Evaluation of a Method for Assaying Sulfonamide Antimicrobial Residues in Cheese: Hot-Water Extraction and Liquid Chromatography−Tandem Mass Spectrometry

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Several sulfonamide antimicrobials (SAAs) are largely used in veterinary medicine. A rapid, specific, and sensitive procedure for determining 12 SAAs in cheese is presented. The method is based on the matrix solid-phase dispersion technique followed by liquid chromatography (LC)−tandem mass spectrometry (MS) equipped with an electrospray ion source. Target compounds were extracted from Mozzarella, Asiago, Parmigiano, Emmenthal, and Camembert cheese samples by 6 mL of water modified with 10% methanol and heated at 120 °C. The addition of methanol to hot water served to improve remarkably extraction yields of the most lipophilic SAAs, that is, sulfadimethoxine and sulfaquinoxaline. After acidification and filtration, 100 μL of the aqueous extract was injected in the LC column. MS data acquisition was performed in the multireaction monitoring mode, selecting two precursor-to-product ion transitions for each target compound. Methanol-modified hot water appeared to be an efficient extractant, because absolute recovery ranged between 67 and 88%. Using sulfamoxole as surrogate analyte, recovery of the 12 analytes spiked in the five types of cheese considered at the 50 ng/g level ranged between 75 and 105% with RSD not higher than 11%. Statistical analysis of the mean recovery data showed that the extraction efficiency was not affected by the type of cheese analyzed. This result indicates this method could be applied to other cheese types not considered here. The accuracy of the method was determined at three spike levels, that is, 20, 50, and 100 ng/g, and varied between 73 and 102% with relative standard deviastions ranging between 4 and 12%. On the basis of a signal-to-noise ratio of 10, limits of quantification were estimated to be <1 ng/g.

KEYWORDS: Sulfonamide antimicrobials; matrix solid-phase dispersion; heated water as extractant; LC-MS/MS

INTRODUCTION

Antibiotics and antibacterial agents in food can provoke allergic reactions in some hypersensitive individuals and may compromise the human immune system (*1*). Even more important, the presence of subtherapeutic doses of the above drugs in foodstuff for long periods has led to the problem of drug-resistant pathogenic bacterial strains (*1*). Sulfonamide antimicrobials (SAAs) comprise a large number of synthetic bacteriostatic compounds. Structures of selected SAAs are presented in **Figure 1**. SAAs 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 SAAs are routinely used in veterinary medicine to treat a variety of bacterial and protozoan infections in food-producing animals. SAAs are also active against some viruses. The presence of SAAs in milk and milk products is a continuing health issue because recent studies have shown that one or more members of this drug class have a potential carcinogenic charac-

ter (*2*, *3*). Thus, any misuse or lack of adherence to withdrawal times may result in the illegal presence of SAA residues in milk and dairy products. Therefore, analytical procedures for analyzing them also in processed milk products are needed.

So far, development of analytical methods able to analyze drug residues in cheese has received little attention (*4*−*11*). Among these works, only one (*11*) describes a procedure based on liquid chromatography (LC)−tandem mass spectrometry (MS) for detecting three SAAs in condensed milk and cream cheese products. Interestingly, the authors detected the presence of sulfathiazole and sulfamethazine in three of six products analyzed.

Extraction procedures adopted in many standardized analytical methodologies for determining contaminants in food are labor-intensive and solvent consuming. To obtain satisfactory analyte recovery, efforts to isolate the compound(s) of interest require repeated extractions of the analytes from the biological matrix, replacement of the solvent with fresh each time, centrifugation, and pooling of the supernatants. This part of the analytical protocol requires the use of relatively large volumes of toxic, expensive, and flammable solvents and the subsequent need to evaporate and dispose of the employed solvent. In many cases,

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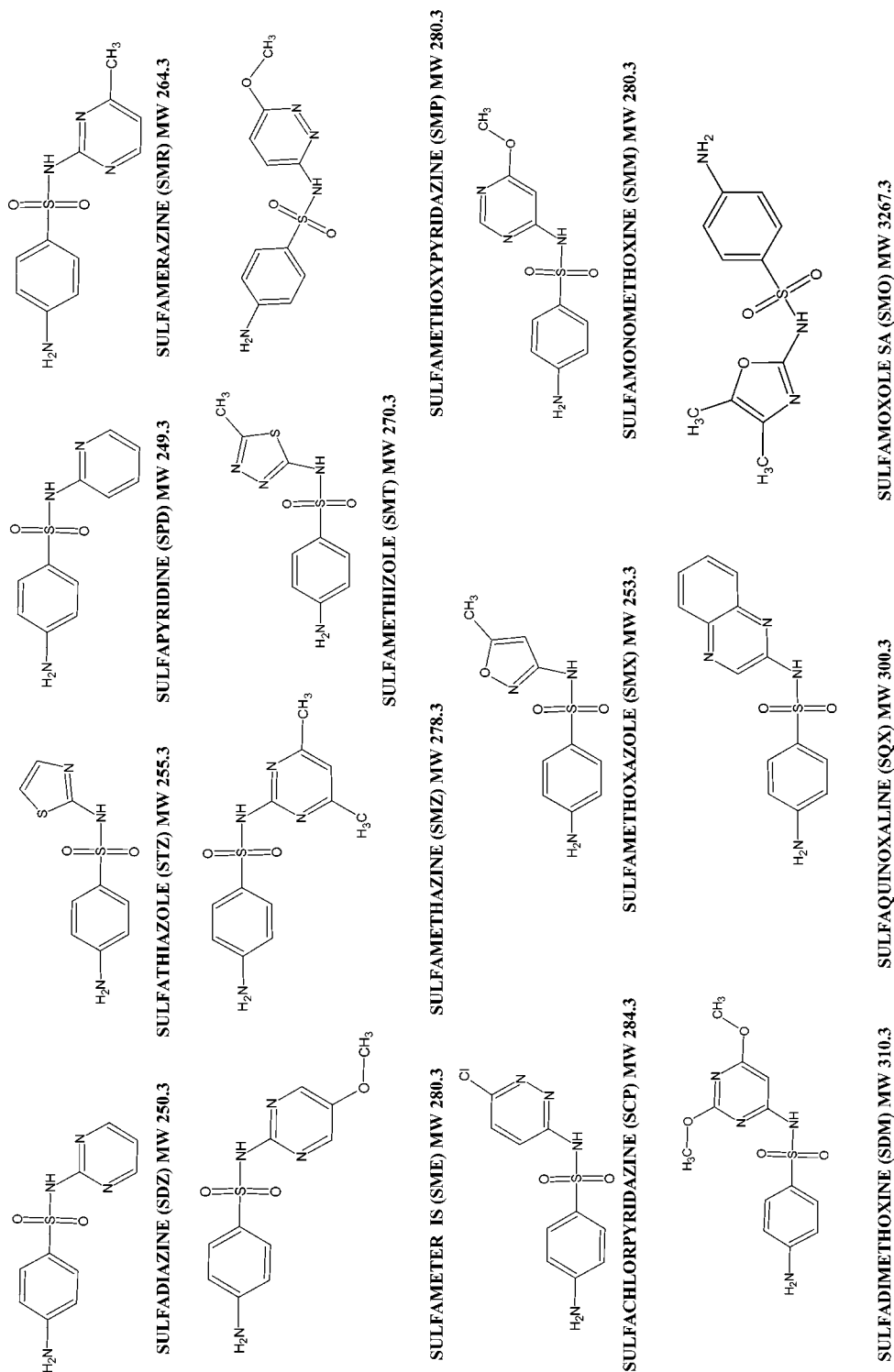**4538** *J. Agric. Food Chem.,* Vol. 54, No. 13, 2006

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Figure 1. Molecular structures of selected sulfonamide antimicrobials.

the sample and solvent combination produces emulsions that may decrease the extraction efficiency and lengthen the time required for the analyst to complete the procedure. As the generally employed organic solvents do not selectively extract targeted compounds, tedious and time-consuming cleanup procedures, involving often the use of a solid-phase extraction cartridge, are needed to partially isolate analytes from the matrix components.

Recently, we have proposed three LC-MS-based methods for determining residues of 12 SAAs in milk and eggs (*12*) and bovine (*13*, *14*) and fish (*13*) tissues. These methods involve simple and rapid sample treatment procedures that couple the

advantages of the matrix solid-phase dispersion (MSPD) technique, that is, simplicity and intimate contact between the extractant and the matrix, to those offered by heated water as extractant. Besides, to be a cheap and environmentally friendly solvent, water is able to selectively extract analytes by suitably controlling the extraction temperature (*15*). In essence, these methods consist of (i) dispersion of the biological matrix onto a solid support by blending the sample and the support with a mortar and pestle, (ii) filling a column with this material, (iii) flowing through the cell a suitable volume of water heated at a selected temperature, and (iv) little manipulation (pH adjustment and filtration) of the aqueous extract and injection of a relatively

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large volume of it into a reversed-phase LC column coupled to a MS detector. It has to be pointed out that the entire sample treatment procedure described above requires no more than 40 min to be completed.

The aim of this work has been to evaluate the feasibility of using the above analytical protocol for a rapid, sensitive, and selective determination of 12 commonly used SAAs in cheese. In this study, we considered some selected hard and soft cheeses, that is, Mozzarella, Camembert, Asiago, Emmenthal, and Parmigiano.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals. Sulfadiazine (SDZ), sulfathiazole (STZ), sulfapyridine (SPD), sulfamerazine (SMR), sulfamoxole, (SMO), 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 and SMO are not used in veterinary medicine and were adopted as internal standard (IS) and surrogate analyte (SA), respectively. We prepared 1 mg/mL stock solutions of each SAA by dissolving 100 mg of the pure analytical standard in 100 mL of methanol. For recovery studies, a composite working standard solution of the target compounds was prepared by mixing the above solutions and diluting with methanol to obtain analyte concentrations of 5 μg/mL. A 10 μg/mL solution of the IS and a 5 μg/mL solution of the SA were prepared by diluting the respective stock solutions with methanol. When unused, all of the above solutions were stored at 4 °C.

Sand (Crystobalite, 40−200 mesh size), a material obtained by heating silica at ≈1500 °C, was from Fluka AG, Buchs, Switzerland. Methanol "Plus" of gradient grade was obtained from Carlo Erba (Milan, Italy). For LC, distilled water was further purified (18 MΩ-cm) by passing it through a Milli-Q Plus apparatus (Millipore, Bedford, MA). Formic acid (>98%, purity) was from Sigma.

Cheese Samples. Cheese samples used for this study were collected from local markets. Before using them for recovery studies, they were analyzed according to this method to ascertain the absence of the drugs considered.

Extraction Apparatus. The design of the homemade extraction apparatus used in this work was very similar to that shown in a previous paper (*16*), with the exception that nitrogen was bubbled in water to eliminate any trace of dissolved oxygen, and the analyte-containing water leaving the extraction cell was collected in a calibrated glass tube instead of a sorbent cartridge. Briefly, the extraction apparatus consisted of a LC pump forcing water to pass through the extraction cell, a gas chromatography oven containing a preheating stainless steel coil and the extraction cell (16 cm × 8.3 mm i.d. stainless steel column). Twenty micrometer pore size polyethylene frits (Alltech, Sedriano, Milan, Italy) were located above and below the matrix/sand material.

Sample Preparation and Extraction. Prior to blending with sand, Mozzarella, Camembert, Asiago, and Emmenthal cheese samples were finely diced by a knife, whereas Parmigiano cheese was grated. For recovery studies, a 2.5 g portion of cheese was put in a porcelain mortar and spiked with variable volumes of the working standard solution and a constant volume of the SA solution, taking care to uniformly spread them on the sample. Intimate contact between the analytes and the sample was obtained by pounding with the pestle for ∼1 min. Then, 1 h was allowed for equilibration, storing the mortar at 4 °C. Thereafter, 10 g of Crystobalite was added to the mortar, and the mixture was blended with the pestle for ∼10 min, until an apparently homogeneous material was obtained. This material was then packed into the extraction cell. To ensure homogeneous packing of the cell, close attention was paid to pour the material into the tube in three or four aliquots; the tube was firmly tapped for 10−15 s after the addition of each aliquot. Any void space remaining after packing of the solid material was filled with Crystobalite. The tube was then put into the oven and heated at 120 °C for 5 min. Six milliliters of a water/methanol solution (90:10, v/v) was then passed through the cell at a 1 mL/min flow rate to extract SAAs. After the addition of 500 ng of the IS, extracts were acidified

Table 1. Time-Scheduled Multireaction Monitoring Conditions for Detecting Sulfonamide Antibacterials in Cheese by Tandem MS

compound	transition, m/z	cone voltage, V	collision energy, eV	dwell time, ms	retention time, min
sulfadiazine	251→92	32	25	50	0–18.3
	251→156		15	50	
sulfathiazole	256→92	27	27	50	0–18.3
	256→156		13	50	
sulfapyridine	250→92	30	25	50	0–18.3
	250→156		15	50	
sulfamerazine	265→156	30	15	50	0–18.3
	265→172		15	50	
sulfamoxole (SA)[a]	268→92	30	25	50	18.3–27
	268→156		15	50	
sulfameter (IS)[b]	281→92	32	25	50	18.3–27
	281→156		17	50	
sulfamethizole	271→92	28	23	50	18.3–27
	271→156		15	50	
sulfamethazine	279→156	32	18	50	18.3–27
	279→186		15	50	
sulfamethoxypyridazine	281→92	32	25	50	18.3–27
	281→156		17	50	
sulfachloropyridazine	285→92	30	28	50	18.3–27
	285→156		13	50	
sulfamethoxazole	254→92	28	25	50	18.3–27
	254→156		15	50	
sulfamonomethoxine	281→92	32	25	50	18.3–27
	281→156		17	50	
sulfadimethoxine	311→92	37	32	100	27–34
	311→156		20	100	
sulfaquinoxaline	301→92	32	30	100	27–34
	301→156		15	100	

[a] Surrogate analyte. [b] Internal standard.

to pH 4.5−4.6 with 10 mol/L formic acid. The precipitate was eliminated by filtration through a glass fiber filter (0.7 μm pore size, 2.5 cm diameter, Alltech, Sedriano, Italy), and 100 μL of the final extract was injected into the LC column. By following the procedure described above, the guard column was replaced with a new one after >300 injections of extracts.

LC-MS/MS Analysis. The liquid chromatograph consisted of a Waters pump (model 600 E, Milford, MA), a 100 μL injection loop, an Alltima 5 μm C-18 guard cartridge (7.5 × 4.6 mm i.d., Alltech), and a C-18 reversed-phase analytical column (250 mm × 4.6 mm i.d., Alltech) thermostated at 35 °C; the chromatograph was interfaced to a benchtop triple-quadrupole mass spectrometer (model Micromass 4 MICRO API, Waters) by an electrospray ion (ESI) source operating in the positive ion mode. To separate analytes, the mobile phase was made of (A) methanol and (B) water. Both phases contained 5 mmol/L formic acid. The mobile phase gradient profile (where *t* refers to time in minutes) was as follows: t_0, A = 10%; t_{20}, A = 36%; t_{29}, A = 50%; t_{30}, A = 100%; t_{33}, A = 100%; t_{34}, A = 10%; t_{43}, A = 10%. Analyte retention times varied by ≤1% over 2 weeks. A diverter valve led the effluent at a 400 μL/min flow rate into the ion source at a defined time of between 10.5 and 35 min of the chromatographic run. High-purity nitrogen was used as drying and curtain gases; high-purity argon was used as collision gas. Nebulizer gas was set at 650 L/h and the cone gas at 50 L/h. The probe and desolvation temperatures were maintained at 100 and 350 °C, respectively. The gas pressure in the collision cell was 3 mbar. Capillary voltage was 3000 V, and extractor voltage was 2 V. Declustering potential, collision energy, and other transmission parameters were optimized for each analyte and are reported in **Table 1**. Mass axis calibration of each mass-resolving quadrupole Q_1 and Q_3 was performed by infusion of a sodium and cesium iodide solution at 10 μL/min. Unit mass resolution was set and maintained in each mass-resolving quadrupole by keeping a full width at half-maximum of ≈0.7 amu. All of the source and instrument parameters for monitoring SAAs were optimized by standard solutions at 5 μg/mL of each drug infused at 10 μL/min by a syringe pump. The multireaction monitoring (MRM) technique was used for quantitation by selecting at least two molecular ion decomposition reactions for each analyte (see again **Table 1**).

At the beginning of every working day, the sample cone was cleaned by immersing it in a water/methanol/formic acid (45:45:10, v/v) solution
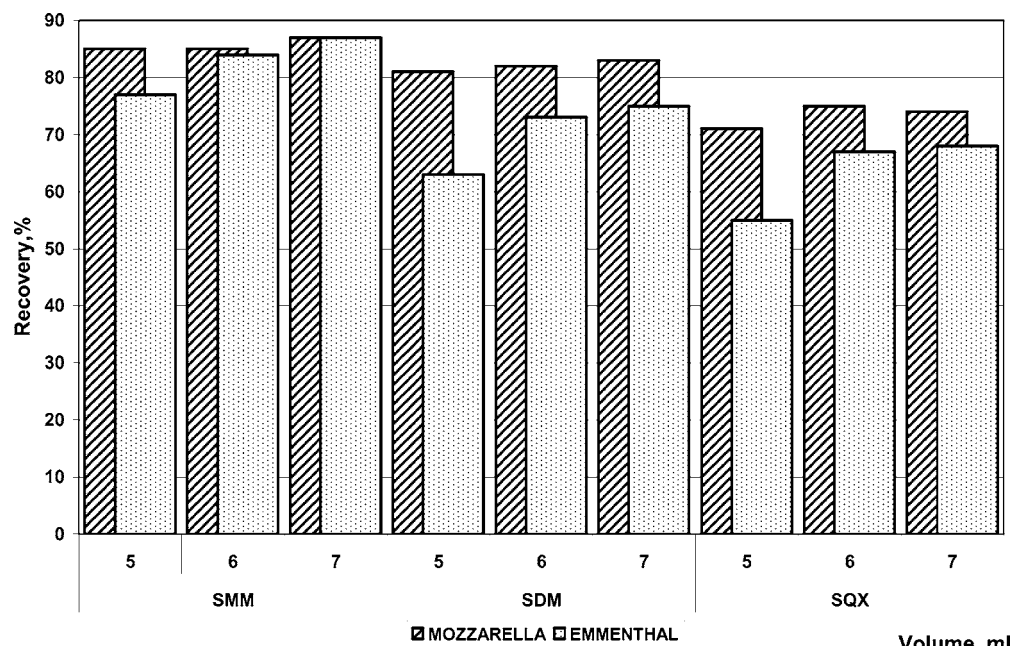
Figure 2. Effect of extractant volume on recovery of three selected sulfonamide antimicrobials added to Mozzarella and Emmenthal cheese samples at 50 ng/g level. SMM, sulfamonomethoxine; SDM, sulfadimethoxine; SQX, sulfaquinoxaline.

and sonicating for 10 min. The 4 MICRO mass spectrometer has a device allowing removal of the sample cone without loss of the vacuum in the MS region. Therefore, after the cleaned sample cone had been placed, the MS instrument was ready to work in a matter of minutes.

Quantitation. Absolute recovery of each analyte and the SA added to any cheese sample was assessed by summing the ion current profiles relative to the transitions considered, normalizing them to the peak area of the IS, and comparing these ratios to those obtained by injecting a related blank sample extract to which the analytes were added postextraction. We followed this procedure to obviate matrix effects that weakened the analyte ion signal intensities, as compared to those observed by injecting a standard mixture solution of SAAs. The validation of the method at any given concentration and for any cheese type considered was performed in an analogous way, with the difference that signals of targeted compounds were normalized to those of the IS and related to that of the SA.

RESULTS AND DISCUSSION

Recovery Studies. Following conditions reported elsewhere (*12*), initial extraction experiments were performed by using pure water heated at 75 °C as extractant. A Parmigiano cheese sample was spiked with SAAs (including that candidate for use as surrogate analyte) at 50 ng/g level and analyzed. Experiments performed in quadruplicate showed that water heated at 75 °C gave low extraction yields of the least polar analytes, that is, SMX, SMM, SDM, and SQX. Increasing progressively the extraction temperature to 120 °C improved recovery of the above-mentioned analytes, but recovery of SDM and SQX was still unsatisfactory. Gentili et al. (*17*) succeeded in obtaining high recovery even of the most hydrophobic SAAs in bovine muscle tissue by extracting with water at 160 °C. In this work, the use of an extraction temperature of >120 °C was precluded because it provoked clogging of the extraction cell, especially when SAAs were extracted from soft cheeses, such as Mozzarella and Camembert. It is known that cheese is a foodstuff rich in fatty substances that are present mainly as globules contained within the protein matrix network. Thus, it is conceivable that contaminants in cheese can be incorporated into fat particles to a larger or lesser extent, depending on their polarity. The partial failure of water heated at 120 °C in

Table 2. Absolute Recoveries of Sulfonamide Antibacterials in Parmigiano Cheese by Extracting Them under Different Conditions (Spike Level = 50 ng/g)

	recovery,[a] % (RSD, %)			
compound	75 °C,[b] 0%[c]	90 °C, 0%	120 °C, 0%	120 °C, 10%
sulfadiazine	87 (6)	85 (5)	84 (7)	85 (6)
sulfathiazole	83 (7)	85 (7)	81 (6)	82 (6)
sulfapyridine	82 (7)	90 (4)	86 (5)	88 (7)
sulfamerazine	90 (5)	85 (7	89 (6	86 (8)
sulfamoxole (SA)[d]	86 (8	83 (6)	86 (6)	88 (5)
sulfamethizole	72 (9)	80 (8)	84 (7)	83 (7)
sulfamethazine	75 (9)	86 (7)	85 (8)	83 (7)
sulfamethoxypyridazine	68 (9)	76 (8)	84 (6)	85 (7)
sulfachloropyridazine	67 (7)	75 (6)	81 (6)	82 (7)
sulfamethoxazole	59 (10)	70 (6)	79 (6)	84 (7)
sulfamonomethoxine	52 (12)	68 (10)	79 (7)	84 (6)
sulfadimethoxine	33 (15)	44 (11)	56 (12)	74 (9)
sulfaquinoxaline	22 (19)	33 (16)	44 (14)	67 (11)

[a] Mean values from quadruplicate measurements. [b] Extraction temperature. [c] Methanol percentage added to water. [d] Surrogate analyte.

extracting the most lipophilic SAAs from cheese was then traced to its inability to dissolve fat globules, so liberating the least polar SAAs. On the basis of this hypothesis, 10% of methanol was added to water. As can be read in **Table 2**, this modification sufficed to increase remarkably recovery of the two most hydrophobic SAAs, that is, sulfadimethoxine and sulfaquinoxaline. The role played by methanol could be that of shifting the partition equilibrium toward the methanol/water liquid phase and/or that of dissolving fat globules with consequent liberation of absorbed analytes. Doubling the methanol percentage resulted in a further increase of the recovery of the two SAAs mentioned above. However, injection of relatively large volumes of a final extract containing 20% methanol into the LC column provoked undesirable peak broadening for the early-eluted SAAs. Definitively, 10% methanol in water heated at 120 °C was adopted for extracting targeted compounds from the cheeses considered in this study, and these conditions were used in subsequent experiments. When SAAs other than sulfadimethoxine and sulfaquinoxaline were assyed in cheese, pure water heated at

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Table 3. Accuracy[a] and Precision[b] Data of Sulfonamide Antibacterials Directly Added to Six Extracts of Each Selected Cheese (Each Type of Cheese Came from Six Different Manufacturers; Spike Level = 50 ng/g)

compound	Mozzarella accuracy, %	Mozzarella RSD, %	Asiago accuracy, %	Asiago RSD, %	Parmigiano accuracy, %	Parmigiano RSD, %
sulfadiazine	−21	8	−37	10	−30	11
sulfathiazole	−11	7	−26	8	−27	9
sulfapyridine	−9	9	−4	10	−11	9
sulfamerazine	−27	8	−18	12	−15	11
sulfamoxole (SA)[c]	+10	7	+5	9	+13	10
sulfameter (IS)[d]	−25	14	−40	8	−14	12
sulfamethizole	−28	9	−33	11	−30	10
sulfamethazine	−16	11	−5	10	−3	13
sulfamethoxypyrid-azine	−29	13	−18	14	−16	12
sulfachloropyrid-azine	−35	10	−29	11	−18	8
sulfamethoxazole	−22	9	−35	10	−21	7
sulfamonometh-oxine	−11	7	−13	8	−8	9
sulfadimethoxine	−8	7	−15	9	−12	11
sulfaquinoxaline	−10	8	−8	7	−13	8

[a] Calculated as [(mean calcd concn − spiked concn)/spiked concn] × 100. The concentration of each sulfonamide (included sulfamoxole and sulfameter candidate for use as surrogate analye and inernal standard, respectively) in every cheese 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, %). [c] Surrogate analyte. [d] Internal standard.

Table 4. Accuracy and Precision of the Method for Analyzing Sulfonamide Antibacterials in Five Types of Cheeses (Spike Level = 50 ng/g)

compound	accuracy,[a] % (RSD, %) Mozzarella	Asiago	Camembert	Emmenthal	Parmigiano
sulfadiazine	102 (7)	93 (6)	100 (7)	95 (6)	97 (5)
sulfathiazole	99 (7)	91 (7)	91 (8)	96 (6)	93 (5)
sulfapyridine	102 (8)	99 (8)	93 (9)	94 (8)	100 (6)
sulfamerazine	99 (5)	103 (7)	100 (9)	95 (9)	98 (7)
sulfamethizole	96 (5)	102 (8)	95 (7)	92 (7)	94 (6)
sulfamethazine	99 (7)	93 (5)	95 (8)	92 (8)	94 (7)
sulfamethoxypyridazine	101 (4)	105 (6)	99 (5)	97 (7)	97 (7)
sulfachloropyridazine	95 (7)	93 (6)	98 (8)	90 (9)	93 (6)
sulfamethoxazole	100 (6)	105 (9)	101 (6)	103 (7)	97 (6)
sulfamonomethoxine	95 (5)	93 (7)	97 (6)	99 (8)	95 (6)
sulfadimethoxine	91 (9)	85 (8)	84 (10)	82 (8)	86 (7)
sulfaquinoxaline	83 (8)	79 (10)	75 (10)	77 (11)	76 (9)

[a] Mean values from quadruplicate experiments.

90 °C could be used as extractant. In this way, cleaner extracts are obtained with the result that the ESI source is less exposed to contamination by endogenous cheese components.

Effect of Extractant Volume on Analyte Recoveries. Besides affecting the extraction yield of the target compounds, the water volume passing through the extraction cell can influence the sensitivity of the method, as this method does not include any concentration step of the extract. For the purpose of finding the minimum volume of water able to extract efficiently the analytes, experiments were performed by spiking each of the matrices considered with the analytes and the surrogate internal standard at a 50 ng/g level and extracting. For each matrix, experiments were performed in duplicate. To save space, only results relative to the three most hydrophobic SAAs in Mozzarella and Emmenthal cheese samples are visualized in **Figure 2**. As can be seen, the extractability of SAAs by the water/methanol solution depended somewhat on the particular matrix, as cheeses required variable extractant volumes for efficient recoveries. Maybe, interaction forces acting

Table 5. Accuracy and Precision of the Method on Analyzing Sulfonamide Antibacterials in Emmenthal Cheese at Three Different Concentrations

compound	accuracy,[a] % (RSD, %) 10 ng/g	50 ng/g	100 ng/g
sulfadiazine	96 (4)	97 (5)	102 (4)
sulfathiazole	91 (8)	93 (5)	96 (6)
sulfapyridine	99 (8)	100 (6)	99 (8)
sulfamerazine	94 (8)	98 (7)	95 (5)
sulfamethizole	95 (10)	94 (6)	96 (8)
sulfamethazine	92 (8)	94 (7)	92 (7)
sulfamethoxypyridazine	93 (8)	97 (7)	98 (8)
sulfachloropyridazine	91 (10)	93 (6)	93 (6)
sulfamethoxazole	94 (9)	97 (6)	99 (5)
sulfamonomethoxine	92 (11)	95 (6)	100 (7)
sulfadimethoxine	83 (11)	86 (7)	89 (7)
sulfaquinoxaline	73 (12)	76 (9)	79 (10)

[a] Mean values from quadruplicate measurements.

between the analytes and some of the nonextracted matrix components vary in intensity by changing the type of cheese. Anyway, 6 mL of extractant was the best compromise in terms of good recovery of all analytes in any matrix considered and minimum extract volume.

Matrix Effect. We observed that ion signals of SAAs (including the IS and SA) added postextraction to any cheese extract were, with one exception, less intense than those obtained when the same compounds were injected from a reference standard solution. In addition, this effect was remarkably dependent on the type of cheese extract to which target compounds were added. When contaminants in foodstuff matrices are analyzed with an ESI source, a "negative" matrix effect or, less commonly, a "positive" matrix effect is the rule more than the exception. To obviate this drawback, many authors have proposed the adoption of analyte-fortified control matrix extracts as reference standards (*12−14, 18−25*). However, it is possible that the extent of the matrix effect can vary by varying the source of a given biological matrix. In this case, using a generic analyte-fortified control matrix extract as reference standard will affect analyte quantitation in incurred samples. For all of the SAAs considered here, including sulfamoxole (surrogate analyte) and sulfameter (internal standard), we conducted a study aimed at assessing if significant variations of the matrix effect occurred by varying the sources of the different cheeses considered. Owing to the difficulty of finding Camembert and Emmenthal cheese samples from six different producers, this study was limited to samples of Italian cheeses. Practically, six cheese samples respectively of Mozzarella, Asiago, and Parmigiano from six different sources were extracted as reported under Experimental Procedures. Prior to LC-MS analysis, the extracts were spiked with the analytes and the SA at 50 ng/g level, whereas the IS was added at a 200 ng/g level. Quantification of the SAAs in final extracts was performed by comparing their absolute peak areas to those of the same compounds injected from a standard solution. For each cheese type considered, results of these experiments for some selected analytes are presented in **Table 3**. Generally, the matrix effect affected significantly the ion signals of all of the SAAs considered, and the extent of this effect was dependent on the particular type of cheese. Vice versa, the extent of the matrix effect for protonated SAAs appeared to be not remarkably dependent on the source of any particular cheese, as relative standard deviations (RSDs) were in all cases not higher than 14%. Therefore, when a cheese sample is analyzed, a corresponding analyte-fortified control extract of the same type of cheese could be used as reference standard to circumvent the
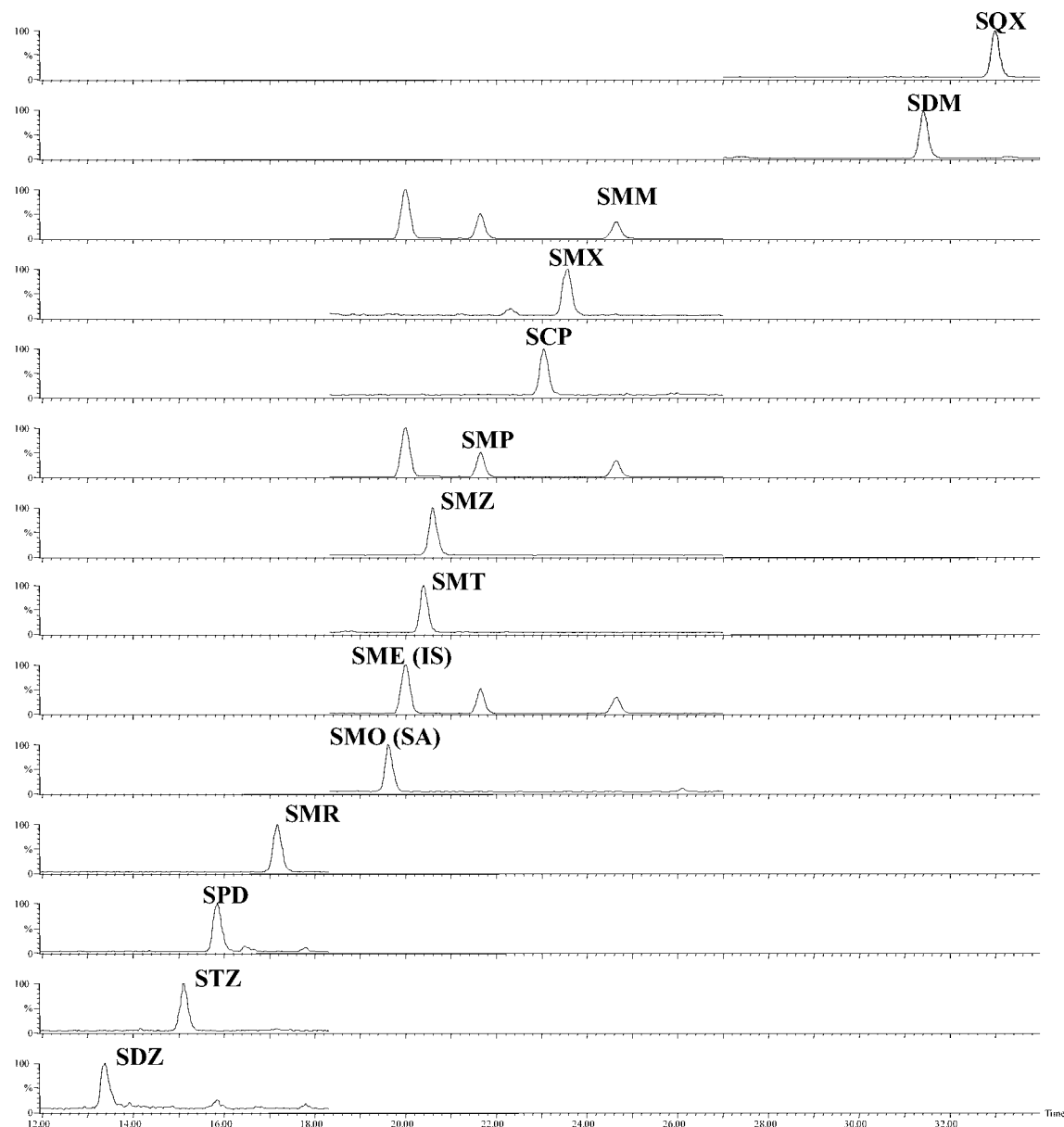
Figure 3. MRM LC-MS/MS chromatogram resulting from analysis of an Emmenthal cheese sample spiked with sulfonamides antibacterials at 10 ng/g level.

matrix effect, so improving the accuracy of the analysis of SAAs in incurred samples.

Accuracy and Precision of the Method. The accuracy and precision of the method were assessed by spiking the five types of cheeses considered with the analytes and the surrogate analyte at the 50 ng/g level and analyzing. In this case, analyte recoveries were estimated by normalizing their signal intensities to that of the IS and relating them to that of the SA. Results are reported in **Table 4**. As can be read, the accuracy of the method varied between 75 and 105% with RSDs not higher than 11%.

To check that the extraction efficiency of SAAs in cheese was not dependent on the particular type of cheese, mean accuracy data were compared among them by using the one-way ANOVA (analysis of variance) test at the $P = 0.05$ significance level. In any case, the calculated $F_{4,15}$ values (not shown here) were lower than the critical value (3.804), showing that the extraction method of any of the SAAs considered was not influenced by the type of cheese. This result indicates that this method could be employed for analyzing SAAs in cheeses other than those considered in this study.

We assessed if the accuracy and precision of the method were dependent on the analyte concentration in cheese by spiking an Emmenthal cheese sample with the analytes at three different concentrations and analyzing. Even in this case, recoveries of the analytes were estimated in the same way as reported above. From the results reported in **Table 5**, it appears that the accuracy of the method was not significantly dependent on the analyte concentrations, as RSDs were within the 4−12% range.

Limits of Detection (LOD) and Quantification (LOQ) of the Method. LOQs of the method were estimated from the MRM LC-MS/MS chromatogram shown in **Figure 3** and resulting from analysis of a Parmigiano cheese extract spiked with SAAs at the 10 ng/g level. After extraction of the sum of the ion currents of the transitions selected for each analyte, the resulting trace was smoothed twice by applying the mean smoothing method (MassLynx 4.0 software, Waters). 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-

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Table 6. Limits of Detection (LOD) and Quantification (LOQ) of the Method for Determining Sulfonamide Antibacterials in Cheese

compound	LOD, ng/g	LOQ, ng/g
sulfadiazine	0.2 (251→92)[a]	0.5
sulfathiazole	0.2 (256→92)	0.3
sulfapyridine	0.1 (250→92)	0.3
sulfamerazine	0.1 (265→172)	0.2
sulfamethizole	0.1 (271→92)	0.3
sulfamethazine	0.1 (279→156)	0.2
sulfamethoxypyridazine	0.1 (281→92)	0.2
sulfachloropyridazine	0.3 (285→92)	0.5
sulfamethoxazole	0.2 (254→92)	0.5
sulfamonomethoxine	0.1 (281→92)	0.3
sulfadimethoxine	0.1 (311→92)	0.1
sulfaquinoxaline	0.2 (301→92)	0.3

[a] *m/z* values of the transitions giving the worst signal-to-noise ratios are reported in parentheses.

to-noise ratio (S/N) of 10. These data are listed in **Table 6**. In the same table, LODs of the method are also presented. When detection with a MS/MS arrangement is performed, the most important condition to be satisfied for ascertaining the presence of a targeted compound is that at least two precursor ion-to-product ion transitions give 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 transition giving the worst S/N. It can be read that SAAs can be quantified in cheese at concentrations of <1 ng/g.

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