Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 89 (2005) 527–532
Food Chemistry
www.elsevier.com/locate/foodchem

Volatile norisoprenoids as markers of botanical origin of Sardinian strawberry-tree (*Arbutus unedo* L.) honey: Characterisation of aroma compounds by dynamic headspace extraction and gas chromatography–mass spectrometry

Federica Bianchi, Maria Careri, Marilena Musci *

Dipartimento di Chimica Generale ed Inorganica, Chimica Analitica, Chimica Fisica, Università degli Studi di Parma, Parco Area delle Scienze 17/A, 43100 Parma, Italy

Received 26 January 2004; received in revised form 4 March 2004; accepted 4 March 2004

Abstract

In order to characterize and authenticate the aromatic profile of strawberry-tree (*Arbutus unedo* L.) honey, a dynamic headspace (DHS) extraction, followed by gas chromatography–mass spectrometry (GC–MS) analysis, was performed on 10 Sardinian strawberry-tree (*Arbutus unedo* L.) honey samples. A total of 28 aroma compounds were identified, but only norisoprenoid compounds such as α-isophorone, β-isophorone and 4-oxoisophorone, were recognized as specific floral origin markers of the strawberry-tree honey. The α-isophorone/β-isophorone ratio varied from 4 to 8, whereas the α-isophorone/4-oxoisophorone ratio was found to range from 11 to 20. The DHS extraction method was proposed as a valid alternative to pollen analysis for floral source detection, especially for products like strawberry-tree honey, characterized by a low pollen content.

Keywords: Honey; Strawberry-tree (*Arbutus unedo* L.); Aroma compounds; Dynamic headspace extraction; Gas chromatography; Mass spectrometry; Norisoprenoid compounds

1. Introduction

Assessment of the botanical origin of honey is of great concern in food analysis, since authenticity guarantees the quality of the product, prevents overpayment and helps to identify frauds. In addition, European Community legislation concerning honey i.e. Directive EEC/74/409 amended by the Proposal COM/95/0722 (1996) requires labelling of the floral origin.

Usually the determination of the botanical origin of honey is carried out by melissopalynological analysis, which is based on the identification of pollen by microscopic examination (Louveaux, Maurizio, & Vorwohl, 1978; Sawyer, 1975).

This analysis is time-consuming, requires a very skilful analyst for data interpretation and sometimes results do not allow a reliable identification of the botanical origin, especially in the case of honey characterized by low amounts of pollen, as is the case with strawberry-tree honey. In addition, for this kind of product, assessment of the botanical origin on the basis of pollen analysis is particularly difficult, due to the presence of secondary pollens, since flowers are in an upside-down position. Consequently, due to difficulties in melissopalynological analysis, great attention has been paid to finding specific chemical markers for proving the botanical source of this honey.

Strawberry-tree honey is a typical product of Sardinia, an insular region of Italy; owing to its distinct fragrance and bitter aftertaste, this product is particularly appreciated. Its high economic value, about six times the price of monofloral honeys of other botanical origins,

* Corresponding author. Tel.: +39-0521-906-023; fax: +39-0521-905-557.
E-mail address: marilena.musci@unipr.it (M. Musci).

0308-8146/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2004.03.009

determined by its peculiar organoleptic characteristics and the limited production, makes this product particularly susceptible to sophistication.

On the basis of these observations, chemical analysis of strawberry-tree honey could offer greater reliability in tracing and differentiating botanical origins. Among the non volatile components of strawberry-tree honey, a phenolic compound, i.e. homogentisic acid, has been previously proposed as a marker of the botanical origin of strawberry-tree (*Arbutus unedo* L.) honey (Cabras et al., 1999).

Since one of the most typical features of honey is its aroma profile, it is used to characterize volatile marker compounds specific to a given botanical origin (Overton & Manura, 1994; Radovic et al., 2001). In particular, the gas chromatographic pattern can be considered as a chemical "fingerprint", since the nature and relative amount of the compounds present in the volatile fraction are a distinctive feature of different floral sources.

Although honeys of different botanical origin, namely chestnut, eucalyptus, lavender, lime and orange, have previously been characterized on the basis of their volatile fraction (Bonaga & Giumanini, 1986; Bouseta, Collin, & Dufour, 1992), to our knowledge only one previous study regarding the semi-volatile aroma fraction of strawberry-tree honey has been published (Dalla Serra et al., 1999).

In this work, the aromatic fraction of 10 Sardinian strawberry-tree (*Arbutus unedo* L.) honey samples was extracted using the dynamic headspace (DHS) technique, followed by gas chromatography–mass spectrometry (GC–MS) analysis. The aim of this study was to obtain a "fingerprint" of the aroma of strawberry-tree (*Arbutus unedo* L.) honey and to find volatile compounds as possible markers of the floral source of this product.

2. Materials and methods

2.1. Honey samples

Ten strawberry-tree (*Arbutus unedo* L.) honey samples produced in Sardinia were analyzed. The floral origin was certified from the producers. In addition, four honey samples of different floral origins, i.e. eucalyptus (*Eucalyptus* spp.), heather (*Erica arborea*), lavender (*Lavandula* spp.) and thyme (*Thymus vulgaris* L.), purchased from a local store, were analyzed. To confirm botanical source these samples were analyzed under conditions adopted in our previous study (Radovic et al., 2001).

Samples were stored in hermetically closed glass jars at 4 °C until analysis.

2.2. Reagents

Authentic substances used for identification were from Sigma Aldrich (Milan, Italy). The following compounds were injected into the GC–MS system for the calculation of Kovàts indices: acetone, 2-butanone, ethanol, 2,3-butanedione, hexanal, 2-methyl-1-propanol, 1-butanol, heptanal, 3-methyl-1-butanol, octanal, 5-hepten-2-one-6-methyl, nonanal, furfural, decanal, benzaldehyde, 2,6,6-trimethyl-1,4-cyclohexenedione (α-isophorone) and 3,5,5-trimethylcyclohex-2-ene-1,4-dione (4-oxoisophorone).

2.3. Honey flavour extraction

Aroma compound extraction was performed by using the DHS extraction.

Preliminary experiments were carried out following the procedure described by Radovic et al. (2001). This method was modified as follows: 1.5 g of honey were placed in a 50 ml round-bottom flask in a water bath maintained at 40 °C. Purified helium was used as the stripping gas at a flow-rate of 40 ml min^{-1} for 30 min; the volatile compounds were adsorbed on a trap consisting of a glass tube (16 × 0.4 cm i.d.) filled with 400 mg of Carbopack™ B 60/80 mesh (Chrompack, Middelburg, The Netherlands), pre-conditioned at 300 °C for 8 h. The adsorbent trap was then back-flushed with the purified gas for 5 min to remove trapped moisture. The volatile compounds were subsequently thermally desorbed and transferred to the gas chromatographic system by using a TCT thermal desorption cold trap (TD800, Fisons Instruments, Milan, Italy). Desorption was performed at 280 °C for 10 min under a helium flow (10 ml min^{-1}), volatile compounds were then cryofocused in a glass lined tube cooled to −120 °C with liquid nitrogen. Volatiles were injected into the GC capillary column by heating the cold trap at 240 °C.

Three independent DHS extractions were performed for each sample.

In order to verify possible environmental contamination, blank analyses were carried out using an empty 50 ml round-bottom flask and following the same procedure.

To assess the presence of carry-over effects, the adsorbent trap was also desorbed before and after each entire sampling procedure.

2.4. Gas chromatography–mass spectrometry

Gas chromatography–mass spectrometry analysis of the honey headspace was carried out with a system consisting of a TRACE GC 2000 gas chromatograph and a TRACE MS quadrupole mass spectrometer (Thermo Electron Corporation, Milan, Italy). The interface and the source temperatures were kept at 230 and 200 °C,

respectively. Electron impact mass spectra were recorded at 70 eV ionization energy (scan time, 0.5 s; electron multiplier voltage, 350 V) scanning the mass spectrometer from m/z 35 to 350. The carrier gas was helium (pressure, 70 kPa). Chromatographic separation was performed on a fused-silica bonded-phase capillary column SUPELCOWAX 10 (30 m × 0.25 mm; df = 0.25 μm) (Supelco, Palo Alto, CA, USA). The temperature program was isothermal at 35 °C for 8 min, then raised to 60 °C at 4 °C min^{-1}, to 160 °C at 6 °C min^{-1} and to 220 °C at 20 °C min^{-1}, holding this temperature for 1 min.

2.5. Data analysis and data evaluation

The mass spectrometer data acquisition was performed using the release 1.2 Xcalibur™ software (Thermo Electron Corporation). The identification of the volatile compounds was achieved by comparing their mass spectra with those stored in the National Institute of Standards and Technology (NIST) US Government library. Identifications were also confirmed by comparing both retention times and mass spectra with those of authentic substances used as references. In addition, Kovàts retention indices (KI) were calculated for the GC peaks, corresponding to identified substances by interpolation of the retention times of normal alkanes (C8–C28) analyzed under the same chromatographic conditions. Calculated KI were compared with those reported in the literature for the same stationary phase (Careri, Mazzoleni, Musci, & Molteni, 1999).

3. Results and discussion

In a previous study on the aroma characterization of honeys of different floral origin, such as chestnut, heather, eucalyptus, lime, rosemary, sunflower, lavender, rape and acacia, we developed and successfully applied a DHS-GC–MS method based on the use of a Tenax TA for the extraction and the analysis of honey volatile compounds (Radovic et al., 2001). That method proved to be inadequate for the extraction of volatile compounds from strawberry-tree honey samples, since a remarkable carry-over effect in correspondence to the peak attributed to α-isophorone was observed. Decreasing the sample amount to be analyzed from 25 to 1.5 g did not eliminate the carry-over effect. In addition, many compounds were not detectable in the GC profile.

A different trapping material was then evaluated by testing the performance of Carbopack™ B (60–80 mesh), a graphitized carbon black indicated for trapping volatiles in the C6–C12 range.

Fig. 1 shows the total ion current GC–MS chromatogram of the volatile fraction obtained by sampling 1.5 g of honey. Using this trap, only a negligible carry-over effect, that could easily be eliminated by a subsequent adsorbent trap bake-cycle, was observed.

Twenty-eight out of 60 volatile compounds separated were identified. Only volatiles occurring in more than four different samples were reported (Table 1). Among the identified compounds 14 ketones, 9 aldehydes, 4 alcohols and 1 furan were detected. Particular attention was paid to norisoprenoids, such as 3,5,5-trimethyl-2-cyclohexen-1-one (α-isophorone), 3,5,5-trimethyl-3-cyclohexen-1-one (β-isophorone) and 3,5,5-trimethyl cyclohex-2-ene-1,4-dione (4-oxoisophorone). Structures and mass spectra of α-, β-isophorone and 4-oxoisophorone are shown in Fig. 2. These compounds were identified in all the samples analyzed, showing abundant signals in the GC chromatograms.

Owing to their attractive sensory properties and low odour thresholds, norisoprenoids, a class of carotenoid-derived compounds, with a 3,5,5-trimethylcyclo-2-hexenic structure (Enzell, 1985), have been found as

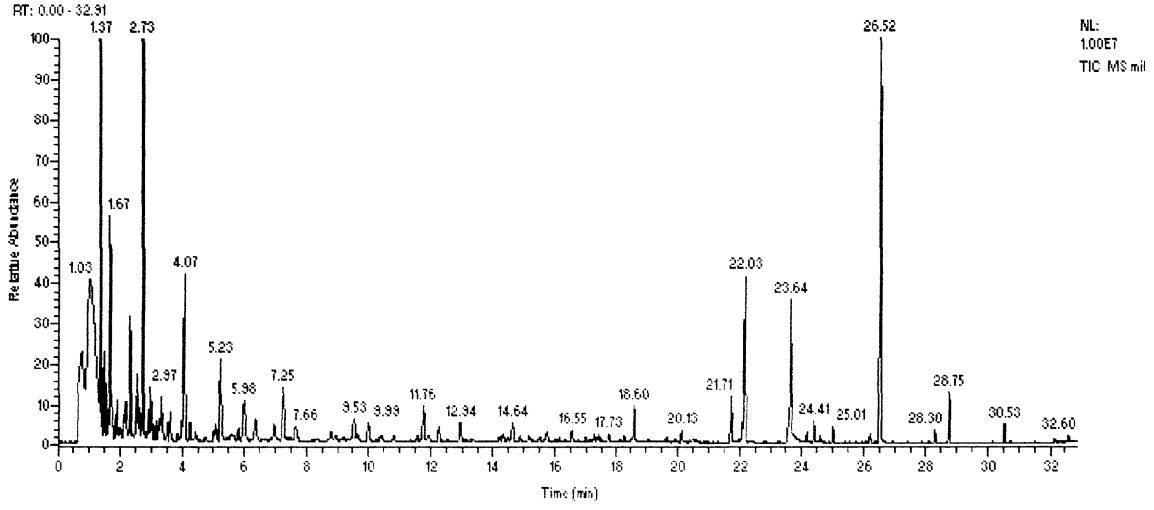

Fig. 1. Total ion current GC–MS chromatogram of the volatile fraction of strawberry-tree (*Arbutus unedo* L.) honey extracted by the DHS technique.

Table 1
Volatile compounds identified in Sardinian strawberry-tree (*Arbutus unedo* L.) honey

No.	Compound	RT (min)	$KI_{calc.}$	ID[a]	Occurrence
1	acetone	2.32	n.c.	MS, RT	9
2	2-butanone	3.30	905	MS, RT, KI	6
3	ethanol	3.98	939	MS, RT, KI	6
4	2,5-dimethylfuran	4.43	965	MS, KI	10
5	2,3-butanedione	5.22	989	MS, RT, KI	10
6	2,3-pentanedione	8.80	1047	MS	9
7	hexanal	9.53	1088	MS, RT, KI	10
8	methyl-2-butenal	9.99	1103	MS	5
9	2-methyl-1-propanol	10.39	1106	MS, RT, KI	5
10	1-butanol	12.34	1155	MS, RT, KI	4
11	heptanal	14.64	1183	MS, RT, KI	10
12	2,4,4-trimethylcyclopentanone	15.62	1211	MS	7
13	3-methyl-1-butanol	15.75	1214	MS, RT, KI	4
14	octanal	18.60	1291	MS, RT, KI	10
15	2,3,4-trimethyl-2-cyclopenten-1-one	19.28	1311	MS	10
16	3-(1-methylethyl)-2-cyclopenten-1-one	19.61	1322	MS	10
17	5-hepten-2-one-6-methyl	20.13	1343	MS, RT, KI	9
18	3,3,5-trimethylcyclohexanone (dihydroisophorone)	21.02	1368	MS	4
19	nonanal	21.71	1397	MS, RT, KI	10
20	3,5,5-trimethyl-3-cyclohexen-1-one (β-isophorone)	22.03	1407	MS	10
21	3-furancarboxaldehyde	22.79	1441	MS	7
22	furfural	23.64	1474	MS, RT, KI	10
23	decanal	24.41	1503	MS, RT, KI	10
24	1-(2-furanyl) ethanone	24.60	1512	MS	9
25	benzaldehyde	25.01	1528	MS, RT, KI	10
26	3,5,5-trimethyl-2-cyclohexen-1-one (α-isophorone)	26.52	1591	MS, RT	10
27	3,5,5-trimethylcyclohex-2-ene-1,4-dione (4-oxoisophorone)	28.75	1698	MS, RT	10
28	3,5,5-trimethylcyclohexan-1,4-dione	30.53	1786	MS	10

n.c.: not calculated.
[a] Method of identification: MS, identification by comparison with NIST mass spectrum; RT, identification by comparison with retention time of authentic reference compound; KI, identification by comparison with KI literature data.

aroma contributors in a number of different matrices, such as tobacco, tea, flower scents, fruits, spices, grapes and wine (Winterhalter & Rouseff, 2002). Substances belonging to this class have also been isolated from honey of different botanical origins as eucalyptus (D'Arcy, Rintoul, Rowland, & Blackman, 1997), thyme (Tan, Wilkins, Holland, & McGhie, 1990), heather (Guyot, Scheirmann, & Collins, 1999; Hausler & Montag, 1989; Tan, Wilkins, Holland, & McGhie, 1989) and strawberry-tree (Dalla Serra et al., 1999).

Since previous studies showed that the types and amounts of norisoprenoids are related to honey botanical origin, they have been proposed as chemical markers of honey floral source (D'Arcy et al., 1997; Guyot et al., 1999; Guyot-Declerck, Chevance, Lermusieau, & Collins, 2000; Hausler & Montag, 1989; Tan et al., 1989, 1990).

The norisoprenoid compounds identified in this work, analyzing aroma of strawberry-tree honey, have previously been identified in the volatile fraction of honey of the same floral source by Dalla Serra et al. (1999); in that work, the extraction of aroma compounds was performed by adsorbing a diluted solution of honey on a XAD-2 resin, followed by solvent extraction and GC–MS analysis. With respect to the previously reported techniques used for extraction of norisoprenoids (Guyot-Declerck et al., 2000), the dynamic headspace-based technique, proposed in this work, proved to be complementary to extract the volatile components of strawberry-tree honey, including the most volatile norisoprenoids.

In order to propose α-isophorone, β-isophorone and 4-oxoisophorone as chemical markers of strawberry-tree honey, we evaluated both their absence in honey samples of different botanical origins and their relative amount in strawberry-tree honey samples. In our previous work (Radovic et al., 2001), α-isophorone and 4-oxoisophorone were found to occur simultaneously only in heather honey samples, whereas β-isophorone was not detected at all. However, it is to be noted that, in that work the sample amount submitted to extraction and analysis was more than 10-fold higher than that used for extraction of strawberry-tree honey volatiles. Consequently, honey samples of other botanical origins were extracted under the conditions developed in this study to verify the presence of norisoprenoids. In particular, eucalyptus (*Eucalyptus* spp.), lavender (*Lavandula* spp.), thyme (*Thymus vulgaris* L.) and heather (*Erica arborea*) honey samples were considered, heather belonging to the same

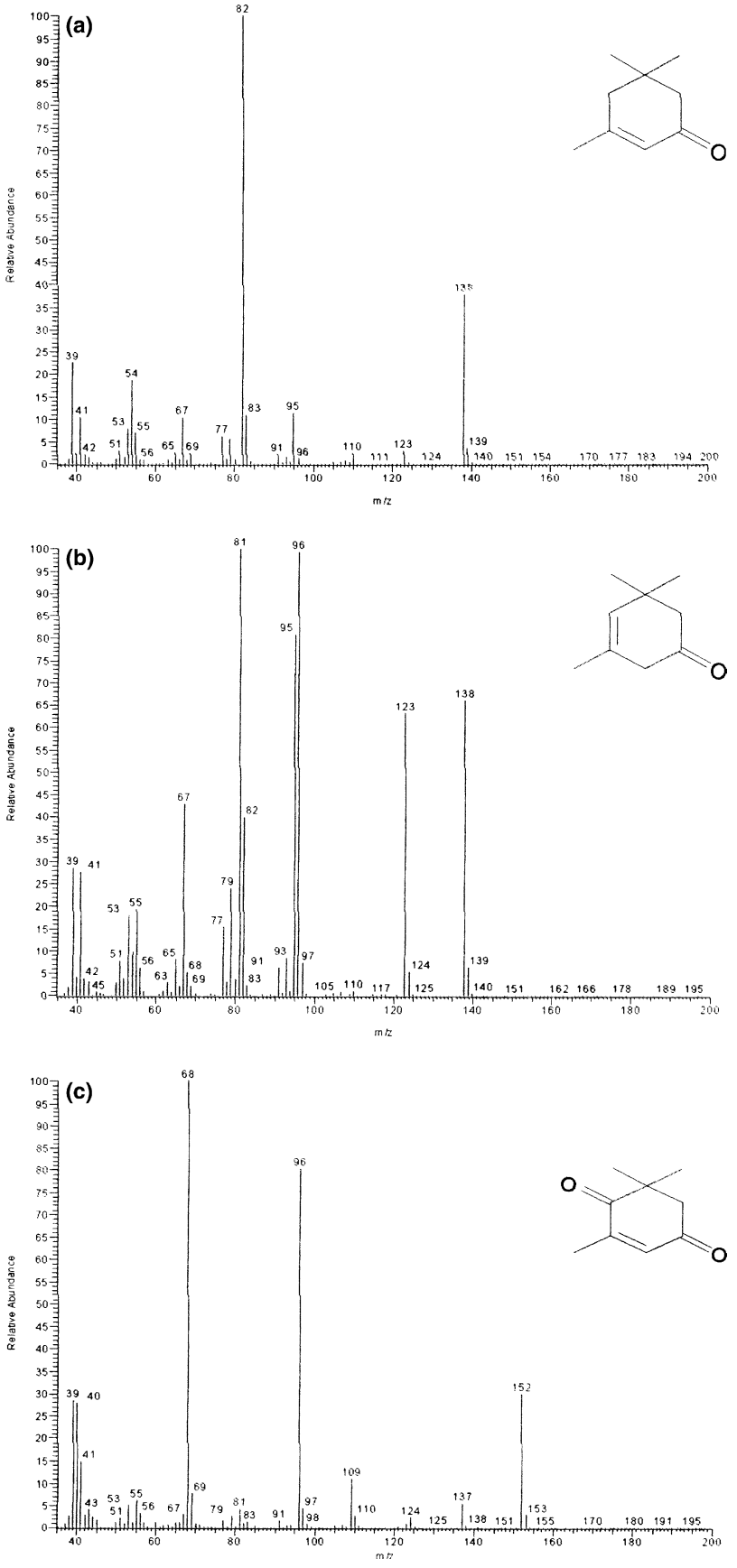

Fig. 2. Structures and mass spectra of: (a) α-isophorone; (b) β-isophorone; (c) 4-oxoisophorone.

botanical family (*Ericaceae*) as strawberry-tree. In general, the obtained GC profiles were characterized by small signals; in particular, norisoprenoids were not identified, even in the heather honey sample. These findings confirmed that these substances had to be present in strawberry-tree honey at levels even higher than those of heather honey, since abundant signals were detected in the GC chromatograms when sample amounts as low as 1.5 g were extracted.

In all the analysed samples, among norisoprenoids α-isophorone quantitatively predominated, followed by β-isophorone and 4-oxoisophorone, which were present in lower concentrations.

In order to quantify the relative amount of α-isophorone, β-isophorone and 4-oxoisophorone in the analyzed honey samples, chromatographic responses in terms of peak areas, were considered. The α- isophorone/β-isophorone ratio varied from 4 to 8, whereas the α-isophorone/4-oxoisophorone ratio was found to range from 11 to 20. Taking into account the great variability of this biological matrix, these ratios appears to be quite constant and characteristic, thus representing useful tool for assessment of the authenticity of Sardinian strawberry-tree honey. A further task will be the study of aromatic profiles of this kind of honey from different production zones, in order to evaluate possible differences attributable to geographical origin.

4. Conclusions

A DHS extraction method, based on the use of Carbopack^{TM}adsorbent traps and GC–MS analysis allowed characterization of the aroma profile of Sardinian strawberry-tree honey and its distinction from honeys of different floral source. Qualitative and quantitative data allowed us to conclude that some norisoprenoid compounds, namely α-isophorone, β-isophorone and 4-oxoisophorone, could be considered as markers of this unifloral honey since they were present in all samples analyzed and they were not detected in other honeys of different botanical origin at all or at comparable levels. Another important finding of this study was the definition of the α-isophorone/β-isophorone and the α-isophorone/4-oxoisophorone ratios in this honey product. These ratio values could be used for authenticity purposes, in particular to recognize and prove possible adulteration of this high economic value product.

Acknowledgements

This work was financially supported by MURST project "Monitoraggio ambientale, proposte di innovazione tecnologica e valorizzazione di risorse alternative nell'area di Porto Torres" (1999–2002).

References

Bonaga, G., & Giumanini, A. G. (1986). The volatile fraction of chestnut honey. *Journal of Apiculture Research, 25*, 113–120.

Bouseta, A., Collin, S., & Dufour, J. (1992). Characteristic aroma profiles of unifloral honeys obtained with a dynamic headspace GC–MS system. *Journal of Apiculture Research, 31*, 96–109.

Cabras, P., Angioni, A., Tuberoso, C., Floris, I., Reniero, F., Guillou, C., & Ghelli, S. (1999). Homogentisic acid: A phenolic marker of strawberry-tree (*Arbutus unedo*) honey. *Journal of Agricultural and Food Chemistry, 47*, 4064–4067.

Careri, M., Mazzoleni, V., Musci, M., & Molteni, R. (1999). Effects of electron beam irradiation on cork volatile compounds by gas chromatography–mass spectrometry. *Chromatographia, 49*, 166–172.

Dalla Serra, A., Franco, M. A., Mattivi, F., Ramponi, M., Vacca, V., & Versini, G. (1999). Aroma characterization of Sardinian strawberry tree (*Arbutus unedo* L.) honey. *Italian Journal of Food Science, 11*, 47–56.

D'Arcy, B. R., Rintoul, G. B., Rowland, C. Y., & Blackman, A. J. (1997). Composition of australian honey extractives. 1. Norisoprenoids, monoterpenes, and other natural volatiles from blue gum (*Eucalyptus leucoxylon*) and yellow box (*Eucalyptus melliodora*). *Journal of Agricultural and Food Chemistry, 45*, 1834–1843.

Enzell, C. (1985). Biodegradation of carotenoids. An important route to aroma compounds. *Pure and Applied Chemistry, 57*, 693–700.

Guyot, C., Scheirmann, V., & Collins, S. (1999). Floral origin markers of heather honeys: *Calluna vulgaris* and *Erica arborea*. *Food Chemistry, 64*, 3–11.

Guyot-Declerck, C., Chevance, F., Lermusieau, G., & Collins, S. (2000). Optimized extraction procedure for quantifying norisoprenoids in honey and honey food products. *Journal of Agricultual and Food Chemistry, 48*, 5850–5855.

Hausler, M., & Montag, A. (1989). Isolation, identification and quantitative determination of the norisoprenoid (S)-(+)-dehyrovomifoliol in honey. *Zeitschrift fur Lebensmittel-Untersuchung und -Forschung, 189*, 113–115.

Louveaux, J., Maurizio, A., & Vorwohl, G. (1978). Methods of melissopalynology. *Bee World, 59*, 139–157.

Overton, S. V., & Manura, J. J. (1994). Flavor and aroma in commercial bee honey. A purge-and-trap thermal desorption technique for the identification and quantification of volatiles and semivolatiles in honey. *American Laboratory, 26*, 45–51.

Proposal COM/95/0722 Official Journal C231, 09/08/1996 P. 0010.

Radovic, B. S., Careri, M., Mangia, A., Musci, M., Gerboles, M., & Anklam, E. (2001). Contribution of dynamic headspace GC–MS analysis of aroma compounds to authenticity testing of honey. *Food Chemistry, 72*, 511–520.

Sawyer, R. W. (1975). Melissopalinology in the determination of the geographical and floral origin of honey. *Journal of the Association of Public Analysts, 13*, 64–71.

Tan, S. T., Wilkins, A. L., Holland, P. T., & McGhie, T. K. (1990). Extractives from New Zealand honeys. 3. Unifloral thyme and willow honey constituents. *Journal of Agricultural and Food Chemistry, 38*, 1833–1838.

Tan, S. T., Wilkins, A. L., Holland, P. T., & McGhie, T. K. (1989). Extractives from New Zealand honeys. 2. Degraded carotenoids and other substances from heather honey. *Journal of Agricultural and Food Chemistry, 37*, 1217–1221.

Winterhalter, P., & Rouseff, R. L. (2002). Carotenoid-derived aroma compounds: An introduction. In P. Winterhalter & R. L. Rouseff (Eds.), *ACS symposium series*, *Carotenoid-derived aroma compounds Winterhalter*. Washington, DC: Published by America Chemical Society.