Method Development for the Analysis of Bee Cuticular Hydrocarbons and Esters by Capillary Gas Chromatography

John Ogden, Claude Pardo*, and **Alain Tchapla**
LETIAM, IUT d'Orsay, Plateau du Moulon, 91400 ORSAY, France

Abstract

This paper describes a method for the determination of honeybee cuticular hydrocarbon and ester composition from a single run using capillary gas chromatography. This method is designed to provide information on individual honeybee profiles with the view to map the quantitative trait loci (QTL) that control their variability. The first part of this work concerns the choice of chromatographic parameters, particularly the comparison of three types of injector: the split/splitless, the on-column, and the recently developed automated solid phase (ASP) injector. The second part refers to the reliability of the results with respect to the above-mentioned objective.

Introduction

The extraction, separation, and identification of honeybee cuticular waxes have recently been reviewed (1). Component extraction has generally been with pentane, hexane, or ethyl acetate, fractionated sometimes by thin-layer or column chromatography prior to being analyzed by packed or capillary gas chromatography (GC) (2–5). Identification of complex mixtures has generally been by coupled mass spectroscopy (MS) (2–6). This work complements a study previously reported by Arnold et al. (5) who used capillary GC coupled to an MS to show that pentane extracts of five-day-old honeybees consisted mainly of C_{21}–C_{33} hydrocarbons, including saturated (linear and branched), unsaturated, and polyunsaturated hydrocarbons. The proportions of the major hydrocarbons were correlated with subfamilies and proposed as labels for subfamily recognition inside the colony. One of the actual objectives was to attempt to include the genes controlling the variation of cuticle hydrocarbon profiles in a global gene map of honeybees.

Anticipating the elution of the low-volatility hydrocarbons considered, we decided to use a capillary column containing an apolar stationary phase capable of operating at high temperatures. Knowing that the quantitative analysis of low-volatility compounds in G[illegible] crimination by the syringe in heated injectors, we decided to compare the split/splitless injector, the on-column injector, and the automated solid phase (ASP) injector, which is an improvement on the falling needle injector generally known as the Ros injector. The interest of this study was emphasized after the examination of the first chromatograms resulting from the combination of the on-column injector and the high-temperature stationary phase. As can be seen in Figure 1, significantly later than the higher molecular weight (MW) hydrocarbon peaks, we obtained the unexpected peaks of even lower-volatility compounds, identified later by coupled GC–MS mainly as palmitic esters of long-chain alcohols $C_{15}H_{31}COOC_nH_{2n+1}$ ($24 \leq n \leq 34$) also found in bees waxes (1). To our knowledge, both hydrocarbons and esters have never been properly separated by a single analysis.

Experimental

Samples

A total of 250 individual extracts (provided by INRA-CNRS [Bures sur Yvette, France] and CNRS laboratories [Gif sur Yvette, France]) of 15-day-old *Apis ligustica ligustica* and *Apis melifera melifera* honeybees and their hybrids were obtained by immersing each bee for 5 min in 1 mL of pentane after the removal of the wax plates. To prevent any evaporative loss of pentane during the extraction step, the glass vials were maintained at 4°C and closed by a PTFE cap. Samples were then stored at –18°C and raised to ambient temperature prior to the analyses.

Instrumentation

The GC was a Hewlett-Packard (Avondale, PA) 5890 series II model equipped with a flame-ionization detector (FID), a split/splitless injector, and an on-column injector fitted with the automated injections adapter. This adapter allowed the easy use of a wide-bore syringe needle that penetrated into the column after piercing a septum. The split/splitless injector

* Author to whom correspondence should be addressed.

Reproduction (photocopying) of editorial content of this journal is prohibited without publisher's permission.

was temporarily replaced by the ASP injector provided by ID Analytical Services (Poitiers, France). Structure identifications by coupled GC–MS were performed with a Varian (Walnut Creek, CA) 3400 GC equipped with an on-column injector and connected to a Finnigan Mat (San Jose, CA) INCOS 50 quadrupole MS.

Table I. Influence of On-Column Injector Temperature on Peak Widths*

On-column injection temperature (°C)	$\omega_{1/2}$ of C_{33}Hyd. (mm)	$\omega_{1/2}$ of C_{46}Est. (mm)
63 (oven track)	2.4	6.3
100	4.0	5.9
150	2.3	6.3
200	2.3	6.1
250	2.5	6.3
300	2.4	6.2
350	2.3	6.0

* Paper speed, 100 mm/min.

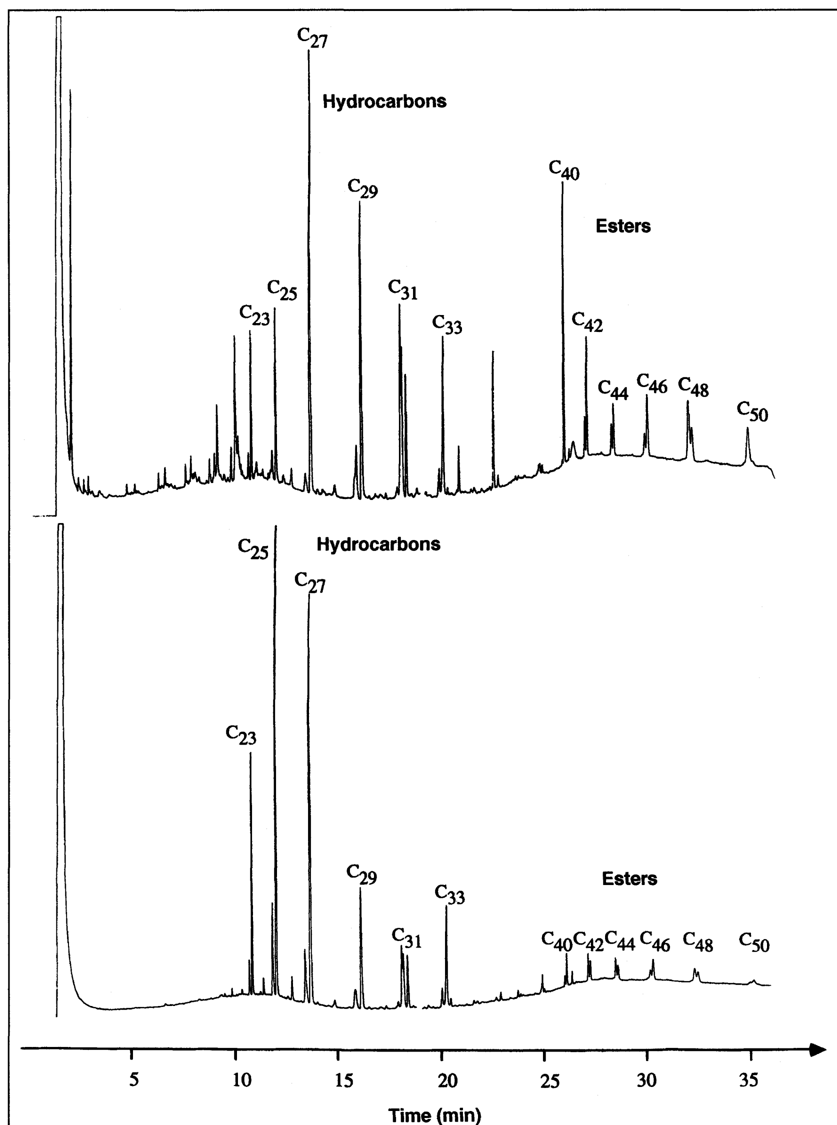

Figure 1. Chromatograms of two different bee extracts obtained with the on-column injector. Ester peaks (starting from C_{40}) were detected significantly later than the expected hydrocarbon peaks. Proportions of peak areas may differ notably from one bee to another.

Chromatographic conditions

The fused-silica capillary column was an SGE (Ringwood, Australia) HT 5 (25 m × 0.32-mm, 0.1-µm film thickness) with an upper temperature limit of 370°C (on cycle). The column was preceded by an SGE deactivated fused-silica retention gap (2 m × 0.53 mm) except when the ASP injector was used. The carrier gas was helium set at 72 kPa (constant pressure). This value was fixed to provide the predetermined optimum linear velocity (ca. 21 cm/s) at the end of the temperature oven program (360°C). Quantitative analyses were performed using a Shimadzu (Tokyo, Japan) CR4 AX integrator. The injection volume was 1 µL of each pentane bee extract.

To prevent injection time and space band-broadening when split/splitless or on-column injectors are used, it is often necessary to combine the solvent effect, thermal focusing by the stationary phase, and the use of a retention gap (7–10). Our extracts were in pentane; the chromatograph was not equipped with cryogenic cooling, so no solvent effect was expected. Starting the oven temperature program at 60°C and using an initial increase rate of 30°C/min to accelerate the elution of the the higher MW hydrocarbons and then using a second increase rate of 10°C/min until the temperature reached 360°C to separate them produced satisfactorily narrow peaks with both injectors.

To elute out of the column the maximum of the aforementioned low-volatility esters, 15 min at 360°C was added to the oven temperature program. The initial rate was also reduced to 20°C/min to minimize column bleeding, and another increase rate was inserted to separate a pair of peaks. The retained program to analyze our 250 extracts was as follows:

$$60°C\ (1\ min) \xrightarrow{20°C/min} 240°C \xrightarrow{1°C/min} 245°C \xrightarrow{10°C/min} 360°C\ (15\ min)$$

This program required 42 min, after which 15–20 min were necessary to cool down the oven and in particular the on-column injector when used in the "oven track mode" (this mode maintains the injector temperature at 3°C above the oven temperature).

Results and Discussion

Injection techniques

In an attempt to save time, an effort was made to reduce the time the on-column

injector took to cool from 363 to 63°C. First the "oven track mode" was disconnected to lower the final temperature reached by the on-column injector at the end of the oven temperature program. Second, the injector's starting temperature was increased. To ensure that the efficiency of the system was not affected by increasing the on-column injector's initial temperature, the peak width was measured at half height ($\omega_{1/2}$) for one of the hydrocarbon peaks and one of the ester peaks at different initial injector temperatures. The results shown in Table I are the averages of three analyses at each temperature.

From these results, it was concluded that using the on-column injector for these compounds at a high temperature had little effect on efficiency, except with regard to the hydrocarbon peak when the injector was initially set at 100°C, which was unexplainable. Setting the on-column injector at 250°C and above produced a series of contaminatory peaks resembling those of a homologous series. This was possibly due to septum bleeding caused by the combined effects of the solvent and the high injector temperature.

Changing the on-column initial temperature may also have had an effect on mass transfer efficiency, causing discrimination for low-volatility compounds, as is well known for the split/splitless injector (7–8). Therefore the peak areas obtained with the same solution injection volumes using the splitless injector set at three different temperatures (250, 300, and 350°C) and the on-column injector set initially at 63°C using the "oven track mode" or set initially at 200°C without the "oven track mode" were compared. All the injections were manual. The sample was taken into the syringe barrel, and before the injection, the emptied needle was allowed to heat up for a few seconds in the injector to minimize sample discrimination. For splitless injections, the purge activation time was set at 30 s. Table II reports the peak areas of the highest MW compounds (i.e., the C_{33} hydrocarbon and the five major esters, the last containing 48 carbons). Each value represents the average of three trials. Table III reports the proportions of these peak areas.

Table II. Average Peak Areas Obtained under Different Injection Conditions

	Area (µV)					
Injection	C_{33}Hyd	C_{40}Est	C_{42}Est	C_{44}Est	C_{46}Est	C_{48}Est
Splitless, 250°C	59412	85592	46994	24017	35610	8702
Splitless, 300°C	54108	122027	79840	56230	113920	75507
Splitless, 350°C	50107	136710	92490	67268	133420	113658
On-column, 63°C (oven track)	73194	164430	106264	74910	152913	135772
On-column, 200°C	94678	198472	123567	87286	177306	158447

Table III. Average Peak Area Proportions Obtained under Different Injection Conditions

	Area proportion (%)					
Injection	C_{33}Hyd	C_{40}Est	C_{42}Est	C_{44}Est	C_{46}Est	C_{48}Est
Splitless, 250°C	22.8	32.9	18.0	9.2	13.7	3.3
Splitless, 300°C	10.8	24.3	15.9	11.2	22.7	15.1
Splitless, 350°C	8.5	23.0	15.6	11.3	22.5	19.1
On-column, 63°C (oven track)	10.3	23.2	15.0	10.6	21.6	19.2
On-column, 200°C	11.3	23.6	14.7	10.4	21.1	18.9

These results show first that peak areas of the less volatile compounds increased as the splitless injector temperature increased. Consequently area proportions were dependent on the splitless injector temperature, which was undesirable. As expected, all the peak areas increased markedly when the on-column injector was used. With this injector, the mass transfer was significantly more effective when the injector was initially set at 200°C, which is an unusual temperature for what is generally called "cool on-column" injection. However, when changing the on-column injector temperature, the peak area proportions were quite the same, although the absolute areas were different.

In conclusion, the use of the on-column injector set initially at 200°C produced narrow peaks and effective mass transfer of the low-

Table IV. Variability of Major Hydrocarbon Peak Area Proportions for a Single-Bee Extract*

	Area proportion (%)									
Run	C_{23}	C_{25}	C_{27}	$C_{29:1}$	C_{29}	$C_{31:1a}$	$C_{31:1b}$	C_{31}	$C_{33:2}$	$C_{33:1}$
1	1.69	5.26	17.73	3.04	19.93	11.31	10.25	12.16	1.84	16.78
2	1.72	5.35	17.85	2.95	20.08	11.28	10.24	11.97	1.78	16.78
3	1.71	5.29	17.79	3.01	19.85	11.37	10.23	12.12	1.84	16.79
4	1.74	5.34	17.74	2.99	19.85	11.40	10.24	12.05	1.85	16.79
5	1.74	5.29	17.70	2.95	19.93	11.65	10.07	12.14	1.79	16.74
6	1.71	5.28	17.71	3.04	19.94	11.41	10.10	12.09	1.87	16.86
Average	1.72	5.30	17.751	3.00	19.93	11.402	10.19	12.088	1.8286	16.79
SD	0.02	0.04	0.06	0.04	0.09	0.13	0.08	0.07	0.04	0.04
RSD	1.14	0.68	0.32	1.37	0.43	1.15	0.80	0.55	2.03	0.22

* $C_{31:1a}$ and $C_{31:1b}$ refer to monounsaturated C_{31} hydrocarbon isomers. The double bond positions a and b were not established.

volatility compounds found in bee extracts along with reducing cooling time between runs. The major constraint of this injector was the low initial oven temperature necessary to produce thermal focusing of the solutes at the beginning of the column. To start the analysis at higher oven temperatures with the aim of reducing time, it was necessary to use a different kind of injector leading to the testing of the ASP injector.

The ASP injector is an improvement on the falling needle injector generally known as the Ros injector. The main difference is the replacement of the glass needle by a tungsten filament on which the solution to be analyzed is placed in the form of a drop. This filament is initially located at the top of the injector and the solvent is allowed to evaporate by the carrier gas flow through an outlet restriction to the atmosphere. Several drops can be successively deposited, permitting substantial amounts of material to be analyzed, especially samples of very low concentration. The filament is then allowed to free-fall into a heated glass inlet at the column head. The solutes are then rapidly desorbed by passing an electrical current through the filament. The filament is finally replaced at the injector top.

The main advantage of this injection technique is that little or no solvent entered into the column, which produced narrow initial band widths and led to high efficiency. Furthermore, stationary phase bleeding and discrimination of low-volatility compounds were markedly reduced. No thermal focusing was needed, so a higher initial oven temperature could be set to reduce analysis time. Band-broadening in space was also eliminated, permitting the withdrawal of the retention gap. With the on-column injector, the union between the column and the retention gap was sometimes a source of leaks, especially after repeated heating and cooling. The main improvements in the ASP injector were the automation of the vertical filament movement and the faster desorption of the solutes by heating the filament briefly but intensively.

Replacing the split/splitless injector by the ASP injector was easy to do, but producing and depositing a drop of our pentane bee extracts onto the filament was more delicate, probably because pentane is too volatile. Drops that missed the filament and fell to the bottom of the heated inlet led to contaminated peaks and often to complete dismantling and cleaning of the injector. To overcome this problem, the syringe was loaded with 1 µL of pentane solution and then with 2 µL of octane. Then a drop of octane was formed on the filament, and the 1-µL pentane solution was deposited into it. The placement of the drop onto the fila-

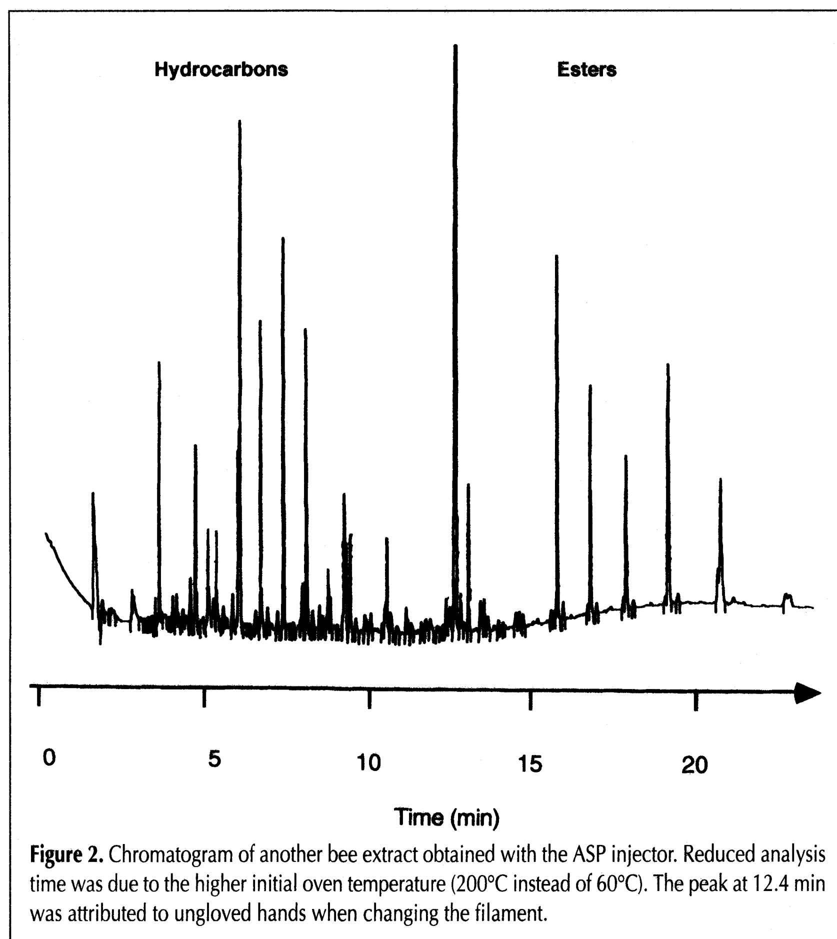

Figure 2. Chromatogram of another bee extract obtained with the ASP injector. Reduced analysis time was due to the higher initial oven temperature (200°C instead of 60°C). The peak at 12.4 min was attributed to ungloved hands when changing the filament.

Table V. Variability of Major Hydrocarbon Peak Area Proportions for Different Bee Extracts

	Area proportion (%)									
Bee	C_{23}	C_{25}	C_{27}	$C_{29:1}$	C_{29}	$C_{31:1a}$	$C_{31:1b}$	C_{31}	$C_{33:2}$	$C_{33:1}$
1	6.63	10.88	32.66	3.38	19.15	5.81	5.60	7.58	1.25	7.06
2	2.69	4.09	17.21	4.55	19.56	15.66	9.44	6.01	1.99	18.80
3	2.12	13.21	42.07	3.50	12.22	5.42	4.34	3.90	1.47	11.74
4	1.02	3.83	8.84	1.75	12.01	11.47	9.51	10.48	2.29	38.81
5	2.61	5.39	13.41	2.51	18.31	12.38	11.74	12.91	1.92	18.83
6	1.73	2.91	11.29	3.61	17.17	17.42	11.63	11.21	2.44	20.58
7	1.72	5.30	17.75	3.00	19.93	11.40	10.19	12.09	1.83	16.79
8	5.02	19.90	34.93	1.66	16.41	3.07	3.50	8.20	0.95	6.36
9	16.52	31.14	32.67	7.29	4.47	2.86	1.51	0.97	0.54	2.03

ment was then slightly easier but remained delicate and time-consuming, especially to analyze the 250 samples.

As can be seen from Figure 2, analysis of a bee extract with the ASP injector produced high-resolution chromatograms similar to those obtained with the on-column injector, with a reduced analysis time due to the permitted higher initial oven temperature (200°C). It must be noted that the chromatogram presented in Figure 2 was obtained from a different bee extract than those presented in Figure 1 and with a different oven program temperature; this may explain the different shape of ester peaks.

Unfortunately, we were not able to produce manually repeatable quantitative results, probably because our extracts were too concentrated, which led to a detrimental injector memory. This problem, combined with the delicacy of placing the pentane drop, led to the decision favoring the on-column injector to analyze the 250 samples. Nevertheless, if used with a less volatile solvent and an automated sampler, the ASP injector should be the best choice for such low-volatility solutes.

Analysis of bee extracts

The peaks obtained when using the on-column injector set at 200°C and the above-mentioned oven temperature program were identified either by injecting standards or by coupled MS. Some peaks were also identified by analogy with other results from the literature (5).

As for previously published studies, the analyzed pentane bee extracts contained saturated and unsaturated hydrocarbons ranging from C_{21} to C_{33} and unexpectedly C_{40} to C_{50} esters of long-chain alcohols. These low-volatility esters were probably also present in other previously studied samples, but the chromatographic conditions were not adapted to elute these compounds correctly.

With the on-column injector and the optimized oven temperature program, each ester peak often appeared as a doublet. After investigation by MS, it was concluded that these doublets corresponded to a partial separation of two isomers: a palmitic ester (resulting from saturated C_{16} acid and C_n alcohol) and an oleic ester (resulting from monounsaturated C_{18} acid and C_{n-2} alcohol). For the same extract, the proportion between the two peaks was reproducible for each doublet and different from one bee to another, as shown in Figure 1. The values in the tables correspond to the second part of the doublets, the peaks of palmitic esters $C_{15}H_{31}COOC_nH_{2n+1}$ ($24 \leq n \leq 34$). Although these doublets did not appear clearly in the chromatogram obtained with the ASP injector presented in Figure 2, a close look at ester peaks does show that every ester peak was preceded or followed by a small one, probably due to isomers being in different proportions in this different bee extract.

Table VI. Variability of Major Ester Peak Area Proportions for a Single-Bee Extract

	Area proportion (%)				
Run	C_{40}Est	C_{42}Est	C_{44}Est	C_{46}Est	C_{48}Est
1	29.13	8.31	14.25	9.34	38.97
2	29.21	8.28	14.23	9.92	38.36
3	29.15	8.49	13.96	9.96	38.45
4	29.05	8.03	14.14	10.57	38.21
5	27.39	7.82	13.76	10.36	40.67
6	29.21	8.03	14.39	9.61	38.76
Average	28.86	8.16	14.12	9.96	38.90
SD	0.72	0.24	0.23	0.46	0.91
RSD	2.50	2.98	1.61	4.58	2.34

Table VII. Variability of Major Ester Peak Area Proportions for Different Bee Extracts

	Area proportion (%)				
Bee	C_{40}Est	C_{42}Est	C_{44}Est	C_{46}Est	C_{48}Est
1	19.40	9.48	12.35	16.82	41.95
2	22.59	17.89	17.70	29.44	12.38
3	10.96	13.52	11.76	18.21	45.54
4	17.29	14.74	16.12	32.43	19.44
5	21.34	11.57	9.59	14.69	42.82
6	24.57	17.70	14.28	25.77	17.69
7	28.86	8.16	14.12	9.96	38.90
8	20.47	14.99	13.57	24.06	26.90
9	14.32	14.98	17.32	35.44	17.94

Method precision

One of the objectives of this work was to determine wether honeybees could be discriminated according to the chromatographic imprint of their cuticular secretions. As can be seen in Figure 1, the chromatograms obtained from different bees can differ markedly in the peak area proportions. When the peak area proportions were not very different, it was assumed that the differences were due to the bees and not to the technique. Therefore, the reliability of the results was tested by injecting nine different bee extracts six times each using the on-column injector. The 10 major hydrocarbon peaks were selected, and their areas were normalized. Table IV reports the area proportions, average area proportions, standard deviations (SDs), and relative standard deviations (RSDs) for a typical sample. The deviations obtained for the major hydrocarbons in this typical analysis are acceptable, especially when compared with the deviations produced between one bee and another, as shown in Table V.

Destined for a separate statistical study, the results of the major ester peaks of the single-bee extract are presented in Tables VI and VII. Because these esters are less abundant than hydrocarbons in cuticular waxes (1,2), determination of their peak areas is often less precise, leading to higher RSDs. Nevertheless, the proportions differ sufficiently from one bee to another (Table VII) to also consider the ester peak profile as another characteristic of each bee subfamily.

Conclusion

This paper outlines the development of a method that allows the determination of bee cuticular hydrocarbon and ester pro-

files from a single run using capillary GC. This method has been optimized by the reasoned choice of the chromatographic parameters and especially the injecting conditions. After comparing three types of injectors, the most suitable for the analysis of the pentane bee extracts has been shown to be the on-column injector initially set at an elevated temperature, permitting improved time analysis and mass transfer to the column while maintaining good efficiency and the same peak area proportions. A simple statistical study was also carried out to ensure the reliability of our results, which can now be utilized for genetic studies of the analyzed bees. This method could be easily adapted to analyze other waxes or complex mixtures of low-volatility compounds.

Acknowledgments

The authors thank Dr. G. Arnold (INRA-CNRS laboratory) and Pr. M. Solignac (CNRS laboratory) for supplying this work, financially supported by Actions Coordonnées Concertées, Sciences de la Vie (ACC SV1). We are also grateful to Mr. P. Parsy (ID Analytical Services) for kindly providing the ASP injector to complete this comparative study and to our colleague, Dr. J. Bleton (LETIAM) for performing the coupled GC–MS analyses.

References

1. D.R. Nelson and G.J. Blomquist. Insect waxes. In *Waxes: Chemistry, Molecular Biology and Functions* . R.J. Hamilton, Ed. Oily Press, Dundee, Great Britain, 1995, pp.1–90.
2. G.J. Blomquist, A.J. Chu, and S. Remaley. Biosynthesis of wax in the honeybee, *Apis mellifera L. Insect Biochem.* **10:** 313–21 (1980).
3. D.A. Carlson and A.B. Bolten. Identification of Africanized and European honey bees, using extracted hydrocarbons. *Bull. Entomol. Soc. Am.* **30:** 32–35 (1984).
4. B.R. Francis, W.E. Blanton, and R.A. Nunamaker. Extraction of surface hydrocarbons of workers and drones of the genus Apis. *J. Apic. Res.* **24(1):** 13–26 (1985).
5. G. Arnold, B. Quenet, J.-M. Cornuet, C. Masson, B. De Schepper, A. Estoup, and P. Gasqui. Kin recognition in honeybees. *Nature* **379:** 498 (1996).
6. C. Lange, J.-J. Basselier, A.-G. Bagnères, P. Escoubas, M. Lemaire, A. Lenoir, J.-L. Clement, A. Bonavita-Cougourdan, M. Trabalon, and M. Campan. Strategy for the analysis of cuticular hydrocarbon waxes from insects using gas chromatography/mass spectrometry with electron impact and chemical ionization. *Biomed. Environ. Mass Spectr.* **18:** 787–800 (1989).
7. P. Sandra. *Sample Introduction in Capillary Gas Chromatography,* Vol. 1. Hüthig, Heidelberg, Germany, 1985.
8. K. Grob. *Classical Split and Splitless Injection in Capillary Gas Chromatography.* Hüthig, Heidelberg, Germany, 1986.
9. K. Grob. *On Column Injection in Capillary Gas Chromatography.* Hüthig, Heidelberg, Germany, 1987.
10. P. Stremple. Troubleshooting in the separation sciences. *J. High Resol. Chromatogr.* **17:** 501 (1984).

Manuscript accepted February 6, 1998.