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# Rapid Confirmatory Assay for Determining 12 Sulfonamide Antimicrobials in Milk and Eggs by Matrix Solid-Phase Dispersion and Liquid Chromatography−Mass Spectrometry

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A rapid confirmatory method for determining 12 sulfonamide (SAs) antibacterials in whole milk and eggs is presented. This method is based on the matrix solid-phase dispersion technique with hot water as extractant followed by liquid chromatography (LC)−mass spectrometry (MS). The LC-MS instrument was equipped with an electrospray ion source and a single quadrupole. After 4 mL of a milk sample containing the analytes had been deposited on sand (crystobalite), this material was packed into an extraction cell. SAs were extracted by flowing 4 mL of water through the cell heated at 75 °C. With some modifications, this procedure was applied also to eggs. After pH adjustment and filtration, 0.5 mL of the final extracts was then injected into the LC column. MS data acquisition was performed in the positive-ion mode and by monitoring at least three ions for each target compound. The in-source collision-induced dissociation process produced confirmatory ions. At the 50 ng/g level, recovery of the analytes in milk and eggs was 77−92% with relative standard deviations ranging between 1 and 11%. Estimated limits of quantification (S/N = 10) were 1−3 ng/g of SAs in milk and 2−6 ng/g in eggs. With both matrices, attempts to reduce the analysis time by using a short chromatographic run time caused severe ion signal suppression for the early-eluted SAs. This effect was traced to competition effects by polar endogenous coextractives, maybe proteinaceous species, which are eluted in the first part of the chromatographic run. This unwelcome effect was almost completely removed by simply adopting more selective chromatographic conditions.

**KEYWORDS: Sulfonamides; milk; eggs; matrix solid-phase dispersion; water as extractant; liquid chromatography−mass spectrometry**

## INTRODUCTION

Sulfonamides (SAs) comprise a large number of synthetic antimicrobial 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. More than 10 SAs are routinely used in veterinary medicine to treat a variety of bacterial and protozoan infections in cattle, swine, and poultry (*1*). SAs are also active against some viruses (*2*). Analysis of SAs in foodstuffs is of particular concern because of their potential carcinogenic character (*3, 4*). To ensure the safety of food for consumers, regulation 281/96 of the EU Commission has set maximum residue limits of 100 ng/g of SAs as a total in milk. Although several SAs are approved for medicinal purposes in chickens, no SAs are 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. Since 1993, the EU (*5*) has stated 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 (*6*−*8*). One of these methods (*6*) is based on the use of the thermospray interface that is no longer commercially available. Doerge et al. (*7*) demonstrated the practicality of using a benchtop single-quadrupole LC-MS instrumentation for sensitive detection of some SAs in milk. Protonated molecules were generated by an atmospheric pressure chemical ionization (APCI) ion source, while fragment (product) ions were obtained by in-source collision-induced dissociation (CID) reactions in the first part of the ion transmission region. Volmer (*8*) elaborated a method based on LC−tandem MS with an electrospray (ESI) ion source

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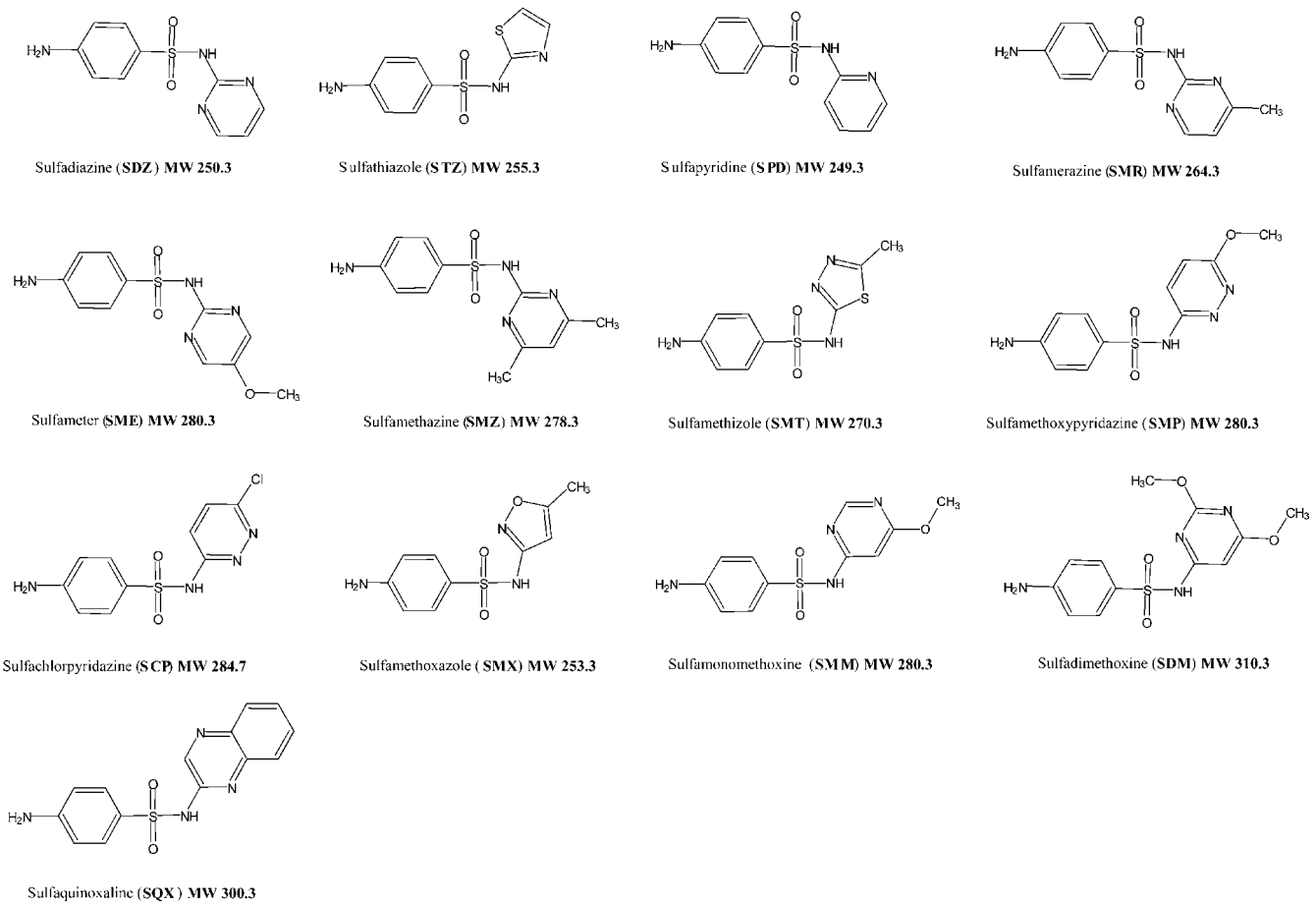


**Figure 1.** Chemical structures and molecular weights of selected sulfonamide antibacterials.

for detecting and quantifying 21 SAs in milk at levels of <1 ng/g. 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.

Little attention has been given in the past to elaborate LC-MS confirmatory methods of SAs in eggs. Tarbin et al. (*9*) developed an LC-APCI-MS method for determining nanograms per gram levels of 16 SAs in whole eggs. Identification and quantification of the analytes relied on MS acquisition of only the molecular ions in the selected ion-monitoring (SIM) mode. 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 (*10*). The authors elaborated a rather lengthy conventional sample treatment protocol, that is, deproteinization/extraction with acetonitrile followed by cleanup with a solid-phase extraction (SPE) cartridge, which contains some critical steps.

Recently, we have elaborated an LC-MS method for analyzing residues of SAs (*11*) in both milk and egg samples. This method involves isolation of the analytes from intact biological matrices by SPE with a Carbograph 4 cartridge.

After the pioneering work of Barker and his colleagues (*12*), many researchers have successfully adopted the so-called matrix solid-phase dispersion (MSPD) technique for extracting contaminants, particularly drugs, from biological matrices (*13*). MSPD offers distinct advantages over classical sample treatment procedures in that (1) the analytical protocol is drastically simplified and shortened; (2) the possibility of emulsion formation is eliminated; and (3) solvent consumption is substantially reduced.

With the exception of a work proposing water at ambient temperature for extracting the highly hydrophilic aminoglycoside antibiotics from bovine kidney (*14*), methods based on MSPD make use of moderate amounts of organic solvents as extractants. This means that problems associated with the use of organic solvents are minimized by MSPD, but not completely removed. Moreover, because no organic solvent is capable of selectively extracting target compounds from complex biological matrices, an extract cleanup step is often included in protocols involving analyte extraction by the MSPD technique. Finally, the use of an organic solvent precludes direct introduction of the eluate into a reversed-phase LC column.

On the basis of MSPD with water as extractant, we have very recently proposed an LC-MS confirmatory method for detecting SA residues in bovine muscle, liver, and kidney tissues (*15*, *16*) and in fish (*15*). Like $CO_2$ used in supercritical fluid extraction, water is an environmentally acceptable solvent, it is cost-effective, and hot water conditions are easily achieved with commercial laboratory equipment. The polarity of water decreases as the temperature is increased. This means that selective extraction of polar and medium polar compounds can be performed by suitably adjusting the water temperature.

The aim of this work has been to extend the use of the MSPD technique with hot water as extractant to the determination of 12 commonly used SA (**Figure 1**) residues in milk and eggs at the EU regulatory levels. After extraction, pH adjustment, and filtration, large aliquots of both milk and egg extracts have been directly introduced into an LC-MS instrument.

## MATERIALS AND METHODS

**Reagents and Chemicals.** 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). A 0.2 mg/mL stock solution of each SA was prepared by dissolving 20 mg of the pure analytical standard in 100 mL of methanol. For recovery studies, a single working composite standard solution was prepared by combining aliquots of each of 12 individual stock solutions and diluting with water/methanol (75:25, v/v) to obtain a final concentration of 2 $\mu$g/mL. A 10 $\mu$g/mL solution of the IS was prepared by diluting the stock solution with methanol. When unused, all of the above solutions were stored at 4 °C.

Sand (Crystobalite, 40−200 mesh size) was from Fluka AG, Buchs, Switzerland. Methanol "Plus" of gradient grade was obtained from Carlo Erba, Milano, Italy.

**Milk and Egg Samples.** Pasteurized, homogenized whole milk and eggs were purchased from retail markets. Preliminary analyses showed they were analyte-free.

**Extraction Apparatus.** The design of the homemade extraction apparatus used in this work was very similar to that shown in a previous paper (*17*), with the exception that the analyte-containing water leaving the extraction cell was collected in a calibrated glass tube instead of a sorbent cartridge. Stainless steel tubes of 8.3 mm i.d. and variable length were used as extraction cells.

**Sample Preparation and Extraction.** *Milk Samples*. For recovery studies, milk samples were spiked with known variable amounts of SAs. Under continuous agitation, 15 min was allowed for equilibration at room temperature. Thereafter, 4 mL of milk was poured in a porcelain mortar containing 12 g of sand, and the mixture was blended with the pestle for <10 min, until an apparently dry material was obtained. This material was then packed into a 16 cm long extraction cell, taking care to tap the tube to avoid loose packing of the particles. Any void space remaining after packing was filled with sand. Polyethylene frits (20 $\mu$m pore size) were located above and below the mixture. The tube was then put into the oven and heated at 75 °C for 5 min. Four milliliters of water at a 1 mL/min flow rate was then passed through the cell to extract the analytes. After the addition of 100 ng of the IS to the extract, the pH of the extract was adjusted to 4.6 with 3 mol/L formic acid and then filtered through a glass fiber (pore size = 0.7 $\mu$m, 25 mm diameter, Whatman, Sigma-Aldrich, Milan, Italy). After filtration, a completely uncolored and transparent solution was obtained. Half a milliliter of the filtrate was injected into the LC-MS apparatus.

*Egg Samples*. For extracting the analytes from eggs, the procedure described above was modified in that (1) 1.2 g of whole egg was mixed with 4.8 g of sand; (2) the extraction cell was 8 cm in length; (3) the extraction temperature was 100 °C and the extraction started after 15 min of equilibration; (4) the pH of the aqueous extract containing the IS was adjusted to 3.1; and (5) the extract was first filtered through a 0.7 $\mu$m pore size glass fiber (25 mm diameter, Whatman, Sigma-Aldrich) and then through a regenerated cellulose filter (pore size = 0.2 $\mu$m, 25 mm diameter, Alltech, Sedriano, Milan, Italy). These modifications were necessary to avoid clogging of the extraction cell and to achieve a transparent final extract. By following the procedures described above, the guard column was replaced with a new one after more than 60 and 100 injections of egg and milk extracts, respectively.

**LC-MS Analysis.** LC was performed by a Thermoquest, Manchester, U.K., model P2000. The analytical (250 mm × 4.6 mm i.d.) and guard (7.5 × 4.6 mm i.d.) columns employed were Alltima (Alltech), filled with 5 $\mu$m C-18 reversed phase packing. A Finnigan AQA benchtop mass spectrometer (Thermoquest) with a single quadrupole was used for identifying and quantifying target compounds in the LC column effluent. Target compounds were chromatographed and detected by following instrumental conditions reported elsewhere (*15*). Briefly, phase A was methanol and phase B was water. Both phases contained 5 mmol/L of formic acid. The mobile phase gradient profile (where $t$ refers to time in min) was as follows: $t_0$, A = 10%; $t_{20}$, A = 36%; $t_{29}$, A = 50%; $t_{30}$, A = 100%; $t_{35}$, A = 100%; $t_{37}$, A = 10%; $t_{45}$, A = 10%. The flow rate of the LC eluant was 1 mL/min, and 120 $\mu$L of the column effluent was diverted to the ESI source. The probe temperature was 170 °C and the capillary voltage 4 kV. 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. Ion signals were acquired by the time-scheduled multiple-ion SIM mode (**Table 1**).

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

| compound | channel,[a] *m/z* (relative abundance) | cone voltage, V | retention window, min |
|---|---|---|---|
| sulfadiazine | 108 (60), 156 (100), **251** (80) | 40 | 0–15.0 |
| sulfathiazole | 108 (40), 156 (100), **256** (50) | 40 | |
| sulfapyridine | 108 (50), 156 (100), *184* (55), **250** (95) | 40 | |
| sulfamerazine | 108 (60), *110* (100), 156 (95), **265** (100) | 42 | 15.0–17.0 |
| sulfameter (IS) | 108 (30), *126* (40), 156 (60), **281** (100) | 40 | 17.0–20.6 |
| sulfamethizole | 108 (40), 156 (100), **271** (50) | 40 | |
| sulfamethazine | 108 (35), *124* (80), 156 (55), **279** (100) | 40 | |
| sulfamethoxypyridazine | 108 (40), *126* (60), 156 (100), **281** (80) | 40 | |
| sulfachloropyridazine | 108 (55), 156 (100), **285** (40) | 41 | 20.6–26.0 |
| sulfamethoxazole | 108 (35), 156 (90), **254** (100) | 41 | |
| sulfamonomethoxine | 108 (30), *126* (40), 156 (75), **281** (100) | 41 | |
| sulfadimethoxine | 108 (40), 156 (100), **311** (50) | 46 | 26.0–33 |
| sulfaquinoxaline | 108 (90), *146* (35), 156 (100), **301** (40) | 46 | |

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

**Quantitation.** Absolute recovery of the analytes added to milk and egg samples at any given concentration was assessed by measuring peak areas resulting from the sum of the interference-free ion current profiles of parent and fragment ions, normalizing them to the peak area of the IS, and comparing these ratios to those obtained by injecting a blank sample extract to which the analytes were added postextraction.

## RESULTS AND DISCUSSION

**Optimization of the Extraction Conditions.** The extraction efficiency of water steadily increases as its temperature is increased. On the other hand, water extraction performed at too high temperatures is expected to give milk and egg extracts containing large amounts of unwanted naturally occurring low-polar species. Moreover, those analytes prone to hydrolytic attack could be rapidly degraded by excessively heated water. Therefore, recovery studies were initially conducted to find the best temperature at which efficient extraction of all of the analytes was obtained. For this purpose, we selected a milk sample spiked with 100 ng/g of each analyte. At each temperature considered, analyses were made in triplicate, and recoveries of selected analytes are reported in **Table 2**. Raising the temperature of the extractant from 50 to 75 °C had the effect of improving significantly the extraction yield of those analytes having the largest hydrophobic moieties, that is, SDM and SQX. The analyte amounts removed from milk did not significantly increase either when the extraction temperature was raised to 100 °C or when the extractant volume was increased. We chose to extract SAs at 75 °C instead of 100 °C, as in the latter case the mass chromatogram showed the presence of a larger number of coextractives.

**Table 2.** Mean Percentage Recoveries of Some Selected Sulfonamides by Extraction from a Milk Sample with Water at Increasing Temperatures (Spike Level = 100 ng/g)

| | recovery,[a] % (RSD) | | |
|---|---|---|---|
| compound | 50 °C | 75 °C | 100 °C |
| sulfadiazine | 75 (5) | 83 (4) | 82 (4) |
| sulfathiazole | 76 (6) | 85 (5) | 86 (4) |
| sulfapyridine | 80 (4) | 86 (5) | 88 (6) |
| sulfamethazine | 77 (5) | 81 (4) | 81 (3) |
| sulfamethoxazole | 70 (5) | 79 (4) | 80 (5) |
| sulfadimethoxine | 66 (6) | 83 (5) | 83 (6) |
| sulfaquinoxaline | 57 (7) | 80 (4) | 81 (4) |

[a] Mean values from triplicate experiments.

When extracting SAs from egg samples, we obtained results analogous to those reported above. With eggs, however, we chose to extract SAs at 100 °C instead of 75 °C, as extracts easier to filter were obtained.

The extraction efficiency was evaluated by varying the flow rate at which water passed through the extraction cell. For this purpose, 4 mL of water heated at 100 °C was used for extracting SAs in an egg sample at flow rates of 0.5, 1, and 2 mL/min. Duplicate measurements indicated that the flow rate did not significantly influence the extraction efficiency. Sometimes, when extracting analytes from eggs at 2 mL/min flow rate, we observed obstruction of the extraction cell. Thus, the best compromise between extraction time and reliability of the method was found to be that of passing the extractant through the cell at a 1 mL/min flow rate.

**Matrix Effect.** To achieve high-throughput determination of analytes in biological matrices, analytical protocols based on LC-ESI-MS with short (3−5 cm) LC columns where analytes are eluted in a few minutes are often adopted. However, numerous examples and studies (*14*−*16*, *18*−*25*) have revealed that the yield of protonation (or cationization) of the analytes in the ESI process can be decreased to a greater or lesser extent by competition effects due to the presence of matrix components. The extent of this unwelcome effect is related to both concentrations and affinities for the proton (or cations) of the coextracted and coeluted matrix components. It was shown that ion suppression of the analytes could be minimized or eliminated by adopting selective extraction methods (*23*) and/or efficient chromatographic separation (*20*, *23*). When trying to analyze SAs in bovine tissues (*15*, *16*) and fish (*15*) extracts with a short chromatographic run, we observed marked ion signal weakening for the earliest eluted analytes, that is, SDZ, STZ, SPD, and SMR. Large amounts of "unseen" polar coextractives eluted in the first part of the chromatogram were considered to be responsible for this effect. To develop a method as rapid as possible for simultaneously determining 12 SAs in milk and eggs, we investigated if the same effect occurred by analyzing targeted compounds in milk and egg extracts with a short chromatographic run time. For this purpose, experiments were designed as follows: (1) repeated extractions ($n = 4$) of both milk and egg samples; (2) postextraction addition of the four SAs mentioned above to the eight final extracts; (3) injections of the spiked extracts into the LC apparatus under various chromatographic conditions obtained by varying the initial concentration of the "strong" solvent, that is, methanol; (4) quantification of the concentrations of the four sulfonamides in any extract by comparing their absolute peak areas to those of the same compounds injected from a standard solution. According to Matuszewski et al. (*23*), we will use the terms "good" and "poor" separation simply to indicate chromatographic conditions by which the analyte is eluted with relatively long and short retention times, respectively. Results reported in **Tables 3** and **4** show that ion signal intensities of the analytes steadily increased as the strength of the LC mobile phase was decreased. Under "good separation" conditions, which are those adopted in this work, little, if any, matrix effect was present. Interestingly, this behavior is similar to that observed when SAs from extracts of bovine and fish tissues are injected (*15*, *16*).

**Table 3.** Effect of Chromatographic Conditions on Ion Signal Intensities of Selected Sulfonamides Directly Added to a Milk Extract (Spike Level = 100 ng/g)

| | poor separation[a] | | fair separation[b] | | good separation[c] | |
|---|---|---|---|---|---|---|
| compound | $t_R$[d], min | rel peak area[e] | $t_R$, min | rel peak area | $t_R$, min | rel peak area |
| sulfadiazine | 5.3 | 0.28[f] (8)[g] | 6.5 | 0.84 (2) | 11.8 | 0.94 (4) |
| sulfathiazole | 5.4 | 0.35 (6) | 8.0 | 0.73 (6) | 13.2 | 0.92 (5) |
| sulfapyridine | 5.9 | 0.47 (8) | 8.7 | 0.85 (5) | 14.1 | 0.97 (2) |
| sulfamerazine | 6.5 | 0.65 (5) | 9.7 | 0.85 (3) | 15.7 | 0.98 (2) |

[a] Gradient elution: 30% methanol to 46% in 10 min. [b] Gradient elution: 20% methanol to 36% in 10 min. [c] Gradient elution: 10% methanol to 36% in 20 min. [d] $t_R$ = retention time. [e] Peak area of the analyte injected from a milk extract relative to that of the same analyte injected from a standard solution. [f] Mean values from four determinations. [g] Standard deviations are reported in parentheses.

**Table 4.** Effect of Chromatographic Conditions on Ion Signal Intensities of Selected Sulfonamides Directly Added to an Egg Extract (Spike Level = 100 ng/g)

| | poor separation[a] | | fair separation[b] | | good separation[c] | |
|---|---|---|---|---|---|---|
| compound | $t_R$[d], min | rel peak area[e] | $t_R$, min | rel peak area | $t_R$, min | rel peak area |
| sulfadiazine | 5.3 | 0.36[f] (7)[g] | 6.5 | 0.57 (9) | 11.8 | 0.91 (5) |
| sulfathiazole | 5.4 | 0.43 (6) | 8.0 | 0.72 (7) | 13.2 | 0.98 (5) |
| sulfapyridine | 5.9 | 0.55 (5) | 8.7 | 0.79 (4) | 14.1 | 1.01 (3) |
| sulfamerazine | 6.5 | 0.71 (4) | 9.7 | 0.87 (4) | 15.7 | 0.97 (5) |

[a] Gradient elution: 30% methanol to 46% in 10 min. [b] Gradient elution: 20% methanol to 36% in 10 min. [c] Gradient elution: 10% methanol to 36% in 20 min. [d] $t_R$ = retention time. [e] Peak area of the analyte injected from an egg extract relative to that of the analyte injected from a standard solution. [f] Mean values from four determinations. [g] Standard deviations are reported in parentheses.

To obviate anomalous effects of ion signal suppressions of target compounds in particular biological matrices, many authors have proposed adoption of analyte-spiked blank sample extracts as reference standards (*14*, *16*, *24*−*30*). This practice can improve the accuracy of the analysis, provided the matrix effect does not significantly vary from sample to sample of a given matrix. In two previous works (*15*, *16*), we analyzed SAs postextraction added to bovine and fish tissue extracts coming from six different sources. For all of the SAs considered, results indicated that the extent of the matrix effect did not vary significantly from sample to sample. Because this behavior cannot be extended a priori to any kind of biological matrix, the same experiment was repeated with milk and egg samples. For this purpose, six milk samples and six egg samples from different producers were extracted as reported under Materials and Methods. Prior to LC-MS quantification under "good separation" conditions (see Materials and Methods), the extracts were spiked with the 13 SAs (12 analytes plus the IS) at the 100 ng/g level. Sulfonamide quantitation was performed in the same way as in the experiment described above. Results of these experiments are presented in **Tables 5** and **6**. As can be read, the matrix effect affected quantitation of several of the SAs considered, particularly SCP, SMX, SDM, and SQX in milk.

**Table 5.** Accuracy[a] and Precision[b] Data of Sulfonamides Directly Added to Milk Extracts from Six Different Sources (Spike Level = 100 ng/g)

| compound | accuracy, % | RSD, % |
|---|---|---|
| sulfadiazine | –7.0 | 3.8 |
| sulfathiazole | –9.4 | 4.8 |
| sulfapyridine | –3.3 | 3.2 |
| sulfamerazine | –2.1 | 2.4 |
| sulfameter | –3.2 | 2.3 |
| sulfamethizole | –10.0 | 4.5 |
| sulfamethazine | –5.3 | 3.2 |
| sulfamethoxypyridazine | –8.2 | 3.4 |
| sulfachloropyridazine | –13.1 | 5.4 |
| sulfamethoxazole | –14.2 | 4.5 |
| sulfamonomethoxine | –8.0 | 5.5 |
| sulfadimethoxine | –15.1 | 5.8 |
| sulfaquinoxaline | –37.2 | 6.3 |

[a] Calculated as [(mean calcd concn – spiked concn)/spiked concn] × 100. The concentration of each sulfonamide (included sulfameter candidate for use as internal standard) in every milk extract was calculated by comparing its absolute peak area to that of the same sulfonamide injected from a standard solution. [b] Expressed as relative standard deviation (RSD, %).

**Table 6.** Accuracy[a] and Precision[b] Data of Sulfonamides Added after Extraction to Egg Extracts from Six Different Sources (Spike Level = 100 ng/g)

| compound | accuracy, % | RSD, % |
|---|---|---|
| sulfadiazine | –6.1 | 5.2 |
| sulfathiazole | +2.3 | 6.0 |
| sulfapyridine | –1.2 | 3.1 |
| sulfamerazine | –2.5 | 3.8 |
| sulfameter | –1.3 | 6.4 |
| sulfamethizole | +8.2 | 6.8 |
| sulfamethazine | +4.3 | 4.3 |
| sulfamethoxypyridazine | +7.7 | 6.0 |
| sulfachloropyridazine | +3.7 | 4.3 |
| sulfamethoxazole | +7.1 | 3.1 |
| sulfamonomethoxine | +8.5 | 5.0 |
| sulfadimethoxine | –12.3 | 4.3 |
| sulfaquinoxaline | –16.5 | 5.7 |

[a] Calculated as [(mean calcd concn – spiked concn)/spiked concn] × 100. The concentration of each sulfonamide (included sulfameter candidate for use as internal standard) in every egg extract was calculated by comparing its absolute peak area to that of the same sulfonamide injected from a standard solution. [b]Expressed as relative standard deviation (RSD, %).

To a substantially lesser extent, ion signal weakening was again observed for some of the analytes when injected from egg extracts. Anyway, the degree of ion suppression for protonated SAs appeared to be independent of the particular milk and egg extract analyzed, as RSDs were in all cases <7%. Therefore, the use of analyte-fortified control milk and egg samples as reference standards could help to improve the accuracy of the analysis for those SAs having signals that are affected by coelution of endogenous components.

**Recovery Studies.** The efficiency of water in extracting SAs from milk was assessed at three spike levels, that is, 50, 100, and 200 ng/g levels. These levels correspond, respectively, to half of the maximum residue limit (MRL), the MRL, and 2 times the MRL set by the EU. Even though, at present, no MRLs exist for SAs in eggs, the same experiment was repeated with an egg sample. At each concentration, six measurements were performed, in both cases. Data are reported in **Table 7**. Analyte quantitation was performed as reported under Materials and Methods by selecting as reference standards pooled ($n = 6$) milk and pooled ($n = 6$) egg sample extracts to which SAs were added postextraction. As can be read, hot water was an excellent solvent for extracting targeted SAs from milk and eggs. With milk, RSDs ranged between 3 and 8% at the safe level. With egg at the same analyte concentration, the precision of the analysis was 2−11%.

**Table 7.** Recovery of the Method at Various Concentrations of Sulfonamides in Milk and Eggs

| | recovery[a] (RSD) | | | | | |
|---|---|---|---|---|---|---|
| | milk | | | egg | | |
| compound | 50 ng/g | 100 ng/g | 200 ng/g | 50 ng/g | 100 ng/g | 200 ng/g |
| sulfadiazine | 87 (6) | 85 (4) | 85 (3) | 90 (11) | 92 (7) | 88 (3) |
| sulfathiazole | 86 (5) | 84 (3) | 83 (6) | 81 (10) | 80 (10) | 77 (7) |
| sulfapyridine | 90 (6) | 84 (4) | 81 (6) | 87 (6) | 93 (8) | 85 (6) |
| sulfamerazine | 80 (7) | 82 (3) | 80 (2) | 89 (5) | 85 (7) | 90 (2) |
| sulfamethizole | 92 (10) | 82 (8) | 81 (8) | 77 (8) | 76 (6) | 81 (9) |
| sulfamethazine | 83 (7) | 82 (6) | 80 (3) | 83 (4) | 79 (4) | 80 (3) |
| sulfamethoxypyridazine | 79 (2) | 80 (3) | 84 (7) | 83 (3) | 83 (5) | 79 (5) |
| sulfachloropyridazine | 78 (3) | 79 (5) | 76 (7) | 80 (4) | 82 (2) | 79 (2) |
| sulfamethoxazole | 80 (7) | 80 (4) | 76 (6) | 83 (3) | 81 (4) | 80 (6) |
| sulfamonomethoxine | 91 (6) | 85 (5) | 82 (7) | 81 (1) | 78 (4) | 79 (6) |
| sulfadimethoxine | 83 (4) | 80 (6) | 86 (5) | 86 (9) | 80 (7) | 79 (7) |
| sulfaquinoxaline | 80 (4) | 79 (7) | 83 (8) | 85 (7) | 83 (11) | 81 (10) |

[a] Mean values from six measurements at any concentration.

**Linear Dynamic Range.** Under the instrumental conditions reported under Materials and Methods, 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 $R^2 > 0.99$.

**Limits of Detection (LODs) and Quantification (LOQs).** LOQs of the method were estimated from the SIM LC-MS chromatograms resulting from analyses of 5 and 15 ng/g of each SA in, respectively, a milk sample (**Figure 2**) and an egg sample (**Figure 3**). At these concentrations, data not reported here indicated that the accuracy and precision of the analysis were very similar to those presented in **Table 7**. 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). For those analytes detected by the monitoring of more than three ions, LOQ estimation was calculated by considering only the three most abundant signals. 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 8**. 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**), LODs were estimated by selecting signals for the parent ion and, among fragment ions, the two giving the

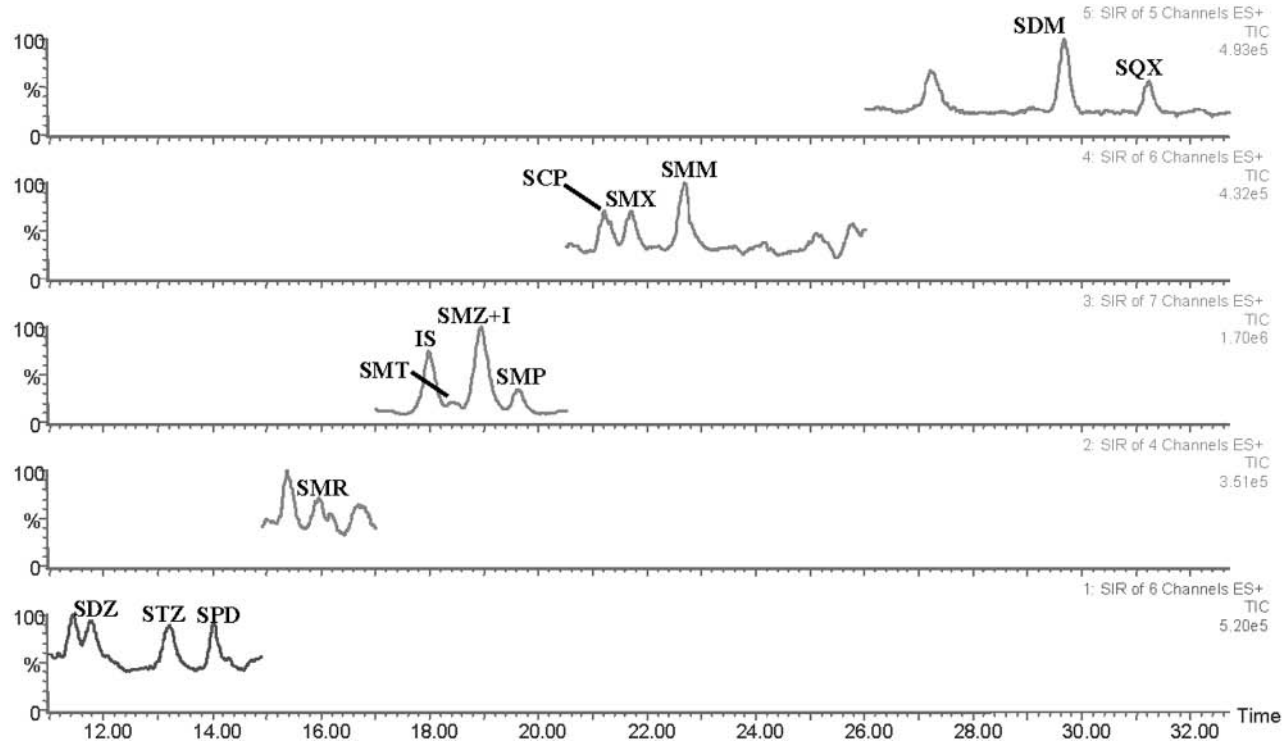


**Figure 2.** LC-ES-MS multiple-ion SIM chromatograms resulting from the analysis of a milk sample spiked with 5 ng/g of sulfadiazine (SDZ), sulfathiazole (STZ), sulfapyridine (SPD), sulfamerazine (SMR), sulfameter (IS = internal standard), sulfamethizole (SMT), sulfamethazine (SMZ), sulfamethoxypyridazine (SMP), sulfachloropyridazine (SCP), sulfamethoxazole (SMX), sulfamonomethoxine (SMM), sulfadimethoxine (SDM), and sulfaquinoxaline (SQX).

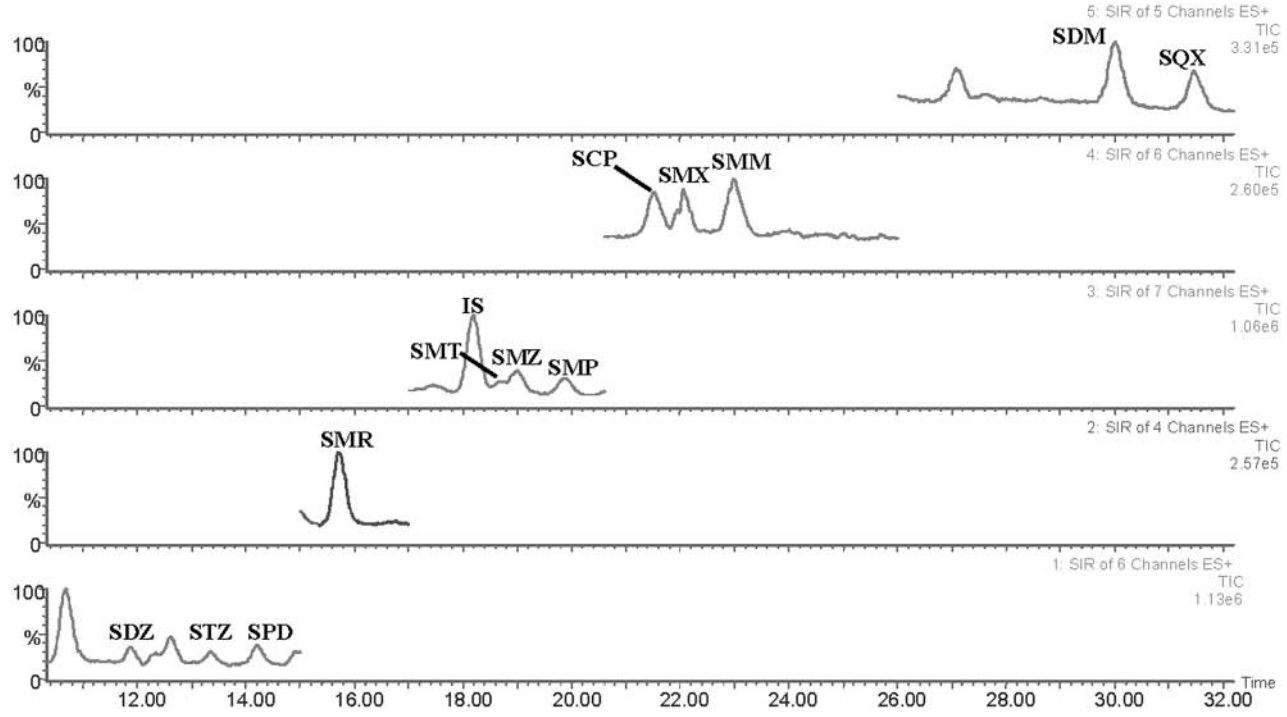


**Figure 3.** LC-ES-MS multiple-ion SIM chromatograms resulting from the analysis of an egg sample spiked with 15 ng/g of sulfadiazine (SDZ), sulfathiazole (STZ), sulfapyridine (SPD), sulfamerazine (SMR), sulfameter (IS = internal standard), sulfamethizole (SMT), sulfamethazine (SMZ), sulfamethoxypyridazine (SMP), sulfachloropyridazine (SCP), sulfamethoxazole (SMX), sulfamonomethoxine (SMM), sulfadimethoxine (SDM), and sulfaquinoxaline (SQX).

best S/N ratios. As estimated by us, LOQs of the method are well below tolerance levels set by the EU for residues of SAs in milk, as well as the safe level set by the U.S. Food and Drug Administration for sulfadimethoxine in milk, that is, 10 ng/g.

**Conclusions.** This work has shown that an environmentally friendly and inexpensive solvent, such as water, can be successfully used for extracting sulfonamide antibacterials from milk and eggs. Compared to other confirmatory methods quoted in the literature, our method is much simpler and faster.

**Table 8.** Limits of Detection and Quantification of the Method for Determining Sulfonamides in Milk and Eggs

| | milk | | egg | |
|---|---|---|---|---|
| compound | LOD, ng/g | LOQ, ng/g | LOD, ng/g | LOQ, ng/g |
| sulfadiazine | 1 (108)[a] | 2 | 3 (108) | 6 |
| sulfathiazole | 1 (108) | 2 | 2 (108) | 4 |
| sulfapyridine | 0.5 (108) | 2 | 1 (108) | 5 |
| sulfamerazine | 1 (110) | 3 | 2 (108) | 3 |
| sulfamethizole | 0.5 (108) | 1.5 | 1 (108) | 3 |
| sulfamethazine | 0.5 (108) | 1 | 2 (108) | 4 |
| sulfamethoxypyridazine | 1 (108) | 1 | 1 (126) | 2 |
| sulfachloropyridazine | 1 (285) | 2 | 2 (285) | 3 |
| sulfamethoxazole | 1 (108) | 1 | 2 (108) | 3 |
| sulfamonomethoxine | 1 (108) | 2 | 3 (108) | 4 |
| sulfadimethoxine | 0.5 (108) | 1 | 1 (108) | 2 |
| Sulfaquinoxaline | 0.5 (301) | 1 | 1 (301) | 4 |

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

Confirmation of the presence of one particular sulfonamide in milk or eggs could be accomplished in <1 h upon sample receipt, after suitable adjustment of chromatographic conditions. Compared to our previous method (*11*), this one offers advantages in that, besides being substantially faster, it does not require particularly skilled personnel for sample treatment and eliminates the use of a toxic solvent, such as methylene chloride.

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