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Contamination of honey by the herbicide asulam and its antibacterial active metabolite sulfanilamide

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A number of antibacterial drugs (antibiotics) like sulfonamides, tetracyclines and streptomycin are used for the treatment of bacterial diseases in beehives. Yet, the finding of sulfanilamide residues in some 15 Swiss honeys out of some 350 samples could not be explained by such apicultural practice. Bees occasionally collect nectar from meadows treated with the herbicide asulam. Such honey is not only contaminated by asulam, but also by its degradation product sulfanilamide. This is the first report that the use of a herbicide causes the appearance of residues of an antibacterial active metabolite belonging to the category of sulfonamide drugs in food. The relevance of this finding lies in the fact that the use of the herbicide asulam might cause unacceptable residue levels of sulfanilamide in a product for human consumption.

Keywords: sulfonamides, sulfanilamide, asulam, honey, metabolite, liquid chromatography-mass spectrometry (LC/MS)

Introduction

Apicultural use of sulfonamides for treatment of disease

Sulfonamides are used against a number of bacterial diseases affecting bees (Frey and Löscher 1996). Sulfathiazole is known to be effective against the American foulbrood (*Bacillus larvae*) (Haseman 1946). A number of other sulfonamides like sulfamethoxazole and sulfamdimethoxine are also used for the same purpose. However, the appearance of resistance against some antibacterially active substances led to the belief that these drugs should be avoided as much as possible in animal husbandry (Wille 1967). In Switzerland, the treatment of bees with antibiotics has not been permitted since 1974 (Bogdanov and Fluri 2000, Zentrum für Bienenforschung 2002). Analytical controls were initiated to monitor the compliance of this regulation.

Honey is a rather complex matrix and the analysis of veterinary residues by classical liquid chromatography with ultraviolet light/vis or fluorescence detection methods might be affected by false-positive findings. It was the use of liquid chromatography coupled with tandem mass spectrometry detection (LC-MS/MS) as an analytical tool that permitted the unambiguous detection and confirmation of a number of antibacterial drug residues (Kaufmann and Guggisberg 2002).

In relation to sulfonamide residues, there were three relevant sulfonamide findings among the 350 Swiss honey samples analysed in the present authors' laboratory:

- Group of beehives with a weakened bee population was treated with sulfathiazole. Bees from neighbouring hives were able to tap the honey from this weak bee population as a food source. This phenomenon, termed 'robbery', can also be observed among healthy bee populations. However, the practice significantly increases when a bee population weakens or even abandons a beehive. Hence, depending on the distance, honey from other beehives showed significant residue levels of sulfathiazole (Seiler and Kaufmann 2002).
- Contamination of a honey with sulfathiazole from another region could be linked to the winter feeding of bees with honey of rather dubious origin. This feeding honey was most likely very old and no

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longer fit for human consumption. It contained some 8000 $\mu g\,kg^{-1}$ sulfathiazole, making it a likely contamination source.

- Several honey samples contained sulfanilamide, an exceptional finding that could not be explained at the time.

Agricultural use of the herbicide asulam

The herbicide asulam (methyl-4-sulfanylcarbamate) in Switzerland is approved to control the growth of a variety of broadleaf weeds (*Rumex* sp., *Dryopteris* sp., *Pteridium aquilinium*) in meadows, pasture, pome and stone fruit orchards. It is used in springtime (April/May) in significant quantities (1–3 $kg\,ha^{-1}$). Asulam is known to be degraded into sulfanilamide (4-aminobenzenesulfonamide; figure 1) and further metabolites (US Environmental Agency 1995). A soil *Flavobacterium* sp. was reported to degrade asulam to sulfanilamide (Walker 1978, Allan and Millward 1984). This bacterium can grow on asulam and sulfanilamide as they act as a carbon and energy source (Walker 1978). The time to degrade 50% of the original asulam concentration in treated soil was less than 7 days (Allan and Millward 1984). Only 2.5% of asulam remained after 15 days (Suzuki and Yaguchi 2001). This degradation proceeds rapidly in topsoil, although adding yeast extract enhanced degradation (Babiker and Duncan 1977). Besides enzymes, iron(III) aqua-complexes were shown to act as photocatalysts in the degradation process of asulam to sulfanilamide (Castastini and Sarkha 2002).

Contamination of the food chain

Residues of active substances in food like honey not only might be caused by the direct use of such drugs by bee-keepers who treat their bees, but also might be caused by robbery or winter feeding with contaminated honey/sugar as described above (Seiler and Kaufmann 2002). There exists the possibility that environmental pollution, e.g. contamination caused by agricultural use of pesticides in different cultivations, is carried into the hive by the bees themselves (Fernandez and Muino 1995, Koch and Weiber 1997).

An accumulation of several sulfanilamide-positive honey samples originating from different beekeepers in the Canton (Province) of Aargau were the starting point for comprehensive investigations. As a result of preliminary research, the present authors stated the hypothesis that the sulfanilamide residues in honey probably originated from the metabolic pathway of the herbicide asulam and were brought in the beehives from the surroundings by the bees themselves (Jahresbericht Kantonales Laboratorium Aargau 2000, 2001).

The present study investigates the finding of sulfanilamide residues in Swiss honey and attempts to elucidate the question whether sulfanilamide is illegally used as an antibiotic to fight bacterial bee diseases or if the sulfanilamide residue levels in honey are indirectly due to the agricultural application of asulam and the honeybees are the crucial vector of this contamination.

Materials and methods

Materials

The following chemicals were obtained commercially: sulfanilamide (Fluka, Buchs, Switzerland); asulam (Promochem, Wesel, Germany); citric acid monohydrate and formic acid 98% (Merck, Darmstadt, Germany); hydrochloric acid 37%; ammonia 25% and acetonitrile (Scharlau, Barcelona, Spain).

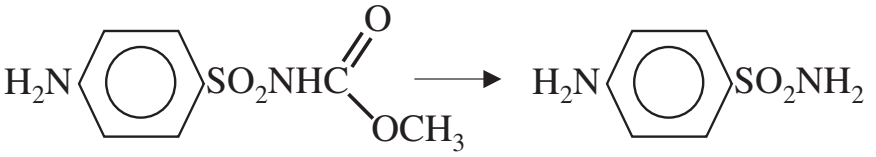

Figure 1. Degradation of asulam into sulfanilamide.

The materials used were as follows: centrifuge tubes, 250 ml, fluted filter paper (Schleicher & Schüll, Dassel, Germany); solid-phase extraction (SPE) columns, OASIS HLB 200 mg (Waters Milford, MA, USA); solid-phase extraction unit, Visiprep (Supelco, Bellefonte, PA, USA); vortex mixer, variable speed; magnetic stirrer, variable speed; pH meter, Metrohm 691 (Herisau, Switzerland); rotary evaporator, Büchi RE 120 (Flawil, Switzerland).

Extraction and sample preparation

Honey (7.5 g) was weighed into a centrifugation tube (the spike solution was added if recoveries were to be determined). It was dissolved in 15 ml 2 mol l^{-1} hydrochloric acid and left for 30 min at room temperature. Citric acid solution (30 ml 0.3 mol l^{-1}) was added, mixed and filtered. An SPE cartridge was moistened with 3 ml acetonitrile and rinsed twice with 2 ml distilled water. Honey filtrate (20 ml) was taken and the pH adjusted to 3.5–4.5 with ammonia. The next step was to proceed immediately. The neutralized honey solution was transferred into the reservoir above the SPE cartridge and the solution sucked through the cartridge within 10–15 min. The cartridge was rinsed three times with 3 ml distilled water and allowed to run dry for about 4 min. The cartridge was eluted with 3 ml acetonitrile into a small, previously weighted conical flask. The solution was evaporated in a rotary evaporator (40°C) to a small volume. Mobile phase A (0.5 ml) was added to the remaining liquid. It was mixed with a vortex mixer and the conical flask was weighed. The extract was transferred without filtration into a high-performance liquid chromatography vial.

Liquid chromatography coupled with tandem mass spectrometry detection (LC-MS/MS)

LC-MS/MS was performed by means of a type Agilent Model 1100, binary pump, autosampler (Agilent, Waldbronn, Germany) coupled to an electrospray ionization (ESI) coupled with tandem mass spectrometry detection, Quattro LCZ with electrospray interface and MassLynx software (Micromass, Manchester, UK) using a Nucleosil 100-5, C18 HD, 50 × 2 mm, 5 µm plus guard column (Macherey-Nagel, Oensingen, Switzerland).

A linear gradient was employed: 0–10 min: 0–30% B; 10–12 min 30% B; 12–12.1 min: 30–0% B; 12.1–19 min 0% B. Mobile phase A: 50 ml acetonitrile and 3 ml formic acid were added into a 1000 ml graduated flask and filled up to the mark with distilled water. Mobile Phase B: 3 ml formic acid were added into a 1000-ml graduated flask and filled up to the mark with distilled water. The column flow was 0.2 ml min^{-1} and the injection volume was 10 µl.

MS parameters were as follows: capillary voltage, 3.25 kV; extractor voltage, 3 V; source temperature, 90°C; desolvation temperature, 250°C; cone gas flow, 50 l h^{-1}; desolvation gas flow, 560 l h^{-1} (nitrogen); nebulizer gas flow, factory preset value; collision cell pressure, 2×10^{-3} mbar (Argon); multiplier voltage, 650 V. Compound specific parameters were as follows: asulam: 231 > 156 transition by applying 10 eV collision energy and 32 V cone potential; sulfanilamide: 173 > 93 by applying 30 eV collision energy and 25 V cone voltage.

Confirmation was based on the monitoring of at least two MS/MS transition ratios. Sulfanilamide: 173 > 156; cone, 30 V; collision energy: 9 eV; asulam: 231 > 108; cone, 15 V; collision energy, 20 eV. A number of samples were analysed by a pseudo-MS/MS/MS approach (cone-induced fragmentation).

Validation

The method underwent validation. Sulfanilamide was validated in three different honey matrices (six levels, three repetition each), while asulam was validated only for one honey matrix (blossom honey).

r^2 were between 0.984 and 0.994. The recoveries (not corrected for signal suppression effects) varied between 45 and 55%. The MS signal was observed to be suppressed by the matrix to a level between 54 and 78% of the sulfanilamide peak area obtained by injecting a pure standard. Hence, recoveries corrected for signal suppression were found between 70 and 83%. The r^2 for asulam was 0.989; recovery (not corrected for signal suppression effects) was 61%. MS signal suppression was 85%. Hence, the recovery corrected for signal suppression was 72%. The limit of detection was 1 µg kg^{-1} (sulfanilamide) and 0.8 µg kg^{-1} (asulam). These limits were calculated based on a 3:1 s/n ratio. Intra- and interday precisions were 6.2 and 9.8% relative standard deviation (RSD) for sulfanilamide and 5.4 and 10.2% RSD for asulam (referring to a blossom honey sample spiked with 50 µg kg^{-1} analyte).

Samples/analysis

Some 350 Swiss honey samples were officially collected from either retail trade or directly from apiculturists. Among the 15 sulfanilamide contaminated honey samples, four corresponding beekeepers were selected for further investigations, in the course of which additional samples were obtained from them the following year, consisting of honey collected by the bees particularly in spring (April/May) and in mid-summer (June/July).

All samples were analysed by the official Food Control Authority of the Canton of Zürich by the LC-MS/MS method described above. The analytical method permitted the detection of 16 different sulfonamides and three tetracyclines. Additional technical details have been published by Kaufmann and Guggisberg (2002). Positive samples were confirmed by monitoring the ratio of at least two analyte-specific MS/MS transitions.

Results and discussion

Fifteen honey samples officially collected from the local market contained measurable concentrations of sulfanilamide in the range 3–227 $\mu g\,kg^{-1}$, whereby in four cases, the sulfanilamide content was above the maximum permitted residue level of 50 $\mu g\,kg^{-1}$, according to the ordinance on foreign substances and constituents in foods (Swiss Federal Office of Public Health 2002). Figure 2 shows a typical chromatogram of a honey containing sulfanilamide and asulam residues.

In the beginning, the positive sulfanilamide findings were not easy to explain. Initially, sulfanilamide was the first discovered drug belonging to the group of sulfonamides. Chemical modifications of the side chain of the molecule produced a large number of derivatives exhibiting clearly stronger antibacterial activities. Hence, it was not evident why an 'old', not very potent drug, which is therefore not anymore easily obtainable, should be used in apiculture.

Since a number of sulfanilamide-positive honey samples originated from the Canton Aargau, the local Food Control Authority questioned the apiculturists involved and investigated in detail the apicultural and agricultural situation surrounding the origin of the contaminated samples. The dismayed beekeepers clearly denied the use of any drugs in their beehives.

If sulfanilamide was used for the treatment of bees, honey samples from these producers should show very high antibacterially effective concentrations of sulfanilamide. However, unlike the mentioned cases concerning sulfathiazole with measured levels up to above 10 $mg\,kg^{-1}$ (Seiler and Kaufmann 2002), only small amounts of sulfanilamide in the $\mu g\,kg^{-1}$ range were found. Such levels are more easily explained by unintentional contamination than by a prohibited therapeutic use.

Further inquires by the Official Food Control Authority of the Canton of Aargau, focused on other possible sources of sulfanilamide, finally referred to the following theory as a 'working hypothesis' (Jahresbericht Kantonales Laboratorium Aargau 2000):

> Sulfanilamide contamination in honey might be closely interrelated to the agricultural use of the herbicide asulam, because the active agent is known to be degraded into sulfanilamide and asulam-containing products are usually applied in cultures and plantations foraged by honeybees.

Consequently, this hypothesis was tested by analysing sulfanilamide-positive honey samples for possible asulam residues. It was shown that the described sample preparation used for the determination of sulfonamides in honey could be used as well for the sample clean up of asulam. The substance survived the hydrolysis step and was also concentrated efficiently on the SPE material. Therefore, all samples containing sulfanilamide residues were (re)analysed with the modified method.

In all the 15 sulfanilamide-positive honey samples, asulam was detected in concentrations of 1–200 $\mu g\,kg^{-1}$ (table 1). One sample contained asulam residue levels reaching the provisional maximum residue level of 200 $\mu g\,kg^{-1}$, which was fixed by the Swiss Federal Office of Public Health (January 2002) after discovering the unexpected asulam contamination in honey. These results showed a clear correlation between sulfanilamide and asulam content (figure 3). Note that among the 350 honeys from the local market, there were no samples where either only asulam or only sulfanilamide was detected. Hence, there is a clear relationship between these two substances. All positive honeys originated from locations where spring flowers in meadows and pas-

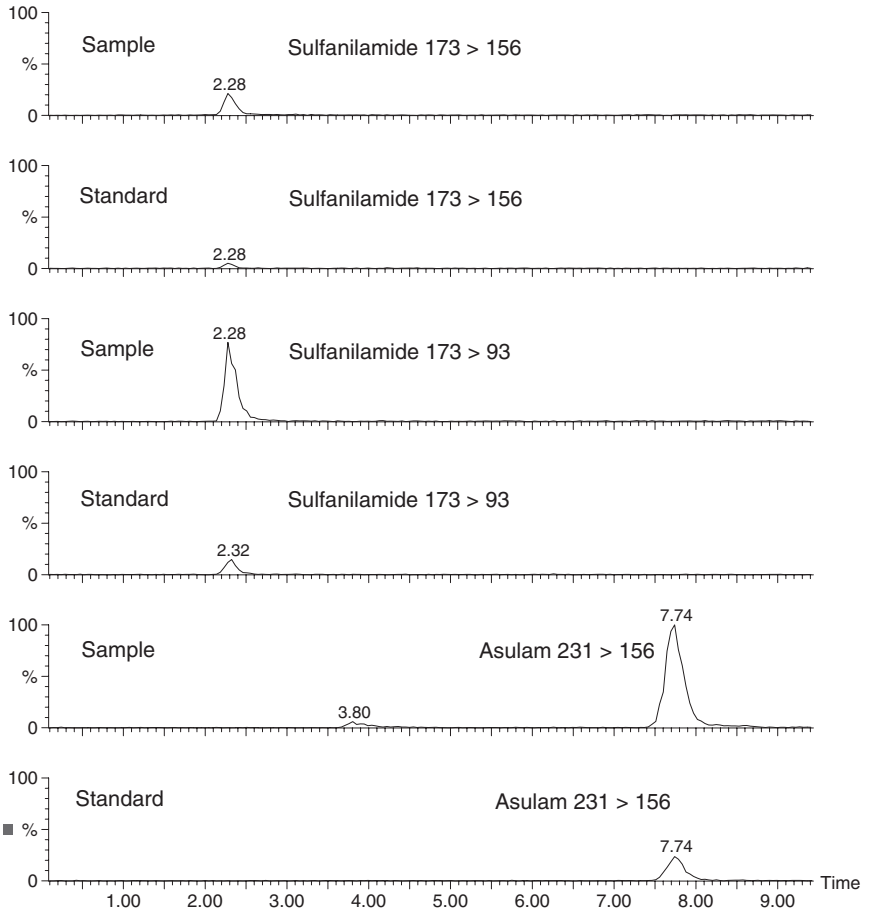

Figure 2. Asulam and sulfanilamide NRM traces of a standard solution and a contaminated honey sample.

Table 1. Sulfanilamide and asulam content of some honey samples.

Sulfanilamide ($\mu g\,kg^{-1}$)	Asulam ($\mu g\,kg^{-1}$)
227	26
190	48
150	200
56	20
26	18
20	2
18	4
15	8
10	1
10	3
10	16
8	9
6	1
4	4
3	1

tures for forage production represented a major nectar yield for bees.

With the additional samples from affected beekeepers, it was discernible that honey collected by the bees in spring (April/May) contained substantially higher levels of sulfanilamide and asulam than honey from the same beehives subsequently collected in mid-summer (June/July). Typical examples are summarized in table 2. This finding corresponds to the period where meadows and pastures are treated with asulam.

The use of asulam for the treatment of integrated production cultures has to be sanctioned by the Official Center of Crop Protection. Yet, no such application has been received from farmers in the neighbourhood of the contaminated beehives. However, it is possible that non-integrated produced meadows were treated with asulam or that asualm

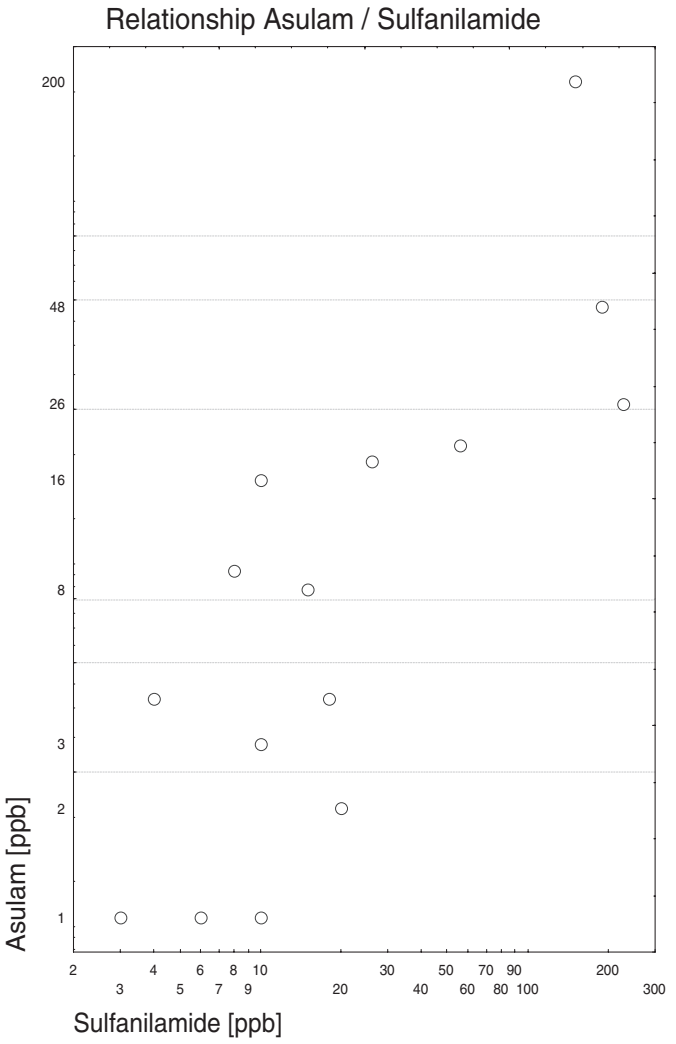

Figure 3. Asulam/sulfanilamide relationship among sulfanilamide-positive honey samples.

was used without official permission. Nevertheless, the main proposition of the present working hypothesis was substantiated by the preliminary result of a project initiated by the Swiss Bee Research Centre following the unexpected and mysterious sulfonamide contamination in Swiss honey. Experimentally controlled application of asulam in pasture in springtime (April) caused analytically measurable sulfanilamide and asulam residues in nearly all blossom honey samples harvested in neighbouring beehives (Bogdanov 2003).

More than 50% of all foreign honey sold in local markets contained traces of at least one antibacterial drug (Jahresbericht Kantonales Labor Zürich 2001a). Some samples contained small amounts of up to five different sulfonamides. However, the present authors could never detect asulam/sulfanilamide in foreign honey. This might be explained by the nature of the final product. Swiss honey is a high-priced product, which is commonly sold directly by the apiculturist and it is rather seldom that such honey is blended with honey from other producers. On the other hand, imported honey mostly comprises mixtures consisting of products from different producers, possibly even from different countries or continents. Hence, the blending of a few strongly contaminated honeys with a majority of uncontaminated honeys may produce products with a number of different antibiotic residues. The possible low level contamination of some honeys

Table 2. Sulfanilamide and asulam content in honey harvested in different seasons.

Sample owner	Collection period	Sulfanilamide ($\mu g\,kg^{-1}$)	Asulam ($\mu g\,kg^{-1}$)
Beekeeper A	April/May	227	185
	June/July	59	20
Beekeeper B	April/May	125	115
	June/July	45	11
Beekeeper C	April/May	44	91
	June/July	39	18
Beekeeper D	April–June	20	7
	July	< 5	< 5

by sulfanilamide and/or asulam is therefore most likely diluted to an extent to make them undetectable by present analytical capabilities. On the other hand, in respect of botanical origin a considerable number of foreign products available in Switzerland are specialty honeys, e.g. acacia, chestnut, lemon, lavender or forest trees. These trees/flowers are probably less likely to be treated with asulam than meadows and pasture.

There also is the possibility that the source of sulfanilamide is related to the manure of animals (pig/cows) treated with sulfonamides. This vector cannot be completely ruled out; however, it is considered to be rather unlikely. The Official Food Control Authority of the Canton of Zürich screens yearly an average of about 800 cow/pig urine samples for the presence of different veterinary drug residues. Positive results were repeatedly found for tetracyclines and the sulfonamides. Sulfadimidin and sulfathiazole residues were rather common, but sulfanilamide was never detected (Jahresbericht Kantonales Labor Zürich 2001b). Although in some cases total sulfonamide concentrations up to $20\,mg\,kg^{-1}$ liquid manure could be found on farms where medicinal feed had been applied (Haller *et al.* 2002), the amount of sulfa drugs spread by contaminated manure on meadows is most likely significantly below the sulfanilamide quantities on meadows resulting from the degradation asulam sprayed in the scale of $1–3\,kg\,ha^{-1}$. Moreover, manure is spread almost throughout the year on mowed meadows, in contrast to asulam which is applied in April/May shortly before the flowering of the pastureland or after the end of August. The preferential appearance of sulfanilamide in spring blossom honey could therefore not be explained by manure as the source of sulfanilamide contamination.

Conclusion

The results and findings of these investigations are in agreement with the present main hypothesis: that sulfanilamide contamination in honey might be closely related to the agricultural use of the herbicide asulam in springtime. This rather unexpected finding should be investigated and clarified in detail by means of further experimental studies and projects. In Switzerland, as a consequence of these findings has been that the approval of asulam for agricultural use, especially with regard to the treatment area, has to be modified by the authorizing administration. For instance, restricting the use of asulam to application in autumn would probably resolve the problem to do with the contamination of honey. However, asulam is used world wide in various geographical environments to treat different 'crops', ranging from potato and sugarcane to Christmas tree cultures. Therefore, different circumstances might require appropriate precautions and measures to prevent a contamination of the food chain by its metabolite sulfanilamide.

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