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Journal of Chromatography A, 1095 (2005) 60–67
JOURNAL OF CHROMATOGRAPHY A
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High performance liquid chromatography post-column chemiluminescence determination of sulfonamide residues in milk at low concentration levels using bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl] oxalate as chemiluminescent reagent

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Received 20 May 2005; received in revised form 20 July 2005; accepted 28 July 2005
Available online 12 September 2005

Abstract

The determination of seven sulfonamides by means of HPLC with chemiluminescence detection is proposed for the first time. The analytes are derivatized with fluorescamine, separated and subsequently they participate in the post-column chemiluminescence (CL) peroxyoxalate system using imidazole as a catalyst. Among the different peroxyoxalates tested, bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl] oxalate provides higher sensitivities and stabilities, avoiding precipitation problems. A rigorous optimization of the significant variables by means of experimental designs has been developed in order to reconcile the chromatographic conditions with the CL reaction. The method provides detection limits in the low $\mu g\,l^{-1}$ range and has been satisfactorily applied to the analysis of spiked raw milk samples.
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Keywords: Sulfonamides; Chemiluminescence; HPLC; Bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl] oxalate; Milk

1. Introduction

Sulfonamides, *N*-derivatives of 4-amino-benzenesulphonamide, comprise a large group of synthetic antibacterial compounds. They have been used in human medicine against a wide variety of microbes, being their current use primarily in the treatment of urinary tract infections. They are also widely used in farm animal feedstuff and fish cultures as veterinary drugs for prophylactic and therapeutic purposes. Furthermore, sulfonamides act as growth promoting substances, and their residues in food are of concern due to their potential carcinogenic character and the possibility of development of antibiotic resistance in humans as well as severe allergic reactions. Thus, the European Union (EU) regulations have set a maximum residue limit of 100 $\mu g\,kg^{-1}$ for the sum of all sulfonamides in animal muscle or in milk [1].

The analytical methods applied to determine sulfonamides in food of animal origin have been comprehensively reviewed [2,3]. These compounds are usually extracted from the matrix into organic solvents or purified by solid phase extraction (SPE) and analyzed by GC or HPLC. Among the HPLC methods, the most commonly used detection modes are UV, diode array, electrochemical or fluorimetric detection after chemical derivatization. The chemical post-column derivatization of 12 sulfonamides with fluorescamine (FR) and 2-mercaptoethanol for their fluorescence detection in an

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doi:10.1016/j.chroma.2005.07.123

HPLC system has been recently reported in the analysis of honey [4], with recoveries ranging 37–67%, quantification limits between 2 and 5 μg kg^{-1} (with a preconcentration factor of 30) and an analysis time of 45 min. Moreover, 10 sulfonamides have been derivatised with FR in acidic medium, separated and detected by HPLC-fluorescence for the analysis of foods of animal origin, with recoveries in the range of 64–75%, quantification limits in the low μg kg^{-1} level (considering a preconcentration factor of 10) and times of analysis of 30 min [5]. There are also several recent publications concerning liquid chromatographic analysis of sulfonamides using MS [6–10] or MS–MS detection [11–13].

Chemiluminescence (CL) is a high sensitive analytical technique that can be used in the determination of different compounds in a great variety of matrices depending on their participation in the CL reaction as precursors, catalysts, inhibitors, oxidants, etc. [14]. Considering the kinetic characteristics of this technique, it can be easily coupled to a flow injection (FI) manifold as detection mode [15]. Among the different CL systems that can be used with analytical purposes, peroxyoxalate (PO) reaction is one of the most efficient, being an indirect or sensitized type of chemiluminescence in which an activate oxalate reacts with hydrogen peroxide leading to the formation of one or more energy-rich intermediate(s) capable of exciting a large number of fluorophores [16] through the CIEEL (chemically initiated electron exchange) mechanism [17], by which the intermediate forms a charge transfer complex with the fluorophore, donating one electron to the intermediate, which is transferred back to the fluorophore raising it to an excited state and liberating an emission typical for the nature of this fluorescent derivative. Recently, the nature of the postulated intermediate, 1,2-dioxetane-3,4-dione, has been confirmed using ^{13}C nuclear magnetic resonance (NMR) spectroscopy [18]. From an analytical point of view, the usefulness of this PO-CL reaction is based on the possibility of detecting native fluorophores or compounds derivatised with fluorescent labels [19]. The advantage of the PO reaction is the wide pH range to carry out the oxidation, generally occurring near neutral values, the main limitation being the need for an organic solvent based on solubility, stability and efficiency considerations. The most widely used oxalate ester is bis-(2,4,6-trichlorophenyl)oxalate (TCPO) followed by bis-(2,4-dinitrophenyl)oxalate (DNPO). However, a problem with PO-CL when applied to post-column detection in liquid chromatography (LC) is the relatively long duration of the light emitting reaction, where even the best known systems results in a light emission profile that lasts for more than 60 s which could produce extra-column band broadening. For this reason, it is very useful the use of a catalyst to speed up the reaction while maintaining a reasonable high quantum efficiency, enabling a "chemical narrowing" of analytes separated in LC and allowing the effective cell volume to become dependent on the speed of mixing of eluate and reagent, rather than on the total detection cell volume. Imidazol (IMZ) has been proved to be an efficient catalyst for the PO-CL reaction [20,21] due to its ability to destabilize the PO by forming an intermediate less stable, which is thus more susceptible to nucleophilic attack by hydrogen peroxide. A general mechanism was proposed by Hadd and Birks for the nucleophilic reaction of IMZ with oxalate esters [22].

Despite the advantages of CL detection, there is scarce literature concerning the CL determination of sulfonamides. Based on the oxidation of these analytes by potassium permanganate in acid medium, the determination of sulfacetamide and sulfafurazole in pharmaceutical dosage forms using sequential injection analysis (SIA) technique with CL detection has been reported, with detection limits ranging 2–8 mg kg^{-1} and quantitative recoveries [23]. Also, the (FL)-CL determination of sulfonamides by means of a photochemical reaction followed by the same oxidation reaction has been recently reported. The method has been applied to the determination of sulfamethoxazole in pharmaceutical preparations, providing a limit of detection of 60 μg l^{-1} [24]. A sulfur chemiluminescence detection (SCLD) system for supercritical fluid chromatography based on ozone-induced CL following a two-step combustion process of consecutive oxidation and reduction of sulfur-containing compounds has also been proposed for different products, including some sulfonamides, although no application was reported [25].

In this paper, a sensitive method for the quantification of seven sulfonamides in raw milk is proposed. The sulfonamides are extracted from the milk, labeled with FR (a selective reagent for primary amines that reacts almost instantly with these substrates while the excess is hydrolyzed to a non-fluorescent product) and then separated by HPLC and subsequently detected by post-column PO-CL reaction. Among the different POs tested, bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl] oxalate (TDPO) has been selected as the most adequate chemiluminogenic reagent, as it provided higher sensitivity and stability than others commonly employed PO. It dissolves well in acetonitrile (ACN), acetone and ethyl acetate in the range of low to sub-molar concentrations (1, 0.8 and 0.4 M, respectively), providing larger concentrations of the intermediate of the reaction and avoiding the precipitation of the reagent in the flow line which produces a good mixing of the solutions to afford a stable baseline [26,27]. However, its analytical applications are reduced; it has been employed in the development of and HPLC method for the determination of dansylamino acids [27] and catecholamines [28,29] by means of post-column CL detection. The method proposed in this paper provides limits of detection in the low μg l^{-1} level for the seven sulfonamides assayed and it has been applied and validated for the analysis of raw milk, providing satisfactory recoveries. As far as we know, this is the first time that the PO reaction has been applied for the analysis of sulfonamides by coupling HPLC with CL detection, optimizing the compatibility of both separation and post-column

reaction conditions. Experimental design methodology has been used for the multivariate optimization in order to select the significant variables and with the aim to consider the significant interactions among the studied variables to reach the optimum experimental conditions, reducing the experimental work. Moreover, the advantages of the use of TDPO instead of the most common TCPO have been reported.

2. Experimental

2.1. Chemicals

All the reagents were analytical reagent grade, solvents were HPLC grade, and sulfonamides were analytical standard grade. Ultrapure water (Milli-Q plus system, Millipore Bedford, MA, USA) was used throughout the work. A stock standard solution of 100 mg l^{-1} of each sulfonamide (namely: sulfapyridine: SPD; sulfadiazine: SDZ; sulfamethazine: SMZ; sulfachloropyridazine: SCP; sulfadoxine: SDX; sulfamethoxazole: SMX; and sulfadimethoxine: SDM) was prepared by dissolving 10 mg of the product (Riedel-de-Haën (Vetranal), Sigma–Aldrich Química, Madrid, Spain) in 100 ml of methanol (Merck, Darmstadt, Germany) in a calibrated flask. The solutions were stable for at least 2 months, stored in the dark at 4 °C. Working standard solutions containing all the sulfonamides were freshly prepared by dilution of the stock solutions with tris-(hidroxymethyl)-aminomethane (Tris, Merck)/chloride acid (Panreac, Madrid, Spain) buffer (10 mM, pH 7.0).

In the post-column reaction, proper working solutions ranging from 1 to 5 mM of TCPO (Sigma–Aldrich Química), DNPO (Wako, Osaka, Japan) and TDPO (Wako) were prepared daily in ACN (Merck) or a mixture of ACN: tetrahydrofurane (THF, Merck), 75:25. A 1 M stock solution of IMZ (Sigma–Aldrich Química) was prepared weekly in water and proper working solutions were prepared daily in ACN and used as catalyst. This solution also contains 500 mM of hydrogen peroxide (from 30% p/v solution, Panreac), used as oxidant.

Working solution of 4 g l^{-1} of FR (Sigma–Aldrich Química) was prepared daily by dissolving 20 mg in 5 ml of acetone (Merck).

A mixture of chloroform (Merck): acetone (Panreac) (2:1) was used for sample extraction, while hexane (Panreac) was used for removing fatty residues and Tris–buffer was used for dissolving residues.

2.2. Equipment

A scheme of the equipment is shown in Fig. 1. The HPLC system consisted on a quaternary high pressure pump (Model PU–2089, Jasco Analítica Spain, Madrid, Spain) coupled to a multichannel UV–vis detector (Model UV-2077, Jasco Analítica Spain) and subsequently to a CL detector (Model CL-1525 detector, Jasco Analítica Spain) operating at gain 10 and short attenuation and equipped with a PTFE spiral detection cell, data control and acquisition program. A C18 Luna separation column (5 μm, 100 Å, 150 mm × 4.6 mm, Phenomenex, supplied by Jasco Analítica Spain), a guard column equipped with a 4 mm disposable cartridge of similar packing (Phenomenex, supplied by Jasco Analítica Spain), a Rheodyne 7725i manual injection valve (Rheodyne, L.P., Bensheim, Germany) with a 100 μl loop, and PEEK and stainless steal tubing and connections were also used in the system.

For the post-column reaction, two high pressure pumps (Model PU-2085 and PU-2080, Jasco Analítica Spain), PEEK and stainless steal tubing and connections were used.

An ultrasound bath (Selecta, Barcelona, Spain) was used for the derivatization reaction and a rotavapor (Büchi RE 121, Büchi Laboratoriums-Technik AG, Flawil, Switzerland) was used for sample preparation.

For statistical treatment of data, ALAMIN software [30] and STATGRAPHICS [31] package were used.

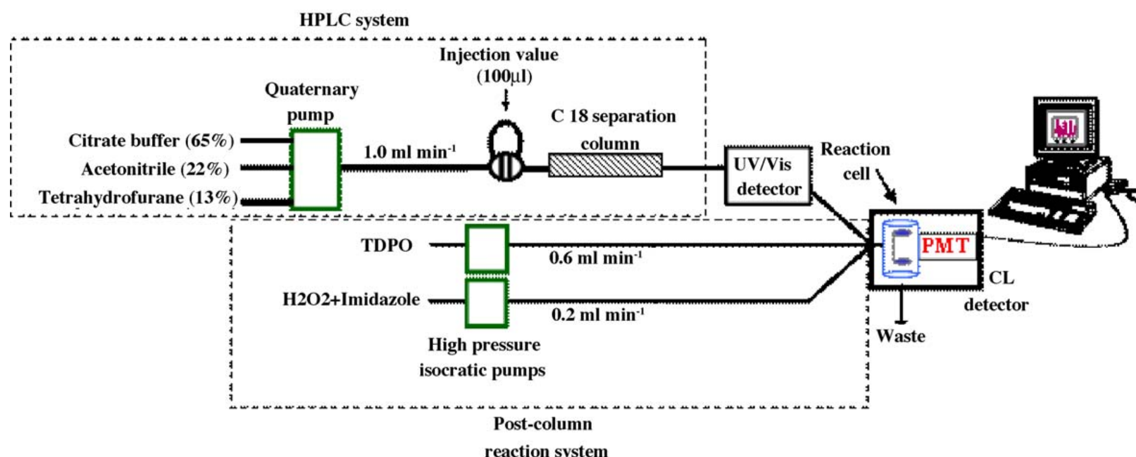

Fig. 1. Scheme of the HPLC-CL system. PMT: photomultiplier tube.

2.3. Experimental procedure

The reversed phase separation was carried out in a C18 column using an isocratic ternary mobile phase consisting on a mixture of tri-sodium citrate $5\frac{1}{2}$ hydrate (Panreac)/citric acid monohydrate (Merck) buffer (10 mM, pH 5.2): ACN: THF (65:22:13) at a flow rate of $1.0\,\mathrm{ml\,min^{-1}}$. All the solvents were filtered through a 0.45 μm nylon filter (Supelco, Barcelona, Spain). After separation and UV–vis detection at a wavelength of 265 nm, the eluate was mixed in a four-way stainless steal connection (Valco International, Schenkon, Switzerland) with the PO solution at a flow rate of $0.6\,\mathrm{ml\,min^{-1}}$, and the IMZ/hydrogen peroxide solution at a flow rate of $0.2\,\mathrm{ml\,min^{-1}}$. Due to the rapid kinetic of the PO reaction, the connection was placed in such a way that the distance with the cell of the CL detector was the minimum (4.5 cm). Then, the CL emission is produced and registered by the CL detector.

2.4. Sample extraction and preparation

AOAC official method for multiple sulfonamide residues in raw bovine milk [32] was followed for sample extraction and validation. Thus, sulfonamides in milk samples are extracted by chloroform–acetone (2 + 1, v/v). After solvent evaporation, residues are dissolved in Tris–buffer. Then, fatty residues are extracted with hexane and the aqueous layer is filtered and analyzed by LC.

3. Results and discussion

3.1. Optimization of the method

Considering the need to obtain a suitable compatibility between the chromatographic separation and post-column CL reaction conditions, a rigorous and sequential optimization of the proposed method was carried out in several steps, according to the different stages of the process.

3.1.1. Configuration of the post-column system

First of all, different configurations for the post-column derivatization system were tested, in order to choose the most suitable one in terms of both sensitivity and stability of the reaction. In order to incorporate the CL reagents to the eluate before the CL detection, two channels were selected, namely: channel 1: PO solution; channel 2: IMZ solution + hydrogen peroxide solution. Thus, the following configurations were tested:

(a) Both channels were mixed with the eluate from the separation column in a four-way connection and placed just in front of the detection cell (distance: 4.5 cm).
(b) Channel 1 was firstly mixed with the eluate in a three-way connection and subsequently mixed with channel 2 in another three-way connection, placed just in front of the detection cell (distance: 4.5 cm).
(c) Similar to (b), but the distance between the second three-way connection and the detection cell was increased to 14 cm.

The best results were obtained with configuration (a), which is shown in Fig. 1.

3.1.2. Optimization of the mobile phase

In this step, 2.5 mM of TCPO (the most common used PO) in ACN:THF (75:25) and a mixture of 10 mM of IMZ and 500 mM of hydrogen peroxide, each one at a flow rate of $0.4\,\mathrm{ml\,min^{-1}}$ were used for the post-column CL reaction. As it has been previously stated, POs are very unstable in aqueous solutions and precipitation of these compounds is a common problem when working with aqueous flow systems. Nevertheless, it has been reported that the addition of THF in the PO solutions increases both the solubility and stability of POs, avoiding the above-mentioned problems [33]. Thus, taking into account that a compromise between separation and post-column reaction conditions was mandatory for obtaining appropriate signals and to avoid precipitation of POs in the system, previous studies were carried out varying the composition of the mobile phase in order to select a first experimental domain for a further optimization. This study comprises the use of different buffers (acetate, phosphate and citrate), pH values (4–9) and organic solvent mixtures (methanol, THF and ACN). Two important conclusions were obtained: (i) at a pH lower than 5, no CL signals were obtained, as the PO reaction requires a pH between 5 and 9, while at pH values above 7, it was impossible to resolve the sulfonamide mixture, as at this pH all of them are negatively charged and their retention times decrease; (ii) the addition of THF in the mobile phase provided a better resolution of the sulfonamides, allowing at the same time the use of higher concentrations of POs and avoiding their precipitation.

Taking into account the results obtained in this previous study, citrate/citric acid pH 5.2 was selected as buffer, and ACN and THF as organic solvents. Then, a univariante optimization of the mobile phase was carried out in order to obtain an optimum resolution for all the selected sulfonamides. First of all, the concentration of the buffer was studied in the range of 10–40 mM, selecting 10 mM as optimum value. Then, the composition of the mobile phase was optimized, testing different ternary mixtures of buffer: ACN:THF, selecting a final composition of 65:22:13, which allows a good resolution of the analytes, without precipitation problems. Finally, a flow rate of $1.0\,\mathrm{ml\,min^{-1}}$ was selected.

3.1.3. Optimization of the post-column CL reaction

After the optimization of the mobile phase, the first step in the study of the post-column reaction was the selection of the most suitable PO. With this purpose, three different POs at a concentration of 2.5 mM in ACN:THF (75:25) were tested, namely: TCPO, DNPO and TDPO. When compared to TCPO (the most commonly used PO), DNPO did not improve the sensitivity, producing a high background signal. By contrast,

Table 1
Optimization of the post-column CL reaction

Variables	Levels		
1. Selected variables and levels in the 2^{5-1} fractional factorial screening design and in the 2^{4-1} plus centered star response surface design			
	−1	0	+1
A: [TDPO] (mM)	1	2.5	4
B: [Peroxide] (mM)	200	500	800
C: [Imidazole] (mM)	5	45	85
D: TDPO flow rate (ml min^{-1})	0.2	0.4	0.6
E: (Peroxide + imidazole) flow rate (ml/min)	0.2	0.4	0.6
2. Selected variables and levels in the 2^3 plus centered star response surface design			
	−1	0	+1
A: log [Imidazole] (mM)	−0.3	0.2	0.7
	0.5	1.6	5.0
B: (Peroxide + imidazole) flow rate (ml/min)	0.050	0.125	0.200
C: TDPO flow rate (ml min^{-1})	0.6	0.7	0.8

it was found that TDPO provided the best results in terms of CL signal (approximately doubled CL intensity compared to TCPO), solubility, as no THF was required for its solubilisation, and stability in aqueous solutions, as no precipitation occurred into the system. Thus, TDPO was selected for the post-column CL reaction, dissolved only in ACN.

The different variables involved in the post-column reaction (that is, concentration of the reagents and flow rates) were optimized following a formal strategy based on the use of sequential experimental designs. Once the different experimental regions were selected, a 2^{5-1} fractional factorial screening design plus three central points was carried out, selecting the variables and levels shown in Table 1. The sum of the peak height intensity of the three smaller peaks of the chromatogram (namely: SDX, SMX, SDM) was used as response variable. The total effects of the different variables as well as their second order interactions were evaluated, finding that the concentration of hydrogen peroxide as well as its second order interactions were non-significant variables. For this reason, the central value of the design, corresponding to 500 mM was selected for further experiences.

The significant variables were optimized in a subsequent 2^{4-1} plus centered face star response surface design, including three central points and using the same experimental domain as in the screening design. From this design, the optimum values obtained were: concentrations of TDPO and IMZ, 4 and 5 mM, respectively, and flow rates for TDPO solution and IMZ plus hydrogen peroxide solution of 0.6 and 0.2 ml min^{-1}, respectively. As can be seen, those optimum values are in the limit of the selected experimental domain, thus, another experimental design was carried out, consisting in a 2^3 plus centered face star response surface design, and selecting the values shown in Table 1. In this case, the concentration of TDPO was excluded from the design, as higher concentrations of TDPO give raise to an increase on the noise. Thus, 4 mM was selected as optimum. The results obtained by means of this design were similar to the previous one, confirming in this way the initial optimum values.

3.1.4. Optimization of the labeling reaction

Once the optimum values for the post-column reaction variables have been fixed, a study of different buffers for dissolving the sulfonamide mixture as a suitable medium for the derivatization reaction was carried out. The following buffers were tested: phosphate buffer 20 mM, pH 3; phosphate buffer 10 mM, pH 7; phosphate buffer 20 mM, pH 7; and Tris–buffer 10 mM, pH 7. The labeling reaction was finally carried out by mixing 1 ml of the sulfonamide standard solution in Tris–buffer 10 mM, pH 7 and 50 μl of FR solution, placing the mixture in an ultrasound bath.

In a further study, a 3^2 plus three central point design was employed for the optimization of the rest of variables involved in the labeling reaction: concentration of FR (ranging from 1 to 4 g l^{-1}) and time of labeling reaction in the ultrasound bath (ranging from 1 to 15 min). The result of this study showed that the best results were obtained for the maximum concentration of FR and the higher time. The concentration of FR could not be increased, as it precipitates, thus 4 g l^{-1} was selected as optimum. In the case of the labeling time, 20 min was also tested, but decay in the CL signal was obtained, so 15 min was selected as optimum.

As a summary, all the optimum values for the variables involved in the CL system are included in Table 2.

3.2. Calibration curve and performance characteristics of the method

Standard calibration (SC) curves were established by triplicate injections of seven sulfonamides (SPD, SDZ, SMZ, SCP, SDX, SMX, SDM) standard solutions with different concentrations for each analyte, ranging 5–60 μg l^{-1} for SPD and SMZ, and 10–120 μg l^{-1} for the rest. Statistical parameters obtained from least squares regression are shown in Table 3. In order to check the influence of the extraction process to be applied for the analysis of real samples on the

Table 2
Optimum values for the variables involved in the proposed HPLC-CL system for the determination of sulfonamides

Mobile phase composition	Citrate buffer (10 mM, pH 5.2): ACN: THF (65:22:13)
Mobile phase flow rate (ml min^{-1})	1.0
Injection volume (μl)	100
[TDPO] (mM)	4
[Peroxide] (mM)	500
[Imidazole] (mM)	5
TDPO flow rate (ml min^{-1})	0.6
(Peroxide + imidazole) flow rate (ml min^{-1})	0.2
[FR] (g l^{-1})	4.0
Time of labelling reaction (min)	15
Buffer in the labelling reaction	Tris (10 mM, pH 7.0)

Table 3
Calibration curves

	Dynamic range ($\mu g\,l^{-1}$)	Calibration equation[a]	$S_{R,c}$	R^2 (%)	Lack of fit *P*-value (%) ($\alpha = 0.05$)	LD ($\mu g\,l^{-1}$)[b]	LQ[b] ($\mu g\,l^{-1}$)
SPD	5–60	CL = 10.44*C* + 85.20	38.7	96.67	17.4	6.2	17.0
SDZ	10–120	CL = 6.88*C* + 45.58	47.0	97.15	49.9	6.9	18.8
SMZ	5–60	CL = 8.68*C* + 65.62	29.4	97.20	25.0	7.1	19.5
SCP	10–120	CL = 6.47*C* + 22.60	42.3	97.39	29.7	13.2	36.5
SDX	10–120	CL = 3.66*C* + 25.60	23.0	97.58	11.6	13.6	37.0
SMX	10–120	CL = 4.17*C* + 12.72	31.7	96.49	7.2	10.4	28.3
SDM	10–120	CL = 3.62*C* + 27.67	18.9	98.33	70.2	9.5	26.0

[a] Obtained from triplicate injections of the standard mixture solutions. CL: height of the chromatographic peak in the CL measurement system; *C*: concentration of sulfonamide in the standard solution ($\mu g\,l^{-1}$).
[b] Calculated from the calibration curves obtained for the lower concentration range; $S_{R,c}$: regression standard deviation; R^2: determination coefficient.

CL signals, another calibration curves were established using the same standard solutions of sulfonamides but applying the extraction process to each standard solution. By comparing statistically both curves, no significant differences were obtained from the intercepts and the slopes. This comparison ensures that there are no significant losses of analytes due to the extraction process, being possible to use directly the SC curves for quantification purposes. The performance characteristics of the method obtained from the calibration data set [30] are shown in Table 3. Detection and quantification limits have been calculated as $\Delta_{\alpha,\beta}S_0$ and $10S_0$, respectively [34], where $\Delta_{\alpha,\beta}$ is the non-central parameter of a non-central *t*-distribution with ν degrees of freedom which depends on both α and β; α is the probability of committing a statistical type I error (false positive, usually 0.05); β is the probability of committing a statistical type II error (false negative, usually 0.05); and S_0 is the standard deviation for "zero" concentration, estimated as an approximate expression which uses the residual standard deviation [35] of a calibration curve obtained from the lower concentration range of the initial SC, near the expected LD, obtaining in this way a better estimation of S_0. Considering that the applied extraction process of the sulfonamides in sample involves a pre-concentration step of 10-fold, the proposed method allows the detection of sulfonamides in the lower $\mu g\,l^{-1}$ range, improving the usual detection limits provided by other typical detection modes.

3.3. Validation of the method

In order to check the applicability of the proposed methodology, a sample of bovine raw milk spiked with a mixture of the seven sulfonamides (SPD, SDZ, SMZ, SCP, SDX, SMX, SDM) at different concentration levels (5, 10 and 20 $\mu g\,l^{-1}$ for each one) was analyzed by duplicate and also injected by duplicate. Previously, the sample was extracted following the AOAC official method of analysis for multiple sulfonamide residues in raw bovine milk [32], and a blank of the sample was also analyzed in order to check that the milk sample was free of sulfonamides. The recoveries obtained were compared, in terms of mean value and standard deviation for each concentration level and for each sulfonamide, with those provided by the official method in a collaborative study [36], for

Table 4
Recovery study at different concentration levels, comparison with the values obtained from the AOAC method

	SPD	SDZ	SMZ	SCP	SDX	SMX	SDM
5 $\mu g\,l^{-1}$ fortified							
R^a (%)	87.13	82.71	73.79	81.47	94.94	93.75	71.44
S^a	4.22	13.17	6.92	8.02	14.71	8.16	6.87
R^b (%)	80.46	74.09	85.38	79.08	n.r.	n.r.	68.03
S_r^b	7.48	6.00	9.26	11.35	n.r.	n.r.	12.08
10 $\mu g\,l^{-1}$ fortified							
R^a (%)	59.33	55.04	67.09	67.05	78.17	41.74	56.38
S^a	3.85	15.35	7.91	9.89	13.21	5.12	11.56
R^b (%)	76.73	75.36	82.36	71.22	n.r.	n.r.	66.54
S_r^b	5.82	5.37	6.78	6.11	n.r.	n.r.	4.23
20 $\mu g\,l^{-1}$ fortified							
R^a (%)	83.03	88.06	86.35	96.13	96.79	53.10	71.79
S^a	11.19	16.66	10.12	22.13	9.96	6.49	8.52
R^b (%)	76.23	75.01	82.87	67.88	n.r.	n.r.	67.05
S_r^b	5.47	5.14	5.78	3.82	n.r.	n.r.	3.18

R: recoveries (%); *S*: standard deviation; S_r: intralaboratory standard deviation.
[a] Values obtained with the proposed method.
[b] Values reported by the collaborative study [36]; n.r.: not reported.

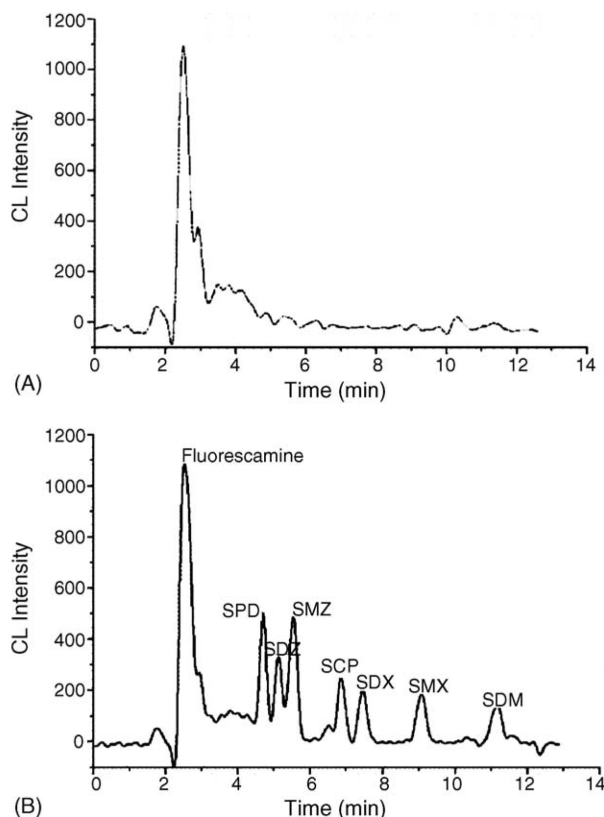

Fig. 2. Chromatogram of a blank sample (A) and a bovine raw milk sample spiked with 20 $\mu g\,l^{-1}$ of each sulfonamide (B). SPD: sulfapyridine; SDZ: sulfadiazine; SMZ: sulfamethazine; SCP: sulfachloropyridazine; SDX: sulfadoxine; SMX: sulfamethoxazole; and SDM: sulfadimethoxine.

the five sulfonamides analyzed by both methods (SPD, SDZ, SMZ, SCP, SDM). Except for SPD and SMZ, both spiked at 10 $\mu g\,l^{-1}$, and SMZ spiked at 5 $\mu g\,l^{-1}$, the applied student *t*-test showed no significant difference between the recoveries obtained by the proposed method and the collaborative study. The results are shown in Table 4, and as can be seen, the proposed method provides better results in terms of both recovery and precision for the lower spiked level (5 $\mu g\,l^{-1}$). Chromatograms of a sample spiked with 20 $\mu g\,l^{-1}$ of each sulfonamide, as well as a blank sample are shown in Fig. 2. No interferences from the matrix were observed. In this sense, it is important to highlight that SPD is well-resolved in the chromatogram corresponding to the analysis of a real sample, solving the problem of interferences that appears in the application of the official method.

4. Conclusions

A sensitive HPLC-CL method for the determination of sulfonamides in raw milk has been developed and validated. The method allows the resolution of seven sulfonamides in less than 12 min, taking advantage of the very sensitive CL detection using the PO system as post-column reaction. TDPO has been selected instead of TCPO, avoiding precipitation problems and increasing sensitivity and experimental convenience mainly considering the compatibility of the chromatographic conditions and the CL variables. The method has been applied to the analysis of spiked raw milk samples, and the results are comparable to that provided by a collaborative study using the AOAC official method, in terms of both recovery and precision. The limits of detection are better than those commonly reported for the analysis of these compounds, allowing the determination of sulfonamides in milk in the very low $\mu g\,l^{-1}$ range. Moreover, the time of analysis is shorter that those reported by other chromatographic methods.

Acknowledgements

The National Institute of Agricultural and Food Research and Technology (INIA, Ministerio de Agricultura, Pesca y Alimentación, Project Ref. CAL03-096-C2-2) supported this work. One of the authors (LGG) is grateful to the *Plan Propio* of University of Granada for a research contract.

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