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Methyl Syringate: A Chemical Marker of Asphodel (*Asphodelus microcarpus* Salzm. et Viv.) Monofloral Honey

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During the liquid chromatographic study of the phenolic fraction of monofloral honeys was detected in the asphodel honey (*Asphodelus microcarpus* Salzm. et Viv.) chromatogram a distinctive peak not detected in other monofloral honeys such as *Arbutus unedo* L., *Hedysarum coronarium*, *Eucalyptus* spp., and *Galactites tomentosa*. After thin layer chromatography (TLC) purification and characterization by NMR and LC-MS/MS, the compound was identified as methyl syringate (MSYR) and confirmed against an original standard. Levels of MSYR were measured in honeys of 2005, 2006, and 2007 by HPLC-DAD analysis. Level determination of MSYR was repeated in 2008 for 2006 and 2007 honeys to evaluate chemical stability of this phenolic compound. Levels of MSYR measured 1 year after the sampling did not show significant statistical differences ($p < 0.05$). The stability of MSYR was also confirmed by 12 asphodel honey samples collected in 2005 that showed amounts of methyl syringate comparable with those found in fresh honey. For the evaluation of MSYR origin, samples of nectars were collected from flowers and the content of MSYR was measured. Levels of MSYR in honeys are originated from the nectar with an average contribution of the nectar to the honey of 80%. Melissopalinological analysis did not allow the attribution of the honey monofloral origin because levels of asphodel pollen were <6% for all analyzed samples. Previously reported levels of MSYR for robinia, rape, chestnut, clover, linden blossom, dandelion, sunflower, thyme, manuka, and fir honeys were <5 mg/kg. For this reason, a minimum level of 122.6 mg/kg for MSYR in asphodel honeys can be considered as a chemical marker and, unlike the melissopalynological analysis, can be used for the origin attribution and to evaluate the percent of asphodel nectar in the honey.

KEYWORDS: Methyl syringate; honey; phenolic compounds; asphodel; HPLC-DAD; HPLC-MS/MS

INTRODUCTION

Asphodelus microcarpus Salzm. et Viv. (Liliaceae) monofloral honey is produced during springtime; it is light-colored, with medium-fine size crystals. The smell is delicate and the taste weak and immediately sweet (*1*−*4*). Of the Sardinian melliferous species, *Asphodelus* is the first to come into flower, starting to bloom in February−March and, in relation to the altitude, continuing to bloom until May. Asphodel honey is very similar to sulla (*Hedysarum coronarium* L.) honey, but the latter has a slightly sour taste. Different from many other Italian honeys (*5*), very few studies dealing with asphodel honey characterization have been published and, so far no descriptive file is available. Melissopalynology, the study of microscopic elements (pollen grains, spores) in the sediment of honey, does not allow the unambiguous determination of the origin of asphodel honey. In fact, in this honey the pollen is underrepresented (class I, < 20000 pollen grains per 10 g of honey), and *Asphodelus* pollen is sporadic, usually < 6%. This can be due to the large size of the *Asphodelus* pollen grain and the fact that during aging it degrades quickly (*4*, *6*). Moreover, the morphology of the flower can contribute to the limitation of nectar contamination. For these reasons asphodel honey is rich in other spring and summer contaminating pollens.

The aim of this work was to develop a direct and accurate HPLC-DAD and LC-MS/MS method to identify possible constituents of the nonvolatile components of *A. microcarpus* Salzm. et Viv. honey in order to use them as markers of the monofloral origin of the honey.

MATERIALS AND METHODS

Chemicals. Methanol, acetonitrile, ethyl acetate, acetic acid, and silica gel preparative TLC plates (20 × 20 cm, 60 F254) were purchased from Merck (Darmstadt, Germany). Sodium carbonate and ferric chloride were purchased from Carlo Erba (Milan, Italy). Standards of methyl syringate, gallic acid, ferrous sulfate, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tris(2-pyridyl)-1,3,5-triazine

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(TPTZ), and Folin−Ciocalteu's reactive were purchased from Sigma-Aldrich, Fluka (Milan, Italy). All chemicals used in this study were of analytical grade. Ultrapure water was distilled and then purified with a Milli-Q apparatus (Millipore, Milan, Italy).

Honey and Nectar Samples. Samples of asphodel honey were collected in triplicate in different areas of Sardinia (Italy) from professional beekeepers in the period 2006−2007. Sensory and melissopalynological analyses were used to attribute the floral origin and, as a first classification attempt, nine asphodel honeys were selected (**Table 1**). Samples were stored at 4 °C in dark glass bottles. The nectar samples were collected from the flowers of asphodel plants growing under environmental conditions of different zones, from central (Borore) to northern Sardinia (Ploaghe), using homemade pipets with two bulbs (*7*). The nectar was collected in April 2008, from 4 to 12 p.m. during the main nectar flow, with a sampling of 2500 flowers.

Melissopalynological Analyses. Qualitative and quantitative melissopalynological analyses were carried out following the method of the International Commission of Bee Botany (*8*). This involves the estimation of the absolute number of elements in the sediment, the identification of the most frequent elements, and the evaluation of the asphodel pollen grains percentage.

Water Content. Honey water content was measured with a portable refractometer that enables the reading of the percentage of water from 12 to 26% (ATAGO Hand Refractometer Honey, Atago Co. Ltd., Tokyo, Japan). Water amount of asphodel nectar samples was assessed by drying for 2 h 100 μL of nectar in a thermostatic oven at 105 ± 1 °C and weighing until constant weight.

Total Polyphenols. The total phenols content was measured through a spectrophotometric determination with a modified Folin−Ciocalteu method (*9*). One hundred microliters of honey diluted with water (1:5, w/v) was purified on a C18 SPE cartridge previously activated with 2 mL of methanol and washed with ultrapure water. Phenolic compounds were eluted with 2 mL of methanol, and to the extract was added 0.5 mL of Folin−Ciocalteu's reactive. After 5 min, 3 mL of 10% Na_2CO_3 (w/v) was added, and the mixture was shaken and diluted with water to a final volume of 10 mL. After a 90 min period of incubation at room temperature, the absorbance was read at 725 nm in a 10 mm quartz cuvette using a Varian Cary 50 Scan spectrophotometer (Varian, Milan, Italy) against a blank. The total polyphenols content results, expressed as milligrams per kilogram of methyl syringate equivalent (MSYRE), was obtained using a calibration curve of a freshly prepared methyl syringate standard solution (10−200 mg/L).

Antiradical Activity (DPPH Test). A spectrophotometric analysis using DPPH and comparison with the Trolox calibration curve was performed (*10*). Fifty microliters of diluted honey (1:5, w/v, with water) was dissolved in 2 mL of DPPH 0.04 mmol/L in methanol. A calibration curve in the range of 0.05−1.0 mmol/L was used for Trolox, and data were expressed as Trolox equivalent antioxidant capacity (TEAC, mmol/kg). Spectrophotometric readings were carried out with a Cary 50 spectrophotometer at 517 nm using a 10 mm quartz cuvette.

Total Antioxidant Activity (FRAP Test). The ferric reducing-antioxidant assay (FRAP) is based on the reduction at low pH of ferric 2,4,6-tris(2-pyridyl)-1,3,5-triazine [Fe(III)-TPTZ] to the ferrous complex followed by spectrophotometric analysis. The reagent was prepared by mixing 10 mM TPTZ with 20 mM ferric chloride in acetate buffer (pH 3.6). Quantitative analysis was done using the external standard method (ferrous sulfate, 0.1−2 mmol), correlating the absorbance (λ = 593 nm) with the concentration. The results were expressed as millimoles per kilogram of Fe^{2+}.

Honey Extraction and TLC Separation. Five grams of well-homogenized honey was solubilized with 5 mL of deionized water in screw-capped 40 mL tubes. Five milliliters of ethyl acetate was added, the mixture was shaken in a rotary shaker for 10 min, and, after separation, the organic phase was separated. The extraction was repeated, and the extracts were collected together and concentrated under a nitrogen flow at room temperature. Preparative thin-layer chromatography (TLC) on silica gel plates was used to separate the compounds from honey extract. The plates were developed in hexane/ethyl acetate (7.5:2.5, v/v), and bands were detected under UV light (254 and 366 nm). Individual bands (R_f = 0.72) were scraped from the preparative TLC plates, extracted with ethyl acetate, and dried under a gentle nitrogen flow. Extracts were dissolved in the eluting mixture, filtered through 0.45 μm PTFE membrane (Acrodisc CR, 13 mm ∅, Pall Life Science, Varese, Italy) to remove silica traces, and finally injected for HPLC-DAD analysis. Once the fraction with the unknown peak was detected, the same extraction procedure was applied on a larger amount of honey. Only the band corresponding to the unknown compound was scraped and analyzed by NMR and HPLC-MS/MS techniques.

Table 1. Characteristics of the Asphodel Honey and Nectar Samples

					water (g/100g)		DPPH (mmol of TEAC[a]/kg)		FRAP (mmol of Fe^{2+}[b]/kg)		total phenols (MSYRE[c] mg/kg)		methyl syringate (mg/kg)			
													time = 0		time = 12 months	
sample		site	year	asphodels pollen (%)	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
1H	honey	Sennariolo (OR)	2006	0.5	17.6	0.5	0.5	0.0	3.8	0.4	287.4	8.1	189.9	7.6	169.4	13.6
2H	honey	Ales (OR)	2006	2.6	18.1	0.8	0.8	0.0	5.7	0.6	394.3	14.8	278.0	12.2	259.9	6.2
3H	honey	Fordongianus (OR)	2006	0.9	17.5	0.6	0.5	0.0	5.2	0.5	299.1	12.9	261.6	11.8	248.8	12.0
4H	honey	Bauladu (OR)	2006	1.3	18.2	0.8	0.7	0.0	6.0	0.6	360.4	18.4	286.7	8.9	280.2	11.3
5H	honey	Villanovatulo (CA)	2006	5.2	17.1	0.4	0.8	0.1	3.8	0.4	287.7	11.6	186.6	8.5	175.9	4.8
6H	honey	Orgosolo (NU)	2006	4.8	17.8	0.5	0.8	0.0	4.9	0.7	315.2	15.9	219.2	13.4	209.9	7.3
													min 186.6 ± 8.5, max 287.7 ± 8.9, av 237.0 ± 44.3			
7H	honey	Orgosolo (NU)	2007	3.3	17.9	0.9	0.8	0.1	5.1	0.2	210.0	8.6	185.6	9.4	176.1	14.6
8H	honey	Ittiri (SS)	2007	3.4	16.7	0.8	0.7	0.0	3.0	0.2	236.5	13.5	188.2	8.6	182.2	9.4
9H	honey	Terralba (OR)	2007	4.5	17.2	0.8	0.9	0.0	5.7	0.4	321.3	14.0	288.4	12.3	279.7	10.4
													min 185.6 ± 9.4, max 288.4 ± 12.3, av 220.7 ± 58.6 samples 2006/2007 av 231.6 ± 46.4			
1N	nectar	Ploaghe (SS) Arpil 26, 2008	2008		46.4	4.2							203.6	7.5		
2N	nectar	Borore (NU) April 25, 2008	2008		56.3	4.9							127.7	10.1		
3N	nectar	Ploaghe IV (SS) April 21, 2008	2008		49.6	5.3							177.2	12.4		
													min 127.7 ± 10.1, max 203.6 ± 7.5 , av 169.5 ± 38.5			

[a] DPPH value is expressed as TEAC millimolar concentration, obtained from a Trolox solution having an antiradical capacity equivalent to that of the dilution of the honeys. [b] FRAP value is expressed as Fe^{2+} millimolar concentration, obtained from a $FeSO_4$ solution having an antioxidant capacity equivalent to that of the dilution of the honeys. [c] MSYRE, methyl syringate equivalent. Values are means of triplicate determinations.

NMR Analysis. NMR spectra were measured at 300 K on a Bruker DRX-600 spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C. Data were processed using the Topspin v. 1.3 software package.

HPLC-DAD. Honey and nectar samples were diluted with ultrapure H_2O (respectively 1:50 and 1:20 w/v) and then filtered through a cellulose acetate GD/X disk (0.45 μm, 25 mm ∅, Whatman, Milan, Italy). An HPLC Varian system ProStar was employed, fitted with a pump module 230, an autosampler module 410 with a 20 μL loop, and a ThermoSeparation diode array detector SpectroSystem UV 6000lp (ThermoSeparation, San Jose, CA) set at 280 nm. The solvents used were 0.2 M phosphoric acid (solvent A) and acetonitrile (solvent B) at a constant flow rate of 1.0 mL/min. The gradient (v/v) was generated decreasing from 90% of solvent A to 85% in 2 min, to 65% in 18 min, to 50% in 30 min, and to 10% in 40 min. Before each injection, the system was stabilized for 10 min with the initial A/B ratio (90:10, v/v). Separation was obtained with a Lichrocart Purosher Star RP-18e column (250 × 4.0 mm, 5 μm, Merck KGaA, Darmstadt, Germany).

Methyl syringate standard solutions were prepared in methanol and working standard solutions in ultrapure water. Calibration curves were built with the method of external standard, correlating the area of the peaks with the concentration. The correlation values were 0.9999 in the range of 0.4−20 mg/kg.

HPLC-MS/MS. An HPLC-MS/MS (Varian Palo Alto, CA) system made of two ProStar 210 pumps, an autosampler ProStar 410 module, and a 1200 L triple-quadrupole mass spectrometer with electrospray ionization source (ESI) was employed. Varian MS workstation version 6.6 software was used for data acquisition and processing.

The solvents used were water and acetonitrile 60:40 (v/v) in isocratic mode, and the analysis time for each sample was 25 min. The flow was maintained at 0.4 mL/min, and the injection volume was 10 μL. Separation was obtained with the Lichrocart Purospher Star RP-18 column (Merck KGaA).

The system was optimized in the negative mode. The electrospray capillary potential was set to −55 V. Air as desolvatation solvent gas was used at 300 °C, whereas the housing API temperature was kept at 44 °C. Deprotonated analyte molecules of the parent compounds were subjected to collision-induced dissociation using argon at 2.18 mTorr in the multiple reaction monitoring (MRM) mode. The transitions observed ion precursor 211.0 (m/z) were m/z 211.0 → 195.8 and 211.0 → 180.1, with energies of collision of + 20 eV for the transition m/z 211.0 → 195.8 and + 30 eV for the transition m/z 211.0 → 180.1. The scanning time was 0.2 s and, the voltage detector was set to 1200 V.

Statistical Analyses. Data were expressed as an average of three replicates, and the statistically significant differences were determined by analysis of variance to one-way (ANOVA) and Duncan's test ($p < 0.05$) using the software GenStat see 7.1 (VSN International Ltd., Herts, U.K.).

RESULTS AND DISCUSSION

Methyl Syringate Identification. During the HPLC-DAD analysis (at 280 nm) of the phenolic fraction of different monofloral honeys, an intense peak (t_R = 17.8 min) was detected in the chromatogram of asphodel honey (**Figure 1a**). This peak was not detected in other analyzed honeys (*Arbutus unedo* L., *Hedysarum coronarium*, *Eucalyptus* spp., *Galactites tomentosa*). The isolation of this compound was conducted by preparative thin-layer chromatography (TLC) on silica gel plates, as previously reported. The pure compound obtained from preparative TLC was characterized by ^{1}H NMR and ^{13}C NMR and, from spectra signals and according to the molecular weight, was identified as methyl 4-hydroxy-3,5-dimethoxybenzoate (benzoic acid, 4-hydroxy-3,5-dimethoxy-, methyl ester, methyl syringate, $C_{10}H_{12}O_5$). The ^{1}H NMR spectrum was particularly revealing because it showed two singlets at δ 3.91 and δ 3.87 indicative for methoxyl groups. Integration showed six protons for the first singlet (indicative of two symmetrical methoxyl groups) and three protons for the second singlet. In the same spectrum, a singlet at δ 7.30 revealed two aromatic protons indicative of two symmetrical methines. A singlet at δ 5.88 was assigned to a phenolic hydroxyl. The pure compound obtained from preparative TLC was analyzed with HPLC-MS/MS. The analysis showed an intense peak at m/z 211 with the two typical transitions of the precursor ion $[M - H]^-$ 211 (m/z) (211.0 → 195.8 and 211.0 → 180.1) corresponding to the loss of a methyl and a methoxyl group, respectively. Confirmation of MSYR was obtained against a commercial standard.

Melissopalynological Analysis and Methyl Syringate Level in Honey and Nectar. Honey samples selected for this investigation were obtained from professional beekeepers, and the sensorial characteristics were evaluated as the first step to determine that samples belonged to asphodel monofloral honeys. The results of the quantitative microscopical analysis agreed with previous works (*4*−*6*), showing an absolute number in pollen grains below 20000 for 10 g of honey (class I). Qualitative melissopalynological analysis of honey samples showed the following most frequent 31 different pollen types: *Asphodelus*, *Brassica f.* < 20; *Capsella*, *Cistus monspeliensis*, *Cistus incanus*, *Cistus salvifolius*, *Citrus*, *Cupressus*, *Cynoglossum*, *Echium*, *Erica arborea gr.*, *Eucalyptus*, *Galactites*, *Graminaceae* < 37; *Hedysarum*, *Lavandula stoechas*, *Leopoldia*, *Ononis spinosa*, *Papaver*, *Pinus*, *Pistacia*, *Plantago*, *Prunus f.*, *Pyrus amygdaliformis*, *Quercus ilex gr.*, *Rhamnus f.*, *Rubus f.*, *Salix*, *Trifolium campestre*, *Trifolium incarnatum*, *Vicia*. The percentages of asphodel pollen in the sediment were in most cases below 3% (from 0.1 to 2.6% in 16 samples on a total of 22), only in six samples the percentage of asphodel pollen was above 3% (from 3.3 to 5.2%). Asphodel pollen is highly underrepresented, confirming that the botanical approach in classifying monofloral honey is sometimes misleading. It is interesting to observe that in some cases, where the asphodel pollen percentage is higher than 3%, the sediment was characterized by the presence of pollen from other nectariferous plants. For instance, sample 5H is the one with the highest amount of asphodel pollen, but it has also a high level of pollen from another good nectariferous plant, lavender (*Lavandula stoechas*); consequently, sample 5H may be considered a mixed asphodel−lavender honey from a melissopalynological point of view. Although in the case of sample 9H a high contamination of a typical over-represented pollen such as *Eucalyptus* was recorded, nevertheless this honey has a good botanical purity and can be classified as an asphodel honey. Samples 1H and 7H are characterized by pollens of several interfering floral sources, such as *Leguminose*, making it hard to define their monofloral origin.

Levels of methyl syringate in the asphodel honeys ranged from 185.6 ± 9.4 to 288.4 ± 12.3 mg/kg (**Table 1**). Nectar samples were taken from asphodel flowers to evaluate if this phenolic compound originated from the nectar. Levels of MSYR were also measured and ranged from 127.7 ± 10.1 to 203.6 ± 7.5 mg/kg. This is a confirmation of the floral origin of this compound found in honey samples (**Figure 1b**). Considering that asphodel nectar and honey humidity are on average 50.8 and 17.6%, respectively, and the methyl

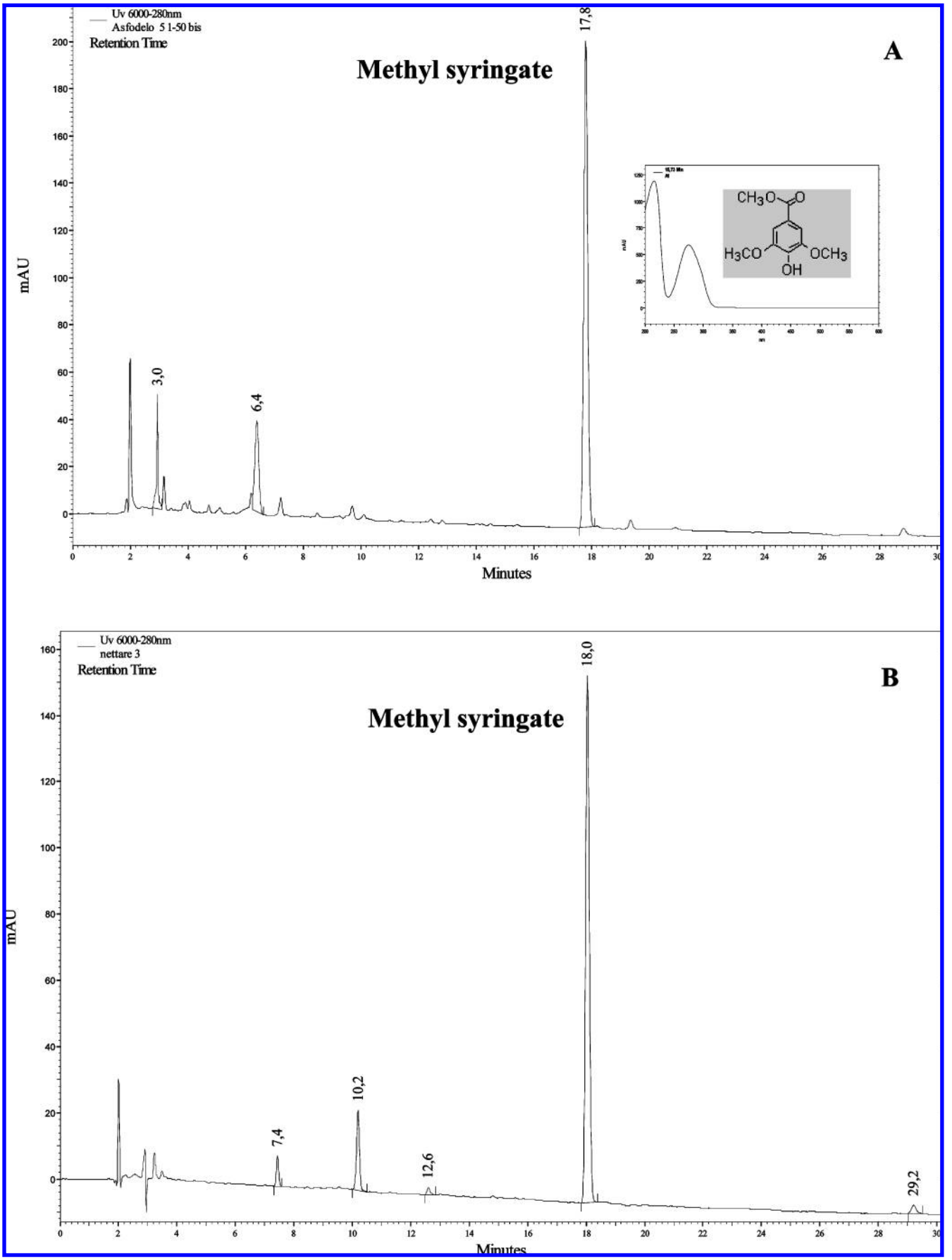

Figure 1. HPLC chromatograms at 280 nm of *Asphodelus microcarpus* Salzm. et Viv. honey (**A**) and nectar (**B**) samples.

Table 2. Characteristics of the Asphodel Honey Samples Collected in Sardinia (Italy) in 2005

		water (g/100 g)		DPPH (mmol of TEAC[a]/kg)		FRAP (mmol of Fe^{2+}[b]/kg)		total phenols (MSYRE [c] mg/kg)		methyl syringate (mg/kg)	
sample	asphodel pollen (%)	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
478	0.5	17.4	0.5	0.5	0.0	3.7	0.1	365.8	25.9	173.9	11.8
479	1.0	17.7	0.5	0.8	0.0	4.2	0.2	393.1	31.9	253.3	3.7
480	0.3	16.7	0.7	0.6	0.1	4.8	0.2	356.0	32.8	222.2	3.0
482	1.3	16.6	0.6	0.6	0.0	5.8	0.2	342.5	16.6	263.3	3.4
484	0.4	16.4	0.6	0.4	0.0	3.2	0.1	348.3	24.8	207.7	3.6
485	0.9	16.8	0.5	0.7	0.1	5.0	0.2	318.0	23.9	240.6	2.8
486	0.1	16.7	0.7	0.9	0.1	6.6	0.3	361.6	11.0	292.5	1.2
487	1.2	16.3	0.6	0.8	0.0	5.9	0.3	425.3	34.2	343.8	5.9
488	0.3	16.3	0.7	0.5	0.0	3.2	0.0	388.1	20.2	226.2	1.1
489	0.3	17.8	0.6	0.4	0.0	4.8	0.2	245.8	8.1	122.6	2.0
490	0.2	16.9	0.5	0.8	0.1	5.6	0.1	403.1	31.5	229.4	4.2
491	0.4	16.0	0.4	0.8	0.0	4.3	0.2	350.5	20.8	174.7	2.9
										min 122.6 ± 2.0, max 343.8 ± 5.9, av 229.2 ± 57.9	

[a] DPPH value is expressed as TEAC millimolar concentration, obtained from a Trolox solution having an antiradical capacity equivalent to that of the dilution of the honeys. [b] FRAP value is expressed as Fe^{2+} millimolar concentration, obtained from a $FeSO_4$ solution having an antioxidant capacity equivalent to that of the dilution of the honeys. [c] MSYRE, methyl syringate equivalent. Values are means of triplicate determinations.

syringate average amounts in nectar and honey being 169.5 and 231.6 mg/kg, asphodel honeys contain on average about 80% of the original methyl syringate found in the nectar; the remaining 20% was accountable for other botanical species nectars.

Levels of MSYR for samples 1H−9H (**Table 1**) measured 1 year after sampling did not show significant statistical differences ($p < 0.05$). The usefulness of MSYR was also confirmed by the analysis of 12 asphodel honey samples collected in 2005 (**Table 2**) that showed amounts of this phenol, ranging from 122.6 ± 2.0 to 343.8 ± 5.9 mg/kg, comparable on average with those found in fresh honey (**Table 1**).

Syringic acid is a common plant constituent (*11*), but the corresponding methyl ester is rare. Methyl syringate was previously found in grapevines (*12*), in *Cestrum parqui* leaves (*13*), and in *Taraxacum formosanum* roots (*14*). Methyl syringate was detected also in robinia, rape, chestnut, clover, linden blossom, dandelion, sunflower, and fir honeys (*15*). Levels of methyl syringate ranged from 0.093 to 5.044 mg/kg, with the rape honey having the highest amount. Moreover, methyl syringate was found in thyme honey (*16*). The presence of methyl syringate in manuka (*Leptospermum scopariu*) honey was described in several papers (*17*−*20*), and Weston et al. (*21*) reported that the percentage of methyl syringate in manuka honey was 70% w/w of the phenolic fraction, with a mean value of 0.6 mg/kg. It is interesting to note that methyl syringate was detected in honeys obtained from plants of different botanical families but only the asphodel honey reached the highest level.

Furthermore, honey levels of methyl syringate are usually determined by GC after methylation. In fact, phenolic compounds, due to their low volatility, need to be transformed into methyl, ethyl, or sylilated derivatives (*11*, *17*, *21*). For this reason, it is not easy to differentiate between the naturally occurring methyl ester and the free acid (*11*, *16*). D'Arcy et al. (*22*) reported the GC and GC-MS analyses of *Eucaliptus leucoxylon* and *Eucaliptus mellidora* honey extracts without derivatization, finding an average level of 0.8 mg/kg of methyl syringate. Also, syringic acid was detected in *Leptospermun polygalifolium* (*23*), *Malaleuca quinquenervia*, *Guioa semiglauca*, *Lephostemon conferta* (*24*), *Castanea sativa* Miller, *Robinia pseudoacacia* L., *Lavandula* sp., *Brassica napus* L., *Helianthus annuus* L., *Eucaliptus* sp. and *Tilia* sp. heather honeys (*25*), and clover and buckwheat honeys (*26*) by HPLC. These data show that in a large number of monofloral honeys levels of MSYR are commonly below 5 mg/kg. Vice versa, levels of MSYR in asphodel honeys were higher than 122.6 mg/kg; for this reason this compound can be used as an origin marker for this honey.

Despite the fact that MSYR was found in several honeys, no data on the corresponding floral origin were published before. Only Russell et al. (*19*) suggested that methyl 4-hydroxy-3,5-dimethoxy-substituted syringic acid is a common fragment of hardwood lignin and that aromatic acids can be originated from the sap of the tree rather than from the nectar. They also proposed that, as the manuka tree is often infested with *Eriococcus orariensis* (a scale insect that collects honeydew) and with *Capnodium walteri* Sacc. (a sooty mold fungus), probably the aromatic acids are derived from precursors collected by the bees from the manuka trees. Moreover, white-rot fungi are able to degrade lignin, a major component of the wood, using ligninolytic enzymes as laccase (*27*). Laccase is an enzyme widely distributed in higher plants, fungi, some insects, and bacteria. The lignin barrier breakdown of ligninocellulose is currently understood as an enzymatic process mediated by small molecules, and syringic acid methyl ester is a typical laccase substrate (*28*). On the other hand, it is well-known that benzoic acid derivates can be produced through the shikimate pathway (*11*), and methyl syringate (*13*, *14*) and its glucoside (*29*) were found in several plants extracts.

The antioxidant and antiradical activities of asphodel honey were also evaluated. Antiradical activity showed an average value of 0.7 mmol of TEAC/kg, whereas the antioxidant activity ranged between 3.0 and 5.7 mmol of Fe^{2+}/kg (**Table 1**). Methyl syringate showed a low antioxidant activity (a 300 mg/L solution has a TEAC value of 0.1 mmol/L) compared to other phenols found in other honeys (data not shown). For this reason, the antioxidant and antiradical activities of this honey cannot be ascribed to the MSYR being the main phenolic compound of the asphodel honey.

In conclusion, analyses of pollen have clearly shown that asphodel honey is a typical underrepresented honey with a very low amount of *A. microcarpus* Salzm. et Viv.

pollen granules, below 6%. These data confirm that the melissopalynological analysis is very uncertain and, therefore, the only analysis of pollen does not allow a classification of *A. microcarpus* Salzm. et Viv. honey as a monofloral honey. The very high amount of methyl syringate (>122.6 mg/kg) can be used as a specific marker for fingerprinting the botanical origin of this honey as methyl syringate is originated from the nectar.

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