UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di *Ingegneria Industriale*

SCUOLA DI DOTTORATO DI RICERCA IN: INGEGNERIA INDUSTRIALE
INDIRIZZO: INGEGNERIA CHIMICA, DEI MATERIALI E MECCANICA
CICLO XXVIII

SUPERCRITICAL CO_2 TECHNOLOGIES FOR THE PRODUCTION OF BIOACTIVE COMPOUNDS FROM NATURAL SOURCES

Direttore della Scuola: Ch.mo Prof. Paolo Colombo
Coordinatore d'indirizzo: Ch.mo Prof. Enrico Savio
Supervisore: Ch.mo Prof. Alberto Bertucco

Dottorando: Miriam Solana Ciprés

"If you want to build a ship, don't drum up the men to
gather wood, divide the work, and give orders. Instead,
teach them to yearn for the vast and endless sea."

— Antoine de Saint-Exupéry

Contents

Abstract

This Ph.D. project was aimed to evaluate the potential of supercritical CO_2 technologies for obtaining natural extracts rich in bioactive compounds from residual agricultural biomass and microalgae. Three different supercritical CO_2 techniques have been investigated with the purpose of optimizing the operating conditions and assessing the possibility to obtain the desired products in a safe, green and efficient way.

First, attention has been focused on supercritical CO_2 extraction (SFE). Experimental work regarding essential fatty acids extraction from microalgae and the application of solubility and kinetic models to the data measured are reported. Another application concerns the SFE of different bioactive compounds from rocket salad. In this case, a sequential extractive approach is proposed, consisting in the extraction of two products by using different co-solvents: the first one rich in phenols and glucosinolates and the second step headed to extract lipids. A profitability analysis of the SFE process is also reported, where data from experimental results have been used together with large scale process simulations. Eventually, SFE is applied to asparagus for the effective recovery of phenolic compounds and is compared with Soxhlet and pressurized liquid extraction methods.

Another investigation has been carried out to obtain a dried natural extract from cherries. In this case, the supercritical anti-solvent technique (SAS) has been investigated, which allows to precipitate compounds non soluble in CO_2 previously extracted by a traditional solvent method. The operating conditions were optimized to obtain a precipitate rich in polyphenols and anthocyanins from cherries extracts.

Eventually, it is presented a study about supercritical counter-current fractionation for the extraction of fat from soy skim, which is the aqueous residue after enzyme-assisted aqueous extraction of soybeans.

In the conclusion, a summary of the potential of CO_2 in natural extracts technology is outlined and some perspectives are discussed for industrial applications in the near future.

Riassunto

La richiesta di estratti naturali e composti di origine vegetale sta aumentando a causa del loro crescente uso in alimenti funzionali, medicina naturale, additivi per la salute, prodotti cosmetici e applicazioni farmaceutiche. Questi settori richiedono prodotti ultra-puliti, verificabili, di alta qualità, apparecchiature affidabili e prezzi competitivi.

Finora, sono state studiate numerose fonti vegetali come materie prime per la produzione di estratti naturali. L'idea della trasformazione dei rifiuti alimentari è sempre più presa in considerazione anche perché è vantaggiosa rispetto ai problemi di contaminazione, di gestione del processo e delle vie economiche. Inoltre, le fonti naturali emergenti come le microalghe contengono elevate quantità di composti di alto valore, la cui estrazione potrebbe essere ambientalmente ed economicamente vantaggiosa.

In questo contesto, è necessario mettere a punto un metodo di estrazione e/o separazione efficiente e "verde" non solo per elaborare gli alimenti non vendibili e i rifiuti e sfruttare le fonti naturali emergenti, ma anche per creare un prodotto molto richiesto nel mercato attuale.

La tecnologia della CO_2 supercritica è emersa come un'importante alternativa ai processi tradizionali con solventi organici e meccanici, grazie alla sua pressione critica moderata, che permette di contenere i costi di compressione, mentre la sua bassa temperatura critica consente l'estrazione di composti termosensibili senza degradazione. Altri vantaggi rispetto ai metodi di estrazione con solventi classici includono il fatto che la CO_2 è inerte, non tossica e rispettosa dell'ambiente. Inoltre, nei processi supercritici, la CO_2 viene facilmente rimossa dopo la depressurizzazione e permette cicli più veloci. D'altra parte, la natura polare della maggior parte dei composti naturali rende necessaria l'aggiunta di co-solventi alla CO_2 supercritica, al fine di migliorare l'affinità del fluido verso composti polari, e il loro effetto è rilevante sulla composizione dell'estratto e di conseguenza sull'economia del processo. In questo caso, è importante utilizzare solventi ecologici, come acqua ed etanolo, al fine di mantenere i vantaggi dei processi supercritici.

L'estrazione supercritica con la CO_2 è il processo più studiato ed applicato tra quelli che usano la CO_2 sotto pressione. Tuttavia, c'è un certo numero di tecnologie supercritiche che vengono

studiate e sviluppate per altre applicazioni interessanti, come la precipitazione di composti polari (precipitazione supercritica con anti-solvente, SAS), che permette di ottenere precipitati secchi naturali, o la separazione di composti in miscele liquide (frazionamento supercritico in contro-corrente) per l'ottenimento di composti bioattivi più puri.

L'ottimizzazione dei processi e delle variabili operative per estrarre composti di interese dalle nuove fonti naturali sono importanti per garantire rese massime di alta qualità e rendere il prodotto finale adatto per l'uso nelle industrie alimentari, cosmetiche e farmaceutiche. Perciò, è fondamentale continuare la ricerca delle tecnologie con la CO_2 supercritica per diversi materiali e generare nuovi dati che possano essere utili per il potenziale scale-up dei processi proposti.

Per tutti questi motivi, l'obiettivo di questo progetto di ricerca è stato quello di valutare il potenziale delle tecnologie supercritiche con CO_2 per ottenere in modo sicuro, verde ed efficiente nuovi estratti naturali ricchi di composti bioattivi da prodotti agricoli e microalghe.

I temi affrontati da questa tesi sono organizzati e suddivisi in capitoli come segue.

Il capitolo 1 è una discussione introduttiva sul mercato degli estratti naturali, la situazione dei diversi metodi di estrazione, le ricerche e gli ultimi risultati riportati per le tecnologie supercritiche con CO_2.

I capitoli 2, 3, 4 e 5 presentano i risultati sperimentali e la modellizzazione eseguite sulla estrazione con CO_2 supercritica da diverse fonti naturali, con lo scopo di verificare l'efficacia di questo metodo per ottenere in modo competitivo estratti naturali ricchi di diversi composti bioattivi. Nel capitolo 2, viene mostrata l'estrazione di acidi grassi essenziali con CO_2 supercritica da tre diverse specie di microalghe. Viene studiato l'effetto delle variabili operative sulla resa di estrazione totale e sulla solubilità. Vengono applicati i modelli matematici sviluppati da Sovová per descrivere le curve di estrazione sperimentali. Nel capitolo 3 viene riportata l'estrazione di frazioni arricchite in diverse classi di composti bioattivi. Secondo i risultati, si propone l'applicazione di un metodo di estrazione sequenziale, utilizzando prima CO_2 + etanolo per l'estrazione dei lipidi e poi l'acqua come co-solvente per ottenere estratti ricchi in composti fenolici e glucosinolati. Il capitolo 4 descrive la valutazione economica di un impianto su scala industriale per la produzione di estratti naturali ricchi di glucosinolati e composti fenolici di rucola. Il software Aspen Plus ™ V8.2 è stato impiegato per la simulazione del processo su larga scala, basandosi sulle misure sperimentali di laboratorio. Viene valutato l'effetto dei parametri operativi sui costi di processo. Il capitolo 5 è imperniato sul recupero dei composti fenolici da asparagi. Ulteriormente, viene esaminato l'effetto di miscele di co-solventi diverse sulla estrazione con CO_2 supercritica per estrarre selettivamente molecole di polifenoli. I risultati sono confrontati con l'estrazione attraverso un liquido sotto pressione (PLE) e con il metodo Soxhlet.

Inoltre, è stato applicato il processo supercritico con anti-solvente (SAS), con l'obiettivo di ottenere un precipitato essiccato ricco di composti antiossidanti. Il capitolo 6 è focalizzato sul processo SAS con CO_2 per ottenere precipitati che sono ricchi di polifenoli e antociani composti da ciliegie. Vengono confrontati il modo continuo e batch di funzionamento. Inoltre, sono discussi l'effetto della pressione e della composizione della CO_2 sui rendimenti di precipitazione di polifenoli e antociani.

Il terzo metodo che è stato studiato è il frazionamento in controcorrente per la separazione dei composti di interesse di una miscela liquida. Nel capitolo 7, viene riportata la verifica del impianto di frazionamento con CO_2 in colonna continua riempita. A tal fine, è stato eseguito il recupero di butanolo da soluzioni acquose. L'influenza delle variabile operative, come il rapporto delle portate del solvente e la soluzione, la temperatura, la pressione e la composizione della soluzione è stato studiato sperimentalmente in termini di efficienza di separazione, percentuale tasso di rimozione del butanolo, rimozione totale e di concentrazione butanolo nell'estratto alla fine del ciclo continuo. Nel capitolo 8 è presentato l'uso della CO_2 in controcorrente come mezzo per ridurre il grasso residuo nella soia dopo l'estrazione acquosa enzimatica assistita della soia. In particolare, vengono analizzati gli effetti del rapporto di solventi da alimentare, dell'aggiunta di etanolo come modificatore e dell'introduzione di un riempimento nella colonna. L'interpretazione dei risultati è stata effettuata mediante l'analisi statistico ANOVA.

Infine, nelle conclusioni, sono discussi la sintesi della tesi e gli aspetti che dovrebbero essere messi a fuoco per garantire il futuro di questa tecnologia.

Foreword

This research project was developed at the Department of Industrial Engineering of the University of Padova (Italy), in the laboratory of High Pressures Technologies, under the supervision of Prof. Alberto Bertucco. Part of the work reported in this Thesis (chapters 7 and 8) has been carried out at the Functional Foods Research Unit of the United States Department of Agriculture (Peoria, Il, USA) under the supervision of Fred J. Eller, PhD.

The fulfilment of the research project presented has involved the financial support of Cassa di Risparmio del Veneto, to whom the author grateful acknowledges. The author is also gretarly acknowledged with the United States Department of Agriculture.

As a tangible result of the work completed during the Ph.D. school, a number of publications and presentations to conferences have been produced, as listed below.

Publications in Refereed Journals

1. **Solana, M.**, Mirofci, K., Bertucco, A., Production of phenolic and glucosinolate extracts from rocket salad by supercritical fluid extraction: Process design and cost benefits analysis, 2016, Journal of Food Engineering, 35-41.
2. **Solana, M.**, Boschiero, I., Dall'Acqua, S., Bertucco, A., A comparison between supercritical fluid and pressurized liquid extraction methods for obtaining phenolic compounds from *Asparagus officinalis* L, 2015, The Journal of Supercritical Fluids 100, 201-208.
3. **Solana, M.**, Boschiero, I., Dall'Acqua, S., Bertucco, A., Extraction of bioactive enriched fractions from *Eruca sativa* leaves by supercritical CO_2 technology using different co-solvents, 2014, The Journal of Supercritical Fluids 94, 245-251.
4. **Solana, M.**, Rizza, C.S., Bertucco, A., Exploiting microalgae as a source of essential fatty acids by supercritical fluid extraction of lipids: Comparison between *Scenedesmus obliquus, Chlorella protothecoides* and *Nannochloropsis salina*, 2014, The Journal of Supercritical Fluids 92, 311-318.

5. Hernandez, D., Riaño, B., Coca, M., **Solana, M.**, Bertucco, A., Garcia-Gonzalez, M.C., Microalgae cultivation in high rate ponds using slaughterhouse wastewater for biofuel applications, 2015, Chemical Engineering Journal, accepted for publication.[1]
6. Hernandez, D., **Solana, M.**, Riaño, B., Garcia-Gonzalez, M.C., Bertucco, A., Biofuels from microalgae: lipid extraction and methane production from the residual biomass in a biorefinery approach, 2014, Bioresource Technology 170, 370-378.[1]

Papers submitted for publication in Refereed Journals

1. **Solana, M.**, Teel, J., Hojilla, M., Bertucco, A., Eller, F., Counter-current carbon dioxide extraction of fat from soy skim, sent to Journal of Supercritical Fluids.
2. **Solana, M.**, Qureshi, N., Bertucco, A., Eller, F., Recovery of butanol by counter-current carbon dioxide fractionation with its potential application to butanol fermentation, sent to Separation and Purification Technology.

Papers or abstracts in Conference Proceedings

1. **Solana, M.**, Bertucco,A., Supercritical Carbon Dioxide Extraction Of Lipids From Microalgae, 6th International Symposium on High Pressure Processes Technology, Belgrade (Serbia), 2013.
2. **Solana, M.**, Dall'Acqua, Boschiero, I., Bertucco, A., Glucosinolates and fatty acids extraction from *Eruca sativa* leaves through supercritical technology, 14th European Meeting on Supercritical Fluids, Marseille (France), 2014.
3. **Solana, M.**, Hernandez, D., Garcia-Gonzalez, M.C., Bertucco, A., Microwave as a pre-treatment to facilitate the supercritical extraction of lipids from microalgae, 14th European Meeting on Supercritical Fluids, Marseille (France), 2014.
4. Hernandez, D., Riaño, B., **Solana, M.**, Garcia-Gonzalez, M.C., Bertucco, A., Growth of microalgal biomass in raceways using agroindustrial wastewater: Biomass production and fatty acids accumulation, 10th International Conference on Renewable Resources and Biorefineries, Valladolid (Spain), 2014.
5. **Solana, M.**, Dall'Acqua, S., Boschiero, I., Bertucco, A., Effect of different co-solvents on the supercritical CO_2 extraction of polyphenols from asparagus, 21th International Congress of Chemical and Process Engineering, Prague (Czech Republic), 2014.

---

[1] *Not discussed in this work because they are part of D.Hernandez PhD thesis (Instituto Tecnológico Agrario de Castilla y León, Valladolid, Spain).*

Introduction

Nowadays, the demand for natural extracts and compounds of vegetable origin is growing due to their increasing use in functional foods, natural medicine, health additives, cosmetic products and pharmaceutical applications. These sectors require ultraclean, verifiable, high quality products, reliable supplies and competitive prices.

So far, a number of vegetable sources have been investigated as raw materials for the production of the natural extracts. The idea of processing food wastes is increasingly taken into account as, on the other hand, gives benefits to pollution, food management and to the economy of food industry. Moreover, emerging natural sources such as microalgae contain high amounts of valuable compounds, whose extraction could be economically and environmentally advantageous.

In this context, an efficient and green extraction and/or separation method is required not only to process the non-saleable and waste food and exploit the emerging natural sources, but also to create a highly demanded product in the current market.

Supercritical CO_2 technology has emerged as an important alternative to traditional solvent and mechanical processes for a number of years, since the CO_2 moderate critical pressure allows for a modest compression cost, while its low critical temperature enables successful extraction of thermally sensitive compounds without degradation. Other advantages over classical solvent extraction methods include the fact that CO_2 is inert, non-toxic and environmentally friendly. Additionally, in supercritical processes, CO_2 is easily removed after depressurization and allows faster cycles. However, the polar nature of most natural compounds makes it necessary the addition of co-solvents to supercritical CO_2, in order to enhance the fluid affinity towards them, and their effect is relevant on the extract composition and consequently the process economics. In this case, it is important to use environmentally friendly solvents, such as water and ethanol, in order to maintain the benefits of supercritical processes in this respect.

Supercritical CO_2 extraction is the most studied and applied process among supercritical CO_2 applications to process industry. However, there is a number of supercritical technologies that are being investigated and developed for other interesting applications such as the precipitation of polar compounds (supercritical anti-solvent precipitation, SAS), which allows to obtain natural dried precipitates, or the fractionation of compounds from liquid mixtures (supercritical counter-current fractionation) for obtaining more pure bioactive products.

Optimization of the processes and material parameters are important to provide maximum yields with highest quality and to make the final product suitable for use in foods, cosmetic and pharmaceutical industries. Thus, it is important to continue the investigation of supercritical CO_2 technologies for different materials and samples in order to generate new data that can be useful for the potential scale-up of the newly proposed processes.

All the reasons above are the motivation of this research project, whose aim is to evaluate the potential of supercritical CO_2 technologies to obtain new natural extracts rich in bioactive compounds from agricultural products and microalgae, in a safe, green and efficient way.

The topics addressed by this thesis are organized and subdivided in chapters as follows.

Chapter 1 is an introductory discussion on natural extracts market, a survey of extraction methods and latest results reported for supercritical CO_2 technologies.

Chapter 2, 3, 4 and 5 report the experimentation and modelling performed on supercritical fluid extraction from different natural sources, to verify the efficiency of this method to obtain natural extracts rich in different bioactive compounds in a competitive way.

In Chapter 2, supercritical fluid extraction of essential fatty acids from three different species of microalgae is investigated. The effect of operating variables on the total extraction yield and solubility were studied. Mathematical models developed by Sovová were applied to describe the experimental extraction curves.

In Chapter 3, the extraction of bioactive enriched fractions containing different classes of valuable compounds is reported. According to the results, the application of a sequential extractive approach is proposed using first CO_2+ethanol for lipid extraction and then water as co-solvent for phenolic and glucosinolate containing extracts.

Chapter 4 describes the economic evaluation of an industrial-scale plant for the production of natural extracts rich in glucosinolates and phenolic compounds from rocket salad. An industrial-scale supercritical extraction plant is designed. The software Aspen Plus™ V8.2 was employed for process simulation which was tuned on laboratory experimental measurements. Afterwards, the effect of operative variables on the process costs is evaluated.

Chapter 5 is focused on the recovery of phenolic compounds from asparagus. The effect of different mixtures of co-solvents on supercritical fluid extraction for selectively extract diverse polyphenols molecules is examined. Results are compared with pressurized liquid extraction (PLE) and Soxhlet methods.

Furthermore, supercritical anti-solvent process (SAS) is applied, with the objective of obtaining a dried precipitate rich in antioxidant compounds. Chapter 6 is focused on SAS process with CO_2 to obtain precipitates from cherries extracts which are rich in polyphenols and anthocyanins compounds. Continuous and batch mode of operation are compared. Moreover, the effect of pressure and composition of CO_2 on the polyphenols and anthocyanins yields is discussed.

The third method that have been studied is counter-current fractionation for the separation of the compounds of interest of a liquid mixture. In Chapter 7, the testing of the counter-current CO_2 fractionation plant is reported. For that purpose, the recovery of butanol from aqueous solutions is performed. The influence of operating variables, such as solvent-to-feed ratio, temperature, pressure and feed solution composition was experimentally investigated in terms of separation efficiency, butanol removal rate, total removal and butanol concentration in the extract at the end of the continuous cycle.

In Chapter 8 the use of counter-current carbon dioxide method investigated as a mean to reduce residual fat in soy skim after the enzyme-assisted aqueous extraction of soybeans. The effects of solvent-to-feed ratio, addition of ethanol as modifier and introduction of packing in the column are also analyzed. The interpretation of the results is carried out by ANOVA analysis.

Finally, in the conclusions, the summary of the thesis and the aspects that possibly need to be focused for developing this technology in the near future are discussed.

CHAPTER 1

State of the art

1.1. Trends in the market for natural extracts from vegetable materials

The natural extract industry is an emerging field of both research and business due to the growing markets of functional foods, natural medicine, health additives and cosmetic products. A natural extract can be defined as a substance rich in bioactive compounds, such as polyphenols, carotenoids, sterols or polyunsaturated fatty acids, that is normally extracted from vegetable materials [1,2]. Pharmaceutical, cosmetic and food are the main industries demanding these substances to be used as additives in their products and in the development of new ones. In this context, the selection of a safe, selective and efficient extraction method is a crucial issue for the mentioned sectors.

The interest of food industries in the market of natural extracts is not only based on the product itself, but also on the potentials of their non-edible residues and wastes. Annually, billions of tons of non-edible residues are generated by food industries, that cause pollution, management and economic problems worldwide [3]. FAO, the Food and Agriculture Organization of the United Nations, estimates that globally around one-third of food produced for human nutrition gets lost or is wasted, which equates to approximately 1.6 billion tons per year [4]. Of this amount, 54% are lost in production steps, postharvest, handling and storage, and other 46 % are caused by steps downstream of entry in the industry, i.e., processing, distribution and consumption [3]. On a weight basis, approximately 30% of cereals, 40-50% of root crops, fruit and vegetables, 20% of oilseeds, meat and dairy products, and 35% of fish are currently wasted [3].

Another advantage for different industrial sectors related to the recovery of bioactive compounds is the production of a wide range of different commercial products, as well as raw materials for secondary processes, substitutes for traditionally used ingredients, or ingredients of new products [5]. Among these products, functional foods, nutraceuticals and essential oils are particularly interesting. A functional food can be defined as a food producing a beneficial effect in one or more physiological functions, increasing the welfare and/or decreasing the risk of suffering a particular disease [6]. Nutraceuticals, usually employed as food supplements, are marketed as tablets and pills, and can also provide important health benefits [6]. Essential oil is the general classification for volatile oils that have traditionally been obtained by steam distillation of plants. They are interesting for numerous applications, such as flavours, fragrances, food ingredients, phytopharmaceuticals and cosmetics, thanks to the presence of bioactive compounds [7].

The demand for single bioactive compounds is also remarkable. For example, Leatherhead Food Research (2009) estimates the current market of polyphenols is worth approximately $200 million a year [8]. The marketing of carotenoids made around 1.07 billion dollars in 2010 [9]. The global market for omega-3 ingredients was estimated to be 24.87 kilo tons in 2013, and is expected to grow at a ACAGR (Compound Annual Growth Rate) of 13.7% from 2014 to 2020 [10]

As regards the sources from which bioactive compounds can be obtained, several plants, food products and by-products have been investigated. One of the most popular is fish, whose extracts are sold as omega-3 fish oil supplements. The research has also been started about other natural sources, such as microalgae and macro algae [1], which are particularly rich in polyunsaturated fatty acids and carotenoids.

To sum up, efficient extraction methods and commercially viable strategies for the provision of natural compounds need to be developed in order to explore and exploit the resources from industrial food wastes or new natural sources, and to probably obtain the novel products demanded by the market.

1.2. Emerging technologies for the production of natural extracts

The extraction of bioactive compounds from natural sources is conventionally performed by hydrodistillation, organic solvent extraction, maceration or other conventional separation technique that usually employ the use of an organic solvent to extract the compound of interest [11]. The drawbacks of these techniques are well known, such as the presence of trace amounts of organic solvents and the thermal degradation of sensitive compounds [12]. The imposed environmental regulations and the necessity of minimizing energy requirements have given impulse to investigate more environmentally friendly methods, such as pressurized-liquid extraction (PLE), supercritical fluid extraction (SFE), microwave, ultrasounds and pulsed electric field [3,13]. Among them, PLE and SFE are gaining popularity due to their ability to increase target molecule specificity and reduce waste solvent production, and have reached the industrial application stage [3]. Microwave, ultrasounds and pulsed electric field are mainly being considered as potentially applicable to enhance extraction yield when using SFE and PLE, and their effects are still under investigation.

The advantages of using supercritical fluids as solvents are numerous and include environmental, health, safety, and chemical benefits. Furthermore, the thermo-physical properties of supercritical fluids (high diffusivity, low viscosity, density, and dielectric constant) can be fine-tuned by changes of operating pressure and/or temperature, and thus supercritical fluid extraction has a great potential as a promising, efficient, and clean alternative method if compared with the conventional methods of distillation and extraction [7,13]. Different substances have been examined as SFE solvents. For example, hydrocarbons such as hexane, pentane and butane, nitrous oxide, sulphur hexafluoride and fluorinated hydrocarbons [14]. However, as a supercritical solvent CO_2 has been used in more than 90% of SFE of compounds from natural sources [2], thanks to its favourable characteristics with respect to the previous ones.

On the one hand, the critical point of CO_2 (Tc= 31.1 °C, Pc= 7.38 MPa) allows application of relatively low operation temperatures so that thermally labile solutes are protected and the extracts better resemble the natural material. In addition, carbon dioxide is non-flammable, non-explosive, cheap, and easily accessible in high purity. Last but not least, CO_2 is a gas at room temperature, so once the extraction is completed, and the system decompressed, a substantial elimination of CO_2 is achieved without residues, yielding easily a solvent-free extract. At the industrial scale, when solvent consumption is high, CO_2 is usually recycled to the extraction step.

Unfortunately, the low polarity nature of CO_2 limits its use for the extraction of polar compounds. However, this limitation can be overcome by the addition of small concentration of polar co-solvents such as methanol, ethanol or water, among others. In a recent review regarding SFE of vegetable matrices [7], it was reported that 38% of 441 publications include at least one experimental assay with modified CO_2. Ethanol was selected in 53% of the works involving entrainers. Ethanol is an innocuous solvent both at human health and environmental levels and this is a great advantage for applications in food, cosmetic or pharmaceutical industries. Methanol was used in 21% of the works reviewed by De Melo *et al.* [7], and is followed by water and dichloromethane, with 5% and 3%, respectively. Despite being more polar than ethanol, methanol raises hazard concerns to human health, a fact that discourages an extended use of it [7]. In cases where water can be technically employed with success as co-solvent, the drying stage can be softer and optimized, leading to energy and utility savings. Besides these savings, water is obviously an inexpensive co-solvent to include in a SFE process, being this a motive that fosters even more its potential inclusion in commercial SFE units [7].

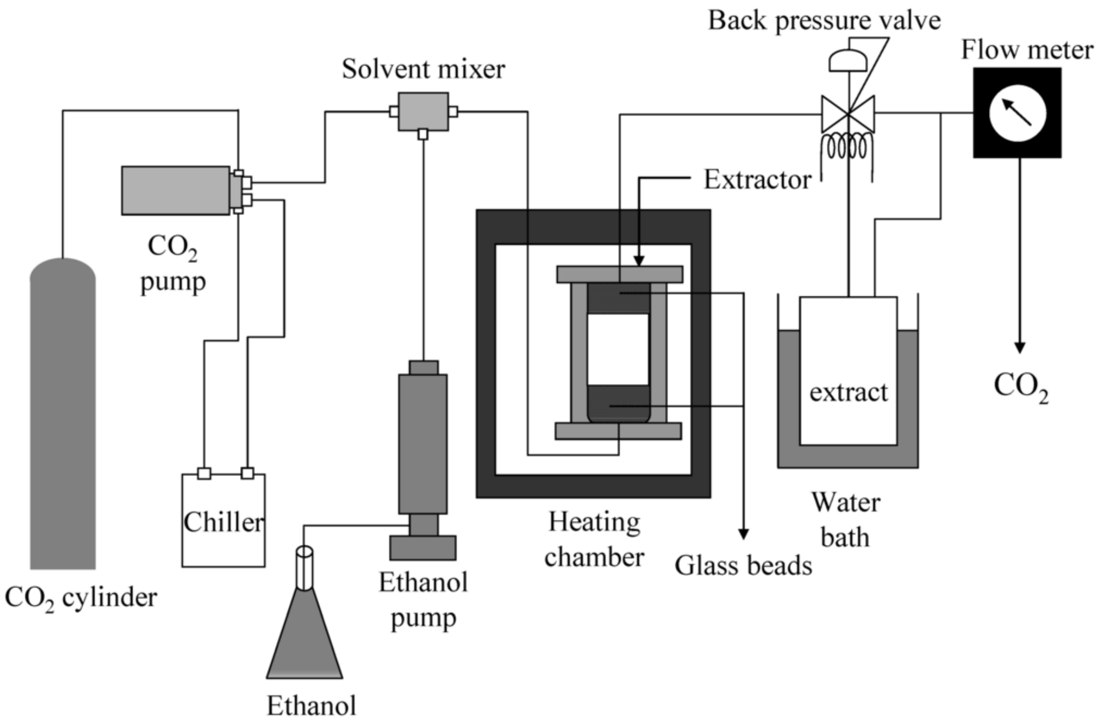

Figure 1.1. Schematic of supercritical CO_2 extraction process (SFE) [16].

SFE extraction is generally carried out as a semi-continuous process. In Figure 1.1, a typical scheme of a SFE process, using a co-solvent (ethanol) for the extraction of polar compounds, can be seen. Vegetable materials, usually dry and disintegrated, are charged into an extraction vessel of cylindrical shape to obtain a fixed bed of particles. The supercritical solvent, fed to the extractor continuously by a high pressure pump at a fixed flow rate, dissolves the required substances [15]. The typical volume of the extractors is from 0.1 to 2 L at the laboratory scale and from 2 to 5 L on the pilot scale [15].

The optimization of SFE requires the study of different parameters. Both solute solubility and diffusion are important to achieve an optimal solute flux. When using SFE to remove unwanted substances from food and natural products, such as pesticide removal from ginseng or desolventization of a vegetable oil, solute mass transfer from the food matrix dominated by diffusive-based processes becomes very important. The onset of the diffusion control region of SFE usually occurs after an optimal solvent/feed ratio has been determined. Below the optimal solvent/feed ratio, the extraction is thermodynamically driven by the solute’s solubility in the supercritical fluid; hence, low solvent/feed values are desired to minimize extraction time and production costs. [17]

In summary, the effect of pressure and temperature upon solubility can be briefly described in this way: (i) concerning pressure, it increases the supercritical fluid density, thus increasing its solvent power; (ii) with respect to temperature, it influences the solvent properties, namely density, and also the solute properties, mostly vapour pressure. Increasing temperature, density decreases (lower solubility) but vapour pressure increases (higher solubility). Depending on this double influence upon solubility, the proper selection of temperature requires a trade-off of these two opposing effects [7].

In PLE, a wide range of organic solvents has been used for the extraction of bioactive compounds from foods and herbs [18–25]. For example, two solvent mixtures (chloroform–methanol 2:1, v/v, and hexane–isopropanol 3:2, v/v) were used for the extraction of oxysterols from spray-dried whole egg, vanilla cake and egg noodlesat different extraction temperatures and pressures (60°C at 15 MPa, 100°C at 15 MPa, 120°C at 20 MPa) [20]. The extraction of capsaicinoids from peppers was carried out with a mixture of methanol and water [19]. Methanol as a solvent was chosen for the extraction of tocopherols and tocotrienols in cereals [26]. Acetone–water mixture was used for the extraction of phenolic compounds from parsley [27]. The extraction of isoflavones from soybean was performed with dimethyl sulphoxide- ethanol-water solvent mixture [28].

Nowadays, the most common solvent used in PLE is water (also called subcritical water), according to the proposal initially done by Hawthorne and Miller in 1994 [29]. PLE can be carried out at a temperature between 100 to 374 °C (the latter being the water critical temperature) under high pressure (usually between 1 to 6 MPa), needed to maintain water in the liquid state [6]. The main advantages of this method are: low extraction times, high quality of the extracts (mostly for essential oils), low costs of the extracting agent, and environmentally friendly. Additionally, the attendant equipment costs are much cheaper than the construction of a SFE plant. However, the aqueous-derived extracts from subcritical water extraction will often require removal of water unless supercritical water extraction can be performed quickly, as noted by King [17].

Subcritical water extraction has been widely used to extract different compounds from several vegetable matrices. Likewise, rosemary has been one of the most deeply studied materials [6]. Subcritical water extraction of several plants such as laurel [29], fennel [30], oregano [31] and kava [32] have also been reported. Furthermore, subcritical water has been demonstrated to be an extremely versatile method by its application to the hydrolysis of vegetable oils to produce fatty acids [33], extraction of flavors, spices [34], and essential oils [35], and degradation of cellulosic and lignin-containing natural polymers to oligomeric carbohydrate mixtures, as well as polyphenolic compounds [36]. In any case, the high temperature reported makes it impossible to apply PLE with water to the recovery of heat-sensitive products.

Other techniques considered as non-thermal, such as ultrasound, pulsed electric field, and microwave assisted extraction have been evaluated as potentially applicable to enhance extraction from various compounds found in food wastes, when using PLE and SFE. In particular, the use of ultrasound power in food industries (UAE) has increased during the last years, since it represents a potentially efficient way of enhancing mass transfer. SFE process is an example in which ultrasonic energy has been demonstrated to be efficient as an auxiliary technique without affecting, or even improving the main characteristics and quality of the products [37].

Microwave assisted extraction technique has gained popularity to recover low molecular weight organic compounds or small molecules from softer matrices such as food and plant sources [38,39]. The basic motivation of performing microwave assisted extraction is to exploit the energy provided by microwaves, which comes from electromagnetic radiation with frequencies from 0.3 to 300 GHz. In order to avoid interference with radio waves, domestic and industrial microwaves usually operate at 2.45 GHz, and occasionally at 0.915 GHz in the USA and 0.896 GHz in Europe. Microwaves are transmitted as waves that can penetrate biomaterials and interact with their polar molecules, like water, releasing heat [38–40]. Thus, the temperature of the starting material is raised, leading to an increase of extraction efficiency [3].

Pulsed electric field (PEF) is a non-thermal method involving the release of high tension electric pulsed (up to 70 kV/cm), for few microseconds, on a material located between two electrodes [41–44]. The objective of this technique is to disrupt the structure of cell membranes to enhance extraction. When the critical electric potential of cell membranes, which depends on the intensity of the external electric field, is exceeded, an electric collapse occurs, leading to a transitory or definitive permeability of the membranes. This happens due to pore formation in the weak regions of the membrane, turning the vegetable substrate into a very porous material. In the recent years, a growing number of researches have been dealing with application of PEF to enhance the permeability of cell membranes and thus improve mass transfer, reducing the extraction time. In all cases, PEF has resulted in important improvements in extraction yield, by easing the release of phytochemicals [3]. As an example, the extraction of anthocyanins from grape by-products assisted by pulsed electric fields was reported by Corrales et al. [45], concluding that the total phenolic content of the extracts is increased by 50% due to the effect of the high tension electric pulsed.

Hou et al. [46] compared the extraction techniques mentioned in this section (ultrasonic-assisted extraction, pulsed electric field, microwave assisted extraction, pressurized liquid extraction), with the exception of SFE, on the recovery of ginsenosides from Panax ginseng. Results indicated that pulsed electric field yield was the highest one. All the pulsed electric field process took less than 1 s, much faster than any other technique. The high efficiency, low process time and energy cost of pulsed electric field could be applied at the industrial scale to recover valuable compounds from food by-products. Corrales *et al*. [45], extracting anthocyanins from grapes, also noted higher efficiency of pulsed electric field over UAE and high hydrostatic pressure. Therefore, pulsed electric field may also work as an enhancement strategy for SFE and PLE, even in sample pre-treatment. However, as well as microwave assisted extraction, PEF is limited to extractions from aqueous media, which are not common in SFE. Consequently, this technique would be more indicated to improve subcritical processes, or SFE with water as co-solvent [3].

1.3. Supercritical CO_2 extraction of natural extracts: an overview

The discovery of the solvent power of pressurized carbon dioxide was made in the 19th century [47], but its practical application for extraction of vegetable substances was first studied in the 1960s when more sensitive analytical methods indicated trace amounts of residual organic solvents in food samples and initiated concern about their impact on human health [15]. The first experiments of supercritical CO_2 extraction of natural products were conducted in a limited number of laboratories, most intensively in Germany [48] and in Russia [49], about 50 years ago. Nowadays, coffee decaffeination and naturals extract are probably the most successful application of supercritical fluids and related compressed fluids [17].

Regarding research works, substances from more than 300 botanical materials have been extracted using supercritical CO_2 in the last 15 years [7]. Supercritical fluids have been mainly applied to the extraction of seeds and leaves. Together, they represent 45% of the plant fractions of all the works considered in a recent review [7], being seeds the biggest fraction (28%), and leaves the second one (17%). They are followed by fruits (10%), roots (7%), flowers (5%), rhizomes (3%) and bark (2%) [7]. SFE extracts obtained from vegetable matrices are typically mixtures of the following family of compounds: triglycerides, fatty acids, fatty alcohols, terpenoids, phytosterols, tocopherols, tocotrienols, and phenolics [7].

Recently, several microalgae species have also been used to produce natural compounds of interest for the food industry using SFE [50–52]. For instance, the extraction of diolefines from *Botrycoccus brauniicells* has been reported [52]. The authors proved that the solubility of these type of compounds in CO_2 increased with pressure, and found that 30 MPa provided its optimum value with respect to yield and extraction speed. SFE has also been used to extract carotenoids from microalgae *Chlorella vulgaris* [51]. They informed that, when microalgae cells were crushed, the carotenoid extraction improved slightly. The optimum extraction conditions were 55 °C and 35 MPa.

Temperature, pressure, solvent flow rate and type of solvent are important process parameters while origin of the plant material, chemical composition and particle size of the material, different types of tissues (leaves, stems, seeds, etc.) and other variables, such as pre-treatment and storage conditions also affect yield and the composition of the extract in supercritical fluid extraction [12]. The optimum pressure and temperature conditions of a SFE process are those that best suit the trade-off between kinetics and equilibrium behaviours. SFE from vegetable materials essays typically focus on pressures from 10 to 40 MPa [7]. Within this range, and taking into account the temperature normally considered (40–60 °C), densities range from 200 to 900 kg m^{-3}[7].

Optimization of the process and material parameters are important to provide maximum yields with highest quality and making the final product suitable for use in foods, cosmetic and pharmaceutical industries [12]. Thus, it is important to continue the investigation of SFE for different samples in order to generate new data that can be useful for the potential scale-up of the newly proposed processes.

1.4. Other supercritical CO_2 processes for food applications

SFE is the most studied and applied process among the supercritical CO_2 ones. However, there is a number of supercritical CO_2 technologies that are being investigated and developed for other interesting applications and deserve to be mentioned. A brief summary is presented in this section, with special attention to supercritical anti-solvent precipitation (SAS) and supercritical counter-current fractionation, which have been experimentally studied and are reported later in this thesis.

The counter-current fractionation process consists of a separation column, where gaseous and liquid phases are contacted counter currently. It includes a separator at the top for separating solvent and extract, and devices for feeding reflux to the column, for recovering top product, for delivering feed to the column, for recovering product at the bottom of the column, and for recycling the solvent. The separation column consists of two separation cascades. In the upper one (enriching section) the bottom product compounds are separated from the top product compounds and rejected to the lower section (stripping section). In the stripping section the top product compounds are separated from the bottom product compounds and transported to the enriching section [62]. A

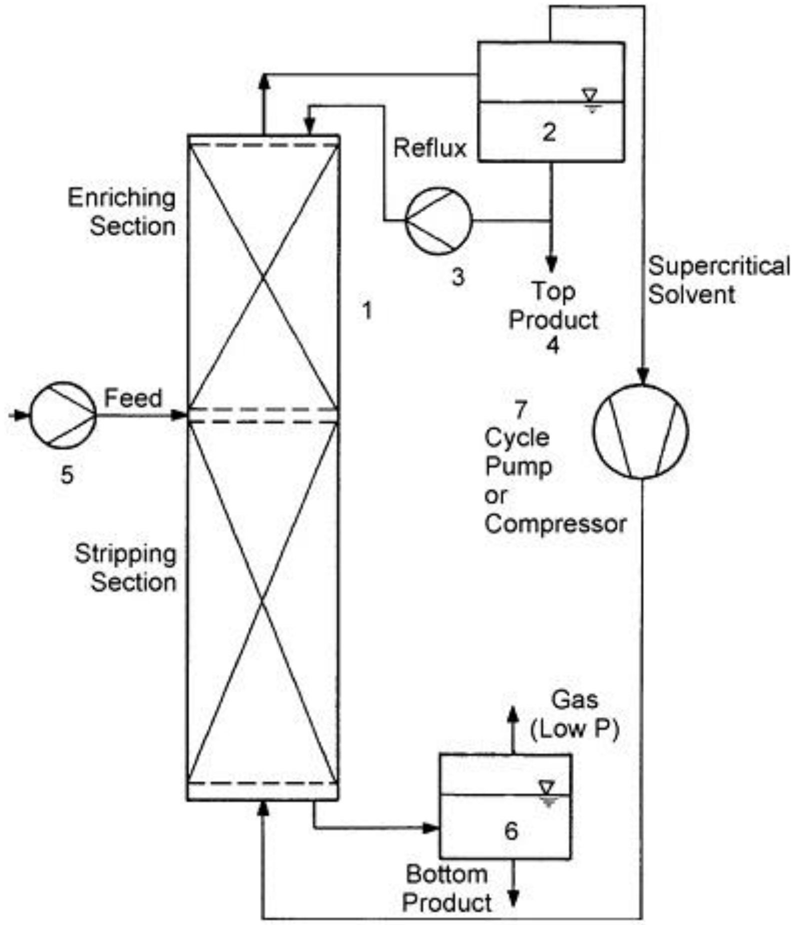

Figure 1.2. Schematic of counter-current process with supercritical fluid for the separation of two components [62].

schematic diagram of a typical with a supercritical CO_2 counter-current process for the separation of two components is presented in Figure1.2.

The advantages of using counter-current mode are the reduction of solvent consumption, increased throughput, and higher oil extract concentrations in the solvent and lower residual concentration in the raffinate (Brunner, 2009). In addition, counter-current fractionation of a liquid feed mixture can be implemented in a continuous mode (Pieck et al., 2015), whereas this cannot be the case for solid substances.

Numerous food-related materials have been fractionated using the columnar approach, such as marine-derived oils [63,64], mixed glycerides [65,66], tocopherols [67], free fatty acids [68] and phytosterols [69].

SAS is a method in which supercritical CO_2 is used to precipitate selected, non-soluble compounds already dissolved in a suitable liquid solvent. CO_2 at relatively high pressure acts as an

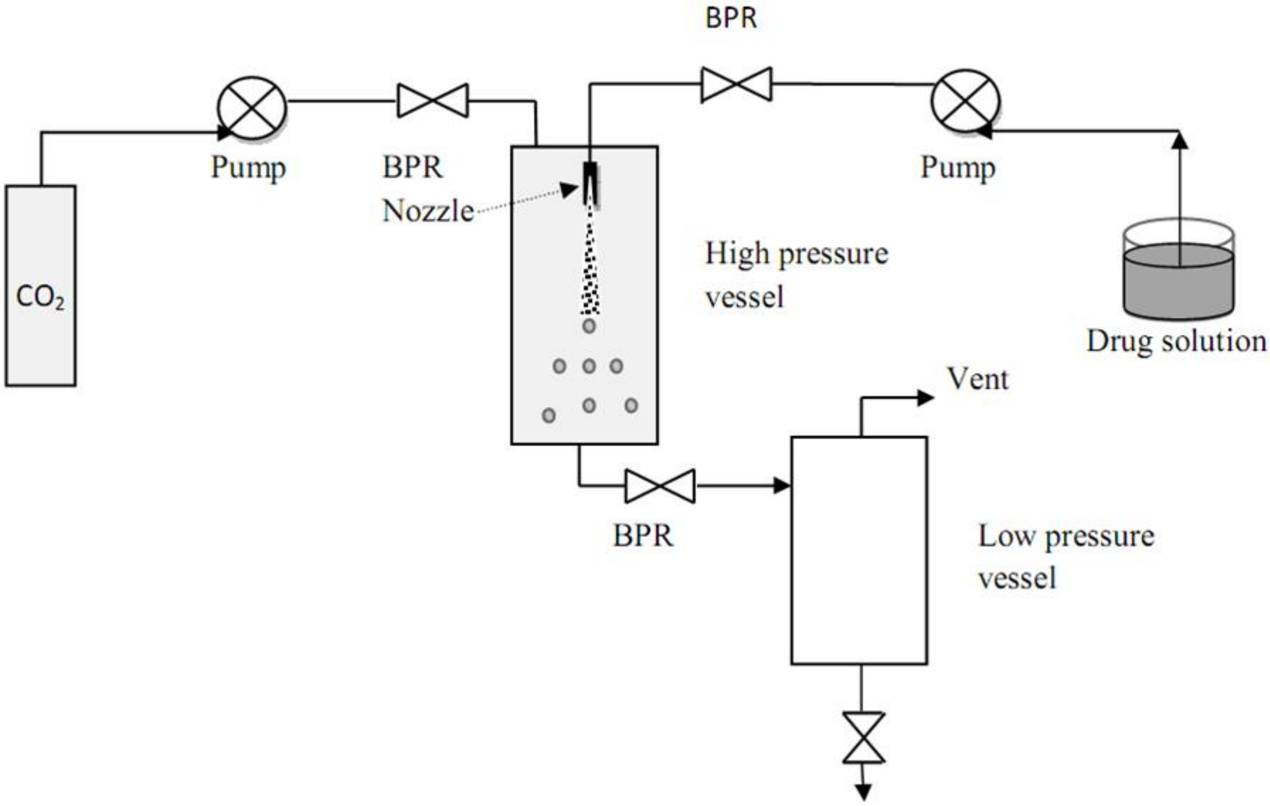

Figure 1.3. Schematic of supercritical anti-solvent (SAS) process [55].

anti-solvent decreasing the solubility of the solid in the mixture and causing a fast supersaturation [53]. To properly perform the SAS process, two requisites have to be respected: (i) the solute has to be soluble in the solvent and not soluble in supercritical CO2, (ii) the organic solvent and supercritical CO_2 have to be completely miscible at the process conditions; i.e., a supercritical solution has to be formed [54].

Figure 1.3 shows a schematic of a typical semi-continuous SAS process. First, supercritical CO_2 is pumped to the top of the high pressure vessel (precipitator) until the system reaches a constant temperature and pressure. Subsequently, the solution containing the substance of interest is sprayed as fine droplets into supercritical CO_2 bulk phase through an atomization nozzle. The large volume expansion of the solution in vessel, due to the dissolution of supercritical CO_2 in the liquid droplets and, subsequently, the super saturation due to reduction in solvent power, lead to the nucleation and formation of small and monodisperse particles with the liquid phase. These particles are collected on a filter at the bottom of the vessel. The supercritical CO_2 and organic solvent mixture flow down to a depressurized tank where suitable temperature and pressure condition allow gas liquid separation. After collection of the particles, the spraying of liquid solution is stopped. Furthermore, to remove residual solvent, pure supercritical CO_2 continues to flow through the vessel until needed. [55]

SAS precipitation of bioactive compounds from food has been experimentally investigated in the last years. For instance, the precipitation of phenolic compounds from mango by-products [56] and from grape residues [57] have been reported. Other antioxidants compounds were obtained in a similar way from grape seeds [58], rosemary [53], marigold flowers [59], Lycium barbarum [60] and microalgae [61].

Other techniques have been developed for the production of particles and/or dried extracts bearing acronyms such as RESS (rapid expansion of supercritical solutions), GAS (gas antisolvent), PGSS (particles from gas-saturated solutions), CPF (concentrated powder form), as well as others based on either dissolution in an SCF or use of the medium to precipitate the targeted material as fine particles or encapsulates having usually very narrow particle-size distributions [17].

RESS process consists of two steps; (a) dissolution of the solid substance in a SCF and (b) formation of particles due to supersaturation. In the RESS process, supercritical CO_2 is first pumped at desired pressure and temperature to extraction chamber containing solid substances through heat exchanger. Supercritical CO_2 percolates and dissolves the solid substances in the extractor and the resulted solution is then depressurized through a heated nozzle or capillary at supersonic speed into a low pressure chamber. The supercritical solution is expanded adiabatically in the chamber, which leads to a rapid drop in temperature and pressure and spontaneous formation of droplets/particles. During the rapid expansion of the supercritical solution, the density and solvent power decrease significantly, resulting in super saturation of the solution and consequently precipitation of desire particles free of a residual solvent [55]. Unfortunately, RESS can be only applied to products that have enough solubility in CO_2.

GAS is an anti-solvent batch process where the precipitator is partially filled with the solution of solute of interest and then the supercritical anti-solvent is pumped into the vessel, preferably from the bottom, until the fixed pressure is reached. Precipitates are obtained as the gas concentration in the solution increases with pressure. After a holding time, the expanded solution is made to pass through a valve to wash and clean the precipitated particles. A clear disadvantage of this technique is the lack of control on the particle formation, which prevent the formation of mono dispersed particles. [55]

In the “Particles Generated from Gas Saturated Solutions” (PGSS) process, CO_2 is employed as a solute. A molten mixture composed of the lipid matrix forming materials and the bioactive molecule is saturated by CO_2 at supercritical conditions. This remarkably reduces the viscosity of the molten mass allowing for its outflow to the expansion chamber through a micrometric nozzle

[62]. CPF involves contacting the liquid form of the bioactive material with the compressed fluid, and then expanding the mixture through a nozzle onto a solid carrier material. These two processes find favour in part due to the ability to operate them continuously, their GMP compatibility, and their low consumption of compressed fluid. [17]

Another property of CO_2 treatment of great interest in food technology is pasteurization and sterilization by high pressure CO_2. The use of supercritical CO_2 in preservation processes has also gained great interest in the scientific field. Since the 1980s, high pressure carbon dioxide process (HPCD) has been increasingly investigated as a promising technique to induce a pasteurizing/sterilizing effect when applied to both solid and liquid matrixes [63]. The process has been applied not only to liquid foodstuff but also to meats (chicken, pork, and beef), vegetables (celery, and spinach), seeds and food powders (alfalfa seeds, cocoa powder, and ginseng), fruits (cut pieces of pears, strawberries, honeydew melon, and cucumber), spices and herbs (chives, thyme, oregano, parsley, and mint), and fish (shrimp, and oyster) [63]. Both microbial and enzymatic deactivation have been demonstrated on liquid food substrates, such as fruit juices, milk, and alcoholic beverages after supercritical CO_2 treatment [12].

1.5. Industrial supercritical CO_2 applications

Currently, there are more than 150 SFE plants operated throughout the world with an extraction volume equal or greater than 500 L, and many of these production plants are dedicated to the extraction of natural products, leading to the recovery of higher-added value compounds [17]. They are mostly distributed in Europe, the USA, Japan, and in the South East Asian Countries [64].

The first SFE applications in the food industry were decaffeination of coffee and isolation of hops extracts for flavouring beer, which led to the construction of sizeable plants for these dedicated purposes [17]. Today, literally all hops extraction for beer industry is done via supercritical CO_2 extraction, whereas coffee decaffeination competes with alternative extraction processes [17]. Other food processing SFE applications include the recovery of aromas and flavours from herbs and spices, the extraction and fractionation of edible oils, and the removal of contaminants [64].

Nowadays, supercritical CO_2 processes are oriented toward extracts or products with much higher added value that those developed using in the past 40 years [16]. For instance, the activity in producing fine particles for use in the pharmaceutical industry started in the late 1990s, but in recent years there has been a focus on incorporating this technology platform into foods and nutraceutical formulations [64].

Despite supercritical CO_2 technologies have been successfully applied in industries for almost four decades, there is still reluctance in some world regions to adopt them because of the wrong perception that they are not fully competitive [19] or because of safety reasons. High capital costs of supercritical CO_2 equipment are usually mentioned among the drawbacks of the process [15]. However, the operating costs are usually lower than those of conventional extraction and can compensate the higher capital costs. In any case, accurate cost estimates would reduce the risk to reluctant companies of investing in the commercial application of SFE. Fully recognizing this, companies that sell industrial SFE plants such as Uhde High Pressure Technologies GmbH in Germany, Separex S.A.S. in France, Thar Process Inc. in the USA, and Natex Prozesstechnologie GesmbH in Austria, support industry with partial information on economics of SFE processes [65].

It is worth mentioning that the performance of the economic evaluation of a supercritical separation process cannot be based uniquely on the statement of fixed and operating costs, which on the other hand is the method currently used for classical and well defined extraction processes where the product is widely accepted by the market without price fluctuations. Indeed, the products obtained after the supercritical process present special characteristics which make them different from current commercial products, or even in some cases, they constitute entirely new products that cannot be manufactured by conventional processes. These new products should fulfil the requirements of the market, which demands high quality, high knowledge about the chemical composition of the final product and exclusivity. Besides, the ease of the new product to adapt itself to changes in market demand, e.g. by modifying its formulation, is also highly desired. All these requirements make it especially difficult to determine the price of the new product, which is also commonly unknown for the market [13]. In this context, the economic evaluation of a supercritical process should be developed using a business plan analysis [11].

The future of production of valuable compounds from vegetable and food residues using sub- and supercritical fluids is definitely promising with the use of combined technologies, such as ultrasound, microwaves, pulsed electric fields, membrane, high-pressure reactors, or integrated formation, among others, which will lead to high quality products [17]. This will be able to lead to higher efficiency and lower processing costs, making it viable the production of valuable compounds at industrial scale. Although most of these techniques are successfully applied in laboratory or pilot scale, and many industrial applications of SFE can be found, numberless other potential uses of SCFs are still to be explored. As well, more advancements should be made on the mentioned techniques in order to make them feasible to process food wastes [3].

Other future issues to be taken into account for the development of supercritical and high pressure extraction techniques are eventually summarized, based on a recent review work by King [17]:

1. Optimizing continuous SCF processing systems should be headed to increase productivity.

2. Removal of residual solvents and toxicants from food and nutraceutical products by SFE needs to be studied further with respect to new regulatory requirements.

3. Cost and life-cycle analysis for supercritical CO_2 technologies versus competitive technologies will be a future requirement and will complement research and development in this field.

4. Production-scale supercritical CO_2 needs further development in terms of its application to producing bioactive concentrates for the nutraceutical or functional food marketplace.

5. Extraction versus reaction conditions to produce functional food ingredients using subcritical fluids such as water needs further investigation in terms of deriving bioactive components.

Finally, it is also important and necessary to improve the marketing strategies, to inform consumers about the advantages of applying supercritical CO_2 technologies to recover valuable products from food wastes, and therefore to gain market in this field [3].

References

[1] M. Herrero, A. del Pilar Sánchez-Camargo, A. Cifuentes, E. Ibáñez, Plants, seaweeds, microalgae and food by-products as natural sources of functional ingredients obtained using pressurized liquid extraction and supercritical fluid extraction, TrAC Trends Anal. Chem. (2015). doi:10.1016/j.trac.2015.01.018.

[2] M.S. Uddin, M.Z.I. Sarker, S. Ferdosh, M.J.H. Akanda, M.S. Easmin, S.H. Bt Shamsudin, et al., Phytosterols and their extraction from various plant matrices using supercritical carbon dioxide: a review, J. Sci. Food Agric. 95 (2015) 1385–1394. doi:10.1002/jsfa.6833.

[3] J. Viganó, A.P.D.F. Machado, J. Martínez, Sub- and supercritical fluid technology applied to food waste processing, J. Supercrit. Fluids. 96 (2015) 272–286. doi:10.1016/j.supflu.2014.09.026.

[4] European Commission, Preparatory Study on Food Waste Across Eu 27, 2010. doi:10.2779/85947.

[5] R. Martínez, P. Torres, M.A. Meneses, J.G. Figueroa, J.A. Pérez-Álvarez, M. Viuda-Martos, Chemical, technological and in vitro antioxidant properties of mango, guava, pineapple and passion fruit dietary fibre concentrate, Food Chem. 135 (2012) 1520–1526. doi:http://dx.doi.org/10.1016/j.foodchem.2012.05.057.

[6] M. Herrero, a Cifuentes, E. Ibanez, Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgaeA review, Food Chem. 98 (2006) 136–148. doi:10.1016/j.foodchem.2005.05.058.

[7] M.M.R. de Melo, a. J.D. Silvestre, C.M. Silva, Supercritical fluid extraction of vegetable matrices: Applications, trends and future perspectives of a convincing green technology, J. Supercrit. Fluids. 92 (2014) 115–176. doi:10.1016/j.supflu.2014.04.007.

[8] W. Paper, Food Additives – A Growing Global Market, (n.d.).

[9] H. Wijngaard, M.B. Hossain, D.K. Rai, N. Brunton, Techniques to extract bioactive compounds from food by-products of plant origin, Food Res. Int. 46 (2012) 505–513. doi:10.1016/j.foodres.2011.09.027.

[10] http://www.iffo.net/node/573, (n.d.).

[11] M.P. Fernández-Ronco, A. de Lucas, J.F. Rodríguez, M.T. García, I. Gracia, New considerations in the economic evaluation of supercritical processes: Separation of bioactive compounds from multicomponent mixtures, J. Supercrit. Fluids. 79 (2013) 345–355. doi:10.1016/j.supflu.2013.01.018.

[12] A. Shilpi, U.S. Shivhare, S. Basu, Supercritical CO 2 Extraction of Compounds with Antioxidant Activity from Fruits and Vegetables Waste -A Review, Focus. Mod. Food Ind. 2 (2013) 43–62.

[13] G. Joana Gil-Chávez, J.A. Villa, J. Fernando Ayala-Zavala, J. Basilio Heredia, D. Sepulveda, E.M. Yahia, et al., Technologies for Extraction and Production of Bioactive Compounds to be Used as Nutraceuticals and Food Ingredients: An Overview, Compr. Rev. Food Sci. Food Saf. 12 (2013) 5–23. doi:10.1111/1541-4337.12005.

[14] R.M. Smith, Supercritical fluids in separation science – the dreams , the reality and the future, 1999.

[15] H. Sovová, R.P. Stateva, Supercritical fluid extraction from vegetable materials, Rev. Chem. Eng. 27 (2011) 79–156. doi:10.1515/REVCE.2011.002.

[16] H. Kanda, Y. Kamo, S. Machmudah, E.Y. Wahyudiono, M. Goto, Extraction of fucoxanthin from raw macroalgae excluding drying and cell wall disruption by liquefied dimethyl ether., Mar. Drugs. 12 (2014) 2383–96. doi:10.3390/md12052383.

[17] J.W. King, Modern supercritical fluid technology for food applications., Annu. Rev. Food Sci. Technol. 5 (2014) 215–38. doi:10.1146/annurev-food-030713-092447.

[18] L.J. S. Santoyo, M. Herrero, F.J. Senorans, A. Cifuentes, E. Ibanez, Func- tional characterization of pressurized liquid extracts of Spirulina platensis, Eur. Food Res. Technol. 224 (2006) 75–81.

[19] C.G.B. G.F. Barbero, M. Palma, Pressurized liquid extraction of capsaici- noids from peppers, J. Agric. Food Chem. 54 (2006) 3231–3236.

[20] E. Boselli, V. Velazco, M. Fiorenza Caboni, G. Lercker, Pressurized liquid extraction of lipids for the determination of oxysterols in egg-containing food, J. Chromatogr. A. 917 (2001) 239–244. doi:http://dx.doi.org/10.1016/S0021-9673(01)00688-4.

[21] A.U. Buranov, G. Mazza, Extraction and purification of ferulic acid from flax shives, wheat and corn bran by alkaline hydrolysis and pressurised solvents, Food Chem. 115 (2009) 1542–1548. doi:10.1016/j.foodchem.2009.01.059.

[22] a. R. Maurí-Aucejo, T. Arnandis-Chover, R. Marín-Sáez, M. Llobat-Estellés, Application of pressurized fluid extraction to determine cadmium and zinc in plants, Anal. Chim. Acta. 581 (2007) 78–82. doi:10.1016/j.aca.2006.08.008.

[23] C.U. Tam, F.Q. Yang, Q.W. Zhang, J. Guan, S.P. Li, Optimization and comparison of three methods for extraction of volatile compounds from Cyperus rotundus evaluated by gas chromatography-mass spectrometry, J. Pharm. Biomed. Anal. 44 (2007) 444–449. doi:10.1016/j.jpba.2006.10.026.

[24] Y.Y. Dang, X.C. Li, Q.W. Zhang, S.P. Li, Y.T. Wang, Preparative isolation and purification of six volatile compounds from essential oil of Curcuma wenyujin using high-performance centrifugal partition chromatography, J. Sep. Sci. 33 (2010) 1658–1664. doi:10.1002/jssc.200900453.

[25] G. Song, C. Lu, J.-M. Lin, Application of surfactants and microemulsions to the extraction of pyrene and phenanthrene from soil with three different extraction methods., Anal. Chim. Acta. 596 (2007) 312–8. doi:10.1016/j.aca.2007.06.004.

[26] M. Bustamante-Rangel, M.M. Delgado-Zamarreño, a. Sánchez-Pérez, R. Carabias-Martínez, Determination of tocopherols and tocotrienols in cereals by pressurized liquid extraction-liquid chromatography-mass spectrometry, Anal. Chim. Acta. 587 (2007) 216–221. doi:10.1016/j.aca.2007.01.049.

[27] D.L. Luthria, Influence of experimental conditions on the extraction of phenolic compounds from parsley (Petroselinum crispum) flakes using a pressurized liquid extractor, Food Chem. 107 (2008) 745–752. doi:10.1016/j.foodchem.2007.08.074.

[28] D.L. Luthria, R. Biswas, S. Natarajan, Comparison of extraction solvents and techniques used for the assay of isoflavones from soybean, Food Chem. 105 (2007) 325–333. doi:10.1016/j.foodchem.2006.11.047.

[29] V. Fernández-Pérez, M.M. Jiménez-Carmona, M.D.L. de Castro, An approach to the static–dynamic subcritical water extraction of laurel essential oil: comparison with conventional techniques, Analyst. 125 (2000) 481–485. doi:10.1039/a907244f.

[30] L. Gámiz-Gracia, M.D. Luque de Castro, Continuous subcritical water extraction of medicinal plant essential oil: Comparison with conventional techniques, Talanta. 51 (2000) 1179–1185. doi:10.1016/S0039-9140(00)00294-0.

[31] R. Soto Ayala, M.D. Luque de Castro, Continuous subcritical water extraction as a useful tool for isolation of edible essential oils, Food Chem. 75 (2001) 109–113. doi:10.1016/S0308-8146(01)00212-6.

[32] D.J.M. and S.B.H. Kubatov, Alenaa, Arnaud J. M. Lagadec, Selective extraction of oxygenates from savory and peppermint using subcritical water, Flavour Fragr. J. (2001) 64–73.

[33] P. DE Eller FJ, Teel JA, Continuous hydrolysis of cuphea seed oil in subcritical water, J. Am. Oil Chem. Soc. 88 (2011) 1455–61.

[34] P. Khuwijitjaru, N. Sayputikasikorn, S. Samuhasaneetoo, P. Penroj, P. Siriwongwilaichat, S. Adachi, Subcritical water extraction of flavoring and phenolic compounds from cinnamon bark (Cinnamomum zeylanicum)., J. Oleo Sci. 61 (2012) 349–55. doi:10.5650/jos.61.349.

[35] M.Z. Ozel, F. Gogus, A.C. Lewis, Subcritical water extraction of essential oils from Thymbra spicata, Food Chem. 82 (2003) 381–386. doi:10.1016/S0308-8146(02)00558-7.

[36] J.E. Cacace, G. Mazza, Pressurized low polarity water extraction of lignans from whole flaxseed, J. Food Eng. 77 (2006) 1087–1095. doi:10.1016/j.jfoodeng.2005.08.039.

[37] E. Riera, Y. Golás, a. Blanco, J. a. Gallego, M. Blasco, a. Mulet, Mass transfer enhancement in supercritical fluids extraction by means of power ultrasound, Ultrason. Sonochem. 11 (2004) 241–244. doi:10.1016/j.ultsonch.2004.01.019.

[38] B. Kaufmann, P. Christen, Recent extraction techniques for natural products: microwave-assisted extraction and pressurised solvent extraction., Phytochem. Anal. 13 (2002) 105–13. doi:10.1002/pca.631.

[39] C.C. Teo, W.P.K. Chong, Y.S. Ho, Development and application of microwave-assisted extraction technique in biological sample preparation for small molecule analysis, Metabolomics. 9 (2013) 1109–1128. doi:10.1007/s11306-013-0528-7.

[40] L. Wang, C.L. Weller, Recent advances in extraction of nutraceuticals from plants, Trends Food Sci. Technol. 17 (2006) 300–312. doi:10.1016/j.tifs.2005.12.004.

[41] H. Wijngaard, N. Brunton, The optimization of extraction of antioxidants from apple pomace by pressurized liquids, J. Agric. Food Chem. 57 (2009) 10625–10631. doi:10.1021/jf902498y.

[42] J. Señorans, E. Ibáñez, A. Cifuentes, New trends in food processing., Crit. Rev. Food Sci. Nutr. 43 (2003) 507–526. doi:10.1080/10408690390246341.

[43] E. Puértolas, E. Luengo, I. Álvarez, J. Raso, Improving Mass Transfer to Soften Tissues by Pulsed Electric Fields: Fundamentals and Applications, Annu. Rev. Food Sci. Technol. 3 (2012) 263–282. doi:10.1146/annurev-food-022811-101208.

[44] N. López, E. Puértolas, S. Condón, I. Álvarez, J. Raso, Application of pulsed electric fields for improving the maceration process during vinification of red wine: Influence of grape variety, Eur. Food Res. Technol. 227 (2008) 1099–1107. doi:10.1007/s00217-008-0825-y.

[45] M. Corrales, S. Toepfl, P. Butz, D. Knorr, B. Tauscher, Extraction of anthocyanins from grape by-products assisted by ultrasonics, high hydrostatic pressure or pulsed electric fields: A comparison, Innov. Food Sci. Emerg. Technol. 9 (2008) 85–91. doi:10.1016/j.ifset.2007.06.002.

[46] J. Hou, S. He, M. Ling, W. Li, R. Dong, Y. Pan, et al., A method of extracting ginsenosides from Panax ginseng by pulsed electric field, J. Sep. Sci. 33 (2010) 2707–2713. doi:10.1002/jssc.201000033.

[47] H.J. Hannay JB, On the solubility of solids in gases., Proc R Soc London A. (1879) 29: 324.

[48] Zosel K., German patent no. 14 93 190, 1964.

[49] P.A. Pekhov AV, Ponomarenko IJ, Patent USSR No. 167798, 1965.

[50] R.L. Mendes, J.P. Coelho, H.L. Fernandes, I.J. Marrucho, J.M.S. Cabral, J.M. Novais, et al., Applications of supercritical CO2 extraction to microalgae and plants, J. Chem. Technol. Biotechnol. 62 (1995) 53–59. doi:10.1002/jctb.280620108.

[51] R.L. Mendes, H.L. Fernandes, J.P. Coelho, E.C. Reis, J.M.S. Cabral, J.M. Novais, et al., Supercritical CO2 extraction of carotenoids and other lipids from Chlorella vulgaris, Food Chem. 53 (1995) 99–103. doi:10.1016/0308-8146(95)95794-7.

[52] R.L. Mendes, B.P. Nobre, M.T. Cardoso, A.P. Pereira, A.F. Palavra, Supercritical carbon dioxide extraction of compounds with pharmaceutical importance from microalgae, Inorganica Chim. Acta. 356 (2003) 328–334. doi:10.1016/S0020-1693(03)00363-3.

[53] a. Visentin, S. Rodríguez-Rojo, a. Navarrete, D. Maestri, M.J. Cocero, Precipitation and encapsulation of rosemary antioxidants by supercritical antisolvent process, J. Food Eng. 109 (2012) 9–15. doi:10.1016/j.jfoodeng.2011.10.015.

[54] I. De Marco, E. Reverchon, Supercritical carbon dioxide+ethanol mixtures for the antisolvent micronization of hydrosoluble materials, Chem. Eng. J. 187 (2012) 401–409. doi:10.1016/j.cej.2012.01.135.

[55] P.S. Rabinarayan Parhi*, SUPERCRITICAL FLUID TECHNOLOGY : A REVIEW, Adv. Pharm. Sci. Technol. (2013) 13–36.

[56] M. a. Meneses, G. Caputo, M. Scognamiglio, E. Reverchon, R. Adami, Antioxidant phenolic compounds recovery from Mangifera indica L. by-products by supercritical antisolvent extraction, J. Food Eng. 163 (2015) 45–53. doi:10.1016/j.jfoodeng.2015.04.025.

[57] T. Floris, G. Filippino, S. Scrugli, M.B. Pinna, F. Argiolas, a. Argiolas, et al., Antioxidant compounds recovery from grape residues by a supercritical antisolvent assisted process, J. Supercrit. Fluids. 54 (2010) 165–170. doi:10.1016/j.supflu.2010.04.006.

[58] J.L. Marqués, G. Della Porta, E. Reverchon, J. a. R. Renuncio, a. M. Mainar, Supercritical antisolvent extraction of antioxidants from grape seeds after vinification, J. Supercrit. Fluids. 82 (2013) 238–243. doi:10.1016/j.supflu.2013.07.005.

[59] P. Boonnoun, H. Nerome, S. Machmudah, M. Goto, A. Shotipruk, Supercritical anti-solvent micronization of marigold-derived lutein dissolved in dichloromethane and ethanol, J. Supercrit. Fluids. 77 (2013) 103–109. doi:10.1016/j.supflu.2013.02.026.

[60] K.-L. Lin, L.-M. Chng, J.C.-T. Lin, S.-L. Hsu, C.-C. Young, C.-J. Shieh, et al., Effect of Anti-solvent Conditions on Low Density Supercritical Fluids Precipitation of Zeaxanthin Palmitates from Lycium Barbarum Fruits, J. Supercrit. Fluids. 87 (2014) 104–110. doi:10.1016/j.supflu.2014.01.001.

[61] B.-C. Liau, C.-T. Shen, F.-P. Liang, S.-E. Hong, S.-L. Hsu, T.-T. Jong, et al., Supercritical fluids extraction and anti-solvent purification of carotenoids from microalgae and associated bioactivity, J. Supercrit. Fluids. 55 (2010) 169–175. doi:10.1016/j.supflu.2010.07.002.

[62] A.S. Pedro, S.D. Villa, P. Caliceti, S. a. B.V. De Melo, E.C. Albuquerque, A. Bertucco, et al., Curcumin-loaded solid lipid particles by PGSS technology, J. Supercrit. Fluids. 107 (2016) 534–541. doi:10.1016/j.supflu.2015.07.010.

[63] G. Ferrentino, S. Spilimbergo, High pressure carbon dioxide pasteurization of solid foods: Current knowledge and future outlooks, Trends Food Sci. Technol. 22 (2011) 427–441. doi:10.1016/j.tifs.2011.04.009.

[64] G. Brunner, Supercritical fluids: Technology and application to food processing, J. Food Eng. 67 (2005) 21–33. doi:10.1016/j.jfoodeng.2004.05.060.

[65] J.M. del Valle, Extraction of natural compounds using supercritical CO2: Going from the laboratory to the industrial application, J. Supercrit. Fluids. 96 (2014) 180–199. doi:10.1016/j.supflu.2014.10.001.

CHAPTER 2

Production of essential fatty acids from microalgae by SFE[2]

Supercritical CO_2 extraction from microalgae is applied with the aim of obtaining an oil rich in α-linolenic (ALA) essential fatty acid and with a low ω6:ω3 ratio. The maximum extraction yield is obtained at 60 °C and 30 MPa with 0.4 kg/h of CO_2 and 5% of co-solvent (ethanol). When the effect of pressure, temperature and density on the supercritical extraction yield and solubility are studied, the thermodynamic cross-over is found at a pressure close to 30 MPa, while the extraction cross-over occurs at around 25 MPa. The experimental solubility data are correlated by literature empirical models. Mathematical models developed by Sovová are applied to describe the experimental extraction curves. Soxhlet extraction of lipids is also carried out, obtaining a similar fatty acids profile but proving to be less selective than $SCCO_2$ method. Among the three species of microalgae examined, results show that *Scenedesmus obliquus* oil is richer in ω-3 fatty acids and ALA than *Chlorella protothecoides* and *Nannochloropsis salina* lipids. The effect of the extraction parameters on ALA content and the fatty acid profile is also analysed, concluding that the ω-3 percentage is favoured by lower temperatures, lower pressures and shorter extraction times.

Abbreviations: ALA, α-linolenic acid C18:3ω3; LA, linoleic acid C18:2ω6; AA, arachidonic acid C20:4ω6; EPA, eicosapentaenoic acid C20:5ω3; DPA, docosapentanoic acid C22:5ω3; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; FFA, free fatty acids

2.1. Introduction

Western diets are deficient in omega-3 fatty acids, and have excessive amounts of omega-6 fatty acids compared with the diet on which human beings evolved and their genetic patterns were established. Excessive amounts of omega-6 polyunsaturated fatty acids (PUFA), and a very high ω6:ω3 ratio, promote the pathogenesis of many diseases, including cardiovascular ones, cancer, and inflammatory and autoimmune diseases, whereas increased levels of omega-3 PUFA exert suppressive effects [1]. Therefore, also due to the difficulty of changing the nutritional habits of a

---

[2] *Part of this chapter has been published as: Solana, M., Rizza, C.S., Bertucco, A., Exploiting microalgae as a source of essential fatty acids by supercritical fluid extraction of lipids: Comparison between Scenedesmus obliquus, Chlorella protothecoides and Nannochloropsis salina, 2014, The Journal of Supercritical Fluids 92, 311-318.*

whole society, in the last years many products enriched with omega-3 as nutritional supplements or functional foods have been developed to integrate the diet and reach a good ω6:ω3 ratio in blood without changing the diet too much [2].

The major current food source of omega-3 is fish. However, global fish stocks are in danger and consequently its production may decrease in the future if large amounts of omega-3 are needed. In addition to this, some fishes, especially marine ones like salmon, sardine, tuna, anchovy, mackerel or hake, are sometimes contaminated with heavy metals, as copper or mercury, and organic pollutants as PCBs or dioxins, which have a toxic effect for human health [3]. Furthermore, they have a limited capacity for synthesis of PUFA, so most of them are simply accumulated from their microalgae diet [4]. The idea of using microalgae to obtain essential fatty acids is increasingly being taken into account.

Besides producing lipids rich in PUFAs, microalgae offer important advantages over conventional oil crops. On the one hand, they can be cultivated under mild conditions, including short growth cycle with very simple nutrients supply, no field and season restrictions, and less extensive care [5,6]. Additionally, there is no need of the use of chemicals such as herbicides or pesticides thus reducing costs and environmental impacts [7]. On the other hand, the growth of microalgae can effectively remove phosphates and nitrates from wastewater and have greater photosynthetic efficiency than terrestrial plants [6]. Specifically, microalgae growth actively utilizes and captures about 1.9 kg of CO_2 for every kg of dry biomass produced [8].

Two of the most abundant fatty acids in microalgae are linoleic acid (18:2ω6; LA) and α-linolenic acid (18:3ω3; ALA), which are considered the two parent essential fatty acids, since human body is able to synthesize the long chain PUFAs from them. In particular, omega-6 arachidonic acid (C20:4ω6; AA) can be synthesized by humans from LA, and omega-3 fatty acids, such as eicosapentaenoic acid (C20:5ω3; EPA), docosapentaenoic acid (C22:5ω3, DPA) and docosahexaenoic acid (C22:6ω3, DHA), from ALA [2]. These essential oils have been traditionally obtained by steam distillation and hydrodistillation. However, these separation techniques suffer a number of problems due to the thermal degradation of some compounds of the essential oil and partial hydrosolubilization and hydrolysis, which can affect its quality [9]. Supercritical fluid extraction has already been demonstrated as a good method in the production of omega-3 oil and omega-6 concentrates, avoiding the use of high temperatures and organic solvents. In addition, since CO_2 is gas at room temperature, it is easily removed when extraction is completed, thus it is safe for food applications and it can safely be recycled, which is an environmental benefit. Other advantage respect to other methods for lipid extraction is the lack of catalyst requirement [7].

The present study investigates the influence of supercritical fluid extraction conditions on the fatty acid profile of lipids from microalgae, with a focus on increasing the omega-3 ALA content. The results of three different species of microalgae are also compared. These data form the basis to analyse the feasibility of exploiting microalgae as a source of omega-3 rich oil, which could become an interesting option for pharmaceutical and food markets.

2.2. Materials and methods

2.2.1. Chemicals and microalgae

Carbon dioxide (4.0 type, purity greater than 99.99%) used as supercritical solvent was provided by Rivoira. Ethanol (≥99.8%), hexane (99.8%), methanol (99.8%) and chloroform (99%) were purchased from Sigma Aldrich.

Scenedesmus obliquus 276-7, *Chlorella protothecoides* 33.80 and *Nannochloropsis salina* 40.85 strains were obtained from SAG-Goettingen. The growth temperature was 24±1 °C, with artificial light (fluorescent tubes) under a continuous photon flux density of 150±10 μE m^{-2} s^{-1}, measured by a photoradiometer (LI-COR, Model LI-189). *C. protothecoides* and *S. obliquus*, freshwater species, were grown in BG11 medium, following SAG indications. *N. salina,* a marine species, was cultured in sterilized sea salts with 22 g L^{-1} solution enriched with f/2 Guillard solution modified by adding an excess of 1.5 g L^{-1} of $NaNO_3$. Medium was buffered with 40 mM Tris–HCl pH 8 to avoid alterations due to excess CO_2 supply. Maintenance and propagation of cultures were performed using the same medium added with 10 g L^{-1} of Plant Agar (Duchefa Biochemie). These conditions had been previously optimized in our laboratory [10,11].

After the harvest, the microalgae suspension was centrifuged at 24°C for 10 minutes and 4425 rpm. The centrifuged microalgae were kept refrigerated at a temperature of -20°C until the oil extraction tests were performed.

Before extraction, microalgae were oven dried at 37°C for two days, to reduce the water content to less than 20 wt%, since it is possible to extract oil from microalgae with water content in the biomass up to 20 wt% [12]. The water content of raw microalgae powder was evaluated in the following way: a mass of 0.1 g of microalgae powder was heated at 80°C in an air flow oven until the mass no longer changed. By measuring the mass before and after the drying, we calculated the water loss. The water content is expressed as percentage with respect to the initial mass. After being dried for two days, a moisture content of 8% was left in *S. obliquus*, 20% in *C. protothecoides* and 16% in *N. salina*. The dried samples were ground and sieved before the tests, obtaining a particle size less than 0.5 mm.

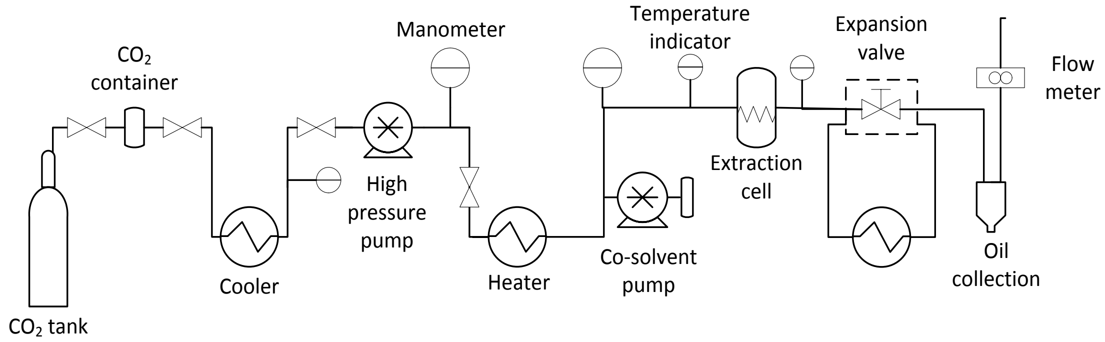

Fig. 2.1. Schematic diagram of the supercritical extraction equipment.

2.2.2. Soxhlet extraction

Solvent extraction was carried out in a laboratory scale apparatus at ambient pressure. A mixture of methanol: chloroform 2:1 was used as solvent. The temperature of extraction was maintained at 105 °C during 18 hours. Then the solvent was removed by a rotary evaporator at 40 °C and the extract was weighted.

2.2.3. Supercritical fluid extraction

A classical supercritical extraction process was specifically designed and constructed to carry out the microalgae oil extraction tests. Fig. 2.1 shows the schematic diagram of the equipment.

The operating procedure was as follows: the stainless steel vessel (extraction cell) was filled with 0.5±0.1 g of dried microalgae powder and heated by a thermoresistance. The temperature of the internal flow was controlled before and after the extractor. The CO_2 was compressed through a high pressure pump and pre-heated before flowing through the vessel. The pressure was controlled by two gauges. The co-solvent (ethanol) was pumped by a HPLC pump (model PU-1580) and mixed with the CO_2 before the extractor.

The choice of ethanol as a co-solvent was based on literature data [13,14,15]. The ethanol concentration was 5%. A constant CO_2 flow rate of 0.4±0.05 kg/h was kept in every run. The designed extraction pressures were 15, 20, 25 and 30 MPa. The minimum temperature was 45°C, as the addition of 5% ethanol to CO_2 increases the critical temperature of the mixture to 42.5°C [16].

After the extraction, the supercritical mixture was expanded by a Medium-Flow High-Pressure Metering Valve inserted in a water bath at 40 °C to avoid CO_2 freezing caused by sudden expansion. Extract samples were periodically collected in 12 mL of ethanol. CO_2 gas at atmospheric temperature passed through a flow meter before being vented. Finally, the system was depressurized. The lipids were collected after evaporating the co-solvent by a rotatory evaporator

Table 2.1. Relative oil extraction percentage from supercritical fluid extraction when compared with Soxhlet extraction.

Method	Extraction time (h)	Temperature (°C)	Extraction yield[a] (wt.%)	FFA conversion[b] (wt.%)	Lipid yield[c] (wt.%)
Soxhlet	18	105	29.03	51.13	14.84
$SCCO_2$	1.5	65	24.67	73.57	18.15

[a] Extraction yield= (mass of microalgae extract/mass of insoluble solid)×100
[b] FFA conversion= (mass of fatty acids/ mass of microalgae extract)×100
[c] Lipid yield= (Extraction yield×FFA conversion)/100

and weighted. At some operating conditions the experiments were made in duplicate to determine experimental error of the yield values, whose average resulted to be 6.00%.

2.2.4. Analysis of FFA

The fatty acids composition of the extracted oil was measured by gas chromatography. The method was performed according to reported procedures [17,18]. A GC Agilent Technologies (Model 7890) with a FID detector was used. The columns were Supelco (75 m × 180 µm × 0.14 µm film thickness) Model 23348-U and J&W (3.8 m × 250 × 0.25 µm film thickness) Model 190915-431. The carrier gas was H_2.

2.3. Results and discussion

2.3.1. Comparison between $SCCO_2$ and Soxhlet extraction

Table 2.1 summarizes the results of supercritical carbon dioxide extraction and conventional Soxhlet extraction with respect to extraction yield (wt.%), FFA conversion (wt.%) and lipid yield (wt.%). The extraction yield is expressed as the ratio between the mass of the total extract collected and the mass of the starting insoluble microalgae powder. It should be noted that the extraction yield not only includes the lipids, but possibly the pigments and waxes contained in the microalgae.

The extraction yield of *S. obliquus* obtained by Soxhlet extraction was 29.0%, higher than the recovery of the oil extracted by supercritical technology. However, only a 51.1% of FFA were found in the Soxhlet extracted oil, in comparison with a 73.6% of the FFA obtained by supercritical extraction. Therefore, the total lipid yield obtained by Soxhlet extraction was lower than the one by $SCCO_2$ (14.8% and 18.2%, respectively). Hence, Soxhlet results to be a less selective method that, apart from requiring toxic solvents, requires longer extraction times.

2.3.2. Extraction curves and modelling

Six fractions of the extracted oil were collected in order to obtain the overall extraction curve. It should be noted that the colour of the extract in the first samples was dark green, however the last samples presented a yellow colour, what indicates that the chlorophyll was extracted at the beginning of the extraction. This can be due to the effect of using ethanol as co-solvent, that is one of the most extended solvents used to extract chlorophyll.

Supercritical extraction curves were made by plotting yield against the ratio between solvent consumption and the insoluble microalgae biomass loaded into the extractor. The curve modelling was performed using the models published by Sovová in 2005 [19] and 2012 [20]. Three models were applied: the approximate one, the complete one and the simplified one based on the determination of characteristic times. The complete model, based on the concept of broken and intact cells, was initially developed to fit experimental data of supercritical fluid extraction of natural products. It independently simulates three extraction periods, the first one governed by phase equilibrium, the second one is a transition period and the third one is governed by internal diffusion within the particles. The adjustable parameters are the grinding efficiency, r, the product between the external mass transfer coefficient and the specific area between broken and intact cells, $k_s a_s$, and the product between the internal mass transfer coefficient and the specific area between broken and intact cells, $k_f a_s$. The approximate model makes a first estimation of the adjustable parameters r and $k_s a_s$, that are used to optimize all the parameters of the complete model. The simplified equations based on the determination of characteristic times consider four extraction steps: internal diffusion, external mass transfer, hypothetic equilibrium extraction without mass transfer resistance and displacement of the solution from the extractor. In this model, the external mass transfer resistance, θ_f, the characteristic time of the internal mass transfer, t_i, and the initial fraction of solute in the broken cells, G, are the unknown parameters.

Mouahid et al. [21] already verified that the modelling by Sovová's equations, initially developed for plants and vegetables, could be applied for the supercritical CO_2 extraction of lipids from microalgae. These authors fitted the experimental extraction curves from the microalgae species *Nannochloropsis oculata*, *Cylindrotheca closterium*, *Chlorella vulgaris* and *Spirulina platensis* subjected to different pre-treatments. In this research, the kinetics at different temperatures and pressures from *Scenedesmus obliquus* are investigated to show that Sovová's equations can also be applied with different solvent densities. The extraction curves obtained from *Chlorella protothecoides* and *Nannochloropsis salina* were also correlated by Sovová's models.

In agreement with Mouahid et al. results [21], only two periods were observed when the extraction curves from microalgae were plotted, instead of the three parts reported by Sovová [19]. Therefore, the first and the third equations of the complete model are enough to fit the experimental

Table 2.2. Parameters evaluated from experimental data on microalgae.

M	P (MPa)	T (°C)	Approximate model			Complete model				Characteristic times (simplified model)			
			r	$k_sa_s(\times10^5)$ s^{-1}	AARD (%)	r	$k_sa_s(\times10^5)$ s^{-1}	$k_fa_0\,(\times10^3)$ s^{-1}	AARD (%)	θ_f	t_i (min)	G	AARD (%)
S.o	15	45	0.22	7.40	1.41	0.23	1.29	1.88	2.58	0.03	17.19	0.43	10.94
S.o	25	45	0.34	8.35	0.88	0.36	18.96	1.84	3.60	0.03	10.46	0.74	9.66
S.o	30	45	0.44	2.71	0.92	0.44	5.35	1.96	1.01	0.03	35.46	0.54	13.56
S.o	15	55	0.18	7.01	1.25	0.18	12.13	1.96	0.76	0.03	21.70	0.42	18.14
S.o	25	55	0.22	4.32	1.97	0.18	7.95	1.84	0.34	0.03	28.02	0.36	7.69
S.o	30	55	0.25	5.31	2.40	0.20	9.99	1.81	1.46	0.03	23.20	0.41	4.04
S.o	15	65	0.15	3.29	2.21	0.14	5.70	1.78	0.60	0.03	39.61	0.28	8.05
S.o	25	65	0.38	6.01	1.96	0.25	17.46	1.83	2.76	0.03	16.08	0.67	13.26
S.o	30	65	0.32	4.36	4.53	0.24	9.35	1.77	4.37	0.03	20.33	0.36	7.27
C.p	30	45	0.25	2.78	2.99	0.16	6.38	2.04	1.33	0.03	51.47	0.41	14.09
N.s	30	45	0.36	2.56	0.99	0.37	4.61	1.73	0.50	0.03	39.21	0.48	15.01

M, microalgae; S.o, *Scenedesmus obliquus*; C.p, *Chlorella protothecoides*; N.s, *Nannochloropsis salina*; r, grinding efficiency; ks, solid-phase mass transfer coefficient; as, specific area between the regions of intact and broken cells; AARD, average absolute relative deviation; kf, fluid-phase mass transfer coefficient; a0, specific surface area per unit volume of extraction bed; θf, dimensionless external mass transfer resistance; ti, characteristic time of the solid phase mass transfer; G, initial fraction of extract in broken cells.

extraction curves. Mouahid et al. [21] opted to use the equations of the approximate model, however in this work both the approximate and the complete models were applied in order to calculate the values of all the parameters, which are summarised in Table 2.2. As can be seen, the values of r, k_sa_s are of the same order of magnitude as the values reported by Sovová [19] and Mouahid [21], for both the approximate and the complete model. The values of k_fa_0, obtained from the complete model, are of the same order of magnitude as those reported by Sovová [19]. The fitting results obtained by using the simplified equations based on the determination of the characteristic times are shown in Table 2.2.

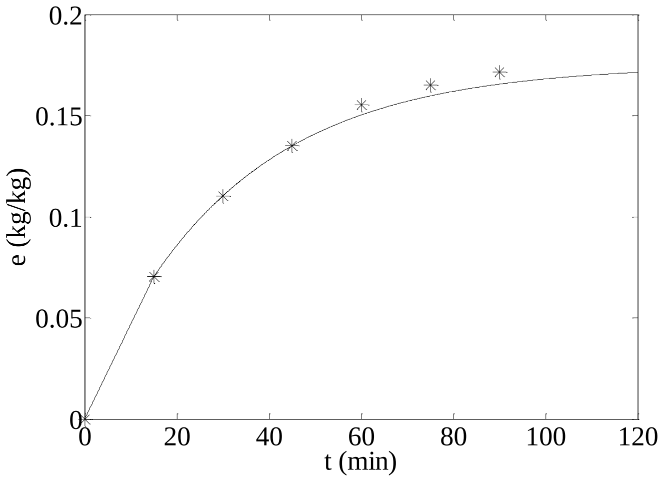

Fig. 2.2. $SCCO_2$ extraction curve of lipids from *Scenedesmus obliquus* at 65°C and 20 MPa, experimental and modelling (equations of characteristic times). The curve represent *e*, the extraction yield ($kg_{extract}/kg_{insoluble\ solid}$) as a function of the time (min).

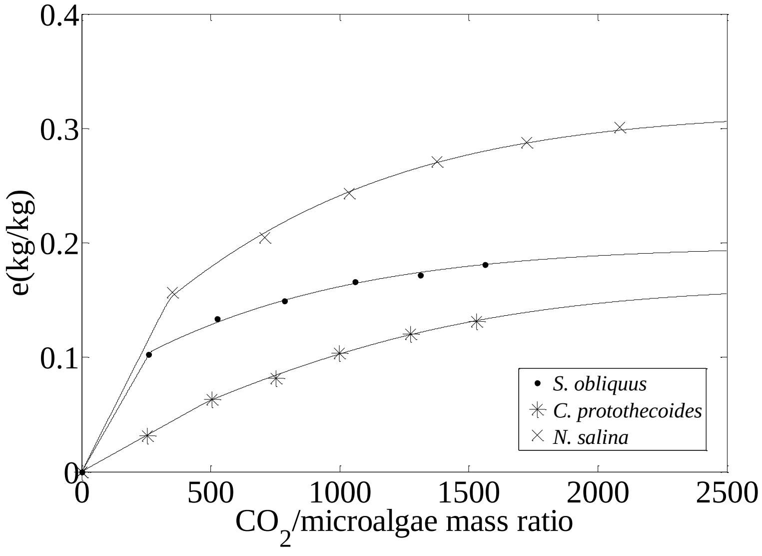

Fig. 2.3. SCCO$_2$ extraction curves of lipids obtained at 30 MPa and 45°C from *S. obliquus*, *C. protothecoides* and *N. salina*, experimental and modelling (complete model). The curves represent *e*, the extraction yield ($kg_{extract}/kg_{insoluble\ solid}$) as a function of the CO$_2$/microalgae mass ratio.

Once again, the values of the adjustable parameters *ti*, *θf* and *G* are of the same order of magnitude as the values reported by Sovová [20] and Mouahid [21]. As an example, Fig. 2.2 illustrates a fitted extraction curve by the equations based on the determination of characteristic times. The experimental data are those at 20 MPa and 65 °C. In this case, as well as in the others, the agreement between experimental data and modelling is satisfactory. The extraction curves fitted by the complete model are shown in sections 2.3.3 and 2.3.4.

2.3.3. Comparison between the three species of microalgae

The experimental curves of three different species of microalgae, *S. obliquus*, *C. protothecoides* and *N. salina*, are shown in Fig. 2.3 and compared with the results of the complete model. They were obtained at 30 MPa and 45°C, with a CO$_2$ flow rate of 0.4±0.05 kg/h and 5% ethanol, during 90 minutes. The highest yield, 30.4%, was achieved with *N. salina*, followed by *S. obliquus*, 18.0%, and *C. protothecoides*, 13.0%. On the other hand, by comparing simulated and experimental curves from Fig. 2.3, it has been detected that the complete model ensures a good representation to experimental data. From Table 2.2, it can be seen that also the simplified and the characteristic times models behave correctly.

2.3.4. Effect of pressure and temperature

Temperature and pressure affect the supercritical CO$_2$ process in a complex way due to their combined effect on solvent density and solute vapour pressure. The solute vapour pressure increases with temperature raising the solubility, while temperature has an opposite effect on the solvent density and solubility. The pressure and temperature dependences of $SCCO_2$ extraction of *S. obliquus* were investigated. As Fig. 2.4 shows, at 15 MPa the extraction yield decreases from 15.7% to 11.5% when raising the temperature from 45 °C to 65 °C. The same behaviour was observed for extractions at 20 MPa and temperature from 45 °C to 65 °C. However, at 30 MPa the yield behaviour is the opposite: an increase in temperature from 45 °C to 65 °C enhances the extraction yield from 18.0% to 24.8%. At 25 MPa the yield was found to be practically the same at three different temperatures investigated (45 °C, 55 °C and 65 °C). This pressure is called cross-over pressure and it suggests that a pressure higher than 25 MPa, the dominant effect which influences the yield is the vapour pressure, instead of the density. Such a phenomenon, supported by a number of experimental studies, is related to the fact that at lower pressures the expected increase in oil fugacity with the increase of T is overcome by the decrease in density of $SCCO_2$ and therefore by the decrease of its solvent power [22]. Determination of crossover is interesting to better understand supercritical fluid phase phenomena in a region where the solubility is extremely sensitive to pressure [23, 24].

Finally, it should be noted that, comparing modelled and experimental curves from Fig. 2.5 and Fig. 2.6, the model can reproduce well the measured data obtained at different pressures and temperatures.

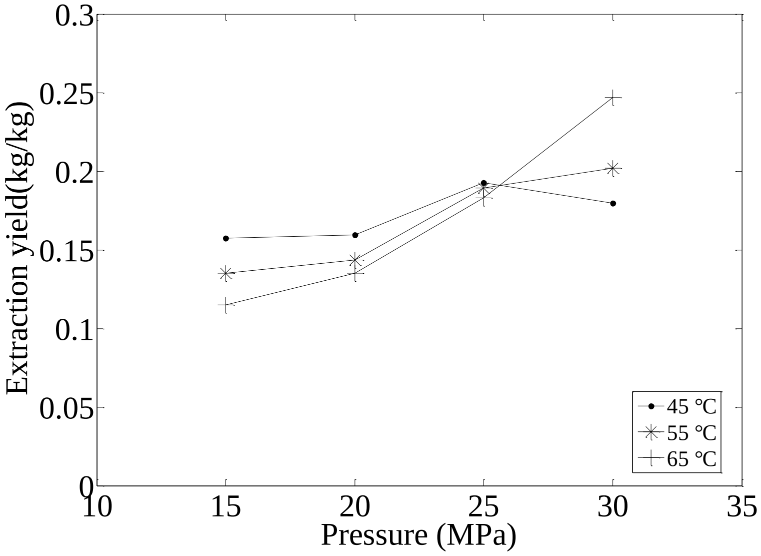

Fig. 2.4. Effect of operating pressure on the yield of the extraction ($kg_{extract}/kg_{insoluble\ solid}$) from *S. obliquus* under different operating temperatures.

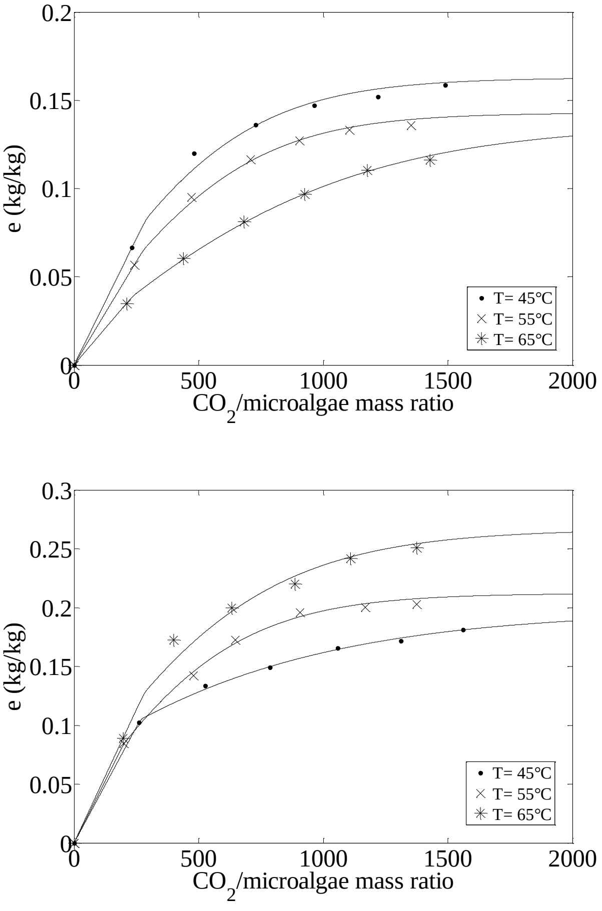

Fig. 2.5. $SCCO_2$ extraction curves of lipids from *Scenedesmus obliquus* at constant pressure (15 and 30 MPa), experimental and modelling (complete model). The curves represent *e*, the extraction yield ($kg_{extract}/kg_{insoluble\ solid}$) as a function of the CO_2/microalgae mass ratio.

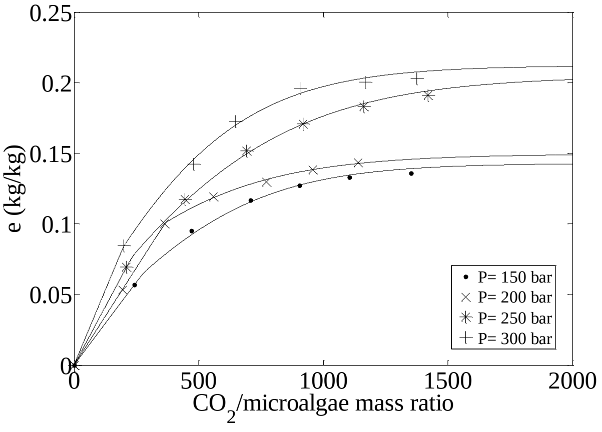

Fig. 2.6. $SCCO_2$ extraction curves of lipids from *Scenedesmus obliquus* at 55°C, experimental and modelling (complete model). The curves represent *e*, the extraction yield ($kg_{extract}/kg_{insoluble\ solid}$) as a function of the CO_2/microalgae mass ratio.

2.3.5. Cross-over and solubility

Once the extraction cross-over has been identified, it is interesting to verify that this phenomenon occurs also when the effect of pressure and temperature on solubility is analysed. In our experiments the feed-to-mass flow rate ratio (R) was 0.075 g min g^{-1}, which appears to be low to ensure saturation of the supercritical solution. Therefore the value of the experimental solubility is lower than the one corresponding to phase equilibrium. However, since R was kept constant in all our experiments, we have reasonably assumed that the decrease of solubility with respect to phase equilibrium is constant. Hence, the effect of density on the experimental solubility can be examined and the thermodynamic cross-over can be semi-quantitatively calculated.

At different conditions of pressure and temperature, the density of CO_2 is determined by applying the Bender equation [25]:

$$P=RT\rho+B\rho^2+C\rho^3+D\rho^4+E\rho^5+F\rho^6+(G+H\rho^2)\rho^3\exp(-a20\rho^2) \qquad (2.1)$$

where P is the pressure in MPa, R is the gas constant, T is the temperature in K and ρ is the density in g/cm3. B, C, D, E, F, G and H depend on the temperature and a_{20} is a constant as reported in reference [25].

Experimental solubilities in $SCCO_2$ (g/kg) are calculated from the slopes of the linear part of the extraction curves representing the yield as a function of the CO_2/microalgae mass ratio.

Three semi-empirical density-based equations are applied to correlate the measured solubility data: Chrastil [26], del Valle-Aguilera [27] and Adachi-Lu [28] models.

The Chrastil model correlates the solubility of the solute to the density of the pure solvent [26]:

$$S = \rho^{A}\exp\left(B + \frac{C}{T}\right) \qquad (2.2)$$

where S is the solubility in g/kg, ρ is the density of the solvent in kg/m^3, A is an association number, B is a function of the association number and molecular weights of the solute and the solvent, C is a function of the enthalpy of solvation and enthalpy of vaporization, and T is the temperature in K.

The Del Valle-Aguilera model assumes the association number as constant, and independent of the solvent density or temperature [27]:

$$S = \rho^{A}\exp\left(B + \frac{C}{T} + \frac{D}{T^2}\right) \qquad (2.3)$$

In the Adachi-Lu model, the association number is changed to a second-order polynomial of the solvent density [28]:

$$S = \rho^{A+E\rho+F\rho^2}\exp\left(B + \frac{C}{T}\right) \qquad (2.4)$$

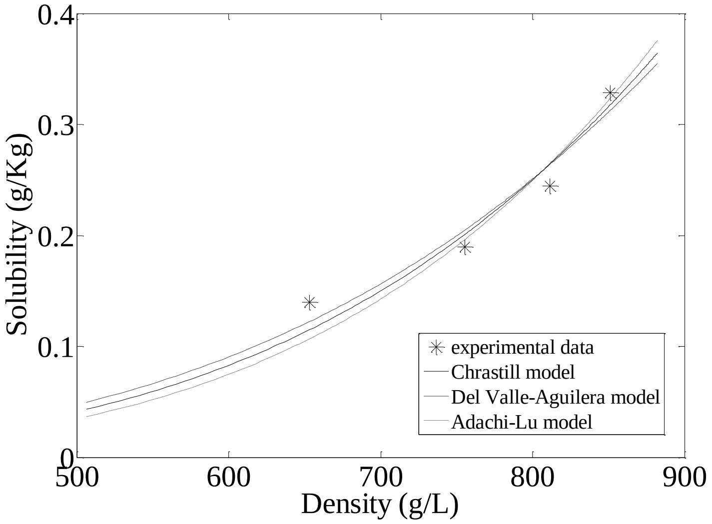

Fig. 2.7. Effect of density on solubility. Experimental data and comparison between the calculated curves by three semi-empirical models.

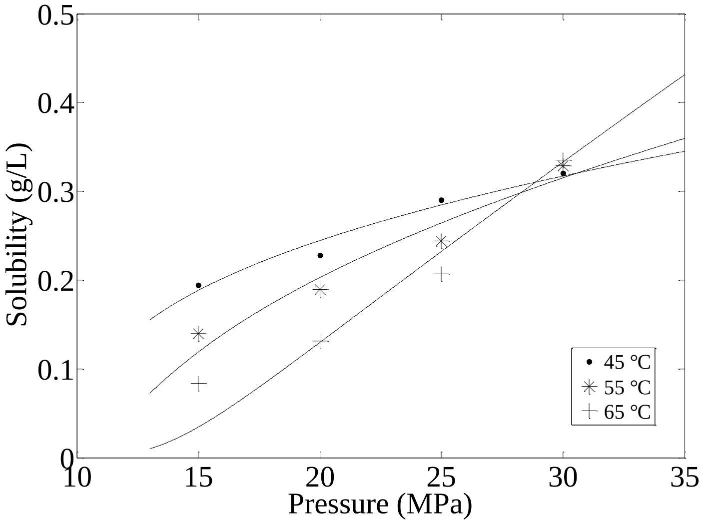

Fig. 2.8. Effect of operating pressure on solubility of microalgae in CO_2 under different operating temperatures modelled by the Del Valle-Aguilera equation.

The solubility model of Del Valle-Aguilera showed the best fits with experimental data, as illustrated in Table 2.3. However, no substantial difference was observed between the fitting of the three models, as illustrated in Fig. 2.7. In fact, the values of *E* and *F* from Adachi-Lu model (Table 3) are so small than they could be neglected and consequently the equation would become the Chrastil one.

The experimental and modelled microalgae solubility data on $SCCO_2$ at different pressures and temperatures are shown in Fig. 2.8. In this case, a thermodynamic cross-over is found at a pressure close to 30 MPa, higher than the already mentioned extraction cross-over pressure.

Table 2.3. Calculated parameters for the semi-empirical solubility models.

Solubility models	Model constants						AARD (%)
	A	B	C	D	E	F	
Chrastil	3.9265	17.7140	-14850.0000	-	-	-	9.09
del Valle-Aguilera	3.7059	-19.0766	-2324.8000	10587.0000	-	-	8.77
Adachi	4.4849	-25.0568	-2041.1000	-	1.04×10^{-10}	1.08×10^{-12}	9.71

AARD, average absolute relative deviation

2.3.6. Comparison of the lipid content

2.3.6.1. Influence of the extraction method on the fatty acids content

As shown in Table 2.4, no significant differences were found between the lipids obtained by supercritical extraction and conventional Soxhlet extraction, even if the extraction temperature was much higher in the second case. In both extracts the main components were palmitic (C16:0), oleic (C18:1ω9), C16:3ω3, C16:4ω3, LA (C18:2ω6) and ALA (C18:2ω3) fatty acids. As presented in Table 2.5, the polyunsaturated content of the oil extracted by Soxhlet was 52.7%, with a total omega-3 content of 36.3% and an ω6:ω3 ratio of 0.31. Very similar values were found in the oils extracted at different conditions by supercritical fluid technology, as shown in Tables 2.4 and 2.5. Regarding the parent essential fatty acids, the Soxhlet extracted oil had a 18.1% of ALA and a 8.9% of LA. Once again, similar values were found in the oil extracted by supercritical CO_2. Therefore, it can be concluded that the fatty acid composition does not change when the two extracting methods are used.

2.3.6.2. Comparison of microalgae fatty acid profiles

Nannochloropsis has traditionally been studied as a source of omega-3 polyunsaturated fatty acids [29, 30, 31] due to its high content in EPA (C20:5, ω3). Indeed, as shown in Table 2.4, *N. salina* lipids have a higher content of EPA (1.50%) than *S. obliquus and C. protothecoides,* where polyunsaturated fatty acids are 0.01% in the best conditions and 0.03%, respectively. Nevertheless, if we focused on the parent omega-3 fatty acid, *N. salina* has a very low content (0.3%), in comparison with *S. obliquus* (13.4%) and *C. protothecoides* (7.1%) at the same conditions of extraction, as illustrated in Table 2.4. Concerning the omega-6 fatty acids, the highest content of LA was observed in *C. protothecoides* (23.6%) and the lowest one in *N. salina* (1.2%). The LA content in *S. obliquus* was 9.3%. In addition, *S. obliquus* oil presents a low ω6:ω3 ratio, which is 0.35 at 30 MPa and 45°C, in comparison with *C. protothecoides* and *N. salina* oils, extracted at the same conditions, where these values are 1.56% and 3.02%, respectively (Table 2.5).

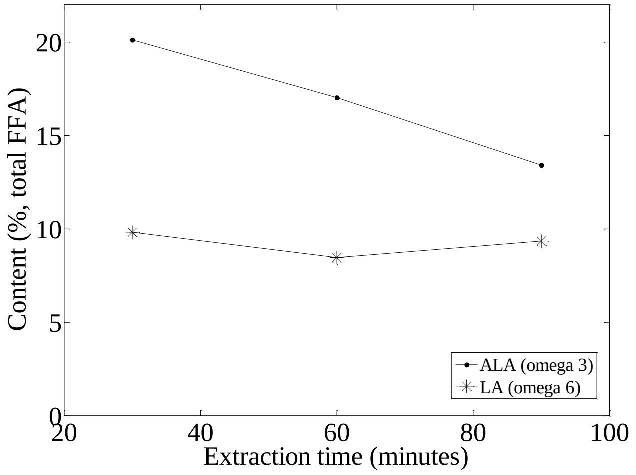

Fig. 2.9a. Influence of the extraction time on essential fatty acids content.

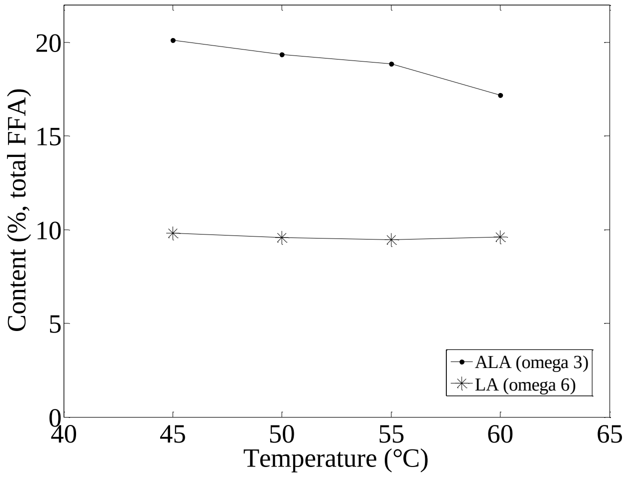

Fig. 2.9b. Influence of the temperature of extraction on essential fatty acids content.

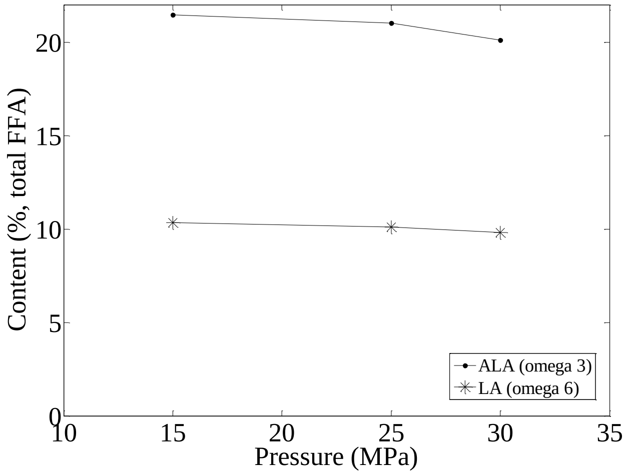

Fig. 2.9c. Influence of the pressure of extraction on essential fatty acids content.

2.3.6.3. Influence of supercritical extraction operating variables on the fatty acids content

Extraction time, temperature and pressure play a crucial role in the economy of the process, as they heavily affect productivity. Therefore, once studied the total yield of the supercritical extraction, the fatty acid profile of the oil extracted at different conditions was analysed.

By looking at Fig. 2.9a, one concludes that the maximum ALA yield is obtained at lower extraction times. However, LA content does not change significantly when extraction time increases. This result suggests that ALA is degraded due to its additional double bound with respect to LA.

A decrease in temperature is relevant towards ALA extraction efficiency, whereas LA yield remains practically constant (Fig. 2.9b).

A slight decrease of the ALA content is also appreciated when the pressure increases (Fig. 9c). Once again, the LA fraction remains practically constant.

It is also noted in Table 2.5 that the ω6:ω3 ratio and the PUFA percentage follows the same tendency that the ALA content. That is, the ω6:ω3 ratio increases and the PUFA content decreases when the extraction temperature, pressure and time are higher.

Table 2.4. Major fatty acids content (expressed as percentage of total fatty acids) in microalgae lipids as a function of the extraction method, species and SFE conditions adopted.

	S. obliquus									*C.protothecoides*	*N.Salina*
	Soxhlet	SCCO$_2$								SCCO$_2$	SCCO$_2$
Pressure (MPa)	1	30	30	30	**15**	**25**	30	30	30	30	30
Temperature (°C)	105	45	45	45	45	45	**50**	**55**	**60**	45	45
Extraction time (min)	1080	**30**	**60**	**90**	30	30	30	30	30	90	90
Saturated											
C14:0	0.54	0.47	0.51	0.82	0.42	0.45	0.49	0.51	0.55	0.76	3.87
C16:0	24.10	19.83	23.53	29.91	18.03	18.89	20.35	21.39	23.68	21.12	52.41
C18:0	0.93	1.10	1.39	1.87	0.97	1.01	1.15	1.45	1.06	1.46	2.29
Monounsaturated											
C16:1 ω9	3.00	2.65	2.73	4.11	2.15	2.45	2.78	2.77	3.19	4.80	0.24
C16:1cis ω7	0.30	0.26	0.36	0.51	0.17	0.24	0.28	0.29	0.34	1.58	29.17
C18:1 ω9	14.22	15.50	14.01	16.22	14.56	14.90	15.62	16.21	16.07	3.54	3.18
Polyunsaturated											
C16:2 ω6	2.23	2.03	2.12	0.00	1.98	2.00	2.22	2.19	2.53	0.00	0.00
C16:2	2.63	2.04	2.53	2.89	2.00	2.02	2.62	2.62	2.99	21.32	0.11
C16:3 ω3	4.03	4.20	4.09	3.83	4.00	4.10	4.02	4.39	3.95	7.60	0.11
C16:4 ω3	11.16	12.31	10.72	7.83	8.89	10.60	11.55	10.63	9.81	0.31	0.18
C18:2 ω6 (LA)	8.91	9.80	8.45	9.34	10.33	10.10	9.59	9.47	9.61	23.59	1.19
C18:3 ω3 (ALA)	18.08	20.10	17.02	13.40	21.47	21.01	19.36	18.86	17.18	7.12	0.30
C18:4 ω3	2.22	2.50	2.15	1.40	3.00	2.75	2.47	2.30	1.94	0.04	0.08
C18:5 ω3	0.74	0.01	1.92	0.00	0.00	0.01	1.11	0.95	0.83	0.00	0.00
C20:5 ω3 (EPA)	0.01	0.59	0.00	0.00	0.01	0.14	0.01	0.00	0.01	0.03	1.50

Table 2.5. Microalgae lipid classes (%) as a function of the extraction method, species and SFE conditions adopted.

	S. obliquus									*C.protothecoides*	*N.salina*
	Soxhlet	SCCO$_2$								SCCO$_2$	SCCO$_2$
Pressure (MPa)		30	30	30	**15**	**25**	30	30	30	30	30
Temperature (°C)		45	45	45	45	45	**50**	**55**	**60**	45	45
Extraction time (min)		**30**	**60**	**90**	30	30	30	30	30	90	90
SFA	27.38	22.75	26.90	34.59	20.33	21.33	23.40	24.65	26.71	25.68	61.25
MUFA	19.95	20.25	19.78	23.69	20.99	20.86	20.93	21.27	22.00	13.12	33.89
PUFA	52.67	57.44	53.32	41.80	58.84	58.08	55.67	54.08	51.28	61.77	5.66
Omega-3	36.32	40.90	36.01	26.59	41.86	41.10	38.63	37.24	33.76	15.13	0.77
Omega-6	11.20	11.00	10.61	9.34	10.65	10.46	11.86	11.71	12.20	23.63	2.32
Omega-6/Omega-3	0.31	0.27	0.29	0.35	0.25	0.25	0.31	0.31	0.36	1.56	3.02

2.4. Final remarks

On the basis of the results obtained in this chapter, *Scenedesmus obliquus* is the best source of ALA essential fatty acid among the three microalgae species studied. While SCCO$_2$ and Soxhlet have shown to be comparable as far as the process yield and fatty acid composition of the extracts are concerned, SCCO$_2$ is faster, more selective and does not require a toxic solvent. Studying pressure and temperature effects on the extraction yield and solubility, it was found that the cross-over phenomenon occurs at a pressure close to 25 MPa. Consequently, the maximum extraction yield was obtained at 30 MPa and 65 °C. The mathematical model and the equations based on the characteristic times developed by Sovová fitted accurately the experimental extraction curves. Experimental solubility data were correlated by Chrastil, del Valle-Aguilera and Adachi-Lu empirical models and a thermodynamic cross-over pressure was found close to 30 MPa. Finally, comparing FFA analysis of the extracted oils, it was found that the omega-3 content is negatively affected by high temperatures, high pressures and long extraction times, and therefore the highest amount of ALA was found at 45°C and 15 MPa after 30 minutes of extraction. An economical evaluation assessment could be useful to calculate the most viable conditions for industrial applications.

References

[1] A.P. Simopoulos, The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp Biol Med (Maywood) 233 (6) (2008) 674-88.

[2] N. Rubio-Rodríguez, S. Beltrán, I. Jaime, S.M. de Diego, M.T. Sanz, J. R. Carballido, Production of omega-3 polyunsaturated fatty acid concentrates: A review, Innovative Food Science and Emerging Technologies 11 (2010) 1–12.

[3] J.L. Domingo, A. Bocio, G. Falcó, J.M. Llobet, Benefits and risks of fish consumption I. A quantitative analysis of the intake of omega-3 fatty acids and chemical contaminants, Toxicology 230 (2007) 219–226.

[4] A.C. Guedes, H.M. Amaro, C.R. Barbosa, R.D. Pereira, F.X. Malcata, Fatty acid composition of several wild microalgae and cyanobacteria, with a focus on eicosapentaenoic, docosahexaenoic, and α-linolenic acids for eventual dietary uses, Food Research International 44 (2011) 2721-2729.

[5] A.M.F. Palavra, J.P. Coelho, J.G. Barroso, A.P. Rauter, J.M.N.A. Fareleira, A. Mainar, J.S. Urieta, B.P. Nobre, L. Gouveia, R.L. Mendes, J.M.S. Cabral, J. Novais, Supercritical carbon dioxide extraction of bioactive compounds from microalgae and volatile oils from aromatic plants, J. of Supercritical Fluids 60 (2011) 21-27.

[6] J. M. Gordon, J. E. W. Polle, Ultrahigh bioproductivity from algae, Applied Microbiology and Biotechnology 76 (2007) 969-975.

[7] R. Rawat, R. Ranjith Kumar, T. Mutanda, F. Bux, Biodiesel from microalgae: A critical evaluation from laboratory to large scale production, Applied Energy 103 (2013) 444-467.

[8] R. Halim, M.K. Danquah, P.A. Webley, Extraction of oil from microalgae for biodiesel production: A review, Biotechnology advances 30 (2012) 709-732.

[9] T. Bruno, C.A.N. Castro, J.F. Hammel, A.M.F. Palavra, Supercritical fluid extraction of biological products, in: J.F. Kennedy, J.M.S. Cabral (Eds.), Recovery Processes for Biological Materials, J.Wiley&Sons, Chichester, 1993 (Chapter 11).

[10] E. Sforza, R. Cipriani, T. Morosinotto, A. Bertucco, G.M. Giacometti, Excess CO_2 supply inhibits mixotrophic growth of *Chlorella protothecoides* and *Nannochloropsis salina*, Bioresource Technology 104 (2012) 523–529.

[11] E. Sforza, M. Enzo, A. Bertucco, Design of microalgal biomass production in a continuous photobioreactor: An integrated experimental and modeling approach, Chemical Engineering Research and Design, doi: 10.1016/j.cherd.2013.08.017.

[12] C. Crampon, A. Mouahid, A. Toudji, O. Lépine, E. Badens, Influence of pretreatment on supercritical CO_2 extraction from *Nannochloropsis oculata*, J. of Supercritical Fluids, doi: 10.1016/j.supflu.2012.12.022.

[13] M.D. Macias-Sanchez, C. Mantell, M. Rodriguez, E. Martinez de la Ossa, Kinetics of the supercritical fluid extraction of carotenoids from microalgae with CO_2 and ethanol as co-solvent, Chemical Engineering J, 150 (2009) 104-113.

[14] S. Tang , C. Qin, H. Wang, S. Li, S. Tian, Extraction of Carotenoids and Fatty Acids from Microalgae Using Supercritical Technology, J. of Supercritical Fluids, 57 (2011) 44-49.

[15] M. Careri, L. Furlattini, A. Mangia, M. Musci, E. Anklam, A. Theobald, Supercritical fluid extraction for liquid chromatographic determination of carotenoids in *Spirulina Pacifica* algae: a chemometric approach, J. of Chromatography, 912 (2001) 61-71.

[16] Hasbay, H.I. Cetin, M.E. Yener, A. Bayindirli, Subcritical (carbon dioxide + ethanol) extraction of polyphenols from apple and peach pomaces, and determination of the antioxidant activities of the extracts, J. of Supercritical Fluids, 43 (2007) 55-63.

[17] T.C. Jenkins, Technical note: Common analytical errors yielding inaccurate results during analysis of fatty acids in feed and digesta samples, J. Dairy Science, 93 (2010) 1170-1174.

[18] P.S. Sukhija, D.L. Palmquist, Rapid method for determination of total fatty acid content and composition of feedstuffs and feces, J. Agric. Food Chemistry, 36 (1988) 1202-1206.

[19] H. Sovová, Mathematical model for supercritical fluid extraction of natural products and extraction curve evaluation, J. of Supercritical Fluids 33 (2005) 35-52.

[20] H. Sovová, Steps of supercritical fluid extraction of natural products and their characteristic times, J. of Supercritical Fluids 66 (2012) 73-79.

[21] A. Mouahid, C. Crampton, S.A. Amine Toudji, E. Badens, Supercritical CO2 extraction of neutral lipids from microalgae: Experiments and modelling, J. of Supercritical Fluids 77 (2013) 7-16.

[22] J.P. Friedrich, Supercritical CO_2 extraction of lipids from lipid-containing materials, US Patent 4, 466, 923 (1984).

[23] S.A.B. Vieira de Melo, G.M.N. Costa, A.C.C. Viana, F.L.P. Pessoa, Solid pure component property effects on modeling upper crossover pressure for supercritical fluid process synthesis: A case study for the separation of Annatto pigments using SC-CO_2, J. of Supercritical Fluids, 49 (2009) 1-8.

[24] G. Brunner, Gas Extraction: An introduction to Fundamentals of Supercritical Fluids and the Application to Separation Process, Springer, Darmstadt, DE (1994) p. 387

[25] U. Sievers, Die thermodynamischen Eigenschaften von Kohlendiosxid, Fortschr.-Ber.VDI-Z, Reihe 6, Nr. 155, Düsseldorf, 1984, pp. 7-8.

[26] J. Chrastil, Solubility of Solids and Liquids in Supercritical gases, The Journal of Physical Chemistry, 86 (1982) 3016-3021.

[27] J.M. Del Valle, J.M. Aguilera, An improved equation for predicting the solubility of vegetable oils in supercritical CO_2, Industrial and Engineering Chemistry Research 27 (1988) 1551-1553.

[28] Y. Adachi, B.C.Y. Lu, Supercritical fluid extraction with carbon dioxide and ethylene, Fluid Phase Equilibria 14 (1983) 147-156.

[29] C.Y Chen, Y.C Chen, H.C Huang, C.C Huang, W.L Lee, J.S Chang, Engineering strategies for enhancing the production of eicosapentaenoic acid (EPA) from an isolated microalga *Nannochloropsis oceanica* CY2, Bioresource technology 147 (2013) 160-167.

[30] N. Zou, A. Richmond, Effect of light-path length in outdoor flat plate reactors on output rate of cell mass and of EPA in *Nannochloropsis sp*., Journal of Biotechnology 70 (1999) 351-356.

[31] N. Zou, A. Richmond, Effect of light-path length in outdoor fiat plate reactors on output rate of cell mass and of EPA in *Nannochloropsis sp*., Progress in Industrial Microbiology 35 (1999) 351-356.

CHAPTER 3

Supercritical CO_2 extraction of bioactive compounds from *Eruca sativa* leaves[3]

Supercritical fluid extraction from freeze-dried *Eruca sativa* leaves is assessed with the aim of studying the feasibility to obtain bioactive enriched fractions containing different classes of valuable compounds. Total extraction yields and compositions using pure CO_2 and CO_2+selected co-solvents are compared. Overall extraction curves, fitted by the model of broken and intact cells developed by Sovová, are reported and the influence of the main parameters that affect the extraction process is analysed. The extract with the highest content in glucosinolates and phenols was collected at 30 MPa and 75°C using 8% (w/w) of water with respect to the CO_2 flow rate, whereas the fraction richest in lipids was obtained using 8% (w/w) of ethanol as co-solvent at 45°C and 30 MPa. A process including a first step with supercritical CO_2 extraction using water as co-solvent followed by a second step, where a fraction rich in lipids is extracted using ethanol as co-solvent, is proposed. $SCCO_2$ results are compared with Soxhlet and other methods that combine organic solvents with ultrasounds.

3.1. Introduction

Rocket salad (*Eruca sativa*) is a vegetable considered as a good source of glucosinolates, phenolic compounds [1] and unsaturated fatty acids [2]. The potential beneficial effects of such natural products, with respect to several diseases (cancer, cardiovascular and neurological diseases) have been recently reported [3,4]. Indeed, some of the health promoting and cancer chemoprotective activity of *Eruca sativa* and the other cruciferous vegetables is widely believed to be associated to their content of minor dietary components such as glucosinolates [5]. It is also well known that polyphenols have antioxidant properties and several other specific biological actions in preventing and or treating diseases [6]. By other hand, erucic acid and other lipids obtainable from

---

[3] *Part of this chapter has been published as: Solana, M., Boschiero, I., Dall'Acqua, S., Bertucco, A., Extraction of bioactive enriched fractions from Eruca sativa leaves by supercritical CO_2 technology using different co-solvents, 2014, The Journal of Supercritical Fluids 94, 245-251.*

cruciferous oil are promising materials due to the physic-chemical characteristics of long chain fatty acids [7]. Extraction of phenolic compounds from plant materials has been traditionally carried out by solvents such as methanol, ethanol, acetone and ethyl acetate [6]. Aqueous methanol at high temperatures has been predominantly used to extract glucosinolates [1,8]. However the use of methanol in food products is limited by strict legal statues [9] and the use of high temperatures causes the thermal degradation and hydrolysis of many compounds, thus affecting their quality. Supercritical carbon dioxide could be an alternative and environmentally friendly technique, that offers several advantages over classical solvent extraction methods, since is inert, non-toxic and allows faster extraction at lower temperature and relatively low pressure. In this study, the polar nature of glucosinolates, phenolic compounds and a number of lipids makes it necessary the use of co-solvents, so to enhance the fluid affinity towards polar compounds. Water and ethanol are generally recognised as safe (GRAS), and environmental benign, and can therefore be used in food extraction processes [10]. In consequence, the use of these co-solvents permit to extract polar compounds without losing the advantages of $SCCO_2$ extraction, resulting in the development of an environmental friendly process to obtain in a safe way components of interest for food and pharmaceutical industries.

Supercritical fluid extraction of phenols from other vegetables has been widely applied [10–12]. However, only one study reporting the glucosinolate content of an extract obtained by $SCCO_2$ has been found, in which supercritical CO_2 extractions from Canola meals both without and with ethanol as co-solvent are applied and compared with conventional methods [13]. This work investigates supercritical fluid extraction from rocket salad with the purpose of obtaining two extracts, the first one rich in phenols and glucosinolates and the second one rich in unsaturated lipids. These data form the basis to analyse the feasibility of extracting valuable compounds from *Eruca sativa* leaves, products which could become an interesting option for the pharmaceutical and food markets, as active ingredients in functional-foods or food supplements. To the best of our knowledge, supercritical CO_2 assisted by water has never been applied for extracting glucosinolates from vegetables so far. Also, as far as we know, this is the first work on supercritical fluid extraction from rocket salad.

3.2. Materials and methods

3.2.1. Raw material

Rocket salad leaves were supplied by the agri-company “La Marostegana”, located in Piazzola sul Brenta (Italy). Raw material was crushed with a kitchen grinder and stored at -25 °C.

Before the extraction tests, crushed rocket salad leaves were lyophilized for 72 hours, milled with a mortar and sieved with a metallic filter (0.5 mm of pore diameter). The total content of water after lyophilisation was measured in the following way: a mass of 0.1±0.2 g of freeze-dried *Eruca sativa* was heated at 80°C in an air flow oven until the mass no longer changed. By measuring the mass before and after the drying, the water loss was calculated. The water content of the freeze-dried leaves, expressed as percentage with respect to the initial mass, resulted to be 4%.

3.2.2. *Chemicals*

CO_2 (4.0 type, purity greater than 99.99%) as supercritical solvent was purchased by Rivoira. Ethanol (99.8%), used as co-solvent for $SCCO_2$, and methanol (99.8%), used both as solvent for Soxhlet and co-solvent for $SCCO_2$, were purchased from Sigma Aldrich. Water used as co-solvent was Milli-Q quality. Methanol, acetonitrile, formic acid and ethanol, used for the measures, were provided by Carlo Erba, Prolabo, J.T. Baker and Lab-Scan.

3.2.3. *Supercritical fluid extraction*

Supercritical extraction tests were performed by a laboratory scale equipment already reported [14]. The operating procedure was as follows: the extraction cell was filled with 0.50±0.05 g of freeze-dried *Eruca sativa* powder. A thermo-resistance around the extractor maintained the desired temperature, which was controlled in the internal flow before and after the vessel. CO_2 was compressed through a high pressure pump and pre-heated before flowing through the extraction vessel. A constant CO_2 flow rate of 0.3 ± 0.05 kg h^{-1} was kept in every test. The co-solvent was pumped by an intelligent HPLC pump (Jasco PU-1580) and mixed with the CO_2 stream before the extractor. After extraction, the supercritical fluid was expanded. The extract and the co-solvent were collected in 12 mL of a solvent (the same used as a co-solvent). CO_2 gas at atmospheric temperature passed through a flow meter before being vented. The extract was filtered through a 0.20 µm filter (Ministart) and the co-solvent was evaporated by a rotary evaporator. The water content of the sample extracted using water as co-solvent was measured to ensure that it was totally evaporated.

Before any experimental run, a pre-treatment consisting in a pure supercritical CO_2 extraction during 15 minutes was carried out, in order to extract the low polarity CO_2-soluble compounds. It has been reported that a pre-treatment of raw material with supercritical CO_2 is essential to remove lipophilic and nonpolar substances and consequently makes polyphenols more available for the extraction [15]. In this case, the objective of the "CO_2 extraction pre-treatment" was also to obtain a first fraction of lipids and to deactivate the enzyme myrosinase, a step which it is necessary in order to achieve a better extraction of the components of interest [16]. During SC-CO_2 extraction, the level of inactivation of the myrosinase enzyme depends on the extraction operating variables [17].

In kinetics evaluation, the extraction curve simulation was performed using the model published by Sovová in 2005 [18]. This model is based on the concept of broken and intact cells and simulates two extraction periods, the first one governed by phase equilibrium and the second one governed by internal diffusion in particles. The first step to develop this model, called complete model, consists in the application of a simplified approximate form of it in order to estimate the parameters. The parameters calculated by both the approximate and the complete models are reported in section 3.4.

3.2.4. Soxhlet extraction

Solvent extraction was carried out by a traditional Soxhlet apparatus. A mixture of methanol: water 7:3 was used as solvent. The temperature of extraction was maintained at 100 °C during 18 hours. Then the solvent was evaporated by a rotary evaporator.

3.2.5. Quantification of phenolic and lipids contents

For the quantification of the phenolic content, the freeze-dried vegetal material (0.025±0.001 mg) was extracted in an ultrasound system for 20 min with 7 mL of methanol 70%. The supernatant was removed after centrifugation. Liquids were collected and the volume was adjusted to 10 mL in a volumetric flask. The final water content of the solution was changed to 50% to increase chromatographic peak resolution. Solutions were filtered through 0.45 μm and used for the HPLC analysis.

For the quantification of the lipids content, the freeze-dried vegetal material (0.050±0.001 g) was extracted with n-hexane (5 mL) in ultrasound bath for 10 minutes in a flask. Supernatant was removed after centrifugation and the liquid was dried under vacuum at 40°C. Residue was dissolved in methanol (2 mL). Solutions were filtered through 0.45 μm and used for the HPLC analysis.

3.2.6. Analysis of the extracts

Quali-quantitative analysis of glucosinolates, phenols and lipids in the extracts were obtained by HPLC-MS. The measurements were performed on a Varian 212 series chromatograph equipped with Prostar 430 autosampler and MS-500 Ion Trap as detector. MS spectra were recorded in positive and in negative ion mode (50–2000 Da). The APCI (Atmospheric Pressure Chemical Ionizationion) source was used for lipid analysis while the ESI (Electron Spray Ionization) was used for phenolic and glucosinolates. Fragmentation of the main ionic species were obtained during the

HPLC run by the turbo data depending scanning (tdds) function, yielding the fragmentation pattern of eluted compounds. As stationary phase Agilent Zorbax C-18 (2.1 × 150 mm) 3.5 µm was used.

For glucosinolates and phenolics compounds, mobile phases solvent A (water 0.1% formic acid) and solvent B (methanol) were used. The elution gradient started at 80% A then decreased to 0% A over 30 min. Quantification of phenolic constituents was obtained using rutin as reference compound. Calibration curves were obtained in the range 0.5-20 ug mL^{-1} at four different concentrations. Rutin calibration curve was (area Y vs concentration X) $Y= 134292 X + 512$ ($R^2=0.9998$). For glucosinolates quantification, gluconapin was used as internal standard. Calibration curve was obtained preparing solutions with different ratio of gluconapin (IS) and glucoerucin from standard solutions at 1.04 mg mL^{-1} of glucoerucin and 1.02 mg mL^{-1} of glucoraphanin. Calibration curve (area ratioY vs concentration ratio X) was $Y = 0.384 X + 0.012$ ($R^2=0.9998$).

For lipid analysis mobile phases were A (acetonitrile) and B (isopropanol), gradient elution was starting 100 % A then in 10 minutes to 40% A isocratic until 20 minutes then 15% A at 22 minutes and isocratic until 30 minutes. As reference compound caprilic dyglyceride was used and the calibration curve was obtained in the range 3-73 ug mL^{-1}. Calibration curve (area Y vs concentration X) was $Y= 4.3\ 10^{-7} X + 5.92$. ($R^2=0.9998$). Analysis were all performed in triplicate.

3.3. Results and discussion

3.3.1. Effect of the co-solvent on the extraction yield and composition of the extract

Preliminary extraction tests were performed with the aim of selecting the co-solvent that extracts better each one of the compounds of interest. First, three co-solvents were tested: water, ethanol and methanol. Then, $SCCO_2$ extraction without any modifier was carried out. All the runs were performed at the same conditions: 30 MPa, 45°C, 0.3 kg h^{-1} of CO_2 and 8% of co-solvent (w/w) with respect to the CO_2 flow rate. Two extractions tests were performed for each co-solvent. Table 3.1 shows the average and the error bars of the extracted amounts on the base of the two measures.

It should be noted that, as calculated by Peng Robinson equation of state and according to literature data [19], the 8% weight fraction of water is well above the solubility of water in CO_2 at the operating conditions of the tests. Consequently, these extractions are carried in the presence of an aqueous liquid phase. In contrast, when 8% of ethanol or methanol are used as co-solvents at these conditions, they are fully solubilized in CO_2 and hence there is only one phase.

Table 3.1: Extraction yield, total lipids content (TLC), total phenolic content (TPC) and total glucosinolates content (TGC) from supercritical fluid extraction at 45°C and 30 MPa using different co-solvents.

Co-solvent	Extraction yield[a] (%)	TLC (mg g^{-1})	TPC (mg g^{-1})	TGC (mg g^{-1})
Water	29.20±0.61	0.00±0.00	0.64±0.08	1.82±0.02
Ethanol	5.54±0.24	87.31±0.76	0.19±0.01	0.00±0.00
Methanol	5.56±0.06	42.09±0.03	0.28±0.01	0.30±0.02
None	1.50±0.07	24.85±0.38	0.00±0.00	0.00±0.00

a Extraction yield= (mass of rocket salad extract/mass of dried rocket salad powder)×10

Results, shown in Table 3.1, indicate that a much higher quantity of extract is obtained when water is used as co-solvent (29.2%), with respect to the values found using ethanol and methanol as co-solvents, and with pure supercritical CO_2. This could be due to the fact that the presence of water increases the density of the fluid mixture, causing swelling of the particles, and therefore improving diffusion process and solubilisation of several compounds [10]. Practically no differences were found between the yields obtained with ethanol and methanol as co-solvents. A low extraction yield (1.5%) was obtained with pure $SCCO_2$, indicating that *Eruca sativa* leaves contains mainly polar compounds, which are not extractable without the use of a co-solvent.

As regards the composition of the extract, the fraction recovered using water as co-solvent was the richest one in phenols and glucosinolates when compared with the other co-solvents or when the extraction was carried out without a modifier, probably due to the hydrophilic nature of such constituents. Particularly, the extract obtained by $SCCO_2$+water contained 0.64 mg g^{-1} of phenolics and 1.82 mg g^{-1} of glucosinolates at 45°C and 30 MPa, as shown in Table 3.1. No lipids were found in this fraction. Table 3.1 also shows that the fraction with the highest content in lipids was extracted using ethanol as co-solvent (87.31 mg g^{-1}) while only traces of phenols were found in it. The extract obtained by $SCCO_2$+methanol contained less lipids than the one obtained by $SCCO_2$ ethanol, but presented a higher content of phenols and glucosinolates. The sample obtained with pure $SCCO_2$ contained 24.85 mg g^{-1} of lipids, whereas phenolic and glucosinolate compounds were no identified, as reported in Table 3.1.

3.3.2. Supercritical CO_2 extraction using water as co-solvent

Once water was demonstrated to be the most efficient co-solvent to extract glucosinolates and phenols from *Eruca sativa*, the effect of the operative conditions on the extraction yields and compositions was studied.

In Figure 3.1a overall extractions curves, experimental and simulated ones, are plotted at temperatures between 45 and 75°C. The runs were performed at 30 MPa using 0.4 mL min^{-1} of co-solvent (8% (w/w)). As illustrated in Figure 3.1a, the effect of temperature is very relevant, as the yield increases from 29.2% to 38.2% when the temperature is varied from 45°C to 75°C. This result can be due to the fact that at 30 MPa the pressure is higher than the called cross-over pressure, where the vapour pressure is the dominant effect which influences the yield, instead of solvent density. In addition, when the temperature of water is increased, its polarity decreases and becomes similar to that of methanol [9]. Therefore, the observed increase of the yield at higher temperatures could be also related to a more efficient extraction of less polar compounds that does not occur at lower temperatures.

As represented in Figure 3.1b, four extraction tests were performed at pressures between 15 and 30 MPa, maintaining the temperature at 65°C, with 0.4 mL min^{-1} of water as co-solvent. Contrary to expectations, no significant changes were found on the total extraction yield after 60 minutes. The lowest extraction yield was obtained at 20 MPa and the highest one at 25 MPa, but there is not a clear tendency and indeed the results are quite similar. As suggested by Farias-Compomanes et al. [20], "the low mass-transfer rates at the high pressures may be partially attributed to the low dispersion coefficient of the fluid". With increasing pressure, the solvent density and viscosity increase, reducing the ability of the fluid to penetrate the raw material and to interact with the extractable components. This effect may have counteracted the increase in the fluid density and solvation power at the higher pressures, reducing the extraction yields.

For the investigation of the influence of the co-solvent dosage, temperature and pressure were maintained at 65°C and 30 MPa, respectively. As shown in Figure 3.1c, a high increase of the extraction yield was found when the flow rate was raised from 0.4 to 0.5 mL min^{-1}. However, when such a flow rate of water was tested, the system got clogged. This could be due to the saturation of CO_2 with water, which is in liquid state at the extraction operative conditions, as mentioned above.

The total content of glucosinolates and phenols at different extraction conditions was measured with the objective of selecting the optimum conditions to extract a fraction rich in these bioactive molecules. Table 3.2 shows the results, together with the density of CO_2 at the different pressures and temperatures. The density was calculated by the equation of Bender [21]. From Table 3.2 it can be seen that the phenolic content increased when the temperature of extraction was higher, following the same tendency of the extraction yield, mentioned above. The glucosinolate content increased when the temperature was varied from 55 to 75°C. Hence, the highest glucosinolate content (1.96 mg g^{-1}) and the highest phenols content (1.48 mg g^{-1}) were obtained at the maximum

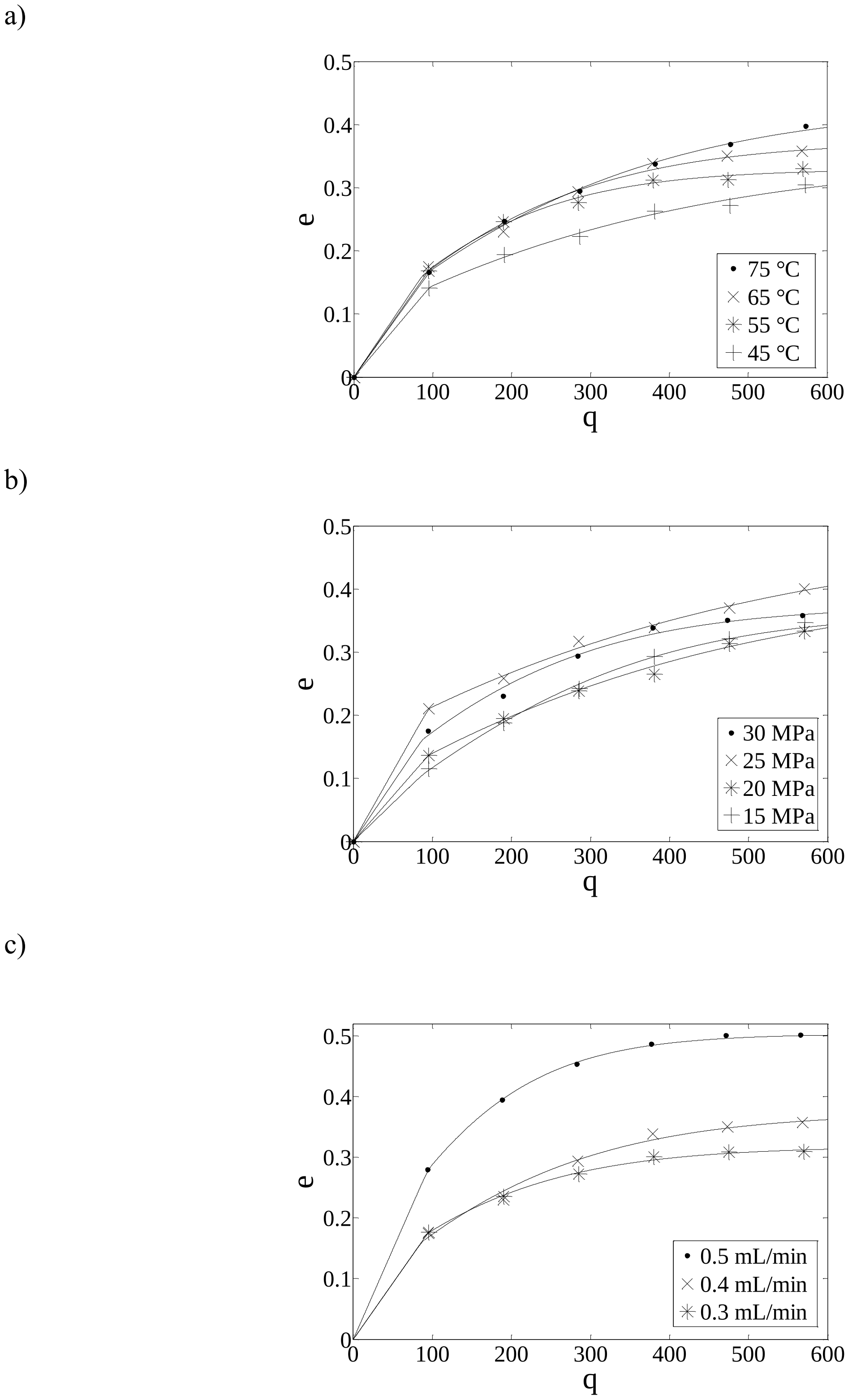

Figure 3.1: $SCCO_2$ extraction curves using water as co-solvent obtained at different operative conditions (temperature (a), pressure (b) and co-solvent dosage (c)), experimental and modelling. The curves represent *e*, the extraction yield ($kg_{extract}\ kg^{-1}{}_{solid}$) as a function of *q*, the relative amount of passed solvent ($kg_{solvent}kg^{-1}{}_{solid}$).

temperature tested, 75°C. On the other hand, the pressure controlled the extraction selectivity of phenols and glucosinolates components of Eruca sativa, as shown Table 3.2, even if the extraction

Table 3.2: Effect of the operating conditions on the total phenolic content (TPC) and total glucosinolate content (TGC) on the extracts obtained by supercritical fluid extraction.

Temperature (°C)	**75**	**65**	**55**	**45**	65	65	65	65	65
Pressure (MPa)	30	**30**	30	30	**25**	**20**	**15**	30	30
Density[a] (kg m^{-3})	767	810	851	891	762	692	554	810	810
Co-solvent dosage (mL min^{-1})	0.4	**0.4**	0.4	0.4	0.4	0.4	0.4	**0.5**	**0.3**
TPC (mg g^{-1})	1.48±0.13	1.42±0.18	1.07±0.08	0.64±0.18	1.23±0.13	1.04±0.11	1.01±0.14	1.31±0.16	1.11±0.13
TGC (mg g^{-1})	1.96±0.03	1.87±0.06	1.54±0.00	1.82±0.02	1.83±0.94	0.76±0.35	0.45±0.02	1.07±0.03	0.11±0.04

yield did not increase significantly when at higher pressure. Indeed, the fraction with the maximum content of bioactive molecules was extracted at the highest pressure studied, 30 MPa. Regarding the co-solvent dosage, the highest values of bioactive molecules content were found with 0.4 mL min-1 of water.

3.3.3. *Supercritical CO_2 extraction using ethanol as co-solvent*

The effect of temperature, pressure and co-solvent dosage on the extractions by $SCCO_2$+ethanol was also investigated, with the objective of selecting the optimum conditions to extract a fraction with the highest content of lipids. First, tests were performed at temperatures between 45 and 65°C and a constant pressure of 30 MPa using 0.5 mL min^{-1} of ethanol (8% (w/w)). As Figure 3.2a illustrates, the overall extraction curves at different temperatures are very similar and the final yields are practically the same. Clearly, at these operative conditions of extraction the cross-over point is around 30 MPa.

The influence of pressure on the extraction yield was studied at a constant temperature of 45°C, with 0.5 mL min^{-1} of ethanol. Figure 3.2b shows clearly that the higher the pressure, the higher the solubility and therefore the extraction yield. This is much more pronounced at higher pressures. As widely studied by $SCCO_2$ researchers, the higher densities ensured by higher pressures increase the solvent power leading to higher extraction efficiency. In this study, its highest value was observed at 30 MPa, which is the pressure limit for the equipment used.

For the investigation of the influence of the co-solvent dosage, temperature and pressure of the tests were maintained at 45°C and 30 MPa, respectively. As shown in Figure 3.2c, 0.5 mL min^{-1} (8% (w/w)) is the minimum flow rate of ethanol that allows to maximize the extraction yield.

Since the values of the yield at different temperatures were practically the same, the lipid content of the samples extracted at 45°C and 65°C was measured in order to select the optimum temperature to obtain the fraction richest in lipids. The total lipids content of the sample extracted at

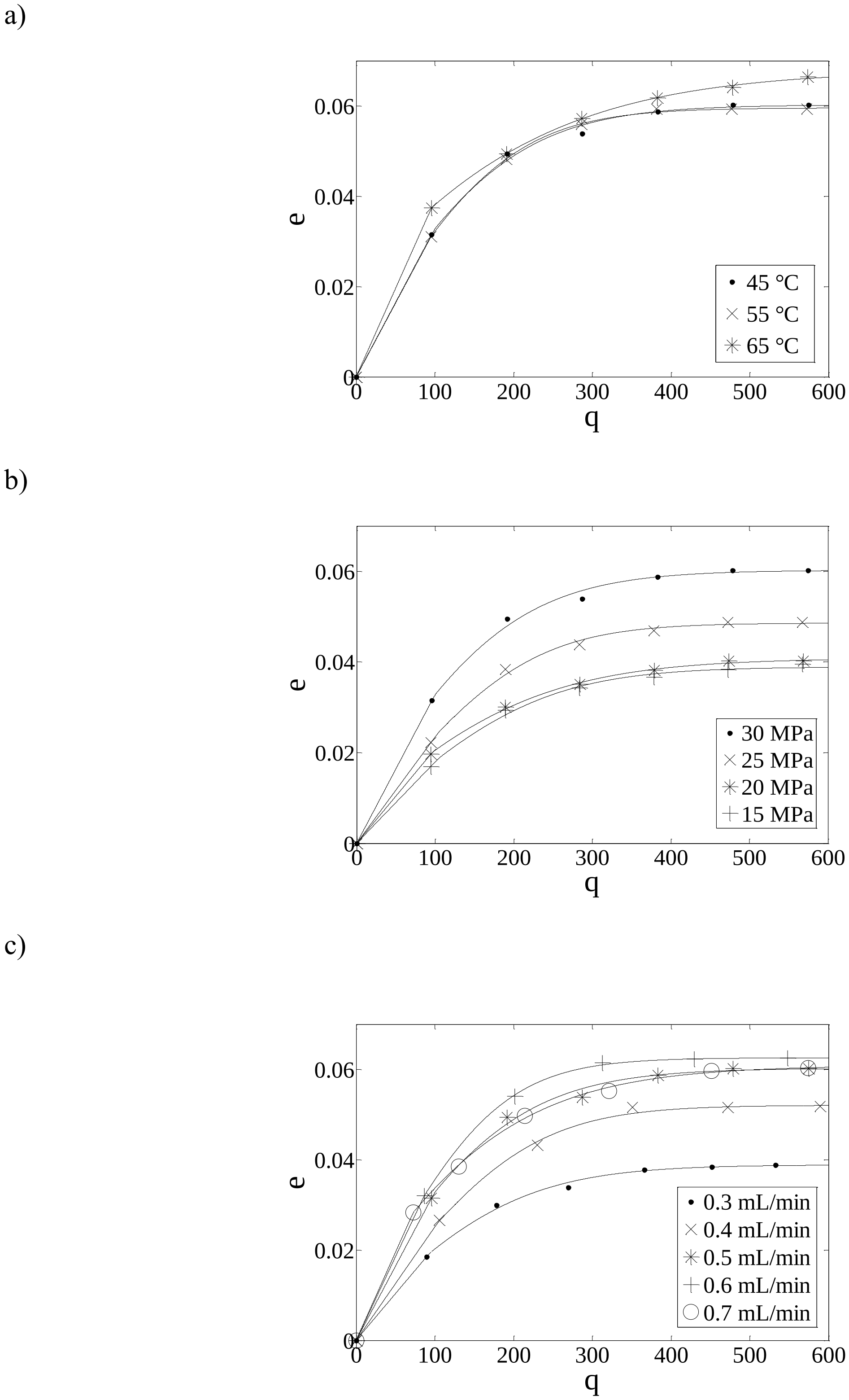

Figure 3.2: SCCO$_2$ extraction curves using ethanol as co-solvent obtained at different operative conditions (temperature (a), pressure (b) and co-solvent dosage (c)), experimental and modelling. The curves represent *e*, the extraction yield ($kg_{extract}\ kg^{-1}_{solid}$) as a function of *q*, the relative amount of passed solvent ($kg_{solvent}kg^{-1}_{solid}$).

Table 3.3. Mass transfer parameters evaluated from experimental data at different operating conditions using the approximate and the complete models.

C	P (MPa)	T (°C)	CD (mL min^{-1})	Approximate model			Complete model			
				r	$k_s a_s (\times 10^5)$ s^{-1}	AARD (%)	r	$k_s a_s (\times 10^5)$ s^{-1}	$k_f a_0$ $s^{-1} m^{-1}$	AARD (%)
water	15	65	0.4	0.18	5.59	3.17	0.14	10.30	1.89	1.31
water	20	65	0.4	0.24	3.21	1.85	0.25	5.14	1.89	1.35
water	25	65	0.4	0.36	2.32	1.46	0.36	3.99	1.89	1.31
water	30	65	0.4	0.16	7.74	3.81	0.29	10.40	1.90	2.49
water	30	45	0.4	0.32	3.22	1.97	0.32	5.48	1.80	2.06
water	30	55	0.4	0.33	7.33	1.57	0.35	13.30	1.81	1.81
water	30	75	0.4	0.26	4.55	1.25	0.27	7.44	1.88	1.16
water	30	65	0.5	0.33	8.96	0.94	0.36	16.38	1.90	0.49
water	30	65	0.3	0.39	6.39	1.27	0.40	12.05	1.80	0.94
ethanol	15	45	0.5	0.20	11.10	0.93	0.21	19.43	1.81	1.89
ethanol	20	45	0.5	0.27	8.63	0.67	0.29	14.92	1.81	0.90
ethanol	25	45	0.5	0.22	12.46	0.78	0.20	22.84	1.81	1.65
ethanol	30	45	0.5	0.26	11.49	1.22	0.29	19.82	1.79	1.49
ethanol	30	55	0.5	0.18	14.04	0.88	0.24	22.96	1.80	0.28
ethanol	30	65	0.5	0.41	5.29	0.47	0.42	10.28	1.79	0.22
ethanol	30	45	0.3	0.22	11.19	1.12	0.25	19.04	1.85	1.38
ethanol	30	45	0.4	0.17	15.97	1.31	0.20	27.57	1.82	1.02
ethanol	30	45	0.6	0.17	17.32	0.57	0.22	29.03	1.82	0.26
ethanol	30	45	0.7	0.29	7.10	1.13	0.30	12.26	1.79	0.68

C, co-solvent; CD, co-solvent dosage; r, grinding efficiency; ks, solid-phase mass transfer coefficient; as, specific area between the regions of intact and broken cells; AARD, average absolute deviation; kf, fluid-phase mass transfer coefficient; a0, specific surface area per unit volume of extraction bed; θf, dimensionless external mass transfer resistance; ti, characteristic time of the solid phase mass transfer; G, initial fraction of extract in intact cells.

45°C was 87.31 mg g^{-1}, as mentioned above. However, the extract obtained at 65°C only contained 44.8 mg g^{-1} of lipids, which indicates that the increase of temperature affected negatively the content of lipids. Considering this result and the ones obtained from the study of the yield, it can be concluded that the best conditions achieved by $SCCO_2$+ethanol were 30 MPa, 45°C using 8% of ethanol with respect to the CO_2 flow rate.

3.3.4. *Mathematical modelling of overall extraction curves*

The adjustable parameters for the model of broken and intact cells are presented in Table 3.3, together with the average absolute deviation obtained for each condition. The values of the parameters reported are of the same order of magnitude as the values reported by Sovová [18] for both the approximate and the complete model. The average absolute deviation are between 0.47 and 3.81 in the approximate model and between 0.22 and 2.49 in the complete model. Hence the

Table 3.4: Comparison among the phenolic content (TPC), glucosinolate content (TGC) and lipid content (TLC) of the fractions recovered in two-step supercritical extractions using water and ethanol as co-solvents.

	$SCCO_2$: (First step 8% water-Second step 8% ethanol)		$SCCO_2$: (First step 8% ethanol-Second step 8% water)	
	First fraction	Second fraction	First fraction	Second fraction
TPC (mg g^{-1})	1.48±0.18	0.24± 0.03	0.19±0.01	0.81±0.09
TGC (mg g^{-1})	1.96±0.03	0.00±0.00	0.00±0.00	1.06±0.03
TLC (mg g^{-1})	0.00±0.00	43.21±0.62	87.31±0.76	0.00±0.00

approximate model reproduced well our experimental data. A slight improvement was obtained with the complete one.

3.3.5. *Process proposal*

Two different two-step methodologies of supercritical fluid extraction were tested in order to optimize the process to extract two fractions from *Eruca Sativa* leaves. First, an experiment using water as co-solvent during 60 minutes at 30 MPa and 75°C, followed by an extraction using ethanol as modifier during 60 minutes at 30 MPa and 45°C was carried out. Then the extractions at the same conditions using first ethanol and then water as co-solvents were performed. In the first case, the raw material was dried in an oven at 37°C for three days before the second extraction. In the second experiment, the raw material was dried at 37°C in an oven for one day. The yield was calculated considering the weight of the raw material loaded into the extractor before the first extraction.

Table 3.4 shows the phenols, glucosinolates and lipids content of the samples extracted by these two steps processes. Results obtained using the two different approaches clearly showed a different composition of the obtained extracts. In particular using 8% water in the first step there is a higher yield in more polar constituents namely polyphenols and glucosinolates, while using ethanol 8% in the first step we observed a lower yield of these compounds but an increased yield of lipids. Thus in order to extract compounds with different polarity different protocols may be useful. As an example, the methodology shown in Figure 3.3 is proposed to recover two fractions of extract from *Eruca sativa* leaves by supercritical technology.

3.3.6. *Comparison between supercritical fluid extraction, Soxhlet and other extraction methods*

Eruca sativa leaves present an ample variability of total glucosinolate and phenol contents [1]. Phenolic contents are affected by biotic stresses (insect attack and pathogen infection) and abiotic stresses (light, temperature, nutrient supplies, water availability, growing conditions and UV radiation) besides storage conditions, post-harvest treatments and the estimation methods. All these

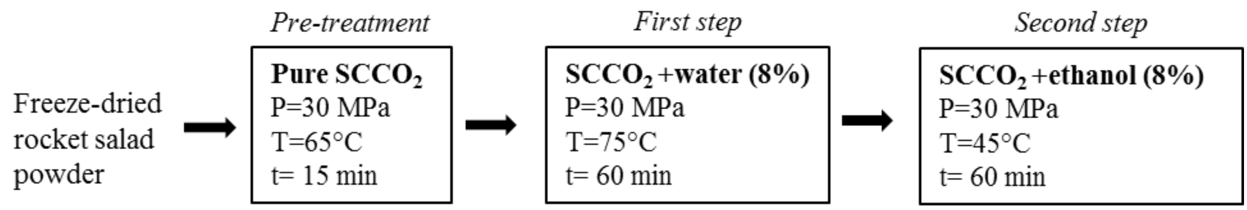

Figure 3.3: Proposal of a two-step methodology for the extraction of two fractions rich in valuable compounds from rocket salad.

Table 3.5: Comparison between $SCCO_2$, Soxhlet and quantification methods in terms of total phenolic content (TPC), total glucosinolates content (TGC) and total lipids content (TLC),.

Method	TPC (mg g^{-1})	TGC (mg g^{-1})	TLC (mg g^{-1})
$SCCO_2$ +water- $SCCO_2$ +ethanol	1.48±0.18	1.96±0.03	43.21±0.62
Soxhlet	0.16±0.04	1.57±0.28	0.00±0.00
Quantification methods	1.96±0.16	0.756-2.459[a]	64.43±5.33

[a] Literature data[1]

factors, together with the biosynthesis of phenolic antioxidant compounds, affect the final concentration of polyphenols in plant tissues [22]. This made it necessary the quantification of phenolic content in the raw material used for the experiments. An ultrasound system with aqueous methanol was the technique used to extract the phenolic compounds. The lipid content was also measured, obtaining the sample by n-hexane in an ultrasonication bath. The glucosinolate content is compared with the data published by Pasini et al. [1], that reported the results of 37 rocket salad accessions extracted by aqueous methanol at 75°C. Table 3.5 shows the results obtained using these extraction methods, together with the ones by Soxhlet and the composition of the extracts with $SCCO_2$+co-solvents. The total content of lipids, glucosinolates and phenols was higher in the fraction obtained by $SCCO_2$+co-solvents than by Soxhlet, as presented in Table 3.5. Comparing the total content of these bioactive molecules in the leaves extracted by $SCCO_2$+water (1.48 mg g^{-1}) with the total content of phenols extracted by ultrasound system with aqueous methanol (1.96 mg g^{-1}), 76% of the phenols were recovered by supercritical fluid extaction. In this extract, 77% of glucosinolates were recovered with respect to the maximum value found in *Eruca sativa* leaves by Pasini et al. [1]. Regarding the lipids content, 87.31 mg g^{-1} was the highest value obtained by $SCCO_2$+ethanol, as shown in Table 3.1. It means that supercritical CO_2 assisted by ethanol was more efficient than Soxhlet and the method that combines n-hexane (5 mL) and ultrasonication. This can be due to the effect of pressurized CO_2, that opens the pores of the cells facilitating the access of the solvent in the core.

3.4. Final remarks

In this chapter, supercritical fluid extraction from *Eruca sativa* leaves, using both pure CO_2 and $SCCO_2$+co-solvents, was investigated. For the extraction of glucosinolate and phenolic compounds, water resulted to be the most efficient co-solvent, whereas the fraction richest in lipids was extracted by $SCCO_2$+ethanol. The model of broken and intact cells developed by Sovová fitted well the experimental data. The effect of the operative conditions on the yield and composition of the extract presented a different behaviour when different co-solvents were tested. The extractions performed by $SCCO_2$+water were favoured by higher pressures and higher temperatures. Accordingly, an extract containing a total phenolic content of 1.48 mg g^{-1} and a total glucosinolate content of 1.96 mg g^{-1} was obtained operating at 30 MPa and 75°C. Nevertheless, the lipids content of the extract obtained by $SCCO_2$+ethanol was negatively affected by temperature and consequently the optimum conditions were found at 45°C and 30 MPa, resulting in a fraction with 87.3 mg g^{-1} of lipids. Thus the application of a sequential extractive approach was proposed using first CO_2+ethanol for lipid extraction and then water as co-solvent for phenolic and glucosinolate containing extracts. The sequential extraction scheme can be attractive for the production of solvent-free products containing health promoting constituents that can be useful as active ingredients in functional-foods or food supplements.

References

[1] F. Pasini, V. Verardo, M.F. Caboni, L.F. D’Antuono, Determination of glucosinolates and phenolic compounds in rocket salad by HPLC-DAD–MS: Evaluation of Eruca sativa Mill. and Diplotaxis tenuifolia L. genetic resources, Food Chemistry 133 (2012) 1025–1033. doi:10.1016/j.foodchem.2012.01.021.

[2] M. Khoobchandani, B.K. Ojeswi, N. Ganesh, M.M. Srivastava, S. Gabbanini, R. Matera, et al., Antimicrobial properties and analytical profile of traditional Eruca sativa seed oil: Comparison with various aerial and root plant extracts, Food Chemistry 120 (2010) 217–224. doi:10.1016/j.foodchem.2009.10.011.

[3] A.P. Vig, G. Rampal, T.S. Thind, S. Arora, Bio-protective effects of glucosinolates – A review, LWT - Food Science Technology 42 (2009) 1561–1572. doi:10.1016/j.lwt.2009.05.023.

[4] A.T. Dinkova-Kostova, R. V Kostov, Glucosinolates and isothiocyanates in health and disease., Trends Molecular Medicine 18 (2012) 337–47. doi:10.1016/j.molmed.2012.04.003.

[5] J.W. Fahey, a T. Zalcmann, P. Talalay, The chemical diversity and distribution of glucosinolates and isothiocyanates among plants., Phytochemistry 56 (2001) 5–51.

[6] J. Dai, R.J. Mumper, Plant phenolics: extraction, analysis and their antioxidant and anticancer properties., Molecules 15 (2010) 7313–52. doi:10.3390/molecules15107313.

[7] L. Lazzeri, M. Errani, O. Leoni, G. Venturi, Eruca sativa spp. oleifera: a new non-food crop, Industrial Crops and Products 20 (2004) 67–73. doi:10.1016/j.indcrop.2002.06.001.

[8] G. Kiddle, R. Bennett, -performance liquid chromatographic separation of natural and synthetic desulphoglucosinolates and their chemical validation by UV, NMR and chemical ionisation-, Phytochemical Analysis (2001).

[9] M.-J. Ko, C.-I. Cheigh, M.-S. Chung, Relationship analysis between flavonoids structure and subcritical water extraction (SWE)., Food Chemistry 143 (2014) 147–55. doi:10.1016/j.foodchem.2013.07.104.

[10] P.C. Veggi, J.M. Prado, G. a. Bataglion, M.N. Eberlin, M.A. a. Meireles, Obtaining phenolic compounds from jatoba (Hymenaea courbaril L.) bark by supercritical fluid extraction, J. of Supercritical Fluids 89 (2014) 68–77. doi:10.1016/j.supflu.2014.02.016.

[11] İ.H. Adil, H. Çetin, M.E. Yener, a. Bayındırlı, Subcritical (carbon dioxide+ethanol) extraction of polyphenols from apple and peach pomaces, and determination of the antioxidant activities of the extracts, J. of Supercritical Fluids 43 (2007) 55–63. doi:10.1016/j.supflu.2007.04.012.

[12] C. Da Porto, D. Decorti, A. Natolino, Water and ethanol as co-solvent in supercritical fluid extraction of proanthocyanidins from grape marc: A comparison and a proposal, J. of Supercritical Fluids 87 (2014) 1–8. doi:10.1016/j.supflu.2013.12.019.

[13] M. Sun, L. Xu, M.D.A. Saldana, F. Temelli, Comparison of canola meals obtained with conventional methods and supercritical CO2 with and without ethanol, J. of the American Oil Chemists' Society 85 (2008) 667–675. doi:10.1007/s11746-008-1239-5.

[14] M. Solana, C.S. Rizza, a. Bertucco, Exploiting Microalgae As A Source Of Essential Fatty Acids By Supercritical Fluid Extraction Of Lipids: Comparison Between Scenedesmus Obliquus, Chlorella Protothecoides And Nannochloropsis Salina, J. of Supercritical Fluids (2014). doi:10.1016/j.supflu.2014.06.013.

[15] A.T. Serra, I.J. Seabra, M.E.M. Braga, M.R. Bronze, H.C. de Sousa, C.M.M. Duarte, Processing cherries (Prunus avium) using supercritical fluid technology. Part 1: Recovery of extract fractions rich in bioactive compounds, J. of Supercritical Fluids 55 (2010) 184–191. doi:10.1016/j.supflu.2010.06.005.

[16] A.M. Ares, M.J. Nozal, J. Bernal, Extraction, chemical characterization and biological activity determination of broccoli health promoting compounds., J. of Chromatography A 1313 (2013) 78–95. doi:10.1016/j.chroma.2013.07.051.

[17] N.T. Dunford, F. Temelli, Effect of Supercritical CO 2 on Myrosinase Activity and Glucosinolate Degradation in Canola, (1996) 2372–2376.

[18] H. Sovová, Mathematical model for supercritical fluid extraction of natural products and extraction curve evaluation, J. of Supercritical Fluids 33 (2005) 35–52. doi:10.1016/j.supflu.2004.03.005.

[19] G.D. Pappa, C. Perakis, I.N. Tsimpanogiannis, E.C. Voutsas, Thermodynamic modeling of the vapor–liquid equilibrium of the CO2/H2O mixture, Fluid Phase Equilibria 284 (2009) 56–63. doi:10.1016/j.fluid.2009.06.011.

[20] A.M. Farías-Campomanes, M. a. Rostagno, M.A. a. Meireles, Production of polyphenol extracts from grape bagasse using supercritical fluids: Yield, extract composition and economic evaluation, J. of Supercritical Fluids 77 (2013) 70–78. doi:10.1016/j.supflu.2013.02.006.

[21] U. Sievers, Die thermodynamischen Eigenschaften von Kohlendiosxid, Fortschr.-Ber.VDI-Z, Reihe 6, N, 1984.

[22] M.E. Cartea, M. Francisco, P. Soengas, P. Velasco, Phenolic compounds in Brassica vegetables., Molecules 16 (2011) 251–80. doi:10.3390/molecules16010251.

CHAPTER 4

SFE of natural extracts from rocket salad: process design and cost benefit analysis[4]

A profitability analysis of a large scale supercritical fluid extraction (SFE) process to obtain natural extracts from rocket salad is presented in this chapter. An industrial-scale SFE plant is designed using process simulation techniques based on experimental data. Afterward, the effect of operative parameters on the process costs is evaluated. At the optimum conditions, the economic and financial analysis is performed, and the influence of the price of rocket salad on the price of the natural extract is assessed. Results indicate that the raw materials account for 67 % of the total production costs. The proposed industrial application could be economically viable with a pay-back period of 10 years if the price of the rocket salad extract is larger than 21.9 US\$ kg^{-1}, considering the current wholesale price in the market, or 17.0 US\$ kg^{-1} if using non-saleable rocket salad. Both scenarios make the process economically sustainable and competitive with the current price of natural extracts obtained by organic solvent extraction process.

4.1. Introduction

Plant extracts is a rapidly growing emerging industry due to the increased interest in the substitution of medicines by natural compounds for the prevention of diseases and the enhancement of our general health status. Main applications of the vegetable extracts include the fields of herbal medicine, food additives, functional food and cosmetics. These markets require ultraclean, verifiable, high quality products, reliable supplies and competitive prices [1]. Supercritical fluid technology is advantageously positioned as a sustainable and safe extraction option for the preparation of plant extracts and has been widely studied in a variety of applications during the last decades [2]. In many cases, the molecules of interest have high polarity such as most of the phenolic and glucosinolate compounds. The extraction of polar compounds by supercritical fluid

---

[4] *Part of this chapter has been published as: Solana, M., Mirofci, K., Bertucco, A., Production of phenolic and glucosinolate extracts from rocket salad by supercritical fluid extraction: Process design and cost benefits analysis, 2016, Journal of Food Engineering 168, 35-41.*

extraction requires the presence of polar co-solvents due to the non-polar nature of CO_2, and their effect is relevant on the extract composition and consequently the economics of the process.

Studies on the economic viability of SFE processes have been reported by several authors along the years. Among others, Fiori investigated this problem for a supercritical extraction plant from exhausted grape marc to obtain grape seed oil [3]. This study concluded that the proposed industrial application could be economically interesting, obtaining a breakeven point of 5.9 US$ kg^{-1} for the price of supercritically extracted grape seed oil. Comim et al. presented an economic analysis of a process to obtain extracts from banana peel oil, which resulted in a production cost of 13.69 US$ kg^{-1} for extractions performed at 30 MPa and 50 °C during 35 min of process [4]. Leal et al. concluded that the cost of raw material of the SFE of sweet basil represented 80% of the process costs, prevailing over both energy and utilities costs [5]. A similar result was reported by Veggi et al., who investigated the production of phenolic-rich extracts from the Brazilian plants *Pyrostegia venusta*, *Inga edulis*, *Heteropterys aphrodisiac* and *Phaseolus vulgaris* L., confirming that the price of raw material has a great influence on the final production cost [6].

Rocket salad (*Eruca sativa*) is a vegetable particularly rich in natural compounds such as glucosinolates [7–9], phenolic compounds [7,8] and unsaturated fatty acids [10,11]. Thus it can be ideal as a starting material for the production of extracts to be used in the market of functional foods or nutraceutical. As regards its numerous health benefits, it is an excellent stomachic, stimulant, diuretic and antiscorbutic [12]. Recent studies have also shown that an extract from rocket salad possesses antisecretory, cyto-protective and anti-ulcer activities [13]. Moreover, the cultivation of rocket salad with respect to other food crops has a number of advantages, since it is a very hardy plant which requires little care, little irrigation and manuring [12], and it easily adapts to climate changes and different soil types [14]. Indeed, rocket grows spontaneously in places modified by humans: abandoned gardens, waysides, tips and among rubble [12].

In our previous study, SFE from rocket salad and the content of the mentioned nutrients in the extracts is reported [15]. Accordingly, the use of rocket salad as a natural source of these compounds appears attractive and reasonably feasible. The present work aims to assess the scaling up, the feasibility and the profitability of the supercritical fluid extraction process to obtain natural extracts from rocket salad at a commercial level. The methodology presented herein could be easily extended to the supercritical fluid extraction of natural extracts from other vegetal materials.

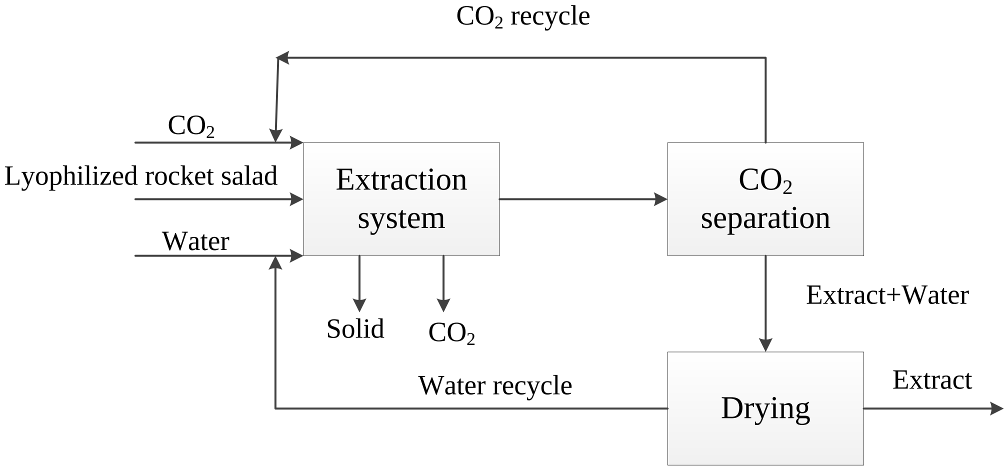

Figure 4.1. Block flow diagram of the SFE process.

4.2. Methodology

A conceptual design of an industrial scale application of the supercritical CO_2 extraction process to obtain natural extracts from rocket salad rich in glucosinolate and phenolic compounds is proposed, based on real data published by our group [15]. In the previous work, a two-step process using first water and then ethanol as co-solvents was proposed, obtaining two extracts rich in phenolic and glucosinolate compounds and lipids, respectively. In the present study, the attention is focused on the first step of such a process, namely water is used as a co-solvent. In this case, the highest extraction yields were obtained at 75°C, 25 MPa and 10% (w/w) of water in the CO_2 flow, whereas the highest content of phenolic and glucosinolates compounds was detected in the extract obtained at 75°C, 30 MPa and 8% of water (w/w). This natural extract presented 1.48±0.13 mg g^{-1} of phenols and 1.96±0.03 mg g^{-1} of glucosinolates. In this work an economical evaluation is performed in order to investigate the cost benefit analysis of such a process and optimizing the operating conditions from the economical point of view. The large scale process has been developed using the simulation software Aspen Plus™ V8.2.

4.2.1. Process model

The SFE plant has been designed for a processing capacity of 100 kg h^{-1} of lyophilized rocket salad, which is assumed as the raw material. Considering that the water content of rocket salad is 85.7 wt% [16] and the moisture of the lyophilized rocket salad of our experiments was 4 wt% [15], 546.45 kg of rocket salad are required to obtain 100 kg of lyophilized rocket salad.

As shown in Figure 4.1, the process consists of three main blocks: the extraction section, the CO_2 separation, recovery and recycle, and the drying step. The components involved in this process are the solid material to be extracted (lyophilized and milled rocket salad), CO_2 and water. The solid

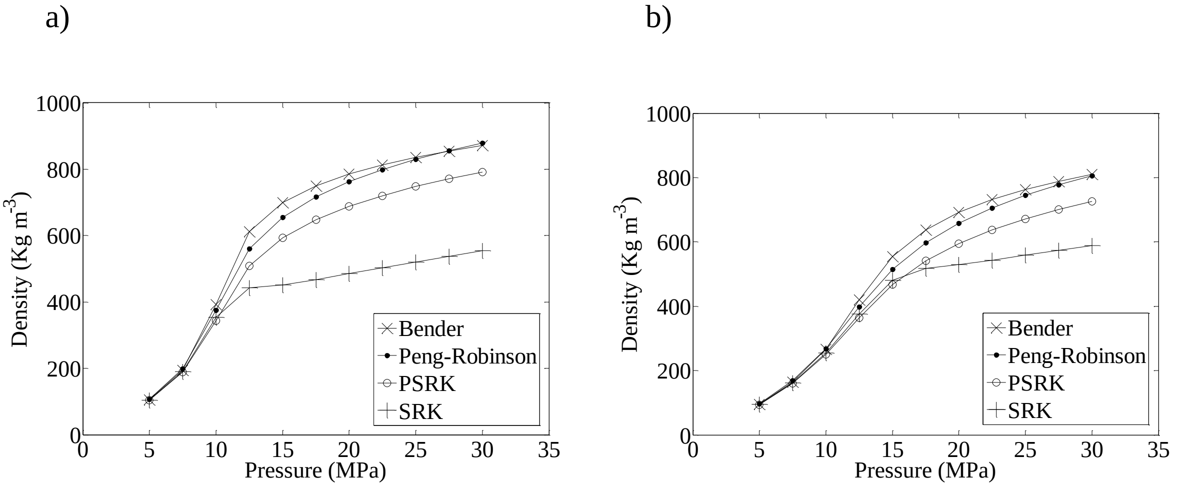

Figure 4.2. Comparison among the CO_2 density at different pressures calculated by the equations of SRK, PSRK, Peng Robinson and Bender at 50°C (a) and 65°C (b).

material was simplified due to the complexity of the composition of vegetal materials. Accordingly, the extractable solid material was approximated with the conventional component oil and the inert matrix was approximated with cellulose. Among the components present in Aspen Plus Process Simulator, cellulose and oil have been chosen to approximate the real material because of their macroscopic similarities with the real material components. We think this approximation does not affect the bulk properties of the process streams. By the other hand, the properties that could be affected by this approximation, such as solubility, have been experimentally measured using the real material, correlated to operating variables and inserted in the simulation.

The extraction system includes two extractors operating in parallel, in order to ensure a continuous throughput. The extract, the solid not extracted and the loss of CO_2 after extraction are considered as process outlets.

Peng-Robinson model was chosen as thermodynamic method [17]. The consistence of this method for the calculation of density was evaluated by comparing the data obtained from Peng-Robinson, PSRK [18] and SRK [19–21] with those of a more precise equation of state, the Bender equation [22]. Results, presented in Figure 4.2, indicate that Peng-Robinson is the most appropriate thermodynamic method for the calculation of the CO_2 density at the supercritical state, at the conditions here described and under the comparisons here performed.

The consistence of Peng-Robinson method to calculate the solubility of water in CO_2 was also evaluated. Data of water solubility in CO_2 obtained with this model were compared to experimental data from literature (Coan and King Jr., 1971; Hou et al., 2013; Wiebe, 1941). For this fitting the

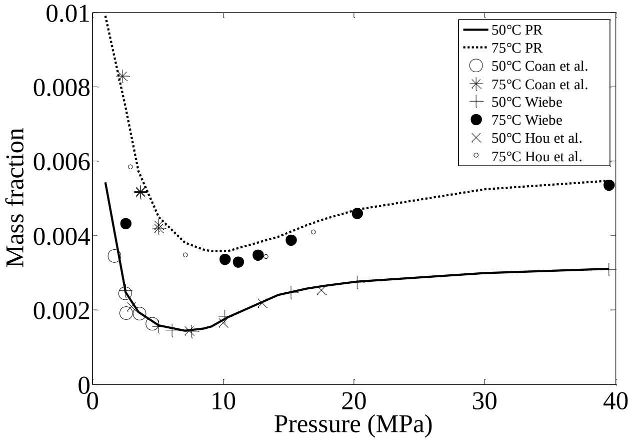

Figure 4.3. Comparison between the water solubility in CO_2 calculated by Peng-Robinson equation and experimental data.

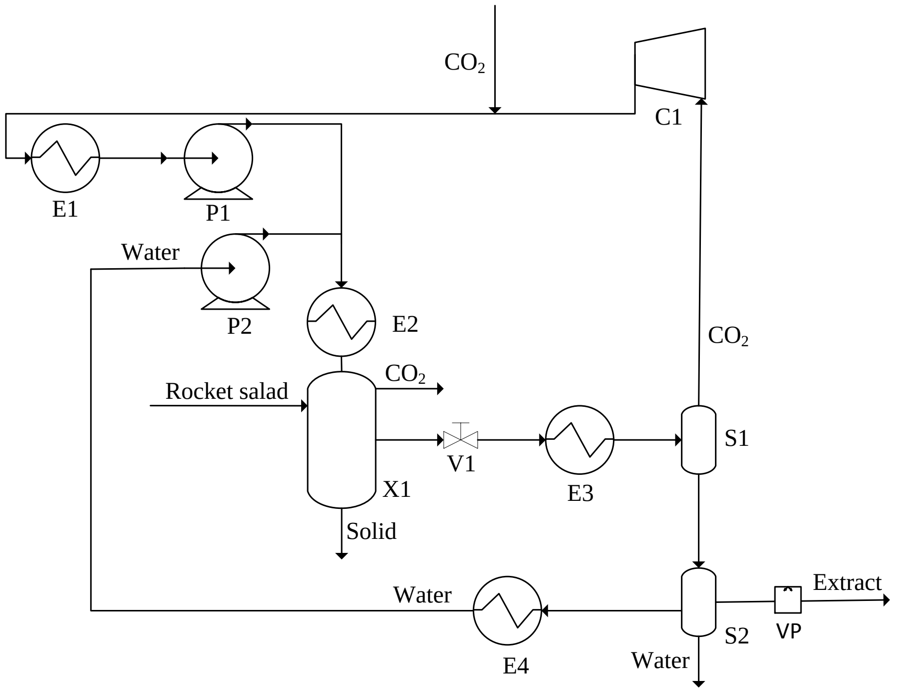

Figure 4.4. Schematic diagram of the SFE industrial plant for the extraction of natural extracts from rocket salad. Range of operating conditions: X1: 45-75°, 15-30 MPa; V1: from extraction pressure to separation pressure (0-7.4 MPa); S1: 75°C; S2: 75°C; VP: 0.01 MPa ;E1: 5°C; E2: 45-75°C; E3: 75°C; E4: 45-75°C; P1: 15-30 MPa; P2: 15-30 MPa; C1: from separation pressure to CO_2 storage pressure (6 MPa). If separation pressure> CO_2 storage pressure C1 will be substituted by an expansion valve.

binary interaction parameters have been adjusted, resulting to be kij(1)=0.20, kij(2)=0 and kij(3)=0. From the results, shown in Figure 4.3, it can be concluded that Peng-Robinson is an appropriate thermodynamic model as regards the calculation of the water solubility in supercritical CO_2 at the conditions of our interest.Figure 4.4 represents the schematic diagram of the process. In the following, the description of the models used to simulate and scale-up the process is summarized.

Table 4.1. Parameters *a* and *b* from the experimental curves obtained at different operating conditions.

P (MPa)	T (°C)	a	b
30	75	61.65	196.55
30	65	47.36	180.72
30	55	36.29	137.39
30	45	29.40	213.31
25	65	50.57	188.94
20	65	47.47	290.22
15	65	56.33	415.79

4.2.1.1.Extraction system

A flow rate of 30,000 kg h^{-1} of CO_2 at 25°C and 6 MPa (pressure of the CO_2 storage of our experiments) is cooled down (E1) in order to prevent the cavitation of the pump. This liquid CO_2 is pumped up (P1) to the extraction pressure. The co-solvent (water), stored at 0.1 MPa and 25°C, is pumped up to reach the extraction pressure by P2. The flow rate of water is 2400 kg h^{-1}, thus 8% (w/w) of water with respect to CO_2 is used, according to our experimental results. The mixture of CO_2 and water is heated (E2) to the extraction temperature before the extractor. A separation block has been used to simulate the extraction cell (X1) in Aspen Plus™ V8.2. An external calculator block calculates the split fraction of the extract and the remained solid at different times, according to the experimental extraction curves reported in our previous work [15]. They are represented by equation 4.1, where y=extraction yield (% $kg_{extract}/kg_{solid}$), x=specific solvent consumption ($kg_{CO2+water}/kg_{solid}$), and parameters *a* and *b* fitted on experimental data at different pressures and temperatures are listed in Table 4.1.

$$y = \frac{ax}{b+x} \tag{4.1}$$

It is worth noting that the applicability of the experimental data is not scale independent and consequently flow patterns would be changed by scaling up. This introduces uncertainty in our calculation. In any case, in this work a constant D/L ratio was kept, according to Duba at al. [27], who recently reported that the rate of $SCCO_2$ extraction mainly depends on extractor D/L ratio.

The losses of CO_2 at the end of the extraction, during the opening and refilling steps (two extractors are operating in parallel), are simulated by a separation block. They are referred to the CO_2 that remains in the extractor when the separation valve is closed, i.e. when the extraction pressure gets equal to the separator pressure. It means that once a cycle is finished, the first separation valve is closed, the extractor is depressurized and then it is cleaned and filled again with the raw material.

Table 4.2. Solubility of the extract in CO_2 at 75°C calculated by Chrastil equation.

P (MPa)	Density (g L^{-1})	Solubility ($g_{extract}$/ g_{CO2})
15	466.4	9.83×10^{-4}
10	233.7	6.19×10^{-5}
8	166.4	1.59×10^{-5}
7	138.5	7.65×10^{-6}
6	113.3	3.42×10^{-6}
5	90.5	1.39×10^{-6}
4	69.6	4.87×10^{-7}

In the meanwhile, the second extractor is operating. Therefore, the pressure and temperature of the CO_2 that remains in the extractor are the same of the extraction. The losses were calculated from the porosity of the solid bed in the laboratory extraction cell: it was measured that the apparent density of the solid is 190 kg m^{-3}, whereas its real density resulted to be 1554 kg m^{-3}. With a density of CO_2 at 7.2 MPa (separation pressure calculated hereafter) and 25°C equal to 1554 kg m^{-3}, the holdup of CO_2 in the extraction cell, which is consequently lost when it is opened after the extraction cycle, is about 3.6 kg_{CO2} kg_{solid}^{-1}. Hence to treat 100 kg h^{-1} of lyophilized rocket salad the losses of CO_2 are estimated to be 360 kg h^{-1}. In addition, the losses of CO_2 were calculated at the separation pressure (7.4 MPa), resulting to be 344 kg h^{-1} instead of 360 kg h^{-1}. In summary, our calculation of CO_2 losses can be considered as a conservative assumption which does not affect substantially the overall result.

4.2.1.2.CO_2 separation

The expansion of the supercritical CO_2 is carried out by means of valve V1. A heat exchanger (E3) is needed to prevent the freezing of CO_2 due to depressurization.

Before the separation block S1, the separation of CO_2 from the extract is simulated. Chrastil equation (4.2) allows to calculate the solubility of the extract in CO_2, where S is the solubility (g kg^{-1}) of extract in CO_2, ρ is the density (g L^{-1}), and T is the temperature of the block E3 (K).

$$S = \rho^4 e^{\left(18-\frac{14824}{T}\right)} \tag{4.2}$$

The values of the parameters were calculated from the experimental data [15]. In Table 4.2, the solubility of the extract in CO_2 at different pressures, calculated by the equation 4.2, is reported.

After being separated from the co-solvent and the extract, CO_2 is pressurized again (C1) to 6.0 MPa to be recycled.

Table 4.3. Price of utilities.

	Price (US$ GJ^{-1})
Common Utilities	
Electricity (110V - 440V)	16.8
Cooling Water (30°C to 45°C)	0.354
Steam	
Low Pressure (5 bar, 160°C)	6.08
Refrigeration	
Moderately Low (5°C)	4.43

4.2.1.3.Drying

The separation between water and the extract is carried out by a drying unit that is simulated by a separation block (S2). Water is assumed to be 99% recycled. To evaluate the duty that should be supplied to evaporate water and to condensate it again, a heat exchanger has been used (E4). The recycled water is sent to the co-solvent pump (P2) to reach the extraction pressure.

4.2.2. Economic and financial evaluation

The economical evaluation method used to assess the profitability of the proposed supercritical fluid extraction process is briefly presented in this section.

4.2.2.1.Operating costs

The operating costs are the expenses related to the operation of the plant. They can be divided in two categories: cost of utilities and cost of raw materials. The utilities needed for the operation of the SFE plant are: electricity, water, steam and refrigeration. The price of the utilities that have been considered in this study, reported by Turton at al. [28], are summarized in Table 4.3.

As regards the raw materials, the price of CO_2 and water are 0.279 and 0.0014 US$ kg^{-1}, respectively (http://www.icis.com). Considering that the average wholesale price of rocket salad is 0.25 € kg^{-1} (0.29 US$ kg^{-1}) [30] and the pre-processing cost (milling and drying) is 0.03 US$ kg^{-1} according to literature estimates [31,32], the price of rocket salad considered in this study is 0.32 US$ kg^{-1}. However, the price of rocket salad depends on some factors that are difficult to predict, and thus a sensitivity analysis to determine the influence of the price of rocket salad is presented in section 4.3.4.

The solid remaining after extraction can be used as a matrix for the extraction of unsaturated fatty acids [15] or for animal feeding [33], among other uses. As a consequence, the wastewater treatment has not been included in the cost evaluation.

4.2.2.2. Capital costs

As capital costs, the investment for equipment has been calculated using the equations proposed by Douglas [34]. Aspen Plus™ V8.2 evaluation options were used for sizing the pieces of equipment.

The following equation was applied to calculate the cost of the heat exchangers:

$$\text{Cins (US\$)} = \left(\frac{(M\&S)}{280}\right) 101.3A^{0.65}(2.29 + Fc) \tag{4.3}$$

where *M&S* is the Marshall &Swift index considered equal to 1469.6 (0.2% per year from the value of 1457.4 of 2010), *A* is the exchange area (ft^2) and *Fc* is a corrective factor. For each extraction vessel, equation 4.4 was used:

$$\text{IC reactor (US\$)} = \left(\frac{(M\&S)}{280}\right) 101D_R^{1.066}L_R^{0.802}(2.18 + Fc) \tag{4.4}$$

where D_R is the diameter (ft) and L_R is the length of the vessel.

For compressors the following equation has been applied:

$$\text{IC Comp(US\$)} = \left(\frac{(\text{M\&S})}{280}\right) 517.5(\text{bhp})^{0.82}(2.11 + \text{Fc}) \tag{4.5}$$

where *bhp* is the brake power (hp).

In equations 3 to 5, *Fc* is 1.55 for the extractor vessels and 1.52 for the rest of the equipment.

Pump, pipes, valve costs were neglected.

4.2.2.3. Cost benefit analysis (CFA)

The CFA of this process has been carried out considering an operation time of 8760 h $year^{-1}$. The standard method proposed by Douglas [34] has been used, according to the hypotheses summarized in Table 4.4. The values of interest and taxation are according to similar analysis on SFE plants [35,36].

The total capital investment (TCI) and the total production cost (TPC) were estimated using the formulas proposed by Douglas [34]:

$$\text{TCI (US\$)} = 2.36\ (\text{DCO}) \tag{4.6}$$

$$\text{TPC}\ \left(\frac{\text{US\$}}{\text{year}}\right) = 1.03(\text{RM} + \text{UT}) + 0.186(\text{DCO}) + 2.13{\cdot}10^{5}(\text{NOP}) + 0.025 \tag{4.7}$$

Table 4.4. Parameters used to evaluate the profitability of the SFE process.

Life of the plant	10 years
Period of start up	2 years
Depreciation (DDB method)	7 years
Taxation	20%
Interest	5%
Fixed capital land	1000000 US$
Residual value	150000 US$

where DCO stands for direct costs onsite, RM for raw materials, UT for utilities, NOP for number of operators and INC for incoming.

4.3. Results and discussion

4.3.1. Operating costs

First, the effect of the separation pressure on the operating cost at different extraction pressures was evaluated. As can be seen in Figure 4.5, the higher the separation pressure the lower the operating costs. Therefore, the critical CO_2 pressure, namely 7.4 MPa, is the most economical separation pressure in the separation step for all the extraction pressures. It should be noted that the similar values of compositions obtained from the experimental extraction measures are the reason of the overlapping of the trends obtained at 25 and 30 MPa.

The effect of extraction pressure on the operating costs at 65°C was investigated. As shown in Figure 4.6a, the lowest operating cost is obtained at 25 MPa, which corresponds to the highest extraction yield. It is worth noting that the content of phenolic and glucosinolate compounds at 25

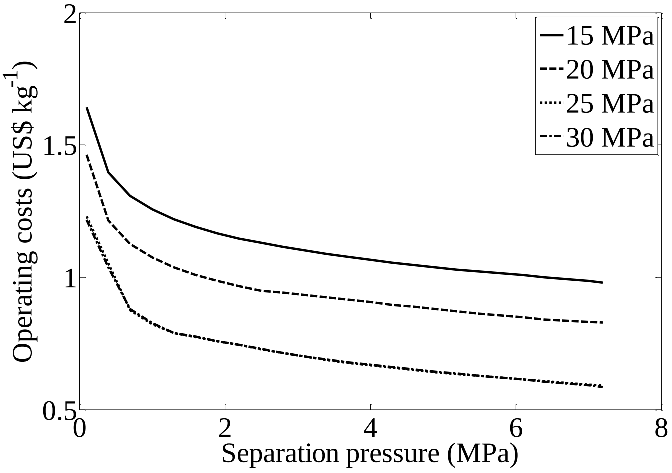

Figure 4.5. Effect of the separation pressure on the operating costs at different extraction pressures.

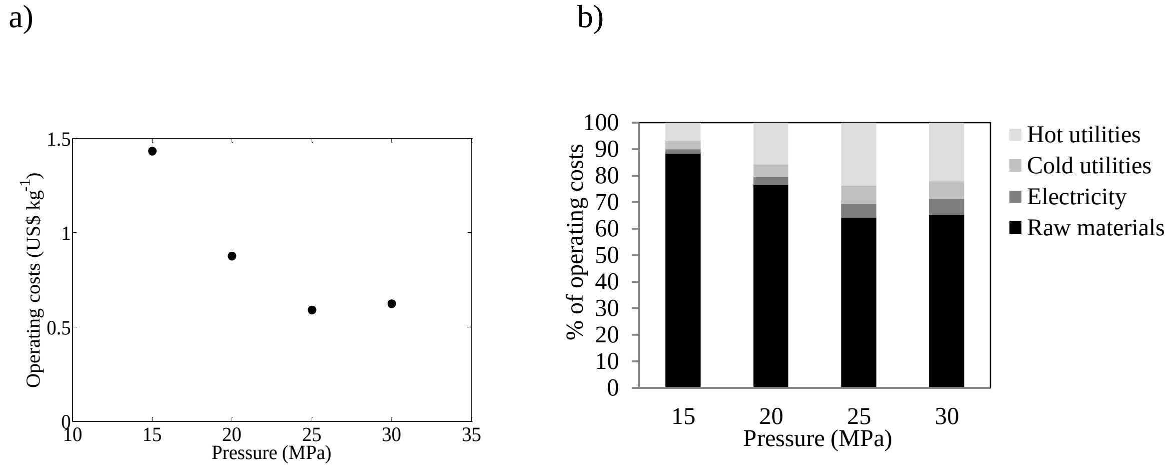

Figure 4.6. Total operating costs (a) and distribution of the operating cost (b) at different extraction pressures and a constant temperature of 65°C.

MPa is slightly lower than at 30 MPa, however results shown in Figure 4.6 indicate that the cost of pumping up to 30 MPa are not compensated by the benefits of the slightly higher content of such natural compounds. The percentage distribution of the operating costs at different extraction pressures is illustrated in Figure 4.6b. The pumping cost is lower at low extraction pressures. Clearly, the cost of raw materials is always the most significant one.

The effect of extraction temperature on the operating costs at 25 MPa has also been investigated. As shown in Figure 4.7a, the higher the extraction temperature the lower the operating costs. There is a significant decrease from 45°C to 55°C due to the increase of the extraction yields in that range. In Figure 4.7b, the distribution of the operating costs at different temperatures is represented. Once again, the cost of raw materials is the most significant one. At 45°C the consumption of raw materials significantly increases due to the decreasing of the extraction yield.

To sum up, the lowest operating costs were obtained at 25 MPa and 75 °C, at a separation pressure of 7.4 MPa. At these conditions, as reported in our previous work [15] between 1.23-1.48 mg g^{-1} of phenolic compounds are present in the natural extract, together with 1.83-1.96 mg g^{-1} of glucosinolates.

4.3.2. Capital costs

The equipment costs were calculated using the equations proposed by Douglas [34]. The direct costs onsite (DCO) at the optimum conditions resulted to be 653,729 $, the number of operators necessary are 14 and the INC has been calculated to be 3,012,564 $/year. Table 4.5 shows the area and the cost of each piece of equipment. The distribution of the equipment cost is represented in Figure 4.8. As can be seen, the cost of the compressor is the most significant one,

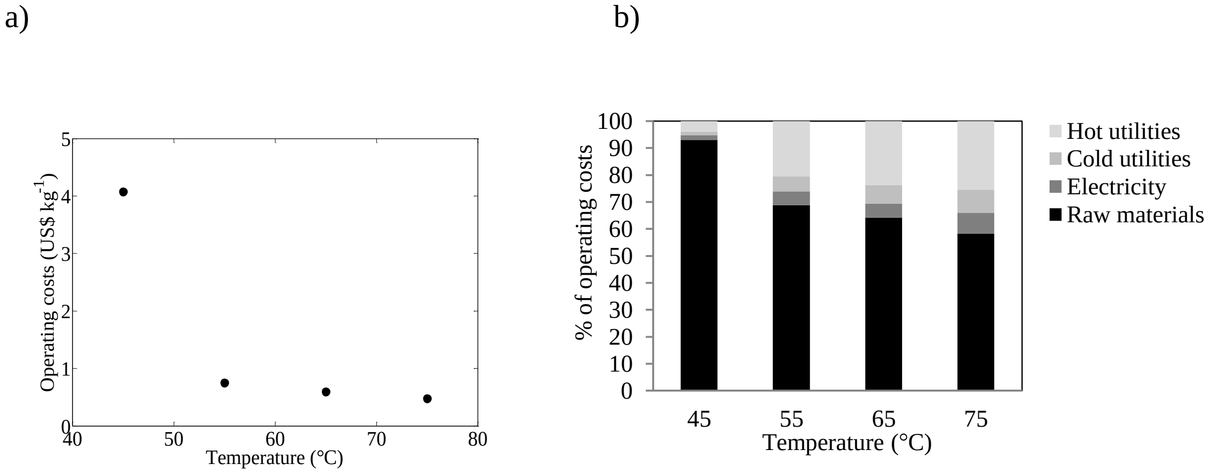

Figure 4.7. Total operating costs (a) and distribution of the operating cost (b) at different extraction temperatures and a constant pressure of 25 MPa.

followed by the one of the heat exchangers.

Eventually, a preliminary analysis of the total production cost distribution (i.e. capital plus operating) at the optimum conditions (25 MPa and 75°C) was considered. The equipment costs are annualized according to Douglas [34], using a capital charge factor (CCF) equal to 1/3. Results are shown in Figure 4.9, where it can be seen that the fixed capital (FCI) contribution is quite low compared to the operating cost. Above all, raw materials are the most expensive production cost (67%). Hot utilities affects the production cost more than cold utilities, while the influence of pumping is quite low.

Table 4.5. Equipment area and cost of the equipment calculated by calculated by Aspen Plus Simulator and Douglas equations, respectively.

	E1	E2	E3	E4	X1	X1b	C1
Area (ft^2)	259.07	188.56	120	39.14	3 × 12	3 × 12	
Cost ($)	75,011	61,04045	45,503	21,966	47,210	47,210	308,547

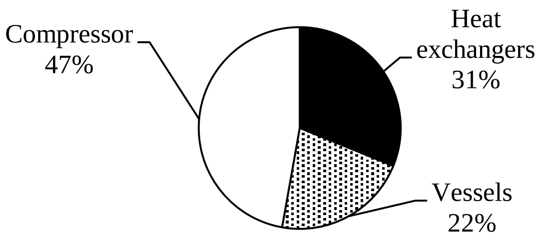

Figure 4.8. Equipment cost distribution for a SFE plant treating 100 kg h-1 of lyophilized rocket salad. Cost of heat exchangers= 203,551 US$; cost of vessels=141,631 US$; cost of compressor= 308,546 US$. Total equipment cost= 653,729 US$.

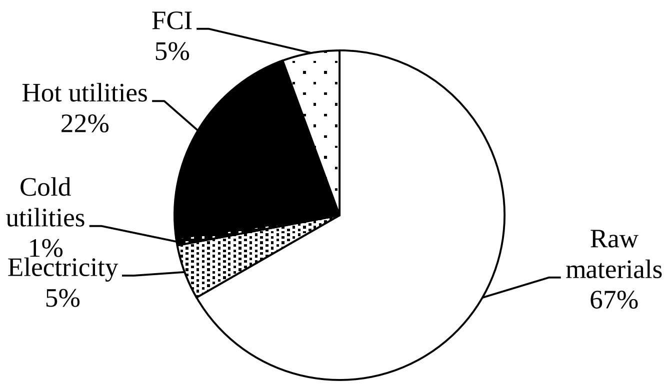

Figure 4.9. Total process costs distribution at the optimum conditions: extraction pressure of 25 MPa and temperature of 75°C.

4.3.3. Cost benefit analysis

Finally, an analysis of the profitability of the process was carried out. The total capital investment and the total operating cost, calculated by the equations of Douglas [34], resulted to be 1,542,802 US$ and 5,344,313 US$ year^{-1}, respectively.

Fiori et al.[35] reported that the indicative cost of a 10 kg h^{-1} plant is 1,600,000 €, 3,900,000 € for a 100 kg h^{-1} plant and 6,300,000 € for a 300 kg h^{-1} plant. Our plant investment cost is comparable to these values.

Considering all the hypotheses mentioned above, the process would have a positive net present value at the end of life if the price of the extract is higher than 21.9 US$ kg^{-1}. Nowadays, the market price of the natural extracts from vegetables such as broccoli or apple varies from 10 to 80 $ kg^{-1} [37] . Therefore, the process under investigation can be considered quite interesting for future developments.

4.3.4. Sensitivity analysis

The price of the rocket salad used as raw material can be influenced by many factors, including the location of the plant, which affects the cost of transportation, or the variation of the price over time. Moreover, using as raw material the non-saleable rocket salad, whose expense is only the pre-processing cost, could be another option. It is therefore interesting to calculate the price of the extract in different scenarios. The cost benefits analysis was repeated for values of rocket salad prices in the range 0-1 US$ kg^{-1}. As illustrated in Figure 4.10, the price of the rocket salad extract that makes the process economically viable after 10 years using non-saleable salad is 17.0 US$ kg^{-1}. On the contrary, for price of rocket salad equal to 1 US$ kg^{-1}, the price of the extract would have to be increase to 33.2 US$ kg^{-1}.

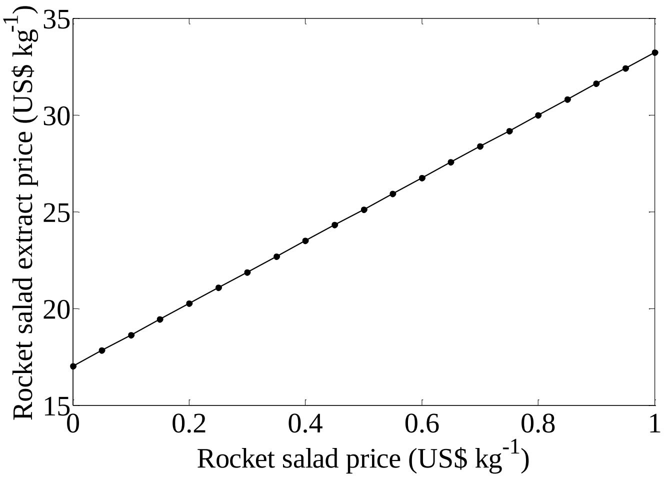

Figure 4.10. Evolution of the rocket salad extract price as a function of different prices of rocket salad.

4.4. Final remarks

A conceptual design of an industrial supercritical fluid extraction process that produces an extract rich in glucosinolates and phenolic compounds from freeze-dried rocket salad, using water and carbon dioxide as the solvents, has been developed. The base for calculation is a plant size able to treat 100 kg h^{-1} of lyophilized rocket salad. The software Aspen Plus™ V8.2 was employed for process simulation which have been turned on laboratory experimental measurements. The influence of the extraction pressure and the extraction temperature have been investigated, obtaining the lowest operating costs at 25 MPa and 75 °C. The effect of the separation pressure on the operating costs has also been studied, obtaining an optimum value at 7.4 MPa. A cost benefit analysis of the profitability of the process was made showing that with an interest of 5% and 10 years of pay-back period, the net present value of the process would be positive if the selling price of the extract was higher than 21.9 US$ kg^{-1}, based on the current wholesale price of rocket salad. In the best scenario, namely using non-saleable salad, the price of the extract would be 17.0 US$ kg^{-1}. The proposed method is general and can be applied for the extraction of natural extracts from other vegetal materials.

References

[1] B. Chung, Natural Plant Extracts-Export Market Opportunities in the USA, Chicago, USA, 2000.

[2] M.M.R. de Melo, a. J.D. Silvestre, C.M. Silva, Supercritical fluid extraction of vegetable matrices: Applications, trends and future perspectives of a convincing green technology, J. Supercrit. Fluids. 92 (2014) 115–176. doi:10.1016/j.supflu.2014.04.007.

[3] L. Fiori, Supercritical extraction of grape seed oil at industrial-scale: Plant and process design, modeling, economic feasibility, Chem. Eng. Process. Process Intensif. 49 (2010) 866–872. doi:10.1016/j.cep.2010.06.001.

[4] S.R.R. Comim, K. Madella, J.V. Oliveira, S.R.S. Ferreira, Supercritical fluid extraction from dried banana peel (Musa spp., genomic group AAB): Extraction yield, mathematical modeling, economical analysis and phase equilibria, J. Supercrit. Fluids. 54 (2010) 30–37. doi:10.1016/j.supflu.2010.03.010.

[5] P.F. Leal, N.B. Maia, Q. a. C. Carmello, R.R. Catharino, M.N. Eberlin, M.A. a. Meireles, Sweet Basil (Ocimum basilicum) Extracts Obtained by Supercritical Fluid Extraction (SFE): Global Yields, Chemical Composition, Antioxidant Activity, and Estimation of the Cost of Manufacturing, Food Bioprocess Technol. 1 (2007) 326–338. doi:10.1007/s11947-007-0030-1.

[6] P.C. Veggi, R.N. Cavalcanti, M.A. a. Meireles, Production of phenolic-rich extracts from Brazilian plants using supercritical and subcritical fluid extraction: Experimental data and economic evaluation, J. Food Eng. 131 (2014) 96–109. doi:10.1016/j.jfoodeng.2014.01.027.

[7] F. Pasini, V. Verardo, M.F. Caboni, L.F. D’Antuono, Determination of glucosinolates and phenolic compounds in rocket salad by HPLC-DAD–MS: Evaluation of Eruca sativa Mill. and Diplotaxis tenuifolia L. genetic resources, Food Chem. 133 (2012) 1025–1033. doi:10.1016/j.foodchem.2012.01.021.

[8] M. Villatoro-Pulido, F. Priego-Capote, B. Alvarez-Sánchez, S. Saha, M. Philo, S. Obregón-Cano, et al., An approach to the phytochemical profiling of rocket [Eruca sativa (Mill.) Thell]., J. Sci. Food Agric. (2013). doi:10.1002/jsfa.6286.

[9] R.N. Bennett, F. a Mellon, N.P. Botting, J. Eagles, E. a S. Rosa, G. Williamson, Identification of the major glucosinolate (4-mercaptobutyl glucosinolate) in leaves of Eruca sativa L. (salad rocket)., Phytochemistry. 61 (2002) 25–30.

[10] L. Lazzeri, M. Errani, O. Leoni, G. Venturi, Eruca sativa spp. oleifera: a new non-food crop, Ind. Crops Prod. 20 (2004) 67–73. doi:10.1016/j.indcrop.2002.06.001.

[11] M. Khoobchandani, B.K. Ojeswi, N. Ganesh, M.M. Srivastava, S. Gabbanini, R. Matera, et al., Antimicrobial properties and analytical profile of traditional Eruca sativa seed oil: Comparison with various aerial and root plant extracts, Food Chem. 120 (2010) 217–224. doi:10.1016/j.foodchem.2009.10.011.

[12] J.. E. Hernández Bermejo, J. Leon, Neglected crops 1492 from a different perspective, FAO Plant, Rome, 1994.

[13] S. Alqasoumi, M. Al-Sohaibani, T. Al-Howiriny, M. Al-Yahya, S. Rafatullah, Rocket “Eruca sativa”: A salad herb with potential gastric anti-ulcer activity, World J. Gastroenterol. 15 (2009) 1958–1965. doi:10.3748/wjg.15.1958.

[14] J. Varga, A. Silviu, E. Laczi, A.I. Apahidean, Studies concerning the sowing period in the arugula (Eruca sativa Mill) plants ’ development Enik ̋, Acta Univ. Sapientiae Agric. Environ. 4 (2012) 5–10.

[15] M. Solana, I. Boschiero, S. Dall'Acqua, a. Bertucco, Extraction of bioactive enriched fractions from Eruca sativa leaves by supercritical CO2 technology using different co-solvents, J. Supercrit. Fluids. 94 (2014) 245–251. doi:10.1016/j.supflu.2014.08.022.

[16] D. Znidarčič, D. Ban, H. Sircelj, Carotenoid and chlorophyll composition of commonly consumed leafy vegetables in Mediterranean countries., Food Chem. 129 (2011) 1164–8. doi:10.1016/j.foodchem.2011.05.097.

[17] D. Peng, D.B. Robinson, A New Two-Constant Equation of State, 15 (1976) 59–64.

[18] T. Holderbaum, J. Gmehling, PSRK: A Group Contribution Equation of State Based on UNIFAC, Fluid Phase Equilib. 70 (1991) 251–265. doi:10.1016/0378-3812(91)85038-V.

[19] G. Soave, Equilibrium constants from a modified Redlich-Kwong equation of state, Chem. Eng. Sci. 27 (1972) 1197–1203. doi:10.1016/0009-2509(72)80096-4.

[20] A. Péneloux, E. Rauzy, R. Fréze, A consistent correction for Redlich-Kwong-Soave volumes, Fluid Phase Equilib. 8 (1982) 7–23. doi:10.1016/0378-3812(82)80002-2.

[21] P.M. Mathias, H.C. Klotz, J.M. Prausnitz, Equation-of-State mixing rules for multicomponent mixtures: the problem of invariance, Fluid Phase Equilib. 67 (1991) 31–44. doi:10.1016/0378-3812(91)90045-9.

[22] U. Sievers, Die thermodynamischen Eigenschaften von Kohlendiosxid, Reihe 6, N, Fortschr.-Ber.VDI-Z, Düsseldorf, 1984.

[23] C.R. Coan, A.D. King Jr., Solubility of water in compressed carbon dioxide, nitrous oxide, and ethane. Evidence for hydration of carbon dioxide and nitrous oxide in the gas phase, J. Am. Chem. Soc. 93 (1971) 1815–1862. doi:10.1021/ja00737a004.

[24] A. Bamberger, G. Sieder, G. Maurer, High-pressure (vapor + liquid) equilibrium in binary mixtures of (carbon dioxide + water or acetic acid) at temperatures from 313 to 353 K, 17 (2000) 97–110.

[25] S.-X. Hou, G.C. Maitland, J.P.M. Trusler, Measurement and modeling of the phase behavior of the (carbon dioxide+water) mixture at temperatures from 298.15K to 448.15K, J. Supercrit. Fluids. 73 (2013) 87–96. doi:10.1016/j.supflu.2012.11.011.

[26] R. Wiebe, The binary system carbon dioxide-water under pressure1, (1941) 475–481.

[27] K.S. Duba, L. Fiori, Supercritical CO2 extraction of grape seed oil: Effect of process parameters on the extraction kinetics, J. Supercrit. Fluids. 98 (2015) 33–43. doi:10.1016/j.supflu.2014.12.021.

[28] R. Turton, R.C. Bailie, W.B. Whiting, J.A. Shaeiwitz, B. Debangsu, Analysis, synthesis, and design of chemical processes, Prentice H, Prentice Hall; 4 edition (July 2, 2012), 2012.

[29] http://www.icis.com/, (n.d.).

[30] CAAT, http://www.caat.it, (2015) accessed 22.01.2015.

[31] P.T.V. Rosa, M.A. a. Meireles, Rapid estimation of the manufacturing cost of extracts obtained by supercritical fluid extraction, J. Food Eng. 67 (2005) 235–240. doi:10.1016/j.jfoodeng.2004.05.064.

[32] C.G. Pereira, P.T.V. Rosa, M.A. a. Meireles, Extraction and isolation of indole alkaloids from Tabernaemontana catharinensis A.DC: Technical and economical analysis, J. Supercrit. Fluids. 40 (2007) 232–238. doi:10.1016/j.supflu.2006.07.001.

[33] N. Mezzomo, J. Martínez, S.R.S. Ferreira, Economical viability of SFE from peach almond, spearmint and marigold, J. Food Eng. 103 (2011) 473–479. doi:10.1016/j.jfoodeng.2010.10.032.

[34] J. Douglas, Conceptual design of Chemical Processes, 1st ed., McGraw-Hill Science/Engineering/Math, New York, 1988.

[35] L. Fiori, M. Manfrini, D. Castello, Supercritical CO2 fractionation of omega-3 lipids from fish by-products: Plant and process design, modeling, economic feasibility, Food Bioprod. Process. 92 (2014) 120–132. doi:10.1016/j.fbp.2014.01.001.

[36] M.P. Fernández-Ronco, A. de Lucas, J.F. Rodríguez, M.T. García, I. Gracia, New considerations in the economic evaluation of supercritical processes: Separation of bioactive compounds from multicomponent mixtures, J. Supercrit. Fluids. 79 (2013) 345–355. doi:10.1016/j.supflu.2013.01.018.

[37] Alibaba, www.alibaba.com, (2015).

CHAPTER 5

Comparison of extraction methods for obtaining phenols from asparagus[5]

Supercritical fluid and pressurized liquid extractions are investigated for effective recovery of phenolic compounds from asparagus. By the one hand, the effect of different co-solvents on supercritical fluid extraction (SFE) is examined. Results confirm that the presence of water and ethanol is essential to obtain a phenolic enriched extract with high antioxidant activity. Operative conditions influence the extraction yield, whereas the phenolic composition of the extract does not vary significantly. By the other hand, the differences among various solvents on pressurized liquid extraction (PLE) is analysed. Once again, the mixture water-ethanol gives the best results in terms of phenolic content, which are comparable with SFE and Soxhlet ones. In total, fourteen phenolic derivatives were identified, being rutin the most abundant compound in all the extracts. Phenolic acids, mainly 3-O-feruloylquinic acid, represent a relevant percentage of the phenolic content of the asparagus extracts. The methods and solvents considered influence diversely the extraction of molecules with different structure.

5.1. Introduction

Extracts of vegetal materials rich in phenolic compounds have gained a growing interest due to their beneficial antioxidant properties. They are mostly used as functional ingredients for the food industry improving the quality and the nutritional value of foods. Other applications involve the production of paints, paper and cosmetic [1]. As a good source of phenolic compounds, asparagus can be considered a product of interest for such industries.

The phenolic content of asparagus is mainly composed by flavonoids [2,3], which are the major dietary constituents of plant-based food. Over 4000 chemically varieties of flavonoids have

---

[5] *Part of this chapter has been published as: Solana, M., Boschiero, I., Dall'Acqua, S., Bertucco, A., A comparison between supercritical fluid and pressurized liquid extraction methods for obtaining phenolic compounds from Asparagus officinalis L, 2015, The Journal of Supercritical Fluids 100, 201-208.*

been reported to date. They are categorized into the class of flavonols, flavones, flavanols, flavanones and isoflavones,based on the additional presence of a C2–C3 double bond, hydroxyl, methoxy groups, glycoside, and different substituents of the molecules [4–7]. In food, flavonoids exit mainly as 3-O-glucosydes and polymers [8]. Among the major flavonoids, asparagus green spears are rich in rutin, the 3-O-rutinoside of the flavonol quercetin [2]. Rutin, also known as vitamin P, exhibits significant pharmacological activities, including anti-oxidant, anti-inflammatory, anti-diabetic, anti-adipogenic and neuroprotective [4]. Currently, over 130 registered therapeutic medicinal preparations are containing rutin in their formulations [4,9]. Asparagus also contain high levels of another quercetin glycoside, isoquercetrin, and phenolic acids as chlorogenic acid, p-coumaric, caffeic and ferulic acids [2].

Nowadays, the search for an extraction technique to obtain phenolic compounds is the focus of numerous researches. The objective of such studies is to find out an economical and safe method to obtain plant extracts with high antioxidant activity, which is directly correlated to their phenolic content [10]. Supercritical fluid extraction (SFE) is advantageously positioned as a sustainable and safe extraction option for the preparation of extracts from vegetal materials, with more than 300 species reported in literature [11]. The polar nature of most natural compounds, such polyphenols makes it necessary the addition of co-solvents to supercritical CO_2, in order to enhance the fluid affinity towards polar compounds. Water and ethanol are inexpensive and green co-solvents that are increasingly taken into account due to the opportunity of direct use in food and pharmaceutical products. Furthermore, the use of water-ethanol mixtures as co-solvents has been demonstrated to be more efficient in extracting phenolic compounds than the corresponding mono-component solvent system [12–17].

Recently, the recovery of valuable compounds by means of extraction with pressurized liquids (PLE), also known as accelerated solvent extraction, has shown promising results [18–20]. A great advantage of this technique is that high pressure solvents remain in the liquid state, even when subjected to temperatures above their boiling points. These conditions enhance the solubility of target compounds in the solvent and the desorption kinetics from solid matrices [19]. Moreover, high pressure permits to accelerate the rate of extraction from sample matrix [4]. As a consequence, shorter extraction times with respect to traditional extraction techniques are needed [18].

Based on the exposed context, this work aims to evaluate the effect of using environmental friendly techniques, such as SFE and PLE, on the extraction of enriched phenolic extracts from asparagus. The differences among the phenolic composition and the antioxidant activity of the extracts are discussed and compared with the ones obtained by traditional organic solvent extraction method. This analysis can be useful to understand the selectivity of different solvents and methods towards different phenolic compounds. In addition, to the best of our knowledge, this is the first work in which phenolic compounds from asparagus have been extracted by SFE and PLE extraction methods.

5.2. Materials and methods

5.2.1. *Raw material*

Asparagus (*Asparagus officinalis* L.) was purchased from a local market in Italy. Raw material was first milled by a knife mill and then stored at -25° until dehydration in a freeze-dryer was performed. Before the extraction tests, lyophilized asparagus were milled with a mortar to obtain a particle diameter less than 0.5 mm.

5.2.2. *Chemicals*

CO_2 (4.0 type, purity greater than 99.99%) was purchased by Rivoira. Ethanol (99.8%) and methanol (99.8%), used for the extraction tests, were purchased from Sigma Aldrich. Water was Milli-Q quality. Methanol, acetonitrile, formic acid ethanol and Rutin standard, used for the measures, were provided by Carlo Erba, Prolabo, J.T. Baker and Lab-Scan.

5.2.3. *Supercritical fluid extraction*

SFE tests were conducted in a laboratory scale equipment reported elsewhere [21,22]. Before extraction with co-solvents, a pre-treatment consisting in pure supercritical CO_2 extraction during 15 minutes at the same conditions of each test was carried out, in order to extract the low polarity CO_2-soluble compounds. This pre-treatment is essential to remove lipophilic and nonpolar substances and consequently making polyphenols more available for the extraction [23].

A constant CO_2 flow rate of 0.25±0.05 kg/h was kept in every experimental run. Water, ethanol, methanol and mixtures of water and ethanol, in a proportion of 8% (w/w) with respect to the CO_2 flow rate, were added to CO_2 as co-solvents. Pressure was varied in a range 10-30 MPa and temperatures between 50 and 80°C were tested. The operating procedure was as follows: 0.50±0.05 g of freeze-dried asparagus powder were place into a stainless steel extraction cell. CO_2 was cooled at 5°C before being compressing by the high pressure pump. Pressurized CO_2 was heated and mixed with the co-solvent, previously pumped by an intelligent HPLC pump (Jasco PU-1580). The mixture of supercritical CO_2 and co-solvent passed through the extraction cell, where the extraction took place. A thermo-resistance maintained the desired temperature, which was measured in the internal flow before and after the vessel. After extraction, the mixture of solvents was expanded by a Medium-Flow High-Pressure Metering Valve inserted in a water bath at 40 °C to avoid CO_2

freezing caused by sudden expansion. CO_2 gas at atmospheric temperature passed through a flow meter before being vented. The extract and the co-solvent were collected in 20 mL of ethanol and they were afterwards separated by rotary evaporation. All experimental runs were carried out in duplicate. The average and the standard deviation were calculated on the base of the two measures.

5.2.4. *Pressurized liquid extraction*

The same experimental setup to SFE system reported in section 5.2.3 was used for PLE experiments. In this method, only the HPLC pump was utilized. Before the test, the extraction cell was filled with 0.50±0.05 g of freeze-dried asparagus powder and the solvent was placed in a reservoir. Water, ethanol and a mixture of water and ethanol 1:1 were tested as solvents. After the temperature of the extraction (65°C) was reached, the solvent flow was started. The pressure was set at 10 MPa. A flow rate of 2 mL/min, controlled by a Medium-Flow High-Pressure Metering Valve, was maintained during 30 minutes. The extracts were evaporated by a rotatory evaporator to remove the solvent.

5.2.5. *Soxhlet extraction*

Solvent extraction was carried out by a traditional Soxhlet apparatus. Methanol was used as solvent. The temperature of extraction was maintained at 100 °C during 4 hours. Then the solvent was evaporated by a rotary evaporator.

5.2.6. *Phenolic analysis of the extracts*

Quali-quantitative analysis of phenolic compounds in the extracts were obtained by HPLC-MS. The measurements were performed on a Varian 212 series chromatograph equipped with Prostar 430 autosampler and MS-500 Ion Trap as detector. MS spectra were recorded in negative ion mode, using ESI (Electron Spray Ionization) as ions source. Full scan spectra were acquired over the range 50-2000 m/z. Fragmentation of the main ionic species were obtained by the turbo data depending scanning (tdds) function, yielding the fragmentation pattern of eluted compounds. As stationary phase Agilent Eclipse Plus C-18 (2.1 × 150 mm) 3.5 μm was used. Elution was carried out with a water 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B) gradient as follow: start 90% A, 23 min 0% A, 23.3 90% A. The length of the run was of 28 min. Rutin was used as reference compound for the quantification of phenolic constituents in the range 0.5-20 μg mL-1 at four different concentrations. Rutin calibration curve was (area Y vs concentration X) $y = 244229 x + 725$.

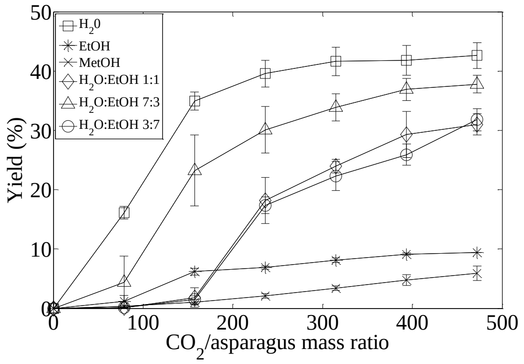

Figure 5.1. Effect of co-solvent nature on SFE curve at 65°C and 30 MPa. The curves represent the extraction yield (% $kg_{extract}/kg_{lyophilized\ mass}$) as a function of the relative amount of passed solvent ($kg_{solvent}/kg_{lyophilized\ mass}$).

5.2.7. DPPH assay

The scavenging activity towards the 1,1-diphenyl-2-picrylhydrazyl (DPPH-Sigma aldrich) radical was measured modifying a previously used protocol [24,25]. Rutin was used as standard reference in order to compare obtained results with a known antioxidant compound. A linear range of concentration vs. % decrease of absorbance was observed and was used for the determination using rutin solutions in the range 0.26-52 µg/mL. Methanol solution of asparagus extracts were used as the assay, and the relative % decrease of absorbance was calculated. The activity was expressed as mg/g equivalent of rutin.

5.3. Results and discussion

5.3.1. SFE: Co-solvent effect on extraction yield and phenolic content

The effect of organic and aqueous co-solvents on SFE yield and phenolic content was studied, setting the pressure at 30 MPa and the temperature at 65°C. As shown in Figure 5.1, the extraction yield is largely affected by the use of co-solvents of different nature, even if its proportion with respect to CO_2 is low (8%). The maximum final yield (42.6 %) was obtained using water as a co-solvent. Looking at Figure 5.1, one can notice that from the beginning of the extraction the amount of extract obtained by SFE with water is notably higher than when using other co-solvents. As already reported, the presence of water can increase the density of the fluid mixture, and it can also cause swelling of the solid particles, improving the internal diffusion process and therefore the solubilisation of several classes of compounds [26]. In addition, at the conditions of these tests, water is in the liquid phase and therefore extractions are carried out in the presence of two phases, supercritical and liquid, whereas ethanol and methanol are fully solubilized in supercritical CO_2 [21,27]. The acidification of liquid water by pressurized CO_2 may help breaking chemical bonds and releasing soluble compounds [19].

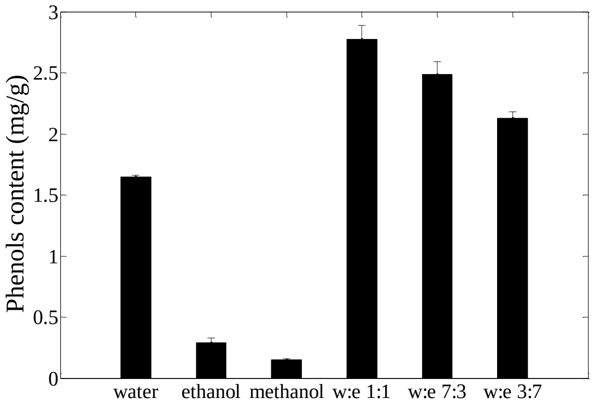

Figure 5.2. Effect of co-solvent nature on phenolic content ($mg/g_{lyophilized\ mass}$) at 65°C and 30 MPa.

The lowest extraction yields were obtained using ethanol and methanol as co-solvents. High yields were achieved with mixtures of water-ethanol, nearer to the values of SFE and water than to the ones obtained by SFE and ethanol. Comparing the proportion of water and ethanol in the co-solvent mixture, the best result in terms of extraction yield was achieved by using water-ethanol 7:3. There were no significant differences between the tests with the mixtures water-ethanol 3:7 and 1:1.

Considering the phenolic content of the obtained extracts, the effect of different co-solvents on SFE was very significant. As shown in Figure 5.2, only 0.15 and 0.29 mg/g of phenolic compounds were recovered using methanol and ethanol, respectively. It is noteworthy that even if water provided the highest yield, the phenolic content was higher when ethanol was added, which suggests that water is able to dissolve a largest variety of compounds leading to lower selectivity [19].

Clearly, adding mixtures of water-ethanol to supercritical CO_2 favours the extraction of phenols, which can be due to the large polarity ranges of phenolic compounds [19]. The addition of water and ethanol in the same proportion gave the highest total content of phenols of the extract, namely 2.78 mg/g. Therefore, this mixture was chosen as co-solvent to study the effect of the operating conditions on the SFE yield and composition in the following sections.

5.3.2. SFE: Effect of pressure and temperature on extraction yield and phenolic content

Once the mixture of water-ethanol 1:1 was proved to be the most efficient one to extract phenolic compounds, the influences of pressure and temperature on SFE yield and extract

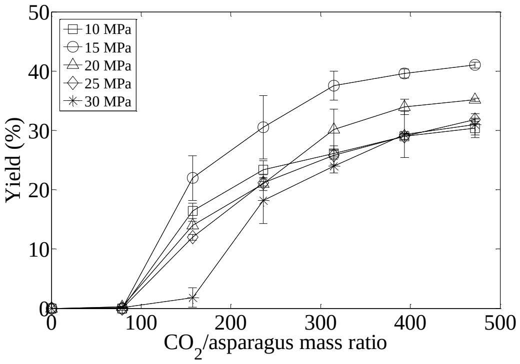

Figure 5.3. Effect of pressure on SFE curve at 65°C using the mixture water-ethanol 1:1 as co-solvent. The curves represent the extraction yield (% $kg_{extract}/kg_{lyophilized\ mass}$) as a function of the relative amount of passed solvent ($kg_{solvent}/kg_{lyophilized\ mass}$).

composition were analysed. In Figure 5.3, overall extraction curves obtained at a constant temperature of 65°C and pressures in a range 10-30 MPa are plotted. As can be noted, the maximum yield (41.0 %) was obtained at 15 MPa. From 15 to 25 MPa the final yield of the extraction decreased at increasing pressure and it remained practically constant from 25 to 30 MPa. However, at 10 MPa the final yield was lower than at 15 MPa.

As reported above, when water is used as co-solvent at 8%, this is present as a liquid phase and its acidification by CO_2 has a significant effect on increasing the overall extraction yield. The same situation happens with water-ethanol 1:1. It is interesting to evaluate the influence of pressure on the liquid composition of CO_2-water-ethanol mixtures. For this purpose, the composition of the liquid phase at different pressures was calculated. The thermodynamic model Predictive Soave-Redlich-Kwong equation of state [28] was chosen as calculation method using Aspen Plus V8.2. Results, shown in Figure 5.4a, indicate that at 10 MPa the liquid phase has a higher proportion of ethanol with respect to pressures greater than 15 MPa. From 15 to 30 MPa it slightly changes. Hence, the lower proportion of water in the liquid phase at 10 MPa could be the reason of the unexpected decrease of the yield when decreasing the pressure.

The influence of temperature on SFE yield was also studied in the range 50-80°C. In Figure 5.5, overall extraction curves obtained at constant pressure (20 MPa) are represented. It can be seen that at 65°C the final yield of extraction (35.2%) was higher than at 50°C and 80°C (32.3 and 34.0 %, respectively). Nevertheless the effect of temperature on extraction yield in the range studied was less significant than the effect of pressure. Figure 5.4b shows that the proportion of water and ethanol in the liquid phase is practically constant at different temperatures, which could be the motive of the similar yields obtained.

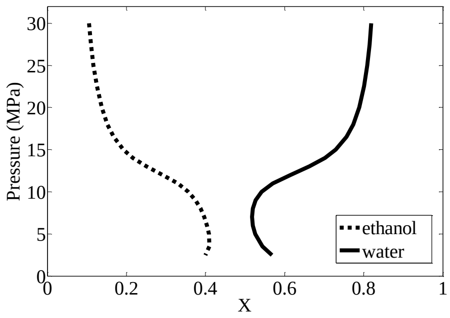

Figure 5.4a. System CO_2-water-ethanol: water and ethanol content in the liquid phase at different pressures and constant temperature of 65 °C.

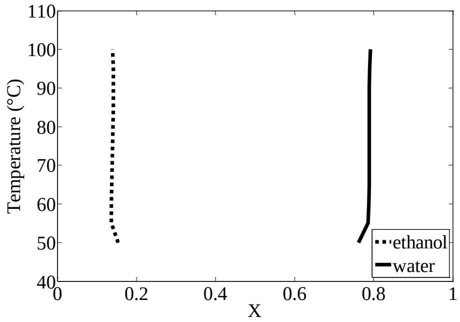

Figure 5.4b. System CO_2-water-ethanol: water and ethanol content in the liquid phase at different temperatures and constant pressure of 20 MPa.

The phenolic composition of the extracts obtained from the SFE experiments reported above was analysed by HPLC-MS. Results revealed that pressure and temperature in the studied range do not have significant effect on the total phenolic content and composition of the extract. Hence, the media of the analysis of all the samples extracted at different conditions using the mixture water-ethanol as co-solvent was calculated and the phenolic compounds extracted in a percentage higher than 1% are illustrated in Figure 5.6. Rutin was the predominant molecule, representing the 67% of the total phenolic content. A derivate of rutin, rutin-4'-glucoside, was found in a percentage of 10%.

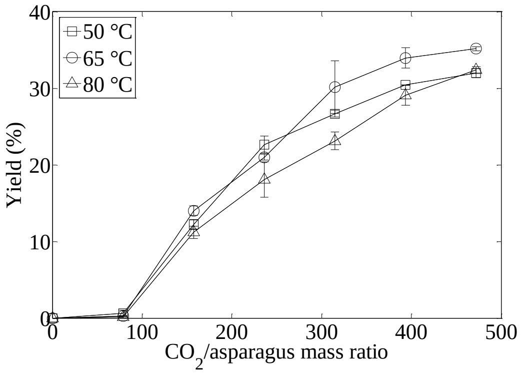

Figure 5.5. Effect of temperature on SFE curve at 20 MPa using the mixture water-ethanol 1:1 as co-solvent. The curves represent the extraction yield (% $kg_{extract}/kg_{lyophilized\ mass}$) as a function of the relative amount of passed solvent ($kg_{solvent}/kg_{lyophilized\ mass}$).

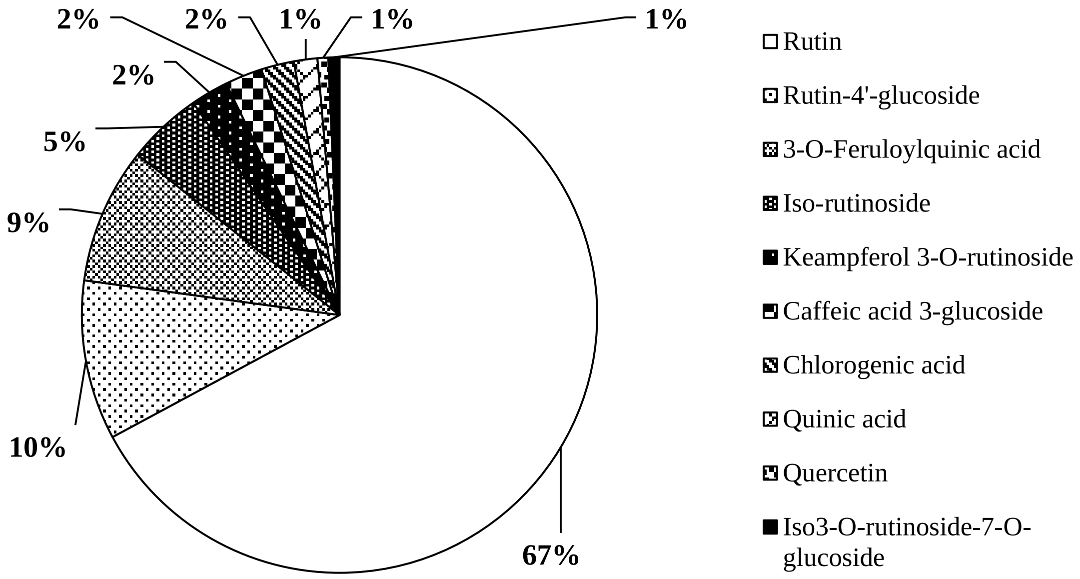

Figure 5.6. Phenolic content (% $kg_{phenolic\ compound}/kg_{total\ phenolic\ content}$) in SFE extract using the mixture water-ethanol as co-solvent from *Asparagus officinialis.* The percentage values corresponds to the media of the results obtained at the different conditions of pressure and temperature tested.

The 9% of the total phenolic content of the extract corresponded to the phenolic acid 3-O-feruloylquinic acid. Other phenolic compounds were detected in proportions lower than 5%.

5.3.3. SFE: Effect of time on the composition of the extract

The phenolic analysis of the samples collected every 10 minutes was compared with the yield extraction curve obtained at the same conditions, 15 MPa and 50°C. As shown in Figure 5.7, this study revealed that the extraction yield is proportional to the total phenolic content of each sample.

The percentage of the major phenolic compounds found in the samples obtained after 20, 30, 40, 50 and 60 minutes of extraction are presented in Figure 5.8. Rutin has not been included to better appreciate the trend of the other compounds, in any case its tendency was similar to Rutin-4'-glucoside and Iso-rutinoside, thus it was found in practically the same proportion during the 60 minutes of extraction. The last sample corresponds to the extract recovered during the depressurization of the equipment, that has not been considered in the extraction curves but it is interesting to know how the gradual decrease of pressure affect the composition of the extract and whether is worth to collect it. In total, around 5% of the phenolic content was found in that sample. As shown in Figure 5.8 this fraction was richer in the phenylpropanoids 3-O-feruloylquinic acid and caffeic acid 3-glucoside. The sample corresponding to the last 10 minutes of extraction was also

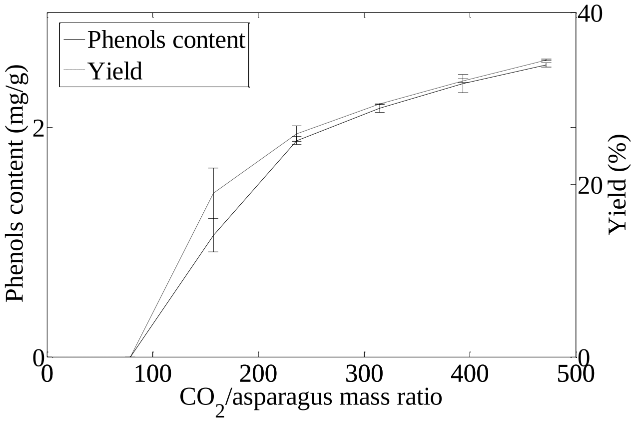

Figure 5.7. Comparison among the SFE curve in terms of phenolic content (mg/$g_{\text{lyophilized mass}}$) and yield of extraction (% $kg_{\text{extract}}/kg_{\text{lyophilized mass}}$) as a function of the relative amount of passed solvent ($kg_{\text{solvent}}/kg_{\text{lyophilized mass}}$). The experimental conditions were: water-ethanol 1:1 as co-solvent, 15 MPa and 50°C.

richer in these two compounds than the previous ones. This could be due to competition of these molecules with more polar components present in asparagus. If the extraction process had been continued for longer, these molecules probably would have been extracted after the exhaustion of the other competitive components, as suggested by Azevedo et al. [29].

5.3.4. Comparison of extraction methods

The results of the analysis of the extracts obtained by SFE, PLE and Soxhlet are summarized in Table 5.1. Looking at the total phenolic contents of PLE extracts, once again the mixture of water

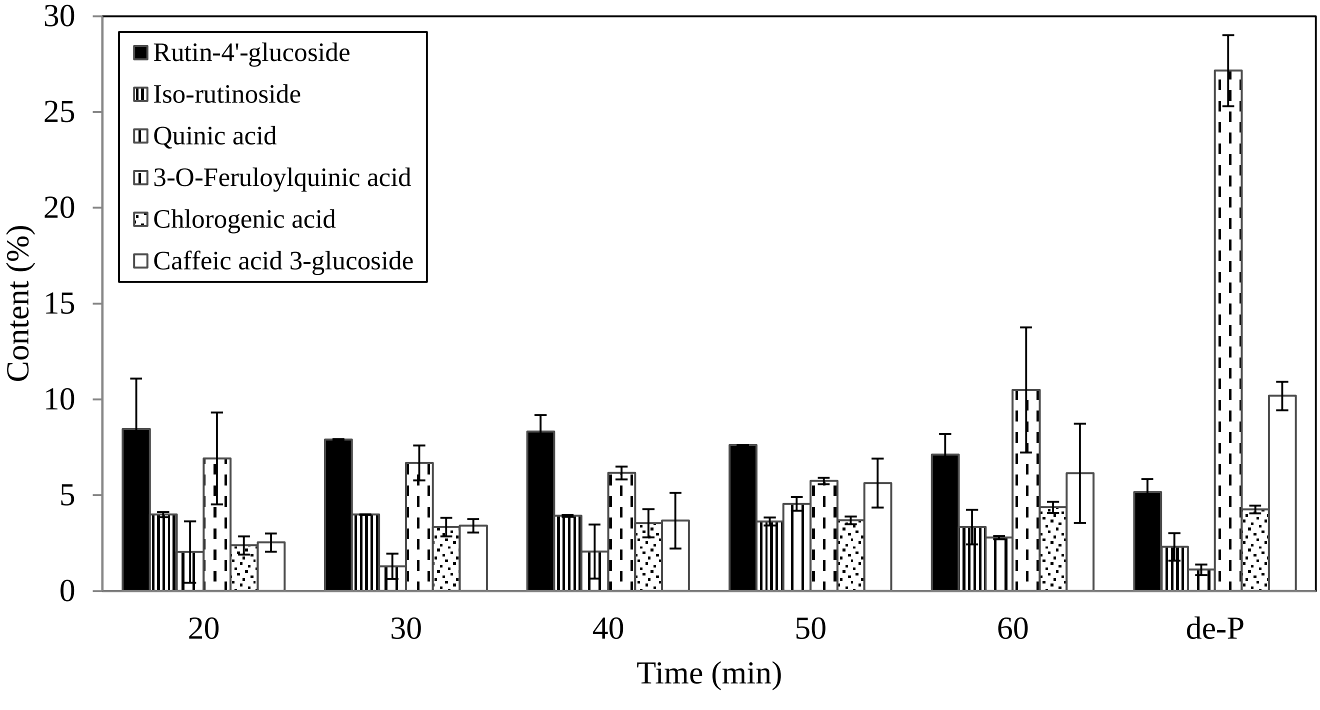

Figure 5.8. Major phenolic compounds (% $kg_{\text{phenolic compound}}/kg_{\text{total phenolic content}}$) of the samples collected at different times of SFE using the mixture of water-ethanol 1:1 as co-solvent at 15 MPa and 50°C, and after the depressurization (de-P).

Table 5.1. Phenolic composition (mg/g$_{\text{lyophilized mass}}$) and antioxidant activity (rutin equivalent mg/g) of the asparagus extracts obtained by SFE, PLE and Soxhlet using different solvents (n.d=not detected).

Group	Compound	Mw (g/mol)	SCCO$_2$+co-solvents				PLE			Soxhlet
			Water	Ethanol	Methanol	W:E 1:1	Water	Ethanol	W:E 1:1	Methanol
Flavonol aglycone	Keampferol	286.23	0.01±0.00	n.d.	n.d.	0.01±0.00	n.d.	n.d.	n.d.	n.d.
	Quercetin	302.24	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.01	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00
	Isoramnetin	316.26	0.01±0.00	n.d.	n.d.	0.01±0.00	<0.01	0.01±0.00	<0.01	0.01±0.00
	Total		0.04	0.02	0.02	0.04	0.02	0.03	0.02	0.03
Flavonol glucosyde	Isoquercetin	464.38	0.01±0.00	n.d.	n.d.	0.01±0.00	n.d.	n.d.	n.d.	n.d.
	Keampferol 3-O-rutinoside	594.52	0.05±0.01	0.01±0.01	n.d.	0.08±0.03	0.04±0.00	0.06±0.00	0.06±0.00	0.07±0.00
	Rutin	610.52	1.04±0.04	0.19±0.03	0.10±0.01	2.28±0.17	0.95±0.02	1.72±0.16	2.17±0.31	2.81±0.06
	Iso-rutinoside	624.56	0.13±0.00	0.03±0.02	0.01±0.00	0.16±0.03	0.07±0.00	0.12±0.00	0.11±0.01	0.14±0.00
	Rutin-4'-glucoside	772.66	0.17±0.00	0.01±0.00	n.d.	0.34±0.03	0.25±0.00	0.11±0.00	0.36±0.05	0.29±0.01
	Iso3-O-rutinoside-7-O-glucoside	786.68	0.02±0.00	n.d.	<0.01	0.02±0.01	0.05±0.00	0.04±0.00	0.05±0.00	0.04±0.00
	Rutin glucosyl rhamnoside	918.80	<0.01	n.d.	n.d.	<0.01	n.d.	n.d.	n.d.	<0.01
	Total		1.42	0.24	0.11	2.89	1.36	2.05	2.75	3.35
Organic acid	Quinic acid	192.17	n.d.	n.d.	n.d.	0.05±0.03	0.11±0.01	0.05±0.00	0.11±0.00	0.05±0.00
Phenylpro panoid	Caffeic acid 3-glucoside	342.30	0.03±0.00	n.d.	n.d.	0.08±0.04	0.09±0.00	0.07±0.04	0.08±0.00	0.12±0.00
	Chlorogenic acid	354.31	0.01±0.00	n.d.	n.d.	0.07±0.04	0.05±0.00	0.05±0.03	0.05±0.01	0.09±0.00
	3-O-Feruloylquinic acid	368.34	0.16±0.02	0.02±0.00	0.02±0.00	0.29±0.05	0.25±0.01	0.26±0.01	0.20±0.07	0.36±0.01
	Total		0.20	0.02	0.02	0.44	0.39	0.38	0.33	0.57
Total			1.65±0.07	0.29±0.06	0.15±002	3.42±0.45	1.88±0.05	2.52±0.24	3.22±0.47	4.01±0.08
Antioxidant activity (DPPH)			1.18	1.75	1.58	2.49	2.08	2.14	1.90	2.25

and ethanol was more efficient than the use of only one solvent. Thus, 3.2 mg/g of phenolic compounds were found in the water-ethanol extract whereas 1.9 and 2.5 mg/g were extracted by water and ethanol, respectively. The difference with respect to SFE is that, by PLE, ethanol extracts more phenolic compounds than water and also differences between the total phenolic content extracted by different solvents are less significant. These results are in agreement with Paes et al. conclusions [19].

Taking as a reference the traditional Soxhlet extraction using methanol as solvent, the total phenolic content extracted by SFE and PLE, both with water-ethanol 1:1, were 85.3 % and 80.3 %, respectively. The great advantage of SFE and PLE methods with respect to Soxhlet is the use of green solvents, which is of utmost importance to the food industry. The comparison between SFE and PLE in economic terms appears to be the key factor for the choice of the most convenient method. Clearly, the investment and operating costs of a PLE plant are lower than the ones of a SFE plant, since in the first case the section of pressurizing CO_2 is not needed, and the extraction is faster. Moreover, the amount of solvent required for the extraction is not a relevant factor as it can be recycled in both cases. Thus, since pressurized solvent extraction takes less time, is cheaper and has the advantages of SFE (green solvents and moderate temperatures), it should be considered as a promising alternative to extract phenolic or other polar compounds. More studies could be interesting to optimize this method and possibly obtain a higher phenolic yield.

5.3.5. *Influence of extraction method and solvent on phenolic compounds extraction*

In Table 5.1, phenolic compounds have been classified according to their groups and the molecular weight of each one is also reported. Four groups of molecules were found: flavonol aglycone, flavonol glycoside, organic acid and phenylpropanoids.

It is noted that using water as co-solvent the extraction of aglycone favonol molecules does not change with respect to SFE with water-ethanol. In particular, kaempferol was extracted only by SFE with these co-solvents. Quercetin was found in the same proportion regardless of the method and solvent. Isoramnetin was extracted by SFE using water and water-ethanol, by pressurized ethanol and by Soxhlet. These results indicate that quercetin having relatively more polar OH substituents was better extracted by all the methods and solvents than isorhamnetin, having the 3’ O-CH_3 group, and kaempferol, missing the 3’-OH group. Ko et al. [7] reported similar tendency concerning the extraction of flavonoids by subcritical water at different temperatures. They found that quercetin was optimally extracted at lower temperature than isorhamnetin and kaempferol.

Higher amounts of flavonoid glycosides were found in all the extracts, such derivatives are organic compounds that have a sugar portion, which increase their polar nature [7], so they are more easily extracted by polar solvents than less-polar aglycones. Particularly, kaempferol 3-O-rutinoside and iso-rutinoside were extracted better by SFE with water-ethanol whereas rutin was found in higher quantity on the Soxhlet extract. Rutin-4'-glucoside and Iso3-O-rutinoside-7-O-glucoside, molecules possessing sugar residues and with the highest molecular weight, were better extracted by pressurized solvents. Traces of Rutin glucosyl rhamnoside and isoquercetin were found in some extracts obtained by SFE with water and SFE with water-ethanol.

Extraction of quinic acid was more efficient by PLE than by SFE and Soxhlet. The water presence was important since water, and the mixture water-ethanol as solvents resulted in the highest quinic acid extracted values.

Phenylpropanoids were found with higher proportions in the extract obtained by Soxhlet. High values were obtained by SFE with water-ethanol and slightly lower proportions were extracted by pressurized solvents. Hence, there is a correlation between the time of extraction and the extracted phenylpropanoid amounts, possibly due to the competition of these molecules with more polar components, as mentioned above.

To summarize, the higher phenolic content of Soxhlet with respect to SFE and PLE was owing to rutin and phenylpropanoids. Aglycone favonols and low molecular weight flavonoid glycosides were efficiently extracted by SFE with water-ethanol whereas PLE extraction resulted in the highest content of high molecular weight glycoside flavonoids and quinic acid.

5.3.6. Influence of extraction method and solvent on the extract antioxidant activity

The antioxidant activity of the extracts obtained by SFE, PLE and Soxhlet are shown in Table 5.1. Focusing on SFE, it was higher in the extract obtained using the mixture water-ethanol as co-solvent, which is significantly richer in phenolic compounds. These results are in agreement with Paes et al. [19]. Hence, the presence of water and ethanol is also essential to obtain an extract with high antioxidant activity. Looking at the results obtained from the mono-component co-solvents, is should be noted that even if the extraction yield and phenolic content of the extracts obtained by ethanol and methanol were much lower than the one collected with water, the antioxidant activities were higher. This result suggests that SFE with ethanol or methanol are very selective to the extraction of antioxidant compounds different from phenols such as polyunsaturated fatty acids.

As regards the PLE extracts, the antioxidant activity was slightly higher in the ethanolic extract, however the differences between the use of different solvents were not very significant. Similar results were obtained by other authors [19].

5.4. Final remarks

The extraction of phenolic compounds from asparagus and the antioxidant activity of the extracts were found to be affected by both the extraction method (SFE, PLE and Soxhlet) and the solvent used. In any case, Rutin was the predominant compound and the presence of phenolic acids was significant.

As regards the SFE extracts, the overall extraction yield and the phenolic content were largely influenced by the use of different co-solvents. The highest phenolic content was obtained using a mixture of water-ethanol 1:1 as co-solvent, and consequently the effect of pressure, temperature and extraction time with this co-solvent was evaluated. The maximum extraction yield (41.0 %) was obtained at 15 MPa and 65°C, being the effect of temperature on extraction yield less relevant than the effect of pressure. No significant differences were found on the phenolic composition of the extracts obtained at different pressures and temperatures. Longer extraction times favoured the extraction of phenolic acids.

When PLE was performed, once again the mixture of water-ethanol 1:1 was more efficient as regard the phenolic content than the use of a single component solvent. The total phenolic content and the antioxidant activity were slightly lower than the ones obtained by SFE and Soxhlet. Nevertheless, since PLE is cheaper, faster and has the main advantages of SFE (environmental friendly at moderate temperatures), it can be considered as a good alternative for the extraction of natural compounds.

References

[1] A. Shilpi, U.S. Shivhare, S. Basu, Supercritical CO_2 Extraction of Compounds with Antioxidant Activity from Fruits and Vegetables Waste -A Review, Focusing on Modern Food Industry 2 (2013) 43–62.

[2] A. Di Maro, S. Pacifico, A. Fiorentino, S. Galasso, M. Gallicchio, V. Guida, et al., Raviscanina wild asparagus (Asparagus acutifolius L.): A nutritionally valuable crop with antioxidant and antiproliferative properties, Food Research International 53 (2013) 180–188. doi:10.1016/j.foodres.2013.04.026.

[3] J.M. Fuentes-Alventosa, S. Jaramillo-Carmona, G. Rodríguez-Gutiérrez, R. Guillén-Bejarano, a. Jiménez-Araujo, J. Fernández-Bolaños, et al., Preparation of bioactive extracts from asparagus by-product, Food and Bioproducts Processing 91 (2013) 74–82. doi:10.1016/j.fbp.2012.12.004.

[4] L.S. Chua, A review on plant-based rutin extraction methods and its pharmacological activities., J. Ethnopharmacology 150 (2013) 805–17. doi:10.1016/j.jep.2013.10.036.

[5] A. Djebbar, C. Nassima, A. Dina, B. Meriem, D. Nadjet, B. Hania, Flavonoids in Human Health: From Structure to Biological Activity, Current Nutrition & Food Science 5 (2009) 225–237. doi:http://dx.doi.org/10.2174/157340109790218049.

[6] K.E. Heim, A.R. Tagliaferro, D.J. Bobilya, Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships, J. Nutritional Biochemistry 13 (2002) 572–584. doi:10.1016/S0955-2863(02)00208-5.

[7] M.-J. Ko, C.-I. Cheigh, M.-S. Chung, Relationship analysis between flavonoids structure and subcritical water extraction (SWE)., Food Chemistry 143 (2014) 147–55. doi:10.1016/j.foodchem.2013.07.104.

[8] J.F. Hammerstone, S.A. Lazarus, H.H. Schmitz, Procyanidin content and variation in some commonly consumed foods., J. Nutrition 130 (2000) 2086S–92S.

[9] I. Erlund, T. Kosonen, G. Alfthan, J. Mäenpää, K. Perttunen, J. Kenraali, et al., Pharmacokinetics of quercetin from quercetin aglycone and rutin in healthy volunteers, European J. Clinical Pharmacology 56 (2000) 545–553. doi:10.1007/s002280000197.

[10] P.C. Veggi, J.M. Prado, G. a. Bataglion, M.N. Eberlin, M.A. a. Meireles, Obtaining phenolic compounds from jatoba (Hymenaea courbaril L.) bark by supercritical fluid extraction, J. Supercritical Fluids. 89 (2014) 68–77. doi:10.1016/j.supflu.2014.02.016.

[11] M.M.R. de Melo, a. J.D. Silvestre, C.M. Silva, Supercritical fluid extraction of vegetable matrices: Applications, trends and future perspectives of a convincing green technology, J. Supercritical Fluids. 92 (2014) 115–176. doi:10.1016/j.supflu.2014.04.007.

[12] R.N. Almeida, R.G. Neto, F.M.C. Barros, E. Cassel, G.L. von Poser, R.M.F. Vargas, Supercritical extraction of Hypericum caprifoliatum using carbon dioxide and ethanol+water as co-solvent, Chemical Engineering and Process Process Intensification 70 (2013) 95–102. doi:10.1016/j.cep.2013.05.002.

[13] C. Da Porto, D. Decorti, A. Natolino, Water and ethanol as co-solvent in supercritical fluid extraction of proanthocyanidins from grape marc: A comparison and a proposal, J. Supercritical Fluids. 87 (2014) 1–8. doi:10.1016/j.supflu.2013.12.019.

[14] J.T. Paula, L.C. Paviani, M. Foglio, I.M.O. Sousa, F. Cabral, Extraction of anthocyanins from Arrabidaea chica in fixed bed using CO_2 and CO_2/ethanol/water mixtures as solvents, J. Supercritical Fluids. 81 (2013) 33–41. doi:10.1016/j.supflu.2013.04.009.

[15] I.J. Seabra, M.E.M. Braga, M.T. Batista, H.C. de Sousa, Effect of solvent (CO2/ethanol/H2O) on the fractionated enhanced solvent extraction of anthocyanins from elderberry pomace, J. Supercritical Fluids. 54 (2010) 145–152. doi:10.1016/j.supflu.2010.05.001.

[16] E.E. Yilmaz, E.B. Özvural, H. Vural, Extraction and identification of proanthocyanidins from grape seed (Vitis Vinifera) using supercritical carbon dioxide, J. Supercritical Fluids. 55 (2011) 924–928. doi:10.1016/j.supflu.2010.10.046.

[17] Z.D. Zulkafli, H. Wang, F. Miyashita, N. Utsumi, K. Tamura, Cosolvent-modified supercritical carbon dioxide extraction of phenolic compounds from bamboo leaves (Sasa palmata), J. Supercritical Fluids. 94 (2014) 123–129. doi:10.1016/j.supflu.2014.07.008.

[18] M. Markom, M. Hasan, W. Daud, H. Singh, J. Jahim, Extraction of hydrolysable tannins from Phyllanthus niruri Linn.: Effects of solvents and extraction methods, Separation and Purification Technology 52 (2007) 487–496. doi:10.1016/j.seppur.2006.06.003.

[19] J. Paes, R. Dotta, G.F. Barbero, J. Martínez, Extraction of phenolic compounds and anthocyanins from blueberry (Vaccinium myrtillus L.) residues using supercritical CO_2 and

pressurized liquids, J. Supercritical Fluids. 95 (2014) 8–16. doi:10.1016/j.supflu.2014.07.025.

[20] Y. Zhang, S. Li, X. Wu, Pressurized liquid extraction of flavonoids from Houttuynia cordata Thunb, Separation and Purification Technology 58 (2008) 305–310. doi:10.1016/j.seppur.2007.04.010.

[21] M. Solana, I. Boschiero, S. Dall'Acqua, A. Bertucco, Extraction of bioactive enriched fractions from Eruca sativa leaves by supercritical CO_2 technology using different co-solvents, J. Supercritical Fluids. 94 (2014) 245–251. doi:10.1016/j.supflu.2014.08.022.

[22] M. Solana, C.S. Rizza, A. Bertucco, Exploiting Microalgae As A Source Of Essential Fatty Acids By Supercritical Fluid Extraction Of Lipids: Comparison Between Scenedesmus Obliquus, Chlorella Protothecoides And Nannochloropsis Salina, J. Supercritical Fluids. (2014). doi:10.1016/j.supflu.2014.06.013.

[23] A.T. Serra, I.J. Seabra, M.E.M. Braga, M.R. Bronze, H.C. de Sousa, C.M.M. Duarte, Processing cherries (Prunus avium) using supercritical fluid technology. Part 1: Recovery of extract fractions rich in bioactive compounds, J. Supercritical Fluids. 55 (2010) 184–191. doi:10.1016/j.supflu.2010.06.005.

[24] S. Dall'Acqua, R. Cervellati, M.C. Loi, G. Innocenti, Evaluation of in vitro antioxidant properties of some traditional Sardinian medicinal plants: Investigation of the high antioxidant capacity of Rubus ulmifolius, Food Chemistry 106 (2008) 745–749. doi:10.1016/j.foodchem.2007.06.055.

[25] S. Dall'Acqua, G. Innocenti, Antioxidant compounds from Chaerophyllum hirsutum extracts, Fitoterapia. 75 (2004) 592–595. doi:10.1016/j.fitote.2004.05.007.

[26] P.C. Veggi, R.N. Cavalcanti, M.A.A. Meireles, Production of phenolic-rich extracts from Brazilian plants using supercritical and subcritical fluid extraction: Experimental data and economic evaluation, J. Food Engineering 131 (2014) 96–109. doi:10.1016/j.jfoodeng.2014.01.027.

[27] G.D. Pappa, C. Perakis, I.N. Tsimpanogiannis, E.C. Voutsas, Thermodynamic modeling of the vapor–liquid equilibrium of the CO_2/H_2O mixture, Fluid Phase Equilibria 284 (2009) 56–63. doi:10.1016/j.fluid.2009.06.011.

[28] T. Holderbaum, J. Gmehling, PSRK: A Group Contribution Equation of State Based on UNIFAC, Fluid Phase Equilibria 70 (1991) 251–265. doi:10.1016/0378-3812(91)85038-V.

[29] A.B.A. De Azevedo, P. Mazzafera, R.S. Mohamed, Extraction of caffeine, chlorogenic acids and lipids from green coffee beans using supercritical carbon dioxide and co-solvents, Brazilian J. Chemical Engineering 25 (2008) 543–552.

CHAPTER 6

Precipitation of polyphenols and anthocyanins compounds from cherries extracts by SAS process

Supercritical anti-solvent (SAS) process with CO_2 is used to obtain precipitates from cherries extracts which are rich in polyphenols and anthocyanins compounds. The compounds of interest were first extracted from milled and lyophilized cherries, using methanol in an ultrasounds bath. Then, the methanolic solution was processed by supercritical anti-solvent CO_2 at various operating conditions. Results showed that continuous mode is more efficient than batch. Moreover, polyphenols and anthocyanins yields of precipitation were favoured by lower pressures (in a range 10-15 MPa) and higher compositions of CO_2 (0.95-0.995). Overall, the maximum yields achieved in our work were 51.0±2.1% (polyphenols) and 86.1±2.0% (anthocyanins), operating in continuous mode at 12.5 MPa, 40°C and 0.995 of CO_2 molar fraction. These results confirm that SAS is effective in producing dried extracts rich in polyphenols compounds, specially anthocyanins, from lyophilized cherries.

6.1. Introduction

Polyphenols compounds are well known for their numerous health-promoting properties, such as their role in the prevention of cardiovascular diseases, cancer, neurodegenerative diseases, diabetes, or osteoporosis [1]. The attention in the last years has been focused on the benefits of flavonoids, a large class of phenolic compounds. Most prominent among the flavonoids are the anthocyanins, universal plant colorants responsible for the red, purple, and blue hues evident in many natural sources, that are of particular interest to the food colorant industry due to their ability to impart vibrant colours to the product [2].

Polyphenols, and in particular anthocyanins, are commonly obtained from agricultural products as natural extracts and are intensively used in functional foods, cosmetic and pharmaceutical industries. Natural extracts are typically marketed in the form of liquid, viscous preparations or powders resulting from the drying of a liquid extract [3]. The dried extract has some advantages over conventional liquid forms, such as lower storage costs and higher concentration and stability of active substances [4].

Polyphenols compounds are practically insoluble in pure CO_2 [5], and therefore a dried extract can be obtained by precipitation using supercritical anti-solvent (SAS) technique. Generally, SAS is defined as a method in which supercritical CO_2 is used to precipitate selected, non-soluble compounds. CO_2 at relatively high pressure acts as an anti-solvent decreasing the solubility of the solid in its mixture with an organic solvent. In this process, a fast supersaturation takes place [3]. The advantage of SAS is that it improves the production capacity and it is able to control the particle size distribution, whereas its disadvantage is that it generally uses an organic solvent [6,7]. However, this limitation is normally overcome by the fact that the complete removal of the solvent can be achieved by the anti-solvent [6].

SAS involves the knowledge of: high-pressure phase equilibria of the binary or ternary system, jet mixing and mass transfer to and from the injected liquid phase [8]. Moreover, the initial concentration of the extract and the ratio of the solvent to the supercritical anti-solvent for a given solid solute are important for the evolution of the precipitation process [3,9,10]. Obviously, the value of pressure at which the operation is performed and the washing step are fundamental [6]. Thus, SAS is complex from both the theoretical and experimental points of view.

SAS has already been applied to precipitate bioactive compounds from natural sources. In Table 6.1, the summary of studies reported in the last years similar to this one is presented. Two works refer the extraction of polyphenols compounds and posteriorly precipitation by SAS, the first one from mango by-products [11] and the second one from grape residues [12]. Other antioxidants compounds were obtained in a similar way from grape seeds [13], rosemary [3], marigold flowers [14], *Lycium barbarum* [15] and microalgae [16].

In our work, cherries were chosen as the vegetable material because of their higher amount of polyphenolic and anthocyanins content with respect to other vegetables. For instance, the phenolic content of cherries were reported to be in the range 290-316 mg/100 g [17,18], whereas other agricultural products such as asparagus and rocket salad contain 80.9 mg/100g [19] and 132 mg/100 g [20], respectively. Moreover, non-saleable cherries were used, with the purpose of having a more economically viable and environmentally friendly process.

As regards the extraction solvents reported in literature, methanol with 1% of HCl and 1% of butylated hydroxytoluene (BHT) was used to extract and analyse phenolic compounds from cherries [21]. Ballisteri *et al.* [22] applied a methanol solution containing 0.5% of HCl. Serra *et al.* [18] extracted a high-valuable product from the cherry culls by supercritical CO_2 using ethanol as co-solvent. However, the complexity of SAS process limits the chosen of extraction solvents, and their thermodynamic behaviour should be carefully analysed.

In this work, SAS processing of non-saleable cherries for the precipitation of polyphenols and anthocyanins compounds extracts is presented for the first time. On the one hand, the comparison of different solvents for the extraction of the compounds of interest is shown. On the other hand, the main operating parameters affecting the SAS process is investigated and discussed.

Table 6.1. Summary of SAS studies to obtain antioxidant compounds reported in the last 5years.

Author	Source	Compounds	Pre-treatment	Extraction	SAS	Results
Meneses, 2013 [11]	Mango by-products	Polyphenols compounds	Dehydration and grinding	0.5 ascorbic acid and 50 mL of acetone solution for 6 h Centrifugation Rotatory evaporator Aqueous solution dissolved 1:4 deionized water Purification step	X_{CO2}=0.98 8-15 MPa 35-45 °C	90% of the total phenolic compounds present in the ethanolic extract were recovered
Floris, 2010 [12]	Grape residues	Polyphenols and anthocyanins	Lyophilisation	Stirred in tartaric buffer Filtered Adsorption and desorption (with methanol)	X_{CO2}=0.97 Up to 20 MPa 40 °C	Recovery= 88.3 % of phenolic compounds and 99.8 % of anthocyanins
Visentin, 2012 [3]	Rosemary	Antioxidants	No specified	Microwave 450 W, 5 min (de-oiled) Stirred in ethanol Filtered (Millipore 0.45 μm) by vacuum Polymer dissolved in the extract 2.5:1	X_{CO2}=0.94 8-12 MPa 25-50 °C	Recovery= 90%
Boonnoun, 2013 [14]	Marigold flowers	Lutein	Grinding and dried	Stirred 500 mL hexane 4 h Rotatory evaporator Dried in an oven KOH, ethanol 4h Na_2SO_4, diethyl eter Water Vacuum oven	X_{CO2}=0.93-0.95 8-12 MPa 55 °C	Composition no measured
Lin, 2014 [15]	*Lycium barbarum*	Zeaxanthin palmitates (carotenoid)	De-glycosided	Ultrasonication stirred in water Filter paper 20-25 μm Freeze dried Ultrasonication stirred in THF Vacuum evaporation Solution 5 mg mL^{-1}	CO_2= 15 L min^{-1} Sol=0.2 mL min^{-1} 12.5-17 MPa 55 °C	Recovery = 71%
Liau, 2010 [16]	Microalgae	Carotenoids	Freeze-dried	Soxhlet with CH_2Cl_2 for16 h	CO_2= 12 L min^{-1} Sol=1.2 mL min^{-1} 20 MPa 40 °C	The contents of Zeaxanthin were enhanced from 11.12 mg g^{-1} to 674 mg g^{-1}

6.2. Materials and methods

6.2.1. *Raw material*

Non-saleable cherries were provided by a local market in Italy. Raw material was first de-hulled, milled by a knife mill and then stored at -25° until dehydration in a freeze-dryer was performed.

6.2.2. *Chemicals*

CO_2 (4.0 type, purity greater than 99.99%) was purchased by Rivoira. Reagents for extraction (methanol, ethanol, DMSO and acetone) and analysis (methanol, acetonitrile, formic acid and ethanol) were provided by Carlo Erba, Prolabo, J.T. Baker and Lab-Scan.

6.2.3. *Extraction*

The freeze-dried vegetal material was extracted in an ultrasound system for 30 min with methanol. The supernatant was removed after centrifugation. Liquids were collected for subsequent treatment by SAS.

6.2.4. *Supercritical anti-solvent precipitation*

The flow diagram of the apparatus built for the SAS experiments is illustrated in Figure 6.1. The operating procedure is as follows: $CO_{2,}$ stored in a reservoir tank (R), is first cooled down to avoid the cavitation of the high pressure pump (P). After being pressurized up to the operating pressure, it is heated up (HE) to the required temperature. Once the flow rate of CO_2 is constant and the operating parameters are stable, the solution is pumped by a chromatographic pump (P) into the precipitator at the desired flow rate. After 30 minutes of continuous CO_2 and solution flow, the pumping of the solution is stopped and the washing step takes place, where only CO_2 flows for 20 minutes. Then, the system is depressurized and the precipitator (PR) is opened. The precipitate is collected from the filter (porous metallic frit with a screen size of 1 μm) and solved in methanol for analysis.

For the experiments in batch mode, the precipitation vessel is loaded with a given quantity of the liquid solution and then the pressure is increased by CO_2, which allows to start anti-solvent precipitation. Finally, a flow of CO_2 is fed at a constant flow rate from the top of the chamber for 30 minutes, followed by 20 more minutes for the washing step. After depressurization of the system, the precipitator is opened and the particles are collected.

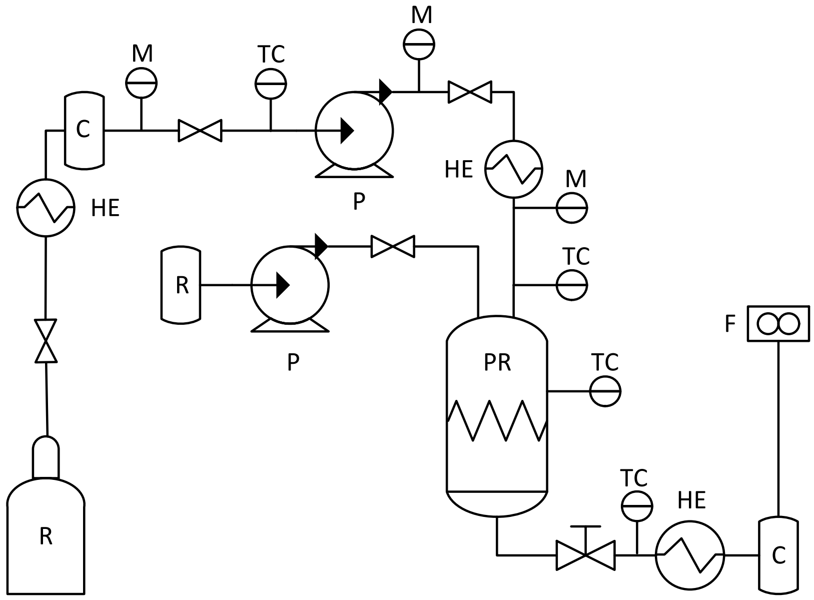

Figure 6.1. Schematic of the supercritical anti-solvent (SAS) apparatus. R: reservoir, HE: heat exchanger, C: container, M: manometer, TC: temperature controller, P: pump, PR: precipitator, F: flow meter.

6.2.5. Polyphenolic analysis of the extracts and precipitates

Quali-quantitative analysis of polyphenols in the extracts were obtained by HPLC-MS. The measurements were performed on a Varian 212 series chromatograph equipped with Prostar 430 autosampler and MS-500 Ion Trap as detector. MS spectra were recorded in positive and in negative ion mode (50–2000 Da). The ESI (Electron Spray Ionization) was used. Fragmentation of the main ionic species were obtained during the HPLC run by the turbo data depending scanning (tdds) function, yielding the fragmentation pattern of eluted compounds. As stationary phase Agilent Zorbax C-18 (2.1 × 150 mm) 3.5 µm was used.

6.3. Results and discussion

Polyphenols and anthocyanins precipitation yields (Y_p and Y_a, respectively) were calculated by using:

$$Y_p = \frac{c_p V}{c_{sp} V_s} * 100 \tag{6.1}$$

$$Y_a = \frac{c_a V}{c_{sa} V_s} * 100 \tag{6.2}$$

Table 6.2. Concentration of polyphenolic compounds (mg g^{-1}) in the extracts obtained using different solvents.

Polyphenolic compounds (mg g^{-1})	EtOH	MeOH	DMSO	DMSO EtOH	DMSO EtOH 7:3	DMSO EtOH 3:7	Acetone	Acetone DMSO 1:1	EtOH Acetone 1:1
Anthocyanins	0.15	0.91	0.95	1.05	0.89	0.86	0.02	0.38	0.18
Daidzein and derivates	0.00	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00
Ferulic and derivates	0.18	0.87	0.65	0.96	0.86	0.94	0.00	0.00	0.00
Chlorogenic	0.06	0.98	0.78	0.85	1.00	0.94	0.00	0.00	0.00
Rutin	0.03	0.23	0.23	0.23	0.19	0.16	0.00	0.00	0.00
Total polyphenols	0.42	3.00	2.62	3.10	2.94	2.90	0.02	0.38	0.18

where C_p is the polyphenols concentration of the precipitate collected after SAS (mg mL^{-1}), V is the volume of methanol used to solve the precipitate for analysis (mL), C_{sp} is the polyphenols concentration in the initial solution (mg mL^{-1}), V_s is the volume of solution injected (mL), C_a is the anthocyanins concentration of the precipitate collected after SAS (mg mL^{-1}) and C_{sa} is the anthocyanins concentration in the initial solution (mg mL^{-1}).

6.3.1. Solution: comparison of solvents and initial concentration

A large number of solvents for extracting phenolic compounds from vegetable materials has been reported in literature. For SAS applications, it is important to take into account not only the extraction yield but also the physicochemical properties of the mixture solvent/anti-solvent as well as its mixing and flow behaviour. As shown in Table 6.1, ethanol, acetone, ascorbic acid, CH_2Cl_2 and methanol are among the extraction solvents that have been demonstrated to be suitable for the subsequent application of the SAS process.

In our work, extractions with different solvents were carried out with the aim of selecting the most effective one for the extraction of polyphenols and anthocyanins compounds from cherries and further SAS precipitation. As shown in Table 6.2, the highest polyphenols and anthocyanins extraction yields were achieved using methanol, dimethyl sulfoxide (DMSO) or mixtures of DMSO and ethanol. According to the results in Table 6.2, preliminary anti-solvent precipitation tests were performed with the extracts obtained using these solvents. No particles were found when using DMSO or mixtures of DMSO and ethanol. Hence, methanol was chosen as the solvent to extract the compounds of interest for all the runs that are reported in the following sections. In Figure 6.2, a chromatogram showing the polyphenolic compounds profile from cherries extracted by methanol is presented.

The concentration of the initial solution is another parameter that affects the SAS process [3]. Initially, two solutions were prepared, starting from 3% and 9% (w/w) of lyophilized cherries.

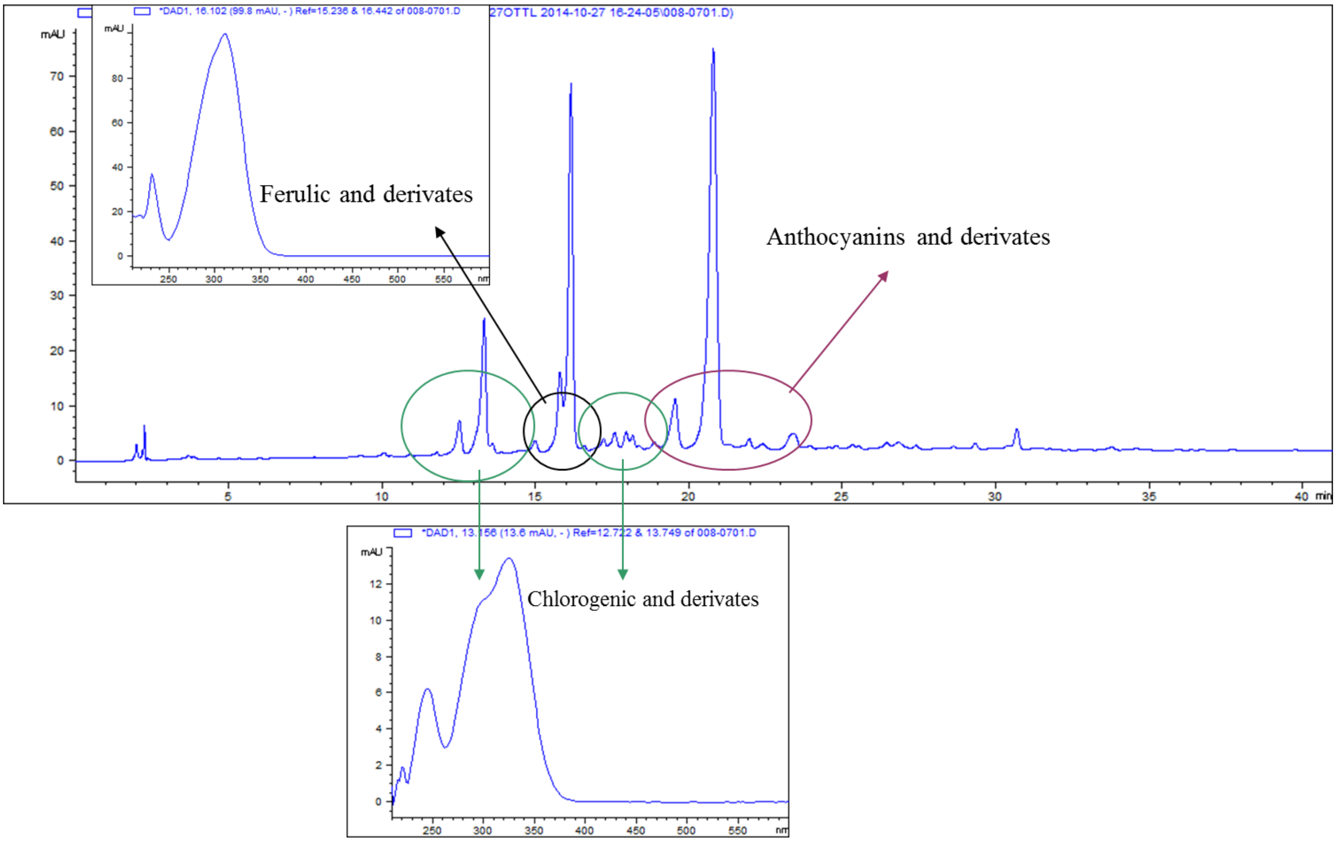

Figure 6.2. Chromatogram showing the phenolic compounds from cherries extracted by methanol.

Considering that the extraction yield (w/w) is around 35-40%, the second solution contained approximately 3% of solids, which is the content that have previously been used by other authors for SAS [3,13,23]. Indeed, preliminary SAS tests demonstrated that this solution is much more effective that the one prepared with 3% of lyophilized cherries. Thus, solutions obtained from 9% of lyophilized cherries in methanol were prepared for the subsequent experiments.

6.3.2. *Equilibrium system CO_2-methanol*

The knowledge of solubility data of the liquid solvents and solids in supercritical CO_2 is very important for the proper selection of SAS process temperature and pressure and the interactions between thermodynamic constrains and mass transfer mechanisms controlling the process performance [24]. Typical SAS precipitation experiments are operated at pressure above the binary mixture critical point [8].

According to the work by Floris *et al.*[12], who also used a methanolic solution, pressure in the range 10-15 MPa was selected (at 40 ºC) as an acceptable compromise between supercritical operation and the lowest possible solubilities of the polyphenolic compounds in high pressure CO_2. In Figure 6.3, the equilibrium curve obtained from experimental data reported in literature for the

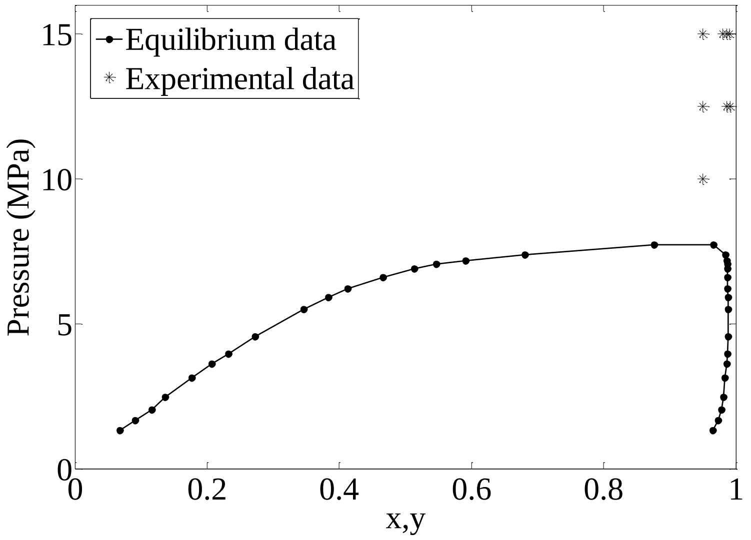

Figure 6.3. Equilibrium system CO_2-methanol at 40°C and location of our experimental data.

system CO_2-methanol [25] is shown, and the location of the points at the conditions we chose for our experiments is evidenced. As mentioned by Martin *et al.* [23], it should be taken into account that the presence of vegetable extract in the CO_2-methanol mixture may change the vapour-liquid equilibrium data of the binary system.

6.3.3. Batch vs. continuous operation

Initially, two experiments were performed with the aim of comparing batch and continuous modes of operation. The advantages of using continuous mode at industrial scale are evident. Nevertheless, at laboratory scale, batch operation is frequently useful to prove the operation of a new process and/or new application and have a first estimation of the operating variables values. In the case of SAS processes, this seems to be especially important due to the number of parameters that are essential for the success of the test, such as the kind of injection device [6], the compound concentration in the initial solution [26] or the molar fraction of CO_2 to trigger precipitation [10,26].

Batch and continuous mode of operation were tested at 40 ºC and 10 MPa. For batch experiments, 250 mL of solution and a CO_2 flow rate of 5 L min^{-1} were used. A molar fraction of 0.95 was set for continuous test. It should be noted that in batch, the pressure increased during the 30 minutes of experiments up to 15 MPa. As presented in Table 6.3, the highest polyphenols and anthocyanins yields were obtained when using continuous mode.

Table 6.3. Comparison between batch and continuous mode of SAS operation in terms of anthocyanins and polyphenols yield at 40 º and 10 MPa.

	Batch	Continuous
Polyphenols yield (%)	39.00±2.21	44.10±0.30
Anthocyanins yield (%)	29.17±2.29	38.52±2.41

6.3.4. *Effect of pressure on anthocyanins and polyphenols yield*

The effect of pressure on anthocyanins and polyphenols yield operating in continuous mode was then investigated. The temperature was maintained constant at 40 ºC in all the experiments and the CO_2 molar fraction was fixed at 0.95. Figure 6.4 shows clearly that the higher the pressure, the lower the anthocyanins and polyphenols yields. The best result (Y_p= 44.1%, Y_a= 38.5%) was obtained at 10 MPa, which corresponds to the close condition to the binary mixture critical point. This result seems to be related to the fact that the higher the pressure, the higher the solubility of the extracts, and therefore the compounds of interest are solubilized by supercritical CO_2 during the washing step are lost in the solvent collected instead of being stopped by the filter.

6.3.5. *Effect of CO_2 molar fraction on anthocyanins and polyphenols yield*

In SAS processes, one of the problems is to find the appropriate ratios of the anti-solvent to the supercritical solvent flow rates for a given solid solute (this is referred to as the CO_2 molar fraction) [10,26]. This effect was studied at two different precipitation pressures: 12.5 and 15 MPa. Results, shown in Figures 6.4 and 6.5, confirm the high influence of the supercritical anti-solvent to solvent ratio on the SAS process, even if the range of the CO_2 molar fraction studied is quite limited (0.95-0.995). Clearly, the higher the CO_2 flow rate injected in the precipitation vessel, the higher the yield of the polyphenolic compounds.

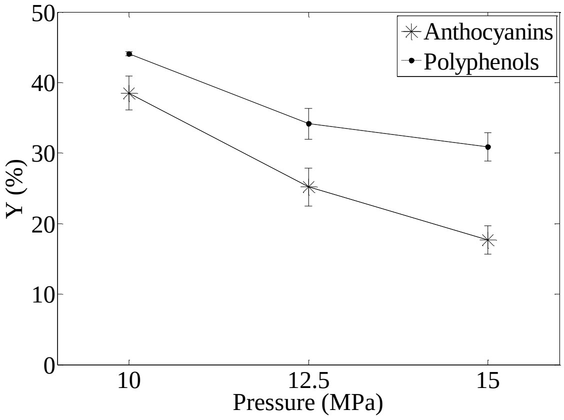

Figure 6.4. Effect of pressure on anthocyanins and polyphenols yield of SAS.

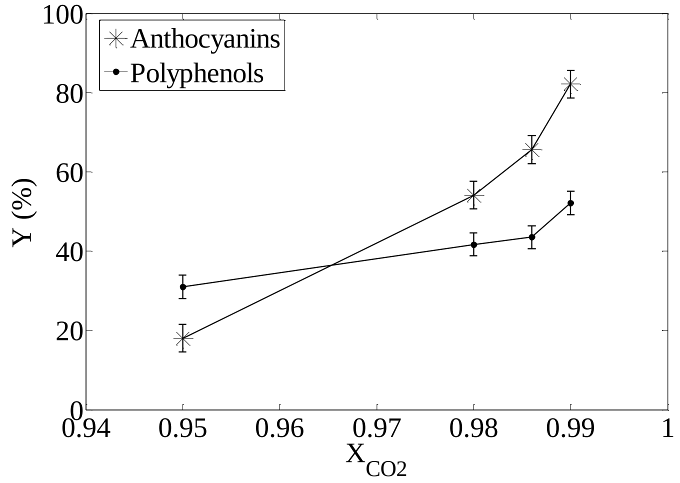

Figure 6.5. Effect of CO_2 molar fraction on anthocyanins and polyphenols yield of SAS at 15 MPa.

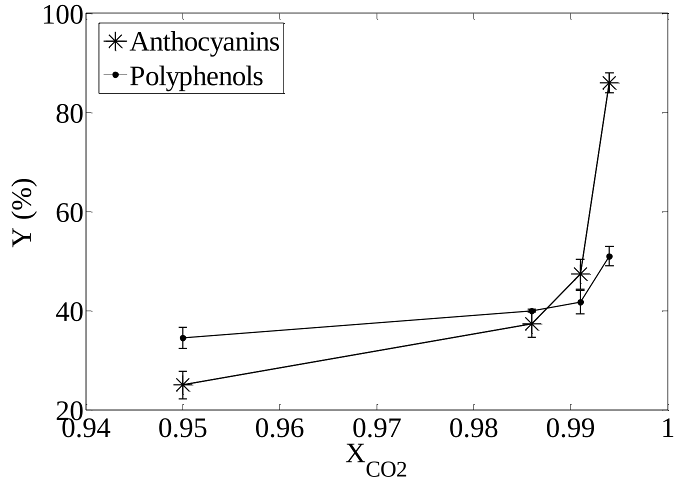

Figure 6.6. Effect of CO_2 molar fraction on anthocyanins and polyphenols yield of SAS at 12.5 MPa.

Looking at Figure 6.5, it is also noted that at a CO_2 molar fraction of 0.95 the polyphenols yield was higher than the anthocyanins one, but at values of CO_2 molar fraction from 0.98 the opposite tendency was found. This trend is also noticeable at 12.5 MPa (Figure 6.6), with the cross-over shifted to molar fraction values around 0.99. It means that, among the phenolic compounds present in the methanolic extracts, the process is selective towards anthocyanins at higher supercritical anti-solvent ratios. Overall, the highest yields were 51.0±2.1% (Y_p) and 86.1±2.0% (Y_a), operating in continuous mode at 12.5 MPa, 40°C and 0.995 of CO_2 molar fraction, as can be seen in Figure 6.6. An image of the precipitate obtained in this experiment is presented in Figure 6.7.

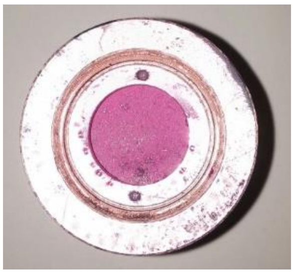

Figure 6.7. SAS of a precipitate from cherries obtained in the experiment performed at 12.5 MPa, 40°C and 0.995 of CO_2 molar fraction.

6.4. Final remarks

Polyphenols and anthocyanins compounds from cherries were successfully precipitated using supercritical anti-solvent (SAS) process with CO_2. Among different solvents tested, methanol was demonstrated to be the most efficient one for obtaining an extract rich in the compounds of interest, while being appropriate for the precipitation by SAS. Continuous mode of operation, at 10 MPa and a CO_2 molar fraction of 0.995 were the optimum conditions for obtaining the highest polyphenols and anthocyanins yields. The maximum yields achieved in our work were 51.0±2.1% (polyphenols) and 86.1±2.0% (anthocyanins). Hence, non-saleable cherries can be considered as raw material for producing dried precipitates rich in polyphenols and anthocyanins by extraction with methanol and subsequent SAS process.

References

[1] A. Scalbert, C. Manach, C. Morand, C. Rémésy, L. Jiménez, Dietary polyphenols and the prevention of diseases., Crit. Rev. Food Sci. Nutr. 45 (2005) 287–306. doi:10.1080/1040869059096.

[2] I. Konczak, W. Zhang, Anthocyanins — More Than Nature ' s Colours, 5 (2004) 239–240.

[3] a. Visentin, S. Rodríguez-Rojo, a. Navarrete, D. Maestri, M.J. Cocero, Precipitation and encapsulation of rosemary antioxidants by supercritical antisolvent process, J. Food Eng. 109 (2012) 9–15. doi:10.1016/j.jfoodeng.2011.10.015.

[4] C.R.F. Souza, I.A. Schiavetto, F.C.F. Thomazini, W.P. Oliveira, Processing of Rosmarinus officinalis Linne extract on spray and spouted bed dryers., Brazilian J. Chem. Eng. 25 (2008) 59–69. doi:10.1590/S0104-66322008000100008.

[5] M. Solana, I. Boschiero, S. Dall'Acqua, a. Bertucco, Extraction of bioactive enriched fractions from Eruca sativa leaves by supercritical CO2 technology using different co-solvents, J. Supercrit. Fluids. 94 (2014) 245–251. doi:10.1016/j.supflu.2014.08.022.

[6] E. Reverchon, Supercritical antisolvent precipitation of micro- and nano-particles, 15 (1999) 1–21.

[7] Y. Chen, Y. Ling, An overview of supercritical fluid extraction in Chinese herbal medicine: from preparation to analysis, J. Food Drug Anal. 8 (2000) 235–247.

[8] E. Reverchon, E. Torino, S. Dowy, a. Braeuer, a. Leipertz, Interactions of phase equilibria, jet fluid dynamics and mass transfer during supercritical antisolvent micronization, Chem. Eng. J. 156 (2010) 446–458. doi:10.1016/j.cej.2009.10.052.

[9] C. Chinnarasu, a. Montes, M.T.F.- Ponce, L. Casas, C. Mantell, C. Pereyra, et al., Precipitation of antioxidant fine particles from Olea europaea leaves using supercritical antisolvent process, J. Supercrit. Fluids. 97 (2015) 125–132. doi:10.1016/j.supflu.2014.11.008.

[10] V.J. Pereira, R.L. Matos, S.G. Cardoso, R.O. Soares, G.L. Santana, G.M.N. Costa, et al., A new approach to select solvents and operating conditions for supercritical antisolvent precipitation processes by using solubility parameter and group contribution methods, J. Supercrit. Fluids. 81 (2013) 128–146. doi:10.1016/j.supflu.2013.05.010.

[11] M. a. Meneses, G. Caputo, M. Scognamiglio, E. Reverchon, R. Adami, Antioxidant phenolic compounds recovery from Mangifera indica L. by-products by supercritical antisolvent extraction, J. Food Eng. 163 (2015) 45–53. doi:10.1016/j.jfoodeng.2015.04.025.

[12] T. Floris, G. Filippino, S. Scrugli, M.B. Pinna, F. Argiolas, a. Argiolas, et al., Antioxidant compounds recovery from grape residues by a supercritical antisolvent assisted process, J. Supercrit. Fluids. 54 (2010) 165–170. doi:10.1016/j.supflu.2010.04.006.

[13] J.L. Marqués, G. Della Porta, E. Reverchon, J. a. R. Renuncio, a. M. Mainar, Supercritical antisolvent extraction of antioxidants from grape seeds after vinification, J. Supercrit. Fluids. 82 (2013) 238–243. doi:10.1016/j.supflu.2013.07.005.

[14] P. Boonnoun, H. Nerome, S. Machmudah, M. Goto, A. Shotipruk, Supercritical anti-solvent micronization of marigold-derived lutein dissolved in dichloromethane and ethanol, J. Supercrit. Fluids. 77 (2013) 103–109. doi:10.1016/j.supflu.2013.02.026.

[15] K.-L. Lin, L.-M. Chng, J.C.-T. Lin, S.-L. Hsu, C.-C. Young, C.-J. Shieh, et al., Effect of Anti-solvent Conditions on Low Density Supercritical Fluids Precipitation of Zeaxanthin Palmitates from Lycium Barbarum Fruits, J. Supercrit. Fluids. 87 (2014) 104–110. doi:10.1016/j.supflu.2014.01.001.

[16] B.-C. Liau, C.-T. Shen, F.-P. Liang, S.-E. Hong, S.-L. Hsu, T.-T. Jong, et al., Supercritical fluids extraction and anti-solvent purification of carotenoids from microalgae and associated bioactivity, J. Supercrit. Fluids. 55 (2010) 169–175. doi:10.1016/j.supflu.2010.07.002.

[17] J. a Vinson, X. Su, L. Zubik, P. Bose, Phenol antioxidant quantity and quality in foods: fruits., J. Agric. Food Chem. 49 (2001) 5315–21.

[18] A.T. Serra, I.J. Seabra, M.E.M. Braga, M.R. Bronze, H.C. de Sousa, C.M.M. Duarte, Processing cherries (Prunus avium) using supercritical fluid technology. Part 1: Recovery of extract fractions rich in bioactive compounds, J. Supercrit. Fluids. 55 (2010) 184–191. doi:10.1016/j.supflu.2010.06.005.

[19] J.A. Vinson, Y. Hao, X. Su, L. Zubik, Phenol Antioxidant Quantity and Quality in Foods : Vegetables, 8561 (1998) 3630–3634.

[20] A. Martínez-Sánchez, A. Gil-Izquierdo, M.I. Gil, F. Ferreres, A comparative study of flavonoid compounds, vitamin C, and antioxidant properties of baby leaf Brassicaceae species., J. Agric. Food Chem. 56 (2008) 2330–40. doi:10.1021/jf072975+.

[21] V. Usenik, J. Fabčič, F. Štampar, Sugars, organic acids, phenolic composition and antioxidant activity of sweet cherry (Prunus avium L.), Food Chem. 107 (2008) 185–192. doi:10.1016/j.foodchem.2007.08.004.

[22] G. Ballistreri, A. Continella, A. Gentile, M. Amenta, S. Fabroni, P. Rapisarda, Fruit quality and bioactive compounds relevant to human health of sweet cherry (Prunus avium L.) cultivars grown in Italy, in: Food Chem., 2013: pp. 630–638. doi:10.1016/j.foodchem.2012.11.024.

[23] L. Martín, a. González-Coloma, R. Adami, M. Scognamiglio, E. Reverchon, G. Della Porta, et al., Supercritical antisolvent fractionation of ryanodol from Persea indica, J. Supercrit. Fluids. 60 (2011) 16–20. doi:10.1016/j.supflu.2011.03.012.

[24] E. Reverchon, I. De Marco, Supercritical fluid extraction and fractionation of natural matter, J. Supercrit. Fluids. 38 (2006) 146–166. doi:10.1016/j.supflu.2006.03.020.

[25] C.C. J, C. Kou-lung, D. Chang-yih, A new apparatus for the determination of P – x – y diagrams and Henry ’ s constants in high pressure alcohols with critical carbon dioxide, 12 (1998) 223–237.

[26] I.J. Joye, D.J. McClements, Production of nanoparticles by anti-solvent precipitation for use in food systems, Trends Food Sci. Technol. 34 (2013) 109–123. doi:10.1016/j.tifs.2013.10.002.

CHAPTER 7

Testing of equipment: Recovery of butanol by counter-current CO_2 fractionation[6]

A counter-current CO_2 fractionation method was studied as a means to recover butanol (also known as 1-butanol or n-butanol) and other compounds that are typically obtained from biobutanol fermentation broth from aqueous solutions. The influence of operating parameters, such as solvent-to-feed ratio, temperature, pressure and feed solution composition was experimentally investigated in terms of separation efficiency, butanol removal rate, total removal and butanol concentration in the extract at the end of the continuous cycle. Results show that the highest separation efficiency (351.5) is obtained at 35 °C and 10.34 MPa, with a removal rate of 0.0011 kg h^{-1}. At these operating conditions, 92.3 % of the butanol present in the feed solution was removed and a concentration of 787.5 g L^{-1} of butanol in the extract was obtained, starting from a feed solution of 20 g L^{-1}. Selectivity was calculated from experimental data, concluding that our column performs much better than a single equilibrium stage. When adding ethanol and acetone to the feed solution, ethanol was obtained in the water rich fraction (raffinate) whereas the highest concentration of acetone was found in the butanol rich fraction (extract).

7.1. Introduction

The increase of oil prices and the depletion of fossil fuels have promoted the development of biofuels such as biobutanol and bioethanol [1]. Efforts to re-commercialize biobutanol are gaining a remarkable attention; its potential to substitute for both ethanol and biodiesel in the biofuel market are estimated to be worth \$247 billion by 2020 [2]. In addition, butanol is an important feedstock for the chemical industry, being used in the production of paint, solvents and plasticizers [3]. In this context, the optimization of the processes to produce and recover biobutanol is of utmost importance.

---

[6] *Part of this chapter has been sent to Separation and Purification Technology as: Solana, M., Qureshi, N., Bertucco, A., Eller, F., Recovery of butanol by counter-current carbon dioxide fractionation with its potential application to butanol fermentation.*

First of all, biobutanol needs to compete in cost (priced on an energy basis) with ethanol [4]. Biobutanol has a higher energy density, is less volatile, less explosive and less hygroscopic (thus does not pick up water) than ethanol. Moreover, butanol can easily mix with gasoline in any proportion, can be used in internal combustion engines, can be transported in existing pipe lines, and is less corrosive [5–7]. However, at present, improving the economics of the butanol production is crucial for its re-commercialization and competition with ethanol [8].

Renewable butanol is produced from the fermentation of carbohydrates in a process often referred to as the acetone-butanol-ethanol (ABE) fermentation [4]. Butyric and acetic acids are first produced by *Clostridium acetobutylicum* or *C. beijerinckii* (acidogenesis), and in the subsequent phase (solventogenesis) butanol, acetone and ethanol are formed [8]. The concentration of butanol typically reached in the fermenter is rather low (10–20 g L^{-1}) because this compound is toxic to the butanol-producing microorganisms [9]. Distillation is traditionally carried out to recover butanol from the fermentation broth. However its cost is high, due to the low concentration of butanol and to the fact that water is the major component, with a boiling point below that of butanol (100 ^{o}C vs 117.7 ^{o}C) [1].

A decrease in product recovery cost and purification from the dilute fermentation broth is included among the four recommendations to revive butanol fermentation and make it a commercially viable biofuel [10,11]. The methods so far proposed for the recovery of butanol include adsorption, liquid-liquid extraction, gas stripping, vacuum fermentation and pervaporation [8,12–20]. Conventional distillation method can still be improved but non-conventional methods are required to significantly reduce energy duty and the associated cost [4].

The alternative we propose in this work consists of using counter-current CO_2 fractionation to recover butanol from the fermentation broth. Carbon dioxide could be an appropriate solvent for the extraction of high molecular weight alcohols from aqueous solutions because high molecular weight alcohols, such as butanols, are less hydrophilic and less volatile than lower molecular weight alcohols. Further, the vapor pressure of butanol (bp=117.7 °C) is significantly less than the vapor pressure of methanol or ethanol, making the separation of the solute from CO_2 easier [21]. Moreover, CO_2 is inert, non-toxic and can be safely recycled, resulting in an economic and environmental benefit. Counter-current operation facilitates the separation since it reduces the amount of solvent necessary, increases the throughput, and enables higher extract concentration in the solvent and lower residual concentrations in the raffinate than does single stage or multistage cross current operation [22].

To the best of our knowledge, only one study reports the recovery of butanol from aqueous solutions by using CO_2 [21]. These authors reported that, using supercritical CO_2 in a mechanically agitated extraction column at 10 MPa and 40°C, approximately 0.02 wt% of butanol concentration in the raffinate and 85–90 wt% butanol in the extract can be obtained.

In this study our objective is to investigate the effects of CO_2 extraction process parameters on separation of butanol from aqueous solutions containing also acetone and ethanol with the ultimate aim to apply this technology to ABE fermentation.

7.2. Materials and methods

7.2.1. Materials

Butanol, acetone and absolute ethanol were supplied by Fisher scientific (Fair Lawn, NJ, USA). Carbon dioxide was provided by ILL-MO products Co. (Jacksonville, IL, USA). Analytical grade acetone, butanol, ethanol, and n-propanol were obtained from Sigma Chemicals (St. Louis, MO, USA).

7.2.2. Fractionation column

The basic design of the counter-current fractionation laboratory scale unit has been described elsewhere [23,24]. In this work, the gas booster pump was substituted by a syringe pump. The column was packed with seventy six vertically-stacked packing Pall Ring pieces, 316-stainless steel, of 0.016 m diameter (AMACS Process Tower Internals. Houston, TX). A schematic of the complete apparatus used for this study is shown in Figure 7.1.

Initially, the system was pressurized and the column was heated at the experimental pressure and temperature. When equilibrium was reached, the feed solution was pumped at 1 mL min^{-1} and the extract collection was started. The extract flask was submerged in dry ice to avoid the losses of butanol (or acetone and ethanol as week when applicable) by evaporation. CO_2 entered from the bottom of the column and the butanol aqueous solution was fed from the top, so as to allow counter current contact of CO_2 with the feed solution. Experiments lasted 300 minutes. Continuous feed solution flow was maintained for 200 minutes (continuous cycle), whereas only CO_2 was pumped during the last 100 minutes. The raffinate was accumulated in the reservoir pump and was drained every 100 minute intervals. One sample of the extract was collected after 200 minutes, namely at the end of the continuous cycle.

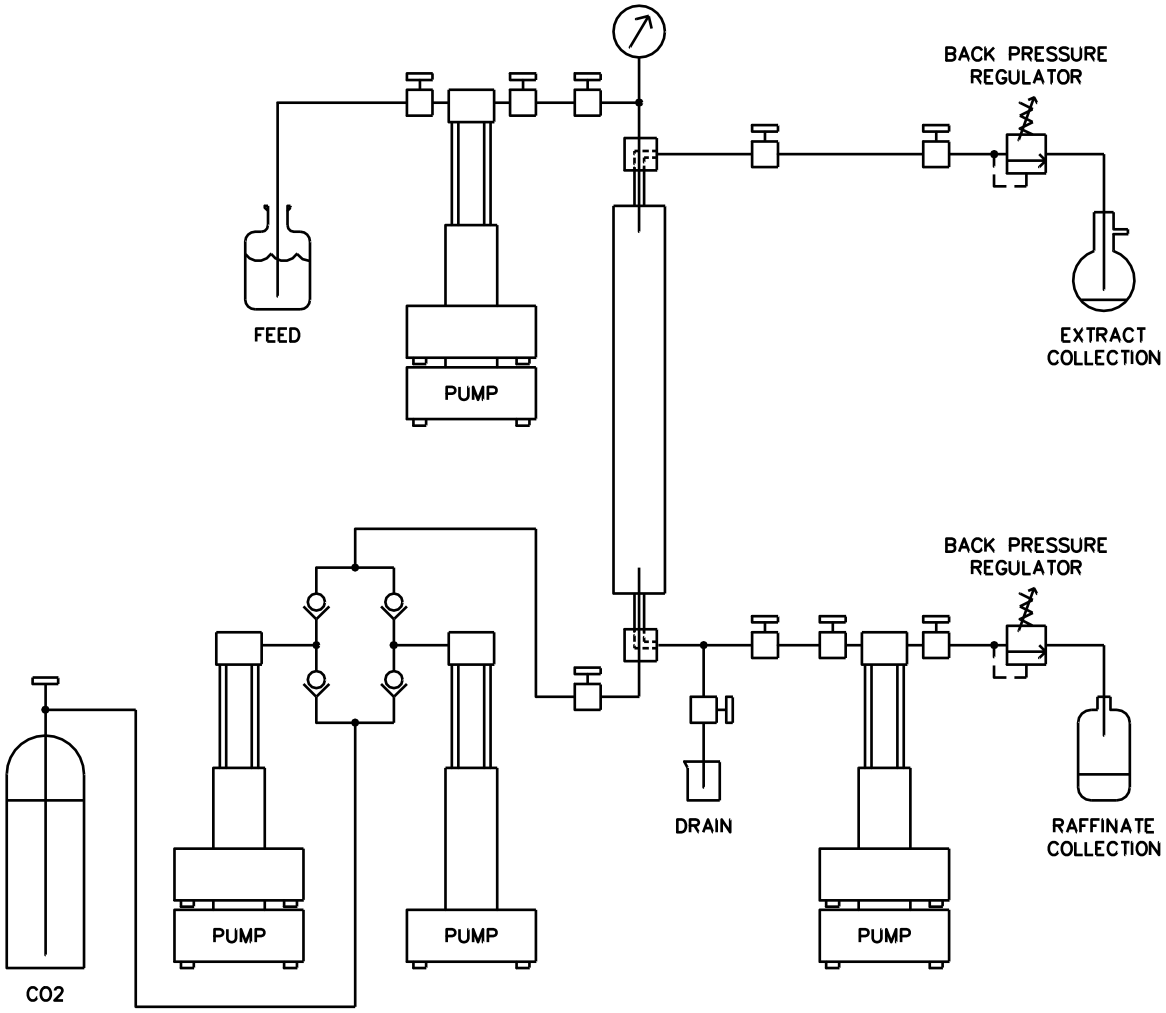

Figure 7.1. A schematic of CO_2 counter-current fractionation system for recovery of butanol.

Tests at 10.34 and 25.16 MPa were performed. Temperature was varied in a range between 25-50 °C. Considering that CO2 critical temperature and pressure are 31.1°C and 7.39 MPa, respectively, for the experiments performed at 25°C CO2 was at liquid state, whereas for the tests at 35 and 50 °C it was at supercritical conditions. CO2 flow rate was varied from 1.25 to 7.5 mL min-1.

Each experimental run was replicated twice. The average and the standard deviation were calculated on the basis of the measures of the two tests.

7.2.3. Analytical method

The compositions of extract, raffinate and feed were determined by gas chromatography as reported elsewhere [16,25].

7.3. Results and discussion

Separation efficiency was calculated as follows:

$$Separation\ efficiency = \frac{X/(1-X)}{Z/(1-Z)} \tag{7.1}$$

where X= weight fraction of butanol in the extract, and Z= average of weight fraction of butanol in the raffinate and in the feed.

Removal rate was calculated using the following equation:

$$Removal\ rate\ (kg_{butanol}\, h^{-1}) = (Feed\ conc. - Raffinate\ conc.) \times Feed\ flow\ rate \tag{7.2}$$

Specific removal, i.e. the removal per unit of solvent used, was calculated as:

$$Specific\ removal\ \ (kg_{butanol}\, kg_{CO2}^{-1}) = \frac{Removal\ rate}{CO_2\ flow\ rate} \tag{7.3}$$

CO_2 space velocity was evaluated by:

$$CO_2\ space\ velocity\ (min^{-1}) = \frac{CO_2\ flow\ rate}{Volume\ of\ extractor} \tag{7.4}$$

The total removal of butanol with respect to the feed solution at the end of the continuous cycle was calculated as:

$$Total\ removal\ (\%) = \left(1 - \frac{Raffinate\ concentration}{Feed\ concentration}\right) \times 100 \tag{7.5}$$

Overall selectivity of butanol in the separation unit was evaluated by:

$$Selectivity = \frac{X/(1-X)}{Y/(1-Y)} \tag{7.6}$$

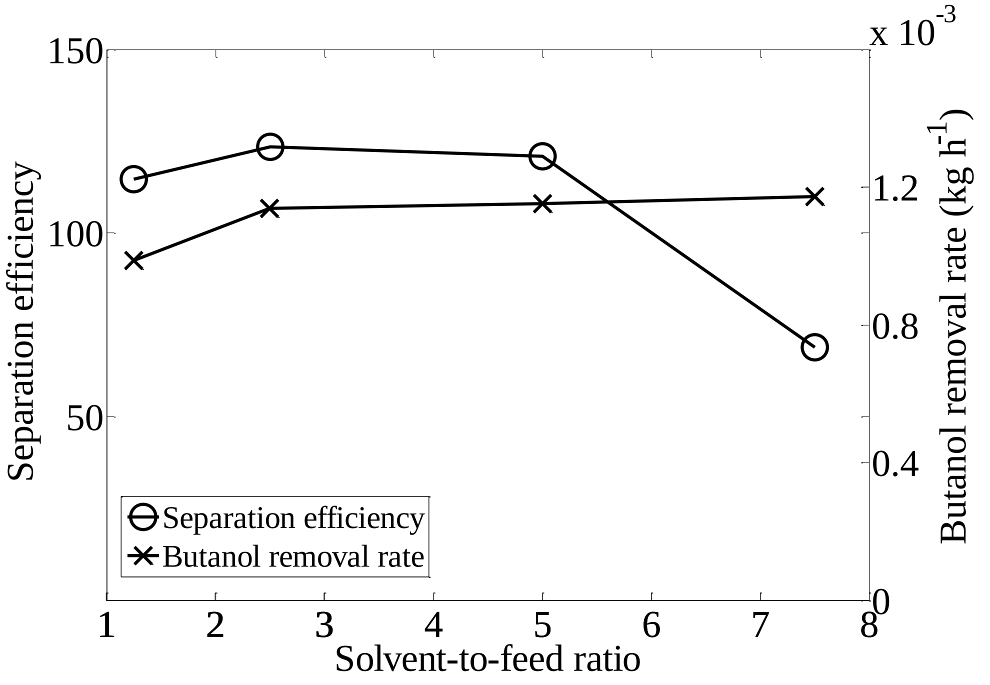

Figure 7.2. Effect of solvent-to-feed ratio on separation efficiency and butanol removal rate (kg h^{-1}) at constant pressure (10.34 MPa) and temperature (25 °C).

where X= fraction of butanol in the extract, and Y= fraction of butanol in the raffinate (Note that the same result is obtained when calculating selectivity from weight or molar fractions).

7.3.1. Effect of solvent-to-feed ratio

Solvent-to-feed ratio is an important parameter that affects the yield and the economics of the fractionation process. The influence of solvent-to-feed was studied at constant pressure (10.34 MPa) and temperature (25 °C) with ratios at 1.25, 2.5, 5 and 7.5. The average concentration of butanol in the feed solution was 20.1±1.0 g L^{-1}. Separation efficiency, removal rate and total removal were calculated from the analysis measures.

As illustrated in Figure 7.2, the effect of solvent-to-feed ratio between 1.5-5 on separation efficiency is not very significant. However, when the solvent-to-feed ratio was increased from 5 to 7.5, the efficiency decreased from 120.8 to 69.0. The removal rate increased from 0.0010 to 0.0011 kg h^{-1} when the solvent-to-feed-ratio was changed from 1.25 to 2.5, and it slightly increased when the flow rate was increased from 2.5 to 7.5.

The highest butanol concentration in the extract (603.2 g L^{-1}) was obtained at a solvent-to-feed ratio of 1.25 and it decreased as the solvent-to-feed ratio increased, as shown in Table 7.1. It means that increasing the CO_2 flow rate, it extracts more water, decreasing the concentration of

Table 7.1. Effect of solvent-to-feed ratio on butanol concentration in extract (g L^{-1}) and total removal (%) after the continuous cycle.

Solvent to feed rate	Butanol concentration in extract (g L^{-1})	Total removal (%)
1.25	603.15±13.22	76.87±7.72
2.5	571.15±42.21	93.01±0.79
5	548.00±82.80	94.01±2.83
7.5	423.06±39.26	96.14±0.17

Values are mean±S.D of duplicate experiments.

butanol. Nevertheless, the total removal of butanol from the feed solution was lower at 1.25 than at higher solvent-to-feed ratio. From 2.5 to 7.5 the total removal increased slightly.

Considering the results presented in this section, a solvent-to-feed ratio of 2.5 was chosen to perform the experiments reported in the following sections. This result is in agreement with the work reported by Laitinen *at al*. [21], who used a solvent-to-feed ratio of 2.7.

Figure 7.3 shows the influence of the CO_2 space velocity (min^{-1}) on the specific removal of butanol ($kg_{butanol}kg_{CO2}^{-1}$) considering the CO_2 consumption. As expected, the higher the CO_2 space velocity, the lower the removal of butanol for the same values of CO_2 consumption.

7.3.2. Effect of pressure and temperature

Studies of pressure and temperature effects of CO_2 processes are essential, since the solving power of CO_2 can vary significantly when these operating variables are changed. On the one hand, two tests were performed keeping constant pressure (10.34 MPa) and varying the temperature from 25 °C (liquid CO_2) to 35 °C (supercritical CO_2). On the other hand, results obtained at constant density of CO_2 (0.842 g mL^{-1}) and diverse temperature and pressure are analyzed. In the latter, liquid CO_2 at 25 °C and 10.34 MPa versus supercritical CO_2 at 50°C and 25.16 MPa were used. In all the runs, CO_2 flow rate was kept constant at 2.5 mL min^{-1} and the feed solution was pumped at 1 mL min^{-1}. The average concentration of butanol in water in the feed solution was 20.0±1.0 g L^{-1}.

Looking at Table 7.2, it can be seen that there are no significant differences on the values of removal rate either when temperature is increased or when both temperature and pressure are changed. It is noteworthy that the separation efficiency was much higher in the experiment performed at 35 °C (351.5). This can be due to the higher temperature with respect to 25 °C or to the lower density with respect to the other two tests.

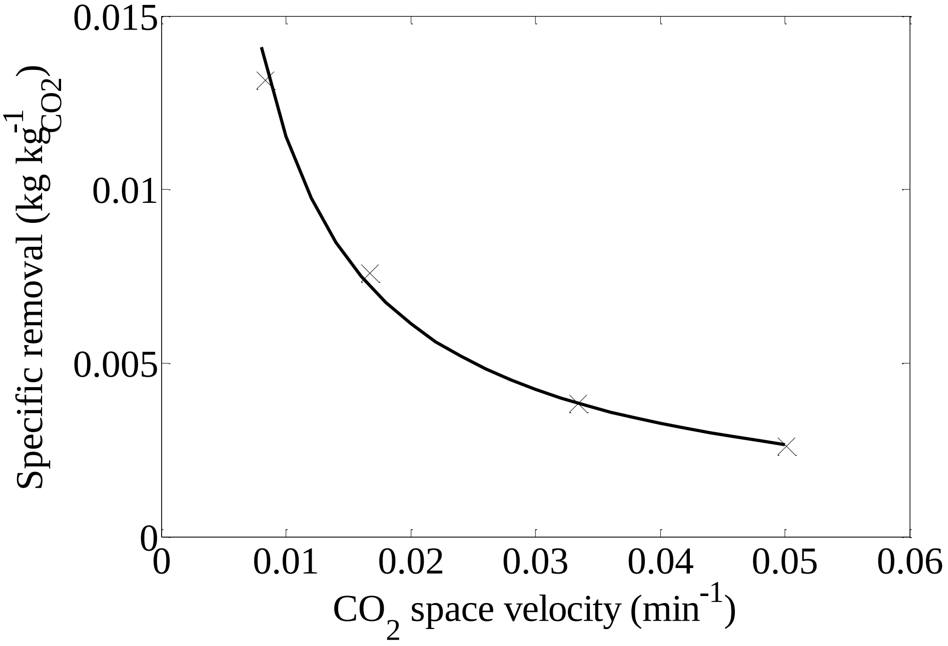

Figure 7.3. Effect of CO_2 space velocity on specific removal (kg$_{butanol}$ kg$_{CO2}^{-1}$) at constant pressure (10.34 MPa) and temperature (25 °C).

With regards to the total removal of butanol from the feed solution at the end of the continuous cycle, values in the range 92.3-96.9 % were obtained. At the highest temperature and pressure, 50 °C and 25.16 MPa, the removal value was slightly higher. The highest concentration of butanol in the extract was 787.5 g L^{-1}, obtained at 35°C and 10.34 MPa.

Based on these results, it can be concluded that supercritical CO_2 at 35°C and 10.34 MPa is more efficient to recover butanol than liquid CO_2. Increasing pressure and temperature up to 50 °C and 25.16 MPa, the total removal is slightly increased, but the separation efficiency is much lower than at 35°C and 10.34 MPa.

Table 7.2. Separation efficiency, removal rate (kg h^{-1}), concentration in extract (g L^{-1}) and total removal (%) using liquid CO_2 (25 °C and 10.34 MPa) and supercritical CO_2 (50 °C and 25.16 MPa).

Temperature (°C)	Pressure (MPa)	Separation efficiency	Removal rate (kg h^{-1})	Butanol concentration in extract (g L^{-1})	Total removal (%)
25	10.34	123.49±34.34	0.0011±0.0002	571.15±42.21	93.01±0.79
35	10.34	351.48±5.74	0.0011±2.04×10^{-5}	787.50±3.54	92.33±0.71
50	25.16	119.15±6.09	0.0012 ±4.16×10^{-5}	552.4±17.11	96.90±0.90

Values are mean±S.D of duplicate experiments.

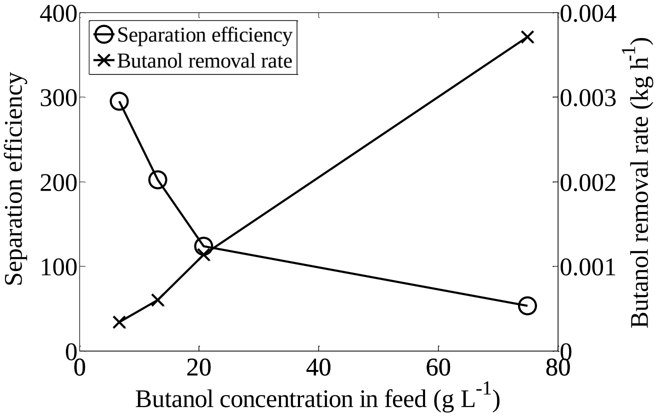

Figure 7.4. Influence of butanol concentration in feed (g L^{-1}) on separation efficiency and removal rate of butanol (kg h^{-1}). Experiments were performed at 25 °C and 10.34 MPa.

7.3.3. Butanol concentration

The concentration of butanol in the fermentation broth of batch reactors can vary from 12-20 g L^{-1} depending on microbial strain and fermentation conditions [26–29]. In the effluents of immobilized cell continuous reactors [30] and integrated reactors where product is recovered simultaneously [31] it can be lower than 12 gL^{-1}. In a recent work [31], the concentration of biobutanol produced from corn stover varied from 3.2 to 9.1 g L^{-1} when using different conditions. Hence, it is important to study a wide range of butanol concentrations in the feed solution and their influence on butanol recovery. For this purpose, experiments with butanol concentrations in the range 6.7-74.9 g L^{-1} were performed. Although 74.9 g L^{-1} butanol concentration is never reached in butanol bioreactors, this concentration is often obtained in the recovered aqueous phase in the product recovery experiments. Butanol separation from this aqueous phase is essential and it results in high product recovery rates. Recycle of this phase has been discussed below. In all the runs, the operating parameters were 10.34 MPa, 25 °C, 2.5 mL min-1 of CO2 and 1 mL min-1 of feed solution.

Results, represented in Figure 7.4, show that the lower the butanol concentration in the feed solution, the higher the separation efficiency. This result suggests that counter current CO2 fractionation would be especially effective when low concentrations of butanol are obtained in the fermentation broth. As predicted, the removal rate increased at higher concentrations of butanol.

As shown in Table 7.3, starting from a solution of 74.9 g L^{-1}, a concentration of 712.5 g L^{-1} of butanol was obtained in the extract, removing 85.6% of the butanol present in the feed solution. However, the butanol concentration in the extract and the total removal rate did not follow a clear trend.

Table 7.3. Butanol concentration in extract (g L^{-1}) and total removal (%) at different concentrations of butanol in the feed solution.

Butanol concentration in feed (g L^{-1})	Butanol concentration in extract (g L^{-1})	Total removal (%)
6.7±0.6	526.99±58.28	84.46±2.38
13.1±0.7	609.2±48.01	77.15±1.50
20.8±1.1	571.15±42.21	93.01±0.79
74.9±5.3	712.5±80.26	85.63±4.93

Values are mean±S.D of duplicate experiments.

7.3.4. Comparison with equilibrium data

The values of butanol selectivity calculated from the results presented above and the phase equilibrium data of the system CO_2-water-butanol reported in literature [32] are illustrated in Figure 7.5. According to the data reported by these authors, our column is clearly performing much better than a single equilibrium stage. In other words, the number of ideal separation stages provided by the column is consistently higher.

7.3.5. Butanol, acetone and ethanol

As mentioned above, acetone and ethanol are also formed in fermentation broth when biobutanol is produced. In the work published by Qureshi *et al.* [31], concentrations of acetone in a range 1.90-11.58 g L^{-1} and concentrations of ethanol from 0.24 to 1.11 g L^{-1} are reported. The aim of the study presented in this section was to find out if the major proportion of these compounds would be separated from butanol or would be obtained in the extract. The experiments were carried out at 10.34 MPa, 35 °C, 2.5 mL min^{-1} of CO_2 and 1 mL min^{-1} of feed rate.

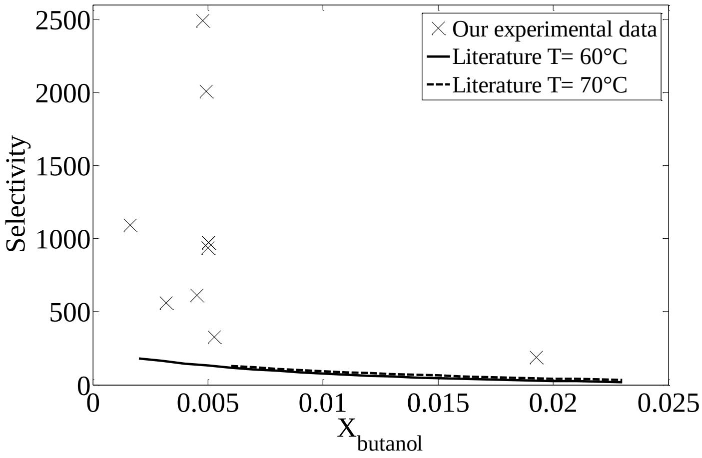

Figure 7.5. System CO_2-water-butanol: selectivity of butanol calculated from reported data (60 and 70°C) and comparison with the selectivity calculated from our experimental results.

.

Table 7.4. Concentration of butanol, acetone and ethanol (g L^{-1}) in the feed, raffinate and extract samples collected after the continuous cycle at 35 °C and 10.34 MPa.

Concentration in feed (g L^{-1})			Concentration in raffinate (g L^{-1})			Concentration in extract (g L^{-1})		
Butanol	Acetone	Ethanol	Butanol	Acetone	Ethanol	Butanol	Acetone	Ethanol
19.83±2.83	-	-	1.49±0.12	-	-	787.50±3.54	-	-
20.09±1.48	6.37±1.37	-	1.74±0.15	1.14±0.11	-	740.73±10.47	24.01±1.34	-
19.34±0.99	4.71±0.38	1.01±0.07	1.61±0.08	0.57±0.04	0.75±0.01	829±85	69.09±0.81	0.00±0.00

Values are mean±S.D of duplicate experiments.

Table 7.4 shows the concentrations of the three compounds measured in the feed, raffinate and extract. In the experiment starting from the mixture of butanol and acetone, these two compounds were mostly collected in the extract flask, at concentrations of 740.7 and 24.0 g L-1, respectively. Only 1.74 g L-1 of butanol and 1.14 g L-1 of acetone were found in the raffinate However, when ethanol was added to the feed solution, traces of it were measured in the extract.

It should be noted that, in all the runs performed in this study, the extract collected presented two phases, the aqueous and the organic ones (butanol). Prior to the analysis, the solution was stirred until the two phases were mixed in order to find out the total concentration of butanol in the extract without any loss of accuracy. Consequently, in large scale recovery units the organic phase (top layer) would be easily decanted off or separated. This phase contains small amount of water and can be dehydrated by removing it using molecular sieves such as silicalite. The aqueous phase contains approximately 78 g L^{-1} butanol which can be recycled to the separation unit for further concentration.

There are a number of reactor types which can be used to produce butanol including: i) free or suspended cell batch reactors [26]; ii) immobilized cell continuous reactors [30,33]; and iii) membrane cell recycle reactors [34]. In suspended cell batch reactors butanol productivities of the order of 0.50 g L^{-1} h^{-1} or less are achieved [29]**.** Application of CO_2 extraction to remove butanol from these reactors would require much smaller recovery units as removal rates of butanol that were observed in the present studies were of the order of 8-9 g L^{-1} h^{-1}. The reactor productivities in most immobilized cell continuous reactors and membrane cell recycle reactors are of the order of 6.5 g L^{-1} h^{-1} [30,33]**.** These high productivity reactors can also be integrated with the CO_2 extraction process simply due to high rates of butanol removal. In some cases reactor productivities over 15 g L^{-1} h^{-1} have been reported [35], however, these can also be integrated with butanol recovery by CO_2 extraction. In such a case, the overall capital and operational costs would still be lower as compared to the systems which offer low productivities.

As mentioned in the introduction section of this article, the methods that have been studied for butanol removal from fermentation broths include adsorption, gas stripping, liquid-liquid extraction, vacuum fermentation, and pervaporation. Among these methods vacuum fermentation and pervaporation appear to be promising. However, in the present studies on CO_2 extraction, superior butanol separation efficiencies (351.5) than pervaporation (209; [36]) have been achieved. Also, the butanol concentration in the range of 787.5-829 g L^{-1} have been obtained while using CO_2 extraction process. These values are greater than the butanol concentration obtained employing pervaporation. Additionally, pervaporation requires membranes which are costly and have limited working life. With these advantages, it is clear that CO_2 extraction process can be applied to effectively recover butanol or ABE from fermentation broth.

7.4. Final remarks

In this work, the recovery of butanol from aqueous solutions by counter-current CO_2 fractionation was studied. Results show that the effect of solvent-to-feed ratio on separation efficiency and removal rate is significant, obtaining the highest separation efficiency at 2.5. The specific removal ($kg_{butanol}\ kg_{CO2}^{-1}$) decreased at increasing the CO_2 space velocity. When studying the effect of pressure and temperature, the highest separation efficiency was obtained at 35 °C and 10.34 MPa, with a butanol concentration of 787.5 g L^{-1} in the extract. At these operating conditions, 92.3 % of the butanol present in the feed solution was removed. Different concentrations of butanol in the feed solution were tested, concluding that the higher the concentration of butanol in the feed solution, the higher the removal rate but the lower the separation efficiency. The comparison of phase equilibrium data of the system CO_2-water-butanol with the experimental data presented in this work showed that our column provides a consistent number of ideal separation stages. Experiments with the other compounds typically obtained in the ABE process were also performed. Ethanol was collected in the raffinate whereas the highest concentration of acetone was obtained in the butanol-rich fraction (the extract). The results obtained in this study form the basis to consider counter-current CO_2 as an alternative method to recover butanol from butanol or acetone-butanol-ethanol (ABE) fermentation broths.

References

[1] V.I. Águeda, J. A. Delgado, M. A. Uguina, J.L. Sotelo, Á. García, Column dynamics of an adsorption–drying–desorption process for butanol recovery from aqueous solutions with silicalite pellets, Sep. Purif. Technol. 104 (2013) 307–321. doi:10.1016/j.seppur.2012.11.036.

[2] pikeresearch, www.pikeresearch.%20com/research/biofuels-markets-and-technologies

[3] D.L. Carvalho, R.R. De Avillez, M.T. Rodrigues, L.E.P. Borges, L.G. Appel, Mg and Al mixed oxides and the synthesis of n-butanol from ethanol, Appl. Catal. A Gen. 415-416 (2012) 96–100. doi:10.1016/j.apcata.2011.12.009.

[4] E.M. Green, Fermentative production of butanol--the industrial perspective., Curr. Opin. Biotechnol. 22 (2011) 337–43. doi:10.1016/j.copbio.2011.02.004.

[5] D. Anton, I. Dobson, DuPont and BP biobutanol update, Ind. Bioprocess. 30 (2008) 7.

[6] S. Balasubramanian, J.D. Allen, A. Kanitkar, D. Boldor, Oil extraction from Scenedesmus obliquus using a continuous microwave system--design, optimization, and quality characterization., Bioresour. Technol. 102 (2011) 3396–403. doi:10.1016/j.biortech.2010.09.119.

[7] P. Dürre, Biobutanol: An attractive biofuel, Biotechnol. J. 2 (2007) 1525–1534. doi:10.1002/biot.200700168.

[8] V. García, J. Päkkilä, H. Ojamo, E. Muurinen, R.L. Keiski, Challenges in biobutanol production: How to improve the efficiency?, Renew. Sustain. Energy Rev. 15 (2011) 964–980. doi:10.1016/j.rser.2010.11.008.

[9] W.L. Luyben, Control of the Heterogeneous Azeotropic n -Butanol/Water Distillation System, Energy & Fuels. 22 (2008) 4249–4258. doi:10.1021/ef8004064.

[10] N. Qureshi, M. A. Cotta, B.C. Saha, Bioconversion of barley straw and corn stover to butanol (a biofuel) in integrated fermentation and simultaneous product recovery bioreactors, Food Bioprod. Process. 92 (2014) 298–308. doi:10.1016/j.fbp.2013.11.005.

[11] K. Kraemer, A. Harwardt, R. Bronneberg, W. Marquardt, Separation of butanol from acetone-butanol- ethanol fermentation by a hybrid extraction- distillation process, Comput. Chem. Eng. 35 (2010) 949–963. doi:10.1016/j.compchemeng.2011.01.028.

[12] V. Saravanan, D. A. Waijers, M. Ziari, M. A. Noordermeer, Recovery of 1-butanol from aqueous solutions using zeolite ZSM-5 with a high Si/Al ratio; suitability of a column process for industrial applications, Biochem. Eng. J. 49 (2010) 33–39. doi:10.1016/j.bej.2009.11.008.

[13] E. A. Fouad, X. Feng, Pervaporative separation of n-butanol from dilute aqueous solutions using silicalite-filled poly(dimethyl siloxane) membranes, J. Memb. Sci. 339 (2009) 120–125. doi:10.1016/j.memsci.2009.04.038.

[14] F. Liu, L. Liu, X. Feng, Separation of acetone–butanol–ethanol (ABE) from dilute aqueous solutions by pervaporation, Sep. Purif. Technol. 42 (2005) 273–282. doi:10.1016/j.seppur.2004.08.005.

[15] J. Huang, M.M. Meagher, Pervaporative recovery of n-butanol from aqueous solutions and ABE fermentation broth using thin-film silicalite-filled silicone composite membranes, J. Memb. Sci. 192 (2001) 231–242. doi:10.1016/S0376-7388(01)00507-5.

[16] N. Qureshi, M.M. Meagher, J. Huang, R.W. Hutkins, Acetone butanol ethanol (ABE) recovery by pervaporation using silicalite-silicone composite membrane from fed-batch

reactor of Clostridium acetobutylicum, J. Memb. Sci. 187 (2001) 93–102. doi:10.1016/S0376-7388(00)00667-0.

[17] A. G. Fadeev, Y. A. Selinskaya, S.S. Kelley, M.M. Meagher, E.G. Litvinova, V.S. Khotimsky, et al., Extraction of butanol from aqueous solutions by pervaporation through poly(1-trimethylsilyl-1-propyne), J. Memb. Sci. 186 (2001) 205–217. doi:10.1016/S0376-7388(00)00683-9.

[18] E. A. Fouad, X. Feng, Use of pervaporation to separate butanol from dilute aqueous solutions: Effects of operating conditions and concentration polarization, J. Memb. Sci. 323 (2008) 428–435. doi:10.1016/j.memsci.2008.06.054.

[19] A.G. Fadeev, S.S. Kelley, J.D. McMillan, Y.A. Selinskaya, V.S. Khotimsky, V. V Volkov, Effect of yeast fermentation by-products on poly[1-(trimethylsilyl)-1-propyne] pervaporative performance, J. Memb. Sci. 214 (2003) 229–238. doi:Doi 10.1016/S0376-7388(02)00550-1.

[20] N. Qureshi, Integrated Bioprocessing and simultaneous product recovery for butanol production, in: A.V. N. Qureshi, D. Hodge (Ed.), Biorefineries Integr. Biochem. Process. Liq. Biofuels, Elsevier, Amsterdam, Netherlands, 2014: pp. 595–603.

[21] A. Laitinen, J. Kaunisto, Supercritical fluid extraction of 1-butanol from aqueous solutions, J. Supercrit. Fluids. 15 (1999) 245–252. doi:10.1016/S0896-8446(99)00011-X.

[22] G. Brunner, Counter-current separations, J. Supercrit. Fluids. 47 (2009) 574–582. doi:10.1016/j.supflu.2008.09.022.

[23] F.J. Eller, S.L. Taylor, J. A. Laszlo, D.L. Compton, J. A. Teel, Counter-Current Carbon Dioxide Purification of Partially Deacylated Sunflower Oil, J. Am. Oil Chem. Soc. 86 (2008) 277–282. doi:10.1007/s11746-008-1340-9.

[24] N.T. Dunford, J. a. Teel, J.W. King, A continuous countercurrent supercritical fluid deacidification process for phytosterol ester fortification in rice bran oil, Food Res. Int. 36 (2003) 175–181. doi:10.1016/S0963-9969(02)00134-5.

[25] T.C. Ezeji, N. Qureshi, H.P. Blaschek, Production of acetone, butanol and ethanol by Clostridium beijerinckii BA101 and in situ recovery by gas stripping, World J. Microbiol. Biotechnol. 19 (2003) 595–603. doi:10.1023/A:1025103011923.

[26] B.M. Ennis, I.S. Maddox, Use ofClostridium acetobutylicum P262 for production of solvents from whey permeate, Biotechnol. Lett. 7 (1985) 601–606. doi:10.1007/BF01026457.

[27] C. Frick, K. Schügerl, Continuous acetone-butanol production with free and immobilized Clostridium acetobutylicum, Appl. Microbiol. Biotechnol. 25 (1986) 186–193. doi:10.1007/BF00253646.

[28] I.S. Maddox, The acetone-butanol-ethanol fermentation: recent progress in technology., Biotechnol. Genet. Eng. Rev. 7 (1989) 189–220. doi:10.1080/02648725.1989.10647859.

[29] T.C. Ezeji, N. Qureshi, S. Liu, Cellulosic butanol production from agricultural biomass and residues: Recent advances in technology, in: J.W. Lee (Ed.), Adv. Biofuels Bioprod., Springer S, New York, 2013: pp. 247–265.

[30] N. Qureshi, I.S. Maddox, Reactor design for the ABE fermentation using cells of Clostridium acetobutylicum immobilized by adsorption onto bonechar, Bioprocess Eng. 3 (1988) 69–72. doi:10.1007/BF00369330.

[31] N. Qureshi, V. Singh, S. Liu, T.C. Ezeji, B.C. Saha, M. a. Cotta, Process integration for simultaneous saccharification, fermentation, and recovery (SSFR): Production of butanol from corn stover using Clostridium beijerinckii P260, Bioresour. Technol. 154 (2014) 222–228. doi:10.1016/j.biortech.2013.11.080.

[32] H. Chen, H. Chang, P. Chen, High-Pressure Phase Equilibria of Carbon Dioxide + 1-Butanol , and Carbon Dioxide + Water + 1-Butanol Systems, (2002) 776–780.

[33] W. Huang, D. Ramey, S. Yang, Continuous Production of Butanol by Clostridium acetobutylicum Immobilized in a Fibrous Bed Bioreactor, Appl. Biochem. Biotechnol. 113 (2004) 887–898. doi:10.1385/ABAB:115:1-3:0887.

[34] A.S. Afschar, H. Biebl, K. Schaller, K. Schiigerl, Production of acetone and butanol by Clostridium acetobutylicum in continuous culture with cell recycle, Appl. Microbiol. Biotechnol. 22 (1985) 394–398. doi:10.1007/BF00252779.

[35] N. Qureshi, J. Schripsema, J. Lienhardt, H.P. Blaschek, Continuous solvent production by Clostridium beijerinckii BA101 immobilized by adsorption onto brick, World J. Microbiol. Biotechnol. 16 (2000) 377–382. doi:10.1023/A:1008984509404.

[36] N. Qureshi, M.. Meagher, R.. Hutkins, Recovery of butanol from model solutions and fermentation broth using a silicalite/silicone membrane1This paper is published as paper no. 12061, Journal Series, Nebraska Agricultural Experiment Station, Lincoln, NE 68583-0704.1, J. Memb. Sci. 158 (1999) 115–125. doi:10.1016/S0376-7388(99)00010-1.

CHAPTER 8

Extraction of fat from soy skim by counter-current carbon dioxide fractionation[7]

This chapter aims to investigate the use of counter-current carbon dioxide method as a means to reduce residual fat in soy skim after the enzyme-assisted aqueous extraction of soybeans. Extractions with liquid CO_2 at 25 °C and 10.34 MPa and supercritical CO_2 at 50 °C and 25.17 MPa are compared. The effects of solvent-to-feed ratio, addition of ethanol as modifier and introduction of packing in the column are also analysed. Results show that the highest reduction of fat content is obtained without modifier and with packing in the column. At these conditions, the total fat content present in soy skim was reduced from 4.4 to 0.7 %, with the protein content practically unaffected. ANOVA was performed to determine effects on fat and protein in soy skim. The fatty acid profile was also analysed, with C18:2 being the predominant fatty acid present in all the soy skim samples. Gel electrophoresis indicated that supercritical CO_2 settings did not affect protein structure; however, higher pressure, temperature, and CO_2 flow significantly increased protein solubility within pH 2 to 10 compared with untreated samples.

8.1. Introduction

Soybean oil is typically produced by direct solvent extraction with a petroleum distillate containing about two-thirds *n*-hexane [1]. However, the increasing concern about safety and environmental emissions produced by organic solvents makes it essential to search for other extraction and separation techniques. Enzyme-assisted aqueous extraction is one of the potential alternatives that is increasingly considered for processing soybean oil. This method uses water and enzymes to recover free and emulsified oil [2,3], based on the insolubility of oil in water instead of

---

[7] *Part of this chapter has been sent to The Journal of Supercritical Fluids as: Solana, M., Teel, J., Hojilla, M., Bertucco, A.,Eller, F., Counter-current carbon dioxide extraction of fat from soy skim.*

the dissolution of oil [4]. The advantage of enzyme-assisted aqueous extraction, in addition to safety and environmental benefits, is that oil and protein are extracted simultaneously [1,5].

Additionally, the oil requires less refining because of its low content of phospholipids [3,4] and also the protein damage is less [1]. Moreover, the capital investment of enzyme-assisted extraction is lower with respect to conventional solvent extraction [1].

The enzyme-assisted extraction method is typically performed in two steps. First, oil and protein are extracted from the high-fiber solids. Then, the extraction mixture is centrifuged to produce oil-rich fraction (free oil and cream emulsion), oil- and protein-lean spent solids, and a protein- and sugar-rich aqueous phase (skim) [6]. The skim is a valuable co-product that is not produced in other extraction processes [5]. A typical soy skim contains about 11% dry matter, of which 56–60% is partially hydrolyzed proteins and a small amount is oil [1,2]. The presence of oil and the nature of the hydrolysis not only make it difficult to purify the skim protein, but also cause a substantial loss of oil product. Indeed, process economic analysis demonstrated that creating value from the skim fraction is decisive to the economic feasibility of enzyme-assisted extraction processes of soybeans [5]. So far, the simplest and most economical method to purify soy proteins is isoelectric precipitation. Nevertheless, the oil has a high binding capacity of soybean protein, which limits purity that can be achieved [5].

The alternative method to purify the soybean proteins that we propose in this work involves separating the oil from the skim fraction through counter-current CO_2 extraction. Previous research demonstrated that CO_2 can effectively solubilize soybean oil [7]. CO_2 is an ideal solvent to be used for food applications since is inert and non-toxic, and no high temperatures that could damage thermo-labile compounds are needed. Besides, CO_2 counter-current fractionation is an environmentally friendly process in which CO_2 can be safely recycled after product separation.

Some applications of CO_2 counter-current separation method applied to oils that have been reported include purification of raffinate rice oil from rice bran [8], fractionation of fish oils [9], extraction of olive oil [10] and fractionation of lemon oil [11]. The advantages of using counter-current mode are the reduction of solvent consumption, increased throughput, and higher oil extract concentrations in the solvent and lower residual concentration in the raffinate [12]. In addition, counter-current fractionation of a feed mixture can be implemented in a continuous mode [13].

In this work, the influence of the main operating parameters affecting the extraction of fat from soy skim by counter-current CO_2 separation was investigated. The amounts, electrophoretic

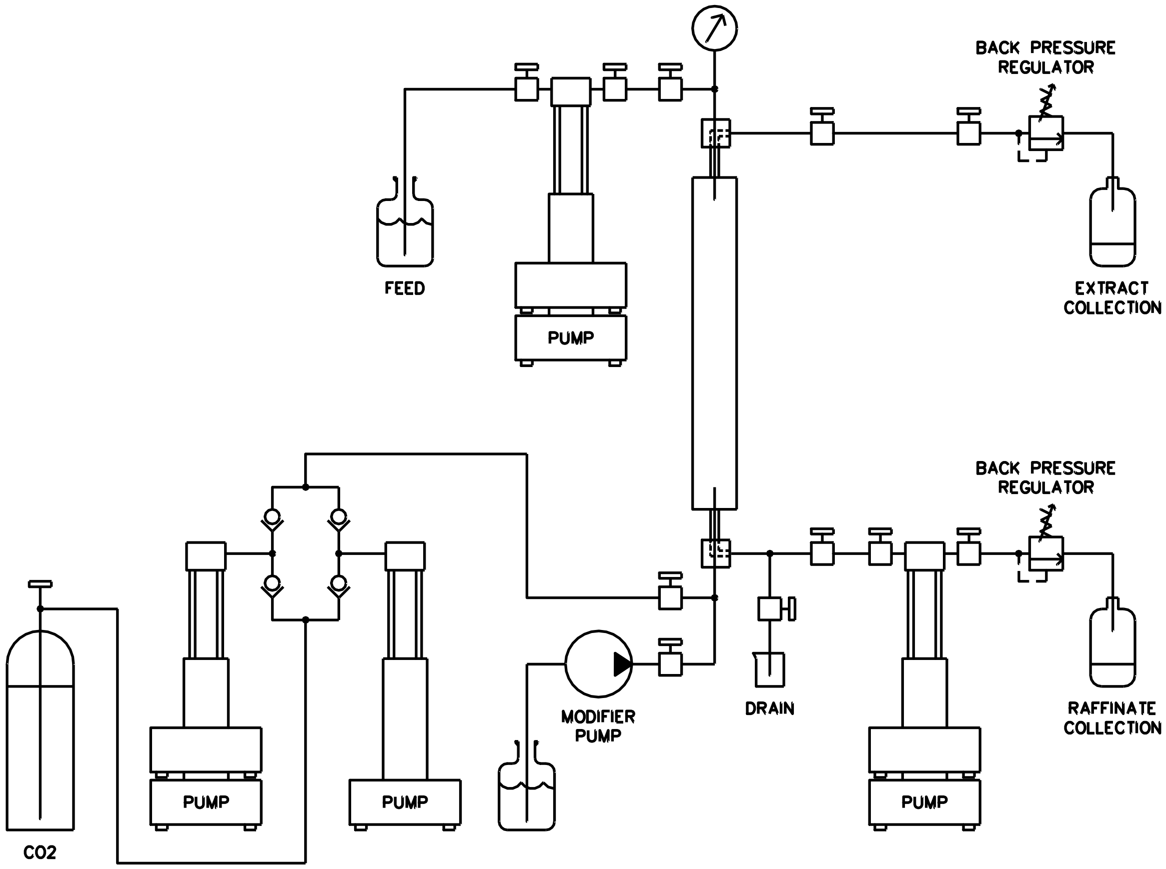

Figure 8.1. Schematic of CO_2 counter-current extraction apparatus.

patterns, and solubility behaviours of proteins in the soy skim samples before and after the extraction were determined, in order to detect any alterations by the CO_2 treatment. The results reported herein would support the development of an environmental and safe process to obtain and separate the oil and the proteins from soybean and soy skim.

8.2. Materials and methods

8.2.1. Materials

Soy skim was provided by Center for Crops Utilization Research, Iowa State University, Ames, IA. Carbon dioxide was provided by ILL-MO products Co. (Jacksonville, IL). Absolute ethanol was supplied by Fisher scientific (Fair Lawn, NJ).

C11 TAG, chloroform, hydrochloric acid, diethyl eter, hexane, toluene, methanol, BF3-in-methanol, sodium hydroxide, sodium sulphate were supplied by Fisher scientific (Fair Lawn, NJ, USA).

8.2.2. Counter-current extraction apparatus

The basic design of the counter-current CO_2 plant has been previously published [14,15]. The stainless steel column is 1.83 m long and 10.2 cm of outer diameter. The original system was modified to include ethanol supply for this study. The gas booster pump was substituted with a syringe pump. Figure 8.1 shows the scheme of the complete apparatus. In some experiments, the column was packed with seventy-six vertically-stacked packing pieces, 316-stainless steel Pall Ring, of 0.016 m (AMACS Process Tower Internals. Houston, TX).

The operating procedure was as follows: the system was pressurized up to the experimental pressure and the column was heated up to the temperature of the run. When a steady temperature and pressure were reached, the pumping of the feed solution and the extract collection were started. In the experiments in which ethanol was used as modifier, the ethanol pump was also activated at the same time. CO_2 entered from the bottom of the column and the soy skim solution was delivered from the top of the column, so as to allow counter current contact of CO_2 with the feed solution. The raffinate was accumulated in the reservoir pump. Continuous feed solution flow was maintained for 200 minutes, whereas only CO_2 was pumped during 100 minutes more. One sample of the extract and one of the raffinate were collected every 100 minutes.

Runs at 10.34 and 25.16 MPa were performed, and temperatures of 25 and 50 °C were tested. Considering that CO_2 critical temperature and pressure are 31.1°C and 7.39 MPa, respectively, for the experiments performed at 25°C CO_2 was at a liquid state, whereas for the tests at 50 °C it was at supercritical conditions. CO_2 flow rate was varied from 2.5 to 10 mL min^{-1}. The feed solution was pumped at 1 mL min^{-1} in all the runs. Tests with and without modifier (ethanol) were carried out. For a number of experiments, the packing was introduced in the column.

8.2.3. GC-FAME analysis

The soy skim samples were hydrolysed, transesterified and fatty acids profile determined -as described by House *et al*. [16].

8.2.4. Moisture, crude fat, and crude protein contents

Moisture, crude fat, and crude protein contents were determined by following AOCS standard methods Ba 2a-38, Am 5-04 and Ba 4e-93, respectively (AOCS, 2009). Samples were weighed before and after drying for 3 hours at 103 °C to determine moisture content. Dried samples were then extracted with petroleum ether at 90 °C for 60 min in an Ankom XT15 Fat Extractor (ANKOM Technology, Macedon, New York).. Samples weighing 0.2 g were analyzed for nitrogen content using a Leco FP-528 Dumas-Type Elemental Analyzer (LECO Corp., St. Joseph, Michigan). A conversion factor of 6.25 was used to calculate percent protein. Determinations were performed in triplicate.

8.2.5. Gel electrophoresis

SDS-PAGE was done according to the method of Hojilla-Evangelista et al. (2013). Reduced proteins from the defatted, freeze-dried soy skim samples were prepared to provide 4 mg protein/mL and then loaded (15 µL) onto a 4-12% pre-cast gradient gel. Protein standards with molecular weight range 6.5-200 kDa were also included in the gel. Native gel electrophoresis was done by following the procedure of Hojilla-Evangelista and Evangelista (2006). Sample concentrations were 4 mg protein/mL in 500 µL of commercial Tris-glycine native sample buffer (pH 6.8, Invitrogen Corp., Carlsbad, CA). Loading volume was 20 µL onto pre-cast Novex^{TM} Tris-glycine 8-16% gradient gel. Protein standards (20 to 1236 kDa) were included in the gel.

8.2.6. Protein solubility

Protein solubility curves were generated by following the method of Myers et al. (1994). Aqueous solutions containing 1% protein (dry basis) were stirred for 10 minutes and adjusted to pH 2, 4, 5.5, 7, 8.5, or 10 by addition of 1.0 M HCl or 1.0 M NaOH. Solutions were then centrifuged at 6000 rpm (4186 *x g*) for 20 minutes. The amount of protein in the supernatant was determined by the Biuret method at 540 nm using a Shimadzu UV-2600 UV/Vis Spectrophotometer. A standard curve was created from solutions of bovine albumin (Sigma A-3059). Determinations were done in duplicate.

8.2.7. Statistical analysis

The mean and the standard deviation were calculated on the basis of the four (fat content) and six (protein content) measures obtained from the two runs. Analysis of variance (ANOVA) of the results was performed using Statistix 7 software (Analytical Software, Tallahassee, FL, USA) and means were compared using least significant difference (LSD) test at P=0.05.

8.3. Results and discussion

The study of the influence of different parameters on the reduction of the fat content of soy skim is presented in this section. In particular, the effect of the solvent-to-feed ratio, CO_2 state, packing of the column and use of ethanol as modifier is discussed. Additionally, the proteins content of the samples is reported.

The fat and protein content of the soy skim solution without any treatment was measured in order to compare it with the solutions obtained as raffinate after the CO_2 treatment. In the following, this sample will be called control sample.

Table 8.1. Influence of pressure, temperature, modifier and packing of the column on the soy skim fat content after the treatment and comparison with the control sample.

Pressure (MPa)	Temperature (°C)	Modifier	Packing	Soy skim fat (%)
25.16	50	yes	no	1.83±0.39
25.16	50	no	no	1.09±0.01
10.34	25	yes	yes	3.29±0.09
10.34	25	no	yes	1.59±0.31
25.16	50	yes	yes	2.33±0.94
25.16	50	no	yes	0.83±0.17
Control sample				4.29±0.15

Values are mean±S.D of two measures of duplicate experiments.

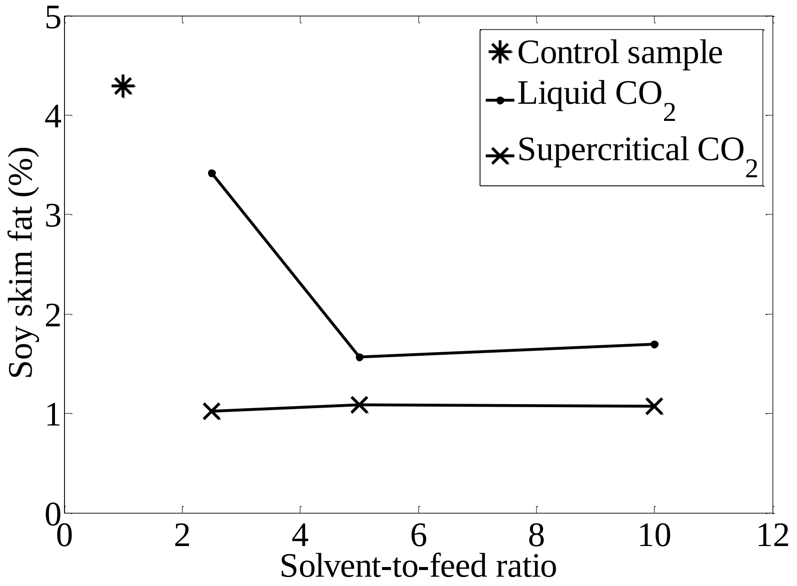

Figure 8.2. Effect of solvent-to-feed ratio on the fat extraction from soy skim using liquid and supercritical CO_2, and comparison with the control sample.

8.3.1. Effect of CO_2 state

The influence of CO_2 state on the extraction of fat from soy skim was studied keeping constant the density of CO_2 (0.842 g mL^{-1}). Results are shown in Table 8.1 and Figure 8.2. The operating conditions of liquid CO_2 were 10.34 MPa and 25 °C, whereas the tests with supercritical CO_2 were carried out at 25.16 MPa and 50 °C. CO_2 was pumped at 5 mL min^{-1} in all the runs.

ANOVA indicated that there is a significant effect of the CO_2 treatment on the extraction of fat from soy skim (P=0.0004), but no significant differences were found among the tests performed at different conditions of pressure and temperature. From Figure 8.2, it can be seen that, regardless of the CO_2 flow rate, the reduction of soy skim fat was slightly higher when supercritical CO_2 was used, however this difference was not statistically significant.

8.3.2. Effect of solvent-to-feed ratio

The average content of soy skim fat after the treatment as a function of the solvent-to-feed ratio is summarized in Figure 8.2, together with the fat content of the control sample. In all the runs, the feed solution flow rate was kept at 1 ml min^{-1}. Three different CO_2 flow rates were tested: 2.5, 5 and 10 ml min^{-1}. First, the effect of the solvent-to-feed ratio was studied at 10.34 MPa and 25 °C. Then, this effect was analyzed using CO_2 at 25.16 MPa and 50 °C. ANOVA indicated that there are significant effects of solvent-to-feed ratio on fat content after the CO_2 treatment (P=0.0005). The highest reduction of fat content (from 4.3 to 1.0 %) was achieved at a solvent-to-feed ratio of 2.5 with supercritical CO_2, as represented in Figure 8.2. However, at these conditions of pressure and temperature, the differences of fat content using diverse flow rates were negligible. As regards the tests with liquid solvent, the fat content decreased significantly when the solvent to feed ratio was increased from 2.5 to 5. It slightly decreased at increasing the CO_2 flow rate from 5 to 10 mL min^{-1}. In conclusion, the minimum solvent to feed ratio to achieve the highest reduction of soy skim fat is 5 with liquid CO_2, whereas the effect of solvent-to-feed ratio is not significant with supercritical CO_2.

8.3.3. Effect of the column packing

In an attempt to enhance mass transfer efficiency by improvement of flow conditions inside the column, stainless steel packing was placed in the fractionation column. Results are presented in Table 8.1. Experiments at liquid and supercritical CO_2 conditions were performed at a constant CO_2 flow rate of 5 mL min^{-1}.

ANOVA indicated that there are significant effects of adding the packing to the column on the fat content in the raffinate (P=0.0002). At 25.16 MPa and 50 °C without modifier, the soy skim fat was reduced from 1.09 to 0.83 % when the packing was introduced in the column, as shown in Table 8.1.

8.3.4. Effect of modifier

Ethanol in a proportion of 5 % (mass) with respect to a CO_2 flow rate of 5 ml min^{-1} was added as a modifier to CO_2 with the aim of increasing the polarity of the solvent and studying its effect on the extraction of fatty acids from soy skim. Tests at liquid and supercritical CO_2, with and without packing, are compared. A constant CO_2 flow rate of 5 mL min^{-1} was maintained in all the runs.

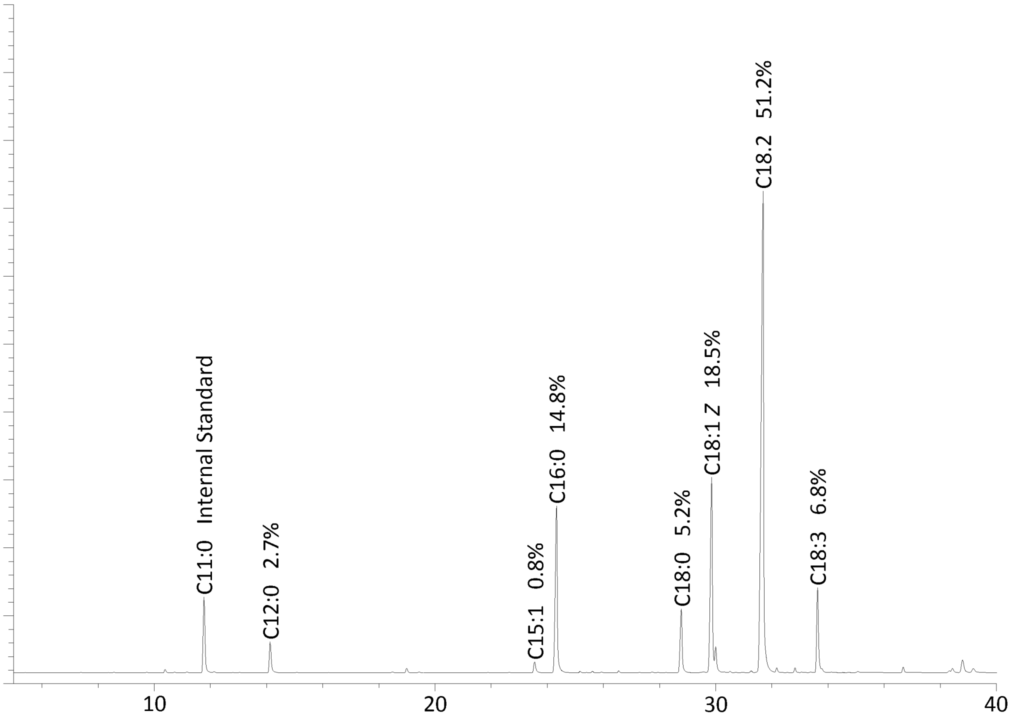

Figure 8.3. Gas chromatogram of fatty acid profile of soy skim.

ANOVA indicated that there are no significant effects of adding the modifier on the fat content in the raffinate, but there are differences with respect to the no treated sample (*P*=0.0018). As shown in Table 8.1, the addition of the modifier to the solvent resulted in a lower decrease of fat content, probably because of the apolar nature of the fatty acids present in the soy skim.

When both modifier and packing in the column were tested, the soy skim fat content in the sample increased from 1.09 to 2.33 %, as shown in Table 8.1. This can be due to the lower density of ethanol with respect to CO_2, that difficult the flow of solvent through the packing of the column or to the affinity at the ethanol to partition into the aqueous phase.

8.3.5. *Fatty acid composition*

The fatty acid composition of all the samples obtained before and after the treatment was analyzed. Results showed that counter-current CO_2 is not selective towards the different fatty acids, since the fatty acid composition did not vary significantly. A chromatogram of one of the samples is shown in Figure 8.3. It can be seen that soy skim is rich in C18:2 (51.2 %) fatty acid. The presence of C12:0 (2.7 %), C15:1 (0.8 %), C16:0 (14.8 %), C18:0 (5.2 %), C18:1 Z (18.5 %) and C18:3 (6.8 %) is also significant. This fatty acid profile is the typical one of soy bean oil [17].

Table 8.2. Protein content of soy skim before (control sample) and after the treatment at different operating conditions.

Test	Pressure (MPa)	Temperature (°C)	Solvent-to-feed ratio	Modifier	Packing	Protein content (%)
1	10.34	25	2.5	no	no	62.30±1.09
2	10.34	25	5	no	no	60.98±0.28
3	10.34	25	10	no	no	60.85±1.24
4	25.16	50	2.5	no	no	59.15±1.32
5	25.16	50	5	no	no	56.57±1.17
6	25.16	50	10	no	no	57.65±2.60
7	25.16	50	5	yes	no	62.78±1.93
8	10.34	25	5	no	yes	59.09±0.49
9	25.16	50	5	no	no	56.57±1.17
10	25.16	50	5	no	yes	57.28±1.51
11	10.34	25	5	yes	yes	56.43±0.61
12	25.16	50	5	yes	yes	58.33±1.00
Control sample						59.91±0.72

Values are mean±S.D of three measures of duplicate experiments.

8.3.6. *Effect of CO_2 treatment on the protein content*

The protein content of the samples collected after the treatments reported above is shown in Table 8.2. A significant F-test was obtained from ANOVA analysis for packing (*P*=0.0006). It seems the packing caused a slight decrease in protein, probably as a result of protein sticking to the packing inside the column. In any event, the decrease was relatively small. There were no differences in protein between the levels of the factors CO_2 state, solvent to feed ratio, or modifier.

Gel electrophoresis results (native and reduced gels) showed no differences in the protein band patterns, indicating that the treatments used did not have effect on protein structure.

8.3.7. *Protein solubility*

Figure 8.4 shows the protein solubility of the samples before and after the CO_2 treatment at different values of pH. It is noteworthy that soy skim contact with liquid CO_2 (tests 1, 2, 3) resulted in slightly higher protein solubilities at pH higher or equal to 7. Protein solubilities were similar to the control at pH values of 2, 4, and 5.5.

Soy skim contact with supercritical CO_2 increased protein solubility at all the values of pH, especially at a CO_2 flow rate of 10 mL min^{-1} (test 6). The effect of modifier or packing was not significant, in any case the protein solubility was higher than the control for all the values of pH.

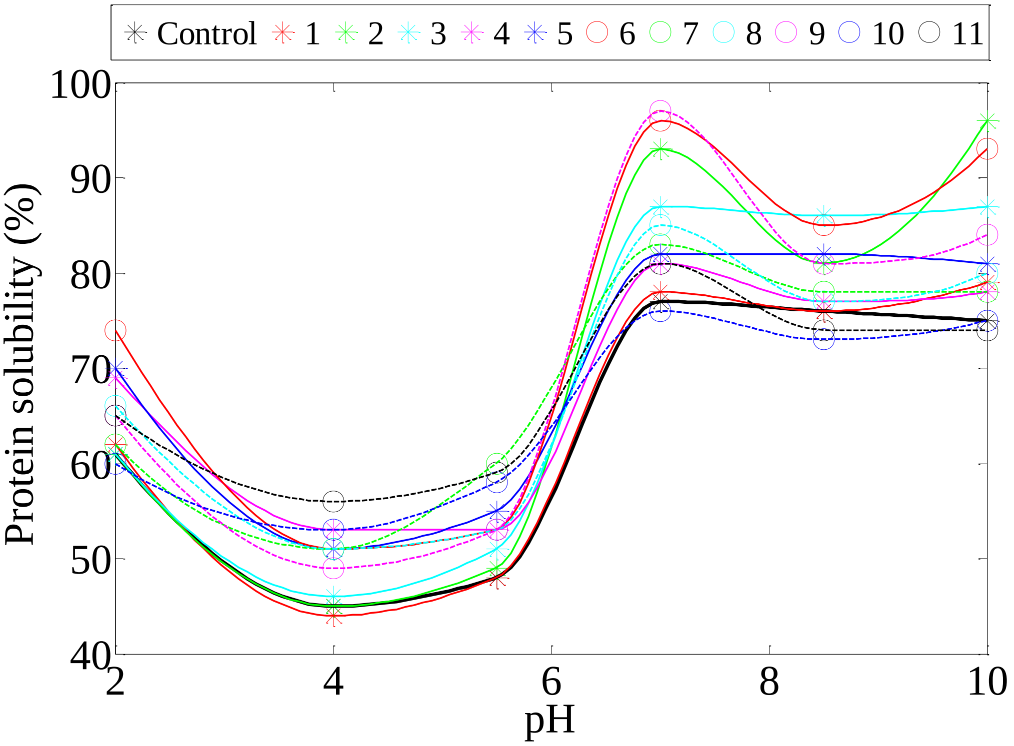

Figure 8.4. Protein solubility (%) of the samples before and after the CO_2 treatment at different values of pH. The numbers that are shown in the legend corresponds to the tests that are enumerated in Table 2, where the conditions of each experiment are specified.

8.4. Final remarks

Counter-current CO_2 extraction of fat from soy skim was studied and the effects of the main parameters were investigated. ANOVA analysis indicated that the effect of CO_2 state is not significant. The influence of solvent-to-feed rate in the range 2.5-10 was significant with liquid CO_2, however no relevant effect was found at supercritical conditions. The introduction of packing in the column improved the results, whereas using ethanol as a modifier resulted in a decrease in soy skim fat. After the extraction at the optimum conditions (25.16 MPa, 50 °C, with packing and without modifier) the fat content of soy skim was reduced from 4.4 to 0.8%. The fatty acids profile of soy skim oil was not significantly altered by the CO_2 treatment, being also the typical one of soy bean oil. A slightly decrease of the protein content was found in the experiments with packing. The protein levels of factors CO_2 state, solvent-to-feed ratio, or modifier were unaffected by CO_2 extraction. In conclusion, counter-current CO_2 extraction is an efficient method to reduce the fat content of soy skim without altering the protein content and could be considered as an alternative to the methods that are currently being applied in the industry.

References

[1] J.M.L.N. de Moura, K. Campbell, a. Mahfuz, S. Jung, C.E. Glatz, L. Johnson, Enzyme-Assisted Aqueous Extraction of Oil and Protein from Soybeans and Cream De-emulsification, J. Am. Oil Chem. Soc. 85 (2008) 985–995. doi:10.1007/s11746-008-1282-2.

[2] L. Yao, T. Wang, H. Wang, Effect of soy skim from soybean aqueous processing on the performance of corn ethanol fermentation, Bioresour. Technol. 102 (2011) 9199–9205. doi:10.1016/j.biortech.2011.06.071.

[3] S. Jung, D. Maurer, L. a. Johnson, Factors affecting emulsion stability and quality of oil recovered from enzyme-assisted aqueous extraction of soybeans, Bioresour. Technol. 100 (2009) 5340–5347. doi:10.1016/j.biortech.2009.03.087.

[4] A. Rosenthal, D.L. Pyle, K. Niranjan, Aqueous and enzymatic processes for edible oil extraction, Enzyme Microb. Technol. 19 (1996) 402–420. doi:10.1016/S0141-0229(96)80004-F.

[5] K. a. Campbell, C.E. Glatz, L. a. Johnson, S. Jung, J.M.N. De Moura, V. Kapchie, et al., Advances in aqueous extraction processing of soybeans, JAOCS, J. Am. Oil Chem. Soc. 88 (2011) 449–465. doi:10.1007/s11746-010-1724-5.

[6] C.M. Cater, K.C. Rhee, R.D. Hagenmaier, K.F. Mattil, Aqueous extraction-An alternative oilseed milling process, J. Am. Oil Chem. Soc. 51 (1974) 137–141. doi:10.1007/BF02639723.

[7] L. Montanari, P. Fantozzi, J.M. Snyder, J.W. King, Selective extraction of phospholipids from soybeans with supercritical carbon dioxide and ethanol, J. Supercrit. Fluids. 14 (1999) 87–93. doi:10.1016/S0896-8446(98)00110-7.

[8] L. Danielski, G. Brunner, C. Schwänke, C. Zetzl, H. Hense, J.P.M. Donoso, Deterpenation of mandarin (Citrus reticulata) peel oils by means of countercurrent multistage extraction and adsorption/desorption with supercritical CO2, J. Supercrit. Fluids. 44 (2008) 315–324. doi:10.1016/j.supflu.2007.09.007.

[9] O.. Catchpole, J.. Grey, K.. Noermark, Fractionation of fish oils using supercritical CO2 and CO2+ethanol mixtures, J. Supercrit. Fluids. 19 (2000) 25–37. doi:10.1016/S0896-8446(00)00075-9.

[10] A.M. Hurtado-Benavides, F.J. Señoráns, E. Ibáñez, G. Reglero, Countercurrent packed column supercritical CO2 extraction of olive oil. Mass transfer evaluation, J. Supercrit. Fluids. 28 (2004) 29–35. doi:10.1016/S0896-8446(03)00004-4.

[11] F. Gironi, M. Maschietti, Supercritical carbon dioxide fractionation of lemon oil by means of a batch process with an external reflux, J. Supercrit. Fluids. 35 (2005) 227–234. doi:10.1016/j.supflu.2005.01.007.

[12] G. Brunner, Counter-current separations, J. Supercrit. Fluids. 47 (2009) 574–582. doi:10.1016/j.supflu.2008.09.022.

[13] C.A. Pieck, C. Crampon, F. Charton, E. Badens, Multi-scale experimental study and modeling of the supercritical fractionation process, J. Supercrit. Fluids. (2015). doi:10.1016/j.supflu.2015.01.021.

[14] N.T. Dunford, J. a. Teel, J.W. King, A continuous countercurrent supercritical fluid deacidification process for phytosterol ester fortification in rice bran oil, Food Res. Int. 36 (2003) 175–181. doi:10.1016/S0963-9969(02)00134-5.

[15] F.J. Eller, S.L. Taylor, J. a. Laszlo, D.L. Compton, J. a. Teel, Counter-Current Carbon Dioxide Purification of Partially Deacylated Sunflower Oil, J. Am. Oil Chem. Soc. 86 (2008) 277–282. doi:10.1007/s11746-008-1340-9.

[16] S.D. House, P.A. Larson, R.R. Johnson, J.W. DeVries, D.L. Martin, Gas chromatographic determination of total fat extracted from food samples using hydrolysis in the presence of antioxidant., J. AOAC Int. 77 (1994) 960–965.

[17] C. Tsukamoto, S. Shimada, K. Igita, S. Kudou, M. Kokubun, K. Okubo, et al., Factors affecting isoflavone content in soybean seeds: Changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development, J. Agric. Food Chem. 43 (1995) 1184–92. doi:10.1021/jf00053a012.

Conclusions

Nowadays the potential of supercritical CO_2 technologies in the food industry is astonishing for a number of reasons: efficiency, selectivity and green quality, among others. However, the economic viability of these technologies is under discussion, as well as their efficiency when applied to new sources and the production of novel products.

This thesis has been focused on the study of three supercritical CO_2 technologies for the production of bioactive compounds from natural sources. Extraction, precipitation and counter-current fractionation processes have been successfully performed by SFE, SAS and a continuous packed column.

First, extraction from different species of microalgae, rocket salad and asparagus have been demonstrated to achieve high yields when using co-solvents, including mixtures of them. The effect of the operative conditions on the yield and composition of the extracts presented a different behavior when different co-solvents were tested. A model typically applied in supercritical fluid extraction, the broken and intact cells model of Sovová, has been successfully applied to the experimental data obtained from new sources and with the addition of co-solvents.

As regards microalgae essential fatty acids extraction, supercritical CO_2 and Soxhlet have shown to be comparable as far as the process yield and fatty acid composition of the extracts are concerned, whereas SFE is faster, more selective and does not require a toxic solvent. Studying pressure and temperature effects on the SFE yield and solubility, it was found that the cross-over phenomenon occurs at a pressure close to 25 MPa. Consequently, the maximum extraction yield was obtained at 30 MPa and 65 °C. Comparing FFA analysis of the extracted oils, it was found that the omega-3 content is negatively affected by high temperatures, high pressures and long extraction times.

The use of supercritical CO_2 for the extraction of new bioactive compounds has also been demonstrated. Particularly, an extract rich in glucosinolates from rocket salad have been obtained by supercritical CO_2 using water as co-solvent. Water resulted to be the most efficient co-solvent also for the extraction of phenolic compounds from rocket salad, whereas the fraction richest in lipids was extracted by $SCCO_2$+ethanol. According to these results, a sequential extraction scheme was proposed, in which the two extracts are produced by using first water and then ethanol as co-solvent.

Considering the experimental results obtained on the supercritical extraction of bioactive compounds from rocket salad, a large scale process has been developed for the production of natural extracts using water as co-solvent. A cost benefit analysis of the profitability of this process has been made showing that with an interest of 5% and 10 years of pay-back period, the net present value of the process would be positive if the selling price of the extract is higher than 21.9 US$ kg^{-1}, based on the current wholesale price of rocket salad. In the best scenario, namely using non-saleable salad, the price of the extract would be lower (17.0 US$ kg^{-1}). These results showed that the SFE extract price is competitive with the current price of natural extracts in the market.

The last study regarding supercritical fluid extraction concerned the comparison of three extraction methods (SFE, PLE and Soxhlet) to produce phenolic compounds from asparagus. It was concluded that the extraction of different phenolic compounds and the antioxidant activity of the extracts are affected by both the extraction method and the solvent used. As regards the SFE extracts, the highest phenolic content was obtained using a mixture of water-ethanol 1:1 as co-solvent. No significant differences were found on the phenolic composition of the extracts obtained at different pressures and temperatures, whereas longer extraction times favoured the extraction of phenolic acids.

Moreover, polyphenols and anthocyanins compounds from non-saleable cherries have been successfully precipitated using supercritical anti-solvent (SAS) process with CO_2. Results showed that continuous mode is more efficient than batch, and that polyphenols and anthocyanins yields of precipitation are favoured by lower pressures and higher compositions of CO_2.

Counter-current CO_2 fractionation plant was first tested. For that purpose, the recovery of butanol from aqueous solutions was performed. The comparison of phase equilibrium data of the system CO_2-water-butanol with the experimental data showed that our column provides a consistent number of ideal separation stages. The effect of the operating parameters, such as solvent-to-feed ratio, temperature, pressure and feed solution composition, were found to be significant on the separation efficiency.

Once counter-current fractionation plant had been tested, counter-current extraction of fat from soy skim was investigated for the first time. Results showed that counter-current CO_2 is an efficient method to reduce the fat content of soy skim without altering the protein content and the fatty acids profile. ANOVA analysis indicated that the effect of CO_2 state is not significant. The influence of solvent-to-feed rate was significant with liquid CO_2, however no relevant effect was found at supercritical conditions. The introduction of packing in the column improved the results, whereas using ethanol as a modifier resulted in a decrease in soy skim fat.

In summary, the future of production of valuable compounds from vegetable and food waste and separation of the compounds of interest using supercritical technologies is definitely promising, the possibilities to improve the results are numerous, and should be the subject of further investigations. It is specially important to continue the investigation on supercritical technologies for different samples in order to generate new data and probably obtain new products, that can be useful for the potential scale-up of the so newly proposed processes.

Acknowledgments

I would like to thank the people who have contributed in some way to the work described in this thesis, in particular:

My supervisor, Alberto Bertucco, for getting excited with each one of my insignificant ideas and works, it gave me the courage and patience to develop this thesis.

Fred Eller, for making me realize that science is not a boring and serious stuff, with his permanent laughs and distinct ideas.

My colleagues in Padova, specially Silvia, Alberto, and Paolo, for bearing with me and giving me all kind of advise.

My colleagues in Peoria, specially Jeff, Judy, Michael and Lynne, for their huge generosity and permanent smile.

Bob, Hector, Ale and Melanie, for making funnier my life in Peoria.

Silu, Saeed, Andrei, Purnendu and Argita, for being really such a family in this way of life called Cariparo.

Loredana, Giorgio and Marco, for opening the doors of their house and their heart since the first day we met.

Mama, papa, Yoli, Cayetano, Pablo and Maria José, for unconditionally encouraging and helping me to achieve my dreams.

Andrea, for his constant patience and unalterable good humour, being the best mate I could have ever found.

To all of you this thesis is dedicated.