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Evaluation and validation of a biochip multi-array technology for the screening of 14 sulphonamide and trimethoprim residues in honey according to the European guideline for the validation of screening methods for veterinary medicines

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Honey could be contaminated by antibiotic residues. There is still a great need for a cheap and single multi-residue method. The Evidence Investigator™ system is a biochip and semi-automated system. The microarray kit I (AM I) for the detection of sulphonamide and trimethoprim (TMP) residues in honey was evaluated, and then validated according to the European decision EC/2002/657 and to the European guideline for the validation of screening methods for veterinary medicines (2010). The method was found to be rapid and able to screen simultaneously 14 sulphonamides and TMP in honey with a very easy sample preparation procedure. The false–positive rate of 4% maximum is very satisfactory. All detection capabilities (CCβ) were well below the recommended concentration (RC) of 50 μg kg^{-1}. The applicability of AM I kit with different types of honey (monofloral or multifloral, liquid or solid, different floral origins, etc.) has been proved.

Keywords: validation; decision 2002/657/EC; multi-array; biochip; screening; sulphonamides; honey

1. Introduction

Antimicrobial compounds are used in food production to treat or prevent animal diseases. Risks related to the presence of antimicrobials in food of animal origin are multiple allergies in humans, processing food problems (milk) and possible development of antibiotic-resistant bacterial strains. Because of these concerns, many countries have banned or restricted the use of antimicrobial compounds in food-producing animals and have established maximum residue limits (MRLs) for antimicrobial residues in food.

No antibiotic has marketing authorisation for the treatment of bees. No MRL is set for honey. So the policy setting thresholds are specific to each country or continent. At European level, the Community Reference Laboratory (European Union-Reference Laboratories, EU-RL) has published recommendations (CRL, Guidance paper of 7 December 2007). The RC for sulphonamide residues in honey is equal to 50 μg kg^{-1}.

Very few methods are available to detect sulphonamide residues in honey. In general, there are specific tests of one sulphonamide or multi-sulphonamides (generic test). These are ELISA tests, radioimmunoassays or receptor tests (dipsticks). There are also microbiological tests (ampoule or microplate) based on the inhibition of bacterial growth of *Bacillus*

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stearothermophilus which have a wide detection spectrum as compared with immunological methods. However, microbiological tests generally have a good sensitivity to β-lactams, but poorer sensitivity to sulphonamides. So a broad spectrum method for sulphonamides in honey, with good sensitivity below the RC, is always looked at for testing.

A multiplex approach should be suitable to simultaneously detect several sulphonamides. The Evidence Investigator™ Biochip Array Technology (Randox, UK) is used to perform a simultaneous quantitative detection of multiple analytes from a single sample (Fitzgerald, Lamont, Mcconnell, & Benchikh, 2005; Mcaleer, Mcphillips, Fitzgerald, Mcconnell, & Rodriguez, 2006; Molloy, Mcconnell, Lamont, & Fitzgerald, 2005). The core technology is a biochip, a solid substrate containing an array of discrete test regions of immobilised antibodies specific to different antimicrobials. A competitive chemiluminescent immunoassay is employed. Until now, to our knowledge, only one article was published in the field of antibiotic residues concerning the detection of four nitrofuran metabolites in honey with an Evidence Investigator™ system (O'mahony et al., 2011).

The objective of this project was to evaluate the performance of this innovative system called Evidence Investigator™ for the detection of sulphonamide residues in honey, and then to validate the method. The AM I test kit is able to detect 14 sulphonamides (sulphadiazine, sulphadimethoxine, sulphaquinoxaline, sulphamethazine, sulphamethoxazole, sulphathiazole, sulphisoxazole, sulphapyridine, sulphamerazine, sulphamonomethoxine, sulphamethoxypyridazine, sulphachlorpyridazine, sulphamethizole and sulphadoxine) and trimethoprim (TMP).

At first, a performance evaluation of microarray I kit will be realised to check the detection limits in honey provided by the supplier. Then, this kit will be validated according to European decision EC/2002/657 (Commission, 2002) and according to the European guidelines for the validation of screening methods for veterinary medicines (2010).

2. Material and methods

2.1. Chemicals and standard solutions

Oxytetracycline (OTC), tylosin, penicillin G, chloramphenicol, sulphadiazine, sulphadimethoxine, sulphamethazine, sulphamerazine, sulphamethizole, sulphanilamide, sulphaguanidine, sulphamethoxypyridazine, sulphadoxine, sulphaquinoxaline, sulphamethoxazole, sulphachlorpyridazine, sulphamonomethoxine, sulphapyridine, sulphisoxazole, sulphathiazole, TMP streptomycin, ceftiofur and sarafloxacine were all from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

2.2. Matrices

Different batches of blank honey (without antibiotic residues) were analysed. Samples of honey were selected to obtain a representative group of varying degrees of colour and general physical appearance (liquid, creamed and crystalline). Twenty different honeypots have been used: acacia, chestnut, rape, sunflower, lime tree, garrigue and multifloral. These homogeneous honeys (not blended) have different geographical origins.Most of the honey samples have been previously analyzed by immunoassays (ELISA test and receptor test) and were detected negative. However, when the presence of antibiotics was suspected following the analyses with Evidence Investigator™ system, a confirmatory analysis was implemented by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2.3. Preparation of standard solutions

Stock standard solutions of each antibiotic were prepared at a concentration of 1 mg mL^{-1} in methanol. Working solutions of each compound in water were prepared. Spiked samples were prepared at different concentrations by fortifying 1.0 ± 0.1 g of blank honey with 100 µL of the working solution.

2.4. Evidence Investigator™ system

2.4.1. Multi-array biochip technology

The core technology of the Evidence Investigator™ system is a biochip, a solid substrate containing an array of discrete test regions of immobilised antibodies specific to different antimicrobials. A competitive chemiluminescent immunoassay is employed. Increased levels of antimicrobial in a sample will lead to decreased binding of antimicrobial labelled with horseradish peroxidase (HRP) and thus a decrease in the chemiluminescence signal emitted (in Relative Light Unit, RLU)

2.4.2. Microarray I kit

The Evidence Investigator™ Anti Microbial Array I is a commercially available kit which will simultaneously and quantitatively test for 14 sulphonamides (sulphadiazine, sulphadimethoxine, sulphamethazine, sulphamerazine, sulphamethizole, sulphamethoxypyridazine, sulphadoxine, sulphaquinoxaline, sulphamethoxazole, sulphachlorpyridazine, sulphamonomethoxine, sulphapyridine, sulphisoxazole and sulphathiazole) and TMP. Fifteen spots are present on each biochip which allows identifying which sulphonamide is present in a sample.

2.4.3. Honey procedure for sample preparation

One gram of honey sample is weighted out. Then 19 mL of diluted wash buffer warmed to 37°C is added. The tubes are placed on a roller for 10 min or until dissolved. The sample is now ready for application to the biochip.

2.5. Evaluation of the kit

The objectives of the evaluation are first to check the detection limits announced by the manufacturer and second to select the target concentrations that will be tested later on during the validation to determine the CCβ of the antibiotics of interest (see validation protocol).

Because of the specificity of each spot for a sulphonamide or TMP (multiplex system), it is possible to spike samples with antibiotic mixtures. However, some cross-reactivities between sulphonamides (between some of the spots) were announced by Randox (Table 1).

So cross-reactions (CRs) have to be checked first. For two days, in a first series (Series 1), four blank samples (four different batches of honey), spiked at concentration 1 (C1) with a mix of the 14 sulphonamides and TMP and at concentration 2 (C2) with a mix of the 14 sulphonamides and TMP, have been analysed.

The evaluation of sensitivities was focused on the 14 sulphonamides and TMP that present 100% cross-reaction. The tested antibiotics and their related concentrations (C1 and C2) are presented in Table 2. Ideally, the concentrations should be lower than the RC

Table 1. CRs announced by the manufacturer.

		Percentages of CRs with the following antibiotics														
		Sulphadi-methoxine	Sulpha-diazine	Sulpha-doxine	Sulpha-methizole	Sulpha-chlorpy-ridazine	Sulpha-methoxy-pyridazine	Sulpha-merazine	Sulphi-soxazole	Sulpha-thia-zole	Sulpha-metha-zine	Sulpha-quinoxa-line	Sulpha-pyridine	Sulphameth-oxazole	Sulpha-monometh-oxine	Trime-thoprim
Spots	Sulphadimethoxine	100													1.7	
	Sulphadiazine		100													
	Sulphadoxine			100												
	Sulphamethizole				100	13.3								1.4		
	Sulphachlorpyridazine				1.2	100										
	Sulphamethoxy-pyridazine					2.1	100									
	Sulphamerazine		4.8					100								
	Sulphisoxazole				2.2	2.9			100							
	Sulphathiazole		6.2							100			1.5			
	Sulphamethazine							1.9			100					
	Sulphaquinoxaline											100				
	Sulphapyridine						3.4			1.6			100			
	Sulphamethoxazole				92	12.1				1.9				100		
	Sulphamonomethoxine														100	
	Trimethoprim															100

Spot Sulphamethoxypyridazine: 56% with sulphaethoxypyridazine.
Spot Sulphapyridine: 12.9% with sulphasalazine.
All blank cells mean that the cross-reaction was determined to be less than 1%.

Table 2. Tested concentrations for 14 sulphonamides and trimethoprim (Series 1) during the evaluation part and selected concentrations for Series 2.

Molecule	Announced LOD (μg kg^{-1})	Tested C1 (μg kg^{-1})	Tested C2 (μg kg^{-1})	Chosen concentration for Series 2 (μg kg^{-1})
Sulphadiazine	5	5	10	5
Sulphadimethoxine	5–10	5	10	10
Sulphamerazine	5	5	10	5
Sulphamethazine	5	5	10	5
Sulphamethizole	5	5	10	5
Sulphachlorpyridazine	5	5	10	5
Sulphamethoxazole	5	5	10	5
Sulphaquinoxaline	5	5	10	5
Sulphathiazole	5	5	10	5
Sulphadoxine	5	5	10	5
Sulphamethoxypyridazine	5	5	10	5
Sulphisoxazole	5	5	10	5
Sulphapyridine	8	8	16	16
Sulphamonomethoxine	20	20	40	20
Trimethoprim	8	8	16	8

(CRL guidance paper, December 2007). The choice of the concentrations was performed according to the supplier data in the kit's booklet (announced detection limits) and according to the RC.

Then, for two days, in a second series (Series 2), one blank honey sample and 15 samples spiked individually with each of the 14 sulphonamides and TMP have been analyzed. The concentrations of Series 2 will be chosen from the results of Series 1.

A calibration curve was analysed at the beginning of the validation. This calibration was used to analyse the quantitative results of the tested samples. In fact, our data analyses were focused on the signal in RLU not on the calculated concentration.

Then, the average value of the signal (in RLU) of the four blank samples will be calculated, as well as the standard deviation (SD) of the signal of the four blank samples. Similarly, the average signal and the SD of the four spiked samples at C1 and C2 will be calculated for each tested antibiotic. Finally, the positivity threshold T will be calculated as follows from the blank samples: T = average RLU signal of blank − 1.64 × SD RLU signal of blank. Moreover, the cut-off value F_m will be calculated as follows from the spiked samples: F_m = average RLU signal of spiked + 1.64 × SD RLU signal spiked.

This evaluation step will allow selecting the target concentrations for the determination of CCβ during the validation. The chosen concentrations (C1 or C2) will be valid if and only if the cut-off value F_m is below the positivity threshold T. Otherwise it is necessary to increase the concentration of antibiotics in the validation step.

2.6. Validation protocol

After the evaluation step, the validation was performed according to the European guideline for the validation of screening methods for residues of veterinary medicines (CRL, 2010) which is based on the criteria of European Decision No 2002/657/EC (Commission, 2002). The performance characteristics to be determined were: practicability, applicability (honeys from different sources), specificity, CCβ, ruggedness and

stability. The analyses have been blindly performed by a technician (random numbers given to the samples).

2.6.1. Practicability

The aim of the practicability study was to check whether the methodology is appropriate or not for routine analysis. It is not a separate study which would need complementary analysis. During the evaluation and validation, practicability was considered by the analysts as the ease of use of the method in routine conditions. So necessary equipment (specific or usual equipments in a lab), reagents (ready to use or not), instruments (specific or usual instruments in a lab) and environmental conditions (large or narrow temperature intervals to implement the kit) are noticed.

2.6.2. Specificity, false-positive rate

Twenty different lots of blank honey (antibiotic-free) from organic apiculture (trade) or control plans should be analysed (different floral origins, different colours, different textures and different levels of crystallisation; cf. Applicability). As in the evaluation step, if the presence of antibiotic residues following the analyses with the Evidence Investigator™ system was suspected, a confirmatory analysis by LC-MS/MS was asked for.

2.6.3. Detection capability

The target concentration was determined after the evaluation. The CCβ have to be determined for the 14 sulphonamides and TMP.

In the case of honey, there are recommendations of the EU-RL. In the European guideline for the validation of screening method (CRL, 2010), it is required to determine the number of samples necessary for the validation in relation to the selected target concentration and the regulatory limits (here RCs) (CRL, Guidance paper of 7 December 2007). If the target concentration is set at half the RC (half MRL), the occurrence of one or no false-compliant result after the analysis of 20 control samples is sufficient to demonstrate that the detection capability is less or equal to the RC (MRL) and less than or equal to the half MRL. Given the detection limits announced by the manufacturer, the number of samples should probably be reduced to $n = 20$ for all sulphonamides. This will be checked during the evaluation phase.

Then, a statistical approach which takes into account the β error of 5% was chosen as it is recommended in the guideline for the validation of screening methods (CRL, 2010). The positivity threshold T and the cut-off value F_m were calculated as it is recommended in the European guideline. Positivity threshold T and cut-off factor F_m are matrix-specific.

$T = B - 1.64 \times SD_B$ and $F_m = M + 1.64 \times SD$, where B is the mean and SD_B the SD of the signal (RLU) of the blank samples; M is the mean and SD is the SD of the signal (RLU) of the spiked samples.

The first basic principle is that the assay is considered valid only if T is higher than F_m. If F_m is higher than T, it means that the spiked concentration is too low because the kit is not able to discriminate between blank and spiked samples, regarding the dispersion (SD) of the results of blank and spiked samples. On the contrary, when T is higher than F_m value, the kit is able to discriminate between blank and spiked samples.

As a second basic principle, T or F_m will be chosen as the cut-off level to conclude on unknown samples if the result is negative or positive. When the response in RLU of an unknown sample is higher than the cut-off level, the sample is declared as negative

sample. On the contrary, when the response in RLU of an unknown sample is lower than the cut-off level, the sample is declared as positive sample. The number of spiked samples with mean signal (RLU) below the cut-off level was identified (positive result). If more than 5% of the spiked samples at the screening target concentration gave signals (RLU) higher than the cut-off level (false–negative result), this concentration chosen for the spiking study should have been too low for validation (the true CCβ is higher than this concentration).

The detection capability CCβ of the method will be equal to the spiking level (screening target concentration) if less than 5% of false–negative results (i.e. 1 or less false–negative results out of 20 spiked samples) are obtained at this concentration, after the analysis of 20 spiked samples. The CCβ should be lower than or equal to the regulatory/action limit.

2.6.4. Applicability

Samples of honey were selected to obtain a representative group of varying degrees of colour and general physical appearance (liquid, creamed and crystalline). Twenty different honeypots have been used: acacia, chestnut, rape, sunflower, lime tree, garrigue and multifloral. These honeys have different geographical origins. The applicability of kits to different types of honey (monofloral or multifloral, different flowers, liquid or solid, etc), will be tested by determining CCβ of the 14 sulphonamides and TMP from 20 different samples.

2.6.5. Cross-reactions

Cross-reactivities towards other sulphonamides (sulphanilamide and sulphaguanidine) were determined from a reduced number of samples. Each sulphonamide was prepared at C1 and was analysed 10 times (10 different batches of honey). Six to ten antibiotics from other major antibiotic families [streptomycin (aminoglycosides), sarafloxacin (quinolones), penicillin G (penicillin), OTC (tetracyclines), ceftiofur (cephalosporins), tylosin (macrolides), CAP (chloramphenicol)] were chosen to be tested at a minimum at a concentration of 10,000 μg kg^{-1} 10 times (10 different batches of honey). All antibiotics were mixed in the same sample, even with sulphachlorpyridazine.

2.6.6. Stability

The stability of analytes in solution and in the matrix will be determined through a literature review.

3. Results and discussion

3.1. Evaluation part

Evaluation of sensitivities was focused on the 14 sulphonamides detected and TMP. The 14 sulphonamides were individually tested (Series 2) and the same 14 sulphonamides in a mixture (Series 1) were tested to see if there was interference between channels.

The results of Series 1 allowed selecting concentrations for Series 2 (Table 2). In the end, all sulphonamides are used at C1, except sulphapyridine and sulphadimethoxine (C2). Overall, the limits of detection (LOD) were similar to those announced by Randox, except for sulphapyridine (chosen concentration was twice the announced LOD).

The concentrations determined from Series 1 have proved adequate to 11 sulphonamides and TMP during this Series 2 where each sulphonamide and TMP was tested individually. On the contrary, the concentrations chosen for sulphadoxine (5 μg kg^{-1}) and sulphapyridine (16 μg kg^{-1}) were not well detected individually in Series 2. Furthermore, 5 μg kg^{-1} of sulphadiazine was not detected at all individually. Therefore, the second day of Series 2, the concentrations were increased for sulphadoxine at 10 μg kg^{-1}, sulphapyridine at 25 μg kg^{-1} and sulphadiazine at 10 μg kg^{-1}.

Comparing the signals obtained with individual sulphonamides or a mixture of sulphonamides between Series 1 and 2 helped highlighting interactions (CR) between sulpha drugs. There was a strong interaction on the spot sulphamonomethoxine with sulphapyridine which was not announced by Randox. Sulphapyridine at 25 μg kg^{-1} gave an equivalent concentration of sulphamonomethoxine 3–10 times higher than a blank sample. Sulphapyridine should be tested at higher concentration to determine precisely percentage of cross-reaction. There was also a strong interaction on the spot sulphamethoxazole with sulphamethizole. The cross-reactivity announced by Randox was equal to 92%. CRs on the spot sulphamethizole with sulphachlorpyridazine (13.3%) and sulphamethoxazole (1.4%) were confirmed. Some CRs have been suspected the first day of analysis but were not confirmed the second day of analysis on the spot sulphisoxazole with sulphadoxine and sulphamethoxypyridazine. Some CRs were announced by Randox but were not observed here probably because the tested concentrations were too low to interfere (on the spot sulphamethoxazole, CR announced 12.1% with sulphachlorpyridazine, on the spot sulphapyridine CR announced 1.6% with sulphathiazole, on the spot sulphapyridine CR announced 3.4% with sulphamethoxypyridazine). There was no visible interaction on 11 spots to detect sulphadiazine, SDMX, sulphaquinoxaline, sulphamethazine, sulphathiazole, sulphayridine, sulphamerazine, sulphamethoxypyridazine, sulphachlorpyrididazine, sulphadoxine and TMP.

As a conclusion of the evaluation step, sulphamethizole should not be mixed with sulphamethoxazole (spot sulphamethoxazole), sulphamethizole should not be mixed with sulphachlorpyridazine, and sulphamethoxazole (spot sulphamethizole) and sulphamonomethoxine should not be mixed with sulphapyridine (spot sulphamonomethoxine). So for determining CCβ, two groups of substances were selected: mixture 1 and mixture 2 (Table 3). The chosen concentration for each antibiotic is valid if and only if the cut-off factor F_m is below the positivity threshold *T*. Otherwise it is necessary to increase the

Table 3. Choice of antibiotics and concentrations for validation in two different mixtures.

Mixture 1		Mixture 2	
Molecule	Concentration (μg kg^{-1})	Molecule	Concentration (μg kg^{-1})
Sulphadimethoxine	10	Sulphadiazine	10
Sulphamethazine	5	Sulphamerazine	5
Sulphamethizole	5	Sulphachlorpyridazine	5
Sulphaquinoxaline	5	Sulphamethoxazole	5
Sulphathiazole	5	Sulphadoxine	10
Sulphisoxazole	5	Sulphamethoxypyridazine	5
Sulphamonomethoxine	20	Sulphapyridine	25
TMP	8		

concentration of antibiotics in the validation step. The choice of concentrations for validation (determination of CCβ) is presented in Table 3.

3.2. Validation part

3.2.1. Practicability

The amount of reagents provided is comfortable. The implementation is easy with a single dilution of the sample of honey 1/20th in buffer. The software is simple in its functionality. The data from the disk provided in the box have to be inserted and loaded when a new batch number is used.

3.2.2. Specificity and false-positive rate

During validation, 20 samples of different blank honey were analysed, and spiked with mixture 1 and mixture 2. Among the blank samples analysed on day 1, some positive results have been obtained that are presented in Table 4.

If T is chosen as the threshold value (T = average RLU blank − 1.64 × SD), one sample was positive for sulphadiazine (sample A very slightly), one sample positive for sulphaquinoxaline (sample A) and one sample clearly positive for TMP (sample B). These three samples were not sent for confirmation by LC-MS/MS. If F_m is chosen as the threshold value (F_m = average RLU spiked + 1.64 × SD), the sample A is negative for all the sulphonamides. On the contrary, the sample B has a lower signal than F_m and is therefore positive for TMP. The F_m was calculated for a TMP concentration equal to 8 µg kg^{-1}. This probably means that the sample contains a concentration greater than or equal to 8 µg kg^{-1} TMP.

For days 2, 3 and 4, whenever the T or F_m is chosen as the threshold value, no false–positive result occurred. As it has already been observed with ELISA kits, taking T as the threshold value increases the occurrence of false-positive results which will lead to expensive confirmatory analyses for which the final result would be compliant sample. Here two samples out of 25 (8%) of false-positives were obtained and even probably one out of 25 (4%) of false-positive because sample B has a high probability of being a true-positive result. If the F_m is chosen as the threshold value, only one positive result (sample B positive for TMP;1/25) is obtained which is very satisfactory. The percentage of false-positives may even drop to 0% if the presence of residues of TMP is confirmed in this sample.

So F_m was chosen as the cut-off value to conclude on the positivity of a sample.

Table 4. Number of false-positive results depending on the choice of the threshold value T or cut-off factor F_m as cut-off value.

	Sulphadiazine	Sulphaquinoxaline	TMP
T value (in RLU) (n = 20)	12,302	10,487	2335
T = cut-off value (n = 20)	1 false-positive	1 false-positive	1 false-positive
F_m value (in RLU) (n = 20)	10,287	6736	2126
F_m = cut-off value (n = 20)	0 false-positive	0 false-positive	1 false-positive

3.2.3. Detection capabilities

For the first 2 days, all the sulphonamides and TMP were detected except sulphadimethoxine at 10 μg kg^{-1}. Therefore, after two days of validation, the concentration of sulphadimethoxine was increased to 25 μg kg^{-1} (instead of 10 μg kg^{-1}). On days 3 and 4, all the sulphonamides and TMP were detected.

During the four days of validation, in the mixture 1 which contains no sulphamethoxazole a strong reaction was observed on the spot sulphamethoxazole. This is due to a CR of sulphamethizole with this spot sulphamethoxazole.

Table 5 presents the summary of the results when F_m is chosen as the cut-off value. The letter C means that the result is compliant, that is to say, F_m is much lower than T and thus the validated concentration is equal to CCβ. No more than 5% of false-negative results (e.g. 1 out of 20 spiked samples) were obtained for nine sulphonamides and TMP. On the contrary, false-negative rates of 5.6% (2/36) were obtained for three sulphonamides (sulphaquinoxaline, sulphamethazine and sulphamonomethoxine) and 9.1% (2/22) for two other sulphonamides (sulphapyridine and sulphamerazine).

Table 6 presents the CCβ obtained during the validation for the 14 sulphonamides and TMP.

The CCβ have not been determined precisely for the five sulphonamides having more than 5% of false-negative results. Regarding the low number of false-negative results, the true CCβ should be very near from the validated concentration. A range of estimation is given in Table 6 for these five sulphonamides, based on the following principle.For the three sulphonamides having a false-negative rate of 5.6% (two false-negative results have been obtained, instead of one after the analysis of 36 spiked samples), the range of estimation was calculated from the validated concentration plus 50%. For the three sulphonamides having a false-negative rate of 9.1% (two false-negative results have been obtained, instead of one after the analysis of 22 spiked samples), the range of estimation was calculated from the validated concentration plus 100%. The CCβ of sulphaquinoxaline, sulphamethazine, sulphamonomethoxine, sulphapyridine and sulphamerazine could be determined more precisely in the future so that target concentrations should be increased a little. However, taking into account the high price of the kits needed for a validation, it was decided to go on with these estimated CCβ for routine analyses because negative and positive quality controls (QCs) will be used in routine analyses. If QCs are not valid, the samples analysed this day have to be reanalysed.

As a conclusion, the CCβ were equivalent to the detection limits announced by Randox for 10 sulphonamides, not for 5 sulphonamides (sulphadimethoxine (2.5–5 times), sulphamethoxazole (three times), sulphapyridine (three times), sulphadoxine (two times) and sulphadiazine (two times) for which the determined CCβ were higher than the announced LOD. In conclusion, all CCβ were well below the RC of 50 μg kg^{-1} (even lower than half RC for 11 sulphonamides).

3.2.4. Cross-reactions

Cross reactions were analyzed in terms of calculated concentrations and not in RLU signal because the quantitative relationship between the antibiotic at 100% of cross-reactivity and the antibiotic that potentially cross-reacts should be determined. Fifteen different antibodies immobilised on the microarray can detect 14 different sulphonamides and TMP. In addition, Randox announced that sulphanilamide and sulphaguanidine are not detected by the AM I kit (CR < 1%).

Table 5. Summary of the results when F_m is taken as the cut-off value.

	SZ	SDM	SM	SMT	SMZ	SCP	SMX	SQ	ST	SD	SMP	SS	SP	SMM	TMP
Concentration ($\mu g\ kg^{-1}$)	10	25	5	5	5	5	5	5	5	10	5	5	25	20	8
T (RLU)	12,302	2599	5442	3591	10,585	8886	4296	10,487	10,160	2271	5215	3901	2493	2539	4202
Mix 1 F_m (RLU)		1914		2089	8977			6736	6935			3581		1877	2265
Mix 2 F_m (RLU)	10,287		3407			8422	3559			1558	4670		2009		
$F_m < T$	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Number of FP	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
FP rate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**2.8**
Number of FN	0	1/22	2/22	2/36	0	0	0	2/36	1/36	1/22	0	1/36	2/22	2/36	0
FN rate	0.0	4.5	**9.1**	**5.6**	0	0	0	**5.6**	2.8	4.5	0	2.8	**9.1**	**5.6**	0

Concerning the false-positive rate, bold characters mean that the false-positive rate was different from zero. Concerning false-negative rates, bold characters mean that the false-negative rate was higher than 5% (the requested beta error).

RLU: Relative light Unit; SZ: sulphadiazine; SDM: sulphadimethoxine; SM: sulphamerazine; SMT: sulphamethazine; SMZ: sulphamethizole; SCP: sulphachlorpyridazine; SMX: sulphamethoxazole; SQ: sulphaquinoxaline; ST: sulphathiazole; SD: sulphadoxine; SMP: sulphamethoxypyridazine; SS: sulphisoxazole; SP: sulphapyridine; SMM: sulphamonomethoxine; TMP: Trimethoprim; FN: false-negative; FP: false-positive.

Table 6. Detection capabilities CCβ.

Mix 1	Announced LOD Randox (µg kg^{-1})	Concentration used for validation (µg kg^{-1})	CCβ (µg kg^{-1})	Mix 2	Announced LOD Randox (µg kg^{-1})	Concentration used for validation (µg kg^{-1})	CCβ (µg kg^{-1})
Sulphadimethoxine	5–10	25	25	Sulphadiazine	5	10	10
Sulphamethazine	5	**5**	**5–7.5**	Sulphamerazine	5	**5**	**5–10**
Sulphamethizole	5	5	5	Sulphachlorpyridazine	5	5	5
Sulphaquinoxaline	5	**5**	**5–7.5**	Sulphamethoxazole	1.6	5	5
Sulphathiazole	5	5	5	Sulphadoxine	5	10	10
Sulphisoxazole	5	5	5	Sulphamethoxypyridazine	5	5	5
Sulphamonomethoxine	20	**20**	**20–30**	Sulphapyridine	8	**25**	**25–50**
TMP	9	8	8				

In bold characters, the CCβ that have not been determined precisely.

Whatever the concentration of sulphanilamide (from 10 to 10,000 μg kg^{-1}), no cross-reactivity was observed with the 14 other sulphonamides or TMP, which confirms the results of Randox. Moreover, sulphanilamide at 10,000 μg kg^{-1} was retested 10 times with different honeys, simultaneously with the mixture of seven other tested antibiotics (seven families of antibiotics different from sulphonamides). It was confirmed that no cross-reaction occurs between sulphanilamide, TMP and the 14 other sulphonamides. A cross-reaction was observed with sulphaguanidine at 1000 μg kg^{-1} on the sulphapyridine spot [giving a concentration equivalent to about 25 μg kg^{-1} of sulphapyridine; 2.5% of cross-reaction) and even more at 10,000 μg kg^{-1} and a slight interaction on the spot sulphamonomethoxine at 10,000 μg kg^{-1} (2216 RLU is equivalent to a concentration greater than 20 μg kg^{-1} of sulphamonomethoxine for 10,000 μg kg^{-1} of sulphaguanidine < 0.2%)].

In addition, there were no CRs with seven other families of antibiotics [penicillins (penicillin G), cephalosporins (ceftiofur), macrolides (tylosin), aminoglycosides (streptomycin), tetracycline (OTC), quinolones (sarafloxacin) and phenicols (chloramphenicol)].

3.2.5. Applicability

The method has proved to be applicable to different floral honeys with different geographical origins. In addition, CCβ were determined from at least 20 different samples and so this value takes into account the variability of honey. A positive result was obtained for TMP with F_m taken as the cut-off value. This sample was not sent to confirmation, so it could be a true-positive or a false-positive result.The applicability of the AM I kit to different kinds of honey has been proved during the specificity study and determination of CCβ. The floral origin, texture and colour of honey did not influence the result. So this AM I kit is widely applicable to all kinds of honey.

3.2.6. QCs in routine analyses

During the validation, there has been considerable variability in the signals and background noise so there is need to analyze two to three negative QC (QC−) and two to three positive QC (QC+) in parallel with unknown samples to conclude on their compliance or not. Two different positive QCs have to be prepared, a first containing seven sulphonamides and TMP (mix 1) and a second containing the seven other sulphonamides (mix 2). The antibiotic concentrations to produce positive QCs will be the CCβ of the 15 validated antibiotics. The T value will be determined from the mean signal in RLU and SD of the negative QC and the F_m value from the mean signal in RLU and SD of the positive QC. Each day of routine analyses, the F_m value should be lower than the T value to declare that the essay is valid. Furthermore, every unknown sample with a signal lower than the F_m value will be declared as positive and conversely.

3.2.7. Stability of sulphonamide residues and TMP

The stability data of sulphonamide residues in solution and in honey have been already presented in a validation study of a receptor test named Sulfasensor® (Unisensor, Belgium) in honey (Gaudin, Rault, & Verdon, 2012). The stability of TMP in solution and in the matrix is not intensively documented. In one study, TMP was proved to be stable in a working solution for a storage time up to 35 days after the preparation in the dark at 2–8°C (Croubels, De Baere, & De Backer, 2003). In this approach, the stability of six aliquots at one data point in time was determined. TMP was also proved stable in pig

Table 7. Analyses of incurred honey samples with the Evidence Investigator™ system and an LC-MS/MS method.

	Evidence Investigator™		LC-MS/MS	
	Identification	Quantification (μg kg^{-1})	Identification	Quantification (μg kg^{-1})
Sample 1	Sulphathiazol	20.62	Sulphathiazol	10.06
Sample 2	Sulphathiazol	25.55	Sulphathiazol	8.80
Sample 3	Sulphathiazol	25.94	Sulphathiazol	9.35
Sample 4	Sulphamonomethoxine	86.94	Sulphamonomethoxine	24

Sulphathiazol CCβ 5 ppb.
Sulphamonomethoxine CCβ 20–25 ppb.

muscle, pig kidney, pig liver and skin and fat at 50 μg kg^{-1} for four months at a temperature lower than –15°C. No data about the stability of TMP in honey could be found in the literature.

3.2.8. Analyses of incurred honey samples

After the validation, the AM I kit was implemented for the analyses of four incurred honey samples. These samples which have already been screened and confirmed non-compliant by LC-MS/MS were of great interest to complete our validation study. The comparative results of the analyses of these four samples with the AM I kit and the LC-MS/MS confirmatory method are presented in Table 7.

The results were very satisfactory because sulphathiazol and sulphamonomethoxine have been identified in three samples and in one sample, respectively, by both methods. The calculated concentrations with the AM I kit were overestimated in comparison with the quantification of the LC-MS/MS method. Finally, these results were in agreement with the CCβ determined for sulphathiazol and sulphamonomethoxine.

3.3. Discussion

Agricultural contamination with pesticides and antibiotics is a challenging problem that needs to be fully addressed. There are different potential origins of contamination of honey with sulphonamides. It could be due to the usage of vet drugs containing sulphonamides to treat bacterial diseases in apiculture or to the transformation of pesticide asulam into a sulphonamide or sulphanilamide which is in this case a degradation product of asulam (Kaufmann & Kaenig, 2004). Sulphonamide residues in honey are very stable (Reybroeck, Jacobs, De Brabander, & Daeseleire, 2010). In fact, sulphonamide-containing beeswax could lead to the contamination of honey.

Examples of screening methods available for the screening of sulphonamides and TMP residues in honey are presented in Table 8 (Bargańska et al., 2011; Galarini et al., 2014; Morlot & Beaune, 2003; Pastor-Navarro, Gallego-Iglesias, Maquieira, & Puchades, 2007; Quintana-Rizzo, Salter, & Saul, 2005; Serra Bonvehi & Lacalle Gutierrez, 2009).

Furthermore, different approaches have been evaluated in our laboratory during the past years for the control of sulphonamide residues in honey. The respective performances of AM I kit (Randox, UK), Sulfasensor® (Unisensor, Belgium),

Table 8. Examples of screening methods for the detection of sulphonamide residues in honey.

Screening method	Screened sulphonamides	Reference
Commercial radioimmunoassay named Charm II® system (Charm Sciences, USA)	French honeys found positive at 2.9%, Spanish honeys at 17.1% and Turkish honeys at 82.1%. In France, 7.2% of the lavender honeys and 5% of the chestnut honeys positive	(Quintana-Rizzo et al., 2005; Morlot & Beaune, 2003)
Commercial radioimmunoassay named Charm II system (Charm Sciences, USA)	567 Basque honey samples (68 samples presumptive positive; 19 non-compliant containing sulphathiazole residues at levels from 20 to 210 µg kg^{-1})	(Serra Bonvehi & Lacalle Gutierrez, 2009)
ELISA test LOD sulphathiazole 4 µg kg^{-1}	Sulphathiazole, sulphamethoxypyridazine, sulphapyridine, sulphamethizole, sulphasalazine and *N*4-phtalylsulphathiazole	(Pastor-Navarro et al., 2007)
ELISA test for 11 sulphonamides CCβ ≤10 µg kg^{-1}	Sulphathiazole and sulphadimethoxine (3 positive samples out of 7) tested	(Galarini et al., 2014)

Table 9. Comparison of performance between LC-MS/MS, AM I kit, Sulfasensor®, Eclipse 50® and Premi® test.

	LC-MS/MS method (CCβ) (µg kg^{-1})	AM I (CCβ) (µg kg^{-1})	Sulfasensor® (CCβ) (µg kg^{-1})	Eclipse 50® (CCβ) (µg kg^{-1})	Premi® test (CCβ) (µg kg^{-1})
Sulphaguanidine	< 20	Around 1000	/	/	/
Sulphadiazine	< 10	10	25–50	300	≤ 25
Sulphathiazol	< 10	5	≤ 25	250	25
Sulphamethazine	< 10	5–10	≤ 12.5	> 1000	≤ 25
Sulphamethoxypyridazine	< 10	5	/	/	/
Sulphamonomethoxine	< 10	20–25	/	/	/
Sulphadoxine	< 10	10	/	/	/
Sulphaquinoxaline	< 10	5–10	≤ 150	> 75	≤ 10
Sulphadimethoxine	< 10	25	25–50	250	≤ 25
Sulphamethizole	< 10	5	1000-2000	> 75	≤ 25
Sulphamethoxazole	< 10	5	1000	25	≤ 10
Sulphanilamide	< 20	> 10000	>100000	>> 1000	≤ 25
Sulphamerazine	< 10	5–10	≤ 12.5	> 300	≤ 25
Sulphapyridine	ND	25–30	≤ 25	/	/
TMP	ND	8	/	/	/
Reference	/	/	(Gaudin et al., 2012)	(Gaudin et al., 2013)	(Gaudin et al., 2013)

ND: not detected; TMP: Trimethoprim.

Premi®Test (r-Biopharm, Germany), Eclipse 50® (Zeu Inmunotech, Spain) and our internal LC-MS/MS method are presented in Table 9.

For the implementation of the French official honey control plan in 2010, the Sulfasensor® was implemented in our laboratory as the screening method for sulphonamides, after its validation (Gaudin et al., 2012). However, it was abandoned in 2011 in favor of an internal screening LC-MS/MS method because Sulfasensor® did not detect all the sulphonamides of interest (like sulphamethizole and sulphamethoxazole). The LC-MS/MS method used to quantify and identify residues of 13 sulphonamides in honey is based on LC-MS/MS. Their detection is performed in positive mode with electrospray interface coupling. The CCβ are much lower than the RC for sulphonamides in honey (50 μg kg^{-1}).

Furthermore, microbiological screening tests based on the inhibition of growth of *B. stearothermophilus* have a wide spectrum of detection and could have been competitive with multi-array biosensors. However, the CCβ determined for nine sulphonamides for the Eclipse® 50 kit were higher than the RCs, except for sulphamethoxazole (Gaudin et al., 2013). On the contrary, the Premi® test kit gave satisfactory CCβ for nine sulphonamides. But, the high false-positive rate (14%) led to a great number of physicochemical confirmations. Therefore, this wide spectrum approach was not kept as an alternative to receptor tests or to LC/MS/MS.

Finally, the CCβ of the AM I kit is better than those of Sulfasensor® for eight sulphonamides. However, they both did not detect sulphanilamide which is detected by the LC-MS/MS method. The AM I kit does not detect sulphaguanidine and sulphanilamide while they are detected by the LC-MS/MS method. On the contrary, the AM I kit detects sulphapyridine and TMP which are not detected by the LC-MS/MS method.

One significant advantage of the AM I kit is that its sample preparation did not include a hydrolysis step, which is very interesting regarding the reduced time of sample preparation. On the contrary, the Sulfasensor® test (Unisensor, Belgium) needed a quick hydrolysis step (90 min including sample preparation and analysis; Gaudin et al., 2012). Up to 40 samples could be analysed during one day with the Sulfasensor® kit and with AM I kit. This hydrolysis step is due to the fact that sulphonamide residues bound to sugars in honey (Schwaiger & Schuh, 2000). It can be hypothetised that the AM I kit is able to detect bound sulphonamides or, the kit detects only free-sulphonamides, but very low CCβ.

Table 10 presents a review of articles dealing with the occurrence of positive honey samples for sulphonamides (Bogdanov, 2006; Economou, Petraki, Tsipi, & Botitsi, 2012; Kaufmann & Kaenzig, 2004; Näumann, Mahrt, Himmelreich, Mohring, & Frerichs, 2012; Reybroeck, 2003; Reybroeck, Daeseleire, De Brabander, & Herman, 2012; Reybroeck et al., 2010; Tölgyesi et al., 2013; Venable, Haynes, & Cook, 2014).

Regarding the list the different sulphonamide residues which were found in honey (Table 10), the only disadvantage of AM I kit (as well as Sulfasensor®) is the lack of detection of sulphanilamide which is sometimes found in honey samples. The others cited sulphonamides (sulphathiazole, sulphamerazine, sulphamethazine, sulphamethaxazole, sulphadiazine, sulphamethoxypiridazine, sulphadoxine and sulphamethazine) are detected below the RC of 50 μg kg^{-1} by the AM I kit. A very recent review has been published very recently on the prevalence of antibiotic residues in honey worldwide from 2008 to 2013 (Venable et al., 2014). The most usual antibiotic families detected and quantified in honey all over the world are in the following order of importance: tetracyclines, sulphonamides (from 3 to 300 μg kg^{-1}), macrolides, quinolones and aminoglycosides. Sulphadimethoxine and sulphathiazol were the two sulphonamides which were most often indentified. Therefore, the scope of the AM I kit is really interesting because the

Table 10. Prevalence of honey samples containing sulphonamide residues.

Source of contamination or provenance of honey	Antibiotics	Reference
Degradation of pesticide asulam	Sulphanilamide	(Kaufmann & Kaenzig, 2004)
Treatment of American foolbrood (AFB) and Nosemosis in a prophylactic way	Sulphathiazole, sulphamethazine	(Reybroeck et al., 2012)
Product approved in Argentina to be used against foulbrood and nosemosis	Sulphadimethoxine, trimethoprim, and oxytetracycline	
Product to prevent nosemosis	Sulphamethazine	(Reybroeck et al., 2010)
Review	Sulphathiazole, sulphamerazine, sulphamethazine, sulphamethaxazole, sulphadiazine, sulphamethoxypiridazine, sulphadoxine, sulphamethazine, sulphanilamide	(Bogdanov 2006)
Honey samples from Belgium and imported honey samples (2003)	Sulphathiazole, sulphamethoxazole, and low concentrations of sulphamethazine and sulphadiazine	(Reybroeck, 2003)
Spiked and incurred honey samples	Sulphadimethoxine, sulphachloropyridazine, and trimethoprim	(Tölgyesi et al., 2013)
30 honey samples (Greek origin and imported)	Sulphamethoxazole, sulphathiazole and TMP	(Economou et al., 2012)
30 honey samples from Germany and 47 imported honeys	Sulphamethoxazole the most frequently found antibiotic (often combined with TMP)	(Näumann et al., 2012)
Honey worldwide (Australia, Canada, China, European Union, South America, India, New Zealand, South Asia, United States) (2008–2013)	Sulphadimethoxine, sulphathiazol, Sulphamethoxazole, sulphadimethoxine, sulphadiazine, sulphamethazine, sulphamonomethoxine	(Venable et al., 2014)

seven sulphonamides identified in these samples could be detected by this method. Moreover, giving the estimated CCβ of the AM I, most of the positive samples of interest should have been detected by this method. Finally, another kit from Randox (UK), the AM I, for the screening of six families of antibiotics in honey could be implemented in parallel with the AM I kit to complete the scope of antibiotics. In this way, the five usual antibiotic families would be covered.

Finally, since 2014, the French honey control plan is implemented using the AM I and AM II kits with the Evidence Investigator™ system as official methods, replacing ELISA kits, receptor test and LC-MS/MS method. The sample preparation for honey is the same for AM I and AM II kits which is saving time and money when both kits are applied on the same samples.

4. Conclusion

To our knowledge, this study represents the first validation of the AM I kit in honey according to the European guideline for the validation of screening methods for veterinary medicines (2010). The AM I kit applied on the Evidence Investigator™ system for the determination of 14 sulphonamides and TMP in honey was validated in terms of its detection capability, specificity, CRs and applicability, according to Commission Decision 2002/657/EC (Commission, 2002). The method was found to be rapid and able to screen simultaneously 14 sulphonamides and TMP in different kinds of honey with a very easy sample preparation procedure. The CCβ were well below the RC of 50 μg kg^{-1} for sulphonamides (even ≤ half RC). .The false-positive rate of 4% maximum is very satisfactory.

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