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Fluorimetric determination of sulphathiazole in honey by means the formation of CDs inclusion complexes

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ABSTRACT

The inclusion complex of sulphathiazole in β-cyclodextrin has been investigated. A 1:2 stoichiometry of the complex was established and formation constants K_2 ($42.83 \pm 3.27\ M^{-1}$) and K_1 ($4.98 \pm 0.36\ M^{-1}$) were calculated by using the changes produced on the native fluorescence of the drug, when included on the hydrophobic cyclodextrin cavity. An enhancement in the fluorescence emission of sulphathiazole and protection of the drug against photochemical reactions has been attained upon inclusion. In solutions of β-CD dual emission (458 nm) was noticed in STZ. Formation of the inclusion complex of STZ should result in dual emission, which is due to a twisted intramolecular charge transfer band (TICT). A fluorimetric method for the determination of sulphathiazole has been proposed and applied in honey without sample treatment. The optimized fluorimetric method showed detection and quantitation limits of 9.74 ng/g and 32.48 ng/g, respectively. Selectivity is high, showing no cross-reactivity to other chemically related antibiotics. The results obtained for blind honey samples (mean recovery 97%), were in good agreement with those obtained by liquid chromatography separation and mass spectrometry detection (LC–MS) (mean recovery 102%), showing that the proposed method might be used for the determination of sulphathiazole residues without expensive equipment.

1. Introduction

The sulphonamide family includes a broad-spectrum of synthetic bacteriostatic antibiotics used against most Gram-positive and many Gram-negative microorganisms and protozoa. These compounds are commonly used in human and veterinary medicine for therapeutic and prophylactic purposes to fight common bacterial diseases (Mandell & Sande, 1990). In addition, in animal husbandry these substances are used as feed additives (Wang, Zhang, Wang, Duan, & Kennedy, 2006). As a result of their wide use, worrying levels of these drugs are detected in commodities (Adesiyun et al., 2005), generating serious problems in human health, such as allergic or toxic reactions (Sanderson, Naisbitt, & Park, 2006). Furthermore, the main risk from the use of antimicrobials in animals is that bacteria may develop resistance to them (Botsoglou & Fletouris, 2001). Moreover, some sulphonamides have been found to be potentially carcinogenic compounds (Littlefield, 1988).

The American foulbrood (caused by spore-forming *Paenibacillus larvae*) and the European foulbrood (caused by *Melissococcus pluton*) are two of the most highly contagious and destructive diseases that affect honeybees (Heyndrickx et al., 1996). However the treatment deals with the use of drugs such as apicicline that contains 0.4% oxytetracycline and 4% sulphathiazole as active compounds. Although sulphathiazole was initially recommended to control foulbrood, at this moment, its use is banned because there were found residues in honey many months after being applied. Nevertheless it is known that those compounds and products with similar chemical structure have still been detected in several honey samples from some countries. For this reason, the analysis of STZ in honey nowadays is very important; to assure that this natural product does not contain residues in quantities that could imply a risk for consumer.

Currently, no maximum residue levels (MRLs) for sulphonamide residues in honey are set in the European Union, what indicates that if present they must be below the limit of quantitation (LOQ) reached by the analytical method (Council Regulation EEC No 2377/90, 1990). Since LOQs differ between laboratories, some countries within the European Union have established action limits or tolerated levels, ranged from 20 to 50 ng/g, referring to the s of all substances within the sulphonamide-group or between 10 and 20 ng/g for one, like sulphathiazole.

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Residues of sulphonamide were found in animal products for human consumption, such as milk, eggs, fish, meat or honey (Adesiyun et al., 2005). The current sulphonamide detection methods are based on chromatography or microbiological growth inhibition (Nouws et al., 1999). Microbial inhibition tests are cheap and easy to perform, but require 2–3 days for microbe growth or may be non-specific or lack the necessary sensitivity for desirable residue monitoring.

Different chromatographic methods have been reported for the determination of multiple sulphonamide residues in honey including sulphathiazole (Bernal, Nozal, Jimenez, Martín, & Sanz, 2009; Hammel, Mohamed, Gremaud, Le Breton, & Guy, 2008). Generally qualified personnel is required, are time-consuming and labour intensive, and therefore of limited use as first-action methods involving cumbersome extraction, concentration and separation protocols, followed by identification and quantitation using specialized tools such as spectrometry. In this sense, rapid, sensitive and specific assays are demanded to detect positive sulphonamide samples in routine analysis, which then can be confirmed by reference methods.

For weakly fluorescent and non-fluorescent analytes, derivatization to convert the analyte into a more detectable product (i.e., better fluorescing), constitutes a means of enhancing the detectability of the analyte. The fluorescence characteristics of different sulphonamides have been studied (Bridges, Gifford, Hayes, Miller, & Thorburn Burns, 1974) and proposed for the determination of several of these compounds.

In addition, the formation of inclusion complexes by using cyclodextrins will be an easy approach to enhance the fluorescence signal of the target molecule.

Cyclodextrins (CDs) are a group of naturally occurring cyclic oligosaccharides derived from starch with six, seven or eight glucose residues linked by $\alpha(1 \rightarrow 4)$ glycoside bonds in a cylinder-shaped structure, and denominated α-, β- and γ-CDs, respectively. The central cavity of these molecules is hydrophobic, while the rims of the surrounding walls are hydrophilic (Martín del Valle, 2004). This hydrophobic cavity forms inclusion complexes with a wide range of organic and inorganic guest molecules (Cai et al., 1990), and the properties of the materials which they complex can be significantly modified, being widely used in many industrial products, technologies and analytical methods. In some cases, the inclusion complex with CDs, enhance the fluorescence signal of the target molecule; which is important in order to develop more sensitive analytical methods and, at the same time, allows working in aqueous media.

In this way, different authors (Antony Muthu Prabhu, Venkatesh, & Rajendiran, 2010; Premakumari et al., 2011) studied the spectral properties of sulpha drug–β-CD complexes by UV–vis and fluorescence, concluding that the formation of the inclusion complex should result in dual emission, 330–340 nm and 430–440 nm.

In the present work, the development and evaluation of a sensitive fluorescence method based on using CDs as fluorescence signal enhancers of sulphathiazole, for rapid testing of sulphathiazole residues in honey samples, is presented.

2. Materials and methods

2.1. Materials

β-CDs were purchased from TCI (Eschborn, Germany). All other chemicals used were of analytical grade. Sulphathiazole (STZ) and structurally related sulphonamides such as sulphadiazine (SDZ), sulphadimethoxine (SDM), sulphamerazine (SMZ), sulphamethizole (SMT), sulphamethoxazole (SMX), sulphamethoxypyridazine (SMP), sulphapyridine (SPD), sulfisoxazole (SSX), quercetin, kaempherol and myricetin were purchased from Fluka–Sigma–Aldrich Química (Madrid, Spain).

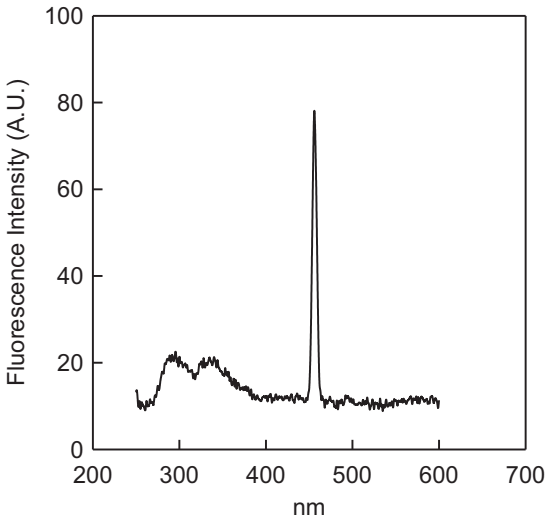

Fig. 1. Fluorescence spectral of STZ λ_{exi} 259 nm.

2.2. Methods

2.2.1. Solubility studies

Phase solubility diagrams were constructed according to Higuchi and Connors (1965). Basically, excess amounts of STZ were added to aqueous solutions of increasing concentrations of β-CDs (up to 15 mM), in 10 mL of acetate buffer 100 mM (pH 5.5). The samples were maintained in an ultrasonic bath for 60 min till reach equilibrium. After that, the aqueous solutions were filtered through 0.45 μm of cellulose acetate membrane filter (Düren, Germany) and diluted in 80% ethanol–water. The STZ (ε_{286} = 11,000 M^{-1} cm^{-1}) concentration was spectrophotometrically determined in a Shimadzu spectrophotometer, model UV-1063.

2.2.2. Fluorescence assays

Steady-state fluorescence measurements were carried out in a Shimadzu RF 5301 PC spectrofluorimeter. Emission fluorescence spectra were acquired in the 435–483 nm range (1 nm step), at a fixed excitation wavelength of 259 nm (Fig. 1). The reaction medium: 100 ng/mL STZ and increasing concentrations of β-CDs, was prepared in sodium acetate buffer 100 mM (pH 5.5), to reach a final volume of 1 mL at 25 °C.

2.2.3. Determination of STZ in honey samples

Honey samples of different origin, i.e., thousand flowers, rosemary, orange, thyme, forest, sunflower, chanteuse, eucalypt, lavender and orange blossom honey, were analyzed in this study. Commercial samples were kindly provided by several honey suppliers from ASEMIEL (Spanish association of honey packers). All samples were stored in dark and dry place at room temperature until assay.

In order to avoid matrix effects, honey samples were simply diluted (1/5, w/v) in assay buffer and a set of standard regression lines were obtained and compared with those carried out in assay buffer [sodium acetate buffer 100 mM (pH 5.5) at 25 °C]. The analysis of honey samples was carried out as follow: briefly, 0.5 g of honey was first dissolved in 2.5 mL of sodium acetate buffer 100 mM (pH 5.5) at 25 °C, containing 13 mM of β-CDs, and maintained in an ultrasonic bath for 60 min to reach equilibrium. After that, the aqueous solution was filtered through 0.2 μm acetate of cellulose membrane filter. One millilitre of the filtrate was placed

in the sample port of the spectrofluorimeter device using a plastic mini Pasteur pipette, and the fluorescence values were analyzed. In order to carry out STZ regression line, standards were prepared as described above, with the exception that honey samples (0.5 g) were spiked with different concentration of STZ (0, 5, 25, 50, 100, 150 and 200 ng/g) before adding assay buffer containing β-CDs. In order to assess assay reproducibility, triplicates of each fortification level were performed.

The samples were also analyzed, for confirmatory purposes, by HPLC–ESI-MS in an Agilent 1100 series LC/MSD Ion Trap (Agilent Technologies, Waldbronn, Germany). To this end, honey samples were extracted as described by Maudens, Zhang, and Lambert (2004), with slight modifications. Briefly, an aliquot of 1.5 g honey was dissolved in 12.5 mL of 1.2 M sodium acetate buffer solution, pH 5.0. The mixture was shaken on an ultrasonic bath for 15 min and the solution was loaded onto a Bond Elut SCX (500 mg, 3 mL, 40 μm) SPE column (Varian, Harbor City, CA, USA), conditioned with 3 mL methanol and 3 mL water. The column was washed with 3 mL sodium acetate buffer solution. Sulphathiazole was eluted with 3 mL acetonitrile and then, the solution was evaporated to dryness at 45 °C under gentle stream of nitrogen. The residue was redissolved in mobile phase and an aliquot of 50 μL injected into the chromatographic system. The separation of sulphathiazole was performed on a ZORVAX C18 column (50 mm × 2.1 mm I.D., particle size 3.5 μm) and running a linear gradient from 100% solvent A (0.5% acetic acid/5% methanol, v/v) at 0 min to 50% solvent A and 50% solvent B (methanol) at 15 min, at a flow rate of 0.4 mL/min. The nebulizer pressure and dry gas flow (350 °C) was set to 40 psi and 10 L/min, respectively. The STZ was detected using electrospray in the positive ionization mode. The only molecular-ion species formed in acidic mobile phase are protonated molecules. Typical MS settings were: needle voltage 3.5 kV; lens 1: 6.8 V and lens 2: −60 V; capillary voltage 110.2, octopole amplitude of 143.8 Vpp, cut-off 69 and amplitude 1.20 V. Two different characteristic fragmentation ions m/z 108 ($[H_2NPhO]^+$) and m/z 156 ($[H_2NPhSO_2]^+$), were chosen in the selected reaction monitoring (SRM) mode using a dwell time of 0.1 s.

2.2.4. Supplementary information

The equilibrium constants between free and complexed STZ were determined using a model involving the sequential binding of two CD molecules to one of STZ (Lucas-Abellán, Gabaldón, Penalva, Fortea, & Núñez-Delicado, 2008):

$$STZ + CD \overset{K_1}{\longleftrightarrow} STZ - CD$$

$$STZ - CD + CD \overset{K_2}{\longleftrightarrow} STZ - CD_2$$

where the complexation constants K_1 and K_2 are defined as:

$$K_1 = \frac{[STZ - CD]}{[STZ]_f [CD]_f} \quad (1)$$

$$K_2 = \frac{[STZ - CD_2]}{[STZ - CD][CD]_f} \quad (2)$$

The mass balance for both STZ and CD in aqueous media may be represented by the following equations:

$$[STZ]_t = [STZ]_f + [STZ - CD] + [STZ - CD_2] \quad (3)$$

$$[CD]_t = [CD]_f + [STZ - CD] + [STZ - CD_2] \quad (4)$$

where subscripts f and t stand for free and total, respectively.

Reorganizing Eqs. (1) and (2), $[STZ - CD]$ and $[STZ - CD_2]$ can be expressed as follow:

$$[STZ - CD] = K_1 [STZ]_f [CD]_f \quad (5)$$

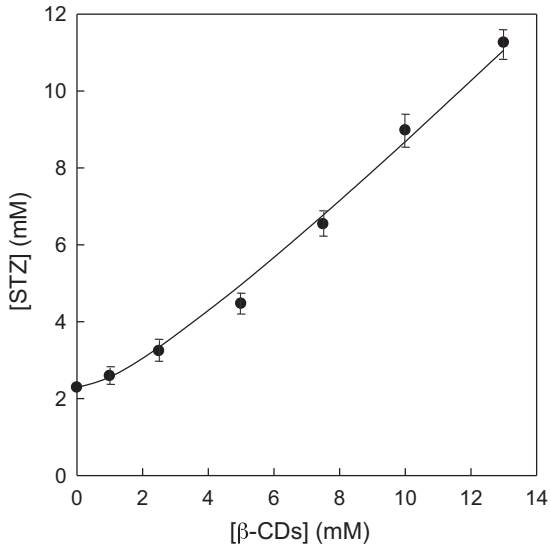

Fig. 2. Phase solubility diagram of SFZ with and β-CDs (●). The lines show the best fits to Eq. (7).

$$[STZ - CD_2] = K_1 K_2 [STZ]_f [CD]_f^2 \quad (6)$$

Then, substituting these two equations into Eq. (3), the following relationship was obtained:

$$[STZ]_t = [STZ]_f + K_1 [STZ]_f [CD]_f + K_1 K_2 [STZ]_f [CD]_f^2 \quad (7)$$

Now, $[CD]_f$ can be deduced from Eq. (4), following substitution of Eqs. (5) and (6) and the appropriate rearrangement:

$$[CD]_f = \frac{-(1 + K_1[STZ]_f) + \sqrt{((1 + K_1[STZ]_f)^2 + (4K_1K_2[STZ]_f[CD]_t))}}{2K_1K_2[STZ]_f} \quad (8)$$

Eq. (8) was used to fit the experimental data, to determine solubility complexation constant.

Another method used to determine the equilibrium constants between free and complexed STZ is the fluorescence method, in which the Eq. (9) is used to fit the experimental data and calculate complexation constant (Lucas-Abellán, Gabaldón, et al., 2008):

$$\frac{1}{(F - F_0)} = \frac{2}{(F_\infty - F_0)} + \frac{1}{(F_\infty - F_0)K_1[CD]_t} + \frac{1}{(F_\infty - F_0)K_1K_2[CD]_t^2} \quad (9)$$

This Eq. (9) showed a lineal relation between $1/(F - F_0)$ and $1/[CD]_t^2$ (Lucas-Abellán, Gabaldón, et al., 2008) for 1:2 complexes.

3. Results and discussion

Firstly, the aqueous solubility of STZ was studied in the presence of increasing concentrations of different types of CDs (β-, HP-β- and G_2-β-CDs) in order to select the best guest in the basis of the higher value of the complexation constant (K_c). Among all CDs tested, only in the case of β-CDs, the solubility of STZ increased with the β-CDs concentration (Fig. 2) and the solubility curve obtained could be classified as A_P type (Higuchi & Connors, 1965), indicating that the stoichiometry of the complexes formed were higher than 1:1.

Assuming that the stoichiometry of the complexes was 1:2, the equilibrium constants between free and complexed STZ were determined, as described in supplementary information, using a model involving the sequential binding of two CD molecules to one of STZ.

Fitting the data of the phase solubility diagram showed in Fig. 2 – using Sigma Plot (Jandel Scientific), by a non-linear regression to Eq. (7) where $[CD]_f$ is substituted by Eq. (8), and taking into account

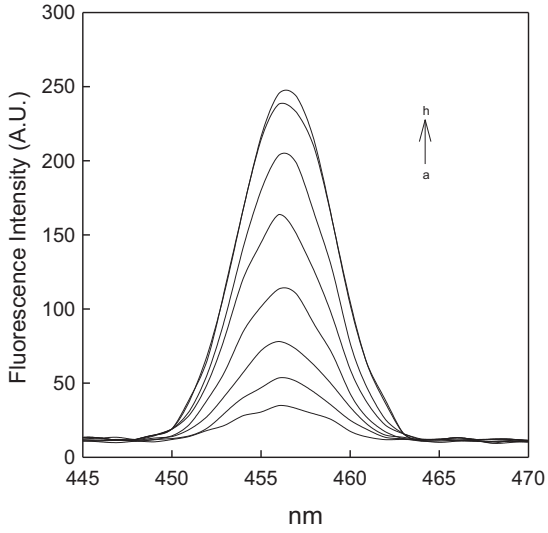

Fig. 3. Fluorescence spectral of STZ with various concentrations of β-CDs λ_{exi} 259 nm: (a) 0, (b) 0.5, (c) 1, (d) 2.5, (e) 5, (f) 7.5 and (g) 10 (h) 13 mM of β-CDS and 0.1 mg/L STZ.

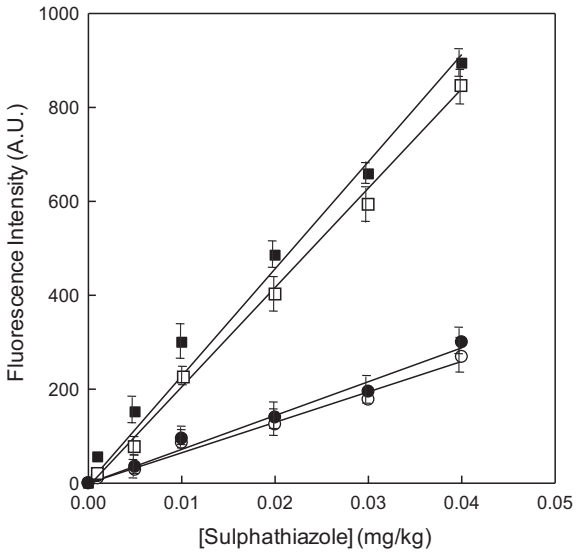

Fig. 5. Fluorescence intensity of STZ (○), STZ in honey (●), STZ in assay buffer and 13 mM β-CDs (□) and STZ in honey and 13 mM β-CDs (■).

that $[STZ]_f$ always refer to the maximum aqueous solubility of STZ (S_0 = 2.29 mM), the values of constants for 1:1 (K_1) and 1:2 (K_2) complexes were obtained. The K_2 value (42.83 ± 3.27 M^{-1}) was significant higher than K_1 value (4.98 ± 0.36 M^{-1}), suggesting that the 1:2 inclusion complexes are the most predominant species in the assayed conditions.

The STZ excitation and emission spectra showed a maximum at 259 and 456 nm, respectively. As can be seen in Fig. 3, for a fixed STZ concentration (100 ng/mL), fluorescence increases with CDs concentration without changes in the excitation and emission wavelengths. In this sense, CDs act as STZ fluorescence enhancer being the fluorescence signal proportional to the CDs concentration and levelled off when all of the STZ has been entrapped in the hydrophobic cavity of the CDs.

To corroborate that the complexes formed between STZ and β-CDs were 1:2 and that the proposed model involving the sequential binding of two CDs molecules to one STZ molecule is correct, the inclusion complexing properties were also studied by photochemically induced fluorescence (PIF). Fig. 4 showed a lineal relation between $1/(F-F_0)$ versus $1/[CD]_t^2$. Confirming that the stoichiometry of the STZ:β-CDs complex is 1:2 (Lucas-Abellán, Gabaldón, et al., 2008) and the K_1 and K_2 values (5.1 ± 0.37 M^{-1} and 46.78 ± 3.52 M^{-1}) were similar to those obtained by solubility studies.

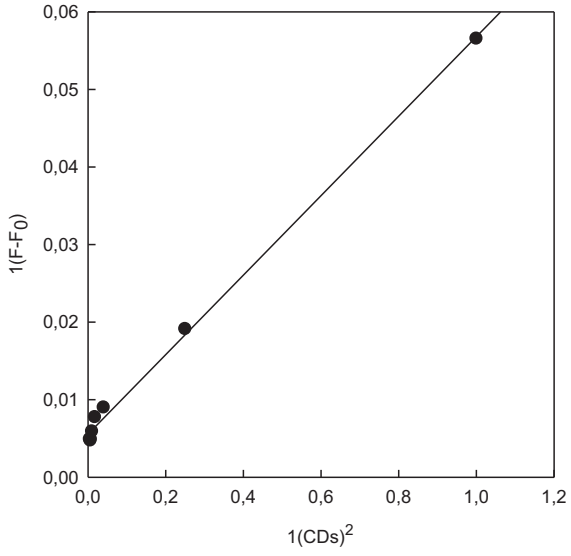

Fig. 4. Lucas-Abellán et al. plot for 100 ng/mL STZ: β-CDs (Eq. (9)).

Once assessed the complexation of STZ with β-CDs, changes in fluorescence spectra of STZ in the presence of β-CDs were evaluated to optimize a new, rapid and more sensitive detection method for the determination of STZ residues in honey samples.

In order to cheek the possible matrix effect on STZ fluorescence spectrum, it was measured in absence and in the presence of increasing concentration of β-CDs, using assay buffer or honey as matrix (Fig. 5).

As described in materials and methods (determination of STZ in honey samples), when increasing concentrations of STZ were added to honey samples in the absence of CDs, and their fluorescence were measured, similar regression lines were obtained for both buffer and honey samples (Fig. 5, filled and open circles). These results indicated that the fluorescence intensity of STZ was not affected by composition of the honey. In contrast, when β-CDs (13 mM) were present, a substantial increase in fluorescence was observed in both cases – buffer and honey samples, even at low STZ concentrations.

As described by other authors (Antony Muthu Prabhu et al., 2010), the formation of the inclusion complex between STZ and β-CDs should result in dual emission. The β-CD dependent emission spectra of sulpha drugs show that the shorter wavelength (SW) band is more sensitive in β-CD solutions, whereas the longer wavelength (LW) band shows a small enhancement. The LW intensity in β-CD medium is lower than the SW intensity. With addition of β-CD both LW and SW intensities are increased; however, the rate of enhancement of the SW emission is greater than that for the LW band.

The enhancement of the SW band in β-CD may be due to lowering of the solvent polarity at higher β-CD concentration. Inside the β-CD cavity sulpha drug molecule feels a much less polar environment and the main non-radiative path of the SW band (through intramolecular charge transfer (ICT) or twisted intramolecular charge transfer (TICT)) is restricted, which also causes an enhancement of the SW band. The authors speculated that the enhanced 430 nm emission in the sulpha drugs should originate from the TICT state. Supporting this implication concluded that the excitation spectra for the 440 nm emission is similar to that of the 340 nm

emission, which suggest that TICT is present in these molecules. In our case, the dual fluorescence wavelength was shifted slightly up to 458 nm, and was used to quantify STZ concentration in honey.

This fact, opened the way for the optimization of a fast and reliable analytical method for STZ detection in honey samples, based in the use of CDs. For that, the fluorescence intensity – in aqueous medium – of different honeys, in the presence of β-CDs was determined.

Since honey is a complex matrix with a large variety in composition due to different proportions of the possible sources, nectar and/or honeydew, coming from a great variety of plants and origins, the robustness of the method was checked on different unifloral and multifloral honeys (thousand flowers, rosemary, orange, thyme, forest, sunflower, chanteuse, eucalypt, lavender and orange blossom honey). No matrix interferences were observed since fluorescence signal for honey samples fortified with STZ and diluted (1/5, w/v) in assay buffer 100 mM (pH 5.5) at 25 °C, containing 13 mM of β-CDs, was similar for all samples tested (data not shown).

In addition, from a blank honey sample (1 kg) as a mix of all honeys tested, previously checked by LC–MS to be free of STZ, different subsamples of 0.5 g were fortified with a STZ standard (100 mg/g) at 0, 5, 25, 50, 100, 150 and 200 ng/g and diluted (1/5, w/v) in assay buffer 100 mM (pH 5.5) at 25 °C, containing 13 mM of β-CDs.

The linear relationship between fluorescence intensity and STZ concentration in different types of honey was calculated (samples were also corroborated by LC–MS), yielding the following equation:

$$y = 17.83 + 23.67x; \quad (r^2)\,0.998 \qquad (10')$$

The limit of detection or least detectable dose (LOD) is the smallest concentration of the analyte that produces a signal significantly different from zero with a stated degree of confidence. There is a general consensus in favour of selecting the analyte dose providing three times the standard deviation (S.D.) from the mean measurement of the blank dose signal. The LOD was estimated by analysis of five sets of 12 replicates of the zero standards (Gabaldón, Maquieira, & Puchades, 2007). The mean fluorescence value plus three fold S.D. corresponded to an estimated limit of detection of 9.7 ± 0.04 ng/g of STZ.

The limit of quantification (LOQ) was estimated from the mean fluorescence blank value plus 10-fold S.D. The LOQ was calculated on the basis of the analysis of 20 honey samples, giving a value of 32.4 ng/g of STZ with a S.D. of 0.30 ng/g.

Assay precision was tested by repeatability (intra-day variation) and reproducibility (inter-day variation) studies. Six honey samples were fortified at 0.2 mg/kg, and the STZ concentration measured six times (by triplicate), on the same day (repeatability) and of five different days (reproducibility). The S.D. and intra and inter-day coefficient of variation (%CV) of the fluorescence intensity were calculated giving values of 2% and 6%, respectively.

To determine the ruggedness of the method, a honey fortified at 0.2 mg/kg of STZ was analyzed for 30 days, during which time the fluorescence intensity presented a CV of 5% (data not shown), confirming the reliability of the assay.

The selectivity of the method for STZ was evaluated in comparison to other analogue compounds: SDZ, SDM, SMZ, SMT, SMX, SMP, SPD and SSX. Stock solutions of each sulphonamide (200 mg/L) and a mix (sulphamix) containing all of them (except STZ), at the same concentration, were prepared in ethanol and stored at 4 °C. From a control honey free of sulphonamides (previously tested by LC–MS), three honey samples of 0.1 kg were fortified as follow: one of them was fortified with STZ (0.2 mg/kg), another with a sulphamix (0.2 mg/kg of SDZ, SDM, SMZ, SMT, SMX, SMP, SPD and SSX), whereas the last of them was fortified with STZ 0.2 mg/kg and a sulphamix (0.2 mg/kg each of them). From each sample, five subsamples were diluted 1/5 with sodium acetate buffer (pH 5.5) solution containing β-CDs at 13 mM and the fluorescence intensity of STZ was measured. The fluorescence intensity of STZ alone and in the presence of a mix of different sulphonamides was similar, indicating that sulphonamides did not interfere in the fluorescence intensity of STZ. It is important to note that the mix of sulphonamides in absence of STZ, showed no fluorescence at the described assay conditions. So, these results indicated that β-CDs acts as a selective guest for STZ versus structurally related compounds. These results are similar to those described previously by other authors (Muñoz de la Peña, Mora Diez, Mahedero García, Bohoyo Gil, & Cañada-Cañada, 2007) for detection of sulphaguanidine in honey in absence of CDs.

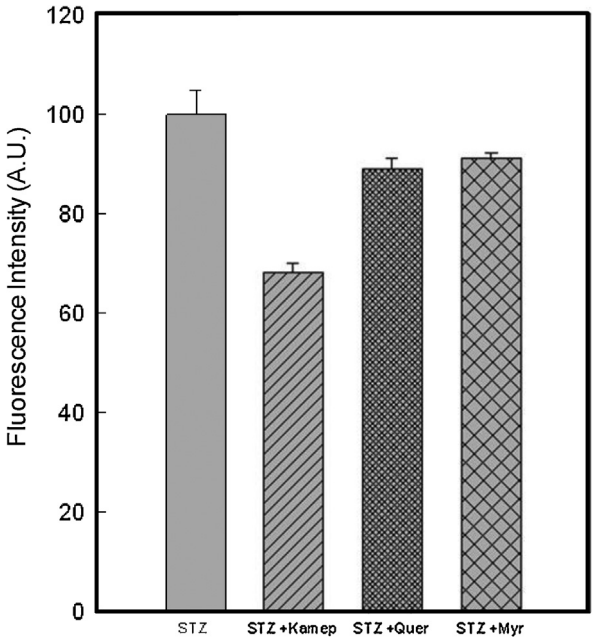

Fig. 6. Interference of phenolic compounds (kaempferol, quercetin and myricetin at 0.2 mg/kg) with fluorescence intensity of STZ 0.2 mg/kg.

In order to demonstrate that the interference in the STZ complexation in CDs by another compounds, could head a decrease in the fluorescence intensity of STZ, the effect of the addition of phenolic compounds – with known K_c values – to the honey samples, was evaluated. For this purpose, the fluorescence intensity of STZ at 0.2 mg/L was compared with the fluorimetric signals in the presence of different phenolic compounds (quercetin, myricetin and kaempferol) at the same concentration level. As can be seen in Fig. 6, the fluorescence intensity of STZ at 0.2 mg/L, in the presence of different phenolic compounds in the reaction medium, were lower than in their absence. These results indicated that phenolic compounds tested compete with STZ by internal cavity of β-CDs. Although kaempferol, quercetin and myricetin showed higher K_c values than STZ for their complexation with β-CDs, giving all phenolic compounds tested 1:1 complexes with β-CDs (1:2 for STZ) (Lucas-Abellán, Fortea, Gabaldón, & Núñez-Delicado, 2008; Mercader-Ros et al., 2010), their content in honeys are lower than the dose tested in interference assay, and are mostly as polyhydroxylated flavonols derived – among others – from kaempferol, quercetin, 3 methyl myricetin and myricetin (Ferreres, Tomás-Barberán, Gil, & Tomás-Lorente, 1991). Anyway, the optimization of the proposed method was carried out in honey samples containing the flavonols tested, but their presence in honey was not enough to avoid the complexation of STZ with CDs. Consequently the composition of honeys would not affect the fluorescence intensity of STZ.

Table 1
Results of the analysis of blind honey samples by LC–MS and fluorimetry.

Honey sample	STZ added (μg/kg)	STZ detected fluorescence[a] (μg/kg)	*R* (%)	STZ detected LC–MS (μg/kg)	*R* (%)
1	0	<LOD	–	<LD	–
2	5	<LOD	–	<LD	–
3	50	48.2 ± 0.3	97	48.6 ± 0.4	97
4	100	94.0 ± 0.6	94	102.0 ± 0.8	102
5	150	145.5 ± 0.9	97	174.5 ± 2.3	116
6	200	192.3 ± 1.6	96	215.0 ± 3.5	107
7	250	255.0 ± 3.8	102	237.3 ± 9.6	95
8	500	475.3 ± 10.4	95	480.4 ± 17.4	96
		R mean (%)	97	*R* mean (%)	102

Values as mean ± S.D. ($n = 3$). LOD, limit of detection; LOQ, limit of quantitation; *R*, recovery.
[a] These values correspond to the STZ detected, multiplied by the dilution factor (5).

In addition, a set of eight blind honey samples was checked by the proposed method. The samples were also analyzed by HPLC–ESI-MS and the results were outlined in Table 1. As can be seen in Table 1, for all honey samples tested, good agreement was observed when STZ was quantified by both methods and all reported results (100%), were in agreement with the expected STZ chromatographic values, considered as true diagnostic.

The average recovery by proposed method was 97% with CV values ranging from 0.6% to 2.2% with most below 1. Such results are considered appropriate for residue methods (Parker, 1991), especially considering that the application of any assay in complex matrices such as honey, is more difficult than in water samples. For LC–MS the recovery values were in the range 95–116%, average 102, with higher coefficients of variation in percentage (0.7–3.6%). Correlation obtained by established LC–MS method and proposed fluorescence method for eight fortification levels tested seem to be satisfactory.

Results obtained demonstrated the potential of the proposed methodology for quantitative analysis of STZ in honey samples in a rapid, sensitive and cost-effective way.

4. Conclusions

The fluorimetric determination of STZ in a complex matrix such as honey with β-CDs, has been demonstrated to be feasible. This method is developed enhancing the fluorescence emission of the aqueous solution of the analyte in sample, which is clearly advantageous. In β-CD solution, the heterocyclic ring of STZ is encapsulated into the β-CD cavity. Formation of the inclusion complex should result in dual emission, which is due to the presence of TICT.

No sample treatment is required, and the assay can be carried out with small volumes of aqueous diluted honey. The method is easy to use, highly portable and the results are obtained in a short time, without the need for expensive equipment, washing and/or separation steps. On the basis of these findings, the LOQ of the method was 32.4 ng/g. Even though, we will discriminate all honey samples having a STZ concentration upper 10 ng/g, since the LOD was 9.7 ± 0.04 ng/g.

The results obtained for fortified honey samples were in good agreement with those yielded by LC–MS. Then, the proposed method shows the possibility of a rapid testing of STZ residues in honey samples, free of interference from the matrix components or structurally related compounds. In fact, the method had a high specificity towards STZ since related sulphonamides, when were present in honey at concentrations around 0.2 mg/kg, did not interfere in the fluorimetric signals of STZ.

The proposed approach, have no equivalence in the market, and could be used by the honey sector to carry out on-site screening for STZ, using a field-portable fluorometer at the beginning of the food chain to improve commercial trade.

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