A Simple and Effective Method for Speciation Analysis of 13 Arsenic Species Using HPLC on a Fluorocarbon Stationary Phase Coupled to ICP-MS

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Abstract

The separation properties of arsenic species was investigated using HPLC-ICP-MS with several commercially available fluorocarbon stationary phases and no ion-pair reagents in HPLC. One pentafluorophenyl column showed the highest potential for the separation of a larger number of arsenic species when using simple acid-based mobile phases. After modification of the operational parameters in HPLC, the speciation analysis of representative 13 arsenic species, including arsenite, arsenate, methylarsonic acid, dimethylarsinic acid, trimethylarsine oxide, tetramethylarsonium, arsenobetaine, arsenocho line, thio-dimethylarsinic acid, oxo-arsenosugar-glycerol, oxo-arsenosugar-phosphate, oxo-arsenosugar-sulfonate, and oxo-arsenosugar-sulfate, was achieved by HPLC-ICP-MS with the column along with a mobile phase of 0.05 % heptafluorobutyric acid-methanol (99:1, volume per volume).

Keywords: HPLC, fluorocarbon, pentafluorophenyl, perfluoroalkyl, ICP-MS, arsenic, arsenosugar.
Introduction

Arsenic (As) speciation analysis has been performed by many research groups using high-performance liquid chromatography (HPLC) coupled to inductively coupled plasma mass spectrometry (ICP-MS). In this technique, each As compound is identified by comparing its retention time with that of a standard (i.e. a commercially available or synthesized As compound). Ion-pair chromatography (IPC) on an octadecylsilica (ODS or C18) column is a type of reversed-phase (RP) HPLC and has been widely used for As speciation analysis in different research fields, such as food\textsuperscript{1-6} and environmental chemistry\textsuperscript{2,7,8} because it allows for the separation of a larger number of ionic As species (i.e. 15 species at most\textsuperscript{7,9}) at one time usually for a considerably shorter time as compared to the case of more frequently-used anion-exchange HPLC (e.g. 7 species mainly including arsenosugars\textsuperscript{10}). In this technique, an ion-pair reagent is added to the mobile phase as a counter ion to promote the formation of an ion pair with the ionic As compound. Most of researchers have used sulfur (S)-containing ion-pair reagent (i.e. sodium alkylsulfonate) for cationic As species, along with another ion-pair reagent (e.g. tetramethylammonium hydroxide) for anionic As species, a buffer solution, and an organic modifier. However, the chemical composition of the mobile phase is complex, and it is costly to make. Some arsenosugars and other As species frequently found in aquatic organisms include phosphorus (P) or S in their chemical structures. In their analysis using HPLC-ICP-MS, the phenomena that retention times can slightly shift due to the presence of matrix components in the sample is known to occur, resulting in the difficulty in identifying the As species. Simultaneous detection of three relevant elements (i.e. As, P, and S) in As species using HPLC-ICP-MS owing to ICP-MS’s multi-element detection capability is a promising approach to increase the accuracy of
the identification and quantification results for P- and S-containing As species. However, the above-mentioned RP-IPC-HPLC is not suitable for such simultaneous detection by HPLC-ICP-MS because the ion-pair reagents for cationic As species include S in their chemical structures in the form of sulfonate group and thereby leads to seriously low signal-to-background (S/B) ratio for S, which hampers the identification and further quantification of the S-containing As species. Therefore, the establishment of a new HPLC-ICP-MS method for speciation analysis of As species using a simple, non-P and non-S containing mobile phase without ion-pair reagents is needed.

As compared to common C18 phases, fluorocarbon stationary phases are known to have unique properties. Pentafluorophenyl (PFP or F5) phases, for example, have the following properties: (1) the strong retention capability for polar compounds (especially polar basic compounds) without any ion-pair reagents, (2) the special selectivity for aromatic compounds, and (3) the ability to be used in various separation modes such as RP, HILIC, and even 100 % aqueous separation modes. While common C18 phases mainly exhibit a hydrophobic interaction potential, PFP phases exhibit several potentials, including the relatively weak hydrophobic interaction potential (usually corresponding to C8 or lighter), strong dipole-dipole potential (i.e. polar interaction) from the carbon-fluorine bonds, π-π interaction potential, charge-transfer interaction potential due to the electronegativity of the fluorine groups, and ion-exchange interaction potential, which result in the unique properties above. In 2014, Baba et al. reported the speciation analysis of five simple As species, including arsenite [As(III)], arsenate [As(V)], methylarsonic acid [MA(V)], dimethylarsinic acid [DMA(V)], and arsenobetaine (AB), using HPLC-ICP-MS with a PFP column (i.e. Discovery HS F5 column) and its application to rice. However, they have never investigated the separation properties of other As species such as arsenosugars when using the column.
and the other commercially available PFP columns. Moreover, the availability of perfluoroalkyl (PFA) phases for the separation of As species have not yet been evaluated.

In this study, we investigated the separation properties of As species frequently found in food and environmental samples using HPLC-ICP-MS with several commercially available fluorocarbon stationary phases and no ion-pair reagents in HPLC and established a simple and effective method for speciation analysis of 13 As species using an acid-based, non-S and non-P containing mobile phase and isocratic conditions.

**Experimental**

*Reagents and chemicals*

Deionized water with a resistivity of 18.2 MΩ cm\(^{-1}\) was obtained using a Simpli Lab-UV (Nihon Millipore, Tokyo, Japan) and used throughout unless otherwise stated. The following commercial products were purchased from Kanto Chemical (Tokyo, Japan): methanol (MeOH), ethanol (EtOH), isopropanol (IPA), and acetonitrile (AcCN) for HPLC grade, formic acid (FA) and acetic acid (AcOH) for special grade, nitric acid (HNO\(_3\), ultrapure grade), pentafluoropropionic acid (PFPA, 97 %), and heptafluorobutyric acid (HFBA, 99 %). Trifluoroacetic acid (TFA, analytical grade) was from Wako Pure Chemical Industries (Osaka, Japan). Inorganic As compounds, including As(III) and As(V), and methylated As compounds, including MA(V), DMA(V), trimethylarsine oxide (TMAO), tetramethylarsonium (TMA), AB, and arsenocholine (AC), were obtained from Tri Chemical Laboratories (Yamanashi, Japan)
and used as representative As species. Thio-dimethylarsinic acid [Thio-DMA(V)] originally synthesized by a research group led by Professor Kevin A. Francesconi (University of Graz, Austria) was kindly donated by Professor Takafumi Ochi (Teikyo University, Japan). Oxo-arsenosugar-glycerol (Oxo-Gly) was synthesized by previously reported procedures. Stock standard solutions of each As compound were prepared in water (from the crystal form) at the concentrations of 1000 μg mL$^{-1}$ (as As) for the eight representative As species and 100 ng mL$^{-1}$ (as As) for the two synthesized As species. A working standard mixture solution of the 10 As species listed above was prepared at the concentration of 10 ng mL$^{-1}$ (as As) for each species. A freeze-dried purified extract of the brown macroalga Fucus serratus containing four oxo-arsenosugars, Oxo-Gly, oxo-arsenosugar-phosphate (Oxo-PO$_4$), oxo-arsenosugar-sulfonate (Oxo-SO$_3$), and oxo-arsenosugar-sulfate (Oxo-SO$_4$), was kindly donated by Professor Francesconi. It was dissolved in 1 mL of water and used for validation purposes (Supporting Information) and, thereafter, analysis of arsenosugars after appropriate dilution, otherwise mixed with the working standard mixture solution to make another solution including all the 13 As species.

**Instrumentation**

The speciation analysis of As species was performed by HPLC-ICP-MS. The HPLC system comprised a carrier reservoir (CR 670; GL Sciences), a metal-free binary pump (PU 611; GL Sciences), a column oven (CO 631; GL Sciences), and an autosampler (MIDAS; Spark Holland) with a 100-μL sample loop made of polyetheretherketone tubing (ca. 1.6 mm OD × 0.25 mm ID; GL Sciences). The vial tray temperature in the autosampler was set at 4 °C to avoid As oxidation state changes. Each prepared solution was transferred into a 0.6-mL polyethylene vial (GL Sciences)
just before analysis, and then injected onto an analytical column through the sample loop. The analytical columns used were four different types of fluorocarbon columns with silica gel-based fluorinated phases, including three PFP columns with C₆F₅ phases and one PFA column with a C₆F₁₃-branched phase. Their main properties are summarized in Table 1. All columns were end-capped. In the case of a Discovery HS F5 column (mainly used in this study as described below), the column with the size of 4.6 mm × 15 cm was used for preliminary investigation and then, it was changed to the one with the size of 2.1 mm × 15 cm for further investigation. The ICP-MS system was ELAN DRC-e (PerkinElmer SCIEX, Concord, Canada) equipped with a micro-concentric nebulizer (MicroMist, 0.2 mL min⁻¹ uptake; Glass Expansion, West Melbourne, Australia) and a cyclonic spray chamber (Glass Expansion). The typical operational parameters are given in Table S1 (Supporting Information). For detecting the As species separated by the column, As⁺ was monitored at a mass-to-charge ratio (m/z) of 75 in standard mode. Only for analyzing samples with the As-P-S simultaneous detection technique, a dynamic reaction cell (DRC) mode was employed with oxygen (O₂) as the reaction gas into the cell. In this mode, the cell-generated oxide ions, AsO⁺ (m/z 91), PO⁺ (m/z 47), and SO⁺ (m/z 48 and m/z 50), were monitored instead of As⁺, P⁺ (m/z 31), and S⁺ (m/z 32 and m/z 34), respectively, in order to prevent isobaric interferences (e.g. As⁺ interferes with ArCl⁺ at m/z of 75).¹⁵ The cell gas (O₂) flow rate was briefly optimized and set at 0.7 mL min⁻¹. The retention time, intensity, and integrated area of each peak in the resulting HPLC-ICP-MS chromatogram were determined with a TotalChrom Workstation software (version 6.2.0; PerkinElmer SCIEX).
HPLC-ICP-MS analysis of the target As species

For investigating the separation properties of the fluorocarbon columns for HPLC-ICP-MS analysis of the target As species, a mobile phase composition (as a major factor that can drastically affect their separation) and relevant operational parameters in HPLC (as minor factors) were changed variously. As one of the relevant operational parameters in HPLC, organic modifiers such as alcohols (i.e. MeOH and EtOH), IPA, and AcCN were used mainly to elute cationic As species faster, and additionally to increase the ICP-MS signal intensity of As due to a well-known carbon-induced signal enhancement phenomenon and production of finer droplets with lower surface tensions by the nebulizer (resulting in higher sample transport efficiency into the plasma). The HPLC conditions giving the best separation of the target As species during the investigation was determined to be the best conditions in this study. Throughout the investigation, it was checked whether there is no difference in the retention time and integrated area between the peaks from single standard solutions and a mixture of standard solutions.

The above-mentioned approach is a simple way to quickly find almost optimum HPLC conditions with the minimum effort and without help from chemometric tools such as factorial designs, which means that the best conditions obtained in this study (described below) have room for further improvement, which would be provided by chemometric tools in a future work.

Results and Discussion

Details on the investigation of the separation properties of a Discovery HS F5 column
with a PFP stationary phase for HPLC-ICP-MS analysis of the target As species are described below because it showed the highest potential for the separation of a larger number of As species when using simple acid-based mobile phases among the tested fluorocarbon columns, while those of the other three fluorocarbon columns (i.e. two PFP columns, Ascentis Express F5 and Kinetex F5, and one PFA column, Wakopak Fluofix-II 120E) are described in Supporting Information.

To achieve the separation of the representative 10 As species (other than Oxo-PO₄, Oxo-SO₃, and Oxo-SO₄) or 13 As species in all using the Discovery HS F5 column with the size of 2.1 mm × 15 cm, water (at pH 7.0), an inorganic acid (i.e. HNO₃ at pH 3.0), and several kinds of organic acids at typical concentrations (i.e. 0.1 % FA, 0.5–1 % AcOH, 0.035–0.05 % TFA, 0.05–0.1 % PFPA, and 0.05–1 % HFBA) in the mobile phase and relevant operational parameters in HPLC, including the flow rate (ranging 0.2–0.3 mL min⁻¹ or controlled manually with time, like a time program, if it was expected to be effective), the temperature (ranging from 35–50 °C), and organic modifier (i.e. 1–5 % MeOH, 1 % EtOH, 1 % IPA, and 1 % AcCN), were investigated. The resulting HPLC-ICP-MS chromatograms giving the best separation of the As species during this investigation are given in Fig. 1. When using water and HNO₃ in the mobile phase, only eight peaks appeared in the chromatograms (Figs. 1a and 1b, respectively). As compared to the case of water, the overall elution of the As species in the case of HNO₃ was more than two times faster, although the separation of the As species (especially that of TMA and AC) was not enough. When using FA in the mobile phase, the separation of 10 As species was not fully achieved because that of TMA and Oxo-Gly was difficult to accomplish (Fig. 1c). Similarly, when using TFA in the mobile phase, the separation of 10 As species was not fully achieved because that of As(V) and As(III) and Thio-DMA(V) and TMAO were difficult to accomplish (Fig. 1d).
Meanwhile, when using AcOH and PFPA in the mobile phase, the separation of 10 As species was achieved at their respective concentrations of 0.5–1 % and 0.05 % within 13 min (Fig. 1e) and 17 min (Fig. 1f), respectively. When using HFBA in the mobile phase, the separation of the 10 As species was achieved at the HFBA concentration of 0.05 %, and furthermore the separation of the eleven As species other than DMA(V) and Oxo-SO₄, which were eluted at the same retention time, was achieved within 30 min under the same analytical conditions (Fig. 1g). To accelerate the elution of DMA(V) and Oxo-SO₄ with an increase in the hydrophobicity of the mobile phase and elute the cationic As species faster, the effect of organic modifiers on them was investigated. When using MeOH at different concentrations along with 0.05 % HFBA in the mobile phase, the separation of DMA(V) and Oxo-SO₄ was improved at the MeOH concentration of 1 % and the elution of the strongly retained cationic As species (i.e. AC and TMA) became faster; thereby, the separation of the 13 As species was achieved within 25 min (Fig. 1h). In addition, this organic modifier increased the signal intensity of As approximately three times as compared to the case without it. Almost similar results were obtained when substituting EtOH for MeOH at the same mixing ratio with 0.05 % HFBA; that is, the separation of the 13 As species was almost achieved within 32 min at the flow rate of 0.2 mL min⁻¹ and the temperature of 40 ℃, while that of Oxo-SO₃ and Oxo-PO₄ still had room for improvement. As can be seen in Figs. 1i and 1j, the other organic modifiers (i.e. EtOH, IPA, and AcCN) exhibited similar effects as the case of MeOH, including the faster elution of the cationic As species (especially in the case of 1 % IPA) and the approximately two to three times increase in the signal intensity of As, although the separation of all the 13 As species was not tested for these organic modifiers in this study. According to the results of the investigation above, the HPLC conditions with a mobile phase of 0.05 % HFBA/MeOH = 99:1 gave the best
separation of the target As species (Fig. 1h). Therefore, the HPLC conditions were determined to be the best conditions with a constant flow rate.

The results above (Fig. 1) showed that the anionic As species such as the inorganic and simple methylated As species were eluted earlier due to their weak retention on the PFP phase and conversely, cations such as TMA and AC were eluted later due to their relatively strong retention on the PFP phase, while the other As species exhibited different elution trends depending on the mobile phase composition. The trends of the anionic and cationic As species would be mainly due to the strong dipole potential (i.e. polar interaction) of the PFP phase from its carbon-fluorine bonds; that is, the anionic As species less interact with negatively-charged (anionic) fluorine groups and conversely, the cationic species strongly interact with them. This might be the major separation mechanism when using water and an inorganic acid (i.e. HNO₃) as a mobile phase. Meanwhile, when using organic acids such as AcOH and fluorinated organic acids (i.e. TFA, PFPA, and HFBA) as a mobile phase, the major separation mechanism might be due to the weak hydrophobicity of the ion pairs between the positively-charged (cationic) As species and the dissociated organic acids. The fluorinated organic acids above may have small hydrophobicity, be partially dissociated, and thus work as pairing ions. In fact, separation of cationic compounds in ion pair chromatography are conducted frequently under acidic condition because more chemicals are protonated by taking positive charge or losing negative charge. Regarding the four arsenosugars examined in this study (i.e. Oxo-Gly, Oxo-PO₄, Oxo-SO₃, and Oxo-SO₄), for example, a dimethylarsinoyl group in their molecules is protonated with pKₐ around 4.⁷ Among them, only Oxo-Gly does not have a negatively-charged group and therefore becomes positive in acidic pH, which may show hydrophobic interaction with the column by making the ion pairs with the dissociated organic acids in the
mobile phase. In acidic pH, Oxo-Gly and probably TMAO in addition to TMA and AC possess positive charge in their molecules and interact with the dissociated organic acids to make the ion pairs. This is the reason why these four As species retained on the column stronger than others. This can be supported by the facts that all of them showed longer retention time when fluorinated organic acids with longer chain are used in the mobile phase, and that all of them showed shorter retention time when a small amount of organic modifier was added to the mobile phase because it effectively weakens the interaction and shortens the retention time.

The results of the investigation (mentioned above) did not lead to finding the HPLC conditions (with a constant flow rate) achieving the separation of Oxo-SO$_3$ and Oxo-PO$_4$. Then, to simply modify the intervals of the retention times of the As species under the above-mentioned best conditions with a constant flow rate and thereby improving the separation of Oxo-SO$_3$ and Oxo-PO$_4$, only the flow rate was manually changed with time. When holding the flow rate at 0.1 mL min$^{-1}$ for 8 min, increasing it to 0.3 mL min$^{-1}$ and then 0.4 mL min$^{-1}$ for 10 s each, and finally holding it at 0.4 mL min$^{-1}$, the appropriate spacing of the peak intervals in the chromatogram and the separation of the 13 As species were achieved at the same time within 25 min (Fig. 2), which was the same duration as the case of a constant flow rate of 0.3 mL min$^{-1}$ (Fig. 1h). Even so, the separation of the 13 As species without changing the flow rate should be accomplished in the future, because small changes in retention time under such changing flow rate conditions may worsen the separation and identification of the peaks in the presence of sample matrix.

The validation of the HPLC-ICP-MS method established as a result of the investigation above, the real sample analysis using the method, and the feasibility study on the As-P-S simultaneous detection technique using the method are described in
Supporting Information.

The established HPLC-ICP-MS method has the following advantages: (1) the separation and detection capability for representative 13 As species at most, (2) no usage of any ion-pair reagents (including S-containing ones), enabling As-P-S simultaneous detection for the As species including these elements, that is, Oxo-PO₄, Thio-DMA(V), Oxo-SO₃, and Oxo-SO₄, and (3) less usage of solvents (i.e. only a small amount of an organic solvent is used), resulting in cost saving. In the RP-IPC-HPLC method¹⁶ generally used for As speciation analysis, the retention times of DMA(V) and Oxo-SO₃ were closely similar, resulting in the co-elution of these compounds. In the SAX-HPLC method¹⁶ frequently used for the determination of arsenosugars, AC and TMA were co-eluted around the void volume and shortly thereafter, As(III), TMAO, AB, and Oxo-Gly were co-eluted. Thus, these methods require attention to such issues and is not universally applicable to speciation analysis of As species including arsenosugars. In contrast, the established method (without need of caring about the issues above) has great potential to be a universal method for As speciation analysis. An improvement in the relatively long separation time (i.e. 25 min) as compared to the case of a conventional RP-IPC is of interest as a future work.

Conclusions

As a result of investigating the separation properties of As species frequently found in food and environmental samples using HPLC-ICP-MS with several commercially available fluorocarbon stationary phases and no ion-pair reagents in HPLC, we established a simple and effective method for speciation analysis of 13 As species using
a simple acid-based mobile phase and isocratic conditions. Since the mobile phase does not contain any S- and P-including compounds, this method enables As-P-S simultaneous detection for the As species including these elements, which has never been performed by using popular S-containing ion-pair reagents due to their increasing the background signal of S and thereby decreasing its S/B ratio. This advantage must be able to contribute to the accurate separation analysis of Oxo-PO₄, Thio-DMA(V), Oxo-SO₃, and Oxo-SO₄ from other non-P and non-S containing As species such as Oxo-Gly and DMA(V) in a variety of real samples. The established HPLC-based method is expected to have a significant contribution to various arsenic-related research fields, particularly food and environmental chemistry, where many types of As species (frequently including arsenosugars) are targets for analysis with HPLC-hyphenated techniques such as HPLC-ICP-MS.

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Supporting Information

Table S1 summarizes the typical ICP-MS operating conditions. Four distinct sections include the following information, respectively: (1) validation of using the dissolved *F.*
serratus extract as an arsenosugar standard, (2) separation properties of PFP columns (other than a Discovery HS F5 column) and a PFA column for HPLC-ICP-MS analysis of the target As species, (3) validation of the established HPLC-ICP-MS method, and (4) real sample analysis using the established HPLC-ICP-MS method. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.
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Table 1  Properties of fluorocarbon columns of the present study classified according to their column chemistry

| Column     | Supplier       | Dimensions/ ID in mm × length in cm | Particle diameter/μm | Pore diameter/Å | Special particle technology |
|------------|----------------|-------------------------------------|-----------------------|-----------------|------------------------------|
| PFP column |                |                                     |                       |                 |                              |
| Discovery  | Supelco        | 4.6 × 15                            | 3                     | 120             | None                         |
| HS F5      |                | 2.1 × 15                            | 3                     | 120             | None                         |
| Ascentis   | Supelco        | 2.1 × 15                            | 2.7                   | 90              | Fused-core                   |
| Express F5 |                |                                     |                       |                 |                              |
| Kinetex F5 | Phenomenex     | 2.1 × 10                            | 2.6                   | 100             | Core-shell                   |
| PFA column |                |                                     |                       |                 |                              |
| Wakopak    | Wako Pure      | 2.0 × 15                            | 5                     | 120             | None                         |
| Fluofix-II | Chemicals      |                                     |                       |                 |                              |
| 120E       | Industries     |                                     |                       |                 |                              |
Figure Captions

Fig. 1  HPLC-ICP-MS chromatograms of the representative 10 or 13 As species obtained using a Discovery HS F5 column with the following different conditions from (a) to (j): mobile phase: indicated in each chromatogram, flow rate: 0.2 mL min$^{-1}$ or 0.3 mL min$^{-1}$ (only in the cases of 0.05 % HFBA and 0.05 % HFBA/MeOH = 99:1), temperature: 35 ℃ (only in the case of 0.5 % AcOH) or 40 ℃, injection volume: 5 µL.

Peak: 1. As(V), 2. As(III), 3. MA(V), 4. DMA(V), 5. AB, 6. TMAO, 7. Thio-DMA(V), 8. TMA, 9. AC, 10. Oxo-Gly, 11. Oxo-PO$_4$, 12. Oxo-SO$_3$, 13. Oxo-SO$_4$.

The insert shows an enlarged view of the chromatogram.

With respect to (a), the peaks have not been formally assigned at the time of analysis.

Fig. 2  HPLC-ICP-MS chromatogram of the 13 As species obtained under the best HPLC conditions with a Discovery HS F5 column.

Mobile phase: 0.05 % HFBA/MeOH = 99:1, flow rate: hold at 0.1 mL min$^{-1}$ for 8 min, increase to 0.3 mL min$^{-1}$ and then 0.4 mL min$^{-1}$ for 10 s each and hold at 0.4 mL min$^{-1}$, temperature: 40 ℃, injection volume: 5 µL, peak: 1. As(V), 2. As(III), 3. MA(V), 4. DMA(V), 5. AB, 6. TMAO, 7. Thio-DMA(V), 8. TMA, 9. AC, 10. Oxo-Gly, 11. Oxo-PO$_4$, 12. Oxo-SO$_3$, 13. Oxo-SO$_4$, UK1. unknown As species 1, UK2. unknown As species 2.
Fig. 1
Fig. 2
Graphical Index