Immunochemical screening for antimicrobial drug residues in commercial honey†

The Analyst

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Honey samples (n = 100; origin: various countries from Eurasia, Oceania, and the Americas) were analysed by enzyme immunoassays (EIA) for tetracyclines, streptomycin, and sulfathiazole. Considering antibody specificity, these EIAs are either quantitative (streptomycin) or qualitative (tetracyclines, sulfathiazole) tests. Honey extract purification was achieved by liquid–liquid partition (tetracyclines), and by solid phase extraction–immunoaffinity chromatography (streptomycin, sulfathiazole). Detection limits were 20 μg kg^{-1} (tetracycline equivalents), 10 μg kg^{-1} (streptomycin), and 50 μg kg^{-1} (sulfathiazole equivalents), with mean recoveries of 100–117%. A total of 42% of the samples was found positive by EIA; 25% were positive in one assay, 13% in two, and 3% were positive in all three tests. In the EIA for tetracyclines, 26% were positive, with 12 samples exceeding a level of 50 μg kg^{-1} (tetracycline equivalents). In the EIA for streptomycin, 19% were positive, with a mean concentration of 19 ± 12 μg kg^{-1}. In the sulfathiazole EIA, 16% of the samples were positive, with 13 samples exceeding a level of 100 μg kg^{-1} (sulfathiazole equivalents). However, when samples which were positive in the sulfathiazole EIA were reanalysed for sulfonamides by HPLC, no sulfa drugs could be detected. Experimental heating (40 °C) of honey spiked with sulfathiazole indicated that the sulfa drug(s) responsible for positive EIA results could be present as sugar derivatives.

Introduction

Contamination of natural honey with residues of antibiotics and sulfonamides may occur after direct treatment of bacterial diseases of honey bees, such as American foulbrood or European foulbrood. Drugs known to be effective against these diseases are tetracylines (oxytetracycline), streptomycin and sulfathiazole.[1-3] In Germany and several other countries, the use of such antimicrobials is not approved for the treatment of honey bees.

More recently, another possible contamination pathway has attracted scientific attention. Some antibiotics, including oxytetracycline and streptomycin, are increasingly used to treat bacterial infections of plants in the orchard environment.[4] Important fruit-tree diseases such as fireblight (caused by *Erwinia amylovora*), which in recent years caused severe losses in several apple producing regions in Germany,[5] or *Pseudomonas* blossom blast,[6] have to be treated mainly during blossom. Contamination of the blossom with high concentrations of antimicrobials implies the risk of a carry-over of residues into the honey.[7] Indeed, contamination of commercial honey with streptomycin has recently been reported.[8,9]

So far, maximum residue limits have not been set for antimicrobial compounds in honey within the European Union. Because of the dual use of some antibiotics both for animals and plants, it is questionable whether residues in natural honey will be dealt with under European Union veterinary drug residue regulation 2377/90.[10] German food law has set a general maximum residue concentration of 10 μg kg^{-1} for plant protective substances if no other specific regulation exists.[11]

Few studies on the occurrence of antimicrobials in honey have been published in the past, which is mainly due to the lack of suitable screening tests. Next to modified microbial inhibition tests,[12,13] method development for drug residue detection in honey has mainly focussed on chromatographic detection of tetracyclines,[14–17] sulfonamides,[18–24] and streptomycin.[9] Microbial inhibition tests are cheap and easy to perform, but weakly sensitive for the compounds which are of concern in honey. Physico-chemical methods for drug detection in honey generally are time-consuming and costly, and therefore of limited use as first-action methods. In such a situation, immunochemical methods offer advantages in test simplicity and costs. For honey analysis, EIA methods for tetracyclines,[25,26] streptomycin,[8,27] and sulfonamides[28,29] have been described. However, extensive surveys on the contamination of commercial honey using one of these methods have not been published. The aim of this study was to obtain an overview on the incidence of tetracyclines, streptomycin, and sulfathiazole in honey from the German market.

Experimental

Materials

Honey sample materials. Honey samples (n = 100) were purchased from retail stores and health food shops in the Munich area between 1996 and 1997. The countries of origin, indicated on the labels of 64 of the samples, included countries of Europe, Asia, Oceania, and the Americas. The price per kilogram ranged from 4.5 to 63 DM.

Immunochemicals. Indirect competitive enzyme immunoassay for tetracyclines was used as described earlier.[30] For the detection of sulfathiazole, a competitive direct EIA originally developed for sulfadiazine, having strong cross-reactivity with sulfathiazole,[31] was used with a sulfathiazole standard curve. Streptomycin was detected by competitive direct EIA as described earlier.[32] The characteristics of these

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tests are summarized in Table 1. Monoclonal antibody-based immunoaffinity chromatography columns (IAC) for streptomycin/dihydrostreptomycin (STM 4E2) and sulfadiazine/sulfathiazole/sulfamerazine (SDA III 2G6) were prepared as described earlier.[33] The characteristics of these IACs are summarized in Table 2.

Chemicals, buffers and solutions. Tetracycline, streptomycin, sulfathiazole, 3,3′,5,5′-tetramethylbenzidine (TMB), and all other chemicals were purchased from Sigma-Aldrich Vertriebs GmbH (Deisenhofen, Germany). Stock solutions (1 mg ml^{-1}) of tetracycline, streptomycin, and sulfathiazole were each prepared in methanol. Water was bidistilled for all purposes throughout the study. For EIA, antibiotic standard solutions, in phosphate buffered saline (PBS; 0.01 mol l^{-1}, pH 7.3; phosphate buffer containing 0.1 mol l^{-1} NaCl) were prepared daily. The dilution buffer for coating microtitre plates (Maxisorp; Nunc, Wiesbaden, Germany) with either antiserum or tetracycline–caseine conjugate was carbonate–bicarbonate buffer (0.05 mol l^{-1}; pH 9.6; 100 µl per well). To block free protein binding sites of the plates, PBS containing 20 g l^{-1} sodium caseinate (Sigma) was used (200 µl per well). The microtitre plate wash solution was distilled water containing 8.5 g l^{-1} NaCl and 0.25 ml l^{-1} Tween 20. Enzyme conjugate dilution buffer was PBS containing 10 g l^{-1} sodium caseinate. Enzyme substrate solution consisted of 0.2 mol l^{-1} potassium citrate buffer (pH 3.9) containing 0.003 mol l^{-1} H_2O_2 and 0.001 mol l^{-1} TMB (100 µl per well). The enzyme reaction stopping solution was 1 mol l^{-1} H_2SO_4 (100 µl per well). Colour development for the EIA reaction was measured at 450 nm with an AT 400 microtitre plate reader (SLT, Crailsheim, Germany). The absorbance data were evaluated and the results calculated using an enzyme immunoassay calculation software developed by Märtlbauer.[34]

Chromatography. For HPLC analysis of sulfonamides in IAC-purified honey extracts, a chromatographic method validated for the separation and determination of sulfonamides in milk extracts[35] was used. The analytical equipment was a Shimadzu LC 10 system (Shimadzu, Duisburg, Germany) with a SPD-M10A diode array detector. The analytical column was a Merck LiChroCART (250 × 4 mm id) filled with Superspher 100 RP-18 material (4 µm particle size) and a LiChroCART (4 × 4 mm id with Superspher 100 RP-18, 5 µm particle size) guard column in a column oven set at 18 °C. The mobile phase was prepared by mixing sodium acetate buffer (0.1 mol l^{-1}, pH 4.6–4.7; 780 ml), acetonitrile (170 ml), and methanol (50 ml) and degassing by ultrasonication before use. The mobile phase flow rate was 0.7 ml min^{-1}. The sample extract injection volume was 50 µl. For solid phase extraction, Chromabond C18-ec cartridges (#730014, Macherey–Nagel, Düren, Germany) were used with a vacuum chamber (#730150, Macherey–Nagel).

Table 1 Test parameters of the EIAs used in this study

Test system	Mean IC_{50} of standard curve/ ng ml^{-1}	Major cross-reactions (%)[a]	Mean detection limit in honey[b]/ µg kg^{-1}
Tetracycline (TC)	1.3	ChlorTC (100); OxyTC (4); RoliTC (110); Doxycycline (5); Minocycline (130); Demeclocycline (40)	20
Streptomycin	2.9	Dihydrostreptomycin (150)	10
Sulfathiazole	67	Sulfadiazine (910); Sulfamerazine (100)	50

[a] Taken from refs. 30–32. [b] Using sample extract preparation as described in text.

Table 2 Characteristics of the IACs used for honey extract cleanup[a]

IAC system	Monoclonal antibody	Antigen capacity/ µg ml^{-1} gel	Gel volume per column/ ml	Selectivity
Streptomycin	STM 4E2	4.47	0.2	Streptomycin Dihydrostrepomycin
Sulfathiazole	SDA III 2G6	3.0	0.3	Sulfathiazole Sulfadiazine Sulfamerazine

[a] Taken from ref. 33.

Sample extract preparation. For tetracycline analysis, approximately 1 g of honey was weighed into a 50 ml beaker. Aqueous sodium chloride solution (20 g l^{-1}) was added to give the fivefold weight, and the mixture stirred for 10 min with a magnetic stirrer. One ml of this solution was transferred into a test tube, ethyl acetate (5 ml) was added, and mixed on a vortex mixer at high speed for approximately 30 s. After separation of the two phases, the upper organic phase was removed, and the aqueous phase was extracted again with another 5 ml portion of ethyl acetate. The organic phases were pooled and the solvent evaporated to dryness under reduced pressure at 50 °C using a rotary evaporator. The residue was dissolved with 2 ml PBS, further diluted with PBS if required, and analysed by EIA.

For streptomycin analysis, a method employing solid phase extraction on C18 reversed phase columns, followed by further extract purification on IAC columns, was used as described in detail.[8,27]

For sulfathiazole analysis, approximately 2.5 g of sample was weighed into a beaker, sodium acetate buffer (0.1 mol l^{-1}, pH 5.0) was added to give the fivefold weight, and mixed for 10 min with a magnetic stirrer. A C18-ec solid phase extraction cartridge was activated with 20 ml of methanol, followed by 20 ml of water. Then, 10 ml of the honey–acetate buffer mixture was slowly added (*ca.* 2–3 ml min^{-1}), followed by 2 ml of water. Then the cartridge was dried for approximately 5 min under reduced pressure. Sulfonamides were eluted with 5 ml of acetonitrile. The organic phase was evaporated at 50 °C under reduced pressure with a rotary evaporator. The residue was redissolved with 5 ml of PBS for EIA analysis and further IAC cleanup. An IAC column was prewashed with 10 ml of PBS, then a portion of the PBS extract was applied to the column. The volume of extract was dependent on the sulfathiazole concentration determined by EIA for the semi-purified solid-phase extract, thus avoiding overloading of the column. The column was washed with 10 ml of PBS, elution was performed with 2 ml of 0.1 mol l^{-1} glycine buffer set at pH 2.5 with 0.2 mol l^{-1} HCl. This extract was neutralized with 0.05 mol l^{-1} sodium bicarbonate buffer, pH 9.6 (850 µl) and analysed by EIA. IAC extracts of honey samples which were positive for sulfathiazole were reanalysed by HPLC. With an injection volume of 50 µl, the detection limit for sulfathiazole in standard solutions was about 50 ng ml^{-1}, the calculated detection limit for sulfathiazole in honey was about 100 µg kg^{-1}.

For all three methods, recovery was routinely checked by addition of the respective analyte to diluted honey samples before extract cleanup.

Experimental heating of honey spiked with sulfathiazole

Honey (10 g) was mixed with PBS (10 ml) containing sulfathiazole (10 µg ml^{-1}) for 10 min using a magnetic stirrer. A small portion (200 µl) was removed for sulfathiazole

analysis, and the mixture incubated in a water bath at 40 °C. Samples (200 µl) were taken after incubation times of 1 h and 4 h. The samples were purified using solid phase extraction–IAC as described above, and analysed both by EIA and HPLC.

Results and discussion

The standard curves of the EIA methods had detection limits in the low ng ml^{-1} range. In order to achieve low detection limits in honey samples, sample extract purification steps were used instead of simple sample dilution to eliminate sample matrix interferences. For streptomycin and sulfathiazole analysis, a combined solid-phase extraction–immunoaffinity chromatography clean-up procedure was used, which gives highly purified extracts. For tetracyclines, IACs were not available, therefore sample extract purification was achieved by a liquid–liquid partitioning procedure. The resulting detection limits in honey (Table 1) ranged from 10 to 50 µg kg^{-1} and were considered sufficiently sensitive. The results of the recovery experiments are listed in Table 3.

It has to be pointed out that considering antibody specificity, the tests for tetracycline and sulfathiazole are qualitative screening tests if the identity of the residue is not known. To our best knowledge, however, the only sulfonamide which is widely used for honey bee diseases is sulfathiazole, and the most important tetracyclines used both for treatment of bacterial diseases in honey bees and plants are oxytetracycline and tetracycline. For the sulfathiazole EIA, overestimation due to presence of sulfadiazine having 910% cross-reactivity is therefore unlikely. For the tetracycline EIA, the risk exists that the concentration of oxytetracycline in a sample would be grossly underestimated due to the relatively low cross-reactivity (4%) of this compound. This means a EIA result of 50 µg kg^{-1} could be caused by oxytetracycline at a concentration of about 1000 µg kg^{-1}. In contrast, the streptomycin EIA can be regarded as a quantitative test, because the only compound with major cross-reactivity, dihydrostreptomycin, is for the moment neither used for honey bees nor for plants.

The EIA results for commercial honey samples (Table 4) indicated that there is a high incidence of residues of tetracyclines, streptomycin, and sulfathiazole in honey from the German market. Out of a total of 100 samples, 42 samples were positive in at least one EIA. Twenty-six samples were positive in only one test system, 13 were positive in two tests, and three samples were positive in all EIAs. When EIA results were arranged according to the origin of the samples (Table 5), only those five samples from Oceania (Australia and New Zealand) were all negative. One out of 10 samples of German origin was positive for tetracyclines. Higher incidences were found for samples from other European countries, and from the Americas. Interestingly, 24 of those 36 samples for which the country of origin was not indicated were positive in at least one EIA, this group accounted for 61% of all positive results. Furthermore, the mean price per kilogram of samples with known origin was 22.1 DM, whereas the mean price was 11.3 DM for products without known origin; most of the cheaper products (4.5–10 DM per kilogram) were in this group. It seems reasonable to assume that cheaper products are a mixture of a large number of batches purchased from various locations, making the risk of contamination more likely.

In the tetracycline EIA, 26 samples were positive, maximum concentrations (tetracycline equivalents) exceeded 50 µg kg^{-1} in 12 samples. Although false-positives could not be completely excluded at a level near the detection limit, at least samples exceeding 50 µg kg^{-1} allowed high extract dilutions for EIA analysis, making false-positives relatively unlikely. Furthermore, similar results after repeated analysis of the same sample further supported the finding that there is a high contamination rate of honey with tetracyclines. If the compound responsible for the positive results were oxytetracycline, this would mean a contamination level in the mg kg^{-1} range. Another possibility which cannot be excluded is the formation of metabolites (*e.g.*, 4-epitetracyclines) in honey having cross-reactivity in the EIA, further emphasising that the results of this test system have to be interpreted as qualitative. The 4-epitetracyclines have been

Table 3 Recovery of tetracycline, streptomycin, and sulfathiazole from artificially contaminated honey samples

Test system	Amount added/ µg kg^{-1}	Recovery (%)	RSD (%)	*n*
Tetracycline	50	117	13.2	4
	100	108	15.3	4
Streptomycin	50	100	8.42	6
Sulfathiazole	200	105	13.9	4
	1000	100	21.8	5

Table 4 EIA results for tetracycline, streptomycin, and sulfathiazole in commercial honey samples (n = 100)

Test system	Number of positive samples	Mean of positives/µg kg^{-1}	Standard deviation of the mean/µg kg^{-1}	Median/µg kg^{-1}	Maximum/µg kg^{-1}
Tetracycline[a]	26	80	89	45	400
Streptomycin	19	19	12	16	63
Sulfathiazole[b]	16	280	140	190	750

[a] Results expressed as tetracycline equivalents. [b] Results expressed as sulfathiazole equivalents.

Table 5 EIA results for tetracycline, streptomycin, and sulfathiazole in commercial honey samples (n = 100) according to product origin as claimed by the distributing company

Origin	Number of samples	Number of positive EIA results		
		Tetracycline	Streptomycin	Sulfathiazole
Germany	10	1	0	0
Other European countries	35	5	3	7
Asia	2	1	0	0
Oceania	5	0	0	0
North America	5	2	1	1
Middle and South America	7	0	1	2
Not indicated	36	17	14	6

Table 6 EIA and HPLC results (IAC extracts) for sulfathiazole in spiked (10 μg g^{-1}) honey after heating at 40 °C

Time/h	EIA/μg g^{-1}	HPLC/μg g^{-1}
0	10.5	7.95
1	10.9	3.33
4	13.3	0.99

found to have about 10–20% cross-reactivity compared with the parent compounds (unpublished data). However, the EIA method described here is the most sensitive test for tetracycline in honey described so far. Therefore it is not surprising that a higher number of positives were found in this study compared with others. Our results are supported by the findings of Jürgens,[14] who, at a detection limit of 0.1–1.0 mg kg^{-1}, reported tetracycline levels from 1.5 to 5.1 mg kg^{-1} in three out of 54 honey samples.

Low concentrations of streptomycin were found in 19 samples. It is in agreement with earlier findings[8,9] that contamination of honey with streptomycin is usually in the range from 10–50 μg kg^{-1}, with maximum values in most cases being below 100 μg kg^{-1}.

After combined solid phase extraction–IAC cleanup, 16 samples were positive in the sulfathiazole EIA, with levels ranging from 70 to 750 μg kg^{-1}. After HPLC analysis of these extracts, neither sulfathiazole nor any other sulfonamide could be detected. Since the sample extract preparation was very selective, the possibility of a reaction of sulfathiazole with reducing sugars[2,3] or other compounds was considered. A preliminary experiment in which honey was spiked with sulfathiazole and incubated at 40 °C showed that EIA results were more or less unaffected, whereas the HPLC peak for sulfathiazole decreased rapidly, and less than 10% of the added sulfathiazole could be detected after 4 h of incubation. Commercial honey is usually a mixture from several producers, and may be stored for extended periods (> 1 year) before filling into jars. Mild heat treatment to redissolve crystallized sugars is then required before preparation of a new batch of jars, similar to in our experiment. Our results support those published by Low *et al.*,[2] who concluded that residues of sulfathiazole in honey are probably not present as free sulfathiazole but in chemically modified form. Therefore the negative HPLC results have to be considered as false-negative. Since literature data indicate that bound sulfathiazole could be converted into free form under acidic conditions,[3] further work would aim on improving the HPLC method.

For immunogen synthesis, the N^4-amino group of sulfadiazine was used for linkage to proteins, therefore the antibodies used in this study, and most other EIA methods for sulfonamides described so far,[3] have therefore strong cross-reactivities with N^4-amino derivatives of all cross-reacting compounds. In our EIA for sulfathiazole, N^4-acetylsulfathiazole, a major metabolite of sulfathiazole in meat products, has about 80% cross-reactivity relative to sulfathiazole (unpublished data). Reaction of sulfathiazole with reducing sugars in honey appears to result in derivatives having slightly more than 100% relative cross-reactivity (Table 6). Further work will aim on the isolation and characterization of these derivatives. Since sulfonamide derivatives are cleaved under acidic conditions,[3] they could thus be transformed back into the active parent compound in the human digestive system.

Although the results obtained by EIA screening may not be acceptable for legal purposes, the methods are still reliable enough to indicate that at present, commercial honey appears to be amongst those foodstuffs with the highest incidence of residues of antibiotics and sulfonamides. The contamination situation appears to be a worldwide problem. Much more intensive control measures are required, and legal steps should be considered to set maximum residue limits for antibiotics and sulfonamides in honey.

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