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Determination of Low Levels of Perchlorate in Lettuce and Spinach Using Ion Chromatography−Electrospray Ionization Mass Spectrometry (IC-ESI-MS)

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A sample preparation method was developed to quantify environmentally relevant (low micrograms per liter) concentrations of perchlorate (ClO_4^-) in leafy vegetables using IC-ESI-MS. Lettuce and spinach were macerated, centrifuged, and filtered, and the aqueous extracts were rendered water-clear using a one-step solid-phase extraction method. Total time for extraction and sample preparation was 6 h. Ion suppression was demonstrated and was likely due to unknown organics still present in the extract solution after cleanup. However, this interference was readily eliminated using a $Cl^{18}O_4^-$ internal standard at 1 μg/L in all standards and samples. Hydroponically grown perchlorate-free butterhead lettuce was spiked to either 10.3 or 37.7 μg/kg of fresh weight (FW), and recoveries were between 91 and 98% and between 93 and 101%, respectively. Five types of lettuce and spinach from a local grocery store were then analyzed; they contained from 0.6 to 6.4 μg/kg of FW. Spike recoveries using the store-bought samples ranged from 89 to 100%. The method detection limit for perchlorate in plant extracts is 40 ng/L, and the corresponding minimum reporting limit is 200 ng/L or 0.8 μg/kg of FW.

KEYWORDS: Perchlorate; ion chromatography; mass spectrometry; sample preparation; internal standard; extraction; ion suppression; plants; lettuce; spinach; vegetables; *Lactuca sativa* L.; *Spinacia oleracea* L.

INTRODUCTION

Perchlorate (ClO_4^-) is an emerging pollutant that has been detected in soil, vegetation, milk, and ground and drinking water supplies at various concentrations across the United States (*1*−*3*). It is used as an oxidant in rocket fuel, some explosives, and pyrotechnics, and extensive pollution is most often associated with defense-related activities (*3*). Perchlorate is chemically similar to iodide and effectively competes with iodide for uptake by the human thyroid (*4*, *5*). Reduced iodide uptake by humans can lead to a lower production of key thyroid hormones, which are needed for proper growth and development. Perchlorate contamination in water and food can thus be detrimental to human health, especially for fetuses and breast-fed infants, who rely on the mother for their iodide supply.

The increased frequency of detection of perchlorate in recent years is due mainly to advancement in analytical instrumentation. New technology has evolved in which perchlorate is effectively retained on certain ion-exchange columns and is thus well-separated from other common anions such as sulfate, nitrate, and chloride. Ion chromatography (IC) using electrical conductivity detection has historically been used to quantify perchlorate, with method detection limits (MDL) in water of ∼1 μg/L (*6*). Using a preconcentration−preelution technique with IC, Tian et al. (*7*) were able to achieve MDLs ranging from 0.052 to 0.77 μg/L in water depending on initial injection volume. The IC method of quantification with electrical conductivity detection is reliable for water matrices containing above ∼1 μg/L perchlorate, but it has some disadvantages for more complex background matrices. To measure perchlorate in biological samples such as plants, an extensive off-line cleanup procedure is required that can be time-consuming and expensive. In addition, an extensive cleanup procedure could result in a loss of perchlorate with each step, especially if perchlorate is present in low concentrations. Finally, IC with electrical conductivity detection relies purely on the retention time of unknowns against standards for identification and, with minor fluctuations in retention times, unambiguous detection and quantification can be challenging.

Ellington and Evans (*8*) developed a method to extract and quantify perchlorate in plants using IC with electrical conductivity detection. Their method involved an extensive, time-consuming cleanup procedure that yielded an MDL of 250 μg/kg of fresh weight (FW). Plants were freeze-dried, ground, boiled in a hot water bath, and cooled and shaken overnight. The extraction and cleanup procedure involved two centrifugations, four filtering steps, and a 20-h alumina interaction prior to analysis. Total time of freeze-drying and extraction was ∼5 days. Variations of their method have been used by several researchers and are seemingly successful, albeit time-consuming,

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for perchlorate quantification in plants with MDLs ranging from 100 to 500 μg/kg of FW (*1*, *9*−*11*). More recently, however, Sanchez et al. (*12*) have achieved minimun reporting levels (MRLs) of 20−30 μg/kg of FW using this method.

Krynitsky et al. (*13*) have recently developed a method using IC-MS/MS to analyze perchlorate in lettuce, cantaloupe, bottled water, and milk with respective limits of quantification of 1.0 μg/kg, 2.0 μg/kg, 0.50 μg/L, and 3.0 μg/L. Other researchers were able to achieve an MDL of 0.0219 μg/L perchlorate in water using LC-MS/MS (*14*). Analyses conducted using IC-MS/MS provide a considerable increase in sensitivity and selectivity for perchlorate detection over IC with electrical conductivity detection, but with a substantial cost increase and a more complex analysis of the mass spectral data.

Recently, ESI-MS detection has been used in conjunction with IC to provide higher sensitivity and selectivity for perchlorate detection in water (*15*). ESI is considered a "soft" ionization technique in which fragmentation of the analyte is insignificant, making spectral data less complicated. With ESI-MS, single-ion mass units can be simultaneously monitored and mass chromatograms of several ions for each run can be generated. When perchlorate is analyzed in this way, both SIM 99 and SIM 101 (corresponding to *m*/*z* 99 and *m*/*z* 101 for the natural stable isotopes of $^{35}ClO_4^-$ and $^{37}ClO_4^-$) are monitored, and identification of unknowns is based not only on the retention times of SIM 99 and 101 being similar to one another and to those of the standards but also on the 3:1 natural isotopic ratio of ^{35}Cl to ^{37}Cl (*15*). Using IC-ESI-MS methods, perchlorate can be detected and quantified in the nanograms per liter range in water with an MDL of ∼50 ng/L and corresponding limit of quantification of ∼150 ng/L (*16*). Using slightly different IC-ESI-MS instrumentation, Roehl et al. (*15*) achieved an MDL of 300 ng/L in water. IC-ESI-MS provides sensitivity comparable to that of IC-MS/MS methods but at a lower cost.

When surveying candidate methods for quantifying perchlorate in plant tissues, it seemed to us that IC-ESI-MS offered the best prospects. IC-ESI-MS provides greater sensitivity and selectivity than IC with electrical conductivity detection, and it is not as costly as IC-MS/MS instrumentation. However, published methods using this approach for plant analysis of perchlorate are lacking. Our goals were twofold: (1) to develop an efficient method of extraction and sample preparation for perchlorate analysis in plant tissues and (2) to unambiguously quantify perchlorate in leafy vegetation using IC-ESI-MS. Although IC-ESI-MS has recently been shown to be successful and more accurate than IC with electrical conductivity detection alone for perchlorate analysis in fish homogenates with an MRL of 63 μg/kg of FW (*17*), to our knowledge this is the first attempt to fully validate IC-ESI-MS for perchlorate in plants.

MATERIALS AND METHODS

Reagents. All solutions were prepared using 18 MΩ·cm water or better, and all salts used for nutrient solutions were ACS certified 99% purity or higher. Perchlorate standards ranging from 0.25 to 30 μg/L were prepared by dilution of a liquid 1000 mg/L perchlorate standard solution (SPEX CertiPrep, Metuchen, NJ). Perchlorate spikes were prepared from the same standard.

Internal Standard (ISTD). The ISTD used in this study was $Cl^{18}O_4^-$ with ion masses of 107 and 109 (Dionex Corp., Sunnyvale, CA). SIM 107 was used for quantification. This ISTD was ideal because it is chemically and thus chromatographically very similar to SIM 99 perchlorate ($^{35}Cl^{16}O_4^-$), yet it is distinguishable by its SIM.

Plant Materials. To obtain perchlorate-free plant material, we hydroponically grew butterhead, romaine, and red leaf lettuces (*Lactuca sativa* L.) in growth chambers within which light intensity, temperature and relative humidity were accurately controlled. Plants were grown for ∼60 days under the following conditions: 65% relative humidity, 25 °C during 16 h of light and 18 °C during 8 h of dark. During light hours, light intensity was ramped to 500 μmol/(m^2s) and held there for 6 h before ramping back down to 0. Complete nutrient solutions (*18*) were prepared to simulate field conditions with regard to nutrient availability and did not contain perchlorate. Upon harvesting, shoots were separated from roots, and each section was enclosed in a plastic bag with air removed and placed in a −22 °C freezer until extraction. Additionally, five types of lettuce—butterhead, romaine, green leaf, red leaf, and crisphead—and spinach (*Spinacia oleracea* L.) were purchased in March 2005 from a local grocery store in Riverside, CA. The vegetables were similarly frozen until extraction.

Extraction and Sample Preparation. Approximately 20 g of frozen plant material was weighed into a Mason jar along with a known volume of water. Due to the various degrees of pigmentation in each type of leafy green, the amounts of frozen plant material and water added were somewhat different for each vegetable, but each was diluted at least 3:1 at this stage. The jar was fitted to a blender (Hamilton Beach, Washington, NC), and the plant mixture was liquefied for ∼3 min. Freezing and macerating the plant material ruptures the plant's cell walls and membranes to allow entrained perchlorate to be released to solution. Approximately 50 mL of plant slurry was placed into a centrifuge tube and allowed to shake for 4 h to release any remaining perchlorate to solution. Samples were then centrifuged at $1.0 \times 10^5 g$ for 1 h. The clear, colored supernatant was vacuum-filtered through a 0.22 μm polyethersulfone filter (Millipore Corp., Bedford, MA), and the pellet was discarded. A 3 mL aliquot of each sample was then passed through a preconditioned ENVI-18 solid-phase extraction (SPE) cartridge (Supelco, Bellefonte, PA) using a syringe pump (Sage Instruments, Freedom, CA) at a flow rate of ∼3 mL/min. After the first 1 mL was discarded, a clear, colorless liquid sample was obtained for analysis. Some samples were subsequently diluted at least 2:1 prior to analysis. Total time of extraction and sample preparation was 6 h.

Aliquots of lettuce extracts before and after the SPE step were analyzed for dissolved organic carbon (DOC) using a TOC-V CSH analyzer (Shimadzu, Torrance, CA). High-purity air was the carrier gas at a flow of 150 mL/min. A four-point standard curve was generated, ranging from 20 to 120 mg/L, using potassium hydrogen phthalate. All lettuce samples were diluted by a factor of 100 to be within the range of the standard curve. After 50 μL of sample was injected, it was automatically acidified to a pH of 2 and purged to remove inorganic carbon prior to catalyst-aided combustion at 680 °C. Electrical conductivity of these extracts was also determined using a CDM 83 conductivity meter (Radiometer America, Inc., Westlake, OH) equipped with a CDC 343 electrode with a cell constant of 1.00 cm^{-1}.

Analysis. All perchlorate analyses were carried out using IC-ESI-MS (Dionex Corp.). Chromatography was performed on a DX500 ion chromatograph equipped with an AS50 autosampler and a GP50 pump run in the isocratic mode using 45 mM NaOH eluent at a flow rate of 0.3 mL/min. An injection loop of 100 μL was used for all analyses. Background conductivity was maintained below 1 μS/cm by using an ASRS Ultra II (2 mm) suppressor in the external water mode and an ATC-HC trap column. Chromatographic separation was achieved using an IonPac AS16 (2 × 250 mm) analytical column equipped with an IonPac AG16 (2 × 50 mm) guard column. Flow from the IC was directed into a Finnigan Surveyor MSQ Plus single-quadropole MS (Thermo Electron Corp., Waltham, MA).

The MS was equipped with an AXP-MS auxiliary pump, pumping 50:50 acetonitrile/water at a flow rate of 0.3 mL/min. Matrix diversion was used to divert all of the IC flow to waste for the first 9 min of each run, and then the IC flow joined the acetonitrile/water so that a total flow rate of 0.6 mL/min was provided to the MS for each analysis. The retention time of perchlorate was ∼13 min under these conditions. Flow to the MS was nebulized through an ESI source using ultrahigh-purity nitrogen gas at a pressure of 550 kPa. The electrospray capillary was held at 450 °C with a needle voltage of 3.5 kV. The entrance cone was held at a voltage of 70 V.

Table 1. Electrical Conductivity (EC) and DOC Content of Lettuce Extracts before and after Treatment with SPE ENVI-18 Cartridges

	EC (dS/m)			DOC (mg/L)		
lettuce variety	before cartridge	after cartridge	% decrease	before cartridge	after cartridge	% decrease
butterhead	3.73	2.03	46	5060	1758	65
romaine	3.88	2.83	27	9066	5348	41
red leaf	3.20	2.63	18	4200	2670	36

Negative ion monitoring of *m*/*z* 99 (±0.5), *m*/*z* 101 (±0.5), and *m*/*z* 107 (±0.5) (corresponding to $^{35}Cl^{16}O_4^-$, $^{37}Cl^{16}O_4^-$, and $^{35}Cl^{18}O_4^-$, respectively) was utilized with a dwell time of 0.30 s per ion. SIM 99 was used for calibration of standards exclusively and, after preliminary results indicated the need, $Cl^{18}O_4^-$ was used as the ISTD at a concentration of 1 μg/L in all standards and samples. Identification of perchlorate in the unknowns was confirmed by retention times of unknown peaks with those of the standards as well as the SIM 99 to SIM 101 ratio of 3:1. Chromeleon version 6.6 (Dionex Corp.) was used to control the instrumentation and to quantify perchlorate.

Spike Recoveries. Perchlorate-free butterhead lettuce was used for spike recovery experiments. Six jars were prepared according to the aforementioned procedure. Three of the six jars were spiked with perchlorate at 10.3 μg/kg of FW when the plant sample and water were added to the Mason jar (designated "spiked at beginning"). All six jars were subjected to the aforementioned extraction and sample preparation procedure and, after centrifugation and filtration but prior to treatment with the SPE cartridge, the remaining three jars were spiked with the same amount of perchlorate (designated "spiked at end"). Another set of perchlorate-free butterhead lettuce was subjected to a similar spike recovery experiment but contained a perchlorate spike of 37.7 μg/kg of FW.

Store-bought lettuce and spinach were also used for spike recovery experiments. An aliquot of extract was spiked with perchlorate after the filtration step at a concentration similar to that found initially in each plant. Each aliquot was passed in triplicate through the SPE cartridges and analyzed concurrently with an aliquot of unspiked extract.

Method Detection Limit (MDL). A widely used method for calculating the detection limit of analytical methods as described in EPA Method 314.0 (*19*) was also utilized here. In short, seven replicate injections of 250 ng/L perchlorate in butterhead lettuce extract were analyzed by IC-ESI-MS, and an MDL was calculated on the basis of the Student's *t* value at the 99% confidence interval. The MDL is given by

$$\mathrm{MDL} = ts \quad (1)$$

where $t = 3.14$ for six degrees of freedom and *s* is the standard deviation of the quantified perchlorate concentration in the seven replicate injections.

Statistics. Standard curves were compared using a *t* test for slopes of the fitted regression equations (*20*). Means of the spiked at beginning and spiked at end perchlorate concentrations were compared with a paired *t* test using SPSS version 12.0 for Windows (SPSS, Inc., Chicago, IL).

RESULTS AND DISCUSSION

Sample Preparation Efficacy. Excellent recoveries were obtained when 10 μg/L perchlorate water standards were passed through SPE cartridges in triplicate, with recoveries of 100.0 ± 0.53% (data not shown). It should be noted that it is necessary to discard the first 1 mL passing through the cartridge before collection of a sample for analysis to minimize sample dilution. Because the SPE cartridges are conditioned with methanol and then water, a small amount of water remains entrained in the cartridge even after vacuum-drying for ~30 s.

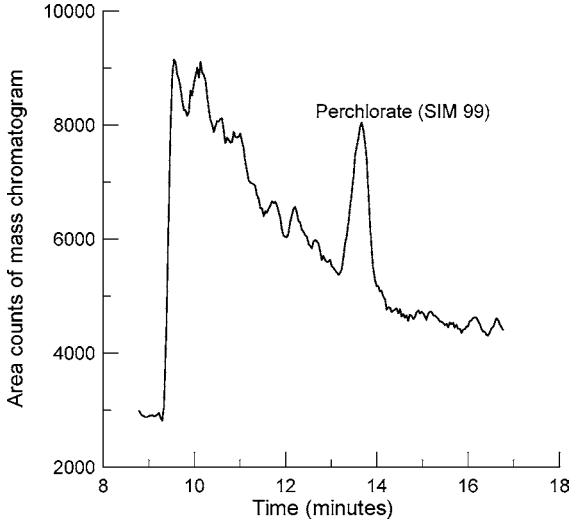

Figure 1. Mass chromatogram of 250 ng/L perchlorate in butterhead lettuce extract at SIM 99.

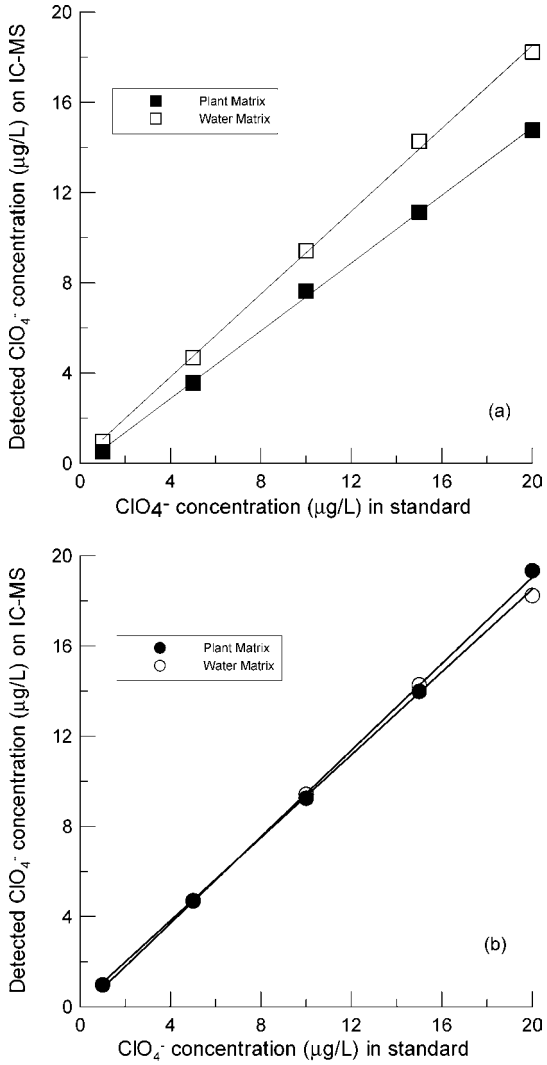

Figure 2. Standard curves for perchlorate analysis using IC-ESI-MS in a water matrix verses in a plant matrix (**a**) without and (**b**) with a $Cl^{18}O_4^-$ internal standard.

Volumetric fractionation of the cartridge eluent revealed that discarding the first 1 mL was sufficient to eliminate this sample dilution.

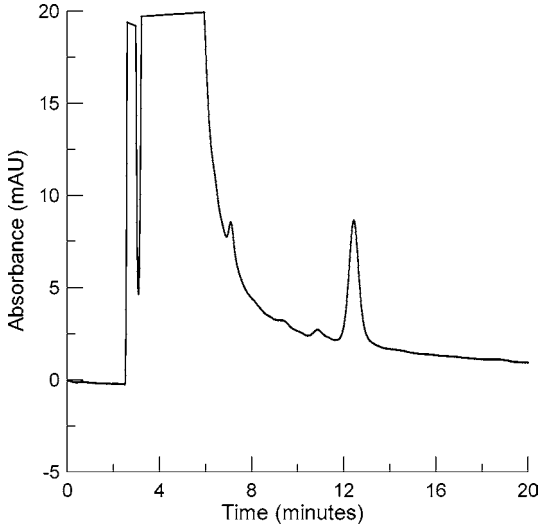

Figure 3. UV absorbance (254 ± 10 nm) ion chromatogram of butterhead lettuce extract after cleanup with ENVI-18 SPE cartridges. Note partial overlap with the perchlorate peak at ~13 min in **Figure 1**.

Table 2. Spike Recovery Data for Initially Perchlorate-free Butterhead Lettuce Spiked with either 37.7 or 10.3 μg/kg of FW (ISTD at 1.0 μg/L Was Used for All Spiked Samples)

sample identification	recovered ClO_4^- spike (μg/kg of FW)	av recovered ClO_4^- spike (μg/kg of FW)	SD	% recovery
spiked at beginning	34.62			
	36.42			
	34.18	35.08	1.188	93
spiked at end	37.69			
	38.23			
	38.26	38.24	0.019	101
spiked at beginning	9.76			
	10.73			
	9.71	10.07	0.577	98
spiked at end	7.06			
	9.35			
	11.73	10.54	1.684	102

The ENVI-18 SPE cartridges were effective in removing pigmentation and other organic compounds from the plant extracts. After the SPE cartridge step, colored plant extracts were rendered water-clear for analysis. Depending on lettuce type, the SPE cartridges removed 41−65% of dissolved organic carbon and reduced electrical conductivity by 18−46% (**Table 1**). Using this sample preparation and analytical procedure, a lettuce extract spiked to 250 ng/L perchlorate is easily detected and quantified (**Figure 1**).

To determine whether water standards were acceptable for accurately quantifying perchlorate in plant matrices, five-point standard curves (1−20 μg/L) in both water and perchlorate-free lettuce extract matrices were compared. The plant matrix yielded consistently low recoveries as compared to the standards in water (**Figure 2a**). The slopes of the lines were compared using a *t* test for slopes (*20*) and were found to be significantly different ($P < 0.001$). Because the standard solutions were prepared simultaneously using the same calibrated pipets, the chance of determinant error was minimized and the difference seen is likely real. This discrepancy suggested that ion suppression is occurring when perchlorate is analyzed in the lettuce extracts.

Ion suppression is the process by which coeluting ions prevent the ionization of analyte during ESI nebulization. It is most likely to happen if another ion coelutes and is present at a concentration higher than that of the analyte of interest (*21*). Because the AS16 column is designed to elute most ions quickly and preferentially retains perchlorate longer, it seems unlikely that common ions present at high concentrations such as chloride, sulfate, or nitrate would cause ion suppression. Because the electrical conductivity detector in-line with the IC-ESI-MS showed no other coeluting ions in the range of perchlorate elution, the substance causing suppression is likely not an ion, but perhaps a molecule that is not detected by electrical conductivity. A full scan on the MS during routine analysis ranged from *m*/*z* 20 to 150 and did not show any evidence of a coeluting species in that mass range. The DOC analysis indicated that a substantial amount of organic carbon was in the water-clear extracts and thus present during analysis (**Table 1**). Thus, it seemed likely that if a coeluting species was present and was causing ion suppression, it likely was an organic molecule with a molecular weight >150.

To confirm the presence of perchlorate-suppressing compounds in plant matrices, prepared butterhead lettuce extracts were run through the IC and directed to a PDA-100 photodiode array detector (Dionex Corp.). It can be seen in **Figure 3** that there are some organic compounds present that absorb in the 254 ± 10 nm UV range. Most of these compounds elute early, but at least one elutes around 12.5 min and partially overlaps with the perchlorate peak at ~13 min; this is likely the cause of the observed ion suppression in **Figure 2a**.

ISTD and Ion Suppression. Internal standards are useful because they provide a correction for MS fluctuation with time as well as for any ion suppression that may occur. The effect of ion suppression was successfully removed with the use of the $Cl^{18}O_4^-$ ISTD. **Figure 2b** shows the same set of water and plant matrix standards fortified with 1 μg/L ISTD. By the aforementioned *t* test for slopes, these two standard curves are not significantly different ($P = 0.20$) when the ISTD is used. This result indicates that a water matrix standard curve can be used when plant samples are analyzed, as long as the ISTD is utilized in all of the samples and standards.

Spike Recoveries of Initially Perchlorate-free Lettuce. Unspiked extracts of hydroponically grown lettuce were ana-

Table 3. Perchlorate Content of Store-Bought Lettuce and Spinach and Spike Recovery Data ($n = 3$)

vegetation type	initial ClO_4^- content (μg/kg of FW)	amount of ClO_4^- spike (μg/kg of FW)	expected recovery (μg/kg of FW)	measured ClO_4^- recovery (μg/kg of FW)	SD	% recovery
crisphead lettuce	2.3	1.8	4.1	4.0	0.30	99
butterhead lettuce	5.4	7.1	12.5	11.2	0.23	90
romaine lettuce	0.7	0.8	1.5	1.4	0.06	90
green leaf lettuce	2.1	2.0	4.1	3.8	0.03	93
red leaf lettuce	0.6	1.2	1.7	1.7	0.06	100
spinach	6.4	6.5	12.9	11.5	0.20	89

Table 4. Determination of the MDL by Seven Replicate Injections of 250 ng/L ClO_4^- in Initially Perchlorate-free Butterhead Lettuce Extract

replicate	ClO_4^- (ng/L)
1	204
2	219
3	240
4	213
5	232
6	228
7	213
av	221
SD	13
PRSD[a]	6
MDL[b]	40
equivalent MDL (ng/kg of FW)	156

[a] Percent relative standard deviation. [b] MDL = 3.14 × SD.

lyzed and did not contain detectable perchlorate (data not shown). Spike recoveries of initially perchlorate-free butterhead lettuce ranged from 93 to 101% and from 91 to 98% for the 37.7 and 10.3 μg/kg of FW perchlorate spikes, respectively (**Table 2**). A *t* test revealed that there were no differences between the spiked at beginning and spiked at end values ($P > 0.05$) for both sets of spikes. These data indicate that no loss of perchlorate occurs during the sample extraction and preparation steps including centrifugation, filtration, and sample transfer.

Store-Bought Lettuce and Spinach. The concentrations of perchlorate in five types of lettuce and spinach ranged from 0.6 to 6.4 μg/kg of FW (**Table 3**). The highest concentrations of perchlorate were found in the butterhead lettuce variety and in spinach, whereas the lowest was found in the red leaf lettuce. After perchlorate initially in the samples was accounted for, spike recoveries of perchlorate in these extracts ranged from 89 to 100% (**Table 3**). These excellent spike recoveries at low concentrations give further indication that this method is very sensitive and is ideally suited to the analysis of perchlorate at low concentrations in leafy vegetation.

MDL and MRL. The MDL for the determination of perchlorate in plant extracts using the method described here is 40 ng/L (**Table 4**). The MRL, however, will be higher than this value. The MRL is usually taken to be a factor of the MDL and is usually determined at the discretion of the analyst. On the basis of visual scrutiny of the mass chromatograms of plant extracts, we feel that an MRL of 5 times the MDL is appropriate. Thus, the MRL for the method described here is 200 ng/L, or 0.8 μg/kg on an equivalent FW basis.

We have established an efficient method to extract perchlorate from lettuce and spinach and prepare the plant extracts for analysis using IC-ESI-MS. The total time of extraction and sample preparation was 6 h. High spike recoveries were achieved when the ISTD was used at 1 μg/L in all standards and samples, and we recommend its use when perchlorate in plant samples is analyzed. The sensitivity of the MRL reported here is comparable to or slightly better than that for lettuce using IC-MS/MS (*13*), and IC-ESI-MS instrumentation costs less and requires less expertise.

ABBREVIATIONS USED

ISTD, internal standard; SPE, solid-phase extraction; DOC, dissolved organic carbon; MDL, method detection limit; MRL, minimum reporting limit; FW, fresh weight; SD, standard deviation.

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