# Extraction kinetics and parameters of *Silybum Marianum* seeds

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## ABSTRACT

In order to respond to the basic requirements of food and pharmaceutical industries, to reduce or eliminate organic solvent residues in the final products, several extraction techniques were tested from the standpoint of performance, with low energy consumption. The aim of this study was to determine biological activities such as antioxidant and antiproliferative activities of flavonolignans from *Silybum Marianum.* Quantitative study of two methods, solvent extraction (Soxhlet) and supercritical $CO_2$ extraction has concluded that the best performance is obtained for the supercritical fluid extraction at high pressure and low temperature. This study enhances this technique which aims to an estimable amount of pure product.

The effect of supercritical extraction parameters on extraction kinetics and yields were studied: pressure (100-220 bars), temperature (40-80°C), particule size, $CO_2$flowrate, contact time (15-90 min), ethanol as cosolvent. The model chosen is those proposed by Reverchon [1] who introduced models based on the differential mass balance for the extractor vessel, this model describes mass transfer between a single spherical porous particles and the supercritical fluid. Fatty acids were analyzed by gas chromatography and flavonolignans (silychristin, silydianin and silybin) by an HPLC method in seed extracts obtained by organic solvents and SC-$CO_2$ with cosolvent.

The antioxidant activity was measured by two complementary tests (DPPH and ABTS) and confirmed that the extract with higher antioxidant effect is the extract obtained by SC-$CO_2$ at 220 bar and 40°C (yield=32.3 %). The biological activity of standards flavonolignans and an extract (220 bar, 40°C, ethanol as cosolvent, yield= 30.8%) was demonstrated with respect to a colon cancer cell line Caco-2.[2]

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## INTRODUCTION

*Silybum Marianum* (family: Asteracae) is native from the Mediterranean area, which has now spread to other warm and dry regions. *Silybum Marianum* (known milk thistle) seeds are classified as major dietary supplements thanks to its oil composition and fatty acids.
The oil extracted from milk thistle seeds essentially contains lipids, vitamin E (50-60 mg/100 g), and flavonoids (0.25%)[1]. Its high content of unsaturated fatty acids (56%poly-unsaturated and 21% mono-unsaturated) allows it to enter the anti-cholesterol diets for cardiovascular disease prevention [2].

Milk thistle seeds contain active compounds such as silymarin. Silymarin has been known since centuries and recommended in traditional European and Asian medicine, mainly for treatment of liver disorders [3]. These seeds contain silymarin complex, which consists of four flavonolignans [4]: silychristin (SCN), silydianin (SDN), silybinin (SBN)and taxifolin (TXF). The anticancer activity of silymarin as well as silybinin was demonstrated against various cancer cells.
The aim of the present work is to compare extracts from organic solvent extraction and from supercritical carbon dioxide extraction considering extraction yield, composition, antioxidant and biological properties.

## MATERIALS AND METHODS

### 1. Plant materials

Sampling was carried out after the passage of the plant by three phases of growth: vegetative, flowering and maturation phases. Harvest was made from May to August in the area of Bizerte (Northern Tunisian). The sheets, stems, roots and impurities were eliminated thanks to a traditional sieve. The recovered seeds are preserved in dry place (95.95% dry material). Then they are crushed with a grinder to obtain a fine powder (mean diameter: $d_p$=310 µm).

### 2. Oil extraction

#### *2.1 Soxhlet Extraction*

Conventional extraction was performed in a Soxhlet apparatus testing various organic solvents classified according to their polarity; hexane (ρ= 0.66g.cm$^{-3}$, 97% Prolabo), petroleum ether (99.8% Fluka), chloroform (ρ= 1.50g.cm$^{-3}$, 99.5% Carlo Erba). At each manipulation we have introduced 30g of *Silybum marianum* seeds powder in Soxhlet apparatus. The extraction solvents were evaporated and the extracts were weighted and analysed. Yield of extraction was calculated by the formula:

$$Yield\ (\%) = \frac{m_{\ extract}}{m_{raw\ material}} x\ 100 \qquad (1)$$

#### *2.2 Supercritical fluid extraction*

The supercritical home-made apparatus (figure 1) mainly consisted of a 125 ml extractor (23 mm of internal diameter and 300 mm of length) and three cyclonic separator vessels operated in series. $CO_2$ circulation was assured by volume metering pump (Dosapro Milton Roy, MilRoyal D) with a maximum liquid flow rate up to 3.2 kg/h and pressure up to 250 bar. The extracts are separated and collected every 15 minutes by a valve located at the base of the separators. Oil extracted was weighed immediately after collection.

The extraction column is a cylindrical form, out of stainless steel. Its closing is carried out by the use of sintered and cerclips which makes it possible to ensure that the *Silybum Marianum* seed powder does not leave the extractor in a solid state. The extremities of the receptacle consist of sintered which play the part of very fine metal filters which prevent the drive of the solid sample. The *Silybum Marianum* seed powder (m=30g) and the balls of glass are placed in the receptacle. The use of balls of glass ( diameter approximately of 1.7 mm) are to increase the surface of contact between supercritical $CO_2$ and the sample, while ensuring a homogeneous distribution of the substances extracted in the autoclave and to avoid the formation of agglomerates in the extractor.

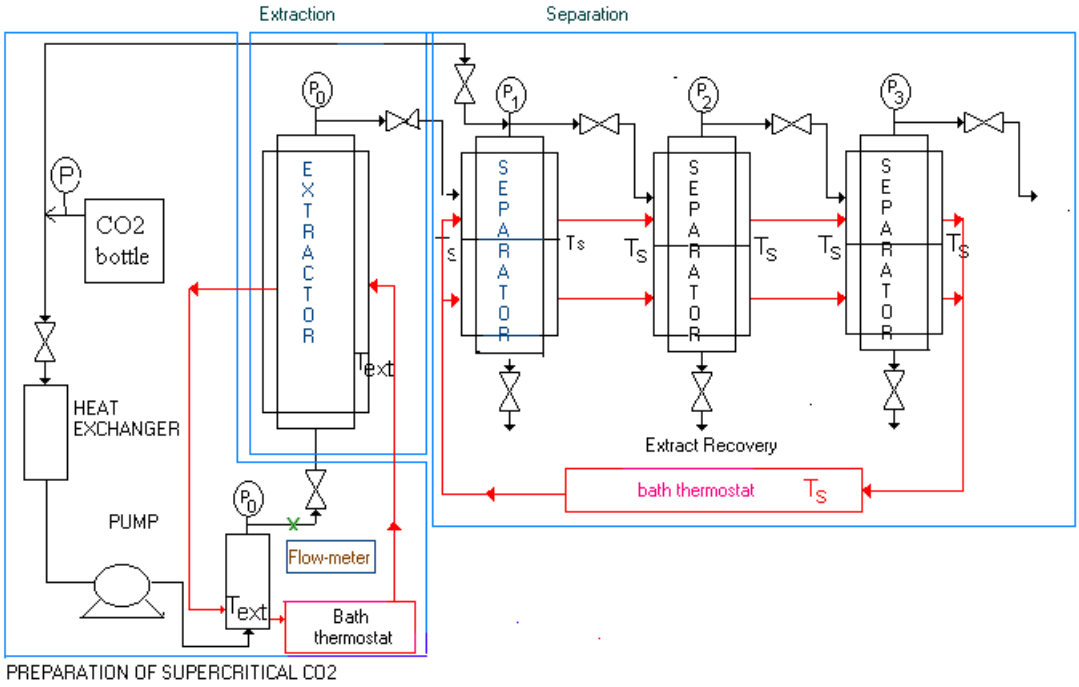


**Figure 1** Schematic flow-sheet of SFE pilot plant.

The method consists in adjusting the $CO_2$ flow near 0.81 kg/h for all the experiments (Flow-meter MicroMotion). Two kinds of experiments were realized: SF-$CO_2$ alone and SF-$CO_2$ with ethanol as co-solvent. The co-solvent was introduced by means of a pump (Gilson) at a rate of 5 ml/min for 15 minutes during the static contact time (30 minutes) of the feed with $CO_2$. SFE temperature and pressure were varied as mentioned in table 3 in the case of $CO_2$ SFE and from 100 bar to 220 bar and from 40°C to 80°C in the case of $CO_2$/EtOH SFE. The raffinate is weighted at the end of experiment to evaluate mass balance:loss from 5 % to 10 %.

## 3 HPLC Analysis

This HPLC method is adapted from Quaglia et al.[5]. A Shimadzu LC-10AT VP chromatograph equipped with a Varian photodiode array detector was used for the analyses. The chromatograph was controlled and the data evaluated by a computer Flyer Pentium, interface D 7000. Sample solutions were injected using a 20ml sample loop. The flavonolignans separation was carried out using stationary phase: C18 pre-column (Alltech) and C18 column (150 mm x 4.6 mm, 5 µm) (poursuite XRs de Varian) maintained at 40 °C. The isocratic conditions [5] (Water, acidified until pH 2.6 with 10% $H_3PO_4$, was mixed with acetonitrile in the ratio of 62:38) have been modified in gradient conditions:
Solvent A: Water, acidified until pH 2.6 with 10% $H_3PO_4$;
Solvent B: acetonitril ;
Solvent C: methanol .
The flow rate was 1 ml $min^{-1}$. The separation analysis is carried out according Table 1.

Table 1. Gradient conditions of HPLC for flavonolignans separation

| Time (min) | % solvent A | % solvent B | % solvent C |
|---|---|---|---|
| 0.0 | 63 | 15 | 22 |
| 7.5 | 63 | 15 | 22 |
| 8.5 | 40 | 20 | 40 |
| 15.0 | 40 | 20 | 40 |

The photodiode array detector conditions were: λ = 330 nm. Acquisition rate of spectra 1600 ms. Spectral bandwidth for each channel 4 nm. Wavelength range: 220–350 nm. The standard solutions were prepared from three flavonolignans (Silybin (SBN) 97.1% tr=12.25 min., Silychristin (SCN) 82.2% tr=8.78 min., Silydianin (SDN) 93.2% tr=9.60 min.) from ChromaDex, France). Flavonolignans extracts were identified by comparison of retention times and quantified with calibration curves of the 3 standards.

### 4. Antioxidant tests

In order to determine the antioxidant activities of the extracts, two methods were chosen: DPPH° radical scavenging activity was measured according to the method described by Brand- Williams, Cuvelier&Berset [6] and $ABTS^{\circ+}$ radical activity according to Miller [7]. The measured using DPPH ° radical scavenging activity is expressed as 50% effective concentration $EC_{50}$ (mol. $L^{-1}$). The antioxidant power of a compound is much higher than the $EC_{50}$ value is low. Regarding the second method, the antiradical power was determined by the TEAC assay (µm), Trolox equivalent antioxidant capacity. More TEAC, the higher the antioxidant power is high.

Caco2 (colon cancer cell line) [8] cells were used to study the cytotoxic activity by the aim of the mortality rate calculation.

## RESULTS

### 1.Soxhlet extraction

Our aim is to obtain an extract with interesting properties, essentially biological activities. The first result of the extraction and easier to obtain, is the extraction yield.
In the case of Sohxlet extraction, the more important experimental parameter is the solvent polarity. On table 2 it is possible to observe that the higher yield is obtained with chloroform, it is higher than in the literature [3]. The results are same order of magnitude with the two other solvents. The yield and the composition of the extracts depend also from the geographic origin of the plant material.

Table 2. Yield : Soxhlet extraction results.

| **Solvent** | **Temperature (°C)** | **Extraction duration (h.)** | **Yield (%)** | **Yield [3] (%)** |
|---|---|---|---|---|
| hexane | 68 | 6 | 21.88 | 20.90 |
| chloroform | 61 | 3.5 | 28.85 | 21.70 |
| Petroleum ether | 137 | 6 | 19.46 | 22.50 |

## 2.Supercritical $CO_2$

Since various parameters (temperature and pressure of $CO_2$-SC, flow-rate, feed water percentage, particle size, separation $CO_2$/Extract,…) potentially affect the extraction process, the optimization of the experimental conditions is a critical step in the development of $CO_2$ supercritical extraction method. As $CO_2$ density is an important parameter, we chose relatively high pressures, 180 and 220 bar and temperature from 40°C to 80°C to observe the influence of those parameters firstly on the yield and also on the extracts composition. The examination of table 3 shows us that the yield is the same order of magnitude than with organic solvents and some times higher. The maximum yield is obtained at P=220 bar and T= 40°C, corresponding to the highest $CO_2$ density in the case of experimental conditions chosen.

Table 3. Yield : Supercritical carbon dioxide extraction results

| **Pressure (bar)** | **Temperature (°C)** | **$CO_2$ density [9] ($kg/m^3$)** | **Yield (%)** |
|---|---|---|---|
| 180 | 40 | 760 | 22.46 |
| | 60 | 620 | 21.70 |
| | 70 | 550 | 24.09 |
| | 80 | 490 | 27.77 |
| 220 | 40 | 810 | 32.37 |
| | 60 | 700 | 30.14 |
| | 70 | 640 | 26.12 |
| | 80 | 580 | 30.25 |

We have withdrawn extracts each quarter hour in order to obtain extraction kinetics curves (Figure 2). Figure 2, we observed the highest efficiency at 220 bar and 40°C and the curves are not typical.

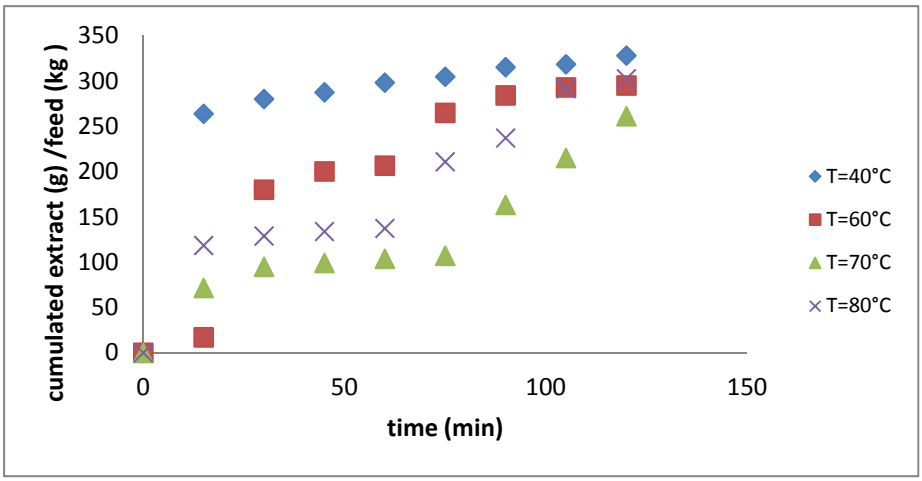


Figure 2. Kinetics extractions at 220 bar.

The adopted model is the model with uniform extraction throughout proposed by Reverchon [10]: this is a two-parameters model (Di and kp), which express the effects of different resistance to internal transfers and equilibrium solute sharing between the solid and the fluid.

We can summarize the assumptions of this model as follows:
• piston-type flow in the bed;
• the resistance due to axial dispersion in the bed, is disregarded;
• resistances to mass transfer are located in the solid and characterized by the coefficient Di
• the partition equilibrium of the solute in the solute between the fluid and the solid surface is linear and characterized by the coefficient kp
• constant interstitial velocity along the bed.

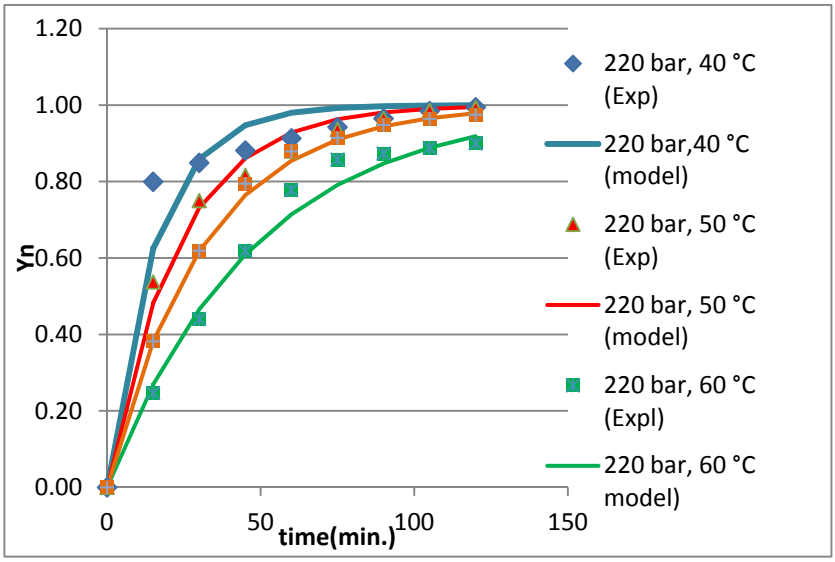


Figure 3. Kinetics of theoretical model [10] and experimental extraction at different temperatures

Generally the curves (Figure 3) obtained by the model have the same trend as those representing the experimental values which shows the reliability of the model chosen.

The analysis of the extracts by GC-FID and GC-MS [11] showed their richness in essential fatty acids but HPLC analysis and a high antioxidant capacity (table 4) but showed the absence of flavonolignans. As these molecules have a high polarity, we decided to introduce a co-solvent, ethanol with $CO_2$-SC.

**2.Supercritical $CO_2$ with ethanol as co-solvent**

At each pressure (100, 120, 140,160, 180 and 220 bar), the temperature was varied from 40 to 80 °C by 10°C. All cumulated extract have been analysed: fatty acids, antioxidant properties (2 tests), flavonolignans HPLC detection. We have selected the best chromatographic conditions for quantifying flavonolignans in samples obtained by extraction with organic solvent and supercritical $CO_2$ with co-solvent (Figure 4).

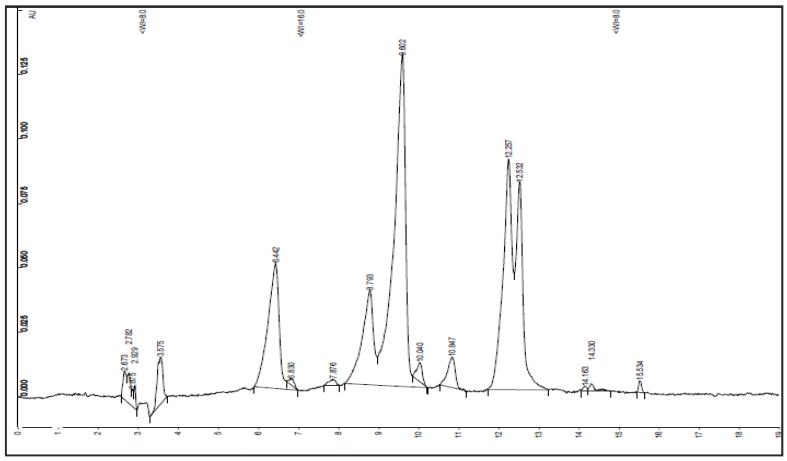

**Figure 4. HPLC extract ($CO_2$ /EtOH) analysis**

We present the results of the extract that seem to be the "best" (Table 4) at 220 bar and 40°C, with ethanol as co-solvent, that means with higher flavonolignans. We have studied the cytotoxic activity of this extract compared to standards (Silybin, Silychristin, Silydianin) : this oil exhibited a high antioxidant activity and induced the highest percentage of mortality of Caco2 Cancer cells (from 43 to71% for concentrations of 10 up to 100µg/ ml).

**Table 4.**

| Extraction | Yield (%) | Fatty acids (%) C18:1 | C18:2 | C18:3 | DPPH $CE_{50}$(µg/ml) | ABTS TEAC(µM) | Flavonolignans (mg/g) extract |
|---|---|---|---|---|---|---|---|
| **hexane** | 21.88 | 20.66 | 56.11 | - | 0.70 | 1.08 | 45.40 |
| **220 bar 40°C** | **32.37** | **27.01** | **65.22** | **0.59** | **0.42** | **3.32** | - |
| **220bar 40°C +EtOH** | 30.87 | 22.01 | 55.13 | 0.44 | 0.87 | 0.97 | **106.75** |

## CONCLUSION

The oily extracts obtained at 220 bars and 40°C of *Silybum marianum* seeds were rich in fatty acids, such as linoleic acid (65.22%), oleic acid (27.01%) and palmitic acid (12.12%). The evaluation of antioxidant activities demonstrated that this extract presented a high antioxidant effect. The present study allowed the identification and quantification of flavonolignans seed extracts obtained by SC-$CO_2$ with co-solvent. Three major flavonolignans were identified as silychristin, silydianin and silybin. Furthermore, biological activity of this extract is demonstrated with respect to a colon cancer cell line Caco-2.


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