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Quantification of Sterols and Aliphatic Alcohols in Mediterranean Stone Pine (*Pinus pinea* L.) Populations

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Individual components of *Pinus pinea* L. oil unsaponifiable matter isolated from seven Mediterranean populations were identified and quantified. *P. pinea* oil unsaponifiable matter contained very high levels of phytosterols (≥4298 mg kg^{-1} of total extracted lipids), of which β-sitosterol was the most abundant (74%). Aliphatic alcohol contents were 1365 mg kg^{-1} of total extracted lipids, of which octacosanol was the most abundant (41%). Two alcohols (hexacosanol and octacosanol), which are usually absent in common vegetable oils, were described for *P. pinea* oils. There were almost no differences in the total unsaponifiable matter of the seven Mediterranean populations studied. However, sterol and aliphatic alcohol contents showed some variability, with Tunisian and Moroccan populations showing very different and higher contents.

KEYWORDS: Mediterranean stone pine; seeds; sterols; aliphatic alcohols

INTRODUCTION

The Mediterranean stone pine *Pinus pinea* L. (gymnosperm, Pinaceae) is much appreciated for its seeds, which are used in food preparation throughout the Mediterranean Basin. Seeds contain 45−50% lipids and 21−23% proteins on a dry weight basis (*1*). Phytosterols (plant sterols) are triterpenes that are important structural components of plant membranes. They are also natural components of human diets. In recent years, with the growing interest in functional foods, the use of phytosterols for reducing serum cholesterol levels has gained considerable attention. In addition to sterols, plants also accumulate aliphatic and triterpenic alcohols. Sterols and triterpenic alcohols are 30-carbon structures that are derived from oxidosqualene. Triterpenic alcohols are not essential, and not all plants accumulate triterpenes or their derivatives (*2*).

Sterols occur ubiquitously as minor constituents in oilseeds. Sitosterol, stigmasterol, and campesterol make up 60−80% of all sterols in almost all plant tissues. Although the biosynthesis of these compounds is complex and our knowledge of their biosynthetic pathway is incomplete, their roles are well-known. Sterols have two functions: they are membrane structural compounds, and they are also signaling molecules (*3*, *4*). They have a considerable dietary importance. Oleaginous seeds are often valuable for their mono- and polyunsaturated fatty acids, and also for their tocopherols (antioxidants) and phytosterols. Some authors consider that sterols are active for lowering cholesterol levels in human serum. Sitosterol, for example, was used in the 1950s as a supplement and a treatment to lower serum cholesterol for hypercholesterolemic patients (*5*). Other authors have suggested that minor compounds protect against cardiovascular complications and could reduce the risk of heart attacks by 15−45% (*6*−*8*).

Seed components and particularly minor lipid compounds are often reliable species-specific biochemical indicators (*9, 10*). For example, numerous studies have approached the problem of determining the varietal and geographical origin of olive oils using chemometric methods (*11*−*13*). Sterols have also been used to differentiate among populations and varieties. For example, they were able to separate *arabica* from *robusta* coffee varieties (*14*). In this case, sitosterol and Δ^5-avenasterol were the two most differentiating sterols. Minor compounds were also able to discriminate between wheat species, varieties, and even geographical origins. Oleate, β-sitosterol, and the ratio oleate/β-sitosterol provided excellent discriminative power to distinguish *Triticum durum* from *Triticum aestivum* (*15*).

To our knowledge, a comprehensive study regarding the unsaponifiable matter levels of stone pine seeds has not yet been reported. The objective of this study is to describe the sterol and aliphatic alcohol contents and compositions of seed samples from *P. pinea* L. for the first time.

MATERIALS AND METHODS

Material. Seeds used were drawn from a bulk seed collection made from 3−4 cones per tree, collected from 20−30 trees per population in the following seven forest stands: Bechateur, Tunisia (BE); Cordillera Central, Spain (E2); Saint-Aygulf, France (F2); Agios Nikolaos, Greece (G2); Feniglia, Italy (I); Mezzine, Morocco (M1); and Izmir, Turkey (T2). All seeds were stored at 4 °C until used.

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Oil Extraction. All solvents were of reagent grade purchased from Merck Chimie S.A.S. and were used without any further purification. 5α-Cholestane-3β-ol and 1-eicosanol were purchased from Sigma-Aldrich. Oil was extracted manually from seeds according to the method of ref *16*, as modified in ref *17*. Seeds (5 g) were fixed with 20 mL of boiling aqueous 1% NaCl (w/v) solution to denature phospholipases. The aliquot was crushed in a mortar, and 20 mL of methanol and 20 mL of chloroform were added. The total chloroform−methanol−NaCl (1%) homogenate was centrifuged at 3000 rpm, and the lower chloroform phase containing the total lipids was saved. The solvents were removed in a rotary evaporator at 50 °C, and total lipids were recuperated and preserved at −20 °C (*18*).

Saponification of the Lipids. To separate the unsaponifiable fraction, oil from *P. pinea* seeds was treated with a potassium hydroxide solution to transform the fatty acyl esters into potassium salts that are soluble in water. Total extracted lipids were treated with 50 mL of 2 M KOH−ethanol solution, and the mixture was refluxed, with constant stirring, for 1 h. Then, 50 mL of water was added. The unsaponifiable fraction was extracted with 3 × 40 mL volumes of diethyl ether. The organic extract was separated and washed with 3 × 40 mL volumes of distilled water. The fraction was then dried over anhydrous sodium sulfate, filtered, and concentrated on a rotary evaporator under reduced pressure at 60 °C. The unsaponifiable fraction was dissolved into chloroform to obtain an approximately 5% (w/v) solution.

Separation of the Sterolic and Aliphatic Alcohol Fractions from Unsaponifiables by Thin-Layer Chromatography (TLC). The unsaponifiable matter (5% in chloroform) was separated on TLC plates (20 × 20 cm) coated with KOH−methanol (2 N) impregnated silica gel (0.25 mm), previously activated by heating at 100 °C for 1 h. The unsaponifiable fraction (250 μL) and internal standards 5α-cholestane-3β-ol and 1-eicosanol (0.2%, w/v) were spotted on the plates. Elution was performed using hexane/diethyl ether 65:35 (v/v) as the mobile phase. The plates were then sprayed with a 0.2% solution of 2′,7′-dichlorofluorescein in ethanol, and the sterol and aliphatic alcohol pink bands appeared under UV light together with the spots of 5α-cholestane-3β-ol and 1-eicosanol used as internal standards. Sterol and aliphatic alcohol bands were scraped off separately and dissolved into warm chloroform (5 mL). The obtained solutions were dried over anhydrous sodium sulfate and filtered through Whatman filter paper. The chloroform was evaporated by nitrogen stream, and sterolic and alcohol fractions were dried in an oven at 103 °C.

GC-FID Analysis. Sterolic and alcohol fractions were treated with a derivatizing reagent obtained from Sigma-Aldrich France Ltd. (pyridine/hexamethyldisilazane/trimethylchlorosilane, 9:3:1, v/v/v). A volume of 0.05 mL of reagent for each milligram of sterol was added. One microliter of this solution was injected into the gas chromatograph. TMS derivatives were analyzed in duplicate by GC in a Hewlett-Packard HP-4890D chromatograph equipped with a HP-5 (5% diphenyl−95% methylpolysiloxane) fused silica capillary column (30 m × 0.32 mm × 0.25 μm film thickness), operated isothermally at 250 °C with an inlet carrier gas (nitrogen) pressure of 100 kPa. The injector with a split ratio of 1:15 was maintained at 230 °C and the flame ionization detector (FID) at 250 °C. Sterols and aliphatic alcohols were identified by GC-MS and by using a known mix of sterols and aliphatic alcohols chromatographed under the same conditions. Sterols and alcohols were expressed as milligrams per kilogram of total extracted lipids by using 5α-cholestane-3β-ol (sterol) and 1-eicosanol (aliphatic alcohols) as internal standards.

GC-MS Analysis. The sample, 1 μL, was injected into the GC system, a Hewlett-Packard 5890 series II connected to an HP 5989A mass spectrometer. The GC system was equipped with a 30 m (0.25 mm i.d., 0.25 μm film thickness) HP-5 fused-silica capillary column coated with a stationary phase of 5% cross-linked phenylmethylsilicone. The oven temperature was as follows: raised from 210 to 250 °C at a rate of 6 °C/min, held at 250 °C for 11 min, then raised from 250 to 310 °C for 12 min. The detector temperature was 350 °C. Split ratio was 1:30. Helium was used as a carrier gas at a pressure of 100 kPa. TMS esters eluted from the column and passed into the mass spectrometer using electron impact with an ion source of 70 eV.

Table 1. Unsaponifiable Content of Mediterranean *Pinus pinea* Populations[a]

population					
name	code	country	latitude	longitude	total unsaponifiables (%)
Saint-Aygulf	F2	France	43° 27′ N	6° 41′ E	1.78 ± 0.33
Agios Nikolaos	G2	Greece	40° 14′ N	23° 34′ E	1.64 ± 0.29
Feniglia	I	Italy	42° 25′ N	11° 17′ E	1.45 ± 0.22
Mezzine	M1	Morocco	36° 06′ N	5° 21′ W	1.85 ± 0.23
Cordillera Central	E2	Spain	40° 30′ N	4° 20′ W	1.52 ± 0.13
Bechateur	BE	Tunisia	37° 14′ N	9° 56′ E	1.32 ± 0.17
Izmir	T2	Turkey	39° 12′ N	26° 57′ E	2.09 ± 0.26
mean					1.66 ±0.26

[a] Each population value is the mean of a duplicate analysis performed on different seed samples.

Statistical and Chemometric Methods. Population values for each compound were compared to the mean of all populations by calculating a confidence interval. An analysis of variance (ANOVA) was used to compare populations grouped according to their geographical origin and thus identify possible regional trends.

RESULTS AND DISCUSSION

Unsaponifiable Content and Characteristics of the Different *P. pinea* Samples. Table 1 clearly shows that there were almost no differences in the seed unsaponifiable matter of the seven Mediterranean populations studied. Mean population values fluctuated between 1.32 and 2.09% and were all included in the 5% confidence interval around the overall mean of 1.66%. These values are in the higher range of those found for oleaginous species, such as sunflower (0.5−1.5%), soybean (0.5−1.6%), or rapeseed (0.7−1.8%) (*19*).

Sterol Contents. One of the most useful analytical methods to determine sterols and aliphatic and triterpenic alcohols combines TLC and GC, and thus it was applied in this work. Qualitatively, TLC plates indicated that *P. pinea* seed oil unsaponifiables contained six spots with different intensities. Phytosterols ($R_f = 0.18$) were resolved from aliphatic alcohols ($R_f = 0.33$) and hydrocarbons (0.9). Other spots, at R_f values of 0.44, 0.54, and 0.69, were not identified. **Figure 1** shows a typical *P. pinea* chromatogram, in which the peaks of sterol TMS derivatives can be observed. Fourteen sterols were identified and quantified (**Table 2**). *P. pinea* contained very high levels of phytosterols (≥4298 mg kg^{-1} of total extracted lipids TL), which is higher than soybean (1610 mg kg^{-1}), almond (1430 mg kg^{-1}), olive oil (2210 mg kg^{-1}), or peanut (2200 mg kg^{-1}), but still substantially less than sesame oil (8650 mg kg^{-1}) or corn oil (9680 mg kg^{-1}) (*20*). This confirms the high nutritional value of *P. pinea* and its potential role in lowering serum cholesterol levels in humans (*7*, *8*). Moreover, *P. pinea* oils showed relatively high contents of Δ^5-avenasterol. This sterol was found to have an essential anti-polymerization effect, which could protect oils from oxidation during prolonged heating at high temperatures (*21*).

β-Sitosterol was the most abundant sterol in all *P. pinea* populations (3208 ± 253 mg kg^{-1} TL) followed by campesterol (661 ± 32 mg kg^{-1} TL), Δ^5-avenasterol (296 ± 67 mg kg^{-1} TL), and $\Delta^{5,24}$-stigmastadienol (21 ± 5 mg kg^{-1} TL). The Moroccan population Mezzine contained the highest level of phytosterols (4773 mg kg^{-1} TL) among the seven populations analyzed and significantly higher levels of campesterol and stigmasterol (**Table 2**). When the ANOVA was used to test for

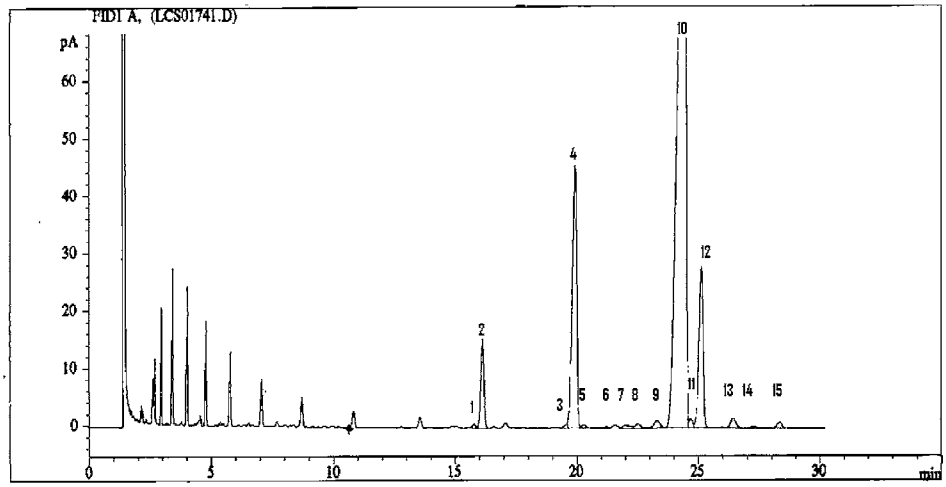

Figure 1. Typical chromatogram of sterol trimethysilyl derivatives prepared from the unsaponifiable matter extracted from *P. pinea* seeds. Analysis was performed on a HP-5 fused-silica capillary column 30 m (0.25 mm i.d., 0.25 μm film thickness) coated with a stationary phase of 5% cross-linked phenylmethylsilicone. Peaks: 1, cholesterol; 2, cholestanol (internal standard); 3, *24-meth*-cholesterol; 4, campesterol; 5, campestanol; 6, stigmasterol; 7, Δ^7-campesterol; 8, $\Delta^{5,23}$-stigmastadienol; 9, clerosterol; 10, β-sitosterol; 11, sitostanol; 12, Δ^5-avenasterol; 13, $\Delta^{5,24}$-stigmastadienol; 14, Δ^7-stigmastenol; 15, Δ^7-avenasterol.

Table 2. Sterol Contents (Milligrams per Kilogram of Total Extracted Lipids) of Mediterranean *Pinus pinea* Populations[a]

	population							
sterol	BE	E2	F2	G2	I	M1	T2	mean ± SD (n = 7)
cholesterol	4.65	9.59	6.51	8.23	12.55	2.47	9.16	7.59 ± 3.11
24-methylcholesterol	0.77	2.62	6.05	1.83	2.69	4.44	3.05	3.06 ± 1.60
campesterol	630.7	641.31	662.33	665.14	663.75	728.89*	640.44	661.79 ± 14.23
campestanol	7.75	4.36	6.51	3.2	6.27	5.93	2.18	5.17 ± 1.84
stigmasterol	3.87	6.54	6.51	7.31	6.27	14.81*	5.23	7.22 ± 1.21
Δ^7-campesterol	8.14	12.64	11.16	21.94	18.38	13.83	22.23	15.47 ± 5.06
$\Delta^{5,23}$-stigmastadienol	8.91	13.08	11.63	6.4	6.72	6.42	8.28	8.78 ± 2.46
clerosterol	22.47	19.18	25.58	19.66	22.41	26.67	17.87	21.98 ± 3.06
β-sitosterol	2792.06	3084.47	3292.09	3305.6	3260.5	3617.78	3109.75	3208.89 ± 234.58
sitostanol	25.18	28.77	17.67	21.94	17.93	20.25	15.69	21.06 ± 4.28
Δ^5-avenasterol	172.4	323.05	390.7	311.31	260.39	296.3	319.56	296.24 ± 62.14
$\Delta^{5,24}$-stigmastadienol	20.53	26.16	28.37	17.37	26.44	14.81	19.62	21.9 ± 4.76
Δ^7-stigmastenol	1.94	4.36	4.65	5.03	1.34	6.91	4.8	4.15 ± 1.77
Δ^7-avenasterol	10.46	20.05	17.67	11.89	12.55	14.32	18.31	15.04 ± 3.39

[a] Each population value is a duplicate GC analysis of the same seed sample. * Values are significantly higher than the mean at p = 0.05.

Table 3. Aliphatic Alcohol Contents (Milligrams per Kilogram of Total Extracted Lipids) of the Different *Pinus pinea* Samples[a]

	population							
aliphatic alcohol	BE	E2	F2	G2	I	M1	T2	mean ± SD (n = 7)
docosanol	75.32	33.87	60.57	99.63	182.5	150.77	29.62	90.33 ± 53.74
tricosanol	49.79	2.89	19.19	36.51	53.54	148.04*	11.56	45.93 ± 20.09
tetracosanol	395.32	34.79	86.84	86.29	244.38	123.56	23.33	142.07 ± 123.47
pentacosanol	105.96	128.45	25.19	28.68	60.21	99.32	5.49	64.76 ± 43.67
hexacosanol	1034.04*	87.96	239.88	145.45	398.13	73.04	29.9	286.91 ± 133.06
heptacosanol	175.74	22.61	55.65	85.17	99.17	72.41	8.02	74.11 ± 51.33
octacosanol	2337.87*	313.45	631.48	287.1	964.38	34.57	61.74	661.51 ± 342.85

[a] Each population value is a duplicate GC analysis of the same seed sample. * Values are significantly higher than the mean at p = 0.05.

the presence of a geographic structure, Δ^7-campesterol appeared as the most discriminative sterol, opposing the eastern populations from Greece (G2), Italy (I), and Turkey (T2) from the others (p value = 0.0033).

Aliphatic and Triterpene Alcohol Contents. Aliphatic alcohols isolated from *P. pinea* oil unsaponifiable matter were identified by GC-MS and quantified by using 1-eicosanol as internal standard (**Table 3**).

Octacosanol was the predominant alcohol in *P. pinea* seeds (661 mg kg^{-1} TL) followed by hexacosanol (286 mg kg^{-1} TL). Interestingly, these two alcohols are usually not found in common vegetable oils, such as olive (*22*). Less abundant alcohols were tetracosanol (142 mg kg^{-1} TL), docosanol (90 mg kg^{-1} TL), heptacosanol (74 mg kg^{-1} TL), pentacosanol (64 mg kg^{-1} TL), and tricosanol (45 mg kg^{-1} TL). The Tunisian population, Bechateur, contained the highest level of aliphatic

alcohols (4174 mg kg^{-1} TL) among the seven populations analyzed and significantly higher levels of hexacosanol and octacosanol (**Table 3**). The aliphatic alcohol profile of population Mezzine from Morocco was different from the seven others, with docosanol as its main alcohol (150 mg kg^{-1} TL) followed by a significantly higher level of tricosanol (148 mg kg^{-1} TL). When the ANOVA was used to test this geographic structure, pentacosanol appeared as the most discriminative aliphatic alcohol, opposing the northern Mediterranean populations of Italy (I), Turkey (T2), France (F2), and Greece (G2) from all others (p value = 0.0031).

The composition of the *P. pinea* lipid unsaponifiable fraction is reported here for the first time. Mediterranean stone pine seeds contain very high levels of sterols (4376 mg kg^{-1} TL), of which β-sitosterol is the most abundant (74%). Aliphatic alcohol content is also high and much diversified, as seven compounds were identified (two more than olive oil, for example). Octacosanol is the main aliphatic alcohol (661 mg kg^{-1} TL) followed by hexacosanol (286 mg kg^{-1} TL). As sterols and other minor unsaponifiables are known to have a wide range of beneficial biological activities and physical properties, the oil unsaponifiable matter of *P. pinea* seeds confirms their nutritional value and dietary importance.

Our GC and GC-MS analyses also showed that *P. pinea* oils are deprived of triterpenic alcohols, which is somewhat not surprising because many of the species do not accumulate these metabolites (*2*).

The Mediterranean stone pine is widely distributed in the Mediterranean Basin. Surprisingly, its genetic diversity was found to be very low for a conifer species, using isozymes (*23*), fatty acids (*24*, *25*) or chloroplast microsatellites (*26*). Interestingly, sterol and aliphatic alcohol contents showed some variability. The two southern Mediterranean populations in our sample had the most unusual sterol and aliphatic alcohol contents and compositions. Population Bechateur from Tunisia had the lowest sterol (3709 mg kg^{-1} TL) and the highest aliphatic alcohol (4174 mg kg^{-1} TL) contents of all populations, with unusually high levels of hexacosanol and octacosanol. Population Mezzine from Morocco had the highest sterol content (4773 mg kg^{-1} TL) of all populations, with significantly higher levels of campesterol and stigmasterol. This population also contained an unusually high level of tricosanol. We were also able to demonstrate using an ANOVA that some populations from the same broad geographic origin tend to cluster together. Eastern Mediterranean populations from Italy, Turkey, and Greece were always significantly grouped, although populations from Morocco and Spain were always isolated. This suggests that, using an appropriate wide-range sample of *P. pinea* populations, sterol and aliphatic alcohol contents could be used for population profiling.

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LITERATURE CITED

(1) Nasri, N.; Triki, S. Analyse des lipides des graines de pins de Tunisie: *Pinus halepensis* Mill. et *Pinus pinea* L. *Riv. Ital. Sostanze Grasse* **2004**, *81*, 244−247.

(2) Chappel, J. The genetic and molecular genetics of terpene and sterol origami. *Curr. Opin. Plant Biol.* **2002**, *5*, 151−157.

(3) Schrick, K.; Mayer, U.; Horrichs, A.; Kuhnt, C.; Bellini, C.; Dangl, J.; Schmidt, J.; Jurgens, G. FACKEL is a sterol C-14 reductase required for organized cell division and expression in *Arabidospsis* embryogenesis. *Genes Dev.* **2000**, *14*, 1741−1784.

(4) Jang, J. C.; Fujioka, S.; Tasaka, M.; Seto, H.; Takatsuto, S.; Ishii, A.; Aida, M.; Yoshida, S.; Sheen, J. A critical role of sterols in embryonic patterning and meristem programming revealed by the fackel mutants of *Arabidopsis thaliana*. *Genes Dev.* **2000**, *14*, 1485−147.

(5) Pollak, O. J. Reduction of blood cholesterol in man. *Circulation* **1953**, *7*, 702−706.

(6) Vanhanen, H. T.; Blomqvist, S.; Ehnholm, C.; Hyvönen, M.; Jauhiainen, M.; Torstila, I.; Miettinen, T. A. Serum cholesterol, cholesterol precursors, and plant sterols in hypercholesterolemic subjects with different apoE phenotypes during dietary sitostanol ester treatment. *J. Lipid. Res.* **1993**, *34*, 1535−1544.

(7) Weststrate, J. A.; Meijer, G. W. Plant sterol-enriched margarines and reduction of plasma total- and LDL-cholesterol concentrations in normocholesterolaemic and mildly hypercholesterolaemic subjects. *Eur. J. Clin. Nutr.* **1998**, *52*, 334−343.

(8) Law, M. Plant sterol and stanol margarines and health. *Br. Med. J.* **2000**, *320*, 861−864.

(9) Heyrman, J.; Mergaert, J.; Denys, R.; Swings, J. The use of fatty acid methyl ester analysis (FAME) for the identification of heterotrophic bacteria present on three mural paintings showing severe damage by micro organisms. *FEMS Microbiol. Lett.* **1999**, *181*, 55−62.

(10) Mongrand, S.; Badoc, A.; Patouille, B.; Lacomblez, Ch.; Chavent, M.; Cassagne, C.; Bessoule, J. J. Taxonomy of gymnospermae: multivariate analyses of leaf fatty acid composition. *Phytochemistry* **2001**, *58*, 101−115.

(11) Pulido, J. G.; Aparicio, R. Triacylglycerol determination based on fatty acid composition using chemometrics. *Anal. Chim. Acta* **1993**, *271*, 293−298.

(12) Lanteri, S.; Armanino, C.; Perri, E.; Palopoli, A. Study of the oils from the Calabrian olive cultivars by chemometric methods. *Food Chem.* **2002**, *76*, 501−507.

(13) Giansante, L.; Vizenzo, Di D.; Bianchi, G. Classification of monovarietal Italian olive oils by unsupervised (PCA) and supervised (LDA) chemometrics. *J. Sci. Food Agric.* **2003**, *83*, 905−911.

(14) Carrera, F.; Leóon-Camacho, M.; Pablos, F.; González, A. G. Authentication of green coffee varieties according to their sterolic profile. *Anal. Chim. Acta* **1998**, *370*, 131−139.

(15) Armanino, C.; De Acutis, R.; Festa, M. R. Wheat lipids to discriminate species, varieties, geographical origins and crop years. *Anal. Chim. Acta* **2002**, *454*, 315−326.

(16) Folch, J.; Lees, M.; Loane-Stanley, G. M. A simple method of isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497−509.

(17) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem.* **1959**, *226*, 497−509.

(18) Vorbeck, M. L.; Marinetti, G. V. Separation of glycosyldiglyceride from phosphatid using silicic acid column chromatography. *J. Lipid Res.* **1965**, *6*, 3−6.

(19) Karleskind, A.; Wolf, J. P. *Oils and Fats Manual*; Lavoisier "Tec and Doc": Paris, France, 1996; Vol. 1, 806 pp.

(20) Abidi, S. L. Chromatographic analysis of plant sterols in foods and vegetable oils. *J. Chromatogr. A* **2001**, *935*, 173−201.

(21) Mohamed, H. M. A.; Awatif, I. I. The use of sesame oil unsaponifiable matter as a natural antioxidant. *Food Chem.* **1998**, *62* (3), 269−276.

(22) Lercker, G.; Rodriguez-Estrada, M. T. Chromatographic analysis of unsaponifiable compounds of olive oils and fat-containing foods. *J. Chromatogr. A* **2000**, *881* (1−2), 105−129.

(23) Fallour, D.; Fady, B.; Lefevre, F. Study on isozyme variation in *Pinus pinea* L.: evidence for a low polymorphism. *Silvae Genet.* **1997**, *46* (4), 201−206.

(24) Nasri, N.; Khaldi, A.; Hammami, M.; Triki, S. Fatty acid composition of two Tunisian pine seed oils. *Biotechnol. Prog*. **2005**, *21*, 998−1001.
(25) Nasri, N.; Khaldi, A.; Fady, B.; Triki, S. Fatty acids from seeds of *Pinus pinea* L.: composition and population profiling. *Phytochemistry* **2005**, *66*, 1729−1735.
(26) Nasri, N. Evaluation biochimique du Pin Pignon (*Pinus pinea* L.): analyse de la diversité génétique. Thèse de Doctorat en Sciences Biologiques, Faculté des Sciences de Tunis, 2006; 169 pp.

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