DRUGS, COSMETICS, FORENSIC SCIENCES

Simultaneous Determination of Chloramphenicol, Florfenicol, and Thiamphenicol Residues in Milk by Gas Chromatography with Electron Capture Detection

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A gas chromatographic (GC) method is described for determining residues of chloramphenicol (CAP), florfenicol (FF), and thiamphenicol (TAP) in raw milk, with *meta*-nitrochloramphenicol (mCAP) as internal standard. Milk is extracted with acetonitrile, centrifuged, evaporated, reconstituted in water, and passed through a C_{18} solid-phase extraction (SPE) column. The SPE column is eluted with 60% methanol, and then the eluate is evaporated and derivatized with Sylon BFT {N,O-*bis*(trimethylsilyl)trifluoroacetamide [BSTFA]–trimethylchlorosilane [TMCS], 99 + 1}. After derivatization, toluene is added directly to the sample, followed by water, to quench the derivatization process. After centrifugation, the organic layer is carefully removed. Analytes are determined by GC with electron capture detection (ECD). Milk was fortified with fenicols (the collective name for CAP, FF, and TAP) at 5, 10, 20, 40 and 80 ng/mL (target level = 10 ng/mL). Overall recoveries were 92, 100, and 104% for CAP, FF, and TAP, respectively. Overall interassay (between-day) variabilities were 6.1, 6.7, and 6.0% for CAP, FF, and TAP, respectively. Raw milk samples containing incurred residues of FF were also analyzed.

Chloramphenicol (CAP) is a broad-spectrum antibiotic that was developed around 1950. It has effective antibacterial properties, as well as serious toxic effects on humans (1). More recently, thiamphenicol (TAP) and florfenicol (FF; Figure 1) have been developed and found also to be effective antibacterials. Despite being a structural analogue of TAP, FF has a wider spectrum of activity and greater potency than TAP (2). The U.S. Food and Drug Administration (FDA) approved the use of FF for treatment of bovine respiratory disease in late 1996 (3). Although none of the fenicols (the collective name for CAP, FF, and TAP) is approved for use in lactating cattle, the possibility exists that one or more may be used to treat a variety of bovine diseases of lactating cattle, resulting in drug residues in milk.

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The UV response factor of each fenicol varies, making it difficult to detect all 3 analytes at similar concentrations by liquid chromatography (LC) with UV detection when only one wavelength is monitored. The only method for simultaneous determination of all 3 fenicols by LC applies only to bovine, swine, poultry, and fish muscle (4). The method (4) detects analytes at 2 wavelengths (225 nm and 270 nm) because of differences in their UV characteristics. The only other method found for all 3 analytes was gas chromatography (GC)/mass spectrometry with selected ion monitoring applied to samples of fortified yellowtail fish muscle (5).

Unlike most methods for fenicols in milk, which use LC detection, the method reported here is practical for simultaneous determination of all 3 analytes. With GC with electron capture detection (ECD), the analytes give essentially similar response factors, thus allowing simultaneous determination. Method detection limits (MDLs) are 1.0, 1.5, and 2.1 ppb for CAP, FF, and TAP, respectively. The limit of quantitation (LOQ) as determined empirically by this method is the lower limit of the standard curve, approximately 5 ppb for each analyte.

METHOD

Apparatus

(**a**) *Pasteur pipet.*—Disposable, glass, 5.75 and 9 in.

(**b**) *Centrifuge tube.*—15 mL, glass, graduated with Teflon stopper No. 13 (Cat. No. 45153-A, Kimble) or equivalent.

(**c**) *Centrifuge tube.*—50 and 225 mL, Falcon Blue Max, disposable, conical, graduated, polypropylene (P/P) with cap (Cat. No. 2070 and 2075, respectively, Becton-Dickinson, Lincoln Park, NJ) or equivalent.

(**d**) *Flasks.*—Pear-shaped (P-S); 50, 100, and 300 mL; 24/40 (Cat. No. 608700-0124, -0224, and -054, respectively, Kontes Co., Vineland, NJ), or equivalent.

(**e**) *C_{18} solid-phase extraction (SPE) columns.*—Disposable, 3 cc/500 mg, Bond Elut (P/N 1210-2028, Varian, Harbor City, CA).

(**f**) *Nitrogen evaporator (N-evap).*—12-sample nitrogen evaporator, 45°C water bath (P/N 11155 Organomation Associates, Inc., Berlin, MA) or equivalent.

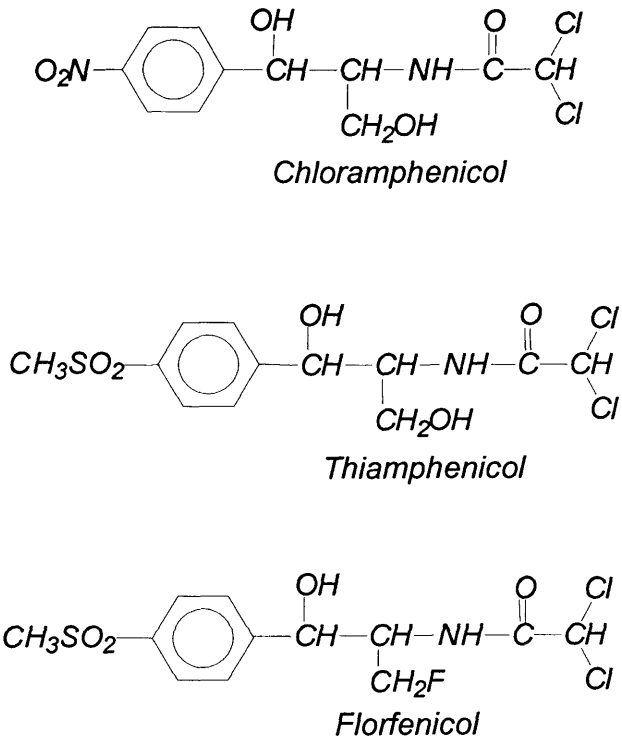

Figure 1. Chemical structures of fenicols.

(**g**) *Rotary evaporator (Rotavapor).*—Buchi R-110 with ice trap, water bath set at 45°C (Brinkmann Instruments, Inc., Westbury, NY), or equivalent.

(**h**) *Centrifuge.*—Model 6000 with Model 823A rotor and Model 259 armature with Model 384 cups (International Equipment Co., Needham Heights, MA) or equivalent.

(**i**) *Pipettor.*—Adjustable 5 mL (No. 851350, Wheaton Manufacturers, Millville, NJ); microadjustable 20–200 µL and 200–1000 µL (V200TE and V1000TE, respectively, Ulster Scientific, Inc., Baxter Co., McGaw Park, IL) or equivalent.

(**j**) *Mixer.*—Vortex-Genie (Model S8223, Baxter Co.) or equivalent.

(**k**) *Heating block.*—Thermolyne Dri-Bath Model DB28125 set at 45°C (Barnstead/Thermolyne, Dubuque, IA) or equivalent.

(**l**) *Gas chromatograph.*—Hewlett-Packard Model 5890 with splitless injector equipped with ^{63}Ni electron capture detector. Injection volume, 3 µL; zone temperatures: inlet, 250°C, detector, 320°C; oven program: equilibration time, 0.5 min; oven initial temperature, 150°C; initial time, 0.5 min; ramp rate 30°C/min; final temperature, 270°C; final time, 7.5 min (Hewlett-Packard Co., Avondale, PA) or equivalent.

(**m**) *Column.*—HP-5 fused-silica capillary column, cross-linked 5% phenyl methyl silicone, 0.33 µm film thickness, 25 m × 0.2 mm id (Hewlett-Packard); carrier gas, He, ca 0.7 mL/min (28.5 cm/s at 150°C) or equivalent.

Reagents

(**a**) *Solvents.*—Distilled-in-glass, pesticide grade, non-spectrophotometric acetonitrile (ACN), isopropyl alcohol (IPA), methanol (MeOH), toluene (Burdick & Jackson Labs, Inc., Muskegon, MI) or equivalent.

(**b**) *Derivatizing agent.*—Sylon BFT {N,O-*bis*(trimethylsilyl)trifluoroacetamide [BSTFA]–trimethylchlorosilane [TMCS], 99 + 1} 1 mL ampules (Cat. No. 3-3148, Supelco, Inc.), or equivalent. If a larger container (25 mL) is purchased, it must be kept tightly sealed and stored in a desiccator.

(**c**) *Standards.*—U.S.P. chloramphenicol (U.S. Pharmacopeia, Rockville, MD), thiamphenicol (Sigma Chemical Co., St. Louis, MO) and florfenicol (graciously supplied by Schering-Plough, Kenilworth, NJ).

(**d**) *Internal standard.*—*meta*-Nitrochloramphenicol (mCAP; 6).

Standard Preparation

(**a**) *Internal standard.*—(*1*) *Stock solution (0.5 mg/mL).*—Weigh ca 5 mg mCAP into 10.0 mL volumetric flask and dilute to volume with ACN. (*2*) *Internal standard curve solution (I_{std} curve soln; 25 µg/mL).*—Pipet 0.50 mL stock solution into 10.0 mL volumetric flask and dilute to volume with ACN. (*3*) *Working solution (I_{std}; 1250 ng/mL).*—Pipet 0.50 mL I_{std} curve soln into 10.0 mL volumetric flask and dilute to volume with ACN.

(**b**) *Standards.*—(*1*) *Individual stock solutions (1 mg/mL).*—Accurately weigh ca 10 mg each fenicol (CAP, FF, and TAP) into individual 10.0 mL volumetric flasks and dilute to volume with ACN. (*2*) *Combined stock solution (0.1 mg/mL).*—Pipet 1.00 mL each individual stock solution into 10.0 mL volumetric flask and dilute to volume with ACN. (*3*) *Intermediate solution (5000 ng/mL).*—Pipet 5.00 mL combined stock solution into 100.0 mL volumetric flask and dilute to volume with ACN.

(**c**) *Standard curve solutions.*—(*1*) *4000 ng/mL standard (80 ppb).*—Pipet 8.00 mL standard intermediate solution and 0.50 mL I_{std} curve soln into 10.0 mL volumetric flask and dilute to volume with ACN. (*2*) *2000 ng/mL standard (40 ppb).*—Pipet 4.00 mL standard intermediate solution and 0.50 mL I_{std} curve soln into 10.0 mL volumetric flask and dilute to volume with ACN. (*3*) *1000 ng/mL standard (20 ppb).*—Pipet 2.00 mL standard intermediate solution and 0.50 mL I_{std} curve soln into 10.0 mL volumetric flask and dilute to volume with ACN. (*4*) *500 ng/mL standard (10 ppb).*—Pipet 1.00 mL standard intermediate solution and 0.50 mL I_{std} curve soln into 10.0 mL volumetric flask and dilute to volume with ACN. (*5*) *250 ng/mL standard (5 ppb).*—Pipet 0.50 mL standard intermediate solution and 0.50 mL I_{std} curve soln into 10.0 mL volumetric flask and dilute to volume with ACN. *Note*: all standards take into account the 5 mL sample size, and 100 µL of each standard curve preparation (including I_{std}) pipeted into an extracted milk matrix. For example, 4000 ng/mL × 0.100 mL – 5 mL = 80 ng/mL = 80 ppb (*see Standard Derivatization*).

(**d**) *System suitability.*—(*1*) *Resolution.*—The resolution (R) between any 2 peaks should be baseline ($R \geq 2$). (*2*) *Calculations.*—All calculations should be based on peak height ratio (PHR) between analyte(s) and I_{std} (or peak area ratio [PAR] if PHR is not possible). (*3*) *Relative standard deviation (RSD).*—The RSD of 5 replicate injections of a standard must be ≤4%. (*4*) *Correlation coefficient.*—The correlation coefficient (r) of a 5-point standard curve must be 0.995 or better for the PHR of

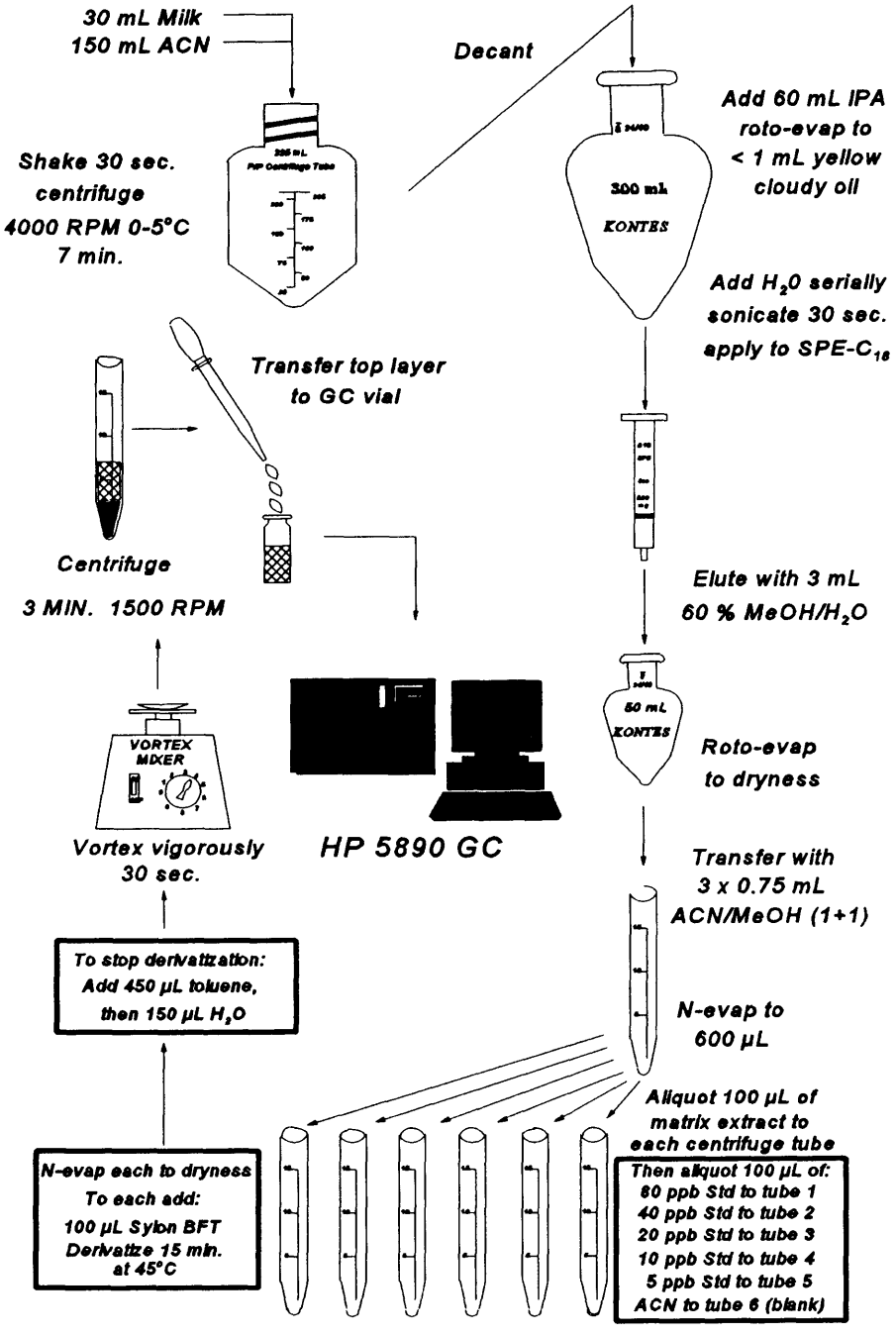

Figure 2. Procedures for preparing milk extract and standard curve.

each analyte being tested. (5) *System stability.*—After a series of samples is chromatographed, a standard should be reinjected that is of a similar concentration to the samples. If the results of the PHRs of the 2 standards vary by more than 10%, the entire series must be rechromatographed after the column is equilibrated by injecting 2 or more 3 µL portions of toluene through the gas chromatograph, ramping the oven temperature to 290°C, and holding that temperature for 30 min.

Milk Extract for Standard Curve Preparation

Figure 2 is a schematic representation of the procedures for preparing the milk extract and the standard curve. Measure 30 mL raw, control milk in a 225 mL P/P disposable centrifuge tube, add 150 mL ACN, shake well for 30–40 s, and centrifuge at 0°–5°C for ca 7 min at 4000 rpm. Decant liquid into a 300 mL P-S flask, add 60 mL IPA to prevent foaming, and evaporate to near dryness (≤1 mL of a yellow, cloudy oil). Add 6 mL H_2O and sonicate for ca 30 s while swirling the contents of the flask. Apply this mixture with a 9 in. Pasteur pipet to a prepared (3 mL MeOH, then 3 mL H_2O) C_{18} SPE column installed on a vacuum manifold (ca –0.6 bar or 1 drop/s) using reservoir if desired. Wash flask twice more with 3 mL H_2O, each time with swirling and sonicating, and apply flask contents to the SPE column. After all of the liquid has run through,

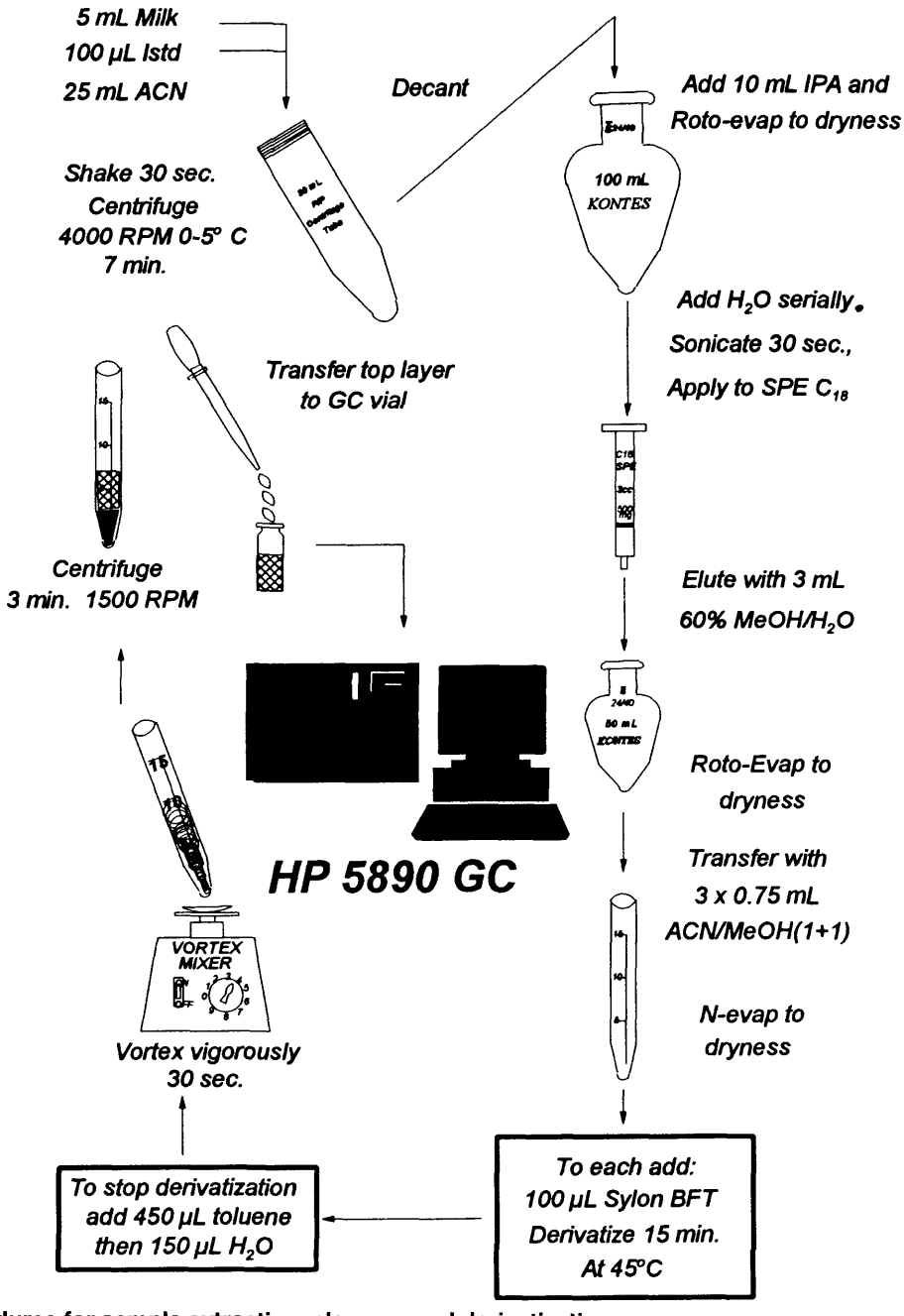

Figure 3. Procedures for sample extraction, cleanup, and derivatization.

allow column to remain under vacuum for ca 1 min. Discard all liquid. Elute the column (to dryness) with 3 mL MeOH–H_2O (60 + 40) into a 50 mL P-S flask. Take this eluant to dryness on a rotary evaporator. Transfer dried extract to a 15 mL tapered centrifuge tube with 3 × 0.75 mL MeOH–ACN (50 + 50). Reduce volume of extract to 600 µL MeOH by using an N-evap. Store unused extract in a freezer at ≤0°C.

Standard Derivatization

Add 100 µL portions of raw milk extract from above into individual 15 mL tapered centrifuge tubes, pipet individual standard mixture (100 µL) into the centrifuge tube, and take these aliquots to dryness by using an N-evap. Add 100 µL derivatizing agent (Sylon BFT), agitate on a Vortex mixer for ca 15 s, stopper the tube, and place the tube in a 45°C heating block for 15 min, agitating once more on a Vortex mixer after ca 8–10 min. Remove tube from heat, and add 450 µL toluene followed quickly by 150 µL H_2O. Agitate on a Vortex mixer vigorously for 30 s to quench derivatization. Centrifuge for 3 min at 1500 rpm. *Note*: It is more important to add the toluene and water quickly than carefully. Too much time exposed to the water will reduce recoveries. Use a Teflon stopper to avoid breaking glass centrifuge tube. Carefully remove the top layer with a Pasteur pipet and place in an autosampler vial for injection on

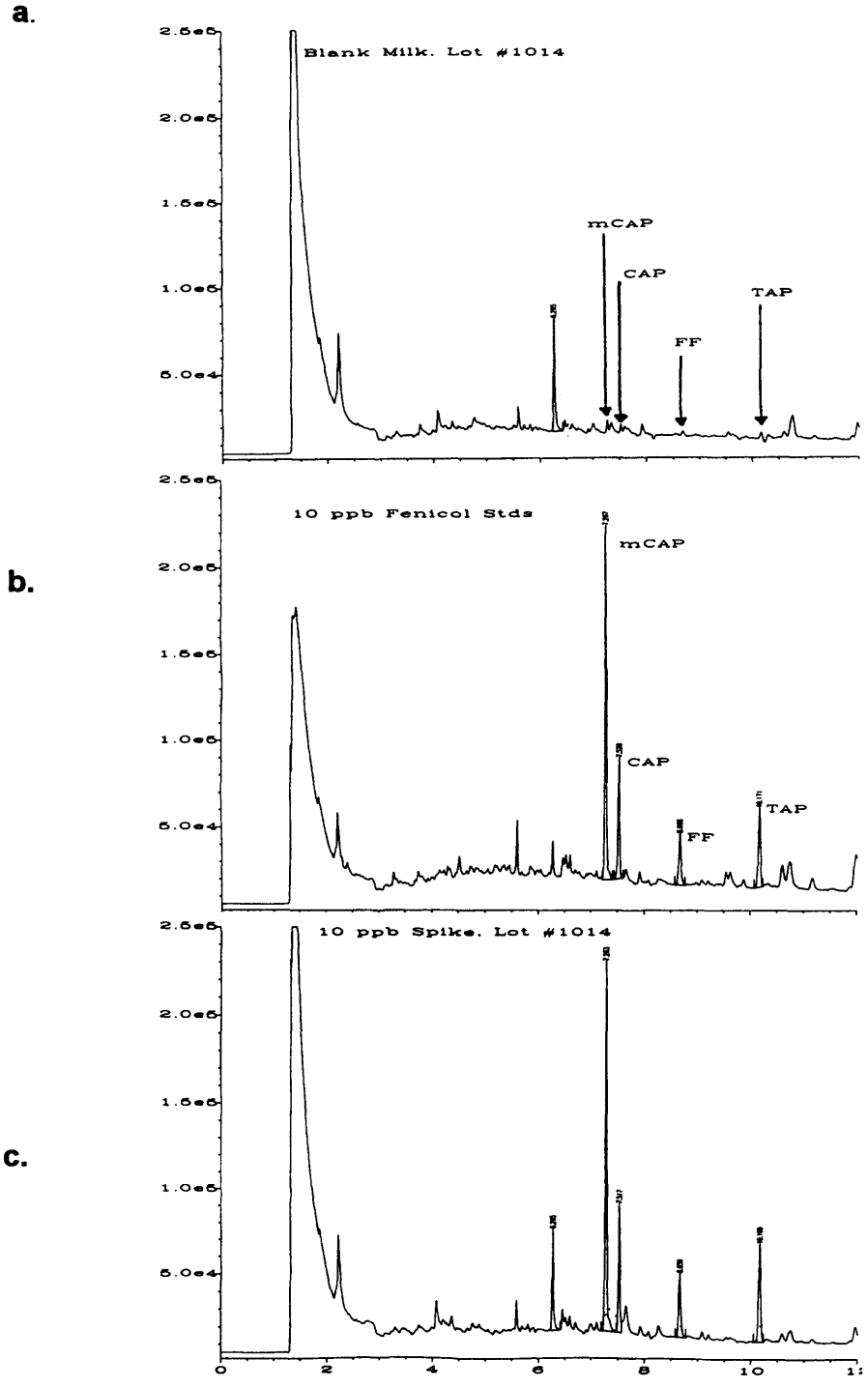

Figure 4. Representative chromatograms: (a) milk blank, (b) 10 ppb fenicol standard, (c) milk sample spiked at 10 ppb.

the gas chromatograph. (Small-volume glass injection vials may be used if it is anticipated that the standards will be used repeatedly.) Sealed derivatized standards will remain viable for at least 10 days if stored in freezer at ≤0°C.

Sample Extraction and Cleanup

Figure 3 is a schematic representation of the procedures for sample extraction, cleanup, and derivatization. Pipet 5.0 mL raw milk into a 50 mL P/P centrifuge tube. If spiking control milk, add the 4 standards (100 µL of desired standard curve preparation solution) and allow to sit at room temperature for 5 min before proceeding. If preparing sample, add 100 µL working solution I_{std} at this time. Add 25 mL ACN, shake well for 30 s, and centrifuge at 0°–5°C for 7 min at 4000 rpm. Decant into 100 mL P-S flask, add 10 mL IPA to prevent foaming, and evaporate to dryness with a rotary evaporator. Add 4 mL H_2O to flask and sonicate for ca 30 s while swirling contents. Apply this mixture to a prepared (3 mL MeOH, then 3 mL

H_2O) C_{18} SPE column installed on a vacuum manifold. Wash the flask twice more with 2 mL H_2O, with swirling and sonicating, and then apply flask contents to the SPE column. After all of the liquid has run through, allow column to remain under vacuum for ca 1 min. Discard all liquid. Elute column to dryness with 3 mL MeOH–H_2O (60 + 40). Collect eluate in a 50 mL P-S flask and dry with a rotary evaporator. Transfer residue to 15 mL centrifuge tube with 3 × 0.75 mL MeOH–ACN (50 + 50) and Pasteur pipet and take to dryness on N-evap. The residue should appear to be a yellow powder at this point. Derivatize from this point as described in *Standard Derivatization*. If derivatized sample is not injected immediately, place in a freezer at ≤0°C for long-term storage. Perform calculations from a 5-point standard curve (*see System Suitability* and *Calculations*).

Calculations

A 5-point standard curve (PHR of analyte to internal standard) is used to calculate amount of each fenicol present in a sample. From calculated linear regression, determine the amount as follows:

$$\text{Amount of fenicol, ng} = \frac{Y - \text{b}}{\text{m}}\, df$$

where Y is PHR, m is slope, b is intercept, and df is dilution factor. To obtain amount in parts per billion, convert as follows:

$$\text{Amount of fenicol, ppb} = \frac{X}{V}$$

where X is amount in nanograms and V is sample volume (mL). *See* Table 1 for an example of standard curve data.

Method Detection Limit

As described by Glaser et al. (7), "The MDL can be presented as an error distribution. The definition of MDL implies that, on average, 99% of the trials measuring the analyte concentration at the MDL must be significantly different from zero analyte concentration. The MDL refers to samples processed through all the steps comprising an established analytical procedure. The fundamental difference between our approach to detection limit and former efforts is the emphasis on the operational characteristics of the definition. MDL is considered operationally meaningful only when the method is truly in the detection mode, i.e., analyte must be present. The method detection limit is defined as the minimum concentration of a substance that can be identified."

Two sets of 7 portions of spiked samples at the X level (level of interest) and $\frac{1}{2}X$ level are analyzed by the entire procedure. The following formula therefore applies:

$$\text{MDL} = 2.681 \times S_{\text{pooled}}$$

where 2.681 is equal to $t_{(12,1-\alpha=0.99)}$ [a statistical value based on the total number of degrees of freedom 2(7 – 1) = 12, which is from the table of analyst's values at the 99% confidence level], and $S_{\text{pooled}} = [(S_A2 + S_B2)/2]^{1/2}$. Here, S_A = standard deviation of each analyte at $1/2X$ (N = 7) and S_B = standard deviation of each analyte at X (N = 7).

Table 1. Example 5-point standard curve data[a]

Analyte	Slope (standard error)	Y intercept (standard error)	Correlation coefficient (r)
CAP	0.026486 (0.000494)	0.078548 (0.0208)	0.9995
FF	0.015196 (0.000275)	0.009072 (0.0115)	0.9995
TAP	0.021069 (0.000351)	0.027453 (0.0154)	0.9996

[a] Range, 5–80 ppb; calculations are for mixed 20 and 80 ppb set.

The calculated MDL values are 1.0, 1.5, and 2.1 ppb for CAP, FF, and TAP, respectively.

Results and Discussion

When we decided to pursue this method, we set several criteria: All 3 analytes had to be detected and quantitated at the low ppb levels, the method had to be rapid, and solvent use should be minimal. The first criterion determined that GC would be the means of detection because LC responses varied considerably from one analyte to another. The other 2 criteria steered the method away from traditional extraction and cleanup steps normally encountered with fenicols, namely, ethyl acetate extraction and hexane defatting (4, 5, 8–11).

A method developed in this laboratory several years ago to determine thionin in milk (12) used an extraction with ACN to produce a remarkably clean liquid, free of milk solids, and with very little fat. The major drawback to that method was that the ACN extract foamed so much that rotary evaporation was not possible. It was found during development of the present fenicol method that addition of IPA reduced foaming to the point that rotary evaporation was possible, as well as reduced evaporation time by 2 h.

Use of a C_{18} SPE column was an effective cleanup for the present method. The minimum concentration of MeOH in H_2O required to elute all of the fenicols from the SPE column was determined through experimentation to be 60%. At this MeOH concentration, some contaminants remain on the SPE column, whereas at 100%, MeOH elutes most of the contaminants with the analytes. The apparent extra step of using the rotary evaporator to dry the SPE eluate was purposefully added to reduce drying time by over 1 h, compared with drying time when using a nitrogen evaporator in conjunction with anhydrous ethanol to evaporate the 40% H_2O present in the eluate.

One problem encountered in developing a GC method was the difficulty in derivatizing FF. With the derivatization procedure described by Munns et al. (8), the resulting FF derivative yields 4 distinct peaks. After experimenting with a number of derivatizing agents, the one used in this method proved to be the most efficient for all 3 analytes and the internal standard, creating only one major peak for each analyte. A time and temperature study was performed to determine optimum conditions for derivatization. Although many temperatures would work, the most reproducible results were obtained at 45°C. Derivatization appears to be complete after about 5 min, but 15 min are allowed to ensure completion of the reaction in all milk

Table 2. Recoveries of fenicols in spiked milk samples

Spike level, ng/mL	Fenicol	*n*	Mean recovery, %	Relative standard deviation, %
5.10[a]	CAP	7	76.4	10.9
5.06[a]	FF	7	99.5	11.4
5.33[a]	TAP	7	90.4	10.4
10.2[b]	CAP	7	96.1	3.2
10.1[b]	FF	7	104	5.2
10.7[b]	TAP	7	112	8.2
20.4[b]	CAP	5	99.8	6.2
20.2[b]	FF	5	96.7	7.9
21.3[b]	TAP	5	110	2.2
40.8[c]	CAP	5	96.3	3.7
40.5[c]	FF	5	98.5	5.3
42.6[c]	TAP	5	101	1.6
81.6[b]	CAP	5	91.9	1.8
81.0[b]	FF	5	102	4.5
85.3[b]	TAP	5	107	6.0

[a] Three sources of control milk, analyzed on 3 separate days.
[b] Two sources of control milk, analyzed on 2 separate days.
[c] Two sources of control milk, analyzed in 1 day.

matrixes. It was also discovered that better results were obtained when standards were prepared in extracted milk residues.

The method originally called for drying the derivatizing agent before adding toluene and injecting, but results were variable, especially the FF and TAP responses. It was observed that adding only toluene also resulted in variable responses for the same 2 analytes. It appeared that the derivatizing agent or the matrix may be reacting with the analyte(s) in the GC injector because of the elevated temperature there. Addition of a small quantity of water quenches the derivatizing agent while reducing the size of the solvent front on the chromatogram and providing a final cleanup step to the procedure by partitioning contaminants into the aqueous phase. The organic phase in the centrifuge tube is clear and nearly colorless, while the aqueous phase is a cloudy yellow; the baseline of the chromatogram is also much quieter.

Standards prepared in matrix produced less variable chromatograms and better linear standard curves than those prepared neat. They also seem to cancel any matrix effect from recoveries. During method development, a new set of standards was prepared for each sample set, but this was time and resource consuming. We found that standards and samples remained viable for a minimum of 10 days if kept in a freezer at ≤0°C in well-sealed injector vials.

Standard curve data for a typical set of standards used to calculate a sample set are presented in Table 1. Calculations of this table and all recoveries are based on PHR between analytes and internal standard. Table 2 is a summary of recoveries from samples spiked at 5 levels: 5, 10, 20, 40, and 80 ppb. All recoveries are calculated from the standard curve. The 80, 20, and 10 ppb sets were performed on 2 different days with 2 different lots of milk. The 40 ppb set was performed in one day with 2 different lots of milk. The 5 ppb set was performed over

Table 3. Analysis of florfenicol in incurred milk samples

Time after dosing, h	Florfenicol detected, ppb			CVM results, ppb
	1	2	Average	
56	139	138	138[a]	77.7
72	41.6	42.0	41.8	22.5
96	9.1	8.9	9.0	6.89

[a] Value is an estimate because the level detected is outside the standard curve.

3 days with 3 different lots of milk. Table 3 is a summary of the results of the analysis of 3 sets of FF-incurred milk samples received from the Center for Veterinary Medicine (CVM) of the FDA. The milk was collected at 56, 72, and 96 h after dosing of the animal. The CVM results are from a single analyte LC method. Samples were frozen for nearly 9 months before they were analyzed by the GC method. Because the samples have not recently been analyzed by the LC method, a direct comparison of the 2 methods may not be accurate. Figure 4 contains 3 representative chromatograms: a milk blank, a 10 ppb standard, and a 10 ppb spiked milk sample.

Conclusion

The method described is very simple and rapid and requires very small quantities of solvents. An experienced analyst can perform a set of standards and 8 samples in 2 days or less and a set of samples each day thereafter. All equipment required is readily available in most analytical chemistry laboratories.

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