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Original article

Determination of residual oxytetracycline in honey with an immunoassay kit

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Summary — The application of the ELISA kit Ridascreen 'Tetracyclins" (from R-Biopharm GmbH, Darmstadt, Germany) for the semiquantitative analysis of oxytetracycline (OTC) residues in honey samples was investigated. To assess the extension of the kit to this use, some OTC-positive honey samples, spiked with OTC at concentrations ranging from 2 to 250 ppb, were prepared, purified and submitted to the ELISA test. For every set of positive honey samples, a set of OTC-standard solutions in water at the same concentration value of the positive samples and an OTC-negative (blank) control honey sample, were tested. Also, the efficiency of three different chemical purification procedures of honey samples was studied and verified: (1) the honey samples were diluted in water; (2) the honey samples were purified on a prepacked reversed-phase column (C-18 cartridges); (3) the honey samples were purified by a two-step system using a C-18 cartridge and a Diol-column. The mean recoveries of OTC were 90.4% (CV = 2.6%) for the test using C-18 (2) and 88.6% (CV = 2.1%) for the test using both C-18 and Diol cartridges (3). The detection limits were lower than 50 ppb.

honey / residue / oxytetracycline / immunoenzymatic assay

INTRODUCTION

To prevent foul brood of honeybees, many antibiotics of the chemical class of tetracyclines, especially oxytetracycline (OTC), are widely used in honeybee culture all over the world. Given to bees in sugared water, OTC can remain in honey. Therefore, for public health purposes, it is necessary to detect its presence and amount in this very peculiar matrix. Several microbiological assays (Lightbown and De Rossi, 1965; Corner and Gochnauer, 1971; Gochnauer and Bland, 1974; Bentler and Frese, 1981; Lehnert and Shimamuki, 1981; Hasselberger, 1993) are presently available for the determination of OTC in biological samples, but their accuracy appears to be variable and their specificity questionable. Moreover, these assays are not suited for the analy-

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sis of OTC in honey, because honey itself has a bacteriostatic action, which affects the results of the tests (White et al, 1963; Sporns et al, 1986).

High performance liquid chromatography (HPLC) analysis of OTC offers a selective low level detection method and many HPLC methods have been reported for the determination of residual OTC in biological samples (Jurgens, 1981; Sporns et al, 1986; Oka et al, 1987; Renon et al, 1988; Moats, 1990). However, these methods are often not very reliable in honey. In fact, the chromatographic profiles of injected honey extracts are often complicated by many spurious peaks, resulting from the interfering substances contained in the matrix (Jurgens, 1981; Sporns et al, 1986; Argauer and Moats, 1991).

In this study, we investigated the extension of an ELISA test kit specifically designed for meat and milk samples (Ridascreen ELISA kit 'Tetracyclines' from R-Biopharm GmbH, Darmstadt, Germany), to the semiquantitative analysis of OTC residues in honey. According to the manufacturers' description, this kit recognizes several compounds of the chemical class of tetracyclines, including OTC, to which it is reported to display cross-reactivity of 5%.

MATERIALS AND METHODS

The assessment procedure of this ELISA test to honey samples has been developed as follows: First, the sensitivity of the test to OTC was determined by analysing a set of water solutions of the standard compound (OTC-HCl, Vetranal, code No 46597) at four different concentrations (0.2, 1.0, 5.0 and 25 ng/mL).

The applicability of the test to honey was then determined by analysing OTC-fortified honey samples, spiked with different amounts of OTC at concentrations ranging from 2 to 250 ppb. The samples were extracted with three different chemical purification procedures and submitted to an ELISA test. All tests were performed using the same batch of honey. For every set of fortified honey samples, a set of OTC-standard water solution at 0, 0.2, 1.0, 5.0 and 25 ng/mL and an OTC-negative control honey sample (blank samples) were tested. Each honey sample determination was carried out twice. The mean values of the absorbance values obtained for the standards and the samples are divided by the absorbance value for the 'zero' standard and multiplied by 100. Before being submitted to the ELISA test, all the purified honey extracts and standard solutions were diluted 1 to 10 with aliquots of the PBS-like buffer (Buffer 1) provided in the kit. The three following purification procedures were tested.

(1) Simple dilution (1:2.5) in distilled water involved adding 2 g honey samples spiked at 50 and 100 ppb ($n = 2$) to 5 mL water and diluted 1 to 10 with Buffer 1 (dilution factor = 25). In the test solution the concentration of OTC was 2 ng/mL (50 ppb) and 4 ng/mL (100 ppb). For study of the matrix effects, the absorbance values of blank honey samples were determined after dilution with water in 1:25, 1:50 and 1:100 proportions.

(2) Extraction with McIlvane Buffer (Renon et al, 1988) at pH 4 (prepared dissolving in water aliquots of EDTA disodium salt 0.1 M, phosphate disodium Salt 0.2 M and Citric acid 0.2 M) and clean-up on C18 Sep-Pak cartridges (from Waters, code No WAT051910) involved spiking the honey samples with OTC at 2, 10, 50, 250 ppb ($n = 2$). The activation of the column was performed by flushing 10 mL of methanol and 10 mL of distilled water. Extraction buffer (20 mL) was added to each 5 g honey sample. The solution was sonicated for 5 min and transferred to a C-18 Water Sep-Pak cartridge. After the sample was loaded, the column was flushed with 10 mL of water (which was discarded) and air-dried under vacuum for 5 min. The samples were eluted with 5 mL of 0.01 M methanolic/oxalic acid. The eluate was diluted 1 to 10 in Buffer 1: the concentration of OTC in the test solution was 0.2, 1.0, 5.0 and 25 ng/mL.

(3) Extraction with McIlvane Buffer at pH 4 and tandem clean-up on C-18 Sep-Pak cartridges and Diol Bakerbond SPE columns (from JT Baker, code No 7094-03). Involved spiking the honey samples with OTC at 10 and 50 ppb ($n = 2$). The C-18 cartridge containing the sample (see (2) above) was fitted into an adapter on a Diol -Bakerbond SPE column already conditioned with 10 mL methanol. OTC was transferred from the C-18 to the Diol-(COHCOH) column by using 10 mL of 0.01 M methanol/oxalic acid. The C-18 cartridge was removed and the Diol-column was washed

with 10 mL methanol. The sample was eluted with 10 mL distilled water. The concentration of OTC in the test solution was 1 (10 ppb) and 5 ng/mL (50 ppb).

RESULTS AND DISCUSSION

The ELISA results are reported in table I. The mean absorbance values (percentage Abs/Abs_0) of the standard solutions at 0.2, 1.0, 5.0 and 25 ng/mL ($n = 3$) were 86% (CV = 7.3%), 66% (CV = 0.7%), 43% (CV = 4.9%) and 29% (CV = 4.8) respectively. The values calculated for the standards have enabled us to establish a sensitivity limit of the kit for OTC in water of approximately 0.2 ng/mL. A comparison of the three chemical purification procedures show false positive reactions of the blank samples purified with the procedure (1). The absorbance values of honey samples diluted with water 1:25, 1:50 and 1:100 were 67.0%, 80.0% and 92.2% of the 'zero' standard, respectively.

Table I. Results of the immunoassay determination of OTC in honey samples submitted to three different procedures of purification.

	Concentration of OTC test solution (ng/mL)	*Abs/Abs_0 (%)* [a]	*CV (%)* [b]	*Concentration OTC honey samples (ppb)*
Standards ($n = 3$)	0	100		
	0.2	86	7.3	
	1.0	66	0.7	
	2.0	nd		
	4.0	nd		
	5.0	43	4.9	
	25.0	29	4.8	
Assay 1. Dilution with water. $n = 2$				
	0 (dil 1:25)	67.0	5.7	0
	0 (dil 1.50)	80.0	3.0	0
	0 (dil 1:100)	92.2	3.89	0
	2 (dil 1:25)	67.1	4.9	50
	4 (dil 1:25)	60.8	5.1	100
Assay 2. C-18 cartridges. $n = 2$				
	0	97.3	2.5	0
	0.2	84.6	6.2	2
	1.0	77.2	4.3	10
	5.0	59.0	4.8	50
	25.0	41.0	2.2	250
Assay 3. C18/Diol cartridges. $n = 2$				
	0	94.8	2.9	0
	2.0	63.0	4;8	10
	5.0	43.0	3.7	50

[a] Abs/Abs_0 = absorbance value of the sample (Abs) divided (/) by absorbance value of the zero standard (Abs_0).
[b] CV = coefficient of variation. dil = dilution; nd = not determined.

As for the procedure (2), the absorbance values of samples purified on C-18 cartridges were approximately the same as the 'zero' standard but the reaction of spiked samples was lower than expected, ie, the absorbance values of spiked samples were higher than the absorbance of OTC standards at the same concentration. We were not able to repeat the test and confirm this result because of the deterioration of kit reagents. Assay (3), using the tandem clean-up with C-18 and Diol-cartridges, was accurate and specific. We concluded that with the tandem clean-up is perhaps possible to obtain a quantitative determination of the presence of OTC in honey with a detection limit lower than 20 ppb. With other procedures only a qualitative estimation of the presence of OTC in honey can be obtained because of the interfering effect of the matrix. The detection limit was approximately 50 and 10 ppb for assays (1) and (2), respectively. As for the reliability of the three different sample preparations, the complex and time-consuming procedure of tandem clean-up is not suited to a routine application such as a screening test. Nevertheless, owing to the excellent purification, the same extract can be use twice both for the screening test with ELISA and for the confirmatory test with HPLC. Owing to its simplicity, the first method proved the most suitable for the screening of honey provided that the dilution of honey samples in water is in a proportion of at least 1:100 to eliminate false-positive reactions. Nevertheless, the results suggest that some trials should be repeated, particularly to confirm the type of matrix effects in this and other types of honey and to check the unexpected results obtained with spiked samples purified on C-18 cartridges.

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Résumé — Détermination des résidus d'oxytétracycline dans le miel : utilisation de la technique Elisa. On a recherché si le kit Elisa Ridascreen 'Tetracyclins', utilisé tout spécialement pour la recherche des tétracyclines dans des échantillons de lait et de viande, pouvait être appliqué à la détermination qualitative et quantitative des résidus d'oxytétracycline (OTC) dans le miel. Pour cela des échantillons de miel contaminés par de l'OTC à des concentrations comprises entre 2 et 250 ppb ont été préparés. Ils ont ensuite été soumis à différents procédés de purification et enfin testés avec le kit Elisa. Pour chaque échantillon de miel positif, des dilutions standard d'OTC en solution aqueuse, à la même concentration que le miel, ont été préparées, ainsi qu'un échantillon de miel sans OTC (témoin). Les échantillons de miel ont été préparés selon trois procédés de purification différents : i) dilution de l'échantillon dans de l'eau à des proportions de 1:2.5 ; ii) purification sur colonne à phase inversée (C18) ; iii) purification en tandem sur colonne à phase inversée (C18) et successivement sur colonne à phase diolique (COHCOH). Les résultats obtenus avec le test Elisa varient en fonction du procédé de purification. Une estimation qualitative est seulement possible avec les deux premiers procédés. En revanche, le procédé 3 permet d'obtenir une détermination qualitative et quantitative d'OTC, mais il est techniquement compliqué et ne convient pas à des analyses de routine. Le seuil de détermination se situe à 100 ppb pour une détermination semi-quantitative et à 50 ppb pour une détermination quantitative précise avec la méthode 3 (purification en tandem).

miel / résidu / oxytétracycline / antibiotique / technique Elisa

Zusammenfassung — Bestimmung von Oxytetracyclinrückständen in Honig durch einen enzymgebunden Immuno-

assay. Es wurde untersucht, ob das für den Nachweis von Tetrazyclinen in Muskel- und Milchproben entwickelte Testkit ELISA Ridascreen 'Tetracyclin' auch für die qualitative und quantitative Bestimmung der Oxytetracyclin-Rückstände (OTC) im Honig geeignet ist. Hierzu wurden Honigproben mit verschiedenen OTC-Konzentrationen (2-250 ppb) hergestellt. Die Proben wurden mit unterschiedlichen Methoden aufbereitet und anschließend mit der ELISA Testkit getestet. Als Kontrollwerte für jeden Test dienten eine wässerige Standardösung mit OTC gleicher Konzentration wie die Honigprobe und eine OTC-freie Honigprobe (Leerprobe). Die Honigproben wurden mit folgenden 3 Verfahren aufbereitet: 1. Verdünnung der Probe mit Wasser im Verhältnis von 1:2,5; 2. Reinigung mit einer reversed phase Säule (C_{18}); 3. Tandem - Reinigung mit einer reversed phase Säule (C_{18}) und anschließend mit einer Säule mit diolischer Phase (COHCOH). Die mit dem ELISA Test erzielten Ergebnisse sind je nach Aufbereitung unterschiedlich. Bei den ersten beiden Verfahren ist nur eine qualitative Bestimmung möglich. Dagegen ist mit Reinigung über eine C18 und Diol-Säule eine qualitative und quantitative Bestimmung des OTC-Gehalts im Honig möglich. Dieses dritte Verfahren ist allerdings technisch kompliziert und für Routineuntersuchungen ungeeignet. Die Nachweisgrenze liegt bei 100 ppb für eine semiquantitative Aussage mit der ersten Methode und unter 50 ppb bei der genauen quantitativen Bestimmung mit der dritten Methode (Tandem- Reinigung mit einer C-18 und einer Diol- Säule).

Honig / Rückstande / Oxytetracyclin / Enzymimmunologisches Testkit

REFERENCES

Argauer RJ, Moats WA (1991) Degradation of oxytetracycline in honey as measured by fluorescence and liquid chromatographic assays. *Apidologie* 22, 109-115

Bentler VW, Frese E (1981) Mikrobielle Beschaffenheit und Rückständuntersuchung von Bienenhonig. *Arch Lebensmittelhyg* 32, 130-135

Corner J, Gochnauer TA (1971) Persistence of tetracycline activity in medicated syrup stored by wintering honeybee colonies. *J Apic Res* 10, 67-71

Gochnauer TA, Bland SA (1974) Persistence of tetracycline activity in medicated syrup stored in honeybee colonies in late spring. *J Apic Res* 13, 153-159

Hasselberger ML (1993) Assay of oxytetracycline in animal feed by liquid chromatography and microbiological plate assay. *J Assoc Off Anal Chem Int* 76, 39-43

Jurgens U (1981) Zur hochdrückflüssigchromatographischen Analyse von Arzeimittelrückständen in Honig. *Z Lebensm Unters Forsch* 173, 356-358

Lehnert T, Shimanuki H (1981) Oxytetracycline residue in honey following three different methods of administering the antibiotic. *Apidologie* 12, 133-136

Lightbown JW, De Rossi (1965) The identification and assay of mixtures of antibiotics by electrophoresis in agar gel. *The Analyst* 90, 89-95

Moats WA (1990) Liquid chromatographic approaches to antibiotic residue analysis. *J Assoc Off Anal Chem* 73, 343-346

Oka H, Yoshimoto I, Kawamura N, Keijchi U, Yamada M (1987) Improvement of chemical analysis of antibiotics. IX: A simple method for residual tetracyclines analysis in honey using a tandem cartridge clean-up system. *J Chromatogr* 389, 417-426

Oka H, Matsumoto H, Kelichi U (1985) Improvement of chemical analysis of antibiotics. VIII. Application of prepacked C18-cartridge for the analysis of tetracycline residues in animal liver. *J Chromatogr* 325, 265-274

Renon P, Bareggi S, Corsico G, Pirola R (1988) Contributo alla determinazione di residui di tetracicline mediante cromatografia liquida ad alta prestazione. *Ingegnieria Alimentare* 3, 9-23

Sporns P, Kwan S, Roth LA (1986) HPLA analysis of oxytetracycline residue in honey. *J Food Prot* 49, 383-388

White JW, Subers MH, Schepartz AI (1963) The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose oxidase system. *Biochim Biophys Acta* 73, 57-70