RESIDUES OF ANTIBIOTICS AND SULPHONAMIDES IN HONEY ON THE BELGIAN MARKET

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ABSTRACT

Samples of locally produced honey and imported honey were monitored for the presence of residues of streptomycins, tetracyclines, sulphonamides, β-lactams and chloramphenicol. Streptomycins, tetracyclines, sulphonamides (whole group) and β-lactams were determined with the respective Charm II test (Charm Sciences Inc., USA). For the determination of chloramphenicol the Chloramphenicol EIA test kit (Euro-Diagnostica b.v., NI) was used. The detection capability was respectively 15 µg/kg streptomycin, 10 µg/kg chlortetracycline, 10 µg/kg sulfamethazine, 10 µg/kg penicillin G and 0.1 µg/kg chloramphenicol.

Residues of veterinary drugs were found in a very limited number of honey samples produced by Belgian (mainly Flemish) beekeepers, namely: streptomycins 4 out of 248 samples (1.6%), tetracyclines 2 out of 72 samples (2.8%) and sulphonamides 3 out of 72 samples (4.2%). No residues of β-lactams (50 samples) and chloramphenicol (93 samples) were found.

However, in imported (industrial and table) honey samples available on the Belgian market, residues were frequently found: streptomycins 51 out of 108 samples (47.2%), tetracyclines 29 out of 98 samples (29.6%), sulphonamides 31 out of 98 samples (31.6%) and chloramphenicol 40 out of 85 samples (47.1%). No β-lactams (18 samples) were found.

Following the European legislation (EEC-Regulation 2377/90 and amendments) no MRLs (Maximum Residue Limits) are fixed for anti-infectious agents in honey and therefore the use of antibiotics is not accepted in apiculture. The Scientific Committee of the Belgian Federal Agency for the Safety of the Food Chain (FAVV) advised the introduction of action limits coupled to an adequate monitoring.

1. INTRODUCTION

Honey is generally considered as a natural and healthy product. Addition of additives or conserving agents to honey is not allowed. However the last years in some publications the problem of residues of antibiotics and sulphonamides in honey was mentioned (Anon., 2000). Antibiotics are mainly used in apiculture for the treatment of bacterial brood diseases, e.g. American foulbrood (*Paenibacillus larvae* subsp. *larvae*) (Spivak, 2000). In Europe this is an illegal practice. In some countries outside Europe the use of tetracyclines, sulphonamides and other antibiotics is legalised for the treatment of American foulbrood. The systematic use of tetracyclines in Canada and the USA has lead to tetracycline resistant strains of *Paenibacillus larvae* subsp. *larvae*. At the Apimondia meeting in 1997 (Antwerp, Belgium) it was mentioned that Mexican beekeepers provide the bees reinforcing products containing streptomycin (and at the same time preventing diseases) directly in the beehives (Bogdanov & Fluri, 2000). Sulphonamides are in some countries also used for the prevention of nosemosis

(*Nosema apis*). Chloramphenicol is used in beekeeping in China. Honey samples positive for chloramphenicol indicate honey of Chinese origin or blending of the honey with honey of Chinese origin.

Low concentrations of streptomycin (< 20 µg/kg) can also be found in fruit honey from nectar collected on pear orchards since the blossoms are sometimes sprayed with streptomycin preparations like Fructocin or Plantomycin for the treatment of fire blight (*Erwinia amylovora*) (Brasse, 2001).

In Belgium there are no professional beekeepers. So the production of honey remains limited to 800-1500 ton per year, while 3000-4000 ton honey is yearly imported. Around 86% is consumed as table honey, 14% as industrial honey. A survey study indicated that 66% of the population is consuming honey. So the average consumption of honey is 500 g per person per year with the highest consumption for children in the age of 4-10 years.

2. LEGISLATION

Regarding the European legislation (EEC Regulation 2377/90 and amendments) the use of antibiotics is not allowed in apiculture: no MRLs (Maximum Residue Limits) are fixed for antibiotics in honey.
Some Member States established action limits. The Scientific Committee of the Belgian Federal Agency for the Safety of the Food Chain (FAVV) advised in 2001 the introduction of action limits coupled to an adequate monitoring. This decision was partly based on monitoring data also included in this paper (Reybroeck *et al.,* 2001). The action limits valid in Belgium are described below (table 1).

Table 1. Action limits valid in Belgium regarding residues of (dihydro)streptomycin, sulphonamides and tetracyclines in honey.

Start date	Action limit (µg/kg)		
	(dihydro)streptomycin	sulphonamides (group)	tetracyclines (group)
1/1/2002	200	50	50
1/7/2002	100	20 (1) (2)	20 (1)
1/1/2003	50	20 (1) (2)	20 (1)
1/7/2003	20 (1)	20 (1) (2)	20 (1)

(1): Action limit based on detection capability
(2): The detection capability (LOQ) of the physicochemical confirmatory method still needs to be verified. The established action limit can possibly be changed to 10 µg/kg.

Chloramphenicol is included in Annex IV of EEC Regulation 2377/90: no MRL could be elaborated what means a zero tolerance in all foodstuffs of animal origin. Chloramphenicol is a banned substance due to the fact that it was shown in epidemiological studies that it could induct an aplastic anaemia. A Minimum Required Performance Limit (MRPL) of the analytical method of detection was established by some Member States, e.g. the MRPL for the detection of chloramphenicol in honey in Belgium is 0.1 µg/kg since July 1st 2002. The previous MRPL was 0.3 µg/kg.

3. METHODOLOGY

3.1. Screening methods

3.1.1. (Dihydro)streptomycin

(Dihydro)streptomycin is determined in honey with the Charm II Streptomycin Honey assay (Charm Sciences Inc., USA). The limit of detection (LOD) for streptomycin is 15 µg/kg and for dihydrostreptomycin 25 µg/kg. The Charm II Streptomycin Honey test is a microbial receptor test with radioactive labelling. A binding reagent is used, consisting of a microbial cell containing a specific receptor.
In the beginning also a few honey samples were screened on the presence of streptomycin using the Streptomycin EIA (Euro-Diagnostica b.v., Nl) with as LOD for streptomycin 15 µg/kg. After an extraction with extraction buffer, the extract was further cleaned over a C18 cartridge. After eluation, the eluate was evaporated to dryness under a nitrogen flow and the evaporated crude extract was resolved in buffer solution and further used in the ELISA.

3.1.2. Tetracyclines

Tetracyclines are screened in honey with the Charm II Tetracyclines Honey assay (Charm Sciences Inc., USA). The limit of detection for chlortetracycline, tetracycline, oxytetracycline and doxycycline is 10 µg/kg. In contrast with most other Charm II tests, antibodies are used in this kit instead of receptors.

3.1.3. Sulphonamides

A broad range of sulphonamides is detected in honey with the Charm II Sulphonamides Honey receptor assay (Charm Sciences Inc., USA). The limit of detection for some sulpha drugs: sulfamethazine, sulfathiazole and sulfacetamide 10 µg/kg; sulfamethoxazole 25 µg/kg and sulfadiazine 50 µg/kg. A special extraction procedure is needed to set free the sulphonamides bound to the sugars in the honey and to prevent interference from sulpha analogues such as para-aminobenzoic acid. By using a specific receptor (and not antibodies) all substances belonging to the group of sulphonamides can be detected with this receptor assay.

3.1.4. β-Lactams

Penicillins and cephalosporins are screened in honey using the Charm II β-Lactam Honey receptor assay (Charm Sciences Inc., USA). The limit of detection for penicillin G is 10 µg/kg.

3.1.5. Chloramphenicol (CAP)

Chloramphenicol is screened in honey using the Chloramphenicol Enzyme ImmunoAssay (EIA) (Euro-Diagnostica b.v., Nl), a microtiter based competitive enzyme immunoassay. In the first period a simple extraction with a buffer was performed as sample pretreatment (limit of detection 0.3 µg CAP/kg).
In May 2002 the extraction procedure was improved in order to improve the test sensitivity. After a chemical extraction with ethyl acetate, the extract is cleaned-up using a mixture of hexane and buffer. After centrifugation, the buffer solution part is further used in the ELISA. The limit of detection for chloramphenicol using a chemical extraction is 0.1 µg/kg.

DVK-CLO is BelTest accredited (ISO 17025) for the determination of (dihydro)streptomycine, tetracyclines, sulphonamides and chloramphenicol in honey and other apiarian products.

3.2. Confirmation methods

Confirmation of suspect samples was performed in external laboratories (Switzerland and Belgium). The confirmation of streptomycin, tetracyclines and sulphonamides was performed with high performance liquid chromatography either with fluorescence detection (HPLC-FL; streptomycin and tetracyclines) either with ultra-violet detection (HPLC-UV; sulphonamides). The limit of quantification for streptomycin and tetracyclines is 10 µg/kg; the limit of quantification for sulphonamides (sulfaguanidine, sulfanilamide, sulfacetamide, sulfadiazine, sulfathiazole, sulfadimidine, sulfamethoxazole) is 20 µg/kg.
Confirmation of chloramphenicol suspect samples was performed with liquid chromatography in combination with mass spectrometry (LC-MS). The limit of quantification for chloramphenicol is 0.1 µg/kg.

4. HONEY SAMPLES: ORIGIN, TYPE AND SAMPLING

4.1. Locally produced honey
In the framework of the Flemish Honey Project (application of Council Regulations 1221/97 and 2300/97) Flemish beekeepers can bring in honey samples for determination of the quality in order to obtain a certificate. The certification is based on the analysis of some physicochemical quality criteria (moisture content, electrical conductivity, hydroxymethylfurfural content and enzyme activity (diastase or invertase)) with test methods approved by the European Honey Commission (Bogdanov *et al*., 1997) and an evaluation of the presentation, the crystallisation structure and organoleptic qualities. Part of these samples was screened on the presence of antibiotics and sulphonamides. Other locally produced honey samples were sampled by food inspectors (FAVV) and the consumer's organisation Test-Aankoop/Test-Achats. Some of these samples originated from the Walloon region.

4.2. Imported honey
Both industrial honey and table honey available on the Belgian market were screened on the presence of antimicrobials. Food inspectors (FAVV), honey importers, honey traders and the consumer's organisation Test-Aankoop/Test-Achats performed the sampling. Of some of the imported honey samples the origin and/or type of honey is known.

5. RESULTS AND DISCUSSION

5.1. Residues in locally (Belgian) produced honey

The results of the determination of streptomycins, tetracyclines, sulphonamides, β-lactams and chloramphenicol in locally (Belgian) produced honey is summarised in table 2.

Table 2. Residues of streptomycins, tetracyclines, sulphonamides, β-lactams and chloramphenicol in locally (Belgian) produced honey in the period 2000-2002 (CAP only determined in 2002)

Group	n	Positive	%
Streptomycins	248	4	1.6
Tetracyclines	72	2	2.8
Sulphonamides	72	3	4.2
β-Lactams	50	0	0
Chloramphenicol	93	0	0

Out of these monitoring and screening data it could be concluded that the frequency of residues of anti-infectious agents in honey from local beekeepers is low. In some cases of a positive Belgian honey the responsible beekeeper granted the addition of foreign honey to his own production.

5.2. Residues in imported (industrial and table) honey

The results of the determination of streptomycins, tetracyclines, sulphonamides, β-lactams and chloramphenicol in imported (industrial and table) honey is summarised in table 3.

Table 3. Results of the determination of streptomycins, tetracyclines, sulphonamides, β-lactams and chloramphenicol in imported (industrial and table) honey in the period 2000-2002 (CAP only determined in 2002)

Group	n	Positive	%
Streptomycins	108	51	47.2
Tetracyclines	98	29	29.6
Sulphonamides	98	31	31.6
β-Lactams	18	0	0
Chloramphenicol	85	40	47.1

In foreign industrial and table honey, present on the Belgian market, the frequency of anti-infectious agents is remarkable high. Even many honey samples contain different residues at the same time. Residues were also found in organic produced honey with an official bio control label.
CAP was also found in Chinese royal jelly.

88 Honey samples were of known origin. The results of the residue analysis of this group of samples is summarised in table 4. Since the analysis of CAP in honey was only started in 2002, not all samples were controlled on the presence of CAP.

Table 4. Results of the determination of streptomycins, tetracyclines, sulphonamides and chloramphenicol in imported honey samples with known origin

Origin	Samples (n)	Positive samples			Samples (n)	Positive samples
		Strepto-mycins	Tetra-cyclines	Sulpho-namides		Chlor-amphenicol
Bulgaria	1	0	0	0	---	---
France	3	0	2	0	1	0
Germany	1	1	0	0	1	0
Hungary	2	0	0	0	2	0
Romania	2	0	1	0	1	0
Spain	6	1	2	2	5	0
Turkey	2	0	1	0	2	0
Cuba	7	0	4	1	1	0
Mexico	6	1	0	3	---	---
Yucatan	5	3	0	5	4	0
Argentina	3	1	3	3	2	0
China	5	5	2	3	40	31
India	2	0	0	1	1	0
Vietnam	1	1	0	0	---	---
New-Zealand	1	0	0	0	---	---
Tasmania	1	0	0	0	---	---

---: not analysed

The data in table 4 show that the residue problem is not restricted to a certain geographical area of origin of the honey, except for CAP (China). The data also prove that in many countries different veterinary products are used at the same time.

5.3. Confirmation results

A part of the positively (streptomycins, tetracyclines or sulphonamides) screened samples were further investigated with physicochemical methods in an external laboratory. Residues of respectively streptomycin, sulphonamides and/or tetracyclines were mostly confirmed. However in some samples the amount of residue quantified was much lower than expected. In some cases the residue was confirmed but could not be quantified. Streptomycin was confirmed in concentrations ranging from spores (< 10 µg/kg) to 71 µg/kg, tetracyclines in concentrations ranging from spores (< 10 µg/kg) to 30 µg/kg (tetracycline), respectively to 11 µg/kg (oxytetracycline). Up to now 4 different sulphonamides were identified in positive honey samples: sulfathiazole (33 - 430 µg/kg), sulfamethoxazole (spores (< 20 µg/kg) – 73 µg/kg) and low concentrations (< 20 µg/kg) of sulfadimidine and sulfadiazine.

Confirmation and quantification of residues of antibiotics and sulphonamides in honey remains a bottleneck in residue analysis in honey. This could also be concluded from the results of a recent international ring trial organised by F.E.E.D.M. (Anon., 2002).

False positive results could occur using Charm II assays. Extreme high HMF (hydroxymethylfurfural) concentrations can lead to false positive streptomycin results. However high HMF values can only occur in adulterated or severely heated honey.
Reasons for not conformity in between screening and confirmation results could be the difference in detection capability, the fact that metabolites are also detected by Charm II assays, the stability of the inhibitory substance in honey, the difference in response, ... The instability of especially oxytetracycline in honey is a known fact. This was studied by Münstedt *et al.* (2002) after applying tetracyclines as a powdered sugar dust. Positive results obtained with the Charm II test for tetracyclines couldn't be confirmed by HPLC. This leads to the conclusion of the authors that oxytetracycline was degraded to products with sterical similarity to the parent compound.

All honey samples giving a positive result for the chloramphenicol screening (ELISA, LOD 0.3 µg CAP/kg) were sent to an external laboratory for physicochemical confirmation. In all these honey samples chloramphenicol was confirmed. China was indicated as country of origin of the 31 positive samples; in the other 9 positive samples (unknown origin) blending with Chinese honey is possible.
Only recently, since the application of a chemical extraction with ethyl acetate (ELISA, LOD 0.1 µg CAP/kg), the presence of CAP in some positively screened samples (concentration < 0.3 µg/kg; data not included in this paper) could not get confirmed. This indicates a difference in response in between the ELISA and the physicochemical method as reported by some authors.

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