GC-FID ANALYSIS OF SUPERCRITICAL FLUID EXTRACTS OF SILYBUM MARIANUM SEEDS

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Abstract

Silybum marianum is a plant, member of sunflower family, native of a narrow area of the Mediterranean region. It is known since two miles years by its value estimable into therapeutic (anti-hepatotoxic and hepatoprotective activity) and used like popular and traditional drug. Considered by its high content in antioxidants, it easy acts against the infections of the liver, hepatitis as well as the cirrhosis. The seed has a great wealth of flavonoïdes (flavonolignanes).

The seed oil of milk thistle was extracted by supercritical carbon dioxide extraction, supercritical carbon dioxide and co-solvent (ethanol) extraction, and Soxhlet procedure using various solvents. The experimental parameters of supercritical carbon dioxide extraction which is classic in these extraction techniques including pressure, temperature, particle size and extraction time were optimized.

The impact of extraction variables have been evaluated on the yield (11.33 to 39.88%) and fatty acid composition (linoleic acid 55.08% and oleic acid 20.86) of the extracted oil.

The analytical methods for the qualitative and quantitative determination of fatty acid composition are gas chromatography–flame ionisation detection (GC-FID).

The *Silybum marianum* oil is rich in linoleic acid (omega-6). This fatty-acid is essential to the good performance of the body. It plays an important role in the nervous system, cardiovascular balance and constitutes a protection against free radicals, responsable for the aging of the skin.

***Keywords**: Silybum marianum oil;Supercritical Fluid Extraction ; oil indices; GC-FID; fatty-acids, omega 6*

INTRODUCTION

Milk thistle (*Silybum marianum L*. Gaertn.; family: Compositae or Asteraceae) is an annual or biennial plant and a recognised medicinal plant that originated in the Mediterranean Basin. As a crop and weed on agricultural plantations, it occurs in many European countries, North Africa, South and North America, Central and Western Asia and southern Australia [1] [2] [3] [4]. Extracts of milk thistle (*Silybum marianum L.)* seed have a long tradition for treating liver ailments [5]. In 2005, milk thistle seed products placed in the top ten of dietary supplements, at about $8.3 million [6]. The seeds contains 20–25% (w/w) lipid [7] [3] which makes one step extraction of silymarin from the fruit impossible. The oil is by-product of silymarin industrial production and has to be removed from seeds prior to the silymarin extraction.

The oil is by-product of silymarin industrial production and has to be removed from seeds prior to the extraction of silymarin and is a by-product of silymarin production [8]. This oil contains essential phospholipids and a relatively high content of vitamin E (0.08%) [9] and a great quantity of the unsaturated fatty acids such as linoleic acid (53.3%) and oleic acid (20.8%) [10]. Therefore, the *S.marianum* seeds would be as an ovel source of the plant oils.

The silymarin is a complex composed of the six flavonolignans silychristin (SC), silydianin (SD), silybinin A (SA), silybinin B (SB), isosilybinin A (ISA) and isosilybinin B (ISB) [11] and it is used for multiple medicinal purposes, due to its various physiological characteristics. Research has confirmed that silymarin extracted from milk thistle seeds can protect healthy liver cells from deterioration, helping cleansing and detoxification, as well as contributing to regeneration of damaged cells [12][13].

The oil from the plant seeds has been traditionally recovered by hydraulic pressure and solvent extraction [14] [15]. Limitations of these processes include the requirements of special instruments and energy consummation.

Supercritical fluids (SCFs) are the most commonly used solvent for the extraction of edible oils from natural products [16] [17]. Particularly, supercriticalCO_2 ($SCCO_2$) is often promoted as an environmentally friendly solvent having useful properties for a wide range of chemical and biochemical processes [18].

In this field, carbon dioxide has been especially adopted since it is essentially non-toxic, non-flammable, inexpensive at the industrial level, can be recycled, has easily

accessible supercritical conditions, and is totally dissipated from extracts at atmospheric pressure avoiding the necessity of further expensive and harmful refining treatments [19][20].

MATERIAL 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 on 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 grinder to obtain a fine powder.

2. Oil extraction

2.1 Extraction by soxhlet with organic solvents

Conventional extraction was performed in a Soxhlet apparatus at different temperatures, using different organic solvents: hexane (ρ= 0.66g.cm^{-3}, 97% Prolabo), petrol ether (ρ= 1.26 g.cm^{-3} ,99.8% Fluka), chloroform (ρ= 1.50g.cm^{-3}, 99,5% Carlo Erba) and methanol (ρ= 0.79g.cm^{-3} , 99% Fluka) . At each manipulation we have introduced 30g of *Silybum marianum* seeds powder with solvent (100ml) in soxhlet at 75°C apparatus during 4h (estimated time after exhaustion of the extractable mass). The extraction solvents were evaporated and the extract was analysed. Yield of extraction was calculated by the formula:

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

Where $m_{\ extract}$ is mass of the extract and $m_{raw\ material}$ is mass of the raw material (seeds of *Silybum marianum*) extracted.

2.2 Supercritical fluid extraction

The supercritical home-made apparatus (figure.1) mainly consisted of a 125 ml extractor (23mm of internal diameter and 300mm of length); and three cyclonic separator vessels operated in series. CO_2 circulation was assured by volume metering pump (Dosapro Milton Roy) capable of liquid flow rate up to 3.2 kg/h and pressure up to 250 bar. The

extracts are separated and collected at any time by a valve located at the base of the separators. Oil extracted was weighed immediately after collection.

The dynamic extraction corresponds to the dissolution of a chemical compound under the effect of a continuous flow of supercritical fluid, crossing a known mass of the sample. The method consists in adjusting the CO_2 flow while preserving the mass of the sample, in order to adjust the contact time (30min) [11].

With an aim of obtaining the best output of extraction several experiments were carried out, while varying the temperature and the pressure, the mass of the extract, CO_2 flow rate and separators pressure were recovered every 15 minutes.

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 consists of sintered which play the part of very fine metal filters which prevent the drive of the solid sample .The *Silybum marianum* seed powder 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.

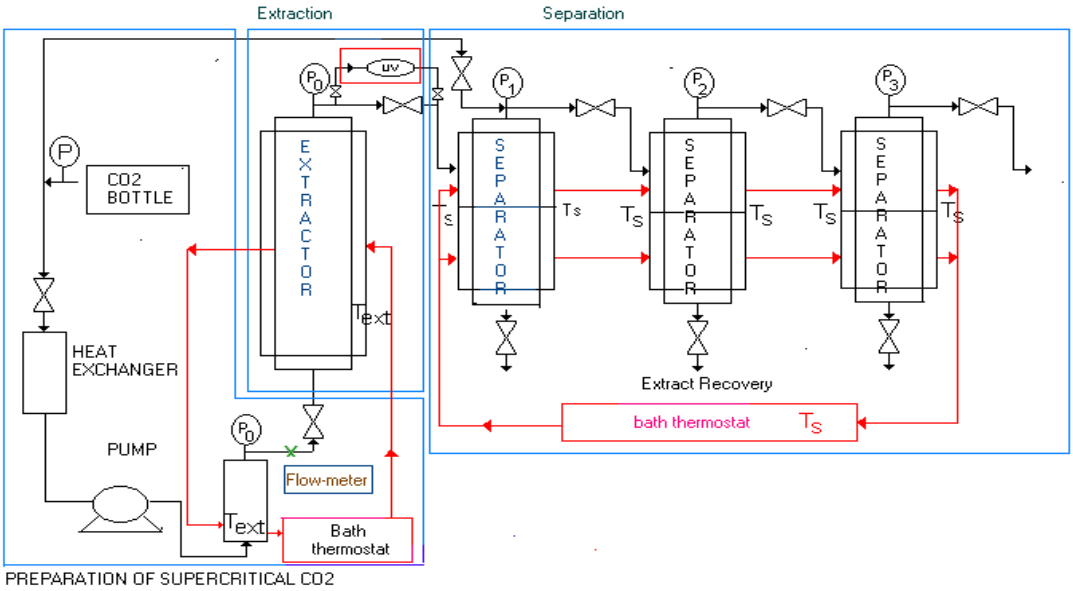

Figure 1 Schematic representation of a pilot plant

Extractions were accomplished in different temperatures (40, 50, and 60°C) and one level pressure (180bar) without co-solvent. The co-solvent is introduced by means of a pump at a rate of 5 ml / min for 15 min) in different temperatures (40, 60, 70 and 80°C) and different pressures (100,120,140 and 160 bars) (Table 1)

The tests are realized using 30g of Silybum marianum seeds at a time. The experimental parameters are given as follow:

Vegetable matter:	*Silybum marianum* seeds
Mass flow:	1.3 kg h^{-1}
Size of particle:	1.7mm ≥ Ø ≥ 250 µm
Contact period of the vegetable matter with CO_2:	0.5h
Co-solvent:	Ethanol
The vegetable matter dries:	95.95 %
Temperature of separation:	30°C
High bed:	18 cm
Volume of bed:	66.537 cm^3
Density of *Silybum marianum* seeds:	415 $kg.m^{-3}$
Fraction of vacuum of extractor:	0.1

3. GC-FID analysis

The chromatographic analysis by GC-FID of the oily extracts obtained by various technics of extraction is carried out with an aim to determinate their fatty-acids composition. Before their introductions in the chromatograph, booth standards (corn germ oil and copra vegetable oil, Sigma Aldrich) and samples are prepared thanks to a method of methyl esters preparation starting from a neutral fatty acid. The measurements by GC-FID are taken with filled column (1.5m*1/8 " *2mm;15% CP sil 84 on chromosorb WHP 100-200 mesh;10% phenylpropylysiloxane +90% cyanopropylpolysiloxane) with a type chromatograph Varian Série 1400 and one detector FID, the type integrator is Spectra Physics SP 4100. Nitrogen as carrier gas was adjusted to a flow rate of 15ml min^{-1}. The column temperature is 190°C, the temperature of the injector is 240°C, the attenuation of the detector DTA is equal to 1 and that of the integrator is equal to 16, the sample injected is 1µl of methylester fatty-acids.

The constituents of seeds oil and extracts were identified comparing their retention times with those of available standards and their mass was calculated from a determined peak area response factor.

RESULTS

We have realized the extraction at various temperatures and pressure in order to determine the various stages of extraction of Tunisian Milk thistle seeds and to determinate the oil yield of this plant and the influence of these extraction parameters on fatty acid composition.

The extraction parameters and oil yield Tunisian Milk thistle seeds are recorded in table 1.

Table1 Extraction parameters and oil yield

Sample	Extraction parameters	Temperature	Pressure	CO_2 mass (kg /h)	Oil yield (%)
E1	*Soxhlet –petroleum ether*	–	–	–	*19.46*
E2	*Soxhlet –chloroform*	–	–	–	*28.85*
E3	*Soxhlet – hexane*	–	–	–	*21.88*
E4	**Supercritical CO_2**	**40°C**	**180 bars**	**0.60**	**32.37**
E5	**Supercritical CO_2**	**50°C**	**180 bars**	**0.95**	**21.70**
E6	**Supercritical CO_2**	**60°C**	**180 bars**	**1.17**	**24.09**
E7	Supercritical CO_2– ethanol	40°C	100 bars	0.92	36.88
E8	Supercritical CO_2–ethanol	60°C	100 bars	0.67	11.38
E9	Supercritical CO_2– ethanol	70°C	100 bars	1.09	24.81
E10	Supercritical CO_2–ethanol	80°C	100 bars	0.80	13.45
E11	Supercritical CO_2– ethanol	40°C	120 bars	1.19	34.55
E12	Supercritical CO_2– ethanol	60°C	120 bars	0.75	17.12
E13	Supercritical CO_2– ethanol	70°C	120 bars	1.11	14.45
E14	Supercritical CO_2– ethanol	80°C	120 bars	1.20	11.33
E15	Supercritical CO_2– ethanol	40°C	140 bars	1.23	15.40
E16	Supercritical CO_2– ethanol	60°C	140 bars	0.78	26.02
E17	Supercritical CO_2– ethanol	70°C	140 bars	0.81	16.58
E18	Supercritical CO_2– ethanol	80°C	140 bars	1.20	22.11
E19	Supercritical CO_2– ethanol	40°C	160 bars	1.28	**39.88**
E20	Supercritical CO_2– ethanol	60°C	160 bars	1.31	12.45
E21	Supercritical CO_2– ethanol	70°C	160 bars	0.86	19.78
E22	Supercritical CO_2– ethanol	80°C	160 bars	0.65	27.14

Since various parameters (extraction solvents, temperature and pressure of supercritical CO_2, flow rate, water percentage, particle size and…) potentially affect the extraction process, the optimization of the experimental conditions represents is a critical step in the development of CO_2 supercritical extraction method. The maximum yield (39.88 %) of extract is obtained with supercritical process at 40°C and 160 bars with co-solvent. It should be noted that the mass of the oil extract varies according to the solvents and the variability of temperature and pressure of CO_2 supercritical extraction. All parameters influence quality and

yield oil. The soxhlet extraction gives the best output yields with chloroform (28.85 %). The highest yields was obtained at 40°C we note that an increase of temperature resulted a decrease in the extraction yield. Another CO_2 supercritical extraction study [12], realized on the aromatic plants revealed that an increase in the temperature involves a reduction of the oil yields. This result can be explained by the reduced CO_2 density at higher temperature consequently it result a decrease solubility of solutes in CO_2 which influence the extraction kinetics. Several studies on the influence of pressure and temperature have showed that increasing pressure increases the yield of extraction, which is due to an increase in the density of the solvent by a constant temperature, does not give the same result, because of the influence of either the density of the solvent and the vapor pressure of compounds extracted.

The fatty acids composition of *Silybum marianum* oil seeds is recorded in table 2 and in figure 1.It was found that this oil is rich in unsaturated fatty acids, which constitute 79.46% of the total fatty acids, this value is higher than that found in El-Mallah and al., [10] (2003) study (75.1%) in Egyptian Milk thistle oil seeds extracted with chloroform-methanol (2:1 v/v).

Among the unsaturated fatty acids, linoleic acid is the major constituent forms (55.08%) of the total composition followed by oleic acid (20.86%). These amounts are approximately the same in Egyptian Milk thistle oil seeds [10]. Whereas sunflower oil contains lower amount of linoleic acid, 42.0 %, but higher amount of oleic acid, 46.0 % (El-Mallah and al., 1999) [23]. Saturated fatty acids, namely, palmitic acid, 10.40 %, and stearic acid, 4.99 % are the main saturated constituents in Silybum marianum oil seeds. Cotton seed oil contains much higher amount of palmitic acid, 24.7 %, which nearly forms the saturated part of the fatty acid profile, whereas, in sunflower oil palmitic acid constitutes 6.2 % of the total fatty acids content.

Table 2 Fatty acids composition

AG	*E1*	*E2*	*E3*	**E4**	**E5**	**E6**	E7	E8	E9	E10	E11	E12	E13	E14	E15	E16	E17	E18	E19	E20	E21	E22	**M**
C12 :0	*ND*	*ND*	*ND*	**ND**	**ND**	**ND**	0.07	ND	ND	ND	ND	ND	ND	ND	0.05	ND	ND	ND	0.08	ND	ND	ND	**0.06**
C14 :0	*0.071*	*traces*	*traces*	**0.05**	**0.06**	**0.06**	0.30	0.11	0.38	0.09	0.16	0.18	0.15	0.26	0.13	0.22	0.28	0.16	0.19	0.32	0.19	0.36	**0.18**
C16 :0	*9.70*	*11.71*	*10.44*	**11.27**	**9.57**	**10.84**	8.27	10.91	11.37	9.84	9.20	10.10	11.75	10.81	9.84	8.74	9.17	10.11	13.01	12.74	8.66	10.88	**10.40**
C16 :1	*ND*	*ND*	*ND*	**ND**	**ND**	**ND**	0.77	0.24	0.13	0.17	0.36	0.22	0.19	0.12	0.31	0.59	0.67	0.14	0.78	0.44	0.19	0.69	**0.37**
C16 :2	*ND*	*ND*	*ND*	**ND**	**ND**	**ND**	3.77	0.50	1.59	ND	ND	ND	0.44	ND	ND	ND	ND	ND	1.59	ND	ND	ND	**1.57**
C18 :0	*5.26*	*2.59*	*4.85*	**4.92**	**5.10**	**5.03**	4.94	12.43	5.53	0.79	5.41	5.12	4.84	5.16	5.55	4.79	5.60	1.13	5.62	5.11	5.09	5.54	**4.99**
C18 :1	*21.75*	*21.09*	*21.84*	**22.01**	**21.90**	**22.02**	20.59	12.77	21.29	26.07	22.26	20.11	23.56	25.58	22.46	19.87	21.21	20.15	27.04	23.51	21.51	20.89	**20.86**
C18 :2	*60.36*	*63.15*	*60.76*	**59.47**	**61.66**	**60.52**	46.39	55.13	50.43	57.53	53.10	56.21	55.14	42.13	49.89	52.33	50.54	48.51	65.16	52.47	55.14	51.03	**55.08**
C18 :3	*0.59*	*traces*	*traces*	**1.25**	**0.42**	**0.43**	0.33	0.60	0.46	0.18	0.12	0.30	0.15	0.28	0.29	0.11	0.31	0.19	0.35	0.17	0.12	0.15	**0.34**
C20 :0	*2.13*	*1.43*	*2.08*	**2.11**	**1.25**	**1.28**	01.74	2.37	3.20	2.53	4.50	1.89	2.66	4.01	3.54	2.28	1.77	2.19	3.33	2.15	3.11	2.01	**2.43**
C20 :1	*ND*	*ND*	*ND*	**ND**	**ND**	**ND**	0.76	0.95	1.07	0.94	1.97	1.11	0.98	0.89	1.02	1.58	0.88	1.19	2.01	1.79	1.84	0.66	**1.23**
C22 :0	*ND*	*ND*	*ND*	**ND**	**ND**	**ND**	1.99	1.93	4.50	1.79	2.87	3.39	2.15	2.09	1.84	4.11	2.39	3.61	4.77	3.85	2.45	2.03	**2.86**

M= Fatty acid Average

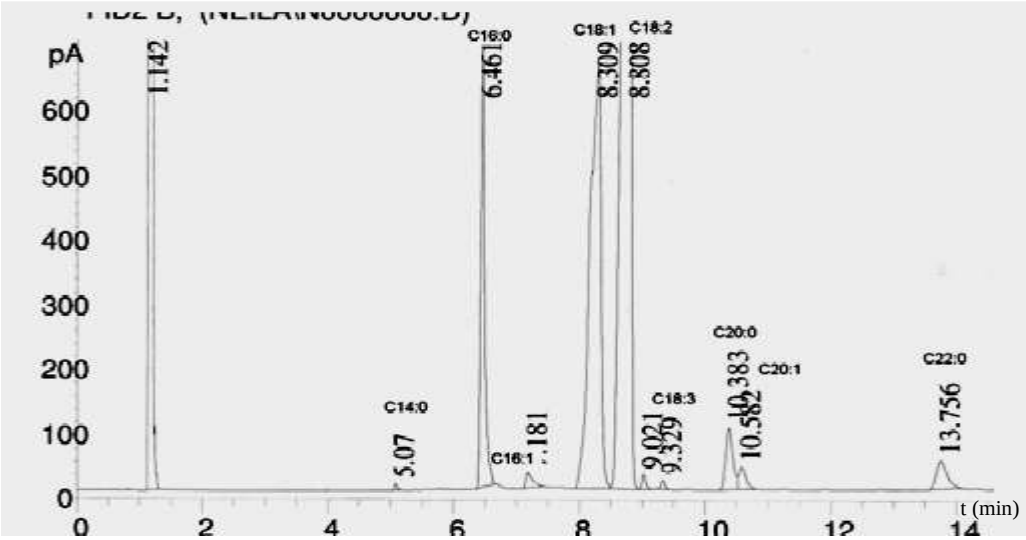

Figure 2. Extract chromatogram (E16 experiment)

CONCLUSION

In this study, different extraction Tunisian Milk thistle seeds carried out according different parameters: extraction method, solvent, temperature, pressure, co-solvent. From this work the following can be deduced that changing of extraction parameters was founded to have an influence on fatty acids composition. The *Silybum marianum* oil is rich in oleic acid (omega 9) and linoleic acid (omega 6). These fatty-acids are essential to the good performance of the human body; they exploit a significant role in the nervous system, cardiovascular balance and constitute a protection against the free radical.

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