Short communication

Application of sugaring-out extraction for the determination of sulfonamides in honey by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A simple sugaring-out assisted liquid–liquid extraction method combined with high-performance liquid-chromatography with fluorescence detection (HPLC-FL) was developed for the extraction and determination of sulfonamides in honey. Sample preparation consisted of acid hydrolysis to release sugar-bound sulfonamides. After derivatization with fluorescamine, the derivatives were partitioned into the organic layer under the honey (sugar)/water/acetonitrile system. The clear organic extract obtained by centrifugation could be injected into the HPLC system either directly or after dilution. Linearity was obtained with the coefficient of determination (R^2) higher than 0.998 from 2 to 200 ng/mL. Under the optimal conditions, recoveries were determined for honey fortified at three levels (5, 20, and 100 ng/g) were 80.9–99.6% with coefficients of variation of 0.3–4.4%. Limits of detection for the sulfonamides studied were found to range from 0.6 to 0.9 ng/g.

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1. Introduction

In honeybees, sulfonamides (SAs) are employed to prevent and treat bacterial bee diseases such as American foulbrood and European foulbrood [1]. Maximum residue levels (MRLs) have not been established for SAs in honey in the European Union or in Taiwan. Some countries within the European Union have set action limits or tolerated levels. Belgium and the United Kingdom have set action limits of 20 and 50 $\mu g\,kg^{-1}$, respectively and Switzerland has established a fixed limit of 50 $\mu g\,kg^{-1}$, for total SAs in honey [2].

Numerous extraction techniques such as dissolution [3,4], liquid–liquid extraction (LLE) [5], solid-phase extraction (SPE) [1], and LLE followed by SPE [2] have been developed for SAs in honey. However, these techniques using high volume of organic solvents and SPE cartridges were usually expensive and environmentally toxic, the performance of rotary evaporation step was also very time-consuming.

For sample preparation, besides the water-immiscible solvents, the high-polarity, water-miscible solvents have also been investigated as an alternative to the conventional LLE. For example, the interest in LLE with MeCN has been reported in the literature by significantly lowering the temperature of the mixture or increasing the salt concentration in the mixture of MeCN and aqueous phase, resulting in the separation of the MeCN phase from aqueous phase, as observed in conventional LLE [6,7]. Recently, the salting-out assisted liquid–liquid extraction (SALLE) approach with MeCN has been used more extensively for the analysis of pesticide and antibiotic residues in food samples [8,9]. Moreover, a novel phenomenon was recently observed and named "sugaring-out", which the phase separation of a MeCN–water mixture can be achieved with triggered by addition of sugars [10].

For honey, the main group of chemicals present comprises sugars (with monosaccharides and oligosaccharides, totaling ca. 77%), with glucose and fructose having average content of 30% and 38%, respectively [11]. For this reason, the aim of this work was to assess whether the sugaring-out approach can be used as a valuable sample preparation for the determination of SAs in honey. Moreover, to keep the analytical steps as few and simple as possible, SA residues were firstly derivatized with fluorescamine in the dissolved honey

matrix and subsequently the final extractant solution obtained from the sugar (honey)/water/MeCN system after centrifuging was directly analyzed by HPLC was also investigated.

2. Experimental

2.1. Reagents and materials

Sulfapyridine (SPD), sulfaguanidine (SGN), sulfachloropyridazine (SCP), sulfamonomethoxine (SMMX), sulfadimethoxine (SDM), sulfisoxazole (SIZ) and fluorescamine were purchased from Sigma–Aldrich (Steinheim, Germany). Sulfamerazine (SMR) and sulfadoxine (SDX) and were purchased from Riedel-de Haën (Sigma–Aldrich, Seelze, Germany).

HPLC-grade acetonitrile (MeCN), acetone and hydrochloric acid (HCl) 37% were purchased from Merck (Darmstadt, Germany). Sodium citrate dehydrate was purchased from Mallinckrodt (Xalostoc, Mexico). Glacial acetic acid was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ, USA). Honey (longan flower) samples were obtained from local grocery stores and stored at room temperature. The stock solutions of sulfonamides were each prepared by dissolving in acetonitrile at the concentration of 0.1 mg/mL and stored at 4 °C. The structures of the sulfonamide compounds studied are presented in Fig. 1. Fluorescamine reagent (0.2%) was prepared by dissolving 10 mg of fluorescamine in 5 mL of acetone.

2.2. Instrumentation

The Hitachi HPLC system (Hitachi, Tokyo, Japan) was used consisting of a L2130 LC pump, an L2200 autosampler and a L2485 fluorescence detector with excitation wavelength $\lambda = 405$ nm and

Fig. 1. Chemical structures of the studied sulfonamides.

emission wavelength $\lambda = 495$ nm. The chromatographic separation was performed on a Cosmosil 5C18-AR-II analytical column (250 mm × 4.6 mm, particle size 5 μm; Nacalai Tesque Inc., Kyoto, Japan) operated at 40 °C. The mobile phase for LC analyses consisted of MeCN and a 2% solution of acetic acid with a total flow of 1 mL/min. The separation gradient was an initial isocratic step 35% MeCN for 2 min, from 2 to 15 min linearly change to 50% MeCN, and maintained for 5 min. The initial gradient conditions were reestablished immediately and the column was equilibrated for 10 min for the next injection. The pH values were measured by Cyberscan 2000 pH meter (Eutech Cybernetics, Singapore, Singapore). Centrifugation of the samples was performed in a Hermle Z206A centrifuge (Hermle Labortechnik GmbH, Wehingen, Germany).

2.3. Sample preparation

A honey sample (1 g) was fortified with the desired quantities of SAs. After remaining for 1 day at room temperature, 0.8 mL of 4 M hydrochloric acid was added to the sample and mixed vigorously until honey is completely dissolved. The mixture was shaken on a mechanical shaker for 30 min and then the pH was adjusted to 3.5 by adding 50 μL 400 mM sodium citrate solution and 300 μL 10 M sodium hydroxide. After hydrolysis SAs were derivatized with 200 μL of 0.2% fluorescamine solution in the honey matrix for 25 min. After that, to keep the volume of the upper organic layer constant with the final value of 1 mL, the mixture was mixed with 1.1 mL MeCN on a vortex mixer for 30 s and then centrifuged for 3 min at 4000 rpm. A 0.5 mL of the upper organic layer was mixed with 0.5 mL of 20 mM citrate buffer (pH 3.5). After filtereation by 0.45 μm membrane, an aliquot (10 μL) were injected into the HPLC.

For the derivatization of standards, the stock solution of SAs was transferred in another centrifuge tube at appropriate microliter aliquots and 400 μL of 20 mM citrate buffer (pH 3.5) was added. A 50 μL of 0.2% fluorescamine solution was added and vortexed for 30 s and then the volume was adjusted to 0.5 mL with 20 mM citrate buffer. After 25 min derivatization, a 0.5 mL MeCN was added into the buffer solution and mixed thoroughly. An aliquot (10 μL) were injected into the HPLC.
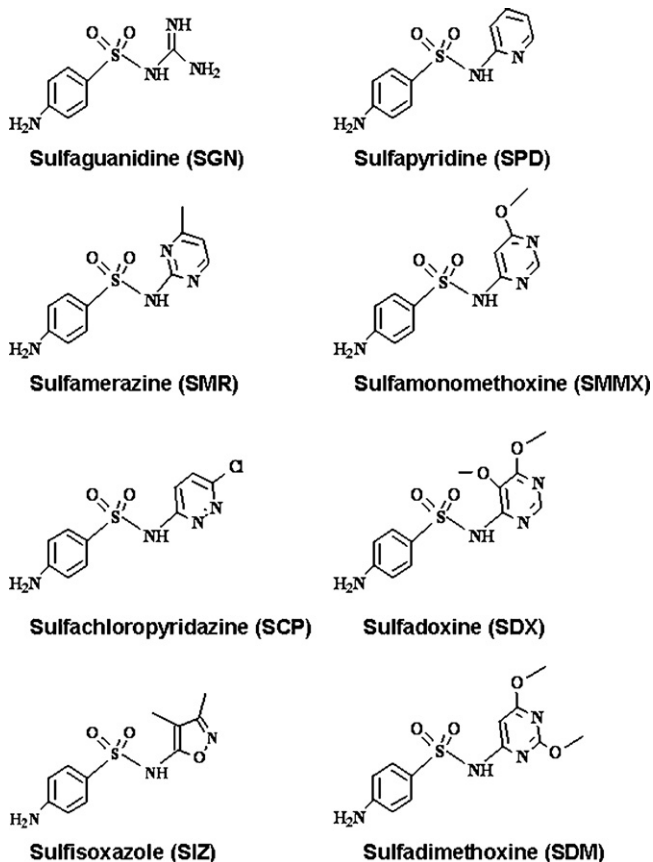
3. Results and discussion

3.1. Sample preparation

Since the average composition consists of approximately 80% sugars in honey, by the addition of MeCN, the phase separation could be easily achieved after centrifuging and LLE could be simply performed in this study. We initially tried to extract the SAs from honey into the upper layer using the sugaring-out assisted liquid–liquid extraction step. Honey samples were spiked for 20 min and dissolved with 1 mL citrate buffer pH 5.5. After centrifuging, the upper organic layer was evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted and SAs were determined by HPLC-FL after pre-column derivatization with fluorescamine. Except for SGN, extraction efficiencies of most SAs were above 70% (shown in Fig. 2). This result revealed that under the sugaring-out condition, most of the SAs could be extracted from the honey sample to the MeCN layer. However, a drawback for this method is that an evaporation step was needed before the derivatization step.

To keep the analytical steps as few and simple as possible, we therefore assessed the possibility whether it could efficiently extract the derivatized SAs from honey into the MeCN layer. After dissolving spiked samples with 1 mL citrate buffer pH 3.5, the SAs were derivatized with fluorescamine in the dissolved honey matrix and then extracted with MeCN. The remarkable improvement in the
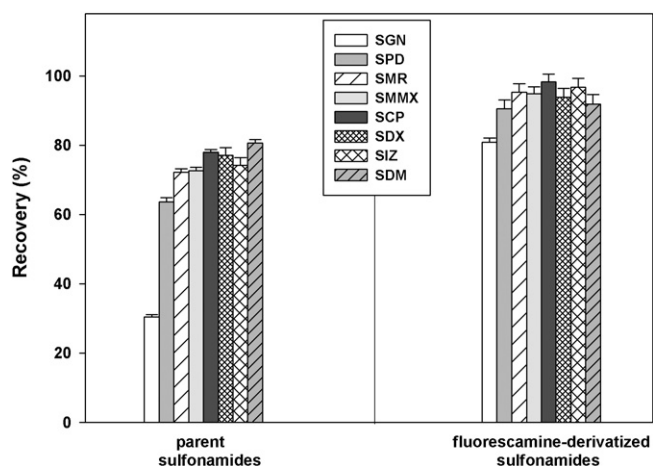
Fig. 2. Extraction efficiencies of the parent sulfonamides or the fluorescamine-derivatized sulfonamides under the sugaring-out condition. Honey samples spiked at 100 ng/g of each sulfonamide for 20 min were extracted with MeCN before or after derivatization with fluorescamine.
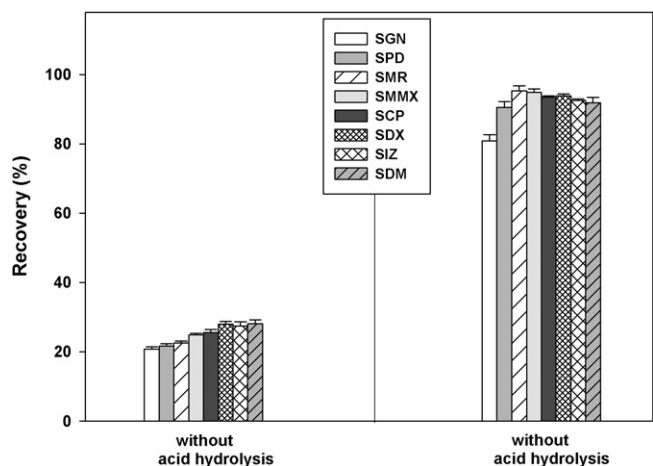
Fig. 3. Extraction efficiencies of sulfonamides in honey spiking at 100 ng/g of each sulfonamide at day 1 without or with an initial acid hydrolysis step prior to pre-column derivatization.

recovery of SGN may indicate that the addition of the more bulky pyrrolidine structure to the SGN increased the hydrophobicity and resulted in their partitioning to the organic layer. In addition, the presence of the bulky pyrrolidine structure may also plays a dominant role in their partition behavior and led to more consistent recoveries (90.6–98.3%) between other seven SAs as compared with those (62.8–81.8%) obtained for parent SAs as described in Fig. 2. To study the effect of the amount of intrinsic sugar on the partition behaviors of the derivatized SAs, the addition of 0.1 g glucose got a similar extraction efficiencies and did not led to change in the volume of the upper organic layer after centrifuging. We also repeated the same experiment by using salt instead of glucose to observe the extraction efficiencies, the results were similar.

A problem for the isolation of SAs in honey is that SAs carry free aromatic amino groups, which can react with reducing sugars in honey to form a variety of different sugar-bound compounds [12]. As described in Fig. 2, when honey samples were spiked for 20 min, the good extraction efficiencies of the SAs could be obtained. However, when spiked samples were stood for 1 day at room temperature, the recoveries decreased dramatically as shown in Fig. 3. If the acid hydrolysis step before extracting SAs in honey was included, this apparent reduction in recoveries was restored again even the spiked samples were stored at room temperature

for 7 days. In this work, an initial acid hydrolysis step was also used to ensure completely releasing of bound SAs in honey.

For the acid hydrolysis step, a 1 g amount of honey was dissolved with 0.5–4 N HCl solution. The sample was shaken for 30 or 60 min and the pH was adjusted to 3.5 with 10 N NaOH followed by the derivatization and extraction steps. It was found that the use of $\geq$2 N HCl could release the bound SAs back to its free form and the hydrolysis time of 30 min was enough to ensure completeness of the reaction. Therefore, in this work, 0.8 mL 4 N HCl was used for acid hydrolysis for 30 min.

3.2. Fluorescamine derivatization

SAs themselves are not fluorescent, but they react easily with flourescamine to form highly fluorescent pyrrolidine-type derivatives at pH 3–4 [13,14]. As described above, since the derivatization step was performed in the dissolved honey matrix prior to the sugaring-out assisted liquid–liquid extraction step, it was therefore necessary to investigate the amount of the derivatization reagent needed in the complex honey matrix. A constant 200 μL acetone containing various amounts of fluorescamine used was tested for the approximate 2 mL of dissolved honey sample at pH 3.5. The highest signal was obtained by use of $\geq$0.05% derivatization reagent when the initial acid hydrolysis step was not included, while $\geq$0.15% as the acid hydrolysis step was included. In this study, for the purpose of using the same concentration of derivatization reagent, a 200 μL 0.2% derivatization reagent was used for the dissolved honey sample (about 2 mL) and 50 μL for the derivatization of standards in 0.5 mL citrate buffer. The optimal incubation period was also examined and found to be 20–40 min, and the reaction was performed for 25 min in our experiments.

For HPLC analysis, sample solvent sometimes plays an important role in the chromatographic separation of sample components. The interaction between sample solvent and mobile phase may results in markedly distorted bands Therefore, after extracting the derivatized SAs into the upper organic layer by LLE, the final extracts were also tested to directly inject into the HPLC system and no significant changes in peak shapes or areas were observed. Although MeCN concentration had little or no effect on the fluorescence signal, due to the high sensitivity of the fluorescence detector, we still diluted the final organic extract with citrate buffer to near 50% solvent composition, which is more consistent with that of standard solution.

3.3. Method evaluation

The standard curves for the 8 SA drugs in the range of 2–200 ng/mL are listed in Table 1. A satisfactory linearity with the R^2 greater than 0.998 was achieved. The absolute recovery and precision of each SA were determined on spiked blank samples at three concentration levels low (20 ng/g), medium (50 ng/g) and high (100 ng/g) during storage at room temperature for 1 day. The mean recoveries were in the range 80.9–99.6% and the relative standard deviations (RSD, n = 4) were from 0.3 to 4.4%. The limits of detection (LODs, ranged from 0.6 to 0.9 ng/g) of the proposed method, were calculated as three times the standard deviation obtained from seven replicate runs of honey sample spiked level at 5 ng/g SAs. Chromatograms of honey samples spiked at 20 ng/g are shown in Fig. 4 using the proposed method. All the steps could be finished in one tube and the main equipment for the proposed method is a benchtop centrifuge. Further, unlike conventional LLE and SPE, it used very little solvent (<1.5 mL) and did not entail the time-consuming evaporation step. The developed method was applied onto the analysis of the 8 SAs in five different brands of commercial longan honey products and they were not detected residues in any of the analyzed samples.

Table 1
Performance of the proposed method under optimized conditions.

Analyte[a]	Linearity range (ng/mL)	R^2	LOD (ng/g)	Mean recovery (%) (storage at room temperature for 1 day)		
				5 ng/g[b]	20 ng/g	100 ng/g
SGN	2–200	0.9987	0.8	89.1 (3.4)[c]	87.8 (3.5)	80.9 (2.3)
SPD	2–200	0.9989	0.8	90.7 (0.3)	95.1 (1.1)	90.6 (3.1)
SMR	2–200	0.9991	0.7	95.8 (1.4)	99.6 (1.4)	95.3 (4.4)
SMMX	2–200	0.9991	0.7	96.9 (3.1)	97.4 (1.1)	94.9 (3.0)
SCP	2–200	0.9995	0.9	95.7 (2.3)	97.0 (1.4)	98.3 (2.8)
SDX	2–200	0.9992	0.7	93.6 (2.7)	89.4 (1.6)	93.8 (2.8)
SIZ	2–200	0.9994	0.6	97.1 (1.7)	92.9 (2.6)	96.8 (3.2)
SDM	2–200	0.9994	0.7	88.4 (2.9)	86.3 (1.4)	91.9 (2.6)

[a] SGN: sulfaguanidine, SPD: sulfapyridine, SMR: sulfamerazine, SMMX: sulfamonomethoxine, SCP: sulfachloropyridine, SDX: sulfadoxine, SIZ: sulfisoxazole and SDM: sulfadimethoxine.
[b] Spike level.
[c] Number in parentheses represents coefficient of variation (%), $n = 4$.
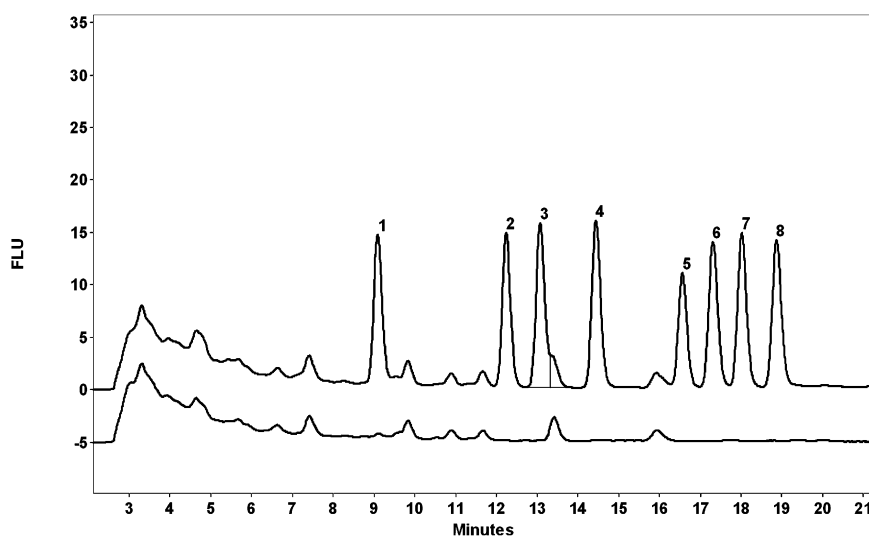
Fig. 4. HPLC chromatograms of blank honey sample (bottom curve) and of a sample spiked with 20 ng/g of each sulfonamide (top curve). The peaks were (1) SGN; (2) SPD; (3) SMR; (4) SMMX; (5) SCP; (6) SDX; (7) SIZ and (8) SDM.

4. Conclusions

In this paper we demonstrate that under the sugar/water/MeCN system, MeCN could be used as an acceptor phase to extract both the parent SAs and the fluorescamine-derivatized SAs. Using sulfonamides as a model, the sugaring-out assisted liquid–liquid extraction procedure could be applied to the determination of SAs at trace levels in honey by HPLC-FL. The proposed method is simple, inexpensive and highly sensitive with low limit of detection.

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