JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Chlorate Analyses in Matrices of Animal Origin

David J. Smith*,† and Joshua B. Taylor‡

†Biosciences Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 1605 Albrecht Boulevard, Fargo, North Dakota 58102-2765, United States

‡U.S. Sheep Experiment Station, Agricultural Research Service, U.S. Department of Agriculture, Dubois, Idaho 83423-5032, United States

ABSTRACT: Sodium chlorate is being developed as a potential food-safety tool for use in the livestock industry because of its effectiveness in decreasing concentrations of certain Gram-negative pathogens in the gastrointestinal tracts of food animals. A number of studies with sodium chlorate in animals have demonstrated that concentrations of chlorate in meat, milk, wastes, and gastrointestinal contents range from parts per billion to parts per thousand, depending upon chlorate dose, matrix, and time lapse after dosing. Although a number of analytical methods exist for chlorate salts, very few were developed for use in animal-derived matrices, and none have anticipated the range of chlorate concentrations that have been observed in animal wastes and products. To meet the analytical needs of this development work, LC-MS, ion chromatographic, and colorimetric methods were developed to measure chlorate residues in a variety of matrices. The LC-MS method utilizes a $Cl^{18}O_3^-$ internal standard, is applicable to a variety of matrices, and provides quantitative assessment of samples from 0.050 to 2.5 ppm. Due to ion suppression, matrix-matched standard curves are appropriate when using LC-MS to measure chlorate in animal-derived matrices. A colorimetric assay based on the acid-catalyzed oxidation of *o*-tolidine proved valuable for measuring ≥20 ppm quantities of chlorate in blood serum and milk, but not urine, samples. Ion chromatography was useful for measuring chlorate residues in urine and in feces when chlorate concentrations exceeded 100 ppm, but no effort was made to maximize ion chromatographic sensitivity. Collectively, these methods offer the utility of measuring chlorate in a variety of animal-derived matrices over a wide range of chlorate concentrations.

KEYWORDS: chlorate, chromatography, gastrointestinal tract, method, milk, serum, urine, feces, mass spectrometry

INTRODUCTION

Since 1901 when its first application was described in the scientific literature,[1] sodium chlorate has found several important agricultural and industrial applications including use as a broad-spectrum herbicide[2] and as a source of chlorine dioxide in the pulp-wood bleaching process and in water-treatment plants.[3] Within the past decade, the potential use of sodium chlorate has been expanded by the discovery[4] that chlorate salts effectively mitigate the presence of pathogens such as *Salmonella* and *Escherichia coli* O157:H7 in the gastrointestinal tracts of food animals[5] including poultry,[6,7] swine,[8,9] sheep,[10,11] and cattle.[12−14]

Coincidental with the investigation of chlorate as a preharvest food safety tool, there has been a need to specifically and accurately measure chlorate in animal tissues, products, and wastes over wide concentration ranges.[15−21] Analytical methods available for the measurement of chlorate have been typically based on visible spectrophotometry, titration, or ion chromatography. More recent analyses have included liquid chromatography coupled with mass spectral detection,[22−24] but these methods have been developed mostly for relatively clean matrices such as water. Methods based on spectrophotometry and titration typically involve the acid-catalyzed reduction of chlorate with the subsequent oxidation of an indicator chromophore. Although colorimetric and titration methods may be fairly useful with high concentrations of chlorate or when chlorate is dissolved in a fairly pure solvent (i.e., water), their sensitivities in biological matrices containing endogenous reductants that can compete with the indicator during oxidation are limited. Ion chromatography with conductivity detection has been successfully used to measure parts per billion (ppb) concentrations of chlorate in drinking water[25] and, more recently, it has been used to measure the disappearance of chlorate salts from ruminal fluid cultures.[26] In the latter study, accurate measurements of chlorate were limited when chlorate concentrations were <25 ppm. For example, recoveries of analyte at 0.5 and 5 ppm were 718 and 145%, respectively.

Because chlorate is highly water-soluble, ionic, inorganic, and is of low molecular weight, typical reversed phase chromatography procedures are not appropriate for chlorate analysis. Preliminary studies in our laboratory using ion chromatography with conductivity detection indicated that sensitivity would not be sufficient to measure chlorate residues in food animal tissues at withdrawal periods approaching 24 h. LC-MS has been used to detect perchlorate in several animal[27−29] and plant[30] matrices. We therefore had reason to believe that a LC-MS method could be developed for chlorate in animal matrices that would allow a limit of quantitation in the parts per billion (ppb) range.

During the course of development work with sodium [^{36}Cl]-chlorate, it was noted that chlorate concentrations in feces, serum, gastrointestinal tissues, and urine would vary considerably (from hundreds of ppm to ≤50 ppb) depending upon the dose and the time of sample collection. For studies in which radiolabeled chlorate was employed, chromatographic separation

Received: November 19, 2010
Accepted: January 13, 2011
Revised: January 10, 2011
Published: February 07, 2011

and quantitation of low chlorate levels could be accomplished even with matrix interferences because quantitation was performed by radiochemical analysis.[15−19] The use of nonradiolabeled chlorate, however, requires that chlorate be quantified in the presence of matrix components. Therefore, the objective of this study was to develop a series of analytical tools suitable for use in animal studies that would be simple, applicable to a variety of matrices (gastrointestinal contents, milk, serum, and urine), and specific for chlorate over a wide range of concentrations.

MATERIALS AND METHODS

General Approach. A LC-MS method was developed for the analysis of samples containing low (typically ≤2.5 ppm) levels of chlorate including milk, urine, and serum samples and for more complex matrices such as gastrointestinal contents and feces. The lower limit of quantitation of the MS-based assay was dependent upon sample size, but was as low as 50 ppb for some applications. For less troublesome matrices containing high quantities of chlorate such as serum and milk, a rapid colorimetric assay based on the work of Couture[31] was developed. Components of urine interfered greatly with the colorimetric assay, even with high chlorate concentrations, so an ion chromatographic method based on the method of Beier et al.[26] was used after some modification. These developed methods were then utilized to measure chlorate in a variety of matrices including sheep gastrointestinal tract contents, feces, serum, urine, and milk.

Collection of Sheep Matrices. Urine, feces, milk, gastrointestinal contents, and blood serum were collected from control (nondosed) and chlorate-dosed sheep (oral administration) at the U.S. Sheep Experimental Station (Dubois, ID) or at the Biosciences Research Laboratory (Fargo, ND). Samples were collected only after studies had been approved by the respective Institutional Animal Care and Use Committees. A detailed description of animal husbandry, dosing, and tissues collection will be reported in a separate paper.

Liquid Chromatography−Mass Spectrometric Assay. *Chemicals.* Sodium chlorate having a purity of no less than 99.6% was obtained from EKA Chemical Co. (Marietta, GA); $H_2{}^{18}O$ (99.3 at. % ^{18}O) was purchased from Sigma-Aldrich (St. Louis, MO); and ACS-grade sodium chloride was purchased from VWR Scientific (West Chester, PA).

Synthesis and Characterization of a $NaCl^{18}O_3$ Internal Standard. An electrolysis cell was constructed using a 5.0 mL Supelco glass reaction vial equipped with a Teflon septum. Platinum wire (18 gauge) electrodes were placed through the septum within the vial cap and passed through additional Teflon−silicone seals above and below the cap to ensure electrode separation (Figure 1). The reaction vessel was charged with 348 mg of sodium chloride and 1 mL of $H_2{}^{18}O$. Sodium chloride was dissolved, and a 10 μL sample was reserved for analysis by ion chromatography before the initiation of electrolysis. Electrolysis (5 V) was timed, and the reaction was stopped at 10, 20, 30, 40, and 50 min to remove samples for analysis by ion chromatography. After 30 min, 250 μL of $H_2{}^{18}O$ was added to the reaction vessel. At the completion of electrolysis, the reaction vial was sealed with a new Teflon−silicone septum and the vial was frozen at −20 °C.

Reaction progress was monitored by diluting each 10 μL reaction aliquot in 990 μL of nanopure H_2O and subsequent analysis of 5 μL aliquots by ion chromatography. The ion chromatograph consisted of a Waters model 600 pump and processor retrofitted with Teflon pump heads and Peek tubing. A mobile phase consisting of 30 mM NaOH was pumped at 1 mL/min through Dionex AG11HC and AS11HC guard and analytical columns (250 × 4 mm), respectively. Ions were detected using a Dionex CD25 conductivity detector with an ASRS Ultra 4 mm ion suppressor. Peaks were recorded and integrated using a Waters 746 model data module.

Figure 1. Schematic of the reaction vessel used for the synthesis of the $NaCl^{18}O_3$ internal standard used in mass spectrometric analyses. Teflon disks shown inside and outside the reaction vial were used to maintain physical separation of each platinum electrode. Each electrode also passed through a Teflon disk within the vial cap (not shown).

To quantify the yield of ^{18}O-chlorate synthesized, the contents of the reaction vial were transferred to a 100 mL volumetric flask and diluted to volume with purified water. Triplicate dilutions (10 μL in 5 mL of water) were prepared, and 25 μL aliquots ($n = 3$) of each dilution were injected onto the ion chromatography system described above. A standard curve was constructed by injecting triplicate 25 μL aliquots of sodium chlorate standards containing 0.5, 1, 5, and 10 μg/mL. The concentration of synthesized chlorate in each dilution was calculated by linear regression analysis, and the reaction yield of chlorate was back calculated.

The purity of the $NaCl^{18}O_3$ internal standard was determined using a Waters Q-TOF Ultima API-US, quadrupole time of flight (QTOF) MS (Waters, Beverly, MA) and an electrospray ionization source. Negative ions of the synthetic product were characterized by infusion (25 μL/min) in the MS and MS/MS modes. Fragmentation was induced by variation of the collision energy from 5 to 35 eV. Isotopic composition of the internal standard was assessed by infusion in the MS mode.

Extraction of Gastrointestinal Fluids and Fecal Material. Sheep gastrointestinal tract materials were weighed (intestine, 0.1 or 0.03 g; cecum, 0.25 g; colon, 0.5 g; and fecal samples, 1 g; wet weights) into 50 mL polypropylene vials. For each set of samples, a set of four blank (control sheep) matrices was also weighed into corresponding tubes. After weighing, all samples were placed on ice to prevent bacterial degradation of chlorate. Each sample was fortified with 27 μL (988 ng) of an aqueous $NaCl^{18}O_3$ internal standard solution, and two of the blank matrices were fortified with 10 μL (1000 ng) of an aqueous $NaClO_3$ solution. After the addition of internal and fortification standards, 5 mL of ice-cold water was added to each tube and vortexed vigorously for a minimum of 60 s. Samples were individually probe-sonicated (Branson Sonifier, Danbury, CT) using a setting of "7" and a duty cycle of 90% for 1 min. After sonication, samples were centrifuged for 15 min at 30600*g* (Sorvall, Ashville, NC). Supernatants were decanted into clean tubes, which were placed on ice, and pellets were resuspended and revortexed in additional 5 mL aliquots of ice-cold water. The resulting suspension was recentrifuged as described above, and the new supernatant was combined with the previous supernatant. The combined supernatants were vortexed, and residual particulates were removed by centrifugation at 3210*g* for 15 min. During centrifugation C-18 SPE tubes (Mega Bond Elute; C18, 1 g, 6 cm^3) were conditioned with 3 mL of methanol followed by 6 mL of purified water. Upon completion of centrifugation, 2.5 mL aliquots of sample extract were applied to respective C18 tubes

and the aqueous solutions were collected into 5 mL volumetric flasks. Each SPE tube was rinsed with 2 mL of nanopure water, and rinsewater was combined with the loading material into 5 mL volumetric flasks. Volumetric flasks were diluted to the 5 mL mark with nanopure water, capped, sealed with parafilm, and inverted 10 times. Aliquots of each dilution were transferred to LC vials for analysis by LC-MS; 20 μL each of unknown samples, blanks, and fortified blanks was injected onto the LC-MS system. Sets of blanks, fortified blanks, and samples were bracketed by samples of a standard curve containing 0, 2.5, 5, 25, 50, 125, and 250 ng/mL $NaClO_3$ containing 50 ng/mL of the $NaCl^{18}O_3$ internal standard.

Serum/Milk/Urine Containing Low Levels of Chlorate. Control or test matrices (50−200 μL) were transferred to 2 mL polypropylene vials, and internal standard (250 ng $NaCl^{18}O_3$) was added to each tube. Sodium chlorate (125 ng) was added to duplicate tubes of blank matrix for each set run. Proteins were precipitated and pelleted by adding 0.2 mL of ice-cold acetonitrile to each tube with subsequent freezing (−20 °C, 1 h) and centrifugation (15000*g*) for 20 min. Aliquots (10−100 μL depending upon the expected chlorate concentration) of supernatant were transferred to 5 mL volumetric flasks and diluted to volume with water. Before LC-MS analysis, individual samples were mixed and filtered through 0.45 μm PTFE syringe filters. Upon analysis, 20 μL aliquots were injected onto the LC-MS system. Matrix-fortified standard curves containing 2% blank serum, 0.5% blank urine, or 0.5% blank milk and 0, 2.5, 5, 25, 50, 125, and 250 ng/mL $NaClO_3$ with 50 ng/mL $NaCl^{18}O_3$ internal standard were prepared. Ion suppression was assessed by comparing the detector response of standard curves prepared in water and matrix.

Liquid Chromatography−Mass Spectrometry. A Waters Acquity UPLC system online with a Waters triple-quadrupole mass selective detector was used for quantitative analyses of chlorate. Data were acquired and processed using MassLynx 4.1 with TargetLynx systems; quantification was effected with the same software. Summed ion chromatograms were constructed for ^{35}Cl and ^{37}Cl isotopes of chlorate ions (m/z 82.9, 84.9) and the ^{35}Cl and ^{37}Cl isotopes of ^{18}O-chlorate ions at m/z 88.9 and 90.9. Sample aliquots (20 μL) were injected from an autosampler maintained at 4 °C onto a Waters Ion-Pak Anion HR column (4.6 × 75 mm) maintained at 40 °C and eluted with an isocratic mobile phase of acetonitrile/water (1:1) at a flow rate of 0.75 mL/min. Ions were detected in the negative ion mode with a capillary setting of 3.00 kV and a cone voltage of 40 V for $Cl^{18}O_3^-$ and 50 V for ClO_3^-; the source and desolvation temperatures were set at 150 and 500 °C, respectively, with cone and desolvation gas flows at 50 and 800 L/h, respectively.

Colorimetric Analyses. The *o*-tolidine method of Couture[31] was adapted for use in the measurement of chlorate in serum and milk. Briefly, serum was thawed, and 100 μL aliquots were transferred to Pall Nanosep (Pall Life Sciences, Ann Arbor, MI) ultrafiltration centrifuge tubes with a 10K molecular weight cutoff. Milk samples were prepared by diluting with an equal volume of ice-cold methanol and freezing for 1 h, followed by ultrafiltration of 200 μL aliquots. Ultrafiltration was effected by centrifugation for 15 min at 15000*g* at 4 °C. Aliquots of serum (15 μL) or milk/methanol (12.5 μL) filtrate were transferred to a microcuvette containing 385 μL of nanopure water. Aliquots (100 μL) of 1.4 M *o*-tolidine−2HCl, dissolved in 25% concentrated HCl, and 0.5 mL of concentrated HCl were sequentially added to each cuvette; color was allowed to develop for a minimum of 10 min, and the absorbance of each tube was read at 448 nm using a Shimadzu UV-1601 UV−visible spectrophotometer (Kyoto, Japan). Minimum color development time was established by measuring absorbance in pentuplicate tubes fortified with 1.2 μg of sodium chlorate in serum at 0, 2, 4, 6, 8, 10, 15, 20, 25, and 30 min after the addition of *o*-tolidine and acid. The effect of milk or serum on absorbance relative to water was determined by substituting various amounts of the water in the reaction mixture with

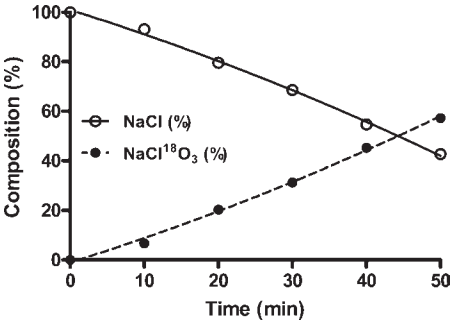

Figure 2. Formation of sodium [^{18}O]chlorate from sodium chloride as a function of time. The reaction was stopped at 50 min because the formation of perchlorate in the reaction media was detected by ion chromatography.

serum or milk (12−400 μL, for example) in tubes fortified with a constant quantity of chlorate. Chlorate in unknown samples of serum was calculated from quadratic regression curves prepared from blank matrices fortified with 0.3, 0.6, 1.2, 2.1, and 3.0 μg of sodium chlorate representing matrix concentrations of 20−200 μg/mL when 15 μL of serum was assayed. For milk, standard curves consisted of matrix fortified with 0.25, 0.5, 1.0, 1.75, and 2.5 μg of sodium chlorate, representing matrix concentrations of 40−400 μg/mL when 6.25 μL of milk was assayed (12.5 μL of milk/methanol). Standard curves in matrices were prepared daily and were processed in the same manner as unknowns. For some serum samples, dilution was required; in these instances matrix aliquots were diluted in water prior to ultrafiltration, and 15 μL of the dilute filtrate was used for the colorimetric assay.

Experiments were conducted to determine the utility of protein removal from serum by precipitation with ice-cold acetonitrile followed by centrifugation (15000*g*; 15 min), rather than by ultrafiltration. In such experiments equal volumes of serum and ice-cold acetonitrile were vortexed and centrifuged. The absorbance of 15 μL of the resulting supernatant was determined as described above for controls and for serum fortified with several levels of sodium chlorate. Experiments were also conducted to determine the utility of the colorimetric method with urine samples, but the presence of urine always quenched the assay regardless of whether samples were prepared for the assay by ultracentrifugation or by C18 solid phase extraction.

Ion Chromatography. Because matrix components of urine completely interfered with the rapid colorimetric chlorate assay and because previous studies had indicated that very high concentrations of chlorate are excreted in urine, the ion chromatographic method of Beier et al.[26] was modified for the detection of chlorate in urine. Briefly, urine samples (50−250 μL depending upon sodium chlorate content) were loaded onto Bond Elut Certify (200 mg; Varian, Lake Forest, CA) SPE cartridges conditioned with 2 mL of methanol followed by 4 mL of water. The unretained liquid component of each urine aliquot was collected into a 5 mL volumetric flask as was a 0.5 mL water rinse of each SPE cartridge. Each volumetric flask was diluted to the mark with nanopure water, capped, and mixed thoroughly. Aliquots of urine extracts (50 μL) were injected on the ion chromatograph described above, except that a 4 × 250 mm Dionex (Sunnyvale, CA) AS9-HC column was used. Chlorate was eluted using an isocratic mobile phase consisting of 9 mM sodium carbonate (1.0 mL/min) and was detected using a conductivity detector operated in the external water mode. Chlorate in urine was quantified using standard curves derived from peak areas of 50 μL injections of 5, 10, 20, and 100 μg/mL sodium chlorate standards dissolved in water (unknowns and standards injected in duplicate).

Statistics. Data generated from the mass spectral analysis of chlorate standards in water and in incurred matrices were best fit to quadratic equations. Parameter (B_1 and B_2) estimates for curves in water and in

Table 1. Possible ^{18}O-Labeled Products of the Reaction of Chloride Ion with 99.3 At. % $H_2{}^{18}O$ and Expected Molecular Masses

		masses of expected isotopic products							
		$Cl^{16}O_3^-$		$Cl^{16}O_2{}^{18}O_1^-$		$Cl^{16}O_1{}^{18}O_2^-$		$Cl^{18}O_3^-$	
oxygen isotopic composition	theoretical percentage[a]	^{35}Cl	^{37}Cl	^{35}Cl	^{37}Cl	^{35}Cl	^{37}Cl	^{35}Cl	^{37}Cl
$Cl^{16}O_3^-$	<0.001	82.95	84.95						
$Cl^{16}O_2{}^{18}O_1^-$	0.01			84.96	86.96				
$Cl^{16}O_1{}^{18}O_2^-$	1.48					86.96	88.96		
$Cl^{18}O_3^-$	97.91							88.96	90.96

[a] Percentage of each isotopic species present is calculated by the binomial distribution $(X + Y)^3$, where X is the atom percentage of $H_2{}^{18}O$ (99.3%) and Y is the atom percentage of $H_2{}^{16}O$ (0.5%) in the electrolytic synthesis.

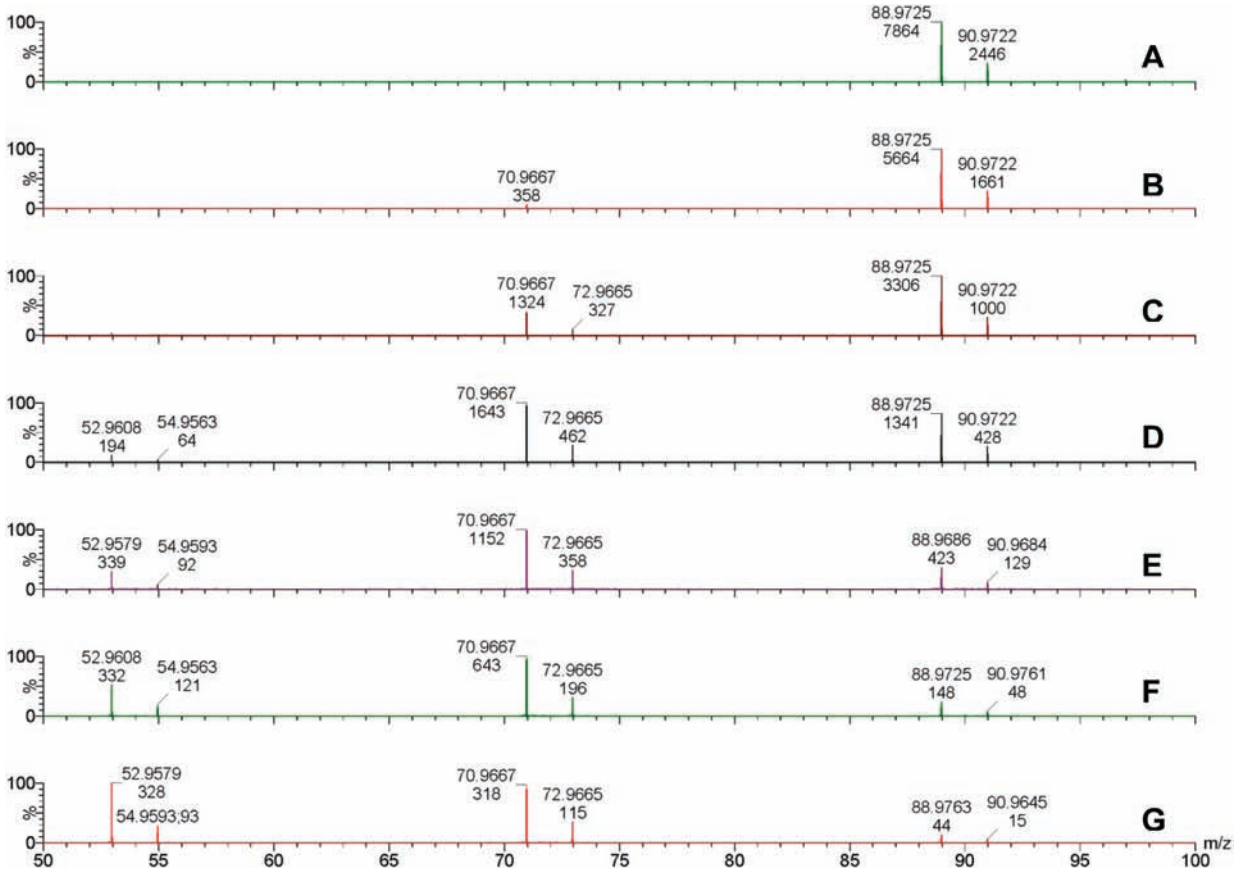

Figure 3. MS-MS fragmentation of the synthetic product, sodium ^{18}O-chlorate internal standard with collision energies of 5, 10, 15, 20, 25, 30, and 35 eV in panels A, B, C, D, E, F, and G, respectively.

matrices were compared using the *F* test. Standard curves generated for chlorate in water, milk, or serum were analyzed by the colorimetric assay and fit to quadratic equations in a similar manner as the mass spectral data and parameter estimates compared using the *F* test (GraphPad Prism 5.00; GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

LC-MS Assay. *Synthesis and Characterization of Na $Cl^{18}O_3$.* Synthesis of an ^{18}O-labeled sodium chlorate internal standard was necessitated because no commercial product was available. Initial efforts to use sodium perchlorate as an internal standard were not satisfactory for two important reasons. First, the mass spectral detector response to the perchlorate ion was about 10 times greater than the detector response to chlorate when equal masses were injected. Second, the chromatographic retention time of perchlorate was sufficiently long, relative to chlorate, so that in matrix perchlorate would not be subject to the same ion suppression effects as chlorate. Standard errors derived from fortified matrix samples were sufficiently large to indicate that perchlorate was not an appropriate internal standard for chlorate.

Previous experience with the synthesis of chloroxyanions at the Fargo Laboratory[21,32] involved the electrolysis of radioactive chloride or chlorate ion ($Na^{36}Cl$ or $Na^{36}ClO_3$) in water to form radioactive sodium chlorate and sodium perchlorate test articles, respectively, for use in metabolism and residue studies. A similar approach was taken in the synthesis of a sodium chlorate internal standard except that unlabeled chloride was used in the presence of 99.3 at. % $H_2{}^{18}O$. Figure 2 shows the progress of the electrolytic

dx.doi.org/10.1021/jf1044684 |*J. Agric. Food Chem.* 2011, 59, 1598–1606

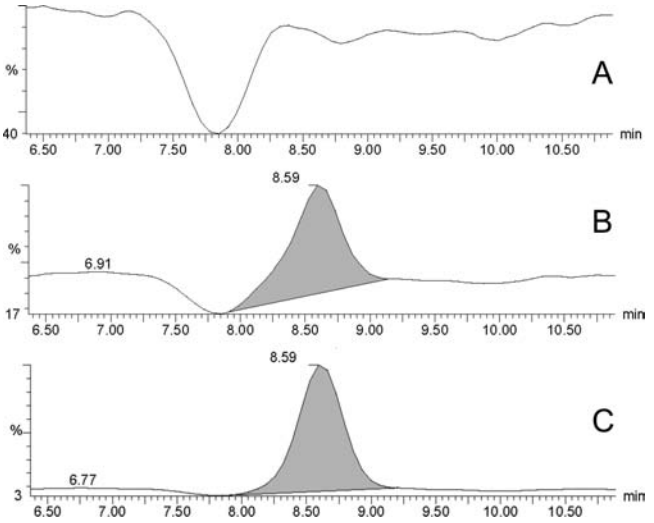

Figure 4. Representative selected ion (m/z 82.9 + 84.9) chromatograms of blank serum (A), serum fortified with 25 ng/mL of chlorate (B), and blood serum collected from a sheep 1 h after being dosed with 17 mg/kg body weight of sodium chlorate (C). (Internal standard not shown.)

synthesis of ^{18}O-chlorate based on the conductivity detection of chloride and chlorate. The reaction was terminated at 50 min because in preliminary test reactions using unlabeled water the formation of perchlorate (ClO_4^-) was detected at 50 min. At the termination of the reaction containing $H_2{}^{18}O$, a total of 328.8 mg of $NaCl^{18}O_3$ had been formed, representing a 51.9% yield from chloride.

Table 1 shows the possible labeled chlorate products of the electrolysis of NaCl with $H_2{}^{18}O$. Because chloride exists naturally as $^{35}Cl^-$ and $^{37}Cl^-$ isotopes and because the water in which chloride was reacted contained a 0.5 at. % ^{16}O impurity, one would expect the formation of eight distinct isotopic chlorate products. However, full-scan TOF MS spectra of the chlorate ion showed no evidence for the presence of $NaCl^{16}O_3$ or $NaCl^{16}O_2{}^{18}O$ in the reaction mixture (data not shown). Such a result was expected because the probability for the formation of either product was ≤0.01% (Table 1). For example, the theoretical maximal amount of $Cl^{16}O_3^-$ that could be formed according to the binomial probability distribution of $(X + Y)^3$, where X is the fraction of $H_2{}^{18}O$ (0.993) and Y is the fraction of $H_2{}^{16}O$ (0.005), is <0.001%. In contrast, $Cl^{16}O_1{}^{18}O_2^-$ was detected at about 1.5% of the total when the reaction product was infused in the MS mode. Even with the presence of the $Cl^{16}O_1{}^{18}O_2^-$, the masses of the $Cl^{18}O_3^-$ internal standard were well separated (6 amu) from the masses of the unlabeled target analyte $Cl^{16}O_3^-$. The $^{37}Cl^{16}O_1{}^{18}O_2^-$ isotope peak does have the same mass as the $^{35}Cl^{18}O_3^-$ isotope peak (m/z 88.96), which would introduce a slight bias (about 1.5%) in the selected ion measurement of the internal standard.

Figure 3 shows MS/MS spectra of the chlorine isotopes of $Cl^{18}O_3^-$ with increasing collision energies. As collision energy was increased, the internal standard fragmented as expected with sequential losses of 18 amu consistent with sequential losses of ^{18}O. In addition, Figure 3 shows the optimal collision energy for monitoring the parent ion in the MS mode would be 5−10 eV; for operating the MS/MS mode, collision energies of 15−20 eV would be appropriate.

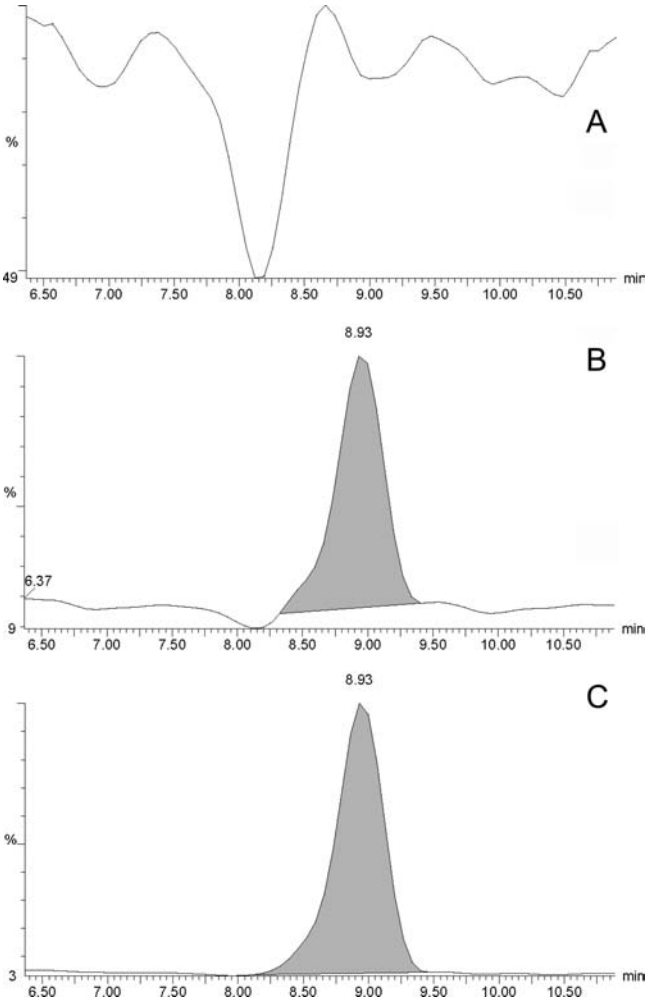

Figure 5. Representative selected ion (m/z 82.9 + 84.9) chromatograms of blank small intestinal contents (A), small intestinal contents fortified with 25 ng/mL of chlorate (B), and small intestinal contents collected from a sheep 1 h after being dosed with 90 mg/kg body weight of sodium chlorate (C). (Internal standard not shown.)

LC-MS Analysis of Chlorate in Animal Matrices. Chlorate present in animal matrices was analyzed in the MS mode. Representative selected ion chromatograms of blank, fortified, and incurred serum and small intestinal fluids are shown in Figures 4 and 5, respectively. Standard curves of chlorate fortified into water, fecal extract, sheep serum (2%), urine (0.5%), and milk (0.5%) fit to quadratic polynomials are shown in Figure 6. Ion suppression effects for serum and urine matrices were clearly present as verified by the fact that the quadratic parameter estimates (B_0, B_1, B_2) differed from the same coefficients of standard curves in water in a highly significant manner ($P < 0.001$). When the parameters of the standard curves prepared from milk and feces were compared with standard curves in water, differences were not detected ($P > 0.05$). These data confirm the requirement to establish, on a matrix-by-matrix basis, whether ion suppression or enhancement effects occur with a LC-MS-based method. However, the use of an isotopically labeled surrogate as the internal standard should correct for any ion suppression events and provide a robust analysis no matter the matrix.

Table 2 shows results of the application of the MS assay to the measurement of chlorate in tissues of sheep dosed with sodium

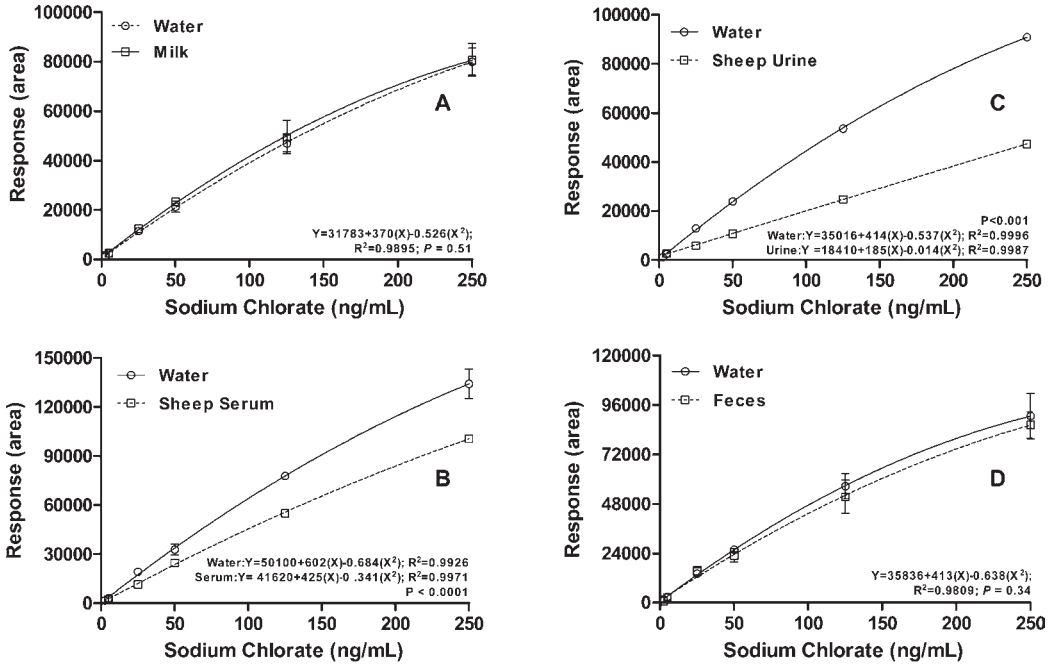

Figure 6. Matrix effects of extracts derived from sheep milk (A), serum (B), urine (C), and feces (D) on the mass spectral analysis of sodium chlorate. Solid lines represent sodium chlorate standards fortified into water, and hatched lines represent sodium chlorate fortified into various sheep matrices. Regression equations and *P* values are shown for each comparison; instances of a single regression indicate that matrix effects were not significant.

Table 2. Incurred Chlorate Residues (Mean ± SD) in Matrices of Sheep Dosed with Sodium Chlorate

			tissue						
dose (mg/kg bw)	exposure period (h)	withdrawal period (h)	intestine[a] (ppm)	cecum (ppm)	colon (ppm)	serum (ppm)	milk (ppm)	urine (ppm)	*n*
			Mass Spectral Assay						
0	none	0				NDR[b]			3
42	bolus, oral	2[d]				15.8 ± 1.6			3
42	bolus, oral	24[d]				0.045 ± 0.018[e]			3
0	none	25.5 ± 1.6	NDR	NDR	NDR				4
60	bolus, oral	25.5 ± 1.6	21.3 ± 14.4	0.67[c]	0.12[c]				4
			Colorimetric Assay						
450	bolus, oral	8[d]				422.5 ± 78.9	287 ± 67		6
450	bolus, oral	24				65.9 ± 31.9	151 ± 61		4
			Ion Chromatography						
450	bolus, oral	0−8						7712 ± 2632	6
450	bolus, oral	40−48						480 ± 244	6

[a] Samples removed from the small intestine of sheep. [b] No detectable residue. [c] Three of four animals had no detectable residue. [d] Sample collected at the indicated number of hours after dosing. [e] One animal had no detectable residue; thus, the limit of quantitation (0.025 ppm) was used to calculate the mean.

chlorate. In sheep provided 60 mg/kg of chlorate and killed 25.5 ± 1.6 (mean ± SD) h after the last exposure to chlorate, measurable residues occurred in the small intestine, cecum, and colon, but levels decreased with passage down the tract. Samples containing >5 ppm of chlorate required reassay or dilution because of the sensitivity of the MS analysis. Lower limits of quantitation (LOQ; Table 3) were defined as the chlorate concentration corresponding to the lowest standard of the standard curve (2.5 ng/mL) having a signal-to-noise ratio of at least 5. Practical LOQs were dependent upon sample sizes and on sample dilution during workup. Of note for the mass spectral assay is that all samples were diluted during workup. No effort was made to increase assay sensitivity by concentration after chlorate extraction.

Colorimetric Determination of Chlorate in Serum, Urine, and Milk. Although the MS method proved to be satisfactory for matrices containing chlorate at concentrations below about 5 ppm, many matrices collected during routine pharmacokinetic and efficacy studies had chlorate concentrations well in excess of 5 ppm. Analysis of such samples by LC-MS required dilutions with appropriate adjustments to the internal standard and was an inefficient use of MS resources. It was reasoned that a colorimetric method, even though insensitive, might serve as a fairly

dx.doi.org/10.1021/jf1044684 |*J. Agric. Food Chem.* 2011, 59, 1598–1606

Table 3. Typical Limits of Quantitation with Various Analytical Techniques

matrix	sample size	LC-MS (ppm)	colorimetric (ppm)	ion chromatography (ppm)
blood serum	0.015 mL		20	
	0.025 mL	0.5		
	0.050 mL	0.250		
	0.1 mL	0.125		
	0.5 mL	0.025		
milk	0.025 mL	0.500		
	0.0063 mL		40	
urine	0.025 mL			1000
	0.050 mL			500
	0.100 mL			100
small intestine	0.05 g	1.000		
	0.5 g	0.100		
cecum	0.25 g	0.200		
colon	0.25 g	0.200		
	0.5 g	0.100		
feces	0.5 g	0.100		

rapid and simple tool that would provide adequate chlorate quantitation of high-concentration samples.

To this end, the photometric method described by Couture,[31] which measures the acid-catalyzed oxidation of *o*-tolidine in water by chlorate, was modified to accommodate urine, serum, and milk samples. Initial efforts in developing the assay employed water, blank sheep serum, or sheep urine fortified with either 2.6 or 5.1 μg of sodium chlorate. Recoveries of chlorate in water samples were excellent, but chlorate fortified into unprocessed serum or urine was not detectable. Through an iterative process it was learned that chlorate fortified into serum or milk could be measured after ultrafiltration through a 10000 molecular weight cutoff filter. In fortified urine samples, ultrafiltration was ineffective at removing the interfering compound(s) and urinary chlorate could not be determined using the colorimetric assay. Likewise, cleanup of chlorate-fortified urine or serum using C18 SPE cartridges was not sufficient for color development.

Figure 7A shows color development of the *o*-tolidine reaction in fortified sheep serum and in water as a function of time. No differences ($P > 0.05$) between absorbance in water or in serum occurred at any of the time points tested at the volume routinely employed (0.012 mL) and the mass of chlorate added (1.2 μg). In serum, optimal development occurred between 4 and 15 min (20−22 °C) with no differences ($P > 0.05$) occurring in absorbencies between 4 and 30 min. For the purposes of standardization, a minimal 10 min development time was selected to provide flexibility in handling sets of 30−40 samples. Matrix volume was a factor in the successful execution of the colorimetric analysis. For example, Figure 7B shows the effect of increasing milk volume on color development in samples fortified with 1.5 μg of sodium chlorate. Figure 7C shows standard curves

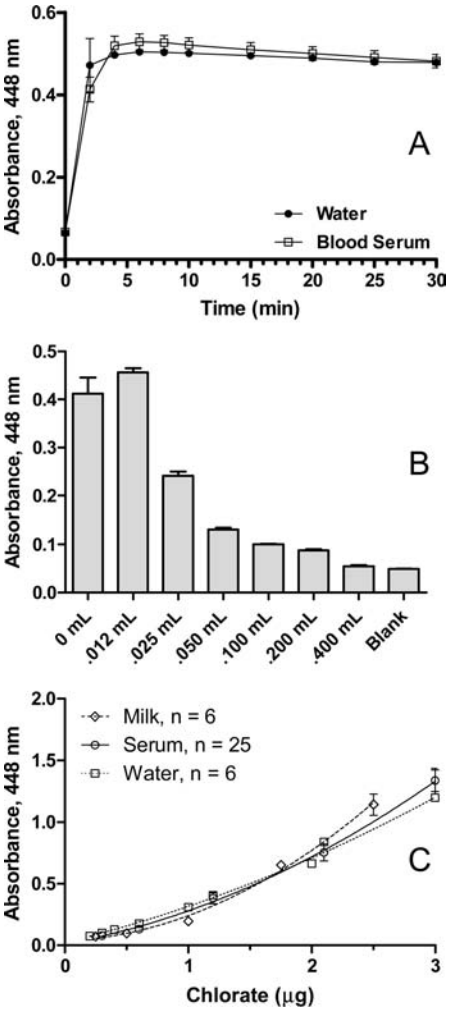

Figure 7. (A) Color development in chlorate (1.2 μg) in fortified water and blood serum as a function of time; (B) color development as a function of blood serum volume at a constant mass of chlorate; (C) standard curves of sodium chlorate in water and sheep blood serum and milk. Regression equations of chlorate in milk and serum differed from that of water ($P < 0.001$).

prepared in milk, serum, and water. Linear regression analyses of standard curves resulted in fairly poor fits as can be intuited from Figure 7C. The nonlinear relationship of absorbance and chlorate concentration was true even for the water matrix. The data were fit (R^2 = 0.9853, 0.9864, and 0.9401 for serum, milk and water, respectively) to a second-order polynomial (quadratic), and unknowns were predicted from the least-squared regression equations. When unknowns were calculated, matrix-fortified standard curves were used because regression parameters (B_0, B_1, B_2) differed ($P < 0.001$) for curves prepared in matrix (serum $[Y = 0.4519 + 0.4288(X) + 0.0865(X^2)]$ or milk $[Y = 0.3209 + 0.4328(X) + 0.1625(X^2)]$) and water $[Y = 0.4456 + 0.3782(X) + 0.0495(X^2)]$. Limits of quantification for the colorimetric assay of serum and milk were 20 and 40 ppm, respectively. Nevertheless, the colorimetric assay was useful for quantifying incurred residues of chlorate in milk and serum from ewes that had been dosed with 450 mg/kg bw of sodium chlorate (Table 2).

Urine proved to be a troublesome matrix for the colorimetric assay. Although urinary concentrations of chlorate can be quite

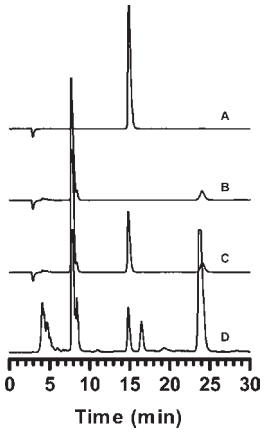

Figure 8. Ion chromatograms of sodium chlorate (A), blank sheep urine sample (B), a chlorate-fortified sheep urine sample (C), and urine from a ewe dosed with 450 mg/kg bw sodium chlorate (D) collected 40−48 h after dosing. Chlorate (retention time, 14.8 min) was measured with the conductivity detector range set at 200 μS full scale. Extraneous peaks, present in urine collected 40−48 h after dosing, were not present in urine collected prior to dosing with sodium chlorate.

high because of its rapid excretion,[15,17,18,21] chlorate fortified into blank urine at high levels could not be detected using the colorimetric assay, even after processing by ultrafiltration or passage through a C18 SPE cartridge. As a consequence, urine samples were analyzed by ion chromatography. Initial ion chromatography efforts employed Dionex AS11HC and AS16HC columns. For lamb urine, chlorate was eluted from these columns using sodium hydroxide mobile phases without interference (data not shown), but when urine from mature animals was analyzed, interferences prevented quantification of chlorate. Beier et al.[26] utilized a Dionex AS9 column to quantify chlorate in diluted (1/10) and filtered, but otherwise unprocessed, ruminal fluid samples. Beier et al.[26] reported recoveries of 718 and 142% at sample concentrations of 0.5 and 5 ppm, respectively. We adopted the chromatographic method of Beier et al.[26] but incorporated a C18 SPE sample cleanup step prior to ion chromatography (Figure 8). The ion chromatographic method was, by necessity, insensitive, with a limit of quantitation ranging from 100 to 1000 ppm, depending upon the volume of urine assayed (typically 50−250 μL). Recovery of chlorate from fortified urine (50 μg) was 91.4 ± 4.6% (n = 7). No attempts were made to optimize the ion chromatographic method to obtain lower limits of quantification in urine. Table 2 shows the magnitude of chlorate residues present in urine of sheep given a large dose of sodium chlorate. In the initial 8 h after dosing, urinary chlorate was excreted in milligram per milliliter quantities with levels dropping off to about 0.5 mg/mL at 40−48 h.

Collectively, the analytical methods described offer simplicity and flexibility to measure parts per billion to parts per thousand concentrations of chlorate in food and other complex animal matrices. Because chlorate concentrations in edible tissues of cattle,[15] swine,[17] and poultry,[18] measured by radiochemical methods were typically below 100 ppb, the mass spectral method could likely be adapted for use in tissue samples. For matrices containing greater quantities of chlorate such as urine, serum, and milk, the ion chromatographic or colorimetric assay would be useful. Gastrointestinal samples are amenable to analysis by LC-MS or ion chromatography depending upon dose.

AUTHOR INFORMATION

Corresponding Author

*Phone: (701) 239-1238. Fax: (701) 239-1430. E-mail: david.j.smith@ars.usda.gov.

DISCLOSURE

The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

ACKNOWLEDGMENT

We acknowledge the able technical skills of Michael Giddings, Grant Harrington, Jason Holthusen, and Tonya Thelen.

REFERENCES

(1) Anonymous. Destruction of prickly pear. *Qeensland Agric. J.* **1901**, *9*, 460−462.

(2) Klingman, G. C.; Ashton, F. M. In *Weed Science, Principles and Practices*; Wiley: New York, 1982, Chapter 20, pp 274−277.

(3) Deshwal, B. R.; Lee, H. K. Manufacture of chlorine dioxide from sodium chlorate: state of the art. *J. Ind. Eng. Chem.* **2005**, *11*, 330–346.

(4) Anderson, R. C.; Buckley, S. A.; Kubena, L. F.; Stanker, L. H.; Harvey, R. B.; Nisbet, D. J. Bactericidal effect of sodium chlorate on *Escherichia coli* O157:H7 and *Salmonella* typhimurium DT104 in rumen contents in vitro. *J. Food Prot.* **2000**, *63*, 1038–1042.

(5) Wales, A. D.; Allen, V. M.; Davies, R. H. Chemical treatment of animal feed and water for the control of *Salmonella*. *Foodborne Pathogens Dis.* **2010**, *7*, 3–15.

(6) Jung, Y.-S.; Anderson, R. C.; Byrd, J. A.; Edrington, T. S.; Moore, R. W.; Callaway, T. R.; McReynolds, J.; Nisbet, D. J. Reduction of *Salmonella* Typhimurium in experimentally challenged broilers by nitrate adaptation and chlorate supplementation in drinking water. *J. Food Prot.* **2003**, *66*, 660–663.

(7) McReynolds, J. L.; Byrd, J. A.; Moore, R. W.; Anderson, R. C.; Poole, T. L.; Edrington, T. S.; Kubena, L. F.; Nisbet, D. J. Utilization of the nitrate reductase enzymatic pathway to reduce enteric pathogens in chickens. *Poult. Sci.* **2004**, *83*, 1857–1860.

(8) Anderson, R. C.; Callaway, T. R.; Buckley, S. A.; Anderson, T. J.; Genovese, K. J.; Sheffield, C. L.; Nisbet, D. J. Effect of oral sodium chlorate administration on *Escherichia coli* O157:H7 in the gut of experimentally infected pigs. *Int. J. Food Microbiol.* **2001**, *71*, 125–130.

(9) Anderson, R. C.; Jung, Y. S.; Oliver, C. E.; Horrocks, S. M.; Genovese, K. J.; Harvey, R. B.; Callaway, T. R.; Edrington, T. S.; Nisbet, D. J. Effects of nitrate or nitro supplementation with or without added chlorate, on *Salmonella enterica* serovar Typhimurium and *Escherichia coli* in swine feces. *J. Food Prot.* **2007**, *70*, 308–315.

(10) Callaway, T. R.; Edrington, T. S.; Anderson, R. C.; Genovese, K. J.; Poole, T. L.; Elder, R. O.; Byrd, J. A.; Bischoff, K. M.; Nisbet, D. J. *Escherichia coli* O157:H7 populations in sheep can be reduced by chlorate supplementation. *J. Food Prot.* **2003**, *66*, 194–199.

(11) Edrington, T. S.; Callaway, T. R.; Anderson, R. C.; Genovese, K. J.; Jung, Y. S.; McReynolds, J. L.; Bischoff, K. M.; Nisbet, D. J. Reduction of *E. coli* O157:H7 populations in sheep by supplementation of an experimental sodium chlorate product. *Small Ruminant Res.* **2003**, *49*, 173–181.

(12) Callaway, T. R.; Anderson, R. C.; Genovese, K. J.; Poole, T. L.; Anderson, T. J.; Byrd, J. A.; Kubena, L. F.; Nisbet, D. J. Sodium chlorate supplementation reduces *E. coli* O157:H7 populations in cattle. *J. Anim. Sci.* **2002**, *80*, 1683–1689.

(13) Fox, J. T.; Anderson, R. C.; Carstens, G. E.; Miller, R. K.; Jung, Y. S.; McReynolds, J. L.; Callaway, T. R.; Edrington, T. S.; Nisbet, D. J. Effect of nitrate adaptation on the bactericidal activity of an experimental chlorate product against *Escherichia coli* in cattle. *Int. J. Appl. Res. Vet. Med.* **2005**, *3*, 76–80.

(14) Anderson, R. C.; Carr, M. A.; Miller, R. K.; King, D. A.; Carstens, G. E.; Genovese, K. J.; Callaway, T. R.; Edrington, T. S.; Jung, Y.-S.; McReynolds, J. L.; Hume, M. E.; Beier, R. C.; Elder, R. O.; Nisbet, D. J. Effects of experimental chlorate preparations as feed and water supplements on *Escherichia coli* colonization and contamination of beef cattle and carcasses. *Food Microbiol.* **2005**, *22*, 439–447.

(15) Smith, D. J.; Oliver, C. E.; Caton, J. S.; Anderson, R. C. Effect of sodium [^{36}Cl]chlorate dose on total radioactive residues and residues of parent chlorate in beef cattle. *J. Agric. Food Chem.* **2005**, *53*, 7352–7360.

(16) Smith, D. J.; Anderson, R. C.; Ellig, D. A.; Larsen, G. Tissue distribution, elimination, and metabolism of dietary sodium [^{36}Cl]chlorate in beef cattle. *J. Agric. Food Chem.* **2005**, *53*, 4272–4280.

(17) Smith, D. J.; Anderson, R. C.; Huwe, J. K. Effect of sodium [^{36}Cl]chlorate dose on total radioactive residues and residues of parent chlorate in growing swine. *J. Agric. Food Chem.* **2006**, *54*, 8648–8653.

(18) Smith, D. J.; Byrd, J. A.; Anderson, R. C. Total radioactive residues and residues of [^{36}Cl]chlorate in market size broilers. *J. Agric. Food Chem.* **2007**, *55*, 5898–5903.

(19) Smith, D. J.; Oliver, C. E.; Shelver, W. L.; Caesar, T.-C.; Anderson, R. C. Chlorate metabolism in pure cultures of *E. coli* O157:H7 pretreated with either nitrate or chlorate. *J. Agric. Food Chem.* **2009**, *57*, 10216–10224.

(20) Oliver, C. E.; Craigmill, A. L.; Caton, J. S.; Anderson, R. C.; Smith, D. J. Pharmacokinetics of ruminally-dosed chlorate in beef cattle. *J. Vet. Pharmacol. Ther.* **2007**, *30*, 358–365.

(21) Hakk, H.; Smith, D. J.; Shappell, N. W. Tissue residues, metabolism and excretion of radiolabeled sodium chlorate ($Na[^{36}Cl]O_3$) in rats. *J. Agric. Food Chem.* **2007**, *55*, 2034–2042.

(22) Charles, L.; Pépin, B. Analysis of oxyhalides in water by ion chromatography—ionspray mass spectrometry. *J. Chromatogr., A* **1999**, *804*, 105–111.

(23) Ahrer, W.; Buchberger, W. Analysis of low-molecular-mass inorganic and organic anions by ion chromatography-atmospheric pressure ionization mass spectrometry. *J. Chromatogr., A* **1999**, *854*, 275–287.

(24) Barron, L.; Paull, B. Simultaneous determination of trace oxyhalides and haloacetic acids using suppressed ion chromatography-electrospray mass spectrometry. *Talanta* **2006**, *69*, 621–630.

(25) Karamarkar, S.; Bahowick, T. J. Enhanced ion chromatographic separation of disinfection by-products in drinking waters by coupling columns of different selectivities. *Am. Lab.* **2002**, *34*, 10–12.

(26) Beier, R. C.; Hume, M. E.; Anderson, R. C.; Oliver, C. E.; Callaway, T. R.; Edrington, T. S.; Nisbet, D. J. HPLC determination of chlorate metabolism in bovine ruminal fluid. *J. Environ. Sci. Health Part B* **2007**, *42*, 717–726.

(27) Dodds, E. D.; Kennish, J. M.; von Hippel, F. A.; Bernhardt, R.; Hines, M. E. Quantitative analysis of perchlorate in extracts of whole fish homogenates by ion chromatography: comparison of suppressed conductivity detection and electrospray ionization mass spectrometry. *Anal. Bioanal. Chem.* **2004**, *379*, 881–887.

(28) Kirk, A. B.; Smith, E. E.; Tian, K.; Anderson, T. A.; Dasgupta, P. K. Perchlorate in milk. *Environ. Sci. Technol.* **2003**, *37*, 4979–4981.

(29) Capuco, A. V.; Rice, C. P.; Baldwin, R. L., VI; Bannerman, D. D.; Paape, M. J.; Hare, W. R.; Kauf, A. C. W.; McCarty, G. W.; Hapeman, C. J.; Sadeghi, A. M.; Starr, J. L.; McConnell, L. L.; Van Tassell, C. P. Fate of dietary perchlorate in lactating dairy cows: Relevance to animal health and in the milk supply. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 16152–16157.

(30) Marinelango, P. K.; Tian, K.; Dasgupta, P. Perchlorate in seawater bioconcentration of iodide and perchlorate by various seaweed species. *Anal. Chim. Acta* **2006**, *567*, 100–107.

(31) Couture, E. Chlorate and chlorite analysis in seawater, chlorate sinks, and toxicity to phytoplankton. M.S. Thesis, Dalhousie University, Halifax, Nova Scotia, Canada, 1998.

(32) Smith, D. J.; Hakk, H.; Larsen, G. L. Tissue distribution, elimination, and metabolism of sodium [^{36}Cl]perchlorate in lactating goats. *J. Agric. Food Chem.* **2006**, *54*, 8829–8835.