

High-Throughput Quantitation of Seven Sulfonamide Residues in Dairy Milk using Laser Diode Thermal Desorption-Negative Mode Atmospheric Pressure Chemical Ionization Tandem Mass Spectrometry

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Sulfonamides are antibiotic compounds widely used in the dairy industry. Their presence in dairy milk poses a risk to public health and may also contribute to the spread of antibiotic resistance in bacteria. Sulfonamide residues in dairy milk were quantified by tandem mass spectrometry (MS/MS) using a novel ionization source based on laser diode thermal desorption-negative mode atmospheric pressure chemical ionization (LDTD-APCI(-)). Seven sulfonamides spiked in milk were extracted with acetonitrile, which yielded high recoveries (77.5–101.5%). Calibration curves in the matrix showed good linearity ($0.9977 \geq R^2 \geq 0.9658$) over the dynamic range (1.6–500 $\mu\text{g L}^{-1}$), and limits of quantitation were between 2 and 14 $\mu\text{g L}^{-1}$, lower than or of the same magnitude as maximum residue criteria set by several regulatory agencies (10–100 ng L^{-1}). In addition, the run time using the LDTD-MS/MS system was 30 s per sample, as compared to actual methods running from 7 to 84 min for the same sulfonamide residue compounds, which gave the method the high screening throughput capacity necessary for monitoring milk production.

KEYWORDS: High-throughput; LDTD; milk; sulfonamides; tandem mass spectrometry

INTRODUCTION

Since the early 1950s, antibiotics have been used in agriculture to treat or prevent infections in food-producing animals. They are also employed as feed additives to reduce animal susceptibility to stress-related diseases or to enhance growth (1). The nontherapeutic application of antibiotics amounts to about 90% of the total agricultural applications in the U.S. (2), and it is estimated that more than 1.6 million kilograms of antibiotics is used annually on cattle in the U.S. alone (3).

In the dairy industry, sulfonamides are widely used and their improper use in lactating dairy cattle may result in drug level residues sufficiently high to pose a risk for consumer health (4). In the U.S., except for the approved label use of sulfadimethoxine, sulfabromomethazine, and sulfaethoxyypyridazine, all other sulfonamide compounds are prohibited for extralabel use in lactating dairy cattle (5). However, the U.S. Food and Drug Administration reported in 2005 the illegal use of sulfonamides in a dairy farm. The agency declared that “the use of a small amount of a sulfonamide drug in a lactating dairy cow can result in the contamination of milk from several hundred cows when mixed in a bulk tank” (4). Even the occurrence of small amounts of antibiotics in food products is of concern, as it can contribute to the spread of antibiotic resistance in bacteria (6). Furthermore,

sulfamethazine (also known as sulfadimidine) is carcinogenic to animals (7). In order to protect consumers, many regulatory agencies in several countries have set maximum residue limits (MRLs) for sulfonamides in dairy milk ranging between 10 and 100 $\mu\text{g L}^{-1}$ (Table SI-1, Supporting Information) (8–11).

Sulfonamides can be rapidly identified in milk samples by dipstick immunoassay techniques, but few techniques are able to quantitate large groups of sulfonamides simultaneously (12). Many multiresidue determination methods of sulfonamide in dairy milk have been proposed so far in the literature, mainly using liquid chromatography (LC). Most of these methods require time-consuming preparation steps such as solid-phase extraction (SPE) (13, 14), with or without liquid–liquid extraction (LLE) (15, 16), followed by evaporation to dryness. Moreover, for the LC techniques using fluorescence or ultraviolet detection, an additional derivatization step is also required prior to analysis to increase the method sensitivity (17). In combination with the time-consuming sample-preparation steps, the chromatographic step requires several minutes (from 7 to 84 min) (13–16), increasing the overall time needed for a sample to be analyzed. Ideally, each dairy production should be tested to avoid the contamination of entire batches by a few adulterated milk samples (4), but the actual analysis turnover limits the number of samples that can be analyzed by inspecting agencies.

We have developed a sensitive, high-throughput screening method of seven sulfonamides (Chart 1) in order to meet the

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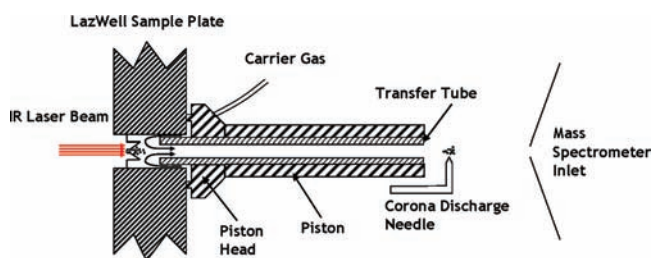
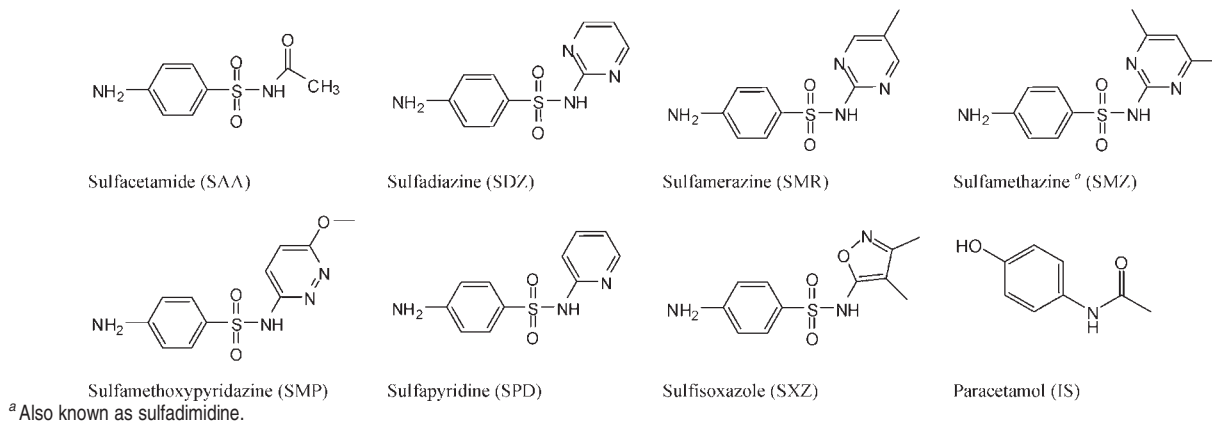
Chart 1. Molecular Structures of the Studied Sulfonamides^a

Figure 1. Diagram showing the laser diode thermal desorption/atmospheric pressure chemical ionization (LDTD-APCI) source.

daily analysis of milk samples required to ensure food safety. The proposed method uses a simple sample cleanup procedure and requires no chromatography before detection. Samples are analyzed in only 30 s using a new laser diode thermal desorption-atmospheric pressure chemical ionization (LDTD-APCI) source coupled to a tandem mass spectrometer.

MATERIALS AND METHODS

Reagents and Materials. Solid sulfonamide standards of sulfacetamide (SAA), sulfadiazine (SDZ), sulfamerazine (SMR), sulfamethazine (SMZ), sulfamethoxy-pyridazine (SMP), sulfapyridine (SPD), sulfisoxazole (SXZ), and paracetamol (internal standard) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (ACN, Accusolv grade) was obtained from Anachemia (Montréal, Canada), and water (HPLC Reagent grade) was obtained from J.T. Baker (Philipsburg, NJ). Dairy milk samples were obtained directly from the Ministère de l'Agriculture, Pêcheries et Alimentation du Québec (MAPAQ), and represent a pooled dairy milk equivalent to 1 month of production from an individual dairy farmer. Adulterated dairy milk samples were provided by the MAPAQ's Food Analysis Laboratory.

We prepared stock solutions of 1000 mg L⁻¹ of the seven sulfonamides in ACN and of 10 mg L⁻¹ of paracetamol (internal standard) in water. Working solutions of sulfonamides were prepared daily by diluting 10 μL of stock solution in 990 μL of H₂O for a final concentration of 10 mg L⁻¹.

Sample Preparation. *Dairy Milk Samples.* We added an 800 μL volume of ACN to 200 μL of whole dairy milk. The samples were mixed with a vortex for 4 min and centrifuged at 24 000g for 5 min using Nanosep 0.2 μm devices (Pall Corporation, Port Washington, NY). We added 10 μL of internal standard stock solution, and the sample was mixed with a vortex for 10 s. A 2 μL volume of the supernatant was transferred to a LazWell 96-well plate manufactured by Phytronix Technologies (Québec, Canada) and dried at 37 °C in a convection oven before LDTD-APCI(-)MS/MS analysis.

Extraction Recovery Samples. Two sets of spiked dairy milk samples (A and B) were prepared to determine the recovery of the extraction. In set A, the analytes were spiked after extraction, while for set B they were spiked before extraction. To prepare A, the same procedure as given in

Table 1. Selected Reaction Monitoring (SRM) Transition Parameters

compd	precursor ion (<i>m/z</i>)	product ion (<i>m/z</i>)	collision energy (V)	tube lens (V)
SAA	213.0	171.0	21	50
SDZ	249.3	185.1	19	62
SMR	263.3	199.1	19	65
SMZ	277.3	106.1	38	65
SMP	252.0	156.1	17	43
SPD	248.3	184.1	20	62
SXZ	266.3	171.0	21	48
PAR(IS)	150.0	107.0	21	50

Dairy Milk Samples was performed, except that before transferring the supernatant to the plate, we added 10 μL of the sulfonamide working solution (100 μg L⁻¹ spike) and the sample was mixed with a vortex for 10 s. A 2 μL volume of the supernatant was then transferred to a LazWell 96-well plate and dried before LDTD-APCI(-)MS/MS analysis. For set B, a 10 μL volume of the sulfonamide working solution was added to 200 μL of whole dairy milk (500 μg L⁻¹ spike, equivalent to 100 μg L⁻¹ after extraction) and mixed using a vortex for 10 s. Spiked samples were allowed to settle for a period of 12 h to be more representative of adulterated milk samples. After the equilibrium period, the Dairy Milk Samples procedure was followed. The A samples were used to determine maximum sulfonamide signal in the milk matrix, while the B samples were prepared to simulate sulfonamide-adulterated milk samples.

Instrumentation. Thermal desorption and ionization was performed using a LDTD source, Model T-960 (Phytronix Technologies, Québec, Canada). This source was mounted on a TSQ Quantum Ultra AM triple quadrupole (Thermo Fisher Scientific, San Jose, CA).

The LDTD source uses the rapid heating of a metal surface induced by a 980 nm laser diode to transfer the dry analytes from the solid to the gas phase. In LDTD, the sample has no contact with the infrared photons emanating from the laser diode; therefore, desorption is induced solely by heat transfer (Figure 1). Once in the gas phase, the volatilized compounds (neutrals) are transported by a carrier gas (air) in a transfer tube to a corona discharge region where ionization occurs at atmospheric pressure by chemical ionization reactions (18).

LDTD-APCI(-) Parameters. The laser power pattern was the following: 0% laser power for 2 s, 0% to 35% in 3 s, 35% to 0% in 0.01 s, and then 0% for 3 s using a 20 W diode laser. Air was used as the carrier gas at a flow rate of 3 L min⁻¹. APCI was performed in the negative mode, and the current was -3 μA.

MS/MS Parameters. The ion sweep gas was set to 0.3 arbitrary unit. The ion transfer capillary temperature was 320 °C. Quantitation was performed in the selected reaction monitoring (SRM) mode. The SRM parameters appear in Table 1. Resolution was set to 0.7 u full width at half maximum, scan width at *m/z* 0.7, and the scan time at 0.01 s.

Method Validation. Six-point calibration curves were performed on spiked dairy milk samples. Each concentration level (3.1, 6.3, 13, 25, 100, and 500 μg L⁻¹) was analyzed in triplicate. The limit of detection (LOD)

Table 2. Method Validation Parameters of the Target Sulfonamides

sulfonamide	recovery % (RSD) ^a	equation	R ²	LOD (μg L ⁻¹)	LOQ (μg L ⁻¹)
SAA	100 (10)	$y = 0.00321 + 0.00099x$	0.9929	3	10
SDZ	82 (7)	$y = 0.00102 + 0.00071x$	0.9776	4	14
SMR	81 (9)	$y = -0.00268 + 0.00178x$	0.9970	1	3
SMZ	102 (9)	$y = 0.00243 + 0.00427x$	0.9744	2	6
SMP	78 (9)	$y = -0.00352 + 0.00175x$	0.9925	2	6
SPD	94 (7)	$y = -0.00269 + 0.00258x$	0.9977	1	4
SXZ	89 (9)	$y = -0.00304 + 0.00104x$	0.9658	0.5	2

^a Spiking level 100 μg L⁻¹. The precision of the method was <10% for all the calibration levels tested.

and limit of quantitation (LOQ) were determined as 3.3 and 10 times, respectively, the standard deviation of the *y* intercept divided by the slope of the calibration curve, as proposed by the International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use (19). Method precision was determined on spiked dairy milk samples by measuring the percent RSD of all calibration curve levels except for the lowest in triplicate measurements. Method blanks (nonspiked dairy milk samples subjected to the entire preparation and analysis procedure) were used to detect sample contamination.

Extraction Recovery Study. Extraction recovery was determined by analyzing the A and B sets of samples and comparing their area ratios. Sulfonamide recovery was therefore calculated using the formula

$$\text{recovery (\%)} = \left[\frac{\left(\frac{\text{sulfonamide area}}{\text{IS area}} \right)_{\text{in B sample}}}{\left(\frac{\text{sulfonamide area}}{\text{IS area}} \right)_{\text{in A sample}}} \right] \times 100\%$$

Three recovery experiments were performed for each individual sulfonamide, and each sample was analyzed in triplicate.

Comparison with LC-MS/MS and Analysis of Adulterated Samples. Dairy milk samples are routinely analyzed for antibiotic adulteration by a validated LC-MS/MS method (20) at the Food Analysis Laboratory of the MAPAQ (details on this method are found in the Supporting Information). Aliquots of the samples testing positive were subsequently prepared and analyzed according to our LDTD-APCI(-)-MS/MS method.

RESULTS AND DISCUSSION

LDTD-APCI(-)-MS/MS. Most of the mass spectrometric methods of determination of sulfonamides (13–16) usually use ionization in the positive mode. However, in order to improve method performance, we also explored APCI in the negative mode (APCI(-)). Analysis of sulfonamides with APCI(-) has seldom been reported in the literature (21, 22). Full scan spectra obtained by LDTD-APCI(-) yielded the deprotonated pseudomolecular ion $[M - H]^-$ with little or no fragmentation. This indicates that LDTD-APCI is a soft ionization source with low thermal degradation, despite desorption being performed by thermal transfer. Also, APCI-LDTD is more efficient than traditional LC-APCI, as high amounts of water and organic solvents reduce the abundance of small hydronium clusters, which are the main reactive species during the ionization process (23). Selected reaction monitoring (SRM) transitions (Table 1) were chosen according to intensity and specificity. Observed fragments corresponded to sulfonamide group specific ions such as *m/z* 156 (24) or to compound-specific ions derived from the moiety attached to the common sulfanilamide group ($H_2NC_6H_4-SO_2NH-$). Because SRM transitions were specific to each target sulfonamide, it was possible to detect each one without interference from the others. The tandem mass spectrometer allows us to rapidly monitor (scan time 0.01 s) these transitions using different collision energies without any cross-talk (25). The positive mode is often chosen over the negative mode because of better signal-to-noise (*S/N*) ratios (21, 24); nevertheless, we observed that sulfonamide precursor ions generated by LDTD-APCI(-)

were intense enough and the product fragments specific enough to allow the unambiguous quantitation of the target sulfonamides.

In the LDTD-APCI source two main interdependent parameters need to be optimized: the laser pattern and the carrier gas flow. The laser pattern is used to control the power of the laser diode radiation (maximum 20 W) applied to the back of the metal well during a short period of time (< 10 s). Therefore, increasing the percentage of laser power will increase the laser radiation power hitting the back side of the sample holder and ultimately the amount of energy transferred to the sample. This fast heat transfer (as high as 3000 °C s⁻¹) induced by the laser, combined with the nanoscale distribution of the analyte into the well surface, allows the thermal desorption of compounds at a lower energy (26). Using spiked dairy milk samples at 100 μg L⁻¹ for all sulfonamides, the laser pattern was optimized to obtain the highest *S/N* ratio for all SRMs. The laser pattern should not be set too high in power in order to keep low the background signal generated by the matrix components' thermal degradation.

For the carrier gas optimization, the same samples were used to determine the highest *S/N* signal. The carrier gas flow has two main functions: (i) transfer of the thermally desorbed analytes from the well to the corona discharge region and (ii) thermalization of the desorbed molecules, which reduces the thermal degradation of the desorbed analytes after vaporization. Set to 3 L min⁻¹, the carrier gas shows the highest *S/N* values for all SRMs.

Dairy milk is a highly complex matrix; therefore, it is essential to use an internal standard (IS) and, moreover, to use it as a volume correction, because it compensates for errors during the transfer of small volumes on the 96-well plates. For the data acquisition, despite the electronics of the MS/MS permitting the monitoring of the 8 SRM transitions (analytes + IS) in a single experiment, operating at a scan time of 0.01 s will yield about 25–38 acquisition points for each transition over the 2–3 s peak width, close to the minimum 10 acquisition points per peak required for good quantitation (27). Therefore, the SRM transitions were pooled in groups of 4 in order to obtain a larger number of MS/MS acquisition points across each analyte peak.

Method Validation. Method validation parameters appear in Table 2. Sample cleanup and extraction using an ACN to milk ratio of 4/1 allows a good extraction of the analytes from the matrix, as shown by the resulting extraction recovery (77.5–101.5% with RSD < 9%). Inferior or comparable values have been reported in most published methods of extraction of sulfonamides from milk (Table 3). While the reported methods use several steps before analysis, the proposed procedure has the advantage of using minimal sample preparation (LLE with no evaporation and reconstitution), thus increasing the method's overall throughput.

Limits of quantitation (LOQ) were between 2 and 14 μg L⁻¹. These LOQ values are lower than or of the same magnitude as maximum residue limits (MRLs) set by regulatory authorities for some of the target sulfonamides in Australia (100 μg L⁻¹),

Table 3. Comparison of Methods for the Determination of Sulfonamides in Milk^c

authors	sample preparation	instrument	extraction recovery % ^a	R ²	precision (RSD)	LOQ ^b ($\mu\text{g L}^{-1}$)	anal. time (min)
Volmer et al. (1996) (15)	(1) acidification, sonication, centrifugation (2) LLE with hexane (3) evaporation, reconstitution	LC-ESI(+)-MS/MS	72–96 (1–10)	≥ 0.998	≤ 5	0.3–3	7
Cavaliere et al. (2003) (13)	(1) dilution with H ₂ O (2) SPE (3) evaporation, reconstitution (4) filtration	LC-ESI(+)-MS	82–104 (2–9)	0.9865–0.9900	N.A.	1–6	44
Mtagati et al. (2004) (16)	(1) filtration (2) dilution with methanol, acidification (3) LLE with acetone/ethyl acetate (1/4, v/v) by centrifugation (4) supported liquid membrane enrichment and cleanup	LC-ESI(+)-MS/MS	34–77 (4–8)	N.A.	2–8	41–80	84
Santos et al. (2005) (14)	(1) acidification, sonication, centrifugation (2) online SPE	CFS-CE-ESI(+)-MS	89–96 (5–7)	0.986–0.999	6–7	2–6	~30
this method	(1) LLE with acetonitrile (2) transfer supernatant to 96-well plate, dry at 37 °C for 5 min	LDTD-APCI(–)-MS/MS	78–102 (7–9)	0.9658–0.9977	≤ 10	2–14	0.5

^a Extraction recovery values were rounded to the closest integer and correspond to the highest spiked concentration. The RSD of the recovery is shown in parentheses. ^b Values expressed as the limit of detection ($S/N = 3$) were multiplied by 3.3 to obtain the limit of quantification. ^c Legend: CFS-CE, continuous-flow system coupled to capillary electrophoresis; N.A., not available.

Canada ($10 \mu\text{g L}^{-1}$), and the European Union ($100 \mu\text{g L}^{-1}$), as well as by international organizations such as the Food and Agriculture Organization and the World Health Organization ($25 \mu\text{g L}^{-1}$) (Table SI-1, Supporting Information).

The dynamic range of the calibration curves extended from 3 to $500 \mu\text{g L}^{-1}$ for most sulfonamides except SDZ (6.3 – $500 \mu\text{g L}^{-1}$). Coefficients of determination (R^2) were between 0.9658 and 0.9977. The method precision was excellent, with $\text{RSD} < 10\%$ for all the tested levels, which is lower than acceptable limits (15–23%) set by regulatory agencies (28, 29). Additionally, these values are comparable to precision reported (2–8%) in most published methods. Although interday precision was not investigated, the long-term precision of the LDTD-APCI source in complex matrices has been proven (30). Analysis of adulterated dairy milk samples previously analyzed by LC-MS/MS and containing low amounts of SDZ ($1.8 \mu\text{g L}^{-1}$) resulted in signals lower than the LOD of our method for that compound ($4 \mu\text{g L}^{-1}$).

LDTD-APCI(–)-MS/MS demonstrated performance similar to that of published methods (Table 3), but the analysis is done 10–150 times faster, giving the method an unmatched high-throughput capacity. Additional advantages of this method include minimal solvent consumption (extraction of a dairy milk sample needed only $800 \mu\text{L}$ of ACN and no LC mobile phase) and reduced instrument maintenance, as only vaporized samples come in contact with the MS/MS inlet.

In conclusion, a high-throughput and sensitive method of determination of seven sulfonamide residues in whole dairy milk using LDTD-APCI(–)-MS/MS was successfully developed. The overall performance (recovery, precision, LOD) of this rapid method is comparable to that of other previously published quantitation methods (Table 3) but offers the advantage of ultrafast analysis (30 s per sample), around 10–150 times faster than similar methods based on capillary electrophoresis-mass spectrometry, LC-MS, and LC-MS/MS. Even though our method is not able to confirm the presence of sulfonamide residues in milk (we did not use a second SRM for confirmation), its high throughput makes it an ideal complement to confirmatory

LC-MS/MS methods. The proposed LDTD-APCI(–)-MS/MS method is therefore efficient for the quantitation and fast screening of sulfonamide residues in dairy milk with minimal sample preparation and without any chromatographic separation.

Future work will focus on method variability while analyzing hundreds of samples during routine application for screening and monitoring purposes.

ABBREVIATIONS USED

ACN, acetonitrile; CFS-CE, continuous-flow system coupled to capillary electrophoresis; ESI(+), electrospray ionization in the positive mode; IS, internal standard; LC, liquid chromatography; LDTD-APCI(–), laser diode thermal desorption and atmospheric pressure chemical ionization in the negative mode; LLE, liquid–liquid extraction; LOD, limit of detection; LOQ, limit of quantitation; MAPAQ, Ministère de l'Agriculture, Pêcheries et Alimentation du Québec; MRLs, maximum residue limits; MS/MS, tandem mass spectrometry; SAA, sulfacetamide; SDZ, sulfadiazine; SMP, sulfamethoxyypyridazine; SMR, sulfamerazine; SMZ, sulfamethazine; SPD, sulfapyridine; SRM, selected reaction monitoring; SXZ, sulfisoxazole.

SAFETY

Sample preparation steps using ACN should be done under a fume hood.

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Supporting Information Available: Tables and a figure giving maximum residue limits (MRL) of the seven target sulfonamides

in dairy milk and LC-MS/MS method details. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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