Preconcentration of sulphonamides in bovine milk by the cloud point extraction method using smartphone-based digital images

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Sulphonamides are a group of synthetic antibiotics used specially in veterinary medicine. Among the procedures employed in the sample preparation for sulphamamide determination are liquid–liquid extraction (LLE) and solid-phase extraction (SPE) that use large volumes of organic solvents. Hence, a clean procedure was developed based on preconcentration and cloud point extraction (CPE) without using organic solvents to quantify total sulphonamides in bovine milk. The procedure was optimized as follows: 2 mL of pre-cleaned milk sample, 2 mL of reagent solution and 1 mL of Triton X-114 7% (m/v) were added to a tube, heated in a water bath at 40 °C for 10 minutes and centrifuged at 2950 rcf for 20 minutes. Digital image acquisition was employed directly at the tube without removing the supernatant/ aqueous phase. The linear response was observed between 10 and 400 μg L⁻¹ of total sulphonamides and described by the following equation: S = 2.50 + 0.0514C (μg L⁻¹) and R = 0.999. The LOD and the CV (n = 11) were estimated to be 10 μg L⁻¹ and 1.3%, respectively. The main interferents present at their usual concentrations in the sample did not interfere with the results. Spike and recovery tests of total sulphonamides were carried out in UHT and pasteurized milk with recovery values between 73 and 106% and the results obtained for this kind of sample were in agreement with those achieved by a high performance liquid chromatography (HPLC) procedure at the 95% confidence level. The analytical procedure presents an adequate sensitivity to determine total sulphonamides in bovine milk and does not require organic solvents, being aligned to the principles of green chemistry.

Introduction

Antimicrobial agents are one of the oldest drugs employed both in human and veterinary medicine. Their use started around 1940 and they were mixed with cattle feed in order to avoid infections. In bovines, a higher incidence of antibiotic residues is related to inadequate handling of these kinds of drugs that are used to treat mastitis, for example, contaminating milk through the udder.

The ingestion of milk that presents residues of veterinary drugs, such as sulphonamides, may cause headaches, meningitis, intestinal disfunctions, and bacterial resistance. According to the European Medicine Agency’s (EMA) Committee for Medicinal Products for Veterinary Use (CVMP) the maximum limit of total sulphonamide residues must not surpass 100 mg kg⁻¹ in animal tissue or 100 μg L⁻¹ in milk. The recommended analysis method to quantify sulphonamides in milk according to the European Agency for the Evaluation of Medicinal Products is HPLC with diode array detection.

Sample preparation is the stage that precedes the quantification of sulphonamides. There are several conventional methods that are employed during this stage to extract and/or preconcentrate the analyte, but the most common ones are LLE and SPE. Some of the LLE advantages are simple execution and usage of accessible solvents. The disadvantages are that there is a possibility of emulsion formation, which makes the extraction slow, besides consuming large volumes of samples and solvents, which can lead to an issue during the discard due to the toxicity of solvents.

Searching for cleaner and more environment-friendly procedures, some extraction alternatives were developed, such as ultrasound assisted, microwave assisted, and CPE. The use of surfactants to aid in the extraction procedure has also been of scientific interest since they can be employed in preconcentration and/or separation stages avoiding the use of organic solvents or costly materials, like in LLE or SPE.

Cloud point extraction and preconcentration procedures can be employed for organic and inorganic samples and for different matrices, such as environmental samples, through the formation of a micellar aggregate organized medium. An
ultrasound-assisted CPE method was employed for sulphonamide (sulfadiazine and sulfamerazine) determination and derivatization with fluorescamine in honey samples; CPE was also employed as a double extraction method for simultaneous determination of sulphonamides in water and urine samples, firstly extracting and preconcentrating in the surfactant rich phase and then it was used with the addition of NaOH for posterior injection in HPLC; and with salting-out effect promoting the denaturation of milk proteins, followed by quantification of sulphonamides through HPLC-UV or ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS).

Aiming simple alternatives in the development of analytical methods, digital images have been used as photometric measurements. The analytical measurements allow color monitoring, quality and food safety results. Furthermore, digital images acquired through a smartphone for analytical purposes are easily accessible, since it is an apparatus widely used, besides being portable. Smartphones’ cameras have sensors that convert photon energy (from colorimetric reactions) into electric energy. These converted signals are aligned in cartesian planes made of pixels that can indicate luminosity changes through light reflection, which allows the acquisition of qualitative and quantitative information. Each pixel acquires a different transmittance from the red, green and blue channels (RGB system), in which the signal is converted into decimal units between 0 and 255 (additive colors) that can be interpreted through free access software or smartphone application.

Maroubo et al. (2021) employed digital image measurements to quantify total sulphonamides in bovine milk. However, their study was based on dispersive liquid–liquid extraction using 1-butanol as solvent, which is not as environmentally friendly as CPE.

In this study, the application of CPE was proposed to preconcentrate and separate the total sulphonamides in bovine milk samples. Digital image analysis was also employed through photometric measurements aiming the development of an alternative procedure that is quick, low cost, and in accordance with green chemistry principles to quantify adulterants in milk.

**Experimental**

**Apparatus**

The measurement chamber was built using a Styrofoam box (14 cm high, 16 cm wide and 10 cm deep). The openings to the 15 mL Falcon® tubes and to the smartphone camera and flashlight (Asus X00ADA, Android 6.0.1, equipped with a 16 MP camera) were made at the top and at the front of the box, respectively. The tubes were disposed in a way that the measurements were taken perpendicularly, ensuring that all measurements were always taken in the same spot. The free access application used to obtain RGB values was ColorGrab (Lomatrix®, 3.6.1 version). A LED emergency light (Elgin 2W) disposed at the inferior part of the measurement chamber and the smartphone flashlight, which can be used with the application simultaneously, controlled the lighting.

A Dubnoff water bath (QUIMIS, Diadema, Brazil; model Q226M1) and centrifuge (QUIMIS, Diadema, Brazil; model Q222T) were used in the heating and phase separation processes, respectively. Vortex (Scientific Industries, New York, USA; model SI-0266 Vortex-Genie 2) and orbital shakers (QUIMIS, Diadema, Brazil; model Q225M) were used during the sample cleanup stage.

**Samples and solutions**

All solutions were prepared using deionized water (18.2 MΩ cm at 25 °C) and analytical-grade chemicals.

Reference solutions of total sulphonamides containing sulfa-methazine (SMZ), sulfadimethoxine (SDM), and sulfathiazole (STZ) (Sigma-Aldrich, St. Louis, USA) were prepared through dilution in deionized water of stock solutions of 10 mg L−1 of each sulphonamide in equal proportions.

Triton X-114 (Sigma-Aldrich, St. Louis, USA) 7% (m/v) solution was prepared in water and kept refrigerated under 20 °C.

A reagent solution of 0.3 mmol L−1 p-DAC (p-dimethylnocinnamaldehyde; Sigma-Aldrich, St. Louis, USA) containing 0.02 mol L−1 SDS (sodium dodecyl sulfate; Merck, Darmstadt, Alemanha) was prepared through dissolution of appropriate quantities of the reagents in 0.3 mol L−1 HCl. A solution of 0.3 mol L−1 trichloroacetic acid (TCA; Sigma-Aldrich, St. Louis, USA) was prepared through dissolution in deionized water.

Solutions of Mg2+, Ca2+, K+, PO43−, SO42−, and Cl− were prepared using the appropriate salt dissolved in milk.

Milk samples were acquired from local markets in the city of Piracicaba (São Paulo, Brazil) and fortified daily.

**Proposed procedure**

For the sample cleanup procedure, 1.5 mL of the fortified sample or standard solutions containing total sulphonamides (50 to 200 μg L−1) in equal proportions and 3.0 mL of TCA were added to a plastic Falcon® tube. All tubes were agitated in an orbital shaker and centrifuged at 2950 rcf, both for 5 minutes. The supernatant was removed to be used later in CPE.

For the CPE procedure, 2 mL of cleaned sample containing the mixture of total sulphonamides, 2 mL of reagent solution, and 1 mL of Triton X-114 were added to a 15 mL plastic Falcon® tube, heated in a water bath at 40 °C for 10 minutes and then centrifuged at 2950 rcf for 20 minutes. Analytical measurements through digital images were taken directly from the tubes.

**Reference procedure**

High performance liquid chromatography with UV detection at 265 nm (Agilent, model 1100 Series, VWD Detector) was adopted as a reference for the analysis of total sulphonamides in milk samples. A column Agilent Eclipse XDB (C18 column, 250 mm × 4.6 mm id × particle size 5 μm) was used for separation. The mobile phase consisted of an aqueous solution of acetonitrile (solvent A) and 0.1% (v/v) formic acid (solvent B) at a flow rate of 1 mL min−1. Gradient elution was performed as follows: initial concentration of A 15% linearly increased to 20% in 9 min and then linearity increased to 40% in 18 min, held at 40% for 4 min, and returned to the initial condition in 3 min.
For sample cleanup, trichloroacetic acid solution and Triton X-114 for the cloud point extraction were employed.\textsuperscript{17} Cleanup preparation and chromatographic measurements were performed in triplicate.

## Results and discussion

The proposed procedure is based on colorimetric determination of sulphonamides through condensation reaction between the secondary amine and the carbonyl group of \( p \)-DAC in acidic medium, forming a pink-colored iminium salt being measured at \( \lambda_{\text{max}} = 557 \) nm in the absorption spectrum (Fig. 1).\textsuperscript{18,19}

Analytical measurements were based on the digital images captured by a smartphone application that converts these images into RGB values. Thus, the data were obtained through reflectance measured on the G (green) channel because the iminium salt is a magenta-color compound, which is the complementary color to green.

### Procedure optimization

**Pre-treatment and cleanup.** Milk contains proteins and fats that interfere with chemical reactions; thus, pretreatment stages are necessary in order to obtain clean samples. Therefore, organic solvents such as acetonitrile, chloroform, or acetone are used. An alternative is trichloroacetic acid (TCA), a weak acid that is efficient in denaturing milk proteins.\textsuperscript{20}

In this study, sample cleanup was achieved through TCA addition followed by agitation and centrifugation, respectively. Sulphonamides were then dispersed in whey, while proteins and fats precipitated.

Optimizations were carried out univariately and in triplicate. TCA concentration was evaluated between 0.3 and 1.0 mol L\(^{-1}\) in the sample fortified between 0.05 and 0.2 mg L\(^{-1}\) of total sulphonamides. The best results in the addition and recovery tests were recoveries estimated as 100.0 ± 0.5% when 3.0 mL of 0.3 mol L\(^{-1}\) TCA was used in 1.5 mL of sample. For this concentration, the volume of TCA evaluated was between 2.0 and 4.0 mL and the best recoveries were estimated as 93.0 ± 0.5% for 3.0 mL of TCA. For lower concentrations and smaller volumes, solutions became cloudy because protein precipitation was impaired, which made digital image measurements impractical.

Vortex and orbital shakers were evaluated for the agitation stage varying from 5 to 20 min, followed by centrifugation at 2950 rcf also varying from 5 to 20 min. The samples were fortified with 100 µg L\(^{-1}\) of the mixture of total sulphonamides (SMZ, SDM, and STZ in equal proportions). The recovery of total sulphonamides after agitation in vortex and centrifugation was estimated to be between 100 and 283%, which occurred due to cloudy whey because of the intensity of agitation, showing sample matrix interference. Recoveries closer to 100% were obtained when there was a less intense agitation and during short periods of time for both agitation and centrifugation (5 min).

**Reaction.** The effects of parameters that affect the development of the reaction (\( p \)-DAC, HCl and SDS concentrations) and of the analyte extraction from the whey (surfactant concentration, temperature and period in the water bath) were investigated in order to obtain maximum efficiency and lower the volume of reagents, which reduces both the amounts of residues and the analysis duration.

\( p \)-DAC concentration was evaluated between 0.0057 and 2.3 mmol L\(^{-1}\), for both standard and blank samples submitted to CPE. The results are presented in Fig. 2A, in which the analytical signal increased up to 2.3 mmol L\(^{-1}\) of \( p \)-DAC. The signal for the blank sample also increased due to the reagent color. The concentration of 0.3 mmol L\(^{-1}\) of \( p \)-DAC was the one in which there is more significant difference between the blank and the standard.

Surfactants can reduce the surface tension or influence the contact surface between two liquids. Their use in a reaction medium can favor analytical sensitivity by increasing the molar absorptivity of the substance of interest. SDS concentration was evaluated between 0.005 and 0.03 mol L\(^{-1}\) (Fig. 2B). An increase of 78% in the analytical signal was observed when 0.02 mol L\(^{-1}\) was added to the reagent solution in comparison to 0.005 mol L\(^{-1}\), without affecting significantly the blank. Higher concentrations of SDS caused a decrease in the analytical signal and increased standard deviations due to cloudiness of the sample. This may have occurred because the mixture of ionic and non-ionic surfactants increases the temperature of the CPE. For example, the addition of 1.0 mmol L\(^{-1}\) of SDS increased the CPE temperature from 25 to 74 °C in a solution of Triton X-114 1% (m/v), which made it more difficult to separate phases.\textsuperscript{21}

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Fig. 1 Reaction between the secondary amine and \( p \)-DAC in acidic medium.
Therefore, all quantifications were performed with an addition of 0.02 mol L\(^{-1}\) of SDS.

In general, sulphonamides are completely protonated under pH 2.0 due to their \(pK_{a1}\) (2.1) and \(pK_{a2}\) (7.0).\(^{22}\) This is the ideal condition that favors the derivatization reaction that forms the iminium salt as well as the separation of phases during CPE by reducing the repulsion between the monomers.\(^{23}\)

HCl evaluated concentrations were between 0.1 and 0.5 mol L\(^{-1}\) and the selected one was 0.3 mol L\(^{-1}\) since it showed the most significant difference between the standard and the blank and resulted in the medium pH around 1.0.

Surfactant concentration can affect both extraction efficiency and phase volume ratio. Triton X-114 was selected because of its high density, which helps in the phase separation during centrifugation, and it induces CPE to occur at temperatures between 23 and 25 °C.

Evaluated concentrations for Triton X-114 were between 5 and 10% (m/v) (Fig. 3A). It was observed that analytical signals for both standard and blank solutions increase linearly for concentrations up to 7% (m/v). Above this concentration there was an increase in the surfactant-rich phase volume affecting the analytical signals. Furthermore, the surfactant concentration selected for CPE was 370 times greater than the CMC (0.35 mmol L\(^{-1}\)), forming 500 μL of surfactant rich-phase.

Incubation time effect for the 40 °C water bath (between 5 and 60 minutes) was evaluated aiming to achieve complete extraction of the iminium salt and efficient phase separation for the minimum period of time. Since there were no significative differences between the standard and blank analytical signals by varying the incubation time in the water bath, 20 minutes was selected to guarantee that the cloud point would be formed.

Centrifugation speeds up the phase separation in the CPE, and hence this parameter needed to be studied and optimized. The evaluated time range was between 5 and 60 min (Fig. 3B) and the centrifugation speed was evaluated between 46 and 2950 rcf (Fig. 3C). In the centrifugation time evaluation, the analytical signal was increased up to 20 min, which demonstrated effective phase separation. Above 20 min of centrifugation, the chromogenic reagent probably went through degradation, which affected the analytical signals. As for the centrifugation speed, the one that presented the best results between the standard and the blank favoring the phase separation was 2950 rcf.

### Table 1  
CPE and digital image measurement optimized parameters to quantify total sulphonamides in bovine milk

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Evaluated range</th>
<th>Selected value</th>
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<tbody>
<tr>
<td>(p)-DAC (mmol L(^{-1}))</td>
<td>0.0057–2.3</td>
<td>0.3</td>
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<tr>
<td>SDS (mol L(^{-1}))</td>
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<td>0.02</td>
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<tr>
<td>HCl (mol L(^{-1}))</td>
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<td>0.3</td>
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<tr>
<td>Triton X-114 (%, m/v)</td>
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<td>7</td>
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<td>Incubation time (min)</td>
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<td>20</td>
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<tr>
<td>Centrifugation time (min)</td>
<td>5–60</td>
<td>20</td>
</tr>
<tr>
<td>Rotation (rcf)</td>
<td>46–2950</td>
<td>2950</td>
</tr>
</tbody>
</table>
Milk SF Acetic acid and Na\textsubscript{2}SO\textsubscript{4} Triton X-100 60 Spectrophotometry 215 2.1 95–102 25
Honey SF — NP-7 110 Fluorescence 0.5 3.7 84–92 7
Urine and water SMZ Triton X-114 70 HPLC-UV 6.1 7.7 85–108 8
Milk SF Acetic acid and ethanol Triton X-114 24 Spectrophotometry 2.8 4.7 95–98 27
Milk Total Trichloroacetic acid Triton X-114 30 Digital images 10 1.3 73–106 This work

Table 2 Analytical features of CPE procedures to quantify sulphonamides\textsuperscript{a}

Table 3 Spike and recovery tests of total sulphonamides in milk samples

Table 1 presents chemical and physical parameters, evaluated ranges, as well as the selected values during the experimental optimization.

Analytical features

Analytical features were estimated after experimental parameter optimization. The proposed procedure presented a linear response between 10 and 400 µg L\textsuperscript{-1} of total sulphonamides as described by the equation $S = 2.50 + 0.0514C$ ($R = 0.999$, $n = 11$), in which $S$ corresponds to the analytical response and $C$ to the concentration of total sulphonamides (µg L\textsuperscript{-1}). LOD, coefficient of variation, and sampling rate were estimated to be 0.01 mg L\textsuperscript{-1}, 1.3%, and 32 quantifications per hour, respectively. The coefficient of variation was estimated for 0.4 mg L\textsuperscript{-1} ($n = 11$) of total sulphonamides, and the sampling rate was estimated by calculating the total time necessary for the sample preparation and posterior quantification of the analyte.

The enrichment factor was calculated by the ratio between the inclinations of the analytical curves with and without CPE for initial and final volumes of 5.0 and 0.5 mL, respectively. The equations were $S = 2.50 + 0.0514C$ ($R = 0.999$, C in µg L\textsuperscript{-1}) and $S = 2.58 + 0.0193C$ ($R = 0.938$, C in µg L\textsuperscript{-1}), with and without CPE, respectively. The estimated enrichment factor was 3.0, which is below the expected value (10.0). One of the factors that may have contributed to this result is the degradation of the iminum salt.

Nevertheless, the employment of CPE in the proposed procedure is carried out in a single step, without the need for removal of the supernatant phase and dilution of the rich phase in ethanol, before photometric measurements. As pointed out by Acevedo et al. (2018), as more steps are involved in sample processing, precision and recoveries can be affected.\textsuperscript{24}

The analytical features of the proposed procedure were comparable to those of other CPE procedures for sulphonamide extraction (Table 2). Acetic and trichloroacetic acids, and ethanol were employed on complex samples, such as milk, as a cleanup step. Time spent in the CPE for the proposed procedure was 3.7 and 2.3 times inferior in relation to the ones used in honey\textsuperscript{7} and urine\textsuperscript{8} samples, respectively. When compared to the application of CPE to the same type of sample, the extraction time\textsuperscript{23} and variation coefficient\textsuperscript{27} were 2 and 3.6 times lower, respectively. Furthermore, the smallest coefficient of variation (1.3%) was estimated for the proposed procedure when compared to the other procedures, which was achieved through digital image measurements taken directly at the tube.

Acceptable recuperation values were presented for all listed procedures and low limits of detection were met, especially for the proposed procedure, which was 10 µg L\textsuperscript{-1} of total sulphonamides.

Another advantage of the proposed procedure is the low use of analytical-grade reagents due to the miniaturization of CPE and the analytical measurements were taken directly at the tube. Furthermore, there was no need to remove the surfactant-poor phase, hence avoiding the dilution commonly needed for this type of extraction, which implied a reduction of the residues generated.
Interferents

The effect of some ions that can be found in milk was evaluated through a solution of 100 μg L⁻¹ of total sulphonamides and interferents at elevated concentrations (100 times higher than the analyte). The signals for total sulphonamides were obtained with and without the presence of Mg²⁺, Ca²⁺, K⁺, Cl⁻, SO₄²⁻ and PO₄³⁻ ions, considering non-interference in an analytical signal variation lower than 5%. None of the ions was considered an interferent in the addition and recuperation test employed on the proposed procedure.

Validation

Milk sample analysis. Spike-and-recovery tests were carried out in skimmed milk, partially skimmed milk, UHT whole milk, type A pasteurized and whole powdered milk. The samples were fortified (n = 3) with the following total sulphonamide concentrations: 50, 100, and 200 μg L⁻¹ according to Table 3. According to international regulatory agencies (e.g., FDA and AOAC), acceptable values in spike-and-recovery tests for complex samples must be between 60 and 115%. The Table 3 results show that all samples are in accordance with the acceptable range, which demonstrates the potential of the proposed procedure. The proposed procedure was then applied to total sulphonamide determination in milk after sample spiking (Table 4). The results agreed with the reference procedure based on HPLC with a 95% confidence level using a paired t-test.

Conclusions

The proposed CPE procedure showed to be a simple, quick and robust alternative to quantify total sulphonamides in milk. It is a procedure with high sampling rate and low reagent consumption and residue generation, aside from not using organic solvents. The proposed procedure does not remove the supernatant phase, which is a stage that could imply an increase of systematic errors.

Established limits for total sulphonamides in milk by regulatory agencies, such as EMEA, were met using digital images. The proposed procedure presented tolerance to ions at concentrations superior to the ones that may be present in milk samples and they did not interfere significantly with results. Spike-and-recovery tests presented acceptable recoveries that are in accordance with regulatory agencies.

Conflicts of interest

The authors declare no conflict of interest.

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