Solid-Phase Microextraction of Honey Volatiles: A Method for the Determination of the Botanical Origin of Honey

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Tiivistelmä — Referat — Abstract

In the present work, a headspace-Solid-Phase Microextraction (SPME) method was developed and optimised in regard to all important criteria for the extraction of honey volatiles, such as fiber coating, sample amount, matrix modification by water and sodium chloride addition, agitation of sample matrix, extraction temperature, extraction time and analyte desorption.
The 50/30 µm divinylbenzene/Carboxene/polydimethylsiloxane coated SPME-fiber proved to extract successfully volatile components within a broad range of chemical characteristics and volatility. Extraction temperature, matrix modification and extraction time were shown to have the most pronounced influence on the extraction rate of honey volatiles. Headspace SPME seems to be a promising technique for the simple and fast determination of honeys volatiles.

The developed method was applied for determination of volatiles in *Brassica rapa* var. *oleifera* subvar. *annua* (spring turnip rape) and *Brassica napus* var. *biennis* (winter rape) honeys from Finland, respectively Switzerland. 26 new compounds could be identified in Brassica honeys. A significant difference was found between the volatile composition of the two honeys. Especially the concentration of 2-furancarbox-aldehyde, benzaldehyde, benzeneacetaldehyde, benzyl alcohol, benzeneethanol, and benzeneacetonitrile in the two honey types was different ($p < 0.05$). On average, the concentration of all these compounds was lower in spring turnip rape honey than in winter rape honey. The concentration of benzeneethanol and benzoic acid can be used to distinguish between rape and turnip rape honey.

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Was hier steht, das Sichtbare, das sind ja nur die Gipfel oder die Gräte – ich aber ging durch das Gebirge, erstieg die ganzen Berge, war in den Bergen, schaue durch die ganzen Berge hindurch.

Ludwig Hohl

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Introduction

1 Determination of botanical origin of honey: a short review

1.1 Introduction to the determination of botanical origin

At present, the beekeepers throughout the world produce various kinds of honeys, some of them expressing very distinct organoleptic properties. These features strongly influence consumer preferences and also honey price. In countries like Italy and France, up to 50% of the honey is sold as unifloral honey. It is generally sold at a higher price than honey blends. In order to be able to compete with cheap imported honey European beekeepers are forced to produce unifloral honeys of high quality.

According to the EU Council Directive relating to honey (2001/110/EC, EU Council 2002), the product names may be supplemented by information related to the botanical origin, if the product comes wholly or mainly from the indicated source and possesses the organoleptic, physico-chemical and microscopic characteristics of the source.

In order to produce unifloral honeys, the beekeepers move empty hives to places with abundant flowering of the plant of the desired nectar source. Under favourable conditions the bees gather nectar predominately from the desired plant. After the flowering period, the ripe honey is harvested and extracted separately.

The precise definition of a specific type of unifloral honey is a very complex problem because the values of individual parameters vary greatly. The variation is due to the fact that bees do not collect nectar from one plant species only. Strictly speaking, absolutely pure unifloral honeys do not exist

or are extremely rare (Persano Oddo *et al.* 2000). However, it is possible to produce experimentally pure unifloral honeys (Demianowicz 1961).

On the following pages, an overview of the analytical methods for the determination of the botanical origin is given.

1.2 Melissopalynology

Honey contains pollen grains and other microscopic particles like fungi spores and algae, originating from the plants from which the nectar or honeydew has been collected by the bees.

The pollen grains are identified by light microscopy and are counted according to the methods of the International Commission for Bee Botany (Louveaux *et al.* 1978). In qualitative analysis, all the pollen, present in honey are identified and their relative amount is determined. For the determination of frequency classes, which is normally done in routine pollen analysis, at least 300 pollen grains and the corresponding honeydew elements are counted. The pollen types found are presented in four frequency classes: predominant pollen (>45%), secondary pollen (16-45%), important minor pollen (3-15%) and minor pollen (<3%). If the frequency of pollen types is presented in percentages, the counting of 500-1000 grains is necessary (Behm *et al.* 1996).

Remark: In an agreement of the International Honey Commission it was decided to use the family names (not written in italics) of the nectar producing plants instead of the common names to denote uniforal honeys: e.g. Citrus, Castanea etc.

1.2.1 Determination of botanical origin

The amount of pollen in nectar is not constant. Therefore the assumption, that the relative amount of a certain pollen type in honey correlates with the nectar contribution of the plant, is not always correct. Some pollen are known to be over-represented, and some are under-represented. This can be explained by the way pollen find their way into honey.

1.2.2 Factors influencing the representation of pollen in honey

Primary dusting

The amount of pollen in the nectar plants depends on the design of the flower i.e. of the position of the anthers in respect to the nectaries. It depends on the amount of pollen, its size and on whether nectar secretion coincides with anther maturation or not. Nectar from dioecious or male sterile plants e.g. cultivars of Citrus spp. will not contain any or very little pollen.

During honey processing, some pollen is removed by the bees proventriculus. The removal depends on the extent of honey processing, pollen size and structure of the pollen exine (Vorwohl 1994).

Secondary dusting

In addition to primary dusting, the so called secondary dusting affects pollen representation as well. Since pollen is the only source of proteins for bees, they collect it and store it in their combs. When pollen and honey are processed by the bees in the hive, it can be transferred from the combs into honey. If the pollen originates from the same plant as the nectar, the pollen content of honey is enriched. On the other hand, the honey may also be contaminated with pollen of other plants.

Tertiary dusting

Pollen can enter the honey by the actions of the beekeeper during uncapping and extraction of the honeycombs. Cells containing pollen are often cut especially during rigorous, mechanical uncapping. A high number of pollen is released from the cut cells into the honey. Some pollen may also be released during extraction. The most severe contamination occurs when honey is extracted by pressing, which is still practiced for the harvest of some honeys, especially Calluna honey (Vorwohl 1994).

Melissopalynological data may cause difficult interpretation problems although pollen representations are described in the literature (Talpay 1985; Persano Oddo *et al.* 1995). Generally the presence of a dominant pollen form (>45%) has been considered to indicate unoflorality. In honeys from under-represented species, the minimum percentage of pollen is often as low as

10% or even lower e.g. Arbutus, Citrus, Taraxacum and Tilia honeys. On the other hand, honeys from over-represented plants e.g. Castanea and Eucalyptus contain usually more than 90% pollen from the unifloral source. Differences in pollen representation resulting from flower design can be taken into account when the results are interpreted, but the influence of pollen contamination in the hive and during extraction can not be controlled. Pollen analysis is relatively time consuming and needs a long experience before it can be reliably performed. The trustworthiness of the results depends very much on the expert's ability. Today it is currently accepted that the determination of botanical origin can not be based solely on pollen analysis (Persano Oddo *et al.* 1995). Pollen analysis becomes useless if the pollen has been removed by filtering during honey processing. According to the present EU honey directive (EU Council 2002), the removal of pollen by filtration is allowed. In honeys without pollen, the determination of the botanical origin by melissopalynology will be no longer possible.

1.3 Physico-chemical routine methods

1.3.1 Electrical conductivity

Electrical conductivity depends mainly on the mineral content of honey. The results are expressed in milli Siemens per centimetre ($mS.cm^{-1}$) (Bogdanov *et al.* 1997). The range of electrical conductivity in honey lies between 0.06 and 1.71 $mS.cm^{-1}$. It is an important tool for the estimation of honeydew in honey. Honeydew honeys have a higher electrical conductivity than 0.8 $mS.cm^{-1}$, blends between blossom and honeydew honeys have conductivity values lying between 0.51 and 0.79 $mS.cm^{-1}$, and pure floral honeys have conductivity values lying between 0.15 and 0.50 $mS.cm^{-1}$.

Among all physical and chemical parameters, electrical conductivity is the best criterion for discrimination between unifloral honeys (Persano Oddo *et al.* 1988; Mateo and Bosch-Reig 1998; Bogdanov *et al.* 1999). However, the conductivity values of many floral honeys lie very close to each

other. A reliable determination of botanical origin is not possible based on this parameter only.

1.3.2 Sugars

Sugar composition is determined by gas or liquid chromatography (Bogdanov *et al.* 1997; Bogdanov and Martin 2002).

Honeydew honeys contain less fructose and glucose and have a higher content of oligosaccharides than blossom honeys. Melezitose can be found in varying quantities in most honeydew honeys, but not in blossom honeys. Honeydew is a sticky syrup, produced by aphids feeding on the phloem sap of plant. The aphids partly transform fructose into oligosaccharides, the trisaccharide melezitose being the main one (Bacon and Dickinson 1957). Honeydew is subsequently collected by bees to produce honeydew honey. However, it is impossible to distinguish between different blossom honeys based on sugar composition. Even using very thorough separation of the sugars, the oligosaccharide patterns of different unifloral honeys are very similar (Low *et al.* 1988). In combination with other characteristics, the fructose/glucose ratio is used for the determination of the botanical origin (Talpay 1985).

An attempt to differentiate between honeydew honeys from various aphids was made by von der Ohe and von der Ohe (1996). Qualitative and quantitative differences in trehalose-, raffinose- and oligosaccharide L2-content were found for the different aphid species. For aphids of the *Coccidae* family, a difference in oligosaccharide L1 content could be observed. The two unidentified oligosaccharides L1 and L2 could be identified by retention time, but the chemical nature of these compounds was not determined. It is supposed that L2 might be manninotriose (a sugar present in the phloem sap) as it is relatively stable to hydrolysis and is present in every honeydew honey.

Recently the oligosaccharide isomelezitose was detected in honey by a gas chromatographic method (Gomez Barez *et al.* 1999) and proposed as an indicator of the oligosaccharide content of honey by Rittig (2001).

1.3.3 Chemometrical classification with routine parameters

Chemometrical methods use statistical methods like prinicipal component analysis, cluster analysis and discriminant analysis to combine different physico-chemical parameters and aim at the classification different of honeys. Chemometrical evaluation of physico-chemical routine parameters like water content, proline content, electrical conductivity, acidity (free and lactone), pH, colour, 5-hydroxy-methyl-furfural (HMF) content, diastase activity and sugar composition has been predominately used for the discrimination between geographical origin (Crecente and Latorre 1993; Sanz *et al.* 1995; Latorre *et al.* 1999; Gomez Barez *et al.* 2000), but there are also studies on botanical origin (Mateo and Bosch-Reig 1998; Piro *et al.* 2002). A proper classification of the samples studied was achieved for many unifloral honeys with a high percentage of correct assignation. Unfortunately the models were often created with a very limited number of samples and were not tested in practical honey control. The findings are thus valid only for the honey types studied and do not allow a generalisation to all unifloral honeys. Because of the enormous variability of these parameters in polyfloral honeys, it is very unlikely that chemometry based on routine parameters is of any use for the determination of unifloral honeys.

1.4 Amino acids and minerals

1.4.1 Amino acids

The composition of free amino acids has mainly been used for the determination of the geographical origin of honey (Davies 1982).

In a study on Lavandula and Eucalyptus honeys, high amounts of phenyl-alanine (906-1830 ppm) and tyrosine (229-382 ppm) were found to be characteristic for Lavandula honey and allowed a differentiation from eucalyptus honeys (Bouseta *et al*. 1996). Unifloral honeys differ to a certain extent in their content of amino acids (Bosi and Battaglini1978; Pirini *et al.* 1992; Sanchez *et al.* 2001), but the differences are small and can not be used as an unambiguous discriminant parameter.

1.4.2 Minerals

Mineral content in honey has been studied by activation analysis with thermic neutrons or with atom-absorption spectrophotometry. Discrimination between light and dark coloured honeys could be attained (Lasceve and Gonnet 1974; Ivanov and Chervenakova 1984). Mineral content does not allow a more de-tailed classification between different unifloral honeys than the measurement of electrical conductivity allows.

In a preliminary study, the mineral content of Robinia honeys from Hungary and France was studied. The Hungarian sample could be distinguished from the samples from France by Rb/Fe- and Rb/Zn-ratios (Lasceve and Gonnet 1974). In a Canadian work where the mineral composition of honeys from different provinces was studied, no discrimination was attained between different floral origins. However, honeys from the coastal provinces with a more humid climate had a higher mineral content than honeys from central provinces with a continental climate (Feller-Demalsy *et al.* 1989). Thus, mineral content seems to be more useful for the determination of geo-graphical origin of honey.

1.5 Phenolic acids and flavonoids

Phenolic acids and flavonoids are plant-derived secondary metabolites. These compounds have been used as chemotaxonomic markers in plant systematics. Recently they have been suggested as possible markers for the determination of botanical origin of honey. The analytical methods are very time consuming as different purification steps are necessary, before the phenolic compounds can be determined. Generally, liquid chromatography is used.

Considerable differences in composition and content of phenolic compounds between different unifloral honeys were found. Dark coloured honeys are reported to contain more phenolic acid derivatives, but less flavonoids than light coloured ones (Amiot *et al.* 1989).

Ferreres *et al.* (1993) studied the flavonoid profile of Citrus honeys and compared their profiles with the profiles of Rosmarinus, Lavandula, Tilia, Helianthus, Prunus dulcis, Castanea, Trifolium repens, Robinia, Erisarum, Rhododendron, Prosopis, Eucalyptus, Calluna and polyfloral honeys. Hesperetin (5,7,3'-trihydroxy-4'methoxyflavanone) was detected in Citrus honeys only and was proposed as a marker substance. The volatile compound methyl antranilate has been proposed as a marker of Citrus honey (Deshusses and Gabbai 1962; White 1966; Serra Bonvehi 1988). Ferreres *et al.* (1994) compared the methyl antranilate and the hesperetin content of Citrus honeys. No consistent relationship could be found. Since hesperetin is more stable than methyl antranilate, it was proposed as a complementary marker for Citrus honey.

In a recent study (Tomas-Barberan *et al.* 2001), the flavonoid profile of 9 European unifloral honeys was analyzed by HPLC. Hesperetin was confirmed as a marker of Citrus honey. No specific compounds could be detected in Robinia and Lavandula honeys. Abscisic acid, formerly reported as a characteristic compound of Calluna honey (Ferrers *et al.* 1994), was also detected in Brassica, Tilia and Robinia honeys in similar concentrations. Erica honey was characterized by the presence of hydroxybenzoic acids, syringic acids, o-coumaric acids and ellagic acids. The gallic acid dimer

ellagic acid was confirmed as marker of Calluna honey. These findings agree with similar results found in heather honeys from *Erica* and *Calluna* species (Andrade *et al.* 1997). All honey samples contained variable amounts of propolis derived compounds that were not helpful for the determination of botanical origin i. e. the flavanones pinobanksin, pinocembrin; the flavones chrysin, galangin, techtochrysin, apigenin and genkwanin, several quercetin and kaempferol methyl ethers and the caffeic acid esters phenyl-ethyl-caffeate and dimethyl-allyl-caffeate (Tomas-Barberan *et al.* 2001).

The determination of flavonoid patterns is useful for the classification of some, but not all unifloral honeys. A considerable drawback of the flavonoid analysis is the remarkable time consumption of the methods used.

1.6 Sensory analysis

Sensory assessment is routinely used for quality control of honey and for the determination of the botanical origin of honey.

To meet minimum quality requirements according to the legislation, honey must not have an off flavour or taste.

Sensory analysis is a very important tool for the determination of botanical origin of honey. The first attempt for descriptive analysis of unifloral honeys was made by Gonnet and Vache (1985) and was further developed by Piana (1995). The sensory description of unifloral honey has been recently improved by the introduction of reference compounds and flavours (Guyot-Declerck 1998; Guyot-Declerck *et al.* 2001; Guyot-Declerck *et al.* 2002). Sensory analysis is generally performed by trained tasters or expert panels.

Generally organoleptic analysis carried out by an expert, provides a fairly precise evaluation. The advantage of organoleptic analysis is that it recognizes the same characteristics as the ones perceived by the consumer.

However, reliable classification of unifloral honeys can only be achieved by well trained panels. Also, the available sensory methods should be further developed and harmonised in panels of different countries in order to obtain more objective and reproducible tools for honey characterisation.

1.7 Analysis of honey volatiles (without SPME)

Volatile compounds derived from the nectar sources are likely to be responsible for the specific aroma of unifloral honeys. Research on honey volatiles began in the early 1960's. From the very beginning, the determination of volatiles was suggested to allow an objective characterisation and classification of unifloral honeys. Various methods have been used.

1.7.1 Thin-layer chromatography and Parnas-Wagner-micro-Kjeldahl-distillation

Deshusses and Gabbai (1962) described a method for the determination of methyl antranilate (MA) in Citrus honey by extraction with petrol ether and subsequent thin-layer chromatography with a mixture of hexane and ethyl acetate (9:1).

White (1966) adapted a distillation method with the Parnas-Wagner-Micro-Kjeldahl-apparatus for the determination of MA in citrus honey. The distillate was diazotized and coupled with 1-naphtol-2-sulfonic acid. The absorbance was photometrically determined.

1.7.2 Liquid extraction and steam distillation

Ten Hoopen (1963) performed vacuum distillation of honey and derivatisation of the carbonyls before separation by GC. In a next step, gas-stream distillation with hydrogen (steamdistillation) was performed at 70°C, respectively 95°C with subsequent concentration of the distillate by ether extraction. About 120 components were detected. About 60 compounds were identified belonging to the groups of alifatic and aromatic alcohols, aldehydes, ketones, esters and acids. The different unifloral honeys were found to be qualitatively very different from each other.

Blank *et al.* (1990) dissolved honey in borate puffer, extracted the neutral substances with dichloromethane (CH_2Cl_2) and isolated subsequently the

volatile fraction by high vacuum transfer. The extract was analysed by capillary GC/olfactometry, and sensory thresholds for several compounds were defined. The volatile fraction of lime tree honeys of different geographical origin were compared with that of Calluna, Trifolium repens, Robinia and Citrus honeys. It was shown that some of the intense aroma compounds are present in all honeys. Characteristic compounds were found for lime tree honey, and some of the substances were detected in lime tree flowers as well. Differences in the quantitative distribution of aroma compounds between lime tree honeys from different geographical origins were observed.

In 1995 solvent extraction and subsequent steam distillation with simultaneous solvent extraction (Likens-Nickerson methodology) was tested (Bouseta and Collin 1995). This method was applied for the identification of volatile marker substances in Castanea, Tilia and Calluna honeys. The volatile composition of nine additional unifloral honeys from various countries (i. e. Abies, Lavandula, Citrus, Brassica, Robinia, Rosmarinus, Helianthus and Trifolium repens) was studied as well. Several marker compounds were identified (Guyot *et al.* 1998; 1999)

With the techniques described above, a large number of volatiles of many unifloral honeys have been described. The methods are useful for thorough research in the field of honey volatiles. Because of the considerable time consumption, none of them is suitable for the routine determination of botanical origin.

1.7.3 Headspace extraction

Dynamic headspace extraction coupled with a GC-MS system was introduced by Bouseta *et al.* (1992). A solution of honey in water (5 g/15 ml) was directly purged with nitrogen at 70°C to a metal capillary cold trap. The qualitative and quantitative composition of the honey volatile fraction of various unifloral honeys was found to be different. The results allowed a classification of Lavandula, Abies, Eucalyptus, Taraxacum and Brassica honeys. It was stated that it would be necessary to extract less volatile

flavour compounds for differentiation between Castanea, Citrus, Tilia and Robinia honeys.

In a recent study, the headspace of a honey was purged at only 45°C for 30 minutes. 110 compounds were identified by GC-MS, and several markers for the determination of botanical and geographical origin were proposed. The authors admit that the results should be confirmed with a larger set of samples (Radovic *et al.* 2001).

Dynamic headspace extraction of honey volatiles seems to be a promising approach for the determination of botanical origin of honey, also suitable for routine analysis. However, extraction conditions should be further optimised in order to extract more semi-volatiles. In laboratories dealing with honey quality control, purge and trap systems are very rare, and thus it is rather unlikely that this technique will be applied in the near future in honey routine control.

Analysis of Volatiles: Conclusions

In the past decades, over 400 different volatile compounds have been identified. For several unifloral honeys, characteristic marker compounds have been proposed, but no quantitative classification limits have been set. With the exception of hesperitine and methyl anthranilate for Citrus honey, no marker compounds are currently used in routine analysis for the determination of unifloral honeys. The marker status of many compounds is questionable because in most studies only a few unifloral honeys have been analysed. Indeed, it is known, that the composition of unifloral honeys, and probably also that of the volatiles, varies greatly. Many compounds found in unifloral honeys are present in all honeys and are therefore not useful as marker e.g. phenylacetaldehyde, higher chain length hydrocarbons and fatty acids all derived from beeswax. For the establishment of compositional criteria, unifloral honeys should be carefully selected on the basis of sensory, melissopalynological and physico-chemical parameters.

1.8 General conclusions on determination of botanical origin

In Annex II of the EU honey directive (EU Council 2002), only very rudimentary criteria for unifloral honeys are given, which do not allow the classification of unifloral honeys. Recently, compilations of analytical parameters for unifloral honeys from different countries have been published. The best example is the description of the main 19 Italian unifloral honeys by physico-chemical, melissopalynological and sensory analytical data (Persano Oddo *et al.* 1995; Persano Oddo *et al.* 2000). The International Honey Commission has gathered routine analytical data from different European countries to cover the main 15 European unifloral honeys. The compilation will be published as a monography (International Honey Commission 2003, Characterisation of European unifloral honeys, Apidologie, in preparation). However, in the present stage of knowledge, a reliable determination can be achieved by a global interpretation of sensory analysis, pollen analysis and physico-chemical data by an analyst with good knowledge of the product. However, the decision on unifloral purity is made by organoleptic evaluation.

Thus, there is need for new methods capable of determining the botanical origin of honey in a reproducible and objective way. The analysis of honey volatiles seems to be the most promising way to achieve an objective characterisation and classification of unifloral honeys. There is a need for development of a fast, reproducible and reasonably priced method for the analysis of honey volatiles. SPME, which has been developed recently, seems to be a promising choice.

2 Solid Phase Microextraction (SPME)

2.1 Introduction

In the late eighties, experiments in laser desorption/gas chromatography were performed by dipping the end of an optical fiber into solvent extract containing the analytes of interest. The fiber was coated with them, and the solvent was removed by evaporation. The fiber tip was introduced in the injection port of a gas chromatograph, and the analytes were desorbed by laser pulses. As the technique developed, it was found that short fibers coated with the analytes can be placed directly in the injection port, where the analytes are desorbed by heat. Polymer coated fibers were developed to improve selectivity of the method. It was shown that this simple and rapid technique enabled a reproducible extraction of the analytes. A device had to be developed to overcome the problems related to the reduced column head pressure, resulting from opening the injector for placing the fiber for desorption. The name SPME originates from the first experiments when the analytes were adsorbed on solid silica fibers. As the later developed, liquid coatings have a solid appearance the name was maintained. The first SPME device was built out of a micro syringe by replacing the thin piston with a stainless steel micro tube. The end of the fused silica fiber was glued into the micro tube. The needle of the syringe served as a protective sheath for the fragile fiber placed inside. When the plunger of the syringe was lowered, the fiber was exposed, and by pulling the plunger, the fiber was withdrawn back into the protective sheath (Pawliszyn 1997).

Since 1993, SPME is produced and marketed by Supelco (Bellefonte USA). In the past years, this technique has become more and more popular and has been applied to many different environmental, clinical, forensic, toxicological, biological, flavour and food applications. Compared to traditional techniques like liquid-liquid extraction, this method presents enormous advantages. SPME is highly sensitive and reproducible. The technique can be applied qualitatively or quantitatively with internal standard

or standard addition methods. SPME is a fast technique as sample preparation steps are reduced or even no more required and analyte preconcentration is done in a single step. In most applications, the compounds of interest can be directly extracted from the sample matrix. This makes the technique environmental friendly as no solvents are required. Another important advantage is that the extraction and desorption process can be automated. SPME is a equilibrium technique, which means that analytes are generally not exhaustively extracted from the sample matrix. The selevtivity is not necessarily a disadvantage. This applies especially when analytes have to be extracted from complex matrices with many interfering compounds. Interfering matrix components are not extracted, and compounds that would otherwise be masked can be identified. The minute design and the portability of the SPME-fiber assembly allows the extraction of compounds from small targets like individual flowers of living plants.

2.2 Theory of SPME

In solid phase microextraction, an equilibration process of analytes between multiple phases takes place. Often the system is complex since an aqueous sample can contain suspended solid particles, which can interact with the analytes of interest. To simplify the system, only three phases will be considered: the fiber coating, the headspace and a homogenous sample matrix such as pure water. The driving force for multiphase equilibrium is the difference between the chemical potential of the analytes in the three phases. Since SPME is a selective equilibrium technique, the recovery of the analytes depends on their partition between the three phases present in the sampling vial. When equilibrium conditions are reached, the mass transfer of analytes into the coating equals the mass transfer of analytes desorbing from it to the headspace. In other words, exposing the fiber for a longer time will not lead to accumulation of more analytes (Pawliszyn 1997).

The total amount of analytes in the system does not change during extraction. Their distribution between the three phases can be described by the following equation:

$$C_0 V_s = C_f^\infty V_f + C_h^\infty V_h + C_s^\infty V_s \qquad (1)$$

There is:

C_0 = initial concentration of the analyte in the matrix

$C_f^\infty, C_h^\infty, C_s^\infty$ = equilibrium concentrations of the analyte in the fiber coating (f), headspace (h) and the sample matrix (s), respectively

V_f, V_h, V_s = volumes of the fiber coating, headspace and sample matrix, respectively

The partition coefficients between fiber coating and headspace (K_{fh}) and between headspace and sample matrix (K_{hs}) can be defined as follows:

$$K_{fh} = \frac{C_f^\infty}{C_h^\infty} \;;\; K_{hs} = \frac{C_h^\infty}{C_s^\infty}$$

The mass of analyte absorbed by the coating, $n = C_f^\infty V_f$, can also be expressed by the formula:

$$n = \frac{K_{fh} K_{hs} V_f C_0 V_s}{K_{fh} K_{hs} V_f + K_{hs} V_h + V_s} \qquad (2)$$

It can be derived from equation 2 that under equilibrium conditions the amount of analyte extracted by the coating is independent of the location of the fiber in the system. The analyte may be adsorbed in the headspace or directly in the sample as long as the volumes of the coating, headspace and sample are kept constant.

After equilibrium is attained, the concentration of the analytes in the fiber coating should be the same whether the fiber is immersed in the liquid or in the headspace (Zhang and Pawliszyn 1993).

SPME combines the extraction and concentration of the analytes in a single step and allows a direct transfer of the compounds of interest to desorption in an analytical instrument.

2.3 SPME practice

2.3.1 Fiber assembly

A commercial SPME fiber assembly consists of a (approx. 6 cm long) hollow stainless steel needle that serves as a protective sheath of the fragile fiber placed inside. The upper end is sealed with a septum mounted on a ferrule to prevent carrier gas leaking through the septum piercing needle. The fiber (1 cm long, 0.11 mm diameter) itself is made of fused silica and is coated with an adsorbent polymer. It is attached to the end of a thin metal rod that has a threaded plastic hub on its upper end. This hub can be screwed on the plunger of a syringe like device, called SPME fiber holder (see figure 1). The holder protects the fiber assembly, allows a simple adjustment of the length of the penetrating needle and enables easy exposure and retraction of the fiber (Shirey 1999).

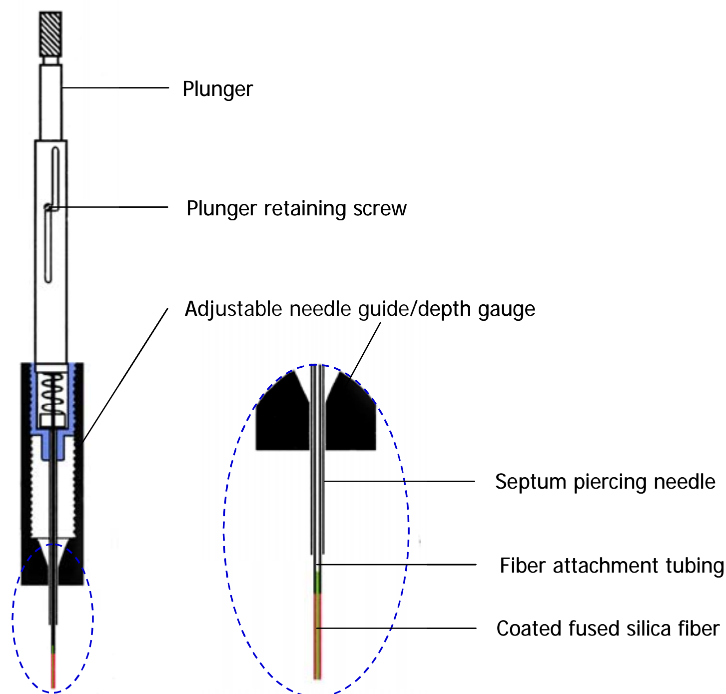

FIGURE 1. Design of a commercial SPME fiber holder (adapted from Zhang and Pawliszyn 1993)

2.3.2 Sampling process

The SPME technique consists of an adsorption and a desorption step. During the first step, the volatile analytes partition between the fiber coating and the sample matrix until equilibrium is attained (see figure 2). In the second step, the analytes are desorbed from the fiber coating in the injection port of an analytical instrument.

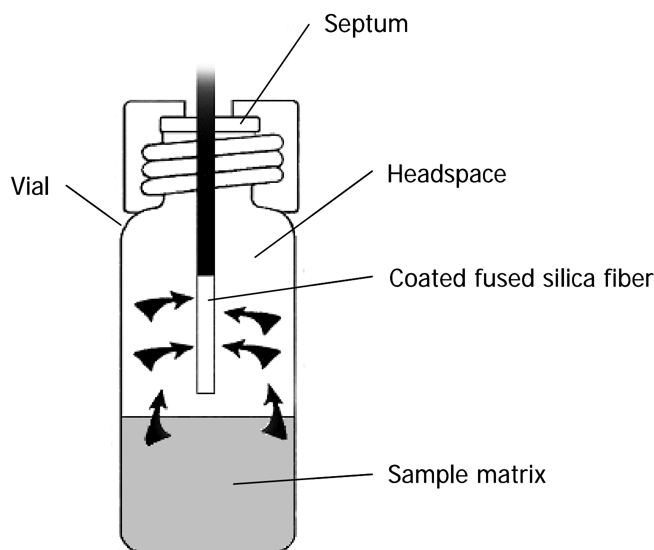

FIGURE 2. Headspace SPME Process (adapted from Scheppers Wercinski 1999)

The sample containing the analytes of interest can be gaseous, liquid or solid. Generally, it is placed in a vial, tightly closed with a septum. For extraction of the analytes, the septum is punctured with the protective needle of the fiber assembly. The fiber is lowered into the liquid or into the headspace of the sample by pushing the plunger off the fiber holder. The analytes begin immediately to migrate between the three phases until equilibrium is reached. In other words, they partition between the sample, the headspace and the fiber coating. After a certain adsorbtion time, the fiber is retracted into the needle and pulled out of the sample vial. The SPME-device is immediately transferred to a GC or HPLC-injector. For GC separation, the extracted analytes are thermally desorbed from the fiber coating that is exposed to the heat of the injection port. For HPLC separation, the analytes are desorbed by solvents. After desorption, the fiber coating contains no

more analytes, and it is ready for the extraction of the next sample (Scheppers Wercinski 1999).

2.3.3 Extraction mode

SPME can be used in three different extraction modes: direct extraction, headspace extraction and membrane protected extraction.

2.3.3.1 Direct extraction SPME

For direct extraction, the coated fiber is directly immersed into the sample matrix that may be either liquid or gaseous. The analytes are directly transported from the matrix to the coating. In liquid samples, stirring is used to enhance diffusion of the analytes and thus to reduce extraction time.

For gaseous samples, no stirring is required as the natural convection of gas is enough for fast equilibration. For compounds with very low volatility, liquid sampling is often preferable.

2.3.3.2 Headspace SPME

Headspace extraction is applied for volatile analytes and samples containing components that may damage or contaminate the fiber i.e. soil particles, sugars or proteins. In order to ensure extraction by the coating polymer, the analytes have to move from the sample matrix to the headspace and from there to the adsorbent layer. Volatile analytes are extracted faster than semi-volatiles since their concentration in the headspace is higher resulting from a faster diffusion through the headspace. The analytes are first adsorbed from the headspace and then from the matrix. An advantage of the headspace mode is that the sample matrix can be modified to favour the extraction of the analytes of interest. This can be done by adjusting the pH of the sample or by salt addition. Equilibration times for volatiles are much shorter in headspace extraction mode than in direct extraction mode.

2.3.3.3 Membrane protected SPME

Membrane protected SPME is suited for the extraction of analytes that are not volatile enough to be extracted by headspace extraction or are present in too dirty matrixes to be extracted by direct immersion. The use of the membrane enhances the selectivity of the extraction process, but slows down its velocity compared to the direct immersion mode (Pawlizyn 1997).

2.3.4 Coating

Equation 2 states that the extraction efficiency depends on the distribution constant K_{hs}. The selectivity of the adsorbent for an analyte versus other components is described by this parameter. The distribution constant of a certain compound can change up to two orders of magnitude when switching from non-polar PDMS coating to a polar polyacrylate film. The adsorption selectivity of the coating follows the chromatographic principle “like dissolves like”. In SPME sampling, the affinity of the fiber coating to the target analytes is important because both matrix and fiber coatings are competing for analytes (Pawlizyn 1997).

Method sensitivity depends also on coating volume. Sensitivity can be enhanced by using a thicker coating, which, however, will lead to longer extraction times.

Today, many SPME fibers with different coatings are commercialy available (see table 1). The coatings can be divided into two main categories: liquid or crystalline phases and solid porous phases. The coating stability is determined by its ability to bind to the fiber and by the crosslinking within the polymer. Non-bonded, cross-linked and bonded phases are commercially available. The non-bonded phases do not contain any crosslinking agents and tend to swell in organic solvents. Their thermal stability is inferior to cross-linked or bonded phases. The cross-linked phases are not bonded to the fused silica core. Only the bonded phases are both cross-linked and linked to the supporting fiber. These coatings show the best thermal stability and resistance to solvents. The thicker the phase, the more difficult it becomes to bond the phase. Consequently, the only bonded phase available is a fiber coated with 7µm polydimethylsiloxane (PDMS) (Shirey 1999).

TABLE 1. Commercially available SPME fiber assemblies

Fiber Coating	Use	Application
7 µm polydimethylsiloxane	GC/HPLC	Nonpolar high molecular weight compounds
30 µm polydimethylsiloxane	GC/HPLC	Nonpolar semivolatiles
100 µm polydimethylsiloxane	GC/HPLC	Volatiles
65 µm polydimethylsiloxane/ divinylbenzene	GC	Volatiles, amines and nitroaromatic compounds
60 µm polydimethylsiloxane/ divinylbenzene	HPLC	Amines and polar compounds
50/30 µm divinylbenzene/Carboxen/ polydimethylsiloxane	GC	Flavor compounds (volatiles and semivolatiles)
50/30 µm divinylbenzene/Carboxen/ polydimethylsiloxane*	GC	Odor compounds
50 µm Carbowax/templated resin	HPLC	Surfactants
65 µm Carbowax/divinylbenzene	GC	Alcohols and polar compounds
70 µm Carbowax/divinylbenzene	GC	Alcohols and polar compounds
85 µm polyacrylate	GC/HPLC	Polar semivolatiles
75 µm Carboxen/polydimethylsiloxane	GC	Gases and low molecular weight compounds
85 µm Carboxen/polydimethylsiloxane	GC	Gases and low molecular weight compounds

* lenght of the fiber 2 cm

The liquid phases are represented by polydimethylsiloxane (PDMS), polyacrylate (PA) and Carbowax (CW) coatings. PDMS is a non-polar coating well suited for the extraction of hydrocarbons. Polar fiber coatings like PA and CW are designed to extract polar analytes like phenols and carboxylic acids. Because of the rigidity of PA at room temperature, the migration of the analytes in the coating is slower. This characteristic results in longer extraction times compared to other coatings. PA fibers are relatively solvent resistant and thermally stable, but are susceptible to oxidation at elevated temperatures. Oxidation taking place at elevated temperatures in presence of oxygen, will turn the fiber completely black. This must not be confused with

the browning of the phase when exposed to temperatures above 280°C (Shirey 1999).

Carboxen (CAR) and Divinylbenzene (DVB) coatings belong to the category of porous phases, which are solid at room temperature. The solid porous particles have to be suspended in a liquid phase before coating onto the fiber. Analytes that fit into the pores are physically retained. Phases containing porous material are therefore generally better suited for the extraction of trace level compounds and analytes with low distribution constants. The pore size of DVB particles is primarily mesoporous (20-500 Å) and is thus ideal for trapping C_6-C_{15} compounds, but will also work with larger molecules. A disadvantage of DVB coatings is their fragility and susceptibility to stripping. The pores of the DVB particles are dead-ended in contrast to the Carboxen particles that have throughput. This feature of the DVB pores leads to stronger retention of the analytes and may result in longer desorption times and in carry over of analytes. Carboxen particles show an even distribution of micro, meso and macro pores and are suited to adsorb a broad range of compounds. Very small molecules like nitrogen or oxygen are not retained, but larger ones, for example ethane, can already be adsorbed. In contrast to DVB particles, Carboxen has pores that travel through the whole particle. This feature enables small analytes to be desorbed rapidly (Shirey 1999).

Phases consisting of blends of different phases express features of all components. They can have advantages over single phase coatings, especially when extracting complex analyte mixtures.

2.3.5 Film thickness

A thicker phase means an increase of coating volume, which will enable the retention of more analyte, thus leading to a broader linear extraction range. On the other hand, extraction with thicker coatings requires longer equilibration time. Reducing film thickness of PDMS phases from 100 µm to 30 µm will therefore considerably reduce extraction time without lessening the linear range. As thinner coatings can be desorbed at higher temperatures

compared to thicker films of the same phase, carry-over is somewhat reduced (Shirey 1999).

2.3.6. Coating polarity and porosity

The commercially available liquid coatings that have a polar nature are Carbowax and polyacrylate. The latter coating is said to be very durable. As polyacrylate is solid at room temperature, analyte up take and desorption is relatively slow compared to other coatings. It is well suited for the extraction of polar compounds and phenols. When the extraction ability of different liquid fiber coatings was compared, it was shown that a polyacrylate coating extracted polar compounds at a higher rate than the non polar PDMS coating. A similar correlation was found when the relatively non polar PDMS-DVB coating and the more polar CW-DVB coating were compared. However, all of these coatings extracted only a fraction of the amount that was extracted with a Carboxen-PDMS fiber. This indicates that the contribution from coating porosity is often greater than from polarity. Mixed phase coatings express complementary properties compared to single phase films, enabling the absorption of a broad range of analytes with different chemical characteristics. The film thickness is considerably enhanced when porous particles are suspended in a liquid coating. Porous coatings are able to extract considerably more analytes than non porous ones, especially when the analytes of interest are highly volatile (Shirey 1999).

2.3.7 Temperature

Extraction temperature is very important, especially for the extraction of semi-volatile compounds. Temperature has a great influence on the vapor pressure of the analytes. Extraction temperature is closely related to equilibration time because an increase of temperature results in an increase of Henry's constant and of the K_{hs} diffusion coefficient of the analytes. This will lead to a decrease of the equilibration time and will accelerate the analytical process considerably. High temperature facilitates also the release of analytes from the sample matrix. An adverse effect of higher temperatures is the decrease

of the amount of analyte extracted at equilibrium. This can be explained by the decrease of the K_{fh} distribution constant when temperature is rising. Thus, extraction temperature should be optimised to the highest possible level where acceptable sensitivity can be achieved (Pawliszyn 2000). When high sampling temperatures have to be used, sensitivity can be significantly enhanced by cooling the fiber with liquid CO_2 as shown by Zhang and Pawliszyn (1995).

2.3.8 Matrix modification

2.3.8.1 Salting-out

Salt addition is used to enhance the extraction rate of organics. In headspace SPME, the partition coefficient of a substance between headspace and liquid is changed in favour of an increase of concentration in the gaseous phase. As a result, the analytes are driven into the headspace. The effect depends on salt concentration and on the chemical nature of the analytes. The influence of salt addition has only been determined experimentally. Generally the salting out effect increases with increasing analyte polarity. Saturation with salt can be used to improve sensitivity and also to normalize random salt concentration in natural matrices, but it has a relatively insignificant effect on non-polar compounds (Penton 1997).

2.3.8.2 pH

Adjustment of pH can improve method sensitivity for basic or acidic analytes as only non ionic species can be extracted from water. By pH adjustment, weak acids or bases can be converted to their neutral form. To insure that more than 99% of the analytes are in their neutral form, sample pH has to be at least 2 units lower (acids), respectively higher (bases), than the pK_a of the analyte (Pawliszyn 2000).

2.3.8.3 Other sample modifiers

The adsorbtion of analytes on the fiber coating necessitates a release of the compounds from the matrix. For headspace SPME, the addition of modifiers can increase the release of target analytes from the matrix. By adding 5-15% water to the sample matrix, the extraction of BTEX-compounds from clay matrix could be significantly enhanced (Zhang and Pawlizyn 1995). If samples contain more water, and if extraction temperatures above 100°C are used, the substantial pressure increase in the vial may cause leakage and danger for the operator. This problem may be overcome by the use of non-volatile modifier agents. The mechanism of the water addition effect is not properly understood. It is supposed that the polar water molecules adsorb in a solid matrix more strongly and displace less polar molecules from their adsorption sites (Zhang and Pawliszyn 1995).

2.3.9 Agitation

For rapid extraction, some agitation is required to transport the analytes from the sample to the vicinity of the fiber. Agitation can be achieved by stirring with a magnetic stir bar in the vial, by vibrating the fiber, rotating the sample tray or by sonication. Depending on the sample matrix and on the analyte of interest, the best agitation method can be chosen. In order to obtain reproducible results, the agitation conditions have to be kept constant. If a magnetic stirrer is used, the plate has to be thermally isolated from the sample vial, e.g. by a Styrofoam plate.

2.3.9.1 Direct extraction

In a non-agitated liquid sample, absorbtion of analytes is very slow because of their limited diffusion in the solution. Agitation provides a considerably faster extraction by enhancing the convection in the liquid. The sensitivity is improved because of increasing the number of collisions between analytes and fiber coating. This results in a shorter extraction time. Generally the

faster the stirring, the more analyte is transported to the coating and the shorter is the equilibrium time (Scheppers Werzinsky and Pawliszyn 1999).

2.3.9.2 Headspace extraction

Stirring of the sample matrix will accelerate the migration of analytes from the liquid to the gaseous phase by constantly generating a fresh surface. When the analytes reach the gaseous phase, they will be rapidly transported to the fiber by air convection. Stirring the liquid will not affect the transport of analytes into headspace. If the absorption of volatiles from the headspace reduces considerably their concentration, the mass transport from the liquid to the headspace becomes the limiting factor of the extraction process. In this case, stirring can reduce the equilibration time by continuously replenishing the headspace concentration. Stirring has little effect on the absorption of highly volatile compounds with small K_{fh} values because of their large headspace capacity. However, agitation has a large effect on compounds with low volatility and high affinity for the fiber coating. Sonication has been shown to be more effective than stirring for headspace extraction of poly-aromatic hydrocarbons from water (Pawliszyn 1997).

2.3.10 Sample and headspace volume

Sample and headspace volumes should be carefully controlled to attain good precision. Volumes of samples and standard solutions used for calibration should be the same.

In direct SPME, the amount adsorbed by the fiber increases with the rise of sample volume (V_s) until it becomes much larger than the product of the distribution constant (between fiber and sample K_{fs}) and the volume of the coating (coating capacity $K_{fs}V_f << V_s$). From this point onwards, method sensitivity does not further increase with increasing volume. The use of larger sample volumes than the limiting volume maximizes not only sensitivity, but leads also to better precision as the variation in sample volume will not considerably affect the results. As the sample can be added more accurately, the variations of analyte concentration are smaller (Pawlizyn 1997).

In headspace extraction, highest sensitivity can be achieved by minimising the volume of headspace. Highly volatile compounds will accumulate in the headspace. If the headspace is large enough, the concentration of the analytes will be low resulting in a considerable loss of sensitivity. Equation 2 indicates also that the detection limit can be lowered by reducing the headspace volume (V_h), which will result in an increase of the mass adsorbed by the coating (Zhang and Pawliszyn 1993).

Headspace capacity depends rather on its volume than on the headspace to sample volume ratio. If the headspace capacity is large compared to the capacity of the coating, the analyte is adsorbed mainly from the headspace. Since diffusion in the gas phase is fast, equilibrium conditions are established quickly. Thus, dramatic differences in extraction time can result if methods are scaled down from larger vials to smaller ones, even if the headspace to sample volume ratio remains the same. Therefore extraction time has to be determined again when sample or headspace volume are altered (Górecki and Pawliszyn 1997).

However, in practice virtually no increase in sensitivity is observed when polar compounds are extracted from a matrix with similar polarity if the phase ratio is changed. But with non-polar analytes, sensitivity can be enhanced if the volume of the liquid phase is enlarged (Penton 1997).

Vial design can have some effect on sample handling and on extraction precision. Larger vials can be more easily filled with solid or very viscous samples. Samples filled in high and narrow vials may be more difficult to stir uniformly, which can result in prolonged extraction times and/or poor precision (Pawliszyn 1997).

2.3.11 Extraction time

The time of exposure of the fiber coating to the analytes plays an important role for analyte adsorption by the fiber.

Immediately after exposure of coating to the analytes, the coating will begin to adsorb them. When detector response is plotted against extraction time, a rapid increase of mass absorbed by the coating is observed in the beginning

of the plot, followed by a slow levelling off. Equilibrium between sample matrix, headspace and fiber coating is reached when the plot reaches a plateau. Under these conditions, best precision is achieved (Penton 1999). Exposing the coating for longer time will not increase any further the amount of analyte extracted by the coating. The time necessary for the extraction to reach equilibrium depends primarily on mass transport conditions, chemical nature of analyte and temperature.

In headspace SPME of a complex analyte mixture, highly volatile compounds will reach equilibrium within seconds, compounds of lower volatility will display a steady increase with time. In the latter case, waiting for equilibrium to establish may not be reasonable, since it is not essential that equilibrium is attained. But because the equilibration curve rises steeply before reaching a plateau, timing is very critical. When extraction is interrupted before equilibration, the longest possible extraction time should be applied as the relative errors are smaller. In order to obtain maximum reproducibility during short time exposures, it is important to control precisely temperature, convection and extraction time (Louch *et al.* 1992).

2.3.12 Desorption

Once extraction is completed, the analyte containing fiber is transferred to the injection port of a GC or HPLC instrument. During the desorption process, the analytes diffuse from the coating into the stream of carrier gas or fluid. In GC, desorption time depends primarily on analyte volatility, temperature and injector design. Non-polar volatiles are generally desorbed within few seconds, but polar semi-volatiles may need several minutes to be completely desorbed. Injector design affects desorption mainly through the linearity of the flow rate and the temperature profile in it. For efficient desorption, it is important to achieve a high linear flow rate. It ensures that the desorbed analyte is immediately removed from the vicinity of the coating. This prevents an interaction with the coating that may slow down the desorption process. Since no expansion of evaporating solvents takes place during desorption of analytes from the coating, the volume of the inlet liner can be reduced to 0.25 ml. When narrow bore liners and splitless injection

mode are used, much narrower peaks and better resolution can be obtained. Special narrow bore liners with an i.d. of 0.75 mm are recommended. The fiber should be desorbed at the nominal injector temperature. In order to position the fiber correctly, it is important to know the actual temperature profile of the injector (Langenfeld *et al.* 1996). Desorption time can be reduced by increasing the injector temperature. The conditions have to be optimised during method development. For maximum coating durability, desorption time and temperature should be minimized, but analyte carry-over has to be prevented (Pawliszyn 1997).

3 Use of SPME for the determination of honey volatiles

3.1 Method described by Guidotti and Vitali (1998)

The first application of SPME for the determination of volatiles of several Italian unifloral honeys was published by Guidotti and Vitali (1998).

3 g of honey were weighed into a 10 ml crimp top vial and sealed with PTFE-faced silicone septa. The sample was incubated for 30 min in a water bath at 70°C. The headspace was subsequently extracted for 20 min with a 100 µm PDMS coated SPME fiber. The analytes were desorbed for 3 min at 240°C in the injection port of a GC/MS instrument in splitless injection mode. Chromatographic separation was achieved on a SUPELCO PTE 5 column (95% dimethyl- and 5% diphenyl polysiloxane) 30 m; 0.25 mm i.d.; 0.25 µm film thickness.

In the Robinia, Castanea, Eucalyptus, Tilia and Thymus honeys studied, 20 to 30 peaks were detected in each chromatogram. The compounds were identified by comparison of the MS spectra with the NIST library. The differences between the chromatographic patterns and also peak heights of the same type of unifloral honeys were considerable. Many of the compounds were detected in all unifloral honeys, but some of them were found in only one type of honey and were considered as markers.

Comment

In this study, a non polar fiber coating was used to extract compounds from a polar matrix containing primarily polar analytes. This resulted in the extraction of relatively few compounds. As a consequence, many of the extracted compounds are non-polar and may be derived from beeswax, thus being non specific for the honey type. More components could have been extracted with porous or more polar fiber coating. Chromatographic resolution could have been enhanced by using a more polar column. The head-space to sample volume ratio is too large for optimal method sensitivity, and no stirring is used to enhance the mass transport of analytes from the matrix to headspace. As no sample matrix modifier and stirring is used, it will take a considerable time for the honey sample to be homogenized, thus limiting the mass transport of analytes to the headspace. No quantitative results can be obtained by this method, and no information on method precision was given.

3.2 Method described by Verzera et al. (2001)

Another SPME method for the extraction of honey volatiles was developed by Verzera et al. (2001). During method development, the extraction rates of 100 µm PDMS and 65 µm PDMS/DVB coatings were compared. The latter fiber was found to be much better suited for the extraction of volatile compounds. 16 g of honey were diluted with 7 ml of water in a 40 ml crimp top vial. 2 g of NaCl were added to the solution in order to drive polar compounds into the headspace. To avoid artefact formation, the sample was equilibrated at 30°C for 30 min followed by a headspace extraction for 25 min under constant stirring. The fiber was desorbed for 3 min at 220°C. Gas chromatographic separation was carried out on CP-Wax 52 CB column (Chrompack) (polyethylene glycol; 60 m; 0.24 mm; i.d.; 0.25 µm film thickness) with subsequent MS-detection and comparison of the obtained spectra with the NIST library.

Direct extraction of the analytes by dipping the SPME fibers into honey solutions were also tested. A higher amount of analytes could be adsorbed, but because of the sugars present in the sample, the fiber deteriorated after

three extractions, although it was rinsed with slightly acidic water before desorption.

Sampling times higher than 25 minutes resulted in the decrease of the absorption of some components. The relative standard deviation for most of the peaks was about 10% with maximum values inferior to 20% for trace compounds. Unique chromatographic profiles were obtained for each type of unifloral honeys studied (Eucalyptus, Citrus, Hedysarum, and Castanea).

Comment

The method described above is to date the most sophisticated. The modification of the sample matrix with water and sodium chloride is an important optimisation of SPME, leading to higher sensitivity. Nevertheless, there is still potential for improvement. The relatively large sample amount is difficult to stir uniformly thus slowing the mass transport from sample to headspace. The amount of sample could be diminished without loss in sensitivity. The extraction temperature is too low, which results in very poor sensitivity for less volatile compounds. GC-temperature programming is relatively slow, and the column length is unnecessarily long.

3.3 Method described by Piasenzotto et al. (2002)

The method described by Guidotti and Vitali (1998) was adapted by Piasenzotto *et al.* (2002). 85 µm PA, 100 µm PDMS and 75 µm Carboxen/PDMS fiber coatings were compared for the extraction of honey volatiles. The PA coated fiber was found to give the best reproducibility for the ten most significant peaks in Castanea honey. A range of relative standard deviations of peak heights between 8% and 52% was reported. The following adaptations were introduced by Piasenzotto *et al.* (2002):

The water in the honey was adsorbed by adding 0.5 g of sodium sulfate. The sample was incubated in water bath for 30 min at 70°C and twice gently shaken by hand. Subsequently the fiber was exposed for 20 min to the headspace of the sample. GC separation with subsequent MS- or FI-

detection was carried out on a polar INNOWax column (Hewlett Packard) (polyethylene glycol; 30 m; 0.32 mm i.d.; 0.5 μm film thickness).

For quantitative analysis, benzophenone was used as internal standard. The internal standard solution was prepared by dissolving 15 mg of benzophenone in 8 ml of acetone in a 25 ml volumetric flask. The solution was diluted to 25 ml with deionized water. The addition of the internal standard was described as follows: "0.5 μl of benzophenone solution is added by means of a GC syringe, and the sample is gently shaken to homogenize it (Piasenzotto *et al.* 2002)."

Many volatile compounds, reported to be present in honey by previous authors working with other methods, were detected in Eucalyptus, Tilia, Citrus, Taraxacum, Castanea and Thymus honeys sampled with SPME. Most of the compounds were present in several types of unifloral honeys, but some were restricted to one unifloral source and could therefore be used as markers. High variability in concentrations of the compounds was reported for different honey samples of the same unifloral source. This variation is supposedly due either to limitations of SPME-technique or to the complex composition of the matrix. Since different samples of unifloral honeys have similar chromatographic profiles, SPME was judged to be a good method for the determination of botanical origin by specific chromatographic fingerprints.

Comment

As the method is adopted from the previous authors, many of the same limitations can be observed. The amount of sample is relatively small compared to the volume of the vial. Without stirring the mass, transport from sample to headspace is very slow. This is especially true for the present method where the water is absorbed by sodium sulphate, leading to a higher viscosity of the honey. Without addition of water, it also takes a relatively long time for crystallized honey to homogenize in the sample vial. The use of internal standard for quantification is doubtful, as reproducible addition of 0.5 μl is impossible. It is impossible to homogenize the viscous honey sample by gently shaking.

3.4 Method described by Pérez et al. (2002)

Another method for the extraction of honey volatiles by SPME is described by Pérez et al (2002). This publication appeared after carrying out the present work.

During method development, Carboxen/PDMS and PDMS/DVB coated fibers were compared. The first was shown to be able to extract more highly volatile compounds. Headspace sampling was tested at 50, 70 and 85°C. At the highest temperature, the increased presence of HMF was detected, and it was decided to use 70°C in order to avoid artefact formation. Equilibration time (before extraction) of 30 and 60 min was compared and decided to use 1 h as more compounds could be extracted this way.

1 g of honey was weighed into a 4 ml vial. To attain equilibrium, the sample was incubated for 1 h at 70°C under stirring. The headspace of the sample was extracted for 30 minutes with a 85 µm Carboxen/PDMS fiber. The fiber was desorbed for five minutes at 270°C. Gas chromatographic separation was carried out on 5MS column (Hewlett Packard) (95% diphenyl- and 5% dimethylpolysiloxane; 30 m; 0.25 mm i.d.; 0.25 µm film thickness) with subsequent MS-detection. Most of the volatiles were quantified by comparing the peak areas in the sample with ratios found for a known concentration of these compounds in an external standard mixture. Quantification of compounds not present in the external standard mixture was performed in respect to benzyl alcohol assuming a response factor equal to 1. The limit of detection was 0.1 ng of benzyl alcohol per kg.

Citrus, Eucalyptus, Rosmarinus, Lavandula and Thymus honeys were studied, and a total of 35 components was detected. 11 of the compound were found in all honeys investigated. Some of the compounds were proposed to be characteristic for certain unifloral honeys. Canonical and stepwise discriminant analysis was performed to find out the characteristic compounds of each unifloral honey studied. The honeys could be correctly classified by using the canonical functions. It is stated that this technique can be used for the authentification of unifloral honeys although further studies are necessary to confirm the utility of the technique.

Comment

To date, it is the only method that enables a quantitative determination of honey volatiles by SPME. Relatively few and only highly volatile compounds were detected. This may be due to the very low sample volume compared to headspace volume resulting in poor sensitivity. GC resolution could be improved if a more polar column would be used. The time needed for sample equilibration before extraction is very long. Crystallized honey is very difficult to weigh into the sampling vial. Unfortunately, no information on method precision is given. Also, it is doubtful if discriminant analysis can be used for routine classification of honeys, as most probably SPME is not a robust method yielding comparable results in different laboratories.

4 Objectives of the present work

SPME is a selective extraction technique. This means that it is not useful for the determination of all volatile compounds in honey. However, with SPME, a characteristic fingerprint of unifloral honeys can be obtained. Therefore, it seems to be a promising technique for the classification of unifloral honeys on the basis of the profile of the volatile components.

As described above, several methods for the extraction of honey volatiles have already been published. However, it seems that none of the existing methods uses optimal conditions.

The present work had two principal objectives:

1. To develop an optimal SPME method for honey volatiles
2. To use the method for the characterisation of Brassica honeys

Method development comprised following points:

- To test the suitability of different fiber coatings for the extraction of honey volatiles
- Optimisation of extraction conditions in regard to sample amount, matrix modification, agitation conditions, incubation and extraction time as well as extraction temperature
- Determination of the repeatability of the method and the durability of the fiber used

Characterisation of Brassica honeys

The second objective of the work was the application of the developed method for determination of volatiles in *Brassica rapa* L. var. *oleifera* subvar. *annua* (spring turnip rape) and *Brassica napus* L. var. *biennis* (winter rape) honeys from Finland respectively Switzerland by GC-MS/FID. The aim was to evaluate if honeys produced from two different Brassica species can be distinguished on the basis of their volatile composition.

Materials and Methods

5 Materials and methods

5.1 Materials

5.1.1 Equipment for manual SPME

- Fiber Holder for manual sampling (Supelco 57330-U)
- SPME fiber-assemblies :
  - 100 µm polydimethylsiloxane (PDMS) (Supelco 57300-U)
  - 85 µm polyacrylate (PA) (Supelco 57304)
  - 50/30 µm divinylbenzene/Carboxene/polydmetylsiloxane, (DVB/CAR/PDMS), 1 cm (Supelco 57328-U)
- Incubator with temperature regulation, maximum 160°C
- Water bath with temperature regulation (maximum 95°C, compatible with magnetic stirrer plate)
- Magnetic stirrer with digital control and infinitely variable speed (up to 1100 rpm)
- Headspace-vials, 10 ml, 23 x 46 mm (Supelco 2-7198)
- PTFE/silicone septa, 0.25 mm PTFE, 2.5 mm total, (Supelco 27237-U)
- Aluminium crimp-seals (Supelco 27200)
- Cross-shaped PTFE magnetic stirrer bar, 10 mm (Semadeni, Ostermundigen-Bern, Switzerland, Nr. 3066)

5.1.2 GC instrumentation

For the separation of the honey volatiles, a HP 5890 series II gas chromatograph was used. Detection was performed with a flame ionisation detector (FID) or with a HP 5971A mass-selective detector (MSD). The equipment was adapted to analysis with SPME.

The split/splitless inlet was equipped with a narrow bore liner specially developed for rapid desorption of analytes from SPME-fibers (Supelco 26375,01). In order to facilitate needle penetration and to prevent SPME fiber, assembly damage pre-drilled inlet septa were used (Supelco 23167).

5.1.3 GC-software and calculations

The FID-chromatograms were integrated using the 3365 ChemStation version A.03.34 software (Hewlett Packard). For all calculations, peak heights were used. For GC-MS, HPG1034C Version C.03.00 software (Hewlett Packard) was used. For tentative identification of the volatiles the mass-spectra obtained were compared with the Wiley- and National Institute of Standards and Technology (NIST) databases integrated in the HP-GC-MS software (Hewlett Packard). The mass-spectrometry data were subsequently re-evaluated using the MassLib Version 8.7 software from MSP Kofel, Koeniz, Switzerland.

Relative retention index

The relative retention index of the components were calculated on the basis of the retention times of the n-alkanes using the formula below:

$$I_x = 100z + 100\frac{(t_R)_x - (t_R)_z}{(t_R)_{z+1} - (t_R)_Z}$$

where:

I_x: Retention index of the component X relative to the homologous n-alkanes

z: Number of C-atoms in the n-alkane eluting before the component x

$(t_R)_x$: Retention time of the component X

$(t_R)_z$: Retention time of the n-alkane eluting before the component x

$(t_R)_{z+1}$: Retention time of the n-alkane eluting after the component x

Statistical analysis

During method development, the statistical significance was evaluated by analysis of variance using STATGRAPHICS Plus version 3.0 software for Windows (Statistical Graphics Corp. 1997) in the multiple samples comparison mode).

For the evaluation of differencies between the Brassica honeys, discriminant analysis was performed using SYSTAT Version 9 software for Windows (SPSS Inc., 1998).

5.1.4 Chemicals and standards

5.1.4.1 Chemicals

n-Alkanes C_6, C_8-C_{31} for the determination of retention index

Hexane, octane, decane, undecane, dodecane, tridecane, tetradecane, tentadecane, hexadecane, heptadecane, octadecane, nonadecane, eicosane, heneicosane, docosane, tricosane, tetracosane, pentacosane, hexacosane, heptacosane, octacosane, nonacosane, triacontane and

hentriacontane were all puriss. grade. They were all obtained from Fluka Chemie, Buchs, Switzerland.

Saccharides

D(-)-fructose; D(+)-glucose monohydrate; D(+)-maltose monohydrate and D(+)-melezitose (all puriss. grade) were supplied by Fluka Chemie, Buchs, Switzerland. Saccharose and raffinose (both reinst grade) were provided by Merck, Darmstadt, Germany. D(+)-Turanose, kojibiose, trehalose, isomaltose (all puriss. grade) were supplied by Calbiochem, Luzern, Switzerland. Nigerose, purum was provided by Sigma Chemie, Buchs, Switzerland. Erlose puriss. was provided by Senn Chemicals, Dielsdorf, Switzerland.

Other chemicals

Benzophenone, NaCl and Na_2SO_4, (all reinst grade) were all supplied by Merck, Darmstadt, Germany. Acetone, HPLC grade, was obtained from Fluka, Buchs.

5.1.4.2 Standards

Retention index standard solution.

The solution was prepared as follows:

- Prepare solutions of each n-alkane in n-hexane (10000 mg/l) in separate vials.
- Pipette 50 µl of each n-alkane solution (C_8 to C_{22}) into the standard vial.
- Pipette 150 µl of C_{23} solution into the standard vial.
- Pipette 200 µl of each n-alkane solution from C_{24} to C_{31} into the standard vial.
- Store the 2.7 ml of standard solution in the refrigerator at –20°C.

Saccharide standard

A solution of 70 g/l of glucose and fructose as well as 5 g/l of each of the following saccharides, saccharose, turanose, nigerose, maltose, kojibiose, trehalose, isomaltose, erlose, melezitose and raffinose, was prepared in a mixture of methanol in water (1+3 parts of volume).

5.1.5 Honey samples

For the development of the SPME-method, a Castanea honey from Switzerland was used. This honey is rich in volatiles and was thus judged appropriate for method development.
In order to test the new method for the extraction of volatiles in spring turnip rape honeys (*Brassica rapa* L. var. *oleifera* subvar. *annua*) and winter rape honeys (*Brassica napus* (L.) var. *biennis* Schübler & Martens) from Finland, respectively Switzerland, harvested between 1998 and 2000, were collected. The samples were stored at 0°C prior to analysis. All the honey samples with most of the corresponding routine analytical parameters measured are listed in table 5 (section 7.1.2).

5.2 Methods

5.2.1 Physico-chemical routine analytical methods, pollen analysis and organoleptical analysis

5.2.1.1 Physico-chemical analysis

Moisture, invertase, HMF (photometrical method), electrical conductivity and sugar composition (HPLC-method with RI-detection) were determined according to the harmonised methods of the International Honey Commission (Bogdanov *et al.* 1997).

5.2.1.2 Pollen analysis

Microscopic pollenanalysis was performed according the method DIN 10760, which is an adaptation of the method described by Louveaux *et al.* (1978). The pollengrains were counted until stabilisation of the results was attained (minimum of 500 pollen counted). The relative pollen frequencies of the nectar plants were expressed in percent of the total pollen.

5.2.1.3 Organoleptic analysis

The difference between spring turnip rape and winter rape honeys was studied with a triangle test according standard method DIN 10951. A mixture of each honey type was prepared from the same samples analyzed for the volatiles by GC-FID/MS. The sample amount was 12 g. The honey samples were presented in light blue coloured plastic jars (30 ml) with a tight lid. The samples were labeled with randomized codes with four digits and presented to the assessors at room temperature (22°C). The sensoric panel, accredited according to EN 45001, consisted of 14 professional acessors.

5.2.2 SPME procedure described by Piasenzotto et al. (2002)

This procedure was used for the choice of the GC-column and the SPME-fiber in the initial part of this work.

5.2.2.1 Preparation before extraction

The vials, septa and Na_2SO_4 were baked over night at 160°C and stored in an exsiccator prior to extraction.

5.2.2.2 Internal standard

For quantitative analysis, benzophenone was used as internal standard. 15 mg of benzophenone were diluted in 8 ml of acetone in a 25 ml volumetric flask that was subsequently filled to mark with ultrapure Milli-Q-water.

5.2.2.3 Extraction procedure

0.5 g of sodium sulphate and 3.00 g of honey were weighed into the 10 ml vial. 0,5 µl of internal standard solution (benzophenone) were added with a microliter GC-syringe. The vial was immediately closed with a PTFE-coated septum by means of a crimp seal. The vial was transferred to a water bath and incubated at 70°C for 30 minutes. During the incubation, the sample was twice gently shaken by hand. For the extraction of the volatiles, a 85 µm

polyacrylate coated fiber was used. It was exposed to the headspace for 20 minutes. The analytes were desorbed in the GC-injection port for 3 minutes.

5.2.2.4 GC-parameters

GC-column	HP-Innowax (95 % polyethylene glycol) 30 m; 0.32 mm i.d.; film thickness 0.5 µm
Carrier gas	He
Carrier gas flow	1.5 ml at 50°C
Injection mode	Splitless; 4 min.
Injection port temp.	250°C
FID-temperature:	300°C

Oven temperature program:

50°C /4min -> 10 °C/min, -> 230 °C/10 min -> 10 ° C/min -> 250 °C/10 min

5.2.3 SPME procedure of the present work

5.2.3.1 Preparations before analysis

The following preparations were performed before analysis:

- Deposit headspace vials and septa at least two hours prior to use in 2% detergent solution of Deconex 11 universal (Borer Chemie, Zuchwil, Switzerland).
- Rinse the vials and septa carefully with deionised water.
- Place the septa side by side in a petri dish (PTFE-coating facing upwards).
- Bake the vials and septa in an incubator at 160°C for at least 8 hours.

- Remove the vials and septa from the incubator and allow them to cool to room temperature in an exsiccator containing dry silica gel and a bowl with 150 g activated charcoal (for adsorbtion of volatiles).
- Store the vials and septa in the exsiccator prior to use to avoid contamination with chemicals present in the laboratory.

5.2.3.2 Preparation of NaCl used for sample matrix modification

The crystalline NaCl was placed in a platinium crucible and baked in a muffel furnace for 4 hours at 400°C to remove volatile contaminants. The salt was allowed to cool down in the same exsiccator where the clean vials were stored. It was kept there before addition to the samples.

5.2.3.3 Water

Ultrapure Milli-Q-Water was obtained from a Millipore-device and placed in a 500 ml Erlenmeyer flask with a glass tap. The water was boiled in the open flask for at least 20 min and allowed to cool down before use. This procedure allowed the removal of volatile contaminants from the water.

5.2.3.4 Conditioning of the SPME-fibres

The SPME-fibres were conditioned according the following procedure:

- Set the inlet temperature according to the conditioning instructions of the manufacturer.
- Adjust the needle depth on the depth gauge to the setting that exposes the fibre to the hottest part of the injector.
- Purge the inlet constantly with carrier gas while leaving the splitter vent open.
- Condition the 50/30 µm DVB/CAR/PDMS fibre for 4 hours at 270°C.
- If many extraneous peaks are observed in the blank run, conditioning is extended for two hours.

- With the 85 µm PA- and the 50/30 µm DVB/CAR/PDMS fiber, some small extraneous peaks were always observed in the blank runs during the whole live span of the fiber although they were well conditioned. Some of them were later identified as siloxanes that were released from the fiber coating or from epoxy glue used to attach the fiber to the supporting rod.

5.2.3.5 Equipment set-up

- Use a laboratory clamp to hold the sample vial, and mount it the way that the vial can be easily lowered into the water and taken out again.
- Mount a glass bowl large enough for placing the sample vial holder in it on a magnetic stirrer plate
- Fill the bowl with deionised water
- Connect a circulating pump between a thermostatically controlled water bath and the bowl.
- Set the temperature of the circulating water to the desired temperature and control it next to the sample vial with a calibrated thermometer.
- Set the revolution speed of the magnetic stirrer to the desired rate.

5.2.3.6 Basic SPME procedure used in the present work for method development

This procedure used for method development (from section 6.2.1.1 onwards) was compiled on the basis of the SPME-literature on honey volatiles cited and discussed above.

Extraction procedure

An equivalent amount of 4.00 g of honey dry matter were weighed into the 10 ml vial. The water content of the sample was adjusted to 36 g/100 g by addition of boiled ultrapure Milli-Q-water.

1,05 g sodium chloride was weighed into the vial (sample matrix was saturated with salt), and the magnetic stirrer was added. The vial was

immediately closed with a PTFE-coated septum by means of a crimp seal. It was transferred to a water bath and incubated at 70°C for 20 minutes. During incubation and extraction, the sample was continuously stirred at 600 rpm. For the extraction of the volatiles, a 50/30 µm DVB/CAR/PDMS coated fiber was used. It was exposed to the headspace for 30 min. The analytes were desorbed in the GC-injection port for 3 minutes.

GC-parameters

GC-column	HP-Innowax (95% polyethylene glycol) 30 m; 0.25 mm i.d.; film thickness 0.5 µm
Carrier gas	He
Carrier gas flow	1.5 ml at 50°C
Injection mode	Splitless, 4 min.
Injection port temp.	270°C
FID-temperature	300°C

Oven temperature program:

50°C /4min -> 10 °C/min, -> 230°C/15 min -> 10° C/min -> 250 °C/10 min

MS-Parameters

Interface temperature	280°C
Ionisation by electrone impact	70 eV
Acquisition mode	scan (TIC)
Scans/sec.	1,6
Mass Range	26-300 amu
MS temperature	185°C
Tuning	Autotune
Electrone multiplier voltage:	~ 1929 V

5.2.3.7 Extraction and desorbtion of alkanes (retention index)

- Pipette 5 ml boiled Milli-Q-Water into a baked sample vial containing the magnetic stirrer.
- Pipette 10 µl of the retention index standard solution into the sample vial.
- Closed the vial immediately with a septum and incubate it for 10 min at 95°C under constant stirring.
- The SPME-fibre is exposed to the headspace for 60 min.
- When the extraction is completed, the fibre is transferred to the injection port and desorbed under the same conditions as the honey volatiles.

Results and Discussion

6 Method development

6.1 Evaluation of GC-Column and SPME-fiber

6.1.1 Evaluation of GC-column

Extraction and desorption of the honey volatiles was carried out according to the method described by Piasenzotto *et al.* (2002; see 5.2.2), and the separation efficacy of two columns were compared (for GC-settings see 5.2.2.4).

1. A non-polar DB-1 column (J & W Scientific; coating: 100% poly-dimethylsiloxane) length 30 m; i.d. 0.25 mm; film thickness 0.25 µm
2. A polar HP-INNOWax column from (Agilent Technologies; coating 95% polyethylene glycol) 30 m; i.d. 0.25 mm; film thickness 0.5 µm

The separation of the majority of the analytes took about 22 min on the DB-1 column, and no column bleeding was observed at higher oven temperatures. The separation on the INNOWax column took about 34 min, and some column bleeding at temperatures above 210°C was detected, but it remained at acceptable level. Relatively poor separation with many overlapping peaks were observed with the DB-1 column. A better resolution was achieved with the INNOWax column. It was decided to use the polar column for the analysis of the honey volatiles.

6.1.2 Evaluation of the SPME-fibers

Test outline

Three different coatings of 1 cm SPME-fibres were compared in order to evaluate their ability to extract volatiles from the headspace of a Castanea honey sample.

A non-polar, 100 µm PDMS coating, an intermediately-polar 50/30 µm DVB/CAR/PDMS coating with molecular sieve properties and a polar 85 µm PA coated fibre were tested (see 5.1.1).

Extraction was performed with all of the fibers according the procedure described by Piasenzotto *et al.* (2002; see method description 5.2.2). Blank runs were conducted, without honey and internal standard, to check for the presence of interfering compounds originating from fiber coating bleed. The separation of the analytes was achieved on the DB-1 column (30 m; i.d. 0.25 mm; film thickness 0.25 µm) with the GC settings described above (see 5.2.2.4).

Results and discussion

The PDMS coated fiber had the least affinity for the analytes, only 16 peaks with a height of 1000 pA being detected in the chromatogram (see figure 3). In the blank run, no interferences were observed.

The DVB/CAR/PDMS coating extracted much more volatiles, and 65 relatively intense peaks were observed in the chromatogram. In the blank run, seven peaks were detected, this coating causing the highest number of interfering compounds. The characteristic peak with extensive tailing at the beginning of the chromatogram could be identified later as benzyl, originating from the DVB coating. This bleeding could be reduced by fiber conditioning for a longer time. Some of the other interfering peaks present in the blank were identified as siloxanes originating from the glue used to attach the fiber to the supporting metal tube (see figure 4).

The PA coating extracted about the same number of compounds as the DVB/CAR/PDMS-fiber, but less volatile compounds were observed (see figure 5). In the blank run one interfering peak was always present and could not be removed by further conditioning. According to the manufacturer, it originates from the glue used to attach the fiber to the supporting metal tube.

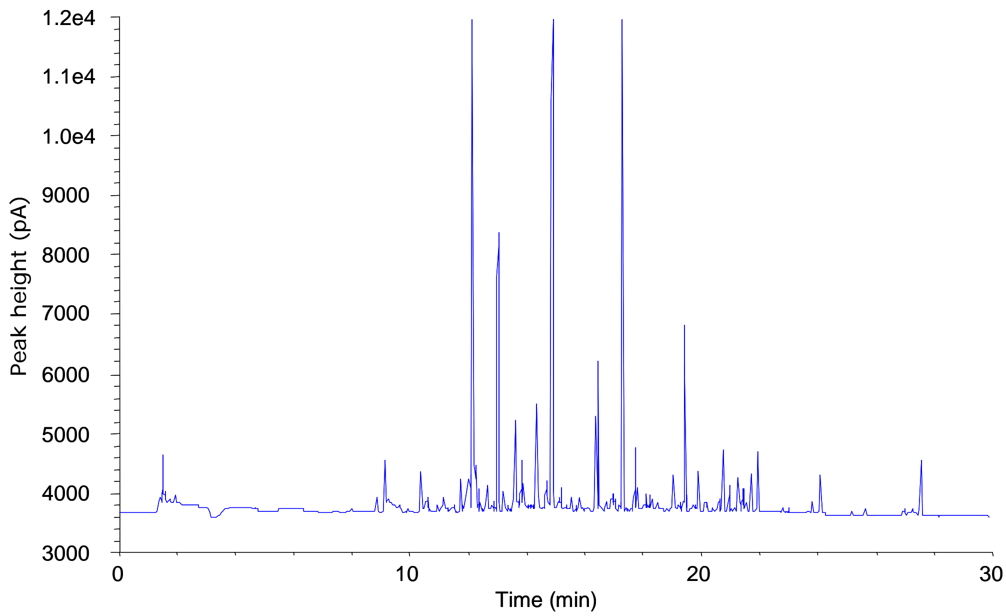

FIGURE 3. Chromatogram of honey volatiles after extraction with 100 µm PDMS fiber

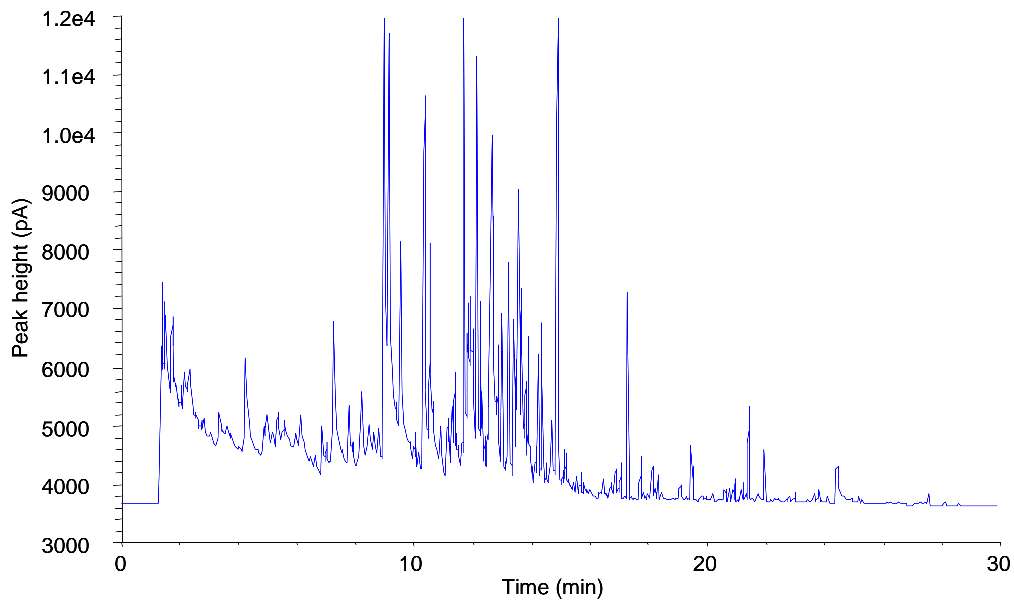

FIGURE 4. Chromatogram of honey volatiles after extraction with 50/30 µm DVB/CAR/PDMS fiber

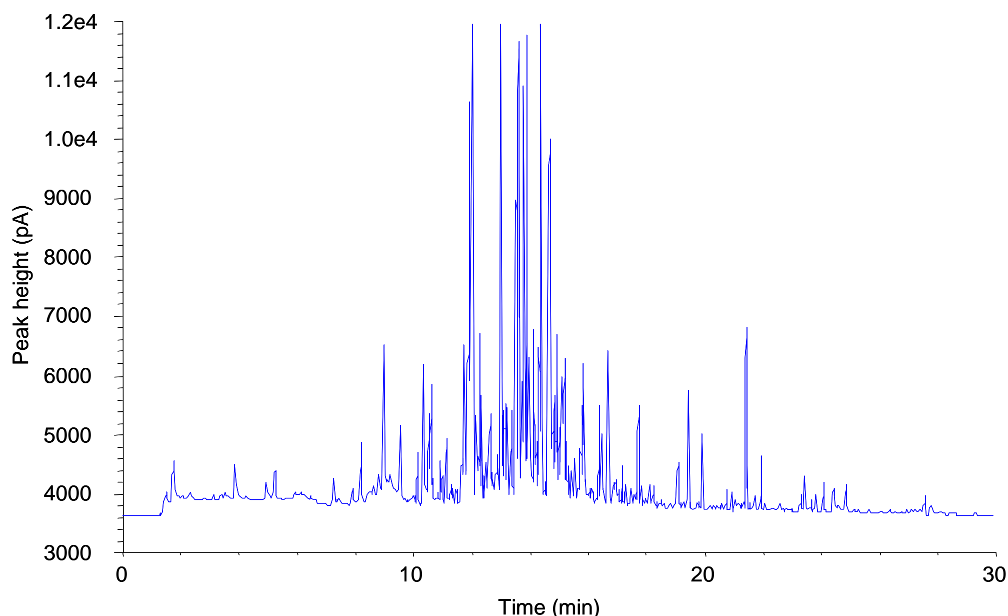

FIGURE 5. Chromatogram of honey volatiles after extraction with 85 µm PA fiber

Despite the presence of some interfering peaks, it was decided to use the DVB/CAR/PDMS-fiber for the extraction of honey volatiles because the broadest range of compounds with the highest concentration could be extracted. This fiber was used in all subsequent tests for method development and extraction of Brassica honey volatiles.

6.2 Optimisation of extraction procedure

In the trials explained below, the method was optimised for different parameters. The trials were conducted in the order given below. The optimal conditions found in a trial were used in the following optimisation step. The direct comparison between different optimisation steps is difficult because the extraction rate was found to be not stable (see section 6.6). For the evaluation of the extraction conditions, 21 major peaks were selected (13 identified compounds and 8 peaks (P1-P8)), which covered different components in respect to volatility and chemical structure. In the figures, the behaviour of some common honey volatiles with different chemical nature to the altered extraction conditions are presented. Because of complex chromatograms and the associated partial low resolution, it was decided to

use the peak heights for all calculations and statistical evaluations presented in this work (Snyder and Kirkland 1979).

6.2.1 Matrix modification

6.2.1.1 Water addition

The water content in honey varies depending on the prevailing climatic conditions, present at the place where the honey is produced. Water content is one of the most varying parameter in honey. According to the EU honey directive (EU Council 2002) and Codex Alimentarius (Codex Alimentarius Commission 2001), the maximum amount for honey is 23 g/100 g. In order to keep sample amount as constant as possible, it was decided to adjust the water content of all honey samples at 23 g/100g.

As different honey samples are heterogeneous in texture, making it difficult to obtain uniform extraction conditions. In addition to this, the high viscosity of honey makes it difficult to stir even at elevated temperatures. On the other hand, efficient stirring enhances the rapid equilibration of the analytes thus increasing analyte evaporation to headspace. Matrix modification by water addition was thus performed in order to lower the viscosity and to allow a fast homogenisation of the sample.

The water addition and the influence of temperature on the time needed to melt the glucose monohydrate crystals was estimated in a preliminary trial using honeys with different texture. A liquid Robinia honey, a very fine crystalline Brassica honey and a Taraxacum honey with very coarse crystals were chosen for the trial. The water content of the three honey samples was determined refractometrically. An equivalent of 4.0 g dry matter of honey were weighed into the 10 ml vial. A cross shaped PTFE-coated magnetic stirrer (diameter 10 mm) was placed in it. The water content of the honey samples was adjusted to the same level (23 g/100 g) by adding ultra pure, boiled water. The vial was sealed with a septum and incubated in a water bath at 50°C, respectively 70°C. The samples were constantly stirred at 600 rpm. Different amounts of water (0.5 ml, 1.0 ml,1.5 ml) were added to the

honey sample, and time was measured until the crystals were completely melted.

The results show that the crystals melted and the sample was homogenized faster with larger amounts of water added and at higher temperatures (see tables 2 and 3). The viscosity of the honey sample was considerably reduced when incubation was performed at 70°C. Thus, this temperature was used during the subsequent trials for method development. As the addition of 1.0 ml of water yielded a honey solution that could be uniformly stirred at a high speed, this was the water addition of choice. The use of more water will decrease the headspace volume and increase the risk of contact between the honey solution and the fibre.

TABLE 2. Time necessary to dissolve all honey at 50°C (values express the mean of two measurements).

Honey type	Amount of water added		
	0.5 ml	1.0 ml	1.5 ml
Robinia	10 min	4 min	3 min
Brassica	20 min	14 min	12 min
Taraxacum	26 min	25 min	20 min

TABLE 3. Time necessary to dissolve all honey at 70°C (values express the mean of two measurements).

Honey type	Amount of water added		
	0.5 ml	1.0 ml	1.5 ml
Brassica	16 min	12 min	10 min
Taraxacum	13 min	10 min	8 min

The possible effect of water addition on method sensitivity due to dilution was studied by extracting the volatiles from honey samples where 0.50 ml respectively 1.00 ml was added to an equivalent of 4 g dry matter of honey. The sample matrix was saturated with sodium chloride and the sample was incubated at 70°C under constant stirring at 600 rpm.

Water addition of 0.5 ml or 1.0 ml seemed not to have a negative influence on the extraction efficacy of honey volatiles.

For eight components, the rise in water content resulted in an increase in extraction efficiency (see appendix 1). A remarkable increase was observed for the components P1 and benzaldehyde. For fife components, the rise of water content resulted in a decline of peak height, benzyl alcohol and benzene ethanol showing the most severe decline. For the remaining 8 components of the total 21 studied, the peak heights were not altered by water addition. Because of the benefits mentioned above and as no dramatic loss in sensitivity was observed, it was decided to adjust the water content to 23.0 g/100g about 1.0 ml of additional water to the sample. This equals in total about a water content of 36.0 g/100 g.

Calculations for the adjustment of water content

The water content was determined refractometrically.

Determination of the sample amount:

$$amount\ of\ honey\ to\ be\ weighed\ (g) = \frac{100 \times 4.00g}{(100 - W)}$$

Adjustment of water content to 36 g/100 g:

$$amount\ of\ water\ to\ be\ added\ (ml) = \frac{100 \times 4.00g}{(100 - 36.0)} - \frac{100 \times 4.00g}{(100 - W)}$$

where is :

4.00 g	=	honey dry matter
36.0	=	water content to be adjusted (g/100 g)
W	=	water content of the honey (g/100 g)

Note

In later experiments not included in this work, the effect of the addition of water to equalize the water content of honeys was tested. No differences were detected with and without equalized water content. From these results, it can be concluded, that an equalisation of water content is not necessary, and that optimal results after addition of 1 ml of water to all honeys can be achieved.

6.2.1.2 Addition of NaCl

Trials were performed to estimate the influence of NaCl addition on the extraction rate of honey volatiles. No salt, 0.53 g NaCl and 1.05 g NaCl were added to the sample matrix. With addition of 1.05 g, the sample was saturated with NaCl. Two replicate extractions were performed for each salt concentration tested.

Most peaks heights increased when NaCl was added to the sample. An increase was recorded for 19 of the 21 components followed during method development (see figure 6 and appendix 2). A steady increase was observed with rising sodium chloride concentration. Without the addition, some compounds like benzoic acid could not be detected at all.

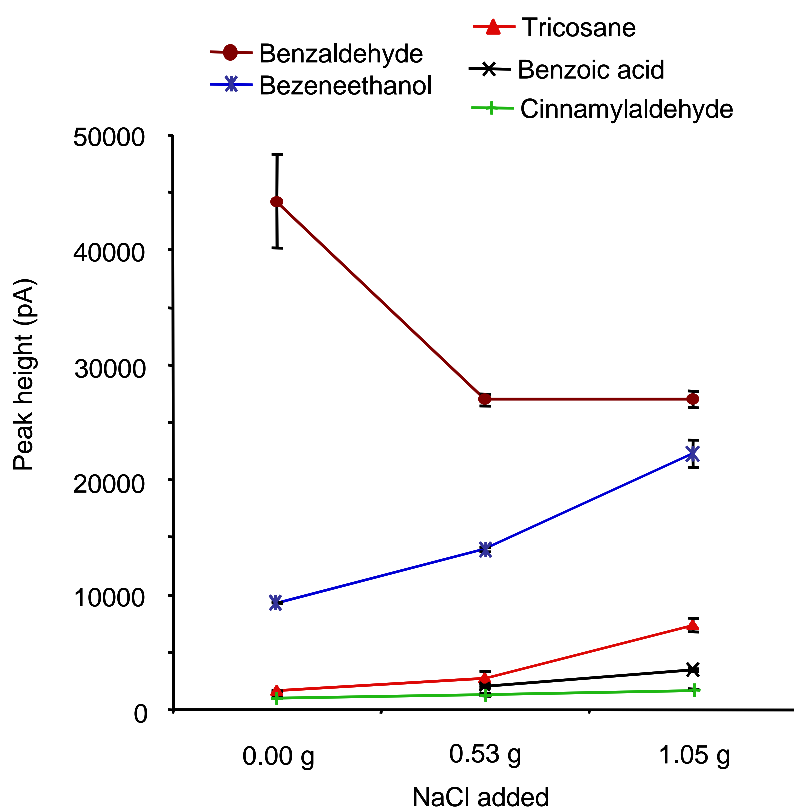

FIGURE 6. Effect of NaCl addition on extraction rate of honey volatiles. Values are means of duplicates, whiskers indicating the range

For four components, the addition of NaCl had an adverse effect. The strongest adverse effects were observed for P1, benzaldehyde and nonanal. The peak height of nonanal showed a steady decrease with rising NaCl

concentration. Benzaldehyde reacted on salt addition by a decrease in peak height, but did not decrease any further when the sample was saturated with salt. Matrix modification with salt seemed to improve repeatability for benzaldehyde. The components where the salt addition had a adverse effect were present in relatively high concentrations. Their decrease will not pose detection problems. As method sensitivity could be considerably enhanced for the majority of the components, it was decided to use a sample matrix saturated with sodium chloride.

6.2.2 Influence of sample amount

For optimum method sensitivity, the headspace to sample volume ratio should be minimized. The effect of sample amount on method sensitivity was studied with 4.00 g and 4.50 g of dry matter. The water content was adjusted in both cases to 36 g/100 g, and the sample matrix was saturated with 1.05 g respectively 1.20 g of sodium chloride and was stirred at 600 rpm. Two replicate extractions were performed for both sample amounts. As there was no increase of absorbed volatiles (see appendix 3), a sample size of 4 g was chosen. The extraction with the larger sample amount was very critical to getting the fiber coating in contact with the sample matrix.

6.2.3 Influence of agitation speed

Stirring enhances the mass transport from sample matrix to headspace by steadily generating a new surface and should be used with viscous samples like honey.

The influence of agitation speed on extraction efficacy was studied with two stirring rates. The sample matrix was stirred during incubation and extraction at a constant speed of 600 rpm, respectively 1100 rpm. Two replicate extractions were performed for both stirring rates.

For 15 of the 21 components studied, the peak heights remained on the same level or showed a slight increase at the stirring rate of 1100 compared to 600 rpm (see figure 7 and appendix 4).The extraction rate rose considerably for the component P1, P3 and P4. The extraction rate diminished for only six compounds, the decrease of benzyl alcohol and benzeneethanol being the most pronounced.

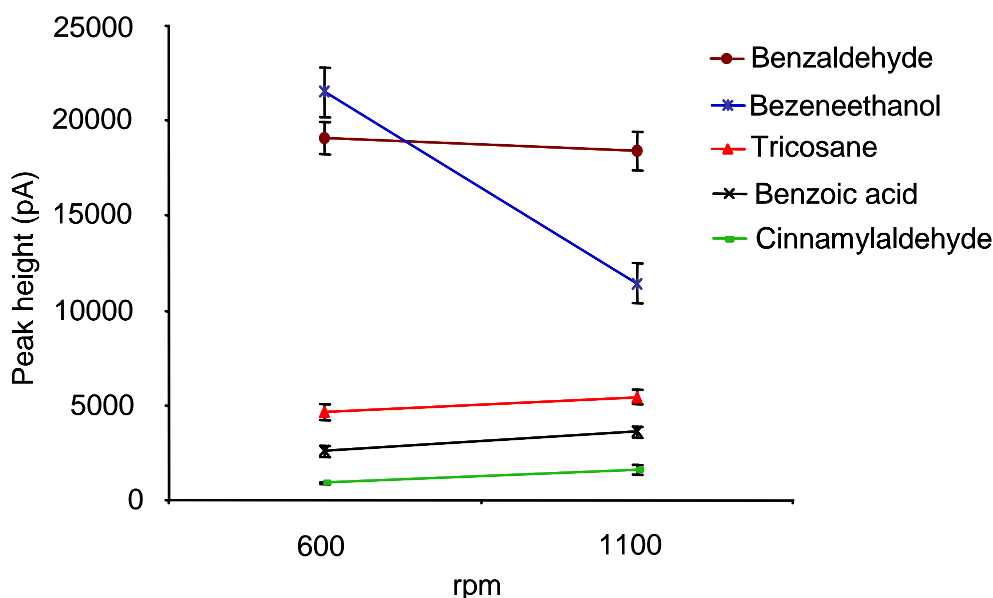

FIGURE 7. Effect of stirring on extraction of honey volatiles. Values are means of duplicated, the whiskers indicating the range

However, in the case of honey volatiles agitation, speed seems not to be very critical as long as the stirring rate is constant and the convection is strong enough to generate a new surface towards the headspace.

6.2.4 Incubation temperature

Two replicate analyses were carried out at 50°C, 60°C and 70°C. Temperature has a great influence on the vapour pressure of the analytes and thus on the extraction rate. This was confirmed by the results. An increase in peak height was recorded for all components with an increase in temperature within the range and extraction time studied (see figure 8 and appendix 5). Some compounds could not be detected at all below 60°C. It was decided to use 70°C for the extraction of honey volatiles as method sensitivity could be substantially enhanced. Higher temperatures than 70°C

were not considered in order to avoid extensive pressure build up in the sample vial and related leaking as well as artefact formation due to Maillard reaction.

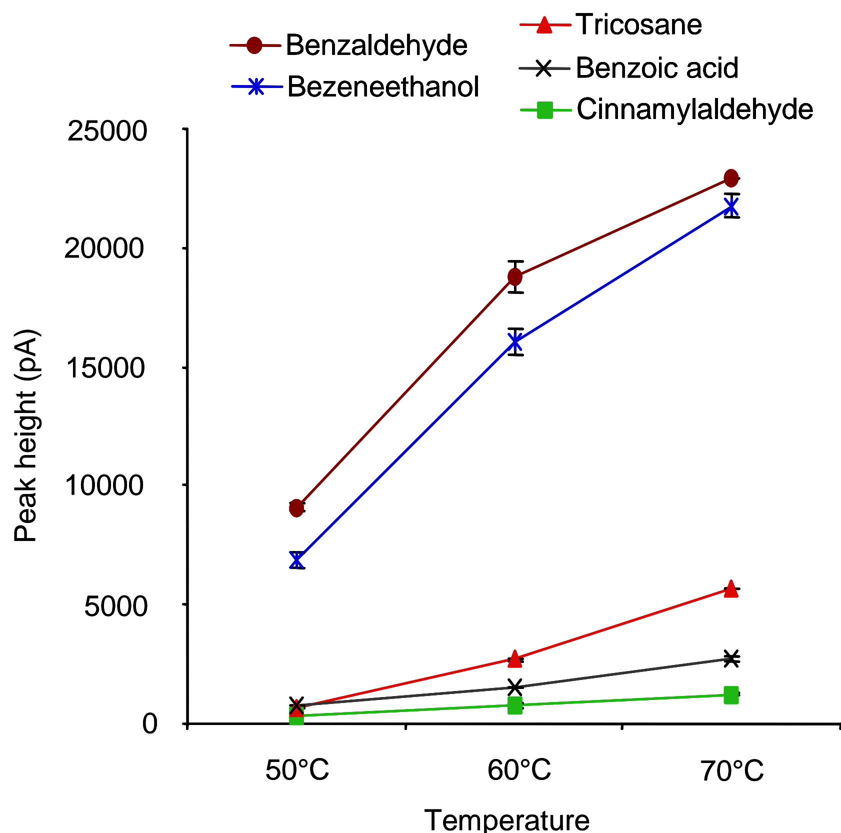

FIGURE 8. Effect of incubation temperature on the extraction rate of honey volatiles. Values are means of duplicates, the whiskers indicating the range

6.2.5 Duration of incubation and extraction

6.2.5.1 Incubation

Analytes equilibrate between sample and headspace during the incubation of the sample before the fiber is introduced. The time needed for equilibration depends on the sample and headspace volumes and on the volatility of the analytes. More volatile compounds will reach equilibrium faster than less volatile ones. In the case of the complex volatile mixture in honey, no ideal conditions can be obtained for all compounds. For highly volatile compounds, equilibrium between coating and headspace may be reached within a few

minutes or even seconds. If the coating is left in the headspace for a longer time some volatiles can be replaced by less volatile compounds that have a higher affinity for the fiber coating. The extraction rate of an individual compound could be further improved by optimisation of incubation and extraction time. If, like in the present study, a broad range of compounds is of interest, a compromise has to be made.

During equilibrium time, glucose crystals melted and honey dissolved in the water added. It needed approximately 20 min until the honey solution reached the temperature of 70°C.

Incubation times of 20 min and 30 min were tested and three replicate analyses were performed for each incubation time.

No significant differences were found between the volatiles, extracted at different incubation times for any of the 22 compounds studied. As the variation in peak height was smaller after an incubation of 30 min. this time was used for the later trials.

6.2.5.2 Extraction

The duration of fiber exposure to the analytes influences the mass of analyte adsorbed by the coating. The chemical equilibrium between sample matrix, headspace and fiber coating is attained when the graph of detector response plotted against extraction time becomes horizontal.

Extraction time was optimised using three different extraction times i.e. 10 min, 35 min and 60 min. Three replicate analyses were carried out for each extraction time studied.

An extraction time of ten minutes yielded for all studied analytes the lowest detector response. The mass of analyte adsorbed could be considerably enhanced for all compounds studied when an extraction time of 35 min was used (see figure 9 and appendix 6). The increase was statistically significant for 16 of the total 22 components. For all the compounds presented in figure 9 the increase from 10 min to 35 min was statistically significant. When the extraction time was prolonged to 60 min the majority of the compounds reached equilibrium. The increases of benzaldehyde and benzeneethanol

(see figure 9) were not significant. For nine of the 22 compounds, a statistically significant increase in peak height could be found between 35 and 60 minutes of extraction. On the other hand, a slight decrease in some highly volatile compound was observed at 60 min (data not shown).

For many compounds, it will take a very long time to reach equilibrium, resulting in increased analysis time. As it is not essential that equilibrium is attained in the SPME process as long as temperature, agitation and extraction time are precisely controlled, it was decided to use 35 min for the extraction of the honey volatiles.

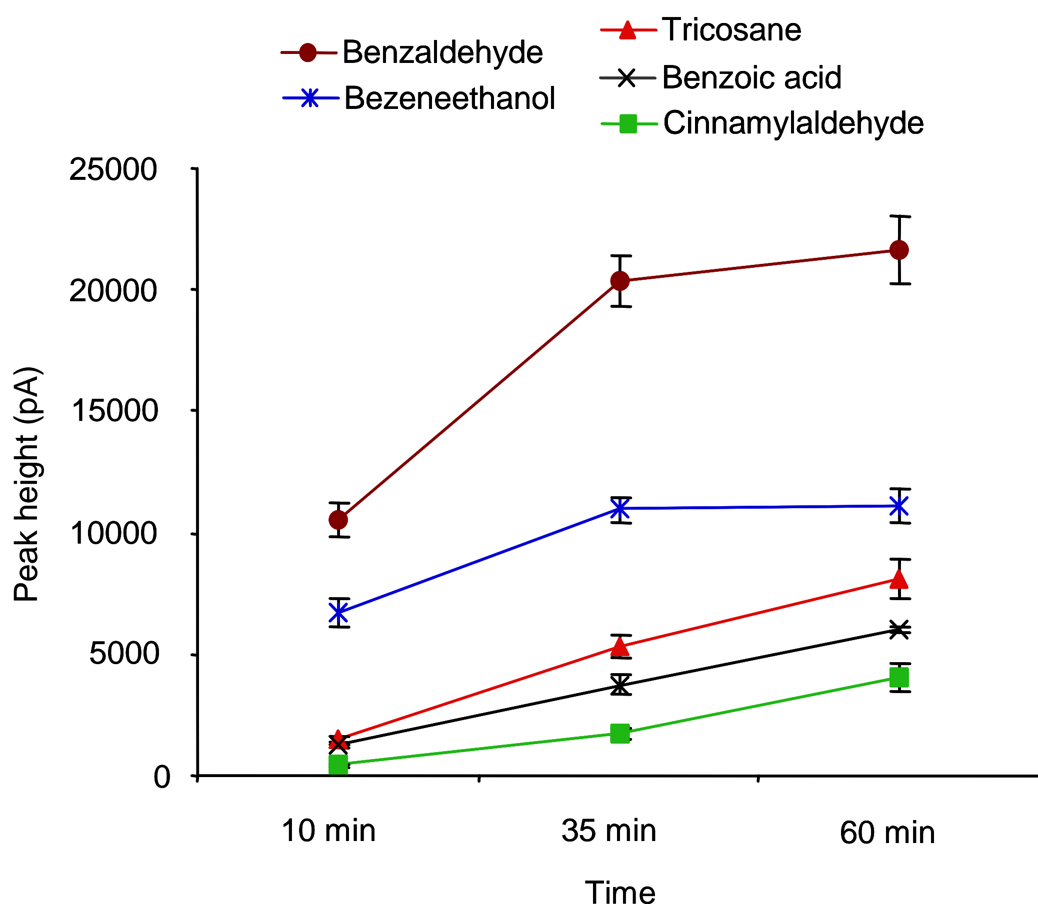

FIGURE 9. Effect of extraction time on the extraction of honey volatiles. The graphs display average values three measurements, the whiskers displaying the standard deviation

6.2.6 Optimisation of analyte desorption

To check for sample carry over after desorption, the fiber was desorbed under the same conditions without the adsorption of new analytes. Initially desorption for 3 minutes at 270°C was found to be enough for the complete desorption of the honey volatiles extracted from chestnut honey. As volatiles from several unifloral honeys were extracted under the same conditions, it was observed that Tilia honey contained the highest concentration of volatiles among the honeys studied. When checked for sample carry over, it was found that after extraction of Tilia honey desorption of all analytes was not completed within three minutes. As desorption temperature was already set to the maximum suitable for the 50/30 µm DVB/CAR/PDMS fiber the desorption time was prolonged. Four, six, eight and ten minutes of desorption were tested. It was found that, for complete desorption, ten minutes were necessary for honeys with a high concentration of volatiles.

6.3 Description of the optimised method for the extraction of honey volatiles by SPME

For determination of dry matter see sections 5.2.1.1 and 6.2.1.1. For materials see section 5.1, and for preparations before analysis and equipment set-up see sections 5.2.3.1 - 5.2.3.5. For extraction of homologous alkanes (retention index) see section 5.2.3.7.

Instruction

- Mix the honey sample carefully by means of a spatula.
- Weigh an equivalent of 4.00 g dry matter of honey with an accuracy of ±0.02 g into a baked 10 ml vial.
- Weigh accurately 1.05 g of NaCl into the vial.
- Adjust the water content of honey sample to 36.0 g/100 g with ultrapure boiled Milli-Q-Water.
- Place the magnetic stirrer into the vial.
- Close the vial by means of a PTFE-coated septa and crimp seal.

- Incubate it for 30 min in a water bath at 70°C (±0,5°C) under constant stirring at 1100 rpm.
- Introduce 50/30 µm DVB/CAR/PDMS/ stableflex fiber into the vial, and expose it for 35 min to the headspace while incubating at 70°C and constantly stirring at 1100 rpm.
- Withdraw fiber from the headspace.
- Adjust the penetrating needle on the depth gauge to 38 mm (setting for HP injector).
- Immediately transfer the fiber to the GC-injector and desorbe it for 10 minutes at 270°C.

GC-Parameters

Injection mode	Splitless, 10 min
Injector port temperature	270°C
FID-temperature	300°C
Carrier gas	Helium
Carrier gas flow	1.5 ml at 50°C

Oven temperature program:

11min, 50°C -> rate 10°C/min, to 230°C, 15 min. -> rate 10° C/min. to 250°C, 10 min.

MS-settings

See section 5.2.3.6

6.4 Structure of the fiber coating

The stableflex DVB/CAR/PDMS coated fiber consists of a 30 µm thick combined carboxen and polydimethylsiloxane layer over which a 50 µm thick divinylbenzene coating is placed. A thorough examination of the fibers showed that at least two of three new fibers coming direcly from the manufacturer show cracks in the divinylbenzene coating. These cracks can be visually detected by the naked eye or at least with the help of a magnifying glass. In order to document the cracks, scanning electron microscope (SEM) photos were taken. Most fibers have cracks at right angles with the length of the fiber but some have longitudinal cracks as well (see figures 10 and 11).

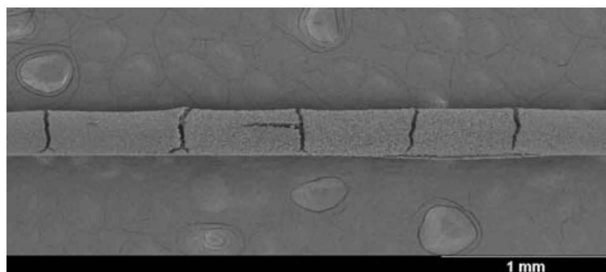

FIGURE 10. SEM photograph of a 50/30 µm DVB/PDMS/CAR stableflex fiber with cracks in the divinylbenzene layer

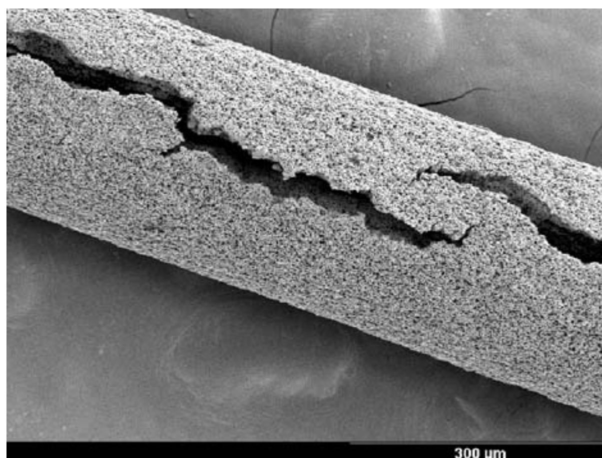

FIGURE 11. SEM photograph of a 50/30 µm DVB/CAR/PDMS stableflex fiber with longitudinal cracks in the DVB-coating

A preliminary examination of other SPME-fibers showed that cracks existed in all the coatings where a porous polymer was used. Fibers coated with liquids or glassy polymers like PDMS or PA had a smooth and even surface without any cracks (see figure 12).

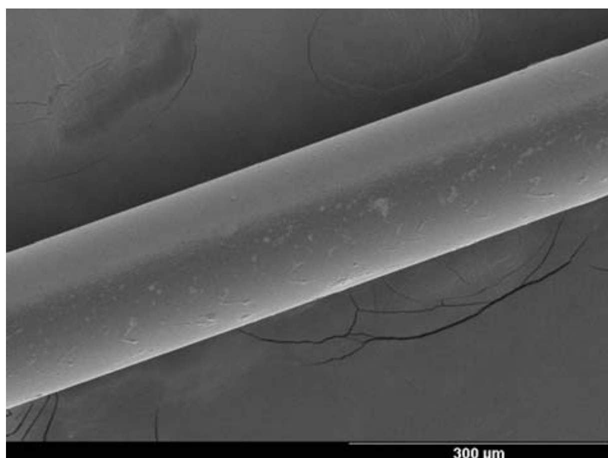

FIGURE 12. SEM photograph of a 85 µm polyacrylate fiber

6.5 Repeatability of the optimised method and the influence of the fiber cracks

Within a day repeatability of the developed method was determined for GC-FID analysis. For 65 components detected, the within a day repeatability for a fiber with a smooth coating (7 replicates) was calculated and are expressed as coefficients of variation (CV) for the individual components

The repeatability was also determined for extractions with a cracked coating (6 replicates) with the same honey sample used for the replicate extractions with the smooth coating.

The CV of the 65 components, extracted by a smooth fiber varied between 4.0 and 18.5%, with a median of 8.6%, that CV of the substances extracted by a cracked fiber varied between 1.8 and 27.3%, with a median of 11.0%. For most compounds method repeatability seems to be slightly poorer with a cracked fiber coating. On the other hand for some compounds like bezoic acid and benzeneacetic acid the repeatability of the method is better with a cracked fiber coating. Generally the repeatability of the method is good and allows a reliable determination of the honey volatiles.

The individual CV of 26 compounds identified by relative retention index and comparison of chromatograms are presented in appendix 7 and for some components in table 4.

When the extraction rate of a smooth and a cracked coating are statistically evaluated, for most of 26 no significant differences are found (see table 4 or appendix 7). But for benzaldehyde, cinnamylaldehyde, 3,5-dimethoxy-benzaldehyde and benzoic acid, the peaks obtained with cracked fibers were significantly higher than with a smooth coating. On the other hand, significantly lower peaks heights were detected with cracked coatings for nonanoic acid and benzyl alcohol.

TABLE 4. Within a day repeatabilities of some honey volatiles extracted with 50/30 µm DVB/CAR/PDMS stableflex fibers with and without cracks in the fiber coating.

*denotes a significant difference in the extraction rate between the smooth and the cracked fiber

Compound	Fibercoating						
	Smooth			Cracked			Sm/Cr
	Mean	STD	CV (%)	Mean	STD	CV (%)	Statistical sig. on 95% level
1-(2-furanyl)-Ethanone	1486	157	10.6	2233	304	13.6	
Benzaldehyde	193322	19366	10.0	279256	46274	16.6	*
Benzene-acetaldehyde	13718	1192	8.7	13693	1342	9.8	
Benzyl alcohol	23508	2522	10.7	11705	1444	12.3	*
Benzeneethanol	27698	2032	7.3	28370	2349	8.3	
Octanoic acid	6512	533	8.2	6058	726	12.0	
Cinnamylaldehyd	5912	401	6.8	7218	1050	14.5	*
n-Tricosane	1713	126	7.4	1291	257	19.9	
Benzoic acid	21774	864	4.0	26846	493	1.8	*
Benzeneacetic acid	1544	93	6.1	1495	39	2.6	

6.6 Durability of the fiber coating

An important quality factor is the number of extractions that can be performed with the fiber. The more robust the fiber, the greater the number of extractions. The initial extraction capability was determined by a 7-fold extraction of a reference winter rape honey with a absolutely smooth fiber coating. Afterwards, other honey samples were extracted and the reference

rape honey was analysed every tenth time until fifty extractions were completed with the same fiber. The experiment was carried out within 4 days.

To evaluate extraction efficacy during the fiber test, the average and standard deviation of the first five extractions were calculated. It was decided that extraction efficacy of the fiber had changed when the peak height lies outside the range of two standard deviations below and above the average.

For some of the compounds like cinnamylaldehyde, extraction efficacy remained relatively stable for the whole duration of the test (see figure13). Only a few compounds like benzyl alcohol showed a relatively steady decrease during subsequent extractions. The majority of the compounds behaved like benzene acetaldehyde. A decrease in peak height was observed during the first three or four extractions, followed by a stabilisation of the extraction efficacy until about 30 extractions. From then onwards, the extraction efficacy decreased considerably. On the other hand, for the most acids like octanoic acid, a dramatic increase in extraction efficacy was observed after 30 extractions (see figure 13). In the previous test where smooth and cracked fibers were tested it was shown that a cracked fiber extracted significantly more benzoic acid than a smooth one. Thus, a reason for the increase in acid adsorbtion may be coating deterioration. On the other hand, there was no difference between smooth and cracked fibers in the adsorbtion of octanoic acid and benzeneacetic acid. For nonanoic acid, the adsorbtion rate was even lower with a cracked coating. No unambiguous explanation for the change in extraction rate after about 30 extractions can be given.

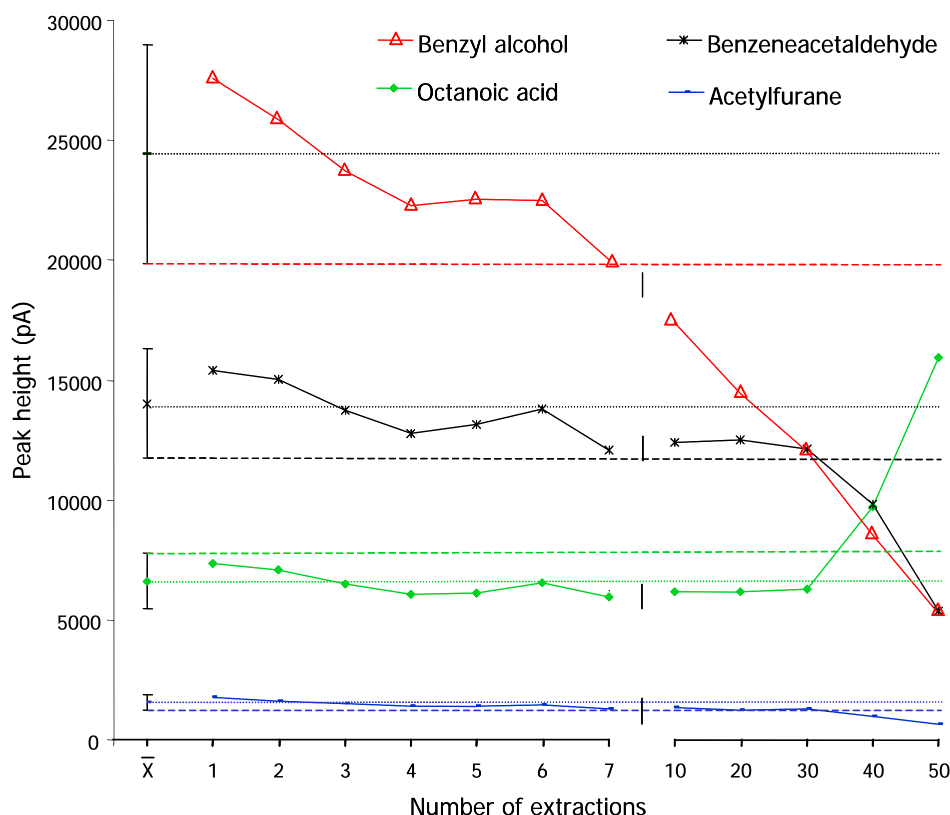

FIGURE 13 Durability of SPME fiber during repeated extraction of honey volatiles

It is known that acids are difficult to determine with GC capillary columns because of the absorption on active sites of the separation phase. On the other hand, experience in the laboratory of the Bee Research Centre showed that similar effects are seen after repeated extraction of wax extracts where the response of many wax constituents increased with increasing number of determinations of wax extracts (S. Bogdanov, personal communication). Thus, the increase of the acid response might also be due to a desactivation effect of honey components on the capillary column, rather than an effect of SPME absorption.

The results show that a perfectly smooth 50/30 µm DVB/CAR/PDMS fiber can be used for about 35 extractions.

The dependence of extraction efficacy on the number of extraction was not tested with fibers with cracks, because optimal extraction conditions were aimed. In the trials described before, we found that the effect of the cracks in the fiber coating on extraction efficacy is not very pronounced. On the other

hand, the durability of some fibers was remarkably reduced as parts of the coating, especially at the tip of the fiber, were stripped of after a few extractions. Thus, I presume that the durability of fibers with cracks is lower, than that of smooth fibers.

7 Determination of volatile compounds of Brassica honey

Up to now, Brassica honey has been regarded as as single unifloral type. It is not further divided into honeys produced different *Brassica*-species. No differencies could be found by routinely used physico-chemical and melissopalynological means between different *Brassica* species.

However, during sensory evaluation of Brassica honeys, it was noticed that not all Brassica honeys (expressing typical physico-chemical and melissopalynological characteristics) show the distinct cabbage like aroma that many consumers dislike. bemerken

When assessing Brassica honeys from Finland and Switzerland by sensory analysis, it was noticed that the finnish Brassica honeys did not show the cabbage like aroma. When the cultivation of oliseed crops in the two countries was studied, it was found that different *Brassica* species are cultivated in the two counties.

Brassica rapa L. var. *oleifera* subvar. *annua* (spring turnip rape) is the main oliseed crop in Finland with an annual cultivation of approximately 63000 hectares covering over 95% of the total oilseed production (Franssila 2000; Antonius-Klemola and Sahramaa 1998).

In Switzerland, the predominant oilseed crop is *Brassica napus* (L.) var. *biennis* Schübler & Martens (winter rape). Annually about 16000 hectares of winter rape are cultivated, other oilseed crops covering an area of only a few hectares (D. Pellet, personal communication).

In the present study, the volatile composition of Brassica honey produced by spring turnip rape and winter rape was studied by Headspace SPME GC-MS.

The winter rape honeys studied were harvested in Switzerland during the seasons 1998, 1999 and 2000. The spring turnip rape honeys analyzed in the present work were harvested in the seasons 1999 and 2000 in Finland.

7.1 Composition criteria

For the analysis of volatiles, the honey samples of the two *Brassica* species were selected on the basis of physico-chemical and melissopalynological criteria currently used for the determination of Brassica honeys (von der Ohe and von der Ohe 1996). Typical winter rape and spring turnip rape honey samples were chosen according to the following criteria.

Composition criteria for Brassica honeys:

Pollenanalysis	>80% Brassica spp. pollen
Electrical conductivity	<0.25 mS/cm
Fructose/Glucose ratio	<1.2
Melezitose	not detected
Sensory analysis	typical for winter rape honey, respectively spring turnip rape honey

7.1.1 Sensory analysis

The individual winter rape and spring turnip rape honeys were tasted and evaluated by trained experts. On this basis, thirteen samples of winter rape honey and twelve samples of spring turnip rape honey were selected for the analysis by Headspace SPME-GC-FID/MS.

In addition, the sensory differencies between rape and turnip rape honeys were studied by means of a triangle test with 14 professional sensory assessors. A significant difference between the two honey types was found on the 99% confidence level. The winter rape honeys expressend a more pronounced cabbage-like aroma than the spring turnip rape honeys.

7.1.2 Results of physico-chemical analysis and pollen analysis

For methods used, see section 5.2.1

TABLE 5. Physico-chemical and pollen analytical results of the parameters measured in the winter rape honeys and spring turnip rape honeys. Abbreviations: FI = Finland, CH = Switzerland, IN = invertase activity expressed as invertase number, HMF = 5-(hydroxymethyl)2-furancarboxaldehyde, F/G = fructose/glucose ratio, Mean = mean of a parameter of all winter rape honeys and spring turnip rape honeys, STD = standard deviation

Year	Country	Physico-chemical parameters measured							
		H_2O	Invertase	HMF	Electrical conductivity	Pollen	Fructose	Glucose	F/G
		g/100g	IN	mg/kg	mS/cm	Brassica sp. %	g/100 g Honig	g/100 g Honig	
1999	FI	14.8	12.5	2.1	0.155	81	38.6	32.5	1.2
1999	FI	15.4	17.9	3.0	0.203	92	37.2	33.6	1.1
1999	FI	15.0	19.4	2.5	0.219	93	36.7	33.0	1.1
2000	FI	18.6	18.4	2.8	0.205	90	34.8	33.3	1.0
1999	FI	17.4	16.5	3.1	0.226	89	37.0	31.9	1.2
2000	FI	15.9	13.3	5.3	0.216	92	35.4	34.0	1.0
1999	FI	15.6	12.1	3.7	0.162	87	36.6	33.4	1.1
1999	FI	16.2	12.5	11.2	0.214	90	36.0	33.2	1.1
1999	FI	16.0	15.4	2.7	0.153	86	37.0	33.3	1.1
2000	FI	16.0	12.7	6.2	0.180	89	33.8	35.9	0.9
2000	FI	17.0	16.4	1.7	0.198	95	35.2	34.9	1.0
2000	FI	18.7	17.8	2.2	0.170	96	35.4	34.6	1.0
2000	FI	16.6	14.8	2.5	0.208	95	35.3	33.3	1.1
1998	CH	15.4	10.9	1.0	0.229	96	39.1	36.3	0.9
1998	CH	15.8	16.3	2.3	0.182	85	37.2	36.8	1.0
1999	CH	15.0	14.6	3.4	0.199	92	37.4	36.1	1.0
1998	CH	15.8	9.2	1.0	0.160	98	36.8	38.0	1.0
2000	CH	16,2	11.7	1.0	0.165	90	35.9	36.0	1.0
1998	CH	15.9	9.4	1.0	0.217	89	35.8	33.9	0.9
1998	CH	15.4	3.2	5.8	0.177	91	34.6	35.2	1.0
1999	CH	16.7	11.7	1.4	0.135	88	36.6	34.3	0.9
2000	CH	17,2	9.5	3.3	0.208	89	37.6	36.4	1.0
2000	CH	15.7	10.6	3.5	0.227	87	35.1	35.2	1.0
1998	CH	16.6	11.3	1.0	0.140	91	37.7	36.0	1.0
1998	CH	15.4	11.6	1.9	0.200	95	36.4	34.8	1.0
1998	CH	16.4	13.1	1.0	0.160	93	37.7	36.5	1.0
Mean		16.1	13.2	2.9	0.189	91	36.4	34.7	1.0
STD		1.0	3.6	2.2	0.029	4	1.3	1.5	0.1
Min.		14.8	3.2	1.0	0.135	81	33.8	31.9	0.9
Max.		18.7	19.4	11.2	0.229	98	39.1	38.0	1.2

7.2. Analysis of spring turnip rape and winter rape honeys by headspace SPME-GC-FID

The volatile compounds of each honey sample were analyzed by headspace SPME-GC/FID according to the method developed and described above (see sction 6.3). A total of 137 different components were detected. Twentyseven compounds could be identified by comparison of the relative retention index and the chromatograms obtained by GC/MS and GC/FID.

Of the total components detected, in winter rape honey, 22 components were found that were not present in spring turnip rape honey. On the other hand, 10 components were detected in spring turnip rape honey only. Most of the components were present in both honey types, and no specific marker compound could be detected for either of the honey types. Statistical analysis was performed on the peak heights of the 27 identified components of both honey types by t-test and Mann-Whitney u-test. A significant difference was found between the two unifloral honeys for 2-furancarboxaldehyde, benz-aldehyde, benzeneacetaldehyde, benzyl alcohol, benzeneethanol and benzeneacetonitrile on the 95% confidence level. On average, the concen-tration of all of these compounds was lower in spring turnip rape honey than in winter rape honey (see appendix 8). However, all compounds except ben-zeneethanol and benzoic acid showed a considerable overlapping of the results. Possibly benzeneethanol and benzoic acid could be used as markers to distinguish between rape and turnip rape honey. The peak height of benzene ethanol in winter rape honey was on average 4.5 times higher than in spring turnip rape. For benzoic acid, the average difference was 1.8 fold in favour of winter rape honey (see figure 14). Two examples of chromatograms of winter rape honey and spring turnip rape honey volatiles are presented in appendix 9.

1,4-dichloro-benzene (PDCB) was detected in 9 of the winter rape honey samples and in none of the turnip rape samples. The reason for this difference is the fact that PDCB is used for the control of *Galleria mellonella* L. (great wax moth) in Switzerland. As all winter rape honey samples come from Switzerland and the compound is not used in Finland,

the PDCB residues are found only in the rape honey samples (see appendix 8).

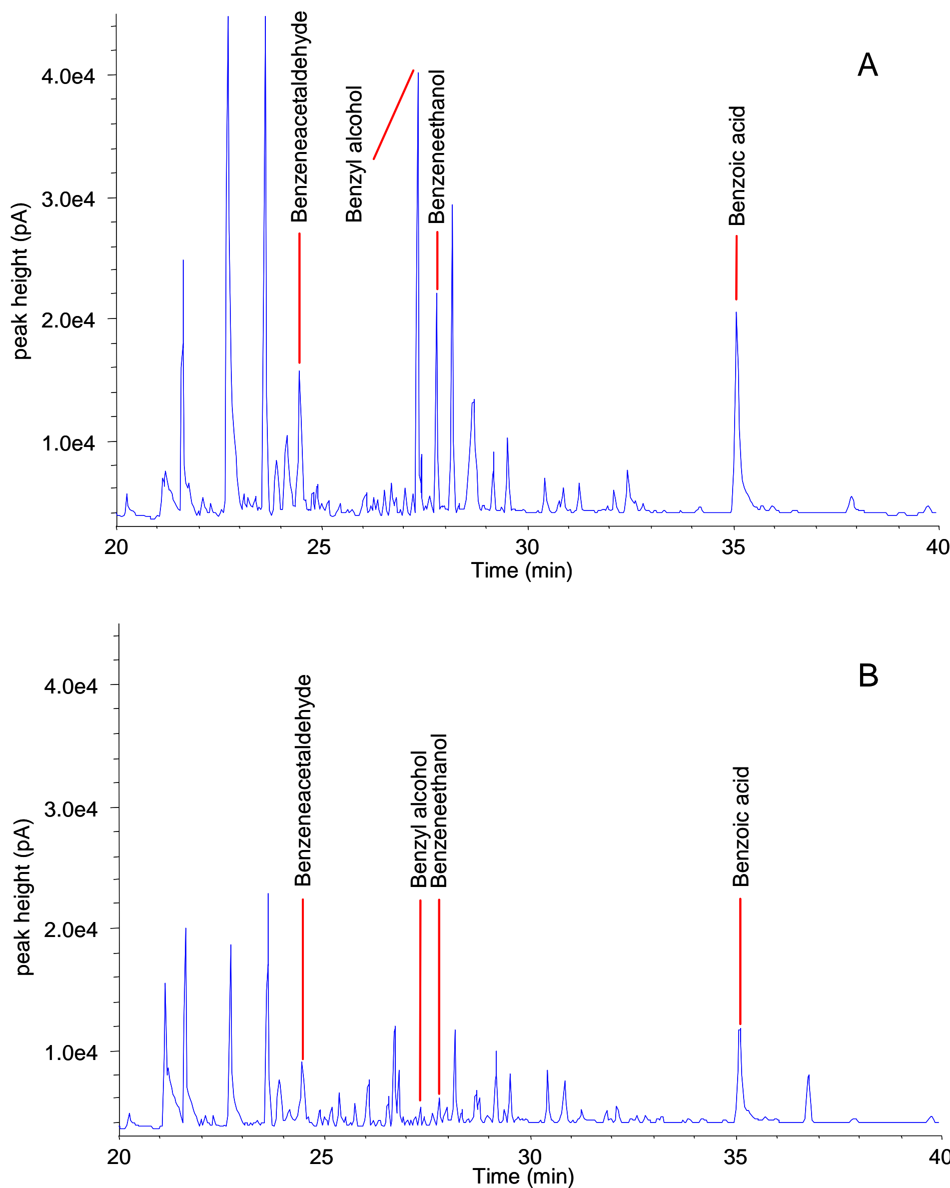

FIGURE 14. Cutout from chromatograms of winter rape (A) and spring turnip rape (B)

If the peak heights of the winter rape and spring turnip rape samples are plotted as box plots for individual components, it becomes evident that the two honey types can be correctly classified by their benzoic acid and/or benzene ethanol concentration (see figure 15). As these compounds are very common volatiles in honey, they may not be useful for the discrimination between Brassica honeys and other unifloral honeys.

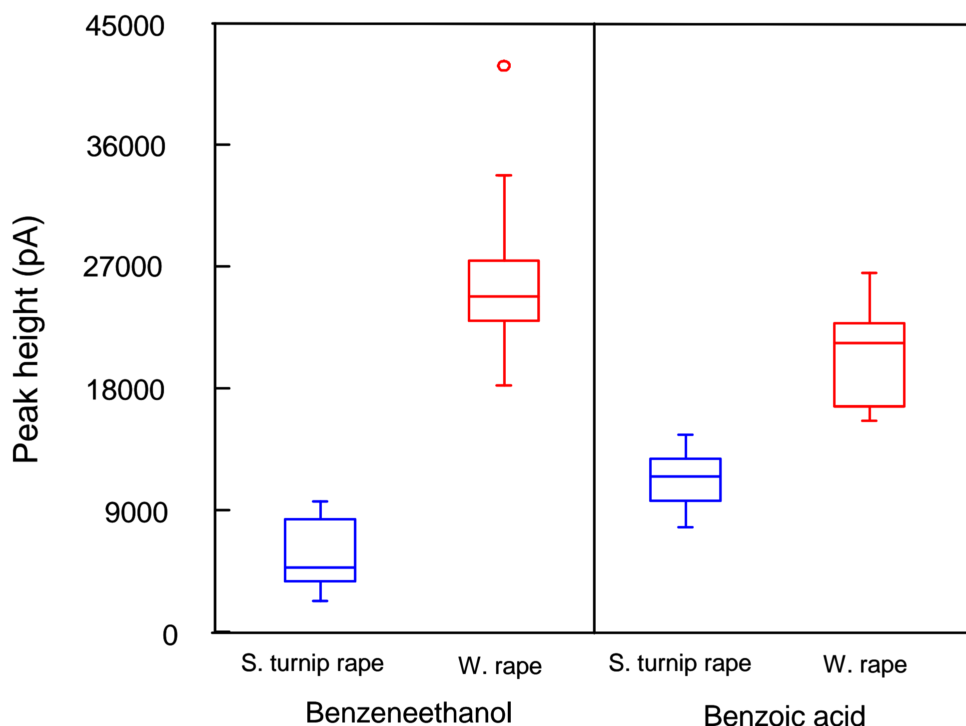

FIGURE 15. Box-plot of peak heights of benzeneethanol and benzoic acid in spring turnip rape (n = 12) and winter rape (n= 13) honeys

7.3 Statistical analysis for the discrimination between winter rape honeys and spring turnip rape honeys

On order to find out mathematically which variables are most useful for discriminating among winter rape honeys and spring turnip rape honeys linear discriminant analysis was performed.

As relatively few honey samples (n=25) were analysed and the number of variables was 137, discriminant analysis had to be performed in several steps. The limit of detection was 200 counts. Only peaks (variables) with an average height of over 1000 counts were considered for the calculation. This limit reduced the number of variables to 51 peaks that were randomly divided into three groups. General linear modelling with stepwise backward removal (alpha-to-enter = 0.010 and alpha-to-remove = 0.01) was performed for each group. Winter rape honey was coded with 1, and spring turnip rape honey was coded with 0. The significant variables of each model were chosen to form a fourth model. This model was based on the peak heights of nonanal,

1,4-dichloro-benzene, 2-furancarboxaldehyde, benzaldehyde, benzene-acetaldehyde, hexanoic acid, benzyl alcohol, 3,5-dimethoxy-benzaldehyde and three unidentified components. The coefficient of determination (R^2) was 97,0%. Discriminant analysis was performed by stepwise backward removal with Alpha-to-Enter = 0.010 and Alpha-to-Remove = 0.01 (for calculations see appendix 10).

When the model was reduced to the three most discriminant peaks, the coefficient of determination was still 88,9%, and the standard error of estimate was 0.182. The two honey types could be clearly discriminated (see figure 16). Whether a honey belongs to turnip rape or rape honey, can be determined by the following linear combination (1) of the heights of three peaks: benzyl alcohol (Peak 86), Peak 84, which is an unidentified component and benzeneacetaldehyde (Peak 58).

Linear combination for discrimination between spring turnip rape and winter rape honeys based on three peak heights:

$$x = -0.61186480 + (0.00001266 \times P58)+(0.00001297 \times P84)+ (0.00058104 \times P86) \quad (1)$$

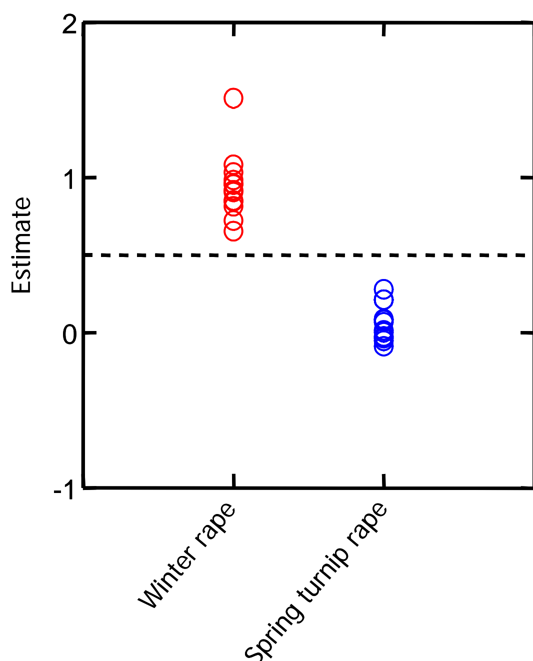

FIGURE 16. Plot of the estimates (x) for the discrimination of winter rape honey and spring turnip rape honey. The limit lies at 0.5

The separation between the two honey types can also be illustrated by plotting the peak heights of benzyl alcohol and peak 84 against each other. The spring turnip rape honey samples exhibit a lower peak heights in both components and can be separated from the winter rape honey samples (see figure 17).

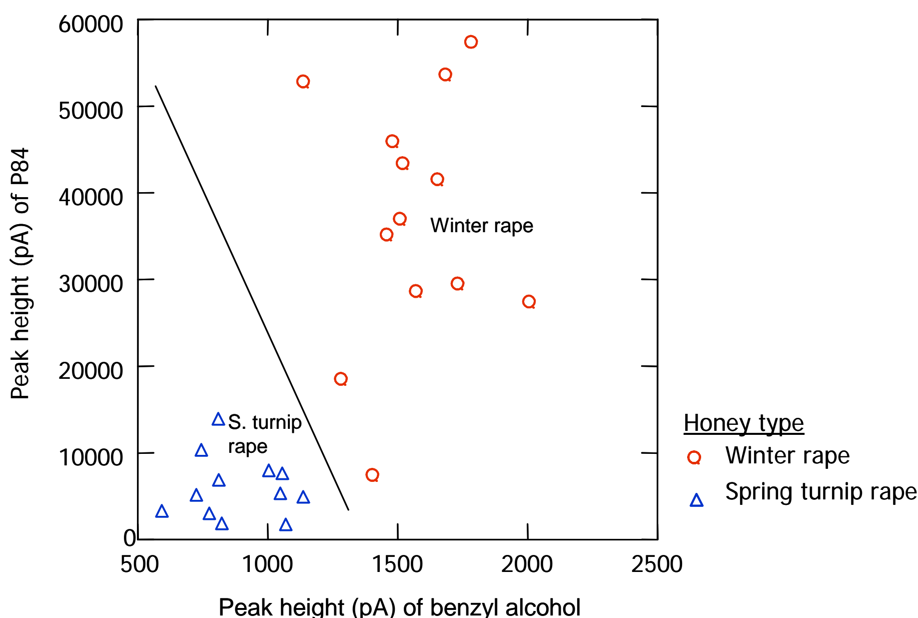

FIGURE 17. Plot of peak heights of peak 84 and peak 86 (benzyl alcohol) and in winter rape honeys and spring turnip rape honeys

When discriminant analysis is performed as a jackknifed classification with the peak heights of benzeneacetaldehyde, benzyl alcohol and peak 84, twelve of the thirteen winter rape honey samples were correctly classified, which equals a correct classification of 92%. 100% spring turnip rape honeys samples were correctly classified. Overall classification was correct in 96% of the samples.

7.4. Volatile compounds of turnip rape honey and rape honey identification by SPME-GC/MS

A pooled sample of winter rape honey was prepared by weighing 25 g of each sample (n = 13) into a glass jar and mixing thoroughly. A pooled spring turnip rape honey sample was prepared in the same manner (n = 12). The

volatiles from winter rape and spring turnip rape honeys were extracted according to the procedure described in section 6.3, for MS-software see section 5.1.3, and the MS-settings were adjusted like described in section 5.2.3.6.

The compounds were tentatively identified by comparison of the mass-spectra with the libraries of Wiley, NIST and MassLib. A number of compounds could be confirmed by injection of reference compounds, and determination of their relative retention index (RI) (for calculations see section 5.1.3) and mass spectra. The compounds identified by GC/MS are listed in table 6.

40 compounds could be tentatively identified by comparison with the library mass spectra. Fifteen of these compounds could be confirmed by comparison of reference spectra obtained with the same system.

The compounds belong to the following groups (number of substances in brackets): aldehydes (13), acids (8), alcohols (8), hydrocarbons (3), nitrogen containing compounds (2), sulphur compounds (2), chlorinated compounds (2), esters (1) and ketones. About 50% of the identified substances were aromates. Twenty-six compounds were detected for the first time in Brassica honey (see table 6).

Of the components identified Octanal, pentadecane, α-terpineol and 5-methyl-2-(1-methylethyl)-phenol were only detected in the spring turnip rape sample. 4-methyl-Phenol and 1,4-dichloro-benzene were only detected in winter rape honey. As mentioned above, the latter compound originates from wax moth control applied in Switzerland. Styrene, cinnamylaldehyde and 3-phenyl-2-propenoic acid could also be detected only in the winter rape honey mixture, but these compounds were also found in individual spring turnip rape samples when the retention indexes were compared of the individual samples analysed by GC-FID. Styrene is most likely derived from plastic honey jars or packages.

TABLE 6. Volatiles identified in winter rape honey and spring turnip rape honey by Headspace SPME-GC-MS. The compounds printed bold were detected for the first time in Brassica honey

Compound				Honey type	
				Spring turnip rape	Winter rape
	CAS reg. no	Relative RI	Identifi-cation*	Relative peak area (%)	Relative peak area (%)
Styrene	100-42-5	1280	a,z		0.05
Octanal	124-13-0	1307	a,x	0.37	
dimethyl-Trisulfide	3658-80-8	1411	a	0.25	0.11
Nonanal	124-19-6	1414	a,b,x	2.17	0.41
2-Furanmethanol, 5-ethenyltetrahydro-a,a,5-trimethyl-,(2R,5R)-rel(9Cl)	34995-77-2	1465	a	1.67	0.92
1,4-dichloro-Benzene	106-46-7	1475	a,b		1.97
2-Furancarboxaldehyde	98-01-1	1495	a,b,x	2.05	1.63
Pentadecane	629-62-9	1500	a	0.71	
Decanal	112-31-2	1522	a,b,x	0.41	0.07
(E,E)-2,4-Heptadienal	4313-03-5	1524	a	0.30	0.07
1-(2-furanyl)-Ethanone	1192-62-7	1539	a,b,x	0.57	0.23
Benzaldehyde	100-52-7	1564	a,b,x,z	13.18	30.53
Dimethyl sulfoxide	67-68-5	1615	a,b	0.18	0.09
Benzeneacetaldehyde	122-78-1	1685	a	1.81	1.62
a-Terpineol	10482-56-1	1725	a	0.12	
2-hydroxy-Benzaldehyde	90-02-8	1729	a	0.24	0.14
3-Pyridinecarboxaldehyde	500-22-1	1755	a	1.28	0.06
3-methyl-Pentanoic acid	105-43-1	1818	a,b	1.55	0.34
2-Hydroxybenzoic acid methyl ester	119-36-8	1830	a	0.59	0.22
Hexanoic acid	142-62-1	1870	a,b	1.51	0.54
Benzyl alcohol	100-51-6	1918	a,x	0.51	0.55
Benzeneethanol	60-12-8	1957	a,x	1.59	2.94
Benzeneacetonitrile	140-29-4	1987	a,b	2.27	1.84
Phenol	108-95-2	2049	a	0.69	0.40
Octanoic acid	124-07-2	2083	a	2.12	1.00
4-methoxy-Benzaldehyde	123-11-5	2093	a	0.80	0.50
Cinnamylaldehyde	104-55-2	2107	a		1.47
4-methyl-Phenol	106-44-5	2124	a		0.13
Nonanoic acid	112-05-0	2188	a,b	2.87	1.13
5-methyl-2-(1-methylethyl)-Phenol	89-83-8	2213	a,v	1.45	
2-Methoxy-4-vinylphenol	7786-61-0	2247	a,b	1.36	0.59
n-Tricosane	638-67-5	2295	a	0.87	0.69
3,5-Dimethoxybenzaldehyde	7311-34-4	2349	a	1.27	5.32
3,5-dimethoxy-Benzoic acid, methyl ester	2150-37-0	2458	a	0.64	0.20
Benzoic acid	65-85-0	2486	a,b,u,y	16.06	14.68
5-(hydroxymethyl)-2-Furancarboxaldehyde	67-47-0	2553	a,b	1.74	0.42
Benzeneacetic acid	103-82-2	2609	a,u,y	0.82	1.01
3-Phenylpropanoic acid	501-52-0	2672	a,b,u	1.29	0.83
3-phenyl-2-Propenoic acid	621-82-9	2899	a		0.06
3,5-Dimethoxy-4-hydroxybenzhydrazide	1443-76-1	2890	a	1.77	2.63

*
a = in the present work by library search
b = in the present work by reference compound
u = Steeg & Montag 1987 by liquid extraction & GC-MS
v = Guyot ey al. 1998, by steam destillation/ solvent extracation GC-MS
x = Radovic et al. 2001 by dynamic headspace GC-MS
y = Speer & Montag 1984 by liquid extraction & GC-MS
z = Bouseta et al. 1992, by dynamic headspace GC-MS

Dimethyl-trisulfide, 5-ethenyltetrahydro-α, α,5-trimethyl-,(2R,5R)-rel (9CI) 2-furanmethanol, (E,E)-2,4-heptadienal, dimethyl sulfoxide, benzeneacet-aldehyde, α-terpineol, 2-hydroxy-benzaldehyde, 3-pyridinecarboxaldehyde, 3-methyl-pentanoic-acid, 2-hydroxybenzoic acid methyl ester, hexanoic acid, benzeneacetonitrile, phenol, octanoic acid, 4-methoxy-benzaldehyde, cinnamylaldehyde, 4-methyl-phenol, nonanoic acid, 2-methoxy-4-vinylphenol, n-tricosane, 3,5-dimethoxybenzaldehyde, 3,5-dimethoxy-benzoic acid methyl ester, 5-(hydroxymethyl)-2-furancarboxaldehyde, 3-phenyl-2-propenoic acid and 3,5-dimethoxy-4-hydroxybenzhydrazide were detected for the first time in Brassica honeys (see table 6). 5-(hydroxymethyl)-2-furancarboxaldehyde (HMF) is a product of Maillard reaction commonly measured for the determination of honey freshness and heat damage. While sample incubation and extraction is performed at 70°C, additional HMF may be formed. It is interesting to note that HMF was not detected before in other works on Brassica honey although the samples were purged at 70°C. (Bouseta *et al.* 1992). This may be due to the shorter exposure to the heat. However, HMF was detected when liquid extraction or solvent extraction and simultaneous steam distillation of honey volatiles was performed (Bicchi *et al.* 1983; Bonaga and Giumanini 1986; To Tan *et al.* 1989; Guyot et al. 1998). Artefact formation caused by the analytical method used is certainly an important issue to discuss (Bicchi *et al.* 1983; Guyot *et al.* 2000). In this study the development of a fast and reproducible method for the discrimination between unifloral honeys was aimed. Thus, avoidance of artefact formation was not of primary interest. However, some furanes seem to be naturally present in honey and are described to be characteristic for Castanea honeys (Bouseta *et al.* 1992; Guyot *et al.* 1998). 2-Furanecarboxaldehyde was also found in Brassica honey before when the volatiles were extracted at only 45°C by a dynamic headspace procedure (Radovic *et al.* 2001).

Following common honey volatiles were detected: octanal, nonanal, decanal, benzaldehyde, benzyl alcohol, benzeneethanol, benzeneacetic and benzoic acid acetaldehyde, isobutanal, 3-methyl-butanal, 2-metyl-butanal, acetone, diacetyl 3-methyl-butanol, 2-metyl-butanol, ethyl acetate, ethyl formate and dimethylsulfide.

Compared to previous studies on Brassica honeys, aromates and volatiles with a relatively high molecular weight were extracted. On the other hand, relatively few ketones were found. The 50/30um DVB/CAR/ PDMS coating seems to be efficient in extracting honey volatiles as 40 components of a total of 96 compounds reported up to now were identified (70 identified in previous works).

In an extract of floral volatiles from *Brassica napus* L. (oilseed rape) flowers of the Topas variety linalool, benzene ethanol, 2-hydroxybenzoic acid methyl ester, benzyl alcohol, (E)-2-hexenal and 1-octen-3-ol were detected (Pham-Delègue et al. 1997). Most likely benzeneethanol and benzyl alcohol detected in winter rape honey and spring turnip rape honey are derived from the plant.

Many compounds detected in winter rape honey and spring turnip rape honey, such as α-terpineol, benzyl alcohol, benzene ethanol, phenol, octanal, nonanal, decanal and benzaldhyde, were also detected in beeswax (Ferber and Nursten 1978). Unfortunately, in this study the volatiles were extracted from beeswax cappings. That means that beeswax had been in intense contact with honey. Therefore, it is not clear, which compounds originate from beeswax. As in the present study only winter rape and turnip rape honeys were studied, only future studies can decide, whether these honeys have markers, that can distinguish them from other unifloral honeys. For the discrimination between rape and turnip rape honeys, benzyl alcohol, benzenacetaldehyde, benzeneethanol and benzoic acid seem to be the most promising.

Glucosinolates are present in all Cruciferae species. They decompose enzymatically by autolysis in presence of water to various nitriles (Lüthy *et al.* 1982) As benzenenitrile was detected in all of winter rape and spring turnip rape honeys and is relatively seldom found in other unifloral honeys, it might be a possible marker compound. 2-methyl-propanenitrile was detected solely in Brassica honey when dynamic headspace extraction of several unifloral honeys was performed (Radovic *et al.* 2001). On the other hand, nitriles have also been identified in Castanea, Citrus and Taraxacum honeys extracted by SPME (Verzera *et al.* 2001; Piasenzotto *et al.* 2002).

TABLE 7. Volatiles detected in Brassica honey by previous authors

Compound group	CAS reg.no	Identi-fied by *
Hydrocarbons		
Hexane	110-54-3	z
Methylbenzene	108-88-3	z
Octane	111-65-9	z
Nonane	111-84-2	z
Styrene	100-42-5	z
Alcohols		
Benzyl alcohol	100-51-7	x
Benzeneethanol	60-12-8	x
3,7-dimethyl-1,5,7-Octatrien-3-ol		x
3-methyl-2-Buten-1-ol	556-82-1	x
3-methyl-3-Buten-1-ol	763-32-6	x
1-butanol	71-36-3	x
Isopropyl alcohol	67-63-0	x
2-methyl-3-Buten-2-ol	115-18-4	x
Ethanol	64-17-5	z,x
3-methyl-Butanol	123-51-3	z,x
1-methyl-Benzenemethanol	98-85-1	v
p-tert-butyl-Phenol	98-54-4	v
5-methyl-2-(1-methylethyl)-Phenol	89-83-8	v
Aldehydes		
2-Furancarboxaldehyde	98-01-1	x
Nonanal	124-19-6	x
Octanal	124-13-0	x
3-methyl-2-butenal	107-86-8	x
Pentanal	110-62-3	x
Acetaldehyd	75-07-0	z
2-methyl-Propanal	78-84-2	z
3-methyl-Butanal	590-86-3	z,x
2-methyl-Butanal	96-17-3	z,x
Hexanal	66-25-1	z,x
Benzaldehyde	100-52-7	z,x
Decanal	112-31-2	x
Ketones		
2,6,6-trimethyl-2-Cyclohexene-1,4-dione	1125-21-9	x
1-(2-furanyl)-Ethanone	1192-62-7	x
1-hydroxy-2-Propanone	116-09-6	x
3-hydroxy-2-Butanone	513-86-0	x
2-Pentanone	107-87-9	x
Acetone	67-64-1	z,x
2,3-Butanedione	431-03-8	z
2-Butanone	78-93-3	z,x
2-Heptanone	110-43-0	z
Acetophenone	98-86-2	v,x
1-(4-methylphenyl)-Ethanone	122-00-9	v
cis-Linalool oxide	11063-77-7	x

Compound group	CAS reg.no	Identi-fied by *
Acids		
Benzoic acid	65-85-0	u,y
Benzeneacetic acid	103-82-2	u,y
3-Phenylpropanoic acid	501-52-0	u
2-hydroxy-Benzoic acid	69-72-7	u
Cinnamic acid	140-10-3	u
B-Phenylmilchsäure		u
4-hydroxy-Benzoic acid	99-96-7	u
4-hydroxy-Benzeneacetic acid	156-38-7	u
Phenylpyruvic acid	156-06-9	u
4-hydroxy-3-methoxy-Benzoic acid		u
3,4-Dihydroxybenzoic acid	99-50-3	u
3-(p-methoxyphenyl)-Acrylic acid	501-98-4	u
4-hydroxy-3-Methoxycinnamic acid	1135-24-6	u
3,4-Dihydroxycinnamic acid	331-39-5	u
4-hydroxy-3,5-Dimethoxybenzoic acid	530-57-4	u
Esters		
Methyl formate	107-31-3	z
Ethyl acetate	141-78-6	z
Formic acid ethyl ester	109-94-4	z
1-methylpropyl-Phenyl acetate		x
Furanes		
Furan	110-00-9	z
2-methyl-Furan	534-22-5	z
2,3-dihydro-4-Methylfurane		x
Chlorinated compounds		
Dichloromethane	75-09-2	z
1,3-dichloro-Benzene	541-73-1	z
Chloroform	67-66-3	x
Sulphur compounds		
Dimethyl sulfide	75-18-3	z
dimethyl-Disulfide	624-92-0	z,x
Nitriles		
2-methyl-Propanenitrile	78-82-0	x

*
z = Bouseta et al., 1992, by dynamic headspace GC-MS
y = Speer & Montag, 1984, by liquid extraction & GC-MS
x = Radovic et al., 2001, by dynamic headspace GC-MS
v = Guyot ey al., 1998, by steam destillation/solvent extracation GC-MS
u = Steeg & Montag, 1987, by liquid extraction & GC-MS

TABLE 8. Volatiles detected in beeswax after Ferber and Nursten (1978)

Compound	CAS reg. no
1-methyl-4-(1-methylethyl)-Benzene	99-87-6
1,2,4,5-tetramethyl-Benzene	95-93-2
1,2,3,5-tetramethyl-Benzene	527-53-7
Undecane	1120-21-4
Dodecane	112-40-3
Tridecane	629-50-5
Tetradecane	629-59-4
Pentadecane	629-62-9
Hexadecane	544-76-3
heptadecane	629-78-7
1-methyl-4-(1-methylethenyl)-, (S)-Cyclohexene	5989-54-8
Naphthalene	91-20-3
1-methyl-Naphthalene	90-12-0
2-methyl-Naphthalene	91-57-6
Acenaphthene	83-32-9
cis-Linalool oxide (furanoid)	11063-77-7
trans-Linalool oxide (furanoid)	34995-77-2
trans-Linalool oxide (pyranoid)	
3,7-dimethyl-1,5,7-Octatrien-3-ol	
a-Terpineol	10482-56-1
2-methoxy-Phenol	90-05-1
Benzyl alcohol	100-51-6
Benzeneethanol	60-12-8
Phenol	108-95-2
Octanal	124-13-0
Nonanal	124-19-6
Decanal	112-31-2
Acetic acid	64-19-7
Benzaldehyde	100-52-7
2-Hydroxybenzoic acid methyl ester	119-36-8
1,4-dichloro-Benzene	106-46-7
Hexanol	111-27-3
3-Phenylpropanol	122-97-4
4-Phenylbutyl alcohol	
Benzoic acid, methyl ester	93-58-3
Benzeneacetic acid, methyl ester	101-41-7
Benzoic acid, ethyl ester	93-89-0
Benzeneacetic acid, ethyl ester	101-97-3
Acetic acid, 2-phenylethyl ester	103-45-7
Decanoic acid, ethyl ester	110-38-3
Acetophenone	98-86-2
4-methoxy-Benzaldehyde	123-11-5

8 Conclusions

The first objective of the present study was to develop and optimise a SPME-method for the extraction of honey volatiles. The other objective was to test the possibilities of the method as a tool for the classification of unifloral honeys on the example of Swiss winter rape and Finnish spring turnip rape.

8.1 An optimized SPME-Method for the extraction of honey volatiles

In the present work, a new headspace-SPME method was developed and optimised in regard to all important criteria for the extraction of honey volatiles such as: sample amount, matrix modification by water and sodium chloride addition, agitation of sample matrix, extraction temperature, extraction time and analyte desorbtion. Extraction temperature, matrix modification and extraction time were shown to have the most pronounced influence on the extraction rate of honey volatiles. A 50/30 µm DVB/CAR/PDMS coated SPME fiber was tested for its use as adsorbent of honey volatiles for the first time. The fiber coating composed of three different polymers, proved to extract successfully volatile components with broad range of chemical characteristics and volatility.

This work reports for the first time on cracks in the CAR/PDMS/DVB coating. The number of cracks varies from fiber to fiber, but generally at least two out of three new 30/50 µm CAR/PDMS/DVB fibers delivered by the manufacturer exhibit cracks in the coating. Significant differences between fibers with cracks and without cracks in regard to absorption of several components were shown. In quantitative analysis, this may be overcome with a calibration for each fiber. However, for most compounds the repeatability of the method is not significantly diminished for fibers with cracked coating.

Compared to present purge and trap and to steam distillation/solvent extraction methods, the present method is less expensive and faster. SPME seems to have also advantages over dynamic head space techniques, as

relatively low volatile compounds can be analysed within a short time. Indeed, it has been postulated (Bouseta *et al.* 1992) that less volatile substances have a higher discriminatory power for the differentiation of unifloral honeys than highly volatile ones. However, properly optimised dynamic headspace methods should also allow the determination of the compounds extracted by SPME.

In summary, the developed method seems to be promising and well suited for routine analysis of honey volatiles. Although the tested fiber has advantages over other ones, e.g. absorbtion capacity for a greater amount of volatiles, it has certain shortcomings, the main one being the relative fragility. Indeed, SPME can be used for routine honey characterisation if it is a robust method, which gives similar results in different laboratories. Future studies should clarify if nonporous fiber coatings are more durable and if they are also efficient in extracting of honey volatiles. Especially the polar polyacrylate coating should be evaluated for the extraction of honey volatiles. The methods developed in future studies should also be adapted to quantitative analysis of honey volatiles. This will be essential for the discrimination between unifloral honeys based on marker compounds or fingerprints. The application seems to be very promising for determination of the botanical origin of honey based on volatile composition. However, SPME is certainly not suitable for the determination of the total volatiles present in honey.

8.2 Application of the SPME Method for the determination of winter rape and spring turnip rape volatiles

Generally, no distinction is made between honeys of winter rape and spring turnip rape, both of these honeys being qualified as Brassica honey. In this study, a comparison of winter rape honey and spring turnip rape honey was carried out for the first time. The results showed that the chemical composition of the two honey types is practically identical. Also, they have a similar content of Brassica-type pollen. However, a difference between the sensory properties of the two honey types was demonstrated. On the other hand, the analysis of volatiles by SPME-FID and SPME-MS showed that

these honeys can be clearly discriminated. With the present technique, 26 new compounds could be tentatively identified in Brassica honeys. The structure of eight of these new compounds could be confirmed by reference compounds.

The present work points out that analysis of volatiles by SPME is a promising approach for the determination of botanical origin of honey. However, there is still a long way to go before the botanical origin of honey can be determined by its volatile composition. For this purpose, the present SPME-method should be adapted to for quantitative determinations of volatiles. In order to establish composition criteria for unifloral honeys, a large number of authentic samples have to be collected and their volatile composition identified and quantified by GC-MS.

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Appendixes

Appendix 1

TABLE 1. Influence of water addition on the extraction rate. In the table the results of 2 replicate determinations are presented.

Compound/Peak	Water added			
	0.50 ml		1.00 ml	
	Mean peak height (pA)	Range (+-pA)	Mean peak height (pA)	Range (+-pA)
Nonanal	3513	61	5468	167
P1	28441	1151	43042	1383
2-Furancarboxaldehyde	36421	1056	36459	825
Decanal	9139	73	9336	723
1-(2-furanyl)-Ethanone	3708	27	3484	154
Benzaldehyde	19486	154	22936	17
2-hydroxy-Benzaldehyde	3650	158	3490	417
P2	15387	275	18227	678
P3	6413	250	8630	171
P4	10565	382	13746	371
Benzyl alcohol	122789	3595	105095	4371
Bezeneethanol	25777	954	21779	472
P5	6432	361	7830	217
Octanoic acid	4993	150	4011	190
Cinnamylaldehyde	1170	10	1236	45
P6	8144	391	8203	174
Nonanoic acid	10762	432	10425	186
P7	7408	130	8269	380
P8	18119	844	19637	109
Tricosane	6686	230	5694	2
Benzoic acid	3365	135	2746	73

Appendix 2

TABLE 2. Influence of NaCl addition on the extraction rate. In the table the results of 2 replicate determinations are presented.

Compound/Peak	NaCl added					
	0.00 g		0.53 g		1.05 g	
	Mean peak height (pA)	Range (+- pA)	Mean peak height (pA)	Range (+-pA)	Mean peak height (pA)	Range (+-pA)
Nonanal	22637	1170	18209	946	12639	1675
P1	129842	1840	126848	9700	103777	16209
2-Furancarbox-aldehyde	21954	1511	31654	385	41638	709
Decanal	10852	183	11499	342	8895	291
1-(2-furanyl)-Ethanone	1878	52	2698	9	3707	154
Benzaldehyde	44253	4116	26974	500	26982	724
2-hydroxy-Benzaldehyde	2833	128	3724	113	4317	187
P2	21927	129	25087	22	23215	1188
P3	7831	109	12074	138	14124	777
P4	10901	557	17869	588	22814	2996
Benzyl alcohol	42485	627	65912	417	100098	4392
Bezeneethanol	9295	34	13947	175	22286	1169
P5	1335	26	2146	145	3438	318
Octanoic acid	2845	166	3366	350	4876	469
Cinnamylaldehyde	990	28	1322	89	1690	121
P6			5907	668	13636	357
Nonanoic acid	4020	199	4493	1082	8824	2710
P7	5662	323	7806	1003	12979	948
P8	11619	625	15772	1965	24266	232
Tricosane	1607	100	2748	544	7394	593
Benzoic acid			2008	161	3487	123

Appendix 3

TABLE 3. Influence of the sample amount on the extraction rate. In the table the results of 2 replicate determinations are presented

Compound/Peak	Honey added (dry matter)			
	4.00 g		4.50 g	
	Mean peak height (pA)	Range (+-pA)	Mean peak height (pA)	Range (+-pA)
Nonanal	5468	167	4936	107
P1	43042	1383	39060	533
2-Furancarboxaldehyde	36459	825	37302	228
Decanal	9336	723	9494	365
1-(2-furanyl)-Ethanone	3484	154	3492	48
Benzaldehyde	22936	17	22771	458
2-hydroxy-Benzaldehyde	3490	417	3734	202
P2	18227	678	17644	20
P3	8630	171	8063	43
P4	13746	371	12223	76
Benzyl alcohol	105095	4371	104653	672
Bezeneethanol	21779	472	22197	56
P5	7830	217	7013	133
Octanoic acid	4011	190	4035	75
Cinnamylaldehyde	1236	45	1114	34
P6	8203	174	7905	348
Nonanoic acid	10425	186	9892	511
P7	8269	380	8136	235
P8	19637	109	19098	286
Tricosane	5694	2	5587	14
Benzoic acid	2746	73	2650	73

Appendix 4

TABLE 4. Influence of agitation speed on the extraction rate. In the table the results of 2 replicate determinations are presented.

Compound/Peak	Agitation speed			
	600 rpm		1100 rpm	
	Mean peak height (pA)	Range (+-pA)	Mean peak height (pA)	Range (+-pA)
Nonanal	3571	339	6631	717
P1	29396	3642	72173	8956
2-Furancarboxaldehyde	31548	1740	31469	2360
Decanal	8560	517	4287	458
1-(2-furanyl)-Ethanone	3192	152	2296	150
Benzaldehyde	19063	855	18400	1014
2-hydroxy-Benzaldehyde	3445	257	3000	424
3-Pyridinecarbox-aldehyde	668		1360	44
P2	14062	1224	13223	2062
P3	5961	833	9110	1890
P4	8515	903	15666	2316
Benzyl alcohol	100299	7488	50643	3441
Bezeneethanol	21495	1344	11432	1071
P5	5430	832	8088	1564
Octanoic acid	4702	232	3979	167
Cinnamylaldehyde	897	75	1625	275
P6	7510	703	11650	1387
Nonanoic acid	10208	1035	5653	385
P7	7599	732	9075	1308
Tricosane	4652	439	5432	406
Benzoic acid	2579	281	3595	282

Appendix 5

TABLE 5. Influence of incubation temperature on the extraction rate. In the table the results of 2 replicate determinations are presented.

Compound/Peak	Incubation temperature					
	50°C		60°C		70°C	
	Mean peak height (pA)	Range (+-pA)	Mean peak height (pA)	Range (+-pA)	Mean peak height (pA)	Range (+-pA)
Nonanal	1099	59	3884	847	5468	167
P1	7179	947	26099	5063	43042	1383
2-Furancarbox-aldehyde	12904	51	27148	92	36459	825
Decanal	2927	271	8257	1449	9336	723
1-(2-furanyl)-Ethanone	1542	11	2691	25	3484	154
Benzaldehyde	9104	154	18780	660	22936	17
2-hydroxy-Benzaldehyde	1847	226	3891	397	3490	417
P2	6699	217	15240	1264	18227	678
P3	1644	184	5601	832	8630	171
P4	3295	3	8754	1066	13746	371
Benzyl alcohol	35484	1747	75648	572	105095	4371
Bezeneethanol	6909	330	16070	564	21779	472
P5	1395	129	4286	471	7830	217
Octanoic acid	1101	46	2539	191	4011	190
Cinnamylaldehyde	294		772	64	1236	45
P6	1202	18	3879	518	8203	174
Nonanoic acid	2622	143	6001	56	10425	186
P7	2033	129	5437	789	8269	380
P8	2923	190	9334	708	19637	109
Tricosane	693	17	2689	47	5694	2
Benzoic acid	795	7	1542	26	2746	73

Appendix 6

TABLE 6. Influence of extraction time on the extraction rate. In the table the results of 3 replicate determinations are presented.

Compound/Peak	Extraction time							
	10 min.		Sig.	35 min.		Sig.	60 min.	
	Mean	STD	10/35	Mean	STD	35/60	Mean	STD
Nonanal	5038			8429	917		8123	1803
P1	55549	7549	*	97014	13583		96592	25103
2-Furancarbox-aldehyde	13068	546		34461	1505		42549	1367
Decanal	4075	233		3471	760		3122	639
1-(2-furanyl)-Ethanone	1285	20	*	2617	159		2683	133
Benzaldehyde	10529	710	*	20367	1028		21629	1431
2-hydroxy-Benzaldehyde	2241	575		2648	297		2646	290
3-Pyridinecarbox-aldehyde				1753	220		1705	260
P2	8389	1168	*	12331	1569		12361	1346
P3	4205	1040	*	7732	340		9330	1076
P4	5432	707	*	16448	1286	*	19520	1149
Benzyl alcohol	29987	732	*	53682	2253		53818	3038
Bezeneethanol	6733	556	*	10944	479		11104	682
P5	2435	377	*	7736	541	*	11408	1086
Octanoic acid	1476	47	*	3677	152		3813	384
Cinnamylaldehyde	432	71	*	1741	233	*	4077	588
P6	3435	317	*	11557	625	*	20007	5193
Nonanoic acid	1762	171		4301	546	*	7440	4565
P7	3282	240	*	8532	211	*	11322	2303
P8	6495	535	*	18932	963	*	25355	1283
Tricosane	1492	93	*	5310	431	*	8098	857
Benzoic acid	1258	66	*	3721	422	*	5996	137

Mean = Mean peak height of three replicate determinations
Sig. = Statistical significance
STD = Standard deviation
* = Statistically significant difference on the 95 % confidence interval between the extraction times tested

Appendix 7

Compound	Fiber coating						
	Smooth			Cracked			Sm/Cr
	Mean	STD	CV%	Mean	STD	CV%	Statistical significance on the 95 % level
Styrene	1702	224.8	13.2	1932	170.6	8.8	
Nonanal	1486	239.1	16.1	1496	266.7	17.8	
5-ethenyltetrahydro-a,a,5-trimethyl-(2R,5R)-rel(9Cl)-2-Furanmethanol,	6756	617.7	9.1	9861	909.4	9.2	
1,4-dichloro-Benzene	12368	1625.9	13.1	13528	2189.1	16.2	
2-Furancarboxaldehyde	19425	1836.1	9.5	22394	2604.6	11.6	
Decanal	783	80.3	10.3	768	97.3	12.7	
1-(2-furanyl)-Ethanone	1486	157.3	10.6	2233	304.0	13.6	
Benzaldehyde	193322	19366.1	10.0	279256	46274.1	16.6	*
Dimethyl sulfoxide	1843	145.1	7.9	2236	246.6	11.0	
Benzeneacetaldehyde	13718	1192.3	8.7	13693	1341.8	9.8	
2-hydroxy-Benzaldehyde	1351	83.8	6.2	1086	143.1	13.2	
3-Pyridinecarboxaldehyde	923	78.3	8.5	1264	132.3	10.5	
Hexanoic acid	3767	395.6	10.5	4570	562.7	12.3	
Benzyl alcohol	23508	2521.7	10.7	11705	1444.5	12.3	*
Benzeneethanol	27698	2031.9	7.3	28370	2349.0	8.3	
Benzeneacetonitrile	15740	977.3	6.2	17418	1209.0	6.9	
Octanoic acid	6512	533.1	8.2	6058	726.4	12.0	
Cinnamylaldehyde	5912	400.6	6.8	7218	1049.6	14.5	*
Nonanoic acid	5731	535.5	9.3	4812	996.7	20.7	*
2-Methoxy-4-vinylphenol	1910	125.7	6.6	1654	91.0	5.5	
n-Tricosane	1713	126.3	7.4	1291	256.5	19.9	
3.5-dimethoxybenzaldehyde	5092	289.0	5.7	7463	300.1	4.0	*
Benzoic acid	21774	863.7	4.0	26846	493.4	1.8	*
Benzeneacetic acid	1544	93.5	6.1	1495	38.9	2.6	
3-Phenylpropanoic acid	1018	64.3	6.3	1107	74.5	6.7	
Benzoic acid 4-hydroxy-3,5dimethoxy-hydrazide	886	89.4	10.1	1161	32.4	2.8	

TABLE 7. Repeatability of the method (within a day). Headspace SPME of honey volatiles was performed with smooth (n=7) and cracked (n=6) 50/30 μm DVB/CAR/-PDMS fiber coatings.

Mean = Mean peak height of the determinations with cracked and smooth fibers

STD = Standard deviation

CV = Coefficient of variation (%)

* denotes a significant difference between the extraction rate of smooth and cracked coatings

Appendix 8

Compound			Winter rape (n=13)					Spring turnip rape (n=12)				
Name	CAS reg. no	RI	*Mean*	STD	CV (%)	Min.	Max.	*Mean*	STD	CV (%)	Min.	Max.
Styrene	100-42-5	1254	*1929*	1170	61	n.d.	4909	*5240*	4077	78	n.d.	14531
Nonanal	124-19-6	1410	*1805*	414	23	n.d.	2520	*6280*	6512	104	1113	22942
5-ethenyltetrahydro-a,a,5-trimethyl-,(2R,5R)-rel(9Cl)-2-Furanmethanol,	34995-77-2	1459	*6895*	4147	60	n.d.	17807	*6420*	3081	48	n.d.	12255
1,4-dichloro-Benzene	106-46-7	735	*17038*	18325	108	n.d.	53557					
2-Furancarbox-aldehyde	98-01-1	1487	*24557*	10836	44	7066	42518	*15505*	8806	57	7075	40204
Decanal	112-31-2	1517	*891*	283	32	578	1605	*1660*	686	41	846	2858
1-(2-furanyl)-Ethanone	1192-62-7	1531	*1437*	445	31	672	2297	*1260*	471	37	858	2339
Benzaldehyde	100-52-7	1557	*117990*	47260	40	50033	2E+05	*38602*	20388	53	9429	77811
Dimethyl sulfoxide	67-68-5	1601	*2459*	830	34	n.d	4424	*977*	417	43	n.d.	2065
Benzeneacetaldehyde	122-78-1	1680	*13683*	13190	96	4898	55254	*6146*	1827	30	3732	9984
2-hydroxy-Benz-aldehyde	90-02-8	1731	*1486*	529	36	927	2912	*1675*	316	19	1170	2312
3-Pyridinecarbox-aldehyde	500-22-1	1746	*1489*	1431	96	n.d	4651	*3014*	2682	89	627	9418
Hexanoic acid	142-62-1	1853	*3408*	894	26	2041	4956	*3999*	1544	39	2274	8172
Benzyl alcohol	100-51-6	1904	*36537*	14802	41	6977	59453	*6057*	3657	60	1450	14085
Benzeneethanol	60-12-8	1944	*25835*	6450	25	18064	41781	*5736*	2621	46	2254	9607
Benzeneacetonitrile	140-29-4	1977	*16715*	13672	82	1316	40567	*7035*	7601	108	787	27790
Octanoic acid	124-07-2	2065	*5509*	1143	21	3444	8035	*6204*	2098	34	3470	10997
Cinnamylaldehyde	104-55-2	2096	*6685*	2081	31	3691	10596	*4009*	1712	43	1956	6932
Nonanoic acid	112-05-0	2171	*4277*	1881	44	2158	8937	*6299*	4330	69	2043	15949
2-Methoxy-4-	7786-61-0	2233	*2146*	774	36	1386	4241	*1824*	562	31	1068	2669
n-Tricosane	638-67-5	2293	*1898*	207	11	1536	2277	*1932*	508	26	1374	3135
3,5-Dimethoxy-benzaldehyde	7311-34-4	2337	*2879*	1653	57	792	5232	*1108*	503	45	400	1945
Benzoic acid	65-85-0	2463	*20395*	3696	18	15681	26627	*11480*	2046	18	7846	14644
5-(hydroxymethyl)-2-Furancarboxaldehyde	67-47-0	2506	*648*	116	18	468	817	*312*	60	19	253	449
Benzeneacetic acid	103-82-2	2585	*1522*	973	64	436	3909	*751*	526	70	n.d.	1684
3-Phenylpropanoic acid	501-52-0	2648	*1003*	247	25	643	1312	*800*	477	60	345	1878
3,5-Dimethoxy-4-hydroxybenzhydrazide	1443-76-1	2996	*945*	238	25	543	1391	*503*	181	36	212	823

TABLE 8.

List of compounds identified in winter rape honeys and spring turnip rape honeys by SPME-GC/FID.

For the compounds printed in bold letters a significant diffe-rentce in peak height between the two honey types was found on the 95% confidence level.

n.d. = not detected

STD = Standard deviation

Mean = Mean of the peak heights

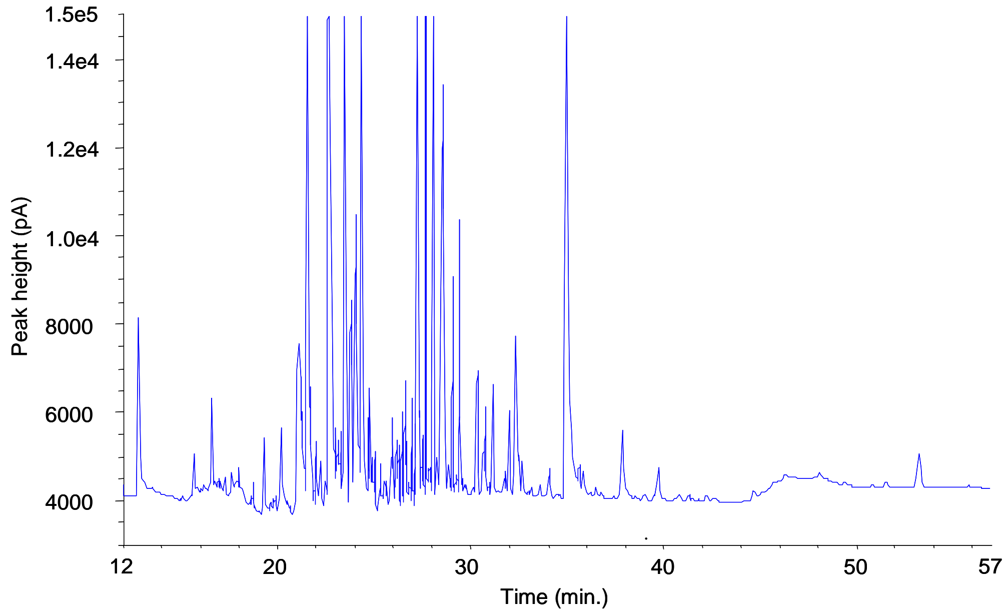
CV = Coefficient of variation (%)

Min. = Minimum

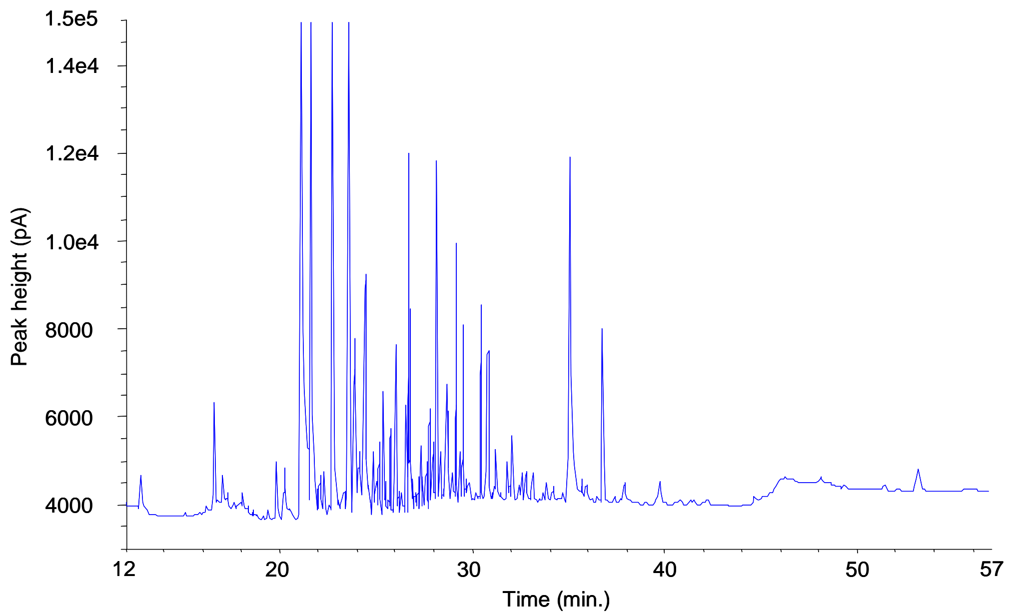
Max. = Maximum

Appendix 9

FIGURE 1. Chromatograms of honey volatiles obtained after extraction with a 50/30 µm DVB/CAR/PDMS coated SPME-fiber according to the method described in section 6.3.



Chromatogram of winter rape honey volatiles.



Chromatogram of spring turnip rape honey volatiles.

Appendix 10

Calculations of general linear modelling with stepwise backward removal of the variables in the fourth (last) model to reduce it to the three most discriminant peaks.

The coefficients for the linear combination for discrimination between spring turnip rape and winter rape honeys based on three peak heights can be found in the end of this appendix together with the plot of residuals against predicted values.

Step # 0 R = 0.985 R-Square = 0.970

Effect	Coefficient	Std Error	Std Coef	Tol.	df	F	'P'
In							
1 Constant							
2 P86	0.001	0.000	0.439	0.06980	1	5.853	0.031
3 P85	0.000	0.000	0.079	0.12873	1	0.346	0.566
4 P118	-0.000	0.000	-0.222	0.05022	1	1.080	0.318
5 P79	-0.000	0.000	-0.094	0.41355	1	1.586	0.230
6 P28	-0.000	0.000	-0.150	0.49807	1	4.874	0.046
7 P50	-0.000	0.000	-0.130	0.08583	1	0.630	0.442
8 P33	0.000	0.000	0.209	0.42579	1	8.113	0.014
9 P58	0.000	0.000	0.260	0.37386	1	10.964	0.006
10 P34	-0.000	0.000	-0.126	0.27974	1	1.931	0.188
11 P84	0.000	0.000	0.198	0.15300	1	2.603	0.131
12 P42	0.000	0.000	0.513	0.07579	1	8.670	0.011
Out	Part. Corr.						
none							

Dependent Variable CH
Minimum tolerance for entry into model = 0.000000
Backward stepwise with Alpha-to-Enter=0.010 and Alpha-to-Remove=0.010

Step # 1 R = 0.985 R-Square = 0.969
Term removed: P85

Effect	Coefficient	Std Error	Std Coef	Tol.	df	F	'P'
In							
1 Constant							
2 P86	0.001	0.000	0.496	0.09687	1	10.853	0.005
4 P118	-0.000	0.000	-0.321	0.12744	1	5.965	0.028
5 P79	-0.000	0.000	-0.114	0.51969	1	3.067	0.102
6 P28	-0.000	0.000	-0.169	0.64080	1	8.336	0.012
7 P50	-0.000	0.000	-0.121	0.08657	1	0.578	0.460
8 P33	0.000	0.000	0.198	0.45773	1	8.178	0.013
9 P58	0.000	0.000	0.252	0.38465	1	11.139	0.005
10 P34	-0.000	0.000	-0.104	0.33715	1	1.663	0.218
11 P84	0.000	0.000	0.210	0.15758	1	3.173	0.097
12 P42	0.000	0.000	0.550	0.08711	1	12.012	0.004
Out	Part. Corr.						
3 P85	0.161	.	.	0.12873	1	0.346	0.566

Step # 2 R = 0.984 R-Square = 0.968
Term removed: P50

	Effect	Coefficient	Std Error	Std Coef	Tol.	df	F	'P'
In								
1	Constant							
2	P86	0.001	0.000	0.419	0.17815	1	14.631	0.002
4	P118	-0.000	0.000	-0.307	0.12974	1	5.741	0.030
5	P79	-0.000	0.000	-0.134	0.62834	1	5.317	0.036
6	P28	-0.000	0.000	-0.175	0.65257	1	9.364	0.008
8	P33	0.000	0.000	0.195	0.45965	1	8.162	0.012
9	P58	0.000	0.000	0.216	0.63266	1	13.857	0.002
10	P34	-0.000	0.000	-0.111	0.34100	1	1.955	0.182
11	P84	0.000	0.000	0.188	0.16745	1	2.787	0.116
12	P42	0.000	0.000	0.532	0.08919	1	11.820	0.004
Out		Part. Corr.						
3	P85	0.139	.	.	0.12984	1	0.275	0.608
7	P50	-0.199	.	.	0.08657	1	0.578	0.460

Step # 3 R = 0.982 R-Square = 0.964
Term removed: P34

	Effect	Coefficient	Std Error	Std Coef	Tol.	df	F	'P'
In								
1	Constant							
2	P86	0.001	0.000	0.487	0.22288	1	23.394	0.000
4	P118	-0.000	0.000	-0.333	0.13242	1	6.487	0.022
5	P79	-0.000	0.000	-0.172	0.79699	1	10.410	0.005
6	P28	-0.000	0.000	-0.188	0.67127	1	10.529	0.005
8	P33	0.000	0.000	0.160	0.52944	1	5.999	0.026
9	P58	0.000	0.000	0.201	0.65473	1	11.730	0.003
11	P84	0.000	0.000	0.136	0.18863	1	1.533	0.233
12	P42	0.000	0.000	0.469	0.09747	1	9.472	0.007
Out		Part. Corr.						
3	P85	-0.024	.	.	0.15774	1	0.009	0.926
7	P50	-0.222	.	.	0.08756	1	0.780	0.391
10	P34	-0.340	.	.	0.34100	1	1.955	0.182

Step # 4 R = 0.980 R-Square = 0.960
Term removed: P84

	Effect	Coefficient	Std Error	Std Coef	Tol.	df	F	'P'
In								
1	Constant							
2	P86	0.001	0.000	0.562	0.34944	1	47.366	0.000
4	P118	-0.000	0.000	-0.419	0.18527	1	13.963	0.002
5	P79	-0.000	0.000	-0.180	0.81099	1	11.333	0.004
6	P28	-0.000	0.000	-0.219	0.82362	1	16.975	0.001
8	P33	0.000	0.000	0.162	0.52963	1	5.930	0.026
9	P58	0.000	0.000	0.169	0.81824	1	9.991	0.006
12	P42	0.000	0.000	0.588	0.16287	1	24.170	0.000
Out		Part. Corr.						
3	P85	0.074	.	.	0.17629	1	0.088	0.771
7	P50	-0.118	.	.	0.09554	1	0.226	0.641
10	P34	-0.207	.	.	0.38414	1	0.713	0.411
11	P84	0.296	.	.	0.18863	1	1.533	0.233

Step # 5 R = 0.973 R-Square = 0.947
Term removed: P33

Effect	Coefficient	Std Error	Std Coef	Tol.	df	F	'P'
In							
1 Constant							
2 P86	0.001	0.000	0.671	0.49866	1	75.588	0.000
4 P118	-0.000	0.000	-0.323	0.21178	1	7.418	0.014
5 P79	-0.000	0.000	-0.204	0.83913	1	11.804	0.003
6 P28	-0.000	0.000	-0.237	0.83878	1	15.812	0.001
9 P58	0.000	0.000	0.139	0.86321	1	5.620	0.029
12 P42	0.000	0.000	0.458	0.20378	1	14.371	0.001
Out	Part. Corr.						
3 P85	0.020	.	.	0.17756	1	0.007	0.934
7 P50	-0.045	.	.	0.09673	1	0.034	0.856
8 P33	0.509	.	.	0.52963	1	5.930	0.026
10 P34	0.011	.	.	0.43773	1	0.002	0.963
11 P84	0.264	.	.	0.18869	1	1.275	0.274

Step # 6 R = 0.964 R-Square = 0.930
Term removed: P58

Effect	Coefficient	Std Error	Std Coef	Tol.	df	F	'P'
In							
1 Constant							
2 P86	0.001	0.000	0.715	0.52863	1	73.101	0.000
4 P118	-0.000	0.000	-0.395	0.22693	1	9.594	0.006
5 P79	-0.000	0.000	-0.187	0.85177	1	8.086	0.010
6 P28	-0.000	0.000	-0.250	0.84598	1	14.278	0.001
12 P42	0.000	0.000	0.510	0.21087	1	14.865	0.001
Out	Part. Corr.						
3 P85	-0.080	.	.	0.18492	1	0.116	0.738
7 P50	0.258	.	.	0.14908	1	1.280	0.273
8 P33	0.321	.	.	0.55874	1	2.066	0.168
9 P58	0.488	.	.	0.86321	1	5.620	0.029
10 P34	-0.016	.	.	0.43900	1	0.005	0.946
11 P84	-0.019	.	.	0.23925	1	0.007	0.935

Step # 7 R = 0.949 R-Square = 0.900
Term removed: P79

Effect	Coefficient	Std Error	Std Coef	Tol.	df	F	'P'
In							
1 Constant							
2 P86	0.001	0.000	0.698	0.53123	1	51.748	0.000
4 P118	-0.000	0.000	-0.476	0.23874	1	10.808	0.004
6 P28	-0.000	0.000	-0.211	0.88248	1	7.889	0.011
12 P42	0.000	0.000	0.638	0.23848	1	19.425	0.000
Out	Part. Corr.						
3 P85	0.114	.	.	0.20678	1	0.252	0.622
5 P79	-0.546	.	.	0.85177	1	8.086	0.010
7 P50	-0.039	.	.	0.18257	1	0.029	0.867
8 P33	0.376	.	.	0.58355	1	3.121	0.093
9 P58	0.339	.	.	0.87622	1	2.465	0.133
10 P34	-0.197	.	.	0.49504	1	0.765	0.393
11 P84	0.081	.	.	0.24702	1	0.125	0.727

```
Step # 8 R =  0.928 R-Square =  0.861
Term removed: P28

Effect             Coefficient   Std Error  Std Coef    Tol.  df       F     'P'

In
___
  1 Constant
  2 P86                  0.001       0.000     0.737 0.54256   1  44.414   0.000
  4 P118                -0.000       0.000    -0.441 0.24054   1   7.032   0.015
 12 P42                  0.000       0.000     0.644 0.23853   1  14.888   0.001

Out                Part. Corr.
___
  3 P85                  0.312          .         .  0.25134   1   2.153   0.158
  5 P79                 -0.345          .         .  0.88852   1   2.700   0.116
  6 P28                 -0.532          .         .  0.88248   1   7.889   0.011
  7 P50                  0.036          .         .  0.18570   1   0.026   0.872
  8 P33                  0.354          .         .  0.58629   1   2.861   0.106
  9 P58                  0.348          .         .  0.88867   1   2.755   0.113
 10 P34                 -0.115          .         .  0.49954   1   0.270   0.609
 11 P84                  0.242          .         .  0.27790   1   1.243   0.278
-------------------------------------------------------------------------------

Step # 9 R =  0.902 R-Square =  0.814
Term removed: P118

Effect             Coefficient   Std Error  Std Coef    Tol.  df       F     'P'

In
___
  1 Constant
  2 P86                  0.001       0.000     0.672 0.57127   1  30.446   0.000
 12 P42                  0.000       0.000     0.306 0.57127   1   6.327   0.020

Out                Part. Corr.
___
  3 P85                  0.564          .         .  0.72654   1   9.785   0.005
  4 P118                -0.501          .         .  0.24054   1   7.032   0.015
  5 P79                 -0.408          .         .  0.94053   1   4.191   0.053
  6 P28                 -0.415          .         .  0.88913   1   4.376   0.049
  7 P50                  0.159          .         .  0.19880   1   0.545   0.469
  8 P33                  0.097          .         .  0.68165   1   0.198   0.661
  9 P58                  0.407          .         .  0.93709   1   4.172   0.054
 10 P34                 -0.187          .         .  0.51563   1   0.759   0.394
 11 P84                  0.405          .         .  0.34158   1   4.123   0.055
-------------------------------------------------------------------------------

Step # 10 R =  0.872 R-Square =  0.760
Term removed: P42

Effect             Coefficient   Std Error  Std Coef    Tol.  df       F     'P'

In
___
  1 Constant
  2 P86                  0.001       0.000     0.872 1.00000   1  72.961   0.000

Out                Part. Corr.
___
  3 P85                  0.484          .         .  0.72707   1   6.727   0.017
  4 P118                 0.075          .         .  0.57609   1   0.126   0.726
  5 P79                 -0.464          .         .  0.99978   1   6.026   0.022
  6 P28                 -0.421          .         .  0.90269   1   4.743   0.040
  7 P50                  0.222          .         .  0.20526   1   1.137   0.298
  8 P33                 -0.033          .         .  0.72533   1   0.025   0.877
  9 P58                  0.341          .         .  0.93839   1   2.894   0.103
 10 P34                  0.162          .         .  0.83239   1   0.593   0.449
 11 P84                  0.548          .         .  0.46091   1   9.433   0.006
 12 P42                  0.473          .         .  0.57127   1   6.327   0.020
-------------------------------------------------------------------------------
```

Step # 10 R = 0.912 R-Square = 0.832
Term entered: P84

Effect	Coefficient	Std Error	Std Coef	Tol.	df	F	'P'
In							
1 Constant							
2 P86	0.001	0.000	0.582	0.46091	1	20.468	0.000
11 P84	0.000	0.000	0.395	0.46091	1	9.433	0.006
Out	Part. Corr.						
3 P85	0.285	.	.	0.53919	1	1.858	0.187
4 P118	-0.007	.	.	0.56345	1	0.001	0.975
5 P79	-0.387	.	.	0.92204	1	3.700	0.068
6 P28	-0.330	.	.	0.82866	1	2.561	0.124
7 P50	0.186	.	.	0.20213	1	0.749	0.397
8 P33	0.054	.	.	0.71048	1	0.062	0.806
9 P58	0.580	.	.	0.88542	1	10.630	0.004
10 P34	-0.081	.	.	0.69302	1	0.140	0.712
12 P42	0.269	.	.	0.42337	1	1.640	0.214

Step # 10 R = 0.943 R-Square = 0.889
Term entered: P58

Effect	Coefficient	Std Error	Std Coef	Tol.	df	F	'P'
In							
1 Constant							
2 P86	0.001	0.000	0.456	0.40837	1	16.044	0.001
9 P58	0.000	0.000	0.252	0.88542	1	10.630	0.004
11 P84	0.000	0.000	0.481	0.43489	1	18.938	0.000
Out	Part. Corr.						
3 P85	0.171	.	.	0.50288	1	0.604	0.446
4 P118	0.096	.	.	0.55149	1	0.187	0.670
5 P79	-0.492	.	.	0.92154	1	6.378	0.020
6 P28	-0.288	.	.	0.80482	1	1.810	0.194
7 P50	-0.309	.	.	0.11679	1	2.105	0.162
8 P33	0.316	.	.	0.63466	1	2.225	0.151
10 P34	-0.136	.	.	0.69130	1	0.375	0.547
12 P42	0.260	.	.	0.41913	1	1.455	0.242

Dep Var: CH   N: 25   Multiple R: 0.94266863   **Squared multiple R: 0.88862415**

Adjusted squared multiple R: 0.87271332   Standard error of estimate: 0.18191904

Effect	Coefficient	Std Error	Std Coef	Tolerance	t	P(2 Tail)
CONSTANT	-0.61186480	0.13520874	0.00000000	.	-4.52533	0.00019
P58	0.00001266	0.00000388	0.25234010	0.8854172	3.26043	0.00374
P84	0.00001297	0.00000298	0.48058014	0.4348925	4.35182	0.00028
P86	0.00058104	0.00014506	0.45647195	0.4083738	4.00550	0.00064

Effect	Coefficient	Lower < 95%>	Upper
CONSTANT	**-0.61186480**	-0.89304676	-0.33068284
P58	**0.00001266**	0.00000459	0.00002074
P84	**0.00001297**	0.00000677	0.00001916
P86	**0.00058104**	0.00027937	0.00088271

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	**F-ratio**	**P**
Regression	5.545	3	1.848	**55.850**	**0.000**
Residual	0.695	21	0.033		

```
*** WARNING ***
Case             6 has large leverage    (Leverage =          0.898)
Case            12 is an outlier         (Studentized Residual =        4.040)

Durbin-Watson D Statistic     1.295
First Order Autocorrelation   0.339
```