

FEASIBILITY OF DIRECT ANALYSIS OF SALIVA AND URINE FOR PHOSPHONIC ACIDS AND THIODIGLYCOL-RELATED SPECIES ASSOCIATED WITH EXPOSURE TO CHEMICAL WARFARE AGENTS USING LC-MS/MS

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Keywords: Saliva, Chemical Warfare Agents, Metabolites, LC-MS/MS

ABSTRACT

A sensitive method based on high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) has shown the feasibility of separation and detection of low-level thiodiglycol-related species in saliva and of nerve agent phosphonic acids in saliva and urine. The analysis of these thiodiglycol-related species and phosphonic acids are of interest since they are specific metabolites of the chemical warfare agents (CWAs) sulfur mustard (HD), Sarin (GB), Soman (GD), GF, and VX. The liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS/MS) and liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) methods developed provide a sensitive and direct approach for determining CWA exposure in non-extracted non-derivitized samples from saliva and urine. Chromatographic separation of the thiodiglycol-related species was achieved using a reverse phase high performance liquid chromatography (HPLC) column with an isocratic mobile phase of 93% water, 20 mM formic acid, 20 mM ammonium formate, and 7% methanol. Chromatographic separation of the phosphonic acids was achieved using a reverse phase HPLC with gradient mobile phases consisting of 0.1% acetic acid in water and 0.1% acetic acid in methanol. Identification and quantification of species was achieved using both atmospheric pressure chemical ionization and electrospray ionization-tandem mass spectrometry monitoring two precursor-to-product ion transitions for each compound, as well as internal standards. The concentration vs response was linear between 10 ng/mL and 500 ng/mL. Instrument detection limits ranged from 10 ng/mL to 50 ng/mL.

INTRODUCTION

Determining the use of chemical warfare agents (CWAs) in times of war or in acts of terrorism requires methods for the rapid analysis of biological media (urine, blood, saliva, skin) to confirm exposure. In the event a large number of people are exposed to a CWA,

analysis of the environment may determine the specific agent of exposure, but that information may not reach the health care providers in a timely fashion. As seen in the recent terrorist attack and police intervention in Russia, victims of an exposure to an unknown chemical could have been treated more successfully if the specific agent, or at least class of agent, could have been determined quickly. In the event of a large number of victims being delivered to several health care facilities, chances are that victims will arrive at health care centers before the identification of the chemical is determined. Although health care providers will treat the symptoms of victims, more definitive treatment can be provided if the chemical causing the symptoms is known. As victims exposed to non-lethal concentrations and showing little to no symptoms begin to report to health care providers, the rapid analysis of short-lived metabolites would aid health care providers to determine which people were exposed and which people were just experiencing a hysteria response.

The development of analytical techniques for the analysis of CWA metabolites in biological fluids has been limited. Several methods have been reported for the analysis of biological media for CWA exposure (Jakubowski et al., 1990; Jakubowski et al., 2000; Shih et al., 1991; Shih et al., 1994; TB MED 296, 1996; Black and Read, 1991; Black and Read, 1988; Black et al., 1992; Black et al., 1999; Fidder et al., 1996; Noort et al., 1997; van der Schans et al., 2002; Karayilanoglu et al., 2001; Lemire et al., 2003; Driskell et al., 2002; Tsushihashi et al., 1998; Zi-Hui and Qin, 2001; Hayes et al., 1999; Graham et al., 2000, US Army SOP), and a review of biomonitoring for exposure to CWAs recently has been published (Noort et al., 2002). All of these methods have labor intensive sample preparation procedures.

The analysis of urine to determine exposure to chemicals, including CWAs, where short- and long-lived metabolites have been identified, has been documented in the literature (Jakubowski et al., 2000; E.M. et al., 1990; Shih et al., 1991; Shih et al., 1994; TB MED 296, 1996; Black and Read, 1991; Black et al., 1992; Lemire et al., 2003; Driskell et al., 2002; Hayes et al., 1999; Noort et al., 2002). Short-lived metabolites are eliminated by the body, usually via the urine, and can only be detected in a period of 24 hours to several days post exposure (Black et al., 1992; Noort et al., 2002). For sulfur mustard exposure, short-lived metabolites include thiodiglycol, thiodiglycol sulfoxide, and possibly thiodiglycol sulfone; for nerve agents, alkyl-phosphonic acids are short-lived metabolites (Shih et al., 1991; Shih et al., 1994; TB MED 296, 1996; Black and Read, 1991; Black, and Read, 1988; Black et al., 1992; Driskel et al., 2002; Tsushihashi et al., 1998; Hayes et al., 1999; Noort et al., 2002). Long-lived metabolites can be found in the urine or blood for days to several weeks after exposure (Black et al., 1999; Fidder et al., 1996; Noort et al., 1997; van der Schans et al., 2002; Noort et al., 2002). For sulfur mustard exposure, long-lived metabolites include β -lyase metabolites, the macromolecule adducts (N1-(2-hydroxyethylthioethyl)-4-methyl imidazole and N7-2-[(hydroxyethyl)thio]ethyl-guanine (N7-HETE-guanine), the DNA adducts N7-(2-hydroxyethylthioethyl)-2'-deoxyguanosine and N7-HETE-guanine, and protein adducts with hemoglobin and albumin (Black et al., 1999; Fidder et al., 1996; Noort et al., 1997; van der Schans et al., 2002; Noort et al., 2002). The detection of metabolites several days to weeks after exposure (long-lived metabolites) may be due to covalent adducts bonding of the agent with proteins and

DNA, but may also be due to the lower detection limits for these compounds compared to the “shorter-lived” metabolites. For nerve agent exposures, long-lived indicators of exposure include the reactivation of phosphorylated binding sites with butyryl cholinesterase, and direct analysis of cholinesterase adducts found in blood and serum (Black et al., 1999; Fidler et al., 1996; Noort et al., 1997; van der Schans et al. 2002; Noort et al., 2002).

Metabolites of CWAs can show up quickly in urine, the traditional biological fluid for testing. Urine sample collection is not invasive; however, collection on demand can be difficult, especially in young children. To complement urine analyses, the analysis of saliva may aid in the chemical identification and thereby aid in the treatment of victims.

Saliva has been used to assess exposure to a number of chemicals, and a review of the use of saliva as an analytical tool in toxicology has been published (Denovan et al., 2000; Hold et al., 1995; Kousba et al., 2003). Saliva, also referred to as oral fluid, is made up of water (~99%), proteinaceous material (~0.3%), mucin (~0.3%), and salts. What potentially makes saliva a good biological fluid to determine exposure is salivary glands have a high blood flow (Haeckel, 1990). High blood flow indicates that xenobiotics can be transported from the blood to the saliva via a number of mechanisms, including ultra filtration, active secretion, and diffusion (Kidwell et al., 1998).

Advantages of using saliva as a biological fluid for testing are the matrix is relatively clean and sample collection is non-invasive. Sampling can be performed in the open, thus reducing risk of sample tampering. Due to pH and ion trapping, the concentration of xenobiotics may be higher than that in the blood, and low protein concentrations make sample preparation procedures simpler. Disadvantages of using saliva are that the concentrations of xenobiotics are usually lower than that found in the urine, oral contamination (e.g., food particles, bronchial fluids, flavor components from gums and candies) may affect results, and the overall detection window is usually shorter than for urine (Schramm et al., 1992; Rivier, 2000). The use of LC-MS/MS techniques can overcome some of the disadvantages by using a highly selective and sensitive ion monitoring technique. In addition, since the CWA metabolites are polar organic compounds, the use of LC-MS/MS negates the need for derivatization techniques required by gas chromatography-mass spectrometry (GC-MS) analysis methods. Several researchers have used LC-MS/MS techniques for the analysis of drugs of abuse and pharmacotherapeutics in saliva (Dams et al., 2003; Wood et al., 2002; Bentley et al., 1999; Phillips et al., 1999; Orтели et al., 2000; Mortier et al., 2001; Mortier et al., 2002). Sample preparation techniques have included simple filtration, protein precipitation with ultra-filtration, and automated solid phase extraction.

The purpose of this study was to demonstrate the feasibility of direct injection of non-extracted non-derivitized saliva and urine samples for the detection of several short-lived CWA metabolites using LC-MS/MS techniques. This technique is an improvement on methods currently found in the literature due to minimal sample preparation while maintaining similar detection limits. The methodology described demonstrates that with

minimal, rapid sample preparation, the selective and sensitive detection of CWA metabolites is possible in saliva and urine.

METHODS AND MATERIALS

Reagents

Thiodiglycol (TDG), methylphosphonic acid (MPA), water (HPLC grade), and acetic acid, acetonitrile (HPLC grade) were purchased from Aldrich, (Milwaukee, WI). Formic acid and ammonium formate were purchased from Sigma Chemical (St. Louis, MO). Methanol and sodium chloride were purchased from Burdick and Jackson (Muskegon, MI). The PTFE filters were purchased from Supelco (Bellefonte, PA). Thiodiglycol sulfoxide (TDGO), ethyl methylphosphonic acid (EMPA), isopropyl methylphosphonic acid (IMPA), cyclohexyl methyl phosphonic acid (CMPA), pinacolyl methylphosphonic acid (PMPA), and deuterated-isopropyl methylphosphonic acid (d_7 -IMPA) were purchased from Cerilliant (Austin, TX). Thiodiglycol sulfone (TDGO2), deuterated thiodiglycol (d_8 -TDG), and deuterated-pinacolyl methylphosphonic acid (d_3 -PMPA) were synthesized in-house.

Analytical Standards

Analytical standards were prepared by weighing 10 mg of each compound using an analytical balance (Mettler AE 100, Mettler Toledo, Columbus, OH) and dissolving the compound in 10 mL of methanol. Prior to weighing, the balance was calibrated to assure accuracy. Standard solutions were prepared by serial dilution of the 1 mg/mL standard using syringes and volumetric flasks. All standards were protected from light and stored at 4 °C or lower for the duration of the study.

LC-MS/MS Analysis

All LC-MS/MS analyses were performed using a Micromass Quattro II triple quadrupole mass spectrometer (Waters, Danvers, MA) interfaced to a Hewlett Packard Model 1100 liquid chromatographic system (Agilent Technologies, Palo Alto, CA). Chromatographic separation of the sulfur mustard degradation products (TDG, d_8 -TDG, TDGO, TDGO2) was achieved using an Allure C18 (150mm X 2.1 mm X 5 μ m) column (Restek, Bellefonte, PA). The isocratic mobile phase consisted of 20 mM formic acid and 20 mM ammonium formate in water (93%) and methanol (7%). The column was equilibrated at 0.6 mL/min, and injection volumes of 100 μ L were used.

Chromatographic separation of the phosphonic acids (MPA, EMPA, IMPA, d_3 -IMPA, CMPA, PMPA, d_3 -PMPA) was achieved using a HIRB-150AM (150mm X 2.1mm X 5 μ m), column (Richard Scientific, Novato, CA). A gradient elution was used with mobile phase A comprised of 0.1 % acetic acid in water, and mobile phase B comprised of 0.1% acetic acid in methanol. Table 1 shows the gradient elution. The column was equilibrated at 0.2 mL/min and injection volumes of 100 μ L were used.

Two ionization modes were used to optimize sensitivity for the two classes of compounds. For the thiodiglycol, thiodiglycol sulfoxide and thiodiglycol sulfone, positive ion atmospheric pressure chemical ionization (APCI) was used. For the phosphonic acids, positive ion electrospray ionization (ESI) was used. For each ionization mode, the cone voltages and collision energies were optimized for each compound and precursor/product ion transition. Table 2 shows the ion transitions used for monitoring.

Integration and quantitation of the data were performed using MassLynx software package (version 3.5 build 004) (Waters, Danvers, MA). Statistical analyses (percent recovery and standard deviations) were performed using Microsoft Excel 2000 (Microsoft, Redmond, WA).

Preparation of Samples

Saliva. Salivation was promoted by stimulation (chewing on a piece of Teflon[®]) and 10 mL of saliva was collected. Standards were added to provide 50 ng/mL (sulfoxy species) or 100 ng/mL (phosphonic acids) spike level. The pH of the saliva was adjusted to 3 using 1–3 drops of formic acid (88%) for the thiodiglycol-related species or 1–3 drops of glacial acetic acid for the phosphonic acids. The acidified samples then were centrifuged (Beckman Coulter Avanti™ J25, Palo Alto, CA) at 2000 x g for 10 min to remove precipitated proteins. After centrifugation, the saliva was put through a PTFE 0.45 µm filter and then placed in an autosampler vial and a 100 µL aliquot analyzed by LC-MS/MS.

Urine – Thiodiglycol-Related Species. Urine from volunteers was collected and analyzed the same day. Urine was spiked with standards to provide a 100 ng/mL concentration. The pH of the urine was adjusted to 3 using formic acid. The spiked, acidified urine was centrifuged at 2000 x g for 10 min, to remove precipitated proteins, then put through a PTFE 0.45 µm filter, and placed in an autosampler vial where a 100 µL aliquot was analyzed by LC-MS/MS.

Urine – Phosphonic Acids – Analysis of Acidified Urine. Urine from volunteers was collected and used the same day of analysis. Urine was spiked with standards to provide the following concentrations: MPA 300 ng/mL, EMPA 100 ng/mL, IMPA 100 ng/mL d₃-IMPA 100 ng/mL, CMPA 50 ng/mL, PMPA 100 ng/mL and d₃-PMPA 100 ng/mL. The pH of the urine was adjusted to 3 using glacial acetic acid. The spiked acidified urine was centrifuged at 2000 x g for 10 min, to remove precipitated proteins, then put through a PTFE 0.45 µm filter, and placed in an autosampler vial and a 100 µL aliquot analyzed by LC-MS/MS.

Analysis of Acetonitrile Extracts of Acidified Urine. Five mL of urine was placed in a centrifuge tube, spiked with standard to provide an approximately 50 ng/mL concentration (specific spike level was compound dependent). The spiked urine was pH adjusted to 3 using glacial acetic acid. To the acidified urine, 2 mL of acetonitrile was added and the mixture vortexed for 15 to 30 sec. Sodium chloride (NaCl) was added to make a supersaturated solution. The sample then was centrifuged at 2000 x g for 10 min

to remove precipitated proteins. The acetonitrile phase was removed and placed in an autosampler vial for analysis by LC-MS/MS.

Recovery

The recovery of the target analytes from both saliva and urine was determined by comparing the concentrations obtained in the samples versus standards analyzed concurrently with the samples. Triplicate samples were prepared for each experiment. The ratio of the sample to standard concentration represented the percentage of analyte recovered by the extraction without correction for the internal standard recovery. Calibrations in the saliva or urine matrix have not been performed yet.

RESULTS

Figure 1 shows the selected ion chromatogram of the quantitation ions for the thiodiglycol-related species analysis using a 50 ng/mL standard prepared in methanol.

Figure 2 shows the selected ion chromatogram of the quantitation ions for the phosphonic acid analysis using a 100 ng/mL standard prepared in water.

The calibration curves prepared in methanol were linear over a 10 ng/mL to 500 ng/mL range. Table 3 shows the detection limits for standards. The detection limits were based on standards with two ions having $\geq 3/1$ S/N (signal to noise ratio) and are not corrected for recovery.

Figure 3 shows the selected ion chromatogram of the quantitation ions for the thiodiglycol-related species from a blank saliva sample. Figure 4 shows the selected ion chromatogram of the quantitation ion for the thiodiglycol-related species from a saliva sample spiked at 100 ng/mL. Figure 5 shows the selected ion chromatogram of the quantitation ions for the phosphonic acids from a blank saliva sample. Figure 6 shows the selected ion chromatogram of the quantitation ions for the phosphonic acids from a saliva sample spiked with 300 ng/mL MPA, 100 ng/mL EMPA, IMPA, PMPA, and 50 ng/mL CMPA.

Table 4 shows the recoveries from the spiked saliva samples.

Figure 7 shows the selected ion chromatogram of the quantitation ions for the phosphonic acids from a blank urine sample. Figure 8 shows the selected ion chromatogram of the quantitation ions for the phosphonic acids from a urine sample spiked with 150 ng/mL MPA, 50 ng/mL EMPA, IMPA, PMPA, and 25 ng/mL CMPA.

Table 5 shows the recoveries from the spiked urine samples. In the urine samples, the thiodiglycol-related species were not recovered. The reasons for this are unknown. Further work will focus on this phenomenon.

DISCUSSION

Assessing use of CWAs in war or acts of terrorism requires validated methods for the rapid analysis of biological media (urine, blood, saliva, skin) to determine exposure to these materials. Urine and saliva can easily be collected in a non-invasive fashion and offer the analytical chemist a relatively clean matrix for analysis. We have shown the feasibility for the detection and quantitation of TDG, TDGO, and TDGO2 in saliva using LC-APCI-MS/MS, and MPA, EMPA, IMPA, CMPA, and PMPA in saliva and urine using LC-ESI-MS/MS.

Salivation was stimulated by chewing on a piece of Teflon[®] for several reasons. First, stimulating salivation allows for larger volumes of saliva to be produced in a short period of time. Secondly, the pH of the stimulated saliva is in a narrow range (approximately 7.4), whereas non-stimulated saliva can have large variability in pH. That may affect the secretion of weakly acidic and basic compounds (Hold et al., 1995). Thirdly, Teflon[®] was used to assure that secreted material was not adsorbed by the stimulating material, and finally, Teflon[®] does not introduce interferences into the saliva.

Detection limits for the thiodiglycol-related species obtained in this study (in methanol standards) are within the range of detection limits for biological samples reported in the literature. The recovery data indicate that for TDG for example, the 50 ng/mL spike in the saliva was recovered $59 \pm 5\%$, indicating that 29.5 ng/mL was observed in the saliva sample. Thiodiglycol has been shown to be present in urine from less than 1 ng/mL to 55 ng/mL (Wils et al., 1985; Wils et al., 1988; Black and Read, 1988; Jakubowski et al., 1990; Jakubowski et al., 2000). The detection limits for our method indicate that it is possible to detect normal background levels in a biological fluid, or above background due to exposure.

Due to the highly toxic nature of CWAs, low detection limits are very important. The LC₅₀ for nerve agents are on the order of 30 to 200 mg/min-m⁻³ and approximately 1500 mg/min m⁻³ for sulfur mustard. Sub-lethal exposures will have less material available for detection.

Detection limits for the phosphonic acids (in methanol standards) obtained in this feasibility study are comparable to detection limits reported in published literature (Driskell et al., 2002; Tsushihashi et al., 1998; Zi-Hui and Qin, 2001; Black and Read, 1997). The only method with lower detection limits is an isotope dilution GC-MS/MS method, which requires extensive sample preparation to concentrate the sample (Driskell et al., 2002). Although the detection limits reported in Table 3 were from standards, the recoveries from the spiked saliva samples (PMPA $95 \pm 2\%$) indicate that the detection limits would be in the same range as those reported in the literature for a biological fluid.

Recoveries for the thiodiglycol-related species in spiked saliva ranged from 56% to 68% with less than 10% relative standard deviation (RSD, Table 4). The recoveries of the phosphonic acids ranged from 95% to 211% with less than 12% RSD (Table 4). Over-recovery ($211 \pm 10\%$) of EMPA was obtained. The reasons for the observed over

recoveries are thought to be due to an ionization enhancement in the electrospray source due to the matrix since the phenomena was observed in multiple precursor/product ion transitions used for monitoring. These over-recoveries may be corrected by using an internal standard specific to EMPA (d_3 -EMPA) as any compound-specific enhancements or degradation in performance can be compensated for accurately.

The thiodiglycol-related species could not be recovered from the urine. Since the thiodiglycol-related species are minimally retained on the chromatographic column (< 7 minutes), further sample clean-up may be necessary to separate native species in the urine that are interfering with the chromatography and ionization of the thiodiglycol-related species.

Two sample preparation procedures were attempted for the analysis of phosphonic acids from urine—the analysis of acidified urine and the acetonitrile extraction of acidified urine (Table 5). The recoveries for the phosphonic acids ranged from 55% to 155% with less than 16% RSD when analyzing acidified urine. These recoveries are better than described in the literature for the GC-MS/MS method (Driskell et al., 2002). The increased recovery is attributed to the minimization of sample preparation steps in our direct analysis method.

In an attempt to increase the recovery of the lower molecular weight species (MPA and EMPA), acetonitrile was used to extract the urine after acidification. The results are shown in Table 5. The acetonitrile extraction method provided higher recoveries for PMPA and better precision for CMPA, but the recoveries of MPA, EMPA, and IMPA were actually lower than the non-extracted samples. Using acetonitrile for extractions appears to improve the recovery of the more aliphatic compounds, at the expense of less aliphatic species.

CONCLUSIONS

The results suggest that the direct analysis LC-MS/MS method has potential for the rapid determination of exposure to sulfur mustard and nerve agents with saliva, and for nerve agent with urine. The detection limits obtained are at or below the background levels for the target analytes. The advantages of this technique are that saliva and urine are more easily obtained than blood, and the sample preparation steps are minimal. The analysis of saliva samples to determine exposure to sulfur mustard and nerve-type agents is feasible. The analysis of urine for sulfur mustard metabolites requires further sample clean-up techniques, but analysis of urine for exposure to nerve agents is feasible. Experiments are underway to determine detection limits in biological fluids and to obtain dose-response relationships for the different CWAs using these techniques.

ACKNOWLEDGMENTS

This program was supported by internal research and development funding by Battelle Memorial Institute.

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Table 1. Gradient used for elution of phosphonic acids

TIME	A%*	B%†
0	98	2
5	98	2
15	5	95
20	5	95
20.1	98	2
30	98	2

* Mobile Phase A consists of water with 0.1% acetic acid.

† Mobile Phase B consists of methanol with 0.1% acetic acid.

Table 2. Ion transitions used for monitoring

COMPOUND	CORRESPONDING AGENT	PRECURSOR ION	PRODUCT IONS*
TDG	HD	123	105, 45
d ₈ -TDG	IS	131	113, 49
TDGO	HD	139	77, 45
TDGO2	HD	155	45, 137
MPA	G-Agents	97	79, 47
EMPA	VX	125	97, 79
IMPA	GB	139	97, 79
d ₃ -IMPA	IS	142	100, 82
CMPA	GF	179	97, 79
PMPA	GD	181	97, 79
d ₃ -PMPA	IS	184	100, 82

* Quantitation ion listed first.

Table 3. Detection limits

COMPOUND	DETECTION LIMITS (ng/mL)	
	THIS STUDY*	LITERATURE VALUES†
TDG	10	5‡; 2§; 3
d ₈ -TDG	10	–
TDGO	25	2§
TDGO2	50	–
MPA	50	200#
EMPA	10	4**; 100††; 500‡‡; 5§§; 10#
IMPA	10	3**; 300‡‡; 2.5§§; 10#
d ₃ -IMPA	10	–
CMPA	10	2**; 200‡‡; 50#
PMPA	20	3**; 100‡‡; 5§§; 50#
d ₃ -PMPA	20	–

– No literature values for a detection limit.

* Detection limits for this study based on standards analyzed with two ions having >3/1 S/N ratios.

† Detection limits in the literature were reported for both standards and matrix samples.

‡ TB MED 296, 1996.

§ Black and Read, 1991.

|| Wils et al., 1985.

Black and Read, 1997.

** Driskell et al., 2002.

†† Tsushihashi et al., 1998.

‡‡ Zi-Hui and Qin, 2001.

§§ Miki et al., 1999.

Table 4. Recoveries of target analytes from saliva

COMPOUND	PERCENT RECOVERY ± STD. DEV. (n=3)*
TDG	59 ± 5
d ₈ -TDG	59 ± 3
TDGO	68 ± 7
TDGO2	56 ± 4
MPA	122 ± 15
EMPA	211 ± 10†
IMPA	118 ± 5
d ₇ -IMPA	90 ± 14
CMPA	95 ± 11
PMPA	95 ± 2
d ₃ -PMPA	70 ± 11

* Sulfoxy species spiked at 50 ng/mL, phosphonic acid species spiked at 100 ng/mL. Recoveries listed are based on external standards and are not corrected for internal standard recoveries.

† Reasons for over-recoveries are unknown at this time. Possible interference or ionization enhancement due to the matrix are theories; a labeled EMPA standard may aid in understanding this phenomena.

Table 5. Recoveries of target analytes from urine

COMPOUND	PERCENT RECOVERY \pm STD. DEV. (n=3) ^{*,†}	
	ACIDIFIED URINE	ACN EXTRACT
MPA	55 \pm 2	0
EMPA	62 \pm 5	10 \pm 1
IMPA	80 \pm 3	1 \pm 1
CMPA	155 \pm 11	60 \pm 5
PMPA	81 \pm 13	95 \pm 8

* Recoveries listed are based on external standards and are not corrected for internal standard recoveries.

† Urine was spiked with standards to provide the following concentrations: MPA 300 ng/mL, EMPA 100 ng/mL, IMPA 100 ng/mL, CMPA 50 ng/mL, and PMPA 100 ng/mL.

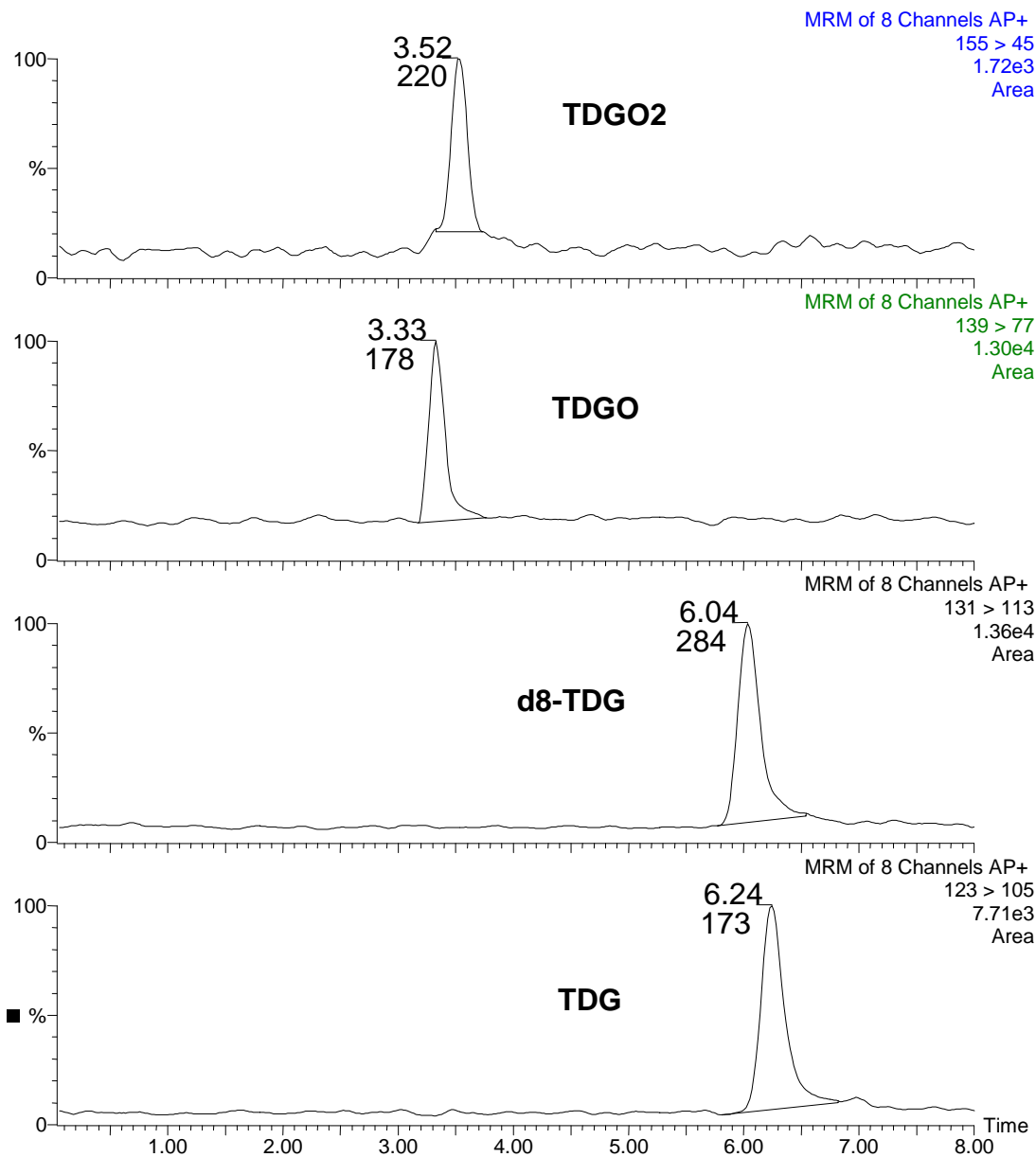


Figure 1. Selected ion chromatogram of the quantitation ions for the thiodiglycol-related species analysis using a 50 ng/mL standard

100 ng/mL Standard

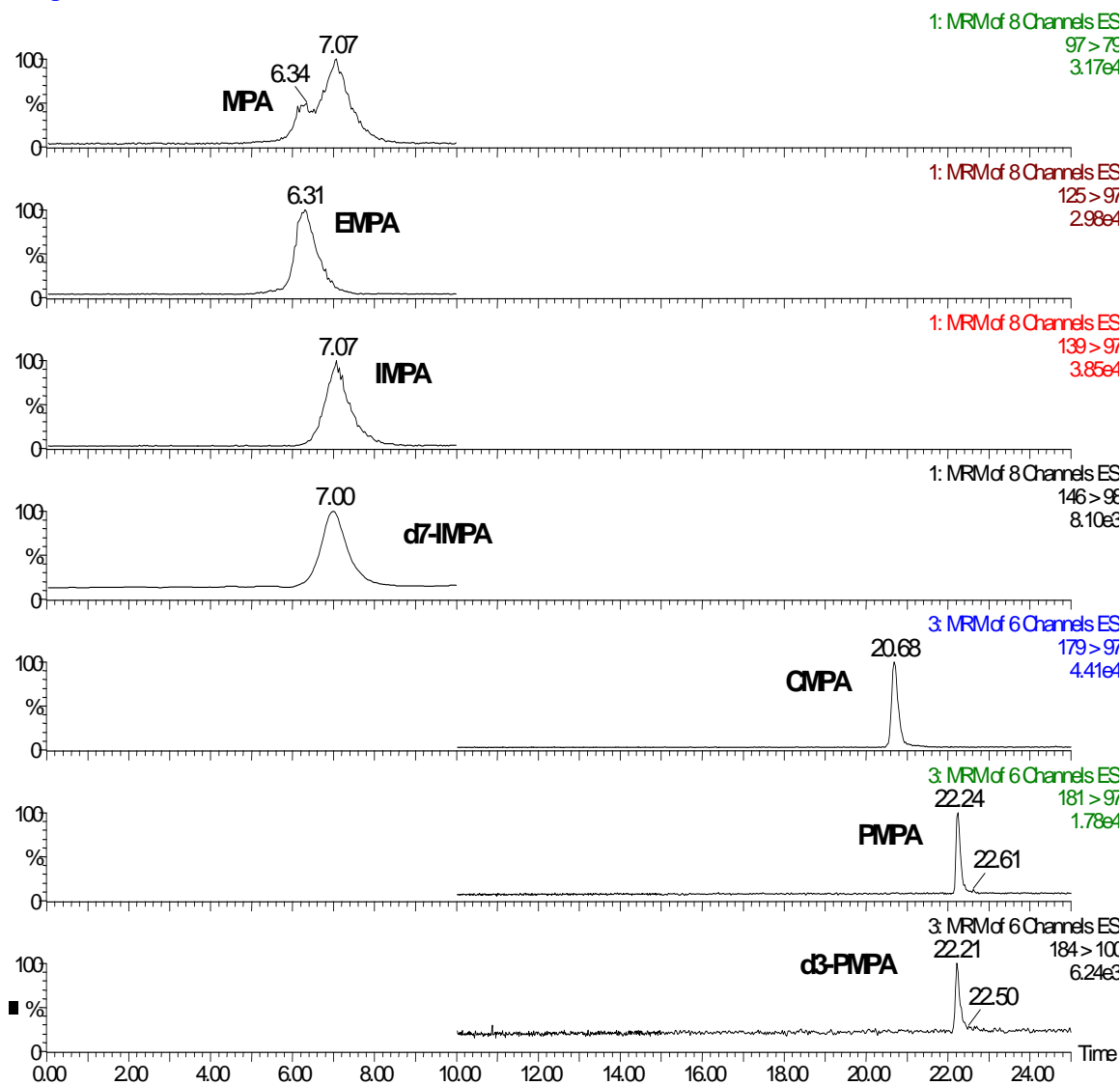


Figure 2. Selected ion chromatogram of the quantitation ions for the phosphonic acid analysis using a 100 ng/mL standard

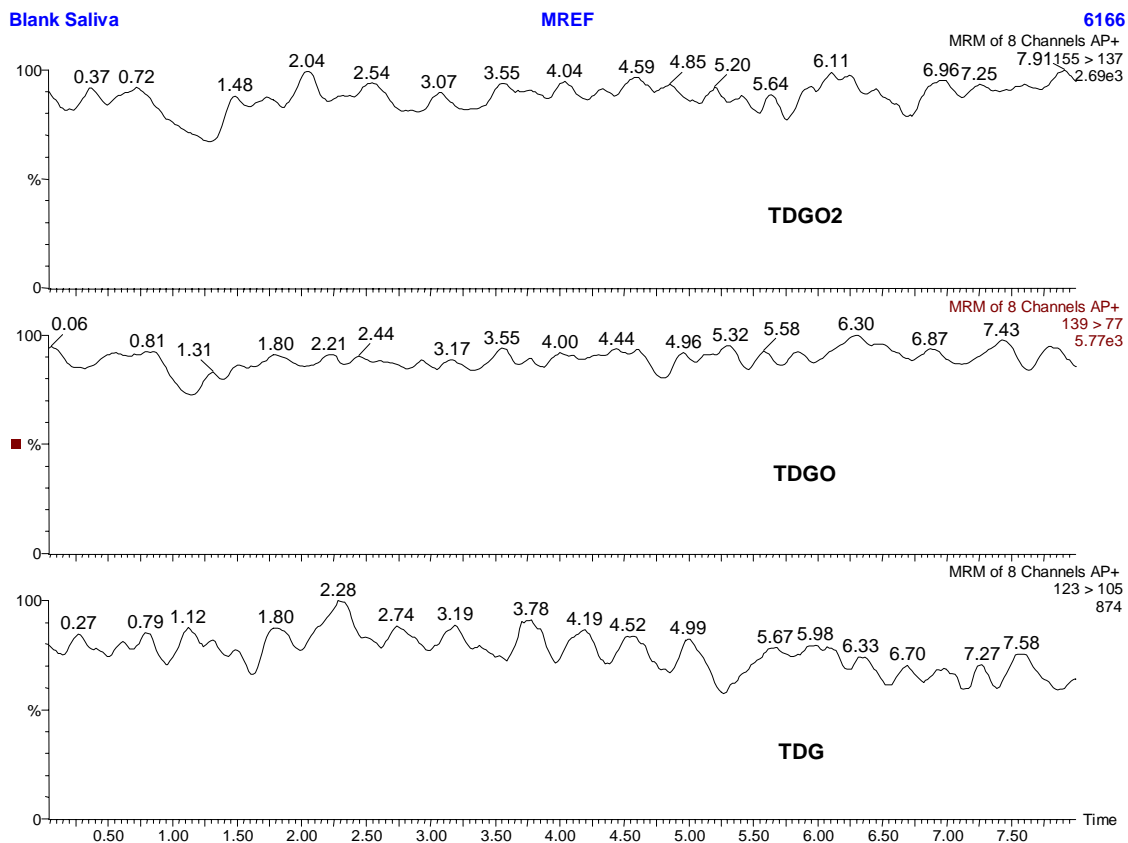


Figure 3. Selected ion chromatogram of the quantitation ions for the thiodiglycol-related species from a blank saliva sample

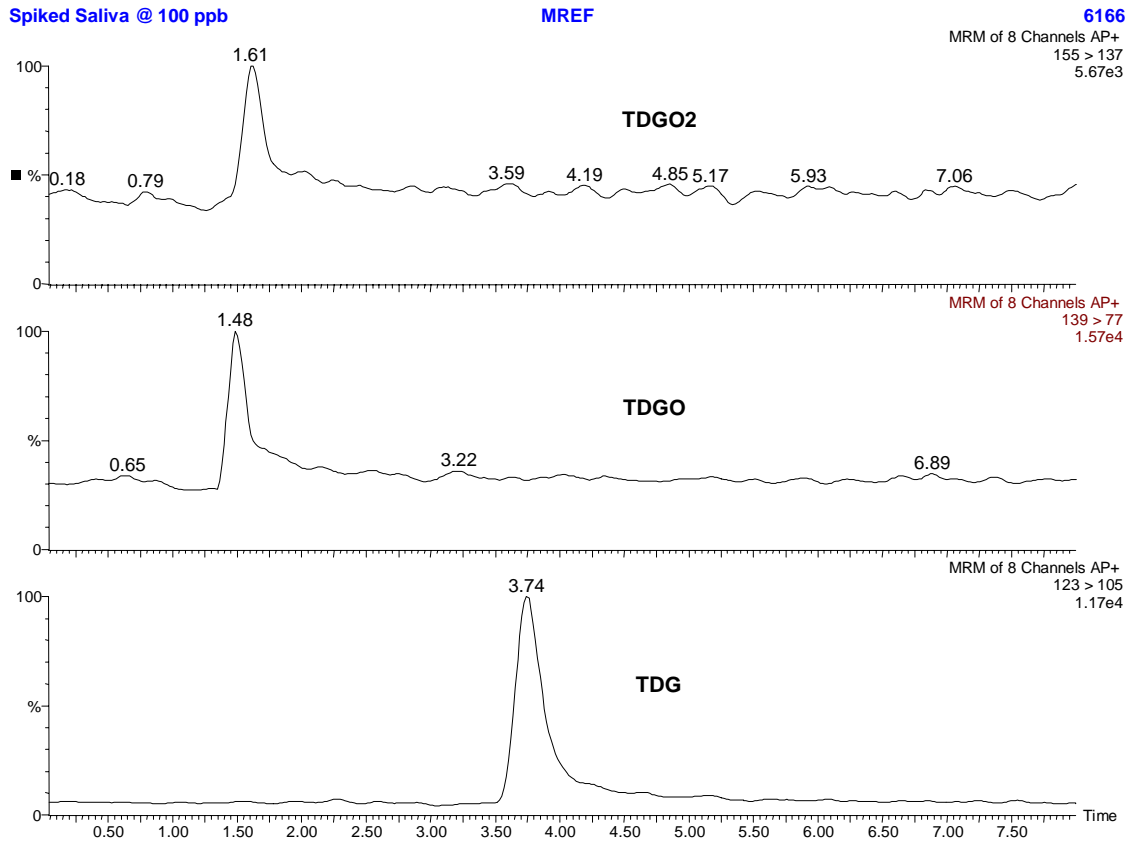


Figure 4. Selected ion chromatogram of the quantitation ion for the thiodiglycol-related species from a saliva sample spiked at 100 ng/mL

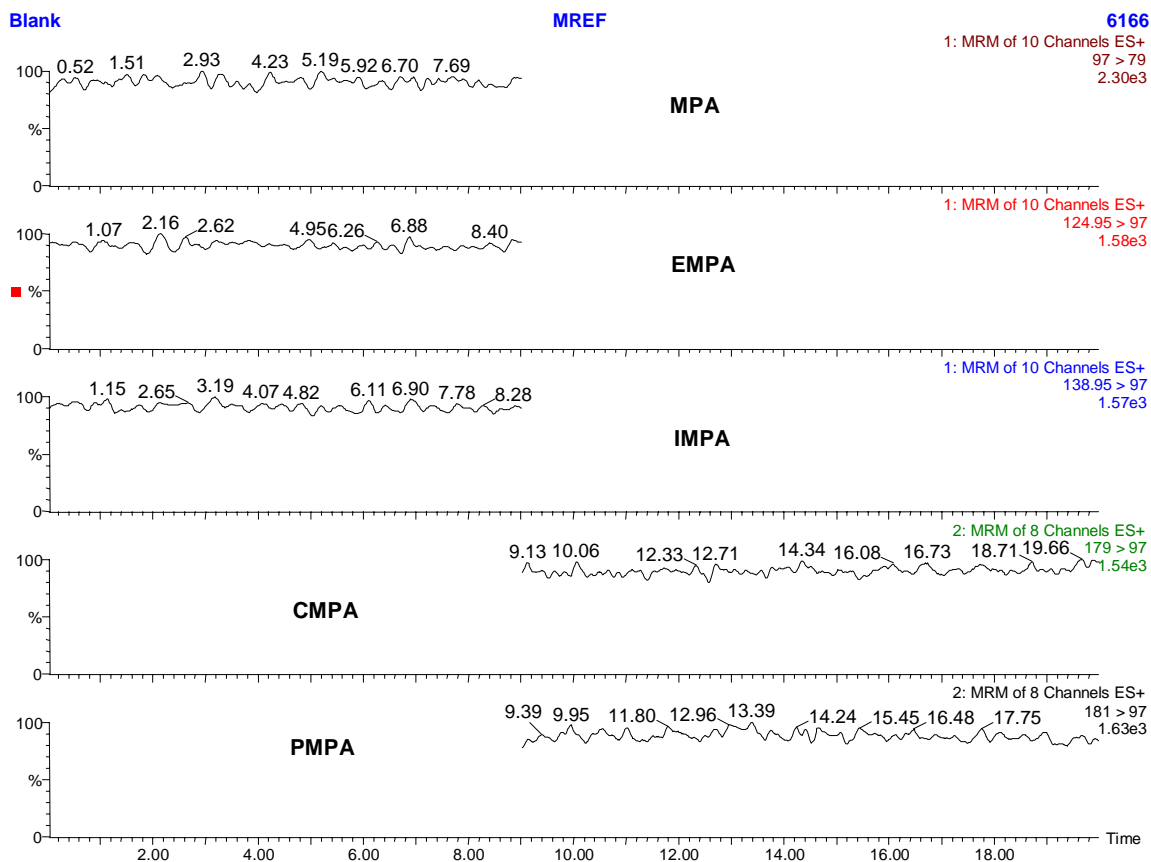


Figure 5. Selected ion chromatogram of the quantitation ions for the phosphonic acids from a blank saliva sample

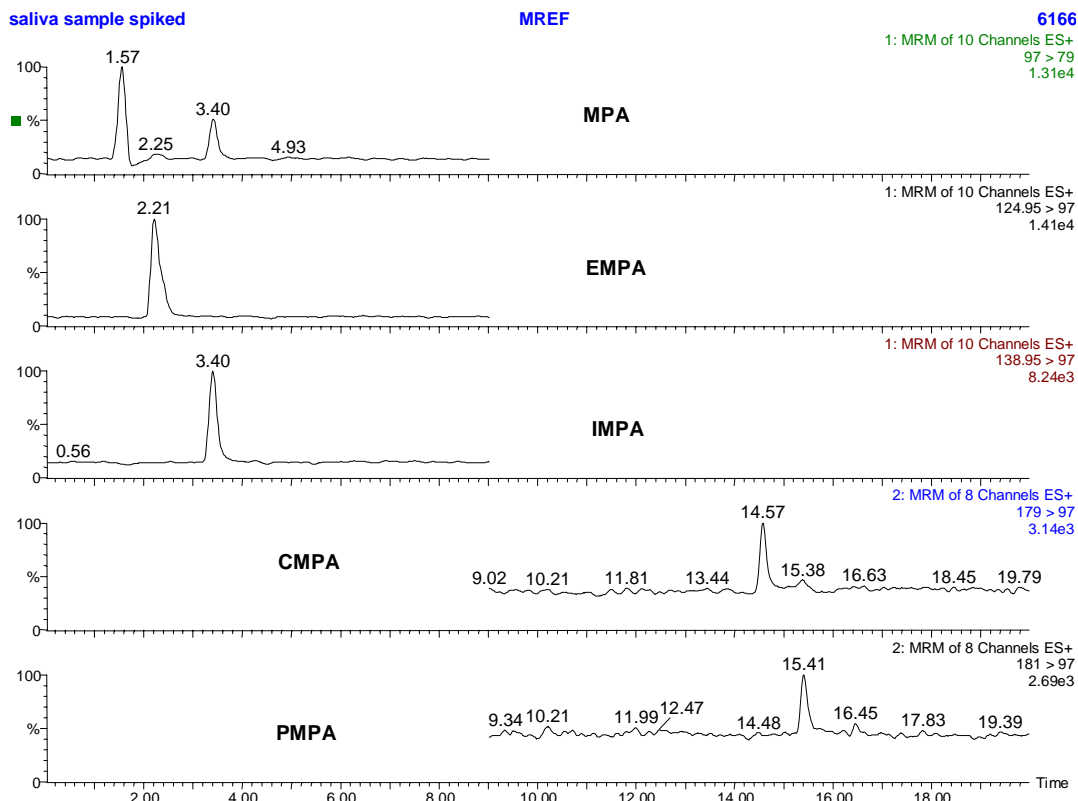


Figure 6. Selected ion chromatogram of the quantitation ions for the phosphonic acids from a saliva sample spiked with 300 ng/mL MPA, 100 ng/mL EMPA, IMPA, PMPA, and 50 ng/mL CMPA

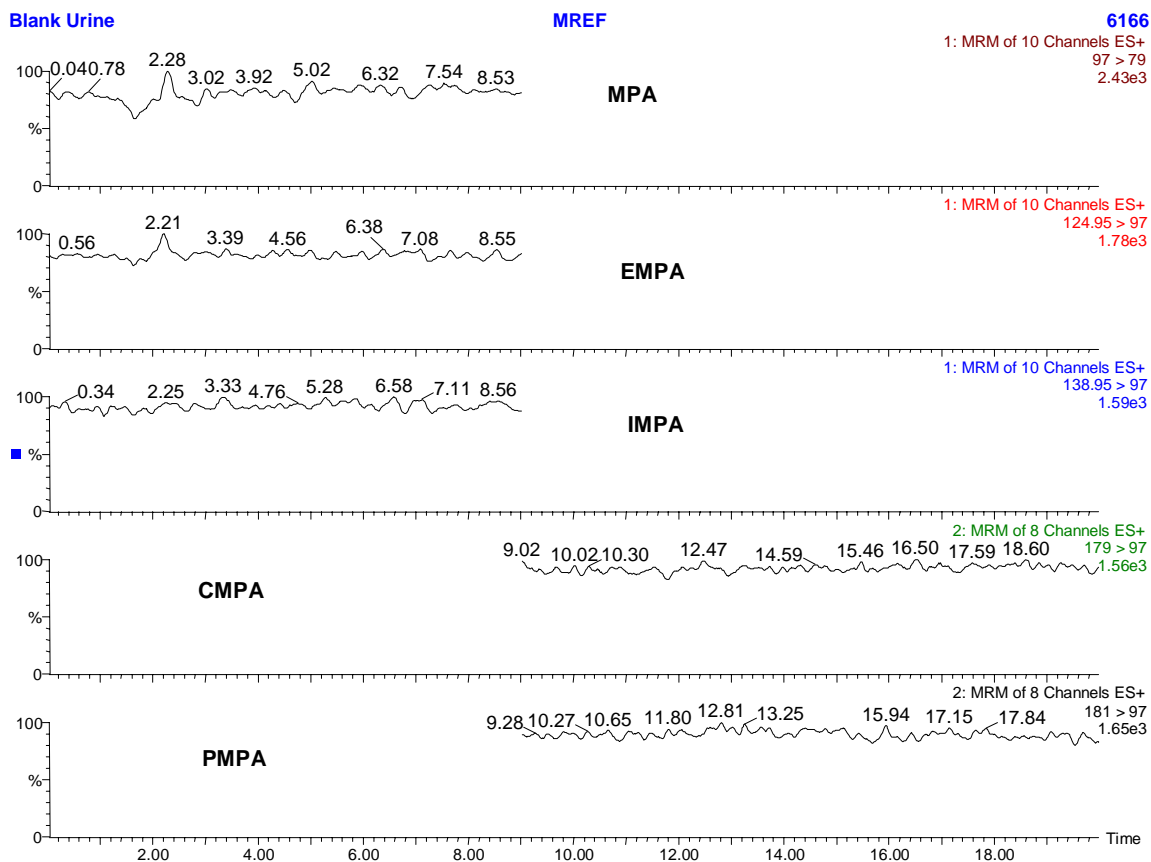


Figure 7. Selected ion chromatogram of the quantitation ions for the phosphonic acids from a blank urine sample

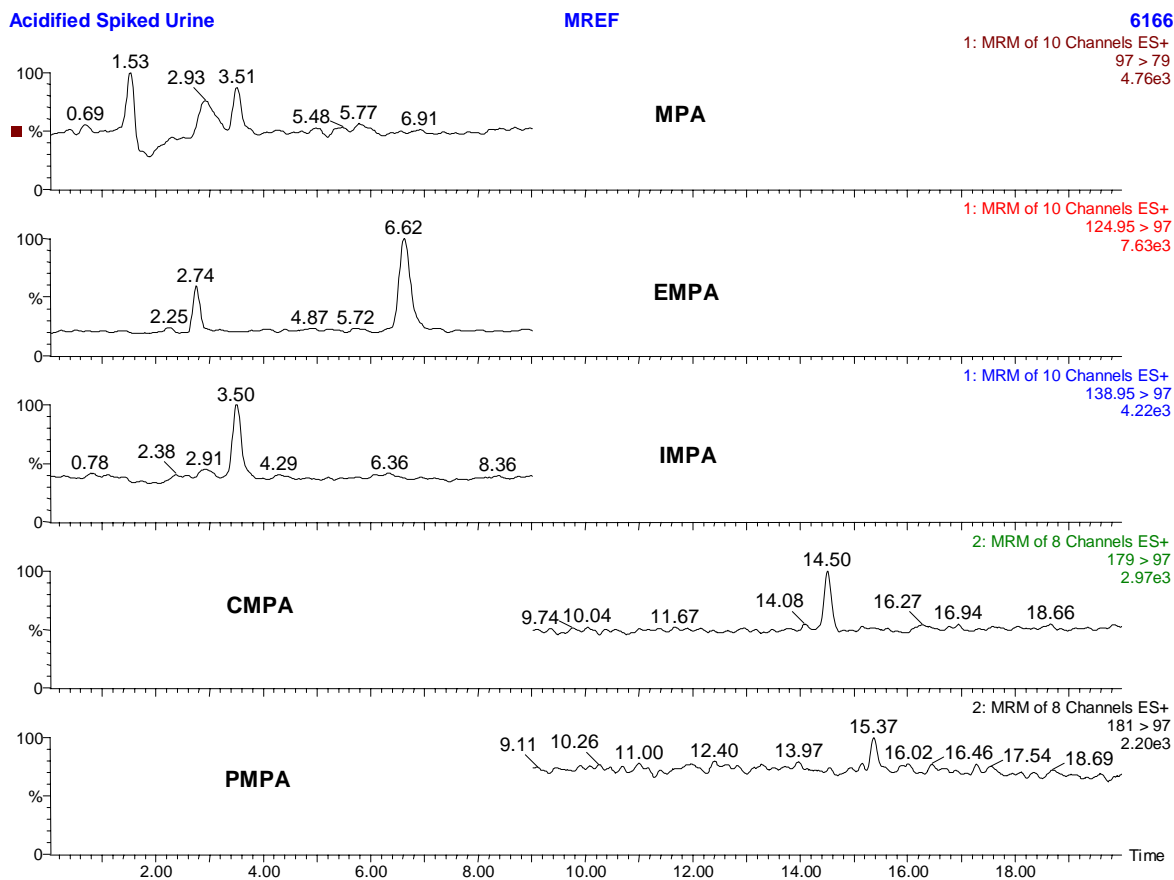


Figure 8. Selected ion chromatogram of the quantitation ions for the phosphonic acids from a urine sample spiked with 150 ng/mL MPA, 50 ng/mL EMPA, IMPA, PMPA and 25 ng/mL CMPA