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Honey for the screening of sulfonamides in honey
according to the Commission Decision 2002/657/EC**

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Validation of a commercial receptor kit Sulfasensor® Honey for the screening of sulfonamides in honey according to the Commission Decision 2002/657/EC

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Abstract:	<p>The Sulfasensor® Honey kit is a receptor test dedicated to the screening of sulphonamide residues respectively in different matrices. The aim of this project was to evaluate and validate this kit according to the CRL guideline for the validation of screening methods European regulation to achieve the French control plan in honey. The test is robust, quick (90 minutes for 40 samples), easy to perform and easy to read. The false positive rate was estimated to 12.5 %. The detection capabilities CCβ of the Sulfasensor® Honey kit were lower than or equal to 25 $\mu\text{g kg}^{-1}$ for sulfamethazineSMZ, sulfamerazine, sulfathiazole, sulfapyridine, and between 25 to 50 $\mu\text{g kg}^{-1}$ for sulfadiazine and sulfadimethoxine, 150 $\mu\text{g kg}^{-1}$ for sulfaquinoxaline and 1000 $\mu\text{g kg}^{-1}$ for sulfamethoxazole and sulfamethizole. Sulfanilamide is not detected by the kit. The kit is applicable to a wide variety of honeys (different floral and geographical origins, liquid or solid). This kit was implementing for the French control plan for the detection of antibiotic residues in honey in 2010 in parallel with an HPLC method. However, in 2011, the Sulfasensor® Honey kit has been replaced by a LC-MS/MS method for the screening and the confirmation of sulfonamide residues in honey, which detect all the sulfonamides of interest.</p>

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3 1 **Validation of a commercial receptor kit Sulfasensor® Honey for the**
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5 2 **screening of sulfonamides in honey according to the Commission**
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8 3 **Decision 2002/657/EC**
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12 5 Valérie GAUDIN*, Annie RAULT, Eric VERDON
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3 21 **ABSTRACT**

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5 22 The Sulfasensor® Honey kit is a receptor test dedicated to the screening of sulphonamide
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7 23 residues respectively in different matrices. The aim of this project was to evaluate and
8
9 24 validate this kit according to the CRL guideline for the validation of screening methods to
10
11 25 achieve the French control plan for honey. The test is robust, quick (90 min for 40 samples),
12
13 26 easy to perform and easy to read. The false positive rate was estimated to be 12.5 %. The
14
15 27 detection capabilities CC_β of the Sulfasensor® Honey kit were lower than or equal to 25 µg
16
17 28 kg⁻¹ for sulfamethazine, sulfamerazine, sulfathiazole, sulfapyridine, and between 25 to 50 µg
18
19 29 kg⁻¹ for sulfadiazine and sulfadimethoxine, 150 µg kg⁻¹ for sulfaquinoxaline and 1000 µg kg⁻¹
20
21 30 for sulfamethoxazole and sulfamethizole. Sulfanilamide was not detected by the kit. The kit
22
23 31 was applicable to a wide variety of honeys (different floral and geographical origins, liquid or
24
25 32 solid). This kit was used to implement the French control plan for the detection of antibiotic
26
27 33 residues in honey in 2010 in parallel with an HPLC method. However, in 2011, the
28
29 34 Sulfasensor® Honey kit has been replaced by a LC-MS/MS method for the screening and
30
31 35 the confirmation of sulfonamide residues in honey, which detect all the sulfonamides of
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33 36 interest.

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38 38 **Keywords:** *Validation; Commission Decision 2002/657/EC; receptor test; screening;*
39 39 *sulfonamides; honey*

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43 INTRODUCTION

44 Honey contains many complex organic compounds: Several natural antibiotic factors, which
45 have potent bacteriostatic activity, that is to say, they prevent the growth of bacteria but do
46 not kill them. Antibiotics have been widely used for animal production for decades worldwide.
47 Bee products can be contaminated from different sources (Bogdanov 2006). The
48 contamination can arise from beekeeping practices or from the environment. Environmental
49 contaminants are the heavy metals i.e. lead, cadmium and mercury, radioactive isotopes,
50 organic pollutants, pesticides (insecticides, fungicides, herbicides and bactericides),
51 pathogenic bacteria and genetically modified organisms. The other contaminants originate
52 from beekeeping. The main ones are acaricides: lipophylic synthetic compounds and non-
53 toxic substances such as organic acids and components of essential oils; and antibiotics
54 used for the control of bee brood diseases, mainly tetracyclines, streptomycin, sulfonamides
55 and chloramphenicol. The major bee diseases, for which antibiotics are indicated, are
56 American and European Foulbrood infection, both due to bacteria and in the fight against
57 Nosema disease, the latter being caused by a microsporidian. The antibiotics are mixed with
58 the food of bees to fight against diseases such as Foulbrood infection. One essential family
59 of antibiotics has been recommended for years in France: the family of tetracyclines (mainly
60 tetracycline and oxytetracycline). However, since the chemistry of antibiotics has made
61 progress and many other substances have emerged, it cannot be forgotten to mention that
62 former bactericidal agents still have an interest in efficiency. Another noteworthy point is that
63 in some countries where antibiotic susceptibility testing methods are implemented to test the
64 susceptibility of Foulbrood bacteria, resistance to tetracyclines for this organism was
65 established as well as sensitivities to other antibiotics. Some strains of *Paenibacillus* larvae
66 have over the years, also been developing resistance to tetracyclines.

67

68 Antibiotics potentially present in honey, or any other food, are unacceptable, even potentially
69 a risk to human health: risk of allergies, even at very low doses in some subjects, but also an
70 issue is the risk of causing selection of resistant strains among human pathogens. No market

1
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3 71 authorization can be issued unless a maximum residue limit (MRL) has been established.
4
5 72 However, no antibiotics have MRLs for honey. The average consumption of honey per
6
7 73 person and per year is very low. So if new MRLs were to be established for honey, they
8
9 74 could be very high. The authorization of the presence in honey of such large quantities of
10
11 75 antibiotics would have a negative impact on the image that the consumer has of honey,
12
13 76 perceived as a natural and healthy product. No one then is in favour of the establishment of
14
15 77 MRLs for antibiotics in honey (Bruneau 2006). Moreover "given the high cost of necessary
16
17 78 scientific studies to establish MRLs, only the most important species called "major" were
18
19 79 studied. Thus, for bees, which are minor species, no antibiotic could be subjected to an
20
21 80 authorization. EU rules on setting MRLs for pharmacologically active substances have been
22
23 81 updated by Regulation No 470/2009 (Commission 2009). The regulation, for the first time,
24
25 82 introduced a mechanism for the extrapolation of MRLs from one species / food matrix to
26
27 83 another ('cascade' system).
28

84

31 85 For now, a lack of harmonization between action limits set by various countries of the EU is
32
33 86 noticeable. Each of the Member States adopts a different position on this issue. Some, like
34
35 87 Italy, prohibit any antibiotic residue and for this rely on the minimum required detection limit
36
37 88 for laboratories to investigate such residues (at least $10 \mu\text{g kg}^{-1}$). Others do not really have
38
39 89 control plans. Some countries have set tolerance levels ranging from 15 to $50 \mu\text{g kg}^{-1}$
40
41 90 (Belgium, United Kingdom, Germany and Austria). In Belgium, FASFC established action
42
43 91 thresholds for tetracyclines, sulfa drugs and streptomycin beyond which the honey must be
44
45 92 removed from sale and destroyed. Each positive result is noted. For other antibiotics (e.g.
46
47 93 fluoroquinolones), the agency intervenes only when the threshold of $20 \mu\text{g kg}^{-1}$ is exceeded,
48
49 94 except for the banned antibiotics (chloramphenicol and nitrofurans).
50

95

53
54 96 In the EU, a technical guide has been published by the EU Reference Laboratories (EU-RL)
55
56 97 (CRL Guidance paper of 7th December 2007). The purpose of this technical guide is to
57
58 98 improve and harmonize the performance of analytical methods for substances for which

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3 99 maximum residue levels (MRLs) have not been set (Commission 2009). These substances
4
5 100 without MRLs include substances partially or totally banned from use in food producing
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7 101 animals. It should be noted that this document should serve as a technical guideline
8
9 102 (recommended levels) for methods of residue analysis. The levels recommended in this
10
11 103 document, however, have no real legal basis. The recommended concentration (RC) for
12
13 104 sulfonamides is set at $50 \mu\text{g kg}^{-1}$.

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17 106 On the Italian market, among the most analyzed substances used, sulfonamides are the first,
18
19 107 followed by tetracycline, streptomycin, tylosin and chloramphenicol (Baggio et al. 2009). In
20
21 108 Belgium, the presence of a family of antibiotics, fluoroquinolones, in Chinese honey was
22
23 109 highlighted recently (Bruneau 2006). In Belgium, the presence of sulfonamides in honey
24
25 110 samples was detected between 2001 and 2003, streptomycin and tetracyclines only in 2000-
26
27 111 2001. It is also worth noting the presence of tylosin in some imported honeys (Reybroeck
28
29 112 2003). Concerning nitrofurans metabolites, some rapid alerts occurred in Europe (e.g. in 2007
30
31 113 for furazolidone (AOZ) in Ukrainian honey) (Vass, 2008).

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35 115 Each year, the technology for the detection of antibiotics improves (Bruneau 2006). The
36
37 116 technical performance has been increased by a factor of 1000 in 10 years. The "Charm II
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39 117 Sulfonamides Honey" kit can detect all sulfonamides (LOD for sulfamethazine, sulfapyridine,
40
41 118 sulfamethopyridazine, sulfadimethoxine at $20 \mu\text{g kg}^{-1}$ and at $30 \mu\text{g kg}^{-1}$ for sulfathiazole and
42
43 119 sulfamethoxazole) (Salter 2003). However radioactive materials are used in the Charm II test
44
45 120 which limits its implementation.

46
47 121
48
49 122 A receptor test called Sulfasensor® Honey developed by Unisensor s.a. (Wandre, Belgium)
50
51 123 is now commercialised for the screening of sulfonamides in different matrices, including
52
53 124 honey. Therefore our objective was to determine if this test could be applicable to the
54
55 125 screening of sulfonamides in honey to implement the French control plan. The aim of this

1
2
3 126 work was to evaluate and to validate the Sulfasensor® Honey kit according to the CRL
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5 127 guideline for the validation of screening methods (Crl 2010) .
6

7 128

8 9 129 **MATERIAL AND METHODS**

10 11 130 ***Chemicals and standard solutions***

12
13 131 Sulfonamides (sulfadiazine, sulfathiazole, sulphamethazine, sulfanilamide, sulfaquinoxaline,
14
15 132 sulfadimethoxine, sulfamerazine, sulfamethizole, sulfamethoxazole) were supplied by Sigma
16
17 133 Aldrich (Saint Quentin-Fallavier, France). Other antibiotics: oxytetracycline,
18
19 134 tylosin, streptomycin, chloramphenicol, erythromycin, benzylpenicillin, enrofloxacin were
20
21 135 supplied by Sigma Aldrich (Saint Quentin-Fallavier, France).
22

23 136

24 25 137 ***Matrices***

26
27 138 During this evaluation, 20 different honeypots have been used: honey rosemary, lavender,
28
29 139 honeydew from Corsica bush, heather, forest, lemon tree, acacia, chestnut, raspberry,
30
31 140 mountain and flowers. These honeys have different geographical origins from Brittany, Isere,
32
33 141 Correze, Corsica and Spain, but are all from certified organic apiculture. In addition, the
34
35 142 consistencies of these 20 honeys were different: liquid / solid and different colours.
36
37 143 Moreover, the applicability of the test to royal jelly was evaluated.
38

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40 41 145 ***Sulfasensor® Honey kit***

42
43 146 The Sulfasensor® Honey kit is a receptor-based assay for rapid screening of sulfonamide
44
45 147 residues present in honey. It is produced by the Unisensor™ firm (Liège, Belgium). The kit at
46
47 148 the time of validation was only a prototype but is now commercialised.
48

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3 149 More information concerning the principle of the receptor kit and the methodology can be
4
5 150 found at <http://www.unisensor.be/>.

6
7 151 ***Evaluation of the receptor test***

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9 152 A preliminary study was conducted on the kit prior to the validation to determine if the kit was
10
11 153 of interest or not, regarding practicability and sensitivity. Furthermore, the evaluation study
12
13 154 was used to determine the concentrations of antibiotics to be spiked in honey samples for
14
15 155 later validation. The recommended concentration (RC) for sulfonamides is set at 50 µg kg⁻¹.
16
17 156 Sulfadiazine, sulfathiazole, sulphamethazine, sulfanilamide, sulfaquinoxaline,
18
19 157 sulfadimethoxine, sulfamerazine, sulfamethizole, sulfamethoxazole have been tested at
20
21 158 various concentrations from 12.5 to 50 µg kg⁻¹ in honey samples with the Sulfasensor®
22
23 159 Honey kit. Ideally, the tested concentrations were half the RC. When the test was not enough
24
25 160 sensitive, regarding the RC, the concentration was increased to assess the true sensitivity.
26
27 161 The concentrations were based on detection limits claimed by the manufacturer. Four days
28
29 162 of analyses were implemented, with 10 to 12 dipsticks per day. For example, 12 samples (4
30
31 163 different blank honey samples and 8 spiked samples near to the claimed CCβ) were
32
33 164 analysed per day.
34

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36
37 166 ***Validation protocol***

38
39 167 Both kits have been validated according to the CRL guideline for the validation of screening
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41 168 methods (Crl 2010) which is based on the criteria of Commission Decision 2002/657/EC
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43 169 (Commission 2002). The performance characteristics to be determined were identical:
44
45 170 practicability, specificity, detection capabilities (CCβ), applicability (honeys from different
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47 171 sources), ruggedness and stability. The analyses have been blindly performed by a
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49 172 technician (random numbers given to the samples).
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174 *Practicability*

175 Practicability was considered as the ease of use combined with the necessary equipment,
176 reagents, instruments and environmental conditions. The aim was to check whether the
177 methodology is appropriate or not for routine analysis.

178 *Specificity, false positive rate*

179 Twenty batches of blank control honeys (organic food) of various floral origins have been
180 used for validation (refer to matrices).

181 *Detection capability $CC\beta$*

182 In the CRL guideline for the validation of screening methods (Crl 2010), it is recommended
183 that the number of samples needed for each substance for the validation (samples spiked at
184 a target concentration or naturally incurred) depends on the degree of statistical confidence
185 required in the result, and the relationship between target concentration and detection limit
186 prescribed. For example:

187 - If the target concentration is set at half the regulatory limit ($\frac{1}{2}$ MRL), the occurrence of one
188 or no false-compliant result after the analysis of 20 control samples is sufficient to
189 demonstrate that the detection capability is less or equal to the regulatory limit (MRL) and
190 less than or equal to the $\frac{1}{2}$ MRL;

191 - "If the target concentration is between 50% and 90% of the prescribed limit, at least 40
192 positive control samples (with 2 or less than 2 false-compliant results) will be sufficient to
193 demonstrate that the $CC\beta$ is below the regulatory limit."

194 - If the screening test sensitivity is such that the target concentration is close to the regulatory
195 limit (10% below the regulatory limit), higher number of samples has to be analyzed. A
196 maximum of 60 repetitions (with 3 or less than 3 false-compliant results) is required to
197 demonstrate that the detection capability is less than or equal to the MRL. These studies
198 can be undertaken by sequential steps, namely that the first 20 fortified samples are tested,

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3 199 and if more than one sample is declared negative, validation may be abandoned at this
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5 200 point or the target concentration should be increased and validation exercise repeated."

6
7 201 The target concentration was determined during the preliminary tests of evaluation. The
8
9 202 sensitivities of each antibiotic with different ranges of concentration were assessed. Then the
10
11 203 chosen concentration for validation is the concentration that gave 95 to 100% of positive
12
13 204 results during the evaluation step.

14
15 205 In the case of honey, there are no MRLs for antibiotic residues, but the recommendations of
16
17 206 the Community Reference Laboratories (CRLs) (Crl Guidance paper of 7th December 2007).

18
19 207 So, the number of samples to analyse will be determined according to the recommended
20
21 208 limits instead of the MRL. A maximum of 60 samples (blank and spiked) will be analyzed to
22
23 209 determine CC β and specificity. After the analysis of 60 spiked (or incurred) samples, the
24
25 210 spiking level (Screening Target Concentration) where $\leq 5\%$ of false compliant results would
26
27 211 be present at the Regulatory/Action Limit, would be the detection capability CC β of the
28
29 212 method (i.e. the concentration at which there are 3 or less false compliant results out of 60
30
31 213 spiked samples).

32
33 214 The CC β of 8 sulfonamides were validated (CC β): sulfamethazine, sulfamerazine,
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35 215 sulfathiazole, sulfapyridine, sulfadiazine, sulfadimethoxine, sulfaquinoxaline and
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37 216 sulfamethoxazole.

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40 41 42 218 *Applicability*

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45 219 We have tested the applicability of both kits for different types of honey (single flower or
46
47 220 multiflower, different flowers, different colours, liquid or solid), identifying the CC β of the
48
49 221 antibiotics from 20 different samples to a minimum.

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51 222

223 *Cross-reactions*

224 Cross-reactivity with other sulfonamides (sulfanilamide, sulfamethizole) has been tested, and
225 also the cross-reactivity with other families of antibiotics (spiked at 10000 $\mu\text{g kg}^{-1}$). The
226 potentially masking effect of the presence of other antibiotics with sulfamethazine for the
227 Sulfasensor® Honey has been evaluated.

228

229 *Ruggedness*

230 Ruggedness studies use the deliberate introduction of minor reasonable variations by the
231 laboratory and the observation of their consequences. The ruggedness study was focused
232 on one representative sulphonamide. Ten blank honey samples and 10 honey samples
233 spiked at the level of interest ($\text{CC}\beta$ of SMZ) were analysed. All the materials were prepared
234 from the same batch of honey for the 8 days. It was a blind test (unknown samples)
235 performed at different days with one operator. An experimental plan which combined the
236 different factors was built to minimise the experiments. Eight factors have to be studied.
237 Therefore, 2 experimental plans have been built. The 8 factors were divided into 2 plans as
238 follows:

239 First plan: Factor A: 1st 600 μl acid buffer $\pm 10\%$, factor B: 1st incubation time in boiling
240 water: 5 ± 1 minutes (20%), factor C: 600 μl neutralisation buffer $\pm 10\%$, factor D: 1800 μl
241 honey buffer $\pm 10\%$.

242 Second plan: Factor A: 200 μl sample $\pm 10\%$ factor B: 1st incubation temperature
243 ($43^\circ\text{C}/37^\circ\text{C}$), factor C: 2nd incubation temperature ($43^\circ\text{C}/37^\circ\text{C}$), factor D: 2nd incubation time
244 at room temperature: 15 ± 3 minutes (20%). The influence of these factors on false positive
245 and false negative rate and the interactions between factors were studied.

246 During the 2nd plan only, a reading was performed immediately as usual. Then a reading was
247 performed after 30 minutes, but without looking at the results of the 1st reading.

248 Ruggedness of the kit was also tested by performing comparative assays between two
249 analysts who were not involved in the evaluation step and in the validation. These 2

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3 250 technicians analysed 2 blank control samples and 2 blank control samples fortified samples
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5 251 (sulfamethazine at $12.5 \mu\text{g kg}^{-1}$) during 3 days of analysis.
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8 252 *Stability*

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10 253 The stability of analytes in solution and in the matrix will be determined through a literature
11
12 254 review.
13

14 255

15 16 256 **RESULTS AND DISCUSSION**

17 257 *Evaluation part*

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19
20 258 The advantages of this receptor test from a technical point of view were: rapidity, easiness of
21
22 259 use and of reading. Moreover only a short extra acidic hydrolysis for the analysis of
23
24 260 sulfonamides. It was observed that the reading of Sulfasensor® Honey dipsticks was a little
25
26 261 trickier than the reading of other tests (i.e. Tetrasensor Honey® dipsticks (Unisensor s.a.,
27
28 262 Liège, Belgium)). The performances of Sulfasensor® Honey kit were compliant with those
29
30 263 claimed by the manufacturer. The assumed detection capabilities seemed to be lower than
31
32 264 the recommended limits for some sulfonamides.
33

34 265

35
36 266 This evaluation step allowed defining the concentrations to be validated and so the number
37
38 267 of samples needed to determine the different detection capabilities. The results of preliminary
39
40 268 tests on Sulfasensor® Honey kit allowed to select the following sulfonamide concentrations
41
42 269 for the validation: sulfamethazine at $12.5 \mu\text{g kg}^{-1}$, sulfadiazine at $25 \mu\text{g kg}^{-1}$,
43
44 270 sulfadimethoxine at $25 \mu\text{g kg}^{-1}$, sulfathiazole at $25 \mu\text{g kg}^{-1}$, sulfamerazine at $12.5 \mu\text{g kg}^{-1}$,
45
46 271 sulfapyridine at $25 \mu\text{g kg}^{-1}$, sulfamethoxazole at $1000 \mu\text{g kg}^{-1}$ and sulfaquinoxaline at $150 \mu\text{g}$
47
48 272 kg^{-1} . The recommended concentration (RC) for sulfonamides is $50 \mu\text{g kg}^{-1}$. Concerning
49
50 273 sulfamethazine, sulfamerazine, sulfadiazine, sulfadimethoxine, sulfathiazole and
51
52 274 sulfapyridine, the requested number is 20 samples (each sample analysed once) because
53
54 275 the assumed $\text{CC}\beta$ is equal or lower than half the recommended concentration (25 % and 50
55
56 276 % of the RC). Concerning sulfamethoxazole and sulfaquinoxaline, the estimated $\text{CC}\beta$ is

277 much higher than the RC. Therefore, the $CC\beta$ did not need to be determined with 60
278 samples. So only 20 samples (each sample analysed once) have been tested. Sulfanilamide
279 gave negative results even at $100000 \mu\text{g kg}^{-1}$.

280

281 ***Validation of Sulfasensor® Honey kit for the detection of sulfonamides***

282 *Practicability*

283 The method is rather quick (90 minutes including sample preparation and analysis) and easy
284 to perform. Up to 40 samples could be analysed during one day.

285 *Specificity and detection capabilities $CC\beta$*

286 Two batches of the kit were used for the validation. 40 blank honey samples from 20 different
287 batches of honey have been analysed during 10 days. So each batch of honey has been
288 analysed twice in total, on 2 different days. For each of the sulfonamides, 20 different
289 batches of honey were spiked for the determination of the $CC\beta$. When 40 samples have to
290 be analysed, each batch of honey has been spiked and analysed twice during the validation.
291 The results are presented in Table 1.

292

Insert Table 1 here

293 The global false positive rate is equal to 12.5 % (5 positive results out of 40 blank samples).
294 The five batches which gave one doubtful or positive results were from rosemary, lime tree,
295 heather, honeydew and acacia. No common profile was observed for the five batches (3
296 yellow honeys (1 solid, 2 liquids), 2 brown honeys (1 solid, 1 liquid). Moreover each batch
297 was analysed twice at different days and each of the 5 batches gave once a negative result
298 and once a doubtful or positive result.

299

300 The results were negative for samples spiked with sulfamethizole at 25 and $50 \mu\text{g kg}^{-1}$. One
301 test at $500 \mu\text{g kg}^{-1}$ gave doubtful results and 2 assays at $1000 \mu\text{g kg}^{-1}$ also. A doubtful result
302 during routine analyses will be considered as a positive result and then will be confirmed by

1
2
3 303 physico-chemical methods. The sensitivity towards sulfamethizole was estimated at 1000 μg
4
5 304 kg^{-1} (data not shown). The sensitivity towards sulphanilamide has been tested with 3
6
7 305 samples (2 assays at 100000 $\mu\text{g kg}^{-1}$ gave negative results) and the conclusion as that
8
9 306 sulphanilamide is not detected by the kit, as it was announced by the manufacturer.

10
11 307

12
13 308 Regarding the structures of the different sulfonamides, the binding to the receptor on the
14
15 309 dipstick is obviously variable (Font et al. 2008). A Spanish team developed 2 direct enzyme-
16
17 310 linked immunosorbent assays (ELISAs) for detection of sulfonamide antibiotic residues in
18
19 311 milk samples. A set of 13 common sulfonamides were assessed using the two
20
21 312 antibody/enzyme tracer combinations. Differences of sensitivity were observed between
22
23 313 sulfonamides, which were linked to the individual structure of each sulfonamide. This could
24
25 314 explain the differences in the detection capabilities between sulfonamides with the
26
27 315 Sulfasensor® Honey kit also. For example, sulfanilamide do not have a cycle linked to the
28
29 316 right NH_2 group, whereas SMZ owns this cycle, like the other sulfonamides with good
30
31 317 sensitivities. Similarly, sulfamethizole and sulfamethoxazole which are detected at 1000 μg
32
33 318 kg^{-1} own a different cycle on the right NH_2 group, different from SMZ which is detected at
34
35 319 12.5 $\mu\text{g kg}^{-1}$. However sulfathiazole is well detected with a cycle which seems very near to
36
37 320 the cycle of sulfamethizole.

38
39 321

40
41 322 The absence of detection of sulphanilamide is a great disadvantage of this test. In fact,
42
43 323 sulphanilamide which is a degradation product of asulam was detected in Swiss honeys and
44
45 324 this could not be explained by the beekeeping (Kaufmann and Kaenzig 2004) (Bogdanov and
46
47 325 Edder 2005). The bees collect nectar from meadows sometimes was treated with the
48
49 326 herbicide asulam. This was the first report on the use of an herbicide that causes the
50
51 327 appearance of active antibacterial residue belonging to the class of sulfonamides in food.
52
53 328 Therefore, the use of the herbicide asulam might cause unacceptable levels of
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55 329 sulphanilamide residues in honey.

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3 330 The determination of the $CC\beta$ was performed with spiked samples and not incurred samples.
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5 331 In fact the production of incurred samples at the needed concentration (estimated $CC\beta$) in
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7 332 honey for each sulfa of interest should have been very difficult. Moreover reference materials
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9 333 in honey are very rare. The difference of spiked and incurred honey samples should have
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11 334 been the binding of sulfa drugs to sugars which could have been higher in incurred samples.
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13 335 However an acidic hydrolysis step is implemented prior to the analysis with the Sulfasensor®
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15 336 Honey kit to break the bond between sulfa and sugars. So it could be hypothesised that the
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17 337 behaviour would be similar between incurred and spiked honey samples. In the case of the
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19 338 availability of this kind of material in the future, the comparison would be of great interest.
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21 339 The Sulfasensor® Honey kit was implemented during the honey control plan in parallel with
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23 340 an HPLC -fluorescence method in 2010. Thirty seven different honey samples (different floral
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25 341 origins) from various areas in France were analysed. Only one positive result was found with
26
27 342 the Sulfasensor® Honey test and it was confirmed complaint by a the HPLC-fluorescence
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29 343 method. The list of sulfa drugs detected by the HPLC-fluorescence method is larger than for
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31 344 the kit: sulfadiazine, sulfathiazole, sulfadimerazine, sulfamonomethoxine, sulfadimethoxine,
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33 345 sulfamethizole, sulfamethoxazole, sulphanilamide, sulfamerazine and sulfameter. The HPLC-
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35 346 Fluorescence method reported 2 positive results at the screening step with traces of
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37 347 sulfamethoxazole. However the concentrations were lower than the $CC\alpha$ at the scr
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39 348 confirmatory step.

41 42 43 349 *Applicability*

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45 350 The applicability of the kit to different kind of honeys has been proved towards the analysis of
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47 351 20 different honeys from certified organic apiculture. The flower origin (rosemary, lavender,
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49 352 honeydew, heather, forest, lemon tree, acacia, chestnut, raspberry, and mountain flowers)
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51 353 and the geographical origins (Brittany, Isere, Correze, Corsica and Spain) were different.
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53 354 Moreover, the consistency of the 20 honeys was different (liquid / solid) and different colours
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55 355 were observed. The results obtained with the 20 different batches were concordant. The $CC\beta$
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3 356 of the 8 sulfonamides were determined with these 20 batches. Therefore, the applicability of
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5 357 the Sulfasensor® Honey to honey samples from various origins has been proved.

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7 358 The applicability of the Sulfasensor® Honey test to royal jelly was also studied. Both negative
8
9 359 (2 blank samples) and positive Quality Controls (2 samples spiked with sulfamethazine at
10
11 360 12.5 µg kg⁻¹) gave positive results. So, the Sulfasensor® Honey kit is not applicable to the
12
13 361 screening of sulfonamides in royal jelly.

14 15 16 362 *Cross-reactions*

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18 363 The specificity of the test towards other families of antibiotics has been studied and the
19
20 364 results are presented in table 2.

21 22 365 **Insert Table 2 here**

23
24 366 Seven antibiotics from different families have been tested at 10000 µg kg⁻¹ and only negative
25
26 367 results have been reported. So the kit is very specific for sulfonamides. When the samples
27
28 368 were spiked with SMZ at 12.5 µg kg⁻¹, plus the other antibiotics at 10000 µg kg⁻¹, the
29
30 369 detection of SMZ was not hidden by the presence of the other antibiotics. So there is no
31
32 370 masking effect of the other antibiotics.

33 34 35 371 *Ruggedness*

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37 372 Because many parameters could have had effect on the performance of the Sulfasensor®
38
39 373 Honey kit, 2 different ruggedness studies have been conducted. During the first ruggedness
40
41 374 study, 4 parameters belonging to the sample preparation (1st part of the protocol) have been
42
43 375 studied. During the second ruggedness study, 4 parameters of the 2nd part of the protocol
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45 376 (analyses on the dipsticks) have been studied. The results are presented in Table 3 for both
46
47 377 ruggedness studies.

48 49 50 378 **Insert Table 3 here.**

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52 379 During the first ruggedness study, negative influence on the performance of the test was
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54 380 observed for the following factors: the volume of acid buffer. In fact, when the volume of acid
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56 381 buffer increased (10 %), the false positive rate and the false negative rate increased. On the

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3 382 contrary, when the 1st incubation time in boiling water increased (20 %) or when the volume
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5 383 of honey buffer (11 %) increased, the false positive and the false negative rate decreased.
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7 384 The volume of neutralisation buffer (± 10 %) has no observed influence on the performance
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9 385 of the test.

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12 387 During the second ruggedness study, the only factor for which a negative influence on the
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14 388 false positive rate and on the false negative rate was observed was the 2nd incubation time.
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16 389 When the 2nd incubation time was increased up to 20 %, the false positive rate and the false
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18 390 negative rate increased. On the contrary, when the volume of sample increased, the false
19
20 391 positive and the false negative rate at the same time when the volume decreased of 10 %. It
21
22 392 is logical that the sensitivity is better when the sample volume is increased. Some factors
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24 393 gave rise to observed effect only on false positive rate. When the 1st incubation temperature
25
26 394 (+7.5 %), the false positive rate decreased. The 1st incubation temperature had also a
27
28 395 reported influence on the false negative rate. When the 1st incubation temperature increased,
29
30 396 the false negative rate increased. The 2nd incubation temperature (± 7.5 %) had no influence
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32 397 on false positive and false negative rates.
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38 399 Finally, the most critical factors during the sample preparation were the volume of acid buffer
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40 400 (± 10 %) because when these factors increased, the false negative rate AND the false
41
42 401 positive rate increased. Therefore the sensitivity and the specificity of the test decreased.
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44 402 Furthermore, the most critical factors during the analysis on the dipsticks were the 1st
45
46 403 incubation temperature (+ 7.5 %), the 2nd incubation time (20 %) because when these factors
47
48 404 increased, the false negative rate increased. Therefore the sensitivity of the test decreased.
49
50 405 Ruggedness of the kit was also tested by performing comparative assays between two
51
52 406 analysts who were not involved in the evaluation step and in the validation. These 2 analysts
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54 407 analysed 2 blank control samples and 2 blank control samples fortified samples
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56 408 (sulfamethazine at 25 $\mu\text{g kg}^{-1}$) during 3 days of analysis. Each analyst obtained the
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3 409 requested results: negative results for blank Quality Controls (QC) and positive or doubtful
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5 410 results for spiked QC.

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7 411 During the 2nd plan only, a reading was performed immediately as usual and a second
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9 412 reading 30 min later. The results were similar when immediate reading and after 30 min.

11 413 *Stability of sulphonamide residues*

14 414 *Stability in solution*

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16 415 The stability of sulfadiazine, sulfamerazine, sulfamethoxydiazine, sulfamonomethoxine,
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18 416 sulfadimethoxine, sulfamethoxazole and sulfaquinoxaline in standard solution and extracts
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20 417 was checked (Chen et al. 2009). Stock solutions of the standards (1 mg mL⁻¹) were
21
22 418 prepared by dissolving each sulfonamide in methanol and stored in a refrigerator at 4°C.
23
24 419 Stock solutions were found to be stable for 2 months. Working standard solutions were daily
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26 420 prepared by diluting the stock solutions with water.

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30 422 Another team studied the stability of the stock solutions of 12 sulfonamides (sulfaguanidine,
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32 423 sulfanilamide, sulfacetamide, sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine,
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34 424 sulfamether, sulfamethazine, sulfamethoxydiazine, sulfachloropyridazine and sulfadoxine)
35
36 425 (Maudens et al. 2004). Individual primary stock solutions of all standards were prepared in
37
38 426 methanol at a concentration of 1 mg mL⁻¹ and stored in the dark at -20°C until use. Under
39
40 427 the stated conditions, stock solutions proved to be stable for at least 6 months.

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44 429 Most often, the extraction of sulphonamide residues from honey includes an acid hydrolysis
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46 430 step to release the sugar-bound sulfonamides. A stability study in acidic conditions was
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48 431 implemented (Thompson and Noot 2005). A darkly coloured, free flowing honey and a lighter
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50 432 coloured, crystallized honey were fortified with 25 µg kg⁻¹ of 8 sulfonamides (sulfathiazole,
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52 433 sulfamethazine, sulfamerazine, sulfapyridine, sulfadiazine, sulfadoxine, sulfadimethoxine,
53
54 434 and sulfachloropyridazine). Based upon stability experiments, it was discovered that it is not
55
56 435 acceptable to store acidified samples at room temperature for periods of more than 7 days.

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3 436 Conversely, sulfonamides were found to be sufficiently stable in acidified honey solutions
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5 437 when stored at +4°C or -20°C for up to 28 days.
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9 439 *Stability in honey*

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11 440 Sulfonamides bound to sugars in honey and that reaction could have consequences on the
12
13 441 residue analysis of honey. A sulfathiazole glucose modelisation, based on spiked honey and
14
15 442 naturally contaminated honey, was studied (Schwaiger and Schuh 2000). The sugar /
16
17 443 sulfathiazole complex could be extracted with organic solvents such as acetonitrile and
18
19 444 dichloromethane. The chromatographic behaviour of the complex is different from that of free
20
21 445 sulfathiazole. Therefore, the probability that the actual contamination of honey is
22
23 446 underestimated is very high. Hydrolysis experiments showed that considerable amounts of
24
25 447 bound sulfonamides may be released in acidic environment. This study demonstrates the
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27 448 need for a hydrolysis step prior to the determination of sulfa drugs for control of residues in
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29 449 honey.
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33 451 Honey samples purified using solid phase extraction-IAC (Immunoaffinity Column) were
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35 452 analysed both by EIA and HPLC (Heering et al. 1998). Sixteen samples were positive in the
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37 453 sulfathiazole EIA, with levels ranging from 70 to 750 µg kg⁻¹. After HPLC analysis of these
38
39 454 extracts, neither sulfathiazole nor any other sulfonamide could be detected. The possibility of
40
41 455 a reaction of sulfathiazole with reducing sugars or other compounds was considered. A
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43 456 preliminary experiment in which honey was spiked with sulfathiazole and incubated at 40°C
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45 457 showed that EIA results were more or less unaffected, whereas the HPLC peak for
46
47 458 sulfathiazole decreased rapidly, and less than 10% of the added sulfathiazole could be
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49 459 detected after 4 h of incubation. Residues of sulfathiazole in honey are probably not present
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51 460 as free sulfathiazole but in chemically modified form. Therefore the negative HPLC results
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53 461 have to be considered as false-negative. Since literature data indicate that bound
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55 462 sulfathiazole could be converted into free form under acidic conditions (Schwaiger and
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57 463 Schuh 2000).

464 *Conclusions*

465 To our knowledge, this work represents the first example of the validation of the
466 Sulfasensor® Honey kit for the analysis of sulfonamide residues in honey according to the
467 CRL guideline for the validation of screening methods (Crl 2010). Efficient screening
468 methods are required for successful control of residues. Regarding the Commission Decision
469 2002/657/EC (Commission 2002), a screening method should have a false negative rate
470 lower than 5 % at the level of interest. Concerning honey, there is no MRL and no authorized
471 antibiotics. Therefore, the level of interest for both kits was the recommended concentration
472 (Crl Guidance paper of 7th December 2007). The Sulfasensor® Honey kit is a robust and
473 selective method, quick (90 min) and easy to perform and easy to read. The global false
474 positive rate was equal to 12.5 %. The Sulfasensor® Honey kit obtained detection
475 capabilities lower than the recommended concentration (RC) ($50 \mu\text{g kg}^{-1}$) for 6 tested
476 sulfonamides (sulfamethazine, sulfamerazine, sulfathiazole, sulfapyridine, sulfadiazine,
477 sulfadimethoxine) which are of interest to be detected in honey samples. However the bad
478 detection of sulfaquinoxaline ($3*RC$), sulfamethoxazole ($20*RC$) and sulfamethizole ($20*RC$)
479 was not satisfactory. The major drawback of the kit is the absence of detection of
480 sulphanilamide, which confirmed the manufacturer information. This receptor test is
481 applicable to a wide variety of honey (mono-flower and multi-flower, different flower origins,
482 different geographic origins and different consistencies (liquid or solid), different colours).

483

484 The next step was the implementation of these tests into the French control plan for the
485 detection of antibiotic residues in honey. The routine application in the National Residue
486 Monitoring Plan in 2010 and the daily analysis of Quality Controls (negative and positive
487 honey samples) have confirmed that this test is a robust and effective screening test for
488 honey. No positive results were obtained out of 50 honey samples analysed. In parallel, the
489 50 samples were tested by an HPLC method. In 2011, the Sulfasensor® Honey kit has
490 been replaced by a LC-MS/MS method for the screening and the confirmation of sulfonamide
491 residues in honey, which detect all the sulfonamides of interest.

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Table 1. False positive, false negative results, detection capability $CC\beta$ and claimed detection limit.

Sulfonamide	Number of false + results	Number of false = results (<u>false negative rate</u>)	$CC\beta$ ($\mu\text{g}/\text{kg}$)	Claimed detection limits (LOD) (by the manufacturer)
<u>SMZ</u> Sulfamethazine	/	0_(0)	≤ 12.5	< 25 $\mu\text{g}/\text{kg}$
Sulfadiazine <u>SDZ</u>	/	4/20 samples (20 %)	25-50	25 $\mu\text{g}/\text{kg}$
Sulfadimethoxine <u>SDMX</u>	/	2/20 samples (10 %)	25-50	25 $\mu\text{g}/\text{kg}$
Sulfathiazole	/	0_(0)	≤ 25	25 $\mu\text{g}/\text{kg}$
Blank	1/20 samples (5 %) (batch 343)	/	/	
Sulfamerazine	/	0_(0)0	≤ 12.5	< 25 $\mu\text{g}/\text{kg}$
Sulfapyridine	/	0_(0)0	≤ 25	< 25 $\mu\text{g}/\text{kg}$
Sulfamethoxazole	/	1/20 samples (5 %)	1000	500 $\mu\text{g}/\text{kg}$
Sulfaquinoxaline	/	0_(0)0	≤ 150	150 $\mu\text{g}/\text{kg}$
Sulfamethoxy pyridazine	/	/	/	< 25 $\mu\text{g}/\text{kg}$
Sulfamonomethoxine	/	/	/	< 25 $\mu\text{g}/\text{kg}$
Sulfachlorpyridazine	/	/	/	25 $\mu\text{g}/\text{kg}$
Sulfacetamide	/	/	/	(> 1000 $\mu\text{g}/\text{kg}$)
Sulfadoxine	/	/	/	(> 1000 $\mu\text{g}/\text{kg}$)
Blank	4/20 samples (20 %) (batches 353, 349, 344, 346)	/	/	

False +: false positive; False -: false negative; SMZ: sulfamethazine; SDZ: sulfadiazine; SDMX: sulfadimethoxine; S___: Sulfa___.

Table 2. Cross-reactions with other families of antibiotics and masking effect.

Antibiotic	Concentration ($\mu\text{g/kg}$)	Without SMZ	With SMZ at 12.5 $\mu\text{g/kg}$
Oxytetracycline	10000	-	+
Tylosine	10000	-	+
Streptomycine	10000	-	+
Chloramphenicol	10000	-	+
Erythromycin	10000	-	+
P Benzympenicillin-G	10000	-	+
Enrofloxacin	10000	-	+
Negative control Batch 344	0	-	-
Negative control Batch 344	0	-	-
Positive control SMZ 12.5 ppb	15	+	/

SMZ: sulfamethazine.

Table 3. Influence of the 4 different factors and interactions of factors during the 1st ruggedness study and during the 2nd ruggedness study.

Response	Factor				Interactions			Mean
	A	B	C	D=ABC	E	F	G	
		1 st						
	Volume of acid buffer (µl)	incubation time in boiling water	Volume neutralisation buffer (µl)	Volume of honey buffer (µl) D=ABC	AB+CD	AC+BD	BC+AD	I
False + rate	0,05	-0,05	0,004	-0,05	-0,05	-0,05	0,05	0,05
False - rate	0,05	-0,05	0,004	-0,05	-0,05	-0,05	0,05	0,05
Response	Factor				Interactions			Mean
	A	B	C	D=ABC	E	F	G	
		1 st	2 nd	2 nd				
	Volume sample (µl)	incubation temperature	incubation temperature	incubation time D=ABC	AB+CD	AC+BD	BC+AD	I
False + rate	-0.01	-0.01	0.001	0.01	0.01	-0.01	-0.01	0.01
False - rate	-0.09	0.09	-0.003	0.04	-0.09	-0.04	0.04	0.09

False + rate: false positive rate; False – rate: false negative rate.