Routine quality control in honey packaging companies as a key to guarantee consumer safety. The case of the presence of sulfonamides analyzed with LC-MS-MS.

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Abstract

One of the main challenges in the Horizon 2020 framework is to ensure sufficient food and feed, while monitoring safety throughout the food chain. In this context, the objective of this paper was to evaluate the efficacy of the routine quality control that honey companies carry out on raw batches (before entering the industrial packaging process) considering the presence of sulfonamides. A total of 279 honey samples were analyzed in this study: 178 raw honey samples were taken on reception in different companies, and 101 samples (from the same industries) were purchased locally. The validation of the methodology applied (LC–MS/MS) before analyzing the samples, confirm the reliability of the results obtained. All the purchased samples were found to be negative for sulfonamides, however, in 9 raw samples sulfathiazole (6 samples) and sulfadiazine (3 samples) were found, which represents 3.4% and 1.7 % of the 178 raw samples analysed, respectively. Therefore, if monitoring is carried out routinely at reception, risk can be decreased to a negligible level. The results confirm that using a suitable analytical methodology and implementing an appropriate routine quality control on reception is

totally effective to avoid the presence of sulfonamides in the commercialized product, thereby ensuring consumer safety.

Keywords: sulfonamides; honey; LC-MS-MS; consumer safety

1. Introduction

Honey is a very healthy, nutritious food, however, in recent years it has been the focus of food alerts due to the presence of chemical hazards such as antibiotics or pesticides. The origin of these residues in honey is mainly veterinary treatments (acaricides, sulfonamides, antibiotics, etc.) required to treat bee parasites and bacterial diseases such as European foulbrood (*Streptococcus pluton*) or American foulbrood (*Bacillus larvae*) which can destroy an apiary, and propagate to other bee-hives very easily; although these compounds are often used in bee-keeping as preventive or therapeutic treatments to protect an apiary (Staub-Spörri, Jan, Cognard, Ortelli & Edder, 2014).

Chemical hazards has become a major concern for the administration and the honey sector due to both the important consequences for public health (allergic reactions, bacterial resistance, changes in intestinal flora, etc.), and the impact on bees. In fact, the European Commission states that if a food-producing animal has to be treated with medicines to prevent or cure disease, the veterinary residues in these food products should not harm the consumer (European Commission, 2007). In the new societal challenges proposed by The EU Framework Programme for Research and Innovation, Horizon 2020 (Commission Decision C 4995 of 22 July 2014, 2014), meeting consumer needs and preferences, but minimising the related impact on health and the environment is included as one of the main goals. The point "Food security, sustainable agriculture and forestry, marine, maritime and inland water research, and the bioeconomy" highlights that research should address food and feed safety, covering the whole food chain and related services

from primary production to consumption. In truth, the control of all stages of the food chain «from farm to fork» is a shared responsibility, including primary production (agricultural and livestock), and industrial processing. It is essential to ensure consumer protection, the last link of the chain. In order to minimize consumer exposure to residues, the Commission requires EU countries to implement residue monitoring plans through official control to monitor the illegal use of substances and misuse of authorized veterinary medicines (Commission Decision C 4995 of 22 July 2014, 2014). Thus, Council Directive 96/23/EC (1996) and Commission Decision 97/747/EC (1997) establish the frequency of sampling and the levels of the groups of substances to be monitored, considering veterinary medicines, pesticides and contaminants in food of animal origin. This situation calls for the development of a quantitative framework based on risk assessment (the tool for science-based decision-making) to estimate the impact on health, and to increase the efficiency and effectiveness of safety evaluations.

Bearing all of this in mind, the objective of the current study was to evaluate the effectiveness of the routine quality control sampling which companies carry out on raw batches of honey (before entering the industrial packaging process) considering the presence of sulfonamides. To this end, both raw samples (unprocessed honey collected randomly from the initial stage of the different industries) and commercialized samples (from the same industries but bought locally) were evaluated. Before analyzing the samples, the methodology applied (LC–MS/MS) was developed and validated to guarantee the reliability of the results. As a first step in the validation process, the matrix effect of the proposed method was studied.

2. Materials and methods

2.1. Chemicals and Reagents

Sulfanilamide, sulfathiazole, sulfamerazine, sulfadiazine, sulfapyridine, sulfamethazine, sulfamethizole, sulfachloropyridazine, sulfamethoxazole, sulfadimethoxine, and sulfaquinoxaline; where purchased from Sigma (Steinheim, Germany), with a purity ≥95% in all cases. Hydrochloric acid (37%), formic acid (FA, 99%), acetonitrile (ACN) and methanol (MeOH) were obtained from Prolabo (VWR, Fontenay-sous-Bois, France); ammonia solution was purchased from Sharlau (Barcelona, Spain) and citric acid monohydrate was acquired from Merck (Darmstadt, Germany). The solid phase extraction (SPE) columns Strata X-CW (33µm, 100 mg, 3mL) were obtained from Phenomenex (Torrance, CA). Ultrapure water was generated in-house from a Milli-Q system (Millipore Corp., Billerica, MA).All reagents were MS, HPLC or analytical grade.

Individual stock solutions of all standards were prepared in methanol at a concentration of 1mg/mL and stored in a freezer at -20ºC, the concentrations were corrected for purity and salt form. The stock solutions were stable for at least 6 months (Kaufmann, Roth, Ryser & Widmer, 2002). A working standard mix solution of the 11 sulfonamides, in a concentration of 1µg/mL, was prepared in water. This solution was used to construct the calibration curves and to prepare the spiking experiments. Before each use it was left to reach room temperature. The stability of the 11 sulfonamides in the mixed working standard solution was checked to ensure that the standard could be stored at +4ºC for at least 3 months, with no decrease in response or degradation.

2.2. Honey samples

A total of 279 multifloral honey samples from the Valencian Region (Spain) were used in this study. 178 of them were taken from the routine quality control sampling which companies carry out on every batch of raw honey before entering the industrial packaging process. The other 101 samples (from the same industries), were purchased locally. All the samples were stored in a dark, dry place at room temperature until analysis.

A mixture of 10 multifloral honeys without the compounds analyzed in this study was selected as a "blank honey" in order to perform the validation procedure of the methodology. Multifloral honeys with very different physicochemical characteristics (colour and texture) were specifically selected in order to cover the widest range of variability, using both light and dark honeys. This is a common procedure used by different authors to obtain a blank honey (Hammel, Mohamed, Gremaud, LeBreton, & Guy, 2008; Martinez Vidal, Aguilera-Luiz, Romero-Gónzalez, & Garrido, 2009; Dubreil-Chéneau, Pirotais, Verdon, & Hurtaud-Pessel, 2014). It is important to point out that our experience on honey analysis, as well as the results observed by other authors, showed that, in general, the types of honey don't affect the accuracy of the method (Dubreil-Chéneau, Pirotais, Verdon, & Hurtaud-Pessel, 2014). Although in specific cases some modifications could occur to certain analyte signals (ion suppression or enhancement) for particular types of honey, these differences are less important than those due to the intrinsic inter-day variation of the method (Dubreil-Chéneau, Pirotais, Verdon, & Hurtaud-Pessel, 2014). Notwithstanding this, in the case of very dark honeys, like chestnut honeys, a matrix effect for some analytes could be observed (Galarini, Saluti, Giusepponi, Rossi, & Moretti, 2014). This may lead to the conclusion that for the specific case of very dark honeys it would be advisable to use this same type of honey as a "blank honey".

2.3. Sulfonamide extraction method in honey

Samples of honey (1.0 g) were placed in beaker flasks. The fortified samples were prepared by adding the mixed working standard solution (1μg/mL) to the blank honey to obtain the appropriate levels for validation of the method. Then, they were shaken well and allowed to stand for at least 1 hour to permit sufficient absorption of the different standards. After addition of 1mL 0.1M HCl, the samples were dissolved using a magnetic

stirrer and left to stand at room temperature for at least 20 minutes to allow hydrolization of the sulfonamides (80-90% of sulfonamides are bound to sugars). Then, 5 mL of 3M citric acid were added and stirred for 30 s. Next, 5mL of the honey solution was passed through the SPE column, previously conditioned with 3mL of MeOH and 3mL of ultrapure water. The cartridges were then washed by adding 3 mL MeOH/ACN (1/1) twice. The cartridges were vacuum drained, by passing air through them, for 2 min at a pressure of 10 mmHg. The elution was accomplished with 3 mL of 2% ammonium hydroxide in MeOH, and the analytes were collected in 6mL glass tubes. The SPE procedure was performed in a Lichrolut vacuum manifold coupled to a vacuum pump (Merck, Darmstadt, Germany). Finally, the eluates were evaporated to dryness under a stream of nitrogen while being maintained at 40 ºC in a thermostatic bath (Grant GR, Cambridge, England). After evaporation, 100 µL of mobile phase was added to each tube, and thoroughly mixed to ensure the complete dissolution of the extract. Finally, the re-dissolved extracts were injected into the LC-MS/MS system.

2.4. LC/MS/MS Analysis

The chromatography system consisted of a HPLC Agilent 1200 Infinity Series coupled to an Agilent 6420 Triple Quadrupole detector, equipped with a source set in positive electrospray ionization mode. The column used was a Zorbax Eclipse XDB-98 (4.6 x 50 mm, 1.8 microns) supplied from Agilent.

Chromatographic separation was carried out with a mobile phase consisting of 0.5% formic acid in water (mobile phase A) and ACN (mobile phase B) with a flow rate of 0.4 mL/min. The gradient used started with mobile phase A at 20%, then at minute 4 was 30%, reaching 40% at minute 7. These conditions were maintained until minute 9. After that, the system was left for 4 min to re-equilibrate before the next injection. The oven column was set at 30 ºC, and the injection volume was 5 µL.

147 The system was equilibrated at the beginning of each day for 1h, and three injections of
148 the standard solution were made to check its stability and the response of the equipment,
149 a solvent blank was then injected to assess the cross-talk. The operating parameters for
150 the mass spectrometer were as follows: capillary voltage 4 kV; source temperature 350
151 ºC; nebulization gas (nitrogen) at a flow rate of 12 L/min and collision gas (nitrogen) at
152 a flow rate of 3 L/min and 40 psi. The optimization of the MS/MS operating parameters
153 were performed by the automatic optimization function of the MS software (Optimizer,
154 Agilent), using direct infusion, without column, of the mixed working standard solution
155 of the 11 sulfonamides, at a concentration of 40 μg/L. The most important LC-ESI-MS
156 parameters for the acquisition and identification of the 11 target compounds are
157 summarized in Table 1.

158 *2.5. Matrix effect evaluation and quantification*

159 Food matrices can vary in terms of complexity and content, and it is well established that
160 co-eluting matrix constituents may interfere with the ionization process of the analytes
161 (Sterner, Johnston, Nicol & Ridge, 2000; Lopez, Pettis, Smith, & Chu, 2008). In order to
162 evaluate the matrix effect, the calibration curve in solvent should be compared with the
163 calibration curve in matrix, and a quantitative measure of the ion suppression or
164 enhancement can be obtained comparing the peak areas of the analyte standards in solvent
165 and the peak areas of the analyte standards spiked in honey before extraction.

166 The calibration method of standard addition was used to quantify the sulfonamides.
167 Therefore, a 7-point standard curve (including zero) was constructed for each
168 sulfonamide by plotting the peak area of the SRM transitions showing the most intense
169 signal of each analyte versus its nominal concentration. As honey has no MRLs
170 (maximum residue levels) for these compounds, the European Commission (Regulation
171 (EC) No 470/2009) states that if sulfonamides are present, they must be below the limit

of quantitation according to the analytical method used. Due to the fact that this limit differs between laboratories and that there is no legislation or official recommendation, in this study the target limit considered was 10 μg/kg, as this is the most demanding action limit or tolerated level found in the literature in Europe (Muñoz de la Peña, Mora Diez, Mahedero García, Bohoyo Gil, & Cañada-Cañada, 2007; Sajid, Na, Safdarb, Lu, Ma, Hec, & Ouyanga, 2013). In addition to this, a further lower level of 5μg/kg was considered in order to evaluate values lower than the target limit.

Therefore, to construct the curves, the blank honey was fortified with 0, 5, 10, 20, 40, 60 and 100 μg/kg of each compound, injected in duplicate into the LC-MS/MS system. This process was carried out in triplicate.

To identify a sample as positive, three criteria were considered: first, the signal-to-noise ratio (S/N) of the product ions selected must be greater than or equal to three; second, the allowable deviation of the retention time of the target matter and that of its corresponding standard should be within ±2.5%; third, the allowable deviation of the relative abundance of the characteristic ions of the target matter and those of the characteristic ions corresponding standard should be within ±20%.

2.6. Validation of the sulfonamide analytical method in honey

The analytical methodology applied in this work was validated as a first step in order to ensure the reliability of the results for every compound in the quantification range considered. The present validation study was performed in accordance with Commission Decision 2002/657/EC (2002). To this end several parameters were studied: selectivity, linearity, recovery, precision (repeatability or intraday precision "RSDr" and reproducibility or interday precision "RSD_R"), accuracy, decision limit (CCα) and detection capability (CCβ).

196 The selectivity, or ability of the method to differentiate and quantify each analyte in the
197 presence of potentially interfering substances in honey samples, was evaluated by
198 analyzing the blank honey 20 times. To this end, the absence of any interference in the
199 segment of the retention window of each product ion was verified for each analyte.
200 Linearity (R^2) was tested in the 0-100 μg/kg range drawing seven-point calibration curves
201 for fortified honey blanks. The accuracy of this method was evaluated through recovery
202 experiments, carried out by spiking a honey blank with aliquots of the mixed working
203 standard solution before the extraction procedure to obtain the seven concentration levels
204 (0, 5, 10, 20, 40, 60 and 100 μg/kg). Six replicates were performed at each level.
205 Recoveries for each analyte were determined by comparing the concentrations obtained
206 from the calibration curves for the fortified blanks with their nominal concentrations.
207 Recovery was measured as a percentage and RSD. The precision of the developed method
208 was evaluated in two stages: intra-day precision (RSDr) and inter-day precision (RSD_R).
209 To determine intraday precision six samples per level were spiked just before analysis
210 and extracted on the same day, at the same levels as for recovery. The experiment was
211 repeated on two further days to determine inter-day precision. Intra-day precision was
212 expressed as the RSD of samples extracted the same day, at the same concentration, inter-
213 day was expressed as the RSD of samples extracted on different days, at the same
214 concentration.

215 CCα (decision limit) was carried out by analyzing the blank honey 20 times and
216 calculating the signal to noise ratio at the time window in which each analyte was
217 expected. Three times the signal to noise ratio was used as the decision limit.
218 CCβ (detection capability) was determined by analyzing the blank honey fortified with
219 the analytes at the decision limit at least 20 times. Detection capability is equal to the

value of the decision limit plus 1.64 times the corresponding standard deviation of the within-laboratory reproducibility.

With the strategy described above, linearity, recovery, and precision were determined through 42 measurements.

3. Results

3.1. Matrix effect

LC-MS/MS detection is considered to be the best tool for good selectivity and speed of analysis (Cirić, Prosen, Jelikić-Stankov, & Durdevic, 2012). However, it should be taken into account that the results may be adversely affected by lack of selectivity (Rogatsky & Stein, 2005) due to ion suppressions or ion enhancement caused by the sample matrix, interferences from metabolites, and "cross-talk" effects (Matuszewski, Constanzer, Chavez-Eng, 2003). Because the matrix effect may compromise the quantitative results as well as the reproducibility of the method, as a first step in the validation process, the matrix effect (ME) of the proposed method was carefully studied and calculated as described in Eq.1. If ME(%)=100, no matrix effect is present; if ME(%)>100 there is a signal enhancement and if ME(%)<100 there is a signal suppression.

$$ME\ \% = \frac{peak\ area\ of\ standard\ in\ solvent}{peak\ area\ of\ standard\ spiked\ before\ extraction} \times 100 \quad (1)$$

Three nominal concentration levels were considered to calculate the matrix effect: low-level=5 μg/kg, medium level= 20 μg/kg and high level= 40 μg/kg (Table 2) (Sajid, Na, Safdarb, Lu, Ma, Hec, & Ouyanga, 2013). All the analytes showed a signal enhancement for the 3 concentration levels to a greater or lesser extent. Sulfanilamide was the least affected by the matrix effect because ME was 100% at the highest concentration level, and very close to it at the lowest and medium concentration levels (113 and 114, respectively). On the contrary, sulfamethoxazole with values of 463, 354 and 316, showed

244 the most marked signal enhancement effect. To estimate the matrix effect it is also
245 possible to compare the slopes of calibration plots built both for the standards in methanol
246 solution and for the standards additions in blank honey samples, which is more visual
247 (Taylor, 2005; Gosetti, Mazzucco, Zampieri & Gennaro, 2010). As an example, Figure 1
248 shows these calibration curves obtained for sulfanilamide and sulfamethoxazole.

249 Due to the fact that an enhancement phenomenon was observed in this study for the eleven
250 sulfonamides studied, it was decided to carry out the quantification step using the standard
251 addition method (that is to say, quantification based on matrix-spiked calibration
252 solution). In this way, the matrix effect was efficiently minimized (Economou, Petraki,
253 Tsipi & Botitsi, 2012).

254 *3.2. In-house validation method*

255 The analytical methodology used to perform the sulfonamide analyses of the honey
256 samples was subjected to an in-house validation method. The selectivity, as mentioned
257 before, was evaluated by comparing 20 chromatograms obtained from the analysis of the
258 corresponding blank honey sample and those obtained from blank honey fortified with
259 11 sulfonamides. Figure 2 shows as an example the selected reaction monitoring (SRM)
260 chromatogram of a blank honey and the same honey fortified at 20 μg/kg with all the
261 sulfonamides studied. The absence of interference, which could compromise the
262 identification and quantitation of the analytes, was verified near the retention time of each
263 sulfonamide. Regarding the linearity, the results demonstrated that in the range studied
264 5-100 μg/kg, the method showed a good linearity for all the sulfonamides, with a linear
265 coefficient between 0.993 and 0.999. This is considered adequate according to the
266 recommendations of regulatory agencies such as the Commission Decision 2002/657/EC
267 (2002)

The data about the other validation parameters are shown in Table 3. These parameters provide information regarding the recovery, precision (repeatability or intra-day precision RSDr and reproducibility or inter-day precision RSD_R), decision limit (CCα) and detection capability (CCβ). The recoveries of all sulfonamides were in a range of 89-114%, complying with the requirements of the Commission Decision 2002/657/EC (2002), which concludes that the proposed method shows good accuracy for all the studied analytes. The repeatability (RSD_r) for all the sulfonamides studied ranged from 3.0 to 19.5%, in agreement with this Commission Decision. In the case of reproducibility (RSD_R), for 6 of the 11 compounds this parameter was below the required value: lower than 20%. In a few cases for the other four compounds, this parameter was exceeded slightly but its value was very close to 20% (always lower than 24%): Sulfanilamide at the 10 μg/kg level showed 22.8 %; sulfamerazine at the 20 μg/kg level showed 22.3%; sulfachloropiridazine 20.8% at the 10 μg/kg level, sulfamethazine at the 10 and the 20 μg/kg levels showed 23.7 and 22.1% and sulfaquinoxaline 22.7 and 22.9% at the 20 and the 40μg/kg level respectively. Therefore it can be concluded that the method used has good precision (repeatability and reproducibility) (Bohm, Stachel, & Gowick, 2013).

The limit of decision (CCα) values ranged from 0.7 μg/kg (sulfamethoxazole) to 4.5 μg/kg (sulfamethazine) and the detection capability (CCβ) limit from 2.3 μg/kg (sulfamethoxazole) to 4.3 μg/kg (sulfadiazine). It is noticeable that in all the cases the 2 limits are below 5 μg/kg, which is the target minimum level in this paper, as mentioned before.

The results of the validation demonstrate that the applied analytical procedure guarantees the quantitative values of sulfonamides in the samples analyzed.

3.3. Samples analyses

Of the 279 honey samples analysed for the presence of 11 sulfonamides, 64% of them were from the routine sampling of every batch of raw honey which companies realize before the industrial packaging process and the other 36% samples (from the same industries), were purchased in local shops. The values of the percentage of positive samples and quantitative results of the sulfonamides found are shown in Table 4. The sulfonamide levels reported are the mean of three replicates obtained by subjecting the sample to the extraction and the analysis process. This was done to confirm that the results were not derived from incidental sample contamination. All the purchased samples had a "negative result" for all the sulfonamides, which means that the values obtained were under the CCα. On the contrary, in 9 raw samples sulfathiazole (6 samples) and sulfadiazine (3 samples) were found, which represents 3.4 % and 1.7 % of the 178 raw samples analysed, respectively. For sulfathiazole the levels ranged between 5 and 9 μg/kg, whereas for sulfadiazine a minimum of 13 μg/kg was found and a maximum that exceeded the maximum limit of quantification (100 μg/kg).

4. Discussion

The "positive" samples found in the present work on raw honey are clearly in violation of current European directives. Although in the Commission regulation (Commission Decision 2010/37/EC, 2010) for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin MRLs are not included for honey, some EU countries have established internal MRLs for some substances. In the case of sulfonamides (based on the sum of sulfonamide family), the permitted level ranges from 20 μg/kg in Belgium to 50 μg/kg in the UK (Maudens, Zhang & Lambert, 2004). There is an obvious discrepancy between countries which affects commercial transactions. At present the limit for sulfonamides and other antibiotics in honey is established taking into account the limit of quantification of the methodology used. For

317 instance, 10 μg/kg for sulfonamide in honey, a value which is only attained by techniques
318 such as LC-MS/MS (Sheridan, Policastro, Thomas & Rice, 2008; Martinez Vidal,
319 Aguilera-Luiz, Romero-Gónzalez & Garrido, 2009). In fact, the afore mentioned Council
320 regulation (Commission Decision 2010/37/EC, 2010) recognizes that it is becoming
321 easier to detect the presence of residues of veterinary medicines in foodstuffs (meat, fish,
322 milk, eggs and honey) at ever lower levels as a consequence of scientific and technical
323 progress.

324 In other studies carried out with a similar detection technique to that used in the present
325 work, sulfathiazole is also one of the most present sulfonamides in honey. For instance,
326 in 116 honey samples analyzed from Eastern Europe sulfathiazole was present in 47% of
327 the cases (Sheridan, et. al, 2008). In a set of honey samples from the USA, Asia and
328 Europe, the presence of sulfadiazine, among other sulfonamides, and the presence of
329 sulfathiazole in 2 samples (Hammel, Mohamed, Gremaud, LeBreton, & Guy, 2008). In
330 the report "Monitoring of veterinary medicinal product residues and other substances in
331 live animals and animal products" published by European Food Safety Authority about
332 the results obtained in 2011 it is noteworthy that the highest frequency of non-compliant
333 samples for antibacterials (including sulfonamides) was observed in honey (European
334 Food Safety Authority (2013). In relation to the specific case of sulfonamides the study
335 mentions positive cases in 4 out of 129 samples from Poland. Sulfadimethoxine was
336 found in 2 out of 67 samples from Hungary and sulfathiazole in 1 out of 5 from Lithuania.
337 However, it is important to mention that in some countries there are specific control
338 programs, applied to different live animals and animal products, which use
339 microbiological tests (inhibitor tests), and sometimes the positive results are not
340 confirmed by the most appropriate technique and thus there is no conclusive
341 quantification of the substance concerned (European Food Safety Authority, 2013).

342 More recent results were reported by Galarini, Saluti, Giusepponi, Rossi, & Moretti,
343 (2014) based on 74 honey samples acquired in the Italian market. The samples had both
344 different botanical and geographical origins (such as Italy, Hungary, Argentina, Bulgaria,
345 Romania, Spain, and other EU and non EU countries). 12% of the samples analyzed by
346 LC-MS/MS had traces of sulfonamides. More specifically, in 5 samples concentrations
347 between 0.3 to 1.7 μg/kg of sulfathiazole, and in 4 samples concentrations between 0.2 to
348 1.7 μg/kg of sulfadimethoxine were confirmed. These authors pointed out that their
349 results were in agreement with those reported by the Italian National Reference
350 Laboratory for Beekeeping. This laboratory analyzed over 1500 honeys during a time
351 period of six years, observing that 11% of the samples contained sulfonamide residues.

352 In Spain the most recent data from the official monitoring of antibiotics in honey are
353 published by the Spanish Agency for Food Safety in the 2012 and 2013 reports (AESAN,
354 2012; AESAN 2013). In both years no antimicrobials were detected in more than 700
355 honey samples analyzed every year.

356 The above mentioned information shows that although the use of sulfonamides in
357 beekeeping is banned in the European Union, the occurrence of residues of these
358 compounds in honey samples is significant when sensitive analytical methods are used.
359 However, when these compounds are present in honey they occur at very low levels, even
360 lower than in the tissues of farm animals. Therefore they are not important from a
361 toxicological point of view (Baran, Adamek, Ziemian, & Sobczak, 2011) given that honey
362 is consumed in very small quantities. In this context, it is possible to estimate the risk to
363 the consumer associated with the presence to this chemical hazard in honey. This risk to
364 the consumer is defined as a combination of the probability of occurrence of a hazard and
365 the severity of this hazard in terms of human health: Risk=Probability*Severity
366 (FAO/WHO 1995; Doménech, Escriche & Martorell, 2007). Asselt, Spiegel, Noordam,

Pikkemaat & Fels-Klerx (2013) considered that this probability must be established as the probability of consumption and the probability of exposure. They estimated the severity of a hazard associated with an antibiotic residue as both the intrinsic toxicity of the antibiotic and the consequences for human health related to the development of antimicrobial resistance. Taking this into account, these authors attributed scores to the above mentioned factors for antibiotics in different foods, including sulfonamides in honey, assigning a value in the range 0-3 (where 0 is low and 3 is high) for every factor. In the case of sulfonamides in honey, they established a value of 1 for the severity. Considering this value and the results obtained in the present paper, the risk to the consumer associated with eating commercial honey samples is 0 because no positive samples were found. However, this value would be 1 if the companies did not monitor the raw honey samples before the industrial packaging process as 3.4% and 1.7% of the analyzed raw honey samples were positive for sulfathiazole and sulfadiazine, respectively. This value concurs with the European Food Safety Authority report (2013), which remarked that a real risk of exposure to different sulfonamides in honey exists. Therefore, if adequate monitoring is carried out routinely at reception, the risk can decrease from 1 to 0 in a range of 0 to 3. This highlights the importance of the routine quality control that each company is expected to carry out.

5. Conclusion

A quantitative LC-MS/MS method for the determination of 11 sulfonamides in honey was validated with good results in agreement with the Commission Decision 2002/657/EC (2002), which confirm the results of sulfonamides obtained in the honey samples analyzed. Monitoring the raw honey samples, before the industrial packaging process, showed that a real risk to the consumer exists due to the presence of sulfonamides. However, the results from honey sampled at retail confirmed that correct

monitoring by the company is able to reduce the risk to an acceptable level. To sum up, the results indicate that using a suitable analytical methodology and implementing the routine quality control that each company is expected to carry out, it is possible to avoid the presence of sulfonamides, and therefore to ensure consumer safety. This can be extrapolated to other chemical hazards that could be present in any kind of honey.

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Table 1. MS/MS operating parameters used in Sulfonamide analysis.

Analyte	Quantification Transition	Confirmation Transition	Fragmentor	CE[a](V)	RT[b] (min)
Sulfanilamide	173.0>93.1	173.0>156.1	100	5/15	1.78
Sulfathiazole	256.0>156.1	256.0>92.1	91	8/24	2.36
Sulfadiazine	251.5>156.0	251.5>92.1	91	12/24	2.45
Sulfapyridine	250.1>156.1	250.1>92.1	121	12/28	2.55
Sulfamerazine	265.1>172.1	265.1>92.1	121	12/28	2.95
Sulfamethazine	279.1>186.1	279.1>124.1	121	12/24	3.40
Sulfamethizole	271.0>156.1	271.0>92.1	120	8/24	3.59
Sulfachloropyridazine	285.0>156.0	285.0>92.1	91	8/24	5.60
Sulfamethoxazole	254.0>156.1	254.0>108.1	91	12/24	6.50
Sulfadimethoxine	311.1>156.1	311.1>124.1	121	16/32	8.60
Sulfaquinoxaline	301.1>156.0	301.1>108.1	121	12/24	8.70

[a] =collision energy
[b] = retention time

546 **Table 2.** Matrix effect of the 11 sulfonamides studied.

	Nominal Concentration		
	Low-level: 5 μg/kg	**Medium-level:** 20 μg/kg	**High-level:** 40 μg/kg
Sulfanilamide	113	114	100
Sulfathiazole	179	206	185
Sulfadiazine	183	173	181
Sulfapyridine	212	168	152
Sulfamerazine	294	262	242
Sulfamethazine	340	278	270
Sulfamethizole	329	313	340
Sulfachloropyridazine	335	322	323
Sulfamethoxazole	463	354	316
Sulfadimethoxine	398	349	241
Sulfaquinoxaline	209	149	135

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551 **Table 3**. Validation parameters for the analytical method.

Analytes	**Nivel (μg/kg)**	**Recovery %**	**RSD_r[a] %**	**RSD_R[b] %**	**CCα (μg/kg)**	**CCβ (μg/kg)**
Sulfanilamide	5	98	15.3	12.4	1.5	3.1
	10	105	16.9	22.8		
	20	95	10.5	17.8		
	40	89	3.5	16.5		
	60	104	10.9	14.8		
	100	104	7.3	9.0		
Sulfathiazole	5	114	4.6	14.2	2.4	4.0
	10	108	4.5	11.6		
	20	96	7.2	14.0		
	40	95	8.6	16.6		
	60	93	7.3	14.6		
	100	98	8.5	10.3		
Sulfadiazine	5	104	9.9	8.9	2.8	4.3
	10	106	13.8	14.8		

	20	97	10.8	18.4		
	40	94	3.5	11.9		
	60	98	5.1	7.2		
	100	104	6.0	8.1		
Sulfapyridine	5	101	6.2	9.6	2.2	3.0
	10	104	6.1	18.9		
	20	98	7.0	18.1		
	40	99	3.9	15.4		
	60	95	5.6	7.3		
	100	102	4.6	13.3		
Sulfamerazine	5	109	7.4	6.8	1.4	3.0
	10	101	4.5	19.5		
	20	96	9.7	22.3		
	40	89	8.8	15.0		
	60	97	6.3	10.1		
	100	101	6.6	14.1		
Sulfamethazine	5	93	7.5	16.3	4.5	4.1
	10	110	6.3	23.7		
	20	96	10.5	22.1		
	40	99	9.2	17.8		
	60	95	6.6	8.1		
	100	101	6.3	17.7		
Sulfamethizole	5	99	5.7	12.5	2.0	3.0
	10	101	6.8	8.1		
	20	96	6.5	13.5		
	40	97	9.6	15.2		
	60	98	16.9	17.1		
	100	102	4.5	7.4		
Sulfachloropyridazine	5	109	10.3	11.5	2.0	3.5
	10	101	6.3	20.8		
	20	95	7.3	18.7		
	40	97	8.8	17.0		
	60	98	8.8	11.9		
	100	101	4.9	16.3		
Sulfamethoxazole	5	101	11.3	11.5	0.7	2.3
	10	101	8.6	16.3		
	20	95	10.2	19.3		
	40	93	6.5	10.5		
	60	99	6.6	10.3		
	100	101	5.4	10.0		
Sulfadimethoxine	5	94	8.6	11.6	2.0	3.5
	10	98	9.4	17.2		
	20	97	11.0	19.9		
	40	99	3.0	13.9		
	60	100	6.9	9.9		
	100	101	3.2	15.7		
Sulfaquinoxaline	5	89	6.4	12.2	2.4	3.9
	10	93	8.5	19.4		
	20	96	8.8	22.7		
	40	100	12.6	22.9		
	60	98	19.5	16.4		
	100	99	7.7	17.9		

552 [a]RSD_r = repeteatability
553 [b]RSD_R = reproducibility

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555 **Table 4.** Samples analyzed, percentage of positives for sulfonamides and concentration range.

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	Honey Samples purchased locally	Raw honey samples from the routine quality control in companies	Concentration range (µg/kg) of the positive compounds
Number of analysed samples	101	178	
Number of positive samples	0	9	
Percentage of *samples positive for sulfathiazole*	0%	3.4%	5-9
Percentage of samples positive for *sulfadiazine*	0%	1.7%	13-(100≤)

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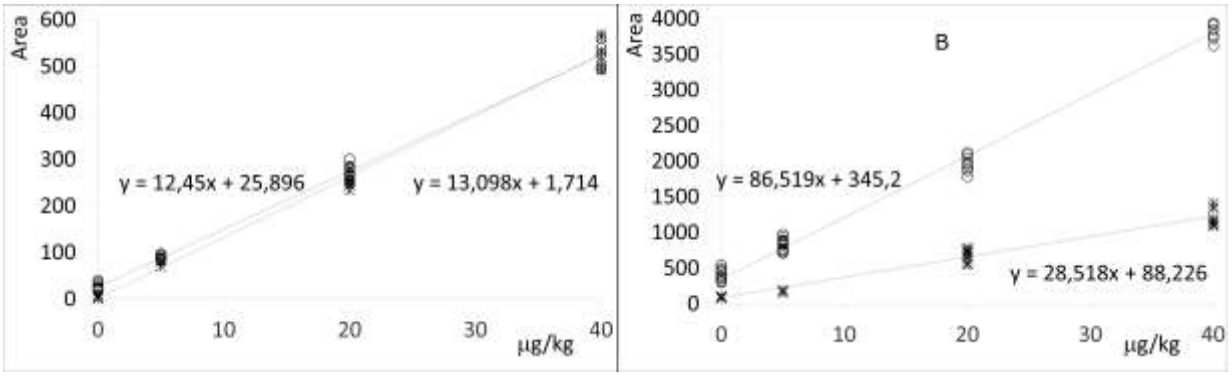

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562 **Figure 1.** Calibration curves obtained for sulfanilamide (A) and sulfamethoxazole (B) in
563 methanol solution (white circles) and in standard additions in blank honey sample
564 (asterisks).

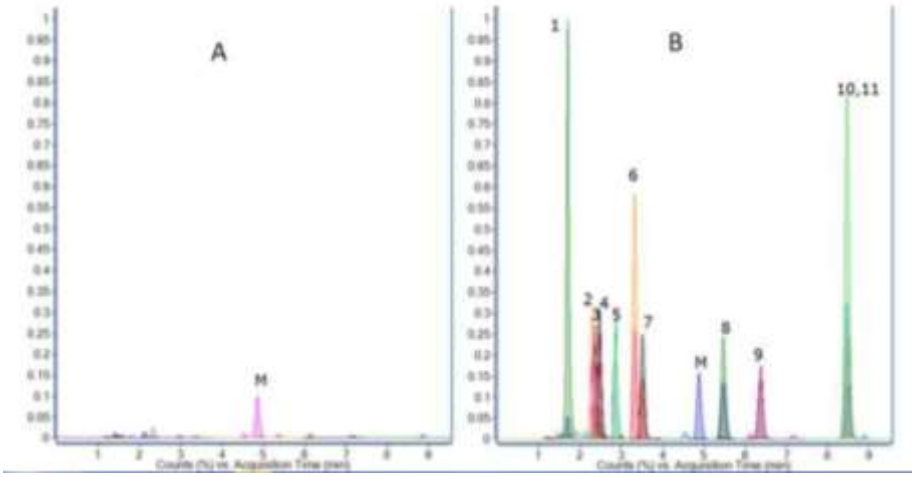
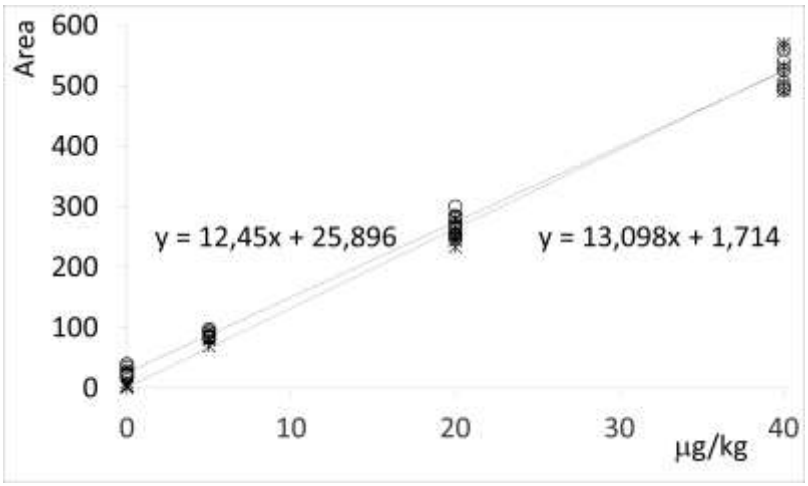


Figure 2. Selected reaction monitoring (SRM) chromatogram of a blank honey extract (A) and the same honey fortified at 20 μg/kg (B) with all the sulfonamides studied. (1) Sulfanilamide; (2) Sulfathiazole; (3) Sulfadiazine; (4) Sulfapyridine; (5) Sulfamerazine; (6) Sulfamethazine; (7) Sulfamethizole; (8) Sulfachloropyridazine; (9) Sulfamethoxazole; (10) Sulfadimethoxine; (11) Sulfaquinoxaline; (M) Matrix


Area
600
500
400
300
200
100
0
y = 12,45x + 25,896
y = 13,098x + 1,714
0
10
20
30
40
µg/kg