ELSEVIER

Journal of Chromatography A, 855 (1999) 601–615

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Investigation of combwax of honeybees with high-temperature gas chromatography and high-temperature gas chromatography–chemical ionization mass spectrometry
I. High-temperature gas chromatography

R. Aichholz[a,*], E. Lorbeer[b]

[a]*Novartis Pharma AG, Research, Core Technology Area, P.O. Box, CH-4002 Basel, Switzerland*
[b]*Institute of Organic Chemistry, University of Vienna, Währinger Strasse 38, A-1090 Vienna, Austria*

Received 2 March 1999; received in revised form 1 June 1999; accepted 7 June 1999

Abstract

The combwaxes of the honeybee species *Apis mellifera*, *Apis cerana*, *Apis dorsata*, *Apis laboriosa*, *Apis florea* and *Apis andreniformis* have been examined by high-temperature gas chromatography. Combwax consists of a complex mixture of homologous neutral lipids. These compounds containing up to 64 carbons were chromatographed intact on a 10 m×0.2 mm high-temperature stable SOP-50-PFD (50%-diphenyl/50%-1H,1H,2H,2H-perfluorodecylmethylpolysiloxane)-coated Duran glass capillary column. The use of this stationary phase results in lower retention values and, at last, in lower thermal stress of the analytes. In order to minimize the discrimination effect due to adsorption and/or degradation, a two-step derivatization was performed resulting in the formation of *tert*.-butyldimethylsilyl esters of the long chain fatty acids and trimethylsilyl ethers of complex hydroxyesters, respectively. The derivatization procedure was optimized using a modification of the extended Donike test. In addition this test allows the quantification of the thermal stability of the derivatives performed. The derivatization procedure was applied for combwax analysis. More than 80 compounds were separated and their peak areas semiquantitatively exploited.

Keywords: Beeswax; Derivatization, GC; *Apis* spp.; Lipids

1. Introduction

Combwax of the honeybees (*Apis* spp.), the so-called beeswax, was originally the only natural wax of commercial interest. A neolithic beeswax sample was dated back to the 4th Millennium B.C. [1] and is evidence of early beekeeping in human settlements. Beeswax, produced by abdominal glands of worker bees, is a valuable substance and has many uses in industry, pharmacy and medicine. This is not the only reason to obtain a comprehensive knowledge of its composition. Chemotaxonomic distinction of the various honeybee species (*Apis* spp.) by the combwax they produce is also of apidological interest. The diversification of the genus *Apis* is based on their morphology, behavior and distribution [2,3]. Today nine honeybee species have been differentiated: *Apis mellifera* (western honeybee), *Apis*

*Corresponding author.
E-mail address: reiner.aichholz@pharma.novartis.com (R. Aichholz)

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00725-6

cerana (eastern honeybee), *Apis koschevnikovi* (red honeybee), *Apis nuluensis*, *Apis nigrocincta*, *Apis dorsata* (giant honeybee), *Apis laboriosa* (rock honeybee), *Apis florea* (dwarf honeybee), and *Apis andreniformis* (bush honeybee). According to Ruttner, the taxonomic rank of *Apis laboriosa* and *Apis dorsata* is not fully confirmed [4].

The chemistry of the combwax lipids produced by *A. mellifera* has been studied extensively by Tulloch [5] and Stránsky and Streibl [6,7]. The structural elucidation of the individual classes of compounds was carried out after thin-layer chromatographic fractionation. Regarding the results of these examinations, beeswax mainly consists of complex mixtures of hydrocarbons, free fatty acids, monoesters, diesters, triesters, hydroxymonoesters, hydroxypolyesters, fatty acid monoesters, fatty acid polyesters and some unidentified compounds. Each compound family is a series of homologues differing in chain length by two carbon atoms. The structures of the principal homologous series of beeswax lipids are shown in Fig. 1.

It was shown earlier that gas chromatography (GC) can be used for the analysis of beeswax [5]. In course of these investigations differences of the elution pattern of four different honeybee species were found. These results are of limited information due to insufficient resolution of the packed columns used. Therefore, only few of the existing compound series could be determined.

The use of wall-coated open tubular capillary columns for the analysis of beeswax by GC has been published in recent years [8–10]. In spite of the considerable progress of column efficiency and inertness of open tubular capillary columns, some of the problems concerning the analysis of beeswax by GC still remain unresolved until today. Free fatty acids and hydroxymonoesters were adsorbed and/or decomposed during the analysis owing to the lack of a derivatization [8]. Conventional GC was used for the detection of adulteration of beeswax. Brüschweiler et al. [9] described a method which results in the discrimination of the complex wax esters series and only approximately 65 to 70% of the wax constituents were taken into account.

Recently we described the analysis of beeswax after derivatization with *N*-methyl-*N*-*tert*.-butyldimethylsilyltrifluoroacetamide (MTBSTFA) by high-temperature GC (HTGC) [10,11]. The main compound series of beeswax, including the hydroxyesters and diesters, could be analyzed successfully for the first time. One prerequisite for this successful application was the use of tailor-made low-bleeding glass capillary columns coated with a highly fluorinated polysiloxane. This stationary phase results in significant lower retention values and an improved signal-to-noise ratio even at high column temperatures. Nevertheless, silylation in context with HTGC can cause some problems as indicated by the extended Donike test [12,13]. Decomposition of trimethylsilyl esters of long-chain fatty acids depends on the column inertness and is a system inherent phenomenon of capillary columns.

Underivatized beeswax was also investigated using supercritical fluid chromatography. Only the hydrocarbon, monoester and diester series were recorded whereby compound families containing exchangeable hydrogen atoms were completely adsorbed during the chromatographic process [14].

In order to extend the knowledge about the composition of combwax of different honeybee species this paper describes a comprehensive investigation using HTGC. The above mentioned problems could be limited by a two-step derivatization procedure to minimize the decomposition of the fatty acid silylester and to enhance the volatility and thermal stability of compounds.

The combwax of the honey bee species *A. mellifera*, *A. florea*, *A. andreniformis*, *A. dorsata*, *A. laboriosa* and *A. cerana* was analyzed on glass capillary columns coated with poly(diphenyl/1H,1H,2H,2H-perfluorodecylmethyl)siloxane [10]. In a first step, flame ionization detection (FID) was used to illustrate the characteristic fingerprint of the combwax of each honeybee species.

Characterization of the individual compounds was performed by HTGC–chemical ionization mass spectrometry (CI-MS) and will be described in a subsequent report [15].

2. Materials and methods

2.1. Gas chromatography

A Carlo Erba Mega 5160 equipped with a FID system (420°C) and a cold on-column injector was used for analysis of the derivatized beeswax samples.

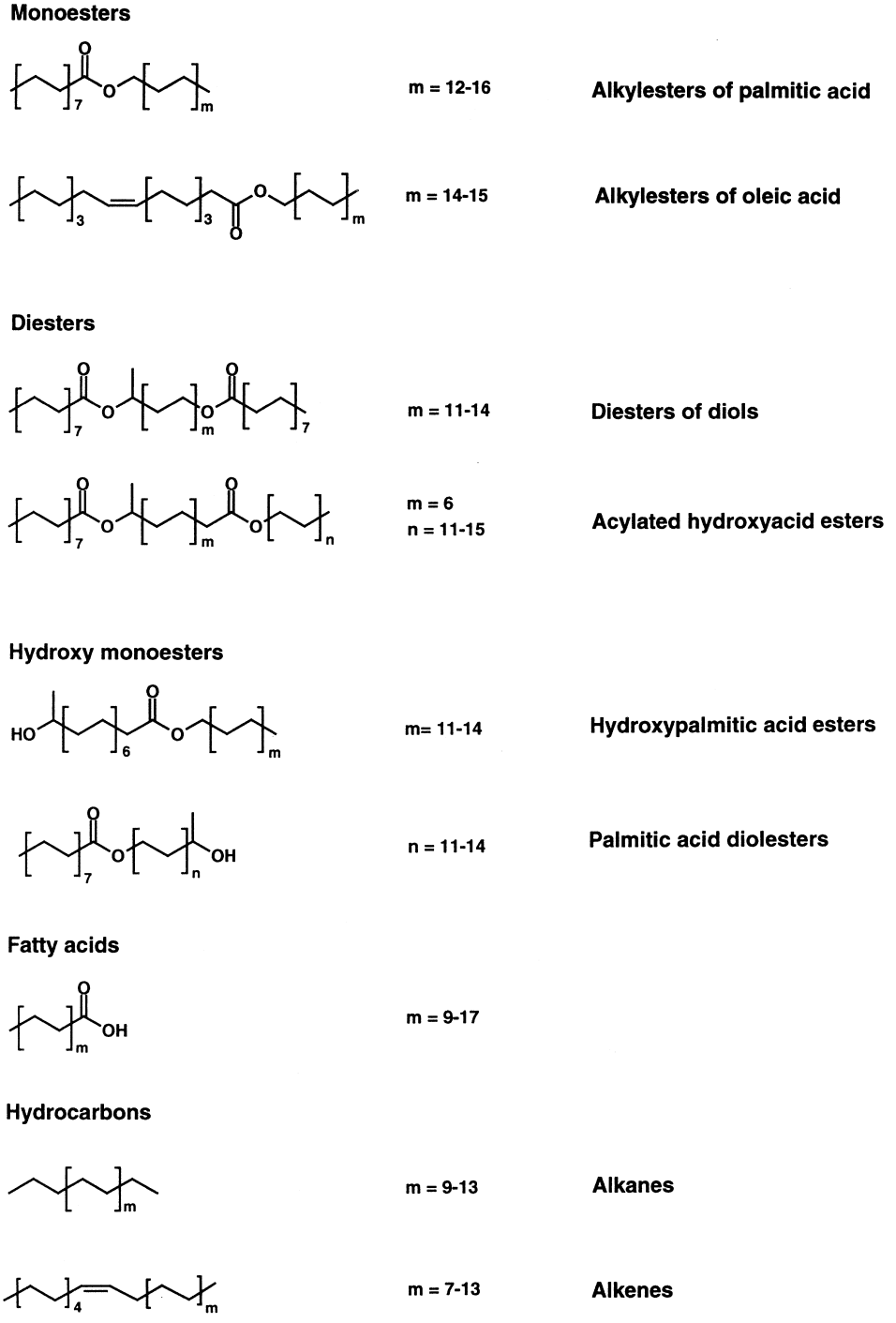


Fig. 1. Structures of the principal homologous series of beeswax lipids.

Optimization of the derivatization procedure by means of the Donike test was carried out on 20 m×0.3 mm Duran glass capillary columns coated with a 0.15 μm film of poly(50% diphenyl/50% 1H,1H,2H,2H perfluorodecylmethyl)siloxane (SOP-50-PFD). The analyses of the individual combwaxes were performed on 10 m×0.2 mm Duran glass capillaries, coated with a 0.10 μm film of SOP-50-PFD, methoxysilane terminated [10]. The synthesis of the stationary phase, preparation of the columns, and testing of the column performance are described in detail elsewhere [10,13,16]. Hydrogen was used as carrier gas with a linear velocity of 0.5 m/s. The oven temperature was programmed as follows: 80–330°C at 5°C/min for the Donike test and 80–380°C at 6°C/min for the combwax analysis. Data acquisition was carried out on a Chromcard system (Carlo Erba).

2.2. Extended Donike test

The extended Donike test described by Blum [13] was used to study the stability of the trimethylsilyl ester (FA-TMS) and *tert*.-butyldimethylsilyl ester (FA-TBDMS) of fatty acids, respectively.

In order to develop a two-step derivatization procedure a set of aliphatic alcohols and 12-hydroxystearic acid were added to the extended Donike test. A 1-mg amount of each compound listed in Table 1 was dissolved in a mixture of 0.8 ml pyridine and 0.8 ml isooctane. The test solution is unstable and has to be used immediately. To 0.1 ml of the clear solution 0.2 ml *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) or *N*-methyl-*N*-*tert*.-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) were added, respectively. In the case of MTBSTFA the sample was heated to 70°C for 20 min. The sample was finally diluted with 0.7 ml pyridine and an aliquot of 0.5 μl injected for analysis.

A two-step derivatization was carried out by adding 0.2 ml MTBSTFA to 0.1 ml test solution. The mixture was allowed to stand at ambient temperature for 3 min followed by adding 0.2 ml BSTFA. After 10 min 0.5 ml pyridine were added and a volume of 0.5 μl were injected for analysis.

2.3. Derivatization of combwax

In order to remove honey residues the combs were treated two times with water in an ultrasonic bath during 20 min, then they were rinsed with a small amount of ethanol and dried at 50°C.

A 5-mg amount of crude beeswax was dissolved in a mixture of 0.5 ml dry pyridine and 0.3 ml MTBSTFA and heated to 70°C for 5 min. Then 0.3 ml BSTFA was added to the hot solution and heated for 5 min at 70°C. The hot derivatization solution was filtered over a 0.5 μm, 13 mm Millex FH_{13} filter cartridge (Millipore, Bedford, MA, USA). A 100-μl volume of this solution was diluted with 500 μl isooctane and an aliquot of 0.5 μl was injected for GC analysis.

2.4. Chemicals and samples

BSTFA, MTBSTFA and all solvents were purchased from Fluka (Buchs, Switzerland). The compounds of the extended Donike test are available from Fluka, except for 1-tetracosanol, 1-octacosanol and 12-hydroxystearic acid, which were obtained from Sigma (Steinheim, Germany; see Table 1). The combwax of the following honey bee species was

Table 1
Composition of the extended Donike test and abbreviations of the individual derivatized compounds (see Fig. 3)

Alkane		Alcohol		Fatty acid	
Name	Abbreviation	Name	Abbreviation	Name	Abbreviation
Hexadecane	C_{16}	1-Dodecanol	A_{12}-TMS	Capric acid	FA_{10}-TBDMS
Eicosane	C_{20}	1-Hexadecanol	A_{16}-TMS	Myristic acid	FA_{14}-TBDMS
Tetracosane	C_{24}	1-Eicosanol	A_{20}-TMS	Stearic acid	FA_{18}-TBDMS
Octacontane	C_{28}	1-Tetracosanol	A_{24}-TMS	Behenic acid	FA_{22}-TBDMS
Dotriacontane	C_{32}	1-Octacosanol	A_{28}-TMS	Cerotic acid	FA_{26}-TBDMS
Hexatriacontane	C_{36}			Melissic acid	FA_{30}-TBDMS
				12-Hydroxystearic acid	TMSO-FA_{18}-TBDMS

investigated: *A. mellifera*, *A. cerana*, *A. dorsata* (Kathmandu, Nepal); *A. laboriosa* (Kodari, Nepal); *A. florea* (Chiang Rai, Thailand); *A.* andreniformis (Borneo, Indonesia).

The collection of the wax samples was supported by local apiarists and/or honey hunters.

3. Results and discussion

3.1. Derivatization

In some instances derivatization of compounds containing exchangeable hydrogen atoms is required prior to HTGC analysis. In practice not only the thermal stability of the analytes is of importance but also the remaining catalytic activity of the capillary columns at elevated temperatures [13]. Some constituents of beeswax lipids contain aliphatic hydroxy groups and aliphatic carboxylic acid groups besides other functional groups. Therefore, an appropriate derivatization is unavoidable.

The different chemical reactivity of the functional groups in context with the stability of the resulting derivative can cause problems during the development of a successful derivatization procedure. Trimethylsilylation is well known for both, aliphatic hydroxy groups and aliphatic carboxylic groups. One drawback of trimethylsilylation is the insufficient thermal stability of long-chain FA-TMS esters at temperatures above 300°C. Blum described the use of an extended Donike test and demonstrated the excessive degradation of the FA-TMS esters on cyanopropyl phases [13]. The column activity was expressed by the activity factor *Q*, which was calculated using the formula shown in Fig. 2. Recently we reported the column performance of SOP-50-PFD-coated Duran glass capillary columns [10]. The FA_{30}-TMS ester shows some unwanted degradation. Therefore a more stable derivative is desirable for the analysis of fatty acids with a carbon number >30. The improvement of the thermal stability of FA-TBDMS ester in comparison to the thermal stability of the respective TMS esters was tested. The extended Donike test was derivatized with BSTFA and MTBSTFA, respectively, which leads to FA-TMS and FA-TBDMS esters. The calculated activity factors (*Q*) versus elution temperature of both fatty acid derivatives are shown in Fig. 2. According to earlier results the FA-TMS

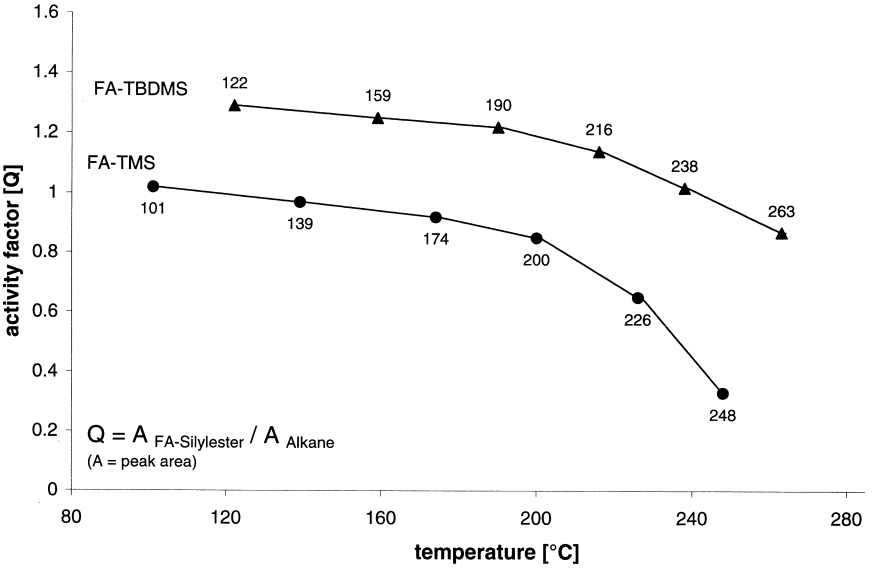


Fig. 2. Activity factors (*Q*) of TMS esters of fatty acids and TBDMS esters of fatty acids obtained by the GC–FID analysis of the Donike test using a 20 m×0.3 mm Duran glass capillary column coated with a 0.15 μm film of poly(diphenyl/1H,1H,2H,2H-perfluorodecylmethyl)siloxane (SOP-50-PFD). Note the increase of thermal stability of the TBDMS esters in comparison to the TMS esters.

esters are subjected to decomposition at temperatures above 200°C. Only a slight decomposition of the FA-TBDMS esters can be observed on the SOP-50-PFD-coated column.

The derivatization of aliphatic alcohols with MTBSTFA requires higher temperatures and/or the addition of *tert.*-butyldimethylchlorosilane to enhance the silylation power of the reagent [17] (e.g., the quantitative derivatization of beeswax constituents with aliphatic hydroxy groups using MTBSTFA was achieved only after heating to 70°C during 16 h [10]). A second drawback of TBDMS derivatives is the distinct increase of the elution temperature (see Fig. 2) in comparison to the respective TMS derivatives. In order to reduce both, the elution temperature of the compounds containing aliphatic hydroxy groups and the time of sample preparation, a two-step derivatization procedure was applied according to the method described by Woollard [18]. This method yields TMS ethers of fatty alcohols and TBDMS esters of fatty acids.

In order to optimize this derivatization procedure, the extended Donike test was modified by addition of a set of aliphatic 1-alcohols and 12-hydroxystearic acid. In a first step MTBSTFA was added to the test mixture to derivatize the more reactive carboxylic groups. In a second step BSTFA was added yielding TMS ethers of the aliphatic hydroxy groups. In order to exclude undesirable side reactions, the success of the derivatization procedure was checked by GC–MS analyses. The resulting chromatogram of the final procedure is shown in Fig. 3. The TMS ether derivatives of the fatty alcohols (A-TMS) show no significant reduction of the peak area even for the higher homologues (e.g., A_{26}-TMS in Fig. 3). An additional indication of the selective derivatization is the complete conversion of 12-hydroxystearic acid to 12-trimethylsilyloxystearic acid-*tert.*-butyldimethylsilyl ester. This two-step derivatization combines both, the advantage of a more stable FA-TBDMS derivative and lower retention times of TMS ethers of compounds containing aliphatic hydroxy groups.

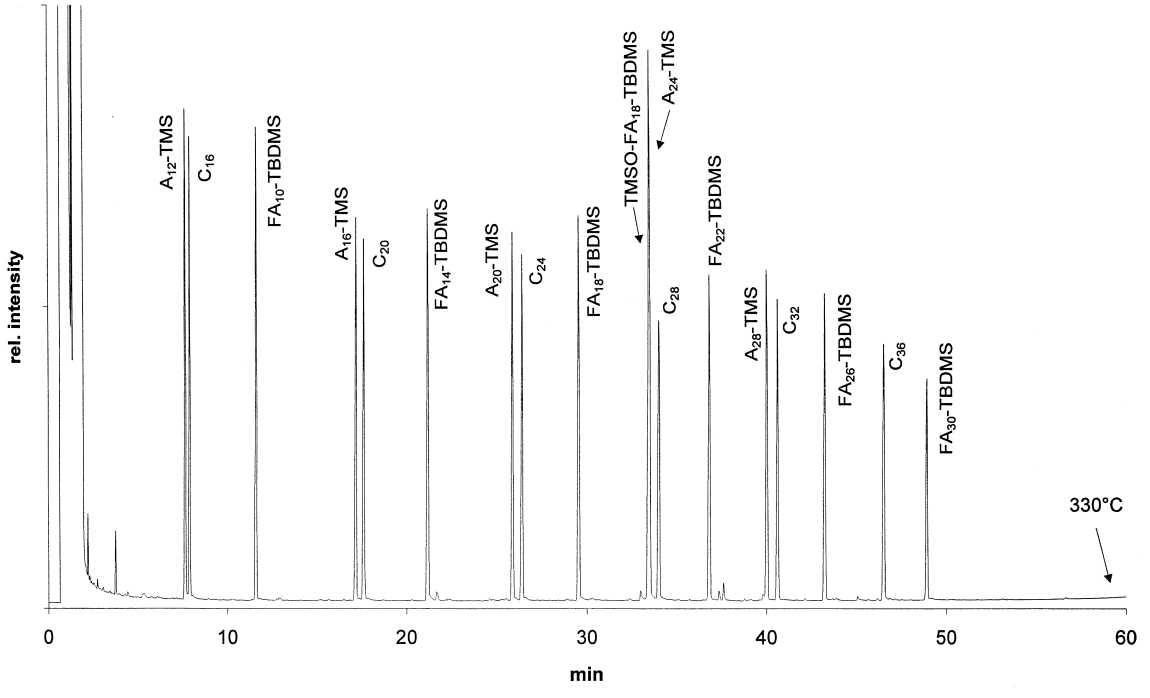


Fig. 3. Extended Donike test gas chromatogram on a 20 m×0.3 mm Duran glass capillary column coated with a 0.15 μm film of poly(diphenyl/1H,1H,2H,2H-perfluorodecylmethyl)siloxane (SOP-50-PFD) conditioned at 330°C (0.5 m/s hydrogen, constant flow, 80–330°C, program rate 4°C/min, on-column injection). The composition of the extended Donike test mixture is shown in Table 1.

3.2. Solubility of combwax

A general problem is the poor solubility of long chain aliphatic lipid compounds in organic solvents. Heterogeneous solutions can cause discriminations during derivatization and finally in the analysis. The use of alkanes (e.g., hexane, isooctane) or halogenated solvents (e.g., chloroform, methylene chloride) in presence of silylation agents, was less successful. Even after heating and/or ultrasonic treatment insoluble constituents remained. The heating of the silylation mixture to 80°C, using pyridine as solvent results in a clear solution. After cooling to ambient temperature the appearance of a precipitate was observed. Therefore, the final dilution prior to analysis must be prepared with the hot silylation mixture.

Crude combwax produced by the honeybees is of different optical appearance. The usual colors varied between white and dark brown. In particular brown colored waxes contain more insoluble constituents of unknown nature, which must be filtered from the hot solution prior to HTGC analysis.

3.3. Stationary phase

Polysiloxane-coated capillary columns are successfully used in the characterization of complex natural lipid mixtures by GC. The selectivity of different stationary phases were recently utilized for the analysis of wax esters, e.g., Refs. [19,20], and steryl esters [21–23]. The separation of compounds within each carbon number group into species with different chain lengths (e.g., 14:0–14:0 and 10:0–18:0) or different degrees of unsaturation have been established using polysiloxanes containing methyl, phenyl and/or cyanopropyl side chains bonded to the polymer backbone. The separation of analytes with different chain length or number of double bonds is just one of the objectives in the characterization of natural waxes. An additional approach is the detection of intact wax components with a molecular mass up to approximately 1000 g/mol. For this reason, the dimensions of the column will be a compromise between the optimum in terms of resolution and the minimized exposure of the analytes at high temperatures. With respect to these requirements, the use of 10 m×0.2 mm columns, coated with a 0.10 μm film of stationary phase, is recommended. For lipid analysis by HTGC, we recently described the use of poly(50% diphenyl/50% 1H,1H,2H,2H-perfluorodecylmethyl)siloxane. This stationary phase shows extraordinary low retention temperatures for apolar lipids like triglycerides and wax esters [11]. Unfortunately, owing to the comparably low selectivity to double bonds, no separation of saturated and unsaturated wax esters was observed. The SOP-50-PFD enabled the investigation of the homologues of diesters and hydroxydiesters at temperatures between 360 and 380°C. Using this phase, under convenient chromatographic conditions, approximately 90% of the volatile beeswax constituents could be characterized by HTGC (Figs. 4–9).

3.4. Combwax analysis

The combwax of the individual honeybee species was investigated with HTGC. In general a characteristic elution pattern of each *Apis* species was observed. This elution order is in good agreement with earlier investigations [5,8]. Alkanes, alkenes, free fatty acids, monoesters, diesters and hydroxymonoesters are the predominant compound families. Fatty alcohols and hydroxydiesters are minor constituents. The concentration of the individual components was derived from the uncorrected peak areas (Table 2). According to the results of Tulloch only a few of the characterized compounds exceed 5% values [5]. These compounds are defined below as major compounds. Some of the peaks are mixtures of compounds with the same carbon number, e.g., saturated and monounsaturated wax esters were eluted in the same peak. Therefore, in Table 2 only the carbon numbers and not the systematic name of the individual wax constituents were used.

3.4.1. Hydrocarbons

Stránsky and Streibl reported three homologous series of alkanes, in which the odd chain *n*-alkanes with a chain length of C_{23}–C_{31} are the most dominant compounds [7]. The degree of unsaturation increases with the chain length of the hydrocarbons. In this study a total content of about 11 to 28% hydrocarbons in beeswax was established. The alkane C_{27} exceeds 5% in the waxes of *A. mellifera*,

Table 2
Comparison of the compound composition of derivatized combwax of *A. mellifera*, *A. cerana*, *A. florea*, *A. andreniformis*, *A. dorsata* and *A. laboriosa* by GC–FID analysis on a SOP-50-PFD column (the peak numbers correspond to Figs. 4–9)

Structure	Peak	*A. mellifera*	*A. cerana*	*A. florea*	*A. andreniformis*	*A. dorsata*	*A. laboriosa*
Alkane C_{23}	1	0.4			1.1	0.4	0.3
n.i. (carbohydrate ?)	2	0.3		0.4	0.5	0.3	0.5
Alkane C_{25}	3	1.5	0.9	1.5	7.0	4.3	3.8
n.i. (carbohydrate ?)	4			0.4	0.3		0.3
n.i. (carbohydrate ?)	5			0.4	0.6		
n.i. (carbohydrate ?)	6			0.3	0.3		0.3
n.i. (carbohydrate ?)	7				0.3		
Alkene C_{27}	8			0.6	0.5		
n.i.	9				0.6		
Alkane C_{27}	10	6.2	8.2	6.3	4.9	3.6	3.6
n.i.	11	0.8			0.9	0.6	
n.i.	12					0.4	
Fatty acid C_{20} ?	13	1.1			0.8	0.8	
n.i.	14	0.4			0.3	0.3	
n.i.	15			0.5	0.4		
Alkene C_{29}	16		0.6	1.0	1.0		
Alkane C_{29}	17	2.6	2.3	3.0	2.8	1.2	1.7
n.i.	18					0.3	
Fatty acid C_{22}	19	0.7				0.3	0.4
n.i.	20						0.4
Alkene C_{31}	21	0.8		2.3			0.3
Alkane C_{31}	22	1.5		1.2	1.8	0.9	1.0
n.i.	23					0.3	
Fatty acid C_{24}	24	6.0				1.4	0.7
Alkene C_{33}	25	2.1	0.4	3.0		0.6	1.9
Alkane C_{33}	26	0.3		0.5	0.5	0.4	0.4
Fatty acid C_{26}	27	2.1	0.5				
Diene C_{35}	28				0.4		
Alkene C_{35}	29		5.4	0.6	1.0		1.7
Alkane C_{35}	30	0.3			0.4		
Fatty acid C_{28}	31	2.6	1.2	0.4	0.5		
Fatty alcohol C_{33}	32	0.3	1.8	0.4			
Diene C_{37}	33				0.9		
Alkene C_{37}	34		1.0		1.4		0.8
Fatty acid C_{30}	35	2.1	1.9	0.4	0.4		
Fatty alcohol C_{35}	36	0.3					
Diene C_{39}	37				1.1		
Alkene C_{39}	38				1.3		0.6
Fatty acid C_{32}	39	1.6			0.2	0.3	0.6
Diene C_{41}	40				1.0		
Alkene C_{41}	41				0.7		
Monoester C_{38}	42					0.5	0.7
Fatty acid C_{34}	43	1.5			0.3	1.4	1.8
Monoester C_{40}	44	6.6	0.7	1.5	1.3	26.8	24.9
n.i.	45				0.3		
Fatty acid C_{36}	46	0.3			0.4	0.7	0.8
Monoester C_{42}	47	4.6	0.9	3.4	1.5	4.7	4.5
Hydroxymonoester C_{40}	48				0.4	3.3	2.3
Hydroxymonoester C_{40}	49	0.9			0.4	9.6	8.4
Monoester C_{44}	50	5.7	4.8	9.7	7.7	0.7	1.0
Hydroxymonoester C_{42}	51					4.0	4.5

Table 2. (*Continued*)

Structure	Peak	*A. mellifera*	*A. cerana*	*A. florea*	*A. andreniformis*	*A. dorsata*	*A. laboriosa*
Hydroxymonoester C_{42}	*52*	*0.8*	*0.4*	*0.4*	*0.8*	*2.5*	*2.6*
Monoester C_{46}	53	11.9	23.7	17.0	10.7	0.9	1.6
Hydroxymonoester C_{44}	54		2.8			1.3	1.3
Hydroxymonoester C_{44}	55	1.8		3.3	4.3	0.5	0.6
Monoester C_{48}	56	9.0	2.2	7.3	4.7	1.7	2.7
Hydroxymonoester C_{46}	57	0.9	9.2			0.4	0.4
Hydroxymonoester C_{46}	58	2.3		2.9	4.7	0.3	0.4
n.i.	59		0.5	0.4	0.6		
Monoester C_{50}	60	2.6	0.6	1.8	1.3	1.2	1.6
Hydroxymonoester C_{48}	61	0.6	4.4			0.3	0.5
Hydroxymonoester C_{48}	62	1.6		1.5	1.9	0.5	0.9
Monoester C_{54}	63	0.4	0.5	0.4	0.3	0.4	0.5
Hydroxymonoester C_{50}	64		0.5		0.8	0.3	0.7
Hydroxymonoester C_{50}	65	0.3	0.8	0.7		0.3	0.5
Hydroxymonoester C_{52}	66			0.3	0.3		0.5
Diester C_{54}	67					1.0	0.6
Diester C_{54}	68	1.2		0.7	0.7	5.6	4.1
Diester C_{56}	69					1.0	0.9
Diester C_{56}	70	1.2	0.6	1.0	1.0	2.4	2.0
Hydroxydiester C_{50}	71		0.7		0.4	1.0	0.7
Diester C_{58}	72			0.8	0.6	0.5	0.3
Diester C_{58}	73	1.4	2.3	5.2	4.2	1.0	0.9
Hydroxydiester C_{52}	74				0.6	0.4	0.4
Diester C_{60}	75		1.1	1.1	0.9		
Diester C_{60}	76	2.0	5.3	4.2	3.4	0.4	
Hydroxydiester C_{54}	77		1.0	1.1	1.6		
Diester C_{62}	78		0.7	0.7			
Diester C_{62}	79	1.2	1.6	1.7	1.6		
Hydroxydiester C_{56}	80		1.0	0.6	0.9		
Diester C_{64}	81	0.4	0.6	0.3	0.5		
Hydroxydiester C_{58}	82		0.3	0.6	0.4		
Total		93.2	91.4	91.8	93.4	92.0	92.9

n.i.=Not identified.

A. cerana and *A. florea*. In the wax of *A. andreniformis* the most dominant alkane is pentacosane ($C_{25}H_{52}$, 7%).

Furthermore, Stránsky and Streibl identified six homologous series of monounsaturated alkenes in the combwax of *A. mellifera* [7]. The most common homologues were identified as odd chain alkenes (C_{27}–C_{39}) with a *cis* double bond at position C_{10}. Upon the results of this study the situation of the unsaturated hydrocarbons seems to be more complex. Some of the alkene peaks are broadened (e.g., peak 29 in Fig. 5) due to the insufficient selectivity of the SOP-50-PFD for the separation of the positional isomers and the *cis*/*trans* isomers, respectively. Therefore, the exact alkene composition in all investigated combwaxes remains open. Heptatriacontene ($C_{37}H_{74}$) was found to be a major compound in the comb wax of *A. cerana* (5.4%). The combwax of *A. dorsata* can be distinguished from the wax of *A. laboriosa* by a comparable low content of alkenes (0.6% in *A. dorsata* versus 5.3% in *A. laboriosa*).

An exception is the wax of *A. andreniformis*. An increase of the chain length of both, the alkanes (up to C_{35}) and alkenes (up to C_{39}) was observed. Additionally, both homologous series were accompanied by a set of various dienes (C_{35}–C_{41}). Due to the broadened peaks (e.g., see peak 39 in Fig. 7) it can be assumed that the composition of the dienes is also complex. The presence of dienes in beeswax was reported elsewhere but without exact determi-

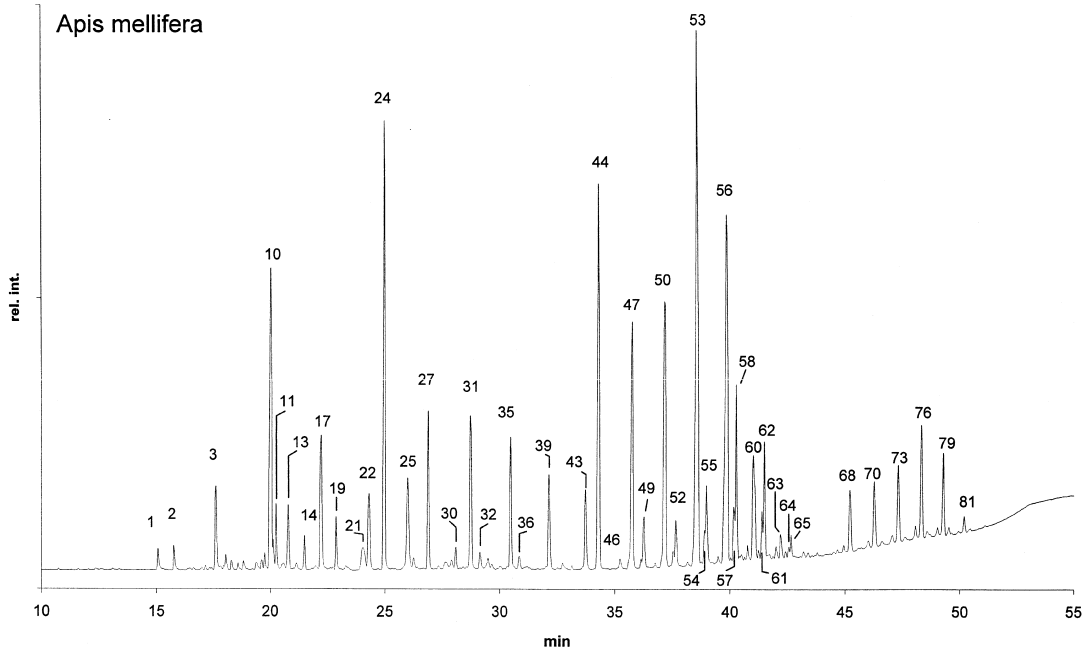


Fig. 4. GC–FID profile of TBDMS/TMS-derivatized crude combwax of *Apis mellifera* on a 10 m×0.2 mm glass capillary column coated with a 0.10 μm film of SOP-50-PFD (0.5 m/s hydrogen, constant flow, 70–380°C, program rate 6°C/min, on-column injection). For peak assignment and uncorrected peak areas, see Table 2.

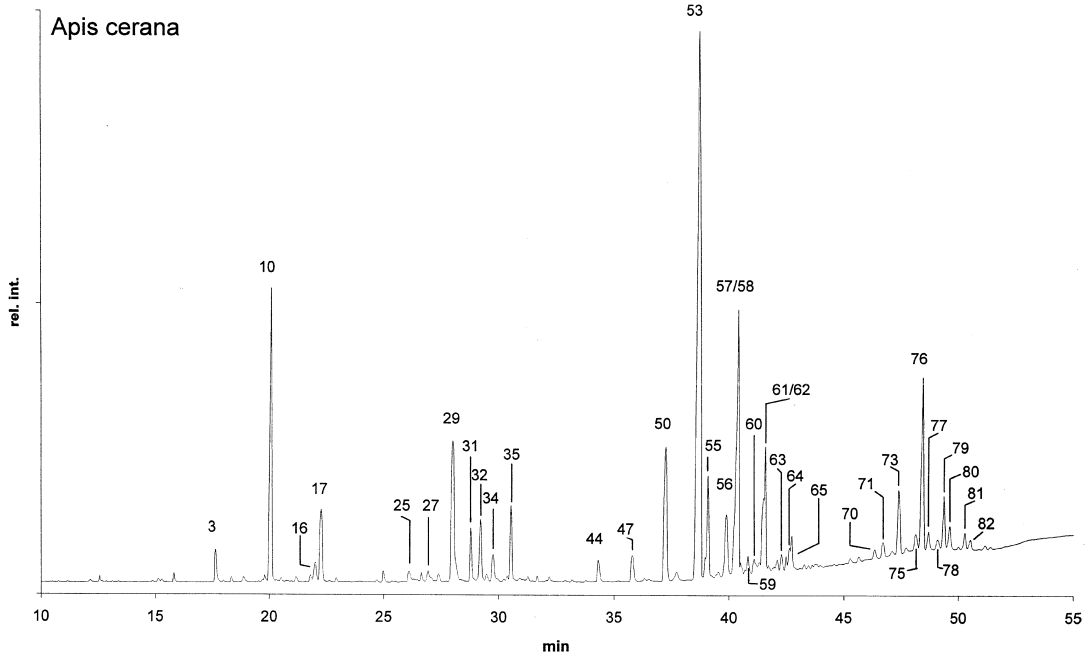


Fig. 5. GC–FID profile of TBDMS/TMS-derivatized crude combwax of *Apis cerana* on a 10 m×0.2 mm glass capillary column coated with a 0.10 μm film of SOP-50-PFD (0.5 m/s hydrogen, constant flow, 70–380°C, program rate 6°C/min, on-column injection). For peak assignment and uncorrected peak areas, see Table 2.

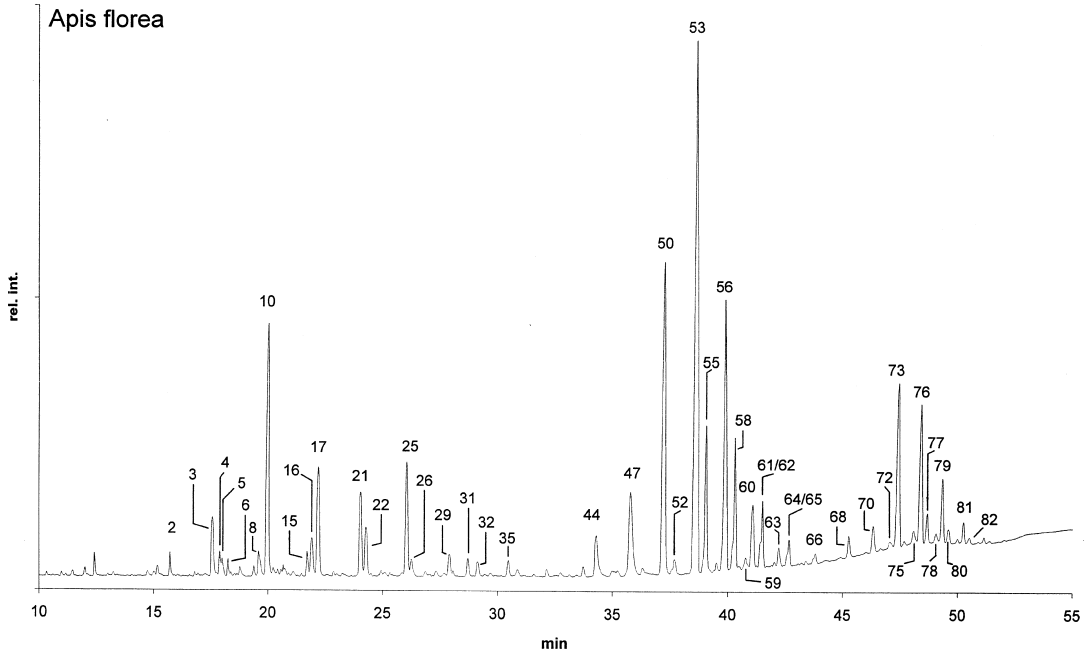


Fig. 6. GC–FID profile of TBDMS/TMS-derivatized crude combwax of *Apis florea* on a 10 m×0.2 mm glass capillary column coated with a 0.10 μm film of SOP-50-PFD (0.5 m/s hydrogen, constant flow, 70–380°C, program rate 6°C/min, on-column injection). For peak assignment and uncorrected peak areas Table 2.

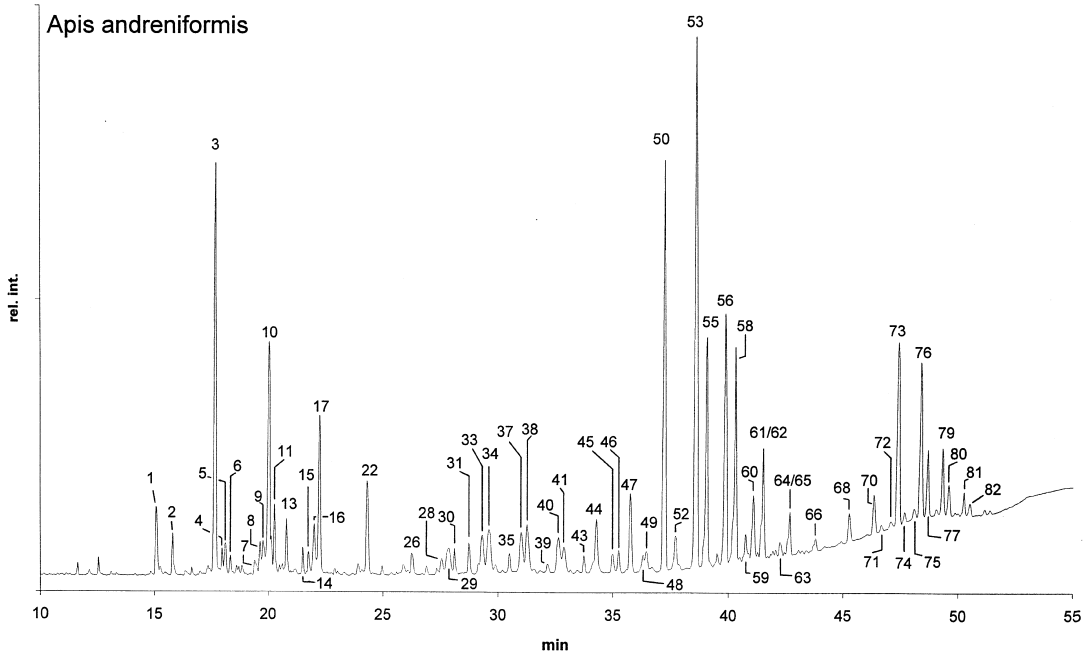


Fig. 7. GC–FID profile of TBDMS/TMS-derivatized crude combwax of *Apis andreniformis* on a 10 m×0.2 mm glass capillary column coated with a 0.10 μm film of SOP-50-PFD (0.5 m/s hydrogen, constant flow, 70–380°C, program rate 6°C/min, on-column injection). For peak assignment and uncorrected peak areas, see Table 2.

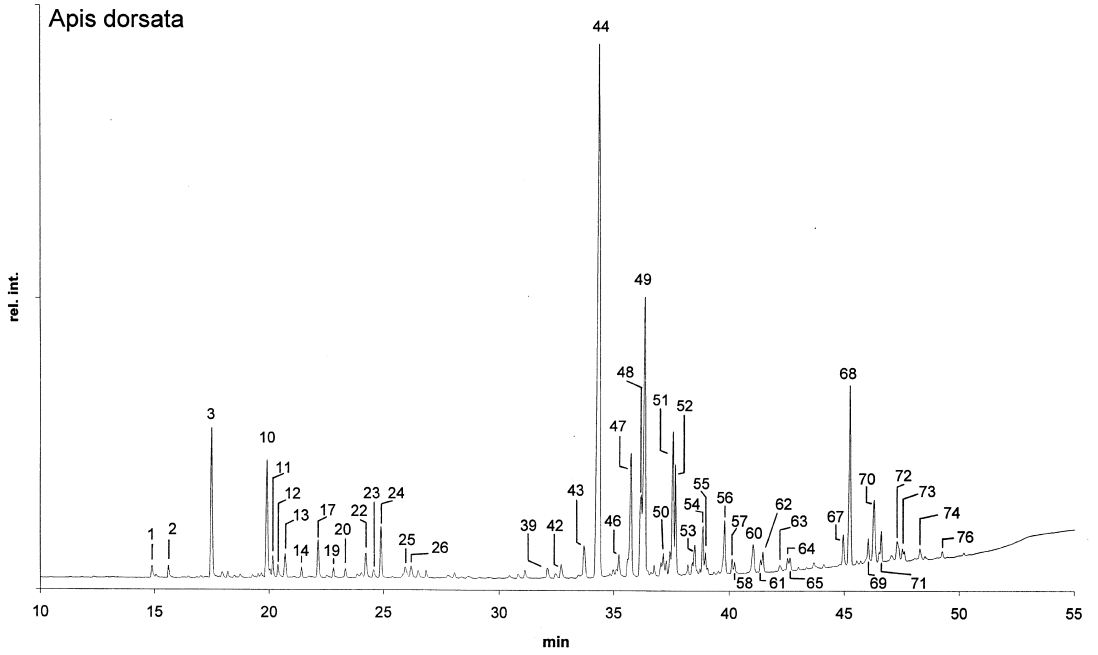


Fig. 8. GC–FID profile of TBDMS/TMS-derivatized crude combwax of *Apis laboriosa* on a 10 m×0.2 mm glass capillary column coated with a 0.10 μm film of SOP-50-PFD (0.5 m/s hydrogen, constant flow, 70–380°C, program rate 6°C/min, on-column injection). For peak assignment and uncorrected peak areas, see Table 2.

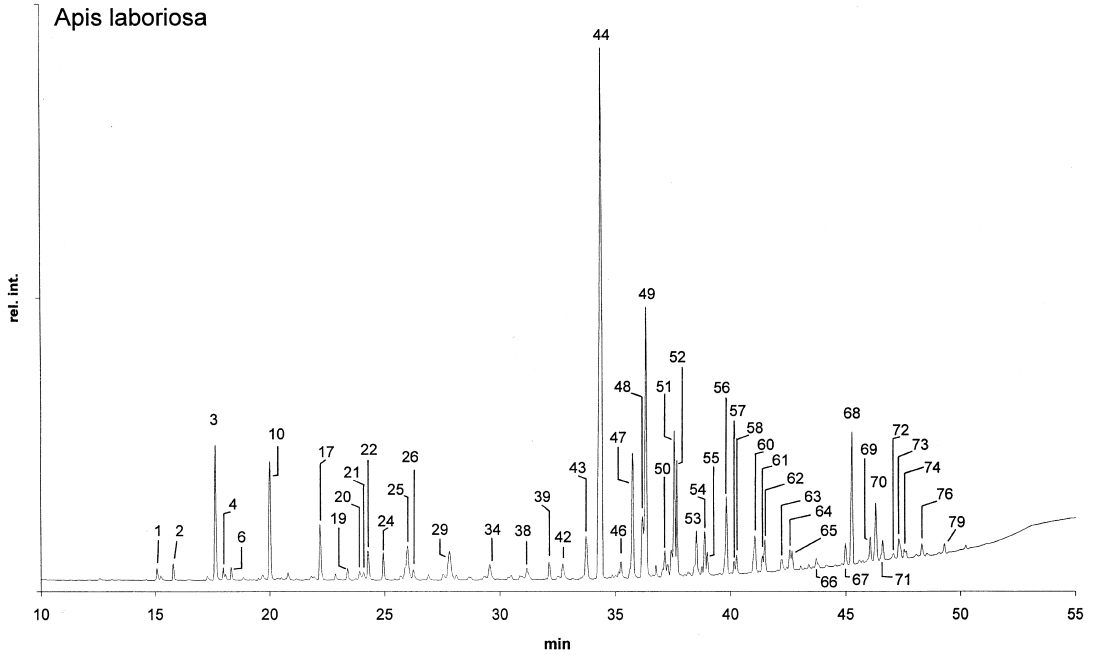


Fig. 9. GC–FID profile of TBDMS/TMS-derivatized crude combwax of *Apis dorsata* on a 10 m×0.2 mm glass capillary column coated with a 0.10 μm film of SOP-50-PFD (0.5 m/s hydrogen, constant flow, 70–380°C, program rate 6°C/min, on-column injection). For peak assignment and uncorrected peak areas, see Table 2.

nation of position and geometry of the double bonds [24].

3.4.2. Free fatty acids

Beeswax contains a homologous series of saturated, unbranched free fatty acids with an even carbon number. In this investigation the free fatty acids from C_{20} to C_{36} were identified as the respective TBDMS derivatives with a total amount of 1% (*A. florea*) to 18% (*A. mellifera*). The remarkably high content of fatty acids in combwax of *A. mellifera* (18%) is reported elsewhere [5]. The most abundant acid of the investigated wax sample of *A. mellifera* is tetracosanoic acid (5.8%). This finding is in agreement with other investigations [5,9].

3.4.3. Monoesters

The monoesters (see Fig. 1) are important compounds of the beeswax lipids. According to Tulloch, they comprise two types of structures: saturated wax esters, which are predominantly alkylpalmitates (C_{38}–C_{52}), and unsaturated esters which are long chain alkyl esters of oleic acid (C_{46}–C_{54}). As reported recently the separation of saturated and unsaturated wax esters with the same carbon number cannot be achieved on capillary columns coated with the stationary phase SOP-50-PFD [10]. The differentiation of the respective compounds was performed by HTGC–CI-MS using methane or ammonia as reagent gas [15].

The total content of the monoesters comprises between 26.9% (*A. andreniformis*) and 40.8% (*A. mellifera*) of the detected peak area. Major compounds are the monoesters C_{46} in *A. mellifera* (11.9%), *A. cerana* (23.7%), *A. florea* (17.0%), *A. andreniformis* (10.7%) and C_{40} in *A. dorsata* (26.8%) and *A. laboriosa* (24.9%).

3.4.4. Hydroxymonoesters

Tulloch characterized hydroxymonoesters in beeswax [25]. There are two types of hydroxyesters present: long chain alcohols, esterified by a hydroxy acid, mainly 15-hydroxypalmitic acid, or esterification of the primary hydroxy group of a diol, mainly with palmitic acid (see Fig. 1). This compound family was successfully determined by HTGC as TMS ether with a chain length in between C_{40} and C_{54}. Two isomers of hydroxymonoesters of each carbon number were identified with HTGC (e.g., peaks 50 and 51). Both structural isomers could be separated on the SOP-50-PFD phase.

The content of the hydroxymonoesters was between 9% (*A. mellifera*, *A. florea*) and 23% (*A. dorsata*, *A. laboriosa*). Two hydroxymonoesters are the major compounds: C_{40} in *A. dorsata* (9.6%) and *A. laboriosa* (8.4%) and C_{46} in *A. cerana* (9.2%).

3.4.5. Diesters

In beeswax two groups of diesters with a chain length between C_{54} to C_{64} are present. One group consists of diol diesters, the second group are acylated hydroxyesters (see Fig. 1) [25]. In spite of their high molecular mass, the determination of the homologous series by HTGC is still possible. The total content of diesters in beeswax is in the range 7% (*A. mellifera*) to 16% (*A. florea*), whereby the diesters C_{54} (*A. dorsata*, 5.6%), C_{58} (*A. florea*, 5.2%), and C_{60} (*A. cerana*, 5.3%) are major compounds. Similar to the hydroxyesters, two types of diesters within nearly all carbon numbers could be distinguished.

3.4.6. Hydroxydiesters

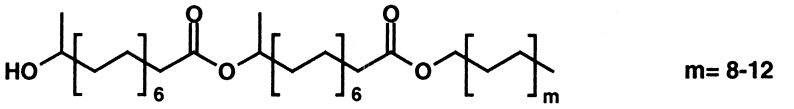
Hydroxydiesters were never reported before and are found in this study in a range between $<0.3\%$ (*A. mellifera*) and 3.9% (*A. andreniformis*). This type of complex esters was analyzed as TMS ether derivatives and they have a chain length between C_{50} and C_{58}. Presumably this class of compounds also consists of two types of structures. Both types of hydroxymonoesters, hydroxypalmitic acid esters and palmitic acid diolesters, are acylated by hydroxypalmitic acid (see Fig. 10). This homologous series appears on the chromatogram in the same region as the diester series.

3.4.7. Fatty alcohols

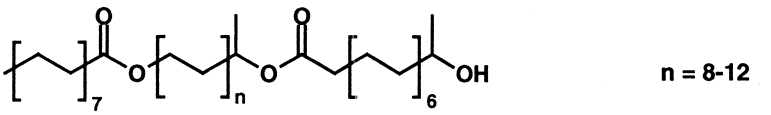
Free fatty alcohols are minor compounds [26]. Two homologous compounds were identified in the wax of *A. mellifera*: C_{33} (0.3%) and C_{35} (0.3%). Moreover in *A. cerana* (1.8) and *A. florea* (0.4%) the C_{33} alcohol was identified.

3.5. Taxonomical considerations

The detailed investigation of the combwax of the species *A. mellifera*, *A. florea*, *A. andreniformis*, *A. dorsata*, *A. laboriosa* and *A. cerana* show remark-



Hydroxypalmitic acid esters acylated by hydroxypalmitic acid



Palmitic acid diolesters acylated by hydroxypalmitic acid

Fig. 10. Proposed structures of the hydroxydiesters.

able differences in the composition. The content of the individual compound classes is, apart of some exceptions, not very expressive (see Table 3). A doubtless more typical feature of the individual honeybee species is the characteristic elution pattern of the individual homologous series (Figs. 4–9). Thirty-four wax samples of *A. mellifera*, five of *A. cerana* and three of *A. dorsata* were analyzed with HTGC and a nearly identical elution pattern of each species was observed. Brand-Garnys and Sprenger reported similar results in an investigation of waxes of different races of the species *A. mellifera* [8]. The authors observed that races of honeybees can only be distinguished after careful calculation of the peak area ratio of selected compounds. Referring to these results and the results of our study, we suppose that the combwax of *A. koschevnikovi*, *A. nuluensis* and *A. nigrocincta* show also a specific chromatographic elution pattern. In order to complete the investigation and confirm the above mentioned statements the

Table 3
Content of the individual principle compound families of combwax of *A. mellifera*, *A. cerana*, *A. florea*, *A. andreniformis*, *A. dorsata* and *A. laboriosa*

Compound family	*A. mellifera*	*A. cerana*	*A. florea*	*A. andreniformis*	*A. dorsata*	*A. laboriosa*
Alkanes total	12.8	11.4	12.5	18.5	10.8	10.8
Alkenes total	2.9	7.4	7.5	5.9	0.6	5.3
Diene total	–	–	–	3.4	–	–
Hydrocarbons total	15.7	18.8	20.0	27.8	11.4	16.1
Fatty acids total	18.0	3.6	0.8	2.6	4.9	4.3
Fatty alcohols total	0.6	1.8	0.4	–	–	–
Monoesters total	40.8	33.4	41.1	27.5	36.9	37.5
Hydroxymonoesters total	9.2	18.1	9.1	13.6	23.3	23.6
Diesters total	7.4	12.2	15.7	12.9	11.9	8.8
Hydroxydiesters total	–	3.0	2.3	3.9	1.4	1.1
Esters total	57.4	66.7	68.2	57.9	73.5	71.0
Total	91.7	90.9	89.4	88.3	89.8	91.4

HTGC analyses of the combwax of these species must still be carried out. An exception is the differentiation of the species *A. dorsata* and *A. laboriosa*, since both waxes show nearly identical elution patterns. Only the low content of alkenes (<2%) in the combwax of *A. dorsata* can be taken into consideration for the differentiation from *A. laboriosa* (see also Table 3). Therefore a clear delimitation of the taxonomic rank of *A. dorsata* and *A. laboriosa* on the basis of HTGC analyses is difficult and should be corroborated by the analysis of additional wax samples.

4. Conclusions

This work demonstrates the feasibility of the investigation on crude beeswax by HTGC. The combwax of six honeybee species (*Apis* spp.) was examined using tailor-made glass capillary columns. Combwax consists of a complex mixture of neutral lipids and the characterization of the intact compounds needs a careful optimized analytical method. The two-step derivatization procedure applied enables the simultaneous detection of complex wax esters like diesters, hydroxyester and hydroxydiesters, beside alkanes, alkenes, fatty acids and monoesters, even at elevated temperatures. With the aid of HTGC nearly 90% of the volatile constituents could be characterized and the uncorrected peak areas of more than 80 compounds were registered. The combwax of *A. andreniformis* was analyzed for the first time. In this combwax dienes were identified as one of the principal compound series.

Referring to the results of this study, the use of HTGC allows the unequivocal and rapid differentiation of individual honeybee species by means of their typical peak pattern of the combwax they produce.

Acknowledgements

The authors are grateful to Dr. H. Pechhacker, Institute of Apiculture, University of Vienna; Lunz am See, Austria, for donating the beeswax samples. The combwax of *Apis andreniformis* was kindly provided by Professor Dr. N. Königer, Institute of Apiculture, University of Frankfurt, Germany.

References

[1] C. Heron, N. Memcek, K.M. Bonfield, D. Dixon, B.S. Ottaway, Naturwissenschaften 81 (1994) 266.
[2] F. Ruttner, Naturgeschichte der Honigbienen, Ehrenwirth, Munich, 1992.
[3] G. Königer, Bienenvater 118 (1998) 9.
[4] F. Ruttner, in: Biogeography and Taxonomy of Honeybees, Springer, Berlin, 1988, p. 10.
[5] A.P. Tulloch, Bee World 61 (1980) 47.
[6] K. Stránsky, M. Streibl, Coll. Czech. Chem. Commun. 36 (1971) 2267.
[7] K. Stránsky, M. Streibl, F. Sorm, Fette, Seifen, Anstrichmittel 68 (1966) 799.
[8] E.E. Brand-Garnys, J. Sprenger, Seifen, Öle, Fette, Wachse 114 (1988) 552.
[9] H. Brüschweiler, H. Felber, F. Schwager, Fat Sci. Technol. 91 (1989) 73.
[10] R. Aichholz, E. Lorbeer, J. Microcol. Sep. 8 (1996) 553.
[11] R. Aichholz, E. Lorbeer, J. High Resolut. Chromatogr. 21 (1998) 363.
[12] M. Donike, Chromatographia 6 (1973) 190.
[13] W. Blum, J. High Resolut. Chromatogr. 8 (1985) 719.
[14] S.B. Hawthorne, D.J. Miller, J. Chromatogr. 388 (1987) 397.
[15] R. Aichholz, E. Lorbeer, J. Chromatogr., manuscript in preparation.
[16] W. Blum, R. Aichholz, in: Hochtemperatur Gas Chromatographie, Hüthig, Heidelberg, 1991, pp. 8–41.
[17] K.D. Ballard, D.R. Knapp, J.E. Oatis, T. Walle, J. Chromatogr. 277 (1983) 333.
[18] P.M. Woollard, Biomed. Mass Spectrom. 10 (1983) 143.
[19] T. Rezanka, M. Podojil, J. Chromatogr. 355 (1986) 399.
[20] Y. Itabashi, T. Takagi, J. Chromatogr. 299 (1984) 351.
[21] N. Limsathayourat, H.-U. Melchert, Fres. Z. Anal. Chem. 318 (1984) 410.
[22] R.P. Evershed, L.J. Goad, Biomed. Environ. Mass Spectrom. 14 (1987) 131.
[23] V.O. Elias, B.R.T. Simoneit, A.S. Pereira, J.N. Cardoso, J. High Resolut. Chromatogr. 21 (1998) 87.
[24] A.G. Giumanini, G. Verardo, P. Strazzolini, H.R. Hepburn, J. Chromatogr. A 704 (1995) 224.
[25] A.P. Tulloch, Chem. Phys. Lipids 6 (1971) 235.
[26] S.L. Puleo, Cosmetics Toiletries 106 (1991) 83.