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Characterization of Isomeric Sulfonamides Using Capillary Zone Electrophoresis Coupled with Nano-Electrospray Quasi-MS/MS/MS†

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The application of capillary electrophoresis/nano-electrospray mass spectrometry to the multi-residue analysis of a large number of sulfonamide antibiotics in milk samples is reported. Tandem mass spectrometric (MS/MS) techniques including precursor ion scans and multiple reaction monitoring were used to identify residues at the low ppb level to the ppt level. Three pairs of isomeric sulfonamides were targeted that differ only in the positions of the nitrogen and oxygen atoms in the heterocyclic aromatic rings of the molecules. Conventional MS/MS analysis yielded no isomer-specific ions. Therefore, a quasi-MS/MS/MS method was applied to overcome these limitations. In-source collision-induced dissociation (CID) was used as a quasi-MS/MS stage to generate ions arising from the heteroaromatic amine moiety. In a second MS/MS step these ions were isolated and made to undergo CID in the collision quadrupole to yield isomer-specific ions. Examples of the application of these methodologies to the analysis of milk extracts are illustrated. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

Sulfonamide antibiotics are commonly used in veterinary medicine for growth promotion and for the treatment of bacterial infections in livestock and poultry, as well as in farmed fin-fish such as salmon. This group of drugs consists of a relatively large number of chemically very similar compounds, including several isomers. Because it is known that at least one member of this group (sulfamethazine) is a thyroid carcinogen,[1] the presence of sulfonamide residues in food products is of concern. Therefore, reliable and accurate analytical methods for their determination at trace levels in food products are needed. The regulatory level for sulfonamides in food products in many countries is 100 ppb in edible animal tissues and 10 ppb in milk.

Several analytical methods for sulfonamides have been described, including thin-layer chromatography (TLC),[2,3] liquid chromatography (LC),[4–6] gas chromatography (GC),[7] and capillary zone electrophoresis (CZE).[8] Because these methods have usually been used in conjunction with non-selective detectors such as ultraviolet (in LC or CZE) and electron-capture (in GC), they all lack the ability to confirm unambiguously the presence of a residue tentatively identified on the basis of retention or migration time alone. Currently, mass spectrometry (MS) is considered to be the confirmatory technique of choice for analytes present at trace levels. GC with mass spectrometric detection (GC/MS) has been used to confirm sulfonamide residues.[9–11] For example, detection limits of 10–50 ppb for six sulfonamides in egg and edible animal tissues by GC/MS have been reported.[10] However, laborious and time-consuming derivatizations are required for the polar sulfonamides. More recently, on-line LC/MS[12–16] and CZE/MS[17–19] have been applied successfully to the analysis of sulfonamides. In these studies, electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI) interfaces were used to interface LC and CZE with the mass spectrometers. Because of the soft ionization characteristics of both ESI and APCI techniques, which usually result in the formation of only protonated molecular ions and/or molecular adduct ions, additional techniques such as in-source collision-induced dissociation (CID)[19,20] or tandem mass spectrometry (MS/MS) have been applied to produce the necessary structurally significant fragment ions for confirmation of identity. Furthermore, because of the large number of different sulfonamide drugs, the need for

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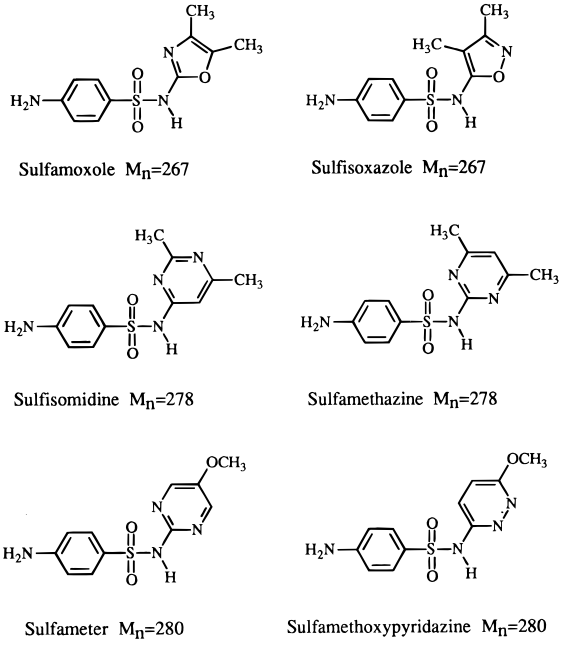


Scheme 1. Isomeric sulfonamide pairs investigated in the present work.

multi-residue methods is obvious. Volmer[21] has recently described a multi-residue method using fast short-column LC coupled with ESI and MS/MS for the simultaneuous determination and confirmation of 21 sulfonamides and two potentiators in milk samples.

In the present study, we have extended our research to the application of CZE/MS to the same group of compounds. A nano-electrospray interface built in this laboratory was utilized for analysis. The nano-electrospray technique was originally developed by Wilm and Mann[22] using gold-coated nanosprayer tips of ~1–2 μm inner diameter. With electrospray tips of such small diameter, the flow rate is dictated by the electrospray process itself and is typically 20–40 nl min^{-1}. Consequently, with such low flow-rates nano-ESI sources have mainly been used for infusion analysis with reduced sample consumption[23,24]. However, efficient coupling of electrospray emitters of very small diameters to CZE or LC is difficult. We have recently described a nano-electrospray technique for interfacing CZE with the MS at sub-microliter per minute flow rates by using disposable microsprayers.[25] This new interface provides ease of operation and enhanced sensitivity compared with traditional on-line CZE/MS interfacing techniques. One aim of this study was to demonstrate the applicability of nano-electrospray CZE/MS to the sensitive multi-residue analysis of a large number of compounds. MS/MS techniques, such as product ion scanning, parent ion scanning and multiple reaction monitoring (MRM), have been used as a means of producing structure-diagnostic ions for screening and confirmation of sulfonamide residues in food samples. Furthermore, special attention has been paid to the analysis of three isomeric pairs of sulfonamides, viz. sulfamoxole and sulfisoxazole (nominal molecular mass, $M_n = 267$), sulfisomidine and sulfamethazine ($M_n = 278$) and sulfamethoxypyridazine and sulfameter ($M_n = 280$). These compounds differ only in the positions of nitrogen and oxygen atoms in the heterocyclic aromatic ring (Scheme 1). The determination of these compounds is challenging because regular MS/MS analysis of the MH^+ ions, on a triple-quadrupole MS, yielded no isomer-specific product ions. We have used a quasi-MS/MS/MS approach to overcome these limitations. First, in-source CID was used as a quasi-MS/MS stage to generate ions arising from the heteroaromatic amine moiety. In a second MS/MS step, these ions were isolated and fragmented in the collision quadrupole to yield isomer-specific ions.

EXPERIMENTAL

Materials

Standards of the sulfonamides were purchased from Sigma (Mississauga, ON, Canada). Methanol, hexane (Caledon, Georgetown, ON, Canada) and purified water (Milli-Q water, Millipore, Bedford, MA, USA) were used as solvents. Formic acid, hexadimethrine bromide (Polybrene) and hydrochloric acid were purchased from Aldrich (Mississauga, ON, Canada). [(Acryloylamino)-propyl]trimethylammonium chloride (BCQ) was obtained from Chemische Fabrik Stockhausen (Krefeld, Germany).

Clean-up of milk samples

Details of the clean-up procedure have been reported elsewhere.[21] Briefly, proteins and lipids were removed from milk samples by acid precipitation and extraction with hexane, respectively. The aqueous extracts were evaporated to dryness at low pressure and the resulting residues were dissolved in methanol. After filtration, the methanol extracts were again evaporated to dryness and the residues re-dissolved in water.

Capillary zone electrophoresis and nano-electrospray interface

CZE/MS experiments were carried out using a Thermo CZE system (Thermo Capillary Electrophoresis, Franklin, MA, USA). Typically, the electrolytes used for nanospray CZE/ESI was 0.1–1 M formic acid. Separations were achieved by applying −20 to −25 kV to the injection end of the column (90 cm × 50 μm i.d.) and ~2.5 kV to the electrospray tip. All fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). The inner diameter of the nanosprayer tips used was 20 μm with CZE flow-rates of 150 nl min^{-1}. Details of the nanosprayer tip construction, the gold coating for the capillary tips and the BCQ and Polybrene capillary coating procedures have been reported elsewhere.[25] Samples were introduced by pressurizing the vial at 100 mbar, resulting in typical injection volumes of 20 nl. CZE/UV analyses were carried out using a P/ACE system (Beckman, Palo Alto,

CA, USA) at a detection wavelength of 280 nm. Separations were achieved by applying 30 kV to the outlet end of an 80 cm × 50 μm i.d. capillary which had previously been coated with a solution of Polybrene (5% w/v) and ethylene glycol (2% v/v).[25] The background electrolyte was 1.0 M formic acid. The sample was introduced by pressurizing the vials at 35 mbar for 10 s, resulting in typical injection volumes of 10 nl.

Nano-electrospray MS and MS/MS

Nano-electrospray data were acquired using an API III$^+$ triple-quadrupole (Q1, Q2, Q3) mass spectrometer (Perkin-Elmer Sciex, Concord, ON, Canada). A separate power supply (Glassman EH Series, Glassman, Whitehouse Station, NJ, USA) was used to provide the electrospray voltage. Mass spectra were acquired using dwell times of 1.5 ms per 0.2 Da step in full mass scan mode. In quasi-MS/MS/MS experiments, in-source collision-induced dissociation (CID) was achieved using an API interface potential difference (orifice–skimmer voltage difference) of 60 V and subsequent MS/MS experiments were conducted in Q2 using a collision-offset voltage of 30 V. The collision gas (argon) thickness was 3.5×10^{15} atoms cm^{-2} in all experiments. Tandem mass spectra in the product ion and parent ion modes were acquired using dwell times of 2 ms per step of 0.5 Da for Q3 and Q1, respectively.

RESULTS AND DISCUSSION

Development of separation conditions for analysis of the investigated sulfonamides

The use of ESI-compatible acidic buffers with bare fused-silica capillaries results in reduced electroosmotic flows (EOF) and adsorption of cationic analytes by surface silanol groups.[26,27] Cationic coating of the capillary eliminates analyte adsorption while providing an EOF compatible with nano-electrospray CZE/MS. We have shown in a previous study[25] that, for peptides, capillaries coated with BCQ or hexadimethrine bromide (Polybrene) yielded numbers of theoretical plates significantly higher than those obtained using other amine coatings and thus increased separation efficiencies. Therefore, BCQ- and Polybrene-coated capillaries were used in this study.

Results of the analyses of sulfonamides using CZE/UV (280 nm) are summarized in Fig. 1 and Table 1. A buffer concentration (formic acid) of 1.0 M was used for these separations. Separation efficiencies, calculated from peak widths at half-height, were typically in the range 200 000–600 000 theoretical plates for most of the investigated compounds as listed in Table 1. CZE/MS experiments used 0.1 M formic acid and resulted in a slight reduction in resolution in comparison with CZE/UV (results not shown).

Application of nano-ESI CZE/MS/MS to the analysis of milk samples

The analysis of complex biological extracts using UV detection has very limited possibilities. First, CZE/UV is usually not sufficiently sensitive to detect sulfonamide residues at trace levels. Furthermore, identification of target analytes is uncertain because the UV spectra of the sulfonamides are very similar and co-migrating analytes and matrix components can make unambiguous confirmation of the presence of such residues impossible. Consequently, more selective and sensitive detection is necessary. MS and MS/MS are the techniques of choice for identification of residues. The application of combined LC/MS/MS for screening, quantification and confirmation of sulfonamide residues in milk samples at levels around 1 ppb has recently been demonstrated.[21]

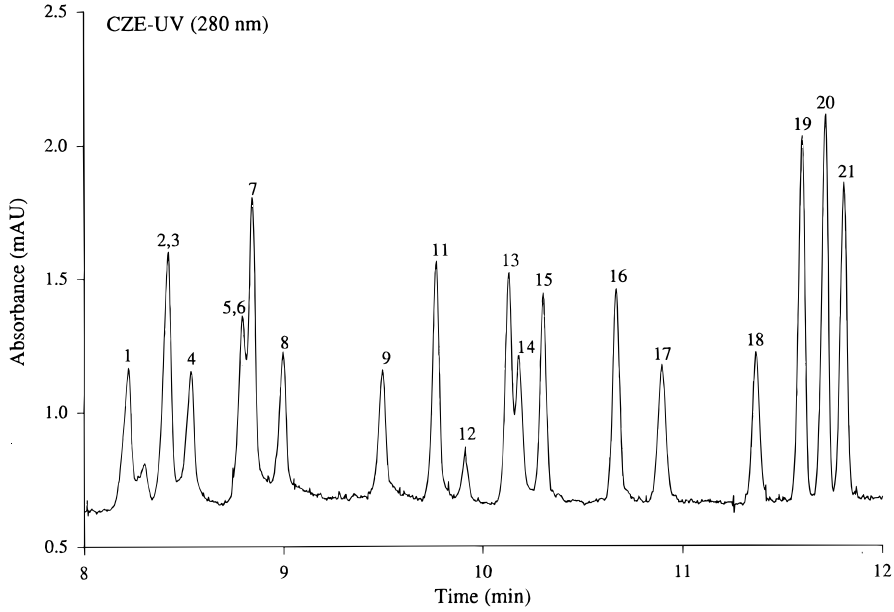


Figure 1. Separation of sulfonamides by CZE/UV (280 nm). For peak assignments, see Table 1. The experimental conditions used are described in the Experimental section.

Table 1. Migration times (t_m), nominal molecular masses (M_n) and number of theoretical plates in CZE (N) for the investigated sulfonamides

No.	Compound	t_m (min)	M_n	N
1	Sulfabenzamide	8.23	276	193 000
2	Sulfacetamide	8.42	214	234 000
3	Sulfisoxazole	8.42	267	234 000
4	Sulfamethoxazole	8.54	253	229 000
5	Sulfadimethoxine	8.80	310	200 000
6	Sulfameter	8.80	280	200 000
7	Sulfaquinoxaline	8.84	300	423 000
8	Sulfachloropyridazine	9.00	284	366 000
9	Sulfamethizole	9.50	270	346 000
10	Sulfadiazine	9.67	250	327 000
11	Sulfamethoxypyridazine	9.77	280	457 000
12	Sulfamoxole	9.92	267	605 000
13	Sulfamerazine	10.13	264	394 000
14	Sulfathiazole	10.19	255	490 000
15	Sulfamethazine	10.31	278	480 000
16	Sulfapyridine	10.67	249	487 000
17	Sulfanilamide	10.90	172	391 000
18	Sulfaguanidine	11.37	214	448 000
19	Trimethoprim	11.61	290	645 000
20	Ormethoprim	11.72	274	621 000
21	Sulfisomidine	11.81	278	596 000

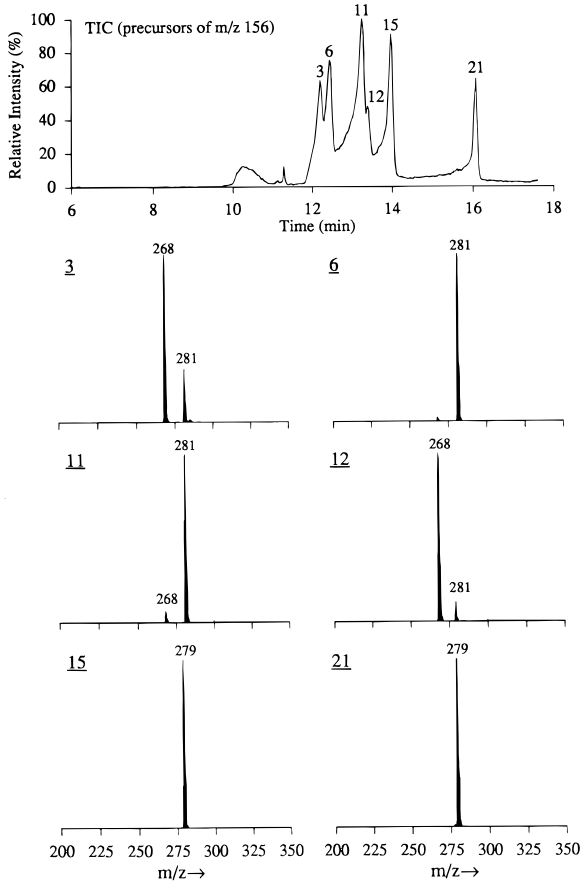


Figure 2. Top: separation of six sulfonamides by CZE/MS/MS in a milk extract using the precursor ion scan mode (precursor ions of m/z 156). The original milk sample was spiked at the 20 ppb level (injected amount, ~3 pg per compound). Bottom: extracted precursor ion mass spectra from the TIC trace. For peak assignments, see Table 1. The experimental conditions used are described in the Experimental section.

In the present study, CZE/MS/MS methodology was used for multi-residue analysis. A nano-electrospray technique developed in this laboratory was used for interfacing CZE with the mass spectrometer at sub-microliter per minute flow rates. Nano-electrospray using disposable microsprayers provides ease of operation and enhanced sensitivity compared with traditional on-line CZE/MS interfacing techniques.[25] MS/MS was applied in all experiments. Generic and compound-specific ions were formed by CID of the MH^+ ions. Two major fragmentation pathways were observed in MS/MS experiments: (i) bond cleavage between the sulfur and the nitrogen atom, with charge retention on the (4-aminophenyl)sulfonyl moiety to give the resonance-stabilized generic ion at m/z 156 (and possible secondary dissociations to m/z 108 and 92[21]), and (ii) S—N bond cleavage and charge retention on the heteroaromatic amine (B) moiety, with hydrogen migration to give compound-specific BH^+ ions. Ions from both pathways were used for analysis. The generic ion at m/z 156 was ideal for screening purposes in precursor ion scan experiments because all investigated compounds exhibited this particular ion. An example is illustrated in Fig. 2, where the CZE/MS/MS precursor ion analysis of a fortified milk sample at the 20 ppb level is shown. Six sulfonamides could be readily identified at this low concentration level. Unfortunately, the CZE peak shapes in milk extracts exhibited fronting for all compounds. This was probably due to co-migrating matrix components. The milk clean-up procedure used was originally designed for reversed-phase HPLC analysis in which residual salts and highly polar compounds passed through the stationary phase without major retention and did not interfere directly with the analyte signals.[21] In CZE, however, residual salts strongly interfered with the separation process, causing erratic peak shapes. Nevertheless, identification at the low ppb level was readily achieved.

In order to compare the sensitivity of this method with that of a previously reported conventional CZE/MS/MS method with a concentric flow interface,[17] multiple reaction monitoring (MRM) was applied for analysis. An example of such an MRM analysis is given in Fig. 3 for a milk sample fortified with six sulfonamides at the 5 ppb level. The transitions $MH^+ \rightarrow 156^+$ were monitored in this experiment. All sulfonamides yielded detection limits at the higher parts per trillion (ppt) level. Thus, the MRM method allowed detection at concentration levels much lower than those reported for the conventional CZE/MS/MS method[17] and can largely be attributed to the new nano-electrospray interface utilized. Even lower detection limits are certainly possible by using improved clean-up procedures designed specifically for CZE analysis and/or preconcentration of the sample.

Identification of isomeric sulfonamides by CZE/quasi-MS/MS/MS

The chemical structures of the three pairs of isomeric sulfonamides investigated are shown in Scheme 1.

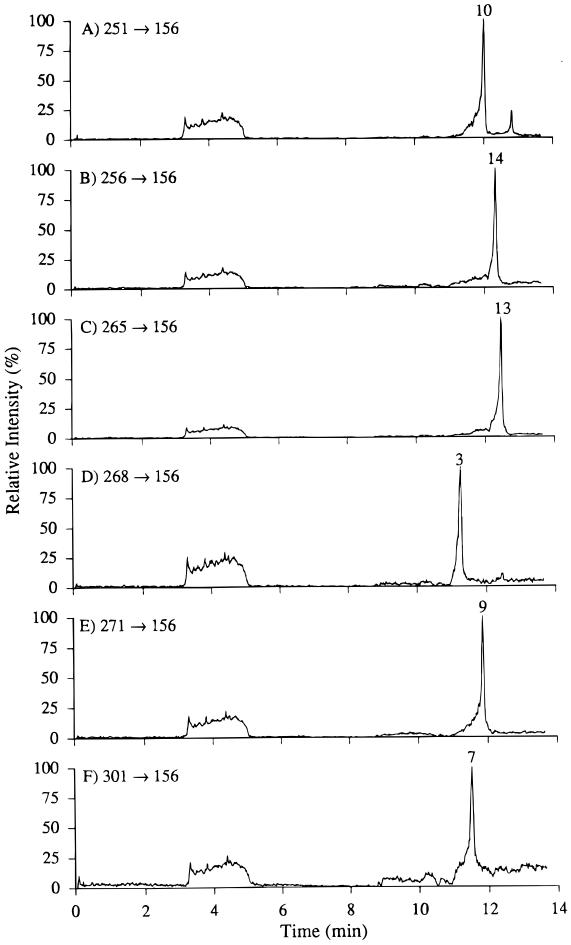


Figure 3. Separation of six sulfonamides by CZE/MS/MS in a milk extract using multiple reaction monitoring of $[MH]^+ \rightarrow 156^+$ transitions. The original milk sample was spiked at the 5 ppb level. For peak assignments, see Table 1. The experimental conditions used are described in the Experimental section.

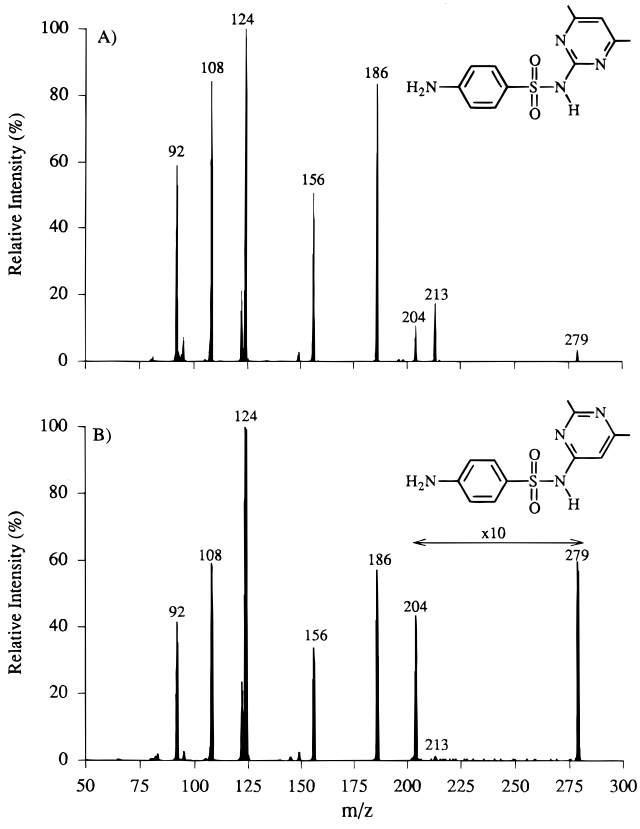


Figure 4. Product ion spectra of the MH^+ ions (*m/z* 279) of the isomers sulfamethazine (A) and sulfisomidine (B) obtained by MS/MS with a collision-offset voltage of 30 V.

Unambiguous identification of these isomers requires mass spectrometric confirmation in addition to tentative electrophoretic confirmation based on the migration times (Fig. 2). Within each isomeric pair, the only structural differences concern the positions of ring-nitrogen and ring-oxygen atoms in the heteroaromatic amine moieties. While CID in a tandem mass spectrometer can often be used to distinguish between isomers, it failed for the investigated compounds because low-energy CID did not lead to fragmentation of the aromatic ring system in the amine moiety of the molecules, but resulted almost exclusively in breakage of the ($-SO_2-NH-$) bonds (see discussion above). That is, the product ion spectra of the isomers within each pair are virtually identical and yielded no isomer-specific ions. This is illustrated in Fig. 4, which shows the product ion spectra of the MH^+ ions (*m/z* 279) of sulfisomidine and sulfamethazine. The MS/MS analyses of the other two isomer pairs also yielded very similar spectra with no isomer-specific ions. Pleasance and coworkers[16,17] suggested that the ion at *m/z* 213 in the product ion spectrum of sulfamethazine is an isomer-specific ion and assigned the transition $279^+ \rightarrow 213^+$ to the loss of H_2 plus SO_2. They did not observe this ion in the MS/MS analysis of its isomer, sulfisomidine. In our experiments, however, the fragment ion at *m/z* 213 was observed in both the product ion and in-source CID spectra of both sulfamethazine and sulfisomidine (Fig. 4). The relative abundances in the spectra of sulfamethazine and sulfisomidine were, however, completely different. Thus, sulfamethazine exhibited *m/z* 213 ions with relative abundances around 20%, but sulfisomidine formed these ions at only low abundances. However, the fragment at *m/z* 213 could clearly be observed in all of the present CID experiments for sulfisomidine and therefore does not appear to be isomer-specific.

In order to overcome this apparent limitation of MS/MS methodology, a quasi-MS/MS/MS approach was used. Specifically, in a first step in-source CID was used as a quasi-MS/MS stage to generate ions corresponding to the heteroaromatic amine moiety (BH^+ at *m/z* 113 for sulfamoxole and sulfisoxazole; *m/z* 124 for sulfisomidine and sulfamethazine; *m/z* 126 for sulfamethoxypyridazine and sulfameter). In a second MS/MS step, the BH^+ ions were isolated and made to undergo CID in the collision quadrupole to yield isomer-specific ions. The results of these experiments are summarized in Table 2 and Fig. 5 (BH^+ ions in the spectra in Fig. 5, formed by CID in the first MS/MS step, are marked with an asterisk). Tentative structure assignments for these second generation product ions are shown in Table 3. All compounds exhibited at least one isomer-specific ion (*m/z* values in italics in Table 2) in the MS/MS/MS process, that is, unambiguous characterization was possible. Unfortunately, the isomer-

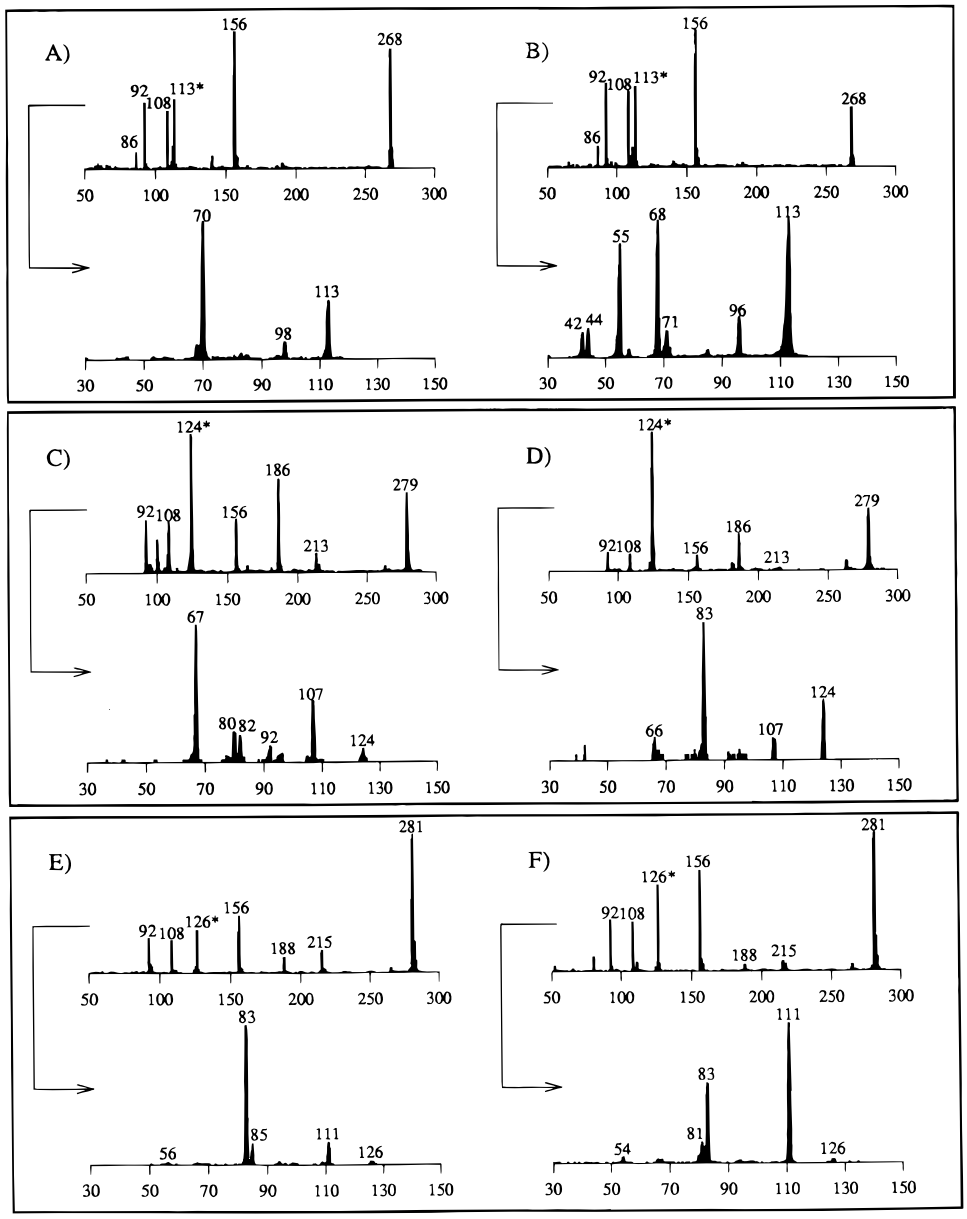


Figure 5. CZE/quasi-MS/MS/MS of three pairs of isomeric sulfonamides. The top mass spectrum of each isomer shows the quasi-tandem mass spectrum (in-source CID). The mass spectrum at the bottom is the tandem mass spectrum of the BH^+ ions isolated from the first quasi-MS/MS step (indicated with an asterisk in the top spectrum). (A) Sulfamoxole, **12**; (B) sulfisoxazole, **3**; (C) sulfamethazine, **15**; (D) sulfisomidine, **21**; (E) sulfameter, **6**; (F) sulfamethoxypyridazine, **11**.

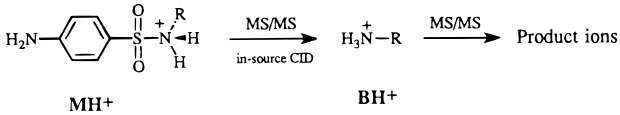
specific ions for sulfamethoxypyridazine and sulfameter, at m/z 81 and 85, respectively, have much lower abundances than their equivalents in the spectra of the other four isomers, where the isomer-specific ions are the base peaks. Nevertheless, the CZE/quasi-MS/MS/MS detection limits for sulfamethoxypyridazine and sulfameter using these ions are still acceptably low, in the higher femtogram range. The other isomers could be detected at the low femtogram level in milk (Table 2).

An application of the nano-ESI quasi-MS/MS/MS technique to the MRM analysis of two of the investigated isomer pairs in a fortified milk sample at the 5 ppb level is illustrated in Fig. 6. The mass spectrometric isomer identification confirms that provided by the relative migration times.

CONCLUSIONS

This study indicates that nano-electrospray CZE/MS/MS can be used for the rapid identification of sulfonamide residues in milk samples. To our knowledge, this is the first demonstration of the use of nano-electrospray in small molecule trace analysis. Simultaneous confirmation of analytes present at the lower ppb level was achieved using precursor ion scan and multiple reaction monitoring experiments. This new CZE technique allowed detection at concentration levels much lower than those reported previously for these compounds using a more traditional CZE/MS interface.[17] On-line enrichment techniques or iso-

Table 2. Major second-generation product ions in the CZE/quasi-MS/MS/MS analysis of sulfonamide isomers



MH^+ BH^+

Isomer	MH^+ *m/z*	BH^+ *m/z*	Product ions of BH^+ [a] *m/z* (% relative abundance)	LOD[b] (fg)
Sulfamoxole	268	113	*70*(*100*), 78(10)	30
Sulfisoxazole	268	113	42(20), 44(25), *55*(*80*), *68*(*100*), 71(20), 96(30)	80
Sulfisomidine	279	124	66(25), *83*(*100*), 107(25)	8
Sulfamethazine	279	124	*67*(*100*), 80(25), 107(50)	15
Sulfamethoxypyridazine	281	126	*81*(*10*), 83(60), 111(100)	~400
Sulfameter	281	126	83(100), *85*(*10*), 111(10)	~400

[a] Isomer-specific ions used in characterization are in italics (see Table 3).
[b] Limit of detection of the isomers in milk samples using the most abundant underlined isomer-specific ion (signal-to-noise ratio = 3).

Table 3. Tentative identification of second-generation isomer-specific ions formed in the quasi-MS/MS/MS analysis of the investigated sulfonamide isomers

m/z	Specific for	Tentative identification
67	Sulfamethazine	$[N{\equiv}C{-}CH_2{-}C{\equiv}N]{-}H^+$
68	Sulfisoxazole	$[N{\equiv}C{-}CH_2{-}CH{=}CH_2]{-}H^+$
70	Sulfamoxole	$[N{\equiv}C{-}O{-}CH{=}CH_2]{-}H^+$
81	Sulfamethoxypyridazine	$[N{\equiv}C{-}N{=}CH{-}CH{=}CH_2]{-}H^+$
83	Sulfisomidine	$[N{\equiv}C{-}CH_2{-}CH{=}CH{-}NH_2]{-}H^+$
85	Sulfameter	$[HN{=}CH{-}NH{-}CH{=}C{=}O]{-}H^+$

tachophoresis preconcentration, in addition to more efficient extraction and clean-up procedures, could further improve detection limits and extend the present method to multi-residue analysis in matrices other than milk, e.g. fish. Because this conventional MS/MS methodology was not able to distinguish between isomeric sulfonamides, reflecting the lack of isomer-specific ions in the product ion spectra, a quasi-MS/MS/MS approach was used to overcome this limitation. The improved method allowed the sensitive identification of isomeric sulfonamides in the low femtogram range. Furthermore, it has been shown that this methodology is capable of confirming the presence of sulfonamide isomers in milk samples at the very low ppb level.

Interestingly, comparison of the new nano-electrospray CZE/MS/MS method with a previously reported LC/MS/MS method for sulfonamides[21] which used conventional ES ionization, revealed similar performance in terms of detection sensitivities despite much smaller injection volumes for CZE (nanoliters compared with microliters in LC). This result can be conveniently discussed by comparing concentration and mass detection limits for both methods. Because of the very small injection volumes, nano-ESI CZE/MS/MS exhibited higher concentration detection limits than LC/MS/MS. This apparent limitation, however, was significantly offset by the lower mass detection limits achieved with nano-ESI in comparison with LC/MS/MS (femtograms rather than picograms).[21] This reflects the much higher ionization efficiency of the new nanospray-ESI interface compared with that of conventional ESI sources.

Acknowledgements

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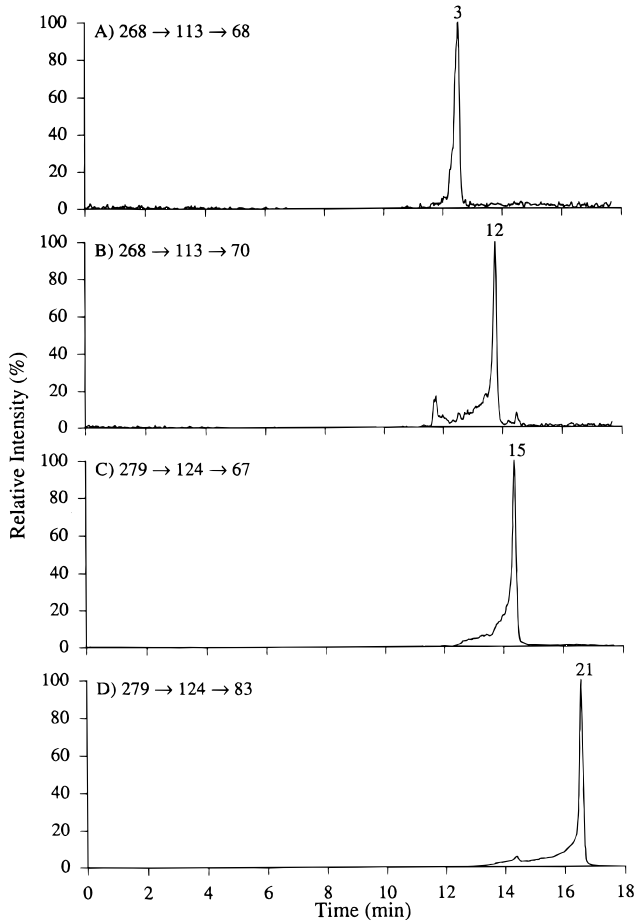

Figure 6. CZE/quasi-MS/MS separation of two pairs of isomeric sulfonamides in milk at the 5 ppb level using multiple reaction monitoring of the $[BH]^+ \rightarrow$ [isomer-specific ion]$^+$ transitions. For peak assignments, see Tables 1 and 2 and Scheme 1.

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