Apimondia journal
APIACTA



# HPLC, CHARM II AND ELISA: ADVANTAGES AND DISADVANTAGES FOR THE ANALYSIS OF TETRACYCLINES IN HONEY

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## INTRODUCTION

HPLC, Charm II and ELISA are the three analytical techniques that have been applied for the analysis of tetracycline residues in honey from a market study with 359 samples and for the analysis of honey with incurred residues after treating honey bees with oxytetracycline and chlortetracycline.

A report is given on the results of these studies and on our experience with the applied analytical techniques especially with regard to costs, sample throughput, detection limit and specificity.

While many countries allow the use of tetracyclines to treat or prevent American foulbrood (AFB) caused by Paenibacillus larvae larvae [1], there is no drug approval of tetracyclines for honey bee medication within the EU and thus honeys with tetracycline residues cannot be marketed in the EU member states [2]. Honey with residues of tetracyclines can be expected when imported from countries with tetracycline approval or by an unauthorized used in apiculture.

## METHODS AND MATERIALS

A commercially available enzyme immunoassay (ELISA) (Ridascreen® Tetracycline, R-Biopharm GmbH, Darmstadt, Germany), the CHARM II test (MCS Diagnostic, Swalmen, NL) and a high-performance liquid chromatographic (HPLC) method were applied to analyse oxytetracycline (OTC) and chlortetracycline (CTC) in honey.

ELISA

The test procedure was carried out according to the instructions given by the test kit manufacturer [3]. Briefly, the honey samples diluted 1:50 with buffer were filled into the microtiter wells coated with a tetracycline-protein-conjugate and anti-tetracycline antibodies were added. Any unbound antibodies were removed by washing steps. After adding enzyme substrate and chromogen the absorption was measured at 450 nm.

Charm II

Sample preparation was restricted to a simple dilution step by dissolving 1 g honey in 19 mL of buffer shipped with the test kit. After suspending the receptor tablet in 300 µL water, 4 mL of the diluted honey and the 3H-labeled tetracycline tablet were added and mixed. The suspension was centrifuged and the unbound tetracyclines were poured off with the supernatant. The sediment was homogenized, mixed with the scintillation fluid, with subsequent measurement of the scintillation counts.

HPLC

The HPLC analyses were performed according to the official method section 35 LMBG (Food and Commodities Act of Germany) [4]. Briefly, 10 g of honey was diluted to 100 g with buffer. An aliquot (50 g) was applied to a copper-loaded chelating sepharose column followed by three washing steps using water and methanol. The analytes were eluted and subsequently trapped by a C18-solid phase extraction cartridge using an EDTA containing buffer. After elution with methanol and evaporation to ca. 200 mg, the residue was re-dissolved and the solution injected onto the HPLC with UV detection with wavelength set to 360 nm.

## MEDICATION STUDY

Formulations of powdered sugar mixed with oxytetracycline or chlortetracycline were prepared for the medication. To prevent inaccuracies due to inhomogeneity of the mixture, 30 g powdered sugar were mixed with 200 mg of antibiotic and administered completely to one hive. According to pharmaceutical practice, 200 mg of antibiotic was first mixed with a small part of the sugar and then successively with increasing amounts of the remaining sugar. These mixtures were stored under dry and dark conditions. Drug administration was carried out by spreading the mixtures completely on the top of the frames. The bees took up these mixtures within one day. Five colonies were treated with oxytetracycline, five with chlortetracycline and two colonies received unmedicated powdered sugar. These two control colonies were located in direct neighbourhood of the treated colonies, while 3 further control colonies were put in a distance of approximately 10 km from the treated colonies. The medication started with the first treatment directly after harvesting spring honey followed by two subsequent administrations 7 and 14 days thereafter. The honeys were harvested 10 weeks after medication started and collected and filtered according to good beekeeping practice. Finally, the honeys were stirred intensively for ten minutes for better homogenization, filled in a jar, capped and stored at ambient temperature until analyses were performed (within 14 days). Additional analyses were performed by Applica GmbH using an online-solid-phase extraction coupled with HPLC [5]. The residues in honeys harvested from bees treated with CTC ranged from 300 to 600 µg/kg (Fig, 2) as the sum of CTC and it's 4-epimer, with an epimerization rate of 38% to 43%.

The honeys from bees treated with OTC gave detectable results with the ELISA and Charm II test. By HPLC a very low residue level could be detected in only one honey, all others were negative (Fig 3).

## MARKET STUDY

A number of 359 honeys of various origin were analysed by ELISA and Charm II test to achieve an overview on the actual situation of contamination of honeys with tetracyclines on the German market. (table I) This study, however, cannot be regarded as representative because some of the samples were supplied as suspects by import companies before blending.

## CHARACTERISATION OF THE APPLIED ANALYTICAL PROCEDURES

Based on our experiences with the chromatographic and the semi-quantitative methods, parameters for their characterisation are given in table II.

## CONCLUSION

ELISA and Charm II tests are useful techniques for semi-quantitative screening of tetracycline residues in honey. HPLC is necessary to identify and reliably quantify the

individual tetracycline. According to HPLC analyses, OTC appeared only in traces as parent compound and 4-epimer in the honey after applying this antibiotic to bees. OTC degradation products, however, could be detected most sensitively with the ELISA test. A positive ELISA (or Charm II) result without signals for the presence of a tetracycline in the HPLC, can therefore be regarded as an indication for an OTC treatment in honey bees.

Fig. 1: Flow diagrams for the HPLC, Charm II and ELISA procedures

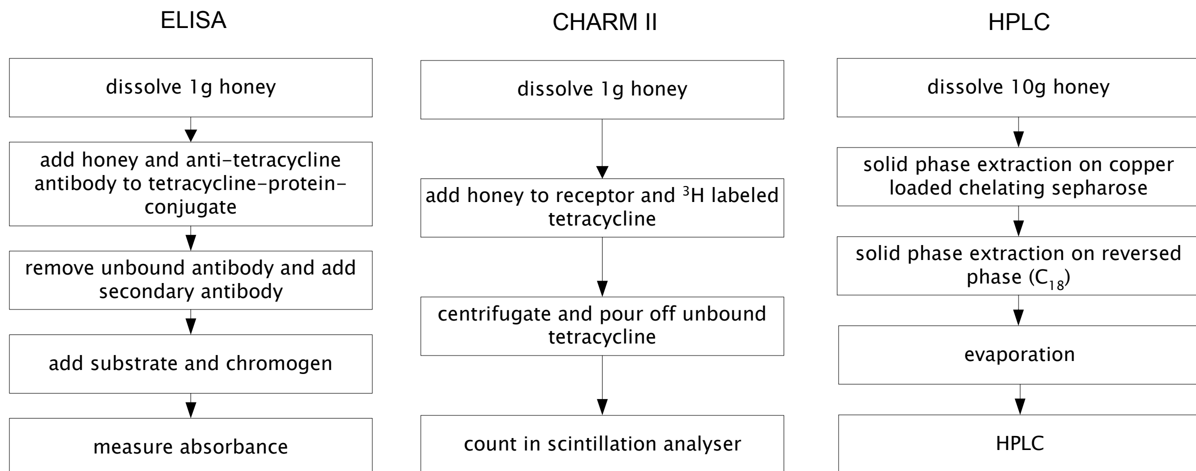


Fig. 2: Residues of CTC (sum of parent compound and the 4-epimer) after medicating colonies with CTC

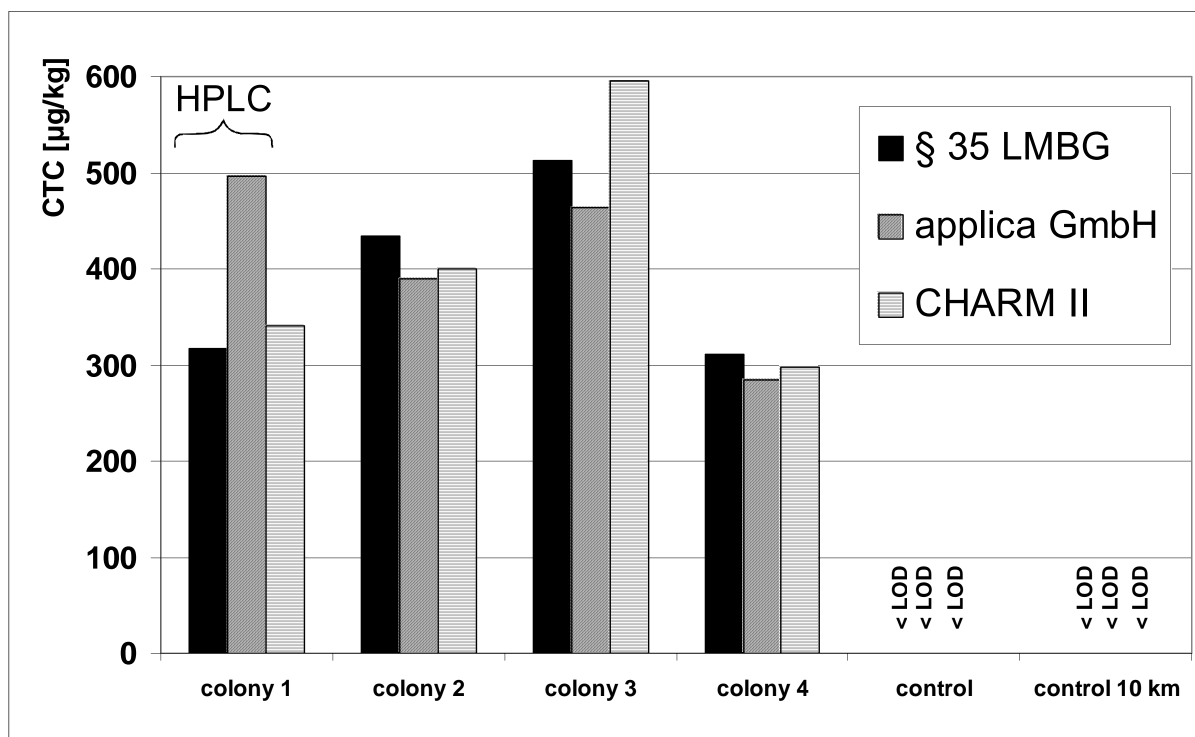

Fig. 3: Results for OTC residue analyses after medicating colonies with OTC.

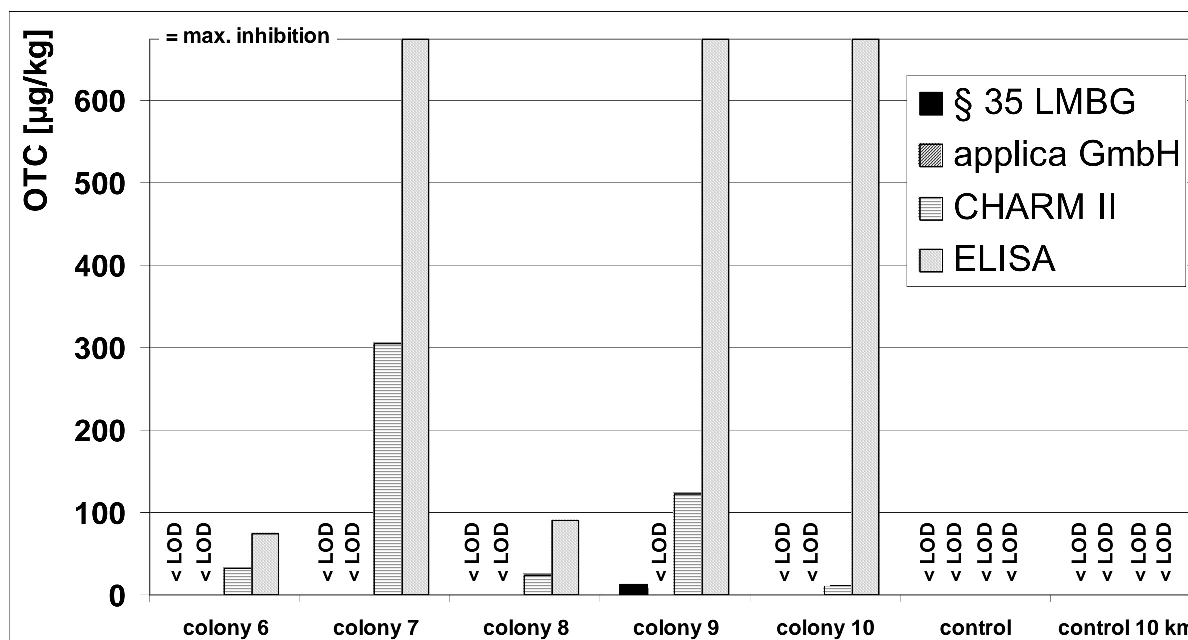


Table I: Results of the market study

| origin of samples | samples | percentage of positive |
|---|---|---|
| Germany | 66 | all negative |
| Europe (without Germany) | 41 | 5% |
| America (North, Central and South) | 79 | 24% |
| Asia | 18 | 11% |
| unknown | 155 | 9% |
| Σ | 359 | 10% |

Table II: Characterisation data of the applied analytical procedures

| | ELISA | Charm II | HPLC |
|---|---|---|---|
| Basic investment | 5.000 € | 20.000 € | 30.000 € |
| Costs per analysis (materials) | 21 € | 11 € | 10 € |
| Suitability for single samples | poor | good | good |
| Practicability | good | good | poor |
| Time (30 samples, duplicate analyses) | 6 h | 8 h | 3 d |
| Limit of detection | 10 µg/kg | 10 µg/kg | 25 µg/kg |
| Cross reactivity | OTC < TC < CTC | OTC < TC < CTC | specific determination |
| Determination of epimers | not possible | not possible | possible |
| Detectability of OTC degradation products | ⊕ | ⊕ | ⊖ |

| | ELISA | Charm II | HPLC |
|---|---|---|---|
| Shelf life of test kit | approx. 6 month (2-8 °C) | approx. 1 year (< 15 °C) | no limitation |

## ACKNOWLEDGEMENT

The authors thank Dr. K. Raezke (Applica GmbH, Bremen) for supplying additional HPLC data, G. Wusterack (Berlin) for beekeeping and the Ministry of Environment and Forestry Rhineland-Palatinate for financial support.

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