SUPERCRITICAL CO_2, HYDRODISTILLATION EXTRACTIONS OF *SALVIA OFFICINALIS* L. INFLUENCE OF EXTRACTION PROCESS ON ANTIOXIDANT PROPERTIES

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Abstract: *Salvia officinalis L.* (Lamiaceae) is a perennial woody shrub, cultivated in the Mediterranean countries, mainly to obtain dried leaves used as raw material in medicine, perfumery and food industry. The aim of this paper is to compare the antioxidant properties and the chemical composition of essentials oils obtain by two extraction process: the hydrodistillation and the supercritical carbon dioxide extraction. The hydrodistillation is a traditional process used to obtain the essential oils from the sage while the supercritical fluid extraction (SFE) is a new technology tested on this plant. The chemical composition has been investigated by the performance of GC and GC/MS, and the antioxidant properties are compared using the DPPH free radical method. In the same time the effects of the extraction parameters on the yield were analysed. The composition of the organic phase obtained by hydrodistillation and SFE methods is widely different. The major constituents of oil obtained by hydrodistillation are: α/β-thujone, 1,8-cineol and camphor. Using the SFE process, the oil is mainly constituted by manool, leden and viridiflorol. Consequently the SFE method improves the antioxidant properties.

INTRODUCTION

The great demand of natural products such as essential oils supports the implantation of aromatic and medicinal plant cultures in Tunisia territory. *Salvia officinalis* L. called sage is a popular plant belongs to the family of Labiatae and native to the Mediterranean region. The sage essential oil has an industrial interest [1, 2] and especially the Dalmatian sage *Salvia officinalis* L. which is considered to possess the finest and the most characteristic aroma [3]. More recent studies on the biological activity of sage showed that the essential oil present some antimicrobial and antioxidant properties [4, 5]. It has tonic stimulant properties and it is used in perfumery, in cosmetics [5] and for liquors. The chemical composition of oil has been the subject of considerable studies [6-9]. Different studies showed that the chemical compositions depend on many parameters as the technique of extraction [10, 11], the geographical origin of the plant [12], and the part of the plant analysed [12, 13]. Although distillation is a very convenient method for extracting essential oils, some disadvantages are caused by the artefacts produced during the process, especially when thermolabile components are involved [14]. Supercritical fluid extraction (SFE) is an interesting technique to resolve the problem occurred by the hydrodistillation, and different study have been reported in the literature [15-19]. Concerning the antioxidants properties of sage [20-23] the different studies developed in the literature conclude that the top of the aerial part contribute mainly to the antioxidant activity.

The aims of our study were: (i) to determine the chemical composition of essential oils of *Salvia officinalis* collected from Tunisia, extracted by hydrodistillation and supercritical fluid extraction (SFE); (ii) to investigate the antioxidant properties of essential oils using the DPPH free radical method [24, 25]; (iii) to conclude on the capacity for the Tunisia to product essential oil from sage with the best antioxidant activity.

MATERIAL AND METHODS

Plant material

Leaves of *Salvia officinalis* was collected in March 2003 at "La Marsa", a maritime site localised in the north of Tunis City (≈ 500 m altitude on sea level). The identification of the plant material has been carried out at the Department of Botanic, Faculty of Sciences. Tunis, Tunisia.

Sample preparation

The plant material has been air-dried until weight stabilisation (water content = 4.8 %). Just before the extraction the vegetal was crushed to product a fine powder.

Hydrodistillation

According to the literature, the chemical composition of the essential oil depends on the extraction process (26, 10). The hydrodistillation have been realized with 1 kg of dried sage and 5 liters of pure water. This technique is the most frequently applied method to produce essential oils from sage. The organic phase has been treated with anhydrous sodium sulphate (Na_2SO_4) to eliminate all water

Supercritical fluid extraction (SFE)

A flow sheet of the supercritical fluid extraction process is shown on the figure 1. The installation is composed of an extraction column, with a stainless steel frit (height: 30 cm, diameter: 23 mm) [10], followed-up by three cyclonic separators. 10 g of plant powder was mixed with 20 g of glass, and was introduced into the extraction reactor. The CO_2 is liquefied at 3.2 °C using a pumped cold exchange (Dosapro Milton Roy - MILROYALD) and heated with an exchanger to obtain the supercritical state, before its introduction in the extractor which contains the leaves of dried sage. The extracted oil is separated from CO_2 by successive decompression. The fall of pressure is accompanied by a significant cooling. The *Salvia* essential oil is recovered at the bottom of the cyclone. This process has been conducted for different pressures: 65 (CO_2 under liquid conditions), 120 and 160 bar. The temperature and the residence time in the extractor were 50 °C and 5 h. The temperature in the separators was 50°C, and the pressures were respectively, 60, 50 and 30 bars in the separators. The supercritical CO_2 flow rate was comprised between 3,5 and 4 g.min^{-1}.

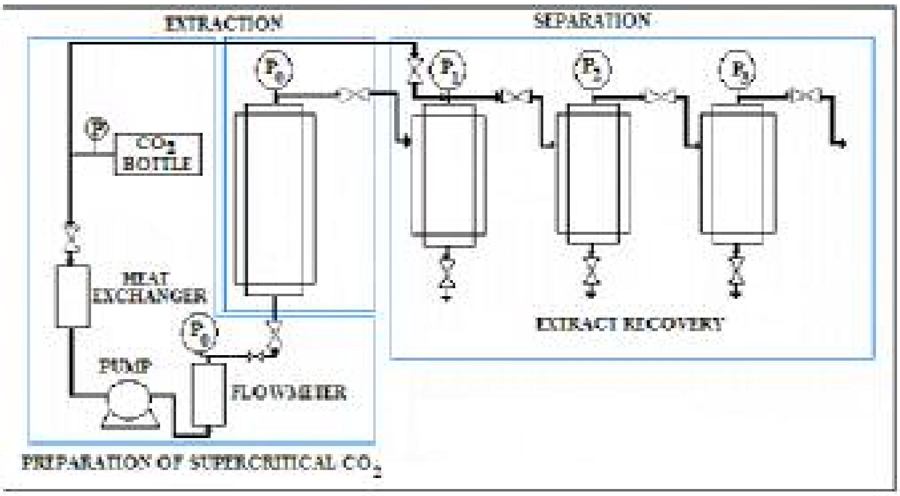

Figure 1: flow sheet, SFE equipment

GC and GC/MS Analysis

The GC analysis of the essential oils was performed using a Hewlett-Packard 5890 gas chromatograph apparatus with FID detector. The carrier gas was helium at a flowing rate of 1.2 ml.min^{-1}. Injector temperature was 240 °C, the injector and detector temperature was 250 °C and the oven temperature 240 °C with a heating rate of 9 °C min^{-1}. The column used was DB-5 fused silica capillary column (25 m x 0.25 mm i.d., 0.25 µm film thickness). The samples were injected in split mode with an injected volume of 0.2 µl. The concentration injection of essential oil was 1% in hexane. The relative amounts of the individual components are based on the peak area obtained with FID response factor correction. The identification of the essential oil components was performed by GC-MS analysis using a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard 5972 Mass Spectrometer under the same conditions as in GC analysis but using a 30 m x 0.25 mm i.d., 0.25 µm film thickness DB-5 column, ionization energy 70 eV, scan time 1 s, mass range 40-300 m/z. Mass spectra correlations were done using Hewlett-Packard Wiley 275.L, or with authentic compounds and confirmed by comparison of their retention indices either with those of authentic compounds or with data published in literature [14].

DPPH radical scavenging assay

The method was originally published by Priyadarsini [27]. Antiradical activities of *Salvia officinalis* essential oil were measured using the stable radical 1.1-diphenyl-2-picrylhdrazyl (DPPH). This method is based on the reaction of transfer of hydrogen atom between DPPH and a hydrogen donating antioxidant (AH) according to the following reaction:

$$\text{DPPH}^{\bullet} + \text{AH} \longrightarrow \text{DPPH-H} + \text{A}^{\bullet}$$

To evaluate the antioxidant activities of oils, methanolic solutions of DPPH at 200 µM and oil at 5% cc (w/w, oil/methanol) were rapidly mixed in the rapid kinetic accessory SFA-11 (Hi-Tech Scientific, S.A. LISBURY, England). Temperature was kept at 30 °C. The scavenging effect was continuously followed by monitoring the rapid change of absorbance at 515 nm as a function of time after mixing equal volumes of the two methanolic solutions in the stopped-flow cell until the reaction reached a plateau with a Perkin Elmer UV/Vis spectrometer Lambda 16. We quantified the antioxidant activity by measuring the half-life "$t_{1/2}$" of DPPH in presence of the extract. Butylhydroxytoluol BHT, a synthetic antioxidant, at 5% cc (w/w, BHT/methanol) was used as a control. All experiments were made in triplicate.

RESULTS AND DISCUSSION

Yields and kinetic extraction of essential oils

Yield of hydrodistillation extraction method is 1.02%. Figure 1 shows the total yields of supercritical CO_2 extraction for the different pressure 65 (liquid CO_2), 120 and 160 bars. We present on the figure 2 the kinetics of the total yield extraction for the three pressures. Around 80 % of the total yield was given by the second separator 77.04% at 65 bar, 84.44% at 120 bars and 82.90% at 160 bars. The maximum total yield depends on the time and pressure for the SFE method, and the total yield obtain by the hydrodistillation (300 min) correspond to that obtain by SFE at 120 bars, 270 min. In all case the best yield is obtain by SFE technique for 160 bars, 150 min.

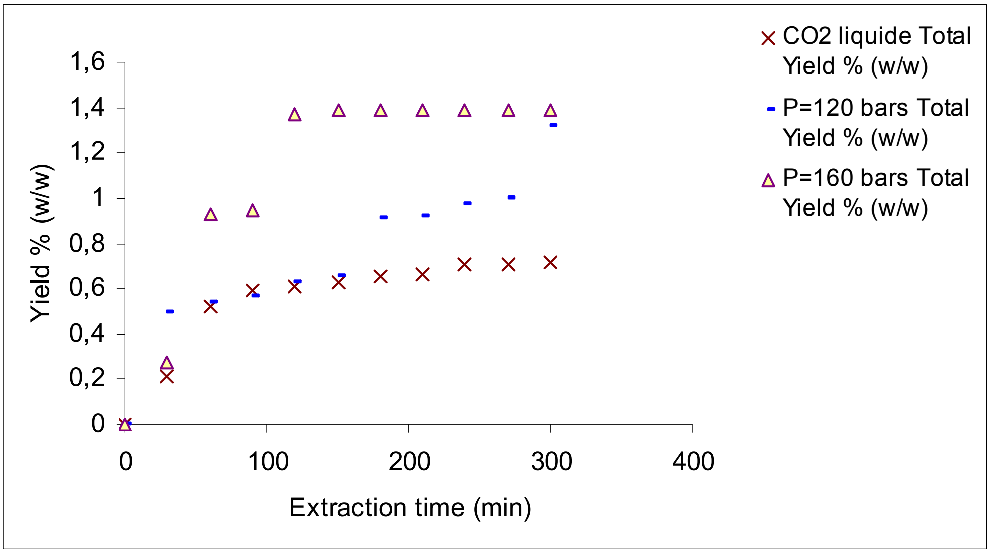

Figure 2: Kinetic CO_2 extraction of essential oil

Essentials oils composition of SFE

The chemical composition of essential oils obtained by CO_2 extraction is reported in table 1, as a function of the pressure. In all cases, the principal components are: manool (32.39 – 56.49%), ledene (4.43- 7.63%), viridiflorol (4.50 – 24.69%), and 4H-naphto(2,3-b]pyran-4,6,9-trione,5,8-dimethoxy-2-methyl (0.03 – 7.18%).

Pressure affects the solubility of pure components in carbon dioxide; therefore the effect of pressure on the composition of SFE extracts is expected to be significant. The effect of pressure was evaluated by M-E. Guvelier *et col* [21].

In the SFE method, as the pressure increases for a given temperature, the carbon dioxide solvent power increases; thus, larger and more polar substances can be co-extracted [22]. The pressure increases, the density, witch commands the solvating power, increases too, but slowly [23]. Figure 3 showed the amount of oxygenated and hydrocarbons compounds at different pressures studied. We can see that the high percentage of oxygenate components was given at 160 bars.

Hydrodistillation Composition results

The GC-MS analysis allowed the identification of 40 compounds representing more than 90% of the total oils. The compounds of the volatile oils and their relative percentages are listed in Table 1, in the order of their retention indices.

The results of the chemical composition presented in table 1 reveal a clear difference for the oils studied. The main composition of “La Marsa” essential oil extracted by the hydrodistillation method is: α-thujone (19.02%), viridiflorol (18.96%), 1-8 cineole (8.58%), limonen (6.56%), trans-carryophyllene (5.20%) and β-thujone (4.09%). Oxygen components present the high percentage of the chemical composition of all essential oils. It represents 72.22% of *Salvia officinalis* essential oil studied.

Table 1: Chemical composition of CO_2 and hydrodistillation extracts from Tunisian salvia officinalis

Components	CO_2 liquid % area	P= 120 bars % area	P= 160 bars % area	Hydrodistillation % area
α-thujene				3.42
α_pinene	0.02	0.07	0.02	0.83
Camphene				0.68
β_pinene	0.05	0.03	0.01	2.19
Myrcene			0.19	0.65
Limonene				6.56
1-8 Cineole	1.79	0.97	0.80	8.58
δ-3-carene		0.17		
Linalool				2.02
α-thujone	1.57	1.00	1.27	19.02
β_thujone	0.78	0.25	0.54	4.09
Camphor	4.45	1.00	1.91	2.10
Borneol	0.53	0.13	0.35	
Trans-ocimene		0.06	0.22	
β_Caryophyllene	2.33	1.39	1.13	5.20
α_Humulène	0.75	0.78	0.79	
Viridiflorol	24.69	10.26	4.50	18.96
δ-Gurjunene	0.11	0.02	0.59	
Aromadendrene	1.01		0.65	
Germacrène D	0.05	0.16	0.21	0.04
E-sesqui-lavandulol				5.04
Ledene	7.63	5.60	4.43	
Manool	32.39	36.01	56.49	
β-silinene		0.17		
α_Gurjunene	0.02	0.20		
14-β-H-pregna	0.67	0.69	0.71	
4H-Naphto[2,3-b]pyran-4,6,9-trione,5,8-dimethoxy-2-methyl	0.03	7.18	4.03	
Estra-1,3,5(10),9(11)-teraen-17-one	0.02	2.84	2.09	

Comparison of essential oil composition of Salvia officinalis obtained by SFE and hydrodistillation methods

The composition of the oils obtained by both processes under different conditions can be observed in table 1.

- Camphene and myrcene are present in a weak rate in the essential oils obtained by hydrodistillation, however they are absent in the SFE essential oil. From another side, on this later oil, α and β-thujone have a low amount (0-4.13%).
- The components that have a high molar mass as α-humulene, α-amorphene, viridiflorol and manool are present with a high amount in SFE essential oil. This result is not expected since these kinds of components are rather present in the hydrodistillation essential oils.
- High molecular weight compounds including esters, fatty acids, and waxy-like compounds are more likely to be extracted by CO_2 rather by hydrodistillation which is more likely to extract the volatile compounds [24-27]

We note too that the oxygenated component represents more than 80% in the essential oils of *Salvia officinalis*. The highest quality of a sage essential oil is linked to the amount of α- and β-thujone (>50%) and camphor (<20%) [16, 17].

Height molecular weight compounds are more likely to be extracted by supercritical carbon dioxide than by distillation. Because the *Salvia officinalis* oil had already been obtained by distillation, these alcohols (manool) are now component with height molecular weight in the mixture, so they are more likely to be extracted with the supercritical carbon dioxide than the present monoterpenes which are present in lower percentages.

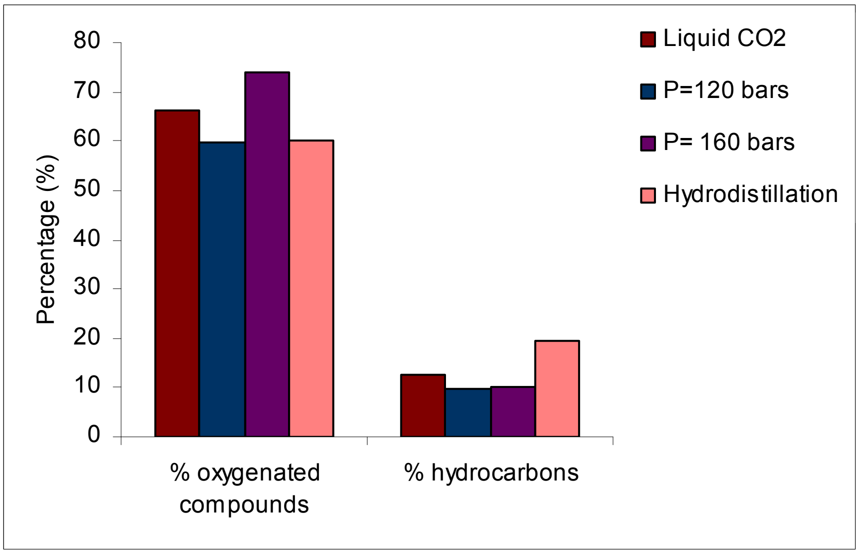

Figure 3 : Percentage of oxygenates compound

Antioxidant activity: free radical scavengers

DPPH is a stable free radical that shows maximum absorbance at 515 nm in methanol. If DPPH encounters a proton-donating substance like antioxidants, its absorbance decreases. Based on this principle, the antioxidant activity of substance can be expressed as its ability in scavenging the DPPH radical. Figure 4 shows the scavenging effect of the different essential oils extracted by hydrodistillation and SFE methods and BHT, illustrated by the decrease of the DPPH absorbance in the course of time. More important is the reaction speed (resulting in the speed of the decrease of the DPPH absorbance) and more the proton-donating substance has antioxidant property. The reason why we quantified the antioxidative activity by measuring the half-life $t_{1/2}$ of DPPH rather than the rate constant of the reaction is that, in our case, the two essential oils from the organic phase studied are complex multi-component mixtures. Let us notice that under our experimental conditions, we work in excess of extracts compared to the DPPH. Thus, we consider that we work under conditions of first pseudo order by suggesting three hypotheses:

(i) The reaction between DPPH and essential oil is order 1 compared to the DPPH; one admits, and one checked it in the case of tocopherol, which it is also of order 1 compared to phenol [28].

$$DPPH^{\bullet} + AH \longrightarrow DPPH\text{-}H + A^{\bullet}$$

(ii) The reverse reaction is negligible

(iii) There are no concurrent reactions.

Under these conditions, $t_{1/2}$ amounts measuring the apparent rate constant reaction of DPPH with the extracts (k'). Indeed the relation:

$$-\frac{d[DPPH]}{dt} = k'.[DPPH]$$

According to: $k' = \frac{\ln 2}{t_{1/2}}$

Actually, the extract is a complex mixture of components and the rate constant of the reaction is not single; though it is, $t_{1/2}$ represents the speed of the reaction. Weaker is $t_{1/2}$; more effective is the antioxidant. One can observe on figure 4 that the BHT is more effective from the antioxidant point of view than the essential oils extracted by the hydrodistillation method, and that extracted by CO_2 process under their liquid conditions. However, in these experiments, oil extracted by CO_2 liquid is near 3 times more potent antioxidant than oil extracted by hydrodistillation with $t_{1/2}$ respectively of 5.3 s and 12 s. Oil extracted by SFE method at 120 and 160 bars is more potent antioxidant; they have $t_{1/2}$ respectively of 0.4 s and 0.5 s. This result revealed the essential oils of *Salvia officinalis* possibly contained substances, more potent antioxidants than BHT, which can be obtained by SFE method at high pressure (120 and 160 bar).

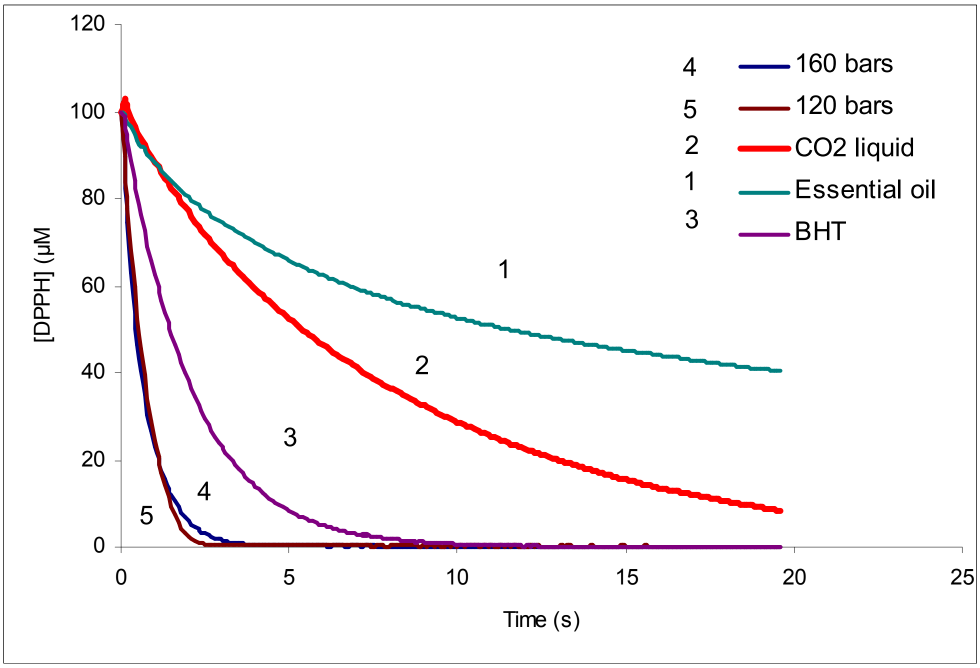

Figure 4: DPPH concentration as a function of time for the antioxidant activity of Tunisian essential oils

(1)

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