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Supercritical fluid extraction of triterpenes and aliphatic hydrocarbons from olive tree derivatives

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Abstract Olive leaves and tree bark were extracted through supercritical fluid extraction (SFE) and the chemical composition of the extracted mixture was determined by Gas Chromatography–Mass Spectrometry (GC–MS). Both samples contain a great number of triterpenes as squalene, which were used since 1997 as a main constituent of the flu vaccine (FLUAD), and the alpha-tocopherol the most biologically active form of vitamin E. We also underline the presence of many aliphatic compounds such nonacosane and heptacosane in low concentrations. The extractions were carried out at 313 and 333 K, at a pressure varying from 90 to 250 bars and using pure carbon dioxide in its supercritical phase. Therefore, their solubilities at equilibrium were numerically optimized via two assumptions and compared with the experimental values. Indeed, a good agreement between several results was shown.

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1. Introduction

Dried leaves and bark of several plants as well as their processed products have been widely used as flavoring agents since ancient times. However, during the last century they have also become the subject of a search for natural antioxidants and antibacterial agent's extraction via supercritical fluid extrac-

tion (De Melo et al., 2012). Due to an increasing interest in the use of olive tree derivatives in many industrial sectors, a systematic assessment of extracts for these properties has become gradually more significant. Nowadays, Olive leaves have been essentially used for the extraction of oleuropein; it has been widely used in folk medicine in Mediterranean regions, (LeFloch et al., 1998) whereas, the olive tree bark has not been the subject of anterior works, (Fig. 1).

Recently, many triterpenes are used frequently in pharmaceutical and agro-alimentary industries. In fact the presence of squalene is considered partly responsible for the beneficial effects of olive oil on human health and its chemo-preventive action against certain cancers (Rao et al., 1998; Smith et al., 1998).

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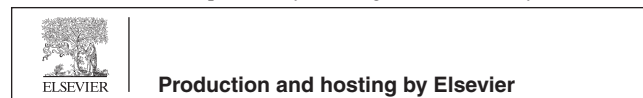




Figure 1 Olive tree: leaves and bark.

Papers concerning the analysis of extracted components from olive tree can be found in the literature, but these investigations concern essentially volatile ones, mostly correlated to their antioxidant properties (see Ghoreishi et al., 2009). Herein, we have qualitatively and quantitatively analyzed by the GC–MS extracts from olive leaves and bark using supercritical fluid extraction. The aim of this study was to develop and optimize a green, simple, reliable, reproducible, and inexpensive procedure for the extraction of many triterpenes from olive leaves and bark, using non-toxic solvents such as carbon dioxide at moderate temperatures, monitored for several organic compounds, (Ksibi, 2004).

2. Experiments

2.1. Plant material preparation

Leaves of cultivars (*Chemlali*) and bark of tree were collected in October 2011 from the region of Sfax (Central Tunisia). These materials have been dried in the shadow at room temperature (25 °C), for 8 days, (see Issaoui et al., 2012). Samples were taken into the laboratory and dusted carefully the same day, and then ground into small pieces (1–3 mm). Approximately 30 g from each sample was prepared to be loaded into the supercritical reactor. The obtained powder was vacuum packed until its use. Before utilization, the material was comminuted. CO₂ (purity 99%) was supplied by SIO (Società Italiana Ossigeno, Cagliari, Italy).

2.2. Extraction method

The supercritical extraction was accomplished in the Department of Chemical Sciences at the University of Monserrato (Cagliari, Italy). Supercritical CO₂ extractions were performed in a laboratory apparatus equipped with a 400 cc extraction vessel, which operated in a single-pass mode by passing CO₂ through the fixed bed of vegetable materials. Two fractions of the extract were recovered in two separator vessels, connected in a series (300 and 200 cc). The cooling of the first separator was achieved by using a thermostatic bath (Neslab, Model CC-100II, accuracy of 0.1 °C). The use of the second

separator allowed the discharge of the liquid product at desired time intervals. The temperature was maintained at the desired value by means of a water thermostatic system connected to the second separator. The solvent holder was contained in a bottle, surmounted by a pipe which allowed the collection of CO₂ in the liquid state. A high pressure diaphragm pump (Lewa, Model EL 1) with a maximum capacity of 6 kg/h, pumped liquid CO₂ at the desired flow rate. The CO₂ was then heated to the extraction temperature in a thermostatic oven (accurate to 0.02 °C). The extraction was carried out in a semi-batch mode: batch charging of vegetable matter and continuous flow of solvent. The CO₂ flow was monitored by a calibrated rotameter (Sho-rate, Model 1355) positioned after the last separator. The total CO₂ delivered during an extraction was measured by a dry test meter. Temperatures and pressures along the extraction apparatus were measured by a thermocouple (Fe/Const 1/8) and Bourdon-tube test gauges, respectively. The pressure was regulated by high pressure valves under manual control, located at different points of the apparatus.

2.3. Identification of the volatile constituents

The qualitative chemical analysis of the obtained samples was necessary to adopt a technique making possible the separation and identification of each component of the mixture via two independent methods. The most appropriate equipment was a gas chromatograph GC coupled with a Mass Spectrometry GC–MS. In fact, this equipment could determine the chemical structure and the molecular weight of each component. The quantitative analysis was performed using a gas chromatograph type Hewlett–Packard 5890-SERIES. It was equipped with a split-splitless injector and a DB5-MS fused silica column of 5% phenyl-methylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 μm. The oven temperature was set at 50 °C for 5 min, and then subjected to an increase of 5°/min to 250 °C. The temperature of the injector and detector was maintained at 250 °C. The carrier gas is nitrogen and the auxiliary gas is hydrogen and the air is free from all impurities. The injection volume was 0.2 ml. The carrier gas was adjusted to a linear velocity of 2 ml/min.

The GC was fitted with a quadrupole mass spectrometer, MS, Model HP 5989 A. MS conditions were as follows: ionization energy 70 eV; electronic impact ion source temperature, 200 °C; quadrupole temperature, 100 °C; scan rate, 1.6 scan/s; and mass range 40–500 amu. Software to handle mass spectra and to record chromatogram was MS ChemStation (Hewlett–Packard) using NIST98, and LIBR (TP) mass spectra libraries. Run samples were diluted in chloroform at a dilution ratio of 1:100 (w/w). Chromatographic results were expressed as area-percentages, calculated without applying any response factor, and were reported as a function of retention times, t_R . Identifications were made by matching both their mass spectra and R_{IK} values, with those reported in the literature and those of pure compounds, whenever it was possible.

3. Results and discussion

The major components in dried powder extracts of olive leaves and bark corresponded to two main groups: aliphatic hydrocarbons (11 compounds, from 20 to 31 C atoms), and

penta-cyclic triterpenes (8 compounds). It is important to note that each bark sample does not necessarily contain all of these triterpenes, a fact which is useful to discriminate among group samples, as will be discussed later. Additional constituents of branch bark extracts were also identified in lower concentrations such as scopoletin and isofraxidin. Table 1 gives details of the experimental runs done at the Department of Chemical Sciences of the University of Monserrato (Cagliari, Italy). Extractions were often performed under high pressure whose level varied from 250 to 300 bars and temperature was maintained at two isotherm values (40 and 60 °C). These operating conditions were chosen as adequate surroundings to extract aliphatic hydrocarbons. Runs of olive leaf and tree bark were conducted in parallel in order to compare the extracted compounds and their amounts in the vegetable matrix (Tables 2 and 3). The extraction of tree bark powder through 90 bars led to the richness of the material with triterpenes and identified their concentrations (Table 4).

The GC–MS spectrum of extracts from olive leaves by supercritical CO₂ at a pressure of 250 bars (Fig. 2) shows the presence of hydrocarbons that occurred after 63 min, heptacosane (63.4 min), nonacosane (70.24 min) squalene (67.36 min) and untriacontane (81.40 min). Quantitative analysis shows that the concentrations of these four hydrocarbons are respectively 0.0188, 0.0905, 0.1202 and 0.2819 g/l. As far as the GC–MS spectrum of samples from the olive bark extracts (Fig. 3) at the same conditions, occurs even the absence of squalene with lower concentrations and other components appeared (heptacosane, nonacosane, and untriacontane).

When the pressure increases from 250 to 300 bars during the supercritical extraction of olive leaves, a decrease of several extracted hydrocarbons quantities is noticed, whereas the CG–MS spectrum (Fig. 2) shows an apparition of a new extract at $t_R = 97.8$ mn which is identified as the alpha-tocopherol (C₂₉H₅₀O₂).

The presence of several components from the olive bark is observed after the lowering of pressure from 250 to 90 bars. In total, eighteen compounds were mainly identified. Analysis of the GC–MS spectrum shows that 14 new extracted components (at 90 bars) appeared after 47 min compared to that of 250 bars where four other components were identified after 63 min, see Fig. 4. These compounds contain polar molecules which are difficult to be analyzed directly and separated by the chromatographic column used in the laboratory. In addition, more polar molecules are trapped at the top of the column and thus are not detected.

Heptacosane concentrations in both leaf and bark extracts respectively, significantly increased when performed under the same conditions, whereas we notice a decrease in the untriacontane and nonacosane concentrations with a total lack of squalene (Fig. 5).

Table 2 Relative compound extract from olive leaf via SFE at $P = 250$ bar.

R_{TK}	t_R (mn)	Compound	Formula	% Air
2700	63.40	Heptacosane	C ₂₇ H ₅₆	3.6882
2825	67.36	Squalene	C ₃₀ H ₅₀	17.7021
2900	70.24	Nonacosane	C ₂₉ H ₅₈	23.4979
3101	81.40	Untriacontane	C ₃₁ H ₆₄	55.1119

Table 3 Relative compound extract from olive leaf via SFE at $P = 300$ bar.

R_{TK}	t_R (mn)	Compound	Formula	% Air
2700	63.346	Heptacosane	C ₂₇ H ₅₆	1.8
2850	66.857	Squalene	C ₃₀ H ₅₀	32.2
2946	69.309	Nonacosane	C ₂₉ H ₅₈	16.4
2988	70.479	N.I.		2.8
3204	78.775	Dotriacontane	C ₃₂ H ₆₆	31.6
3224	97.798	Alpha tocopherol	C ₂₉ H ₅₀ O ₂	15.2

4. Solubility calculation

4.1. Introduction

The solubility of substances in supercritical fluids has been described according to different methods. Two examples of these methods will be included in this paper. One method utilizes: solubility parameters and the other is based on process modeling. Each of these methods has its benefits and drawbacks. In addition, the solubility parameter is also influenced by the equation of state, which is used to calculate some variables needed for the solubility parameter. The equation of state was used is the Peng–Robinson EoS model.

4.2. Mathematical model

The fitting aspect of solubility experimental data is based on the molecular interaction coefficient as the adjustable solute parameter. The solute properties necessary to effectively correlate solubilities need a suitable choice of an equation of state, accurate molar volume estimation, and a saturated vapor pressure model (as a function of temperature).

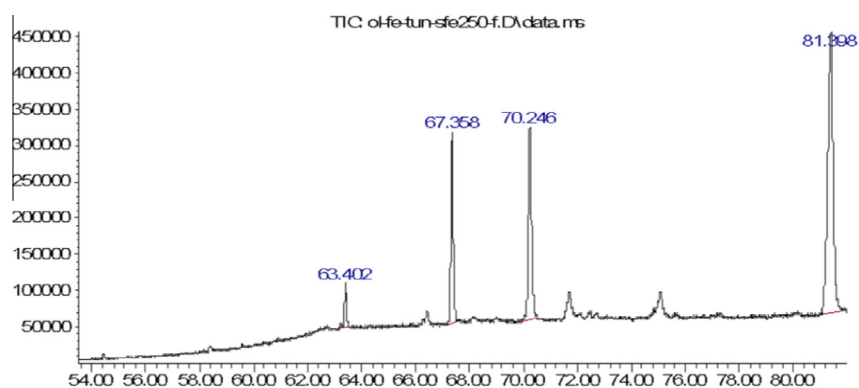
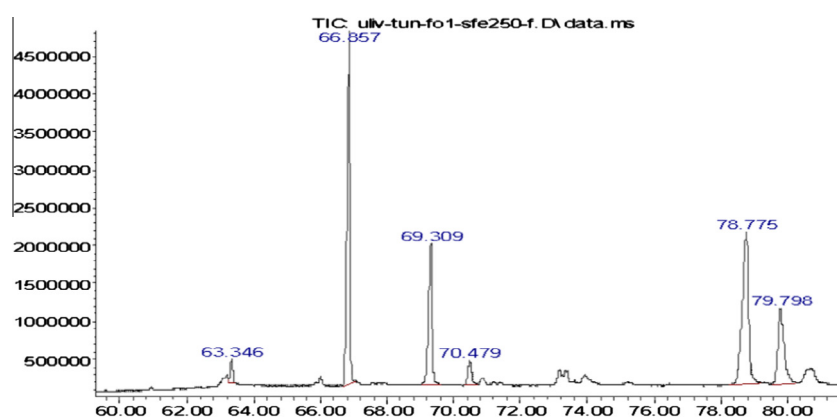
In this numerical calculation, we have used the modified Peng–Robinson EOS and the Van der Waals (VDW) mixing rules, (Subra et al., 1997; Ksibi and BenMoussa, 2007). The “golden section search” optimization technique is suitable to determine the interaction parameter k_{ij} by a certain number of iterations. Indeed, it is shown as a function of characteristic

Table 1 Experimental runs.

Run n°	Materials	Pressure (bar)	Main extracts
1	Olive leaf	250	Heptacosane, Squalene, Nonacosane, Untriacontane
2	Olive leaf	300	Heptacosane, Nonacosane, Untriacontane
3	Tree bark	90	Cinnamylcinnamate, Pentacosane, Nonacosane, Tetracosane, Hexacosane, Heptacosane
4	Tree bark	250	Heptacosane, Nonacosane, Untriacontane
5	Tree bark	300	Heptacosane, Squalene, Nonacosane, Untriacontane, Dotriacontane, Alpha Tocopherol

Table 4 Relative compound extract from olive tree bark via SFE at $P = 90$ bar.

R_{IK}	t_R (mn)	Compound	Formula	% Air
2099	47.336	Heneicosane	$C_{21}H_{44}$	1.1436
2112	47.695	Phytol	$C_{20}H_{40}O$	2.1998
2155	48.941	N.I.		2.738
2300	53.080	Tricosane	$C_{23}H_{48}$	6.0442
2350	54.436	4,8,12,16-Tetramethylheptadecan-4-olide	$C_{21}H_{40}O_2$	0.9915
2399	55.782	Tetracosane	$C_{24}H_{50}$	0.8949
2402	55.861	Cinnamylcinnamate	$C_{18}H_{16}O_2$	1.9329
2500	58.396	Pentacosane	$C_{25}H_{52}$	18.8583
2547	59.550	N.I.		1.588
2595	60.770	9-Hexacosene	$C_{26}H_{52}$	0.9237
2600	60.892	Hexacosane	$C_{26}H_{54}$	1.4989
2701	63.428	Heptacosane	$C_{27}H_{56}$	32.5034
2824	67.354	Squalene	$C_{30}H_{50}$	10.6882
2845	68.136	N.I.		2.3857
2867	68.980	N.I.		1.787
2900	70.234	Nonacosane	$C_{20}H_{60}$	5.894
2919	71.157	N.I.		2.6623
3100	81.330	Untriacontane	$C_{31}H_{64}$	5.2656

**Figure 2** CG-MS of olive leaf sample at pressure $P = 250$ bar.**Figure 3** CG-MS of olive leaf sample at pressure $P = 300$ bar.

parameters of the mixture, saturated vapor pressure and the temperature, (Ksibi and BenMoussa, 2007).

$$y = \frac{P^{sat} \varphi^{sat}}{\phi^F \cdot P} \exp\left(\frac{V^s(P - P^{sat})}{RT}\right)$$

where P^{sat} is the vapor pressure of the pure solid solute at the temperature of interest; φ^{sat} is the fugacity of the equilibrium vapor phase at the vapor pressure (usually very near unity since the vapor pressure is usually quite low); and the exponential is the Poynting factor, which involves the molar volume of

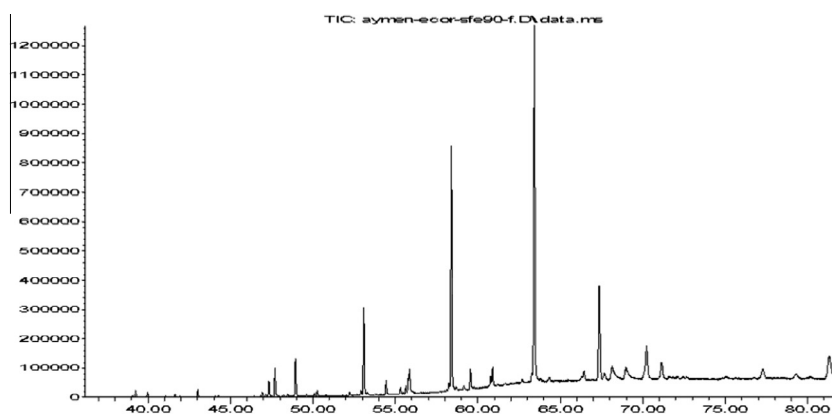


Figure 4 CG-MS of olive tree bark sample at pressure $P = 90$ bar.

Table 5 Critical coordinates and acentric factors as estimated via group contribution methods.

		T_c (K)	P_c (MPa)	w	T_b
Squalene		799.239	08.692	1.90	651.38
Heptacosane		859.762	11.098	1.84	701.05
Nonacosane		880.582	08.529	1.87	713.80

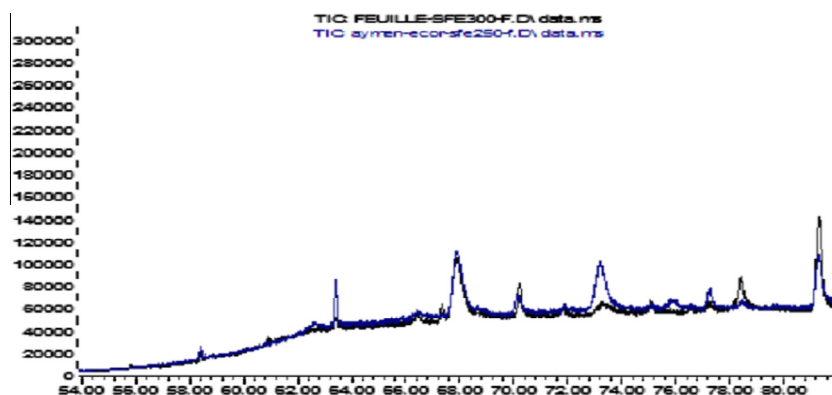


Figure 5 Comparison of CG-MS of extract samples of olive leaf and bark at pressure $P = 300$ bar.

the pure solid solute V^s . The fugacity coefficient at supercritical state ϕ^F is determined as follows:

$$\ln(\phi^F) = \frac{1}{RT} \left(\int_{V^F}^{\infty} \left[\left(\frac{\partial P}{\partial n_2} \right)_{T,V} - \frac{RT}{V} \right] \cdot dV \right)$$

The integration of the fugacity coefficient necessities fluid mixing rules which stipulate the parameters of a mixture through the following expressions:

$$a = \sum \sum y_i y_j (a_{ij} a_{jj})^{0.5} (1 - k_{ij}) \quad \text{and} \quad b = \sum y_i b_i$$

where k_{ij} is the binary interaction parameter, which is usually recovered by using the experimental data. In this field there exists several optimization of the binary interaction presented as a function of operating temperature and polarity, (Issaoui et al., 2011).

4.3. Critical coordinates determined from group contributions

As the critical coordinates of different studied hydrocarbons were undetermined in the literature, several group contribution methods were used to compute these essential parameters for each solubility optimization. The Lydersen method is the most simple and is known for the estimation of critical properties such as temperature (T_c), pressure (P_c) and volume (V_c). The Lydersen method is the prototype for and ancestor of many new models likes Joback, Klincewicz, Ambrose, and others, (see Marrero and Gani, 2001). Comparing different obtained data and choosing the suitable ones, we estimated P_c , T_c , w and T_b (burning temperature) as given in Table 5.

Experimental measurement values were deduced from Tables 2–4 and some published experimental solubility data concerning the designed hydrocarbons in supercritical carbon dioxide.

4.4. Solubility of squalene in supercritical CO₂

Measurements of squalene solubility at equilibrium were given at two isotherms 40 and 60 °C. The calculations were performed via two assumptions without considering binary interaction and taking into account its dependence on temperature and solute polarity.

Figs. 5 and 6 show the evolution of squalene solubility at equilibrium in supercritical carbon dioxide at 40 and 60 °C respectively, Table 5. The modified Peng–Robinson model (PR) can produce accurately the experimental data by optimizing the binary interaction coefficient (k_{ij}) except the first point measured at 100 bars (see Table 6).

4.5. Solubility heptacosane in supercritical CO₂

Secondly, numerical measurements of heptacosane solubility at equilibrium and at 40 °C are shown in Table 7. The calculations were performed with the same model by using the appropriate data describing heptacosane as a solute in dilute

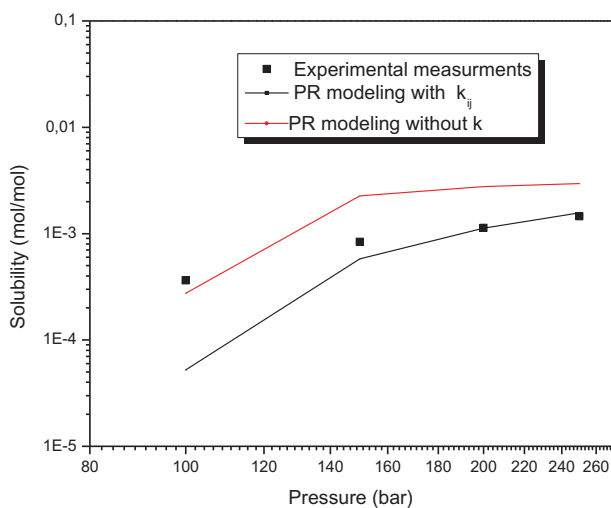


Figure 6 Solubility of squalene as a function of pressure at 313.15 K: comparison of two PR models.

Table 6 Experimental and calculated Squalene solubility as a function of pressure and temperature.

P_{exp} (bar)	$Y_{exp} 10^4$	$Y_{calc} PR 10^4$	$Y_{calc} PR (k_{ij} = 0) 10^4$
$T = 313.15 K$			
100	3.632	0.524	2.75
150	8.369	5.794	22.59
200	11.3	11.28	27.70
250	14.53	15.70	29.50
100	3.632	0.524	2.75
$T = 333.15 k$			
100	0.02	0.003	0.008
175	7.354	2.739	13.46
250	8.345	9.474	31.72
270	11.17	11.16	33.35
100	0.02	0.003	0.008

Table 7 Experimental and calculated heptacosane solubility as a function of pressure.

P_{exp} (bar)	$Y_{exp} 10^4$	$Y_{calc} PR 10^4$	$Y_{calc} PR (k_{ij} = 0) 10^4$
$T = 313.15 K$			
100	1.00	0.229	0.272
130	1.90	1.500	3.474
146	2.10	2.090	6.282
154	2.40	2.340	7.865
176	2.60	2.801	12.57

Table 8 Experimental and calculated nonacosane solubility as a function of pressure.

P_{exp} (bar)	$Y_{exp} 10^4$	$Y_{calc} PR 10^4$	$Y_{calc} PR (k_{ij} = 0) 10^4$
$T = 313.15 K$			
124	0.41	0.467	2.948
133	0.58	0.557	4.040
161	0.69	0.690	6.86
165	0.66	0.694	7.16
178	0.73	0.693	7.951

supercritical solution. The binary interaction coefficient is implemented following the two assumptions explained before.

Similarly to the first calculation of squalene solubility in supercritical CO₂, the experimental data of heptacosane concentration at equilibrium were given at 40 °C and compared with the calculated ones following the two methods; with and without considering binary interaction effects. In fact, Fig. 6 shows the accurate concordance between the experimental and numerical values at high pressure level.

4.6. Solubility nonacosane in supercritical CO₂

Finally, the solubility of nonacosane in supercritical carbon dioxide was a matter of interest in this research. Utilizing the predictive method of solubility in supercritical fluids, which

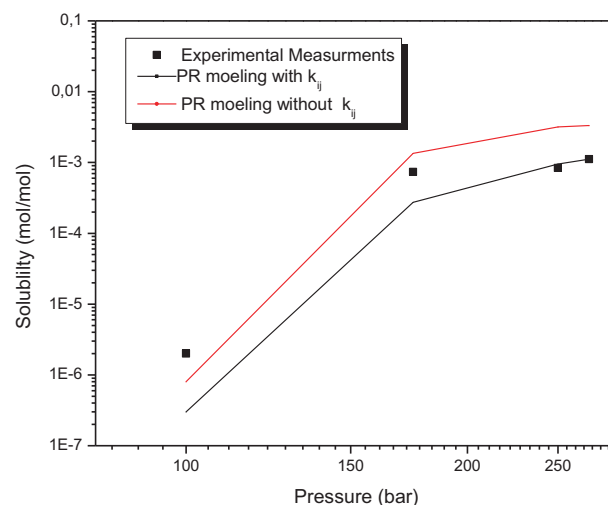


Figure 7 Solubility of squalene as a function of pressure at 333.15 K: comparison of two PR models.

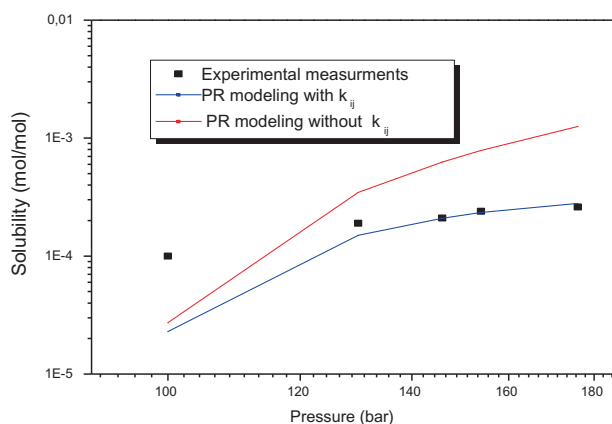


Figure 8 Solubility of heptacosane as a function of pressure at 313.15 K: comparison of two PR models.

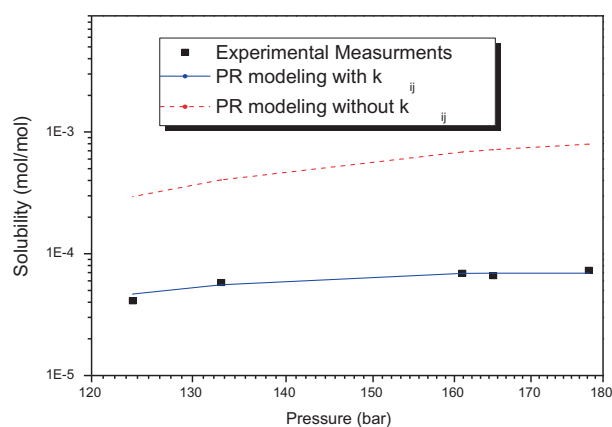


Figure 9 Solubility of nonacosane as a function of pressure at 313.15 K: comparison of two PR models.

was detailed before, we calculated nonacosane concentration at 40 °C at several high pressures, Table 8. From Fig. 7, the results showed that considering the dependence of binary interaction coefficient on temperature is more accurate in solubility prediction than the other assumption for all high pressure values (see Figs. 8 and 9).

5. Conclusion

In this work the supercritical fluid extraction of olive leaves and bark using carbon dioxide at 40 and 60 °C, and 90, 250 and 300 bars has been accomplished. The effect of the operating conditions upon the yields of triterpenes and aliphatic hydrocarbons were analyzed. The maximum extraction yield of several hydrocarbons was obtained in this study at a high pressure of 300 bars, whereas the extraction yield of many tri-

terpenes such as squalene increased significantly at moderate pressure of 90 bars. The optimization of solubility values of many solutes in supercritical carbon dioxide showed the accuracy of the implemented numerical model and allowed the interpolation at other pressures and temperatures. It was shown that treated leaf and bark with supercritical carbon dioxide extractions can produce different extracts with high VAT. Future works will focus on developing methods able to fractionate the desired compounds from these vegetable matrices.

Acknowledgments

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