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Determination of arsenic species: A critical review of methods and applications, 2000–2003

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We review recent research in the field of arsenic speciation analysis with the emphasis on significant advances, novel applications and current uncertainties.

1 Introduction

Analytical chemistry has long played a major role in many areas of scientific investigations. In addition to providing established tools and techniques to facilitate experimental studies, analytical chemistry can also open up new areas of research. Examples include atomic absorption spectrometry and its huge impact on diverse fields ranging from mineralogy to clinical analysis, and the new research, particularly in the biological sciences, emanating from recent advances in ionisation techniques for mass spectrometry.

In the field of arsenic research there are also examples of analytical chemistry opening up new scientific areas. In Scotland in the early 1830s, the scientific evidence of forensic chemist Dr Marsh in a case of suspected arsenic poisoning was dismissed as being inconclusive. This knock to Dr Marsh's professional pride spurred him on to develop a sensitive and reliable analytical method[1], based on the generation of volatile arsine, for determining traces of inorganic arsenic. The Marsh method quickly became of great importance in forensic science, and stimulated research into the environmental, biological, and toxicological chemistry of arsenic. These research fields were given a further boost in 1973 by Braman and Foreback[2] who adapted the Marsh method to enable

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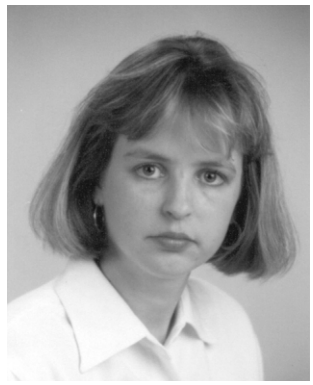
Doris Kuehnelt received her MSc in Chemistry followed by a PhD in Analytical Chemistry at the Karl-Franzens-University Graz. Since 2001 she has been University Assistant at the Institute of Chemistry at the Karl-Franzens-University Graz. Her research interests lie in the field of speciation analysis. They are focused on the determination of organometallic compounds in biological systems with the emphasis on arsenic and selenium compounds using high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry or electrospray mass spectrometry.



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the determination of simple methylated arsenicals in addition to inorganic arsenic species. The work of Braman and Foreback may be seen as heralding the field of arsenic speciation analysis.

In the subsequent years, arsenic research grew steadily in accord with the discovery of new arsenic species and the need to understand their complex environmental and biological chemistry. Recently, interest in arsenic has been heightened by toxicological issues, in particular the carcinogenic effects of inorganic arsenic in drinking water and the consequent worldwide human health implications.[3] In addition, arsenic is also attracting renewed clinical interest and is currently posting some remarkable successes in the treatment of a certain type of leukaemia.[4] These apparent contrasts in the effects and efficacy of arsenic highlight the urgent need to understand the precise underlying mechanisms, and a complete understanding will require identification of the active arsenic metabolites. Advances in arsenic speciation analysis may provide the tools to finally unravel these toxicological conundrums and other intriguing questions regarding arsenic's varied roles in biological systems. This review provides a critical evaluation of the latest analytical methods for determining arsenic species.

2 Previous reviews

We refer the reader to the articles[5–13] listed in Table 1 for recent complementary reviews on specific aspects of arsenic research. As a starting point to understanding the complexity of arsenic's environmental and biological chemistry, the scholarly and comprehensive review of Cullen and Reimer[14] is essential reading, and a good starting point for the novice arsenic researcher.

3 This review: Goals and content

Although this review deals primarily with recent developments and applications of analytical methods, it is not aimed solely at an analytical audience but is intended for all scientists with an interest in arsenic species research. It is hoped that the information will be accessible to, and useful for, scientists in diverse fields such as toxicology and environmental chemistry. We hope to direct these researchers to the most useful techniques for a given application, and, at times, perhaps, dissuade them from following less suitable techniques.

We will begin with a description of the relevant terms and arsenic compounds, followed by a brief history of arsenic speciation research in the 20th century and a synopsis of the situation at year 2000. A compilation of relevant methods reported since 2000 will then be presented before we take a selective but detailed look at recent work in particular areas such as sample preparation, separation and detection. The review attempts to cover the relevant work reported from the beginning of 2000 up to the end of 2003.

4 Abbreviations and definitions

We will follow the terms proposed by Templeton *et al.*[15] which offer useful distinctions between related research areas and terms (*e.g.* chemical species, speciation, speciation analysis, fractionation). The term "coupled technique" features strongly in speciation analysis, no doubt reflecting the early days of the methods when they combined two instruments which at that time were unfamiliar partners in inorganic analyses (*e.g.* GC and AAS). We will not use the idiosyncratic term "hyphenated technique".

Abbreviations to techniques are given here; abbreviations for arsenic species are given in Section 5.

AAS	Atomic absorption spectrometry
AFS	Atomic fluorescence spectrometry
ASE	Accelerated solvent extraction
CE	Capillary electrophoresis
CID	Collision induced dissociation
CT	Cold trapping or cryogenic trapping (see description below)
ES	Electrospray (ionisation)
EXAFS	Extended X-ray absorption fine structure
FTICR (MS)	Fourier transform ion cyclotron resonance (mass spectrometry)
GC	Gas chromatography
HG	Hydride generation (see description below)
HPLC	High performance liquid chromatography
IC	Ion chromatography
ICPAES	Inductively coupled plasma atomic emission spectrometry
ICPMS	Inductively coupled plasma mass spectrometry
MS	Mass spectrometry
SEC	Size exclusion chromatography
TOF (MS)	Time-of-flight (mass spectrometry)
XANES	X-ray absorption near edge structure
XAS	X-ray absorption spectroscopy

Hydride generation (HG)

A general term describing the conversion of an element or elemental species to a volatile analyte (usually a hydride); for

Table 1 Some recent review articles dealing with aspects of arsenic speciation relevant and complementary to this review

Title	Comments	Reference
Speciation of tin, lead, mercury, arsenic and selenium compounds by capillary electrophoresis	Describes the determination of arsenic species (and other elemental species) by capillary electrophoresis coupled to various detectors	5
The cellular metabolism and systemic toxicity of arsenic	Covers recent research on the metabolism and toxicity of arsenic. The focus is on methylated metabolites, in particular methylated As(III) species	6
Microbial methylation of metalloids: arsenic, antimony, and bismuth	Provides an interesting and readable historical account of microbial transformations of arsenic	7
Arsenic speciation analysis	Reports recent work on separation and detection methods for arsenic species, and sample handling techniques	8
Arsenic round the world: a review	Summarises the occurrences of arsenic species, and discusses aspects of arsenic's impact on human health; ends with a compilation of episodes of arsenic poisonings "round the world"	9
Mechanisms of arsenic biotransformation	A succinct overview of biological arsenic transformation processes and the implications for human health	10
The speciation of natural tissues by electrospray-mass spectrometry. I: biosynthesised species, As and Se	Delivers an account of the use of electrospray ionisation mass spectrometry for the identification of arsenic compounds. The focus is on tandem MS methods with many examples clearly demonstrating the power of the technique for arsenic speciation analysis	11
The potential of organic (electrospray- and atmospheric pressure chemical ionisation) mass spectrometric techniques coupled to liquid-phase separation for speciation analysis	A beautifully clear and balanced review which has a large section on arsenic species	12
The speciation of arsenic in biological tissues and the certification of reference materials for quality control	Focuses on the processes for certification of reference materials for arsenic species	13

arsenic speciation analysis, the hydride generation method is used almost exclusively for the determination of As(III)/As(V), MA/MA(III), and DMA/DMA(III) after conversion to their respective hydrides (or, more specifically, their respective arsines), namely arsine (AsH_3, b.p. −55 °C), methylarsine (CH_3AsH_2, b.p. 2 °C), or dimethylarsine ($(CH_3)_2AsH$, b.p. 36 °C); TMAO is also accessible by this method giving the volatile trimethylarsine ($(CH_3)_3As$, b.p. 52 °C), which is clearly not a hydride. A previous general term for this technique was "vapour generation" which avoids the confusion between hydride and arsine, as well as easily accommodating volatile derivatives of other elements formed in this way.

Cold trapping or cryogenic trapping (CT)

Used in conjunction with hydride generation to trap the generated volatile arsines prior to their introduction to the detector.

Hydride-active

A term applied to chemical species (in our case arsenic species) that produce volatile analytes using the hydride generation method.

5 Naming the various arsenic species, and some suggestions for uniformity

In Fig. 1, we provide chemical structures together with names, abbreviations and/or structure numbers for the arsenic species of relevance to this review. The nomenclature and abbreviations for arsenic species is a messy area with only moderate agreement on the terms used by different research groups. This can lead to confusion and possibly impedes literature searches based on key words. Often, there are no clear reasons to choose one set of terms over another, and no single system adequately describes all compounds. Nevertheless, we propose the use of the terms shown in Fig. 1, and briefly describe our rationale for these assignments.

Most of the arsenic species exist in environmental or biological samples in ionic form (arsenous acid, $As(OH)_3$ is an exception), and are analysed accordingly, usually by ion exchange chromatography or ion pairing chromatography. The majority of these species are analysed as anions, and thus in this review we refer to them as such. For simplicity, the structures have been drawn in their most deprotonated form (*e.g.* AsO_4^{3-} rather than $HAsO_4^{2-}$ or $H_2AsO_4^-$).

This approach has been generally adopted by most researchers for inorganic arsenic species where the abbreviations in common use are As(III) for arsenite and As(V) for arsenate. Similarly, we suggest that the abbreviations MA and DMA be used for the simple methylated species methylarsonate and dimethylarsinate rather than for their respective acids. We note that the fairly widespread use of MMA or MMAA (monomethylarsonic acid) has no strong basis since in chemical nomenclature "mono" would be redundant. For the reduced methylated arsenicals, we favour the terms MA(III) and DMA(III) when referring to methylarsonite and dimethylarsinite, respectively. Readers may wish to consult IUPAC *Nomenclature of Inorganic Chemistry* (1990) recommendations I-4.4.1 and I-5.5.2.2 for further information on the conventions for indicating oxidation state. We note that strict adherence to these rules, however, would result in the use of arsenite(III) and arsenate(V) rather than As(III) and As(V).

We prefer the use of AB and AC, for arsenobetaine and arsenocholine, respectively, over the often used alternatives AsB and AsC which rather untidily mix a chemical symbol with a word abbreviation. The name arsenobetaine, the arsenic analogue of (glycine) betaine [$(CH_3)_3N^+CH_2COO^-$], is so descriptive that its chemical name trimethylarsonioacetate is rarely used. This does not apply, however, to a second arsenic betaine, trimethylarsoniopropionate, and this lack of a trivial name has lead to the use of two abbreviations: one, AB2, denotes its arsenic betaine structure and number of methylenes, and the other, TMAP, simply abbreviates its chemical name. Although the former abbreviation may be more descriptive, our feeling is that the latter, TMAP, will age better and accordingly we advocate its use.

The use of TMAO for trimethylarsine oxide appears to present no discord among arsenic analysts, and TETRA for tetramethylarsonium ion is also well accepted. Some of the newer simple compounds also appear to have staked claims to a consistent abbreviation. Thus dimethylarsinoylethanol is usually referred to as DMAE, and dimethylarsinoylacetate as DMAA.

Reference to the more complicated arsenicals should be accompanied by a named or numbered chemical structure because it is otherwise too difficult to convey the structure in a clear unambiguous manner. This is certainly the case for the arsenosugars, although the early attempts in the literature to do this had a mildly amusing consequence. We refer to the paper by Shibata and Morita[16] which described the analysis of 15 arsenicals including six arsenosugars. To each arsenical, the authors assigned a number (they use Roman instead of Arabic numerals, but otherwise committed no sin), and thereafter in their article referred to the compounds by their respective numbers. But, almost inexplicably, other authors in subsequent articles used the exact numbers assigned to the arsenosugars by Shibata and Morita, often without providing accompanying structures, as if the number by itself somehow described the compound!

The advantage of using an abbreviated term, rather than just a structure number, in the text or on a chromatogram is that it can quickly convey information about the compound without constant reference to a structure diagram. For this reason, we suggest that the four common arsenosugars be assigned short names to convey their main distinguishing structural feature, in addition to having their structures reproduced in the scientific article. Thus, we suggest that arsenosugars **1–4** (Fig. 1) be termed glycerol sugar, phosphate sugar, sulfonate sugar, and sulfate sugar, respectively. We should not forget, however, the chemical inadequacies of such terms, and accordingly, use them carefully.

6 A very brief account of arsenic speciation research in the 20th century

The need for arsenic speciation analysis was apparent even 100 years ago following the discovery by French researchers of high arsenic concentrations in foods of marine origin (readers may consult Francesconi and Edmonds[17] for a fuller account of this period). This so-called seafood arsenic was considered to be in an organic form (and non-toxic), although there were no analytical techniques to test this assumption. Methods were developed for measuring inorganic species As(III) and As(V), and these early techniques were applied to the analysis of seawater and other natural water samples. The first real advance in arsenic speciation analysis came in 1973 with the application of the arsine (hydride) generation technique by Braman and Foreback[2] for the separate determination of inorganic arsenic [(As(III) and As(V)] and simple methylated arsenic species (MA and DMA), which allowed for the first time the determination of these important arsenicals at environmentally relevant concentrations. The method was widely used in studies on cycling of arsenic in natural waters, and for the determination of arsenic metabolites in urine where it provided a wealth of valuable data on methylation capacity for different animal species and for human populations. In most cases, the generated arsines were trapped cryogenically, and their separation was effected mainly by differences in volatility; AAS was often used as the arsenic selective detector.

It soon became apparent, however, that the hydride generation method was not suitable for the determination of the arsenic species found in organisms, and progress in this area took the slow and labour intensive route of the natural products chemist.[18] Attempts to couple HPLC with optical spectroscopic methods such as AAS and AES met with limited success; although chromatographic systems had been developed to separate many of the arsenic

species, the detector sensitivity was too poor to be of use for most biological samples. The situation changed dramatically from the mid-1980s onwards when ICPMS instruments became commercially available and were soon after used in combination with liquid chromatographic systems. The ensuing technique, HPLC-ICPMS, provided good separation of arsenic compounds together with

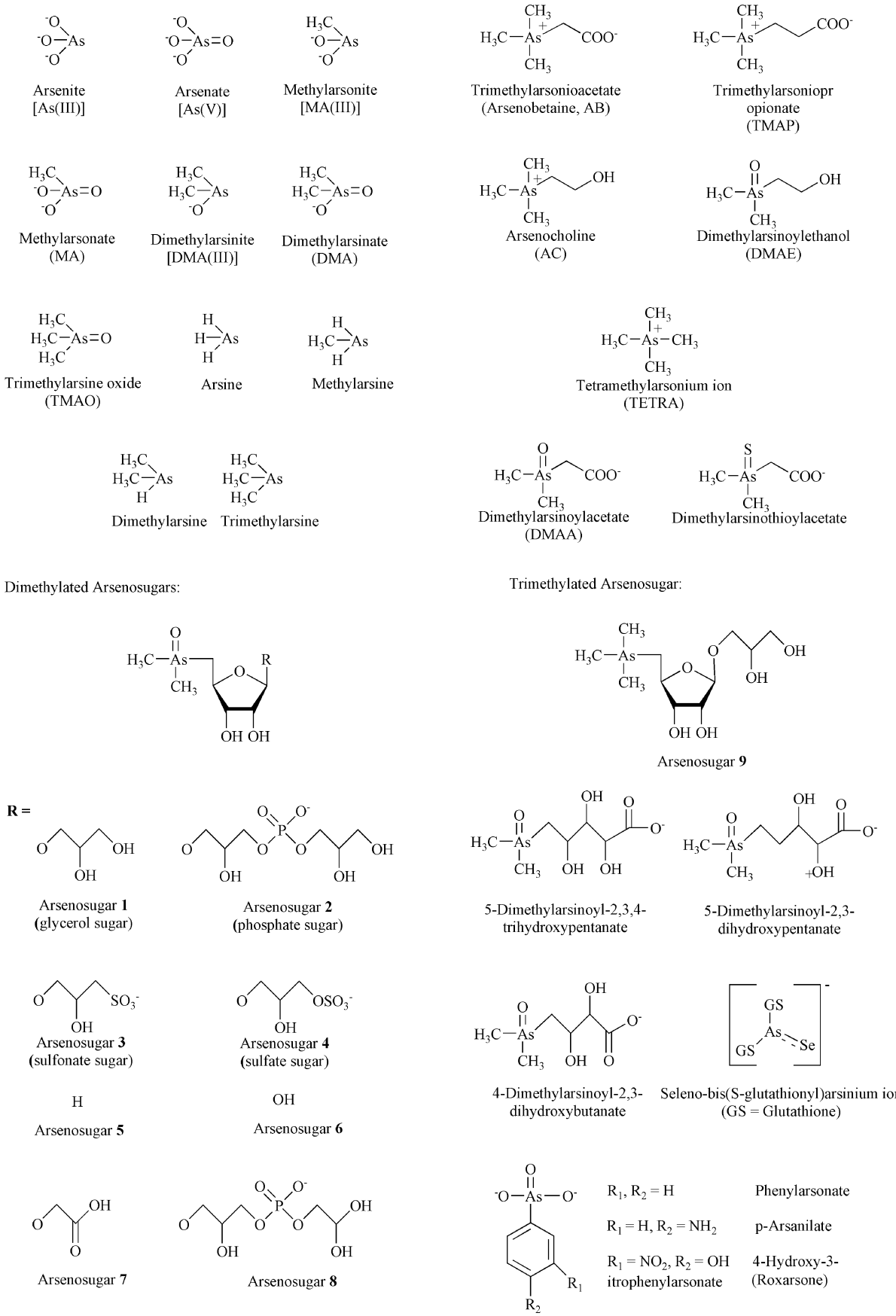

Fig. 1 Chemical structures with names, abbreviations and/or structure numbers for arsenic species of relevance to this review.

excellent detector sensitivity thereby allowing investigation of natural samples with minimal sample preparation. Today, HPLC-ICPMS is the mainstay of arsenic speciation analysis.

Techniques for determining arsenic species based on optical spectroscopic detection (AAS, AES, AFS) also underwent developments through the 1980s and 1990s. In particular, the range of compounds accessible to the methods was increased by incorporating a decomposition step after the separation step. This converted the various arsenicals to a common product, As(v), which was then converted to AsH_3 by the hydride generation method and detected in the normal way. The best of these methods was AFS in combination with hydride generation because it provided low detection limits almost matching those of conventional ICPMS.

A new player, namely molecular mass spectrometry, also contributed to arsenic speciation analysis in the late 1990s. The early work of Sui *et al.*[19] applying electrospray ionisation mass spectrometry to the identification of organoarsenic compounds was, surprisingly, not readily taken up by other researchers. Following a seminal paper by Corr and Larsen[20] in 1996, however, the potential of the method was realised and molecular mass spectrometric techniques are becoming increasingly important in arsenic speciation analysis.

7 The situation at year 2000

We provide here a brief synopsis of the methods often used in arsenic speciation analysis. It is aimed at the non-specialist who wants a quick overview of the available techniques, before reading about the developments since 2000.

Spectrophotometric techniques

Simple inexpensive methods based on a colour reaction selectively involving As(III) or As(v); has some applications for natural water samples.

Chemical/physical separation (fractionation)

Methods based on the selective separation of particular arsenic species prior to determination as arsenic; for example conversion of arsenite to $AsCl_3$ (reasonably volatile, non-polar compound) which is then separated from organoarsenicals by distillation or by solvent partitioning.

HPLC-AES

Sometimes used for investigations into chromatography of standard arsenic compounds, but the detector is insufficiently sensitive to deal with real samples.

HG-(CT)GC-AAS and HG-(CT)GC-AFS

Inexpensive techniques applicable only to hydride-active arsenicals; suitable for water and urine samples because these contain mainly As(III), As(v), MA and DMA which are all hydride-active; the arsines produced are usually trapped cryogenically (liquid nitrogen) in a tube containing solid GC support, which is then gradually heated whereby the trapped arsines are sequentially volatilised and introduced to the AAS or AFS detector.

HPLC-HG-AAS and HPLC-HG-AFS

Suitable only for hydride active arsenicals, and hence restricted mainly to water samples and urine samples; with AFS as detector the method has detection limits almost comparable with those obtained from HPLC-ICPMS.

HPLC-decomposition-HG-AAS and HPLC-decomposition-HG-AFS

The incorporation of a decomposition step before the HG step extends the range of accessible compounds; the arsenicals are separated by HPLC in the usual way, then passed through a reaction coil and decomposed by UV/oxidising agents to the hydride active species As(v), which is then reduced to AsH_3 and introduced directly to the detector; the efficiency of the decomposition step varies with the arsenicals, and is strongly matrix dependent.

HPLC-ICPMS

Robust and sensitive technique; suitable for aqueous samples and for aqueous extracts of environmental and biological samples; able to detect all arsenic species with essentially uniform response which greatly facilitates quantification of the various species; spectral interferences (*e.g.* $^{40}Ar^{35}Cl$) can occur but these can be readily overcome by chromatography, or by use of reaction/collision cell technology or high resolution mass analysers; the technique is generally restricted to mobile phases with low organic content and hence has not yet been applied to the determination of non-polar arsenic species (*e.g.* arsenolipids); assignment of arsenicals is by chromatographic comparison with standards (retention time matching), and thus the method depends on the availability of standard compounds; provides no structural information and hence is greatly restricted in its ability to identify novel arsenic compounds.

HPLC-HG-ICPMS

Provides the advantages mentioned above for HPLC-ICPMS with improved detection limits for hydride-active arsenicals; mainly used for the analysis of water or urine samples which contain mostly hydride-active arsenicals.

GC-MS

Provides excellent separation and detection for volatile arsenicals but applications are relatively few, however, because most naturally-occurring arsenic species are non-volatile; possible future applications for determining volatile derivatives of arsenicals.

HPLC-ESMS and HPLC-ESMS-MS

Increasingly being used to confirm identification of arsenic species, and to provide structural information on novel arsenic compounds; can suffer from large matrix effects and quantification is difficult.

CE-UV

Used to develop conditions for separating arsenicals but lacks the sensitivity and selectivity to deal with real samples.

CE-MS

Provides selectivity and improved sensitivity compared with UV detection for arsenic species, but poor detection limits (resulting from low sample volumes) currently preclude the application of the method to real samples.

X-ray spectroscopic methods

Used to look at arsenic species by recording *in situ* primary data about the chemical environment of the arsenic atom; to a certain extent this information is independent of other forms of arsenic present in the sample, and hence species information can be obtained without the need to separate the species; can handle solid samples and hence the methods are not restricted to extractable arsenic species; a major technique for examining sediments and soils, and is increasingly being applied to those biological samples containing sufficiently high arsenic concentrations.

8 Compilation of papers reporting arsenic speciation analysis, 2000 to 2003

More than 400 research articles have been published from 2000 to the end of 2003 reporting the development or application of arsenic speciation analysis — good news for the journals perhaps, but it is a heavy reading load for the arsenic scientist. Table 2 provides a

Table 2 Research articles in the field of arsenic speciation analysis

	Method development	Natural waters	Marine organisms	Terrestrial and freshwater organisms	Soil/sediment/ minerals and mineral wastes	Sewage/ wastewater	Drinking water	Food and related items	Reference materials	Human urine, blood, cells and tissues	Biotrans-formation/ conversion studies	Other
Spectrophotometry	Refs. 21–27	Refs. 21,24,28					Refs. 21–24			Ref. 23		
Separation with off-line detection (including chemical/physical separation/fractionation)	Refs. 29–43	Refs. 29,43–49	Refs. 50–53		Refs. 42,54–59	Refs. 40,41	Ref. 41	Refs. 60–65		Refs. 66,67	Refs. 68,69	Refs. 38,70
HG-AAS (usually with prior on-line HPLC separation)	Refs. 43,71–79	Refs. 43,49, 73,74,80–85	Refs. 78,87	Ref. 84,86	Refs. 82,88,89			Refs. 64,65, 90–96	Refs. 77,79, 93,97	Refs. 67,87, 98–113	Refs. 92,97, 114–124	
ICPAES (usually with prior on-line LC or HPLC separation)	Refs. 125–129	Ref. 130								Ref. 131	Ref. 132	
HG-AFS (usually with prior on-line HPLC separation)	Refs. 133–139	Refs. 46,133, 134,140–146	Refs. 145, 147–150	Refs. 151–154	Refs. 145,153, 155,156	Ref. 157	Ref. 146	Refs. 92–94, 158–161	Refs. 79,150, 153,156, 161–163	Refs. 100,134, 137,164–168	Refs. 114,139, 169–178	Refs. 145, 179,180
HPLC-ICPMS	Refs. 181–193	Refs. 82,187, 191–201	Refs. 112,185, 202–229	Refs. 154,200, 201,230–250	Refs. 42,82, 187,199–201, 233,236,238, 246,251–257	Ref. 258	Refs. 197, 259–262	Refs. 185, 263–279	Refs. 184, 188–190,207, 216,223,225, 280–287	Refs. 189,225, 272,280,282, 288–300	Refs. 201,263, 301–326	Refs. 219, 220,229, 327–335
HG-ICPMS (usually with prior on-line HPLC separation)	Refs. 189, 336–339	Refs. 145,336, 338–341	Ref. 145		Refs. 145,156				Refs. 156,189, 280,283, 342–344	Refs. 189,280,343	Refs. 304,345	Ref. 145
GC-MS (including GC-ICPMS)	Refs. 346–350				Refs. 350,351			Ref. 352		Ref. 346	Refs. 123,319, 353,354	Refs. 355–358
ES MS (usually with prior on-line HPLC separation)	Refs. 127, 359–362		Refs. 147,202, 206,221,222, 363–366	Refs. 239–241, 243,247				Refs. 264,272, 276–279,367	Ref. 286	Refs. 165,291	Refs. 303,308, 311,315,320, 326	Ref. 332
CE-MS (including CE-ICPMS)	Refs. 181, 368–371				Refs. 253,371							
CE/detection other than MS	Ref. 369, 372–380	Ref. 381			Ref. 382				Ref. 378			Ref. 374,380
X-ray methods	Refs. 127, 383,384	Refs. 85,385		Refs. 240, 386–389	Refs. 57,89, 195,384,385, 390–406		Ref. 404		Ref. 407		Refs. 408,409	Refs. 407, 410
Other separation or detection systems	Refs. 380, 411–416	Refs. 416–425			Refs. 57,351, 390,392,418, 426–428	Ref. 425	Refs. 425,429		Refs. 283,413, 430		Refs. 123, 431–434	Refs. 380, 435

compilation of these methods (refs. 21–435) by grouping them in broad categories depending on the instrumentation/technique and the type of sample. The ensuing discussion will deal with just some of the papers listed in Table 2. First, however, it may be of interest to look at some trends in the development and application of the methods as revealed by the Table.

Development methods are those that aim to describe a new method or considerable improvements to an existing technique. Usually, these reports deal with standard compounds and often include some real samples to test the method. CE features rather strongly here, but, when we look across the table, we find very few applications of the technique. Without a dramatic improvement in detection limits for CE methods, which could lead to its application to real samples, one might expect in the future to see less research interest in the use of CE for determining arsenic species. In contrast, and in relative terms, there was only moderate interest in purely method development for HPLC-ICPMS (because it has already mostly been done), but the method was applied in many studies across all sample types. In fact, HPLC-ICPMS was used in about 40% of the reported investigations. The only other technique that had application for all sample types was HG-AFS, usually with prior on-line HPLC separation, which was used in about 13% of all studies.

X-ray spectroscopic methods dominated solid sample analyses. This is not surprising since all the coupled techniques require the arsenic species to be in solution, and suitable extraction procedures have not yet been developed for sediments and soils. Indeed sample extraction is becoming one of the key issues in arsenic speciation analysis, and we begin the descriptive part of this review with some recent work in the area.

9 Sample extraction

A summary of research papers focusing on extraction methods for arsenic species is presented in Table 3. Before discussing some of that research, however, we will briefly discuss some common misconceptions about arsenic species which may be restricting work in this area. Until quite recently most researchers used methanol/water (or methanol) as the extraction solvent, and refer to the early paper of Shibata and Morita[16] as if it provides some sound rationale for this choice. In fact, no systematic study on extraction was carried out in those early days. Almost certainly, methanol was used because it extracts fewer non-arsenical compounds and it is easy to remove by evaporation. These factors are important when arsenicals are to be extracted and purified using a classical natural products chemistry approach (involving kilograms of material) as adopted in the early work on the isolation and identification of arsenic compounds.[18] Methanol, however, is a poor solvent for extracting inorganic arsenicals, as first reported by Edmonds *et al.*,[436] and hence is not suitable for samples containing such species. This raises two related points. First, a general misconception is that all organoarsenicals will prefer methanol to water as an extraction solvent because they are organic! But, with one exception, all the naturally-occurring arsenic species identified so far are polar and very water-soluble (some are hygroscopic) and they would mostly favour water over methanol as an extraction solvent. This is particularly true for the arsenosugars, some of which are very polar. For these reasons, the best general solvent for extracting arsenic species is probably water, provided it can penetrate the sample matrix. Second, no serious attempt has been made to extract non-polar arsenicals (arsenolipids). Even reported methods using methanol, which might extract some non-polar arsenic, include a step to remove the methanol and redissolve the residue in water prior to analysis. Non-polar arsenicals would be "lost" at this stage.

9.1 Solvents

The goal of the studies listed in Table 3 is complete extraction of all arsenicals with no changes to the species. In comparison with the analysis of other elemental species (*e.g.* organotin compounds), the arsenic speciation analyst had a fairly easy entry point because most of the early samples were from marine animals and rich in arsenobetaine. This small stable molecule is soluble in both water and methanol, and when these solvents or mixtures thereof, were used, extraction efficiencies $>90\%$ were commonly obtained. Marine algae presented a slighter tougher assignment, and very often $<80\%$ of the total arsenic was extracted. This has lead researchers to try different conditions in order to increase the extraction yields. The (unwritten) assumption was that all arsenic species in all samples should be extractable under one set of conditions.

The futility of such an approach can be seen from the study of Tukai *et al.*,[205] who examined extraction efficiencies for three types of marine algae. Using a chemometrics approach, they found that optimal methanol percentages varied from 56% to 78% depending on the type of alga. Despite the fact that the statistical validity of this apparent difference was not established, the data raise the question of the usefulness of carefully determining and describing optimal extraction conditions for a single sample, as is often reported. Presumably, the optimal conditions may change even for different samples of the same organism.

As the focus in the 1990s turned from marine to terrestrial organisms, it became apparent that the water/methanol based extraction procedures were often extracting only a small portion of the total arsenic. The use of water alone produced considerably better results, and this was the solvent of choice for examining the high arsenic burden in a fern species (up to 8000 mg kg^{-1}) with extraction efficiencies generally better than 60%.[233] Interestingly, however, for peach leaves,[151] a methanol/water ratio of 3:1 was purported to be better than water alone (and methanol/water 1:1) for extracting arsenic [mainly As(v)], although the efficiency reduced sharply at a methanol/water ratio of 4:l. Similar results were obtained with water/methanol mixtures for a freeze-dried apple sample, this time with statistical data suggesting that some of the observed small differences may have been significant.[265] Un-

Table 3 Investigations focusing on the extraction of arsenic species

	Extraction process					
Extraction solution	Shaking/mixing	Sonication	MW-assisted heating	Sub/ supercritical fluid	ASE	Soxhlet
Water	Refs. 151,153,163, 223,250,437	Refs. 153,265, 287,437	Ref. 153,437	Ref. 437	Refs. 250,264,266, 269,284	Ref. 437
Methanol		Ref. 344	Ref. 344		Ref. 266,269	Refs. 344,437
Methanol/Water Mixtures	Refs. 151,153, 223,250,437	Refs. 153,265,266, 274,287,344,437	Refs. 148,153, 205,285,344,437		Refs. 250,264, 266,269,284	Ref. 344
Ionic Extractants	Refs. 151,223, 266,437	Refs. 153,265,274,437	Refs. 153,162,163,437			
Enzymes	Refs. 265,266	Ref. 274				
Other		Ref. 274	Ref. 342	Ref. 437		

fortunately, there is no consistent pattern in the data that might allow a plausible explanation, not one based on solubility of the arsenic species at least. Perhaps a particular methanol ratio enabled the solvent to better penetrate the sample matrix. Furthermore, there are no data reported on the actual arsenic species extracted from the freeze-dried apple sample under the different sets of conditions, and this omission precludes a meaningful discussion of the data. The apple samples were also extracted in combination with enzyme treatment, and arsenic species were determined on that occasion. One concern in terms of the general application of the method was that the extraction efficiency varied over a wide range (80.6–104%) depending on the type of freeze-dried apple sample.

In contrast to the above studies, Milstein *et al.*[274] reported that varying the ratio of methanol/water had no influence over the efficiency of extracting arsenicals from food composite samples (arsenobetaine was the major arsenical present). The extraction efficiencies, however, were generally low (about 60%). Refreshingly, the authors also provided mass balance data; the values were generally about 80% indicating some unaccountable losses.

Some terrestrial plants, however, do not readily give up their arsenic to water or mixtures of water/methanol. For example, extraction of rice with water/methanol mixtures removed only 24–36% of the arsenic in one study,[266] and 7–18% in another.[263] Consequently, both these studies, performed by different research groups, investigated the use of aqueous trifluoroacetic acid and reported greatly improved extraction efficiencies (> 80%). Bohari *et al.*[153] also reported poor extraction of arsenic from plant material with water or water/methanol, but extraction efficiency was improved by using an aqueous phosphoric acid solution which was thought to assist breakage of native As–S bonds. Quite different results, however, were obtained by Pizarro *et al.*[268,287] who reported that a single extraction of rice with water/methanol removed 62–82% of the arsenic. A second extraction step was claimed to remove more arsenic, but inspection of their experimental protocol suggests that this was just a carry-over effect of arsenic present interstitially in the pellet obtained from the first extraction. The consequences of such an extraction protocol have been discussed elsewhere.[438]

Most studies investigating effects of solvents on extraction efficiency deal with dried samples, and, because different results are obtained from the various solvent compositions (usually water/methanol mixtures), the optimal conditions found for a dry sample may not apply to a fresh sample which can contain up to 80–90% water by mass. There has been little work focused on this topic. In view of the increasing importance of arsenic speciation analysis in food items, further work in this area appears warranted.

9.2 Extraction systems

In addition to the effect of solvents, the effect of different extraction systems has also been investigated (Table 3). The main methods are mixing/shaking, sonication, and pressurised extraction systems. The last-named can be achieved in commercial digestion products employing microwave-assisted heating in closed chambers, or in a dedicated instrument which goes under the trade name of accelerated solvent extraction (ASE). Gallagher *et al.*[264,284] reported results using ASE from two separate studies. For the ASE system, the sample must be dispersed in an inert medium, and when homogeneous dispersion was not achieved a large reduction in extraction efficiency was observed.[284] Additionally, arsenicals are retained by some of the agents used as dispersion medium. Although these problems can be minimised, and Gallagher *et al.*[284] adopt a fairly optimistic view on the matter, they will certainly limit the applications for ASE. The technique appears to have few advantages over much simpler methods (*e.g.* sonication) and we do not envisage ASE becoming a routine method for extraction of arsenic species.

There have been only few reports on the systematic comparison of various extraction conditions. Brisbin and Caruso[437] compared the extraction efficiency of arsenic and some other analytes from lobster tissue (TORT-2) using soxhlet, mixing, sonication, microwave-assisted heating, and super- and sub-critical fluid extraction with carbon dioxide or water. The stated ultimate goal of this work was to develop mild techniques to quantitatively extract arsenic species from samples without altering their chemical form. Although speciation analysis was not actually performed on the extracts, and hence changes to species could not be assessed, the study produced some interesting results in terms of extraction yields. With the exception of supercritical carbon dioxide extraction, all procedures showed good recoveries of arsenic, although microwave-assisted extraction was favoured largely for practical reasons.

9.3 Extraction of soils and sediments

Mild methods, such as those using methanol/water, extract only a small percentage (typically < 5%) of the arsenic in soil and sediment samples. Accordingly, methods for soils and sediments and other abiotic samples are often based on those used in classical fractionation studies, ie using aqueous solutions of varying ionic strengths/pH/redox potential to release arsenic bound to various mineral phases in the samples. Such methods come under the term fractionation techniques,[15] and they will not be expanded on in this review. As an example, however, we will mention the study of Montperrus *et al.*[163] These researchers compare extraction of sediments, soil and sewage sludge with four extractants: water; 0.1 M hydroxylammonium hydrochloride; 0.2 M ammonium oxalate; and 0.3 M orthophosphoric acid. Water extracted little arsenic from sludge (9.7% of total), and only traces (< 1%) from soil and sediments. The other extractants performed much better (10–94% efficiency) but there were large differences between soil and sediment or sludge. Presumably different types of soil or sediment might also exhibit different extraction efficiencies. This sort of information is used to interpret fractionation data for sediments/soils in the classical way because it reflects how strongly the arsenic is bound. So attempts by the speciation analyst to find conditions that will remove all the arsenic may not be of much use to the soil scientist. Such an extraction would reveal that arsenic is present as As(III) and/or As(V), which the soil scientist knows already, and the finer shades of information such as how it is bound to the mineral phases would be lost.

9.4 Future work in sample extraction

The way ahead in the difficult area of sample extraction is not clear. We believe, however, that the continued piecemeal approach adopted by researchers so far will reap few benefits. Possibly, a thorough study of extraction of a particular group of samples (*e.g.* a type of seafood or terrestrial plants) under a variety of conditions may yield data to reasonably define the problem. Probably, however, the results from such a study will simply show that no single extraction procedure is suitable for all samples and all arsenic species — something most researchers know already. We are not saying that the field of sample extraction is unimportant. Rather, we believe that the sample extraction procedure should be tailored to the particular application and goals of the study, and these will vary with each study depending on a range of factors. In other words, discovering the most efficient extraction procedure for a certain group of samples is a necessary *part* of the broader study producing information about arsenic species.

We end this section on sample extraction with a comment on what is not extracted! While efforts are made to increase the extraction yield of polar arsenicals, little work has been done on the arsenic that is left behind, so-called protein bound arsenic or lipid arsenic. In the period covered by this review there was hardly a single report that focused on this arsenic fraction. More research effort is required in this neglected but important area of arsenic speciation analysis.

10 Sample clean-up procedures

The area of sample clean-up has generally been avoided by arsenic speciation analysts, possibly because of the (justified) fear that any clean-up step before the actual measurement may result in selective loss, or concentration, of some species, and hence produce a speciation pattern different from that in the original sample. The clean-up procedures generally involve chromatography (we include here solid phase microextraction). We also consider as clean-up procedures those methods variously referred to in the recent literature as bi-, tri- or multidimensional chromatography depending on the number of individual steps. These terms can be misleading implying that the chromatography is part of a coupled analytical system. In reality, the early chromatographic steps are performed off-line and simply form part of the clean-up procedure so that speciation analysis can be performed in a less complex matrix. Such clean-up steps are necessary for most sample matrices especially when ESMS is to be used, and their application has been impressively shown in the recent study describing several novel arsenicals in clam kidney.[221] We note here, however, that clean-up procedures involving several chromatographic steps in which "peak fractions" are carried through to the next stage cannot produce reliable quantitative data, and the precision and accuracy of quantitative data reported using such methods should be questioned.

The study of Yalçin and Le[134] provides the clearest application of sample clean-up and its potential use in quantitative analyses. With the aim of developing a simple method for determining low levels of As(III) and As(V) in water, these authors tested a range of solid phase extraction cartridges. Cartridges containing alumina completely retained As(III) and As(V) in addition to MA and DMA. Subsequent elution of As(III) and As(V), however, could only be achieved with hydrofluoric acid and this corrosive agent had to be removed before analysis by HPLC-HG-AFS. Nevertheless, application of the technique to a urine sample resulted in a 20-fold increase in concentration of the arsenic species, enabling previously undetectable arsenicals As(III) and MA to be analysed.

We see an increasing need and interest in clean-up procedures targeting particular arsenic species. This approach can be viewed as the antithesis of the complete extraction approach discussed above. The species targeted should be those arsenicals with known or suspected toxicity, or those which are currently ignored by common extraction methods. We provide three examples that warrant future study. First, techniques are needed to concentrate inorganic arsenic species from extracts of food samples. These species have clear toxic concerns but their quantification in foods, particularly seafoods, can be difficult because of high concentrations of other (non-toxic) arsenicals. Second, newly reported trivalent methylated arsenicals in urine samples appear to have considerable toxicological significance.[6] Many of the reports of their presence, however, are based on tenuous data close to the detection limit. Sample preparation methods to selectively concentrate these arsenicals would greatly facilitate their analysis. Third, methods are needed to concentrate lipid-soluble arsenicals from extracts. The toxicological properties and biochemical significance of these compounds is currently unknown; their analysis will be difficult but might be made accessible with an efficient clean-up procedure.

11 Stability of arsenic species: collection, storage, extraction and chromatography

Data from speciation analysis recorded in the laboratory should ideally accurately represent the situation at the point of sampling. Sampling problems such as loss of analyte or contamination have long plagued trace element analyses, but are today reasonably well understood and controlled. The situation with arsenic speciation analysis is much more complex, and for many types of samples/species we are still a long way from adequately addressing the problems. In the following, we consider only those studies that focused on understanding possible changes to the species between collection and end analysis (Table 4); stability of arsenicals during digestion (or cooking) are not considered here.

11.1 Arsenic transformations in water samples

We begin with the simplest situation — a study examining inorganic arsenic transformations. Bednar *et al.*[199] investigated the effects of different additives and light on the stability of As(III) and As(V) in pure water, and then in ground water and mine drainage samples. When no additives were used, As(V) and As(III) remained unchanged for at least 24 h under both light or dark conditions; after four days (the next sampling point), As(V) had been totally converted to As(III). The authors thought that this conversion was microbially mediated. Addition of EDTA to the experimental solutions preserved the identity of As(III) and As(V) for up to five days in light or dark conditions. Addition of H_2SO_4 was almost as effective as EDTA; HNO_3 was similarly effective but only when light was excluded (reported to be because nitrate is photochemically reduced to nitrite with concomitant oxidation of As(III)). Hydrochloric acid, on the other hand, was reported as being ineffective at preventing the oxidation of As(III) — a rather surprising result. Unfortunately, the details of the experimental conditions used for the acidic storage were not provided, and it is difficult to formulate explanations for these conversions based on the chemistries and relative stabilities of As(III)/As(V).

Interconversion of As(III) and As(V) in water samples was also noted by Le *et al.*[146] and reported to be matrix dependent. To overcome this problem they extended their earlier work on solid phase extraction[134] to include on-site sampling of water. In this way As(III) and As(V) were separated from each other by passing a portion (typically 15 ml) of the water sample through an anion-exchange cartridge immediately after collection. The separated species could then be quantified at leisure in the laboratory.

A study[157] on the stability of inorganic arsenic and simple methylated arsenic species in waste water samples concluded that As(V), MA, and DMA were stable for at least four months (period of the study) at temperatures of 4 °C, 20 °C and 40 °C and pH values of 1.6 and 7.3. The situation with As(III), however, was quite different: its stability depended on pH, temperature and whether the

Table 4 Investigations focusing on the stability of arsenic species

	Arsenic species			
Sample type	As(III), As(V)	As(III), As(V), MA, DMA	MA(III), DMA(III)	Other arsenicals
Standards		Ref. 289,322	Ref. 164	Ref. 148,322
Natural water	Refs. 199,419	Ref. 193		
Waste water		Ref. 157		
Gastric juice				Ref. 320
Basic aqueous solution				Ref. 326
Urine		Ref. 289,322	Ref. 164	Ref. 289,322
Biota		Refs. 250,287,322		Refs. 96,148,250,287,322
Sediments/soil		Refs. 156,162,287,322		Ref. 322
Gases				Ref. 348

sample was raw or treated wastewater. In treated wastewater, As(III) was stable at pH 7.3 for the four month study period at 40 °C. At pH 1.6, however, As(III) was almost completely transformed into As(V) within two months at 40 °C. Unfortunately, the acidic reagent used to lower the pH of the wastewater was not stated, and hence the results cannot be meaningfully compared with those of Bednar *et al.*[199] who reported that As(III) oxidation was affected by acid type. The raw waste water was an even more hostile environment for As(III), and at pH 7.6 it was completely converted to As(V) within 50 days, even at 4 °C (low pH storage was not reported for raw waste water). Interestingly, the authors report that there was no correlation between organic content of the waste waters and As(III) stability. The biological and chemical oxygen demand of raw wastewater was, as expected, very much greater than that for the treated sample.

Roig-Navarro *et al.*[193] found that As(V), MA and DMA in surface waters did not undergo changes after 15 day storage at 4 °C. As(III), however, was converted to As(V) — a process that was strongly influenced by sample matrix. The samples were not acidified; the pH of the natural samples was not reported.

Collectively, the above mentioned studies on water samples indicate that problems of species stability occur mainly with As(III), which under certain conditions is readily oxidised to As(V). The presence of other ions in solution may catalyse the observed conversions. Storage of these samples in the dark at low pH (but not with HCl!) appears a convenient and practical solution to the problem. Probably, there is little more to be gained from further studies on the stability of As(III) and As(V) in synthetic solutions or isolated water samples; the interconversion is clearly matrix dependent and any investigation or survey of arsenic species in water would first need to establish the optimal storage conditions for the particular sample matrix and the purposes of the study.

As a final comment on As(III)/As(V) conversions, we convey unpublished observations made in our laboratory. From time to time we have noticed that analytical standards of As(III) and As(V) can rapidly and apparently randomly interconvert. The most striking example of this has recently been seen by our colleague Dr Walter Goessler. Standards of As(III), MA, DMA, and As(V) with concentrations ranging from 10–20 μg As l^{-1} were prepared in water in sealed vials in readiness for HPLC-ICPMS analysis. Within the space of 36 h at room temperature, As(III) in one of the solutions was completely converted to As(V), and the As(V) in another solution had completely converted to As(III)! This single observation should be sufficient to dissuade researchers in future from merely reporting changes in inorganic arsenic species with storage. On the other hand, a fundamental study of the conversion processes involved would be most interesting. But, we suspect, dauntingly difficult.

11.2 Stability of arsenic species in extractant solutions

The stability of arsenicals following extraction has also been investigated and serves to illustrate the complexity of the problem in regard to matrix effects. Vergara Gallardo *et al.*[162] examined orthophosphoric acid extraction of sludge, sediment and soil for determination of As(III), As(V), MA and DMA. For As(V), MA, and DMA recoveries were good and there was no transformation of species. For As(III), however, a 20–60% conversion to As(V) was observed for sediment and soil extracts, while As(III) in sludge was stable. The study unfortunately stopped short of investigating the factors behind such large matrix-related differences in behaviour.

Vilano and Rubio[148] observed that MA, DMA and arsenobetaine were stable to an extraction process with methanol/water (1:1) using microwave-assisted heating followed by drying under an infrared lamp (the reason for drying in this unusual manner was not stated). The test, however, was carried out on pure (standard) compounds and whether the results also hold for a sample matrix remains unproven. The authors also tested extracts of oyster tissue maintained in solution at 4 °C for 72 h and noted no changes in native arsenic species. Such results, of course, are highly dependent on microbe populations in these samples, and completely different results might be obtained on another occasion. Arsenic species present in oyster (mainly arsenobetaine with small quantities of arsenosugars and DMA) were stable when dried (this time in an electric oven) at 40 °C for 48 h, but some decomposition was recorded at 70 °C after 24 h.

Gamble *et al.*[326] investigated the stability of four individual arsenosugars on treatment with tetramethylammonium hydroxide under various conditions, or with sodium hydroxide. The work is very similar to the earlier study[320] with gastric juices by this same group. The authors state that tetramethylammonium hydroxide was investigated because it efficiently extracts arsenicals from "problematic seafoods". Unfortunately, no data were presented to support this statement (a 2001 reference given to a poster abstract does not contain relevant information), and thus one cannot evaluate the relative importance of extraction efficiency and stability. Despite these shortcomings, the paper does contain some interesting data, in particular, the observation that the sulfate sugar (arsenosugar **4**) was considerably more labile than the other three arsenosugars, and under certain conditions was almost completely degraded to DMA.

The study of Pantsar-Kallio and Korpela[348] is of interest because they looked at the less studied topic of gaseous arsenicals. They found that trimethylarsine in water was unstable and was converted within minutes to uncharacterised water-soluble species. In air, however, trimethylarsine was reasonably stable and only 30% was oxidised to TMAO after 9 days. In contrast, arsine (AsH_3) in air was unstable and was completely oxidised to arsenic oxides after 120 h. Interesting results regarding the chemical formation of arsines following treatment with tetrahydroborate were also reported by these authors. At pH 1–2, most of the DMA was converted to the corresponding dimethylarsine. At pH < 1, however, arsine, methylarsine and trimethylarsine were formed, and under highly acidic conditions (pH < −0.3) all DMA was converted to trimethylarsine. The authors' claim that "all" DMA was converted to TMA appears unsound because, since there was no other source of methyl groups, the reaction probably proceeds by transmethylation and hence quantitative conversion to TMA is not possible. No data were presented to support this part of the study. Arsine and methylarsine were stable under these conditions. This study is likely to have relevance to the broader area of hydride generation methods used in arsenic speciation analysis.

Although maintaining the correct *in situ* inorganic arsenic speciation will always be useful for geochemical studies, this requirement has less relevance for human health studies. Here, the arsenic species present at the time of ingestion, rather than collection, is of greater significance. But even then, for the inorganic forms of arsenic ingested in water at least, the distinction may not be too important because they interconvert in the body.[439] Of considerable importance, however, is the species of arsenic being excreted because this provides information on the biotransformation processes taking place in the body. Accordingly, stability of arsenic species in urine is a major issue. An earlier study[440] reported that As(III), As(V), MA and DMA in urine were stable for up to two months when stored at 4 °C. Renewed interest in urine metabolites has followed the identification of two novel arsenic species MA(III) and DMA(III) which are thought to be key, and perhaps toxic, intermediates in the biotransformation of ingested inorganic arsenic.[6] We will now discuss work on the stability and storage of MA(III) and DMA(III) in some detail.

11.3 Stability of urinary metabolites MA(III) and DMA(III)

Gong *et al.*[164] investigated the stability of MA(III) and DMA(III) in deionised water and urine at −20 °C, 4 °C and 25 °C for up to 114 days and monitored the arsenic species by HPLC-HG-AFS. For MA(III) in deionised water (pH 6.0, 25 °C), 15% of the MA(III)

converted to MA after 3 days, and the MA proportion increased with time reaching a maximum value of 80% after about 25 days. As(III) was also detected in the samples after about 20 days, and remained at approximately 4–10% of the total arsenic concentration up to the end of the experiment (114 days). Although the authors offer no explanation, this result is rather unexpected because it implies that MA(III) is demethylated in deionised water. MA is unlikely to be the source of As(III): because MA is a common standard which has been used in arsenic speciation analysis since the early 1970s, one would expect such a conversion to have been previously observed. Gong *et al.*[164] also noted that storage at 4 °C or −20 °C slowed the conversion of MA(III) to MA so that after about 35 days MA constituted about 10% and stayed at that level for the rest of the storage period (114 days). The authors describe this as an equilibrium, in which case the situation at 25 °C storage could also be described as such since the ratio of MA(III) to MA remained constant at about 1:8 from day 20 to day 114. If this were in fact a true equilibrium, then MA might also be expected to equilibrate under the same conditions giving small quantities of MA(III). Such a conversion in deionised water has yet to be reported. When MA(III) was added to urine stored at 25 °C, it was quickly (3 days) converted to MA; after longer storage (4 months), traces of As(III) and DMA were also detected together with MA (> 96%). This interesting result, indicating both demethylation and methylation taking place in the same urine sample has important implications and requires confirmation. MA(III) in urine is also readily converted to MA at 4 °C storage, and even when stored at −20 °C only about 8% of MA(III) remained after 114 days, the rest being converted mainly to MA. Based on the chromatograms presented, As(III) and DMA also appear to be products from storage at 4 °C and −20 °C in that study.[164]

The results from the investigation into DMA(III) stability were even more dramatic.[164] With storage at 25 °C in water, DMA(III) was completely converted to DMA after 10 days, and frozen storage (−20 °C) slowed this conversion only marginally. In the urine matrix at 25 °C, DMA(III) was extremely unstable and completely converted to DMA in just 90 min! The authors state that this result was confirmed with (an unspecified number of) additional urine samples. Frozen storage helped only a little — DMA(III) was completely converted to DMA within just 17 h. The authors note that the lability of DMA(III) in urine matrices may explain why this species is not often detected in human urine samples. Indeed, the question might be asked how can such an unstable species be detected at all! The work carried out so far on urinary metabolites MA(III) and DMA(III) is of great importance; it also contains some inconsistencies that require clarification. We expand on this topic later in the review.

11.4 Stability/lability of arsenosugars in simulated gastric juice

Several studies have looked at the degradation of arsenicals during various digestion procedures, but, as mentioned above, we do not discuss those studies here because their specific goal was to find conditions to completely convert all arsenicals to a common analyte, usually As(V). The study of Gamble *et al.*[320] deals with the fate of arsenosugars during extraction with simulated gastric juices and acidic media, and thus seems to fall somewhere between studies dealing with stability and those dealing with degradation. Gamble *et al.*[320] tested strong chemical extractants on arsenosugars to evaluate the balance between likely increased extraction efficiency and possible unwanted chemical changes to the original arsenicals. They examined the rate of degradation of the four major arsenosugars **1–4** when heated (38 °C or 60 °C) with simulated gastric juices (pH 1.1) or mineral acid (HNO_3 or HCl) solutions purportedly at the same acid equivalents. All four compounds degraded to the same product, the free dimethylarsinoylribose, arsenosugar **6**. The rate of degradation was the same for the four compounds and for the three sets of acidic media tested; at 38 °C this was reported as 1.4% h^{-1} and at 60 °C it was about 12% h^{-1}. These data indicated that the gastric enzyme pepsin was not involved, and the authors provided a mechanism for acid catalysed hydrolysis of the aglycones which would yield a common product from the four arsenosugars. Interestingly, there was no formation of DMA; studies on human and animal metabolism show that DMA is the major metabolite from arsenosugars.[291,309,441] Collectively these studies indicate that the conversions of arsenosugars in the gut are not induced solely by the chemical environment, as discussed by Gamble *et al.*[320]

The study of Gamble *et al.*[320] did not, unfortunately, investigate actual algal samples containing arsenosugars, so we still do not know if the acidic conditions applied to their standard arsenosugars would in fact increase extraction efficiency. Consequently, it is not possible to evaluate the balance between extraction yield and loss of speciation information through degradation. The authors do, however, touch on a related interesting aspect. Presumably, extension of the extraction/heating period would result in complete conversion of mixtures of arsenosugars to the common product, the free dimethylarsinoylribose, arsenosugar **6**. This single product might be more easily identified and quantified than the original mixture of arsenosugars, and its analysis could provide data for "total arsenosugars" in a sample. Although this might be seen as resulting in a loss of speciation information, in terms of toxicological relevance the data may in fact be at least as useful as quantitative analysis of individual arsenosugars.

11.5 Stability of arsenic species during chromatography

It has been generally accepted that arsenic species are stable in water/methanol mixtures, and hence changes do not occur during extraction with these solvents. This assumption, however, has recently been questioned by using solid phase analysis techniques (*e.g.* XANES) to study the arsenic species *in situ*, and comparing the results with those from HPLC-ICPMS of methanol extracts.[240] The data indicated that despite HPLC-ICPMS analysis showing the presence of As(III) (as arsenite) in earthworms, XANES data indicated that the intact tissues contained As bound to S. It was not known whether the transformation was taking place during the extraction procedure, storage, or the chromatography. Possibly, the changes occur during chromatography. Schmöger *et al.*[442] reported that complexes between S-rich tripeptides and arsenic can be disrupted during gel filtration chromatography, an accepted gentle separation procedure, yielding arsenite. Interestingly, the situation with the arsenic accumulating fern *Pteris vittata* appears to be different from that in the earthworm. Although the first report[232] that *P. vittata* contains its arsenic burden mainly as arsenite was based on HPLC-ICPMS, and hence was subject to the same doubts raised by the work of Langdon *et al.*,[240] a separate study[387] with EXAFS has demonstrated that arsenite is indeed present as the major arsenical in the living fern.

In addition to chromatography possibly effecting changes in arsenic species, it may also influence the observed speciation pattern by partially or completely retaining one or more species. For this reason, many papers now contain a statement about the recovery of arsenic from chromatographic columns, *i.e.* the total quantity of arsenic eluting from the column as one or more species expressed as a percentage of the quantity of arsenic actually injected onto the column. This is most helpful information, and should be provided wherever possible.

Quality of arsenic speciation data is critically dependent on good sampling and storage procedures, and more work is required in this important area. It would appear that the results of most value to the arsenic analytical community would come from a thorough systematic study of sample type/arsenic species/storage conditions which may be able to identify some fundamental processes eliciting the observed changes.

12 Advances in separation

The majority of papers published on arsenic speciation analysis in the last four years use some form of separation procedure, with HPLC being by far the most common method. Those publications reporting significant advances or reporting something novel in terms of separation are recorded in Table 5. Although ion-pairing with reversed-phase HPLC has a solid core of adherents, ion-exchange methods have been more widely adopted by researchers (we include here methods termed ion chromatography). In anion-exchange chromatography, the Hamilton PRP-X100 column is most commonly used; mobile phases employing phosphate buffers are usual when the HPLC is coupled to an ICPMS, and volatile buffers such as ammonium carbonate are preferred for ESMS. Dionex anion-exchangers are also commonly used. No single cation-exchange column dominates the user market; good separations are obtained with both resin-based and silica-based columns. The review of Gong *et al.*[8] provides an overview of the various chromatographic conditions used for arsenic speciation analysis.

12.1 Separations with HPLC

Improved separations with HPLC have come with the use of gradient elution, and two good examples have been reported recently. The first by Kohlmeyer *et al.*[188] is not novel since it is based on the method of Londesborough *et al.*,[443] but it does break new ground in terms of the number and types of arsenicals separated. The method employed anion-exchange (Ionpak AS7 from Dionex) with nitric acid eluents (pH 3.4 and 1.8) together with a doubly-charged ion-pairing agent (0.05 mM benzene 1,2-disulfonate). This enabled arsenic anions to be separated in the usual way, and arsenic cations, which combined with benzene 1,2-disulfonate forming negatively-charged ion pairs, were also retarded by the column. Thus the method has the advantage of determining anions and cations together in a single chromatographic run. The authors established the method with just seven standard arsenicals, but when they applied it to several marine samples they reported the separation of up to 17 arsenicals (in an extract of oyster tissue). Assumptions were made to assign these compounds, some of which appear unjustified (see below). Although the long elution time (18 min) may deter some users, the separation reported is good and provides a useful alternative to previously reported methods.

A particularly interesting claim by Kohlmeyer *et al.*[188] was that retention times of the arsenic species were unaffected by the matrices, and they state that this fact was established by spiking experiments. Presumably, such spiking experiments could only be attempted with the seven standard arsenicals available to that research group, and extension of the statement to cover all 17 detected arsenicals remains to be justified.

One other aspect of the results by Kohlmeyer *et al.*[188] is worth noting. In all five marine samples, including two certified reference materials, MA was found as a significant arsenical (up to about 10% of total arsenic species). One of those reference materials (DORM-2) has been analysed by several other groups,[285] none of which has detected MA. It will be interesting to see if other groups will now be able to detect MA in their samples, perhaps after adopting the Kohlmeyer *et al.* method. Alternatively, MA may have been incorrectly assigned by Kohlmeyer *et al.*, and we consider this the more likely explanation of the current discord in the published results.

The "record" of separating 17 arsenicals stood for a surprisingly short time — only months after the Kohlmeyer *et al*. study, Sloth *et al.*[216] reported that they could separate 23 organoarsenicals in one chromatographic run of 25 min using cation-exchange (Ionospher-5C) and aqueous pyridine formate with gradient elution. In stark contrast to the earlier report of Kohlmeyer *et al.*,[188] Sloth and co-workers[216] found that retention times of arsenicals in their system experienced large matrix effects. Repeated injection of the same sample extract (DORM-2) revealed that retention times for arsenicals were very consistent (RSD < 0.5%, n = 8), but large variations were evident when the matrix type or matrix loading was changed. For example, a fourfold increase in sample loading for TORT-2 resulted in the retention time for arsenobetaine being reduced from 10 min to about 7 min. Each arsenical behaved differently with respect to its relative retention time, so that, as discussed by Sloth *et al.*,[216] elution order can change depending on sample type (and concentration). The practical limitations of such effects for the determination of arsenic species in biological samples are evident.

The studies of Kohlmeyer *et al.*[188] and Sloth *et al.*[216] present contrasts in terms of reported matrix effects. It would be interesting to see if these apparent discrepancies are completely explained by the different types of chromatography used in the two studies, and a comparative investigation may reveal some novel fundamental processes. The system of Sloth *et al.*[216] appears to provide the better separation. For example they were able to separate TMAO and TMAP in a DORM-2 extract whereas in the Kohlmeyer *et al.*[188] system these two arsenicals appear to have co-eluted leading to incorrect assignments and quantifications, as previously discussed.[216]

Another method able to determine arsenic anions and cations in the one chromatographic run was reported by Sakai *et al.*[295] They used anion- and cation-exchange columns in series with 4 mM phosphate buffer (pH 2.6) to separate eight arsenicals. The resolution was good, but the long chromatographic run time of 40 min is likely to limit the application of the technique. The same group also used ion exclusion chromatography to effect good separations, but again with a 40 min chromatographic run time. Ion exclusion chromatography was also investigated for the separation of arsenic species by Nakazato *et al.*[189] By varying the pH, the relative influence of ion exclusion and ion exchange mechanisms could be manipulated to effect good resolution of seven common arsenicals. Long chromatographic run times (up to 60 min) were again a disadvantage, but the method offers another separation option which may be suited to some samples and groupings of arsenicals.

Gradient elution employing anion-exchange columns (Dionex AS7, AS14, and AS16) and various eluent combinations was also used to separate the two poultry feed additives *p*-arsanilate and roxarsone from inorganic arsenic, MA and DMA.[328] Excellent resolution was obtained with aqueous NaOH (and column AS16), but, not surprisingly, some on-column oxidation of As(III) to As(V) was noted. The strongly basic conditions, however, did have the advantage of retarding the elution of As(III) (pK_1 9.3) so that it eluted after the solvent front.

A narrow bore reversed-phase HPLC column with ion-pairing was used by Wangkarn and Pergantis[185] to effect a fast separation (about 2 min) of the anionic arsenicals As(III), As(V), MA, and DMA. Similarly, Sun *et al.*[190] used a microbore anion exchange column to separate these same four arsenic species, and applied the method to a urine reference material. The use of micro columns forms part of these authors' aims of reducing sample size and solvent consumption. These factors are likely to become increas-

Table 5 Investigations focusing on the separation of arsenic species

Ion exchange HPLC (including ion chromatography)	Ion exclusion	Reversed phase HPLC	Capillary electrophoresis	Other
Refs. 188,203,207,216,249,251, 253,262,270,282,295,328	Refs. 189,295	Refs. 183,185,249,282	Refs. 253,368,373–376	Refs. 30,41

ingly important, and we expect that more work will be reported in this area. The paper by Wangkarn and Pergantis[185] is also notable for introducing a novel nomenclature, "Aite" and "Aate", for arsenite and arsenate! Thankfully, this nomenclature has not yet been adopted by the arsenic speciation community.

Finally, although the paper of Edmonds[183] is not about chromatography (rather it focuses on the biogenetic origin of organoarsenic compounds), he briefly describes the separation of two diastereoisomeric arsenosugars using reversed phase HPLC (Inertsil ODS-2). The separation is of interest because several arsenosugars have been reported as diastereomeric "pairs", the most common being arsenosugar **3** (the sulfonate sugar) diastereomeric at C-2 of the glycerylsulfonic acid moiety.[18] Possibly, HPLC might also be able to separate these two diastereoisomers. There have been, however, no reports so far of these two compounds separating on HPLC; researchers could perhaps be attentive to a broadened signal for arsenosugar **3** (and other arsenosugars as well) which might be indicative of the presence of two very similar compounds such as diastereoisomers.

12.2 Other separation techniques

Latva *et al.*[30] considered that common speciation analytical methods such as HPLC-ICPMS were expensive and time consuming, and in an effort to develop an alternative method, they used activated charcoal with various metal loadings to selectively absorb particular arsenic species from solutions. The species were then released by addition of alkali or acid and total arsenic determined by graphite furnace AAS. The steps are tedious and the process appears to be very time consuming (we estimate about 4 h per sample). When performed with As(III), As(V), DMA and phenylarsonic acid, each at 1000 μg As l^{-1} recoveries of 100–101% and RSDs of 1.7–3.0% were reported. The method, however, was not tested with real samples, and we do not expect to see too many applications of this procedure in the future.

Interest has been growing in the use of CE separations for arsenic speciation analysis since the development of interfaces suitable for coupling CE to a mass spectrometer. Investigative studies, however, often still use conventional spectrophotometric detection. The separating strength of CE techniques is well illustrated by the work of Sun *et al.*[374] in a study using nine standard arsenic compounds. As mentioned earlier, the problem of poor detection limits continues to preclude the application of CE techniques to the analysis of real samples.

13 Advances in atomic detection

AAS, AES and AFS

Not surprisingly, long-established techniques such as AAS and AES have not undergone any significant improvements in the recent past, at least none that has had an impact on arsenic speciation analysis. AFS coupled to HG for detecting arsenic species was developed in the 1990s, largely through the efforts of a single commercial supplier, and is now a well-established technique. It has great sensitivity for arsenic, almost rivalling that of ICPMS, and because of its low purchase and operating costs, can be an attractive alternative to mass spectrometric techniques. Its major application is for urine and water analysis — samples dominated by arsenicals that readily give volatile arsines.

AFS can also be used for more recalcitrant arsenicals when a pre-HG decomposition step is included. The consistent efficiency of such systems across all matrix types, however, has not yet been established, and the reliability of such techniques must be questioned. We provide one example to illustrate this concern. Sanchez-Rodas *et al.*[147] used a UV photooxidation step followed by HG to determine arsenic compounds in an oyster extract. The main purpose of the study was to identify an unknown arsenical which appeared to be the major arsenical (> 80% of the sum of As species), based on the UV decomposition-HG-AFS chromatograms. The study identified this unknown as arsenosugar **2** (the phosphate sugar), but when quantification was performed this arsenical accounted for only about 30% of the total arsenic in the extract. The authors report that the only other significant signal in the chromatogram probably corresponded to arsenobetaine, and that its response by UV-HG-AFS was strongly depressed by the matrix. Although the authors do not elaborate on this aspect, a clear interpretation from the data presented is that the "missing" arsenic in the extract resulted from a gross underestimation of arsenobetaine (perhaps by up to a factor of five) because of matrix effects. The UV decomposition step in these HG-AFS procedures is critically dependent on the matrix, as well as the arsenic species, and accordingly, when dealing with biological extracts, standard additions should always be used to provide reliable quantitative data.

ICPMS

ICPMS continues to show improvements in sensitivity and stability, and researchers now report detection limits well below 1 μg As l^{-1} for the various arsenic species with new single quadrupole instruments coupled to HPLC. These detection limits can be further improved for some species by incorporating a HG step which has been applied to urine[189] and seawater samples.[339] The addition of a post-column photo-oxidation step to decompose recalcitrant arsenicals before HG has also been reported, and the technique was applied to the analysis of urinary arsenic species.[280] The advantages of this HG-ICPMS system compared with ICPMS detection with direct nebulisation, included increased sensitivity, reduced matrix interferences and additional selectivity because the system can distinguish hydride-active from non-hydride active arsenic species.[280] Increased instrumental complexity and reagent contamination (mainly from $NaBH_4$) were seen as disadvantages of the HG system.

Advances have been made in terms of reducing interferences in ICPMS by use of reaction/collision cell technology. For certain samples, seawater and urine for example, analysis of total arsenic by atomic mass spectrometry suffers from polyatomic interference from $^{40}Ar^{35}Cl^+$ species, and collision cell technology has clear advantages in this area. Even chloride-rich samples, however, do not usually present a problem when chromatography is employed. Hence, collision cell technology provides fewer advantages for arsenic speciation analysis, and its use must be balanced against the likely loss in sensitivity. In this regard, the study of Xie *et al.*[186] was surprising because they claim improved detection limits using the collision cell, but their work was based on detection of five arsenic standards in deionised water or river water (it was not clear which). Their real "test" samples also appear inappropriate because they analysed water samples from an arsenic toxicity test carried out in freshwater which had been spiked with up to 2000 μg As l^{-1}.

14 Advances in molecular detection

In the last few years molecular mass spectrometry has been the biggest contributor to new information and methods in the area of arsenic speciation analysis. The purists may disagree that such techniques fall under the banner of arsenic speciation analysis, because, in contrast to atomic spectrometric techniques, a complete "arsenic picture" can never be provided. And the organic analysts might wonder why the term speciation analysis is necessary at all — they have been doing this sort of work with other elements for many years without using the term. Nevertheless, name quibbling aside, molecular mass spectrometry has proved a powerful tool for particular applications of arsenic speciation analysis, and we now discuss the methods reported in the last four years.

A simple single quadrupole mass analyser has been used for arsenic speciation analysis in several studies examining arsenic metabolism, and for the identification of two novel arsenicals, as recently reviewed.[444] The electrospray ionisation process can be

performed (essentially) simultaneously at low and high energies to produce, respectively, protonated molecular species and bare As^+ ions from the arsenicals. When coupled to HPLC, the technique enables arsenic-containing peaks to be located during a chromatographic run, and also provides molecular mass information for the peak. It has been shown recently,[359] however, that the generation of the bare As^+ ion is greatly reduced when oxygen is present in the nitrogen collision gas used in the CID process. The use of nitrogen with $< 0.1\%$ oxygen was necessary for production of bare As^+ ions; at higher oxygen concentrations the diatomic species AsO^+ was the dominant product ion. The work has indicated that we know relatively little about the processes taking place during electrospray ionisation, particularly at the higher energies. Some fundamental research in this area is likely to lead to an understanding of processes useful for arsenic speciation analysis.

Although the work with single quadrupole mass analysers has produced some interesting results, most molecular mass spectrometric studies of arsenic species utilise tandem MS systems — either tandem MS in space with triple quadrupole or quadrupole/TOF combinations, or tandem MS in time with ion trap instruments (quadrupole or FTICR). Most early work was done with triple quadrupole systems, and it was quickly realised that the electrospray ionisation process is strongly matrix-affected and that samples had to be cleaned-up before injection into the HPLC-MS-MS instruments. Organic analysts, of course, would not be surprised by this, but it took the arsenic speciation analyst some time to accept. What followed were several laboured publications dealing with known arsenicals describing clean-up operations and displaying ever-improving MS spectra. The strength of tandem MS techniques, however, lies in their ability to identify novel arsenic compounds, and this was subsequently demonstrated in studies using triple quadrupole tandem MS.[286,320] A more convincing display of the strength of tandem MS, however, was provided by a quadrupole/TOF combination in a study of arsenic constituents in clam kidney reported by McSheehy *et al.*[221] The greatly improved mass resolution afforded by the TOF mass analyser facilitated identification, with varying degrees of rigour, of four new arsenic compounds which were then placed in a solid biosynthetic context.

Tandem MS in time techniques have also been reported for arsenic speciation analysis.[360,363,366] An ion trap quadrupole mass analyser was used to collect fragmentation data for standard arsenic compounds,[360] and has also been used in combination with HPLC for identifying known compounds in extracts of algae.[363] FTICR mass spectrometry has also been used on one occasion to identify a known arsenosugar in an algal extract.[366] The excellent mass resolution of the FTICR mass analyser allows the assignment of the empirical formulae of arsenicals from mass measurements alone. It remains to be seen if such sophisticated instrumentation will make a significant impact in the area of arsenic speciation analysis. The chances would be considerable higher, however, if future work focused on heftier targets, arsenic-containing macromolecules for example, that would more fully utilise the capabilities of FTICR mass spectrometry.

The (unspoken) goal of at least some arsenic molecular mass spectrometrists appears to be to do away with the chromatographic part of speciation analysis, and thus end the coupling that has been in use in various forms for 30 years. The idea is simply to perform the separation (or isolation) of arsenic species in the mass spectrometer, and then to detect them selectively. The instrumentation can certainly do this, aided by the mass deficient status of arsenic, as shown in several studies.[360,361,366] The application to real samples, however, has so far proven difficult — although MS without chromatography has produced good qualitative results with partially purified samples, there have been no reports of its successful application to crude samples, or for the identification of novel compounds. The technique may find some applications with unstable metabolites including those arsenic species that chromatograph poorly or not at all.

15 X-ray spectroscopic techniques

X-ray spectroscopic methods such as EXAFS and XANES are being increasingly used for arsenic speciation analysis. They are more often used for geological samples[393,400,401] but can also be applied to arsenic-rich biological samples.[240,387] The information imparted by EXAFS is limited in that it only relates to the immediate environment around the target element, in our case arsenic. EXAFS can provide precise inter-atomic distances (±0.01 nm) and hence by comparison with standard compounds, the technique can say which atoms are bound to arsenic. But the method provides only imprecise (±30%) information about the number of atoms bound to arsenic. Its ability to deal with samples containing several arsenic species at comparable concentrations must also be questioned. Nevertheless, valuable information is forthcoming, and X-ray methods can alleviate concerns regarding changes to arsenic species during extraction and/or chromatography, as discussed earlier in this review.

A most impressive application of X-ray analysis was reported by Gailer and coworkers[408,409] who identified a novel arsenic-selenium metabolite, seleno-bis(*S*-glutathionyl)arsinium ion, in rabbit bile. The assignment was assisted by the complementary X-ray data sets for both arsenic and selenium.

16 New arsenic species, novel metabolites, and advances in lipid arsenic research

The impact of molecular MS over the last four years can most readily be seen in the area of new arsenic species. Most of the arsenic compounds commonly encountered in environmental and biological samples were identified by a classical natural products chemistry approach, usually involving NMR spectroscopy, and these compounds have subsequently served as standards for arsenic speciation analysis, in particular for HPLC-ICPMS. However, in the 15 or more years since the first application of HPLC-ICPMS to arsenic speciation analysis, there were only two reports of new arsenicals, namely DMAA[445] and a trimethylated arsenosugar, arsenosugar **9**[446] identified by this technique in combination with chemical synthesis. In contrast, in the last four years molecular MS has been involved in the identification of six new arsenic compounds, and several new metabolites in urine and in biotransformation studies.

A new arsenic containing betaine, trimethylarsoniopropionate (TMAP) was first identified in fish using single quadrupole ESMS coupled to HPLC,[222] and this compound was subsequently shown to be present in a wide range of organisms.[447] TMAP is also present in the certified reference material DORM-2,[222] and consequently, this material provides a form of "standard" so that TMAP can now be determined by HPLC-ICPMS without the pressing need of pure standard compound.

A single quadrupole ESMS was also used to identify the major arsenical in kidney of the clam *Tridacna derasa* as 5-dimethylarsinoyl-2,3,4-trihydroxypentanate,[364] a result subsequently confirmed by McSheehy *et al.*[221] using tandem MS techniques. In addition, McSheehy *et al.*[221] identified a further 14 arsenicals in the *T. derasa* kidney in a fine demonstration of the power of tandem MS. Although these authors report that four of the identified compounds were novel, they do not clearly indicate which ones these are. From our reading of the data presented, two of the compounds, 5-dimethylarsinoyl-2,3-dihydroxypentanate and 4-dimethylarsinoyl-2,3-dihydroxybutanate, are certainly novel and appear to have been assigned on reasonable MS data and biosynthetic grounds; and possibly *three* other compounds, arsenosugars **6–8**, also appear new although the data on which their assignments are based are less rigorous. We note that DMAA, identified in the clam kidney,[221] had previously been reported as a natural product in several marine samples.[445] The novel arsenosugar **5** (which might also be considered as a substituted dihydroxyfuran) was first tenuously reported in oyster tissue[286] where it was incorrectly referred to as

5-dimethylarsinoyl-β-ribofuranose, and it was also present in the kidney of *T. derasa*.[221]

We refer now to novel arsenic metabolites which we define as compounds reported for the first time or in novel circumstances. Seleno-bis(*S*-glutathionyl) arsinium ion was detected by X-ray methods in the bile of rabbits administered both arsenic and selenium.[408] The structure of this metabolite, showing an As–Se linkage, seems to neatly explain the protective effect of selenium against arsenic toxicity. DMAA was shown to be an intermediate in the degradation of arsenobetaine to DMA,[311,448] and it was recently shown to be a urinary metabolite from sheep ingesting large quantities of algae (and hence arsenosugars).[308] DMAE was also shown to be a human urinary metabolite after ingestion of arsenosugars,[291] and it was also present in the sheep urine.[308] Arsenosugar **6**, a proposed natural constituent of *T. derasa* kidney,[221] was identified as a degradation product of arsenosugars in controlled hydrolysis experiments.[320]

A recent study by Hansen *et al.*[247] (first reported at a conference in September 2003 but published in 2004) identified the sulfur analog of DMAA, namely dimethylarsinothioylacetate, in the urine from the "algae eating" sheep. This is a very important result (justifying its late inclusion in this 2000–2003 review) because it seems likely that S analogs of other arsine oxides may also occur as arsenic metabolites in urine and elsewhere, as noted by Hansen *et al.*[247] Yoshida *et al.*[315] had earlier reported an uncharacterised S-containing arsenical in rat urine. Future work could benefit by focusing on thio-arsenic compounds as possible candidates for the many unidentified arsenicals reported in natural samples and as metabolites in biotransformation studies. One can anticipate interesting results in this area in the next few years, building on the ground-breaking work of Hansen *et al.*[247]

Finally, we look at recent work dealing with lipid arsenic. Although no studies have analysed the arsenolipids *per se*, and no novel identifications have been made, three investigations dealing with lipid hydrolysis products are of relevance. The approach taken by all three studies was to degrade the arsenolipids to water-soluble products that were amenable to arsenic speciation analysis by HPLC-ICPMS. Thus, Hanaoka *et al.*[210] reported the possible presence of at least six arsenolipids in tissues of a shark, *Musterus manazo*, some of which contained a dimethylated arsenic moiety or arsenocholine. Similarly, Ebisuda *et al.*[228] working with the blubber of the ringed seal, *Pusa hisida*, showed the presence of lipid arsenic which yielded DMA on alkaline hydrolysis. In the third investigation, Devalla and Feldmann[324] examined the lipid arsenic in a brown alga, *Laminaria digitata*, and tissues from sheep that feed almost exclusively on *L. digitata* and related algal species. They found that the lipid arsenic from *L. digitata* (constituting about 1% of the total arsenic) had a structure based on arsenosugar **2** (the phosphate sugar), a result consistent with the early definitive work of Morita and Shibata,[449] which identified the intact arsenolipid, and Edmonds *et al.*,[450] which examined hydrolysed arsenolipids from lobster. In contrast to the results for *L. digitata*, the major hydrolysis products from the sheep tissues were DMA and MA.[324] Although studies based on hydrolysis products of lipid arsenic fractions are providing some information, real progress in this difficult area will only come from direct analysis of the intact arsenolipids, and must await advances in analytical techniques able to handle these types of compounds.

17 Areas of particular interest and concern

Much research has been performed over the recent years in the field of arsenic speciation analysis, and impressive advances have been registered. There is, however, still a need for considerable improvement in some aspects of this analysis. Because of the human health issues associated with certain arsenic species, analysts need to be particularly vigilant when developing and applying the methods, and reporting the data. We wish to end this review on the determination of arsenic species by discussing a selected few of the problems we see in this research field.

17.1 Reduced methylated arsenic species MA(III) and DMA(III) in human urine

One of the most important issues currently being investigated in the area of arsenic research is the biotransformation products from ingested inorganic arsenic. Recent work has shown the presence in human urine of reduced methylated arsenicals, referred to as MA(III) and DMA(III), and these compounds appear to have considerable toxicity.[6] A complete understanding of these biotransformation products is essential to explain the role of arsenic as a human carcinogen, as demonstrated in several epidemiological studies.[439] In addition, such knowledge may shed light on the reasons for arsenic's efficacy as a treatment for acute promyelocytic leukaemia.[4] The topic impacts on many disciplines including biochemistry, toxicology, and epidemiology, but it is totally dependent on good analytical chemistry. The recent activity in the area of urine metabolites MA(III) and DMA(III) has stemmed from developments in speciation analysis which enabled these species to be detected and quantified. Because of the important implications of this research, we believe that it is worthwhile to examine in some detail the rigour of the analytical techniques and the way they are being applied.

DMA(III). We begin by examining the data for DMA(III) in human urine. There have been three reports of DMA(III) or a DMA(III) complex being a natural constituent of human urine.[98,167,292] In addition, there has been one thorough investigation of the stability of this species in urine and water.[164] We have already discussed the stability study in detail, but the main point is worth repeating: DMA(III) is extremely unstable in urine and even at −20 °C it is completely converted to DMA within 17 h.

The first report of DMA(III) as a natural constituent of urine was made by Le *et al.*[167] using HPLC-HG-AFS. The urine was collected from subjects who consumed 300 mg of 2,3-dimercaptopropanesulfonate (DMPS, $HSCH_2CH(SH)CH_2SO_3^-$); DMA(III) was not detected in urine from subjects before DMPS ingestion. The basis of the assignment of DMA(III) by Le *et al.*[167] was retention time matching (standard with sample) under *one* set of chromatographic conditions. In addition, for one urine sample, DMA(III) standard was added and an enhanced signal was obtained for one of the sample peaks. But, interestingly, this same peak was also enhanced when As(V) was spiked to the sample, a result which places some doubt on the rigour of the spiking experiments.

The second report was of a DMA(III)-glutathione complex in urine samples from six individuals from central Mexico.[98] The analysis used HG-AAS and was based on a selective reduction of trivalent arsenic compounds at pH 6. In their reporting of the work, the authors, for convenience, often refer to the DMA(III)-glutathione complex simply as DMA(III), but their data do not specifically indicate the presence of "free" DMA(III). Nevertheless, they report[98] that up to 9% of total urinary arsenic was present as DMA(III) complex.

The third paper of relevance to this discussion is that by Mandal *et al.*[292] which examined the urinary arsenic metabolites from 428 subjects from West Bengal, India. These authors used HPLC-ICPMS and hence their analytical technique was not subject to some of the vagaries of selective reduction efficiencies associated with hydride generation techniques. They reported the presence of DMA(III) in 72% of the subjects at levels up to 21% of the total urinary arsenic. The proportion of DMA(III) in the urine did not appear to be related to total arsenic ingested or excreted. Indeed, in one subject drinking "safe" water (< 3 μg As l^{-1}), 92% of the total urinary arsenic was present as DMA(III), and (representative?) chromatograms of urine samples displayed in the paper clearly show a dominant signal (up to 80% of the total arsenic) which was assigned as DMA(III). In the light of this observation alone, one

may wonder why DMA(III) had not previously been reported in the many other studies of urinary arsenic metabolites.

Of crucial importance to the interpretation of the results from Mandal *et al.*[292] is their collection and storage procedure, bearing in mind the report that DMA(III) stored frozen in a urine sample was completely converted to DMA within 17 h. Samples in the Mandal *et al*. study were collected, stored in salt/ice then frozen for transport to Japan. After two months of frozen storage, samples were again transported (72 h) in a salt/ice mixture, and then analysed. The inappropriateness of such sample storage to the problem at hand, determination of a highly labile urine metabolite, is clear. In view of these analytical shortcomings, the work of Mandal *et al.*[292] might be considered unreliable, and consequently, the main outcome from the study, that DMA(III) was present in the urine of 72% of the 428 subjects investigated, must also be questioned.

MA(III). The first report of MA(III) in an organism was by Gregus *et al.*[173] who found this arsenic species in rat bile. The presence of MA(III) in human urine was subsequently reported in several publications emanating from the same core of authors.[100,166–168] The paper of Aposhian *et al.*[100] justifiably claims the very first report — they found MA(III) in urine from subjects from inner Mongolia whom had been subjected to the DMPS arsenic challenge test. But details of the analytical chemistry on which the identification was based were not reported; rather, it was stated that MA(III) was measured by an unpublished HPLC-HG-AFS procedure. Considering that the detection of MA(III) in human urine was a novel result, one would expect the analytical chemistry to be verified (*i.e*. to be published in the analytical literature) before making such a claim. Of particular concern is that the samples were stored frozen for six months before analysis.

Two subsequent papers,[166,167] provided the full analytical details for the Aposhian study.[100] These two papers report work on the same samples (164 urine samples from 41 subjects from inner Mongolia) using the same technique (HPLC-HG-AFS), but interestingly the results differ in some respects. One paper[166] reports the presence of MA(III) in the samples, and presents chromatograms showing a dramatic increase in the concentration of MA following administration of DMPS. The other paper[167] reports MA(III) and also the presence of DMA(III) in the samples (but only in two of them), and an increase in MA is not apparent in the displayed chromatograms. The discrepancies in the data sets are not discussed.

Closer inspection of the analytical method[166,167] reveals that MA(III) was identified on the basis of a poorly resolved peak eluting just after the solvent front (and As(III)). Probably, a more rigorous identification was called for. In addition, both papers reported preliminary results indicating that MA(III) was labile, and, accordingly, the sample storage and preparation must be carefully scrutinised. In those studies, urine was stored frozen for about 6 months before analysis — conditions later reported[164] to result in conversion of MA(III) to MA. Furthermore, before arsenic speciation analysis was performed, all urine samples were digested in HCl.[100] Unfortunately, the fate of MA (and DMA), treated in this manner was not investigated. Bearing in mind that DMPS, a dithiol and a reducing agent, was also present in these samples, the possibility that reduced artefacts, or perhaps thio-arsenic species, were formed during the treatment cannot be discounted.

We digress here momentarily to discuss the possible effect of the HCl pre-treatment, in the presence of DMPS, on DMA and DMA(III). In a recent study,[139] Le and coworkers clearly described a sample preparation procedure without the HCl/heating step, and still reported the presence of DMA(III) in the urine of rats that had been exposed to DMA in their food. The chromatography, however, was performed on a column pre-conditioned with DMPS, and consequently, there must remain a small but lingering doubt about the rigour of the assignment of DMA(III) in the original urine. Interestingly, the authors adopted a different view by suggesting that, because of the instability of DMA(III), the concentration of DMA(III) they recorded probably represented only a small fraction of that originally present in the urine (*i.e*. in "fresh" urine). One could envisage quite simple experiments to test this hypothesis.

A fourth paper in the series dealing with MA(III) investigated urine from subjects from Romania.[168] The interesting aspect of this study was that MA(III) was found in the urine of the subjects even though they did not ingest DMPS (or any other arsenic complexing agent). It is not clear why this result should be so different from the earlier reports by the same researchers which demonstrated that MA(III) was not produced unless DMPS was administered.[100,166,167]

Possibly with a view to explaining this enigma, Aposhian and coworkers investigated the properties of the complex between MA(III) and DMPS, and developed an analytical method to measure it.[165] The complex was not detected (hydride inactive) under the usual hydride generation conditions, but it was efficiently decomposed when treated with NaOH solutions to a hydride active species, presumably MA(III). This work, however, is difficult to follow because a table of data clearly shows that even with no NaOH treatment, the complex is decomposed with 18.5% efficiency, and hence should be easily detectable in the urine samples in which it was claimed to constitute about 60% of the total arsenic.

The above studies are all based on data produced from the same analytical technique (HPLC-HG-AFS). Del Razo *et al.*[98] also used hydride generation and a selective reduction scheme with AAS detection to identify MA(III) in urine. Mandal *et al.*[292] used HPLC-ICPMS to identify MA(III) in urine and hence their study might be seen as providing confirmatory evidence for this species. The experimental design of the Mandal *et al.* study,[292] however, appears to be inadequate in terms of sample storage and interpretation of the chromatographic data. In addition, the standard used in that study[292] must be questioned because the authors reduce MA with sodium thiosulfate to give MA(III), a treatment previously shown to produce two arsenical products in equal amounts,[291] and hence is unsuitable for preparing standard MA(III).

In summary, the early reports of MA(III) and DMA(III) in human urine appear to be based on inappropriate analytical protocol, particularly in terms of sample storage and pre-treatment. Consequently, the results reported so far show inconsistencies which require clarification. The arsenic metabolites MA(III) and DMA(III) may play a crucial role in the biotransformation and toxicology of inorganic arsenic, and their presence and quantification in human urine should be confirmed with reliable arsenic speciation analysis and adequately characterised standard compounds. Researchers in this new field, however, should also be aware of the recent report[247] of a thio-arsenic metabolite in sheep urine, and consider the possible significance of this and perhaps other As–S metabolites on results reported so far for human urine.

17.2 Use of standards and quantification in arsenic speciation analysis

It is probably fair to say that the majority of people performing arsenic speciation analysis have come from a background of inorganic (total element) analysis. Standards for inorganic analyses are generally commercially available in convenient forms such as standard stock solutions. Relatively few arsenic compounds, however, are commercially available and this has created some problems. In particular, there is a tendency to use uncharacterised compounds as "standards". The source of such materials and the manner in which "standard solutions" are prepared from them, should be clearly stated. This practice would provide some sort of traceability: future work may reveal that some of the arsenic standards, obtained in only trace amounts from natural sources, may have been assigned incorrect structures. Perhaps in response to these issues, a paper[451] was published in 1999 describing the certification of an arsenobetaine solution!

The fact that the ICPMS response for arsenic is essentially uniform for all (known) arsenic species allows this detector, in most cases, to quantify the various arsenicals by comparison with simple standards such as As(v) or arsenobetaine. This situation, however, does not apply to other detection methods used in speciation analysis and quantification of a particular arsenical cannot be performed without the specific standard. Techniques such as HPLC-HG-AFS in combination with a decomposition step may claim that quantification is possible without the specific standard, but the result will always be subject to matrix effects and varying degradation efficiencies of the arsenicals, as discussed above. In the case of molecular mass spectrometry, of course, quantification of a sample compound is impossible without the standard, even though the compound may be able to be identified with some confidence. The relative strengths and weaknesses of atomic and molecular MS techniques in terms of identification and quantification means that they complement each other perfectly, and together form a formidable partnership for arsenic speciation work.

The use of ICPMS, however, does not in itself result in quantitative data. One must ensure the analytical rigour of all the other steps in a speciation analysis such as extraction, clean-up, and chromatography. As mentioned above, many molecular MS analyses use preparative chromatography to clean-up arsenic-containing fractions, and at each stage a "peak fraction" is transferred to the next stage. The procedure is designed to prepare samples, concentrated in particular arsenicals, prior to structural elucidation by molecular MS. And, with assumptions, the ICPMS data of the preparative chromatographic fractions can provide estimates of the quantities of arsenicals in the original extract. Such an experimental protocol, however, cannot provide accurate quantitative data.

17.3 "Real samples" and interpretation of the ensuing data

The common, indeed almost mandatory, practice in the reporting of a "new" speciation analysis is to test the method on so-called real samples. This is sound analytical practice and should be encouraged by journals (and reviewers). Researchers should endeavour, however, to choose real samples that suitably challenge the proposed new technique. Furthermore, the data obtained from the real samples should be used only for their intended purpose — to check precision, and perhaps accuracy, of the proposed method when applied to a (complex) sample matrix compared with standard solutions. Very often, however, the few data reported (sometimes just one sample) are discussed in broader environmental, biological or toxicological terms without a proper scientific basis. The previously mentioned study of Xie *et al.*[186] provides a good example, but there are others. In that study, water, spiked with 2000 μg As l^{-1}, from a toxicity test with freshwater organisms was analysed by collision cell ICPMS, employed ostensibly to minimise $^{40}Ar^{35}Cl$ interference on ^{75}As. Based on just a few chemical analyses, and in the absence of experimental details of the toxicity testing, the authors conclude that "their findings have important implications for As environmental risk assessment". Toxicologists might well wish to know the precise experimental protocol and see the full data set before accepting such a statement.

18 Summary

From 2000 to the end of 2003, more than 400 scientific publications have appeared dealing with some aspect of the determination of arsenic species. This review attempts to draw out the most topical or useful of these reports, and we provide now a synopsis of some interesting areas and trends.

Extraction efficiencies vary greatly between samples, thus it appears unrealistic to strive for a universal optimal extraction procedure for arsenic species. Rather, sample extraction procedures should be tailored to the particular application and desired analytes. Most arsenicals are reasonably stable and can be stored frozen for long periods. Some, however, are labile even when stored frozen, and their rate of conversion depends on the sample matrix. There is a need for a thorough study of stability of arsenicals/storage conditions which could provide information on the fundamental transformation processes at work. Such a study should be based on existing information on physico-chemical properties of redox reactions such as stability diagrams as a function of pH and redox potentials.

A neglected area of arsenic speciation research is that dealing with the "insoluble" fraction which is thought to comprise "protein-bound" arsenic and/or "lipid-arsenic". Research in these areas is currently hindered by lack of suitable analytical methods, and increased attempts should be made to overcome these problems. Application of the right analytical strategy is likely to lead to novel and interesting results.

Improvements in chromatographic separation have been reported using HPLC with gradient elution, but the application of these techniques to real samples may be limited because of severe matrix effects. CE provides excellent separation of many arsenicals but the small sample sizes preclude its use for real samples, and this restriction is unlikely to be overcome in the near future.

HPLC-ICPMS is the most powerful and commonly used method for arsenic speciation analysis, and this usage should continue to grow as the required (but expensive) instrumentation becomes more widespread. The method provides reliable quantitative data for arsenic species at environmentally relevant levels in various and diverse matrices.

Methods based on HPLC coupled to AAS, AES or AFS, and used in conjunction with HG, provide lower cost options for arsenic speciation analysis. The application of these techniques to refractory arsenicals (arsenobetaine for example) through the use of a decomposition step should be handled cautiously because the degree of degradation is strongly influenced by the matrix. We expect a decline in the coming years in research investigations into arsenic speciation analysis using optical detection systems. The application of these techniques to study various aspects of arsenic speciation, however, is likely to continue.

Molecular mass spectrometric methods have provided the most interesting recent results in terms of structural elucidation of new metabolites, and they hold the promise of identifying more novel arsenicals and thereby better explaining arsenic's fate and role in biological systems. Molecular MS is also increasingly being used to provide verification of chromatographic peaks detected and quantified with ICPMS. The use of molecular MS alone, however, is likely to stay mainly in qualitative analysis. An exception may arise if there is a need to routinely analyse a specific arsenical, for toxicological purposes for example, which would encourage development of a targeted sample preparation procedure applicable to molecular MS analysis.

X-ray spectroscopic methods, long used for abiotic samples, are now being applied to biological samples and they have already provided interesting data on novel arsenic species and arsenic species *in situ*. Their ability to handle solid samples removes most concerns regarding sample extraction and sample storage which can reduce the usefulness of the traditional coupled techniques based on chromatography. X-ray spectroscopy is currently restricted to arsenic-rich samples but further developments in fluorescence detection methods may extend its use.

The most topical issues on arsenic speciation research are the reduced species MA(III) and DNA(III) in human urine, and the recently reported thio arsenic species in sheep urine. On closer inspection, the data presented so far for MA(III) and DNA(III) are not totally convincing and require confirmation with appropriate sample collection, storage and preparation procedures. On the other hand, the data showing the presence of thio arsenic species look sound and there is the likelihood that other As–S compounds will be found. Given the current uncertainty surrounding MA(III) and DMA(III), the possibility that arsenic species assigned as such may in fact be thio arsenic species should be considered.

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