DETERMINATION OF ACARICIDE RESIDUES IN BEESWAX: COLLABORATIVE STUDY

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Introduction

Persistent acaricides like bromopropylate (BP), fluvalinate (FV), coumaphos (CM), flumethrin (FM) have been regularly used world-wide, during the last years (BOGDANOV et al., 1998; WALLNER, 1999). Thus, beeswax, used in beekeeping is contaminated by these substances.

The determination of acaricides in beeswax is of increasing importance for the control of beeswax quality. That is why, at the meeting of the International Honey Commission in September 2001 in Athens it was decided to carry out a collaborative study.

The results of the trial are now reported to the participants. The present report will be sent by e-mail to each participating laboratory, together with the Lab Nr., which was given to each participant. In this manner every laboratory can compare its performance with the performance of the other laboratories.

A short summary of the results will be presented as a poster at the Apimondia Symposium in Celle, 10-11th of October, 2002. The results will be also discussed at the meeting of the IHC in Celle.

Materials and methods

Materials

- Ultrasound bath.
- Cooled centrifuge, equipped with an fixed angle rotor, glass tubes.
- Sample clean-up glass columns with a coarse frit and teflon stopcock.
- Rotary evaporator.
- Florisil 60-100 mesh (e.g. Merck 12518), heated during 12 hours at 600 ^{o}C, cool down add 5 g water to 95 g of Florisil (5 % water content, w/w) and store in an exsicator for a maximum of 10 days. Check the absorption and the elution capacity of each lot by passing of standards through the column.
- The analytical standard of a mixture of bromopropylate (BP), fluvalinate (FV), coumaphos (CM), flumethrin (FM) can be supplied by Ehrenstorfer Labs, Germany. Contact the lab per Internet to demand the standard solution.
- A 5 mg/l reference standard prepared by diluting the original standard into a blank beeswax extract.
- All other chemicals are of analytical grade. Sodium sulfate, sicc., water-free, corned, e.g. Merck 6639

Method

The method for the quantification of acaricides in wax was originally published by ZIMMERMANN et al., 1993 and modified as follows by BOGDANOV et al., 1998.

Extraction and purification procedure

- weigh 1.000 g of sample into a centrifugation tube, add 10.0 ml hexane, close with a stopper. Secure stopper by Parafilm to prevent opening and eventual evaporation;
- extract within a ultrasonic bath for 45 minutes, shake by hand 2-3 times. At the end the extraction wax is completely dissolved;
- place sample for at least 1.5 hours in a deep-freezer;
- centrifugation for 15 minutes at 10.000 g_{max} and –5^{o}C;
- decant supernatant in another centrifuge tube;
- place sample for at least 4 hours in a deep-freezer;
- centrifugation for 15 minutes at 10.000 g_{max} and –5^{o}C;
- decant supernatant in a glass tube, close with a stopper, warm till room temperature (20-25 ^{o}C);
- Florisil column: fill with hexane, add 0.1 g sodium sulfate, then add 1.5 g Florisil over it, do not dry out;
- add 5.0 ml of extract to column;
- wash two times with 10 ml hexane;
- elute two times with 10 ml hexane/acetone (1:1 v/v);

- evaporate to dryness with rotary evaporator in a water bath at 40 °C;
- dissolve in 2 ml isooctane and place for at least 2 hours in the deep-freezer;
- filter through a 0.45 µm filter directly into a 2 ml autosampler vial, ready for analysis.

GC analysis

- 1 µL extract is injected "on column" by an autosampler on a 30 m DB 1 (J+W) 0.25 mm id, 0.25 µm film thickness capillary column;
- on column temperature: 100 °C;
- ECD detector temperature: 300 °C;
- GC program (can be adapted according to the column);
- 1 min 100 °C, 100-180 °C rate 10 °C/min; 180-320 °C rate 3 °C/min to 320 °C, finally isotherm for 30 min;
- carrier gas: hydrogen, gas flow: 1.7 ml/min at 100 °C;
- quantification is done by the external standard method.

Calculation of results:
Concentration (c) in sample in mg/kg = (c) in extract (mg/l) x 4 (dilution factor)
This method has been successfully used for 8 years in the accredited laboratory of the Swiss Bee Research Centre and has been validated in the range between 0.4 mg to 50 mg/kg:
Recovery of acaricides, added to wax (after BOGDANOV et al., 1998):

mg/kg added	n		BP	CM	FV	FM
0.4, 1, 2, 5, 10, 20, 50	35	Recovery (mean)	101	95	80	88
		% RSD	10	10	7	12

The detection limit, tested in our laboratory was 0.1 for BP and 0.25 mg/kg for the other substances.

Collaborative study

In January 2002 information was sent to different laboratories with the detailed determination method. Fourteen laboratories agreed to participate in the collaboration trial. Two blank beeswax samples of about 10 g each were spiked with 1 and 4 mg/kg of the following acaricides: bromopropylate, coumaphos, fluvalinate and flumethrin. Samples were sent in March 2002 and analysis was carried out in triplicate in 12 laboratoires by the end of June 2002. Seven laboratories were from Italy, 3 from Germany and one from France and Switzerland (n=12, see table below).
Two laboratories used a different determination methods. The results of these two laboratories are not included in the calculation of the precision parameters, as these are valid for a definite method only. Three laboratories did not determine flumethrin.

Participating laboratory	Differences to the prescribed method
Roberta Gallarini Istituto Zooprofilattico Sperimentale Umbria e Marche Laboratoria di Chimica Via G. Salvemini 1 06126 Perugia, Italy E-mail: *r.galarini@pg.izs.it*	Used prescribed method, use of ethion as an internal standard.
Anne Claire Martel AFSSA, Site de Sophia Antipolis BP 111 105, Route de Chappes FR-06902 Sophia Antipolis-France E-mail: *ac.martel@sophia.afssa.fr*	Same method, Column: RT_x–5 (30 m 0.32 mm x 0.5 µm) for ECD and PE-XLB (30 m 0.25 mm x 0.25 µm) for NPD detector, carrier gas: He
Roberto Colombo Istituto Nazionale di Apicoltura Via di Saliceto N 80 IT-40126 Bologna E-mail: *istnapic@inapicoltura.org*	Fluvalinate from Riedel de Haen. Split injection (1:20) at 230° Carrier gas: He, Program: 180° to 300 °C, with 2 °C/min program.
Jurgen Wehlitz Institut für Honiganalysen Flughafendamm 9 a DE-28199 Bremen 1 E-mail: *info@quain.de*	**Different method:** Extraction with water, acetone and petroleum benzine in Ultraturrax, clean-up with celite with acetonitrile, determination with a Varian Saturn 2000 GC-MS/MS.
Handels und Umweltslabor Wiertz -Eggert-Jörissen GmbH Stenzelring 14 b DE-21107 Hamburg E-mail: *laborwej@aol.com*	Same method

Roberto Piro Ist. Zooprofilattico Sperimentale delle Venezie Via Romea 14 A Legnaro – PD, 35020, Italy E-mail: *chimica@izsvenezie.it*	Centrifugation at 2000 g-max and 5 °C for 40 minutes GC columns used: ZB 1 and ZB 1705, carrier gas: He
Paolo Matteini Progetto Natura Via Marradi Giovanni, 41 59100 Prato, Italy E-mail: *matteini.p@progettonatura.com*	Injection split-splitless at 280 °C on 30 m DB5 0.25 mm x 0.25 µm, carrier gas: He
Livia Persano Oddo Istituto Sperimentale per la Zoologia Agraria, Sezione di Apicoltura Via L. Rech 36 00156 Roma, Italy E-mail: *livia.persano@apicoltura.org*	0.2 g wax disolved in 3 ml hexane at 60 °C. Clean-up with 500 mg Florisil SPE columns, elution with 3 ml acetone: hexane 1:1, disolving in 1 ml hexane. Column: DB-1 0.53 mm id, injection of 0.5 µl. Carrier: H_2 5.5 ml/min.
Italo Commissati Chelab Via Fratta, 25 31023 Resana (TV), Italy E-mail: *i.commissati@chelab.it*	**Different method** Wax disolved in hexane, clean-up with GPC, residue dissolved in acetone/hexane/acetonitrile. Detection with GC-MS/ECD. Flumethrin was determined by HPLC.
Stefan Bogdanov Swiss Bee Research Centre, FAM 3003 Bern-Switzerland E-mail: *stefan.bogdanov@fam.admin.ch*	Same method
Enrica Ferretti Istituto Zooprofilattico Sperimentale Lombardia ed Emilia Romagna Via Bianchi 7, 25100 – Brescia E-mail: *eferretti@bs.izs.it*	0.2 g of wax per florisil column. Use of a commercial Florisil column: IST Florisil, 1 g.
Klaus Wallner Universität Hohenheim Landesanstalt für Bienenkunde August von Hartmannstr. 13 70593 Stuttgart, Germany E-mail: *bienewa@Uni-Hohenheim.de*	Same method, extraction by hexane: heating at 60 °C. Injection: split (1:20)

Evaluation of precision parameters

The precision parameters (repeatability r and reproductibility R) of the method, were calculated with robust statistics using the method of the Swiss Food Manual. Only the data of the 10 laboratories, which used the same method were considered for the calculation. Besides, the performance of all laboratories was graphically represented.

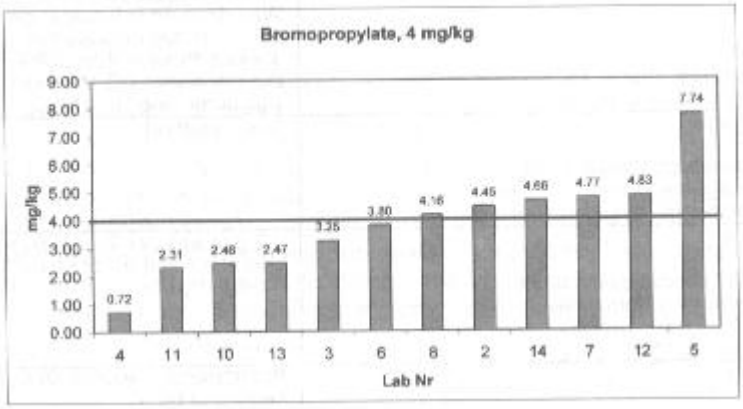
Results

Average concentration of acaricides in beeswax measured in the different laboratories

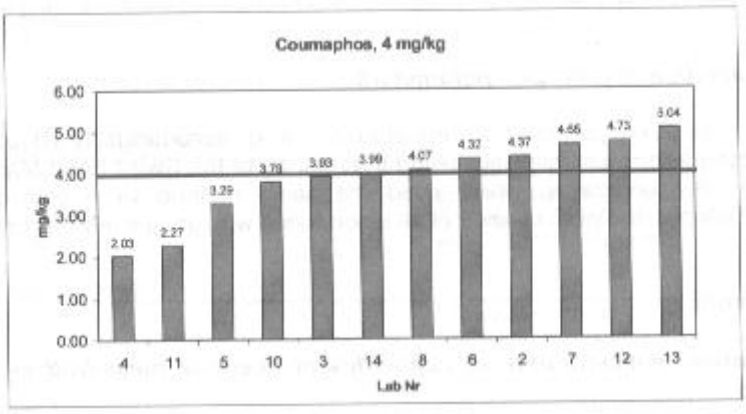
	Sample A, 4 mg/kg				Sample B, 1 mg/kg			
Lab	BP	CM	FV	FM	BP	CM	FV	FM
2	4.45	4.37	3.48	5.50	1.25	1.02	0.80	2.99
3	3.25	3.93	5.54		0.53	0.51	1.58	
5	7.74	3.29	6.09	4.74	3.01	1.07	2.02	1.23
6	3.80	4.31	3.60	6.24	0.91	0.93	0.79	1.73
7	4.77	4.65	6.57	5.80	1.70	1.57	2.05	1.95
8	4.16	4.07	3.19	4.50	0.96	0.95	0.76	1.46
10	2.46	3.79	2.21		0.77	0.96	0.65	
11	2.31	2.27	4.18		0.64	0.53	0.94	
12	4.83	4.73	4.34	4.21	1.21	1.25	0.91	0.90
13	2.47	5.04	1.35	2.60	0.65	0.95	0.26	1.29
4	0.72	2.03	1.56		0.27	8.88	0.64	
14	4.66	3.99	3.35	3.86	1.09	1.10	1.21	0.77

BP: bromopropylate, CM: coumaphos, FV: fluvalinate, FM: flumethrin

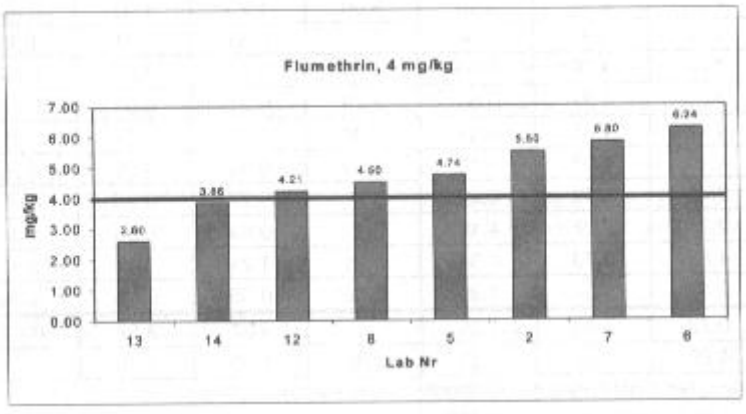
Lab 4 and 14 used another method.
The results of the different laboratories are given in the figures.



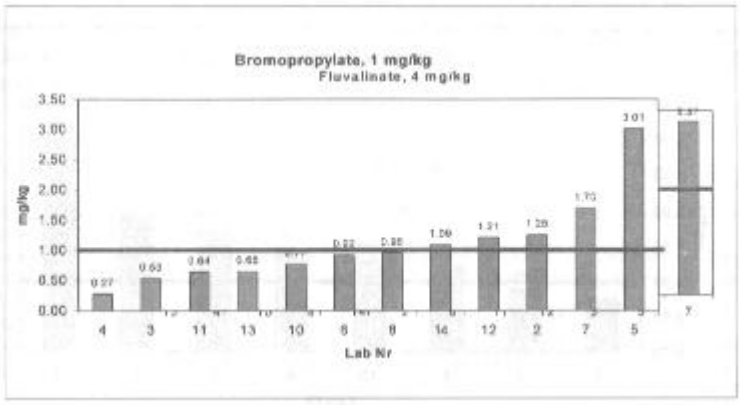
Bromopropylate, 4 mg/kg



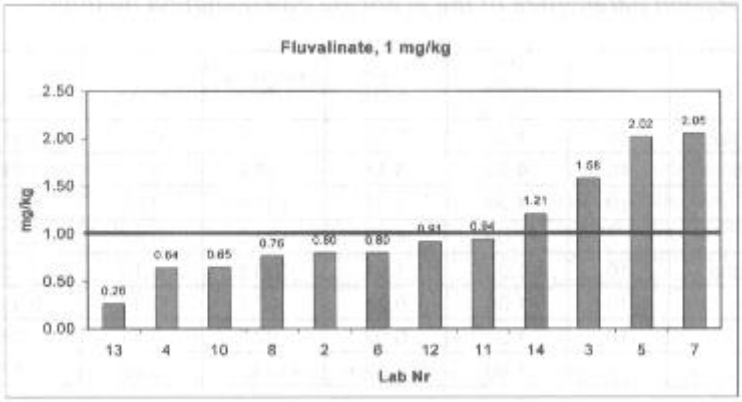
Coumaphos, 4 mg/kg



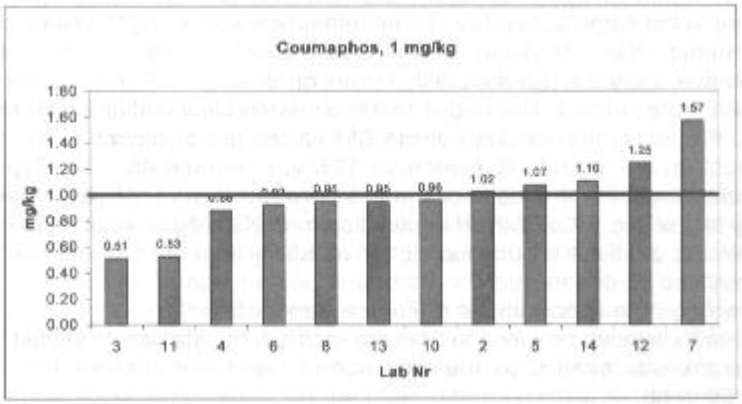
Flumethrin, 4 mg/kg



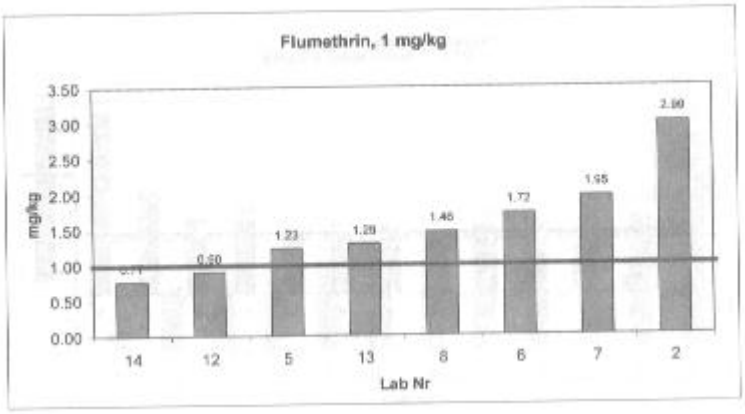
Bromopropylate, 1 mg/kg
Fluvalinate, 4 mg/kg



Fluvalinate, 1 mg/kg



Coumaphos, 1 mg/kg



Flumethrin, 1 mg/kg

Precision parameters of the acaricide determination method

	n	True value mg/kg	Mean mg/kg	Recovery %	r mg/kg	R mg/kg	RSD_R %
Bromopropylate	10	4.00	3.78	94.4	0.72	3.41	31.9
Coumaphos	10	4.00	4.17	104.2	0.97	1.52	12.9
Fluvalinate	10	4.00	4.07	101.8	0.97	5.26	45.7
Flumethrin	7	4.00	4.89	122.2	0.88	3.23	23.4
Brompropylate	10	1.00	1.01	100.8	0.19	1.25	43.6
Coumaphos	10	1.00	0.98	97.6	0.30	0.43	15.6
Fluvalinate	10	1.00	0.92	92.3	0.24	0.86	32.9
Flumethrin	7	1.00	1.52	152.1	0.49	1.38	32.2

Discussion

The repeatability r of the determination is satisfactory (repetition of the measurement in the same laboratory, measurement carried out by the same person).

Generally, the reproducibility R is 2 to 3 times greater than the repeatability r. In this collaborative study the reproducibility R was on average 3.7 times greater than r, which is poorer than the expected. This is due to the considerable variation of the measurement of BP, FV and FM, while the variation of the CM values are acceptable. The RSD_R % value was also high, on average 30 % (minimum 12.9%, maximum 45.7 %). Typically, RSD_R % values of determinations of substances in this concentration range lies between 10 and 20 % (see ring trial of the IHC, HMF determination in BOGDANOV et al., 1997). On the other hand, beeswax is a difficult matrix and higher variability than in honey can be expected. This high variability can be due to:

- wrong calibration with the reference standards;
- matrix effects: as mentioned in the method, the standards should be dissolved in blank wax extract, as the response to standards, dissolved in solvent is quite different;
- losses during the injection (split-splitless injection);
- different separation characteristics of the GC-columns.

Analysts are advised to carry out regularly recovery experiments during the routine analysis of acaricides in wax. In our laboratory, analysis of a refererence wax, containing a certain amount of acaricides, is carried out in each determination series.

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