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Determination of Six Sulfonamide Residues in Honey by HPLC with Fluorescence Detection

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Abstract. Six sulfonamides (sulfanilamide, sulfacetamide, sulfadiazine, sulfathiazole, sulfamethazine, sulfamethoxazole) were separated and quantified in honey of different botanic origin using an HPLC-fluorescence detection method. After the acidic hydrolysis, sulfonamides were extracted from honey with a mixture of acetonitrile and dichloromethane, further clean-up on a C18 SPE cartridge and pre-column derivatization with fluorescamine. LC separation was performed on a RP C8 column in gradient mode, in 30 min. The limit of detection was determined at 2µg/kg for sulfadiazine and sulfamethazine, 3µg/kg for sulfanilamide, sulfacetamide and sulfathiazole and 5µg/kg for sulfamethoxazole. The sample clean-up was efficient and recoveries of the analytes in spiked honeys ranged from 47 to 79%.

Keywords: liquid chromatography, fluorescence, sulfonamides, honey

INTRODUCTION

The presence of sulfonamides in honey is originated from the treatment applied to the bees if those were diagnosed whit European or American Foulbrood. The *Paenibacillus larvae* and *Melissococcus plutonius* bacterium cause those diseases. The names of these diseases do not give an indication of their geographic distribution, as both of them are found in most regions of the world (Sheridan and col., 2008). In any unmotivated intervention in beehive, the bees are aggressed, and theirs immunity is destroyed (Tarta, 2003).

The consumption of bee products containing sulfonamide residues represents a real danger for human health, because they can produce resistance to antibiotics, as well as allergic reactions. Some sulfonamides are known to be carcinogenic. For example, the sulfamethazine is considered a thyroid carcinogenic agent (Msagati and col., 2004).

For this reason, it is very important the development of reliable techniques capable to identify and quantify those contaminants. No maximum residue level (MRL) for sulfonamides in honey is established in Europa. This means that sulfonamides must be below the limit of quantification of the analytical method used. Some countries within the European Union have established action limits or tolerated levels. Belgium and the United Kingdom have set action limits of 20 and 50 µg/kg, respectively and Switzerland has established a fixed limit of 50 µg/kg, for total sulfonamides in honey (Maudens and col., 2004).

Sulfonamides, broad-spectrum synthetic antibiotics, are derivatives of sulfanilamide, an amide of paraaminobenzensulfonic acid, and have amphoteric properties: the weak basic characteristics depend on anilinic nitrogen, while weak acid characteristics are due to the N-H bond of the sulfonamidic group (Gentili and col., 2005).

Many liquid chromatographic methods have been published for the determination of sulfonamides in honey: LC coupled with UV, DAD, fluorescence detection or mass spectrometry.

This paper describes a method for the quantitation of six sulfonamides (sulfanilamide, sulfacetamide, sulfadiazine, sulfathiazole, sulfamethazine, sulfamethoxazole) in honey samples by high-performance liquid chromatography (HPLC) with fluorimetric detection. The method involves an acidic hydrolysis step before extracting sulfonamides, a SPE procedure for clean-up and pre-column derivatisation with fluorescamine.

MATERIALS AND METHODS

The main purpose of this study was the development of an analytical method for sulfonamides determination in honey with different botanical origin, in which the other compounds do not interfere with the analytes.

The measurements were carried out on a Shimadzu VP series LC system (Japan) composed of ternary LC-10 AD pump, DGU-14A degasser, SLC-10S system controller, CTO-10AS column oven, SIL-10AF automatic injector and a fluorescence detector RF-10A XL with excitation wavelength of 405 nm and emission wavelength of 495 nm. The analytical column was a Phenomenex Luna C8 (250 x 4,6 mm, 5µm). The mobile phases were acetonitrile (A), methanol (B) and ammonium acetate buffer solution pH = 4,5 (C), in a gradient mode, at a total flow rate of 1 ml/min. The temperature of the column was 40°C. Injection volume was 50 µl.

The individual standard stock solutions of sulfonamides (1000 ppm) were prepared by dissolving the standard antibiotics in methanol and stored at –20°C. The working standard solutions were prepared from this stock by serial dilution in water.

The honey sample (5g) was weighed in a centrifuge tube and dissolved with 5ml of 2M hydrochloric acid. The sample was shaken on a rotary mixer for 30 min. The pH was adjusted to 5, using solutions of sodium hydroxide (10M, 5M, 1M, 01M). Samples were extracted with 10ml of acetonitrile-dichloromethane: 80:20 (10 min on a rotary mixer) and centrifuged afterwards for 10min at 4000 rpm. The upper organic layer was transferred into a round bottom flask, the sample was again extracted with the same solvent and the organic phases combined. The extract was evaporated to dryness in a rotary evaporator and the residue was reconstituted in 5ml 0,1M acetic buffer solution (pH = 5) and loaded onto a C18 (500mg, 3ml, 45µm) SPE column conditioned with 3ml of methanol, 3ml of water and 5 ml of 0,1M acetic buffer (pH = 5). The SPE cartridge was washed with 6ml of water and dried under vacuum. The sulfonamides were eluted with 5ml acetonitrile, which was then evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 1ml water and derivatizated in a dark vial using 1,6ml of sodium citrate buffer (pH =3) and 0,6ml of 0,02% fluorescamine in acetone. The final solution was vigorously mixed with a vortex mixer. The sample was let to stand 30 min in the dark, at room temperature. After filtering through a 0,22 µm nylon filter, the sample was analyzed.

RESULTS AND DISCUSSION

The method was applied to different types of honey: acacia, multifloral and honeydew honey. No interferences in the analyses of sulfonamides were observed, as it is shown the next figures.

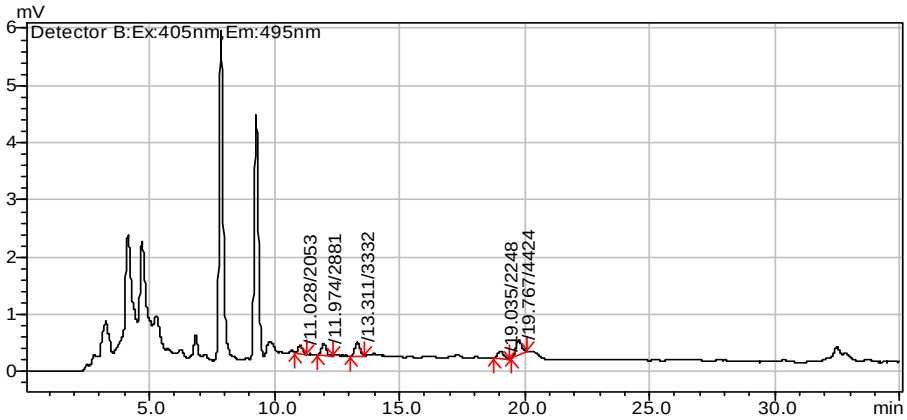

Fig. 1. HPLC chromatogram of acacia honey sample

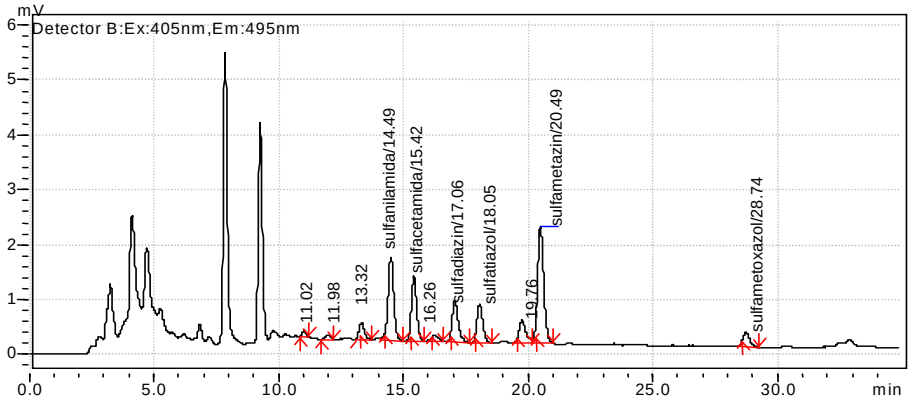

Fig. 2. HPLC chromatogram of acacia honey spiked with 20 µg/kg standard mixture

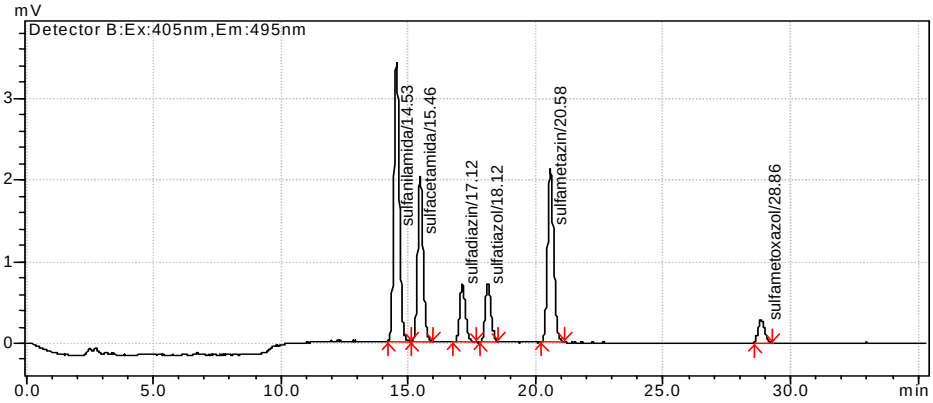

Fig. 3. HPLC chromatogram of 20 µg/kg sulfonamide standard mixture

Levels of sulfonamides in honey are known to decrease over time when the honeys are kept at room temperature. This apparent reduction is attributed to the formation of glucose adducts (Verzegnassi and col., 2002). Therefore, acidic hydrolysis is required to insure complete release of bounded residues in the matrix.

The amphoteric sulfonamides would therefore form positive, neutral as well as negative ions depending on the pH of the environment. At high pH, the amine group, which is not directly attached to the ring, losses its proton to create a negative charge on the molecule.

At low pH values, the amine group, which is attached directly to the ring, is protonated and therefore a positive charge to the molecule is formed. Between these two extremes there will be a range for neutral uncharged species (Msagati and col., 2004). The pH of the hydrolized solution was adjusted at 5 before the first step of liquid-liquid extraction, at a value which can permits the simultaneous extraction of all 6 compounds with an acceptable recovery. Their extraction efficacy is dependent on their individual pKa values, which range from 5 to 8,6 (Verzegnassi and col., 2002). We tested the efficiency of four extracting solvents: acetonitrile-dichloromethane (80-20); acetone-dichloromethane (80-20); acetonitrile; 0,1M acetic buffer, pH=5. The best mixture for the isolation of sulfonamides from the matrix was acetonitrile-dichloromethane (80-20).

Two different stationary phases for solid - phase extraction were tested: C18 octadecyl-modified silica and polymeric Oasis HLB. Best results were obtained when using C18 octadecyl-modified silica over Oasis HLB, because the interferences were eliminated, and the quantification of sulfonamides could be made clearly. When we used polymeric cartridge, the sample clean up was insufficient, which made reliable quantification impossible.

Fluorescamine (non-fluorescent) reacts with primary amines to form a fluorescent product. The resulting fluorescence is proportional to the amine concentration. The intensity of fluorescence emission is higher when the pH is near 3 (Martel and Zeggane, 2003). Another original aspect was automatisation of derivatization step in order to obtain good reproductibility.

Calibration curves were performed for each sulfonamide in the range of 3-75 µg/kg. Correlation coefficient values were higher than 0.995. All compounds were in baseline separation, with a good resolution. The limits of detection were calculated using HPLC soft. Recoveries of the analytes were determined at a fortification level of 30 µg/kg (Table1).

Tab.1

Some of validation parameters for developed method

Analyte	Retention time (min)	Curve correlation coefficient	LOD (µg/kg)	% Recovery
sulfanilamide	14.49	0.9989	3	47
sulfacetamide	15.42	0.9995	3	61
sulfadiazine	17.06	0.9992	2	75
sulfathiazole	18.05	0.9956	3	79
sulfamethazine	20.49	0.9996	2	69
sulfamethoxazole	28.74	0.9985	5	72

CONCLUSIONS

The proposed method in this study is able to separate and quantify six sulfonamides from different types of honey.

The sample clean-up was efficient, in order to remove potentially co-elute with interest analites.

Satisfactory recoveries percentages were obtained, because there are many, but necessary steps in the sample preparation in order to eliminate the possible interferences in the chromatograms.

REFERENCES

1. Gentili, A., D. Perret, S. Marchese (2005). Liquid chromatography-tandem mass spectrometry for performing confirmatory analysis of veterinary drugs in animal-food products. Analytical Chemistry, XX(X).
2. Martel, A.C. and S. Zeggane (2003). HPLC determination of sulfathiazole in French honeys. J. LC & Related Technologies, 26(6): 953-961.
3. Maudens, K. E., G. F. Zhang, W. E. Lambert (2004). Quantitative analysis of twelve sulfonamides in honey after acidic hydrolysis by high-performance liquid chromatography with post-column derivatization and fluorescence detection. J. Chromatogr. A, 1047: 85-92.
4. Msagati, T. A. M. and M. M. Nindi (2004). Multiresidue determination of sulfonamides in a variety of biological matrices by supported liquid membrane with high pressure liquid chromatography-electrospray mass spectrometry detection. Talanta, 64: 87-100.
5. Sheridan, R., B. Policastro, S. Thomas and D. Rice (2008). Analysis and occurrence of 14 sulfonamide antibacterials and chloramphenicol in honey by solid-phase extraction followed by LC/MS/MS analysis. J.Agric.Food Chem. 56: 3509-3516.
6. Tarta, E. (2003). De ce, de unde apar si se mentin antibioticele in miere. Revista Romania apicola, 5:10.
7. Verzegnassi, L., M. C. Savoy-Perroud, R. H. Stadler (2002). Application of liquid chromatography-electrospray ionization tandem mass spectrometry to the detection of 10 sulfonamides in honey. J. Chromatogr. A, 977: 77-87.