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Analysis of erythromycin by liquid chromatography/mass spectrometry using involatile mobile phases with a novel atmospheric pressure ionization source

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A critical limitation of electrospray ionization (ESI) liquid chromatography/mass spectrometry (LC/MS) sources is the susceptibility to blockage of interface orifices due to the deposition of involatile components from the sample and/or mobile phase. These components, including salts, buffers, and ion-pairing agents, can be essential to the performance of the chosen analytical method. We report here the performance enhancements provided by a novel atmospheric pressure ionization (API) source in the analysis of erythromycin A (ERY) using mobile phases that contain involatile components. The enhanced robustness of the new source is derived from the use of a continuous flow of aqueous solvent at the sampling cone orifice that maintains unobstructed ion transmission. The ESI mass spectral responses measured for ERY, using an LC separation that incorporates 10 mM sodium phosphate with and without 10 mM octane sulfonate, were monitored by repeated injections over 13–15 h total analysis time. Minimal effects on ESI mass spectral responses (integrated peak area) or chromatographic performance (peak shape, retention time) were observed during these studies. In the absence of the aqueous cleaning flow, complete loss of mass spectral responses and total blocking of the sampling cone was observed in less than 30 min. Responses for ERY spiked into chicken and beef liver, and catfish muscle at or below the regulatory level of interest (100 ppb), were quantified by internal standard calibration using this procedure. These results demonstrate the ability of a novel API-MS ion source to perform analyses that require the use of involatile mobile phase additives. **Copyright © 2000 John Wiley & Sons, Ltd.**

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Pioneers and current users of LC/MS have always advocated the use of volatile chromatographic buffers for routine analyses using this technique. In the early days, this approach was primarily driven by the limited tolerance of thermospray and particle beam interfaces to involatile buffers. This was due to physical blockage of interface orifices. More recently, these techniques have been surpassed by atmospheric pressure ionization (API) interfaces that generally offer greater reliability, improved limits of detection, and a more universal response over a wide range of analyte classes. Nevertheless, most commercial API interfaces still suffer from limited tolerance to involatile buffers. This can include ionization suppression, increased chemical noise, and physical blockage of interface orifices.[1] In this report, we describe a novel ion source for API-MS (see Fig. 1) whose design eliminates the problem of physical blockage of orifices caused by involatile components. The performance was demonstrated by an application to a problematic analysis of antibiotic residues in food that uses phosphate buffer and an ion-pairing reagent in the mobile phase.

Erythromycin A (ERY), a macrolide antibiotic often used in animal husbandry (see Fig. 2), presents analytical difficulties using conventional detectors because it contains no selective UV-vis chromophore, fluorophore, or readily oxidizable functional group. These limitations have hindered the development of a sensitive and selective analytical method needed for monitoring the presence of ERY in tissues of food-producing animals. Several LC/API-MS[2] or ECD[3,4] methods have been reported for measurement of ERY in tissues at or above 1 μg/g; however, none have reported results at the FDA level of interest of 100 ng/g. In this laboratory, initial developments of an LC separation for electrochemical detection of ERY required buffering and an ion-pairing reagent in the mobile phase. Although these LC conditions were not compatible with our existing LC/MS instrumentation, the novel API-MS source described in this report was used successfully for the analysis of ERY in several edible tissue samples.

EXPERIMENTAL

Tissue sample preparation

Chicken/beef livers and catfish fillets were obtained from local grocery stores and stored at −70 °C following homogenization in a food processor. Frozen samples (5 g) were allowed to thaw at room temperature after which ERY was added in a solution containing the internal standard (IS), oleandomycin (0.5 μg each in 0.1 mL acetonitrile). Solvent

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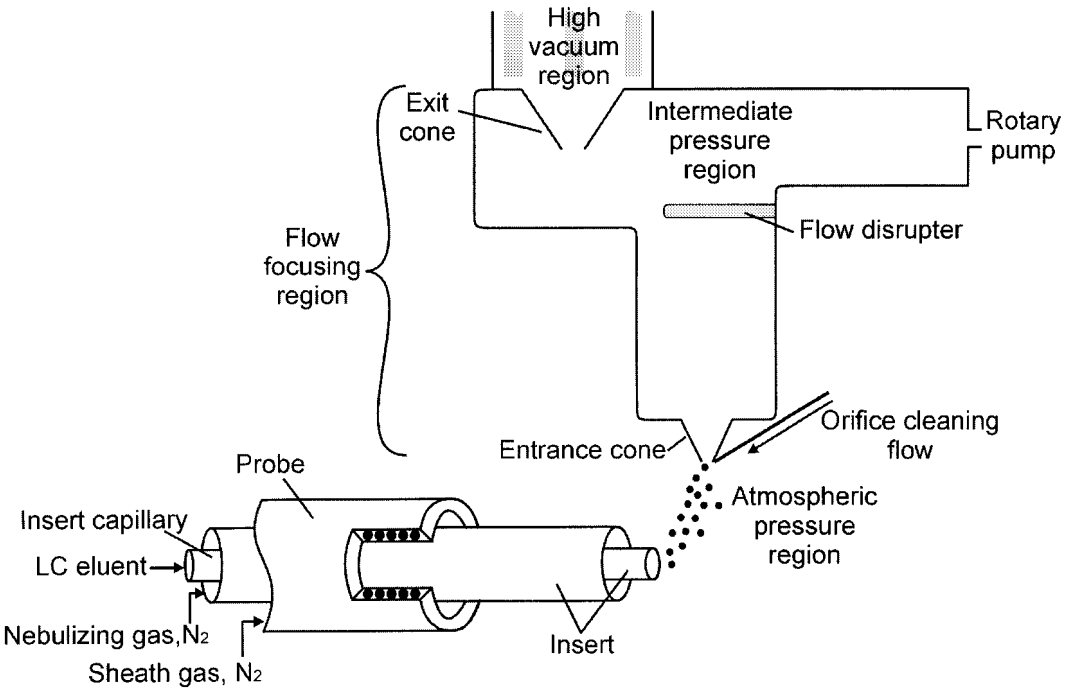

Figure 1. Diagram of the AQA API source/probe.

only was added for control samples. The spiked tissue samples were homogenized three times with ten volumes of aqueous ion-pairing solution (20 mM KH_2PO_4 plus 20 mM sodium octane sulfonate, pH 4.5) containing 20% v/v acetonitrile using 1 min intervals with a tissue homogenizer. The combined liquid was centrifuged at 4500 rpm and the liquid portion (ca. 30 mL) was collected.

Liquid/liquid partitioning

The aqueous extract was subjected to liquid/liquid partitioning by extraction with 2×10 mL volumes of hexane to remove fats. The pH of the solution was then adjusted by addition of 1 mL of 1 M KOH, and extracted three times with 10 mL portions of ethyl acetate. After centrifugation, the bulk of the ethyl acetate layer was transferred to a round-bottom flask and the solvent removed *in vacuo* at 35 °C. The residues were dissolved by treatment with 3×2 mL of CH_2Cl_2.

Solid phase extraction

Aminopropyl-silica SPE cartridges (BondElut-NH2, 3 cc/500 mg, Varian Co., Walnut Creek, CA, USA) were conditioned by washing with CH_2Cl_2 (3×2 ml). The combined sample residue extract was then transferred to the SPE cartridge and the unretained fraction was discarded. ERY and the internal standard were eluted with 2 mL of 2% methanol in CH_2Cl_2 (v/v) into a glass tube and the solvent removed in a dry nitrogen stream. The sample extracts were

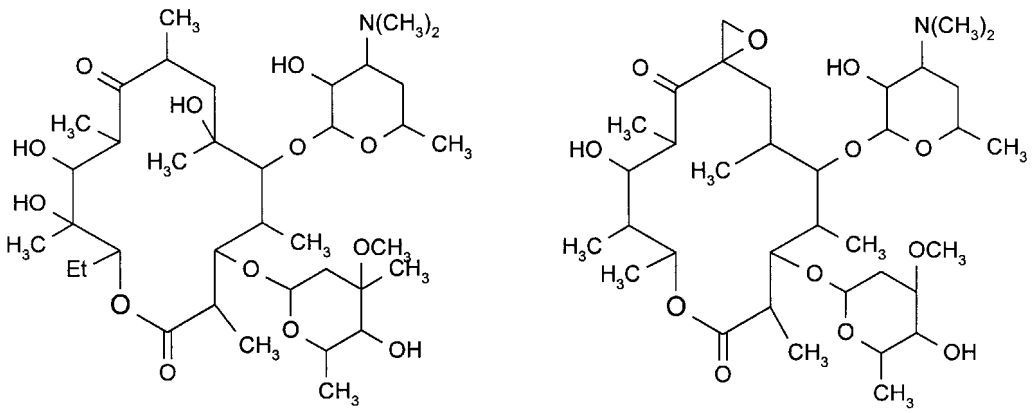

Figure 2. Structures of erythromycin A and oleandomycin.

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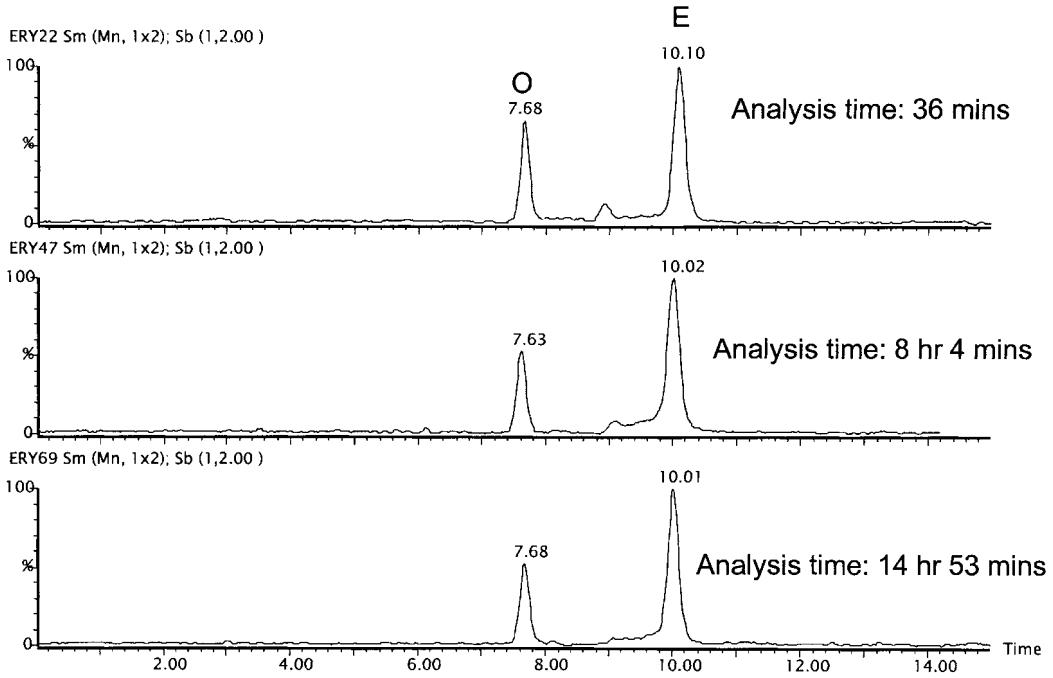

Figure 3. Chromatograms for erythromycin (E) and oleandomycin (O) during an overnight analysis. A constant amount of ERY and oleandomycin standards (1 ng each on-column) was injected every 30–35 min. The chromatograms show MS-SIM responses for ERY and IS at various time points.

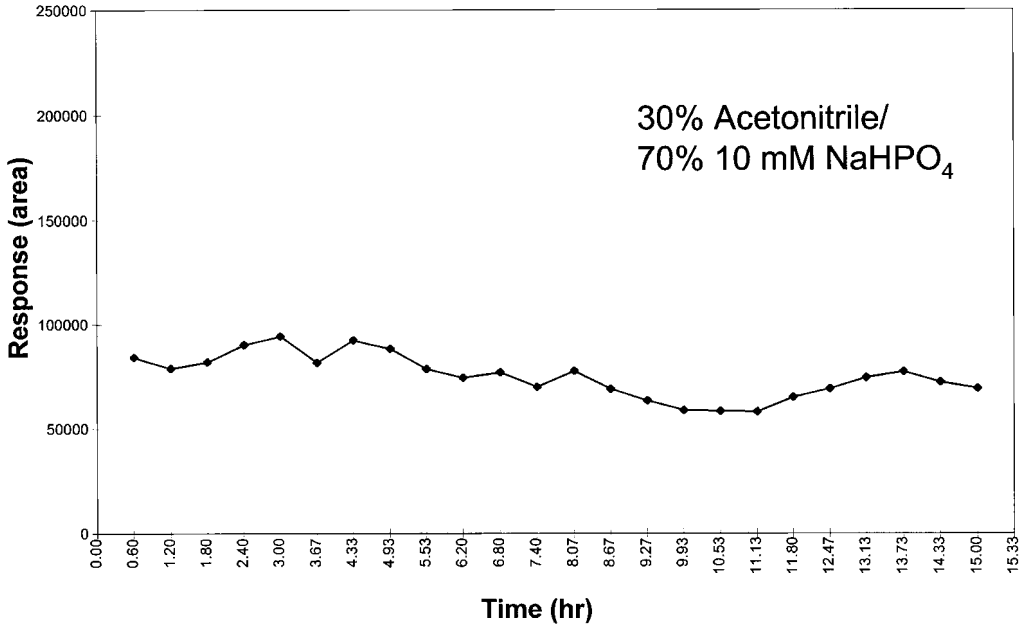

Figure 4. Mass spectral response for ERY during an overnight run using phosphate buffer in the mobile phase. The integrated MS-SIM peak area for the $[M + H]^+$ peak (*m/z* 734) of ERY was determined following repeated injections of ERY as described in Fig. 3 while using 0.01 M potassium phosphate buffer (pH 4.5) in the mobile phase.

stored at – 20 °C until analyzed. Just before analysis, the residue was dissolved in 0.5 mL of mobile phase.

Liquid chromatography

Separations were performed using a Prodigy ODS column (150 × 4 mm, Phenomenex, Torrance, CA, USA) using mobile phases containing 30% by volume acetonitrile and 0.01 M sodium phosphate with and without 0.01 M sodium octane sulfonate, adjusted to pH 4.5. The flow rate was 1 mL/min and the entire column effluent was introduced into the mass spectrometer. Injections of 10 µL were made onto the column.

Mass spectrometry

A Finnigan AQA single quadrupole mass spectrometer (Finnigan MassLab, Manchester, UK) was used with an ESI probe temperature of 450 °C. Positive ions were acquired in selected ion monitoring (SIM) mode (dwell time = 0.3 s,

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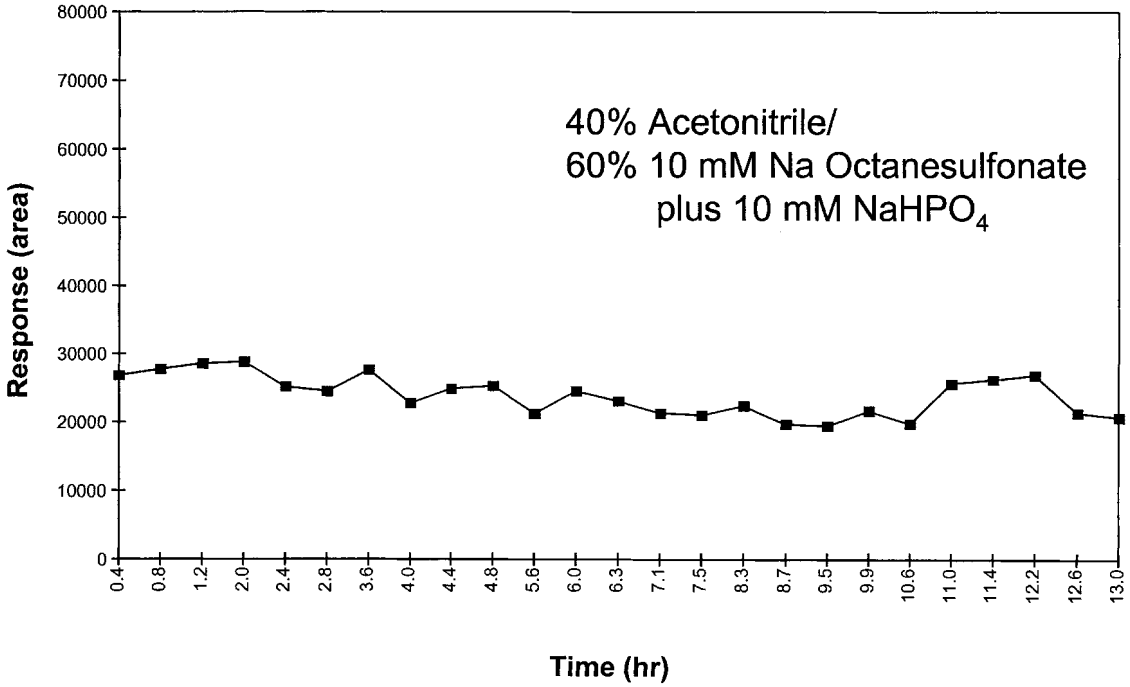

Figure 5. Mass spectral response for ERY during an overnight run Using phosphate buffer and ion-pairing agent in the mobile phase. The integrated peak area for the $[M + H]^+$ peak (*m/z* 734) of ERY was determined as described in Fig. 3 following repeated injections of ERY while using 0.01 M potassium phosphate buffer plus 0.01 M sodium octane sulfonate (pH 4.5) in the mobile phase.

span = 0 Da and interchannel delay time = 0.03 s). The MS method monitored the $[M + H]^+$ ions for ERY and oleandomycin at *m/z* 734 and 689, respectively. A sealed reservoir was pressurized with nitrogen gas to produce a constant flow of HPLC grade water to the sampling cone orifice at approximately 40 μL/min. Mass spectral data were collected using SIM of the protonated molecules for ERY (*m/z* 734) and the IS (*m/z* 689).

RESULTS AND DISCUSSION

API-MS Source Design

All API sources include an ion inlet orifice that forms a boundary between the API region and the low pressure region of the source or mass analyzer. This orifice is generally small (<0.5mm in diameter) owing to the finite pumping speed of the vacuum system and the requirement of a low pressure in the mass analyzer region (typically 10^{-5} mbar). During prolonged periods of analysis by LC/MS, involatile components in the mobile phase are deposited on the periphery of the ion inlet orifice which eventually leads to a partial or complete blockage and concomitant loss in sensitivity of the mass spectrometer.

Previous commercial API sources have incorporated sacrificial counterelectrodes[5] or used orthogonal source geometries that are intended to protect the ion inlet orifice from the build up of involatile compounds. Although a sacrificial counterelectrode does extend the time to eventual blockage, it also typically reduces source sensitivity. In the case of orthogonal ESI, the spray is directed away from the inlet orifice (see Fig. 1). However, for the high flow rates used in LC/MS (typically 1mL/min), both the ions and charged liquid droplets containing involatile components are deflected by the electric field towards the inlet orifice by the ESI capillary voltage. This deflection effect also eventually leads to a blocked orifice. A partial solution to this problem can be effected by either reducing the distance between the probe tip and the inlet orifice or reducing the ESI potential. Unfortunately, these two measures lead to a reduction in sensitivity of the source.[6]

The problem of orifice blocking was eliminated in the present study, without loss of source sensitivity, by the addition of a constant flow of aqueous solvent at the point of initial deposition of involatile substances, i.e. on the outer upstream side of the orifice cone.[6] In practice, this is achieved by incorporating a fused silica capillary that makes contact with the outer conical surface of the orifice member in the immediate vicinity of the orifice (see Fig. 1). A suitable solvent (typically water) is constantly pumped through the fused silica line at a flow rate of 40 μL/min and is drawn into the orifice by the pressure gradient at this point. The constant flow of liquid over the edge of the orifice has no detrimental effect on the focusing of ions into the source and thus maintains conditions of optimal sensitivity while preventing the occurrence of source blockage in the presence of involatile chromatographic buffers. The analysis of ERY in food presented an opportunity to evaluate the performance of the novel source design in an LC/MS method that required both sensitive/selective detection and the use of involatile mobile phase components.

Source performance in the analysis of ERY

Under the chromatographic conditions used, ERY was well separated from the IS and no interferences from sample matrices were observed in the MS-SIM responses. The internal standard ratio for MS-SIM responses from ERY vs. oleandomycin (IS) standards was linear over the concentration range needed for this study (10–500 μg/mL ERY in the presence of 200 μg/mL IS, $R^2 = 0.997$, data not shown). The

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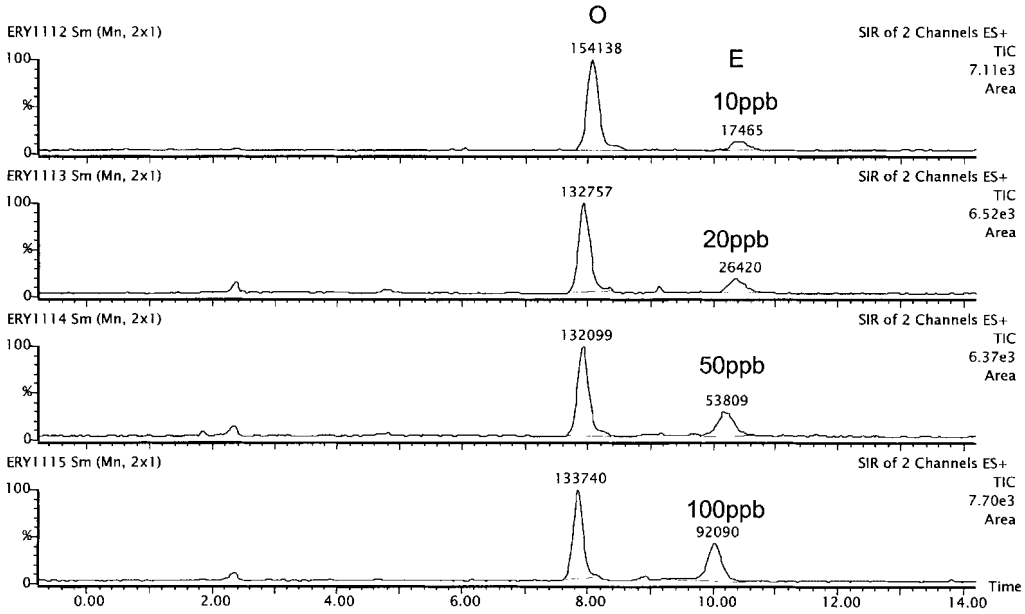

Figure 6. MS-SIM chromatograms for erythromycin (E) and oleandomycin (O) in fortified chicken liver extracts. Blank chicken liver samples were extracted as described in the experimental section, fortified with varying amounts of ERY (10–100 ppb) with a constant amount of IS (200 ppb), and the MS-SIM response determined from integrated peak areas for both $[M + H]^+$ peaks.

performance of the AQA source/probe design was investigated using automated overnight analysis of repeated injections of ERY/IS standards when using a chromatographic mobile phase containing 0.01 M phosphate buffer or 10 mM phosphate buffer containing 10 mM sodium octane sulfonic acid. It was determined that addition of phosphate buffer to the mobile phase caused a small suppression of the average mass spectral response for repeated injections of ERY (16%, data not shown). Figure 3 shows MS-SIM chromatograms for ERY and IS, of the respective $[M + H]^+$ trace, at 0.5, 8, and 15 h time points during the overnight analysis in the presence of phosphate buffer alone. Figure 4 shows the instrument performance shown as the integrated peak area from 1 ng injections of ERY over the entire time range. Similar chromatographic (not shown) and mass spectral response performance were observed when an ion-pairing agent was added to the mobile phase (i.e., minimal decrease in signal intensity over the time range, see Fig. 5). However, the absolute signal intensity was about 50% lower than seen with phosphate buffer alone (see Fig 4), presumably as a result of ionization suppression by the ion-pairing reagent. Control experiments in which the self-cleaning flow was turned off during phosphate buffer elution showed that the signal for ERY fell by more than 50% within 30 min of operation (data not shown).

Liver (chicken and beef) and catfish fillet tissue samples spiked with a known amount of ERY (100 ppb) were analyzed using the phosphate buffer LC method. Figure 6 shows a typical example for a chicken liver extract fortified at 10–100 ppb ERY. Beef liver and catfish extracts showed very similar chromatograms. The corresponding blank chromatograms for all tissue samples showed no responses at the masses of ERY or IS (not shown). The ERY/IS ratio plot for concentrations of ERY fortified into blank chicken liver extracts ranging from 10–500 ppb and IS (200 ppb) was linear over the entire range ($R^2 = 0.997$, data not shown). The MS-SIM chromatogram for 100 ppb ERY was equivalent to 1 ng injected on-column, because the absolute recovery of ERY spiked at 100 ppb into these tissues was approximately 10%. Improving the extraction efficiency for ERY is a priority before this method can be fully validated.

CONCLUSIONS

This study demonstrates the effectiveness of using a self-cleaning flow to extend API mass spectrometric analysis time when involatile mobile phase components are required. When analysis by LC/MS of ERY was performed in the absence of a cleaning flow to the orifice tip, rapid deterioration of performance was observed concomitant with the build up of involatiles. When a cleaning flow was used, the presence of involatile buffers minimally affected the ERY response over extended time periods. The response during LC/MS for ERY was linear with respect to concentration for ERY standards and for chicken liver extracts fortified with ERY at concentrations below the regulatory level of interest (100 ppb).

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