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Validation of the Tetrasensor Honey Test Kit for the Screening of Tetracyclines in Honey

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Regarding anti-infectious agents, no maximum residue limits are fixed for honey in the European legislation. Discussions are being conducted in order to set working limits at the European level; for example, for tetracyclines, 20 μg/kg was proposed. The Tetrasensor Honey test kit is a receptor-based assay using dipsticks for a rapid screening (30 min) of honey on the presence of tetracyclines. The test was validated according to Commission Decision 2002/657/EC. The test detects tetracycline, oxytetracycline, chlortetracycline, and doxycycline in honey in a specific and sensitive way. Depending on the type of tetracycline, detection capabilities (CCβ) between 6 and 12 μg/kg were obtained (4–7 μg/kg for dried dipsticks). The test is rugged and participation with the test in an international ring trial gave compliant results. It can be concluded that the Tetrasensor Honey test kit is a simple and reliable test that can even be used at the production site.

KEYWORDS: Tetracyclines; honey; screening; rapid tests; residues

INTRODUCTION

Honey is generally considered as a natural and healthy product. The addition of additives or conserving agents to honey is not allowed. However, in recent years, the problem of residues of antibiotics in honey has been mentioned in some publications (*1, 2*). Antibiotics, for example, tetracyclines, are used in apiculture for the treatment of bacterial brood diseases like American foulbrood (*Paenibacillus larvae* subsp. *larvae*) (*3, 4*) and European foulbrood (*Melissococcus pluton*) (*5, 6*). This practice is illegal in Europe. However, oxytetracycline is used in Great Britain in the statutory treatment of European foulbrood since this is considered by the authorities as within the cascade system for veterinary medicines under minor uses and minor species (*6*). The intensive use of tetracyclines in professional beekeeping in the United States and South America resulted in tetracycline-resistant *Paenibacillus* strains (*7, 8*).

Tetracyclines are broad-spectrum bacteriostatic antibiotics with a long history in veterinary medicine and are used for the treatment and control of a wide variety of bacterial infections. When used in beekeeping, important concentrations up to a milligram per kilogram level could be found in the honey of the treated hives (*6, 9*) with a slow depletion and degradation (a half-life time for oxytetracycline of 9–44 days (*6*) and a half-life time of 65 days for tetracycline in honey from supers (*9*)). The research conducted by Adams et al. on the fate of chloramphenicol, furazolidone, streptomycin, and tylosin in honey after administration to bee colonies resulted in similar conclusions (*10*).

Reliable screening methods are needed in order to check honey for the presence of antibiotics. In general, Charm II receptor assays are used, but the rate of false-positive results could be relatively high (*1, 11, 12*). A new rapid receptor-based screening test for the detection of tetracyclines in honey was developed by Unisensor s.a. (Liège, Belgium), namely, the Tetrasensor Honey.

The use of antibiotics in apiculture is not authorized in the European Union. No maximum residue limits are fixed for tetracyclines in honey in the European legislation (EEC Regulation 2377/90 and modifications (*13*)). Some member states established action limits in order to make the situation more clear for honey producers, traders, and food inspectors. In Belgium, action limits for residues of antibiotics and sulfonamides in honey were introduced in 2002, taking into account analytical possibilities and available toxicological data. During the first period of 6 months, the action limit for the group of tetracyclines was preliminarily set at 50 μg/kg. Since July 1, 2002, this value has been fixed at 20 μg/kg. France applies a nonconformity limit for tetracyclines in honey of 15 μg/kg, the reporting limit in Great Britain (Central Science Laboratory, DEFRA, GB) is 50 μg/kg, while the tolerance levels in Switzerland are 20 μg/kg. Discussions are being held at the European level to set working limits for residues of antibiotics in honey. The community reference laboratory proposed 20 μg/kg as the recommended concentration for the screening of tetracyclines in honey (*14*).

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At the present, it is generally accepted that the screening level for tetracyclines in honey should lie within the range of 10–20 μg/kg. To adapt their screening test to this level, Unisensor improved the sensitivity of the Tetrasensor Honey test kit in 2004. All data in this study are based on test kits with an improved detection capability (second generation). The kits of this generation carry "Detection limit at 10 μg/kg" on the label.

The aim of this work was to perform a validation study of the Tetrasensor Honey (second generation) screening method on the basis of validation criteria set in Commission Decision 2002/657/EC (*15*). Since the Tetrasensor Honey is a qualitative screening kit for tetracyclines, only the following parameters had to be investigated for validation purposes: the specificity of the test kit, the detection capability, and the test ruggedness (*15*).

MATERIALS AND METHODS

Reagents and Standards. The tetracycline (T3383), oxytetracycline (O5875), chlortetracycline (C4881), doxycycline (D9891), penicillin G (PENNA), cephapirin (C8270), sulfadiazine (S8626), neomycin (N1876), and erythromycin (E6376) were all from Sigma-Aldrich (Bornem, Belgium). The enrofloxacin (17849) was from BioChemika (Bornem, Belgium).

Standard stock solutions of 100 mg/L were made in water and kept below 4 °C. Dilutions of 1 and 0.1 mg/L were freshly prepared on a daily basis.

The Tetrasensor Honey kits were from Unisensor s.a. (Liège, Belgium). In general, lot TH00616-042405/4, expiration date November 23, 2005, was used for the evaluation study; for some parts, such as the study of batch-to-batch differences and the stability of the reagents, lot TH00624-041907/2, expiration date January 19, 2006, was also used.

The Charm II Tetracyclines Honey kits were from Charm Sciences Inc. (Lawrence, MA).

A mixture of different honey samples of known (organic) origin and of different compositions (liquid and solid, flower and honeydew, Belgian and imported) was used as blank honey. Each honey from the blank mixture was tested individually as negative with the Charm II Tetracyclines Honey (detection capability for tetracycline, oxytetracycline, chlortetracycline, and doxycycline in honey $\leq$ 10 μg/kg).

Material. For the instrumental reading, a QuantiSensor (Matest Systemtechnik GmbH, Mössingen, Germany), a small reader device with specially designed QuantiSensor software (release 345, version 2003), was used. A QuantiSensor Control dipstick (batch 051307/01, expiration date July 13, 2008) was used daily to check whether the reader system functioned properly.

Test Protocol and Interpretation of the Results. For liquid and semisolid honey, there is no sample preparation requested. Solid honey can be made liquid by heating in a glass test tube in a water bath at 37 °C. The lid of the plastic vial is filled with honey so that a correct amount of honey (around 600 mg) is diluted with the buffer content of the vial (1.8 mL). A total of 200 μL of diluted honey sample is added to the lyophilized receptor present in a glass vial and incubated at room temperature (20 $\pm$ 5 °C) for 15 min. During this first incubation period, tetracyclines possibly present in the honey bind with the specific receptor. After 15 min, the dipstick is dipped into the vial, and a second incubation at room temperature takes place for 15 min. When the liquid passes through the green capture lines, a red color appears. The first line captures the remaining active receptor, and the second line takes a certain amount of the excess reagent that passed through the first line. The second line serves as a control line and always has to become visible; otherwise, the test is invalid. This is shown in figure **1**.

Results were read both visually and using the Quantisensor, comparing the color intensity of both capture lines. The visual interpretation is as follows: when the color of the test line is more intensive than the color of the control line, the honey sample is negative ("vis neg"). In all other cases, the honey is contaminated with tetracyclines ("vis pos"). The visual interpretation is always done before the instrumental reading in order to prevent an influence on the judging by the technician.

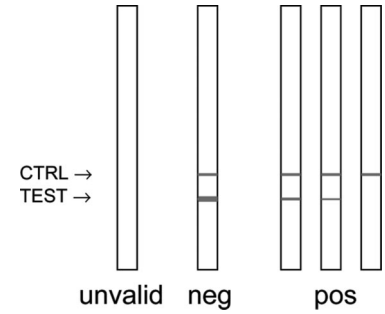

Figure 1. Visual interpretation of Tetrasensor Honey dipsticks.

For the instrumental reading, the intensity of the color formation is measured, and the result is expressed as the ratio of the color intensity of the test line to the color intensity of the control line. Honey samples with a ratio of $\geq$ 1.40 are free of tetracyclines ("neg"); honey samples with a ratio of $\geq$ 0.90 and $<$ 1.40 are slightly contaminated ("low pos"), and honey samples with a ratio of $<$ 0.90 are more heavily contaminated ("pos"). When testing honey samples in a routine, samples giving a ratio $\geq$ 1.40 are considered free from residues of tetracyclines; samples giving a ratio $<$ 1.40 are considered suspect for the presence of tetracyclines.

RESULTS AND DISCUSSION

The most recent EU legislation concerning residue analysis (EEC Regulation 2377/90 and modifications (*13*)) was used as a guideline for the validation of the method.

Stability of Tetracyclines in Honey. Tetracycline is rather stable in honey so long as the honey is stored in the dark, since tetracyclines are light-sensitive. As part of a collaborative trial, Martel et al. implemented a stability study by storing honey with an analyte (tetracycline) for 2 months at 4 °C. No loss of analyte content could be observed (*16*). Münstedt et al. spiked honey with 500 μg/kg of oxytetracycline, chlortetracycline, and tetracycline. Its high-performance liquid chromatography (HPLC) analysis after 10 months of storage at ambient temperatures still showed more than half of the original concentration of chlortetracycline and tetracycline, but no detectable oxytetracycline, proving an instability of oxytetracycline in honey (*17*).

In the study about the false-positive rate for a new Charm II Tetracyclines Honey kit with adapted sensitivity, the incurred samples of the ring trial (*11*) were retested after 1 year of storage in the dark in a cool room by liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS), and nearly identical concentrations of tetracycline were measured (data not shown).

Test and Reader Repeatability. A blank and four incurrent positive (import table honey) honey samples were analyzed 20 times. The color of the test line was evaluated at the end of each assay (wet dipstick) and a second time after 30 min (dry dipstick). The results were used to calculate the test repeatability on the basis of wet or dry dipstick readings.

To calculate the repeatability of the reader, only dry dipsticks were measured 20 times since the ratio still shifts slowly during the drying of the strips. This was done at three different levels, namely, for a blank, a low, and a high positive strip. The results are shown in **Table 1**.

The test repeatability was good and even improved as the concentration of tetracyclines in the honey increased. In general, the standard deviations of repeatability decreased when the dry dipstick readings were considered in comparison to the wet dipstick reading, except for the blank honey sample. The reader repeatability also improved as the concentration of tetracyclines in the honey increased and lower ratio values were obtained.

Table 1. Test and Reader Repeatability (Wet and Dry Dipstick Reading)

test repeatability ($n = 20$) (wet dipstick reading)				
sample	minimum ratio	maximum ratio	mean ratio	s_r[a]
honey 1 (blank)	2.72	3.99	3.28	0.38
honey 2	0.84	1.26	1.09	0.11
honey 3	0.50	1.40	1.06	0.23
honey 4	0.16	0.93	0.58	0.21
honey 5	0.02	0.07	0.05	0.01

test repeatability ($n = 20$) (dry dipstick reading)				
sample	sample	sample	sample	sample
honey 1 (blank)	1.90	3.74	2.96	0.44
honey 2	0.55	0.82	0.69	0.07
honey 3	0.24	0.65	0.49	0.12
honey 4	0.19	0.68	0.40	0.14
honey 5	0.01	0.05	0.03	0.01

reader repeatability ($n = 20$) (dry dipstick reading)				
sample	minimum ratio	maximum ratio	mean ratio	s_r
honey 6 (blank)	1.64	2.15	1.80	0.14
honey 7	0.85	1.08	0.98	0.07
honey 8	0.06	0.16	0.11	0.03

[a] s_r: standard deviation of repeatability.

The consistency in visual judging by the technicians was also checked. It needs to be emphasized that the technicians all received training in the reading of dipsticks as part of the accreditation procedure and that they all had very much experience in the color interpretation of analogue dipsticks (βeta-s.t.a.r. and Tetrasensor Tissue). Real negative and positive honey samples were never wrongly classified by any technician; only very occasionally and only for samples giving a borderline result (both test lines equal in intensity) was a nonconform result between two different persons obtained (data not shown).

Specificity. The specificity or the ability of the method to distinguish between the analyte being measured (tetracycline residues) and other substances was first investigated by spiking blank honey *in duplo* with some other relatively high concentration anti-infectious agents (antibiotics and chemotherapeutics). The Tetrasensor Honey test kit was used for the analysis. One substance was chosen from each of the most important groups: penicillin G (penicillins), cephapirin (cephalosporins), sulfadiazine (sulfonamides), enrofloxacin (quinolones), neomycin (aminoglycosides), and erythromycin (macrolides); spiking was performed at 100 times the Belgian action limit for tetracyclines (= 2 mg/kg). The color of the test line was evaluated directly at the end of the assay and after 30 min (dry dipstick).

All honey samples doped with sulfonamides or antibiotics other than tetracyclines provided negative ratios, and visually the results were also all interpreted as "vis neg". In general, following the drying of the dipsticks, the ratio values dropped, but the results all remained negative.

From the results, it can be concluded that the analysis is not disturbed by anti-infectious agents, which are different from tetracyclines. The Tetrasensor Honey kit is very specific for the analysis of tetracyclines.

Detection Capability. Another important validation parameter is the detection capability for the most important tetracyclines [tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), and doxycycline (DC)].

Therefore, starting from the detection capability concentrations obtained from the manufacturer, blank honey was spiked with the investigated tetracycline at different concentrations: in the range of 1–10 μg/kg in steps of 1 μg/kg and in the range of 10–20 μg/kg in steps of 2 μg/kg. The doped samples were blind coded before analysis. For each investigated tetracycline, the lowest concentration giving 19 (low) positive test results on 20 test results was determined. When a certain concentration tested negative two times, we directly tested a higher concentration since 19 positive test results on 20 test results was no longer achievable, in order to save time and reagents.

Since the strips were read both visually and by using a reader system, the detection capability was determined for both means of strip reading. Moreover, the strips were not only read immediately (wet dipstick reading) but also after 30 min of drying (dry dipstick reading). The results are shown in **Tables 2** and **3**.

First of all, no differences in detection capability were noticed between the visual and the instrumental reading. Second, the detection capabilities obtained from the dry dipstick reading were lower than those obtained from the wet dipstick reading. So, by postponing the reading, the sensitivity of the test increased. A summary of the detection capabilities is given in **Table 4**.

Test Ruggedness. Honey is a complex matrix with a large variety in composition due to different proportions of the possible sources, nectar and/or honeydew, coming from a great variety of plants. So it is important to check the robustness of the Tetrasensor Honey test kit on different unifloral and multifloral honeys.

Impact of the Nature (Type, Origin, Physical Parameters, etc.) of the Honey on the Test Sensitivity. As a starting point, we took the detection capability for tetracycline (TC) of 9 μg/kg (wet dipstick reading, **Table 4**), since this concentration is just at the top of the dose-response curve. Within the group of tetracyclines, tetracycline was the most obvious choice since it is the most frequently detected tetracycline in honey on the Belgian market (*1*).

When testing different types of honey, a comparison was run to see whether the same test detection capability was obtained.

The following types of honey were compared in this study: Belgian honey versus imported honey (**Table 5**), blossom honey versus honeydew honey (**Table 6**), rape (*Brassica spp.*) honey (high glucose content) versus black locust (*Robinia pseudoacacia* L.) honey (high fructose content) (**Table 7**), and solid honey versus liquid honey (**Table 8**).

Regarding flower honey, no significant differences were observed between the Belgian and the imported honey, since all of the honey samples (spiked with 9 μg/kg TC) gave low-positive to positive results. For the Belgian honey samples, this ratio ranged from 0.58 to 1.15; for the imported honey samples, it ranged from 0.67 to 1.27 (both wet dipstick readings).

Regarding honeydew honey, a difference was observed between the Belgian and the imported honeydew honey. All Belgian honeydew honey samples spiked with 9 μg/kg TC gave low positive to positive results, while the Spanish honeydew honey sample spiked with 9 μg/kg TC gave a negative result (wet dipstick reading). So, the detection capability of 9 μg/kg tetracycline was not valid for the Spanish honeydew honey. It is worth noting that the results became "low pos" once the dipstick was dry.

Electrical conductivity could be used for differentiation between honeydew and blossom honeys (except chestnut honey) since electrical conductivity correlates well with the mineral content of honey (*18*). Regarding the composition criteria for honey (*19*), blossom honey should have an electrical conductivity below 0.8 mS/cm, while the electrical conductivity of honeydew and chestnut honey should be higher than 0.8 mS/

Table 2. Visual and Instrumental Reading of the Testing of Honey Doped with the Most Important Tetracyclines, Wet Dipstick Reading

substance spiked in blank honey	concentration (μg/kg)	visual reading (n positive/n analyzed)	instrumental reading			
			n (low) positive/n analyzed	average ratio	lowest ratio	highest ratio
tetracycline	8	1/5	2/5	1.44	1.12	1.61
	9	20/20	20/20	1.09	0.84	1.26
oxytetracycline	10	2/5	3/5	1.37	1.02	1.70
	12	20/20	20/20	1.06	0.50	1.39
chlortetracycline	4	13/20	14/20	1.23	0.59	1.74
	5	20/20	20/20	0.88	0.50	1.06
doxycycline	5	3/5	3/5	1.34	1.13	1.64
	6	20/20	20/20	0.93	0.58	1.27

Table 3. Visual and Instrumental Reading of the Testing of Honey Doped with the Most Important Tetracyclines, Dry Dipstick Reading (after 30′)

substance spiked in blank honey	concentration (μg/kg)	visual reading (n positive/n analyzed)	instrumental reading			
			n (low) positive/n analyzed	average ratio	lowest ratio	highest ratio
tetracycline	6	6/9	6/9	1.28	1.01	1.60
	7	20/20	20/20	1.14	0.37	1.36
oxytetracycline	6	13/15	13/15	1.27	1.03	1.50
	7	19/20	19/20	1.22	1.05	1.49
chlortetracycline	3	3/9	4/9	1.43	0.73	1.85
	4	20/20	20/20	0.90	0.41	1.25
doxycycline	3	0/2	0/2	1.50	1.44	1.56
	4	20/20	20/20	1.12	0.96	1.31

Table 4. Detection Capability (CCβ) of the Tetrasensor Honey Test Kit for the Most Important Tetracyclines: Wet Dipstick Reading and Dry Dipstick Reading (after 30′)

substance spiked in blank honey	detection capability (μg/kg) (visual and instrumental reading)	
	wet dipstick reading	dry dipstick reading
tetracycline	9	7
oxytetracycline	12	7
chlortetracycline	5	4
doxycycline	6	4

cm. In the validation study, we compared the detection of tetracycline in blossom and honeydew honeys. No differences were observed between the blossom honey and the honeydew honey: all honey samples spiked with 9 μg/kg TC gave low positive to positive results. For the blossom honey samples, this ratio ranged from 0.58 to 1.15; for the honeydew honey samples, it ranged from 0.41 to 1.07 (both wet dipstick reading).

Among the European unifloral honeys, rape honey (*Brassica* spp.) and black locust honey (*Robinia pseudoacacia* L.) differ in composition to an extreme extent. Rape honey is light in color and always comes in a crystallized form (solid). Rape honey contains an average of 40.5 g/100 g glucose and 38.3 g/100 g fructose, and the mean fructose/glucose ratio amounts to 0.95. Black locust honey is very light in color and flavor with 26.5 g/100 g glucose and 42.7 g/100 g fructose and a fructose/glucose ratio of 1.61 (*20*).

No differences were observed in detection capability (CCβ) in the examination of rape and black locust honeys, despite the serious differences in composition (main type of sugar) and in the texture of both honeys.

Honey normally becomes solid due to a natural crystallization process caused by the high percentage of sugars, especially glucose. In the validation study, the detection in honey of both liquid and solid forms was studied. For the solid honey samples, the ratios obtained ranged from 0.75 to 1.15; for the liquid honey samples, the ratios ranged from 0.58 to 1.37 (both wet dipstick reading). No differences were observed between the solid honey and the liquid honey: all honey samples spiked with 9 μg/kg TC gave low positive to positive results. It is worth noting that, while the liquid honey sample 2, which when undoped, gave an extremely high ratio of 6.55, a (low) positive result was also obtained when it was doped with 9 μg/kg of tetracycline.

Batch-to-Batch Differences and Reagents' Stability Regarding the Detection Capability. It was examined whether the same detection capabilities for the four most important tetracyclines were obtained when using two completely different batches (reagents and strips). The results are shown in **Tables 9** and **10**. Lot B, used shortly after production, gave a mean ratio value of 3.98 (wet dipstick reading) and 3.42 (dry dipstick reading) for the blank honey.

In the first experiment, doped honey samples were tested on the same day with two different batches, namely lot TH00616-042405/4 (A) and lot TH000624-041907/2 (B). It is worth noting that lot B was used shortly after production and was marked as "young", while lot A was already several months old. From **Tables 9** and **10**, it can be concluded that, in this experiment, differences in detection capability were obtained for tetracycline, oxytetracycline, and chlortetracycline (wet dipstick reading). The detection capabilities claimed in **Table 2** were not reached with lot B "young". However, the ratio values were close to the cut-off value of 1.40 (the highest ratio value obtained for the wet dipstick reading is 1.79). All ratio values for doped honey samples were far below the ratio values for the blank honey (wet dipstick reading: lot A, mean ratio = 4.18; lot B, mean ratio = 3.98). So the differences in test capability between the two different batches remained limited.

In this experiment, both tested batches had a different production date, so it should be further made clear whether the small differences are related to a different production or related to a different age at the moment of use. So it was decided to store batch B for more than a year at 4 °C and to retest the same concentrations of tetracyclines doped in the same blank honey just before the expiration date of the reagents. The results

Table 5. Visual and Instrumental Reading of the Testing of Belgian Honey versus Imported Honey[a]

	blank honey				TC 9 μg/kg spiked in blank honey			
origin	ratio on $t = 0$	vis on $t = 0$	ratio on $t = 30'$	vis on $t = 30'$	ratio on $t = 0$	vis on $t = 0$	ratio on $t = 30'$	vis on $t = 30'$
				Flower Honey				
Belgium 1	2.83 (neg)	vis neg	2.13 (neg)	vis neg	0.84 (pos)	vis pos	0.52 (pos)	vis pos
					1.13 (low pos)	vis pos	0.48 (pos)	vis pos
Belgium 2	3.17 (neg)	vis neg	2.22 (neg)	vis neg	0.58 (pos)	vis pos	0.36 (pos)	vis pos
					0.67 (pos)	vis pos	0.34 (pos)	vis pos
Belgium 3	3.69 (neg)	vis neg	2.32 (neg)	vis neg	1.15 (low pos)	vis pos	0.57 (pos)	vis pos
					0.88 (pos)	vis pos	0.44 (pos)	vis pos
Cuba	4.68 (neg)	vis neg	3.38 (neg)	vis neg	1.07 (low pos)	vis pos	0.54 (pos)	vis pos
					1.23 (low pos)	vis pos	0.57 (pos)	vis pos
Chili	2.77 (neg)	vis neg	2.49 (neg)	vis neg	1.27 (low pos)	vis pos	0.66 (pos)	vis pos
					1.15 (low pos)	vis pos	0.65 (pos)	vis pos
India	2.26 (neg)	vis neg	1.43 (neg)	vis neg	0.67 (pos)	vis pos	0.28 (pos)	vis pos
					0.75 (pos)	vis pos	0.30 (pos)	vis pos
				Honeydew Honey				
Belgium 1	2.43 (neg)	vis neg	1.61 (neg)	vis neg	0.54 (pos)	vis pos	0.27 (pos)	vis pos
					0.41 (pos)	vis pos	0.27 (pos)	vis pos
Belgium 2	2.48 (neg)	vis neg	2.03 (neg)	vis neg	0.71 (pos)	vis pos	0.48 (pos)	vis pos
					1.05 (low pos)	vis pos	0.57 (pos)	vis pos
Belgium 3	3.42 (neg)	vis neg	2.04 (neg)	vis neg	0.95 (low pos)	vis pos	0.57 (pos)	vis pos
					1.07 (low pos)	vis pos	0.56 (pos)	vis pos
Spain	2.99 (neg)	vis neg	2.80 (neg)	vis neg	1.43 (neg)	vis neg	1.17 (low pos)	vis pos
					1.61 (neg)	vis neg	1.21 (low pos)	vis pos

[a] vis: visual reading. neg: negative. pos: positive.

Table 6. Influence of the Botanical Origin of the Honey on the Detection Capability: Blossom Honey versus Honeydew Honey, Both of Belgian Origin[a]

		blank honey				TC 9 μg/kg spiked in blank honey			
identification	conductivity (μS/cm)	ratio on $t = 0$	vis on $t = 0$	ratio on $t = 30'$	vis on $t = 30'$	ratio on $t = 0$	vis on $t = 0$	ratio on $t = 30'$	vis on $t = 30'$
					Blossom Honey				
sample 1	167	2.83 (neg)	vis neg	2.13 (neg)	vis neg	0.84 (pos)	vis pos	0.52 (pos)	vis pos
						1.13 (low pos)	vis pos	0.48 (pos)	vis pos
sample 2	395	3.17 (neg)	vis neg	2.22 (neg)	vis neg	0.58 (pos)	vis pos	0.36 (pos)	vis pos
						0.67 (pos)	vis pos	0.34 (pos)	vis pos
sample 3	241	3.69 (neg)	vis neg	2.32 (neg)	vis neg	1.15 (low pos)	vis pos	0.57 (pos)	vis pos
						0.88 (pos)	vis pos	0.44 (pos)	vis pos
					Honeydew Honey				
sample 1	988	2.43 (neg)	vis neg	1.61 (neg)	vis neg	0.54 (pos)	vis pos	0.27 (pos)	vis pos
						0.41 (pos)	vis pos	0.27 (pos)	vis pos
sample 2	1091	2.48 (neg)	vis neg	2.03 (neg)	vis neg	0.71 (pos)	vis pos	0.48 (pos)	vis pos
						1.05 (low pos)	vis pos	0.57 (pos)	vis pos
sample 3	1063	3.42 (neg)	vis neg	2.04 (neg)	vis neg	0.95 (low pos)	vis pos	0.57 (pos)	vis pos
						1.07 (low pos)	vis pos	0.56 (pos)	vis pos

[a] vis: visual reading. neg: negative. pos: positive.

Table 7. Influence of the Type of the Honey on the Detection Capability: Rape Honey versus Black Locust Honey[a]

	blank honey				TC 9 μg/kg spiked in blank honey			
identification	ratio on $t = 0$	vis on $t = 0$	ratio on $t = 30'$	vis on $t = 30'$	ratio on $t = 0$	vis on $t = 0$	ratio on $t = 30'$	vis on $t = 30'$
rape honey	3.79 (neg)	vis neg	2.40 (neg)	vis neg	0.89 (pos)	vis pos	0.62 (pos)	vis pos
					0.75 (pos)	vis pos	0.50 (pos)	vis pos
black locust honey	4.27 (neg)	vis neg	2.98 (neg)	vis neg	1.17 (low pos)	vis pos	0.45 (pos)	vis pos
					0.81 (pos)	vis pos	0.32 (pos)	vis pos

[a] Vis: visual reading; neg: negative; pos: positive.

of this additional testing with the reagents marked as "old" are also summarized in **Tables 9** and **10**.

From the stability testing data, we remarked a tendency of a small improvement of the testing capacity of the reagents during the shelf life. The reagents of lot B, used just before the expiration date, gave results comparable to the results obtained with lot A.

Impact of Drying of the Strips. The impact on the test results of drying of the strips was investigated throughout the validation by comparing direct (wet dipstick) readings of the test strips and reading after at least 30 min of drying.

Detailed results are provided in the separate tables. The ratio values always decreased when the reading was postponed (longer time for the color formation and the dipsticks become dry); so the detection capability of the test increased by postponing the reading. At the same time, throughout the drying of the strips, the color formation at both capture lines became more pronounced, which facilitated visual reading.

Table 8. Influence of a Physical Parameter of the Honey (Form) on the Detection Capability: Solid Honey versus Liquid Honey, Both from Belgian Origin[a]

	blank honey				TC 9 μg/kg spiked in blank honey			
identification	ratio on $t = 0$	vis on $t = 0$	ratio on $t = 30'$	vis on $t = 30'$	ratio on $t = 0$	vis on $t = 0$	ratio on $t = 30'$	vis on $t = 30'$
solid honey 1	2.83 (neg)	vis neg	2.13 (neg)	vis neg	0.84 (pos)	vis pos	0.52 (pos)	vis pos
					1.13 (low pos)	vis pos	0.48 (pos)	vis pos
solid honey 2	3.69 (neg)	vis neg	2.32 (neg)	vis neg	1.15 (low pos)	vis pos	0.57 (pos)	vis pos
					0.88 (pos)	vis pos	0.44 (pos)	vis pos
solid honey 3	3.86 (neg)	vis neg	1.98 (neg)	vis neg	0.75 (pos)	vis pos	0.28 (pos)	vis pos
					0.83 (pos)	vis pos	0.28 (pos)	vis pos
liquid honey 1	3.17 (neg)	vis neg	2.22 (neg)	vis neg	0.58 (pos)	vis pos	0.36 (pos)	vis pos
					0.67 (pos)	vis pos	0.34 (pos)	vis pos
liquid honey 2	6.55 (neg)	vis neg	3.59 (neg)	vis neg	1.12 (low pos)	vis pos	0.67 (pos)	vis pos
					1.33 (low pos)	vis pos	0.79 (pos)	vis pos
liquid honey 3	4.90 (neg)	vis neg	2.82 (neg)	vis neg	0.80 (pos)	vis pos	0.48 (pos)	vis pos
					1.37 (low pos)	vis pos	0.68 (pos)	vis pos

[a] vis: visual reading. neg: negative. pos: positive.

Table 9. Batch-to-Batch Differences and Kit Stability Regarding the Detection Capability, Wet Dipstick Reading

		instrumental reading			
substance spiked in blank honey and lot of reagents[a]	concentration (μg/kg)	*n* (low) positive/ *n* analyzed	average ratio	lowest ratio	highest ratio
tetracycline lot A	9	20/20	1.09	0.84	1.26
tetracycline lot B "young"	9	8/20	1.41	1.02	1.79
tetracycline lot B "old"	9	20/20	0.81	0.21	1.39
oxytetracycline lot A	12	20/20	1.06	0.50	1.39
oxytetracycline lot B "young"	12	18/20	1.23	0.96	1.52
oxytetracycline lot B "old"	12	20/20	0.75	0.19	1.35
chlortetracycline lot A	5	20/20	0.88	0.50	1.06
chlortetracycline lot B "young"	5	17/20	1.05	0.36	1.78
chlortetracycline lot B "old"	5	20/20	0.43	0.19	1.09
doxycycline lot A	6	20/20	0.93	0.58	1.27
doxycycline lot B "young"	6	20/20	0.58	0.16	0.93
doxycycline lot B "old"	6	20/20	0.63	0.22	1.02

[a] Lot A: TH00616-042405/4. Lot B: TH000624-041907/2.

Table 10. Batch-to-batch Differences and Kit Stability Regarding the Detection Capability, Dry Dipstick Reading

		instrumental reading			
substance spiked in blank honey and lot of reagents[a]	concentration (μg/kg)	*n* (low) positive/ *n* analyzed	average ratio	lowest ratio	highest ratio
tetracycline lot A	9	20/20	0.69	0.55	0.82
tetracycline lot B "young"	9	20/20	0.87	0.56	1.22
tetracycline lot B "old"	9	20/20	0.47	0.35	1.12
oxytetracycline lot A	12	20/20	0.49	0.24	0.65
oxytetracycline lot B "young"	12	20/20	0.62	0.50	0.76
oxytetracycline lot B "old"	12	20/20	0.39	0.02	0.89
chlortetracycline lot A	5	20/20	0.64	0.38	0.78
chlortetracycline lot B "young"	5	20/20	0.72	0.38	1.08
tetracycline lot A′	9	20/20	0.69	0.55	0.82
chlortetracycline lot B "old"	5	20/20	0.39	0.02	0.89
doxycycline lot A′	6	20/20	0.58	0.40	0.85
doxycycline lot B "young"	6	20/20	0.40	0.19	0.68
doxycycline lot B "old"	6	20/20	0.29	0.11	0.57

[a] Lot A: TH00616-042405/4. Lot B: TH000624-041907/2.

Test for False Negative/False Positive Results. To investigate the possibility of false-negative results, naturally incurred honey samples from our collection were retested using the Tetrasensor Honey test kit. All samples with a known concentration above the detection capability also gave positive screening results when using the Tetrasensor Honey test kit.

The test, BELAC accredited since the end of 2004, has also been used at T&V-ILVO routinely over the past 3 years.

Table 11. Tetrasensor Honey Results of an International Proficiency Test: Control of False Negative Results of Honey Contaminated Naturally with Tetracyclines[a]

	Tetrasensor Honey result			
concentration of tetracycline (LC-MS, in μg/kg)	ratio on $t = 0$	vis on $t = 0$	ratio on $t = 30'$	vis on $t = 30'$
3	1.90 (negative)	negative	1.34 (low positive)	negative
4	1.12 (low positive)	positive	0.47 (positive)	positive
6	1.37 (low positive)	positive	0.54 (positive)	positive
10	0.48 (positive)	positive	0.13 (positive)	positive
25	0.23 (positive)	positive	0.04 (positive)	positive
38	0.05 (positive)	positive	0.02 (positive)	positive
72	0.00 (positive)	positive	0.02 (positive)	positive
77	0.10 (positive)	positive	0.03 (positive)	positive

[a] vis: visual reading. LC-MS results by Jean-Marc Diserens, Lausanne, CH.

Positively screened samples were sent to an external laboratory for confirmation. Out of the data about the concentrations confirmed in the positively screened honey samples for tetracyclines, we have no indication that honeys with tetracyclines above the detection limits found in this validation study were missed in the screening. Moreover, the concentration determined by LC-MS is sometimes far below the detection limit of the Tetrasensor Honey screening test.

In 2004, our laboratory used the Tetrasensor Honey test kit in an international proficiency test regarding tetracyclines, organized by P. Beaune (Famille Michaud Apiculteurs, Gan, France). Our Tetrasensor Honey results in this proficiency test (*11*) are shown in **Table 11**, with exceptions from the 10 blanks, which were all found to be negative (no false-positive samples). The blind-coded positive honey samples in the proficiency test, which were naturally contaminated with 4 μg/kg tetracycline or higher, all gave (low) positive results (wet dipstick reading) for the Tetrasensor Honey test kit. Only one honey sample, naturally contaminated with 3 μg/kg tetracycline, gave a negative result (wet dipstick reading); however, when we read the dipstick after 30 min, the same sample already gave a low positive result.

In 2005, our laboratory also participated in another international collaborative trial on antibiotic residues in honey, organized by the Laboratoire d'Etudes et de Recherches sur les Petits Ruminants et les Abeilles de l'AFSSA (Sophia Antipolis, France) (*16*). Three samples contained no residues above the limit of detection of the LC-MS reference analysis. These samples all tested negative for Tetrasensor Honey. In this collaborative trial, sample 6, containing 8.7 μg/kg tetracycline, yielded a negative result directly at the end of the test (wet reading). However, the result became "low positive" after half an hour, following a dry dipstick reading. The other positive

samples with concentrations of tetracycline ranging from 19.8 to 31.7 μg/kg (LC-MS) were all detected as positive even during wet reading. Five out of seven laboratories using high-performance liquid chromatography with a diode array detector reported sample 6 as negative; of the 22 laboratories using LC-MS, two laboratories reported sample 6 as not being detected and one laboratory as <5 μg/kg.

So in both trials, no false-negative or false-positive results were obtained with the Tetrasensor Honey test kit. In the 2004 proficiency test, even concentrations of tetracycline below the detection capability of 9 μg/kg yielded "low positive" results with the Tetrasensor Honey test kit. This could be explained by the dose-response results as shown in **Tables 2** and **3**: concentrations just below the detection capability could sometimes result in a positive result. Another explanation could also be the time delay between the Tetrasensor analysis and the physicochemical confirmation, which possibly resulted in a degradation of incurred tetracycline in honey to degradation products with sterical similarity to the parent compound (*17*). Finally, it is worth noting that the Tetrasensor Honey test kit detects not only the parent compound but also the epimers.

If the data of the screening of 100 table honeys from different countries are considered (*21*), no false-positive or false-negative results were obtained when the Tetrasensor Honey test kit was used as screening method, whereas a rate of 8% false-positive results was obtained for the same honey when the Charm II Tetracyclines Honey was used. In this study, two samples with a tetracycline concentration below the detection capability tested positive for Tetrasensor Honey; the presence of tetracyclines in both samples (4 and 6 μg tetracycline/kg) was confirmed by LC-MS/MS.

From this work, it can be concluded that Tetrasensor Honey is a suitable test kit for the screening of tetracyclines in honey. The test takes 30 min. In general, no differences were noticed between a visual and an instrumental reading of the dipsticks. So a reader system is not required for screening purposes. Since no special equipment (incubator, reader, etc.) is required, the test can be performed at the production site even by the beekeeper himself.

The test detects the tetracyclines in honey in a specific and sensitive way. Depending on the type of tetracycline concerned, a detection capability between 6 and 12 μg/kg was obtained directly after the second incubation period. When reading dry dipsticks, detection capabilities of between 4 to 7 μg/kg were obtained, so when the dipstick becomes dry, the detection capability improves.

The test procedure is very simple, and the test is rugged. No influence on the test capability was noticed, with regard to the geographical or botanical origin or by physical parameters (solid versus liquid). Only small problems were encountered with a Spanish honeydew honey.

We noticed differences in the test capability between two different batches. However, the differences remained limited, and when the tests were repeated 16 months later using the second test kit, significant differences were no longer observed. A stability study showed a slight increase of the test sensitivity during storage.

No false negative and no false positive results were obtained during two international proficiency tests and a study of 100 table honey samples.

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