GRAPE SEED OIL: SUPERCRITICAL EXTRACTION, CHEMICAL ANALYSIS AND FRACTIONATION

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Abstract

Grape seed oil is a product resulting from the processing of grape marc. It is ever more appealing for the cosmetic and pharmaceutical industries: the pharmaceutical activities concern its antioxidant properties, due to the large content of unsaturated fatty acids.
Grape seed oil could be extracted mechanically by pressing or chemically by means of solvents. Among the latter, supercritical CO_2 represents an interesting possibility. The utilization of the supercritical technology to extract oil seems economically justified when obtaining an added-value final product, as could be grape seed oil enriched in poly-unsaturated fatty acids. Actually, the supercritical extraction process could be coupled to a partial fractionation of the extracted matter based on the different solubility of the oil constituents. About the experimental extraction conditions, pressure ranged between 200-500 bar while the temperature was maintained constant at 40°C.
This research aimed at investigating the possibility of a sequential separation of supercritical extracted oil into various fractions, and in analysing their chemical composition by using the modern chromatographic and spectroscopic methodologies. In particular, Nuclear Magnetic Resonance measurements (NMR) allowed us establishing not only the mean unsaturation degree, but also the molar ratio of every lipid class (glycerides, sterols, free fatty acids) present in every sample. Triacylglycerols (TAG) are by far the most abundant lipids in the oil and an accurate quali-quantitative profile of their distribution has been obtained by matching ^{1}H- and ^{13}C-NMR analysis both of the crude oil and of the fatty acid methyl esters (FAME), the latter obtained by direct transesterification of the crude oil. Before analysis, FAMEs have been purified by Silica-gel flash chromatography, identified by NMR and/or Mass spectroscopy and finally quantified by GC-FID.
The results seem to indicate that the sequential fractionation of the oil during extraction is not effective in separating fractions of different composition: the differences in composition resulted almost insignificant.

Keywords: distillery-waste reuse; oil fractionation; chromatographic and spectroscopic methodologies.

INTRODUCTION

Winemaking residues, considered traditionally an economic and environmental problem, are now becoming increasingly recognised as valuable commodities for the production of added value products [1].

Production of grapes generally is located in moderate-warm climate zones, e.g. Italy (9,200,000 tons/year), France (6,800,000 tons/year), USA (6,400,000 tons/year), Spain (5,900,000 tons/year) but also China (5,700,000 tons/year) – data relevant to vintage 2006 [2].

The main characteristic of grape seed oil is its high content in polyunsaturated fatty acids, such as linoleic acid (72–76%, w/w), which exceeds those in other vegetable oils: safflower oil (70–72%), sunflower oil (60–62%) and soybean oil (50–55%) [3]. This makes it interesting for cosmetic and pharmaceutical industries. Moreover, it is a high-quality nutritional oil, which exhibits properties for prevention of thrombosis, cardiovascular diseases, for the reduction of cholesterol in serum, dilation of blood vessels and regulation of autonomic nerves [4].

Grape seed oil could be extracted mechanically by pressing or chemically by means of solvents. Among the latter, supercritical CO_2 represents an interesting possibility, due to the fact that it is a clean, inexpensive, non-flammable and non-toxic solvent. Moreover, the quality of grape seed oil extracted by SFE is similar to the quality of oil extracted by organic solvent and then refined, so SFE of grape seed oil could be potentially more economical than conventional liquid extraction (and mechanical extraction), because the last two stages of oil refining can be eliminated [5].

In the present study, the effect of the supercritical extraction time in the fatty acids composition was assessed. In every extraction, different fractions were sequentially separated and in the following analysed. This could be a possibility to fractionate the extracts, based on the different solubility of the oil constituents. Moreover, supercritical extraction and the classical extraction method using hexane have been compared.

MATERIALS AND METHODS

Materials

CO_2 (4.0 type, purity greater than 99.99 %) used as supercritical solvent was purchased by Rivoira. *n*-Hexane for the soxhlet extraction was purchased from Fluka (USA). All chemicals and solvent used were analytical “Reagent grade”.
Dried grape seeds were furnished by a distillery located in Trentino, Italy.

Extraction procedure

Prior to the extraction the seeds were milled by a grinder (Sunbeam Osterizer blender), and then sieved to obtain seed particles having a diameter not grater than 1 mm. Actually, the seed particle size represents one of the most influent parameters of the extraction process [6].
The supercritical extraction equipment and procedure have been previously detailed [7]. The temperature was maintained constant for the various tests, whereas the pressure was varied in the range 200-500 bar. Solvent flow rate was fixed at about 10 g/min. For each tests, about 45 g of grape seeds were extracted. Every 10 min, during extraction, the collected oil was weighed in order to obtain the extraction kinetic curve.
Every 1-1.5 grams extracted, the vessel where the oil was collected was changed to separate the different fractions. In Table 1 the extraction time and the weigh of the different fractions are given.

The exhausted matrix resulting from supercritical extraction was later extracted by solvent at atmospheric pressure.

Table 1
Extraction time and weigh of the different fractions extracted

Fraction	P= 200 bar		P= 500 bar	
	time (min)	weigh (g)	time (min)	weigh (g)
1	60	1.19	30	1.21
2	110	1.34	50	1.68
3	160	1.11	90	1.36
4	260	0.7	140	0.31

The conventional extraction was carried out by means of the Randall technique. The equipment is a conventional soxhlet extractor modified in some aspects to reduce the extraction time. In the new extractor, a porous sample container is directly immersed in the boiling solvent. The immersion step allows a fast wetting of the sample and a faster extraction of soluble components, obviating the need for long periods of condensed vapour extraction [8]. The solvent used was *n*-hexane. The extraction temperature was maintained at 69°C (boiling temperature of the solvent) and the duration of the extraction was 2 h.
The duration of the extraction for the soxhlet extraction performed after supercritical extraction was 6 h.

Synthesis of FAMEs
The transesterification reactions of different samples were carried out on 200μL of crude oil both in basic and in acid media, in the former case at room temperature by adding 5mL of a 0.5M solution of KOH in methanol for 3 h and in the latter by adding 5mL of a solution of a 3mM H_2SO_4 in methanol at 70°C for 24 hours. Both the reactions were carried out avoiding any contamination of water and were monitored using TLC (*n*-hexane/ethyl acetate 93:7). The process carried out in basic conditions was finally chosen as routinely transesterification method for the different batches of seed oil. After neutralization of the basic solution with sulphuric acid and i.v. evaporation of the organic solvent (yield of crude reaction products: 90%) the FAMEs were isolated by subjecting crude reaction material to a flash chromatography (FC) on Silica gel with *n*-hexane/ethyl acetate gradient elution. FAMEs were collected in the first fractions (overall yield in FAMEs: 85%) whereas free fatty acids and sterols in the last ones.

NMR analysis
NMR spectra (^{1}H NMR, ^{13}C NMR) were recorded on a Bruker-Avance 400 MHz NMR spectrometer by using a 5 mm BBI probe with 90^0 proton pulse length of 8 μs at a transmission power of 0 db. All spectra were taken at 298K in 50-100 mM solution in $CDCl_3$ (700 μL) on the crude grape seeds oil or on the purified FAME fraction as obtained after FCa. The chemical shift scales (δ) were calibrated on the residual signal of $CDCl_3$ at δ_H 7.26 ppm and δ_c 77.00 ppm.

Mass spectroscopy analysis
MALDI-TOF measurements were performed on Bruker Daltonics Ultraflex MALDI-TOF-TOF mass spectrometer equipped with a 337-nm nitrogen laser and with a reflectron. The

acceleration voltage was set at 20 kV. For desorption of the components, a nitrogen laser beam (λ= 337 nm) was focused on the template. The laser power level was adjusted to obtain high signal-to-noise ratios, while ensuring minimal fragmentation of the parent ions. All measurements were carried out in the delayed extraction mode, allowing the determination of monoisotopic mass values (m/z; mass-to-charge ratio). After crystallization at ambient conditions positive ion spectra were acquired in the reflectron mode, giving mainly sodiated adducts ($[M+Na]^+$). Samples were directly applied onto the stainless-steel spectromter plate as 1 μL droplets, followed by the addition of 1 μL of DHB-matrix solution (0.5 M of 2,5-DHB in methanol). Every mass spectrum represents the average of about 100 single laser shoots.

Chromatographic analysis

The fatty acid composition after transesterification was determined by gas chromatography.
A Carlo Erba mega series gas chromatograph, model 5300, equipped with a flame ionization detector, was used. The carrier gas was Helium.
The chromatographic column used was a DB-WAX 30 m x 0.324 mm x 0.25 μm. The temperature of the column, injector and detector were maintained constant at 200 °C, 250 °C and 300 °C, respectively.

RESULTS AND DISCUSSION

Extraction

The extraction yield Y is expressed as the ratio between the amount of extracted oil and the amount of grape seed placed in the extractor vessel. The extraction curves were obtained by plotting Y against the ratio between solvent consumption and seed charge (m_{CO2}/m_{seed}).
Fig. 1 shows typical results of extraction of oil from seeds: in the first part of the extraction the yield increase (the slope of the curve) is constant, then it lowers until the maximum yield is approached. The latter is about 0.095 for both tests. Considering the tests in their linear part, increasing the pressure the slope of the curve also increases. This aspect is emphasized in Fig. 2, where a selection of data of the previous figure, together with their linear trend-line and the determination coefficient R^2, are reported.

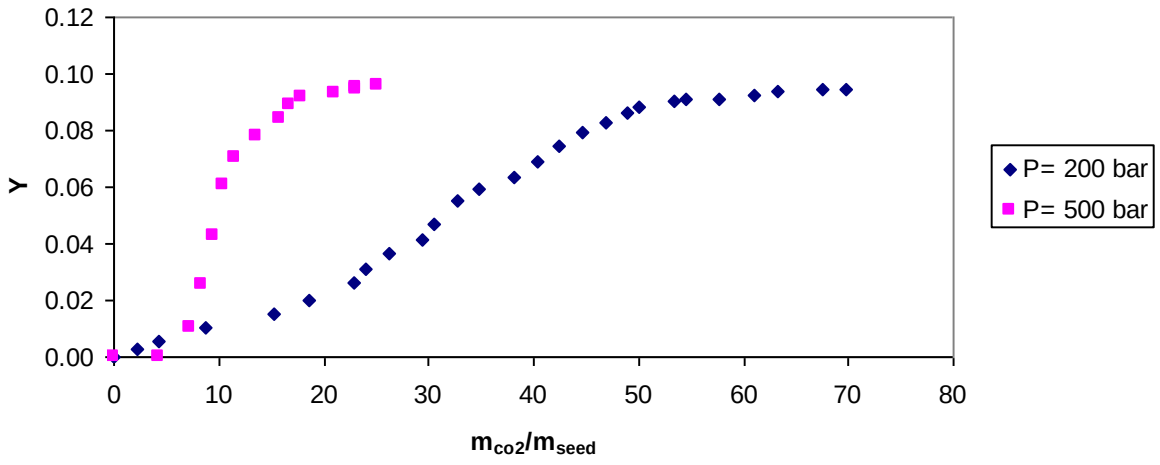

Fig. 1. Extraction curves for varying pressures

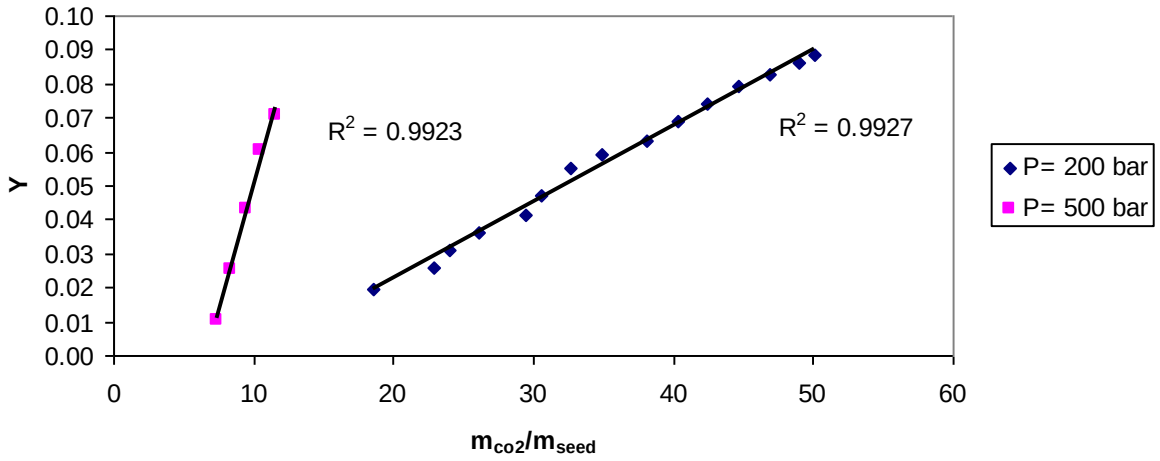

Fig. 2. Linear part of the extraction curves of Fig.1.

The exhausted matrix from the supercritical extraction at 200 °C was then extracted by hexane. Considering the two extraction in series, the total yield was 0.16. A similar value has been obtained for the matrix firstly extracted at 500 bar.

The extraction yield by hexane extraction with the Randall technique was equal to 0.12, higher than the yield obtained by supercritical extraction, but lower than the yield obtained with the double extraction.

Characterization of extracted oils

NMR analysis of the oil

From the ^{1}H-NMR spectrum (Fig. 3) of the raw oil extract as obtained from the soxhlet extraction we obtained: i) the mean unsaturation index UI (1.60 ± 0.02); ii) the relative content (89%) of unsaturated TAGs; iii) the evidence that no appreciable amount of ω-3 lipids, monoacylglycerols (MAG), diacylglycerols (DAG) and free fatty acids (FFA) was present, while only very low amount (<0.5%) of cholesterol was detected.

If we consider that our sample is constituted only by TAGs bearings mono- (16:1 and 18:1) and di-unsaturated chains (18:2), by area integration of suitable ^{1}H-NMR resonances the relative molar ratio of di-unsaturated (mainly linoleic acid chain), mono-unsaturated and saturated chain must be 72%, 17% and 11 % (± 1 %), respectively.

Concerning the extraction procedure, the supercritical method leads to almost the same molar ratios among TAGs species as the classical soxhlet extraction even if the average unsaturation index of all the fractions collected at different time seems a little higher (7%) by SCF procedure than by Soxhlet extraction, whereas the small differences among UIs of the different consecutive SCF fractions are within the measurement errors.

The ^{13}C-NMR (proton decoupled) confirms these results, speaking for the presence of the dominant 18:2 chains with minor amount of 18:1 and 16:0; diacyl- (DAG), monoacylglycerols (MAG) and free fatty acids (FFA) are not detectable by ^{13}C-NMR in our oil, thus must be below the detection limit (1%) of the technique.

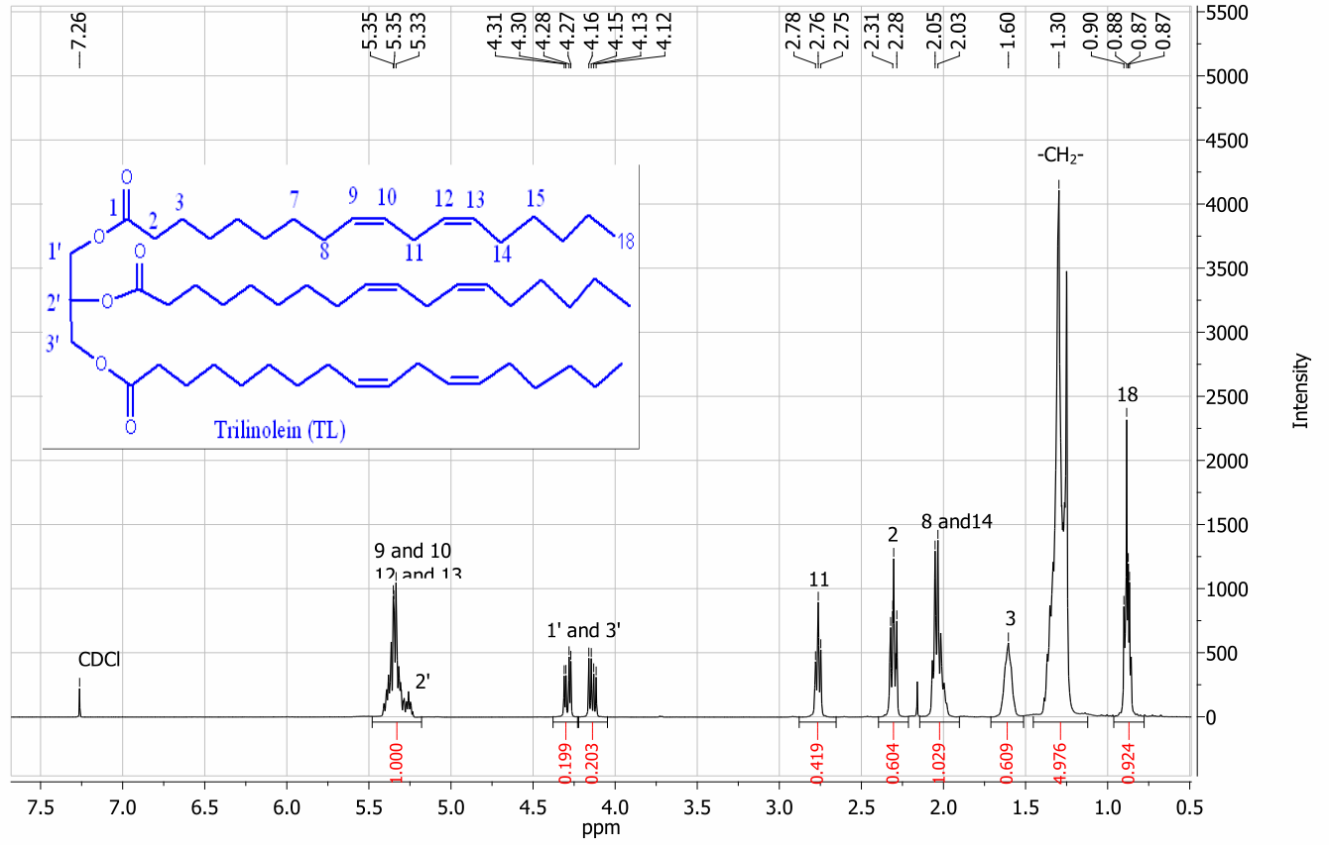

Fig. 3. 400 MHz 1H-NMR spectrum in CDCl3 at 298 K of the Soxhlet organic extract obtained from grape seeds

NMR analysis of the FAMEs

From the ^{1}H-NMR spectrum of the purified fractions containing FAMEs we obtained i) the mean unsaturation index (1.60 ± 0.02), ii) the relative content (89%) of unsaturated chains; iii) the overall unsaturations leading to a relative molar ratio of 73 ± 1 % for diunsaturated 18:2 linoleic acid chain and a 16 ± 1 % for all the mono-unsaturated (18:1, mainly), thus leaving at 11 % the population of the all saturated chains.

MALDI-TOF measurements of the oil

Recently, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF-MS) has emerged as a useful tool for TAG determination in oils, since it offers fast and easy sample preparation without the need for analyte derivatization [9].

The major TAG identified by positive ion-mode MALDI-TOF measurements of the raw grape seeds oil in HCCA matrix components are consistent with NMR data above discussed. In fact, most of the major TAG molecules contain linoleic (18:2) acid. A total of 7 TAGs has been identified of which trilinolein (LLL) is the most abundant detected as Na^{+} adduct at m/z 901.8. Among the others, only two (triolein, OOO and POO) do not contain a linoleic acid moiety. The major TAGs are: PLL (16:0,18:2,18:2) at m/z 877.8, POL (16:0, 18:1,18:2) at m/z 879.8, POO (16:0,18:1,18:1) at m/z 881.8, LLL (18:2,18:2,18:2) at m/z 901.8, OLL (18:1,18:2,18:2) at m/z 903.8, OOL (18:1,18:1,18:2) at m/z 905.8 and finally OOO (18:1,18:1,18:1) at m/z 907.8, in fair agreement with NMR analysis.

The MALDI-TOF mass spectrum also showed small amount of ions at m/z 917.8 and 919.8 attributable to oxidative products of LLL and OLL, respectively.

GC-FID

A representation of a typical chromatogram of grape seed oil is presented in Fig.4.

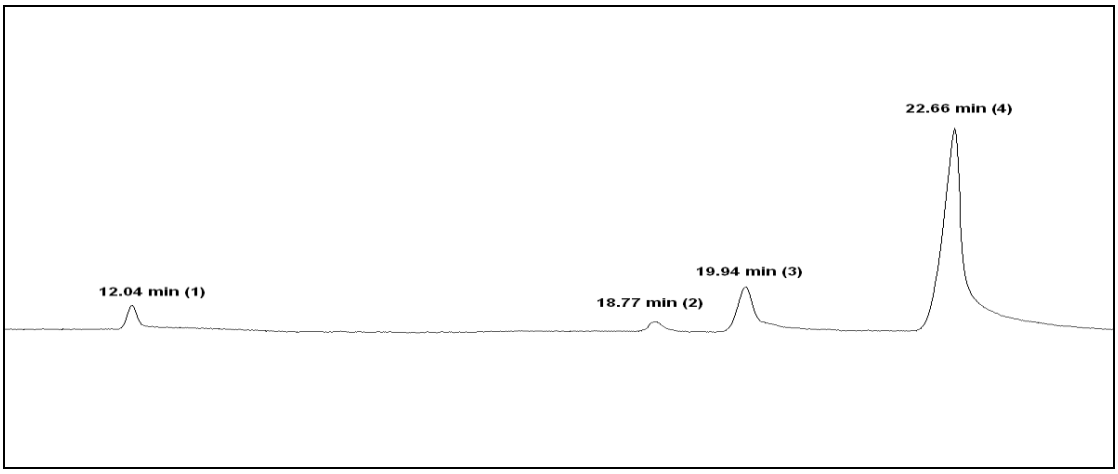

Fig. 4. Chromatogram of grape seed oil extract. Peak numbers and the correspondent representative FA are: (1) 16:0, (2) 18:0, (3) 18:1, (4) 18:2.

It was assumed that the ratio of single component peak area to total peak area is the mass fraction of the component. Each GC analysis was performed in triplicate.

Tables 2 and 3 report the fatty acid composition of the different fractions resulting from the SCE at different pressure. Palmitoleic acid was only extracted in fraction 1 when the extraction was carried out at 200 bar. Moreover, this fraction contains the highest amount of linoleic acid (81.3%).

Nonetheless, there were no significant differences between the oil extracted by supercritical carbon dioxide at different times and at different pressures.

Table 2
Fatty acid composition of the various oil fractions extracted using supercritical CO_2. P=200 bar

Fraction	Fatty acid composition (%)				
	Palmitic c16:0	Palmitoleic c16:1	Stearic c18:0	Oleic c18:1	Linoleic c18:2
1	4.4 ± 0.5	0.2 ± 0.1	1.6 ± 0.1	12.5 ± 0.8	81.3 ± 1.2
2	4.9 ± 0.1		2.1 ± 0.3	13.3 ± 0.5	79.7 ± 0.7
3	4.6 ± 0.2		2.6 ± 0.2	14.5 ± 0.7	78.3 ± 1.1
4	3.7 ± 0.1		3.1 ± 0.2	14.5 ± 0.8	79.24 ± 0.8

Table 3
Fatty acid composition of the various oil fractions extracted using supercritical CO_2. P=500 bar

Frazione	Fatty acid composition (%)			
	Palmitic c16:0	Stearic c18:0	Oleic c18:1	Linoleic c18:2
1	6.0 ± 0.1	2.8 ± 0.1	14.8 ± 0.6	76.4 ± 0.7
2	5.5 ± 0.2	2.7 ± 0.3	15.0 ± 0.3	76.8 ± 0.8
3	5.5 ± 0.4	3.1 ± 0.1	15.5 ± 0.7	75.9 ± 0.7
4	5.2 ± 0.2	3.0 ± 0.1	15.0 ± 0.5	76.8 ± 0.4

In Table 4 the fatty acid compositions of the grape seed oil obtained using carbon dioxide and by soxhlet extraction with hexane are given. The oils are not significantly different. Considering the fatty acid composition, the relative amount of linoleic acid extracted by us is higher than those previously reported in the literature. On the other hand, the soxhlet extraction time used in our tests is shorter than in the reference literature (24 h). The lower percentage of linolenic acid could be due to longer extraction times or to the different typology of the grape seeds utilized.

Table 4
Fatty acid composition of FAME as obtained from basic and acid transesterifications of oils extracted by soxhlet (hexane) and from basic transesterification of oil extracted by supercritical carbon dioxide at different pressures

Oil	Fatty acid composition (%)			
	Palmitic c16:0	Stearic c18:0	Oleic c18:1	Linoleic c18:2
soxhlet basic catalysis	6.10	2.64	14.60	76.66
soxhlet acid catalysis	6.78	2.76	13.80	76.66
soxhlet 24 h [10]	8.5	3.9	15.6	71.7
SCE 200 bar	4.51	2.23	13.59	79.60
SCE 500 bar	5.61	2.88	15.10	76.41
SCE 350 bar [4]	8.03	5.07	19.06	67.39

Table 5 shows that the unsaturated fatty acids contents in grape seed oils, calculated from the fatty acid profile, represents about 91% of the total fatty acids. The result is very similar to the result obtained by NMR analysis, 89%.
The unsaturation index was also calculated from the fatty acid profile and the results (about 1.7), also given in Table 5, is very similar to the results obtained by NMR analysis, 1.6.

Table 5
Lipids classes (% of total fatty acids) in the grape seed oil extracted by supercritical CO_2 at two pressures and by soxhlet transesterified by different catalyst

	SCE 200 bar	SCE 500 bar	soxhlet basic catalyst	soxhlet acid catalyst
saturated fatty acids	6.7	8.5	8.7	9.5
unsaturated fatty acids	93.3	91.5	91.3	90.5
saturated/unsaturated	0.07	0.09	0.10	0.11
unsaturation index	1.73	1.68	1.68	1.67

CONCLUSIONS

In this paper, experimental results concerning grape seed oil extraction are reported.
Two extraction techniques have been utilized and compared: supercritical CO_2 extraction and soxhlet extraction with *n*-hexane as solvent.
Various analytical techniques have been utilized to analyze the extracted oils: some more common (GC-FID), others more advanced (NMR, MALDI-TOF). These techniques allowed having a complete picture of the grape seed oil chemical composition: the relative amount of FFA, MAG, DAG and TAG, the FFA profile, the mean insaturation index have been measured.
The possibility of a sequential separation of supercritical extracted grape seed oil into various fractions was also studied. The overall results indicate that there are not much differences in the fatty acids composition between oils from SFE and solvent extraction, and among the fractions obtained at different times during the supercritical extraction process. Given the above, the sequential separation of different oil fraction during extraction does not seem practicable for an effectively fractionation of the oil in samples with significantly different composition.

ACKNOWLEDGMENTS

The authors greatly acknowledge the support of the 'Federazione delle Cooperative Trentine'.

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