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Accelerated Solvent Extraction and Confirmatory Analysis of Sulfonamide Residues in Raw Meat and Infant Foods by Liquid Chromatography Electrospray Tandem Mass Spectrometry

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This paper describes a new method for the rapid extraction and unequivocal confirmation of 13 sulfonamides (SAs) in raw meat and infant foods. The highly automated extraction procedure is based on accelerated solvent extraction followed by liquid chromatography−tandem mass spectrometry (LC-MS/MS) as a confirmatory analysis. After 1 g of food matrix was blended with 2 g of C18 as a solid support material, the mixture was packed into the extraction cell and the SAs were extracted with 10 mL of hot water at 160 °C and 100 atm; 100 μL of the extract was directly injected into the LC-MS system. The analytes were ionized in an electrospray interface operating in the positive ion mode and were identified by selecting two multireaction monitoring transitions, which guaranteed method specificity. Typical recoveries from crude meat and baby food samples ranged from 70 to 101% at a fortification level of 100 ppb, corresponding to the maximum residue limits established by the European Union and the U.S. Food and Drug Administration. The interday method precision was less than 8.5%, and the limits of detection were below 2.6 ppb. This study has taken matrix-induced suppression of ionization into account, by comparing standard and matrix-matched calibration curves. Four of the 13 monitored SAs have been detected in some baby foods and raw meat samples, bought from Roman supermarkets and butchers' shops, using the described methodology.

KEYWORDS: Sulfonamides; LC-ESI-MS; matrix solid phase dispersion; accelerated solvent extraction; infant food; crude meat

INTRODUCTION

In modern systems of livestock breeding, veterinary drugs with antimicrobical activities are employed for the prevention and treatment of diseases and, at subtherapeutic doses, as growth promoters of food-producing animals (*1*, *2*). The irresponsible use of these substances, such as the administration of doses that are higher than needed and the failure to respect proper withdrawal times before butchering, can result in the occurrence of unwanted residues in edible products (*1*, *3*). Negative repercussions for consumer health are connected with the intrinsic toxicity of a drug and its metabolites and with the selection of resistant bacteria that through the food chain, can be transferred to humans (*3*).

SAs are chemotherapeutics commonly used in veterinary practice, because of their inexpensiveness and wide spectrum of activity, to prevent or to treat acute and chronic bacterial infections; they are also added to animal feed to promote growth (*4*). To explicate their bacteriostatic action, short-life SAs are mixed with food several times a day, while long-life SAs are parenterally administered, with an increasing risk of residues in animal tissues. The presence of SA residues in food is of a toxicological and regulatory concern as some SAs could be carcinogenic, cause allergic hypersensitivity reactions, and reduce the therapeutic effectiveness of these drugs on humans (*5*).

Within the European Union, the Commission Regulation 508/1999/EC (*6*), amending the Council Regulation 2377/90/EEC (*7*), establishes maximum residue limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin; in particular, for all substances pharmacologically active belonging to the SA group, the total level of residues should not exceed 100 μg/kg. This MRL is valid for target tissues (muscle, fat, liver, and kidney) and for the milk of all food-producing species. In the United States, the Food and Drug Administration (FDA) has approved the use of SA veterinary drugs for therapeutic, prophylactic, and/or growth-promoting purposes in cattle, calves, swine, turkeys, chickens, and some minor species, such as trout. The FDA has established a tolerance of 0.1 ppm for most SAs in edible animal tissues and has specified liver as the target tissue (*8*).

Therefore, the toxicological risk and the possibility to develop antibiotic resistance have induced the European and American

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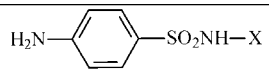
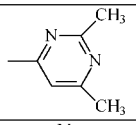
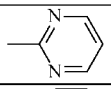
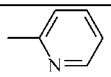
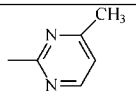
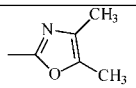
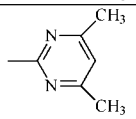
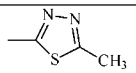
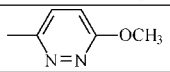
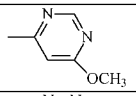
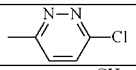
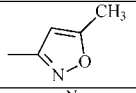
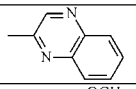
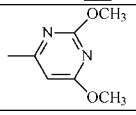
Table 1. Names, Structures, and LC-MS/MS Parameters for Examined SAs

CHEMICAL STRUCTURE 				
Compound	**-X**	**MRM transitions**	**Retention time (min)**	**Collision potential (V)**
Sulfisomidine (SIM)		279/124 279/186	11.3	-40 -43
Sulfadiazine (SDZ)		251/92 251/156	12.9	-46 -32
Sulfapyridine (SPD)		250/92 250/156	13.9	-48 -33
Sulfamerazine (SMR)		265/108 265/156	14.7	-47 -34
Sulfamoxole (SMO)		268/92 268/156	14.9	-48 -32
Sulfamethazine (SMZ)		279/124 279/186	15.8	-34 -55
Sulfamethizole (SMT)		271/92 271/156	16.0	-49 -30
Sulfamethoxypiridazine (SMP)		281/92 281/156	16.2	-55 -35
Sulfamonomethoxine (SMM)		281/92 281/156	17.8	-52 -36
Sulfachloropyridazine (SCP)		285/92 285/156	18.9	-52 -32
Sulfamethoxazole (SMX)		254/92 254/156	20.1	-33 -49
Sulfaquinoxaline (SQX)		301/92 301/156	22.1	-54 -35
Sulfadimethoxine (SDM)		311/92 311/156	22.2	-58 -40

institutions to regulate drug residue levels in raw materials used in food manufacture through the establishment of MRLs, but these may not be suitable for direct application to finished infant products. In fact, infants aged 4 months to 2 years old, corresponding to the age range that consumes baby foods, have a potentially higher sensitivity to toxicants and are more likely to be exposed to certain environmental contaminants than other population groups because, on a body weight basis, they consume 3−4-fold more food than adults and in addition their food sources are less varied (*9*). Only recently, the European Commision has decided to submit stringent rules on pesticide residues in baby food (Commision Directives 1999/50/EC, 2003/14/EC, 99/39/EC, and 2003/13/EC), but at present, there are no regulation-defining limits of drug residues in infant foods.

Several methods have been developed for the determination of SAs in raw materials (tissues, milk, eggs, fish, and honey) (*1*, *5*, *10*). Earlier procedures for measuring the total content of SAs are spectrophotometric techniques based on the Bratton−Marshall colorimetric reaction (*11*); they lack sensitivities and do not identify specific SAs. Other analytical methods are based on bioassay, TLC, GC, and high-performance liquid chromatography (HPLC) (*5*). As screening methods for controlling SA residues, microbiological and immunoassay tests are used in the European Union, but they may give false positive results and supply qualitative nonspecific information. The Commission Decision 2002/657/CE (*12*), implementing Council Directive 96/23/EC (*13*) concerning the performance of analytical methods and the interpretation of results, states that "methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods." Within the U.S. Department of Agriculture, the Food Safety and Inspection Service uses, to monitor sulfa drug residues, TLC as a screening method and GC-MS as a confirmatory method. Various GC-MS methods have been developed, but they have required the polar SAs to be chemical derivatized, due to their low volatility (*14*, *15*); others methods,

based on the coupling of HPLC and capillary electrophoresis (*4*, *16*) to a mass spectrometer, have employed ionization techniques such as thermospray, electrospray (ESI), and atmospheric pressure chemical ionization (*4*, *17*).

At present, five LC-ESI-MS methods have been reported in the literature for determining SA residues in raw meat (*18*−*22*), but none has been published about the detection methods and the presence of these drugs in baby foods. As regards extraction technique, some of these methods require elaborate and time-consuming preliminary cleanup procedures. ASE is an innovative technique, which, by using solvents at an elevated temperature and pressure, allows one to achieve a rapid recovery of analytes from solid and semisolid matrices (*23*, *24*). Till now, ASE has been applied mostly to solid environmental samples such as sediments and soils to recover organic pollutants, while there are few applications on food samples: pesticides in baby food (*25*), quinolones in fish and swine feed (*26*), and PCBs and dioxines in food and biological tissues (*27*).

This paper describes the development of a rapid and highly reproducible extraction procedure of 13 commonly used SAs for veterinary purposes (**Table 1**) from crude meat and infant foods, based on ASE; the use of water at high pressures and temperatures as the extractant allows one to inject the sample directly, without the need to filter the extract; also, the elevated selectivity and sensibility of the developed LC-ESI-MS/MS method could be used so as to avoid unacceptable levels of such residues entering the food chain, especially that of infants.

MATERIALS AND METHODS

Chemicals. SMX, SMO, SPD, SMT, SCP, SMP, SDZ, SMR, SMZ, SIM, SMM, SDM, SQX, and SME were purchased from Sigma-Aldrich S.r.l. (Milano, Italy). For its retention time and because SME is not a veterinary drug, it was adopted as the internal standard. Stock solutions of the individual standards mixtures were prepared by dissolving each analyte in methanol at 1 μg μL^{-1} (except SDZ prepared at 0.5 μg/μL), and by these was prepared the composite working standard solution at a concentration of 50 ng μL^{-1}. All of the solutions were stored at 4 °C when unused.

For LC, distilled water was further purified by passing it through the Milli-Q Plus apparatus (Millipore, Bedford, MA). Acetonitrile and methanol were RS-Plus grade; acetone, dichloromethane, and hexane were RS grade. All of these solvents were purchased by Carlo Erba (Milano, Italy). Formic acid was purchased by Merck (Darmstadt, Germany).

For extraction studies, the solid support materials were as follows: C-18-bonded silica with particles of 35−70 μm diameter and a porosity of 60 Å, supplied by Alltech Associates Inc. (Deerfield, IL), and diatomaceous earth SPE-ED MATRIX 38 supplied by Applied Separations (Allentown, PA).

Baby Foods and Fresh Meat Samples. Bovine (tissues of veal, tender beef, and beef), porcine, and poultry raw meat and several brands of baby foods, whose formulations are based on bovine (veal and beef), porcine (pig and ham), and poultry meat (chicken and turkey), were bought from Roman supermarkets and butchers' shops.

Sample Preparation for Extraction Procedure. Samples of infant foods, which consist of 40% homogenized meat and of rice starch, maize starch, sunflower oil, and cooking water for the remaining 60%, were ready subjection to the extraction procedure, while the crude meat samples had to be prepared. To this purpose, tissue samples were put in a beaker with an adequate quantity of milliQ water and homogenized by means of an ULTRA-TURRAX T8 homogenizer (Staufen, Germany), starting with a speed of 5000 rpm slightly increased to 25000 rpm, for 15 min. The excess of water was then removed by a gentle nitrogen flow.

For recovery studies, 1 g of homogenized sample was put in a glass mortar and fortified with variable volumes of the working standard solution, leaving the analytes in contact with the meat sample for 30 min. After 2 g of C18 was added, the matrix solid phase dispersion technique was applied (*28*, *29*): the food matrix and the solid support were blended with a glass pestle so that a complete dispersion was obtained (the whole mixture took a uniform color and consistency). Thereafter, the blend was prepared for packing into the steel cell for ASE extraction. Before dispersion, the C_{18} sorbent was submitted to a cleaning procedure: 10 g of sorbent was put into a polypropylene tube (i.d. 2.5 cm, capacity 20 mL), washed with 30 mL of methanol, and dried with a gentle nitrogen flow.

ASE. The extractions were carried out using a Dionex ASE 200 Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, CA); this technique is also referred to as pressurized liquid extraction or pressurized fluid extraction. The system, automated and capable of sequential extractions, was made up of stainless steel extraction cells where the programmed parameters (temperature and pressure) were kept at their specified values by electronically controlled heaters and pumps. By pressurizing the sample cell (up to 200 atm), it was possible to keep the solvent in a liquid phase even at the relatively high extraction temperatures (up to 200 °C).

The extraction process consisted of the following steps: (i) preheating the cell, (ii) filling the cell with extraction solvent, (iii) heating the cell for a fixed time to reach thermal equilibrium, (iv) static extraction at constant pressure and temperature, (v) transferring the extract into the collection vial and washing the cell with fresh solvent (flushing step), and (vi) final solvent purging with nitrogen gas (purging step).

In this work, extraction cells of 5 mL were used and packed with 3 g of the blend consisting of homogenized meat and C18 in the ratio of 1:2, as described above. The remaining empty space was filled with diatomaceous earth. Quantitative recoveries have been achieved by using water at 160 °C and 100 atm with an extraction time of 15 min shared among preheating (1 min), heating (8 min directly set by microprocessor on the ground of selected temperature value), a static step (5 min), and purging (1 min).

The extracts (10 mL) were cooled at −18 °C for about 1 h so as to allow the fat and other matrix compounds, coextracted with analytes, to precipitate due to the reduced solubility at a low temperature. After centrifugation at 10000 rpm for 5 min (at −4 °C), 100 μL of the supernatant was injected onto the LC-MS/MS system.

LC and MS. LC was carried out by means of a HPLC/Autosampler (equipped with a 100 μL loop)/vacuum degasser system Perkin-Elmer Series 200 (Perkin-Elmer, Norwalk, CT). The mobile phase was also used as a washing solution for the autosampler. The analytes were chromatographed on an Alltima 25 cm × 4.6 mm i.d. column filled with 5 μm C-18 reversed phase packing (Alltech, Sedriano, Italy) and equipped with an Alltima guard column.

A PE Sciex API 2000 tandem triple-quadrupole mass spectrometer (Perkin-Elmer) equipped with a TurboIonSpray source operated in the PI (positive ionization) mode was used for the work described herein. The ion spray voltage was 5000 V. Nitrogen gas was used as a curtain and collision gas nebulizer, while air was used as a nebulizer and drying gas. The settings for the nebulizer, drying, and curtain were 30, 75, and 30 on API-2000. For each analyte, two multireaction monitoring (MRM) transitions were selected for the quantitation after observing collision-induced dissociation (CID) spectra obtained by full scan product ion experiments.

The mass axis calibration of each mass-resolving quadrupole Q_1 and Q_3 was performed by infusion of a polypropylene glycol solution at 10 μL min^{-1}. The unit mass resolution was established and maintained in each mass-resolving quadrupole by keeping a full width at half-maximum of approximately 0.7 Da. All of the source and instrument parameters for monitoring analytes were optimized by standard solutions of 100 pg mL^{-1} (containing 1 mmol L^{-1} of formic acid) infused at 10 μL min^{-1} by a syringe pump.

In separating the analytes, phase A was acetonitrile and phase B was water; because of the neutral acid character of the analyzed SAs (pK_a between 5 and 7.6), both solvents contained 1 mmol L^{-1} HCOOH. Gradient elution was performed by linearly increasing the percentage of the organic modifier from 10 to 50% in 20 min. The flow rate of the mobile phase was 1 mL min^{-1}. A total of 200 μL min^{-1} of the LC column effluent was diverted to the ES source. The TurboIonSpray probe temperature was maintained at 300 °C, while the gas pressure in

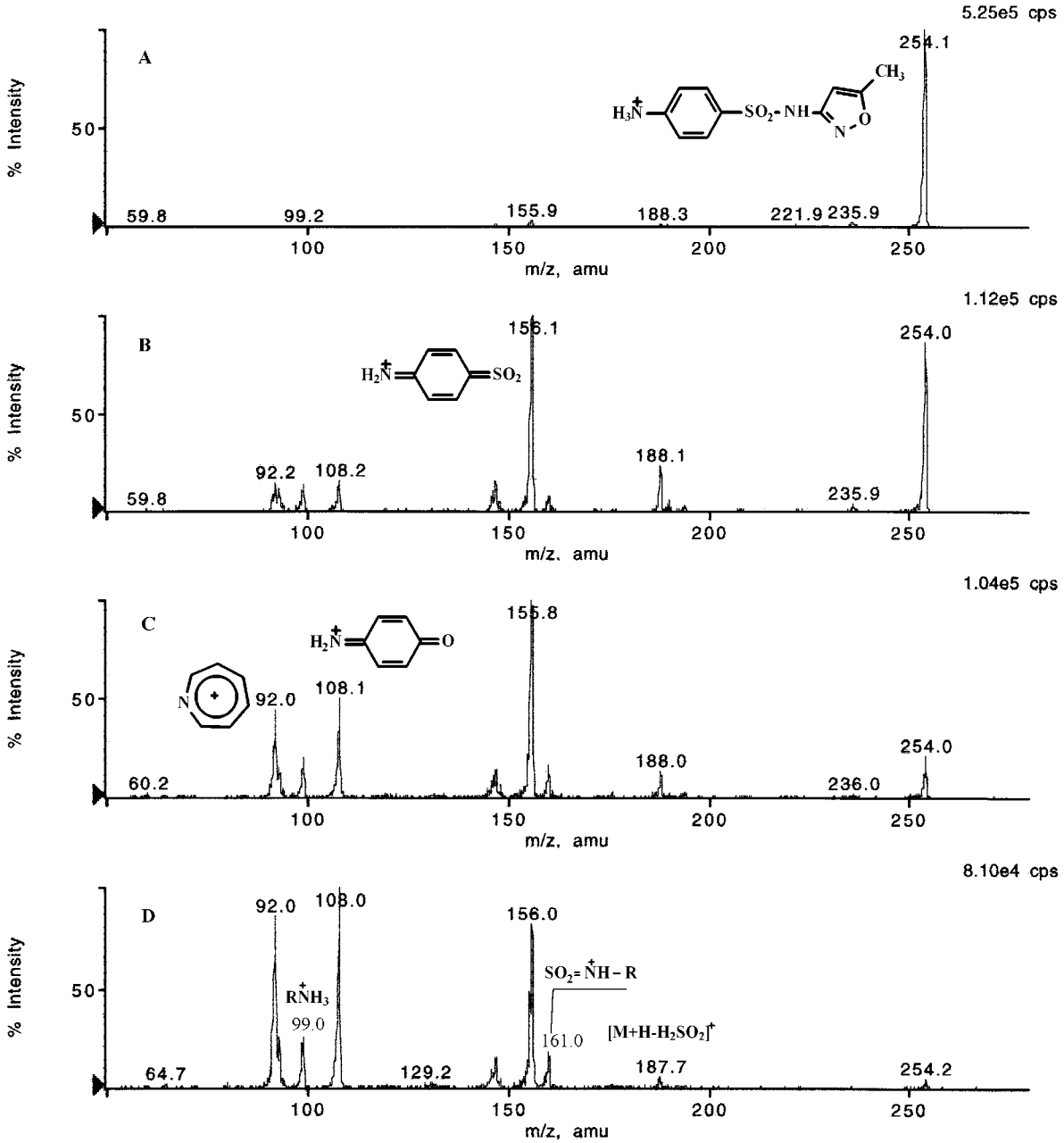


Figure 1. CID mass spectra, acquired in product ion scan, for SMX as a typical example of SA fragmentation increasing collision energy (**A**, 20 eV; **B**, 30 eV; **C**, 35 eV; and **D**, 40 eV).

the collision cell was set at 3 mTorr. The experimental conditions for the MRM LC-MS acquisition were reported in **Table 1**.

The peak areas for selected MRM transitions were determined using PE Sciex package Multiview 1.4.

RESULTS AND DISCUSSION

Optimization of the MS/MS Conditions. A preliminary study of fragmentation was carried out with a 2-fold purpose: (i) to tune the instrumental parameters to improve sensitivity and (ii) to identify unequivocally low amounts of analytes in meat samples. By using a low declustering energy, "up-front CID" was minimized to obtain the maximum transmission of the precursor ion into the collision cell. To this end, for all analytes was selected a declustering potential of 20 V. The CID MS/MS mass spectra, collected in the PI mode, exhibited the protonated molecular ion $[M + H]^+$ as the base peak (**Figure 1A**); increasing the collision energy, fragment ions at 156 ($[M - RNH_2]^+$), 108 ($[M - RNH_2 - SO]^+$), and 92 *m/z* ($[M - RNH_2 - SO_2]^+$) were found to be common to all SAs and were also found to be the most abundant (**Figure 1B−D**); the other fragment ions, characteristic of each substance, were derived from the amide group and differently substituted (**Figure 1D**): RNH_3^+ ($[M + H - 155]^+$), SO_2NHR^+ ($[M + H - 93]^+$), and $[M + H - H_2SO_2]^+$ ($[M + H - 66]^+$). The general scheme for sulfonamide fragmentation and the postulated structures of relative ions was proposed and explained by Pleasance et al. (*18*).

Unlike the majority of SAs, the two structural isomers SIM and SMZ with $[M + H]^+$ at 279 *m/z* showed the compound specific fragment ions at 186 and 124 *m/z* to be the most intense, probably due to the better charge delocalization on the heterocyclic ring. Although SIM and SMZ have the same values of *m/z* for the pseudomolecular ion and the compound specific fragments, they are spectrometrically distinguishable because, under the same collision energy, the intensities of two characteristic product ions are different (**Figure 2**). In the

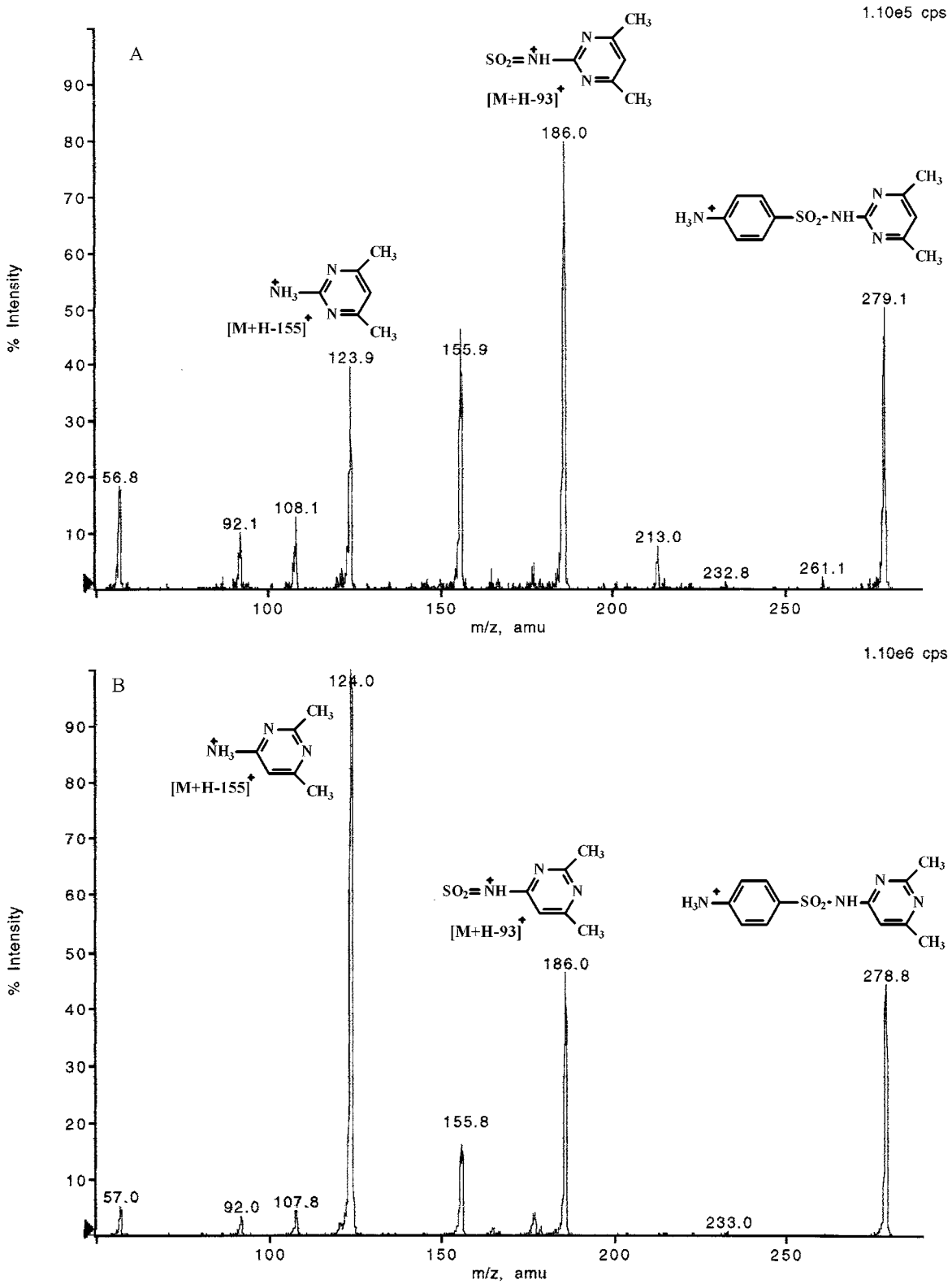


Figure 2. Fragmentation spectra, acquired in product ion scan, for isomers SMZ (**A**) and SIM (**B**).

same way are distinguishable the two isomers SMP and SMM. However, the unambiguous identification of these isomers is guaranteed by the different retention times (**Table 1** and **Figure 3**).

Up to four of the most abundant product ions, observed in full scan MS/MS mass spectra, were selected for the MRM transitions. Among those, only two transitions were chosen on the basis of the best chromatographic S/N with a minimum interference from matrix components. On the basis of Commission Decision 2002/657/EC (*12*), when mass spectrometric detection is performed by fragmentography, specifically by the MRM mode, the pseudomolecular ion shall preferably be selected as the precursor ion, and a system of identification points shall be used to interpret the data; for the confirmation of SAs, listed in Group B of Annex I of Directive 96/23/EC (*13*), a minimum of three identification points shall be required.

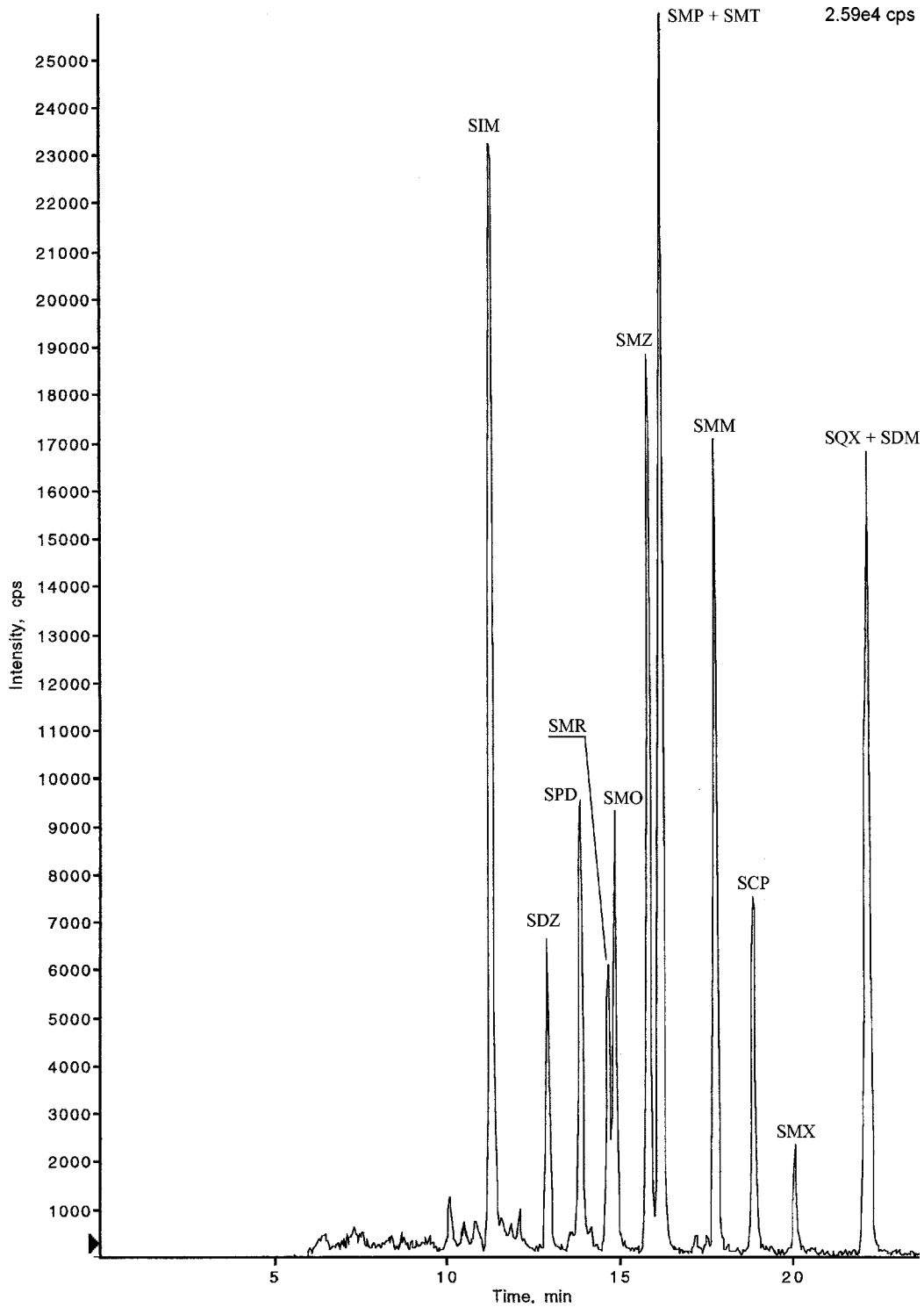


Figure 3. MRM LC-MS chromatogram, acquired in PI with TurboIonSpraysource, of an extract of beef crude meat, fortified at 50 ppb, with 13 SAs selected for this study (0.5 ng injected).

The selection of two MRM transitions, corresponding to one precursor ion and two product ions, consents to earn four identification points. This choice guarantees the specificity of this analysis method.

Optimization of the LC Conditions. The TurboIonSpray source utilizes a heated probe that directs hot nitrogen flow toward the spray aerosol favoring solvent evaporation and ion spray process efficiency; to improve the sensitivity for this multiresidue study, the temperature was set up at 300 °C. For the SAs, studied with this source, the use of drying gas enabled the increase of the S/N ratio by approximately five times.

Despite a slightly lower sensitivity, acetonitrile was chosen as the organic solvent rather than methanol because it allowed one to improve chromatographic separation. The investigated SAs are ampholytes with weakly basic and acidic characteristics (pK_a between 5 and 7.6): the weakly basic characteristics arise from the nitrogen of the anilinic substituent, designated to protonation for mass spectrometric detection, whereas the acidic characteristics arise from the N−H linkage of the sulfoamidic group.

Table 2. Recoveries (RSD) of SAs from Various Meat Matrices at a Fortification Level of 100 ppb

	bovine meat (beef)		porcine meat		poultry meat	
analyte	raw meat	baby food	raw meat	baby food	raw meat	baby food
SPD	89 (5)	90 (4)	92 (5)	94 (3)	91 (4)	93 (3)
SDZ	92 (4)	91 (3)	93 (4)	95 (4)	94(3)	94 (4)
SMX	92 (4)	94 (3)	94 (5)	93 (3)	96 (4)	95 (3)
SMR	99 (4)	101 (4)	98 (6)	99 (4)	98 (6)	100 (5)
SMO	70 (6)	71 (4)	72 (5)	74 (5)	76 (5)	75 (5)
SMT	86 (6)	87 (5)	90 (6)	92 (5)	91 (4)	93 (4)
SIM	94 (4)	97 (4)	98 (5)	100 (4)	99 (3)	98 (4)
SMZ	88 (5)	91 (5)	90 (5)	89 (4)	92 (4)	95 (4)
SMP	86 (6)	84 (4)	85 (6)	85 (5)	88 (5)	87 (3)
SMM	90 (6)	92 (5)	92 (5)	91 (5)	94 (5)	95 (5)
SCP	79 (4)	83 (5)	85 (5)	84 (6)	82 (6)	86 (4)
SQX	81 (5)	84 (4)	82 (6)	82 (5)	85 (5)	87 (5)
SDM	85 (6)	88 (5)	90 (5)	92 (5)	93 (4)	91 (4)

Table 3. Interday Precision (RSD) of Method for Analyzed Meat Matrices at a Fortification Level of 100 ppb

	bovine meat (beef)		porcine meat		poultry meat	
analyte	raw meat	baby food	raw meat	baby food	raw meat	baby food
SPD	8	7	8	6	7	7
SDZ	7	7	8	6	7	6
SMX	8	6	7	8	8	6
SMR	9	7	9	8	8	7
SMO	8	8	9	7	8	7
SMT	7	8	8	8	7	6
SIM	7	6	7	7	6	6
SMZ	8	7	8	6	8	7
SMP	9	7	9	8	7	6
SMM	8	8	9	8	7	7
SCP	8	7	7	8	8	6
SQX	9	7	8	8	9	6
SDM	8	8	9	7	8	7

Because of more marked acidic properties, chromatographic separation was performed in ion suppression by adding formic acid to the mobile phase, which also assisted electrospray ionization.

The effect of the pH of the LC eluent on the LC-MS analysis of the considered SAs was evaluated by adding varying amounts of formic acid so as to obtain pH values of 2.88, 3.03. 3.23, 3.38, 3.53, and 3.88 as measured (pH meter) for water. For each pH value, the same amounts of it were added to acetonitrile. These pH conditions corresponded to the following concentrations of formic acid: 10, 5, 2, 1, 0.5, and 0.1 mmol L^{-1}. By increasing the pH, S/N increased but analyte separation proved unsatisfactory at acid concentrations lower than 0.5 mmol L^{-1}; the acid concentration value in correspondence with retention times was brought closer (in particular coeluted SMT + SMZ + SMP and SQX + SDM) and advanced (analyzing real samples, this could cause a lessening of S/N for the SAs that elute first and that suffer the matrix interfering species eluting with dead volume). The best compromise in terms of sensitivity and analyte separation for analyzing SAs was afforded by the 1 mmol L^{-1} concentration of formic acid. **Figure 3** shows a representative MRM LC-MS chromatogram of a beef crude meat extract.

The use of a diverter valve, with which the mass spectrometry is equipped, allowed one to divert the LC eluent to waste at the beginning and the end of the chromatographic run, to maintain the source cleanliness for a long period of time, and to avoid sudden shrinkages of sensibility, processing real samples.

Table 4. LODs and LOQs of the Method for Analyzing SAs in Beef Raw Meat and Infant Foods

	raw meat		infant food	
analyte	LOD (ppb)	LOQ (ppb)	LOD (ppb)	LOQ (ppb)
SPD	1.4	4.2	1.2	3.6
SDZ	1.6	4.8	0.8	2.4
SMX	2.6	7.8	1.4	4.2
SMR	1.9	5.7	1.5	4.5
SMO	1.1	3.3	1.1	3.3
SMT	1.1	3.3	0.4	1.2
SIM	1.7	5.1	1.7	5.1
SMZ	2.1	6.3	1.6	4.8
SMP	0.7	2.1	0.4	1.2
SMM	0.9	2.7	0.9	2.7
SCP	1.3	3.9	0.5	1.5
SQX	1.2	3.6	1.2	3.6
SDM	0.6	1.8	0.5	1.5

Quantitative Analysis. The developed analytical method was validated by evaluating recovery, precision, linear dynamic range, sensitivity, limit of detection (LOD), and limit of quantitation (LOQ). In particular, the study addresses the impact of the food matrix on ion suppression by comparing standard and matrix-matched calibration curves.

Recovery Studies. Recovery experiments were carried out using blank matrix fortified at 50, 100, and 150 ppb, corresponding to 0.5, 1, and 1.5 times the MRL set by both the EU and FDA. Six replicates were made at each of the indicated levels. The percentage recovery was calculated vs a simulated solution, i.e., an eluate fortified after having been extracted from a blank sample; such a solution was used as the reference material to obviate the matrix effect, responsible for analyte ion suppression.

Among solvents available for SAs extraction from bovine tissues, water was chosen as the extracting phase because of its low affinity toward fatty matter and the polar character of analytes.

At the beginning, the temperature was fixed at 70 °C and the effect of each instrumental parameter (pressure, time, temperature, and extraction cycles) was evaluated in extractive efficiency. The matrix chosen for these preliminary tests was the beef baby food spiked with analytes at a concentration 100 ppb; each trial was triplicated.

Maintaining the temperature at 70 °C and increasing the pressure from 100 to 200 atm, no significant variations (within 2.7%) were observed in recoveries that were comprised between 6 and 70%. Pressure changes have a little impact on analyte recovery, because the pressure effect is to maintain the extracting phase as a liquid when above its atmospheric boiling point and to move the fluids through the system. The pressures used in ASE are well above the thresholds required to maintain the solvents in their liquid states; thus, it does not appear to be a critical parameter.

Increasing the duration of the static phase, the probability that analytes (but also the possible interfering compounds) diffuse from matrix to extraction solvent increases. For this reason, the extraction time was increased from 5 to 15 min resulting in a recovery improvement of less than 10%.

Keeping the pressure at 100 atm and the extraction time at 5 min, the remaining experiments were carried out at the following temperatures: 40, 70, 100, 130, and 160 °C. The recoveries progressively improved as the temperature was

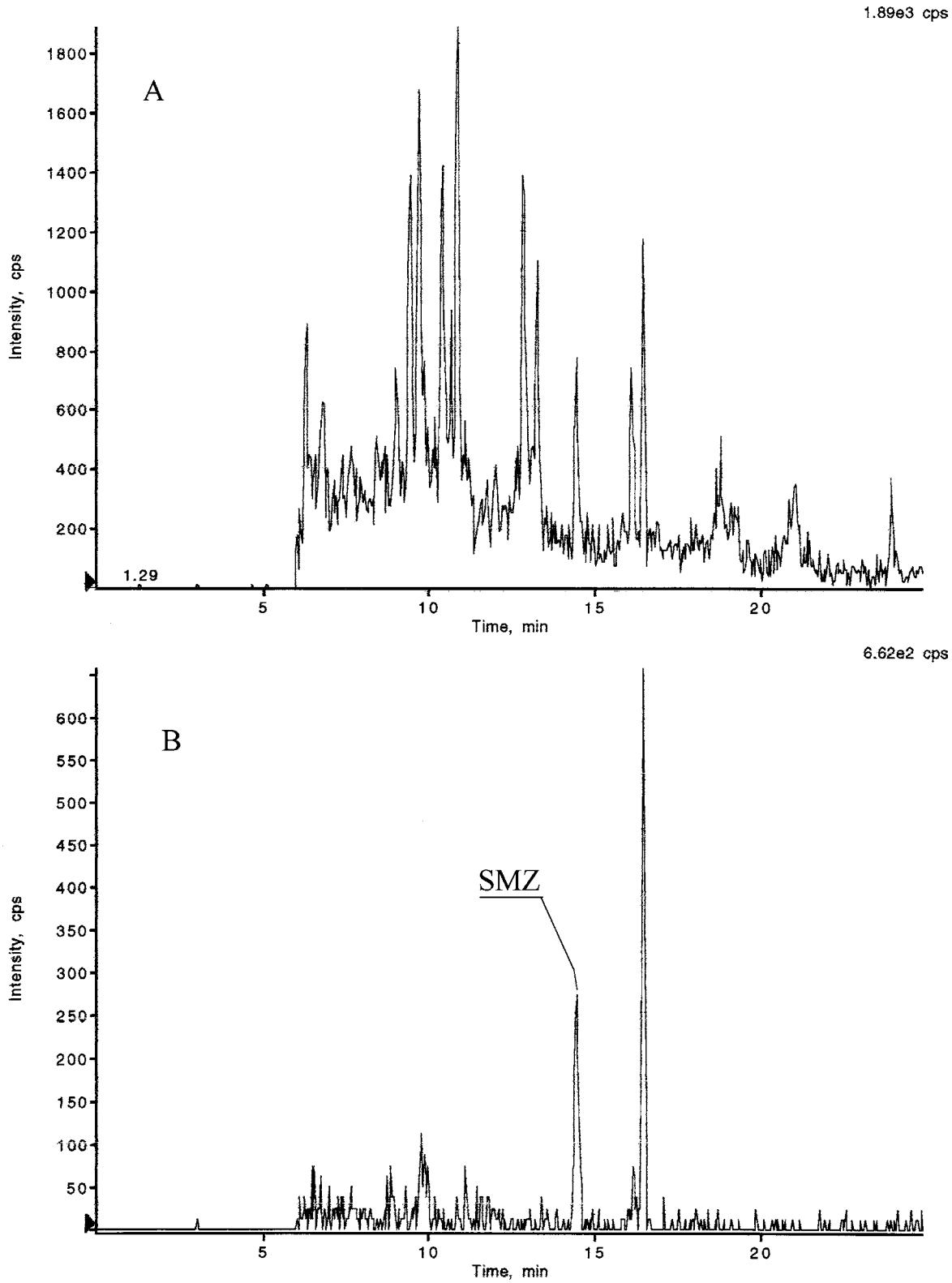


Figure 4. MRM LC-MS chromatogram of baby food of ham, positive for SMZ; panel **A** is the total ion chromatogram; panel **B** is the chromatogram of extracted ion currents 279.3/186 + 279.3/124 of SMZ.

increased, with good results for all analytes except SMO (63%) at 130 °C. For these analytes, the quantitative recoveries (>70%) were reached at 160 °C. No negative effects on analyte stability were observed at this high temperature. Thus, temperature is the most important parameter in ASE extraction. The physicochemical background, according to Richter et alt. (*23*), is that high temperatures reduce the viscosity of solvent, thereby increasing its ability to wet the matrix and solubilize analytes; the thermal energy also assists in disrupting analyte−matrix interactions and aids analyte diffusion through the matrix. Increasing the number of extraction cycles did not lead to improvements in recovery, and so, only one cycle was chosen for the extraction method.

As compared with diatomaceous earth (data not shown), C18 has proved to be the best dispersing mean, as it allowed one to obtain high recoveries of SAs and polar molecules and to reduce

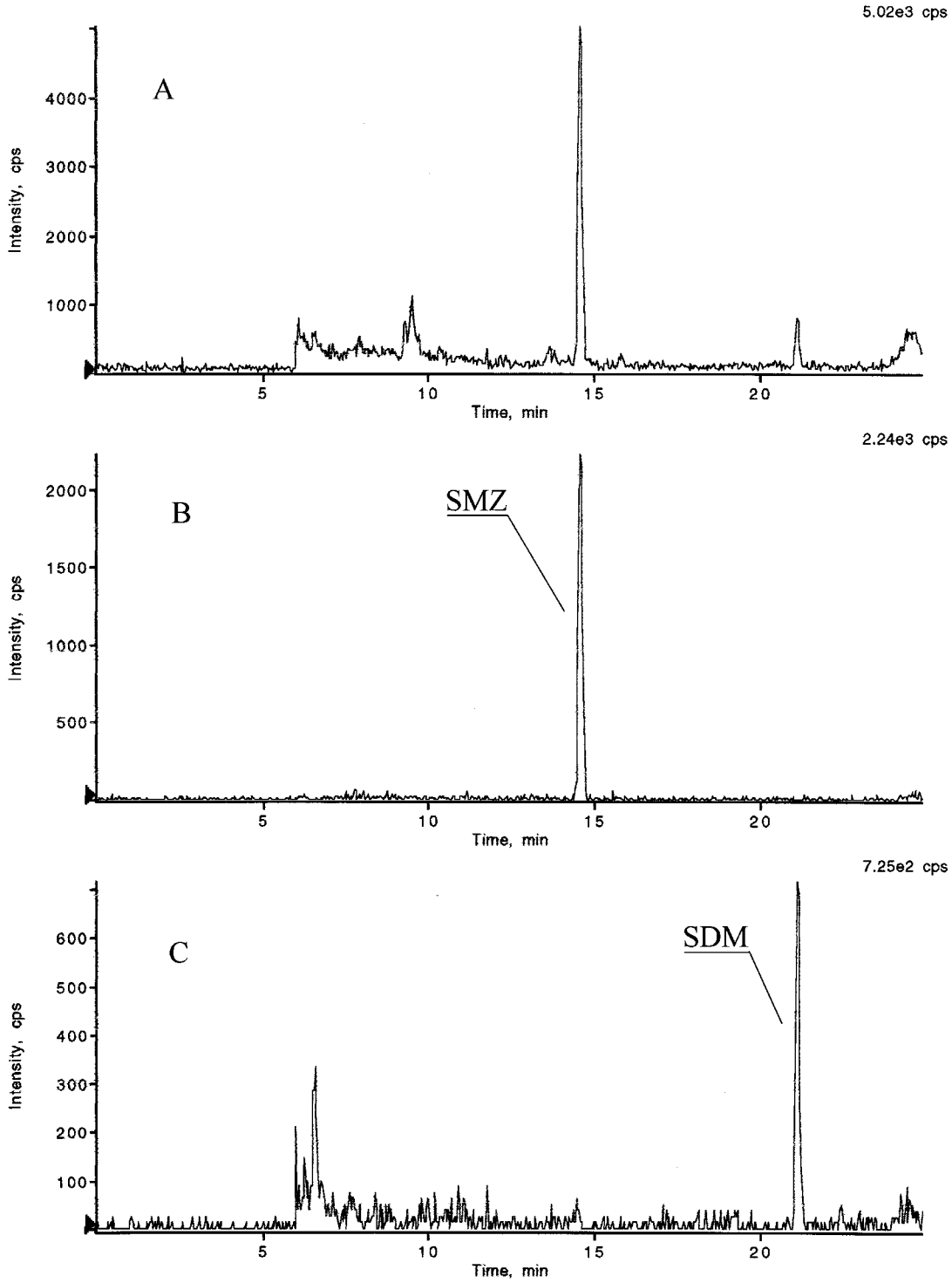


Figure 5. MRM LC-MS chromatogram of a pig meat sample, positive for SMZ and SDM; panel **A** is the total ion chromatogram; panel **B** is the chromatogram of extracted ion currents 279.3/186 + 279.3/124 of SMZ; panel **C** is the chromatogram of extracted ion currents 311.2/156 + 311.2/92 of SMZ.

the matrix effect due to lipid material, which was partly retained from this lipophilic phase.

After having optimized extraction parameters, the recovery trials were carried out on the three meat matrices (bovine, porcine, and poultry) at the above specified fortification levels; the results, reported in **Table 2**, are referred to spiked level of 100 ppb. The experiments proved that recoveries were independent by applied fortification levels.

The ASE resulted in an advantage for several reasons: (i) high reproducibility, due to the total automation and standardization of the procedure; (ii) shorter extraction times; (iii) lower consumption of the extracting solvent as the high temperature increases its solubility; and (iv) the extraction at high temperature and pressure enables the use of water as a good extracting medium for polar compounds as SAs; this solvent is cheap, nontoxic, and nonpolluting. The storage of extracts at −18 °C allowed fat precipitation, due to reduced solubility, as well as elimination of a filtration step and a reduction of the matrix effect.

Precision of Method. The precision of the method was valued by preparing a set of samples of identical matrices, fortified with the analytes to yield concentrations equivalent to 0.5, 1, and 1.5 times the MRL. At each level, the analysis was performed with six replicates. The intraday precision was calculated repeating three independent tests within the same day, while the interday precision was calculated on three different days. The results of intraday precision are showed in **Table 2** near recovery data, while those of interday variability, for the three meat matrices spiked at 100 ppb, are listed in **Table 3**.

Calibration Curve: Linear Dynamic Range, Sensitivity, and Matrix Effect. The analytes were quantified by the external standard quantification procedure. The linear dynamic range was verified between 0 and 25 ng, using calibration standards at 0.25, 1.0, 2.5, 10, 50, 150, and 250 pg μL^{-1}. To exclude memory effects, the first series of data, obtained starting from a lower concentration, was compared with the second, obtained starting from a higher concentration, for a total of four replications for each point. The calibration curves were constructed by applying the least-squares method and using the equation $y = mx + q$ as the regression model. For calibration with internal standards, the peak area ratio of analyte to internal standard was plotted vs the injected amount, while for external calibration, the peak area of analyte was plotted vs the injected amount. The method is sufficiently robust as the intra- and interday variations of the signal intensities for the analytes were similar with and without the use of internal standard. This also means that the compounds can be analyzed successfully without the use of the internal standard.

To evaluate the matrix effect, instrumental calibration curves ("calibration curve in solvent") were compared with calibration curves obtained by blank extracts spiked with analytes ("calibration curve in matrix"). With reference to this, the results for the same matrix were different between raw meat and finished product (baby food). The two calibration curves were nearly superimposable for all meat matrices of baby foods analyzed, while for raw meat the calibration curve displayed a slope, which differed from that observed in the solvent of about 30% for beef bovine and porcine meat and about 10% for veal bovine and poultry meat. It is probable that the fatty material of the meat matrix rather than the proteinous material was responsible for the ion suppression; as reported in the literature (*30*), solvophobic compounds, dispersed in water with a volatile cosolvent, spontaneously form a film on the surface of aerosol droplets, with only the hydrophilic ends of the molecule bound to the substrate liquid; such films are known to be a barrier that inhibits ion evaporation. The regression coefficients for all calibration curves in matrix were not less than 0.9986.

LODs and LOQs. The LOD was set at three times the noise level of the baseline in the chromatogram (S/N = 3), while the LOQ was set at three times the LOD. The noise level depends on the matrix; therefore, there are different LODs for different samples. In reference to this, LODs were estimated from the MRM chromatogram referring to the analysis of 50 ppb of each SA in baby food and raw meat, respectively, of several meat matrices. Higher LODs and LOQs were calculated for beef raw meat; they are listed in **Table 4** together with those of beef baby food. The LOQs of this method are well below the MRLs set by the EU and FDA for residues of SAs in bovine, porcine, and poultry meat.

Analysis of Products on the Market. This method has been used to detect the incidental presence of SAs in baby foods and raw meat bought from Roman supermarkets and butchers' shops. Among 30 analyzed infant foods, one, whose formulation was based on veal meat, was positive to SMT (1.4 ppb) and SDM (<LOQ, roughly around 0.8 ppb); another, based on ham, was positive to SMZ (<LOQ, roughly around 3.5 ppb). Among raw meats, the residues of SMP were found in a veal meat sample coming from Holland (<LOQ, roughly around 1.0 ppb), while an Italian pig meat sample was positive to SMZ (56.2 ppb) and SDM (6.3 ppb).

Figures 4 and **5** show MRM LC-MS chromatograms of the ham-based baby food and of the pig meat sample, whose results were positive to specified SAs.

The total level of found residues did not exceed 100 ppb, the MRL set by the EU and FDA; therefore, all positive samples that resulted were lawful. However, if this is not sufficient evidence to cause worry on behalf of the health of the adult consumer, surely one would at least expect serious concern for infants to be expressed. The positivity of determinate SAs can be explained on the basis of their absorption and metabolism in certain animal species. SMZ, which was found both in a ham-based baby food and in a pig meat sample, is the most frequently revealed SA in pig tissues, as assessed by the Food Safety and Quality Service of the U.S. Agricultural Department. These data can be explained by the wide use of SAs added to pig feed but also by the longer biological life of SMZ in this species than in the others: oxidation of benzenic or heterocyclic rings is an important metabolic pathway for some animal species. Ovine and bovine hydroxylate the pyrimidinic ring of SMZ, while pig seems unable to do so. The SDM and SMP residues, detected in both veal and tender beef, can be explained by the slow gastrointestinal absorption in bovine, for which adequate haematic levels are reached by means of stronger administration of these drugs with increased probability of bovine sample contamination.

ABBREVIATION USED

SAs, sulfonamides; ASE, accelerated solvent extraction; GC-MS, gas chromatography−mass spectrometry; LC-MS/MS, liquid chromatography−tandem mass spectrometry; TLC, thin-layer chromatography; SMX, sulfamethoxazole; SMO, sulfamoxole; SPD, sulfapyridine; SMT, sulfamethizole; SCP, sulfachloropyridazine; SMP, sulfamethoxypyridazine; SDZ, sulfadiazine; SMR, sulfamerazine; SMZ, sulfamethazine; SIM, sulfisomidin; SMM, sulfamonomethoxine; SDM, sulfadimethoxine; SQX, sulfaquinoxaline; SME, sulfameter.

LITERATURE CITED

(1) Aerts, M. M. L.; Hogenboom, A. C.; Brinkman, U. A. Th. Analytical strategies for the screening of veterinary drugs and their residues in edible products. *J. Chromatogr. B* **1995**, *667*, 1−40.

(2) Fidler, D. P. Legal challenges posed by the use of antimicrobicals in food animal production. *Microbes Infect.* **1999**, *1*, 29−38.

(3) Anadòn, A.; Martìnez-Larrañaga, M. R. Residues of antimicrobial drugs and feed additives in animal products: regulatory aspects. *Livest. Prod. Sci.* **1999**, *59*, 183−198.

(4) Niessen, W. M. A. Analysis of antibiotics by liquid chromatography−mass spectrometry. *J. Chromatogr. A* **1998**, *812*, 53−75.

(5) Agarwal, V. K. High-performance liquid chromatographic methods for the determination of sulfonamides in tissue, milk and eggs. *J. Chromatogr.* **1992**, *624*, 411−423.

(6) Commission Regulation 508/1999/EC amending Annexes I to IV to Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Off. J. Eur. Commun.* **1999**, *L 224*, 16−52.

(7) Council Regulation 2377/90/EEC laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Off. J. Eur. Commun*. **1990**, *L 224*, 1−8.
(8) Food and Drug Administration. *New Animal Drugs for Use in Animal Feed*; Code of Federal Regulations; FDA: Washington, DC, 1988; Title 21, Part 558.
(9) Schilter, B.; Renwick, A. G.; Huggett, A. C. Limits for pesticide residues in infant foods: a safety-based proposal. *Regul. Toxicol. Pharmacol.* **1996**, *24*, 126−140.
(10) Verzegnassi, L.; Savoy-Perroud, M.-C.; Stadler, R. H. Application of liquid chromatography-electrospray ionization tandem mass spectrometry to the detection of 10 sulfonamides in honey. *J. Chromatogr*. **2002**, *977*, 77−87.
(11) Bratton, A. C.; Marshall, E. K., Jr. A new coupling component for sulfonamide determination. *J. Biol. Chem*. **1939**, *128*, 537−550.
(12) Commission Decision 2002/657/CE implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Commun*. **2002**, *L 221*, 8−36.
(13) Council Directive 96/23/CE on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC. *Off. J. Eur. Commun*. **1996**, *L 125*, 10−32.
(14) Matusik, J. E.; Sternal, R. S.; Barnes, C. J.; Sphon, J. A. Confirmation of identity by gas chromatography/tandem mass spectrometry of sulfathiazole, sulfamethazine, sulfachloropyridazine, and sulfadimethoxine from bovineor swine liver extracts after quantitation by gas chromatography/electron-capture detection. *J. Assoc. Off. Anal. Chem.* **1990**, *73*, 529−533.
(15) Takatsuki, K.; Kikuchi, T. Gas chromatographic-mass spectrometric determination of six sulphonamide residues in egg and animal tissues. *J. Assoc. Off. Anal. Chem.* **1990**, *73*, 886−892.
(16) Bateman, K. P.; Locke, S. J.; Volmer, D. A. Characterization of Isomeric Sulfonamides by Capillary Zone Electrophoresis coupled with Nano-Electrospray Quasi-MS/MS/MS. *J. Mass Spectrom.* **1997**, *32*, 297−304.
(17) Kennedy, D. G.; McCracken, R. J.; Cannavan, A.; Hewitt, S. A. Use of liquid chromatography−mass spectrometry in the analysis of residues of antibiotics in meat and milk. *J. Chromatogr.* **1998**, *812*, 77−98.
(18) Pleasance, S.; Blay, P.; Quilliam, M. A.; O'Hara, G. Determination of sulphonamides by liquid chromatography, ultraviolet diode array detection and ion-spray tandem mass spectrometry with application to cultured salmon flesh. *J. Chromatogr*. **1991**, *558*, 155−173.
(19) Porter, S. Confirmation of sulfonamide residues in kidney tissue by liquid chromatography−mass spectrometry. *Analyst* **1994**, *119*, 2753−2756.
(20) Fuh, M.-R. S.; Chan, S.-A. Quantitative determination of sulphonamide in meat by liquid chromatography-electrospray-mass spectrometry. *Talanta* **2001**, *55*, 1127−1139.
(21) Bogialli, S.; Curini, R.; Di Corcia, A.; Nazzari, M.; Samperi, R. A liquid chromatography−mass spectrometry assay for analyzing sulfonamide antibactericals in cattle and fish muscle tissues. *Anal. Chem.* **2003**, *75*, 1798−1804.
(22) Bogialli, S.; Curini, R.; Di Corcia, A.; Nazzari, M.; Sergi, M. Confirmatoty analysis of sulfonamide antibactericals in bovine liver and kidney: extraction with hot water and liquid chromatography couplet to a single- or triple-quadrupole mass spectrometer. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1146−1156.
(23) Richter, B. E.; Jones, B. A.; Ezzell, J. L.; Porter, N. L. Accelerated solvent extraction: a technique for sample preparation. *Anal. Chem.* **1996**, *68*, 1033−1039.
(24) Björklund, E.; Nilsson, T. Pressurisez liquid extraction of persistent organic pollutants in environmental analysis. *Trends Anal. Chem.* **2000**, *19*, 434−445.
(25) Chuang, J. C.; Hart, K.; Chang, J. S.; Boman, L. E.; Van Emon, J. M.; Reed, A. W. Evaluation of analytical methods for determining pesticides in baby foods and adult duplicate-diet sample. *Anal. Chim. Acta* **2001**, *444*, 87−95.
(26) Pecorelli, I.; Galarini, R.; Bibi, R.; Floridi, A.; Casciarri, E.; Floridi, A. Simultaneous determination of 13 quinolones from feeds using accelerated solvent extraction and liquid chromatograph. *Anal. Chim. Acta* **2003**, *483*, 81−89.
(27) Djien Liem, A. K. Important developments in methods and techniques for the determination of dioxins and PCBs in foodstuffs and human tissues. *Trends Anal. Chem.* **1999**, *18*, 499−507.
(28) Barker, S. A. Matrix solid-phase dispersion. *J. Chromatogr. A* **2000**, *885*, 115−127.
(29) Barker, S. A. Applications of matrix solid-phase dispersion in food analysis. *J. Chromatogr. A* **2000**, *880*, 63−68.
(30) Fenn, J. B. Ion formation from charged droplets: Roles of geometry, energy and time. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 524−535.

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