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# SIMULTANEOUS SEARCH OF MULTIPLE GROUPS OF ANTIBIOTICS AND THEIR DIFFERENT COMPONENTS IN THE HONEY

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**Abstract**

The honey is a bee (*Apis mellifera*) product formed by the chemical change of the nectars that the bees collect from the flowers. Honey is more or less affected by the antibiotics used in bee diseases. This contagion has vital importance in terms of public health. For this reason, simultaneous and rapid detection of different antibiotic groups in the remnants of used veterinary medications is important. Simultaneous identification of different groups of antibiotics depends on the detection method of each group, the performances of the devices, and the success of the validation. This study was carried out to realize the identification, validation and method development of the multiplex-group antibiotic residues, in the direction of EU Directive 2002/657 EEC. A different method has been developed for a total of 35 antibiotics from many groups, and the detection limits, deviations, LOD, LOQ and validations are presented. It has been identified in this study that the simultaneous detection and validation of multiplex-groups of antibiotics, which have not yet been routinely practised, is possible. With this study, a unique method and a validation technique, which may be used by national competent authorities, has been developed.

**Keywords:** Honey, Residue, Antibiotic, Honeybee, Method

## 1. Introduction

Honey is a very valuable bee (*Apis mellifera*) product that has a high nutritional value and thus has a high consumption rate. It is a nutritional material, which is roughly composed of 90-95% (monosaccharides 15-20%, disaccharides, 0.1- 4.8, oligosaccharides) 0.5-6%, minerals, 0.1-2%, amino acids, 0.2-0.7%, acids, 3.5-6.5% and 3-10% other substances in the sugar dry matter (Derebaşı et al., 2014).

It has been suggested that toxic contamination can be caused by antibiotics and pesticides used for the treatment of bee diseases, or through agricultural pollution, in the direction of industrial and technological development that takes place in our era (Kay, Addlestone, & Arnold, 2009; Lewicki, Reeves, & Swan, 2009; Nebot et al., 2012; Guo-Fang Pang et al., 2003). The Acceptable Daily Intake (MIA) and Maximum Residue Level (MRL) for each of the residues in all food items, including honey, are determined in accordance with EFSA (European Food Safety Authority) and FDA (Food and Drug Administration), which are considered to be the sources. The methods of detection of these criteria in the laboratory, the

lowest amounts that the contamination can be detected (Limit of Detection = LOD), detection limits and standards for these limits have also been determined (Bogdanov, 2006; Galarini, Saluti, Giusepponi, Rossi, & Moretti, 2015; Gómez-Pérez, Plaza-Bolaños, Romero-González, Martínez-Vidal, & Garrido-Frenich, 2012; Granja et al., 2009; Hammel, Mohamed, Gremaud, LeBreton, & Guy, 2008; Jing et al., 2009; Joint & Additives, 2006; Kay et al., 2009).

Each country has its own criteria, which are identified with regulations, for the reference values of contaminants in honey. These reference values are generally based on FDA and EFSA and are the end result of extended laboratory analyzes. Despite all prohibitions and restrictions, antibiotic residues were found on an average of 75% in honey in the European Union (EU) countries. 90% of the 1714 samples tested for the sulfonamide group had an average of 10-11 ppb sulfadimidine, and 90% of the 1425 samples tested for the tetracycline group had tetracycline content lower than 13.65 ppb (Adams et al., 2007; Benetti, Piro, Binato, Angeletti, & Biancotto, 2006; Bernal, Nozal, Jiménez, Martín, & Sanz, 2009; Joint & Additives, 2006; Kay et al., 2009; Schutz, Moy, Kaferstein, & Organization, 1998).

The European Union (EU) announced that it does not allow the Maximum Residue Level (MRL) value for any drugs other than licensed medicines in honey and other beekeeping products (Joint & Additives, 2006; Olaitan, Adeleke, & Iyabo, 2007). Despite the prohibitions, antibiotics such as tetracycline and chloramphenicol, especially streptomycin and sulphonamides, have been reported to be present in the contents of about 20-50% of the imported honey in France, Belgium and Switzerland (Bargańska, Namieśnik, & Ślebioda, 2011; Bogdanov, 2006; Gómez-Pérez et al., 2012; Nozal et al., 2008). It has also been determined that about 20-50% of the imports to France, Belgium and Switzerland contain antibiotics such as streptomycin, sulfonamide, tetracycline and chloramphenicol (Bogdanov, 2006; Hammel et al., 2008; Moreno-Bondi, Marazuela, Herranz, & Rodriguez, 2009).

Today, reference values in almost all over the world are based on the values determined by FDA and EFSA, prepared according to extended laboratory analyzes and are enacted as the result of successive processes. The obtained residual values were determined for each member of different group contaminants separately and by different methods. Technical considerations such as verifiability, repeatability, precision, reproducibility, measurement and value of the determined quantity, and the smallest quantifiable unit in case of repetition have been taken into account as well as the accuracy of the obtained data.

Analyzes made on honey were in the form of searching for the same group of antibiotics, as well as searching for many groups simultaneously. However, it has also been suggested that analytical conditions must be provided separately for each group, after searching for more complicated and simultaneous different extractions of residues of many groups of substances (Gaudin, Hedou, & Verdon, 2013; Olaitan et al., 2007; Guo-Fang Pang et al., 2003; G-F Pang et al., 2006; Peres, Rath, & Reyes, 2010).

In recent years, numerous studies have been carried out on the identification and determination of multiple groups by working on all of them together. For example, since testing several members of several groups together requires different analytical procedures, although it is relatively easy to identify the values of the group members of each group such as amphenicols, macrolides, tetracyclines, macrolides, sulfonamides, it is suggested that it poses serious problems both in terms of the methods to be used and the laboratory analytics (Joint & Additives, 2006; Kochansky, 2004; Song et al., 2014).

In our study, residues of 41 different types of antibiotics from 7 different groups (9 quinolones, 4 tetracyclines, 2 penicillins, 18 sulfonamides, 3 amphenicols, and 2 others), among the bee products (bee pollen, royal jelly, propolis, bee venom and apiair), which are extremely important in terms of community health and pediatrics and literally used as

medicines in a way helping the cure and supporting the nutrition, together with honey. This is the first study of researching 41 types of antibiotics from different groups in our country on the approach that they can be investigated simultaneously. When the literature is considered, this is the first study to analyze the highest number of group members in the same groups (quinolones and sulfonamides) in multiple analyzes. Regarding this study on developing a new method, the EU norms 2002/657/EC have been taken as the basis. All the operations that are to be done in the development of a method have been applied in stages by interrogating and controlling each of the step.

## 2. Materials and Methods

### *2.1. Material*

Important antibiotics used in the treatment of honey were extracted according to the following method and analyzed by the liquid chromatography-mass spectrometer (LC-MS/MS Triple Quadrupole). The analysis and extraction method are validated in accordance with the 2002/657 EEC Directive with regard to the obtained data.

### *2.2. Methods*

#### *2.2.1. Extraction method*

After taking 5 g of honey from the sample, it was placed in 50 ml of polypropylene tubes, heated at 50°C for 30 minutes, and the internal standard and 5 ml of deionized water was added. It was then dried at 50°C for 30 minutes, and then vortexed for 30 minutes. After adding 10 ml of acetonitrile into this mixture, the new mixture was vortexed again for 20 seconds. Then, 2 g of NaCl was added and the mixture was stirred for at least 30 seconds. Then, it was centrifuged at 4350 G for 10 minutes, and the supernatant was transferred to 15 ml polypropylene tube. The next step was to re-elute the unextracted molecules. 5 ml of hexane was added again and vortexed for 30 sec, then the hexane part was discarded. This process has been repeated twice. It was then evaporated under nitrogen at 60°C. After evaporation, the residue obtained was dissolved in 200 µl of deionized ACN-water mixture and was injected into the LC-MS/MS device.

By using this extraction method, 35 different groups of antibiotics were analyzed and the analysis of the members of the indicated antibiotic groups was completed and confirmed at once. The obtained data were evaluated according to the directive of the European Commission, dated August 2, 2002 and named *"Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results"*, and the method of analysis was verified.

In our study, we used consumables such as Demeclocycline hydrochloride hydrate (Vetranalfluka®), roxithromycin for system suitability (EuFluka®), 25 ml and 50 ml centrifuge tube (pp-screw capped), 10 ml standard bottle (glass, screwcapped), HPLC vial (300 µL); and the analytical standards such as Josamycin, Oxolinic acid, Sarafloxacin, Norfloxacin, Difloxacin, Oxacillin, Chloramphenicol, Sulfamethazine, Sulfisoxazole, Sulfamethizole, Sulfadoxine, Sulfamonomethoxine, Sulfamethoxypyridazine, Sulfachloropyridazine, Norfloxacin.

Antibacterials to be analyzed were optimized in LC-MS/MS device and determinations about MRM (multiple reaction monitoring) values were performed in the first place. In this quantitation study, it was attempted to specifically obtain the transitions of the parent ion and fragment ion couple, which is specifically identified for the analyte. This has enabled us to scan the m/z values of only the desired ions during the scanning process. Determinations regarding the time of arrival of the degradation products were made by using the obtained data and the antibacterials.

### *2.2.2. LC-MS/MS Optimization and Analysis*

The graphs and tables of the linear values obtained from the injections are presented together with the optimization values (Table 1-2). The requirements such as the device used for the study, analyte, column, and method randomization scales are also given below. The determination of target analytes was performed using a Liquid Chromatography Mass Spectrophotometer (LC) coupled to an electrospray ionization Tandem Mass Spectrometer (LC-ESI-MS/MS). Chromatographic separation was carried out using Agilent Technologies 1260 Infinity series HPLC with a dual pump, a degasser and a thermostatted column section. Firstly, the target analytes were optimized. Optimized standards were mixed and sent to the HPLC column (Waters Acquity UPLC C18 1.7µm 2.1x100 mm). A gradient method was performed for the dissolution of the materials. Chromatographic separation for both methods was carried out at 400°C using an Acquity UPLC C-18 column (2.1x100 mm, 1.7µm) and using 0.01% formic acid (FA), 1 mM oxalic acid and 1 mM Ammonium Formate (A) in ionised water and 0.02% FA (B) in ACN as the mobile phase. The flow rate of the solvents was adjusted 2 ml/min, as 0 - 15% B for 5-7 minutes, 20% B for 7-11 minutes, 40% B for 11-15 minutes, 60% B for 15-16 minutes, and 10% B for 16-20 minutes for the gradient separation program, with a total analysis time of 20 minutes. 5 µL injection was then performed in the LC-MS/MS and all mass analyzes (in LC-MS/MS) were performed using Mass Hunter software. Mass spectrometry analyses were performed with Agilent Technologies 6460 Triple Quadrupole Mass Spectrometer, which is capable of ESI ionization having the Agilent Jet Stream Technology. The electrospray ionization source was operated under the operating conditions that are given below. Data collection for measurement was performed in dynamic MRM (DMRM) mode.

| |
|---|
| Capillary voltage ……..3.5 Kv, |
| Source and sheath gas temperature ……300 and 350 °C |
| Source and sheath gas flow rate …….11 and 12 L/min |
| Nebulizer pressure ……..set as 45 psi. |

First, 1 g of honey was taken and 29 g of NaCl was added. Then 2 ml of molar potassium dihydrogen phosphate pH:8 was added, it was mixed with vortex, and 10 ml of acetonitrile (ACN) was added when it was ensured that it mixed well, and it was kept in an ultrasonic bath at 60°C for 30 min. It was then centrifuged at 4000 rpm for 30 min. After centrifugation, 9 ml of the supernatant was removed, and the supernatant was evaporated under nitrogen at 50°C. The residue was dissolved in 20 µL 70:30 (water-MeOH) solution. The obtained solution was injected into the LC-MS-MS device and the process was completed.

Using this extraction method, the honey was loaded from the antibiotic mix pool as 6 parallels, which did not contain any antibiotic residues at 25 ng, 50 ng, 100 ng, 200 ng levels. With the extinction method determined, it has been found that there were problems in the extraction of the quinolone group antibiotics, and the studies were intensified in this direction. As the result, it has been decided that the extraction pattern of the quinolone group should be further studied. During this process, the search for increasing the productivity levels of the other groups has been continued with a little more intense. The optimization values obtained in Table 1-2 were given to the device in different mobile phases by creating the analytical conditions of liquid chromatography (LC) and mass spectrometry (MS/MS) in Tables 3, 4, 5 and 6.

### *2.2.3. Validation of the Method*

**I**n this study, two types of extractions, both basic and acidic extractions, have been tried and achieved. For each method, 1 µg/ml of the standard solution of the antibiotic mixture was added to the samples known to be negative at 25, 50, 100, 200 ng/ml levels as 6 parallels (n = 6). Separate basic and acidic extraction orders were formed for these samples. The samples were separately extracted and validated by basic and acidic extraction as shown below (Table 7 and 8). This distinction was also considered to be important in subsequent operations. Results of the basic and acidic extraction validations are presented in tables (Table 7-8).

#### *2.2.3.1. Basic extraction sample preparation*

First, the honey sample was prepared by heating at 40°C. 1 g was taken from the sample and put into a 50 ml PP tube. Then, 1 µg/ml of the prepared internal standard mixture was added. After that, 3 g NaCl, and 2 ml 1M KH2PO4 (pH=8) and 3.75 ml 0.1 M EDTA, respectively, were added and vortexed thoroughly. 10 ml of ACN was added to the mixture and was vortexed thoroughly again. After this average mixing time was about 5 minutes, the following operations were carried out respectively. First, the mixture was held in an ultrasonic bath at 60°C for 20 minutes. After taking it from the ultrasonic bath, it was centrifuged at 4000 rpm for 30 minutes. 9 ml of the supernatant was taken and evaporated under nitrogen. It was then dissolved in 50 µL Water-ACN (80-20 v/v) and made ready by passing through a 0.2 µm filter.

#### **2.2.3.2. Asidic extraction sample preparation**

The honey sample was prepared by heating at 40°C. 1 g of the prepared sample was weighed into a 50 ml polypropylene tube and the internal standard was added to the mixture. Then, the following operations were applied respectively. 3 g of NaCl, 3.75 ml of Mcllvaine Buffer (pH=3), and 75 ml of 0.1 M EDTA were added and mixed. 10 ml of ACN was added to the mixture and the mixture was vortexed for 5 min. It was then held in an ultrasonic bath at 60°C for 20 minutes. The sample mixture was centrifuged at 4000 rpm for 30 minutes. 9 ml of the supernatant was taken and evaporated to dryness under nitrogen, and it was dissolved in 50 µL Water-ACN (80-20 v/v). It was analyzed by passing it through 0.2 µm filters.

Acquity UPLC C-18 column (2.1x100 mm, 1.7µm) was used for chromatographic separation of the extracted samples. As the mobile phase, 0.01% formic acid (FA), 1 mM oxalic acid (OA), 1 mM ammonium formate (A) and 0.02% FA (B) in ACN was used in the deionized water. As the gradient separation program, the flow rate of the solvents was adjusted to 0.2 ml at 15% B for 5-7 minutes, 20% B for 7-11 minutes, 40% B for 11-15 minutes, 60% B for 15-16 minutes, and at a total analysis time of 20 minutes. Under these conditions, 5 µL injection was made into the LC-MS/MS device. All mass analyzes were read on the LC-MS/MS using Mass Hunter® software. Analyzes have been completed in line with EU Directive 2002/657 EC and the results are presented.

## 3. Results

In our study, findings in accordance with the 2002/657 EEC directive were obtained using the analysis and extraction method in the direction of the data obtained by extraction of 41 antibiotics and these were presented in tables and charts. Firstly, the optimization values of crude data obtained by extraction are shown in Tables 1 and 2. Based on the values in Tables 1 and 2, the LC and MS/MS analytical flow conditions are obtained in Tables 3, 4, and 5, and the analytical conditions of liquid chromatography (LC) and mass spectrometry (MS/MS) are obtained and the data are presented as separate mobile phases (Table 6).

**Table 1**. Optimization analysis data

| Cpd Name | ISTD? | Prec Ion | MS1 Res | Prod Ion | MS2 Res | Frag (V) | CE (V) | Cell Acc (V) | Ret Time (min) | Ret Window | Polarity |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 001-Sulfathiazole | No | 256 | | 156 | | 90 | 10 | 4 | 5.6 | 10 | + |
| 001-Sulfathiazole | No | 241 | | 92 | | 90 | 24 | 4 | 5.6 | 10 | + |
| 002Oxytetracycline | No | 461.3 | | 443.1 | | 120 | 9 | 4 | 7.9 | 10 | + |
| 002Oxytetracycline | No | 461.3 | | 426.3 | | 120 | 15 | 4 | 7.9 | 10 | + |
| 003-Enrofloxacin | No | 360.1 | | 342.3 | | 120 | 17 | 4 | 8.1 | 10 | + |
| 003-Enrofloxacin | No | 360.1 | | 316.4 | | 120 | 15 | 4 | 8.1 | 10 | + |
| 003-Enrofloxacin | No | 360.1 | | 245 | | 120 | 25 | 4 | 8.1 | 10 | + |
| 004Sulfadimethoxi | No | 311.1 | | 245 | | 120 | 16 | 4 | 8.2 | 10 | + |
| 004Sulfadimethoxi | No | 311.1 | | 156 | | 120 | 18 | 4 | 8.2 | 10 | + |
| 005-Tetracycline | No | 445 | | 410 | | 120 | 20 | 4 | 7.8 | 10 | + |
| 005-Tetracycline | No | 445 | | 153.9 | | 120 | 25 | 4 | 7.8 | 10 | + |
| 006-Ciprofloxacin | No | 332.1 | | 314 | | 120 | 17 | 4 | 8 | 10 | + |
| 006-Ciprofloxacin | No | 332.1 | | 288 | | 120 | 13 | 4 | 8 | 10 | + |
| 007-Erythromycin | No | 734.4 | | 576.3 | | 180 | 14 | 4 | 9.6 | 10 | + |
| 007-Erythromycin | No | 734.4 | | 158.2 | | 180 | 30 | 4 | 9.6 | 10 | + |
| 008Sulfamethoxaz | No | 254.1 | | 156 | | 100 | 10 | 4 | 8 | 10 | + |
| 008Sulfamethoxaz | No | 254.1 | | 92 | | 100 | 26 | 4 | 8 | 10 | + |
| 009Sulfamethazin | No | 311 | | 218 | | 140 | 15 | 4 | 8.2 | 10 | + |
| 009Sulfamethazin | No | 311 | | 156 | | 140 | 15 | 4 | 8.2 | 10 | + |
| 010-Sulfadiazine | No | 251 | | 185 | | 120 | 15 | 4 | 4.85 | 10 | + |
| 010-Sulfadiazine | No | 251 | | 156 | | 120 | 10 | 4 | 4.85 | 10 | + |
| 012dıcloxacıllın | No | 470 | | 311 | | 100 | 10 | 4 | 9.9 | 10 | + |
| 012-dıcloxacıllın | no | 470 | | 160 | | 100 | 8 | 4 | 9.9 | 10 | + |
| 013-Sarafloxacin | No | 386.1 | | 368.1 | | 140 | 18 | 4 | 8.3 | 10 | + |
| 013-Sarafloxacin | No | 386.1 | | 299 | | 140 | 27 | 4 | 8.3 | 10 | + |
| 014-Doxycycline | No | 445.2 | | 410 | | 110 | 20 | 4 | 7.8 | 10 | + |
| 014-Doxycycline | No | 445.2 | | 154 | | 110 | 30 | 4 | 7.8 | 10 | + |
| 016-Tilmicosin | No | 869.5 | | 696.4 | | 320 | 44 | 4 | 9.3 | 10 | + |
| 016-Tilmicosin | No | 869.5 | | 174.1 | | 320 | 49 | 4 | 9.3 | 10 | + |
| 017-Tylosin | No | 916.4 | | 772.4 | | 280 | 30 | 4 | 9.5 | 10 | + |
| 017-Tylosin | No | 916.4 | | 174.2 | | 280 | 40 | 4 | 9.5 | 10 | + |
| 018-Sulfadimidine | No | 279 | | 186 | | 120 | 12 | 4 | 7.5 | 10 | + |
| 018-Sulfadimidine | No | 279 | | 124 | | 120 | 22 | 4 | 7.5 | 10 | + |
| 019-Lincomycin | No | 407.2 | | 359 | | 150 | 15 | 4 | 6.83 | 10 | + |
| 019-Lincomycin | No | 407.2 | | 126 | | 150 | 30 | 4 | 6.83 | 10 | + |
| 020Sulfachloropyri | No | 285 | | 156 | | 100 | 14 | 4 | 7.7 | 10 | + |
| 020Sulfachloropyri | No | 285 | | 91.8 | | 100 | 28 | 4 | 7.7 | 10 | + |
| 021Chlortetracycli | No | 479 | | 462 | | 120 | 15 | 4 | 4.9 | 10 | + |
| 021Chlortetracycline | No | 479 | | 197 | | 120 | 35 | 4 | 4.9 | 10 | + |
| 022-Spiramycin | No | 843.4 | | 539.8 | | 270 | 35 | 4 | 8.8 | 10 | + |
| 022-Spiramycin | No | 843.4 | | 174.1 | | 270 | 40 | 4 | 8.8 | 10 | + |
| 023-AMPICILINE | No | 350 | | 192 | | 110 | 10 | 4 | 8.6 | 10 | + |
| 023-AMPICILINE | No | 350 | | 160 | | 100 | 5 | 4 | 8.6 | 10 | + |
| 025-Florfenicol | No | 356 | | 336 | | 120 | 4 | 4 | 8.11 | 10 | - |
| 025-Florfenicol | No | 356 | | 185 | | 120 | 12 | 4 | 8.11 | 10 | - |
| 026-Danofloxacin | No | 358 | | 340 | | 120 | 31 | 4 | 8 | 10 | + |
| 026-Danofloxacin | No | 358 | | 314 | | 120 | 20 | 4 | 8 | 10 | + |
| 029-Thiamphenicol | No | 354 | | 290 | | 110 | 2 | 4 | 6.8 | 10 | - |
| 029-Thiamphenicol | No | 354 | | 185 | | 110 | 8 | 4 | 6.8 | 10 | - |
| 034-Trimethoprim | No | 291.1 | | 230.1 | | 120 | 22 | 4 | 6.9 | 10 | + |
| 034-Trimethoprim | No | 291.1 | | 123.1 | | 120 | 22 | 4 | 6.9 | 10 | + |
| 035-Demeclocycline | Yes | 465 | | 448 | | 120 | 12 | 7 | 8.36 | 10 | + |
| 036Sulfaquinoxaline | No | 301 | | 155.8 | | 170 | 18 | 7 | 9 | 10 | + |
| 036Sulfaquinoxaline | No | 301 | | 91.8 | | 170 | 30 | 7 | 9 | 10 | + |
| 037-Marbofloxacine | No | 363 | | 320 | | 120 | 18 | 7 | 6.9 | 10 | + |
| 037-Marbofloxacine | No | 363 | | 72 | | 120 | 20 | 7 | 6.9 | 10 | + |
| 038-OxalinicAcide | No | 262 | | 244 | | 100 | 12 | 7 | 9 | 10 | + |
| 038-OxalinicAcide | No | 262 | | 216 | | 100 | 28 | 7 | 9 | 10 | + |
| 040-Jasamycine | No | 828.5 | | 174 | | 150 | 30 | 4 | 9.8 | 10 | + |
| 040-Jasamycine | No | 828.5 | | 109 | | 150 | 46 | 4 | 9.8 | 10 | + |
| 041-Sulfamethiazole | No | 271 | | 156.1 | | 90 | 8 | 4 | 7.3 | 10 | + |
| 041-Sulfamethiazole | No | 271 | | 92 | | 90 | 26 | 4 | 7.3 | 10 | + |
| 042-Sulfadoxin | No | 311.1 | | 156 | | 140 | 12 | 4 | 8.2 | 10 | + |
| 042-Sulfadoxin | No | 311.1 | | 92 | | 140 | 30 | 4 | 8.2 | 10 | + |
| 043-Sulfisoxazole | No | 268 | | 155.8 | | 120 | 13 | 4 | 8.34 | 10 | + |
| 043-Sulfisoxazole | No | 268 | | 91.8 | | 120 | 28 | 4 | 8.34 | 10 | + |
| 044-Roxhitromycin | Yes | 837.4 | | 158.1 | | 190 | 34 | 4 | 9.9 | 10 | + |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 045-Sulfamethazine | No | 279.1 | 186 | 90 | 12 | 4 | 7.5 | 10 | + |
| 045-Sulfamethazine | No | 279.1 | 92.1 | 90 | 28 | 4 | 7.5 | 10 | + |
| 046-Difloxacin | No | 400.2 | 356 | 130 | 14 | 4 | 8.2 | 10 | + |
| 046-Difloxacin | No | 400.2 | 299 | 130 | 26 | 4 | 8.2 | 10 | + |
| 047Sulfamonomet | No | 281.1 | 156 | 110 | 12 | 4 | 7.6 | 10 | + |
| 047Sulfamonomet | No | 281.1 | 92 | 110 | 28 | 4 | 7.6 | 10 | + |
| 048Sulfamonopyrida | No | 281.1 | 156 | 100 | 12 | 4 | 7.6 | 10 | + |
| 048Sulfamonopyrida | No | 281.1 | 92.1 | 100 | 28 | 4 | 7.6 | 10 | + |
| 049-Norfloxacin | No | 320.1 | 276.1 | 120 | 12 | 4 | 7.8 | 10 | + |
| 049-Norfloxacin | No | 320.1 | 233.1 | 120 | 20 | 4 | 7.8 | 10 | + |
| 050-Norfloxacin-D5 | Yes | 325.1 | 307.1 | 130 | 16 | 4 | 7.8 | 10 | + |
| 051Chloramphenicol | No | 321 | 257 | 120 | 2 | 4 | 8.8 | 10 | - |
| 051Chloramphenicol | No | 321 | 152 | 120 | 8 | 4 | 8.8 | 10 | - |
| 052Chloramphe-D5 | No | 326.1 | 262.1 | 120 | 2 | 7 | 7.05 | 10 | - |
| 052Chloramphe-D5 | No | 326.1 | 157.2 | 120 | 8 | 7 | 7.05 | 10 | - |
| 052-Flumequine | No | 262.1 | 202.1 | 100 | 32 | 4 | 5.6 | 10 | + |
| 052-Flumequine | No | 262.1 | 174 | 100 | 35 | 4 | 5.6 | 10 | + |
| 057-Dapson | No | 249 | 156.1 | 120 | 10 | 7 | 7 | 10 | + |
| 057-Dapson | No | 249 | 92 | 120 | 26 | 7 | 7 | 10 | + |
| 058-OxacillineXX | No | 402.1 | 243.1 | 150 | 15 | 7 | 9.9 | 10 | + |
| 058-OxacillineXX | No | 402.1 | 160 | 150 | 12 | 7 | 9.9 | 10 | + |
| 060-Gamitromycine | No | 777.6 | 619.3 | 200 | 30 | 7 | 9.2 | 10 | + |
| 060-Gamitromycine | No | 777.6 | 601.3 | 200 | 34 | 7 | 9.2 | 10 | + |
| 301SulfamethoxaD4 | Yes | 258.1 | 160 | 100 | 10 | 7 | 8 | 10 | + |

**Table 2**. Optimization analysis data

**Timetable**

| | Time | A | B | Flow | Pressure |
|---|---|---|---|---|---|
| 1 | 0.00 min | 95.0 % | 5.0 % | 0.200 mL/min | 600.00 bar |
| 2 | 0.50 min | 95.0 % | 5.0 % | 0.200 mL/min | 600.00 bar |
| 3 | 2.00 min | 75.0 % | 25.0 % | 0.200 mL/min | 600.00 bar |
| 4 | 5.50 min | 10.0 % | 90.0 % | 0.400 mL/min | 600.00 bar |
| 5 | 7.00 min | 10.0 % | 90.0 % | 0.400 mL/min | 600.00 bar |
| 6 | 7.01 min | 95.0 % | 5.0 % | 0.200 mL/min | 600.00 bar |
| 7 | 10.00 min | 95.0 % | 5.0 % | 0.200 mL/min | 600.00 bar |

**Table 3** LC analytical flow conditions

**Table 4.** MS/MS analytical flow conditions

**LC MS MS-ESI ANALYSIS**

**LC**

**Column:** Acquity UPLC C-18 column (2.1x100 mm, 1.7µm) at 40° C

**Injection volume:** 5 µL

**Mobile phase:**

A: % 01 FA + 1mM OA + 1mM Ammonium Formate in $H_2O$

B: % 02 FA in ACN

**Total flow time:** 20 min

**MS/MS**

**Capillary voltage:** 3.5 Kv

**Gas (Source and sheath gas):** nitrogen

**Gas temperature (Source and sheath):** 300 and 325° C

**Gas flow rate:** 6 ve 11 L $min^{-1}$

**Nebulizer gas:** nitrogen

**Nebulizer gas pressure:** 45 psi

**Table 5.** A and B mobile phase

| Time(Min) | **A (%)** | **B (%)** |
|---|---|---|
| 1 | 9.0 | 10 |
| 5 | 85.0 | 15 |
| 7 | 80.0 | 20.0 |
| 11 | 60.0 | 40.0 |
| 15 | 40.0 | 60.0 |
| 16 | 40.0 | 10.0 |
| 20 | 95.0 | 1.0 |

The obtained data were evaluated statistically. Alternative methods have been tried for certain antibacterials that have been analyzed but suspected to be problematic at the extraction stage, and these results are shown in Table 6.

**Table 6**. The MRM parameters and retention times (Rt) for all analytes and labelled standards. **Deuterium labelled internal standards.**Internal standardsAbbreviation; RT: retention time*

| **Compound** | **Precursor Ion (m/z)** | **Product Ion 1 (m/z)** | **Collision Energy (Ev)** | **Product Ion 2 (m/z)** | **Collision Energy (Ev)** | **Fragmentor (V)** |
|---|---|---|---|---|---|---|
| SDZ | 251 | 185 | 15 | 156 | 10 | 120 |
| STZ | 256 | 156 | 10 | 92 | 24 | 90 |
| SMZ | 279.1 | 186 | 12 | 92.1 | 28 | 90 |
| SMT | 271 | 156.1 | 8 | 92 | 26 | 90 |
| SMM | 281.1 | 156 | 12 | 92 | 28 | 110 |
| SMX | 281.1 | 156 | 12 | 92.1 | 28 | 100 |
| SCP | 285 | 156 | 14 | 91.8 | 28 | 100 |
| SDX | 311.1 | 156 | 12 | 92 | 30 | 140 |
| SMH | 254.1 | 156 | 10 | 92 | 26 | 100 |
| SDM | 311.1 | 245 | 16 | 156 | 18 | 120 |
| SQX | 301 | 155.8 | 18 | 91.8 | 30 | 170 |
| SFX | 268 | 155.8 | 13 | 91.8 | 28 | 120 |
| SPI | 843.4 | 539.8 | 35 | 174.1 | 40 | 270 |
| ERY | 734.4 | 576.3 | 14 | 158.2 | 30 | 180 |
| TYL | 916.4 | 772.4 | 30 | 174.2 | 40 | 280 |
| JOS | 828.5 | 174 | 30 | 109 | 46 | 150 |
| TIL | 869.5 | 696.4 | 44 | 174.1 | 49 | 320 |
| GAM | 777.6 | 619.3 | 30 | 601.3 | 34 | 200 |
| OTC | 461.3 | 443.1 | 9 | 426.3 | 15 | 120 |
| TC | 445 | 410 | 20 | 153.9 | 25 | 120 |
| DXC | 445.2 | 410 | 20 | 154 | 30 | 110 |
| CTC | 479 | 444 | 20 | 153.9 | 28 | 120 |
| THI | 354 | 290 | 2 | 185 | 8 | 110 |
| FLF | 356 | 336 | 4 | 185 | 12 | 120 |
| CMP | 321 | 257 | 2 | 152 | 8 | 120 |
| DIF | 400.2 | 356 | 14 | 299 | 26 | 130 |
| MAR | 363 | 320 | 18 | 72 | 20 | 120 |
| OXO | 262 | 244 | 12 | 216 | 28 | 100 |
| FLU | 262.1 | 202.1 | 32 | 174 | 35 | 100 |

| | | | | | | |
|---|---|---|---|---|---|---|
| SAR | 386.1 | 368.1 | 18 | 299 | 27 | 140 |
| NOR | 320.1 | 276.1 | 12 | 233.1 | 20 | 120 |
| ENR | 360.1 | 342.3 | 17 | 316.4 | 15 | 120 |
| DAN | 358 | 314 | 26 | 96 | 36 | 120 |
| CIP | 332.1 | 314 | 17 | 288 | 13 | 120 |
| TRI | 291.15 | 230.1 | 22 | 123.1 | 22 | 120 |
| LIN | 407.2 | 359 | 15 | 126 | 30 | 150 |
| SMH $D_4$* | 258.1 | 160 | 10 | | | 100 |
| ROX** | 837.4 | 158.1 | 34 | | | 190 |
| DEM** | 465 | 448 | 12 | | | 120 |
| NOR $D_5$* | 325.1 | 307.1 | 16 | | | 130 |

After obtaining LC and MS/S, the validation values of both the basic and acidic extracts of 41 antibiotics in the samples to were determined. The mentioned values are shown in Tables 7 and 8.

**Table 7.** Basic extraction validation results

| | 25 (ng g1-) | | 50 (ng g1-) | | 100 (ng g1-) | | 200 (ng g1-) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Recovery(%) | RSD (%) | Recovery(%) | RSD (%) | Recovery(%) | RSD (%) | Recovery(%) | RSD (%) | Linearity(R2) | LOD (µg/kg) | LOQ (µg/kg) |
| SDZ | 119 | 39.25 | 164 | 31.68 | 113.7 | 10.2 | 102 | 2.73 | 0.9741 | 38.83 | 118 |
| STZ | 128.4 | 25.99 | 151.3 | 28.13 | 111.7 | 11.69 | 98.9 | 6.26 | 0.9807 | 28.662 | 86.855 |
| SMZ | 116.8 | 6.3 | 137.3 | 27.41 | 127.6 | 18.85 | 101.5 | 4.85 | 0.9787 | 5.974 | 18.1 |
| SMT | 108.8 | 30.16 | 135.5 | 30.79 | 118 | 11.77 | 97.5 | 1.36 | 0.9821 | 27.881 | 84.487 |
| SMM | 120.4 | 31.01 | 123.3 | 27.18 | 103.5 | 0.68 | 98 | 2.16 | 0.9957 | 32.03 | 97.06 |
| SMX | 109 | 2.85 | 134.8 | 27.6 | 128.2 | 17.36 | 99.6 | 15.57 | 0.9743 | 2.562 | 7.763 |
| SCP | 130 | 15.23 | 129.1 | 25.49 | 130.3 | 20.26 | 103.8 | 2.93 | 0.9822 | 15.731 | 47.669 |
| SDX | 117.2 | 11.1 | 134.4 | 25.43 | 124.1 | 18.32 | 99.2 | 1.91 | 0.9796 | 10.842 | 32.855 |
| SMH | 146 | 36.81 | 128.4 | 22.41 | 119.4 | 14.53 | 103 | 2.7 | 0.9915 | 48.303 | 132.735 |
| SDM | 117.4 | 12.77 | 141.3 | 27.92 | 123.7 | 16.81 | 99.7 | 2.53 | 0.9783 | 12.48 | 37.818 |
| SQX | 105.5 | 5.53 | 104.3 | 4.14 | 104.2 | 4.83 | 100.1 | 0.63 | 0.9995 | 4.808 | 14.57 |
| SFX | 101.7 | 5.92 | 46.2 | 102.55 | 101.5 | 3.48 | 102.2 | 2.21 | 0.9787 | 4.732 | 14.339 |
| SPI | 128 | 4.42 | 97.2 | 2.69 | 87 | 10.39 | 101.8 | 0.35 | 0.9909 | 4.705 | 14.257 |
| ERY | 150 | 9.43 | 105.3 | 4.78 | 79.4 | 6.37 | 97.6 | 10.76 | 0.9791 | 10.064 | 30.497 |
| TYL | 169.6 | 24.68 | 120 | 14.14 | 94.7 | 17.56 | 98.8 | 8.23 | 0.9882 | 36.843 | 111.644 |
| JOS | 103.5 | 9.35 | 103 | 10.31 | 102.7 | 3.32 | 107.7 | 6.67 | 0.9994 | 7.419 | 22.481 |
| TIL | 154.8 | 24.48 | 103.6 | 16.41 | 99.9 | 7.44 | 95 | 10.42 | 0.994 | 33.963 | 102.917 |
| OTC | 387.2 | 49.7 | 64.3 | 5.06 | 97.3 | 14.58 | 95.2 | 6.04 | 0.79 | 192.4 | 583 |
| TC | 120 | 37.71 | 73 | 17.44 | 96 | 9.94 | 107.5 | 4.19 | 0.9894 | 34.747 | 105.293 |
| DXC | 77.8 | 0.36 | 97.3 | 19.74 | 89.7 | 7.17 | 103.8 | 3.28 | 0.9949 | 0.224 | 0.68 |
| CTC | 98.5 | 15.03 | 92.2 | 11.36 | 105.4 | 5.72 | 102.9 | 6.91 | 0.9989 | 11.755 | 35.621 |
| THI | 100.4 | 10.1 | 54.7 | 18.77 | 52.4 | 51.61 | 105.5 | 82.94 | 0.924 | 8.126 | 24.6 |
| FLF | 40 | 28.28 | 48.3 | 50.01 | 53.7 | 47.48 | 89.3 | 68.92 | 0.9575 | 10.338 | 31.327 |
| CHL | 123.4 | 0.23 | 90.9 | 8.62 | 76.7 | 16.41 | 101.5 | 0.7 | 0.9786 | 0.237 | 0.718 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| DIF | 113.3 | 14.98 | 105.4 | 9.68 | 111 | 7.04 | 99.8 | 3.52 | 0.9967 | 14.01 | 42.455 |
| MAR | 105.6 | 11.86 | 107.5 | 3.99 | 102.9 | 5.8 | 99.8 | 4.63 | 0.9995 | 10.396 | 31.503 |
| OXO | 108.7 | 9.44 | 115.1 | 12.18 | 101.4 | 1.38 | 98.2 | 7.41 | 0.9981 | 8.712 | 26.4 |
| FLU | 108.1 | 8.95 | 107.2 | 7.45 | 104.7 | 2.92 | 101 | 2.16 | 0.9996 | 7.928 | 24.025 |
| SAR | 118.7 | 5.15 | 100 | 6.93 | 99.8 | 2.85 | 106.3 | 8.31 | 0.9982 | 4.783 | 14.495 |
| NOR | 90.5 | 11.28 | 94.4 | 8.33 | 96.7 | 7.27 | 101 | 6.18 | 0.9741 | 8.31 | 25.183 |
| ENR | 122.7 | 26.56 | 107.2 | 11.15 | 112.2 | 10.27 | 100.1 | 4.12 | 0.996 | 26.929 | 81.602 |
| DAN | 96.1 | 70.18 | 109.1 | 13.25 | 109 | 3.01 | 97.2 | 0.7 | 0.9953 | 56.835 | 172.227 |
| CIP | 85.8 | 10.3 | 105.5 | 6.3 | 95.9 | 8.94 | 104 | 3.82 | 0.9978 | 7.013 | 21.252 |
| TRI | 125.6 | 3.6 | 86.9 | 9.7 | 78.7 | 16.1 | 102.6 | 1.24 | 0.9798 | 2.992 | 9.065 |
| LIN | 37 | 95.55 | 27.7 | 79.71 | 22.2 | 81.46 | 103 | 0 | 0.9126 | 57.778 | 175.084 |

**Table 8**. Asidic extraction validation results

| Analyte | 25 (ng $g^{1-}$) | | 50 (ng $g^{1-}$) | | 100 (ng $g^{1-}$) | | 200 (ng $g^{1-}$) | | Linearity($R^2$) | LOD (µg/kg) | LOQ (µg/kg) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Recovery(%) | RSD (%) | Recovery(%) | RSD (%) | Recovery(%) | RSD (%) | Recovery(%) | RSD (%) | | | |
| SDZ | 90.7 | 3.97 | 97.2 | 48.59 | 100.5 | 12.28 | 100.2 | 12.9 | 0.9998 | 2.949 | 8.936 |
| STZ | 78.9 | 10.59 | 107.9 | 14.92 | 111.2 | 13.07 | 113.6 | 26.05 | 0.9987 | 5.977 | 18.112 |
| SMZ | 81.8 | 4.9 | 95.4 | 17.78 | 104.6 | 15.65 | 109.2 | 8.07 | 0.9986 | 2.986 | 9.05 |
| SMT | 73 | 38.27 | 79.8 | 14.29 | 102.5 | 11.04 | 108.7 | 10.98 | 0.9957 | 20.697 | 62.719 |
| SMM | 68.6 | 11.25 | 87.9 | 21.24 | 105.3 | 17.54 | 109.6 | 6.26 | 0.997 | 5.67 | 17.183 |
| SMX | 78.1 | 9.78 | 93.3 | 16.41 | 105.4 | 15.27 | 116.8 | 8.2 | 0.9961 | 5.308 | 16.085 |
| SCP | 95.2 | 6.26 | 100.8 | 14.83 | 105.9 | 11.12 | 109.9 | 5.38 | 0.9994 | 4.441 | 13.459 |
| SDX | 81.1 | 3.79 | 96.6 | 15.2 | 104.5 | 13.89 | 110.9 | 9.91 | 0.9983 | 2.259 | 6.844 |
| SMH | 94.2 | 6.15 | 94.2 | 15.9 | 101 | 14.5 | 104.7 | 4.06 | 0.9993 | 4.534 | 13.739 |
| SDM | 79.5 | 3.37 | 94 | 15.59 | 104 | 13.9 | 108.3 | 8.28 | 0.9985 | 2.008 | 6.085 |
| SQX | 140 | 9.21 | 118 | 25.46 | 115 | 30.22 | 116 | 10.37 | 0.9992 | 9.286 | 28.141 |
| SFX | 124.7 | 15.64 | 113 | 17.22 | 101.3 | 18.13 | 103.7 | 5.02 | 0.9986 | 15.778 | 47.811 |
| SPI | 54.7 | 46.63 | 64.7 | 85.63 | 72.8 | 67.41 | 29.1 | 131.12 | 0.6322 | 68.883 | 208.74 |
| ERY | 122 | 15.59 | 91.9 | 16.58 | 85.6 | 17.86 | 109.3 | 12.32 | 0.9841 | 11.717 | 35.507 |
| TYL | 106.3 | 18.14 | 95.7 | 80.46 | 73.5 | 39.92 | 59.9 | 44.78 | 0.9704 | 27.829 | 84.332 |
| JOS | 90.7 | 7.2 | 119 | 42.21 | 105.3 | 35.39 | 123 | 21.15 | 0.994 | 4.379 | 13.271 |
| TIL | 111.3 | 14.99 | 104.7 | 13.33 | 124 | 63.69 | 176 | 66.91 | 0.9738 | 8.471 | 25.669 |
| OTC | 98.5 | 9.52 | 62.3 | 40.04 | 76.1 | 19.65 | 91.8 | 6.06 | 0.9858 | 8.473 | 25.674 |
| TC | 80.3 | 4.24 | 88.1 | 26.64 | 93.5 | 11.64 | 109.7 | 15.98 | 0.9937 | 2.54 | 7.696 |
| DXC | 95.4 | 3.61 | 109 | 15.06 | 102.1 | 10.2 | 102 | 17.7 | 0.9995 | 2.782 | 8.431 |
| CTC | 88 | 13.48 | 104 | 31.03 | 90.2 | 18.47 | 100.4 | 7.92 | 0.9964 | 9.806 | 29.714 |
| THI | 156.5 | 5.1 | 79.2 | 87.5 | 111.5 | 46.86 | 151 | 79.08 | 0.9699 | 4.375 | 13.258 |
| FLF | 47.1 | 37.13 | 114.4 | 33.04 | 150.3 | 88.8 | 227.5 | 106.7 | 0.9671 | 6.154 | 18.647 |
| CHL | 131.3 | 23.86 | 98.7 | 25.63 | 153.5 | 89.24 | 201.8 | 78.82 | 0.9768 | 12.568 | 38.086 |
| DIF | 123.4 | 2.46 | 106.2 | 23.61 | 102.8 | 22.65 | 97.7 | 17.31 | 0.9986 | 2.594 | 7.86 |
| MAR | 110.7 | 4.65 | 97.3 | 12.62 | 101.5 | 15.88 | 96.4 | 11.52 | 0.999 | 4.416 | 13.382 |
| OXO | 89 | 5.95 | 102.4 | 10.21 | 116.8 | 9.94 | 114.8 | 12.74 | 0.9985 | 3.733 | 11.313 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| FLU | 73.3 | 7.3 | 95.7 | 14.19 | 116.5 | 5.45 | 115.2 | 11.33 | 0.9969 | 3.725 | 11.289 |
| SAR | 122.4 | 244.95 | 108.8 | 244.95 | 109 | 159.38 | 98.9 | 41.34 | 0.997 | 4.556 | 13.805 |
| NOR | 94.3 | 0.32 | 92.3 | 6.46 | 94.9 | 7.6 | 92.7 | 7.49 | 0.9998 | 0.268 | 0.811 |
| ENR | 110.9 | 12.34 | 98.2 | 15.38 | 101.2 | 19.05 | 94.9 | 7.76 | 0.9987 | 11.942 | 36.189 |
| DAN | 134 | 109.78 | 93.3 | 47.35 | 150.5 | 98.6 | 181.5 | 85.41 | 0.9833 | 139.59 | 422.99 |
| CIP | 109.5 | 5.19 | 94.9 | 4.36 | 97.4 | 6.14 | 90.8 | 7.2 | 0.9984 | 5.192 | 15.735 |
| TRI | 111.1 | 3.85 | 98.4 | 33.09 | 98.7 | 39.14 | 94.7 | 26.13 | 0.9993 | 3 | 9.091 |
| LIN | 57.3 | 7.45 | 47.2 | 17.59 | 55.7 | 61.87 | 142.1 | 83.34 | 0.9845 | 4.929 | 14.937 |

After the recovery% and RSD% values and the linearity, LOD and LOQ values of the acidic and basic extraction solvents were determined at 25, 50, 100 and 200 ng $g^{-1}$, the effects of their pH on antibiotics were also determined (Figure 1)

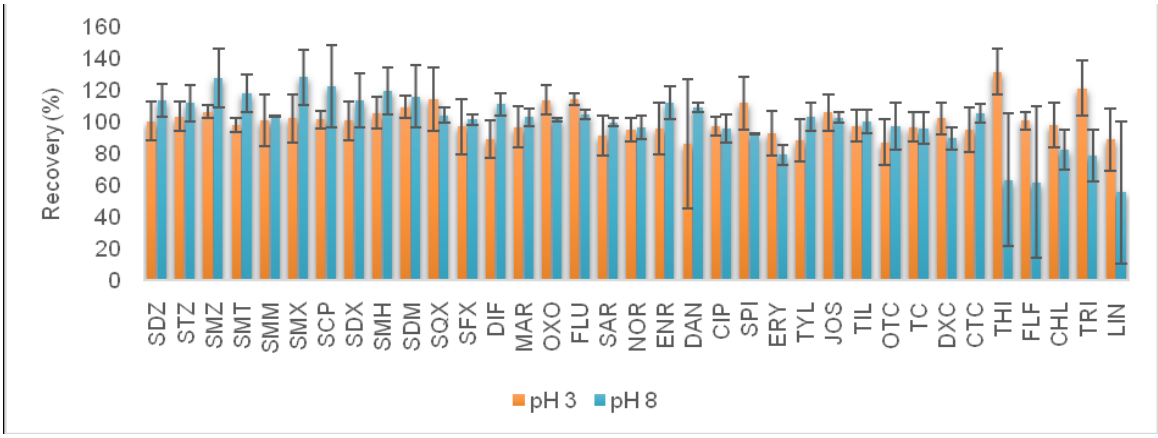


**Figure 1**. Effect of pH of extraction solvents on antibiotics (100 ng/g)

In addition, a study was carried out on the holding the honey sample in the ultrasonic bath for 10 minutes, and it was observed that there was no significant difference between shaking and the ultrasonic bath process (Figure 2). It was evaluated that the reason for not having difference between the mechanical shaking and the ultrasonic bath was the rapid homogenization of the mixture distribution.

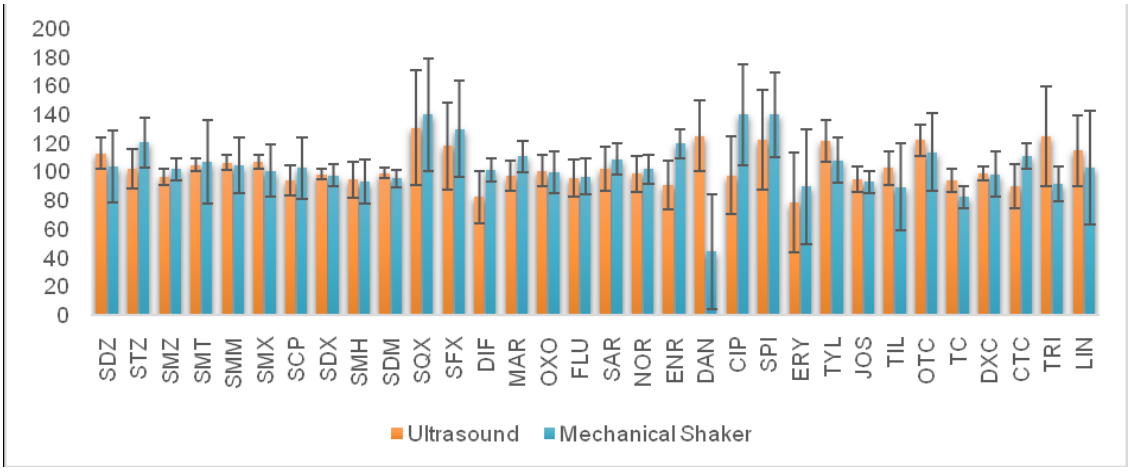


**Figure 2.** Keeping the 100 ng/g antibiotic spiked honey samples in ultrasonic bath for 10 min and the effect of mechanical shaking on the analytes

During our studies, negative effects of the oxalic acid on the masses with negative ionization were detected (low absorbance), thus, positively ionized masses excluding the amphenicols were selected and analyzed. The effect of oxalic acid on analytes is given in Chart 3. The existence of oxalic acid was found to be an essential requirement particularly for the analysis of tetracyclines, in the study carried out regarding the addition of oxalic acid to HPLC solvents

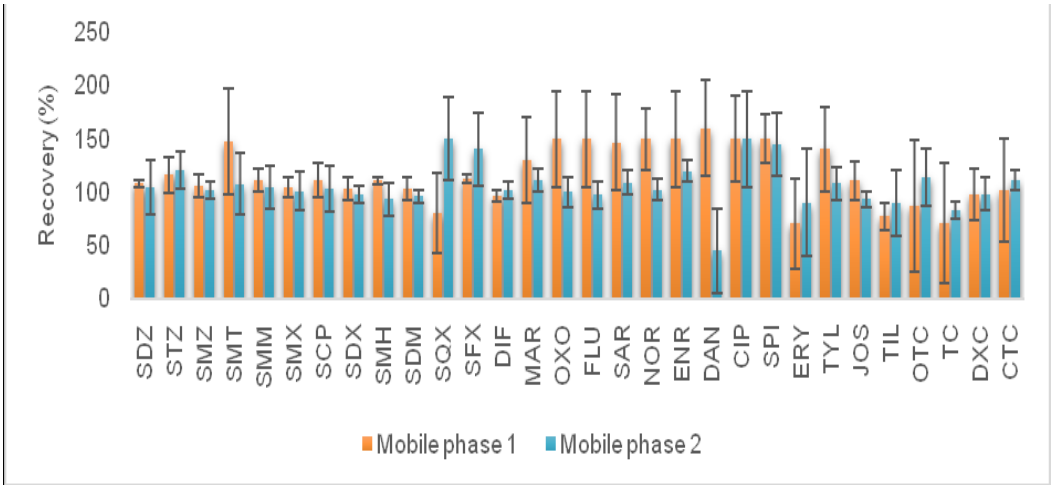


**Figure 3**. Effect of changes of extraction solvents on analytes. Mobile phase 1 -A 0.02% Formic acid and 1 mM Oxalic acid in deionized water, B- Acetonitrile 0.01% Formic acid, Mobile phase 2 -A 0.02% Formic acid in deionized water Mobile phase B Acetonitrile 0.01% Formic acid comparison.

We have also found in our study that there was no response to analyzes of tetracycline compounds without oxalic acid, even at low concentrations. Apart from this, tetracyclines have been found to cause an increase in the responses of all other compounds except for the quinolones, while quinolone compounds cause negligible decay in peak shapes (Figure 4). It was thought that studies may be carried on this situation.

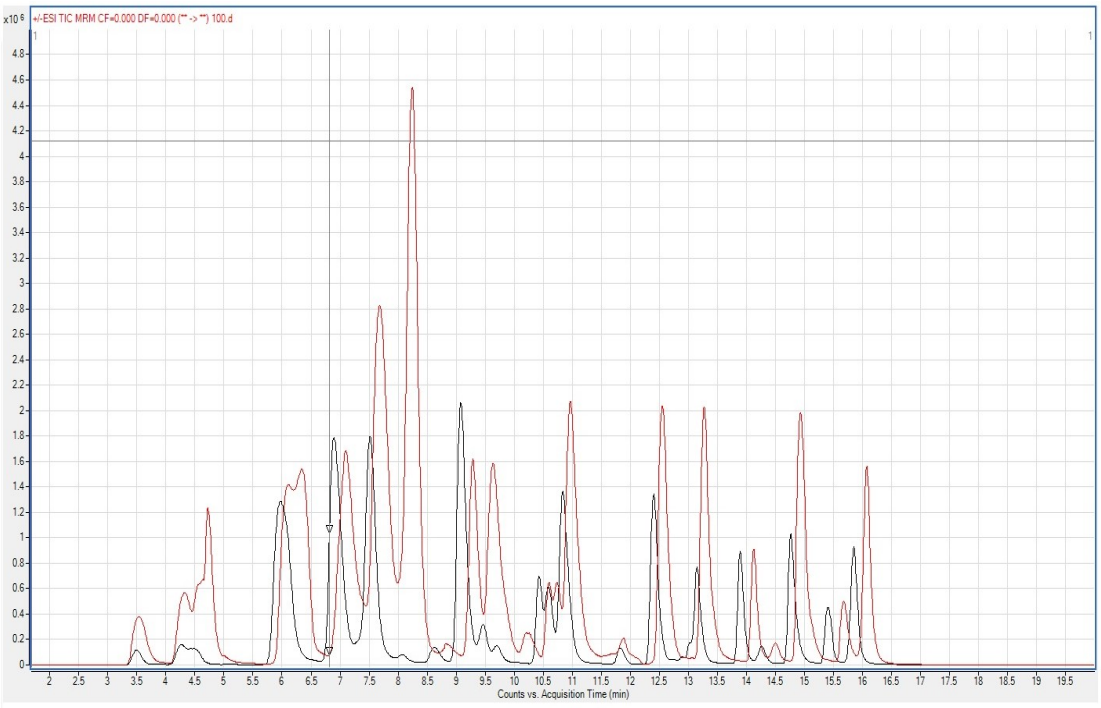


**Figure 4.** Comparison of HPLC solvents: Chromatograms of the HPLC solvent of 100 ng/g spiked sample with and without Oxalic acid

## 4. Discussion

Honey is a very valuable bee (*Apis mellifera*) product with a high nutritional value and therefore it has a high consumption potential, and different substances have been identified at certain rates in its content (Kay et al., 2009). However, it has been argued that a contamination may occur due to antibiotics and pesticides or agricultural chemicals used for the treatment of bee diseases, depending on the industrial and technological developments of our era (Kay et al., 2009; Lewicki et al., 2009; Nebot et al., 2012; Guo-Fang Pang et al., 2003).

Each country has its own criteria, which are identified with regulations, for the reference values of contaminants in honey. These reference values are generally based on FDA (Food and Drug Administration) and EFSA (European Food Safety Authority) and are the end result of extended laboratory analyzes (Adams et al., 2007; Benetti et al., 2006; Bernal et al., 2009; Joint & Additives, 2006; Kay et al., 2009; Schutz et al., 1998). For many different methods, different data have been obtained with different sensitivities depending on the use of various technological laboratory devices. If it is to be worked on with many different molecular structures belonging to several different groups, this have created a considerably complicated situation, even though the technical details such as verifiability, repeatability, precision, reproducibility of the data as well as their accuracy, measurement and value of the identified quantity, and the smallest quantifiable unit in case of repetition are also extremely important (Gaudin et al., 2013; Olaitan et al., 2007; Guo-Fang Pang et al., 2003; G-F Pang et al., 2006).

In recent years, a large number of studies have been conducted on the detection and determination of multiple groups by using them together. Despite the fact that detection of the members of each group, such as amphenicol, tetracyclines, macrolides, and sulphonamides, is relatively easy, it has been suggested that the analysis of various group antibiotics together would cause serious problems both in terms of the methods to be used and laboratory analytics, since testing several members of several groups together requires different analytical procedures (Joint & Additives, 2006; Kochansky, 2004; Thompson & van den Heever, 2012).

While Bruijinsvoort et al. (2004) stated that they obtained satisfactory results in terms of optimization, chromatographic sensitivity, efficacy, validation and recovery in their study conducted on milk and honey samples in Netherlands on streptomycin and dihydrostreptomycin using LC-MS/MS, in accordance with the EU 2002/657, the LOQ was found to be 2 µg/kg lower in honey, 10 µg/kg lower in milk for streptomycin, and one unit lower for dihydrostreptomycin (Benetti et al., 2006).

In addition, in their simultaneous research using LC-MS/MS with positive electrospray ionization, Galarini et al (2014) found that 27 antibiotics from different classes were effective, fast and reliable in the honey. They noted that they could not detect the quinolone group, especially including the nitroimidazoles, which are dangerous residues in honey specimens, but that the sulfonamide residues were present in the rate of 12%, and that the method can also be used in the routine studies of the national authorities (Galarini et al., 2015).

Guadin et al. (2013) found CCP for chlortetracycline, oxytetracycline and doxycycline to be 5-15 µg/kg in their study on tetracycline residues in honey (according to EU screening methods, with the kit used with the commercial name of Tetrasensor®). They noted that the test was easy, fast, and robust, suggesting that cross-reactions with other antibiotics were negligible and that false positive results were around 9.4% (Gaudin et al., 2013).

Granja et al. (2009) found that the validation samples were 10, 15, 20 µg/kg, and the LOD was 3 µg/kg and 7 µg/kg,

respectively, in their study searching streptomycin residues in honey with LC-MS. They stated that the method is simple, can be transferred to routine, precise, low cost and in accordance with the norm of 2002/657/EC.

In our study, the analysis of the antibiotics in the honey and basic and acidic extractions of antibiotics were performed by both methods. The analysis of each individual antibiotic has also saved much more time in the basic excretion, while this process takes a long time since it is necessary to make the appropriate internal standard selection for each compound separately so the analyzes can be transferred to the routine and made commercially viable. The transfer of the method to the routine will also be possible if the internal standard preparation process for basic extraction becomes temporally practicable.

In acidic extraction, the selected internal standards were determined to be sufficient and it was concluded that the analysis can be more optimal, repeatable and simple to use. Yet, as a result of this study, the effect of the use of basic and acidic solvents on the analytes during the extraction phase is also shown in the figure (Figure 4). Even though it is certain that it is faster and more practical than basic extraction, it is possible to improve both. This practice will allow the common extraction and analysis of all antibiotics, perhaps not only for 35 antibacterials but also for all of the groups. It has been suggested that the analysis of hundreds of pesticides can be done in a single step, as well as the extraction and analysis of antibacterial (Venable, Haynes, & Cook, 2014).

In addition, in our study, various experiments were conducted on HPLC at the gradient program, and it was determined that the sugar was not emitted for the short gradient programs of the HPLC column, especially due to the high amount of sugar in the honey. However, it was observed that there were differences in the retention time (RT) of the pressure, depending on the presence of the high amounts of sugar compounds in the column and the increase in pressure (hyperbaric state). With this analysis, irregularities in the peak shapes of the compulsive factor were also detected. Successful results were obtained by adding a high amount of water-phase solution in the HPLC column for 5 minutes at the beginning of the analysis and providing the emittance of the solubilized sugar compounds out of the column in order to solve this problem.

Within the scope of all these studies, it has been determined that the use of oxalic acid in multiple antibiotic analyses, and for low concentrations, the gradient program to be applied to the column, as well as the HPLC column, are important. It has been concluded that this analytical method can be conveniently carried out routinely for the specified analytes using pH: 3 extraction solvent.

In our study, it has been considered that solvent changes will be the basis for both multiple-antibacterial residue screening and validation, and in biphasic applications, in order to achieve the unique optimum conditions. A similar study in the same field was not found among the studies that we could reach in the literature. In the light of this study, it is possible to analyze several groups of antibacterial in biphasic single extraction, and practically, acidic extraction can be immediately adapted to the routine. Adaptability of basic extraction to the routine is possible, however, it is not to the desired extent in terms of saving time. While it is not longer than the process of analysis in the current routine, it is

concluded that it is not at the speed that we desire. However, we believe that basic extraction can be made more efficient by different analyzes and process changes. In addition, it was determined that similar results were obtained in previous studies and these results were found to be on the same axis (Adams, Fussell, Dickinson, Wilkins, & Sharman, 2009; Bargańska et al., 2011; Cronly et al., 2010; Dubreil-Chéneau, Pirotais, Verdon, & Hurtaud-Pessel, 2014; Galarini et al., 2015; Hammel et al., 2008; Thompson & van den Heever, 2012; van Bruijnsvoort, Ottink, Jonker, & de Boer, 2004; Zhou, Lavorato, Mathews, & Countryman). However, in all of these studies, the analytical effects of parameter changes were not given by carrying out bi-directional extraction. In the results of the study, the total antibacterial analysis parameters of the researchers named in Table 9 were examined in numerical values and by a detailed comparison. In this sense, our work has been a primary study also in principle (Table 9) (Adams et al., 2009; Adams et al., 2007; Bargańska et al., 2011; Bohm, Stachel, & Gowik, 2012; Cronly et al., 2010; Dubreil-Chéneau et al., 2014; Galarini et al., 2015; Hammel et al., 2008; Lafontaine, Shi, & Espourteille, 2009; Moreno-Bondi et al., 2009; Nebot et al., 2012; van Bruijnsvoort et al., 2004; Zhou et al.).

As the result of the study, the evaluations were revised over 35 types, because, the number of antibacterial extractions, which was initially designed to be 41, has changed due to the difficulty of the stages, solvent strength and hyperbaric column problems. After the sampling, extraction, validation and determination of the boundaries, it was concluded that the method can be used in the routine and it is possible to apply it as described above. Additionally, new ground is broken in the field of multiple-group antibacterial analysis, with the analysis of 35 antibacterials from different groups. We believe that our study, together with the new techniques and methods to be developed in the future, will lead to fast, reliable and short-term analysis of antibiotics and foreign substances in honey and other food products.

**Table 9.** Comparison of LOD and LOQ values

**COMPARISON TABLE**

| Researcher | Antibacterial | LOD(µgkg$^{-1}$) | LOQ (µgkg$^{-1}$) | Our Results | | |
|---|---|---|---|---|---|---|
| | | | | LOD(µgkg$^{-1}$) | | LOQ(µgkg$^{-1}$) |
| **Barganska et.al. (2011)** | Chloramphenicol<br>Thiamphenicol<br>Florphenicol | - | 0.1-20<br>0.1-20<br>0.1-20 | 0.237<br>4.375<br>6.154 | | 0.718<br>13.258<br>18.647 |
| **Barganska et.al. (2011)** | Oxytetracycline | - | - | 8.473 | | 25.674 |
| | Tetracycline | - | 0.2-1.1 | 2.54 | | 7.696 |
| | Doxycycline | - | - | 0.224 | | 0.68 |
| | Chlortetracycline | - | - | 9.806 | | 29.714 |
| **Benetti et.al. (2006)** | Erythromycin<br>Josamysine<br>Lincomycin | - | < 0.26<br>< 0.26<br>< 0.26 | 10.064<br>4.379<br>4.929 | | 30.497<br>13.271<br>14.937 |
| **Benetti et.al.(2012)** | Spiramycine | - | < 2.1 | 4.705 | | 14.257 |
| **Bruijinsvoort et al. (2004)** | Streptomycin | | 2 | - | | - |
| **Bohm et.al. (2012)** | Macrolide gr.<br>Lincosamide | 7.5-12.9<br>7.5-12.9<br>7.5-12.9<br>7.5-12.9 | 9.4-19.9<br>9.4-19.9<br>9.4-19.9<br>9.4-19.9 | SPİ<br>ERY<br>TYL<br>JOS | 4.705<br>10.064<br>27.829<br>4.379 | 14.257<br>30.497<br>84.332<br>13.271 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | 7.5-12.9 | 9.4-19.9 | TİL | 8.471 | 25.669 |
| | | 7.5-12.9 | 9.4-19.9 | LİN | 4.929 | 14.937 |
| **Carrasco et.al. (2008)** | Tetracycline | 0.002-1.03 | - | OTC | 8.473 | 25.674 |
| | Chlortetracycline | 0.002-1.03 | | | | |
| | Doxycycline | 0.005-0.76 | | TC | 2.54 | 7.696 |
| | Oxytetracycline | 0.005-0.76 | | | | |
| | | | | DXC | 0.224 | 0.68 |
| | | | | CTC | 9.806 | 29.714 |
| **Cheneau et al. (2014)** | 13Different Sulphonamide | 1.8-15.5 | 2.3-17.4 | SDZ | 2.949 | 8.936 |
| | | 1.8-15.5 | 2.3-17.4 | STZ | 5.977 | 18.112 |
| | | 1.8-15.5 | 2.3-17.4 | SMZ | 2.986 | 9.05 |
| | | 1.8-15.5 | 2.3-17.4 | SMT | 20.697 | 62.719 |
| | | 1.8-15.5 | 2.3-17.4 | SMM | 5.67 | 17.183 |
| | | 1.8-15.5 | 2.3-17.4 | SMX | 2.562 | 7.763 |
| | | 1.8-15.5 | 2.3-17.4 | SCP | 4.441 | 13.459 |
| | | 1.8-15.5 | 2.3-17.4 | SDX | 2.259 | 6.844 |
| | | 1.8-15.5 | 2.3-17.4 | SMH | 4.534 | 13.739 |
| | | 1.8-15.5 | 2.3-17.4 | SDM | 2.008 | 6.085 |
| | | 1.8-15.5 | 2.3-17.4 | SOX | 4.808 | 14.57 |
| | | 1.8-15.5 | 2.3-17.4 | SFX | 4.732 | 14.339 |
| **Cronly et al. (2010)** | Chloramphenicol | 0.008 | 0.13 | 0.237 | | 0.718 |
| **Guadin et.al. (2013)** | Chlortetracycline | - | 5-15 | OTC | 8.473 | 25.674 |
| | Oxytetracycline | | 5-15 | TC | 2.54 | 7.696 |
| | Doxycycline | | 5-15 | DXC | 0.224 | 0.68 |
| | | | 5-15 | CTC | 9.806 | 29.714 |
| **Gomez-Perez et al. (2012)** | Macrolide | 1-50 | - | SPİ | 4.705 | 14.257 |
| | Penicilline | 1-50 | | ERY | 10.064 | 30.497 |
| | | 1-50 | | TYL | 27.829 | 84.332 |
| | | 1-50 | | JOS | 4.379 | 13.271 |
| | | 1-50 | | TİL | 8.471 | 25.669 |
| **Granza et al. (2009)** | Streptomycin | 2.33 | 1.64 | - | | - |
| **Nebot et al. (2012)** | 7 Sulphonamide | 7.9-13.3 | 6.3-10.7 | SDZ | 2.949 | 8.936 |
| | | 7.9-13.3 | 6.3-10.7 | STZ | 5.977 | 18.112 |
| | | 7.9-13.3 | 6.3-10.7 | SMZ | 2.986 | 9.05 |
| | | 7.9-13.3 | 6.3-10.7 | SMT | 20.697 | 62.719 |
| | | 7.9-13.3 | 6.3-10.7 | SMM | 5.67 | 17.183 |
| | | 7.9-13.3 | 6.3-10.7 | SMX | 2.562 | 7.763 |
| | | 7.9-13.3 | 6.3-10.7 | SCP | 4.441 | 13.459 |
| | | 7.9-13.3 | 6.3-10.7 | SDX | 2.259 | 6.844 |
| | | 7.9-13.3 | 6.3-10.7 | SMH | 4.534 | 13.739 |
| | | 7.9-13.3 | 6.3-10.7 | SDM | 2.008 | 6.085 |
| | | 7.9-13.3 | 6.3-10.7 | SOX | 4.808 | 14.57 |
| | | 7.9-13.3 | 6.3-10.7 | SFX | 4.732 | 14.339 |
| **Nozal et al. (2008)** | Fumagilline | 1-45 | - | - | | - |
| **Reybroeck et al.(2007)** | Oxytetracycline | 4-12 | - | OTC | 8.473 | 25.674 |
| | Tetracycline | 4-12 | | TC | 2.54 | 7.696 |
| | Doxycycline | 4-12 | | DXC | 0.224 | 0.68 |
| | Chlortetracycline | 4-12 | | CTC | 9.806 | 29.714 |
| **Sajid et al. (2013)** | Sulphonamide | 0.1-1 | 3 | SDZ | 2.949 | 8.936 |
| | | 0.1-1 | 3 | STZ | 5.977 | 18.112 |
| | | 0.1-1 | 3 | SMZ | 2.986 | 9.05 |
| | | 0.1-1 | 3 | SMT | 20.697 | 62.719 |
| | | 0.1-1 | 3 | SMM | 5.67 | 17.183 |
| | | 0.1-1 | 3 | SMX | 2.562 | 7.763 |
| | | 0.1-1 | 3 | SCP | 4.441 | 13.459 |
| | | 0.1-1 | 3 | SDX | 2.259 | 6.844 |
| | | 0.1-1 | 3 | SMH | 4.534 | 13.739 |
| | | 0.1-1 | 3 | SDM | 2.008 | 6.085 |
| | | 0.1-1 | 3 | SOX | 4.808 | 14.57 |
| | | 0.1-1 | 3 | SFX | 4.732 | 14.339 |
| **Thompson et al. (2009)** | 7 Sulphonamide | 0.5-2 | - | SDZ | 2.949 | 8.936 |
| | | 0.5-2 | | STZ | 5.977 | 18.112 |
| | | 0.5-2 | | SMZ | 2.986 | 9.05 |
| | | 0.5-2 | | SMT | 20.697 | 62.719 |
| | | 0.5-2 | | SMM | 5.67 | 17.183 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | 0.5-2<br>0.5-2<br>0.5-2<br>0.5-2<br>0.5-2<br>0.5-2<br>0.5-2 | | SMX<br>SCP<br>SDX<br>SMH<br>SDM<br>SOX<br>SFX | 2.562<br>4.441<br>2.259<br>4.534<br>2.008<br>4.808<br>4.732 | 7.763<br>13.459<br>6.844<br>13.739<br>6.085<br>14.57<br>14.339 |
| | Erythromycin | 0.0012 | 0.0041 | ERY | 10.064 | 30.497 |
| | Anhydroerythromycin | 0.008 | 0.0028 | - | | - |
| | Erythromycin A enol eter | 0.0011 | 0.0038 | - | | - |
| **Vidal et al. (2009)** | Tylosin | - | 1 | TYL | 27.829 | 84.332 |
| | Enrofloxacin<br>Difloxacin | - | 2<br>2 | ENR<br>DİF | 11.942<br>2.594 | 36.189<br>7.86 |
| | Sulfachlorinepridiazin | - | 3.3 | SCP | 4.441 | 13.459 |
| | Sarafloxacin<br>Sulfadimethoxine<br>Tilmicosin<br>Josamysine | - | 0.7<br>0.7<br>0.7<br>0.7 | SAR<br>SDM<br>TİL<br>JOS | 4.556<br>2.008<br>8.471<br>4.379 | 13.805<br>6.085<br>25.669<br>13.271 |
| | Marbofloxacin<br>Tetracycline<br>Danofloxacin<br>Chlortetracycline<br>Sulfaquinoxaline<br>Doxycycline<br>Erythromycin | | 0.3<br>0.3<br>0.3<br>0.3<br>0.3<br>0.3<br>0.3 | MAR<br>TC<br>DAN<br>CTC<br>SQX<br>DXC<br>ERY | 4.416<br>2.54<br>56.835<br>9.806<br>4.808<br>0.224<br>10.064 | 13.382<br>7.696<br>172.227<br>29.714<br>14.57<br>0.68<br>30.497 |

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