Universitat Autònoma de Barcelona

DEVELOPMENT AND CHARACTERIZATION OF PRODUCTS DERIVED FROM SEA BUCKTHORN (Hippophae rhamnoides) BERRIES

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2021

The present thesis report is presented by Arnau Vilas Franquesa to obtain the title of Doctor of Philosophy in Food Science from the Universitat Autònoma de Barcelona

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Industrial doctoral project partnering

&

In collaboration with

Als meus pares

“The important thing is not to stop questioning.”

Albert Einstein

Acknowledgements

This thesis has been performed at the "Universitat Autònoma de Barcelona" (UAB), in collaboration with the University of Reading. The project has been financially supported by the Industrial Doctorate Program of the "Agència de Gestió d'Ajuts Universitaris i de Recerca" (AGAUR) under the grant code 2017DI079. This thesis emerged as a result of a collaboration agreement between the UAB and the sponsor company Vitae Health Innovation S.L., which also provided financial support.

This thesis project finishes as an accomplished dream after several years of effort and resilience, but also after years of worries and stress. This project would not have been possible if it was not for all the persons that built up this research project, and for those who contributed to my mental health and comfort.

Gràcies a en Raimon Cortada i a Vitae Health Innovation S.L. per donar-me la oportunitat de desenvolupar aquest projecte de recerca, i per la confiança dipositada en mi des del primer moment.

Moltes gràcies a la meva tutora i directora de tesi Bibiana Juan, i al meu director de tesi Jordi Saldo, per ser uns supervisors exemplars, per donar-me llibertat alhora d'investigar i per estar sempre a disposició quan tenia dubtes o problemes. Gràcies per les hores que heu dedicat de forma

incessant a revisar meticulosament la tesi i per la rapidesa del feedback proporcionat. No només gràcies per les tot l'esforç, sinó per ser alguna cosa més que els meus supervisors, amb qui he pogut gaudir de més d'un moment fora de la feina i de qui he pogut rebre sempre bons consells.

Gràcies a totes les persones del Departament de Ciència Animal i dels Aliments que m'han ajudat o donat suport i han fet més distesos els dies més estressants. En especial gràcies a la Sònia i la Dolors, per estar sempre disponibles i ser tant eficients i properes.

Gràcies als meus companys de departament de Vitae Health Innovation S.L., per fer cada dia una mica millor. En especial gràcies a la Eli per compartir moments de riures irrepetibles i moments desestressants en parella fora de la feina, sempre necessaris en el transcurs del doctorat.

Gràcies també als meus ex-companys de feina que van seguir camins diferents; a l'Aleix i les Martes, gràcies per integrar-me al departament i per tants moments divertits que hem passat junts.

At last, thanks to Dimitris Charalampopoulos and Afroditi Chatzifragkou, for allowing me to perform part of my research on the Food and Bioprocessing Sciences Research Group from the University of Reading. Thank you for the quick adaptation to the pandemic situation and to allow me to follow my work plan through a virtual stage.

Abstract

The minimal use of processing technologies is a quite extended strategy to avoid an excessive damage on food ingredients, especially in food supplements, where the ingredients generally contain high amounts of bioactive compounds which are critical for the physiological functionality of the final product. Raw ingredients with great proportions of bioactive compounds gain then importance. One of these raw ingredients is sea buckthorn. Sea buckthorn berries are constituted by an aqueous fraction and an oil fraction. The aqueous fraction registers one of the greatest concentrations of vitamin C, whereas the oil fraction registers high percentage of palmitoleic fatty acid, which is very rare in the plant kingdom. Considering the highly valuable nutritional profile of sea buckthorn berries, an in-depth study has been performed in the present work.

First, an extensive review explains in detail the full phytochemical composition of the berries and the products which could derive from sea buckthorn berry processing, as well as potential applications of these products on the food and feed industry. Furthermore, the most recent advances on health promoting effects of sea buckthorn berries and products have also been reviewed, wrapping up with a discussion for future trends. The literature review gains more interest with the following part of the

research, consisting of a full study of the phytochemical composition of commercially available berries. In addition, the study also includes a monitoring over different harvesting years of the berries of an orchard located in Spain.

While the potential health effects of the berry oil have been previously published, the understanding of the impact of extraction technologies on the nutritional profile of the resulting oil is still limited. The present work attempts to fill that gap by investigating the use of green technologies (i.e. supercritical CO_2) using different conditions to extract sea buckthorn oil from dried berries. In addition, green solvents have also been investigated to extract sea buckthorn oil. The phytochemical analysis of the obtained oil served as basis for the comparison and discussion of the results.

The nutritional profile of the aqueous fraction of sea buckthorn berries have also been widely investigated. Yet again, the impact of the technological processes to which this fraction could be submitted is scarce. The present work applies concentration techniques as well as biotechnology processes to sea buckthorn juice aimed at improving the nutritional profile of the original product. The use of membrane osmotic concentration, cryoconcentration and evaporation have been thoroughly investigated to concentrate sea buckthorn juice. Moreover, fermentation by probiotic lactic acid bacteria has been explored in sea buckthorn juice as a bioprocess to

achieve a positive modification in the nutritional profile of the juice. In both processes, the phytochemical analysis of the resulting product has been exhaustively studied.

Despite the phytochemical changes that may occur during sea buckthorn juice processing, the juice contains high amounts of organic acids, partly conferring the juice its characteristic sourness and astringency. The present work also investigates the ideal formulation of a sea buckthorn-based juice to mask these negative organoleptic characteristics for a greater consumer acceptance.

Overall, this project could be considered as a handbook of sea buckthorn berries processing, where not only takes place the full understanding of the phytochemical profile of sea buckthorn berries, but also the investigation of the applicability of different food processing techniques aimed at the obtention of sea buckthorn-based products with improved nutritional profiles. The present work might be of interest for a broad audience interested in food science, including food scientists, food technologists, nutritionists or pharmacologists, especially those working in the fruit juice industry, food (supplements) industry or involved in fractionation processes of bioactive compounds.

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List of abbreviations

2-MTHF: 2-Methyltetrahydrofuran

AIJN: European Fruit Juice Association

ANOVA: Analysis of variance

ASE: Accelerated solvent extraction

BHT: Butylated hydroxytoluene

C16:0: Palmitic acid

C16:1-ω7: Palmitoleic acid

C18:0: Stearic acid

C18:1- ω9: Oleic acid

C18:2- ω6: Linoleic acid

C18:3- ω3: α-linolenic acid

CECT: Spanish Type Culture Collection

CFU: Colony-forming Units

CSBJ: Clarified sea buckthorn juice

CTA: Cellulose triacetate

CWOYE: Sample with berries from 'Cerdanya' (Spain) without yeast extract

CYE: Sample with berries from 'Cerdanya' (Spain) with yeast extract

DM: Dry matter

DPPH: 1,1-diphenyl-2-picrylhydrazyl

EFSA: European Food Safety Authority

FO: Forward osmosis

FRAP: Ferric reducing antioxidant power

FSBJ: Filtered sea buckthorn juice

GAE: Gallic acid equivalents

GC: Gas chromatography

HPLC: High-performance liquid chromatography

IDF: Insoluble dietary fiber

KMO: Kaiser-Meyer-Olkin

LSD: Least Significant Difference

LWOYE: Sample with berries from Latvia without yeast extract

LYE: Sample with berries from Latvia with yeast extract

MAE: Microwave assisted extraction

MRS: de Man, Rogosa, Sharpe

MS: Mass spectrophotometer

MTHF: Methyltetrahydrofuran

MUFA: Monounsaturated fatty acids

PC1: Principal Component 1

PC2: Principal Component 2

PCA: Principal component analysis

PRI: Population Reference Intake

PTFE: Polytetrafluoroethylene

PUFA: Polyunsaturated fatty acids

SB: Sea buckthorn

SBB: Sea buckthorn berries

SBJ: Sea buckthorn juice

SBO: Sea buckthorn oil

SC: Suspension crystallization

SC: Suspension crystallization

SD: Standard Deviation

SDF: Soluble dietary fiber

SFA: Saturated fatty acids

SPTA: Food Technology Plant Service

TC: variety ‘Tatjana’ from ‘Cerdanya’ (Spain)

TDF: Total dietary fiber

TE: Trolox Equivalents

TL: variety ‘Tatjana’ from Latvia

TMP: Transmembrane pressure

TSS: Total soluble solids

UAE: Ultrasound assisted extraction

YE: Yeast extract

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PART I

INTRODUCTION TO SEA BUCKTHORN, PROJECT FRAMEWORK AND METHODS

Chapter 1: Potential of sea buckthorn-based ingredients for the food and feed industry – a review

An exhaustive literature research has been performed on the applicability of ingredients derived from sea buckthorn berries processing in the food and feed industry. Loads of articles had been recently published on the applicability of different ingredients derived from the use of these berries, yet no review article has attempted to summarize these findings. The first part of the present research aimed at methodically retrieving these articles and presenting and explaining their results related. An article was published derived from this part of the research and it is herein used as the first introductory chapter. The following pages include the full disclosed article, published in the *Food Production, Processing and Nutrition* journal on August of 2020.

Food Production, Processing and Nutrition

REVIEW Open Access

Potential of sea buckthorn-based ingredients for the food and feed industry – a review

Arnau Vilas-Franquesa[1], Jordi Saldo[1,2*] and Bibiana Juan[1]

Abstract

Food industries seek to incorporate nutritious ingredients as they could bring added value to the final food products. One of the most interesting options is that sea buckthorn contains high concentrations of vitamin C, carotenoids, tocopherols, and other bioactive compounds, in addition to the unique lipid profile in the berry pulp, seed, and peel. This review summarizes the state-of-the-art of potential applications of sea buckthorn within the food and feed industry based on previously described applications. Products such as cheese, yoghurt or beverages already benefit from its application. Moreover, using sea buckthorn in feed products also derives into higher quality final products (e.g. meat quality, egg quality). Poultry, pig, and fish farming have been studied for that purpose. Despite all the accumulated articles depicted in the present review, the use of this fruit in food product formulation is nowadays scarce. New options for food product development with sea buckthorn are herein discussed.

Keywords: Food science, Feed additive, Product development, Health, Bioactive compounds, Added value, Sea buckthorn

Introduction

Sea buckthorn (*Hippophae rhamnoides* Linnaeus) is a flowering plant (Angiosperm) of the order Rosales and Elaeagnaceae family. Sea buckthorn (SB) is morphologically described from a bush to a small tree, with different growing thorns all around the plant, and it naturally grows in locations near to the sea, specific traits which build up its name. It is stated that its latin name *Hippophae rhamnoides* comes from ancient Greece, from the words 'hippo' – horse – and 'phaos' – shine –, for the horses fed with leaves from this plant developed a shining coat and weighed more (Kalia et al. 2011; Li and Hu 2015).

The plant naturally grows in cold and dry regions around the globe. Himalaya is the region with the highest density of this plant (Kalia et al. 2011). It also grows on cold desert areas of China, Russia, North America, India, and Europe among others (Li and Hu 2015; Rousi 1971). Its highly adaptable characteristics allow the plant to grow in very different environmental situations, being able to grow at temperatures ranging from -40 to +40 °C (Kalia et al. 2011) and high altitudes (Ma et al. 2016). It could endure dry, alkaline or high salinity soils, and inundations (Kalia et al. 2011). The plant normally flowers around March and gives fruits around September. The fruit is a small (geometric average diameter measurements of nine varieties grown in Estonia ranged from 8.64 to 12.57 mm (Lougas et al. 2006)), orange-to-yellow berry weighing 375 mg as average (Beveridge et al. 1999).

Research on SB has grown considerably in the last two decades and several subspecies have been confirmed by means of new phylogenetic techniques. The division of

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H. rhamnoides L. into 8 subspecies – confirmed by Bartish et al. (2002) – seems to be the most currently used and accepted classification (Sun et al. 2002; Ma et al. 2016).

As SB gains more importance, more contributions are being made on the topic, especially on the composition of the berry (Zielińska and Nowak 2017; Kaur et al. 2017) and its health effects (Olas 2018). Similarly, the number of research articles investigating the application of SB ingredients on current food products is increasing as well. However, there is no such a review on the application of SB ingredients in food or feed products. Therefore, the aim of the present review is to give an overview of all the investigated applications of SB on current food products and to discuss the potential of future research and applications in the field. Articles related to the use of SB for the development of food supplements do not suit the aim of the present review and have therefore been excluded after initial screening.

Sea buckthorn products

SB can be easily processed into valuable products. Once harvested, the first clear division is the leaf and the fruit. The leaf itself can be easily processed to obtain tea (Ma et al. 2019) or aqueous extracts, shown to have antioxidant, cytoprotective, and antibacterial effects (Upadhyay et al. 2010). However, its application to food production is difficult since it has not been recognized as a food product in specific areas of the world (i.e. Europe). In contrast, the berry is the most consumed part of the plant worldwide, and therefore, the present review will focus on SB berry rather than on leaf-derived products.

SB berry is the most consumed part of the plant worldwide. It could be conveniently processed into various products as well. The fruit consists of a hard peel, the pulp, and a seed. By using a worm-driver, the aqueous part of the fruit (i.e. the juice) can be separated from the seed, the peel, and some residues of the pulp (Cenkowski et al. 2006). Both products resulting from this extrusion can be further processed. On the one hand, the juice can be clarified by centrifugation. The clarification by centrifugation gives out three different products, namely the clarified juice (main layer), the oily part of the pulp (supernatant), and the residue left at the bottom, which is usually constituted by seeds and peel. On the other hand, the seed and peel can be separated firstly by drying, and later by using a mechanical sieve. SB products could then be classified by their fatty nature (i.e. oil from seeds, pulp, and peel) or aqueous nature (i.e. clarified juice). The yield percentage for juice extraction is about 70% (Cenkowski et al. 2006). The yield percentage for seed oil extraction is approximately 12% whereas the peel and the pulp give out an approximate yield percentage value of 6% (Dulf 2012).

Most important components of sea buckthorn

Aqueous fraction

The juice coming from SB berry processing is a complex product but can be easily further processed to obtain a clarified juice. The clarified juice is the only source of hydrophilic compounds.

The most stand-out trait of SB is high content of vitamin C. Beveridge et al. (1999) reviewed vitamin C values from 360 to as high as 1676 mg/100 g of berry, whereas Tiitinen et al. (2006b) reported values from 128 to 1300 mg/100 ml of berry juice, which is clearly higher than the concentration naturally found in naturally vitamin C rich fruits, such as lemons, oranges (Christaki 2012) or even kiwis (Dumbravă et al. 2016). The highest concentrations are only comparable with exotic fruits like acerola (Cefali et al. 2018). Thus, SB emerges as a great source of vitamin C after considering that one of the lowest values found in literature is 80.58 mg of vitamin C/100 g of fresh berries (Teleszko et al. 2015).

According to the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations, the recommended vitamin C intake (RNI) for an adult is about 45 mg/ day, and for a lactating woman the requirement increases to 70 mg/ day (World Health Organization and Food and Agriculture Organization of the United Nations 2004). If we consider the lowest value found in literature, by only eating 50 g of fresh SB berries, one adult person would meet the recommended dietary intake.

Nevertheless, vitamin C is rapidly degraded under certain processing conditions. Light, temperature, pH, enzymes, metallic catalyzers, and oxygen are parameters that can severely accelerate vitamin C degradation (Gutzeit et al. 2008; Santos and Silva 2008). The starting concentration of vitamin C in the raw product is a major factor affecting the final concentration of this vitamin after processing. The high concentration values reported for SB allows obtaining high vitamin C products even after processing. This is of great importance since a lot of products have difficulties in conserving adequate vitamin C levels after processing.

Along with vitamin C, polyphenols confer SB fruit its high antioxidant activity (Kim et al. 2011). The polyphenolic fraction of SB could be one of the factors contributing to the bactericidal potential of SB extracts. Total polyphenolic concentration is most of the time quantified through mg of gallic acid equivalent, one of the simplest polyphenols – although other polyphenols can be used depending on the major phenolic present in the sample (Singh et al. 2016). Cioroi et al. (2017) have recently reported values from 78 to 95 mg gallic acid equivalent (GAE)/ g dry weight, depending on the origin of the berry. These values are relatively high compared to a polyphenol-rich product like coffee. Hečimović

et al. (2011) studied different coffee varieties and roasting temperatures and found the highest value of medium roasted coffee beans was 43 mg GAE/ g. According to Zadernowski et al. (2005), the main phenolic acid present in the non-flavonol glycoside fraction of SB was salicylic acid, reaching values as high as 1500 mg GAE/ kg of dry matter of berries, closely followed by gallic acid (Arimboor et al. 2008). Reported values of flavonol glycosides in fresh berries range from 23 to 250 mg/ 100 g (Ma et al. 2016), making it the most important phenolic fraction of the fruit (Arimboor et al. 2008).

Oil fraction

The fruit contains two different oil fractions; one obtained from the seed and one retained within the pulp. Unsaturated fatty acids and tocopherols are the major compounds of both oily fractions. Nevertheless, they differ significantly in their concentration. The seed oil contains greater concentrations of tocopherols (Kallio et al. 2002) and alpha-linoleic acid whereas the pulp oil has greater concentrations of palmitoleic acid.

Tocopherols are unequally distributed in SB seed, peel and pulp oil. For instance, α-tocopherol is found at higher concentrations in seed than in the full fruit whereas δ-tocopherol is found at greater concentrations in peel rather than pulp or seed oil (Burčová et al. 2017). Levels of total tocopherols can reach values of more than 160 mg/100 g of seed oil (Beveridge et al. 1999). These values are close to other oils valued for their high concentrations of tocopherols, such as soybean oil (100–200 mg/100 g soybean oil (Carrera and Seguin 2016)) or sunflower oil (50–150 mg/100 g sunflower oil (González Belo et al. 2017)), and much higher than other high-quality oils, such as olive oil, containing 21.24 mg/ 100 g of oil (Gimeno et al. 2002). Conversely, peel oil has been found to contain greater concentrations of δ-tocopherol rather than α-tocopherol (Burčová et al. 2017). β-tocopherol has been identified as the least present in either seed and pulp and peels oil (Burčová et al. 2017; Kallio et al. 2002).

Similarly, saturated fatty acids in SB are unevenly distributed in seed and the rest of the fruit. The seed contains a residual amount of saturated fatty acids (Yang and Kallio 2001) whereas the peel and pulp oil contain as much as 40% (Fig. 1; Dulf 2012). Almost all the fraction of the saturated fatty acids present in pulp oil is built by palmitic and stearic acids, common fatty acids within the plant kingdom (Dulf 2012).

The fraction of unsaturated fatty acids is also very different between pulp and peel when compared to seed oil. Pulp and peel oil contain greater amounts of monounsaturated fatty acids whereas seed oil contains a large fraction of polyunsaturated fatty acids (68%, Fig. 1). The main fatty acids present in seed oil are linoleic (C18:2 ω-6), α-linolenic (C18:3 ω-3) and oleic acids (C18:1 ω-9), accounting for approximately 40, 30 and 16% of the total fatty acids in seed oil, respectively (Fig. 2, (Dulf 2012; Teleszko et al. 2015)), whereas palmitoleic fatty acid (C16:1 ω-7) is found in negligible amounts in seed oil (about 0.5% of total fatty acids). Although oleic fatty acid is found at relatively acceptable concentrations in SB seed oil (13–20% of total fatty acids (Dulf 2012)), other vegetable oils such as olive oil contain much higher concentrations (70% of total fatty acids (USDA Food Composition Database 2018)). Contrarily, pulp and peel oil contain greater amounts of palmitoleic and oleic acids (Fig. 2).

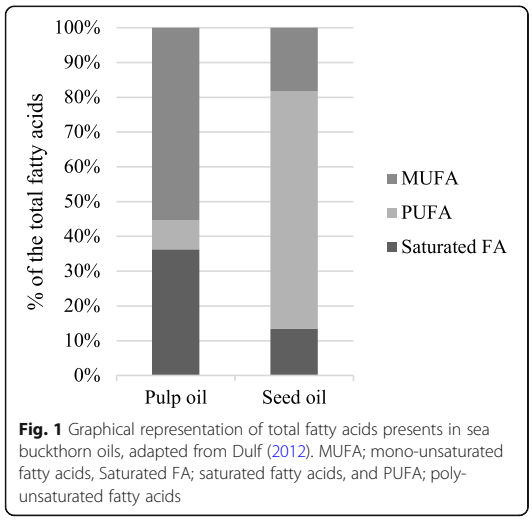

Fig. 1 Graphical representation of total fatty acids presents in sea buckthorn oils, adapted from Dulf (2012). MUFA; mono-unsaturated fatty acids, Saturated FA; saturated fatty acids, and PUFA; poly-unsaturated fatty acids

The role of ω-6 and ω-3 unsaturated fatty acids in human health have been extensively investigated over the years. Different reviews have been published on that field, showing the great implication of unsaturated fatty acids on human health and their importance in any diet (for instance; Innes and Calder 2018; Russo 2009; Zárate et al. 2017).

Palmitoleic acid is the only ω-7 fatty acid, and its presence within the plant kingdom is very rare. SB, together with macadamia nuts contain great amounts of palmitoleic fatty acid when compared to other vegetable oils (11 to 27% of the total fatty acids in peel and pulp oil (Dulf 2012), and 24 to 36% of total fatty acids (Aquino-Bolaños et al. 2017), respectively). Palmitoleic acid intake has been associated with improvements in insulin sensitivity, cholesterol metabolism (Marsiñach and Cuenca 2019), or acceleration of wound healing due to its potential anti-inflammatory effect on skin (Weimann et al. (2018)).

Besides tocopherols and fatty acids, SB contains considerable amounts of different carotenoids. Carotenoids

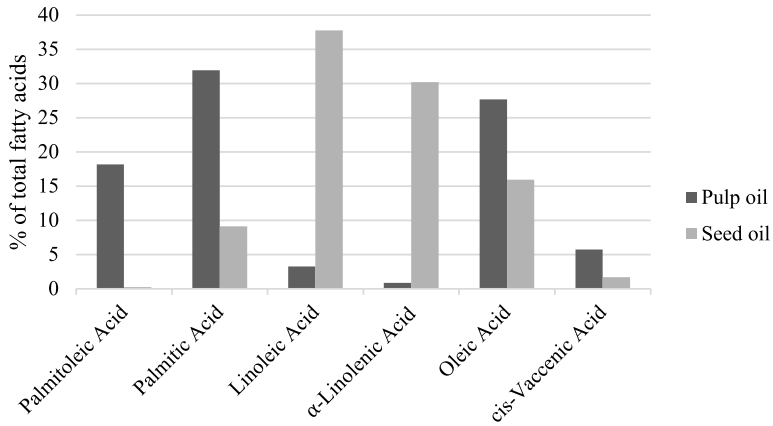

Fig. 2 Graphical representation of specific saturated and unsaturated fatty acids presents in sea buckthorn oils, adapted from Dulf (2012)

are present in pulp oil, conferring the fruit its characteristic orange-bright color. Carotenoids in SB can reach approximate concentration values of 12 mg/ 100 g of fresh weight (Teleszko et al. 2015). Although these concentration values are much lower than those found in oils known for being good sources of carotenoids, such as crude palm oil (54 mg/100 g (Manorama and Rukmini 1992)), these concentrations are higher than those found in other berries, such as black currant, blueberry or strawberry (Marinova and Ribarova 2007).

Health benefits of sea buckthorn: recent advances

Consumer trend towards healthier food choices is unquestionable. As more evidence is added to the field, consumers can take a more informed and healthy decision upon many different food products. Food industries are constantly adapting to fulfill the rapidly changing consumer wishes. In turn, food ingredients are being designed to give an added value to the food product and possibly trigger its choice. As naturalness is more related to values of nutrition and health, food components or ingredients coming from a natural source are becoming an important tool for the development of food products. Sea buckthorn builds up a clear example of what would be easily incorporated as a food ingredient. Having a clear natural origin and the nutritional quality herein reported, sea buckthorn is gaining importance as a promising plant source of several ingredients (either coming from the pulp or the seed).

Research on the association between sea buckthorn consumption and health has been ongoing for several years. The interest of late research has been on the polyphenolic fraction as well as the effects of its oil (either from the pulp or from the seed) on indicators of several diseases. The beneficial effects of isolated compounds that may be found in great proportion and are essential to human life (such as vitamin C or tocopherols) are well known. Therefore, research on SB focused on newly produced extracts (for instance a polyphenolic-rich fraction from the pomace after the extraction of the juice).

The phenolic fraction can be sourced from several SB products and has recently been a matter of study due to its possible attributed health effects. According to Ma et al. (2016), one of the major aglycones from sea buckthorn is isorhamnetin. Isorhamnetin, primarily found in the aqueous fraction of the fruit, had been shown to present a high antioxidant activity, even more than that exerted by ascorbic acid, at least in various chemical assays (FRAP, DPPH), as reported by Pengfei et al. (2009). Nowadays, many food companies thrive to achieve clean-label products, and the phenolic fraction of sea buckthorn emerges as a possible natural antioxidant substitute. Besides, the phenolic fraction of SB fruit had been shown to significantly decrease the peroxidation of plasma induced by hydrogen peroxide and increase the clotting time in a test-tube study, therefore showing an interesting anticoagulant activity (Olas et al. 2018).

In addition, isorhamnetin has been studied for its possible role as immunosuppressive therapy. Shi et al. (2018) conclusively proved that isorhamnetin could effectively suppress dendritic cell's maturation and trafficking. Dendritic cells are major targets of immunosuppressive therapies, and therefore, isorhamnetin could be used in the prevention and treatment of inflammatory and autoimmune diseases, including cases of transplantation rejection. Subsequently important, isorhamnetin had also been found to be more biologically active after digestion when compared to its pure form, at least on the regard of its antioxidant and antiproliferative activity (Guo et al. 2017 b). Indeed, several authors have studied

the bioavailability and transformation of polyphenols from sea buckthorn juice in the gastrointestinal tract. As Attri et al. (2018) conclusively showed, sea buckthorn juice experienced an increase in total polyphenols after gastric and small intestine digestion, doubling the original value in the latter. The original polyphenolic compounds may probably be digested and transformed by beneficial bacteria, resulting in a final increase in caffeic and chlorogenic acids, rutin, and quercetin. However, results were different from those previously found by other authors (Guo et al. 2017 b). The polyphenolic fraction of sea buckthorn juice promoted the growth of beneficial bacteria groups *Bacteroides, Prevotella*, and *Bifidobacteria* in a significant manner (Attri et al. 2018).

After its oil extraction, sea buckthorn seed residue could be used as a source of polyphenols, since the oil extraction usually does not include the extraction of polar compounds. Wang et al. (2014) demonstrated that a procyanidin extract from SB seed powder showed a powerful inhibitory effect against fatty acid synthase (FAS), therefore inducing cell apoptosis in the human cancer cell line MDA-MB-231, which shows specially overexpressed FAS activity. Recently, Wang et al. (2016) tested the efficacy of orally administered procyanidin extract from SB seed powder against visible light-induced retinal degeneration in rabbits. The intake of the studied extract effectively maintained the retinal structure and reduced the effect of inflammatory cytokines, induced by light exposure.

Polyphenols constitute a great fraction of SB, and extracts from different sources (berry juice, pomace, or seed residues) show a different polyphenolic profile. Extracts with different polyphenolic profiles have been tested depending on the main polyphenol found in it, and most of the studies show interesting results, as herein presented. SB emerges as a good source of natural antioxidants as the concentration of polyphenols in the raw matrix is already high, and benefits from the putative antioxidant effects of vitamin C. Also, SB may also be the source of purified extracts constituted primarily of polyphenols. These extracts may prove to be useful to the formulation of food supplements, which benefit from the addition of functional compounds. However, clear epidemiological evidence on the effects of polyphenols extracted from SB is still missing and further research should address to fill that gap.

In addition to polar components such as polyphenols, SB is the source of many other different non-polar compounds, primarily found in SB oil, either coming from the seed or the pulp and peel. Several studies have explored the consuption of SB oil to understand the health implications or, in some cases, of its external application. Pulp and peel oil – also known as fruit oil – is rich in palmitoleic fatty acid, as reported in the previous section, a rare fatty acid in the plant kingdom. Research has shown that palmitoleic acid may have a role in glucose homeostasis as well as in the metabolism of fatty acids. Gao et al. (2017) investigated the effect of sea buckthorn fruit oil in vitro and in vivo and found that SB oil intake could significantly improve glucose homeostasis, insulin sensitivity, and liver injury in HepG2 cells and SD male rats. The oil is partly present in SB juice and therefore SB juice may have also an effect on improving insulin sensitivity and postprandial glycaemia. However, Mortensen et al. (2018) did not found a significant effect on postprandial glucose nor insulin concentration by using a sea buckthorn smoothie before a meal in overweight and obese male subjects. The smoothie, however, consisted of 35 g of added sucrose and protein, which may not be ideal to see an improvement in glucose homeostasis.

Oral administration of pulp oil has recently been reported to effectively reduce tear secretion by 80 and 93% in stress-induced dry eye rats and mice, respectively, compared to tear secretion before oil intake (Nakamura et al. 2017). These results would possibly explain the hydration and protective capacity of SB fruit oil. Also, Hou et al. (2017) proved that treatment with SB oil could suppress the development of atopic dermatitis-like lesions in mice, possibly proving the regenerating capacity of SB fruit oil.

Smida et al. (2019) have interestingly proved the efficacy of SB pulp oil as a mouthwash product. They found that a preparation containing SB pulp oil could have a bactericidal and anti-biofilm activities against oral bacteria species, although antifungal activities were not proved.

Seed oil, on the other hand, has been recently used to investigate the association between its intake and cardiovascular risk factors (Vashishtha et al. 2017). The authors performed an animal and a human study. They found that SB seed oil, administered at dosages of 0.75 ml significantly reduced total cholesterol levels, oxidized low-density lipoproteins, and triglycerides in plasma in hypercholesterolemic human subjects. Besides, Hao et al. (2019) have lately proved that supplementation with SB seed oil could positively modulate the relative abundance of beneficial gut bacteria groups, and together with an improvement in intestinal cholesterol excretion. It would be effective in reducing the blood cholesterol in hypercholesterolemic hamsters. These studies add up to a very interesting outcome that sea buckthorn could be used as a potential therapy against cardiovascular events possibly by inhibiting cholesterol deposition in the arteries (Olas 2016). The seed oil has also been tested on humans suffering from dry eye symptomatology. Larmo et al. (2019) have recently found that a four-times-a-day dosage of a sprayable solution containing 0.4% of SB

seed oil could significantly decrease the symptomatology related to dry eye when compared to a control solution without SB seed oil. The potential in skin hydration and regeneration from SB either attributed by seed or pulp oil has been proven more than once, probably due to the high content of unsaturated fatty acids, tocopherols, and carotenoids (depending on the oil source).

Reviews of SB and health have lately increased in number. Most of the published reviews aimed at summarizing the high number of publications related to a specific sea buckthorn health benefit. For instance, Olas et al. (2018) published a review focusing on the health benefits derived from oil consumption, highlighting its cardioprotective and hepatoprotective properties, anticarcinogenic potential, antioxidant capacity, and dermatological benefits. More interestingly, Guo et al. (2017a) published a meta-analysis of 11 independent randomized controlled trials. The review aimed at elucidating the relationship between the consumption of sea buckthorn and changes in blood lipid profiles.

Recent health advances show the most interesting paths to keep making research on the effects of SB components. The role that the polyphenolic fractions of SB have on human health, either from fruit pomace or from seed residue, varies depending on the phenolic profile of the extract. Several extracts have been reported to exert positive benefits on human health. However, most of them are performed using a highly pure extract, which does not match the real concentration in SB raw materials. Most of the health outcomes herein depicted from this fraction could interestingly be used by pharmaceutical or nutraceutical industries, in which the product developed relies exclusively on its potential health attributes. The fact that the residues could be processed further may also benefit the sustainability and cost of the production process. Nonetheless, SB oil, either from the fruit or from the seed, has been recently used to demonstrate possible health implications derived from its consumption. The oil consumed as such is already proven worthwhile for its use, making the extraction process quicker and simpler compared to other extracts from SB. Even though SB oil may not be included in food products to see a positive health benefit after its consumption. SB oil still possesses great antioxidant activities due to the high presence of bioactive compounds such as tocopherols and carotenoids, as already discussed. Likewise, instead of adding polyphenol extracts to food products, the juice itself could be used to tackle the costly production process of the extract.

Sea buckthorn in new food product development

New product development is a technique most of the food industries nowadays use in order to be competitive in the market. This strategy allows them to develop food products according to consumer's wishes. Consumers are every time more aware of their lifestyle. A healthy lifestyle involves several aspects, one of which is following healthy food habits. Thus, food companies are leading their new product development strategies towards more healthy and nutritious products. SB has emerged as one of the most promising ingredients for food companies, because of the already detailed physic-chemical profile and its derived health benefits. Nevertheless, in sensory quality terms, SB has a well-defined sour and astringent taste (Tiitinen et al. 2005), which makes the formulation of food products with SB quite a challenge. In an attempt to describe the origin of this sourness and bitterness, some authors have added evidence highlighting the importance of malic acid in the sourness profile of SB juice (53.8–74.1% of the total acid content) (Ma et al. 2017). In addition, they also found that ethyl β-D-glucopyranoside, an alkylated glucose of SB, may play a complex and prominent role in the bitterness of SB juice. Sweetness and fruity flavor are also poorly present features in SB juice (Tiitinen et al. 2005). The astringency, also an important attribute of SB juice, could be derived from specific phenolics: proanthocyanins or condensed tannins, as detailed by Lesschaeve and Noble (2005). Nevertheless, to the best of our knowledge, there has been no investigation on the proanthocyanins or condensed tannins in formulated SB-based products.

Fermentation of sea buckthorn juice

Some of the main components contributing to the sourness of SB juice are its organic acids. Malic acid and quinic acid constitute up to 90% of organic acids in the juice, and malic acid is the most prevalent (Zeb 2004). To tackle this issue, Tiitinen et al. (2006a) studied the effect of malolactic fermentation on SB juice. By inoculating *Oenococcus oeni* at a cell density of 10^9 CFU/mL they achieved a moderate increase in pH (from 2.8 to 3.1) after only 1 day of fermentation, and the final juice contained only 3 g/L of malic acid (instead of its original 16 g/L). After 24 h of fermentation, there were significant reductions in sourness and astringency and an increase in sweetness. One of the drawbacks the authors pointed out was the development of unwanted off-flavors, which increased upon increasing fermentation time. One year later, the same authors published more results related to this project (Tiitinen et al. 2007). Interestingly, they subjected four different varieties of SB to the malolactic fermentation detailed in their previous work (at a cell density of 109 CFU/mL over 18 h at 28 °C). This short fermentation time impeded certain compounds to be formed and decreased the off-flavors derived from fermentation. The fermentation rate was found to differ between varieties. Two of the varieties showed a higher conversion rate of malic acid into lactic

acid, a determinant factor when evaluating the fermentation of SB juice. In the study mentioned above, they found that the fruity flavor was enhanced due to the production of esters during the fermentation process. Besides, as found in their previous work, they observed a reduction in sourness and astringency after fermenting the juice in all studied varieties.

Using sea buckthorn in fermented food products

Besides attempting to tame the harsh flavor of the juice by fermentation, other authors have investigated the use of SB juice preparations in different fermented food products. Recent findings suggest that SB juice promotes the development of different beneficial gut bacteria, probably due to its prebiotic characteristics (Attri et al. 2018). Indeed, Selvamuthukumaran and Khanum (2015) already showed that SB may have a positive effect on the proliferation of different lactic acid bacteria. Their work consisted of including SB syrup in the stage prior to yoghurt fermentation. They studied how different concentrations of SB syrup and milk powder affect the sensory, physical, and functional quality of yoghurt (the latter being prebiotic bacterial counts at the final product). The optimum addition of SB syrup in yoghurt was 15%. This concentration achieved higher counts of *S. thermophiles* and *L. bulgaricus*, as well as an improvement in taste. More importantly, the developed SB yoghurt contained higher amounts of vitamin C, E, carotenoids, phenols, and anthocyanins when compared to commercial variations containing other fruits. One of the most important drawbacks was the addition of sugar in the development of SB syrup, which included 50 g of sugar per 100 g of syrup. This could explain the higher taste acceptance found in yoghurt containing SB syrup. Similarly, Gunenc et al. (2016) studied the effect of SB whole fruit, and purified mucilage addition in yoghurt on the final bacterial count after 28-day storage at 4 °C. Just as Selvamuthukumaran and Khanum (2015) previously found, homogenization with SB whole fruit or mucilage prior to incubation was found to increase the final bacterial viability in terms of bacterial counts. Addition of SB berries or mucilage also derived in a decrease in pH and an increase in titratable acidity after 28 days of storage at 4 °C. This marked drop probably happened due to the addition of pure SB fruit and mucilage, which sours the product if not treated before, especially due to its high content in organic acids.

Using SB in the development of yoghurts seem to have great potential, for different authors have provided evidence on the prebiotic ability of SB on lactic acid bacteria (Selvamuthukumaran and Khanum 2015; Gunenc et al. 2016). The berry juice was found by other authors to promote the growth of lactic acid bacteria and bifidobacteria as well as enhance the ratio Bacteroides/Prevotella, groups of bacteria classified as beneficial for the organism (Attri et al. 2018). Thus, SB-based yoghurt could be a good product to invest in, although more research is needed to find out means to improve the organoleptic characteristics of the final product.

Other authors studied the inclusion of SB as an ingredient in cheese. Terpou et al. (2017) used SB berries to study the symbiotic effect with a probiotic strain of *Lactobacillus casei* (ATCC 393) included in a feta-type cheese. They used dry SB berries as an immobilization carrier for the probiotic strain. Briefly, they mixed 10 g of dry sea berries with different weights of bacteria biomass and 500 ml of Man, Rogosa, and Sharpe (MRS) broth. After fermentation, the bottom biomass solution was used as an immobilized carrier. This mixture was added after the cheese coagulum derived from rennet action was cut into 1 cm size blocks. The addition of SB positively contributed to the aroma profile of the cheese, raising the concentration of esters, terpenes, and carbonyl compounds. Interestingly, the sour taste of SB berries was masked by the strong flavor of feta cheese, and the addition of immobilized probiotics gave a soft and smooth taste according to sensory evaluation. It is interesting to note that with strong-flavoring products, successful masking of the sour and bitter taste of SB could be achieved. Recently, Terpou et al. (2019) also used SB berries as a probiotic cell immobilization carrier for the development of functional frozen yogurt. They compared their results with frozen conventional yoghurt and frozen yoghurt prepared with just the probiotic strain. Immobilization of the strains improved their survival rate during storage, and protected the probiotic against gastric conditions in vitro, as shown in their previous study (Terpou et al. 2017). In addition, after performing a hedonic test, the yoghurt with SB as an immobilization carrier was the most overall accepted. The citrus taste was scored higher for SB yoghurt, an attribute derived from its addition. However, color and dairy flavor scored hedonically lower for SB. This is understandable, since SB may mask the dairy flavor and add some orange-like color, non-characteristic of a frozen yoghurt.

Prior products focused on the potential prebiotic ability of SB derived ingredients. Nevertheless, there are other fermented food products to which SB had been included to ultimately achieve a change – in most cases improvements – in its structure, flavor, antioxidant capacity, or shelf-life, among other characteristics. Specifically, SB was included in the production process of beer and bread, achieving a higher antioxidant capacity than its former product (Adadi et al. 2017; Sturza et al. 2016; Guo et al. 2019). Other

main findings of these studies are depicted in Table 1.

Beverages

Albeit having a poor taste mainly due to its sourness and bitterness, SB juice production is for many a serious chance for new product development due to its great concentration of vitamin C and other already mentioned bioactive compounds. Tang et al. (2001) developed a hedonic experiment with the juice of different varieties of SB. SB juice was diluted to 1:5 with tap water and sucrose was added as a sweetener. The sweetener was always added at the same concentrations (6.5%). Natural occurring sugars in SB made the difference between samples. Astringency, sourness, bitterness, sweetness, and color were the measured factors. The sweetness was identified as the major feature affecting the likeliness of the juice. Besides, a reduction in acidity accentuated the sweet taste in the juice and made it more pleasant.

Recently, more studies have emerged on the development of SB berry-based juices. Geertsen et al. (2016) investigated the hedonic characteristics of several newly developed SB berry beverages on the Danish consumer population. SB juice was mixed at different concentrations with locally grown rosehip, fennel, pear, aronia, beetroot, and redcurrant as novel products. The authors concluded that SB novel beverages were evenly liked by Danish consumers. Nevertheless, it was difficult for Danish consumers to include it in their day-to-day food habits. This study was interesting since it is an example of a current consumer response to hedonic features of novel SB juice preparations. Other authors have used SB to produce a highly acceptable berry squash – evaluating the most important features affecting its flavor (Selvamuthukumaran and Farhath 2017) – or by mixing sea berry squash (concentrated syrup of the fruit with the addition of sucrose, pH regulators and stabilizers) with other fruit nectars (e.g. apricot (Naik et al. 2017). Findings from these articles are presented in Table 1.

Other food products

SB is also interesting to ameliorate the negative effect that certain food products may trigger on consumers, or in other words, to give the product added value. Such is the case of sweet products. For instance, Stolzenbach et al. (2013) tested several aspects of different newly produced kinds of honey against traditional honey locally marketed in Denmark. They provided only the information on the classic and novel-produced honey. Novel kinds of honey included ingredients such as apple, SB, peppermint, mustard, or horseradish. Crushed SB was stated to be an ingredient of the SB honey. The main problem of SB honey for consumers was the probably disgusting and surprising taste, which was novel enough to trigger fear among the respondents. However, the authors highlighted the possibility that the results could be influenced by the way SB was said to be added in the honey. Further, the model used for the results explained only 79% of the variance, a percentage that could potentially be changed after a tasting experience of the real product.

Muffins are products in which SB has been added as an ingredient as well (Ursache et al. 2018). Briefly, SB carotenoids were extracted according to the method performed

Table 1 Additional articles investigating SB-juice-based beverages and other fermented food products

Authors and year	Product	Parameters evaluated	Conclusion
Naik et al. (2017)	Apricot nectar blended with SB berry squash in six different proportions	Total soluble solids (%), acidity (%), reducing sugars (%), total sugars, Brix acid ratio, and organoleptic score	A proportion of SB:apricot nectar of 40:60 was the best for consumer acceptance given the parameters measured.
Selvamuthukumaran and Farhath (2017)	Optimization of SB squash	Total soluble solids, acidity, total sugars, reducing sugars, carotenoids, anthocyanins, polyphenols, antioxidant activity, and vitamin C and E	Developed product was superior in all measurements performed when compared to pineapple or grape squash
Adadi et al. (2017)	Kölch beer with SB berries	Physicochemical parameters, microbiological stability, volatile compounds, and antioxidant capacity	SB Kölch beer showed a higher antioxidant capacity than the original Kölch beer. Hedonics were also better for SB beer as expressed by professional panelists. There were no observable changes in other parameters
Sturza et al. (2016)	Gingerbread and sponge cakes with 2 to 4% of SB flour by total flour weight	Structural and mechanical, physicochemical, and microbiological properties, and antioxidant capacity	SB flour addition to gingerbread and sponge cakes considerably improved their antioxidant capacity, conferred greater microbiological stability, improved appearance, color, and consistency but it also contributed to a greater moisture loss.

before by Ursache et al. (2017) and dissolved in blackcurrant oil. The carotenoid extract in oil was then mixed with whey protein in water solution to get oil in water emulsion. 1% w/v of gum acacia was then carefully added to the emulsion, pH was adjusted at constant stirring and temperature and finally the temperature was reduced, and samples were freeze-dried. This microencapsulated extract was then added to the mixing step of the muffin production process in a ratio of 6% of flour. The addition of microencapsulated powder made the muffin firmer and chewier, strongly correlated with the porosity of the food matrix. Contrarily, the addition of SB extract decreased cohesiveness and elasticity. Sensory analysis was statistically no significant when compared to normal muffins. Antioxidant activity was higher in the value-added muffin due to the high content of carotenoids and other bioactive compounds. Furthermore, the microbiological stability of the SB muffin was higher than the normal muffin, probably due to the marked antimicrobial effect of SB extracts.

The antimicrobial effect could be derived from specific compounds in SB berry, such as phenolics. However, the antimicrobial effect might be enhanced by the low pH of the berry juice. Nonetheless, products with a naturally low pH, such as fruit jam, are not much affected by the additional sourness brought by SB. Indeed, some countries have commercially available SB-derived jam. Fruit jelly containing ground SB cryopowder had also been developed and tested. Cryopowders were used by Gubsky et al. (2016) in the development of the fruit jelly. Cryopowders were produced by freeze-drying prior to low-temperature grinding. The study focused on the antioxidant capacity of the fruit jelly with a defined percentage of different cryopastes and cryopowders, one of which included SB-whole berry cryopowder. Among the 9 cryopowders used in their study, SB presented the second-highest total antioxidant activity employing bromine assay, preceded only by rosehip cryopowder. Fruit jelly was developed by mixing a syrup of approximately 80% of solids content consisting of pectin and sugar with a selected cryopaste. Cryopowder was added to the mixture after cooling of cryopaste addition. Thereupon, the developed SB fruit jelly also contained another cryopaste ingredient. SB berry jelly fruit was developed with either carrot or pumpkin cryopaste. Again, total antioxidant capacity was the second highest after any rosehip cryopowder jelly fruit developed.

Sea buckthorn in feed products

The development of food products often relies on ingredient sourcing and expensiveness. For example, the value of animal-derived ingredients is most affected by the growth rate of these animals, which is clearly influenced by the type of composition in their feed, among other variables. Different feeding products may have a different impact on the development of the animals and ultimately on the quality of the end-product or ingredient, leading to different end-point quality. Due to the interesting nutrient profiling of SB oils and juice, several authors have investigated its effect on the final quality of different animal products. Egg quality, broiler or pig meat quality, and sturgeon growth performance are few of the studied outcomes of including SB as a supplement in the feeding products.

Poultry farming

Broiler production efficiency is quite high per se, for the growth and development of a broiler to full potential does not take much more than 30 days. However, different and more natural strategies are needed to achieve the same or higher production efficiency, plainly because of the increasing consumer awareness on the feeding products. In that line, Vlaicu et al. (2017) studied the effect of three different diets on the carcass development, blood, and production parameters of broiler chicks. They compared three different diets in the growing and finishing stages of broiler chicks against a conventional diet, one including rapeseeds and grape meal and another including flaxseeds meal and SB meal. The diet was different in the two studied stages of broiler production (growing and finishing). Related to the overall meat quality, those broilers who were fed a diet with SB and flaxseed meals showed significantly higher concentrations of omega-3 polyunsaturated fatty acids when compared to the control or the group fed with rapeseeds and grape meal. However, by the end of the third phase of feeding (finished), the authors observed differences in the feed conversion ratio between the broilers fed with SB and the control-fed group. In other words, the final weight of SB-fed broilers was significantly lower than those fed with either a common diet or rapeseed and grape meal.

Another recent study performed by Pathak et al. (2015) interestingly used different parts of SB, including a leaf extract, pulp, and seed oil. In contrast to what Vlaicu et al. (2017) had later claimed to observe, supplementation with either leave extract, pulp, or seed oil increased the final bodyweight of the broiler. As reported earlier (Ma et al. 2015), supplementation with SB (any part) increased breast intramuscular fat, but contrarily decreased thigh intramuscular fat when compared to control. The authors found it reasonable to conclude that SB leaves, pulp, and seed oil could be introduced to a normal broiler's diet to help achieve better productivity and better overall carcass traits. Other authors also investigated SB as a potential ingredient for feeding

broilers, at different concentrations. Some of the authors also used flavones extracted from SB instead of SB fruit or derived products. Feeding flavones extracted from SB berries lead to the higher intramuscular fat content in thigh and breast tissue and final body weight (Ma et al. 2015). However, one of the main drawbacks of the study is the variability of flavonoid content in SB berries. The most common flavone is isorhamnetin, but no information on the feeding quantity was provided in the experiment. Thus, the effects on broiler meat quality or broiler productivity (i.e. broiler weight) cannot be attributed to a specific flavone but the flavones of the specific variety of berries used therein. As well, extraction of flavones from sea berries does not seem the most applicable way to provide SB's advantages in animal farming because of the extraction and probably costly step.

Other studies on the productivity and other hedonic parameters of broilers fed with SB supplements (berries, fruit residues) also claim to observe different beneficial effects. However, one of them claimed to observe a lower weight of broilers fed with berry residues when compared to the control group (Ben-Mahmoud et al. 2014), different than what was observed in other already detailed studies. The main findings of the mentioned articles appear textually depicted in Table 2.

Egg quality

Egg quality is an important feature to be assessed. As already depicted in Table 2, some authors have already assessed egg quality by production ratio (number of eggs per hen) or by weight. However, there are other features to be measured when speaking of egg quality. One of the main important traits is the color of the yolk, which has a significant impact on the overall egg quality. The color of the egg strongly relies on the diet with which the broiler has been fed. Different compounds could influence yolk color. For instance, carotenoids can influence the color of the eggs, when included as a supplement of a normal diet (e.g. Alay and Karadas 2017). As described before, SB contains considerable amounts of carotenoids, mainly present in the juice and the fruit residues of the berry. Thus, using either SB fruit extract or juice residues could have an influence on yolk color. In that line, Dvořák et al. (2017) and Shaker et al. (2018) have recently published evidence on the relationship between SB pomace (or fruit cake residue) and different parameters related to egg quality from old hens.

Dvořák et al. (2017) used SB pomace (fruit residue from juice extraction) as a supplement in different concentrations (2, 5, and 10%) to evaluate viscosity and color of egg yolk. They allocated 20 old hens in 4

Table 2 Additional articles investigating the effect of SB on broiler overall quality

Reference	Product	Parameters evaluated	Conclusion
Ma et al. (2015)	Diet supplementation during 42 days with flavones from SB fruits at 0,05, 0,10 or 0,15% of the total daily intake	Growth performance, carcass quality, fat deposition, and lipid metabolism	Supplementation of flavones from SB fruits at all ranges leads to an improved daily gain and final body weight. Intramuscular fat content in thigh and breast tissue was higher in broilers fed with flavones from SB fruits, yet abdominal fat percentage was significantly reduced in these groups when compared to control.
Kang et al. (2015)	Ad libitum diet supplemented with 0,1% vitamin C; 0,1% SB; 0,5% SB and 1% SB for 4 weeks	Egg production, feed conversion ratio, intake, egg weight, carcass yield, partial ratio (breast and neck), level of leukocytes, and erythrocytes. In addition, they measured DM, crude protein, crude fat, and crude ash as proximate analysis, and meat color, water holding capacity, cooking loss, and fatty acid concentrations.	Supplementation of SB in old laying hens for 4 weeks resulted in increased intake and increased partial ratio (breast/neck) only in 0.5% SB-fed hens. Egg weight was higher in basal and vitamin C-fed hen group. Other measured parameters did not show significant differences between groups.
Ben-Mahmoud et al. (2014)	Broilers included in the study were classified into 3 groups and were fed with diets supplemented with either natural color "Avizant Yellow 20S", 5% of SB fruit residues, or with no supplement (control).	Total weight, intake and feed conversion rate, health and mortality, dressing percentage, skin color, and metabolizable energy.	Broilers fed with 5% supplemented SB fruit residues diet showed a higher pigmentation of their skin. Weight was higher during the starter and grower diets when the diet was supplemented with SB fruit residues but lower at the finisher stage when compared to the control group. Skin pigmentation, final live weight, and feed conversion were surprisingly higher in the group fed with the colorant when compared to both other groups.

distinct groups, three of them receiving each one different concentration of SB pomace in their feed and the other acting as a control. The color of egg yolk changed significantly and could be visually appreciated and was more intense in the group fed with a higher amount of SB pomace. Egg yolks from hens supplemented with SB had a more intense red-yellow color. These eggs also showed lower viscosity. The authors firmly stated that both traits found on the eggs from SB pomace-fed hens were those preferred by consumers.

Similarly, Shaker et al. (2018) found that supplementing 5% of the wheat from a conventional diet with SB fruit residue leads to a darker and more intense yolk color of the eggs, a trait of importance by the consumers. As well, the authors found differences in the total number of laid eggs at the end of the experiment, the hens fed with 5% supplementation of SB had higher number of eggs. However, this difference became insignificant when adjusted by the number of hens. All other measured parameters to test egg quality did not give significant differences between groups.

Pig farming

Pigs are other important animals to consider investigating for meat quality. Meat producers use most of the carcass to produce meat and products thereof, swine rearing is a very profitable practice. The quality of the products derived from swine rearing is of utmost importance for the benefit of the companies who are producing it and for the consumers. Similar to what has been the base of supplementing with SB the broilers' diet, swine's diet could be similarly manipulated to investigate the overall resulting quality of the meat.

Nuernberg et al. (2015) recently investigated the effect of SB pomace supplementation at concentrations of 4, 8, and 12% in the finishing performance of a specific breed of meat-producing pigs. They investigated different meat quality parameters and intramuscular fatty acid and vitamin C contents in the muscle of pigs. None of the studied values showed significant differences between feeding diets. However, the authors observed a slight difference in the concentration of ω-3 fatty acids. The highest concentration was found in pigs fed with 12% supplementation of SB pomace. The authors conclude that the overall meat quality, including meat color, pH, or nutrient composition, was not different between experimental groups. They suggested a need of reformulating the pigs' diet to see a true and pronounced effect.

Lately, Dannenberger et al. (2018) used the results from the previous study (Nuernberg et al. 2015) to investigate the effect of supplementation of SB fruit pomace on circulating fatty acids, peripheral immune parameters and mRNA expression of different inflammatory-related hormones (namely corticotropin-releasing hormone, mineralocorticoid receptor, and glucocorticoid receptor) and receptors in hypothalamus and spleen of growing pigs. The experimental design was the same as previously reported (Nuernberg et al. 2015). Pigs fed with 12% SB for 8 weeks were selected for their study. The supplementation with SB turned in an increased concentration of linoleic acid in plasma and subsequently in an increase in ω-6/ω-3 ratio when compared to the control group (fed with 0% SB, same time span). No significant differences were observed on any other parameter related to circulating fatty acids, peripheral immune parameters, or mRNA expression of inflammatory-related hormones and receptors in the hypothalamus and spleen. The authors pointed out that effects derived from supplementation with a rich source of ω-3 fatty acids (such as SB) might be more appreciable on stressful situations.

Fish farming

Rearing fish to produce fish-meat at a competitive price is already a reality. Different from conventional fishing, fish farming does not involve the destruction of the marine ecosystem, a practice encouraged by many ecosystem protection organizations. In addition, productivity and quality of the final product are outcomes that can be changed through the fish feed, something unachievable by conventional fishing. There are several types of fish that can be reared in a confined space. Some of the already reared fish species with a noticeable profit which could be found in the market are salmon or sturgeon, among others. The use of SB has been spread to fish farming, mainly to investigate the effect of SB consumption in productivity and mortality of the reared fish. The weight of the fish is very important for greater efficiency in production and better quality in the end-product. Also, some studies have shown interesting results on lipid peroxidation (Antache et al. 2013), an important matter in fish quality, considering the high percentage of unsaturated fatty acids found in fish meat.

Not only SB, but other plant extracts are of interest when investigating the performance of fish farming. In that line, Antache et al. (2013) investigated the influence of rosemary, SB, or ginger supplementation on oxidative stress of reared Nile tilapia. They classified all the fish for the experiments in either control (receiving none of the experimental supplementations), 1% rosemary, 1% SB or 1% ginger powder group. Groups were fed for 6 weeks. Oxidative stress was assessed by the concentration of malondialdehyde and total antioxidant capacity from muscle, liver, gut, and plasma and reduced glutathione from the blood. SB supplementation to the feeding of Nile tilapia derived in a decrease in malondialdehyde concentration in blood plasma. Malondialdehyde concentration is often referred to as a great index

to assess lipid peroxidation. The total antioxidant capacity did not show significant differences between groups. The following year, Antache et al. (2014) published a second article evaluating other important biochemical indexes, such as hemoglobin concentration, hematocrit percentage, or cortisol concentration in blood. The experiment was the same as previously described (Antache et al. 2013). The authors observed that supplementation with 1% SB leads to an overall improvement of the physiological status in Nile tilapia most likely due to its influence on several biochemical blood parameters.

More recently, SB has been used as a supplement in the diet for different species of fishes. Dorojan et al. (2015) used a supplementation percentage of 1% on sturgeon juveniles with different genetic backgrounds. Understood as a positive control, they added vitamin E at concentration values of 500 mg/kg feed. Sturgeons were fed by 60 days, 3 times per day, with a daily ration of 2.6% of the fish body weight. Different from what previous authors had measured, the authors evaluated the growth performance of sturgeons. They found that the beneficial effect of SB supplementation was similar between groups with a genetically different background. After supplementing the fish feed with 1% SB/ kg feed and part of vitamin E (500 mg / kg feed), all groups showed a significant increase in the final weight and total length of the fish.

Discussion

Sea buckthorn as a food ingredient

There is a continuous growing awareness of healthy eating in our society. The consumption of plant-derived products is taking over the consumption of other products from animal origin, plainly due to emerging evidence proving their health-related benefits. SB is a very interesting plant fruiting little berries to which several health-related improvements have been attributed. Not only this, but SB has many applications within the food and feed industry. This review has summarized recent advances in the field. Indeed, SB derived food supplements are already commercialized, probably because of its old habit to be used as a natural medicine against several health problems. More interestingly, due to the well-established health benefits resulting from consuming SB, the addition of SB-derived ingredients to normal food products could serve to turn them into value-added food products. These value-food products, if marketed, would ameliorate the harmful effect of certain consumption patterns on human health. Besides, adding such a valuable ingredient into already marketed food products could open a wide window for product development and could be used by any company as a new spot in the market where to settle.

However, considerations should be taken when addressing the real needs of the consumer market. One of the most interesting opportunities that could be considered is the juice market. In 2015, fruit juice consumption was 9.6 billion liters in the European Union (EU), which translates to 18.9 l per capita. The global consumption of fruit juice and nectar consumption was 38.5 billion liters, with the EU having the greatest consumers, followed by North America (AIJN European Fruit Juice Association 2016). The likely tendency following the growing demands for natural fruit and vegetable juices is to find a spot where to include SB juice as part of a multi-fruit juice. This will exaggeratedly raise the content of vitamin C of the juice and will confer it a very nice strong-orange color (depending on the added amount). A multi-fruit juice is a great product benefiting from SB juice, but there are many other applications within the juice industry. For instance, when developing a long shelf-life fruit juice, it has to undergo elevated temperatures to achieve a microbiologically safe product. This treatment will inevitably damage several compounds naturally retained in the fruit, such as vitamin C. The addition of SB juice would lead to reduced damage on the vitamin C content due to its additional incorporated vitamin C concentration and its derived very high antioxidant activity (Ursache et al. 2017; Guo et al. 2017b ; Papuc et al. 2008). It is important to note that the inclusion of SB juice will also bring a strong astringent taste, which could be difficult to mask. As explained before, strong flavors could help hide this specific astringent taste (Tiitinen et al. 2006b).

The antioxidant activity of SB, together with its antimicrobiological activity (Michel et al. 2012) could be used for product formulation. Many different products have shelf-life problems that SB could help tackle, for instance, a ready-to-eat food product. Sturza et al. (2016) highlighted the significant improvement in the microbiological stability of their studied food product – in that case, bread – when adding an SB-derived ingredient into the food matrix. Besides helping extend its shelf-life due to its natural composition, the SB ingredients would also bring an added value to the products where it is incorporated.

SB contains great amounts of different polyphenols and other compounds which have a demonstrated antimicrobiological effect. The composition and nature of the berry make the use of SB-based ingredients unsuitable for fermented food products. However, as some authors already claimed, SB does not only affect the growth of other beneficial bacteria but also improves it. This promoting benefit of adding SB has been observed in yoghurt (Selvamuthukumaran and Khanum 2015). In addition, it also serves as an instrument to upgrade the phytochemical characteristics and aroma profile of feta

cheese if applied as an immobilization carrier for specific strains of *L. casei* (Terpou et al. 2017). It is not clear yet why SB exerts these promoting effects on probiotic bacteria strains. Undoubtedly, it is one of the fields with great potential for SB, since any resulting product would also bear the antioxidant capacity of SB.

The application of SB ingredients is strongly dependent on industry interests and therefore, research in this field would most likely be reliant on industries' new product development programs. These programs also focus on the profitability of the entire process, which becomes strongly dependent on the residue produced. From SB juice production, as in many other berry processing, a major residue is formed, the berry pressed cake. The pressed cake has a great antioxidant capacity and relatively high amounts of phenolic compounds when compared to other common Finnish berries, which makes it a good value-added by-product for its further use within the food industry. It could be either sold as a food ingredient or as a feed ingredient. Either way, it could be seen the potential of SB to be included as a food ingredient, not only for its versatile applicability in many different food products but for the profit its by-products result in.

Sea buckthorn as a feed ingredient

There is an increasing demand worldwide for high protein products. This high demand evokes the high competitiveness of this market, where the overall product quality gains more importance than ever. On top, a high protein product demand inevitably turns into more research focusing on improvements in animal farming productivity.

Besides having several interesting health-benefits derived from its direct consumption, SB has been recently investigated due to its promising potential of improving the performance of animal production when supplemented in the feed (Table 2). As disclosed in the present review, several parameters have been subject to investigation for the performance and health of different animals to achieve better productivity. The most studied animals in terms of animal husbandry which have been subject to supplementation with SB are poultry. This is reasonable, for broilers usually grow to its full potential within a month, making it possible to study the final effect of SB supplementation in a short period.

Most of the published studies investigating broiler productivity remark the positive effect of SB supplementation. Most of the herein presented scientific research showed positive results, yet many others did not find a significant contribution to different evaluated parameters (i.e. final weight) when using SB as a feed ingredient. The observed controversy could be influenced by the type of broiler used to perform the study as well as the type of SB ingredient supplied to them. One the one hand, different species of broilers have different metabolic rates and feed conversion ratios. On the other hand, every SB product has its own profile and its own physiological implications in broilers.

Parts of the positive effects observed on broiler also seem to be true for swine production. SB supplementation could lead to an increased concentration of intramuscular fat, mainly unsaturated fatty acids in pigs (Dannenberger et al. 2018). However, other parameters such as improvement in productivity have only been appreciated in broilers. Swine production needs a longer time span to achieve a full-grown individual, ready to be butchered. In addition, the metabolic rate of swine and boilers differ drastically. The nature of the animal is a critical factor to consider the feeding time, quantity, and concentration of SB to be used.

Another important quality factor for animal products is the color. Different techniques can be used to achieve better color in meat. However, SB has not been used as such, since its color improvement ability is proven to be very limited (i.e. skin pigmentation increased (Ben-Mahmoud et al. 2014)). SB has been used to change colors of animal products strongly dependents of their carotenoid composition. Previously detailed, SB fruit contains significant amounts of carotenoids (Pop et al. 2014). Carotenoids are uniquely produced by plants, bacteria, and fungi and are considered precursors of vitamin A. Carotenoids are responsible for the orange color that can be observed in different fruits and vegetables or even in deciduous trees on specific seasons. Carotenoids can be absorbed by animals and can be stored in the fat tissue due to its lipophilic nature. Orange colors of some animal products are derived from increased carotenoid consumption and from the greater ability to store it. This is the case of eggs. SB supplementation in egg-producing hens influences egg yolk color, deriving in more intense orange color (Dvořák et al. 2017). Egg yolk color is one of the main factors affecting egg overall quality (Roberts 2004). The more intense the color is, the better the quality is perceived by consumers. Most of the studies herein discussed egg quality highlighted the efficiency of using SB as a feed ingredient for egg-producing hens when evaluating egg yolk color. Carotenoids from SB are principally found in the oily part of the pulp – although some can be found in the peel. Therefore, the use of SB pulp oil on the development of feeding ingredients for egg-producing hens is a newly explored field to increase egg quality.

Additionally, other orange-colored animal products could also benefit from the use of SB pulp oil. Salmon

flesh quality benefits from carotenoid concentration. Flesh color is the most important attribute by consumers when buying the captive salmonid (Scientific Committee on Animal Nutrition 2002). Salvage salmon gets its orange color from ingesting krill and other shell-fish retaining carotenoids. On the contrary, farmed salmon does not have this advantage. It is thus important to incorporate carotenoids in captive salmon's feed to obtain an approximate quality. To the best of our knowledge, no study has been conducted to evaluate the possible incorporation of SB pulp oil (or a carotenoid extract) to salmon's feed to achieve a stronger orange color.

Salmon flesh and egg yolk color are two highly commercialized food products that could benefit from SB addition as an ingredient. Usually, the feed used in the egg and salmon industries is efficient because they bear acceptable concentrations of carotenoids. These carotenoids can be used gently at a low cost. However, the addition of these compounds is continuously evaluated due to the awareness of their sourcing and stability. For instance, canthaxanthin has been widely used for this purpose and has been strictly evaluated because of that, resulting in different limitations (European Food Safety Authority 2014). The use of SB would be a natural and effective alternative to help improve the color of the aforementioned animal colors.

SB supplementation in fish farming has been recently used for other purposes as well. Recent studies have demonstrated that SB could be included as a feed supplement to improve the overall health in Nile tilapia (Antache et al. 2013) and to possibly improve growth performance in sturgeons (Dorojan et al. 2015). However, it should be noticed that studies investigating this improvement in productivity are scarce. Results coming from this usage in fish farming should be further studied to evaluate their significance. Nevertheless, when it comes to fish farming, the most interesting field of study is salmon rearing, because of the already discussed reasoning.

Conclusion

The shift towards healthy food and life habits among consumers is now a reality. Consumers constantly look for healthy products in the market, and most recently to what is commonly designed to as "superfoods". Superfoods are those food products that have a very high concentration of one or more bioactive compounds to which certain health benefits have been attributed. This is the case of SB. Even though it is narrowly produced – and consumed – in specific geographical areas, where it has been used for a long time. SB has an enormous exploitability within the food industry. There have been different applications of SB in food product development (e.g. SB-based yoghurt) whereas others are new ways of innovation. Specific bioactive compounds (e.g. carotenoids and egg yolk color) are advantages of SB application in food industry. Either way, its impressive phytochemical profile makes it suitable for applications such as those discussed herein and many others still unexplored. Thus, the use of SB in the food industry is highly encouraged and should be widely exploited.

Abbreviations

SB: Sea buckthorn; WHO: World Health Organisation; FAO: Food and Agriculture Organisation of the United Nations; ω-7: Omega-7 fatty acid; ω-6: Omega-6 fatty acid; ω-3: Omega-3 fatty acid; AIJN: European Fruit Juice Association; MRS: Man-Rogosa-Sharpe culturing medium; CFU: Colony Forming Units; GAE: Gallic Acid Equivalents

Acknowledgments

Authors would like to express their gratitude to "Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR)", a public entity attached to the "Departament d'Empresa i Coneixement" of the "Generalitat de Catalunya", from which the first author received a predoctoral studentship grant coded '2017 DI 079'.

Authors' contributions

A. V.-F. designed and conceived the review and all the tables and figures in it. B. J. and J. S. contributed to the final revision of the manuscript. The author(s) read and approved the final manuscript.

Funding

The authors declare that no funding was provided for the present work.

Availability of data and materials

Data and material used include all the original reviewed articles and are available.

Competing interests

The authors declare that they have no competing interests.

Received: 20 February 2020 Accepted: 29 July 2020
Published online: 17 August 2020

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Publisher's Note

Chapter 2: Aims and objectives

According to the European Food Safety Authority (2021), dietary supplements are defined as "concentrated sources of nutrients or other substances with a nutritional or physiological effect that are marketed in 'dose' form (e.g. pills, tablets, capsules, liquids in measured doses)". The dietary supplements market size was estimated to be USD 14.95 billion in 2019 and was projected to grow to USD 33.80 billion by 2027 (Fortune Business Insight, 2019).

Food supplements are developed with a wide range of ingredients. Some supplements are made of very specific bioactive compounds, but the vast majority consist of powder extracts or concentrated liquids. In the latter, the cost of the final product will change according to the cost of the ingredients, which will be usually low when compared to the former. Therefore, companies seek to incorporate raw ingredients which contain high amounts of bioactive compounds so that, with minimal processing, a highly valued ingredient could be obtained. In addition, minimal processing is also an important attribute contributing to the naturalness of the final product, and subsequently upgrading its inner quality.

In that line, sea buckthorn berries emerged some years ago as a very interesting ingredient for the food supplement industry, because highly valuable ingredients could be obtained with minimal processing. Vitae

Health Innovation S.L., a company focused on the formulation and production of food supplements, had spotted the advantage of working with this berry in the past. In fact, certain food supplements containing sea buckthorn oil are already being commercialized by the company (e.g. Oliovita®, Oliovita® Protect, Oliovita® Intima, BucoVitae® or Oliovita® Balm). Precisely due to the industrial interest of the sponsoring company, a region in 'La Cerdanya', a county from Catalunya (Spain) was adapted for the growth of this plant to achieve a sustained production and supply of sea buckthorn berries over the years.

Resulting from the founded interest of the company on this berry, the present project arose as an industrial PhD partnering the sponsoring company with the Universitat Autònoma de Barcelona (UAB). The aim of the present work was:

- To maximize the use of all fractions from sea buckthorn berries for the development of diverse food supplements with unique nutritional profiles.

In order to achieve this ambitious and broad aim, the following objectives were set:

1. Understand the full nutritional profile of commercially available berries from different origins harvested on the same year, including berries harvested by the company.

2. Study of the nutritional profile of berries exclusively obtained from the orchard located in 'La Cerdanya' over three consecutive years.
3. Extraction of oil from dried sea buckthorn berry with green and conventional solvents at different temperatures.
4. Study of the optimal conditions for the extraction of sea buckthorn berry oil using supercritical CO_2 technology.
5. Study of the concentration of sea buckthorn juice by two non-thermal technologies and their comparison with a conventional thermal concentration.
6. Hedonic evaluation and physicochemical quantification of a newly formulated sea buckthorn-based fruit juice.
7. Study of the fermentation of sea buckthorn juice by lactic acid bacteria to improve its nutritional profile.

To understand how important the set objectives were to the successful development of the thesis and to get a visual representation of the study framework, a scheme was developed (Figure 1). The objectives were divided in different parts depending on the broader aim they were addressing; the objectives 1 and 2 were detailed in the second part of the thesis, the objectives 3 and 4 were explored in the third part of the thesis, and the objectives 6, 7 and 8 were studied on the fourth part of the present thesis project.

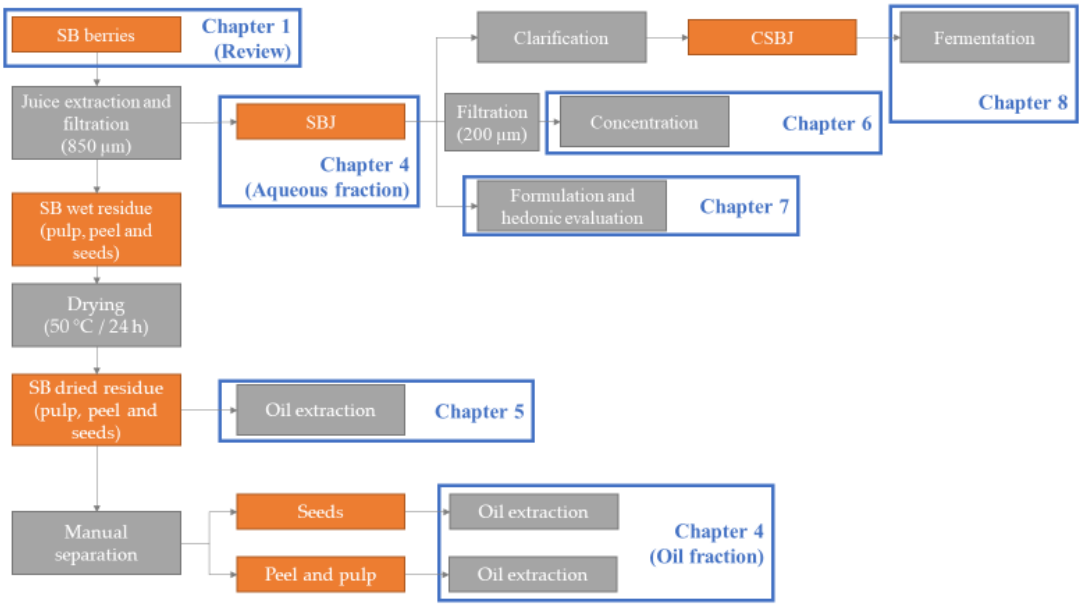

Figure 1. Schematic representation of the aim of the present industrial PhD project divided by chapters. In orange: products; in grey: processes. SB: sea buckthorn; SBJ: sea buckthorn juice; CSBJ: clarified sea buckthorn juice.

2.1. References

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Chapter 3: Materials and methods

3.1. Management of the samples

The term ‘sample(s)’ is used to refer to any sample obtained from an experimental process which had to be submitted to physical or phytochemical (analytical) quantification. All methodological processes herein indicated were performed in every sample from the same study. Any variation during sample preparation or management can be found in the corresponding chapter.

Samples were stored at different temperatures according to the needs. Samples that were to be submitted to physical parameters evaluation were stored at -30 ºC until the day of the analysis. Samples that were to be submitted to analytical quantification – either from the aqueous fraction or from the lipidic fraction – were stored at -80 ºC until the day of the analysis. No samples were stored for more than 3 months before the analysis. All samples were analyzed immediately after thawing. Thawing was performed overnight at 4 ºC, if not stated otherwise. Samples were processed thrice for each analysis, except when indicated otherwise.

3.2. Physical parameters

3.2.1. Total soluble solids (TSS)

Total soluble solids were measured as ˚Brix directly from sea buckthorn juice (SBJ) by using a manual refractometer (Serie 300, Auxilab S. L., Navarra, Spain).

3.2.2. Quantification of dry matter

Approximately 5 g of sample was pipetted in a stainless-steel reservoir suitable to be placed in the oven. After exposing the sample to 100 ºC for 24 hours using a lab-oven (universal use, Memmert GmbH + Co. KG, Schwabach, Germany), the sample was weighed again. Dry matter was calculated by weight difference.

3.2.3. pH

pH was measured from liquid solutions using a Manual pH meter Basic 20 (Crison Instruments S. A., Catalunya, Spain). Calibration of the equipment was performed prior to each analysis by using Hach calibration pH buffers of pH 4.01, 7.00 and 10.01 (Hach, Colorado, U. S.).

3.2.4. Quantification of ashes

Approximately 3 g of sample was pipetted in an oven-suitable ceramic crucible and place it inside a Select-Horn-TFT Muffle Furnace oven (J. P. Selecta, Catalunya, Spain) and left at 500 ºC for 24 hours. Ashes were quantified by weight difference.

3.2.5. Color

Color was measured with a manual MiniScan™ XE color meter (Hunter Associates Laboratory Inc., Virginia, U. S.), illuminant D65 and 10° standard observer. A total of 30 ml of sample were placed in a pre-designed container suitable for the used color meter, and covered with a black, opaque cover. The $L*$ (luminosity, 0-100 (black/white)), $a*$ (greenness/redness, negative to positive), and $b*$ (blueness/yellowness, negative to positive) values of the samples were measured using CIELAB coordinates.

3.3. Phytochemical analysis of the aqueous fraction

3.3.1. Total chloride

Total chloride quantification was performed by using the Model 926 Chloride Analyzer. A total of 0.5 ml of the sample was pipetted inside the reaction vessel containing a Combined Acid Buffer, in which the electrodes

and an electric stirrer were submerged. The reading was provided electronically as mg Cl^-/ L of solution. Calibration was performed using a Chloride Meter Standard of 200 mg Cl^-/ L. The equipment and all the reagents herein detailed for the chloride analysis were purchased from Sherwood Scientific Ltd. (Cambridge, U.K.).

3.3.2. Mineral profile

SBJ filtered at 200 µm was used for mineral content analysis. Briefly, after a vigorous agitation, 0.25 g of sample were weighed accurately and subsequently digested in a microwave oven (Milestone, Ultrawave model) with concentrated nitric acid (ACS reagent 70%, Merck KGaA, Darmstadt, Germany). Samples were then used for analysis using an inductively coupled plasma atomic emission spectroscopy (ICP-OES) (model Optima 4300DV, Perkin-Elmer Ltd., Massachusetts, U. S.)

3.3.3. Sugar and organic acids profile

A simultaneous methodology of quantification of mono-, di- and oligosaccharides and organic acids was performed following the methodology previously developed by Schweiger, Baier, Persicke, & Müller (2014). Briefly, the frozen samples (-80 ºC) were thawed just before analyses, centrifuged for 5 min at 12,000 rpm and diluted 1/25 (v/v) in a solution of 1/1 methanol:mili-Q water (v/v) containing tartaric acid (>99%,

Panreac Química S. L. U., Catalunya, Spain) at 0.75 mg/mL as internal standard. The samples were then evaporated to dryness under a continuous flux of nitrogen at room temperature. Derivatization was conducted by incubating the dried extracts at 37 ºC with O-methylhydroxylamine hydrochloride (Merck KGaA, Dramstadt, Germany) dissolved in pyridine (Merck KGaA, Dramstadt, Germany) during 90 min, and later with N-methyl-N-trimethylsilyltrifluorocetamide (Merck KGaA, Dramstadt, Germany) during 30 min. The samples were then immediately injected to a gas chromatograph 7820A (Agilent Technologies, California, U.S.) coupled with an autosampler.

A capillary column VF5-ms (30 m with 10 m EZ Guard x 250 µm x 0.25 µm, Agilent Technologies, California, U.S.) was used for the chromatographic analysis and helium was used as the carrier gas at 1.2 ml/min. The oven temperature was raised from 80 ºC to 325 ºC at 5 ºC/min for a total run time of 55 min. Samples were injected in a pulsed-split mode (ratio 1:10). The most abundant peaks were identified in comparison with commercial standards (all of them purchased from Merck KGaA (Dramstadt, Germany)). The analytes were quantified by their peak areas using the OpenLab EZChrom edition (Agilent Technologies, California, U.S.) and data were then aligned using the R package ‘GCalignR’. Peaks present in three or more blank samples and those present in less than four samples were removed from the final dataset. For metabolites that formed

different analytes during derivatization (i. e., fructose and glucose), the peak areas of the analytes were summed up. Concentrations of the identified peaks were calculated based on the peak areas of the internal standard corrected by the corresponding response factor for each compound.

3.3.4. Ascorbic acid and vitamin C

Ascorbic acid and total vitamin C content were quantified according to a method previously described by Odriozola-Serrano, Hernández-Jover, & Martín-Belloso (2007). Briefly, 5 g of sample (SBJ) were diluted with 5 ml of metaphosphoric acid (ACS reagent chips 33.5-36.5%, Merck KGaA, Dramstadt, Germany) at 4.5%, homogenized for 5 minutes and centrifuged at 14,000*g* for 15 min at 4 ˚C. This was the processed sample. The sample was taken and passed through a 0.45 µm mixed-ester cellulose filter (Labbox Labware, S. L., Catalunya, Spain) and injected directly to the HPLC system (Dionex Corporation, California, U. S.). Concentrations were checked against a calibration curve developed from a stock solution of 0.05 mg/L of L-ascorbic acid (99%, Merck KGaA, Darmstadt, Germany).

For total vitamin C quantification, 0.2 ml of the reducing agent 1, 4-dithiothreitol (DTT) (Merck KGaA, Darmstadt, Germany) at concentration rate of 20 mg/ml were added to an aliquot containing 1 ml of the processed sample. The solution was then kept from light exposure for 2 hours at room

temperature. Immediately after, the solution was filtered and injected to the HPLC.

The HPLC methodology was the same for both ascorbic acid and vitamin C quantification. The HPLC system consisted of a P680 HPLC Pump attached to an ASI-100 Automated Sample Injector and a Thermostatted Column Compartment TCC-100. Mobile phases were placed in a Solvent Rack SOR-100. The wavelength detection was performed with a UVD170U. All the parts of the HPLC were from Dionex Corporation (California, U.S.). A reverse-phase C18 Tracer Extrasil ODS2 stainless steel column (5 µm pore size, 4.6 mm x 250 mm) from Teknokroma Analítica S. A. (Catalunya, Spain) was used as the stationary phase. The mobile phase consisted of a pH-adjusted (2.4-2.6) acidic aqueous solution of 0.01% sulfuric acid (98% AGR, Labbox Labware, S. L., Catalunya, Spain). An exact amount of 20 µl of the sample was injected to the HPLC system, with a flow rate of 1 ml/min and operating at room temperature. The detection wavelength was set at 245 nm.

3.3.5. DPPH assay

This methodology is based on the colorimetric quantification of the reduced product DPPHH form its former hydrazine 1,1-diphenyl-2-picrylhydrazyl (DPPH·), which is an indirect quantification of the reducing agents in a sample. The methodology was adapted from Suárez-Jacobo et al. (2011).

SBJ was first diluted in milli-Q water at 1:10 (v/v). Diluted SBJ was then clarified at 14,000 *g* for 15 min at 4 ˚C and filtered using a Whatman® qualitative filter paper grade 1 (Merck KGaA, Darmstadt, Germany). An exact aliquot of 50 µl of the clarified and filtered solution was pipetted to a 2.5 ml spectrophotometric cuvette. Immediately after, 1.95 ml of methanolic DPPH· solution at 0.1 mM was added to the cuvette (methanol GC/HPLC GGR was purchased from Labbox Labware S. L. (Catalunya, Spain); DPPH· from Merck KGaA (Darmstadt, Germany)). The cuvettes were then gently shaken and kept at complete darkness for one hour. Absorbance was then read at 515 nm using a UV 3210 digital spectrophotometer (Dinko Instruments, Catalunya, Spain). The results were checked against a five-point calibration curve developed from a 2 mM methanolic stock solution of (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, from Merck KGaA, Darmstadt, Germany).

3.3.6. FRAP assay

This methodology is based on the sample's capacity to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), which translates into a notable chromatographic change. The methodology was adapted from Suárez-Jacobo et al. (2011). The FRAP solution consisted in 2.5 ml of 2,4,6-tripyridyl-s-triazine (TPTZ, from Merck KGaA, Darmstadt, Germany) 10 mM solution in hydrochloric acid (37% AGR ISO, Labbox Labware S. L.,

Catalunya, Spain), 2.5 ml of $FeCl_3$ (reagent grade, 97%, Merck KGaA, Darmstadt, Germany) 20 mM aqueous solution, and 25 ml of acetate buffer (pH 3.6) for a total volume of 30 ml. The acetate buffer was prepared by mixing the appropriate amounts of acetic acid glacial (99.5% GLR, Labbox Labware S. L., Catalunya, Spain) and sodium acetate (ACS reagent, ≥ 99.0%, Merck KGaA, Darmstadt, Germany). The buffer solution could be stored for up to 6 months. The FRAP solution was freshly prepared each day of analysis and warmed up to 37 ˚C prior to its use.

SBJ was diluted 1:10 with mili-Q water. The diluted sample was then centrifuged at 14,000*g* for 15 min at 4 ˚C, then filtered using a Whatman® qualitative filter paper grade 1 (Merck KGaA, Darmstadt, Germany) and pipetted (85 µl) to a 3 ml cuvette, where was mixed with 8.82% (265 µl) of mili-Q water and 88.24% (2.7 ml) of warmed FRAP solution, as indicated by previous authors (Stracke, Rufer, Weibel, Bub, & Watzl, 2009). The cuvettes were stored at room temperature under dark conditions for 30 min. The absorbance was then read at 593 nm and the resulting value was compared against a five-point calibration curve obtained from a 350 µM methanolic stock solution of Trolox (Merck KGaA, Darmstadt, Germany).

3.3.7. Total polyphenols

The method was adapted from the validated method developed by Singleton, Orthofer, & Lamuela-Raventos (1999). SBJ was firstly clarified

at 14,000*g* for 15 min at 4 ˚C and filtered using a Whatman® qualitative filter paper grade 1 (Merck KGaA, Darmstadt, Germany). The sample was then dissolved 1:10 with distilled water.

200 µl of the dissolved solution were added to different cuvettes for spectrophotometric analysis. The cuvette was subsequently filled until 2.5 ml total volume with 75.2% (1.88 ml) of mili-Q water, 4.4% (110 µl) of Folin-Ciocalteau reagent (2 N solution, Merck KGaA, Darmstadt, Germany) and 12.4% (310 µl) of a 20% aqueous solution of $NaCO_3$ (anhydrous AGR, Labbox Labware S. L., Catalunya, Spain). The cuvettes were gently mixed and kept in complete darkness for 60 min at room temperature. The sample absorbance was read at 760 nm using a UV2310 spectrophotometer (Dinko Instruments, Barcelona, Spain).

3.3.8. Total proanthocyanins

Proanthocyanins were quantified as condensed tannins according to the method described by Rösch, Bergmann, Knorr, & Kroh, (2003). An acidic reagent and a blank reagent were used for the analysis. The acidic reagent was an iron (III) sulfate hydrate (97%, Merck KGaA, Darmstadt, Germany) solution in 1-butanol (99.5% AGR, Labbox Labware S. L., Catalunya, Spain) at concentration rate of 0.7 g/L. Concentrated hydrochloric acid (37% AGR ISO, Labbox Labware S. L., Catalunya, Spain) was used to fully

dissolve iron (III) sulfate hydrate and built up a total of 5% of the final acidic reagent. Blank reagent consisted only of 1-butanol.

SBJ was firstly clarified at 14,000g for 15 min at 4 ˚C and filtered using a Whatman® qualitative filter paper grade 1 (Merck KGaA, Darmstadt, Germany). The juice was then dissolved 1:10 with distilled water. 1 ml of dissolved SBJ was poured in a Pyrex®-glass test tube and 6 ml of acidic reagent or blank was added. Tubes were heated in a water bath at 95 ˚C during 50 min. After heating, tubes were cooled down by sinking them in a water bath set at room temperature. The solution was then centrifuged at 10,000g for 5 min at room temperature. The supernatant was pipetted out to a cuvette. Absorbance was read at 550 nm using a UV2310 spectrophotometer (Dinko Instruments, Barcelona, Spain).

The final concentration of proanthocyanins was calculated by using the Lambert-Beer equation and the molar extinction coefficient of the cyanidin, previously published by Rösch et al., (2003) at 550 nm, who used butanol as a reagent for the quantification among other solvents. Therefore:

$$Abs\ (550\ nm) = 17{,}360 \frac{L}{mol \cdot cm} \cdot\ 1\ cm\ \cdot [cyanidin](\frac{mol}{L})$$

3.4. Phytochemical analysis of the lipid fraction

3.4.1. Quantification of α-tocopherol and β-carotene

A simultaneous quantification of β-carotene and α-tocopherol was successfully adapted from Gimeno et al., (2000). Briefly, 400 mg of sea buckthorn oil (SBO) were mixed with 0.2 g of L-ascorbic acid (99%, Merck KGaA (Darmstadt, Germany), 15 ml of absolute ethanol and 4 ml of a 76% potassium hydroxide solution in that order in a centrifugation screw-capped tube. The tubes were then incubated at 70 ˚C for 30 minutes with slow constant stirring. After cooling, 5 ml of sodium chloride at 25 g/L were added and the solution was vigorously mixed. The resulting product was extracted two times with 20 ml portions of *n*-hexane (≥95%, HPLC grade, Merck KGaA, Darmstadt, Germany) and ethyl acetate (≥99.5% ACS reagent, Merck KGaA, Darmstadt, Germany) at a ratio of 85:15 (*v*/*v*). The organic phase was recovered and brought to dryness at 40 ˚C under vacuum conditions. Finally, the residue was resuspended with 3 ml of methanol and passed through a 0.45 μm filter and directly injected to the HPLC.

The HPLC system consisted of a P680 HPLC Pump attached to an ASI-100 Automated Sample Injector and a Thermostatted Column Compartment TCC-100. Mobile phases were placed in a Solvent Rack SOR-100. The wavelength detection was performed with a UVD170U. All the parts of the HPLC were from Dionex Corporation (California, U. S.).

HPLC oven temperature was set at 45 ˚C, sample volume was 50 µl and injected at 1.325 ml/min. Timespan was efficiently set at 20 min, and a gradient profile was 97:3 of solution A and solution B, respectively, for 6 min, then linear gradient to 100% A in 2 min, to an isocratic step of 100% solution A for 10 min and finally a linear gradient to solution A and B (97:3) in 2 min, where solution A was methanol:butanol (92:8, respectively), and solution B was mili-Q water 100%. The wavelength for detection of α-tocopherol was set at 292 nm and wavelength detection of β-carotene was set at 450 nm. DL-α-tocopherol acetate (HPLC standard, Merck KGaA, Darmstadt, Germany) was used as internal standard, giving steady recovery values of 70%. α-tocopherol (synthetic, ≥96%, HPLC standard) and β-carotene (synthetic, ≥93%, analytical standard) from Merck KGaA (Darmstadt, Germany) were used as standard.

3.4.2. Quantification of fatty acids

A rapid extraction methodology developed by Lamba, Modak, & Madras (2017) was adapted to sea buckthorn oil. Briefly, 0.15 g of extracted SBO was poured into a 15 ml screw-capped test tube and mix it with 2 ml of *n*-hexane (≥95%, HPLC grade, Merck KGaA, Germany) and 1 ml of 2 M methanolic KOH solution (methanol GC/HPLC GGR from Labbox Labware S. L. (Catalunya, Spain); KOH pellets ACS reagent ≥85% from Merck KGaA (Darmstadt, Germany)). The mix was shaken vigorously for

30 seconds and held in a water bath previously warmed at 70 ˚C for 2 minutes. The tube was then taken out of the bath and cooled at room temperature for 2 minutes, and immediately after 1.2 ml of HCl 1 N (Panreac Química S. L. U., Catalunya, Spain) was added to the tube. Gently stirring was applied for 10 min and then the mix was left undisturbed. Separation in two phases occurred after 15 min. An aliquot of the upper phase was directly injected to the gas chromatograph for analysis.

A capillary column VF-5 ms, 30 m x 0.25 mm with 0.25 µm film thickness containing 5% phenyl-methylpolysiloxane attached to a gas chromatograph 6890 (Agilent Technologies, California, U. S.) was used for the chromatographic analysis. Helium was used as the carrier gas at 1.7 ml/min, and oxygen and hydrogen served as fuel gases. The oven temperature was raised from 75 to 240 ºC at 5 ºC/min and held at 240 ºC for 20 min. The split value was 1:40 and isopropanol (≥99.5 ACS reagent, Merck KGaA, Darmstadt, Germany) was used as a rinsing agent.

Peak identification was performed using different techniques. First, a standard mix of alkanes (Standard Connecticut ETPH Calibration Mixture (15 Components, C9 to C36 at concentration rate of 1,000 µg/mL dissolved in methylene chloride), Restek, Bellefonte, U.S.) was used to selectively isolate and identify the hydrocarbons of the sample with the GC 6890 system. Secondly, the retention time of each fatty acid of interest appearing

in the chromatogram was compared with the known Kovats retention index. Finally, peak identification was performed with a GC System 7890A attached to a MS triple-axis detector 5975C (Agilent Technologies, California, U. S.) with the Wiley library and by comparison of the retention time of bought standards of methylated fatty acids (Supelco 37 Component FAME Mix, Merck KGaA, Darmstadt, Germany).

3.5. References

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PART II

EXPERIMENTAL STUDY OF THE PHYTOCHEMICAL PROFILE OF THE BERRY

Chapter 4: Phytochemical analysis of sea buckthorn berries from different varieties grown at different locations and harvested at different years

4.1. Introduction

Due to their highly valuable nutritional profile, sea buckthorn berries (SBB) are gaining more importance within the food and feed industries. In fact, this is the reason why sea buckthorn has been used already for some years in the development and production of food supplements. Its oily fraction is the most important in this field, especially that coming from the pulp since it has a high percentage of palmitoleic acid, an uncommon fatty acid in the plant kingdom (Vilas-Franquesa, Saldo & Juan (2020), Chapter 1). The uncommon concentrations of palmitoleic fatty acid, together with the high concentrations of other important fatty acids (namely linolenic, linoleic and oleic fatty acids), and other bioactive compounds such as tocopherols and carotenoids, constitute a highly nutritional vegetable oil highly adequate for the development of food supplements.

Although there could be an average value as reference for all the bioactive compounds, there are different factors that can affect the nutritional quality

of the berries. The nutritional quality is not affected in terms of overall compositional profile, meaning that all sea buckthorn berries possess relevant quantities of the compounds previously deemed as important (i.e. palmitoleic acid, tocopherols and carotenoids). Nevertheless, the nutritional quality can be affected in terms of proportion. For instance, one specific berry from a specific plantation may have higher amounts of vitamin C than other berries from a different plantation. Yet both plantations fruit out berries with great amounts of vitamin C, therefore having different nutritional profile but very similar nutritional quality.

Different factors could have an effect on the nutritional profile of the berry. These factors include but are not limited to temperature, sunlight hours, moisture, latitude and altitude, subspecies, rainfall and maturation of the berry.

The temperature depends greatly on the location and altitude where the plantation is set. Plants can present a higher or lower adaptation to variations in temperatures. The nutritional profile of fruits can be widely affected depending on the average temperature during the year. In sea buckthorn, the temperature has been reported to be a factor that could modulate the concentration of certain bioactive compounds, such as vitamin C. The sum of temperature – the temperature of a specific time-lapse summed all together – has been positively linked to glucose concentration

yet negatively associated with organic acid and vitamin C concentration (Kortesniemi, Sinkkonen, Yang, & Kallio, 2014). One of the most important traits of sea buckthorn is the high concentration of vitamin C, surpassing lots of fruits that are well-known for having great concentrations of the compound (see Chapter 1). Thus, the temperature of the region where the sea buckthorn plant grows is a factor that could modulate the concentration of vitamin C. Besides, average temperatures of more than 5 ˚C have also been negatively associated with glycosylated flavonoid concentration (Zheng, Kallio, & Yang, 2016). Proanthocyanidin concentration can also decrease when temperature increases, probably due to the fact that at low temperatures plants can maintain high photosynthetic rates and produce secondary metabolites in abundance (Yang, Laaksonen, Kallio, & Yang, 2017).

Sunlight exposure, high temperatures and environmental moisture also influence the concentration of certain compounds. Radiation, more than the sunlight hours, had been shown to positively modulate the concentration of glucose, yet it had been negatively associated with the concentration of vitamin C and other organic acids (Kortesniemi et al., 2014).

Rainfall is yet another environmental variable that can affect fruit composition. Rainfall, together with environmental moisture, have been positively associated with the berry weight (Zheng et al., 2012), probably

due to the extra water retention in the fruit. Berry weight is an important trait when it comes to its commercialization. Most likely derived from the increase in water content, rainfall and environmental moisture levels have been negatively associated with organic acid concentrations, including malic acid (Zheng, Yang, Trépanier, & Kallio, 2012).

Latitude and altitude could also be important factors affecting the nutritional profile of the berry. For example, glycosylated flavonoids concentration – in particular proanthocyanidins – increased at higher latitudes (Zheng et al., 2016, 2012), probably due to the low-temperature exposure, as described earlier. Latitude was also negatively associated with the sugar/acid ratio and the total acid content (Zheng et al., 2012). Altitude could also be a factor modulating the nutritional profile, including the concentration of monosaccharides – i.e. glucose, fructose – or the concentration of organic acids such as malic acid and vitamin C (Yang, Zheng, & Kallio, 2011).

4.1.1. Demarcation

Among all the possible – environmental and non-environmental – factors, plant genetics takes the cake. The subspecies is the most direct appreciation for the variability, considering phylogenetic differences trigger different biological pathways that may result in distinct nutritional profiles. Recent investigations suggest that the greatest differences are not those derived

from external factors but from genetic variability, which has a direct impact on the biosynthesis and the composition of the fruit (Kortesniemi et al., 2014; Vuorinen et al., 2015). As detailed in Chapter 1, the species *Hippophae rhamnoides* comprise eight different subspecies (as confirmed by Bartish, Jeppsson, Nybom, & Swenson (2002)). The eight accepted subspecies (ssp.) are ssp. *sinensis*, spp. *yunnanensis*, spp. *carpatica*, spp. *rhamnoides*, spp. *fluviatilis*, spp. *mongolica*, spp. *caucasica* and spp. *turkestanica*. When determining differences between subspecies it is important to make sure these are cultivated in the same conditions so that the differences rely on the genetics of the plant rather than on other factors. Likewise, a comparison of outcomes from different articles is also difficult to make. Nonetheless, some authors have investigated differences between subspecies. Ohkawa, Kanayama, Chiba, Tiitinen, & Kanahama (2009) found significant differences in the ratio of sugar/organic acids when comparing the ssp. *mongolica* against ssp. *rhamnoides*, being higher in the former. The subspecies were cultivated in the same region under the same conditions. In addition, the ssp. *sinensis* was found to be the ssp. presenting higher quantities of sugars when compared to ssp. *rhamnoides* or ssp. *mongolica*, all of them cultivated under the same conditions (Yang, 2009). The concentration of vitamin C has also been studied in four different subspecies – namely *sinensis*, *yunnanensis*, *mongolica*, and *turkestanica* – and found that the ssp. *sinensis* was the one having greater concentrations.

The ssp. *turkestanica* was the one showing lower values of vitamin C (Guo, Guo, Li, Fu, & Liu, 2017). These studies add up to the fact that differences in genes derive into differences in the proportions of the bioactive compounds in the berry. Yet in order to observe that, the plants must be cultivated in the same conditions – obviating differences between individual plants. Therefore, although ssp. *turkestanica* may yield less vitamin C / g of berry than spp. *sinensis* under the same conditions, cultivated in different conditions it may yield higher concentrations of the same compound.

Besides variability between subspecies, variability exists also between varieties. Varieties emerge from crossing different subspecies. Varieties allow a perfect adaptation of a specific plantation to the cultivated soil, yielding the best nutritional profile by maintaining the most relevant parts of each subspecies used. At industrial levels, the adaptability of the plantation in terms of yield (i.e. kg of berries from the plantation) and in terms of the nutritional profile from SBB are the most important parameters. Crossing subspecies – and sometimes even varieties – help overcome adaptability problems and help yield the most out of a specific plantation of sea buckthorn. Studying varieties or subspecies and crossing them would take loads of resources that industries are not willing to make, except when something relevant may be extracted from the results (i.e. seed-free grape as a result of a directional research). Nowadays, many

different varieties are being cultivated in many different parts of the world. Some of them are more capable of adapting at higher latitude, others at higher temperatures or even lower rainfall. The study of their nutritional profile could be used as a basis to understand the potential of each variety to be cultivated in a specific region.

Even though variability plays a major role, the age of the plant is also an important factor to consider. A younger plant would always yield more fruit and probably with a better nutritional profile than a plant that is about to die. In that line, some authors have recently shown that sea buckthorn berries' composition does not only change over large periods of time but also yearly (Zheng, Kallio, Linderborg, & Yang, 2011). Thereupon, the potential of each variety should be always considered together with the age of the plant and its adaptability and evolution over time.

4.1.2. Objective

The objective of the present work was to evaluate and compare the physical and phytochemical properties of SBB from four different commercially available varieties and three different locations harvested in the same year (2018) to identify the one with the best nutritional quality. Additionally, a cross-study was performed aiming at understanding the adaptability of sea buckthorn plants in a specific location near 'Bellver de Cerdanya' (Catalunya, Spain).

In the present work, four varieties of commercially available sea buckthorn berries from three different geographical origins (Latvia, Spain, Romania) were compared according to the phytochemical analysis of their aqueous and lipidic fraction. Furthermore, the variety grown in Spain was studied over three different harvesting years (2018, 2019 and 2020).

4.2. Experimental design

4.2.1. Origin of the berries

A total of four different varieties of sea buckthorn berries were acquired from three different orchards across Europe on late 2018. Berries from the subspecies *caucasica* were purchased from a local harvester of the north-western region in Romania. Berries from variety 'Mary' and 'Tatjana', which were a cross between subspecies *mongolica, rhamnoides* and *fluviatilis* (unequivocally both varieties having genes from these three subspecies in different percentage) were purchased from BRUwell, a local harvesting firm located in Latvia. Finally, sea buckthorn berries from the variety 'Tatjana' were collected from a local orchard located at 'Bellver de Cerdanya', a local shire from the Catalan Pyrenees. This orchard was the youngest of all berries used and was planted after receiving the 'Tatjana' variety from BRUwell, Latvia, in 2005. The orchard from 'Bellver de Cerdanya' was subsequently studied over the years 2018, 2019 and 2020 because of industrial interests.

4.2.2. Sample management and preparation

Berries were harvested and immediately frozen at -20 ºC. Berries were conserved at this temperature until processing. The interest remained in the aqueous and the oily fraction of the berry, and therefore the processing – including juice and oil extraction – of the berry was a necessary step.

4.2.2.1. Juice extraction

Sea buckthorn juice (SBJ) was extracted using a screw-drive extruder located at the Pilot Plant of Universitat Autònoma de Barcelona (UAB, Bellaterra, Spain). SBJ was immediately filtered after extraction through an 800 µm mesh size filter. Juice samples were stored at -80 ˚C until analysis, but no longer than six months. Juice samples were thawed the night before the analysis at 4 ˚C. The thawed juice was then clarified at 14,000*g* for 15 min at 4 ˚C. The clarified juice was used for analysis. All the physic-chemical analysis and the phytochemical analysis of the aqueous fraction were performed with the clarified juice as a working matrix.

4.2.2.1. Oil extraction

The seed and the pulp were dried at 50 ˚C for 24 hours. The dried mass was then ground with a Thermomix® TM 21 (Vorwerk, Wuppertal, Germany) at speed level 9 for 3 seconds, in three different times, to avoid seed damage. The seed was then manually separated from the pulp. Oil from the

seed and from the pulp were extracted separately by an Accelerated Solvent Extractor 200 (Dionex Corporation, Sunnyvale, California), equipped with a Solvent Controller. Oil extraction of all parts was performed at 60 ºC using hexane as extraction solvent as detailed in Chapter 5, as these were concluded to be the optimal conditions. All oil extraction procedures were performed thrice.

4.2.3. Sample analysis

The physical and phytochemical analysis of both aqueous and lipidic fractions were performed as detailed in Chapter 3. The weight, width and depth of the berry were measured before processing. The physical analysis was performed on the aqueous fraction (SBJ) and included the quantification of total soluble solids (TSS), dry matter and ashes and measurements of pH and color. The phytochemical analysis of the aqueous fraction included the quantification of total, soluble and insoluble fiber, ascorbic acid and vitamin C, polyphenols, proanthocyanidins and the antioxidant capacity (FRAP and DPPH assays), and the profile analysis of minerals.

The phytochemical analysis of the extracted sea buckthorn oil (SBO) included the quantification of β-carotene and α-tocopherol, yield, and the fatty acid profile analysis.

The morphological attributes of the whole berry (length, width, and weight) were measured on thirty samples. The quantification of fiber was only performed twice in each sample. All other analyses were quantified as indicated in Chapter 3.

4.2.4. Statistical analysis

All statistical analysis was performed with the software R-4.0. Assumptions were checked by first visually interpreting the Q-Q and boxplots from all analysis. Normality was additionally checked by Shapiro-Wilk and Levene's test, and a conjoint conclusion on the normality of the data was withdrawn. Subsequently, the statistical analysis of the data was performed. A one-way ANOVA was run on normally distributed parameters. When significant differences were spotted, the Tukey's *post hoc* test was used to specifically investigate which values differed. A Kruskal-Wallis test was performed when the data was considered to follow a non-normal distribution. For both statistical analyses, the significance level was set at $p<.05$.

4.3. Results and discussion

4.3.1. Physical and phytochemical analysis of four different varieties from three different orchards

4.3.1.1. Physical analysis of the fruit

Physical evaluation of fruits is important to understand possible differences in the phytochemical concentration of different compounds, but also for the efficiency of fruit processing. For instance, a higher volume of the fruit could translate into a higher juice yield. It had been made previously clear that in sea buckthorn, physical differences between different varieties are a reality (Li et al., 2020). In addition, physical differences between berries from different origins were previously reported (Tang & Tigerstedt, 2001). In the present experiment, differences were expected to be observable. Table 1 displays the physical attributes of the analyzed berries. Regarding the physical attributes of the berry, it was clear that two of the selected berries presented the highest berry volume, as all attributes (berry length, width, and weight) were statistically different when compared to the two other varieties. The origin of the berries was a clear factor affecting their physical quality. Interestingly, the two varieties displaying a larger volume were from the same orchard in Latvia. Although they were from a different variety, it was clear that the management in the orchard led to a berry volume above average. The berries looked turgid and displayed a firm skin,

indicating consistency. The bigger volume was attributed to a higher percentage of water in the berry, equivalent to a lower percentage of dry matter (Tang & Tigerstedt, 2001). Indeed, the lowest dry matter percentage was shown by the berries from the Latvia orchard, evidencing the positive association between higher volume and higher moisture content in that case (Table 1, 3). The width of the fruit was the only physical parameter statistically different between the variety from Cerdanya (Spain) and the variety from Romania, an attribute that did not translate into statistical differences in the berry weight (Table 1).

Table 1. Physical attributes of the studied berries from different varieties and origins.

Subspecies/ variety	**Origin**	**Length (mm)**	**Width (mm)**	**Weight (g)**	**Length (mm)**
Caucasica	Romania	$9.70^{b} \pm 0.63$	$7.08^{c} \pm 0.82$	$0.292^{b} \pm 0.104$	5.44 ± 0.68
Tatjana	Spain	$10.22^{b} \pm 0.75$	$7.96^{b} \pm 0.49$	$0.316^{b} \pm 0.048$	5.73 ± 0.50
Tatjana	Latvia	$12.72^{a} \pm 0.99$	$10.07^{a} \pm 0.39$	$0.620^{a} \pm 0.108$	5.63 ± 0.39
Mary	Latvia	$12.11^{a} \pm 1.29$	$9.57^{a} \pm 0.37$	$0.573^{a} \pm 0.147$	5.75 ± 0.37

Mean values ± standard deviation.

No significant differences were observed between berries from different varieties and growing locations when analyzing the physical characteristics of the seed, at least in seed length and width (Table 2). In other words, the seed volume seemed to be similar across all origins and varieties. Little evidence had been previously reported on the possible changes of the seed

morphology between varieties and origins. Evidence on the field has been recently added by Li et al. (2020), who showed no statistical differences in seed length and seed width between the berries from different varieties, in agreement with the results found in the present experiment. They, however, found differences in seed thickness; an attribute that was not measured in the present experiment due to its resemblance to the seed width. The seeds were seen to display a uniform oval shape. Nevertheless, the weight was found to be significantly different when comparing all varieties, with this trend confirmed by the values obtained from the berries of the same variety grown at different orchards (variety 'Tatjana' grown in Spain and Latvia). Since the volume of the seed was similar across all berries studied herein, this could indicate that the compacity of the seed, or the seed density, was significantly different across varieties. The seed density could be an important factor to consider when extracting seed oil and analyzing the presence of bioactive compounds.

Table 2. Physical attributes of the seeds from the studied berries from different varieties and origins.

Subspecies/ variety	Origin	Length (mm)	Width (mm)	Weight (g)	% of seed (in weight)
Caucasica	Romania	5.44 ± 0.68	2.43 ± 0.41	0.014^{c} ± 0.001	5.40^{b} ± 1.83
Tatjana	Spain	5.73 ± 0.50	2.52 ± 0.48	0.016^{b} ± 0.002	5.31^{b} ± 1.07
Tatjana	Latvia	5.63 ± 0.39	2.57 ± 0.45	0.016^{b} ± 0.003	2.72^{a} ± 0.99
Mary	Latvia	5.75 ± 0.37	2.65 ± 0.35	0.018^{a} ± 0.003	3.40^{a} ± 1.11

Mean values ± standard deviation.

The differences between varieties in the berry volume not only affected the dry matter content (Table 3) but also affected all other parameters that could be consequently affected by the percentage of dry matter. The seed percentage (as g of seed / 100 g of fruit) was subsequently affected (Table 2). The berry from Romania and the berry from Spain were significantly smaller and lighter than the berries from the Latvia orchard, and the seed weighted less for the orchard in Romania compared to other orchards. The percentage of seed (% of weight) was therefore affected. The percentage of seed from the two former orchards was significantly higher when compared to the berries from the Latvia orchard. This indicated that (a) the berry weight was the factor mostly influencing the final percentage of seed from the fruit, which was in turn influenced by the water content of the berry and that (b) the differences in seed weight were significantly different when

comparing some varieties but the differences were not enough to achieve some similarities in the percentage of seed from the fruit.

4.3.1.2. Physic-chemical analysis of the fruit

Besides the physical dimensions of the fruit, other physic-chemical analyses were performed on the berries. These physic-chemical analyses were color, pH, TSS in terms of ºBrix, and dry matter. These analyses are important because they are relatively fast and simple, and they could be used as indicators to forecast the nutritional profile of the berry. Besides, they could also bring information about the maturity stage of the fruit (Tang, 2002).

Dry matter was studied in terms of water content and was found to be significantly different between all studied varieties (Table 3). The results showed that the varieties grown on the Latvia orchard had the highest water content when compared to the other two varieties from other origins. These results support the theoretical reasoning previously disclosed that the bigger the fruit, the higher the water content (or the lower the dry matter content) and thereupon the higher the juice volume obtained from the berry. The 'Mary' variety had significantly lower water content when compared to the 'Tatjana' variety from the same orchard. This indicated that water content may be dependent not only on the origin but also on the variety, as other authors previously reported (Tang & Tigerstedt, 2001). Although those

factors could influence water content, the stronger effect on the water content of the berry is shown by the amount of water the plant receives in the latter stage of fruit maturity, which could come either from rainfall or irrigation (Zheng et al., 2012).

TSS was also significantly different when comparing the values obtained from all varieties. TSS quantification through refractive index measurement is widely used to quantify in a direct, yet unprecise manner, the amount of sugars presents in a fruit juice. It is unprecise because the obtained value may be biased by other molecules or solids which could influence the reading. Nevertheless, the value obtained, although biased, made evident the differences in the nutritional profile of each variety and each origin. Both variety and origin seemed to account for the differences in the TSS values. The variety 'Tatjana' from Latvia showed the lowest score for this parameter. The value obtained was less than half compared to the highest value of more than 8 ºBrix obtained by the subspecies *caucasica* from Romania (Table 3). The variability in this parameter is often high, with registered values of more than 10 ºBrix (Beveridge, Harrison, & Drover, 2002) while other studies reported values as low as 4 ºBrix, as shown in the present research. TSS are an important attribute for fruit juices, for the sweetness attribute is highly related to the TSS and it is a crucial attribute for the acceptability of fruit juices (Chapter 7; Tuorila-Ollikainen, Mahlamaki-Kultanen, & Kurkela, 1984). The fact that the same variety

grown in Spain or Latvia gave significantly different values would mean that TSS could be highly affected by other factors rather than only the variety. The bushes' age could be a factor influencing the nutritional and physic-chemical composition of the fruit, with differences observable after only one year (Tang & Tigerstedt, 2001). The maturity stage in which each fruit was harvested could also be an important factor influencing the ºBrix value and other parameters (Tang, 2002). Yet the berry weight and water content, directly related to the irrigation and rainfall, could also have played an important role, as already explained.

The acidity also displays different values depending on the variety and origin. However, the most valued attributed which is also related to the sourness of the juice is its pH (Tang, Kälviäinen, & Tuorila, 2001). Besides, the pH measurement often is a more fast-forward technique, highly valued within the food industry. Interestingly, the pH value was significantly different across all growing locations, yet not across all varieties. Soil composition had been identified to considerably change the concentration of certain bioactive components of SBJ, but also of the physic-chemical parameters, including the pH value (Nowakowska, Ochmian, & Mijowska, 2017), which in turn could explain the similarities observed in the values of different varieties grown at the same orchard. Furthermore, the pH could also give a solid base to make an approximate guess of the total organic acids. Whereas there could be some organic acids present in the juice that

would not contribute to lowering the pH on a large extent, in general a correlation could be made between organic acids and pH (Wiese & Dalmasso, 1994). The lower pH value observed in the *caucasica* subspecies from Romania could then indicate a higher amount of organic acids. The organic acid of major interest was ascorbic acid, which according to this proven relationship, should be in higher amount in the juice with the lower pH value.

Figure 1. Vial of SBJ after centrifugation. Upper part of the vial: oil juice phase, middle part of the vial: clarified SBJ, lower part of the vial: rests of seeds and peel and pulp.

Color was the last physic-chemical attribute measured in the present analysis. Color was the only attribute that showed differences between the clarified and the unclarified SBJ. Table 3 depicts the results from the analyses of both clarified and unclarified SBJ. The clarification of SBJ results in a loss of the oily phase of the juice (Figure 1). The oily phase provides the juice with a lot of solids, but also with great amounts of interesting lipophilic compounds such as carotenoids or certain fatty acids. It is well known that carotenoids contribute substantially to the color of fruits and vegetables, and therefore a loss of carotenoids may imply a loss of color, most likely in terms of redness. In fact, the clarification of the juice translated in a great reduction of the *a** attribute of the CIE*L*a*b** scale. The

*a** value, which accounts for redness of the sample, decreased as much as three times – in some samples even more (those from Latvia) – from the former values obtained by the unclarified juice (Table 3). The *a** value was significantly higher in those varieties from the Latvia orchard when compared to the other two varieties herein analyzed. This could indicate that there could be more presence of coloring compounds, especially those influencing the redness value, such as carotenoids or proanthocyanidins. The *L** value was the other value showing great differences between clarified and unclarified SBJ. This value accounts for the luminosity, or lightness of the sample. The *L** value was higher in the *caucasica* subspecies and lower in the varieties grown at the Latvia orchard.

Table 3. Physic-chemical attributes of the studied berries from different varieties and origins.

Variety/ Subspecies	Origin	Moisture content (%)	pH	TSS	Unclarified			Clarified		
					L*	***a****	***b****	***L****	***a****	***b****
Caucasica	Romania	$86.44^{d} \pm 0.04$	$2.50^{c} \pm 0.01$	$8.9^{a} \pm 0.1$	$60.41^{a} \pm 0.10$	$29.04^{d} \pm 0.03$	$43.01^{c} \pm 0.49$	$45.61^{c} \pm 0.07$	$8.00^{b} \pm 0.10$	$40.57^{b} \pm 0.41$
Tatjana	Spain	$88.71^{c} \pm 0.06$	$2.93^{a} \pm 0.01$	$7.6^{b} \pm 0.1$	$54.01^{b} \pm 0.13$	$34.69^{c} \pm 0.51$	$68.75^{b} \pm 0.87$	$48.74^{b} \pm 0.03$	$10.93^{b} \pm 0.14$	$52.21^{a} \pm 0.41$
Tatjana	Latvia	$93.77^{a} \pm 0.01$	$2.80^{b} \pm 0.01$	$4.0^{d} \pm 0.0$	$51.69^{d} \pm 0.10$	$51.69^{b} \pm 0.44$	$73.90^{a} \pm 0.39$	$49.07^{ab} \pm 0.45$	$14.49^{a} \pm 1.33$	$55.89^{a} \pm 5.94$
Mary	Latvia	$89.63^{b} \pm 0.03$	$2.81^{b} \pm 0.01$	$6.5^{c} \pm 0.0$	$53.90^{c} \pm 0.07$	$53.90^{a} \pm 1.18$	$68.06^{b} \pm 1.15$	$49.67^{a} \pm 0.14$	$9.00^{b} \pm 1.32$	$52.68^{a} \pm 0.36$

Mean values ± standard deviation. TSS: Total Soluble Solids measured as ºBrix.

4.3.1.3. Phytochemical analysis of the juice

The quantification of all the compounds and determination of the antioxidant capacity of the juice was analyzed by the dry matter weight of the juice. This approach was taken after checking that the water content of all studied varieties was significantly different (Table 3). The results obtained per ml of juice could lead to misinterpretation and therefore a standardization had to be performed. In the food supplements industry, the final product is usually presented with reduced water content, either as a powder or as a concentrated liquid (among different formats of water-soluble products). Therefore, the use of a dry matter basis to quantify the compounds of interest gained even more importance.

As explained in Chapter 1, one of the most important traits of sea buckthorn is its outstanding levels of vitamin C. Vitamin C can be found as ascorbic acid or dehydroascorbic acid in sea buckthorn berries, with the great majority being in the ascorbic acid form (Tiitinen, Yang, Haraldsson, Jonsdottir, & Kallio, 2006). The results from the present experiment aligned with some of the highest results reviewed by Beveridge, Li, Oomah, & Smith (1999), yet only for some of the varieties studied. The statistical analysis revealed significant differences in the concentration of vitamin C across all samples (Table 4). The ascorbic acid concentration was also significantly different across all samples. The ranking of samples was the same in terms of concentration of ascorbic acid and vitamin C content.

Conclusively speaking, the concentration of total vitamin C and ascorbic acid tend to follow the same pattern across different varieties and origins. The levels were higher when analyzing the total vitamin C, since part of it was present in the sample as dehydroascorbic acid, as already explained. The highest values of vitamin C were reported for the *caucasica* subspecies, which gave values more than two-fold from those obtained after analyzing the 'Mary' variety from Latvia (Table 3). Interestingly, the same variety cultivated at two different locations gave very different values on the vitamin C content, being higher in the 'Tatjana' variety grown in Spain. The sample with the higher concentration of vitamin C and ascorbic acid was also the sample registering the lower pH values (Table 3, 4). Although there could be many other compounds affecting the pH, the greater amounts of ascorbic acid could be an important contributing factor, as explained before.

Table 4. Phytochemical profile of the juices from the studied berries from different varieties and origins.

Origin	Romania	Spain	Latvia	
Variety/ Subspecies	*Caucasica*	*Tatjana*	*Tatjana*	*Mary*
Ascorbic acid	$1.580^{a} \pm 0.027$	$1.390^{b} \pm 0.041$	$1.001^{c} \pm 0.194$	$0.788^{d} \pm 0.054$
Vitamin C	$1.884^{a} \pm 0.093$	$1.523^{b} \pm 0.108$	$1.175^{c} \pm 0.164$	$0.843^{d} \pm 0.537$
Total polyphenols	$16.977^{d} \pm 0.156$	$19.463^{c} \pm 0.462$	$36.035^{a} \pm 0.840$	$30.511^{b} \pm 0.402$
Proanthocyanidins	$0.974^{a} \pm 0.009$	$0.577^{b} \pm 0.025$	$0.408^{c} \pm 0.024$	$0.404^{c} \pm 0.008$
DPPH	$158.967^{a} \pm 3.403$	$75.703^{b} \pm 2.170$	$66.761^{c} \pm 1.688$	$47.252^{d} \pm 2.299$
FRAP	$123.774^{a} \pm 2.170$	$85.734^{b} \pm 3.670$	$69.001^{c} \pm 0.640$	$30.942^{d} \pm 1.368$
TDF	29.073 ± 1.407	32.143 ± 1.317	27.447 ± 5.221	39.140 ± 2.863
SDF	4.238 ± 0.677	8.424 ± 0.627	10.834 ± 1.702	15.135 ± 2.863
IDF	24.835 ± 0.730	23.719 ± 0.690	16.613 ± 4.086	24.004 ± 5.726

Mean values ± standard deviation. TDF: Total Dietary Fiber; SDF: Soluble Dietary Fiber; IDF: Insoluble Dietary Fiber.
Ascorbic acid and Vitamin C content expressed as g/ 100 g of dry matter.
Antioxidant assays DPPH and FRAP expressed as µmol/ g dry matter.
Total polyphenols expressed as mg gallic acid equivalent (GAE)/ g dry matter.
Proanthocyanidins expressed as mg cyanidin/ g dry matter.
TDF, SDF and IDF expressed as mg/ g dry matter.

In fruit juices, especially citrus juices, the concentration of vitamin C accounts for more than 65% of its antioxidant potential (Gardner, White, McPhail, & Duthie, 2000). The results from the antioxidant capacity herein explored add evidence to the substantial contribution of vitamin C in this parameter (Table 4). The statistically significant values reported for antioxidant capacity were observed to follow the same pattern as the vitamin C content, although the total phenolic quantification followed a

completely different pattern. It could be concluded that the most important component of sea buckthorn berries to preserve the antioxidant capacity was vitamin C.

The antioxidant activity of fruit juices depends upon the array of bioactive compounds showing this antioxidant activity (Ozgen, Reese, Tulio, Scheerens, & Miller, 2006). It was therefore important to analyze the antioxidant capacity through different assays because different assays express different aspects of the antioxidant phenomenon (Benzie & Devaki, 2017). The antioxidant values of FRAP and DPPH, which measure reducing power and free radical scavenging respectively, showed the same ranking pattern, but not the same behavior across all samples. In other words, samples scored differently depending on the antioxidant assay used. Interestingly, this could be derived from the antioxidant profile of the juice. Vitamin C, together with the total polyphenol content, are the most important contributors to the antioxidant capacity. In general terms, the FRAP assay correlates better with the amount of vitamin C in the juice, since the assay aims at analyzing the reducing capacity of a specific compound by the reduction of Fe^{3+} to Fe^{2+} (Huang, Boxin, & Prior, 2005). The DPPH assay, on the other hand, analyses the scavenging ability of the compounds in the juice against a free radical (DPPH·). Some of the most important compounds with scavenging ability in the vegetable kingdom are polyphenols. Results from the present experiment showed that the varieties

containing more quantity of polyphenols had a similar or even higher antioxidant capacity in terms of DPPH when compared to the FRAP assay (Table 4), adding evidence on the well-stablished fact that the polyphenols have a strong influence in the DPPH assay due to their radical scavenging activity. Nonetheless, the very high quantities of vitamin C present in SBJ could bias the potential contribution of polyphenols in this assay, as it happened when analyzing the samples from Romania and Spain. This could derive from the reducing potential of the ascorbic acid, which could reduce the polyphenols so that they could be again able to reduce other radicals. In addition, other authors already reported high correlations between total phenolic content and vitamin C with the antioxidant assays DPPH and FRAP in methanolic solutions (Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Hawkins Byrne, 2006). Besides, in a matrix were one of the compounds exerting antioxidant activity is in such great proportion, the relations between antioxidant capacity and other compounds could be difficult to elucidate. The use of two antioxidant assays provided higher evidence of the great antioxidant activity of the fruit and made evident the differences between the studied samples.

Polyphenol concentration was significantly different between all studied samples. The samples presenting greater polyphenol concentration were those from the orchard located in Latvia. The variety ‘Mary’ had a higher polyphenol concentration when compared to the ‘Tatjana’ variety. These

results indicated that polyphenol concentration not only seems to be different between different varieties but also between different orchards. The concentrations of the variety 'Tatjana' almost doubled the score obtained by the *caucasica* subspecies grown in Romania and surpassed by more than 10 mg of GAE/ g of dry matter the values obtained by the same variety grown in Spain. Polyphenols are compounds formed by secondary metabolic pathways from plants, which could explain the differences between varieties. In addition, polyphenols are molecules produced in higher proportions when the plant faces situations of stress, such as hydric deficiency, soil alkalinity or shortage of sunshine hours (Stracke, Rufer, Weibel, Bub, & Watzl, 2009). This could explain the marked differences between regions. Since no scientific evaluation was made over any of the factors, it was difficult to understand which factor, or a mixture of them, could more severely affect the concentration of polyphenols in SBJ. Total phenolic content was in line with what other authors previously reported for sea buckthorn (Guo, Chang, et al., 2017; Teleszko, Wojdyło, Rudzińska, Oszmiański, & Golis, 2015). The total phenolic content may also be affected by more controllable factors such as the use of efficient irrigation methods, the use of organic farming methods (Ponder & Hallmann, 2019) or even the maturity of the fruit (Fawole & Opara, 2013). This should be taken into consideration to build a strong phenolic profile of the berry without compromising its quality regarding other important constituents.

One important group of polyphenols presents in SBJ, conferring part of the color and contributing to its antioxidant activity is the proanthocyanidins. Proanthocyanidins are common in some berries, conferring the fruit with the blue-to-red color depending on the maturity stage of the fruit and other factors. In addition, the proanthocyanidins had been shown to contribute to the development of astringency (Basalekou et al., 2019). The proanthocyanidin concentration was significantly different between all the samples, except when comparing the samples from the same orchard located in Latvia (variety 'Tatjana' and 'Mary'). As a group of polyphenols, proanthocyanidin concentration also seemed to be more influenced by the conditions and location of the orchard rather than the variety. The concentration of proanthocyanidins did not follow the same pattern as the concentration of polyphenols. In fact, the highest concentration of proanthocyanidins was found in the samples from Romania followed by those from Spain. It should be noted that the value indicating redness of the sample was significantly higher on the unclarified juice from Latvia (either variety, Table 3) when compared to the samples from Romania and Spain. This suggested that the carotenoids were probably the compounds most largely affecting the color of the juice rather than the proanthocyanidins.

Fiber analysis revealed no significant differences between any sample on any parameter studied (including total dietary fiber, soluble fiber and insoluble fiber, Table 4). Yet the results point at great differences between

the samples analyzed. The robustness of the statistical analysis was probably affected when reducing the number of repetitions per sample (only two), deriving in a decreased power and an increased chance of type II error. The total dietary fiber (TDF) values were in line with what was previously reported for sea buckthorn berries (between 0.274 and 0.391% of the total dry matter across all studied samples; Linderborg, Lehtonen, Järvinen, Viitanen, & Kallio, 2012). From the TDF, the soluble dietary fiber (SDF) accounted for the lower percentage of fiber, ranging from 14.5% in the variety from Romania to 38.7% in the 'Mary' variety from Latvia. The rest was analyzed as insoluble dietary fiber (IDF). The variation in the values of SDF and IDF were higher than the variation in TDF, suggesting that the variation in the type of fiber could be more likely used to discriminate between origins and varieties rather than the total fiber values. Nonetheless, the results should be carefully treated as the statistical analysis indicated no differences between any of the studied samples.

The mineral composition was also analyzed because it could serve as an indicator of soil composition. K, P, Mg, Ca, Na, Fe and Cu were the analyzed minerals because they were found at higher amounts according to previous research (Sabir, Maqsood, Ahmed, Shah, & Khan, 2005; Zeb, 2004). The mineral profile was seen to be very different across all the analyzed samples (Table 5). The variety 'Tatjana' from Spain had the highest concentration of K as well as P, and the subspecies *caucasica* from

Romania had the lowest concentration of both minerals. This proportion was not observed in previous research analyzing different varieties (Sabir et al., 2005). The variety from Spain registered significantly higher values of Mg when compared to all other varieties, except the variety ‘Mary’ from Latvia. The variety from Romania scored the lowest for Mg value. Interestingly, the variety from Romania achieved values of Na ten-fold of those obtained in other varieties. The concentration of Cu and Fe was residual, and Fe concentration was statistically non-different across all varieties analyzed. Mineral composition depends, as it was previously explained for the concentration of certain compounds, on many factors. The soil composition, the variety and the location of the orchard could influence the mineral composition, among different factors. The latter, related to soil composition, could greatly affect the concentration of some minerals (as it can be seen in the results of berries from different origins depicted in Table 5), and the modulation of supplementation of the soil had been used as techniques to positively contribute to the fruit quality (in terms of mineral composition) or even to the growth of the plant (Wang & Lin, 2002).

Table 5. Mineral composition of the studied berries from different varieties and origins.

Origin	Romania	Spain	Latvia	
Variety/ Subspecies	*Caucasica*	*Tatjana*	*Tatjana*	*Mary*
K	$9.507^{d} \pm 0.113$	$19.773^{a} \pm 0.135$	$16.693^{b} \pm 0.000$	$16.693^{b} \pm 0.000$
P	$0.766^{d} \pm 0.043$	$1.747^{a} \pm 0.000$	$1.284^{b} \pm 0.000$	$1.051^{c} \pm 0.000$
Mg	$0.401^{c} \pm 0.008$	$0.494^{a} \pm 0.002$	$0.466^{b} \pm 0.002$	$0.491^{a} \pm 0.015$
Ca	$0.287^{d} \pm 0.009$	$0.491^{c} \pm 0.004$	$0.545^{b} \pm 0.002$	$0.653^{a} \pm 0.017$
Na	$1.051^{a} \pm 0.014$	$0.145^{c} \pm 0.000$	$0.147^{c} \pm 0.000$	$0.194^{b} \pm 0.002$
Fe	12.064 ± 0.371	14.515 ± 2.026	11.524 ± 0.056	13.371 ± 3.198
Cu	$2.189^{c} \pm 0.170$	$3.547^{b} \pm 0.089$	ND	$3.846^{a} \pm 0.093$

Mean values ± standard deviation. ND: Non detected.

K, P, Mg, Ca, Na values expressed by mg/ g dry matter

Fe, Cu values expressed by µg/ g dry matter

4.3.1.4. Phytochemical analysis of the oil

Seed and pulp oil were analyzed separately because the nutritional profile had been previously reported to differ significantly (Dulf, 2012). Oil yield was the first parameter analyzed. Both seed oil yield and pulp and peel oil yield showed significant differences across the studied samples (Table 6, 7). The oil content in the seed was notably lower than the oil content in the peel and pulp in all samples analyzed, consistent with previous findings (Burčová et al., 2017; Yang & Kallio, 2001). The seed oil yield seemed to be more consistent across varieties and origins than the pulp and peel oil

yield (Table 6). The highest seed oil yield was reported for the variety 'Tatjana', with no observable differences between orchards. The lowest seed oil yield resulted from the analysis of the 'Mary' variety.

Table 6. Yield and composition of seed oil of different berries.

Variety/ Subspecies	**Origin**	**Oil yield**	**α-tocopherol**	**β-carotene**
Caucasica	Romania	$6.51^{b} \pm 1.018$	$1.140^{b} \pm 0.220$	$0.141^{b} \pm 0.008$
Tatjana	Spain	$9.00^{a} \pm 0.020$	$1.415^{a} \pm 0.124$	$0.613^{a} \pm 0.055$
Tatjana	Latvia	$9.63^{a} \pm 0.237$	$1.579^{a} \pm 0.040$	$0.620^{a} \pm 0.079$
Mary	Latvia	$5.67^{b} \pm 0.707$	$0.640^{c} \pm 0.163$	$0.284^{b} \pm 0.160$

Mean values ± standard deviation.
Oil yield expressed as g of sea buckthorn oil/ 100 g of raw matrix (seed, or pulp and peel).
α-tocopherol and β-carotene expressed as mg/ g of sea buckthorn oil.

The results could suggest similarities between varieties rather than origins. The results also indicated that seed density could be an important factor influencing oil yield, since the variety obtaining lower oil yield values was the variety with greater seed density (Table 1, 6). Other studies already pointed out differences between varieties (Yang & Kallio, 2001). Nevertheless, the pulp and peel oil yield did not report the same differences. The oil yield from this fraction ranged from 19 to 52% of the raw matrix (Table 7). The highest value was reported for the 'Mary' variety, contrasting with its poor oil content in the seed, whereas the lowest values were observed in the varieties grown in Spain and Romania. Other authors already reported similarities in the pulp oil of different subspecies (Tiitinen,

Hakala, & Kallio, 2005). Statistical differences were found between the same variety grown in Latvia or Spain, which indicated that the variety of the plant could not explain part of the differences between pulp and peel oil content.

Table 7. Yield and composition of the oil from the pulp and peel of different berries.

Variety/ Subspecies	**Origin**	**Oil yield**	**α-tocopherol**	**β-carotene**
Caucasica	Romania	$19.66^{c} \pm 1.548$	$0.439^{c} \pm 0.090$	$0.467^{c} \pm 0.168$
Tatjana	Spain	$19.74^{c} \pm 0.668$	$2.232^{a} \pm 0.463$	$11.295^{a} \pm 1.304$
Tatjana	Latvia	$35.88^{b} \pm 0.443$	$1.849^{a} \pm 0.320$	$4.015^{b} \pm 0.756$
Mary	Latvia	$52.18^{a} \pm 1.382$	$0.826^{b} \pm 0.058$	$3.627^{b} \pm 0.263$

Mean values ± standard deviation.

Oil yield expressed as g of sea buckthorn oil/ 100 g of raw matrix (seed, or pulp and peel).

α-tocopherol and β-carotene expressed as mg/ g of sea buckthorn oil.

Besides oil yield, the nutritional composition of the oil was of great importance. The greater oil yield found in some samples was to be contrasted with the nutritional profile of the recovered oil. The vitaminic compounds α-tocopherol and β-carotene were quantified together with the fatty acid profile of the SBO from each fraction. The statistical analysis showed that the concentration of both analytes was significantly different across samples in both SBO fractions. The concentration of α-tocopherol was variable in the seeds and the pulp, whereas the concentration of β-carotene was found to be greater in the pulp and peel oil than in the seed oil

(Table 6, 7). These results were in line with those found by Burčová et al. (2017), who showed that differences in the concentration of tocopherols depend on the part of the plant, which could have different tocopherol profile. Interestingly, the content of both vitaminic compounds was statistically not different between the same variety (‘Tatjana’) grown at different locations (Spain and Latvia). The seed nutritional profile seemed to be more dependent on the variety rather than the location, in line with previous findings (Kallio, Yang, Peippo, Tahvonen, & Pan, 2002). Interestingly, the variety ‘Tatjana’ showed significantly higher values of the two studied compounds when compared to the other two samples.

Pulp and peel oil composition gave similar results, although the concentration of β-carotene was much greater than the concentration of α-tocopherol. In addition, the concentration of the β-carotene was also much higher than the concentration of the same compound in the seed oil. Again, the concentration of α-tocopherol in seed oil was significantly higher in the ‘Tatjana’ variety regardless of its origin compared to the other studied varieties (Table 6). Nevertheless, the concentration of β-carotene in seed oil showed a completely different proportion depending on the origin. The variety ‘Mary’ from Latvia had statistically similar concentrations when compared to the variety ‘Tatjana’ harvested in the same orchard. Interestingly, the variety ‘Tatjana’ cultivated in Spain resulted in significantly greater values of β-carotene concentration in the extracted

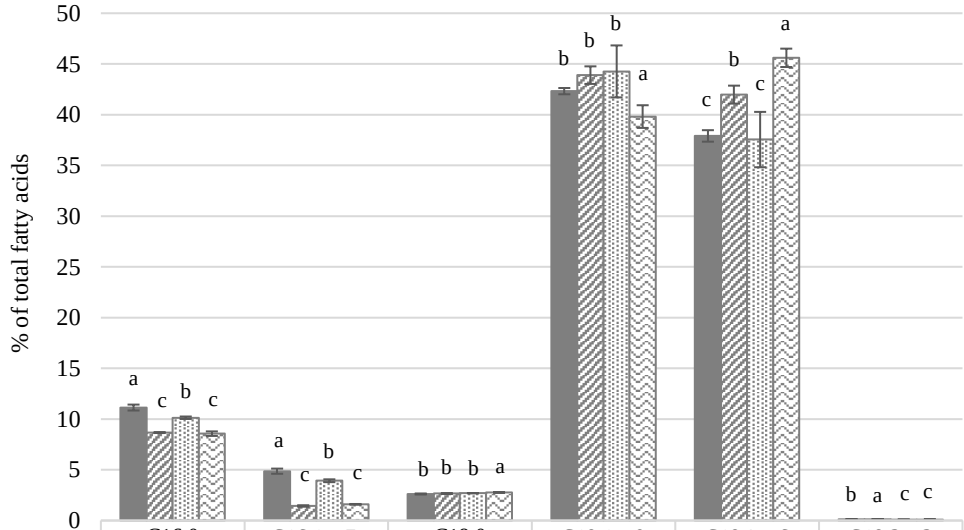
seed oil when compared to all other samples (Table 6). The standard deviation indicated that the results were similar in different repetitions, accounting for the possible human error that could led to these outstanding values. Andersson, Olsson, Johansson, & Rumpunen (2009) already reported very different values of carotenes and total carotenoids depending on the variety analyzed, showing doubled values in some cases. The carotenoid composition found by Andersson et al. (2009) however was very different when compared to the values found in the present study for the variety 'Tatjana' grown in Spain. Additionally, other authors reported great differences in the β-carotene composition between different varieties, in some cases even observing a difference as much as three times higher (Pop et al., 2014).

The fatty acid profile was undoubtedly of great interest since some of the fatty acids presents in SBO had been investigated for their potential health benefits (Chapter 1). There had been previously reported great differences between seed oil and pulp and peel oil, with great variations on the fatty acid profile (Dulf, 2012). Figure 2 and Figure 3 show the different nutritional profile of the seed oil and the pulp and peel oil, respectively. Undoubtedly, oleic and linolenic acid were found to be the major fatty acids constituting the seed oil profile, whereas palmitic and palmitoleic were the two major fatty acids constituting the pulp and peel oil profile.

There were statistically significant differences between at least two studied samples in all the analyzed fatty acids. The two major fatty acids in the seed (oleic and linoleic fatty acids) were found at different levels depending on the sample analyzed (Figure 2). Oleic acid (C18:1ω-9) was found to be significantly lower when comparing the variety of 'Mary' to all other varieties analyzed. Nevertheless, the concentration of linoleic acid (C18:2ω-6) was found to be at significantly higher concentrations in this variety when compared to all other samples.

The levels of oleic acid in both the variety from Romania and the 'Tatjana' from Latvia were higher when compared to the lower levels of linoleic acid. This could indicate a certain relationship between the concentration of these two fatty acids in the seed oil depending on the variety, as reported by Yang & Kallio (2001). However, they reported concentration values of α-linolenic similar to those of oleic and linoleic acids in seed oil (around 30% of total fatty acids), concentrations that were not observed in the present experiment in any of the studied samples. Other authors reported high percentages of α-linolenic acid in seed oil as well (Dulf, 2012). The percentage of α-linolenic acid was reported to be the lowest in comparison to the other studied fatty acids, at concentrations of 0.1% of the total fatty acids of seed oil (Figure 2). This contrasted with the relatively higher concentrations of oleic and linoleic fatty acids obtained in the present experiment compared to previous research. The concentration of palmitic

acid and palmitoleic acid was significantly higher in the *caucasica* subspecies when compared to other varieties. The level of stearic acid was similar across all varieties, except for the 'Mary' variety, which showed significantly higher values.



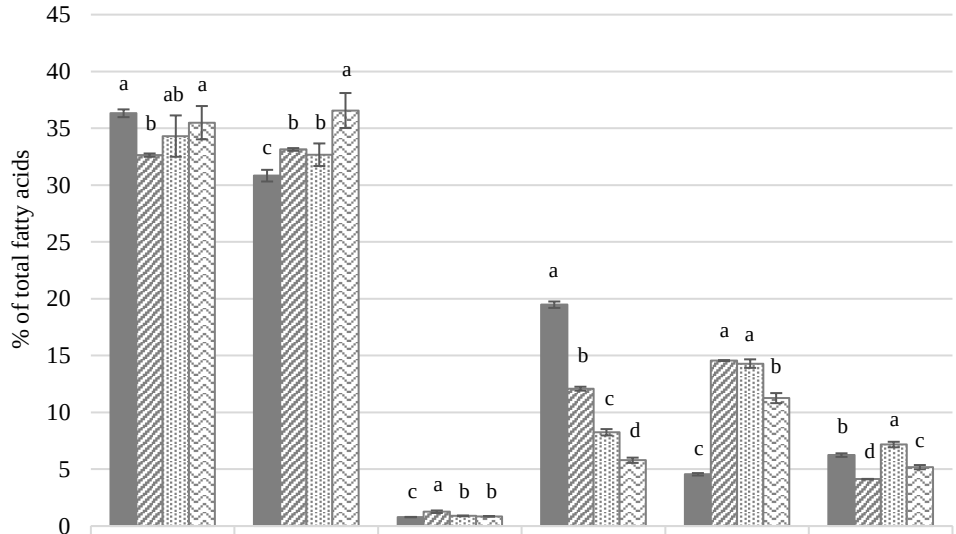
	C16:0	C16:1ω-7	C18:0	C18:1ω-9	C18:2ω-6	C18:3ω-3
Caucasica Romania	11.137	4.862	2.617	42.323	37.909	0.116
Tatjana Spain	8.672	1.436	2.664	43.901	41.981	0.127
Tatjana Latvia	10.132	3.923	2.696	44.268	37.550	0.091
Mary Latvia	8.562	1.599	2.767	39.816	45.606	0.099

Figure 2. Fatty acid profile of SB seed oil of the studied varieties expressed as a percentage of total fatty acids. Error bars show SD. Statistically significant differences are represented with different letters. C16:0 palmitic acid; C16:1ω-7 palmitoleic acid; C18:0 stearic acid; C18:1ω-9 oleic acid; C18:2ω-6 linoleic acid.

The concentrations of α-linolenic acid were considerably higher in the pulp oil than in seed oil (Figure 3), differing from what other authors had previously reported (Burčová et al., 2017; Dulf, 2012; Yang & Kallio, 2001). In general terms, the two major fatty acids present in the pulp and

peel oil were palmitoleic (C16:1-ω7) and palmitic (C16:0) acids, in line with previous research (Dulf, 2012). Palmitoleic acid is by far the most interesting fatty acid in sea buckthorn peel and pulp oil (Chapter 1). The greatest concentration of palmitoleic acid was found in the 'Mary' variety, which was significantly higher than in all other varieties. The variety grown at the Romania plantation showed the lowest concentration of palmitoleic acid, whereas the same variety had the highest concentration of palmitic acid in the pulp and peel oil (Figure 3). The 'Mary' variety showed statistically non-significant values when comparing the palmitic acid content with the values obtained from the 'Tatjana' variety grown at the same orchard. Interestingly, the variety grown in Spain had the lowest content of palmitic fatty acid. The greater variation was observed for oleic acid, and the percentage ranged from almost 20% for the *caucasica* subspecies to as low as 5% for the 'Mary' variety (Figure 3). Dulf (2012) reported differences in oleic acid from the pulp and peel oil of as high as 25% when comparing varieties. Yang & Kallio (2001) also reported great differences in this specific fatty acid. The high proportion of oleic fatty acid in the variety from the Romanian orchard affected greatly the concentration of linolenic acid, which accounted for less than 5%. The other studied varieties surpassed the threshold of 10% in oleic fatty acid concentration. The concentration of α-linolenic acid was significantly different in all

studied samples as well, showing the highest concentration in the 'Tatjana' variety from Latvia and the lowest for the 'Tatjana' variety from Spain.



	C16:0	C16:1ω-7	C18:0	C18:1ω-9	C18:2ω-6	C18:3ω-3
Caucasica Romania	36.319	30.830	0.788	19.476	4.552	6.243
Tatjana Spain	32.635	33.136	1.257	12.085	14.561	4.128
Tatjana Latvia	34.311	32.672	0.891	8.250	14.291	7.169
Mary Latvia	35.491	36.562	0.853	5.787	11.256	5.181

Figure 3. Fatty acid profile of SB pulp and peel oil of the studied varieties expressed as a percentage of total fatty acids. Error bars show SD. Statistically significant differences are represented with different letters. C16:0 palmitic fatty acid; C16:1ω-7 palmitoleic fatty acid; C18:0 stearic fatty acid; C18:1ω-9 oleic fatty acid; C18:2ω-6 linoleic fatty acid.

The fatty acid profile of SBO from either fraction (pulp and peel or seed) was different across all studied varieties and origins. Some of the fatty acids were seen to be similar in the same variety of SB, and some others in different varieties from the same origin. Nevertheless, clear differences and variabilities in the proportion of fatty acids across all the samples were observed, and further research should be performed in order to elucidate the association between the fatty acid profile and the variety or origin of the SB

plant. The research should also consider the use of controllable variables (irrigation systems, soil composition analysis…) and uncontrollable variables (sunlight hours, temperature…) to get more reliable results. In view of the full oil fraction, it seemed that the results of the vitaminic compounds were steadier and followed an association pattern with the varieties and, eventually, with the origins of SB berries. Yet again, further research should be made to elucidate the true association between these two factors.

4.3.2. Physical and phytochemical profile of the variety 'Tatjana' grown in Spain over three consecutive years (2018, 2019 and 2020)

4.3.2.1. Physical analysis of the fruit

The physical shape of the fruit and its seed, together with many other physic-chemical and phytochemical components constituting the juice and the oil, had been shown to differ not only between varieties or origins but also between different harvesting years (Tiitinen et al., 2005). Interestingly, the present experiment showed no statistically significant differences between the length nor the weight of the berries from the same orchard harvested at different years (Table 8). The only observed significant difference was on fruit width. This information did not provide a basis to assume that the fruit might have contained more water and thereupon more juice, as it was observed in the analysis of the varieties (4.3.1.1. Physical

analysis of the fruit). In other words, significant differences in berry width did not translate into differences in berry weight, as previously reported in the comparison between varieties (Table 1). Other authors however reported significant changes when comparing berry weight from two consecutive years, showing a significant increase of more than 5% in berry weight (Tang & Tigerstedt, 2001). Certain techniques, such as implementing an efficient irrigation system, may help increase the water content of the berry and therefore its weight, leading to probable differences over different years.

Seed morphology was observed to be similar in terms of length and width (Table 8). Significant differences were observed when comparing the seed weight of different years. The Tukey's *post hoc* test revealed the difference to be significant when comparing the seed from 2018 against the seed from 2020. Other authors previously reported significant differences between seed weight of the same plantation over two different and consecutive years (Tang & Tigerstedt, 2001). Significantly different seed weight translated into greater percentage of seed weight (Table 8). Nevertheless, this difference was non-significant. It could be possible that since the berry weight was not statistically significantly different between berries harvested in different years, the percentage of seed in terms of weight of the whole fruit was non-significant.

Table 8. Physical attributes of the studied berries from the variety ‘Tatjana’ harvested in Spain on different years.

Year	Berry			Seed			
	Length (mm)	Width (mm)	Weight (g)	Length (mm)	Width (mm)	Weight (g)	% of seed (in weight)
2018	10.22 ± 0.75	7.96^{a} ± 0.49	0.316 ± 0.049	5.73 ± 0.50	2.52 ± 0.48	0.016^{b} ± 0.002	5.31 ± 1.07
2019	10.20 ± 0.82	7.13^{b} ± 0.52	0.319 ± 0.050	5.60 ± 0.61	2.47 ± 0.45	0.018^{ab} ± 0.003	5.73 ± 1.47
2020	10.25 ± 1.33	6.92^{b} ± 0.72	0.324 ± 0.054	5.62 ± 0.45	2.62 ± 0.43	0.019^{a} ± 0.003	5.92 ± 2.20

Mean values ± standard deviation.

4.3.2.2. Physic-chemical analysis of the fruit

The physic-chemical profile of the fruit was important to be measured since it could give information about the nutritional profile of the berry at every stage of its maturity and therefore be used to get a very close fruit quality over different years. In addition, it could also be used as the first indicator to investigate the impact of newly implanted techniques on the berries’ nutritional quality.

Despite the fact that fruit weight showed statistically non-significance, the moisture content of the fruit was significantly different when comparing the same orchard over three consecutive years. The results from the *post-hoc* test revealed the differences to be significant between all samples. Overall, the water content decreased after the first year (Table 9). The moisture content from the first harvesting year was significantly higher when

compared to the fruits from 2019 and 2020. It should be noted, however, that in 2019 some problems in the irrigation system were registered, possibly triggering the sudden decrease of more than 4% in the dry matter content of the fruit. The moisture content studied by other authors were also shown to be very different over different years, in some cases increasing (Tang & Tigerstedt, 2001) and others decreasing (Tang, 2002), pointing to a possible climatic cause of these variations.

The pH value obtained over different years was also significantly different. Again, the highest pH value was measured in the berry from 2018, followed by the berry from 2020 (Table 9). The lowest value was registered in the berry harvested in 2019. These results suggested a positive relationship between the moisture content and the pH value. In fact, the higher the moisture content of the fruit, the more dissolved could the organic acids be in the juice, and thereupon the higher the pH. This could be reasoned provided that the acid organic profile does not change excessively over the years (Tiitinen et al., 2005). Nevertheless, the lower pH registered on the berry from 2019 may also indicate a higher amount of organic acids, as explained before (4.3.1.2. Physic-chemical analysis of the fruit).

PART II

Table 9. Physic-chemical attributes of the studied berries from the variety ‘Tatjana’ harvested in Spain on different years.

Year	Moisture content (%)	pH	TSS	Unclarified			Clarified		
				L*	***a****	***b****	***L****	***a****	***b****
2018	$88.71^{a} \pm 0.06$	$2.93^{a} \pm 0.01$	$7.5^{c} \pm 0.1$	$54.01^{c} \pm 0.13$	$34.69^{b} \pm 0.51$	$68.75^{a} \pm 0.87$	$48.74^{a} \pm 0.03$	$10.93^{b} \pm 0.14$	$52.21^{a} \pm 0.41$
2019	$84.63^{c} \pm 0.04$	$2.82^{c} \pm 0.02$	$11.0^{a} \pm 0.0$	$56.57^{b} \pm 0.12$	$36.22^{a} \pm 0.13$	$69.08^{a} \pm 1.16$	$45.89^{b} \pm 0.13$	$13.88^{a} \pm 0.13$	$36.81^{b} \pm 0.92$
2020	$86.14^{b} \pm 0.02$	$2.88^{b} \pm 0.01$	$8.0^{b} \pm 0.0$	$58.42^{a} \pm 0.01$	$29.97^{c} \pm 0.02$	$50.08^{b} \pm 0.03$	$40.23^{c} \pm 0.11$	$4.93^{c} \pm 0.05$	$17.01^{c} \pm 0.06$

Mean values ± standard deviation. TSS: Total Soluble Solids measured as ºBrix

The suggested positive effect shown by the moisture content over the pH value of SBJ over different years was not observed when analyzing the TSS values (Table 9). In fact, the sample showing the highest TSS value was from the berries of 2019, followed by the berries in 2020 and finally the berries of 2018. The results seemed to indicate a negative relationship between moisture content and the TSS content which, in turn, potentiated the explanation that a positive relationship could exist between moisture content and pH. This negative association could also derive from the dilutive effect that the higher the moisture content the more dissolved the sugars in the juice and therefore the lower the TSS value. More evidence should be provided on the same orchard to understand this negative association. This could provide the basis to work on different techniques aiming at decreasing the water content of the fruit and therefore obtaining a fruit juice with higher values of TSS, which could be useful on the development and acceptability of fruit juices (Chapter 7). One of those techniques could be the use of a lower volume of water in the latter stages of fruit development.

In view of the results obtained after measuring the color of the clarified and unclarified SB juice from different varieties (Figure 1, Table 3), the color was also measured in the clarified and unclarified SB juice over the years 2018, 2019 and 2020. The results were similar: the clarified juice displayed lower values of the CIE*L*a*b** scoring system – especially on the *a** value

– when compared to the unclarified SB juice, which derives from the removal of the oil fraction of the juice (Table 9). All the values from the CIE*L*a*b** measuring scale system and both fractions showed statistically significant differences between the analyzed samples. The *a** and *b** value – accounting for redness and yellowness of the sample – from the year 2020 were considerably lower when compared to the same orchard in 2018 and 2019. This could indicate that the production of coloring compounds such as carotenoids (influencing both redness and yellowness) or proanthocyanins by the sea buckthorn plant was somehow negatively affected during the fruit development stage of 2020. It was clear that there was no pattern associated with any other studied parameter, which in turn made it difficult to understand possible factors affecting the development of color, especially when the factors were not quantified. Sunlight exposure could be very variable and identified as one possible factor affecting the color quality of the fruit (Marini, Sowers, & Marini, 2019).

4.3.2.3. Phytochemical analysis of the juice

The results from all the phytochemical analysis of the juice had also been presented by dry matter, considering the moisture content of each fruit juice, as explained in the phytochemical analysis of the juice from different varieties and origins (4.3.1.3. Phytochemical analysis of the juice).

The vitamin C content, as well as the ascorbic acid content, was significantly different between the fruit sample from 2018 and the fruit sample from 2019 and 2020, being lower in the latter two. No significant differences were observed between the samples from the latter two years. It had been previously made clear that a decrease in pH could derive from an increase in the organic acid concentration. Since ascorbic acid is one of the most – if not the most – important organic acids in SBJ with great presence, the association between pH and the concentration of this organic acid was inevitable. Nevertheless, although this association was true when comparing berries' juices from different origins and varieties, it was not observable when comparing berries' juices from different growing seasons (2018, 2019 and 2020; Table 9, 10). The concentration of vitamin C, and subsequently of ascorbic acid, was significantly higher in the berries harvested in 2018 when compared to those harvested in 2019 or 2020.

No significant differences were observed in either concentration when comparing the fruit samples from 2019 and 2020, although the latter showed lower concentrations of both vitamin C and ascorbic acid than the berries from 2019 (Table 10). Although there could be many factors influencing this big drop in vitamin C content (Magwaza, Mditshwa, Tesfay, & Opara, 2017), the problem detected on the irrigation system of the orchard in 2019 could be an important factor contributing to the lower concentration of vitamin C obtained in the fruits on 2019, and subsequently

in 2020 (Ahmed, Yu, Yang, & Jiang, 2014). Nevertheless, it should be also mentioned that the relatively young age of the plants and their adaptability process could also influence these values, along with many other factors herein unexplored.

Table 10. Physic-chemical attributes of the studied berries from the variety ‘Tatjana’

Year	Ascorbic acid	Vitamin C	Total polyphenols	Proanthocyanidins	DPPH	FRAP	TDF	SDF	IDF
2018	$1.390^{a} \pm 0.041$	$1.152^{a} \pm 0.108$	$19.463^{a} \pm 0.462$	$0.577^{a} \pm 0.025$	$75.703^{a} \pm 2.170$	$85.734^{a} \pm 3.670$	32.143 ± 1.317	$8.424^{a} \pm 0.627$	23.719 ± 0.690
2019	$0.975^{b} \pm 0.076$	$1.108^{b} \pm 0.069$	$15.030^{b} \pm 0.264$	$0.552^{a} \pm 0.005$	$59.058^{c} \pm 1.025$	$55.330^{c} \pm 3.047$	39.076 ± 9.210	$6.513^{b} \pm 0.002$	32.564 ± 9.210
2020	$0.898^{b} \pm 0.088$	$1.014^{b} \pm 0.024$	$12.230^{c} \pm 0.131$	$0.419^{b} \pm 0.074$	$62.369^{b} \pm 2.184$	$77.669^{b} \pm 2.494$	46.902 ± 5.102	$7.216^{ab} \pm 0.001$	39.687 ± 5.102

Mean values ± standard deviation. TDF: Total Dietary Fiber; SDF: Soluble Dietary Fiber; IDF: Insoluble Dietary Fiber.

Ascorbic acid and Vitamin C content expressed as g/ 100 g of dry matter.

Antioxidant assays DPPH and FRAP expressed as µmol/ g dry matter.

Total polyphenols expressed as mg gallic acid equivalent (GAE)/ g dry matter.

Proanthocyanidins expressed as mg cyanidin/ g dry matter.

TDF, SDF and IDF expressed as mg/ g dry matter.

The concentration of vitamin C could be a good indicator of the antioxidant capacity of fruit juices (4.3.1.3. Phytochemical analysis of the juice). A significantly greater concentration of vitamin C was correlated with a significantly greater antioxidant capacity (Table 10). Nevertheless, both values from the DPPH and FRAP antioxidant capacity assays showed statistically significant differences across all samples, including the comparison between the samples from 2019 and 2020, differences that were not observed for vitamin C or ascorbic acid content. The antioxidant ranking for DPPH was the same as that observed for FRAP values, being higher in the fruits from 2018 and lower in the fruits from 2019, even though the FRAP assay correlates better with the total vitamin C content of the fruit (Huang et al., 2005). Despite the fact that the concentration of vitamin C and ascorbic acid was higher in the fruits harvested in 2019 when compared to the fruits harvested in 2020, the antioxidant capacity was higher in the latter (Table 10). These results suggested there would be other compounds that could influence the antioxidant capacity of the juice on both FRAP and DPPH antioxidant assays. Nevertheless, it was stated before that the association between antioxidant capacity and compounds other than vitamin C could be difficult in matrices where the vitamin C is found at such high concentrations (4.3.1.3. Phytochemical analysis of the juice).

Other compounds which could contribute to the fruit's antioxidant capacity are the polyphenols. The concentration of the polyphenolic content was

significantly different between the berries from all studied years (Table 10). Polyphenols are widely studied molecules that are produced by secondary metabolic routes in different parts of the plant, and they are produced in higher amounts when the plant is submitted to higher stress levels (Stracke et al., 2009). Thereupon, consistent with the adaptation of the plant to the new geographical region, the polyphenolic fraction decreased over the years as the plant settled and grew healthy in the new environment. The failure of the irrigation system in 2019 that seemed to influence the concentration of other phytochemical molecules did not seem to influence the concentration of polyphenols since its concentration decreased consistently over the years of study. The total phenolic compounds found in the fruit every year were consistent with those previously reported (Guo, Chang, et al., 2017; Teleszko et al., 2015).

Besides contributing to the antioxidant capacity of the fruit, proanthocyanidins are molecules that could substantially contribute to the fruit color and fruit astringency (Basalekou et al., 2019). Statistically significant differences were observed in the proanthocyanidin concentration when comparing the fruits from 2020 against the years 2018 and 2019 (Table 10). The results indicated there were no statistically significant differences when comparing the proanthocyanidin content of the berries from 2018 against the berries from 2019. The concentration of proanthocyanidins did not follow the same pattern as the concentration of

polyphenols, as happened when analyzing the concentration of proanthocyanidins of fruits from different varieties (Table 4). The greater concentration of proanthocyanidins was related to greater *a** values from the CIE*L*a*b** score system (Table 9, 10). Although proanthocyanidins contribute to the color of the fruit (especially to the *a** value), the differences in color should not be only attributed to its concentration. Carotenoids may also be present at higher amounts in berries from 2018 and 2019, thus also granting higher observable *a** values.

The TDF showed no statistically significant differences between samples, like what also was observed when analyzing IDF (Table 10). The analysis of SDF showed statistically significant differences between the fruits from 2018 when compared to the fruits from 2019, being higher in the former. Although the differences between samples regarding the TDF and the IDF were evident (Table 8), the standard deviation of the values from IDF was very high, which in turn probably affected the robustness of the statistical analysis, like what was observed on the analysis of fiber from different varieties and origins (4.3.1.3. Phytochemical analysis of the juice). The high difference between the results on the IDF probably affected the TDF. Like what was previously reported in fruits of different varieties and origins (Table 3), the SDF fraction accounted for the lowest percentage of the TDF. Although the differences were non-significant, the TDF and the IDF were

found to increase over the years, reporting the highest values in the fruit of 2020 (Table 10).

The mineral composition was also analyzed over years. The concentration of all studied minerals differed statistically significantly across the samples from all the studied years. (Table 11). The most relevant elements (K and P) were found in significantly higher concentrations in the fruit from 2018 when compared to the fruit from other annuities (2019 and 2020). There was an observable and statistically significant decreasing tendency in the concentration of K and P. Besides, Mg and Ca, two of the minerals in high presence in sea buckthorn berries, also followed the decreasing pattern in their concentration in the berries (Table 11). The Na and Fe content experienced highly variable concentrations over the years, both varieties registering the highest value in the fruit from 2020. Interestingly, the Fe content of the fruit from 2020 was more than three times higher than the second-highest value, registered in the fruit from 2018. The Cu content was residual, but the decreasing tendency over years was also observed. The mineral profile of the berry could be effectively influenced by supplementing the soil and changing its composition (Wang & Lin, 2002), and the results of the present experiment suggest that soil supplementation may be effective to mitigate the decreasing tendency observed by several of the minerals herein analyzed.

Table 11. Mineral composition of the studied berries from the variety 'Tatjana' harvested in Spain on different years.

Year	2018	2019	2020
K	$19.717^{a} \pm 0.135$	$16.095^{b} \pm 0.135$	$12.678^{c} \pm 0.042$
P	$1.771^{a} \pm 0.000$	$1.063^{b} \pm 0.038$	$0.866^{c} \pm 0.000$
Mg	$0.494^{a} \pm 0.002$	$0.462^{b} \pm 0.013$	$0.371^{c} \pm 0.001$
Ca	$0.490^{a} \pm 0.004$	$0.447^{b} \pm 0.019$	$0.321^{c} \pm 0.002$
Na	$0.145^{b} \pm 0.000$	$0.084^{c} \pm 0.000$	$0.157^{a} \pm 0.000$
Fe	$14.492^{b} \pm 2.024$	$7.852^{c} \pm 1.499$	$49.821^{a} \pm 3.561$
Cu	$3.542^{a} \pm 0.089$	$2.907^{b} \pm 0.263$	$2.815^{b} \pm 0.000$

Mean values ± standard deviation. ND: Non detected.

K, P, Mg, Ca, Na values expressed by mg/ g dry matter

Fe, Cu values expressed by µg/ g dry matter

4.3.2.4. Phytochemical analysis of the oil

The seed and pulp oil were analyzed separately to understand the evolution of each lipidic fraction over the years of study. Oil yield was important to investigate whether the obtained oil quantity could be affected over different years and whether it could be modulated to get a higher yield. Both seed oil yield and pulp and peel oil yield showed statistically significant differences across different years (Table 12). Interestingly, the oil yield from the first year (2018) differed significantly from the extraction yield obtained in 2019 and 2020, being higher in the former. The oil yield was

related to the observed increase in the berry width, which was also higher in berries harvested in 2018 (Table 8).

The latter two did not show statistically significant differences. Coherent with the results from the analyses of both oil yields in berries from different varieties, the oil content in the seed was clearly lower than the oil content from the peel and pulp (Table 6, 7, 12). Other authors reported differences in the pulp and peel oil extraction over two consecutive years (Tiitinen et al., 2005), yet their difference was much lower than the one observed when comparing the oil yield from 2018 and 2019. Nevertheless, the pulp oil yield disclosed by the same authors was about 2 to 3%, average values very far from those observed in the present experiment. Interestingly, the seed oil yield experienced a drop of almost 2% when comparing the fruit from 2018 and the fruit from 2019 or 2020, whereas the pulp and peel oil yield experienced an increase of more than 5% when performing the same sample comparison. The most important liposoluble vitaminic compounds in seed oil are tocopherols, mainly because the concentration of carotenoids in seed oil is scarce. The statistical analyses showed significant differences between all values of α-tocopherol in seed oil (Table 12). This was the only vitaminic and liposoluble parameter that was statistically significantly different between all three annuities. The greatest concentration of α-tocopherol was found in the berries from 2019.

Table 12. Yield and composition of the oil of the berries from the variety 'Tatjana' harvested in Spain on different years.

	Seed oil			Pulp and peel oil		
Year	**Oil yield**	**α-tocopherol**	**β-carotene**	**Oil yield**	**α-tocopherol**	**β-carotene**
2018	$9.00^{a} \pm 0.02$	$1.415^{b} \pm 0.124$	$0.613^{a} \pm 0.055$	$19.74^{b} \pm 0.67$	$2.232^{a} \pm 0.463$	$11.295^{a} \pm 1.304$
2019	$7.35^{b} \pm 0.35$	$1.731^{a} \pm 0.018$	$0.257^{b} \pm 0.022$	$25.49^{a} \pm 2.02$	$1.843^{a} \pm 0.321$	$3.560^{b} \pm 0.725$
2020	$7.03^{b} \pm 0.09$	$1.187^{c} \pm 0.065$	$0.675^{a} \pm 0.043$	$26.88^{a} \pm 0.49$	$1.344^{b} \pm 0.121$	$3.968^{b} \pm 0.642$

Mean values ± standard deviation.

Oil yield expressed as g of sea buckthorn oil/ 100 g of raw matrix (seed, or pulp and peel).

α-tocopherol and β-carotene expressed as mg/ g of sea buckthorn oil.

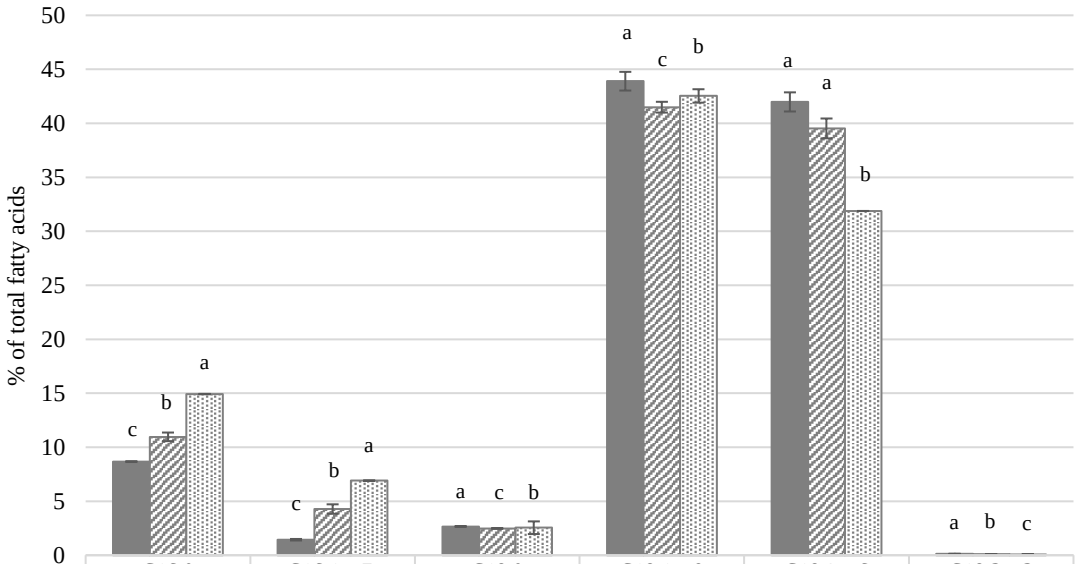
Curiously, the same berries yielded the lowest concentration of β-carotene. When looking at the other results from 2018 and 2020, it seemed that there was a negative association between the concentration of α-tocopherol and the concentration of β-carotene (Table 12). Nevertheless, these types of associations gain reliability with a greater number of samples (i.e. from more years) and the present experiment was performed only over three consecutive years. Thereupon the importance of keep studying the annual analysis of the fruit of the same orchard.

In contrast, this negative association was not observable when comparing the concentration of both studied vitaminic compounds in the pulp and peel oil. Notably, the concentration of β-carotene in the pulp and peel oil of berries from 2018 was more than three-fold the values obtained in the other years. The possible dilutive effect could explain part of the results (Tang &

Tigerstedt, 2001), as the lower oil extraction yield was reported in 2018. The concentration of α-tocopherol in the pulp and peel oil also decreased over the years, yet the concentration of this compound reduced significantly only when comparing the results from 2020 to those of any other previous year (Table 12). The reduction in the concentration of α-tocopherol in the extracted oil could be more likely attributed to the dilutive effect derived from a greater oil yield. It should be however noted that the dilutive effect could only be used as reasoning when the increase in oil yield was attributed to other compounds as well rather than only one bioactive compound.

The fatty acid profile was similar across all years in terms of proportionality of the fatty acids. That is, the palmitic and palmitoleic acids were the two major fatty acids found in the pulp and peel oil whereas the linoleic and oleic acids were the two major fatty acids found in the seed oil (Figure 4, 5). This was in line with what was found in the first part of the present experiment (Figure 2, 3) and with what other authors previously reported (Dulf, 2012). The two major fatty acids in seed oil were found at statistically significantly different levels depending on the harvesting year (Figure 4). The greatest percentage of both fatty acids was reported in the seed oil of the fruit from 2018. Interestingly, while the oleic acid values were reported very close in the seed oil of the fruit from 2019 and 2020 (differences of around 1%), the difference for linoleic acid was almost 8% when comparing the same annuities. The lower percentage of linoleic acid in the seed oil

from 2020 seemed to have an impact on the percentage of other fatty acids analyzed herein. The palmitic and palmitoleic fatty acids were found at significantly higher levels in the seed oil from 2020, which seemed to be in agreement with the lower percentage obtained for the linoleic acid (Figure 4). It was interesting to see that palmitic and palmitoleic acids increased their percentage over the studied years, jumping from 8 to almost 15% and from 1 to almost 7% from 2018 to 2020, respectively. These results suggested that a fatty acid profile with lower percentages of the most relevant C18 fatty acids in seed oil could derive in greater percentages of the most relevant C16 fatty acids (Figure 4). Therefore, the interesting increase in the percentage of palmitoleic acid came along with the unwanted increase of palmitic acid.

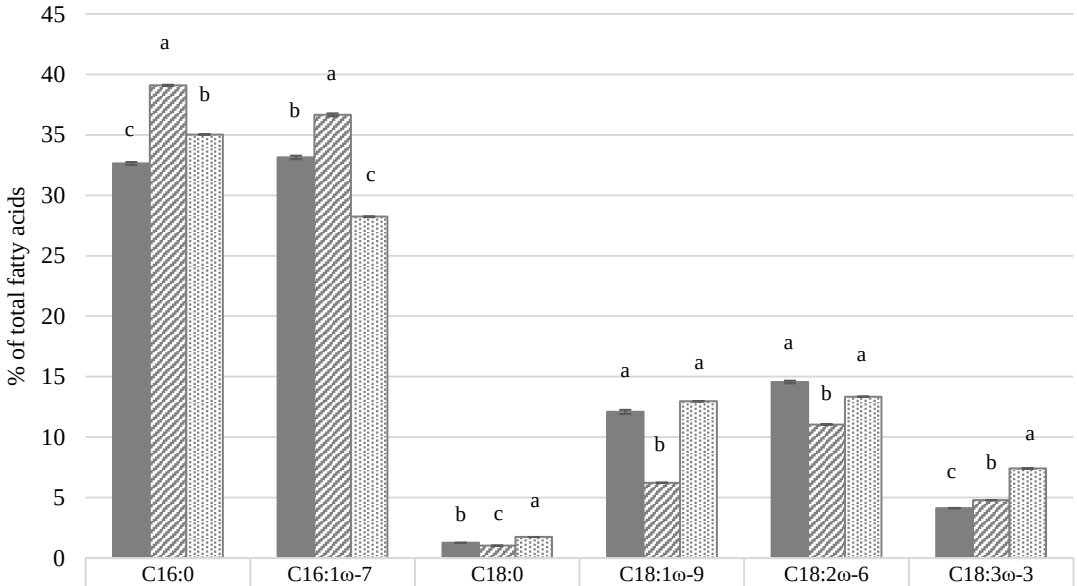


	C16:0	C16:1ω-7	C18:0	C18:1ω-9	C18:2ω-6	C18:3ω-3
2018	8.672	1.436	2.664	43.901	41.981	0.127
2019	10.964	4.278	2.481	41.495	39.528	0.108
2020	14.928	6.909	2.548	42.534	31.881	0.080

Figure 4. Fatty acid profile of SB seed oil of the 'Tatjana' variety from Spain over three consecutive years expressed as percentage of total fatty acids. Error bars show SD. Statistically significant differences are represented with different letters. C16:0 palmitic acid; C16:1ω-7 palmitoleic acid; C18:0 stearic acid; C18:1ω-9 oleic acid; C18:2ω-6 linoleic acid.

Palmitic acid and palmitoleic acid were the two major fatty acids found in the peel and pulp oil (Figure 5). Nevertheless, all fatty acids were found to be present at statistically significantly different levels over the studied years. The fruits from 2019 yielded an oil with a statistically significantly greater concentration of both palmitic and palmitoleic acids when compared to the other two harvesting seasons. The harvest from 2018 yielded an oil with the lowest amounts of palmitic acid whereas the fruit harvested in 2020 yielded an oil with the lowest amounts of palmitoleic acid. This indicated that there was no clear association with the percentage

of both C16 fatty acids. However, as it happened in the seed oil, there seem to be an association between the percentage of C16 fatty acids and the percentage of certain C18 fatty acids (those found at greater concentrations). For instance, the great values of palmitic and palmitoleic fatty acids found in the pulp and peel oil from the fruit harvested in 2019 contrasted with the lowest percentage of linoleic and oleic fatty acids found in the pulp and peel oil from the same annuity (Figure 5). In addition, the variability in the concentration of palmitic and palmitoleic acids observed in the pulp and peel oil when comparing the fruit of 2018 and 2020 led to a similar concentration of linoleic and oleic acids. The concentration of α-linolenic acid accounted for 4 to 7% of the total fatty acids, being significantly greater in the pulp and peel oil of the fruit from 2020. It should be however noted that the percentage of α-linolenic acid was greater in the pulp and peel oil when compared to the seed oil (Figure 5), different from what other authors previously reported (Dulf, 2012; Yang & Kallio, 2001) yet in line with what was observed when comparing different varieties and origins (Figure 3).



	C16:0	C16:1ω-7	C18:0	C18:1ω-9	C18:2ω-6	C18:3ω-3
2018	32.635	33.136	1.257	12.085	14.561	4.128
2019	39.092	36.659	1.022	6.229	11.049	4.782
2020	35.035	28.236	1.737	12.960	13.348	7.401

Figure 5. Fatty acid profile of SB pulp and peel oil of the 'Tatjana' variety from Spain over three consecutive years expressed as percentage of total fatty acids. Error bars show SD. Statistically significant differences are represented with different letters. C16:0 palmitic fatty acid; C16:1ω-7 palmitoleic fatty acid; C18:0 stearic fatty acid; C18:1ω-9 oleic fatty acid; C18:2ω-6 linoleic fatty acid.

The fatty acid profile of both seed and pulp and peel oil was very variable and did not follow a clear pattern over the studied years. Similarly, other parameters also showed very different values over the years. It is thus of importance to continuing the study of the nutritional profile of the orchard to understand its change over more years. This will help implement agricultural techniques to improve the nutritional profile of the berry.

4.4. Conclusions

The complete characterization of different commercially available varieties and the comparison of the obtained results was successfully performed. The results showed great differences in the nutritional profile of the berry depending on the origin and variety analyzed. It was clear that the bigger the fruit the greater the water content, which in turn triggered a dilutive effect for some of the investigated compounds. The most affected varieties by the dilutive effect were those from Latvia. The overall results helped understand differences between varieties and were used to acquire the plant with the most adequate nutritional profile with the aim of expanding the orchard from the sponsoring company.

The complete characterization of the orchard grown in 'Bellver de Cerdanya' (Catalunya, Spain), was performed over three consecutive years to elucidate the evolution of the plants – and thus the fruits – during this period. The results showed important drops in the concentration of vitamin C, which is the most important component of the aqueous fraction. In addition, the results suggested an interesting increase in oil yield from the pulp and peel, without implying an increase in the percentage of palmitoleic acid, the most interesting compound of this fraction. It was difficult from only three values to obtain a clear tendency of any of the studied parameters, also considering the relatively young age of the plants

constituting the studied orchard. Hence, it is of utmost importance to continue the study of the same orchard over several years to investigate any observable tendency and subsequently use the data to improve the nutritional profile of the berries.

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PART III

EXPERIMENTAL STUDY OF OIL EXTRACTION PROCESSES

Chapter 5: Sea buckthorn oil (SBO) extraction using green solvents and green technology

5.1. Solvent extraction of SBO

5.1.1. Introduction

The commercialization of essential oils has grown considerably over the last years. Market reports still predict a more important growth in the coming years, reaching an approximate market share of USD 16,172 million by 2026, with Europe being a leading contributor to the global market revenue (Market Study Report, 2018).

Essential oils are most of the time described as aromatic lipophilic liquids that can be extracted from different parts of plants, such as peels, seeds, flowers, barks or leaves (Tongnuanchan & Benjakul, 2014). Essential oils are widely used by the pharmaceutical, nutraceutical or food industries – among others – in the development of new products. Most of these oils are used in the development of supplements or food additives due to their unique nutritional profile, which translates into health benefits, such as SBO.

As previously presented in Chapter 1 of the present thesis, one of the most relevant products from sea buckthorn is its oil. The oil from sea buckthorn

can be extracted from two parts: (1) the seed and (2) the pulp and peel. The intake of either one or both sea buckthorn (SB) oils has beneficial effects on human health, such as improvements in blood lipid profile (Guo, Yang, Cai, & Li, 2017) modulation of hypoxia, cardioprotective properties and other antioxidant properties (Olas, 2018). These attributes could derive from its unique nutritional profile, which is high in unsaturated fatty acids, especially the seed oil. The most relevant fatty acids in sea buckthorn peel and pulp oil are palmitic and palmitoleic acids, whereas in seed oil the most important fatty acids are linoleic, α-linolenic and oleic acids. All of the unsaturated fatty acids from sea buckthorn have demonstrated health benefits (Marsiñach & Cuenca, 2019).

SB seed oil is also rich in tocopherols. Among all the tocopherols present in the seed oil, α-tocopherol appears to be the most relevant because it is found at greater concentrations (Burčová et al., 2017). In contrast, SB pulp and peel oil is a great source of carotenoids. Among them, the xanthophylls, zeaxanthin and cryptoxanthin are found in great concentrations, together with β-carotene (Pop et al., 2014).

The oil can be extracted using different techniques and different solvents, which could ultimately lead to important variations in the nutritional profile, antifungal and antibacterial activities or the overall oil extraction yield.

5.1.1.1. Essential oil extraction techniques

Several techniques have been exploited over the years for the recovery of essential oils. The most exploited extraction methodologies include cold pressing, distillation or solvent extraction (Reyes-Jurado, Franco-Vega, Remírez-Corona, Palou, & López-Malo, 2015). Cold pressing is based on the use of mechanical force to extract all the possible oil from the plant material. This technique is mainly used to extract oil from oil-rich plant materials. Cold pressing normally gives out poor oil yield and the oil shows a relatively high level of impurities (compounds that are not wanted in the final product). However, the nutritional quality of this oil is quite high, since the technique does not involve the use of heat. In contrast, distillation does include heating. The technology is based on the use of boiling water for the extraction of the volatile fraction of the oil. The plant material can be either submerged in the boiling water or in contact with the steam. The steam could be originated in the same system by boiling water under the extraction container (water/steam distillation) or from an external source (steam distillation) (Reyes-Jurado et al., 2015). Therefore, the extraction of essential oil occurs at temperatures close to the boiling point of water. The system's pressure could be modified to achieve a recovery of the essential oil at lower temperatures. Steam distillation is the most used distillation and widely exploited by essential oil producers. Although the oil yield is generally higher using solvent compared to cold pressing (Ixtaina et al.,

2011), distillation usually causes degradation of thermolabile compounds, and it is normally associated with great losses of time and energy. Finally, solvent extraction has been recently used as the main extraction system of essential oils. Using solvents instead of water allows for a better extraction of hydrophobic compounds – which is usually the case on essential oils – and due to the lower boiling temperatures of the solvents – which are often under 100 ˚C – little heat damage occurs. A well-known example is Soxhlet extraction. The solvent is evaporated and goes through a pipe to a condenser, right up to the extraction container. The solvent condensates and drops onto the extraction container, where it mixes with the sample and solubilizes the hydrophobic compounds. When it reaches a certain volume, the solvent with solubilized compounds flushes back to the boiling recipient through a syphon system. The distilled pure solvent is recycled continuously. Although it is mostly used at lab-scale, solvent extraction of essential oils has also been used at industries, since it is less energy and time-consuming than a normal distillation procedure. The major drawback is the possible contamination of the sample, which can be tackled by exhaustive evaporation after extraction, olfactory check and chemical evaluation (Reyes-Jurado et al., 2015).

Other solvent-using techniques have been developed and optimized in order to spend less time and energy in the recovery of oil from plant materials. The use of high-pressure during extraction has been used as a green

approach to obtain high-quality plant oils, reducing the time of contact between the plant sample and the solvent. Applying high-pressure allows using the same solvent in its liquid form at higher temperatures, which leads to the obtention of plant extracts in less time (Mustafa & Turner, 2011). Pressurized liquid extraction has been widely used at a lab-scale to compare the effects of different solvents on the recovery of several compounds from different plant matrices (Dunford & Zhang, 2003; Khattab & Zeitoun, 2013; Moreau, Powell, & Singh, 2003). The use of accelerated solvent extractions (ASE) is common among these studies, allowing the use of small samples. ASE emerged as one interesting way of fast, reliable and low-cost analysis of fats and oils which could be adapted either for animal or vegetable matrices.

Other interesting techniques include the use of ultrasounds or microwaves to assist during the extraction, which in turn reduces both time and energy compared to the conventional extraction (Vinatoru, Mason, & Calinescu, 2017). Ultrasound-assisted extraction (UAE) has been used as an alternative technique to improve the extraction of oils or bioactive molecules with solvents. For instance, ultrasound technology had been applied to the conventional Soxhlet methodology, showing an improvement in the final yield (Chemat et al., 2017). In addition, UAE has been considered as an efficient methodology to achieve greater recovery of bioactive compounds at lower temperature and pressure (Wang & Weller,

2006). UAE makes the bioactive compounds readily available by breaking cell walls efficiently in short exposure time (Chemat et al., 2017). It is noteworthy that the use of ultrasound technology is limited to the zone around the ultrasound-producing focus, making difficult the extraction of oil or the compounds of interest. Moreover, ultrasounds could even cancel or potentiate each other, creating an unwanted gradient of exposure to different levels of ultrasounds during extraction.

Microwave-assisted extraction (MAE) has been also widely used in the extraction of oil or bioactive compounds from vegetable matrices. In that case, electromagnetic radiations are produced towards the plant sample, inducing vibrations in its molecules and consequently generating heat. MAE also breaks cell walls, improving recovery of the compounds found inside (Wang & Weller, 2006). MAE is strongly dependent on the conductivity and dielectric susceptibility of the matrix and solvent. A high-conductivity solvent will produce a lot of heat and it is possible that important bioactive compounds will be negatively affected. Therefore, the solvent selection is specifically important in this technique. MAE also leads to an improved yield and a lower extraction time when compared to conventional methodologies (Wang & Weller, 2006). In this case, however, the application of heat is intrinsic to the technology and therefore there is no need of using an external energy source to heat-up the process.

5.1.1.2. Essential oil extraction solvents

The selection of the solvent to be used in the extraction will strongly affect the composition, yield and organoleptic quality of the resulting oil. Due to their lipophilic nature, fuel-derived solvents are greatly valued to achieve a good oil extraction. In addition, fuel-derived solvents are generally cheaper than their greener analogue. The most widely exploited solvents to extract essential oils are hexane and other apolar solvents (i.e. diethyl ether), which allow for the extraction of the most non-polar fraction of the oil (mostly fatty acids and tocopherols esters or carotenoids). This is especially important for those essential oils, such as SBO, whose benefits come from long-chain fatty acids, which happen to be highly soluble in fuel-derived solvents. Also, long-chain fatty acids are generally non-volatile, making difficult their extraction with distillation or similar techniques. In addition, most fuel-derived solvents have a low boiling point (i.e. hexane boils at 68 $^{\circ}$C, diethyl ether boils at 34.6 $^{\circ}$C and chloroform boils at 61.2 $^{\circ}$C, just to name a few), which facilitates its separation from the extracted oil, also avoiding excessive heat damage to the bioactive molecules that may be present in the oil. Oil extraction using fuel-derived solvents also varies depending on the solvent used. For instance, Wu et al. (2011) used different fuel-derived solvents (namely petroleum ether, ethyl ether, chloroform and hexane) to extract oil from peach kernel. They found that even when using solvents of the same nature, they lead to very different phytochemical

profiles of the oil. Diethyl ether was the solvent that extracted the greater amount of oil and monounsaturated fatty acids when compared to hexane. The additional extraction weight could be derived from a greater extraction of more polar compounds, such as some carotenoids (Craft & Soares, 1992), although they did not quantify them. This information is to be considered when extracting the oil from SB, rich in monounsaturated fatty acids as well as carotenoids.

Although fuel-based solvents are widely used, green solvents are becoming nowadays more relevant, as global warming is becoming an immediate and evident issue and the consumer is seeking for sustainable products and production processes. In addition, green solvent extraction brings an added value to the final product. Green solvents generally include but are not limited to ethanol, ethyl acetate, water or the combination of water and a water-soluble solvent (Castejón, Luna, & Señoráns, 2018; Danh et al., 2013), and recently terpenes (Kumar et al., 2017). Ethanol and water are used because of their low-cost, and in cases in which the interest remains in obtaining somewhat polar compounds (e.g. extraction of more polar fatty acids such as butanoic acid, or other more polar molecules such as phospholipids). However, using a mixture of ethanol and water leads to an increase in the boiling point of ethanol (formerly 78.37 ˚C), which translates into higher temperatures to separate it from the extracted oil. Using mixtures of miscible non-polar and polar solvents is not uncommon for

those matrices in which there are a significant fraction of polar and non-polar compounds to be extracted. Ethanol itself, at temperatures of 150 ˚C had been shown to yield the same amount of oil and slightly higher amounts of polyunsaturated fatty acids when compared to hexane (Castejón et al., 2018).

Another interesting green solvent that has been studied recently is 2-methyltetrahydrofuran (2-MTHF). 2-MTHF is produced out of carbohydrates from lignocellulose biomass, which represent the largest terrestrial biomass resource (Sicaire et al., 2014). Besides being biodegradable and renewable (produced from biomass), this solvent is also non-corrosive and non-carcinogenic, facilitating its application in several processes. Its relatively low boiling point (80 ˚C), together with its slightly higher polarity when compared to other non-polar solvents, makes it an interesting solvent to work with (Sicaire et al., 2015). Previous works reporting extractions with 2-MTHF had yielded oils with (a) higher monounsaturated fatty acid levels when compared to hexane, obtaining a similar overall yield (such is the case of fennel and anise (Rebey et al., 2019)), (b) oils with a slightly higher polyunsaturated fatty acid concentration in rapeseed oil (Sicaire et al., 2015), or (c) inclusive higher overall oil yield in caraway seeds when compared to hexane (Bourgou, Tounsi, Ksouri, Fauconnier, & Sellami, 2019; de Jesus, Ferreira, Fregolente, & Filho, 2018).

5.1.1.3. Demarcation

The number of studies comparing different techniques and solvents in the extraction yield or the recovery of bioactive compounds from plant matrices has been increasing lately. Most of the studies show variations on the oil yield depending on the technique used (among different evaluated parameters), as well as on the bioactive compounds analyzed (Castejón et al., 2018; Nayak et al., 2015; Zengin et al., 2020). The extraction of bioactive compounds from SB seeds using UAE and MAE has also been recently explored and optimized by Isopencu et al. (2018). They stated the optimal conditions to be 13.77 W/cm^2 of ultrasonic intensity, 40 ˚C and 10 min for UAE and 225 W, 15 ml/g (liquid/solid ratio) and 20 min for MAE, achieving an extraction efficiency of 87% for UAE and 89% for MAE when compared to a standard extraction with hexane using Soxhlet. Previous research showed similar results, with Soxhlet yielding more oil from the seed, leaves, pulp and fruit than MAE or UAE. However, extracts from MAE showed greater phenolic content and radical scavenging activities and used less time and solvent than the UAE counterpart (Sharma et al., 2008). The results could derive from the solvent used, which allows the recovery of more compounds contributing to the antioxidant activities than other solvents (Sharma et al., 2008).

The use of novel technologies to improve the extraction of SBO has been already investigated, showing some interesting results herein explained. Nevertheless, comparative review studies of different techniques should also focus on the parameters of the process (e.g. temperature) instead of focusing solely on the solvent. Besides, in order to see differences in the results obtained from using alternative solvents, different articles must be meticulously checked, and consideration should be made on the bias that the same technique would not be applied equally by different authors.

SBO is considered a valuable source of physiologically active biomolecules (Chapter 1). Therefore, its extraction from the original matrix should be carefully studied. One of the most used techniques nowadays is the combination of solvent, time and temperature to extract the oil, and hexane has been widely exploited for that purpose as it is traditionally considered one of the most efficient solvents. Nonetheless, other solvents have been used in the extraction of other essential oils, with remarkably similar or even improved outcomes when compared to hexane. Yet to the best of our knowledge, a comparative study of different solvents on the extraction of SBO has never been performed.

The present work comparatively evaluates oil yield, the concentration of α-tocopherol and β-carotene (as the most important vitamers in SB seed and pulp oil), and the fatty acid profile of SBO extracted from SB dried berries

by an accelerated solvent extractor using different solvents (hexane, diethyl ether, ethanol and 2-MTHF) at different temperatures (60, 90, 120 and 150 °C). Soxhlet extraction was used as a reference methodology.

5.1.1.4. Objective

The objective of the present work was to evaluate which solvent and which temperature would be the most efficient in extracting SBO from SB dried berries considering the nutritional quality of the resulting oil. Special attention was given to the extraction of the selected green solvents since its use could be extended to the food industry.

Additionally, a subsequent objective of the present research was to elucidate which extraction technique (from those herein explored) would be the most convenient to extract SBO from SB dried berries considering the nutritional quality of the resulting oil as well as the easiness and rapidness of the technique.

5.1.2. Experimental design

5.1.2.1. Sample preparation

A sack of sun-dried sea buckthorn berries weighing 15 kg was purchased from a local harvester from Romania. The cultivar is specifically located in

the north-east region of Romania. The berries were categorized as the subspecies *caucasica*.

Dried sea buckthorn berries were grinded down with a Thermomix® TM 21 (Vorwerk, Wuppertal, Germany). The particle size distribution of the powder was measured by gravimetry using sieves of different mesh size. Particle size distribution was quantified twice (by "Centro Nacional de Tecnologia Alimentaria" (CNTA, Navarra, Spain) and Ofice, S.L. (Barcelona, Spain)).

Approximately 8 g of dried and ground sea buckthorn berries were mixed thoroughly with diatomaceous earth (DE) at a ratio of 4:1. An ASE extraction cell was filled with a cellulose membrane and the mix, in that order. The cell was closed and inserted in the ASE cell rack. For Soxhlet extraction, the same amount of sample was wrapped in a medium lab-working filter paper (Letslab delivering solutions S.L.U., Catalunya, Spain). The wrapped sample was placed directly in the sample compartment of the Soxhlet set-up.

5.1.2.2. Oil extraction

A total of four solvents and four temperatures were tested in the present research using ASE methodology, summing up a total of 16 experimental conditions with two independent variables. The experimental run with Soxhlet methodology made it up to 17 experimental conditions.

Oil was extracted from sea buckthorn dried berry powder by using an Accelerated Solvent Extractor 200 (Dionex, Thermo Fisher Scientific, California, U.S). The pressure was set at 1,500 psi, static time at 10 min, preheating at 5 min, flushing volume at 30% of the total cell volume, purging time at 30 s and only one cycle was used. The temperature was set at 60, 90, 120 or 150 ˚C.

SBO was also extracted from the samples by using hexane in a Soxhlet experimental set-up. Solvent (hexane) was allowed to run free through the system for 5 consecutive hours until no more oil was dragged from the sample.

After the extraction was finished (either ASE or Soxhlet), the extracted solution was poured into a spherical ball flask. The flask was then immediately attached to a rotavapor for 45 minutes to allow the solvent to evaporate at 45 ˚C and 150 mbar. The flask was then allowed to cool and subsequently weighed in an analytical balance for the yield. Immediately after, the oil was stored in amber tinted chromatography vials at -80 ˚C for further analysis.

Solvents and material

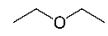
Hexane, ethanol, diethyl ether and 2-methyltetrahydrofuran were used for the experiment. *n*-Hexane (with isomers) 99% purity (HPLC grade) was purchased from Labbox Labware, S. L., Catalunya, Spain. 2-

Methyltetrahydrofuran (2-MTHF) stabilized with 2,6-di-tert-butyl-4-methylphenol was purchased from Merck KGaA, Darmstadt, Germany. Diethyl Ether, stabilized with 6 ppm of BHT and Ethanol absolute (99.8 %) were purchased from Panreac Química S. A. U., Catalunya, Spain. Solvent characteristics are depicted in Table 1.

Polytetrafluoroethylene (PTFE) O-rings, PEEK seals, cell frites, 20 mm cellulose filters and PTFE-lined silicone septa for the ASE were purchased from Restek Corporation, Pennsylvania, U.S.

Ethanol and 2-MTHF dried extracts were further mixed with water (at room temperature) since during the extraction some polar compounds were dragged out (see 5.1.3.2.). The mixture of SBO extracted with ethanol and water resulted in a clear solubilization. The oil was recovered from the walls of the lab flask by dissolving it with hexane. Dissolution was complete and SBO was obtained after solvent drying at 45 ºC using a rotary evaporator. The mixture of SBO extracted with 2-MTHF and water resulted in a fuzzy mixture. The content of the lab-flask was poured into a centrifugal vial and submitted at 10,000 rpm for 10 min at room temperature. SBO was recovered from the upper phase using a glass Pasteur pipette.

Table 1. Characteristics of the solvents used in the present study.

Solvent	Density (g/cm3)	Molecular weight (g/mol)	Purity (%)	Boiling point (ºC)	Polarity*	Molecule scheme
Diethyl ether	0.71	74.12	≥99.5	34.60	5.77	
Hexane	0.66	86.18	≥99	68.00	2.56	
2-MTHF	0.85	86.13	≥99	80.20	6.99†	
Ethanol	0.79	46.07	≥99.5	78.37	8.05	

*Polarity according to the Spectral Polarity Index developed by Freed, Biesecker, & Middleton (1990)

†Polarity value from tetrahydrofuran (THF). According to Aycock (2007), solvent polarity and Lewis base strength properties of 2-MTHF is somewhere between THF and diethyl ether.

5.1.2.3. Yield

The extraction yield was measured by weight difference after the solvent containing the oil coming from the extraction was fully evaporated.

5.1.2.4. Sample analysis

Phytochemical analysis of the extracted SBO was performed. β-carotene and α-tocopherol were analyzed using a simultaneous methodology by HPLC. Fatty acid profile was measured as the percentage of total fatty acids using a gas chromatography technique. The analytical methodology is presented in Chapter 3. Every sample analysis procedure was performed

thrice, and measurements by the appropriate equipment were performed in duplicate.

5.1.2.5. Statistical analysis

All statistical analysis was performed with the software R-4.0. Assumptions were checked by first visually interpreting the Q-Q and boxplots from all analysis. Normality was double-checked by the Shapiro-Wilk test, which gave non-significant values for all the ran analysis, therefore proving the normality of the whole data. Subsequently, the statistical analysis of the data was performed. A two-way ANOVA was run first to understand the importance of the interaction between the temperature and the solvent on the final extraction yield, and concentration of α-tocopherol and β-carotene. Further analysis involved the use of Tukey's *post hoc* tests to understand possible significant differences within variables. To test the single effects of each solvent, a one-way ANOVA was performed to each solvent's results, to understand differences between temperatures.

The fatty acid profile was analyzed by using the principal component analysis (PCA). The PCA is a useful technique when analyzing the fatty acid profile in different samples, as it allows to discriminate between samples using a two-dimensional graph through a dimension reduction of the variables. Further analysis included a two-way ANOVA to test for the effect of solvent and temperature on the extraction of different fatty acid

groups from SB dried berries as well as to test the possible interaction effect. In addition, one-way ANOVA tests were run with all the results obtained with each solvent separately to better understand how one solvent behaves across all studied temperatures in terms of the fatty acid profile of SBO.

5.1.3. Results and discussion

5.1.3.1. Particle size

Particle size plays a major role in oil extraction, as it could greatly influence the outcome yield or the analysis of the specific bioactive compounds. Particle size was measured by two independent laboratories. The average particle size is shown in Table 2. The vast majority of the particles measured less than 2000 μm, and almost half of them measured less than 800 μm.

Table 2. The average particle size of dried SB berries powder used for the extractions.

Particle size	% of the total mass
< 2500 µm	100
< 2000 µm	97.80
< 1000 µm	60.46
< 800 µm	48.73
< 500 µm	16.22

5.1.3.2. Extraction yield of SBO extracted with different solvents

The effect of solvent and temperature on the extraction yield

A significant interaction effect between the solvent and the temperature used during the extraction was observed on the percentage of the extracted SBO (g of oil / g of dried fruit), F (9, 34) = 10.355, p<.05. The SBO yield using hexane, ethanol, diethyl ether and 2-MTHF were affected differently by temperature. The overall yield of SBO did not follow a similar pattern for all the solvents across all temperatures. In fact, the slope of the temperature line differs across solvents, translating in this significant effect. Changing temperature may affect solvents differently, modifying their physical properties and therefore changing their diffusivity and solubility within SB dried berries. At each temperature, each solvent would show

specific physical properties, with changed solubility and therefore with an improved or decreased oil yield at a specific temperature (Figure 1).

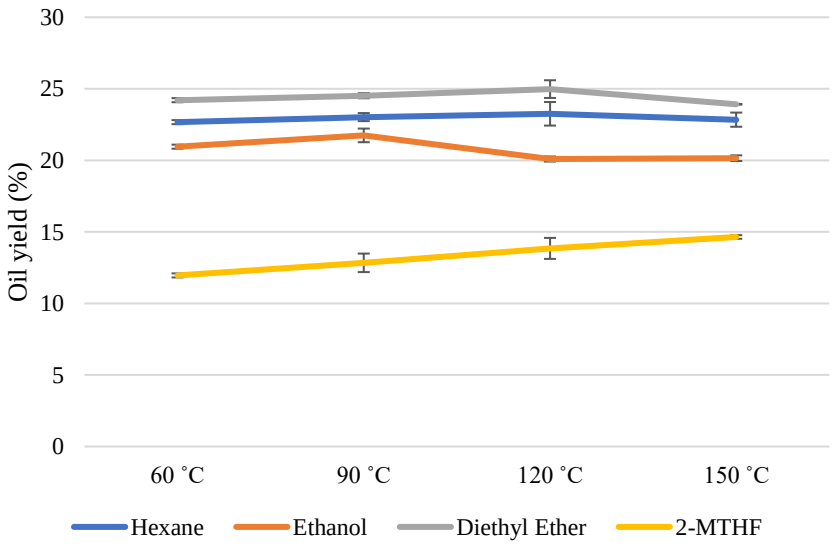


Figure 1. SBO yield as a function of temperature using different solvents. The variable tendency of the lines depending on the solvent indicates an interaction effect between solvent and temperature.

The most efficient temperature for extracting SBO was different depending on the solvent used, being 120 ˚C for hexane and diethyl ether (23.25% and 24.98% yield, respectively), 90 ˚C for ethanol (21.75% yield), and 150 ˚C for 2-MTHF extraction (14.65% yield) (Figure 1). The efficiency of extraction seemed to be greater by increasing the extraction temperature when using 2-MTHF, which was partly true when using diethyl ether as extraction solvent (Figure 1). However, the latter achieved the poorest extraction yield at 150 ˚C, making evident the different behaviour of each solvent across different temperatures. Results of the extraction yield

reported from other authors concluded that the greater the temperature of the extraction, the greater the extraction yield, although this was not true for all the solvents assayed in the present experiment. In echium seed oil, the greatest extraction yield was obtained at temperatures of 150 ˚C when using ethanol or hexane as extraction solvents (Castejón et al., 2018) when compared to lower temperatures. Interestingly, the pattern clearly pointed out that the more polar the solvent was, the more was it affected by changes in temperature (Figure 1). It is important to note that the solvent 2-MTHF was strongly and significantly influenced by changes in temperature when compared to the rest of the solvents.

There was also a significant main effect of the solvent in the extraction yield of SBO (in g of SBO / g of dried berry), F (3, 34) = 1,554.311, p<.05. The Tukey's *post hoc* test revealed that all the solvents had significantly different extraction yields for SBO (Figure 2). Interestingly, using the Soxhlet technique (control) instead of ASE (using hexane as solvent) yielded significantly more oil. Despite the fact that this difference was significant, this difference translated only into 0.65% more oil extracted by the former technique. This difference seemed to be in line with what previous authors found using hexane on flaxseeds (42.40% using Soxhlet and 41.90% using ASE (Khattab & Zeitoun, 2013)) or echium seeds (31.3% using Soxhlet and 31.2% using ASE (Castejón et al., 2018)). One of the possible reasons behind this could be the longer time the sample is left

during Soxhlet extraction (more cycles of extraction, each with renewed, distilled solvent), which may help to extract more oil when compared to the usually short static time employed in ASE extractions.

When comparing the same technique (ASE), diethyl ether was the solvent achieving significantly greater oil yield when compared to ethanol, hexane or 2-MTHF (Figure 2). The results indicated that SBO may contain mostly non-polar compounds that can be extracted by using a non-polar solvent such as hexane (Table 1). However, there may be other polar compounds at a lower presence that would benefit from the extraction with diethyl ether. Similar results were reported using Soxhlet extraction with diethyl ether on peach kernels, which achieved significantly greater oil extraction yield when compared to all other solvents, including hexane (Wu et al., 2011), or on potato peel oil extracted with different organic solvents, including ethanol, hexane and diethyl ether (Zia-ur-Rehman, Habib, & Shah, 2004).

The extractions using the most polar solvents, namely 2-MTHF and ethanol, yielded significantly lower oil yield when compared to the "non-green" solvents. Due to the relatively high polarity of both green solvents, the extraction yield was affected greatly. Ethanol yielded less oil than hexane using pressurized liquid extractions at all temperatures (Figure 2), similar to what was found in echium seeds at different temperatures (Castejón et al., 2018), probably due to the lipophilic nature of the extract.

Ethanol can extract the part of lipids that have a slightly polar behaviour, such as phospholipids, waxes or proteins (Dunford & Zhang, 2003). In the case of SBO, proteins or phospholipids are not expected to interfere greatly. Nevertheless, ethanol could also extract other water-soluble components present in dried berries. An important group constituted of hydrophilic molecules in dried SB berries is carbohydrates. SB dried berries are a great source of sugars, mostly fructose and glucose, but also rhamnose (Chapter 8). SB sugars could be mostly extracted when using ethanol, yielding up to 75% of the initial sugar content (Baümler, Carrín, & Carelli, 2016). The applied further treatment allowed the confirmation of the theoretical reasoning. Great values of extraction (around 50%) were achieved during the first exploration with ethanol, but when water was added to rinse the outcoming oil, the water drained most of the extracted soluble compounds, including sugars, obtaining the final values represented in Figure 2.

Finally, the lowest yield was recorded for the extraction with 2-MTHF, roughly achieving an extraction yield of 13% (Figure 2). Lower extraction yield using 2-MTHF was recorded previously by other authors (Ben-youssef, Fakhfakh, Breil, Abert-vian, & Chemat, 2017), but higher extraction yields were recorded for anise seeds when using 2-MTHF (Rebey et al., 2019). Consonant to what resulted by using ethanol, 2-MTHF effectively achieved a preliminary extraction of higher yield from SB dried berries. The original extraction yield (around 32%) dropped to 13% after

applying the water rinsing process. Thus, it was considered that most of the 'extra' yield derived from the extraction of sugars or other soluble compounds. The difference between the final yield of 2-MTHF and ethanol could be because of two main reasons. First, it could be a consequence from the polarity difference (Table 1); ethanol then extracted a greater polar fraction than 2-MTHF, and part of it could remain in the final oil yield (after rinsing it with water) due to its great amount. Second, it could be a consequence of distinct further processing. While in ethanol a normal rinse would suffice, with 2-MTHF a centrifugation was needed to allow phase separation, leading to possible residues of oil left in the centrifuge vial.

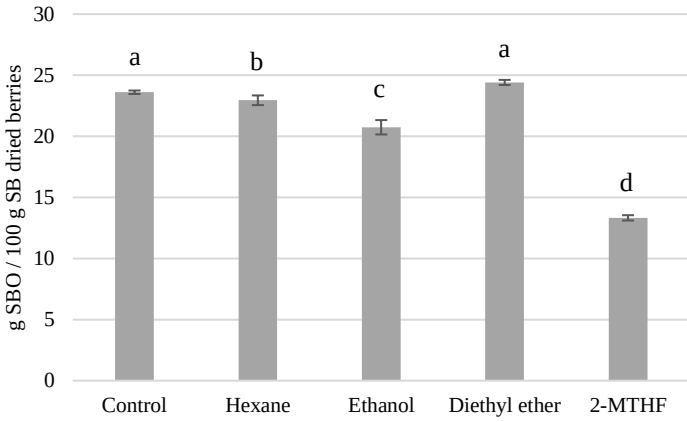


Figure 2. Extraction yield of SBO by ASE using different solvents at high pressure compared to Soxhlet used as a conventional method (Control)).

There was also a significant main effect of temperature on the extraction yield of SBO, F (3, 34) = 4.992, p<.05. The Tukey's *post hoc* tests revealed significant differences in yield between the control group and the rest of the

temperatures, being greater in the former. These results did not allow to discriminate between techniques, since ASE was used for all the solvents and Soxhlet (control group) was only used with hexane. The most important fact of the resulting analysis is that no significant differences were observed in the extraction yield of SBO extracted at different temperatures by using the ASE technique with different solvents.

Individual effect of each solvent on the extraction yield

One of the main interests of the present research was to investigate the effect of the temperature on several specific solvents. In other words, understand how the temperature would affect the extraction of the compounds of interest using the same solvent. Therefore, one-way ANOVAs were run for each solvent to test for differences in the outcomes at a wide range of temperatures, and in that way exclude the control extraction of the analysis, which was used to test differences between overall solvent extractions and temperatures.

There was no significant effect of the temperature on the extraction yield of SBO when using hexane as a solvent, F (3, 8) = 1.142, p = .389 (Table 3). In other words, increasing the extraction temperature did not lead to a significant increase in oil yield, an effect previously reported on the extraction of corn and oat oil (Moreau et al., 2003), amaranth seed oil (Kraujalis, Venskutonis, Pukalskas, & Kazernavi, 2013) and echium seed

oil (Castejón et al., 2018). The temperatures of 90 and 120 ˚C achieved a higher non-significant extraction yield when compared to the other two temperatures used, 90 ˚C being the most efficient.

Table 3. SBO extraction yield (in g of SB oil / 100 g of dried SB berries) split by solvent and temperature used.

	Solvent			
Temperature (ºC)	Hexane	Ethanol	Diethyl ether	2-MTHF
60	22,67 ± 0.28	$20,96^{ab}$ ± 0.82	$24,21^{b}$ ± 0.50	$11,960^{d}$ ±0.16
90	23,56 ± 0.48	$21,75^{a}$ ± 0.18	$24,52^{ab}$ ± 0.20	$12,84^{c}$ ± 0.15
120	23,25 ± 0.18	$20,09^{b}$ ± 0.62	$24,98^{a}$ ± 0.02	$13,85^{b}$ ± 0.19
150	22,84 ± 0.65	$20,15^{b}$ ± 0.73	$23,92^{b}$ ± 0.13	$14,65^{a}$ ± 0.37

Values expressed as mean ± SD

Different letters mean significant differences at $p<.05$.

Comparisons were made in each solvent column. Cross-comparison between solvents were not included in the present table (for differences between solvents and temperatures refer to the results obtained from the two-way ANOVA)

There was a significant effect of the temperature on the extraction yield of SBO extracted with ethanol, F (3, 8) = 4.464, $p<.05$ (Table 3). The *post hoc* tests revealed that there were significant differences in the extraction yield of ethanol at 90 ˚C when compared to 120 ˚C or 150 ˚C, being lower in the latter two. No significant differences were observed between 60 ˚C and any other temperature. The extraction at 90 ˚C yielded more oil than the extraction at 60 ˚C, although the difference was non-significant. Extracting

corn and oat oil with ethanol at 100 ˚C also yielded more oil than at 40 ˚C, although this difference was significant (Moreau et al., 2003). Results from other authors, however, indicated that the higher the extraction temperature, the higher the oil yield when using ethanol as extracting agent (Castejón et al., 2018; Jablonsky, Haz, & Andrea, 2015). Consideration should be taken to optimize the extraction of water-soluble compounds from SBO extracted with ethanol to see if this step could have been affecting the oil yield results. However, it was extracted three times per each of the solvents, indicating that probably the optimal temperature for the extraction of SBO using ethanol was 90 ˚C instead of using higher temperatures. It may be possible that certain compounds are more efficiently extracted by a highly polar solvent at this temperature rather than at higher temperatures.

There was a significant effect of the temperature on the extraction yield of SBO extracted with diethyl ether, F (3, 8) = 8.251, p<.05 (Table 3). The *post hoc* tests revealed that there was a significant difference between the extraction yield obtained at 120 ˚C when compared to the extraction yield obtained at 60 and 150 ˚C, being lower in the latter two. No significant differences were observed in oil yield extracted at 90 ˚C compared to that extracted at 120 ˚C, although the latter temperature achieved greater yield. The yield was also higher extracting the oil at 90 ˚C when compared to 60 ˚C. The extraction at 150 ˚C seemed to be the less efficient extraction temperature in terms of yield (Table 3). Literature did not provide enough

evidence on the variation in vegetable oil yield using diethyl ether as the extraction solvent. However, it may be again that diethyl ether had an optimum range of temperature when extracting SBO, which was somewhere around 120 ˚C.

At last but not least, there was a significant effect of the temperature on the extraction yield in SBO extracted with 2-MTHF, F (3, 8) = 75.005, p<.05 (Table 3). Tukey's *post hoc* test revealed statistically significant differences in the yield of all extraction temperatures. The greater extraction yield was achieved at 150 ˚C, and in a decreasing order at 120 ˚C, 90 ˚C and 60 ˚C. The difference between the latter and the former was more than 2%. The results showed this solvent to be clearly dependent on temperature changes. The extraction of oil was positively associated with temperature. Results from other scientific publications used only one temperature, most of them performing extractions with 2-MTHF using a Soxhlet set-up (Bourgou et al., 2019; Rebey et al., 2019; Sicaire et al., 2015). Interestingly, 2-MTHF was used to extract limonene from orange peel at different times (30, 60, 90, 120, 150 and 180 min) and temperatures (30, 50, 70 and 90 ˚C) and found an increasing trend in the limonene yield when increasing either the extraction time or temperature (Ozturk, Winterburn, & Gonzalez-Miquel, 2019). The limonene recovery yield jumped from around 0.5% at 30 ˚C to almost 1.5% at 70 ˚C, with a slight decrease when the temperature rose to

90 ˚C. The increasing trend in SBO extraction yield was also observed in the present experiment.

5.1.3.3. Concentration of α-tocopherol and β-carotene in SBO

The effect of solvent and temperature on the concentration of α-tocopherol and β-carotene

The concentration of α-tocopherol in SBO extracted with hexane, ethanol, diethyl ether and 2-MTHF were affected differently by temperature, F (9, 85) = 23.344, $p<.05$. In other words, the concentration of α-tocopherol in SBO did not follow the same pattern across temperatures when using different solvents for the extraction (Figure 3). There was also a significant interaction effect between the solvent and the temperature used during the extraction, on the concentration of β-carotene in the extracted SBO, F (9, 85) = 56.457, $p<.05$ (Figure 4).

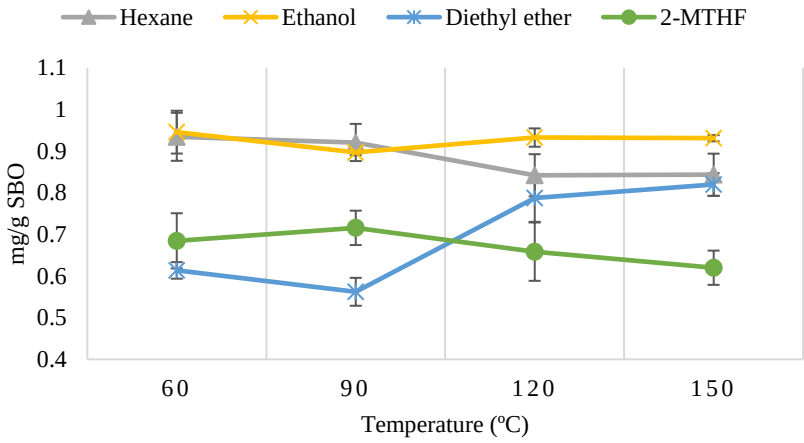


Figure 3. α-tocopherol concentration in extracted SBO as a function of temperature using different solvents. The variable tendency of the lines depending on the solvent indicates an interaction effect between solvent and temperature.

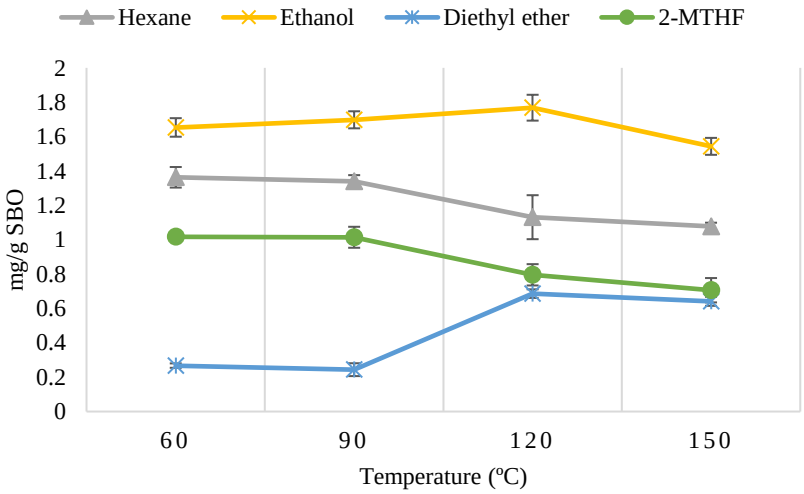


Figure 4. β-tocopherol concentration in extracted SBO as a function of temperature using different solvents. The variable tendency of the lines depending on the solvent indicates an interaction effect between solvent and temperature.

When looking at the values obtained from the extraction with petroleum-based solvents, it appears that temperatures of 60 ºC and 90 ºC would be somewhat more appropriate for the recovery of α-tocopherol and β-carotene

using hexane yet it would be the worst choice when extracting the oil with diethyl ether (Figure 3, 4). Ethanol and 2-MTHF, the most polar and green solvents used in the present work, did not seem to show great differences in concentration of α-tocopherol and β-carotene in the extracted oil after applying different temperatures. The green solvents herein used were thus less affected by changes in the extraction temperature when compared to diethyl ether (Figure 3, 4). Nevertheless, this conclusion was something that needed testing. One-way ANOVAs were thus run for each solvent and each analyte to test for differences in the outcomes across all temperatures.

There was a significant main effect of the solvent used in the extraction, on the concentration of α-tocopherol in the extracted oil, $F(3, 85) = 242.887$, $p<.05$ (Figure 5). The Tukey's *post hoc* test revealed that all the solvents differed significantly on the recovery of α-tocopherol from SBO. The concentration of α-tocopherol in extracted SBO was greater in the oil extracted with ethanol than the oil extracted with hexane (although statistically significantly equal), and both were significantly greater than the oil extracted with Soxhlet (control). The lowest concentration of α-tocopherol was observed in SBO extracted with diethyl ether or 2-MTHF. A matrix of methanol/chloroform also extracted higher amounts of different tocopherols when extracting oil from almond, Brazil nuts, hazelnuts or pecan nuts when compared to other less polar compounds (Miraliakbari & Shahidi, 2008). In addition, other authors reported a higher extraction of

tocopherols – including α-tocopherol – when using ethanol as compared to the extraction with hexane (Baümler et al., 2016). They reasoned that this difference may reside in the difference between solvent polarities. Overall concentrations of extracted α-tocopherol ranged from 0.6 to 1.0 mg/ g SBO, values similar to what previous authors found in SB (Kallio, Yang, & Peippo, 2002). It is important to mention that the values of the present research correspond to SB dried berries instead of different fractions from the fruit (i.e. peel and seeds), differing from values found by other authors.

The concentration of α-tocopherol in SBO was greater in the samples extracted by ASE (hexane) when compared to the same solvent and Soxhlet technique (control). The longer extraction times in Soxhlet extraction may trigger the destabilization of α-tocopherol to a relevant extent in which differences between these two groups become significant. In contrast, the concentration of α-tocopherol in SBO extracted with diethyl ether or 2-MTHF was significantly lower than the concentration in control samples (Figure 5). No significant differences were observed in the concentration of α-tocopherol in oils extracted with diethyl ether or 2-MTHF. This was interesting since previous authors found greater saponification values from peach kernel oil extracted with ethyl ether rather than oil extracted with hexane, translating into a greater extraction of saponifiable matter, including tocopherols and carotenoids (Wu et al., 2011). However, other authors reported higher antioxidant values of the oil (using the DPPH

technique) extracted with hexane rather than the oil extracted with 2-MTHF (Rebey et al., 2019). Antioxidant activity may be subjected to higher polyphenolic content but most importantly to the vitaminic content of the oil. Therefore, greater antioxidant capacity in fennel oil as reported by Rebey et al. (2019) would translate into higher vitaminic content, probably tocopherols (although other compounds may be also present). The values reported herein would support the theory of a greater concentration of α-tocopherol in SBO oil extracted with hexane when compared to 2-MTHF.

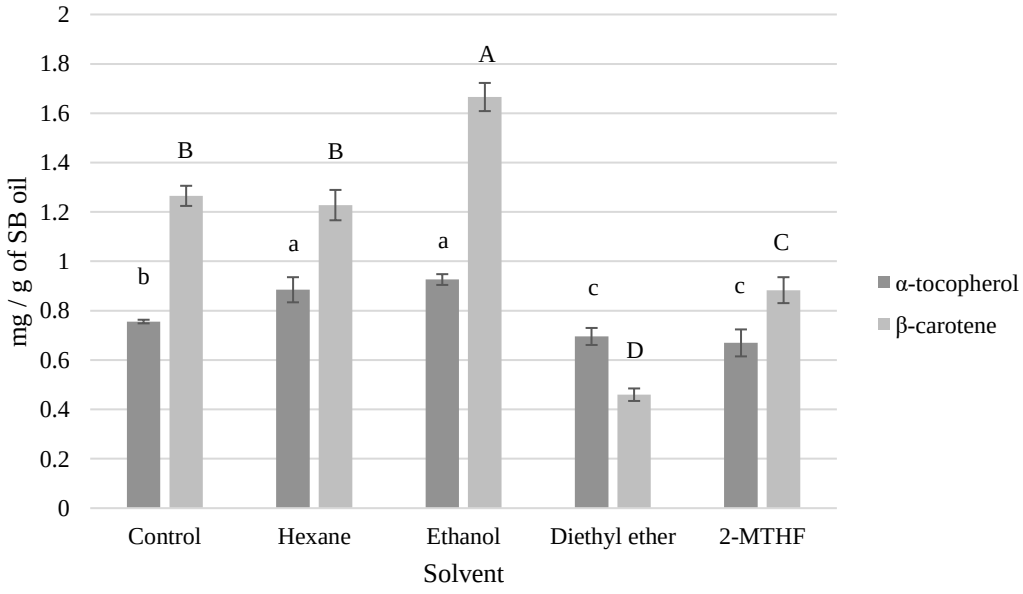


Figure 5. Average concentration of alfa-tocopherol and beta-carotene in SBO extracted with different solvents. Error bar show SD. Different letters mean significant differences at $p<.05$. Comparisons are not made across upper- and lower-case letters (i.e. across vitamers).

The solvent effect was also significant on the concentration of β-carotene in the extracted oil, F (3, 85) = 1945.677, $p<.05$ (Figure 5). The Tukey's

post hoc test revealed that all the solvents differed significantly on the recovery of β-carotene from sea buckthorn oil, except the oil extracted with hexane using the technique ASE when compared to the same solvent using the Soxhlet methodology (control). It could be therefore drawn that the use of different solvents may be more relevant than the use of different techniques. However, more solvents should be tried in the Soxhlet extraction in order to get more reliable results to support this conclusion.

The concentration of β-carotene in extracted SBO was greater in the oil extracted with ethanol than the oil extracted with hexane and the oil extracted in control samples. The lowest concentration was found in oil extracted with diethyl ether. Carotenoids had also been extracted in significantly greater amounts when using ethanol as the extraction solvent instead of other conventional solvents such as diethyl ether (Lichtenthaler & Wellburn, 1983) or acetone (Marsili & Callahan, 1993). Ethanol was also found to be the most efficient solvent for the extraction of β-carotene when compared to other green alternatives, such as ethyl acetate and ethyl lactate (Ishida & Chapman, 2009). Likewise, the values herein reported add up to this fact, being higher the values from ethanol extraction when compared to 2-MTHF. However, ethanol as a polar solvent was expected to extract lower amounts of carotenoids, since these are highly non-polar compounds (Yara-Varón et al., 2016). One of the possible reasons could be that a great deal of carotenoids may be present in SBO as a carotenoid glycoside, or

bound to other polar compounds, making the extraction with ethanol more efficient for their recovery.

Like what was observed for α-tocopherol, the concentration of β-carotene in extracted SBO was significantly greater in the control samples when compared to the samples extracted with diethyl ether or 2-MTHF. However, in this case, differences were not observed between the two extractions with hexane (control samples and ASE extraction), contrarily to what had been reported by Saini & Keum (2018).

Wu et al. (2011) did not analyze the content of carotenoids specifically, but they found greater antioxidant capacity (in terms of DPPH and Trolox equivalent antioxidant capacity assay (TEAC)) in the peach kernel oil extracted with hexane when compared to the oil extracted with ethyl ether. Greater antioxidant capacity does not imply greater vitamin concentration, yet vitamins could contribute significantly to this attribute, as explained before. Hexane could then extract oil with greater antioxidant activity, and thus probably with greater vitamin concentration, as observed in the present experiment. Nevertheless, different vitamins could be present in the peach kernel (tocopherols, carotenoids (Wu et al., 2011)) and it is thereupon difficult to assume whether the greater antioxidant capacity could come from one vitamin or another.

The concentration of β-carotene extracted with diethyl ether was significantly lower compared to the values obtained by 2-MTHF extraction. Diethyl ether is a less polar solvent when compared to 2-MTHF, closer to the polarity of hexane. Rebey et al. (2019) reported higher antioxidant values of fennel and anise oils extracted with hexane compared to oils extracted with 2-MTHF. β-carotene, together with lycopene, are highly lipophilic non-polar carotenoids mainly because of the lack of a functional polar groups in their structure (Saini & Keum, 2018), therefore making them theoretically chemically more prone to their extraction with apolar solvents.

Nonetheless, ethanol extraction achieved the greatest concentration of β-carotene in the present experiment, which although in line with what other authors observed, those results are completely opposed to the chemical structural theory reported above. The results of the present study could be influenced by the two-step extraction performed after the extraction with the more polar and green solvents. However, a two-step extraction would yield a lower concentration of certain compounds – because of the possible dragging of compounds during the second extraction. The two-step extraction would therefore yield less concentration of vitamins, an eloquent conclusion that was not observed in the ethanol extraction.

There was a significant main effect of temperature on the concentration of α-tocopherol in the extracted SBO, F (3, 85) = 3.051, p<.05. The Tukey's *post hoc* tests revealed significant differences in the concentration of α-tocopherol between the control group and the extraction at 120 and 150 ˚C, being higher at the latter two. No significant differences were observed in the concentration of α-tocopherol between the SBO extracted at 120 and the SBO extracted at 150 ˚C. The greater the temperature, the greater the recovery of α-tocopherol, which achieved a plateau after the extraction temperature was set at 120 ºC (Figure 6). This was in line with what other authors observed, although using only one solvent (hexane) and observing the steady recovery of tocopherols above 100 ºC (Sanagi, See, Ibrahim, & Naim, 2005). Greater temperatures generally allow getting better mass transfer rates between the solvent and the sample matrices, which directly increase the capacity of the solvent to solubilize analytes (Sanagi et al., 2005). Lower recoveries of α-tocopherol were observed in the control extraction, which may come from the longer extraction times and the subsequent oxidation of the analytes.

There was also a significant main effect of temperature on the concentration of β-carotene in the extracted SBO, F (3, 85) = 20.099, p<.05. Significant differences were observed in the concentration of β-carotene between the control group and the extraction at any other temperature, being higher in the former (Figure 6). It seems that the degradation of β-carotene during

extraction was slightly more influenced by the temperature rather than by the exposure of the compound to the light and oxygen during the extraction. This was clearly in contrast with what was observed during the extraction of α-tocopherol (Figure 6). The concentration of β-carotene was significantly lower in the oil extracted at 150 ˚C when compared to all other temperatures. No significant differences were found between any other temperature (60, 90 and 120 ˚C). Other authors already reported losses of antioxidant capacity of carotenoids extracts – from *Haematococcus pluvialis* microalga – when increasing the extraction temperature (Jaime et al., 2010). The loss of antioxidant capacity was attributed to the loss of important carotenoid fractions, being severe above 100 ºC. The present work supports this theory, with a stable extraction of β-carotene, significantly diminishing the concentration of the analyte in the oil extracted at 150 ºC, when compared to 120 ºC or lower temperatures (Figure 6). The effect of the temperature is shown herein in more detail as the aforementioned work included temperatures of 50, 100, 150 and 200 ºC.

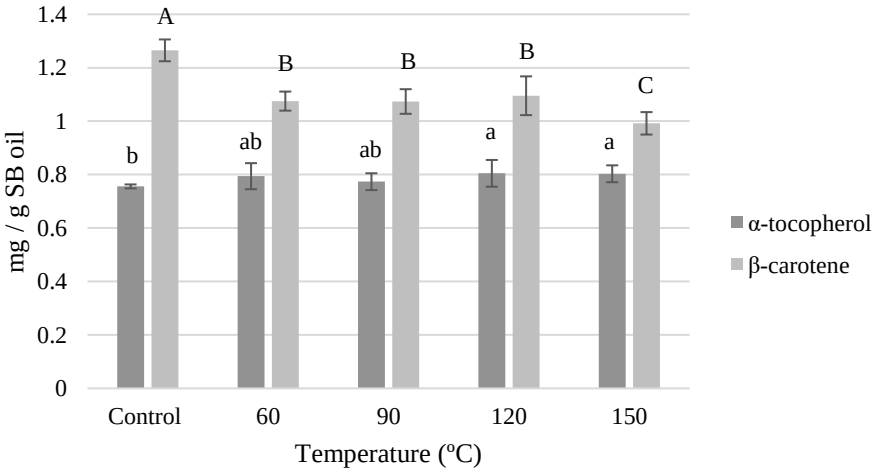


Figure 6. Average concentration of alfa-tocopherol and beta-carotene in SBO extracted at different temperatures. Error bars show SD. Different letters mean significant differences at $p<.05$. Comparisons are not made across upper- and lower-case letters (i.e. across vitamers).

Individual effect of each solvent on the recovery of α-tocopherol

The oil yield was higher when using non-polar solvents when compared to polar, greener solvents (Figure 1). This would lead to believe that using diethyl ether or hexane, a greater number of lipophilic compounds (including tocopherols) would be extracted more efficiently. However, the optimal extraction temperature on the recovery of these compounds should be determined. As shown in Table 4, there was a significant effect of the temperature on the concentration of α-tocopherol in SBO extracted with hexane, $F(3, 20) = 6.701$, $p<.05$. Using extraction temperatures of 60 ˚C and 90 ˚C the recovery of α-tocopherol from SBO was significantly greater than using higher temperatures of 120 ˚C and 150 ˚C (Table 4). As reported earlier by Moreau et al. (2003), higher temperatures can achieve higher

amounts of tocopherols using hexane as the extraction solvent. However, the original food matrix may play a role in the extraction of these non-polar compounds. Moreau et al. (2003) showed that, while the recovery of γ-tocopherol from corn extracts was higher at higher temperatures, the recovery of the same compound from oat extracts was lower at higher temperatures. The same authors explained however that the reported values were very similar and increasing the extraction temperature did not yield a significant improvement on the recovery of γ-tocopherol. In the present work, results were significantly different depending on the temperature used when using hexane as extraction solvent. The recovery of α-tocopherol from SBO using hexane was higher at lower extraction temperatures (Table 4), making the extraction similar to that observed by Moreau et al. (2003) for oat extracts. Different food matrices imply that tocopherols may be found in different conformations, explaining the differences of tocopherol extraction at different temperatures (Marquardt, Van Oosten, Ghelfi, Atkinson, & Harroun, 2016). A more linear, saturated conformation would be more likely to be extracted by hexane rather than with other solvents since this conformation would benefit from using a non-polar solvent for the extraction. However, explorations regarding the extraction using hexane at different temperatures have not yet been investigated. It would be plausible to assume that as temperature rises, the solubility with hexane increases as well, since the solubility and diffusivity increases in heated

solvents. Nevertheless, here we show that at lower extraction temperatures, the best results of α-tocopherol were achieved. Besides, no significant differences were found between the higher (120 and 150 ˚C) nor the lower temperatures (60 and 90 ˚C) in the present study. Among the lower temperatures, the extraction of SBO at 60 ˚C resulted in an oil with a slightly greater concentration of α-tocopherol.

Table 4. α-tocopherol concentration in SBO (g of α-tocopherol oil / 100 g SBO) extracted with different solvents and temperatures.

	Solvent			
Temperature (ºC)	Hexane	Ethanol	Diethyl ether	2-MTHF
60	$0.934^{a} \pm 0.058$	$0.945^{a} \pm 0.051$	$0.613^{b} \pm 0.020$	$0.684^{ab} \pm 0.660$
90	$0.920^{a} \pm 0.045$	$0.897^{b} \pm 0.007$	$0.562^{b} \pm 0.034$	$0.715^{a} \pm 0.041$
120	$0.842^{b} \pm 0.051$	$0.932^{a} \pm 0.022$	$0.787^{a} \pm 0.057$	$0.658^{ab} \pm 0.070$
150	$0.843^{b} \pm 0.051$	$0.931^{ab} \pm 0.007$	$0.820^{a} \pm 0.027$	$0.620^{b} \pm 0.041$

Values expressed as mean ± SD

Different letters show significant differences at $p<.05$. Comparisons were made in each solvent column. Cross-comparison between solvents were not included in the present table (for main differences between solvents and temperatures refer to the results obtained from the two-way ANOVA, Figure 3, 5 and 6)

Temperature significantly affected the concentration of α-tocopherol in SBO extracted with ethanol as well, F (3, 20) = 5.708, $p<.05$. At temperatures of 60 ˚C and 120 ˚C, the recovery of α-tocopherol from SBO was statistically significantly greater than extracting the oil at 90 ˚C (Table 4). High temperatures have not been used until the moment to determine the recovery of α-tocopherol from vegetable oils using ethanol. Some

authors used lower temperatures (50 and 60 ˚C) to extract tocopherols from sunflower collets (Baümler, Carrín, & Carelli, 2017), yet no clear difference was observed between both temperatures on the recovery of tocopherols. The approximate same pattern and same values were drawn from both extractions, also after increasing the extraction time. The previous year, Baümler et al. (2016) already investigated the differences in the recovery of tocopherols from sunflower oil after extraction with hexane and ethanol at two different temperatures (i.e. 50 and 60 ºC). They showed a higher recovery of tocopherols when extracting the oil at 60 ˚C when compared to the extraction at 50 ˚C. A slight increase in the extraction temperature derived into a higher recovery of tocopherols from the oil using ethanol. Different results have been observed in the present study. Higher temperatures decreased the concentration of α-tocopherol when compared to the lowest temperature used (Table 4). However, higher temperatures involved a temperature rise of 30 ºC, the lower jump being from 60 to 90 ºC which are values far from what Baümler et al. (2016) studied and therefore could explain part of the differences obtained herein. In general terms, extracting the oil at 60 ˚C lead to a higher recovery of α-tocopherol than extracting at higher temperatures, although some temperatures may lead to non-significant differences (Table 4). The extraction at 90 ˚C resulted in the lowest amount of α-tocopherol. Interestingly, no significant differences were observed on the concentration of α-tocopherol between

the temperatures of 150 ˚C and 60 ˚C or 120 ˚C, nor between extractions at 150 ˚C and 90 ˚C.

Diethyl ether was the solvent that extracted more oil (Figure 2). It did not translate in a greater recovery of the vitamers herein analyzed, deriving in significantly higher amounts in the oil extracted with hexane (Figure 5), its less polar, petroleum-based analog. Differences in the concentration of α-tocopherol were then to be explored across temperatures, to understand if the temperature was a limiting factor on the extraction of this compound when using diethyl ether as extraction solvent. There was a significant effect of the temperature on the concentration of α-tocopherol in SBO extracted with diethyl ether, $F(3, 20) = 69.689$, $p<.05$. Significant differences were observed between the concentration of α-tocopherol in SBO extracted at 60 and 90 ˚C when compared to the oil extracted at 120 and 150 ˚C. Thus, the greater the extraction temperature, the greater the efficiency in recovering α-tocopherol. As temperature rises, the solvent viscosity and density go down and solvent diffusivity increases, subsequently yielding greater mass transfer and therefore increased recovery of α-tocopherol (Dey & Rathod, 2013). To the best of my knowledge, there are no publications to the date studying the effect of temperature on the extraction of tocopherols using diethyl ether. The novel investigation on different extraction temperatures allows to understand differences between petroleum-based solvents (i.e. hexane and diethyl

ether) and to understand the effect that the temperature may have upon the extraction of valuable compounds. It should be noted that the recovery of α-tocopherol was higher when using extreme temperatures, meaning that it was higher when using 60 ˚C (low extreme) rather than 90 ˚C, or using 150 ˚C (high extreme) rather than 120 ˚C.

The behavior of 2-MTHF was expected to be similar to that of ethanol, according to polarity. However, the behavior of 2-MTHF was closer to that of diethyl ether. When investigating the effect of temperature on the recovery of α-tocopherol, a significant effect of the temperature on the concentration of α-tocopherol in SBO extracted with 2-MTHF was spotted, F (3, 20) = 3.107, p<.05. Tukey's *post hoc* test highlighted statistically significant differences in the α-tocopherol concentration in SBO extracted at 90 ˚C when compared to the extraction at 150 ˚C, being higher in the former (Table 4). The temperature did not negatively affect oil yield – it increased (Figure 1) – but it negatively affected oil composition, yielding lower amounts of α-tocopherol at higher temperatures when compared to lower temperatures. The results were in line with what was observed in the extractions of SBO using hexane, also decreasing the nutritional quality of the oil as the extraction temperature rose. This could derive from the similar technical properties between hexane and 2-MTHF (Sicaire et al., 2015). At 60 ˚C, the recovery of α-tocopherol was lower than at 90 ˚C but slightly

higher than in the oil obtained at 120 ˚C, clearly showing the temperature-dependency of its recovery when using 2-MTHF.

Individual effect of each solvent on the recovery of β-carotene

Carotenoid extraction gains more interest when apolar solvents are used, since carotenoids are highly non-polar compounds. Nonetheless, as it has been shown in Figure 5, SBO extracted with hexane yielded lower concentrations of β-carotene than ethanol. When looking at the hexane extractions, it was clear that temperature the concentration of β-carotene in the extracted SBO was statistically significantly affected by changes in the extracting temperature, F (3, 20) = 23.055, p<.05. Using temperatures of 60 ˚C and 90 ˚C the recovery of β-carotene from SBO was significantly greater than using higher temperatures (Table 5). No significant differences were found between the higher (120 and 150 ºC) nor the lower temperatures (60 and 90 ºC).

There was also a significant effect of the temperature on the concentration of β-carotene in SBO extracted with ethanol, F (3, 20) = 15.999, p<.05. The oil extracted at 120 ˚C achieved the greatest recovery values of β-carotene from SBO when compared to the oil extracted at any other temperature (Table 5). The difference was only significant when comparing the values obtained extracting the oil at 120 ˚C against the values form the oil extracted at 60 ˚C or 150 ˚C. The progressive observable increase in the concentration

of β-carotene from 60 to 120 ºC dropped dramatically when extracting SBO at 150 ºC. The results showed a clear improved concentration of β-carotene when increasing the temperature except for the extraction at 150 ˚C, at which the concentration of β-carotene dropped out significantly. This drop may be derived from the possible degradation of the analyte at higher temperatures. The results obtained from the recovery of β-carotene across different temperatures (when analyzing it as an isolated factor) already suggested a possible degradation of the analyte at the highest temperatures (Figure 6).

There was a significant effect of the temperature on the concentration of β-carotene in SBO extracted with diethyl ether, F (3, 20) = 216.274, p<.05. Tukey's *post hoc* tests showed significant differences between the concentration of β-carotene in SBO extracted at 60 and 90 ˚C when compared to the oil extracted at 120 and 150 ˚C. The greater the extraction temperature, the greater the efficiency in recovering β-carotene, except when comparing the extraction at 120 ºC against that at 150 ºC, being higher in the former (Table 5). The results of diethyl ether were clearly opposed to the overall results since at the highest temperatures (120 and 150 ºC) the extraction of β-carotene was proportionally more efficient when compared to the extraction at the same temperatures using other solvents. However, as already mentioned and as observed in the use of other solvents, the extraction at 150 ˚C led to a drop in β-carotene concentration when

compared to the previous temperature (120 ˚C). The big rise in the concentration of β-carotene was relevant. The use of 120 ˚C almost triple-folded the results obtained at 60 or 90 ˚C (Table 5).

Table 5. β-carotene concentration in SBO (in g of β-carotene / 100 g SBO) extracted with different solvents and temperatures.

	Solvent			
Temperature (ºC)	Hexane	Ethanol	Diethyl ether	2-MTHF
60	$1.363^{a} \pm 0.060$	$1.653^{b} \pm 0.054$	$0.267^{c} \pm 0.013$	$1.017^{a} \pm 0.016$
90	$1.339^{a} \pm 0.036$	$1.698^{ab} \pm 0.050$	$0.244^{c} \pm 0.037$	$1.014^{a} \pm 0.061$
120	$1.131^{b} \pm 0.128$	$1.768^{a} \pm 0.075$	$0.686^{a} \pm 0.025$	$0.796^{b} \pm 0.062$
150	$1.077^{b} \pm 0.022$	$1.543^{c} \pm 0.049$	$0.641^{b} \pm 0.026$	$0.706^{b} \pm 0.071$

Values expressed as mean ± SD

Different letters show significant differences at $p<.05$. Comparisons were made in each solvent column. Cross-comparison between solvents were not included in the present table (for main differences between solvents and temperatures refer to the results obtained from the two-way ANOVA, Figure 3, 5 and 6).

Finally, there was a significant effect of the temperature on the concentration of recovering β-carotene in SBO extracted with 2-MTHF, $F(3, 20) = 46.086$, $p<.05$. Tukey's *post hoc* test highlighted statistically significant differences in recovering β-carotene from SBO extracted at 60 and 90 ˚C when compared to the extraction at 120 and 150 ˚C, being higher in the former temperatures (Table 5). The greater the temperature used in the extraction, the lower the concentration of the analyte in the extracted SBO. Although the differences were non-significant, the extraction at 120

˚C achieved greater concentrations of β-carotene in SBO when compared to the extraction at 150 ˚C. These results were in accordance with all other solvents, suggesting that the drop in the recovery of β-carotene at 150 ºC was indeed more subject to temperature change rather than to solvent choice. It was interesting to note when using 2-MTHF that the oil yield was negatively associated with the β-carotene concentration. In other words, at higher temperatures the extraction yield improved (Figure 1) in detriment to the recovery of β-carotene (Table 5).

5.1.3.4. Principal Component Analysis of fatty acids in SBO

A principal component analysis (PCA) was conducted on the six (6) fatty acids with no rotation applied. The Kaiser-Meyer-Olkin (KMO) measure verified the sampling adequacy for the analysis. Bartlett's test of sphericity indicated that correlations between items were sufficiently large for PCA. An initial analysis was run to obtain eigenvalues for each of the components in the data. Two of the components showed eigenvalues over Kaiser's criterion of 1 and in combination explained 85.91% of the variance (Table 6). The scree plot confirmed the first two components to be the most relevant for explaining the variance of the statistical model. Therefore, the first two components were retained for the final analysis. No rotation was applied since the first two principal components showed a great fit with the raw data. The items that clustered on the first principal component were

palmitic, palmitoleic, oleic and stearic fatty acids. The items that clustered on the second were linolenic and α-linolenic fatty acids. This was interesting, since the dimensions seemed to account for the saturated and mono-unsaturated fatty acids for the first principal component and the poly-unsaturated fatty acids for the second principal component (Table 7).

Table 6. Principal components and their respective eigenvalue and variance explained by the model. Eigenvalues greater than 1 were used to draw the PCA, according to Kaiser's criterion.

Dimension	**Eigenvalue**	**Variance explained (%)**	**Variance explained (%, cumulative)**
1	3.78	63.05	63.05
2	1.37	22.87	85.92
3	0.58	9.66	95.58
4	0.15	2.56	98.14
5	0.09	1.50	99.64
6	0.02	0.36	100.00

Table 7. Contribution of every variable analyzed (fatty acid) to Dimension 1 and Dimension 2 of the PC model.

Variable analyzed	Contribution to Dimension 1 (%)	Contribution to Dimension 2 (%)
Palmitoleic	25.107	0.522
Palmitic	23.355	0.004
Linoleic	0.683	50.448
Oleic	25.301	0.588
Linolenic	1.841	46.203
Stearic	23.714	2.235

The clustering on PCA was performed on the two main extracted principal components (PC1 and PC2) and the two independent factors (the solvent used and the temperature). The PCA main graph shows results from individuals in a scattered plot with PC1 in the X-axis and PC2 in the Y-axis. Individuals are plotted in the same cluster depending on the solvent used during the analysis, to investigate differences between solvents. Figure 7 shows a clear difference using ASE or using Soxhlet as a technique to extract fatty acids from SBO if we compare the groups “Hexane” – extracted using hexane and ASE – against the “Control” group – extracted with the same solvent using the Soxhlet technique. According to the results from PCA, the extraction using Soxhlet achieved a greater extraction of the components building up PC1, those being oleic, stearic, palmitic and

palmitoleic fatty acids, whereas the ASE extraction did not achieve great concentrations of those fatty acids. However, ASE extraction did achieve larger concentrations of the polyunsaturated fatty acids (PC2).

Looking at the main PCA, it was clear that the oil extracted with different solvents showed different fatty acid profiles. Specifically, there seemed to be a clear difference in the oils obtained by using diethyl ether, ethanol and hexane. However, the oil from the extractions using 2-MTHF did not separate in a new, isolated cluster. Instead, the cluster containing all the individual extractions with 2-MTHF was mixed up with all other clusters from the individual extractions using all other solvents (Figure 7). The solvent 2-MTHF seemed to behave both as the most polar (ethanol) and the most non-polar solvents (hexane, diethyl ether). Interestingly, the overall average extraction with 2-MTHF resulted in an oil with a greater concentration of poly-unsaturated fatty acids (linoleic and α-linolenic fatty acids) when compared to the ethanol and hexane extractions. Although in a non-significant manner, a higher extraction of polyunsaturated fatty acids was reported by Sicaire et al. (2015) on rapeseed oil when using 2-MTHF for the extraction and comparing the results to other solvents'. The extraction with 2-MTHF achieved, on average, higher concentrations of saturated and mono-unsaturated fatty acids when compared to the extractions using hexane. The oil extracted with 2-MTHF contained lower amounts of poly-unsaturated fatty acids when compared to the extraction

using diethyl ether, and similar concentrations of saturated and mono-unsaturated fatty acids.

The most polar solvent, ethanol, appeared in the main PCA (Figure 7) as a more clearly isolated cluster when compared to all other ASE extractions. The most relevant difference relied on the extraction of fatty acids from PC2, which was on average higher when the oil was extracted using hexane or diethyl ether. Results however showed a possible higher concentration of poly-unsaturated fatty acids in SBO extracted with ethanol when compared to the extraction with Soxhlet, as other authors have previously reported (Castejón et al., 2018). Nevertheless, this higher concentration of poly-unsaturated fatty acids was most likely strongly dependent on the temperature of the extraction. Contrarily to what observed by Pieber, Schober, & Mittelbach (2012), pressurized liquid extraction using ethanol lead to higher amounts of saturated and mono-unsaturated fatty acids and lower amounts of poly-unsaturated fatty acids. As the poly-unsaturated fatty acids are more polar compared to saturated fatty acids of the same length, the expectations were to achieve higher amounts when using ethanol as a solvent. However, lower extractions were reported in the present experiment. This could be due to the bonding of these specific fatty acids. If the poly-unsaturated fatty acids were generally linked to a triglyceride molecule, the positioning of these fatty acids would most likely be the β position of the triglyceride molecule (Amate, Ramírez & Gil, 1999). The

apolarity of the triglyceride may difficult their extraction using polar solvents, therefore reducing the final concentration of them in the extracted SBO when using ethanol. This could explain the lower percentage of poly-unsaturated fatty acids when compared to the non-polar solvents used, which could extract greater number of triglycerides due to its apolarity (Figure 7).

Diethyl ether was the solvent that, in all the performed extractions, achieved a higher amount of poly-unsaturated fatty acids in the resulting oil. Compared to hexane, the extraction with diethyl ether was expected to achieve higher amounts of poly-unsaturated fatty acids due to the slightly more polar nature of the solvent (Freed et al., 1990).

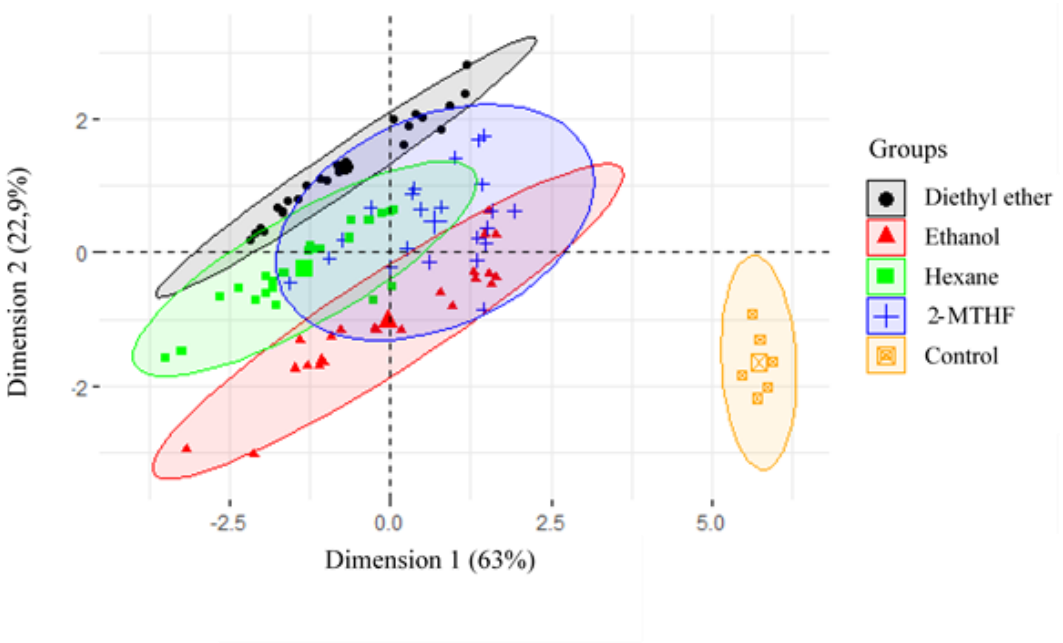


Figure 7. Mapping of the samples in two different dimensions. Samples were grouped according to the solvent used during the ASE extraction, and the control (Soxhlet).

The temperature did not have a significant effect on the fatty acids on the resulting oil (Figure 8). Therefore, the most important factor to consider in the extraction of fatty acids from SBO is the solvent rather than the temperature when using pressurized liquid extractions. There was a clearly separated cluster from the extraction using Soxhlet. As it is depicted in Figure 8, control samples showed clearly higher amounts of saturated and poly-unsaturated fatty acids presents in SBO when compared to ASE (scored greater values on Dimension 1).

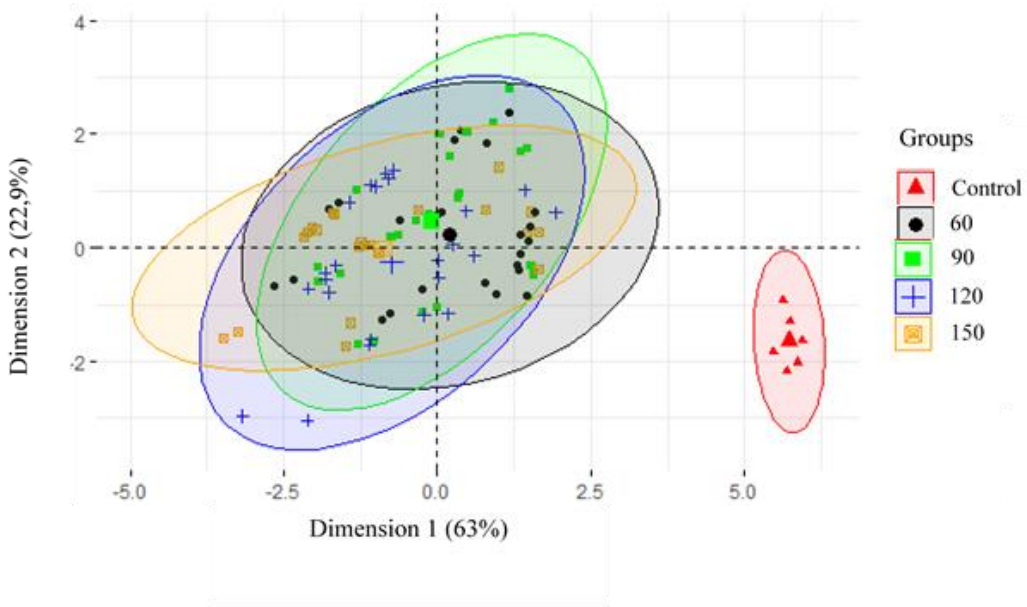


Figure 8. Mapping of the samples in two different dimensions. Samples were grouped according to the temperature used during the ASE extraction, and the Control (Soxhlet).

5.1.3.5. Concentration of monounsaturated (MUFA), polyunsaturated (PUFA) and saturated (SFA) fatty acids in SBO

Even though the PCA analysis can give an overview of the effect of the solvent or temperature on the fatty acid profile, the PCA groups fatty acids in two dimensions regardless of their nature. The grouping in saturated, monounsaturated and polyunsaturated fatty acids allows understanding the nutritional profile of SB oil in more detail. This is important because one of the most important fatty acid constituting the nutritional profile of SB oil is the palmitoleic acid (monounsaturated). Saturated and polyunsaturated fatty acids were also included. A two-way ANOVA was run for each group of fatty acids, also considering the interaction effect.

One of the most important parts of this research was to separately study which temperatures and solvents might be relevant for the extraction of each fatty acid group from SBO. The two-way ANOVA helped investigate the interaction effect between solvent and temperature and gave the first insight into the effect of the solvent and temperature separately. A one-way ANOVA was run as further analysis with the results obtained from each solvent extraction, to deeply understand the variability in the concentration of the aforementioned fatty acid groups in SBO extracted at different temperatures with the same solvent.

The interaction effect on the concentration of SFA, MUFA and PUFA in SBO

SFA in extracted SBO with any of the four solvents was affected differently by temperature, F (9, 81) = 2.781, p<.05 (Figure 9). The same significant result was observed for MUFA, F (9, 81) = 2.922 (Figure 10), and PUFA concentration in SBO, F (9, 81) = 5.084 (Figure 11), both considered significant at p<.05. Thus, the recovery of SFA, MUFA and PUFA did not follow the same pattern across temperatures when using different solvents for the extraction. Considering two of the solvents herein used are somewhat more polar than the other two, there were reasons to think that they could behave differently. However, results suggested that each solvent behave differently across temperatures, making clear there were more

interactions besides the polarity of the solvent. The use of ethanol and hexane on the extraction of SBO seemed to behave similarly – they had a similar tendency at different temperatures – when comparing the results of all the fatty acid groups analyzed (Figure 9, 10, 11).

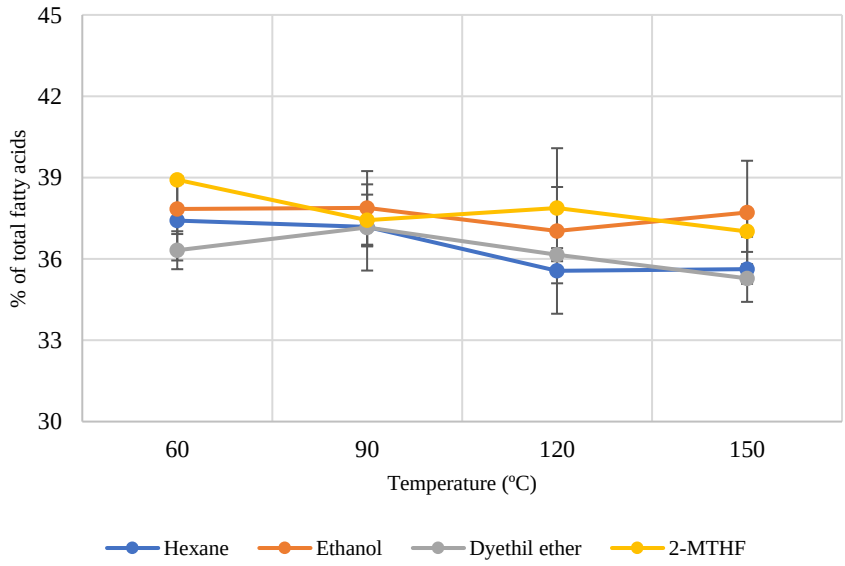


Figure 9. Saturated fatty acid (SFA) content in SB0 (percentage of the total fatty acids) as a function of temperature and separated by solvent.

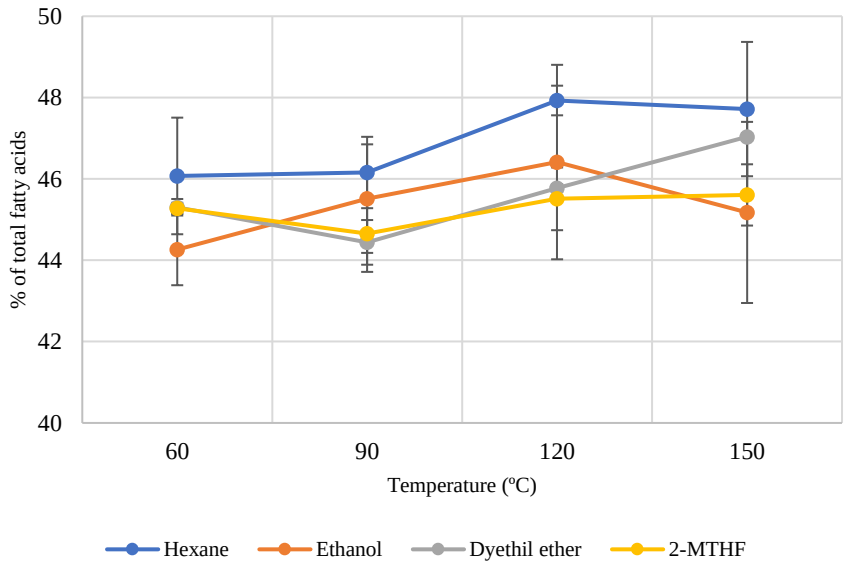


Figure 10. Monounsaturated fatty acid (MUFA) content in SB0 (percentage of the total fatty acids) as a function of temperature and separated by solvent.

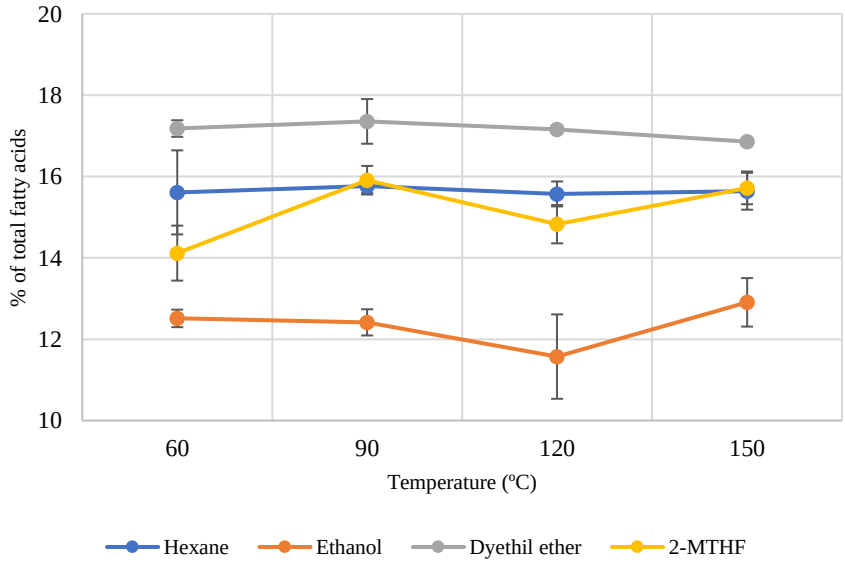


Figure 11. Polyunsaturated fatty acid (PUFA) content in SB0 (percentage of the total fatty acids) as a function of temperature and separated by solvent.

The effect of solvent and temperature on the recovery of SFA, MUFA and PUFA

The results showed significant differences in the recovery of the SFA from the extracted SBO when different solvents were used, F (4, 81) = 92.050, p<.05. Considering the extraction with ASE – and excluding Soxhlet extraction (Control) –, using less polar solvents yielded less SFA than using more polar solvents (Figure 12). Due to the saturation degree of the SFA, apolar solvents should have been the solvents extracting more quantity (Tir, Dutta, & Badjah-Hadj-Ahmed, 2012). This result could be partially explained by the fact that the extraction time was not enough when using apolar solvents, which could not penetrate through the vegetable cell wall and solubilize the fatty acids inside (Tir et al., 2012). Obtaining almost 10% more SFA after Soxhlet extraction when compared to ASE extraction using hexane may serve as a proof that time could be an important factor when considering the extraction of SFA from SB dried berries. At the extraction conditions used in the present research, it could be concluded that ethanol and 2-MTHF are suitable for the extraction of SFA from SBO, yielding significantly greater concentrations than hexane or diethyl ether, two solvents widely used for that purpose (37.62 and 37.81% compared to 36.44 and 36.23% respectively, Figure 12). The exhaust of the sample caused by flushing hexane through it over several extraction samples (control

samples, using Soxhlet) increased the quantity of SFA in the resulting oil (Figure 12).

The extraction of SBO using different solvents also yielded significantly different concentrations of MUFA, F (4, 81) = 7.561, at p<.05. The greatest concentration of MUFA in SBO was observed in the samples extracted with hexane – both extracted with hexane and Soxhlet –, followed by all other extractions (Figure 12). Soxhlet extraction had been also previously reported to extract greater amounts of MUFA than other techniques, including supercritical fluid extraction and ASE, probably due to the higher extraction time (Castejón et al., 2018; Rebey et al., 2019; Reddy, Moodley, & Jonnalagadda, 2012). Among the solvents used in the ASE extractions, hexane yielded significantly higher amounts of MUFA when compared to the rest of the solvents. This was in line with what other authors observed (Bourgou et al., 2019). In the present study, MUFA included palmitoleic and oleic fatty acids, both being long-chain fatty acids. According to Mezzomo, Mileo, Friedrich, Martínez, & Ferreira, (2010), the reason for greater extraction may rely on the polarity index of the solvent, which is lower in apolar solvents when extracting long-chain fatty acids. The fact that diethyl ether did not achieve similar extraction results for MUFA may be due to its higher polarity index (4.4, compared to hexane (0) and ethanol (5.2) (Mezzomo et al., 2010)).

There were also significant differences when comparing the concentration of PUFA in the extracted SBO using different solvents, F (4, 81) = 613.031, at p<.05. All solvents achieved different concentrations of PUFA in the resulting SBO (Figure 12). Control samples (Soxhlet) extracted significantly lower amounts of PUFA when compared to any ASE extraction. Other authors also reported lower amounts of PUFA in oil from caraway (*Carum carvi*) seeds extracted by using Soxhlet technique (and hexane as solvent) when compared to other techniques and greener solvents (Bourgou et al., 2019). The longer extraction time when using Soxhlet may be a great barrier for the efficient recovery of PUFA in SBO. The long extraction time implies prolonged contact with air and prolonged temperature exposure of the sample, two critical factors when extracting polyunsaturated molecules. The results from the ASE extraction indicated that the less polar solvents achieved greater amounts of PUFA in the extracted SBO when compared to greener solvents (i.e. ethanol, 2-MTHF). The greater extraction of more polar lipids with apolar solvents may be due to the form in which these are found in the original matrix, which may be bond to a triglyceride molecule and thus more difficult for them to be extracted using polar solvents (as explained before). The extract using diethyl ether yielded more quantity of PUFA when compared to its non-polar counterpart hexane, probably due to its relatively higher polarity (Mezzomo et al., 2010; Tir et al., 2012). Nevertheless, other authors have

found hexane to yield higher amounts of PUFA in extracted oils when compared to other non-polar solvents (Wu et al., 2011), although the extraction was performed using a Soxhlet apparatus, differing from what had been used in the present experiment. Extracting SBO with 2-MTHF yielded significantly higher PUFA when compared to ethanol. Ethanol is stated to be slightly more polar than 2-MTHF (Table 1), which in turn could influence the efficiency in recovery of fatty acids, especially PUFA, as shown in the results (Figure 12).

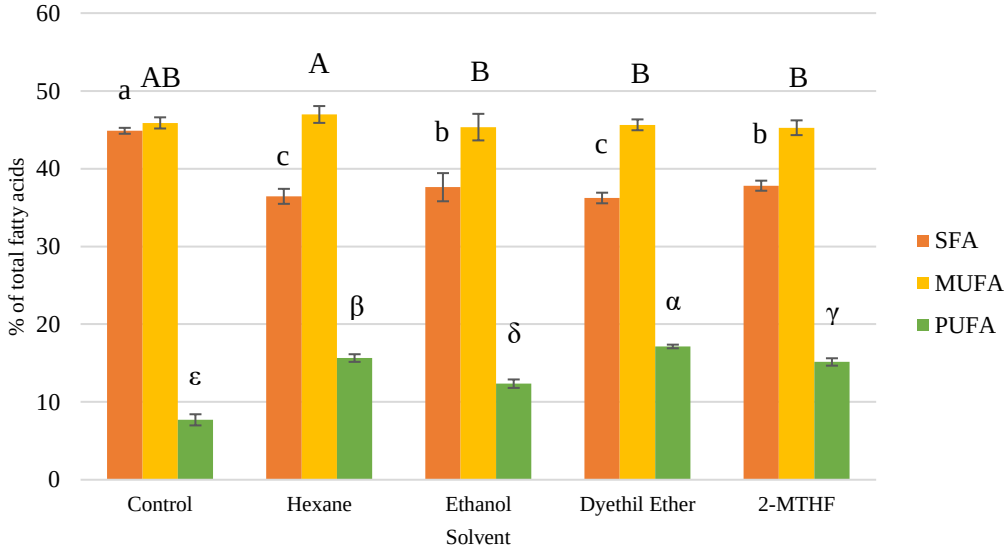


Figure 12. Average concentration of SFA, MUFA and PUFA in SBO extracted using different solvents. Error bars show SD. Different letters mean significant differences at $p<.05$. Comparisons are not made across upper-, lower-case and Greek letters (i.e. across groups of fatty acids).

The effect of temperature on the concentration of SFA, MUFA and PUFA

The results showed significant differences between the tested temperatures on the recovery of SFA, MUFA and PUFA from SBO. Differences in the recovery of SFA were observed with F (3, 81) = 8.703, at $p<.05$. SFA concentration in control samples was greater when compared to all other samples (Figure 13). In fact, greater extraction cycles (from Soxhlet) seemed to benefit the extraction of SFA from SBO, despite being in prolonged contact with air. When it comes to ASE extractions, temperatures of 120 and 150 °C achieved statistically significantly lower concentrations of SFA when compared to temperatures of 60 and 90 °C (36.66 and 36.41% compared to 37.62 and 37.42% respectively, Figure 13). In the case of polar solvents, such as ethanol and 2-MTHF, the polarity of the solvent is reduced at higher temperatures (Lu, Boughner, Liotta, & Eckert, 2002), which could then trigger a greater extraction of SFA at higher temperatures. Results herein show otherwise, indicating a drop in the concentration of SFA at higher temperatures (Figure 13). There may be the need of rising more the temperature in order to see improvements in the extraction of SFA from SBO. Even though it may have an influence, it is also noteworthy that the results show the overall extraction of SFA using all the solvents, not only focusing on the extraction using the most polar solvents.

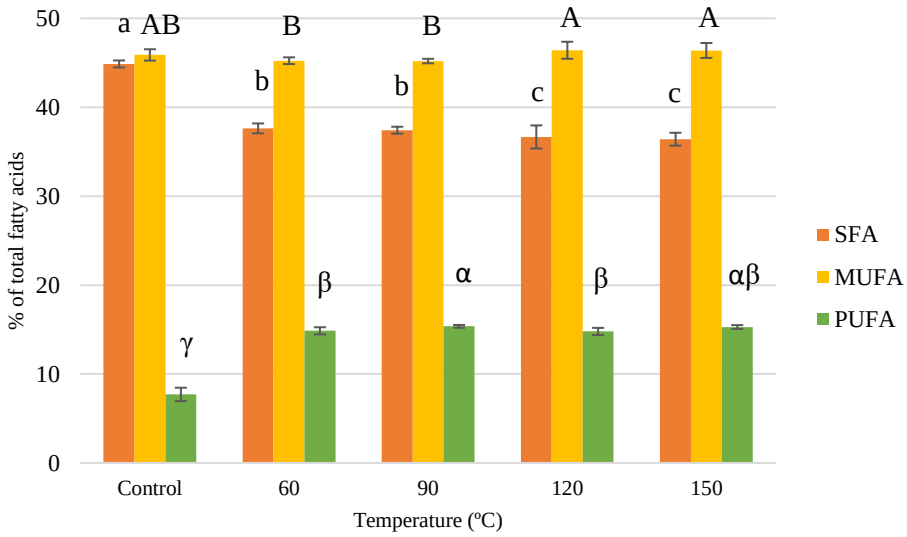


Figure 13. Average concentration of SFA, MUFA and PUFA in SBO extracted using different temperatures. Error bars show SD. Different letters mean significant differences at $p<.05$. Comparisons are not made across upper-, lower-case and Greek letters (i.e. across groups)

The concentration of MUFA in extracted SBO was significantly different across the studied temperatures at F (3, 81) = 10.547, at $p<.05$. No differences in the concentration of MUFA were observed between control samples and any temperature (Figure 13). The greater quantity of cycles performed in Soxhlet extraction did not seem to help extracting more quantity of MUFA, probably due to the unsaturation degree of the molecules. In contrast with what resulted from SFA recovery, extractions at higher temperatures (120 and 150 ºC) yielded a greater concentration of MUFA when compared to extractions at lower temperatures (60 and 90 ºC). The fact that higher temperatures may decrease the polarity of polar solvents may be the reason that could explain why at higher temperatures the extraction of MUFA was higher when compared to lower temperatures.

However, all solvents may influence the results in the temperature, making it closer to a conjecture than to a conclusion.

PUFA concentration in extracted SBO was also statistically significant at different temperatures, F 83, 81) = 5.517, at $p<.05$. The concentration of PUFA in the SBO from control samples was significantly lower than any other temperature (Figure 13). PUFAs are the most oxidizable fatty acid molecules herein explored and are especially vulnerable at high temperatures, which makes them prone to degradation. The use of longer processing times in more cycles when using the Soxhlet extraction technique and the high extraction temperatures employed in this technique could explain the lower concentration obtained in the resulting oil. In fact, other authors already observed lower recoveries of PUFA from Soxhlet when compared to ASE extractions (Castejón et al., 2018). From all other temperatures, the recovery of PUFA seemed steady, except for 90 ºC, in which the recovery of PUFA was significantly greater compared to 60 or 120 ºC. Interestingly, the concentration of PUFA in SBO extracted at 150 ºC was non-significantly different from that obtained at 90 ºC (Figure 13). This seemed to indicate that there could exist an ideal temperature value for the extraction of PUFA from SBO.

Individual effect of each solvent on the recovery of SFA, MUFA and PUFA at different temperatures

It has been already made clear that the different solvents studied in the present experiment were differently influenced by temperature when it came to the extraction of fatty acids from SBO. Therefore, the interest remained in exploring the behavior of a specific solvent across the studied temperatures in terms of the extraction of each fatty acid group using the same solvent.

SBO extracted with hexane gave significantly different percentages of fatty acids depending on the extraction temperature. Statistically different results were observed in the SFA distribution, F (3, 19) = 12.400, at $p<.05$ and in the MUFA distribution, F (3, 19) = 7.556, at $p<.05$. The percentage of PUFA in the resulting oil extracted with hexane was not affected by using different temperatures (Table 8). The recovery of MUFA and SFA followed an opposite pattern across all extractions. Using lower temperatures (60 and 90 ºC) during extraction resulted in an oil with a significantly greater percentage of SFA yet a lower percentage of MUFA when compared to the oil extracted at higher temperatures (120 and 150 ºC). Thus, the greater the temperature, the greater the concentration of MUFA in the resulting SBO and the lower the concentration of SFA. In contrast, Moreau et al. (2003) showed that lower temperatures of extraction (40 ºC) achieved greater

percentages of free linoleic acid (included in the MUFA group herein) from corn samples when compared to higher temperatures (100 ºC). The lower temperatures in the present experiment are slightly higher than those used by Moreau et al. (2003) (60 ºC compared to 40 ºC), yet it could be seen that the results indicate a similar rising behaviour at temperatures of 120 and 150 ºC.

ASE ethanolic extraction showed differences in the percentage of PUFA across the studied temperatures, F (3, 19) = 4.467, at p<.05. No significant differences were observed in the distribution of MUFA and SFA across temperatures in the ethanol-extracted SBO. SBO extracted with ethanol at 150 ºC yielded significantly more PUFA when compared to the oil extracted at 120 ºC (Table 8). The extraction at 150 ºC resulted in an extracted SBO with greater percentage of PUFA when compared to all other temperatures, which may derive from the decrease in polarity at higher temperatures, as previously explained (Lu, Boughner, Liotta, & Eckert, 2002). However, no differences were observed between other temperatures, meaning that the extraction at 120 ºC yielded the oil with the lowest percentage of PUFA and the extraction at 150 ºC yielded the higher percentage of the same group of fatty acids. The fact that the lowest extraction value in the recovery of PUFA was spotted at 120 ºC could simply derive from the greater standard deviation obtained from the same values at 120 ºC. As none of the values obtained at 120 ºC was an outlier,

none of them were ruled out of the statistical analysis. This led to understand that extractions using ethanol may lead to greater recovery of PUFA at lower and higher temperatures than 120 ºC, although more research should be made on the ideal extraction temperature of PUFA from SBO using ethanol as extraction solvent to confirm that.

Diethyl ether was the only solvent yielding significantly different percentages in all the groups of analyzed fatty acids. Differences were observed in SFA, F (3, 19) = 15.720, in MUFA, F (3, 19) = 22.520 and in PUFA, F (3, 19) = 9.350, all of them at $p<.05$. SFA constituted a greater part of the total fatty acids when the extraction was performed at 90 ºC when compared to all other temperatures (Table 8). The obtained data pointed to a maximum extraction temperature (90 ºC), which decreased as extraction temperature rose. There were no statistically significant differences between the percentage of SFA in SBO extracted at 60 and 120 ºC. However, extraction at 150 ºC yielded an oil with significantly lower amounts of SFA when compared to all other temperatures. Likewise, the percentages of MUFA in SBO extracted with diethyl ether also led to one temperature achieving the highest values (150 ºC) when compared to all other temperatures. However, the percentages of MUFA in extracted SBO followed a completely opposite pattern when compared to the extraction profile of SFA. An increasing pattern was observed on MUFA concentration over temperature whereas a decreasing pattern was observed

on SFA concentration in extracted SBO. This was observed to be in line with what was reported with hexane. In line with SFA concentration, the highest temperature was the worst when analysing the percentage of PUFA in the extracted oil, yielding an oil with the lowest percentage of that fatty acid group. No differences were observed when comparing other temperatures of extraction. PUFA are more prone to be oxidized due to their high degree of unsaturation and high temperatures may help oxidize the molecule and therefore to recover lower amounts, at least when using diethyl ether as extraction solvent, as it is shown in the present experiment.

Extractions using 2-MTHF yielded significantly different percentages of SFA, F (3, 19) = 9.514, and PUFA, F (3, 19) = 17.110, both at $p<.05$. No significant differences were observed between the percentages of MUFA in the oil extracted with 2-MTHF when comparing different temperatures. The greater percentage of SFA in the oil extracted with 2-MTHF was obtained at 60 ºC, meaning that higher temperatures could lead to lower extraction of SFA and therefore, lower presence in the oil. In fact, the extraction at the highest temperature (150 ºC) resulted in an oil with a lower percentage of SFA. Although the difference was sometimes non-significant, the behaviour of all the solvents showed a slight decrease in the percentage of SFA at the highest temperature. In addition, like what was observed with ethanol, the highest temperature yielded significantly higher percentages of PUFA when compared to the extraction at 120 ºC, but similar amounts

when compared to the extraction at 90 ºC. Therefore, results could indicate a possible drop in the recovery of PUFA in SBO by polar solvents at some temperature around 120 ºC. However, this should be further investigated to add more evidence on the field. It was however clear that the highest extraction temperature did not achieve the poorest extraction of PUFA either using ethanol or 2-MTHF as the extraction solvents, suggesting the adequacy of the theoretical approach of the decrease in polarity of the most polar solvents at high temperatures (Lu, Boughner, Liotta, & Eckert, 2002).

Table 8. Average percentage value of each fatty acid group in SBO extracted by ASE using different solvents at different temperatures.

Fatty acid group	Temperature (ºC)	Solvent			
		Hexane	Ethanol	Diethyl ether	2-MTHF
SFA	60	$37.412^{a} \pm 1.471$	37.846 ± 0.929	$36.320^{\beta} \pm 0.699$	$38.919^{*} \pm 0.132$
	90	$37.180^{a} \pm 0.721$	37.882 ± 1.357	$37.160^{\alpha} \pm 1.590$	$37.429^{*\#} \pm 0.942$
	120	$35.561^{b} \pm 0.461$	37.030 ± 3.053	$36.156^{\beta} \pm 0.241$	$37.881^{\#} \pm 0.771$
	150	$35.617^{b} \pm 1.202$	37.712 ± 1.908	$35.283^{\gamma} \pm 0.201$	$37.011^{\#} \pm 0.753$
MUFA	60	$46.073^{b} \pm 1.434$	44.263 ± 0.876	$45.303^{\beta} \pm 0.688$	45.281 ± 0.585
	90	$46.157^{b} \pm 0.878$	45.516 ± 1.335	$44.440^{\gamma} \pm 1.424$	44.655 ± 1.384
	120	$47.928^{a} \pm 0.364$	46.414 ± 2.391	$45.776^{\beta} \pm 0.373$	45.511 ± 0.928
	150	$47.717^{a} \pm 1.651$	45.175 ± 2.227	$47.036^{\alpha} \pm 0.310$	45.607 ± 0.907
PUFA	60	15.611 ± 1.033	$12.512^{AB} \pm 0.216$	$17.181^{\alpha} \pm 0.203$	$14.117^{\#} \pm 0.676$
	90	15.767 ± 0.174	$12.414^{AB} \pm 0.322$	$17.356^{\alpha} \pm 0.549$	$15.910^{*} \pm 0.352$
	120	15.572 ± 0.307	$11.573^{B} \pm 1.038$	$17.158^{\alpha} \pm 0.094$	$14.829^{\#} \pm 0.471$
	150	15.639 ± 0.454	$12.907^{A} \pm 0.596$	$16.859^{\beta} \pm 0.091$	$15.722^{*} \pm 0.403$

Values expressed as mean ± SD. Results are statistically different at $p<.05$

Comparisons are not made across different fatty acids groups (e.g. MUFA vs SFA)

$^{a, b, c}$ depict statistically significant differences in hexane extractions; $^{A, B}$ depict statistically significant differences in ethanol extractions; $^{\alpha, \beta, \gamma}$ depict statistically significant differences in diethyl ether extractions; $^{*, \#, \#\#}$ depict statistically significant differences in 2-MTHF extractions.

5.1.4. Conclusions

The extraction using green solvents achieves nutritionally good values when compared to the petroleum-based solvents, especially when using ethanol. The values of β-carotene concentration are far better in SBO after ethanol extraction when compared to all other solvents. However, the fatty acid profile of SBO oil extracted with green solvents is worse than petroleum-based solvents, with the noteworthy greater extraction of SFA.

The high polarity of the green solvents herein studied (ethanol, 2-MTHF) makes them unsuitable for the extraction of SBO from the peel and pulp, since they also extract part of the sugars present in the fruit. The extraction of SB peel and pulp oil with polar solvents must undergo further processing to purify the lipid fraction, which reduces the oil yield and ultimately poses a paradox on the processing using greener solvents. The use of the green solvents herein studied in the extraction of SB seed oil could be a good choice to minimize the overall environmental impact considering the nutritional value of the extracted oil. They could also be useful as co-solvent of other extraction techniques as the extraction of certain compounds of interest may benefit from its use.

The extraction of PUFA achieves the greatest values at the highest temperatures (150 ºC) when using ethanol or 2-MTHF. The possible loss of polarity at high temperatures of these polar green solvents could partly

explain the results. This phenomenon may help understand the applicability of high temperatures on PUFA extraction using polar solvents.

The best rapid technique to apply when extracting SBO from SB dried berries is the ASE with hexane at 60 ºC. Although the extraction yield is lower, the ASE technique demands less amount of time when compared to traditional extraction techniques (Soxhlet). Hexane extraction does not require an extra processing extraction step since the extracted SBO is mainly constituted with apolar compounds. In addition, the outcoming oil is richer in bioactive compounds when compared to the oil extracted with diethyl ether, another apolar solvent used herein. Finally, extracting with hexane at 60 ºC leads to less energy and resources expenditure, and the resulting oil has more concentration of β-carotene and α-tocopherol, and probably greater concentration of other carotenoids and tocopherols, when compared to SBO extracted using higher temperatures.

5.2. Supercritical CO_2 extraction of SBO

5.2.1. Introduction

Green solvents have been widely investigated for their positive results on oil extraction, mostly on the nutritional quality of the extracted oil. As it was analyzed earlier in the present chapter, green solvents could interestingly be used, when possible, to extract oil from vegetable sources conserving a higher oil quality depending on the temperature used during extraction. Differently from not using solvents at all (cold pressing extraction), using solvents usually lead to higher yield, which is an important factor when choosing the oil extraction methodology.

Furthermore, certain emerging technologies have been recently used to optimize the overall yield and the extraction of certain bioactive compounds when using green solvents. These technologies include aqueous enzymatic extraction (Kumar et al., 2017), the use of pulsed electric fields or high voltage electrical discharges (Puértolas, Koubaa, & Barba, 2016), ultrasound or microwave-assisted extractions (Castejón et al., 2018; Wang & Weller, 2006; Zengin et al., 2020).

Besides, supercritical fluid extraction has been also used for some years in lipid extraction. The extraction technique is based on the use of a supercritical fluid (mainly carbon dioxide) to extract oil or the compounds

of interest from a raw matrix. Supercritical CO_2 extraction has different applications in many different industries, from sterilization processes in pharmaceutical industries to the production of everyday food products (e.g. decaffeinated coffee or tea) (Sahena et al., 2009). One of the most interesting applications is the extraction of vegetable essential oils with the aim to preserve their highly volatile compounds or their high nutritional profile (Wang & Weller, 2006).

The supercritical state of the fluid is achieved when the temperature and pressure are changed above its critical point. In general terms, the resulting supercritical fluid would have the diffusivity of gas and the solubility of the liquid (Wang & Weller, 2006). Compared to the widely used solvent extraction, supercritical fluid technology does not need an evaporation process after the extraction process. Only by changing the pressure of the system the state of the supercritical fluid could be easily changed to gas, therefore achieving a clear and efficient separation from the extracted solutes – since they would not be solubilized in gas. Another advantage of using supercritical fluids is the temperature of extraction. For CO_2, the supercritical state could be achieved at only 30.85 ºC at 73 bar of pressure (Wang & Weller, 2006). These working temperatures are far from those considered ideals for solvent extraction (see 5.1. Solvent extraction of SBO). Nevertheless, the process is usually optimized by using higher

pressures and temperatures (Correa et al., 2017; Gustinelli, Eliasson, Svelander, Alminger, & Ahrné, 2018).

Supercritical CO_2 extraction allows for a very efficient recovery of the solvent (CO_2) when compared to the recovery of other solvents (e.g. ethanol or hexane), making the process very efficient and almost self-sustainable. Besides, CO_2 comes at a low cost when compared to other solvents. Nutritionally speaking, supercritical CO_2 extraction could potentially reduce the oxidation of lipids during processing, achieve equal or higher extraction yields or maintain the antioxidant capacity of the raw matrix of which the oil is being extracted, when compared to the conventional extraction with solvents (Danh et al., 2013; Wang & Weller, 2006).

The major disadvantage of this technique is the initial investment cost of the system. The system must be built so it could operate at very high pressures and maintain it steady over the extraction process, which inevitably rises the cost of the material used as well as the control system and the energetic cost. Furthermore, when using supercritical CO_2 during the extraction, losses of solvent – although they are minimal – translates into the release of CO_2 into the atmosphere, and the detrimental contribution of CO_2 on the greenhouse effect has been well documented.

5.2.1.1. Demarcation

Supercritical CO_2 extraction has been widely used to extract oils or actives from vegetable parts. The temperature and pressure used are the most important independent factors to optimize the extraction process. A temperature of 42.35 ºC combined with 250 bar of extraction pressure was found to be the most efficient combination when extracting bioactive compounds from the cooking banana (Correa, Mesomo, Pianoski, Torres, & Corazza, 2016). Other temperatures (60 ºC) and pressures (200 bar) were found to be more adequate to achieve a better extraction yield from sapucaia nut oil (Teixeira, Ghazani, Corazza, Marangoni, & Ribani, 2018) or to get the best recovery of vitamin E from bilberry seed oil (Gustinelli et al., 2018). The temperature and pressure used during the extraction are the most important factors to achieve a greater oil quality. Yet as it has been shown, differences also depend on the raw matrix to be used in the extraction.

Supercritical CO_2 extraction technology has been recently applied to sea buckthorn berries and seeds. Extraction from berries yielded an acceptable amount of oil (Pavlović, Lendić, Miškulin, Moslavac, & Jokić, 2016) when compared to previously described and reviewed extraction yields (Dulf, 2012; Zielińska & Nowak, 2017). The extraction was performed using 300 bar of pressure at 40 ºC, probably working at high pressure to avoid raising the temperature in excess. The nutritional profile of the resulting oil

obtained by Pavlović et al. (2016) was slightly better than the oil extracted with the conventional method Soxhlet, achieving higher concentrations of α-linolenic and linoleic acid and lower concentrations of the saturated palmitic acid. No other experimental conditions using supercritical CO_2 were used. Recently, Zheng, Shi, Zhao, Jin, & Wang (2017) extracted oil from the pulp and from the seed separately, at 40 ºC and 300 bar, and compared the extract with other techniques (subcritical butane extraction). Results showed that extracting with supercritical technology achieved higher antioxidant values when compared to subcritical technology.

To the best of my knowledge, only one work aimed at investigating different pressures and temperatures of extraction using supercritical CO_2 technology. Kagliwal, Patil, Pol, Singhal, & Patravale (2011) extracted SBO from sea buckthorn seed powder at different pressures and temperatures and showed that extracting the oil at higher temperatures (65 ºC) resulted in an oil with lower nutritional profile when compared to the extraction at lower temperatures, derived from a lower recovery of carotenes. Increasing the extraction pressure (400 bar) yielded an oil with higher amounts of carotenes and tocopherols but lower antioxidant capacity when compared to lower pressures (100 bar).

The extraction of SBO using supercritical CO_2 is a clear greener methodology of extracting the oil, and the temperatures used could be

helpful to reduce the damage on the thermolabile compounds. Industrially speaking, the use of this technology would bring an added value to the final product and could make the difference in the marketing and selling processes compared to other competitors that may be using other conventional and cheaper extracting technologies. However, the effect of the pressure and temperature on the extracted SBO should be clear in order to understand the behavior of the process and optimize it.

After careful revision, the best combination of pressure and temperature on the extraction of SBO and its bioactive compounds is not yet clear. It is therefore important to study the effect of different temperatures and pressures on the supercritical CO_2 extraction of SBO.

5.2.1.2. Objective

The objective of the present work was to evaluate which temperature and pressure would be the most efficient in extracting SBO from SB dried berries in a supercritical CO_2 extraction set up, considering the yield and nutritional quality of the resulting oil.

SBO could be extracted from either the residual pulp and peel or from the seed. The sub-objective of the present work was to evaluate which conditions would be the most suitable to extract oil from different parts of the berry, considering the nutritional quality of the oil from each fraction.

5.2.2. Experimental design

5.2.2.1. Sample preparation

Samples were prepared as previously detailed in the first part of the chapter (5.1.2.1. Sample preparation).

5.2.2.2. Oil extraction

SBO was extracted from SB dried berries using a total of six experimental conditions in a supercritical CO_2 lab-scale extraction system from the CNTA (Navarra, Spain). Temperature and pressure were the two parameters changed within the different experimental conditions, as showed in Table 9. Approximately 50 g of dried SB berries were used in each experimental condition. Every extraction was run only once. CO_2 flux was maintained constant at [redacted] L/h and the extraction time was set at [redacted] min, according to reports from other extractions using the same technology. No modifier or cosolvent was used in any experimental condition.

Table 9. Conditions tested in the experimental set up of supercritical CO2 extraction of SBO from SB dried berries.

Experimental condition	Pressure (bar)	Temperature (ºC)
Run 1	[redacted]	[redacted]
Run 2	[redacted]	[redacted]
Run 3	[redacted]	[redacted]
Run 4	[redacted]	[redacted]
Run 5	[redacted]	[redacted]
Run 6	[redacted]	[redacted]

The system was similar to that represented previously by Wang & Weller (2006) and represented in Figure 14. The system consisted of a sealed extraction chamber, the CO_2 feeding pump, the heater and two separators. The first separator operated at [redacted] bar and [redacted] ºC and the second separator operated at [redacted] bar and [redacted] ºC in all experimental conditions. The residual solvent was evaporated in the condenser, where the last fraction of the product came out. The evaporated CO_2 was recompressed feed again into

the system. The obtained fractions in the two different separators were analyzed separately. Only one run was performed in each condition.

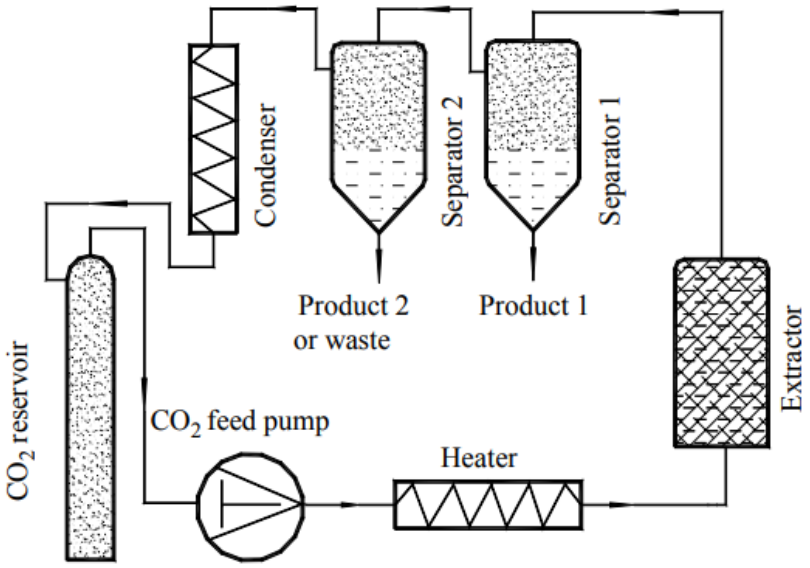


Figure 14. Schematic representation of the System used for the supercritical CO2 extraction of SBO from SB berries. Copied from (Wang & Weller, 2006)

5.2.2.3. Yield

The extraction yield was measured by weight difference before and after the extraction process of every run. Only one value of extraction yield was taken per each experimental condition. Differences were thus statistically non-measurable.

5.2.2.4. Sample analysis

Phytochemical analysis of the extracted SBO was performed. β-carotene and α-tocopherol were analyzed using a simultaneous methodology by HPLC. The fatty acid profile was measured as the percentage of total fatty acids using a gas chromatography technique. The analytical methodology is presented in Chapter 3. Every analysis was performed twice on every sample from every separator, therefore having a total of 12 samples measured twice. All samples were injected twice.

5.2.2.5. Statistical analysis

All statistical analysis was performed with the software R-4.0. Even though each experimental condition was performed once, statistical analysis was possible from the multiple quantification of the compounds of interest. Assumptions were checked by first visually interpreting the Q-Q and boxplots from all analysis. Normality was double-checked by the Shapiro-Wilk test, which gave non-significant values for all the ran analysis, therefore proving the normality of the whole data. Subsequently, the statistical analysis of the data was performed. A two-way ANOVA was run first to understand the importance of the interaction between the temperature and the pressure on the final extraction yield, and concentration of α-tocopherol and β-carotene. Further analysis involved the use of Tukey's *post hoc* tests to understand possible significant differences within

variables. Statistical analysis and significances were resolved after comparing each experimental condition.

The fatty acid profile was analyzed by using a principal component analysis (PCA), a similar procedure as per the analysis of fatty acids from solvent extraction of SBO. Differently from the extraction with solvents, further analysis included a two-way ANOVA to test for the effect of pressure and temperature on the extraction of every specific fatty acid from SB dried berries as well as to test the possible interaction effect. The latter statistical analysis was performed on each fatty acid due to industrial interest in the technology and to deeply understand its effect on each fatty acid, especially in palmitoleic acid.

The samples were separately quantitatively analyzed but statistically treated altogether. In other words, the results from the quantitative analysis of different separators were merged for their statistical analysis.

5.2.3. Results and discussion

5.2.3.1. Particle size

Results from particle size analysis can be found in the part of the chapter on solvent extraction of SBO. The summary of the results is shown in Table 2.

5.2.3.2. Extraction yield of SBO extracted with supercritical CO_2

The extraction yield is represented in Figure 15. There was an observable positive association between pressure and oil yield, showing a greater and exponential extraction yield from [redacted] to [redacted] bar, which translated into a much greater recovery of oil at the highest extraction pressure. Other authors already reported higher oil extraction when using higher pressures in avocado oil (Corzzini, Barros, Grimaldi, & Cabral, 2017) or bilberry seed oil (Gustinelli et al., 2018). The same association had been previously reported on sea buckthorn berries extracted at pressures of 200 to 400 bar, reaching a plateau after surpassing the 300 bar threshold (Xu, Gao, Liu, Wang, & Zhao, 2008). The CO_2 density increases with the pressure, resulting in an increased – and improved – oil solubility, which in turn increases the yield (Fiori, de Faveri, Casazza, & Perego, 2009; Xu et al., 2008; Yu, Rizvi, Zollweg, & Engineering, 1994).

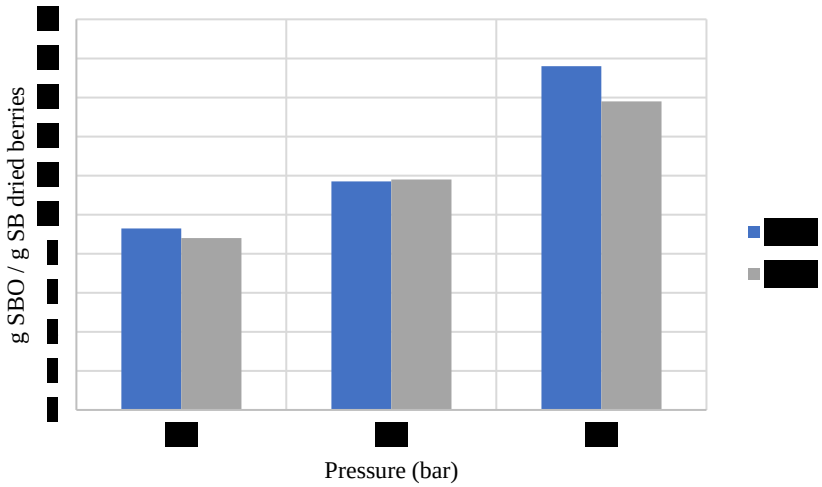


Figure 15. SBO yield after supercritical CO_2 extraction at different temperature and pressure.

The temperature did not greatly affect the amount of oil extracted from sea buckthorn berries, at least at lower pressures ([redacted] and [redacted] bar). At pressures of about [redacted] bar, the difference in oil yield seemed to be relevant, as the difference accounted for almost [redacted]%, being higher at [redacted] ºC (Figure 15). Xu et al. (2008) performed a surface plot for oil yield as a function of pressure and temperature after supercritical extraction of SBO from dried berries at 40 to 60 ºC and 200 to 400 bar of pressure. They found that working at pressures lower than 320 bar, the difference in SBO yield was evident when using either different pressures or different temperatures. Results from their study suggested a strong effect of temperature using pressures of 200 bar, leading to differences in yield about 5 %, being higher at lower temperatures (40 ºC). [redacted]

[redacted], differing from what Xu et al. (2008) previously reported.

Xu et al. (2008) reported oil yield values around 11% at 60 ºC and above 16% at 40 ºC when working at 200 bars. They reported the highest oil yield value at the highest pressure (400 bar), which was around 19% across all temperatures used. The maximum yield herein reported was almost [redacted]% (Figure 15), [redacted]. Other authors reported values of oil yield of 11.6% extracted at 300 bar and 40 ºC (Pavlović et al., 2016), [redacted] (Figure 15). Nonetheless, the difference may be derived from the raw matter quality which, depending on the variety and other important factors, may have greater oil content (Chapter 4).

5.2.3.3. Concentration of α-tocopherol in SBO extracted with supercritical CO_2

The concentration of α-tocopherol in SBO extracted with supercritical CO_2 at different pressures was affected differently by temperature, F (2, 6) = 16.39, at $p<.05$. In other words, the concentration of α-tocopherol in SBO did not follow the same pattern across pressures when using different temperatures for the extraction, clearly pointing to a relevant interaction effect between temperature and pressure. Whereas no difference was

observed in the concentration of α-tocopherol at [redacted] ºC when increasing the working pressure, increasing the extraction pressure decreased the concentration of α-tocopherol when working at [redacted] ºC, clearly showing different tendencies (Figure 16).

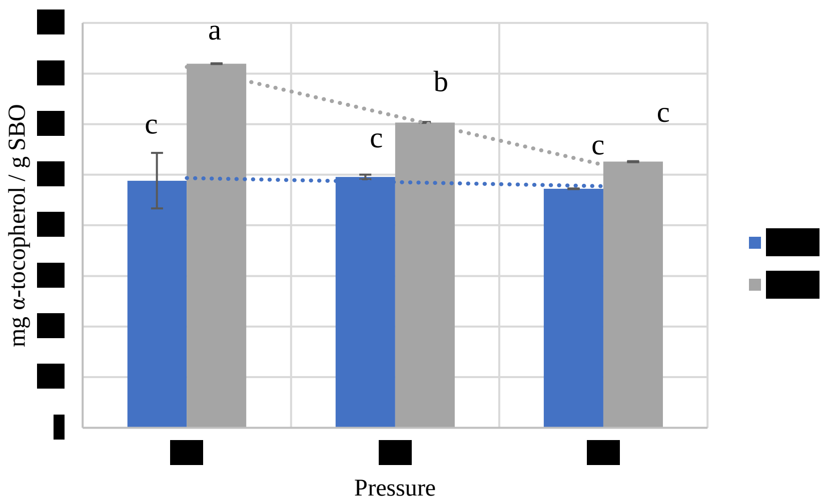


Figure 16. α-tocopherol concentration in SBO extracted with supercritical CO_2 at different pressure and temperatures. Error bars show standard deviation. Different letters show statistical differences at *p*<.05.

There was a significant main effect of temperature on the concentration of α-tocopherol in the extracted SBO, F (1, 6) = 101.59, at $p<.05$, as well as a significant main effect of pressure on the concentration of α-tocopherol, F (2, 6) = 21.76, at $p<.05$. Higher temperatures achieved higher concentrations of α-tocopherol, and lower pressures also resulted in higher concentrations of α-tocopherol. No differences were observed when comparing the extractions using different pressures at [redacted] ºC (Figure 16). It was however interesting to note that over increasing pressure the recovery

of α-tocopherol seemed to diminish when extracting at [redacted] ºC. Other authors reported increased recovery of α-tocopherol when working at 40 ºC and increasing the pressure from 200 to 300 bar, although the difference was about 0.02% (Xu et al., 2008). The flux may be one of the factors affecting the results, since Xu et al., (2008) developed a surface plot of vitamin E yield based on a constant CO_2 flow rate of 15 L per hour, which is [redacted] than the flux used in the present experiment. As the same authors demonstrated in their study, an increase in CO_2 flow rate leads to an improved recovery of total tocopherols. Pavlović et al., (2016) extracted SBO from SB dried berries at 40 ºC and 300 bar [redacted] leading to the conclusion that factors other than the pressure and temperature could strongly influence the recovery of α-tocopherol.

Nevertheless, other studies show otherwise. Kagliwal et al. (2011) showed improvements in recovery of total tocopherols of more than 20% in sea buckthorn seeds when increasing the working pressure from 200 to 300 bar (Kagliwal et al., 2011). They worked at 35 ºC and used a CO_2 flux of around 1.2 L per hour. Their results also suggested that working at lower temperatures could also influence the concentration of α-tocopherol in the

resulting oil over different pressures, [redacted].

Significant differences were observed between the extraction at [redacted] bar and [redacted] ºC when compared to all other extractions, being higher in the former. The difference was also significant when comparing the extraction at [redacted] bar and [redacted] ºC to all other extractions performed at higher pressure or lower temperature (Figure 16). These results undoubtedly suggested that (1) performing the extraction at [redacted] ºC yielded greater amounts of α-tocopherol when compared to [redacted] ºC, and (2) that when working at [redacted] ºC, increasing the pressure significantly reduced the amount of α-tocopherol in the resulting oil. [redacted] higher temperature yielded an oil with greater antioxidant capacity resulting from important contributions coming from the recovery of α-tocopherol (Correa et al., 2017; Kagliwal et al., 2011). The negative tendency between the concentration of α-tocopherol at [redacted] ºC and pressure [redacted], who did not observe any tendency when extracting oil from bilberry seeds at [redacted] ºC, [redacted]. [redacted] Bravi et al. (2007), who showed that the concentration of α-tocopherol was improved in the oils extracted at higher temperatures when compared to lower temperatures at low operational pressures. In addition, they showed that whereas extracting at higher temperatures resulted in less

yield, the concentration of α-tocopherol was significantly higher at higher temperatures, a phenomenon that could be derived from the selectivity of supercritical CO_2 in extracting certain active compounds (Chrastil, 1982; Gustinelli et al., 2018). In addition, the negative association between pressure and concentration of α-tocopherol could be partly explained because at lower extraction yields the levels of α-tocopherol would be constant, but an increase in oil yield at higher pressures would translate into a decreased relative concentration of that compound due to a dilution effect (Gustinelli et al., 2018). The same explanation could be used to discuss the constant concentrations of α-tocopherol at [redacted] ºC operating at different pressures.

Levels of α-tocopherol obtained in the present experiment after extracting SBO with supercritical CO_2 [redacted]. Nevertheless, the behavior of the concentration of α-tocopherol at different conditions was only partly in line with what other authors previously reported. Factors affecting its concentration could be many, for instance, different extraction time or different CO_2 flux. It was however clear that the best extraction conditions to obtain oil with the highest α-tocopherol content was at [redacted] ºC and [redacted] bars, and these conditions were detrimental to the yield.

5.2.3.4. Concentration of β-carotene in SBO extracted with supercritical CO_2

The concentration of β-carotene in SBO extracted with supercritical CO_2 at different pressures was also affected differently by temperature, F (2, 6) = 18.27, at $p<.05$. In other words, the concentration of β-carotene in SBO did not follow the same pattern across pressures when using different temperatures for the extraction, also pointing to an interaction effect. In contrast to what resulted from the analysis of α-tocopherol, the concentration of β-carotene was positively associated with pressure using either [redacted] or [redacted] ºC as extraction temperature. The difference resided in the tendency of the line, which presented a greater slope at [redacted] ºC extractions when compared to the [redacted] ºC extractions. [redacted] extractions at [redacted] ºC resulted in a plainer tendency, translating into little mean variation over changing the operational pressure (Figure 17).

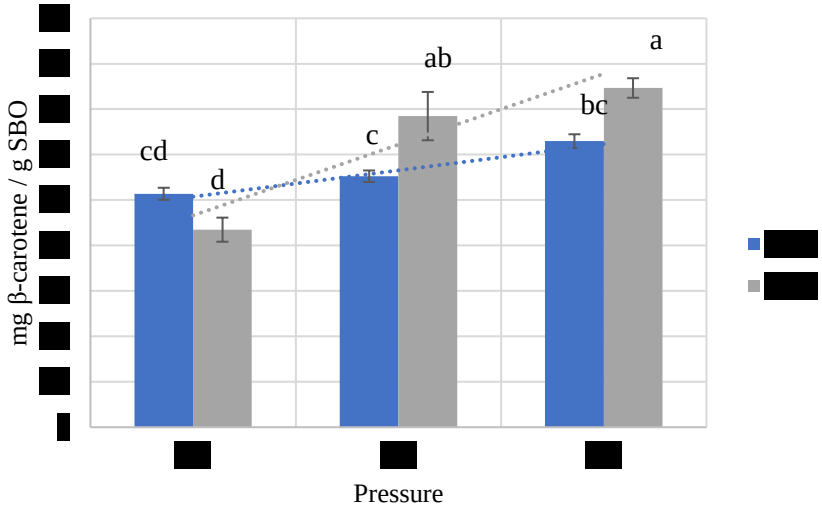


Figure 17. β-carotene concentration in SBO extracted with supercritical CO_2 at different pressure and temperatures. Error bars show standard deviation. Different letters show statistical differences at $p<.05$.

There was a significant main effect of temperature on the concentration of β-carotene in the extracted SBO, F (1, 6) = 12.71, at $p<.05$, as well as a significant main effect of pressure on the concentration of β-carotene, F (2, 6) = 62.62, at $p<.05$. Higher extraction temperatures also resulted in an oil with a greater concentration of β-carotene (Figure 17). Xu et al. (2008) already demonstrated that the carotenoid concentration was very dependent on the temperature used. [redacted] At some point around 250 bars, the tendency was swapped and at higher pressures, a greater concentration of carotenoids was observed for the greatest temperatures used in their experiment (60 ºC). [redacted]

[redacted].

In that case, however, increasing the extraction pressure lead to an increase of β-carotene concentration, although the tendency was only significant when the extraction was performed at [redacted] ºC when comparing the concentration values of [redacted] against the values of [redacted] bar (Figure 17). No differences were observed on β-carotene content when comparing different pressures at [redacted] ºC, although the mean increased positively with pressure. [redacted] Xu et al. (2008), who developed a surface plot of total carotenoids as a function of temperature and pressure. The difference between [redacted] and [redacted] bar at [redacted] ºC was reported to be milder than the difference at [redacted] ºC[redacted]. Besides, the flux they used was greater than the one used in the present experiment, and therefore their work showed stronger differences. However, they identified the CO_2 flux as the least important factor for carotenoid extraction. The extraction of carotenoids showed a tendency similar to the one observed for oil yield, which was not surprising since carotenoids could be greatly extracted together with the berry oil (Xu et al., 2008). [redacted] positive association between pressure and carotene concentration at [redacted] ºC (Kagliwal et al., 2011), which could be explained because rising the

pressure at constant temperature translates into a higher density of the solute and higher solubility of the analytes. [redacted]

5.2.3.5. Principal Component Analysis of fatty acids from SBO extracted with supercritical CO_2

A principal component analysis (PCA) was conducted on the 6 fatty acids with no rotation applied. The Kaiser-Meyer-Olkin measure verified the sampling adequacy for the analysis. Bartlett's test of sphericity indicated that correlations between items were sufficiently large for PCA. An initial analysis was run to obtain eigenvalues for each of the components in the data. Two of the components showed eigenvalues over Kaiser's criterion of 1 and in combination explained 84.46% of the variance (Table 10). The scree plot confirmed the first two components to be the most relevant for explaining the variance of the statistical model. Therefore, the first two components were retained for the final analysis. No rotation was applied since the first two principal components showed a great fit with the raw data. The items that clustered on the first principal component were mainly unsaturated; [redacted] acids. The items that clustered on the second was mainly [redacted], although slightly explained by the [redacted] acid. The dimensions in that case seemed to account for the [redacted] for the first principal component, and

for the [redacted] for the second principal component (Table 11). It should be noted that [redacted] acid contributed over 21% to the first PC. Nonetheless, the contribution of this specific fatty acid on the total fatty acids was about [redacted]%, which reduces the impact of this specific fatty acid on the PCA. This allowed making the aforementioned assumption that the first PC was build up mainly by [redacted].

Table 10. Principal components from the analysis of supercritical CO_2 extraction and their respective eigenvalue and variance explained by the model. Eigenvalues greater than 1 were used to draw the PCA, according to Kaiser's criterion.

Dimension	Eigenvalue	Variance explained (%)	Variance explained (%, cumulative)
1	3.334	55.569	55.569
2	1.733	28.896	84.465
3	0.583	9.711	94.176
4	0.329	5.486	99.662
5	0.020	0.329	99.991
6	0.001	0.009	100.000

Table 11. Contribution of every variable analyzed (fatty acid) from the analysis of supercritical CO_2 extraction to Dimension 1 and Dimension 2 of the PC model.

Variable analyzed	Contribution to Dimension 1 (%)	Contribution to Dimension 2 (%)
Palmitoleic	[redacted]	[redacted]
Palmitic	[redacted]	[redacted]
Linoleic	[redacted]	[redacted]
Oleic	[redacted]	[redacted]
Linolenic	[redacted]	[redacted]
Stearic	[redacted]	[redacted]

PCA was performed on the two main extracted principal components (PC1 and PC2) and the two independent factors (pressure and temperature). The PCA main graph shows results from individuals in a scattered plot with PC1 in the X-axis and PC2 in the Y-axis. Individuals were plotted in the same cluster depending on the pressure used during the extraction, to investigate differences between temperatures. Figure 18 shows the PCA resulting from the data obtained after the CO_2 extraction of SBO.

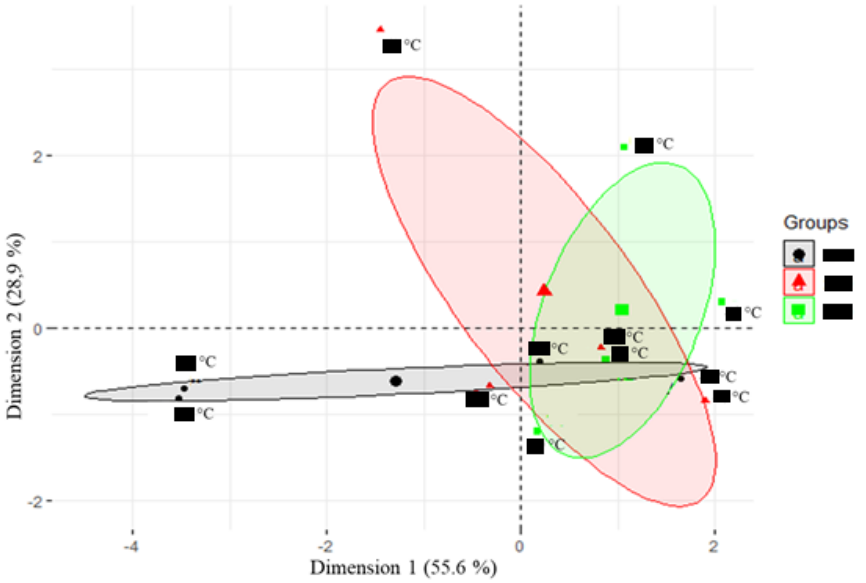


Figure 18. Mapping of the samples in two different dimensions. Samples were grouped according to the pressure used during the supercritical CO_2 extraction. The temperature of the extraction can be found next to every spot. Bigger spots represent the mean.

PCA map shows different extraction conditions clustered in pressures (Figure 18). It seemed that the extraction at different pressures gave a slightly different nutritional profile on the resulting oil. Different nutritional profile was also observed in the PCA map when comparing different temperatures. Notably, the oil extracted at [redacted] bar seemed to present the greatest variation in the fatty acid profile when comparing temperatures. The variation came from Dimension 1, translating into greater variation in [redacted], especially on [redacted] (Figure 18). It appeared that at [redacted] bar, [redacted] acids, as well as [redacted] acid, were found in greater concentrations in the oil extracted at the [redacted] temperature ([redacted] ºC) when compared to the [redacted] temperature ([redacted] ºC)

(Figure 18, Table 12). Interestingly, the results at [redacted] bar and [redacted] ºC gave very different results in both fatty acid extractions (Figure 18). The great differences came from Dimension 2, which registered very different values. The high variation probably came from the high difference in the values of [redacted] (Table 12).

In addition to these specific results, it seemed that the extractions at all other pressures showed the same pattern in terms of Dimension 1, that is, [redacted], excluding [redacted] (Figure 18). As the pressure [redacted], the clusters in the PCA map showed a slight tendency of moving right on the Dimension 1. The [redacted] association between pressure and [redacted] recovery is numerically represented in Table 12. Despite this tendency, the use of [redacted] or [redacted] bar during the supercritical CO_2 extraction of SBO did not give clear separation clusters in terms of PCA analysis, therefore making difficult the evaluation of the fatty acid profile.

The temperature seemed to also cluster the values separately (Figure 18). In fact, extracting at [redacted] ºC seemed to achieve greater concentrations of Dimension 1 fatty acids when compared to the extraction at [redacted] ºC. The numerical values of each fatty acid supported this reasoning (Table 12).

Table 12. Average mean percentage of each fatty acid in SBO extracted with supercritical CO_2 using different pressures and temperatures.

Pressure (bar)	Temperature (ºC)	Fatty acid (% of total fatty acids)					
		[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	34.276 ± 0.556	28.701 ± 0.125	0.911 ± 0.169	25.601 ± 0.064	3.078 ± 0.176	6.676 ± 0.043
	[redacted]	35.179 ± 0.014	31.102 ± 0.043	0.654 ± 0.000	23.760 ± 0.079	2.459 ± 0.042	5.888 ± 0.001
[redacted]	[redacted]	34.189 ± 0.665	28.238 ± 0.229	0.907 ± 0.127	26.090 ± 0.169	3.077 ± 0.031	6.788 ± 0.062
	[redacted]	37.211 ± 3.334	27.627 ± 1.553	0.784 ± 0.006	24.619 ± 1.826	2.807 ± 0.298	6.330 ± 0.298
[redacted]	[redacted]	35.969 ± 1.819	27.111 ± 0.756	0.848 ± 0.000	25.304 ± 0.749	3.332 ± 0.130	7.025 ± 0.187
	[redacted]	34.625 ± 0.009	27.920 ± 0.048	0.821 ± 0.007	26.630 ± 0.513	2.663 ± 0.431	6.678 ± 0.102

Values expressed as mean ± SD. C16:0 palmitic fatty acid; C16:1ω-7 palmitoleic fatty acid; C18:0 stearic fatty acid; C18:1ω-9 oleic fatty acid; C18:2ω-6 linoleic fatty acid. None of the results are statistically different at $p<.05$. Results from the two-way ANOVA performed on each fatty acid can be found in Table 13. Comparisons are not made across different fatty acids (e.g. Palmitic vs Palmitoleic)

5.2.3.6. Concentration of individual fatty acids in SBO extracted with supercritical CO_2

The palmitoleic fatty acid is one of the major industrial interests in SBO. Its recovery using supercritical CO_2 extraction aligned with the interests and values of the sponsor and was therefore critical to elucidate the effect of pressure and temperature on its extraction. A two-way ANOVA was performed on each fatty acid for that purpose.

The effect of pressure and temperature on the extraction of individual fatty acids from SBO extracted with supercritical CO_2

There was no significant interaction effect between the pressure and the temperature used during the extraction with supercritical CO_2 on the concentration of any fatty acid in the extracted SBO at $p<.05$ (Table 13). These results indicated that the supercritical CO_2 extraction of SBO at [redacted], [redacted] or [redacted] bar were not differently affected by temperature, translating into a similar result in the fatty acid profile of SBO when extracting at different pressures and different temperatures. Therefore, the values of Table 12 gave no significant differences on the results, because they are individual values for each pressure/temperature employed in the present study. In spite of the observed results, the two-way ANOVA model resulted in a good fit, since it explained most of the variance (in the worst case it explained as much as 84.82% of the variance, in the best case about 99.95 %). Therefore, the

individual effect of the pressure and temperature on the extraction of individual fatty acids was performed using the results from the two-way ANOVA.

Table 13. *p*-values of the two-way ANOVA from the factors and dependent variables used in the supercritical CO_2 extraction.

		Fatty acid					
Factor	**Df**	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Pressure	2	0.701	0.007**	0.574	0.175	0.344	0.006**
Temperature	1	0.385	0.080	0.034*	0.219	0.005**	0.001**
Pressure:Temperature	2	0.232	0.065	0.244	0.070	0.403	0.186

C16:0 palmitic fatty acid; C16:1ω-7 palmitoleic fatty acid; C18:0 stearic fatty acid; C18:1ω-9 oleic fatty acid; C18:2ω-6 linoleic fatty acid;

* Values are statistically significant at $p<.05$

** Values are statistically significant at $p<.01$

The effect of pressure on the extraction of individual fatty acids from SBO extracted with supercritical CO_2

There was a significant main effect of the pressure used during the extraction on the concentration of some fatty acids in the resulting SBO. Significant values at $p<.05$ were observed for [redacted] and [redacted] acids, $F = 12.751$ and $F = 13.891$ respectively (Table 13). The concentration of [redacted] acid was significantly higher in the SBO extracted at [redacted] bars, when compared to the other two pressures. [redacted]

[redacted] from germ oil more efficiently at lower pressures of 200 and 250 bar when compared to the higher pressure of 300 bar (Piras et al., 2009). The difference herein reported was only significant and clearly observable at [redacted] bar when compared to the extractions at other pressures (Table 12). In a more recent study, Liu, Xu, Gong, He, & Gao (2012) obtained a pomegranate seed oil with a greater concentration of palmitoleic fatty acid when extracting at 150 bar when compared to extractions at 300 or 450 bars. Nevertheless, in both studies, the palmitoleic fatty acids constituted less than 1% of the total fatty acids, which could make it difficult to understand the difference in palmitoleic concentration at different pressures. In the present experiment, [redacted] acid accounts for as much as [redacted]% in the best extraction, and the [redacted] association between the concentration of [redacted] fatty acid and pressure was shown once more, bringing more evidence to the field. It should be interesting to investigate whether at pressures between [redacted] or [redacted] bar the extraction could be similar to that of [redacted] bar and put together the benefits of a good [redacted] acid extraction with a higher oil yield (Figure 15).

In contrast, [redacted] acid showed greater concentrations in SBO extracted at [redacted] bars, compared to the other [redacted] pressures. Gustinelli et al. (2018) [redacted] concentrations of [redacted] acid when extracting at [redacted] pressures (at least 350 bars) in bilberry seed oil. Indeed, in the work of Gustinelli et al. (2018), the percentage of [redacted] acid in

the extraction at [redacted] bar was the [redacted] among all the extraction conditions. In addition, greater concentrations of α-linolenic acid were also observed at 300 bar after extraction of germ oil using pressures of 200 and 250 bars, although in that case, the difference was not significant (Piras et al., 2009). It is noteworthy to know that the concentration of other fatty acids was not significantly affected by changes in the extraction pressure.

The effect of temperature on the extraction of individual fatty acids from SBO extracted with supercritical CO_2

There was a significant main effect of the temperature used during the extraction on the concentration of some fatty acids in the resulting SBO. Significant values at $p<.05$ were observed for [redacted] acids, $F = 7.450$, $F = 18.139$ and $F = 36.328$ respectively (Table 13). In the case of all [redacted] fatty acids, the extraction with supercritical CO_2 at [redacted] ºC achieved greater concentrations when comparing to the extractions at [redacted] ºC (Table 12). Supercritical CO_2 extractions could be performed at any temperature range as long as the supercritical condition of the CO_2 molecule is maintained. Lower temperatures help maintain a higher nutritional profile of the resulting oil by preventing the damage of readily oxidable molecules, such as unsaturated fatty acids. Results from the present experiment show that the application of [redacted] temperatures during CO_2 extraction led to an SBO with [redacted] concentrations of [redacted] and [redacted]

[redacted] acids, [redacted] fatty acids in sea buckthorn seeds (Kallio, Yang, Peippo, Tahvonen, & Pan, 2002). The temperature was [redacted] the most important factor affecting the fatty acid profile of soybean oil, showing its influence on the concentration of unsaturated fatty acids and little effect on the saturated fatty acids (Jokic et al., 2013). [redacted] acid was also extracted at higher amounts when extracting oil from hemp at [redacted] (Aladić et al., 2015).

Like what had been reported by increasing pressure, increasing the extraction temperature also increases the solubility of CO_2 and subsequently the solubility of the solvent with the fatty acids (either in free form, esters or triglycerides among others) (Yu et al., 1994). Thereupon, one may expect the temperature of [redacted] ºC to show higher percentages of fatty acids instead of lower temperatures. Nonetheless, Table 13 and Table 12 showed otherwise. The fatty acids significantly affected by temperature were the [redacted] but also the fatty acids with [redacted] (Table 12). MUFA and PUFA are generally more affected by temperature, as the unsaturations could be readily degraded after applying higher temperatures, directly translating into lower fatty acid yield in the resulting oil. It would be possible that at lower residence time the impact on these fatty acids could be lower, since higher residence time have a negative impact on the content of fatty acids, especially on the

unsaturated fatty acids (Liu et al., 2012). Yet this should be studied in future research.

5.2.4. Conclusions

SBO was successfully extracted from SB dried berries using temperatures of [redacted] and [redacted] ºC and pressures of [redacted], [redacted] and [redacted] bars. The higher the pressure, the higher the oil yield, in detriment of certain bioactive compounds, including α-tocopherol and palmitoleic fatty acid. On the other hand, higher pressures achieved higher concentrations of β-carotene and α-linolenic acid. The information herein reported may serve as the basis to use different parameters depending on the part of sea buckthorn that is being used as raw matter for the oil extraction.

The best extraction conditions for sea buckthorn seed oil are [redacted] bar and [redacted] ºC. At higher temperatures, the extraction of α-tocopherol could be higher, but the extraction of important bioactive unsaturated fatty acids would be reduced. The extraction at [redacted] bar allows for an average extraction yield without heavily compromising oil quality.

The best extraction conditions for sea buckthorn pulp oil are [redacted] bar and [redacted] ºC as well. The extraction at this temperature and pressure allows for a good balance between the recovery of β-carotene and the efficient extraction of palmitoleic acid, the two most valuable bioactive compounds

from sea buckthorn peel and pulp oil. If a higher palmitoleic acid concentration wants to be achieved, higher pressures should be applied.

5.3. Overall discussion

A comparative statistical study was jointly performed considering the two techniques used in the present chapter for SBO extraction. The comparative study was performed with the best results of the different extraction conditions. A total of four conditions were compared:

1. The supercritical extraction at [redacted] bar and [redacted] ºC. It was previously concluded to be the best extraction condition of SBO [redacted], respecting the balance between extraction yield and the nutritional profile of the resulting oil.
2. ASE extraction with ethanol at 90 ºC. It was the green solvent selected for comparison because of its great extraction yield of SBO, one of the greatest values of β-carotene concentration and great PUFA extraction without compromising the quality of MUFA.
3. ASE extraction with hexane at 60 ºC. It was concluded to be the best rapid and less energy consuming technique due to the lower temperature and time used, and the resulting oil presented a higher nutritional profile than other temperatures.

4. Soxhlet extraction. Included for its comparison as a reference method, extensively used in lab setups for oil extraction.

The statistical comparison focused on the most important components of SBO extracted from dried berries. Those were identified to be β-carotene and palmitoleic fatty acid for the peel and pulp part of the fruit, and α-tocopherol and α-linolenic fatty acid for the SB seed oil. Extraction yield was also used as a dependent factor for comparison, yet it was not possible to include it in the statistical analysis due to lack of repetitions when extracting the oil using supercritical CO_2 technology. A one-way ANOVA was run for every compound of interest.

Table 14 shows the average values from the recovered SBO using different conditions and the statistical difference. Yield appears to be greatly affected depending on the employed extraction technique. Supercritical CO_2 extraction of SBO achieved about half of the yield obtained using ASE or the classic Soxhlet extraction. The extraction using supercritical CO_2 also yielded lower yield compared to Soxhlet technique in flaxseed oil (Pradhan, Meda, Rout, Naik, & Dalai, 2010), lavender oil (Danh et al., 2013) or sapucaia nut oil (Teixeira et al., 2018), although the difference was lower. The highest value for supercritical CO_2 extraction considering all the extraction conditions was, at a higher pressure, about 18%, which was closer to the value observed by other techniques (Table 14) and more in line with what other authors previously reported. The extraction with ethanol

yielded the highest amount of oil. It is every time more common to optimize supercritical extractions by adding co-solvents, and ethanol is extensively used. In that line and considering the great oil quality resulting from ethanol extraction, ethanol could be studied as a co-solvent for the extraction of SBO using supercritical CO_2 technology.

Table 14. Summary of the nutritional profile of extracted SBO using traditional techniques and green alternatives.

Technique	Yield* (%)	α-tocopherol (mg / g SBO)	β-carotene (mg / g SBO)	C16:1ω-7 (% of TFA)	C18:3ω-3 (% of TFA)
Soxhlet	23.60 ± 0.14	0.756^{b} ± 0.002	1.292^{b} ± 0.031	34.63^{a} ± 0.33	3.66^{c} ± 0.39
ASE Hexane	22.67 ± 0.28	0.937^{a} ± 0.058	1.365^{b} ± 0.059	23.96^{b} ± 2.81	7.85^{a} ± 2.64
ASE Ethanol	21.75 ± 0.18	0.897^{a} ± 0.005	1.699^{a} ± 0.050	25.68^{b} ± 2.34	6.42^{b} ± 2.72
Supercritical CO_2	11.70 ± NA	0.496^{c} ± 0.004	0.552^{c} ± 0.013	28.24^{b} ± 0.23	6.79^{ab} ± 0.67

TFA: Total Fatty Acids. Values show the mean ± SD. Different letters show statistical differences at $p<.05$.

*A proper statistical comparison was not performed due to the lack of values from the Supercritical CO_2 extraction.

Supercritical CO_2 extraction of SBO also yielded an oil with a lower concentration of α-tocopherol and β-carotene, halving the values obtained using other techniques (Table 14). In contrast, Liu, Xu, Hao, & Gao (2009) obtained an oil richer in α-tocopherol after extracting with supercritical CO_2 when compared to oil extracted by Soxhlet. This could derive from differences during extraction using supercritical technology, either from the flux or the temperature used. In addition, Danh et al. (2013) observed higher

antioxidant activity in the essential oil extracted by supercritical CO_2 when compared to Soxhlet extraction. The difference was more than 50%, which probably derived from the degradation of antioxidant lipophilic compounds during Soxhlet extraction, such as α-tocopherol. The presence of β-carotene was also significantly lower when comparing the extraction with supercritical technology and other extractions, including the extraction using the green solvent ethanol. Again, a combination of technologies could result in an oil with interesting yield and great concentrations of α-tocopherol and β-carotene. In addition, sample pre-treatment could also help to extract a higher amount of oil with a higher nutritional profile when using supercritical technology (Gutiérrez, Ratti, & Belkacemi, 2008).

The percentage of the relevant fatty acids was high in all extraction technologies. Interestingly, the recovery of palmitoleic fatty acid was higher in supercritical extraction when compared to the use of green solvents or the use of the ASE extraction technique using hexane. The difference was not significant but probably derived from the high standard deviation observed for the latter techniques when compared to the supercritical extraction analysis. Interestingly, Soxhlet extraction yielded significantly higher percentages of palmitoleic fatty acid than any other technique. Mendes, Reis, & Palavra (2006) achieved also higher concentrations in palmitoleic acid using a traditional extraction (Bligh and Dyer method) [redacted]

[redacted]. Nevertheless, they also studied the use of 10% ethanol as co-solvent for the supercritical extraction and interestingly found that using ethanol as co-solvent increased the proportion of different neutral lipids, including palmitoleic fatty acid. The results were different when performing the extraction of glycolipids or phospholipids from the same raw matter.

One of the most important parameters from SB seed oil is α-linolenic acid. The higher proportion of α-linolenic acid in SBO resulted from the extraction using ASE extraction with hexane (Table 14). Interestingly, there was no significant difference when comparing the oil extracted by ASE with hexane and the oil extracted with supercritical technology. The lower extraction yield for this fatty acid was observed for Soxhlet, probably due to the degradation of one or more of its unsaturations because of the higher temperatures and residence times. Pradhan et al. (2010) also showed higher recovery of α-linolenic acid with supercritical CO_2 from flaxseed when compared to other Soxhlet or even the conventional cold-press technique.

5.4. Overall conclusion

The use of supercritical CO_2 technology for the extraction of SBO is feasible and can be applied to pulp and peel oil extraction or to seed oil extraction to achieve an oil with a great fatty acid profile. Nevertheless, the extraction yield and the concentration of liposoluble vitamins or pro-

vitamins using this green technology seem to be lower when compared to other green alternatives, such as using ethanol at high temperatures.

The extraction of SB seed oil using supercritical CO_2 could be optimized by combining other green solvents during the extraction, such as ethanol. Thereupon, the supercritical extraction of SB seed oil could benefit from the greater extraction of α-tocopherol as well as an improved yield. The same application could overcome the low recovery of β-carotene in SB pulp and peel oil. Nonetheless, further research is needed on this specific optimization to understand the possible dragging effect of other polar compounds that could be present in SB pulp and peel oil, such as sugars, which could be detrimental to the overall extraction.

5.5. References

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PART IV

PROCESSING TECHNOLOGIES APPLIED TO THE JUICE AND FORMULATION OF NEW PRODUCTS

Chapter 6: Concentration of filtered sea buckthorn juice by forward osmosis, evaporation and cryoconcentration

6.1. Introduction

Fruit juices are an important source of water-soluble vitamins and minerals which are, in most cases, readily available and highly absorbed. There are a lot of fruit juices in the market, each with a different nutritional profile depending on the compounds that can be found in each liquid matrix. Some juices, for instance, are rarely constituted by a good quantity of lipophilic compounds. This is the case of sea buckthorn juice (SBJ), which contains great amounts of carotenoids as well as important fatty acids (i.e. palmitoleic), as explained in Chapter 1 and shown in Chapter 4. All the compounds are immersed in the same matrix and bring value to the final nutritional profile, yet they could be found in different phases. SBJ is presented as a two-phase juice, since the lipophilic compounds will be found in the upper phase (namely the oily phase) when the juice is left undisturbed.

Most of the fruit juices, when extracted, have a short shelf life if not processed adequately. Their high-water activity makes them ideal for its

spoilage by microorganisms. Some juices, however, may have a longer shelf life due to their characteristics. For instance, the low pH of SBJ makes it more difficult to be spoiled by microorganisms. Yet in the end, all fruit juices are deemed to spoilage if left unprocessed. Thermal processing has been used for a long time as a technique to achieve a final safe product. Nevertheless, using high temperatures could lead to a degradation of important bioactive compounds present in the raw juice matrix. The most worrying compounds are vitamins because most of them are highly thermolabile. Certain fruits are known to have high amounts of specific vitamins and strongly depend on their concentration in the final product for its successful sell (i.e. orange juice and vitamin C).

Thermal processing reduces dramatically the original concentration of bioactive compounds. Therefore, the greater concentration of vitamins in the original matrix, the greater the residual amounts in the final product. In SBJ, the initial vitamin content is very high (Chapter 1, 4). Above all, vitamin C concentration is the most relevant. However, high processing and storage temperatures could also lead to significant loss of vitamins in SBJ (Gutzeit et al., 2008). In view of the damaging effects of thermal processing, other specific technologies have been recently developed to tackle this problem (i.e. cold pressing).

In the world of food supplements, fruit juices are widely exploited. The vast range of fruit juices available on the market makes them very useful for the formulation of many different food supplements. Yet products containing fruit juices are mostly presented in capsules or as concentrated juices. The capsules are easy to take and carry, while the concentrated juices can open a new window of applicability, bringing flavor in meals or beverages. The dilution of concentrated fruit juices in water is very common among the food supplements.

The concentration of fruit juices helps in the process of achieving a safe product by reducing its water content and therefore its water activity. Concentrated fruit juices are not only important for the food supplements industry: fruit juices are concentrated to make more efficient the logistics and storage of the juice. Again, thermal processing has been used for several years as the main technique to achieve juice concentration. As it happened with the thermal processing of fruit juices to achieve a safe product, the use of temperature to concentrate the fruit juice also damages the nutritional profile of the raw matrix, including but not limited to bioactive hydrosoluble compounds. In addition, the concentration could also lead to negative changes in the juice flavor. Even though it is not proportional, it is safe to say that the concentration leads to a higher content of bioactive compounds per milliliter of juice, therefore providing a

nutritionally more concentrated product, which is directly beneficial in the formulation of food supplements.

Thermal processing, or evaporation, has been used for many years. To minimize the detrimental changes in the nutritional profile and flavor occurring upon heating, other techniques have been developed. The concentration of liquid matrices by membrane technology or cryo-technology is now a reality.

6.1.1. Evaporation

Concentration by evaporation is understood as a technique in which heat is applied to remove water from a liquid matrix. Evaporation has been widely used because of the simplicity of the technology. Nonetheless, heating a fruit juice (the liquid matrix) triggers unwanted changes and leads to a poor quality of the final product (Maskan, 2006). Initially, evaporation was applied without controlling the exposure of the liquid matrix to the environment. Nowadays, the technique has been improved to minimize the operating temperature and reduce the exposure to light and air of the liquid matrix. By reducing the operating pressure of the system, one could dramatically improve the nutritional quality of the end-product by avoiding the degradation of the compounds of interest (i.e. vitamins). Operating with a vacuum pump is almost mandatory nowadays to achieve an acceptable final product. Reducing the pressure of the system during evaporation

minimizes the air inside the system – and so does the contact of the air with the liquid matrix – and allows to evaporate the water from the matrix at lower temperatures compared to evaporation at environment pressure.

In this type of classic concentration, the clear disadvantage is the application of elevated temperatures, leading to already explained and unwanted changes in the original matrix. Although the concentration would be performed, the efficiency of the process would be very low due to the proportionally high losses in terms of flavor and nutritional profile. The energy expenditure in the process is also an important disadvantage, although one could mitigate this waste of resources by adding more stages to the evaporator, therefore using the heat from the water evaporating in the first stage to heat the liquid entering the second stage and so on. However, the waste of energy to heat up the initial steam as well as the energy to keep the vacuum pump and other pumps active (when needed) is inevitable.

The major advantage is that the process has been widely used in the food industry for several years and its application is well known for a great variety of fruit juices. In addition, the technique has been optimized in the past years to become a more efficient process. Moreover, evaporation could make easier the concentration of the product, reaching values of 45-65 ^{o}Brix, as nothing is impeding the evaporation of water besides the

continuous and inevitable rise on the boiling point of the fruit juice that is being concentrated (Jiao, Cassano, & Drioli, 2004).

6.1.2. Forward Osmosis

Membrane technology embraces a broad range of processes. It could be further classified in filtration technology and osmotic technology. The basics of the process are different, yet in both cases a membrane constitutes the fundamental part of the process. On one hand, membrane filtration technology processes include microfiltration, ultrafiltration or reverse osmosis, among others. The type of process mostly depends on the pore size of the membrane, having a direct impact on the application to certain food products. For instance, microfiltration is used in the fruit juice industry to clarify and remove suspended solids from the juice (including fat and high molecular weight proteins), whereas ultrafiltration is usually used to fractionate milk for cheese generation (Bhattacharjee, Saxena, & Dutta, 2017). Although all of the filtration technology processes allow to concentrate a liquid matrix in a way (for instance in milk fractionation to concentrate proteins), the separation of water from all other components (concentration as it is described in the present Chapter) is reserved to reverse osmosis technology. In fact, reverse osmosis aims at separating the water from a solution despite the normal osmotic pressure. Reverse osmosis, even though is not as efficient as evaporation, it has been used in

fruit juice industry as a technique to preconcentrate the juice (Al-Obaidi, Kara- Zaïtri, & Mujtaba, 2017; Bhattacharjee et al., 2017).

On the other hand, osmotic technology processes make use of membranes to facilitate the separation of a specific compound by osmotic pressure. In other words, the separation occurs due to the transport of water from a region of higher water chemical potential to a region of lower water chemical potential by using a selectively permeable membrane (Hameed, 2013). The process of concentration by osmosis is also known as forward osmosis (FO). In this process, the use of a highly saturated solution is mandatory to create an osmotic gradient so water can flow through the membrane to the appropriate direction. The saturated solution is a critical factor depending on the use of the concentrated solution. Fruit juices demand the use of food-grade salts, therefore limiting its choice. Different salts have been studied with different efficiency to concentrate fruit juices (Hameed, 2013).

The major disadvantage of using membrane technology in juice processing is the sensibility of the membrane. Most of the fruit juices, when extracted, could present several residual solids from the pulp, which could damage the membrane. Therefore, most of the time it would be required to perform a pretreatment to ensure a greater shelf life of the membrane. Even though the solids may be extracted using a clarification methodology (i.e.

prefiltration, pectin treatment, centrifugation), a major obstacle of membrane technology is still the quick decline of the permeate flux as a result of the membrane fouling (Guo, Ngo, & Li, 2012). In addition, residual migration of the extraction solution to the food solution could also occur during concentration (Babu, Rastogi, & Raghavarao, 2006).

The advantages of using membrane technology to concentrate fruit juices, or the use of them to preconcentrate the juice are (1) a better separation quality, (2) a reduction of heat damage (compared to evaporation) and (3) lower capital requirements – both for the initial investment and for the lower cost of maintaining the process (Bhattacharjee et al., 2017).

6.1.3. Cryoconcentration

Another technique which uses heat transfer as the basic physical parameter to concentrate fruit juices is the concentration by freezing, or cryoconcentration. During cryoconcentration, ice crystals are formed in the juice after applying very low temperatures (which temperature will depend on the juice). The crystals are thereafter separated by filtration, centrifugation or simple decantation. There is every time more cryoconcentration techniques that make a cut; suspension crystallization, progressive cryoconcentration, eutectic cryoconcentration, partial block cryoconcentration or complete block cryoconcentration. Differences mostly remain in the size of the ice crystals. Cryoconcentration by

suspension crystallization uses the formation of small ice crystals, which makes the process and the after-process more complicated than other types of cryoconcentration techniques (Vincze, Bányai-Stefanovits, & Vatai, 2009). Eutectic freeze crystallization is a technique used to separate inorganic salts from its aqueous solution by achieving simultaneous crystallization of both salt and ice (Himawan, Vaessen, Kramer, Seckler, & Witkamp, 2002). In contrast, in block freeze concentration the aim resides in forming a big ice block and then separate it from the concentrated fraction by gravitational thawing (Vincze et al., 2009). Suspension crystallization, partial block, and complete block cryoconcentration have been used in the food industry in the concentration of fruit juices.

The disadvantages of applying cryoconcentration are several. The crystal formation and morphology are very important to get an optimal separation of the ice. The presence of impurities or suspended solids may be detrimental to a good crystallization process. In addition, the ice fraction could drag some of the hydrosoluble and interesting compounds which one may want to recover with the concentrated fraction. Finally, the cost of applying such technology is still very high, both the initial investment and the processing costs.

The advantages of cryoconcentration are the very fair separation quality and most importantly the use of very low temperatures, allowing a great

retention of the nutritional quality and aromatic compounds, even when the concentration is performed in presence of air (Vincze et al., 2009).

6.1.4. Demarcation

All technologies herein explained have been used to concentrate different fruit juices. The concentration of SBJ has been attempted using membrane technologies (Vincze et al., 2009) and thermal processes (Gutzeit et al., 2008; Xu, Hao, Yuan, & Gao, 2015).

Gutzeit et al. (2008) concentrated filtered SBJ with a five-stage evaporator, from 80 to 85 ºC. In addition, the concentrate was thermally treated at 90 ºC for 45 s. The concentration of SBJ by evaporation yielded a juice with half of the content of total ascorbic acid in terms of percentage of the original juice. Although SBJ may present high contents of ascorbic acid, the thermal treatment critically reduces its content. Consideration should be paid on the fact that concentration of SBJ did not lead to greater preservation of vitamin C through different storage temperatures and times. SBJ was concentrated by evaporation and uncontrolled environment, with full exposure to oxygen. Xu et al. (2015) investigated the effect of different temperatures (90, 100, 110 and 120 ºC) on the content of ascorbic acid in SBJ. They used equipment in which is not possible to control the contact of samples with oxygen (i.e. autoclave). They found that using temperatures of 120 ºC decreased dramatically the content of ascorbic acid over time,

and the tendency suggested a full depletion of ascorbic acid after 70 minutes. Any temperature decreased dramatically the content of ascorbic acid in SBJ except for the trial at 90 ºC, which was somehow maintained steady (although slightly decreasing) over the 300 min of the duration of the experiment.

Vincze et al. (2009) studied the use of different membrane separation methods on SBJ. Two concentration methodologies were tried. On one hand, SBJ was first clarified and then concentrated by nanofiltration. On the other hand, raw, unfiltered SBJ was directly concentrated using reverse osmosis. The concentration by nanofiltration achieved 15% of total solids (initial content of 4%) whereas the concentration of unclarified SBJ by reverse osmosis achieved 13.4% (initial content of 5%). The content of vitamin C dropped in half when concentrating the juice by reverse osmosis (either clarified or unclarified). The concentrated juice kept most of its vitamin C content when concentrating by nanofiltration. The vast quantity of solids in the raw juice could be decreased by a previous step of microfiltration. The microfiltration membrane fouled rapidly, but with appropriate cleansing was usable again.

To the best of my knowledge, no attempt has been made to study the cryoconcentration of SBJ by any technique. The possible problems that may arise from the total soluble solids present in the raw matrix – the same

problem as observed by Vincze et al. (2009) when concentrating the juice by membrane technology – could have supposed a critical point against the use of this technique.

SBJ is a matrix which is naturally constituted by two fractions: a lipid fraction (pulp oil) and a polar fraction (clarified juice) (Chapter 1, 4). Depending on the variety of the berry and its processing, the stability of this natural mixture will be longer or shorter. One of the characteristics constituting the identity of SBJ is that it is a juice with these two fractions and therefore, any concentration technique should be addressed at maintaining these two fractions instead of removing the lipid fraction by previous steps. Thereupon, the present chapter aims at exploring the different herein explained concentration techniques applied to raw SBJ, with the subsequent discussion of the possible mishaps and the overall results. A value of 30 °Brix was used as a final setpoint to compare the different employed concentration techniques. The value was set at 30 °Brix because it was a coherent value considering the results obtained with similar products using the same techniques (Hameed, 2013; Orellana-Palma, Petzold, Pierre, & Pensaben, 2017) and because it was considered to be half-way of the final target value (65 ºBrix), in which most fruit juices are usually concentrated in the food industries.

6.1.5. Objective

The objective of the present study was to concentrate raw, unclarified SBJ by three different concentration techniques – namely forward osmosis, evaporation and cryoconcentration (i.e. block freeze, progressive and suspension crystallization) – to the target value of 30 ºBrix and compare the nutritional profile of the resulting concentrated juice (in terms of concentration of specific bioactive compounds). The ultimate outcome would elucidate which technique would be the most interesting for its use in the development of food supplements.

6.2. Experimental design

6.2.1. Sample preparation

Sea buckthorn berries of the variety 'Tatjana' (a crossed variety with genes from different subspecies, namely *mongolica*, *rhamnoides* and *fluviatilis*) were purchased from BRUwell, SIA, a local harvesting firm located on western Latvia. Sea buckthorn berries of the subspecies *caucasica* were purchased from S.C. Bios S.R.L. Italia Sucursala (Bucarest, Romania). Sea buckthorn juice (SBJ) was extracted using a screw-drive extruder located at the Food Technology Plant Service (SPTA) of Universitat Autònoma de Barcelona (UAB, Bellaterra, Spain). In order to reduce the particle size of the juice and thus to avoid undesired retention of compounds during

filtering, SBJ was further grinded with a Krups conventional blender (Solingen, Germany) at maximum power for 60 seconds to reduce the particle size of the solids within. Thereafter, SBJ was filtered through a 200 µm pore size filter, to obtain the filtered sea buckthorn juice (FSBJ) used for the experiments. The variety from Latvia was the one used in all the experiments, except when stated otherwise.

6.2.2. Evaporation system setup

A pre-designed distillation equipment was purchased from Vidrio Industrial Pobel, S.A. (Ref: 100308184) and adapted to the needs of the evaporation system. The pre-designed distillation equipment was constituted for a Kjeldahl distillation ball, the evaporation chamber, a ground-glass screw-capped exit from the evaporation chamber, the thermometer and the adapter that connected it to the condenser, and the condenser itself.

Silicone tubes (Ref: SILT-006-005), grounded-glass connections to the silicone tube (Ref: ADS3-002-001), the second Kjeldahl grounded-glass ball (Ref: SPB3-001-001), the glass connection prior to the second Kjeldahl ball (Ref: DIH3-003-001), the vacuum tube (Ref: RUTT-006-002) and the heat exchanger (Dimroth cooler, Ref: DROT-400-001) were purchased from Labbox Labware, S.L. (Barcelona, Spain). The exit of the product from the evaporation chamber was performed trough a 1 ml glass pipette, which connected externally with a silicone tube.

Two electric heaters were attached to the largest water bath. The Clifton heater was purchased from Nickel Electro Ltd. (Bournville, U.K.). The JP Selecta heater was purchased from J.P. Selecta S.A. (Barcelona, Spain). The second water bath carried a Corio CD model of electric heaters, purchased from Julabo Labortechnik (Baden-Württemberg, Germany).

The evaporator was designed to work in a continuous way at reduced pressure so that short residence time and a low boiling temperature of SBJ could be achieved. It consisted of the already explained and predesigned distillation system with major modifications (Figure 1). Briefly, the FSBJ was pumped from the feeding reservoir (7) to the heat exchanger (1) by the partially achieved vacuum of the system (by using a vacuum pump (9)), where it was heated using hot water at 80 °C, to thereafter being subsequently pumped to the evaporation chamber (2) by the partial vacuum, where the evaporated water was separated from the juice. The concentrated product was recollected and helped back to the main SBJ feed reservoir by a peristaltic pump (8). The vapor was allowed to separate from the juice – where the temperature was checked with a thermometer (5) – and led to a second heat exchanger (3), where it condensated with the help of a stream of tap water (cool water, 10 °C) connected to it. The condensate was taken out of the system by a peristaltic pump (8) to avoid air popping in the system and was continuously collected in a reservoir (6). Silicone tubes (Ø interior

6 mm and Ø exterior 10 mm) were used as non-glass connections because of its flexibility.

Kjeldahl distillation bulbs were strategically added within the system to prevent any foam from being dragged from the system (4). The first Kjeldahl bulb was added directly above the evaporation chamber and the second was added just before the vacuum pump, to ensure no dragged foam could damage it.

Hot water was used instead of steam to heat the liquid up to its boiling point. Water was previously heated at 80 °C in a water bath. The water bath was filled with 4 L of water and two heaters were attached. One of the heaters aimed at recirculating and heating the water bath whereas the other was aimed at pumping water through a silicone tube to the heat exchanger, where the FSBJ was effectively heated during its residence time. A second water bath was used to maintain the temperature in the evaporation chamber. The water bath was filled with 2 L of hot water and helped maintain a temperature of 80 °C with a third electric heater.

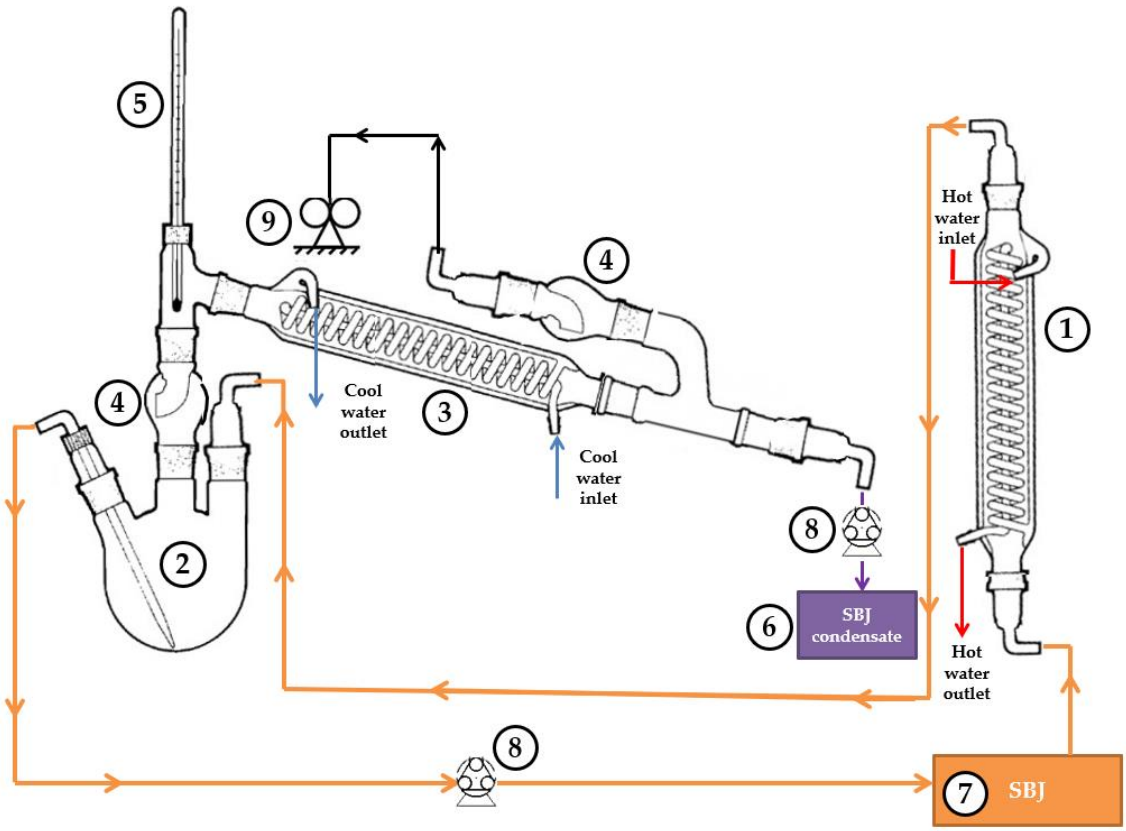

Figure 1. Schematic representation of the system used to concentrate FSBJ by evaporation.

6.2.3. Forward osmosis system setup

FO technique is based on the migration of water through a semi-permeable membrane (Figure 2). Its efficiency strongly depends on two important factors: the membrane characteristics and the efficiency of the saturated solution to draw water from the original matrix. The osmotic gradient pressure originated is what guides the concentration process. The higher the osmotic gradient pressure, the more water would pass through the

membrane and therefore the more concentrated would be the resulting matrix.

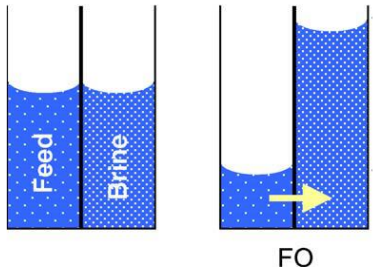

Figure 2. Scheme of the FO process. Copied from Cath, Childress, & Elimelech (2006)

Different salts have been investigated to understand the osmotic gradient pressure that each salt was able to achieve. Cath et al. (2006) clearly defined two salts of being able to create great osmotic pressures as concentration increased, namely $MgCl_2$ and $CaCl_2$ (Figure 3). $MgCl_2$ was selected as the salt to use in the present experiment because the pressure gradient that could create was slightly better than $CaCl_2$. In addition, the chosen salt was food grade, an essential prerequisite before starting any food-related experiment.

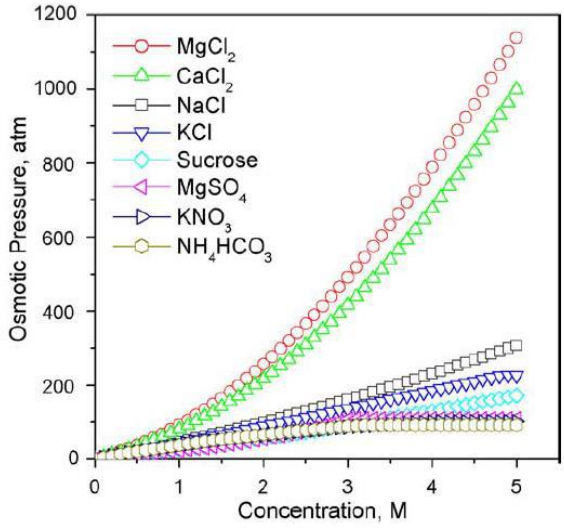

Figure 3. Osmotic pressure as a function of concentration with different salts and osmotic agents. Copied from Cath et al. (2006)

The process of concentrating FSBJ by FO was performed with a system from Leitat Technological Center (Terrassa, Barcelona, Spain). The system has been schematically represented in Figure 4. The system consisted of two tanks, one containing 5 L of FSBJ and the other containing 15 L of $MgCl_2$ 2 M (extraction solution). Both tanks were equipped with balances (2) with decimal precision to check for weight variation during the experiment (Figure 4). Both solutions were propelled with the use of a peristaltic pump (4) to the osmotic membrane. The membrane 'Forward Osmosis Element HFF02-220' (8) was purchased from Aquaporin A/S (Denmark) and was placed in a membrane cell (5). The membrane consisted of an active layer of polyamide thin film composite with integrated

aquaporin proteins. Other characteristics of the membrane are depicted in Table 1.

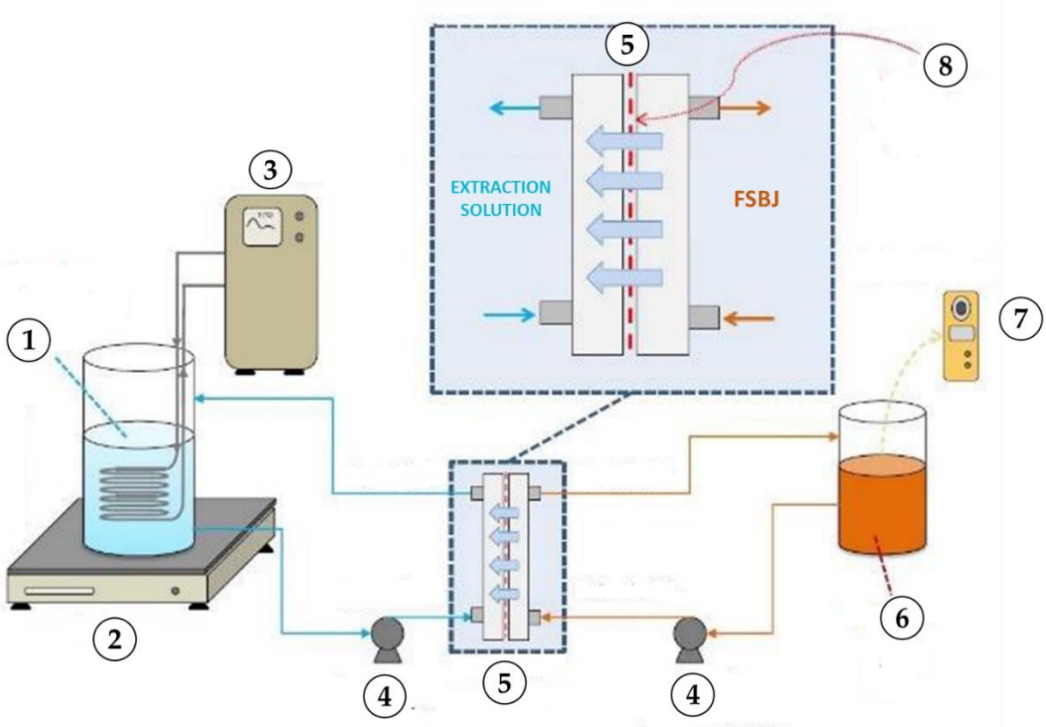

Figure 4**.** Schematic representation of the equipment used to concentrate FSBJ by forward osmosis (courtesy from Leitat Technological Center). 1, extraction solution; 2, analytical balance, 3, thermometer; 4, peristaltic pumps; 5, membrane cell; 6, FSBJ container; 7, refractometer; 8; osmotic membrane.

Table 1. Characteristics of the membrane used for forward osmosis concentration according to the manufacturer.

	Membrane area (m^2)	**Fiber internal diameter (mm)**	**Permeate flux (L/h)**	**Water flux ($L/m^2/h$)**	**Specific reverse salt flux (g/L)**
SFF02	2.3	0.2	>34.5	11 ± 1.5	0.15 ± 0.05

6.2.4. Cryoconcentration

6.2.4.1. Block-freeze centrifugal-assisted cryoconcentration

FSBJ was used for all methodologies at first but resulted unsuccessful for block cryoconcentration (see 6.3.1.3. Cryoconcentration from the section 6.3. Results and discussion). In that specific case, filtered SBJ was clarified by centrifugation in vials of 45 ml at 14,000*g* during 10 min at 4 °C (Cenkowski, Yakimishen, Przybylski, & Muir, 2006) to understand if the suspended solids were causing the problem. The clarified sea buckthorn juice (CSBJ) was separated from the oily fraction (supernatant) and used for the block freeze concentration.

Block freeze concentration procedure was the same as applied by previous authors (Orellana-Palma et al., 2017; Guillermo Petzold, Moreno, Lastra, Rojas, & Orellana, 2015). Briefly, the CSBJ was placed in 45 ml aliquots, and later surrounded by a 1 cm thick Styrofoam jacket which covered all the surface of the aliquot. The top was left uncovered with Styrofoam so that the heat transfer occurred unidirectionally (Figure 5). The freezing

temperature was set to -20 ºC because it was previously stated to be the most efficient temperature to obtain a solute-rich cryoconcentrate by using this technique (Orellana-Palma et al., 2017).

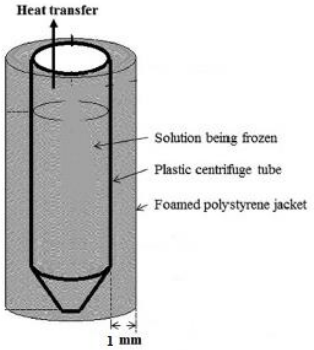

Figure 5. Graphical representation of the freezing cylinder where the aliquot sample was placed in. Copied from Orellana-Palma, Petzold, Pierre, & Pensaben (2017).

Samples were kept in the freezer overnight. The day after, the aliquots were taken out of the freezer and immediately placed in a centrifuge rack. The temperature of the centrifuge was previously set at 20 ºC. Samples were centrifuged for 25 min at 4,000 rpm (Orellana-Palma et al., 2017). Concentrated juice was separated from the ice using an 850 µm pore size filter.

The whole operational procedure was repeated three times more to increase the concentration of the solution and to reach the desired concentration in

terms of total soluble solids (TSS) (Figure 6), or at least as close as possible to the setpoint (30 ºBrix).

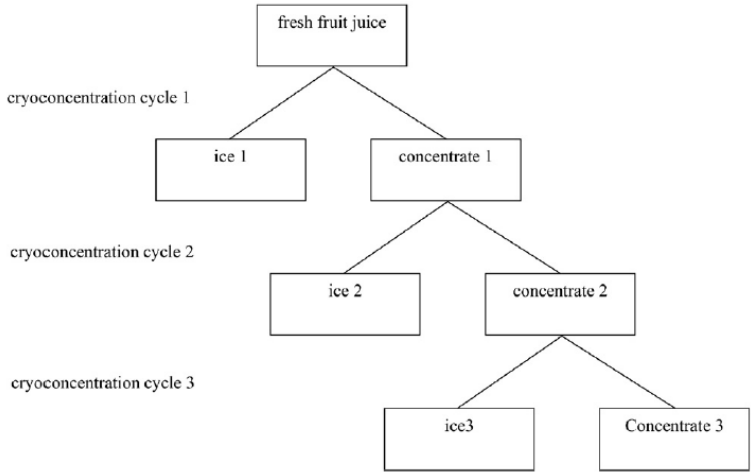

Figure 6. Schematic representation of the experiment. Copied from Petzold, Moreno, Lastra, Rojas, & Orellana (2015).

6.2.4.2. Suspension crystallization cryoconcentration

The methodology of cryoconcentration by suspension crystallization was performed in collaboration with the Beta Technological Center from the Universitat de Vic (UVic, Vic, Barcelona, Spain). The cryoconcentration by this methodology consisted of two steps (Figure 7); (A) the formation of ice crystals while constantly mixing up the solution and (B) the separation of those formed ice crystals by filtration. A suspension of ice crystals is formed with this system, which later on has to be physically separated from the concentrated matrix. The separation was performed with a filter of 0.5 mm and a vacuum pump was used to speed up the process.

Figure 7. System employed for the cryoconcentration by crystallization (A) and the separation system, consisting of a filter (850 µm) and a vacuum pump (B).

The technology is widely used in the food industry as the main cryoconcentration technique, differing from the block-freeze cryoconcentration which to the best of my knowledge, is in the lab-scale process. In addition, it can be used – when adapted – as a continuous concentration technology. However, the present lab-scale used methodology from the Beta Technological Center was not adapted to work in a continuous flow and therefore cryoconcentration cycles were used instead. Using the suspension crystallization methodology, it was not possible to control and infer on the agitation velocity nor in the temperature of the refrigerant. The ability of the technique to concentrate was measured by valorization of the product coming out of each concentration cycle. A total of two cryoconcentration cycles were performed due to the limiting factor, which was the volume of the raw juice. The first cryoconcentration cycle was performed using the raw juice in three different batches. The second cryoconcentration cycle was performed at once by putting together

all three different cryoconcentrates from the first cycle (Figure 8). Suspension crystallization cryoconcentration was not performed more than once from the first concentrate (concentrated SBJ 1ABC from Figure 8) due to a shortage in the raw volume. Every cryoconcentration cycle derived into the concentrated fraction and the diluted fraction.

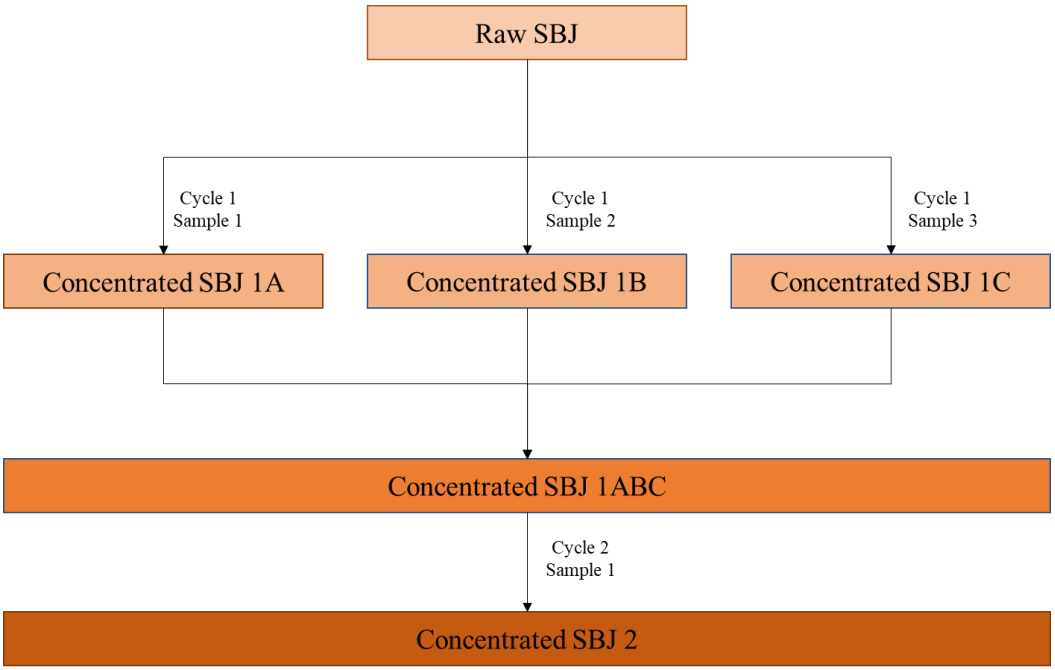

Figure 8. Schematic representation of the experimental design followed up during suspension crystallizatio The diluted fraction from each cryoconcentration cycle was not used as raw matter for the application cryoconcentration cycles.

6.2.5. Feasibility of the concentration techniques in FSBJ

Feasibility is a very important parameter for food companies. If a project is not feasible, it may never see the light of day. In other words, a product that is not feasible may never be launched. Feasibility studies include many different factors, such as the efficiency of the processes, economical

evaluation, production capacity, etc. In the present study, concentration techniques were applied to FSBJ in order to get a concentrated product which could later be used in the formulation and development of food supplements. A target value of 30 ºBrix was established as a set point to consider the concentration process as successful. Some of the techniques herein presented had never been applied to FSBJ. Thus, the results of the process were yet unknown. The capacity of the technique to concentrate FSBJ to 30 ºBrix was considered as the main tool to evaluate the feasibility of the process. In addition, any difficulty observed during concentration of the FSBJ was also reported and discussed.

6.2.6. Sample analysis

Sample analysis was performed on the concentrated SBJ and therefore only those analysis related to the aqueous fraction – as detailed in Chapter 3 – were used in the present matrix. Total soluble solids (TSS, in terms of ºBrix) and dry matter were the two physical parameters herein explored. Total quantification of chloride was performed on all the concentrates to understand the possible migration of the extraction solution during FO. The yield was calculated as the weight difference of the product before and after its concentration. Spontaneously, yield could be calculated against the weight difference of previous cycles (e.g. block-freeze cryoconcentration).

The yield was calculated only once due to the impossibility of repeating the concentration procedure in most cases.

The phytochemical analysis of the sample included (besides total chloride analysis) the quantification of total vitamin C, total polyphenols and total proanthocyanins as well as the antioxidant capacity of the concentrated products by means of DPPH and FRAP assays. Information of these quantitative methodologies can be found in Chapter 3. Besides the dilutions detailed in Chapter 3, concentrated samples were diluted 1:2 and 1:10 with distilled water for total chloride analysis and the rest of analysis, respectively. Vitamin C analysis demanded a greater dilution of the samples (1:50). Every sample analysis procedure was performed thrice, and measurements by the appropriate equipment were performed in duplicate.

6.2.7. Statistical analysis

All statistical analysis was performed with the software R-4.0. Assumptions were checked by first visually interpreting the Q-Q and boxplots from all analysis. Normality was double checked by Shapiro-Wilk test. Subsequently, the statistical analysis of the data was performed. A one-way ANOVA was conducted for every parameter of interest from the phytochemical analysis of the concentrated samples. Further analysis involved the use of Tukey's *post hoc* tests to understand possible significant

differences between groups (concentrated product using different techniques).

6.3. Results and discussion

6.3.1. Feasibility of the process

There is a lot of knowledge on the techniques herein used to concentrate fruit juices. However, each fruit juice behaves differently, each technique being more feasible with a specific fruit juice, or another. The feasibility was herein measured as the capacity of the FSBJ – filtered at 200 µm – to achieve the target value of 30 ºBrix. The feasibility was evaluated in each concentration technique separately, including evaluation of the changes occurring in the FSBJ during the concentration process.

6.3.1.1. Concentration by evaporation

First evaporation experiment

A first approach was performed to get an overview of the time needed to concentrate FSBJ to 30 °Brix. On the first approach, FSBJ at 200 µm was used considering previous observations during concentration of SBJ by FO and cryoconcentration technology. FSBJ was concentrated for 30, 60 and 120 minutes using the lab-scale single-effect evaporator specifically designed for this purpose. At each mentioned time, a sample of the product

was pumped out of the system and the ºBrix were read when the product achieved a temperature of 20 °C. After 120 min, the target value of 30 ºBrix was not achieved and thus the evaporation was performed for two more hours. After that time, the ºBrix were checked again. After a total time of 240 min of evaporation, the concentrated juice only reached 25 ºBrix. From that point, samples were acquired more frequently until 30 ºBrix were achieved at the final time of 300 min (Figure 9). During sample extraction, the system was unwantedly filled with air.

In the end, FSBJ was concentrated from 4.5 to 30 °Brix under reduced pressure in a total timespan of five hours in a designed lab-scale evaporator. A total of 3.831 kg of filtered sea buckthorn juice were used for the experiment. FSBJ was pumped into the system at an approximate rate of 7.38 kg/h. The FSBJ was allowed to flow freely throughout the heat exchanger, where its temperature rose from 18 °C to the evaporation temperature of the liquid at the specific conditions of the system. FSBJ was not allowed to boil in the heat exchanger but in the evaporation chamber. Vapor separated from the juice at a constant pressure of 200 mbar and 56 °C throughout the process.

Results from the first approach suggested a progressive concentration of FSBJ. However, the progressive concentration apparently showed abnormal behavior. The concentration of FSBJ first showed a rapid increase

in the ºBrix (6 ºBrix in 30 min) and then reached a plateau, showing only an increase of 4 ºBrix over 90 min. The abnormal behavior still accentuates at 240 and 300 min, showing a clear and very high concentration after two hours until 30 ºBrix value was reached at 300 min, or five hours (Figure 9). Other acidic juices, such as passion fruit juice, had been previously successfully concentrated to 60 – 70 ºBrix (Yu & Chiang, 1986). The concentration to this ºBrix value was achieved after 60 min of evaporation. The authors recorded the concentration in terms of ºBrix and it increased exponentially over time. They also applied techniques to minimize the suspended solids in an attempt to improve the evaporation of the juice. Interestingly, they found out that reducing the total suspended solids by centrifugation, enzyme treatment or ultrafiltration lead to a more linear concentration and to a major concentration of the juice in terms of ºBrix. This finding was very important for sea buckthorn juice concentration. Sea buckthorn juice has a lot of suspended solids and therefore, the concentration of the juice by evaporation may be especially difficult. These results would explain the abnormal concentration behavior of FSBJ. In addition, heat diffusion through the matrix as well as water evaporation becomes difficult when more solids happen to be in the original working matrix. Thereupon, it may be more difficult to evaporate water from FSBJ than it would be, for instance, for grape juice. However difficult, the aim of

the present experiment was to concentrate as much as possible all the nutrients contained in the original matrix without clarifying the juice.

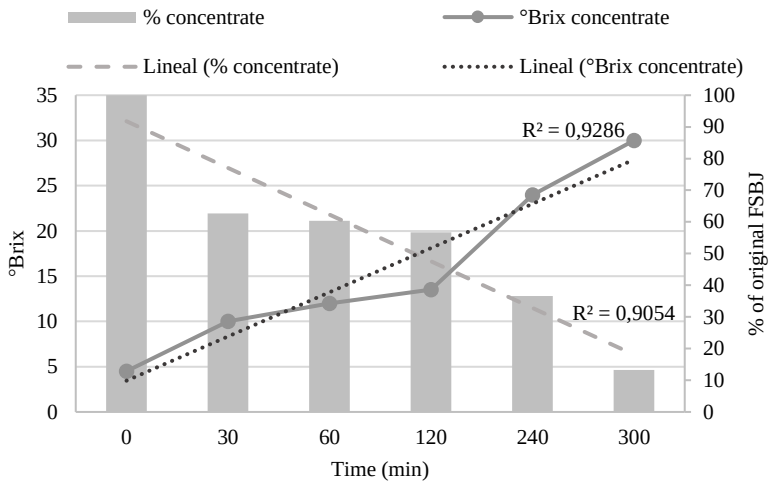

Figure 9**.** Brix degrees and yield (in % of the original FSBJ) as function of time during evaporation.

Additionally, it would also be possible that breaking the vacuum of the system very often (every 30 min at first) translated into more damage to the juice and more mishaps in the continuous concentration of FSBJ. The system did not allow for the continuous extraction of samples, therefore making difficult the real progression of ºBrix and the quantification of the process yield over time. The ideal system would have allowed for the continuous extraction of samples from the juice, therefore allowing the rest of the batch to keep up with the process. In the end, the process of concentration by evaporation of FSBJ to 30 ºBrix gave a 13% of yield.

Second evaporation experiment

The first experiment showed improper behavior during the concentration of the juice, since a great part of the evaporation occurred after two hours of processing. The main interest in this second experimental was to obtain a juice concentrated at 30 ºBrix, minimizing the exposure to air – therefore not breaking the vacuum often – and minimizing the evaporation processing time. The system was allowed to work for 120 minutes until the first sample was taken out to check Brix degrees (Table 2). After that, the Brix degrees were checked every 20 min to get a closer estimate. To overcome the initial problems of the evaporator, its design was checked and improved as follows:

- Silicone tubes were changed by isolated vacuum-resistant tubes.
- The tubes transferring the heated FSBJ from the calandria to the evaporation chamber were shortened to save the amount of energy transferred in the former part.
- The calandria itself was isolated with black Styrofoam, preventing an exaggerated loss of energy and preventing the light from oxidizing important compounds present in the juice.

After the improvements were made, another sample of FSBJ juice was concentrated. A total of 3.812 kg of filtered sea buckthorn juice was used for the experiment. FSBJ was pumped into the system at an approximate

rate of 7.453 kg/h. The FSBJ was allowed to flow freely throughout the heat exchanger, where its temperature rose from 18 °C to the evaporation temperature of the liquid at the specific conditions of the system. A total of 0.480 kg of concentrated juice were recovered from the original volume, leading to a concentration yield of 12.60%. FSBJ was not allowed to boil in the heat exchanger but in the evaporation chamber. The vapor was separated from the juice at a constant pressure of 150 mbar and 40 °C throughout the process, a greater improvement compared to the first experiment. A total of 3 hours was enough to reach the TSS value of 30 ºBrix in FSBJ. Concentration of TSS as a function of time was now steadier, achieving an R^2 of 0.9838 (Table 2). The problem here was that the first 120 min no values of ºBrix were reported since the experiment was believed to behave as the first one at least during the first couple of hours. It could not be assumed that the linearity observed in the second experiment would be correct because of the missing values. However, the obtained linearity during the second experiment resembled that observed for forward osmosis concentration (Figure 9).

Table 2. Progressive concentration of the FSBJ in terms of °Brix during the second concentration by evaporation

Time (min)	TSS (ºBrix)
0	4.5
120	19.5
140	23.0
160	28.5
180	30.0

With the process optimization herein applied, the reduction of evaporation time was made evident, suggesting further improvements in the evaporation technology may be useful to reduce the damage to the raw juice. In addition, evaporation processes used nowadays in the food industry are much more optimized, making the evaporation process shorter and more efficient. Evaporation could be a good technique for the concentration of FSBJ in terms of TSS concentration (measured by º Brix). Although the suspended solids in FSBJ did seem to have an effect during the concentration of FSBJ, they did not suppose an impediment to concentrate the juice to the established final value. Filtering the juice using a filter with smaller pore size may be useful to reduce the overall suspended solids of the juice, probably reducing the evaporation time. A reduction in the evaporation

time could also increase the concentration of vitamins and other thermolabile compounds in the end-product, making the process more efficient.

6.3.1.2. Concentration by forward osmosis

FSBJ was concentrated from 4.5 to 30 °Brix under atmospheric pressure in a total timespan of 60 min in a lab-scale system of forward osmosis. Initial trials were performed using non-filtered SBJ and suggested the imperative use of a filter. Fouling was a major issue with non-filtered SBJ, translating into an impossibility of concentrating SBJ to 30 ºBrix. The subsequent and final experiment of concentration by forward osmosis was performed with SBJ filtered at 200 µm.

A total of 5.9 kg of FSBJ were used for the experiment. Both FSBJ and the extraction solution were propelled towards the osmotic membrane at a flow rate of 1 L/min. The experiment was conducted at room temperature under atmospheric pressure. Both liquids were returned to the former container once they exited the membrane cell and were forced to recirculate. The extraction of FSBJ samples was easily performed and allowed for a clear understanding of the whole process over time in terms of ºBrix and % of the original FSBJ (process yield). During concentration, °Brix and weigh of the concentrate were checked once every 5 min (Figure 10).

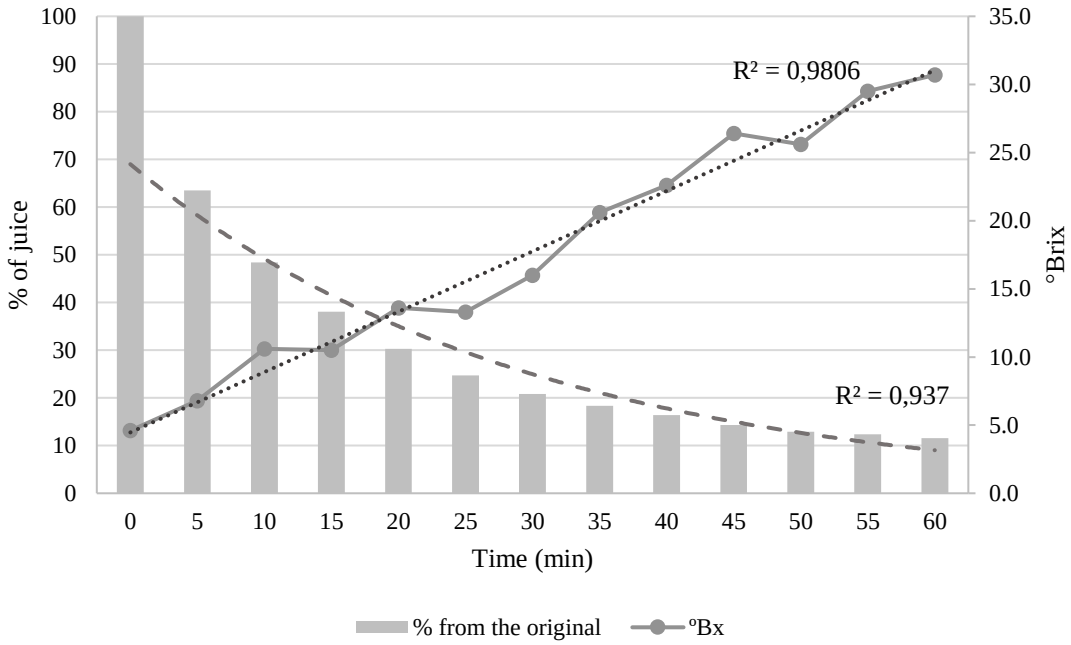

Figure 10. Brix degrees and yield as a function of time during forward osmosis concentration, where ºBx: ºBrix; % of juice: percentage of juice obtained from the original volume.

The concentration of FSBJ over 60 min led to a progressive and linear concentration in terms of ºBrix (Figure 10). Linear concentration was also previously observed during nanofiltration of SBJ (Vincze, Bányai-Stefanovits, & Vatai, 2007; Vincze et al., 2006), yet the results from nanofiltration could only reach values of 15 ºBrix after 72 min of concentration. This probably resulted from the membrane technology employed. FO technology uses a completely different practical approach when compared to nanofiltration or other types of filtration, since it is entirely based on osmotic pressure rather than filtering pressure. This leads to the use of specific membranes. There is a broad range of membranes available for FO, each having a different efficiency depending on the raw

matrix. The use of the membrane employed in the present experiment, which is based on polyamide thin film composite (see section 6.2.3 Forward osmosis system setup), is limited in previous works studying the concentration of fruit juices. Petrotos, Quantick, & Petropakis, (1999) also used a polyamide thin composite membrane to concentrate tomato juice. They achieved a similar product, consisting of tomato juice concentrate of about 30 ºBrix, but the process of concentration was not disclosed, so the evolution of the ºBrix and any other parameter of interest remained hidden. In a recent study, Kim, Gwak, Zhan, & Hong (2019) applied FO for the dewatering process of grapefruit juice by using a polyamide film thin membrane. They studied the concentration of grapefruit juice to 70 ºBrix. They also reported values of concentration to 19, 25 and 35 ºBrix. Although the reported values, together with the total vitamin C analyzed suggested a good effectiveness in concentrating grapefruit juice, the concentration in ºBrix as function of time was not reported in the study. Therefore, it was not possible to know whether using a similar membrane the concentration of FSBJ would follow the same pattern. To the best of my knowledge, these were the two only projects exploring the concentration of fruit juices by using a polyamide thin-film composite membrane.

Other works explored the concentration of fruit juices by using other membranes rather than polyamide based. For instance, other authors studied the concentration of sweet lime by FO. The concentration of sweet

lime juice led to an exponential increase concentration in terms of ºBrix (Chanukya & Rastogi, 2017). Concentration of sweet lime juice was performed to 50 ºBrix and a pronounced exponential effect was observed after reaching 30 ºBrix which was the target value of the present research. Although the membrane type was different than that employed in the present research (Table 3), the concentration of FSBJ to 30 ºBrix gave no information of its possible exponentiality during the concentration and after the target value. The exponential behavior of grape fruit juice also becomes evident after the concentrated product reaches values around 30 ºBrix (Nayak et al., 2011). Other fruit juices, nonetheless, show an exponential behavior in terms of ºBrix during concentration after reaching values of only 10 ºBrix. Such are the cases of beetroot and pineapple juices (Nayak et al., 2011). The behavior of each fruit juice during concentration by FO is clearly different, depending mainly on the raw matrix, but different membranes could also lead to differences during concentration. In fact, it would be possible that the concentration of FSBJ may also become exponential after 30 ºBrix, which was something that required further testing.

Table 3. Reported performances of FO in juice concentration and its comparison with FSBJ. Adapted from (Kim et al., 2019).

Fruit juice	Membrane type	Draw solution	Draw solution concentration	Initial concentration (ºBrix)	Final concentration (ºBrix)	Time of concentration to reach 30 ºBrix (hours)	Total time of concentration (hours)	Reference
Grape	CTA	NaCl	6.0 M	4.4	54.0	17.5	24	Nayak, Valluri, & Rastogi (2011)
Beetroot	CTA	NaCl	6.0 M	2.3	52.0	10	12	
Pineapple	CTA	NaCl	6.0 M	4.4	54.0	11	18	
Kokum	CTA	NaCl	6.0 M	2.0	52.0	15	18	Nayak & Rastogi (2010)
Pineapple	NS	Sucrose and NaCl	Sucrose 40% (w/w), NaCl 12% (w/w)	12.4	60	NS	18	Babu et al. (2006)
Sweet lime	CTA	NaCl	6.0 M	12.7	50	17	21	Chanukya & Rastogi (2017)
FSBJ	PFT	$MgCl_2$	2.0M	4.6	30.7	1	1.5	NA

CTA: cellulose triacetate; NS: Non-specified; NA: Not applicable

The same experiment with FSBJ was kept ongoing since the membrane fouling was not yet severe at any time. From 60 min forward (to the final time to which the experiment was performed (80 min)), linearity did not describe the behavior of the juice. When all the values from the 80 min of the experiment were considered, the tendency resembled an exponential association rather than a linear association between time and ºBrix (Figure 11). The clear difference on the R2 between the concentration of 60 min (Figure 10; R2 = 0.9806) and 80 min (Figure 11; R2 = 0.9614) could be used to understand that some linearity was lost when the concentration was kept ongoing. In addition, the yield of the concentration process (in terms of % of original FSBJ) also became steady from 60 to 80 min of concentration. This translated into a loss of suitability of an exponential equation to the results – from R2 = 0.9370 (Figure 10) to R2 = 0.8937 (Figure 11).

One of the most important parameters to be checked during concentration is its duration. The higher the time, the higher the damage on the nutritional profile of the processed matrix (fruit juice), as its most sensitive compounds are more exposed to the environment (i.e. oxygen, light…). The duration of the concentration to 30 ºBrix was notably faster than other authors previously reported for other matrices such as sweet lime (Chanukya & Rastogi, 2017), grape, beetroot (Nayak et al., 2011) or pineapple (Babu et

al., 2006; Nayak et al., 2011) (Table 3). Only 60 min were needed to concentrate FSBJ to 30 ºBrix (Figure 10, 11).

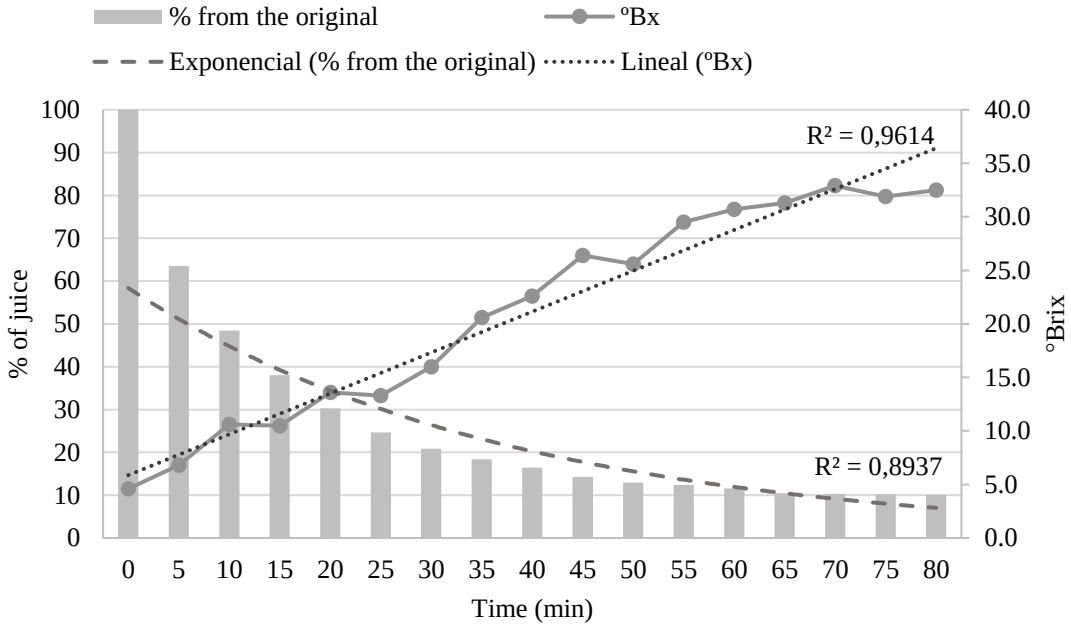

Figure 11. Brix degrees and yield as function of time including the extended experimental during FO concentration. Where ºBx: ºBrix; % of juice: percentage of juice obtained from the original volume. The black line marks the separation between the experimental to 30 ºBx and the following minutes in which the experimental was kept running.

Membrane flux and fouling

One of the critical factors when using membrane technology is the fouling of the membrane. The fouling of the membrane translates into a rapid decline of the permeate flux over time (Guo et al., 2012). The fouling can occur due to an increase in transmembrane pressure (TMP) when a steady flux is used or due to a reduction in the flux when the system operates at constant pressure (Guo et al., 2012). Usually, the former is the most

common as the flux is normally what is easier to control. In the present experiment, the flux was maintained over time by a peristaltic pump and therefore, the fouling occurred due to an increase in the TMP.

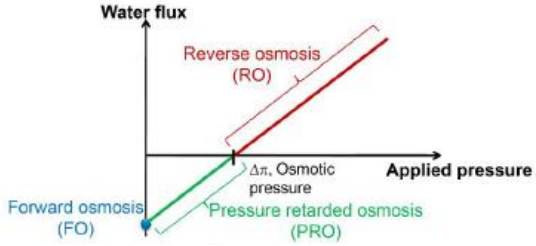

Figure 12. Water flux as a function of the applied pressure during different membrane technology processes. Copied from Chun, Mulcahy, Zou, & Kim (2017)

FO technology presents a lower fouling tendency resulting from the lower operating pressure when compared to other membrane technologies (Figure 12, i.e. nanofiltration, reverse osmosis…). The lower fouling tendency makes it more suitable for the concentration of solutions with a high content of soluble solids, such as FSBJ. In addition, the low fouling tendency increases the efficacy of membrane cleansing and re-usability (Chun et al., 2017).

The high presence of soluble solids in FSBJ still negatively influenced the transmembrane flux over time (Figure 13). Results from the first 30 minutes suggested a rapid decrease in transmembrane flux as a result of increased pressure. Other fruit juices, such as beetroot, grape or pineapple, obtained a linear decrease in transmembrane flux over a period of 6 hours (timespan

of each cycle (Nayak et al., 2011)). Yet the fruit juices employed in FO by Nayak et al. (2011) were previously diluted 1:2 with water and filtered using a muslin cloth. The dilution of the juice may be the reason why the transmembrane flux was kept steadier over time. Nevertheless, other authors used non-diluted pineapple juice filtered by a muslin cloth (Babu et al., 2006), also obtaining a decrease in transmembrane flux after several hours, suggesting that the rapid decline in transmembrane flux observed in the present experiment would result from other causes rather than the dilution. Even though a 200 μm pore size filter was employed to achieve the FSBJ used in the present experiment, the filter did not retain the liposoluble fraction, which consists of larger and heavier molecules than the hydrosoluble fraction. The lipophilic fraction of the juice also displays different viscosity and density values, which makes it prone to be retained in the surface of the membrane (Chun et al., 2017). The molecules of the lipidic fraction rapidly aggregated together to form an obstructive layer on the surface of the membrane, which was visually appreciable, therefore increasing the transmembrane pressure and reducing the transmembrane flux considerably over the first 30 min (Figure 13).

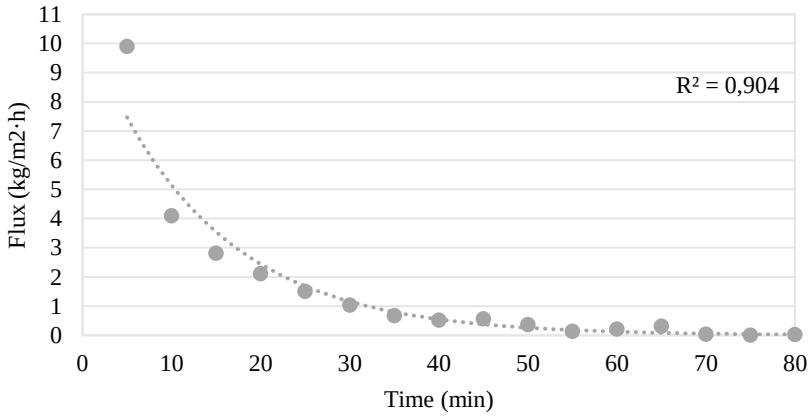

Figure 13. Transmembrane flux through the membrane as a function of time. Transmembrane flux was exponentially reduced during the first 30 min of processing.

A first experiment was performed with the juice filtered at 850 µm instead of 200 µm. The membrane fouling was then so severe that the concentrated did not reach the target value of 30 ºBrix. Filtering the juice at 200 µm achieved a successful concentration of SBJ to 30 ºBrix. The time needed to concentrate FSBJ to the target value was significantly lower when compared to the concentration of other fruit juices by the same technique, reducing the concentration-time by 90% (Table 3). Nonetheless, the reduction of the concentration-time was made as evident as the rapid fouling of the membrane, with a 90% reduction of the transmembrane flux over the first 30 min of processing. Moreover, membrane washing was also complicated due to retention of lipid components, made evident by its color.

In terms of ºBrix, concentration of FSBJ by FO technology could be a good choice, since the target value could be achieved in about 60 min of total processing time. The fact that no temperature is used over the experimental

procedure also becomes an advantage against other temperature-dependent techniques such as evaporation. Nevertheless, membrane fouling is a major disadvantage when concentrating FSBJ, made evident by the results of the transmembrane flux over time in the present experiment. Washing of the membrane also becomes difficult and may suppose a major cost due to its subsequent limited re-usability. Filtering the juice using a smaller pore-size filter may be useful to reduce the suspended solids from the lipidic part of the juice, therefore reducing membrane fouling and extending its shelf-life.

6.3.1.3. Cryoconcentration

Block-freeze centrifugal-assisted cryoconcentration: first experiment

FSBJ was unsuccessfully concentrated by block freeze concentration technology. The variety from Romania (*Hippophae rhamnoides* spp. *caucasica*) was used for the first exploration. Results from initial trials using the same variety led to the imperative use of FSBJ rather than on raw SBJ. Like what was observed in FO technology, block-freeze concentration was heavily affected by the high presence of solids in non-filtered SBJ. Since heat transfer occurred unidirectionally, the process of reaching freezing temperatures was more slowly and phase separation occurred (Figure 14). Filtering SBJ at 200 μm overcame this problem and allowed for the application of this technique.

Figure 14. Phase separation of non-filtered SBJ after 24h of freezing with the styrofoam jacket (as it is schematically represented in Figure 5).

The initial measure of FSBJ was 9 ˚Brix, increasing to a total of 15 ˚Brix at the end of the three cryoconcentration cycles. There was an expected rise in ºBrix from the raw juice to the first cryoconcentration cycle (Figure 15). However, the second and third cryoconcentration cycles did not give the expected values, contrarily to what other authors found in blueberry and pineapple juices (Orellana-Palma et al., 2017; Guillermo Petzold et al., 2015). Maximum values of Brix degrees in FSBJ were around 15 (Figure 15) whereas Guillermo Petzold et al. (2015) achieved values as high as 30 ˚Brix in three cryoconcentration cycles with pineapple juice.

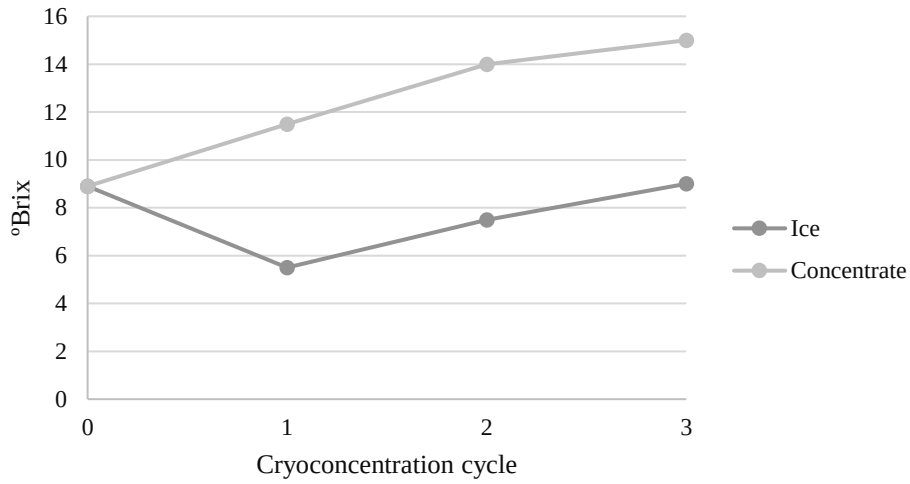

Figure 15. Progression in concentration in terms of TSS (measured as ˚Brix) against the cryoconcentration cycle. Each concentration cycle was performed with the concentrated product of the previous cycle.

By filtering SBJ, particles of more than 200 µm were retained in the filter, avoiding them to interfere during cryoconcentration. However, some particles passed through the 200 µm filter and remained in the juice. Most of the particles remaining in the juice are important to achieve a concentrated product with a greater nutritional profile. These particles are mainly constituted of lipophilic compounds and may be one of the problems when concentrating the juice by block freezing centrifugal-assisted cryoconcentration. During block freezing, these particles remain in the ice matrix and formed a vitreous paste, which differs from a normal ice block, and it did not allow the efficient separation of solutes from the ice. Thus, the block remains as an entire fraction with lots of solids in it, and the concentrate only gains a small fraction of these solutes (Figure 15).

Block-freeze centrifugal-assisted cryoconcentration: second experiment

The first experiment of block-freeze centrifugal-assisted cryoconcentration called in question the usability of FSBJ for block-freeze cryoconcentration technology due to its high presence of lipophilic compounds. Even when filtered at 200 µm – a solution that worked for the other applied concentration techniques –, the still high content of TSS in sea buckthorn juice made it unsuitable for the technique, for the formed ice block did not allow for an adequate separation of the concentrate. A subsequent experiment was planned to test whether the cryoconcentration of SBJ was not possible because of the TSS in the FSBJ or because of other components present in the matrix. The second experiment was run with clarified SBJ (CSBJ).

CSBJ was concentrated using the same methodology. The first three cryoconcentration cycles were especially interesting, since previous results suggested the impossibility of concentrating SBJ to more than 15 ºBrix using this technology. The results obtained indicated that it did not matter whether block cryoconcentration was applied to FSBJ or CSBJ; the ºBrix were very similar using either one matrix or the other (Figure 16). Therefore, there should be more factors other than the solids present in SBJ that may influence the low concentration rate after applying only three cryoconcentration cycles. The methodology herein applied involved the

separation of concentrate from the ice fraction by gravitational force. Yet other authors used vacuum force to aid in the separation of the concentrate from the ice in other food matrices (Orellana-Palma et al., 2017). The use of vacuum in this step may help recover more concentrate from the ice and therefore obtain higher values of ºBrix after only three cryoconcentration cycles. The melting of the ice block when using gravitational force limits the quality of the concentrated product that could be recovered, a problem that could be overcome by aiding the process with a vacuum pump.

After observing similar results from FSBJ and CSBJ, the CSBJ was subsequently submitted to more cryoconcentration cycles to further explore its behavior using this technology with the application of gravitational force during the separation of the concentrate (Figure 16).

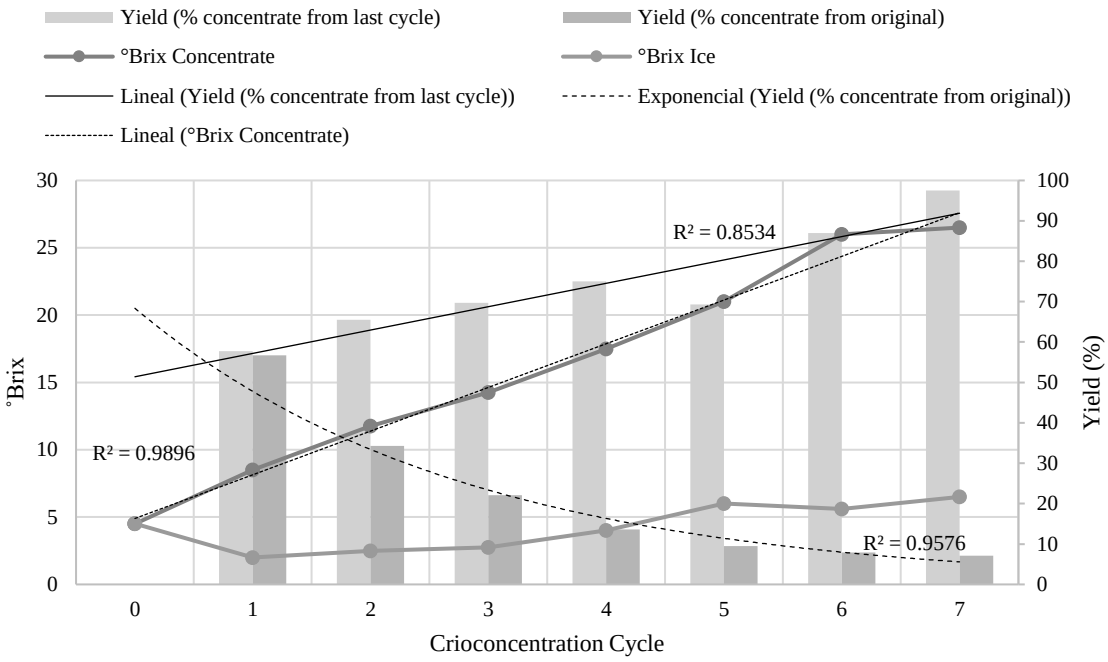

Figure 16. Evolution of °Brix and yield over different cryoconcentration cycles during block-freeze centrifugal-assisted cryoconcentration applied to CSBJ.

Block-freeze centrifugal-assisted cryoconcentration of CSBJ displayed a linear tendency in terms of ºBrix (R^2 = 0.990) after 7 cycles of cryoconcentration. The linear tendency was evident after 6 cryoconcentration cycles, reaching values of 26 ºBrix. Nonetheless, performing a seventh cryoconcentration cycle derived into a stabilization of the ºBrix, interrupting the observed linearity. Interestingly, the more cryoconcentration cycles were applied, the greater the yield from the last cycle (Figure 16). Nevertheless, this did not imply a greater concentration in terms of ºBrix. After values of 90% of yield from the last cycle were achieved, the ºBrix and the concentration yield from the original did not

increase. Petzold et al. (2015) also achieved similar values, obtaining an increase in yield from the last cycle of around 20% in the case of blueberry and pineapple juice. This effect could be explained by the increased viscosity derived from increasing the solute concentration after each cryoconcentration cycle (Welti-Chanes, Bermúdez, Valdez-Fragoso, Mújica-Paz, & Alzamora, 2004), which in turn makes more difficult the separation of the ice block after centrifuging. As it can be observed in Figure 16, the seventh cryoconcentration cycle derived into a slight increase of 0.5 ºBrix (from 26 to 26.5 ºBrix) and an increase in the yield from the last cycle of about 10% (from 87 to 97.5%). The separation of the ice crystal after centrifugation was easy as the thin crystal derived from the seventh cryoconcentration cycle occupied only 2.5% of the total volume obtained from the sixth cryoconcentration cycle. An eighth cryoconcentration cycle was carried out to double check this effect, considering this would be the highest ºBrix value achieved after the block-freeze cryoconcentration of CSBJ. The ice block formed overnight melted completely during centrifugation and therefore, the yield from the last cycle reached 100% and the ºBrix did not increase.

Using the present experimental conditions, block-freeze centrifugal-assisted cryoconcentration of CSBJ could not achieve values higher than 26.5 ºBrix. The concentration of CSBJ was linear over all the cryoconcentration cycles, leading to what was observed to derive into an

exponential curve when the solution achieved saturation values of 26 ºBrix. CSBJ could be concentrated to these values with higher number of cycles of cryoconcentration compared to previous research (Orellana-Palma et al., 2017; Orellana-Palma, Petzold, Torres, & Aguilera, 2018; Petzold & Aguilera, 2013; Guillermo Petzold et al., 2015). Block-freeze centrifugal-assisted cryoconcentration did not reach the established concentration value of 30 ºBrix and therefore the methodology was ruled-out from the effective SBJ concentration technologies list. Furthermore, even though the CSBJ behaved like the unclarified FSBJ in the first three cryoconcentration cycles, there was the need of using at least 6 cryoconcentration cycles to get values close to 30 ºBrix.

Block-freezing is a non-continuous cryoconcentration methodology, so concentration must be performed in batches. This increases the processing time and reduces the efficiency of the process considerably, leading to a clear problem when scaling-up the process. Finally, block-freeze cryoconcentration could be very expensive at high volumes, since the energy needed to reduce the temperature of greater volumes is larger than lower volumes. An alternative to this excessive cost would be splitting the batch to smaller volumes, yet again the solution would translate into greater processing times and lower efficiency of the process.

Suspension crystallization cryoconcentration

FSBJ was concentrated by suspension crystallization (SC) to a total of 20 ºBrix using only two cycles. The variety from Latvia (variety 'Tatjana') was used for the concentration in suspension crystallization. The fact that only two cycles were enough to concentrate to 20 ºBrix indicated that the concentration of FSBJ could be easier when using another technique rather than block-freeze cryoconcentration. Block-freeze cryoconcentration of CSBJ achieved and surpassed 20 ºBrix after 5 cycles of processing (Figure 16). It is important to note that during cryoconcentration, two different varieties were used for SBJ, accounting for the observable difference in the TSS from the original matrix (4.5 ºBrix in block-freeze cryoconcentration compared to 10 ºBrix in suspension crystallization) and for the possible differences during cryoconcentration. As it had been disclosed in Chapter 4, the variety 'Tatjana' from Latvia had a higher water content when compared to all other varieties, including that purchased from Romania (spp. *caucasica*). The higher water content would be beneficial for the application of freeze concentration technology, as the TSS could be more dispersed, therefore facilitating the process of the formation of ice crystals (Aider & de Halleux, 2009).

Similar yield values (% from the original juice) were obtained in block-freeze concentration when compared to suspension crystallization. When

comparing the two first cycles of cryoconcentration – performed by suspension crystallization –, the first cycle achieved an average yield of 59.80% from the raw juice (Table 4), a similar value compared to block-freeze cryoconcentration (56.72%, Figure 16). The second cycle led to values of 39.29% by suspension crystallization and values of 34.27% by using block-freeze concentration. Although the values were similar, the difference in the concentration in terms of ºBrix was evident in the second cycle, block-freeze concentration obtaining values of around 12 ºBrix whereas the concentration by suspension crystallization led to a concentrate of 20 ºBrix. This clear difference in ºBrix could help illustrate the efficiency of suspension crystallization, extracting more concentrate and more concentrated in terms of ºBrix. However, since the experiment was executed only once, the differences were non-significant, only observational. Besides, the difference in TSS from the original juice should always be present when comparing these two techniques.

Table 4. Concentration yield over different suspension crystallization cryoconcentration cycles.

Cryoconcentration cycle	Sample	Original volume (L)	Concentrate volume (L)	DM original (%)	DM concentrated (%)	Yield (% from the original)
	A	3.25	2.00	13.08	16.31	61.54
First	B	3.26	2.00	13.08	16.84	61.35
	C	0.92	0.52	13.06	19.26	56.52
Second	N/A	4.2	1.65	16.88	21.28	39.29

DM (%): g dry matter / 100 g concentrate. N/A: Not applicable. Only one sample was used in the second cycle

When the cryoconcentration technique consists of creating loads of ice crystal nuclei – which is the case in suspension crystallization –, there exists a risk of Ostwald ripening. The ripening happens when, at a certain point, ice crystal nuclei begin to merge, forming larger ice crystals and subsequently making difficult the adequate extraction of the concentrate from the ice. This is the main reason why suspension crystallization is facing difficulties in the food industry (Sánchez, Ruiz, Raventós, Auleda, & Hernández, 2010). Despite this fact, the suspension crystallization technique is the most used technique nowadays in the food industry, due to its constant optimization (Sánchez, Ruiz, Auleda, Hernández, & Raventós, 2009).

Suspension crystallization had been previously used in apple juice concentration with great results. The concentration of apple juice reached

35 ºBrix after three cryoconcentration cycles (Ding et al., 2019), showing a good separation of the concentrate from the ice and an adequate ice crystallization when compared to a saturated sucrose solution. Although the apple juice was more concentrated every cycle, the concentration ratio was lower. This probably derived from the crystallization inhibition of solute content during Ostwald ripening (Ding et al., 2019).

Results from the present study suggest a tendency with a lower slope (Figure 17). FSBJ was cryoconcentrated using suspension crystallization technology, yet after two cryoconcentration cycles only 20 ºBrix were achieved (Figure 17), differing to what was reported by Ding et al. (2019) (26 ºBrix achieved after two cryoconcentration cycles). Since there was a limitation in original juice volume, the juice was only submitted to two cryoconcentration cycles. Nonetheless, the values of the third cryoconcentration cycle could be hypothesized.

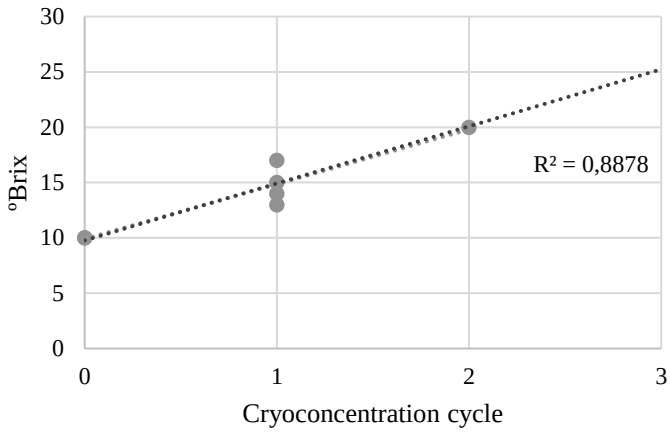

Figure 17. Cryoconcentration of FSBJ over different cryoconcentration cycles using suspension crystallization. Note that only two cryoconcentration cycles were performed. The value from the third cryoconcentration cycle was calculated through the tendency from the first two cryoconcentration cycles.

As shown in Figure 17 and according to what was observed during the first two cryoconcentration cycles, performing a third cycle would help concentrate the FSBJ to 25 ºBrix, a closer value to the target 30 ºBrix value. It would require up to five cycles to concentrate SBJ to more than 30 ºBrix (target value), considering that each concentration cycle would follow the same concentration tendency. The low slope in the curve may be derived from the higher soluble solids present in the juice, since the lipidic fraction was left in the juice before processing due to its nutritional interests. It would be possible that clarifying SBJ would lead to a concentration curve with much higher slope, similar to the concentration tendency that other authors previously observed (Ding et al., 2019).

FSBJ did not present the same difficulties of concentration using suspension crystallization when compared to block-freeze concentration, probably due to the high total soluble solids suspended in the raw matrix. The fact that the ice crystals are much smaller during suspension crystallization may be critical for the development of ice crystals with lower amounts of total solids. Even though the cryoconcentration by suspension crystallization did not reach the target value, the tendency of concentrated FSBJ herein depicted shows promising results, suggesting a concentration to the target value of 30 ºBrix after 4 cryoconcentration cycles. The fact that temperatures below the freezing point of the pure water are used during cryoconcentration becomes a greater advantage than using other techniques which operate at room temperature (e.g. FO). Nonetheless, the solids present in the matrix could be a problem when concentrating at high TSS values. In addition, using smaller pore-size filters (< 200 μm) prior to cryoconcentration may help reduce the greater particles present in the juice and thus optimize the process.

6.3.2. Phytochemical analysis of concentrated FSBJ

Samples were collected from all the technologies that allowed for a full concentration of FSBJ to 30 ºBrix. In addition, samples from the suspension crystallization cryoconcentration were also taken since the limiting factor in that case was only the volume of the original juice rather than the

possibility of reaching the target Brix value. Whereas it could not be assumed that the FSBJ would reach the target ºBrix value (although it had been hypothesized in the previous section), it could give an overview on the quality of the product concentrated at 20 ºBrix.

Moreover, since the disposal of samples from cryoconcentration was wide, every analysis was run in each sample from all cycles. This would give different values of which would be plausible to calculate the tendency, leading to a hypothetical value at 30 ºBrix. The hypothetical value was subsequently contrasted to the previous value obtained at 20 ºBrix and other values from other concentration techniques. This was nothing more than a hypothetical value but served as likely-to-achieve value when concentrating FSBJ by suspension crystallization.

6.3.2.1. Total chloride

Total chloride is an important factor when concentrating liquid foods by FO, as usually the extraction solution contains chloride salts that could migrate to the concentrated product (Sant'Anna, Marczak, & Tessaro, 2012). This was the case of the FO system used in the present experiment. The total chloride content was significantly different when comparing the results from all concentrated products, $F = 2649$ at $p<.05$. The salt migration from the extraction solution to the concentrated SBJ was evident, as the concentrated product showed a difference of more than 200 mg of

chloride ions per liter when compared to the extraction using evaporation (Figure 18). It is noteworthy to mention that values of dry matter of concentrated SBJ by FO were lower than values of concentrated SBJ by evaporation (38.57 and 43.80 g of dry matter / 100 g of product, respectively), which would translate in much higher difference when corrected by dry matter, making more evident this difference.

The results show that the results from SBJ cryoconcentrated to 20 ºBrix led to a product with almost half of the total chloride content when compared to values obtained after concentrating SBJ by evaporation (Figure 18). Nevertheless, the cryoconcentrated product reached values of dry matter of 21.28 g / 100 g of product, also half of the content when compared to that obtained from evaporation of SBJ, which would explain the difference in the total chloride content. The increase in total chloride content related to the increase in the dry matter could be also seen when comparing the cryoconcentrated SBJ to the original juice, with the former having doubled the content of total chloride as well as the content in dry matter.

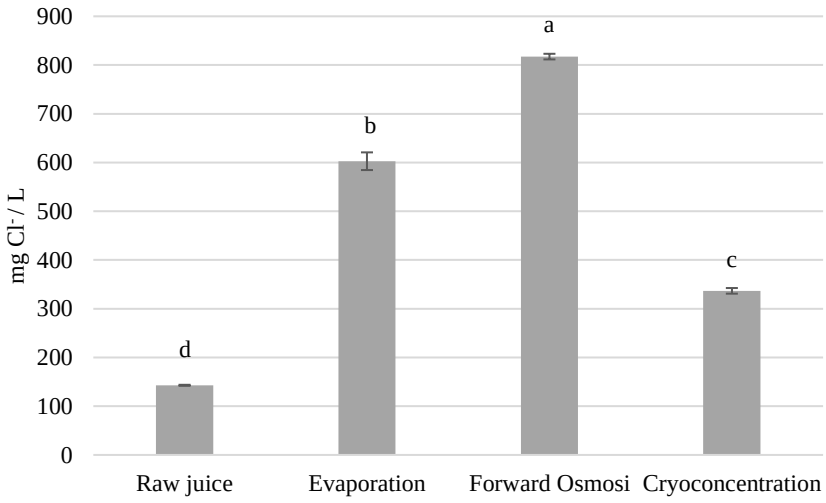

Figure 18. Chloride concentration in raw and concentrated SBJ by different techniques.

To resolve the problem that migration could pose in the concentration of fruit juices (such as SBJ), some authors suggest the combination of sodium chloride (in that case would be magnesium chloride) with sucrose to overcome the drawback of either sucrose or sodium chloride (Babu et al., 2006), or even the use of sodium or potassium bicarbonate as the drawback of bicarbonate would not affect the original matrix's flavor significantly (Achilli, Cath, & Childress, 2010). However, the difference in total chloride when comparing SBJ concentrated by FO when compared to all other techniques may not be so critical regarding the flavor of the juice, since the matrix has already been documented to have a very strong flavor, including astringency and sourness (Chapter 1, 7), which could hinder the taste occasioned from salt migration (Wrolstad et al., 1993).

6.3.2.2. Vitamin C

Total vitamin C was the most important parameter studied in the concentrated SBJ by different techniques. Vitamin C is one – if not the most – important bioactive compound found in the aqueous part of the juice (Chapter 1). Thereupon, it was of utmost importance to keep the total vitamin C content of the concentrated juice as high as possible. In addition, vitamin C would be an ideal indicator to understand the possible impact of the concentration process in other bioactive compounds present in the juice (e.g. carotenoids from the lipidic part of the juice).

Total vitamin C was calculated as the percentage of vitamin C lost after processing. Dry matter of the original and the concentrated products was used to calculate the real loss of vitamin C during processing. Percentage of lost vitamin C was [redacted] across the techniques used in the present study (Figure 19), [redacted]. Tukey's *post hoc* test showed [redacted]. The use of heat to concentrate SBJ [redacted] (Figure 19). The thermolability of vitamin C is well known and had been previously documented in sea buckthorn juice as well (Gutzeit et al., 2008), with great losses observed after storing the juice during 7 days at 6, 25 and 40 ºC, with observed halved values at the higher storage temperatures when compared

to the lower ones. Interestingly, Guillermo Petzold, Orellana, Moreno, & Valeria (2019) recently investigated and compared the impact of cryoconcentration and evaporation on orange juice, and found total vitamin C content reduced by almost 40%, being higher when using cryoconcentration. (Petzold et al., 2019). Herein the concentration of vitamin C in concentrated SBJ [redacted] (Figure 19). The greater difference herein observed may account for the 10 ºBrix of difference when comparing both concentrates. Besides, the use of block freeze concentration by Petzold et al. (2019), as compared to the concentration by suspension crystallization used in the present experiment, [redacted]. Nonetheless, it was clear that the use of heat to concentrate SBJ [redacted]

The percentage of vitamin C [redacted]. The use of temperature [redacted]. Although the processing time was three times higher in evaporation when compared to FO, the processing pressure was five times lower when concentrating by evaporation. [redacted]

[redacted]

[redacted] Previous work by Gutzeit et al., (2008) reported a significant loss of vitamin C of more than 50% when concentrating SBJ by using a five-stages evaporator working from 80 to 85 ºC.

Using other filtration technologies [redacted] showed minimal losses of vitamin C from SBJ (Vincze et al., 2007). The authors suggested the reduction of temperature during processing to help maintain the high concentration values of vitamin C. [redacted]

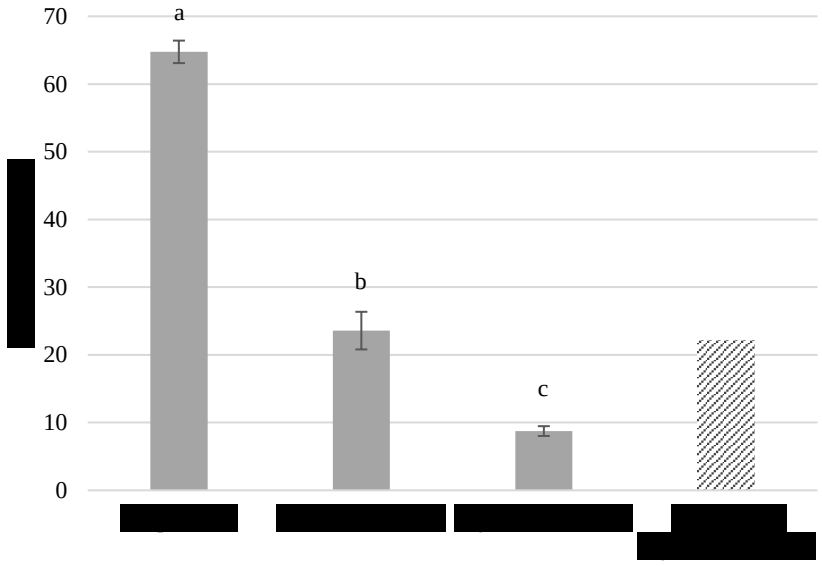

Figure 19.

, since it was the only technology which did not reach 30 g dry matter per 100 g concentrated product (approximately 30 ºBrix). In other words, it would be interesting to investigate whether reaching the concentration target value

The cryoconcentration by suspension crystallization is a technique which allows the user to work in a continuous flow. Nevertheless, the lab-scale experiment was performed in different cycles, just as the block-freeze cryoconcentration technique. This would allow quantifying the vitamin C content and dry matter after two different working cycles (only two were applied), and then estimate the content in future cycles. [redacted] Figure 20 shows the real vitamin C content after 0, 1 and 2 cycles, [redacted] It is important to note that the standard deviation for the first calculation [redacted] the standard deviation was quite high, limiting the use of this theoretical approach to draw reliable conclusions. These values derive from the double quantification of vitamin C and more specifically due to the oxidation of the vitamin in the HPLC carrousel. Nevertheless, a tendency could be and was calculated.

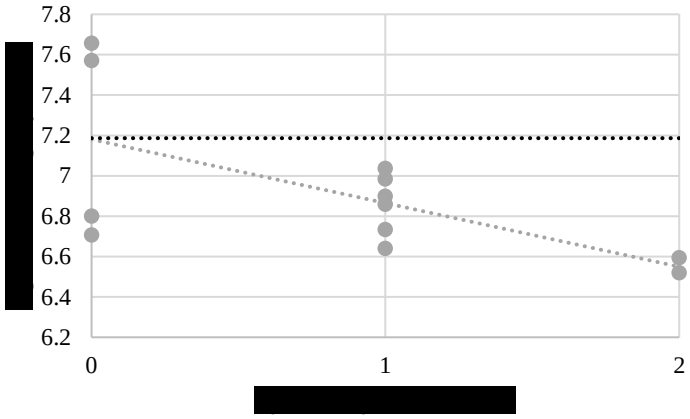

Figure 20.

A total of five cycles would be necessary to concentrate SBJ to the target concentration of 30 ºBrix. represented in Figure 19. Following the tendency represented in Figure 20, the concentrated product would achieve dry matter values of 33.96 g dry matter / 100 g of concentrated product (after five cycles) , which again supports previous findings in orange juice (Petzold et al., 2019). previously reported in apple juice after concentrating by suspension crystallization when compared to the use of heat (Ding et al., 2019).

[redacted]. The pressure and time of concentration by evaporation could explain [redacted] since the evaporation occurred at 0.01 MPa and 60 min and achieved similar dry matter values. It is also important to note when comparing both works, that working with apple juice makes the concentration process [redacted] since the solutes contained in the juice are much lower compared to SBJ.

6.3.2.3. Total polyphenols

Total phenolic content was also quantified in the concentrated SBJ. Total polyphenols also constitute an important fraction of SBJ (Cioroi, Chiriac, & Stefan, 2017; Mendelová, Mendel, Czako, & Mareček, 2016) and may also be important contributors to the total antioxidant capacity of the juice (Gutzeit et al., 2008). It was therefore important to understand the effects of concentration processing to the overall polyphenolic content.

The concentration of polyphenols [redacted] (Figure 21), [redacted]. Results from the literature suggest that thermal degradation of polyphenols occurs at least at 60 ºC for several polyphenols, being severe at shorter times and higher temperatures (Bakowska-Barczak & Kolodziejczyk, 2011; Volf, Ignat, Neamtu, & Popa, 2014). In addition, greater stability of phenolic

compounds was observed in tea polyphenols stored at 25 ºC when compared to storage at 100 ºC after only 24 hours (Zeng, Ma, Li, & Luo, 2017). For some studied polyphenols (e.g. catechin) the thermal stability time was even shorter – reduction in its concentration after only one hour of storage. Therefore, using high temperatures to concentrate FSBJ, lower concentration of phenolic compounds was expected when compared to other non-thermal techniques such as FO. [redacted]. In addition, the concentration of FSBJ to the target 30 ºBrix led to differences in dry matter content when comparing both techniques (see 6.3.1. Feasibility of the process), [redacted]. High molecular weight compounds may be trapped within the porous structure of the osmotic membrane (as explained before), [redacted]. Even though the phenolic compounds may be found in free and bound form (e.g. glycosides), it could be possible that, due to the high

fouling observed during the concentration of FSBJ, [redacted] other authors previously reported (Nayak & Rastogi, 2010).

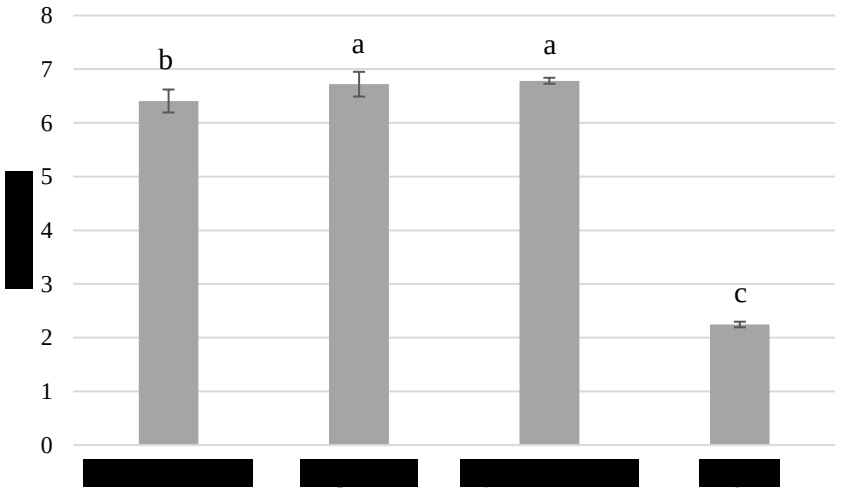

Figure 21. [redacted]

To the best of my knowledge, cryoconcentration processes had never been previously applied to FSBJ and therefore the impact of the process on the total phenolic content had never been investigated before. [redacted] (Figure 21). It should be noted that the concentration by cryo-technology

was only possible until 20 ºBrix, differing from the 30 ºBrix obtained after evaporation. [redacted] reported very high polyphenolic retention (best retention results of 98.4% from the original juice) when applying cryoconcentration to blueberry juice (Orellana-Palma et al., 2017), [redacted]. Over all different types of polyphenols and their possible bounding with other molecules, rutinoside and rhamnoside glycosylated polyphenols were identified as being the most relevant polyphenols present in sea buckthorn juice (Chen, Zhang, Xiao, Yong, & Bai, 2007). Rutinoside and glucoside were some of the flavonols that showed an increase in their concentration after applying freeze-concentration of the must as a step previous to its fermentation (Wu et al., 2017). Not only this but freeze concentration as intermediate step to winemaking also increased the concentration of anthocyanins considerably.

[redacted]

6.3.2.4. Antioxidant capacity

The quantification of vitamin C or total phenolic content may be used to understand the effect of concentration processes on these specific compounds. In addition, vitamin C and phenolic compounds are two important contributors to the antioxidant activity of fruit juices (Dudonné, Vitrac, Coutiére, Woillez, & Mérillon, 2009; Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Hawkins Byrne, 2006). Besides these two contributors, other compounds may also significantly contribute to the antioxidant capacity of the juice, such as carotenoids or tocopherols. Although it may be more difficult, the concentration of FSBJ instead of clarified SBJ would result in a final product with a great variety of bioactive compounds, since the oily fraction still constituted an important part of the juice. It was therefore interesting to investigate the effect of the concentration on the antioxidant capacity of the resulting juice.

Table 5 shows [redacted]. Statistical analysis suggested that [redacted]

[redacted]. The concentration of FSBJ led to [redacted]. In other words, [redacted].

Table 5. Antioxidant capacity expressed by means of DPPH and FRAP.

Concentration technique	**Antioxidant capacity assay (µmol TE/ L) ***	
	DPPH	FRAP
Evaporation	[redacted]	[redacted]
FO	[redacted]	[redacted]
Cryoconcentration	[redacted]	[redacted]
Original SBJ	[redacted]	[redacted]

*The statistical analysis (ANOVA) was performed for each antioxidant assay

The total antioxidant capacity by DPPH assay gave interesting results. [redacted]

[redacted] (Figure 19). [redacted]. The antioxidant activity was precisely measured by two different techniques to understand any possible differences that may emerge. In matrices where polyphenols are the major source reducing agents, they could significantly influence the antioxidant capacity when using either DPPH, FRAP or other antioxidant assays (Dudonné et al., 2009). [redacted]. Results from DPPH assay would strongly be influenced by the amount of polyphenols in the matrix (Danilewicz, 2015), whereas the FRAP assay would be strongly influenced by the amount of vitamin C in the product (Benzie & Szeto, 1999; Contreras-Calderón, Calderón-Jaimes, Guerra-Hernández, & García-Villanova, 2011). This could be partly explained by the greater ability of phenolic compounds to neutralize oxidative damaging compounds such as DPPH, compared to the same ability of vitamin C. [redacted] (Figure 21). [redacted]

[redacted] (Figure 21). [redacted]

The information acquired by the total antioxidant activity by FRAP assay showed consistency with the theoretical approach that different bioactive compounds may contribute in a different manner depending on the antioxidant assay performed. [redacted] (Figure 19), thus suggesting [redacted]

[redacted]

[redacted] reported very high FRAP antioxidant activity in concentrated SBJ using membrane technology, which was clearly linked to a very high retention of vitamin C in the concentrate (Vincze et al., 2007). [redacted]

[redacted]

6.4. Conclusions

Raw, FSBJ (200 μm) was used as the object of concentration instead of clarified juice in order to maintain as much of bioactive compounds as possible in the concentrated product. FSBJ can be successfully concentrated by evaporation and forward osmosis to 30 ºBrix. Concentration to 20 ºBrix was also possible using suspension crystallization cryoconcentration, showing potential for further concentration.

Due to the very high presence of solids in FSBJ, advice should be given against the use of certain technologies for its concentration. Even though all the processes herein used were feasible, concentration by FO derives into a rapid fouling of the osmotic membrane and a subsequent retention of the compounds of interest, and concentration by freeze-concentration technologies pose difficulties on the efficiency of the process, as the oil of the juice may constitute an important barrier for the correct separation of the concentrated product.

Nutritionally speaking, the use of heat for the concentration of FSBJ [redacted]. Nevertheless, concentration by evaporation does not suppose [redacted] and therefore the antioxidant capacity of the concentrated [redacted]

Concentrated FSBJ can have many applications in the food supplement industry since it becomes an even more valuable food ingredient. Its subsequent use would be liquid or concentrated food supplements because spray drying of the concentrated FSBJ would be counterproductive. Due to the problems aroused by the high presence of solids in the working matrix, the concentration of FSBJ by non-thermal techniques would be costlier than concentrating by evaporation, [redacted].

[redacted].

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Chapter 7: Development of a sea buckthorn-based fruit juice

7.1. Introduction

Sea buckthorn was previously presented as a berry, consisting of different fractions with different nutritional profiles (Chapter 1). One of the most important fractions is its juice, for it contains high amounts of aqueous bioactive compounds such as vitamin C (around 1,000 mg/ 100 g of dry matter, Chapter 4), but also lipophilic compounds, such as carotenoids. In fact, carotenoids could be found in sea buckthorn juice (SBJ) at levels of 10.28 mg / 100 g of juice (Beveridge et al., 1999), which resemble those levels found in carrot juice (Chen et al., 1995). Together with the other bioactive compounds (including total polyphenols and vitamin C), they constitute a good raw matrix to work with. In terms of new research, bioprocesses have been applied in the present project to investigate possible beneficial changes of the juice (Chapter 8). In terms of using the original matrix, which is already highly valuable, concentration techniques have been used to investigate the behavior of the juice (Chapter 6).

Companies are already marketing sea-buckthorn based products. The oil gets most of the attention of these companies, which use it for cosmetics and skin lotions, or as a food supplement (Chapter 5). Some companies also

sell and distribute sea buckthorn juice as raw juice, or as a multivitamin-shot product. Nevertheless, the product success is usually based upon the nutritional value of the product instead of considering its sensorial value. Only in the countries where sea buckthorn naturally grows (e.g. Finland, China), and it is usually marketed, would consumers buy it because of its flavor.

7.1.1. Juice consumption in Spain

Spain is a worldwide producer of fruits and vegetables because of its climate diversity and producing regions. The total volume of fresh fruits consumed during 2019 was about 6.47 M Tn, building up an increase of 1.4% when compared to the last year, and the vegetables constituted total consumption values around 2.6 M Tn, showing an increase of 0.9% (Ministerio de Agricultura Pesca y Alimentación, 2019). Spain is a great producer of peaches, apples and oranges (constituting 0.14, 0.44 and 0.75 M of Tn produced only in 2019, respectively) (Ministerio de Agricultura Pesca y Alimentación, 2019). In addition, according to a report developed in 2017 by the European Fruit Juice Association (AIJN), Spain was the 5th European country with the most consumption of juice per capita, making up a total of 808 M of liters (17.4 L per capita yearly). According to the same study, the juice taste is one of the most important factors for its ultimate choice, and orange juice is one of the best valued (36.5% in terms

of preference), followed by multifruit juices (19.2%) and apple juices (15.7%).

The juice market is constituted by fresh juice, concentrated juice, and nectars, among the commonly used categories of fruit juice. Spain's market juice is constituted by 46% of nectars, 24% of concentrated juice and 18.2% of freshly prepared and refrigerated juice (Ministerio de Agricultura Pesca y Alimentación, 2019), with a tendency to shift towards the concentrated and fresh juice due to the increasing consumer consciousness of the impact that nectars could pose on health. From all fruit juices, the multi-unit juice is one of the most consumed, as detailed before, probably due to the great concentration of different micronutrients or the complexity and exoticness of its flavor. Multifruit juices could be formulated to include exotic flavors, since the ultimate taste could be modeled by changing the fruit composition. One of the fruit juices which could benefit from this formulation would be SBJ.

7.1.2. Demarcation

The development of new products by food companies is needed to survive in a very competitive and global market. It is estimated that 90% of new products would last less than a year in the market and will be withdrawn because of (1) bad choice to evaluate if the product would be attractive for the consumer, (2) the development of too similar or too different products,

and (3) the insufficient knowledge of the target population for the developed product (Lesschaeve & Bruwer, 2010). In an attempt to launch a successful product, hedonic studies are vital to

investigate the sensorial properties and perceptions of the consumer towards the newly developed product, especially on fruit juices (Geertsen et al., 2016).

SBJ is sour and bitter, and it could be difficult to consume as it is (Geertsen et al., 2016), probably because of its low sugar/acid ratio (Ma et al., 2018). Its flavor becomes even more important when considering its exportation and marketing to another country where the plant does not grow, or it is not well-known. Spain is a good example. The integration of SBJ in a pre-existing strong market in Spain could lead to an unsuccessful project. In addition, in fruit juices, sweetness is one of the most important factors contributing to the acceptability of the flavor of sweet beverages (Tuorila-Ollikainen et al., 1984). The old way of sweetening a beverage or juice is to add sugars in any form (e.g. syrup, solid). However, consumers are every time more aware of what they eat, which in turn could influence their health, and sugars are on the target point.

Yet other strategies could be adapted to overcome the hedonic barrier that juices such as SBJ face on a developed fruit market. The use of a multifruit juice formulation could be the solution to that problem. The fact that

multifruit juices have several fruits in their ingredient's list is interesting, since then the product could be adapted when adding a new ingredient, such as is SBJ. The fruit juice could be formulated so that besides SBJ, other very sweet fruit juices were used as ingredients to mitigate the possible negative impact of SBJ consumption. This could reduce the use of sweeteners or other compounds usually included to improve the product's flavor.

It could be possible that the strategy of adding sweet fruit juices would not result in the desired acceptability outcome and therefore lead to failure after launching the product. It is therefore of utmost importance to develop and test a product using hedonic experiments with potential consumers.

7.1.3. Objective

The objective of the present work was to investigate the adequate proportions of SBJ that could be included in a newly formulated multi-fruit juice by elucidating its hedonic response with a representative sample of potential consumers. A subsequent objective involved the quantification of the bioactive compounds of interest (phytochemical profile) as well as the physic-chemical properties of the formulated juices.

7.2. Experimental design

A total of seven fruit juices were formulated in two different experimental designs. Each newly formulated fruit juice had different proportions of sea buckthorn juice. The first experimental aimed at producing a multi-fruit juice containing the percentage of sea buckthorn juice that was stated to be optimal for its acceptability in previous research. The outcome of the first experimental setup help to understand the potential of the resulting product to be reformulated in order to maximize the industrial efficiency and the resources and costs of production. This was important considering that the sponsoring company owns an orchard of sea buckthorn berries, as detailed in Chapter 4. Thereupon, the first formulation was only exploratory, whereas the second formulation was attempted at elucidating the impact of a finished product.

7.2.1. SBJ extraction

SBJ was extracted from the berries using a worm-drive extruder located at the Food Technology Plant Service (SPTA) of Universitat Autònoma de Barcelona (UAB, Bellaterra, Spain). In order to reduce the particle size of the juice and thus to avoid undesired retention of compounds during filtering, SBJ was further ground with a Krups conventional blender (Solingen, Germany) at maximum power for 60 s to reduce the particle size of the solids within. Thereafter, SBJ was filtered through an 850 μm pore

size filter, to obtain the clear sea buckthorn juice used for the experiments. SBJ was then stored under freezing conditions at -30 ºC until usage (not more than a week after extraction). SBJ was thawed overnight at 4 ºC and batch-pasteurized at 63 ºC for 30 min the day prior to its use.

For the first experimental formulation, sea buckthorn juice was obtained from berries from the 'Tatjana' variety (a crossed variety with genes from different subspecies, namely *mongolica*, *rhamnoides* and *fluviatilis*). The berries were purchased from BRUwell, SIA, a local harvesting firm located in western Latvia. For the second experimental formulation, sea buckthorn juice was obtained from two different origins: the 'Tatjana' variety from Latvia, and the 'Tatjana' variety from the orchard located at Bellver de Cerdanya (Catalunya, Spain), owned and managed by Vitae Health Innovation S.L. The harvesting year of the berry from Latvia was 2018 whereas the harvest of 2019 was used from the berry cultivated in the Catalan region. The berries were kept at -30 ºC until the juice was formulated and produced.

7.2.2. Juice formulations

7.2.2.1. First experimental formulation

In the first experiment, a percentage of 10, 20 and 30% of SBJ in the final product were tested, since it was previously studied to be percentages

achieving good acceptability of newly developed sea buckthorn-based beverages (Geertsen et al., 2016). Peach, apple and grapefruit juice were selected because of its wide presence in the Spanish market (as indicated in section 7.1.1. Juice consumption in Spain) and because of the sweetness they could incorporate into the final juice. Pomegranate was added due to its great antioxidant capacity (Gil et al., 2000). Carrot juice was added to bring more orange color, in case the addition of pomegranate juice caused a deviation in the original color of the fruit juice, which was considered to be an important attribute. Finally, a small fraction of passion fruit was added in an attempt to make the newly developed fruit juice more exotic and closer to the public, since the flavor of passion fruit is already well known in Spain. The percentage of each fruit was maintained proportionally over the different concentrations of SBJ. The percentage of the used base-fruit juice is represented in Table 1. Pasteurized peach, grapefruit and carrot juice from the brand 'Delizum' were purchased at the online shop 'Planeta Huerto' (Valencia, Spain). Pasteurized apple and pomegranate juices were purchased at 'El Corte Inglés' and were from the same brand, and passion fruit reconstituble puree (reconstitution was performed adding 50% of water) from the brand 'Vaper' was purchased at a local shop from Barcelona, Spain. None of the purchased juices contained added sugars.

Table 1. Percentage of the base-fruit juice used in the first experiment prior to the addition of different percentages of SBJ

Fruit/ Vegetable juice	Percentage in the mixture
Peach	34.6
Apple	32.1
Grapefruit	18.5
Pomegranate	8.6
Carrot	3.7
Passion fruit	2.5

7.2.2.2. Second experimental condition

The first experiment provided solid evidence to back up a second experiment, consisting of a new formulation with a greater percentage of SBJ (7.3.1.3. Hedonic analysis). Thereupon, the new experiment would test higher percentages of SBJ in the final product, maximizing the use of the juice by using fewer resources (since the SBJ would be provided from the orchard located in 'Bellver de Cerdanya', Spain). The percentages were set at 50 and 80% of SBJ. The second experimental set up included two SBJ varieties, making a total of four samples to test, differing from the only three tested in the former experiment. The use of two different varieties aimed at understanding the possible effect that they could have on the final

juice flavor and acceptance, since different varieties have been shown to have a completely different physic-chemical profile (Chapter 4).

The second experiment would also benefit from the observations made evident during the first experiment. Major changes were applied to the former formulation from the first experiment. As the color was an important attribute greatly affected by pomegranate juice addition, this was removed from the formulation. Carrot juice was removed to ultimately build up a fruit juice rather than a vegetable and fruit juice. Passion fruit juice was also removed due to the possible sourness additionally brought by its addition. In the second experiment, greater emphasis was put on the sweetness of the final juice. To that end, mango juice was added to the mixture. The constituents of the base-fruit juice for the addition of different percentages of SBJ is depicted in Table 2. All juices used during the second experimental condition – except SBJ – came from 'Delizum' brand without sugars added and were purchased at the online shop 'Planeta Huerto' (Valencia, Spain).

Table 2. Percentage of each fruit juice constituting the base-fruit juice mixture used in the second experiment prior to the addition of different percentages of SBJ

Fruit juice	Percentage in the mixture
Mango	34
Peach	27.5
Grapefruit	22
Apple	16.5

7.2.3. Juice preparation

All juices were prepared following the same algorithm. Briefly, extracted and pasteurized SBJ was mixed with the base-fruit juice mixture at the proportions established for each experimental condition (Table 3). The final fruit juice was stored at 4 ºC for its use the following day.

Table 3. Percentages of the developed fruit juices in each experimental condition

Fruit juice	First experimental			Second experimental*			
	1	2	3	1	2	3	4
Sea buckthorn	10	20	30	50	80	50	80
Mango	-	-	-	17	6.8	17	6.8
Peach	31.1	27.7	24.2	13.75	5.5	13.75	5.5
Apple	28.9	25.7	22.5	8.25	3.3	8.25	3.3
Grapefruit	16.7	14.8	13.0	11	4.4	11	4.4
Pomegranate	7.8	6.9	6.0	-	-	-	-
Carrot	3.3	2.9	2.6	-	-	-	-
Passion fruit	2.2	2.0	1.7	-	-	-	-

*Formulations 1 and 2 were produced with the variety 'Tatjana' from Cerdanya (Spain), and formulations 3 and 4 from the same variety from Latvia.

7.2.4. Sample analysis

Sample analysis was performed on the formulated sea buckthorn-based multi-fruit juice and therefore only those analysis related to the aqueous fraction – as detailed in Chapter 3 – were used in the present matrix. Total soluble solids (TSS; in terms of ºBrix), pH and color were the three physic-chemical parameters herein explored.

The phytochemical analysis of the sample included the quantification of total vitamin C and DPPH antioxidant capacity. Information on these

quantitative methodologies can be found in Chapter 3. Every sample analysis procedure was performed thrice, measurements by the HPLC methodology were performed in duplicate and antioxidant capacity reads were performed in triplicate. On the second experiment, only one extraction was performed for the DPPH assay and the vitamin C quantification, with two instrumental readings of each extraction.

7.2.4. Recruitment and hedonic evaluation

The purpose of the hedonic or sensorial analysis was to elucidate the acceptability of a newly developed sea buckthorn-based multi-fruit juice with a representative sample of potential consumers. The test was performed over two consecutive days in the Department of Animal and Food Science, at the Universitat Autònoma de Barcelona (Bellaterra, Spain).

The samples were taken out of the fridge at least two hours before the sensorial analysis. The samples were coded resulting in a single-blinded hedonic experiment. The samples were provided at once. Water was provided *ad libitum* together with guidelines to rinse the oral cavity gently after every tasting every sample. A total of eight sensory attributes were used in the analysis: general aspect, color and odor were the attributes used before the taste, and sweetness, sourness, astringency, bitterness and mouthfeel were the attributes evaluated after the volunteer tasted the juice.

The list of attributes was kept short to avoid potential consumer idiosyncrasy (Jaeger et al., 2015). A 7-level Likert-type scale was used on each attribute, 1-value indicating “extremely disliked” and 7-value indicating “strongly liked”, with a neutral value of 4. The hedonic evaluation of the fruit juices was performed for all the experimental designs in the same time span, with slight variations.

7.2.4.1. First experimental hedonic evaluation

In the first experimental design, a total of 16 volunteers were recruited for the sensorial analysis, which basically consisted of analyzing the indicated sensorial attributes of the three provided samples (fruit juices containing 10, 20 and 30% of SBJ). The analysis focused on the likeness of the attribute rather than an objective quantification of it. For instance, a high value on the 7-points Likert scale in sweetness indicated that the attribute was overall liked among the respondents rather than the formulated juice was highly sweet. The questionnaire included a blank spot after every attribute intentionally left for additional comments from the volunteers, which in turn would provide information for the likely subsequent reformulation.

7.2.4.2. Second experimental hedonic evaluation

In the second experimental design, a total of 30 volunteers were recruited for the sensorial analysis. The volunteers were asked to evaluate the sensory

attributes of the four provided juice samples (50% and 80% formulated with berries from Latvia and Cerdanya). Equally to the first experimental hedonic evaluation, the volunteers were asked to rate the personal liking of each attribute using the 7-points Likert scale.

7.2.5. Statistical Analysis

Data were statistically analyzed by IBM SPSS Statistics (IBM Corp., Armonk, US). An exploratory analysis was run on the data to check for normality and homogeneity of variances within groups. Normality was double-checked with a Q-Q plot of the residuals and the Shapiro-Wilk test. Homogeneity of variances was checked with Levene's test. A one-way ANOVA was performed for each dependent variable, when normality allowed for, and a non-parametric Kruskal-Wallis test was carried out when any of the assumptions of normality was violated. If the one-way ANOVA test gave statistical significance at $p<0.05$, a Bonferroni pairwise comparison was carried out to account for the increased type I error (which LSD does not take into consideration). When the Kruskal-Wallis test gave statistically significant results at $p<0.05$, the Mann-Whitney's test was carried out to further investigate possible differences between groups.

7.3. Results and discussion

7.3.1. First experimental

7.3.1.1. Physic-chemical parameters

The physic-chemical properties were measured not only on the newly developed fruit juices but on the pasteurized SBJ to be added to the mixture. The pH of the original SBJ was similar to that reviewed by Beveridge et al. (1999) (Table 4). The pasteurization did not influence the pH value of the SBJ. The addition of SBJ resulted in a significant reduction in the pH values of the final juice, F (4, 10) = 767.2 at p<.05. Any additional percentage of SBJ resulted in a statistically significant drop in the pH value of the resulting juice (Table 4). Important consideration should be made to that change since little differences in pH could be organoleptically detected (Amerine et al., 1965), especially in juices where malic acid is a predominant organic acid, which is the case of SBJ (Beveridge, Harrison, & Drover, 2002).

TSS is another important attribute of beverages. It contributes to the sweetness of the final product and had been previously identified as an important attribute that positively contributes to the taste acceptance of refreshing beverages (Tuorila-Ollikainen et al., 1984). The addition of SBJ had a significant effect on the overall TSS readings, F (4, 10) = 4.067E+37

at $p<.05$. A total of 2.5 ºBrix were reduced at the juice formulated with 30% SBJ when compared to the original fruit juice. However, it should be noted that the used SBJ had an intrinsic ºBrix value of 4.5 (Table 4), and it is a value strongly dependent on several factors, such as variety or plantation latitude and altitude, among many others. Other varieties analyzed during the present research project yielded out a juice with more than 6 ºBrix, others surpassed the 10 ºBrix (Chapter 4), which could lead to milder TSS differences.

Table 4. Results from the physic-chemical analysis of the produced juices

Juice formulation[+]	**pH**	**TSS**	**Color (CIE *L*a*b**)**		
			L*	***a****	***b****
Fruit juice[++]	3.70[a] ± 0.02	12.5[a] ± 0.0	30.95[a] ± 0.12	8.91[a] ± 0.43	22.27[a] ± 1.66
10% SBJ	3.42[b] ± 0.03	12.0[b] ± 0.0	36.24[b] ± 0.46	18.27[b] ± 0.48	37.15[b] ± 0.67
20% SBJ	3.26[c] ± 0.00	11.0[c] ± 0.0	36.77[b] ± 0.41	19.81[bc] ± 0.29	39.35[b] ± 1.34
30% SBJ	3.15[d] ± 0.02	10.0[d] ± 0.0	37.80[c] ± 0.39	20.27[c] ± 0.29	41.54[b] ± 2.02
100% SBJ	2.71[e] ± 0.03	4.5[e] ± 0.0	55.54[d] ± 0.43	33.75[d] ± 0.77	69.72[c] ± 3.18

Mean values ± standard deviation. TSS: Total Soluble Solids measured as ºBrix.

[+]Juice formulations are detailed in Table 3

[++]Fruit juice shows values of the fruit juice without the addition of SBJ

Color was a parameter that resulted of interest for the present research. The fact that adding SBJ at concentrations of 10, 20 and 30% could improve the color of a predesigned base-fruit juice was appealing in terms of research, but most importantly in terms of consumer acceptance. Previous research showed that slight color variation in orange juice reduced the consumer acceptance of the juice (Tepper, 1993). Thus, the orange color of SBJ could be an important attribute of the formulated juice, and a slight variation could negatively affect consumer acceptance. Interestingly, the addition of SBJ was associated with a significant increase of all values of color measurement in terms of CIE$L^*a^*b^*$ score (Table 4; F (4, 10) = 1828, F (4, 10) = 971.4 and F (4, 10) = 250.6 at $p<.05$ from L^*, a^* and b^* values respectively). Significant differences were most notably observed between the base-fruit juice and any formulated fruit juice with SBJ, and between any formulation with SBJ and the SBJ 100%. The differences between the provided samples for the hedonic analysis was only significant when comparing the formulated juice 10% SBJ against the formulated juice 30% SBJ, which translated into observable visual difference (Figure 1).

Figure 1. Visual aspect of the fruit juices of the present research. From left to right: base-fruit juice without SBJ, 10% SBJ, 20% SBJ, 30% SBJ and SBJ.

Significant differences were observed between 30% SBJ and all other percentages in terms of *L** values, which is the value for the perceptual lightness of the sample. Thereupon, there seemed to be a switching point in which the lightness of SBJ would start to significantly contribute to the lightness of the sample. It could also be possible that the lower amount of base-fruit juice added (70% instead of 80 or 90%) did not translate into a negative effect on lightness. *L** values from 100% SBJ averaged 55.54 in all readings (Table 4). This possibly indicated that the addition of SBJ in the final formulation of the juices was not as powerful as the reduction of the percentage of the base-fruit juices (the *L** values range from 0 to 100).

Similarly, significant differences were observed between the samples of 10% SBJ and 30% SBJ in terms of *a** value. This difference was not seen between any other formulated juice (e.g. between 10 and 20% SBJ). Positive *a** values indicate red whereas negative values indicate green. It was interesting to see that the addition of SBJ could proportionally raise this value, yielding more redness to the overall juice, derived from the strong orange color of the original SBJ. Even though the differences between different formulated fruit juices were not too clear, the formulation of a juice with 30% SBJ led to an increase in all CIE*L***a***b** values (Table 4), which could be visually observed (Figure 1).

No significant differences were observed between the developed juice samples in terms of the b^* values. Nevertheless, the differences were significant when comparing the developed fruit juices and both the base-fruit juice and the 100% SBJ, which shows an increase in the yellowness of the juice when increasing the percentage of SBJ.

7.3.1.2. Phytochemical analysis

The antioxidant capacity of fruit juices is an indirect measure of the antioxidant compounds, most of them exerting also certain bioactivity, such as ascorbic acid, carotenoids or polyphenols (Stella et al., 2011). SBJ contains high amounts of these components (Chapter 1, 4) consequently leading to a high antioxidant capacity when compared to other juices (Nowak et al., 2018). The antioxidant capacity as means of DPPH was measured for the newly formulated fruit juices, and the juice without SBJ and the 100% SBJ. The antioxidant capacity of the analyzed juices was significantly different, F (4, 40) = 31.55 at p<.05 (Figure 2). The addition of SBJ improved the antioxidant capacity of the formulated juice, becoming significantly different at 30% when compared to the former juice or the 10% SBJ sample. From the results, it was clear that adding at least 30% of SBJ led to a statistically significant improvement in the antioxidant capacity of the juice. Nonetheless, it should be noted that it was not possible to elucidate which minimum percentage (from 20% onwards) could derive in

a statistically significant difference. Further consideration should be taken to those specific intervals if the formulation would lead to the development of an SBJ containing similar percentages of SBJ. In addition, a different mixture of fruit juices could also influence the boost of SBJ on the antioxidant capacity of the resulting juice in a positive way, an effect that should be tested and was not observed in the present experiment due to the already high antioxidant capacity of the fruit juice without SBJ.

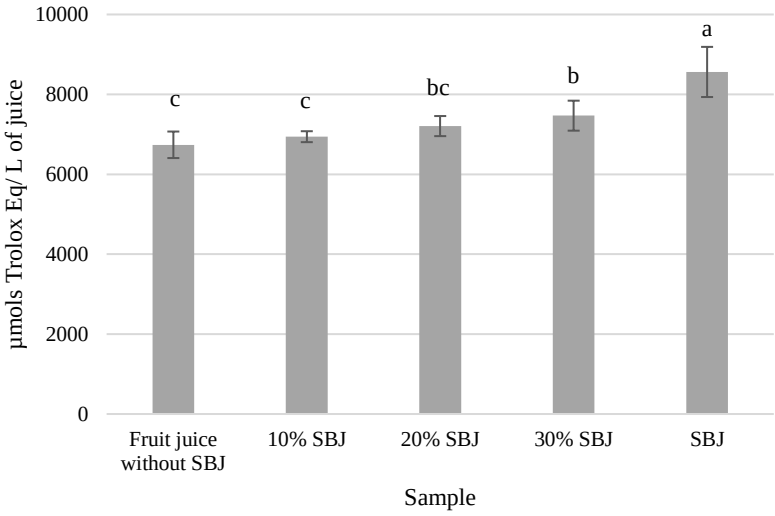

Figure 2. Antioxidant capacity of the newly formulated fruit juices containing SBJ. SBJ: sea buckthorn juice. Error bars show SD. Different letters show statistical differences.

7.3.1.3. Hedonic analysis

The hedonic analysis was only performed on the three formulated fruit juices. The SBJ and the mixture of fruit and vegetable juices were ruled out of the hedonic experiment because they could influence the score of the

other juices and bias the results. All results were clustered together in the same chart (Figure 3). The only attributes in which significant differences were observed were color and sweetness, F (2, 45) = 4.277 and 4.329 respectively, at $p<.05$. In color, the *pos hoc* analysis indicated that significant differences were observed between the 30% and 10% SBJ fruit juices, being more liked the former juice formulation. As indicated in Table 4, L^* and a^* values were significantly greater in the juice at 30% content of SBJ when compared to the 10% formulation. These results clearly indicate that color values in term of redness and luminosity were highly important attributes for the liking of this attribute. However, it was not clear which of both attributes had a major impact on the liking of the juice. The juice was not as orange as 100% SBJ, yet from 10 to 30% some differences could be visually spotted (Figure 1). Color seemed to be an important factor for the visual appeal of the juice, adding evidence to what other authors found with orange juice (Tepper, 1993).

Sweetness was the second and last attribute showing statistically significant differences between one or more samples. The *post hoc* analysis indicated that the differences resided between the formulated samples at 20 and 10% of SBJ (Figure 3). These results were interesting since no linear association was found between the TSS (in terms of ºBrix) and the appeal of the juice. Among the volunteers participating in the study, the juice formulated with 20% of SBJ was more liked in terms of sweetness than was the juice

formulated at 10% of SBJ, leading to the thought that the consumers preferred a less sweet juice (since adding 10% more of SBJ led to a decrease of 1 ºBrix in the formulated juice (Table 4)). Nevertheless, the increase of 10% more of SBJ in the formulated juice – that is, from 20 to 30% – drastically dropped the liking of the juice, even though it also translated in a reduction of 1 ºBrix (Table 4). Yet it should be noted that increasing the percentage of SBJ also significantly reduces the final pH (Table 4). The total pH value was reduced from 3.42 to 3.26 and 3.15 when SBJ changed from 10 to 20 and 30%, respectively. The reduction of pH could be attributed to the high amount of organic acids that constitute the raw SBJ (Tiitinen et al., 2005). The decrease in pH could explain the sudden drop in the liking of the sourness of the juice when adding 30% of SBJ. Besides sourness, the greater percentage of SBJ could bring additional compounds contributing to the astringency and bitterness of the juice formulated with 30% of SBJ. From the present experiment it was not possible to determine the effect of adding more SBJ to each attribute that was negatively affected by this addition (sourness, astringency and bitterness). Previous research showed the contribution of quinine from SBJ to the negative acceptance of the juice because of the increased bitterness (Hartvig et al., 2014), and the positive contribution of the specific compound β-D-glucopyranoside to the astringency of the juice (Ma et al., 2017).

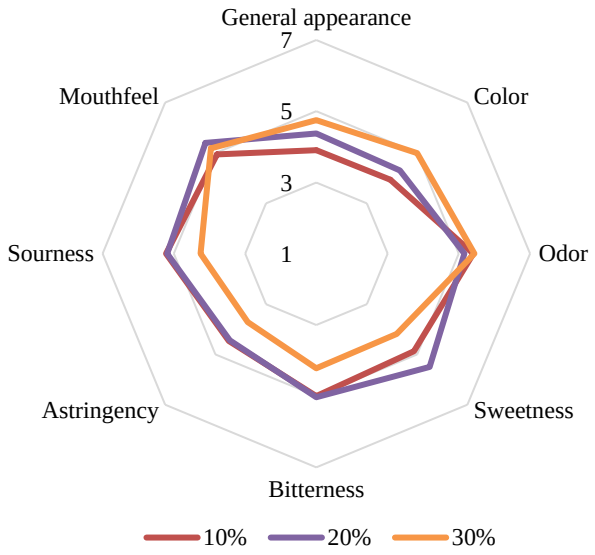

Figure 3. Results from the hedonic analysis of the formulated fruit juices in the first experiment using a 7-point Likert scale system. A 7-level Likert-type scale was used on each attribute, 1-value indicating "strongly disliked" and 7-value indicating "strongly liked", with a neutral value of 4. Percentages indicate the content of SBJ in the formulated juice. Color and sweetness were the only two attributes showing statistically significant differences.

Mouthfeel and odor were the two only attributes that were similar across all the developed juices. This could be interpreted because the different additions of SBJ herein used were not different enough for the consumer to rate it higher or lower, thereupon making it less relevant than other attributes that were identified as critical for the liking of the overall juice. The general appearance is a common attribute to measure the liking of a product without tasting it (Imram, 1999), and had been previously used for other juices (Sabbe et al., 2009). The general appearance usually consists of the observed visual cues, including color or sedimentation. From the

results herein obtained, a positive association between general appearance and color seemed to be true, although it was not checked for. The higher the percentage of SBJ in the final juice, the greater the redness and the luminosity of the juice – stronger orange observable color – and possibly the greater the liking of the general appearance. In that case, it could be interesting to investigate whether the 100% SBJ would be visually more accepted, thereupon adding evidence on the association between color and general acceptance.

Finally, the volunteers were asked to rank each juice based on their overall liking, including before and after-taste attributes (Figure 4). The first ranked juice was the 20% SBJ, probably because of the balance between sweetness and sourness, astringency and bitterness, and because the color was closer to the most liked (30% SBJ). Interestingly, the 10% SBJ choice was the second most-liked juice. However, this juice was the less liked in terms of color and general appearance, which are key attributes for the consumer when purchasing the product. The juice containing 30% SBJ was the last ranked, which in turn poses it as a risky bet. The world of fruit juices benefits greatly from the general aspect of the juice and the overall appearance, two attributes positively valued in the 30% SBJ, although for a successful integration in the market the juice must also be liked in terms of the other attributes herein identified.

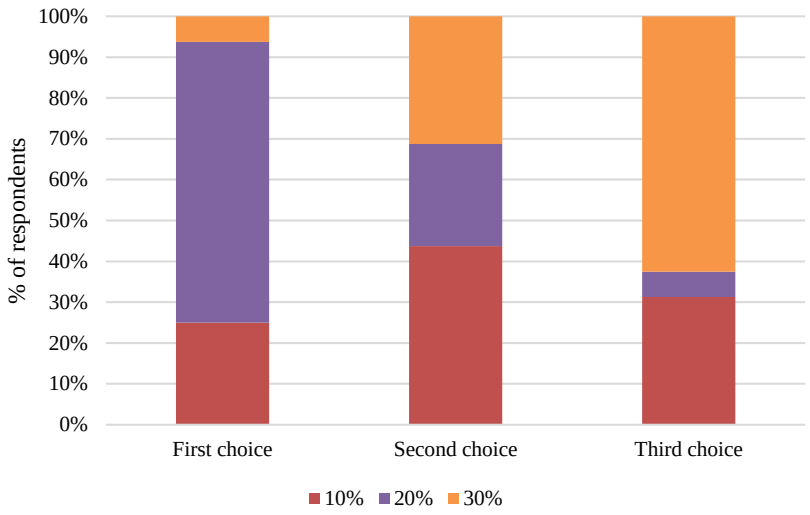

Figure 4. Ranking of the juices according to volunteers' preferences.

The percentage of SBJ studied in the present experiment resulted from previous investigations on the same juice, and results show only slight differences between the developed juices. Thereupon, it should be interesting to explore higher percentages of SBJ added to a formulated fruit juice, to elucidate if very high percentages could negatively influence the acceptance of this juice. In addition, the color was an attribute highly valued in the first experimental and adding more SBJ to the final product could increase the overall liking of this attribute. In the world of food supplements, the nutritional value of the developed product would be the most important attribute when purchasing, despite the fact that the product would be more or less astringent, sour or even bitter. Thus, a greater concentration of SBJ could be used as a juice formulation in that field.

7.3.2. Second experimental

The first experimental showed interesting results to work on subsequent research. The second experimental aimed at elucidating the consumer acceptance of a fruit juice formulated with very high percentages of SBJ and determine the effect of using different varieties in the final product.

7.3.2.1. Physic-chemical parameters

The physic-chemical properties were measured on the developed juices and the pasteurized SBJ from each berry origin (Table 5). The statistically significant differences in the present experiment were more evident, because of the high differences in terms of the addition of SBJ. The use of different proportions of SBJ in the formulation derived into significant differences on the pH value of the formulated juices, F (5, 12) = 400.200 at p<.05. The *post hoc* tests showed that statistically significant differences resulted from all the samples except when comparing the values from the juices at 80% and 100% produced with the berries from Cerdanya (Table 5). The berries from Cerdanya had a slightly higher pH (2.70 vs 2.60 from 100% Latvia origin). It was interesting to note that the formulation with 80% of SBJ from different varieties translated into different pH changes. The formulation with the variety from Cerdanya at 80% led to no significant variation in pH, whereas the formulation with the variety from Latvia at 80% led to a statistically significant variation in the pH when compared to

their respective raw juices. This effect could be explained by two different phenomena: (1) the pH of the added fruit juice (without SBJ) was closer to the pH of the berry juice from Cerdanya, making it more difficult to observe a significant shift in pH after adding only 20% of the fruit-base mixture juice, or (2) the organic acid profile, that could be very different between varieties (Tiitinen et al., 2005; Yang et al., 2011), could trigger different pH responses to the addition of more basic agents (in that case the base-fruit juice mixture, which had a higher pH than SBJ).

Table 5. Results from the physic-chemical analysis of the produced juices on the second experiment

			Color (CIE *L*a*b**)		
Juice	**pH**	**TSS**	***L****	***a****	***b****
Latvia 100%	$2.60^{e} \pm 0.00$	$5.33^{e} \pm 0.29$	$56.45^{ab} \pm 3.11$	$38.20^{a} \pm 1.62$	74.87 ± 3.06
Latvia 80%[+]	$2.65^{d} \pm 0.01$	$6.67^{d} \pm 0.29$	$54.36^{bc} \pm 0.50$	$37.57^{a} \pm 0.29$	73.05 ± 1.35
Latvia 50%[+]	$2.80^{b} \pm 0.00$	$7.80^{c} \pm 0.10$	$52.46^{c} \pm 0.54$	$33.96^{c} \pm 0.59$	71.76 ± 0.51
Cerdanya 100%	$2.70^{c} \pm 0.02$	$11.50^{a} \pm 0.50$	$57.00^{ab} \pm 0.49$	$36.65^{ab} \pm 0.36$	72.09 ± 0.60
Cerdanya 80%[+]	$2.72^{c} \pm 0.01$	$10.77^{ab} \pm 0.25$	$58.10^{a} \pm 0.16$	$36.80^{ab} \pm 0.06$	73.49 ± 0.77
Cerdanya 50%[+]	$2.83^{a} \pm 0.01$	$10.17^{b} \pm 0.29$	$55.43^{b} \pm 0.82$	$34.93^{bc} \pm 0.26$	71.10 ± 1.58

Mean values ± standard deviation. TSS: Total Soluble Solids measured as ºBrix.

[+]Juice formulations are detailed in Table 3

The sweetness of newly formulated fruit juices is an attribute positively associated with their acceptance, as previously explained. As the amount of TSS decreases, other traits of the juice become more relevant, such as the juice's sourness or bitterness. The formulation of fruit juices with different

percentages of SBJ resulted in significant changes in the juice's TSS (measured as ºBrix), F (5, 12) = 191.800 at p<.05. Like what had been observed for the pH values, the origin of the berries seemed to strongly influence the differences between proportions. The Tukey's *post hoc* test revealed that the statistically significant differences were found between all studied values, except for that sourcing from the Cerdanya region at 50% when compared to 80%, and for those of the same origin at 80% and 100% (Table 5). The results of TSS were interesting since the values of the berry juice 100% from the Cerdanya origin were more than two-fold the values of the juice from Latvia 100%. This statistically great difference could be critical when evaluating the sensory attributes of the juice. Although the TSS of the fruit juice without the addition of SBJ was not measured, it was clear that it was a value closer to that obtained for the variety of Cerdanya 100% because the change of the TSS in this variety when reducing the amount of SBJ (to 80%) was non-significant. In addition, the change of TSS was always to lower values when reducing the percentage of SBJ, indicating that the TSS of the added base-fruit juice was lower than the original SBJ. In contrast, the TSS of the juices formulated with the variety of Latvia grew significantly over diminishing the proportion of SBJ, reaching the highest value of 7.80 ºBrix at 50% of SBJ (Table 5). Since the TSS, and therefore the sweetness of the juice, would be very low, it was expected that the juice formulated with the variety cultivated in Latvia

would be more negatively rated when it came to the hedonic response, adding evidence to the positive association between juice sweetness and its acceptance.

The CIE*L*a*b** values followed a similar pattern when compared to the formulated juices in the first experimental setup. The *b** value was the only value that did not give significant differences between samples. This was the same value as that did not show significant differences between the formulated juices in the first experiment. Nevertheless, in the present experiment, no significant differences were observed even when comparing the values from 100% SBJ and the formulated juices. The luminosity and the redness of the juice were the two values from CIE*L*a*b** significantly affected by different proportions of SBJ, F (5, 12) = 6.588 and 14.330 respectively at $p<.05$. Compared to all the fruit juices added in the present formulation, the addition of SBJ could positively contribute to the overall procyanidin content (Yang, Laaksonen, Kallio, & Yang, 2017), directly contributing to the redness of the juice and subsequently to the increase of the *a** value from the CIE*L*a*b** color scale. The results obtained herein make evident this association. Adding 30% more SBJ to the formulated juice (from 50 to 80%, using berries from either origin) significantly increased the *a** value (redness) of the juice. It should be noted that the juice formulated with 80% SBJ did not differ significantly from the pure SBJ in terms of redness. The SBJ of berries from Latvia brought more

redness to the mixture because the *a** value of the SBJ 100% was greater than the variety from the Cerdanya. Differences in polyphenol – or more specifically to procyanidin content – may influence this value, which depends on the variety, origin or even the genetic background of the plant (Yang, Laaksonen, Kallio, & Yang, 2016). Although the differences between varieties did not seem to be significant (Table 5), possible differences in the polyphenolic and procyanidin profile should be considered, as they have an important role in the sensory quality of sea buckthorn (Ma, Yang, et al., 2017).

In contrast, luminosity did not give clear patterns depending on the concentration of SBJ in the final formulated juice. The growing pattern when adding SBJ was observed for the berry juice of Latvia origin, although the significant differences were only observed between the concentration of 50% SBJ when compared to the 100% SBJ, not between the formulated juices (at 50 and 80%). The juices formulated with berries from the orchard located in Cerdanya (Spain) did not follow the same pattern. The highest *L** value was observed for the SBJ 80%, yet it was not significantly different from the 100% SBJ of the same variety. From the formulated juices which were to be subsequently tested, the only significant difference was observed for the 80% SBJ originated from Cerdanya's orchard, showing higher values of luminosity than the latter (Table 5). This could

also be an interesting factor to consider when analyzing the results from the hedonic experiment.

7.3.2.2. Phytochemical analysis

In the present experiment, not a lot of results were pooled from the phytochemical profile of the juice, due to a lack of repetition during extraction. Only two values of each extraction and quantification were obtained. Nevertheless, this was enough to evaluate the results since the repeatability of the method was previously tested and successfully used in previous chapters (Chapter 4, 6). In addition, the differences were expected to be so great derived from the addition of SBJ that the smallest number of replicates would account for the expected high change in both phytochemical analyses performed in the frame of the present experiment.

Antioxidant capacity was different across all formulated juices, also including the 100% SBJ from both varieties, F (5, 12) = 801.10 at p<.05. The Tukey's *post hoc* test revealed that all results differed statistically between each other, excluding the antioxidant value from the sample Cerdanya 50%, which was significantly non-different from the antioxidant value obtained from the sample Latvia 80% (Figure 5). This indicated that the SBJ of the berries from the Cerdanya region had a higher antioxidant value in terms of DPPH assay than the berries from Latvia. This could be double-checked by looking at the values of the sample 100% SBJ from

Cerdanya, which were significantly greater than those of 100% SBJ from Latvia (Figure 5), values that were previously described in Chapter 4 (yet the comparison in Chapter 4 was made on berries harvested on the same year).

The pattern of the antioxidant capacity of all formulated juices show clear and significant drops in the antioxidant capacity after reducing the concentration of SBJ in the final juice regardless of the berry's origin. The drop was more pronounced when comparing the concentration of 80 against 50% of SBJ. This indicates that SBJ appears as the most important juice in the mixture contributing to the antioxidant capacity. The antioxidant capacity of SBJ in terms of DPPH had been previously studied and reported to be very high when compared to other juices (Nowak et al., 2018). The fact that the major effect on the antioxidant activity of the juice may come from the major or lesser presence of SBJ could be also derived from the fact that the other fruit juices were treated thermally more aggressively, leading to a significant loss of certain bioactive compounds and subsequently a loss of the antioxidant capacity.

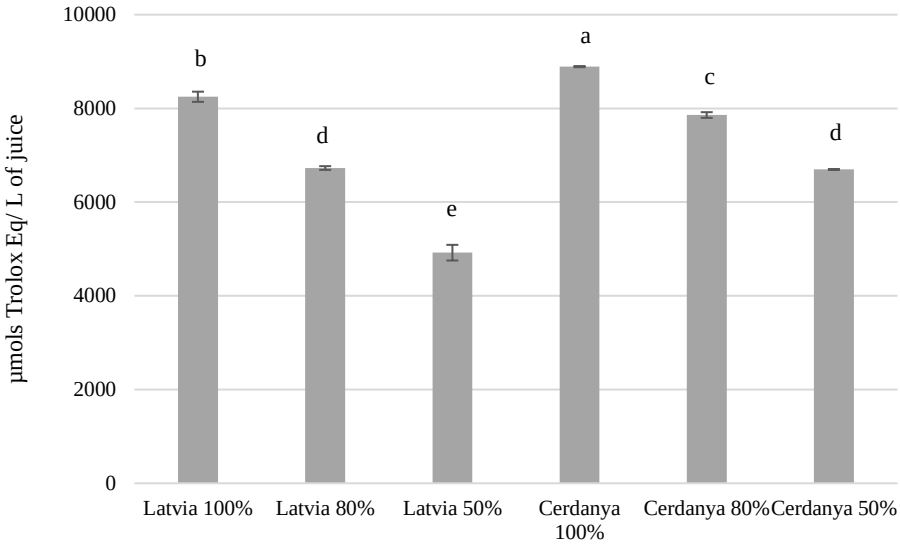

Figure 5. Antioxidant capacity of the developed fruit juices with different varieties in terms of DPPH assay. Error bars show SD. Different letters show statistical differences.

Vitamin C is an important contributor to the antioxidant capacity of fruits (Du et al., 2009). The higher contents of vitamin C in SBJ make it the most relevant bioactive compound contributing to its antioxidant activity (Nowak et al., 2018). From the results obtained in the quantification of the antioxidant activity in the developed fruit juices, it could be subsequently reasoned that the vitamin C content of the fruit would follow a similar pattern, negatively affected when reducing the quantity of SBJ in the final product.

In fact, vitamin C content was different in the formulated fruit juices, F (5, 12) = 1176.000 at p<.05. The *post hoc* analysis revealed the differences to be between all fruit juices. The analysis included the 100% SBJ from each origin. The observed differences in the content of vitamin C in the juice

followed a similar pattern when compared to the antioxidant capacity of the same juice (Figure 6). In that case however, the vitamin C content of the formulated juice using the SBJ from Cerdanya at 50% was significantly lower when compared to the formulated juice using SBJ from Latvia at 80%. This was the only difference between antioxidant capacity and vitamin C content. The results added evidence to the association between vitamin C content and the overall antioxidant capacity of the resulting juice.

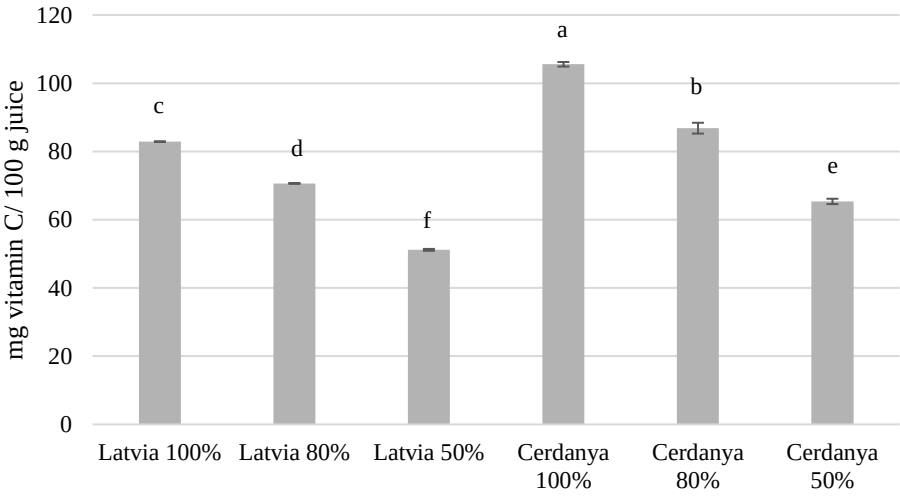

Figure 6. Vitamin C content in the developed fruit juices with different varieties. Error bars show SD. Different letters show statistical differences.

Increasing the SBJ content from 50% to 80% in the formulated fruit juices led to a significant increase in the vitamin C content of the final juice of approximately 20 mg per 100 g of juice when using the berries from Latvia, and about the same for the berries from Cerdanya. The drop in vitamin C content from 80 to 50% of SBJ was greater than the drop from 100 to 80%

SBJ (from the results of berries from both origins) because the former drop was from a higher percentage difference in SBJ (30 vs 20%). This was more obvious in the juices formulated with the berries from Latvia. An approximate value of 40 mg / 100 g juice was the difference in the amount of vitamin C between the sample of 50% SBJ and 100% SBJ, which was true for both SBJ origins. This would translate into the assumption that 40 mg of vitamin C was brought by SBJ to the formulation at 50% as well, translating into only 10 to 20 mg of vitamin C / 100 g of juice brought by the other fruit juices. Although this was just a theoretical reasoning, the low amount of vitamin C brought by other juices could be consequence of the heat treatment to which they were submitted.

The SBJ from Latvia's berries had a significantly lower concentration of vitamin C when compared to the berries from Cerdanya. Nevertheless, the formulated juice containing 50% SBJ from Latvia achieved 50 mg vitamin C / 100 g juice, which translates into half of the Population Reference Intake, or PRI, as established by the EFSA. Taking only 160 g of the juice formulated with 80% of SBJ (from Latvia origin), or 130 g of juice formulated with 80% of SBJ (from Cerdanya origin) would fulfill the requirements of vitamin C.

7.3.2.3. Hedonic analysis

A total of 30 volunteers took part in the hedonic analysis of the formulated fruit juices in the second experiment. The importance of the second experiment was to elucidate if a high percentage of SBJ could be used in the development of fruit juices and achieve great acceptability. The same sensorial attributes were investigated because they were identified to be key in the sensory evaluation of new products, especially on SBJ.

The results point to interesting directions. Among all the studied attributes, sweetness, astringency, sourness and mouthfeel were the attributes showing statistically significant differences between the developed juices, F (3, 116) = 8.284, 3.243, 4.590 and 4.097 at $p<.05$, respectively. Interestingly, in the present hedonic analysis, the juices were similarly rated in terms of color, differing from the previous hedonic experiment. Although significant color changes were observed, the differences were non-significant between the formulations of 50 and 80% SBJ from each variety. The statistical color differences measured instrumentally (L^* and a^*, Table 5) were not enough to obtain significant differences in the visual evaluation.

Again, the sweetness was a hedonic value statistically different across the developed fruit juices. Sweetness is an important value for the acceptance of newly developed juices and SBJ seemed to strongly influence this value (Figure 7). This made sense since the values of TSS showed statistically

significant differences when comparing the formulated fruit juices (Table 5). Specifically, the formulations with SBJ from Latvia led to significant differences between the developed fruit juices (50 and 80% SBJ), whereas the formulations using SBJ from Cerdanya seemed to influence TSS values to a lesser extent (Table 5). In addition, the same results indicated that SBJ from Cerdanya was significantly sweeter than that coming from Latvia, showing a difference of more than 5 ºBrix (Table 5). This could lead to believe that juices formulated with SBJ from Cerdanya would be more positively rated in terms of sweetness when compared to the formulations using SBJ from Latvia. The *post hoc* test did show significant differences but only between the juice at 80% SBJ from Cerdanya and the juice at 50% SBJ from Latvia. Strangely enough, no significant differences were observed between the juice formulated with 80% SBJ from Latvia and the 50% SBJ from the same origin. The SBJ from Cerdanya did show significant differences between both formulations (Figure 7). The chart represented in Figure 7 shows that the formulation with 80% SBJ from Cerdanya was the worst valued, and the juice formulated with 50% SBJ from the same origin was the best rated in terms of sweetness. The fact that 80% SBJ from Cerdanya was more negatively rated than the formulation with 80% SBJ from Latvia was rare, since the difference in ºBrix was still very high, being higher in the former. It became evident that other compounds may be interfering in the sweetness perception of the juice,

compounds which could actively contribute to the sourness or astringency of the juice (Ma, Laaksonen, et al., 2017; Ma, Yang, et al., 2017). Interestingly, the juice formulated with 80% SBJ from Cerdanya was more negatively rated in terms of bitterness and astringency than the rest of the juices, which could add evidence to this theoretical reasoning (Figure 7).

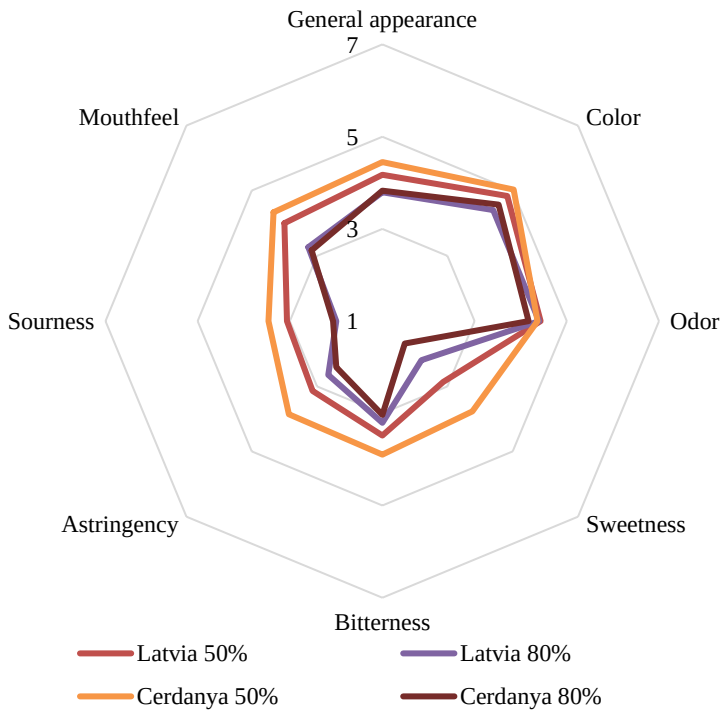

Figure 7. Results from the hedonic analysis of the formulated fruit juices in the second experiment using a 7-point Likert scale system. A 7-level Likert-type scale was used on each attribute, 1-value indicating "extremely disliked" and 7-value indicating "strongly liked", with a neutral value of 4. Percentages indicate the content of SBJ in the formulated juice. Astringency, sourness, mouthfeel and sweetness were the only two attributes showing statistically significant differences.

Astringency and sourness of the juice play an important role since they were significantly affected by changing either SBJ percentage or the origin of the

berries, or both. Tukey's *post hoc* tests revealed that the differences in astringency were significant only when comparing the formulations at 50 and 80% using SBJ from Cerdanya. Again, the juices formulated with Cerdanya's variety were the worst and the best-rated juices in terms of astringency (depending on the proportion of SBJ). The differences in sourness became significant when comparing the values of the juice formulated at 80% with SBJ from both origins and the juice formulated at 50% with SBJ from Cerdanya (Figure 7). Sourness was the attribute worst rated in juices formulated with 80% SBJ with berries from both origins. It was clear that, together with sweetness and astringency, SBJ brought also significant changes in the juice when the SBJ constituted 80% of the total mixture of fruit juices. The value of sourness was nearly 1, which is the lower value of the 7-point Likert scale used in the present experiment. The sourness could derive from the organic acid profile of the juice, thereupon increasing when rising the SBJ content (Tiitinen et al., 2005).

Interestingly, the mouthfeel attribute also led to statistically significant differences. The *post hoc* tests showed that the differences were the same as observed for sourness, becoming statistically significant when comparing the juice at 50% SBJ from Cerdanya and the juice at 80% from both origins. The greater presence of SBJ might also play a critical role in the texture or mouthfeel of the juice since the juice was filtrated with an 850 μm sieve. Whereas the addition of low percentages of SBJ did not

influence this parameter (as shown in the first experiment), higher percentages of SBJ brought more particles to the juice, most likely with greater particle size, therefore probably triggering the observable rejection effect for the two formulated fruit juices having higher percentages of SBJ.

Overall, formulated juices with SBJ at 80% led to lower scores in all the sensorial attributes herein investigated. The sweetness, astringency, bitterness and sourness of the juices developed with 80% SBJ were the most negatively affected when compared to the juices formulated with 50% SBJ. This was because the addition of SBJ reduced the TSS in terms of ºBrix and this was stated to be an important factor contributing to consumer acceptance of newly developed fruit juices (Tuorila-Ollikainen et al., 1984). In addition, higher percentages of SBJ brought more compounds, such as organic acids, which may negatively influence the juice's preference in terms of sourness, bitterness and astringency. Differences in varieties were also observed, and the juice formulated with 50% SBJ from Cerdanya was the highest rated among all fruit juices. It should be noted that the average value given to any fruit juice did not surpass the 4-threshold point, possibly indicating that SBJ itself may be bringing important changes in flavor that results in a relative lower liking of the juice. Nevertheless, in order to test this hypothesis, further research should focus on the hedonic analysis of developed sea buckthorn-based juice and compare it against a juice without sea buckthorn.

7.4. Conclusions

Sea buckthorn berries were used as a novel ingredient in the development of a fruit juice. The inclusion of SBJ in a final juice product was successfully performed at different proportions in view of previous research and industrial interests. Overall, the addition of sea buckthorn juice during the formulation of a fruit juice positively contributed to the general appearance and color. The great amount of carotenoids, conferring the natural and strong orange color to sea buckthorn juice, might be the principal factor influencing the positive hedonic response of both color and general appearance. Other compounds, such as proanthocyanidins, can also influence these parameters.

At the highest percentages of sea buckthorn juice in the final product, the attributes bitterness, sourness, astringency and sweetness were poorly rated when compared to the formulations at lower proportions of sea buckthorn juice. This probably derives from the lower TSS value or the lower pH, modified by the greater presence of organic acids, or by the greater presence of other compounds that could negatively affect the liking of this attributes.

In terms of phytochemical analysis, the use of sea buckthorn juice as an ingredient in the development of fruit juices dramatically increases the vitamin C and the antioxidant capacity content of the final juice. These results bring more support to the use of sea buckthorn juice in the

development of liquid food supplements because this type of developments focus on the nutritional profile rather than on the sensorial characteristics of the resulting product.

The use of percentages of sea buckthorn juice of 10, 20 and 30% in the final product derived in results from the hedonic evaluation to be equal or above the average point of 4, therefore suggesting a certain liking of the final product. The fact that the fruit product was developed with pomegranate potentiated the effect of the addition of sea buckthorn juice in the color attribute. Percentages of 30% obtained the worst ratings in the taste-related attributes yet the best ratings in color liking.

The use of percentages of sea buckthorn juice of 50 and 80% in the final product derived in negative results from the hedonic evaluation (below the average point of 4), especially in the concentrations of 80%. Notably, using sea buckthorn berries from the same variety but different origins significantly affects the liking of the sensory attributes of the final fruit juice, due to the differences in their nutritional and sensorial quality.

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Chapter 8: Fermentation of clarified sea buckthorn juice by probiotic lactic acid bacteria

8.1. Introduction

Fermentation is a food processing technique consisting in using microorganisms which, under specific conditions, can produce chemical changes in a food matrix by using organic substrates. Fermentation is already important in many existing food products – just to name a few, yoghurt, cheese, bread, beer – and very important for the development of flavors and in new food product formulations. As such, fermentation has been applied to fruit juices to understand the fermentation kinetics but also to develop functional food products. Lactic acid bacteria are the most commonly used microorganisms, mainly (1) because of the potential probiotic role of most of the strains building up this microbiological group, and (2) for the easiness of proliferation and fermentation showed by strains of that group. A great deal of articles investigating the fermentation of different fruit juices have been published, because of the suitability of the medium to become an object of fermentation (i.e. high-water content, rich in nutrients), with the subsequent recent reviews on the topic (for instance: Al Daccache et al., 2020; Anal, 2018).

Besides the probiotic role of the strain used to ferment the fruit juice, chemical changes arise within its matrix. Lactic fermentation of fruit juices results (1) in the partial or full elimination of possible anti-nutritional factors which may naturally occur in the juice, (2) in a production of by-products from the fermentation with a biological positive impact (bioactive peptides, exopolysaccharides…), (3) in a possible improvement of the bioavailability of the molecules through its degradation (hydrolysis of polymers), and (4) in a possible increase in mineral, vitamin and phenolic compounds (leading to an increase in the antioxidant capacity of the fruit) (Septembre-Malaterre, Remize, & Poucheret, 2018).

Lu, Tan, Chen, & Liu (2018) fermented star fruit with three commercial probiotic strains (*L. helveticus* L10, *L. paracasei* L26, *L. rhamnosus* HN001). All strains grew well with final cell counts of 10^8 CFU/ml. *L. rhamnosus* produced the highest amount of lactic acid, resulting in a significant lower pH (4.41) than that of *L. helveticus* (4.76) and *L. paracasei* (4.71). Ketones, alcohols and fatty acids were produced at varying levels that could impart different aroma notes to the beverages. Malic acid, one of the most important acids in sea buckthorn juice, was reduced in all three fermentations after 8 days due to the malolactic fermentation. Natural aroma compounds were reduced to low or undetectable levels. The results suggested that fermentation of star fruit would lead to a probiotic-based beverage.

High carotenoid-containing food products, as sea buckthorn, have been investigated for fermentation as well. Kun, Rezessy-Szabó, Nguyen, & Hoschke (2008) used strains of *Bifidobacterium ssp.* to ferment a freshly prepared 100% carrot juice. The problem with *Bifidobacterium* is that they are generally very difficult to grow under extreme conditions. Even though sea buckthorn has also high carotenoid content, there is an impediment as the pH of carrot juice had been stablished around 6 and the pH of sea buckthorn juice has been reported to be around 3 or less (Chapter 4). Authors from this particular study on carrot juice inoculated *Bifidobacterium* at different concentrations and evaluated the changes in the food matrix as well as their ability to grow and colonize the fermented juice. All three varieties of *Bifidobacterium* grew well under the conditions and the matrix used. Carotenoids were degraded in 15% and 45% for alpha and beta-carotene, respectively. The amount of lactic acid increased exponentially until reaching levels of 15 to 17 mg/ml after 24 hours of fermentation. The results suggested that pure carrot juice would be a good matrix to be fermented and therefore provide a probiotic beverage to those who cannot ingest dairy products. The interesting part of this study is that the carotenoid content is negatively affected after 24 hours of fermentation by three different strains of *Bifidobacterium*. The authors suggested that the carotenoids could be an important contributor to the normal metabolism of the *Bificobacterium* that were inoculated, meaning that the loss of

carotenoids may be counterbalanced by the proliferation of these bacteria. This is an important point to consider when attempting a fermentation upon sea buckthorn juice, which is a good source of carotenoids.

A recent study also investigated the potential of a different matrix to be fermented by different strains of *Lactobacillus spp.* (Ricci et al., 2018). In this study, fifteen different strains of *L. plantarum*, *L. rhamnosus* and *L. casei* species were used to ferment elderberry juice. Interestingly, the fruit aroma improved in all three fermentations for the elderberry juice. This is important, since maybe some aroma compounds may increase in sea buckthorn juice as well, and this could help modulate its final aroma profile and its acceptance.

Cherry juice has been recently fermented as well. Ricci et al. (2019) fermented cherry juice with lactic acid bacteria. They found that different lactic acid bacteria had different effects, some improving the aromatic profile of the juice and others modulating the polyphenolic profile of the same juice. Interestingly, a specific strain of *L. plantarum* did produce significant amounts of dihydrocaffeic acid, which possess great positive biological effects (i.e. antioxidant, anticancer potential (Santana-Gálvez, Castrejón, Serna-Saldívar & Jacobo-Velázquez, 2020).

Another recent study investigated the potential of a specific strain of *L. plantarum* to ferment pomegranate juice with the aim of developing a

functional juice product (Mantzourani et al., 2019). The use of this specific lactobacillus strain to ferment pomegranate juice derived in a higher total phenolic content after 24 hours of fermentation, but also during and after the full fermentation period (4 weeks). The difference was more than 150 mg GAE/100 mL when compared to that non-fermented juice. The antioxidant activity followed the same patron over time. By the end, the antioxidant activity of the fermented pomegranate juice was more than 3 times higher than this of the non-fermented pomegranate juice. Alcohol was produced in residual amounts and the inoculated strain retained its viability in levels above 8.8 log CFU/ mL (also true in previous studies such as (Mousavi, Mousavi, Razavi, Emam-Djomeh, & Kiani, 2011)). In addition, by the end of the 4-week period of fermentation, consumers hedonically preferred the fermented juice against the non-fermented one. The aroma profile was also improved in terms of acceptance. The same strain was previously used to ferment cowpea seed flour in water (Dueñas, Fernández, Hernández, Estrella, & Muñoz, 2005). Fermentation modified the content of phenolic compounds, modulating their profile. Additionally, the antioxidant activity was increased, resulting in foods with higher functionality.

Antioxidant activity was also increased in a recent publication on the fermentation of mixed berry juice by lactic acid bacteria, after 24 hours. The juice consisted of different berries, including raspberry, blackberry,

cranberry and blueberry among others. The most notable changes were observed in different volatile compounds, changing the aroma of the product after fermentation. Benzoic acid and benzaldehyde concentration increased by 64 and 188 %, respectively, after 24 hours of fermentation (Park et al., 2017). Interestingly, at the fermentation mid-point, a significant increase in the antioxidant capacity of the fermented juice was spotted, being clearly higher than the non-fermented juice at the same fermentation time. Nevertheless, after 24 h they observed a clear drop in the antioxidant capacity when compared to the 12 h fermentation time. They reported a final antioxidant capacity value of the fermented juice at 24 h statistically similar to the value observed on the unfermented juice at 0h.

8.1.1. Fermentation of sea buckthorn juice

Sea buckthorn juice fermentation had also been investigated, and for different reasons. For instance, Tiitinen, Vahvaselkä, Hakala, Laakso, & Kallio (2006) investigated the use of malolactic fermentation to reduce the sourness of the berry juice. By using the lactic acid bacteria *Oenococcus oeni* at concentrations of 10^9 CFUs/ml, they achieved a 50% reduction of malic acid to lactic acid and CO_2 in 12 h of fermentation, without observable losses of vitamin C, pulp oil or sugars. The following year, Tiitinen, Vahvaselkä, Laakso, & Kallio (2007) investigated the same fermentation technique to observe differences between different varieties

of sea buckthorn berries. Fermentation decreased sourness and astringency in all varieties and increased fruity and fermented flavours. The larger the fermentation time, the larger the changes in the berry juices.

In these studies, sea buckthorn juice had only been fermented in an attempt to reduce its sourness by using the malolactic fermentation of *O. oeni*. Nevertheless, in a recent study, other lactic acid bacteria were used to investigate changes in the phytochemical profile and antioxidant activity of the juice rather than focusing solely on potentiating the organoleptic acceptance of sea buckthorn juice (SBJ). The lactic acid bacteria used included the same bacteria studied by Mousavi et al. (2011) (*Lactobacillus plantarum)* that gave interesting results on the antioxidant capacity of pomegranate juice. The results from the SBJ fermentation were interesting after 48 and 72 h of fermentation, with an increase in flavonols and antioxidant activity of the juice (Tkacz, Chmielewska, Turkiewicz, Nowicka, & Wojdyło, 2020).

Sea buckthorn juice has also been included in the production process of existing fermented food products, such as cheese or yoghurt (Vilas-Franquesa, Saldo & Juan (2020), Chapter 1). In most of the cases, the proliferation of the starter culture was being affected by the addition of sea buckthorn juice, or derived products. In yoghurt, sea buckthorn juice led to higher proliferation in culturing bacteria, as well as improvements in taste

and higher nutritional profile, yet it also brought some additional sourness to the product (Selvamuthukumaran & Khanum, 2015). Increased aroma profile in feta-type cheese as well as increase in esters, terpenes and carbonyl compounds derived from fermentation was observed in the final product of cheese production (Terpou et al., 2017). Furthermore, the addition of a probiotic strain in whey-protein-enriched sea buckthorn juice derived in longer shelf life when intentionally spoiled with a specific strain of *E. coli*, besides being an effective probiotic drink (Sireswar, Dey, Sreesoundarya, & Sarkar, 2017).

In addition, sea buckthorn juice has been recently observed to be a good source of prebiotic substrate in terms of the proliferation of beneficial gut microbiota, for it increased the ratio Bacteroides/Prevotella by 71% and the lactic acid concentration and Bifidobacteria by 35% and 17%, respectively (Attri, Sharma, Raigond, & Goel, 2018).

8.1.2. Demarcation

Fermentation technology is now gaining more importance in the juice industry, because of the potential for the development of functional products. Fermentation of fruit juice could lead to the development of a probiotic beverage, since the inoculated microorganism is usually selected by its probiotic capacity and become a real choice for those who see their choice of fermented food products reduced due to, for instance, a lactose

intolerance. In addition, fermentation of fruit juices may lead to a more stable product without the need to apply a thermal treatment, because of the natural growth and viability of the strains inoculated to the juice. Furthermore, depending on the strains used, the fermentation of a fruit juice could result in a product with a higher antioxidant capacity. Besides, the aroma profile could also be modulated in fruit juices, in some cases improving the overall aroma profile and acceptance of the product. This could be an important attribute from the fermentation, as sea buckthorn contains high amounts of organic acids and other compounds that increase its sourness (Chapter 4, 7), reducing its acceptance.

Results from fruit juice fermentation are mostly positive and generally interesting, which makes researchers dig further on that topic, investigating new strains, new matrices. In that case, the use of fermentation technology in sea buckthorn juice emerges as a new challenge and a promising field. By itself, sea buckthorn juice has clearly high contents of vitamins and lots of bioactive compounds. While it has been shown that carotenoid content may decrease over fermentation time, vitamin C content remains quite stable, with a derived natural degradation over time. Vitamin C content is a very valuable characteristic of sea buckthorn juice and, if unvaried, may boost even further the antioxidant capacity of the fermented fruit juice. In addition, sea buckthorn juice has a unique lipophilic profile that most fruit juices do not have, turning it into an interesting object of fermentation.

Several short-chain fatty acids, which could be produced during the fermentation of sea buckthorn juice, have a positive impact on human health and could derive into a clearly higher nutritional value from the fermented product.

Although some studies have been recently published on the use of different lactic acid bacteria for the fermentation of SBJ, the number of those articles is limited, and more studies should be performed to investigate fermentation of SBJ by lactic acid bacteria to improve the nutritional profile of the juice. This study aims at filling that gap. Moreover, the results from the present study could give a framework for a symbiotic product development, which could derive in both a probiotic and a prebiotic positive impact on the gastrointestinal tract of the targeted consumer population.

8.1.3. Objective

The aim of the present experiment is to ferment sea buckthorn juice with probiotic lactic acid bacteria strains to improve its already high nutritional composition, either because of the formation of biologically active compounds that were not previously found in the raw juice or incrementing the already high antioxidant capacity of sea buckthorn juice. Or any new way in which fermented sea buckthorn juice may be considered as a value-added beverage.

8.2. Experimental design

8.2.1. Groundwork experiment

The SBJ has a very low pH (Chapter 4), which difficult the growth of certain bacteria, especially lactic acid bacteria. A preliminary experiment was carried out to investigate at which pH the lactic acid bacteria could grow at the same levels if compared to its optimal growing conditions (MRS medium). The results indicated that the ideal pH for the growth and therefore to obtain a possible fermentation of SBJ by lactic acid bacteria was [redacted].

Additionally, yeast extract contains high amounts of amino acids, vitamins, and minerals that the lactic acid bacteria could use to promote its growth. Yeast extract had previously been reported to improve the growth of several probiotic strains (Saxelin et al., 1999). Besides, no other study investigating the fermentation of SBJ used yeast extract to potentiate the growth of the inoculated microorganisms. Therefore, yeast extract was used in the same solutions of the experiment as an ingredient to potentiate the growth of the inoculated lactic acid bacteria and therefore optimize the fermentation of SBJ.

8.2.2. Growing medium and reagents

MRS agar (CM0361), peptone water (CM0009) and yeast extract (LP0021) were purchased from Oxoid Ltd. (Hampshire, U.K.). MRS broth was purchased from Laboratorios Conda S. A. (Madrid, Spain). Anaerogen™ 2.5 L bags (Thermo Fisher Scientific Inc., Madrid, Spain) were used to generate an anaerobic working atmosphere when required by the employed strain. The solution of sodium hydroxide 2 M used to rise the pH of the juice was acquired from Panreac Quimica S.L.U. (Barcelona, Catalunya, Spain).

8.2.3. Strain propagation and storage

Lyophiles of *Lactobacillus plantarum* spp. *plantarum* CECT 748 and *Lactobacillus paracasei* spp. *paracasei* CECT 4583 were purchased from the Spanish Type Culture Collection (CECT, Valencia, Spain). *L. plantarum* and *L. paracasei* were resuspended in MRS broth and sowed in MRS agar plates. Culture conditions are depicted in Table 1.

Table 1. Growing conditions of the strains used for CSBJ fermentation.

Strain	Growth medium	Optimum pH	Incubation time (h)	Atmospheric needs	Growth temperature (°C)
L. plantarum spp plantarum	MRS	6.2 – 6.5	24	AGS	30
L. paracasei spp paracasei	MRS	6.2 – 6.5	24	A	30

MRS: de Man, Rogosa, Sharpe medium. AGS: anaerobic generating system. A: aerobic

L. paracasei and *L. plantarum* were purified using MRS broth and MRS agar. After purification, single colonies were picked to check their morphology. After a successful check, isolated and pure colonies were stored at -80 ºC using vials containing cryobeads (Deltalab, Barcelona Spain).

8.2.4. Sample preparation

Sea buckthorn berries of the variety 'Tatjana' (a crossed variety with genes from different subspecies, namely *mongolica*, *rhamnoides* and *fluviatilis*) were purchased in 2018 from BRUwell, a local harvesting firm located in western Latvia. Sea buckthorn berries of the variety 'Tatjana' were harvested from the local plantation of Vitae Health Innovation S. L. located on 'Bellver of Cerdanya' (Catalunya, Spain). SBJ was extracted using a Thermomix® TM21 (Vorwerk, North Rhine-Westphalia, Germany) and filtered at 850 µm to separate the solids with greater volume. The juice from both varieties were used separately for inoculation with both strains.

SBJ was divided into working aliquots of 40 ml and subsequently clarified at 14,000*g* for 15 min at 4 ºC. Clarified SBJ (CSBJ) was further filtered using a Whatman™ filter paper grade 1 (Buckinghamshire, U. K.). A total of four aliquots were obtained (two per each variety used for the fermentation, Figure 1). Yeast extract was added at [redacted] g/L to one aliquot of each variety, and the pH of all aliquots was risen to [redacted] using an aqueous solution of NaOH 2 M. All aliquots of CSBJ were then placed in a water bath for batch pasteurization at [redacted] ºC for [redacted] min. The samples were prepared in duplicate.

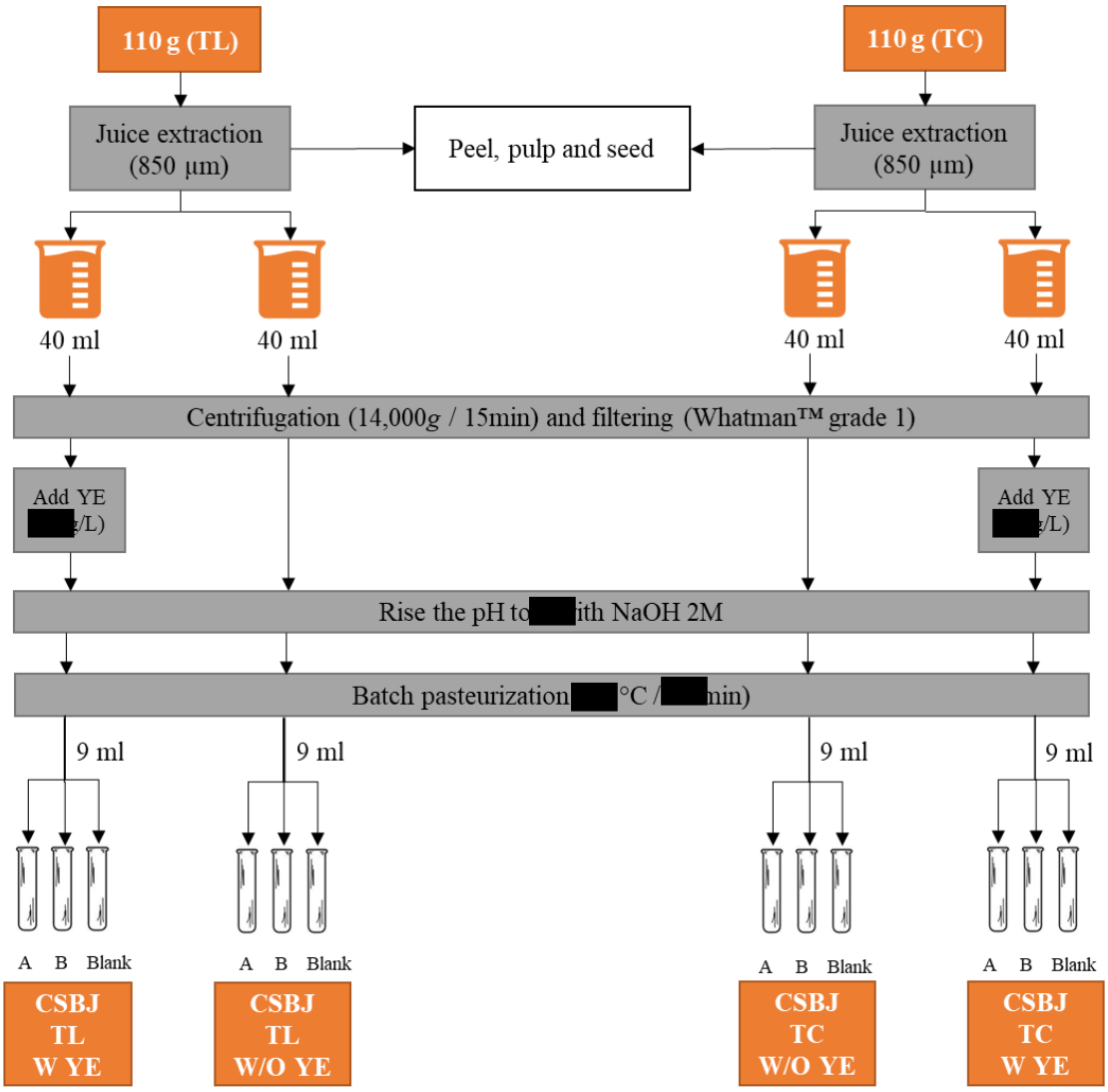

Figure 1. Schematic representation of the juice preparation. TL: 'Tatjana' from Latvia; TC: 'Tatjana' from Catalunya; YE: yeast extract; CSBJ: clarified sea buckthorn juice; W YE: with yeast extract; W/O YE: without yeast extract.

8.2.5. Juice inoculation

Two cryobeads of each strain were placed in 5 ml of MRS broth (modified or unmodified) and placed in the incubation oven at the time and temperature indicated in Table 1. The outcoming vials were merged in one when the growth of the strain was observed visually. The optical density (OD) of the solution was checked at 600 nm (UV 3210 digital spectrophotometer (Dinko Instruments, Catalunya, Spain)) to get an estimate of the strain concentration in the solution. Concentration was estimated after comparing the obtained OD against a calibration curve of different dilutions.

The MRS broth was transferred to sterile centrifuge vials and centrifuged at 3,200*g* for 15 min. The supernatant (MRS solution) was decanted. The resulting pellet was washed and resuspended in 10 ml of 0.9 % sterile saline solution (Tkacz et al., 2020). An exact aliquot of 1 ml of the resuspended solution of each strain was inoculated to all samples of CSBJ (Figure 2). The strain was inoculated to a final concentration (in CSBJ) of 10^8 CFU/ ml. Each sample was inoculated in duplicate, obtaining a total of 4 vials for each solution (provided that solution A and B from the Figure 1 were inoculated twice each). Blank consisted in the samples without inoculation. All inoculated samples were incubated at 30 ºC for 48 hours. The *L.*

plantarum was incubated in an anaerobic generating system and the *L. paracasei* was incubated in aerobic conditions.

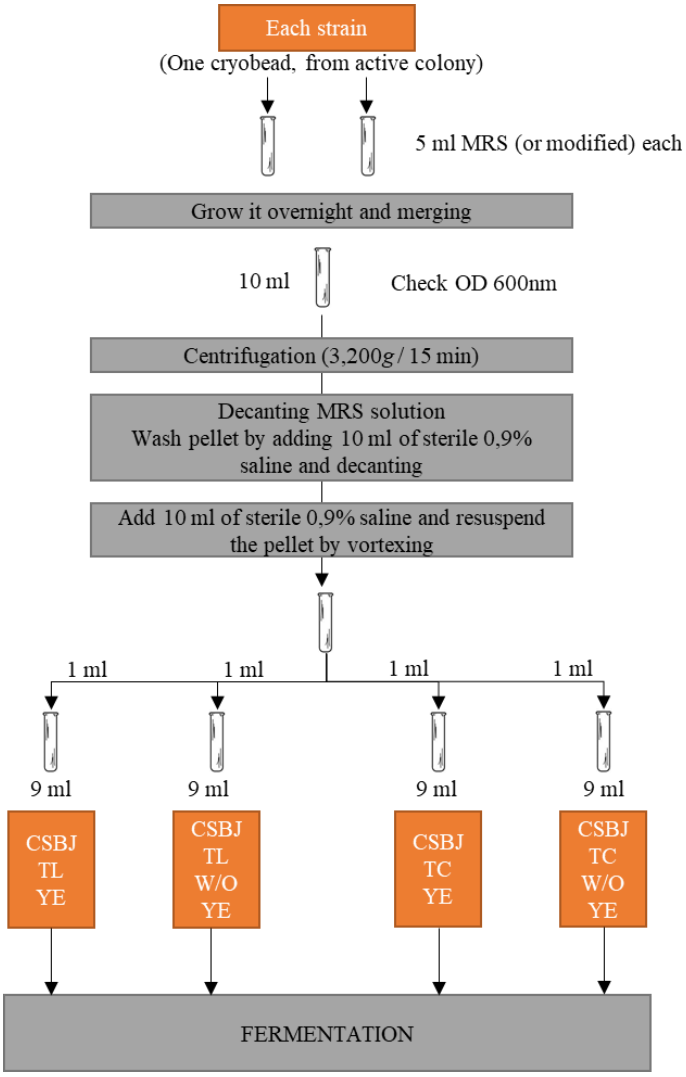

Figure 2. Schematic representation of the strain inoculation to different samples of CSBJ. OD: optical density; TL: 'Tatjana' from Latvia; TC: 'Tatjana' from Catalunya; YE: yeast extract; CSBJ: clarified sea buckthorn juice; W YE: with yeast extract; W/O YE: without yeast extract.

8.2.6. Sample analysis

After the fermentation, the samples were immediately placed in cryovials and stored at -80 ºC for further analysis. Sugars and organic acids were analyzed simultaneously by the gas chromatography technique as detailed in Chapter 3. Total polyphenols and antioxidant capacity of the sample – DPPH assay – were also measured. The growth of each strain was quantified by sowing 200 µl of a dilution of 10^{-6} and 10^{-7} from the fermented CSBJ to MRS agar plates and counting the CFU after incubation of the plates as indicated in Table 1. The plates were sowed immediately after the fermentation. pH and ºBrix were also measured the same day after fermentation. All the analytical quantifications or physic-chemical evaluations were performed at the fermentation's endpoint.

8.2.7. Statistical analysis

All statistical analysis was performed with the software R-4.0. Assumptions were checked by first visually interpreting the Q-Q and boxplots from all analysis. Normality was additionally checked by Shapiro-Wilk and Levene's test, and a conjoint conclusion on the normality of the data was withdrawn. Subsequently, the statistical analysis of the data was performed. A one-way ANOVA was run for all attributes, and samples were treated separately. While the two-way ANOVA could indicate some differences in the variables (the effect of the addition of yeast extract, the effect of using

different varieties and the interaction effect), interest remained in analyzing the data as a whole, including the comparison with the blank at 48 h but also with the blank at 0 h to spot for any relevant statistical difference. Further analysis involved the use of Tukey's *post hoc* tests to understand possible significant differences between samples.

8.3. Results and discussion

8.3.1. Survival and growth rate of the inoculated microorganisms

Pre-fermentation experiments were used to set the pH value in which the growth of the microorganisms herein used would not be very different from the growth of the same microorganisms using the optimal growing conditions (8.2.1. Groundwork experiment). The pH value was established to be [redacted]. This was the reason for only using CSBJ with the pH adjusted to [redacted] for the present experiment.

The growth rate of the microorganisms used was also checked in the present experiment. There were no significant differences between the growth rate of *L. plantarum* in any of the solutions used when compared to the growth at its optimal conditions (Figure 3). However, these results could be explained by the huge standard deviation registered for the growth of this specific bacteria, probably due to the variance in plate growth, affecting

subsequently its count. Taking a closer look at the results, the highest growth mean (in CFU/ml) was registered [redacted], achieving a higher growth when compared to the control. All other growths were registered to be [redacted]. Again, these differences were non-significant.

The growth of *L. paracasei* was more accurately registered. The standard deviation for the growth of this microorganism was much lower, facilitating the reading of the results. The most important growth of the microorganism was registered for the [redacted]. Both solutions with the CSBJ from berries originally from [redacted], contrasting with the results obtained in the solution made with berries from [redacted], for which the solution [redacted] registered the same growth than the control (Figure 3).

Yeast extract was added to improve the growing conditions for both microorganisms, to facilitate its adaptation and growth rate and ultimately increase the fermentation performance (Saxelin et al., 1999). Interestingly, the results of the obtained CFU of *L. paracasei* [redacted] (Figure 3). The growth of the *L. paracasei* in the CSBJ [redacted] was significantly [redacted] than the growth of the same microorganism in solutions [redacted]. The [redacted] growth rate was also

observable for *L. plantarum* in the CSBJ [redacted], but only on the CSBJ formulated with berries from [redacted]. The results seemed to indicate that the concentration of the yeast extract in the fermentation object (CSBJ) was probably higher than the optimal concentration for these two studied microorganisms.

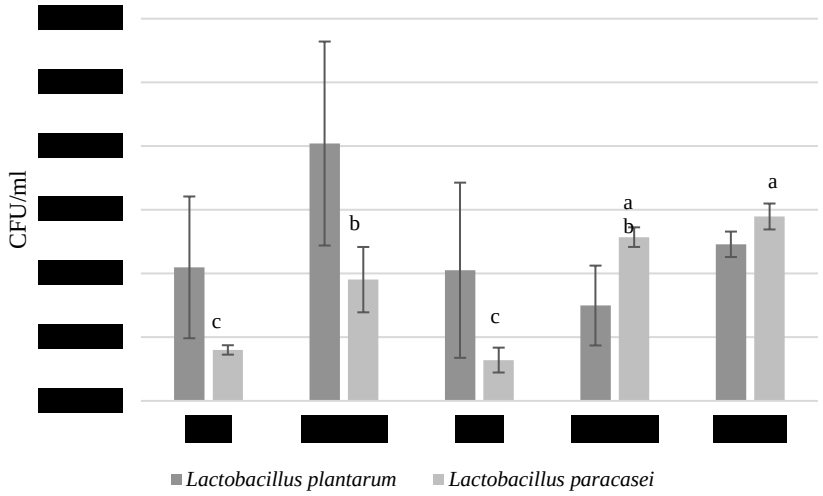

Figure 3. Growth rate of the two microorganisms used in the fermentation of CSBJ and its comparison to the control. Error bars show SD. CFU: colony-forming units. Control: MRS medium. LYE: Latvia variety with Yeast Extract. LWOYE: Latvia variety WithOut Yeast Extract. CYE: 'Cerdanya' variety with Yeast Extract. CWOYE: 'Cerdanya' variety WithOut Yeast Extract.

8.3.2. Physic-chemical analysis of the fermented juice

The pH and total soluble solids (TSS, measured as ºBrix) were the two physic-chemical attributes measured in all the fermentation conditions. On one hand, the pH could give an overview of the possible changes in the organic acid profile after fermentation (i.e. it had been previously

associated with the concentration of lactic acid and malic acid in sea buckthorn juice fermentation (Tkacz et al., 2020)). On the other hand, the reading of the TSS could give an overview of the possible changes in the sugar profile after fermentation and thus on the success of the process.

The pH value was set at [redacted] in all the initial solutions (Table 2, non-fermented CSBJ (0 h)). High pH values (around [redacted]) in other juices had been previously reported to be beneficial for the metabolic activity of certain bacteria strains (Gao, Vasantha Rupasinghe, & Pitts, 2013), and the groundwork experiment (8.2.1. Groundwork experiment) indicated that it was an important parameter for the survival and proper growth of lactic acid bacteria in SBJ.

The pH was not steady over the storage of the uninoculated juice. Slight increments in pH were registered in blank samples after storage at 30 ºC for 48 h. These differences may have occurred after batch pasteurization, since the initial pH value was obtained before this step.

The fermentation of CSBJ by *L. plantarum* led to a solution with a [redacted] pH value when compared to the juice fermented by *L. paracasei*. Different strains of lactobacilli could naturally lead to very different products, including differences in the final pH value of the fermented juice (Di Cagno, Coda, De Angelis, & Gobbetti, 2013). Most of the values obtained by using *L. plantarum* were [redacted] than the value obtained in CSBJ at 0 h

(starting value). It did look as if the strain of *L. plantarum* could somehow modulate the pH to [redacted] values, an ability that was not observed after *L. paracasei* fermentation (Table 2). [redacted]. In addition, different strains may also have different metabolic rates, also influencing the pH value during and after fermentation (Markkinen et al., 2018).

The TSS content was also investigated. This reading could elucidate the success of the fermentation, as it had been previously seen to decrease over the fermentation process (Chniti et al., 2017). The use of CSBJ aimed at two different things: (1) to facilitate dissipation of the inoculated strain over the fermentation process, and (2) to facilitate the TSS reading and make it closer to the sugar content. The TSS values were found to be non-significantly different when comparing both blanks, showing that storing the CSBJ at 30 ºC during 48 h did not affect the TSS present in the matrix. In contrast, the inoculated juice with either *L. plantarum* or *L. paracasei* led to a drop in the TSS value, being significant for all the varieties and all the variations in the fermented solution (i.e. yeast extract addition). This clearly showed that the inoculation of both strains had an effect on some compounds constituting the TSS value. In other words, the results suggested that the fermentation of CSBJ was successfully performed. The sugar content would help to elucidate this theoretical conclusion.

8.3.3. Phytochemical composition of the fermented juice

8.3.3.1. Sugars

The phytochemical composition of the fermented juice was performed on the organic acid and the sugar profile. The methodology successfully identified all the important sugar and organic acids from the juice (Figure 4, 5). Most of the derivatized sugars and organic acids eluted between the minute 27 and 32, with all peaks being easily identifiable. The addition of yeast extract brought additional compounds to the chromatogram, but the identity of these compounds was not analyzed further. The fermentation did not considerably increase any unknown compound.

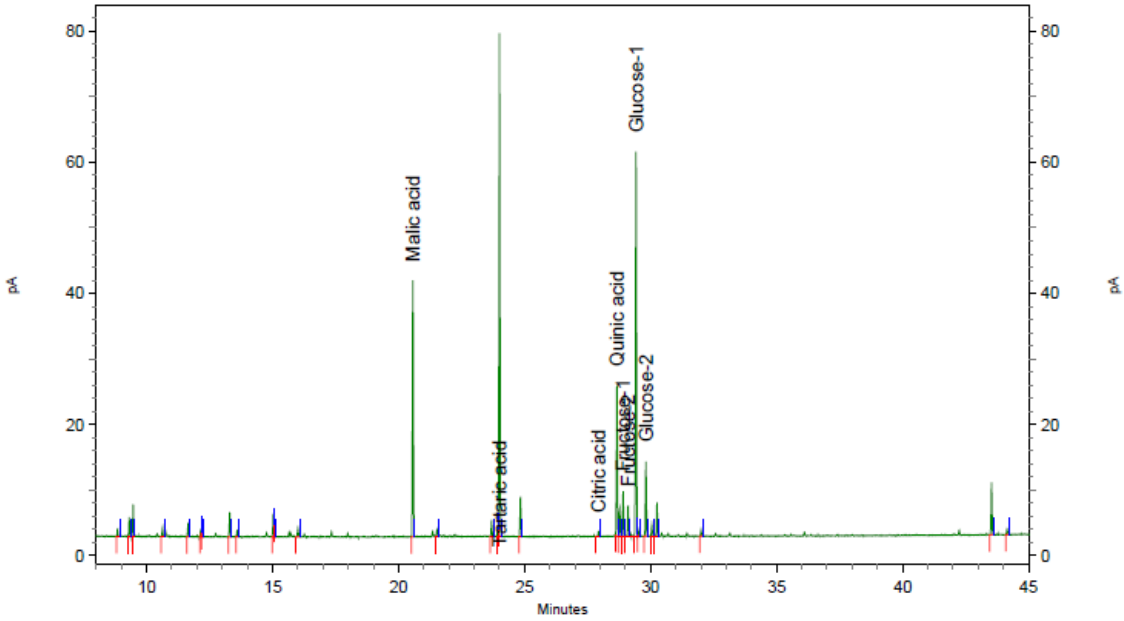

Figure 4. Chromatogram of the non-fermented CSBJ without yeast extract (0h). Tartaric acid was used as IS.

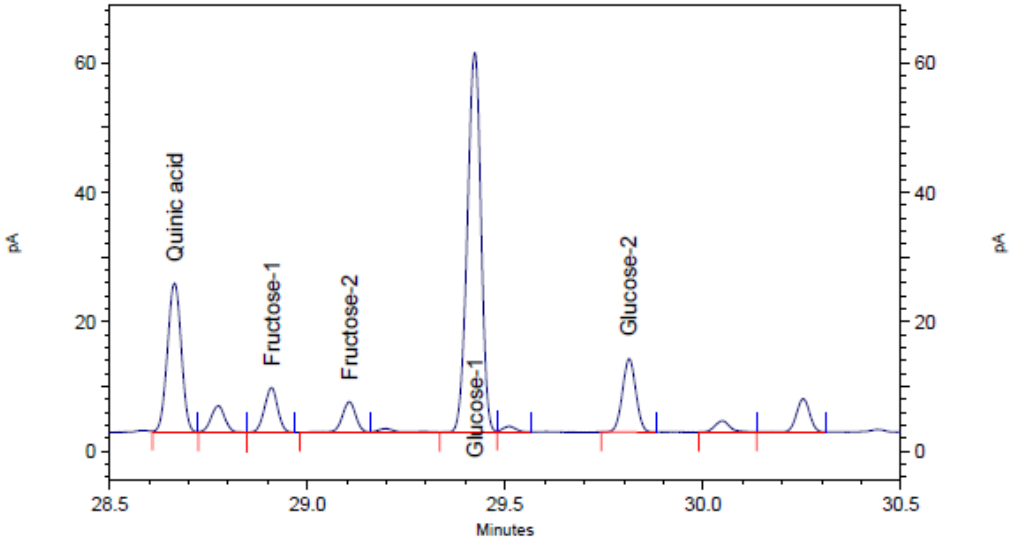

Figure 5. Magnified region of the chromatogram from Figure 4.

To understand the real changes in the sugar profile and concentration after the fermentation, the most abundant sugars in CSBJ were quantified (i.e. glucose and fructose). There were slight differences between the blanks at 0 h and 48 h, in most cases even showing statistical significance. Yet the results were very close, and differences could have emerged derived from the low standard deviation of the results.

Overall, there was a statistically significant decrease in glucose concentration when comparing any fermented solution (by *L. plantarum* or *L. paracasei*) with the corresponding uninoculated solution at 48 h (Table 2). This reduction was not observed in the sample of Spain with yeast extract, which can be explained by the inexplicably low value found in the blank sample from Spain and yeast extract at 48 h. This result difficulted the full understanding of the tendency for this specific solution, which could derive from a sampling management error during analysis or during fermentation. Other authors reported an average decrease in glucose concentration of 0.1 g / 100 ml in SBJ of different varieties fermented with another species of lactobacilli (*O. oeni*) together with an average drop in ºBrix of 0.5 after only 18 hours of fermentation (Tiitinen et al., 2007). Other authors also reported small variations of the total sugars after fermenting sea buckthorn juice with *L. plantarum* for 72 hours (Markkinen et al., 2018). [redacted]

[redacted], suggesting a successful fermentation of the CSBJ.

Interestingly, the concentration of glucose in berries from the variety of Spain almost two-folded the concentration of glucose in berries from the same variety grown in Latvia (Table 2). Yet this did not affect the drop in glucose concentration after its fermentation. Besides, the addition of yeast extract did not influence glucose concentration in any sample. Yeast extract was expected to bring important amounts of vitamins, minerals and other trace elements as well as an important protein fraction among other components. Carbohydrates in yeast extract were previously reported at levels of 12 g / 100 g of dry product (Vieira et al., 2016), although very low quantities would be identified as sugar compounds.

The second most important sugar found in sea buckthorn juice was fructose (Figure 4, 5). Results from the analysis of sugars showed [redacted] CSBJ solution after 48 hours of incubation (Table 2). [redacted]. The decrease in fructose concentration seemed to range from [redacted] of CSBJ. It was interesting to see that the fermentation by *L. plantarum* achieved [redacted] whereas the fermentation by *L. paracasei* achieved a [redacted]

[redacted] of fructose content in the fermented juice, therefore proving the different metabolic activity of both microorganisms used. The addition of yeast extract [redacted]. It should be noted that the abnormality observed in the glucose concentration of the solution with berries from Spain with yeast extract when compared to the same solution without yeast extract was also spotted when analyzing the fructose content (Table 2). This added further evidence that this drop was derived from a methodical or experimental error from this specific sample.

Fructose concentration [redacted] in previous studies after only 18 hours of fermentation by other lactobacilli in different varieties, although the differences were very small (Tiitinen et al., 2007). Nevertheless, in a recent study, fructose nor glucose concentration were affected after fermentation of SBJ by different *L. plantarum* subspecies when compared to the uninoculated juice after 72 hours of incubation (Tkacz et al., 2020), [redacted]. However, to the best of my knowledge, this was the first experiment involving a previous chemical modulation of pH to potentiate the growth of the microorganisms used and thus optimize the fermentation of the juice. This could be the reason why [redacted].

Finally, the sum of glucose and fructose concentration did not achieve the values of TSS reported in the present experiment, which indicated that there were other soluble solids at a high percentage that could influence the reading in terms of ºBrix. For instance, other more complex carbohydrates may not be analyzed using the present technique. Yet it seemed clear that the drop in TSS was derived from the consumption of fructose and glucose by the microorganisms used.

8.3.3.2. Organic acids

The organic acid profile was of great interest, since sea buckthorn juice has great concentrations of organic acids, being quinic acid and malic acid the most relevant (Tiitinen, Hakala, & Kallio, 2005). In addition, fermentation with lactic acid bacteria was expected to influence the concentration of different organic acids. More specifically, a negative association was expected on the final concentration values of malic acid and lactic acid as a result of the malolactic fermentation (Markkinen, Laaksonen, Nahku, Kuldjärv, & Yang, 2019; Tiitinen et al., 2007).

Although the changes in quinic acid were not expected to be relevant to a high degree, quinic acid was still one of the major organic acids found in sea buckthorn juice (Tiitinen, Yang, Haraldsson, Jonsdottir, & Kallio, 2006) (Figure 4). Therefore, the concentration of quinic acid was also to be monitored. Quinic acid was found to be at similar levels when compared to

previous fermentation studies (Tiitinen et al., 2006; Tiitinen et al., 2007; Tkacz et al., 2020). Quinic acid did not follow a clear tendency over the fermentation of CSBJ at 30 ºC for 48 h (Table 2), showing a large variation in the concentration levels in all samples. The fermentation by either strain did not show an important and constant variation on the quinic acid concentration when analyzing the concentration in all different solutions, although the fermentation by *L. plantarum* did result in overall lower quinic acid values when compared to the fermentation by *L. paracasei*. Other authors did not observe a variation in quinic acid concentration after 18 hours of fermentation by *O. oeni* (Tiitinen et al., 2007) or after 72 hours of fermentation by different strains of *L. plantarum* (Tkacz et al., 2020). The small, yet in some cases statistically significant, variations in the concentration of this organic acid in the studied solutions show that the concentration of quinic acid would not be easily forecast when fermenting CSBJ by probiotic lactic acid bacteria.

In contrast, the concentration of malic acid and lactic acid was expected to change dramatically due to malolactic fermentation. Although none of the strains used in the present experiment was selected by its strong ability to perform the malolactic fermentation, the strains were reported to have the ability of conversion of malic to lactic acid, and thus the monitoring of malic and lactic acid could also give some clue on the performance of the fermentation. On one hand, malic acid [redacted]

[redacted] (Table 2), subsequently indicating that the lactobacilli [redacted]. The concentration of malic acid after storage at 30 ºC for 48 h was slightly different than the values at 0 h (Table 2). Nevertheless, the differences accounted for less than [redacted]. [redacted] only occurred after the fermentation of CSBJ. [redacted] malic acid was observed in all [redacted] during fermentation besides the use of lactic acid bacteria. [redacted] reported a partial depletion of malic acid during fermentation by lactobacilli (Tiitinen et al., 2007) or by different strains of *L. plantarum* (Markkinen et al., 2019; Markkinen et al., 2018; Tkacz et al., 2020). [redacted]. Yet again, the modification of CSBJ pH before fermentation may have been helpful for the lactic acid bacteria to grow [redacted]. [redacted]

[redacted] lactic acid was expected to rise as a consequence of the metabolic activity of lactic acid bacteria. Some authors suggested the rise in lactic acid concentration to be proportional to the

degradation of malic acid after fermentation of sea buckthorn juice (Tiitinen et al., 2007). [redacted] (Table 2). Lactic acid was not present in either blank, [redacted] differences in the conversion ratio of lactic: malic acids depending on the strain used for the fermentation (Markkinen et al., 2018; Tkacz et al., 2020). No differences were observed between solutions [redacted]. As happened with the depletion of malic acid, [redacted]. The results indicated that when rising the pH of CSBJ to [redacted], the [redacted] or the use of [redacted] did not influence the conversion of malic to lactic. Nevertheless, other [redacted] and [redacted] should be used to further test this conclusion. Besides, the malolactic fermentation performed by different lactic acid

bacteria may be beneficial to the overall organoleptic taste of CSBJ, most importantly to its sourness (Tiitinen et al., 2006).

The analysis of sugars and organic acids also yielded other non-relevant peaks which summed together achieved relevant concentration values (Table 2). Nevertheless, the higher values were reported for those samples containing yeast extract. Evidently, the addition of yeast extract was adding some compounds that could be derivatized and were efficiently eluted using the same analytical technique. Nevertheless, the fermented samples did not show to have great amounts of these specific compounds (Table 2). Furthermore, no other compound was identified as a relevant peak which could derive from the fermentation process, and therefore samples were not further treated.

Table 2. Results from the physic-chemical and phytochemical analysis of fermented and unfermented CSBJ.

	Spain w/o YE				Latvia w/o YE			
	Non-fermented (0 h)	**Non-fermented (48 h)**	***L. plantarum***	***L. paracasei***	**Non-fermented (0 h)**	**Non-fermented (48 h)**	***L. plantarum***	***L. paracasei***
Physic-chemical								
pH	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
TSS	$8.0^{c} \pm 0.1$	$7.9^{c} \pm 0.1$	[redacted]	[redacted]	$6.0^{d} \pm 0.0$	$6.0^{d} \pm 0.1$	[redacted]	[redacted]
Sugars								
Glucose	$3.654^{a} \pm 0.050$	$3.421^{ab} \pm 0.248$	[redacted]	[redacted]	$2.348^{d} \pm 0.012$	$1.961^{e} \pm 0.051$	[redacted]	[redacted]
Fructose	$0.755^{a} \pm 0.014$	$0.738^{a} \pm 0.053$	[redacted]	[redacted]	$0.519^{c} \pm 0.003$	$0.444^{cd} \pm 0.008$	[redacted]	[redacted]
Organic acids								
Quinic acid	$0.954^{a} \pm 0.013$	$0.908^{ab} \pm 0.067$	[redacted]	[redacted]	$0.873^{b} \pm 0.005$	$0.779^{c} \pm 0.019$	[redacted]	[redacted]
Malic acid	$2.053^{bc} \pm 0.018$	$2.010^{bc} \pm 0.153$	[redacted]	[redacted]	$2.413^{a} \pm 0.014$	$2.119^{b} \pm 0.051$	[redacted]	[redacted]
Lactic acid	ND	ND	[redacted]	[redacted]	ND	ND	[redacted]	[redacted]
Ascorbic acid	$0.099^{a} \pm 0.003$	$0.070^{c} \pm 0.010$	[redacted]	[redacted]	$0.082^{bc} \pm 0.011$	$0.050^{d} \pm 0.002$	[redacted]	[redacted]
Other compounds								
Polyphenols	$1.392^{f} \pm 0.020$	$1.161^{g} \pm 0.022$	[redacted]	[redacted]	$1.006^{h} \pm 0.006$	$0.856^{i} \pm 0.036$	[redacted]	[redacted]
Other sugar and organic acids	$0.643^{h} \pm 0.003$	$0.622^{h} \pm 0.077$	[redacted]	[redacted]	$0.355^{i} \pm 0.005$	$0.319^{i} \pm 0.000$	[redacted]	[redacted]

Missing value. TSS: Total Soluble Solids measured as ºBrix.

w/o YE: without yeast extract; w YE: with yeast extract.

Sugars and organic acids expressed in [redacted] ml of juice. Polyphenols expressed as mg GAE / ml of juice.

Table 2 (*continued*). Results from the physic-chemical and phytochemical analysis of fermented and unfermented CSBJ.

	Spain w YE				Latvia w YE			
	Non-fermented (0 h)	**Non-fermented (48 h)**	***L. plantarum***	***L. paracasei***	**Non-fermented (0 h)**	**Non-fermented (48 h)**	***L. plantarum***	***L. paracasei***
Physic-chemical								
pH								
TSS	11.8^{a} ± 0.3	11.8^{a} ± 0.3			10.3^{b} ± 0.6	10.0^{b} ± 0.0		
Sugars								
Glucose	3.363^{b} ± 0.012	2.224^{de} ± 0.031			2.442^{d} ± 0.045	1.874^{e} ± 0.004		
Fructose	0.681^{b} ± 0.001	0.478^{c} ± 0.004			0.525^{c} ± 0.012	0.414^{d} ± 0.002		
Organic acids								
Quinic acid	0.860^{b} ± 0.000	0.575^{f} ± 0.007			0.879^{b} ± 0.017	0.717^{d} ± 0.000		
Malic acid	1.855^{d} ± 0.008	1.275^{e} ± 0.012			2.443^{a} ± 0.051	1.968^{c} ± 0.019		
Lactic acid	ND	ND			ND	ND		
Ascorbic acid	0.097^{ab} ± 0.008	0.039^{e} ± 0.000			0.073^{c} ± 0.005	MV		
Other compounds								
Polyphenols	2.865^{a} ± 0.070	2.274^{c} ± 0.021			1.958^{d} ± 0.029	1.922^{d} ± 0.033		
Other sugar and organic acids	1.743^{bc} ± 0.09	1.074^{f} ± 0.008			1.705^{c} ± 0.049	1.297^{e} ± 0.054		

Missing value. TSS: Total Soluble Solids measured as ºBrix.

w/o YE: without yeast extract; w YE: with yeast extract.

Sugars and organic acids expressed in g / 100 ml of juice. Polyphenols expressed as mg GAE / ml of juice.

8.3.3.3. Ascorbic acid, total phenolics, and antioxidant activity

One of the most important organic acids of sea buckthorn is ascorbic acid (Chapter 1). Ascorbic acid is an important contributor to the antioxidant capacity of fruit juices (Chapter 4, 7). The organic acids and sugars analysis showed the concentration in ascorbic acid, which is not the same as the total vitamin C content (which also includes dehydroascorbic acid). Nevertheless, ascorbic acid is found at greater concentrations than dehydroascorbic acid, making the concentration of ascorbic acid a good indicator of the total vitamin C content (Chapter 4). Regardless of the fermentation aim, the monitoring of the most important bioactive compound in SBJ was justified, even more when the compound is highly oxidizable. In fact, the drop in ascorbic acid concentration after the uninoculated solutions were incubated at 30 ºC for 48 h was statistically significant for all the solutions (Table 2), indicating a significant loss of this component due to oxidation.

Interestingly, the fermentation by lactic acid bacteria [redacted]. Since the concentration of ascorbic acid [redacted]

[redacted]

Surprisingly, the ascorbic acid concentration at 0 h [redacted] (Table 2). A statistically significant [redacted]

The fermentation with *L. paracasei* led to solutions with overall [redacted] when compared to the solutions fermented by *L. plantarum* (Table 2). [redacted]

These results showed that (1) the variety of the berries could have an impact [redacted], being the reduction [redacted] in the variety having a [redacted], and (2) that the fermentation with both [redacted]

[redacted]

[redacted]

Polyphenols are also important bioactive compounds naturally found in sea buckthorn berries and actively contribute to the antioxidant capacity of the juice (Chapter 4, 7). Polyphenols are generally found in food matrices bound to other components. Flavonoid rhamnoglucosides are the most common in sea buckthorn (Guo, Guo, Li, Fu, & Liu, 2017). One of the lactic acid bacteria herein used was previously reported to break down these specific flavonoid glycosides (Mueller et al., 2018). Not only this, but the strain of *L. plantarum* used had also been shown to modify the polyphenolic fraction of pomegranate juice (Mantzourani et al., 2019). Thus, the total phenolics were checked before and after the fermentation.

There was an overall tendency of [redacted] (Table 2). Nevertheless, these [redacted] reported drops in the phenolic fraction of SBJ, after only 24 h of storage at 30 ºC, but found no statistically significant differences when comparing the total polyphenols form the inoculated samples (Tkacz et al., 2020).

The present results also showed statistically significant differences in the total phenolics when comparing the samples with yeast extract to those without yeast extract, being higher in the former. The increase in phenolic content could be derived from the nutritional profile of yeast extract, which is high in these compounds (Vieira et al., 2016).

Finally, the antioxidant capacity assay by means of DPPH was also investigated. This analysis also helped understand the variation of vitamin C or the total phenolics. The antioxidant capacity was observed to be statistically significantly different across the studied samples (Figure 6). Interestingly, the antioxidant capacity was [redacted] when comparing the same solution before and after 48 h of storage or [redacted] – with some exceptions –, indicating that the storage or [redacted] its antioxidant capacity, in line with previous studies (Tkacz et al., 2020).

The statistically significantly [redacted] when compared to the [redacted] juice was not observed in the antioxidant DPPH assay. Nevertheless, a tendency to lower values (although non-significant) in [redacted], which showed the important contribution of ascorbic acid on the total antioxidant capacity, as previously reported (Chapter 4, 6). The tendency could probably derive

from a [redacted] obtained in the [redacted] samples. This tendency of the [redacted] samples to show [redacted] content when compared to the blanks at 48 h was not reported in previous studies (Tkacz et al., 2020). In fact, Tkacz et al. (2010) reported a drop in the antioxidant activity over the whole fermentation period when compared to the original solution. Nevertheless, the authors reported an increase of 25% in the antioxidant activity after 72 h of fermentation of sea buckthorn juice using the same *L. plantarum* strain used in the present experiment. This strain was the same as previously reported to increase the antioxidant capacity of pomegranate juice in 72 h (Mousavi et al., 2011). The fermentation time herein used was 48 h, [redacted], and further research should address to give answer to that question.

The contribution or tendency observed may be limited by the important role of other antioxidant compounds, such as polyphenols, [redacted]. In any case, the incubation of the inoculated and uninoculated juices for 48 h at 30 ºC was [redacted] in the antioxidant capacity. Other authors reported changes in the antioxidant capacity of orange juices after 6 months of storage, related mainly to the drop in the vitamin C concentration (Klimczak, Małecka, Szlachta, & Gliszczyńska-Świgło, 2007).

At last, the antioxidant activity was statistically significantly different depending on the variety analyzed (Figure 6). These results were in line with previous findings in the present research (Chapter 4). In that case, the positive association between antioxidant activity and ascorbic acid content was much clearer (Figure 6, Table 2). The solutions containing yeast extract also yielded statistically significantly higher antioxidant activity when compared to the corresponding solutions without yeast extract. Even though the yeast extract did not add to the high ascorbic acid content of CSBJ, it brought other compounds, such as polyphenols (Vieira et al., 2016), in great quantities, which could explain the higher antioxidant activity observed in the solutions containing this ingredient.

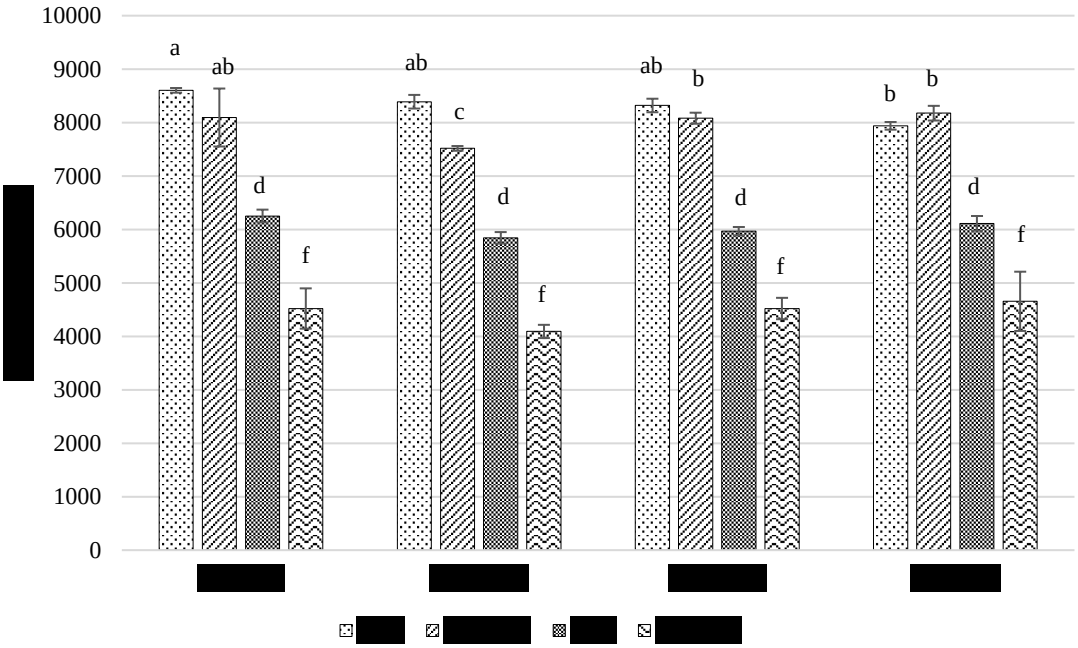

Figure 6. Antioxidant capacity measured as DPPH activity of the inoculated and uninoculated CSBJ. LYE: Latvia variety with Yeast Extract. LWOYE: Latvia variety WithOut Yeast Extract. CYE: 'Cerdanya' variety with Yeast Extract. CWOYE: 'Cerdanya' variety WithOut Yeast Extract.

8.4. Conclusions

The fermentation of CSBJ by probiotic lactic acid bacteria was successfully performed in the present study. The present study shows that the fermentation of CSBJ could derive in a product with a similar phytochemical quality than the original matrix, with the added value of containing a great amount of lactic acid probiotic bacteria. Future fermentation studies of CSBJ using these bacteria should focus on the organoleptic quality of the resulting product.

The fermented CSBJ yielded a product with no presence or residual sugar concentration, in line with the metabolic rate of the inoculated bacteria. The reduction of sugar in the matrix was negatively correlated to the amount [redacted], and together with the [redacted] indicates a successful fermentation of the juice [redacted].

There were no great differences between the samples fermented by *L. plantarum* and the samples fermented by *L. paracasei*, except for the [redacted], the former being higher and the latter being lower in the samples fermented by *L. paracasei*.

One of the most interesting outcomes from the present study was the observed [redacted]. In addition, the phenolic fraction and the antioxidant activity [redacted] the original juice, with a slight drop which could be attributed to the storage of the juice at 30 ºC during 48 h.

Referring to the other independent factors from the present study, the addition of yeast extract to the medium [redacted]. The only benefit of adding yeast extract was found to be in the added antioxidant compounds that the ingredient naturally contains, resulting in the pH to be

the most relevant factor when studying the fermentation of SBJ. Besides, the variety of the berry used did not influence the fermentation activity of any strain herein used.

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PART V

CONCLUSIONS, LIMITATIONS, AND FUTURE RESEARCH

Chapter 9: Conclusions, limitations, and future research

9.1. Conclusions

With the aim of maximizing the use of all fractions from sea buckthorn berries on the development of food supplements, several existing processing techniques have been applied on different fractions from sea buckthorn berries. In addition, the present project has also presented in full detail the theoretic and the empiric composition of sea buckthorn berries, which serves as the base to understand the applicability of the processing techniques.

Based on the exhaustive study of different varieties of sea buckthorn berries from different origins, and the study of the same variety over consecutive years, the following conclusions can be made:

- Both the variety and the growing region of the plant are two of the most important factors affecting the nutritional quality of the aqueous and the oil fraction of the resulting berry. These factors could directly affect the final nutritional composition of the resulting food supplements.

- The dilutive effect mostly observed on the aqueous fraction of the fruit plays a critical role in food supplements production, where the concentration of bioactive compounds is key for the success of any product. Nonetheless, the dilutive effect seems to be more dependent on other factors (i.e. rainfall) rather than on the variety and origin of the plant.
- The in-depth phytochemical profile from the aqueous and the oil fraction of berries from commercially available plants serves as a strong basis for the choice of the plant with the most interesting nutritional profile. This is very relevant for the expansion of the orchard of the sponsoring company.
- The analysis of the fruits cultivated in the orchard located in 'Cerdanya' (Spain) over three consecutive years was enough to understand that the nutritional profile of sea buckthorn, as in many other fruits, can significantly change over the years. In the present research, an important drop has been observed on vitamin C content over the studied years. This information will help to understand which techniques could be applied to improve the overall nutritional quality of the fruit.

In the light of the experimental results obtained from the extraction of sea buckthorn oil from sea buckthorn dried berries with green solvents and green technology, the following conclusions can be made:

- The use of polar solvents for the extraction of sea buckthorn pulp oil is unsuitable, as they can also drag out polar components present in the berries, such as sugars.
- The use of green polar solvents for the extraction of sea buckthorn oil poses a paradox, since the use of green solvents mitigate the environmental impact of the extraction process, yet it also favors further processing to wash out the dragged polar compounds and purify the lipid fraction.
- The extraction of sea buckthorn oil from dried berries with hexane at 60 ºC with ASE technology is the best technique for the rapid evaluation of the nutritional profile of the oil. It demands less amount of time compared to the conventional Soxhlet technique and the outcoming oil has a greater concentration of the most relevant bioactive compounds of sea buckthorn oil.
- The extraction pressure during supercritical CO_2 extraction of sea buckthorn oil from dried berries is positively related to higher oil yield and higher concentrations of β-carotene and α-linolenic acid in the resulting oil. In contrast, pressure is negatively related to the concentration of α-tocopherol and palmitoleic fatty acid in the sea buckthorn oil.
- The extraction temperature during supercritical CO_2 extraction of sea buckthorn oil from dried berries is positively related to the

concentration of β-carotene and it does not affect the concentration of α-tocopherol in the resulting oil. The concentrations of palmitoleic and oleic acids are similarly not affected by changes in temperature, yet stearic, linoleic, and α-linolenic acid concentration are negatively associated with temperature.

- The best extraction conditions for the extraction of both seed oil and pulp oil are [redacted] bars and [redacted] ºC. The extraction at this temperature and pressure allows for a good balance between the recovery of all bioactive compounds of interest in both oils and the extraction yield.
- The extraction of sea buckthorn oil from dried berries using supercritical CO_2 extraction is less efficient in terms of yield and recovery of most of the bioactive compounds of interest when compared to ethanol extraction at high temperature.

Finally, based on the experimental studies performed on sea buckthorn juice, the following conclusions can be drawn:

- The high presence of solids in filtered sea buckthorn juice makes unsuitable its concentration by forward osmosis due to the prone membrane fouling. The high presence of solids also difficult the separation of the concentrate from the ice in block-freeze or suspension crystallization cryoconcentration.

- The concentration of filtered sea buckthorn juice is possible up to 30 ºBrix by evaporation. Even though the solids can reduce the heat transfer, the efficiency of the process is not as reduced as in forward osmosis or cryoconcentration. [redacted]
- The use of different varieties in the formulation of juices or liquid food supplements can have an implication on the overall consumer acceptance due to the nutritional and sensorial differences of the raw sea buckthorn berries.
- The fermentation of clarified sea buckthorn juice by probiotic lactic acid bacteria can be fully performed when rising the pH of the original juice to [redacted]. The fermentation of clarified sea buckthorn juice with the pH stabilized to [redacted] leads to a product with little to no presence of [redacted] nor [redacted], high density of probiotic lactic acid bacteria cells and high [redacted] content, while having a little effect on glucose concentration.
- There exists a certain [redacted] concentration by the lactic acid bacteria on clarified, fermented sea buckthorn juice. This [redacted] may be the reason for the [redacted].

- There exists a certain protective effect of the ascorbic acid concentration by the lactic acid bacteria on clarified, fermented sea buckthorn juice. This protective effect may be the reason for the unreduced antioxidant activity after the fermentation.

To sum up, the present project has brought evidence on the processing of different fractions of sea buckthorn berries by studying the applicability of industrially available and scalable processing techniques, accompanied by in-depth studies of the resulting products. The project brings essential information on the processing of fractions derived from sea buckthorn berries which can be subsequently used to maximize their use in the development of food supplements.

The objectives and aims of this industrial doctoral thesis have been met, opening several fields on which the sponsoring company can now focus its research. Not only this, but most of the information provided by the results from this thesis could be extended to other ingredients, maximizing the use of the processing techniques herein investigated. This thesis allows the sponsoring company to achieve a top position in the field of sea buckthorn processing and product production, which is very interesting due to the high competitivity found in the food supplements market.

9.2. Limitations

All the work developed during the years 2018 to 2021 in the working frame of the doctoral project is also subject to limitations. The following are the most relevant flaws of the project:

- The phytochemical study of different commercially available varieties was only performed one year, being more than possible the change of the phytochemical profile over the years.
- The study of the same variety over three years give a clue on the evolution of the phytochemical profile, but more data should be acquired to make an assertive guess on any tendency observed.
- Most of the processing techniques herein investigated for the production of sea buckthorn oil or sea buckthorn juices concentrates were lab-scale. Although this is a necessary first step, the use of lab equipment does not allow for the control of certain parameters that could be controlled on the same industrial process.
- The studies were performed on low amounts of sample, and therefore the parameters observed and reported could vary over scaling up the process.
- The fermentation study was performed in lab test tubes, without constant low stirring to ensure a homogeneous fermentation.

9.3. Future research

Considering the interesting results from the project, future research should address the objects that were not investigated here either because of timing or because of resources. Future research should focus on:

- Investigating the changes in the phytochemical profile of the berries harvested from the orchard in Spain, which will help understand the evolution of this profile and establish different quality criteria for the development of food supplements.
- Investigating the use of co-solvents in the extraction of sea buckthorn berry oil with supercritical CO_2 technology.
- Scaling up the process of sea buckthorn oil extraction from dried sea buckthorn berries and separate the extraction of the seed oil from the extraction of the pulp oil.
- Investigating the phytochemical profile of the cake left after sea buckthorn oil extraction.
- Scaling up the process of sea buckthorn juice concentration by evaporation, monitoring all the possible parameters and working at lower processing pressures.
- Developing a liquid food supplement based on sea buckthorn juice and the hedonic experiment performed in the present project.

- Performing a scale-up of the fermentation process with a special focus on obtaining a homogeneous fermentation, and study all the possibilities of the resulting fermented product, including microencapsulation, spry-drying, or other established techniques for the development of food supplements.
- The extraction of sea buckthorn seed oil with supercritical CO_2 could be optimized by using ethanol or another green solvent as co-solvent during the extraction process. The extraction of sea buckthorn pulp oil may not be optimized the same way due to the polarity problems posed by the use of polar solvents.

These are the guidelines for future research considering the results of the present work and the interests of the sponsoring company. Nevertheless, as we live in an ever-changing, fast-paced world, the future research may also be adapted to the needs in every scenario.

Personal acknowledgements

Primer de tot, moltíssimes gràcies a la meva promesa Aïda Gabarró, sense la qual aquesta tesi mai hagués progressat amb la perseverança ni la facilitat amb que ho ha fet. Gràcies per estar-hi sempre, per fer dels moments més durs els més fàcils, per convertir els drames en alegries, les ofuscacions en lucideses i les dèries en relaxacions, per apagar tots els focs que han cremat amb paraules i amor. De tu en podria escriure una tesi més llarga que aquesta. Gràcies.

Gràcies de tot cor als meus pares. Gràcies Jordi i Montse, per ser qui sou, per haver-me donat totes les facilitats (que no són poques) perquè hagi pogut arribar on soc avui i culminar amb aquesta tesi, un somni que sense vosaltres no hagués estat possible. Gràcies pels esforços de suportar-me quan era insuportable. Gràcies per acompanyar-me sempre i per criar-me amb els valors dels que avui em sento orgullós de tenir. Us estimo.

També gràcies de tot cor als meus germans. Alba, moltes gràcies per ser una germana model. Gràcies per ensenyar-me a lluitar pels meus somnis, a seguir el camí que més he desitjat, i el més important, a fer-lo realitat. Si ho pots somiar, ho pots fer. Pere, moltes gràcies per ser el germà gran dels dos. Gràcies per acompanyar-me i ensenyar-me tant, per ajudar-me a descobrir mons nous com els castells, per ser el meu comodí, el meu aliat. Gràcies per tants moments de riures i de parides compartits, que m'han donat vida

per arribar on soc ara. Gràcies també a en Xavi per contribuir en aquests moments sempre divertits i necessaris. Sou els millors, us estimo molt.

Encara que no siguin aquí, sempre les porto al cor a les meves dues àvies Maria. Moltes gràcies àvia Maria Cabana, per ensenyar-me la capacitat de resiliència i perseverança que m'ha encaminat i ajudat a aconseguir aquest gran objectiu al llarg de tres llargs anys. Gràcies per ser tant afectuosa amb mi. Moltes gràcies a l'àvia Maria, per endinsar-me en el món del coneixement i la curiositat, per ser la veu de la raó, per preocupar-se per mi i motivar-me a fer les coses que més em costaven i que ara em recompensen més. M'hagués agradat que assistissis el dia de la meva defensa de tesi. Sempre us portaré al cor, estic molt content d'haver-vos gaudit tot el que ho he fet.

Moltes gràcies als 'titus'. A tots ells, Robert, Aleix, Cesc, Balde, Biti, Benet, Miki, Riki i Artur. Gràcies per els incomptables bons moments, tant a les terrasses dels bars quan es podia anar a sopar (encara que em costés), com a les terrasses privades els dies de pandèmia. Amb vosaltres, la tesi, i la pandèmia, han sigut incontestablement plàcides i fàcils de portar. Gràcies per contribuir positivament a la meva salut mental en les tardes de birres, jocs de taula, pàdels o escalada, o en les espectaculars nits de CoD que sempre quedaran en la memòria. Gràcies per tant. També gràcies a en Gianni i en Gabri, que encara que us vegi menys per coses de la vida, sé

que sempre heu estat i sempre estareu allà quan us necessiti, igual que jo per vosaltres. Sou la meva segona família.

Permanentment al meu cap, al germà del carrer Salut, moltes gràcies Isart per ser-hi sempre que t'he necessitat. M'hagués agradat compartir més moments amb tu a U.K., però la pandèmia em va fer abandonar el projecte abans d'hora. Gràcies per ajudar-me amb la mudança tant quan vaig anar com quan vaig tornar. Espero que ens tornem a veure ben aviat!

Gràcies a la Paulina i a en Diego, per contribuir substancialment al meu benestar mental en incomptables tardes plenes de riures i jocs de taula, així com per modificar els plats més carnívors per opcions vegetarianes. Espero que algun dia les opcions vegetarianes també siguin per vosaltres. Seguirem lluitant!

També gràcies als nous veïns des del 2019. Gràcies Lea i Benet per ser els millors veïns que poguéssim tenir. Gràcies per cuidar d'una terrassa que m'ha salvat en determinats moments, gràcies per les nits de jocs, per les *raclettes*... Gràcies per tant, gràcies per ser-hi.

Finalment, moltes gràcies a totes les persones del despatx al que vaig poder treballar a la Universitat de Reading, qui van fer de la meva estada curta una estada millor. Moltes gràcies per ajudar-me en l'adaptació i compartir els dinars amb mi.

Valoració personal

Recordo que al grau em costava posar-m'hi. Però després tot va canviar. L'aventura de fer un màster a l'estranger, inculcada pels meus pares i amb el suport incondicional de l'Aïda, que també s'aventurava a ser una part imprescindible d'aquest èxode, va ser un punt d'inflexió en la meva vida. Tot va passar molt ràpid. L'acceptació a Wageningen University, fer l'IELTS a corre cuita amb la nota justa per entrar, tota la burocràcia que es precisava... Ho recordo tot perfectament però molt ràpid.

Al segon any del màster, vaig fer dos coses que van ser el punt d'inflexió per decidir, just després d'acabar el màster, fer un doctorat. O almenys intentar-ho. El curs d'estadística i la tesi de màster d'investigació em van fer veure el que volia. Mai he sigut una persona amb les idees clares. Sempre he tingut els meus dubtes en totes les decisions que he pres al llarg de la meva vida. Però això si que ho tenia clar. Volia fer un doctorat. Però no en l'àmbit en el que havia fet el màster que era la qualitat. Així, derivat de les meves ganes de canviar l'enfoc de la meva carrera professional, vaig decidir fer el màster en nutrició i metabolisme, que em va obrir les portes a fer un doctorat en el grup de recerca 'Antioxidants naturals: polifenols', liderat per la Rosa, a qui sempre agrairé que em donés aquesta oportunitat.

Però la vida tenia un altre pla per mi. Així, es va obrir un doctorat industrial a la UAB en col·laboració amb Vitae, al que vaig sol·licitar plaça. El

doctorat el vaig començar a cegues, amb moltes coses per investigar d'un sol producte: l'arç groc. En molts casos al llarg del doctorat, m'he plantejat la idea de si realment això és un doctorat com a tal. Molts doctorats, per no dir tots els que conec, son investigacions molt minucioses d'un tema en concret. És com si agaféssim un capítol dels que he estudiat al llarg de la tesi i en féssim una investigació molt més profunda. Però aquesta tesi ha sigut totalment diferent, i això em preocupava. Realment un projecte tan ampli podia ser defensat com a tesi doctoral?

Vist en perspectiva, la idea que hagi tocat tants camps no només m'enriqueix personalment, sinó que obre un munt de ventalls professionals inesgotables alhora d'investigar diferents processos de tractaments d'aliments. I no només per l'arç groc, sinó per moltes fruites i productes similars que es podrien beneficiar de l'aplicació de qualsevol de les tècniques estudiades en aquesta tesi. Així que, en comptes de donar-me una única opció de treball molt específica i seguir una línia d'investigació tancada, aquest doctorat m'ha obert les portes a una formació àmplia en diverses tècniques de processament d'aliments.

Aquesta tesi és, per mi, un objectiu assolit, la meta pel que he estat tots aquests anys lluitant de forma incansable, imparable. La tesi arriba després de molts dies i moltes hores de dedicació, després de moltes situacions i èpoques d'estres i d'angoixa. Personalment, la tesi ha suposat una constant

preocupació mental, però ho he anat superant amb totes les persones del meu voltant a les qui he agraït profundament la seva presència en aquests moments difícils. De fet, durant la tesi no només he patit per aquesta, sinó que he tingut un dels moments més tristos i difícils de la meva vida amb la mort sobtada de la meva àvia Maria, qui m'hagués agradat que hagués pogut ser aquí el dia de la defensa. Qui es pensaria que el petó que li vaig donar a l'hospital un dia d'agost del 2018 fora l'últim. Àvia, allà on siguis, espero que estiguis orgullosa de mi.

Però la tesi també ha portat coses bones. Moments de felicitat, moments de creixement professional (amb l'ajuda concedida del CDTI) i moments d'alegria. Encara que si haig de fer un balanç, per mi han pesat més les preocupacions i angoixa que els moments feliços. De forma breu, no tornaria a fer una segona tesi. Tot i que ara estigui molt content d'haver arribat on soc, no ha sigut fàcil. I el millor d'haver-hi arribat, és compartir aquest moment amb les persones que més estimo.

Espero que després d'acomplir aquesta fita, en vinguin moltes altres iguals o més interessants, per les que seguir lluitant, formant-me i guanyant una experiència que em permetrà seguir creixent personal i professionalment en el món que més m'agrada: la ciència dels aliments.

To the challenges of the future I say: I am ready!