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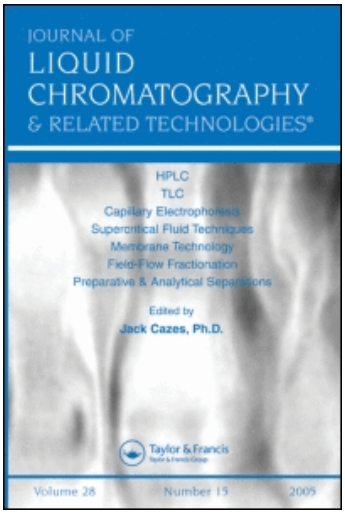

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HPLC Determination of Sulfathiazole in French Honeys

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HPLC Determination of Sulfathiazole in French Honeys

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ABSTRACT

A rapid reversed-phase high-performance liquid chromatographic method, with fluorimetric detection, is described for routine analysis of sulfathiazole (STZ) residues in honey. Samples were dissolved in 1 M hydrochloric acid and submitted to pre-column derivatization with fluorescamine. Liquid chromatographic analysis of the fluorescent derivative is performed on a C_{18} column using a mobile phase of 2% acetic acid/acetonitrile (60 : 40, v/v). The quantification limit obtained for STZ in honey was 10 μg/kg.

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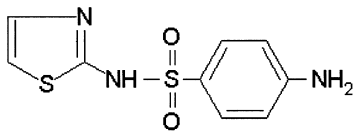
Key Words: Antibiotics; Sulfathiazole; Honey; HPLC.

INTRODUCTION

Sulfonamides are used for the prevention and treatment of a disease known as American foulbrood (*Bacillus larvae*) in honeybees. Sulfathiazole (STZ) (Fig. 1) is one of sulfonamide drugs widely used in beekeeping. The two principal methods of application are to feed honeybees on medicated sucrose syrup (usually 50% by weight sucrose) and to dust them with a mixture of dispersed drug in powdered sucrose. The use of STZ (4-amino-*N*-2-thiazolyl benzenesulfonamide) inside beehives implies a risk of contamination of the honey, so various methods have been developed for the quality control of honeys because the presence of drug residues in honey is undesirable from the standpoint of food sanitation.

Sheth et al.[1] presented a simple enzyme immunoassay developed for screening honey samples for STZ. But immunotests are very specific for a molecule and are expensive. Liquid chromatography (LC) was performed to simultaneously analyze sulfonamide residues. Sulfathiazole, sulfamerazine, and sulfamethazine were analyzed in honey by LC/UV detection at 254 nm after acetone extraction.[2] Horie et al.[3] developed a method for the simultaneous determination of 10 sulfonamides in honey by LC/UV detection. Samples were dissolved in 30% sodium chloride and extracted with dichloromethane, followed by a clean up on a Florisil cartridge. The extracts were analyzed at 275 nm and the limits of detection (LOD) were 50 μg/kg for STZ and other drugs. An LC with micellar mobile phases of sodium dodecyl sulfate (anionic surfactant) method for the determination of 11 sulfonamides, of which the STZ in milk and honey was developed, which allows the direct injection of the samples into the chromatographic system.[4] In chicken tissues, the sulfonamides are extracted with chloroform, partitioned into hydrochloric acid, and submitted to pre-column derivatization with fluorescamine,[5] followed by LC analysis which was performed at 30°C. Schwaiger and Schuch[6] included an acidic hydrolysis step before extracting STZ and 10 other



Sulfathiazole

Figure 1. Structure of sulfathiazole.

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sulfonamides in honey to ensure that sugar-bound sulfonamides in honey are included in the final result. The hydrolysis step converts the bound STZ to free STZ. Pre-column derivatization with fluorescamine increased the sensitivity and the selectivity using LC with fluorescence detection.[5,6] The LC conditions were suitable for the simultaneous detection of 11 sulfonamides and the positive samples were confirmed by gas chromatography/mass spectrometry (GC/MS) after methylation with diazomethane.[6] The low volatility of sulfonamides dictates the suitability of LC over GC methods. So, quantitative LC/MS/MS determination of sulfonamides in honey was developed.[7] This method is sensitive and selective, but the instrumentation is still expensive. All laboratories do not possess this technique.

This paper describes a rapid method for the evaluation of STZ residue levels in honey samples, which involves a liquid-phase extraction and pre-column derivatization before analysis by high-performance liquid chromatography (HPLC) with fluorimetric detection (FLD).

Referring to the sum of the initial substances (sulfonamides and their metabolites), Switzerland has established a limit of 100 μg/kg for meat and 50 μg/kg for honey. But, in the EEC, no maximum residue limits (MRLs) are fixed for sulfonamides in honey, contrary to the other matrices (for milk, meat, liver, and kidney, the MRL is 100 μg/kg). In France, antibiotics are not authorized as bee treatments, but a veterinarian can prescribe the use of antibiotics. Honey is a "pure natural product" by definition, so the consumer does not expect any contamination with antibiotics.

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EXPERIMENTAL

Instrumentation

High-performance liquid chromatography was performed on an Agilent 1100 from Agilent (Karlsruhe, Germany) consisting of a quaternary pump, an autosampler, and a fluorimetric detector.

Materials and Reagents

An agitation system for centrifuge tubes from New Brunswick Scientific Co. (NJ) and the GR 4.11 centrifuge from Jouan (Saint-Herblain, France) were used. A Vortex-mixer, with variable speed, from Fisher Bioblock Scientific (Illkirch, France) was employed.

All solvents used (methanol, acetone) were of ultra-pure for pesticides analysis grade and were bought from Merck Eurolab-Prolabo (Fontenay-sous-Bois, France). Acetonitrile was of HPLC grade and was obtained from Fisher

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Scientific Labosi (Elancourt, France). Hydrochloric acid (35% min, RP Normapur for analysis), citric acid monohydrate crystallized (RP Normapur), and acetic acid (Rectapur) were purchased from Merck Eurolab-Prolabo. Sodium acetate (Analypur) was obtained from Fisher Scientific Labosi. We used laboratory-distilled water.

Fluorescamine reagent was obtained from Interchim (Montluçon, France). The standards STZ and sulfamethizole (SMZ) (Fig. 2) were purchased from Sigma Aldrich (Saint-Quentin-Fallavier, France). A solution of fluorescamine was prepared at a concentration of 200 mg/L in acetone and kept at +4°C for one month in a dark bottle. Standard solutions of STZ and SMZ were prepared at a concentration of 400 mg/L in methanol and kept at +4°C for one week. The solution of STZ was diluted to the required concentrations with 1 M HCl. The solution of SMZ (internal standard) was diluted in 1 M HCl to obtain 0.52 mg/L.

The 1.25 M sodium acetate was prepared by dissolving 2.56 g of sodium acetate in 25 mL of distilled water. The sodium citrate solution was composed of 21 g of citric acid monohydrate and 50 mL of 4 M sodium hydroxide and the solution was adjusted to 100 mL with distilled water. The sodium citrate buffer was prepared with 30 mL of 0.1 M HCl and 18 mL of distilled water. This solution was adjusted to pH = 3 with the sodium citrate solution. Those solutions were stored at +4°C when not in use, during one month.

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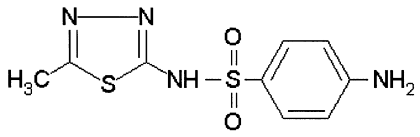
Sample Preparation

The extraction procedure used in this investigation was based on that reported by Schwaiger et al.[6] and Dardenne et al.[8]

A 5 g amount of honey was mixed with 5 mL of 1 M HCl and was vortexed for 2 min. The solution of honey was agitated during 30 min at room temperature on the agitation system. Then, the extract was centrifuged at 3290 g during 20 min at room temperature. A 0.5 mL volume was submitted to the derivatization procedure.



Sulfamethizole

Figure 2. Structure of sulfamethizole.

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Pre-column Derivatization Procedure

In dark vials, 0.5 mL of honey extract, 0.45 mL of 1.25 M sodium acetate, and 0.5 mL of sodium citrate buffer were added to 0.07 mL of internal standard (SMZ). The final solution was vigorously mixed, under vortexing, with 0.3 mL of fluorescamine reagent (200 mg/L). The reaction was left to proceed for 25 min at room temperature before a 100 μL aliquot is injected into the LC system. Derivatization of standards STZ were also performed using 0.5 mL volumes of each of the working solutions and mixing them with 0.45 mL of 1.25 M sodium acetate, 0.5 mL of sodium citrate buffer, 0.07 mL of SMZ, and 0.3 mL of fluorescamine reagent, as above.

Chromatographic Conditions

The liquid chromatographic column was a Hypersil BDS C_{18}, 5 μm, 250 × 4.0 mm I.D. from Merck Eurolab-Prolabo. The mobile phase (pH = 4.5) was 2% acetic acid/acetonitrile (60 : 40, v/v) at a flow-rate of 1 mL/min. Detection of STZ and SMZ was done using an excitation wavelength of 405 nm and an emission wavelength of 495 nm. The injection volume was 100 μL and an internal calibration was employed. The chromatographic runs were made at room temperature.

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RESULTS AND DISCUSSION

Analytical Characteristics

A calibration curve was constructed from peak areas vs. antibiotic concentrations. The concentration of internal standard (SMZ) was 20 μg/L. Good linearity was observed ($r = 0.999$, at least) for STZ from 1.37 to 27.47 μg/L. The determination of STZ was achieved by using the internal standard method.

Limits of detection and quantification (LOQ) were calculated by a signal to noise ratio of 3 : 1 and of 10 : 1.[9] The recovery of STZ was checked by adding 0.125 mL of STZ standard solution prepared in 1 M HCl at different levels to untreated samples of honey. Blank samples from the same honey, without fortification, were treated and analyzed at the same time with spiked honey samples. Honey samples were spiked just before analysis. The STZ was extracted and derivatized as described in the Experimental section and the sample solutions were analyzed by HPLC/FLD under conditions described in the Experimental section. The solute was spiked at three different concentrations and five repetitions were carried out for each fortification level. Profiles

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are shown in Fig. 3. The recoveries ranged from 60.6 to 71.8% and relative standard deviation (RSD) varied from 0.5 to 2.9% (Table 1).

The estimated detection limit for extracts obtained from a honey sample of 5 g and a final volume of 1.82 mL was 2 μg/kg. The LOQ was 10 μg/kg for sulfathiazole in honey.

These results indicate that the present method can be applied on samples of honeys from market. Furthermore, this method allows the determination at levels lower than residues tolerances proposed by Switzerland (50 μg/kg).

Analysis of Sulfathiazole

High-performance liquid chromatography analysis with UV detection was first chosen, with detection at 290 nm (maximal absorbance for STZ) with the external method. But many interferences were observed and the method was not sensitive enough. So, HPLC with fluorimetric detection was preferred to determine residues in honey.

The HPLC method is based on the reaction of fluorescamine (non-fluorescent) with primary amines to form a fluorescent product.[5,10]

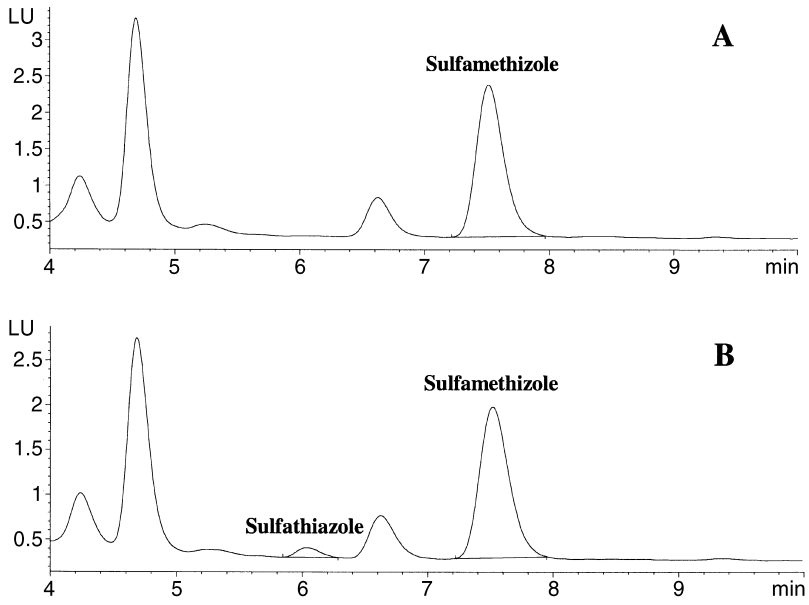

Figure 3. (A) Chromatogram of a blank extract of honey; (B) chromatogram of honey spiked with 10 μg/kg of sulfathiazole.

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Table 1. Recoveries obtained for sulfathiazole at various fortification levels.

Fortification level (μg/kg)	Mean recovery[a] (%)	RSD (%)
10	68.8	2.9
	65.6	
	64.4	
	65.5	
	60.6	
50	71.4	0.5
	71.1	
	71.1	
	70.9	
	70.0	
100	70.6	1.0
	69.9	
	69.8	
	71.7	
	71.8	

[a]$n = 5$.

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Fluorescamine, a fluorogenic reagent, reacts directly with sulfonamides at room temperature to form pyrrolinone derivatives (Fig. 4) which, upon excitation at 405 nm, emit strong fluorescence at 495 nm.[5] The resulting fluorescence is proportional to the amine concentration[8,10] and the fluorophors are stable over several hours.[10] The interferences detected by UV detection are not observed by fluorimetric detection. The intensity of fluorescence emission is higher when the pH is near 3[5] and the speed to achieve the maximal fluorescence is dependent upon the fluorescamine concentration.[8]

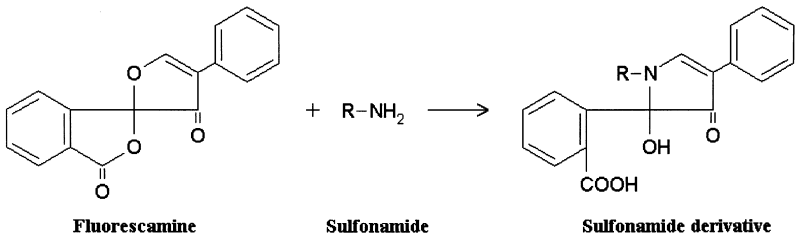

Figure 4. Reaction of fluorescamine with primary amines.

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The use of a fluorescamine solution which is increasingly concentrated for the same concentration of sulfonamides allows reducing, considerably, the duration of the reaction to achieve the level of maximal fluorescence.[8]

So, this method permits rapid analysis of the STZ with a preparation of sample without clean up. This method is used for all honeys, i.e., even if the geographic origins are different. To avoid an underestimate of the residues, the extraction must be realized at acidic pH by adding HCl because STZ is combined with sugars.[6] The hydrolysis step converts the bound STZ to free STZ[6] and STZ itself is stable under acidic conditions.

The analysis of 148 honey samples collected in 2001 from beehives in diverse areas of France (national sampling) with information about the treatments applied[11] shows that STZ was present in amounts above the LOD and STZ was detected in 19 of 148 analyzed samples. The concentrations of nine samples range from 10 μg/kg to 6127 μg/kg. The positive samples (5/19) result essentially from the Northwestern regions of France where the STZ has been used by beekeepers for many years.

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CONCLUSION

An HPLC method has been applied for determination of the antibiotic STZ in honey using reversed-phase chromatography with fluorimetric detection after a dilution in 1 M HCl and pre-column derivatization. This method is very rapid and highly sensitive. For sample extraction, the method described in this paper was applied to honeys from various floral origins with satisfactory recoveries.

ACKNOWLEDGMENT

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REFERENCES

1. Sheth, H.B.; Sporns, P. Enzyme immunoassay for screening of sulfathiazole in honey. J. Assoc. Off. Anal. Chem. **1990**, *73* (6), 871–874.
2. Belliardo, F. Determination of sulphonamide residues in honeys by high-pressure liquid chromatography. J. Apic. Res. **1981**, *20* (1), 44–48.

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3. Horie, M.; Saito, K.; Nose, N.; Nakazawa, H. Simultaneous determination of sulfonamides in honey by liquid chromatography. J. AOAC Int. **1992**, *75* (5), 786–789.
4. Caballero, R.D.; Torres-Lapasio, J.R.; Baeza-Baeza, J.J.; Garcia-Alvarez-Coque, M.C. Micellar chromatographic procedure with direct injection for the determination of sulfonamides in milk and honey samples. J. Liq. Chrom. & Rel. Technol. **2001**, *24* (1), 117–131.
5. Simeonidou, E.J.; Botsoglou, N.A.; Psomas, I.E.; Fletouris, D.J. Liquid chromatographic analysis of multiple sulfonamide residues in chicken muscle using pre-column derivatization and fluorescence detection. J. Liq. Chrom. & Rel. Technol. **1996**, *19* (14), 2349–2364.
6. Schwaiger, I.; Schuch, R. Bound sulfathiazole residues in honey–Need of a hydrolysis step for the analytical determination of total sulfathiazole content in honey. Deutsche Lebensmittel-Rundschau **2000**, *3*, 93–98.
7. Kaufmann, A.; Roth, S.; Ryser, B.; Widmer, M.; Guggisberg, D. Quantitative LC/MS/MS determination of sulfonamides and some other antibiotics in honey. J. AOAC Int. **2002**, *85* (4), 853–860.
8. Dardenne, P.; Fumière, O.; Romnee, J.M.; Berben, G. Developpement de Systèmes Analytiques pour le Contrôle de l'Authenticité de Viandes Certifiées (Rapport Final). Contrat NP/42/022. Ministère des Classes Moyennes et de l'Agriculture, Centre de Recherches Agronomiques de Gembloux: http://www.belspo.be/belspo/ostc/geninfo/publ/pub_ostc/NP2/rNP22via_fr.pdf.
9. Directive 91/414/EEC modified by Directive 96/46/CE and Explantory Note EEC/844/87, European Union, Brussels.
10. Udenfriend, S.; Stein, S.; Böhlen, P.; Dairman, W.; Leimgruber, W.; Weigele, M. Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. Science **1972**, *178*, 871–872.
11. Note DGAL/SDSPA/SDRRCC/N2001-8023, according to European Union Directive 96/23/EC, Ministry of Agriculture, Paris.

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