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Determination of sulphanilamide in milk by first-derivative and second-derivative spectrofluorimetry

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Abstract: First-derivative and second-derivative spectrofluorimetric methods have been developed for the analysis of sulphanilamide (SAN) in milk, using an ethanol–water (60:40, v/v) medium. Linear calibration plots were obtained over a concentration range of 30–120 ppb, with correlation coefficients greater than 0.997. Relative standard deviations were within the range 1.6–3.6%. Limits of detection were 0.7–2.7 ppb. Recoveries of 90–102% were obtained. The derivative fluorescence methods are simple, rapid and sensitive and there is no interference from fluorescent impurities in the determination of low levels of SAN in milk.

Keywords: *Sulphanilamide; first-derivative spectrofluorimetry; second-derivative spectrofluorimetry; milk.*

Introduction

Sulphonamides are currently used in medicine and veterinary practice. These compounds are rapidly absorbed and the therapeutic ranges are 30–150 mg l^{-1} in plasma and 500–1000 mg l^{-1} in urine [1]. The addition of sulphonamides to animal food for prophylactic treatment or growth stimulation constitutes a widespread practice, which has allowed the present development of intensive animal husbandry. As a consequence of this current veterinary practice, there is a risk of contamination of food products by sulphonamide residues [2–6]. Therefore, it is of great importance to be able to evaluate the total amount of these compounds in food.

Several methods have been proposed for the determination of sulphonamides. Molecular spectroscopic methods are the most frequently used [7–16]. The fluorimetric characteristics of various sulphonamides have been studied and applied to the analysis of these compounds [17]. Sulphanilamide (SAN) can be determined by reaction with homophthalaldehyde [18]. Its analysis has also been performed in food and pharmaceutical compounds using the fluorescamine reaction [19]. Recently, SAN has been evaluated in milk and pharmaceuticals by a FIA method with fluorescence detection [20]. TLC has also been used for the analysis of SAN [21] and capillary electrophoretic separation of several sulphonamides, using β-cyclodextrin as separation buffer, has been proposed [22]. HPLC techniques have been applied to the determination of sulphonamides, with photometric [23–26] and fluorimetric detection [27].

The goal of this work was to develop new first-derivative and second-derivative spectrofluorimetric methods for the determination of sulphanilamide residues in milk, using a simple ethanol–water mixture.

Experimental

Apparatus

All fluorescence spectral measurements were performed on a Kontron model SFM-25 spectrophotofluorimeter, interfaced with a Samsung microcomputer for acquisition of the fluorescence spectra. For the treatment of the spectral data, Beckman Data Leader software (version 3.0) [28] was used; a program was developed in Basic in order to convert the SFM-25 spectra file to the BSF file format, through the X.Y ASCII converter included in the software.

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Reagents

All experiments were performed with analytical grade chemicals and solvents. Stock solutions of 10^{-3} M SAN (Sigma) were prepared by dissolving the compound in ethanol. Standard solutions were prepared by suitable dilutions.

Procedure for determining sulphanilamide in milk

An aliquot of the sample solution was diluted with water and ethanol to produce a 60% (v/v) ethanolic solution containing 30–120 ppb of SAN. The fluorescence emission spectra of samples were recorded between 300 and 400 nm, using an excitation wavelength of 272 nm, against a blank of ethanol–water (60:40, v/v), at a speed of 450 nm min^{-1}. The first-derivative and second-derivative spectra were obtained with a bandwidth of 11 and 23 nm, respectively, by the Savitzky and Golay method [29, 30].

The SAN content was determined by measuring the first-derivative and second-derivative signals (at zero-crossing point for milk), at 293 and 327 nm, respectively, and comparing the values with the appropriate calibration graph. For determining SAN in milk, the samples were fortified with SAN, followed by dilution 1:2500 in calibrated flasks.

Results and Discussion

Derivative spectrofluorimetric methods

The fluorescence excitation and emission spectra of solutions of SAN in ethanol–water (60:40, v/v) showed excitation and emission maxima at 272 and 330 nm, respectively. The emission spectra of solutions of SN of different concentrations were recorded (Fig. 1). Using these data, linear calibration plots were found for 30–120 ppb of SAN. The following regression equation and correlation coefficient (r) were obtained:

$$I_F = 7492\ (\pm 314)\ c + 43.5\ (\pm 23.9) \quad (r = 0.998) \qquad (1)$$

where c = SAN concentration (in ppm). Values for the limit of detection (LOD) and limit of determination (LOQ) were 10 and 27 ppb, respectively. The LOD was defined as the concentration of the solution giving a signal-to-noise (S/N) ratio of 3. The LOQ was defined as the concentration of analyte giving a S/N ratio of 10.

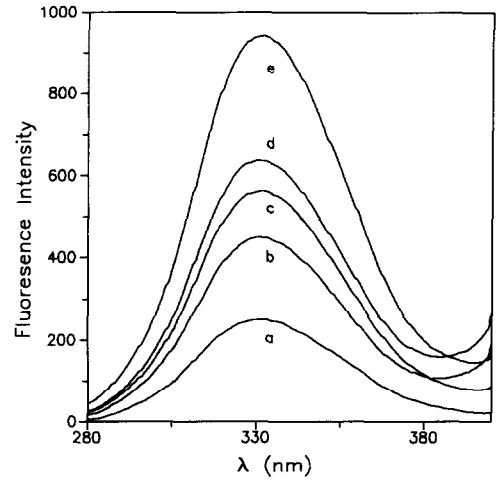

Figure 1
Fluorescence spectra of SAN solutions. (a) 30 ppb; (b) 50 ppb; (c) 70 ppb; (d) 80 ppb; (e) 120 ppb.

In order to obtain first-derivative and second-derivative fluorescence emission spectra, two variables were optimized, these were the scanning speed of the monochromator and the bandwidth ($\Delta\lambda$) used in the differentiation of the fluorescence spectra of SAN. For the first-derivative spectra, a scanning speed of 450 nm min^{-1} was selected, and $\Delta\lambda$ values assayed were 5, 7, 9, 11, 13, 15 and 17 nm. A value of $\Delta\lambda$ = 11 nm was selected as giving the best signal-to-noise ratio. First-derivative spectra presented a maximum at 312 nm and a minimum at 357 nm (Fig. 2).

The second-derivative spectra were recorded with a scanning speed of 450 nm min^{-1} and bandwidths of 11, 15, 19, 23 and 25 nm. The

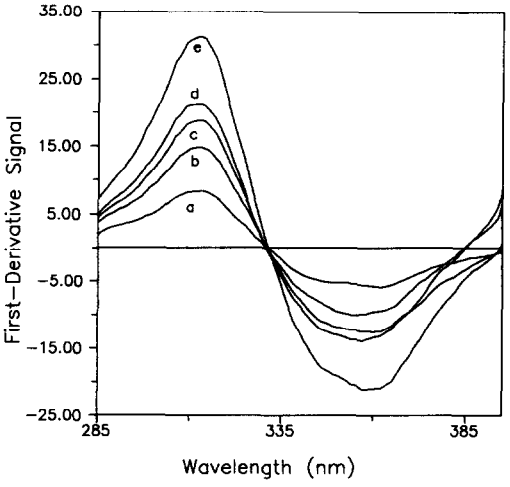

Figure 2
First-derivative fluorescence spectra of SAN solutions. (a) 30 ppb; (b) 50 ppb; (c) 70 ppb; (d) 80 ppb; (e) 120 ppb.

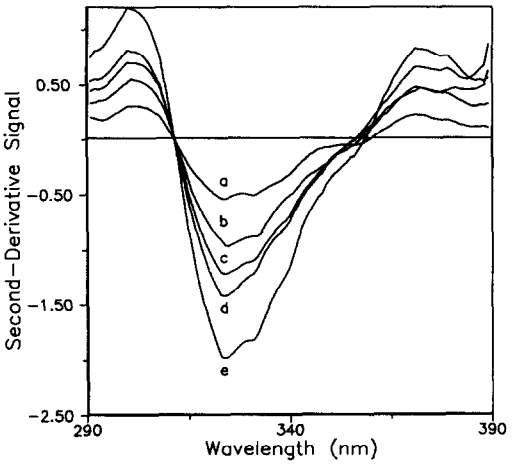

Figure 3
Second-derivative fluorescence spectra of SAN solutions. (a) 30 ppb; (b) 50 ppb; (c) 70 ppb; (d) 80 ppb; (e) 120 ppb.

best signal-to-noise ratio was obtained for $\Delta\lambda = 23$ nm. Second-derivative spectra showed a maximum at 300 nm and a minimum at 324 nm (Fig. 3).

Calibration graphs and analytical figures of merit

For the first-derivative spectra, calibration graphs were constructed by measuring the amplitude of the signal at 312 nm ($^1D_{312}$) and 357 nm ($^1D_{357}$), respectively. Linear relationships were established for the range 30–120 ppb. The following regression equations and correlation coefficients (r) were found:

$$^1D_{312} = 247.8\ (\pm 6)c + 1.5(\pm 0.7) \quad (r = 0.998) \qquad (2)$$

$$^1D_{357} = 168.5\ (\pm 4.3)c + 0.76(\pm 0.3)\ (r = 0.997) \qquad (3)$$

with c = SAN concentration (in ppm).

Values for the relative standard deviation (RSD), LOD and LOQ were, respectively, 1.6%, 0.7 ppb and 2.2 ppb at 312 nm, and 2.7%, 2.4 ppb and 8.0 ppb at 357 nm.

For the second-derivative spectra, calibration graphs were drawn by measuring the amplitude of the signal at 300 nm (maximum) ($^2D_{300}$) and 324 nm (minimum) ($^2D_{324}$), respectively. The following regression equations and correlation coefficients were obtained:

$$^2D_{300} = 9.60(\pm 0.3)c + 0.03(\pm 0.01) \quad (r = 0.999) \qquad (4)$$

$$^2D_{324} = 15.8(\pm 0.7)c + 0.12(\pm 0.05) \quad (r = 0.998). \qquad (5)$$

The RSD, LOD and LOQ were, respectively, 3.6%, 2.7 ppb and 9.0 ppb at 300 nm, and 2.0%, 2.0 ppb and 7.0 ppb at 324 nm. It can be seen that both first-derivative and second-derivative spectrofluorimetric techniques give similar analytical figures of merit.

Analytical applications to milk samples

The first-derivative and second-derivative spectrofluorimetric methods were applied to the determination of SAN in milk. Figures 4 and 5 show the first-derivative and second-derivative spectra of SAN and milk. It can be seen that milk contains fluorescent impurities,

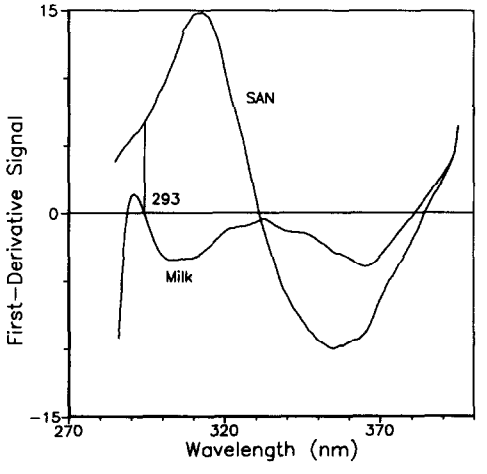

Figure 4
First-derivative fluorescence spectra of SAN (50 ppb) and milk.

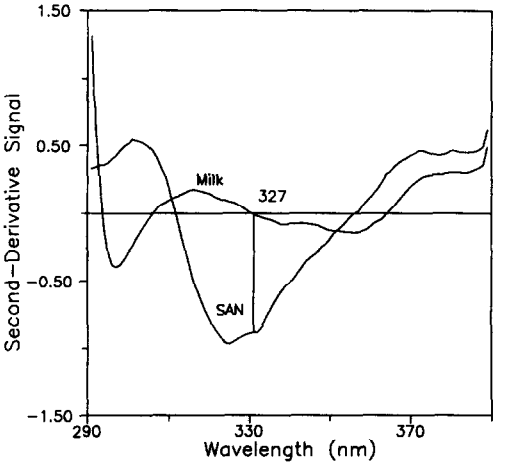

Figure 5
Second-derivative fluorescence spectra of SAN (50 ppb) and milk.

Table 1
Statistical data for the determination of SAN in milk by first- and second-derivative fluorimetry*

Parameter	First-derivative	Second-derivative
Equation	$^1D_{293} = 108c + 0.03$ (± 2.4) (± 0.01)	$^2D_{327} = 15c + 0.04$ (± 0.5) (± 0.03)
Correlation coefficient	$r = 0.999$	$r = 0.998$
RSD† (%)	2.6	1.2
LOQ‡ (ppb)	10.3	17
LOD§ (ppb)	3.0	6

*For each calibration curve, measurements were performed for five concentrations in duplicate.
†RSD = Relative Standard Deviation.
‡LOQ = limit of quantification (see definition in text).
§LOD = limit of detection (see definition in text).

Table 2
Recovery of SAN in milk by first- and second-derivative fluorimetry

	SAN (ppm)		
Method	Added	Found	Recovery (%)
First-derivative	—	—	—
λ = 293 nm	0.03	0.023	97.6
	0.05	0.049	98.4
	0.07	0.068	97.4
	0.10	0.102	102.0
Second-derivative	—	0.006	—
λ = 327 nm	0.03	0.027	90.0
	0.05	0.047	94.0
	0.07	0.067	95.7
	0.10	0.092	92.0

which do not interfere significantly with first-derivative or with second-derivative spectra of SAN. The possible presence in the milk samples of other strongly fluorescent sulphonamides such as sulphacetamide and sulphaguanidine [17] could produce significant interference which may reduce the specificity of the derivative methods. In this event, it would be useful to check preliminarily the content of the milk sample by means of a quick chromatographic test.

Calibration graphs were constructed by measuring the amplitude of the derivative signal at the zero-crossing of milk at 293 and 327 nm, respectively, for the first-derivative and second-derivative spectra. The 293 and 327 nm wavelengths were chosen for quantitative analytical measurements because of the absence of a fluorescence signal from the milk samples under study at these wavelengths. However, there is no evidence to show that the zero-crossing wavelengths are at these same values for *all* samples of milk. The consistency of these zero-crossing wavelengths is important for the method to be applicable to real samples of milk containing SAN. Therefore, it is recommended that the validity of these values be verified for each milk sample, before applying the derivative methods to real samples.

The regression equations, correlation coefficients, RSD, LOD and LOQ are given in Table 1. The linearity of the calibration plots for the determination of SAN in milk is good, as shown by the correlation coefficient values close to 0.999, and the reproducibility is satisfactory, as demonstrated by the RSD of 1.2–2.6%. The sensitivity of the method is high, as indicated by the detection limits of 3–6 ppb.

The standard addition procedure was used for evaluating recoveries of SAN in milk (Table 2). Satisfactory results were obtained with recoveries of 97.4–102.4% for the first-derivative method and 90.9–95.7% for the second-derivative method.

Conclusions

The results demonstrated that the first-derivative and second-derivative spectrofluorimetric methods can be applied to the deter-

mination of sulphanilamide in milk without significant interference by the other constituents present in the samples investigated. These methods are simple, sensitive, rapid, and they do not require any preliminary purification or treatment of the biological sample containing sulphanilamide.

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