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Simple and Selective Determination of Free Chlorine in Aqueous Solutions by an Electrophilic Aromatic Substitution Reaction Followed by Liquid Chromatography Coupled with Mass Spectrometry

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Abstract: We developed a selective technique to rapidly measure free chlorine, which is the sum of elemental chlorine (Cl_2), hypochlorous acid (HOCl), and hypochlorite (OCl^-) in water samples via an electrophilic aromatic substitution reaction hyphenated with liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). Sample preparation involved derivatization at 25 °C for 15 min with 3,4,5-trimethoxyphenylacetic acid (TMPAA) in an aqueous solution prior to analysis. Several parameters were evaluated to determine the optimized reaction and for the production of informative MS/MS spectrum of the derivatization product, 2-chloro-3,4,5-trimethoxyphenylacetic acid (Cl-TMPAA). The resulting Cl-TMPAA derivative displayed an informative ESI-MS/MS spectrum characterized by product ions at *m*/*z* 232.0142, 200.0245, and 185.0009 from the precursor ion at *m*/*z* 259.0379. The linear dynamic range of the method (0.1–10 µg/mL) was fitted to concentration levels relevant to forensic toxicology issues. Compared with other analytical techniques, this newly established LC-MS-based method demonstrated specificity, simplicity, and rapidity. This method enables the detection of free chlorine for forensic investigations in criminal cases.

Keywords: free chlorine; aqueous media; derivatization; electrophilic aromatic substitution; LC-ESI-MS/MS

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1. Introduction

Gaseous chlorine is toxic and classified as a pulmonary irritant. Chlorine gas was used as a chemical weapon during the First World War, the Iraq War, and the Syrian Civil War, leading to many casualties [1,2]. Chlorine is partially soluble in aqueous solutions, and it forms hypochlorous acid (HOCl) in equilibrium with chlorine and hydrochloric acid (HCl) when added to water. Depending on the pH, hypochlorous acid can dissociate into hypochlorite ions (ClO^-). The sum of these species (ClO^-, Cl_2, and HOCl) is defined as free chlorine, and these species are extensively used as disinfectants due to their strong oxidizing properties [3]. The World Health Organization (WHO) suggests that free chlorine concentrations of up to 5 mg/L are safe for drinking water [4]. A low level of free chlorine is insufficient to effectively disinfect water from parasites, viruses, and bacteria. Conversely, an excessively high level of free chlorine can produce harmful chlorinated byproducts, which may be toxic and pose potential dangers to both humans and animals [5,6]. Concentrated free chlorine solutions can lead to mild respiratory issues, such as asthma and allergies, and have also been associated with more serious health concerns, such as congenital abnormalities and cancers. Therefore, it is essential to monitor residual chlorine to ensure that the desired levels are achieved.

Because free chlorine is relatively inexpensive and easily accessible, it is often misused for criminal activities, such as contaminating food and drink. Consequently, it plays a

significant role in forensic toxicology. Several analytical techniques have been reported for the determination of free chlorine, and numerous approaches have been established for that purpose, including iodometric titration [7], ion chromatography [8,9], colorimetry [10–13], chemiluminescence [14,15], liquid chromatography [16,17], flow injection analysis [5], and gas chromatography-mass spectrometry [17–19]. Most recently, dual-mode determination (colorimetric and fluorometric) of hypochlorite has been developed [20]. In addition, a comprehensive review was recently published, cataloging diverse analytical techniques for chlorine detection in both aqueous and gaseous phases [21].

LC-ESI-MS/MS, with its combined sensitivity and selectivity, is an attractive analytical technique over non-MS methods for the determination of free chlorine after chemical derivatization. Compared with those of GC-MS, the major advantages of this method are its sensitivity and method simplicity, as it eliminates the need for extensive sample preparation and reduces processing time.

The synergy of high selectivity, sensitivity, speed, and quantitative accuracy positions LC-MS as a formidable analytical instrument for chlorine detection, which can serve as a viable alternative to traditional techniques such as colorimetric, photometric, and electrochemical methods. However, to fully leverage the advantages of LC-MS for chlorine analysis, an effective derivatization step is crucial. Derivatization has the potential to enhance the compatibility of chlorine with LC-ESI-MS/MS, resulting in improved sensitivity, greater specificity, and relatively fast analysis time. In this study, we introduce a novel approach: the implementation of a derivatization step preceding LC-MS/MS analysis for free chlorine determination.

This research aimed to develop a rapid, simple derivatization method with selective LC-ESI-MS/MS detection for free chlorine in water at poisoning-relevant concentrations

2. Materials and Methods

2.1. Chemicals, Substances, and Sample Preparation

2.1.1. Chemicals and Substances

Bleach was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and 2-(3,4,5-trimethoxyphenyl)acetamide (TMPA) and 3,4,5-trimethoxyphenylacetic acid (TMPAA) were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). LC-MS-grade water, methanol, and ammonium formate were obtained from Biolab (Jerusalem, Israel). In addition to LC-MS-grade water, we utilized municipal tap water that had been boiled for one hour prior to use.

2.1.2. Electrophilic Aromatic Substitution of Free Chlorine

A diluted bleach sample (1 mL) was placed in an Eppendorf vial. To this, 3,4,5-trimethoxyphenyl acetic acid (1 µg) was directly added. The vial was then subjected to stirring for 15 min at r.t. Following this incubation period, the sample was analyzed using LC-MS/MS.

2.1.3. Preparation of Solutions

A stock solution of free chlorine was prepared by diluting an aqueous sodium hypochlorite ion mixture with water to a concentration of 1 mg/mL. This stock solution was standardized via the iodometric method [11] and was diluted to various concentrations with water. Each standard (blank, 0.1 µg/mL, 1 µg/mL, 5 µg/mL and 10 µg/mL) was prepared in triplicate.

2.2. Instrumentation

2.2.1. HPLC

Chromatographic separation was carried out using an Agilent 1290 high-performance LC system (Palo Alto, CA, USA). The system comprised a 1290 Infinity Binary Pump with a Jet Weaver V35 Mixer, a 1290 Infinity Autosampler, and a 1290 Infinity Thermostatted Column Compartment (all from Agilent Technologies Inc., Palo Alto, CA, USA). A Fortis

C18 reversed-phase column (Fortis Technologies Ltd., Neston, UK) (100 mm × 2.1 mm ID, 2.1 μm particle size) was employed for separation. The column temperature was maintained at 40 °C. The mobile phases consisted of water with 1 mM ammonium formate (A) and methanol with 1 mM ammonium formate (B). The gradient was as follows: 0 min 5% B, 0–5 min: linear increase to 90% B, 5–5.01 min: rapid decline to 5% B, and 5.01–8.01 min: hold at 5%. The total LC run time was 11 min. The injection volume was 3 μL.

2.2.2. QTRAP MS Instrument

MS2 experiments were conducted via an Applied Biosystems 6500+ QTRAP LIT quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA) operated with Analyst software (version 1.7.2) and equipped with a Turbo V ion source in positive ESI mode. The ESI inlet conditions were as follows: gas 1 (air) at 60 psi (2.7 bar), gas 2 (air) at 30 psi (4.0 bar), ion spray voltage at 5500 V, ion source temperature at 500 °C, and curtain gas (nitrogen) at 35 psi (2.4 bar). For the enhanced product ion (EPI) (MS/MS) experiments, the collision gas was set to "medium", and the collision energy ranged from 10 V to 50 V.

2.2.3. LC-ESI-MS/MS Characterization of the Cl Derivative (Cl-TMPAA)

Several product ions were observed in the negative ESI-MS/MS spectrum of the Cl derivative (Cl-TMPAA), and three MRM transitions were established: (m/z 259→200, CE 20 V; m/z 261→202, CE 20 V; and m/z 259→185, CE 30 V). The declustering potential (DP) was set to 40 V. The MRM transition at m/z 259→200, with the highest signal-to-noise (S/N) ratio, was chosen as the quantifier transition. To enhance selectivity, the two aforementioned MRM transitions were also selected.

2.2.4. Orbitrap MS Instrument

The Q-Exactive+Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was equipped with a heated electrospray ionization (HESI) system operating in positive mode. The parameters included an electrospray voltage of 1.25 kV, sweep gas set to 2 (arbitrary units), an auxiliary gas heater temperature of 400 °C, a sheath gas flow rate of 45 (arbitrary units), and a capillary temperature maintained at 275 °C. The collision energy ranged from 20 V to 40 V. All analytes were initially analyzed in full-scan mode from m/z 60 to m/z 900, followed by data-independent acquisition (DIA) MS2 experiments with collision energies varying from 10 V to 40 V and a normalized collision energy (NCE) of 35 V.

High-resolution MS (HR-MS) of the chlorine derivative (Cl-TMPAA) revealed several fragment ions at m/z 232.0144, m/z 200.0246, and m/z 185.0011. Plausible fragment ion structures were proposed based on MS/MS exact mass measurements and pre-established fragmentation rules (<3 ppm error). The chlorine derivative also displayed a characteristic isotope distribution pattern consistent with the presence of chlorine (^{35}Cl:^{37}Cl isotope ratio of 3:1).

3. Results and Discussion

3.1. Derivatizing Reaction Strategy

Free chlorine species (Cl_2, ClO^-, and HOCl) cannot be reliably detected in their native forms via LC-MS. Therefore, an appropriate chemical transformation must be performed prior to analysis. Electrophilic aromatic halogenation is a type of electrophilic aromatic substitution. Chlorination of a simple benzene ring, or one with electron-withdrawing groups, typically requires a Lewis acid catalyst. However, if the benzene ring contains a strongly activating substituent (electron-donating group), such as –OH or –OR (R = alkyl), a catalyst is not necessary. Since the substituents on the aromatic ring influence the substitution rate, we selected derivatizing agents containing benzene rings with multiple electron-donor substituents, which would increase the electron density of the aromatic ring, thereby accelerating the chlorination reaction. A search of the literature revealed that trimethoxy benzene (TMB) is an effective free chlorine quencher in aqueous chlorination experiments

and can be quantified via gas chromatography-mass spectrometry (GC-MS) analysis after conversion into Cl-TMB followed by liquid-liquid extraction [18]. However, to ensure compatibility with LC-MS/MS, it is necessary to incorporate a protonation or deprotonation site into the molecule for better detectability. Therefore, for the derivatization reaction, we chose two commercially available candidates: 2-(3,4,5-trimethoxyphenyl)acetamide (TMPA) and 3,4,5-trimethoxyphenyl acetic acid (TMPAA). The derivatization reactions of free chlorine with both 2-(3,4,5-trimethoxyphenyl)acetamide and TMPAA, which was ultimately selected as the derivatizing agent, are depicted in Figure 1.

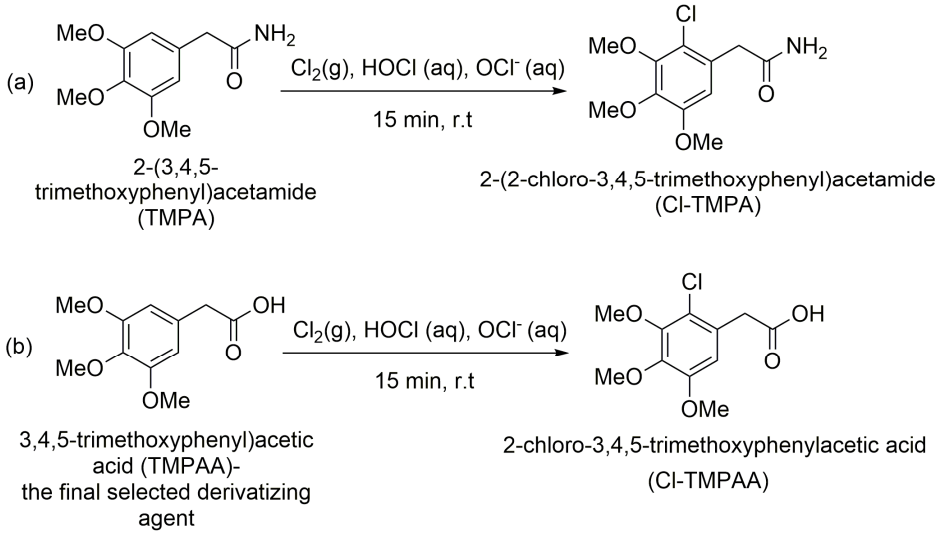


Figure 1. Reaction scheme for free chlorine derivatized by 2-(3,4,5-trimethoxyphenyl)acetamide (TMPA) (**a**) and 2-(3,4,5-trimethoxyphenyl)acetic acid (TMPAA) the final selected derivatizing agent (**b**).

These compounds feature three electron-donating methoxy groups and either an acetamide or acetic acid group, respectively, which are used primarily for increasing the sensitivity of positive or negative ESI-MS/MS, respectively. Furthermore, we predicted that the MS/MS of the acetamide derivative would lead to the formation of a xylyl-based product ion from the favorable loss of formamide as a neutral loss from the precursor ion, whereas carbon dioxide loss is a favorable neutral loss for the acetic acid derivative.

3.2. Structurally Informative MS/MS Spectra of the Two Derivatizing Agents and Their Monochlorinated Products

Orbitrap-ESI-MS/MS experiments were conducted on the two derivatizing agents and their corresponding chlorine derivatives, modifying the collision energy from 10 eV to 50 eV.

The Orbitrap-ESI-MS/MS spectrum of free chlorine after derivatization with 2-(3,4,5-trimethoxyphenyl)acetamide presented five major product ions at m/z 215.0469, m/z 185.0364, m/z 182.0128, m/z 137.0597, and m/z 91.0541 (Figure S1). Figure S2a displays the proposed ion structures of 2-(3,4,5-trimethoxyphenyl)acetamide after chlorination. The ion at m/z 215.0469 (benzylic cation) corresponds to the loss of formamide from the $M+H^+$ precursor ion. The ion at m/z 185.0364 is attributed to the loss of formaldehyde from m/z 215.0469. The ion at m/z 182.0128 is attributed to the loss of two formaldehyde groups and ammonia from the $[M+H]^+$ precursor ion.

The Orbitrap-ESI-MS/MS spectrum of free chlorine after derivatization with TMPAA to generate Cl-TMPAA was acquired in negative ion mode and revealed three fragment ions at m/z 232.0142, m/z 200.0245, and m/z 185.0009 from the $M\text{-}H^-$ precursor ion (Figure 2).

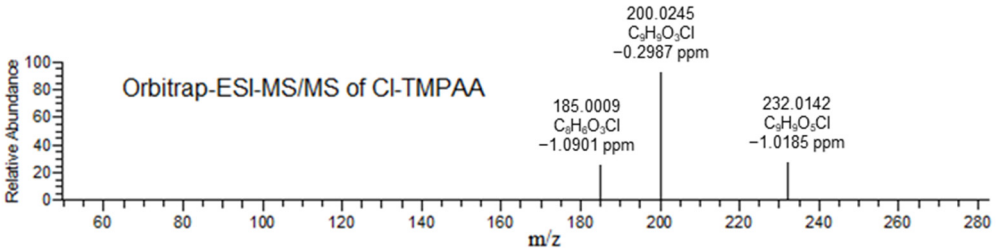


Figure 2. Orbitrap-ESI-MS/MS chromatograms of the Cl derivative generated after derivatization of bleach at a concentration of 100 ng/mL with TMPAA at a normalized collision energy of 35 eV.

The fragment ion at m/z 232.0144 is due to the loss of the C_2H_3 radical. The fragment ion at m/z 200.0246 is due to the loss of two groups (CO_2 and a methyl radical). The ion at m/z 185.0011 was probably generated via elimination of the methyl radical from m/z 200.0246. Loss of CO_2 is a common dissociation pathway of carboxylic acids [22]. In addition, homolytic oxygen-carbon (O-CH_3) cleavage with elimination of a methyl radical is considered a low-energy process [23] in both negative and positive ion modes in several compounds, such as flavonoids [24,25]. Figures 3b and S1b display the proposed ion structures of the two derivatizing agents, 3,4,5-trimethoxyphenylacetic acid and 2-(3,4,5-trimethoxyphenyl)acetamide, respectively, prior to chlorination.

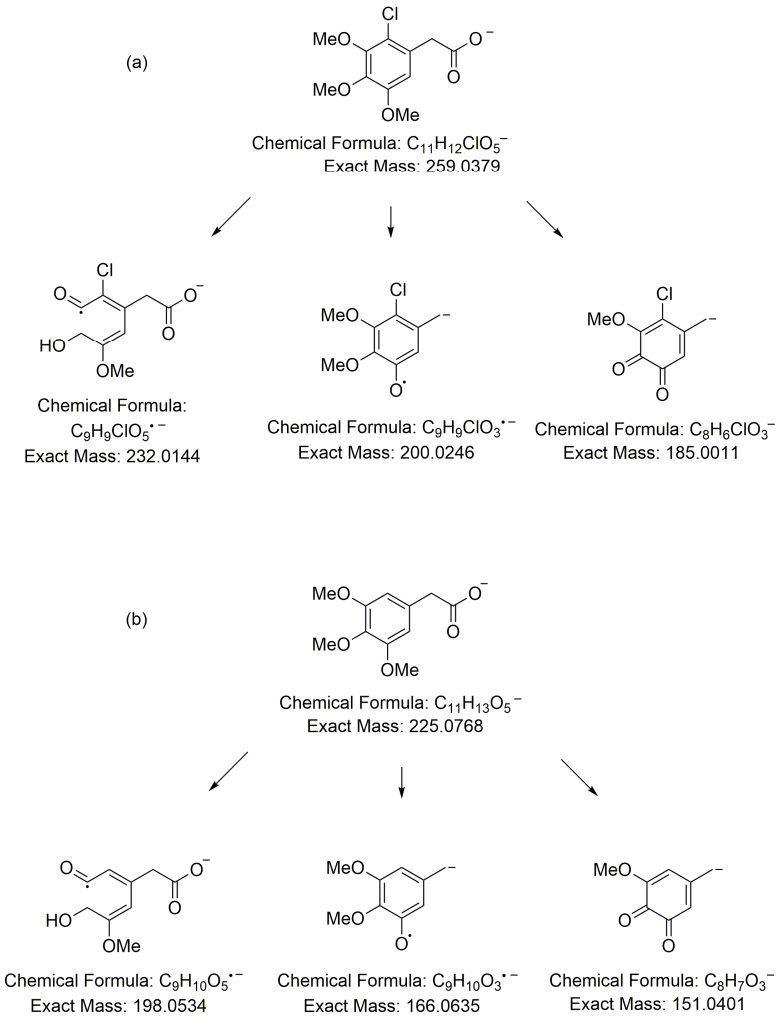


Figure 3. Plausible structures for the product ions observed in the ESI-MS/MS spectra of the derivatizing agent after chlorination (Cl-TMPAA (**a**)) and the derivatization agent prior to chlorination (**b**).

Similar fragmentation pathways were observed for the derivatizing agents prior to chlorine attachment. For example, the fragment ions of TMPAA at m/z 198.0534, m/z 166.0635, and m/z 151.0401 were observed from the M-H-precursor ion at m/z 225.0768. This strengthened our proposed ion structures for the chlorinated derivatization products. This phenomenon can be attributed to the persistence of the chlorine–carbon bond, which remains intact during fragmentation due to its considerable strength. Consequently, the dissociation process of the derivatization reagent and the derivatization reagent after chlorination is identical. The primary difference between the original reagent and its chlorinated reagent is the substitution of a hydrogen atom with a chlorine atom, while the overall molecular fragmentation pattern remains largely similar. For the derivatization product after chlorination (Cl-TMPAA), three MRM transitions, namely, −259→200, −261→202 (the isotopic chlorine), and −259→185, with signal intensity ratios of approximately 21:7:1 and a defined retention time (RT = 1.6 min), were selected for detection and verification. We decided to use TMPAA since the chromatograms of Cl-TMPAA were clean and showed no peak interference.

3.3. Parameters for Chemical Derivatization-Formation of a Monochlorinated Product (Cl-TMPAA)

Several reaction parameters were adjusted for derivatization with TMPAA. A large excess of the derivatizing agent (1 mg of reagent) was added to 1 mL of bleach diluted in water (LC-MS grade). A high concentration of derivatizing agent was used to ensure high yields. In addition, the minimal time required to achieve optimal signal intensities at ambient temperature was tested; therefore, we performed the reaction for 1 min to 60 min. We found that 15 min was sufficient for the quantitative conversion with the highest signal intensities observed. We noted that stirring the reaction mixture for 15 min at 50 °C resulted in a similar reaction yield. Decreasing the derivatizing agent concentration by 10-fold resulted in decreased MRM signal intensities. Therefore, the optimized derivatization conditions included the addition of 1 mg of the derivatizing agent to an Eppendorf tube containing aqueous samples (1 mL) and shaking of the vial for 15 min at ambient temperature (25 °C) prior to LC-ESI-MS/MS analysis. Notably, the dichlorinated product was not observed under the selected conditions.

3.4. Method Validation

The method was validated with tap water. The method's performance was assessed by estimating its linearity, repeatability according to both intraday and interday precision, limit of identification (LOI), and limit of quantification (LOQ) of free chlorine after it was converted to Cl-TMPAA. The derivatization reactivity of TMPAA toward free chlorine in tap water was assessed. In LC-MS analysis, ionization suppression, low derivatization efficiency, and product instability influenced by the matrix are commonly observed and are the primary reasons for signal reduction.

The effects of the matrices were calculated by comparing the recoveries in tap water fortified with bleach at concentrations of 0.1 μg/mL, 1 μg/mL, 5 μg/mL, and 10 μg/mL with those in water of LC-MS grade, followed by LC-ESI-MS/MS analysis after derivatization with TMPAA.

We compared the height of the most prominent MRM transition (m/z −259 > 200) of the monochlorinated derivative product (Cl-TMPAA) in tap water and in LC-MS-grade water, and a negligible matrix effect was anticipated. A comparison of the signal intensity at a concentration of 1 μg/mL is shown in Figure 4.

The calibration curve for the monochlorinated derivative product (Cl-TMPAA) in both types of water (LC-MS-grade and tap water) was linear in the range of 0.1 μg/mL to 10 μg/mL, with correlation coefficients (R^2) greater than 0.99 (Figure S3). Reproducibility was evaluated in triplicate with samples fortified at concentrations of 0.1 μg/mL to 10 μg/mL. The relative standard deviations (RSDs) of the intraday repeatability and interday reproducibility of the Cl derivatives were less than 20%.

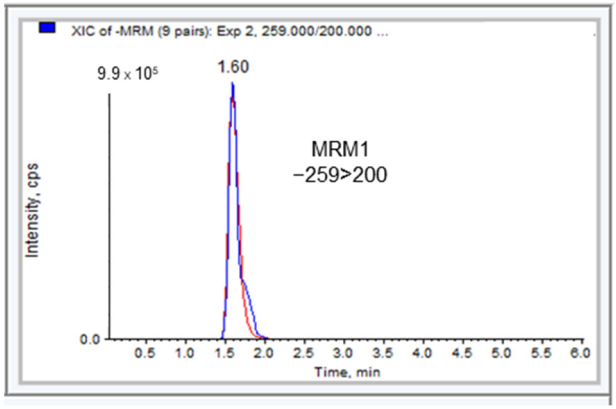


Figure 4. Extracted ion chromatograms (EICs) of the main MRM transition (m/z −259 > 200) of the mono-chlorinated derivative (Cl-TMPAA, RT = 1.6 min) in water (LC-MS grade, red) and tap water (blue). The sample was fortified to achieve a concentration of 1 µg/mL in an Eppendorf vial, followed by derivatization and shaking for 15 min prior to LC-MS/MS analysis.

The limit of identification and limit of quantification were determined as the minimum concentration of Cl-TMPAA with chromatographic signals three times and ten times higher than the background noise peak-to-peak, respectively. The limit of quantitation (S/N ≥ 10) was 0.1 µg/mL. The limit of identification was found to be 0.1 µg/mL as well (S/N ≥ 3), where the three specific MRM transitions are detectable, and the signal intensity ratio of approximately 24:8:1 was preserved. The validation parameters are displayed in Table 1.

Table 1. Validation data: linear dynamic range, R^2, LOI, LOQ, and precision.

Analyte	Linear Range (µg/mL)	R^2	LOI (µg/mL) (S/N ≥ 3)	LOQ (µg/mL) (S/N ≥ 10)	RSD (%)
ClO^-, HOCl, Cl_2	0.1–10	0.99	0.1	0.1	<20

The MRM transition (−259→200) was used as a quantifier transition, and the MRM transitions (−261→202, −259→185) were used as identifier transitions.

The stability of Cl-TMPAA was evaluated, revealing no observable degradation within 48 h after derivatization at 25 °C. The MRM quantifier transition (−259 > 200) and the two qualifier MRM transitions (−261 > 202 and −259 > 185) of the free chloride after its conversion to Cl-TMPAA in tap water at 0.1 µg/mL (LOI) were compared to those of the blank samples, as described in Figure 5. The MRM signals did not display background peaks for the analyte and were dominated by Cl-TMPAA.

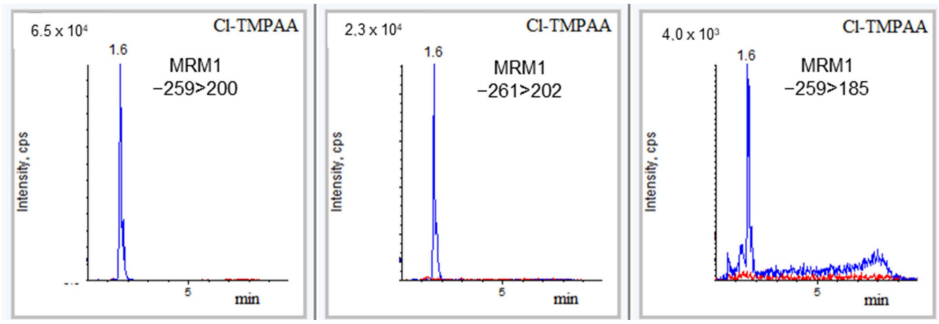


Figure 5. Extracted ion chromatograms (EICs) of the three dominant MRM transitions (m/z −259 > 200, m/z −261 > 202 and m/z −259 > 185) of the mono-chlorinated derivative (Cl-TMPAA, RT = 1.6 min). Spiking was carried out to obtain a concentration of 0.1 µg/mL (LOQ level, S/N ≥ 10, in blue), and the mixture was stirred for 15 min before LC-MS/MS analysis. The negative control sample (in red) was prepared in the same manner.

4. Summary and Conclusions

We have developed an innovative, straightforward, and highly selective LC-MS/MS method for detecting and confirming the presence of free chlorine in aqueous solutions. The sample preparation contains the addition of 3,4,5-trimethoxyphenyl acetic acid directly into an Eppendorf vial containing a diluted bleach sample. Overall, we achieved swift sample preparation and rapid analysis times. The chlorine derivative exhibits remarkable stability. When subjected to MS/MS analysis, it generates distinctive and informative product ions, enabling verification with a high degree of confidence. This developed analytical technique allows for the assessment of whether or not exposure concentration were toxicologically relevant. This method utilizes an electrophilic aromatic substitution reaction with TMPAA prior to analysis, and it has advantages in terms of selectivity over non-MS methods as well as an advantage in terms of a simple sample preparation over GC-MS methods, because the developed protocol includes only the addition of TMPAA directly into an Eppendorf vial containing the free chloride sample before analysis. The simplicity of this method allows for its use in routine laboratory analysis to selectively detect free chlorine in forensic investigations. Additional research to assess chlorine levels in the air is currently in progress.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/org5040032/s1, Figure S1: Orbitrap-ESI-MS/MS chromatograms of the Cl derivative generated after derivatization of bleach at a concentration of 100 ng/mL with 2-(3,4,5-trimethoxyphenyl, 1-chloro)acetamide at a collision energy of 30 eV; Figure S2: Plausible structures for the product ions observed in the ESI-MS/MS spectra of the derivatizing agent (-(2-chloro-3,4,5-trimethoxyphenyl)acetamide) after chlorination (a) and the derivatizing agent prior to chlorination (b); Figure S3: The linear dynamic range of the derivatization product (Cl-TMPAA) in pure water (blue) and tap water (brown). The indicated concentration is an average of spiked bleach in triplicate at 0.1, 1, 5 and 10 µg/mL, followed by derivatization and LC-ESI-MS/MS analysis.

Author Contributions: Conceptualization, A.W.; methodology, A.W.; formal analysis, A.S. and T.S.Y.; validation, M.M. and A.S.; investigation, A.S., T.S.Y. and M.M.; data curation, A.S. and M.M.; writing—original draft preparation, A.W., M.M. and A.S.; writing—review and editing, A.W.; visualization, M.M. and A.W.; supervision, A.W. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study supporting the results are available in the main text and Supplementary Materials. Additional data are available upon request from the corresponding authors. The data of this study are available via request from the corresponding author.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

TMPAA	3,4,5-trimethoxyphenylacetic acid
Cl-TMPAA	2-chloro-3,4,5-trimethoxyphenylacetic acid
TMPA	2-(3,4,5-trimethoxyphenyl)acetamide
Cl-TMPA	2-(2-chloro-3,4,5-trimethoxyphenyl)acetamide

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