The enantiomer distribution of major chiral volatile organic compounds in Slovakian monofloral honeys

ALEXANDRA PAŽITNÁ – ANTÓNIA JANÁČOVÁ – IVAN ŠPÁNIK

Summary

In this work, the distribution of enantiomers of selected chiral volatile organic compounds in 45 monofloral honey samples was studied by gas chromatography (GC). The volatile organic compounds were extracted from Slovakian rapeseed, acacia, sunflower basswood and raspberry honeys by solid phase microextraction followed by gas chromatography – mass spectrometric (GC-MS) analysis. The chiral compounds present at higher contents were selected from 230 organic compounds found in studied samples for determination of their isomeric ratios. It was found that one-dimensional GC with chiral stationary phases provided excellent enantiomer separations; however, the resolved enantiomers often co-eluted with other non-chiral or already separated enantiomers of other organic compounds. Thus, two-dimensional GC with two independent thermostats and proper column setup was required to determine correct isomeric ratios. Finally, the isomeric ratios of linalool, *cis*- and *trans*-furanoid linalool oxides, hotrienol and four isomers of lilac aldehydes were determined. It was found that distribution of enantiomers in honey samples partially depended on their botanical origin. The differences in ratios of lilac aldehyde isomer B and hotrienol were observed for acacia honey that allowed us to distinguish this type of honey from others. Similarly, a different isomeric ratio of *trans*-furanoid linalool oxide was found for sunflower honeys.

Keywords

derivatized cyclodextrin stationary phase; enantioselective gas chromatography; enantiomeric separation; honey; multidimensional gas chromatography

Honey belongs to the most appreciated natural products. Based on its origin, honey can be divided into two categories. Monofloral honey is made preferably from nectar that originates from the flowers of the same botanical species. Various honeys with different botanical origin have been extensively studied, e.g. citrus [1], cotton [2], rosemary [3] produced in old EU member states [4, 5], Tunisia [6] and Australia [7]. The second category, polyfloral honeys, are prepared by mixing of flower nectar obtained from various botanical species.

One of the most characteristic features of each honey is its aroma profile that reflects the organoleptic quality as well as the botanical origin of honey. The aroma profile of honey often contains more than 150 constituents that belong to various chemical classes e.g. aliphatic and aromatic hydrocarbons, monoterpenes and their oxygenated derivatives or furan derivatives. Such complex mixture of different organic compounds can serve as a fingerprint to define the botanical origin of honeys [8]. Many volatile organic compounds (VOC) present in honeys are chiral, i.e. may exist as two enantiomers. Chiral compounds in plants and animals are synthesized by various enzymatic reactions. Based on stereoselectivity of used enzymes, chiral compounds in nature occur as pure enantiomers (amino acids, saccharides) or a mixture of enantiomers with specific ratio (flavours, carboxylic acids, alcohols) [9]. The presence of specific isomeric ratio of chiral substances in a particular food commodity depends also on the used raw material, processing technology or ageing. Any changes in these ratios could indicate illicit manipulation with products, incorrect treatment procedure or addition of synthetically produced chemicals. The enantiomer distribution

Alexandra Pažitná, Antónia Janáčová, Ivan Špánik, Institute of Analytical Chemistry, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, SK 812 37 Bratislava, Slovakia.

Correspondence author:
Ivan Špánik, Tel: +421 2 59325 283, Fax: +421 2 52926043, e-mail: ivan.spanik@stuba.sk

of VOC responsible for characteristic flavour was e.g. applied to authentication of orange juices as possible markers of geographical origin, production technology or adulteration by synthetic aromas [10, 11].

There are two ways how to perform chiral separations. The first one is to use one-dimensional gas chromatography with chiral stationary phases and the second one is to use a system with two independent GC ovens – multidimensional gas chromatography. The one-dimensional GC with a chiral stationary phases often has a limited separation efficiency in complicated matrices. This problem can be solved by using a two-dimensional GC system. Multidimensional gas chromatography was firstly described by SIMMONS and SNYDER [12] in 1958. Its principles have been described in several studies [13–15].

Recently, solid phase microextraction (SPME) has become the most frequently used method for extraction of volatile and semi-volatile compounds from honey [16–19]. This technique is based on reaching the equilibrium between the concentration of VOC in the vapour phase and their concentration on a SPME fibre. SPME followed by gas chromatography – mass spectrometry (GC-MS) was used for the analysis of volatiles in Spanish honeys. In this work, some of the compounds were proposed to distinguish the different types of honey. For example, the presence of chiral lilac aldehydes (2,5-dimethyl-5-ethylenyl-2-tetrahydrofuran acetaldehydes) with a typical floral odour, was characteristic of the orange honey [20]. Similarly, DE LA FUENTE et al. [21] investigated 21 eucalyptus, 35 rosemary, 33 heather and 15 citrus honeys collected within 3 years in different Spanish regions. Samples were analysed by SPME followed by GC-MS. In total, 83 volatile compounds were identified in 110 Spanish honeys of four floral origins. Many of them were chiral, thus could exist in at least two enantiomeric forms, e.g. α-pinene, limonene, linalool oxides, linalool, lilac aldehydes, α-terpineol, hotrienol (3,7-dimethylocta-1,5,7-trien-3-ol). The same chiral compounds were identified also in rape honey [22].

The aim of this work was to identify chiral organic compounds present in Slovakian honeys of various botanical origins, and to determine their isomeric ratios.

MATERIALS AND METHODS

Samples

A selection of 46 monofloral honeys commonly available on market in Slovak Republic was studied. These were honey from acacia (*Robinia pseudoacacia*, 27 samples), honey from basswood (*Tilia cordata*, 8 samples), honey from rapeseed (*Brassica napus*, 5 samples), honey from sunflower (*Helianthus annuus*, 4 samples) and honey from raspberry (*Rubus idaeus*, 2 samples). All honeys originated from Slovakia were obtained from local beekeepers in various Slovakian regions.

Sample treatment

VOC from honey samples were extracted by SPME using Agilent autosampler GC 80 (Agilent Technologies, Santa Clara, California, USA). In total, a 5 g honey sample together with NaCl (0.5 g) were dissolved in deionized water in a 20 ml clear glass vial and the solution was stirred with a polytetrafluoroethylene-coated (PTFE) magnetic stir bar at 450 Hz. Vials were sealed with holecaps and PTFE/silicone septa. A stirred sample solution was heated at 60 °C for 30 min, in order to establish equilibrium between liquid and vapor phases. VOC were extracted to SPME fibre coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) sorbent (50/30 μm thickness) obtained from Supelco (Bellefonte, Pennsylvania, USA). The fibre was conditioned prior to use by heating in the injection port of GC under the conditions recommended by the manufacturer. The adsorption of VOC from honey samples on SPME fibre took 30 min at 60 °C, the solution being stirred at 450 Hz. Desorption was performed in GC injector in splitless mode at 220 °C for 2 min.

Instruments

An Agilent 7890A gas chromatograph connected to Agilent 5975C MS detector was used for GC-MS analyses. The gas chromatograph was connected via Dean's microfluidic switching system (Agilent Technologies) to the second gas chromatograph Hewlett Packard 5890 (Hewlett Packard, Palo Alto, California, USA), which was equipped with a flame ionization detector (FID). Helium of the purity of 99.995% was used as a carrier gas at a flow rate, in the nitroterephthalic acid-modified polyethylene glycol (DB-FFAP) column, of 1 ml·min^{-1}. The first oven temperature programme started at 60 °C, then the temperature increased with a gradient of 2 °C·min^{-1} to 150 °C, followed by an increase at 10 °C·min^{-1} to 250 °C. A 30 m DB-FFAP column (Agilent J&W Column, Agilent Technologies) with i.d. 0.25 mm and film thickness 0.25 μm was used in GC-MS analyses to separate VOC based on their polarities. The carrier gas flow was switched in Dean's microfluidic

device either to a restrictor (length 8.8 m, i.d. 0.18 mm) that ended in MS detector, or, if chiral compounds eluted from DB-FFAP column, to a 20 m capillary column with 0.25 mm i.d., coated with permethyl-β-cyclodextrin anchored to silicone polymer (ChirasilDex; Chrompack International, Bergen op Zoom, The Netherlands) inserted in the second GC oven. Separations of enantiomers of selected compounds were performed isothermically at 75 °C.

Chemicals

Anhydrous NaCl used in the sample treatment procedure was obtained from Mikrochem (Pezinok, Slovakia). Deionized water was prepared using NANOpure device from Wilkem Werner (Leverkusen, Germany). The racemic mixtures of linalool, *cis*- and *trans*-linalool oxides (furanoid form) and L-linalool were obtained from Sigma Aldrich (St. Louis, Missouri, USA). The mixture of racemates of lilac aldehydes A (2'S, 2S, 5S), B (2'R, 2S, 5S), C (2'R, 2R, 5S) and D (2'S, 2R, 5S) were prepared by the procedure described by MOSANDL et al. [23]. The elution order on DB-FFAP column was taken from literature [24]. Racemic hotrienol was prepared from linalyl acetate by the procedure described by YUASA and KATO [25].

Percentage of the first-eluting enantiomer (E_1) of the chiral organic compound was calculated according to equation:

$$E_1 = \frac{A_1}{A_1 + A_2} \times 100 \quad (1)$$

where A_1 is a peak area obtained for 1st eluted enantiomer and A_2 is a peak area obtained for 2nd eluted enantiomer. Similarly, the percentage of the second-eluting enantiomer (E_2) was calculated by replacing A_1 in numerator by A_2.

Isomeric ratios are expressed as

$$Isomeric\ ratio = \frac{E_1}{E_2} \quad (2)$$

RESULTS AND DISCUSSION

The first experiments were aimed at characterization of VOC profile in honey samples by GC-MS with achiral DB-FFAP stationary phase. The obtained chromatograms (Fig. 1) contained more than 230 compounds that belonged to various chemical classes, e.g. aliphatic and branched alkanes, alcohols, aldehydes, ketones, carboxylic acids and their methyl and ethyl esters, while linalool oxides, linalool, hotrienol, phenylethanol, lilac aldehydes and methyl esters of carboxylic acids were present at the highest content [26]. Since most of the mentioned compounds are chiral and are present at sufficiently high contents, we were interested in the distribution of enantiomers of the mentioned chiral terpenes. The easiest way how to perform chiral separation is to separate VOC by GC-MS on a chiral stationary phase. Based on data published in literature [27, 28], enantiomers of furanoid derivatives of *cis*-linalool oxide (2R, 5S and 2S, 5R) and *trans*-linalool oxide (2R, 5R and 2S, 5S) , linalool, hotrienol and lilac aldehydes can be separated on permethylated-β-cyclodextrin stationary phase. In these experiments, temperature programme started at 40 °C and was held for 15 min, then increased to 200 °C with a slow temperature gradient of 2 °C.min^{-1}. The studied compounds were identified by comparison of the

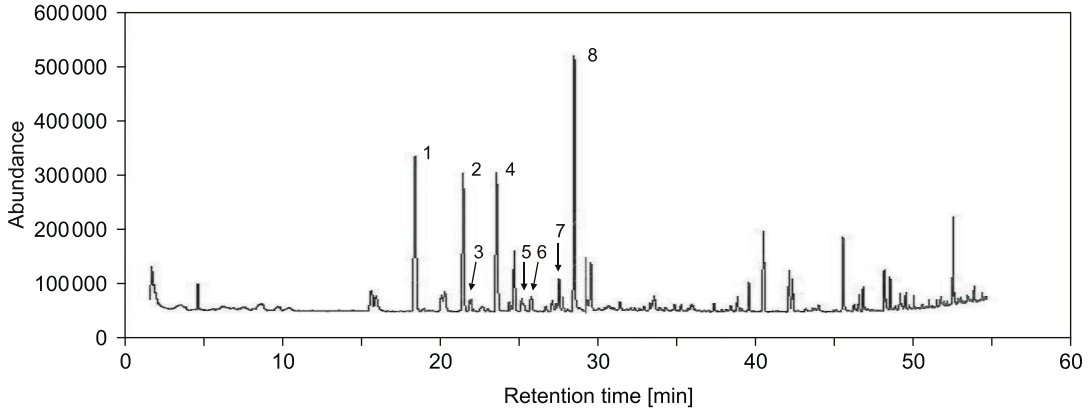

Fig. 1. SPME-GC-MS chromatogram of acacia honey VOC fraction obtained on DB-FFAP stationary phase.

1 – *cis*-linalool oxide, 2 – *trans*-linalool oxide, 3 – lilac aldehyde A, 4 – linalool, 5 – lilac aldehyde B, 6 – lilac aldehyde C, 7 – lilac aldehyde D, 8 – hotrienol.

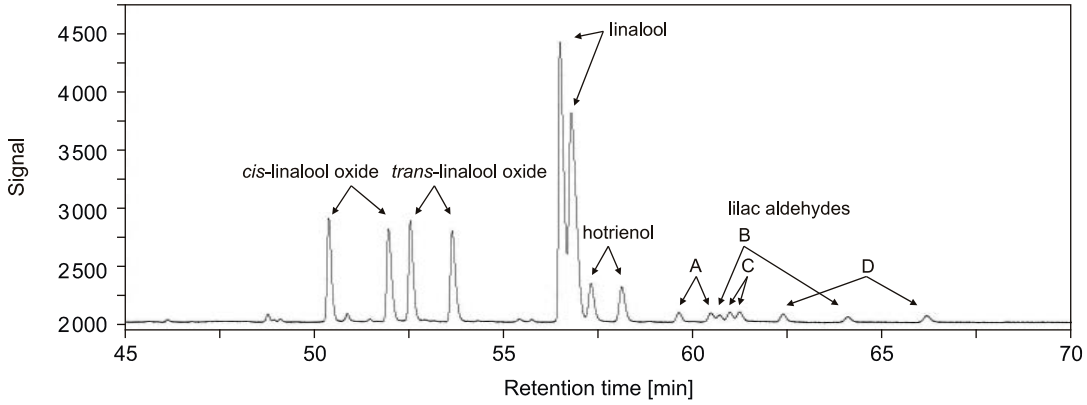

Fig. 2. The enantiomer separation of VOC standards obtained on ChirasilDex stationary phase at 75 °C.

Tab. 1. Percentages of the first-eluting enantiomers of the chiral organic constituents of the honey samples of different botanical origin.

Compound	Percentage [%]				
	Acacia	Rapeseed	Raspberry	Sunflower	Basswood
trans-linalool oxide	58–73	66–75	55–60	65–78	75–80
cis-linalol oxide	44–59	53–55	58–51	66–70	37–60
R-linalool	19–37	52–60	48–59	32–45	25–37
Hotrienol	68–82	13–25	52–58	6–15	7–12
Lilac aldehyde, isomer A	61–70	66–72	64–72	60–68	77–82
Lilac aldehyde, isomer B	7–15	30–32	26–32	28–34	21–24
Lilac aldehyde, isomer C	27–41	38–41	33–39	37–41	28–38
Lilac aldehyde, isomer D	37–45	41–45	36–40	33–39	17–24

Percentage of the first-eluting enantiomer of the chiral organic compound was calculated according to Eq. 1.

measured retention times and mass spectra with those obtained for racemic mixtures of standards injected under the same conditions. The enantiomers of *trans*-linalool oxide, hotrienol and lilac aldehydes were successfully separated, while these experimental conditions did not facilitate separation of enantiomers of *cis*-linalool oxide and linalool. Unfortunately, the second eluted enantiomer of *trans*-linalool oxide and some enantiomers of lilac aldehydes co-eluted either with other organic compounds or with already separated enantiomers. Thus, only isomeric ratio of hotrienol 67:33 in acacia honey samples was successfully determined by this approach.

Since the approach based on separation of organic compounds on a chiral stationary phase failed, multidimensional chromatography was used to determine the isomeric ratios of selected chiral compounds in the next experiments. In the multidimensional approach, VOC extracted from honeys were first separated on an achiral stationary phase in the first GC oven. When chiral compound eluted from the first column, a switching system switched this compound to the second GC oven with a chiral stationary phase, where enantiomers were separated at isothermal conditions. The optimization of working conditions for chiral separations included the separation temperature and the number of sample "cuts" from the first GC, which had to be performed to determine the enantiomer ratios of selected compounds. It was found that enantiomers of linalool oxides, hotrienol and all isomers of lilac aldehydes were baseline-separated, while only a partial resolution of a linalool enantiomer was achieved at 75 °C. On the other hand, these experimental conditions allowed us to separate enantiomers of all studied compounds in one GC run (Fig. 2). The elution order of linalool was determined by injection of a racemic mixture enriched by pure R-linalool. The elution order of other compounds on permethylated-β-CD was taken from previously pub-

lished results [27, 28], except of lilac aldehydes and hotrienol that, according to our best knowledge, were not available in the literature. In order to find correct isomeric ratios, the enantiomers of VOC were separated in four independent GC runs. In the first GC run, isomeric ratios of linalool oxides, hotrienol, and lilac aldehydes isomer A and isomer D were determined. The isomeric ratios of lilac aldehydes B and C were determined separately in the second and third GC run. Finally, in the last GC run, the enatiomers of linalool were separated at 50 °C, where almost baseline enantiomer separation was achieved.

The obtained content in percent of the first-eluted enantiomers of the chiral organic constituents of the honey samples is shown in Tab. 1. As can be seen, the obtained isomeric ratios varied within different ranges for individual compounds and in some cases reflected the botanical origin of honey. For example, the first-eluted enantiomer of linalool oxide could be found in a wide range in acacia, rapeseed and sunflower honeys. On the contrary, all enantiomers of lilac aldehyde isomers were found in a narrow range, where differences between honey samples from the same botanical origin usually did not exceed 9%. Additional differences were observed for the enantiomer distribution of lilac aldehydes based on botanical origin of honey. In acacia honeys, the second-eluted enantiomer of lilac aldehyde B was present in a higher purity than in other studied samples. This could be used to distinguish between acacia and rapeseed honeys, which is interesting as these plants are blossoming in the same period. The basswood honey could be distinguished from other monofloral honeys based the content of the first-eluted enantiomer of lilac aldehyde isomer D. Similarly, the sunflower honeys were characteristic by a higher enantiomer content of the first-eluted enantiomer of *cis*-linalool oxide, compared to other studied samples. The isomeric ratios found for hotrienol were in a good agreement with those observed from the one-dimensional analysis.

CONCLUSIONS

The isomeric ratios of furanoid linolool oxides, linalool, hotrienol and all isomers of lilac aldehydes were determined in Slovakian honeys of various botanical origins. The one-dimensional GC with chiral stationary phases was found to have a limited capacity to determine the enantiomer distribution in complex matrices. In such cases, even if the chiral stationary phase facilitated excellent separation of enantiomers, the resolved enantiomers often co-eluted with other non-chiral or already separated enantiomers of other organic compounds. This lack of separation efficiency could be overcome by the two-dimensional approach, in which two independent GC ovens equipped with a proper switching system (e.g. heart-cut) and a specific column setup, namely, the first column having an achiral stationary phase and the second column having a chiral stationary phase. Such GC system was more suitable for the determination of enantiomer composition of organic compounds present in very compex matrices. Finally, the two-dimensional GC system was used to determine the isomeric ratios of selected volatile organic compounds in Slovakian honeys. It was found that determination of the isomeric ratio of chiral organic compounds in honey has a potential to distinguish samples of different botanical origin.

ACKNOWLEDGEMENT

This work was supported by VEGA grant no. 1/0710/10.

REFERENCES

1. Castro-Vazquez, L. – Diaz-Maroto, M. C. – Perez-Coello, M. S.: Aroma composition and new chemical markers of Spanish citrus honeys. Food Chemistry, *103*, 2007, pp. 601–606.
2. Alissandrakis, E. – Tarantilis, P. A. – Harizanis, P. C. – Polissiou, M.: Aroma investigation of unifloral greek citrus honey using solid-phase microextraction coupled to gas chromatographic mass spectrometric analysis. Food Chemistry, *100*, 2007, pp. 396–404.
3. Castro-Vazquez, L. – Perez-Coello, M. S. – Cabezudo, M. D.: Analysis of volatile compounds of rosemary honey: Comparison of different extraction techniques. Chromatographia, *57*, 2003, pp. 227–233.
4. de la Fuente, E. – Martinez-Castro, I. – Sanz, J.: Characterization of Spanish unifloral honeys by solid phase microextraction and gas chromatography-mass spectrometry. Journal of Separation Science, *28*, 2005, pp. 1093–1100.
5. Alissandrakis, E. – Kibaris, A. C. – Tarantilis, P. A. – Harizanis, P. C. – Polissiou, M.: Flavour compounds of Greek cotton honey. Journal of the Science of Food and Agriculture, *85*, 2005, pp. 1444–1452.
6. Martos, I. – Cossentini, M. – Ferreres, F. –Tomás-Barberán, F. A.: Flavonoid composition of Tunisian honeys and propolis. Journal of Agricultural and Food Chemistry, *45*, 1997, pp. 2824–2829.
7. D'Arcy, B. R. – Rintoul, G. B. – Rowland, C. Y. – Blackman A. J.: Composition of Australian honey extractives. 1. Norisoprenoids, monoterpenes, and other natural volatiles from blue gum (*Eucalyptus leucoxylon*) and yellow box (*Eucalyptus melliodora*) honeys. Journal of Agricultural and Food Chemistry,

45, 1997, pp. 1834–1843.
8. Radovic, B. S. – Careri, M. – Mangia, A. – Musci, M. – Gerboles, M. – Anklam E.: Contribution of dynamic headspace GC-MS analysis of aroma compounds to authenticity testing of honey. Food Chemistry, *72*, 2001, pp. 511–520.
9. Mosandl, A.: Authenticity assessment: a permanent challenge in food flavor and essential oil analysis. Journal of Chromatographic Science, *42*, 2004, pp. 440–449.
10. del Castillo, M. L. R. – Caballero, E. G. – Blanch, G. P. – Herraiz, M.: Enantiomeric composition of filbertone in hazelnuts and hazelnut oils from different geographical origins. Journal of the American Oil Chemists' Society, *79*, 2002, pp. 589–592.
11. del Castillo, M. L. R. – Caja, M. M. – Blanch, G. P. – Herraiz, M.: Enantiomeric distribution of chiral compounds in orange juices according to their geographical origins. Journal of Food Protection, *66*, 2003, pp. 1448–1454.
12. Simmons, M. C. – Snyder, L. R.: Two stage gas-liquid chromatography. Analytical Chemistry, *30*, 1958, pp. 32–35.
13. Bertsch, W.: Methods in high resolution gas chromatography. I. Two dimensional techniques. Journal of High Resolution Chromatography and Chromatography Communications, *1*, 1978, pp. 85–90.
14. Krock, K. A. – Wilkins, C. L.: Recent advances in multidimensional gas chromatography. Trends in Analytical Chemistry, *13*, 1994, pp. 13–17.
15. Schomburg, G. – Weeke, F. – Muller, F. – Oreans, M.: Multidimensional gas chromatography (MCD) in capillary columns using double oven instrument and newly designed coupling piece for monitoring detection after pre-separation. Chromatographia, *16*, 1982, pp. 87–91.
16. Guidotti, M. – Vitali, M.: Identificazione di composti organici in miele mediante SPME e GC/MS. Industrie Alimentari, *37*, 1998, pp. 351–353.
17. Soria, A. C. – Martinez-Castro, I. – Sanz, J.: Analysis of volatile composition of honey by solid phase microextraction and gas chromatography mass spectrometry. Journal of Separation Science, *26*, 2003, pp. 793–801.
18. Soria, A. C. – Sanz, J. – Martinez-Castro, I.: SPME followed by GC-MS: a powerful technique for qualitative analysis of honey volatiles. European Food Research and Technology, *228*, 2009, pp. 579–590.
19. Plutowska, B. – Chmiel, T. – Dymerski, T. – Wardencki, W.: A headspace solid-phase microextraction method development and its application in the determination of volatiles in honeys by gas chromatography. Food Chemistry, *126*, 2011, pp. 1288–1298.
20. Pérez, R. A. – Sánchez-Brunete, C. – Calvo, R. M – Tadeo, J. L.: Analysis of volatiles from Spanish honeys by solid-phase microextraction and gas chromatography-mass spectrometry. Journal of Agricultural and Food Chemistry, *50,* 2002, pp. 2633–2637.
21. de la Fuente, E. – Martínez-Castro, I. – Sanz, J.: Characterization of Spanish unifloral honeys by solid phase microextraction and gas chromatography-mass spectrometry. Journal of Separation Science, *28*, 2005, pp. 1093–1100.
22. Kaškonienė, V. – Venskutonis, P. R. – Čeksterytė, V.: Composition of volatile compounds of honey of various floral origin and beebread collected in Lithuania. Food Chemistry, *111,* 2008, pp. 988–997.
23. Kreck, M. – Mosandl, A.: Synthesis, structure elucidation, and olfactometric analysis of lilac aldehyde and lilac alcohol stereoisomers. Journal of Agricultural and Food Chemistry, *51*, 2003, pp. 2722–2726.
24. Matich, A. J. – Young, H. – Allen, J. M. – Wang, M. Y. – Fielder, S. – McNeilage, M. A. – MacRae, E. A.: *Actinidia arguta*: volatile compounds in fruit and flowers. Phytochemistry, *63*, 2003, pp. 285–301.
25. Yuasa, Y. – Kato, Y.: A practical and convenient synthesis of hotrienol, an excellent fruity smelling compound. Journal of Agricultural and Food Chemistry, *51*, 2003, pp. 4036–4039.
26. Špánik, I. – Janáčová, A. – Šusterová, Z. – Jakubík, T. – Jánošková, N. – Novák, P. – Chlebo, R.: Characterisation of VOC composition of Slovak monofloral honeys by GC×GC-TOF-MS. In: Chemical Papers [online]. October 2012 [cit. 30 October 2012], <http://link.springer.com/article/10.2478/s11696-012-0254-z>. ISSN 1336-9075. doi 10.2478/s11696-012-0254-z.
27. Demyttenaere, J. C. R. – Willemen, H. M.: Biotransformation of geraniol, nerol and citral by sporulated surface cultures of *Aspergillus niger* and *Penicillium* sp. Phytochemistry, *47*, 1998, pp. 1029–1036.
28. Baigrie, B. D. – Chisholm, M. G. – Mottram, D. S.: The effect of processing on chiral aroma compounds in fruits and essential oils. In: Taylor, A. J. – Mottram, D. S. (Ed.): Flavour science: recent developments. Cambridge: Woodhead Publishing, 1996, pp. 151–158. doi 10.1533/9781845698232.3.151.

Received 21 August 2012; revised 30 October 2012; accepted 23 Novemvber 2012.