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University of Coimbra
Faculty of Sciences and Technology
Chemical Engineering Department

Extraction of valuable compounds from agro-residues of elder (*Sambucus nigra*), pine (*Pinus pinaster*) and tara (*Caesalpinia spinosa*)

A Thesis submitted in partial fulfillment of the
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Inês José Abrantes de Oliveira Seabra

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Abstract

Agricultural processing inevitably goes along with the production of large amounts of agro-residues, which may represent a major waste disposal problem. Legislation regulating the management of waste materials has appeared throughout the European Union and has significantly contributed to the introduction of sustainable waste management procedures. The reuse and recycling of agro-residues has been highly encouraged and new technologies applying environmentally clean processes have been playing a central role within this context. These strategies may boost both the environmental and economic profiles of the implied industries, since they may create value in the entire chain-production pipeline.

Typically, a substantial part of the agro-residues produced during the handling and processing of fruits, vegetables and forest resources still comprises important amounts of the original plant materials, such as fruit skins, fruit seeds, flowers, leaves, stems, barks, and roots. High-value natural compounds can be found in most of these vegetable residues, many of them having health-promoting characteristics. It is the case of, for example, phenolic compounds (which have been associated to the alleged health-promoting effects related to the consumption of fruits and vegetables), since they are preferentially biosynthesised in the external vegetable tissues. Usually, and despite their significant potential value, these residues are often underexploited and thus their potential value as natural products is frequently lost. Efficient techniques can thus be explored for separation and isolation of oils, antioxidants, fragrances, colorants, biocides and other bioactive substances of natural origin. Therefore, with some additional and adequate processing steps, these materials can be easily transformed, from a residual low-value status into a very interesting high-value status, in terms of well-known and consumer high-value and accepted uses in the food, cosmetic and pharmaceutical industries, among others.

This Thesis is focused on the extraction of potentially valuable compounds from agro-residues of elder and maritime pine (Portuguese native plants) and also from tara (a Peruvian native plant). Solvents and techniques generally regarded as “environmental friendly” were applied. Despite conventional methods such as hydrodistillation and Soxhlet extraction had

been employed, special attention was given to high pressure extraction procedures, namely supercritical and pressurized solvent extractions using, respectively, carbon dioxide and mixtures of carbon dioxide, ethanol and water as solvents. In general terms and besides other particular aspects, the effect of several process variables/conditions on the yields and on the characteristics of the obtained extracts, such as composition and antioxidant and anti-inflammatory activities, was analyzed. Extract fractionation, solvent flow rate and composition, in addition to extraction time, temperature and pressure, were among these studied process variables/conditions. Characterization of extracts involved the use of diverse analytical techniques including spectrophotometric and chromatographic ones.

Elderberry pomace and elderflower were evaluated as sources of potentially useful natural compounds. Elderberry pomace is a juice-processing industry residue which comprises the fruit skins and seeds, and is known to be rich in anthocyanins and other phenolic compounds. It was subjected to high pressure extractions at 313 K and ~20 MPa, applying supercritical carbon dioxide in a first step and pressurized solvent mixtures of carbon dioxide, ethanol and water in a second step. Extract fractionation was achieved by such a methodology. While the first step led to extracts rich in lipids and other apolar substances, obtained second step extracts revealed high contents of anthocyanins and presented high antioxidant activities, which were strongly influenced by the employed solvent mixture composition. The results of this study highlight the potential of elderberry pomace as a source of valuable compounds, and may assist in the development of strategies for integral exploitation of elderberries, reducing the environmental impact of elderberry juice processing industries. Elderflower was, in its turn, subjected to supercritical carbon dioxide extraction. The effect of solvent density (300-900 kg/m^3) and of extraction temperature (308-328 K) on extracts characteristics was analyzed. Most of the obtained extracts revealed high contents of apolar compounds. Their phenolic compounds contents and the resulting antioxidant activities were relatively low, but they may possibly be increased if ethanol and water mixtures are added as cosolvents to carbon dioxide (which is planned to be performed in the near future). Nevertheless, obtained results revealed that elderflower extracts achieved by supercritical fluid technology may be added as natural aromatic additives to cosmetic and food systems.

Maritime pine bark is an abundant residue from pine wood processing industries which is rich in condensed tannins. This residue was subjected to conventional solid-liquid aqueous-based extractions as well as to supercritical and pressurized solvent extraction procedures in order to obtain efficient vegetable tanning agents and bioactive phenolic-based extracts, respectively. Conventional aqueous extractions were optimized by adding small amounts (up

to 15%, v/v) of ethanol. This methodology was selective for condensed tannins and the obtained extracts revealed adequate characteristics for leather tanning applications. Concerning supercritical fluid extraction experiments, carbon dioxide and a mixture of carbon dioxide and ethanol (10%) were used in two consecutive steps, varying extraction pressure in the range 10-30 MPa, and extraction temperature in the range 303-323 K. The employed fractionated extraction methodology showed that it was possible to obtain different extract fractions having diverse antioxidant activities from maritime pine bark. Moreover, it showed that the process could be further optimized through the usage of different solvent mixtures as well as other operational conditions. Therefore, pressurized solvent extractions were consecutively performed varying some process variables such as solvent mixture composition (carbon dioxide and ethanol, with up to 90% of ethanol) and flow rate. Extraction kinetics and extracts composition (in terms of phenolic compounds in general, and of condensed tannins in particular) were considerably affected by the solvent mixture composition. The results of these studies showed that both conventional and high pressure extraction methodologies may be feasible strategies for the valorization of maritime pine bark, therefore reducing the environmental impact and increasing the profit of the involved industries.

Finally, tara seed coat, which is a residue of tara powder and of tara gum processing industries, was evaluated as a source of phenolic compounds in general, and of hydrolysable tannins, in particular. Pressurized solvent extractions using homogeneous solvent mixtures of carbon dioxide, ethanol and water were performed at 313 K and 20 MPa. An experimental mixture design was applied in order to optimize the solvent mixture composition in terms of selectivity towards the above referred compounds, as well as in terms of antioxidant activities and lipoxygenase inhibition activities (indicative of their anti-inflammatory capacities) of the whole extracts. The achieved mixture models permitted to discern the contribution of the three individual solvents used: while water revealed to be the most effective solvent to obtain high extraction yields, ethanol-rich mixtures originated the maximum extracts´ phenolic contents. Moreover, the obtained extracts presented quite relevant antioxidant and lipoxygenase inhibition activities. Tara seed coat is, therefore, a rich source of bioactive compounds, which may be applied as natural additives in food, cosmetic or pharmaceutical goods, increasing their shelf-lives and/or acting as human health promoters.

In conclusion, instead of being disposed as waste of environmental concern or utilized in low value applications, agro-residues of elder, maritime pine and tara are promising sources of important constituents and should be considered for valorization. Environmentally clean methodologies and solvents may be applied for the extraction of natural colorants, flavors,

aromas, preservatives, among others, some of them presenting bioactive properties. The choice of the appropriate extraction methodology and extraction conditions is of extreme importance for the selectivity of the extraction towards the intended compounds and for the resulting properties of the obtained natural extracts, defining their future potential applications. The successful exploitation of the studied agro-residues still requires further research and development, besides the one presented in this Thesis. A more precise identification of the natural compounds present in the obtained extracts is still required and will be addressed in future works. Moreover, other issues like the toxicity, metabolism, bioavailability and physiological activities of the extracted compounds also need to be considered. Nevertheless, a contribution has been given to the sustainability of elder, maritime pine and tara processing industries.

Resumo

O processamento industrial de produtos agrícolas e florestais origina enormes quantidades de resíduos que podem constituir um sério problema ambiental, se forem sujeitos a procedimentos incorrectos de aproveitamento e/ou eliminação. Frequentemente, estes resíduos são apenas parcialmente valorizados, sendo utilizados para alimentação animal, como fertilizantes ou transformados em biocombustível. Contudo, nos últimos anos tem-se verificado uma tendência crescente para a sua valorização, acompanhando a evolução de uma legislação ambiental cada vez mais restritiva. Neste contexto, a reutilização e reciclagem destes resíduos naturais têm sido encorajadas, contribuindo para agregar valor económico na linha de produção das empresas do sector, podendo mesmo constituir uma excelente oportunidade de negócio.

Tipicamente, uma parte substancial dos resíduos produzidos durante o processamento de frutos, vegetais e recursos florestais consiste da matéria-prima original, incluindo a pele e as sementes dos frutos, flores, folhas, caules, casca de árvores e raízes. Na maior parte destes resíduos existem quantidades substanciais de compostos com elevado valor acrescentado, muitos deles possuindo propriedades benéficas para a saúde humana. De entre eles, destacam-se os compostos fenólicos (que têm sido associados aos benefícios relacionados com o consumo de frutos e vegetais), uma vez que são preferencialmente sintetizados nos tecidos vegetais exteriores. Usualmente, e apesar do seu valor significativo, estes resíduos são comummente sub-explorados, e o seu potencial valor como produto natural é frequentemente perdido. Sendo assim, uma das áreas com maior potencial de exploração é o aproveitamento de substâncias com elevado valor acrescentado. De facto, após o tratamento adequado das matérias-primas, seguido de técnicas de separação adequadas, a maior parte dos resíduos agro-industriais pode constituir uma fonte rica de antioxidantes, corantes, fragrâncias e biocidas naturais, entre outros, com enorme interesse para as indústrias farmacêutica, cosmética ou alimentar.

Neste trabalho foi estudada a extracção de compostos com potencial valor acrescentado a partir de resíduos de três plantas: o sabugueiro e o pinheiro-bravo (nativas de Portugal), e a tara (nativa do Peru). Foram usadas técnicas e solventes de extracção considerados “amigos

do ambiente". Apesar de terem sido usadas técnicas convencionais de extracção, como a hidrodestilação e a extracção por Soxhlet, foi dado especial destaque à extracção supercrítica e à extracção com solvente pressurizado, usando como solventes, respectivamente, o dióxido de carbono supercrítico e misturas pressurizadas de dióxido de carbono, etanol e água. Em termos gerais, e para além de outros aspectos particulares, foi estudado o efeito de diversas variáveis/condições de processo (fraccionamento de extracção, composição do solvente, temperatura e pressão de operação, caudal de solvente, entre outros) nos rendimentos e nas características dos extractos obtidos, nomeadamente em termos de composição e actividades antioxidante e anti-inflamatória. Para a caracterização analítica dos extractos foram usadas diversas técnicas analíticas, incluindo espectrofotométricas e cromatográficas.

O bagaço e a flor de sabugueiro foram avaliados como possíveis fontes de compostos naturais de interesse. O bagaço de sabugueiro, um resíduo proveniente da indústria de processamento de sumo, é constituído essencialmente pelas peles e grainhas das bagas e é uma fonte rica de antocianinas e outros compostos fenólicos. Este resíduo foi sujeito a extracção a alta pressão a 313 K e a ~20 MPa, usando dióxido de carbono supercrítico numa primeira etapa, e misturas pressurizadas de dióxido de carbono, etanol e água numa segunda etapa. Esta metodologia de extracção permitiu obter o fraccionamento do extracto. A primeira etapa de extracção originou extractos contendo elevados teores de lípidos e outras substâncias apolares. Os extractos obtidos na segunda etapa revelaram ter elevados teores de antocianinas e apresentaram uma elevada actividade antioxidante, com uma acentuada influência da composição do solvente. Os resultados deste estudo evidenciaram o potencial do bagaço do sabugueiro como fonte de compostos com elevado valor acrescentado, podendo contribuir para o desenvolvimento de estratégias de utilização integral das bagas, reduzindo o impacto ambiental das indústrias do sector. Por outro lado, a flor foi sujeita a extracção supercrítica usando dióxido de carbono como solvente. Foi avaliado o efeito da massa específica do solvente (300-900 kg/m^3) e da temperatura de extracção (308-328 K) nos rendimentos e nas características dos extractos obtidos. A maior parte dos extractos obtidos revelaram ter um elevado teor de compostos apolares. No entanto, os seus teores de compostos fenólicos e as suas actividades antioxidantes foram relativamente baixos, podendo eventualmente ser melhorados se misturas de etanol e água forem usadas como co-solventes (o que se prevê que venha a ser feito em trabalhos futuros). Os resultados obtidos revelaram que os extractos de flor de sabugueiro obtidos recorrendo à tecnologia de extracção supercrítica podem, eventualmente, ser usados como aditivos aromáticos naturais em produtos alimentares e cosméticos.

A casca do pinheiro-bravo, um resíduo abundante da indústria madeireira e rico em taninos condensados, foi sujeita a extracções convencionais (com o objectivo de obter extractos eficazes na curtimenta de peles de animais) e a extracções supercríticas e com solvente pressurizado (com o objectivo de obter extractos bioactivos). Nas extracções convencionais, foi utilizada água como solvente e foi optimizado o aditivo usado. Verificou-se que uma pequena percentagem de etanol (até 15%, v/v) foi selectiva para os taninos condensados e que os extractos obtidos tinham condições adequadas para serem usados na curtimenta de pele de animais. Nas extracções supercríticas foram estudadas as condições de pressão (10-30 MPa) e temperatura (303-323 K), usando o dióxido de carbono e uma mistura de dióxido de carbono e etanol (10%) como solventes, em dois passos consecutivos. A metodologia de extracção aplicada mostrou que é possível obter fracções de extracto com actividades diversas a partir da casca de pinheiro-bravo. Para além disso, mostrou que o processo podia, ainda, ser optimizado utilizando diferentes condições experimentais, entre as quais a composição do solvente. Sendo assim, foram efectuadas extracções com solvente pressurizado variando algumas condições de processo, tais como a composição do solvente (misturas de dióxido de carbono e etanol, contendo até 90% de etanol) e o caudal de solvente. Tanto as cinéticas de extracção como a composição dos extractos (em termos de compostos fenólicos, em geral, e de taninos condensados, em particular) foram consideravelmente afectados pela composição da mistura do solvente. Este estudo mostrou que, tanto a metodologia de extracção convencional como a de alta pressão, podem ser estratégias viáveis de valorização da casca do pinheiro-bravo, contribuindo para a redução do impacto ambiental e para o aumento do lucro das indústrias do sector.

Finalmente, foi avaliada a possibilidade de aproveitamento da casca da semente de tara (um resíduo proveniente do processamento do fruto para a obtenção de pó e goma de tara) para a recuperação de compostos fenólicos, em geral, e de taninos hidrolisáveis, em particular. Foram efectuadas extracções pressurizadas usando misturas homogéneas de dióxido de carbono, etanol e água, a 313 K e a 20 MPa. Foi utilizado um desenho experimental para optimizar a composição do solvente de extracção, em termos de selectividade para com os compostos referidos, assim como em termos de actividade antioxidante e de inibição da lipoxigenase (indicativa da actividade anti-inflamatória). Os modelos de mistura obtidos permitiram avaliar a contribuição de cada um dos solventes individuais usados: enquanto que a água foi eficaz na promoção de elevados rendimentos de extracção, o etanol foi selectivo para os compostos fenólicos. Os extractos obtidos mostraram ter um elevado teor de compostos fenólicos e apresentaram relevantes actividades antioxidante e anti-inflamatória.

Sendo assim, a casca da semente de tara é uma fonte rica de compostos bioactivos que podem ser usados como aditivos naturais em produtos alimentares, cosméticos e farmacêuticos, aumentando a sua vida útil e/ou actuando como substâncias promotoras da saúde humana.

Em conclusão, os resíduos obtidos a partir de sabugueiro, pinheiro-bravo e tara são fontes promissoras de substâncias importantes devendo, por isso, ser considerados para valorização, em vez de serem encarados como causadores de problemas ambientais, ou serem usados em aplicações de baixo valor acrescentado. Neste estudo foram aplicadas metodologias e solventes "amigos do ambiente" para a extracção de corantes, aromas e antioxidantes, entre outros, de origem natural, alguns deles apresentando potenciais propriedades biológicas. A escolha da metodologia adequada e das condições particulares de extracção é de extrema importância para a selectividade da extracção para com os compostos de interesse, assim como para as propriedades evidenciadas pelos extractos naturais obtidos, definindo as suas potenciais aplicações futuras. O sucesso da exploração dos resíduos agro-industriais considerados requer mais investigação e desenvolvimento, para além dos apresentados neste estudo. Nomeadamente, requer uma identificação mais precisa dos compostos naturais presentes nos extractos obtidos, que irá ser abordada em trabalhos futuros. Outras questões tais como o metabolismo, a bio-disponibilidade e a actividade fisiológica dos compostos presentes nos extractos também necessitam, eventualmente, de ser considerados. No entanto, neste estudo foi dada uma contribuição para a sustentabilidade das indústrias de processamento de sabugueiro, pinheiro-bravo e tara.

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List of publications

Published papers that resulted from the research work presented in this Thesis:

1. Seabra, I.J., Braga, M.E.M., Batista, M.T.P., De Sousa H.C. (2010) Fractioned high pressure extraction of anthocyanins from elderberry (*Sambucus nigra* L.) pomace. *Food and Bioprocess Technology: An International Journal 3(5)*: 674-683.

2. Seabra, I.J., Braga, M.E.M., Batista, M.T.P., De Sousa, H.C. (2010) Effect of solvent (CO_2/ethanol/H_2O) on the fractionated enhanced solvent extraction of anthocyanins from elderberry pomace. *Journal of Supercritical Fluids 54*: 145-152.

3. Braga, M.E.M., Santos, R., Seabra, I.J., Facanali, R., Marques, M.O.M., De Sousa, H.C. (2008) Fractioned SFE of antioxidants from maritime pine bark. *Journal of Supercritical Fluids 47*: 37-48.

Papers that resulted from the research work presented in this Thesis and that will be submitted for publication in 2011:

4. Seabra, I.J., Gomes, O., Dias, A.M.A., Braga, M.E.M., De Sousa, H.C. High pressure extraction of *Pinus pinaster* bark: study of fractionation, solvent flow rate and solvent composition. In preparation, to be submitted to LWT - Food Science and Technology.

5. Seabra, I.J., Braga, M.E.M., Salgueiro, P., De Sousa, H.C. Aqueous extraction of tannins from *Pinus pinaster* bark for leather tanning applications - influence of the solvent additive. In preparation, to be submitted to Bioresource Technology.

6. Seabra, I.J., Braga, M.E.M., De Sousa, H.C. Statistical mixture design investigation of CO_2/ethanol/H_2O high pressure extractions from tara seed coat. In preparation, to be submitted to Journal of Supercritical Fluids.

7. Seabra, I.J., Braga, M.E.M., De Sousa, H.C. Separation of volatile fractions from elderflowers by supercritical CO_2 extraction. In preparation, to be submitted to Journal of Supercritical Fluids.

Published book chapters that resulted from the research work presented in this Thesis:

1. Seabra, I.J., Braga, M.E.M., Salgueiro, P., De Sousa, H.C. Extracção da casca de pinheiro (*Pinus pinaster*): influência de aditivos no solvente de extracção, in Aplicaciones Industriales de los Taninos Vegetales, Eds. E. Cassel, M.F. Vargas, E-book - CYTED and CNPq. EDIPUCRS, Porto Alegre, Brazil, 2008 (ISBN 978-85-7430-809-8).
2. Seabra, I.J., Braga, M.E.M., Batista, M.T.P., De Sousa, H.C. Extracção fraccionada de taninos condensados de subprodutos de *Pinus pinaster* e *Sambucus nigra* L., in Aplicaciones Industriales de los Taninos Vegetales, Eds. E. Cassel, M.F. Vargas, E-book - CYTED and CNPq. EDIPUCRS, Porto Alegre, Brazil, 2007 (ISBN 978-85-7430-674-2).

Other published and submitted papers not directly related with the work presented in this Thesis:

1. Dias, A.M.A., Seabra, I.J., Braga, M.E.M., Gil, M.H., De Sousa, H.C. (2010) Supercritical solvent impregnation of natural bioactive compounds in *N*-carboxybutyl chitosan membranes for the development of topical wound healing applications. *Journal of Controlled Release 148*: e21-e56.
2. Serra, A.T., Seabra, I.J., Braga, M.E.M., Bronze, M.R., De Sousa, H.C., Duarte, C.M.M. (2010) Processing cherries (*Prunus avium*) using supercritical fluid technology. Part 1-Recovery of extract fractions rich in bioactive compounds. *Journal of Supercritical Fluids 55*: 184-191.
3. Durães, L., Moutinho, A., Seabra, I.J., Costa, B.O., De Sousa, H.C., Portugal, A. Characterization of iron(III) oxide/hydroxide nanostructured materials produced by sol-gel technology based on the $Fe(NO_3)_3{\cdot}9H_2O$-C_2H_5OH-CH_3CHCH_2O system. Submitted to Materials Chemistry and Physics, September 2010.
4. Sánchez Martín, J., Beltrán de Heredia, J., Seabra, I.J., Braga, M.E.M., De Sousa, H.C. Cationic surfactant removal by a novel tannin-based adsorbent. Submitted to Journal of Wood Science & Technology, September 2010.

Preamble / Thesis organization

The current Thesis is the result of a PhD research work entitled "Extraction of valuable compounds from agro-residues of elder (*Sambucus nigra*), pine (*Pinus pinaster*) and tara (*Caesalpinia spinosa*)". The Thesis is divided into five Parts (A, B, C, D and E).

In Part A, which is divided in three Chapters, is provided an introduction to the main subjects that are covered in this Thesis. Chapter 1 intends to present the motivation, objectives and scope of the Thesis. The issue of efficient and integral utilization of agro-residues to recover valuable constituents is addressed. The three studied agro-residues are described and the motivations that led to their evaluation as agro-residues with potential for valorization are presented. Finally, the main objectives of the Thesis are described. Chapter 2 focuses on phenolic compounds (in general) and on the most important theoretical and practical aspects of extraction from vegetable raw materials. A brief overview on phenolic compounds structure and classification is provided, emphasizing flavonoids and tannins. Furthermore, special attention is given to fundamentals aspects of high pressure extraction methodologies, namely supercritical fluid extraction and pressurized solvent extraction. Chapter 3 presents specific details on the three plants that were studied, giving a botanical description, referring their production and main uses, as well as their typical phenolic and non-phenolic composition. An overview of the current state-of-the-art on extraction from each one of the studied plants and residues is provided as well as the contribution of this work for the current state-of-the-art.

Part B comprises three Chapters that include all the performed research on elder. Chapters 4 and 5, which concern two already published articles, refer to elderberry pomace, which is an agro-residue originated at the elderberry juice processing industry that is quite rich in anthocyanins. Chapter 4 resulted from the initial stages of the research program. It reports the employment of a successful extract fractionation methodology that consisted in changing the solvent polarity, from supercritical CO_2 (in a first extraction step) to a high pressure solvent mixture of CO_2, ethanol and water (in a second extraction step). A preliminary study on the effect of some process variables was performed. These variables were raw material humidity and composition of the second step employed solvent mixture. In Chapter 5, a more systematic evaluation of the influence of this CO_2-ethanol-water solvent mixture composition on the extracts yields, composition and resulting antioxidant activities was performed. The extraction of volatile compounds from elderflowers using supercritical CO_2, for possible

applications as flavor or aromatic ingredients in food, cosmetic and pharmaceuticals products is the subject of Chapter 6. A 3^2 factorial experimental design was applied to determine the effects of extraction temperature and of solvent density on the composition and characteristics of the obtained extracts. This study will be published in a scientific journal after conclusion of the experimental work, namely the detailed analysis of the CO_2 extracts composition by gas chromatography and gas chromatography coupled to mass spectrometry. Moreover, further extractions using CO_2 plus a cosolvent will be performed in order to improve the extraction of phenolic compounds and therefore the bioactivity of the obtained extracts.

Part C concerns the research performed on maritime pine bark, which is an abundant wood industry residue. It comprises three Chapters, referring to distinct extraction methodologies: conventional extraction is addressed in the first one of these Chapters, while fractionated high pressure extraction is the topic of the two other Chapters. Therefore, Chapter 7 is focused on the optimization of a solvent additive (sodium hydroxide, formic acid, ethanol and sodium sulfite) to be used in conventional aqueous extractions from maritime pine bark and in order to obtain tannin-rich extracts. This study was done in collaboration with a Portuguese leather tanning company (Curtumes J.B. Salgueiro Lda.) which is interested in reducing the usage of chromium salts in leather tanning processes by applying less pollutant vegetable tanning agents. This study was also supported by "Programa CYTED - Ciencia y Tecnologia para el Desarrollo", under the scope of the Project "Aplicaciones Industriales de los Taninos Vegetales". This study will be published in a scientific journal after conclusion of the size exclusion chromatography analysis, which will allow the evaluation of the molecular weight distribution of the compounds present in the extracts. Chapter 8 concerns an already published paper, and in which attention is given to the fractionated supercritical extraction of antioxidants from pine bark using CO_2 and mixtures of CO_2 and ethanol. Studied operational conditions were extraction pressure and temperature, at constant solvent flow rate. Composition and antioxidant activity of the obtained extracts were compared to those obtained by hydrodistillation and by ethanolic Soxhlet extraction. Chapter 9 is a further development of this study: other process variables were assayed, namely fractionation, solvent flow rate and solvent composition. Extraction kinetics were studied as well as extracts composition and antioxidant and anti-inflammatory activities, so as to evaluate their potential applications in the food, pharmaceutical and nutritional fields. The text that comprises this Chapter will be submitted for publication in a scientific journal after conclusion of the high performance liquid chromatography analysis, which will allow the quantification of some compounds that are present in the extracts.

Extractions from tara fruit seed coat is the topic of Part D, which only comprises Chapter 10. This subject arose from the above mentioned Project ("Aplicaciones Industriales de los Taninos Vegetales") and as the result of the contacts established with a Peruvian tara powder and tara gum producer company (Transformadora Agrícola S.A.C). This company is interested in the valorization of a residue that is believed to be highly appropriate for those purposes where antioxidant properties should be relevant. A mixture design study was performed so as to evaluate the effect of a (CO_2/ethanol/water) solvent mixture composition on the yields and characteristics (in terms of composition and bioactivity) of extracts obtained by high pressure extractions. The text that comprises this Chapter will be submitted for publication in a scientific journal after conclusion of the high-performance liquid chromatography analysis, which will allow the quantification of some compounds which are present in the extracts and may play an important role in their bioactivities.

Finally, the major conclusions that may be addressed from the work developed within this Thesis, as well as some suggestions for future work are presented in Part E (Chapters 11 and 12).

List of contents

List of figures

List of tables

Part A. Introduction

1. Thesis motivations and scope

This Chapter intends to present the motivations and the scope of this Thesis. The subject of the efficient and integral utilization of agro-residues to recover valuable constituents is addressed. The studied agro-residues are briefly described and the motivations that led to their specific evaluation as vegetable raw materials with potential for valorization are presented. Finally, the objectives of the developed doctoral work are described.

1.1. Motivations and scope of the Thesis

Large amounts of residues are produced from agricultural processing which, without a proper treatment and disposal, may create serious environmental problems (Capasso et al., 1992; Louli et al., 2004; Schieber et al., 2001). For example and in particular, several tonnes of agro-industrial residues are generated every year as the result of the transformation of fruits and vegetables into food and drink products. In Europe, the amount of byproducts and waste produced in food processing activities accounts for approximately 2.5×10^8 tonnes per year (AWARENET, 2004, as cited by Federici et al., 2009). A substantial part of these residues still comprises important amounts of the original raw materials, since up to 75% of the original vegetables and fruits may end up as a solid residue (Kumar, 2004). Such waste streams are only partially valorised as animal feed, transformed into biomass fuel, or subjected to composting, whereas the main volumes are managed as wastes of environmental concern. In many countries, these environmental concerns gave rise to specific legislation regarding waste minimization and disposal. It is not unreasonable to assume that future legislation regarding agro-industrial wastes will become even more demanding, thus increasing the associated costs of their waste management. Besides, making a correct use of discarded natural materials may be a profitable form of entrepreneurial recycling, creating value in the entire chain-production pipeline. In recent years new technologies have been

proposed for more efficient utilization of agro-industrial residues, not only for their re-use in agriculture, but also for the production of common and novel products for other sectors and applications. One of the most interesting areas of exploitation is the utilization of these residues to recover valuable constituents (Laufenberg et al., 2003), with special attention to bioactive substances, where phenolic compounds play an important role (Schieber et al., 2001). Indeed, after the appropriate pretreatment of the raw material, followed by tailored recovery procedures (usually extraction), most natural residues obtained from agricultural processing can provide value-added natural oils, antioxidants, colorants, fragrances, preservatives, biocides and other bioactive substances of enormous interest to the pharmaceutical, cosmetic, food or even other industries. Recent research and development has produced many viable products from this type of residues which are finding an increasing commercial acceptance. For example, an array of natural extracts is commercially available, many of them being advertised as dietary supplements, which are believed to improve overall health and prevent disease. Prices of these extracts cover a wide range depending on the concentration of active compounds, some reaching hundred of euros per kilogram.

In this Thesis, agro-residues from elder (*Sambucus nigra*), pine (*Pinus pinaster*) and tara (*Caesalpinia spinosa*) were evaluated as raw materials to recover valuable constituents.

Elder is a shrub that produces creamy-white flowers and small deep purple berries, which has been used for generations in traditional herbal medicine. In Portugal, elder plantation is mostly concentrated at the north of the district of Viseu. Nearly all Portuguese elderberry production is currently exported to Germany. Both elderflower and elderberry pomace can be considered for valorisation. Elderflower is an agro-residue that results from the elder plant pruning process, commonly performed to enhance posterior fruit production. This flower contains various substances potentially useful for valuable applications, such as phenolic and volatile compounds. On the other hand, elderberry pomace is the agro-industrial residue that results from elderberry juice production. It is known to contain high levels of anthocyanins, besides other potential useful phytochemicals like tannins and unsaturated fatty acids.

Maritime pine is the third most important tree species in Portugal and still represents an important component of the Portuguese forest economy, despite the crisis that has been recently affecting the pine production sector. The main industrial activities associated with maritime pine are related to its wood. In these industries, pine bark constitutes a residue. The valorization of this abundant, low-cost and not-perishable residue could give a quite positive contribution to the Portuguese pine sector. Pine bark contains some important phytochemicals

constituents, in particular condensed tannins, and therefore some interesting possibilities of utilization can arise straightforwardly.

Finally, tara is a small tree native to the Cordillera region of Bolivia, Peru and northern Chile, which is commonly used in the popular medicine of those countries. Tara tree produces flat oblong pods which are a source of diverse products having several applications in the food, cosmetic, pharmaceutical and leather industries. The main commercial product that is originated from tara pods is tara powder which is obtained by crushing the de-seeded pods, and is widely used as a natural tanning agent in the leather industry. Tara seeds, which are considered a byproduct of the tara powder industry, do have commercial applications as well: the ground endosperm is the raw material for the production of tara gum which is used as a thickener, stabilizer and gelling agent in some food applications. Tara seed coat constitutes an agro-residue of these processing industries and may represent an opportunity for an additional valorization of this vegetable material due to the well documented rich hydrolysable tannins content of tara pods.

1.2. Objectives of the Thesis

The main objective of the research activities developed during this doctoral training period was to evaluate if, with some additional and proper processing, agro-industrial residues of elder (elderberry pomace and elderflower), maritime pine (tree bark) and tara (fruit seed coat) could to be transformed from a residual low-value status into a quite interesting high-value one, for consumer well-known and appealing uses. The extraction of valuable phytochemicals was explored, with special attention to phenolic compounds, using environmental friendly and food/pharmaceutical accepted solvents and techniques. Therefore, high pressure extraction methodologies using supercritical carbon dioxide and mixtures of carbon dioxide, ethanol and water were profusely used and compared to conventional extraction methodologies (also using environmental friendly and food/pharmaceutical accepted solvents: water and ethanol) .

Extraction conditions were optimized so as to achieve extracts with high contents of the desired and specific phytochemicals that are known to be present in the original raw material and that may have some potential interest for high-value applications, like in food, pharmaceutical and cosmetic industries. Among the extraction conditions that were optimized, one can distinguish extraction pressure and temperature, solvent composition and

solvent flow rate. For the extracts characterization, chromatographic, spectrophotometric and other analyses were applied.

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2. Extraction of phenolic compounds from vegetable matrices

This Chapter focuses on phenolic compounds in general and on the most important theoretical and practical aspects of extraction from vegetable raw materials. A brief overview on phenolic compounds structure and classification is provided, emphasizing flavonoids and tannins. Furthermore, special attention is given to fundamentals aspects of supercritical fluid extraction and pressurized solvent extraction methodologies.

2.1. Plant secondary metabolites

Plant cells synthesize a vast supply of natural compounds that are not strictly necessary for their growth and reproduction, which are known as secondary metabolites and have a wide range of chemical, physical and biological activities (Gottlieb, 1990). The total number of plant secondary metabolites exceeds 100000 and may be grouped into three main groups: *(i)* phenolic compounds, *(ii)* nitrogen containing compounds, and *(iii)* terpenes (Reichling, 1999). They are present in different parts of the plants (roots, leaves, barks, seeds, fruit skins, etc.) and exhibit diverse functions, including plant chemical defense and attraction of beneficial animals for pollination and seed dispersal. The contents of secondary metabolites vary among plant species (they may contain as little as 1% or as much as one-third of their dry weight) and plant tissues, also varying from season to season. Plant metabolites have been used by humans for thousands of years as dyes, flavors, fragrances, stimulants, hallucinogens, poisons and medicines. However, it was the potential use of plant secondary metabolites in health care and personal care products, and in the development of novel drugs, that led to a huge interest in their isolation and characterization from major plant species over the past few decades. Special attention has been given to phenolic compounds due to their antioxidant properties and their probable role in the prevention of various diseases associated with oxidative stress,

such as cancer and cardiovascular and neurodegenerative diseases (Harborne and Williams, 2000; Kroon and Williamson, 2005).

2.1.1. Phenolic compounds

Phenolic compounds are plant secondary metabolites originated from the pentose phosphate, shikimate, and phenylpropanoid patwways in plants (Randhir et al., 2004). These compounds play an important role in plant growth and reproduction, providing protection against ultraviolet radiation and pathogens (Bravo, 1998). Structurally, phenolic compounds comprise an aromatic ring bearing one or more hydroxyl substituents, and range from simple phenolic substances to highly polymerized compounds (Bravo, 1998). They are usually found as conjugates with mono- and polysaccharides, linked to one or more of the phenolic groups, and may also occur as functional derivatives such as esters and methyl esters (Harborne et al., 1999). These compounds may be classified into different groups as a function of the number of phenol rings that they contain and of the structural elements that bind these rings to one another, as illustrated in Table 2.1.

Table 2.1. Classes of phenolic compounds in plants (Harborne et al., 1999).

Class	Structure
Simple phenolics, benzoquinones	C_6
Hydroxybenzoic acids	C_6-C_1
Acethophenones, phenylacetic acids	C_6-C_2
Hydroxycinnamic acids, phenylpropanoids (coumarins, isocoumarins, chromones, chromenes)	C_6-C_3
Napthoquinones	C_6-C_4
Xanthones	C_6-C_1-C_6
Stilbenes, anthraquinones	C_6-C_2-C_6
Flavonoids, isoflavonoids	C_6-C_3-C_6
Lignans, neolignans	$(C_6$-$C_3)_2$
Biflavonoids	$(C_6$-C_3-$C_6)_2$
Lignins	$(C_6$-$C_3)_n$
Condensed tannins (proanthocyanidins or flavolans)	$(C_6$-C_3-$C_6)_n$

Flavonoids comprise the largest group accounting for over half of the eight thousand naturally occurring phenolic compounds (Harborne and Baxter, 1999). Flavonoids share a common structure consisting of two aromatic rings (A and B) that are bound together by 3 carbon atoms that form an oxygenated heterocycle (ring C) (Figure 2.1).

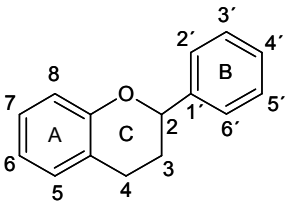


Figure 2.1. Generic structure of a flavonoid molecule (Balasundram et al., 2006).

Variations in the substitution pattern to ring C result in the major flavonoid classes, namely, flavonols, flavones, flavanones, flavanols (or catechins), isoflavones, flavanonols, and anthocyanidins (Figure 2.2) (Balasundram et al., 2006). Substitutions to rings A and B give rise to different compounds within each class of flavonoids (Pietta, 2000). These substitutions may include oxygenation, alkylation, glycosilation, acylation, and sulfation.

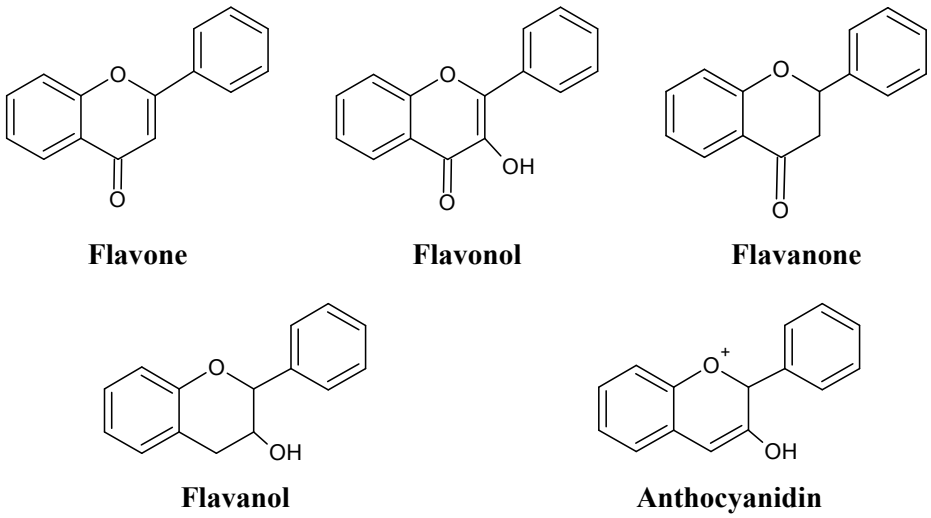


Figure 2.2. Generic structure of the major classes of flavonoids.

Among flavonoids, anthocyanins and catechins, which are known collectively as flavans because of the lack of the carbonyl group in the 3-position, are important. Anthocyanins are glycosidically bound anthocyanidins present in many flowers and fruits. These water-soluble pigments are responsible for the bright red, blue and violet colors of fruits and other foods. They exist in different chemical forms, both colored and uncolored, according to pH. Although they are highly unstable in the aglycone form (anthocyanidins), while they are in plants they are resistant to light, pH, and oxidation conditions that are likely to degrade them. Degradation is prevented by glycosilation, generally with a glucose molecule at position 3, and esterification with various organic acids and phenolic acids. In addition, anthocyanins are stabilized by the formation of complexes with other flavonoids (copigmentation). In the

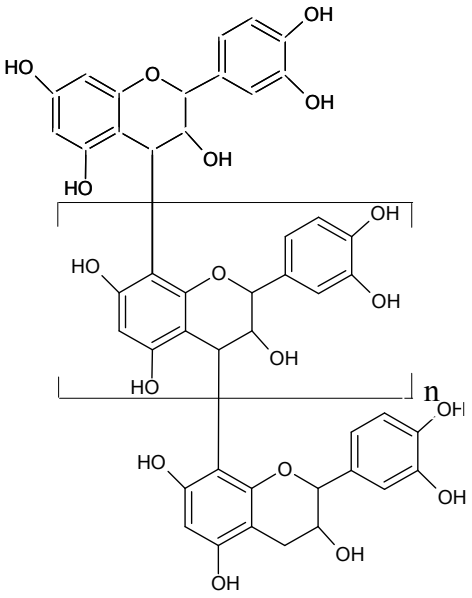
human diet, anthocyanins are mainly found in fruits, especially in berries, where they mainly occur in fruit skins (Manach et al., 2004).

Tannins are relatively high molecular weight compounds which constitute an important group of phenolics, being synthesized by a wide diversity of plants. They can occur in wood, bark, leaves, fruits and galls and are subdivided into hydrolysable and condensed tannins. The former are esters of gallic acid (gallo- and ellagi-tannins), while the latter are polymers of flavan-3-ol monomers, also known as proanthocyanidins (Figure 2.3). The name proanthocyanidins came from the characteristic oxidative depolymerization reaction in acidic medium, which yields colored anthocyanidins (Hümmer and Schreier, 2008).

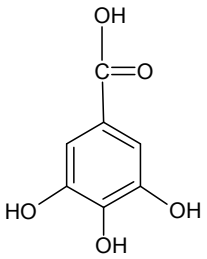
There are a variety of different classes of condensed tannins depending on the substitution pattern of the monomeric flavan-3-ol units. The condensed tannins that exclusively consist of (epi)catechin units are designated procyanidins, the most abundant condensed tannins in plants. The flavan-3-ol subunits may carry acyl or glycosyl substituents, the most common being gallic acid to form 3-*O*-gallates. Condensed tannins are quite sensitive to oxygen, light, acids and alkalis (Shi et al., 2005). Monomers and dimmers, for example, rapidly degrade at pH higher than 9.0, forming brown colored degradation products (Zhu et al., 2002).

A very large number of hydrolysable tannins exist in nature. The structural variations amongst these compounds are caused by oxidative coupling of neighboring gallic acid units or by oxidation of aromatic rings. Gallotannins consist of a central polyol, such as glucose, which is surrounded by several gallic acid units. Ellagitannins, which have a more complex structure, also derive biosynthetically from pentagalloylglucose by oxidative reactions between the gallic acid units (Mueller-Harvey, 2001).

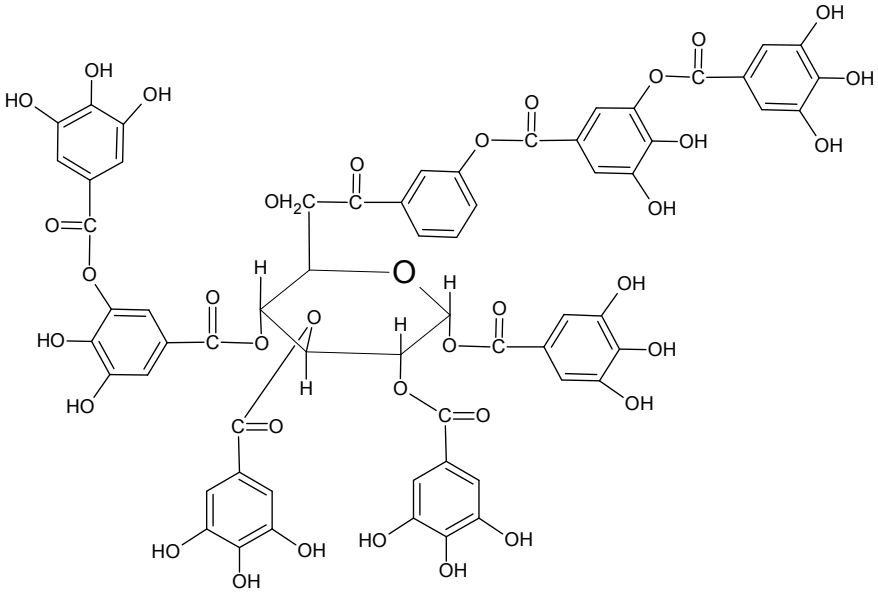
Phenolic compounds are known to possess several biological and chemical properties, including antioxidant activity. The antioxidant activity of phenolic compounds resides mainly in their ability to donate electrons or hydrogen atoms (Heim et al., 2002). Phenolic compounds possess ideal structural chemistry for this activity and have been shown to be more effective *in vitro* than vitamins E and C on molar basis (Rice-Evans et al., 1997). However, the possible health benefits derived from dietary phenolic compounds depend on their absorption and metabolism (Parr and Bolwell, 2000), which in turn are determined by their structure, including their conjugation with other phenolics, degree of glycosilation/acylation, molecular size and solubility (Bravo, 1998). Anthocyanins, for example, appear to be absorbed in their original glycosylated forms and are bioavailable to humans (Cao and Prior, 1999; Milbury et al., 2002).



Condensed tannin



Gallic acid



Hydrolysable tannin

Figure 2.3. Generic structure of condensed and hydrolysable tannins.

Though phenolic compounds are present in almost all foods of plant origin, fruits, vegetables and beverages are the major sources of these compounds in the human diet (Hertog et al., 1993). Agro-industrial byproducts are also good sources of phenolic compounds and have been explored as sources of natural antioxidants. However, in order to efficiently explore these byproducts as a source of phenolic compounds, practical aspects need to be considered, such as extraction efficiency, availability of sufficient raw material, and toxicity or safety considerations (Balasundram et al., 2006).

2.2. Solid-liquid extraction methodologies

Several extraction techniques have been developed to isolate bioactive compounds from plant material resources. Given the wide diversity of real-world matrices and the thousands of compounds that typically constitute them, there is not a universal strategy for all intended analytes and matrices. The adequate extraction technique should maximize the extraction of the target metabolites and, at the same time, minimize the co-extraction of undesirable compounds that may interfere negatively with their activity, avoid degradation of the target metabolites (like thermal and light degradations), minimize the presence of toxic solvents, should be economically feasible and not very time-consuming. Therefore, the choice of the adequate extraction methodology is vital for the potential applications of the resulting extract and the success of the whole process.

There are quite a few extraction techniques available, namely (Rosa et al., 2009):

1. Steam distillation, which is a modified distillation process used for the recovery of high boiling point volatile compounds from an inert matrix, using steam as a separation and energy agent.
2. Low-pressure solvent extractions, where conventional, microwave assisted and ultrasound assisted extractions are included.
3. High-pressure solvent extractions, where supercritical fluid and pressurized solvent extractions are included.

2.2.1. Fundamental aspects of solid-liquid extraction

Solid-liquid extraction is a separation process involving the transfer of solutes from a solid matrix to a solvent. From an engineering point of view, it is a multicomponent, multiphase and unsteady state mass transfer operation (Aguilera, 2003). In the extraction of natural compounds from plants, the chemical species being recovered are ill-defined and the solute is usually referred to as extract.

During extraction, a series of parallel and consecutive steps occur between the solute-containing particle and the solvent, including (Aguilera, 2003):

1. Entrance of solvent in the solid matrix;
2. Solubilization of compounds;
3. Transport of the solute to the outer surface of the solid matrix (diffusion is the most important transport mechanism);

4. Migration of the extracted compounds from the external solid surface to the bulk solution;
5. Separation of the extract and solvent.

In multicomponent systems, different chemical species migrate through the plant matrix with different transport rates. Moreover, since these steps occur at their own rate and in some cases sequentially, the overall rate of the extraction process is determined by the step having the slowest rate, i.e., the rate-controlling step.

The ability to remove target compounds from a plant matrix depends on the compounds solubility in the selected solvent, on compounds-matrix interactions, on the matrix porosity and on the compounds location within the matrix. Therefore, solvent selection and structural features of the plant tissue to be extracted are of major importance.

2.2.1.1. Selection of solvents for extraction

There are no simple rules for choosing solvents for extraction of natural compounds from plant matrices, because the current understanding of the various interactions that compete in such a multi-component system is still incomplete. However, solvent selection is based on several aspects, among which the most relevant are the following (Aguilera, 2003):

1. Solubility of the specific compound (or mixture of compounds) in the solvent. However, the knowledge of the target compounds solubility does not allow us to predict the effectiveness of the extraction methodology chosen. This is especially significant if dynamic extraction is being performed, if the target compounds are present in minor or trace amounts, and if there are significant quantities of co-extracted compounds (Taylor, 1996).
2. Facility of removal of the solvent in post-extraction preparation steps since residual levels of solvent in extracts must very often be minimized. Recycling of solvent is also of particular importance, due to economic reasons and effluent treatment policies.
3. Interfacial tension and viscosity. The solvent should be capable of wetting the solid matrix and its viscosity should be sufficiently slow so that it can flow easily. Wettability is also important if the solvent must penetrate through pores in the plat matrix.
4. Ideally, the solvent should be nontoxic, stable, nonreactive, nonflammable, harmless to the environment and economically affordable.

2.2.1.2. Structural features of vegetable matrices

Plant materials (leaves, flowers, stem, wood, bark, etc.) have an intricate microstructure formed by cells, intercellular spaces, capillaries and pores. The initial distribution of the desired solutes within the plant tissue influences extraction kinetics, since the compounds may me present inter- or intracellularly. In the first case, intact cell walls and adhering membranes constitute a major resistance to diffusion, affecting the permeability of solutes so that small molecules pass at a faster rate than larger ones, resulting in selective mass transfer (Aguilera, 2003).

Transport paths are shorter and mass transfer is enhanced if the plant material is crushed and cells and cell structures are destroyed. Therefore, when a plant material is collected, it is usually dried to avoid degradation of the components (by air or by microorganisms), and crushed to smaller particles in the sequence. In these processes, care should be exercised so as to avoid the excessive loss of volatile components as they may also exhibit interesting biological activities.

2.2.1.3. Theoretical overall extraction curve

Extraction can be accomplished using a static, dynamic or coupled static/dynamic mode. In static extraction, a fixed amount of solvent interacts with the plant matrix whereas in dynamic extraction fresh solvent is continuously passed over through the plant matrix. Dynamic extraction is usually preferred since a more exhaustive extraction is frequently accomplished. Nevertheless, a combination of an initial static period followed by a dynamic one is gaining popularity, especially for situations where solvated compounds must diffuse to the matrix surface to be extracted (Taylor, 1996).

Figure 2.4 shows the theoretical extraction profile of an extract from a solid matrix. The y axis represents the amount of extracted compounds; the x axis represents the amount of solvent or time in a dynamic system, or time in a static system (Brunner, 1994; Taylor, 1996).

The initial extraction of material (corresponding to *part I*) occurs rapidly and is dependent upon the solubility of the extract in the solvent. During this period, quasi-equilibrium conditions govern the partition of the solute into the solvent. *Part II* corresponds to an intermediate region where the extraction process is enthalpically controlled (i.e., analyte-matrix interaction must be disrupted), showing a slower rate of extraction. A transition to diffusion-controlled kinetics takes place in this region. *Part III* represents the portion of the extraction process that is truly diffusion limited, either due to the limited diffusivity of the

target compounds within the matrix or to the limited access of the solvent to the target compounds.

For a static extraction, as time increases, equilibrium between extracted compounds in the matrix and in the solvent establishes and the diffusion-limited process is no longer in one direction, as in the dynamic case (Taylor, 1996). Furthermore, during a dynamic extraction, fluid is constantly removing any extracted compounds away from the matrix, so diffusion back into or onto the matrix ideally does not occur. Therefore, exhaustive extraction via the static mode does not occur and it is usually used in combination with a dynamic mode.

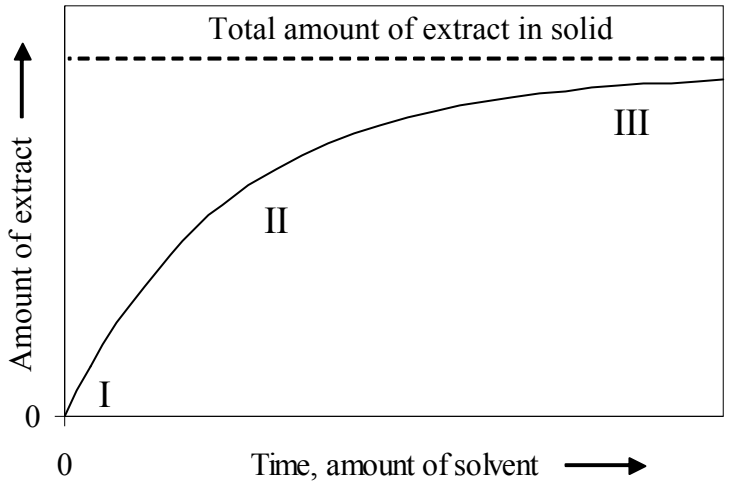


Figure 2.4. Theoretical extraction curve. *Part I* is linear. *Parts II* and *III* are nonlinear.

2.2.2. Supercritical fluid extraction

A fluid is considered to be in the supercritical state when the system pressure and temperature are above the ones corresponding to its critical point. A supercritical fluid displays unique characteristics that enable its usage as a solvent. One of these characteristics is its relatively high density and its consequent high solvation power. Another one is the extreme sensitivity of its density to temperature and to pressure near the critical point that permits selective separation of one or more active constituents out of the total extractables from a plant material, and allows an easy solvent-solute separation process. The separation is usually achieved by decreasing the pressure of the mixture that leaves the extraction column. Other important characteristics are the relative low surface tension and viscosity as well as high diffusivity that permit high extraction rates when these fluids are applied, when compared to conventional solid-liquid extraction (Aguilera, 2003).

The solubility behavior of a solute in a supercritical solvent is mostly density dependent. At higher densities, the molecular interactions between the solvent and the solute are enhanced and as a result, more solute is dissolved. The exponential density increase in the vicinity of the supercritical point allows an isothermal exponential solubility enhancement with pressure. However, density alone does not give the complete explanation of solubility enhancement: the volatility of the solid solute is also responsible for its solubility. The retrograde solubility behavior, characterized by a decrease in solubility caused by an isobaric increase in temperature, is explained by the relative influence of the density effect and the volatility effect (Mamata, 2000).

However, the solvent capacity of supercritical solvents depends on their physicochemical properties, such as polarisability[1] and polarity, besides density and other thermodynamic properties. Differences in solvent capacities of different supercritical solvents can not be attributed merely to differences in their densities or polarities. Moreover, chemically similar solutes dissolve in a supercritical solvent according to their respective volatility. Further, solubility of compounds in a mixture of solutes is different from those of pure solutes and synergistic solubility enhancements of the components in a mixture are sometimes observed. Therefore, solute-solute and solute-solvent interactions should be considered for the design of supercritical separations.

A striking feature of a supercritical solvent is that it can selectively dissolve certain specific compounds from a mixture of compounds having even similar volatility but different chemical structures. In general, the selectivity of separation depends on the mixture composition, temperature, pressure and solvent characteristics such as molecular structure and properties. Usually, lower selectivities occur for higher solubilities.

By far, the most widely used solvent in supercritical fluid extraction (SFE) is carbon dioxide. It has a mild critical pressure of 7.38 MPa and low critical temperature (304 K), which makes it attractive for the extraction of heat sensitive compounds. In addition, it is an inert, non-flammable, non-explosive, inexpensive, odorless, colorless, clean solvent that leaves no solvent residue in the product. Due to these characteristics, it is also generally accepted as a harmless ingredient in pharmaceuticals and food products (Mamata, 2000).

Because the CO_2 molecule has no dipole moment, it is usually regarded as nonpolar. However, CO_2 has some affinity for polar solutes at relatively high pressures, due to its high quadrupole moment. Nevertheless, the presence of hydroxyl, amino and nitro groups is known

[1] Polarisability can be defined as the degree by which the electron cloud of a molecule is distorted in the presence of an electric field.

to diminish solubility, especially if two or more of these groups are present in the solute molecule (Taylor, 1996). The solubilities of such molecules may be greatly enhanced by the addition of small amounts (typically less than 10%, v/v) of a polar cosolvent. The role of a cosolvent in SFE is to increase the polarity and solvent strength while retaining the sensitivity of solubility with respect to pressure and temperature. Cosolvents are usually chosen to interact with targeted solutes through hydrogen bonding, acid-base interactions or strong dipole-dipole interactions. Hexane, benzene, chloroform, isopropanol, methanol, ethanol, acetone and water have all been used as cosolvents. However, ethanol is the preferred cosolvent for food, pharmaceutical and cosmetic applications since it is nontoxic and is approved as a GRAS (generally recognized as safe) component (Dunford et al., 2003).

The phase equilibrium behavior between the supercritical fluid and the solutes being extracted is a very important information to be considered in the design of SFE experiments. The extraction system is quite complex, comprising the supercritical solvent, a mixture of different compounds that forms the solute and a solid structure where the solute is initially distributed (Rosa et al., 2009). When the supercritical solvent contacts the solute at conditions near the critical point of the solvent plus the solute mixture, there may be occurrence of multiple phases involving vapor, liquid and solid phases, depending on the mixture composition and temperature and pressure conditions. For practical reasons, it is desirable to avoid regions of multiple phases in the pressure-temperature-composition space. Accordingly, a designer needs to select the neighborhood of temperature and pressure at which processing of natural materials and separation of their extracts are technically and economically attractive (Mamata, 2000).

A typical diagram of a supercritical extraction unit is shown in Figure 2.5. It is most commonly operated in a semi-continuous mode, with the extraction cell filled with the raw material to be extracted and the solvent passing through it, either in a dynamic mode or a combination of static followed by dynamic mode. CO_2 is pumped into the system as a liquid. A cooler is necessary to keep it in the liquid phase. Depending on the process requirements, a system for cosolvent addition will be incorporated usually containing a high pressure liquid pump. The fluid is then pressurized and heated to the desired processing conditions. The temperature and pressure controlled extraction cell is packed with the raw material that contains the desired solutes. A high-pressure metering valve can control the flow rate of the fluid.

After extraction, the recovery of extracted compounds is usually done by lowering the pressure, as illustrated in Figure 2.5. The decrease in the solvent pressure leads to a reduction

in its density and a consequent reduction in its solvent power. The solvent pressure reduction may also be accompanied by a temperature reduction, to further diminish the extracted compounds solubility in the exiting solvent. Fractional separation of extracted solute mixtures may, therefore, be achieved in a series of collection vessels, successively lowering pressure on each vessel. Cyclones have been effectively used for this purpose (Brunner, 1994; Dunford et al., 2003). If the extracted compounds are very volatile, extract recovery by depressurization may result in the lost of significant part of the extract with the expanded CO_2. In this case, adsorption is also very effective with respect to removing the extract. Adsorption may be also employed as additional operation for removing residual quantities of extracted compounds from the gaseous solvent (Clifford, 1999).

Increasing the extract temperature at constant pressure may also reduce solvent power and is another alternative for extract recovery, mainly for nonvolatile compounds. This method improves feasibility of a supercritical extraction process by eliminating recompression of the solvent, if it is to be recycled (Brunner, 1994; Clifford, 1999; Dunford et al., 2003).

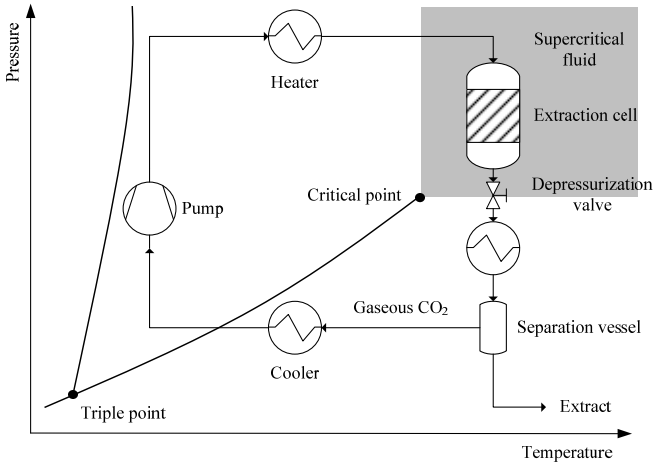


Figure 2.5. Diagram of a SFE process (adapted from Rosa et al., 2009).

The solvent separated from the extract by means of one of the methods mentioned above can be either vented out to atmosphere or recycled to the system (as illustrated in Figure 2.5) depending on the size of the operation. For small-scale equipment, such as those in laboratories, solute-free gas is vented out to the atmosphere, due to the higher cost of installing a recycling system as compared to the cheaper gas cost (Dunford et al., 2003). To recycle, the gaseous CO_2 is condensed by the decrease of temperature, the pressure is increased to a value above the critical point, and it is again transformed into a supercritical fluid at the extraction temperature by flowing through a heat exchanger (Rosa et al., 2009).

Very frequently, the recovery of diverse fractions from the same vegetable material is desired. There are a few strategies to accomplish fractionation of the extract:

1. One of them is the sequential collection of fractions during the extraction. This strategy succeeds if the extract composition changes over the time, due to changes in composition of the residual material in the extraction cell. Compounds having a higher solubility under the initial processing conditions will be selective extracted in the beginning. Then, as these compounds are depleted from the matrix, the extraction selectively will eventually shift to other compounds.
2. Another strategy may be the increase of solvent density over time, which changes solvent power and selectivity, as already discussed.
3. Fractional separations of extracted solute mixtures can also be achieved in a series of collection vessels by successively lowering pressure on each vessel.
4. The usage of different solvents or solvent mixtures at consecutive steps of the extraction process is also possible. In this case, a first CO_2 extraction step is usually performed to separate low polar compounds, followed by a second step, where CO_2 plus a more polar cosolvent are introduced in the system. In this way, fractionation of the extract is mainly based on the polarity of the solvent mixture.

As a concluding remark, one can say that SFE offers considerable versatility for the extraction of natural compounds from plant matrices. Improved equipment design and operational dynamics are making SFE processes much more efficient and competitive. However, for some product applications, SFE technology still faces competition from conventional techniques, though greater selectivity is potentially available from SFE.

2.2.3. Pressurized solvent extraction

Pressurized solvent extraction (PSE), also designated by enhanced solvent extraction, is a solid-liquid extraction technique which has been developed as an alternative to current extraction methods such as Soxhlet, maceration, percolation or reflux, offering advantages with respect to extraction time, solvent consumption, extraction yields and reproducibility (Kaufmann and Christen, 2002). PSE involves the use of H_2O or of organic solvents at considerable elevated temperatures (313-473 K) and pressures (3.3-20.3 MPa). It offers the possibility to perform efficient and “enhanced” extractions due to its improved characteristics in terms of mass transfer and of solvating properties. If high temperatures are applied, viscosity of the liquid solvent diminishes, diffusivity of the solvent trough the plant matrix is

improved and consequently extraction kinetics is accelerated. Moreover, high pressure forces the solvent into the matrix pores and hence should facilitate extraction of target compounds (Kaufmann and Christen, 2002). Therefore, this extraction technique takes advantage of the beneficial combination between typical liquids solvation properties and the advantageous transport properties of supercritical fluids (Chamblee et al., 2004). However, a liquid separation step is additionally required in the post-extraction preparation steps, which represents a disadvantage relatively to the SFE methodology.

H_2O and organic solvents usually applied in PSE are able to establish intermolecular interactions with plant compounds with hydroxyl, amino and nitro functional groups that are not soluble in supercritical CO_2, even with small quantities of a polar cosolvent. In particular, the OH groups of phenolic compounds interact favorably with alcoholic solvents, since they provide polarity and a site for accepting and donating hydrogen bonds. Several types of intermolecular interactions between solute molecules, between solvent molecules and between solute and solvent molecules will compete with each other and will determine solubility. Therefore, it may be necessary to try several solvents to achieve the maximum specificity for a given system.

If CO_2 is used in combination with H_2O or with an organic solvent, a gas-expanded liquid is formed. A unique and potentially useful property of CO_2-aqueous and CO_2-alcohol gas-expanded liquids is the *in situ* generation of carbonic acid and alkyl carbonic acid, respectively (Figure 2.6) (West et al., 2001; Weikel et al., 2006). The pH decrease that follows the formation of these acids may be beneficial or detrimental to the extraction of the desired compounds and should be taken into consideration.

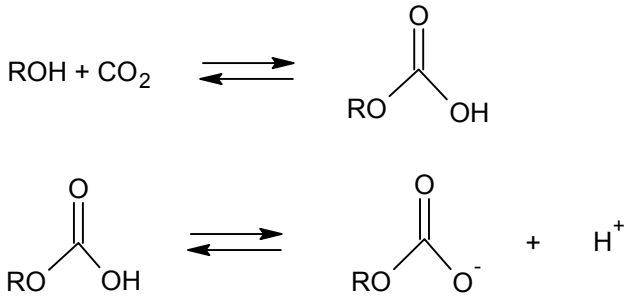


Figure 2.6. Formation of carbonic and alkyl carbonic acids.

There are also some other aspects that should be considered in an PSE process, such as the thermodynamic properties of the individual solvent or solvent mixture applied, like density and viscosity. However, due to the typical difficulties of performing thermodynamic measurements at high pressures, there are not that many experimental values available in

literature. Moreover, if a solvent mixture is applied, like an aqueous-alcoholic one, the phase-equilibrium diagram at the operation conditions of pressure and temperature should be verified. Experimental conditions of pressure, temperature and solvent mixture composition may be chosen so as to avoid a two- or three-phase equilibrium, since the presence of a gaseous phase in the extraction cell may be detrimental to process dynamics and target compounds solubility.

PSE may be performed using a SFE apparatus equipped with a high-performance liquid chromatography pump to introduce the liquid solvent or solvent mixture into the system. Raw material preparation steps are similar and semi-continuous operation is usually performed, with the extraction cell being filled at the beginning of the operation. Usually, an initial static period of 10-15 minutes is accomplished, followed by a dynamic one. CO_2 may be used to flush the liquid solvent out of the cell at the end of the extraction.

As opposed to SFE, fractionation in a PSE process does not rely mostly on solvent density which, in turn, depends on the experimental conditions of pressure and temperature chosen. Actuality, since selectivity in the PSE process is based on the capacity of the solvent to establish molecular interactions with solutes possessing functional groups, the usage of different solvents or solvent mixtures (having different compositions) at consecutive steps may be the proper choice to achieve fractionation. In this case, a first CO_2 extraction step may be performed to separate low polar compounds. In some cases this strategy may render the remaining vegetable material compounds more available for the consecutive extraction steps (Pinelo et al., 2006).

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3. Studied vegetable raw materials: elder, maritime pine and tara

This Chapter presents specific details on the three plants that were studied, giving a botanical description, referring their production and main uses, as well as their typical phenolic and non-phenolic composition. An overview of the current state-of-the-art on extraction from each one of the studied plants and residues is provided as well as the contribution of this work for the current state-of-the-art.

3.1. Elder (*Sambucus nigra*)

3.1.1. Botanical description of elder plant

The elder plant is a member of the *Caprifoliaceae* family which comprises more than 25 species. In Portugal, as well as in central and northern Europe, the most common species is *Sambucus nigra* L., also known as black or European elder (Sansdrap, 2000). Portuguese common names for elder are *sabugueiro-negro, sabugueiro-preto, rosa-de-bem-fazer* and *sabugo* (Cunha et al., 2003). *Sambucus canadensis* is the main species occurring in North America, being often referred to as American elder (Charlebois, 2007).

Elder is a deciduous multi-stemmed shrub with brittle branches that easily bend under the weight of its fruit clusters (Figure 3.1.a). This plant tolerates relatively poor soil conditions and is often found on moist, well-drained sunny sites. Elder plants may flower and fruit after only 2-3 years and can reach full size in 3-4 years. Aging of the shrub is accompanied by the death of old branches, a process preventing the plant from reaching extreme heights, usually 3-5 m (Wright et al., 2007; Charlebois et al., 2010). In late spring, creamy-white flowers gather into large terminal clusters and this is followed by the production of bunches of small deep purple almost black berries (Figures 3.1.b and 3.1.c) that are 5-7 mm in diameter (Wright et al. 2007; Charlebois, 2007). Harvest is done by hand and the plant can rapidly be

striped of its fruits. In order to preserve fruit quality, elderberries should be refrigerated as soon as possible while processing can be delayed many months if fruits are kept frozen (Charlebois, 2007).

(a) **(b)** **(c)**

Figure 3.1. Elder shrub **(a)**, cluster of elderberries **(b),** and detail of elderberries **(c)**.

3.1.2. Marketable products from elder plant

Elderberry has been used for generations in traditional herbal medicine as a remedy for colds, sinusitis, and herpes. Nowadays, elderberries are almost exclusively commercially available in a processed form (Cejpek et al., 2009), since they have an odd taste, even when sweetened. Both flowers (usually dried) (Figure 3.2) and berries (preferentially fresh) are suitable for processing being widely used in central and northern Europe, as well as in North America. Elderberry juice concentrate is a natural alternative to artificial food dyes due to its high content of anthocyanins (Kaack and Austed, 1998). The elder products that reach consumers can be divided into two main categories: food and beverages, and health products. Elderberries are used to prepare jams, jellies, pies, sauces, juices, soft drinks, cordials and wines (Charlebois, 2007). To a less extent, distillates and extracts of elder flowers are used as natural flavor ingredients in alcoholic and non-alcoholic beverages, fruit brandies and other spirits, as well as yoghurts and ice creams (Kaack, 2008). The pharmaceutical and natural products industries are also taking advantage of the high contents of phenolic compounds of elder berries and flowers. Several cosmetic products, including shampoos and body lotions are available to consumers. In addition, a full array of products including syrups, herbal teas, extracts and supplements are also available, all capitalizing on diverse health benefits. The most popular one is Sambucol® which is an elderberry extract formulation that activates the human health immune system (Barak et al., 2002).

(a) **(b)**

Figure 3.2. Fresh **(a)** and dried **(b)** elderflowers.

3.1.3. Portuguese elderberry production

In Europe there are many countries, such as Germany, Austria, Denmark, Italy, Hungary, Poland and Switzerland that sustain both an important fruit production and processing industry (Charlebois, 2007). In Portugal, the elder plant is mainly found in the north of the district of Viseu, at the south of the Douro River, in the Douro-Sul region which includes the counties of Tarouca, Armamar, Lamego, Tabuaço and Moimenta da Beira (Figure 3.3). This region is surrounded by several mountains that create a favorable microclimate for the development of this species (OPAV, 2009).

Figure 3.3. Portuguese Douro-Sul region, where elder is mainly found (ARS-Norte, 2008).

Elder cultivation in Portugal can not be dissociated from the Port wine production's history. By the 1730s, the port industry was blighted by scandal: sugar was being added and elderberry juice being used to give color to poor, overstretched wines. In 1756 the Marquis of Pombal created the Douro Wine Company to regulate the Port wine trade. One of the company's first regulations was the delineation of the Douro wine region as the only sanction area that could produce wine labeled and sold as "Port" (Robinson, 1994). The company also banned the use of elderberry juice and other adulterants, going so far as to mandate that all elder shrubs in the Douro be ripped out (Stevenson, 2005). This is probably the main reason

why elder has not been very popular in Portugal and remains relatively unknown. Moreover, little information has been published on the market potential and production volumes and costs of elderberries. Indeed, elder is not included in the official statistics of fruit production by the Portuguese National Statistical Institute, INE.

Until a few years ago, elder shrubs would only be found along agricultural edges and fence lines (sometimes to delimitate vineyards), as well as on moist areas along rural walking paths and water lines close to small villages. Birds, which are attracted by the colorful elder fruits, played an important role in elder colonization. However, elder cultivation is slowly picking up momentum in Portugal and is being recognized as an alternative to traditional cultures, like the apple and olive ones. Elder was even considered a priority culture by the Portuguese government in 2008, along with other fruit crops, and incentives were given to improve production (OPAV, 2009).

In 2008 there were in the Douro-Sul region 850 elder producers, which owned approximately 700 hectares of orchards that yielded 3500 tonnes of elderberries. Among these producers, 443 sold their 1200 tonnes of berries to the local agricultural cooperative that exported the entire production to Germany (OPAV, 2009). A considerable increment in elderberry production was observed in the last few years. Indeed, between 2002 and 2004 the cooperative received an average of 654 tonnes of elderberries per year, corresponding to a commercial transaction of 261 thousand euros (per year) and representing a significant complement to the income of elder producers (OPAV, 2009). Portuguese elderberries, raised under full sun, are considered of good quality and are very appreciated by Central European countries, which use them in a myriad of food products with health-promoting connotations. Indeed, there is a trend towards higher flavonoid levels, including anthocyanins, in plants grown under warmer and sunnier climates when compared to cooler regions (Aherne and O'Brien, 2002).

In February of 2010, an elderberry refrigerating and stalk remover facility was inaugurated in Tarouca, and there are now rigid guidelines for harvesting, trucking and cooling berries, which are then sent to Germany in refrigerated trucks. The traditional way of preserving elderberries by drying is now abandoned. Elderberries used to be dried under sun after harvesting, using a laborious procedure during one or two weeks, that included removing of stalks using a sieve, dropping berries in a terrace or a threshing-floor, covering them at night to avoid nighttime humidity and regular mixing to avoid berries to become sticky. The success of this operation depended on the weather conditions - a heavy rain could spoil if not all, at least part of the annual production. In addition, the high temperatures most certainly

attained by such a big dark mass of fruits under the hot summer sun, and the molds that most probably developed were certainly detrimental to elderberries quality, compromising their future industrial applications.

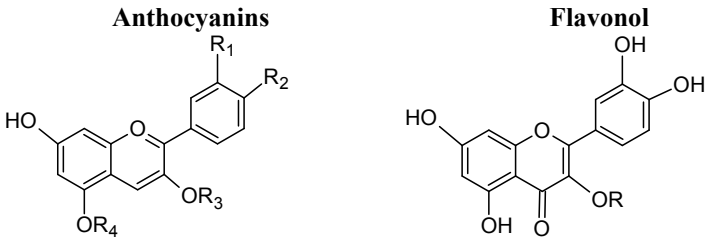
The increasing demand for healthy food and the publication of numerous papers pointing at the health potential of elderberries are expected to create a renewed interest in processing elderberries inside Portugal, and an efficient and profitable elderberry production system will probably be assembled.

3.1.4. Phenolic composition of elder plant

3.1.4.1. Elderberries

There is now sufficient amount of scientific evidence to sustain most of the enthusiastic commercial claims of elder products. Elder medicinal potential comes from its superior antioxidant activity which in turn comes from its high content of phenolic compounds. Among most common fruits and vegetables, and even among berries, elderberry is one of the richest in terms of total phenolic compounds and total anthocyanins, and one of the strongest in terms of antioxidant capacity (Lugasi and Hovari, 2003; Bermúdez-Soto and Tomás-Barberán, 2004; Wu et al., 2004a; Nakajima et al., 2004). Wu and co-workers (2004b) quantified anthocyanins in over 100 common fruits (29 fruits, 26 vegetables, 14 nuts and dry fruits, 15 spices and 19 other foods) and found out that elderberry had the second highest amount of anthocyanins (1374 mg per 100 g fresh weight) only surpassed by chokeberry (1480 mg per 100 g fresh weight) and far above all the other tested. Even grapes, a common source of rich-anthocyanin extracts, presented an anthocyanin content of 27-120 mg per 100 g fresh weight, depending on the variety. Similar results have been reported by Bridle and Timberlake (1997).

Cyanidin-3-glucoside and cyanidin-3-sambubioside are the main anthocyanins present in elderberries. Also present in elderberries, but in lower amounts, are cyanidin 3-sambubioside-5-glucoside, cyanidin 3,5-diglucoside, cyanidin 3-rutinoside, pelargonidin 3-glucoside and pelargonidin 3-sambubioside (Figure 3.4) (Braga et al., 2002; Nakajima et al., 2004; Wu et al., 2004a). After anthocyanins, flavonols are the second most important phenolic compounds present in elderberry. Quercetin 3-rutinoside (rutin) (Figure 3.4) is the most representative flavonol (Bermúdez-Soto and Tomás-Barberán, 2004), having a highly effective antioxidant activity (Zheng and Wang, 2003).



Name				
Anthocyanins	**R_1**	**R_2**	**R_3**	**R_4**
Cyanidin-3-glucoside	OH	OH	Glucose	H
Cyanidin-3-sambubioside	OH	OH	Xylose-glucose	H
Cyanidin 3-sambubioside-5-glucoside	OH	OH	Xylose-glucose	Glucose
Cyanidin 3,5-diglucoside	OH	OH	Glucose	Glucose
Cyanidin 3-rutinoside	OH	OH	Rutinose	H
Pelargonidin 3-glucoside	H	OH	Glucoside	H
Pelargonidin 3-sambubioside	H	OH	Xylose-glucose	H
Flavonol	**R**			
Quercetin-3-rutinoside	Rutinose			

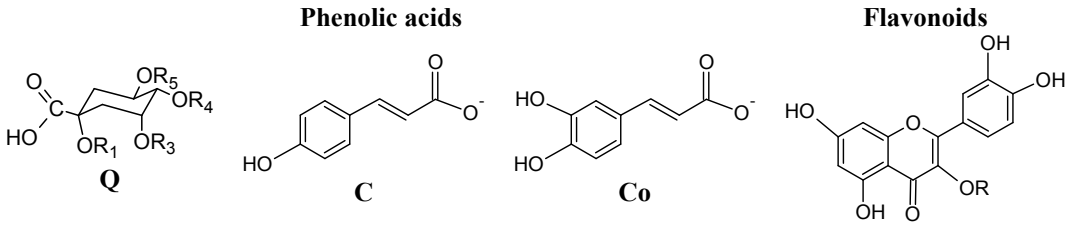
Figure 3.4. Chemical structures of anthocyanins and flavonol in elderberries.

3.1.4.2. Elderflowers

Eleven phenolic acids and six flavonol glycosides were detected in fresh elderflowers by Christensen and co-workers (2008) (Figure 3.5).

The flavonol glycosides quercetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside and isorhamnetin-3-*O*-rutinoside are the major flavonoids in elderflowers comprising over 90% of the total flavonoid content, whereas the major phenolic acids are 5-*O*-caffeoylquinic acid and 1,5-di-*O*-caffeoylquinic acid comprising over 70% of the total phenolic acid content. Moreover, the concentration of flavonoids in fresh elderflowers was found to vary from 21.0 mg/g (dry weight) for quercetin-3-*O*-rutinoside to 0.5 mg/g for isorhamnetin-3-*O*-glucoside (Christensen et al., 2008).

Besides phenolic compounds, more than 100 different volatile compounds were identified by some authors, despite they only agree on a few, maybe due to the differences among the analyzed elder cultivars. Moreover, the different extraction and concentration techniques that have been used for the isolation of the volatile compounds probably influenced their results (Toulemonde and Richard, 1983; Joulain, 1987; Christensen et al., 2008). Christensen and co-workers (2008) identified that the important contributors to the floral and elderflower flavor of the obtained extracts were rose oxides, hotrienol, linalool, linalool derivatives and *α*-terpineol. They also identified that the fruitiness and freshness of the extracts were mainly due to non-oxidized monoterpenes, aliphatic aldehydes and alcohols.



Q = quinic acid, C = caffeoyl, Co = *p*-Coumaroyl

Name				
Phenolic acids	**R_1**	**R_3**	**R_4**	**R_5**
3-*O*-Caffeoylquinic acid	H	C	H	H
4-*O*-Caffeoylquinic acid	H	H	C	H
5-*O*-Caffeoylquinic acid	H	H	H	C
1,5-Di- *O*-Caffeoylquinic acid	C	H	H	C
3,5-Di- *O*-Caffeoylquinic acid	H	C	C	H
3,4-Di- *O*-Caffeoylquinic acid	H	C	C	H
4,5-Di- *O*-Caffeoylquinic acid	H	H	C	C
3-*O*-*p*-Coumaroylquinic acid	H	Co	H	H
5-*O*-*p*-Coumaroylquinic acid	H	H	H	Co
Flavonoids	**R_1**	**R_2**	**R_3**	
Quercetin-3-*O*-rutinose	OH	OH	Rutinose	
Quercetin-3-*O*-glucoside	OH	OH	Glucose	
Quercetin-3-*O*-6´´-acetylglucoside	OH	OH	6´´-acetylglucoside	
Kaempferol-3-*O*-rutinoside	H	OH	Rutinose	
Isorhamnetin-3-*O*-rutinoside	OCH_3	OH	Rutinose	

Figure 3.5. Chemical structures of phenolic acids and flavonoids in elderflowers.

3.1.5. Health benefits of elder berries and flowers

The high phenolic contents of elderberries (in particular, of anthocyanins) have motivated diverse scientific studies aiming to demonstrate the health benefits of these red fruits. There are several studies on elder focusing on antioxidant activity and consequent health-promoting abilities of elderberries (either specific phenolic compounds present in elderberries, juice, extracts, or elderberry-rich commercial formulations, such as Sambucol®) (Table 3.1). Moreover, elderflowers, which are known in the pharmaceutical field as Sambuci Flos, are described in pharmacopeias as diaphoretic agents for the treatment of fever and chills, and as expectorant for the treatment of mild inflammation of the upper respiratory tract (WHO, 2002). While many of the reported effects lack adequate scientific validation, there is an increasing number of studies supporting important therapeutic properties associated with elder flowers, as reported in Table 3.1.

Table 3.1. Scientific studies on health benefits of elder berries and flowers.

Activity	Reference
Elderberry juice	
High total phenolic compounds content and high antioxidant capacity	Bermúdez-Soto and Tomás-Barberán, 2004
Elderberry extract	
Antioxidant activity (*in vitro* assays)	Abuja et al., 1998 Wang et al., 1997 Seeram and Nair, 2002
Antioxidant activity (*in vivo* assays - human aortic endothelial cells)	Youdim et al., 2000
Immune response enhancer	Barak et al., 2001 Zakay-Rones et al., 1995
Anti-influenza activity (human clinical trials)	Zakay-Rones et al., 2004
Anticarcinogenic potential	Thole et al., 2006
Anti-influenza virus (H1N1) activity	Roschek Jr. et al., 2009
Sambucol	
Antiviral properties, especially against the human influenza virus *(in vitro* and *in vivo* assays)	Zakay-Rones et al., 1995 Burge et al., 1999 Zakay-Rones et al., 2004
Activate the healthy immune system by increasing inflammatory and anti-inflammatory cytokines production	Barak et al., 2002
Synergistic formula comprising six berry extracts, including elderberry	
High ORAC[1] value, low cytotoxicity, superior anti-angiogenic[1] and anti-carcinogenic potentials	Bagchi et al., 2004
Antioxidant activity (*in vivo* assays - rats, mice, and rabbits)	Bagchi et al., 2006
Elderflowers	
Anti-inflammatory	Mascolo et al., 1987 Yesilada et al., 1997
Antiviral versus influenza types A and B and herpes simplex virus type 1	Serkedjieva et al., 1990
Diuretic	Beaux et al., 1998
Antidiabetic	Gray et al., 2000

[1]ORAC - oxygen-radical absorbing capacity. Anti-angiogenic - the ability to reduce unwanted growth of blood vessels which can lead to varicose veins and tumor formation.

3.1.6. Extraction from elder berries and flowers

Table 3.2 reports the current state-of-the-art for extractions performed using the elder plant, either applying conventional or other extraction methodologies. Emphasis is given on the studied process conditions.

Table 3.2. State-of-the-art for extractions from elder plant.

Objective	Process parameters studied	Reference
Flowers, berries and leaves		
Optimization of accelerated solvent extraction (ASE) for HPLC analysis of rutin and isoquercitrin	*ASE* Solvent: aqueous methanol (20-100%, v/v) T: 20-200 ºC P: 40-200 bar Static extraction time: 5-30 min	Dawidowicz et al., 2003
Evaluate antioxidant properties of alcoholic extracts and relate to composition	*Pressurized liquid extraction* Solvent: aqueous ethanol (80%) T: 20-200 ºC P: 60 bar Static time: 10 min	Dawidowicz et al., 2006
Elderflowers		
Study the influence of genotype and conventional solvent extraction (CSE) conditions on extracts composition, adequate for human consumption	*CSE* Solvent: aqueous acetonitrile (50%); 3 sugar levels; 4 acid citric levels; sodium benzoate; potassium sorbate T: 20-200 ºC	Christensen et al., 2008
Elderberries		
Obtain extracts from grape marc and elderberries with high anthocyanin contents, stable during storage and potentially interesting for commercial applications	*Single-step CSE* Solvent: aqueous ethanol, ethyl-acetate and acetone T: 20, 40, 60ºC pH: 1-5 *Two-step extractions: SFE (CO_2) followed by CSE*	Vatai et al., 2009

3.1.7. Elderberry pomace: agro-residue from elderberry juice production

Processing of elderberries into juice generates huge amounts of elderberry pomace which comprises pressed skins, disrupted cells from the berry pulp and seeds, and corresponds to 25 to 40% of the initial berry weight (Brønnum-Hansen et al., 1985). This residue has potential for valorization due to its high phenolic compounds and oil contents. Since anthocyanins biosynthesis is stimulated by light (Manach et al., 2004), they can be mainly found in the skins of fruits. It is estimated that 75 to 98% of total berry anthocyanins remain in the pomace after pressing (Brønnum-Hansen et al., 1985), which definitely is an invaluable opportunity for valorization and may represent a further business opportunity. Furthermore, considering that phenolic-rich agro-residues represent a major disposal problem (Capasso et al., 1992), it would also represent an improvement in elderberry processing residues management policies.

Additionally, elderberry seeds hold over 15% of oil. The unique available study on elderberry seeds oil composition dates from 1902 (Byers and Hopkins, 1902). As stated by these authors, elderberry seeds oil contains high levels of unsaturated fatty acids (~68% of oleic acid and ~6% of linoleic acid) and a lower proportion of saturated fatty acids (~22% of

palmitic acid), resembling olive oil composition (67, 5 and 28% for the three acids, respectively). This composition makes elderberry seeds oil rather appropriate to be used in the food industry, since the intake of unsaturated fatty acids goes within guidelines for a healthy diet. In fact, unsaturated fatty acids are known to reduce low-density lipoprotein (LDL or "bad" cholesterol) levels and to slightly raise high-density lipoprotein (HDL or "good" cholesterol) levels, providing an anticholesterol effect and protecting against heart diseases (Hu et al., 2001). Grape seeds oil is an example of a well established marketed product, which is obtained by pressing seeds of grape pomace (a byproduct of the winemaking industry). Like elderberry oil, grape seeds oil has also a high oleic acid content (Beveridge et al., 2005) which makes it interesting as a good anticholesteremic and dietetic oil (Oomah et al., 1998), with proven ability to reduce LDL and raise HDL levels. It is also used in other high-value applications such as a cosmetic ingredient or as a nutritional supplement. Therefore, and due to its oil composition, elderberry seeds oil can also be envisaged for the same applications as grape seeds oil.

3.1.8. Contribution of the Thesis to the state-of-the-art in extraction from elder pomace and flowers

The contribution of this Thesis to the state-of-the-art in extraction from elder may be divided into two major parts. The first one corresponds to the raw material itself, and the second one corresponds to the applied high pressure extraction methodology. This Thesis suggests for the first time the valorization of elderberry pomace, a residue of elderberry juice processing industries, for the production of phenolic-rich extracts, using environmental friendly solvents and processes.

The developed work permitted to conclude that elderberry pomace does not need to be treated as a solid effluent of environmental concern. Instead, it has great potential to be valorized as a raw material for the production of high-valuable products with consumer acceptance in the pharmaceutical, food, nutraceutical and cosmetic fields. In addition, elderflower, an agro-residue that is originated from the elder plant pruning process (commonly performed to enhance fruit production), was also evaluated as a raw material for the production of potentially useful extracts.

Extraction experiments were performed using different methodologies, with particular focus on high pressure extraction processes using supercritical carbon dioxide and pressurized solvent mixtures of carbon dioxide, water and ethanol. Operational extraction conditions were

evaluated in an attempt to find a correlation with the characteristics of the obtained extracts in terms of composition and antioxidant activity. Special emphasis was given to the composition of the high-pressure solvent mixture. The work developed in this Thesis provided a deep understanding of the extraction process itself and may possibly be extended to other vegetable matrices.

3.2. Maritime pine (*Pinus pinaster*)

3.2.1. Botanical description of pine tree

Maritime pine (*Pinus pinaster* Ait.) is a conifer native to south-western Europe and north-western Africa, with major forestry developments on the Atlantic coast of southern France, Spain and Portugal (Figure 3.6). The *P. pinaster* species, a member of the *Pinaceae* family, is sometimes split into two or three subspecies (Farjon, 1998), but the differences between them are small (minor details of leaf anatomy). In Portugal, the dominant subspecies is the *atlantica*. This species can be found in quite different environments, from sea level to high elevations and from regions characterized by a heavy annual rainfall to dry regions (Alía and Martín, 2003).

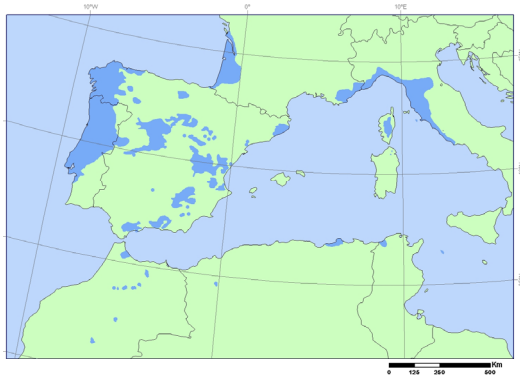


Figure 3.6. Natural distribution area of *P. pinaster* (Alía and Martín, 2003).

Maritime pine is a medium-size tree (Figure 3.7) reaching 20 to 40 m tall and with a trunk diameter of 40 to 50 cm. Pine trees can live as long as 200 years, though they do not usually surpass 100 years. When compared to other Mediterranean pine species, maritime pine has longer needles (that occur in pairs), and larger cones (Figure 3.7). Its bark is reddish brown, thick and deeply fissured at the base of the trunk, somewhat thinner in the upper crown (Figure 3.7) (Alía and Martín, 2003; Correia et al., 2007).

Figure 3.7. Maritime pine trees and detail of pine needles, cones and bark.

3.2.2. Situation of the pine sector in Portugal

The evolution of the Portuguese pine forest area since 1874 is reported in Table 3.3. The low value corresponding to 1874 is attributed to an underestimation of the real pine forest area, due to the use of different classification criteria (Leite and Martins, 2000). Between 1902 and 1974, the area of pine forest expanded by over 36%, mainly due to a systematic reforestation program whose main objective was to secure coastal sand dune areas and forestation of mountain regions. From 1974 onwards, pine forest area has reduced, especially after 1985, due to widespread forest fires and to a strong trend for the substitution of certain areas of conifers by fast growing broad-leaved species, like eucalyptus.

Pine wilt, which is a disease of the *Pinus* species caused by the pinewood nematode (*Bursaphelenchus xylophilus*), has also been responsible for vast and irreversible damages in the Portuguese pine forest. Pinewood nematode was first found and identified in Portugal in 1999, in the Setúbal Peninsula (Mota et al., 1999). Since then, several measures have been established in order to delineate the extent of the infested area and to prevent the spread to the remainder of the country, as well as to other European Union state members. However, those measures did not prevent the spread of pine wilt disease in Portugal, and an increasing number of trees have been affected since 1999, reaching more than 250000 trees in 2006 (Mota et al., 2009).

All these factors have contributed to the reduction of the Portuguese pine forest area, which represented approximately 46% of the total Portuguese forestry area in 1974 while in 2006 it represented only 23% (Leite and Martins, 2000; DGF, 2006).

Table 3.3. Evolution of the Portuguese pine forest area (Leite and Martins, 2000; DGF, 2006).

Year	1874	1902	1928	1956	1974	1985	1995	2000	2006
Portuguese pine forest area, × 1000 ha	210	1020	1198.6	1309	1388	1343.6	1080.8	976.1	710.6

Despite the crisis that has been affecting the pine sector in the last 30 years, it still represents an important component of the total Portuguese forest economic value (around 17%), being the third most important species after eucalyptus and cork oak (Mendes, 2007). The industrial forestry sector based on maritime pine is characterized by thousands of small companies, mostly of family structure, distributed in the north and center of the country. The main industrial activities related with pine are sawmills, wood panels, pulp and paper, carpentry, packing and furniture (De Sousa, 2000). The sawmill industry is the primer transformer of pine wood, providing to other industries a vital supply of residual products. In 2004, the amount of wood (with no bark) that was produced in Portugal was 4.1 million cubic meters, of which 2.1 millions were used by sawmills and 1.8 millions were used by the paper and pulp, and wood panel industries (Mendes, 2007).

Portuguese pine sector industries need to develop in order to increase competitiveness in international terms and to stop the under-utilization of the economic potential of pine forest in Portugal. Strategies for this may include the resolution of those problems that affect forest production (like the degradation of national forest resources) as well as the technological development of the existent industries (De Sousa, 2000). Exploitation of pine bark, a residue from the pine wood-processing industries, could give a quite positive contribution to this industry as well.

3.2.3. Pine bark: agro-residue from pine wood processing industries

Pine bark is an abundant residue since it represents 10 to 20% of the pine tree trunk. It presents various favorable features such as some important phytochemicals constituents, low price and long-term stability that together make the usage of this waste highly attractive (So and Eberhardt, 2006). Nevertheless, at present it is almost exclusively used as fuel, being also subjected to composting, utilized to cover public gardens, or simply thrown away on landscapes (Fradinho et al., 2002).

Bark is a very complex tissue that is composed by two main zones: the inner bark (phloem) and the non-living outer bark (rhytidome). The inner bark includes all tissues from the vascular cambium to the innermost periderm being composed of several types of cells. The outer bark includes the tissues outside this periderm and serves the function of protecting the living inner bark. Its thickness varies greatly between and within species and with the age of the bark (Rowell et al., 2005).

3.2.4. Chemical composition of pine bark

The chemical composition of pine barks varies between and within species, depending on several factors, such as localization in the tree (base, middle and top), age, growing conditions of trees (like soil, climate and solar exposition) and method of sampling (Labosky, 1979; Karchesy and Hemingway, 1980; Harun and Labosky, 1985; So and Eberhardt, 2006). In general, chemical constituents of pine barks can be classified into four major groups (Rowell et al., 2005):

- polysaccharides (cellulose, hemicellulose, and pectic materials),
- lignin and other phenolic compounds,
- hydroxy acid complexes (suberin),
- and extractives (fats, oils, phytosterols, resin acids, waxes, tannins, terpenes, phlobaphenes, and flavonoids).

Table 3.4 illustrates the typical chemical composition of *P. pinaster* bark.

Table 3.4. Average chemical composition of *P. pinaster* bark (Nunes et al., 1996).

Component	Percent oven-dry weight
Polysaccharides	41.7 ± 0.9
Lignin and other phenolic compounds	43.7 ± 2.4
Suberin	1.5 ± 0.2
Extractives	11.4 ± 2.2

The extractive contents of tree barks are quite high when compared to the corresponding contents of wood, and these bark extractives do interfere in polysaccharides and lignin analyses. Therefore, procedures for elucidation of the chemical composition of barks usually begin with an extraction protocol that consists of sequential extraction solvents of increasing polarity (Rowell et al., 2005), as elucidated in Table 3.5.

Table 3.5. Extraction steps for analysis of tree barks (Rowell et al., 2005).

Step	Extraction solvent	Extractives
1	Diethyl ether	Waxes, fatty acids, fats, resin acids, phytosterols, and terpenes
2	Ethanol	Condensed tannins, flavonoids, and other phenolics
3	Hot water	Condensed tannins and water-soluble carbohydrates
4	1% aqueous NaOH	Flavonoids, hemicelluloses, and suberin monomers

Vázquez et al. (1987a) performed successive extractions of five different samples of *P. pinaster* outer bark with hexane plus benzene, ether, ethanol, water and 1% aqueous sodium hydroxide. The obtained extract yields are reported in Table 3.6. The hexane and benzene soluble fractions were markedly hydrophobic yellow colored wax-like materials. Paper chromatography of these fractions revealed a quite complex mixture of compounds, where stilbenes were included. Paper chromatography of the other organic and aqueous extracts revealed the presence of flavonoids. These authors confirmed that the phenolic compounds present in these extracts corresponded to polyflavonoids with phloroglucinol A rings that gelify very fast in the presence of formaldehyde. The interest of these authors in the hydroxylation pattern of the A rings of the flavonoids was related to the possible application of pine bark in wood adhesives production. Considering that, Vázquez et al. (1987a) also subjected *P. pinaster* bark to direct extraction with a 1% aqueous sodium hydroxide (with no pretreatment with organic or inorganic solvents). The portion of bark weight solubilized by alkaline lixiviation was 39.3%, corresponding approximately to the sum of all extract fractions reported in Table 3.6. Roughly 80% of this extract were formaldehyde-condensable polyphenols, and the remaining 20% were suberin and wax degradation products, uronic acids, polysaccharides (and their degradation products) among other non-specified compounds.

Table 3.6. Extract content of *P. pinaster* bark (Vázquez et al., 1987a).

Extraction solvent	Extract yield (%, w/w)
Hexane + benzene	2.2 - 3.4
Ether	0.8 - 1.0
Ethanol	10.5 - 12.6
Water	2.0 - 6.6
1% aqueous sodium hydroxide	20.0 - 25.6

Vázquez et al. (1987a) also determined that the polysaccharides content in *P. pinaster* bark was approximately 29%, with glucose accounting for half of the reducing compounds. Moreover, extracts aromatic content (which provides an indication of all the polyphenols present in bark) was 60%, and its Klason lignin (which is the acid-insoluble lignin) was 30%.

The most representative phenolic compounds of *P. pinaster* bark are (+)-catechin, (-)-epicatechin, dihydroquercetin, phenolic acids and, most of all (approximately 65%), condensed tannins dimers, trimers, oligomers and polymers having a mean polymerization degree of 10.6 (Jerez et al., 2007b; Jerez et al., 2009).

3.2.5. Market potential of pine bark products

There are quite a few possible applications of pine bark widely reported in scientific literature. Most of them are related with its rich phenolic composition, mainly with condensed tannins. The two most reported ones are its use as a phenol substitute in the formulation of adhesives for wood derivatives (Pizzi, 1980; Pizzi and Merlin, 1981; Pizzi, 1982; Yazaki and Collins, 1994; Pizzi et al., 1994; Li and Maplesden, 1998; Jorge et al., 2002; Kim et al., 2003) and as a source of alternative (natural) antioxidants (Pietta et al., 1998; Kähkönen et al., 1999; Wood et al., 2002; Vuorela et al., 2005; Touriño et al., 2005; Guri et al., 2006; Jerez et al., 2007a; Jerez et al., 2007b; Ku et al., 2007; Weber et al., 2007). Additionally, condensed tannins from different pine species have received considerable attention in the fields of nutrition, health, and medicine owing to their physiological and biological activities, namely antibacterial, antiviral, anticarcinogenic, anti-inflammatory and cardiovascular system diseases prevention (Karonen et al., 2004; Cos et al., 2004; Vuorela et al., 2005; Koleckar et al., 2008). There is even a patented aqueous ethanolic extract from the bark of French maritime pine, Pycnogenol®, which is marketed worldwide as a food supplement or as an herbal-based medication (Rohdewal, 2002). Clinical studies have indicated that Pycnogenol® is effective in the treatment of several health conditions, such as the treatment of chronic venous insufficiency (Arcangeli, 2000), the improvement of endothelial function of hypertensive patients (Liu et al., 2004b) and the lowering of plasma glucose levels of diabetic patients (Liu et al., 2004a).

However, pine bark extracts have potential for other applications rather than the ones already referred, such as a chromium substitute in leather tanning applications (Kurth and Hubbard, 1951; Covington, 1997; Saravanabhavan et al., 2004), and as an adsorbent to treat residual effluents (Vázquez et al., 2002; Brás et al., 2004).

Besides phenolic compounds, polysaccharides contained in *P. pinaster* bark are also potentially useful for the manufacture of other marketable products. Enzymatic hydrolysis can be carried out to recover those polysaccharides existing in the lignocellulosic waste, after extraction of phenolic compounds. The sugars thus obtained could provide a fermentation medium to be used for production of many chemicals (Vázquez et al., 1986; Vázquez et al., 1987b). Therefore, an integrated process could be envisaged and developed, taking advantage of both carbohydrates and phenolic compounds present in *P. pinaster* bark.

3.2.6. State-of-the-art in extraction from pine bark

The scientific literature on extraction from bark of different pine species is vast and may be divided into two main groups, depending on their applications: *(i)* extraction of formaldehyde-condensable polyphenols for adhesives formulations to apply in the wood panels industry, and *(ii)* extraction of phenolic compounds with high antioxidant activity for food, nutraceutical, cosmetic and pharmaceutical applications. Conventional solid-liquid extraction methodologies at low pressure have been typically applied in both cases. However, aqueous alkaline solutions either or not with sulfite salts have been preferentially used in the first case, while aqueous alcoholic solvents have been frequently used in the second one. Table 3.7 reports the most relevant scientific studies in the field.

Table 3.7. Relevant scientific studies on extraction from pine bark.

Objective	Process parameters studied	Reference
Adhesives formulation		
Study of the effect of the solvent polarity on: extract yield, molecular weight, molecular weight distribution, viscosity and gel time.	*CSE* Different pine species Type of solvent: water, aqueous alkaline, sulfite	Weissmann, 1983
Study of the effect of alkaline extraction conditions on extraction yield and composition.	*CSE* T Solvent: NaOH concentration	Vázquez et al., 1986
Study of the effect of alkaline extraction conditions on the yield of formaldehyde-condensable polyphenols.	*CSE* T Time Solvent: alkali type and concentration	Vázquez et al., 1987a
Examination of the chemical nature of the NaOH extracts.	*CSE* Solvent: NaOH concentration	Yazaki and Aung, 1988
Minimize auto-condensation and self-polymerization of tannins during aqueous extractions of pine bark.	*CSE* Solvent: metabisulfite and urea concentration Lab and industrial scale	Sealy-Fisher and Pizzi, 1992
Determination of the rheological characteristics of pine bark extracts obtained by successive extractions using aqueous NaOH solutions of increasing alkalinity.	*CSE* Solvent: NaOH concentration	Kim and Mainwaring, 1995
Study of the influence of geographical origin and anatomic characteristics on alkaline extracts composition, in terms of type of phenolic compounds present.	*CSE* Size of pine bark particles T Solvent: NaOH concentration	Vázquez et al., 2000
Study of the effect of alkaline extraction conditions on the yield of formaldehyde-condensable polyphenols and reactivity of extracts.	*CSE* T Solvent: NaOH concentration Solid/liquid ratio	Vázquez et al., 2001
Development of a NMR-based method to determine the extent of extracted material removed from bark.	*CSE* Storage time of milled bark Lab and pilot scale Solvent: sulfite concentration	Grigsby et al., 2003

Table 3.7. Continuation.

Objective	Process parameters studied	Reference
Antioxidant activity formulation		
Determination of the nature and content of condensed tannins in the barks of some European tree species.	*CSE* Fractionation of the water: methanol (1:1, v/v) extract	Matthews et al., 1997
Study of the influence of some critical extraction variables on the phenolic yield and antiradical activity of the resultant ethanolic extracts.	*CSE* T: 25-50ºC Time: 60-90 min Liquid/Solid ratio: 5:1 and 10:1	Jerez et al., 2006
Evaluate the antioxidant efficacy of extracts obtained from six pine species.	*CSE* Fractionation of the chloroform: methanol (3:1, v/v) extract	Guri et al., 2006
Composition analysis		
Determination of phenolic contents of barks from eleven pine species and correlation with antioxidant activity.	*CSE* T Solvent: water, ethanol:benzene (1:2, v/v), 1% NaOH	Ku et al., 2007
Estimate the condensed tannin content and composition of the bark from two varieties of pine and their antiradical activity.	*CSE* Different pine species Fractionation of the ethanolic extract	Jerez et al., 2007a
Characterize different fractions obtained from ethanolic extracts of pine bark for tannin content, degree of polymerization and antiradical activity.	*CSE* Different pine species Fractionation of the ethanolic extract	Jerez et al., 2007b
Optimize SFE process parameters and obtain extracts in both laboratory and pilot scale under optimized conditions; predict the supercritical CO_2 extractability of the chosen phenolic substances in pine bark by sonication with different solvents.	*SFE (CO_2)* P: 100-300 bar T: 27.5-80 ºC Solvent: Ethanol 1% and 3% Solvent/solid ratio: 30:1 and 50:1 Lab and pilot scale *Sonication* Type of solvent	Yesil-Celiktas et al., 2009
Analyze condensed tannins of two varieties of pine by MALDI –TOF MS[1].	*CSE* Different pine species Fractionation of the ethanolic extract	Jerez et al., 2009

[1]MALDI –TOF MS: Matrix-assisted laser desorption and ionization time-of-flight mass spectrometry.

3.2.7. Contribution of the Thesis to the state-of-the-art in extraction from maritime pine bark

In this Thesis, new alternatives were suggested for utilization of maritime pine bark, an abundant, not perishable, all-year available, inexpensive and tannin-rich residue of pine wood industries. Environmental friendly extraction processes and solvents were used to separate fractions to be applied as vegetable tanning agents in leather industry, as well as fractions possessing high antioxidant and anti-inflammatory activities adequate for other valuable applications like the pharmaceutical, nutraceutical, cosmetic and food ones.

Pine bark extracts appropriate for leather tanning applications were achieved using conventional extraction. The optimization of the applied aqueous solvent additive was performed considering the desired extracts characteristics for these applications. This work was performed in collaboration with a Portuguese tanning company (Curtumes J.B. Salgueiro Lda.), which was interested in reducing the usage of chromium salts and consequently its pollution burden, and was eager to diversify and improve the quality of its final leather products. The conclusions of this research had direct implications on the company's production.

Additionally, maritime pine bark fine extracts were obtained by high pressure extraction methods, using carbon dioxide and mixtures of carbon dioxide and ethanol, either or not in the supercritical state. Several experimental conditions were studied, like pressure, temperature, fractionation, solvent flow rate and composition of the solvent mixture. The characteristics of the achieved extracts confirmed that pine bark is a raw material that has a great potential to be processed into consumer high-valued products for several applications.

3.3. Tara (*Caesalpinia spinosa*)

3.3.1. Botanical description of tara plant

Tara, whose scientific name is *Caesalpinia spinosa* (Mol.) O. Kuntz, is a small leguminous tree of the *Caesalpinaceae* family, indigenous to the Cordillera region of Bolivia, Peru and northern Chile. This tree is also found in Ecuador, Colombia, Venezuela and Cuba, and nowadays it is also grown in Morocco and East Africa (Wielinga, 2010). Tara has been used since the pre-Hispanic era in popular medicine, being known by a wide diversity of common names in Spanish speaking countries such as *taya*, *algarroba*, *huarango*, *guaranga*, and *caranca* (De la Cruz Lapa, 2004). Tara tree is 2 to 5 m high, has alternate, evergreen leaves that consist of 3-10 pairs of primary leaflets under 8 cm in length, and has yellow to orange flowers with 6-7 mm petals. Tara tree produces flat oblong pods, which are 6-12 cm long and 2.5 cm wide, being orange to red when ripe (Figure 3.8). Every pod contains 4 to 7 rigid round seeds that are black when mature (Figure 3.8) and that correspond to approximately 33% of the weight of the pod. Tara seeds contain 28% by weight of coat, 34% of endosperm (Figure 3.8) and 37.5% of germ (De la Cruz Lapa, 2004).

Figure 3.8. Tara pods, whole seeds and de-coated seeds (endosperm halves).

3.3.2. Peruvian production of tara pods

The main commercial interest in tara tree remains on its pods, which are a source of diverse products for various applications in the food, cosmetic, pharmaceutical and leather tanning industries. Peru is the world's largest producer of tara pods with 21072 tonnes in 2009 (MINAG, 2010), representing 80% of the world wide production. The 2009´s tara pods production represents an increase of almost 20% relatively to the 2005´s production and almost 200% relatively to the 1993´s one (Li Pereyra, 2006). This production increase is the reflex of an accentuated rise in tara products demand (over 90% between 2006 and 2008) by some developed countries such as the United States, Germany, Spain and Italy, which contributed to the development of a US$23 million industry in Peru (I-Dev International, 2009). Even though much of tara Peruvian production still relies on wild collection, the number of farmers who actively cultivate tara grew from several hundred to many thousand in the last few years, following initiatives driven to promote this crop in this country. International demand had increased so much that a flood of new players, including new processors, exporters and whole-sale distributors entered the tara market and profit from buying, processing and selling tara (Li Pereyra, 2006).

3.3.3. Popular uses of tara pods and commercial products available

In Peru, tara pods are used by local inhabitants to prepare folk medicine remedies for treating various health problems. Gargling infusions of tara pods provide a natural medication for inflamed tonsils, washing wounds, fevers, colds and stomach aches. Tara is also used in eyewash for its anti-bacterial properties (Duke, 1981; De la Cruz Lapa, 2004). However, there is little scientific evidence on the medicinal properties and uses of this plant. Nevertheless, tara pods are known to contain up to 25% of gallic acid (Galvez et al., 1997), which has a well-known antimicrobial activity (Adesina et al., 2000; Panizzi et al., 2002) and certainly contributes to the observed tara pods medicinal properties.

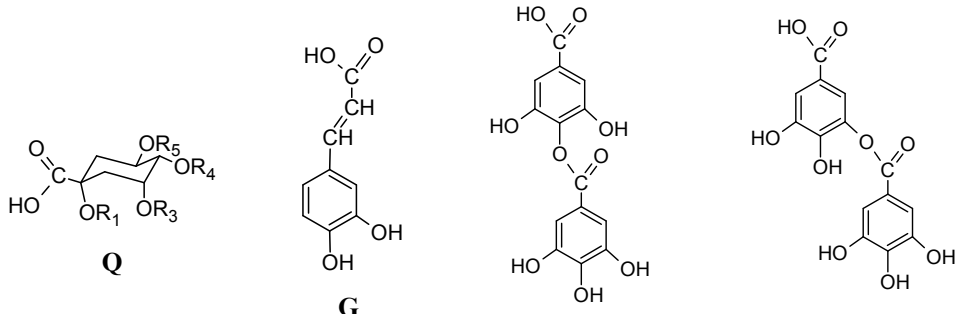
The main commercial product that is originated from tara pods is tara powder, which is obtained by crushing the de-seeded pods that corresponds to approximately 67% of the total pods weight. Tara powder contains around 54% of tannins and is used as a natural tanning agent in the leather industry being very much appreciated due to its light color, therefore giving quite bright, light-resistant leather (De la Cruz Lapa, 2004; ProFound, 2009). The crushed de-seeded pods are also extracted using hot water, followed by extract filtration, concentration and spray-drying. This extract contains 66-71% of tannins and can be further purified in order to obtain tannic acid, gallotannic acid or gallic acid which can lead to a wide range of applications in the food and pharmaceutical industries (De la Cruz Lapa, 2004).

Tara seeds, also considered a byproduct of the tara powder industry, do have commercial applications as well. The ground endosperm of these seeds (Figure 3.8) can be a raw material for the production of tara gum, and it was approved in 1996 by the European Union (as additive E417) for use as a thickener, stabilizer and gelling agent in food (Wielinga, 2010). Tara gum is a white to off white powder with a neutral flavor, odor and color, and consists of polysaccharides of high molecular weight which are composed mainly by galactomannans (80%).

3.3.4. Tannin composition of tara pods

Tara tannins are of the hydrolysable type, being predominantly gallotannins based on a galloylated quinic acid structure whose main constituent is gallic acid (Haslam and Haworth, 1962). Thus, they differ from other members of the hydrolysable tannin group which are based upon a galloylated or ellagoylated hexose. Clifford et al. (2007) performed LC-MS^n on commercial tara tannin and detected that the galloylquinic acids of tara tannin can be divided into three groups: (i) mono-, di-, tri- and tetragalloylquinic acids that lack depsidic galloyl residues; (ii) depsides[1] related to the 3,4,5-trigalloyl structure previously considered to be the major components of tara tannin (Haslam and Haworth, 1962); and (iii) depsides related to the 1,3,4,5-tetragalloylquinic acid. Figure 3.9 illustrates the major tara tannins and respective chemical structures.

[1] Depsides - A class of compounds formed by condensation of a phenolic carboxylic acid (such as gallic acid) with a similar compound, the reaction being between the carboxylic acid group on one molecule and a phenolic OH group on the other.



Q = quinic acid, G = gallic acid, diG = digalloylquinic acid

Name	R_1	R_3	R_4	R_5
3-*O*-galloylquinic acid	H	G	H	H
5-*O*-galloylquinic acid	H	H	H	G
4-*O*-galloylquinic acid	H	H	G	H
3,4-di-*O*-galloylquinic acid	H	G	G	H
3,5-di-*O*-galloylquinic acid	H	G	H	G
4,5-di-*O*-galloylquinic acid	H	H	G	G
3,4,5-tri-*O*-galloylquinic acid	H	G	G	G
1,3,4,5-tetra-*O*-galloylquinic acid	G	G	G	G
4-*O*-galloyl, 5-*O*-(digalloyl)quinic acid	H	H	G	diG
5-*O*-galloyl, 4-*O*-(digalloyl)quinic acid	H	H	diG	G

Figure 3.9. Major tara tannins and respective chemical structures (Clifford et al., 2007).

3.3.5. Tara seed coat, an agro-residue from tara pods processing

In tara gum production there are a few methods to accomplish the separation of the endosperm from the seed germ and coat. In the thermo-mechanical process, the endosperm is separated by differential grinding, sifting and sieving of the seed, which are purely mechanical operations that keep the process cost at low levels. Alternative methods may employ extraction with water followed by precipitation with alcohol and drying, or chemical treatment with hot sulphuric acid, followed by intense and efficient washing with water (Duke, 1981; Wielinga, 2010).

In the thermo-mechanical process, tara germ and coat are recovered as byproducts. Tara germ is mainly used as animal feed or as a source of protein hydrolysates (Del Re-Jiménez and Amadò, 1989). Tara coat does not have commercial applications, thus representing an agro-residue of tara processing industries.

3.3.6. Contribution of the Thesis to the state-of-the-art in extraction from tara seed coat

In this Thesis, the usage of tara seed coat, an abundant residue of tara pods processing industries, was suggested for the first time. This research was performed in collaboration with a Peruvian company (Transformadora Agrícola S.A.C.) that uses tara pods to produce tara

powder and tara gum. This company showed interest in the valorization of this residue so as to improve profitability of the whole production process. Considering the high tannin content of the whole tara pods, a methodology typically applied for the separation of fractions rich in phenolic compounds, adequate for food, cosmetic and pharmaceutical applications was studied. Therefore, a pressurized solvent extraction methodology using environmental friendly solvents was applied. The solvent mixture composition effect on the final characteristics of the obtained extracts was investigated using statistical mixture design. Conclusions of this study highlighted the potential that this agro-residue has to be processed into valuable products.

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Part B. Elder (*Sambucus nigra*) pomace and flower

4. Fractionated high pressure extraction of anthocyanins from elderberry pomace - preliminary study

This Chapter resulted from the initial stages of the research program. It reports the employment of a successful extract fractionation methodology that consisted in changing the solvent polarity, from supercritical CO_2 (in a first extraction step) to a pressurized solvent mixture of CO_2, ethanol and water (in a second extraction step). A preliminary study on the effect of some process variables was performed. These variables were raw material humidity and composition of the second step employed solvent mixture. The contents of this chapter are published in: Seabra, I.J. Braga, M.E.M, Batista, M.T.P., De Sousa, H.C (2010) Fractioned high pressure extraction of anthocyanins from elderberry (*Sambucus nigra* L.) pomace. *Food and Bioprocess Technology: An International Journal 3*: 674-683.

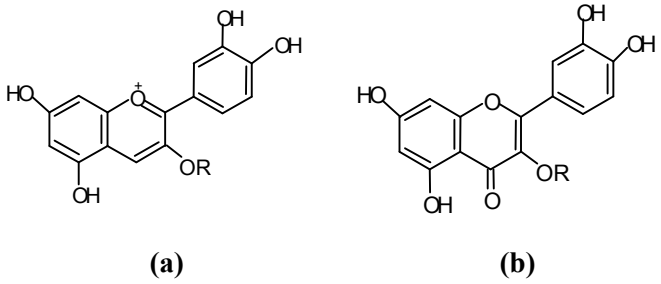
4.1. Abstract

Fractionated high pressure extractions from dry and *in natura* elderberry pomace were performed in order to obtain anthocyanin rich extracts. Experiments were carried out using CO_2 supercritical fluid extraction (SFE) followed by pressurized solvent extraction (PSE) with CO_2/ethanol-H_2O mixtures (1-100%, v/v), to obtain anthocyanin rich fractions in the 2^{nd} step, at 313 K and ~20 MPa. Higher extract yields, anthocyanin contents and antioxidant activities occurred by the presence of water, both in the raw material and in the solvent mixture. The CO_2 dissolved in the PSE solvent mixture favored either anthocyanin contents or antioxidant activities, which were not directly related. Comparing to the literature data for elderberries and grapes, these fractions had higher anthocyanins contents. From these results, an added economical value to this agro-industrial residue is proposed, using solvents and techniques "generally regarded as safe" in the food and pharmaceutical industries.

4.2. Introduction

Presently, one of the most important trends in food and pharmaceutical industries is the growing demand for valuable natural sources of antioxidant compounds. Among common fruits and vegetables, elderberry (*Sambucus nigra* L.) is one of the richest in total phenolics and anthocyanins and, consequently, in antioxidant capacity (Bermúdez-Soto and Tomás-Barberan, 2004; Wu et al., 2004). Cyanidin 3-glucoside (CyG) and cyanidin 3-sambubioside (CyS) are the main anthocyanins present (Braga et al., 2002) and quercetin 3-rutinoside (rutin) is the most representative flavonol (Bermúdez-Soto and Tomás-Barberan, 2004) (Figure 4.1).

Most part of literature focus essentially on elderberry fruit studies and few work has been devoted to elderberry pomace regardless of its high anthocyanin content (75-98% of total berry anthocyanins) when compared to its weight (25-40% of total berry weight) (Brønnum-Hansen et al., 1985). Despite its significant potential value, elderberry pomace does not own an important economic high value as it is traditionally used as animal feed or as an organic fertilizer. However, with some additional and proper processing, this byproduct could be easily transformed, from a residual low-value status into a very interesting high-value one, for consumer-accepted uses in food, cosmetic and pharmaceutical industries.



(a) **(b)**

Figure 4.1. Main flavonoids present in elderberries: **(a)** cyanidin 3-glucoside (R - glucose) and cyanidin 3-sambubioside (R - xylose-glucose); **(b)** quercetin 3-rutinoside (R - rutinose).

Anthocyanins are polar molecules which are normally extracted from raw plant tissues by conventional solvent extraction (CSE) methodologies, using polar solvents slightly acidified with acids or sulfites. For some applications, high pressure extraction processes, like supercritical fluid extraction (SFE) and pressurized solvent extraction (PSE), already proved to represent valuable alternatives to CSE. SFE is an attractive "tunable" technique which may remove selectively various active constituents from plant matrices. Moreover, it can be particularly attractive for the extraction of antioxidants and anthocyanins because it may avoid their thermal degradation and, due to the absence of light and oxygen, it can prevent oxidation reactions (Díaz-Reinoso et al., 2006). In food and pharmaceutical applications, CO_2 is the most employed supercritical solvent. The main drawbacks of CO_2 are its non-polar

nature and its inability to extract high molecular weight compounds which might limit the extraction performance. Even though the addition of suitable cosolvents such as ethanol (EtOH) is the most frequent employed strategy to overcome these problems, a more recent employed approach is its association with other methodologies, such as PSE. PSE involves the use of H_2O or organic solvents, at considerable elevated temperatures (313-473 K) and pressures (3.3-20.3 MPa). It offers the possibility to perform efficient and "enhanced" extractions due to its improved characteristics in terms of mass transfer and of solvating properties, which can even be improved by the utilization of gas-expanded liquids, obtained upon the dissolution of CO_2 in H_2O or in an organic solvent. This method takes advantage of the beneficial combination between typical liquids solvation properties and the advantageous transport properties of supercritical fluids (Chamblee et al., 2004), and it was already applied to the extraction of polar solutes (Yuan and Olesik, 1997). Another unique and potentially useful property of CO_2-aqueous and CO_2-alcohol gas-expanded liquids is the generation of *in situ* carbonic acid and alkyl carbonic acid, respectively (Towes et al., 1995; West et al., 2001). In anthocyanins extraction this fact can be an important advantage: there will be a temporary reduction in the extraction medium pH value and this will increase anthocyanins stability and cell membrane permeability, leading to higher diffusivities (Türker and Erdoğdu, 2006; Norton and Sun, 2008). Moreover, it also can inactivate unwanted enzymes (Kamat et al., 1995; Norton and Sun, 2008) and microorganisms (Foster et al., 2003) that may destroy these pigments (Delgado-Vargas and Paredes-López, 2003; Garcia-Palazon et al., 2004). On the other hand, the presence of undesired compounds in several vegetal matrixes, which may be co-extracted or which may interfere negatively with the extraction of desired substances, decreasing extraction yield and selectivity, is a typical situation in natural products extraction methodologies (conventional and supercritical). A common way to overcome this situation is to employ extraction fractionated procedures (Pasquel et al., 2000; Reverchon, 1997; Reverchon et al., 1999), using different solvent mixtures at different stages of the extraction process.

In this work we explored the use of high pressure fractionated extraction of elderberry pomace, using environmental friendly and food/pharmaceutical accepted solvents and techniques, in order to obtain anthocyanin rich extracts which can be used as natural colorants, dietary supplements or as phytochemical products. For all extraction experiments, a CO_2 SFE was employed in a first extraction step and, subsequently, different CO_2/EtOH/H_2O mixtures were employed in a PSE step. The influence of the polar PSE solvent mixture

composition and of the raw material humidity were studied concerning extract yield, anthocyanin content and antioxidant activity, and compared to the results obtained by CSE.

4.3. Materials and methods

4.3.1. Raw material

In natura elderberries, provided by Cooperativa Agrícola do Vale do Varosa (Tarouca, Portugal), were collected in August 2004 according to Neto and Monteiro (2002) and stored under vacuum, at 255 K. Elderberry pomace was obtained by mechanical pressing (Hafico, Neuss, Germany) and dehydrated in a fluidized bed dryer (MK II, Sherwood Scientific, Cambridge, England) at 308 K, in the absence of light, for 12 hours. Pomace was milled in a grinder (Braun, KSM 2, Kronberg, Germany) for 2 minutes, conditioned under vacuum at 255 K, and its H_2O content was determined gravimetrically (triplicate assays).

4.3.2. Chemicals

Carbon dioxide (99.998%, Praxair, Madrid, Spain), ethanol (99.5%, Panreac Quimica S.A., Barcelona, Spain) and distilled H_2O were used for the extraction experiments. Chemicals/solvents employed for extract analyses were: ethanol (p.a.), methanol (Lichrosolv), formic acid (98-100%), hydrochloric acid (p.a.), glacial acetic acid (p.a.), n-hexane (96%) and ethyl acetate (99.5%), purchased from Merck (Darmstadt, Germany), p-anysaldehyde (Sigma-Aldrich Inc., MO, USA), 2-aminoethyl diphenylborinate (97%, Fluka, Steinheim, Germany), 2,2-diphenyl-1-picrylhydrazyl (~90%, Sigma-Aldrich Inc., Steinheim, Germany) and Ultra pure Milli Q water. Standards used for TLC analyses were quercetin dehydrate (≥98%), rutin hydrate (≥95%), D-(+)-catechin hydrate (98%), and gallic acid (≥98%), purchased from Sigma-Aldrich Inc. (Steinheim, Germany) and (-)-epicatechin (≥90%, Fluka, Buchs, Switzerland). Standards used for HPLC analysis were cyanidin 3-glucoside chloride (analytical grade, Extrasynthèse, Genay, France) and rutin (extra pure, Merck, Darmstadt, Germany).

4.3.3. Experimental extraction procedures

4.3.3.1. Conventional solvent extractions

These extractions were carried out using either an EtOH/H_2O (8:2, v/v) mixture or H_2O, at 313 K. Dried elderberry pomace was homogenized using an Ultra-turrax (Ystral, D79282, Ballrechten-Dottingen, Germany) slowly increasing the rotational velocity from 8000 to

24000 rpm, during the ~3 min extraction time, and employing a 1:10 (w/v) solid-to-solvent ratio. The resulting slurry was then centrifuged (3000 rpm, 15 min) and filtered. The filter cake was re-extracted for four times, until depletion of its anthocyanin characteristic color, and filtrates were combined and concentrated using a rotary evaporator (BÜCHI Rotavapor R-114, Flawil, Switzerland) at 313 K. Later, extracts were lyophilized (FTS Systems Inc., N.Y., USA) and kept at 255 K. All procedures were made in the absence of light. The experimental conditions employed are reported in Table 4.1.

4.3.3.2. Fractionated high pressure extractions

These extractions were carried out using the apparatus presented in Figure 4.2. Liquid CO_2 and EtOH, EtOH/H_2O (8:2, v/v) or H_2O were delivered to the extraction cell using high pressure liquid pumps (respectively, home-built and L-6200A, Hitachi, Merck, Darmstadt, Germany). Solid-to-solvent ratios utilized are reported in Table 4.1. A stainless steel extraction cell (~30 mL) was filled with elderberry pomace in three layers separated by glass beads (4 mm diameter), in order to achieve a uniform distribution of the solvent flow and a reduction of the dead space in the cell. Cotton wool was placed on both endings of the cell, to prevent line obstructions. Extraction cell was placed into a water bath with temperature controlled by an immersion circulator (±0.1 K, DC30, Thermo Haake, Karlsruhe, Germany) and pressure was maintained by a back-pressure regulator (26-1762-24-090, Tescom, Selmsdorf, Germany) and measured by a pressure transducer (C204, Setra, Boxborough, USA). Extracts were recovered in a recovering flask and a trap, placed in an ice bath, and the expanded CO_2 flow was measured by a wet gas meter (DM3C ZE 1411, G.H. Zeal Ltd., London, England).

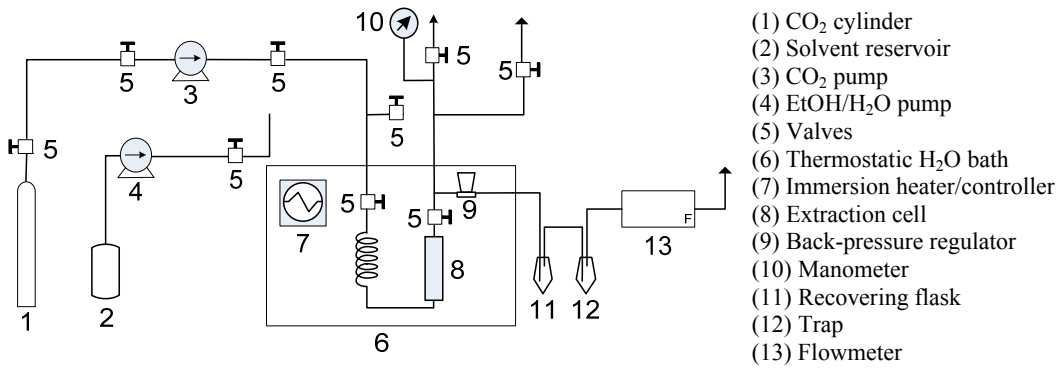


Figure 4.2. Schematic diagram of the employed high pressure extraction apparatus.

A two-step fractionated high pressure extraction methodology was employed, comprising: *i*) a 1st CO_2 SFE step in order to remove the low polarity CO_2-soluble compounds (15 min static + 40 min dynamic period); tubing line was cleaned with EtOH; *ii*) a 2nd PSE step (45 min), in order to extract polar compounds like anthocyanins, and wherein CO_2 and/or EtOH, EtOH/H_2O (8:2, v/v) or H_2O were introduced into the system; different amounts of EtOH and EtOH/H_2O (90 and 100%, v/v) were assayed for *in natura* and for dried elderberry pomace. These solvent mixtures will be named ethanolic solvents. CO_2 and H_2O mixtures were also assayed with dried elderberry pomace, employing different percentages of H_2O (10-100%, v/v). Operational conditions of both steps (313 K and 20 MPa) were selected based on the literature information concerning the solubility (in supercritical CO_2) of the main components of vegetable oils, triglycerides (Bamberger et al., 1988), and on the anthocyanin stability (Jackman et al., 1987). Extracts were concentrated at 313 K, under vacuum and in the absence of light, and kept at 255 K. For the 1st SFE step, extraction curve was determined in order to define the extraction time that assured a high lipophilic material removal from the plant material. Some PSE assays were duplicated, in order to determine the experimental error of the yield values; the others were single. Employed experimental conditions are reported in Table 4.1.

4.3.4. Characterization of elderberry pomace extracts

4.3.4.1. Thin layer chromatography

TLC analyses were performed using silica gel plates with a 254 nm fluorescent indicator (20 cm × 20 cm × 0.2 mm) (Fluka, Steinheim, Germany). Same extract concentrations were chromatographed. For low polarity compounds analysis, hexane-ethyl acetate (8:2, v/v) and an anisaldehyde solution (Wagner et al., 1984) were used as the mobile phase and the spray reagent, respectively. For the analysis of phenolic compounds, the mobile phase was ethyl acetate-formic acid-glacial acetic acid-H_2O (100:11:11:27, v/v) solution and a NP solution (methanol-2-aminoethyl diphenylborinate, 99:1, v/w) was used for detection of phenolic compounds, at 365 nm.

4.3.4.2. High-performance liquid chromatography

Quantification of anthocyanins and rutin was performed in a Gilson apparatus equipped with a diode-array detector. An ODS-2 column (250×4.6 mm i.d., 5 μm, Spherisorb S5, Waters, MA, USA), at 298 K and a C18 guard cartridge (30×4 mm i.d., 4 μm, Hichrom, Berkshire, UK) were used. A mobile phase, constituted by aqueous formic acid (5%, v/v) (A)

and methanol (B), was used with a discontinuous gradient of 5-15% B (0-10 min), 15-25% B (10-15 min), 25-50% B (15-40 min) and 50-80% B (40-50 min), followed by an isocratic elution during 10 min, at a flow rate of 1 mL/min. Samples were adjusted to pH ~2 with HCl and microfiltered (0.20 μm) before HPLC injection. Anthocyanins were identified from their chromatographic and UV spectral properties and the major anthocyanins, cyanidin 3-glucoside (CyG) and cyanidin 3-sambubioside (CyS), by comparing with the CyG standard and according to literature data (Brønnum-Hansen and Hansen, 1983; Wu et al., 2004). Quantification of anthocyanins (CyS, CyG and total anthocyanins, TA) and rutin (R) was carried out using external standards (CyG and R), at 520 nm and 360 nm, respectively. The correlation between peak area/concentration was assessed by the least-squares regression model. One of the extracts was injected three times to determine the standard deviation of the assay.

4.3.4.3. Antioxidant activity: DPPH assay

The antioxidant activity of the extracts was evaluated by using the DPPH method (Blois (1958). Aliquots (100 μL) of extracts, were added to 500 μL of a methanol solution (500 μM) of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical in the presence of 100 mM acetate buffer, pH 6.0 (1 mL) and Methanol (1.4 mL). After mixing for 30 seconds, the reaction mixtures were kept, in the dark, at room temperature, for 30 min and the absorbance was measured, at 517 nm, on a UV-Vis spectrophotometer (U-2000, Hitachi, Tokyo, Japan). Triplicate assays were performed. The reducing capacities of the samples were estimated from the observed absorbance decrease and expressed as IC_{50} values, defined as the amount of elderberry extract (d.b.) that decreased, by 50%, the initial absorbance of the DPPH radical solution, at the referred wavelength.

4.3.5. Calculation procedures

Global yields were obtained considering the total extracted mass divided by feed mass in a dry basis (d.b.). For both high pressure extraction steps, the total extract mass was determined by the sum of the extract obtained in the recovering flask and trap; for 1st step the extract recovered from tubing was also considered. The overall SFE curve was constructed using the accumulated mass of extract, collected at a given extraction time interval. The data was fitted by a curve formed by two lines. The fitting was done by minimizing the least regression error in the least squares sense, using the fminsearch function of Matlab (R2007a). The first line

was identified with the constant extraction rate period (CER) and the corresponding kinetic parameters were calculated (M_{CER}, t_{CER} and Y_{CER}), according to Rodrigues et al. (2002).

4.4. Results and discussion

Elderberry pomace represented around 25% (w/w, d.b.) of the total fruit weight. Two lots of dry and one lot of *in natura* pomace were employed, with humidity (± standard deviation, s.d.) of 8.5±0.1, 6.1±0.1 and 61±2% (w/w, d.b.), respectively (Table 4.1).

The elderberry pomace SFE (1st step) exhibited a typical overall extraction curve profile (Figure 4.3), with 5.3×10^{-7} kg/s for M_{CER}, 12.1 min for t_{CER}, and Y_{CER} of 2.0×10^{-3}. For all SFE experiments, extraction was prolonged for 40 minutes to guarantee the diffusional period. The mass of extract recovered from tubing cleaning represented ~9% of the total obtained extract mass in this step. Global yields standard deviation (1.2%) was calculated from three SFE assays. For PSE (2nd step) duplicated assays, higher standard deviations (Table 4.1) were obtained for *in natura* pomace, probably due to the non-homogeneity of the solvent and the H_2O from the raw material.

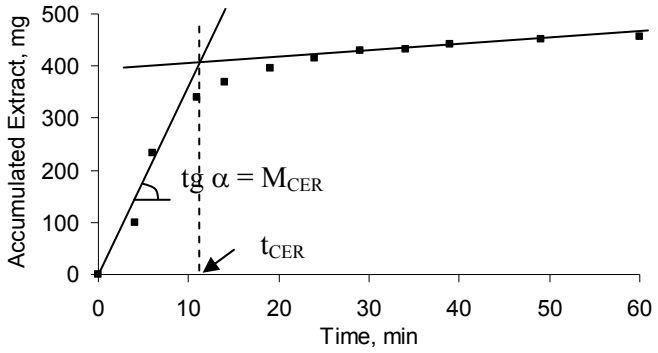


Figure 4.3. Kinetics of the 1st step (SFE) elderberry pomace extraction, at 20.6±0.6 MPa and 313±0.1 K, and at a flow rate of 27.3×10^{-5} kg/s.

The obtained global yields are represented in Figure 4.4. For fractionated high pressure extractions with ethanolic mixtures in the 2nd step (PSE), higher yields of 1st + 2nd steps were always obtained for dried elderberry pomace (~24-35%) when compared to those using *in natura* pomace (~16-18%). The main contribution to this difference was essentially due to the 1st step, wherein the less polar substances were extracted. This could have happened because, for *in natura* pomace, the seeds were not efficiently comminuted, and H_2O may have acted as a barrier to diffusion and may have increased the overall polarity of the solvent (Temelli, 2000; Reverchon and Marrone, 2001). Therefore, drying the byproduct seems to permit a more efficient SFE of low polarity compounds. Since anthocyanins are mainly located in

outermost layer of elderberry skin cell walls (Manach et al., 2004) wherein the fatty acids and waxes exist (Pinelo et al., 2006), an effective lipophilic substances removal, in a 1st step SFE, can make polar material more available for extraction.

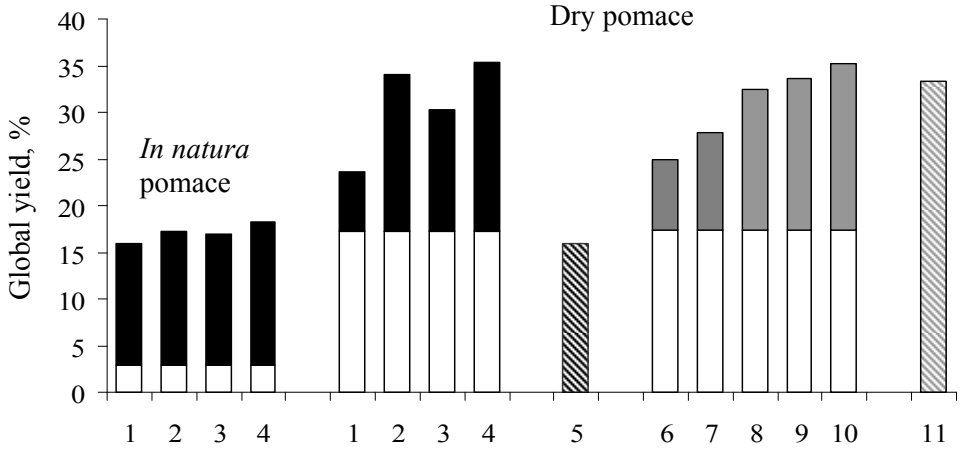


Figure 4.4. Obtained global yields (d.b.) for elderberry pomace extractions. □ SFE-CO_2; Ethanolic extracts ■ PSE: 1 EtOH+CO_2 (10%), 2 EtOH/H_2O+CO_2 (10%), 3 EtOH, 4 EtOH/H_2O; ▨ CSE: 5 EtOH/H_2O; Aqueous extracts ■ PSE: 6 H_2O+CO_2 (90%), 7 H_2O+CO_2 (80%), 8 H_2O+CO_2 (50%), 9 H_2O+CO_2 (20%), 10 H_2O; ▨ CSE: 11 H_2O.

For the 2nd step ethanolic PSE, the solvent mixture, which was in a single homogeneous phase according to Durling et al. (2007), did not significantly affect global yields for the *in natura* pomace (~13-15%), while they had a greater variation for the dry pomace (~6-18%) (Figure 4.4). The initial high *in natura* material H_2O content improved the extraction ability of the solvent mixture, suggesting that the presence of H_2O in the extraction medium could also provide higher yields of more polar compounds, namely anthocyanins, by increasing the solvent mixture density and polarity. In fact, for both raw materials (*in natura* and dried) the presence of H_2O in the solvent mixture resulted in an increment in polyphenol contents (anthocyanins and rutin), and in the antioxidant activity of the extracts (Table 4.1).

The dissolved CO_2 negatively affected PSE yields (decrease of 1-7%) and so, the possible positive influence of the enhanced transport properties of gas-expanded liquids (Chamblee et al., 2004) and the decrease in the solvent mixture pH were not so significant as the negative influence of the decrease in the solvent polarity that happened when CO_2 was added to the solvent (Weikel et al., 2006). However, an increase of TA contents, from 105 to 149 mg/g for *in natura* pomace and from 111 to 120 mg/g for dried pomace (Table 4.1), occurred for the CO_2/EtOH/H_2O gas-expanded mixtures and so, not only the higher solvent mixture density

Table 4.1. Elderberry pomace extractions at 313 K: experimental conditions, global yield, composition and antioxidant activity of obtained extracts.

	Operational conditions			Global yield±s.d.[a], %, (d.b.)	Phenolic[b], mg/g (d.b.)				Ant. activity (IC_{50}, µg)
	Solvent mixture[c], %	Solid-to-solvent ratio	Pomace humidity		CyS	CyG	TA	R	
Conventional solvent extraction at P_{atm}									
CSE (~15min)	EtOH/H_2O	1:33	8.5%	15.9	61	78	153	13	48
	H_2O (100)	1:50		33.3	30	31	67	6	86
Fractionated high pressure extraction at 20±0.6 MPa and 313±0.1 K									
SFE (55 min)	CO_2 (100)	1:200	61%	2.8	-	-	-	-	-
PSE (45 min)	EtOH (90) + CO_2(10)	1:140		13.1	24	38	68	8	122
	EtOH/H_2O (90) + CO_2(10)			14.4±3.2	59	72	149	14	63±16
	EtOH (100)			14.2±1.8	33	43	85	8	110±7
	EtOH/H_2O (100)			15.4±2.1	44	48	105	9	77±10
SFE (55 min)	CO_2 (100)	1:200	8.5%	17.2	-	-	-	-	-
PSE (45 min)	EtOH (90) + CO_2 (10)	1:100		6.4	33	24	62	9	150
	EtOH/H_2O (90) + CO_2(10)			16.8±0.3	52±1.1	54±0.5	120±1.1	10±0.1	57±5
	EtOH (100)			12.7±1.3	24	35	66	8	171±30
	EtOH/H_2O (100)			18.2±0.5	45	55	111	10	57±7
SFE (55 min)	CO_2 (100)	1:200	6.1%	17.4±1.2	-	-	-	-	-
PSE (45 min)	H_2O (10) + CO_2 (90)	1:167		7.5	18	16	46	10	46
	H_2O (20) + CO_2 (80)			10.5	25	24	65	12	40
	H_2O (50) + CO_2 (50)			15.1	15	14	39	9	41
	H_2O (80) + CO_2 (20)			16.3	20	22	52	8	44
	H_2O (100)			17.9	37	41	89	9	52

[a] s.d. - standard deviation. [b] CyS: cyanidin-3-sambubioside; CyG: cyanidin-3-glucoside; TA: total anthocyanins; R: rutin. HPLC evaluation of polyphenol contents using external standards (CyG and R). The correlation peak area/concentration was assessed by least-squares regression model. [c] EtOH/H_2O mixture had always a fixed proportion of 8:2 (v/v).

and polarity but also the solvent's pH drop, can have important roles in the extraction of these polar substances (West et al., 2001). Therefore, it seems that, for this ternary solvent mixture, CO_2 played a similar role as sulfites and weak acids in the conventional solid-liquid extraction of anthocyanins from natural matrixes. In fact, for both raw materials, the highest TA contents and antioxidant activities were obtained when the solvent was the CO_2/EtOH/H_2O gas-expanded mixture, presenting *in natura* pomace similar TA content (149 mg/g) as the CSE ethanolic extract (153 mg/g) that was the highest obtained. Similar antioxidant activities were verified for the *in natura* and the dried pomaces: IC_{50} of 63±16 μg and 57±5 μg, respectively.

When EtOH/H_2O was used as the extracting solvent, a 2% increase in yield was obtained for the PSE relative to the CSE, and so, high pressure was more favorable to the extraction yield than simultaneous milling, stirring and extraction. The lower solid-to-solvent ratio used in PSE (1:100) when compared to the one used in CSE (1:33) also favored extraction yield. For this solvent mixture (EtOH/H_2O, 8:2 v/v), and at these particular conditions of pressure and temperature, PSE seems to be not an advantageous alternative to CSE, if the objective is to obtain anthocyanin rich fractions having high antioxidant activities.

Dawidowicz et al. (2006) performed pressurized liquid extraction of *Sambucus nigra* berries, using an EtOH/H_2O (8:2, v/v) mixture as the extracting solvent, at 6 MPa and 293 K, and obtained an extract with ~1.1 mg/g of rutin and ~5 mg/g of CyS + CyG. The difference between the phenolic compositions in that extract and the ones obtained in this study from elderberry pomace can be related to the different extraction methodologies (no fractionation, lower operational pressure and temperature) and the raw materials nature.

Because H_2O seemed to have a positive influence on the observed PSE global yields, this solvent was tested for dry pomace, just employing CO_2 and H_2O (10-100%) on the 2nd extraction step. Higher global yields occurred when the H_2O content was incremented in the PSE solvent mixture (Figure 4.4), that increased the relative amount of the H_2O rich liquid phase (a gas-expanded liquid) but did not change the composition of the two phases present, which remained constant (Perakis et al., 2006). Therefore, the observed raise in PSE yields was just due to an increment in the amount of the high density and polarity phase, which presented a better capacity to dissolve polar substances like anthocyanins. This raise is similar to the one that occurs in conventional solid-liquid extraction when solvent-to-solid ratio increases (Cacace and Mazza, 2002). As happened with the ethanolic solvent, when no CO_2 was added, a maximum PSE yield was attained (~18%), showing a negative CO_2 influence on extraction yield.

In contrast, there was not a direct relationship between the H_2O content in the pressurized solvent mixture and the TA and rutin extract contents, or antioxidant activities. Even though the highest amount of TA (89 mg/g) was obtained when no CO_2 was added to the solvent mixture, the extracts obtained with the CO_2/H_2O gas-expanded liquid showed higher antioxidant activities (IC_{50} 40-46 μg). These results suggest that the pH drop that followed the possible carbonic acid formation (pH may decrease down to 2.8, according to Towes et al. (1995)) did not influence positively the amount of extracted anthocyanins, but possibly influenced their stability, once these pigments are known to be at the most stable state at pH 1 to 3 (Delgado-Vargas and Paredes-López, 2003). In fact, PSE aqueous extracts IC_{50} values were close to the ethanolic CSE value (48 μg), despite having around three times less anthocyanins. However, rutin contents and other eventually extracted substances, like proanthocyanidins, may have also contributed to the extracts antioxidant activity.

Comparing the aqueous extraction methodologies, and in opposite to what happened with the ethanolic experiments, extraction from a non defatted raw material and simultaneous milling and extraction was more favorable to extraction yield than high pressure (Table 4.1). Although, the obtained high CSE yield was due to the co-extraction of compounds that did not contribute to the extract antioxidant activity, as is confirmed when TA and rutin contents (67 and 6 mg/g respectively), and antioxidant activity (IC_{50} ~ 86 μg) are compared to ethanolic CSE ones (153 and 13 mg/g respectively, and IC_{50} ~ 48 μg).

The presence of low polarity compounds in extracts was monitored by using anisaldehyde sprayed TLC plates (Figure 4.5). For PSE, the presence of zones that migrated with the solvent (II and III) in some extracts indicates that lipophilic compounds were not completely extracted during SFE. The lipophilic composition of these extracts varied with the nature of the employed solvent, being almost absent in aqueous extracts (Figure 4.5). Strong zones at the solvent front (III) appeared in those PSE ethanolic extracts in which H_2O was not used in the solvent mixture, which means that the presence of H_2O avoided the co-extraction of high lipophilic compounds, playing an anti-solvent role. Despite the fact that the low polarity substances were not completely extracted, TLC analysis was an indication that CO_2 SFE was capable of extracting lipophilic/low polarity compounds, which resulted in the concentration of phenolic compounds and other polar substances in the vegetal matrix for the subsequent extraction step.

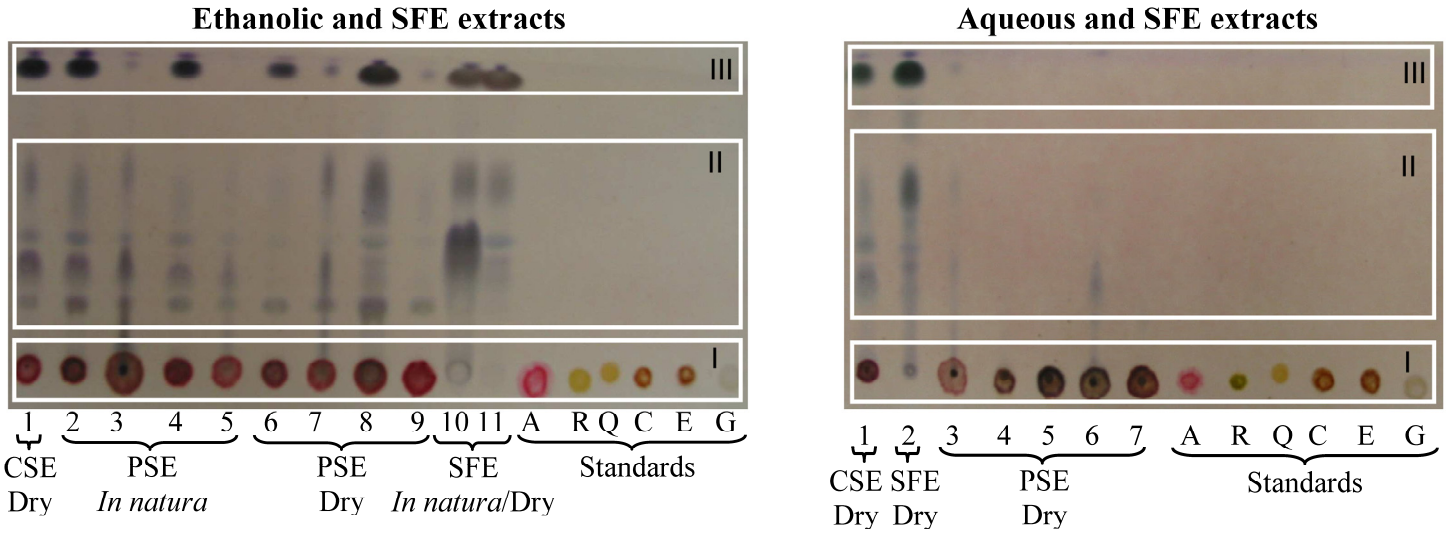


Figure 4.5. Analysis of low polarity compounds by anisaldehyde sprayed TLC. Conventional extracts at 313 K and high pressure extracts at 20.6±0.6 MPa and 313±0.1 K. Ethanolic and SFE extracts: 1 CSE-EtOH/H_20; 2, 6 PSE-EtOH+CO_2; 3, 7 PSE-EtOH/H_2O+CO_2; 4, 8 PSE-EtOH; 5, 9 PSE-EtOH/H_2O; 10, 11 SFE-CO_2. Aqueous and SFE extracts: 1 CSE-H_20, 2 SFE-CO_2, 3 PSE-H_20 (10%), 4 PSE-H_20 (20%), 5 PSE-H_20 (50%), 6 PSE-H_20 (80%), 7 PSE-H_20 (100%). Standards: A Cyanidin 3-glucoside, R Rutin, Q Quercetin, C Catechin, E Epicatechin, G Gallic acid.

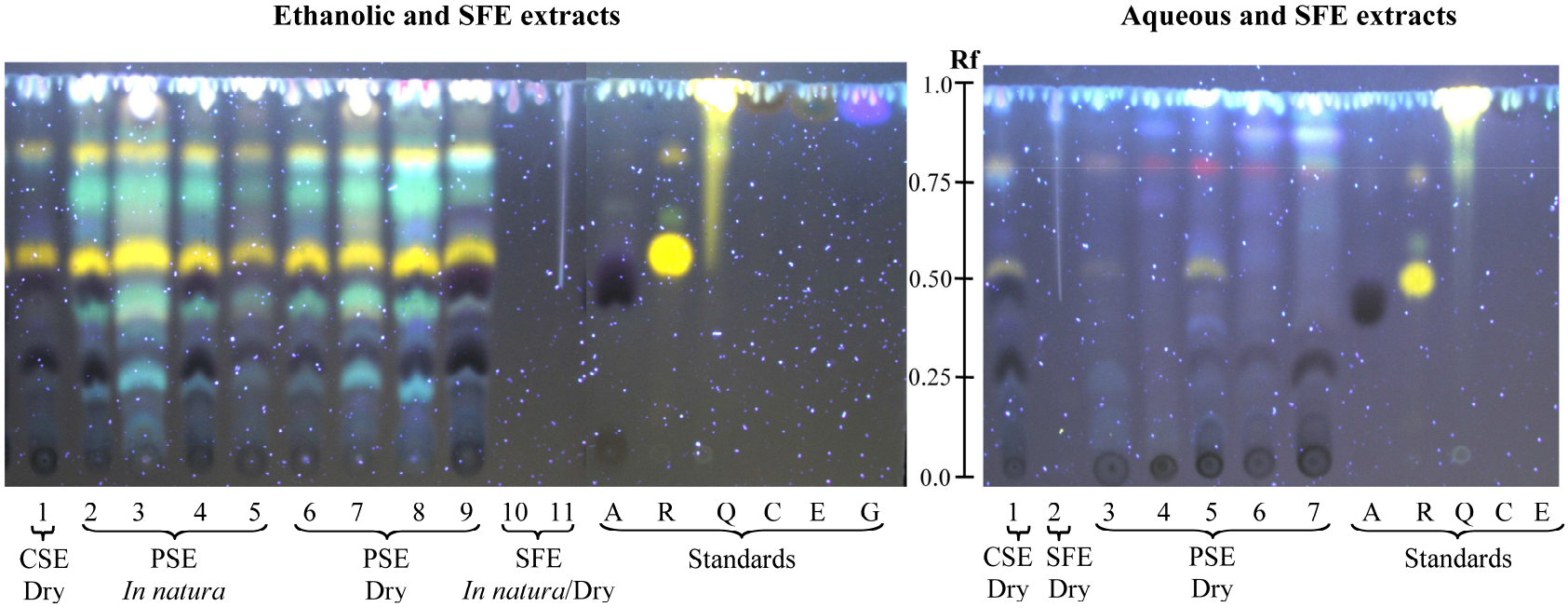


Figure 4.6. Analysis of high polarity compounds by NP sprayed TLC plates, observed at 365 nm. Conventional extracts at 313 K and high pressure extracts at 20.6±0.6 MPa and 313±0.1 K. Ethanolic and SFE extracts: 1 CSE-EtOH/H_20; 2, 6 PSE-EtOH+CO_2; 3, 7 PSE-EtOH/H_2O+CO_2; 4, 8 PSE-EtOH; 5, 9 PSE-EtOH/H_2O; 10, 11 SFE-CO_2. Aqueous and SFE extracts: 1 CSE-H_20, 2 SFE-CO_2, 3 PSE-H_20 (10%), 4 PSE-H_20 (20%), 5 PSE-H_20 (50%), 6 PSE-H_20 (80%), 7 PSE-H_20 (100%). Standards: A Cyanidin 3-glucoside, R Rutin, Q Quercetin, C Catechin, E Epicatechin, G Gallic acid.

The overall phenolic composition of the extracts was monitored by using NP sprayed TLC plates (Figure 4.6). Several zones appeared in both ethanolic and aqueous CSE and PSE extracts, with higher intensity for the ethanolic ones: two main dark blue zones with Rf 0.28 and Rf 0.48 (CyG), five weaker light blue zones (corresponding to non identified anthocyanins), two orange fluorescent zones at Rf 0.55 (R) and at Rf 0.8, that may correspond to quercetin glycosides, and two light blue fluorescent, that may be assigned to phenol carboxylic acids (Wagner et al., 1984), among others. The other standards used in the analysis appeared at the solvent front and were not identified in extracts.

For most extracts, the major anthocyanin was found to be CyG and the sum of CyS and CyG contents represented around 90% of the TA contents (Table 4.1, Figure 4.7). These results are similar to those already reported in literature for elderberries (Wu et al., 2004). In general there was not a direct relationship between TA contents and antioxidant activity of the extracts, which is in agreement with the results obtained by Kähkönen et al. (2001) and Nakajima et al. (2004) for berry extracts obtained by CSE, using different solvents.

There was not an evident and direct relationship between rutin contents and the antioxidant activity of the extracts, either, although extracts with higher antioxidant activity (aqueous PSE (20% H_2O) - IC_{50} of 40 µg, and ethanolic CSE – IC_{50} of 48 µg) were among the ones with higher rutin contents. These results are in accordance with the fact that the relationship between antioxidant activity of berry extracts and their phenolic composition is complex (Kähkönen et al., 2001) and, in addition, synergism can have a significant effect on the antioxidant response of plant extracts.

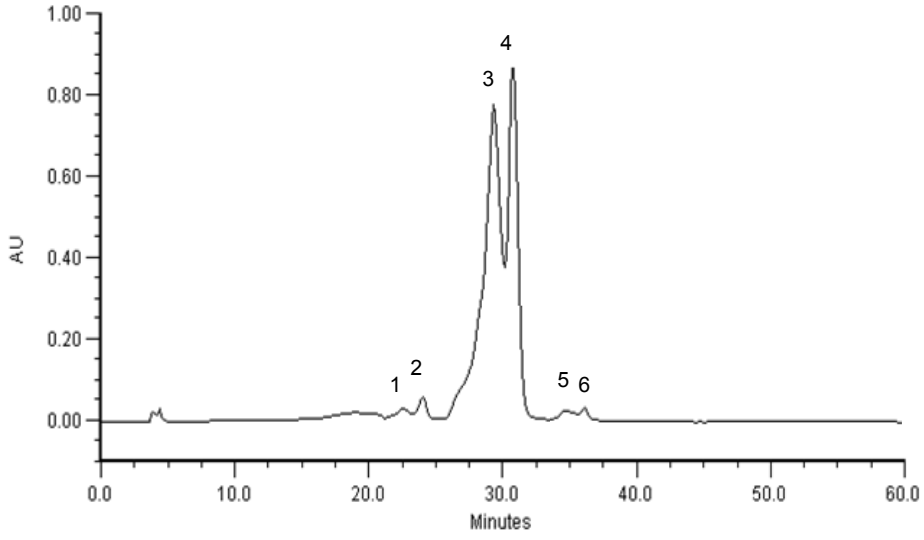


Figure 4.7. HPLC profile of *in natura* elderberry pomace extract obtained by PSE using EtOH (90%) + CO_2 (10%), at 313 K and ~20 MPa recorded at 520 nm. Peaks: 1 and 2, anthocyanins; 3, cyanidin 3-sambubioside; 4, cyanidin 3-glucoside; 5, quercetin glycoside; 6, rutin.

Total anthocyanin content in the elderberry pomace extracts obtained in this work (39–153 mg/g) were considerably higher than total anthocyanin content in the dry red grape skin extracts (41-57 mg/g), obtained by Ju and Howard (2003) and using different solvents (acidified alcohols, H_2O and acetone mixtures) at 323 K and 10.1 MPa. This is probably the best anthocyanin content that can be obtained using grape pomace as raw material, since the authors claim that the employed grape variety was exceptionally rich in TA and, like in elderberry pomace, these were more concentrated in grape skin than in the whole grape. These results show that elderberry pomace is a quite good source of anthocyanins, when compared to wine industry byproducts.

4.5. Conclusions

Anthocyanin rich elderberry pomace extracts were obtained, employing a fractioned high pressure methodology. During the first step CO_2 SFE, that was more efficient for the dry raw material, essentially lipophilic compounds were extracted. For ethanolic PSE assays, the presence of H_2O in the solvent mixture resulted in an increment in global yields and in TA contents, with a consequent improvement in the extracts antioxidant activity. Moreover, for the PSE assays using EtOH/H_2O in the solvent mixture, the dissolved CO_2 favored TA contents and antioxidant properties. For the aqueous PSE assays, even though global yields increased with the H_2O content increase in the solvent mixture, there was not a direct relationship with TA contents or antioxidant capacities. Antioxidant activity of these aqueous PSE extracts were high and comparable to the ethanolic CSE extract, despite their low TA and phenolic (observed by TLC) contents.

Relatively to the methodology used, PSE has several additional advantages over CSE, such as the possibility of extract fractionation and a higher extraction flexibility, which is offered by the possibility of modifying solvent dissolution capability just by changing operational conditions as dissolved CO_2, and temperature and pressure, which can also be explored.

The presented results clearly indicate that it is possible to obtain anthocyanin rich extracts from elderberry pomace possessing high antioxidant activity and, in this way, adding economical value to this agroindustrial residue. These was done using solvents and techniques considered as “acceptable” and “generally regarded as safe” in the food and pharmaceutical industries.

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5. Effect of solvent (CO_2/ethanol/H_2O) on the fractionated high pressure extraction of anthocyanins from elderberry pomace

This Chapter is a development of the research work presented in the previous Chapter. A more systematic evaluation of the influence of the second step CO_2/ethanol/H_2O solvent mixture composition on the extracts yields, composition and resulting antioxidant activities was performed. The text that comprises this chapter is published in: Seabra, I.J., Braga, M.E.M, Batista, M.T.P., De Sousa, H.C. (2010) Effect of solvent (CO_2/ethanol/H_2O) on the fractionated enhanced solvent extraction of anthocyanins from elderberry pomace. *The Journal of Supercritical Fluids 54*: 145-152.

5.1. Abstract

The management of agro-industrial residues is an important issue for environmental reasons and the reuse of byproducts represents a good alternative, especially if it is conjugated with green technologies and the production of valuable products. Portuguese elderberry pomace is rich in anthocyanins with therapeutic properties that confer to this byproduct potential to be applied in the food and pharmaceutical industries. Fractionated high pressure extractions from elderberry pomace were performed using supercritical CO_2 extraction, followed by pressurized solvent extractions (PSE) with diverse CO_2/ethanol/H_2O solvent mixtures (0-90, 0.5-100, 0-95, % v/v/v), at 313 K and 21 MPa, in order to obtain anthocyanin-rich fractions. The PSE solvent mixtures had a substantial effect on extract's yield and composition. The maximum extraction yield (24.2%), total phenolic compounds (15.8% gallic acid equivalents), total flavonoids (8.9% epicatechin equivalents), total anthocyanins (15.0% cyanidin-3-glucoside equivalents) and antioxidant activity (IC_{50} of 21 μg) achieved highlight the great potential of elderberry pomace for valuable applications.

5.2. Introduction

Elder (*Sambucus nigra* L.) berry juice processing unavoidably generates large amounts of residues (25-40% of total berry weight) (Brønnum-Hansen and Jacobsen, 1985) that are mainly used as animal feed, fertilizer, or simply thrown away, creating environmental problems, despite their potential for recycling into useful products, due to their high phenolic and anthocyanin contents (75-98% of total berry anthocyanins) (Brønnum-Hansen and Jacobsen, 1985). In northern Portugal, the elder tree takes advantage from excellent edaphoclimatic conditions, and its berries contain high anthocyanin contents (Braga et al., 2002) and, consequently, potential to originate high quality final processed products (Lee and Finn, 2007). The extraction of these compounds does not compromise the common usage of this residue if toxic solvents are avoided, such as in green technologies. On the contrary, it would represent an improvement in the residues management policy, by reducing their phenolic burden and consequently the hazard that some of these substances may represent to cultivated lands or drinking and irrigation water (Capasso et al., 1992).

Elderberries contain a high phenolic content and antioxidant activity when compared with other fruits and even with other berries (Lichtenthäler and Marx 2005; Lugasi and Hóvári, 2003). In fact, Lugasi and Hóvári (2003) studied several red and white wines, beers and fruits and vegetables juices, and detected the highest phenolic content in elderberry juice, significantly higher than the best quality red wines, and also the second highest *in vitro* antioxidant capacity, only beat by Pinot Noir. Pietta and Bruno (1992) and Rice-Evans et al. (1996) reported that the antioxidant properties of *Sambucus nigra* extracts are mainly related with the presence of flavonols and anthocyanins. Rutin is the most representative flavonol for this plant (Pietta and Bruno, 1992), while cyanidin-3-glucoside (CyG) and cyanidin-3-sambubioside (CyS) are the main anthocyanins, being cyanidin-3-sambubioside-5-glucoside (CySG) and cyanidin-3,5-diglucoside also present (Nakajima et al., 2004).

Anthocyanins are polar vacuolar pigments that are traditionally extracted from plant materials by conventional methods, such as solid-liquid extraction, using slightly acidified aqueous alcoholic solvents. Nevertheless, there is an increasing demand for faster extraction procedures with reduced organic solvent consumption and lower pollution burden (Wang and Weller, 2006). Pressurized solvent extraction (PSE), usually performed at 313-473 K and 3.3-20.3 MPa, combines the advantages of typical liquids solvation properties and the transport properties of supercritical fluids (Chamblee et al., 2004). The high extraction pressure facilitates the breakdown of the vegetable tissue, opening up cells, further increasing

extraction yield. Carbon dioxide can be used in combination with water and/or an alcohol (forming a gas-expanded liquid), to extract polar compounds like anthocyanins. The pH decrease that occurs due to the generation of *in situ* carbonic acid and/or alkyl carbonic acid (Towes et al., 1995; West et al., 2001) can have a positive impact on anthocyanins stability, besides increasing cell membranes permeability which leads to higher diffusivities (Türker and Erdoğdu, 2006). Furthermore, the absence of oxygen in the extraction cell is an advantage of this process, because its presence can lead to structural changes in phenolic compounds that can, in turn, result in altered properties, as has been demonstrated by Pinelo et al. (2005), who proved the formation of flavan-3-ols polymers in the extractor bulk, when grape skins were subjected to a continuous ethanolic extraction. PSE can be easily used in combination with supercritical fluid extraction (SFE), in order to perform a fractionated process, in which the raw material is previously extracted with CO_2, to remove lipophilic compounds, and subsequently with water, alcohol or the mixture of both to obtain a phenolic rich fraction. This methodology was already applied to elderberry pomace (Seabra et al., 2010), resulting in lipophilic and anthocyanin-rich extracts. Although not leaving the vegetable matrix free of low polarity compounds, the first CO_2 extraction step was important to concentrate the phenolic and other polar compounds for the subsequent extraction step.

A high pressure extraction methodology such as the one described has other advantages that should be considered, such as the fact that native enzymes, which degrade phenolic compounds, are inhibited by increasing extraction pressure and CO_2 addition (Kamat et al., 1995; Castellari et al., 1997), and that supercritical fluid processed materials do not require additional sterilization steps (Díaz-Reinoso et al., 2006). Even though the choice of the appropriate polar solvent is a key factor for the success of anthocyanin-rich fractions extraction procedures, its influence on the extract characteristics is not always clear, due to the diverse structure and composition of plant materials, and so, each material-solvent system shows a different behavior, which can not be predicted.

In this work, pressurized solvent extractions of elderberry pomace, previously delipidified with supercritical CO_2, were performed in order to obtain anthocyanin-rich extracts with potential applications in the pharmaceutical and food areas. Several CO_2/ethanol/H_2O solvent mixtures were assayed, varying the proportions of CO_2, ethanol (EtOH) and H_2O over a wide range, in order to discern their influence on the yield, composition and antioxidant activity of the obtained extracts.

5.3. Materials and methods

5.3.1. Raw material

Ripe elderberries were collected at the experimental field of Cooperativa Agrícola do Vale do Varosa (Portugal) in August 2004, and stored under vacuum, at 255 K. Elderberries were harvested from several trees belonging to the Ucanha population, which were selected amongst 15 elders belonging to 5 different populations from the Varosa Valley, based on their high anthocyanin contents in August 1999, which was 37.2±2.5 g/kg (dry basis, d.b.) (Braga et al., 2002; Carvalho, 2000). The juice was separated using a mechanic press (Hafico, Germany) and the elderberry pomace (peels, pulp residues and seeds) was dehydrated in a fluidized bed dryer (MK II, Sherwood Scientific, England) at 308 K, in the absence of light, for 12 hours. It was then milled in a knife mill (Braun, KSM 2, Germany) avoiding sample overheating. The dried elderberry pomace was conditioned under vacuum in a plastic bag at 255 K, prior to extraction. Its humidity was gravimetrically determined by drying sample in an oven (J.P. Selecta, Spain) at 376 K, until a constant weight achievement.

5.3.2. Chemicals

Carbon dioxide (99.998%, Praxair, Spain), ethanol (99.5%, Panreac Quimica SA, Spain) and distilled water were used for the extraction experiments. Chemicals and solvents employed for extracts analyses were: ethanol (p.a.), methanol (Lichrosolv), formic acid (≥ 98%) and Folin-Ciocalteu's phenol reagent from Merck (Germany), sodium carbonate (p.a.) and sodium nitrite (p.a.) from Pronalab (Portugal), anhydrous aluminum chloride (98%), potassium chloride (p.a.), sodium acetate 3-hydrate (p.a.) and potassium metabisulfite (p.a.) from Panreac (Spain), sodium hydroxide anhydrous pellets (p.a., Carlo Erba, Italy), 2,2-diphenyl-1-picrylhydrazyl (DPPH radical) (~ 90%, Sigma-Aldrich, Germany) and distilled deionized water (ddH_2O), obtained with a Ultra pure Milli Q water system. Standards used for spectrophotometric and HPLC analyses were: gallic acid (≥ 98%, Sigma-Aldrich, Germany), (-)-epicatechin (≥ 90%, HPLC grade, Fluka, Switzerland), cyanidin-3-glucoside chloride (analytical grade, Extrasynthèse, France) and rutin (extra pure, Merck, Germany).

5.3.3. Experimental fractionated high pressure extractions procedure

The fractionated high pressure extraction assays were performed using the extraction unit described by Seabra et al. (2010), comprising a ~30×10^{-6} m^3 stainless steel extraction cell

placed inside a controlled temperature water bath. High pressure liquid pumps were used to deliver the liquefied CO_2 (home-built) and the EtOH/H_2O mixture (L-6200A, Hitachi, Merck, Germany). Extracts were recovered in a flask placed in an ice bath and a trap was used to prevent any losses with the expanded CO_2, which flow was measured by a wet gas meter.

Fractionated extractions were performed at 20.9±0.4 MPa and 313±0.1 K, in two steps: *i)* a 1st step (SFE) (15 min static + 40 min dynamic period), wherein CO_2 was used in order to remove low polarity CO_2-soluble compounds. At the end, extraction cell was depressurized to ~10 MPa, to avoid a pressure increase above 20 MPa caused by the following step liquid solvent entrance. Tubing line was cleaned with EtOH, and the extract recovered was considered for the extraction yield calculation. *ii*) a 2nd step (PSE) (45 min dynamic period), in order to extract polar compounds like anthocyanins, and wherein diverse CO_2/EtOH/H_2O solvent mixtures were introduced into the system, varying the volumetric percentage (10-100%) of the liquid EtOH/H_2O mixture and its composition. Volumetric percentages of CO_2, EtOH and H_2O varied in the range 0-90, 0.5-100, and 0-95, respectively. The central experimental point was triplicated to get the experimental error. Total solvent flows were $12.3\pm1.4\times10^{-5}$ and $7.20\pm0.37\times10^{-5}$ kg/s for the 1st and 2nd steps, respectively, corresponding to solvent to solid ratios of 1:229±25 and 1:135±11 (w/w, d.b.). Extracts were vacuum evaporated (BÜCHI Rotavapor R-114, Switzerland) at 313 K, and those with higher H_2O contents were subsequently lyophilized (Labconco, model 77560, Missouri, USA) to dryness, and kept at 255 K until analyzed. Global yields were obtained considering the total extracted mass divided by raw material mass in dry basis.

5.3.4. Characterization of elderberry pomace extracts

5.3.4.1. Spectrophotometric analyses: total phenolic compounds (TP), total flavonoids (TF), total monomeric anthocyanins (TMA) and polymeric color (PC)

For total phenolic compounds and total flavonoids analyses of the CO_2/EtOH/H_2O elderberry pomace extracts, solutions were prepared by dissolving the dried extracts in EtOH (for TP) or methanol (MeOH) (for TF), and then adding ddH_2O until a 50:50 (v/v) final proportion.

Total phenolic compounds in these extracts were quantified according to the Folin-Ciocalteu's method, and following the procedure proposed by Singleton and Rossi (1965) with some modifications, based on Cheung et al. (2003). Up to 0.5 mL aliquots of each extract sample were introduced into test tubes, and aqueous EtOH (50:50) was added as

necessary to bring the total volume to 0.5 mL. The Folin-Ciocalteu's reagent (0.5 mL) was added and the mixture vortexed. After 3 min, 0.5 mL of saturated Na_2CO_3 (~17%) was added to the mixture and it was made up to 5.0 mL by adding distilled water. The reaction was kept in the dark for 90 min, after which absorbance was read at 725 nm. Results were designated by TP and expressed as gallic acid equivalents (GAE).

Total flavonoids in the PSE extracts were measured by an assay developed by Zhishen et al. (1999). Up to 1.0 mL aliquots of each extract sample were introduced into test tubes containing 4 mL ddH_2O, and aqueous MeOH (50:50) was added as necessary to bring the total volume to 5.0 mL; 0.3 mL of aqueous $NaNO_2$ (5:95) was added to the flask, followed by 0.3 mL of aqueous $AlCl_3$ (10:90) and 2 mL of 1 M NaOH. The reaction solution was immediately diluted with the addition of 2.4 ml of ddH_2O and thoroughly mixed, and its absorbance was measured at 510 nm. Results were designated by TF and expressed as epicatechin equivalents (ECE).

The CO_2/EtOH/H_2O elderberry pomace extracts were also analyzed by the pH differential method (Giusti and Wrolstad, 2001) to quantify total monomeric anthocyanins (TMA) and polymeric color (PC). For TMA quantification, extracts were diluted in potassium chloride buffer pH 1.0 and in sodium acetate buffer pH 4.5 using the appropriated dilution factor. Absorbance measurements were taken at 520 and 700 nm, after 15 min. TMA contents were determined considering the molar extinction coefficient and molecular weight of cyanidin-3-glucoside chloride (26900 L/(cm.mol) and 449.2 g/mol, respectively), and expressed as CyG equivalents (CyGE). For PC evaluation, extracts were diluted in distilled water using the appropriated dilution factor. Potassium metabisulfite solution and water were added to two separate samples and absorbances were measured at 420, 520 and 700 nm, after 15 min.

An UV/VIS spectrophotometer (Jasco V-530, Japan) was employed for all measurements which were run in triplicate, and results were expressed in percentage (w/w, d.b.).

5.3.4.2. Analysis by high-performance liquid chromatography, coupled to photodiode array and electrospray ionization mass spectrometry detectors (HPLC-PDA-ESI/tandem MS)

Anthocyanins and rutin identification for the CO_2/EtOH/H_2O (0:80:20) extract was carried out on a SURVEYOR LC equipped with a Surveyor MS Pump (MSPUMP) and a PDA detector Surveyor PDA Plus (Thermo Finnigan) and interfaced with a QITMS mass spectrometer (LCQ Advantage Ion Max MS/MS, Thermo Finnigan) equipped with an API-ES ionization chamber, which were controlled by the LCQ Xcalibur software.

Separation was performed on an Spherisorb ODS2 column, 150×2.1 mm, 3 μm particle size and a Spherisorb ODS2 guard cartridge, 10×4.6 mm, 5 μm particle size (Waters, MA, USA) at 293 K, using 2% aqueous formic acid (A) and methanol (B) as mobile phase. A discontinuous gradient of 5-15% B in A (0-10 min), 15-25% B (10-15 min), 25-50% B (15-40 min), 50-80% B (40-50 min) and 80% B (50-60 min) isocratically was used at a flow rate of 200 μL/min. The first detection was made with a PDA in a wavelength range 200-600 nm, followed by a second detection in the mass spectrometer.

Mass analyses were obtained in the negative ion mode. The mass spectrometer was programmed to perform two consecutive scans: full mass (*m/z* 50-2000) and MS^2 of the most abundant ion in the full mass. Source voltage was 5 kV and the capillary voltage and temperature were -10 V and 563 K. Nitrogen was used as sheath and auxiliary gas at flow rate of 13 and 2 (Finnigan arbitrary units), respectively. The normalized energy of collision was 35% using helium as collision gas.

5.3.4.3. HPLC-PDA analysis

PSE extracts were analyzed in a Gilson apparatus equipped with a diode-array detector and data treatment was carried out with software Unipoint® 2.10 Gilson. An ODS-2 column (250×4.6 mm i.d., 5 μm, Spherisorb S5, Waters, MA, USA) at 298 K and a C18 guard cartridge (30×4 mm i.d., 4 μm, Hichrom, Berkshire, UK) were used. A mobile phase, constituted by aqueous formic acid (5%, v/v) (A) and MeOH (B), was used with a discontinuous gradient of 5-15% B (0-10 min), 15-25% B (10-15 min), 25-50% B (15-40 min) and 50-80% B (40-50 min), followed by an isocratic elution during 10 min, at a flow rate of 1 mL/min. Samples were adjusted to pH ~2 with HCl and microfiltered (0.20 μm) before HPLC injection. Anthocyanins were detected from their chromatographic and ultraviolet (UV) spectral properties and the major anthocyanins, CyG, CyS and CySG, were identified by comparing with the HPLC-PDA-ESI/tandem MS results. Quantification of CyG, CyS, CySG and total anthocyanins (TA) was carried out from a calibration curve prepared using the CyG standard, at 520 nm, and expressed as cyanidin-3-glucoside equivalents (CyGE), in percentage (w/w, d.b.). Quantification of rutin (R) was carried out by using a calibration curve prepared from the rutin standard, at 360 nm. The assay standard deviation was obtained by the central experimental point extract injected three times.

5.3.4.4. Antioxidant activity: DPPH assay

The method described by Blois (1958) was employed in which aliquots (100 μL) of extracts were added to 500 μL of a methanolic solution (500 μM) of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and 1.4 mL of methanol. After mixing for 30 s, the reaction mixture was incubated in the dark at room temperature for 30 min and the absorbance measured at 517 nm on a spectrophotometer (U-2000 model, Hitachi, Tokyo, Japan). The extracts reducing activities were estimated from the decrease in absorbance, and the results expressed as IC_{50} values, defined as the amount of extract that decreased by 50% the initial absorbance of the DPPH radical solution, at 517 nm. All assays were performed in triplicate.

5.4. Results and discussion

5.4.1. Extraction yields

The CO_2 SFE (1st step) from the elderberry pomace containing 5.2±0.1% (w/w) of humidity resulted in a lipophilic fraction with yield equal to 17.1±0.3%. This result is similar to the one obtained by Seabra et al. (2010), that also performed fractionated high pressure extractions from dried elderberry pomace, using the same experimental conditions in the 1st step.

In order to obtain anthocyanin-rich extracts, CO_2/EtOH/H_2O solvent mixtures were introduced into the system for the PSE (2nd step), at the same experimental conditions (313 K and ~21 MPa). These solvent mixtures were either in a homogeneous liquid phase or in vapor and liquid equilibrium phases (represented by L and V-L in Table 5.1, respectively), according to Durling et al. (2007) and depending on the different overall CO_2, EtOH and H_2O volumetric percentages used. The corresponding equilibrium molar fractions, as well as the quotient between the vapor and liquid phases molar flows, Q_V/Q_L, are also reported on Table 5.1.

During the first few minutes of the PSE continuous process (2nd step), an extract fraction presenting a yellow-green color, without anthocyanins, was obtained for most cases. These fractions should contain the remaining low polarity compounds still present in the vegetable matrix (Seabra et al., 2010) that were extracted by the rich CO_2 gaseous phase, while the liquid solvent phase was absorbed/adsorbed by the vegetable matrix. Extraction yields of these yellow-green fractions are reported on Table 5.1, as well as the subsequent anthocyanin-rich fractions yields. When no CO_2 was used in the PSE, and when CO_2/EtOH/H_2O (80:1:19)

and (60:8:32) solvent mixtures were used, corresponding to very high anthocyanin-rich fraction yields (Table 5.1), no yellow-green fractions were obtained.

The 45-min extraction time was not sufficient to obtain anthocyanin-rich fractions for some assays performed with high CO_2 amounts and with no H_2O or with low H_2O amounts, for which extraction yields equal to zero are reported on Table 5.1. The partial depressurization to ~10 MPa between the two extraction steps lead to a low desorption rate of CO_2, not creating empty spaces within the raw material that could favor a faster 2^{nd} step solvent adsorption/absorption. Besides, the complete CO_2 desorption from vegetable matrices is known to take more than 45 minutes (Stamenic et al. 2010). Therefore, for some assays, there was not enough time for the CO_2, provided from the first step, to diffuse out of the raw material bed, and for the solvent mixture to soak the whole raw material bed. The complete depressurization of the 1^{st} step could have improved the 2^{nd} step mass transfer (Stamenic et al. 2010). Soaking times were higher for higher CO_2 percentages in the overall solvent mixture, due to higher Q_V/Q_L values, and for higher EtOH percentages in the EtOH/H_2O mixtures, because of the lower EtOH density, comparatively to the H_2O density. For similar vegetable matrixes soaking times in the range 11-60 minutes were reported (Mantell et al., 2003). The expected low diffusivities of solutes with high molar masses, such as anthocyanins, may also have contributed to this result.

Amongst the solvent mixtures that resulted in anthocyanin-rich fractions there were considerable differences in extraction yields, which varied between 4.0% and 24.2%. Since a forty five minutes extraction time was previously established, better yields could possibly have been achieved if the diffusion-controlled mass transfer region of the kinetic curve was attained. The diverse solvent mixtures probably caused different transport rates of solutes, from the matrix to the bulk of the extraction fluid, and consequently different extraction yields.

Anthocyanin-rich fractions extraction yields and Q_V/Q_L values were not inversely related, as could be expected due to the presence of a higher amount of a higher density and polarity phase for lower Q_V/Q_L values, which was expected to have an enhanced capacity to dissolve polar substances, like anthocyanins. Moreover, the presence of a higher amount of a vapor phase (that corresponds to a Q_V/Q_L value higher than one) or of a higher amount of a liquid phase in the cell (that corresponds to a Q_V/Q_L value lower than one) were not decisive to obtain low and high extraction yields, respectively.

Table 5.1. PSE solvent mixtures composition, at 313 K and 20.9 MPa (CO_2, EtOH, and H_2O volumetric percentages, molar fractions in the liquid and vapor equilibrium phases, and quotient between the vapor phase and liquid phase molar flows) and extraction yields.

CO_2/EtOH/H_2O (%, v:v:v)	Phases in equilibrium[b]	Solvent composition[c]						Q_V/Q_L[d] (n/n)	2nd step extraction yield (%, d.b.)		
		Liquid phase			Gaseous phase						
		x_{CO_2}	x_{EtOH}	x_{H_2O}	y_{CO_2}	y_{EtOH}	y_{H_2O}		*Yellow-green fraction*	*Anthocyanin-rich fraction*	Total yield
90:10:0	L	0.909	0.091	0.000	-	-	-	0.0	2.4	0.0	2.4
90:8:2	V-L	0.162	0.274	0.564	0.925	0.060	0.015	14.06	1.7	0.0	1.7
90:5:5	V-L	0.040	0.040	0.920	0.985	0.005	0.010	4.90	1.6	4.3	5.9
90:2:8	V-L	0.040	0.040	0.920	0.985	0.005	0.010	3.64	1.8	17.9	19.7
90:0.5:9.5	V-L	0.065	0.09	0.843	0.972	0.020	0.008	3.33	1.9	8.1	10.0
80:20:0	L	0.821	0.179	0.000	-	-	-	0.0	2.0	0.0	2.0
80:16:4	V-L	0.202	0.320	0.478	0.880	0.092	0.028	4.37	4.3	4.0	8.3
80:10:10	V-L	0.092	0.167	0.741	0.954	0.037	0.008	2.01	1.7	5.2	6.9
80:4:16	V-L	0.037	0.050	0.913	0.980	0.010	0.010	1.60	0.2	9.4	9.6
80:1:19	V-L	0.083	0.131	0.786	0.967	0.024	0.009	1.35	0.0	20.4	20.4
60:32:8	V-L	0.288	0.375	0.337	0.850	0.120	0.030	0.82	1.4	11.2	12.6
60:8:32	V-L	0.045	0.062	0.893	0.980	0.010	0.010	0.55	0.0	20.3	20.3
50:50:0	L	0.527	0.473	0.000	-	-	-	0.0	2.2	0.0	2.2
50:40:10	V-L	0.295	0.377	0.328	0.845	0.121	0.034	0.34	1.0	11.5	12.5
50:25:25[a]	V-L	0.110	0.200	0.690	0.940	0.045	0.015	0.38	2.9±0.6	17.4±1.1	20.3±0.6
50:10:40	V-L	0.045	0.062	0.893	0.980	0.010	0.010	0.35	1.1	5.5	6.6
50:2.5:47.5	V-L	0.035	0.015	0.950	0.990	0.005	0.005	0.06	0.3	11.5	11.8

[a] For central point, data are reported as mean value ± standard deviation of three extraction experiments. [b] L - liquid and V - vapor. [c] x, y - molar fractions in liquid and vapor phases, respectively. [d] Q_V/Q_L - quotient between the vapor and liquid phases molar flows.

Table 5.1. Continuation.

CO_2/EtOH/H_2O (%, v:v:v)	Phases in equilibrium[b]	Solvent composition[c]						Q_V/Q_L[d] (n/n)	2nd step extraction yield (%, d.b.)		
		Liquid phase			Gaseous phase				*Yellow-green fraction*	*Anthocyanin-rich fraction*	Total yield
		x_{CO_2}	x_{EtOH}	x_{H_2O}	y_{CO_2}	y_{EtOH}	y_{H_2O}				
40:48:12	V-L	0.316	0.380	0.304	0.843	0.123	0.035	0.03	3.1	14.2	17.2
30:56:14	L	0.246	0.418	0.336	-	-	-	0.00	0.0	16.2	16.2
20:80:0	L	0.214	0.786	0.000	-	-	-	0.0	2.1	8.0	10.0
20:64:16	L	0.160	0.465	0.375	-	-	-	0.00	0.9	15.9	16.8
20:40:40	V-L	0.115	0.208	0.677	0.940	0.045	0.015	0.01	3.0	18.3	21.3
20:16:64	V-L	0.045	0.062	0.893	0.980	0.010	0.010	0.05	0.4	10.6	11.0
20:4:76	V-L	0.035	0.015	0.950	0.990	0.005	0.005	0.01	0.5	14.4	14.9
10:72:18	L	0.073	0.514	0.413	-	-	-	0.00	1.0	16.5	17.5
0:100:0	L	0.000	1.000	0.000	-	-	-	0.0	0.0	13.9	13.9
0:80:20	L	0.000	0.554	0.446	-	-	-	0.00	0.0	17.7	17.7
0:50:50	L	0.000	0.237	0.763	-	-	-	0.00	0.0	15.6	15.6
0:20:80	L	0.000	0.072	0.928	-	-	-	0.00	0.0	24.2	24.2
0:5:95	L	0.000	0.02	0.984	-	-	-	0.00	0.0	15.2	15.2

[a] For central point, data are reported as mean value ± standard deviation of three extraction experiments. [b] L - liquid and V - vapor. [c] x, y - molar fractions in liquid and vapor phases, respectively. [d] Q_V/Q_L - quotient between the vapor and liquid phases molar flows.

For example, for CO_2/EtOH/H_2O (90:2:8) and (50:10:40), yields were 17.9 and 5.5%, and Q_V/Q_L values were 3.64 and 0.35, respectively. Instead, solvent composition, particularly the liquid phase composition, had a probable great effect on the anthocyanin-rich fractions extraction yields, though the individual contribution of any of the solvents used (CO_2, EtOH and H_2O), in terms of molar fractions or any combination of them, was not possible to evaluate. This is not a surprising result, if the complex nature of the vegetable matrix is considered, as well as the complex nature of the high pressure solvent mixtures applied, in terms of physical and chemical characteristics, which were not studied and are not possible to predict with accuracy.

The highest 2nd step yield (24.2%) was obtained when no CO_2 was present in the extraction solvent (0:20:80). The proportion of EtOH/H_2O (20:80) gave a considerably higher yield than that obtained by pure EtOH (13.9%) and almost pure H_2O (liquid molar fraction of 0.984) (15.2%), since both polar and less polar compounds were extracted together. Furthermore, there were high PSE extraction yields obtained with high CO_2 amounts, in particular CO_2/EtOH/H_2O (90:2:8) and (80:1:19). That can be very advantageous in terms of process economy due to the possibility of CO_2 recycling and to the reduced effort of removing lower liquid solvent amounts in the final extract preparation steps.

5.4.2. Extracts composition and antioxidant activity

The PSE extracts compositions are reported on Table 5.2 for total phenolic compounds (TP), total flavonoids (TF), cyanidin-3-glucoside (CyG), cyanidin-3-sambubioside (CyS), cyanidin-3-sambubioside-5-glucoside (CySG), total anthocyanins (TA) and rutin (R). Identification of anthocyanins and rutin was confirmed by HPLC-PDA-ESI/tandem MS.

Total phenolic compounds of green extract fractions reached 3.1% GAE, though no flavonoids could be detected by the spectrophotometric assay. The low TP contents, when compared to the anthocyanin-rich fractions contents that reached 15.8% GAE, and the apparent lack of flavonoids, may be explained by the fact that these fractions were probably constituted by low polarity compounds, still present in the elderberry pomace previously extracted with CO_2, and soluble in a CO_2 rich gaseous phase, as already discussed.

The PSE solvent mixtures applied resulted in elderberry pomace anthocyanin-rich extracts with very diverse phenolic compositions. CyS and CyG accounted for 70.8-96.4% of total anthocyanins present in the extracts. The presence of H_2O in the liquid phase was important to promote the extraction of phenolic compounds, in general, and of anthocyanins and rutin, in

particular. In fact, the extracts obtained with no H_2O in the solvent mixtures, i.e., CO_2/EtOH/H_2O (20:80:0) and (0:100:0), revealed to have 2.7% GAE of TP, 3.4 and 1.4% CyGE of TA, respectively, and 0.5% of R, which were amongst the lowest values obtained. These two extracts also showed very low antioxidant activities (IC_{50} of 262 and 211 μg, respectively), when compared to the others (21-80 μg), which is most probably related to their low phenolic contents, essentially anthocyanins and rutin, reinforcing the important role of these compounds in the antioxidant activity of elderberry extracts (Pietta and Bruno, 1992; Rice-Evans et al., 1996). However, PSE yields were relatively high for these solvent mixtures and consequently, it can be concluded that they promoted the extraction of non-phenolic substances with low antioxidant activity.

Extracts obtained using solvent mixtures with low liquid EtOH fractions (0.015-0.020) presented low TP, TF and TA contents, though rutin contents were similar to others, varying over the range of 0.6 up to 1.2% (Table 5.2). Therefore, and likewise H_2O, higher EtOH molar fractions in the liquid solvent mixture were required to efficiently extract phenolic compounds, in general, and anthocyanins, in particular, which have low solubility in H_2O, according to Metivier et al. (1980). Corrales et al. (2009) studied the influence of EtOH concentration (20-100%) on the extraction of anthocyanins from grape skins at 600 MPa, and found a positive relation between the EtOH solvent concentration and the amount of anthocyanins recovered. Such a correlation was not observed in the present study, eventually due to differences on raw materials and extraction procedures employed. Nevertheless, these extracts presented low IC_{50} values (31-54 μg), reflecting their high antioxidant activities, which may be another indication of the important role of rutin as an antioxidant promoter, and the presence of other eventually extracted substances (soluble in aqueous rich media) with high antioxidant activity, like proanthocyanidins. Seabra et al. (2010) performed fractionated high pressure extractions from elderberry pomace using CO_2/H_2O solvent mixtures in the 2nd step (with no EtOH), and obtained extract fractions with similar total anthocyanins (3.9-8.9%) and rutin (0.8-1.0%) contents, showing low IC_{50} values (40-52 μg).

The important role of EtOH and H_2O in the extraction of fractions with biological activity was also observed by Roschek Jr. et al. (2009), who performed fractionated high pressure extractions from the whole elder fruit - using CO_2 (333 K, 30 MPa) in a first step, and EtOH/H_2O (40:10, v/v) and EtOH in a second step - and obtained extracts with anti-influenza virus (H1N1) activities comparable to the ones of the commercial drugs Oseltamivir (Tamiflu®) and Amantadine.

Table 5.2. Composition of the PSE elderberry pomace extracts, obtained with different CO_2/EtOH/H_2O solvent mixtures, at 313 K and 20.9 MPa, evaluated by spectrophotometric assays, HPLC-PDA and HPLC-PDA-ESI/tandem MS, and their antioxidant activities.

PSE solvent	***Green fraction***	***Anthocyanin-rich fraction*** [b]							
CO_2/EtOH/H_2O (%, v:v:v)	**TP (% GAE)**	**TP (% GAE)**	**TF (% ECE)**	**CyG (%)**	**CyS**	**CySG**	**TA_{HPLC}**	**R (%)**	**IC_{50} (µg extract)**
					(% CyGE)				
90:10:0	2.9±0.2	-	-	-	-	-	-	-	-
90:8:2	2.3±0.1	-	-	-	-	-	-	-	-
90:5:5	2.7±0.2	3.5±0.1	6.3±0.6	5.9	4.6	1.3	11.6	1.0	61±0.8
90:2:8	2.2±0.2	9.0±0.3	4.1±0.0	2.1	1.9	1.2	5.0	0.8	80±1.2
90:0.5:9.5	3.1±0.3	7.3±0.1	5.6±0.2	1.0	1.1	0.9	2.9	0.6	70±1.4
80:20:0	2.6±0.1	-	-	-	-	-	-	-	-
80:16:4	2.4±0.1	11.7±0.4	7.4±0.2	5.2	4.8	1.2	11.0	1.2	72±1.4
80:10:10	2.7±0.1	15.8±0.7	6.0±0.3	4.3	3.5	1.1	8.8	0.9	65±1.6
80:4:16	n.d.	9.3±0.7	7.2±0.6	6.3	4.9	2.2	13.3	1.4	33±0.3
80:1:19	-	9.1±0.5	6.1±0.2	0.8	0.8	0.7	2.4	0.6	53±0.8
60:32:8	1.2±0.1	14.5±1.6	5.8±0.1	6.0	4.8	1.3	12.0	1.3	65±1.5
60:8:32	-	8.7±0.8	7.0±0.5	1.9	1.8	1.0	4.8	0.6	57±1.2
50:50:0	2.5±0.1	-	-	-	-	-	-	-	-
50:40:10	1.9±0.2	12.2±0.8	4.8±0.1	6.6	5.5	1.4	13.2	1.4	59±2.1
50:25:25[a]	2.0±0.2	8.9±1.6	7.5±0.2	3.6±0.06	3.1±0.07	1.1±0.1	7.6±0.3	0.8±0.06	55±4.0
50:10:40	2.1±0.1	8.2±0.3	7.0±0.3	6.2	5.3	3.1	15.0	2.0	21±0.7
50:2.5:47.5	1.7±0.1	8.5±0.8	5.9±0.6	2.1	2.2	2.0	6.1	1.2	31±0.7
40:48:12	1.5±0.2	8.6±0.6	5.7±0.3	6.5	5.1	1.3	12.7	1.2	60±2.5
30:56:14	-	6.8±0.2	5.5±0.6	6.4	4.7	1.2	12.1	1.1	64±0.8
20:80:0	2.6±0.2	2.7±0.1	3.4±0.2	1.2	2.0	0.2	3.4	0.5	262±14
20:64:16	2.2±0.2	12.8±1.1	6.3±0.5	6.0	4.6	1.2	11.5	1.0	48±1.2
20:40:40	2.8±0.2	8.8±0.5	7.6±0.6	4.5	3.5	1.4	9.6	1.0	49±1.0
20:16:64	1.5±0.1	7.9±0.8	7.9±0.1	1.7	1.5	1.3	4.5	0.9	45±0.7
20:4:76	2.2±0.1	8.1±0.7	5.7±0.5	2.6	2.5	1.6	6.6	1.0	36±0.5
10:72:18	1.7±0.1	8.7±0.6	6.2±0.3	6.8	5.3	1.4	13.3	1.1	52±1.1
0:100:0	-	2.7±0.3	5.7±0.2	0.3	1.3	0.1	1.4	0.5	211±17
0:80:20	-	10.1±0.3	6.1±0.5	6.7	5.3	1.4	13.2	1.0	50±2.2
0:50:50	-	10.9±0.4	8.9±0.4	7.5	5.2	1.6	14.3	1.1	45±0.1
0:20:80	-	7.6±0.3	5.5±0.1	2.7	2.4	0.9	5.8	0.6	73±1.5
0:5:95	-	7.4±0.4	5.9±0.4	2.4	2.3	1.2	5.7	0.8	54±0.6

[*] TP - total phenols, TF - total flavonoids, CyG - Cyanidin 3-glucoside, CyS - Cyanidin 3-sambubioside, CySG - cyanidin 3-sambubioside-5-glucoside, TA - total anthocyanins, R - rutin. n.d. - non detected.

[a] Data are reported as mean value ± standard deviation; for central point, three extraction experiments were considered. [b] MS and MS^2 (*m/z*) for CYG (449 and 287), CYS (581 and 449, 287) and CYSG (743 and 581, 449, 287), and MS (*m/z*) for R (611).

No considerable differences were observed between the compositions of the extracts obtained with and without CO_2 in the solvent mixture, despite the enhanced transport properties of gas-expanded fluids and the decrease in the solvent mixture pH, with a consequent improvement of cell membranes permeability and anthocyanins stability (Türker and Erdoğdu, 2006).

Total anthocyanin (TA) contents determined by HPLC and total monomeric anthocyanins (TMA) determined by the pH differential method did not coincide, as shown in Figure 5.1. Higher contents were detected from HPLC for most extracts. This was also the tendency

observed for elderberries by Lee and Finn (2007) and for diverse berry juices by Lee et al. (2008): HPLC values were, respectively, 2.0-2.3- and 1.98-4.26-fold higher than spectrophotometric values. For the extract obtained with pure EtOH (0:100:0), a considerable lower content was detected from HPLC (TA was 0.3 times TMA value), which may be related to its low anthocyanin content (TA of 1.4% CyGE, Table 5.2). This trend was also observed by Lee and Finn (2007). The apparent general underestimation of anthocyanins by the pH differential method may be related with the presence of anthocyanins with the glycosidic substitution at the 3rd and 5th positions (cyanidin-3-sambubioside-5-glucoside and cyanidin-3,5-diglucoside), and also with the presence of polymerized anthocyanins, which are known to be more resistant to color change (Lee et al., 2005; Stintzing et al., 2002).

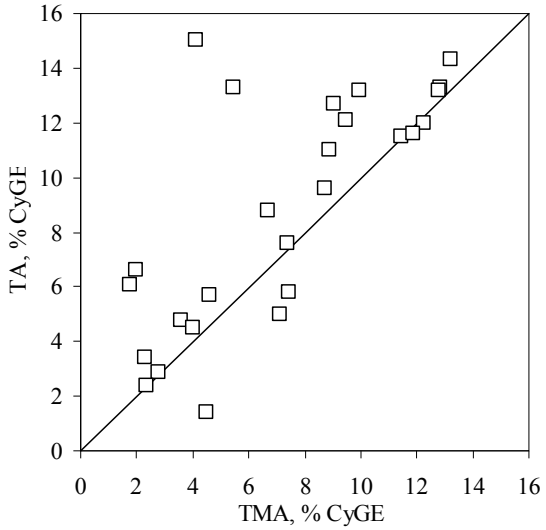

Figure 5.1. Total anthocyanin contents determined by HPLC (TA) *versus* total monomeric anthocyanin contents determined by the pH differential method (TMA) of elderberry pomace extracts, obtained by fractionated PSE with diverse CO_2/EtOH/H_2O solvent mixtures.

Figure 5.2 shows TMA contents and polymeric color (PC) of the PSE extracts obtained. As can be observed, for a fixed CO_2 percentage, an increase in water amount in the solvent mixture resulted in a decrease in TMA contents (represented by bars) and an increase in PC (represented by empty square symbols), resulting in greater contribution of polymers to color. This general behavior may be an indication that molecular condensation reactions occurred between anthocyanins or with other phenolic compounds. It could have happened within solvent mixtures containing higher water amounts, probably due to a higher water activity that is known to decrease anthocyanins stability (Stintzing and Carle, 2004). Some exceptions were observed for the lowest water amounts. The high antioxidant activities of some of these

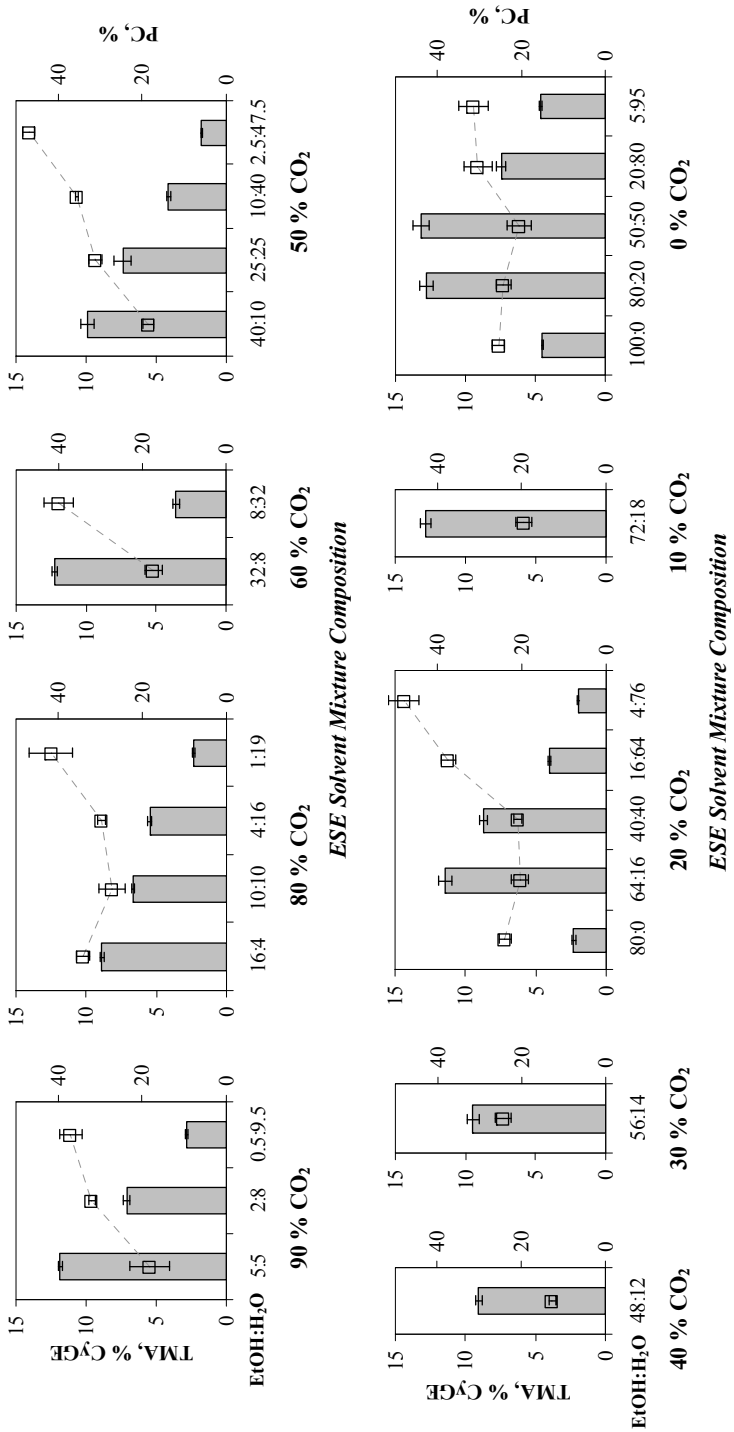

Figure 5.2. Total monomeric anthocyanin contents (TMA) and polymeric color (PC) of elderberry pomace extracts, obtained by fractionated PSE with diverse CO_2/EtOH/H_2O solvent mixtures.

extracts (50:10:40, 50:2.5:47.5 and 20:4:76, with IC_{50} of 21, 31 and 36, respectively - Table 5.2) suggest that the polymerized anthocyanins presence, which are known to present enhanced stability and favorable biological activities, may have contributed to these results (Stintzing and Carle, 2004; McDougall et al., 2005).

The highest TMA contents (~13%) were achieved for the extracts obtained using solvent mixtures with no CO_2 and EtOH:H_2O proportions of 80:20 and 50:50. However, the extract obtained using 90% of CO_2 and a EtOH:H_2O proportion of 5:5 achieved a similar TMA content (12%). Considering the solvent nature and the applied technology, the process condition that used 90% of CO_2 has some advantages over the others that used less CO_2 due to ease of solvent separation from the extract, reducing post-extraction preparation steps and resulting in an extract with lower solvent traces. Moreover, the high CO_2 availability and low price may reduce extraction costs with no detriment to extract's quality.

No direct relationship was observed between IC_{50} values and total phenolic contents, or even with any other compound or family of compounds quantified. The different phenolic compounds present in the extracts may have diverse abilities to reduce the Folin-Ciocalteu's reagent (Appel et al., 2001) and dissimilar antioxidant capacities. Moreover, synergistic, antagonist or even additive effects arising from phenolic compounds mixtures may contribute to the extract antioxidant ability (Seeram, 2008). Nevertheless, the extract (50:10:40) with the highest TA and R contents (15.0% and 2.0%, respectively) also showed the lowest IC_{50} value (21 μg). However, this extract was not the one with the highest TP content which, once again, confirms the important contribution of anthocyanins and rutin to the extracts antioxidant activities.

Vatai et al. (2009) performed a conventional extraction from lyophilized elderberries previously extracted with supercritical CO_2 (at 30 MPa), using EtOH/H_2O (50:50) and obtained an extract containing 7.5% GAE of total phenols and 0.43% CyGE of total anthocyanins. The CO_2/EtOH/H_2O high pressure elderberry pomace extracts obtained in the present study reached considerably higher contents, 15.8% GAE and 15.0% CyGE, that may be attributed to the usage of elderberry pomace that keeps 75-98% of total berry anthocyanins (Brønnum-Hansen et al., 1985) along with differences in the extraction methodology, namely operational pressure (0.1 MPa *vs* 20.9 MPa), temperature (323 K *vs* 313 K), solvent mixture (EtOH/H_2O (50:50) *vs* diverse CO_2/EtOH/H_2O solvent mixtures) and solid-to-solvent ratio (1:20 (w/v) *vs* 1:135 (w/w)).

5.5. Conclusions

High antioxidant activity anthocyanin-rich extracts were successfully obtained from elderberry pomace using CO_2 and diverse CO_2/EtOH/H_2O mixtures in a fractioned high pressure extraction methodology. CO_2/EtOH/H_2O solvent composition had a great influence on extract yield and composition, in terms of total phenolic compounds, total flavonoids, anthocyanins and rutin. The presence of EtOH and H_2O was important to promote the extraction of anthocyanins, even if their presence was not directly related with the extract antioxidant activity. Nevertheless, high anthocyanin contents were also achieved with high CO_2 amounts in the solvent mixture, with advantages to the fractionation process through the improvement of the solvent-extract separation step and the quality of the extract. The results of this study are useful to perform a complete exploitation of elderberries, by finding new applications for elderberry pomace, namely the production of anthocyanin-rich extracts, which may supply high antioxidant power to several formulations, with health-promoting advantages to consumers.

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6. Separation of volatile fractions from elderflowers by supercritical CO_2 extraction

The subject of this Chapter is the extraction of volatile compounds from elderflowers using supercritical CO_2, for possible applications as flavor or aromatic ingredients in food, cosmetic and pharmaceuticals products. A 3^2 factorial experimental design was applied to determine the effects of extraction temperature and of solvent density on the composition and characteristics of the obtained extracts. The text that comprises this chapter was published in Prosciba 2010 - II Iberoamerican Conference on Supercritical Fluids (Natal, Brazil) as a poster presentation, in the proceedings and CD-rom. This study will be published in a scientific journal after conclusion of the experimental work, namely the detailed analysis of the CO_2 extracts composition by gas chromatography and gas chromatography coupled to mass spectrometry. Moreover, further extractions using CO_2 plus a cosolvent will be performed in order to improve the extraction of phenolic compounds and therefore the bioactivity of the obtained extracts.

6.1. Abstract

Elder (*Sambucus nigra* L.), a plant native to most of Europe, northwest Africa and southwest Asia, is used either as an ornamental plant or a medicinal plant. Fresh, frozen or dried elderflowers have been used to produce distillates and extracts to be applied as natural ingredients in diverse alcoholic and non-alcoholic beverages, yoghurts, ice creams, cakes and candies. In recent years, elder cultivation is significantly increasing in Portugal. Besides the pleasant flavor that elderflower extracts impart to the food to which they are added to, their high phenolic acids and flavonoids contents may contribute to reduce the risk for some virus-induced and infectious diseases. Isoquercitrin, hiperoside, rutin and ferulic, caffeic and chlorogenic acids are among the phenolic compounds identified in these flowers. Terpenoid

alcohols and oxides constitute the major part of the volatile compounds present in their extracts. In this work, the extraction of volatile compounds from dried elderflowers was studied using supercritical carbon dioxide and following an experimental design where temperature (308-328 K) and solvent density (300-900 kg/m^3) were varied in a 3^2 factorial experiment. The composition of the extracts was explored using TLC and GC, and their potential to act as antioxidants was inferred by the coupled reaction of linolenic acid and β-carotene. Several volatile compounds were observed in CO_2 extracts, though no phenolic compounds could be detected. Extracts had pro-oxidant or low antioxidant activities and therefore, the use of ethanol as cosolvent should be explored in future works in order to obtain extracts with higher antioxidant activities. Obtained extracts may thus be used as natural aromatic flavor additives in pharmaceutical, cosmetic or food products, which suggest new high-value applications for elderflowers.

6.2. Introduction

Elder (*Sambucus nigra* L.) is a deciduous shrub native to most of Europe, northwest Africa and southwest Asia, that has had a common use in Europe since ancient times to prevent or cure numerous ailments and health problems (Ross, 2003). Almost all its parts are employed for different purposes, but berries, flowers and leaves are the most commonly used ones. The most relevant applications of this plant are in foods (pies, jellies, jams), beverages (wines, juices, teas) and medicinal products (lotions, aerosols, poultices, syrups and distillates).

Elderflowers, an agro-residue that is originated from the elder plant pruning process (commonly performed to enhance fruit production), are known in the pharmaceutical field as Sambuci Flos. In pharmacopeias, elderflowers are described as a diaphoretic for treatment of fever and chills, and as an expectorant for treatment of mild inflammation of the upper respiratory tract (WHO, 2002). While many of the reported effects lack adequate scientific validation, there is an increasing number of studies supporting important medicinal or therapeutic properties associated with elderflowers, namely anti-inflammatory (Mascolo et al., 1987; Yesilada et al., 1997), antiviral versus influenza types A and B and herpes simplex virus type 1 (Serkedjieva et al., 1990), diuretic (Beaux et al., 1998) and antidiabetic (Gray et al., 1999).

Elderflowers are a rich source of potential bioactive flavonoids and phenolic acids that exhibit a wide range of biological activities arising mainly from their antioxidant activities

(Rice-Evans et al., 1996; Lopez-Lazaro, 2002; Steinberg et al., 2003). Six flavonol glycosides were identified in elderflowers by Christensen and co-workers (2007). Quercetin-3-*O*-rutinoside (~21.0 mg/g dry weight of fresh flowers), isorhamnetin-3-*O*-rutinoside (~7.5 mg/g) and kaempferol-3-*O*-rutinoside (~2.0 mg/g) were the most important ones, representing over 90% of their total flavonoid content. Nine phenolic acids were also identified by the same authors, the major ones being 5-*O*-caffeoylquinic acid (chlorogenic acid, ~15.0 mg/g) and 1,5-di-*O*-caffeoylquinic acid (~12.2 mg/g), that comprised over 70% of their total phenolic acid content.

Other major elderflower secondary metabolites include volatile compounds that confer to these flowers typical sensory characteristics in terms of aroma and flavor. Kaack and co-workers (2006) identified and quantified 59 volatile compounds emitted from aqueous extracts of 86 different elder genotypes, from different European countries, and detected great variations among them. Terpenoid alcohols and oxides were the major volatile compounds detected, as well as other compounds such as non-oxidized terpenes, fatty acid derivatives, aldehydes and aromatic compounds. These authors also identified the important contributors to the floral and elderflower flavor (rose oxides, hotrienol, linalool, linalool derivatives and α-terpineol), and to the fruitiness and freshness of the extracts (mainly non-oxidized monoterpenes, aliphatic aldehydes and alcohols) and concluded that it is possible to produce elderflower products with different flavors in order to satisfy diverse consumer preferences.

The usual process to isolate volatile compounds from vegetable matrices, hydro-distillation, typically leads to very low yields. Toulemonde and co-workers (1983) obtained the essential oil from dry elderflowers on a modified Clevenger apparatus, during 24 h and obtained 0.053% (w/w) of yield. Conventional solid-liquid extraction methods using a sequence of liquid solvents with different polarities is also an alternative, but it is time consuming and most of the times it involves the utilization of pollutant and/or toxic organic solvents. Toulemonde and co-workers (1983) applied this sequential methodology to dry elderflowers, performing a previous ethyl alcohol (45%) extraction (to obtain a volatile essence) and subsequently a liquid-liquid extraction using isopentane; the yield of this last extraction was 0.015% (w/w).

Supercritical fluid extraction (SFE) represents a good alternative to these traditional methodologies and CO_2 was already applied with success in the extraction of vegetable and essential oils from innumerous vegetable matrixes (Reverchon, 1997; Reverchon and De Marco, 2006). The oxygen-free medium, the usually low extraction temperature, and the low activity of some enzymes at high pressure conditions considerably reduce the possibility of

oxidative, thermal and enzymatic degradation of those bioactive and labile compounds present in the raw material. In addition, supercritical CO_2 extraction can be optimized in terms of solvent power and selectivity, just by choosing the appropriate conditions of temperature and solvent density, and thus reducing to a minimum the co-extraction of undesired compounds.

The aim of the present study was to obtain volatile extracts from elderflowers, through supercritical CO_2 extraction, which may be applied in the food, pharmaceutical or cosmetic fields as flavor or aromatic ingredients. An experimental design was used to analyze the CO_2 selectivity effect on the extracts composition and antioxidant activity, varying the extraction temperature and solvent density.

6.3. Materials and methods

6.3.1. Raw material

Elderflowers were picked at the experimental field of Cooperativa Agrícola do Vale do Varosa (Portugal) in May of 2006. Flowers were cut off with about 5 cm stalk and dried in a fluidized bed dryer (MK II, Sherwood Scientific, England) at 313 K, in the absence of light, for approximately 5 hours. Stalks were removed and flowers were kept under vacuum in plastic bags, out of sunlight, until used in this study. Elderflowers were milled in a knife mill (Braun, KSM 2, Germany) and particle size distribution was obtained using a sieve series (50-18 mesh), under mechanical stirring (Retsch, Germany). Dried elderflowers humidity was determined by the Jacobs xylene distillation method (Jacobs, 1973), with duplicate assays.

6.3.2. Chemicals

Carbon dioxide (99.5%) and ethanol (99.5%) were used for the extraction experiments. Analytical grade chemicals and solvents employed for extract analyses were: methanol (99.9%), formic acid (~98%), ethyl acetate (99.9%), glacial acetic acid (p.a.), diphenylboric acid-β-ethylamino ester (p.a.), polyethyleneglycol-4000 (ultra), toluene (HPLC), *p*-anysaldehyde, sulfuric acid (95-98%), β-carotene (Type I, ~95% UV), Tween 40, linolenic acid (≥98.5%), chloroform (99.9%) and bi-distilled water. Employed standards for TLC analysis were rutin hydrate (≥95%), isoquercitrin (≥90%), astragalin (HPLC), quercetin dehydrate (≥98%), chlorogenic acid (≥95%), p-cumaric-acid (predominantly trans isomer), p-caffeic acid (99%) and ferulic acid (≥98%).

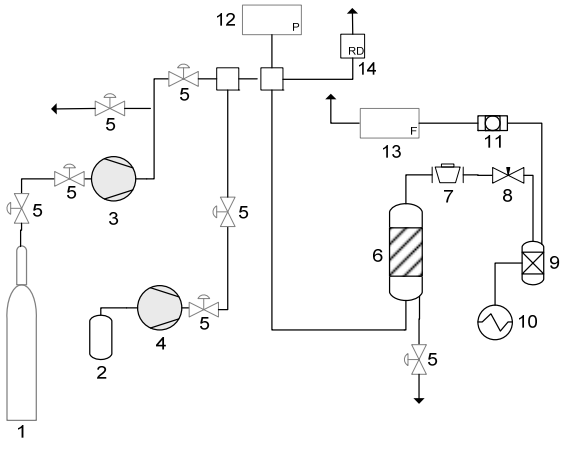
6.3.3. Experimental extraction procedures

6.3.3.1. Conventional extractions

The volatile oil was obtained by hydrodistillation (duplicate assays) in a Schilcher apparatus for 180 min, at the solvent boiling point and with a solid-to-solvent ratio of 1:20 (w/v). Conventional solid-liquid ethanol extraction (duplicate assays) was performed during 360 min, under magnetic stirring (800 rpm), at approximately 313 K, and applying a solid-to-solvent ratio of 1:10 (w/v). During this extraction, the emitted volatile compounds were collected by head-space: an adsorbent packed column (Porapak Q 80/100 mesh) was placed on the top of the Erlenmeyer flask where the extraction occurred, and eluted with ethyl acetate at the end of the extraction, to recover the volatile compounds.

6.3.3.2. Supercritical extractions

Supercritical CO_2 extractions were performed in a laboratory equipment (LAB SFE 20 mL, Separex, Champigneulles, France), represented in Figure 6.1. An experimental design was followed, in which temperature (308-328 K) and solvent density (300-900 kg/m^3) were varied in a 3^2 factorial experiment. Extraction time was 240 min and solid-to-solvent ratio was 1:151±2 (w/w, dry basis, d.b.). For all experiments extracts were collected at approximately 268 K, and an adsorbent packed column was placed at the solvent exit to prevent extract losses. At the end of the extractions, the empty extraction cell and tubing line were cleaned with 200 mL of ethanol. The recovered extracts were evaporated at 313 K and in the absence of light, using a rotary evaporator with vacuum control (BÜCHI Rotavapor R-210, Switzerland). Dried extracts were kept at 255 K until further analysis. The central point of the experimental design was duplicated to obtain the extraction error.



1	CO_2 cylinder
2	Cosolvent reservoir
3	CO_2 pump
4	Cosolvent pump
5	On-off valve
6	Extraction vessel
7	Back-pressure regulator
8	Micrometric valve
9	Refrigerated recovering flask
10	Heat exchanger
11	Adsorbent packed column
12	Flow meter
13	Manometer
14	Rupture Disk

Figure 6.1. Schematic diagram of the employed supercritical extraction equipment.

6.3.4. Characterization of elderflower extracts

6.3.4.1. Thin layer chromatography analysis

Thin Layer Chromatography (TLC) of volatile and phenolic compounds in CO_2 and ethanol extracts was performed on silica gel plates (20 cm × 20 cm, thickness 0.2 mm) (Merck, Germany). To observe the volatile compounds, a toluene-ethyl acetate (93:7, v/v) mixture and an anisaldehyde-sulfuric acid solution were used as the mobile phase and spray reagent, respectively (Wagner et al., 1984). To develop the phenolic compounds (at 365 nm), an ethyl acetate-formic acid-glacial acetic acid-water (100:11:11:27, v/v) and NP/PEG solution were used as the mobile phase and spray reagent, respectively (Wagner et al., 1984). Besides the extracts (400 μg), the following phenolic standards were applied: rutin hydrate, isoquercitrin, astragalin, quercetin dehydrate, chlorogenic acid, p-cumaric-acid, p-caffeic acid and ferulic acid.

6.3.4.2. Gas chromatography analysis

A gas chromatograph (Tremetrics 9001, Thermo Scientific, Austin, USA), equipped with a fused capillary column (15 m × 0.32 mm i.d., 0.45 μm, CP-Sil 8 CB, Varian, Middelburg, The Netherlands) was used. Carrier gas was helium (1.4 mL/min) and 2 μL of sample (10 mg/mL, in ethyl acetate) was injected. The injector temperature was 503 K and that of the detector was 533 K. Temperature program was 306 K (for 1 min), 306-403 K (at 2 K/min), 403-523 K (at 10 K/min) and 523 K (for 10 min) (Jørgensen et al., 2000).

6.3.4.3. Antioxidant activity: β-carotene and linolenic acid coupled reaction assay

Antioxidant activities of extracts were determined by a method based on the coupled oxidation of the β-carotene/linolenic acid system (Hammerschmidt and Pratt, 1978). Reactions were monitored by absorbance readings at 470 nm, at 0, 1, 2 and 3 h of reaction, and results were expressed as oxidation inhibition (in percentage).

6.3.5. Calculation procedures

The mean geometric diameter of elderflower particles was calculated according to the American Society of Agricultural Engineers ASAE S319.2 method (ASAE, 1993). Total yields were calculated as the ratio between the total extract mass and the raw material mass, on a dry basis (d.b.). For supercritical experiments, the total extract mass was determined summing the extract mass collected in the recovering flask, the extract mass retained in the adsorption column and the one recovered from cell and tubing line cleaning.

6.4. Results and discussion

Elderflowers having a mean geometric particle diameter of 0.82 mm and 7.2±0.7% (w/w, d.b.) of humidity were used for the extraction experiments. Extract yield was 0.042±0.0007% (w/w, d.b.) for hydrodistillation and 0.021±0.005% for the volatile compounds collected by head-space.

Total extraction yields of CO_2 supercritical extractions (Figure 6.2) varied between 0.46% (328 K, 300 kg/m^3) and 3.96% (318 K, 900 kg/m^3). As expected, there was a direct relationship between CO_2 density and total extraction yield, for all tested temperatures, due to the larger solvent power at higher densities. While all the essential oil components are largely soluble in supercritical CO_2 at mild operational pressures (9-10 MPa) and temperatures (313-323 K), other components, like cuticular waxes and fatty acids exhibit a low solubility at these conditions (Reverchon and De Marco, 2006). On the contrary, there was not an apparent relationship (direct or indirect) between extraction temperature and yield, at a constant solvent density, and therefore there were other factors, besides the compounds vapor pressure, that governed the amount of extracted substances, probably related to mass transfer phenomena, like compounds diffusivities and solvent viscosity (Brunner, 1994).

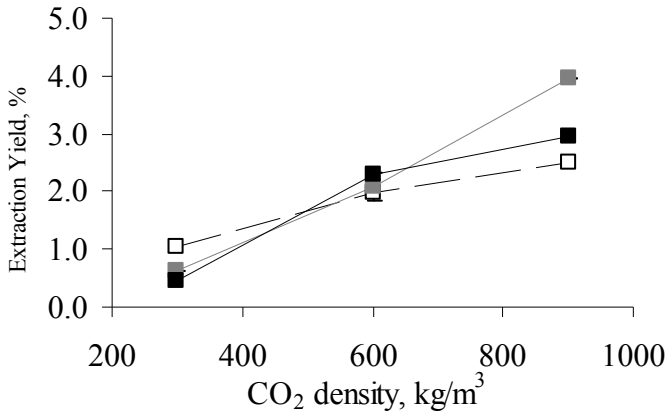


Figure 6.2. Total extraction yields of supercritical CO_2 extractions of dry elderflowers obtained at 308 K (□), 318 K (■) and 328 K (■), and different CO_2 densities: 300, 600 and 900 kg/m^3.

The TLC plate for the analysis of volatile compounds in elderflowers extracts (Figure 6.3.a) was characterized by a series (at least 18 distinct compounds) of blue, green, yellow, violet and grey zones extending from Rf 0 to the solvent front. Most of the zones were observed at Rf values bellow ~0.75, indicating the predominance of higher polarity volatile compounds, such as terpene alcohols (Rf 0.15-0.3), aldehydes (Rf 0.4-0.65) and ketones (Rf 0.45-0.7) (Wagner et al., 1984). The same zones were detected in the ethanol and in the

supercritical extracts, though with different intensities. Different intensity zones were also detected amongst CO_2 extracts, in particular the violet zone at Rf 0.6 that was very weak in the extracts obtained at 308 K and 300 kg/m^3, and at 328 K. Furthermore, the intensity of the dark grey zone at the solvent front (that corresponds to a low polarity compound or mixture of compounds) seemed inversely related with the extraction temperature and solvent density. The decrease in solubility of some volatile compounds present in elderflowers (cineole, limonene, camphor, benzaldehyde, linalool) with the increasing CO_2 temperature (Gupta and Shim, 2007) could possibly contributed to this behavior.

Only traces of phenolic compounds could be detected in 400 μg of the CO_2 extracts applied to the TLC plate, which might be explained by the low solubility of these compounds in supercritical CO_2 when no cosolvent is used (Gupta and Shim, 2007). In contrast, rutin hydrate, isoquercitrin, astragalin, quercetin dehydrate, p-caffeic and ferulic acids were detected in the ethanol extract (Figure 6.3.b).

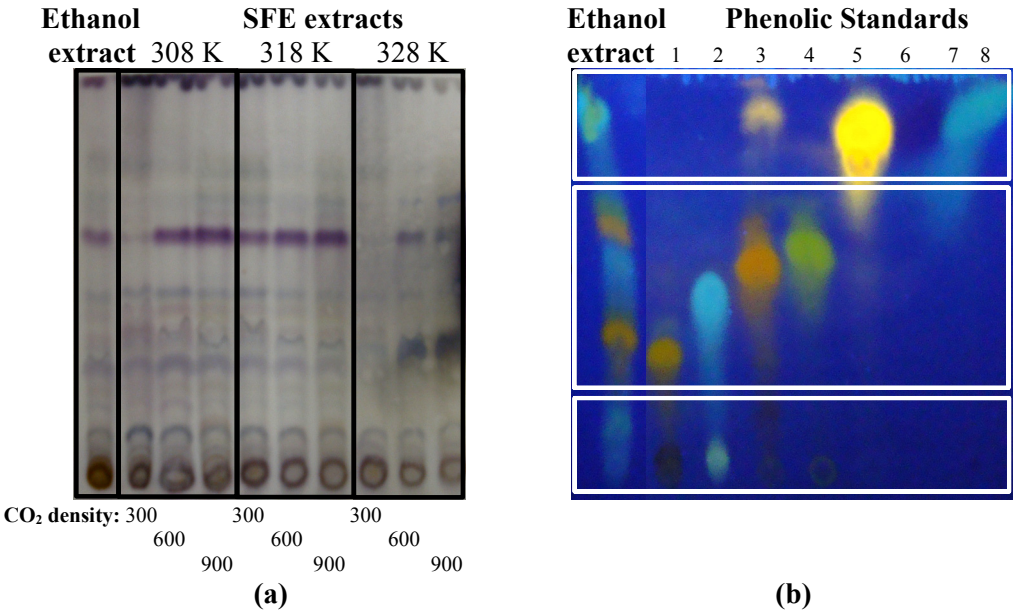


Figure 6.3. Analysis of volatile compounds **(a)** and phenolic compounds **(b)** in elderflower extracts, by anisaldehyde-sulfuric acid solution and NP/PEG solution sprayed TLC plates. Phenolic standards: 1-rutin hydrate, 2-isoquercitrin, 3-astragalin, 4-quercetin dehydrate, 5-chlorogenic acid, 6-p-cumaric-acid, 7-p-caffeic acid, 8-ferulic acid.

The gas chromatographs of the CO_2 extract obtained at the central point (318 K; 600 kg/m^3), the one obtained by hydrodistillation and the one corresponding to the volatile compounds trapped in the adsorbent column, during the conventional ethanol extraction (i.e., collected by head-space) are shown in Figure 6.4 (lower, middle, and upper lines, respectively). The first two are similar in terms of peaks retention times that were above 50

minutes, for the majority of the peaks, while the latter presented lower retention time peaks. This analysis reveals some similarities in the volatile composition of the supercritical extract obtained at 318 K and at a CO_2 density of 600 kg/m^3 and the hydrodistillation extract. The lower retention time peaks in the upper chromatogram revealed the presence of volatile compounds in elderflowers that were not extracted by hydrodistillation and by supercritical extraction, at the referred experimental conditions. Jørgensen et al. (2000) presented a gas chromatograph of volatiles collected from an elderflower drink, by dynamic head-space sampling, and identified 76 peaks, with retention times above ~ 8 min. In order to identify the compounds present in the extracts obtained in this study (by GC-MS), a previous fractionation of the extracts obtained by supercritical extraction is required.

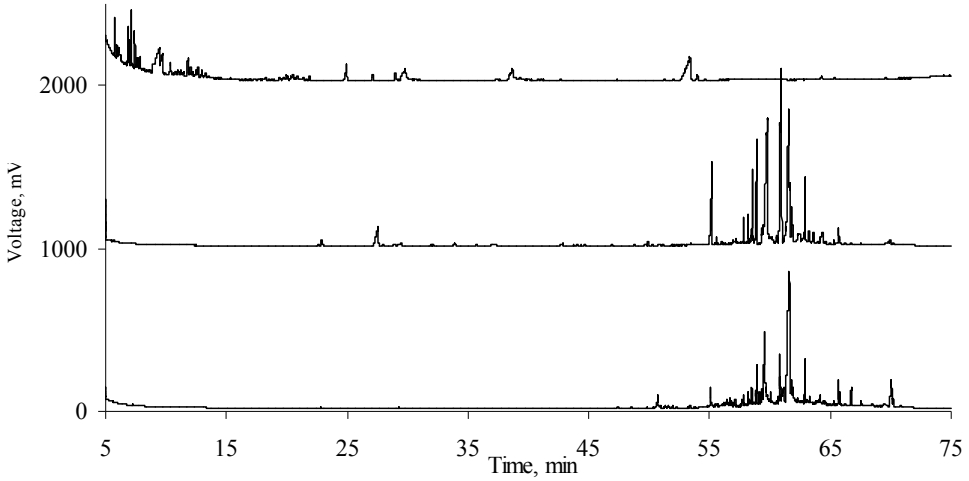


Figure 6.4. Gas chromatograph of elderflowers extracts obtained by SFE (318 K, 600 kg/m^3) (*lower line*), hydrodistillation (*middle line*) and volatile compounds collected by head-space during conventional ethanol extraction (*upper line*).

Oxidation inhibitions of the elderflowers CO_2 extracts are reported in Figure 6.5. The extracts obtained at the lowest CO_2 density (300 kg/m^3) and the one obtained at 328 K and with a CO_2 density of 600 kg/m^3 presented pro-oxidant activities, while the others presented low antioxidant activities. Considering the high solubility of most essential oil compounds in supercritical CO_2 at mild conditions of temperature and pressure, it seems that the elderflowers essential oil components did not confer antioxidant activity to the extracts. This result is in accordance with the findings of Ruberto and Baratta (2000), which found low antioxidant activities, if any, for terpenoid alcohols and oxides, and non-oxidized terpenes, the main essential oil components of elderflowers (Kaack et al., 2006).

The extracts obtained at different temperatures and with the highest CO_2 density presented very similar oxidation inhibition capacities (6.0-8.2%), which may reflect their more

homogeneous composition, when compared to the ones obtained at lower solvent densities that presented more diverse activities. This behavior may possibly be related to an increase in the CO_2 solvent power with density, and consequently to a selectivity decrease.

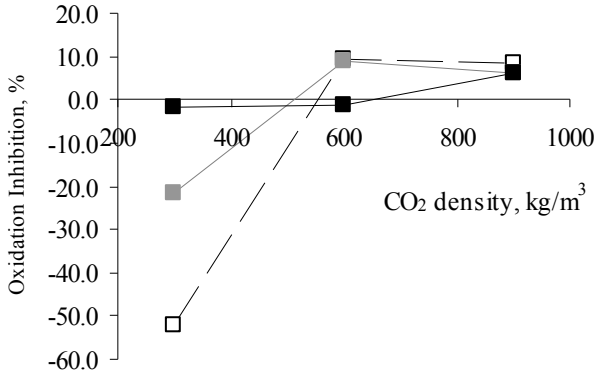


Figure 6.5. Oxidation inhibitions (after 3 h of reaction) of supercritical CO_2 extracts of elderflowers obtained at 308 K (□), 318 K (■) and 328 K (■), and different CO_2 densities: 300, 600 and 900 kg/m^3.

The oxidation inhibition of the ethanol extract was 44.3±1.2%, after 3 h of reaction. The presence of phenolic compounds in this extract (as detected by TLC analysis, Figure 6.3.b) can certainly explain this result, as these specific compounds are well known for their high antioxidant activities. Therefore, the utilization of ethanol as a cosolvent in the SFE from elderflowers should be explored in future works, in order to obtain extracts with higher antioxidant activities.

6.5. Conclusions

Elderflower extracts were obtained by CO_2 SFE, hydrodistillation, and conventional ethanol extraction. CO_2 SFE was performed at different conditions of temperature (308-328 K) and of solvent density (300-900 kg/m^3). Extraction yields increased with CO_2 density, reflecting the solvent power increase, and similar extracts oxidation inhibitions were observed at the highest CO_2 density, which may reflect the extracts similar composition and thus, the lower solvent selectivity. Volatile compounds were observed in both CO_2 and ethanol extracts by TLC analysis, while phenolic compounds were only observed in the conventional ethanol extract. Moreover, CO_2 extracts revealed to have pro-oxidant or low antioxidant activities, while the ethanol extract revealed a moderate antioxidant activity, and therefore, higher antioxidant activity extracts may be obtained by SFE, if ethanol is used as a cosolvent.

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Part C. Pine (*Pinus pinaster*) bark

7. Aqueous extraction of condensed tannins from maritime pine bark for leather tanning applications: influence of the solvent additive

The topic of this Chapter is the optimization of a solvent additive (sodium hydroxide, formic acid, ethanol and sodium sulfite) to be used in conventional aqueous extractions from maritime pine bark and in order to obtain tannin-rich extracts. This study was done in collaboration with a Portuguese leather tanning company (Curtumes J.B. Salgueiro Lda.) which is interested in reducing the usage of chromium salts in leather tanning processes by applying less pollutant vegetable tanning agents. This study was also supported by "Programa CYTED - Ciencia y Tecnologia para el Desarrollo", under the scope of the Project "Aplicaciones Industriales de los Taninos Vegetales". This study will be published in a scientific journal after conclusion of the size exclusion chromatography analysis, which will allow the evaluation of the molecular weight distribution of the compounds present in the extracts.

7.1. Abstract

Pinus pinaster bark, a waste residue that represents approximately 10-20% of the tree trunk, is almost exclusively used as fuel, in spite of being a rich source of condensed tannins and phenolic compounds, suggesting a wide range of fine applications. In order to obtain condensed tannins-rich *P. pinaster* bark extracts (for later application in leather tanning), conventional aqueous extractions were performed varying the type and the amount of the employed solvent additives: NaOH (0.5, 1.0 and 1.5%, w/v), HCOOH (0.5, 1.0 and 1.5%, v/v) and EtOH (5, 10 and 15%, v/v), with and without 1.0% (w/v) of $NaSO_3$. The most favorable additive in terms of total phenolic compounds and condensed tannins was EtOH, and extracts with a maximum of 34.8% of gallic acid equivalents, for the former, and 62.8% of catechin

monohydrate equivalents, for the latter, were obtained. Moreover, the favorable pH (~3.5) and relatively low viscosities of the aqueous extract solutions prepared revealed the potential of these EtOH extracts for leather tanning applications, as well as for other applications, like those in the food and pharmaceutical industries. The small amount of employed EtOH may represent a strong advantage when an industrial process is considered. The results of this study demonstrate that high valuable extracts can be obtained from *P. pinaster* bark (an abundant and low-cost agro-industrial residue) by the use of a conventional extraction methodology with water as the major solvent.

7.2. Introduction

Pinus pinaster (maritime pine) is native to the western Mediterranean region, being one of the most important trees in forestry in Portugal, Spain and France. The Portuguese National Forest Inventory (DGF, 2006) reports around 710600 ha for *P. pinaster* in 2006, which corresponds to 23% of the total Portuguese forestry area. This species is commercially explored essentially for its wood, and its main applications are pulp and paper production, construction, chipboards, floor boards and palettes. In 2006, the Portuguese production of *P. pinaster* coniferous logs reached 2100000 m^3 (DGF, 2006). Its bark, which represents 10-20% of the trunk, is almost exclusively used as fuel, despite its high polyphenol content (Jerez et al., 2006; Jerez et al., 2009). In fact, its rich polyphenol composition together with other favorable features, such as low price, high availability and long-term stability, makes the use of this waste residue highly attractive. Furthermore, the well known disposal problems of polyphenol rich vegetable matrices (Capasso et al., 1992) may be attenuated by its industrial usage.

As opposed to wood, the chemical composition of *P. pinaster* bark is complex and it is characterized by a high yield of extractives and phenolic compounds, essentially condensed tannins. Successive extractions of *P. pinaster* bark with hexane and benzene, ether, ethanol, water and aqueous NaOH (1%, w/v) resulted in 2.5, 0.9, 11.6, 4.1 and 22.7% of yield, respectively. The lipophilic fraction consisted mainly of waxes while the hydrophilic fraction consisted mainly of polyflavonoids with phloroglucinol A-rings (Vázquez et al., 1987a), that exhibit high reactivity towards formaldehyde (Pizzi, 1980). The polysaccharides in *P. pinaster* bark are roughly 29% and its aromatic content (which provides an indication of all the phenolic compounds present in bark) is 60.0%. Finally, Klason lignin (which is the acid-insoluble lignin) is around 30% (Vázquez et al., 1987a).

The condensed tannins in *P. pinaster* bark consist of mixtures of oligomers and polymers with an average degree of polymerization of 10.6 (Jerez et al., 2007b), containing (+)-catechin and/or (-)-epicatechin units linked mainly through C4-C8 and/or C4-C6 bonds. Some of these condensed tannins are non-extractable, due to the existence of covalent bonds to cell walls or to other cell components (Appel et al., 2001). However, others are extractable (though partially water insoluble) due to their weak chemical linkages with non-extractable condensed tannins, polysaccharides and/or proteins (Mattews et al., 1997; Xu et al., 2006). Non-extractability of condensed tannins is related to their role of protecting bark underlying living tissues against invasion by pathogens or rots (Mila et al., 1996). In order to this protection be effective, condensed tannins must resist leaching by rainwater despite their well known water solubility when in a free form.

The interest in the use of pine bark arose from the oil crisis of the 1970s, due to the possibility of making a variety of chemicals traditionally obtained from petroleum. Both the extract and the raffinate obtained from the alkaline lixiviation of pine bark have found applications: the former being used as a phenol substitute in the production of wood adhesives, and the latter offering a high content of polysaccharides. Several scientific studies have been published on the extraction of condensed tannins from pine bark, but most of them were motivated by their application in adhesive formulations (Jorge et al., 2001; Sealy-Fisher and Pizzi, 1992; Vázquez et al., 1987b; Vázquez et al., 2001). Recently, some studies were published concerning the use of *P. pinaster* bark extracts in potent antioxidant formulations (Jerez et al., 2007a; Jerez et al., 2007b; Touriño et al., 2005). However, there are other possible applications for pine bark condensed tannins, in particular as a chromium substitute in leather tanning. The elimination of chromium (III) salts, which currently applied in the production of 80-90% of the world leather, would represent an important step in the development of an eco-friendly process, since the poor chromium leather uptake (50-70%) leads to material wastage and to the corresponding environmental concerns. Chromium levels in untreated tannery wastewater are typically 100-400 mg/L, while international limits for discharge are increasing stringent, typically less than 2 mg/L (World Bank, 1998).

Molecular weight of vegetable condensed tannins for leather tanning should be between 500 and 3000 Da: lower molecular weight compounds have weak protein complexation and polymerization abilities (Yao et al., 2006) while higher molecular weight compounds poorly penetrate into rawhides and rawskins, and, consequently, do not bind or bind very weakly to collagen (Mavlyanov et al., 2001).

Even though there are advantages in the usage of vegetable tanning agents, due to their natural and renewable origin, the co-extraction of undesirable compounds is an inconvenient that makes the final tanning process hard to control (Covington, 1997). Furthermore, the protein precipitation capacity of tannin rich fractions increases with their tannin contents (Yao et al., 2006). The selectivity of the extraction process depends on the choice of the appropriate extraction conditions, like temperature, solid-to-solvent ratio and type of solvent, among others. The employment of solvent chemical additives, such as sodium hydroxide, trisodium phosphate, sulfanol, phenol and sulfite salts is recommended in some cases in order to increase tannin extraction yields. In particular, sulfite salts are used with the intention of improving tannin aqueous solubility and of reducing its viscosity, as a result of the cleavage of tannin interflavanoid bonds with the generation of lower molecular weight procyanidin-4-sulphonated derivatives (Foo et al., 1983). Nevertheless, in the production of leather tanning agents, it is important to restrict the severity of this reaction, since dimeric and trimeric procyanidins have poor tanning abilities (Foo et al., 1983). The usage of organic solvents, such as ethanol, though originating higher tannin extraction yields, has been avoided at an industrial scale due to their high cost (Sealy-Fisher and Pizzi, 1992). Moreover, when selecting a solvent to extract tannins for leather tanning, the dissolution of the obtained tannin rich extract in aqueous media should be always considered. Small amounts of an extraction additive may substantially enhance the extraction of tannins without causing the process to be commercially unattractive, and simultaneously maintain the extract solubility in water, allowing its application in leather tanning.

In the Portuguese leather industry there is an increasing consciousness of the need to substitute chromium salts for organic tanners. Pine bark extracts have been used for centuries for leather tanning in Portugal, typically in small family-operated businesses. However, the choice of the aqueous extraction solvent additives usually applied - sodium hydroxide and sulfites - is not supported by strong scientific knowledge in the field, but rather on the practice of the pine adhesives industry, where sodium hydroxide and sulfites are commonly used as solvent additives. Bearing that in mind, the purpose of this work was to study the influence of small amounts of a base (sodium hydroxide, 0.5-1.5%), of an acid (formic acid, 0.5-1.5%), and of an organic solvent (ethanol, 5-15%), with and without the addition of sodium sulfite (1.0%), on the characteristics of *P. pinaster* bark aqueous extracts, considering their possible leather tanning applications. Extraction yields, as well as total phenolic compounds and tannins contents, pH values, viscosities and colors of the extracts were determined and

compared to diverse tannin-rich products, among which we included some commercially available, frequently used tanning agents.

7.3. Materials and methods

7.3.1. Raw material

Comminuted *P. pinaster* bark from Pinhal de Leiria (Portugal) was provided by Curtumes J.B. Salgueiro Lda., a Portuguese leather company, and stored at room temperature. Its humidity was determined by the Jacobs xylene distillation method (Jacobs, 1973), with triplicate assays, and its particle size distribution was obtained using a sieve series (500 mesh – 3-1/2 mesh) under mechanical stirring (Retsch, Germany).

Commercial extracts of quebracho and wattle (rich in condensed tannins), an extract of chestnut and tara powder (rich in hydrolysable tannins), and a synthetic tannin product (Figure 7.1) were also employed in order to compare to the *P. pinaster* bark extracts obtained in this study. Three lots of wattle extract (from Mimosa Extract Company Ltd. and from Tanac S.A. (2008 and 2009)) and two lots of tara powder (from Colorobbia Italia S.p.A. and from Transformadora Agrícola S.A.C.) were used. Curtumes J. B. Salgueiro Lda. company provided quebracho and wattle extracts (from Unitan and from Mimosa Extract Company Ltd., respectively), chestnut extract and tara powder (from Colorobbia Italia S.p.A.), and synthetic tannin (from Clariant).

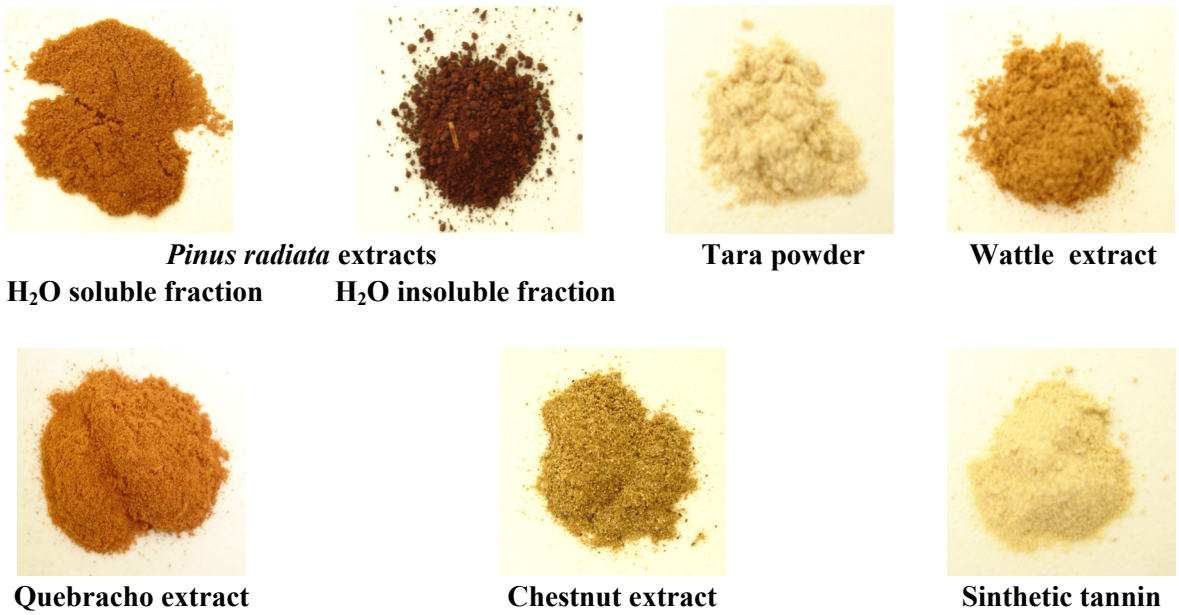

Figure 7.1. *Pinus radiata* extract fractions and commercial tannin-rich products considered in this study.

The features of these tannin-rich products, as provided by their respective producers, are reported in Table 7.1. Two extract fractions of *Pinus radiata* D. Don bark were kindly supplied by Professor Alex Berg of Universidade de Concepção (Chile). *P. radiata* bark was extracted with methanol 80% (w/w) at 393 K, and the obtained extract was subsequently fractioned by distillation into two fractions: a H_2O insoluble fraction (soluble in methanol) and a H_2O soluble fraction (insoluble in methanol). The humidity of all tannin-rich materials was determined gravimetrically (triplicate assays).

7.3.2. Chemicals

For the extraction experiments, distilled water, formic acid (~98%, Fluka, Steinheim, Germany), sodium hydroxide anhydrous (p.a., Carlo Erba, Milan, Italy), ethanol (99.5%, Panreac Quimica S.A., Barcelona, Spain) and anhydrous sodium sulfite (97%, Esseco S.p.A., Italy) were employed. For extracts analyses, the following analytical grade chemicals and solvents were used: ethanol (99.5%, Panreac Quimica S.A., Barcelona, Spain), Folin-Ciocalteu's phenol reagent (Merck, Darmstadt, Germany), sodium carbonate (Pronalab, Lisbon, Portugal), vanillin (José M. Vaz Pereira S.A., Sintra, Portugal), methanol (Fluka, Buchs, Switzerland), hydrochloric acid (37%) and potassium iodate from Riedel-de-Haën (Seelze, Germany), and o-xylene (97%), anhydrous methanol (99.8%), ethanolamine (99%), ammonium acetate (97%) and sulfuric acid (95-98%) from Sigma-Aldrich Inc. (Steinheim, Germany). Employed standards for phenolic compounds and condensed and hydrolysable tannins quantification were, respectively, gallic acid (≥98%) and D-(+)-catechin monohydrate (≥98%) (from Sigma-Aldrich Inc., Steinheim, Germany), and methyl gallate (≥98%) (from Fluka, Buchs, Switzerland).

7.3.3 Experimental conventional extractions procedure

Conventional solid-liquid extractions, at atmospheric pressure and at the solvent boiling temperature, were performed in a round-bottom flask without stirring, attached to a cooled condenser to reflux the uprising solvent. A 1:10 (w/v) solid-to-solvent ratio was employed and extraction time was 2 hours. Aqueous extractions were performed using, as additives, NaOH (0.5, 1.0 and 1.5%, w/v), HCOOH (0.5, 1.0 and 1.5%, v/v), EtOH (5, 10 and 15%, v/v) with and without Na_2SO_3 (1.0%, w/v). Afterwards, *P. pinaster* bark extracts were vacuum filtered, freeze-dried (Labconco, model 77560, USA), and kept at 255 K until further analyses.

Table 7.1. Characterization of the commercial tannin-rich products (extracts of quebracho, wattle and chestnut, tara powders and synthetic tannin) as provided by their respective producers.

Commercial product		Producer	Country	Tannins, %	Non-tannins, %	Insolubles, %	Humidity, %	pH (Aq. sol. conc.)
Condensed tannins rich products	Quebracho extract	Unitan	Argentina	70.4 (Min)	21.5 (Max)	0.4 (Max)	8.1	4.93 (6.7 ºBé)
	Wattle extract	Mimosa Extract Company, Ltd.	South Africa	67.5 (Min)	24.0 (Max)	0.4	7.3	4.75 (6.7 ºBé)
		Tanac S.A. (2008 and 2009)	Brazil	74.0±2.0 (d.b.)	-	0	10.0	4.0-5.0 (20%)
Hydrolysable tannins rich products	Chestnut extract	Colorobbia Italia S.p.A.	Italy	67-70	20-28	1-2	5-8	3.5±0.5 (10%)
	Tara powder	Colorobbia Italia S.p.A.	Italy	60±3	18±1	20	9-10	3.3-3.5 (10%)
		Transformadora Agrícola S.A.C.	Peru	> 48	-	0	8-12	-
Synthetic tannin		Clariant	Switzerland	-[a]	-	-	-	2.9-3.4 (5%)

[a]Active substance - approximately 92%.

7.3.4. Characterization of maritime pine bark extracts

7.3.4.1. Quantification of total phenolic compounds

Total phenolic compounds in pine bark extracts and in commercial tannin-rich products were quantified according to the Folin-Ciocalteu's method, following the procedure proposed by Singleton and Rossi (1965) with some modifications based on Cheung et al. (2003). Synthetic tannin product was not considered for this analysis. Extracts solutions were prepared using ethanol (10%, v/v) and up to 0.5 mL aliquots were reacted with the Folin-Ciocalteu's reagent. Saturated Na_2CO_3 (~17%) and distilled water were added and the reaction was kept in the dark for 90 min, after which absorbances were recorded at 725 nm using a UV/VIS spectrophotometer (Jasco V-530, Japan). Results were expressed as gallic acid equivalents (GAE), in percentage (mg GAE/mg extract × 100 in a dry basis, d.b.).

7.3.4.2. Quantification of condensed tannins

The vanillin-H_2SO_4 methodology (Sun et al., 1998) was used to determine condensed tannins contents in pine bark extracts and in commercial tannin-rich products. Synthetic tannin was not considered for this analysis. Samples were diluted in methanol, centrifuged during 5 minutes at 3000 rpm, and the supernatant analyzed. Aliquots of each sample were reacted with 1.25 mL of the vanillin reagent (1% (w/v) vanillin in methanol) followed by 1.25 mL of the acid solution (25% (v/v) H_2SO_4 in methanol) at 303 K.

A non-vanillin-containing sample was run for each sample. Absorbances were recorded at 500 nm after 15 min, and results were expressed as (+)-catechin monohydrate equivalents (CME), in percentage (mg CME/mg extract × 100, d.b.).

7.3.4.3. Quantification of hydrolysable tannins

Hydrolysable tannins were assayed by reaction with KIO_3, after a methanolysis step to release methyl gallate moieties, as described by Hartzfeld et al. (2002). Samples were reacted at 85 °C for 20 h in methanol and sulfuric acid, centrifuged, and the supernatant was analyzed. Ethanolamine was added and pH was adjusted to 5.5, followed by oxidation of the methyl gallate by KIO_3 at 30 °C. Absorbances at 525 nm were recorded and results were expressed as methyl gallate equivalents (MGE), in percentage (mg MGE/mg extract × 100, d.b.).

7.3.4.4. Quantification of total tannins: hide powder assay

The experimental procedure followed for the quantification of total tannins by the hide powder assay was based on a method standardization development performed in the scope of the Cyted Project, in which several Ibero-American laboratories participated. It started with the preparation of 250 mL of aqueous solutions of pine bark extracts and tannin-rich products. Total solids and soluble solids in these samples were determined gravimetrically by drying (at ~373 K) the original solutions (5 mL) and previously vacuum filtered solutions (25 mL - using a 0.45 μm cellulose acetate filter) until achieving a constant weight. The hide powder (2.0 g per sample) was placed inside a white cotton fabric and covered with distilled water. Afterwards, it was mixed, allowed to stand for 15 minutes and squeezed. This process was repeated two more times. Sample solutions (33 mL) were added to the hide powder and the suspension was stirred (50-60 rpm) for 30 minutes, and finally vacuum filtered through a regular Whatman filter paper. The filtrate was evaporated in order to determine the non-tannins. Total tannins (%) were calculated as the difference between soluble solids (%) and non-tannins (%). If the tannin concentration in the initial aqueous extract solution was not between 3.75 and 4.25 g/L, the analysis was repeated, adjusting the extract concentration.

7.3.4.5. pH and rheological characterization

Aqueous solutions of pine bark extracts and of commercial tannin-rich products were prepared at two different concentrations, namely 20 and 50% (w/v), to cover the concentrations applied in their most common industrial applications (Garnier et al., 2001). Mixtures were moderately stirred at room temperature using a magnetic stirrer. A Meterlab PHM210 standard pH Meter (Radiometer Analytical, France) was used to measure the pH of the 20% solutions. Rheological tests were carried out in a controlled stress rheometer (Model RS1, Haake, Germany) at 293 K. A cone-plate sensor (60 mm diameter) at a gap of 0.052 mm was used for all extract solutions, except for the solutions of *P. pinaster* bark extracts obtained with NaOH as the solvent additive at 50% concentration, given their higher viscosities. For these samples a plate sensor (20 mm diameter) at a gap of 1 mm was used. Flow tests were performed in triplicate with upward ramps and limit viscosities at high shear rates were reported.

7.3.4.6. Color measurements

Color (CIE L*, a*, and b*) values of pine extracts and of the commercial tannin-rich products considered in this study were measured using a hand-held Minolta CR-200b

colorimeter (Osaka, Japan). Chroma (c*) and hue angle (h*) values were then calculated. The reported results are the average of at least ten measurements.

7.3.5. Calculation procedures

The mean geometric diameter of *P. pinaster* bark particles was calculated according to the American Society of Agricultural Engineers ASAE S319.2 method (ASAE, 1993). Extraction yields were calculated as the ratio between the total extract mass and the raw material mass, on a dry basis. The results reported in this study are the averages of at least three measurements, and the coefficients of variation, expressed as the ratio between standard deviations and mean values (in %), were found to be less than 10% in all cases.

7.4. Results and discussion

7.4.1. Extraction yields

P. pinaster bark particles of mean geometric diameter of 0.81 mm, containing 5.5±0.4% (w/w, d.b.) of humidity, were used in the extraction experiments. Figure 7.2 represents the extraction yields (%, d.b.) obtained from the two-hour long extractions, using a solid-to-solvent ratio of 1:10 and modified aqueous solvents with NaOH, HCOOH (both at 0.5-1.5%) and EtOH (5-15%), either or not with 1.0% of Na_2SO_3. The presence of NaOH in the extraction medium was favorable in terms of the extraction yield, resulting in 21.5-38.5%, while for HCOOH and EtOH values were in the 5-7% range. An increase of the extraction yield with the additive amount was observed when NaOH and HCOOH were used, though this effect was considerably more evident for the alkaline additive. In contrast, extraction yield was not significantly affected by the amount of organic additive applied (for the tested concentrations).

NaOH is typically employed in the extraction of formaldehyde condensable phenolic compounds from pine bark, resulting in extraction yields usually above 15%, depending on several factors like particle size distribution, extraction temperature, solid-to-solvent ratio and NaOH concentration (Jorge et al., 2001; Vázquez et al., 2001). Vázquez et al. (2001) also observed an extraction yield increase with the NaOH concentration, which was attributed to an increase in the polysaccharides extraction yield, essentially hemicelluloses (Vázquez et al., 1987c), that may be credited to an increased decoupling of lignin-carbohydrate ester linkages (Sjöström, 1993). Moreover, lignin is relatively soluble in alkaline solutions (Kurth, 1947),

and significant amounts of lignin (~23%) were already detected in *P. pinaster* bark extracts obtained with NaOH aqueous solutions (Fradinho et al., 2002).

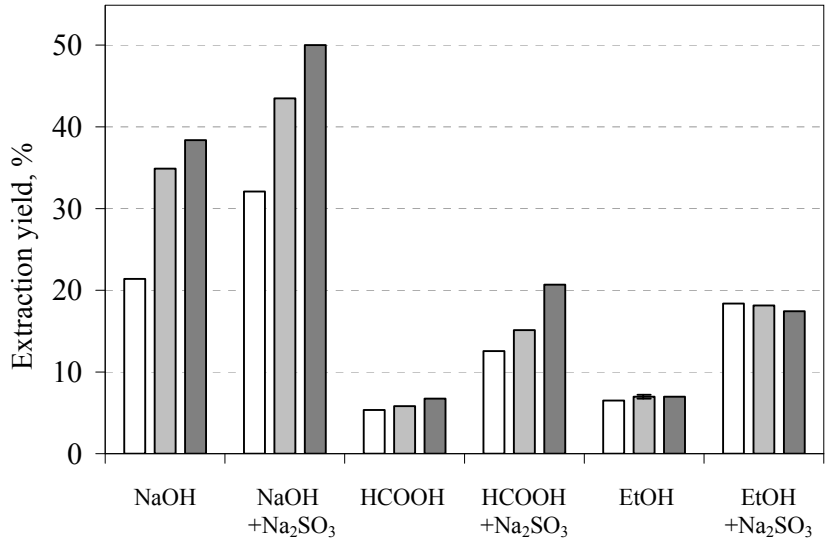

Figure 7.2. *P. pinaster* bark extraction yields (%, d.b.) obtained by two-hour long aqueous extractions at the solvent boiling point, using a 1:10 solid-to-solvent ratio. Additive amount: □ 0.5% for NaOH/HCOOH and 5% for EtOH; ▒ 1.0% for NaOH/HCOOH and 10% for EtOH; ■ 1.5% for NaOH/HCOOH and 15% for EtOH; Na_2SO_3 was always applied at a 1.0% proportion.

For the 1.0% Na_2SO_3 extraction experiments, enhanced yields were obtained, in particular for the acidic and the organic additives, resulting in extraction yields ~1.4 fold higher for NaOH, 2.3-3.1 fold higher for HCOOH, and ~2.7 fold higher for EtOH, when compared to the ones performed without sulfite addition (Figure 7.2). This extraction yield increase may be explained by the increase in the amount of lignin and of carbohydrates in obtained extracts, due to decoupling of lignin-carbohydrate ester linkages, in an analogy to the acid sulfite process of wood delignification (Sjöström, 1993).

7.4.2. Total phenolic compounds

The quantification of total phenolic compounds, expressed as gallic acid equivalents (GAE, % d.b.) is reported in Table 7.2 for *P. pinaster* extracts and for the tannin-rich products considered in this work (extracts of barks of *P. radiata*, quebracho, wattle and chestnut, and tara powder).

The EtOH modified aqueous solutions led to the extracts with the highest contents of phenolic compounds, reaching 34.8% GAE. Considering the corresponding low extraction yields (Figure 7.2), EtOH was clearly selective for these phytochemicals which is in agreement with other published studies (Laks, 1991), being related to its ability to set up hydrogen bonds with the abundant hydroxyl groups of phenolic substances. The NaOH and

HCOOH aqueous solutions led to extracts with maximum contents of 32.0% and 20.7% GAE, respectively. For the alkaline additive, the phenolic compounds contents decreased with the additive concentration increase (as also observed by Vázquez et al., 2001) while the corresponding extraction yields increased, indicating that this additive promoted the extraction of other non-phenolic substances, which may include polysaccharides and lignin, as already discussed.

Table 7.2. Total phenolic compounds, condensed tannins and total tannins (hide powder assay) of the *P. pinaster* bark extracts obtained by aqueous extractions using NaOH, HCOOH, EtOH and Na_2SO_3 as additives, and of the tannin-rich products considered in this study.

	Extraction Additive			Phenolic compounds, % GAE	Condensed Tannins, % CME	Total Tannins, %
	Type		%[a]			
P. pinaster extract	NaOH		0.5	32.0±0.4	1.2±0.1	55.3±1.2
			1.0	26.4±1.9	0.9±0.02	51.2±1.0
			1.5	23.5±0.7	1.1±0.02	35.1±0.8
	$NaOH + Na_2SO_3$		0.5	25.2±0.4	1.2±0.1	47.2±1.1
			1.0	23.2±1.1	0.9±0.1	35.4±0.9
			1.5	20.4±0.7	0.7±0.01	45.3±2.3
	HCOOH		0.5	19.1±1.9	7.1±0.3	37.0±1.9
			1.0	16.2±1.5	6.8±0.6	32.9±1.9
			1.5	20.7±1.1	7.0±0.3	29.9±0.3
	$HCOOH + Na_2SO_3$		0.5	14.6±1.4	18.0±1.1	35.0±2.2
			1.0	25.2±0.3	12.5±1.2	50.2±2.7
			1.5	15.4±1.3	13.6±1.4	37.4±0.1
	EtOH		5	33.3±2.0	30.9±1.0	60.6±4.4
			10	32.2±1.7	51.2±3.0	61.2±1.6
			15	34.8±3.1	62.8±0.6	56.0±1.1
	$EtOH + Na_2SO_3$		5	34.0±2.1	10.5±0.2	66.7±2.0
			10	28.0±2.5	37.6±3.6	64.0±1.4
			15	30.8±1.8	35.2±0.3	67.1±1.7
Tannin -rich product	*Condensed tannins*	*P. radiata* extract	HO soluble	37.3±0.4	40.8±1.2	56.2±4.2
			H_2O insoluble	31.6±0.7	28.6±0.5	n.d.[b]
		Quebracho extract		49.1±1.8	29.8±0.1	81.5±5.4
		Wattle extract	Mimosa Extract Company	41.4±0.3	24.4±0.4	70.7±3.2
			Tanac (2008)	41.0±0.2	29.8±1.4	70.4±2.8
			Tanac (2009)	41.0±0.3	24.2±0.4	76.3±0.5
	Hydrolysable tannins	Chestnut extract		26.0±0.1	7.5±0.2	54.8±4.3
		Tara powder	Colorobbia Italia	33.9±0.2	0.6±0.001	43.9±1.6
			Transformadora Agrícola	46.6±0.2	1.6±0.1	56.3±5.6

[a] Na_2SO_3 was always applied at a 1.0% concentration; [b] n.d. - Non detected.

In general, 1.0% of Na_2SO_3 in the extraction solvent had a negative influence on total phenolic compounds contents (drop of 3.1-6.8% GAE, Table 7.2), as opposed to the extraction yield behavior and, consequently, Na_2SO_3 promoted the extraction of non-phenolic substances from *P. pinaster* bark, as observed before. The only exception to this observation was the extraction performed with 1% of HCOOH, for which an increase of 9.0% was observed.

The total phenolic compounds contents of the tannin-rich products considered in this study varied between 26.0% for chestnut extract (which is rich in hydrolysable tannins) and 49.1% for quebracho (an extract rich in condensed tannins).

The results reported in this study may not reflect the real amount of total phenolic compounds in the samples, but rather the reducing activity of the whole extract towards the Folin-Ciocalteu reagent. Several factors may have contributed to this effect, namely: *(i)* the usage of the same commercial standard for all different tannin sources – standards purified from the vegetable source reflect better real contents; *(ii)* different phenolic compounds, namely condensed and hydrolysable tannins, are known to have different abilities to reduce the Folin-Ciocalteu reagent; *(iii)* other plants constituents as well as Na_2SO_3, which is easily oxidized, may interfere in the redox reactions with the Folin-Ciocalteu reagent (Appel et al., 2001; Hummer and Schreier, 2008).

Deviations from real contents may be especially noticed in the alkaline extracts due to the presence of significant amounts of non-phenolic compounds, as verified by Ku et al. (2007) in aqueous extracts from barks of various pine species.

7.4.3. Condensed and hydrolysable tannins

Condensed tannins contents of *P. pinaster* bark extracts, expressed as (+)-catechin monohydrate equivalents (CME), are also reported in Table 7.2. Extremely low values were obtained for the NaOH and for the NaOH + Na_2SO_3 extracts, which may incorrectly express real contents in these extracts, because of their alkaline character. In fact, the acid concentration (which plays a catalytic role in the reaction between vanillin and flavonoids) was found to be the most important factor, among several studied, influencing the vanillin-H_2SO_4 assay results (Sun et al., 1998). Lower acid concentrations decrease color intensity and, therefore, condensed tannin contents were most likely underestimated for these alkaline extracts. We repeated the vanillin assay making a previous correction of the pH of all of the extracts to the same value, in order to verify if this correction would lead to different results.

We did not verify any difference and attributed this fact to the similar pH values of the reaction media, which is controlled by the high H_2SO_4 concentration. Moreover, since most (65-87%) of the phenolic compounds present in *P. pinaster* bark are condensed tannins (Jerez et al., 2007b), higher contents of these compounds should be expected, given the Folin-Ciocalteu results.

The acidic (HCOOH) solvent resulted in extracts with approximately 7% CME, showing no influence of the additive level, but reflecting a positive influence of sulfite. For pine bark extractions in acidic media there are favorable conditions for the formation of phlobaphenes, formed by the condensation of various polymers, including carbohydrates and tannins (Sealy-Fisher and Pizzi, 1992). Their intermolecular linkages can be broken by the presence of Na_2SO_3, resulting in higher solubilization in aqueous solvents.

The extracts obtained with the organic additive exhibited high tannins contents, which doubled when the additive dose varied from 5 up to 15%, reaching 62.8% CME. Similar extraction yields were obtained, confirming the selectivity of ethanol towards these phytochemicals, as observed by other authors (Nakamoto et al., 2004). We believe that two factors contributed to this selectivity, namely: *(i)* the hydrogen bonds established with the abundant polar hydroxyl groups of condensed tannins and *(ii)* the acidification that is verified with the addition of the organic additive that shifted the equilibrium of the condensed tannins towards the solvent medium (Hummer and Schreier, 2008). Nevertheless, the possible presence of monomers in these extracts may have lead to the slight overestimation of tannins contents, as they are known to react faster with vanillin when compared to oligomers and polymers (Sun et al., 1998). The lower tannins contents detected for the EtOH + Na_2SO_3 extracts were probably underestimated due to the alkaline nature of sulfite, as happened with the NaOH ones. However, the higher extraction yields achieved in the presence of sulfite suggest, once again, that this chemical promoted the extraction of extraneous materials, including probably polysaccharides and lignin.

Ku et al. (2007) performed hot water extractions from the outer bark (which is known to concentrate tannins in tree bark tissues) of a variety of *Pinus* species that did not include *P. pinaster*, and obtained condensed tannin contents that varied from 2.1 up to 48.9% CME. These authors obtained extracts with high antioxidant activities that were directly related to their condensed tannins contents and, therefore, the extract obtained in this study from *P. pinaster* whole bark using 15% of EtOH might be a good candidate to be applied in high added value formulations with potent antioxidant activity. Moreover, this ethanolic extract presented 22% more tannins than the H_2O soluble *P. radiata* bark extract fraction, which

presented the maximum (40.8% CME) among all analyzed products (Table 7.2). Taking into account the higher amount of organic solvent (methanol 80%), the higher temperature (393 K) and the extra fractionation step used to obtain this H_2O soluble *P. radiata* extract fraction, *P. pinaster* extracts obtained in this study using 5-15% of EtOH revealed to be a very good compromise between experimental extraction effort and extract composition.

The low contents of quebracho and of wattle samples, rich in 5-deoxy condensed tannins, when compared to the producers values, is certainly related to their condensed tannins molecular structures, since vanillin reacts more intensively with meta-substituted flavonoids (Haggerman, 2002).

As expected, products rich in hydrolysable tannins exhibited low condensed tannins contents (0.6-7.5%). On the other hand, hydrolysable tannins, quantified as methyl gallate equivalents, MGE, were only detected in tara powder among all samples considered in this study, being 13.6±0.34% MGE for Colorobbia Italia tara powder. Considering the producers' quantification (Table 7.1), underestimation of tannins contents may be attributed to the usage of a low molecular weight standard, when compared to the average molecular weight of the hydrolysable tannins of tara powder.

7.4.4. Total tannins: hide powder assay

Total tannins were quantified by the hide powder assay and are reported in Table 7.2. In general, higher tannin contents were obtained by this procedure when compared to the vanillin-H_2SO_4 assay results, which may be attributed to the underestimation of tannin contents by the vanillin assay, as already referred, and to the fact that the hide powder assay is gravimetric and all substances that adhere to the hide powder, by chemical linkages or merely physical associations, were quantified. This situation may explain the great differences between quantification of tannins in the extracts obtained in the alkaline media: 35.1-55.3% for the hide powder assay, decreasing with the NaOH dose, and 0.9-1.2% for the vanillin assay. Three factors might have contributed (as a whole) to these results, namely: *(i)* the probable polysaccharides and lignin extraction increase with the NaOH concentration (as discussed above and that also explains the increase in extraction yield and drop in phenolic contents), *(ii)* the association forces that exist between tannins and polysaccharides (Garnier et al., 2001), and *(iii)* the similarities between chemical structures of lignin and condensed tannins, indicating that lignin has a strong affinity to leather (Thorstensen, 1993).

Similar statements can be made for the acidic extracts as for the alkaline ones, considering that higher tannin contents were obtained for the hide powder assay (29.9-37.0%) with a

negative correlation with the additive amount. The glycosidic linkages between lignin and carbohydrates are easily cleavage by acids (Sjöström, 1993), and therefore they are likely present in the acidic extracts.

For the EtOH extracts, the discrepancies in the quantification of tannins were not so drastic (30.9-62.8% CME for condensed tannins *vs* 56.0-61.2% for total tannins), probably due to the higher selectivity of organic solvents towards lower molecular weight condensed tannins and to the presence of less extraneous substances. Moreover, the positive influence of the EtOH concentration observed in the condensed tannins assay was not observed in the total tannins assay. Considering this fact and also that extraction yields were approximately the same for the three levels of additive (Figure 7.2), there is an indication that the amount of extraneous materials decreased with the EtOH concentration, which probably adhered to the hide powder. Additionally, low molecular weight tannins (which are, most likely, more abundant in the 15% EtOH extract) have low affinity to associate with proteins (Foo et al., 1983; Jerez et al., 2006) and consequently with hide powder.

Sulfite had a positive effect on the quantification of total tannins by the hide powder assay for most extracts, which may be an indication of the extraneous material adhesion to hide powder. Moreover, sulfite is an antioxidant and increases the capacity to precipitate proteins (Hummer and Schreier, 2008).

No tannins were detected in the *P. radiata* H_2O insoluble extract, since this sample corresponded to the methanol soluble extract fraction which contains low molecular weight condensed tannins, with poor affinity with hide powder. Besides, we noted that the aqueous soluble fraction had 56.2% of tannins (Table 7.2). For tested commercial products, the hide powder assay resulted in higher tannin contents when compared to the vanillin assay results (as occurred with *P. pinaster* extracts). Although similar results to the ones reported by the respective producers (Table 7.1) were obtained for the condensed tannins rich products, lower tannin contents were found for the hydrolysable tannins rich products (chestnut extract and tara powders).

7.4.5. pH values and limit viscosities

Table 7.3 present the obtained values of pH and of limit viscosities at high shear rates of 20% and 50% (w/v) aqueous solutions of pine bark extracts and of tested commercial tannin-rich products, including synthetic tannin, obtained from controlled stress rheometer flow tests. Limit viscosities of the 20% solutions of *P. pinaster* bark extracts obtained with no sulfite varied in the range 10.5-18.9 mPa.s, being slightly higher for the alkaline extracts. However,

viscosities of the 50% extracts solutions were 325.0 and 820.2 mPa.s for the 0.5 and 1.0% NaOH extracts, respectively, which are considerably higher than the acidic and organic ones. The alkaline solutions high viscosities are characteristic of the presence of polymeric carbohydrates derived from degraded hemicelluloses, which heavily contribute to the extract's viscosity due to secondary force associations between tannins and carbohydrates (Garnier et al., 2001; Laks, 1991). The possible presence of carbohydrates in these extracts was mentioned above when phenolic compounds and tannins contents were discussed. Carbohydrates are probably also present in the acidic extracts, but the sensitivity of glycosidic linkages towards acidic hydrolysis (Sjöström, 1993) may have caused their partial depolymerization, with the consequent viscosity decrease.

The presence of 1.0% of Na_2SO_3 originated a viscosity decrease for most of the obtained *P. pinaster* bark extracts, which may be explained by the sulfite contribution to hydrolysis of polymeric carbohydrates, as reported by Pizzi and Merlin (1981). A particular accentuated decrease was noticed for the alkaline extracts which can be most certainly attributed to minimization of autocondensation reactions of condensed tannins in the presence of sulfite, as explained by Pizzi (1980). A considerable viscosity increase was detected in the 50% solution of the 5 and 15% EtOH extracts, which may be an indication of the presence of higher amounts of carbohydrates, as already discussed.

The high viscosities of alkaline extracts solutions may compromise their efficient application in leather tanning, due to the need to use low extracts concentrations in order to obtain solutions that can penetrate into raw hides and skins, together with the high steric hindrances of the tannin molecules that may impede the establishment of linkages with proteins. Moreover, the pH of the aqueous solutions prepared from the acidic and organic extracts with no sulfite (3.3-3.9, Table 7.3) is adequate to be applied in leather tanning, once vegetable tannins are firmly fixed to animal skin proteins at pH below 4 (Covington, 1997), thus avoiding the usage of alkalis or acids to adjust the pH and, consequently, reducing the overall costs and the effluents pollution burden. Their low viscosity also represents an advantage by allowing the application of highly concentrated solutions in the tanning process, making it more efficient (Yao et al., 2006). Therefore, the employment of sulfite salts to reduce the vegetable extract viscosity (Pizzi and Merlin, 1981), which is a common procedure at an industrial scale, appears to be unnecessary.

Table 7.3. pH values, limit viscosities of aqueous solutions (20% and 50%, w/v) and CIE lightness (L*), hue angle (h*) and chroma (c*) values of the *P. pinaster* bark extracts.

	Extraction Additive			pH[b]	Limit Viscosity, mPa.s		Color attribute[d]		
					Solution Concentration, %		Chroma (c*)	Hue, ° (h*)	Lightness (L*)
	Type		%[a]		20	50			
P. pinaster extract	NaOH		0.5	9.0	17.5±0.7	325.0±21.2	10.7	33.6	13.5±0.3
			1.0	9.9	17.3±0.6	820.2±40.4	3.3	-49.9	12.6±0.2
			1.5	11.0	18.9±1.6	-[c]	16.0	31.1	14.0±0.2
	$NaOH + Na_2SO_3$		0.5	9.6	12.7±1.2	83.3±8.4	22.3	33.3	20.8±0.1
			1.0	10.6	10.0±0.2	63.0±2.1	4.1	-18.2	17.7±0.1
			1.5	12.2	11.6±0.7	36.1±1.1	15.5	34.2	21.2±0.1
	HCOOH		0.5	3.4	11.7±0.6	52.9±2.1	16.0	38.4	19.9±0.2
			1.0	3.9	10.5±0.6	32.6±1.3	11.7	68.5	35.0±0.1
			1.5	3.3	12.9±1.2	38.1±2.8	14.7	41.7	20.0±0.1
	$HCOOH + Na_2SO_3$		0.5	5.6	12.9±0.3	20.7±1.1	21.2	47.0	32.4±0.0
			1.0	4.7	8.9±0.2	23.2±0.8	1.4	84.8	30.7±0.1
			1.5	3.9	10.8±0.6	17.0±0.7	16.8	47.3	32.7±0.1
	EtOH		5	3.7	14.5±0.1	35.3±3.6	18.7	60.3	36.4±0.0
			10	3.6	14.7±0.1	58.8±5.3	41.9	44.5	37.7±0.1
			15	3.4	12.3±0.1	26.8±2.6	34.0	42.9	30.0±0.1
	$EtOH + Na_2SO_3$		5	6.8	10.6±1.2	65.1±8.2	5.9	39.0	28.2±0.1
			10	7.1	12.1±1.1	44.0±7.6	30.0	41.9	30.6±0.1
			15	7.0	12.1±1.0	57.7±1.3	24.1	40.1	30.9±0.1
Tannin-rich product	*Condensed tannins*	*P. radiata* extract	H_2O soluble	3.1	3.1±0.1	18.5±0.6	33.3	51.6	37.3±0.1
			H_2O insoluble	3.7	2.6±0.2	23.1±0.5	17.1	31.2	18.9±0.1
		Quebracho extract		5.2	2.4±0.1	12.6±0.3	39.4	57.9	49.4±0.1
		Mimosa extract	Mimosa Extract Company	4.4	3.6±0.04	31.7±0.2	33.1	63.5	46.3±0.1
			Tanac (2008)	5.0	3.7±0.1	31.9±0.4	30.0	60.0	42.3±0.1
			Tanac (2009)	4.6	3.9±0.05	46.7±0.1	22.0	50.3	30.5±0.1
	Hydrolysable tannins	Chestnut extract		3.3	4.2±0.2	34.7±0.05	23.8	75.8	48.0±0.0
		Tara powder	Colorobbia	3.4	6.9±0.5	130.1±9.2	24.0	84.9	75.8±0.0
			Transformadora Agrícola	3.5	5.6±0.1	104.3±8.0	10.9	80.5	33.7±0.0
	Synthetic tannin			8.4	1.7±0.1	3.7±0.1	27.2	89.5	77.7±0.1

[a] Na_2SO_3 was always applied at a 1.0% concentration; [b] pH of 20 % (w/v) aqueous solutions; [c] No viscosity measurement was possible due to the paste like consistency of the sample. [d] $c^* = \sqrt{(a^*)^2 + (b^*)^2}$, $h^* = \arctan\left(\frac{b^*}{a^*}\right)$.

The usage of an organic additive also avoids the usage of acids and alkalis, which lead to the generation of salts as well as to increase effluent treatment costs (Saravanabhavan et al., 2005). Although ethanol is a relatively high cost solvent, the small involved amounts (5-15%)

and the advantageous features of the obtained extracts may justify its application in the production of condensed tannin rich extracts.

Limit viscosities of *P. radiata* bark extract fractions and of commercial tannin-rich products varied in the ranges 1.7-6.9 mPa.s and 3.7-130.1 mPa.s for the 20% and 50% solutions, respectively (Table 7.3). The lowest values correspond to the synthetic tannin and the highest ones to the tara powder from Colorobbia Italia. With the exception of the synthetic tannin solutions, which showed an alkaline pH value, all other tannin-rich products exhibited acidic pH values (3.1-5.2).

7.4.6. Color attributes

CIE L* (lightness), h* (hue angle) and c* (chroma) values, which give an accurate description of the color of a sample, are shown in Table 7.3, and a* (red) and b* (yellow) coordinates of *P. pinaster* extracts are represented in Figure 7.3. The main contributors to the extracts color should be the condensed tannins whose color depends on pH, changing from light orange to brown, increasing in darkness, with the pH increase above 9.0 (Hummer and Schreier, 2008; Jorgensen et al., 2004), as well as the chromophoric groups of lignin which present a brown color. Hemicelluloses are inherently white and do not contribute to color (Sjöström, 1993).

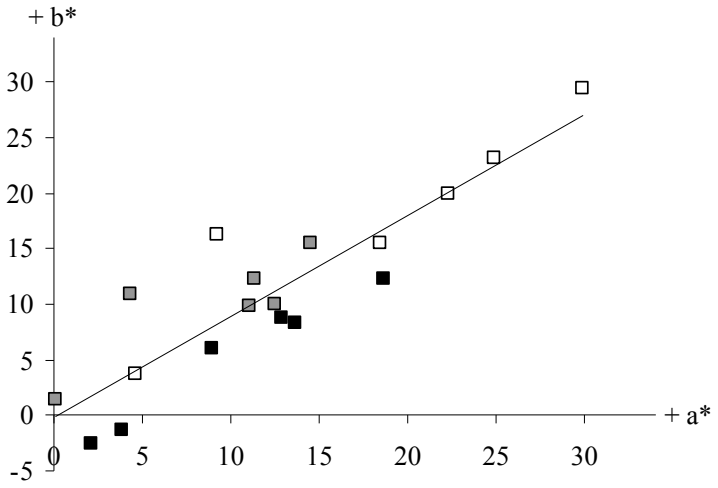

Figure 7.3. CIE a* and b* coordinates of *P. pinaster* bark extracts obtained by two-hour long aqueous extractions at the solvent boiling point, using as additives NaOH ■, HCOOH ■ and EtOH □, with or without Na_2SO_3.

The type of employed solvent additive in extraction experiments did not have a pronounced effect on hue angle values, which were close to 45 º for the majority of the extracts (Figure 7.3), reflecting their orange-red color. The NaOH extracts exhibited the

lowest lightness values (12.6-14.0) and the 1% NaOH ones (with and without Na_2SO_3) showed negative values for hue, corresponding to a red-blue color. The color of these alkaline extracts is typical of the presence of oxidation products of condensed tannins, characterized by a higher degree of inter-flavonoid linkages, as observed by Jorgensen et al. (2004) for grape seeds and skins. Moreover, lignin should also be present, as previously noticed when discussing other extracts features.

The highest a* values were observed for the organic extracts, indicating a higher contribution of red to color that may be related to the presence of phlobaphenes which are reddish colored and soluble in ethanol (Sealy-Fisher and Pizzi, 1992).

Chroma values were below 24 for most of the extracts, indicating low color intensity. EtOH was the additive that resulted in the highest chromacity extracts, with Na_2SO_3 having a negative influence on this color parameter. The lightness values of *P. pinaster* extracts varied between 12.6 and 37.7, which reveals their dark colors, increasing along the ordering NaOH < HCOOH < EtOH (Na_2SO_3 increased the lightness of the alkaline and the acidic extracts, but not of the organic extracts one).

These differences in the extracts color attributes reflect dissimilarities on their chemical composition, which was conferred by the different employed solvent additives. The extract color is a feature that may have some impact on its future applications, namely in leather tanning processes, giving a specific tonality to the leather and defining whether or not artificial coloring is necessary.

The tannin-rich products studied showed hue angle values between 50 and 90 º reflecting a high contribution of yellow to color (in particular for tara powder and synthetic tannin). The exception to this pattern was the H_2O insoluble *P. radiata* extract fraction which showed a hue angle value of 31.2 º (Table 7.2). Chroma and lightness values varied in the range 10.9-39.4 and 18.9-77.7, respectively.

7.5. Conclusions

Aqueous extractions from *P. pinaster* bark were performed using low amounts of an alkaline (NaOH), an acidic (HCOOH) and an organic (EtOH) additive, with and without sulfite (Na_2SO_3), and in order to obtain extracts with potential applications in leather tanning. Alkaline and acidic additives were not selective for condensed tannins and promote the extraction of extraneous materials, among which carbohydrates and lignin are possibly included. On the contrary, ethanol was selective for these phytochemicals. The achieved low

extraction yield with this additive is a disadvantage that may not be very limitative due to the low cost and abundance of *P. pinaster* bark. Furthermore, the applied low amount of EtOH may render the extraction process industrially viable. The pH and viscosity of these organic extracts are adequate for application in leather tanning, avoiding the use of acids, alkalis and sulfites. Additionally, their high phenolic and condensed tannins contents suggest that these extracts may be applied in other applications besides leather industries, like those in the pharmaceutical and food industries.

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8. Fractionated supercritical fluid extraction of antioxidants from maritime pine bark

In this Chapter attention is given to the fractionated supercritical extraction of antioxidants from maritime pine bark using CO_2 and mixtures of CO_2 and ethanol (EtOH). Studied operational conditions were extraction pressure and temperature, at constant solvent flow rate. Composition and antioxidant activity of the obtained extracts were compared to those obtained by hydrodistillation and ethanolic Soxhlet extraction. The text that comprises this Chapter is published in: Braga, M.E.M., Santos, R., Seabra, I.J., Facanali, R., Marques, M.O.M., De Sousa, H.C. (2008) Fractionated SFE of antioxidants from maritime pine bark. *Journal of Supercritical Fluids 47*: 37-48.

8.1. Abstract

Maritime pine (*Pinus pinaster*) is an important Portuguese forest species and its bark is an abundant furniture industry residue. In this work, Fractionated Supercritical Fluid Extraction (FSFE) experiments were carried out using CO_2 and CO_2:EtOH (90:10, v/v) mixtures in two consecutive steps. Different pressures (from 10 up to 30 MPa) and temperatures (303, 313 and 323 K) were assayed. FSFE extracts were compared with hydrodistillation (HD) and Soxhlet (SoE) extracts. Gas Chromatography (GC) was used to characterize the obtained volatile oils while catechin and epicatechin quantified in ethanolic extracts by HPLC. Antioxidant activities were determined spectrophotometrically. Around 84% of the total extract was obtained in the 1st CO_2 extraction step and this volatile oil rich extract presented lower oxidation inhibitions (~29-62%) than those obtained at the 2nd CO_2 + EtOH extraction step (60-84%). The catechin + epicatechin yields of these 2nd step ethanolic extracts were higher (0.051-0.346 μg/mg, d.b.) than the ones obtained for SoE (0.039 μg/mg, d.b.). The increment

in catechin + epicatechin extract content influenced the corresponding extract antioxidant activity, although not directly. Pressure and temperature affected the extraction kinetic parameters for both extraction steps and the obtained catechin + epicatechin contents for the 2nd CO_2 + EtOH extraction step.

The employed high pressure fractionated extraction methodology showed that it was possible to obtain different extract fractions having diverse antioxidant capacities from *Pinus pinaster* bark. Furthermore, this process can be further optimized in order to improve extraction yields, selectivities and antioxidant activities, by the use of different solvent mixtures compositions and of other different operational conditions. Therefore, the reuse of this abundant Portuguese agroindustrial residue may originate several possible high-value extracts for different applications like, for example, the use the volatile oil fractions in the aroma/flavor industries, and/or the use of phenolic fractions in the food and pharmaceutical industries.

8.2. Introduction

Maritime pine (*Pinus pinaster*) is one of the most important Portuguese native forest species which comprises approximately 23% of all the Portuguese forest area and achieved an annual production of 671000 tonnes during the year of 2006 (DGF, 2006). Considering that, for southern pines of pulpwood size, bark volumes are usually between 12% and 24% of total produced pine volume (Koch, 1972, as cited by Gao et al., 2006), there are huge amounts of pine bark residues which are readily available at low prices. Therefore, these residues can be used as a raw material following the worldwide tendency of recovering, recycling and upgrading wastes. When compared to other common residues generated by the Portuguese agricultural activities, like grape pomace, pine bark presents some important advantages such as its all year availability, its low price, its long-term stability and its easy way to handle and process (Selga and Torres, 2005).

In addition, and due to its high polyphenolic content, *Pinus pinaster* bark (which is mostly used as firewood for energy production) has also a strong potential to be used as a raw material for the production of high-value bioactive substances.

The most common phenolic compounds present in maritime pine bark are (+)-catechin, (-)-epicatechin, dihydroquercetin and numerous phenolic acids, most of them being procyanidin dimers, trimers, oligomers and polymers (Wood et al., 2002), i.e., proanthocyanidins (or commonly known as condensed tannins). Like other pine barks, *Pinus pinaster* bark also

contains fatty acids, aliphatic acids, resin acids, sterols, as well as other lipophilic compounds (Hafizoglu et al., 2002) which have been reported to present antioxidant activity and also to cause synergistic effects with other antioxidants (Díaz-Reinoso et al., 2006).

Presently, a commercially available pine bark extract, Pycnogenol®, is exclusively obtained from maritime French pine (*Pinus pinaster*) using several consecutive extraction steps involving H_2O and EtOH as solvents, in a patented, fully automated extraction process (Rohdewald, 2002). The resulting extract is rich in numerous phenolic compounds such as phenolic acids, procyanidins (having chain lengths ranging from 2 up to 12 monomeric units), catechin and taxifolin. This extract has been reported to present excellent radical scavenger properties which may originate various health promoting properties namely cardioprotective, anticancer and antihypertensive properties (Rohdewald, 2002; Packer et al., 1999).

For food, pharmaceutical and cosmetic applications, supercritical fluid extraction (SFE) represents an attractive option to conventional solid-liquid extraction methodologies, offering a number of well-known advantages concerning selectivity, separation, purification and contamination-free extracts (Díaz-Reinoso et al., 2006; Mukhopadhyay, 2000). Moreover, SFE is recognized to preserve the extracts with respect to the vegetable matter starting composition (Reverchon and Della Porta, 1995) and already has been employed successfully to extract numerous functional ingredients from food industry byproducts (Herrero et al., 2006).

In addition, SFE offers the possibility of extract fractionation, avoiding some post extraction purification steps, by the use of successive changes in solvent density or composition (and polarity), and which will lead to corresponding different solvent natures and capacities. For example, this stepwise modification of solvent density has been used quite often to separate the volatile oil fraction (which was soluble in CO_2 with ~0.6 g/cm^3 of density) from the other high molecular weight lipophilic compounds, namely fatty acids and their derivatives, aliphatic aldehydes and sterols (which were soluble at higher densities) (Reverchon and Della Porta, 1995). Rosemary leaves (Ibáñez et al., 1999) and coriander seeds (Yepez et al., 2002) were also subjected to such a fractionated extraction procedure, in order to obtain rich antioxidant fractions. To isolate phenolic antioxidants, a polar organic modifier has to be added to increase their solubility in supercritical CO_2. In this case, extract fractionation can be achieved by performing a first extraction step, wherein 100% CO_2 is used and where lipophilic compounds are extracted, followed by a CO_2 + polar organic modifier extraction, where phenolic compounds are preferentially extracted. This strategy was also employed by Palma and Taylor (1999) for grape seeds extraction and in order to fractionate

oil and phenolic fractions. Authors used CO_2 and CO_2/methanol (5:1, v/v) mixtures as consecutive extracting fluids, at 308 K and at 45 MPa. Catechin and epicatechin were the main components present in the obtained phenolic fractions. Other authors also used CO_2 fractionated extraction of polyphenolics and procyanidins, using CO_2/methanol (Ashraf-Khorassani and Taylor, 2004) and CO_2/ethanol (Pinelo et al., 2004) mixtures. Finally, substances such as ar-turmerone and curcuminoids, which have selective anticancer activities, were the main compounds extracted from turmeric at a 1st (CO_2) extraction step and at a 2nd CO_2/ethanol + isopropyl alcohol step, respectively (Braga and Meireles, 2007; Braga et al., 2003).

Berna et al. (2001) and Cháfer et al. (2002) studied the solubility of (+)-catechin and (-)-epicatechin (the main phenolic compounds known to be present in pine bark extracts), in CO_2 + EtOH (5-30%, v/v) mixtures, at 8-12 MPa, while Murga et al. (2000) studied their solubility at 20 MPa but at lower ethanol compositions (2-10% v/v). These authors concluded that the solubility of these compounds was increased with the raise in extraction pressure and cosolvent composition.

The main objective of this work was to obtain and characterize rich antioxidant fractions from *Pinus pinaster* bark, using a fractionated supercritical fluid extraction (FSFE) methodology and using CO_2 and a CO_2:EtOH (90:10, v/v) mixture as consecutive extraction solvents, for a sequential recovery of extracts. The effects of extraction pressure and temperature were studied on total extraction yields, on composition profiles and on extracts antioxidant activities. Results were compared to the ones obtained by conventional extraction methods such as hydrodistillation (HD) and Soxhlet (SoE) extraction.

8.3. Materials and methods

8.3.1. Raw material

Maritime pine (*Pinus pinaster*) bark was collected in Beira Litoral, Portugal, in February 2006, and was comminuted using a hammer mill (Bauknecht, Stuttgart. Germany). Particle size distribution was obtained by Light Scattering using a Laser Malvern Mastersizer (Hydro 2000 MU, Worcestershire, UK). The particles present in higher amounts were separated using a sieve series (120–18 mesh), under mechanical agitation (Retsch, Germany), and were stored in plastic bags at approximately 263 K. Pine bark humidity was determined by the Jacobs xylene distillation method (Jacobs, 1973) with duplicate assays.

8.3.2. Chemicals

Carbon dioxide (99.998%, Praxair, Madrid, Spain), ethanol (99.5%, Panreac Quimica S.A., Barcelona, Spain) and distilled water were used for the extraction experiments. Chemicals and solvents employed for extract analyses were: ethanol (99.5%, Panreac Quimica S.A., Barcelona, Spain), methanol (99.9%, Chromasolv, Sigma-Aldrich Inc., Steinheim, Germany), formic acid (~98%, Fluka, Steinheim, Germany), ethyl acetate (99.9%, Chromasolv Plus, Sigma-Aldrich Inc., St. Louis, USA), hexane (96%, Merck, Darmstadt, Germany), glacial acetic acid (p.a., Merck, Darmstadt, Germany), p-anysaldehyde (Sigma-Aldrich Inc., St. Louis, USA), 2-aminoethyl diphenylborinate (97%, Fluka, Steinheim, Germany) and bi-distilled water.

Employed standards were quercetin dehydrate (≥98%, HPLC grade), rutin hydrate (≥95%, HPLC grade), D-(+)-catechin hydrate (98%) and gallic acid (≥98%) which were purchased from Sigma-Aldrich Inc. (Steinheim, Germany). (-)-epicatechin (≥90%, HPLC grade) was obtained from Fluka (Buchs, Switzerland). Standards for GC analysis were alkane standard solutions: C_8-C_{40} (Fluka, Buchs, Switzerland). Chemicals used for antioxidant activity tests were: β-carotene (Type I, ~95% UV, Sigma-Aldrich Inc., Buchs, Switzerland), Tween 40 (Sigma-Aldrich Inc., Steinheim, Germany), linolenic acid (≥98.5%, p.a., Fluka, Buchs, Switzerland) and chloroform (99.9%, HPLC grade, Sigma-Aldrich Inc., Dorset, England).

8.3.3. Experimental extraction procedures

8.3.3.1. Hydrodistillation and Soxhlet ethanol extraction

The volatile oil was obtained by hydrodistillation (HD) in a Schilcher apparatus following the AOAC 962.17 method (A.O.A.C., 1984). Extraction was performed for 120 min, at the solvent boiling point (triplicate assays) and with a solid/solvent ratio of 1:33 (w/v). The oleoresin (ethanol extract) was obtained in a Soxhlet apparatus, employing a 1:50 (w/v) solid/solvent ratio The system was kept under reflux for 120 min at the solvent boiling point (triplicate assays). Ethanol was evaporated from the extract using a rotary evaporator with vacuum control.

8.3.3.2. Fractionated supercritical fluid extractions

The fractionated supercritical fluid extraction assays (FSFE) were performed using the apparatus schematically represented in Figure 8.1 (Braga et al., 2007). Extraction bed was constituted by the raw material (pine bark) and glass beads (3 mm diameter) were placed at the extraction cell entrance in order to facilitate solvent dispersion. Extraction cell (~30×10^{-6}

m^3, stainless steel cell) was placed into a temperature controlled water bath (±0.1 K, DC30, Thermo Haake, Karlsruhe, Germany). Pressure was maintained by a back-pressure regulator (26-1762-24-090, Tescom, Selmsdorf, Germany) and was measured by a pressure transducer (C204, 0-67 MPa, Setra, Boxborough, USA). CO_2 and EtOH were delivered using high pressure liquid pumps (respectively, home-built and L-6200A, Hitachi, Merck, Darmstadt, Germany).

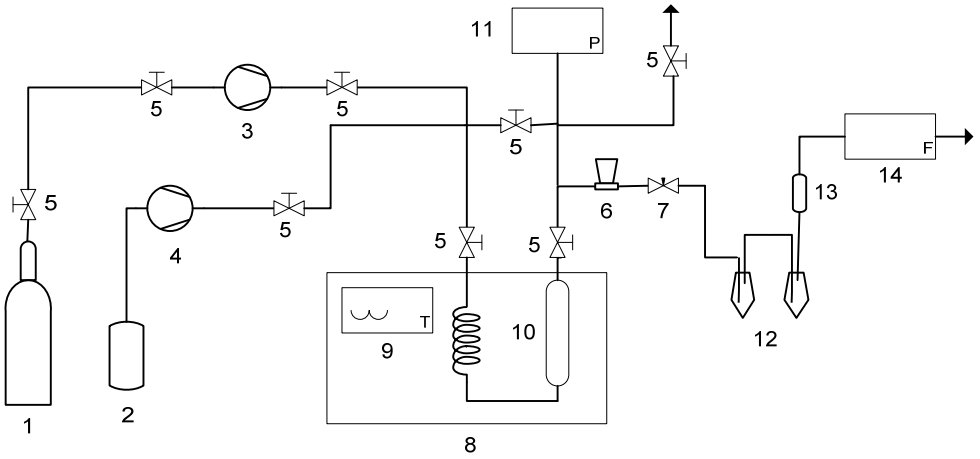

Figure 8.1. Schematic diagram of the employed SFE apparatus. (**1**) CO_2 cylinder; (**2**) EtOH reservoir; (**3**) CO_2 pump; (**4**) Cosolvent pump; (**5**) Valves; (**6**) Back-pressure regulator; (**7**) Micrometering valve; (**8**) Thermostatic water bath; (**9**) Immersion heater/controller; (**10**) Extraction cell; (**11**) Manometer; (**12**) Glass flasks; (**13**) Adsorbent column; (**14**) Wet gas meter.

The employed solvent flow rates (Q) are reported in Table 8.1. Operational pressure and temperature conditions were chosen taking in consideration the available literature solubility studies for (+)-catechin and (-)-epicatechin (Berna et al., 2001; Cháfer et al., 2002) and were a combination of a low ethanol composition and of high operational pressures, in order to avoid evaporation to recover high ethanol amounts after the extraction process. A two-step FSFE methodology was employed at 303, 313 and 323 K and approximately at 10, 15, 20, 25 and 30 MPa, comprising: *i*) A first extraction step in which pine bark was extracted with CO_2, in order to remove low polarity CO_2 soluble compounds, employing a 10 min static period followed by a 90 min dynamic period; *ii*) A second dynamic extraction step, for 90 minutes, in order to remove polar compounds, and wherein CO_2:EtOH (90:10, v/v) mixture was introduced into the system. Extracts were recovered in different glass flask traps, placed in an ice bath, for every 15 minutes. An adsorbent packed column (Porapak Q 80/100 mesh, lot: 134, Supelco, Bellefonte, USA) was placed after the trap in order to prevent extract losses in the solvent stream and the expanded CO_2 flow was measured by a wet gas meter (DM3C ZE 1411, G.H. Zeal Ltd., London, England). The tubing line was always cleaned with ethanol after the 2nd extraction step. Ethanol-containing extracts were then evaporated using a rotary

evaporator with vacuum control (B-480, Büchi, Flawll, Switzerland) at 313 K and in the absence of light. Dried extracts were kept at approximately 255 K until further analysis. The central point of experimental design was duplicated to get the extraction error.

8.3.4. Characterization of pine bark extracts

8.3.4.1. Thin layer chromatography

TLC analyses of FSFE extracts were performed using silica gel plates (20 cm×20 cm, thickness 0.2 mm) (Merck, Germany). A hexane-ethyl acetate (8:2, v/v) mixture was used as the mobile phase. To observe the CO_2 soluble volatile oil compounds, an anisaldehyde solution was used as the spray reagent while a NP solution (methanol-2-aminoethyl diphenylborinate, 99:1, v/v) was used for the analysis of ethanolic extracts phenolic compounds (at 365 nm) (Wagner et al., 1984). Plates were drawn using the ACD/TLC Plate Tool for ChemSketch, Freeware version 10.02 (Advanced Chemistry Development, Inc.).

8.3.4.2. Gas chromatography

The compositions of the extracts obtained by hydrodistillation (HD) are presented in terms of relative area (%) and were determined using a GC-MS apparatus (QP-5000, GC-MS, Shimadzu, Tokyo, Japan). The system was equipped with a fused silica capillary column (30 m×0.32 mm i.d., 0.45 μm, OV-5, Ohio Valley Specialty Company, Ohio, USA). Helium was employed as the carrier gas (1.0 mL/min) and 1 μL of sample was diluted in dichloromethane and injected. The injector temperature was 513 K and that of the detector was 503 K. The oven temperature was raised from 333 to 553 K, at 3 K/min, and maintained at 553 K for 20 min. The identification of compounds was done based on the comparative analyses between the mass spectra of the substances with those present on the GC-MS system database (Nist. 62 lib.), on literature (McLafferty and Stauffer, 1989) and on the Kovats retention indexes (Adams, 1995). These were obtained by co-injection of a standard mixture of *n*-alkanes (C_8-C_{24}) and applying the van den Dool and Kratz equation (Van Den Dool and Kratz, 1963). GC-MS analyses were performed at Laboratório de Produtos Naturais, Centro de P&D Recursos Genéticos Vegetais, Instituto Agronômico de Campinas, Brazil.

The chemical compositions of the CO_2 SFE extracts (1st step FSFE) and of Soxhlet extracts (SoE) are also shown in Table 8.2 (in terms of relative area (%)) and were determined using a gas chromatograph (Tremetrics 9001, Thermo Scientific, Austin, USA), equipped with a fused capillary column (15 m × 0.32 mm i.d., 0.45 μm, CP-Sil 8 CB, Varian, Middelburg, The Netherlands). The carrier gas was helium (2-3 mL/min) and 2 μL of sample

(0.1 mg/mL, in ethyl acetate) was injected. The injector temperature was 503 K and that of the detector was 523 K. The temperature program was 323 K (for 5 min), 323-553 K (at 5 K/min) and 553 K (for 5 min). Kovats indexes were determined relatively to the retention times of a series of *n*-alkanes and using the Kovats' method (Adams, 1995). The identification of substances was done based on the comparison of their Kovats indexes with those of the hydrodistillation (HD) extracts and also based on their chromatogram profiles.

In order to obtain relative area standard deviation values, GC analyses were done in duplicate for HD and for SoE samples. For FSFE samples and despite the identification have been made for all the obtained kinetic point samples, only the total composition profiles are presented in Table 8.2 because it was difficult to obtain a significant value variation since there were differences between the vials compositions.

8.3.4.3. High-performance liquid chromatography

HPLC analyses were performed, at room temperature, in a C18 column (250×4 mm i.d., 5 μm, Eurospher, Berlin, Germany) equipped with a pre-column and coupled to an UV detector (WellChrom k-2500, Kanuer, Berlin, Germany) and to a HPLC pump (WellChrom Maxi-Star k-1000, Knauer, Berlin, Germany). A mobile phase, constituted by methanol/acidified water (5% formic acid, v/v) in a proportion of 8:2 (v/v), was employed in an isocratic elution (80 min), at a flow rate of 1 mL/min. Samples were microfiltered (0.20 μm) before injection. For all samples, chromatographic profiles were measured at 280 nm and the concentrations (expressed as % (w/w) on a dry basis) of catechin and epicatechin were calculated from previously determined duplicated calibration curves.

8.3.4.4. Antioxidant activity: β-carotene and linolenic acid coupled reaction assay

The antioxidant activities of the obtained extracts were determined by the coupled reaction of linolenic acid and β-carotene (Hammerschmidt and Pratt, 1978). Reaction were monitored at 470 nm by UV-vis absorbance readings (of extracts and control samples - the reaction medium with no extract) at 0 h (Abs^{t_0}) and after 1, 2 and 3 h (Abs^{t_n}) of reaction. Antioxidant activities were expressed as oxidation inhibition percentages (Equation 8.1).

$$\text{Oxidation Inhibition (\%)} = \left[1 - \frac{Abs^{t_0}_{extract} - Abs^{t_n}_{extract}}{Abs^{t_0}_{control} - Abs^{t_n}_{control}}\right] \times 100 \qquad \text{(Equation 8.1)}$$

8.3.5. Calculation procedures

The mean geometric diameter (d_{mg}) of pine bark particles was calculated according to the American Society of Agricultural Engineers ASAE S319.2 method (ASAE, 1993), using sieves of 18/120 mesh (through 18 mesh and on 120 mesh).

$$d_{mg} = \exp\left[\frac{\sum_{i=1}^{n}\left(w_i \log \overline{d_i}\right)}{\sum_{i=1}^{n} w_i}\right] \qquad \text{(Equation 8.2)}$$

In this equation, $\overline{d_i}$ is the geometric mean length of particles on the i^{th} screen $= (d_i.d_{i+1})^{0.5}$, d_i is the (diagonal) screen opening of the i^{th} screen (mm), d_{i+1} is the (diagonal) screen opening in the next larger than i^{th} screen (mm) and, w_i is the particle mass on i^{th} screen.

For all employed extraction methods, total yields were calculated as the ratio between the total extract mass and the feed mass, on a dry basis (d.b.). For FSFE experiments, the total extract mass was determined summing the extracted masses and the extract masses retained in the adsorption column. Extract masses recovered from tubing line washing were also considered for the calculation of 2^{nd} extraction step yields.

The overall kinetics extraction curves were constructed using just the accumulated masses of extracts which were collected at a given extraction time interval. Therefore, the masses collected from the adsorption column, as well as those collected in the cleaning process, were not considered for the kinetic representations.

For the 1^{st} FSFE step, a linear regression analysis was used to fit experimental data while, for the 2^{nd} extraction step, each overall high pressure extraction curve was fitted by a curve formed by two straight lines. Second step fitting was done by minimizing the least regression error (in the least squares sense) using the fminsearch function of Matlab (R2006a), and the first line was identified as the constant extraction rate period (CER). The corresponding kinetic parameters were calculated according to Rodrigues et al. (2002) (Rodrigues et al., 2002): mass transfer rate, M_{CER} (Kg/s); mass ratio of solute in the solvent phase at measuring-cell outlet, Y_{CER} (kg/kg) and duration of it, t_{CER} (s). For the 1^{st} step FSFE, and because the CER period was not yet entirely completed, t_{CER} was fixed at 90 minutes and, consequently, the mass transfer rates and mass ratios of solute in solvent phase were calculated just for 90 minutes of extraction (M_{90min} and $Y_{90min,}$ respectively). The extract yields corresponding to these two consecutive extraction periods were denoted by R_{90min} and R_{CER} (kg/kg), respectively.

8.4. Results and discussion

8.4.1. Extraction kinetics

Pine bark particles having a mean geometric particle diameter of 0.66 mm were used for all the extraction experiments. Operational conditions, experimental total yields, and correlation results are presented in Table 8.1. Humidity was found to be 8.6±0.1% (w/w, d.b.).

Figure 8.2 shows the overall extraction curves obtained for FSFE at 313 K and at ~20 MPa. As can be seen, for the 1st extraction step, the obtained curve exhibited a constant extraction rate for the entire 90 minutes extraction period, indicating that the raw material still had more CO_2 soluble compounds to be extracted. On the right axis, it is represented the 2nd step extraction curve (90 minutes) which exhibited the typical falling rate (FER) period and the beginning of the diffusion controlled rate period (DP). This was probably due to the obtained low extraction yields (the maximum yield was ~10 times lower than the 1st step extraction yields) which almost permitted to exhaustively extract the raw material bed after the 90 minutes period. The same kinetic behaviour was observed for all other FSFE kinetics.

As already referred, the correlated kinetic parameters (M_{90min}, Y_{90min}, M_{CER} and Y_{CER}), which were defined in section 2.6, are also presented in Table 8.1, as well as the extract yields obtained during the CER periods (R_{90min} and R_{CER}). Because the diffusion controlled rate periods were not attained in the 1st extraction step, the achieved extraction yields, were probably not the maximum extraction yields that could be attained at each extraction condition.

Despite the small variations in solvent flow rates, which were due to some specific characteristics of the extraction apparatus, results show that there was some dependence of M_{90min} and M_{CER} on the extraction pressure and temperature operational conditions. The obtained values at 303 K were similar to the ones obtained at 313 K (~4-6×10^{-8} kg/s for 1st step and ~1-3×10^{-8} kg/s for 2nd step), which can be ascribed to some of the fluid characteristics that may influence mass transfer (like viscosity and diffusivity).

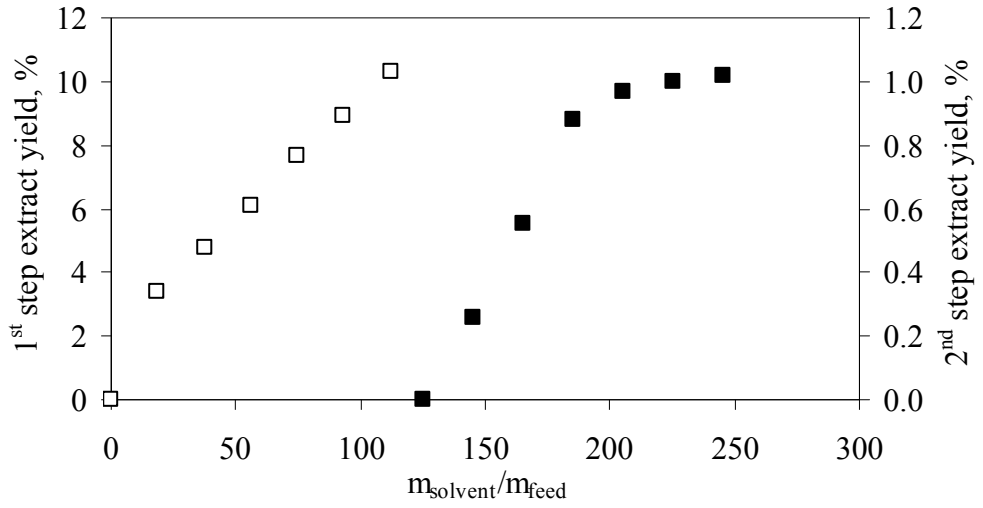

Figure 8.2. Pine bark FSFE kinetics results. Experiments at 303 K and at ~20 MPa: (□) 1st step CO_2 extraction and (■) 2nd step CO_2:EtOH (90:10) extraction.

High values for M_{90min} were obtained for the experiments carried out at 11 and 20 MPa (at 323 K). However, in these conditions, correlations presented relatively low R^2 values (0.87 and 0.78, respectively). For the 15.4 MPa and at 303 K, it was not possible to fit a straight line to the obtained 1st step results. Nevertheless, these properties had quite similar values for the three tested temperatures despite the different employed solvent physical states (liquid for 303 K and supercritical for the other temperatures (NIST, 2007)). For all experimental conditions, the mass transfer rate values (M_{90min}) achieved for the 1st step periods were higher than those obtained at the 2nd step CER periods (M_{CER}), which means that the solutes were rapidly transported from the substrate into the solvent bulk phase, maybe because they were more available for extraction. This is in accordance with the obtained higher yield values for this 1st step. The most favorable conditions for mass transfer were achieved at 313 and 323 K (for 1st step) and at 303 K (for 2nd step), at 10 MPa (Table 8.1). For the 1st step period, this corresponds to the extraction experiments performed with the lower viscosity and higher diffusivity solvent conditions which are known to favor mass transfer. Furthermore, at this temperature, the higher vapor pressures of solutes will lead to higher extract solubilisation which will also contribute to this behavior. On the other hand, and for the 2nd step period, the cosolvent addition changed solvent's polarity and thus the targeted compounds to be extracted were different. Therefore, the increment in those solvent properties which favor mass transfer (and were promoted by higher temperatures) seemed to not have such a positive effect on mass transfer during the CER period.

Table 8.1. Pine bark extraction total yields (obtained by different methodologies) and the corresponding correlated kinetic parameters of extraction curves for 1st and 2nd FSFE steps.

Process[a]	Pressure ±0.2-0.7 (MPa)	1st step					2nd step							
		$Q^b \times 10^5$ (kg/s)	$M_{90min} \times 10^8$ (kg/s)	$Y_{90min} \times 10^4$ (kg/kg)	R_{90min} (%, db)	Total yield (%, db)	$Q^b \times 10^5$ (kg/s)	$M_{CER} \times 10^8$ (kg/s)	$Y_{CER} \times 10^4$ (kg/kg)	R_{CER} (%, db)	$t_{CER}/60$ (s)	Fitting error[d] $\times 10^3$	Total yield (%, db)	Cat+Epi/extract (µg/mg, db)
FSFE 303 K	11.6	5.7 ± 1.8	4.7	8.2	9.9	10.0	6.4 ± 1.9	2.7	4.2	0.9	16	4.5	1.8	0.243±0.007
	15.4	7.5 ± 2.3	-	-	13.8	14.2	7.5 ± 2.2	1.5	2.0	0.5	16	1.7	1.4	0.149±0.011
	20.7	8.0 ±2.4	3.8	4.8	6.8	7.1	6.9 ± 1.5	1.7	2.5	0.6	16	1.5	1.5	0.107±0.008
	25.6	8.2 ± 2.8	4.1	5.0	7.8	7.9	6.7 ± 1.7	2.5	3.8	0.9	17	1.2	2.1	0.346±0.008
	30.4	7.3 ± 2.6	5.0	6.8	9.4	9.8	6.2 ± 2.1	1.7	2.7	0.6	18	1.8	1.8	0.097±0.000
FSFE 313 K[c]	11.1	10.0 ± 5.5	4.0	4.0	7.4	7.5	4.7 ± 1.8	1.5	3.2	0.6	19	2.8	1.7	0.117±0.003
	15.4	6.6 ± 3.5	6.7	8.6	10.3	10.5	5.9 ± 2.2	0.83	1.4	0.6	35	26.5	3.0	0.188±0.003
	20.4	5.9 ± 1.6	4.5±1.9	8.4±2.2	8.1±3.8	8.2±2.3	5.6 ± 1.4	0.71±0.21	1.3±0.4	0.7±0.3	43±6	53.8	2.0±0.01	0.231±0.014
	25.2	7.4 ± 2.7	5.7	7.6	9.8	9.8	5.9 ± 2.2	1.0	1.7	0.3	16	9.2	1.5	0.051±0.057
	30.3	7.9 ± 2.5	4.0	5.0	7.7	7.8	6.1 ± 1.6	2.2	3.6	0.6	12	2.1	1.7	0.110±0.001
FSFE 323 K	11.6	6.9 ± 1.7	7.7	11.1	13.9	14.2	6.0 ± 1.0	1.6	2.6	0.6	17	7.2	1.1	0.177±0.005
	15.4	7.3 ± 2.6	4.5	6.2	8.1	8.1	6.2 ±1.0	1.4	2.3	0.4	15	20.7	1.7	0.131±0.003
	20.6	7.4 ± 0.8	6.8	9.2	12.0	12.2	6.2 ± 0.8	1.6	2.6	0.7	21	2.6	1.5	0.165±0.000
	25.5	7.1 ± 1.4	5.5	7.8	10.3	10.4	6.8 ± 2.3	1.4	2.1	0.3	12	9.3	2.4	0.212±0.007
	30.3	7.4 ± 1.3	5.2	7.1	9.4	9.8	6.6 ± 2.2	1.4	2.1	0.5	17	3.0	1.6	0.171±0.001
HD	~0.1	-	-	-	-	0.022±0.004	-	-	-	-	-	-	-	-
SoE	~0.1	-	-	-	-	-	-	-	-	-	-	-	9.7±0.9	0.039±0.002

M_{90min}: mass transfer rate until 90 min of extraction; Y_{90min}: mass ratio of solute in the solvent phase until 90 min of extraction; R_{90min}: accumulated extract yield after 90 min of extraction.
[a]Solid/solvent ratio: 1st step FSFE (1:137); HD (1:33); SoE (1:50); [b]Average solvent (CO_2) flow rate; [c]Values are presented as mean value ± standard deviation;

[d] $\text{Fitting error} = \frac{1}{N}\sqrt{\sum\left(\frac{y^{calc} - y^{obs}}{y^{obs}}\right)^2}$.

Experimental pressure and temperature also had an effect on the mass ratio of solute in the solvent phase, Y_{90min} and $Y_{CER,}$ (Table 8.1). These effects may be associated to the simultaneous effect of temperature on the extracted compounds vapor pressures and on the solvent density, which were not so significant for the 2nd step 323 K isotherm (Y_{CER}: 2.1-2.6×10^{-4}). Thus, isothermal inversions can be observed for both extraction steps (Figure 8.3). For the CO_2 1st step, at 30 and 313 K, an isobaric increase in temperature originated an extract concentration increase for pressures between ~14 and ~28 MPa. The opposite occurred for pressures below ~14 MPa and above ~28 MPa. For the CO_2 + EtOH 2nd step, pronounced temperature inversions occurred at ~15 MPa for the 303 and 323 K isotherms and at ~29 MPa for the 303 and 313 K isotherms.

Total yields are also resented in Table 8.1 for both extraction steps. Extract yields recovered from adsorption columns represented 0.14-1.21% and 30-80% of total yields, for 1st and 2nd steps, respectively. This high variation for the 2nd step can be explained by compounds capture when ethanol was used at high solvent flow rates in the 2nd step and demonstrates an inefficient extract recovery. Extract recovered in the tubing cleaning (performed in the end of the FSFE) represented 0.2-6.5% of the total extract yield.

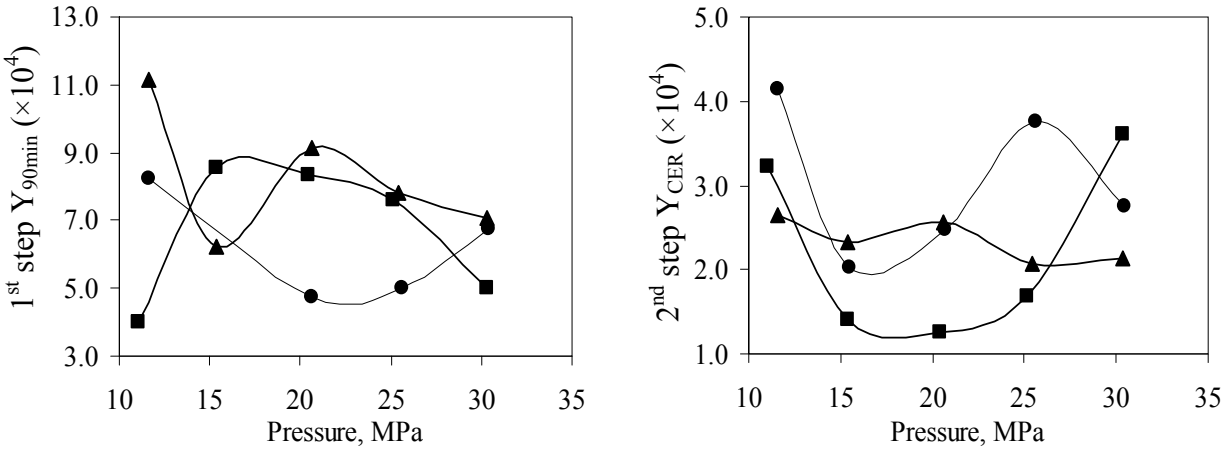

Figure 8.3. Mass ratio of solute in the solvent phase ($Y_{90\ min}$ and Y_{CER}) for 1st step (**a**) and 2nd step (**b**) FSFE: (◆) 303 K; (■) 313 K; (▲) 323 K.

For the three tested temperatures, total yields were higher for the CO_2 extraction step (~7-14%) when compared to the CO_2 + EtOH extraction step (~1-3%). The existence of few compounds soluble in CO_2 + EtOH in a vegetable matrix exhaustively depleted of CO_2 soluble compounds as well as mass transfer phenomena may explain the lower yields obtained for the 2nd extraction step. The operational pressure effect on total yields was different for each isotherm and for both extraction steps. For the CO_2 1st step, highest yields were obtained

at 303 K and 15 MPa, and at 323 K and 10 MPa. For the CO_2 + EtOH 2nd step, the highest yield (3.0%) was obtained at 313 K and at 15 MPa. However, the long constant extraction rate period (35 min) achieved in these conditions may be a drawback and may lead to the choice of other better experimental conditions, such as 323 K and 25 MPa, which conduced to a similar total yield (2.4%) but with a lower t_{CER} (12 min).

Hydrodistillation and Soxhlet extraction experiments may be compared to the 1st and 2nd SFE steps, respectively, due to the overall nature of the extracted substances in each of these methodologies (lipophilic and phenolic compounds, respectively). Analyzing extraction methodologies data (Table 8.1), HD extraction yield was much lower than the obtained higher 1st SFE step yield, probably due to the pine bark lower volatile oil content which can be associated to the tree old age (Iwai et al., 1991). Furthermore, some volatile compounds losses in the Schilcher apparatus may have also contributed to this low yield. On the other hand, for the ethanolic extractions, SoE yield was five times higher than the obtained higher 2nd FSFE yield, due to the SoE solvent composition and to temperature which favored the extraction of higher molecular weight substances.

8.4.2. TLC analysis

Figure 8.4.a shows the anisaldehyde sprayed TLC plate performed for the analysis of the volatile fraction in the CO_2 FSFE extracts (1st step), obtained at 303 K. The same zones appeared and with approximately similar intensities in the extracts obtained at different pressures, showing that pressure did not have a significant effect on the lipophilic extracts composition. Similar behavior was observed at 313 K and 323 K (results not shown). Temperature also did not have a major effect on the compositions. The TLC plates were characterized by intense colored zones at Rf 0, which are indicative of the presence of high polarity substances. The standards used (rutin and quercetin) also remained at the origin, and their color (light orange/brown) is an indication that they were probably present in the extracts. There were also zones that migrated with the solvent, indicating that this extraction methodology conduced to the presence of lipophilic/low polarity compounds in the extracts, associated with the lipophilic nature of the solvent (CO_2). There were two stronger violet zones, with Rf 0.05-0.15, which may correspond to terpene alcohols, and three intense blue zones, with Rf 0.4-0.6, that may correspond to terpenes hydrocarbons, according to Wagner et al. (1984). The concentration of terpenes hydrocarbons was lower in the solvent front.

This TLC analysis was an indication that 1st step CO_2 FSFE was capable of extracting lipophilic/low polarity compounds, which resulted in the concentration of phenolic

compounds and of other polar substances in the vegetal matrix for the subsequent extraction step.

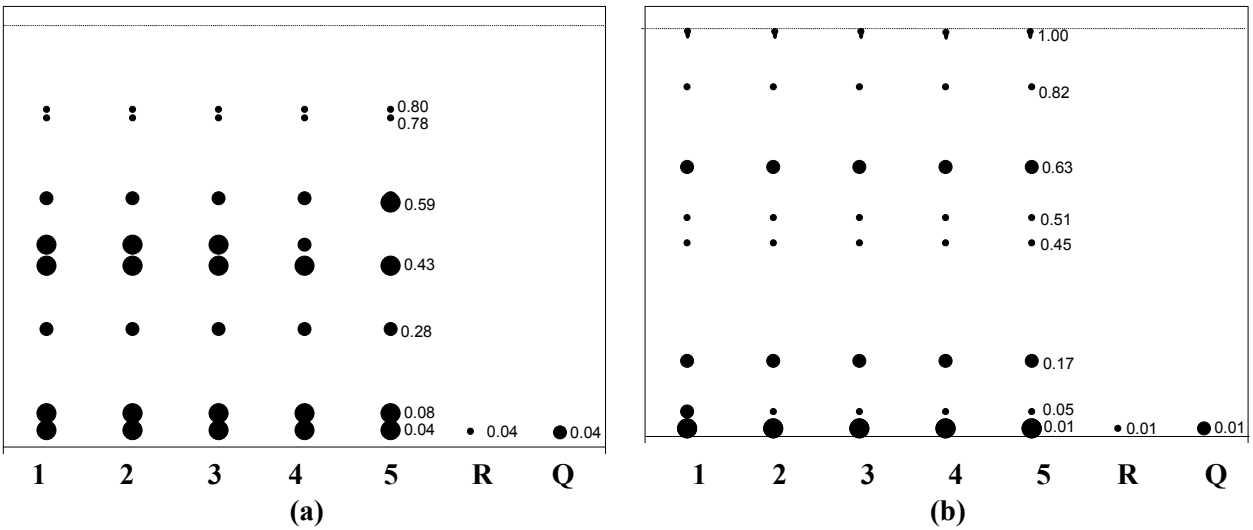

Figure 8.4. TLC analysis of pine bark FSFE extracts obtained at 303 K. Results were drawn using ACD/TLC Plate Tool for ChemSketch, Freeware version 10.02: **(a)** Anisaldehyde sprayed TLC plate for analysis of volatile compounds in 1st step CO_2 FSFE extracts; (**b**) NP sprayed TLC plate, observed at 365 nm, for analysis of phenolic compounds in 2nd step CO_2 + EtOH FSFE extracts: **(1)** 10 MPa; (**2)** 15 MPa; (**3)** 20 MPa; (**4)** 25 MPa; (**5)** 30 MPa; **Standards: R** Rutin and **Q** Quercetin.

NP sprayed TLC plates, observed at 365 nm, were done for the analysis of phenolic compounds extracted at the 2nd step CO_2 + EtOH FSFE. The obtained plate (at 303 K) is presented in Figure 8.4.b. Similarly to the TLC plate for volatile compounds, the same zones appeared, and with approximately the same intensity, for the extracts obtained at different pressures and temperatures (results not shown), showing that neither pressure nor temperature had a significant effect on the phenolic CO_2 + EtOH extracts composition. All chromatograms were characterized by four light blue fluorescent zones at Rf 0.05, 0.17, 0.63 and 1 (in the solvent front), which may be assigned to phenol carboxylic acids (according to Wagner et al., 1984), and a red zone, at Rf 0.45, that was not identified. The standards used in the analysis appeared at Rf 0 and were not identified in extracts.

8.4.3. GC analysis

Chromatograms corresponding to the volatile oil composition profile of 1st step CO_2 FSFE, HD and SoE extracts are presented in Figure 8.5. Three groups of compounds, with retention times in the ranges of 10-25, 25-45 and 45-60 minutes, can be distinguished in all the samples.

The first group of compounds, which corresponds to the most volatile substances, is strongly represented in the HD sample, presenting many compounds and high intensities, and

weakly in the 1st step FSFE and SoE extracts, with few compounds with low intensities. The second group also appears with higher intensities for the HD sample and, for the SoE extract, there are two non identified pronounced compounds. The third group, corresponding to the heavier compounds, appears with more peaks with better resolution for the 1st step FSFE extracts, followed by the SoE sample. These compounds were also observed (without high resolution) in the HD extract chromatogram (Figure 8.5).

As already referred, the volatile oil composition profiles of the extracts are reported in Table 8.2. Nine compounds were identified and they were present in most of the samples, being diterpenes, oxygenated sesquiterpenes, fatty acids (saturated and unsaturated) and esters of fatty acids (Figure 8.6). The identified compounds quantities accounted for just 8-28% of total contents and the highest one corresponded to the SoE extract. For the 1st step FSFE, the non identified compounds had higher retention times (~38-57 min) (Table 8.2).

Among the identified compounds, the ones obtained in higher percentages were palmitic acid, (z)-9-octadecenoic acid, ethyl palmitate, and abietatriene. The CO_2 solubilities (molar fractions) of myristic and palmitate acids are reported in literature (Iwai et al., 1991) for operational conditions close to the ones used in this study, and are 4.03×10^{-3} (19.7 MPa and 308 K) and 4.82×10^{-4} (20.6 MPa and 308 K), respectively.

For CO_2 FSFE, different fractions were collected at different extraction periods (6 fractions, collected at 15 min interval time) and were analysed by GC. For all assays, there was a general increment tendency in the retention indexes of the extracted compounds (for each successive fraction recovered) which is associated with higher diffusion times of higher molecular weight substances.

In general terms, for the 1st step FSFE extracts obtained at 303 and 313 K, it can be observed a pronounced effect of extraction pressure on the composition profile of the identified compounds (with retention times lower than ~32 minutes) as well as on the non-identified separated compounds after ~40 minutes (Table 8.2). At 323 K, the pressure effect was not so pronounced and so, the differences in extracts compositions were not so marked, which is in accordance with the mass ratio of solute in the solvent phase data reported in the kinetic parameters discussion. With the temperature increment, there was a decreasing tendency of the relative amounts of the heavier compounds in the 1st step FSFE extracts, which are associated with the decrease of the solvent's density and which shows a higher ability of a liquid solvent in solubilizing heavier compounds, as reported by Mukhopadhyay (2000).

Table 8.2. Composition profiles of pine bark extracts obtained by HD, SoE and CO_2-FSFE at 303, 313 and 323 K.

Time (min) ± 1-4	Kovats index	Substances	Extraction process, Relative area (%)						
			HD	SoE	FSFE, 303 K				
			Area ± sd (%)		10 MPa	15 MPa	20 MPa	25 MPa	30 MPa
18.1	1519	Oxygenated sesquiterpenes	0.6±0.1	0.29±0.01	0.84	0.77	1.53	0.8	1.89
20.5	1617	Oxygenated sesquiterpenes	tr	0.43±0.13	tr	tr	tr	tr	tr
21.6	1667	14-hydroxy-9-epi-(E)-caryophyllene	2.2±0.2	0.34±0.14	2.38	3.69	1.79	1.88	2.05
24.4	1791	Myristic acid	0.9±0.2	0.18±0.08	2.56	0.85	tr	tr	tr
25.9	1864	Hexadecanol	1.1±0.3	0.42±0.10	1.10	0.92	4.94	tr	tr
28.7	2006	Palmitic acid	3.9±0.3	0.50±0.09	2.24	3.75	tr	3.70	tr
29.4	2044	Ethyl palmitate	1.6±0.3	1.04±0.23	2.21	tr	15.48	tr	tr
30.2	2084	Abietatriene	3.66±0.01	1.25±0.15	4.37	3.68	3.07	4.14	1.50
31.0	2128	7-ethenyl-1,2,3,4,4a,4b,5,6,7,8,10,10a Dodecahydro-4a,7-dimethyl-1-methyl phenanthrene	1.63±0.04	0.60±0.24	2.52	3.52	tr	2.25	tr
31.8	2170	n.i.	5.0±0.4	29.9±6.5	7.24	3.57	2.83	tr	2.98
32.32	2199	(Z)-9-Octadecenoic acid	0.7±0.1	8.11±0.59	1.20	0.69	tr	4.95	tr
32.7	2222	(Z,Z)-9,12-octadecadienoic acid ethyl ester	2.0±0.1	14.33±3.51	0.69	1.50	tr	tr	tr
36.1	2421	n.i.	1.7	5.43±1.59	tr	tr	tr	tr	tr
37.9	2535	n.i.	3.5	4.15±0.53	tr	tr	tr	tr	tr
38.8	2593	n.i.	1.2±0.1	4.13±0.29	tr	tr	tr	tr	tr
38.3	2561	n.i.	tr	tr	5.92	tr	tr	4.81	tr
41.3	2762	n.i.	tr	tr	3.89	6.06	4.28	3.32	tr
49.1	3352	n.i.	tr	tr	11.08	8.47	6.30	12.86	9.48
57.2	-	n.i.	tr	tr	12.02	28.58	26.85	33.60	63.68
		Identified, %	*18.2*	*27.69*	*20.11*	*19.37*	*26.81*	*17.72*	*8.42*
		Non-identified, %	*81.8*	*72.31*	*79.89*	*80.63*	*73.19*	*82.28*	*91.58*

tr: traces < 0.18; n.i.: non identified substance

Table 8.2. Continuation.

Time (min) ± 1-4	Kovats index	Substances	Extraction process, Relative area (%)									
			FSFE 313 K					FSFE 323 K				
			10 MPa	15 MPa	20 MPa	25 MPa	30 MPa	10 MPa	15 MPa	20 MPa	25 MPa	30 MPa
18.1	1519	Oxygenated sesquiterpenes	1.02	1.57	0.68	0.86	0.8	0.65	0.31	0.52	1.31	1.18
20.5	1617	Oxygenated sesquiterpenes	tr	tr	tr	tr	0.7	tr	tr	tr	tr	tr
21.6	1667	14-hydroxy-9-epi-(E)-caryophyllene	1.11	1.57	1.72	0.93	1.51	0.59	0.17	0.85	1.23	1.5
24.4	1791	Myristic acid	0.43	tr	tr	tr	tr	1.28	tr	tr	tr	tr
25.9	1864	Hexadecanol	tr	tr	0.75	2.04	1.31	0.51	0.43	0.79	1.39	0.78
28.7	2006	Palmitic acid	5.73	tr	tr	1.98	3.33	2.32	1.80	2.38	3.59	2.74
29.4	2044	Ethyl palmitate	2.32	tr	tr	0.7	0.98	tr	0.53	0.48	2.56	tr
30.2	2084	Abietatriene	tr	4.26	6.32	6.09	4.77	6.01	4.49	5.02	4.79	4.35
31.0	2128	7-ethenyl-1,2,3,4,4a,4b,5,6,7,8,10,10a Dodecahydro-4a,7-dimethyl-1-methyl phenanthrene	3.19	2.30	3.10	2.92	2.72	3.00	2.68	5.78	2.10	2.28
31.8	2170	n.i.	5.66	10.67	12.1	11.67	15.12	17.33	18.48	16.10	14.97	14.93
32.32	2199	(Z)-9-Octadecenoic acid	tr	tr	tr	1.65	3.02	2.59	2.79	3.23	1.73	1.90
32.7	2222	(Z,Z)-9,12-octadecadienoic acid ethyl ester	tr	tr	tr	0.42	tr	0.81	1.38	1.32	4.68	7.45
36.1	2421	n.i.	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
37.9	2535	n.i.	tr	tr	tr	tr	tr	tr	tr	0.36	tr	tr
38.8	2593	n.i.	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
38.3	2561	n.i.	tr	4.03	5.03	4.88	5.45	4.35	8.95	5.09	tr	tr
41.3	2762	n.i.	1.51	3.53	6.72	7.58	4.00	3.01	9.99	8.66	4.02	8.79
49.1	3352	n.i.	1.52	5.76	12.92	9.63	8.28	13.32	4.17	9.69	9.24	8.31
57.2	-	n.i.	32.88	35.11	20.16	9.73	7.07	19.14	10.90	10.15	3.67	9.47
		Identified, %	*13.8*	*9.70*	*12.57*	*17.59*	*19.14*	*17.76*	*14.58*	*20.37*	*23.38*	*22.18*
		Non-identified, %	*86.2*	*90.3*	*87.43*	*82.41*	*80.86*	*82.24*	*85.42*	*79.63*	*76.62*	*77.82*

tr: traces < 0.18; n.i.: non identified substance

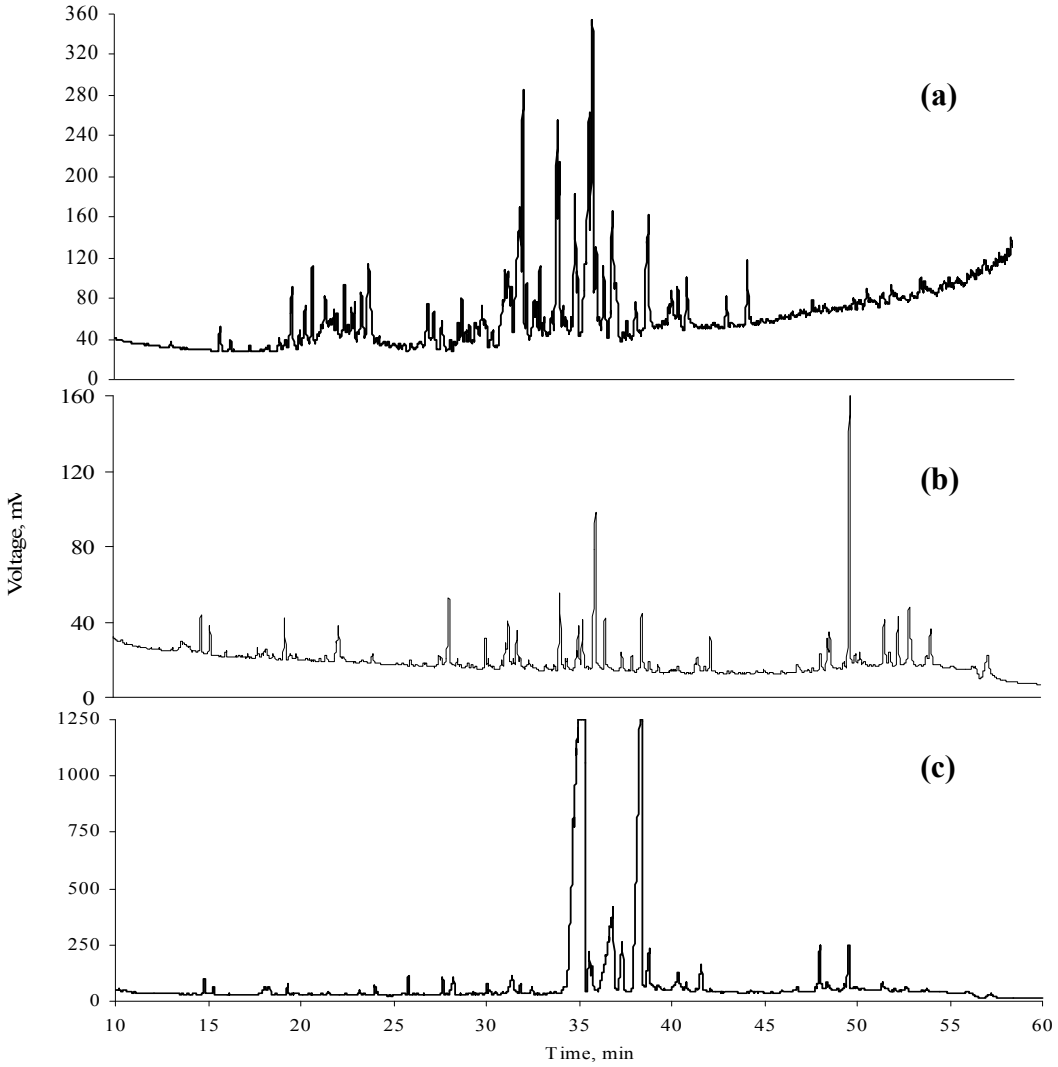

Figure 8.5. GC chromatograms obtained for pine bark extract samples: hydrodistillation (**a**); 1st step CO_2-FSFE, 303 K/10 MPa (**b**) and Soxhlet (**c**).

No additional phytochemical studies have been reported on volatile oil composition of *Pinus pinaster* bark. Other parts of the tree, such as needles, branches and cones have essential oil contents of 0.1-0.2% (w/w) (Macchioni et al., 2003) and wood has an oleoresin having approximately 30% of monoterpenes, 3% of sesquiterpenes and ~60% of resin acids (Arrabal et al., 2005).

It can also be seen that, for some operational conditions, 1st step FSFE was selective for some compounds like ethyl palmitate, abietatriene, palmitic acid and myristic acid. These substances are known to have some biological properties with industrial pharmaceutical applications: ethyl palmitate is used for the treatment of some diseases including cancer (Mann and Staba (1986) cited by Kumar et al., 2004); myristic acid revealed to be a potent enzyme inhibitor (Paige et al., 1990); abietatriene diterpenes were also extracted from Salvia and showed antibacterial and antioxidant activities (Marrero et al., 2002). Although fatty acid

esters, through esterification, may be transformed into nonionic surfactants to be used in cosmetic, pharmaceutical and food industries as emulsifiers (Sabeder et al., 2005; Otake et al., 2004), the oil separation and fractionation steps, with sequential recovering of palmitic acid (as antioxidant) is important for some industries, like the palm oil production (Gordillo et al., 2004).

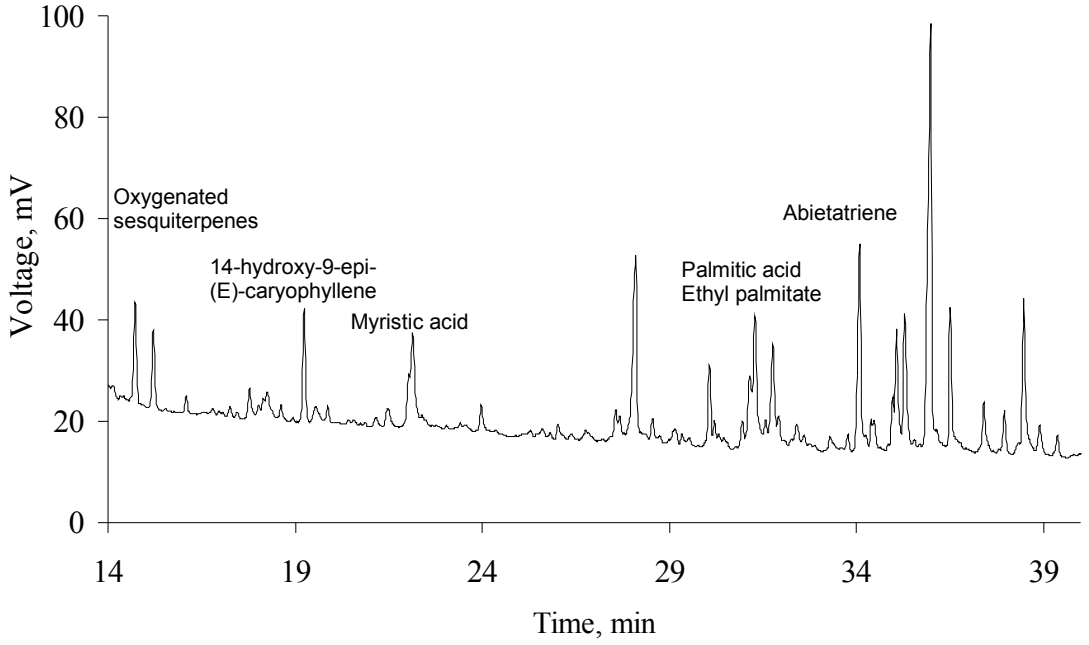

Figure 8.6. Zoomed GC chromatogram obtained for pine bark extract sample obtained by 1st step CO_2-FSFE, at 303 K and 10 MPa.

The presence of fatty acids in the 1st step FSFE extracts volatile oil may be an indication that the raw material was obtained from an old pine tree, or that there was a somehow process inefficiency on the recovering of the lighter extract fractions. It can also explain the obtained low extract yield for HD (0.02%) and explain its composition profile (shown in Figures 8.5 and 8.6). This low volatile oil yield indicates that the higher yields obtained for FSFE and for SoE extracts are essentially composed by high molecular weight substances. According to the literature (Fradinho et al., 2002), these substances may be holocellulose, hemicellulose (A and B), klason lignin, dioxane lignin, neutral sugars as arabinose, mannose and uronic acids. Furthermore, some procyanidins, as tannins, are found in pine bark extracts, mainly to be applied in the tanning and adhesives industries (Fradinho et al., 2002; Seabra et al., 2007). These monomers, oligomers and polymers are essentially formed by units of catechin, epicatechin, gallic acid and other phenolic compounds (Selga and Torres, 2005; Fradinho et al., 2002).

8.4.4. HPLC analysis

For the 2nd step FSFE ethanolic extracts, the obtained catechin and epicatechin profiles were similar for each tested temperature (Figure 8.7), which should be associated to their chemical similarity (these substances are isomers). However, catechin content was between 1.6 and 5.8 times higher than the obtained epicatechin amount, being around twice its amount for most assays. At 313 K and 25 MPa, and at 323 K and 20 MPa, catechin contents were 5.8 and 5.4 times higher than the epicatechin ones, respectively. Therefore, we can assume these were selective extraction conditions which can be used to partially separate these two isomers.

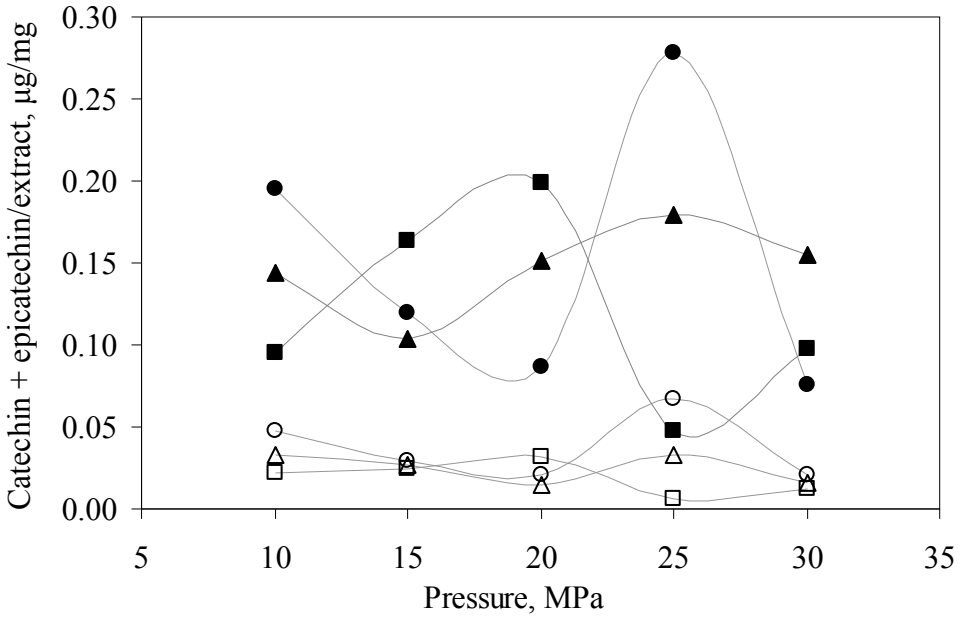

Figure 8.7. Characterization of pine bark 2nd step FSFE CO_2:EtOH (90:10, v/v) extracts by HPLC: catechin contents (µg/mg, d.b.) ● 303 K; ■ 313 K; ▲ 323 K, and epicatechin contents (µg/mg, d.b.) ○ 303 K; □ 313 K; Δ 323 K.

Catechin + epicatechin concentrations are also presented in Figure 8.8, but now as a function of CO_2 density, which is considered to be close to the solvent mixture (CO_2:EtOH (90:10)) density. To the best of our knowledge, there are no available data from literature for experimental catechin and epicatechin solubilities in CO_2:EtOH (90:10%) for all the assayed values of pressure and temperature. Although, and from the available data at 8-11.1 MPa and at 313 K (Berna et al., 2001; Cháfer et al., 2002), data showed a solubilisation increase tendency with pressure. However, in this work such behaviour was not observed for the ethanolic extracts and in terms of catechin + epicatechin contents. This was probably due to the complex mixture composition of pine bark extracts. Instead, a temperature inversion was observed for the three isothermal curves, as happened with Y_{CER} data (Figure 8.3) and already

explained in terms of the possible relative effects of volatility and solvent mixture density. Pressure effect on catechin + epicatechin concentrations was not so pronounced at 323 K, which is also in good agreement with Y_{CER} data. These substances probably influenced the overall extract solubility and, consequently, the obtained extract yields (although not directly). As a consequence, there were not such significant variations on catechin + epicatechin contents for 2nd step CO_2 + EtOH FSFE extracts obtained at 323 K (Table 8.1), as those obtained for the 1st step CO_2 FSFE extracts and volatile oil profiles (Table 8.2).

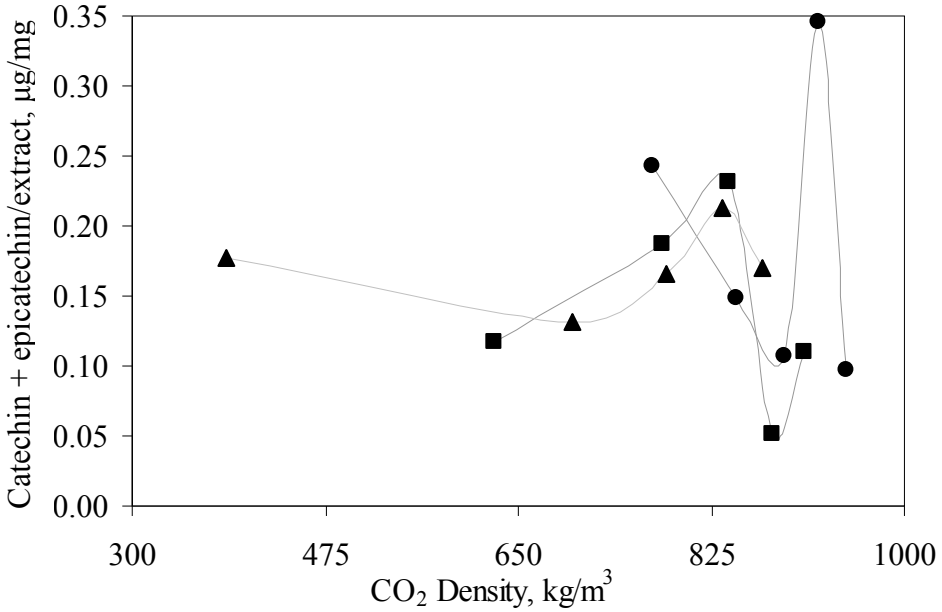

Figure 8.8. Catechin + epicatechin concentration (µg/mg, d.b.) as a function of CO_2 density for pine bark 2nd step FSFE CO_2:EtOH (90:10, v/v) extracts. ●303 K; ■ 313 K; ▲323 K.

The most favourable condition to obtain an extract rich in these phenolic substances was found to be 303 K and 25 MPa, which corresponds to the obtained highest mass ratio of solute in the solvent phase (Y_{CER}) and mass transfer rate (M_{CER}) CER period values, and to a CER period duration (t_{CER}) of 17 minutes (Table 8.1).

Comparing the different employed phenolics extraction methodologies, the catechin + epicatechin contents obtained in the SoE extract (0.039 µg/mg, d.b.) were 24-88% lower than those obtained at 2nd step FSFE extract contents, which clearly demonstrates that CO_2+EtOH extraction procedures increased catechin + epicatechin extract contents.

8.4.5. Antioxidant activity results

The antioxidant activity isobaric profiles (which were measured after 3 hours of oxidation inhibition assays), for the 1st and 2nd step FSFE extracts, are represented in Figure 8.9. For the

majority of the obtained extracts, antioxidant activities can be considered as approximately constant during the 3 hour assays. The exceptions were the extract obtained at 303 K and 30 MPa, which presented ~40% increase, and the extract obtained at 323 K and 25 MPa, which presented a ~10% decrease during oxidation inhibition assays.

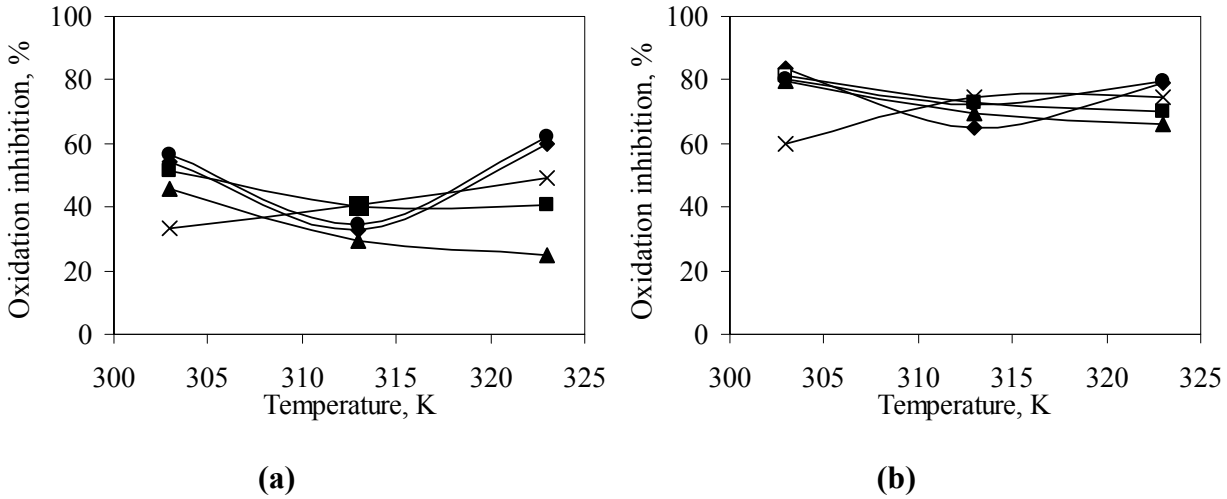

Figure 8.9. Isobaric oxidation inhibition profiles (obtained after 3 hours inhibition assays) for pine bark extracts. 1st step FSFE CO_2 (**a**) and 2nd step FSFE CO_2:EtOH (90:10, v/v) (**b**): ◆ 10 MPa; ■15 MPa; ▲20 MPa; ●25 MPa; × 30 MPa.

As can be seen, the obtained antioxidant activities where lower for the 1st step FSFE CO_2 extracts (29-62%), (A), when compared to the 2nd step FSFE CO_2:EtOH (90:10) ones (60-84%), (B), which indicates that, for the two tested FSFE conditions, ethanol had an important role in the extraction of compounds with higher antioxidant activities. It is also interesting to notice that 1st step and 2nd step extracts presented similar inhibition profiles at a same pressure. Higher activities were obtained for the extracts obtained at 303 K and at lower pressures, which correspond to the extracts having higher catechin and epicatechin contents. Furthermore, it seems that there was some relationship between antioxidant activities of the obtained 2nd step FSFE extracts and their catechin and epicatechin contents (Figure 8.10), because the increment in catechin + epicatechin contents was accompanied by the increment in the antioxidant activity of extracts, though they were not linearly related. In fact, some extracts having considerably different catechin + epicatechin contents had approximately the same oxidation inhibition, which means that the presence of other substances certainly affected (promoted/inhibited) the resulting antioxidant activities. For example, 2nd step FSFE extracts obtained at 303 K and at 20 MPa and 25 MPa, presented the same oxidation inhibition (~80%) though having considerably different catechin + epicatechin contents

(0.107 and 0.346 μg/mg, d.b., respectively). This behavior is quite typical and, for example, Pinelo et al. studied the effect of several process variables (time, temperature and liquid/solid ratio) on the ethanolic SoE extraction from pine (*Pinus pinaster*) sawdust and concluded that there was not an explicit correlation between the extracts polyphenolic contents and their antioxidant activities (measured by the DPPH method).

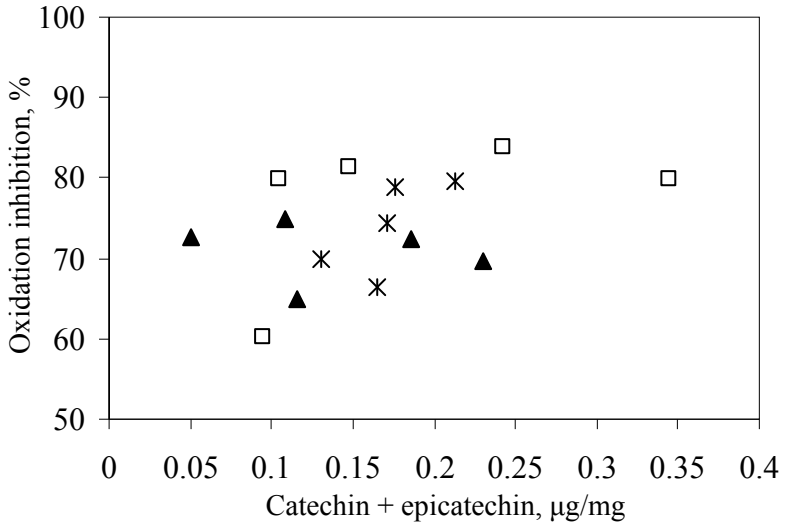

Figure 8.10. Isobaric oxidation inhibition (%) for pine bark 2nd step FSFE CO_2:EtOH (90:10, v/v) extracts, as a function of catechin + epicatechin contents: □ 303 K; ▲ 313 K; ✳ 323 K.

For the SoE extract, the oxidation inhibition was found to be 63±3% (after the third reaction hour) and an 11% reduction was observed between the first and the third reaction hour. This value is comparable to the lower range values obtained for the 2nd step FSFE extracts (60-84%). However, 2nd step FSFE extractions can be "tuned" and it was possible to manipulate high pressure extraction conditions in order to obtain pine bark extracts with higher antioxidant activities (which is not possible to do in SoE experiments). For the HD extract, the observed oxidation inhibition was -39±0.7%, for the first hour, with a 17% increment at the end of the reaction, reaching -22±0.3%. This negative value can be explained by a pro-oxidant activity and this behavior has been observed for other raw materials such as cilantro, for example (Wong and Kitts, 2006).

8.5. Conclusions

Different pine (*Pinus pinaster*) bark extracts were obtained using a fractionated supercritical fluid extraction procedure. Around 84% of the total extract was obtained in the

1st CO_2 extraction step and this volatile oil rich extract presented lower oxidation inhibitions (~29-62%) than those obtained at the 2nd CO_2 + EtOH extraction step (60-84%). The catechin + epicatechin yields of these 2nd step ethanolic extracts were higher (0.051-0.346 μg/mg) than the ones obtained for SoE (0.039 μg/mg). The increment in catechin + epicatechin extract content influenced the corresponding extract antioxidant activity, although not directly.

Pressure and temperature affected the extraction kinetic parameters (M_{90min}, Y_{90min}, M_{CER}, Y_{CER} and t_{CER}). In terms of the mass ratio of solute in the solvent phase, these results may be associated to the simultaneous effect of temperature on the extracted compounds vapor pressures and on the solvent density. The same possible effects may explain the obtained catechin + epicatechin contents for the 2nd CO_2 + EtOH extraction step.

Therefore, and as a main conclusion, the employed high pressure fractionated extraction methodology showed that it was possible to obtain different extract fractions having diverse antioxidant capacities from *Pinus pinaster* bark. Furthermore, this process can be further optimized in order to improve extraction yields, selectivities and antioxidant activities, by the use of different solvent mixtures compositions and of other different operational conditions.

Finally, the reuse of this agroindustrial residue may be an important factor for the Portuguese economy, and these methodologies follow the current worldwide tendency of recycling this type of residues. Possible envisaged applications of the different obtained extracts are, for example, the use the volatile oil fractions in the aroma/flavor industries, and/or the use of phenolic fractions in the food and pharmaceutical industries.

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9. High pressure extraction of maritime pine bark: study of fractionation, solvent flow rate and solvent composition

This Chapter is a further development of the research work presented in the previous Chapter. Other process variables were assayed, namely fractionation, solvent flow rate and solvent composition. Extraction kinetics were studied as well as extracts composition and antioxidant and anti-inflammatory activities, so as to evaluate their potential applications in the food, pharmaceutical and nutritional fields. The text that comprises this Chapter will be submitted for publication in a scientific journal after conclusion of the high-performance liquid chromatography analysis. This analysis will allow the quantification of some compounds that are present in the obtained extracts, and which may contribute to their bioactivity.

9.1. Abstract

Pinus pinaster bark, an abundant Portuguese residue rich in high-value phenolic compounds, was subjected to fractionated and non-fractionated high pressure extractions (F-HPE and NF-HPE, respectively). Supercritical carbon dioxide ($scCO_2$) was the chosen solvent to extract the pine bark low-polarity fraction and ethanol (EtOH) was added to $scCO_2$ to recover the phenolic fraction. The effect of the solvent flow rate was studied on first step (CO_2) and on second step (CO_2:EtOH (90:10), v/v) F-HPE kinetics. Due to the obtained low first step yield (0.6-1.0%, d.b.) HPE was performed with no fractionation at 303 K and ~25 MPa. The flow rate that yielded the highest global yield (~7×10^{-5} kg/s) was chosen to carry out NF-HPE with different EtOH compositions (30-90%, v/v). The HPE results were compared with hydrodistillation and with Soxhlet extraction results in terms of global yields, extracts composition and extracts antioxidant activities. Kinetic parameters were obtained

using Matlab. Fractionation, solvent flow rate and solvent composition affected extraction kinetics and the characteristics of the extracts. In particular, the NF-HPE solvent composition had a considerable influence on extracts total phenolic compounds and condensed tannins contents, which varied in the ranges 3.5-25.6% gallic acid equivalents and 1.0-19.8% catechin monohydrate equivalents. In particular, the solvent composition CO_2:EtOH (30:70) led to the extract with the highest contents of total phenolic compounds and of condensed tannins (25.6% and 19.8%, respectively), being similar to the ones achieved by Soxhlet extraction (26.0% and 18.2%, respectively). The HPE methodology takes advantage over the conventional methodology due to the reduced EtOH consumption, lower solvent-to-solid ratio, lower extraction temperature, and oxygen-free medium in which it occurs.

9.2. Introduction

Maritime pine (*Pinus pinaster*) is one of the most important forest species used in Portugal by the furniture, wood and pulp and paper industries. Its byproduct (pine bark) is a very promising source of high-value phenolic compounds which can have important applications in food, cosmetic, pharmaceutical, leather tanning and adhesives industries. Typical phenolic compounds present in pine bark are (+)-catechin, (-)-epicatechin, dihydroquercetin, as well as phenolic acids. Most of these compounds are procyanidin dimers, trimers, oligomers and polymers (Wood et al., 2002; Karonen et al., 2004). Pycnogenol is a commercially available French maritime pine bark extract that was reported to have a potent antioxidant activity and, consequently, potential health promoting properties (Rohdewald, 2002).

The choice of the extraction process, solvents and operational conditions is always conditioned by the required extract quality and by other particular specifications, like extraction yield and presence of undesired compounds. Conventional solid-liquid extraction with water, alcohols and/or acidified alcohols is usually employed for extraction of condensed tannins. However, and because natural products usually contain a wide variety of low and high molecular weight phenolic compounds (and their complexes), natural extracts from these materials will always contain a mixture of different classes of these phenolic substances, depending on the chosen extraction solvent and on the particular employed operational conditions. Usually, additional steps are then required in order to purify/concentrate the desired compounds and to remove the undesired phenolic and other non-phenolic substances. For food and pharmaceutical applications, high pressure solvent extraction (HPE) represents an attractive option to conventional solid-liquid extraction methods and, in some cases,

supercritical fluid extraction can also be applied, offering several advantages in terms of selectivity, separation conditions and on the use of environmental friendly technology and solvents.

The main purpose of this study was to extract phenolic compounds from pine bark using high pressure extraction methodologies and to compare these procedures to Soxhlet extraction (SoE) and to hydrodistillation (HD). Supercritical carbon dioxide ($scCO_2$) was the chosen solvent to extract the pine bark low-polarity fraction, and ethanol (EtOH) was added to $scCO_2$ in order to obtain phenolic-rich fractions. The effects of solvent flow rate and of solvent mixtures compositions were studied on the extraction kinetics results as well as on the CO_2:EtOH extracts composition.

9.3. Materials and methods

9.3.1. Raw material

Comminuted *P. pinaster* bark was provided by a wood processing company from Beira Litoral, Portugal. Particles presenting a size distribution between 60 and 18 mesh were separated using sieves under mechanical stirring (Retsch, Germany). Light scattering experiments were performed using a Laser Malvern Mastersizer (Hydro 2000 MU, Worcestershire, UK) so as to find the particle size distribution. Finally, the raw material humidity was determined by the xylol distillation method of Jacobs (1973) with triplicate assays.

9.3.2. Chemicals

Extraction experiments were performed using carbon dioxide (99.998%, Praxair, Madrid, Spain), ethanol (99.5%, Panreac Quimica S.A., Barcelona, Spain) and distilled water. For TLC and spectrophotometric analyses of extracts, the following analytical grade chemicals and solvents were used: ethanol (99.5%, Panreac Quimica S.A., Barcelona, Spain), Folin-Ciocalteu's phenol reagent (Merck, Darmstadt, Germany), sodium carbonate (Pronalab, Lisbon, Portugal), vanillin (José M. Vaz Pereira S.A., Sintra, Portugal), methanol (Fluka, Buchs, Switzerland), formic acid, ethyl acetate, hexane, glacial acetic acid, p-anisaldehyde, hydrochloric acid (37%) from Riedel-de-Haën (Seelze, Germany), o-xylene (97%), anhydrous methanol (99.8%), 2-aminoethyl diphenylborinate (97%, Fluka, Steinheim, Germany), sulfuric acid (95-98%) from Sigma-Aldrich Inc. (Steinheim, Germany), and distilled water. Chemicals used for HPLC analysis were formic acid (98-100%, Sigma-Aldrich Inc.,

Steinheim, Germany), water (HPLC grade, Carlo Erba, Milan, Italy), and acetonitrile (HPLC grade, Fisher Scientific, Leicestershire, UK).

Standards used for TLC and spectrophotometric analyses were quercetin dehydrate (≥98%, HPLC grade), rutin hydrate (≥95%, HPLC grade), D-(+)-catechin hydrate (98%) and gallic acid (≥98%) from Sigma-Aldrich Inc. (Steinheim, Germany). Standards used for the GC analysis were alkane standard solutions C_8-C_{20} and C_{21}-C_{40} (Fluka, Buchs, Switzerland).

9.3.3. Experimental extraction procedures

9.3.3.1. Hydrodistillation and Soxhlet extraction

Pine bark essential oil was separated by hydrodistillation (HD) in a Schilcher apparatus, following the AOAC 962.17 method (1984), with a 33:1 (v/w) solvent-to-solid ratio. Soxhlet extraction was performed using ethanol and a solvent-to-solid ratio of 50:1 (v/w). Both systems were kept under reflux for 120 min at the solvent boiling point (triplicate assays). EtOH was removed from the extracts using a rotary evaporator with vacuum control. The pine essential oil and dried Soxhlet extracts were kept at 255 K until further analysis.

9.3.3.2. High pressure extractions

Fractionated and non-fractionated high pressure extractions (F-HPE and NF-HPE, respectively) were performed using a supercritical fluid extraction apparatus previously described by Braga et al. (2008). The selection of pressure and temperature conditions was based on the previous work developed by these authors and taking into consideration already obtained pine bark extracts composition as well as mass transfer coefficients. Table 9.1 reports the operational conditions of the HPE experiments performed in this study. The comminuted pine bark was always placed inside a stainless steel cylindrical 120-mesh screen thus avoiding any particle loss with the exit solvent stream. However, and in order to evaluate if this cylindrical screen affected extraction kinetics, two extraction experiments were also performed keeping the same experimental conditions (323 K, ~20 MPa, 12.5×10^{-5} kg/s of CO_2) except that in one the raw material was placed directly inside the extraction cell, and in the other one the cylindrical screen was used.

The F-HPE methodology comprised: *(i)* a first step in which pine bark was subjected to extraction with CO_2 in order to remove low-polarity compounds, employing a 10 min static period followed by a 360 min dynamic period; *(ii)* a second step in which CO_2:EtOH (90:10, % v/v) was used to extract polar compounds, such as condensed tannins, and employing a 360 min dynamic period. Three solvent flow rates were assayed for these fractionated extractions,

varying between ~7×10^{-5} and ~18×10^{-5} kg/s, corresponding to solvent-to-solid ratios from 234:1 up to 660:1 (w/w, on a dry basis, d.b.). Approximately the same flow rate was used in each step of the same experiment.

In order to evaluate the fractionation effect on the characteristics of the extracts obtained with CO_2 and EtOH, NF-HPE experiments were assayed using the same experimental conditions of the fractionated second step (CO_2:EtOH (90:10), 303 K, 20 MPa, 360 min and three levels of solvent flow rate, Table 9.1). Then, the optimum flow rate was identified and was used to perform another set of non-fractionated extractions, varying the composition of EtOH in the solvent mixture between 30% and 90% (v/v) (Table 9.1). For these assays, extraction time was reduced to 210 min. The extraction performed with CO_2:EtOH (30:70) was quadruplicated in order to determine the experimental error.

Table 9.1. Experimental conditions tested for HPE methodologies performed with *P. pinaster* bark.

Extraction Methodology	Fractio_nation	Solvent (%, v/v)		T±0.1 (K)	P±0.3-0.6 (MPa)	Time (min)	Q×10^5 (kg/s)	Solvent-to-solid ratio
F-HPE	Yes	*1st step*	CO_2	323	20.5	370	6.9±2.3	234:1
		2nd step	CO_2:EtOH (90:10)	303	24.3	360	8.0±2.6	274:1
		1st step	CO_2	323	20.4	370	12.4±5.2	439:1
		2nd step	CO_2:EtOH (90:10)	303	25.4	360	12.1±2.9	442:1
		1st step	CO_2	323	19.9	370	17.6±5.7	629:1
		2nd step	CO_2:EtOH (90:10)	303	24.5	360	18.3±4.8	660:1
NF-HPE	No	CO_2:EtOH (90:10)		303	25.3	360	7.68±2.3	280:1
		CO_2:EtOH (90:10)			24.9	360	14.0±0.5	511:1
		CO_2:EtOH (90:10)			25.6	360	20.8±0.5	761:1
		CO_2:EtOH (70:30)			25.0	210	7.9±1.5	175:1
		CO_2:EtOH (50:50)			25.4	210	8.1±1.0	177:1
		CO_2:EtOH (30:70)			24.9	210	7.4±0.2	158±10:1
		CO_2:EtOH (10:90)			25.5	210	7.4±1.0	168:1

For the NF-HPE using CO_2:EtOH (30:70), indicated standard deviations are for two extraction experiments.

For all experiments, extract fractions were recovered in a glass flask (placed in an ice bath) every 15 min during the first hour of extraction, and every 30 min afterwards. A trap (another glass flask) was used to prevent any extract losses with the expanded CO_2 whose flow was measured by a wet gas meter (DM3C ZE 1411, G.H. Zeal Ltd., London, England). An adsorbent (Porapak Q 80/100 mesh, lot: 134, Supelco, Bellefonte, USA) packed column was placed after the trap to reinforce the extract loss prevention. Tubing line was cleaned with EtOH after each extraction step or extraction assay. Finally, ethanol was evaporated from the CO_2:EtOH extract fractions using a rotary evaporator. In order to prepare the extracts for future analyses, the fractions recovered during the extraction kinetics were mixed together after being weighted. Dried extracts were stored away from light, at 255 K.

9.3.4. Characterization of pine bark extracts

9.3.4.1. Thin layer chromatography: analysis of low polarity compounds, phenolic compounds and condensed tannins

TLC analyses of the extracts were performed using silica gel plates (20 cm×20 cm, thickness 0.2 mm; Merck, Germany). Table 9.2 reports the mobile phases and the spray reagents that were applied for the analysis of low polarity compounds, phenolic compounds and condensed tannins (Wagner et al., 1984). Two groups of extracts were considered for the analysis of low polarity compounds: *(i)* HD, Soxhlet, and F-HPE first step (CO_2) and second step (CO_2:EtOH (90:10)) extracts; *(ii)* F-HPE second step extracts (CO_2:EtOH (90:10)) and NF-HPE extracts (CO_2:EtOH (90:10)). This latter group was also considered for the analysis of phenolic compounds, where the Soxhlet extract was also included. The used standards in these two analyses were quercetin dehydrate, rutin hydrate, gallic acid, D-(+)-catechin hydrate and (-)-epicatechin. For the analysis of condensed tannins, the extracts obtained by NF-HPE using different EtOH compositions in the solvent mixture (10-90%, v/v) and approximately the same solvent flow (7.3-8.1×10^{-5} kg/s) were considered, as well as the Soxhlet extract. Employed standards were D-(+)-catechin hydrate and (-)-epicatechin. The same extract quantities were chromatographed in each analysis.

Table 9.2. Mobile phases and spray reagents used for the TLC analysis of low polarity compounds, phenolic compounds and condensed tannins of the *P. pinaster* bark extracts.

Compounds analyzed	Mobile Phase	Spray Reagent	Detection
Low polarity compounds	Hexane-ethyl acetate (8:2, v/v)	Anisaldehyde solution	Visible
Phenolic compounds	Ethyl acetate-formic acid-glacial acetic acid-H_2O (100:11:11:27, v/v/v/v)	NP solution (MeOH-2-aminoethyl diphenylborinate, 99:1, v/w)	365 nm
Condensed tannins	Toluene-acetone-formic acid (3:6:1, v/v/v)	Ethanol-vanillin (95:5, v/w) solution acidified with HCl (10%)	Visible

9.3.4.2. Gas chromatography

The composition of the extract obtained by hydrodistillation was determined using a GC-MS apparatus (QP-5000, GC-EM, Shimadzu, Tokyo, Japan). The system was equipped with a fused silica capillary column (30 m×0.32 mm i.d., 0.45 μm, OV-5, Ohio Valley Specialty Company, Ohio, USA). The carrier gas was helium (1.0 mL/min) and 1 μL of the diluted sample (in dichloromethane) was injected. The temperature of the injector was 513 K and that of the detector was 503 K. The oven temperature was raised from 333 to 553 K at 3 K/min and maintained at 553 K for 20 min. Identification of compounds was based on the

comparative analyses of mass spectra of the substances with those of the database of the system GC-EM (Nist. 62 lib.), literature (McLafferty, 1989) and Kovats retention indexes (Adams, 1995), which were obtained by co-injection of a standard mixture of *n*-alkanes (C_8-C_{24}) and by the application of the Van den Dool and Kratz equation (Van den Dool, 1963). This analysis was performed at Laboratório de Produtos Naturais, Centro de P&D Recursos Genéticos Vegetais, Instituto Agronômico de Campinas, Brazil.

The chemical composition of the fractionated first step $scCO_2$ extracts was determined using a gas chromatograph (Tremetrics 9001, Thermo Scientific, Austin, USA). The extract fractions recovered in the flask, the trap and the adsorbent column were analyzed individually. The system was equipped with a fused capillary column (15 m×0.32 mm i.d., 0.45 μm, CP-Sil 8 CB, Varian, Middelburg, The Netherlands). The carrier gas was helium (2-3 mL/min) and 2 μL of the sample (0.1 mg/mL, in ethyl acetate) was injected. The temperature of the injector was 503 K and that of the detector was 523 K. The temperature programming was 523 K (5 min), 323–553 K, 5 K/min and 553 K (5 min). Components Kovats indexes were determined relatively to the retention times of a series of *n*-alkanes, using the Kovats' method. The identification of the substances was based on the comparison of their Kovats indexes with those of the HD extract and also on the chromatogram profiles. The composition profiles of the samples are presented as relative area (%). This analysis was performed in duplicate.

9.3.4.3. Quantification of total phenolic compounds

Total phenolic compounds in pine bark extracts (obtained by Soxhlet ethanolic extraction, 2nd step F-HPE and NF-HPE) were quantified according to the Folin-Ciocalteu's method. The procedure followed was the one proposed by Singleton and Rossi (1965) with some modifications based on Cheung et al. (2003), being described in detail by Seabra et al. (2010). Results were expressed as gallic acid equivalents (GAE) in percentage (mg GAE/mg extract × 100, d.b.). This analysis was performed in triplicate.

9.3.4.4. Quantification of condensed tannins

The vanillin-H_2SO_4 methodology (Sun et al., 1998) was used to determine condensed tannins contents in pine bark extracts obtained by Soxhlet ethanolic extraction, 2nd step F-HPE and NF-HPE. Samples were diluted in methanol, centrifuged during 5 min at 3000 rpm, and the supernatant was analyzed. Aliquots of each sample were reacted with 1.25 mL of the vanillin reagent (1% (w/v) vanillin in methanol) followed by 1.25 mL of the acid solution (25% (v/v) H_2SO_4 in methanol) at 303 K. A non-vanillin-containing sample was run for each

sample. Absorbances were recorded at 500 nm after 15 min, and results were expressed as (+)-catechin monohydrate equivalents (CME), in percentage (mg CME/mg extract × 100, d.b.). This analysis was performed in triplicate.

9.3.4.5. High-performance liquid chromatography

The procyanidin profile of the Soxhlet ethanolic extract and those of the 2nd step F-HPE and NF-HPE extracts were screened by reversed-phase HPLC, following the method developed by Karonen et al. (2004), with some modifications. Samples were dissolved in ethanol, microfiltered (0.20 μm), vacuum dried and redissolved in methanol to a final concentration of 40 mg/mL. The HPLC system was equipped with a LiChrosorb RP18 column with precolumn (250 x 4 mm i.d., 5 μm), an UV detector (WellChrom k-2500) and a HPLC pump (WellChrom Maxi-Star k-1000) from Knauer (Berlin, Germany). The binary mobile phase consisted of (A) water and formic acid (99.5:0.5, v/v) and (B) acetonitrile. A linear gradient elution was performed: 0 min: 100% A; 2 min: 100% A; 19 min: 91% A, 9% B; 35 min: 70% A, 30% B; 43 min: 30% A, 70% B. Column temperature was 308 K, flow rate was 1 mL/min, detection wavelength 280 nm and injection volume was 20 μL. Concentration of catechin and of epicatechin in extracts (eluted at the same time) were calculated from a previously determined calibration curve of epicatechin and expressed as % (w/w) on a dry basis. This analysis was performed in duplicate.

9.3.4.6. Antioxidant activity: β-carotene and linolenic acid coupled reaction assay

Antioxidant activity of NF-HPE and Soxhlet extracts was determined by the coupled oxidation of β-carotene and linolenic acid (Hammerschmidt and Pratt, 1978), following the procedure described by Braga et al. (2003). The reaction was monitored continuously during 6 hours and the antioxidant activities were expressed as oxidation inhibitions (in %):

$$\text{Oxidation Inhibition (\%)} = \left[1 - \frac{\text{Abs}_{\text{extract}}^{t_0} - \text{Abs}_{\text{extract}}^{t_n}}{\text{Abs}_{\text{control}}^{t_0} - \text{Abs}_{\text{control}}^{t_n}}\right] \times 100$$

where Abs^{t_0} is the absorbance reading at the beginning of reaction and Abs^{t_n} is the corresponding one at t_n ($0\ \text{h} < t_n \leq 6\ \text{h}$).

9.3.5. Calculation procedures

For all employed extraction methods, total yields were calculated as the ratio between the total extract mass and the raw material mass, on a dry basis. For the HPE experiments, the

total extract mass included the fractions collected during the extraction kinetic, the ones retained in the trap and adsorbent column, as well as the one recovered from tubing line cleaning with EtOH.

The overall extraction curves were set up considering the accumulated mass of extract (collected at a fixed time interval). Therefore, the extract fractions retained in the trap and adsorbent column, as well as the ones collected in the cleaning process were not considered for the kinetic representations. Each overall 1st step and 2nd step F-HPE curve, and NF-HPE curve was fitted by a curve formed by two straight lines using the fminsearch function of Matlab (R2007a), as described by Seabra et al. (2010). The kinetic parameters of the constant extraction rate (CER) period were calculated according to Rodrigues et al. (2002): *(i)* mass transfer rate for the constant extraction rate period, M_{CER}; *(ii)* mass ratio of solute in solvent phase at measuring-cell outlet, Y_{CER}, and *(iii)* duration of the constant extraction rate period, t_{CER}.

9.4. Results and discussion

9.4.1. Extraction kinetics

Pine bark presenting 3.9±0.17% (w/w, d.b.) of humidity and a mean particle size of 0.76×10^{-3} m were extracted with $scCO_2$ followed by a homogeneous high pressure CO_2:EtOH (90:10) mixture at different mass flow rates. Figure 9.1 shows the two overall extraction curves that were achieved using 12.5×10^{-5} kg/s of $scCO_2$, at 323 K and ~20 MPa, and placing the comminuted raw material directly in the extraction cell or in a cylindrical mesh screen. No considerable differences were observed between the two curves, and so the cylindrical screen was used for all the forthcoming extraction experiments.

Figures 9.2.a and 9.2.b represent the overall extraction curves of the F-HPE assays for, respectively, the first and second steps. In these figures, the extraction yield was reported as a function of the specific mass of employed solvent, i.e., mass of solvent per pine bark mass, on a dry basis. Higher specific masses of solvent were achieved for higher flow rates, since extraction time was maintained the same for all experiments.

Considering the first step extraction curves (Figure 9.2.a), for a fixed specific mass of solvent, higher yields were achieved for lower $scCO_2$ flow rates. This is experimental evidence that the limiting phenomenon controlling extraction rate is internal mass transfer. Keeping $scCO_2$ in the extraction cell for longer periods favors extraction yield, since the

intraparticle diffusion of the solvent, as well as the solubilization of the extracted substances within the micropores require a longer contact time between solvent and solutes.

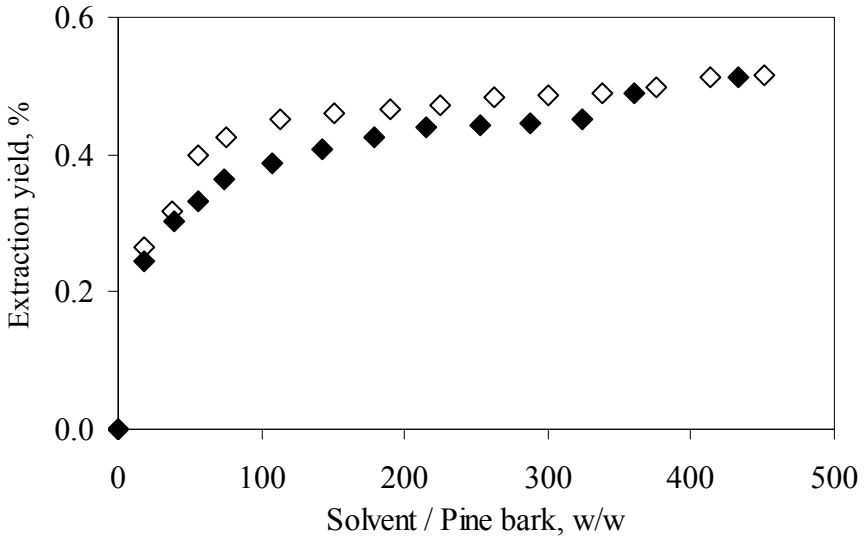


Figure 9.1. Overall curves of *P. pinaster* bark HPE at 323 K, ~20 MPa, with 12.5×10^{-5} kg/s of $scCO_2$ and placing comminuted raw material directly in the extraction cell (◆) or in a cylindrical 120-mesh screen (◇).

For the second step (Figure 9.2.b), where a high pressure CO_2:EtOH (90:10) homogeneous mixture was used to extract phenolic compounds, for a given specific mass of solvent the highest yield was also achieved for the lowest flow rate, corresponding to the highest solvent residence time. However, similar yields were achieved for the two higher solvent flow rates, with even slightly superior values for the highest one. This tendency was even more accentuated for the NF-HPE using CO_2:EtOH (90:10) (Figure 9.2.c). The highest flow rate may have favored extract yield at the solvent outlet, when compared to the medium flow rate, by favoring the effective diffusion coefficient which increases with the increase in fluid velocity (Brunner, 1994). Moreover, it may have promoted the faster depletion of extracted compounds in the bulk of the fluid which, in turn, caused a higher concentration gradient between the interior of the cell and the solvent bulk and, consequently, a higher mass transfer rate. In addition, at the highest flow rate, the solvent may have disrupted the boundary layer surrounding pine bark particles, thereby reducing external mass transfer resistance and favoring extraction yield for a particular mass of solvent.

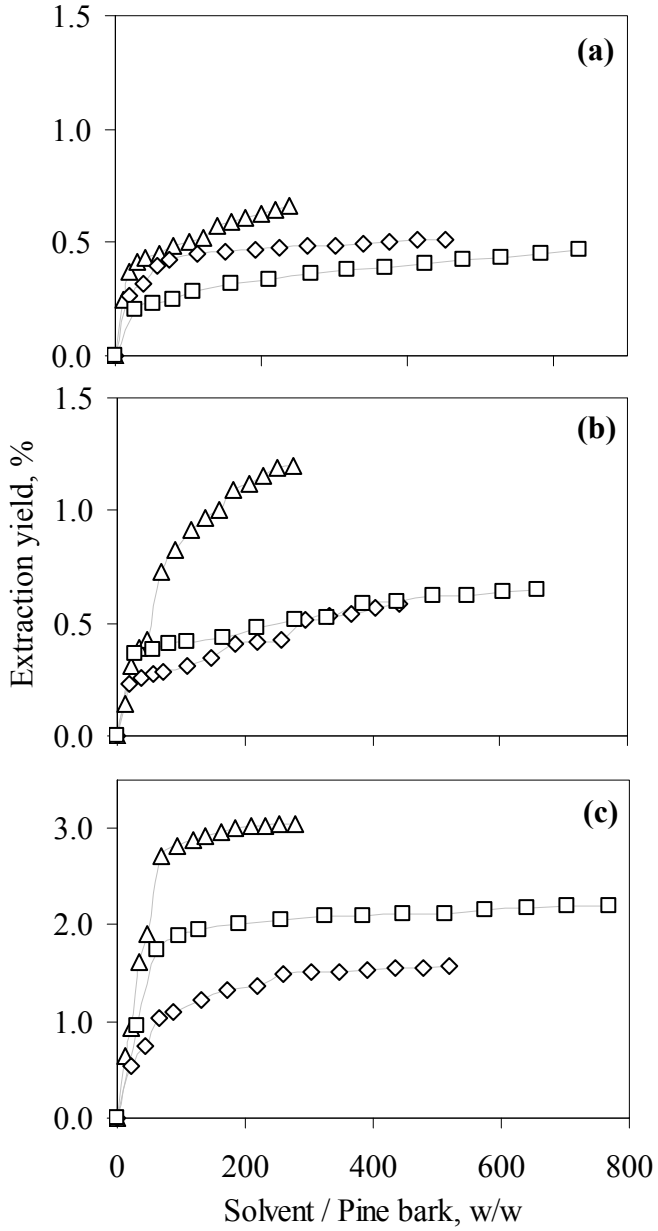


Figure 9.2. Overall curves of *P. pinaster* bark HPE using three different flow rates (Δ low, ◊ medium and □ high): **(a)** F-HPE - first step under the conditions of 323 K and ~20 MPa and using $scCO_2$, **(b)** F-HPE - second step under the conditions of 303 K and ~25 MPa and using CO_2:EtOH (90:10, v/v), and **(c)** NF-HPE at 303 K and ~25 MPa, using as solvent CO_2:EtOH (90:10, v/v).

Considering the higher extraction yield reached for the lowest solvent flow rate, it was chosen to perform NF-HPE varying the composition of EtOH in the range 30-90% (v/v). Figure 9.3 illustrates the corresponding overall extraction curves, where the one corresponding to CO_2:EtOH (90:10) (represented in Figure 9.2.c) was also included for comparison. Under the fixed conditions of temperature and pressure used in these extraction experiments (303 K and 25 MPa), the diverse applied CO_2 and EtOH mixtures were in homogeneous phases below the corresponding supercritical points (Secuianu et al., 2008). The diverse EtOH concentrations led to different polarities of the solvent mixtures and, therefore, were capable to extract different target compounds. The extractions performed with 30% and 50% of EtOH originated similar kinetic curves, achieving identical extraction yields

(~4.1%), higher than the one corresponding to 10% of EtOH (3.0%). The kinetic curves corresponding to 70% and 90% of EtOH almost overlapped, reaching higher extraction yields (respectively 6.4±1.2% and 5.8%) when compared to the ones that used less EtOH.

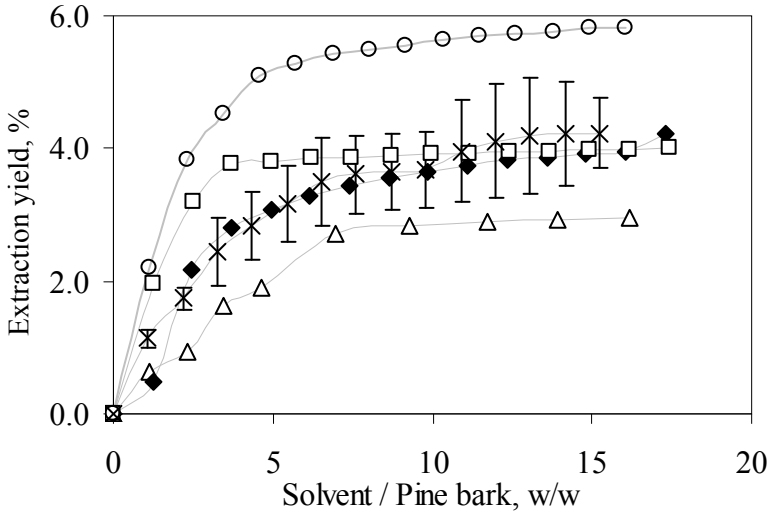


Figure 9.3. Overall extraction curves of HPE of *P. pinaster* bark at 303 K and ~25 MPa, at a solvent flow rate of ~7.5×10^{-5} kg/s and diverse EtOH volumetric compositions (%) in the solvent mixture: △ 10, ◆ 30, □ 50, × 70 and ○ 90.

The F-HPE and NF-HPE overall extraction curves (Figures 9.2 and 9.3) exhibited the typical constant extraction rate period (CER), the falling rate period (FER) and the diffusion controlled rate period (DP). Table 9.3 reports the calculated kinetic parameters (M_{CER}, Y_{CER} and t_{CER}), total yields corresponding to the CER period, R_{CER}, and total yields obtained during the complete extraction processes (which include the extract fractions recovered in the glass and trap flasks, adsorbent column and tubing line cleaning).

In general, fractionation and solvent flow rate influenced kinetic parameters. For the F-HPE first step, $scCO_2$ flow rate and extract solubility in the CER period (Y_{CER}) were inversely related, indicating the non-saturation of the solvent for high flow rates and that internal mass transfer is governing extraction rate, as already discussed. On the other hand, for the second step, extract solubility (1.00-1.31×10^{-4}, w/w) from a previously $scCO_2$-extracted raw material was almost independent of the solvent flow rate indicating that, at the beginning, extraction rate was mainly limited by the solubility of the extracted substances in the solvent phase. This situation lasted until the depletion of these substances from the lower portion of the raw material bed and the consequent non-saturation of the solvent at the cell outlet.

Table 9.3. Kinetic parameters of fractionated and non-fractionated HPE of *P. pinaster* bark, and total yields achieved for high pressure and conventional extractions.

Extraction methodology			M_{CER} ×10^8 (kg/s)	Y_{CER}×10^4	t_{CER} /60 (s)	R_{CER} (%, d.b.)	Fitting error [a]	Total yield (%, d.b.)
	Q×10^5 (kg/s)	Solvent						
F-HPE	6.9	$scCO_2$	1.75	2.53	24	0.38	0.01	1.37
F-HPE	12.4	$scCO_2$	1.74	1.40	22	0.39	0.02	0.97
F-HPE	17.3	$scCO_2$	0.78	0.45	32	0.25	0.05	1.12
F-HPE	8.0	CO_2:EtOH (90:10)	0.80	1.00	106	0.79	0.11	1.22
F-HPE	12.1	CO_2:EtOH (90:10)	1.54	1.27	16	0.25	0.01	0.77
F-HPE	18.3	CO_2:EtOH (90:10)	2.39	1.31	16	0.39	0.02	0.65
NF-HPE	7.7	CO_2:EtOH (90:10)	3.17	4.11	85	2.7	0.26	3.08
NF-HPE	14.0	CO_2:EtOH (90:10)	2.14	1.53	51	1.1	0.31	1.57
NF-HPE	20.8	CO_2:EtOH (90:10)	5.64	2.71	33	1.9	0.12	2.27
NF-HPE	7.6	CO_2:EtOH (70:30)	6.51	8.24	49	3.3	0.61	4.48
NF-HPE	7.6	CO_2:EtOH (50:50)	10.4	13.0	34	3.7	0.45	4.06
NF-HPE	7.6	CO_2:EtOH (30:70)	15.9±3.4	22.1±5.2	37±1.5	5.7±1.2	0.71±0.03	6.51±1.20
NF-HPE	7.6	CO_2:EtOH (90:10)	9.83	13.3	48	4.8	1.39	5.86
SoE	-	EtOH	-	-	-	-	-	6.58±0.4
HD	-	H_2O	-	-	-	-	-	0.014±0.003

For the NF-HPE performed using CO_2:EtOH (30:70), standard deviations are of four extraction experiments.

[a] $$\text{Fitting error} = \frac{1}{N}\sqrt{\sum\left(\frac{y^{calc} - y^{obs}}{y^{obs}}\right)^2}$$

Comparing F-HPE to NF-HPE using the same solvent mixture (CO_2:EtOH (90:10)) and with similar flow rates (~8-21×10^{-5} kg/s), the presence of different substances in the raw material led to higher solubilities (1.53-4.11×10^{-4}, w/w), that were also influenced by the solvent mixture flow rate. For these experiments, Y_{CER} decreased in the following order: low > high > medium flow rates. As expected, a lower flow rate implied longer residence time per weight unit of solvent and hence more favorable diffusive behavior through the matrix of bark. On the other hand, when using the highest flow rate, the solvent may have swept the boundary layer surrounding pine bark particles which, in turn, may have contributed to higher extracted compounds solubility in the solvent mixture. This behavior was previously discussed when presenting Figure 9.2.

The duration of the CER period, t_{CER}, was also affected by the employed solvent flow rate, especially for those experiments that used CO_2:EtOH (90:10). For these extractions (both fractionated and non-fractionated) considerable higher values were achieved for the lowest mass flow rate (106 min and 85 min, respectively), which corresponded to higher R_{CER} values and total yields. Channeling through the bed at higher flow rates may have contributed to the

observed lower extraction yields. The high duration of the CER period achieved in this condition may be a drawback, especially for the F-HPE second step, for which the increment in the solvent flow rate resulted in a decrease in t_{CER} from 106 min to 16 min. This tendency was not so marked for the NF-HPE (t_{CER} decreased from 85 min to 33 min).

Total yields obtained during the NF-HPE that used CO_2:EtOH (90:10) were higher than F-HPE second step yields (Table 9.3) using the same solvent mixture. They were even higher than the sum of total yields of the first and second steps, for the two extreme flow rates, which were 2.59 and 1.77%, for ~7×10^{-5} and ~18×10^{-5} kg/s, respectively.

Considering the solvent composition effect on M_{CER} and Y_{CER} obtained for NF-HPE using 7.6×10^{-5} kg/s of solvent flow, the increment in the EtOH percentage from 10 to 70% had a positive influence on these kinetic parameters. Y_{CER} varied in the range 4.1×10^{-4} to $22.1\pm5.2\times10^{-4}$. The higher solubility achieved with the higher EtOH amount in the solvent mixture should be related with the existence of more compounds soluble in the employed solvent mixture which were readily available for extraction (and where phenolic compounds may be included). R_{CER} values and global yields followed the same behavior, while t_{CER} values followed approximately the opposite behavior. Therefore, the choice of CO_2:EtOH (30:70) as solvent mixture seems to be the most appropriate one to obtain the highest extract amount in the shortest time, from pine bark at 303 K and ~25.4 MPa, at the employed flow rate.

Extract yield recovered in the glass flask, the trap and lines cleaning process represented, in average, 88.3, 2.0 and 9.7% of the total obtained extract, in that order.

Comparing the F-HPE first step ($scCO_2$) yield with that of HD (0.010±0.005% yield), it can be concluded that supercritical fluid extraction is an efficient process for pine bark low polarity compounds extraction. Soxhlet extract yield was 6.85±0.4%, close to the one achieved with NF-HPE using 70% of EtOH (6.51±1.20%).

The work developed in this paper was based on a previous work performed by authors (Braga et al., 2008). In that work, pine bark was subjected to fractionated supercritical fluid extractions where pressure and temperature were varied over the range 303-323 K and 11-30 MPa, using consecutive extraction steps with $scCO_2$ and with CO_2:EtOH (90:10), at a low mass flow rate (~7×10^{-5} kg/s). In that study, the total achieved yield in the first step, at 323 K and 20 MPa (the same conditions of the first extraction step presented in this paper) was 12.2%, which was considerable higher than the one obtained in this work (which was 1.37%). Pine barks used in the two studies came from distinct geographical origins with diverse edaphoclimatic conditions (though both from Beira Litoral, in the center of Portugal). Such

high dissimilarities in extraction yields were certainly related to the dissimilarities in their original bark compositions which were, in turn, related to the tree age, radial location of the collected bark (i.e., phloem, newly formed outer bark or old outer bark), as well as sample position in the tree of the collected bark (i.e., base, middle or top) (Hemingway and Graw, 1976; Karchesy and Hemingway, 1980). Therefore, and when optimizing extraction process conditions using vegetable raw materials from the same species but from different geographical origins, the variability between raw materials should always be considered, despite the expected similarities within the same species.

9.4.2. TLC analysis

The presence of low polarity compounds in the F-HPE extracts was monitored by using anisaldehyde sprayed TLC plates (Figure 9.4.a). The presence of only two zones that migrated with the eluent in the second step extracts (one at Rf 0.05 and the other one at Rf 0.42) indicates that lipophilic compounds were almost completely extracted by $scCO_2$ during the first supercritical step, for the three tested mass flow rates. The lipophilic composition of the $scCO_2$ extracts did not vary with the solvent flow rate: the same zones appeared with Rf values ranging from 0.0 up to 1.0, with a higher incidence of substances with lower retention indexes. These latter substances did not appear in the HD extract, which consisted of the pine bark essential oil, typically obtained by this conventional technique and characterized by low molecular weight compounds. At the employed conditions of temperature (323 K) and pressure (20 MPa) in the F-HPE first step, $scCO_2$ density was 784.29 kg/m^3. At this density, CO_2 has a considerably high solvent power and low selectivity, and thereby several compound families were simultaneously extracted, where fatty acids, fatty acid methyl esters, pigments and other higher molecular weight compounds were possibly included (in addition to the essential oil) (Reverchon et al., 1995). The Soxhlet ethanolic extract also contained some low polarity compounds, with a composition analogous to the one of the first step $scCO_2$ extracts. However, a strong zone appeared at the origin, indicating the presence of a high amount of polar compounds, where condensed tannins may be included.

The fractionation effect on the low-polarity composition profile of the extracts acquired with CO_2:EtOH (90:10) can be visualized in Figure 9.4.b. In this figure, the F-HPE second step extracts were included again for comparison purposes.

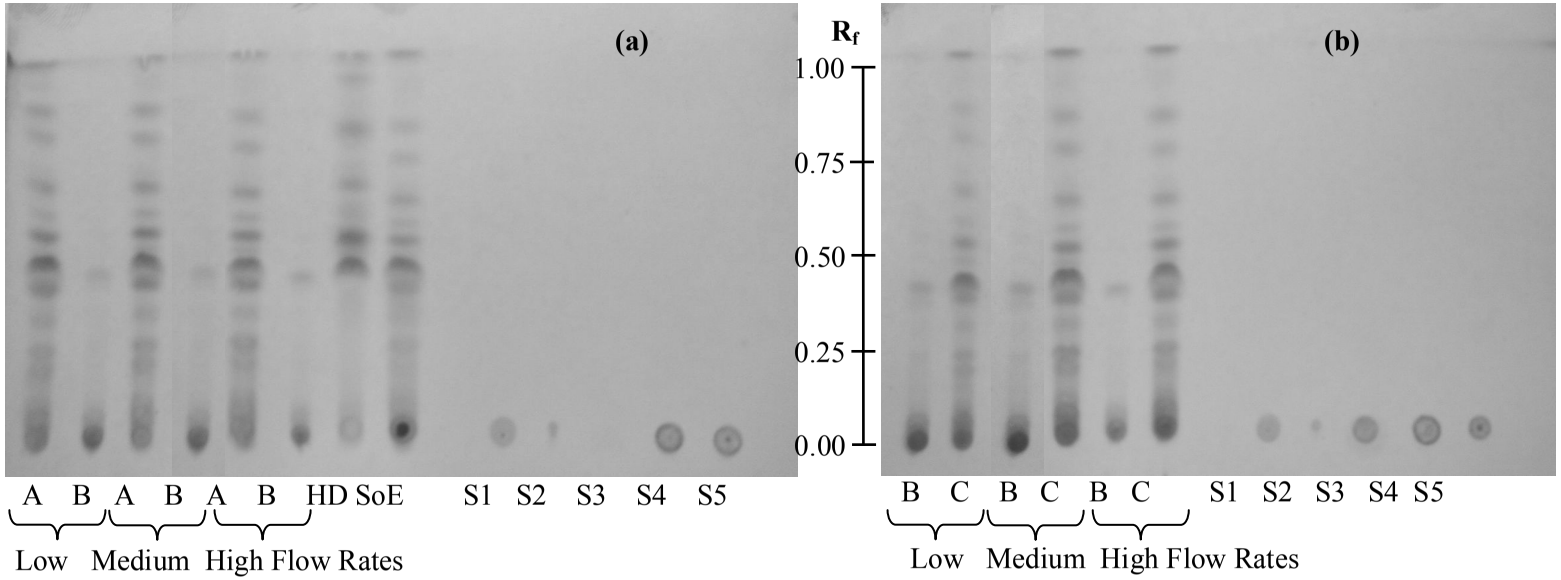


Figure 9.4. Analysis of low polarity compounds by anisaldehyde sprayed TLC plates of: **(a)** first and second steps fractionated high pressure extracts acquired at different flow rates, hydrodistillation and Soxhlet extracts, and **(b)** second step fractionated and non-fractionated high pressure extracts acquired with CO_2:EtOH (90:10) at different flow rates. Extract samples: A -fractionated first step extract; B - fractionated second step extract; C - non-fractionated extract; HD - hydrodistillation extract; SoE - Soxhlet extract. Standards: S1 - quercetin dehydrate; S2 - rutin hydrate; S3 - gallic acid; S4 - catechin hydrate; S5 - epicatechin.

There are several zones that migrated with the eluent in the non-fractionated extracts showing that it was the fractionation methodology (and not the solvent) that avoided the presence of lipophilic compounds in these extracts. The lipophilic composition of these non-fractionated CO_2:EtOH extracts is close to the fractionated $scCO_2$ extract fractions (Figure 9.4.a).

The performed TLC plate for the analysis of phenolic compounds (not shown) in the fractionated and non-fractionated CO_2:EtOH (90:10) and in Soxhlet extracts only permitted to identify the presence of gallic acid. However, there was also another yellow green zone with Rf close to 1.0 that was not identified.

The non-fractionated CO_2:EtOH and the Soxhlet extracts were also analyzed by TLC for screening of condensed tannins (not shown). Catechin and epicatechin, which appeared as red zones at the solvent front, were not identified in these extracts. However, red zones appeared at Rf 0.0 with a higher intensity for the CO_2:EtOH (70:30) and Soxhlet extracts, which suggests the presence of condensed tannins that appear as red colored zones in this TLC analysis (Wagner et al., 1984).

9.4.3. GC analysis

Table 9.4 reports the composition profiles of the essential oil of pine bark isolated by HD and that of the first step CO_2 volatile oil fractions collected in the glass flask, trap and adsorbent column, for the three tested solvent flow rates. At the highest flow rate (17.6×10^{-5} kg/s), the adsorbent column was dragged out with the exiting gaseous CO_2, the adsorbed volatile oil was lost and therefore it was not included in the GC analysis. Figure 9.5 illustrates the GC chromatograms of first step $scCO_2$ extract fractions obtained at 323 K, 20.5 MPa and 6.9×10^{-5} kg/s recovered in the glass flask and retained in the adsorbent column.

In the HD pine bark essential oil forty two compounds were detected. Among these, 16 compounds were identified and 12 others were identified as being oxygenated sesquiterpenes, totalizing 76% of all the detected substances. Caryophyllene alcohol, a tricyclic sesquiterpenol was the major detected compound (12.2%), followed by elemol (6.2%), a monocyclic sesquiterpene alcohol. Oxygenated sesquiterpenes was the major compounds group (21 compounds, 48.8%). Sesquiterpene alcohols were the major subgroup of oxygenated sesquiterpenes (5 compounds, 18.4%). A cyclic monoterpene (alpha-terpineol), a fatty alcohol (hexadecanol), a diterpenoid (abietatriene) and a diterpene (7-ethenyl-1,2,3,4,4a,4b,5,6,7,8,10,10a-dodecahydro-4a,7-dimethyl-1-methyl phenanthrene) were also present but in much lower amounts.

Table 9.4. Composition profile of the essential oil of pine bark isolated by HD and that of the first step $scCO_2$ volatile oil fractions collected in the glass flask, trap and adsorbent column, for the three solvent flow rates tested.

Kovats Index	Substance	Relative area (%)							
		Extraction Process							
		HD	FHPE-1st step, Low flow			FHPE-1st step , Medium flow			FHPE-1st step, High flow	
			Glass Flask	Trap	Adsorbent Column	Glass Flask	Trap	Adsorbent Column	Glass Flask	Trap
1154	n.i.	-	2.90±0.77	0.92±0.05	tr	1.94±0.51	tr	-	-	0.61±0.06
1187	alpha-terpineol	0.75	0.52±0.15	tr	-	-	-	-	-	tr
1552	**Elemol**	**6.21**	tr	tr	tr	-	-	-	-	tr
1556	Longicamphenylone	0.83	tr	-	-	tr	tr	tr	-	-
1567	**Caryophyllene alcohol**	**12.21**	1.37±0.36	0.98±0.02	0.70±0.12	2.16±0.13	0.88±0.48	tr	-	1.01±0.19
1575	Oxygenated sesquiterpenes	1.76	-	-	-	-	-	-	-	-
1579	Caryophyllene oxide	2.83	-	-	-	-	-	-	-	-
1581	Beta-copaen-4-alpha-ol	2.55	tr	-	-	-	-	-	-	tr
1593	Longiborneol	4.15	-	-	-	-	-	-	-	-
1597	Oxygenated sesquiterpenes	2.42	-	-	-	-	-	-	-	-
1605	Oxygenated sesquiterpenes	2.05	tr	tr	-	-	-	-	-	-
1609	Oxygenated sesquiterpenes	0.91	-	-	-	-	-	-	-	-
1611	1,10-di-epi-cubenol	1.00	-	-	-	-	-	-	-	-
1626	1-epi-cubenol	4.91	0.50±0.10	-	-	-	-	0.78±0.02	-	-
1632	Oxygenated sesquiterpenes	1.56	-	-	-	-	-	-	-	-
1639	epi-alpha-cadinol	2.02	-	-	-	-	-	-	-	-
1643	Alpha-muurolol	2.87	-	-	-	-	-	-	-	-
1654	Oxygenated sesquiterpenes	3.24	tr	-	-	0.53±0.03	tr	tr	-	tr
1663	Oxygenated sesquiterpenes	0.87	0.55±0.10	tr	tr	-	-	-	-	-
1668	14-hydroxy-9-epi-(E)- Caryophyllene	5.47	tr	-	-	-	-	-	-	-
1673	Oxygenated sesquiterpenes	5.51	-	-	-	-	-	-	-	-
1718	Oxygenated sesquiterpenes	0.79	-	-	-	-	-	-	-	-
1736	cedr-8(15)-en-9-alpha-ol acetate	0.99	0.50±0.36	tr	-	-	-	-	-	-
1744	Oxygenated sesquiterpenes	0.87	-	-	-	-	-	-	-	-
1753	Oxygenated sesquiterpenes	2.95	-	-	-	-	-	-	-	-
1764	Oxygenated sesquiterpenes	0.86	0.51±0.06	tr	-	-	tr	tr	-	-
1878	Hexadecanol	0.78	-	-	-	-	-	-	-	-

tr: traces < 0.18; n.i.: non identified substance

Table 9.4. Continuation.

Kovats Index	Substance	Relative area (%)							
		Extraction Process							
		HD	FHPE-1st step, Low flow			FHPE-1st step , Medium flow			FHPE-1st step, High flow
			Glass Flask	Trap	Adsorbent Column	Glass Flask	Trap	Adsorbent Column	Glass Flask	Trap
1900	Oxygenated sesquiterpenes	0.91	tr	-	-	0.54±0.07	tr	tr	-	tr
1929	n.i.	0.75	-	-	-	-	tr	tr	-	-
1980	n.i.	-	2.15±0.36	1.51±0.09	1.03±0.09	1.91±0.12	1.06±0.27	0.84±0.04	-	1.00±0.13
2012	n.i.	1.90	tr	tr	-	-	-	tr	-	-
2040	n.i.	1.61	tr	-	-	tr	0.21±0.03	tr	-	tr
2052	Abietatriene	1.03	1.48±0.58	1.22±0.01	1.49±0.30	-	0.20±0.01	-	-	1.26±0.13
2070	7-ethenyl-1,2,3,4,4a,4b,5,6,7,8,10,10a-dodecahydro-4a,7-dimethyl-1-methyl phenanthrene	2.67	tr	-	-	2.76±0.42	1.18±0.51	1.40±0.10	-	-
2103	n.i.	3.61	-	-	-	-	-	-	-	-
2116	n.i.	3.64	1.01±0.11	0.51±0.01	tr	1.30±0.21	0.58±0.11	0.55±0.08	-	0.56±0.07
2125	n.i.	1.05	-	-	-	-	-	-	-	-
2150	n.i.	-	4.30±0.14	1.93±0.05	2.29±0.21	3.52±0.85	2.41±0.05	1.10±0.03	-	1.39±0.23
2174	n.i.	0.83	tr	tr	0.50±0.01	0.51±0.04	0.55±0.03	tr	-	tr
2198	n.i.	1.06	tr	-	-	-	-	tr	-	tr
2205	n.i.	-	0.92±0.08	1.21±0.36	0.81±0.07	2.35±0.57	1.16±0.01	0.77±0.10	-	0.89±0.11
2232	n.i.	1.54	tr	-	-	-	-	-	-	-
2247	n.i.	4.40	-	-	-	-	-	-	-	-
2264	n.i.	1.55	tr	tr	tr	1.21±0.13	tr	tr	-	tr
2304	n.i.	1.35	8.39±0.15	6.65±0.02	7.83±0.11	9.37±0.64	7.23±0.15	6.93±0.50	-	5.87±1.13
2336	n.i.	0.72	0.71±0.07	0.64±0.12	0.50±0.08	tr	0.72±0.01	tr	-	tr
2336	n.i.	0.72	0.71±0.07	0.64±0.12	0.50±0.08	tr	0.72±0.01	tr	-	tr
2376	n.i.	-	5.52±0.11	4.67±0.07	1.87±0.27	4.03±0.37	5.30±0.10	3.18±0.10	-	4.38±0.74
2445	n.i.	-	25.71±2.32	26.26±0.17	33.25±6.89	22.61±2.54	26.76±0.47	27.26±0.51	3.66±0.92	24.03±4.08
2475	n.i.	-	3.94±0.36	3.52±0.06	2.17±0.88	3.95±0.28	4.45±0.01	3.08±0.01	-	3.25±0.57
2516	n.i.	-	0.99±0.02	2.12±0.07	1.04±0.21	-	-	0.57±0.03	-	-
2534	n.i.	-	tr	-	3.52±0.62	1.19±0.22	2.63±0.03	2.37±0.11	-	3.23±0.33
2582	n.i.	-	1.47±0.44	4.04±0.16	6.32±0.79	1.53±0.31	3.74±0.11	3.84±0.17	-	3.49±0.45

tr: traces < 0.18; n.i.: non identified substance

Table 9.4. Continuation.

Kovats Index	Substance	Relative area (%)								
		Extraction Process								
		HD	FHPE-1st step, Low flow			FHPE-1st step , Medium flow			FHPE-1st step, High flow	
			Glass Flask	Trap	Adsorbent Column	Glass Flask	Trap	Adsorbent Column	Glass Flask	Trap
2691	n.i.	-	0.95±0.39	3.63±0.05	4.56±1.85	-	2.64±0.17	4.59±0.06	-	3.67±0.31
2770	n.i.	-	-	3.61±0.02	3.08±0.18	-	-	-	-	-
2788	n.i.	-	-	-	tr	-	3.16±0.05	2.80±0.14	0.84±0.05	2.82±0.25
3020	n.i.	-	-	tr	0.70±0.34	0.79±0.11	1.19±0.13	1.20±0.19	2.66±0.15	1.30±0.16
3165	n.i.	-	0.57±0.24	1.13±0.45	1.99±0.33	1.66±0.08	tr	1.71±0.03	4.97±0.06	1.39±0.38
3239	n.i.	-	1.07±0.87	3.72±0.08	3.50±0.99	-	tr	3.06±0.38	4.60±0.03	3.01±0.41
3286	n.i.	-	1.20±0.70	0.87±0.41	1.82±0.64	4.28±0.38	3.50±0.06	1.20±0.20	2.73±0.61	1.27±0.47
3325	n.i.	-	-	tr	1.37±0.11	2.18±0.27	0.69±0.06	tr	7.51±1.06	tr
3392	n.i.	-	4.01±0.04	4.03±1.31	2.92±0.26	4.51±0.43	4.38±1.26	3.50±0.04	2.98±0.82	3.79±1.51
3455	n.i.	-	0.99±0.50	tr	tr	1.96±0.61	0.82±0.44	1.11±0.01	7.51±0.15	tr
3530	n.i.	-	0.89±0.61	1.61±0.45	0.99±0.13	-	-	-	9.87±2.17	3.39±0.45
3542	n.i.	-	-	-	1.79±0.04	tr	tr	1.73±0.38	-	-
3567	n.i.	-	1.34±0.02	1.03±0.08	2.00±0.02	2.35±0.59	1.75±0.38	1.28±0.51	1.67±1.22	-
3591	n.i.	-	-	1.76±0.20	0.65±0.13	-	-	-	7.79±3.39	2.00±0.14
3620	n.i.	-	2.42±0.13	0.91±0.30	1.73±0.25	3.52±0.01	2.40±0.14	1.62±0.43	3.64±0.45	5.11±0.50
3633	n.i.	-	4.78±1.08	2.16±0.05	3.01±0.03	-	-	5.00±0.14	-	-
3655	n.i.	-	-	-	1.34±0.18	0.54±0.04	-	1.04±0.02	0.51±0.04	2.37±0.20
3655	n.i.	-	-	-	1.34±0.18	0.54±0.04	-	1.04±0.02	0.51±0.04	2.37±0.20
3675	n.i.	-	-	1.58±0.53	1.29±0.27	4.37±0.18	10.09±1.25	0.59±0.07	tr	4.96±0.82
3695	n.i.	-	0.98±0.25	-	1.11±0.26	tr	1.25±0.19	0.83±0.12	8.21±2.83	1.16±0.03
3707	n.i.	-	-	-	0.55±0.21	tr	-	3.16±0.26	-	-
3721	n.i.	-	2.97±1.75	4.04±0.29	0.76±0.14	2.21±0.83	-	0.62±0.02	10.69±3.15	3.42±0.07
3736	n.i.	-	-	2.83±0.02	1.72±0.71	2.36±0.04	1.50±0.03	1.03±0.01	-	-
3763	n.i.	-	tr	0.51±0.07	0.83±0.07	1.81±0.61	2.15±0.38	-	4.89±0.98	0.62±0.06
3837	n.i.	-	1.73±0.89	3.23±0.50	8.66±0.77	0.68±0.05	1.91±0.20	6.19±0.84	13.72±4.49	2.09±0.31
	Identified, %	*76.0*	*5.4*	*2.2*	*2.2*	*6.0*	*2.4*	*2.2*	*0.0*	*2.3*
	Non-identified, %	*24.0*	*94.6*	*97.8*	*97.8*	*94.0*	*97.6*	*97.8*	*100.0*	*97.7*
	Total number of compounds detected	*42*	*63*	*56*	*56*	*45*	*46*	*56*	*20*	*55*

tr: traces < 0.18; n.i.: non identified substance

Most of the substances detected in the pine bark essential oil were not detected in the analyzed $scCO_2$ (volatile oil) fractions. As mentioned before, at the experimental conditions applied in the F-HPE first step, $scCO_2$ had a considerable high solvent power towards all low polarity compounds present in the vegetable matrix due to its high density (784.29 kg/m^3). Therefore, higher molecular weight substances, presenting higher Kovats indexes were also extracted when compared to the essential oil substances. The almost complete absence of the essential oil lower molecular weight substances may be explained by a deficient recovering of these substances from the exiting gaseous CO_2 in the glass flask, trap and adsorbent column. This situation may also explain that, with the raise in the solvent flow rate: (i) the number of substances detected by GC analysis decreased, *(ii)* substances with lower Kovats indexes, corresponding to more volatile substances, not so easily precipitated from a high flow rate CO_2 stream, were less represented in the volatile fractions, and *(iii)* the number of substances retained in the trap (56, 46 and 55 for low, medium and high flow rates, respectively - Table 9.4) increased relatively to the ones retained in the glass flask (63, 45 and 20 for low, medium and high flow rates, respectively - Table 9.4). Therefore, and considering the achieved corresponding total yields and extraction kinetics (Figure 9.2.a, Table 9.3), the lowest flow rate (6.9×10^{-5} kg/s) may be considered as the most appropriate condition to recover the volatile oil from pine bark in the employed extraction apparatus and at the applied particular conditions of temperature and pressure. Nevertheless, the recovering efficiency is a parameter of maximum importance and should be considered for future process optimization.

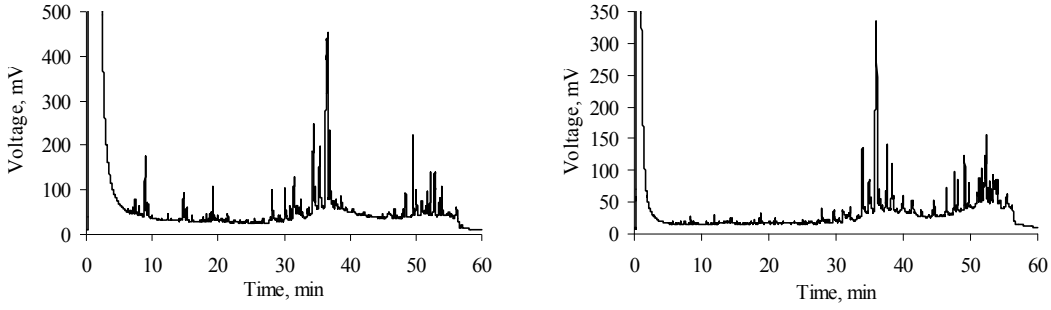


Figure 9.5. GC chromatograms of *P. pinaster* bark fractionated first step $scCO_2$ extracts obtained at 323 K, 20.5 MPa and 6.9×10^{-5} kg/s: **(a)** extract recovered in the glass flask; **(b)** extract retained in the adsorbent column.

9.4.4. Total phenolic compounds and condensed tannins contents

Table 9.5 reports total phenolic compounds and condensed tannins contents of the CO_2:EtOH F-HPE and of the NF-HPE extracts, as well as those of the Soxhlet ethanolic extract.

Table 9.5. Total phenolic compounds and condensed tannins contents of *P. pinaster* extracts obtained by F-HPE and NF-HPE, and oxidation inhibition of extracts obtained by NF-HPE.

Extraction methodology			Phenolic compounds	Condensed tannins	Oxidation inhibition (%)					
	$Q \times 10^5$ (kg/s)	Solvent (v/v)	(% GAE)	(% CME)	1 h	2 h	3 h	4 h	5 h	6 h
F-HPE	~7.5		3.3±0.2	1.2±0.07	-	-	-	-	-	-
F-HPE	~12.0	CO_2:EtOH (90:10)	2.4±0.1	1.3±0.06	-	-	-	-	-	-
F-HPE	~18.0		8.2±0.5	4.0±0.1	-	-	-	-	-	-
NF-HPE	~7.5		3.5±0.1	1.0±0.07	40.7±1.7	40.9±1.6	35.6±2.6	32.0±1.1	28.7±1.8	27.0±2.4
NF-HPE	~12.0	CO_2:EtOH (90:10)	2.1±0.2	1.1±0.05	40.6±1.8	36.7±1.3	34.5±1.1	30.4±0.7	27.9±1.0	27.3±0.9
NF-HPE	~18.0		3.3±0.1	1.1±0.06	35.4±1.9	34.3±0.9	33.7±1.2	35.0±0.8	34.6±1.1	34.4±1.2
NF-HPE	7.6	CO_2:EtOH (70:30)	10.5±0.5	3.5±0.3	34.5±1.0	36.0±0.9	35.7±1.0	35.1±0.7	34.6±0.8	33.9±0.7
NF-HPE	7.6	CO_2:EtOH (50:50)	17.5±1.5	9.0±0.8	74.1±2.0	79.9±0.6	77.1±0.4	74.7±0.2	72.7±1.0	71.0±1.4
NF-HPE	7.6	CO_2:EtOH (30:70)	25.6±2.0	19.8±0.3	78.7±2.3	75.7±2.1	71.5 ±1.5	67.5±1.8	64.0±0.6	61.6±1.3
NF-HPE	7.6	CO_2:EtOH (10:90)	15.0±1.5	9.3±0.4	41.4±3.3	46.4±1.4	43.0±1.7	40.7±2.1	37.6±1.9	36.1±1.4
SoE	-	EtOH	26.0±0.4	18.2±0.6	75.2±4.1	72.0±3.9	68.3±3.2	64.8±2.3	62.4±1.5	60.6±2.2

Experimental results are presented as average values ± standard deviations of three replicates, for total phenolic compounds and condensed tannins contents and four replicates for antioxidant activity values. For the NF-HPE performed using CO_2:EtOH (30:70), standard deviations are of four extraction experiments.

For the two lower solvent flow rates, total phenolic compounds and condensed tannins contents (2.1-3.5% GAE and 1.0-1.2% CME, respectively) were approximately the same for the fractionated and non-fractionated extracts, with little influence of solvent mixture flow rate. However, when the highest solvent flow rate was applied, the obtained extract from a raw material previously extracted with $scCO_2$ had considerably higher total phenolic compounds and condensed tannins contents (8.2% GAE *vs* 3.3% GAE and 4.0% CME *vs* 1.1% CME for fractionated and non-fractionated extracts, respectively). The particular mass transfer conditions associated with high solvent flow rates (such as high effective diffusion coefficients and low external mass transfer resistances) together with possible chemical and physical changes of the vegetable matrix (that occurred during the first step $scCO_2$ extraction) may have influenced high molecular weight compounds solubility and diffusivities which led to the particular extract compositions achieved in the F-HPE second step.

The CO_2:EtOH solvent mixture composition had a considerable influence on total phenolic compounds and condensed tannins contents of non-fractionated *P. pinaster* extracts, varying in the range 3.5-25.6% GAE and 1.0-19.8% CME, respectively. Extracts contents and EtOH amount in the solvent mixture were positively related for EtOH compositions in the range 10-70%, decreasing afterwards. In this range, a linear correlation was observed for total phenolic compounds contents and a quadratic one was observed for condensed tannins (Figure 9.6). For both correlations, R^2 was 0.9987. The observed positive effects should be associated with an increase in the solvent mixture density and polarity, with a consequent increase in its capacity of dissolving high molecular weight and polar substances.

The drop in the extract contents that was observed when EtOH amount was increased to 90% (and CO_2 amount was reduced to 10%) seems to indicate that the dissolved CO_2 may also contributed to the observed behavior. In fact, when CO_2 is dissolved in an organic solvent, a gas-expanded liquid is formed presenting enhanced transport properties (Chamblee et al., 2004). Moreover, a decrease in the solvent mixture pH is observed due to the generation of *in situ* alkyl carbonic acid (West et al., 2001). This temporary reduction in the extraction medium pH value may increase cell membrane permeability, leading to higher diffusivities (Norton and Sun, 2008).

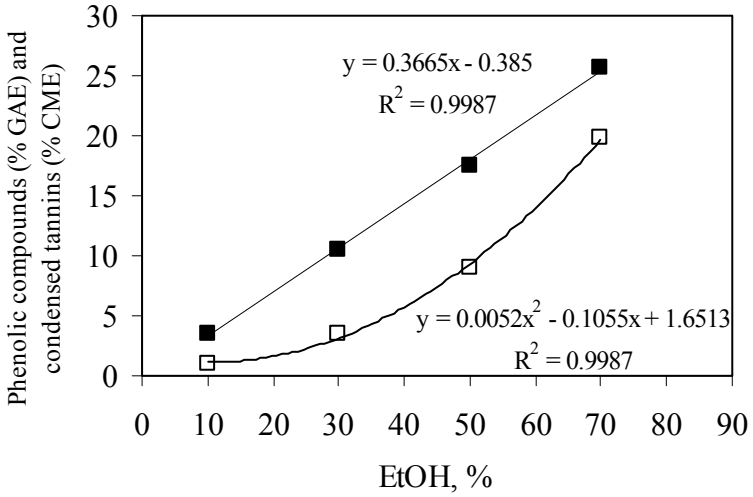


Figure 9.6. Total phenolic compounds (■) and condensed tannins (□) of *P. pinaster* bark non-fractionated high pressure extracts obtained using CO_2:EtOH solvent mixtures with 10, 30, 50 and 70% of EtOH, at 303 K, 25 MPa and 7.6×10^{-5} kg/s.

9.4.5. HPLC analysis

Reversed-phase HPLC analysis was performed based on the work of Karonen et al. (2004), who isolated condensed tannins from pine bark crude extract into three fractions of increasing degree of polymerization. These fractions were characterized using HPLC with DAD and ESI-MS. While an extremely good separation of individual condensed tannins from monomers to trimers (first fraction) was achieved, the separation of oligomers higher than trimers was not successfully achieved. Indeed, oligomers from dimers to decamers (second fraction), as well as polymers (third fraction), eluted as broad and unresolved humps at times higher than 15 and 20 minutes, respectively.

Reversed-phase HPLC profiles of the CO_2:EtOH (90:10) F-HPE and NF-HPE extracts (obtained under increasing solvent mixture flow rates) are illustrated in Figure 9.7. Considering that most phenolic compounds in pine bark are condensed tannins (Karonen et al., 2004), these HPLC traces are indicative of the procyanidin profiles in extracts. For all extracts, broad and unresolved humps were also detected at times higher than 35 minutes, indicating the presence of oligomers and polymers. The number and intensity of detected peaks, i.e., the number of compounds and their concentrations in extracts, increased with the solvent flow rate, in particular for the fractionated methodology. Therefore, the higher solvent-to-solid ratios and the favorable mass transfer conditions associated with higher solvent flow rates favored the extraction of condensed tannins, in particular when the raw material was previously extracted with $scCO_2$. The HPLC profile of the NF-HPE extract achieved using the highest flow rate is in good agreement with the quantification of total

phenolic compounds and condensed tannins (Table 9.5) that was higher for this extract when compared to the others.

HPLC profiles of the NF-HPE extracts achieved using diverse CO_2:EtOH solvent mixtures and of the Soxhlet extract are illustrated in Figure 9.8. For EtOH compositions between 10% and 70%, a direct correlation was observed between the EtOH amount in solvent mixture and the number of detected compounds and the peaks intensity in extracts, which appeared essentially as broad and unresolved humps for times higher than 25 minutes. Hence, EtOH favored the solubilization of higher molecular weight compounds (oligomers and polymers, according to Karonen et al., 2004). Two factors may have contributed to this situation: the increase in the solvent mixture density and polarity, and the increase of hydrogen bonds established between EtOH and the abundant polar hydroxyl groups of condensed tannins. These HPLC profiles are in good agreement with the previously reported total phenolic compounds and condensed tannins contents (Table 9.5, Figure 9.6).

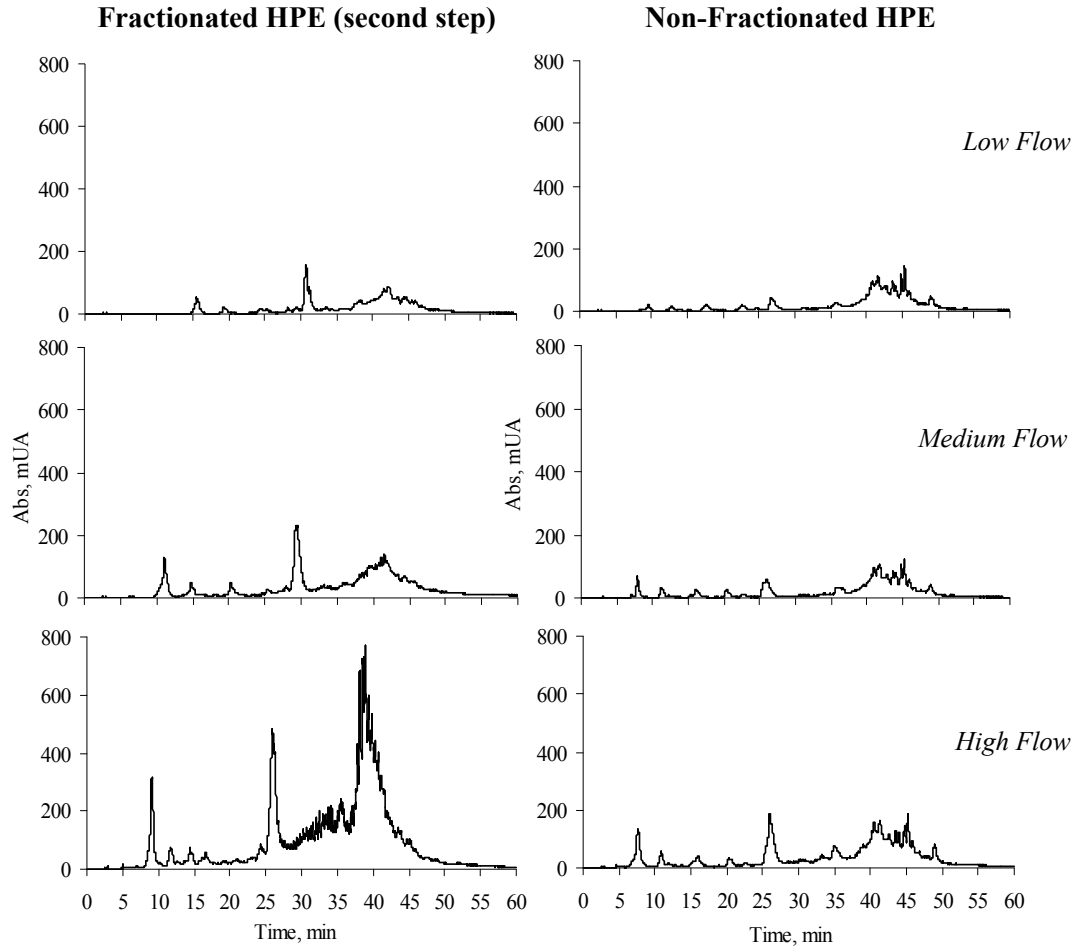
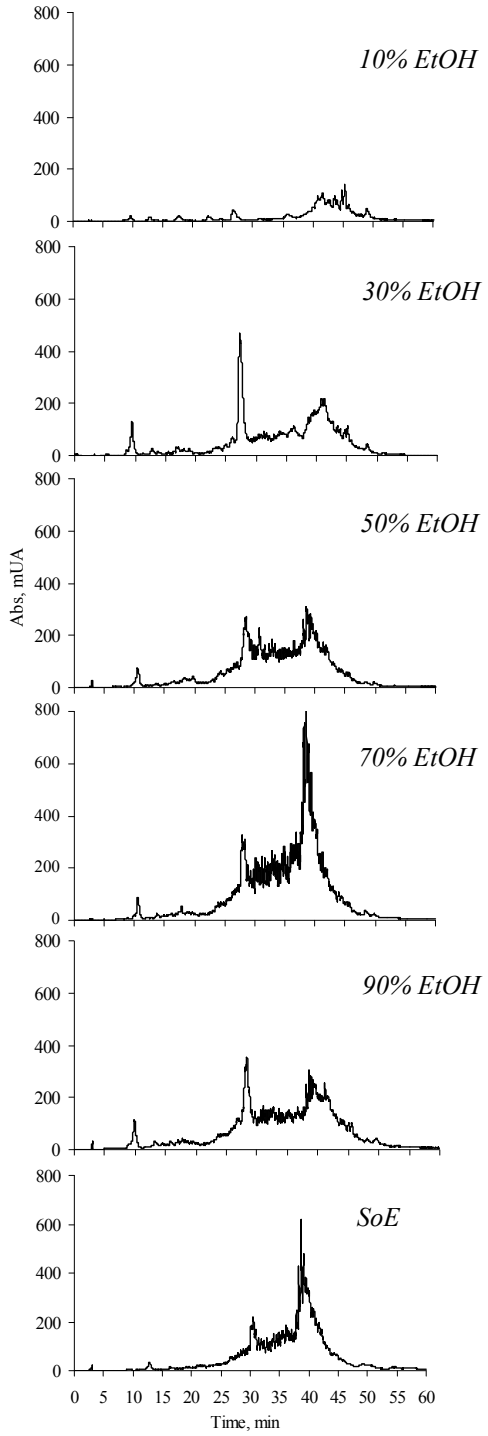


Figure 9.7. Reversed-phase HPLC traces of *P. pinaster* bark F-HPE and NF-HPE extracts obtained using CO_2:EtOH (90:10) solvent mixtures at different flow rates: low (~7.5×10^{-5} kg/s), medium (~12×10^{-5} kg/s) and high (~18×10^{-5} kg/s). Concentration of injected samples was kept constant and equal to 40 mg/mL.



The decrease in peaks intensity that was observed when the amount of EtOH was raised to 90% (and the one of CO_2 lowered to 10%) indicates that CO_2 also played a positive role in the extraction of condensed tannins from pine bark. This positive CO_2 effect is possibly related with the decrease in the solvent mixture pH that is believed to increase cell membranes permeability (leading to higher diffusivities of raw material compounds as already referred), and to shift the equilibrium of condensed tannins towards the solvent medium (Hummer and Schreier, 2008).

HPLC profile of Soxhlet extract is similar to the CO_2:EtOH (30:70) NF-HPE extract, though with lower intensity. However, the quantification of total phenolic compounds and condensed tannins by spectrophotometry revealed similar contents.

Figure 9.8. Reversed-phase HPLC traces of *P. pinaster* bark NF-HPE extracts obtained using CO_2:EtOH solvent mixtures with 10, 30, 50 and 70% of EtOH (at 303 K, 25 MPa and 7.6×10^{-5} kg/s) and of Soxhlet ethanolic extract. Concentration of injected samples was kept constant and equal to 40 mg/mL.

9.4.6. Antioxidant activity results

Oxidation inhibition of NF-HPE and of Soxhlet extracts (measured by the coupled oxidation of β-carotene and linoleic acid) are reported in Table 9.5 for up to 6 hours of analysis. In general terms, oxidation inhibitions decreased between 0.6 and 17.1% during the 6 hour assay.

Oxidation inhibitions of the NF-HPE extracts achieved with 10% of EtOH in the solvent mixture were in the range 27.0-34.4% after 6 hours of assay, with little influence of the solvent mixture flow rate. On the other hand, the oxidation inhibition of the pine bark extract obtained in our previous work (Braga et al., 2008), and using the same experimental conditions of pressure, temperature, solvent composition and solvent flow rate, was 70% after 3 hours of assay. The higher antioxidant activity of that extract may be ascribed to the applied fractionated methodology (pine bark was previously extracted with $scCO_2$) and to the distinct geographical origins of pine barks used, as previously referred when interpreting extraction yield results.

For NF-HPE extracts obtained under the lowest solvent flow rate (7.6×10^{-5} kg/s) higher oxidation inhibitions were achieved when the solvent mixture contained 50 and 70% of EtOH, being 71.0±1.4% and 61.6±1.3% after 6 hours of analysis, in that order (Table 9.5). No direct and clear correlation was observed between the antioxidant activity of these NF-HPE extracts and their phenolic or condensed tannins contents, as well as with their HPLC profiles. Indeed, the extract with the highest antioxidant activity (corresponding to CO_2:EtOH (50:50)) was not the one with the highest content of total phenolic compounds and condensed tannins (corresponding to CO_2:EtOH (30:70)). Moreover, extracts containing similar contents of condensed tannins and total phenolic compounds revealed quite different oxidation inhibition values (the ones corresponding to CO_2:EtOH (50:50) and (10:90)). The opposite was also verified, i.e., there were extracts presenting similar oxidation inhibition values with quite different contents of condensed tannins and total phenolic compounds (such as the ones achieved with CO_2:EtOH (70:30) and (10:90)). The lack of significant correlation between total phenolic content and antioxidant activity of plant extracts has been observed by other authors when studying pine bark and other plant materials extracts (Ku et al., 2007; Zhang and Hamauzu, 2004).

The 6-hour oxidation inhibition of the Soxhlet extract was 60.6±2.2%, close to the one of the NF-HPE CO_2:EtOH (30:70) extract, which was 61.6±1.3%. These two extracts also had similar total phenolic compounds and condensed tannins contents.

9.5. Conclusions

This study focused on the effect of HPE fractionation, solvent flow rate and CO_2:EtOH solvent mixture composition on the extraction kinetics and on the composition of the obtained *P. pinaster* bark extracts. It was verified that these experimental variables are of major

importance when defining HPE operational conditions best suitable to achieve extracts with high contents of phenolic compounds and of condensed tannins (and that simultaneously possess high antioxidant activities). In particular, the CO_2:EtOH solvent mixture composition revealed to be of major importance. The choice of the particular solvent composition CO_2:EtOH (30:70) combines the optimum characteristics of extract composition and extraction kinetic behavior since, as discussed above, this was also the solvent mixture most appropriate to obtain the highest extract amount in the shortest time. Moreover, this NF-HPE condition was able to achieve similar total phenolic compounds and condensed tannins contents to the ethanolic Soxhlet extract ones, which were 26.0% GAE and 18.2% CME (Table 9.5). The high pressure methodology takes advantage of using lower EtOH consumption (70% instead of 100%), lower solvent-to-solid ratio (15:1 instead of 50:1) and lower temperature (303 K instead of EtOH boiling point). These conditions contribute to reducing the effort of removing an organic solvent in the post-extraction preparation steps. Furthermore, the reduced extraction temperature and the oxygen-free environment characteristic of the HPE methodology may be advantages in preserving the quality of an extract that has phenolic compounds which are typically degraded at higher temperatures and in contact with oxygen.

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Part D. Tara (*Caesalpinia spinosa*) seed coat

10. Statistical mixture design investigation of CO_2/ethanol/H_2O pressurized solvent extractions from tara fruit seed coat

In this Chapter, the optimization of the solvent mixture composition applied on the pressurized solvent extractions from tara fruit seed coat is presented. This subject arose under the scope of the Project "Aplicaciones Industriales de los Taninos Vegetales" of "Programa CYTED - Ciencia y Tecnologia para el Desarrollo". It was a result of the contacts established with a Peruvian tara powder and tara gum producer company (Transformadora Agrícola S.A.C.), which is interested in the valorization of a residue that is believed to be highly appropriate for those purposes where antioxidant properties should be relevant. A mixture design study was performed so as to evaluate the effect of a (CO_2/ethanol/H_2O) solvent mixture composition on the yields and characteristics (in terms of composition and bioactivity) of extracts obtained by pressurized solvent extractions. The text that comprises this Chapter will be submitted for publication in a scientific journal after conclusion of the high-performance liquid chromatography analysis, which will allow the quantification of some compounds which are present in the obtained extracts.

10.1. Abstract

Tara (*Caesalpinia spinosa*) fruit seed coat is an abundant Peruvian agro-industrial residue that could be explored as a raw material for the production of valuable phenolic- and antioxidant-rich extracts. In this work, a mixture design was applied to optimize solvent mixtures for selective extraction of phenolic compounds from tara fruit seed coat. Pressurized solvent extractions (PSE) were performed at 313 K and 20 MPa, using homogeneous CO_2, ethanol (EtOH) and H_2O solvent mixtures. Extraction kinetics were obtained and phenolic contents, antioxidant activities (measured by the coupled oxidation of β-carotene and linoleic acid) and anti-inflammatory activities (measured by the lipoxygenase inhibition activity) of

extracts were evaluated. A linear mixture model predicted total yields and it was verified that H_2O was the most effective solvent to obtain high extraction yields. A quadratic model predicted phenolic extracts contents and it was verified that H_2O and CO_2 had an antagonistic effect and that maximum phenolic contents can be expected for EtOH-rich mixtures. A special cubic model predicted anti-inflammatory activity and the maximum activities were also predicted for EtOH-rich mixtures. For the antioxidant activity, no model revealed to be significant (at a 95% confidence level). Nevertheless, high antioxidant activities were observed after 3 hours of assay (above 80% of oxidation inhibition for nine out of eleven extracts) and even after 6 hours of assay (above 70%). The obtained results strongly suggest that tara seed coat is a residue that has a great potential to be valorized for the production of phenolic-rich extracts presenting high anti-inflammatory and antioxidant activities.

10.2. Introduction

Plant cells synthesize a great variety of secondary metabolites which play important roles in plant protection. Scientific studies suggest that the long-term intake of some secondary plant metabolites can have a positive impact on human health. In particular, phenolic compounds, due to their well known and profusely documented antioxidant properties, have health promoting characteristics that contribute to protecting the body against cancer and cardiovascular illnesses (Block et al., 1992; Campbell et al., 2006; Engler and Engler, 2006).

The biggest challenge when extracting phenolic compounds, or other secondary metabolites from plant matrices, is to be able to isolate them with chemical and biological activity, while keeping the extraction process environmentally safe and economically feasible. The success of the extraction process is, therefore, dependent on the chosen specific extraction methodology and on the applied process conditions. Pressurized solvent extraction and supercritical extraction are methodologies that may be attractive to produce extracts for fine applications, like the pharmaceutical ones, being a possible alternative to conventional extraction. The initial high capital investment required may be compensated by the quality/purity of the obtained extracts, since these technologies are able to minimize the co-extraction of undesirable compounds and the extracts contamination with solvent impurities. These advantages are usually related with the usage of supercritical CO_2, whose physicochemical properties are easily tuned with small changes in pressure and temperature, which permit to adjust its solvent power and selectivity. Additionally, supercritical CO_2 extraction is performed in a light- and oxygen-free medium, avoiding oxidation reactions that

may be detrimental to the extract's quality (Díaz-Reinoso et al., 2006). However, the extractability of compounds with supercritical CO_2 depends on the presence of specific functional groups in these compounds, on their molecular weights, and on their polarities. However, most phenolic compounds, which are characterized by the presence of several hydroxyl groups as well as other functional groups, and have high molecular weights, are scarcely soluble in supercritical CO_2.

Pressurized solvent extraction (PSE) which involves the usage of H_2O and/or organic solvents at considerable elevated temperatures (313-473 K) and pressures (3.3-20.3 MPa) represents an attractive option to extract phenolic compounds from plant matrices. It has improved characteristics in terms of mass transfer and of solvating properties (Chamblee et al., 2004) that can even be improved by the utilization of gas-expanded liquids (usually obtained upon the dissolution of CO_2 in H_2O or in an organic solvent). Moreover, the utilization of a mixture of two miscible solvents with different polarities and with different abilities to establish specific interactions with the target compounds is a procedure that can selectively extract substances from plant matrices. The versatility of the solvent mixture may still be improved with the addition of a third solvent. Very frequently, the intermediate mixture of solvents will give maximum solubility (Barwick, 1997).

CO_2, ethanol (EtOH) and H_2O are solvents with different polarities (Barwick, 1997) that, when mixed in different proportions allow obtaining homogeneous solvent mixtures (depending on the particular temperature and pressure and the individual solvents molar fractions) with polarities that go from the one of the less polar CO_2 to the one of the most polar H_2O. Moreover, EtOH is a strong proton-donor and plays an important role in the formation of hydrogen bonding associations with the diverse functional groups of phenolic compounds. The role of the OH group in the phenol-$(H_2O)_n$ cluster can be either proton-donating or -accepting. For small molecules, only the proton-donating OH conformer has been observed. However, as the carbon sidechain gets longer in the aromatic alcohol, the acidity of the hydroxyl group may decrease (Ahn et al., 2003). The role of solvent-solvent interactions in competition for the solvation of solutes is also an important issue to consider (Marcus, 1993). In particular, the H-bond type interactions established between solutes´ functional groups and solvent molecules may become weaker as the amount of CO_2 increases in the ternary mixture, as verified by Sala et al. (2006) when studying solute-solvent interactions governing solvation phenomena of acetaminophen in CO_2-expanded organic solutions. In combination, all these factors may allow the selective extraction of phenolic compounds from plant matrices.

The investigation of the effect of the composition of a ternary mixture in extraction should be addressed by techniques of experimental design for mixtures, also known by mixture design. Besides helping to minimize the number of experiments to be performed and to maximize the amount of acquired information, these designs permit the identification and characterization of synergistic and of antagonistic interaction effects between the different solvents. Extraction studies would beneficiate if this technique were applied more frequently, though some studies had appeared on the investigation of solvent composition through this technique in the last years (Dingstad et al., 2004; Soares et al., 2007, Soares et al., 2009).

Tara (*Caesalpinia spinosa*), a native tree from Peru, produces around 20 to 40 kg of pods per year, that have been used by the local population since the pre-Hispanic era due to their proved medicinal and nutritional benefits (Lapa, 2004). Tara pulp, which represents approximately 62% of the total pod weight, is extremely rich in tannins (40-60%) which are predominantly of the hydrolysable type and which are based on a galloylated quinic acid structure (Haslam and Haworth, 1962), whose main constituent is gallic acid (Galvez et al., 1997). Tara pulp has diverse applications such as in leather tanning (as tara powder, produced by crushing the pod pulp), in the manufacture of plastics and adhesives, in the pharmaceutical industry (as a therapeutic agent for treating conditions likely to be associated with microorganisms) (Lapa, 2004; Kloucek et al., 2005).

Tara seeds consist of 34% of endosperm, 37.5% of germen, and 28% of coat (by weight) (Lapa, 2004). Whereas the ground endosperm is a commercially important stabilizer and thickening agent (approved by the European Union as a food additive, E417), the germen is recovered as a byproduct and is mainly used as animal feed or as a source of protein hydrolysates (Wieling, 2000). Hence, tara seed coat constitutes a residue of these industries. To the best of our knowledge, no scientific study has been published on the chemical composition of tara seed coat. However, considering that in several legumes, such as diverse beans and lentils, phenolic compounds are located essentially in their seed coat (Takahata et al., 2001; Ranilla et al., 2007; Dueñas et al., 2003) and that the whole tara pod has a rich tannin content, there is a strong possibility that tara seed coat is by itself a rich source of phenolic compounds. Therefore, instead of being treated as a residue of environmental concern, tara seed coat could be used to recover natural compounds that could be potentially useful for pharmaceutical, cosmetic and food industries. Besides being an opportunity to improve the economic profit and competitiveness of the involved companies, this strategy would probably be well-accepted by most consumers. Indeed, nowadays consumers tend to

prefer products containing natural ingredients and additives in their formulation, instead of prefer products containing artificial or chemical ingredients.

In this work, a mixture design has been applied to the PSE (at 313 K and 20 MPa) of phenolic compounds from tara seed coat, varying the ternary CO_2/EtOH/H_2O solvent mixture composition. Employed compositions (molar fractions) were picked taking into consideration the utilization of a homogeneous high pressure phase. Extracts phenolic contents and antioxidant and anti-inflammatory activities were evaluated, and statistical mixture models were developed.

10.3. Materials and methods

10.3.1. Raw material

Tara seed coat was provided by Transformadora Agrícola S.A.C. This Peruvian company produces tara powder from tara de-seeded pods for the leather tanning industry, and tara gum from tara seeds endosperm to be used as a thickener and stabilizer in food industry. Tara seed coat is not included in their marketed products and therefore constitutes a residue. Raw material was comminuted using a knife mill (Braun, KSM 2, Germany) and particles having a size distribution between 48 and 24 mesh were separated using sieves under mechanical stirring (Retsch, Germany). The comminuted tara seed coat was conditioned in a plastic bag under regular atmosphere conditions, prior to extraction. Its mean geometric diameter was determined by Light Scattering using a Laser Malvern Mastersizer (Hydro 2000MU, Worcestershire, UK). Raw material humidity was determined by the xylol distillation method of Jacobs (Jacobs, 1973) employing triplicate assays. Real density of tara seed coat particles was determined using an helium gas pycnometer AccuPyc 1330 (Micromeritics Instrument Corporation, USA), with ten replicates. Apparent density of the extraction bed was calculated from the mass used to fill the extraction cell. Bed porosity was defined using the real density of the particles and the apparent density of the bed.

10.3.2. Chemicals

Solvents used for extraction experiments were: carbon dioxide (99.998%), from Praxair (Spain), ethanol (99.5%), from Panreac Quimica S.A. (Spain) and distilled water.

Analytical grade chemicals and solvents employed for extract analyses were ethanol (99.5%), from Panreac Quimica S.A. (Spain), bi-distilled water and: *(i)* Folin-Ciocalteu's phenol reagent, from Merck (Germany) and sodium carbonate, from Pronalab (Portugal), for

total phenols quantification, *(ii)* β-carotene (Type I, ~95% UV), tween 40 and chloroform (99.9%, HPLC grade), from Sigma-Aldrich Inc. (Germany), and linolenic acid (≥98.5%), from Fluka (Switzerland), for the antioxidant activity spectrophotometric experiments and *(iii)* soybean lipoxygenase from BioChemika (Switzerland), linoleic acid (99%), from Aldrich (Germany) and sodium tetraborate (≥99%), from The British Drug Houses Ltd. (England) and hydrochloric acid (37%), from Panreac Quimica S.A. (Spain) (these last two reagents were used to prepare the borate buffer 0.1 M pH 9), for the anti-inflammatory activity spectrophotometric experiments. Standard employed for spectrophotometric analysis was gallic acid (≥98%) from Sigma-Aldrich Inc. (Germany).

10.3.3. Experimental pressurized solvent extractions procedure

Pressurized solvent extractions from tara seed coat were performed using the extraction apparatus described by Braga et al. (2008), with some modifications in the extraction cell. A diffuser was introduced at the cell inlet to guarantee the solvent dispersion through the raw material bed, and a filter was introduced at the cell outlet to avoid raw material particles to be dragged out with the solvent stream. This reduced volume cell (~17.8×10^{-6} m^3) was filled with comminuted tara seed coat and placed inside a controlled-temperature water bath. Initially, a supercritical CO_2 extraction kinetic was performed in triplicate, at 313 K and 20 MPa, to decide if a two-step methodology would be applied in the forthcoming extractions. The solid-to-solvent of this supercritical extraction was 1:65 (w/w, dry basis, d.b.) and its duration was 240 minutes (20 min static period followed by a 220 min dynamic one). Since this CO_2 extraction resulted in an extremely low extraction yield, it was decided to proceed just with one-step PSE experiments at 313 K and 20 MPa, and applying diverse CO_2/EtOH/H_2O mixtures. Molar fractions of CO_2 and H_2O varied between 0.0 and 0.6 and the ones of EtOH varied between 0.4 and 1.0. The solid-to-solvent ratio was 1:48 and extraction duration was 210 minutes (10 min static period followed by a 200 min dynamic one). High pressure liquid pumps were used to deliver the EtOH/H_2O mixtures (L-6200A, Hitachi, Merck, Germany) and the liquefied CO_2 (home-built). Temperature was chosen taking into consideration phase equilibrium correlations as well as thermal sensitivity of phenolic compounds. Extracts were recovered in a flask, placed in an ice bath, every twenty minutes. Dried ethanolic extracts were obtained by vacuum evaporation and dried aqueous extracts were freeze dried. Finally, they were stored at approximately 263 K until further analysis.

10.3.4. Characterization of tara seed coat extracts

10.3.4.1. Quantification of total phenolic compounds

Total phenolic compounds in tara seed coat extracts were quantified according to the Folin-Ciocalteu's method, following the procedure proposed by Singleton and Rossi (1965) with some modifications based on Cheung et al. (2003). Extracts solutions were prepared using ethanol (10%, v/v) and up to 0.5 mL aliquots were reacted with the Folin-Ciocalteu's reagent. Saturated Na_2CO_3 (~17%) and distilled water were added and the reaction was kept in the dark for 90 min, after which absorbances were recorded at 725 nm using a UV/VIS spectrophotometer (Jasco V-530, Japan). Results were expressed as gallic acid equivalents (GAE) in percentage (mg GAE/mg extract × 100, d.b.).

10.3.4.2. Antioxidant activity: β-carotene and linolenic acid coupled reaction assay

The antioxidant activity of extracts was determined by the coupled oxidation of β-carotene and linolenic acid (Hammerschmidt and Pratt, 1978). The reaction was continuously monitored during 6 hours by taking absorbance readings of extract and control (the reaction medium without extract) at 470 nm, every 5 minute. Antioxidant activities were expressed as oxidation inhibition (Equation 10.1):

$$\text{Oxidation inhibition (\%)} = \left[1 - \frac{\text{Abs}_{\text{extract}}^{t_0} - \text{Abs}_{\text{extract}}^{t_n}}{\text{Abs}_{\text{control}}^{t_0} - \text{Abs}_{\text{control}}^{t_n}}\right] \times 100 \qquad \text{(Equation 10.1)}$$

where Abs^{t_0} is the absorbance reading at the beginning of reaction and Abs^{t_n} is the corresponding one at t_n ($0\ \text{h} < t_n \leq 6\ \text{h}$).

10.3.4.3. Anti-inflammatory activity: lipoxygenase assay

The lipoxygenase inhibition activity of tara seed coat extracts was measured in order to evaluate their anti-inflammatory activities. Therefore, the loss of soybean lipoxygenase activity was measured in the presence of three concentrations of extract, using linoleic acid as the substrate. The enzyme and substrate solutions were prepared by dissolving lipoxygenase and linoleic acid in potassium borate buffer to achieve 1.25×10^{-2} mg/mL and 100 µM, respectively. The concentrations of the three prepared extract solutions (by dissolving in ethanol) depended on the extract's inhibition activity, and were chosen in order to allow interpolation for the calculation of IC_{50}.

Aliquots (15 µL) of tara seed coat extracts solutions were added to 3.0 mL of substrate solution, and the mixture was then stirred. The enzymatic reaction was initiated by the

addition of 75 µL of the lipoxygenase solution. A control run was also performed using ethanol instead of the inhibitor. Kinetic absorbance measurements were taken at 234 nm, during 15 minutes at 293 K. The initial reaction rate (V_i) was determined from the slope of the straight line portion of the curve. The calculation of the percentage inhibition of the enzyme activity was based on the comparison between the samples being analyzed and the control sample using the following equation:

$$\text{Inhibition, \%} = \frac{V_{i,control} - V_{i,inhibitor}}{V_{i,control}} \times 100 \qquad \text{(Equation 10.2)}$$

where $V_{i,control}$ and $V_{i,inhibitor}$ are the initial reaction rates corresponding to the control and extract, in that order.

Each inhibitor concentration was tested in quadruplicate and the concentration that gave 50% inhibition (IC_{50}) was interpolated from the outline of the inhibition percentage reported as a function of the inhibitor concentration. Gallic acid and dexamethasone were also assayed for comparison.

10.3.5. Kinetic parameters calculation

Total extraction yields were calculated as the ratio between the total extract mass (the sum of all fractions collected during the extraction kinetic) and the raw material mass, on a dry basis. The overall extraction curves were set up considering the cumulated mass of extract collected at a pre-defined time interval and were fitted by a curve formed by two straight lines. The fitting was done by minimizing the least regression error in the least squares sense, using the fminsearch function of Matlab (R2007a). The first line was identified with the constant extraction rate period (CER) and the corresponding kinetic parameters were calculated according to Rodrigues et al. (2002): *(i)* mass transfer rate for the constant extraction rate period, M_{CER}; *(ii)* mass ratio of solute in solvent phase at measuring-cell outlet, Y_{CER}, and *(iii)* duration of the constant extraction rate period, t_{CER}.

10.3.6. Experimental design and statistical analysis

In order to evaluate the influence of the CO_2/EtOH/H_2O solvent mixture composition on the chemical composition and on the antioxidant and anti-inflammatory activities of tara seed coat extracts, an experimental design was considered to take into account quadratic responses and interactions based on CO_2, EtOH and H_2O molar fractions. EtOH molar fraction values were chosen higher than 0.4 (and consequently the ones of CO_2 and H_2O were lower than 0.6) so as to obtain homogeneous high pressure mixtures (Durling at al., 2007). The experimental

design, which is illustrated in Figure 10.1, included three vertices, three center of edges, three axial check blends, one interior check blend, plus one overall centroid. The number of randomized experiments was 11 plus one replication of each of the three vertices, totalizing 14 extraction experiments.

Linear, quadratic and special cubic mixture models were used to fit total extraction yield (Y), total phenols (TP), 6-hour oxidation inhibition (6-h OI) and IC_{50} of tara seed coat extracts. The special cubic model, which includes linear, quadratic and cubic components, is represented by Equation 10.3:

$$Y = \sum_{i=1}^{3} b_i x'_i + \sum_{i=1}^{3}\sum_{j\neq i}^{3} b_{ij} x'_i x'_j + \sum_{i=1}^{3}\sum_{j\neq i}^{3}\sum_{k\neq j}^{3} b_{ijk} x'_i x'_j x'_k + \varepsilon \qquad \begin{cases} x'_i \geq 0 \\ \sum_{i=1}^{3} x'_i = 1 \end{cases} \qquad \text{(Equation 10.3)}$$

where *bi*, *bij* and *bijk* are regression coefficients calculated from the experimental data by multiple regression.

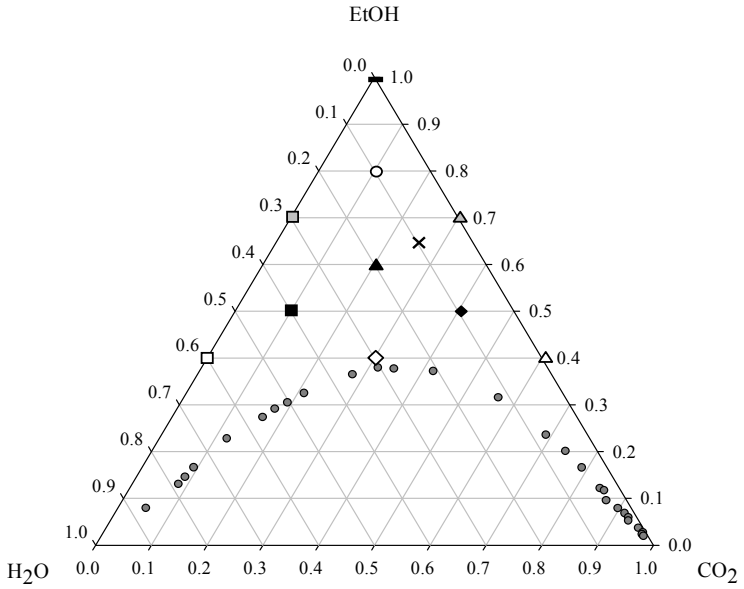

Figure 10.1. Representation of the assayed solvent mixtures with diverse CO_2-EtOH-H_2O molar fractions: □ (0.0-0.4-0.6), ◇ (0.3-0.4-0.3), △ (0.6-0.4-0.0); **(b)** ■ (0.1-0.5-0.4), ▲ (0.2-0.6-0.2), ◆ (0.4-0.5-0.1), × (0.25-0.65-0.1), ○ (0.1-0.8-0.1); **(c)** ▩ (0.0-0.7-0.3), ◬ (0.3-0.7-0.0), ▬ (0.0-1.0-0.0), and of experimental phase equilibrium values for the ternary system at 313 K and 20 MPa as determined by Durling et al. (2007) (●).

To allow for scale-independent comparisons of the parameter estimates, the component settings were recoded to so-called pseudo-components:

$$x'_i = \frac{x_i - L_i}{\text{Total} - L} \qquad \text{(Equation 10.4)}$$

Here, x'_i stands for the *i*'th pseudo-component, x_i stands for the original component value, L_i stands for the lower constraint for the *i*'th component, L stands for the sum of all lower constraints for all components in the design, and *Total* is the mixture total.

For each response variable, the analysis of variance tables were generated for the three models. The significance of the regression models was evaluated by considering their F-values and their p-values. Subsequently, the acceptance of the model depended on the lack of fit test, which compares the residual error, i.e., the error associated with the fitted model, to the pure error from replication. If residual error significantly exceeds pure error, the model shows significant lack of fit. Therefore, the regression models were accepted when the F-values of the model were higher than the corresponding critical F-values and the F-values of the lack of fit were lower than the corresponding critical F-values. Alternatively, the models were accepted when the p-values of the models were lower than 0.05 (ideally lower than 0.001) and the p-values of the lack of fit higher than 0.05. The regression coefficients of the most significant model of each response variable were determined and the significances of all terms were judged statistically by computing their F-values at a p-value of 0.05. The regression coefficients were then used to make statistical calculations to generate contour maps from the regression models. Statistica software was used to design the experiments and to model and to analyze the results.

10.4. Results and discussion

The humidity of tara seed coat was 4.9±0.5% (w/w, d.b.). The particles mean diameter was 960±6 μm and their real density was $1.5\pm0.004\times10^3$ kg/m^3. Considering that the bed apparent density was $7.0\pm0.9\times10^4$ kg/m^3, its porosity was then 0.47±0.08.

10.4.1. Pressurized solvent extraction kinetics

A preliminary supercritical CO_2 extraction of tara seed coat was performed, at 313 K and 20 MPa, in order to evaluate if a two-step extraction methodology (where the CO_2/EtOH/H_2O PSE would be the second step) would be justified. The corresponding overall kinetic curve is illustrated in Figure 10.2. The obtained quite low extraction yield (0.07±0.006%) was the criteria chosen to not perform this previous supercritical CO_2 extraction. Considering that the raw material did not include the internal seed tissues, this was not a surprising result since those tissues comprise the plant reservoir which is mainly comprides by carbohydrates and lipids.

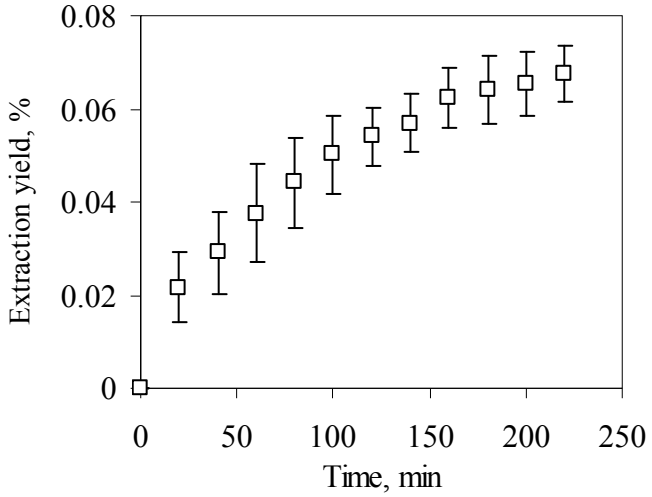

Figure 10.2. Overall curve of supercritical CO_2 extraction from tara seed coat at 313 K and 20 MPa.

Overall PSE curves of tara seed coat are represented in Figure 10.3. In this figure, extraction yields were reported as a function of the specific mass of solvent used, i.e., mass of solvent per mass of tara seed coat, on a dry basis.

Curves exhibited the typical constant extraction rate (CER) period, the falling rate (FER) period and the diffusion controlled rate period (DP), indicating a nearly complete exhaustion of the extraction bed. Table 10.1 reports the calculated kinetic parameters and extraction yields corresponding to the CER period (M_{CER}, Y_{CER}, t_{CER} and R_{CER}), as well as the extraction yields obtained during the complete extraction process. Solvent composition affected both extraction kinetics and yields, which indicates the probable selectivity of the solvent mixture towards different tara seed coat substances.

The two extraction experiments performed with H_2O plus EtOH and no CO_2 (CO_2-EtOH-H_2O molar fractions of (0.0-0.4-0.6) and (0.0-0.7-0.3), Figure 10.4, symbol □), showed higher M_{CER} and Y_{CER} values (45.6-60.7×10^{-8} kg/s and 76.0-101.1×10^{-4}, respectively) when compared to the others (8.0-32.2×10^{-8} kg/s and 13.3-53.6, respectively). On the contrary, the extraction experiments carried out without H_2O or with low H_2O composition (Figure 10.4, symbol ■) showed lower M_{CER} and Y_{CER} values (8.0-16.3×10^{-8} kg/s and 13.3-27.1×10^{-4}, respectively) (Table 10.1). Intermediate values were observed for the other experimental points (Figure 10.4, symbol ▣). Therefore, it seems that tara seed coat contains substances that present high solubility towards H_2O and that are readily available for extraction, maybe due to their localization in the external cell membranes. On the contrary, the substances which are more soluble in a lower density and polarity solvent phase, i.e., a solvent mixture without H_2O or with low H_2O amount, were probably located in the interior cell tissues.

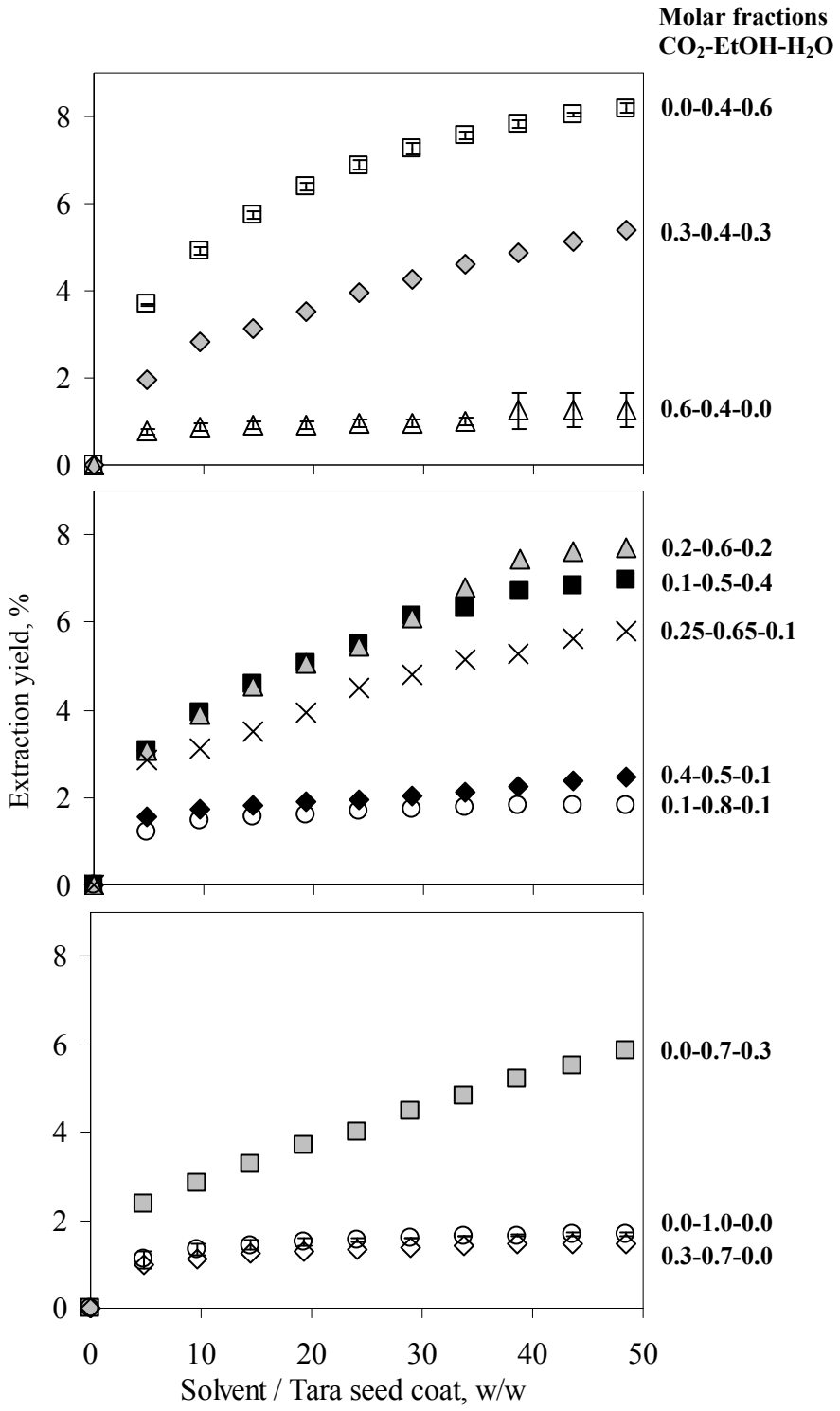

Figure 10.3. Overall curves of PSE from tara seed coat at 313 K, 20 MPa and diverse CO_2, EtOH and H_2O molar fractions in the solvent mixture.

Table 10.1. Kinetic parameters, total yields, phenolic contents, oxidation inhibition and lipoxygenase assay IC_{50} values of tara seed coat extracts obtained by PSE at 313 K and 20 MPa with diverse CO_2/EtOH/H_2O solvent mixtures.

Solvent composition			Kinetic parameter					Total yield (%)	Phenolic compounds (% GAE)	OI_{6h} (%)	IC_{50} (mg/mL)
x_{CO_2}	x_{EtOH}	x_{H_2O}	$M_{CER}\times10^8$ (kg/s)	$Y_{CER}\times10^4$	$t_{CER}/60$ (s)	R_{CER} (%, d.b.)	Fitting error$\times10^{2a}$				
0.30	0.40	0.30	24.2	40.3	27.7	2.7	1.32	5.4	17.9±0.5	76.4±1.8	36.8
0.20	0.70	0.10	15.0	25.1	24.5	1.5	1.13	1.8	39.4±2.4	82.8±2.6	12.0
0.40	0.50	0.10	16.3	27.1	20.7	1.6	0.82	2.5	33.3±0.8	74.2±0.2	12.7
0.60[b]	0.40	0.00	8.0±0.8	13.3±1.3	21.5±0.1	0.8±0.1	0.97±0.55	1.3±0.2	31.0±0.2	72.0±7.7	13.5±1.0
0.00[b]	1.00	0.00	13.8±2.5	22.9±4.1	24.9±2.3	1.4±0.1	1.38±0.61	1.7±0.03	39.2±1.6	66.0±11.7	11.8±1.1
0.00[b]	0.40	0.60	45.6±0.4	76.0±0.6	28.2±0.3	5.2±0.1	2.89±0.05	8.2±0.1	28.7±1.9	87.5±5.1	21.7±2.1
0.00	0.70	0.30	60.7	101.1	9.5	2.3	2.02	5.9	31.8±1.6	72.5±5.9	15.7
0.10	0.50	0.40	32.0	53.3	25.8	4.0	2.67	7.0	24.7±1.1	71.8±0.5	23.8
0.20	0.60	0.20	32.2	53.6	23.4	3.6	1.93	7.7	32.7±0.6	70.4±6.4	15.0
0.25	0.65	0.10	30.2	50.3	20.6	2.9	2.20	5.8	31.3±0.8	75.7±4.9	17.3
0.30	0.70	0.00	12.5	20.8	23.0	1.2	1.49	1.5	37.0±1.0	51.4±1.0	15.5

[a] $\text{Fitting error} = \frac{1}{N}\sqrt{\sum\left(\frac{y^{calc} - y^{obs}}{y^{obs}}\right)^2}$; [b] Experimental results are presented as average values ± standard deviations of two replicates.

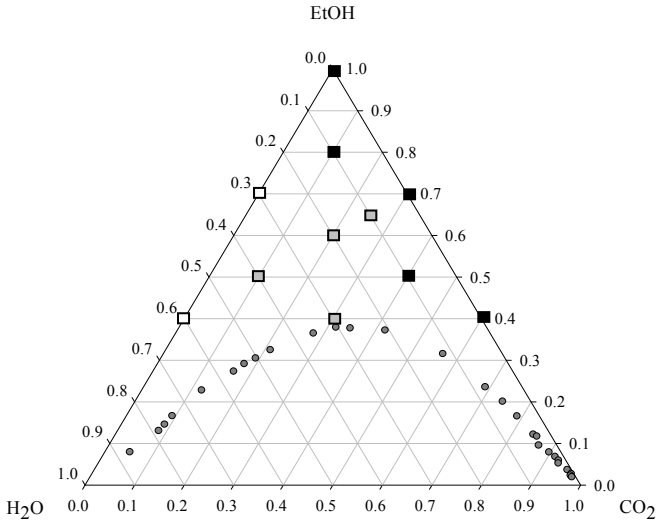

Figure 10.4. Representation of the solvent mixture group of points that led to similar M_{CER} and Y_{CER} values: □ high values, ▒ medium values and ■ low values.

10.4.2. Mixture regression models

Linear, quadratic and special cubic mixture models were used to fit extraction yields and extracts´ total phenolic compounds, 6-hour oxidation inhibition and IC_{50} data which are also reported in Table 10.1. Analysis of variance (ANOVA) was used to test for model significance and lack of fit. For the extraction yield response variable, the simultaneous test for all parameters of the linear model was statistically significant ($F(2,11)=18.88;\ p<.05$). The addition of model parameters for the quadratic and for the special cubic models did not significantly improve the fit of the surface. For the extracts´ total phenolic compounds response variable, though the simultaneous test for all parameters of the linear model was also statistically significant, the addition of the three quadratic model parameters significantly improved further the fit of the model ($F(3,8)=5.19;\ p<.05$). However, adding the parameters for the special cubic model did not significantly improve the fit of the surface. For the extracts´ oxidation inhibition response variable, none of the models revealed statistically significant at the 95% confidence level. Finally, for the extracts´ IC_{50} response variable, all the three models were statistically significant. However, the addition of the quadratic and of the special cubic parameters improved the fit of the surface ($F(1,7)=7.41;\ p<.05$), and so the special cubic model was further analyzed in order to check if it could provide an adequate IC_{50} data fit.

Table 10.2 reports the ANOVA results for the validated regression models. All have regression mean square/residual mean square ratios that are larger than the corresponding F

distribution value at the 95% confidence level, showing that all of them describe significant dependencies of the response variables on the employed solvents compositions.

Table 10.2. ANOVA for the regression results of the preferred models predicted for the extraction yield and total phenols and IC_{50} values of tara seed coat pressurized solvent extracts.

Response variable	Sum of squares	Degrees of freedom	Mean square	Calculated F-value[a]	Probability[b]
Extraction yield - Linear model					
Regression	122.00	2	61.00	23.60	0.00011
Residuals	28.43	11	2.58		
Lack of fit	21.84	8	2.73	1.24	0.47
Pure error	6.59	3	2.20		
Total adjusted	150.43	13	11.57		
Extracts´ total phenolic compounds - Quadratic model					
Regression	401.46	5	80.29	9.54	0.0032
Residuals	67.33	8	8.42		
Lack of fit	61.33	5	12.27	6.13	0.083
Pure error	6.00	3	2.00		
Total adjusted	468.79	13	36.06		
Extracts´ IC_{50} - Special cubic model					
Regression	554.60	6	92.43	10.10	0.0037
Residuals	64.03	7	9.15		
Lack of fit	57.42	4	14.36	6.52	0.078
Pure error	6.60	3	2.20		
Total adjusted	618.63	13	47.59		

[a] Lack of fit mean square/pure error mean square ratio. Corresponding critical F values at the 95% confidence level for the linear model fit is $F_{2,11,0.05} = 3.98$, for the quadratic model fit is $F_{3,8,0.05} = 4.07$, and for the special cubic model fit is $F_{1,7,0.05} = 5.59$. [b] Significance probability level.

The extraction yield linear model has calculated lack of fit mean square/pure error mean square ratio less than the F distribution critical value at the 95% significance level, showing that it provides an excellent fit to the experimental data. However, the total phenolic compounds quadratic model and the IC_{50} special cubic model presented significant lack of fit at the 95% confidence level, since the lack of fit mean square/pure error mean square ratios were 6.13 and 6.52, respectively, which are higher than the $F_{3,8}$ and $F_{1,7}$ critical values of 4.07 and 5.59, respectively. However, all the three models can be considered acceptable since the p-values of lack of fit were higher than 0.05 (Table 10.2). In addition, obtained residuals have random behavior, which also supports the validity of the models.

The linear regression model that fits tara seed coat extraction yield (Y) (reported in Table 10.1) is represented in Equation 10.5 in terms of the pseudo-components molar fractions:

$$Y = \mathbf{10.17}\, x'_{H_2O} + \mathbf{1.29} x'_{CO_2} + \mathbf{2.15} x'_{EtOH} \quad \text{(Equation 10.5)}$$

(*p-values of significant coefficients*: H_2O - 3.42×10^{-7}, EtOH - 4.64×10^{-2})

Bold-faced coefficients indicate those that are significant at the 95% confidence level. The corresponding p-values are presented in parenthesis directly below their model coefficients.

The value of adjusted R^2, which is a measurement for fitness of the regressed model, is 0.78, showing that experimental data are in reasonable agreement with predicted values. Each regression coefficient describes the individual contribution of each solvent to total extraction yield. H_2O presented a highly significant (p-value of 3.42×10^{-7}) positive regression coefficient of 10.17, meaning that H_2O was the most effective solvent in extracting compounds from tara seed coat in a high pressure extraction methodology such as the one applied in this study. Regression coefficient of EtOH was also significant (p-value of 4.64×10^{-2}) and positive (2.15). Figure 10.5.a illustrates the mixture response surface for the model in Equation 10.5, in terms of pseudo-components molar fractions. Therefore, the two lower vertices stand for binary mixtures of H_2O and EtOH (0.6 and 0.4 molar fractions, respectively) and of CO_2 and EtOH (0.6 and 0.4 molar fractions, respectively), while the upper vertex stands for pure EtOH.

Total phenolic compounds of tara seed coat extracts, also reported in Table 10.1, varied in the range 17.9-39.4% GAE, being higher than 31% for eight out of eleven extracts. These contents are high even when compared to the ones of other well known raw materials having a rich phenolic composition, like grape and elderberry byproducts (Louli et al., 2004; Vatai et al., 2009; Seabra et al., 2010). These results confirm our initial suppositions that this would be a raw material quite rich in phenolic compounds, being a strong motivation to the future utilization of this residue to produce high-valuable phenolic-based products.

The quadratic regression model that fits total phenolic compounds (TP) of tara seed coat extracts is represented in Equation 10.6:

$$TP = \mathbf{28.10} x'_{H_2O} + \mathbf{31.24} x'_{CO_2} + \mathbf{39.09} x'_{EtOH} - \mathbf{39.09} x'_{H_2O} x'_{CO_2} - 2.32 x'_{H_2O} x'_{EtOH} + 19.07 x'_{CO_2} x'_{EtOH}$$

(*p-values of significant coefficients*: H_2O - 6.73×10^{-7}, CO_2 - 2.94×10^{-7}, EtOH - 5.73×10^{-8}, $H_2O\times CO_2$ - 9.13×10^{-3})

The value of adjusted R^2 is 0.77 and thus experimental data and predicted values are in reasonable agreement. All linear coefficients are significant and positive, meaning that all pure solvents contributed positively to the extraction of phenolic compounds, with special

emphasis to EtOH that has the highest coefficient with the lowest p-value. The only significant binary interaction is antagonistic and is the one between H_2O and CO_2 (-39.39). Therefore, maximum phenolic compounds contents can be expected for mixtures mostly rich in EtOH, as observed in Figure 10.5.b. The affinity between phenolic compounds and alcohol solvents has been widely reported in literature by several authors (Cacace and Mazza, 2003; Canals et al., 2005) being related to the important role that these solvents play in the formation of hydrogen bonding associations with the diverse functional groups of phenolic compounds.

The special cubic regression model that fits IC_{50} of tara seed coat extracts, which varied between 12.0 and 36.8 mg/mL (Table 10.1), is represented in Equation 10.7:

$$IC_{50} = \mathbf{22.04}x'_{H_2O} + \mathbf{12.66}x'_{CO_2} + \mathbf{11.88}x'_{EtOH} + \mathbf{72.98}x'_{H_2O}x'_{CO_2} - 2.09x'_{H_2O}x'_{EtOH} + 11.41x'_{CO_2}x'_{EtOH} - \mathbf{253.28}x'_{H_2O}x'_{CO_2}x'_{EtOH}$$

(*p-values of significant coefficients*: H_2O - 1.57×10^{-5}, CO_2 - 5.29×10^{-4}, EtOH - 8.39×10^{-4}, $H_2O\times CO_2$ - 8.81×10^{-4}, $H_2O\times CO_2\times$EtOH - 2.56×10^{-2})

The value of adjusted R^2 is 0.81, showing that the experimental data were in reasonable agreement with predicted values. All linear coefficients are significant and positive. The highest one corresponds to H_2O, meaning that this is the solvent that gave the highest contribution to lower the anti-inflammatory activity of the extract (i.e., to raise its IC_{50} value). The only significant binary interaction involves H_2O and CO_2. Since the corresponding coefficient is positive (equal to 72.98, considerably higher than the linear ones), these two solvents, when present together in the solvent mixture, also contribute to raise the IC_{50} value of the extract, i.e., to lower its anti-inflammatory activity.

On the other hand, there is a strong antagonistic effect among the three studied solvents, with a low negative value of -253.28. So, it can be expected that tara seed coat extracts with higher anti-inflammatory activities are obtained with solvent mixtures having lower H_2O and CO_2 contents and higher EtOH contents. This fact may be clearly visualized in Figure 10.5.c, which illustrates the mixture response surface for the model in Equation 10.7.

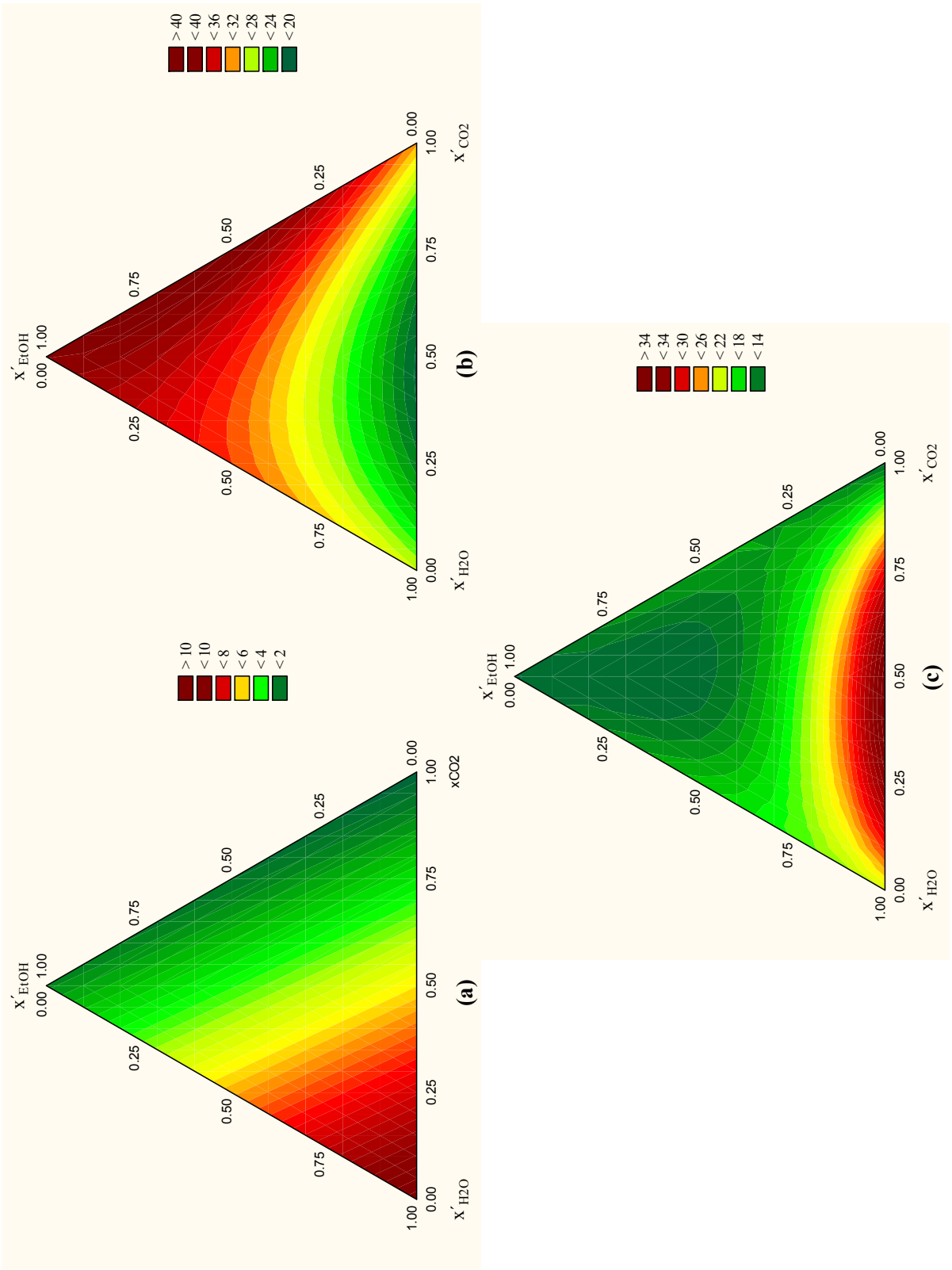

Figure 10.5 . Response surface contour plot curve for the **(a)** extraction yield, **(b)** extracts´ total phenolic compounds and **(c)** extracts´ IC_{50} values of tara seed coat pressurized solvent extracts.

A linear correlation was observed between IC_{50} values and phenolic compounds in extracts (Figure 10.6), suggesting that they do play an important role in the anti-inflammatory activity of tara seed coat extracts. The anti-inflammatory activities of these tara seed coat extracts may be explained by the potent inhibitory effects of their phenolic compounds on arachidonic acid metabolism through the lipoxygenase pathway. Indeed, studies have implicated oxygen free radicals in the process of inflammation and phenolic compounds may block arachidonic acid metabolism by inhibiting lipoxygenase activity, or may serve as a scavenger of reactive free radicals which are produced during arachidonic acid metabolism (Sreejayan and Rao, 1996).

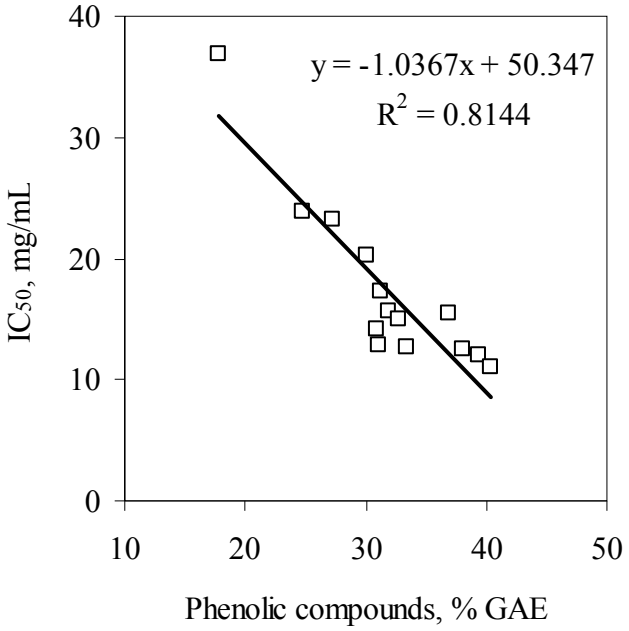

Figure 10.6. Lipoxygenase IC_{50} values *versus* phenolic compounds contents of tara seed coat extracts obtained by PSE at 313 K and 20 MPa with diverse CO_2/EtOH/H_2O solvent mixtures.

10.4.2. Antioxidant activity results

Antioxidant activity of tara seed coat extracts, expressed by their oxidation inhibition in the β-carotene and linolenic acid system, is represented in Figure 10.7. In general, all extracts presented high antioxidant activities even 6 hours after the beginning of the assay. Nine out of eleven extracts maintained their oxidation inhibitions above 80% after 3 hours, and above 70% after 6 hours of the beginning of the assay. As mentioned before, none of the linear, quadratic or special cubic models revealed to be statistically significant at the 95% confidence level, and therefore there was not an obvious influence of solvent mixture composition on

antioxidant activity of the extracts. There was neither a direct correlation with the phenolic contents of the extracts nor with their anti-inflammatory activities whatsoever.

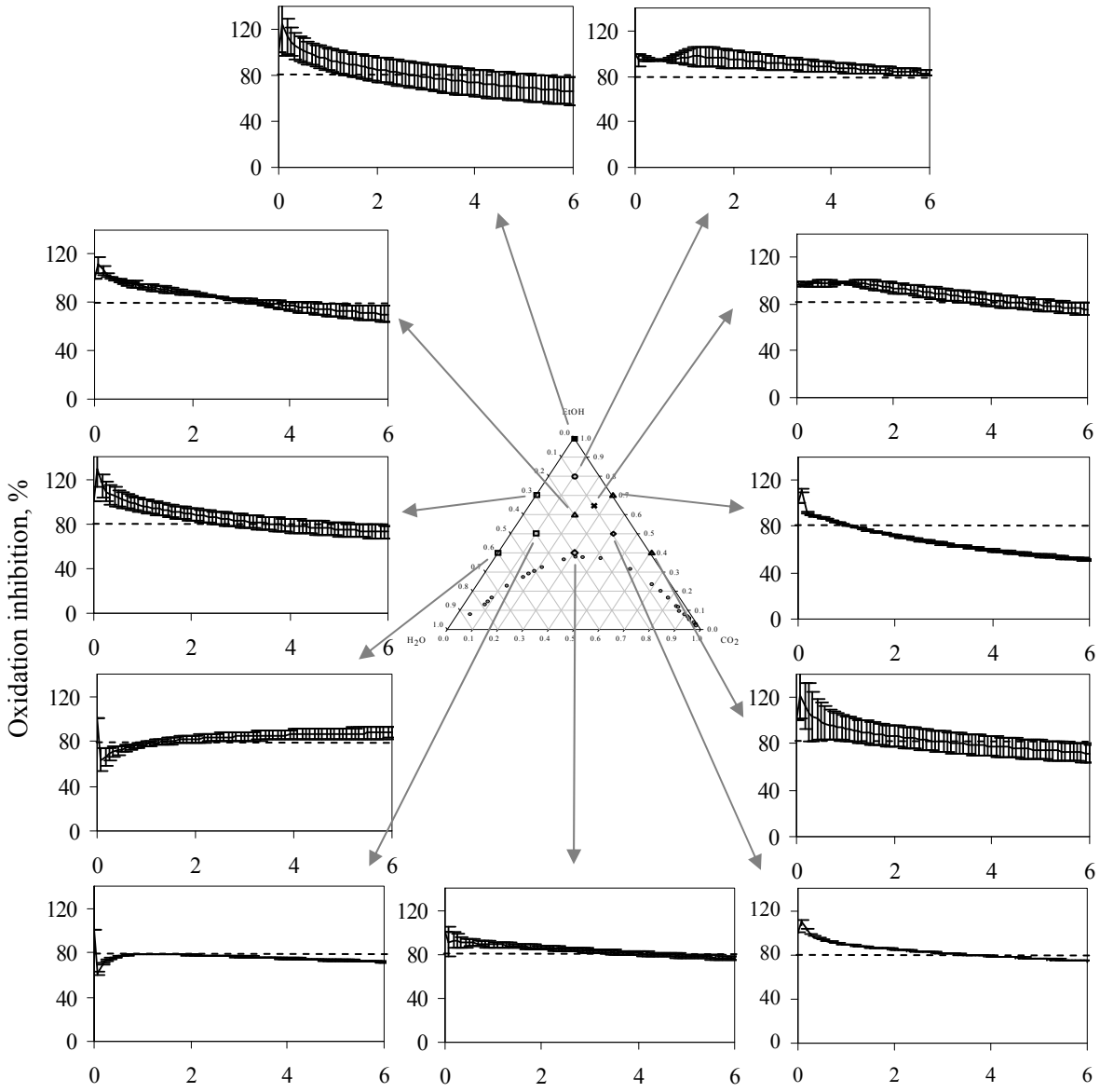

Figure 10.7. Oxidation inhibition of tara seed coat extracts obtained by PSE at 313 K, 20 MPa and diverse CO_2, EtOH and H_2O molar fractions.

Many authors have failed to find direct relationships between total phenolic contents and antioxidant activities of plant extracts (Kähkönen et al., 2001; Trouillas et al., 2003; Seabra et al., 2010). Some explanations have been given, including: (i) reagents that depend on redox reactions, such as the Folin-Ciocalteau, do not produce reliable absolute quantification of phenolic compounds (Appel et al., 2001), (ii) antioxidant activity of phenolic compounds is known to be correlated to their chemical structures (Rice-Evans et al., 1996; Lien et al., 1999)

and then the overall activity of extracts does not depend on total amount of phenols presented but instead on specific compounds present, (iii) synergistic, antagonist or even additive effects are known to occur between phenolic compounds, affecting antioxidant activity of the whole extract (Seeram, 2008). Further investigations (such as HPLC analysis) are required to determine specific phenolic compounds present in these extracts. Only then, new insights into possible correlations between the specific phenolic compounds present and the antioxidant activity (and even anti-inflammatory activity) of the extracts may be discerned.

10.5. Conclusions

The overall results confirmed that tara seed coat is a residue quite rich in phenolic compounds with potent lipoxygenase inhibition and antioxidant activities. Pressurized solvent extraction applying CO_2, EtOH and H_2O, at 313 K and 20 MPa, proved to be a feasible extraction methodology to achieve potentially useful extracts.

Solvent composition affected extraction kinetics, extraction yields, and composition and activity of extracts. Whereas the presence of H_2O in the extraction medium favored extraction yield, the presence of EtOH favored the extraction of phenolic compounds with high antioxidant activity, though no direct correlation with antioxidant activity of extracts could be determined. The most remarkable features of the obtained tara seed coat extracts are their high total phenolic compounds contents and antioxidant activities. Tara seed coat is, therefore, a residue that has great potential to be valorized trough the extraction of compounds that may be applied as natural additives in food, cosmetic or pharmaceutical goods, increasing their shelf-lives and/or acting as human health promoters.

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Part E. Final remarks

11. Conclusions

In this Thesis, agro-residues of elder (*Sambucus nigra* L.), pine (*Pinus pinaster* Ait.) and tara (*Caesalpinia spinosa* (Mol.) O. Kuntz) were evaluated as a source of potentially valuable compounds for food, pharmaceutical, cosmetic and nutraceutical applications, among others.

Elderberry pomace was found to be a quite promising source of phenolic compounds in general and of anthocyanins in particular. A fractionated high pressure extraction methodology applying supercritical CO_2 in a first step and diverse CO_2/EtOH/H_2O solvent mixtures in a second step was performed. Supercritical CO_2 was able to solubilize most lipophilic compounds of elderberry pomace, avoiding their presence in the anthocyanin-rich extracts achieved in the subsequent extraction step, which involved the usage of a higher density and polarity solvent mixture. Indeed, anthocyanin-rich extracts were achieved during this second step, whose yield and composition were quite affected by the concentrations of CO_2, EtOH and H_2O in the solvent mixture. High antioxidant activities were observed for most extracts, with no direct correlation with any specific phenolic compound, or even with total phenolic compounds contents. Even though EtOH and H_2O were both important to promote the extraction of anthocyanins, some solvent mixtures containing high CO_2 concentrations originated extracts with high anthocyanin contents as well. These particular solvent mixtures confer advantages to the whole process due to the reduced effort of removing liquid solvent traces in the post-extraction preparation steps.

Elderflower was also evaluated as a raw material suitable to obtain phenolic-rich extracts. Supercritical CO_2 extraction was studied and compared to conventional ethanolic extraction. A factorial experimental design was applied to study the effect of temperature and solvent density on the characteristics of the resulting supercritical extracts. Solvent density and extraction yield were directly related, reflecting the solvent power increase with CO_2 density. Whereas volatile compounds were observed by TLC in both CO_2 and conventional ethanolic extracts, phenolic compounds were only observed in the latter one. A more detailed investigation of the composition of these supercritical extracts is needed in order to conclude about solvent selectivity. However, since CO_2 extracts revealed to have pro-oxidant or low

antioxidant activities and the ethanolic extract revealed a moderate antioxidant activity, higher antioxidant activity extracts may be obtained by SFE, if ethanol is used as a cosolvent.

Pine bark, a condensed tannin-rich residue of pine wood processing industries was subjected to conventional solid-liquid and high pressure extraction methodologies so as to obtain effective leather tanning agents and extracts containing biologically active components with potential applications in pharmaceutical, cosmetic and food industries.

Regarding the conventional methodology, the optimization of the aqueous solvent additive applied in minor concentrations (NaOH, HCOOH and EtOH, either or not with Na_2SO_3) was performed. Overall results revealed that, as opposed to the alkaline and acidic additives, EtOH was selective for condensed tannins. In addition, pH values and viscosities of these extracts indicate that they may be adequate for leather tanning, avoiding the usage of acids, alkalis and sulfites which contribute to the overall pollution burden of leather tanning industries. Their high phenolic compounds and condensed tannins contents indicate that they may find alternative applications besides leather tanning, such as those where compounds of natural origin are particularly valorized, like pharmaceutical, cosmetic and food industries, increasing shelf-life of products and/or promoting human health.

As previously mentioned, maritime pine bark was also subjected to high pressure extraction applying supercritical CO_2, to recover the volatile oil, and supercritical and subcritical mixtures of CO_2 and EtOH, to recover phenolic compounds, including condensed tannins. Diverse process parameters were studied, like pressure, temperature, fractionation, solvent flow rate and CO_2/EtOH solvent mixture composition. Extraction kinetics were evaluated and composition of extracts was determined by spectrophotometric and chromatographic techniques. Comparison with hydrodistillation and ethanolic Soxhlet extraction methodologies was carried out. Pressure, temperature and CO_2/EtOH solvent mixture composition revealed to be of major importance to extraction kinetics, and also to composition and antioxidant activity of maritime pine bark extracts. Catechin plus epicatechin contents of CO_2:EtOH (90:10) fractionated extracts influenced antioxidant activities, though not directly, being higher than the ethanolic Soxhlet extract content. The solvent mixture composition study revealed that the particular solvent composition CO_2:EtOH (30:70) combined the optimum characteristics of extract composition and of extraction kinetic behavior. Moreover the corresponding extract had similar total phenols and condensed tannin contents when compared to the ethanolic Soxhlet extract ones. The lower EtOH consumption, lower solvent-to-solid ratio and lower temperature applied in the high pressure methodology are advantages over the conventional Soxhlet methodology, due to the known thermal-

sensitiveness of phenolics compounds and to the reduced effort of removing an organic solvent in the post-extraction preparation steps. Possible envisaged applications of the different obtained extracts are, for example, the use the volatile oil fractions in the aroma/flavor industries, and the use of phenolic fractions in the food and pharmaceutical industries.

Finally, the overall results performed on tara seed coat, a residue of tara powder and of tara gum processing industries, confirmed that it constitutes a rich source of phenolic compounds with potent antioxidant and lipoxigenase inhibition activities. Moreover, high pressure extraction using homogeneous solvent mixtures of CO_2, EtOH and H_2O proved to be a feasible methodology to achieve potentially useful extracts. A mixture design investigation was performed so as to explore solvent composition effect on extraction kinetics, extraction yields, and composition and activity of obtained extracts. Whereas the presence of H_2O in the extraction medium favored extraction yield, the presence of EtOH favored the extraction of phenolic compounds with high antioxidant activity, though no direct correlation could be determined between antioxidant activities and total phenolic contents. The high antioxidant and lipoxigenase inhibition activities of the obtained extracts suggest that they may be applied as natural additives in food, cosmetic and pharmaceutical systems. Therefore, this study revealed that managing tara seed coat as a waste is a real misuse of a rich vegetable resource, and strategies should be implemented in order to supply tara processing industries with new and sustainable technologies in order to transform tara seed coat into marketable products.

In conclusion, the three studied agro-residues revealed to be important sources of valuable nutraceutical substances like phenolic compounds (which present anti-ageing, anti-proliferative and anti-inflammatory activities as well as protection for cardiovascular diseases), antioxidants for food, cosmetic and pharmaceutical industries (which can replace several synthetic and potentially toxic and carcinogenic antioxidants like BHA and BHT), colorants, fragrances, and vegetable leather tanning agents (which can replace chromium salts and reduce the environmental impact of leather tanning industries). The implementation of agro-residues valorization strategies is in accordance with several national and international environmental regulations, following the current worldwide tendency of recycling this type of residues. Furthermore, these strategies represent an opportunity for creating additional value and increasing profit of the involved agro-industries. Therefore, with some additional and appropriate processing, elderberry pomace, maritime pine bark and tara seed coat can be changed from a residual low-value status into a very interesting high-value one, for well-known and consumer high-value and accepted uses in the food, cosmetic and pharmaceutical

industries, among others. Solvents and techniques considered as “acceptable” and “generally regarded as safe” in the food and pharmaceutical industries were proposed for valorization of these agro-residues.

The work developed during this doctoral training period still needs to be completed, if a real recycling and valorization strategy is to be implemented. Besides those issues related to market demands (including evaluation of consumers´ needs and acceptance of new products) and to the economic viability of the whole process (including processing costs evaluation, such as initial equipment investment and raw-material handling and preservation costs), other scientific and technological issues still need additional developments. To be precise, the most promising extracts need further analytical characterization studies, in order to identify important bioactive compounds. Moreover, biological tests also need to be implemented, so as to discern the possible applications of the obtained extracts in bioactive formulations. Other studies focusing on their particular practical applications will certainly become necessary, as new applications emerge. Some of these issues will be addressed in future works, in preference with the support of interested companies of the sector which may implement a real-world application, with benefits to their environmental and economical sustainability as well as with benefits to consumers.

12. Suggestions for future work

The work developed during the doctoral training period has opened some possibilities to be explored in the future. Among several suggestions, some may be considered more relevant and are described below. Nearly all of these suggestions are related to a deeper analytical characterization of the most promising extracts separated, and also to studies focusing on some of their feasible applications.

Elder (*Sambucus nigra*) pomace and flower

The elderberry pomace supercritical CO_2 lipophilic-rich extract that was separated in the first fractionated extraction step, at 313 K and 20 MPa, corresponds essentially to the elderberry seed oil, known to contain high levels of unsaturated fatty acids, resembling olive oil composition. Moreover, this oil was obtained by a process that might be competitive with conventional organic solvent extraction, since it considerably simplifies the oil refinement stages and completely eliminates the solvent distillation stage, which are the most costly processing stages in terms of energy consumption. Therefore, this extract may have some interesting applications as an alimentary oil of vegetable origin, as a cosmetic ingredient, and as a nutritional supplement, similarly to other vegetable oils, like grape, primrose, borage, and currant seed oils. In order to confirm this supposition, physicochemical characterization of elderberry pomace oil needs to be carried out according to the legislation for the analysis of oils for human consumption, and its fatty acid composition needs to be determined by GC analysis.

The elderberry pomace anthocyanin-rich extracts (achieved using CO_2/EtOH/H_2O solvent mixtures in the second high pressure extraction step) which reached higher anthocyanins and phenolic compounds contents, and also higher antioxidant activities were found to be of particular interest for valuable applications. In order to confirm this supposition, these extracts do need to be subjected to further studies. It would be important to evaluate their *in vitro* antioxidant activities using other methods besides the DPPH method used in this work, since

only one assay does not truly reflect the total antioxidant capacity of a particular sample. Moreover, *in vivo* tests would also be useful, since activity measured by *in vitro* tests may not reflect *in vivo* effects of antioxidants. Therefore, several *in vitro* biocompatibility and biological activity tests will be carried out on some selected extracts, namely: blood compatibility and interaction tests, cytotoxicity tests and immune response tests. These tests will help to clarify potential applications of elderberry pomace extracts.

Regarding elderflower, a more detailed investigation of the CO_2 extracts composition is needed in order to publish this work in a scientific journal. The whole extracts have been analyzed by GC and it was not possible to separate and identify individual substances, even after optimization of the carrier gas flow rate and the temperature program. Therefore, fractionation of the extracts to separate higher molecular weight compounds such as cuticular waxes, fatty acids, fatty acid methyl esters and pigments from odoriferous compounds like terpenes, oxygenate terpenes, sesquiterpenes and oxygenate sesquiterpenes will be performed, followed by GC analysis of individual fractions. Considering the possible utilization of these elderflower extracts as attractive odor and flavor ingredients of food products, organoleptic analysis performed by a trained panel would also be useful to obtain an overall characterization of the extracts in terms of color, odor and taste. Finally, further supercritical extractions using CO_2 plus a cosolvent (probably ethanol) will also be carried out in order to evaluate the possibility of obtaining phenolic-rich extracts from this raw material, possessing high antioxidant activity.

Pine (*Pinus pinaster*) bark

Pine bark conventional extracts achieved using water plus low amounts of ethanol revealed appropriate characteristics to be applied as leather tanning agents. So as to confirm this supposition, the best extraction condition needs to be performed at a pilot-plant scale and the respective extract used in leather tanning. The resultant leather should then be compared to chromium-tanned leather and subjected to thermomechanical analyses (so as to determine, for example, tear resistance, tensile strength, and shrinkage temperature), Scanning Electron Microscopy to determine leather morphology, color measurements, etc. Official Methods of Analysis for Physical Testing of Leather will be preferred. Part of these tests will be undertaken at CTIC-Centro Tecnólogico das Indústrias do Couro, a Portuguese leather research center in Alcanena, Portugal. This characterization is of crucial importance to evaluate possible future industrial applications of pine bark extract tanned leather.

On the other hand, pine bark high pressure extracts achieved at some particular experimental conditions showed good chances to be used as valuable ingredients in food, nutraceutical, cosmetic and pharmaceutical products. Considering this scenario, and similarly to what was above mentioned for anthocyanin-rich elderberry pomace extracts, further *in vitro* and *in vivo* studies are needed in order to evaluate their possible activities such as anti-inflammatory, antiviral and anti-cancer.

Tara (*Caesalpinia spinosa*) seed coat

The most remarkable features of tara seed coat extracts achieved in this Thesis using high pressure CO_2/EtOH/H_2O solvent mixtures were their high total phenolic contents and antioxidant activities. So as to determine possible correlations between specific phenolic compounds present in the extracts and their antioxidant activities (and even anti-inflammatory activities), further investigations on extracts composition are required, namely HPLC analysis. Only then this work will be considered complete and will be submitted for publication on a scientific journal.