Determination of Sulfamethazine and Trimethoprim in Liquid Feed Premixes by HPLC and Diode Array Detection, with an Analysis of the Uncertainty of the Analytical Results

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Sulfamethazine (SMZ) and trimethoprim (TMP) are antibacterials used in veterinary practice. This paper describes a method for their determination in veterinary liquid feed premixes that is based on liquid chromatography with diode array detection. Gradient elution with methanol and ammonium acetate achieved excellent separation of the two analytes within 15 min without any interference from the matrix. Absorbance of the column effluent was monitored at 264 nm for SMZ and at 230 nm for TMP. Detailed analyses of the uncertainties of determinations afford estimated expanded uncertainties of, respectively, 0.2 and 0.1 w/v % for typical SMZ and TMP concentrations of 10.7 and 2.1 w/v %, respectively. At the lower end of the calibrated range of the method, the dominant source of uncertainty is the preparation of standards and the construction of the calibration line.

Keywords: *Antibiotics; diode array; feed premix; liquid chromatography; sulfamethazine; trimethoprim; uncertainty*

1. INTRODUCTION

Sulfamethazine, 4-amino-*N*-(4,6-dimethyl-2-pyrimidine)benzenesulfonamide (SMZ, Figure 1a), and trimethoprim, 5-(3,4,5-trimethoxybenzyl)pyrimidine-2,4-diamine (TMP, Figure 2a), are antibacterial compounds used to treat livestock diseases such as gastrointestinal and respiratory tract infections. SMZ is commonly used in combination with TMP because they act synergistically at different points of the same bacterial metabolic pathway: SMZ depresses dihydrofolic acid synthesis, whereas TMP interferes with folic acid metabolism by inhibiting dihydrofolate reductase. This double action is effective against a range of Gram-positive and Gram-negative organisms, and less resistance is encountered than when either agent is used alone (*1*).

SMZ and TMP are usually added as veterinary medicinal components in solid form during animal feed production. This way of production poses a serious cross-contamination problem between medicated feeds and nonmedicated blank feeds when the production line is the same. To overcome this problem, the antibiotic addition could be performed by spraying the veterinary medicinal components in liquid solution onto the granulated feed. The use of these liquid veterinary premixes requires a well-defined and standardized analytical method for the determination of their antibiotic contents.

Methods frequently used for the determination of SMZ in pharmaceutical preparations include mainly high-performance liquid chromatography (HPLC) (*2*, *3*). In addition, Mahedero and Aaron (*4*) have developed a method involving flow injection and photochemically induced fluorescence. Methods used to determine TMP in pharmaceutical preparations include also HPLC (*5*−*8*). Determination of SMZ and TMP can then be carried out by HPLC as specified by the *United States Pharmacopeia* (*9*).

We propose a rapid and interference-free HPLC method developed for the quality control department of veterinary pharmaceutical companies producing liquid feed premixes with SMZ and TMP. This method uses inexpensive mobile phase components (methanol and ammonium acetate), which are compatible with atmospheric pressure ionization techniques of mass spectrometric detection. LC mobile phases containing inorganic mineral acids, nonvolatile buffers, and high levels of additives (>100 mM) are generally not recommended for liquid chromatography−mass spectrometry (LC-MS) because they can deposit on the ion source. With these exceptions, most LC-MS systems are compatible with a wide range of aqueous and organic solvents and mixtures thereof and also with volatile pH control agents such as buffers (e.g., ammonium acetate), acids (e.g., formic, acetic, and trifluoroacetic), and bases (e.g., trialkylamines and ammonia). The LC mobile phase we selected during method development is then appropriate for MS interfaces such as ApcI probes, which are normally used to confirm the presence of an analyte.

The method allows verification of the purity of the chromatographic peaks by means of the optimization of diode array detection conditions. The method as described determines SMZ and TMP separately because their concentrations in the commercial feed premix of interest differed by a factor of ∼5, and the sample dilutions that were optimal for the determination of each analyte differed accordingly; if use of the same sample dilution for both analytes is considered to be acceptable, they can be determined simultaneously with no added difficulty. The description of the method is accompanied by a detailed analysis of the uncertainty

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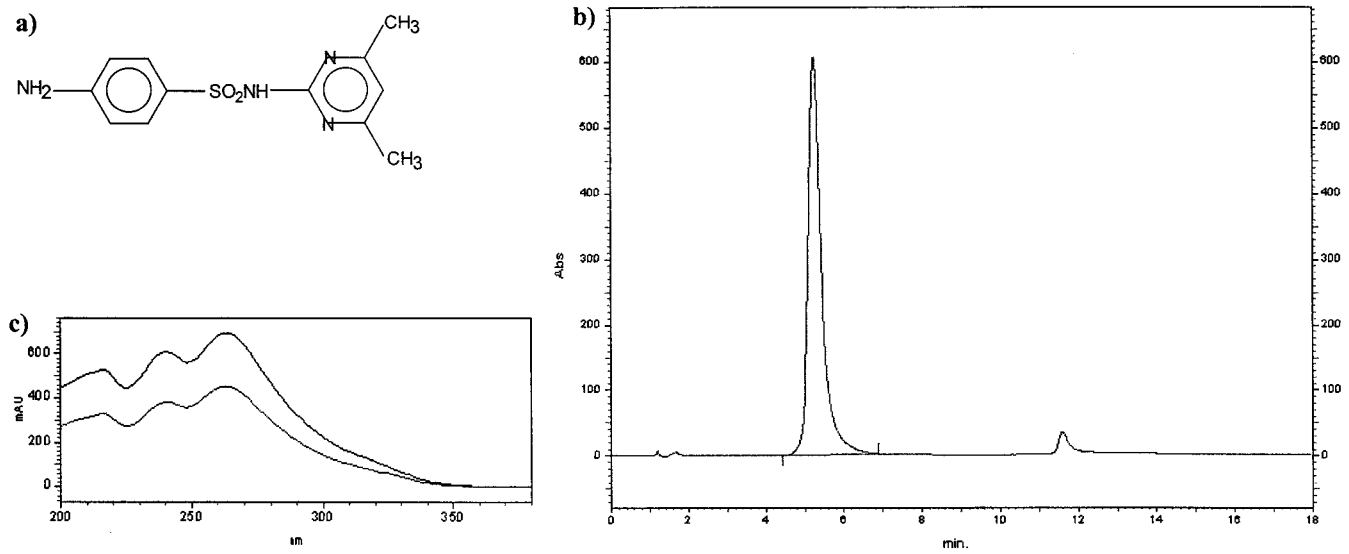

Figure 1. (a) Structure of sulfamethazine. (b) HPLC chromatogram of commercial feed premix obtained by diode array detection at 264 nm. (c) UV spectra of commercial feed premix and sulfamethazine standard (40 mg/L) at the apex of the chromatogram peak (5.5 min).

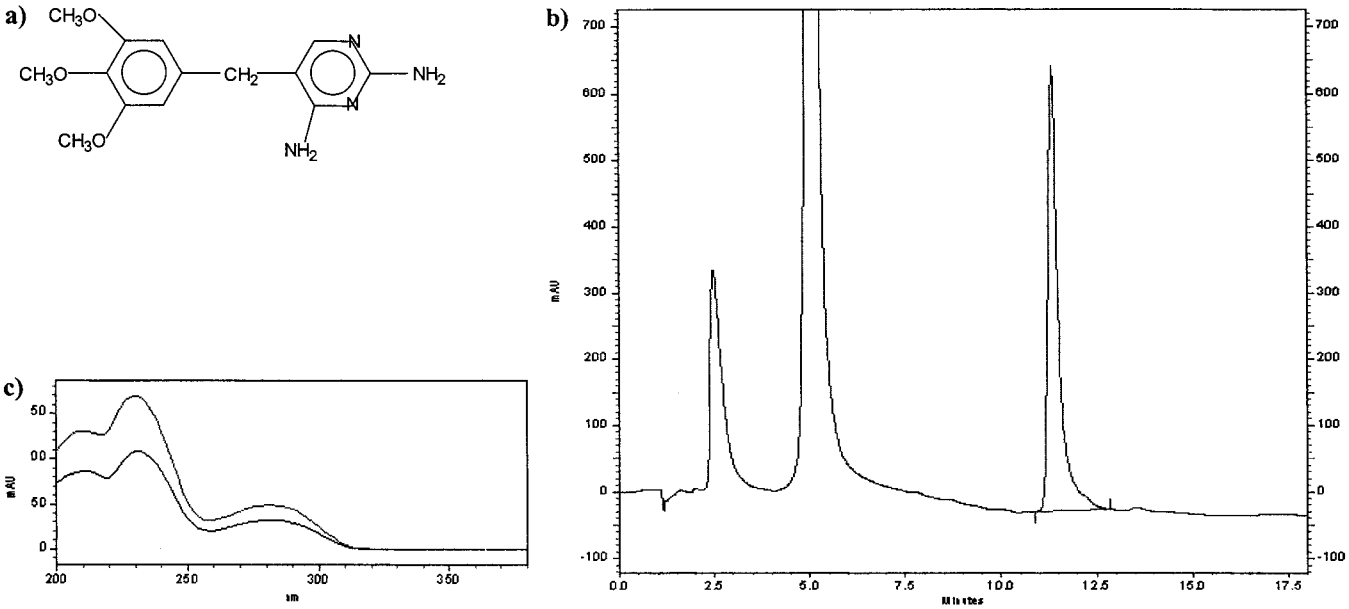

Figure 2. (a) Structure of trimethoprim. (b) HPLC chromatogram of commercial feed premix obtained by diode array detection at 230 nm. (c) UV spectra of commercial feed premix and trimethoprim standard (40 mg/L) at the apex of the chromatogram peak (12.0 min).

of its results. Uncertainty estimation allows one to detect systematic errors in the different stages of the analytical procedure and, consequently, to improve this procedure.

2. EXPERIMENTAL PROCEDURES

2.1. Chemicals and Reagents. Sulfamethazine (purity = 99.8%) and trimethoprim (purity = 99.5%) were purchased from Sigma (St. Louis, MO) and Riedel de Haën (Seelze, Germany), respectively. Their purities were tested by the chemical supplier by HPLC and NMR. Methanol of HPLC grade was supplied by Sigma, and analytical grade ammonium acetate by Panreac (Barcelona, Spain). HPLC grade water was obtained from a Milli-Ro water purification system (Millipore, Bedford, MA).

Separate ~1000 mg/L stock solutions of SMZ and TMP were prepared by dissolving 0.1 g of SMZ or 0.05 g of TMP in a small amount of methanol and diluting to 100 or 50 mL, respectively, with the same solvent. These solutions were stored in stoppered flasks at 4 °C in the dark.

2.2. Sample Preparation. Liquid feed premix [a solution of dimethylacetamide, SMZ (nominal 10% w/v) and TMP (nominal 2% w/v) in distilled water] was purchased from a veterinary firm. For HPLC determination of SMZ, samples were successively diluted with Milli-Ro water 100 and 20 times, giving a final concentration of ~50 mg/L. For determination of TMP the two successive dilution factors were 100 and 5, giving a final concentration of ~40 mg/L. Pipet and flask volumes are specified below in the analysis of uncertainty under Results and Discussion.

2.3. Chromatography. HPLC analyses were performed on a Thermo Separation Products HPLC system comprising a P2000 gradient pump, an AS1000 autosampler, and a UV6000LP diode array detector; on the basis of scans in the range of 200−380 nm, wavelengths of 264 and 230 nm were used for quantification of SMZ and TMP, respectively. Peak areas were obtained using the program ChromQuest 2.51 (Thermo Quest). The analytical column (15 cm × 4.6 mm i.d.)

Table 1. Performance of the Proposed Method for Determination of Sulfamethazine (SMZ) and Trimethoprim (TMP) in Veterinary Feed Premixes

	SMZ	TMP
t_R (min)	5.5	12.0
regression line		
intercept term (a) (counts)	−56097	303548
slope (b) (counts L/mg)	276665	299950
r^2	0.9996	0.9992
linear range (mg/L)	1.0−100	1.0−100
LOD (mg/L)	0.3	0.4
HPLC repeatability		
RSD (%)	0.3	1.0
method repeatability		
RSD (%)	2.6	1.0
feed premix analysis		
C_{premix} (w/v %)	10.7	2.1
$\pm S$ (w/v %)	0.3	0.02

was packed with Ultracarb 5 μm ODS 30% C from Phenomenex (Torrance, CA) and the guard column (5 cm × 4.6 mm i.d.) with dry 40 μm Pelliguard LC-18 from Supelco (Gland, Switzerland).

In each run, 20 μL of prepared sample was injected into the column and eluted with methanol (A) and ammonium acetate (B) in accordance with the following program: 23:77 A/B for 5 min; 23:77 to 44:56 over 5 min; 44:56 for 10 min; 44:56 to 23:77 over 1 min; 23:77 for 10 min. All analyses were performed at a constant flow rate of 1.5 mL/min and at room temperature.

2.4. Calibration. Calibration lines (peak area versus concentration) were constructed using standards prepared from the stock solutions by dilution with HPLC grade water. For SMZ 8 standards with approximate concentrations of 1, 3, 10, 20, 30, 40, 80, and 100 mg/L were used, and for TMP 10 standards with approximate concentrations of 1, 3, 5, 10, 20, 30, 40, 60, 80, and 100 mg/L were used.

3. RESULTS AND DISCUSSION

3.1. Performance of the Method. The performance of the method was evaluated following the rules established by the VICH Expert Working Group (*10*). The results are summarized in Table 1.

3.1.1. Specificity. The SMZ and TMP peaks were well separated, with retention times of 5.5 and 12.0 min, respectively, and had acceptable symmetry (*United States Pharmacopeia* asymmetry factors of 1.4 for SMZ peak and 1.6 for TMP peak) under the conditions used (Figures 1b and 2b). For both samples and standards prepared from the stock solutions, absorption spectra recorded between 200 and 380 nm at different points of the chromatogram peak (scan rate = 1 Hz, step = 2 nm, bandwidth = 3 nm) were identical in form with those recorded at the apex of the peak, and the spectra of samples and standards were likewise identical in form. Figures 1c and 2c show the spectra recorded at the apex of the peaks. In particular, for each compound the wavelength of maximum absorbance at the apex of the chromatogram peak was the same for sample and standard to within ±2 nm.

3.1.2. Detection Limits (LODs). LODs were estimated following the recommendations of the American Chemical Society (*11*). The LOD for SMZ was 0.3 mg/L, and the LOD for TMP was 0.4 mg/L.

3.1.3. Repeatability. HPLC repeatability was evaluated in terms of the relative standard deviation (RSD %) of six analyses of a single liquid feed premix performed on the same day. For neither analyte was the RSD % >1%.

Method repeatability was evaluated in terms of the RSD % of six values obtained on the same day, corresponding to duplicate HPLC runs of each of three solutions that were obtained, independently of each other, as described above in section 2.2. For neither analyte was the RSD % >3%.

3.1.4. Linearity and Interferences. The coefficients of determination of the calibration line were 0.9996 for SMZ and 0.9992 for TMP. No systematic trends or other indications of nonlinearity were observed (*12*). The absence of interference was shown by the values of the intercepts of the calibration lines not differing significantly from zero.

3.1.5. Stability of Stock Solutions. After storage in the dark at 4 °C for 3 months, the stock solutions used for the preparation of calibration standards showed no signs of instability, and neither did feed premix stored in the dark at a constant 20 °C for the same period.

3.2. Analysis of the Commercial Feed Premix. Analyses of the commercial feed premix using samples prepared in triplicate and duplicate HPLC determinations for each sample afforded SMZ and TMP concentrations of 10.7 ± 0.3 and 2.10 ± 0.02% w/v, respectively.

3.3. Estimation of Uncertainty. Estimation of the uncertainty of analytical results is mandatory for laboratories accredited under EN45001 (*13*). Detailed analysis of the accumulation of uncertainty during the various stages of an analytical determination can pinpoint critical stages on which uncertainty-reducing efforts should be focused. The *EURACHEM/CITAC Guide* (*14*) was followed to quantify uncertainty in the analytical measurement.

3.3.1. Preliminaries. (1) To first order, the error Δy in the value of a parameter y that is calculated, in the course of an analytical determination, from an expression $y = G(x_1, ..., x_n)$, is given by

$$\Delta y = \Sigma_i(\partial G/\partial x_i)\Delta x_i \quad (1)$$

Assuming that the Δx_i are statistically independent of each other, the uncertainty u_y of y is therefore given in terms of the uncertainties u_{xi} of the independent variables by

$$u_y^2 = \Sigma_i (\partial G/\partial x_i)^2 u_{xi}^2 \quad (2)$$

If $y = G(x_1, ..., x_n) = x_1^{m1} x_2^{m2} ... x_n^{mn}$, then $\partial G/\partial x_i = m_i y/x_i$ and hence

$$u_y^2 = y^2 \Sigma_i (m_i u_{xi}/x_i)^2 \quad (3)$$

that is

$$\hat{u}_y^2 = \Sigma_i (m_i \hat{u}_{xi})^2 \quad (4)$$

where $\hat{u}_\alpha = u_\alpha/\alpha$ is the relative uncertainty of α.

(2) The uncertainty u_α of a parameter α determined by averaging n measurements can be taken to be the estimated standard deviation of the mean

$$u_\alpha = wS/n^{1/2} \quad (5)$$

where S is the standard deviation of the n measurements and w is a correction factor that differs from unity only when n is <10. In this work we used factors $w = 1.2$ for $n = 8$ and $w = 1.3$ for $n = 6$ (*14*).

(3) The uncertainty of a parameter assumed to have a uniform distribution of width $2a$ is taken to be the standard deviation of that distribution, $a/\sqrt{3}$. The

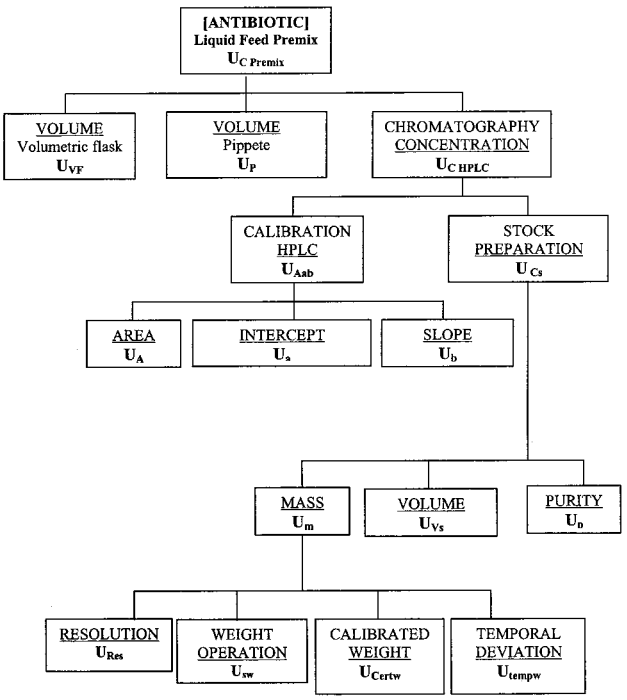

Figure 3. Diagram of the accumulation of uncertainty in the determination of SMZ and TMP by the proposed method.

Table 2. Analysis of the Uncertainty Associated with the Use of Volumetric Flasks and Pipets

V (mL)	$S_{V\alpha}$ (mL)	$T_{V\alpha}$ (mL)	$\beta_{V\alpha}$ (mL)	$u^2_{V\alpha}$	$\hat{u}^2_{V\alpha}$
Volumetric Flasks					
25	0.0459	±0.04	0.0163	0.0024	3.80 E−06
50	0.0846	±0.06	0.0245	0.0077	3.10 E−06
100	0.0815	±0.10	0.0408	0.0083	8.31 E−07
Pipets					
1	0.0038	±0.011	0.0045	3.46 E−05	3.45 E−05
5	0.0078	±0.015	0.0061	9.85 E−05	3.94 E−06

uncertainty of a parameter assumed to have a triangular distribution of width $2a$ is similarly taken to be $a/\sqrt{6}$.

3.3.2. Uncertainty of Determinations of SMZ and TMP. The accumulation of uncertainty in the analytical method described in this paper is represented diagrammatically in Figure 3. The final calculation in each determination uses the equation

$$C_{\text{premix}} = C_{\text{HPLC}}(V_{\text{F1}}/V_{\text{P1}})(V_{\text{F2}}/V_{\text{P2}}) \times 10^{-4} \qquad (6)$$

where C_{premix} is the concentration of analyte in the liquid premix (in % w/v), C_{HPLC} is the concentration of analyte in the sample injected into the HPLC apparatus (in mg/L), and $V_{\text{F}i}$ and $V_{\text{P}i}$ ($i = 1, 2$) are the volumes of the flask and pipet used for the ith dilution of the premix sample in preparing it for HPLC. Hence, by eq 4

$$\hat{u}_{C\text{premix}}{}^2 = \hat{u}_{C\text{HPLC}}{}^2 + \hat{u}_{V\text{F1}}{}^2 + \hat{u}_{V\text{F2}}{}^2 + \hat{u}_{V\text{P1}}{}^2 + \hat{u}_{V\text{P2}}{}^2 \qquad (7)$$

In what follows we describe the estimation of each of the terms on the right-hand side of eq 7.

3.3.2.1. Estimation of $\hat{u}_{VFi}$ and $\hat{u}_{VPi}$. We used 25 and 100 mL volumetric flasks and 1 and 5 mL pipets (see Table 4) with certified tolerances $T_{V\alpha}$ ($\alpha = 0.04$ for F_1, 25 mL; 0.1 for F_2, 100 mL; 0.011 for P_1, 1 mL; or 0.015 for P_2, 5 mL). In each case, the 10 vessels were calibrated by weight at 20 °C using ultrapure water, and $u_{V\alpha}{}^2$ was calculated as the estimated mean squared error of a single measurement

$$u_{V\alpha}{}^2 = S_{V\alpha}{}^2 + \beta_{V\alpha}{}^2 \qquad (8)$$

where $S_{V\alpha}$ is the standard deviation of the 10 measurements and $\beta_{V\alpha} = T_{V\alpha}/\sqrt{6}$ is the uncertainty due to the error of the volumetric mark, which was assumed to have a triangular distribution of width $2T_{V\alpha}$. These quantities are listed in Table 2 together with the corresponding values of $u_{V\alpha}{}^2$ and $\hat{u}_{V\alpha}{}^2$.

3.3.2.2. Estimation of $\hat{u}_{C\text{HPLC}}$. Given a calibration line of slope b and intercept a, the concentration C_{HPLC} estimated for an HPLC sample affording a peak area A is given by

$$C_{\text{HPLC}} = (A - a)/b \qquad (9)$$

The error ΔC_{HPLC} is given by

Table 3. Analysis of the Uncertainty in the Determination of the Concentration of Analyte in the HPLC Sample (Equation 12)[a]

	SMZ	TMP
derivation of $\hat{u}_{Aab}$		
HPLC area		
A (counts)	14827912	12909376
S_A (counts)	38687	126444
u_A (counts)	20532	67107
intercept term (a)		
a (counts)	−56097	303548
S_a (counts)	110176	145980
u_a (counts)	46744	46163
slope (b)		
b (counts L/mg)	276665	299950
S_b (counts L/mg)	2159	3051
u_b (counts L/mg)	916	965
u_{Aab} (mg/L)	0.256	0.303
C_{HPLC} (mg/L)	53.0	41.3
$\hat{u}_{Aab}$	**0.0048**	**0.0073**
derivation of $\hat{u}_{Cs}$		
m (μg)	104700	50100
u_{sw} (μg)	137.0	527.5
u_m (μg)	158.2	533.4
$\hat{u}_m$	0.0015	0.0106
V_s (mL)	100	50
u_{Vs} (mL)	0.091	0.088
$\hat{u}_{Vs}$	0.00091	0.0018
p (parts per unity)	0.998	0.995
u_p (parts per unity)	0.0012	0.0029
$\hat{u}_p$	0.0012	0.0029
$\hat{U}_{Cs}$	**0.0021**	**0.0112**
derivation of $\hat{u}_{C\text{HPLC}}$		
$\hat{U}^2_{Aab}$	2.34 E−05	5.39 E−05
$\hat{U}^2_{Cs}$	4.45 E−06	1.25 E−04
$\hat{U}^2_{C\text{HPLC}}$	2.79 E−05	1.79 E−04
$\hat{U}_{C\text{HPLC}}$	**0.0053**	**0.0134**

[a] Data refer to results obtained upon analysis of a feed premix with typical nominal concentrations of 10 and 2 w/v % for SMZ and TMP, respectively.

Table 4. Analysis of the Uncertainty of Determinations of SMZ and TMP by the Proposed Method

	SMZ	TMP
$\hat{u}^2_{V\text{F1}}$ (100 mL of SMZ and TMP)	8.31 E−07	8.31 E−07
$\hat{u}^2_{V\text{F2}}$ (100 mL of SMZ and 25 mL of TMP)	8.31 E−07	3.80 E−06
$\hat{u}^2_{V\text{P1}}$ (1 mL of SMZ and 5 mL of TMP)	3.45 E−05	3.94 E−06
$\hat{u}^2_{V\text{P2}}$ (5 mL of SMZ and TMP)	3.94 E−06	3.94 E−06
$\hat{u}^2_{C\text{HPLC}}$	2.79 E−05	1.79 E−04
$\hat{u}^2_{C\text{premix}}$	6.80 E−05	1.92 E−04
$\hat{u}_{C\text{premix}}$	**0.0082**	**0.0138**
$\hat{u}_{\text{expanded}C\text{premix}}$	**0.0165**	**0.0277**
$C_{\text{premix}} \pm u_{\text{expanded}C\text{premix}}$	**10.7 ± 0.2**	**2.1 ± 0.1**

$$\Delta C_{\mathrm{HPLC}} = \Delta_{Aab} + (C_{\mathrm{HPLC}}/C_{\mathrm{s}})\Delta C_{\mathrm{s}} \quad (10)$$

where C_s is the nominal concentration of the stock solution from which calibration standards are prepared, ΔC_s is the error in this concentration, and Δ_{Aab} is the further error introduced in constructing the calibration line and in obtaining C_{HPLC} from A and the line (see eq 9). Therefore, assuming Δ_{Aab} and ΔC_s to be statistically independent of each other

$$u_{C\mathrm{HPLC}}{}^2 = u_{Aab}{}^2 + (C_{\mathrm{HPLC}}/C_{\mathrm{s}})^2 u_{C\mathrm{s}}{}^2 \quad (11)$$

or

$$\hat{u}_{C\mathrm{HPLC}}{}^2 = \hat{u}_{Aab}{}^2 + \hat{u}_{C\mathrm{s}}{}^2 \quad (12)$$

where $\hat{u}_{Aab} = u_{Aab}/C_{\mathrm{HPLC}}$.

From eq 9, the squared uncertainty $u_{Aab}{}^2$ due to the uncertainty u_A in A and the uncertainties u_a and u_b of the calibration line parameters is given by eq 2 as

$$u_{Aab}{}^2 = b^{-2}\{u_A{}^2 + u_a{}^2 + u_b{}^2[(A - a)/b]^2\} \quad (13)$$

where u_A, u_a, and u_b may be calculated from the corresponding standard deviations using eq 5. The top panel of Table 3 lists the values of u_A, u_a, u_b, and u_{Aab} obtained in this work, in which the value of n in eq 5 was 6 for u_A (corresponding to duplicate determinations of triplicate samples) and for u_a and u_b was 8 for SMZ and 10 for TMP. Also listed are the values of A, a, and b themselves, their standard deviations, C_{HPLC}, and the relative uncertainty $\hat{u}_{Aab}$.

Because the stock concentration $C_s = mp/V_s$, where m is the weight of drug used, p its purity, and V the volume in which it was dissolved, $u_{Cs}{}^2$ is given by eq 14 as

$$\hat{u}_{C\mathrm{s}}{}^2 = \hat{u}_m{}^2 + \hat{u}_{V\mathrm{s}}{}^2 + \hat{u}_p{}^2 \quad (14)$$

The term $\hat{u}_{Vs}{}^2$ was calculated in the same way as described for the uncertainties $\hat{u}_{V\alpha}$ in section 3.3.2.1; for TMP it is the value for 50 mL flasks in Table 2 and for SMZ the value for 100 mL flasks. To obtain the term $\hat{u}_p{}^2$ in eq 14, u_p was calculated by assuming the error Δp to have a uniform distribution of width $(100 - p)/100$. To obtain the term $\hat{u}_m{}^2$, $u_m{}^2$ was decomposed as

$$u_m{}^2 = u_{\mathrm{res}}{}^2 + u_{\mathrm{MSEw}}{}^2 \quad (15a)$$

$$= u_{\mathrm{res}}{}^2 + u_{S\mathrm{w}}{}^2 + u_{\mathrm{w}}{}^2 \quad (15b)$$

$$= u_{\mathrm{res}}{}^2 + u_{S\mathrm{w}}{}^2 + u_{C\mathrm{ertw}}{}^2 + u_{\mathrm{tempw}}{}^2 \quad (15c)$$

where u_{res} is the uncertainty due to the limited resolution of the balance and $u_{\mathrm{MSEw}}{}^2$ the mean squared error of the weighing operation, which was estimated as the sum of the variance $u_{S\mathrm{w}}{}^2$ of 10 weighings of a weight calibrated at the Galician Official Metrology Laboratory (Ourense, Spain); the squared uncertainty $u_w{}^2$ of this weight (a 100 mg weight was used for SMZ and a 50 mg weight for TMP), $u_w{}^2$, was in turn estimated as the sum of $u_{C\mathrm{ertw}}{}^2$ and $u_{\mathrm{tempw}}{}^2$, where $u_{C\mathrm{ertw}}$ is the uncertainty in the calibrated weight declared on its calibration certificate (0.070 mg) and u_{tempw} the uncertainty due to possible changes in the weight between calibrations. The terms $u_{\mathrm{res}}{}^2$ and $u_{\mathrm{tempw}}{}^2$ were calculated from the resolution of the balance ($R = 0.0001$ g) and the estimated between-calibration weight deviation stated on the weight calibration certificate ($D = 0.04$ mg) by assuming the corresponding errors to have uniform distributions of widths R and $2D$, respectively [$u_{\mathrm{res}} = R/(2\sqrt{3})$, $u_{\mathrm{tempw}} = D/\sqrt{3}$].

The values of u_m, $u_{V\mathrm{s}}$, u_p, $\hat{u}_m$, $\hat{u}_{V\mathrm{s}}$, and $\hat{u}_p$ obtained in this work are listed in the middle section of Table 3 together with the corresponding values of m, V_s, and p and the values of $u_{C\mathrm{s}}$ and $\hat{u}_{C\mathrm{s}}$. The values of $u_{C\mathrm{HPLC}}$ and $\hat{u}_{C\mathrm{HPLC}}$ given by eqs 11 and 12 are listed in the bottom section of Table 3.

$U_{C\mathrm{premix}}$ and $\hat{u}_{C\mathrm{premix}}$ of SMZ and TMP are shown in Table 4. The final stage of uncertainty estimation is to multiply the uncertainty by the chosen coverage factor k in order to obtain an expanded uncertainty (see Table 4). The expanded uncertainty is required to provide an interval that may be expected to encompass a large fraction of the distribution of values which could reasonably be attributed to the measurand. For most purposes it is recommended that k is set to 2, giving an interval containing ~95% of the normal distribution of values (*14*).

In the analysis of different sources of uncertainty in Table 4, it is easy to see that in relative terms expanded uncertainty is about two times higher for TMP than for SMZ. This is caused by the use of lower volume flasks in the case of TMP analysis but mainly by its higher uncertainty due to C_{HPLC}. The reason for its higher C_{HPLC} uncertainty (see Table 3) is the weight of a lower amount of TMP (50 versus 100 mg). The uncertainty due to the preparation of standards and the construction of the calibration line is already kept to a minimum. It is then recommended to use flask and pipet volumes and to weigh solid amounts as large as possible.

Finally, by eqs 7 and 12

$$\hat{u}_{C\mathrm{premix}}{}^2 = \hat{u}_0{}^2 + u_1{}^2/C_{\mathrm{HPLC}}{}^2 \quad (16a)$$

$$= \hat{u}_0{}^2 + u_1{}^2/(fC_{\mathrm{premix}})^2 \quad (16b)$$

where $f = 5$ (SMZ) or 20 (TMP), and if the errors ΔA are homocedastic neither $\hat{u}_0{}^2$ ($= \hat{u}_{V\mathrm{F1}}{}^2 + \hat{u}_{V\mathrm{F2}}{}^2 + \hat{u}_{V\mathrm{P1}}{}^2 + \hat{u}_{V\mathrm{P2}}{}^2 + \hat{u}_{C\mathrm{s}}{}^2 + (u_b/b)^2$) nor $u_1{}^2$ [$= (u_A{}^2 + u_a{}^2)/b^2$] depends on the analyte concentration. For the values of $\hat{u}_0$ and u_1 obtained in this work, $\hat{u}_{C\mathrm{premix}}$ ranges from 0.1783 (for SMZ) or 0.2726 (for TMP) w/v % at the lower end of the calibrated range to 0.0077 (for SMZ) or 0.0125 (for TMP) w/v % at the upper end (see Figure 4). This relationship may be used to decide whether, for given nominal SMZ and TMP concentrations, it is acceptable for SMZ and TMP to be determined in the same HPLC sample or not.

Under the selected method conditions maximizing sensitivity for SMZ and TMP, note in Figure 4 that the uncertainty due to the preparation of standards and the construction of the calibration line is kept to a minimum in the calibration range 20−100 mg/L (4−20 w/v % for SMZ for a factor f = units factor/dilution factor = $10^4/2000 = 5$, and 1−5 w/v % for TMP for a factor $f = 10^4/500 = 20$). The dilution factors for SMZ and TMP were selected to provide a solution to measure a final concentration around the middle of the calibration range (~40−50 mg/L). With this in mind, and assuming homocedastic errors in y between 20 and 100 mg/L according to Figure 4, for the feed premix tested (with an SMZ/TMP ratio of 5) a factor $f = 10^4/1000 = 10$ could have been used for the simultaneous determination of

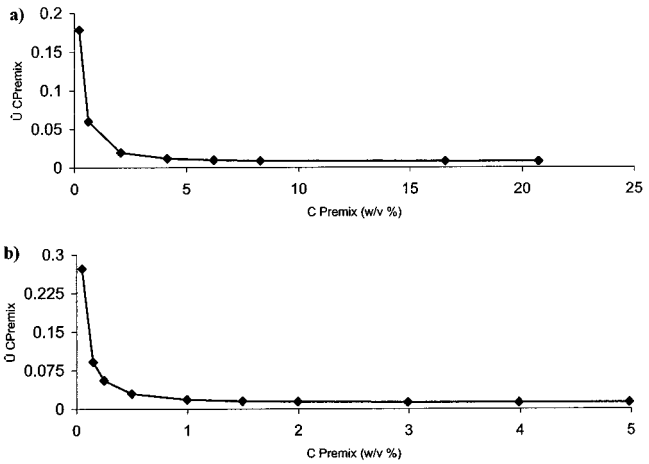

Figure 4. Graphs of $\hat{u}_{C\text{premix}}$ as a function of C_{premix} (eq 16b): (a) SMZ; (b) TMP.

SMZ and TMP to give a final concentration to measure 100 mg/L for SMZ and 20 mg/L for TMP, upper and lower ends of the suitable calibration range, respectively. For feed premixes with higher SMZ/TMP ratios, a common factor could also be used, but in this case it is necessary to reduce sensitivity to SMZ by selecting a wavelength for which it shows lower absorption (see Figure 1c).

4. CONCLUSIONS

The above HPLC method for the determination of SMZ and TMP in veterinary liquid feed premixes is reliable and fast, uses only inexpensive solvents, suffers no interference from the matrix, and allows simultaneous determination of SMZ and TMP if their concentrations in the matrix are sufficiently similar. The use of diode array detection allows confirmation of the identities of the analytes. For the typical SMZ and TMP concentrations determined in this work (approximately 10 and 2 w/v %, respectively) the expanded uncertainties of their separate determinations are estimated as 0.2 and 0.1 w/v %, respectively. The detailed analysis of uncertainties carried out in section 3.3 should aid decisions as to whether it is acceptable to determine the two drugs simultaneously and orient any uncertainty-reducing modifications that might be called for in particular laboratory circumstances.

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