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Abstract: Due to the low cost of sulfonamides, they are widely administrated to livestock but their residues in food could be a risk for human health. Litters of milk are daily monitored for the presence of veterinary drugs, including sulfonamides, in the milk industry. A rapid and simple liquid chromatographic-tandem mass spectrometry method for the simultaneous detection of 10 sulfonamides in milk samples is presented in this article. Sulfonamides are extracted with a very simple and fast liquid-liquid extraction. After evaporation, the reconstituted extract is directly injected into the HPLC/MS/MS system and the analytes identified by their retention time and 2 SRM transitions. The method was validated according to Commission Decision 657/2002/EC and maximum residue limits established in the Commission Regulation 37/2010. The limits of detection of all sulfonamides in milk were above 50 μg Kg-1 and recoveries at this concentration were above 90% and relative standard deviation RSD below 15%.

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Dear Dr.:

My name is Carolina Nebot, and I am enclosing here a manuscript entitled "A rapid and simple method for quantification of ten sulfonamides in milk using HPLC/MS/MS and without using SPE" for possible evaluation and publication Journal Dairy Science and Technology. The article has been written by C. Nebot, P. Regal, A. Iglesias, J. Miranda, A. Cepeda and C. Fente of the Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Veterinary Medicine of the University of Santiago de Compostela in Lugo, Spain.

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All the authors are fully aware of the submission of this paper which is our original unpublished work and it has not been submitted to any other journal for reviews.

Sulfonamides are widely administrated to livestock due to their low cost, however their residue in food of animal origin are of great concern. Residue analysis laboratories which control the presence of residues of veterinary medicines in food of animal origin monitor frequently the presence of sulfonamides in milk. The work presented in this article is a simple and fast method to identify and quantify ten sulfonamides in milk. The method save processing time and cost as solid phase extraction is not employed. The method was fully validated according to the guideline described in the European Commission Decision 657/2002. With the present method sulfonamides could be identify and quantified at concentration below the Maximum Residue Level (100 μg Kg^{-1}). This article contributes to the field by improving previous reported methods which are more laborious, complicated and expensive.

The method was successfully applied in a total of 125 milk samples (raw and commercial milk).

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The authors believe that they do not have conflict of interest with any experts.

Waiting for hear from the journal,

Best Regards,

Carolina Nebot

Highlights

- Rapid and simple method for simultaneous extraction of ten sulfonamides from milk samples.
- The method was completely validated at the MRL established by the European legislation.
- LOD and LOQ achieved were below the MRL established for sulfonamides in milk in Europe.
- The method was tested in raw milk samples collected from ten farms located in the North of Spain.

The advantage of the method is the reduction of time processing and cost of material and solvents.

A rapid and simple method for quantification of ten sulfonamides in milk using HPLC/MS/MS and without using SPE

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ABSTRACT

Due to the low cost of sulfonamides, they are widely administrated to livestock but their residues in food could be a risk for human health. Litters of milk are daily monitored for the presence of veterinary drugs, including sulfonamides, in the milk industry. A rapid and simple liquid chromatographic-tandem mass spectrometry method for the simultaneous detection of 10 sulfonamides in milk samples is presented in this article. Sulfonamides are extracted with a very simple and fast liquid-liquid extraction. After evaporation, the reconstituted extract is directly injected into the HPLC/MS/MS system and the analytes identified by their retention time and 2 SRM transitions. The method was validated according to Commission Decision 657/2002/EC and maximum residue limits established in the Commission Regulation 37/2010. The limits of detection of all sulfonamides in milk were above 50 µg Kg^{-1} and recoveries at this concentration were above 90% and relative standard deviation RSD below 15%.

Key words: Sulfonamides, milk, high-performance liquid chromatography, mass Spectrometry.

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1. Introduction

Sulfonamides are one class of antimicrobial agent widely administrated to livestock to prevent and treat bacterial infections. They are used extensively because they are inexpensive, readily available and present a wide spectrum of activity. Two types of sulfonamides are normally administrated: short-life and long-life. While, short-life sulfonamides are mixed with the feed several times per day to prevent bacterial contamination, long-life sulfonamides are injected into the animals at high levels to increase animal growth which is an illegal practice. Regardless of their use, legal or illegal, these substances could remain in the animal and consequently in the food endangering consumer health. Residues of sulfonamides in milk are of great concern because of their potential carcinogenic character and the possibility of the development of antibiotic resistance in human (Balizs & Hewitt, 2003).

To protect consumer's health, maximum residue limits (MRLs) in food of animal origin have been established for sulfonamides in muscle, fat, lever kidney and milk obtained from bovine, caprine and ovine. Their evaluation lays down in the Regulation 470/2009 which repeals Regulation 2377/90 and amends Directive 2001/82 and the Regulation 726/2004. The maximum residue limits in foodstuffs of animal origin are listed in Table 1 of the Annex of Regulation 37/2010.

Cost and effectiveness of analytical methods are becoming an important issue for all laboratories involved in residue analysis. Therefore, there is a need for the development of a simple, rapid, precise, inexpensive, and capable method to detect residues of sulfonamides below MRL in milk. Several analytical methods have been reported for the analysis of sulfonamides in milk, these method employed high performance liquid chromatography (HPLC) (Chung, Lee, Chung & Lee, 2009, Potter, Burns, van de Riet, North & Darvesh, 2007, Serra Bonvehí & Lacalle Gutiérrez, 2009), hydrophilic interaction chromatography (Zheng, Zhang, Peng & Feng, 2008), gas chromatography (Cannavan, Hewitt, Blanchflower & Kennedy, 1996, Chiavarino, Elisa Crestoni, Di Marzio & Fornarini, 1998), thin-layer chromatographyc (Van Poucke, Depourcq & Van Peteghem, 1991). Mass spectrometry detectors are becoming the most commonly way to detect sulfonamides replacing ultraviolet detection due to MS higher sensitivity identification. The development of HPLC/MS/MS techniques opened a new era in qualitative and quantities analysis of veterinary drugs allowing identification of analytes of the same class, something that could not be fully

resolved with chromatography only. In addition to specificity, HPLC/MS/MS is a sensitive technique which sometimes requires less than 1 pg of analyte injected in column for its detection.

Milk is a complex matrix and several pre-treatment methods have been described for the extraction of sulfonamides: liquid-liquid extraction (Kishida & Furusawa, 2004, Tarbin, Clarke & Shearer, 1999, Volmer, 1996), solid phase extraction (SPE) (Vargas Mamani, Reyes Reyes & Rath, 2009, Wu, Li, Liu & Shen, 2007) , solid phase micro extraction (SPME) (Lu, Chen & Lee, 2007, Wen, Zhang, Zhao & Feng, 2005, Zheng, Zhang, Peng & Feng, 2008), stir bar sorptive extraction (SBSE) (Arancibia, Valderrama, Rodriguez, Hurtado & Segura, 2003) and microwave-assisted extractions (MAE) (Akhtar, Wong, Crooks & Sauve, 1998).

All of these protocols are laborious and time consuming. Therefore, to significantly reduce sample manipulation and analysis time several authors have proposed the use of a pre or post column derivatisation. However, this technique introduces a source of variation and may complicate the methodology (Akhtar, Wong, Crooks & Sauve, 1998, Salisbury, Sweet & Munro, 2004).

The aims of the presented study was to developed a rapid, simple and reliable method for the identification of 10 sulfonamides in milk samples by HPLC/MS/MS (Figure 1). The method employs a single extraction protocol without using SPE cartridges or similar materials and with small amount of milk (100 µL). Once they have been extracted, sulfonamides are detected and quantified by HPLC/MS/MS within 36 min. The method was validated according to the Commission Decision 2002/657/EC (EU,The European parliament and the Council of the European Union, 2002), an limits of detection achieved were 10 times below the MRL established for sulfonamides in milk in the Regulation 37/2010 (EU,The European parliament and the Council of the European Union, 2010).

2. Materials and methods

2.1. Chemicals

Sulfachlorpyridazine, sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethizole, sulfamethoxazole, sulfamethoxypyridazine, sulfapyridine, sulfaquinoxaline and sulfathiazole the internal standard (IS) sulfadoxine-d_3 (purity > 98 %) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, methanol and dichloromethane were purchased from Scharlau Chemie (Barcelona, Spain) and formic acid (purity > 99% for analysis) from Acros Organics (Geel, Belgium). Purified water was made in-house with a Milli-Q water system (Millipore, Bedford, MA, USA).

Acidify dichloromethane was prepared by adding to clean 100 ml volumetric flask 25 mL of dichloromethane and 1 mL of formic acid. The volume was then made up to the mark with more dichloromethane, sonicated for 15 min and shaken.

2.2. *Instrumentation*

The HPLC system consisted of a quaternary pump, degasser and auto-sampler model 1100 from Agilent Technologies (Waldbronn, Germany). The HPLC was connected to a mass spectrometer (MS) Qtrap 2000™ from Applied Biosystems, MSD Sciex (Toronto, Canada) which integrates a TurboIonSpray® for molecules ionization. Data acquisition and control were carried out using Analyst 1.4.1 software package (MDS SCIEX). Gas nitrogen was supplied by a nitrogen generator (Peak Scientific Instruments Ltd., Chicago, IL, USA). Nitrogen was employed as curtain gas; nebulizer and collision gas on the MS. Nitrogen was also employed for extracts evaporation on a turbo-evaporator (Turbo Vap® II from Zyrmark, Hopkinton, MA, USA). The HPLC column employed was a Synergi 4 µ Polar-RP 80A (50 mm × 2.00 mm) used in conjunction with a security guard cartridge Polar-RP (4.0 mm × 2.0 mm) both from Phenomenex (Macclesfield, UK). The centrifuge was a 5415D from Eppendorf (Hamburg, Germany).

2.3. Standard Solutions

Stock solutions of individual analytes (sulfonamides) were prepared at 1 mg/mL in 0.1% of formic acid in methanol. A standard solution mixture of sulfonamide was prepared by mixing aliquots of each sulfonamide. This solution was then diluted several times with 0.1% of formic acid in methanol to obtain a series of standard solution with the following

concentrations: 25, 50, 75, 100, 150, 200, 250 and 500 µg L^{-1}. The IS prepared with 0.1% formic acid in methanol at 1 mg mL^{-1}.

The analysis of real samples was conducted simultaneously with 4 types of control samples: blank sample (bovine milk known to be negative), fortified samples (bovine milk spiked to a known concentration of sulfonamides), blank of reagent (only reagents, no milk), fortified reagents (reagents spiked to a known concentration of sulfonamides).

2.4. *Sample Preparation and Extraction*

Homogenized bovine raw milk sample (100 µL) was mixed with 10 µL of IS solution and 800 µL of dichloromethane acidified with 1% of formic acid. The mixture was vortex, sonicated (10 min) and centrifuged at 3500 rpm (10 min), the organic phase was then transferred into a 10-mL Pyrex® glass conical tube. The extraction procedure was repeated with additional 800 µL of acidified dichloromethane. The 2 extracts were mixed and evaporated to dryness at 40ºC. The dried extract was reconstituted with 100 µL 0.1% of formic acid in methanol and transferred into an Ultrafree-MC centrifugal filter (Millipore, Bedford, MA). After filtration the extract was transferred into an ambar HPLC-vial containing a 100 µL insert and the vial kept at -18ºC until sample analysis by HPLC/MS/MS.

2.5. *Quality Control Samples*

During routine analysis, 4 control samples were processed in addition to the samples: a blank sample (milk known to be negative for sulfonamides), a fortified sample (milk spiked to a known concentration of sulfonamides), a blank of reagent (reagents only, no milk) and fortified reagents (reagents spiked to a known concentration of analytes).

2.6. HPLC/MS/MS Analysis

The ultra-filtrated extract was injected (10 µL) into the HPLC column and analytes were eluted with 2 mobile phase components: 0.1% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B). Elution was performed with a gradient mode where the percentage of phase A and B varied as follow: 0- 2 min, 98% A; 2- 6 min, 70% A; 6- 9 min,

60% A; 9- 14 min, 55% A; 14- 17 min, 40% A; 17- 18 min, 15% A; 18- 19 min, 0% A; 21-22 min, 98% A; 22- 30 min, 98% A. The flow rate was held at 0.150 mL/min.

The effluent from the HPLC column was directed into the electrospray source of the MS which was working on positive ion-mode. Optimum signal for sulfonamides ions was obtained with the source temperature at 400ºC, vacuum gauge 2.2 atm, ion spray 5500 V, curtain gas 25 psi, ion source 1 to 55 psi, ion source 2 to 50 psi. The transitions were monitored with a dwell time of 20 mis to achieve, at least, 6 data points across the respective HPLC chromatographic peak. Sulfonamides were identified by their retention times (Rt) and by 2 or more selected reaction monitoring (SRM) transitions. Precursor and product ions selected for each analyte as well as the declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE), and cell exit potential (CXP) employed for their detection are summarized in Table 1.

2.7. *Validation*

Validation was carried out in terms of selectivity, specificity, linearity, recoveries, repeatability (inter and intra-day), decision limit (CCα) and detection capability (CCβ) according to the specifications laid down in the Decision Commission 2002/657 (EU,The European parliament and the Council of the European Union, 2002). . CCα and CCβ are intended to replace the following method characteristics: limit of detection (LOD) and limit of quantification (LOQ) (Stolker, Zuidema, Nielen & Nielen, 2007). The decision limit (CCα) is the lowest concentration level at which a method can discriminate with a statistical certainty of 5 – α that a particular analyte is present. The detection limit (CCβ) is defined as the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. For this study, CCα and CCβ were calculated with equation 1 and 2.

$$CC\alpha = MRL + 1.64 \times SDrep \qquad \text{Equation 1}$$

$$CC\beta = CC\alpha + 1.64 \times SDrep \qquad \text{Equation 2}$$

Where SDrep is the standard deviation within-laboratory reproducibility calculated with the recoveries obtained at the MRL during the three days of the validation.

Blank milk samples found to contain no detectable concentrations of sulfonamides were used as matrix matched. A homogeneous milk sample was divided into 63 sub-samples (100 µL). During 3 consecutive days (Day 1, 2, and 3), 21 samples were fortified as follow: 1 sample was not spiked (Blank sample), 6 samples were spiked 0.5 X MRL, 6 samples were spiked 1 X MRL, 6 samples were spiked 1.5 X MRL, 1 sample at 2 X MRL and 1 sample at 5 X MRL. On Day 4, 10 milk samples obtained from different farms were divided in 2 set of 10 samples. The first set was analysed as blank sample and the second set was fortified at 1 X MRL. Samples (fortified and blank) were processed according to the protocol described for routine analysis.

Limit of detection (LOD) and limit of quantification (LOQ) of the sulfonamides were also calculated with signal/noise ratios (S/N) of 3 and 10, respectively.

Each day of the validation and for each analyte, a calibration graph was built with standard mixture solution containing 25, 50, 75, 100, 150, 200, 250, 500 µg Kg^{-1} of each sulfonamide. Calibration graphs were constructed by representing analyte concentrations in the standard solution against the ratio of peak areas analyte/IS which resulted in a curve that can be described by the equation $y = m \cdot x + b$.

2.8. *Applicability of the Method in Milk Samples Collected from un-treated Animals*

Milk samples employed to investigate the applicability of the method were kindly provided by 10 dairy farms. The farms were located in the North-West of Spain and provided 1 milk sample, collected from a milk tank, during 10 days of 5 consecutive weeks. After collection, samples were frozen at −20°C until analysis.

3. Results and discussion

3.1. *HPLC/MS/MS Determination and Quantification*

For the detection of each sulfonamide by the MS, standard solution of individual compounds (1000 ng g^{-1} in 0.1% formic acid in methanol) was infused directly into the MS.

The objective was to select representative ions (precursor and product ions) and to tune the MS to optimise the detection of the sulfonamides. Sulfonamides fragmentation produced 3 common fragmentation ions: $[M\text{-}RNH_2]^+$ 196 (*m/z* 156), $[M\text{-}RNH_2\text{-}SO]^+$ (*m/z* 108) and $[M\text{-}RNH_2\text{-}SO_2]^+$ 197 (*m/z* 92). It was though that these ions could complicate the correct identification of the sulfonamides due to signal contamination. Once, MS signal was optimised, the next step was to develop an HPLC method which could give satisfactory resolution of each sulfonamide. Several gradient profiles were tested. The best resolution was achieved starting with 98% of phase A and 2% of phase B. Even if sulfonamides eluted at about 65% of phase B, the percentage was increased to 100% to clean the HPLC column. When a standard solution containing a mixture of sulfonamides at 50 µg/L were analysed no signal contamination was observe (Figure 2). Identification of the sulfonamides was conducted according to the specification laid down in the Commission Decision 657/2010. Four identification points were earned, 2 SRM transitions corresponding to 1 precursor and 2 daughters. In addition to the SRM sulfonamides were identified by their Rt.

3.2. Sample Extraction Procedure

To develop an extraction protocol as short as possible for the extraction of the selected sulfonamides from milk samples different organic solvents were tested. Extraction was performed on 1 mL of sample with solvents immiscible in milk and capable of extracting sulfonamides such as dichloromethane, hexane, ethyl-ether and ethyl acetate, the less greaser extract was obtained with dichloromethane. Recoveries of dichloromethane and acidified dichloromethane were compared. Different acids (formic acid, acetic acid and sulphuric acid) and at different percentage (0.1, 0.5 and 1%) were employed to improve sulfonamide recoveries. The best results were obtained with dichloromethane acidified with formic acid at 1%.

HPLC/MS/MS permits identification of analytes in complex matrices such as milk. However, when a large amount of sample is employed laborious extraction processes are required. Available extraction protocols for sulfonamides employed different sample volumes 10, 5, 1 mL (Huang, Yuan & Huang, 2007, Volmer, 1996, Wen, Zhang, Zhao & Feng, 2005).

The inconvenient of using large volume of sample is that more interference could be extracted and more specific cleanup procedure is required for their elimination. Different volumes of milk were tested (3, 2, 1.5, 1, 0.5, 0.1 mL) with different volumes of 1% of formic acid in dichloromethane, recoveries of sulfonamides at the MRL level were compared. The increase of the sample volume did not increase the signal intensity due to matrix effects. The best results were obtained with samples of 0.1 mL, certainly due to a lower matrix effect. Figure 3 and 4 shows SRM chromatograms of sulfonamides in milk samples fortified with the sulfonamides at 0.5 and 1 X MRL.

3.3. Method Validation

According to the Commission Decision 2002/657/EC (EU,The European parliament and the Council of the European Union, 2002) mass spectrometric methods are suitable for consideration as confirmatory methods only following on-line chromatographic separation. Four identification points were earned for each sulfonamides following the Commission Decision 2002/657/EC requirements (EU,The European parliament and the Council of the European Union, 2002). The specificity of the method was evaluated on Day 4. Ten milk samples collected from different farms were analysed for the presence of any interfering peak at the Rt of the sulfonamides. The absence of any chromatographic signal at sulfonamides Rts indicated that no matrix or chemical compounds could gave a false positive signal. The selectivity, investigated by comparing signal intensity ratios of the 2 SRM transition in fortified and blank samples, resulted to be reliable and within the criteria laid down in the European Commission.

Instrument calibration curves (ICC) were built, for each sulfonamide, with the standard mix solutions. They were constructed by representing the ratio (analyte peak area / IS peak area) against the correspondent concentration of the pharmaceutical in milk samples expressed in µg/L. All graphs were described by the equation $y = mx + b$. These curves were used to calculate recoveries of sulfonamides in fortified samples. Samples calibration curves (SCC) were built by representing the ratio (analyte peak area / IS peak area) against the concentrations of the sample. SCCs were employed to calculate CCα and CCβ and ICCs to

calculate mean recoveries, variation coefficients and uncertainty resulted from inter-day and intra-day experiments conducted over a 4 day period.

Correlation coefficients (R^2) of CCI were above 0.985 (see Table 2), R^2 of SCC were < 0.980. These results indicated a good linearity of the method for sulfonamides detection in milk samples and at concentration between 0 and 500 µg/L. Recoveries of sulfonamides were calculated at all concentrations (0.5, 1, 2 and 5 X MRL),

Intra- and inter-day assay recoveries were calculated by replicate analysis of quality control samples containing known amounts of sulphonamides (0, 0.5, 1, 1.5, 2 and 5 X MRL). Six replicates samples were employed for concentrations of 0.5, 1 and 1.5 X MRL level, the experiment was repeated over 3 consecutive days to investigated inter-day variability. Acceptable quantification results were obtained for all analytes in fortified milk samples, as similar results were obtained at the different concentration levels only the results obtained for 0.5 X MRL are presented in Table 2. Intra and inter-day recoveries of sulfamethoxypyridazine, sulfachlorpyridazine, sulfadimethoxine and sulfaquinoxaline were approximately 100%, those of sulfamethazine, sulfamethoxazole and sulfapyridine were between 95 and 103%. Inter- and intra-day RSD of sulfonamides recoveries were acceptable; inter-day RSD were < 10% and intra-day < 15%.

CCα, CCβ, LOD and LOQ achieved are summarised in Table 2. Sulfapyridine and sulfadiazine resulted to have the lowest CCα 106.1 and 108.2 µg Kg^{-1}, respectively. On the other hand, the highest CCα was achieved for sulfadimethoxine. To calculate LOD and LOQ milk samples fortified with sulfonamides at different concentration and the S/N ration of each sulfonamide was measured. LOD of sulfachlorpyridazine, sulfamethizole, sulfamethoxazole and sulfamethoxypyridazine were higher than 25 µg Kg^{-1} as S/N ratio was higher than 10 for this concentration. Even if at 12.5 µg Kg^{-1}, the S/N ratio were higher than 10 for sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethizole, sulfamethoxypyridazine, sulfaquinoxaline, sulfathiazole it was decided to reported a LOD of 12.5 for these substances (Table 2) as the method was not investigated at lower concentration (Table 2).

3.4. Applicability

One hundred samples collected from 10 different farms were analysed for the presence of sulfonamide. Two samples resulted to be compliant for sulphonamides; one was positive for sulfadiazine (0.021 mg Kg^{-1}) and the other for sulfamethoxypyridazine (0.063 mg Kg^{-1}). Figure 5 shows SRM chromatograms of sulfonamides in a muscle samples which resulted to be compliant and negative.

4. CONCLUSIONS

This work presents a suitable method for the simultaneous extraction, detection of 10 sulfonamides in bovine milk samples. Sample process time and cost were reduced using small sample size (100 µL). Sulfonamides were identified and quantified by HPLC/MS/MS employing 2 SRM and their Rt. The method was validated according to the Commission Decision 657/2002. The CCα achieved were above MRL for all sulfonamides and LOD below 50 µg Kg^{-1}. The method was successfully in real milk samples. The method presented has a number of advantages compared to other available methods: short time of analysis (within 3 hours the sample could be processed and analysed), reduction of solvents required, save on SPE cartridges or similar material, the total cost of the sample process has been reduced considerable by the reduction of the sample size.

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Figure capitation

Figure 1. Structures of the sulfonamides selected for the study

Figure 2. SRM chromatograms of a standard mix of sulfonamides at 50 µg Kg: (a) sulfachlorpyridazine, (b) sulfadiazine, (c) sulfadimethoxine, (d) sulfamethazine, (e) sulfamethizole, (f) sulfamethoxazole, (g) sulfamethoxypyridazine, (h) sulfapyridine, (i) sulfaquinoxaline and (j) sulfathiazole

Figure 3. SRM chromatograms of a milk samples spiked at 0.5 x MRL of sulfonamides: (a) sulfachlorpyridazine, (b) sulfadiazine, (c) sulfadimethoxine, (d) sulfamethazine, (e) sulfamethizole, (f) sulfamethoxazole, (g) sulfamethoxypyridazine, (h) sulfapyridine, (i) sulfaquinoxaline and (j) sulfathiazole

Figure 4. SRM chromatograms of a milk sample compliant for sulfonamides: (a) sulfachlorpyridazine, (b) sulfadiazine, (c) sulfadimethoxine, (d) sulfamethazine, (e) sulfamethizole, (f) sulfamethoxazole, (g) sulfamethoxypyridazine, (h) sulfapyridine, (i) sulfaquinoxaline and (j) sulfathiazole

Tables

Table 1. Precursor ion, product ions, declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE), cell exit potential (CXP) and retention time (Rt) selected to identify and quantify the sulfonamides

Analyte	Precursor $[m/z]^+$	Product $[m/z]^+$	DP	EP	CEP	CE	CXP	Rt (min)
Sulfachlorpyridazine	284	156[a]	21	6	14	17	4	13.8
		92	21	6	14	34	4	
		108	21	6	14	33	4	
		165	21	6	14	61	4	
Sulfadiazine	251	92 [a]	21	6	14	17	4	11.8
		156	21	6	14	33	4	
		108	21	6	14	31	4	
		96	21	6	14	59	4	
Sulfadimethoxine	311	156 [a]	31	7	14	17	4	14.8
		92	31	7	14	41	4	
		108	31	7	14	37	4	
		245	31	7	14	67	4	
Sulfamethazine	279	186 [a]	26	5	14	19	4	12.7
		92	26	5	14	39	4	
		124	26	5	14	29	4	
		108	26	5	14	75	4	
Sulfamethizole	271	156 [a]	21	6	16	15	4	13.0
		92	21	6	16	35	4	
		108	21	6	16	33	4	
		116	21	6	16	59	4	
Sulfamethoxazole	254	92 [a]	21	6	14	35	4	14.1
		156	21	6	14	35	4	
		108	21	6	14	31	4	
		99	21	6	14	59	4	
Sulfamethoxypyridazine	281	156 [a]	26	7	14	17	4	13.1
		92	26	7	14	37	4	
		108	26	7	14	35	4	
		126	26	7	14	65	4	
Sulfapyridine	250	92 [a]	21	5	14	17	4	11.9
		156	21	5	14	33	4	
		108	21	5	14	33	4	
		184	21	5	14	59	4	
Sulfaquinoxaline	301	92 [a]	26	7	14	17	4	15.0
		156	26	7	14	41	4	
		108	26	7	14	31	4	

Analyte	Precursor $[m/z]^+$	Product $[m/z]^+$	DP	EP	CEP	CE	CXP	Rt (min)
		184	26	7	14	67	4	
Sulfathiazole	256	156 [a]	26	7	14	15	4	12.1
		92	26	7	14	33	4	
		108	26	7	14	29	4	
		104	26	7	14	55	4	

406 [a]Product ion employed for quantification

407

408

Table 2. Regression coefficients (R^2), decision limits (CCα), detection capabilities (CCβ), Limit of detection (LOD), Limit of quantification (LOQ), standard deviation (SD) and relative standard deviation (RSD) of sulfonamides in milk samples.

	ICC	SCC	[a]Intra-day (n=6)			[b]Inter-day (n=18)			Critical levels			
	(r2)	(r2)	Mean recoveries (%)	SD (%)	RSD (%)	Mean recoveries (%)	SD (%)	RSD (%)	CCα (µg Kg^{-1})	CCβ (µg Kg^{-1})	LOD (µg Kg^{-1})	LOQ (µg Kg^{-1})
Sulfachloropyridazine	0.995	0.993	102	9.3	9.2	101	5.9	5.9	109.8	119.6	30.0	36.0
Sulfadiazine	0.993	0.979	99	8.0	8.1	90	4.9	5.5	108.2	116.3	12.5	12.5
Sulfadimethoxine	0.991	0.989	107	10.3	9.6	101	13.3	13.7	122.8	145.7	12.5	12.5
Sulfamethazine	0.992	0.988	96	7.6	7.9	103	9.3	9.1	115.4	130.8	12.5	25.0
Sulfamethizole	0.995	0.995	90	6.8	7.5	92	11.3	12.3	118.6	137.2	40.0	45.0
Sulfamethoxazole	0.987	0.976	102	5.4	5.3	97	7.0	7.2	111.5	123.0	25	35.0
Sulfamethoxypyridazine	0.988	0.98	101	5.7	5.7	108	8.9	8.2	114.6	129.2	38	45
Sulfapyridine	0.989	0.982	104	10.0	9.6	95	3.7	3.9	106.1	112.2	12.5	25.0
Sulfaquinoxaline	0.985	0.98	110	9.2	8.4	105	13.7	12.4	121.4	142.9	12.5	12.5
Sulfatiazole	0.981	0.98	90	6.4	7.1	95	11.9	12.5	119.6	139.2	12.5	12.5

ICC: Instrument calibration curves; SCC: Sample calibration curves; [a]Intra-day results of milk samples fortified with 50 µg Kg^{-1} of each sulfonamides; [b]Inter-day results of milk samples fortified with 50 µg $Kg^{-1}$of each sulfonamides

Figure 1

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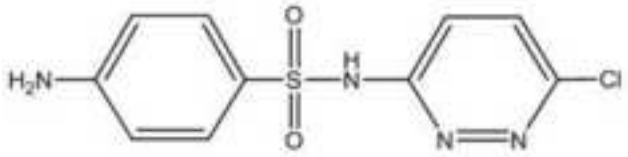
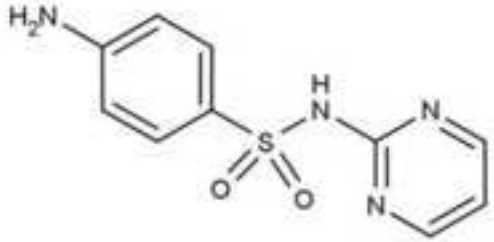
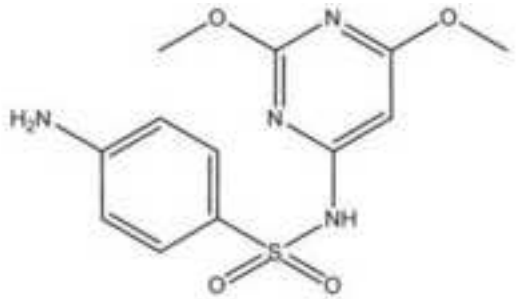


Sulfachloropyridazine



Sulfadiazine



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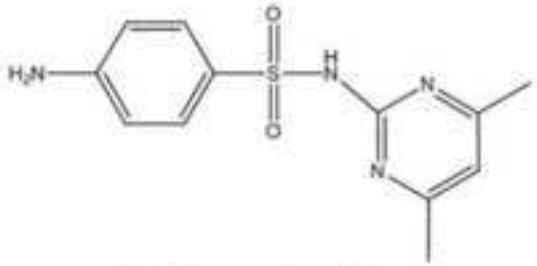

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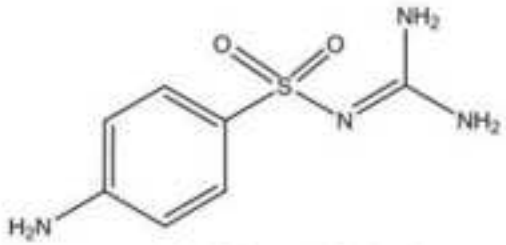

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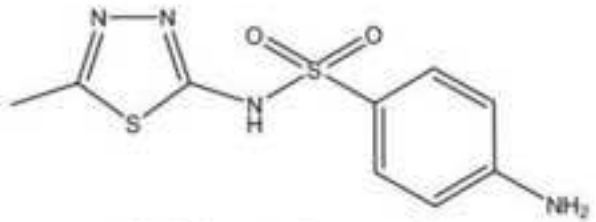

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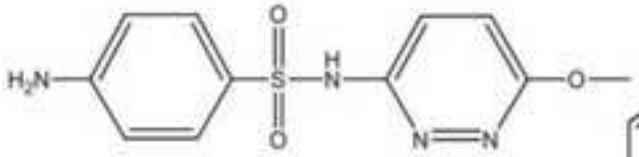
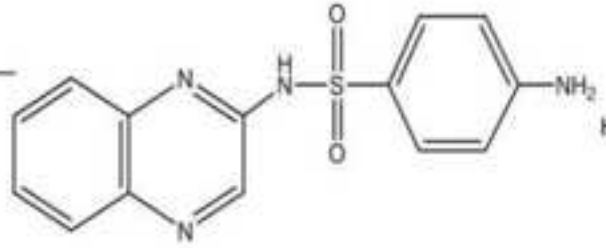
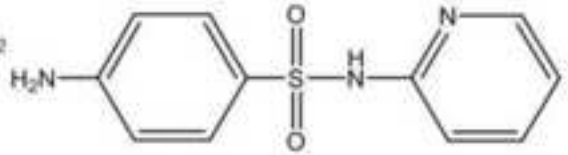


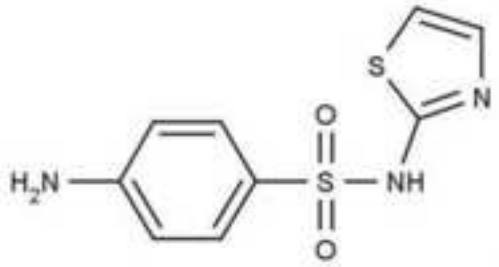
Sulfamethoxypyridazine



Sulfaquinoxaline



Sulfapyridine



Sulfathiazole

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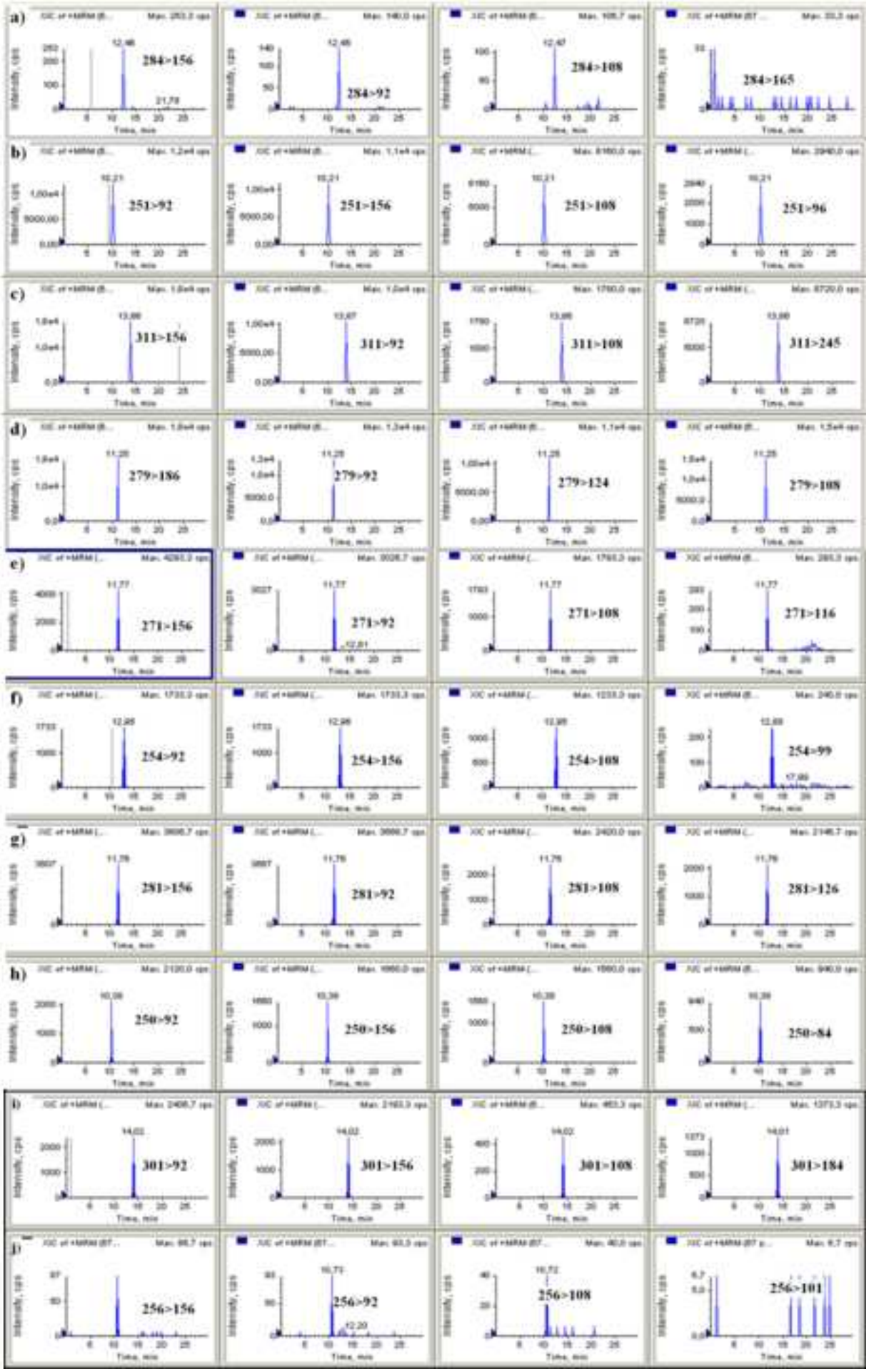

Figure 3

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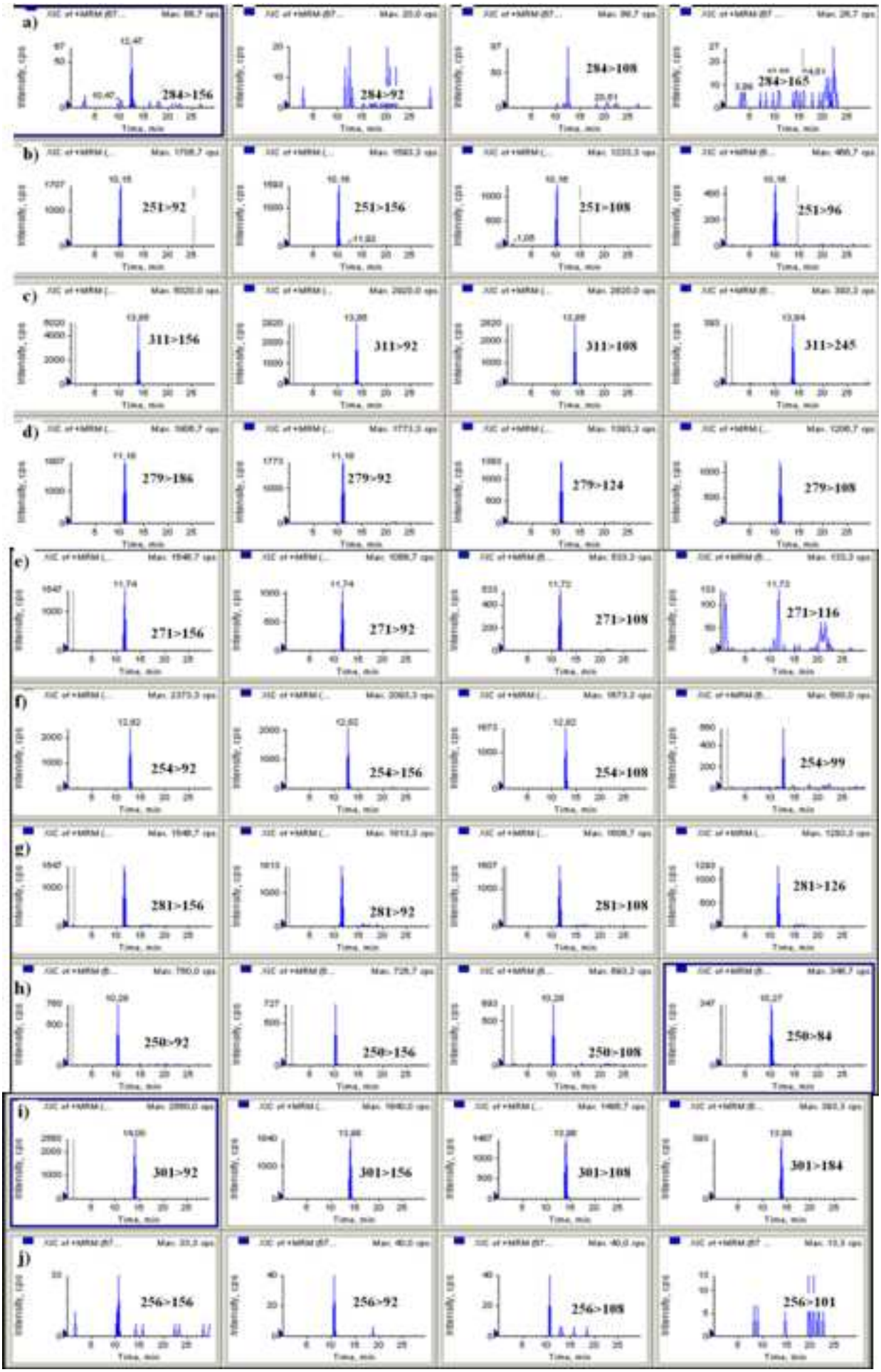

Figure 4

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