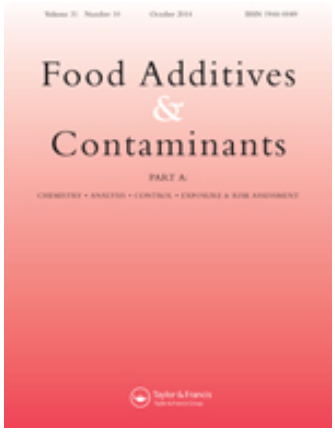

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Evaluation and validation of biochip multi-array technology for the screening of six families of antibiotics in honey according to the European guideline for the validation of screening methods for residues of veterinary medicines

Valérie Gaudin[a], Celine Hedou[a], Christophe Soumet[a] & Eric Verdon[a]
[a] ANSES, Laboratoire de Fougères, Fougères Cedex, France
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Valérie Gaudin*, Celine Hedou, Christophe Soumet and Eric Verdon

ANSES, Laboratoire de Fougères, Fougères Cedex, France

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The main chemicals used against varoa are acaricides, and the antibiotics used for the control of bee bacterial diseases are mainly tetracyclines, streptomycins, sulfonamides and chloramphenicol. No maximum residue limits (MRLs) have been set for any antibiotics in honey. Therefore, in the European Union, minimum recommended concentrations (RC) for the analytical performance of methods to control a certain set of these non-authorised chemicals in honey were published by the European Union Reference Laboratory (EU-RL) in 2007. Concerning the strategy for the control for antibiotic residues in honey, there is still a great need for a cheap and single multi-residue method. Biochip array technology is an innovative assay technology for the multi-analyte screening of biological samples in a rapid and easy-to-use format. A multi-array system, called Evidence Investigator™ (Randox, Crumlin, Co., Antrim, UK), was evaluated in our laboratory. It is a semi-automated biochip system designed for research, clinical applications and veterinary use. A competitive chemiluminescent immunoassay is employed for the detection of antimicrobials. The MicroArray II kit (AM II) dedicated to the screening of six different families of antibiotic residues was validated according to the European guideline for the validation of screening methods for residues of veterinary medicines. The specificity was proven to be very satisfactory, and applicability to different kinds of honey was demonstrated. The detection capabilities (CCβ) of six antibiotic residues were determined and were below the RCs when exist. The AM II kit could detect at least six quinolones, four tetracyclines and three epimers, three aminoglycosides, three macrolides, thiamphenicol, florfenicol and ceftiofur along with one of its stabilised metabolites, the desfuroylceftiofurcysteine disulfide (DCCD).

Keywords: validation; Decision No. 2002/657/EC; multi-array; biochip; screening; antibiotic, honey

Introduction

Antimicrobial compounds are used in food production to treat or prevent animal diseases. Antibiotics potentially present in honey, or any other food, are inappropriate, even presenting risks to human health. There is a risk of allergies, even at very low doses in some subjects; there is also a risk of causing the selection of resistant strains among human pathogens. Because of these concerns, many countries have banned or restricted the use of antimicrobial compounds in food-producing animals and have established MRLs for antimicrobial residues in food.

The major bee diseases for which antibiotics are indicated are American and European foulbrood infection, both due to bacteria, and Nosema disease, the latter caused by a protozoan. The antibiotics are mixed with the food of bees to fight against diseases such as foulbrood infection. Bee products can be contaminated from different sources (Bogdanov 2006). The main contaminants originating from beekeeping are acaricides and antibiotics used for the control of bee brood diseases, mainly tetracyclines, streptomycin, sulfonamides and chloramphenicol. On the Italian market, for example, among the most analysed substances used, sulfonamides are the first, followed by tetracycline, streptomycin, tylosin and chloramphenicol (Baggio et al. 2009). No market authorisation can be issued unless an MRL has been established previously. But given the high cost of necessary scientific studies to establish the MRL files, only the most important species called "major" were studied. Thus, for bees, minor species by excellence, no antibiotic could be subjected to an authorisation. European Union rules on setting MRLs for pharmacologically active substances have been updated by Regulation (EC) No. 470/2009 (European Commission 2009). For the first time, the regulation introduced a mechanism for the extrapolation of MRLs from one species/food matrix to another.

In the European Union, no veterinary medicinal product containing antibiotics is permitted in beekeeping. In fact, no antibiotic ever has an MRL in honey. For now, each member states adopts a different position on this issue. In the European Union, a technical guide was published by the European Union Reference Laboratories (EURLs) (CRL Guidance Paper of 7th December 2007). The purpose of this technical guide is to improve and harmonise the performance of analytical methods for substances for which MRLs have not been set. These

*Corresponding author. Email: valerie.gaudin@anses.fr

substances without MRLs include those partially or totally banned from use in food-producing animals. It should be noted that this document should serve as a technical guideline (recommended levels) for methods of residue analysis. The levels recommended in this document, however, have no real legal basis. The recommended concentrations (RCs) are 40 μg kg^{-1} for streptomycin, 20 μg kg^{-1} for macrolides (tylosin and erythromycin), 50 μg kg^{-1} for sulphonamides, and 20 μg kg^{-1} for tetracyclines. Therefore, a screening method for antibiotic residues in honey should detect antibiotic residues at least at these levels and even lower. The evaluation and validation hereafter will determine if the AM II kit can detect the concerned substances that are in the scope of the kit at or lower than the RCs.

There are very few methods to detect antibiotic residues in honey. In general, there are specific tests of an antibiotic or a family of antibiotics (generic test). These are ELISA kits (tylosin, streptomycin/DHS, sulfonamides) or receptor tests (strips Tetrasensor® or Sulfasensor® (Unisensor, Liège, Belgium)). The Charm II kit for honey, a test based on microbial receptor and radiolabelling developed by Charm Sciences Co. (Lawrence, MA, USA), allows a relatively quick analysis of streptomycin and dihydrostreptomycin (DHS) (Salter 2003). There are also microbiological tests (ampoule or microplate) based on the inhibition of bacterial growth of *Bacillus stearothermophilus* (i.e. Premi®Test (r-Biopharm, Darmstadt, Germany), Eclipse® 50 (Zeu Inmunotech, Zaragoza, Spain)) that have a wide spectrum as compared with immunological methods. However, microbiological tests generally have a good sensitivity to beta-lactams, but poorer sensitivity to tetracyclines, macrolides, sulfonamides and especially poor detection of aminoglycosides and quinolones. Therefore, there is a great need for broad spectrum analytical methods and good sensitivity dedicated to the screening of antibiotic residues in honey samples. A biosensor method based on surface plasmon resonance (SPR) named "Biacore™" (GE Healthcare Europe GmbH, Velizy-Villacoublay, France) was developed and validated for the detection of streptomycin and DHS in honey (Ferguson et al. 2002). Simple dilution was applied to honey samples. The LOD in honey was estimated at 15 μg kg^{-1}. Similarly, a biosensor method was developed and validated for the screening of tylosin in honey (Caldow et al. 2005). The detection capability was estimated at 2.5 μg kg^{-1}. Moreover, HPLC methods have been developed and validated for the screening of antibiotics in honey (Kochansky 2006). Confirmatory MS-based methods have also been developed and validated (Kaufmann et al. 2003; Granja et al. 2009), even multiresidue methods (Vidal et al. 2009).

A multiplex approach should be suitable to detect simultaneously several families of antibiotics. The Evidence Investigator™ Biochip Array Technology (Randox, Crumlin, Co., Antrim, UK) is used to perform simultaneous quantitative detection of multiple analytes from a single sample (Fitzgerald et al. 2005; Molloy et al. 2005; McAleer et al. 2006). 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. The Evidence™ Investigator system is a biochip, semi-automated, designed for research, clinical, forensic and veterinary applications. Until now, to our knowledge, only one article has been published in the field of antibiotic residues concerning the detection of four nitrofuran metabolites in honey with an Evidence™ system (O'Mahony et al. 2011).

The MicroArray II (AM II) kit can detect six families of antibiotics: tetracyclines, quinolones, some macrolides, some aminoglycosides, thiamphenicol and florfenicol, and ceftiofur and its metabolite.

The objective was to evaluate the performance of an innovative system called Evidence™ Investigator for the detection of antibiotic residues in honey. At first an evaluation of the performance of the microarray kit II (AM II) in honey was realised to check the detection limits provided by the supplier. This kit was then validated according to European Decision No. CE/2002/657 (European Commission 2002) and according to the European guideline for the validation of screening methods for the residues of veterinary medicines (CRL 2010). This guideline was written by the EURLs involved in the screening and confirmation of veterinary medicines in food of animal origin and by DGSANCO (European Union Directorate General for Health and Consumers). The objective was to help national reference laboratories to perform the validation of screening methods for veterinary medicines according to Decision No. EC/2002/657, which was lacking detailed information concerning the screening methods. It was published in January 2010 on the DGSANCO website (http://ec.europa.eu/food/food/chemicalsafety/residues/lab_analysis_en.htm).

Materials and methods

Chemicals and standard solutions

Chlortetracycline (CTC), oxytetracycline (OTC), tetracycline (TTC), tylosin, tilmicosine, spiramycin, sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethizole, sulfanilamide, sulfathiazole, streptomycin, DHS, gentamicine, paromomycin, spectinomycin, sarafloxacine, ciprofloxacine, enrofloxacine, oxolinic acid, danofloxacine, ceftiofur, cefalonium, cephapirin, cefacetril, cefazoline, florfenicol and thiamphenicol were all from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Marbofloxacine, doxycycline, erythromycin, cefquinome and bacitracine were from Cluzeau Info Labo (Sainte-Foy la Grande, France).

Norfloxacine was supplied by Dr. Ehrenstorfer (Augsburg, Germany). Neospiramycin was supplied by Wako (Neuss, Germany). Gamycthromycin was supplied by Merial (Toulouse, France). Epimers of CTC, TTC and OTC were supplied by ACROS Organics (Geel, Belgium). Desmycosin was prepared according to a published protocol (Adams et al. 2007). Tylosin A in acidic solution is hydrolysed overnight at 50°C to produce desmycosin.

Matrices

Samples of honey were selected to obtain a representative group of varying degrees of colour and general physical appearance (liquid, creamed and crystalline). Sixty different honeypots were used which were from 14 different floral origins: rosemary, heather, forest, acacia, chestnut, rhododendron, rape, orange tree, sunflower, scrubland, lime tree, garrigue, mountain and multifloral. These honeys have different geographical origins from France, Italy and Sicily, and Spain.

Preparation of standard solutions

Stock standard solutions of each antibiotic were prepared at a concentration of 1 mg ml^{-1} in methanol. An intermediate standard solution containing 10 µg ml^{-1} in water was prepared from the individual stock standards. This intermediate standard solution was further diluted to prepare working solutions of each compound. Spiked samples were prepared at different concentrations by fortifying 1.0 ± 0.1 g of blank honey with 100 µl of the working solution.

Evidence Investigator™ system

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 RLU – relative light unit). This term is used because luminometers typically do not yield a measurement directly in units of photons. This is because some luminometers measure the current output by the photomultiplier tube, which cannot be translated into photons. Additionally, a calibration factor is typically used to normalise the response of the instrument to a calibrated light standard. The light signal generated from each of the test regions on the biochip is detected using state-of-the-art digital imaging technology and compared with that from a stored calibration curve. The concentration of analyte present in the sample is then calculated from the calibration curve.

MicroArray II kit (AM II)

The Evidence Investigator™ Anti Microbial Array II is a commercially available kit that will quantitatively test for quinolones, ceftiofur, thiamphenicol, streptomycin, tylosin and tetracyclines, simultaneously. Each kit includes six carriers, nine points of calibration, buffers required to reconstitute and other reagents (conjugate, chemiluminescent solution). Each carrier is composed of nine microarrays (1 ×1 cm). So with a kit, one calibration carrier and five carriers of samples (45 samples) could be analysed. A carrier can be divided into three × three biochips, so a number of samples multiplied by three will be analysed.

Honey procedure for sample preparation

A total of 1 g of honey sample is weighted out. Then 19 ml of diluted wash buffer warmed to 37°C are added. The tubes are placed on a roller for 10 min or until dissolved. The sample is now ready for application to the biochip.

Evaluation of the kit

The objectives of the evaluation are, firstly, to check the detection limits announced by the manufacturer and, secondly, to select the target concentrations that will be tested later on during the validation to determine the detection capabilities (CCβ) of the antibiotics of interest (see the validation protocol). During our evaluation it was not required to analyse a calibration curve for each test day. 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 the data analyses were focused on the signal in RLU not in the calculated concentration in ppb.

Different batches of blank honey without antibiotic residues were analysed. Most honey samples were previously analysed by immunoassay (ELISA test and receptor test) and were detected to be negative. However, when the presence of antibiotics was suspected following the analyses with Evidence ™ system, a confirmatory analysis was implemented by LC-MS/MS.

Because of the specificity of each spot for a family of antibiotics (Multiplex system), it is possible to spike samples with antibiotic mixtures, providing a mix of up to six antibiotics (six different families).

The evaluation of sensitivities was focused on six antibiotics that showed 100% cross-reaction with the antibody and on six antibiotics that showed a minimum percentage of cross-reaction and six antibiotics with the lowest cross-reactions. The tested antibiotics and their

Table 1. Antibiotics and related concentrations to be tested at the evaluation step.

First series	Norfloxacine	Ceftiofur	Florfenicol	DHS	Tylosin	OTC
Claimed LOD (μg kg^{-1})	5	3	1	4.5	2	18
Concentration 1 (μg kg^{-1})	5	3	1	5	2	15
Concentration 2 (μg kg^{-1})	10	6	2	10	4	20
Second series	Sarafloxacine	DCCD	Thiam	Strepto	Tilmico	Doxy
Claimed LOD (μg kg^{-1})	83	–	2	8	5.5	39
Concentration 1 (μg kg^{-1})	100	100	2	10	5	n.d.
Concentration 2 (μg kg^{-1})	150	1000	4	20	10	

Note: DHS, dihydrostreptomycin; OTC, oxytetracycline; DCCD, desfuroylceftiofur cysteine disulfide; Thiam, thiamphenicol; Strepto, streptomycin; Tilmico, tilmicosin; Doxy, doxycycline; LOD, limit of detection.

related concentrations are presented in Table 1. Ideally, the chosen concentrations should be lower than the RC (CRL Guidance Paper of 7th December 2007) when it exist. The choice of concentrations was performed according to the supplier data in the kit booklet (announced detection limits) and according to the RCs when they exist.

During 2 days, four blank samples (four batches of honey), the same four samples spiked at concentration 1 with the first series of antibiotics and the same four samples spiked at concentration 2 with the same antibiotics were analysed. The same protocol was then repeated with the second series of antibiotics. In parallel, each antibiotic was tested individually to assess whether or not there is interference between spots.

Regarding cephalosporins, the kit is deemed to detect only ceftiofur. No tests have been done to date on the major metabolite of interest: desfuroyl ceftiofur. Desfuroyl ceftiofur is very unstable; it is impossible to obtain it. By consequence, DCCD (desfuroyl ceftiofur cysteine disulfide), which is a cysteine conjugated metabolite of ceftiofur, will be tested. Two concentrations will be tested initially and additional testing may be required depending on the initial results.

The average value of the signal (in RLU) of the four blank samples is then calculated, as well as the SD of the signal of the four blank samples. Similarly, the average signal and SD of the four spiked samples at concentrations 1 and 2 is calculated for each tested antibiotic. Finally, the positivity threshold T is calculated as follows from the blank samples:

$$T = \text{average RLU signal of blank} - 1.64 * \text{SD RLU signal of blank}$$

Moreover, the cut-off value Fm is calculated as follows from the spiked samples:

$$\text{Fm} = \text{average RLU signal of spiked} + 1.64 * \text{SD RLU signal spiked}$$

At the end of the evaluation step, the target concentration is selected for the determination of detection capabilities (CCβ) during the validation (see the validation protocol). The chosen concentration (concentration 1 or 2) is valid if and only if the cut-off value Fm is below the positivity threshold T. Otherwise, it is necessary to increase the concentration of antibiotics in the validation step.

In addition, if blank honey is detected to be positive during the two series, this sample will be confirmed by LC-MS/MS.

Validation protocol

After the evaluation step, one kit for each family was selected and validated according to the European guideline for the validation of screening methods for the residues of veterinary medicines (CRL 2010), which is based on the criteria of European Decision No. 2002/657/EC (European Commission 2002). The performance characteristics to be determined were the same: practicability, applicability (honeys from different sources), specificity, detection capabilities (CCβ), ruggedness and stability. The analyses were blindly performed by a technician (random numbers were given to the samples).

Practicability

The aim of the practicability study was to check whether or not the methodology is appropriate 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 laboratory), reagents (ready-to-use or not), instruments (specific or usual instruments in a laboratory) and environmental conditions large or narrow temperature intervals to implement the kit are noticed.

Specificity, false-positive rate

Sixty different lots of blank honey (antibiotic free) from organic apiculture (trade) or control plans should be

analysed (different floral origins, different colours, different textures, different levels of crystallisation) (cf. Applicability). As in the evaluation step, if the presence of antibiotic residues following the analyses with the Evidence™ system was suspected, a confirmatory analysis by LC-MS/MS was asked for.

Detection capability (CCβ)

Detection capabilities were determined for six antibiotics (the least sensitive antibiotics in each detected family) (cf. Table 3).

In the European guideline for the validation of screening method for the residues of veterinary medicines (CRL 2010), it is recommended that the number of samples needed for each substance for the validation (samples spiked at a target concentration or naturally incurred) depends on the degree of statistical confidence required in the result, and the relationship between target concentration and detection limit prescribed. If the target concentration is set at half the RC (½ 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 ½ MRL. If the target concentration is between 50% and 90% of the prescribed limit, at least 40 positive control samples (with two or less than two false-compliant results) will be sufficient to demonstrate that the CCβ is below the RC. If the screening test sensitivity is such that the target concentration is close to the RC (10% below the RC), a higher number of samples have to be analysed. A maximum of 60 repetitions (with three or less than three false-compliant results) is required to demonstrate that the detection capability is less than or equal to the MRL. These studies can be undertaken by sequential steps, namely that the first 20 fortified samples are tested, and if more than one sample is declared negative, validation may be abandoned at this point or the target concentration should be increased and the validation exercise repeated.

The target concentration was determined during the preliminary tests of evaluation. The sensitivities of each antibiotic with different ranges of concentration were assessed. The chosen concentration for validation is then the concentration that gave 95–100% of positive results during the evaluation step. In the case of honey, there are no MRLs for antibiotic residues, but the recommendations of the EURL AFSSA-Fougeres LERMVD (CRL Guidance Paper of 7th December 2007). Therefore, the number of samples to be analysed will be determined according to the recommended limits instead of the MRL. A maximum of 60 samples (blank and spiked) will be analysed to determine CCβ and specificity.

A statistical approach that takes into account the β error of 5% was then chosen as it is recommended in European Decision No. EC/2002/657 (European Commission 2002). The positivity threshold T and the cut-off value Fm were calculated as recommended in the guideline. Positivity threshold T and cut-off factor Fm are matrix-specific:

$$T = B - 1.64 \times \mathrm{SD_B}$$

$$\mathrm{Fm} = M + 1.64 \times \mathrm{SD}$$

where B is the mean and $\mathrm{SD_B}$ is the standard deviation of the signal (RLU) of the blank samples; and M is the mean and SD is the standard deviation of the signal (RLU) of the spiked samples.

The first basic principle is that the assay is considered valid only if $T > \mathrm{Fm}$. If $\mathrm{Fm} > T$, it means that the spiked concentration is too low because the kit was 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 $T > \mathrm{Fm}$, the kit can discriminate between blank and spiked samples.

As a second basic principle, T or Fm 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 a 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 a positive sample. The number of spiked samples with a mean signal (RLU) below the cut-off level will be identified (a positive result). If more than 5% of the spiked samples at the screening target concentration gave a signal (RLU) higher than the cut-off level (a false-negative result), this concentration chosen for the spiking study should be 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 fewer than 5% of false-negative results (i.e. three or fewer false-negative results out of 60 spiked samples) are obtained at this concentration after the analysis of 60 spiked samples. The CCβ should be lower than or equal to the regulatory/action limit.

Applicability

Samples of honey were selected to obtain a representative group of varying degrees of colour and general physical appearance (liquid, creamed and crystalline). Sixty different honeypots were used: rosemary, heather, forest, acacia, chestnut, rhododendron, forest, rape, orange tree, sunflower, scrubland, lime tree, garrigue, mountain and multifloral. These honeys have different geographical origins from France, Italy and Sicily, and Spain. The CCβ of 12 antibiotics were determined from at least 60 different samples.

Cross-reactions

Cross-reactivity (CR) for molecules of the same family are determined from a reduced number of samples. Six mixtures of six antibiotics from the six detected families were prepared (cf. Table 5). Each mixture is prepared at three concentrations and each concentration is analysed three times. Regarding cephalosporins, the kit is deemed to detect only ceftiofur. During the evaluation phase, tests are performed on the conjugate metabolite DCCD. Depending on the results, the CCβ of ceftiofur or its metabolite is determined. That for which the CCβ has not been determined is tested for cross-reactions. Among the antibiotics tested, some families such as sulfonamides should not be detected by this kit, which will be checked. Six sulfonamides were chosen to be tested at concentrations of 100, 1000 and 10 000 μg kg^{-1}: sulfamethazine, sulfadimethoxine, sulfadiazine, sulfathiazol, sulfanilamide and sulfamethizole.

Stability

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

Results and discussion

Evaluation

The evaluation of sensitivities focused on six antibiotics that are 100% cross-reaction and six antibiotics that have a minimum percentage of cross-reaction. Before several antibiotics were individually tested, the same antibiotic mixture was tested to see if there was interference between channels.

Variability of quantitative results was observed (in terms of concentration) in samples spiked with several antibiotics (SD = 5–35%). The spiked samples with a single antibiotic were not repeated. When comparing the quantitative results (concentration) of samples spiked with a single antibiotic (individual) and the results for the same antibiotic mixed with other antibiotics (that are "known" detected in other spots than the spot of this antibiotic), there is no significative difference in the calculated concentrations. So it seems that mixing several antibiotics had no impact on the quantitative results. The molecule for which the greatest variability was observed was norfloxacin (35%). This is the molecule for which the greatest difference between the concentration of the mixture and the concentration of the antibiotic alone was observed.

As a conclusion from this evaluation there was no interference between the different antibiotics detected (different spots). Therefore, several antibiotics could be mixed in the same sample to determine CCβ and thus the number of samples to be analysed and the number of kits is reduced.

The chosen concentration is valid if and only if Fm < *T*. Otherwise, it is necessary to increase the concentration of antibiotics in the validation step. The choice of concentrations for validation is presented in Table 2.

Oxytetracycline was detected at 10 (at this concentration, *T* was already higher than Fm, but quantitative values were very close) and 15 μg kg^{-1}; DHS was not detected at either 5 or 10 μg kg^{-1}; ceftiofur was not detected at either 3 or 6 μg kg^{-1}; tylosin was detected at 4 μg kg^{-1}; florfenicol was not detected at either 1 or 2 μg kg^{-1}; and norfloxacin was detected at 10 μg kg^{-1}. Therefore, the chosen target concentrations for ceftiofur, florfenicol and dihydrostreptomycin were too low. The detection capability CCβ is higher than 6 μg kg^{-1} for ceftiofur (the highest tested concentration for ceftiofur in the evaluation part), higher than 2 μg kg^{-1} for florfenicol, and higher than 10 μg kg^{-1} for dihydrostreptomycin because, at these concentrations, *T* < Fm. These three antibiotics were not included in the determination of detection capabilities. Therefore it is only an estimation of the performance of the kit at this level of the evaluation study. The concentrations of sarafloxacin have to be increased compared with the announced LOD (83 μg kg^{-1}) because it was not detected at 100 μg kg^{-1}. On the contrary for DCCD, concentrations were decreased. Sarafloxacin was detected at 150 μg kg^{-1}; DCCD was detected at 10 μg kg^{-1}; thiamphenicol was detected at 6 μg kg^{-1}; streptomycin was detected at 30 μg kg^{-1}; tilmicosin was detected at 10 μg kg^{-1} (close); erythromycin was not detected at 20 μg kg^{-1}; and doxycyclin was detected at 40 and 60 μg kg^{-1}.

Table 2. Concentrations chosen for the validation.

	Norfloxacin	Ceftiofur	Florfenicol	DHS	Tylosin	OTC
Claimed LOD (ppb)	5	3	1	4.5	2	18
Concentration for validation (ppb)	10	> 6	> 2	> 10	4	15
	Sarafloxacin	DCCD	Thiam	Strepto	Tilmico	Doxy
Claimed LOD (ppb)	83	–	2	8	5.5	39
Concentration for validation (ppb)	150	10	6	30	10	30

Note: DHS, dihydrostreptomycine; OTC, oxytetracycline; Thiam, thiamphenicol; Strepto, streptomycin; Tilmico, tilmicosin; Doxy, doxycycline; LOD, limit of detection.

During the evaluation step, one honey sample was detected positive for tylosin and/or streptomycin (for streptomycin there were inconsistent results). It was confirmed by implementing ELISA for streptomycin and for tylosin. It was found negative by ELISA for streptomycin (< 20 µg kg^{-1} equivalent streptomycin). However, it was detected positive by ELISA for tylosin (i.e. > 20 µg kg^{-1} equivalent tylosin). This sample could not be confirmed by LC/MS-MS.

Validation part

Practicability

The amount of reagents provided is comfortable. The implementation is easy with a single dilution of the sample of honey by 1/20th in a 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.

During validation, it was decided always to work with fresh conjugate, not with frozen conjugate because the background noise was very high with frozen conjugate.

Specificity and false-positive rate

The specificity of the ELISA kit was determined after the analysis of 60 blank samples of honey from different origins. After 3 days of validation, day 3 was a problem because a lot of background noise was observed with the blank samples for the screening of streptomycin. Three of these samples were analysed by ELISA for streptomycin to detect any presence of streptomycin or DHS. The result was negative for streptomycin/DHS, while the CCβ of streptomycin in ELISA is 20 µg kg^{-1} (10 µg kg^{-1} for the DHS). In addition, the background noise increased significantly for all antibiotics. Therefore the results were not consistent. All the results were not valid when cumulating the results of the 3 days of validation (Fm > *T*). It was suspected that the frozen conjugate could cause this problem. Therefore, on day 4 fresh and frozen conjugates were compared. After some further testing, the conclusion was that the reconstituted frozen conjugate was the cause of a lot of background noise (although the preparation was made only 11 days before analysis) and not the freshly reconstituted conjugate. So from day 4 a newly and fresh reconstituted conjugate was always used. A warning was added to the practicability evaluation. Following the analysis of these 60 blank samples on six different days, the value of *T* (positivity threshold) was determined for each family of antibiotics (cf. Table 3).

Taking *T* as the cut-off value, the test will be more sensitive (because of fewer false-negative results), but the increase of the false rate will lead to expensive confirmatory analyses for which the final result would be a compliant sample. The choice of Fm as the cut-off level is a compromise between detection capabilities, low enough to reach the respective RC and a reasonable false-positive rate. Therefore, Fm was chosen as the cut-off value to conclude on the positivity of a sample.

Four false-positive results were obtained for quinolones (6.7%), one false-positive (1.7%) for tetracyclines, one false-positive (1.7%) for aminoglycosides and no false-negative positive for the three other families antibiotics. Three of the four positive samples for quinolones (two chestnut honeys and one multifloral honey) (concentrations were estimated by the Evidence™ system at about 30 µg kg^{-1}, while background noise was about 1 µg kg^{-1}) were confirmed by a multi-residue method (LC-MS/MS) for quinolones. The honeys were confirmed to be compliant for quinolones. However, there is the presence of a peak (Tr = 4.20 min) on the major transition of flumequine and/or oxolinic acid with a molar mass of the parent ion at 262.2 g mol^{-1} and a molar mass of son ion at 244 g mol^{-1}. The retention times of flumequine and oxolinic acid are 9.42 and 7.59 min respectively. It would be interesting to ask for a non-targeted analysis on the Orbitrap to identify the interfering molecule. It is suspected that some honeys (chestnut and multifloral) contain a natural substance that interferes with the detection of quinolones. This is probably a substance that has a structure close to quinolones. This hypothesis should be confirmed in a subsequent project.

The presence of tetracycline was suspected in one sample (multifloral honey) (32 µg kg^{-1} of equivalent tetracycline). It was detected negative with the Tetrasensor® (Unisensor) (Gaudin, Rault et al. 2013). Then it was

Table 3. Number of false-positive and false-negative results depending on the choice of the threshold value *T* or cut-off factor Fm as cut-off level.

	Sara	DCCD	Thiam	Streptomycin	Tilmicosin	Doxycycline
T (*n* = 60)	1210	3284	6283	5438	18 753	6822
Cut-off = T (*n* = 60)	6 false-positive 0 false-negative	1 false-positive 0 false-negative	3 false-positive 0 false-negative	2 false-positive 0 false-negative	3 false-positive 0 false-negative	3 false-positive 1 false-negative
Fm (*n* = 60)	1060	1817	4829	4445	11 315	5427
Cut-off = Fm (*n* = 60)	4 false-positive 0 false-negative	0 false-positive 2 false-negative	0 false-positive 1 false-negative	1 false-positive 1 false-negative	0 false-positive 3 false-negative	1 false-positive 2 false-negative

Note: Sara, sarafloxacine; DCCD, desfuroylceftiofur cysteine disulfide; Thiam, thiamphenicol.

confirmed by LC-MS/MS and the sample was found to be compliant for tetracyclines. The presence of tetracycline was also suspected in sample 11F003024 (sunflower honey) (> 70 µg kg^{-1} of equivalent tetracycline). It was analysed with Tetrasensor® and was also found to be positive with this receptor test. It was then confirmed by LC-MS/MS and it was declared non-compliant for tetracyclines (quantification 488 µg kg^{-1} tetracycline and 390.4 µg kg^{-1} epitetracycline).

Detection capabilities (CCβ)

Twelve antibiotics were evaluated (two series) (Table 2). However, the detection capabilities CCβ were determined for six antibiotics only – the least sensitive antibiotics (but still detected) in each family – with 60 different batches of honey (cf. Table 4). The distribution of the results in RLU for the 60 negative samples and the 60 samples spiked with the six validated antibiotics are presented in Figure 1.

The detection capabilities CCβ were determined for six antibiotics – the least sensitive antibiotics (but still detected) in each family – with 60 different batches of honey. This figure represents the distribution of the signal in RLU after the analyses of 60 blank samples of honey from different origins and the analyses of the same 60 samples fortified at different concentrations of antibiotics. Figure 1a presents the results of sarafloxacin at 150 µg kg^{-1}; Figure 1b DCCD at 10 µg kg^{-1}; Figure 1c thiamphenicol at 6 µg kg^{-1}; Figure 1d streptomycin at 30 µg kg^{-1}; Figure 1e tilmicosin at 10 µg kg^{-1}; and Figure 1f doxycycline at 30 µg kg^{-1}.

The following Table 4 shows the detection capabilities CCβ for the six validated antibiotics. The letter C means that the result is compliant, i.e. Fm is much lower than *T* and thus the validated concentration is equal to CCβ. In fact, no more than 5% of false-negative results (three out of 60 spiked samples) were obtained for the six tested antibiotics, which are among the least detected antibiotics in each family.

Globally the detection capabilities were a little higher than those announced by Randox, except for doxycycline. For substances having a RC (streptomycin, tilmicosin and doxycycline), the detection capabilities were lower than the RC. The results were very satisfactory because the validated antibiotics were the least detected antibiotics in the family. It means that the other detected antibiotics in the family (dihydrostreptomycin, tylosin, tetracycline, OTC, CTC and the three epimers) are much better detected and by consequence lower than the RC.

Cross-reactions

Cross reactions were analysed in terms of calculated concentration and not in the RLU signal because we wanted to determine the quantitative relationship between the antibiotic at 100% of CR and the antibiotic that potentially cross-reacts. The results of CRs in each family are summarised in Table 5.

The calculated cross-reactivities (CR%) were very close to those reported by Randox. The following antibiotics were not detected: cephapirin, cephalonium, florfenicol-amine, CAP, neomycin, bacitracin, paromomycin, spectinomycin, erythromycin, spiramycin, neospiramycin and gamythromycin. On the contrary, cefquinome (12% CR), gentamicin (14.6% CR) and desmycosin (24.4% CR) were detected. The determination of CR with this kit with desmycosin was of great interest because it was demonstrated that honey destined for human consumption should be analysed for both tylosin A and desmycosin (tylosin B) rather than the parent antibiotic alone (Thompson et al. 2007). None of the sulfonamides was detected by the AM II kit. There is no interference with the detection of six other families of antibiotics.

Applicability

An influence of honey origins was hypothesised because honey is a very complex matrix and with antioxidant

Table 4. Summary of the results.

	Sara	DCCD	Thiam	Strepto	Tilmico	Doxy
Concentration (µg kg^{-1})	150	10	6	30	10	30
T	1210	3284	6283	5438	18 753	6822
Fm	1060	1817	4829	4445	11 315	5427
Fm < *T*	C	C	C	C	C	C
When cut-off = Fm	0 false-negative	2 false-negative	2 false-negative	1 false-negative	3 false-negative	2 false-negative
Recommended concentration (ppb)	–	–	–	40	20	20
Claimed LOD (µg kg^{-1})	83	–	2	8	5.5	39
CCβ (µg kg^{-1})	150	10	6	30	10	30

Note: Sara, sarafloxacin; DCCD, desfuroylceftiofur cysteine disulfide; Thiam, thiamphenicol; Strepto, streptomycin; Tilmico, tilmicosin; Doxy, doxycycline; LOD, limit of detection; C, conform.

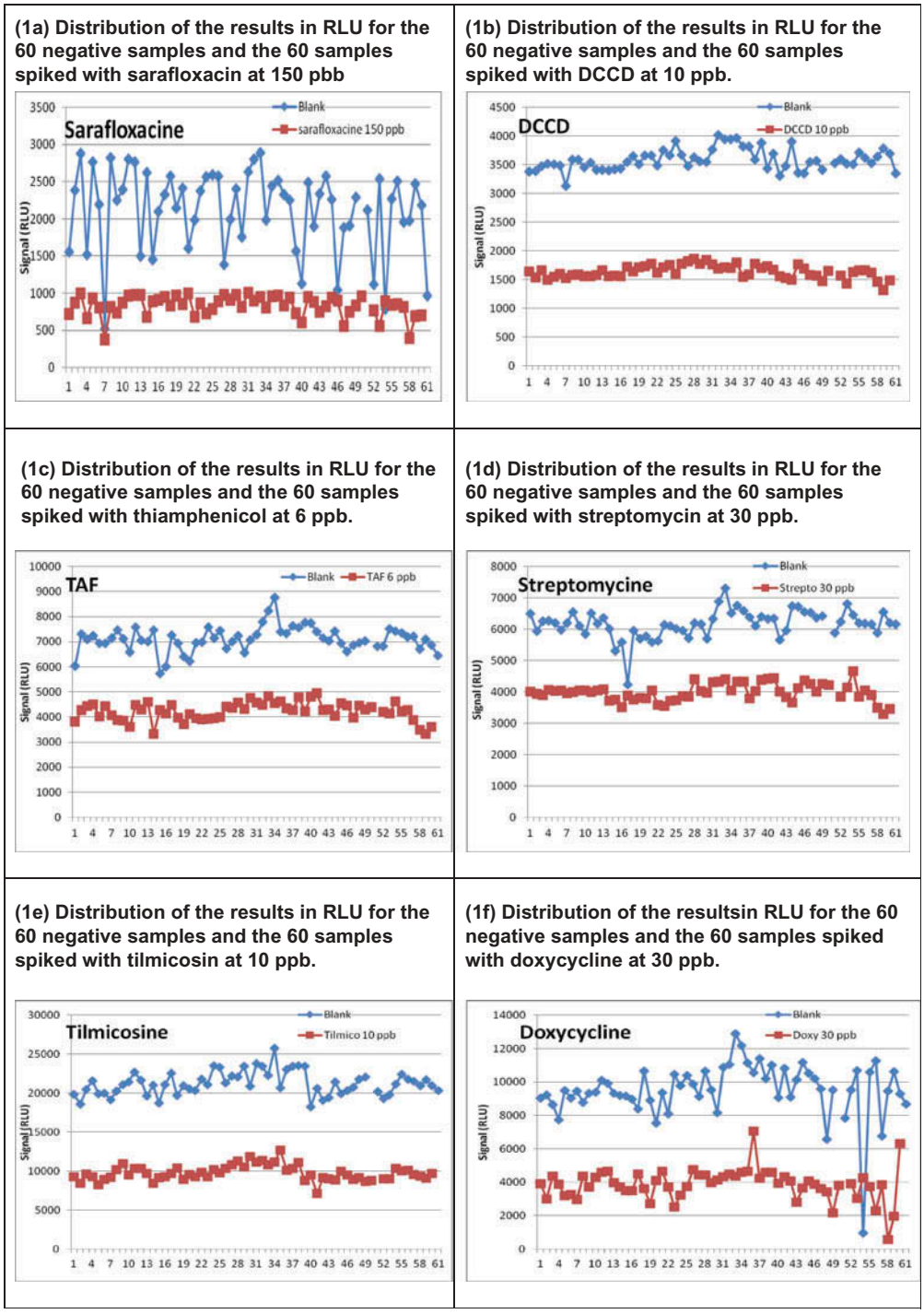

Figure 1. (colour online) Distribution of the results in RLU for the 60 negative samples and the 60 spiked samples.

properties. The method has proved to be applicable to different floral honeys with different geographical origins. One false-positive (1.7%) was obtained for tetracyclines, one false-positive (1.7%) for aminoglycosides and no false-positive for the three other families of antibiotics, which is very satisfying. However, a restriction must be made for the detection of quinolones. In fact, four false-positive results were obtained for quinolones (6.7%). It is suspected that some honeys (chestnut and multifloral) contain a natural substance that interferes with the detection of quinolones. This is probably a substance that has a structure close to quinolones. This hypothesis should be

Table 5. Results of cross-reactions in the six different mixtures of six antibiotics each.

Mixture 1	Norfloxacine	Ceftiofur	Florfenicol	DHS	Erythromycin	OTC
Mean calculation CR%	139.3	129	98.8	115.1	0	73.5
Claimed CR (%)	100	100	100	182	n.d.	52
Mixture 2	Ciprofloxacine	Cephapirine	Florfenicolamine	Neomycin	Tylosin	TTC
Mean calculation CR%	36.4	0.1	0	0	56.3	97.9
Claimed CR (%)	59	n.d.	n.d.	n.d.	< 1	100
Mixture 3	Danofloxacine	Cephalonium	CAP	Gentamycin	Desmycosin	CTC
Mean calculation CR%	29.6	0.3	0	14.6	24.4	59.3
Claimed CR (%)	20	n.d.	n.d.	n.d.	n.d.	51
Mixture 4	Enrofloxacine	Cefquinome		Spectinomycin	Spiramycin	Epimer TTC
Mean calculation CR%	67.6	12.0		0	0	112.7
Claimed CR (%)	76	n.d.		n.d.	n.d.	87
Mixture 5	Marbofloxacine	Cefacetril		Bacitracin	Neospiramycin	Epimer OTC
Mean calculation CR%	17.8	0.1		0	0	69.6
Claimed CR (%)	16	n.d.		n.d.	n.d.	52
Mixture 6	Oxolinic acid	Cefazolin		Paromomycin	Gamythromycin	Epimer CTC
Mean calculation CR%	9.7	0.1		0	0	30.6
Claimed CR (%)	12	n.d.		n.d.	n.d.	20

Note: Mean calculation CR (%) = mean calculated cross-reaction (%); announced CR (%) = announced cross-reaction by the manufacturer of the kit; n.d., not detected.

confirmed in a subsequent project using non-targeted LC-MS/MS methods. In addition, CCβ was determined from 60 different samples and so this value takes into account the variability of honey.

Quality controls in routine analyses

During validation there was considerable variability in the signals and background noise so there is a need to analyse two to three negative quality controls (QC–) and two to three positive quality controls (QC+) in parallel with unknown samples to conclude on their compliance, or not. The antibiotic concentrations to produce QC+ will the detection capabilities of the six validated antibiotics (the least sensitive in their family). The *T* value is determined from the mean signal in RLU and SD of QC– and Fm value from the mean signal in RLU and SD of QC+. On each day of routine analyses, the Fm value should be lower than *T* to declare that the essay is valid. Furthermore, every unknown sample with a signal lower than Fm will be declared as positive, and conversely.

Stability of antibiotic residues

The stability data of tylosin, streptomycin and DHS in solution and in honey were already presented in a validation study of two ELISA kits for the screening of tylosin, streptomycin and DHS in honey (Gaudin, Hedou et al. 2013). Moreover, data about the stability of tetracyclines in solution and in honey were collected during the validation study of a receptor test for tetracyclines in honey (Gaudin, Rault et al. 2013).

The stability of frozen stock solutions of beta-lactams and quinolones was studied with a diffusion test based on *Bacillus subtilis* and data were presented in a validation study of two microbiological tests in honey for the screening of antibiotic residues (Gaudin, De Courville et al. 2013). No data are available on the stability of quinolones and beta-lactams in honey. The stability of quinolones in muscle was presented, even if it cannot be extrapolated completely with honey (Bailac et al. 2006). The quinolones (ciprofloxacine, enrofloxacine, danofloxacine, sarafloxacine, difloxacine, flumequine and acid oxolinic) are stable for at least 3 months in chicken muscle at –20°C.

No data are available on the stability of thiamphenicol (TAP) and florfenicol (FFC) in honey. The stabilities of TAP and FFC in solution were assessed after 1, 2, 4 and 8 weeks of storage in a freezer at –18°C (Evaggelopoulou & Samanidou 2013). Spiked fish samples at 50 µg kg^{-1} for TAP and at 1000 µg kg^{-1} for FFC stored at –18°C in the absence of light were analysed and stability was demonstrated for 2 weeks for all analytes. The stability of the two amphenicols in fish samples was investigated after four freeze–thaw cycles (–18°C to RT). Degradation was decided using the –10% criterion. TAP and FFC were stable for three cycles.

Discussion

A review was published recently on the prevalence of antibiotic residues in honey worldwide (Australia, Canada, China, the European Union, South America, India, New Zealand, South Asia and the United States) from 2008 to 2013 (Venable et al. 2014). The most usual

Table 6. Detection capabilities (μg kg^{-1}) of different commercial kits for the screening of antibiotic residues in honey.

Antibiotic family	Commercial kit (manufacturer, country)	Principle of the test	CCβ (μg kg^{-1}) ≤ RC (μg kg^{-1})	CCβ (μg kg^{-1}) > RC (μg kg^{-1})	RC (μg kg^{-1})	Reference
Tetracyclines	Tetrasensor® (Unisensor)	Dipstick	TTC, OTC, CTC and doxycycline respectively at 15, 15, 10 and 5 μg kg^{-1}	Epi-TTC and epi-CTC 1000 μg kg^{-1}, epi-OTC not detected even at 1000 μg kg^{-1}	20	Gaudin, Rault et al. (2013)
	Eclipse® 50 kit (Zeu Inmunotech)	Microbiological	–	TTC, OTC and CTC > 100, ≤ 200 and > 75 μg kg^{-1}. Epimers not validated		Gaudin, De Courville et al. (2013)
	Premi®Test kit (r-Biopharm)	Microbiological	TTC, OTC, CTC respectively at ≤ 10, 10 and >10 μg kg^{-1}	Epimers not validated		Gaudin, De Courville et al. (2013)
	AM II (Randox)	Chemiluminescent biosensor	TTC, OTC and CTC < 15 μg kg^{-1}, doxycycline (30 μg kg^{-1}) and three epimers < 20 μg kg^{-1}	–		–
Macrolides	Kit tylosin (TECNA, Trieste, Italy)	ELISA	Tylosin A 10 μg kg^{-1}	Tylosin B (desmycosin) only at 200 μg kg^{-1}	20	Gaudin, Hedou et al. (2013)
	Eclipse® 50 kit (Zeu Inmunotech)	Microbiological	–	Tylosin A ≤ 200 μg kg^{-1}, tylosin B > 400 μg kg^{-1}		Gaudin, De Courville et al. (2013)
	Premi®Test kit (r-Biopharm)	Microbiological	Tylosin A ≤ 10 μg kg^{-1}, tylosin B ≤ 15 μg kg^{-1}	–		Gaudin, De Courville et al. (2013)
	AM II (Randox)	Chemiluminescent biosensor	Tylosin A 4 μg kg^{-1}, tylosin B 8–10 μg kg^{-1}, tilmicosin 10 μg kg^{-1}	–		–
Aminoglycosides	Kit streptomycin/DHS (Europroxima, Arnhem, the Netherlands)	ELISA	Streptomycin 10 μg kg^{-1} and DHS ≤ 10 μg kg^{-1}	–	40	Gaudin, Hedou et al. (2013)
	Eclipse® 50 kit (Zeu Inmunotech)	Microbiological	–	Streptomycin and DHS > 10 000 μg kg^{-1}		Gaudin, De Courville et al. (2013)
	Premi®Test kit (r-Biopharm)	Microbiological	–	Streptomycin > 400 μg kg^{-1}, DHS around 100 μg kg^{-1}		Gaudin, De Courville et al. (2013)
	AM II (Randox)	Chemiluminescent biosensor	Streptomycin 30 μg kg^{-1}, DHS around 20–30 μg kg^{-1}	–		–

Note: RC, recommended concentration; DHS, dihydrostreptomycin; tylosin B, desmycosin; TTC, tetracycline; OTC, oxytetracycline; CTC, chlortetracycline; ELISA, enzyme-linked immunosorbent assay.

antibiotic families detected and quantified in honey all over the world are in order of importance: tetracyclines (mostly oxytetracycline from 0.21 to 250.4 μg kg^{-1} and tetracycline from 0.28 to 61 μg kg^{-1}), sulfonamides, macrolides (tylosin, 124 positive samples from 0.34 to 139.2 μg kg^{-1}; erythromycin, nine positive samples from 2.9 to 280.3 μg kg^{-1}), quinolones from 0.5 to 144.8 μg kg^{-1} (enrofloxacin, ciprofloxacin, norfloxacin) and aminoglycosides (streptomycin only from 23 to 127 μg kg^{-1}). Therefore, the scope of the AM II kit is really interesting because four of the five antibiotic families of interest could be detected by this method. Moreover, regarding the quantified concentrations, the very low concentrations could not have been detected when they were lower than the estimated detection capabilities of the AM II kit (i.e. lower than 4 μg kg^{-1} for tylosin). Many of the samples were lower than the respective RC of tetracyclines, macrolides and aminoglycosides. Giving the estimated detection capabilities of the AM II, most of the positive samples of interest should have been detected by this method. Finally, another kit from Randox (Crumlin, Co.), the MicroArray I (AM I), for the screening of 14 sulfonamides and trimethoprim in honey could be implemented in parallel with the AM II kit to complete the scope of antibiotics. In this way, the five main antibiotic families would be covered.

Even if few methods exist for the screening of antibiotic residues in honey, different approaches have been evaluated in our laboratory during the past few years. The detection capabilities CCβ of different commercial kits for the screening of tetracyclines, some macrolides and some aminoglycosides are summarised in Table 6. These results were extracted from our in-house validations of commercial kits in honey and these data have been published (Gaudin, De Courville et al. 2013; Gaudin, Hedou et al. 2013; Gaudin, Rault et al. 2013).

The AM II kit is more performing than Tetrasensor® because it is sensitive enough not only for the four tetracyclines but also for the three epimers. Concerning macrolides and aminoglycosides, both validated ELISA kits have had detection capabilities lower than the RC for tylosin A and for streptomycin (Gaudin, Hedou et al. 2013). The performances of the AM II kit were similar, except that tilmicosin was also detected with the AM II kit. Therefore, the AM II kit could be able to replace ELISA kits for the screening of macrolides and aminoglycosides in honey. An additional advantage of the AM II kit is that each spot is specific for a family of antibiotics so six families of antibiotics are detected simultaneously (a wider spectrum of detection) and could be discriminated. Microbiological screening tests based on the inhibition of growth of *Bacillus stearothermophilus* have a wide spectrum of detection and could have been competitive with multi-array biosensors. However, the detection capabilities CCβ determined for 16 antibiotics of the following families (tetracyclines, macrolides, sulphonamides, penicillins and lincosamides) for the Eclipse® 50 kit (Zeu Inmunotech) were all higher than the RCs when they exist, even though they had a low false-positive rate of 5%. On the contrary, the Premi®Test kit (r-Biopharm) gave satisfactory sensitivity with respect to a great number of antibiotics of interest (tetracyclines, sulfonamides and macrolides), but the estimated detection capabilities were higher than the RC for streptomycin and dihydrostreptomycin. Moreover, a 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 the classical tests (ELISA tests and receptor tests).

Finally, the AM II kit seems to be a good compromise between the sensitive approach of specific methods like ELISA or receptor tests and wide-spectrum methods like microbiological tests. In fact, low detection capabilities were reported for the AM II kit in honey and the multi-array system allowed the simultaneous detection of six families of antibiotics. Moreover, technically speaking, implementing two ELISA kits and one receptor test for the screening of three families of antibiotics is time-consuming and costly in relation to the cumulative time for analyses. The extraction protocol is also much easier than those of ELISA kits. Finally, the AM II kit could be satisfactorily replacing the use of the three different kits for screening antibiotics in honey.

Conclusions

The Antimicrobial Array II kit applied on the Evidence Investigator™ system for the determination of six families of antibiotics in honey was validated in terms of its detection capability, specificity, cross-reactions and applicability, according to Commission Decision No. 2002/657/EC (European Commission 2002). The method was found to be rapid and able to screen six families of antibiotics simultaneously in different kinds of honey with a very easy sample preparation procedure. The detection capabilities for tetracyclines, some macrolides and some aminoglycosides were lower than the respective RCs. The results clearly prove that the proposed method is adequate for the purpose of screening analyses of six families of antibiotics in honey.

To our knowledge, this study represents the first validation of the MicroArray II kit in honey according to the European guideline for the validation of screening methods for the residues of veterinary medicines (CRL 2010). The AM II kit will continue to be evaluated in our laboratory. Indeed, the 200 samples of the French honey control plan will be analysed with the Evidence Investigator™ system in parallel with the official tests (ELISA, receptor tests and LC-MS/MS).

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