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**Tesi di Laurea Magistrale in Ingegneria Chimica
e dei Processi Industriali**

SUPERCRITICAL CO_2 EXTRACTION FROM FOOD MATRICES WITH OPTIMIZATION OF MAIN OPERATING VARIABLES

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Riassunto

Il processo di estrazione condotto mediante fluidi in condizioni supercritiche si pone come una buona alternativa ai sistemi classici di separazione con solvente, garantendo numerosi vantaggi che riguardano soprattutto l'assenza di solventi residui nel prodotto e la riduzione dell'impatto ambientale. La SFE (supercritical fluid extraction) è da quarant'anni il campo di maggior impiego per i fluidi supercritici. Un fluido è considerato supercritico quando la sua temperatura e la sua pressione sono superiori a quelle critiche. In questo stato non c'è distinzione tra fase liquida e gas e alcune delle sue proprietà sono simili a quelle dei liquidi, come la densità, mentre altre sono più somiglianti a quelle dei gas, come la viscosità e la diffusione molecolare. A temperatura costante, ad una variazione di pressione corrisponde una variazione di densità, che comporta cambiamenti di alcune proprietà come la solubilità e la costante dielettrica. La possibilità di poter "controllare" la densità dei fluidi supercritici, ovvero la loro capacità solvente, con una semplice modifica di temperatura e pressione, li ha resi particolarmente adatti nell'estrazione di sostanze da prodotti naturali. Essi infatti possono penetrare efficacemente la matrice offrendo un potere solvente che può essere adattato alla solubilizzazione del componente chimico di interesse.

In tal senso, l'anidride carbonica è il fluido supercritico più usato. Le sue condizioni critiche sono infatti facilmente raggiungibili e tecnologicamente sostenibili: temperatura di 31.1°C e pressione di 72.8 bar. Inoltre essa è chimicamente inerte, altamente pura, economica, non infiammabile e non tossica. Essendo apolare, è più adatta ad estrarre composti apolari ma con l'aggiunta di un co-solvente polare come acqua, etanolo o miscele acqua-etanolo, è idonea anche per l'estrazione di sostanze polari. Dopo l'estrazione, la CO_2 può essere completamente recuperata per semplice depressurizzazione, non lasciando alcuna traccia nell'estratto, ed è per questa sue peculiarità che si presta molto bene come solvente da usare anche nell'industria alimentare e/o farmaceutica.

L'obiettivo della SFE, riguarda l'eliminazione di alcune sostanze presenti nella matrice o le sostanze che vengono recuperate nell'estratto.

Anche in campo alimentare si possono ritrovare queste due diverse esigenze. Alla prima appartiene il caso della decontaminazione da pesticidi quali, ad esempio, quelli organofosforici. Il Malathion, che appartiene a questo gruppo, è usato nelle coltivazioni soprattutto per controllare una grande varietà di insetti. Così come agisce negativamente sugli insetti, è tossico anche per l'uomo tanto che la legislazione ha instituito dei valori massimi limite (MRL) di Malathion presente in diverse matrici naturali.

La seconda situazione, invece, interessa la richiesta di alcuni composti, presenti in cibi o piante, che possono avere applicazioni specifiche, per esempio l'estrazione di polifenoli

dall'asparago. I polifenoli sono sostanze presenti nella pianta dell'asparago, (in particolare dell'*Asparagus Officinalis*) le cui buone proprietà antinfiammatorie sono molto importanti nel settore farmaceutico.

In questo lavoro di ricerca sono state effettuate prove di SFE per entrambi gli ambiti di interesse, cercando di ottimizzare alcune variabili operative che incidono sull'estrazione supercritica. L'elaborato può essere suddiviso in due parti: la prima riguardante la decontaminazione di ceci da Malathion e la seconda inerente l'estrazione di polifenoli da asparagi.

Gli esperimenti di decontaminazione sono stati condotti nel Laboratorio della Prof. Lourdes Calvo, all'interno del Departamento de Ingenieria Quimica presso L'Universidad Complutense de Madrid. Innanzitutto è stata fatta una valutazione sulla procedura di contaminazione, utilizzando delle sferette di silicone contaminate con la stessa concentrazione, ma con modalità differenti. L'analisi chimica è stata effettuata con un metodo spettrofotometrico e si è riscontrata un'elevata differenza tra la concentrazione teorica e quella reale di Malathion, evidenziando così la presenza di problemi di perdita di pesticida al momento della contaminazione. La procedura in cui tale perdita è risultata minore, è stata quella nella quale un preciso volume di soluzione standard di Malathion in acqua distillata contenente tracce di acido acetico, veniva spruzzato sulla superficie delle particelle sferiche. In seguito sono stati contaminati i ceci e seguendo l'analisi con lo stesso metodo spettrofotometrico. Quest'ultimo ha però misurato un'assorbanza anche nei ceci non contaminati (corrispondente ad una concentrazione di Malathion pari a circa 7 ppm), probabilmente dovuta alla presenza di qualche sostanza interferente all'interno dei ceci stessi. Pertanto è sorta l'esigenza di utilizzare un'altra tecnica analitica, e ci si è indirizzati verso la GC-MS (gascromatografia accoppiata ad uno spettrometro di massa quale rivelatore).

Non essendo però disponibile un gascromatografo all'interno del laboratorio dapprima sono state condotte tutte le prove, cambiando i diversi parametri e, solo alla fine, sono state effettuate le analisi dando la possibilità di calcolare la resa dell'estrazione. Le condizioni di estrazione indagate sono state: il tempo di estrazione (15-120 min), la temperatura (40-80°C), la pressione (150-360 bar), la portata di CO_2 (1-4 g min^{-1}) e la percentuale di umidità nella matrice all'interno dell'estrattore (0-12%). La concentrazione di Malathion nei ceci, introdotti nell'estrattore interi e con una massa di circa 20 g, era di 20 ppm per permettere al gascromatografo di rilevarla.

In tutti i casi, la resa di estrazione del Malathion è stata maggiore del 90% non consentendo di fatto di ottimizzare le condizioni operative. Tuttavia, la letteratura è stata di grande aiuto per l'individuazione degli intervalli di operatività e per capire l'effetto che queste grandezze hanno sull'estrazione.

Le prove di estrazione di polifenoli dagli asparagi sono state, invece, condotte nel Laboratorio di alta pressione del Prof. Bertucco, all'interno del Dipartimento di Ingegneria Industriale.

Circa 0.5 g di asparagi, prima ridotti a particelle di diametro di 0.5 mm, sono stati introdotti nell'estrattore dell'impianto pilota e i parametri indagati sono stati essenzialmente temperatura (50-80°C) e pressione (100-250 bar). Si è utilizzata una miscela di etanolo e acqua 1:1 come co-solvente in quanto studi effettuati precedentemente avevano riscontrato che il suo utilizzo portava a maggiori rendimenti di estrazione. Il tempo di estrazione è rimasto fisso a 60 minuti. Per valutare l'efficienza dell'estrazione così da individuare il valore ottimale dei parametri da usare, si sono calcolate due grandezze: la resa di estrazione definita come la quantità di estratto totale (espressa in percentuale rispetto alla massa caricata nell'estrattore) e la concentrazione totale di fenoli (e dei vari tipi di fenoli) presenti nell'estratto stesso. Quest'ultima analisi è stata condotta all'interno del Dipartimento di Farmacia dell'Università degli studi di Padova con un HPLC-MS (cromatografia liquida ad alta prestazione accoppiata ad uno spettrometro di massa quale rivelatore). I valori misurati per queste due grandezze sono spesso risultati in contrasto. Per esempio, se con una pressione di 150 bar si otteneva una resa di estratto totale maggiore, con una di 100 bar si raggiungeva una concentrazione maggiore di polifenoli. Dato però che lo scopo dell'indagine era di recuperare la maggior quantità di fenoli, la loro concentrazione nell'estratto, indice della selettività del processo, è da considerarsi la grandezza più importante ai fini dello studio. La temperatura non ha avuto grande influenza sulla resa di estrazione e il suo incremento ha portato ad una quantità di fenoli solo leggermente maggiore. Successivamente, alla temperatura di 50°C e con una pressione di 150 bar è stata fatta una verifica della concentrazione dei fenoli estratti, a intervalli di 10 minuti. E' risultato evidente che la maggior parte dell'estrazione avveniva tra i 10 e i 20 minuti, dopo i quali la concentrazione di polifenoli estratti decresceva.

A scopo di confronto, sono state condotte anche estrazioni di polifenoli da asparagi con la tecnica del PLE (pressurized liquid extraction) nel quale il tempo necessario per effettuare l'estrazione era di 30 minuti e non veniva più utilizzata la CO_2 supercritica con un co-solvente, ma solamente pompato un liquido come acqua, etanolo o loro miscele. Da ultimo, si è aggiunto un ulteriore confronto con la tecnica di estrazione tradizionale Soxhlet. Nonostante gli alti rendimenti raggiunti dal PLE usando una miscela di acqua e etanolo 1:1, le concentrazioni di polifenoli ottenute mediante estrazione con Soxhlet, sono quelle maggiori (tra i 6 e i 7 mg/g partendo da una quantità di asparagi di 0.5 g). Non molto minori sono quelle risultanti dalla tecnica SFE operante a 65°C e 100 bar (circa 6 mg/g).

Si è così potuto concludere che l'estrazione con CO_2 supercritica e co-solvente funziona bene come alternativa ai metodi classici di estrazione (Soxhlet) garantendo alte concentrazioni di fenoli estratti e soprattutto assicurando un metodo di estrazione a minore impatto ambientale.

Abstract

SFE is a new promising technique studied in recent years as an alternative to older ones that involve the use of toxic solvents and have a greater environmental impact.

The aim of this work is to carry out two types of supercritical CO_2 extraction from food matrices and to optimize the main important extraction variables.

The first one is the extraction of an organophosphorous pesticide (Malathion) from chickpeas. The effects of extraction time, temperature, pressure, CO_2 flow rate and humidity of the matrix were studied. Some parameters' ranges, found in literature, were investigated. The experiments were mainly performed for 30 min, at a temperature of 70°C; a pressure of 350 bar; a CO_2 flow rate of 2 g min^{-1} and in absence of humidity inside the matrix.

The samples after the extraction were analyzed by GC-MS method and the recovery of Malathion was more than 90% in all the conditions investigated.

The second part collects the experiments and results of the extraction of polyphenols from asparagus. In this case the effect of temperature and pressure were deeply investigated. These results, analyzed by HPLC-MS method, are compared with the ones obtained with pressurized liquid extractions (PLE) using a mixture of water ethanol, ethanol only, water only and with Soxhlet extraction.

It was found that with the SFE technique, operating at 100 bar, 50-65°C, 60 min, with co-solvent water:ethanol 1:1, CO_2 flow rate of about 0.2 kg/h it was possible to reach extracted phenols concentrations which were interestingly high.

Acknowledgments

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Introduction

Supercritical fluid extraction (SFE) had a growing interest in the past few years. It offers an alternative to traditional methods of solvent- based extractions and in recent years there has been an increasing interest in using supercritical carbon dioxide.

A supercritical fluid is a substance at a temperature and pressure above its critical point, and its main characteristic is that it has similar density to liquids, but lower viscosity and higher diffusion coefficient. The solubility of specific matrix component in a supercritical fluid can be varied by employing different extraction pressures and temperatures to effect a change in density; therefore little variations can modify mass transfer and heat transmission properties.

The use of supercritical carbon dioxide promises a number of advantages which include quite low critical conditions: pressure (73.8 bar) and temperature (31.35°C), cheapness, greater potential for automation, short extraction times, small solvent quantities and non-toxicity.

This thesis deals with the applicability of SFE in food technology.

The first part of the work aimed to extract an organophosphorous pesticide (Malathion) from chickpeas using a supercritical CO_2 equipment, placed in Madrid at the Laboratory of Chemical Engineering Department of Universidad Complutense (UCM). At first, the research of the best operating conditions of extraction was carried out and the extracts were analyzed by a spectrophotometric analysis, and by a GC-MS.

The interest in this field has emerged during the last 10 years, as a result of a number of new extraction technologies which had been assessed to be applied in the analysis of pesticide residues in foods, and the technology of supercritical fluid extraction (SFE) is one generating the biggest interest in this area of analytical chemistry. ([1])

The second part of the work was carried out in Padova at the Laboratory of Industrial Engineering Department of Università degli Studi di Padova and had the purpose of extracting phenols from asparagus, using a supercritical CO_2 plant. The analysis of the extracts were made by the Department of pharmacy by HPLC-MS.

In both works a series of experiments have been done in order to learn how to work with high pressure and critical conditions and to find the optimum values of the main extraction parameters including pressure, temperature, co-solvent type and concentration and carbon dioxide flow rate.

The discussion has been divided into four chapters.

Chapter 1 provides a description of supercritical fluids in general and the SFE method with its characteristics. It also describes some characteristics about Malathion which was extracted from chickpeas, and phenols which were extracted from asparagus.

Chapter 2 is about the materials and methods that were used to perform the SFE tests and analysis of the extracts.

Chapter 3 reports the results obtained in the extraction of Malathion from chickpeas and expands with their discussion. Furthermore, it provides a comparison with other results from literature.

Chapter 4 deals with the results acquired during the extraction of polyphenols from asparagus. They are compared with the ones reached with other extraction techniques, as the PLE one and the Soxhlet one.

Lastly, some conclusions are drawn.

Chapter 1

Supercritical fluids and SFE method

Supercritical fluid extraction (SFE) is the method used in this work

First of all, it is necessary to define a supercritical fluid with its characteristics; in particular carbon dioxide, which is the most used supercritical fluid. In our case, CO_2 was used to extract Malathion (an Organophosphorous pesticide) from chickpeas, which were previously contaminated, and phenolic compounds from asparagus. This chapter also describes the SFE method with its characteristics, Malathion and how it is present in chickpeas and polyhenols contained in asparagus.

1.1 Supercritical fluids

Supercritical fluids are substances at pressures and temperatures above their critical values. The most important one is carbon dioxide, which is used to extract chemical compounds from food matrices for various reasons, such as its low critical values and no toxicity.

1.1.1 Characteristics and definition of supercritical fluids

Figure 1.1 ([2]) shows a P-T diagram where the three phases (solid, liquid and gas) are separated by phase boundary lines that are equilibrium phase transition lines. For example liquid-vapor equilibrium is represented by a curve that starts at the triple point (TP) and ends at the critical point. In the latter one the densities of the two phases become identical and gas and liquid are not distinguishable. Above this point there is an area named supercritical state where a unique phase exists. As a consequence, a supercritical fluid has some properties more similar to liquids, like density, and others similar to gas, like viscosity and diffusion coefficient.

The aspect of supercritical phase can be illustrated in Figure 1.2 ([3]), where a carbon dioxide phase diagram is represented. It can be seen the differences between the image of liquid-vapor equilibrium where the two phases are distinguishable, and the picture of supercritical state, where there is a unique phase.

The interdependence of volume, temperature and pressure is important for gas extraction, because the variation of properties of supercritical compounds with conditions of state is the basis for many applications.
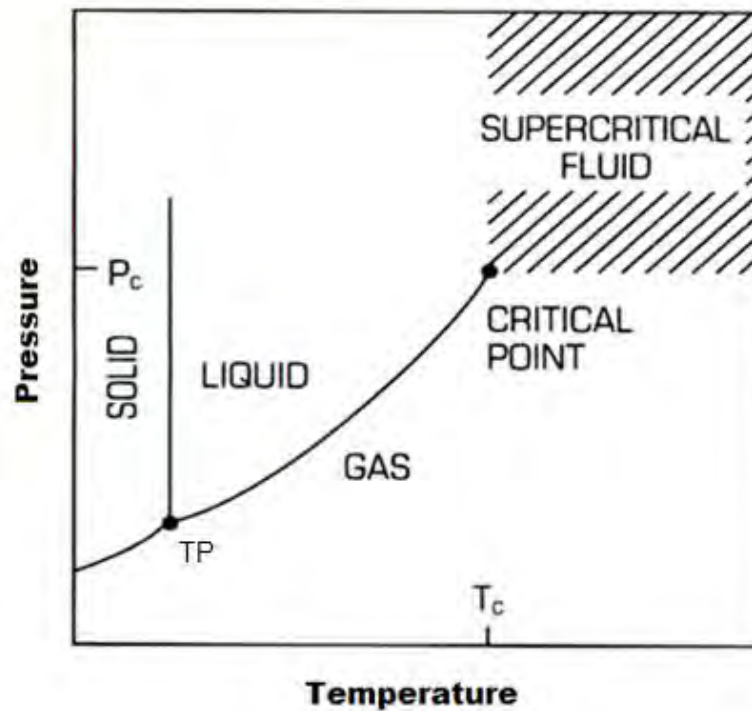
Figure 1.1 *P-T diagram for a pure component: TP=triple point; T_c=critical Temperature; P_c=critical Pressure*

Figure 1.3 ([4]) is a P-V-T diagram that shows the dependence of volume on pressure for different temperatures. There are three different behaviors for fluid that depend on temperature:

1) At $T < T_c$ (isotherm a) the volume of the gas decreases rapidly with the increase of pressure, until point a_2 where the phase boundary line is reached. Crossing this line at a constant pressure, the decrease of total volume causes the formation of liquid drops of specific volume a_3. Points within the two-phase region represent mixtures of a gas and a liquid, coexisting at constant pressure and temperature: a gas of volume a_2 and a liquid of volume a_3.

2) At T=Tc the critical isotherm (isotherm b) has a horizontal tangent at the critical point. At this point, the liquid phase boundary line and the gas one meet and the two phases become identical.

3) At $T > T_c$ (isotherm c), the compression of a gas (c_1) until the point c_2 occurs without visible change of phases. In T_c the isotherms are flat in the vicinity of the critical temperature. Compressibility in this region is high. Small changes in pressure or temperature cause large variations of (specific) volume or density.
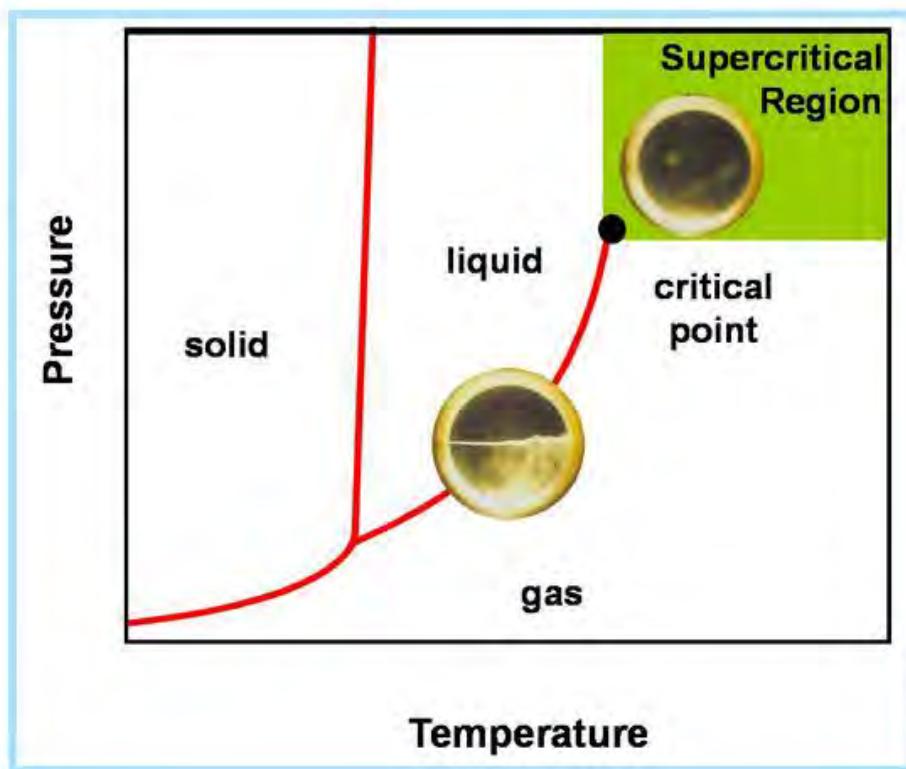
Figure 1.2 *Phase diagram of Carbon dioxide whit images of liquid-vapor equilibrium and supercritical state*
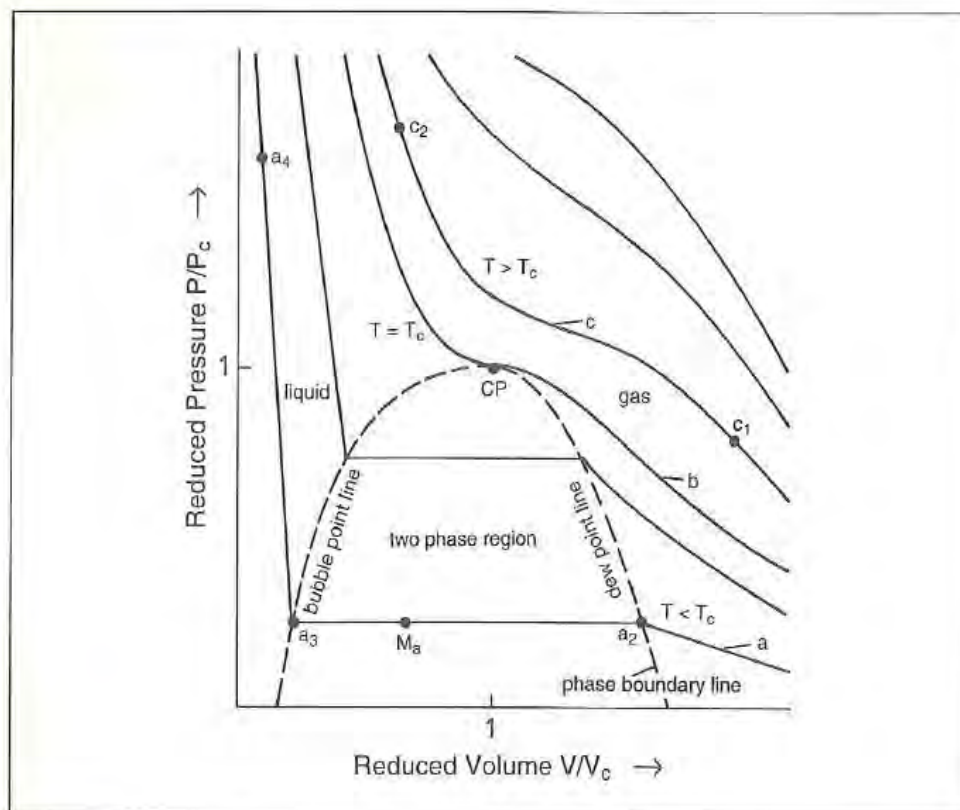
Figure 1.3 *P-V-T diagram of a fluid (e.g. carbon dioxide)*

At low pressure and high temperature it is possible to describe the relation between pressure
P, molar volume V and temperature T with the ideal gas law (equation 1.1):

$$PV = RT$$ (1.1).

For quantitative representation of the P-V-T behavior of supercritical fluids it is necessary to
use an equation of state (EOS) which takes into consideration the proprieties of individual
molecules.

The compressibility of supercritical fluids is one of the main differences between them and
conventional solvents. The latter, in the liquid phase, requires very large pressure to change
the density, whereas for supercritical fluids, very significant changes in density can be
achieved by small pressure and/or temperature changes, particularly around the critical point
(Figure 1.4). ([5]).

The density of supercritical fluids, that is the solvent power of the fluid, can be adjusted by
correctly choosing both pressure and temperature. It is lower than conventional solvents and
can lead to problems of reduced solubility in some cases. On the other hand, its lower
viscosity leads to a significantly greater diffusivity. This can result in meaningfully faster
reaction rates if diffusion is rate limiting.
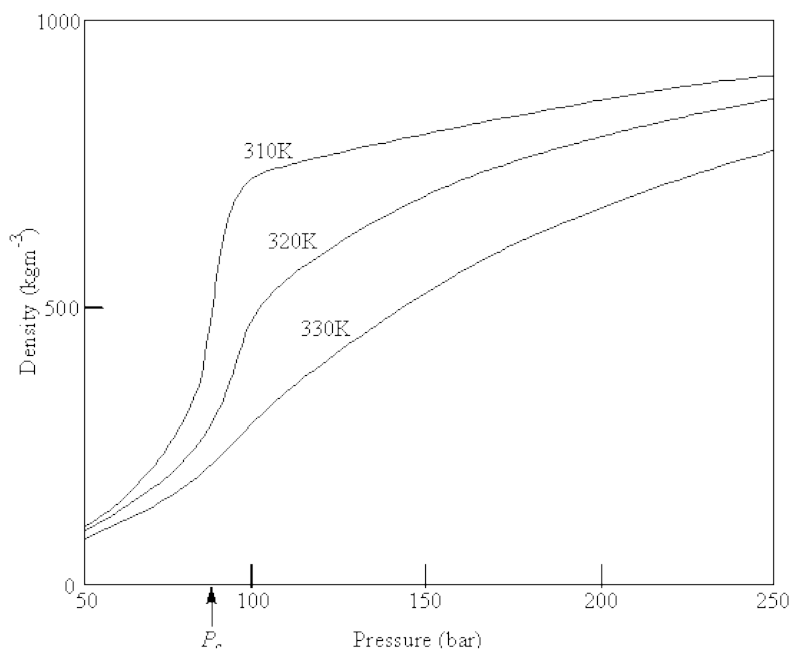
Figure 1.4 *Variation of carbon dioxide density with pressure.*

1.1.2 Supercritical CO$_2$

The supercritical fluid which has played a major role in SFE is supercritical Carbon dioxide,
for a lot of reasons. All the advantages related to using CO$_2$ are analyzed in this chapter.

First of all its supercritical conditions are easily accessible: P_c =72.8 bar and T_c = 31.1°C. Figure 1.5 is a P-T diagram of CO_2 and in Figure 1.6 the critical points of some fluids are represented (H_2O,S,Cs,Br,NH_3,Cl,CH_3CH_2OH,H_2SO_4,CH_4,O_2,N,H_2).

Moreover, CO_2 is non-flammable with a TLV (= threshold limit value for airborne concentration at 25°C to which it is believed that nearly all workers may be repeatedly exposed to day after day without adverse effects) of 5000 ppm . This value means that it is less toxic than many other organic solvents such as acetone, which has a TLV of 750 ppm, pentane (600 ppm), chloroform (10 ppm).

Carbon dioxide is also relatively inert towards reactive compounds (however not completely inert), highly pure, gaseous at atmospheric pressure, cheap and defined "environmentally friendly" because, if it can be removed from the environment, employed in a process, then returned to the environment 'clean', without environmental detriment accrues.

CO_2 is also a greenhouse gas, but it is a naturally abundant material that can be theoretically extracted from the atmosphere or collected as a by-product of some processes. However, while CO_2 could in theory be extracted from the atmosphere (or the stack gas of a combustion based power plant), most of the CO_2 employed in processes today is collected from the effluent of ammonia plants or derived from naturally occurring deposits. ([6])
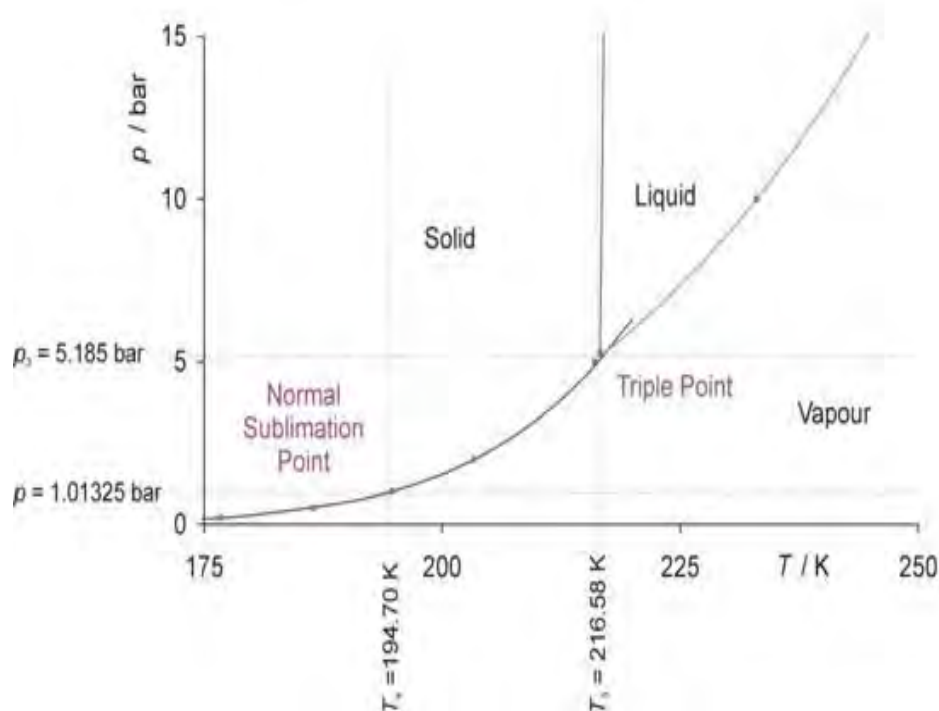
Figure 1.5: *CO_2 p-T diagram*
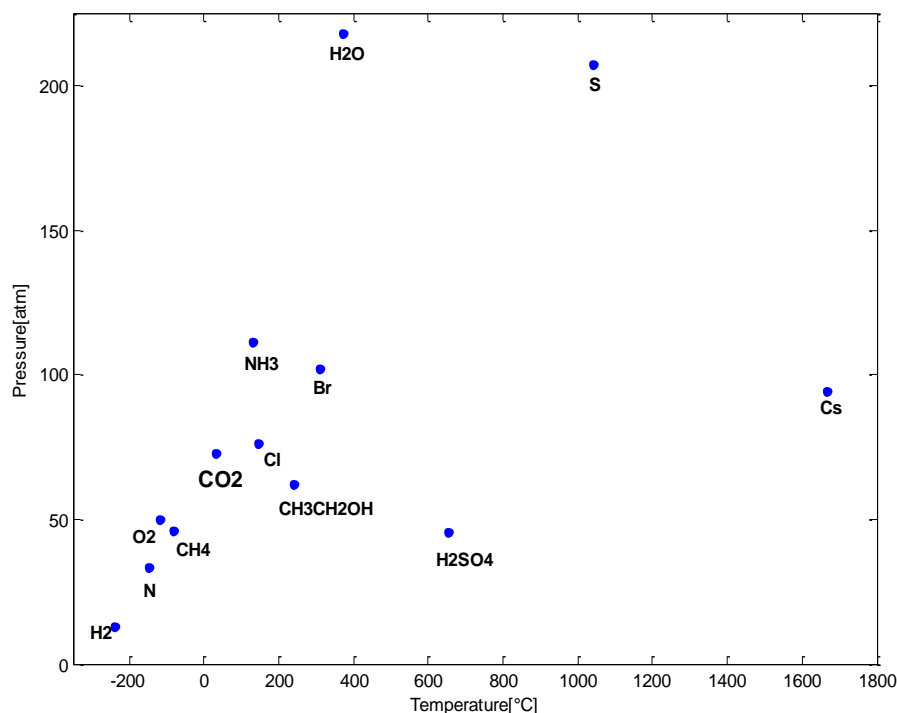
Figure 1.6: *Critical points of some fluids*

Another advantage of using carbon dioxide is that its solving power is easily manipulated by changes in pressure and temperature. In addition it is a good solvent for the extraction of no-polar compounds and substances which have low molecule weight, such as alcohols, ketones, aldehydes and esters. The most frequent limitation of carbon dioxide as an extraction solvent is that its polarity is often too low to obtain efficient extraction for polar compounds. In order to overcome this problem, a polar co-solvent or mixture of co-solvents can be added to increase compounds solubility to supercritical CO_2 extraction. All the polar co-solvents such as methanol, ethanol, acetonitrile, acetone, water and dichloromethane could be used. The type of co-solvent depends on the compounds to be extracted and the matrix. ([7])

1.2   SFE method

Supercritical fluid extraction (SFE) has faced a growing interest in the past few years. Here below its brief history and its main characteristics are explained.

1.2.1 Brief History

The first two researchers who defined a supercritical fluid (SCF) were Hannay J. B. and J. Hogarth. ([8]) At a meeting of the Royal Society (London) in 1879, they described their work which was focused on the pressure dependent dissolving power of supercritical fluids: when increasing the pressure their dissolving power gets higher. The solubility behavior of

supercritical fluids found by Hannay and Hogarth was not exploited until many years later, but it is of historical interest.

Starting in the early 1900s many research groups, and E. Büchner in 1905, studied the properties of supercritical fluids, investigating primarily thermodynamic phase behavior, and then extending the list of gases and solutes.

In the early 1950 a paper at the Annual AIChE meeting in San Francisco started to suggest that supercritical fluids could be used in an extraction process.

By the 1950s, the first description of a supercritical fluid extraction (SFE) process appeared in a US scientific journal. Meanwhile in Europe, especially in Germany, many engineers were spearheading the industrial development of SFE.

During the '60s many research groups, primarily in Europe and later in the U.S.A., examined SCFs for developing "advanced" extraction processes. At first, European researchers emphasized extraction from botanical substrates such as spices, herbs, coffee and tea, using predominantly supercritical carbon dioxide; later their attention was focused also in polymers, lubricants, pharmaceuticals and specialty chemicals.

By the 1980s there were several large SCF extraction processes in operation in Germany, in UK and in USA, for decaffeinating coffee and tea, and also extracting flavors and essential oils from hops, spices, and herbs. One of the major motivations for developing these SCF processes was the complete elimination of residual solvents in the products. (^{9})

Nowadays SFE ensures a number of advantages over traditional solvent-based methods:

- supercritical fluids have solvating powers similar to liquid organic solvents, but higher diffusivities, lower viscosity, and lower surface tension similar to gases;

- changing the pressure or temperature it is possible in order to adjust the solvating power, therefore the separation of extracts from solvent is fast and easy;

- the polarity of a supercritical fluid can be changed by adding modifiers to have more selective separation power,

- there are no solvent residuals problems in food or pharmaceutical industries;

- SCFs are generally cheap, simple and many are considered safe.

However there are also difficulties in developing a new SFE method due to the great number of parameters that need to be taken into account: its effectiveness is also strongly dependent on the physical nature of the matrix. (^{10})

1.2.2 Characteristics of SFE

The extraction of components from solid material is carried out by putting the solid substrate in contact with a continuous flow of the supercritical solvent. Through this solid substrate, the supercritical gas flows and extracts the product components.

The interest of the extraction could be either on the extract, or on the elimination of some substances presented in the matrix. For this reason, natural material to be extracted can be divided into two categories.

The first group includes materials which allow pretreatment and where the extracted substances represent the main product, like in the case of high value-added products: flavors, spices, polyphenols. For these kinds of processes the extract's revenues must cover the raw-material's treatment cost, and therefore only highly valuable extracts justify the application of CO_2 technology.

The second category is composed by raw materials, whose geometry must be maintained during the process, and/or only undesired substances are removed. This is the largest category in terms of tonnage and it includes the following applications:

- decaffeination of green coffee, black and green tea;
- defatting of cocoa press cake and nuts;
- removal of plant protective materials or pesticides (e.g. Malathion) from foods and pharmaceutical (e.g. ginseng);
- reducing alcohol content in beverages.

Such processes require a high selectivity for the substances to be removed, in order to maintain the flavor, appearance, smell, and shape of the treated feed material which represents the main product. Since CO_2 has selective solubility proprieties, which can be altered to some extent, its use may often be feasible. If by-products are recovered, such as caffeine, they can improve the process economy. ([11])

SFE can be regarded as a five-stage process:

1) The matrix absorbs the supercritical solvent and the co-solvent (if this is used). Mass transport resistance is lowered.

2) The compounds to be extracted are dissolved by the solvent.

3) The dissolved compounds are transported to the outer surface of the solid. Diffusion is the most important mechanism.

4) The dissolved compounds pass through the outer surface and a phase change may occur at that place.

5) The compounds are transported from the surface layer into the bulk of the supercritical solvent and are subsequently removed with the solvent from the bulk of the solid material. Each part of the process has to be carefully optimized in order to obtain quantitative and reproducible recoveries. Majority of time, the first step remains the most difficult to control, as solute-matrix interactions are very hard to hinder and predict.

The behavior of SFE can be assessed by determining the amount of extract against the time of extraction. This amount accumulated during the course of the extraction will in principle follow the curve shown in Figure 1.7.

While the first part of the curve (I) may be a straight line, the second part (II) is nonlinear.

The I strecht corresponds to a constant extraction rate and the II part may approach a limiting value, given by the amount of extractible substances. ([4])
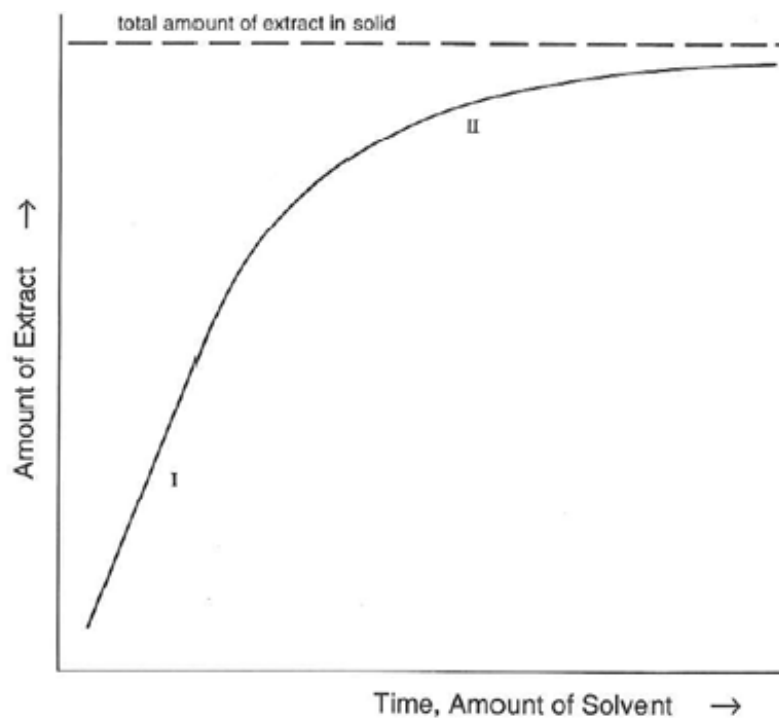
Figure 1.7: *Integral extraction curve. Total amount of extract against time of extraction.*

The basic principles for using a supercritical fluid as an extraction medium are the solubility and phase equilibrium of the substances in the compressed gas. The compounds to be extracted must be soluble in the supercritical fluid at a moderate pressure and temperature. The solubility and phase- equilibrium can be varied over a wide range by changing the pressure and temperature. Besides the solubility behavior, mass-transfer plays an essential role in extraction processes. At the beginning, the extraction efficiency is limited by the solubility in the available amount of fluid. The second phase of extraction is controlled by diffusion and this leads to long extraction times.

For the calculation of the extraction yield in the solubility phase, Brunner gives the following correlation for solid raw materials (1.2):

$$E = k_s * a_s * V_t * \Delta c_m \tag{1.2}$$

Where k_s = mass transfer coefficient [m s^{-1}]; a_s= specific interfacial area [m^2/m^3]; V_t= solid bed volume [m^3]; Δc_m= mean concentration gradient.

The mass transfer coefficient can be calculated by a correlation with Sherwood number (relation 1.3):

$$Sh = k_s * \frac{d}{D_{12}} = 2 + 1.1 * Sc^{1/3} * Re^{0.6} \qquad \text{for } 3<Re<3000 \quad (1.3)$$

Where **Sc** is the Schmidt number and *Re* the Reynolds number, defined as (1.4) :

$$Re = \frac{v*d}{v} \qquad\qquad\qquad (1.4)$$

Where v= the mean velocity of the fluid [m s^{-1}]; d= the characteristic travelled length [m]; v= the kinematic viscosity [m^2 s^{-1}]. By relation 1.3 the influence of the diffusion coefficient *D_{12}* and *Re* on the mass transfer coefficient is evident. ([11])

1.3   SFE for extracting Malathion from chickpeas

Applications of pesticides SFE from food matrices have been recently reviewed. As an example, SFE has been applied to the determination and extraction of organophosphorus pesticides in food such as wheat grains, crops, bread, flour, rice, meat, potatoes, cucumbers, apples, oranges, strawberries, cereals. The analysis of pesticides residues in food intended for human consumption or in the production of fermentation-derived chemicals, is of increasing importance because of tolerance and revised-action levels developed by regulatory agencies.

1.3.1 Pesticides

As defined by the US Environmental Protection Agency, the term "pesticide" is often used to designate "any substance or mixture of substances intended for: preventing, destroying, repelling, or mitigating any pest". ([12])

The use of pesticides increased tremendously since the 1960s and it helped reducing crop losses and improving the yield of crops such as corn, maize, vegetables, potatoes and cotton. Despite the beneficial effects of pesticides, adverse effects on environmental quality and human health have also been found. Residues of pesticides contaminate soils and water, persist in the crops, enter the food chain and finally are ingested by humans with foodstuff and water. Furthermore, pesticides can contribute to biodiversity losses and deterioration of natural habitats ([13]). Pesticides can be divided into three big groups: Fungicides, Herbicides and Insecticides. The latter includes four other types: organochloride (OC), organophosphorous (OP), carbamate and pyrethroid.

Organophosphorous pesticides (OP) are the most widely used group of pesticides in the world. They are characterized by the structural group in Figure 1.8:
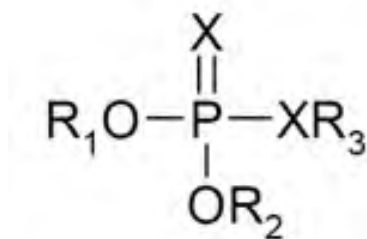
Figure 1.8: *Structural group of Organophosphorous pesticides (OP)*

where:

- X= O,S;
- R_1 and R_2 = alkyl groups (Me, Et);
- R_3 = electron donor group.

OP are moderately polar and are organic esters of phosphoric acid ($O=P(OH)_3$), thiophosphoric acid ($C_3H_9O_3PS$) and other phosphoric acids. They have been widely used in agricultural fields to control pests, weeds, diseases and to increase harvest productivity for the last five decades. However, they are toxic, due to the prevention of neural impulse transmission by their inhibition of cholinesterase. They are dangerous and more toxic for human and mammals health than organochlorine compounds. The researchers have recently found that most toxic incidents are linked to the intoxication of OP, especially OP residues in vegetables, fruits and cereals; the dangerous exposition is possible by inhalation, ingestion and absorption in skin. The European Union (EU) legislation has established maximum residues limits (MRL) for the pesticides. ([14])

Three types of OP can be distinguished:

1. Type A where X=O
2. Type B where X =S for the double bound and X=O for the single bound
3. Type C where X= S

Malathion belongs to this last group.

The structural formula of Malathion is in Figure 1.9
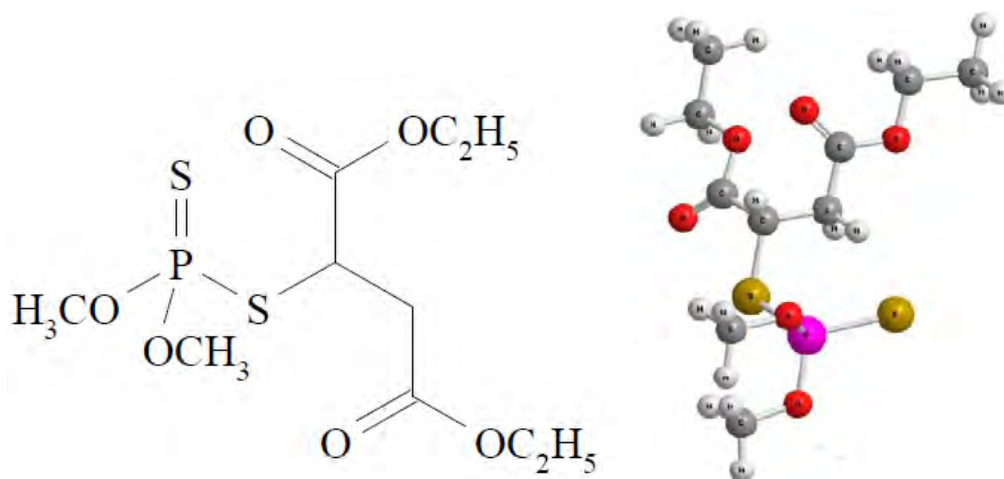
Figure 1.9: *Strctural formula of Malathion*

Its main charachteristics are listed in Table 1.1([15]):

Table 1.1 *Main characteristics of Malathion*

ISO common name	Malathion
IUPAC name	O,O- dimethyl dithiophosphate of diethyl mercaptosuccinate
Molecular formula	$C_{10}H_{19}O_6PS_2$
Molecular Weight	330.36
Appearance	Colourless to amber liquid with a skunk-or garlic-like odor.
Melting Point	2.85°C
Vapor Pressure	5.3 mPa at 30°C
Specific Gravity	1.23 at 25°C
Henry's Law Constant	4.89×10^{-9} atm/m^3 mol
Water Solubility	148.2 mg/l at 25°C
Soluble	Readily soluble in hydrocarbons, esters and alcohols. Moderately soluble in aliphatic hydrocarbons (62 g/l in n-hexane)
Octanol-Water Partition coefficient (log K_{ow})	2.75; 2.36-2.89
Thermal stability	Stable at ambient temperatures (below 25°C); decomposes rapidly at temperatures above 100°C
Stability	Stable for at least two years when stored at ambient temperatures in the unopened original container.
Min. purity	95%.
Main impurities	*O,O,S*-trimethyl phosphorothioate.(Malaxon)

Malathion is an Organophosphorous pesticide first registered in 1956 by the United States Departament of Agriculture (USDA). It is now regulated by the United States Environmental Protection Agency (U.S.EPA).

Malathion has numerous commercial agricultural, industrial, governmental, and homeowner uses. It is used to control a variety of outdoor insects in both agricultural and residential settings and it is registered for use on food, feed, ornamental crops in mosquito and fruit fly. Malathion is also a shampoo ingredient regulated by the United States Food and Drug Administration (FDA) to control head lice and to kill fleas on pets.

It kills insects by preventing their nervous system from working properly. Like other Organophosphorous pesticides, it binds to the enzyme Acetylcholinesterase (AChE) at nerve endings throughout the bodies of insects and other organisms. Under normal circumstances, AChE binds with the neurotransmitter Acetylcholine (ACh) at the nerve junction, ending effectively the stimulation of the next neuron. When AChE is bound by malathion's metabolite Malaoxon, ACh accumulates at the nerve junction and results in overstimulation of the nervous system. This causes the nerves to signal each other without stopping. The constant nerve signals make it so the insects can't move or breathe normally until they die.

Malaxon is Malathion's metabolite due to the conversion of the latter, under certain conditions, via oxidation of the P=S bond to P=O. Its structural formula is in Figure 1.10
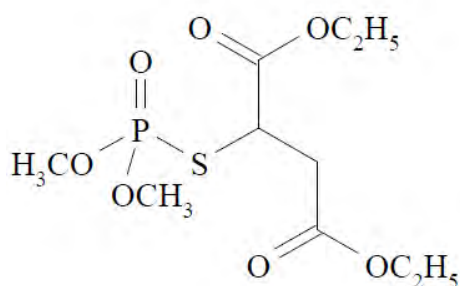
Figure 1.10: *Structural formula of Malaxon*

Malathion is considered toxic via skin contact, ingestion, and inhalation exposure. It can become more toxic if it has been sitting for a long time, especially in a hot place.

Nevertheless, the general population is not likely to be exposed to high levels of it: exposition occurs through ingestion of contaminated food or water and affects especially people living near areas where Malathion is sprayed. They are under a greater risk of exposure through dermal contact with contaminated plants and soils, inhalation of mist formed during application, and ingestion of residues in food or water. Also workers involved in the production, formulation, handling, and application of Malathion are likely to have the highest levels of exposure. For example, farm workers who enter treated fields prior to the passage of the appropriate restricted entry intervals may also be exposed to high levels of it.

In Table 1.2 the toxicity classification of Malathion is reported.

Table 1.2 : *Toxicity classification of Malathion ([16])*

*A common measure of acute toxicity is the lethal dose (LD_{50}) or lethal concentration (LC_{50}) that causes death (resulting from a single or limited exposure) in 50 percent of the treated animals. LD_{50} is generally expressed as the dose in milligrams (mg) of chemical per kilogram (kg) of body weight. LC_{50} is often expressed as mg of chemical per volume (e.g., liter (L)) of medium (i.e., air or water) the organism is exposed to. Chemicals are considered highly toxic when the LD_{50}/LC_{50} is small and practically non-toxic when the value is large. However, the LD_{50}/LC_{50} does not reflect any effects from long-term exposure (i.e., cancer, birth defects or reproductive toxicity) that may occur at levels below those that cause death.

	High Toxicity	*Moderate Toxicity*	*Low Toxicity*	*Very low Toxicity*
Acute Oral LD $_{50}$ *	≤ 50 mg/kg	>50-500 mg/kg	>50-500 mg/kg	>5000 mg/kg
Inhalation LC_{50}*	≤ 0.05 mg/L (aereosol)	>50-500 mg/L	>0.5-2 mg/L	>2 mg/L (dust)
Dermal LD_{50}*	≤ 200 mg/kg	>200-2000 mg/kg	>2000-5000 mg/kg	>5000 mg/kg
Primary Eye Irritation	Corrosive (irreversible destruction of ocular tissue) or corneal involvement or ittitation persisting for more than 21 days	Corneal involvement or other eye irritation clearing in 8-21 days	Corneal involvement or other eye irritation clearing in 7 days or less	Minimal effects clearing in less than 24 hours
Primary Skin Irritation	Corrosive (tissure destruction into the dermis and/or scarring)	Severe irritation at 72 hours (severe erythema or edema)	Moderate irritation at 72 hours (moderate erythema)	Mild or slight irritation at 72 hours (no irritation or erythema)

People who were exposed to enough Malathion to become sick felt nauseated or vomited, had muscle tremors, cramps, weakness, shortness of breath, a slowed heart rate, headache, abdominal pain and diarrhea.

About its carcinogenicity, researchers found no evidence of increased cancer in the treated animals. Other studies using higher doses of Malathion in rats and mice found that they developed liver cancer. The United States Environmental Protection Agency (EPA) has determined that there is a "suggestive evidence of carcinogenicity but not sufficient to assess human carcinogenic potential by all routes of exposure", for Malathion.

There were no studies showing that children are more sensitive to Malathion than adults. While children may be especially sensitive to pesticides compared to adults, there are currently no data showing that children have increased sensitivity specifically to it.

Malathion enters the environment from spraying on crops or for public health in urban or nonresidential areas.

Most of Malathion will stay where it is applied, but some may be distributed to other locations by rain, fog or wind. Bacteria in the soil may break down Malathion and sunlight can break down it in the air. It will mix with water and can move quickly through soil.

Because of these properties, it can be found in surface waters such as streams, and sometimes it is found in well water. The time it takes for Malathion to break down to half of the original amount (half-life) in soil is about 17 days, depending on the soil type. In water, Malathion has a half-life between 2 and 18 days, depending on conditions like temperature and pH. Its vapor may also move long distances in air or fog. ([16])

In EU Pesticides Database, the MRL values of products are reported, and some of them are listed in Table 1.3. The Agency for Toxic Substances and Disease Registry (ATSDR) in 2003, decided the environmental levels admitted, listed in Table 1.4 .([17,18])

Table 1.3 *: Pesticides maximum residue levels from Eu Pesticides Database*

Groups and examples of individual products to which the MRLs apply (a)	*Malathion (sum of malathion and malaoxon expressed as malathion)* *Pesticide residues and maximum residue levels (mg/kg)*
1. Fruit fresh or frozen nuts	0.02*
2. Vegetables fresh or frozen	0.02*
3. Pulses, dry	0.02*
4. Oilseeds and oilfruits	0.02*
5. Cereals	8
6. Tea, Coffee, Herbal Infusions and Cocoa	0.5
7. HOPS (dried)	0.02*
8. Spices	0.02*
9. Sugar plants	0.02*
10. Products of animal origin- terrestrial animals	0.02*

(*) Indicates lower limit of analytical determination

Table 1.4 *: Environmental levels of Malathion*

Medium	*Malathion level admitted*
Air	In samples collected along the Mississippi River was 0.23 ng/m^3 with a range of 0.14–4.6 ng/m^3.
Sediment and soil	In soil samples from residential areas after aerial spraying was estimated to be 1.4 μg/g in subsurface soil (1 cm) and 14.1 μg/g in surface soil.
Water	Concentrations in groundwater in 3 states ranged from 0.007 to 6.17 μg/L.

1.3.2 SFE of pesticides literature

Since the end of the '80s, the application of SFE to pesticide residue analysis has been demonstrated for some pesticides in sediment, soil and agricultural products. In the past few years, publications have appeared on this type of extraction applied to different matrices.

The matrix used in the extraction publications about the extraction of Organophosphorous pesticide and in particular Malathion, can be classified in four big groups:
- Fruits and vegetables;
- Soils;
- Biological tissues;
- Other applications.

The main characteristics of SFE of Organophosphorous pesticides from fruits and vegetables, written in some of past publications, are reported in Table 1.5.

From the papers listed in Table 1.5 is possible to identify the main ranges of operating variables:
- Regarding the pretreatment of the matrix, it was sometimes grinded and/or dried (with lyophilization or addition of drying components) to remove water presented. Other times the presence of water is used as a modifier; it depends on the matrix and on the pesticide.
- The pressure range goes from 120 to 690 bar, a large range depending on the matrix, the pesticide and the equipment used.
- The temperature is in the range of 40- 80°C, always above the critical temperature of CO_2.
- The time of extraction is quite low: never more than 60 min.
- Finally, the presence of modifier could be important due to the polarity of the Organophosphorous pesticides that need extraction. The most used modifiers are Methanol and Acetone, but water is used too.

Table 1.5 was the starting point to the operating conditions choice of extraction of Malathion from chickpeas, reported in Chapter 3.

Table 1.5 *Main characteristics of SFE of organophosphorous pesticides from fruit and vegetables.*

Matrix belonged to fruit and vegetables	Pre-treatment	Best SFE conditions					Reference
		P [bar]	T [°C]	Flow rate of CO_2 [mL min^{-1}]	t [min]	Modifier	
Wheat, bread, flour, rice, potatoes, cucumbers, tomatoes, apple, orange	Mixed with hydromatrix to obtain powered sample	123-202	50-80	3	3 static time + 30 dynamic time	-	[19]
Strawberries	-	276	50	1-1.5	20	Acetone in hexane	[20]
Wheat, maize, rice	-	245	70	1	5 static time + 35 dynamic time	-	[21]
Wheat flour	-	206.8	60	0.7-1.4	60	-	[22]
Cereals, strawberries, fruit and vegetables	Grinding and lyophilized	200-214	50-60	1.2 - 2- 3	5 static time + 10 dynamic time	10% Methanol-10% Acetone	[23]
Tomato	20 g blended fresh vegetable sample with 28 g of anhydrous magnesium sulfate	300	50	15 mL CO_2	1 static time	Methanol	[24]
Grain	-	137-344-689	40-60-80	5	20-30	-	[25]
Meat,grain	-	680--250	50-80	10	20 min dynamic	-	[26]
Grain, potatoe	-	250 -350 -320	40-50-60	0.84	15 static time +30 dynamic -2 static time +10 dynamic time	-	[27]
Gazpacho	-	300-350	50-60	-	-	200 μL of Methanol as static modifier	[1]

1.4   SFE for extracting phenolic compounds from asparagus

Extracts from aromatic herbs, spices and medicinal plants are used to impart flavor and functional properties to food products. Some of the properties presented by plant extracts include antioxidant, antiulcerogenic, antimalarial, anticancer and anti-infiammatory, among others. In particular, antioxidants are substances that are able to prevent or retard the oxidation of lipids, proteins and DNA and they also protect the compounds or tissues from damage caused by oxygen or free radicals. For these reasons their health promoting effects reduce the risk of various diseases and their recovery from food or by- products of food processing plants has gained importance. The antioxidant activity of plant extracts is mainly attributed to the presence of phenolic compounds, both volatile and non- volatile. ([28, 29]). Therefore, another application of SFE in food matrices could be the extraction of these phenolic compounds from Asparagus.

1.4.1 Asparagus and phenolic compounds

There are eight type of asparagus presented and diffused in Italy. The most important and used one is *Asparagus officinalis* (Figure 1.11). It belongs to the Liliaceae family, which is a plant family numbering several thousand species of as many as 300 genera, widely distributed all over the earth and particularly abundant in warm, temperate and tropical regions. They are native to the East Mediterranean area and now naturalized over a big part of the world.

Asparagus has been considered a type of food that can promote good health, since ancient Grecians and Romans, who used it for its diuretic properties. It helps flush out the kidneys and prevent the formation of kidney stones. Hippocrates highly regarded asparagus as a medicinal plant that was used for treating all kinds of conditions from toothaches to healing certain types of cancer. Chinese pharmacists save the best Asparagus roots for their families and friends, believing that it will increase feelings of compassion and love. In India, it is used to promote fertility, reduce menstrual cramping, and increase milk production in nursing mothers. This plant contains vitamins A, B1, B2, C and E, magnesium, phosphorus, calcium, iron and folic acid. Its stems is rich in the amino acids asparagine, tyrosone and arginine, plus succinic acid and a methylsulfonium derivative of methionine. Other constituents of asparagus include essential oil, flavonoids (kaempferol, quercitin, rutin), resin, and tannin. Two sulfur-bearing mercaptans, S-methylthioacrylate and S-methyl 3-(methylthio) thiopropionate accounts for the distinctive odor of the urine shortly after consuming a mass of the sterm. ([30])

Figure 1.11: *Asparagus officinalis*

In Table 1.6a and b, nutritional data and soluble sugar of its spear are reported. The values are the results of a study ([31]), are means (± SD) of triplicate analyses (n=3) and are expressed on a fresh weight basis.

Table 1.6: *(a) Nutritional data of Asparagus Officinalis spear (b) Soluble sugar in the Asparagus Officinalis spear*

(a)

Moisture [g/100 g]	Proteins [g/100 g]	Lipids [g/100 g]	Phenols [mg/100 g]	Folic acid [g/100 g]	Ascorbic acid [mg/100g]
92.20 ±4.61	3.62±0.09	0.33 ±0.01	27.62 ±1.11	80.6 ±4.03	23.05±1.15

(b)

Ribose [g/100 g]	Arabinose [g/100 g]	Xylose [g/100 g]	Fructose [g/100 g]	Mannose [g/100 g]	Glucose [g/100 g]	Galactose [g/100 g]
0.15 ±0.01	0.39±0.06	0.14±0.01	0.17±0.01	0.49 ±0.02	1.21±0.07	0.19±0.01

In this type of asparagus, as it is written in the same study ([31])and others([32]), some of the phenols that can be found are: Cinnamic acid; Syringaresinol; Salicylic acid; Benzoic acid; Ferulic acid; Coumaric acid; Syringic acid; p-hydroxybenzoic acid; catechecol; prothocathecuic acid; Gallic acid; Caffeic acid; Chiorogenic acid; Catechin; Isoquercetrin; rutin. Their structural formulas are represented in Figure 1.12:
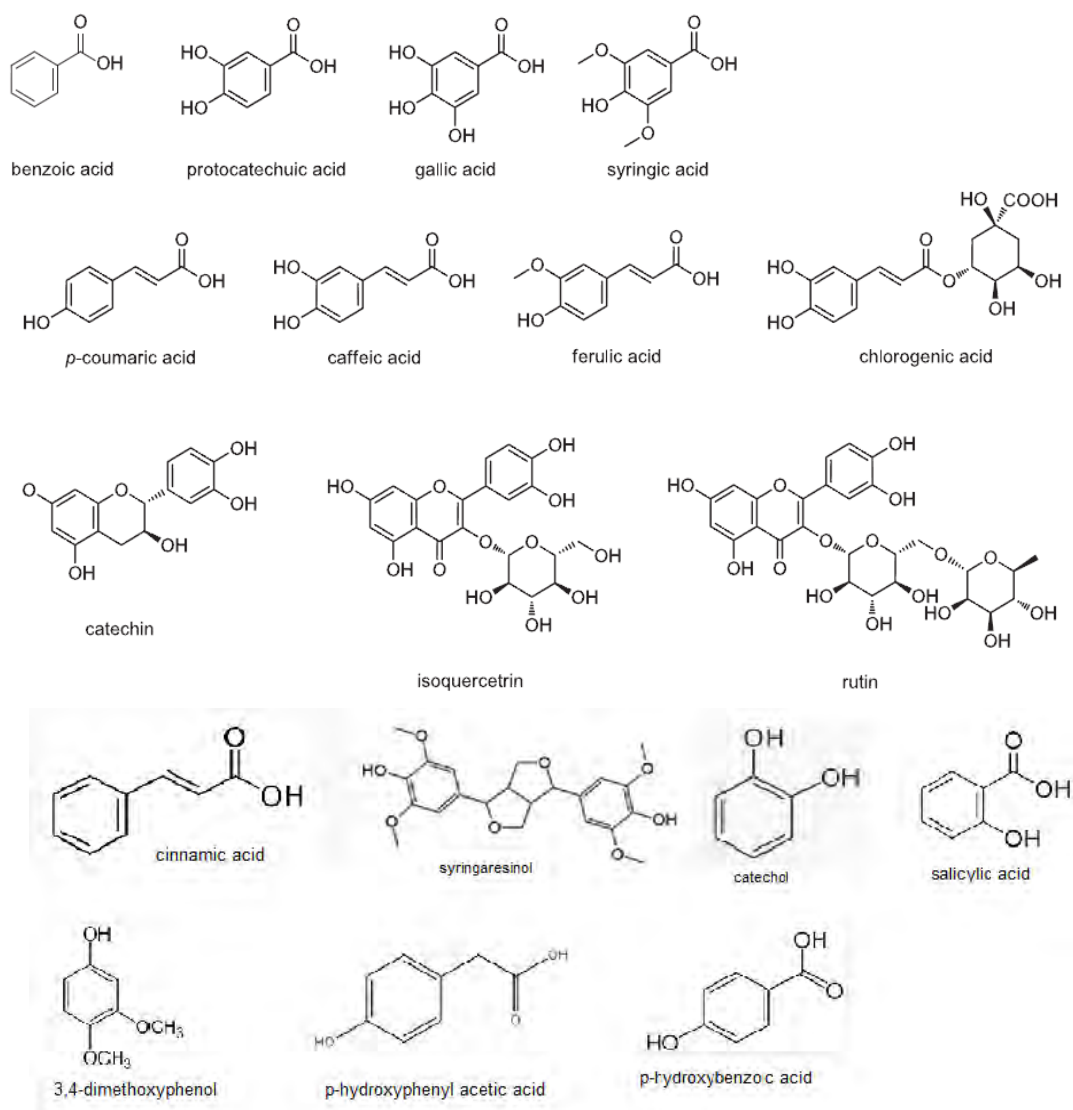
Fig 1.12: *Structures of the main phenolic compounds presented in Asparagus Officinalis*

Figure 1.13 shows the percentage of phenolic components of *Asparagus Officinalis* reported by Ferrara et al. ([31]).
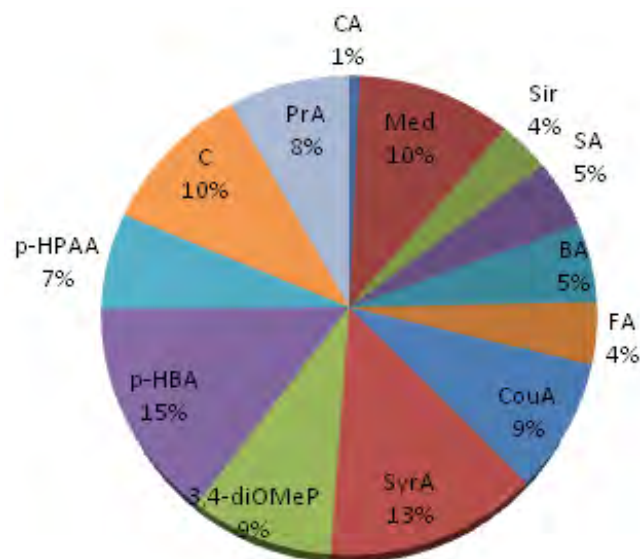
Figure 1.13: *Phenolic compounds of Asparagus Officinalis. Values are reported as mg/100 g fresh weight ±SD.*
CA= cinnamic acid; Med= mediaresinol; Sir=siringaresinol; SA=salicylic acid; BA=benzoic acid;
FA= ferulic acid; CouA= coumaric acid; SyrA= syringic acid; 3,4-diOMeP=3,4-dimethoxyphenol;
p-HBA= p-hydroxybenzoic acid; p-HPAA= p-hydroxyphenyl acetic acid; C=cathecol;
PrA=prothocathecuic acid.

Chemical structures of the different classes of polyphenols are represented in Figure 1.14.
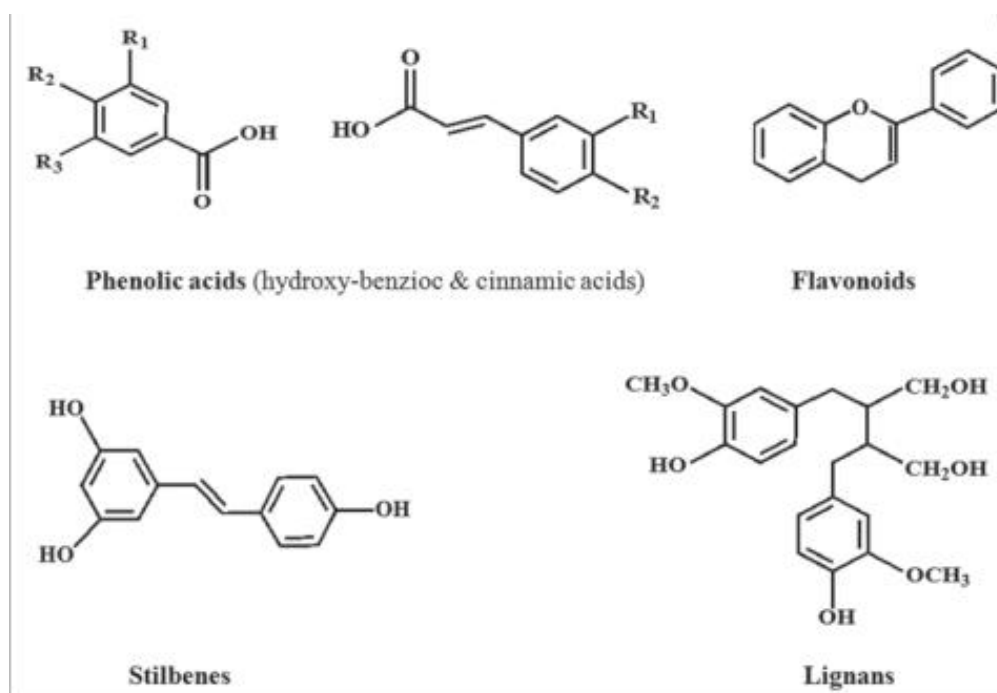
Figure 1.14: *Chemical structures of the different classes of polyphenols.*

Polyphenols are classified on the basis of the number of phenol rings that they contain and of the structural elements that bind these rings to one another. They are broadly diveded in four classes; phenolic acids, flavonoids, stilbenes and lignans, as it represented in Figure 1.14.

Phenolic acids are further divided into hydroxyl benzoic and hydroxyl cinnamic acids. Phenolic acids account for about a third of the polyphenolic compounds in our diet and are found in all plant materials, but they are particularly abundant in acidic-tasting fruits. Caffeic acid, gallic acid, ferulic acid are some common phenolic acids. Flavonoids are a class of plant secondary metabolites, that are mostly distributed in fruits, vegetables and the leaves of herbal plants. Their name derives from the Latin word "flavus" meaning yellow (their colour in nature) due to be the most important plant pigments for flower coloration. They are polyphenolic compounds that are often added to medicines as antioxidants. Their nuclear structure comprises two benzene rings connected by a pyrene ring containing oxygen. Usually, as it is represented in Figure 1.15 ([33]), they are classified into sub groups including flavonols, flavones, flavanols, flavanones, and isoflavones based on the additional presence of a C2-C3 double bond, hydroxyl, methoxy groups, glycoside, and different positions of molecules.

Stilbenes contain two phenyl moieties connected by a two-carbon methylene bridge. Most stilbenes in plants act as antifungal phytoalexins, compounds that are synthesized only in response to infection or injury. Lignans are di-phenolic compounds containing a 2,3-dibenzylbutane structure that is formed by the dimerization of two cinnamic acid residues. ([34])

Figure 1.15 : *Structures of the major classes of flavonoids*

The antioxidant properties of polyphenols have been widely studied.

Despite the distribution of polyphenols in plants, the health effects of dietary polyphenols have come to the attention of nutritionists only rather recently. Until the mid-1990s, the most studied antioxidants were antioxidant vitamins, carotenoids, and minerals. Only after 1995, as it can be seen in Figure 1.16, researches on flavonoids, other polyphenols, their antioxidant properties, and their effects in disease prevention began. ([35])
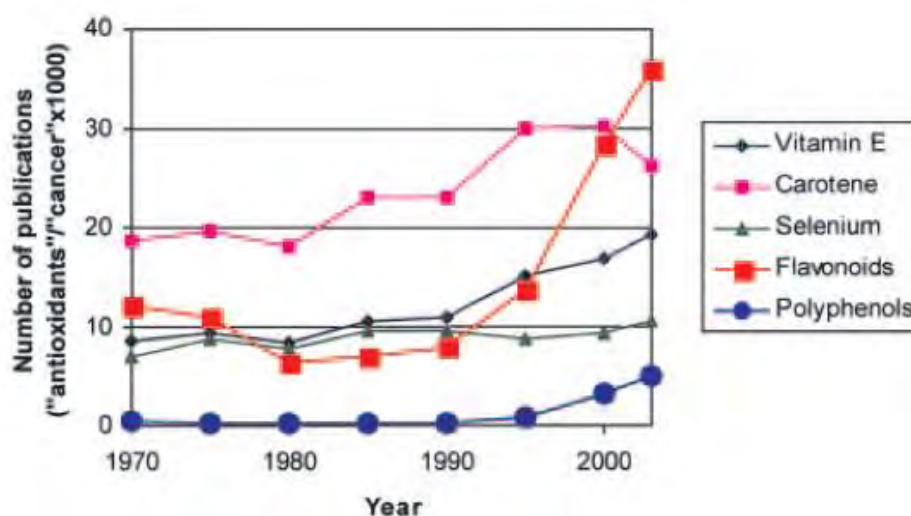
Figure 1.16: *Increase in the number of publications regarding various antioxidants in the past 30 y. Publications were those registered in the Medline database. Values were determined as follows: number of results from the query "compound X" AND "year n"/number of results from the query "cancer" AND "year n"_1000. The key word cancer was used as a reference, to take into account the general increase in the number of publications. ([35])*

Polyphenols, in general, are molecules which are secondary metabolites of plants. They are generally involved in defense against ultraviolet radiation or aggression by pathogens and may also contribute to the bitterness, astringency of the food.

Towards the end of the 20th century, epidemiological studies and associated meta-analyses strongly suggested that long term consumption of diets rich in plant polyphenols offered some protection against development of several chronic diseases such as cardiovascular diseases (CVDs), cancer, diabetes, infections, aging, asthma, osteoporosis and neurodegenerative diseases etc (Figure 1.17). Researchers have explored that these molecules are very good antioxidants and may neutralize the destructive reactivity of undesired reactive oxygen/nitrogen species produced as byproduct during metabolic processes in the body. ([34])
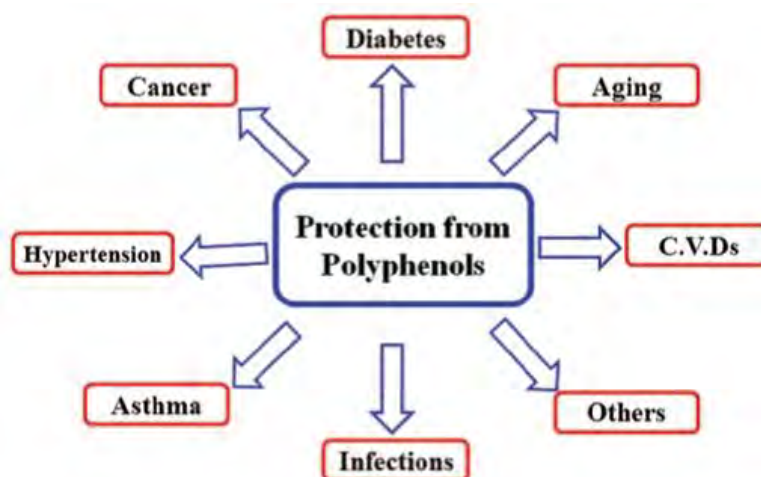
Figure 1.17: *Health beneficial effects of dietary plant polyphenols*

1.4.2 SFE of polyphenols literature

The applications of SFE to extract components from food and plant, especially pholyphenols, have been studied since the born interest in them. The main characteristics of extractions written in past publications are listed in Table 1.7

Table 1.7 *Main characteristics of SFE of polyphenols from fruit and vegetables*

Fruit and vegetables	Best SFE conditions				References
	P [bar]	**T [°C]**	**t [min]**	**Modifier**	
Apple and peach pomaces	546-570 -506-510	55.7-58.4-50.9-52.3	40	20% Ethanol	[29]
Jatoba (Hymenaea courbaril L.)	350	50	-	CO_2:Water 9:1	[28]
Pomegranate (Punica granatum L.) leale	300	50	-	-	[36]
Grape peel	137-147-157-167	37-43-46	30	Ethanol 5-6-7-8%	[37]
Guava seed	100-200-300	40-50-60	120	Ethyl acetate, Ethanol 10%	[38]
Grape pomace	80-350	35-50	60	Ethanol (0-8%)	[39]
Apple and peach pomaces	200-600	40-60	10-40	Ethanol (14-20%)	[29]
Wine industry waste (red grape pomace)	100-150-250	45	20	Methanol (5%)	[40]
Green tea leaves	310	60	-	Ethanol (18-70-95-99-8%)	[41]
Guava seeds	100-200-300	40-50-60	120	Ethyl acetate and ethanol (10%)	[42]
Olive leaves	155-334	80-100-120	5-140	Methanol and Ethanol(0-20%)	[43]
Pistachio hulls	100-200-350	35-45-55	15-25-40	Methanol (0-5-15%)	[44]
Rosemary leaves	350	100	40	Methanol (5%)	[45]
Wheat germ	148-602	40-60	10-60	-	[46]

From Table 1.7 is possible to identify the main ranges of operating variables.

- First of all the presence of a modifier is essential due to the polarity of polyphenols: the most used modifiers are methanol and ethanol, but also water is used.

- For what concerns the pressure range, it goes from 80 to 570 bar. It is a large range depending on the matrix and the modifier used.
- The temperature utilized is inside the interval of 35- 120°C.
- The time of extraction is never more than 120 min.

Table 1.7 was the starting point for the choice of operating conditions of extraction of polyphenols from asparagus, reported in Chapter 4.

Chapter 2

Materials and methods

The work is divided into two parts. The first one is about the supercritical extraction of Malathion from chickpeas. The supercritical extraction tests and analysis were done in Prof. Calvo's laboratory located in the Chemical Engineering department of University Complutense de Madrid. The second part concerns the extraction of phenols from asparagus. The respective experiments and analysis were conducted in Prof. Bertucco's high-pressure laboratory located in the Industrial Engineering department of Università degli studi di Padova.

The equipments used in both parts of the work and the analysis methods applied are described below.

2.1 Supercritical fluid extraction of Malathion from chickpeas

The chemicals and reagents, the way and type of contamination, the apparatus used for the extraction of Malathion from chickpeas and the successive analyses are described below.

2.1.1 Chemicals, reagents and contamination

Malathion (99.5%, Chem Service Inc.), glacial acetic acid (analytical grade, Scharlau), acetone (≥99.5%, Sigma Aldrich), potassium bromide (reagent grade, Scharlau), amaranth (85%, Acros Organics), N-bromosuccinimide (NBS) (99%, Acros Organics), hydrochloric acid (37% Scharlau).

2.1.1.1. Stock solution preparation

Malathion stock solutions were prepared mixing Malathion in milli-q water and a little bit of glacial acetic acid (necessary to make the solution a little acid to permit the dissolution of Malathion). The concentrations used were 100 and 110 ppm. There were stored at 4°C.

To simulate a food matrix contaminated by Malathion, chickpeas were chosen. They were bought in a classical supermarket (Alipende) and are illustrated in Figure 2.1.

They were used without being milled previously, because the idea was to decontaminate chickpeas with the extraction and make them edible food.

 Also, Malathion was spiked on silicon balls of a diameter equal to 0.1 cm, to choose the best method for chickpeas contamination.

Figure 2.1: *Chickpeas contaminated*

2.1.1.2. Silicon balls contamination

At first, a contamination of silicon balls was carried out. Thirty grams of silicon balls were contaminated at the same theoretical concentration (30 ppm) but in different ways: drop wise, by spraying and by immersion using Malathion stock solution of 100 ppmv.

2.1.1.3. Chickpeas contamination

Then, chickpeas were contaminated. They were put into a flat pan. Then, the calculated volume of Malathion stock solution was sprayed on top of the chickpeas surface. They were placed on the sun to allow the complete evaporation of the water. In all cases, a Malathion stock solution of 110 ppmv was used.

To determine the initial and remaining amount of Malathion after the supercritical extraction, 20 mL of acetone were added to 20 g of chickpeas and the mixing was made by an ultrasonic bath (Elmasonic S 15 H, Elma), for 15 minutes and at room-temperature. Then, the samples were evaporated to dryness in rotary evaporator at room pressure, fixing the water bath temperature at 60°C (R-210, Buchi, Figure 2.2). The time to permit the complete evaporation of the solvent was more than 12 hours. The residue was dissolved in 1 mL of acetone, filtrated through 0.22 μm membrane, and then directly analyzed by GC–MS.

Figure 2.2: *Rotavapor used*

2.1.2 Extraction

The extraction of Malathion from chickpeas was carried out using the plant schematized in Figure 2.3. A picture of the pilot plant employed is shown in Figure 2.4.

First of all it was necessary to learn how to work under high-pressure and with this equipment. A protocol for operation was defined. (Appendix A).

The SFE plant is a semi continuous flow apparatus consisting of a CO_2 feeding line, a 0.1 L capacity 316 SS extractor, a pressure control device and a sample collection system. The laboratory scale system was previously described in other works ([47]). First, the CO_2 (99% by Carburos Metálicos S.A) is fed from the bottle in liquid phase, then cooled in a temperature-controlled bath (Selecta, Frigiterm-30) to 263 K to avoid cavitation and impelled by a cooled-head membrane pump (Milroyal D, Dosapro Milton Roy). The pressurized CO_2 is pre-heated in a coil placed inside a heating jacket, prior to entering the extractor. The extractor is also thermally conditioned by another heating jacket, both with a temperature control of $\pm 1/2$ K and recorded by a type K thermocouple located inside the reactor in direct contact with the fluid and the solid. Pressure is read by a Bourbon manometer with an accuracy of ± 3 bar. The controls of the pressure and flow rate are achieved by a heated micrometering valve (Tescom, Serie 26-1700) and the pump, respectively. A safety rupture disk, set at 380 bar, avoids the pressure from exceeding the prescribed values. The amount of CO_2 per unit of time is determined in a mass flow meter (Alicat Scientific, M-10SLPM-D) connected at the end of the line with an accuracy of ± 0.5 g min^{-1}. An activated carbon bed was placed after the BPR, to adsorb the extracted Malathion.

The extractor used is stainless, type 3106, seamless, designed in 2012, following the legislation ASME section VIII (division 1) -code. Other specifications are reported in Appendix B.

The solid sample (chickpeas contaminated by Malathion) was put inside the extractor with a small amount of cotton at the top and the extractor was closed and pre-heated. After that, the CO_2 was pumped in, and, once the desired pressure was reached, the back pressure regulator (BPR) was opened, providing a continuous flow through the bed.

When the established treatment time was over, the apparatus was depressurized and the total amount of CO_2 circulated was read by the mass flow meter. Then the extractor was removed from the plant, and the matrix (chickpeas) inside the extractor was analyzed.

To control the temperature inside the extractor, a control system temperature calibration was done between the set temperature, the one inside the extractor and the one relating to the heating jacket. It is reported in Appendix C.

The extraction tests were made changing temperature, pressure, time of extraction, flow rate of CO_2, type and quantity of modifiers, to find the optimum values of the main parameters.
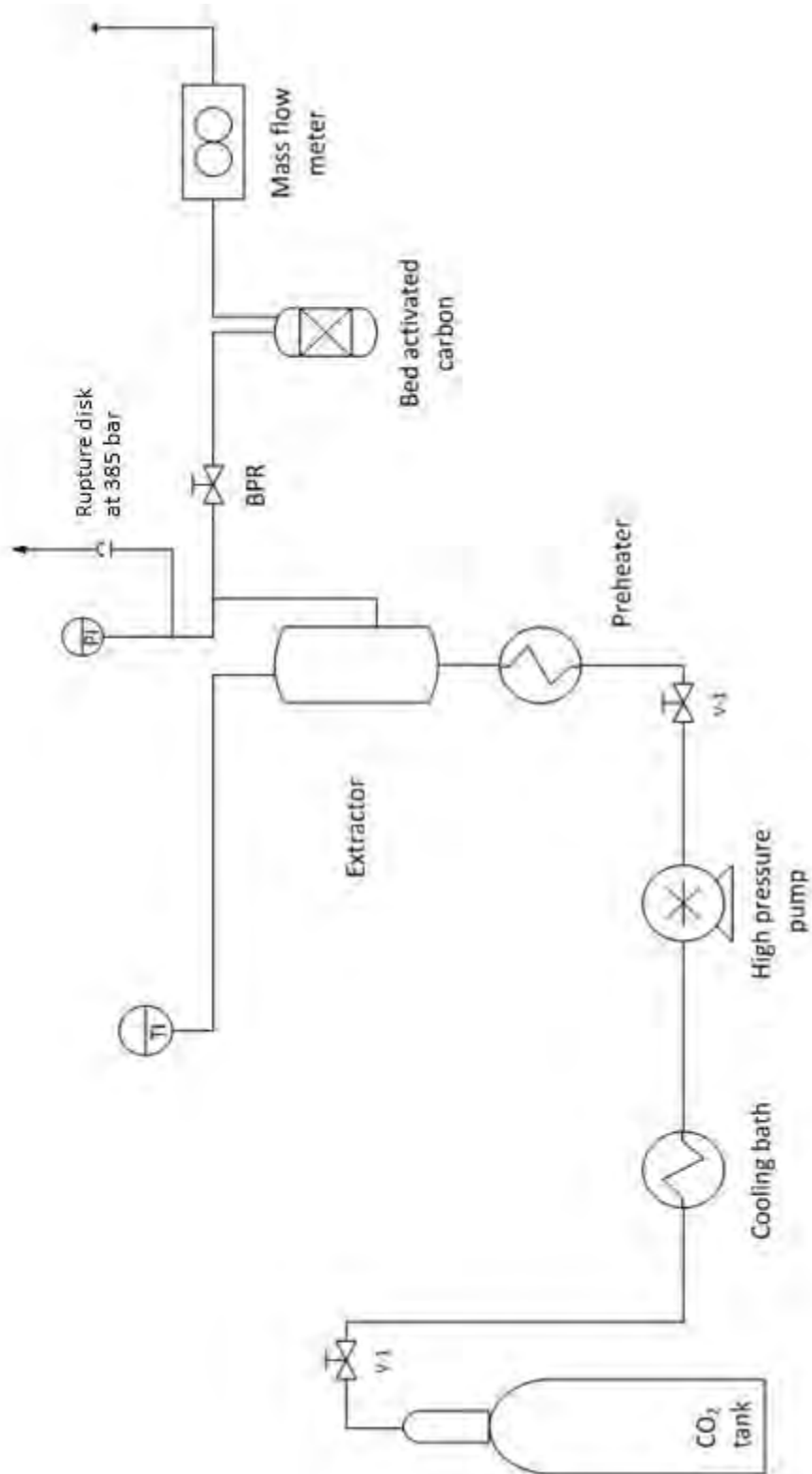
Figure 2.3. *Supercritical fluid extraction equipment used to perform the extraction of Malathion from chickpeas*
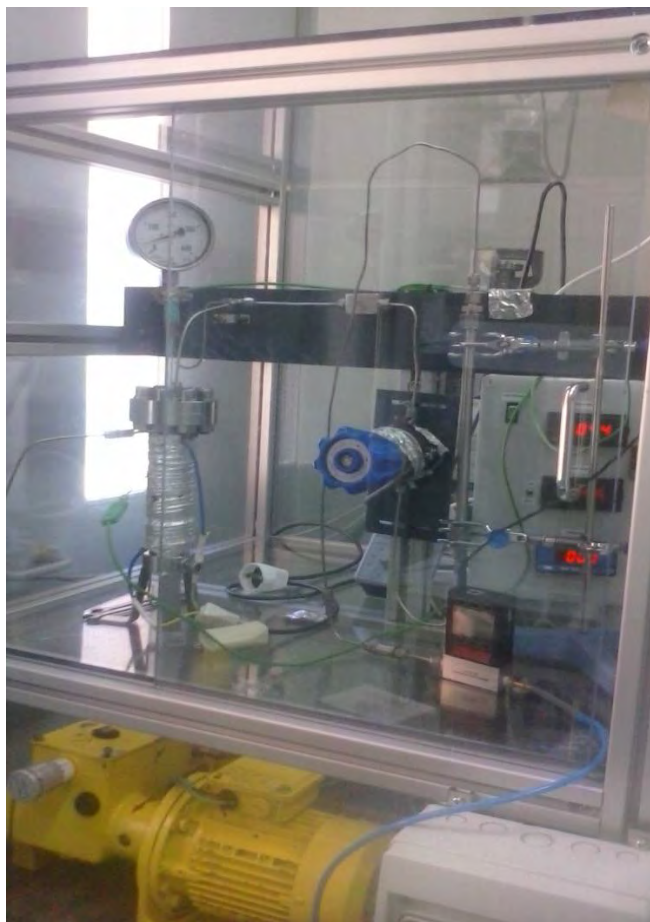
Figure 2.4. *Picture of the pilot plant used to perform the extraction of Malathion from chickpeas*

2.1.3 Analysis

The removal of Malathion after the supercritical extraction was determined by spectrophotometry and gas chromatography.

2.1.3.1 Spectrophotometric analysis

The spectrophotometric analysis was conducted, based on the previous studies made by Ayman A. Gouda et al. (2010) ([48]) It was used a Spectrophotometer, type MRC V-1600/1800 UV 1600/1800. The following solutions were prepared: an aqueous solution of 0.01% (w/v) N-bromosuccinimide (NBS) that it had to be fresh, one of 4.0 M hydrochloric acid , one of 1.0% KBr and one of 1.0×10^{-3} M amaranth (AM).

First of all it was necessary to prepare a calibration, according to the steps listed below.

An aliquot of the sample solution containing known concentration of Malathion was transferred into a series of 25 mL calibrated flasks, of which 3.0 mL consisted of 0.01% (w/v) NBS, 2.0 mL of hydrochloric acid (4.0 M); also 2.0 mL of KBr were added; the solution was kept aside- with occasional shaking- for about 10 min at ≈25- 30°C and then 0.75 mL of AM

were added. The volume was completed to 25 mL with water. The absorbance was measured at λ_{max} of 520 nm. A blank without insecticide (dye and NBS) and the dye (devoid of insecticide and NBS) were prepared in a similar manner and then absorbances were measured against milli-q water. A decrease in absorbance, corresponding to the consumed oxidant which reflects the insecticide concentration, was obtained by subtracting the decrease in absorbance of the test solution (dye minus test) from that of the blank solution (dye minus blank). A calibration graph was prepared by drawing the decrease in absorbance of the dye (ΔA) against the amount of the insecticide, for some known concentrations.

Table 2.1 shows the absorbance measured at different concentrations of Malathion.

Table 2.1. *Absorbance measured in different concentration of Malathion*

Concentration [ppm]	Average absorbance	Standard deviation	Dye- test	(Dye-blank)-(dye-test)= ΔA
10	0.767	0.009	-0.020	0.725
6	0.742	0.002	0.006	0.700
5	0.621	0.001	0.127	0.579
4	0.533	0.002	0.215	0.490
3	0.409	0.001	0.339	0.366
2	0.263	0.001	0.485	0.221
1.5	0.095	0.001	0.653	0.053
1.25	0.085	0.001	0.663	0.042
1	0.055	0.001	0.693	0.012
0.8	0.066	0.001	0.682	0.024
0.5	0.048	0.001	0.700	0.006
0.2	0.044	0.001	0.704	0.001
References				
Only dye (dye)	0.748	0.002		
Dye+NBS (blank)	0.043	0.001		
water	0.036	0.001		

The measured absorbance corresponding to a concentration of 10 ppm was higher than the dye one. So the detection range of the spectrophotometric method was 0-6 ppmv.

To determine the contamination of Malathion in the silicon balls by this method the procedure was the following: the balls were washed with 150 mL of milli-q water and a small quantity of glacial acetic acid. They subsequently passed through a colander and a vacuum filter and, lastly 17 mL of this solution were put inside of a 25 mL calibrated flasks. After this HCl, NBS, KBr, AM and milli-q water were added.

Figure 2.5 represents the correlation and the calibration line of the spectrophotometer.

The absorbance was measured at different concentrations of Malathion and each measure was made three times. The differences on absorbance are evident by looking at the color of the solutions in Figure 2.6. The red color was visible until 1.25 ppmv more or less.
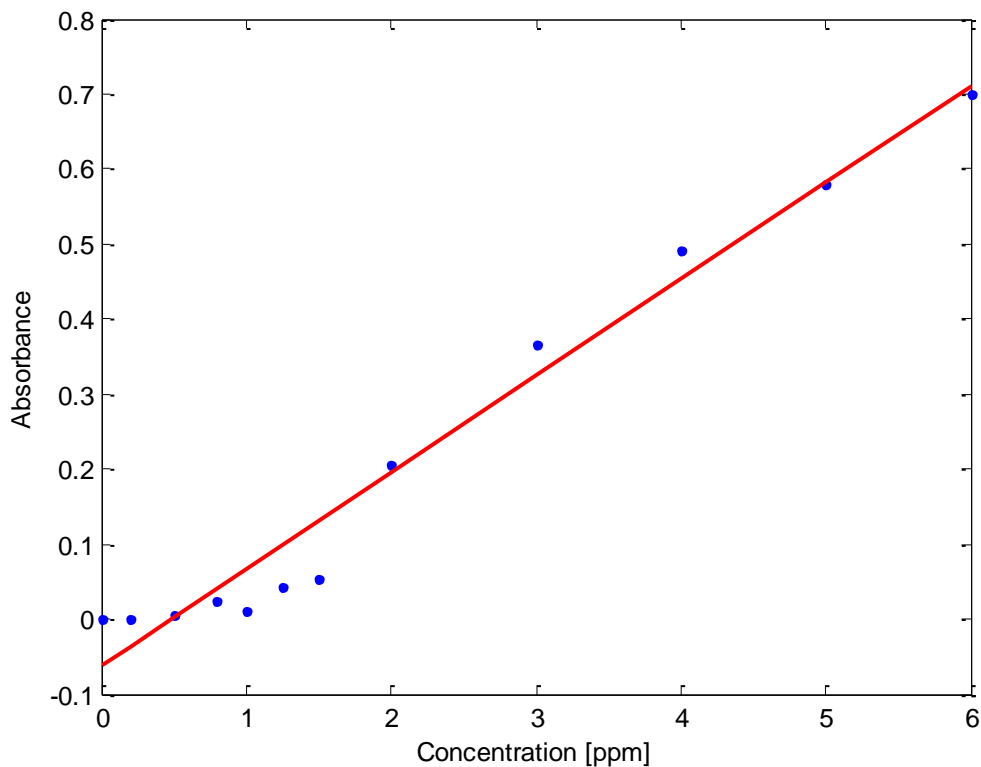
Figure 2.5 *Correlation of calibration*

Figure.2.6. *Comparison between the absorbance in different concentration of Malathion. From left to right: only dye-10-6-5-4-3-2-1.5-1.25-1-0.8-0.5-0.2-dye and NBS ppmv.*

2.1.3.2 GC-MS analysis

A 7890A Gas Chromatographer from Agilent Technologies coupled to a GCT Premier Mass Spectrometer with Time of Flight as analyzer was used. Chromatographic separation was conducted with a DB-5MS capillary column (30 m x 0.25 mm I.D., film thickness of 0.25 μm). Helium (purity 99.999%) was used as carrier gas at a constant flow of 1.0 mL min^{-1}. The temperature program was set initially at 40ºC for 3 min and then raised to 200ºC at a rate of

15ºC min $^{-1}$ (held for 2 min), finally, raised to 290ºC at a rate of 25ºC min^{-1} (held for 18 min). Injector temperature was maintained at 270ºC, and the injection volume was 1.0 µL in a 5:1 split ratio. The ion source and interface temperatures were 200 ºC and 250 ºC, respectively, and electron impact ionization energy was 70 eV. The mass spectrometer was operated in scan mode in the range 40-800 amu.

First of all it was necessary to do a calibration method. It was made starting to a stock solution of Malathion in acetone with a concentration of 240 ppmv. Different dilutions were prepared. The areas corresponding to each concentration are listed in Table 2.2 and the straight line resulted from this calibration is represented in Figure 2.7.

Table 2.2*: Corresponding area of Malathion concentration necessary to do the calibration*

Malathion concentration [ppmv]	*Malathion area*
10	ND
25	77
86	379
144	714
240	1169
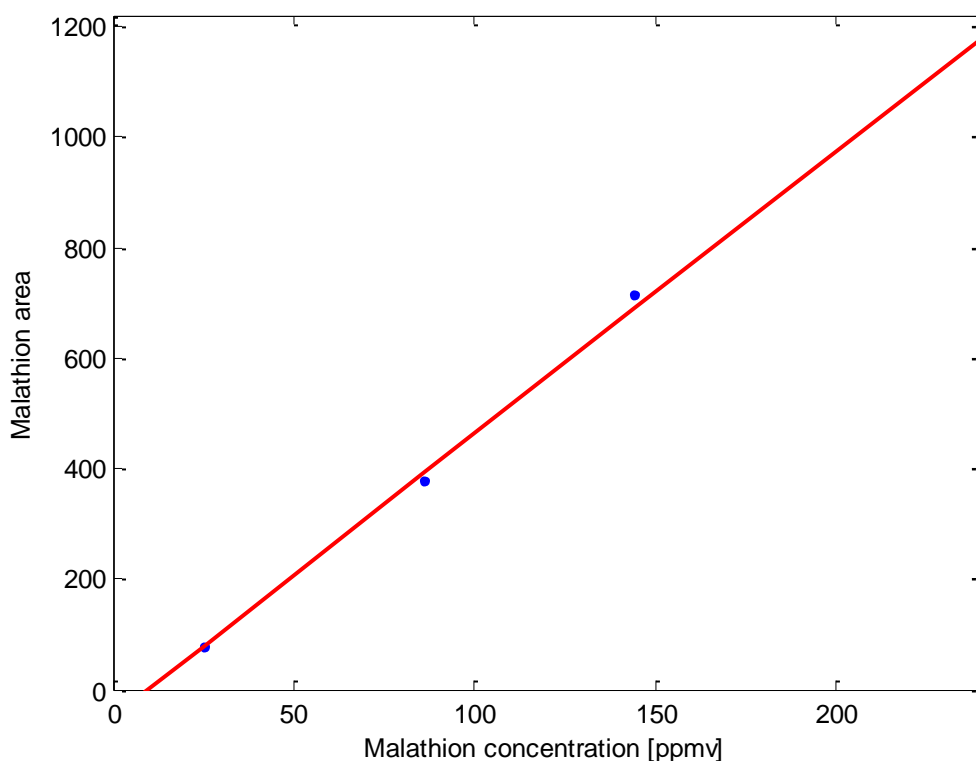
Figure 2.7*: Malathion calibration to do the GC- MS analysis*

Since the minimum Malathion concentration detected was 10µg in 1 ml acetone, this amount meant 0.5 ppm on 20 g of chickpeas.

2.2 Supercritical fluid extraction of phenols from asparagus

The extraction of phenols from asparagus can be divided in four sections. The first one is the extraction carried out with a laboratory scale plant, using CO_2; the second one concerns the pressurized solvent extractions which were conducted using the same plant, with only pressurized solvents, without CO_2. The third one describes another type of extraction done i.e. Soxleth; the last part concerns the analysis method of samples.

The matrix chosen to be extracted was asparagus due to its content of polyphenols.

2.2.1 Extraction

The process flow diagram of the extraction plant used for the SFE from asparagus is illustrated in Figure 2.8. Figure 2.9 shows a picture of the pilot plant employed.

CO_2 (99.998%, by Rivoira) is stored in a tank and passes through a container equipped with a valve. Then, before being pumped, CO_2 is cooled in a cooling bath with ethylene glycol, to make it liquid and to prevent pump from cavitation. The temperature of the CO_2 before being pumped is 5°C. The desired pressure of the pump is fixed and a manometer verifies that the pressure is reached.

Before the extractor there is another heat exchanger to permit CO_2 to reach the supercritical temperature. A temperature regulator ensures a constant temperature and a manometer measures the pressure before the extractor.

A pump, HPLC pump (Jasco PU-1580), for the co-solvent is also placed before the extractor. The co-solvent, stored in a little vessel, is a pure component such as methanol, ethanol, water or a mixture of them.

The supercritical CO_2 and co-solvent flow through the extractor where the matrix (asparagus) has been previously placed. The mass of solid charged in the tests was 0.49±0.05 g.

Asparagus (brought at a supermarket) were previously lyophilized to reduce their content of water. Then, they were milled and filtered with a metallic sieve to obtain a diameter of 0.5 mm.

The characteristics of the extractor are reported in Appendix D .

A jacket is located around the extractor to maintain the desired temperature. The temperature of the extractor is controlled in the internal flow. The depressurization valve, placed after the extractor, regulates the CO_2 flow rate. A water bath avoid the freezing of the CO_2 due to depressurization.

A collector was placed after the valve to collect the extract. The extract was collected with Ethanol (99.5%, Sigma Aldrich).

The flow rate of CO_2 was read by a flow meter. The extracts were collected in each experiment every 0.02 m^3 of flowing CO_2. Therefore, after an extraction of 60 minutes, there were six test tubes containing the extract and ethanol. Figure 2.10 shows an example of six samples collected during a test.
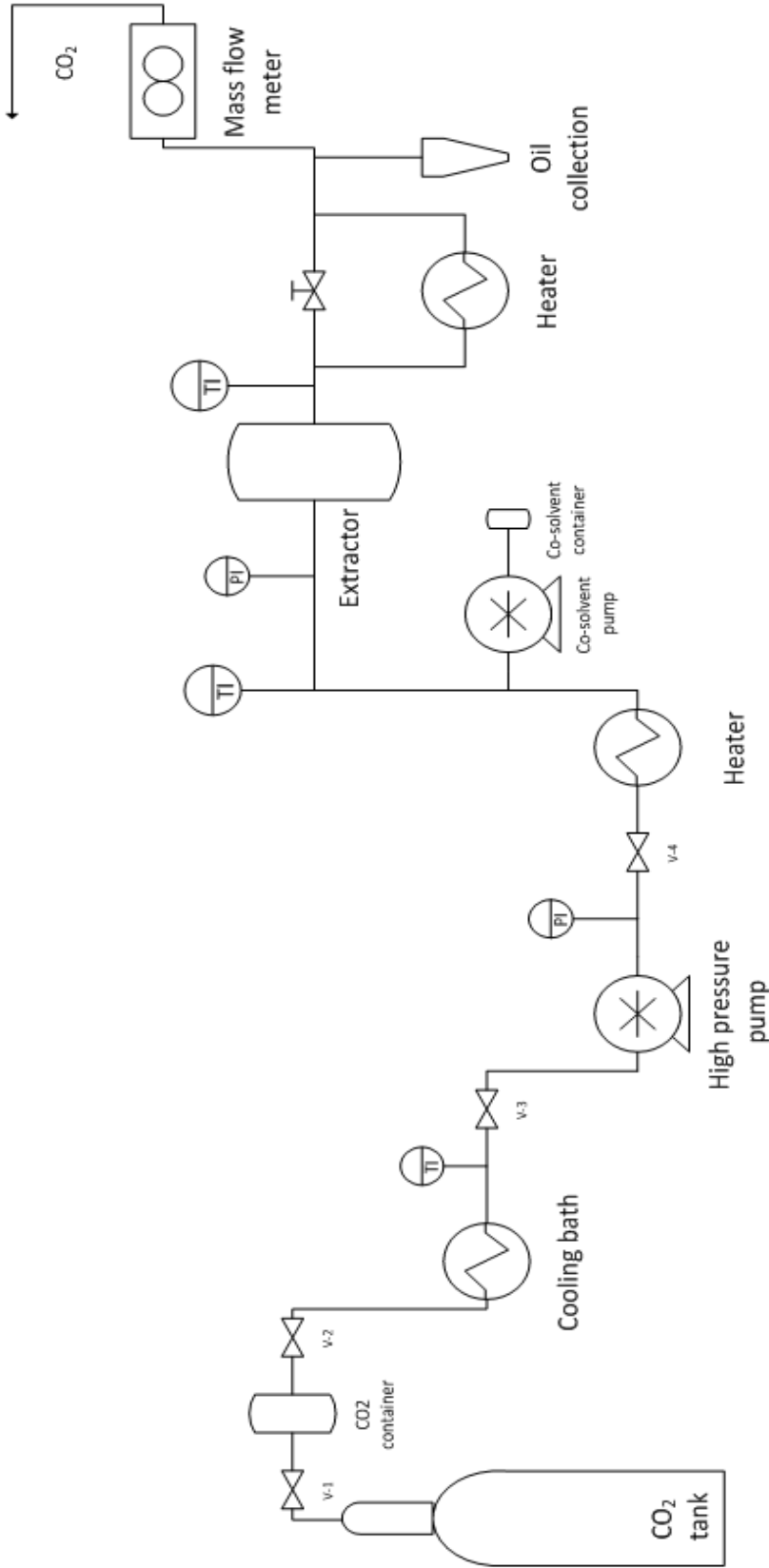
Figure 2.8: *Supercritical fluid extraction equipment used to perform the extraction of phenols from asparagus*

Figure 2.9: *Picture of the pilot plant used to perform the extraction of phenols from asparagus*
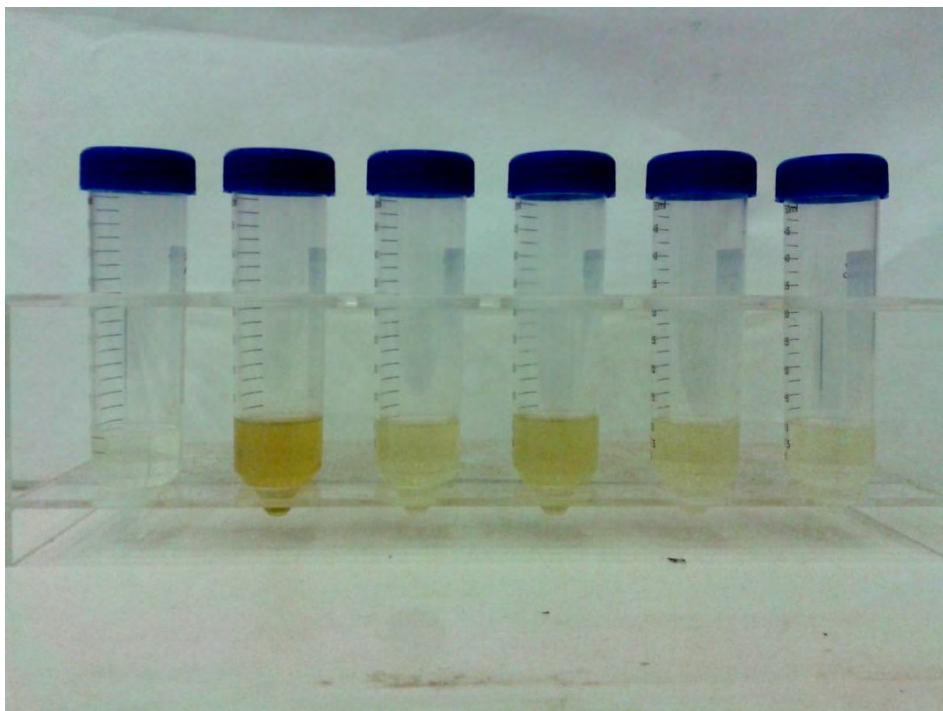
Figure 2.10: *Extracted samples with the same quantity of Ethanol*

2.2.2 Pressurized solvent extraction

The plant described before was also used to perform extractions without CO_2, using only a pressurized liquid solvent (PLE). Three different solvents were tested: a mixture of water and Ethanol 1:1, water and ethanol.

In this method, only the pump of co-solvent was utilized.

The mass of solid charged in the tests was 0.49±0.05 g. Asparagus (brought at a supermarket) were previously lyophilized to reduce their content of water. Then, they were milled and filtered with a metallic sieve to obtain a diameter of 0.5 mm (like in supercritical CO_2 extraction). After the temperature of the extraction (65°C) was reached, the solvent was pumped. The pressure was fixed at 10 MPa. A flow rate of 2 mL min^{-1} was maintained during 30 minutes. The extracts were evaporated by a rotatory evaporator to remove the solvent.

2.2.3 Soxhlet

A typical Soxhlet apparatus is represented in Figure 2.11.
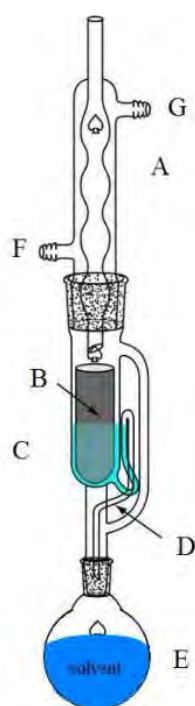
Fig.2.11 ([49])*Soxhlet extractor where A= Water-cooled condenser; B= sample thimble; C= upper reservoir, D= return tube; E= round bottom flask ;F=cold water in; G=cold water out*

As can be seen in Figure 2.11, the Soxhlet apparatus consists of a round bottom flask, an extractor (soxhlet extractor) and a condenser. The bottom flask was filled with the solvent (methanol, 99.8% Sigma Aldrich) and placed in a heated bath. The vaporized solvent flows to the condenser and after its condensation, it passes through the matrix. The extractable solid (asparagus) was previously placed in a paper thimble. The extract is finally collected in the round bottom flask. The extraction tests were carried out for 3 hours.

2.2.4 Analysis

A rotary evaporator (Figure 2.12) was used to evaporate the co-solvent present in every sample contained in the tubes after the extractions.
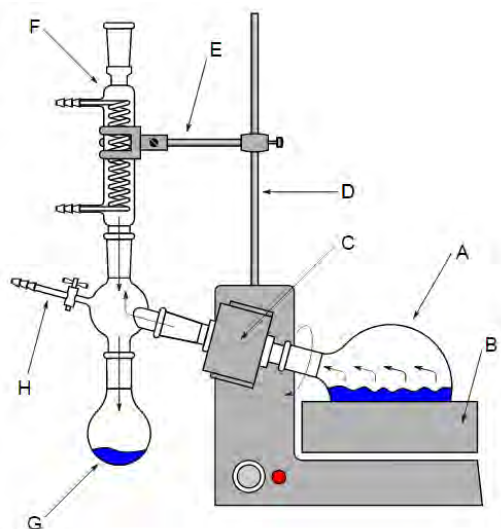
Figure 2.12 ([50]) *Rotary evaporator. Where A= rotative flask , B= Water bath, C=speed control D= tripoid, E=paw tripod, F= solvent condenser, G= collection flask, H= tap Olive*

The flask was weighed when the solvent was completely evaporated.

Afterwards, the second sample was poured in the same flask and it was placed in the rotary evaporator until it reached the total evaporation of the solvent. After it was weighed, the same operations written previously were performed until the last sample. The total amount of extract was calculated with the difference between the final weight and the empty flask.

After being weighted, 10 mL of water and 10 mL of ethanol were added in the flask containing the total extract. Then samples were delivered to the laboratory of Pharmaceutical and Pharmacological Science Department where the GC-MS analysis was carried out.

2.2.4.1 HPLC-MS analysis

HPLC-MS measurements were obtained on a Varian 212 series chromatograph equipped with Prostar 430 autosampler and MS-500 Ion Trap as detector. MS spectra were recorded in positive and in negative ion mode (50-2000 Da). The APCI ion source was used for lipid analysis while the ESI was used for phenolic and glucosinolates. Fragmentation of the main ionic species were obtained during the HPLC run by the turbo data depending scanning (tdds) function, yielding in fragmentation pattern of eluted compounds. As stationary phase Agilent Zorbax C-18 (2.1 x 150 mm) 3.5 μm was used. As mobile phases solvent A (water 0.1% formic acid) and solvent B (methanol) were utilized. The solvent gradient started at 80% A then decreased to 0% A over 30 min.

Chapter 3

Extraction of Malathion tests: results and discussion

This part of the work deals with the experiments related to the extraction of Malathion from vegetables. First, the most appropriate contamination method was decided. Then the most important extraction parameters were explored.

3.1 Selection of the contamination method

Malathion contamination of silicon balls was done first to simulate a solid matrix. A same quantity of 30 g of silicon balls was contaminated at the same theoretical concentration (30 ppm) but in different ways:

1) Dropwise contamination that consisted in pouring the defined volume of stock Malathion solution with a pipette drop by drop, shacking the balls continuously;
2) Immersion contamination where the defined volume of stock Malathion solution was poured in a glass and then the silicon balls were put inside the glass;
3) Contamination with acetone which is similar to contamination #2, but with the difference of the presence of acetone, which is more volatile, and thus faster to evaporate;
4) Spray contamination in which the defined volume of stock Malathion solution was sprayed on the silicon balls.

In each case, the silicon balls were dried after contamination, until the total evaporation of water of the Malathion solution or of acetone (in case 3) was reached. The analysis method applied was the spectrophotometric one.

Making a proportion between the concentration of Malathion present in the silicon balls and the volume of the water and acetic acid mixture used to wash the sample, the theoretical concentration should be 4 ppm. The different types of contamination with the relative measured absorbances are listed in Table 3.1.

Table 3.1: *Tests about different contamination ways of silicon balls, starting from the same theoretical Malathion concentration of 4 ppm*

Type of contamination	Measured absorbance	Average measured concentration [ppm]
Dropwise contamination	0.174 0.141 0.116	1.3
Immersion contamination	0.238 0.236 0.109	1.7
Acetone contamination	0.986 0.909 0.799	7.2
Spray contamination	0.251 0.316 0.199	2.1

From these results, it can be deduced that the third contamination method was probably affected by the presence of acetone because the measured concentration was higher than the initial one and out of spectrophotometric detection limits. For this reason, this type of contamination was dismissed.

Then, it was evident that a quantity of Malathion solution did not wet on the balls surface or was washed away, since the measured concentration was always lower than the theoretical one. The spectrophotometric analysis was not precise but was useful for a first and fast analysis. By its measures, it was found that the best method was the last one, in which the contamination was achieved by spraying a Malathion solution on the silicon balls.

Once decided the best contamination method, the experiments were done with chickpeas. First of all, the chickpeas were contaminated with a Malathion stock solution (Malathion and water and a little bit of acetic acid) of 110 ppm. Pouring different volumes of this solution on a known quantity of chickpeas, it was possible to achieve various contamination concentrations.

Then it was tried to confirm the achieved contamination with the spectrophotometric method, but it was not possible. Even non contaminated chickpeas had an absorbance that would correspond to more or less 7 ppm concentration of Malathion. It seems that a component of the chickpeas interfered with the spectrophometric method. Therefore, it was necessary to change the method to evaluate the Malathion content.

The most appropriate and accurate method was the GC-MS one, described in Chapter 2. By its calibration and the measured areas, it was possible to calculate a difference between the theoretical contamination and the real one. These data are listed in Table 3.2 where ND (not detected) means that chickpeas concentration is less than 0.5 ppm which was the detection limit of GC-MS method.

Table 3.2*: Degree of Malathion contamination in chickpeas used as raw material*

Theoretical concentration [ppm]	Measured area	Real concentration [ppm]	Average of real concentration [ppm]
20	278	6	5
20	139	4	
10	ND	<0.5	-
10	ND	<0.5	-
10	64	1	1

By these results it was evident that the contamination of the chickpeas was less than expected. The difference between the theoretical and the real values was very significant, probably due to the loss of the stock solution of Malathion during the atomization process. Since in practice, the contamination by Malathion will be higher, only samples contaminated with 5 to 6 ppm were used as raw material.

3.2 Extraction experiments and effects of process parameters

In general, supercritical CO_2 extraction of soluble compounds from solid plant material proceeds in main four and consecutive steps:

1. The plant matrix absorbs the $SCCO_2$ causing the cell swelling. In this step the internal mass transport resistance is lowered.
2. The extract compounds are dissolved.
3. The dissolved compounds are transported to the outer solid surface.
4. The compounds are transported from the surface layer into the bulk and subsequently removed. [4]

The valuable substance can be located within the plant cell, or on the surface, or absorbed in the solid matrix. First, the location of the substances must be determinated. Further, it is necessary to know if the desired substance is bound in any chemical reaction to the matrix or bound to a complex. [11]

In the case of chickpeas, the Malathion, was laid down on the particle surfaces and it was non-chemically bounded with the matrix. Weak adsorption may occur though. Therefore,

only the two last steps are applicable. The Malathion had to be solubilized by the supercritical CO_2 and transported to the bulk phase.

Consequently, the conditions that will affect the process are those that will affect the solvent power of the fluid and those that will reduce the external mass transport resistance:

- The CO_2 mass flow-rate;
- The extraction time;
- The ratio between extractor length and diameter because it affects the axial dispersion and the contact time. ([11]) In these experiments this ratio was fixed at 8.
- Temperature;
- Pressure.

Although also the effect of a different particles size could be an important condition to be investigated, in this case, the chickpeas were used without being milled. This decision was taken because the idea was to decontaminate chickpeas with the extraction and make them edible food.

Starting from the contaminated chickpeas described before, extraction experiments were carried out. For each group of experiments only one parameter was change, to see its effect on the extraction. A summary of the tests performed is shown in Table 3.3.

Table 3.3: *Amount of residual Malathion on chickpeas at different experimental process conditions. The initial contamination was six ppm*

t [min]	T [°C]	P [bar]	CO_2 Flow rate [g min^{-1}]	Matrix humidity [% w/w]	CO_2 consumption [kg/kg]	CO_2 density [g/mL]	Final concentration [ppm]
				Effect of humidity			
30	70	350	2	**Initial**	3	0.826	<0.5
30	70	350	2	**12**	3	0.826	<0.5
				Effect of temperature			
30	**40**	350	2	Initial	3	0.935	<0.5
30	**55**	350	2	Initial	3	0.881	<0.5
30	**70**	350	2	Initial	3	0.826	<0.5
30	**80**	350	2	Initial	3	0.792	<0.5
				Effect of pressure			
30	70	**150**	2	Initial	3	0.515	<0.5
30	70	**250**	2	Initial	3	0.737	<0.5
30	70	**350**	2	Initial	3	0.826	<0.5
				Effect of operation time			
15	70	350	2	Initial	1.5	0.826	<0.5
30	70	350	2	Initial	3	0.826	<0.5
60	70	350	2	Initial	6	0.826	<0.5
90	70	350	2	Initial	9	0.826	<0.5
120	70	350	2	Initial	12	0.826	<0.5
				Effect of solvent flow rate			
30	70	350	**1**	Initial	2	0.826	<0.5
30	70	350	**2**	Initial	3	0.826	<0.5
30	70	350	**4**	Initial	6	0.826	<0.5

3.2.1. Effect of humidity presented in the matrix

The first parameter that was investigated was the humidity of chickpeas, when they were loaded inside the extractor. A test with 12% humidity was carried out and compared with chickpeas used as received. Other conditions were maintained constant: temperature (70°C), pressure (350 bar), CO_2 flow rate (2 g min^{-1}), initial contamination (6 ppm) and quantity of chickpeas (around 20 g).

Water in the sample often causes strong effects on SFE ([26]). In fact, operating as a modifier, it is suspected to favor the swelling of the matrix, thereby enhancing diffusion of the fluid within the matrix when the solute is inside. If the solute is a surface contaminant, as in this case, the humidification of the matrix may promote its desorption.

Previous investigations conducted by Lack et al. ([11]) regarding current extractions of pesticides from rice demonstrated that an initial water content between 10 and 15% was necessary in order to achieve pesticides reduction to values below the ones marked by the legislation. This humidity acted as matrix modifier helping the detaching of the pesticides.

Modifiers in general, could be added either continuously to the fluid, or directly into the matrix, just before the extraction. The second way, adopted in this experiment, requires a static period to allow the solvent to interact with the matrix ([10]). In this case, the time to achieve the required extraction time was used, typically in the order of minutes.

As shown in Table 3.3, no impact of the water addition could be detected on the removal of Malathion from the chickpeas, since both extractions yielded recoveries higher than 90%. Other researchers found similar results. Several organophosphorous pesticides, like Malathion, were quantitatively recovered by pure CO_2 from an inert matrix, without the use of any co-solvent ([27]).

However, the water helped to preserve their aspect. In assays where dry chickpeas were used, they appeared broken and crumbled with a lot of powder after the high CO_2 pressure extraction. Contrary, with the addition of water, the chickpeas were unbroken and intact.

The next assays were run to determine the influence of the process parameters on extraction.

3.2.2. Effect of temperature

The next parameter to be optimized was temperature: 40°C, 55°C, 70°C and 80°C were explored. The tests were carried out at constant pressure (350 bar) and CO_2 flow rate (2g min^{-1}).

In general, if the extraction temperature is increased at a constant pressure, the density of supercritical CO_2 will decrease. Therefore, increasing the temperature would reduce the solubility of compounds in the fluid. On the other hand, for volatile compounds, the increase of volatility favors the extraction; thus, several studies report a dramatic increase in extraction efficiencies as the temperature is elevated. This has been attributed to the more efficient desorption of the compounds from the active sites of the matrix at higher temperatures, as well as to a higher volatility.

However the use of high temperatures in SFE is often limited by either the maximum operating temperature (generally around 150°C) or by the thermal degradation of the pesticide investigated: for Malathion this is above 100°C. Consequently moderate temperatures should be used whenever possible ([27])

In this work a temperature of 40°C was sufficient to obtain an extraction yield superior to 90%; probably because of the high pressure used.

3.2.3. Effect of pressure and density

Fluid pressure is an essential parameter in SFE, as the fluid density is directly related to it. In fact, at a given temperature, the fluid density increases with pressure, so that higher pressure is beneficial dissolve the pesticide into the fluid.

Whatever the solute, the relations between solubility and pressure are similar: at a low pressure, the solute has little solubility in the fluid; above the "crossover pressure" same solubility is measured, and it further increased with pressure; lastly, the maximum solubility for a given temperature is attained. ([4, 26, 27]).

From the pesticides extraction literature (Chapter 1), a wide range of pressure was identified (120-690 bar), depending on the characteristics of the equipments too.
 Decreasing values from 350 to 150 bar were explored.

The tests were carried out at the same temperature (70°C), CO_2 flow rate (2 g min^{-1}) and extraction time (30 min). At this temperature, the CO_2 density varied in the range from 0.506 and 0.826 g/mL. A previous study on the removal of Malathion and other 87 pesticides from a diatomaceous earth (Celite) showed that by increasing the CO_2 density in a range from 0.3 to 0.85 g/mL, the recoveries increased too ([53]). The other constant parameters were: temperature = 40°C, CO_2 flow rate 2 mL min^{-1}; static extraction =3 min and dynamic extraction =20 min. Another one ([11]) the best performances were reached at 320 bar.

As it can be seen in Table 3.3., a pressure of 150 bar was sufficient to recover more than 90% of the pesticide. The CO_2 density corresponding to 250 bar and 70 °C was 0.506 g/mL. This quite low density value indicates the fairly high solubility of Malathion in CO_2.

It is necessary to remember that the extractions of this research were conducted without any information about their yield and efficiency. Therefore, the basis for the next experiments were literature and the knowledge about the effect of pressure on extraction, on which there was an improvement of recovery with an increase of pressure.

In the following, the pressure was maintained constant at 350 bar. The costs relating to the high pressure were the flaws; in fact it would be better to use the lowest pressure possible in order to reduce the costs associated with high fluid compression and pressure ratings on the equipment.

3.2.4 Effect of extraction time

In general terms, the dynamic time is essentially a measure of the total volume of fluid passing through the extractor (as determined by the flow rate). Pesticides that are hardly extracted (i.e. polar pesticides, or compounds that strongly interact with the matrix) may require large volumes of extraction fluid, and thus more time. [10]

In some previous studies conducted by Lehotay [26], the static time was also considered important. It was either considered long enough to allow the sample to reach the temperature of extraction or as a parameter to be changed. In fact for pesticides that were not easily extracted, increasing the static time, led to longer recoveries. However, if the pesticide was poorly extracted using a short static step, longer and repeated static steps did not dramatically increase recoveries. [26]

In this work the static time was only the time necessary to reach the desired operating temperature. For the first experiments of the day it was 30 min, for the others, more or less five minutes. During this static time all the heater exchangers were switched on and the CO_2 filled the vessel and reached its pressure on the bottle (approx. 60 bar). On the other hand, the dynamic times explored in this work were from 15 to 120 min. The tests were conducted at the same temperature (70°C), pressure (350 bar), CO_2 flow rate (2 g min^{-1}), initial contamination (6 ppm) and quantity of chickpeas (around 20 g) used as received .
It was found that proceeding like this and using a continuous flowing time of only 15 min minutes gave an extraction yield higher than 90%. Similarly, most pesticides, operating at 275 bar, and 50°C, were recovered in the first 10 minutes from Karma L. Pearce et al. (1997). The running time of 20 min was considered optimal. [20]

The next section presents the results when the flow rate was modified using a fixed operation time of 30 min.

3.2.5 Effect of CO$_2$ flow rate

The effects of the CO$_2$ flow rate concern the external mass transfer (steps 3 and 4 of supercritical extraction of soluble compounds from solid plant material).

By the equations 1.1., 1.2, (Chapter 1), it is evident that a strong relation between extraction yield, mass transfer coefficient and velocity of the supercritical fluid (by Re definition in Eq. 1.3) exists. For increasing extraction yields is to use higher flow rates because, at constant extractor diameter, the velocity increases, causing an increasing of the Reynolds number.

The interval of CO$_2$ flow rates explored in the extraction of organophosphorous pesticides from fruits and vegetables ([19,21,22,23,25,27]) ranges go from 0.7 to 10 mL min^{-1}. Based on the CO$_2$ density at 70°C and 350 bar, which is 0.826 g/mL, the range becomes 0.6-8 g min^{-1}. This work explores the range from 1 g min^{-1} to 4 g min^{-1} and compared with the ones done previously done at 2 g min^{-1}. The results (Table 3.3) show that a CO$_2$ flow rate of 1 g min^{-1} was sufficient to give an extraction yield higher than 90%.

3.3 Extraction conclusions

From the results obtained in this work, it can be concluded that supercritical CO$_2$ extraction of Malathion from chickpeas is very effective. This may occur because of the high solubility of this compound in CO$_2$; or it could have been caused by the type of contamination which was performed artificially. The latter, in fact, led to a surface contamination only, not establishing strong adsorption bonds. For this reason, the extraction yields obtained were 90%, in all explored conditions.

Clearly, a real contaminated samples investigation and validation should be done. If the data presented in this work were confirmed, it would imply that the extraction could be performed under lower conditions of temperature and pressure, and consequently lower energy costs.

Although it is not necessary for the extraction itself, chickpeas should be previously wetted to preserve their quality. A part of the natural water present in chickpeas is extracted during the supercritical process. This causes an excessive drying on the chickpeas and therefore their damage.

Chapter 4

Extraction of polyphenols tests: results and discussion

This second part of research work regards the extraction of polyphenols from asparagus with supercritical CO_2. The data and analysis are presented and the effects of the main parameters affecting the extraction, such as pressure and temperature, are evaluated.

Furthermore, the technique of SFE is compared with extractions conducted without the use of CO_2, like pressurized solvents (named also PLE= pressurized liquid extraction) and Soxhlet.

The experiments are divided into seven groups. In each one, a different parameter was changed (e.g. temperature, pressure, use of CO_2 or only solvents and type of co-solvent…) to study its effect on the extraction yield and composition of the extract.

4.1 Supercritical CO_2 extraction

SFE is an alternative use of toxic organic solvents or other extraction methods that apply high temperature.

In the following, the extraction yield was calculated by:

$$Yield[\%] = \frac{Mass\ of\ extract\ [g]}{Mass\ of\ initial\ matrix\ inside\ the\ extractor\ [g]} * 100 \qquad (4.1)$$

Every 0.02 m^3 of CO_2 flow (corresponding to around 10 minutes of extraction), the extract was collected in a glass with ethanol, it was put inside a flask and the ethanol was evaporated with a rotavapor. The difference between the initial mass of the flask and its mass after the evaporation with rotavapor, gave the total amount of extract. In this way it was possible to calculate not only the final yield of the extraction, but also the yield every 10 minutes.

After the solvent was evaporated and the samples collected every 10 min were weighed, 10 mL of water and 10 mL of ethanol were poured into the flask to store them until analysis. There, by HPLC-MS analysis, the phenols extracted (expressed in mg/g) where calculated and the composition was analyzed.

The extraction curves reported in this chapter represent the cumulative yield behavior versus mass of cumulated CO_2. This last parameter is calculated with:

$$CO_2 = \frac{CO_2\ Volume\ passed * CO_2\ density[kg]}{Mass\ of\ initial\ matrix\ inside\ the\ extractor[kg]} \qquad (4.2)$$

4.1.1. Effect of pressure

The effect of pressure on the extraction was studied at 65°C and 50°C. First, the effect of pressure at 65°C is reported.

In the tests belong to the extractions at 65°C, the mass of asparagus (around 0.50 g), type and flow rate of co-solvent (W:E 1:1 with flow rate equivalent to 8% of CO_2) and quantity of CO_2 flow (corresponding to 0.12 m^3 every 10 minutes and a time of extraction of around 60 min), were constant. Pressures tested were 100 bar, 150 bar and 250 bar. The characteristics of the test and their final extraction yield (calculated as written before) are listed in Table 4.1.

Table 4.1: *Experiments conducted at different pressure and constant temperature= 65°C*

Matrix and weight[g]	*CO$_2$ [kg/h]*	*Co-solvent*	*T [°C]*	*P [bar]*	*Time [min]*	*Final average yield [%]*
Asparagus 0.4998	0.280	W:E 1:1	65	**100**	51	
Asparagus 0.5072	0.226	W:E 1:1	65	**100**	63	30.34
Asparagus 0.4973	0.285	W:E 1:1	65	**150**	50	
Asparagus 0.4968	0.285	W:E 1:1	65	**150**	50	41.01
Asparagus 0.4984	0.259	W:E 1:1	65	**250**	55	
Asparagus 0.4990	0.274	W:E 1:1	65	**250**	52	30.57

The corresponding extraction curve is plotted in Figure 4.1. From the behavior of the curves at different pressure and constant temperature (65°C), it can be seen that the highest yield was obtained at 150 bar. From 100 bar to 150 bar an increase of pressure corresponded to an increasing of yield. On the contrary from 150 bar to 250 bar an increase of pressure corresponded to a decreasing of yield. The final yield of 250 and 100 bar were very similar, even though their kinetics resulted quite different.
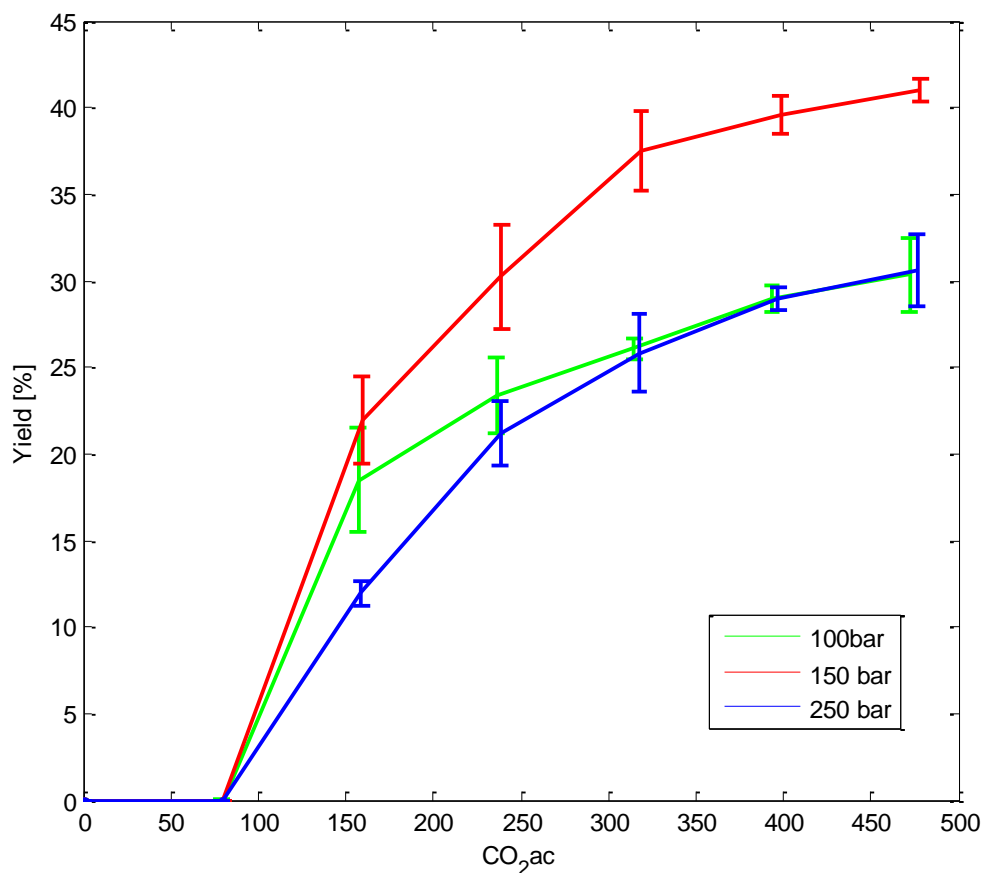
Figure 4.1 : *Extraction curve at constant T=65°C and different P (100,150,250 bar)*

The phenolic compounds of the samples are listed in Table 4.2.

Total phenols contents at different pressures, are shown in Figure 4.2a. The most selective extraction was the one carried out at 100 bar, in which the quantity of phenols extracted is the highest one.

From 100 to 150 bar, an increase of pressure corresponded to a decrease of extracted phenols; the opposite occurred when increasing pressure from 150 bar to 250 bar.

Figure 4.3 shows the quantity of major phenolic compounds (Rutin, Rutin + glucose, Ferulic acid, Chlorogenic acid and Caffeic- glucoside acid), at different pressures. Rutin was the one found in higher proportion in this extraction and followed a behavior similar to the total phenols extracted, with the highest quantity at 100 bar, represented in Figure 4.2b.

The content of the other phenolic compounds, is shown in Figure 4.3, although their quantities were small and their tendency is not clear.

Table 4.2: *Phenolic compounds at constant T=65°C and different P (100,150,250 bar)*

Pressure [bar]	100		150		250	
Phenolic compounds	Average mg/g	St. dev	Average mg/g	St. dev	Average mg/g	St. dev
Quercetin	0.108	0.001	0.103	0.003	0.104	0.001
Q glucoside	0.083	0.001	0.082	0.002	0.084	0.000
Rutin	**2.963**	0.037	**2.711**	0.314	**2.748**	0.088
Rutin + glu	**0.512**	0.032	**0.511**	0.081	**0.485**	0.005
Rutin + glu + Ramn	0.077	0.000	0.079	0.000	0.078	0.001
Keampferol	0.080	0.000	0.081	0.001	0.082	0.001
K 3rd rutinoside	0.152	0.000	0.145	0.001	0.143	0.000
Isoramnetina	0.088	0.002	0.084	0.002	0.088	0.002
Iso-rutinoside	0.253	0.005	0.231	0.007	0.235	0.005
iso-rutinoside + glu	0.112	0.003	0.109	0.001	0.114	0.002
Quinic acid	0.130	0.001	0.194	0.001	0.136	0.004
Ferulic acid	**0.711**	0.033	**0.590**	0.066	**0.624**	0.008
Chlorogenic acid	**0.396**	0.027	**0.335**	0.015	**0.370**	0.014
Caffeic-glucoside acid	**0.369**	0.010	**0.312**	0.009	**0.339**	0.001
TOT mg/g	**6.035**	0.072	**5.567**	0.498	**5.630**	0.085
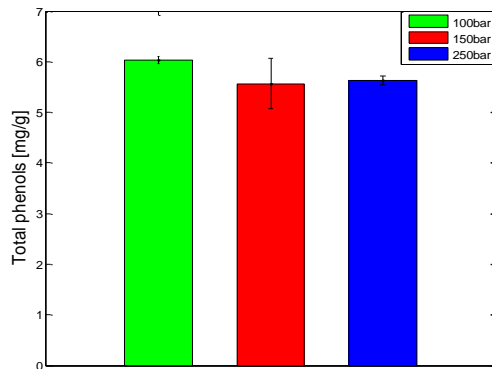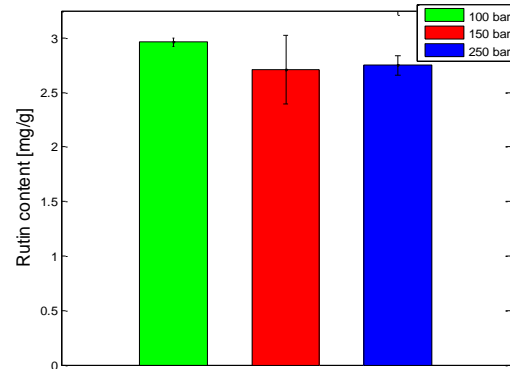
(a)   *(b)*

Figure 4.2: *At constant T=65°C and different P (100,150,250 bar): (a) Total phenols content; (b) Rutin content*
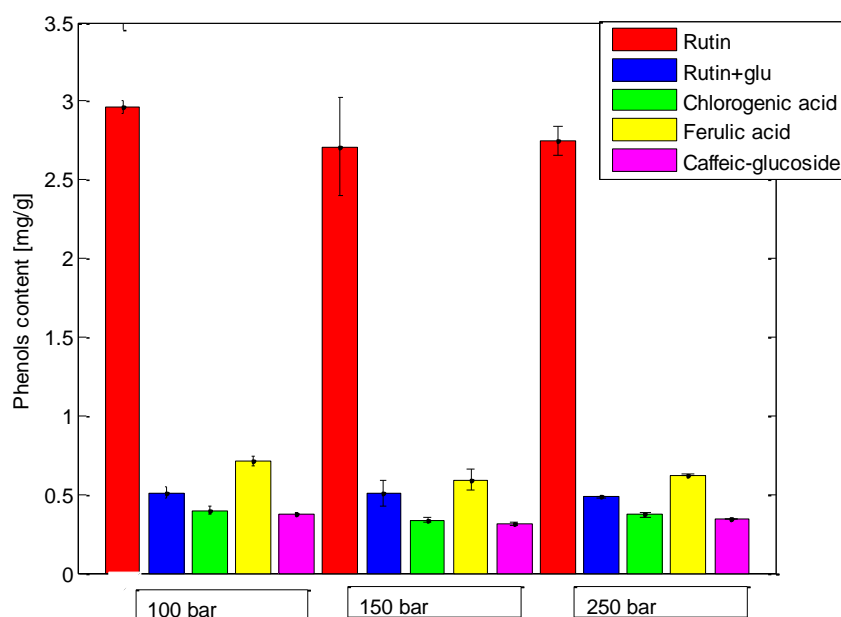
Figure 4.3: *Major phenolic compounds at constant T=65°C and different P (100,150,250 bar);*

Another series of experiments at different pressures were performed at 50°C, mass of asparagus (around 0.50 g), type and flow rate of co-solvent (W:E 1:1 with flow rate equal to 8% of CO_2 one) and quantity of CO_2 flow (corresponded to 0.12 m^3 every 10 minutes and a time of extraction of around 60 min). In this case the pressures were 150 and 200 bar. The characteristics of the tests and their final extraction yield are listed in Table 4.3.

Table 4.3: *Experiments conducted at different pressure and constant temperature = 50°C*

Matrix and weight[g]	CO_2 [kg/h]	Co-solvent	T [°C]	P [bar]	Time [min]	Final average yield [%]
Asparagus 0.4999	0.250	W:E 1:1	50	**200**	57	32.55
Asparagus 0.4999	0.280	W:E 1:1	50	**150**	51	
Asparagus 0.4982	0.274	W:E 1:1	50	**150**	52	34.11

The corresponding extraction curve is plotted in Figure 4.4. From the behavior of the curves at different pressure and constant temperature (50°C), it can be seen that, like in the previous group of tests, the major yield was reached at 150 bar.

The previously drawn conclusions about the extraction yields when increasing from 150 bar upward, were confirmed in this case. In fact at 200 bar the yield was lower than the one reached at 150 bar.
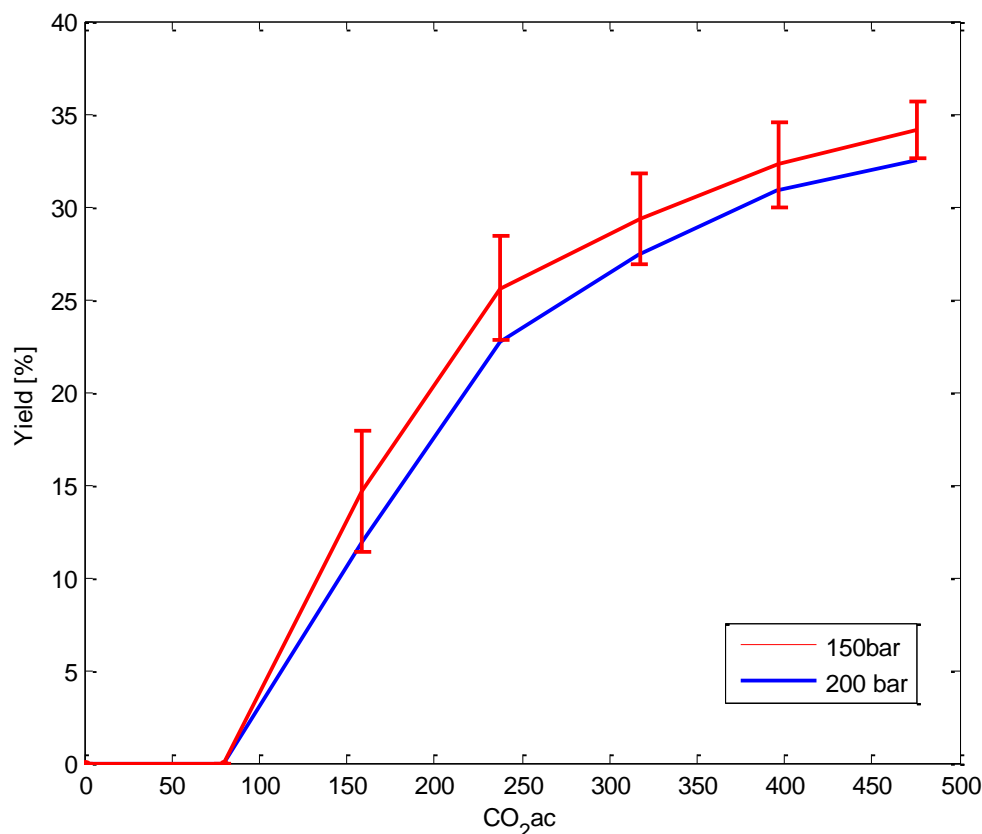
Figure 4.4: *Extraction curve at constant T=50°C and different P (150,200 bar)*

Table 4.4 lists the phenolic compounds and their quantity analyzed, related to this group of test and Figure 4.6 represents some of them.

Table 4.4: *Phenolic compounds at constant T=50°C and different P (150, 200 bar)*

Pressure [bar]	150		200	
Phenolic compounds	*Average mg/g*	*St. dev*	*Average mg/g*	*St. dev*
Quercetin	0.097	0.008	0.096	0.003
Q glucoside	0.078	0.002	0.080	0.001
Rutin	**1.992**	0.778	**2.569**	0.012
Rutin + glu	**0.384**	0.148	**0.483**	0.026
Rutin + glu + Ramn	0.000	0.000	0.000	0.000
Keampferol	0.040	0.057	0.079	0.001
K 3rd rutinoside	0.135	0.007	0.136	0.009
Isoramnetina	0.083	0.002	0.085	0.001
Iso-rutinoside	0.213	0.009	0.153	0.092
iso-rutinoside + glu	0.137	0.035	0.130	0.001
Quinic acid	0.168	0.041	0.172	0.007
Ferulic acid	**0.555**	0.029	**0.595**	0.028
Chlorogenic acid	**0.396**	0.067	**0.300**	0.000
Caffeoilglucoside acid	**0.328**	0.051	**0.354**	0.005
TOT mg/g	**4.607**	0.926	**5.232**	0.173

The total phenols content at different pressures is represented in Figure 4.5a. The most selective extraction was the one at 200 bar in which the quantity of phenols extracted was the highest, but the extraction yield was the lowest, as it can be seen in Figure 4.4. Above 150 bar an increase of pressure improved the quantity of phenols extracted.

Figure 4.6 shows the quantity of major phenolic compounds (Rutin, Rutin + glucose, Ferulic acid, Chlorogenic acid and Caffeic- glucoside acid). Rutin was the phenolic compound found in higher proportion in these extractions and it followed a similar behavior to the total phenols extracted with the highest quantity at 200 bar, as shown in Figure 4.5b.

(a)   *(b)*

Figure 4.5: *At constant T=50°C and different P (150.200 bar): (a) Total phenols content; (b) Rutin content*

Figure 4.6 *Major phenolic compounds at constant T=50°C and different P (150.200 bar)*

In literature, studies about the solubility of ferulic and caffeic acids in supercritical carbon dioxide are available ([50]). These authors concluded that the solubility of such phenolic
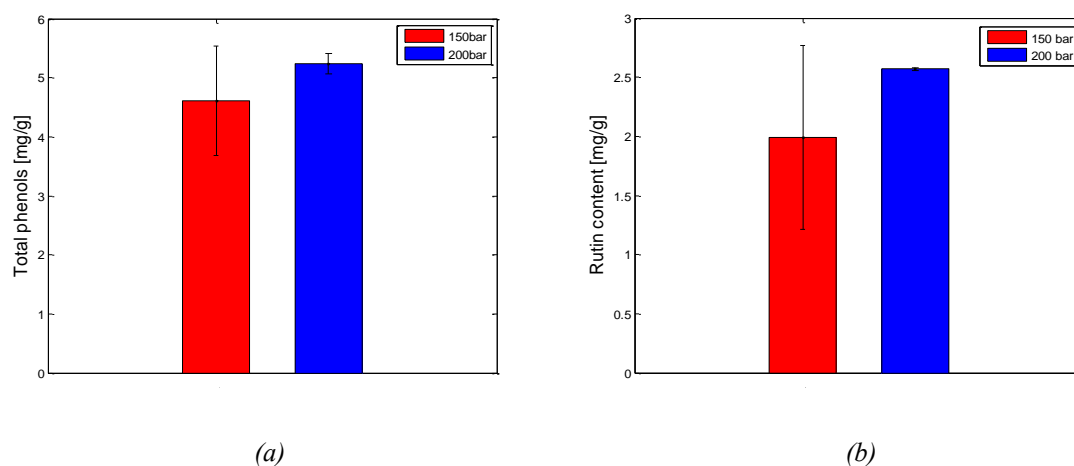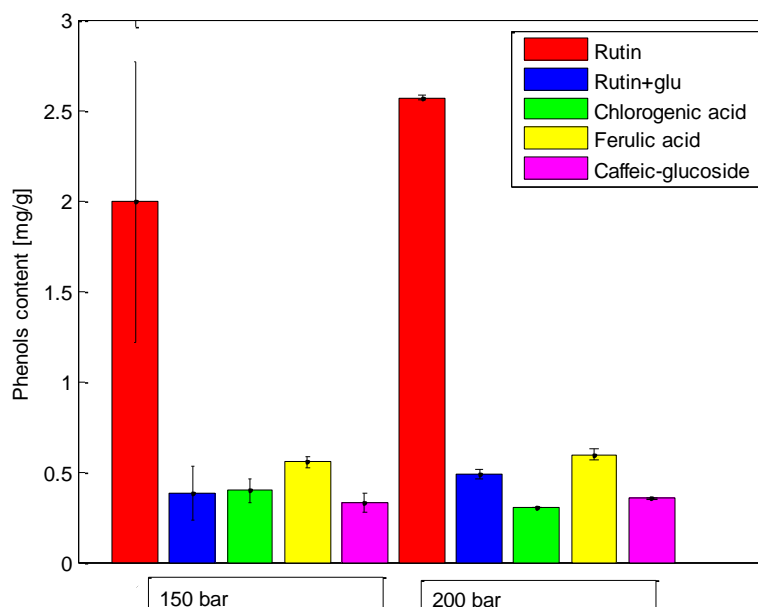
compounds increased with pressure, at constant temperature, in all cases. Another research ([51]) justified this result by saying that the increase of CO_2 density with pressure caused a decrease of the intermolecular distances which increased the solute-solvent interactions. The consequence of these investigations is that to an increase of pressure the extracted content of the two acids should be enhanced.

In general, the influence of pressure on the extraction can finds a reason for the solvent capacity which normally increases with pressure at constant temperature, at process conditions of gas extraction([4]). Therefore, the increase of SFE yield with pressure, like it happened from 100 bar to 150 bar was due to the enhancement of solvation power of CO_2 with density.

On the other hand, above 150 bar an increase of pressure reduced the extraction yield. It competed with the extraction selectivity, whose behavior was the opposite with respect to the yield one.

In conclusion, the highest extraction yield was obtained at 150 bar and it was in the range of 35-40%. However the total phenols extracted was found at 100 bar: 6 mg/g.

4.1.2. Effect of temperature

To evaluate the role of temperature in the extraction, a group of experiments at constant pressure (200 bar), mass of asparagus (around 0.50 g), type and flow rate of co-solvent (W:E 1:1 with flow rate equal to 8% of CO_2 one) and quantity of CO_2 flow (corresponded to 0.12 m^3 every 10 minutes and a time of extraction of around 60 min), was performed. The temperatures tested were 50°C, 65°C and 80°C. The operating conditions of the tests and their final extraction yield are listed in Table 4.5.

Table 4.5: *Experiments conducted at different temperature and constant pressure= 200 bar*

Matrix and weight[g]	CO_2 [kg/h]	Co-solvent	T [°C]	P [bar]	Time [min]	Final average yield [%]
Asparagus 0.4999	0.250	W:E 1:1	50	200	57	32.55
Asparagus 0.4991	0.285	W:E 1:1	80	200	50	32.02
Asparagus 0.5027	0.226	W:E 1:1	65	200	63	33.96
Asparagus 0.5039	0.226	W:E 1:1	65	200	63	

The corresponding extraction curves are plotted in Figure 4.7. From the behavior of the curves at different temperature and constant pressure (200 bar), it can be seen that the highest yield was reached at 65°C.

The difference of the final yield at 50°C, 65°C and 80°C was not so significant. However, their kinetics were different in this range of temperature, as illustrated in Figure 4.7.
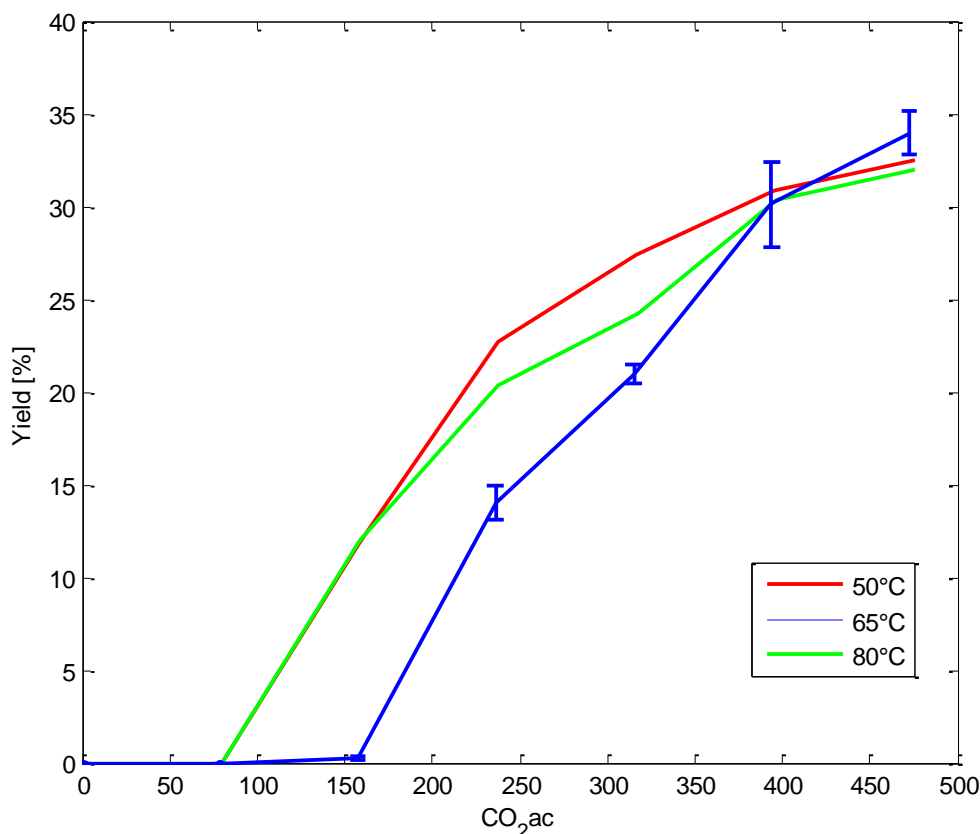
Figure 4.7: *Extraction curve at constant P=200bar and different T (50, 65, 80°C)*

Table 4.6 lists the phenolic compounds analyzed and their amounts.

Table 4.6: *Phenolic compounds at constant P=200 bar and different T (50, 65, 80°C)*

Temperature [°C]	50		80	
Phenolic compounds	Average mg/g	St.dev	Average mg/g	St.dev
Quercetin	0.096	0.003	0.103	0.004
Q glucoside	0.080	0.001	0.082	0.001
Rutin	**2.569**	0.012	**2.493**	0.208
Rutin + glu	**0.483**	0.026	**0.474**	0.019
Rutin + glu + Ramn	0.000	0.000	0.000	0.000
Keampferol	0.079	0.001	0.080	0.001
K 3rd rutinoside	0.136	0.009	0.135	0.003
Isoramnetina	0.085	0.001	0.082	0.002
Iso-rutinoside	**0.153**	0.092	**0.201**	0.002
iso-rutinoside + glu	0.130	0.001	0.108	0.004
Quinic acid	0.172	0.007	0.147	0.002
Ferulic acid	**0.595**	0.028	**0.580**	0.007
Chlorogenic acid	**0.300**	0.000	**0.342**	0.002
Caffeoilglucoside acid	**0.354**	0.005	**0.348**	0.007
TOT mg/g	**5.232**	0.173	**5.173**	0.233

Total phenols content at different temperatures is represented in Figure 4.8a. The difference between the total phenols extracted at operating values of 50°C or 80°C was not significant. Figure 4.9 shows the quantity of the major phenolic compounds (Rutin, Rutin + glucose, Ferulic acid, Chlorogenic acid and Caffeic- glucoside acid), extracted at constant pressure. Rutin was the phenolic compound found in higher proportion in these extractions and it followed a behavior similar to the total phenols, not having significant differences between the two temperatures, represented in Figure 4.8b.

The content of other phenolic compounds, is shown in Figure 4.9(Table 4.6) .

(a)   *(b)*

Figure 4.8: *At constant P=200 bar and different T (50, 65, 80°C): (a) Total phenols content; (b) Rutin content*
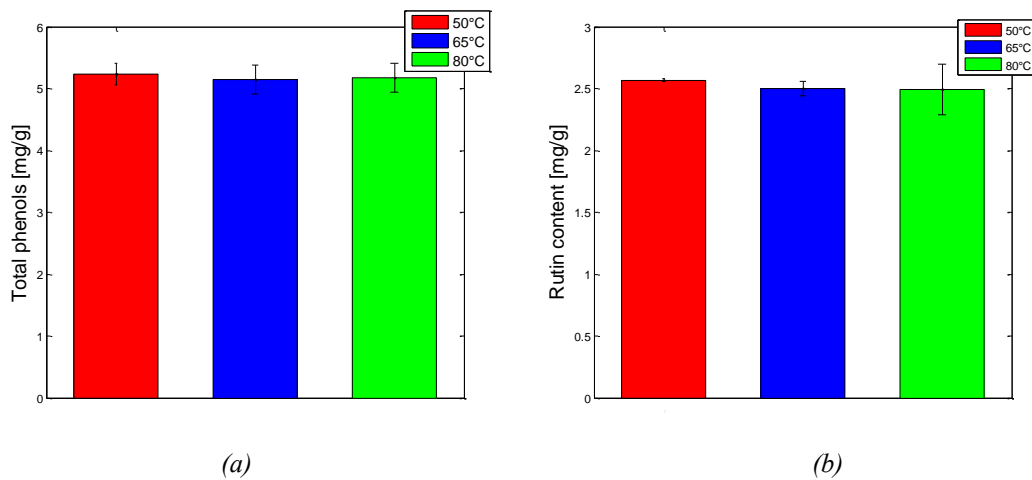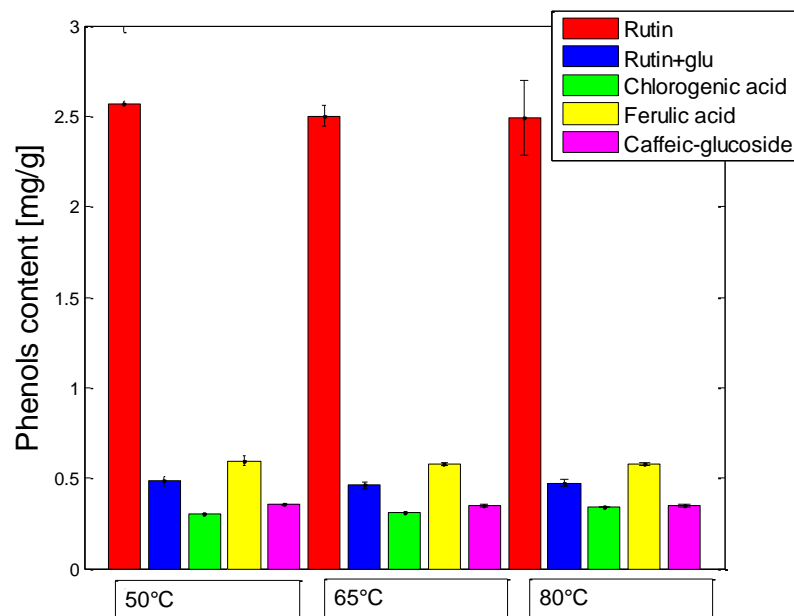

Figure 4.9 *Major phenolic compounds at constant P=200 bar and different T (50, 65, 80°C)*

4.1.3. Behavior of phenolic extraction, every 10 minutes

At fixed conditions, it was interesting to study the quantity of phenols extracted every 10 minutes and the influence of the use of rotavapor.

The temperature and pressure chosen for the fourth group of experiments were respectively 50°C and 150 bar. The operative parameters and results of these conditions, are reported in Table 4.7 and the extraction curve is represented in Figure 4.10. The latter was calculated taking into account the yield after depressurization. Table 4.8 lists the phenolic compounds and their quantity analyzed; in Figure 4.11a the total phenols content is represented and Figure 4.12 shows the quantity of major phenolic compounds (Rutin, Rutin + glucose, Ferulic acid, Chlorogenic acid and Caffeic- glucoside acid). The concentration of Rutin, which was the phenolic compound found in higher proportion, is shown in Figure 4.11b.

Table 4.7: *Experiments conducted at T=50°C and P=150°C*

Matrix and weight[g]	*CO_2 [kg/h]*	*Co-solvent*	*T [°C]*	*P [bar]*	*Time [min]*	*Final average yield [%]with depressurization)*
Asparagus 0.4999	0.280	W:E 1:1	50	150	51	
						37.4
Asparagus 0.4982	0.274	W:E 1:1	50	150	52	
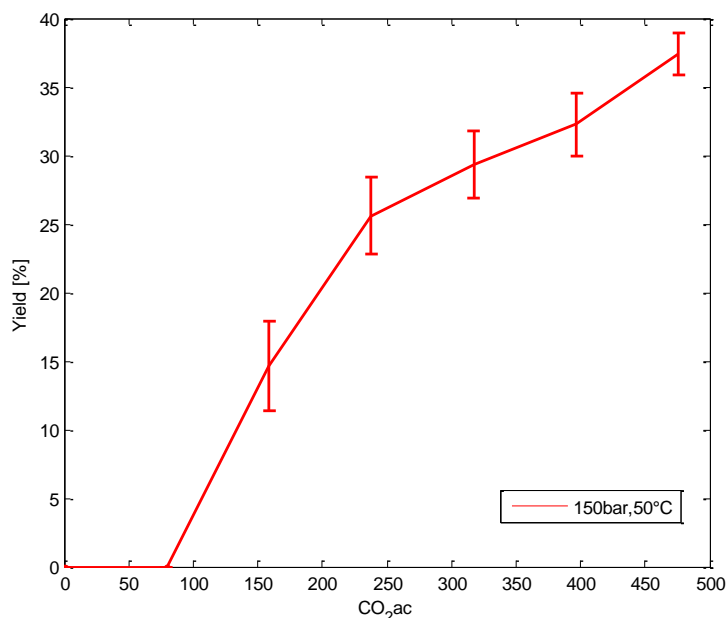
Figure 4.10: *Extraction curve at T=50°C and P=150 bar*

The final average yield without depressurization was 34% (Table 4.3) and 37% with depressurization (Table 4.7). It is interesting to analyze the phenolic content of the sample

collecting during this step, when the pressure decreases from 150 to 0 bar, to decide if it has to be considered.

Table 4.8: *Phenolic compounds at T=50°C P=150 bar*

Phenolic compounds	Average mg/g	St. dev
Quercetin	0.097	0.008
Q glucoside	0.078	0.002
Rutin	**1.992**	0.778
Rutin + glu	**0.384**	0.148
Rutin + glu + Ramn	0.000	0.000
Keampferol	0.040	0.057
K 3rd rutinoside	0.135	0.007
Isoramnetina	0.083	0.002
Iso-rutinoside	0.213	0.009
iso-rutinoside + glu	0.137	0.035
Quinic acid	0.168	0.041
Ferulic acid	**0.555**	0.029
Chlorogenic acid	**0.396**	0.067
Caffeoilglucoside acid	**0.328**	0.051
TOT mg/g	**4.607**	0.926

(a)   *(b)*
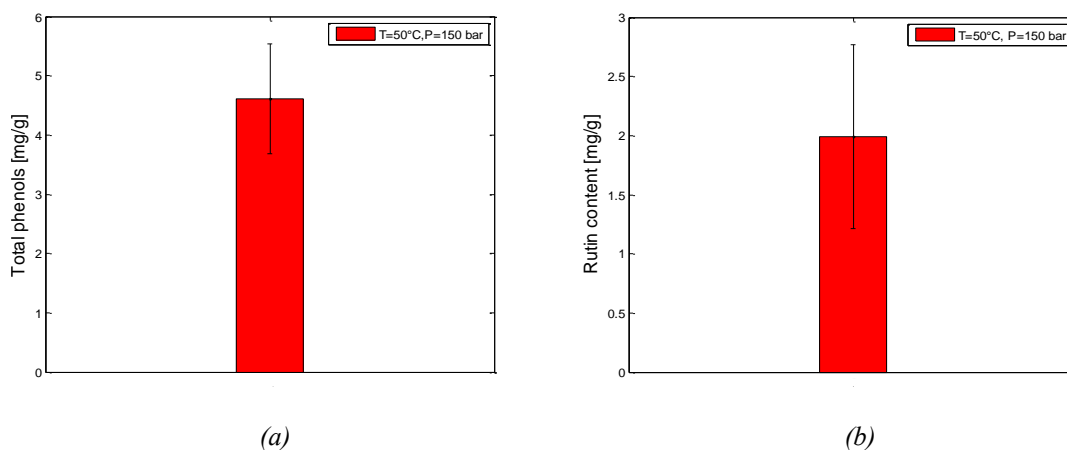
Figure 4.11: *At T=50°C P=150 bar (a) Total phenols content; (b) Rutin content*
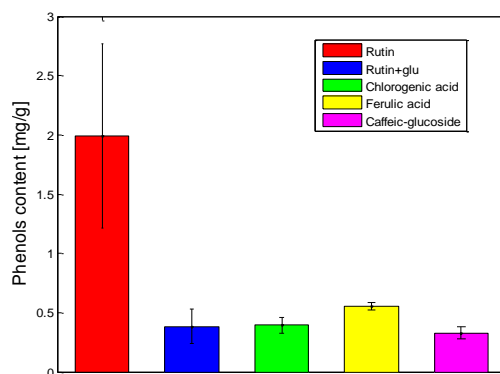
Figure 4.12: *Major phenolic compounds at T=50°C P=150 bar*

In the extractions performed at 50°C and 150 bar, the extracts were collected in the same volume of an equimolar mixture of water and ethanol (10 mL), every 0.02 m^3 of flow CO_2, and they were analyzed without the evaporation of ethanol with rotavapor. The characteristics of the extractions are listed in Table 4.9.

Table 4.9: *Experiments conducted at T= 50°C and P= 150 bar where the extracts were collected and analyzed every 10 minutes*

Matrix and weight [g]	CO_2 [kg/h]	Co-solvent	T [°C]	P [bar]	Time [min]
Asparagus 0.4999	~ 0.24	W:E 1:1	**50**	**150**	59.30
Asparagus 0.4992	~ 0.24	W:E 1:1	**50**	**150**	54

Each different time was identified with a letter, 10 min correspond to A; 20 min to B; 30 min to C; 40 min to D; 50 min to E; 60 min to F; and the depressurization to G.

Two experiments were carried out at these conditions.

Figures 4.13a and 4.13b report the first experiment, on the other hand Figures 4.14a and 4.14b report the second one.

(a)   (b)
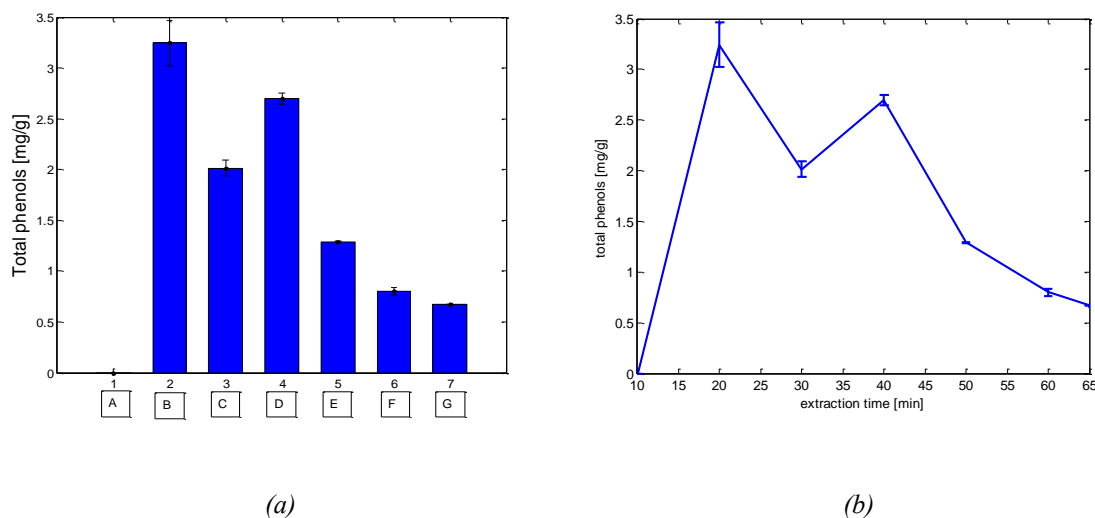
Figure 4.13: *At T=50°C P=150 bar, where the extract was collected and analyzed every 10 minutes: (a)Histogram about Total phenols content (b) Total phenols content (first test)*

(a)   *(b)*

Figure 4.14: *At T=50°C P=150 bar, where the extract was collected and analyzed every 10 minutes: (a)Histogram about Total phenols content (b) Total phenols content (second test)*

In Table 4.10 the averages of the quantities of phenols extracted every 10 minutes between the two experiments are reported. The average of the total content of phenols are represented in Fig 4.15 and Figure 4.16.

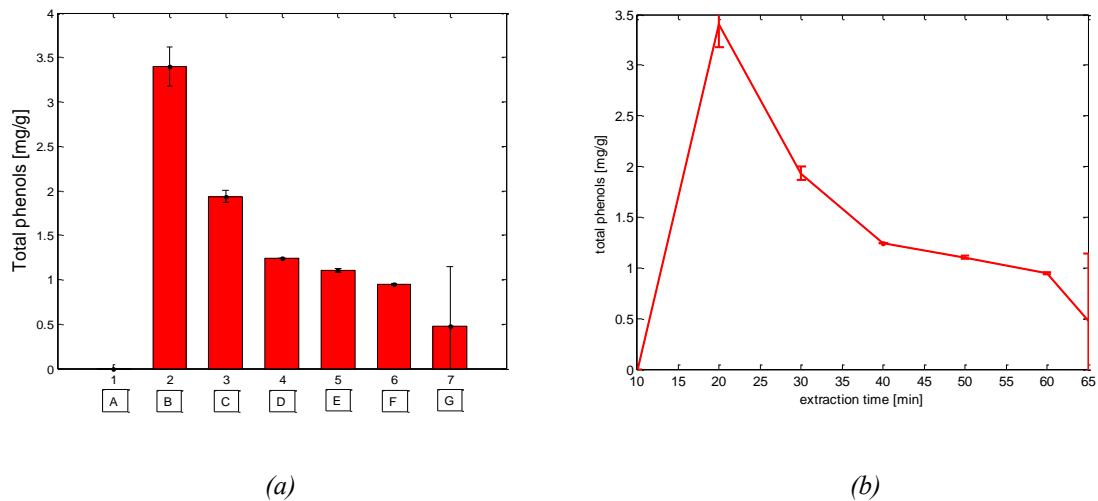**Table 4.10**: *Phenolic composition of the extracts collected every 10 minutes of extraction, at T=50°C and P= 150 bar*

Phenolic compounds	Average A mg/g	St.dev	Average B mg/g	St.dev	Average C mg/g	St.dev.	Average D mg/g	St.dev.	Average E mg/g	St.dev.	Average F mg/g	St.dev.	Average G mg/g	St.dev.
Quercetina	0.000	0.000	0.059	0.000	0.047	0.001	0.048	0.011	0.042	0.003	0.000	0.000	0.000	0.000
Q glucoside	0.000	0.000	0.049	0.000	0.043	0.001	0.043	0.007	0.040	0.003	0.000	0.000	0.000	0.000
Rutina	**0.000**	0.000	**1.758**	0.000	**0.848**	0.057	**0.857**	0.614	**0.362**	0.063	**0.236**	0.100	**0.125**	0.047
Rutina+glu	**0.000**	0.000	**0.199**	0.000	**0.134**	0.006	**0.143**	0.087	**0.077**	0.009	**0.061**	0.010	**0.041**	0.008
Rutina+glu+ramn	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Keampferol	0.000	0.000	0.051	0.000	0.043	0.000	0.024	0.034	0.000	0.000	0.000	0.000	0.000	0.000
K 3o rutinoside	0.000	0.000	0.085	0.000	0.061	0.000	0.064	0.024	0.048	0.004	0.044	0.000	0.032	0.014
Isoramnetina	0.000	0.000	0.050	0.000	0.045	0.000	0.045	0.008	0.041	0.003	0.010	0.015	0.000	0.000
Iso-rutinoside	0.000	0.000	0.141	0.000	0.088	0.003	0.087	0.040	0.057	0.007	0.050	0.004	0.035	0.012
iso-rutinoside+ glu	0.000	0.000	0.068	0.000	0.053	0.003	0.052	0.013	0.044	0.004	0.020	0.028	0.010	0.014
Ac. chinico	0.000	0.000	0.119	0.000	0.057	0.009	0.071	0.040	0.061	0.005	0.048	0.001	0.033	0.013
Ac. feruilchinico	**0.000**	0.000	**0.223**	0.097	**0.220**	0.008	**0.211**	0.082	**0.147**	0.015	**0.146**	0.016	**0.113**	0.063
Ac. clorogenico	**0.000**	0.000	**0.179**	0.000	**0.167**	0.012	**0.162**	0.033	**0.133**	0.009	**0.126**	0.002	**0.092**	0.041
Ac. caffeoilglucoside	**0.000**	0.000	**0.195**	0.000	**0.168**	0.002	**0.164**	0.035	**0.146**	0.005	**0.132**	0.007	**0.097**	0.046
TOT mg/g	**0.000**	0.000	**3.321**	0.109	**1.974**	0.057	**1.971**	1.028	**1.197**	0.131	**0.874**	0.104	**0.575**	0.139
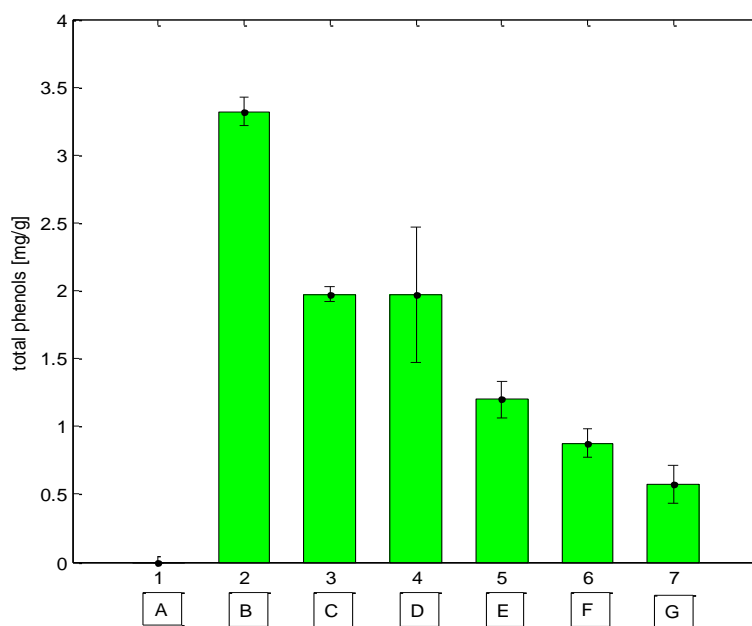
Figure 4.15: *Total phenols content at T=50°C P=150 bar, every 10 minutes (average of the two tests)*
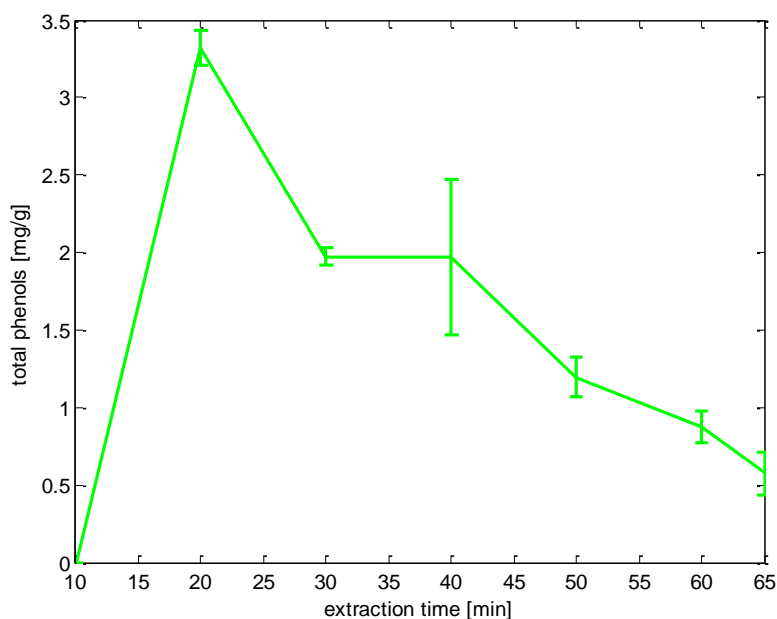
Figure 4.16: *Total phenols content at T=50°C P=150 bar, every 10 minutes (average of the two tests)*

From these graphs it can be seen that the longer amount of phenols was extracted after 20 min (corresponding to letter B). During the first 10 minutes there was no phenols extraction, and after B the quantity of extract decreased gradually. Looking at Figure 4.17, that represents the major phenolic compounds extracted, it is evident that the conclusions above, are confirmed also for Rutin, Rutin + glucose, Ferulic acid, Chlorogenic acid and Caffeic- glucoside acid,

whose concentrations decreased during the time of extraction and they reached the peak after 20 minutes.
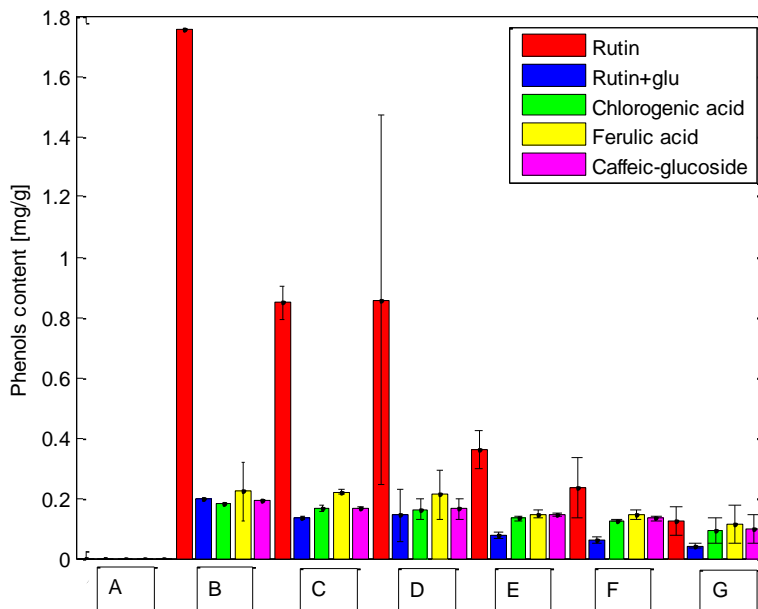
Figure 4.17: *Major phenols compounds at T=50°C P=150 bar, every 10 minutes (average of the two tests)*

4.2 Pressurized solvent extraction

Some extractions without CO_2 using ethanol and water as solvents were also tested, operating at high pressures. This technique is named Pressurized liquid extraction (PLE).

First of all, the use of a mixture of water-ethanol 1:1 was tested. The pressure was changed and other parameters were maintained constant, such as the temperature at 65°C, the mass of asparagus at around 0.50 g, the type of solvent W:E 1:1 and the solvent flow rate (2 mL min^{-1}). The pressures that were tested were 100 bar and 150 bar.

For these experiments the final yield (calculated by in Equation 4.1) was obtained.

The operative parameters and the final extraction yields are listed in Table 4.11.

PLE required less time (30 min) than superctitical fluid extraction (60 min).

In Figure 4.18a the final yields, which correspond to 100 and 150 bar are represented. The yield corresponding at 100 bar was higher than the one reached at 150 bar.

The total phenols content are tabulated in Table 4.12 and are represented in Figure 4.18b.

Table 4.11: *Experiments conducted using only solvent W:E 1:1 at different pressures*

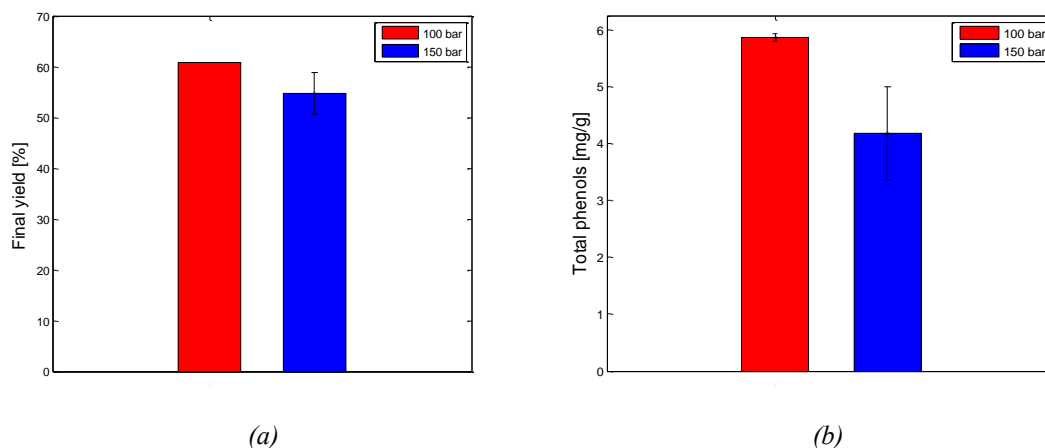| Matrix and weight[g]	CO_2 [mL min^{-1}]	Co-solvent and type	T [°C]	P [bar]	Time[min]	Final average yield [%](no after depresuriz)
Asparagus 0.4999	NO	W:E 1:1 2 mL min^{-1}	40-65	**150**	60	54.75
Asparagus 0.4996	NO	W:E 1:1 2mL min^{-1}	65	**150**	30	
Asparagus 0.4992	NO	W:E 1:1 2mL min^{-1}	65	**100**	34	60.8

(a)   *(b)*

Figure 4.18: *Using only co-solvent W:E 1:1 at constant T=65°C and different P (100, 150 bar): (a) Final extraction yield (b) Total phenols content*

Table 4.12: *Phenolic compounds using only co-solvent W:E 1:1 at T=65°C and different P (100, 150 bar)*

Phenolic compounds	Average mg/g	St.dev.	Average mg/g	St.dev.
Quercetin	0.099	0.006	0.094	0.006
Q glucoside	0.086	0.001	0.078	0.001
Rutin	**2.927**	0.026	**1.827**	0.786
Rutin + glu	0.555	0.010	0.353	0.146
Rutin + glu + Ramn	0.000	0.000	0.000	0.000
Keampferol	0.079	0.000	0.080	0.003
K 3rd rutinoside	**0.150**	0.001	**0.152**	0.007
Isoramnetina	0.088	0.002	0.042	0.059
Iso-rutinoside	0.264	0.002	0.236	0.001
iso-rutinoside + glu	**0.129**	0.004	**0.139**	0.014
Quinic acid	0.236	0.003	0.213	0.008
Ferulic acid	0.638	0.018	0.418	0.049
Chlorogenic acid	0.311	0.003	0.235	0.024
Caffeoilglucoside acid	0.303	0.012	0.314	0.007
TOT mg/g	**5.864**	0.071	**4.181**	0.817

The total phenols extracted followed the same tendency that the extraction yield. In fact the major quantity of total phenols extracted corresponded to the extraction conducted at 100 bar. It is also evident when analyzing the graphic in Figure 4.19, where the concentrations of Rutin, Rutin + glucose, Ferulic acid, Chlorogenic acid and Caffeic- glucoside acid are represented.
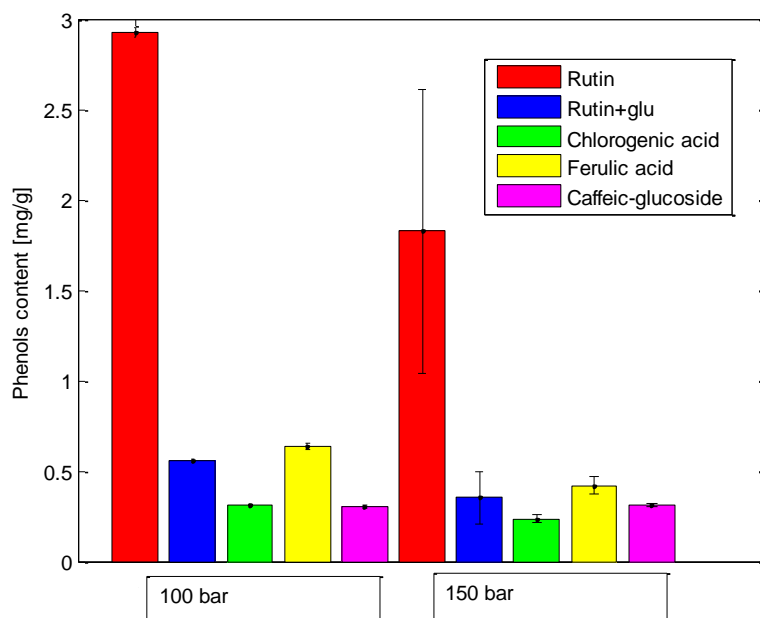
Figure 4.19: *Major phenols compounds extracted using only co-solvent W:E 1:1 at constant T=65°C and different P (100, 150 bar)*

Experiments with only water and only ethanol were made in order to make a comparison between extractions performed with different liquids. The experiments were carried out at the same temperature of 65°C, pressure of 100 bar, mass of asparagus (around 0.50 g) and solvent flow rate (2 mL min^{-1}). The characteristics of the tests and their final extraction yields are listed in Table 4.13.

Table 4.13: *Experiments conducted using different solvents (no CO_2)*

Matrix and weight[g]	CO_2 [mL min^{-1}]	Co-solvent and type	T [°C]	P [bar]	Time[min]	Final yeld [%](no after depresuriz)
Asparagus 0.4992	NO	**W:E** 1:1 2mL min^{-1}	65	100	34	60.8
Asparagus 0.4998	NO	**Water** 2mL min^{-1}	65	100	30	56.1
Asparagus 0.4202	NO	**Ethanol** 2 mL min^{-1}	65	100	21	58

Figure 4.20 represents the yields related to the extractions made with different liquids. The difference between them was not so high but the best performance corresponded to the extraction made with the mixing of W:E 1:1 and conducted at 100 bar.

These results suggested that phenolic compounds of asparagus are more soluble in a mixture of ethanol and water, rather than in the pure solvents.
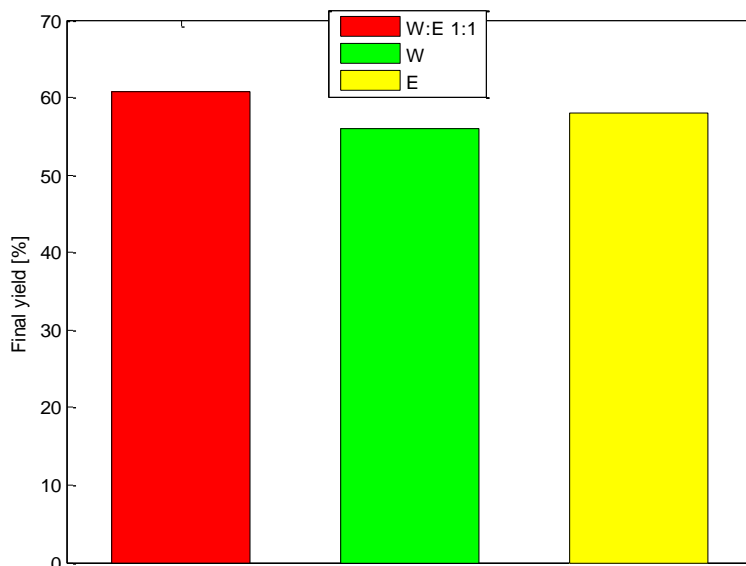
Figure 4.20: *Final extraction yield using only solvents at T=65°C and P= 100 bar: W:E 1:1; Water; Ethanol*

For the total phenols content the comparison is shown in Figure 4.21 where it can be seen that the best result was reached with the extraction made with W:E 1:1 at 100 bar. The one corresponding to the use of water gave the worst result. This indicates that water is able to dissolve a largest variety of compounds from the substrate, according to result obtained of extraction yield, but leading to lower selectivity in the extraction and of phenolic compounds. Figure 4.22 illustrates the concentrations of the major phenolic compounds: Rutin, Rutin + glucose, Ferulic acid, Chlorogenic acid and Caffeic- glucoside acid obtained by pressurized solvents.

Figure 4.21: *Total phenols extracted using only solvents at T=65°C and P= 100 bar: W:E 1:1; Water; Ethanol*
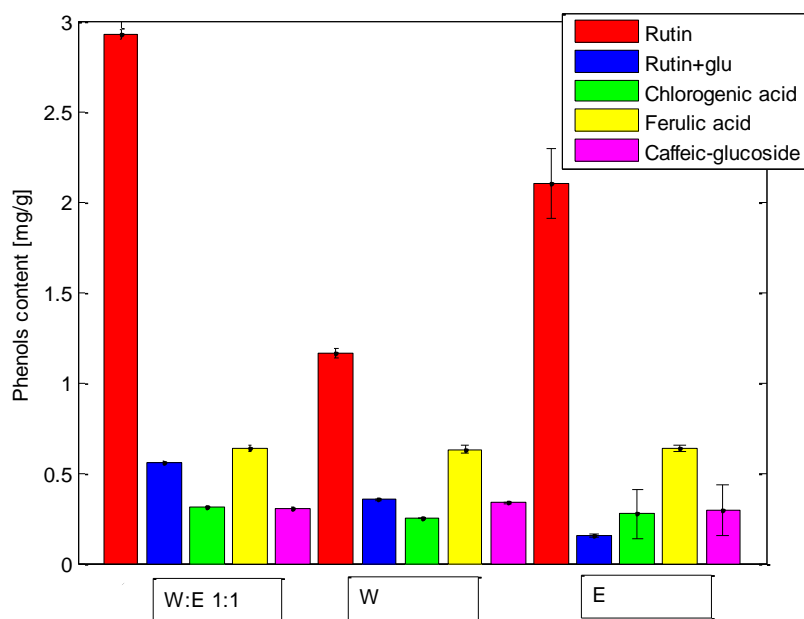
Figure 4.22 *Major phenols compounds extracted using only solvents at T=65°C and P= 100 bar: W:E 1:1; Water; Ethanol*

4.3 Soxhlet extraction

Last, an extraction using Soxhlet with Methanol was conducted. The operative parameters of the extraction are listed in Table 4.14

Table 4.14: *Experiments conducted using Soxhlet*

Matrix and weight[g]	CO_2 [mL min^{-1}]	Co-solvent and type	T [°C]	P [bar]	Time[min]	Final yeld [%](no after depresuriz)
Asparagus 0.4977 g	NO	Methanol	105	1	3 h	32.37

Yields and total phenols that obtained by pressurized solvents are compared with the ones of Soxhlet extraction in Figure 4.23a and Figure 4.23b.
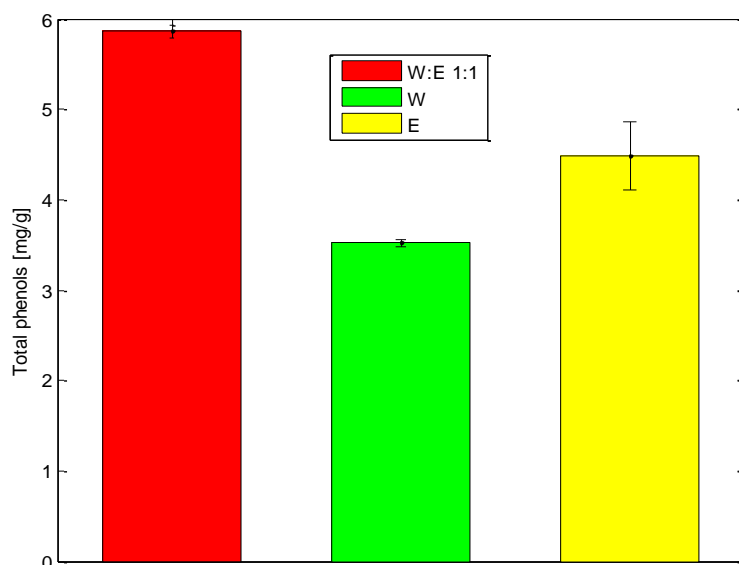

(a)   *(b)*
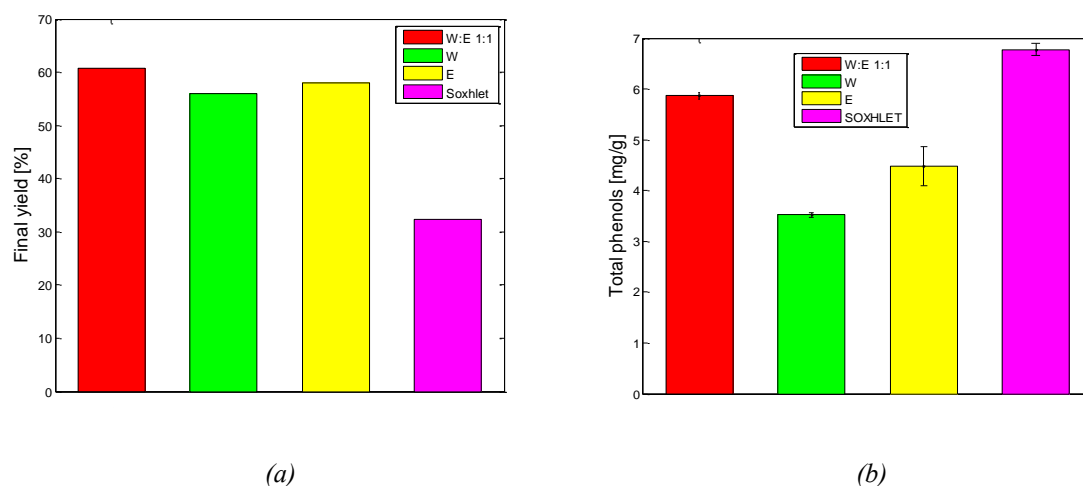
Figure 4.23: *Extraction using only solvents:at T= 65°C and P= 100 bar: W:E 1:1; Water; Ethanol; and Soxhlet (a) Final extraction yield; (b) Total phenols extracted*

Despite the lowest yield corresponded to Soxhlet extraction (32%), this technique permitted to have the highest selectivity, compared with other solvents. The aim was to extract polyphenols from asparagus and Soxhlet achieved the best value for it, as it can be seen in Figure 4.23b.

At this point it was important to compare the pressurized solvent extractions with supercritical fluid extractions that used CO_2 and a mixture of W:E 1:1 as co-solvent. The comparison in terms of extraction yield is illustrated in Figure 4.24. The highest value was connected to the extraction done using only solvent W:E 1:1 and it was above 60%. The lowest was the one conducted with supercritical CO_2 (about 30%).
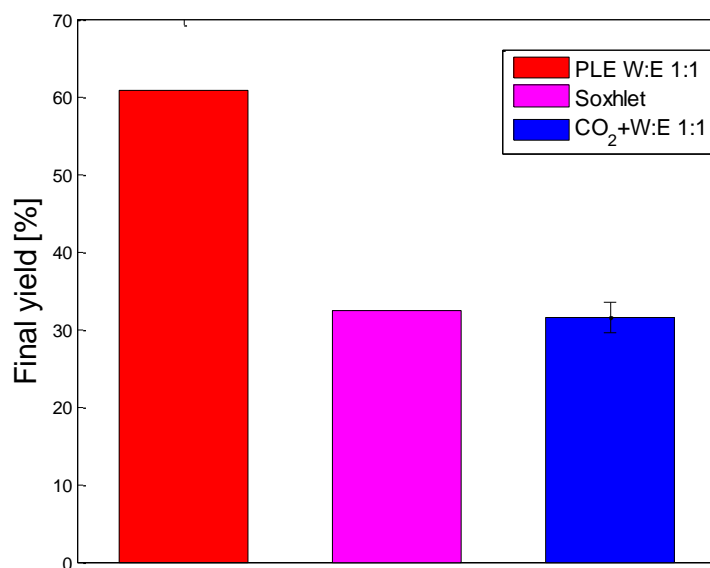
Figure 4.24: *Final extraction yield using only solvents:at T= 65°C and P= 100 bar: W:E 1:1; CO_2 + W:E 1:1 and Soxhlet*

Figure 4.25 is a representation of the total phenols extracted by different extraction methods.
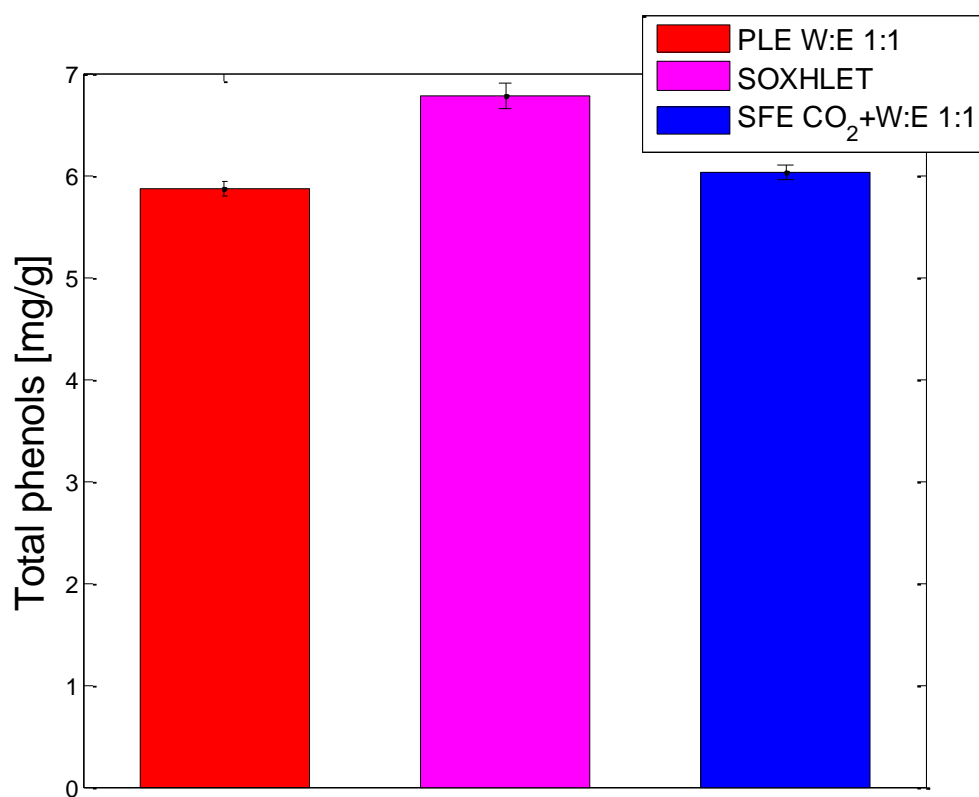
Figure 4.25 *Total phenols extracted using only solvents:at T= 65°C and P= 100 bar: W:E 1:1; CO_2 + W:E 1:1 and Soxhlet*

The best result was obtained by Soxhlet, followed by SFE, but methanol is a toxic solvent and its use is limited in food products. Pressurized liquid extraction can be considered as an economical and environmental friendly alternative as it is cheaper than supercritical CO_2 extraction without losing its advantages.

Conclusions

Supercritical carbon dioxide extraction of Malathion from chickpeas and of polyphenols from asparagus were two types of extraction from food matrices addressed in this thesis.

For the first problem it was not possible to optimize the extraction parameters such as extraction time, temperature, pressure, CO_2 flow-rate and percentage of humidity inside the matrix. Their effects on the extraction were studied, but the results gave a Malathion recovery of 90%, in all cases, so that a comparison between the different operating conditions was not possible. A real contaminated samples investigation should be done. In fact, the high extraction yield value could be caused by the solubility of Malathion in supercritical CO_2, but also by the artificial and surface contamination.

On the other hand, in order to optimize both pressure and temperature related to the SFE applied to asparagus, it was noted that they have an opposite effect on the two parameters studied: extraction yield and extraction selectivity. The aim of this work was to extract polyphenols. The extraction yield is the quantity of the total extract; the extraction selectivity gives information on how many polyphenols are contained in this extract.

In terms of total phenols extracted, the best performances were reached at 100 bar and 65°C with 6 mg/g, always using a mixture of ethanol and water 1:1 as co-solvent and a CO_2 flow-rate equal to about 0.2 kg/h. A comparison with the techniques of pressurized liquid extraction (PLE) and Soxhlet was done. It was shown that the highest total phenols concentrations, at the end of the extraction processes, was the one reached with Soxhlet (6-7 mg/g with an asparagus quantity of 0.5 g). But when using the traditional Soxhlet, the extraction time required is longer than the one needed for SFE.

The polyphenol recovery obtained with SFE is not much lower than Soxhlet, thus confirming the effectiveness of this new and "green" extraction method.

The strong reduction in the use of organic solvents makes this technique particularly suitable to food applications.

However, a cost benefit analysis of the SFE process is essential before going to the commercialization step.

Appendix A

Protocol for switching on the supercritical CO_2 equipment.

1. Switch on the cold bath (-10°C) manually or by a programmer long before the starting of the extraction (there is a time of about 4 hours for the cooling).
2. Switch on the potentiometer (to 10%) which heats the BPR.
3. Load the extractor: solid samples are loaded and placed inside the extractor with a small amount of cotton above the sample
4. Fit taper and close the STUD cross.
5. Close CO_2 input and output connections with two keys (fixed and mobile). The cone connections and counter-cone shall be entirely fitting and most linear possible. First hand screwed everything and then the set of keys.
6. Put on the heating jacket.
7. Switch on the heating jacket:
   - Adjust the temperature by the red display
   - Temperature heating jacket measurement by the green display.
8. Connect the probes:
   - One for the internal extractor temperature.
   - One for the heating jacket
9. Close the BPR valve (blue) to start de pressurization
10. Open CO_2 bottle and the bypass valve. Verify that the BPR is closed, flow = 0.
11. Check the initial pressure (50-60 bar of the CO_2 bottle) and close the bypass valve
12. Ensure that the mass flow meter is at 0, if it is not press "Reset".
13. Wait to reach of the desired temperature.
14. Once the desired temperature is reached, open the bypass valve and switch on the pump by pressing the black button, to an initial pump capacity of almost 50%.
15. With soap and water leaks are checked.
16. Wait until the desired operating pressure is reached and then open the BPR valve slowly to allow the exit of a small amount of CO_2 and keep the pressure constant. Decrease the flow value for the pump by changing the level of the pump.
    - NEVER EXCEED 350 BAR PRESSURE BECAUSE THE RUPTURE DISC IS SET TO 380 BAR.
17. Note variables.
    - Time to reach desired heating jacket T
    - Time to reach operating pressure.
    - Time of high pressure treatment.

- Depressurization time.
- At interval times:
    i.   Extractor internal T (TC_{22} green)
    ii. Heating jacket T (TC_{21} green)
    iii. Internal P (gauge)
    iv. CO2 flow rate ($g\ min^{-1}$) (flowmeter)

Protocol for switching off the supercritical CO_2 equipment.

1. Stop CO_2 pump by pressing the red button.
2. Close the inlet equipment valves and / or CO_2 bottle (check if someone else is using it)
3. Depressurize slowly by opening the BPR valve (blue), controlling:
    a. CO_2 flow rate is not excessive ($Q < 10\ g\ min^{-1}$)
    b. The fall of the internal temperature is not excessive (to prevent freezing of the sample in the extractor or the BPR plugging).
4. Switch off the temperature controllers.
5. Disconnect the probes.
6. Switch off the potentiometer.
7. Remove the heating jacket.
8. When the pressure = 0, open CO_2 input connections with keys (fixed and mobile).
9. Unscrew the STUD and open the extractor to take the solid sample.
10. Clean the extractor.

Appendix B

The main characteristics of extractor used in SFE of Malathion are defined in Table B.1

Table B.1 *Main characteristics of extractor used in Supercritical equipment of Malathion*

Vessel function	Extractor
Material to be treated	Solid matrices with supercritical CO_2
Maximum operating pressure	400 bar
Maximum operating temperature	100°C
Construction material	Stainless steal 316L
Volume	100 ml
L/D	8
Shape	Cylindric
Orientation	Vertical
Type of closure	Screw flat plate
Needed connections	1 lower and 2 upper
Accesories	Spiral tube 1/8 heating jacket Support
Internal diameter	25 mm
Wall thickness	10 mm
External diameter	35 mm
Internal length	200 mm
Bottom thickness	10 mm
Cap thickness	26 mm
Total length	235 mm
Cap diameter	119 mm

In Figure B.1 is represented a picture of the extractor.

Figure B.1 *Extractor of the plant used to perform the SFE of Malathion from chickpeas*

Appendix C

Table C.1 reports the values of temperature inside the extractor (Tin) and of the heating jacket (Thj), when the set temperature (T set) was changed (from 40 to 80°C) every around 20 min, at constant pressure (150 bar). Figure C.1 represents the straight line calibration.

Table C.1: *Values of temperature inside extractor when the set temperature was changed, at constant pressure*

T set [°C]	T in [°C]	Thj [°C]	t [min]
40	45	48	5
40	45	46	10
40	45	47	15
45	47	55	5
45	47	53	10
45	48	53	15
45	48	52	20
50	50	61	5
50	51	61	10
50	52	60	15
50	53	62	20
55	55	65	5
55	55	62	10
55	56	67	15
55	56	62	20
60	59	72	5
60	60	70	10
60	61	73	15
60	62	67	20
70	64	76	5
70	65	79	10
70	67	78	15
70	67	76	20
80	69	79	20
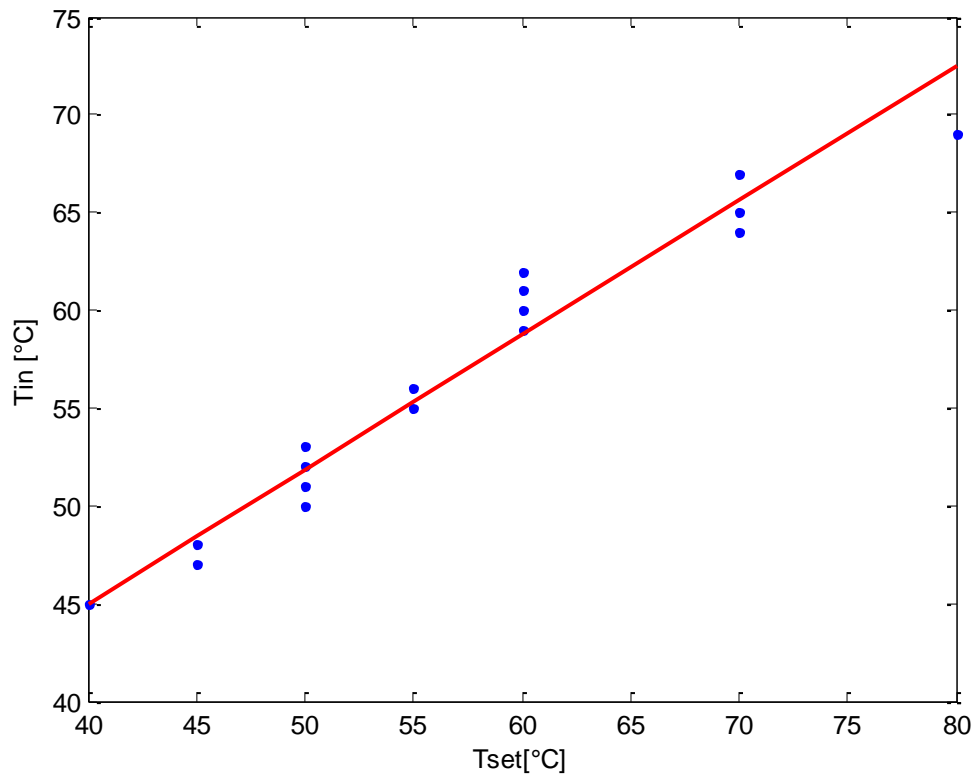
Figure C.1: *Temperature calibration*

Appendix D

The main characteristics of extractor used in SFE of phenols plant are defined in Table D.1

Table D.1. *Main characteristics of extractor used in Supercritical equipment of phenols*

Vessel function	Extractor
Construction material	Stainless steel
Shape	Cylindric made up of two screwed parts: -one bigger cylinder -one inner and smaller cylinder
Orientation	Vertical
Total diameter	5 cm
Bigger cylinder diameter	3 cm
Inner cylinder diameter	1.5 cm
Diameter of hollow tube where solvent flows	0.5 cm
Total lenght	11.5 cm
Inner cylinder lenght	1.5 cm
Wall thickness	1 cm

In Figure D.1 is represented a picture of the two screwed parts of the extractor (a) and of the total extractor (b)

(a)

(b)

Figure D.1 *(a) Two screwed parts of extractor (b) Extractor of the SFE used to extract phenols from asparagus*

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