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A Simple and Sensitive Liquid Chromatography−Mass Spectrometry Confirmatory Method for Analyzing Sulfonamide Antibacterials in Milk and Egg

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A simple and specific method able to identify and quantify traces of 14 sulfonamide antibacterials (SAs) in milk and eggs is presented. This method uses a single solid-phase extraction (SPE) cartridge for simultaneous extraction and purification of SAs in the above matrices. Milk and egg samples are passed through a Carbograph 4 sorption cartridge. After analyte desorption, an aliquot of the final extract is injected into a liquid chromatography−mass spectrometry (LC-MS) instrument equipped with an electrospray ion source (ESI) and a single quadrupole. MS data acquisition is performed in the positive-ion mode and by a time-scheduled multiple-ion selected ion monitoring program. Compared to two published methods, the present protocol extracted larger amounts of SAs from both milk and egg and decreased the analysis time by a factor of 3 with milk samples and by a factor of 2 with egg samples. Recovery of SAs in milk at the 5 ppb level ranged between 76 and 112% with relative standard deviations (RSDs) of ⩽13%. Recovery of SAs in egg at the 50 ppb level ranged between 68 and 106% with RSDs of ≤12%. Estimated limits of quantification (S/N = 10) of the method were 1−6 ppb of SAs in whole milk and 5−13 ppb of SAs in eggs. Analyses of eggs from three layer chickens treated with sulfoquinoxaline revealed this antibacterial was still present at the 150 ppb level 1 week after withdrawal.

KEYWORDS: Sulfonamides; milk; egg; SPE extraction; LC-MS

INTRODUCTION

Sulfonamides (SAs) comprise a large number of synthetic bacteriostatic compounds. They act by competing with *p*-aminobenzoic acid in the enzymatic synthesis of dihydrofolic acid. This leads to a decreased availability of the reduced folates that are essential in the synthesis of nucleic acids. No fewer than 10 SAs are routinely used in veterinary medicine to treat a variety of bacterial and protozoan infections in cattle, swine, and poultry. Analysis of SAs in foodstuffs is of particular concern because of the potential carcinogenic character (*1*, *2*). To ensure the safety of food for consumers, Regulation 281/96 of the EU Commission has laid down maximum residue limits of 100 ppb of SAs as a total in milk. Although several SAs are approved for medicinal purposes in chickens, no SAs is approved for use in laying hens. Violative residues in eggs could result from giving SAs intended for broilers to laying hens.

Public health agencies in many countries rely on detection by mass spectrometry (MS) for unambiguous confirmation of xenobiotics in foodstuffs. EU Commission Decision 93/256/EEC states that "Methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods". Liquid chromatography (LC)−MS is thus the ideal technique to determine nonvolatile, polar compounds such as sulfonamide antibacterials.

At present, three LC-MS methods are quoted in the literature for determining SA residues in milk (*3*−*5*). One of these methods (*3*) is based on the use of the thermospray interface that is no longer commercially available. Doerge et al. (*4*) demonstrated the practicality of using a benchtop single-quadrupole LC-MS instrumentation for sensitive detection of SAs in milk. Protonated molecules were generated by an atmospheric pressure chemical ionization (APCI) ion source, whereas fragment (product) ions were obtained by in-source collision-induced dissociation (CID) reactions in the first part of the ion transmission region. Volmer (*5*) elaborated a method based on LC−tandem MS with an electrospray (ESI) ion source for detecting and quantifying 21 SAs in milk at levels of <1 ppb. A drawback of this method is that it is time-consuming, as one of the steps of the sample treatment involves evaporation of ∼15 mL of water.

Despite the fact that SAs are widely used for poultry, little attention has been given in the past to elaborate LC-MS confirmatory methods of these antibacterials in egg. Tarbin et al. (*6*) elaborated an LC-APCI-MS method for determining parts per billion levels of 16 SAs in whole egg. Identification and

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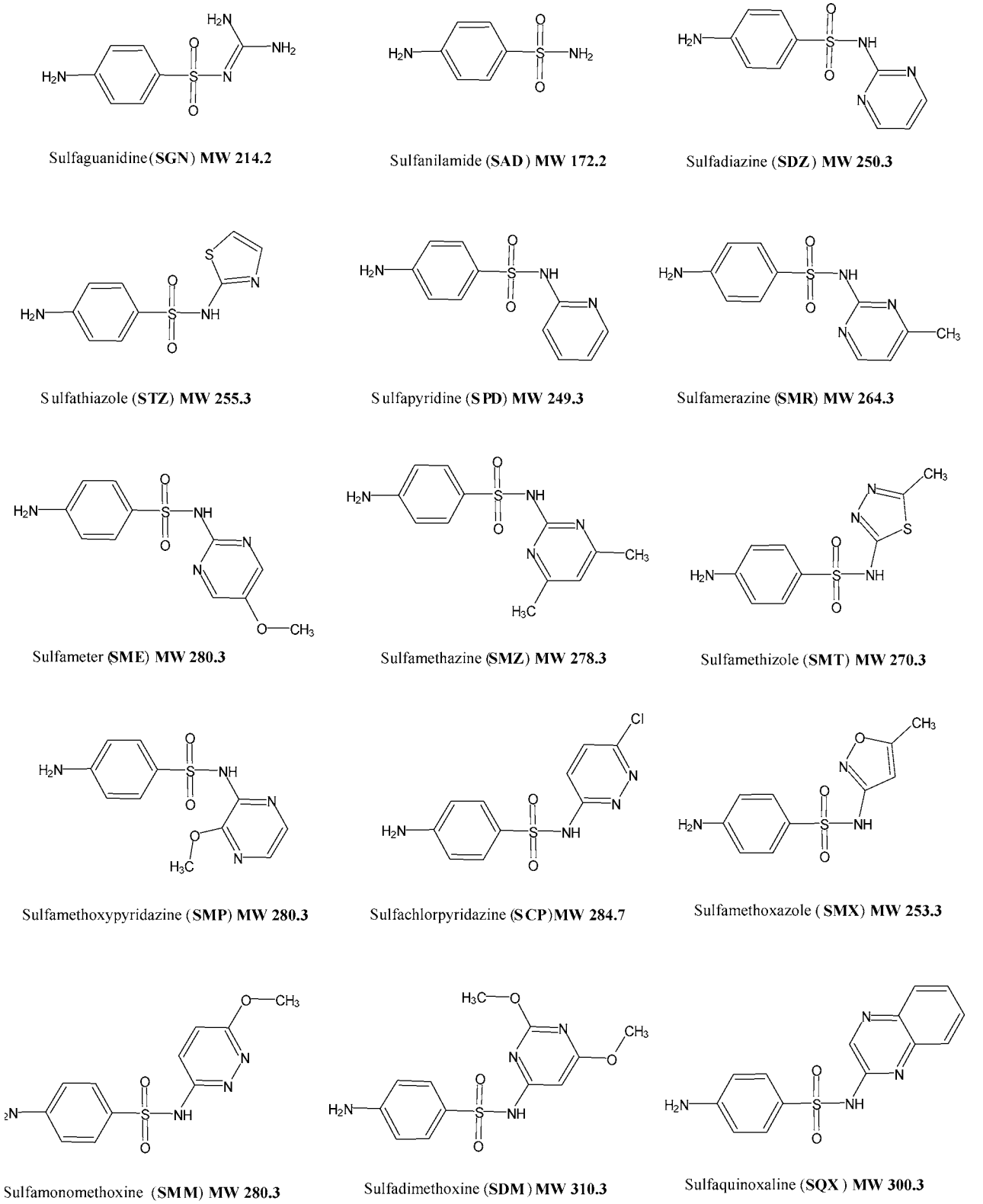

Figure 1. Chemical structures of sulfonamide antibacterials.

quantification of the analytes relied on MS acquisition of only the molecular ions in the selected ion monitoring (SIM) mode. Very recently, a work devoted to determining residues of SAs in eggs by LC-MS/MS with an ion trap and an ESI source appeared in the literature (*7*). A rather lengthy conventional sample treatment protocol, that is, deproteinization/extraction with acetonitrile followed by cleanup with a solid-phase extraction (SPE) cartridge, containing some critical steps is proposed by the authors.

Recently, we have elaborated two simple sensitive and relatively inexpensive LC-MS methods for analyzing residues of 10 β-lactam antibiotics in milk (*8*) and 4 commonly used tetracyclines in both milk and egg samples (*9*). These methods involve isolation of the analytes from intact biological matrices

by SPE with a Carbograph 4 cartridge and identification and quantification by LC-ESI-MS with a single quadrupole. Product ions are generated by in-source CID reactions.

The purpose of this work was to extend the above methodology to the analysis of residues of 14 widely used SAs (**Figure 1**) in milk and egg.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals. Sulfaguanidine (SGN), sulfanilamide (SAD), sulfadiazine (SDZ), sulfathiazole (STZ), sulfapyridine (SPD), sulfamerazine (SMR), sulfameter (SME), sulfamethizole (SMT), sulfamethazine (SMZ), sulfamethoxypyridazine (SMP), sulfachloropyridazine (SCP), sulfamethoxazole (SMX), sulfamonomethoxine (SMM), sulfadimethoxine (SDM), and sulfaquinoxaline (SQX) were obtained from Sigma-Aldrich, Milwaukee, WI. SME is not used in veterinary medicine and was adopted as internal standard (IS). We prepared 1 mg/mL stock solutions of each SA by dissolving 100 mg of the pure analytical standards in 100 mL of methanol. For recovery studies, a single working composite standard solution was prepared by combining aliquots of each of the 14 individual stock solutions and diluting with methanol to obtain a final concentration of 2 μg/mL. A 20 μg/mL solution of the IS was prepared in a similar way. While not in use, all of the above solutions were maintained at 4 °C.

Methanol "Plus" of gradient grade was obtained from Carlo Erba, Milano, Italy. Trifluoroacetic acid (TFA) was from Aldrich. All other solvents and chemicals were of analytical grade (Carlo Erba) and were used as supplied.

Apparatus. Extraction cartridges filled with 0.5 g of Carbograph 4 and drilled cylindrical Teflon pistons with indented bases and Luer tips for analyte elution in the back-flushing mode (*10*) were supplied by LARA, Rome, Italy. Carbograph 4 is an example of the graphitized carbon black sorbent family having a surface area of ~200 m^2/g. It is commercially referred to also as "Carboprep" (Restek, Bellefonte, PA). The SPE cartridge was fitted into a sidearm filtration flask, and liquids were forced to pass through the cartridge by vacuum (water pump). Before milk and egg samples were processed, the cartridge was washed with 20 mL of water acidified with HCl (pH 2) followed by 5 mL of distilled water.

Milk and Egg Samples. Pasteurized, homogenized whole milk and eggs were purchased from retail markets. Preliminary analyses showed they were analyte-free. Three ~3.5 kg layer chickens in mid-lay were purchased from a local supplier. The birds were individually housed in wire-floored cages and were allowed access to fresh water at all times from nipple drinkers. This acclimation period lasted for 1 week. Thereafter, following the directions of the manufacturer (Aviochina, Vetem, Agrigento, Italy), a solution containing 3.4% SQX was dissolved in the fresh water to give a 0.25 g/L final concentration of SQX. The birds drank this water for 2 days, and then the SQX administration was interrupted for 3 days; finally, the birds were again treated with SQX for other 2 days. After treatment and once daily (when possible), eggs were collected for 9 days, amended with 1000 ppb of SMM used as a surrogate internal standard, homogenized, and stored at −16 °C until analysis.

Sample Preparation. *Milk Samples.* Ten milliliters of each milk sample was spiked with known variable amounts of SAs. Under continuous agitation, 15 min was allowed for equilibration at room temperature. Then, the milk was diluted with 100 mL of distilled water. Again, 10 min was allowed for equilibration with stirring facilitated with the aid of a magnetic stirrer. Thereafter, this mixture was passed through the SPE cartridge at a flow rate of 20 mL/min. Washings of the cartridge and analyte back elution were carried out as described elsewhere (*8*), with the exception that 1.5 mL of methanol followed by 6 mL of a methylene chloride/methanol (80:20, v/v) solution acidified with 10 mmol/L TFA was used to re-extract SAs. Analytes were collected in a 1.4 cm i.d. glass vial with a conical bottom. Before solvents were removed in a water bath at 40 °C under a nitrogen stream, 40 μL of the solution containing the IS was added to the eluate. To avoid some analyte loss, solvent removal was stopped when the vial contained still ~100 μL of the eluate. After 700 μL of an ammonium acetate aqueous solution (0.1 mol/L) had been added, the extract was passed through a Teflon filter (pore size = 0.45 μm, 13 mm diameter, Alltech, Sedriano, Milan, Italy). After filtration, a completely uncolored and transparent solution was obtained. Twenty-five microliters of the final extract was then introduced into the LC analytical column.

Egg Samples. After homogenization, 2-g aliquots of whole egg were spiked with known and variable amounts of SAs. After 10 min of equilibration, the egg sample was diluted with 200 mL of distilled water. Again, 10 min was allowed for equilibration while the suspension was stirred with a magnetic stirrer. The sample was then forced to pass through a stack of two large-pore-size paper filters (black ribbon, 125 mm diameter, Scheicher & Schuell, Dassel, Germany) by the aid of vacuum (water pump). Note that this filtration step was necessary to avoid clogging of the SPE cartridge. After filtration, the same procedure as that for milk was followed, with the exception that no care was taken to control the flow rate at which the water-diluted egg sample passed through the SPE cartridge (~40 mL/min). With egg extracts, solvent removal was stopped when the vial contained still ~50 μL of the eluate. After 350 μL of a 0.1 mol/L ammonium acetate aqueous solution had been added, the extract was filtered as reported above. After filtration, a completely uncolored and transparent final extract was obtained. Twenty-five microliters of the final egg extract was then introduced into the LC analytical column.

SQX in incurred egg samples was analyzed as reported above with the exception that SMM was used as surrogate internal standard.

Method Comparison. In terms of recovery of the analytes and speed of analysis, the preparation procedures of the milk and egg samples described here were compared with two conventional procedures. These procedures are here briefly described. According to various authors (*3*, *5*), proteins in milk samples spiked with 100 ppb of SAs were precipitated by sample acidification, whereas lipids were removed with hexane. After water had been eliminated by evaporation, the residue was reconstituted with methanol that, after centrifugation, was removed by evaporation. Finally, the analytes were dissolved in water before LC-MS analysis. Following a recently reported procedure (*7*), SAs added to egg samples at a level of 100 ppb were extracted with acetonitrile. After dilution with water, the extract was purified by a C-18 SPE cartridge. The eluate (acetonitrile) was diluted with water and filtered, before LC-MS analysis.

LC-MS Analysis. LC was performed by a Thermoquest, Manchester, U.K., model P2000. Analytes were chromatographed on an Alltima 25 cm × 4.6 mm i.d. column filled with 5 μm C-18 reversed phase packing (Alltech). The column temperature was maintained at 35 °C. For fractionating the analytes, phase A was methanol and phase B was water. Both phases contained 5 mmol/L formic acid. The mobile phase gradient profile (where t refers to time in min) was as follows: t_0, A = 1%; t_8, A = 9%; t_{30}, A = 50%; t_{32}, A = 100%; t_{34}, A = 100%; t_{36}, A = 1%; t_{44}, A = 1%. By following conditions reported above, retention times of the analytes did not differ by >0.2% during a working day. The flow rate of the LC eluant was 1 mL/min, and 250 μL of the column effluent was diverted to the ESI source. A Finnigan AQA benchtop mass spectrometer (Thermoquest) consisting of a pneumatically assisted ES interface and a single quadrupole was used for detecting and quantifying target compounds in the LC column effluent. The probe temperature was 250 °C, and the capillary voltage was 4 kV. Nitrogen was used as drying and nebulizer gases at flow rates of 300 and 50 L/h, respectively. The ESI/MS system was operated in the positive ionization (PI) mode. For each analyte, diagnostic fragment ions were obtained by in-source CID of the protonated molecule $[M + H]^+$ by suitably adjusting the voltage of the skimmer cone. Unless otherwise specified, ion signals were acquired by the time-scheduled multiple-ion SIM mode (**Table 1**).

Quantification. Recovery of each analyte added to milk and egg samples at any given concentration was assessed by selecting the sum of the ion current profiles for both parent and fragment ions, measuring the peak area relative to that of the IS, and comparing this result with that obtained for a reference solution containing the same nominal analyte quantities and the internal standard. Reference solutions were prepared by dissolving known and appropriate volumes of the working standard solution in the eluent phase used for re-extracting analytes from the Carbograph 4 cartridge and then following the rest of the procedure reported above.

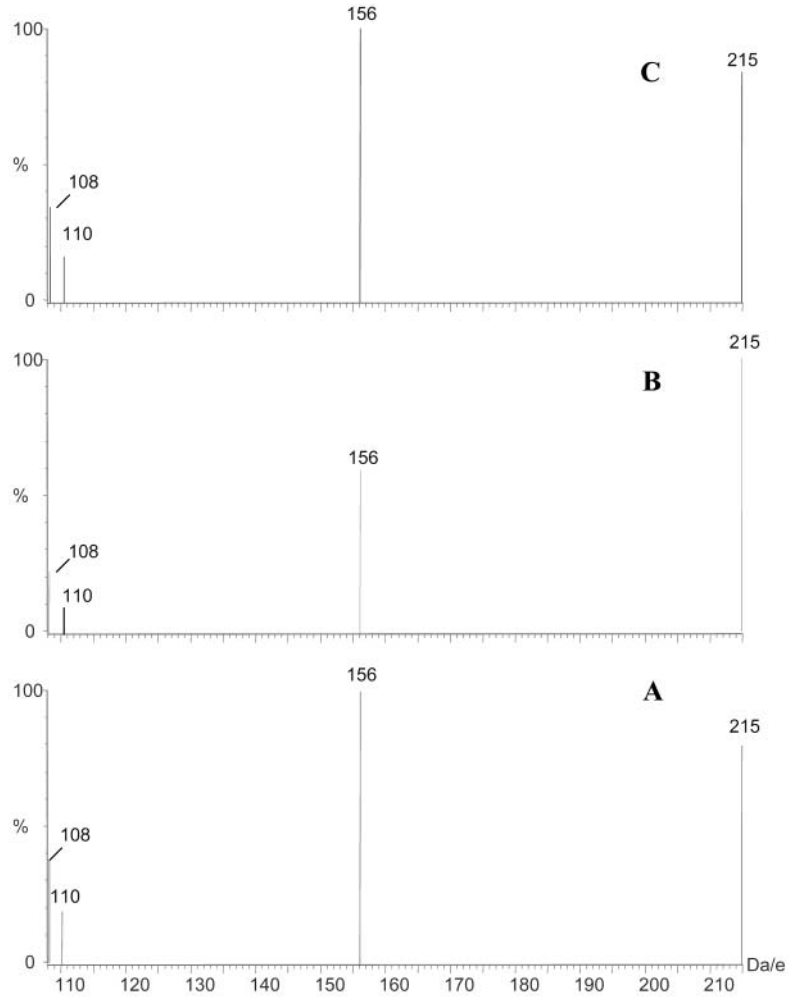

Figure 2. In-source CID spectra of sulfaguanidine resulting from injection of (A) a reference solution, (B) 6.2% of the final extract of milk, and (C) 3.1% of the final extract of milk.

Table 1. Time-Scheduled Multiple-Ion SIM Conditions for Detecting Sulfonamides in Milk and Egg

compound	channel,[a] *m/z* (relative abundance)	cone voltage, V	retention window, min
sulfaguanidine	108 (35), 156 (100), **215** (80)	35	0–13
sulfanilamide	108 (35), 156 (100), **173** (40)	35	
sulfadiazine	108 (35), 156 (50), **251** (100)	45	13–19
sulfathiazole	108 (70), 156 (100), **256** (90)	45	19–22.2
sulfapyridine	*95* (100), 108 (70), 156 (70), *184* (40), **250** (70)	45	
sulfamerazine	108 (55), 110 (100), 156 (60), **265** (60)	48	22.2–3.7
sulfameter (IS)	108 (60), *126* (65), 156 (70), **281** (100)	45	23.7–27.8
sulfamethizole	108 (70), 156 (100), **271** (50)	45	
sulfamethazine	108 (60), *124* (100), 156 (50), **279** (55)	45	
sulfamethoxypyridazine	108 (70), *126* (70), 156 (100), **281** (60)	45	
sulfachloropyridazine	108 (55), 156 (100), **285** (40)	45	27.8–33
sulfamethoxazole	108 (65), 156 (100), **254** (70)	45	
sulfamonomethoxine	108 (60), *126* (55), 156 (80), **281** (100)	45	
sulfadimethoxine	108 (35), 156 (100), **311** (50)	48	33–41
sulfaquinoxaline	108 (55), 156 (100), **301** (70)	48	

[a] Compound-specific product ions and molecular ions are reported, respectively, in italic and boldface type.

Analysis of SQX in incurred egg samples was performed by using SMM as surrogate internal standard. After estimation of the SQX molar response factor relative to that of SMM, quantification of SQX was carried out by comparing its peak area to that of the surrogate.

The MS data acquisition and processing system used was the "Mass Lab" software from Thermoquest.

RESULTS AND DISCUSSION

Optimization of the MS Conditions. The ESI process produces very simple ESI mass spectra displaying signals for protonated molecules and low-abundant sodium adduct ions. However, it is generally accepted that sufficient confirmatory evidence for the presence of a target compound by the LC-MS technique is obtained when, in addition to other less stringent conditions, the spectrum displays the molecular ion plus at least two characteristic fragment ions. When using LC-ESI-MS equipped with a single quadrupole, in-source fragmentation of protonated molecules can be induced by raising the sample cone voltage (*4*). The effect of varying the sample cone voltage in the 20−50 V range on the production of fragment ions was investigated. At any selected cone voltage, a full-scan mass chromatogram was generated by injecting 200 ng of each SA from a composite standard solution into the LC column, under chromatographic conditions reported in Experimental Procedures. The quadrupole was scanned over the *m/z* 90−360 range in 2 s. SAs differ only in the heterocyclic base attached to a sulfonamide moiety. The observed fragmentation produced, in addition to class-specific fragment ions, compound-specific fragment ions for several SAs. Similarly to the CID process obtained by tandem MS (*5*), in-source fragmentation of all SAs produced generic fragment ions at *m/z* 92, 108, and 156. Postulated structures of these ions have been already reported elsewhere (*5*). In addition, all compound-specific product ions obtained for several SAs by the conventional CID process (*5*)

were also generated by the in-source one. Unlike CID spectra of SAs, in-source CID spectra of all SAs displayed rather weak signals for the ion at *m*/*z* 92 at any sample cone considered. Thus, this ion was not considered in this study for detecting and quantifying SAs. Another difference observed between conventional CID spectra of SAs (*5*) and the in-source CID spectra obtained by us was that the latter ones displayed a fairly abundant class-specific signal ion at *m*/*z* 110, which was tentatively assigned to protonated *p*-aminophenol. However, acquisition of this ion was disturbed by a high noise level, and it was used for monitoring only SMR. In this case, the ion at *m*/*z* 110 comes also from specific formation of protonated 2-amino-5-methylpyrimidine that, at the cone voltage of 48 V, was the base peak in the SMR spectrum.

According to legal criteria, the presence of a contaminant in a given matrix is demonstrated if, compared with a reference standard analyzed under the same instrumental conditions, the absolute relative abundances of the ion signals agree within 20%. In a previous work (*11*) aimed at monitoring penicillins in environmental waters, we observed that, compared to in-source CID spectra of penicillins injected from a standard solution, those of penicillins injected from a river water extract displayed significantly less abundance of the product ions. This difference was more accentuated for the early-eluted penicillins and increased by injecting increasing volumes of the final extract. This effect was traced to an anomalous behavior of the in-source CID process occurring when analytes enter the ESI source together with large amounts of matrix components. Probably, coextractive molecular ions interfered with the in-source CID process by acting as a shield, partially hindering collision of molecular ions of the analytes with nitrogen molecules. This unwelcome effect was also observed in this work for the earliest eluted SAs, that is, SGN, SAD, SDZ, and STZ, when the aliquot of the milk final extract injected into the LC column was doubled (see Experimental Procedures). Exemplary CID spectra of SGN obtained by injecting a standard solution and two different aliquots of a milk final extract are shown in **Figure 2**. Except for SGN and SAD, this particular matrix effect could be attenuated also by slowing the gradient elution program in the first part of the chromatographic run.

Recovery Studies. The effect of the flow rate at which water-diluted milk and egg samples passed through the Carbograph 4 cartridge on SA extraction efficiency was briefly investigated. Although the flow rate did not affect recovery of SAs in egg, progressive losses of the analytes in milk were experienced as the flow rate was gradually increased from 20 to 60 mL/min. The lost fractions of SAs were found in the sample effluents. If some analyte−protein binding persists even after abundant water dilution, analyte loss occurring at elevated milk sample flow rates could be then explained by the fact that insufficient time is allowed for the analytes to migrate from proteins to the sorbent surface.

With a view to developing a method able to determine SA trace levels in milk, increasing milk volumes spiked with 50 ppb of each analyte were carried through the procedure reported under Experimental Procedures. At each milk volume considered, duplicate measurements were performed, and results for some selected SAs are shown in **Figure 3**. At sample volumes >10 mL, a remarkable decrease of the recovery of some SAs, such as SQX, SCP, and SMZ, was already observed, whereas loss of the other analytes occurred when milk volumes >20 mL were analyzed. For only a few SAs was recovery not affected by the milk volume processed. This result was rather surprising to us, considering that SAs dissolved in 2 L of water

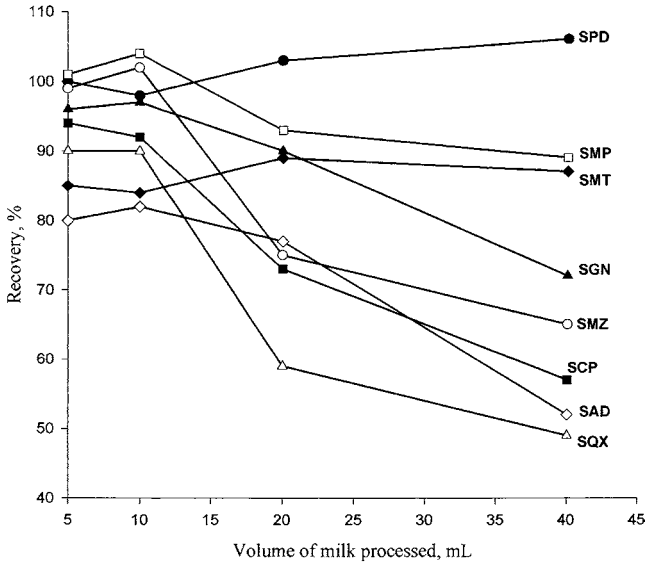

Figure 3. Variation of the recovery of some selected SAs at increasing volumes of milk processed (see **Figure 1** for acronym explanation).

Table 2. Accuracy and Precision of the Method at Various Concentrations of Sulfonamides in 10 mL of Whole Milk and 2 g of Whole Egg

	recovery[a] (RSD)					
	milk			egg		
	5 ppb	100 ppb	300 ppb	50 ppb	100 ppb	300 ppb
sulfaguanidine	110 (13)	97 (8)	92 (4)	70 (11)	76 (3)	80 (6)
sulfanilamide	93 (6)	82 (7)	83 (9)	71 (6)	69 (13)	66 (8)
sulfadiazine	105 (10)	98 (4)	104 (2)	106 (5)	104 (4)	93 (3)
sulfathiazole	109 (6)	98 (1)	103 (4)	78 (10)	82 (2)	76 (8)
sulfapyridine	112 (6)	98 (8)	96 (7)	96 (3)	106 (4)	103 (4)
sulfamerazine	97 (9)	99 (3)	102 (2)	90 (5)	95 (2)	97 (3)
sulfamethizole	76 (6)	83 (3)	82 (3)	68 (11)	79 (4)	81 (6)
sulfamethazine	96 (10)	102 (5)	100 (3)	97 (6)	99 (4)	96 (1)
sulfamethoxypyridazine	109 (5)	104 (1)	103 (4)	95 (7)	93 (2)	97 (2)
sulfachloropyridazine	105 (9)	92 (4)	96 (1)	80 (4)	84 (5)	86 (12)
sulfamethoxazole	82 (5)	85 (2)	87 (5)	80 (10)	81 (4)	77 (6)
sulfamonomethoxine	97 (10)	96 (5)	99 (4)	90 (9)	86 (4)	91 (6)
sulfadimethoxine	104 (4)	92 (5)	94 (2)	74 (9)	81 (9)	75 (5)
sulfaquinoxaline	93 (8)	90 (7)	88 (6)	71 (12)	75 (2)	71 (3)

[a] Mean values from five measurements.

were quantitatively extracted by the sorbent cartridge (data not shown here). In the past, we observed that a large fraction of proteins pass through a Carbograph cartridge unretained (*12*). To a greater or lesser extent, SAs are bound to proteins by various types of interactions. Bearing this in mind, it is conceivable that, when excessive milk volumes are passed through the SPE cartridge, certain analytes having a particular affinity for binding to proteins are partially carried away by them and lost in the milk effluent. In any case, processing 10 mL of milk sufficed to quantify most of the SAs at levels of a few parts per billion (see below).

Variations of the analyte recovery by varying the weight of the egg sample processed could not be obtained as, when egg amounts >2 g were analyzed, the sorbent cartridge obstructed.

For both milk and egg samples, the method was validated at three different concentrations of SAs in both matrices. At any analyte concentration and for the two matrices considered, five measurements were performed, and the results are reported in **Table 2**. These data show that the accuracy in determining SAs in milk and eggs does not significantly depend on the contamination levels. Recovery of SAs in milk at the 5 ppb level ranged

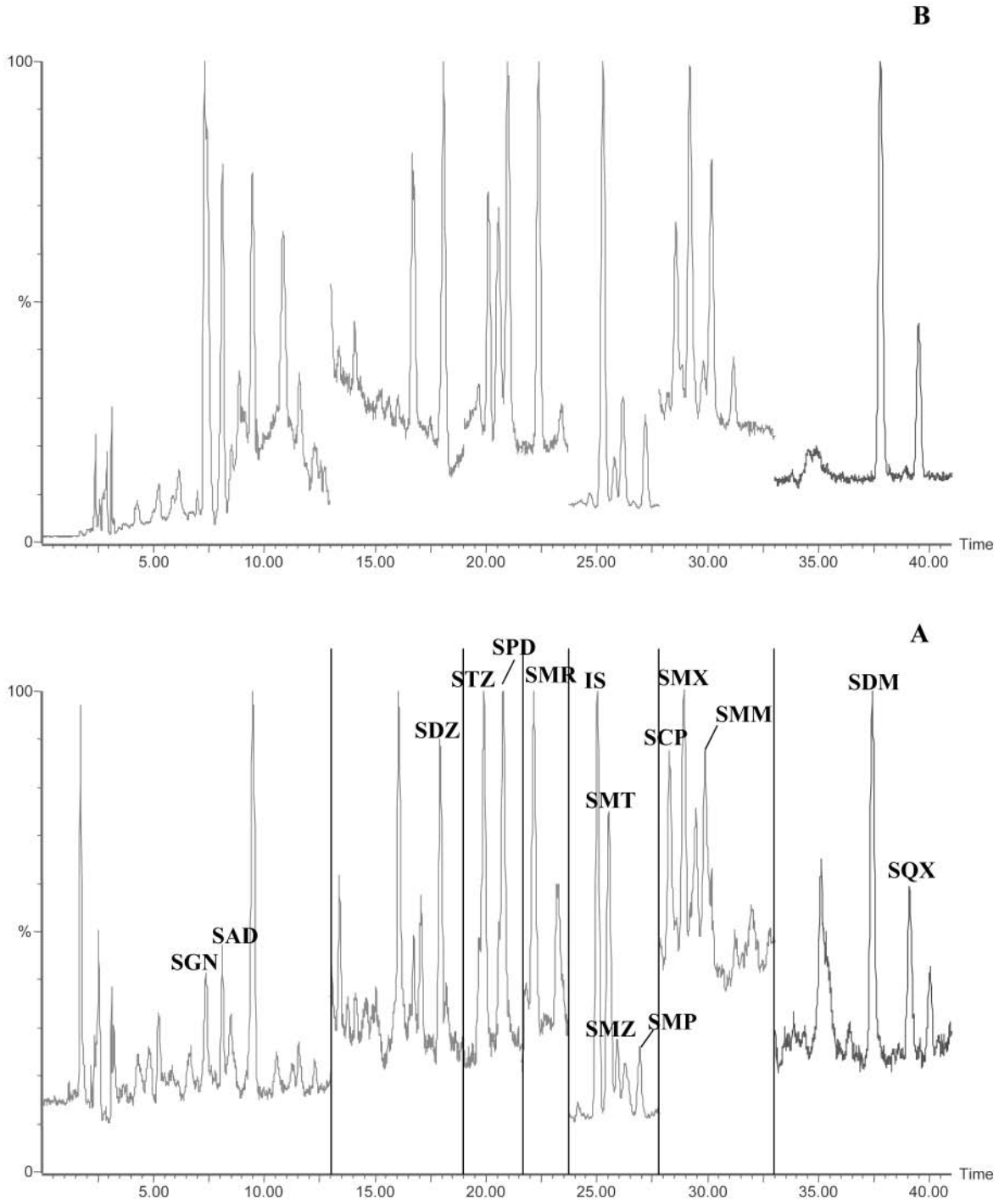

Figure 4. LC-ES-MS multiple-ion SIM chromatograms resulting from the analysis of (A) whole milk sample spiked with 5 ppb of sulfonamides and (B) egg sample amended with 50 ppb of sulfonamides (see **Figure 1** for acronym explanation).

between 76 (SMT) and 112% (SPD) withrelative standard deviations (RSDs) between 4 (SDM) and 13% (SGN). Recovery of SAs in egg at the 50 ppb level ranged between 68 (SMT) and 106% (SDZ) with RSDs between 3 (SPD) and 12% (SQX). Parts A and B of **Figure 4** show typical LC-MS SIM chromatograms resulting from analyses of pasteurized whole milk and whole egg samples spiked, respectively, with 5 and 50 ppb of each SA. More specifically, **Figure 5** shows the individual ion current profiles of the $[M + H]^+$ ions and selected fragment ions measured for 5 ppb of SPD in milk.

Linear Dynamic Range. Under the instrumental conditions reported in Experimental Procedures, the linear dynamic range of the ES/MS detector was estimated for all of the analytes. Amounts of each analyte varying from 10 to 500 ng and a constant amount of 50 ng of the internal standard were injected from suitably prepared standard solutions into the LC column. At each injected analyte amount, two replicate measurements were made. Signal versus amount-injected curves were then constructed by averaging the peak areas resulting from the sum of the signals for parent and fragment ions of each analyte and relating this area to that of the internal standard. For all analytes results showed that ion signals were linearly correlated with injected amounts up to 300 ng, with R^2 ranging between 0.9865 and 0.9900.

Limits of Detection (LODs) and Quantification (LOQs). LOQs of the method were estimated from the LC-MS SIM

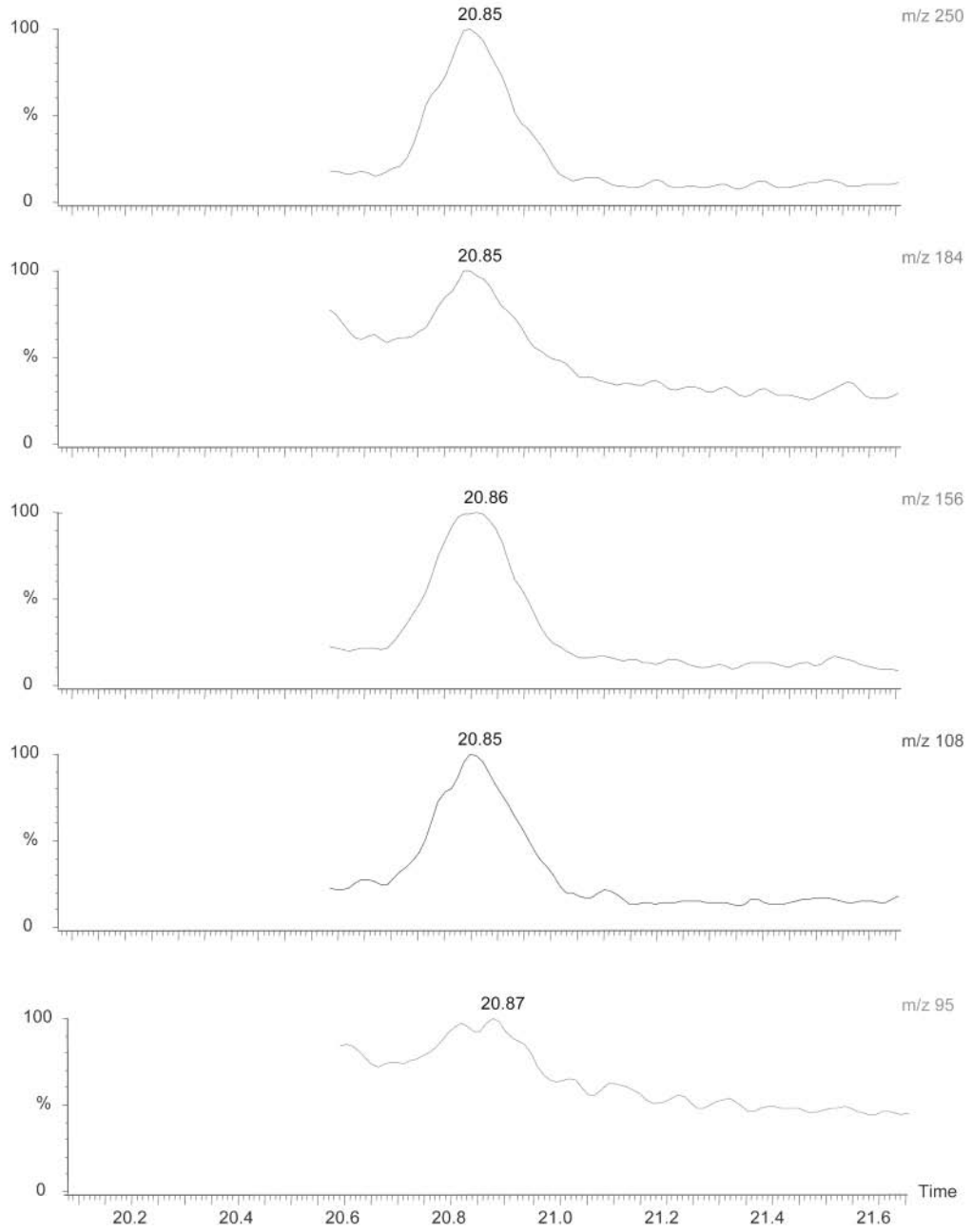

Figure 5. SIM profiles for $[M + H]^+$ and product ions of sulfapyridine resulting from analysis of 10 mL of milk fortified with 5 ppb of sulfapyridine.

chromatograms resulting from analyses of 5 and 50 ppb of each SA in, respectively, milk and egg samples. After extraction of the sum of the ion currents of both precursor and fragment ions relative to each analyte, the resulting trace was smoothed twice by applying the mean smoothing method (Mass Lab software, Thermoquest). Thereafter, the peak height-to-averaged background noise ratio was measured. The background noise estimate was based on the peak-to-peak baseline near the analyte peak. LOQs were then calculated on the basis of a minimal accepted value of the signal-to-noise ratio (S/N) of 10. These data are listed in **Table 3**. In the same table, LODs of the method are also presented. When using an MS detector, the first condition to be satisfied for ascertaining the presence of a targeted compound is that the precursor ion and at least two product ions produce signals distinguishable from the background ion current. Accordingly, a definition of LOD (S/N = 3) of each analyte was adopted, considering in each case the ion giving the worst S/N. When more than three ions were selected for analyte identification (see **Table 1**), the LODs were estimated by selecting signals for the parent ion and, among fragment ions, the two giving the best S/N ratios. As it makes sense to quantify an analyte only if its presence is confirmed, it follows that the LOQ of the method, like the LOD, is dictated by the signal intensity of the least abundant ion. In other words, an LOQ is correctly estimated only if its value is larger than or equal to the LOD estimated as mentioned above. As can be seen in **Table 3**, LOQs of the analytes determined as described above were generally larger than the corresponding LODs, except for some SAs in both milk and egg. In these cases, LOQs of these analytes should be more correctly increased to equal the respective LODs.

Method Comparison. For analyzing contaminants in foods by physicochemical techniques, sample treatment methods are almost invariably based on deproteinization/extraction by various agents followed by various cleanup steps, which often involve the use of SPE cartridges filled with various sorbent materials

Table 3. Limits of Detection and Quantification (LOD and LOQ, Respectively, in Parts per Billion) of the Method for Determining Sulfonamides in Milk and Egg

	milk		egg	
compound	LOD[a]	LOQ	LOD[a]	LOQ
sulfaguanidine	4 (108)	6	10 (108)	13
sulfanilamide	3 (108)	3	6 (108)	9
sulfadiazine	4 (108)	3	7 (108)	7
sulfathiazole	1 (108)	2	4 (108)	8
sulfapyridine	1 (184)	2	3 (184)	7
sulfamerazine	2 (265)	3	5 (265)	12
sulfamethizole	4 (271)	3	5 (108)	7
sulfamethazine	2 (156)	4	5 (156)	9
sulfamethoxypyridazine	2 (126)	4	7 (108)	10
sulfachloropyridazine	2 (285)	4	6 (285)	10
sulfamethoxazole	1 (254)	1	4 (108)	5
sulfamonomethoxine	1 (126)	3	6 (126)	10
sulfadimethoxine	3 (108)	2	7 (108)	6
sulfaquinoxaline	3 (108)	3	9 (108)	11

[a] *m*/*z* values of the ions giving the worst S/N ratio are reported in parentheses.

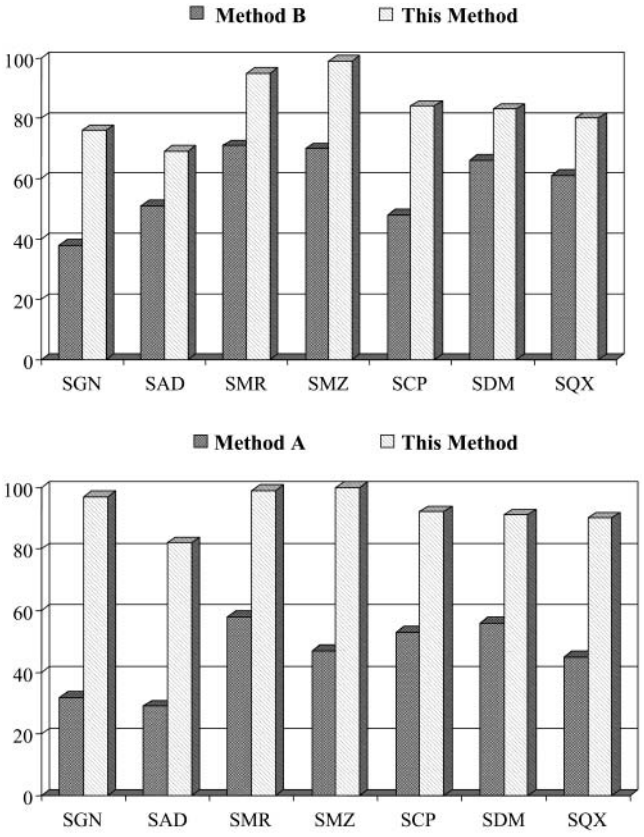

Figure 6. Mean recovery ($n = 2$) of some selected sulfonamides spiked at the 100 ppb level in milk (bottom histogram) and egg (top histogram) by following different sample treatment protocols: method A, ref *3*; method B, ref *7*.

(*13*−*15*). However, the method described here is based on simultaneous extraction and purification of SAs by a single Carbograph 4 cartridge. In terms of recovery and speed of analysis, we compared the two sample preparation strategies (see Experimental Procedures) to determine which was more convenient for analyzing SAs in milk and egg. In any case, two measurements were performed and results in terms of analyte recovery are shown in **Figure 6**. Compared to previously reported methods (*3*, *5*, *7*), our method extracted substantially larger amounts of the analytes from both milk and egg samples.

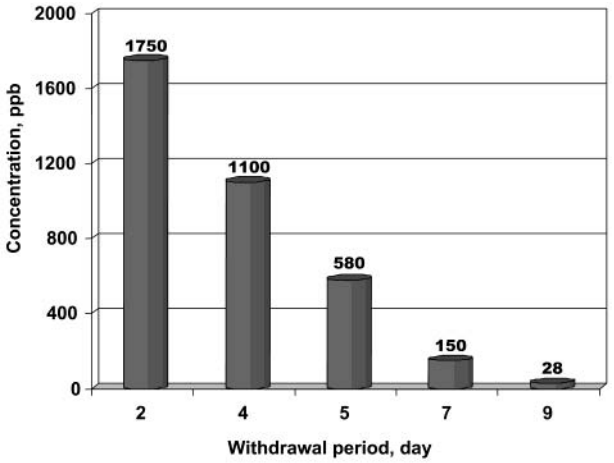

Figure 7. Depletion of sulfaquinoxaline from eggs of layer chickens ($n = 3$) following withdrawal of medication (0.25 g/L of sulfaquinoxaline in fresh water). Relative standard deviations associated with any measured concentration of sulfaquinoxaline were $\leq 20\%$.

In addition, the present procedure required only one-third and half of the times needed by the conventional procedures for preparing final extracts of milk and egg, respectively.

Application to Real Samples. The effectiveness of this method in measuring trace levels of SAs was checked by analyzing eggs from three layer chickens treated with sulfoquinoxaline (see Experimental Procedures). **Figure 7** shows the variation with time of the mean concentration of SQX in eggs following withdrawal of medication. In the EU, SAs are not approved for use in layer chickens and an action level has been set at 100 ppb in eggs. Our data show that, 1 week after the end of the treatment, the SQX concentration in eggs still exceeded the action level.

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