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Gas Chromatography

MICRO-QUANTITATIVE METHOD FOR ANALYSIS OF STEROL LEVELS IN HONEYBEES AND THEIR POLLEN LOADS

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A Gas Chromatography-Flame Ionization Detector method for characterization and micro-quantification of sterols was developed. The applicability of this method was evaluated for determining sterolic compounds in pollen loads, larvae, and adults of the honeybee. After a multi-step procedure to extract and purify sterols, the compounds were identified on the basis of their retention data and quantified on the basis of peak areas from analyses. Quantifications were feasible with a limited amount of samples, allowing single specimen analyses and estimation of inter-individual variation. The reliability of this micro-quantitative method for sterol analysis was also investigated.

Keywords: Biological material; GC analyses; Micro-quantification; Sterols

INTRODUCTION

Insects use sterols in a variety of key metabolic pathways, such as for the synthesis of ecdysteroid molting hormone in larvae and for the synthesis of ecdysteroid hormone for ovariole maturation in females (Nation 2002). Because insects do not produce endogenous sterols, they must assimilate these compounds from their food. Requirement of dietary sterols was first described in the fly *Lucilia sericata* (Hobson 1935), and has since been verified in many different insect orders (Behmer and Nes 2003). While cholesterol alone is usually sufficient for an insect's sterol

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requirements, some species require very specific dietary sterols, such as δ7-stigmasten-3β-ol in *Drosophila pachea* (Kircher et al. 1967), ergosterol and 7-dehydrocholesterol in *Xyleborus ferrugineus* (Norris and Baker 1967) or fungus sterols (ergosterol, 85.2%) in *Losebia botrana* (Mondy and Corio-Costet 2000). Therefore, knowledge about sterol composition in insect food is often needed to analyze insect diets.

Sterols are an important group of unsaponifiable matter. Currently, the analytical methods used for the analysis of these compounds include gas chromatography (GC) and high performance liquid chromatography (HPLC). In spite of its simplicity, the HPLC technique suffers from insufficient selectivity and sensitivity (Lognay et al. 1992). Except for the determination of ergosterol, the routine application of HPLC to sterol analysis is still limited (Osswald, Höll, and Elstner 1986; Schwadorf and Müller 1989). Although GC requires both thermally stable columns and chemical derivatization prior to analysis, it is recommended by official bodies (Anonym 1983; IUPAC 1987). In fact, GC is recognized as the method of choice for sterol analysis and provides a powerful alternative for characterization and quantification of sterols found in low amounts in complex matrices. Sterol analysis in oil has been described by Lognay et al. (1992) and adapted for insects and pollen matrices by Regali (1996) and Rasmont et al. (2005). The procedure includes various steps and can be summarized as follows: (1) saponification of the samples with alcoholic potassium hydroxide, (2) extraction of the unsaponifiable (USM) part with diethylether and several water-washings of the organic phase, (3) evaporation of solvent, (4) USM fractionation into its components using thin-layer chromatography (TLC), (5) derivatization of the sterols (scraped from the silicagel) into trimethylsilyl ethers (TMS), and (6) separation of TMS by Gas Liquid Chromatography (GLC). The main advantage of this procedure is to determine accurately and selectively sterolic profiles for samples with a low sterol concentration. However, the adapted multi-step protocol of Lognay et al. (1992) requires a minimum of two hundred milligrams of pollen and ten bee specimens (Rasmont et al. 2005), making it impossible to evaluate individual bees. Furthermore, it limits analysis of some matrices that are available only in very small amounts, such as pollen that has been painstakingly hand-collected.

The aim of this paper is to detail a new analytical procedure that presents the advantage of accurate determination of sterolic profiles with very low amounts of natural matrices.

EXPERIMENTAL SECTION

Chemicals and Reagents

Methanol, ethanol, hexane, and chloroform were purchased from E. Merck (Darmstadt, Germany) and diethyl ether was obtained from Fisher Scientific (United Kingdom). Each solvent was of analytical grade. Potassium hydroxide was obtained from UCB (Leuven, Belgium). Anhydrous Na_2SO_4 (E. Merck, Darmstadt, Germany) was used to filter and dry the extracts. Cholesterol (minimum 99%), betulin (minimum 98%), and anhydrous pyridine (99.8%) were purchased from Sigma Aldrich NV/SA (Belgium). The 2′,7′-dichlorofluorescein was obtained from Fisher Scientific, Acros Organics (Tournai, Belgium). Silyl-991 BSTFA-TMCS [(99:1) bis (trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) used

as 99:1 mixture for silylation] was purchased from Filter Service, Macherey-Nagel (Eupen, Belgium). Ultrapure water was obtained with a Milli-Q system (Millipore) and was employed for all aqueous solutions.

Analytical TLC plates (20 cm × 20 cm) were made before each analysis with silica gel G60 purchased from E. Merck (Darmstadt, Germany). The capillary column VF-5ms used for sterol GC analyses was obtained from Varian (Middelburg, Netherlands).

Instrumentation

All quantitative analyses were made with a FISON GC 8000 top gas chromatograph. The injections were performed with an on-column Thermo Finnigan AS 2000. Instrumental parameters were controlled by Chrom-Card software.

Identifications were made with a GC-MS system including 7890A GC and a 5975C inert XL EI/CI MSD with Triple-Axis Detector (Agilent Technologies). The injections were performed manually. Instrumental parameters were controlled by ChemStation software.

Sample Preparation and Procedure

Reliability of the micro-quantitative method.

Honeybee pollen loads. We used pollen loads collected by placing pollen traps in hives at the University of Mons (Belgium). Botanical composition of pollen loads was all-pure *Salix caprea* L. Before each analysis, pollen loads were carefully lyophilized and homogenized. Each sample unit contained only twenty milligrams of pollen (mean weight about 20.59 ± 0.37 mg). Samples were saponified with 2.5 ml of 2 M methanolic KOH for 1 h at 80°C. After cooling and addition of 1 ml of internal standard (ethanolic solution of 0.05 mg/ml betulin), the solution was diluted with 2.5 ml of milli-Q water. The methanolic solution was transferred into a separatory funnel. To extract sterol, the saponification flask was rinsed with 5 ml of diethyl ether, which was then added to the separating funnel. The separator was shaken and allowed to stand to separate the phases. The solution was extracted three times this way and the successive ether layers were carefully transferred into another flask. The pooled ether extracts were washed with three successive 5 ml portions of Milli-Q water and dried over anhydric sodium sulfate. The diethyl ether was evaporated under reduced pressure at 35°C until completely dry. After addition of 0.5 ml of chloroform, the crude extract was fractionated into its components using TLC [eluant: chloroform/diethyl ether/ammonia water (880 ammonia; i.e., 28% w/w) 90:10:0.5]. The sterols were detected on the TLC plate ($R_F = 0.45$) under UV (254 nm) after spraying with an ethanolic 0.2% 2′,7′ – dichlorofluorescein solution. The sterols were scraped off the plate and extracted from the silicagel with 5, 3, and 3 ml of chloroform, respectively. The solvent was then evaporated under reduced pressure and under a gentle stream of nitrogen. The recovered sterols were finally derivatized with 100 µl of a (1:1 v/v) mixture of anhydrous pyridine and silylation reagent (BSTFA + 1% TMCS) at 90°C for 30 min after mixing on a vortex mixer. The reagents were evaporated under nitrogen. Chemical quantifications were then performed through GC and n-hexane (200 µl) was used as solvent. The capillary

column specifications were the following: VF-5ms (5%-phenyl-methylpolysiloxane stationary phase; 30 m column length; 0.25 mm inner diameter; 0.25 μm film thickness). The initial temperature of the column was maintained 1 min at 60°C, then programmed to 290°C at 30°C min^{-1} and maintained for 22 min. After this isotherm at 290°C, the temperature was increased to 325°C (30°C min^{-1}) then isothermal at 325°C for 5 min. The carrier gas used was helium at 1 ml/min and the injection mode was "on-column". Quantification of the compounds was performed with Chrom-Card software. All chromatograms have been subjected to manual integration in order to improve the baseline for each peak.

The compounds were identified by comparing the relative retention times (β-sitosterol – TMS = 1.00) with those of oil reference (sunflower oil with well-known composition). These identifications were corroborated by GC/MS (Gas Chromatograph/Mass Spectrometer) analyses in similar conditions as with GC. The capillary column specifications were the following: HP-5ms (5%-phenyl-methyl-polysiloxane stationary phase; 30 m column length; 0.25 mm inner diameter; 0.25 μm film thickness). The initial temperature of the column was maintained 1 min at 60°C, then programmed to 290°C at 30°C min^{-1} and maintained for 22 min. After this isotherm at 290°C, the temperature was increased to 320°C (30°C min^{-1}) then isothermal at 320°C for 5 min. The carrier gas used was helium at 1 ml/min and the injection mode was "splitless" at 280°C. The mass range scanned was from 50 a.m.u. to 600 a.m.u. The identifications were performed by careful examination of the fragmentation data of sterol-trimethylsilyl ethers. The main and characteristic fragments were interpreted on the basis of literature data and the fragmentation patterns were compared to the Wiley275.L Mass Spectral Library.

Repetability of the method. The entire process was repeated fifteen times on pollen loads to give a measure of the variability in sample preparation. Three batches of pollen were firstly homogenized separately to reduce variability due to pollen variation. These batches were divided into five equal aliquots, which were processed separately. Each single aliquot (fifteen aliquots in total) was run on GC three times. The repeated injections of each sample give a measure of the variability in analytical procedure (injection/GC-run).

Qualitative and quantitative controls. In order to confirm that all analytes separated well, sample solutions and an extract of sunflower oil were injected alternately. This oil sample was prepared according to Lognay et al. (1992).

In order to guard against undetected drift, the response factor was monitored between internal standard and sterols by injecting the calibration and sample solutions alternately. This response factor was determined on the basis of peak areas from GC-FID analysis of a cholesterol/betulin solution 0.05 mg/ml. Cholesterol was used because it was the only sterol for which a standard of high purity was available. Cholesterol and betulin of the standard solution were both silylated in the same method as the different samples.

Application to Honeybee Workers and Larvae

Honeybee workers and larvae were collected at the same time from the same hive in Mons (Belgium). Before each analysis, wings and legs were removed from

the worker body. Because the larvae have a blind gut, it was necessary to remove it as well as the cuticle before each analysis. The multi-step protocol was the same as for pollen, regardless of weight (worker mean weight of about 103 ± 23.38 mg and larvae mean weight of about 114.13 ± 7.01 mg). In all analyses, the measurements were repeated on three different specimens to ensure sufficient precision in spite of intraspecific variability.

RESULTS AND DISCUSSION

Method Originality

The originality of our adapted method is mainly to successfully analyze a low quantity of biological material with trace amounts of sterols. Our methodological approach is quite similar to the method of Lognay et al. (1992). No step has been added or eliminated. However, we had to adapt the GC columns, temperature program, solvent/reagent volumes, and internal standard concentration to make possible the analyses of very small samples. The new GC column (VF-5ms, 30 m × 0.25 mm i.d., 0.25 μm film thickness instead of CPSIL-19CB, 25 m × 0.32 mm mm i.d., 0.2 μm film thickness) combined with the new temperature program (60–290°C at 30°C min^{-1}, isothermal for 22 min, 290–325°C at 30°C min^{-1}, isothermal for 5 min instead of 60–285°C at 30°C min^{-1}) increased the sensitivity of the method. The use of this chromatographic column with high efficiency and selectivity is recommended for the best achievable separation of sterols and the temperature program improves the peak resolution. Volumes of different solvents and reagents have been reduced as well as the internal standard concentration.

Analysis of Pollen Loads

The concentration of total sterols in pollen loads is 4.44 ± 0.47 mg per g of lyophilized matter ($n = 15$). Pollen of *Salix caprea* L. contains a high concentration of C29 sterolic compounds like β-sitosterol and δ5-avenasterol (Fig. 1a). The second most abundant compound is unknown, but its detection is confirmed by a previous study on the pollen of *Salix caprea* L. (Regali, 1996) (Tables 1, 2, and 3). Thus, the present results are in line with previous studies. However, this new microquantitative method represents a significant improvement over the previous method because it enables the investigation of very small amounts of pollen ten times lower than in Rasmont et al. (2005), and enables novel applications, such as the analysis of small quantities of hand-collected pollen or of pollen loaves, as well as comparisons between pollen from the same flower species collected by bees and by hand.

Repeatability of the Method (Pollen Loads)

The results for repeatability of the analytical procedure and repeatability of the sample preparation procedure are summarized in Tables 4 and 5. Only the compounds with a concentration higher than the limit of quantification (LOQ; 9.6 ng/1.2 μl injected) were considered. To estimate the repeatability for the minor compounds, the concentration of the internal standard had to be adapted to produce a peak height similar to that of the minor compounds.

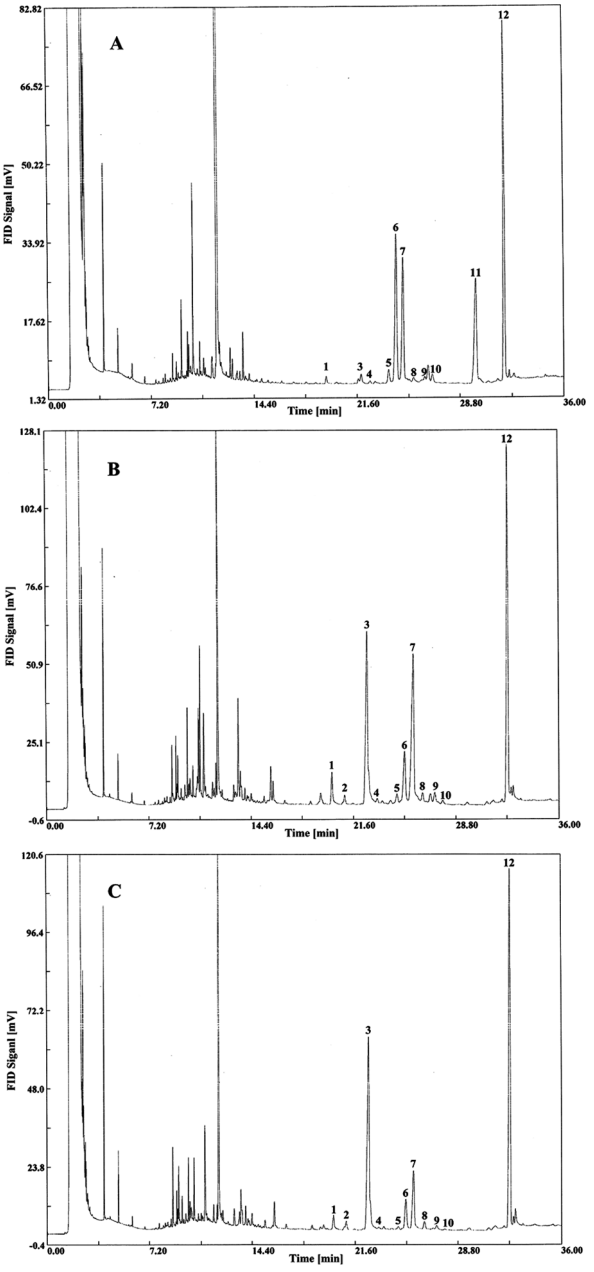

Figure 1. Chromatograms of the sterols of willow pollen loads (a), honeybee worker (b) and honeybee larvae (c). Experimental conditions: column, VF-5ms, 30 m X 0.25 mm i.d., 0.25 μm film thickness. "On-column injector. Temperature program: 60–290°C at 30°C min^{-1}, 290°C for 22 min, 290–325°C at 30°C min^{-1} and 325°C form 5 min. Peak identification, 1, cholesterol; 2, desmosterol; 3, 24-methylenecholesterol and campesterol; 4, stigmasterol; 5, unknown 1 (mass TMS = 484); 6, β-sitosterol; 7, δ5-avenasterol; 8, cholestenone; 9, δ7-stigmasterol; 10, δ7-avenasterol; 11, unknown 2 (mass TMS = 496); and 12, betulin (ISTD). The peak succession before cholesterol contains no sterolic compound but does contain residues from silylation (except the first peak, which corresponds to the solvent).

Table 1. Sterolic compounds from pollen loads (batch A)

Sterol	Aliquot 1 (20.7 mg)	Aliquot 2 (20 mg)	Aliquot 3 (20.1 mg)	Aliquot 4 (20.2 mg)	Aliquot 5 (20.9 mg)	Mean for batch A
Cholesterol	83.61 (1.77)	40.69 (0.99)	42.15 (0.91)	67.98 (1.54)	127.52 (2.42)	72.39 (1.52)
24-Methylenecholesterol + Campesterol[a]	131.39 (2.78)	100.31 (2.43)	119.51 (2.58)	113.54 (2.58)	147.25 (2.79)	122.40 (2.63)
Stigmasterol	31.08 (0.66)	41.70 (1.01)	21.87 (0.47)	16.01 (0.36)	18.42 (0.35)	25.82 (0.57)
Unk.1 (mass TMS = 484)[b]	142.57 (3.02)	112.32 (2.72)	129.69 (2.80)	126.41 (2.87)	154.83 (2.94)	133.17 (2.87)
β-Sitosterol	**1625.32 (34.39)**	**1324.74 (32.08)**	**1582.99 (34.23)**	**1479.31 (33.61)**	**1885.65 (35.73)**	**1579.60 (34.01)**
δ5-Avenasterol	**1208.43 (25.57)**	**1020.81 (24.72)**	**1235.33 (26.72)**	**1129.72 (25.66)**	**1336.52 (25.29)**	**1186.16 (25.59)**
Cholestenone	59.02 (1.25)	72.08 (1.75)	54.79 (1.18)	66.80 (1.51)	119.72 (2.27)	74.48 (1.59)
δ7-Stigmasterol	74.98 (1.59)	91.76 (2.22)	43.57 (0.94)	51.97 (1.18)	39.19 (0.75)	60.29 (1.34)
δ7-Avenasterol	110.04 (2.33)	94.12 (2.28)	74.90 (1.62)	104.95 (2.39)	99.77 (1.90)	96.76 (2.10)
Unk.2 (mass TMS = 496)[c]	**1259.22 (26.65)**	**1230.49 (29.80)**	**1319.33 (28.53)**	**1245.85 (28.30)**	**1348.48 (25.56)**	**1280.67 (27.77)**
Total	4725.66	4129.02	4624.12	4402.53	5277.34	4631.74

[a]Under the analytical conditions applied, campesterol and 24-methylenecholesterol are nearly impossible to separate; the results are therefore pooled.

[b]Unknown 1; unknown compound.

[c]Unknown 2; unknown compound specific of *Salix* spp. pollen.

For each aliquot, the provided values are the mean of three successive injections. The concentrations are expressed in μg/g in dry matter (lyophilization) and as percent total sterolic compounds in brackets. The three major sterols in the investigated samples are printed in bold.

Table 2. Sterolic compounds from pollen loads (batch B)

Sterol	Aliquot 1 (20.8 mg)	Aliquot 2 (20.5 mg)	Aliquot 3 (20.4 mg)	Aliquot 4 (20.4 mg)	Aliquot 5 (21.2 mg)	Mean for batch B
Cholesterol	74.20 (2.20)	121.92 (2.99)	57.08 (1.33)	87.08 (2.05)	89.58 (2.19)	85.97 (2.15)
24-Methylenecholesterol + Campesterol[a]	82.60 (2.44)	99.70 (2.45)	106.08 (2.46)	110.50 (2.61)	94.78 (2.32)	98.73 (2.46)
Stigmasterol	23.13 (0.69)	15.29 (0.38)	11.00 (0.26)	13.31 (0.31)	12.82 (0.31)	15.11 (0.39)
Unk.1 (mass TMS = 484)[b]	106.82 (3.17)	128.57 (3.16)	136.00 (3.16)	128.04 (3.02)	111.27 (2.72)	122.14 (3.05)
β-Sitosterol	**1176.90 (34.82)**	**1438.16 (35.33)**	**1518.03 (35.23)**	**1433.43 (33.83)**	**1314.80 (32.18)**	**1376.26 (34.28)**
δ5-Avenasterol	**825.91 (24.38)**	**961.93 (23.63)**	**989.80 (22.96)**	**1028.33 (24.28)**	**1032.96 (25.29)**	**967.78 (24.11)**
Cholestenone	77.36 (2.28)	75.39 (1.85)	76.15 (1.77)	70.25 (1.66)	106.04 (2.60)	81.04 (2.03)
δ7-Stigmasterol	45.72 (1.35)	51.51 (1.27)	35.97 (0.84)	36.69 (0.87)	29.19 (0.71)	39.82 (1.01)
δ7-Avenasterol	74.96 2.21)	86.73 (2.13)	79.32 (1.84)	84.65 (1.99)	72.03 (1.76)	79.54 (1.99)
Unk.2 (mass TMS = 496)[c]	**893.85 (26.46)**	**1091.60 (26.82)**	**1296.82 (30.15)**	**1244.51 (29.38)**	**1222.53 (29.91)**	**1149.86 (28.54)**
Total	3381.47	4070.80	4306.24	4236.77	4086.00	4016.25

[a]Under the analytical conditions applied, campesterol and 24-methylenecholesterol are nearly impossible to separate; the results are therefore pooled.

[b]Unknown 1; unknown compound.

[c]Unknown 2; unknown compound specific of *Salix* spp. pollen. For each aliquot, the provided values are the mean of three successive injections. The concentrations are expressed in μg/g in dry matter (lyophilization) and as percent total sterolic compounds in brackets. The three major sterols in the investigated samples are printed in bold.

Table 3. Sterolic compounds from pollen loads (batch C)

Sterol	Aliquot 1 (21.2 mg)	Aliquot 2 (20.3 mg)	Aliquot 3 (20.7 mg)	Aliquot 4 (20.7 mg)	Aliquot 5 (20.8 mg)	Mean for batch C
Cholesterol	104.26 (2.30)	94.81 (2.06)	131.83 (2.92)	152.68 (3.45)	184.45 (3.52)	133.61 (2.85)
24-Methylenecholesterol + Campesterol[a]	113.80 (2.51)	113.61 (2.47)	122.48 (2.72)	118.91 (2.69)	142.45 (2.72)	122.25 (2.62)
Stigmasterol	15.19 (0.33)	13.33 (0.29)	14.99 (0.33)	16.28 (0.37)	18.67 (0.36)	15.69 (0.34)
Unk.1 (mass TMS = 484)[b]	132.06 (2.91)	121.67 (2.65)	127.22 (2.82)	120.98 (2.74)	141.52 (2.70)	128.69 (2.76)
β-Sitosterol	**1492.50 (32.91)**	**1478.88 (32.17)**	**1593.78 (35.30)**	**1489.07 (33.69)**	**1830.87 (34.94)**	**1577.02 (33.80)**
δ5-Avenasterol	**1126.13 (24.83)**	**1158.40 (25.20)**	**1081.57 (23.95)**	**1070.55 (24.22)**	**1361.50 (25.98)**	**1159.63 (24.84)**
Cholestenone	124.49 (2.74)	119.45 (2.59)	45.57 (1.01)	82.27 (1.86)	102.02 (1.95)	94.76 (2.03)
δ7-Stigmasterol	43.42 (0.96)	31.92 (0.69)	26.69 (0.59)	32.35 (0.73)	32.56 (0.62)	33.39 (0.72)
δ7-Avenasterol	90.79 (2.00)	79.63 (1.73)	78.13 (1.73)	76.12 (1.72)	79.44 (1.51)	80.82 (1.74)
Unk.2 (mass TMS = 496)[c]	**1292.69 (28.50)**	**1385.46 (30.14)**	**1292.96 (28.64)**	**1260.42 (28.52)**	**1346.29 (25.70)**	**1315.56 (28.30)**
Total	4535.32	4597.16	4515.22	4419.64	5239.78	4661.42

[a]Under the analytical conditions applied, campesterol and 24-methylenecholesterol are nearly impossible to separate; the results are therefore pooled.
[b]Unknown 1; unknown compound.
[c]Unknown 2; unknown compound specific of *Salix* spp. pollen.
For each aliquot, the mentioned values are the mean of three successive injections. The concentrations are expressed in μg/g in dry matter (lyophilization) and as percent total sterolic compounds in brackets. The three major sterols in the investigated samples are printed in bold.

Table 4. Results for variability of the analytical procedure (injection/GC-run) expressed by the variation coefficient (%)

Sterols											
Variation coefficient (%)		Cholesterol	24-Methylenecholesterol + Campesterol[a]	Stigmasterol	Unk.1 (mass TMS = 484)[b]	β-Sitosterol	δ5-Avenasterol	Cholestenone	δ7-Stigmasterol	δ7-Avenasterol	Unk.2 (mass TMS = 496)[c]
Batch A	Aliquot 1	1.81	4.37	–	2.05	0.51	0.56	–	–	2.84	0.43
	Aliquot 2	–	1.36	–	2.26	0.09	0.46	–	1.84	2.89	0.74
	Aliquot 3	–	1.13	–	3.46	0.48	1.04	–	–	–	1.68
	Aliquot 4	–	0.68	–	3.62	0.72	1.53	–	–	9.45	0.59
	Aliquot 5	1.92	1.79	–	12.52	0.23	4.91	9.02	–	14.93	1.92
Batch B	Aliquot 1	–	2.75	–	9.79	1.47	7.64	–	–	–	4.18
	Aliquot 2	3.30	10.55	–	4.70	0.52	0.82	–	–	2.92	0.95
	Aliquot 3	–	1.84	–	4.81	1.90	3.72	–	–	–	3.41
	Aliquot 4	9.27 (n = 2)	2.27	–	0.80	0.41	1.03	–	–	3.26 (n = 2)	0.79
	Aliquot 5	0.19 (n = 2)	8.42	–	10.49	2.16	10.18	27.13 (n = 2)	–	0.64 (n = 2)	5.30
Batch C	Aliquot 1	5.73	7.25	–	1.44	0.66	0.30	11.55	–	1.36	0.51
	Aliquot 2	2.68	3.04	–	2.77	0.83	0.04	13.93	–	–	1.53
	Aliquot 3	3.56	2.09	–	5.15	0.26	1.08	–	–	2.41 (n = 2)	1.49
	Aliquot 4	6.37	4.63	–	1.18	0.54	0.35	3.91	–	–	1.10
	Aliquot 5	3.33	4.37	–	2.82	0.23	0.12	8.81	–	5.40 (n = 2)	0.47

[a]Under the analytical conditions applied, campesterol and 24-methylenecholesterol are nearly impossible to separate; the results are therefore pooled.
[b]Unknown 1; unknown compound.
[c]Unknown 2; unknown compound specific of *Salix* spp. pollen.
Only the values higher than LOQ were considered (three repeated injections for each aliquot, except for the n mentioned in brackets).

Table 5. Results for variability in sample preparation procedure expressed by the variation coefficient (%)

	Sterols									
Variation coefficient (%)	Cholesterol	24-Methylenecholesterol + Campesterol[a]	Stigmasterol	Unk.1 (mass TMS = 484)[b]	β-Sitosterol	δ5-Avenasterol	Cholestenone	δ7-Stigmasterol	δ7-Avenasterol	Unk.2 (mass TMS = 496)[c]
Batch A	–	5.79	–	4.04	3.90	2.84	–	–	7.78 (n = 4)	6.01
Batch B	18.79 (n = 3)	4.17	–	6.27	3.84	3.63	–	–	4.78 (n = 3)	6.19
Batch C	23.16	4.60	–	3.77	3.91	3.25	18.27 (n = 4)	–	8.75 (n = 4)	5.69
All batches combined (n = 3)	19.72 (n = 2)	3.84	–	4.95	0.70	2.99	<0.01 (n = 2)	–	9.54	1.40

[a]Under the analytical conditions applied, campesterol and 24-methylenecholesterol are nearly impossible to separate; the results are therefore pooled.
[b]Unknown 1; unknown compound.
[c]Unknown 2; unknown compound specific of *Salix* spp. pollen.
Only the values higher than LOQ were considered (five aliquots for each batch, except for the n mentioned in brackets).

This method seems to be highly reliable. The variation coefficients (V.C.) of the measured sterol levels ranged from 0.04 to 4.91% in all samples except those with sterol levels lower than 3%. In these samples, the variation coefficients were larger, and ranged from 5.15 to 27.13%. Furthermore, the variation coefficients of sterol levels in the independently prepared samples followed this same pattern: the sterols with low concentrations (<3% total sterols) were assigned higher variation coefficients than those samples with abundant sterolic compounds. Regarding the minor constituents (<3%), the repeatability ranged from 3.77 to 23.16%. For the major sterols (>3%), the variation coefficients range between 2.84 and 6.19% total sterols. Given the limited quantity of investigated samples, the variation coefficients are satisfactory. The variation coefficients between the three batches of each sterol level ranged from <0.01 to 19.72%.

The results obtained for the different analyses are satisfactory both qualitatively and quantitatively. In terms of qualitative analysis, the VF-5ms column provided the ideal compromise between separation and thermal resistance, and yielded a high resolution between different sterolic compounds, allowing a preliminary identification of sterols based on retention times. These identifications were corroborated by GC-MS analysis. With regards to the quantitative analysis, the repeatability of the analytical procedure is more than satisfactory, and the experimental repeatability is acceptable in view of the micro-quantities investigated (variation coefficients for major peaks are <7% but greater for minor compounds).

Analysis of *Apis mellifera* Workers and Larvae

The 24-methylenecholesterol and campesterol (same fraction) were the most abundant sterols in honeybee workers (Table 6, Fig. 1b). The δ5-avenasterol and β-sitosterol were also abundant in GLC profiles. Desmosterol, stigmasterol, and δ7-avenasterol concentrations were low. The concentration of total sterols in *A. mellifera* adults is 1.10 ± 0.12 mg per g of fresh matter.

Honeybee larvae had a lower sterolic content than workers, with a total sterol concentration of 0.64 ± 0.11 mg per g of fresh matter (Table 6, Fig. 1c). The 24-methylenecholesterol + campesterol fraction was again the most abundant sterolic compound followed by δ5-avenasterol and β-sitosterol. Stigmasterol, δ7-avenasterol and δ7-stigmasterol concentrations were low.

According to these results, 24-methylenecholesterol is the primary sterol present in honey bee larvae as well as in adults. This is in line with a previous study by Svoboda et al. (1980). However, with the method presented here, only one specimen was required to attain these results instead of a pool of 15 individuals.

The study of insect sterol nutrition and physiology is very important. Most insects cannot directly use phytosterols, but must dealkylate them into cholesterol through one of several pathways (Svoboda, Weirich, and Feldlaufer 1991; Svoboda and Feldlaufer 1991). Cholesterol is then metabolized to obtain ecdysteroids, the major insect molting hormones. Because *Apis mellifera* lacks a dealkylation mechanism and uses an uncommon 28-carbon molting hormone, makisterone A, the honey bee does not use C28 and C29 sterols to obtain C27 cholesterol and ecdysone. This insect uses a special pathway that probably includes 24-methylenecholesterol

Table 6. Sterolic compounds from workers and larvae of the honeybee

Sterol	*A. mellifera* workers (n = 3; 103 ± 23.38 mg)	*A. mellifera* larvae (n = 3; 114.13 ± 7.01 mg)
Cholesterol	38.82 ± 21.63 (3.44 ± 1.55)	28.21 ± 7.90 (4.35 ± 0.61)
Desmosterol	21.52 ± 3.09 (1.99 ± 0.45)	15.36 ± 1.69 (2.43 ± 0.35)
24-Methylenecholesterol + Campesterol[a]	**499.69 ± 52.56 (46.09 ± 9.11)**	**381.19 ± 47.28 (59.77 ± 2.48)**
Stigmasterol	12.48 ± 4.85 (1.14 ± 0.45)	4.58 ± 3.19 (0.67 ± 0.37)
Unk.1 (mass TMS = 484)[b]	43.35 ± 42.98 (4.19 ± 4.47)	23.11 ± 31.70 (3.19 ± 4.11)
β-Sitosterol	**120.20 ± 44.98 (10.83 ± 3.69)**	**55.74 ± 4.21 (8.80 ± 0.98)**
δ5-Avenasterol	**306.93 ± 131.70 (27.33 ± 9.06)**	**108.44 ± 7.90 (17.10 ± 1.64)**
cholestenone	14.09 ± 4.44 (1.28 ± 0.36)	13.63 ± 5.87 (2.09 ± 0.60)
δ7-Stigmasterol	30.36 ± 21.41 (2.76 ± 1.99)	8.29 ± 1.65 (1.29 ± 0.05)
δ7-Avenasterol	10.42 ± 8.38 (0.95 ± 0.78)	1.95 ± 0.66 (0.30 ± 0.07)
Total	1097.86 ± 115.26	640.50 ± 105.16

[a]Under the analytical conditions applied, campesterol and 24-methylenecholesterol are nearly impossible to separate; the results are therefore pooled.

[b]Unknown 1; unknown compound.

The concentrations are expressed in μg/g in fresh matter and as percent total sterolic compounds in brackets. The three major sterols in the investigated samples are printed in bold.

related to makisterone A. The sterol composition of *Salix caprea* L. pollen therefore seems suitable to the metabolic requirements of *A. mellifera* (Tables 1, 2, and 3).

Perspectives

The micro-quantitative method described here permits new analytical possibilities for understanding the relationship between sterols and insects. It will shed new light on how insect–plant interactions are shaped.

During this study, we managed to quantify the sterolic compounds in very low quantities of pollen and individual larvae and adults, approaching the quantification limit of the method proposed by Lognay et al. (1989; 1992).

In the future, we will attempt to make individual measures on extremely low sterol quantities in order to describe the extent of variability of sterol levels within a population of bees. Furthermore, we hope to extend this method to examine inter- as well as intraspecific variation in further studies.

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