol and (C) 100% methanol, as extraction solvents, at 280 nm.*

3.2.2. Beetroot juice

The beetroot juice, from the production of which the beetroot pomace derives as waste, was analysed with the HPLC-DAD and LC-MS techniques. The HPLC-DAD chromatograms of the beetroot juice are presented in Figure 3.2. In particular, the analysis revealed the presence of betanin, isobetanin, neobetanin, 2-Decarboxy-2,3-dehydro-neobetanin and vulgaxanthin I, as well as of an undetermined compound with a retention time at 6 min, which has also been found in the beetroot extracts. Apart from those compounds, one more peak was further observed in the HPLC-DAD chromatograms, which had not been found before in the beetroot extracts, with a retention time at 21.5 min. This compound had a UV_{max}=325 nm and was not detected in the LC-MS chromatograms. In view of those limited peak data, the qualitative

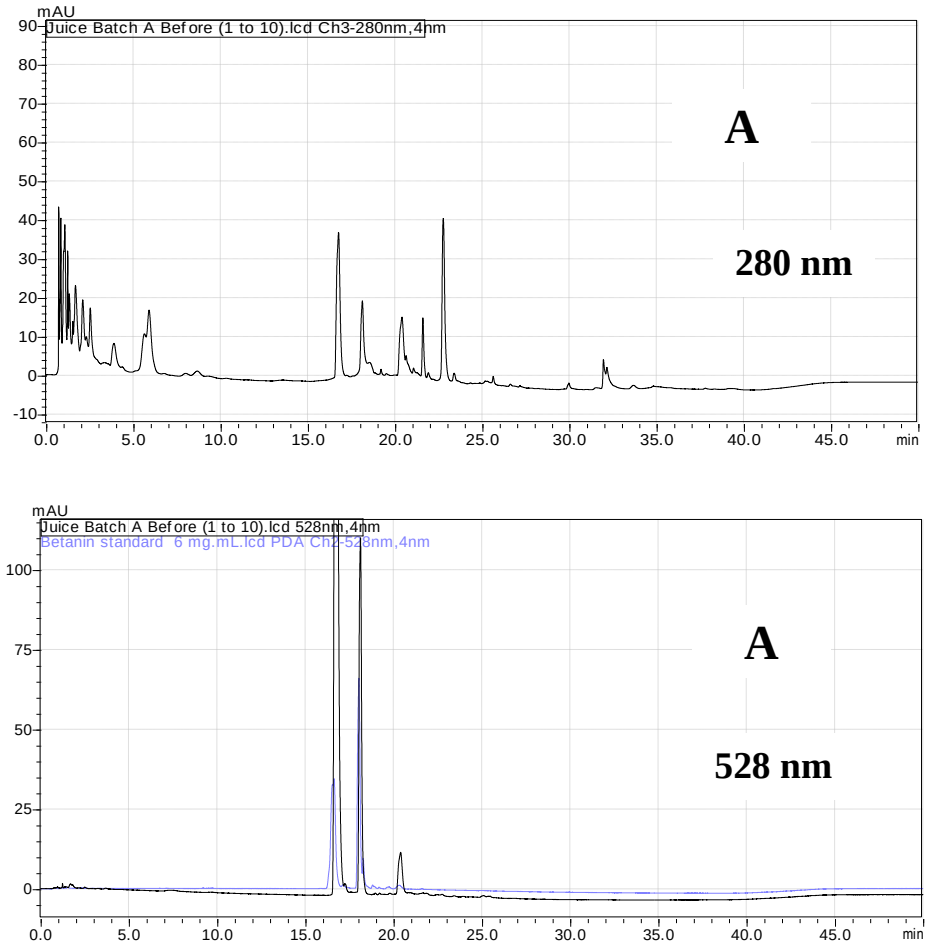


Figure 3.2 *The HPLC-DAD chromatograms of the beetroot juice, at (A) 280 and (B) 528 nm.*

determination of the compound could not be achieved, only based on its retention time and UV_{max}, thus the compound remained unidentified. A synopsis of the retention times and the identification of the peaks found in beetroot juice is given in Table 3.1.

Table 3.1 *A synopsis of the retention times (rt.) of the peaks found in each of the raw beetroot extracts and in beetroot juice, along with their qualitative determination.*

Peak rt. (min)	Identification	Raw beetroot extracts			Beetroot juice
		Methanol extract	15% Ethanol extract	100% Ethanol extract	
1.7	Vulgaxanthin I	✓	✓	✗	✓
6	-	✗	✓	✗	✓
17	Betanin	✓	✓	✗	✓
18	Isobetanin	✓	✓	✗	✓
20.5	Neobetanin	✓	✗	✗	✓
21.5	-	✗	✗	✗	✓
23	2-Decarboxy-2,3-dehydro-neobetanin	✗	✓	✗	✓

3.3. Lab-scale extractions of beetroot pomace

Initial experiments of this project involved the extraction of the beetroot pomace in laboratory-scale. This can offer several advantages, such as testing a large number of experimental conditions in minimum time and cost. In these experiments, the enzymatic-aided and the conventional extractions of the beetroot pomace were performed at various conditions. The aim of these experiments was mainly the optimisation of the extraction conditions, in order to obtain maximum compound yields.

The enzymatic-aided extraction was initially performed in two subsequent steps; firstly, the commercial enzyme mixture was added in the beetroot pomace in order for the hydrolysis to take place, and, secondly, the organic phase of the extraction solvent (i.e. ethanol) was added inside the enzyme mixture, in order for the extraction to occur. In this way, the enzymatic-aided extraction was executed in the simplest possible manner.

3.3.1. Enzymatic hydrolysis & subsequent extraction without intermediate separation of the hydrolysis medium from the pulp

3.3.1.1. Preliminary results

The lab-scale preliminary experiments of enzymatic-aided and conventional extractions were performed at three different conditions using a single batch of beetroot pomace. After the extraction, the extracts were collected by centrifugation and were then analysed using the Folin-Ciocalteu assay and the HPLC-DAD methods. The effect of the enzymes on the decomposition of the pulp was apparent after the centrifugation. The pulp appeared to consist of smaller particles and formed easily a pellet which was strongly settled at the bottom of the tube in the case of the enzymatic extraction, as compared to the conventional extraction (Figure 3.3).

Figure 3.3 *The pulp after the centrifugation of the hydrolysis mediums (i) for the conventional extraction (left) and (ii) for the enzymatic extraction (right).*

<u>Folin-Ciocalteu assay</u>

The Folin-Ciocalteu analysis of the extracts initially demonstrated that the pure enzyme mixture (blank) exhibits strong Folin responses. Therefore, the correction of the enzymatic extracts' Folin response was necessary in order to acquire valid TPC estimations; this correction entailed subtracting the Folin response of the pure enzyme mixture at the same enzyme concentration as in the enzymatic extract.

Table 3.2 *The Folin results of the conventional and enzymatic extraction of beetroot pomace; (i) extraction with Batch A (Top) and (ii) extraction with Batch B (Bottom). Negative values were observed for the enzymatic extraction following the enzyme correction, using both batches (red boxes) ["B" indicates conventional extraction, "E" indicates enzymatic extraction, numbers 1,2 and 3 indicate repetitions of the extraction] (Conditions: T=45°C / pH = 4.5 / treatment time = 360 min / Enzyme activity = 250 U/mL·g)*

							Amounts				Enzyme				with Enzyme Correction			
Sample	**Volume (mL)**	**Dilution**	**A1**	**A2**	**A Average**	**A STDEVA**	**TPC 1 (µg)**	**TPC 2 (µg)**	**Average**	**STDEVA**	**TPC 1 (µg)**	**TPC 2 (µg)**	**Average**	**STDEVA**	**TPC 1 (µg)**	**TPC 2 (µg)**	**Average**	**STDEVA**
1B	9,6	1/5	0,532	0,527	0,530	0,004	795,0	787,8	791,4	5,1	-	-	-	-	795,0	787,8	791,4	5,1
2B	9,0	1/5	0,391	0,427	0,409	0,025	554,2	603,0	578,6	34,5	-	-	-	-	554,2	603,0	578,6	34,5
3B	9,5	1/5	0,464	0,456	0,460	0,006	689,5	678,0	683,7	8,1	-	-	-	-	689,5	678,0	683,7	8,1
1E	10,5	1/50	0,759	0,792	0,776	0,023	12285,3	12807,2	12546,2	369,0	24854,7	28262,9	26558,8	2410,0	-12569,4	-15455,8	-14012,6	2041,0
2E	10,2	1/50	0,709	0,770	0,740	0,043	11166,2	12103,3	11634,8	662,6	26408,5	27862,0	27135,2	1027,8	-15242,2	-15758,7	-15500,5	365,2
3E	10,0	1/50	0,710	0,765	0,738	0,039	10962,3	11790,7	11376,5	585,7	26859,6	28363,2	27611,4	1063,2	-15897,2	-16572,5	-16234,9	477,5

							Amounts				Enzyme				with Enzyme Correction			
Sample	**Volume (mL)**	**Dilution**	**A1**	**A2**	**A Average**	**A STDEVA**	**TPC 1 (µg)**	**TPC 2 (µg)**	**Average**	**STDEVA**	**TPC 1 (µg)**	**TPC 2 (µg)**	**Average**	**STDEVA**	**TPC 1 (µg)**	**TPC 2 (µg)**	**Average**	**STDEVA**
1B	9,4	1/5	0,447	0,439	0,443	0,006	658,1	646,8	652,5	8,0	-	-	-	-	658,1	646,8	652,5	8,0
2B	9,5	1/5	0,468	0,470	0,469	0,001	695,2	698,0	696,6	2,0	-	-	-	-	695,2	698,0	696,6	2,0
3B	9,5	1/5	0,454	0,462	0,458	0,006	675,2	686,6	680,9	8,1	-	-	-	-	675,2	686,6	680,9	8,1
1E	10,3	1/50	0,712	0,718	0,715	0,004	11322,2	11415,3	11368,8	65,8	24854,7	28262,9	26558,8	2410,0	-13532,5	-16847,6	-15190,1	2344,1
2E	10,3	1/50	0,754	0,807	0,781	0,037	11973,8	12795,9	12384,8	581,3	26408,5	27862,0	27135,2	1027,8	-14434,7	-15066,1	-14750,4	446,4
3E	10,4	1/50	0,788	0,860	0,824	0,051	12622,5	13750,2	13186,4	797,4	26859,6	28363,2	27611,4	1063,2	-14237,0	-14612,9	-14425,0	265,8

Indicative results of the Folin responses for both conventional and enzymatic extracts are given in Table 3.2. In particular, the results demonstrated that negative values are obtained for the enzymatic extracts after the pure enzyme mixture's Folin correction, thus leading to invalid results. These results were fully repeatable and were observed in all three tested conditions. The experiments were also reproduced using beetroot pomace from another batch, however negative values were again obtained for the enzymatic extracts.

HPLC-DAD analysis

The HPLC-DAD analysis of the enzymatic and the conventional extracts did not unveil the presence of peaks, which could be attributed to compounds deriving from the beetroot pomace. More specifically, only peaks entirely related to the enzyme mixture

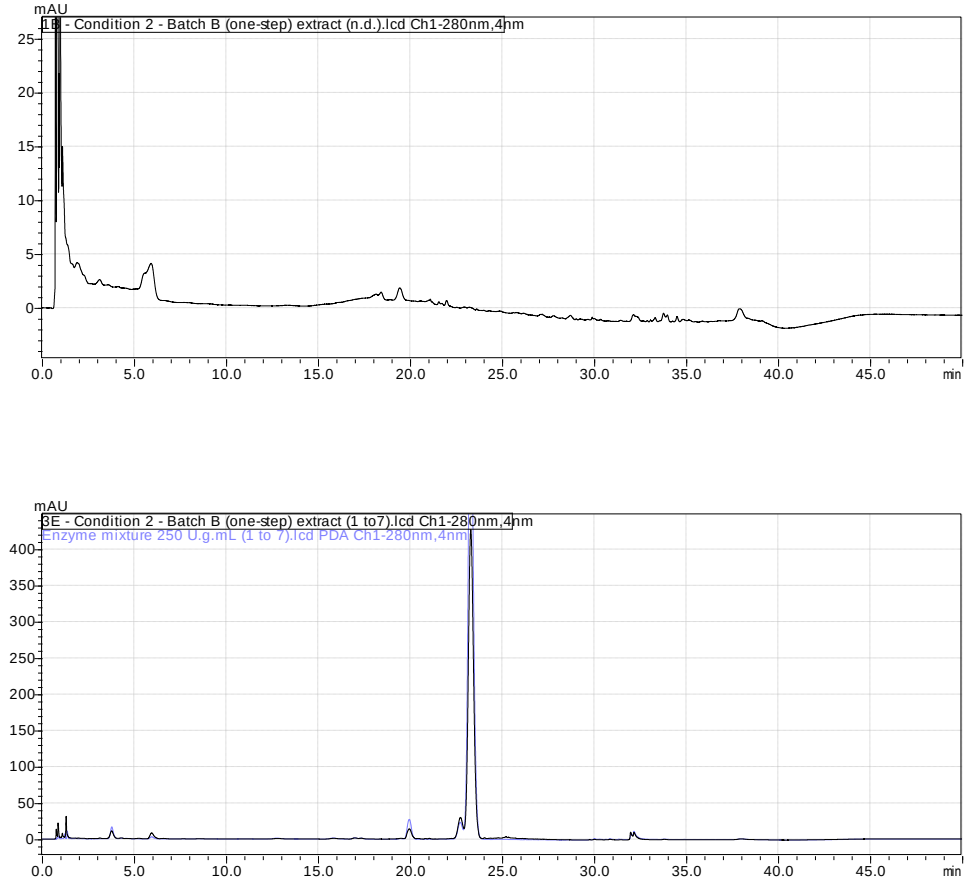


Figure 3.4 *The HPLC-DAD chromatograms of the beetroot pomace extract; (i) the conventional extraction (Top) and (ii) the enzymatic extraction (Bottom), at 280 nm.*

could be observed in the enzymatic extracts, while no peaks were detected in the conventional extracts, for all three tested conditions. Similar results were observed using beetroot pomace from another batch.

Representative HPLC-DAD chromatograms of both enzymatic and conventional extracts are given in Figure 3.4. It should be noted that the conventional extracts were injected undiluted in the HPLC-DAD instrument, while the enzymatic extracts were run at a dilution of 1:7 to avoid the overload of the HPLC column with the enzyme.

3.3.1.2. Investigation of the decreased Folin responses

The decreased Folin values of the enzymatic extracts were repeatable and led to invalid negative results. The evaluation of the extracts was not rendered possible with the Folin-Ciocalteu assay, at least not by using the employed extraction and sample procedure. There is therefore the need to investigate the reasons for the decreased Folin absorbances of the enzymatic extracts and to modify the extraction and sample preparation procedure, in order to render this analytical technique suitable for the evaluation of phenolic compounds in the extracts.

There are three possible scenarios that could explain the lower Folin absorbances of the enzymatic extracts in comparison to the pure enzyme mixture blanks. These are the following:

1. Interactions between protein-phenol and carbohydrate-protein
2. Enzyme denaturation
3. Adsorption of the enzyme in the beetroot pomace

The above scenarios are investigated with the performance of suitable experiments, that will be presented in the following subchapters.

3.3.1.2.1. Protein-phenol & carbohydrate-protein interactions

Protein-phenol or carbohydrate-protein interactions could be a possible reason for the observation of decreased Folin absorbances in the enzymatic extracts.

According to Arts et al. (2002), the antioxidant capacities of phenol and protein components are not additive in protein-phenol mixtures, as a part of the total antioxidant capacity is masked by their interaction, as measured with the ABTS assay. The masking of the antioxidant capacity was observed between phenolic compounds of tea (catechin, epicatechin, epigallocathechin, gallic acid etc.) and proteins found in milk (α-, β-, κ-caseins and albumin). The authors reported that the highest masking was observed in mixtures of β-casein with epigallocathechin and gallic acid. It was finally concluded that the addition of milk in tea is expected to decrease the antioxidant capacity of the antioxidants present in tea, and that this may occur to generally other systems consisting of protein and phenol substances.

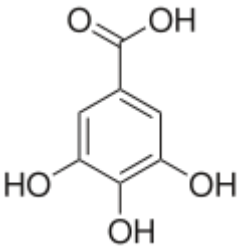
The protein-phenol interactions have also been reported by using different analytical techniques: (1) the formation of protein-phenol adducts has been validated with LC-MS techniques (Rawel, Rohn and Kroll, 2003; Schilling et al., 2010; Tantoush et al., 2011), (2) the formation of protein-phenol aggregates has been confirmed with SDS-PAGE, by the appearance of bands with higher molecular weights in protein-phenol mixtures, which were absent in their pure solutions (model systems) (Rawel, Czajka, et al., 2002; Rawel, Rohn, et al., 2002; Rawel, Rohn and Kroll, 2003; Rohn, 2014), (3) changes in the UV-Vis spectrums of protein and phenol solutions compared to their mixture (Rawel, Rohn and Kroll, 2003; Seifert et al., 2004), (4) the drift in the retention times of proteins in their mixtures with phenols using RP-HPLC (Rawel, Czajka, et al., 2002; Rawel, Rohn and Kroll, 2003) and finally (5) changes in the peak characteristics of proteins and phenols and also the appearance of protein-phenol complexes by using Gel Filtration Chromatography (Bartolomé, Estrella and Hernández, 2000; Prigent et al., 2003).

The decreased Folin absorbances observed in the enzymatic extracts compared to the pure enzyme mixture's Folin absorbance are due to the lower amount of enzymes which have reacted with the Folin reagent. This could be possibly attributed to interactions of

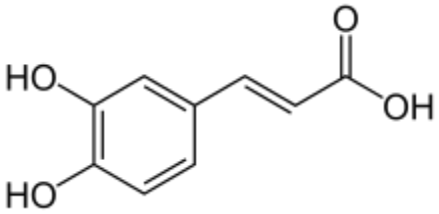
the enzymes with extracted phenols, which can impede their reaction with the Folin reagent. Although the protein-phenol interactions have not been previously studied using the specific proteins (i.e. cellulase and pectinase enzymes) employed in this work, in combination with various phenols, such interactions have been clearly proven to exist in similar systems to the enzymatic beetroot pomace extracts, with regard to containing relatively high amounts of proteins (i.e. enzymes), sugars and polysaccharides (i.e. lignocellulose), and potentially phenolic compounds. Furthermore, those interactions have been observed by using similar analytical techniques (Arts *et al.*, 2002), since ABTS and Folin-Ciocalteu assays are categorised in the same electro-transfer based category of the antioxidant capacity assays (Huang, Ou and Prior, 2005; Apak *et al.*, 2007). The hypothesis herein is therefore that the groups of phenols and proteins which normally react with the Folin reagent, may participate in the protein-phenol interactions or may be generally impeded from reacting with the Folin reagent due to these interactions.

In order to investigate the occurrence of such protein-phenol interaction and also of potentially protein-carbohydrate interactions, the experiments as described in section 2.3.5.1. were conducted between model systems of the used enzymes, i.e. Celluclast 1.5 L and Pectinex Ultra Mash, in combination with glucose (sugar), carboxyl methyl cellulose (CMC; polysaccharide) and phenolic compounds commonly reported to be present in beetroot (gallic acid, vanillic acid, caffeic acid and rutin), based on the literature (Figure 1.4), both in pure solutions and their mixtures (Figure 3.5).

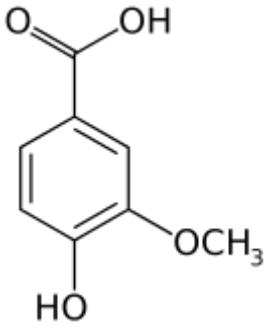
These phenolic compounds were also selected on the basis of certain characteristic, such as: (a) their molecular weight (gallic, vanillic and caffeic acid have a MW of 168-180 g/mol, while rutin has 610 g/mol), (b) the number of hydroxy groups bound to the phenyl ring (i.e. one –OH in vanillic acid, two –OH in caffeic acid and three -OH in gallic acid) and (c) the number of phenyl rings (one phenyl ring in gallic, vanillic and caffeic acid, whereas two phenyl rings in rutin), and finally (d) the presence of glycosides in the phenolic structure (no glycosides in gallic, vanillic and caffeic acid, while two glycosides in rutin) (Figure 3.5).



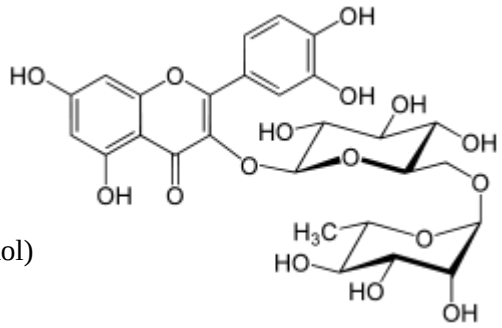
Gallic acid
(Mr = 170 g/mol)



Caffeic acid
(Mr = 180 g/mol)



Vanillic acid
(Mr = 168 g/mol)



Rutin
(Mr = 610 g/mol)

Model systems:	1) **Gallic acid** (200 µg/mL)	–	**Celluclast/Pectinex** (125 U/mL)	**Phenol - Protein**
	2) **Vanillic acid** (120 µg/mL)	–	**Celluclast/Pectinex** (125 U/mL)	
	3) **Caffeic acid** (50 µg/mL)	–	**Celluclast/Pectinex** (125 U/mL)	
	4) **Rutin** (saturated)	–	**Celluclast/Pectinex** (125 U/mL)	
	5) **Glucose** (1% w/v)	–	**Celluclast/Pectinex** (125 U/mL)	**Carbohydrate -Protein**
	6) **CMC** (0.25% w/v)	–	**Celluclast/Pectinex** (125 U/mL)	

*CMC = carboxyl methyl cellulose

Figure 3.5 *Top: The structures of the phenolic compounds that were used as standards for the protein-phenolic interactions experiments, Bottom: The phenol-protein and carbohydrate-protein model systems which were used for the investigation of interactions, along with their concentrations.*

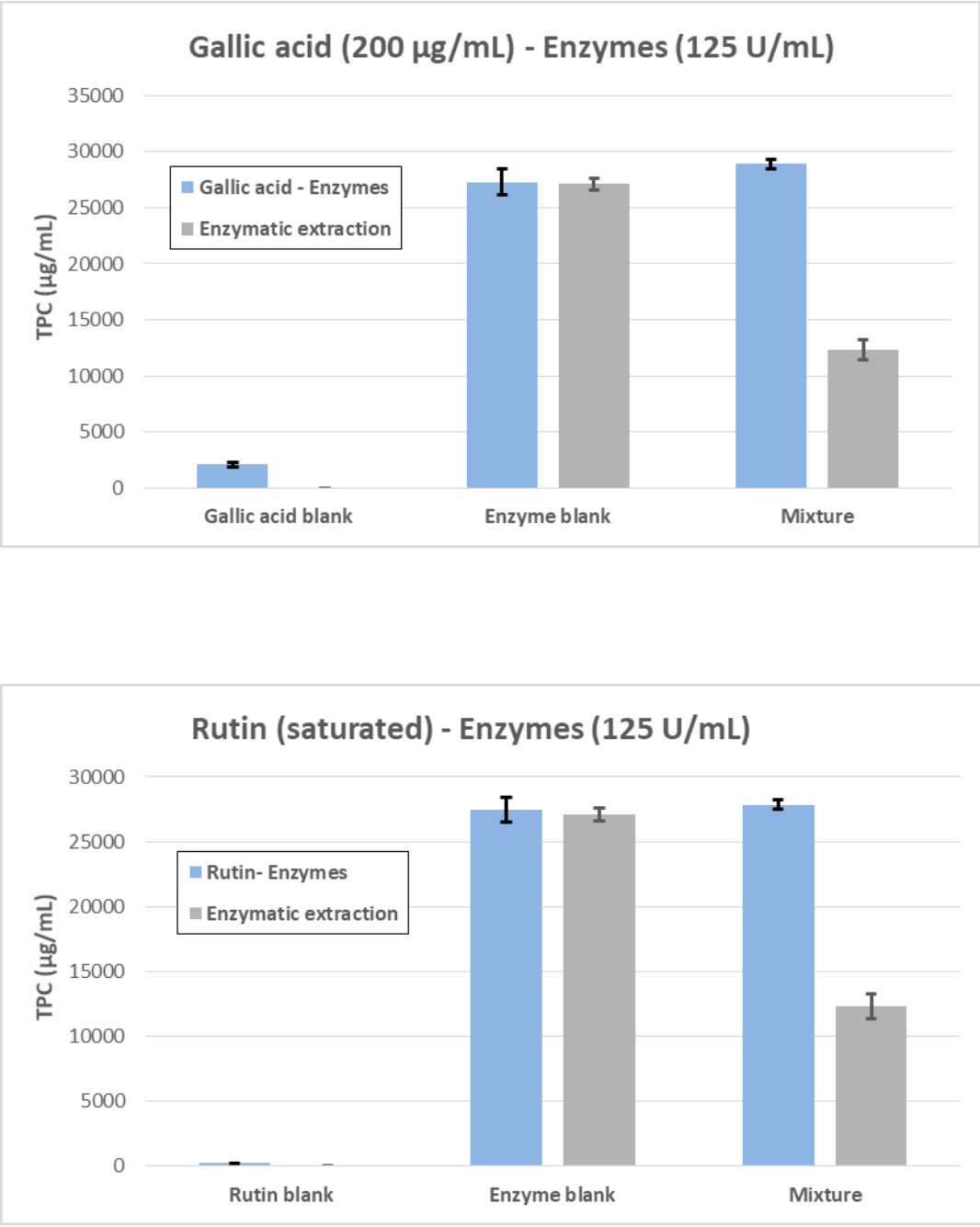


Figure 3.6 *The Folin results of the gallic acid-enzymes (Top) & the rutin-enzymes (Bottom) model systems (in blue), as compared to the enzymatic extraction.*

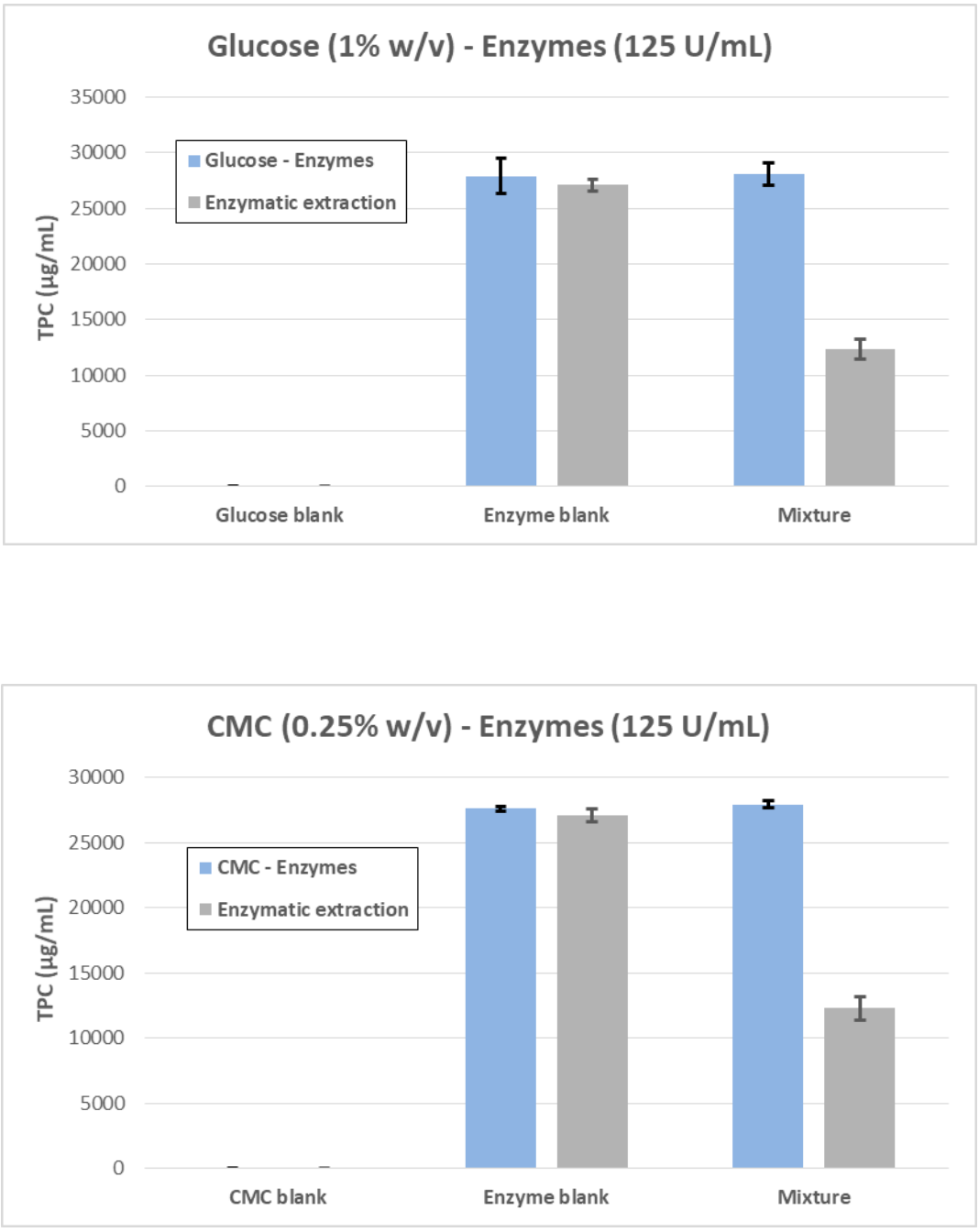


Figure 3.7 *The Folin results of the glucose-enzymes (Top) & the CMC-enzymes (Bottom) model systems (in blue), as compared to the enzymatic extraction.*

The pure and mixture solutions were analysed and compared using the Folin assay, so as to simulate similar sample conditions in the enzymatic beetroot pomace extracts with the same analytical method, but this time using standard enzyme-phenol and enzyme-carbohydrate solutions of known composition. The final aim was to observe a decrease in the Folin response of any of the mixtures in comparison to the pure solution, which would indicate the occurrence of interactions and, eventually, would justify the decreased Folin absorbances in the enzymatic extracts compared to the pure enzyme mixture.

The results of those experiments, however, indicated that Folin absorbances are not being decreased in any of the mixture solutions when compared to the pure solutions. Indicative results of these experiments are given in Figures 3.6 & 3.7, including the average GAE values and their standard deviations. Furthermore, the GAE values of an enzymatic beetroot pomace extract and its enzyme mixture blank are given in the same figures, as measured during the extractions performed in section 3.3.1.1, in order to compare the enzymatic extracts with the model systems. As the results suggest, the decreased Folin absorbances of the enzymatic beetroot extracts are not attributed to phenol-protein or carbohydrate-protein interactions.

To validate further those results and conclusions, the UV-Vis analysis of the pure and mixed solutions of gallic acid and the enzymes was performed. Any interaction between gallic acid and the enzymes could potentially change the UV-Vis spectral/absorbance characteristics of those substances, through either the formation of an adduct with a different extinction coefficient (i.e. molar absorptivity) and spectrum fingerprint, or the change in the structure of those compounds caused potentially by their reaction, which could shift the UV-Vis maxima of the substances at different wavelengths. On the other hand, the UV-Vis absorbance of a two-compound mixture is known to be equal to the sum of the absorption of their single component solutions at the same concentration, when those compounds do not interact with each other (Inscoe et al., 1958). The applicability of this law in the UV-Vis spectrums of the gallic acid-enzymes model systems would indicate that no interactions between those compounds are taking place.

The experiments were performed with mixtures at the following concentrations: 12.5 U/mL enzymes – 150 μg/mL gallic acid, 12.5 U/mL enzymes – 30 μg/mL gallic acid,

12.5 U/mL enzymes – 15 μg/mL gallic acid, 12.5 U/mL enzymes – 3 μg/mL gallic acid, for reaction times of 2, 4 and 6 hours.

The results of the UV-Vis experiments suggest clearly that no interaction is taking place between gallic acid and the enzyme mixture comprising of Celluclast 1.5 L and Pectinex Ultra Mash, as the spectrums of their mixtures were identical to the sum of spectrums

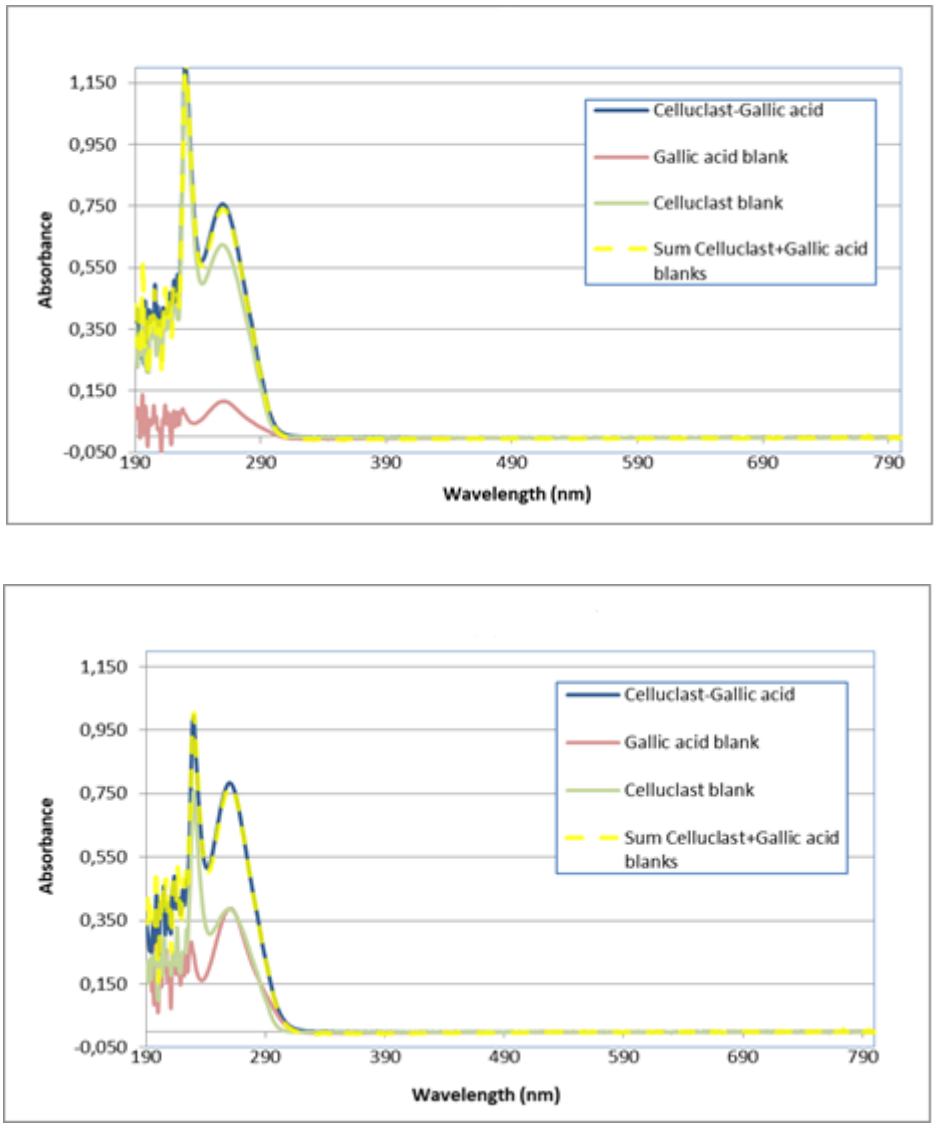


Figure 3.8 *The UV-Vis spectrums of the gallic acid-enzymes model system, at (i) 12.5 U/mL enzymes – 30 μg/mL gallic acid (Top) and (ii) 12.5 U/mL enzymes – 150 μg/mL gallic acid (Bottom), both at a reaction time of 6 hours [**blue line**: the mixture's spectrum, **dotted yellow line**: the calculated sum of the spectrums of each individual component in the mixture]*

of their pure solutions at the same concentrations, in all examined concentration ratios and reaction times (Figures 3.8).

3.3.1.2.2. Investigation of the enzyme denaturation

The decreased Folin responses of the enzymatic extracts in comparison to the pure enzyme mixture could be also explained by the denaturation of the enzymes in the enzymatic extracts. In particular, the enzymes can be denatured, i.e. lose their structure (secondary, tertiary or quaternary) and consequently their activity, due to extreme extraction conditions (e.g. temperature, pH). The conditions that can cause the denaturation of the enzymes depend on the stability characteristic of that specific enzyme. The denaturation can cause the complete or partial degradation of the enzymes, which could affect their level of reaction with the Folin reagent.

The potential changes that the denaturation can cause in Folin absorbances are: (i) an increase of the Folin absorbance, possibly by increasing the availability of certain groups of the enzyme to react with the Folin reagent after the protein chain unfolding, (ii) a decrease of the Folin absorbance, e.g. caused by the degradation or changes in the amino acids and their groups, which can lead to lower levels of reaction of the enzyme with Folin reagent, and finally (iii) no change in the Folin absorbance, as the denaturation of the enzymes can lead to the loss of their structure, however perhaps without changing the reactivity of their building blocks with the Folin reagent.

The above assumptions were tested by performing experiments with the enzyme mixture at several temperatures. According to the manufacturer company, the Celluclast 1.5L enzyme mixture has an optimum activity at 65^{o}C and pH 5, and shows stability at temperatures below 68^{o}C and pH ranges of 3.5-6.5. Furthermore, the Pectinex Ultra Mash enzyme mixture has an optimum activity at 50^{o}C and pH 4, and is stable at temperatures below 68^{o}C and pH ranges of 2.8-6. The conventional and enzymatic extraction of beetroot pomace, described in section 3.3.1.1., were performed at mild temperatures of 45-55^{o}C and at a pH range of 4.5-6; those fall within the stability ranges of the enzymes. Nevertheless, experiments were performed at three different temperatures: (i) 55^{o}C, (ii) 65^{o}C and (iii) 90^{o}C (at maximum exposure time of 6 h).

The experiments performed at 90ºC demonstrated that the enzymes are denaturing by forming a viscous gel, even after a short exposure of 2-5 min. The denaturation was irreversible; i.e. the gel form was retained even after cooling the enzyme mixture to ambient temperatures. The experiments at 55ºC and 65ºC showed that there is no gel formation, indicating no denaturation of the enzymes takes place after the exposure of 6 h. Also, no differences were witnessed before and after the heat treatment in the Folin absorbances; the differences in the Folin values measured were lower than their standard deviations, indicating that these are not significant and are attributed to the repeatability of the analysis (Figure 3.9). Consequently, it is concluded that no change is observed in the Folin absorbance of the enzymes during extraction and that the decreased Folin values of the enzymatic extracts are not due to the denaturation of the enzymes.

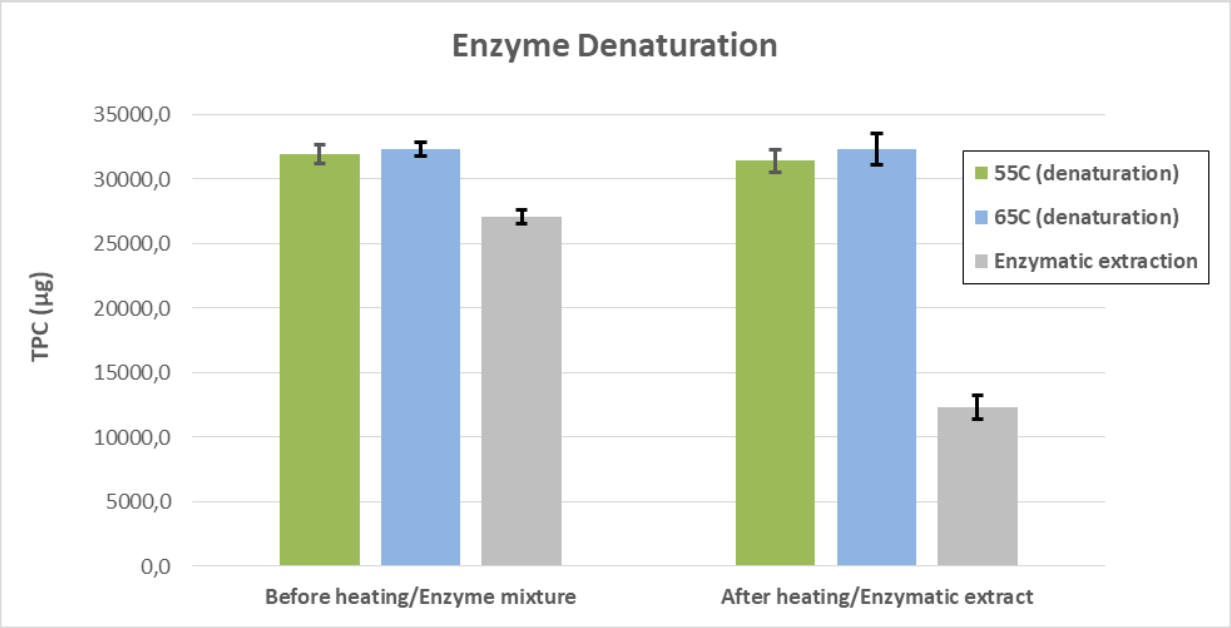


Figure 3.9 *The Folin results of the pure enzyme mixture after the exposure of 6 hours at 55ºC (green) and 65ºC (blue), as compared to the enzymatic extraction (grey).*

3.3.2. Enzymatic hydrolysis & subsequent extraction with intermediate separation of the enzymes from the pulp using washings with DI water

The interactions and the denaturation experiments were not able to explain the occurrence of the decreased Folin absorbances of the enzymatic extracts of beetroot pomace in comparison to the pure enzyme mixture's respective absorbances. The last hypothesis about the reasons for the observed decreased Folin values of the enzymatic extracts would be the adsorption of the enzymes on the beetroot pomace. As the enzymes are hydrolysing the pomace substrate, they tend to attach on the substrate's surface, where they remain attached even at the end of the hydrolysis. This attachment leads to an overconcentration of enzymes on the pulp and to a simultaneous decrease of concentration in the extract's liquid bulk. Due to this strong attachment, the enzymes are subsequently removed along with the solid pomace from the liquid extract during centrifugation. This decrease in the concentration of the enzymes inside the extract's liquid bulk leads to the decreased Folin responses and to the negative results following the value correction of the total enzyme amount.

To examine this hypothesis, experiments were performed which involved the washing of the beetroot pomace with DI water, after the enzymatic hydrolysis step of the lab-scale extraction procedure, as described in section 2.3.5.3., with the aim of retrieving the entire amount of potentially attached enzymes, by measuring the total TPC of all washings (using the Folin assay) and comparing it to the respective value of the pure enzyme mixture. A total TPC value equal to the pure enzyme mixture value could indicate that the entire enzyme would have been washed out of the pulp, while a surplus value would certainly and safely indicate the presence of phenolic compounds.

The results of the total TPC value of all washings for the conventional and enzymatic extraction, and that of the pure enzyme mixture are given in Figure 3.10. The pure enzyme mixture, which is assigned with the green-coloured line, has been included in the graph to serve as the upper level of the total enzyme amount. The graph of the value difference between the sum of all washings of the enzymatic extracts and the pure enzyme mixture is also given in Figure 3.10, along with the sum value of all washings of the conventional extraction, for reasons of comparison.

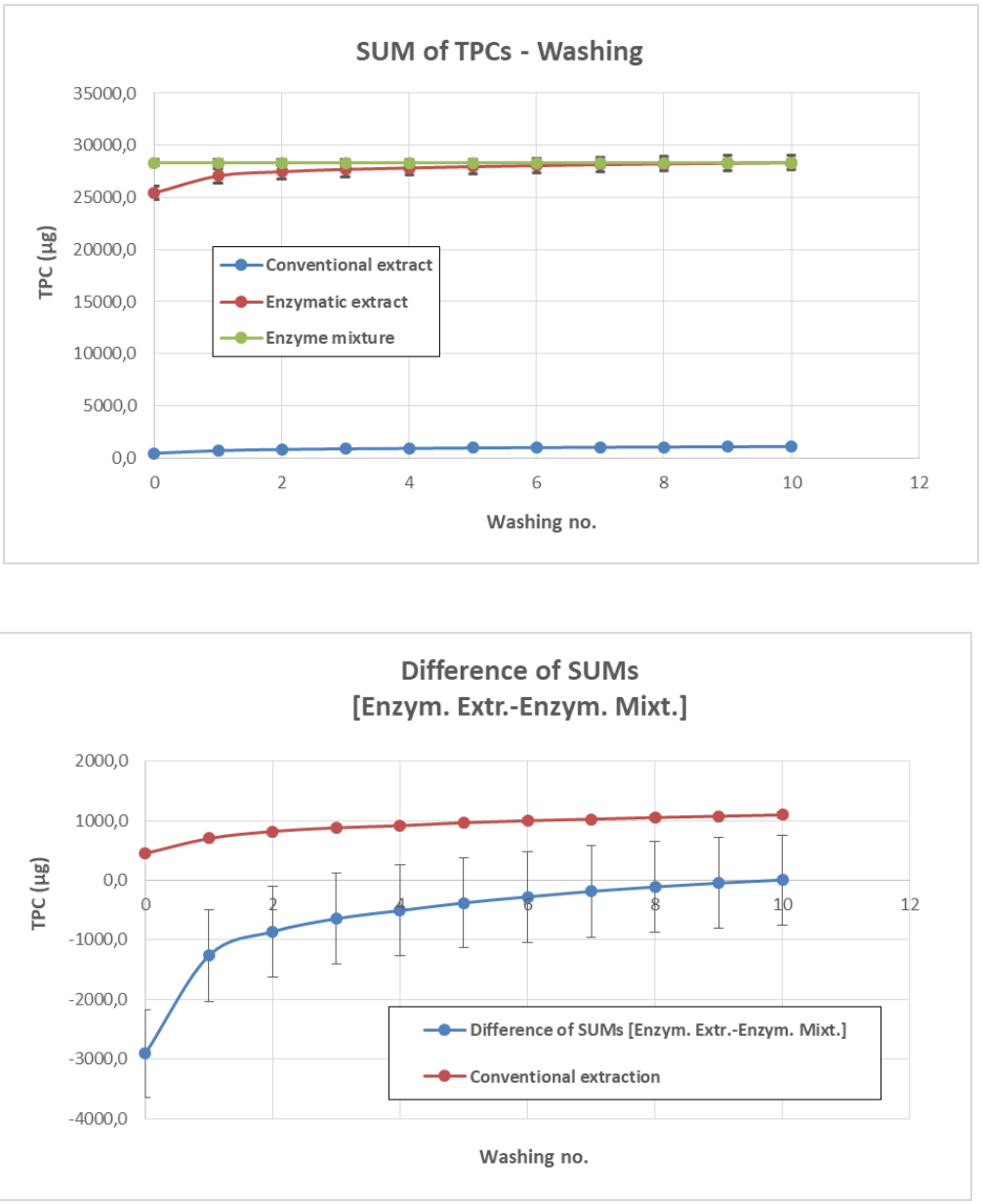


Figure 3.10 *The Folin results of the washing experiments; Top: The sum of the Folin responses of all the washings for the conventional and the enzymatic extraction, and Bottom: The sum of Folin responses of all washings by including the enzyme correction of the enzymatic extracts.*

The graphs demonstrate that the TPC of the hydrolysis medium of the enzymatic extract has a lower value compared to the total amount of enzymes. Similarly, the sum of TPCs of all washings remains lower than the total amount of enzymes almost for all washings, except for the final 10th washing that is equal to the enzyme's total amount. The curve

is practically flattened after the sixth washing, as the percentage change of value is less than 0.40% (equal to 0.37%).

Firstly, it can be observed from the graphs that the TPC value of the hydrolysis medium is equal to 89.7% of the total enzyme amount. In the extraction experiments of section 3.3.1.1., the TPC values of the enzymatic extracts varied between 12-45% of the total enzyme amount. This shows that the loss in TPC value of the extracts was significantly higher in the extraction experiments of the section 3.3.1.1. This significant loss cannot be directly attributed to the addition of ethanol during extraction, as the same loss of value was not observed in the pure enzyme mixture blanks before and after the addition of the same concentration of ethanol. Therefore, it can be assumed that this loss is possibly attributed to the enhanced adsorption of the enzyme on the beetroot pomace substrate in the presence of ethanol.

According to the experimental results, the enzymes are possibly fully washed out of the beetroot pomace only after the final 10^{th} washing. The washing of the enzymes, therefore, takes place in a slow rate and is labour and time intensive. Since the total TPC of all washings for the enzymatic extract never exceeds the total enzyme amount, there is no indication to support the recovery of phenolic compounds from the beetroot pomace by using this methodology.

Further experiments were also performed aiming to investigate whether varied conditions and different batches of beetroot pomace would give more promising results. These experiments involved the conventional and enzymatic extraction of the beetroot pomace with two incorporated washings of the pulp after the hydrolysis step and a final extraction step of the washed pulp, which were performed at three hydrolysis/extraction conditions and using two different batches. The sum of the TPCs was measured for all liquids (hydrolysis medium, two washings, extract). Indicative results for the conventional and the enzymatic extraction at one of the tested conditions are given in Table 3.3. The results demonstrated that only negative values are obtained for the enzymatic extraction, after applying the enzyme correction, at all tested conditions and for both beetroot pomace batches. These results indicate that the enzymes are not fully washed out of the pulp and that there is no safe evidence to confirm the recovery of phenolic compounds from the beetroot pomace.

Table 3.3 *The total Folin values of the conventional and enzymatic extraction of beetroot pomace, using the washing methodology with two washings; Negative values were observed for the enzymatic extraction following the enzyme correction (red boxes) ["B" indicates conventional extraction, "E" indicates enzymatic extraction, numbers 1,2 and 3 indicate repetitions of the extraction]*
(Conditions: T=45°C / pH = 4.5 / treatment time = 360 min / Enzyme activity = 250 U/mL·g)

Condition 3 **(T=45°C / pH = 4.5 / t = 360 min / E = 250 U/mL·g)**

								Concetration		Amounts			
Sample		Volume (mL)	Dilution	A1	A2	A Average	STDEVA	TPC 1 (µg/mL)	TPC 2 (µg/mL)	TPC 1 (µg)	TPC 2 (µg)	Average	STDEVA
1B	Extract	9,4	1/3	0,058	0,059	0,059	0,001	6,9	6,9	64,5	65,3	64,9	0,6
	Hydrolysis medium	6,1	1/10	0,262	0,262	0,262	0,000	84,3	84,3	514,3	514,3	514,3	0,0
	1st wash	5,6	1/10	0,125	0,119	0,122	0,004	43,0	41,2	241,0	230,9	236,0	7,2
	2nd wash	5,4	1/5	0,071	0,060	0,066	0,008	13,4	11,7	72,3	63,4	67,8	6,3
	Enzyme	-	-	-	-	-	-	-	-	-	-	-	-
	Total	-	-	-	-	-	-	-	-	892,1	873,9	883,0	12,9
2B	Extract	9,0	1/3	0,048	0,063	0,056	0,011	6,0	7,3	53,6	65,8	59,7	8,6
	Hydrolysis medium	6,2	1/10	0,24	0,251	0,246	0,008	77,7	81,0	481,6	502,2	491,9	14,5
	1st wash	5,6	1/10	0,118	0,107	0,113	0,008	40,9	37,6	229,2	210,7	220,0	13,1
	2nd wash	5,8	1/5	0,080	0,073	0,077	0,005	14,7	13,7	85,5	79,4	82,5	4,3
	Enzyme	-	-	-	-	-	-	-	-	-	-	-	-
	Total	-	-	-	-	-	-	-	-	850,0	858,0	854,0	5,7
3B	Extract	9,5	1/3	0,051	0,059	0,055	0,006	6,2	6,9	59,1	66,0	62,6	4,9
	Hydrolysis medium	5,8	1/10	0,261	0,262	0,262	0,001	84,0	84,3	487,2	489,0	488,1	1,2
	1st wash	4,8	1/10	0,122	0,132	0,127	0,007	42,1	45,2	202,3	216,7	209,5	10,2
	2nd wash	3,6	1/5	0,106	0,095	0,101	0,008	18,7	17,0	67,2	61,2	64,2	4,2
	Enzyme	-	-	-	-	-	-	-	-	-	-	-	-
	Total	-	-	-	-	-	-	-	-	815,8	832,9	824,4	12,1
1E	Extract	11,0	1/3	0,026	0,008	0,017	0,013	4,0	2,3	43,6	25,7	34,7	12,7
	Hydrolysis medium	9,5	1/200	0,383	0,391	0,387	0,006	2415,1	2463,3	22943,1	23400,9	23172,0	323,7
	1st wash	9,0	1/10	0,346	0,363	0,355	0,012	109,6	114,7	986,5	1032,6	1009,5	32,6
	2nd wash	9,7	1/5	0,032	0,071	0,052	0,028	7,5	13,4	72,9	129,9	101,4	40,3
	Enzyme	-	-	-	-	-	-	-	-	26259,0	29572,3	27915,7	2342,8
	Total	-	-	-	-	-	-	-	-	-2213,0	-4983,2	-3598,1	1958,9
2E	Extract	11,6	1/3	0,000	0,006	0,003	0,004	1,6	2,2	18,8	25,1	21,9	4,4
	Hydrolysis medium	9,5	1/200	0,394	0,417	0,406	0,016	2481,3	2619,9	23572,6	24888,9	24230,7	930,7
	1st wash	9,6	1/10	0,347	0,355	0,351	0,006	109,9	112,3	1055,1	1078,3	1066,7	16,4
	2nd wash	9,5	1/5	0,055	0,063	0,059	0,006	11,0	12,2	104,3	115,7	110,0	8,1
	Enzyme	-	-	-	-	-	-	-	-	27463,9	29632,5	28548,2	1533,5
	Total	-	-	-	-	-	-	-	-	-2713,1	-3524,6	-3118,8	573,8
3E	Extract	11,5	1/3	0,011	0,000	0,006	0,008	2,6	1,6	30,0	18,6	24,3	8,1
	Hydrolysis medium	9,5	1/200	0,428	0,437	0,433	0,006	2686,1	2740,4	25518,4	26033,4	25775,9	364,2
	1st wash	9,8	1/10	0,362	0,389	0,376	0,019	114,4	122,6	1121,4	1201,1	1161,2	56,4
	2nd wash	10,0	1/5	0,040	0,062	0,051	0,016	8,7	12,0	87,2	120,3	103,8	23,4
	Enzyme	-	-	-	-	-	-	-	-	27222,9	29873,5	28548,2	1874,3
	Total	-	-	-	-	-	-	-	-	-465,9	-2500,0	-1483,0	1438,4

The HPLC-DAD analysis was also performed for all liquids of the conventional and enzymatic extraction. The aim was to observe possible remnants of the enzyme mixture in the washings and the final extracts, which could indicate the adsorption of the enzymes on the pulp, and to detect potential compounds from the beetroot pomace. The results have shown that enzyme peaks are still found in the washings and extracts, although in decreasing intensities, and that no beetroot pomace peaks are present, apart from those peaks corresponding entirely to the enzyme (Figure 3.11.).

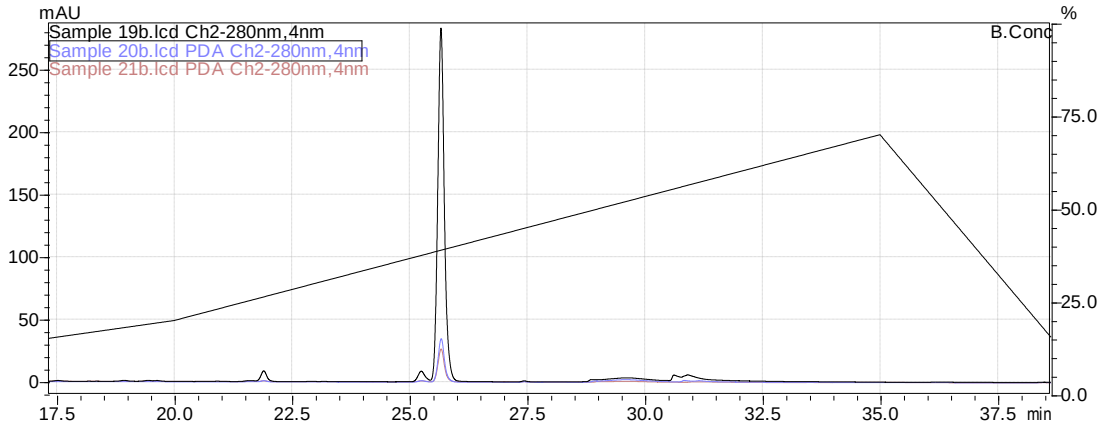


Figure 3.11 *The HPLC-DAD chromatograms of (i) the hydrolysis medium (black; sample 19b), (ii) the 1st washing (purple; sample 20b) and (iii) the 2nd washing (red; sample 21b), of the enzymatic extraction.*

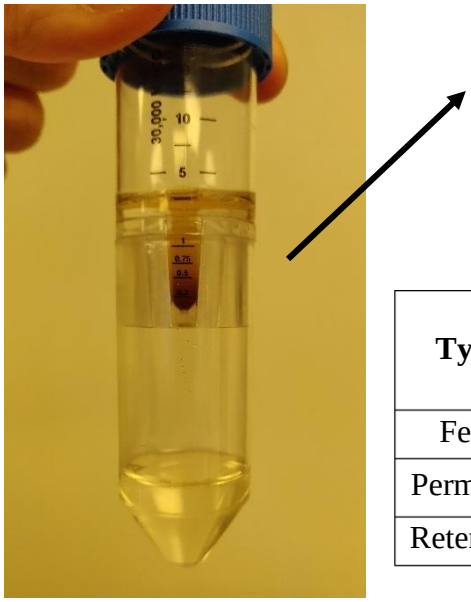
Based on these findings, it is safe to conclude that the decreased Folin absorbances of the enzymatic extracts are attributed to the adsorption of the enzymes on the beetroot pomace. This conclusion is supported by: (i) the lower TPC values of the enzymatic extracts in comparison to the total enzyme amount, (ii) the exclusion of any other hypothesis, i.e. the phenol-protein and carbohydrate-protein interactions and the enzyme denaturation hypothesis, (iii) the presence of enzyme compounds in the washings, which indicates the existence of enzyme remnants in the pulp, and finally (iv) the considerable TPC values of the washing samples that were equal in total to the TPC value of the pure enzyme mixture, indicating the ability to retrieve possibly the entire added enzyme amount from the pulp with the washes.

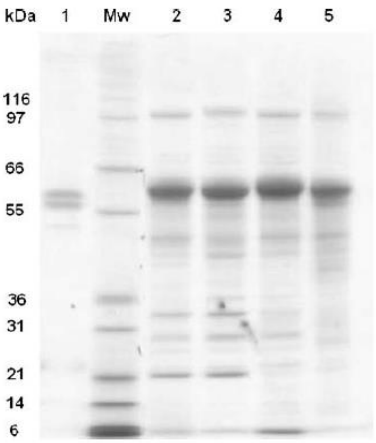
In view of the above, it becomes apparent that the recovery of any potentially present compound in the beetroot pomace is not possible to be achieved by performing the

enzymatic extraction with the aforementioned examined methodologies. A new sample preparation approach needs, therefore, to be introduced prior to analysis, by also considering the adsorption of the enzymes on the pomace.

3.3.3. Investigation of the enzyme removal using UF filtration

An alternative methodology of sample preparation was required to be able to validly measure the phenolic content with the Folin assay. An alternative approach could be to remove the entire remaining enzyme from the liquid phase of the extract, instead of attempting to retrieve the entire adsorbed enzymes from the pulp to the extract.





Type	Areas– Concentrations (after the dilution correction)	Volumes (mL)	Areas - Quantities
Feed	95.632	7	669.424
Permeate	86.872	6	521.232
Retentate	81.967	1	81.967

Figure 3.12 (A) UF filter tube used for the filtration of the enzyme mixture (Left), (B) the retentate of the enzyme mixture's UF filtration (Top Center), (C) the SDS-PAGE analysis of the Celluclast 1.5L (Band 5) (from Sipos et al., 2009) (Top Right) and (D) the peak's areas of the HPLC-DAD analysis before and after the filtration of the enzyme mixture (Bottom Right).

A suitable methodology for removing the enzymes from the liquid phase could be Ultrafiltration (UF). The commercial enzyme in use (Celluclast 1.5 L, Novozymes) has a MW of approx. 55 kDa (Sipos et al., 2009), thus a UF filter of 30 kDa MWCO should be able to retain the enzyme, while allowing the smaller target compounds of the extraction (e.g. phenolic compounds and pigments; normally having a maximum MW of around 1500 Da) to permeate. Moreover, other compounds with molecular weight greater than 30 kDa, such as mainly polysaccharide chains deriving from the enzymatic degradation of cellulose in the beetroot pomace, would be also retained. The UF filter tube which was used for the sample filtration (Vivaspin® 20 Ultrafiltration unit PES 30 kDa, Sartorius AG, Germany) is illustrated in Figure 3.12. The filtration takes place inside a centrifuge (7000 rpm, 2 h, 6 ^{o}C). At the end of the filtration both a retentate and a permeate can be collected.

The UF filtration of the pure enzyme mixture and the enzymatic extract was performed and the collected liquids (feed, permeate and retentate) were analysed with the Folin assay and HPLC-DAD analysis.

HPLC-DAD

The purpose of the HPLC-DAD analysis of the pure enzyme mixture and the enzymatic extract, following their UF filtration, was to understand the nature of the enzyme's compounds which were present in previous HPLC-DAD chromatograms, since it was uncertain whether they were directly attributed to the enzyme itself or to other types of compounds present in the commercial enzyme mixture, such as to impurities from the enzyme's crude production (Rosales-Calderon, Trajano and Duff, 2014).

In the case that the peaks of those compounds would be absent in the HPLC-DAD chromatograms of the pure enzyme mixture's UF permeates, this would mean that those peaks would correspond directly to the retained enzymes, while their presence in the permeate would indicate that those belong to lower MW impurities contained in the enzyme mixture deriving from its crude production.

The HPLC-DAD chromatograms indicated that the enzyme peaks are still present in both the retentate and the permeate of the pure enzyme mixture. In particular, the areas of the peaks were relatively similar in the feed, the permeate and the retentate, thus suggesting non-selective separation and retention from the UF filter (Figure 3.12). Therefore, it can be concluded that those peaks correspond to impurities inside the enzyme mixture and not to the enzyme itself.

Finally, the chromatograms of the UF samples (feed, permeate and retentate) of the enzymatic extracts were identical to the respective chromatograms of the pure enzyme mixture (Figure 3.13), and thus they did not unveil the presence of compounds deriving from the beetroot pomace.

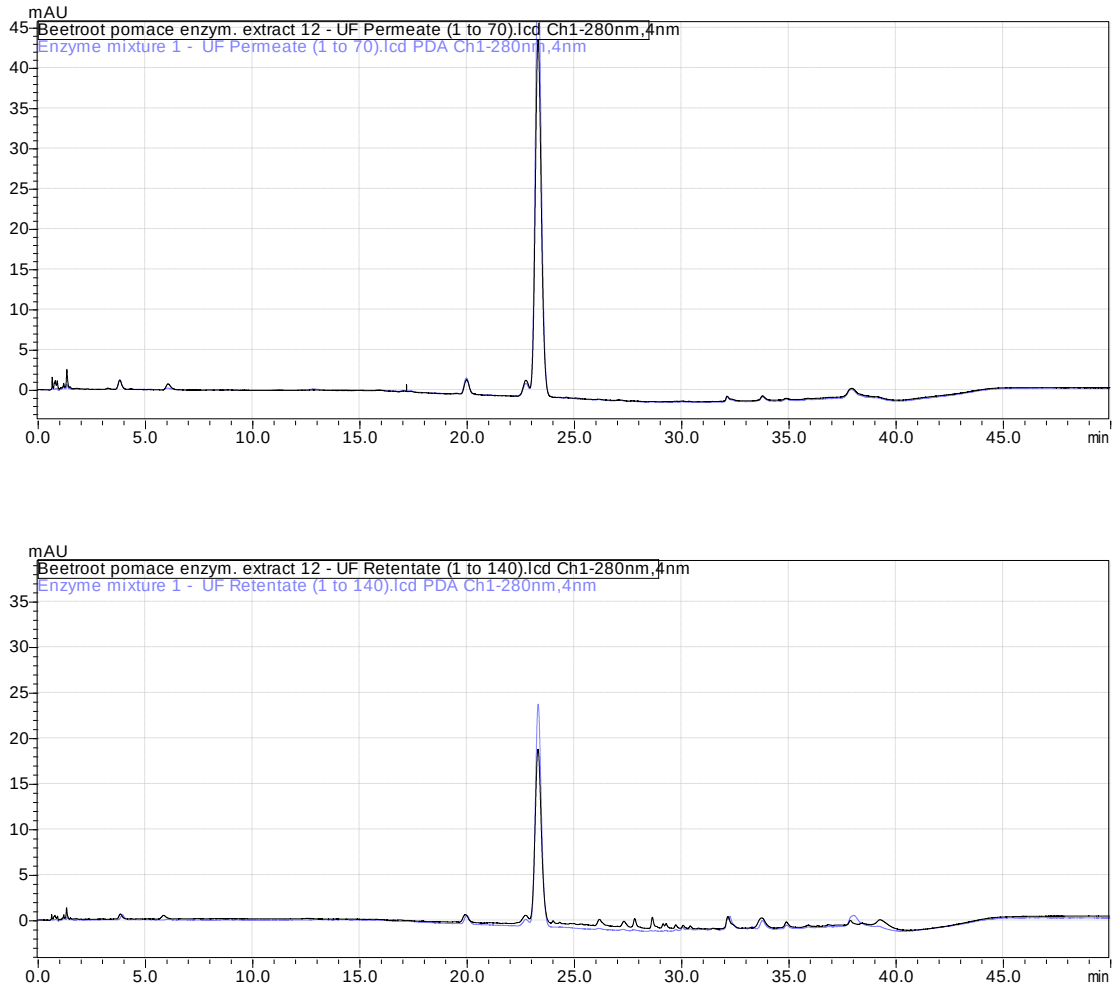


Figure 3.13 The HPLC-DAD chromatograms of the retentate (top) and permeate (bottom) from the UF filtration of the enzymatic extracts (in black), in comparison to the respective chromatograms of the pure enzyme mixture (in purple).

Folin-Ciocalteu assay

The Folin-Ciocalteu assay was initially performed for analysing the feed, permeate and retentate of the pure enzyme mixture, with the aim of providing additional information about the retention of the enzymes. In particular, a stronger Folin absorbance of the retentate in comparison to the permeate would suggest the selective retention of the enzymes from the UF filter, while a relatively similar Folin response would indicate that no selective retention of the enzyme is taking place.

The concentration results of the Folin analysis of the pure enzyme mixture are given in Figure 3.14. As it can be noticed from the graph, the concentration of the retentate was

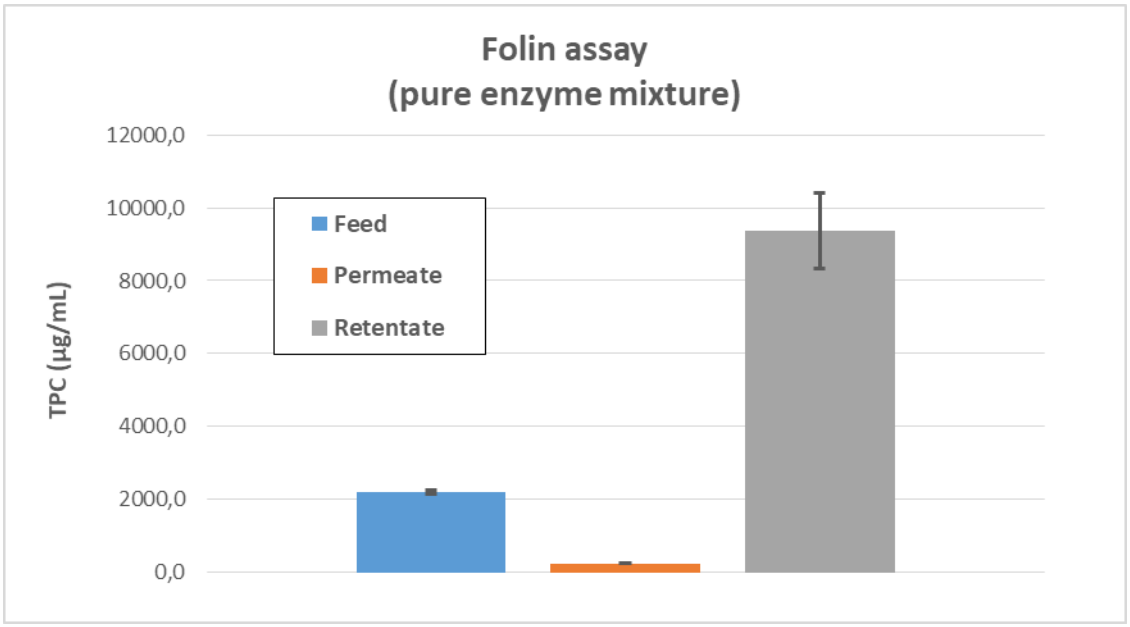


Figure 3.14 The Folin concentration results of the feed, retentate and permeate samples from the UF filtration of the pure enzyme mixture.

Table 3.4 The Folin values of the low MW compounds in the enzymatic extracts, corrected by the values of the low MW compounds in the pure enzyme mixture.

	Enzyme mixture		Enzyme extract		Difference	
Repetition	**TPC average (µg)**	**STEDEVA**	**TPC average (µg)**	**STEDEVA**	**TPC average (µg)**	**STEDEVA**
1	6277,0	86,8	6258,3	85,6	-18,7	105,3
2	6111,8	80,0	6002,8	89,2	-109,1	105,6
3	6170,9	101,2	6174,5	134,7	3,7	152,5

significantly higher than the feed's concentration (i.e. 4.27-fold greater). On the contrary, the permeate concentration was significantly lower than the feed's concentration (i.e. 11,2% less). Based on these results, it can be safely concluded that there is an overconcentration of high MW compounds in the retentate and thus a decrease in concentration in the permeate, attributed most probably to the selective retention of the enzymes by the UF filter.

The response of the permeate of the pure enzyme mixture was low, but considerable, and can be presumably attributed to the low MW impurities, which were determined in the chromatograms of the HPLC-DAD.

The determination of phenolic compounds from the beetroot pomace was also attempted using the Folin assay, by analysing the feed, permeate and retentate of the enzymatic extract. Firstly, the Folin concentration of the low MW compounds was analysed in the permeates of both the enzymatic extract and the pure enzyme mixture, and then their quantities were calculated based on the total feed volume. The difference in the quantity of low MW compounds between the enzymatic extract and the pure enzyme mixture was finally estimated; any surplus in the enzymatic extract would presumably indicate the presence of phenolic compounds recovered from the beetroot pomace.

According to the results, there was no difference observed in the quantities of the low MW compounds between the enzymatic extracts and the pure enzyme mixture (Table 3.4). Therefore, the recovery of phenolic compounds from the beetroot pomace could not be safely confirmed.

Gel Filtration Chromatography

The retention of the enzymes from the UF filter was also investigated by using Gel Filtration Chromatography (GFC) coupled with a Refractive Index Detector (RID). The feed, retentate and permeate of the pure enzyme mixture were analysed by using a size-exclusion column with a nominal fractionation range of MW 10 kDa-100 kDa. The GFC chromatograms clearly showed the presence of two peaks in the feed and retentate of the pure enzyme mixture, however those peaks were absent from the permeate (Figure

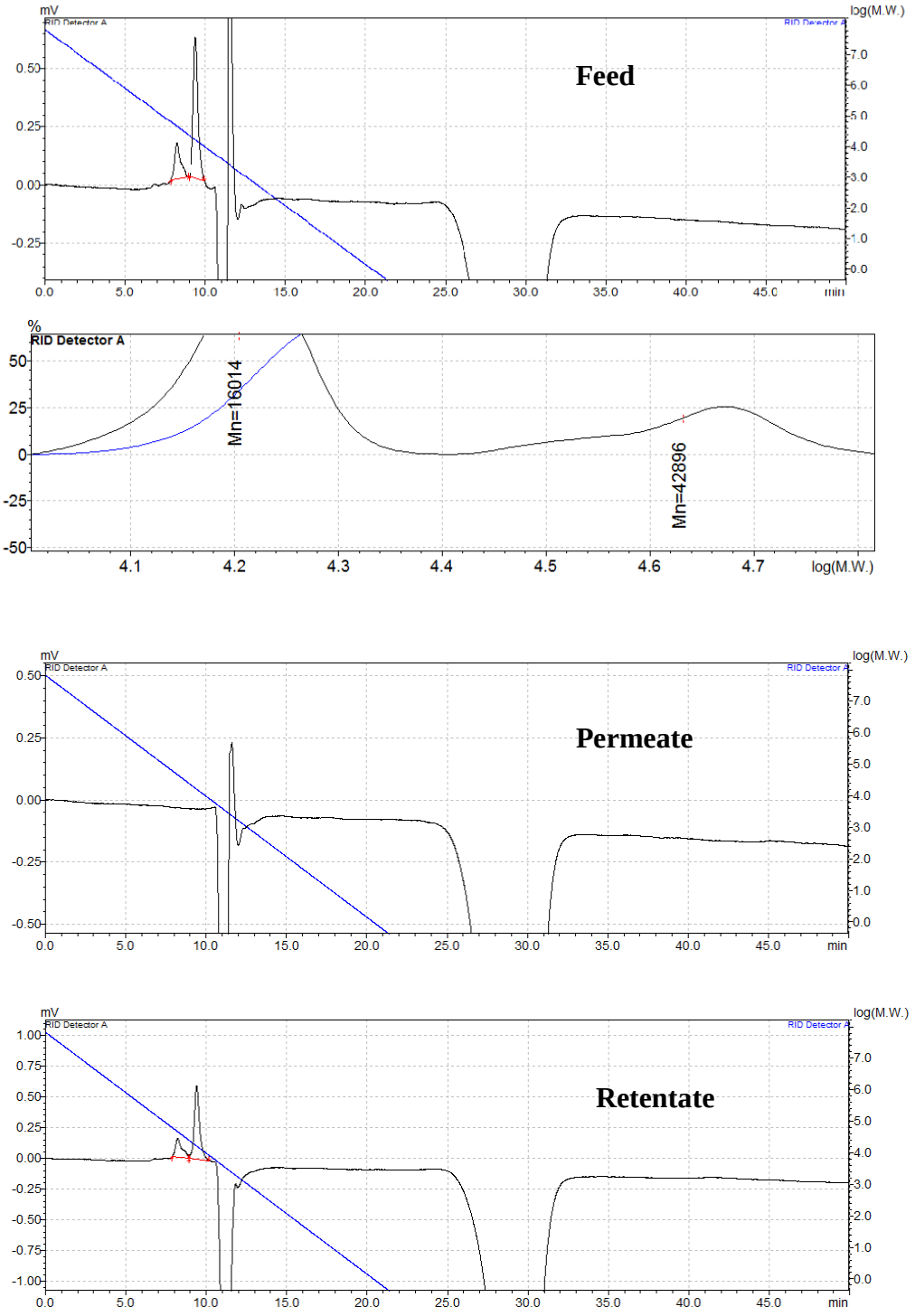


Figure 3.15 The results from the Gel Filtration Chromatography of the feed, retentate and permeate samples of the UF filtration of the pure enzyme mixture. The presence of a peak with a MW of 42 kDa (peak range: 24-67 kDa) in the feed and retentate, but not in the permeate, indicates that this corresponds to the enzyme and validates its retention by the UF filter.

3.15). According to the post-run data analysis of these results, the peaks appeared to have an estimated average MW of 15 kDa (actual peak range: 7-22 kDa) and 42 kDa

(actual peak range: 24-67 kDa). Consequently, the latter peak corresponds most probably to the Celluclast 1.5 L enzymes (cellulase). The presence of this specific peak in both the feed and the retentate in combination with its absence inside the permeate, further validates the successful retention of the enzyme by the UF filters.

Apart from the GFC and Folin analysis, the retention of the enzymes from the UF filter was also confirmed by visual observation. More specifically, the retentate of the pure enzyme mixture appeared to have the same viscous and semi-solid texture with the original enzyme solution in its commercial packaging (Figure 3.12). This semi-solid texture is attributed most probably to the increase in the concentration of the high MW compounds of the enzyme mixture, mainly of the enzymes, in the retentate side, due to their selective retention by the UF filter and the constant removal of solvent in the permeate. Therefore, the above observation is another supportive evidence to the efficient retention of the enzymes by the UF filter.

The retention of the enzyme could have also been effectively evaluated by conducting relevant enzyme activity assays, however they were not performed due to budget restrictions.

3.4. Semi-pilot scale extractions & membrane cascade processing

The lab-scale experiments performed did not lead to the recovery of any compounds in either the conventional or the enzymatic extracts of the beetroot pomace, even though several hydrolysis/extraction conditions and different batches of the beetroot pomace were tested. A possible explanation might be that potentially present compounds may have been recovered, yet at lower concentrations to the detection limits of the analytical methods applied (Folin, HPLC-DAD). In that case, the employed lab-scale extraction methodology would not be sufficient for the determination of such compounds. Semi-pilot scale experiments are, therefore, required to investigate this hypothesis.

The experiments described in this section involve the semi-pilot scale conventional and enzymatic extraction of the beetroot pomace, followed by the concentration of the extract using the UF/NF membrane cascade processing and subsequently the rotary

evaporation of the collected membrane streams (permeate/retentate). In this way, potentially recovered compounds may increase their concentrations up to levels that would exceed the detection limits of the applied analytical techniques.

3.4.1. Preliminary results

The semi-pilot scale conventional and enzymatic extractions of the beetroot pomace were performed and, then, the extracts were subjected to membrane cascade processing using three UF (Helix 5mm, HFS, UF075) and three NF (dNF80, dNF40, dNF20) filtration modules, predominately for the fractionation and the concentration of the potentially recovered compounds. In the retentate streams, the concentration of the rejected compounds is achieved due to the gradual removal of solvent to the permeate side. All streams collected from the NF processing are subsequently subjected to rotary evaporation for further concentration of the potentially extracted compounds and are, then, analysed with HPLC-DAD and LC-MS chromatographic methods.

The pure enzyme mixture was also subjected to the aforementioned processing methodology, to serve as a blank for reasons of comparison to the processing of enzymatic extract.

HPLC-DAD

Representative HPLC-DAD chromatograms from the membrane processing streams of the conventional and enzymatic extracts are given in Figures 3.16 & 3.17. In general, the chromatograms of the enzymatic extraction and of the respective pure enzyme mixture samples appeared to be almost identical. There was only one peak, eluting at a retention time of 6 min, which could be clearly attributed to the beetroot pomace; this peak was also observed in the raw beetroot extracts and the beetroot juice samples, but its identification was not feasible.

Apart from this beetroot pomace peak, few early-eluting peaks were also observed and appeared to have significantly higher intensities in almost all the chromatograms of the

NF streams of the enzymatic extracts in comparison to the pure enzyme mixture. As a consequence, it is safe to conclude that those peaks correspond most probably to

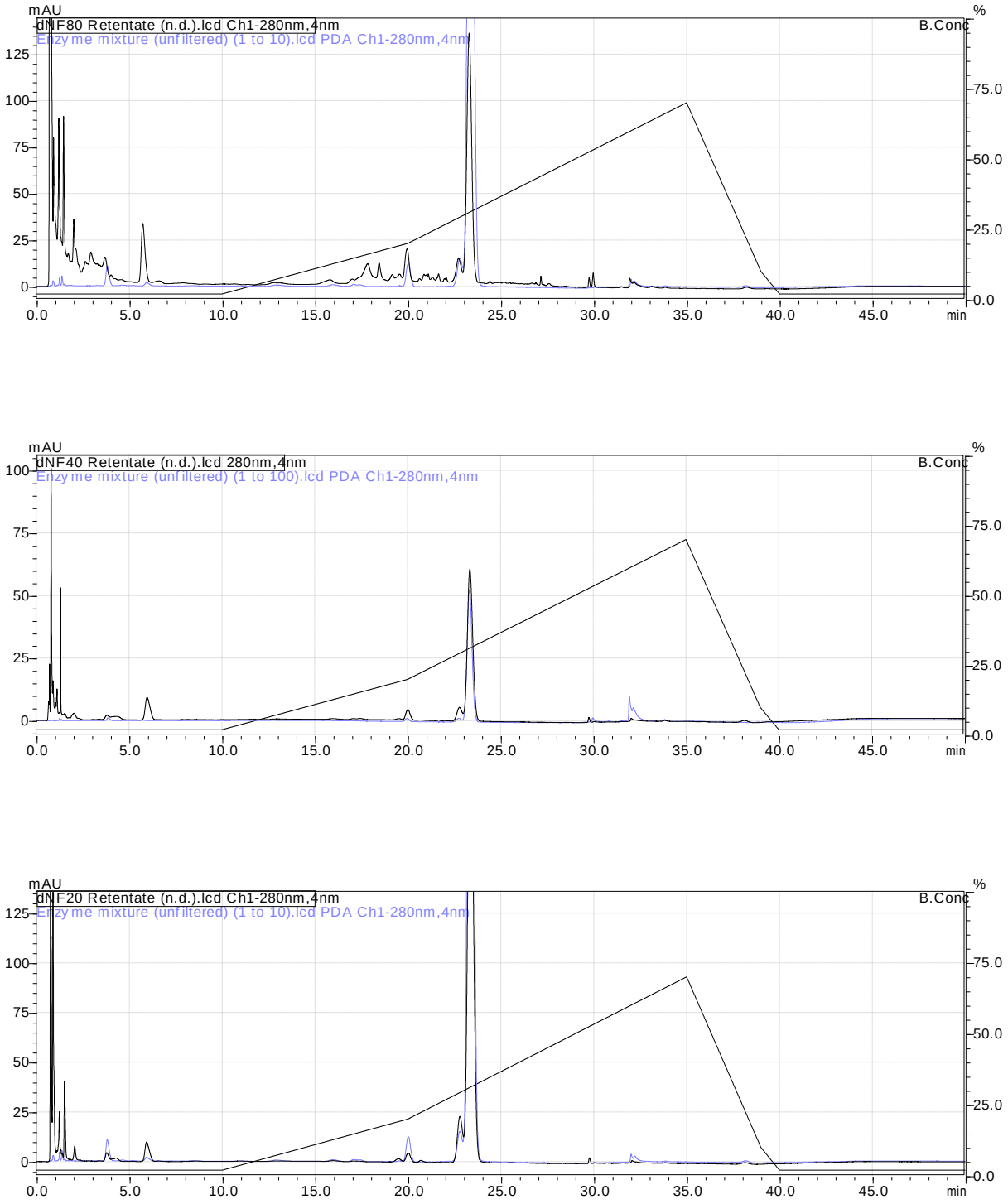


Figure 3.16 The HPLC-DAD chromatograms of the dNF80 retentate (top), dNF40 retentate (middle), dNF20 retentate (bottom) streams of the enzymatic extraction at 280 nm, in comparison to the pure enzyme mixture.

compounds of the beetroot pomace, however their actual evaluation was impossible due to their inadequate separation.

All the remaining peaks found in the NF streams of the enzymatic extracts were entirely associated to the pure enzyme mixture. No peaks of betalain pigments were observed in any of the chromatograms (Figure 3.17).

In regard to the conventional extraction, the chromatograms did not reveal the presence of any beetroot pomace compound, except for few early-eluting peaks, the evaluation of which was impossible due to insufficient separation.

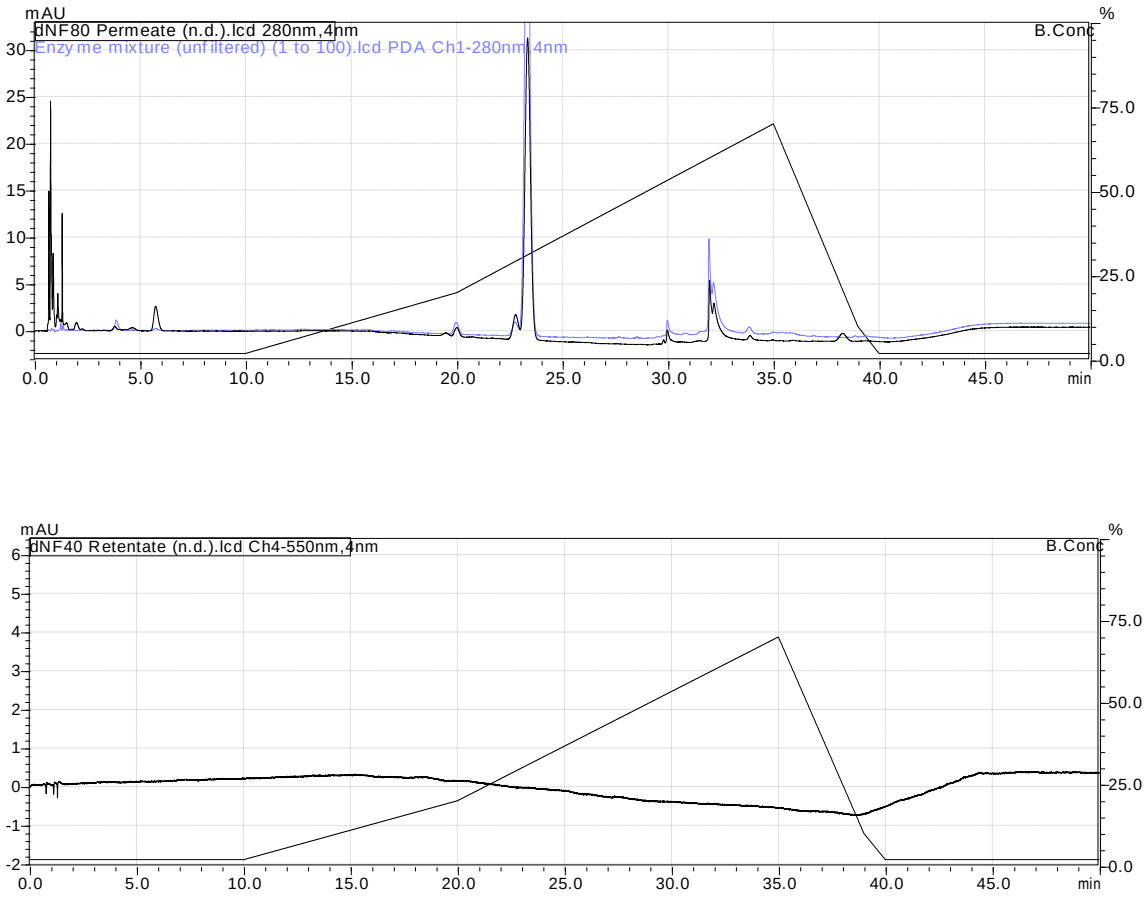


Figure 3.17 The HPLC-DAD chromatograms of the dNF80 permeate (top) at 280 nm, in comparison to the pure enzyme mixture, and of the dNF40 retentate (bottom) at 550 nm.

LC-MS

The LC-MS chromatograms did not unveil the presence of compounds from the beetroot pomace inside the NF processing streams of the enzymatic extracts. In particular, the Total Ion Current (TIC) chromatograms (in both the positive and negative modes) were found to be almost identical for the enzymatic extract and the pure enzyme mixture; no major differences could be seen. Those chromatograms for both samples were practically flat, with only few early-eluting peaks being able to be observed. Some representative TIC chromatograms from the analysis of the NF streams of the enzymatic extracts and the pure enzyme mixture are given in Figure 3.18.

The "manual scan" of the LC-MS data was also performed in the hope of detecting ions that would correspond to compounds only present in the NF streams of the enzymatic extracts, but not in the pure enzyme mixture. The manual scan was performed with additional attention drawn towards the early parts of the chromatograms, as greater intensities of ions were noticed in those parts and due to the fact that early-eluting peaks

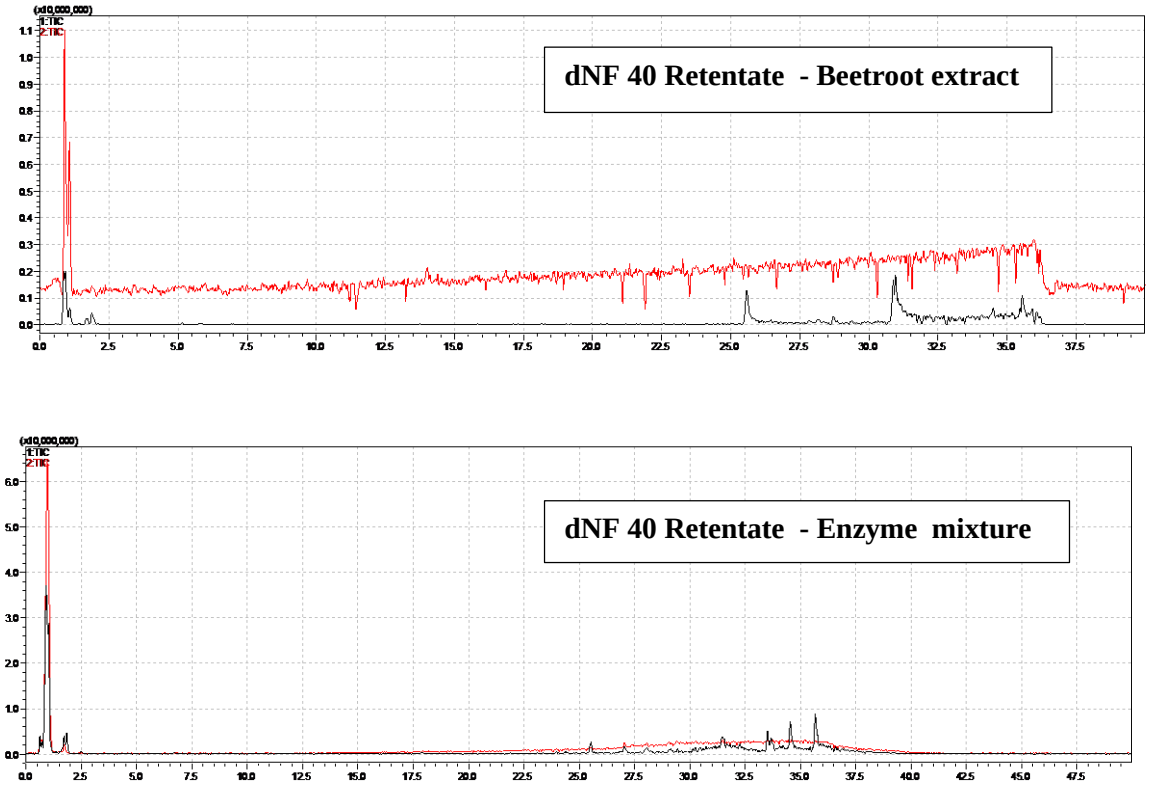


Figure 3.18 The TIC chromatograms from the LC-MS analysis of the dNF40 retentate stream of the enzymatic beetroot pomace extraction (top) and of the same stream of the pure enzyme mixture (bottom).

corresponding most probably to the beetroot pomace had been previously seen in the HPLC-DAD chromatograms. However, no ions were detected in any part of those graphs, that could be associated to the beetroot pomace.

Visual observation

The visual observation of the NF streams of the enzymatic and conventional extracts and of the pure enzyme mixture led to an interesting conclusion. A colour change was observed between the different retentate streams of both the conventional and enzymatic extracts of the beetroot pomace, which was not, however, seen in the case of the pure enzyme mixture (Figure 3.19 & 3.20). The dNF80 retentate streams of the beetroot extracts displayed a characteristic green colour, whereas the colour of the dNF40 and dNF20 retentate streams was blue. As the colour change was apparent only for the beetroot pomace extracts, it can be assumed that this is most probably due to pigment compounds, which were extracted from the pomace.

Based on the previous observation, it could be hypothesised that the beetroot extract contains a mixture of pigments, with each pigment being responsible for a specific colour; the colour of the extract is the result of the contribution of all pigments in the mixture and hence depends on the types of pigments and their concentration inside the extract. As those pigments have most probably different MWs, they are separated accordingly in the various streams of the membrane cascade processing. As a consequence, each stream contains those pigments in different concentrations, which leads to the diverse colouration of those streams. Finally, the colouration appears to be more intense in the retentate streams, as those contain the rejected pigments in increased concentrations, due to the removal of solvent from the feed to the permeate.

It should be noted that, in the semi-pilot scale experiments, concentration of the extracts is taking place in two stages: (i) the membrane processing; where the retentates are concentrated 4-6 times, i.e. the maximum VRF that could be achieved based on the membrane system configuration in use, and (ii) the rotary evaporation; where every stream of the NF processing is concentrated 4-7 times. In particular, the rotary

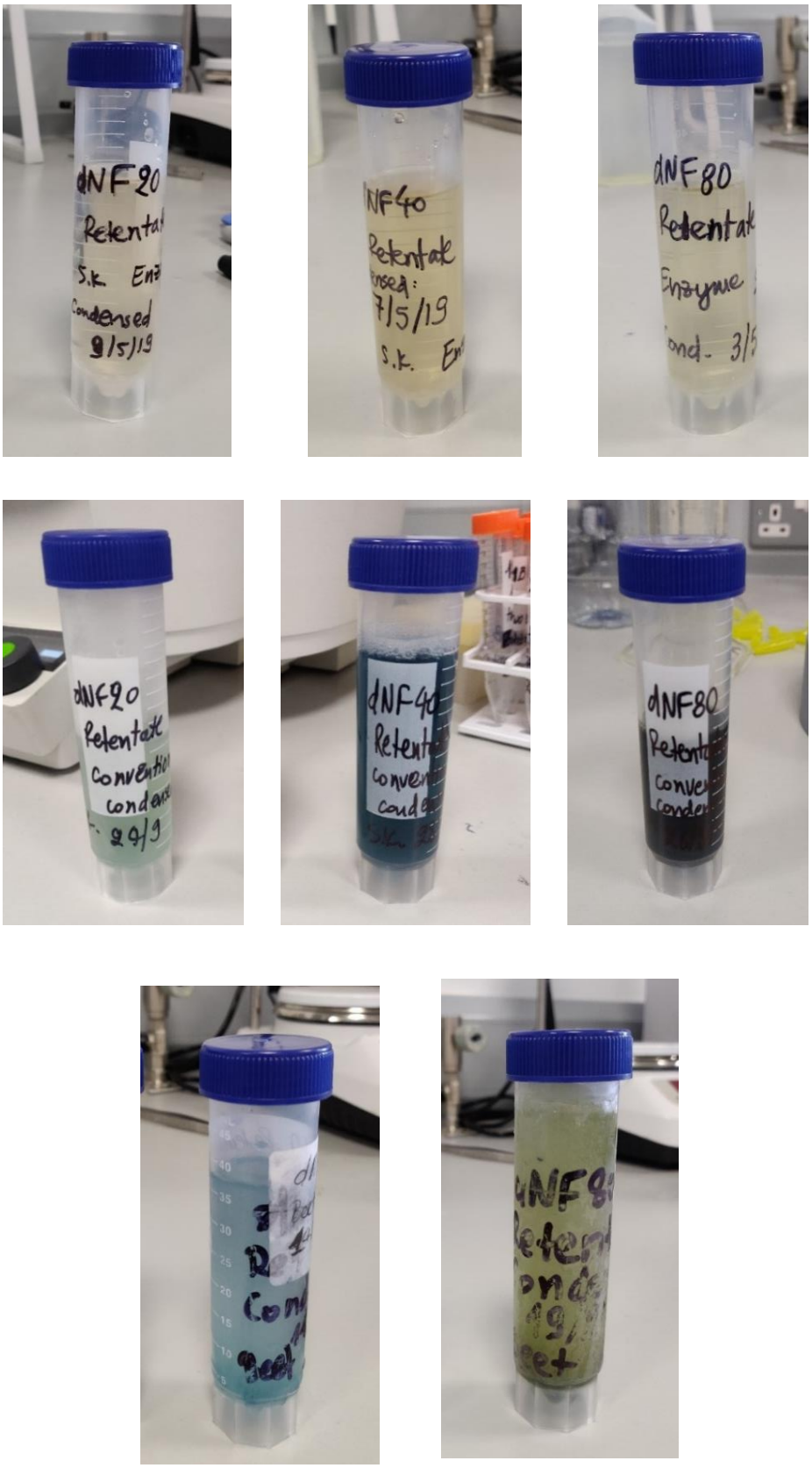


Figure 3.19 The NF retentate streams of the pure enzyme mixture (1st row), the conventional extraction (2nd row) and the enzymatic extraction (3rd row), after the final evaporation (left: dNF 20, middle: dNF40, right: dNF80).

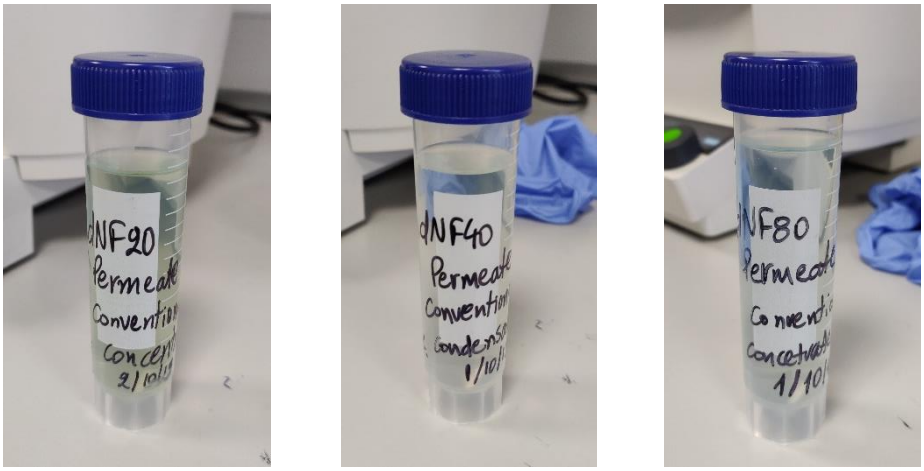


Figure 3.20 The NF permeate streams of the conventional extraction, after the final evaporation (left: dNF 20, middle: dNF40, right: dNF80).

evaporation was performed until almost complete dryness in the case of the conventional extraction, achieving concentrations of 30-50 times. Overall, the rejected compounds from the NF processing of the enzymatic and conventional extracts were concentrated up to 12-24 times and 65-123 times, respectively.

Despite these exceptionally high concentrations achieved, the HPLC-DAD and LC-MS chromatograms of the NF streams of the conventional and enzymatic extracts failed to unveil the presence of pigments that would demonstrate characteristic strong absorbances in the visible spectral region, or of any other type of compound originating from the beetroot pomace (Figure 3.17). In view of the above, the colour change of those NF streams could not be safely attributed to the recovery of pigments out of the beetroot pomace.

Folin-Ciocalteu assay

The Folin-Ciocalteu assay was not employed for the analysis of the membrane streams of the beetroot extract. As it is a non-selective and of low qualitative value analytical method, it cannot be solely used for the evaluation of the NF processing streams.

In particular, the membrane cascade is expected to fractionate both phenolic compounds and compounds of the commercial enzyme mixture in different permeate and retentate streams. The Folin analysis of each stream will give a "total" absorbance value, which will correspond to both phenolic and enzyme compounds; it will be impossible to technically assess which part of the Folin response corresponds to the phenolic compounds, unless a correction is made with the respective membrane processing streams of the pure enzyme mixture at identical process conditions.

However, the performance of membrane cascades of the pure commercial enzyme mixture is expected to increase the volume and time of experiments in a way that is impossible to perform the optimisation of the membrane cascade process (which is among the aims of the project). Furthermore, the comparison of the membrane cascade processing streams of beetroot pomace extracts to the respective of the pure enzyme mixture blank might not be completely accurate, as the fouling of the membranes will be more intense in the case of beetroot pomace extract, which is likely to change the separation result.

In view of the above, the Folin-Ciocalteu method cannot be the single, and thus the principal, analytical method used for the determination of phenolic compounds in the membrane streams of the enzymatic beetroot pomace extracts.

3.4.2. Investigation of the separation of the early-eluting peaks

The chromatograms of extracts obtained during the semi-pilot scale experiments showed the presence of few early-eluting peaks deriving from the beetroot pomace; however, these remained unseparated according to the applied chromatographic method and, hence, their evaluation was impossible. In this chapter, the separation of those peaks is attempted by alternating the chromatographic conditions

The chromatographic analysis was performed so far by using the "Shim-Pack XR-ODS" column (L. 50 x I.D. 3 mm, C18, particle size 2.2 μm; Shimadzu Europa GmbH, UK). This is a reversed-phase column and, as such, it is considered suitable for separating the target compounds which, in these extractions, are mainly phenolic compounds and

pigments (betanin, vulgaxanthin I etc.) (Koubaier *et al.*, 2014; Slatnar *et al.*, 2015), potentially present in the beetroot plant. The separation efficiency of the column was further validated through running an analysis of standard solutions of several phenolic compounds (i.e. gallic acid, vanillic acid, caffeic acid, rutin) and of the betanin pigment (Figure 3.21). As shown in the graph, the column is able to efficiently retain all the phenolic compounds tested, as well as betanin.

The efficient separation of various other phenolic compounds by using this specific column has also been supported in published papers (Wang et al., 2014; Bataglion et al., 2015). As the column was shown to retain efficiently the standard compounds, it is concluded that it does not demonstrate any retention or separation performance defect.

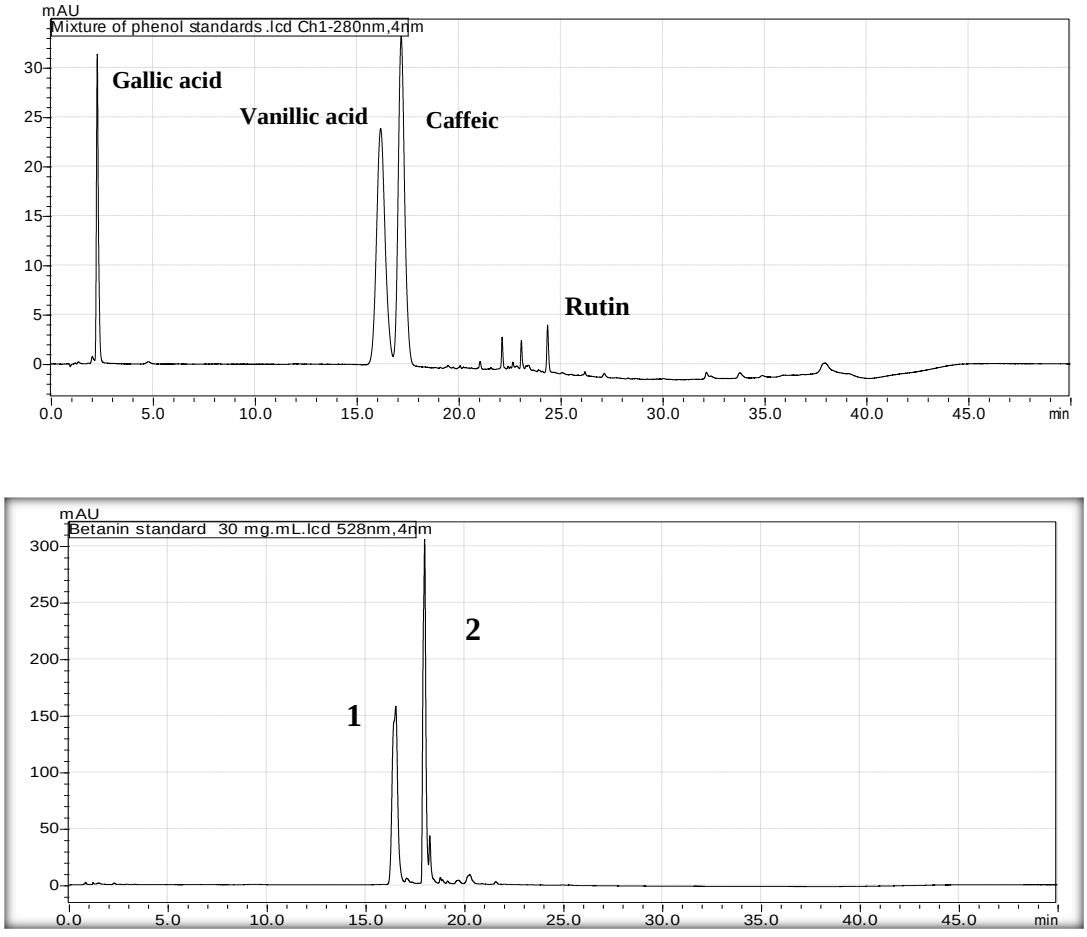


Figure 3.21 The HPLC-DAD chromatograms of several phenolic compounds (top) and of the betanin standard (bottom; 1-betanin, 2-isobetanin), which shows that the column achieves the efficient retention of those compounds.

It must be also noted that the chromatographic analysis was run with the maximum possible concentration of the aqueous component in the mobile phase during the initial conditions, which ensures the maximum retention of compounds with the specific column in use. Therefore, the aforementioned evidence leads safely to the conclusion that the observed early-eluting peaks of the HPLC-DAD chromatograms are not attributed to phenolic compounds or pigments, since they should have otherwise been retained by the column. The identity of the early-eluting peaks still remained unknown.

In order to achieve the efficient separation of the early-eluting peaks that would allow their safe evaluation, the chromatographic analysis was examined using different method approaches, such as the use of mobile phases adjusted to different pH and the use of columns with different chemistry of stationary phase and different dimensions. In particular, the new chromatographic conditions are synopsised in Table 3.5., and were as follows: (i) the use of the same "Shim-Pack XR-ODS" column in combination with a mobile phase adjusted at pH 7 (instead of pH 2); this is expected to affect the ionisation state of both the compounds and the stationary phase, which may improve the retention of the early-eluting compounds in the column, (ii) the use of a reversed-phase column with a different chemistry of stationary phase, more specifically of the "Agilent Zorbax SB-Phenyl" (L. 100 x I.D. 2.1 mm, particle size 3.5 μm, Agilent Inc., USA) column; this column demonstrates improved retention selectivity to hydrophilic and hydrophobic compounds which possess phenyl groups and therefore it might be able to separate the early-eluting compounds, in the case that they contain phenyl rings, (iii) the use of a cation exchange column, in particular of the "Agilent Hi-Plex H" (L. 300 x I.D. 7.7 mm, particle size 8.8 μm, Agilent Inc., USA) column; this column demonstrates improved retention selectivity to cationic compounds and, thus, it could be able to retain the early-eluting compounds, in the case these have a positive charge, and finally (iv) the use of a reversed-phase column with larger dimensions, in particular of the "Phenomenex Gemini 5u C18" (L. 250 x I.D. 4.6 mm, particle size 5 μm, Phenomenex Inc., USA) column; the larger size of the column, mainly its increased length, could improve the retention of the early-eluting peaks. As the identity of the early-eluting peaks is unknown, this investigation cannot be based on choosing a particular pre-selected chromatographic condition and mechanism for the HPLC-DAD analysis, but it must be performed using various conditions, as it was done in the "trial-and-error" experiments which are described above.

Table 3.5 The HPLC-DAD chromatographic conditions (i.e. columns, mobile phases) used for the investigation of the separation of the early eluting peaks in the chromatograms of the beetroot pomace extracts.

Condition	Column	Mobile phase
1	**Shim-Pack XR-ODS (50 x 3 mm, 2.2 µm)**	**Eluent A**: H_2O **[pH 7]** **Eluent B**: ACN / Meth / H_2O 0.8 : 0.1 : 0.1 **[pH 7]**
2	**Agilent Zorbax SB-Phenyl (100 x 2.1 mm, 3.5 µm)**	**Eluent A**: H_2O (+ 0.1% formic acid) **[pH 2]** **Eluent B**: ACN / Meth / H_2O 0.8 : 0.1 : 0.1 (+ 0.1% formic acid) **[pH 2]**
3	**Agilent Hi-Plex H (300 x 7.7 mm, 8 µm)**	**Eluent**: H_2O **[pH 7]**
4	**Phenomenex Gemini 5u C18 (250 x 4.6 mm, 5µm)**	**Eluent A**: H_2O (+ 0.1% formic acid) **[pH 2]** **Eluent B**: ACN / Meth / H_2O 0.8 : 0.1 : 0.1 (+ 0.1% formic acid) **[pH 2]**

According to the chromatograms obtained from these tests, there was no apparent improvement in the efficiency of the separation of the early-eluting peaks (Figure 3.22). In particular:

- the "Shim-Pack XR-ODS" column combined with a mobile phase adjusted at pH 7 and the "Agilent Zorbax SB-Phenyl" column did not have any retention effect on the early-eluting peaks; these continued to be eluted unseparated in the early parts of the chromatograms.

- The early-eluting peaks were not observed with the use of the "Agilent Hi-Plex H" column, and only one broad peak was able to be spotted approximately 2 min later in the chromatogram, indicating possibly co-elution of those peaks and, generally, a bad retention performance from the column.

- In addition, the enzyme peaks interestingly disappeared from the main parts of the chromatograms at all the above chromatographic conditions involving mobile phases of pH 7, possibly due to the change in the ionisation state of the compounds, which affects their retention.

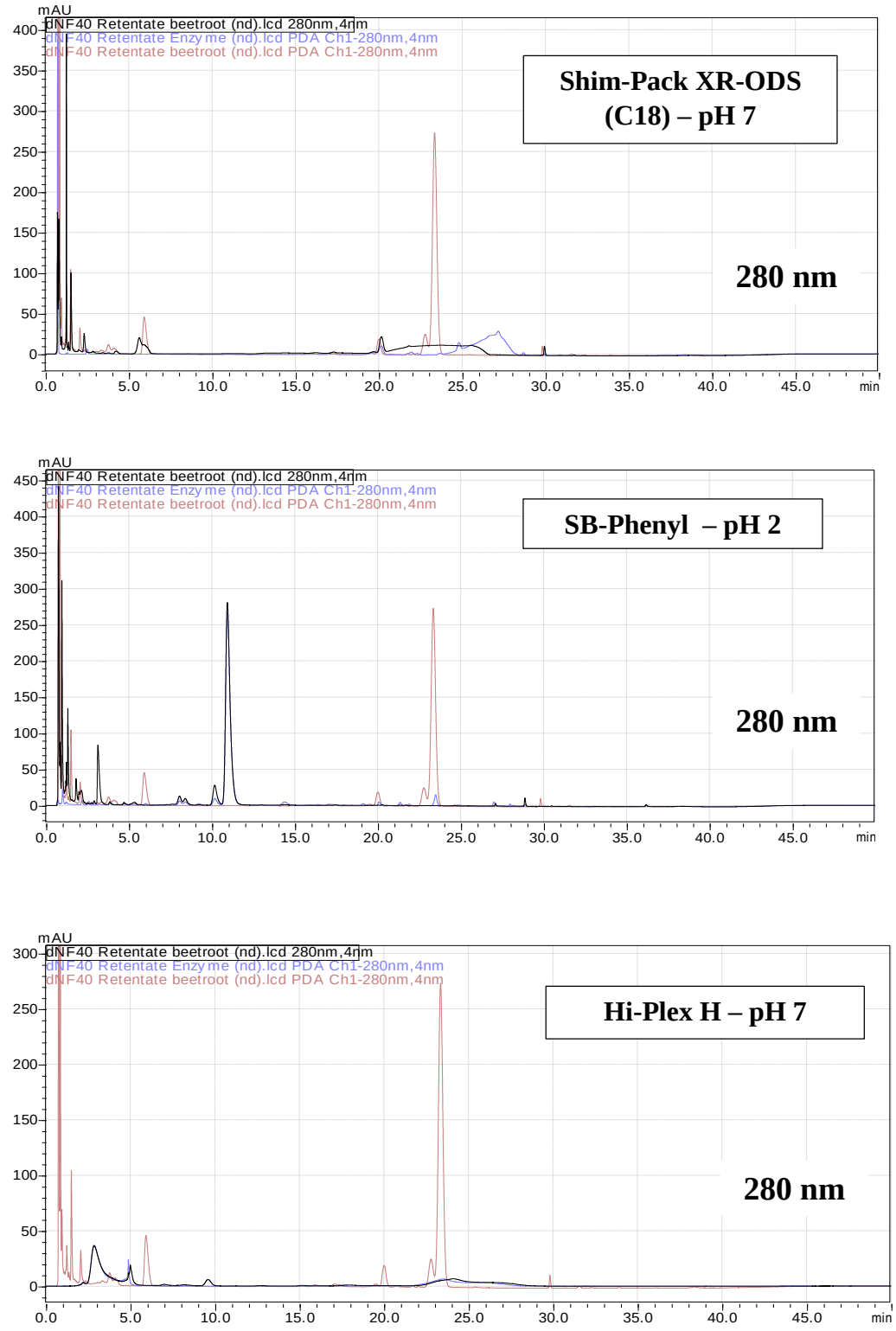


Figure 3.22 The HPLC-DAD chromatograms of the different chromatographic conditions used for the separation of the early eluting peaks; (i) Shim-Pack XR-ODS (C18) pH 7 (Top), (ii) SB-Phenyl pH 2 (Middle), (iii) Hi-Plex H pH 7 (Bottom)

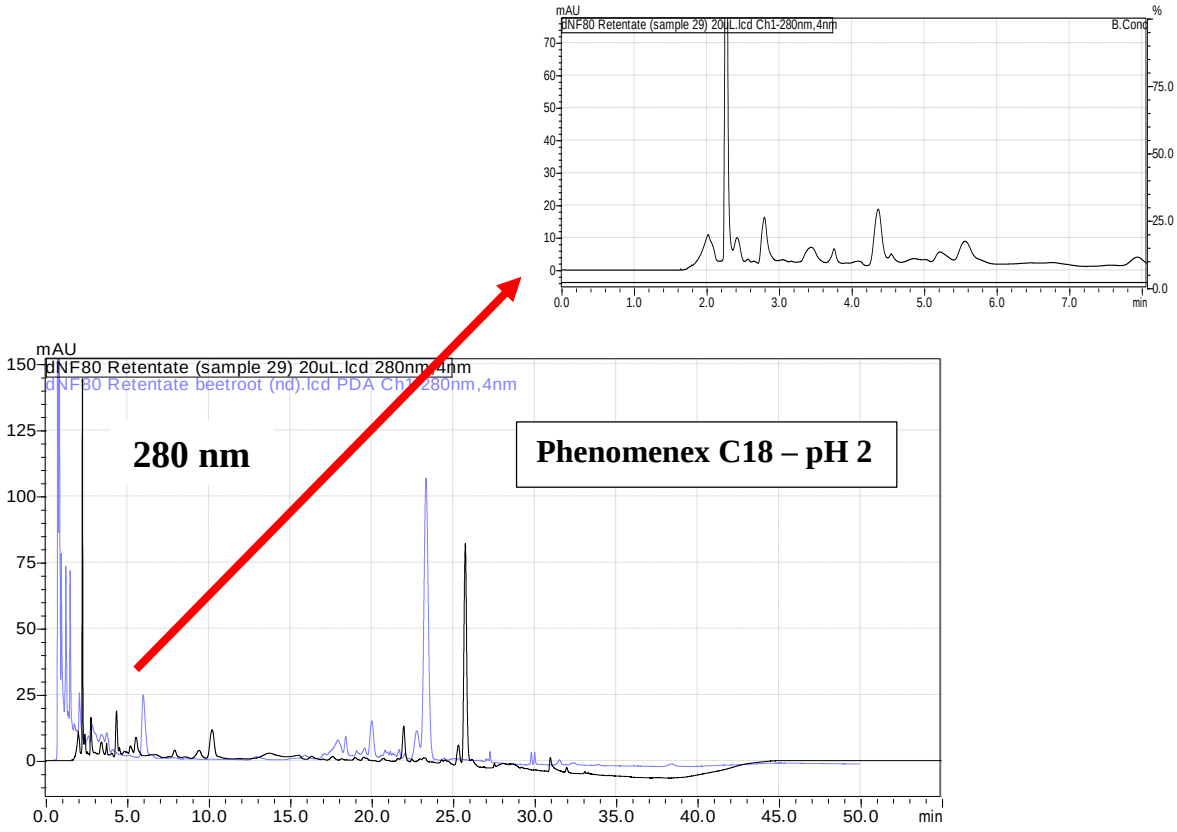


Figure 3.23 The HPLC-DAD chromatogram of the Phenomenex C18 column with mobile phase adjusted to pH 2, used for the separation of the early eluting peaks.

- It appears that the "Phenomenex Gemini 5u C18" column demonstrates the best separation performance; however this separation was not sufficient and did not show the presence of sharp and intense peaks (Figure 3.23).

It seems that those early-eluting peaks appear to belong presumably to polar compounds, as none of the aforementioned conditions were able to retain them. Consequently, the use of an HILIC column would be possibly a more appropriate chromatographic approach for the efficient separation of those early-eluting peaks, since it combines the effective retention of polar compounds using aqueous based mobile phases.

3.5. Conventional extraction with pure organic solvents

The described semi-pilot scale extractions of the beetroot pomace were not able to confirm the presence of extracted compounds by using standard chromatographic techniques. Different extraction and analytical approaches were investigated in order to validate the presence of recovered compounds, but they were all proven unsuccessful. The lack of extracted beetroot pomace compounds was apparent for both the conventional and enzymatic extractions.

A possible explanation for the absence of recoverable compounds might be the selection of the extraction solvent. In particular, the extraction solvent used in these experiments was an aqueous-based solvent consisted of 30% v/v ethanol. However, since the beetroot pomace is the remaining solid mass from the juice production of raw beetroot, and juice is practically an aqueous matrix, it is very likely that the majority of hydrophilic compounds would have migrated from raw beetroot to the beetroot juice, thus leaving the pomace depleted in those compounds. In that case, the use of an aqueous-based solvent would not be expected to extract any hydrophilic compound in sufficiently high yields.

Generally, the target compounds of the extraction are betalains and phenolic compounds; betalains are polar (i.e. hydrophilic) in nature (Gonalves *et al.*, 2012; Tesoriere *et al.*, 2013), whereas phenolic compounds demonstrate varying polarities with some being non-polar (Noubigh, Abderrabba and Provost, 2009), and other more polar in nature (Ray and Das, 1996; Mojzer *et al.*, 2016).

According to the literature, methanol is the most suitable solvent for the conventional extraction of betalains (Kujala, Loponen and Pihlaja, 2001). Regarding the phenolic compounds, the non-polar types are commonly optimally extracted using pure organic solvents; methanol is more suitable for low MW polyphenols, while acetone for higher MW flavonols. Polar phenolic compounds are better extracted using aqueous solutions of organic solvents (Mojzer *et al.*, 2016).

As the presence of betalain pigments was validated inside raw beetroot and the beetroot juice, the absence of betalain and phenolic compounds in the beetroot pomace might

only be explained by the following scenarios: (i) either the pomace was depleted in those compounds, due to their entire transfer from the fruit to the juice, or (ii) the inefficiency of the aqueous-based solvent to extract those compounds, in the event that these are indeed present in the pomace in detectable amounts.

The selection of the aqueous-based solvent was made on the basis of certain processing limitations, which are associated with (i) the enzyme hydrolysis and (ii) the membrane processing of the extracts. More particularly:

(1) the first limitation is related to the enzyme hydrolysis: The majority of the enzymes exhibit their strong activities under aqueous environments, and are often denatured in organic solvents (Bisswanger, 2014; Labuda *et al.*, 2018). Hence, the combination of the enzymes with organic solvents is often forbidding. To test their compatibility with such solvents, the enzymes in use were attempted to be diluted in pure methanol and ethanol. Nevertheless, those enzymes remained undiluted, forming a semi-solid precipitate, in the case of ethanol, and a highly turbid and almost dense solution in the case of methanol (Figure 3.24). In contrast, the enzymes were perfectly diluted in water, forming a transparent yellow solution (Figure 3.24). Therefore, the enzymes in use are generally soluble in water, whereas they appear to be incompatible/insoluble in organic solvents.

(2) the second limitation is related to the compatibility of the solvent with the membrane material: In specific, the materials of the filtration modules are: (i) modified Polyethersulfone (PES) for the dNF80, dNF40 and dNF20 modules, (ii) a combination of PES and sulfonated PES (SPES) for the HFS module, and (iii) a combination of polyvinylidene difluoride (PVDF) and Polyvinylpyrrolidone (PVP) for the Helix 5mm module. Although the majority of the membrane manufacturers generally advise that those membrane materials are compatible with ethanol and methanol (GE Healthcare Life Sciences, 2013; Pall Life Sciences; Membrane Solutions; TPP Techno Plastic Products AG, 2016), there are few who do not recommend the use of these solvents (Restek Co., 2014). Furthermore, the manufacturer of the particular filtration modules in

use did not provide specifications for the compatibility of the membranes with pure organic solvents.

Hence, the operation of the membranes modules with pure organic solvents was not attempted.

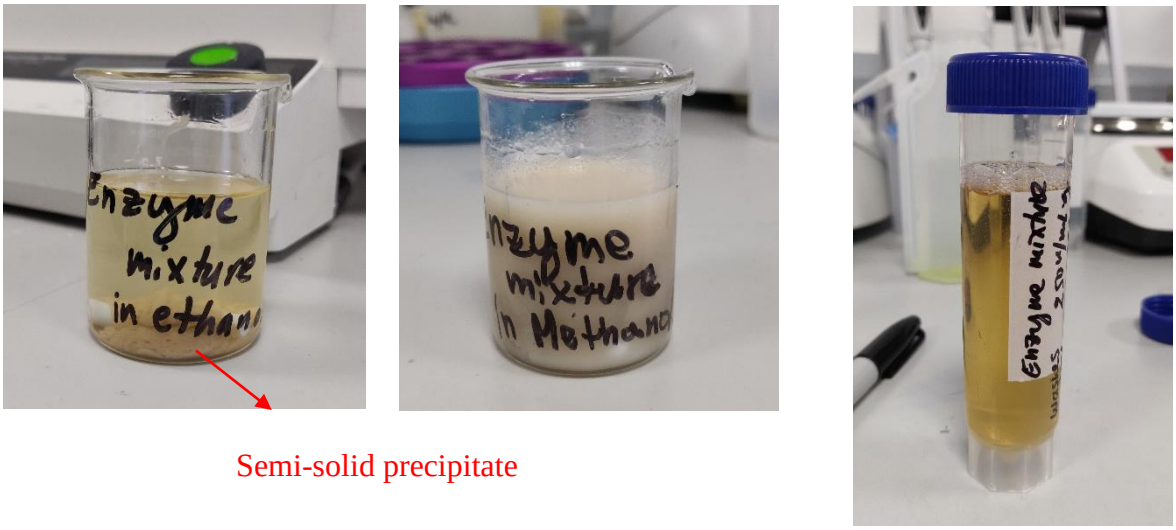


Figure 3.24 The solutions of the Celluclast 1.5L/Pectinex Ultra Mash enzymes in three different solvents; (i) pure ethanol (left), (ii) pure methanol (centre) and (iii) pure water (right).

In view of the above, the combination of organic solvents with the enzymatic-aided extraction of the beetroot pomace and the membrane processing of the extract is technically infeasible. The highest concentration of organic solvent, which could be used for the extraction without any apparent problem of incompatibility with the enzyme and the membrane, was 30% v/v of ethanol. However, as mentioned previously, this solvent did not retrieve any compound from the beetroot pomace.

In order to validate the presence of compounds in the beetroot pomace, the conventional extraction of the pomace was finally attempted using three pure organic solvents, i.e. using pure ethanol, methanol and acetone, without the use of enzymes and any membrane processing. The conventional extracts were subsequently analysed with the HPLC-DAD chromatographic method. The results of the analysis are presented in Figure 3.25, and indicate that there are no major peaks related to the beetroot pomace

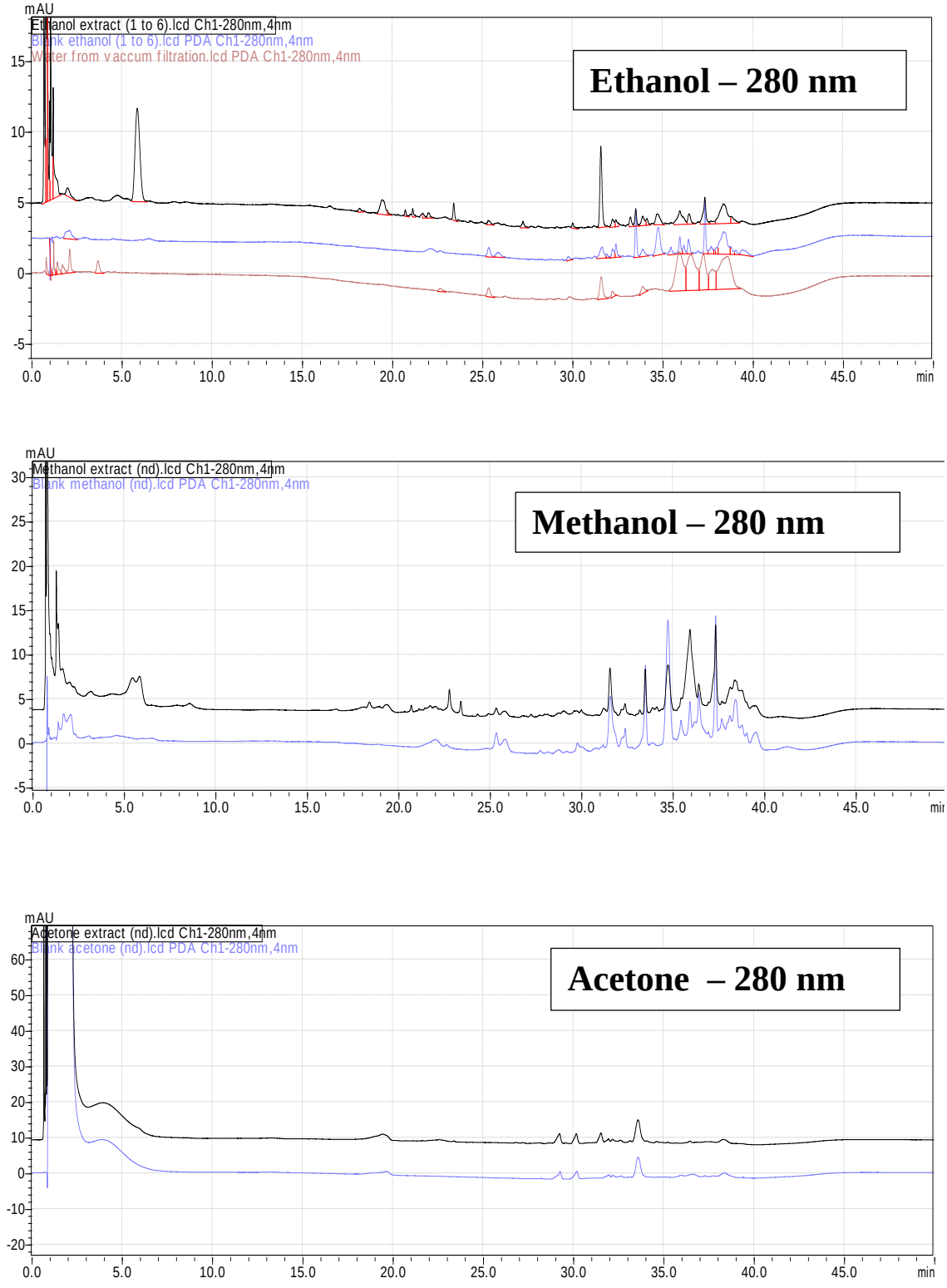


Figure 3.25 The HPLC-DAD chromatograms of the beetroot pomace extracts, using three different solvents; (i) pure ethanol (Top), (ii) pure methanol (Centre) and (iii) pure acetone (Bottom), at 280 nm.

in any of the pure organic solvent extracts, apart from one peak with rt. 6 min, which has been also observed inside raw beetroot extracts, the beetroot juice and in semi-pilot scale NF streams of the beetroot pomace extract, but was not possible to be qualitatively determined. In all chromatograms, some early-eluting peaks and several other peaks related to impurities of the solvent were also observed.

Interestingly, the presence of betanin was observed in methanol extracts, although with a low, yet clear, signal (S/N = 29.5>10[1]). The recovered betanin was measured at 0.06 mg/mL or 0.63 mg/g beetroot pomace (Figure 3.26). The beetroot juice, from the production of which the pomace is collected as waste, has been estimated to contain a betanin concentration of 433.21 mg/mL. The recovered amount of betanin found in the beetroot pomace could be worth 27,78 £/kg of pomace (based on the 42.50 £/g market price of betanin[2]). These results further support that methanol is a more efficient solvent for the extraction of betalain pigments.

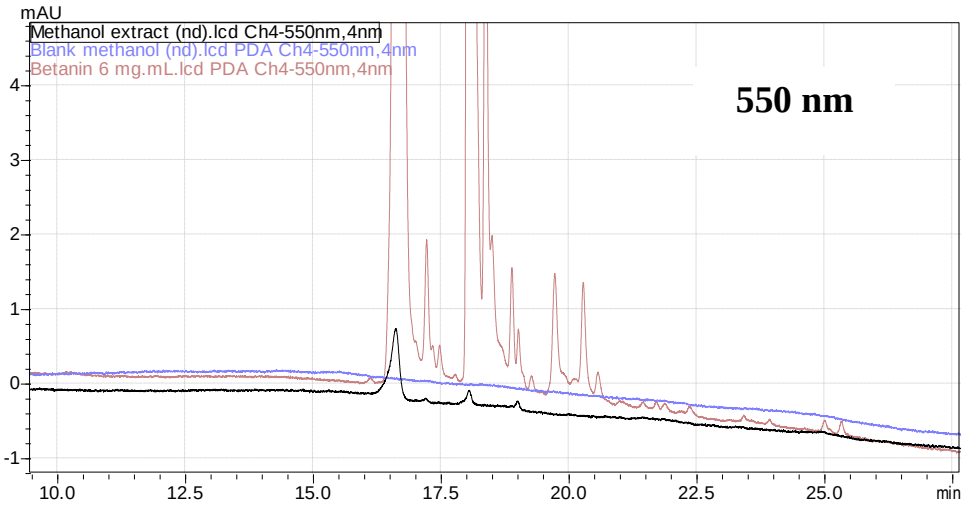


Figure 3.26 The HPLC-DAD chromatogram of the beetroot pomace methanol extract at 550 nm (in black), as compared to the betanin standard (in red), showing the presence of betanin and isobetanin in the extract.

[1] The Limits of Detection (LOD) are calculated based on a Signal-to-Noise (S/N) ratio equal to 3 and the Limit of Quantification (LOQ) on a S/N equal to 10 (Ha *et al.*, 2006; Nour, Trandafir and Cosmulescu, 2013; Zhang *et al.*, 2014). Since the S/N is above 10, the quantification of this betanin peak can be safely applied. It has to be noted that the S/N is quite close to the LOQ limit. Therefore, the detected amount is close to the detection limit.

[2] According to Merck & Co, Inc., USA (prices reported on 07/04/2020) <https://www.sigmaaldrich.com/catalog/search?term=Betanin&interface=All&N=0&mode=partialmax&lang=en&region=GB&focus=product>

All in all, the processing methodology of the beetroot pomace followed in this work could not validly prove the recovery of betalains or phenolic compounds in detectable amounts. In particular, it appears that the beetroot pomace is partly depleted in the majority of the compounds found to be present inside raw beetroot and the beetroot juice. The recovery of betanin was confirmed, yet only using conventional extraction techniques with methanol as a solvent. As the conventional extraction of the beetroot pomace has, however, been studied in the literature and, also, because methanol cannot be combined with the processing strategy initially designed for this work, involving the combination of the enzymatic-aided extraction with membrane processing, it appears that the current research work has fulfilled its initial hypothesis investigation and has no further objectives.

Chapter 4

Conclusions

The proposed processing methodology, which was examined during this work, was shown to be inefficient for the recovery of added-value compounds from the tested beetroot juice industry wastes. The early screening experiments demonstrated the absence of any beetroot compounds, such as of betalain pigments and phenolic compounds, in the pomace, at least at amounts which would be detectable by well-established analytical techniques.

The experiments performed investigated the potential causes for the absence of such detectable beetroot compounds, including (i) the use of different processing conditions and (ii) of different beetroot pomace batches, and (iii) the investigation of potential interactions between commonly found beetroot compounds and the employed hydrolytic enzymes, which could impede the beetroot compounds' detection. The up-scaling of the process with the subsequent concentration of the extracts, via membrane cascade processing and evaporation, was also examined, as it was expected to increase the concentration of possibly present beetroot pomace compounds in the extracts, up to

levels which could potentially render them detectable with the applied analytical techniques. None of these investigated hypotheses appeared to be responsible for the absence of detectable compounds in the beetroot pomace.

The absence of detectable beetroot pomace compounds using the enzymatic-aided extraction indicated that there are no compounds whose extraction could be optimised, neither there is a mixture of compounds which could be concentrated or fractionated using membrane cascade processes. Therefore, the optimisation of the extraction process, as well as the investigation for the evaluation and the optimisation of the membrane cascade processing were both rendered impossible, and, thus, they had to be suspended.

The conventional extraction of the beetroot pomace using pure methanol was the only examined processing strategy, shown to be able to extract betanin pigment from the pomace, at an amount of 0.63 mg betanin/g beetroot pomace that was estimated to be worth 27,78 £/kg of pomace. This finding confirmed that methanol is a more efficient solvent than water for the extraction of betalains from beetroot, in accordance with the already published literature (Kujala, Loponen and Pihlaja, 2001). It also validated the presence of betalain pigments in the examined beetroot pomace and the inefficiency of the suggested processing strategy, involving the enzymatic-aided extraction and the membrane cascade processing of the extract, to extract them.

The beetroot pomace was found to contain relatively lower amounts of betanin in comparison to raw beetroot, while the beetroot juice was found to contain significant amounts of betalains. Both raw beetroot extracts and the beetroot juice contained several types of betalain pigments, including vulgaxanthin I, betanin, isobetanin and neobetanin, whereas the beetroot pomace was found to contain only betanin. The reason for not detecting as high and diverse betalain pigments in the pomace could be due to either (a) the degradation of these compounds in the pomace, during or after juice production processing, or (b) the migration of the majority of these compounds from raw beetroot to the juice, during juice production. The presence of significant amounts of betalains in the beetroot juice denotes that a great part of those compounds is transferred from raw beetroot to the juice, rendering the beetroot pomace partially

depleted, and hence a cheaper source, of those high-value pigments following juice production.

Any modification in the suggested processing strategy, that would involve its combination with the use of methanol as an extraction solvent, would not be feasible, since the hydrolytic enzymes are not stable in organic environments, such that of methanol, and, also, due to concerns in regard to the compatibility of the membranes in use with organic solvents.

In conclusion, the conventional extraction of beetroot pomace using pure methanol appeared to be a more suitable, effective and therefore promising processing methodology for industrial application, than the enzymatic-aided extraction, for the recovery of betalain pigments from the beetroot pomace.

As methanol cannot be combined with the enzymatic-aided extraction and the membrane cascade processing, the performance of the simple conventional extraction of the beetroot pomace alone using pure methanol cannot constitute the base for an innovative research work.

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