Simone Braeuer, BSc

Arsenic and Co(ws)
Arsenic speciation in urine and tissue samples of ruminants

MASTERARBEIT

zur Erlangung des akademischen Grades einer

Master of Science

in

Chemie

[gemeinsames Studium im Rahmen von NAWI Graz]

eingereicht an der

Technischen Universität Graz

Betreuer:

Ao. Univ. Prof. Mag. Dr. Walter Goessler

Institut für Chemie, Karl-Franzens Universität Graz

Graz, März 2014

Die vorliegende Arbeit wurde am Institut für Chemie - Bereich Analytische Chemie der Karl-Franzens Universität Graz in der Zeit von Jänner 2013 bis März 2014 unter der Leitung von Ao. Univ. Prof. Dr. Mag. Walter Goessler durchgeführt.

Abstract

The Chaco-Pampean plain in Argentina is not only known for its numerous cattle herds, but also for very high arsenic concentrations in the groundwater (up to 2500 µg As/L). To evaluate a possible health risk for humans through consumption of bovine products from this area (e.g. liver or kidney), we investigated the total arsenic concentrations in groundwater samples as well as tissue samples of cattle that had been grazing on these landscapes. In addition to the Argentinean samples, we also analyzed the total arsenic concentrations in bovine livers and kidneys from Austria. While the total arsenic levels in the kidneys were very similar for Austrian and Argentinean samples, the Argentinean liver samples contained more total arsenic than the Austrian ones. There were also other elements present in higher concentrations in the Argentinean samples compared to the Austrian samples. For example, we found very high vanadium concentrations in water and tissue samples from Argentina.

We also determined the arsenic speciation in bovine liver and kidney extracts. Until now, little to nothing has been reported in literature about the arsenic compounds present in tissue samples of ruminants. It is believed that dimethylarsinic acid (DMA) is the major arsenical in tissues of terrestrial mammals. Surprisingly, we found that methylarsonic acid (MA) was the dominating arsenic species in bovine liver and kidney extracts. DMA and inorganic arsenic were also present in significant amounts. Additionally we detected one to three unknown arsenic compounds in some of the extracts.

Since most of the arsenic is excreted via the urine, we also investigated the total arsenic concentrations and the arsenic speciation in urine samples of cows of two Styrian farms and of various mammals of the Schönbrunn Zoo. The total arsenic concentrations ranged from 5 to 125 µg/L. The major arsenic species was DMA, followed by inorganic arsenic and MA, similar to the arsenic speciation that has been reported for the urine of other terrestrial mammals. However, there were also significant amounts of three unknown arsenicals, which have not been reported in literature so far. We tried to identify at least one of them by applying different chromatographic methods and co-chromatography with various standard solutions of arsenic species. We also started to isolate and concentrate the unknowns via preparative HPLC for future analysis with molecular mass spectrometry.

Zusammenfassung

Die Pampa in Argentinien ist nicht nur bekannt für ihre vielen Rinderherden, sondern auch für extrem hohe Arsenkonzentrationen im Grundwasser. Werte bis weit über 2500 µg/L wurden gemessen. Um das Gesundheitsrisiko für Menschen, abschätzen zu können, analysierten wir die Totalarsenkonzentrationen in einigen Grundwasser- und Rindergewebeproben der Pampa. Zusätzlich bestimmten wir den Totalarsengehalt von Lebern und Nieren österreichischer Rinder. Die Arsenkonzentrationen der österreichischen und argentinischen Nieren waren sehr ähnlich. Bei den Rinderlebern wiesen die Proben aus Argentinien einen etwas höheren Totalarsengehalt auf als die österreichischen Proben. Auch andere Elemente kamen in den argentinischen Proben in höheren Konzentrationen vor als in österreichischen. Beispielsweise fanden wir extrem hohe Vanadiumwerte in Wasser- und Gewebeproben aus Argentinien.

Weiters analysierten wir die Arsenspeziation in Extrakten der Gewebeproben. In der Literatur wurde bis jetzt kaum über Arsenspezies in Wiederkäuern berichtet, aber es wird generell davon ausgegangen, dass Dimethylarsinsäure (DMA) die Hauptarsenverbindung in Landsäugetieren darstellt. Überraschenderweise fanden wir in den Extrakten unserer Proben Methylarsonsäure (MA) als dominierende Arsenspezies, gefolgt von DMA und anorganischem Arsen. Außerdem konnten wir in einigen Proben ein bis drei unbekannte Arsenverbindungen detektieren.

Da Arsen hauptsächlich über den Urin ausgeschieden wird, bestimmten wir auch die Totalarsenkonzentration und die Arsenspeziation von Urinproben von Kühen zweier steirischer Bauernhöfe und von verschiedenen Säugetieren des Tiergarten Schönbrunn. Die Proben enthielten zwischen 5 und 125 µg As/L. Die Hauptarsenspezies war DMA. Außerdem fanden wir signifikante Konzentrationen von anorganischem Arsen und MA. Generell war die Arsenspeziation sehr ähnlich zu Literaturwerten für Urin von Landsäugetieren, jedoch fanden wir zusätzlich noch größere Mengen von zumindest drei unbekannten Arsenverbindungen, die bis jetzt für keine Urinproben publiziert worden waren. Wir versuchten diese Verbindungen mittels unterschiedlicher chromatographischer Methoden und Co-Chromatographie mit verschiedenen bekannten Arsenspezies zu identifizieren. Schließlich begannen wir mit der Isolierung und Aufkonzentrierung der unbekannten Arsenverbindungen mit präparativer HPLC, um diese dann zukünftig mit molekularer Massenspektrometrie analysieren zu können.

Danksagung

Ich möchte dir, **Walter**, für dieses spannende und originelle Masterarbeitsthema und für die Betreuung danken. Ein besonderes „Danke!“ für die Möglichkeit, nach Argentinien (und in den Tiergarten Schönbrunn) zu reisen. Nicht viele Leute können von sich behaupten, im Rahmen ihrer Masterarbeit um die halbe Welt gereist zu sein.

Matthias, du hast mich nicht nur in Argentinien, sondern auch auf meinem restlichen Weg durch die Höhen und Tiefen, die so eine Masterarbeit mit sich bringt, ausgehalten. Danke, dass du immer für mich da bist.

Liebe **Mama**, lieber **Papa**: Danke, dass ihr mich so geduldig unterstützt habt. Nicht nur finanziell, sondern auch mental. Danke auch meinen **Geschwistern**, speziell **Susi,** fürs Nerven (und damit Anspornen) „wann ich denn endlich fertig werde“.

Vielen Dank an **Lisa, Kathi, Melanie, Oliver und die weiteren Mitglieder der ACHE** fürs Zuhören, Gedanken austauschen und für das angenehme Arbeitsgruppenklima.

Ein großer Dank gilt **Lisa, Walter, Sarah und Sophia**, die für mich von Kuh zu Kuh gerannt sind und Proben für mich „aufgefangen“ haben.

Bianca und Fripi gebührt ein spezieller Dank dafür, dass ich ihre ohnehin schon endlos langen Sequenzen des Öfteren noch um ein oder auch zehn Proben verlängern durfte.

Vielen lieben Dank auch **Jaqui, Hanna, Michi, Sarah und Gabriela,** für die Unterstützung bei der Probenvorbereitung.

Centro de Estudios Transdisciplinarios del Agua

I also want to thank the people from the research group in Buenos Aires for welcoming me in Argentina, especially **Alejo, Cynthia and Cristina**.

TIERGARTEN SCHÖNBRUNN www.zoovienna.at

Bedanken möchte ich mich außerdem beim **Tiergarten Schönbrunn**, speziell bei **Regina Pfistermüller,** für die gute Kooperation und die Bereitstellung der Proben.

OeAD

Ich danke auch der **OeAD** für die Finanzierung der Argeninien-Reise (WTZ Österreich Argentinien, Projekt 08/2013).

Table of Contents

1 Scope of this master thesis

- Determination of the total arsenic concentration in groundwater from the Chaco-Pampean plain, Argentina
- Determination of the total arsenic concentrations in Argentinean and Austrian bovine livers and kidneys with ICPMS to evaluate a possible bioaccumulation of arsenic in cattle exposed to high total arsenic levels in their drinking water
- Determination of the total arsenic concentrations in urine samples of ruminants and other terrestrial mammals with ICPMS
- Arsenic speciation analysis of extracts of the bovine livers and kidneys as well as of the urine samples with HPLC-ICPMS to gain information about the arsenic metabolism of ruminants
- Investigation of unknown arsenic species in the urine samples
- Determination of the concentrations of various other elements in the water, liver, kidney and urine samples

2 Introduction

2.1 Arsenic

2.1.1 A famous poison and drug

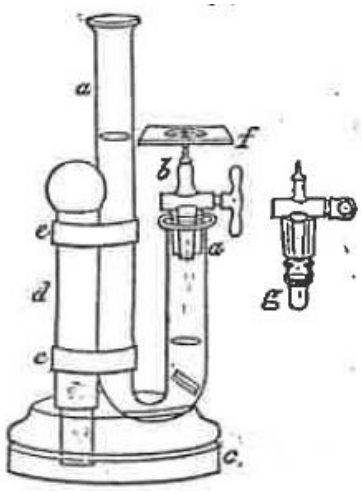


Figure 1: Apparatus described and used by J. Marsh, 1836

It is said that already Hippocrates used arsenic containing medications around 400 B.C. [1]. Through history arsenic became well known as a very effective poison. It was even given infamous titles like “king of poisons” and “poison of kings”. Arsenic poisoning was very easily achieved and the murderers could not be convicted, because As_2O_3 is taste- and odorless, was easily available, and moreover there was no good analytical technique to detect arsenic in human samples for a long time [1]. Finally, in 1836, James Marsh developed a method to detect even small amounts of arsenic by converting it into a volatile hydride and then depositing it in its metallic state on a cold glass plate [2] (the original apparatus is shown in Figure 1): His method reduced the number of arsenic murders drastically and became famous as the “Marsh test”.

Beside its reputation as a very “comfortable” toxic substance, arsenic has also been used through the ages in order to increase health. In the Victorian age, people became somewhat obsessed with arsenic and used it for treatment of almost everything, from syphilis to coarse skin [3]. One famous tincture was Fowler’s solution, which contained 1 % potassium arsenite and, besides its use for all kinds of illnesses, was probably the first chemotherapeutic drug against leukemia [4]. Paul Ehrlich synthesized a new organoarsenic compound, Salvarsan, which was used from 1910 onwards as an effective drug against syphilis until it was replaced by penicillin around 1940.

There are recorded cases of arsenicophagy, the behavior of eating large amounts of arsenic on purpose on a regular basis. Especially people from Styria, Austria, were known to consume mainly arsenic trioxide from the 17th century onwards [5]. They ate it weekly to daily, usually together with some bread or beacon. One dose could contain up to 400 mg, which is about 200 % of the commonly known lethal dose of 100 – 200 mg [6].

During the 19th and the early 20th century, arsenic compounds, for example Paris Green (copper acetoarsenite, also utilized as a pigment), were used as insecticides and pesticides, and some organoarsenic compounds are still in use today [3]. For example, the arsenic based compound roxarsone is given to poultry as a feed additive [7]. Concerning cattle, no

arsenic based additive is usually fed nowadays. However, there are reported cases of cattle that had been poisoned by arsenic based pesticides which happened to be stored on the pasture [8, 9].

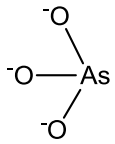
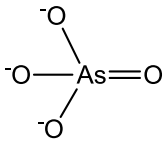
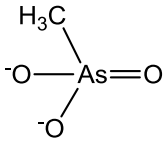
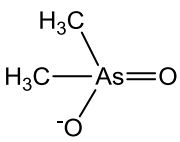
There are some medical applications of arsenic that are currently in use. The medication Trisenox® (arsenic trioxide) is applied against acute promyelocytic leukemia, and there are attempts to use it against other types of cancer as well [1]. Melarsoprol, an organic arsenic containing compound, is still applied in some countries for treatment of African trypanosomiasis (sleeping sickness) [3].

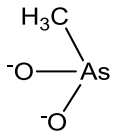
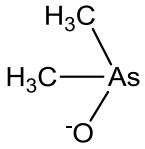
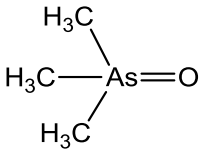
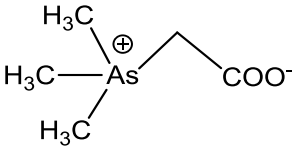
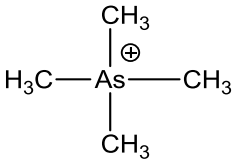
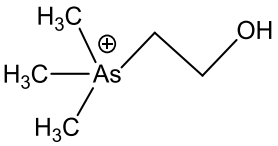
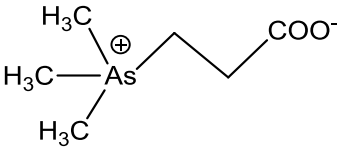
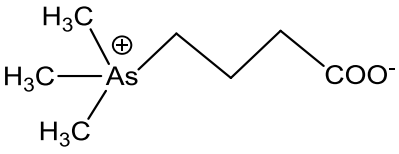
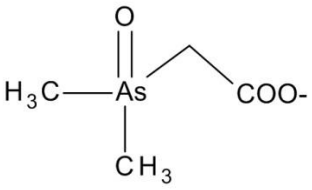
Despite of its reputation as one of the most potent poisons, it is possible that arsenic might actually be essential for mammals in very small amounts [10, 11]. Still, this has not been proven yet, and there is need for further investigations on this topic.

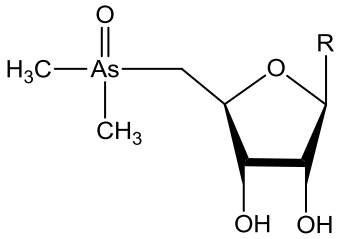
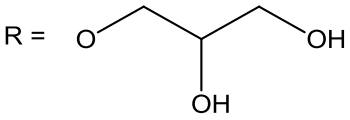
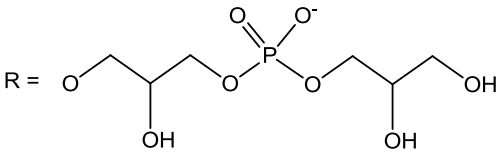
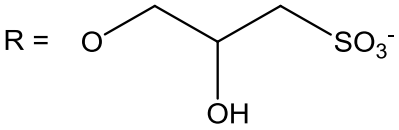
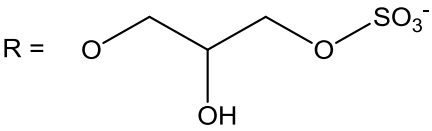
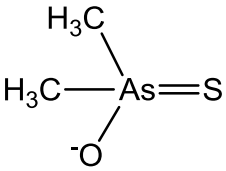
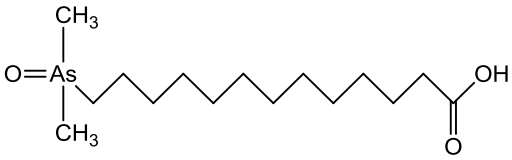
2.1.2 Different Arsenic compounds and toxicity

There are more than 100 different naturally occurring arsenic compounds known at the moment. The most important ones (especially with regard to this thesis) are listed in Table 1.

Table 1: Common arsenicals

Name	Abbreviation	Structure	pK values
Arsenite (arsenous acid)	As(III)		$pK_1 = 9.2$ $pK_2 = 13.5$ $pK_3 = 14.0$
Arsenate (arsenic acid)	As(V)		$pK_1 = 2.2$ $pK_2 = 6.9$ $pK_3 = 11.5$
Methylarsonate (monomethyl-arsonic acid)	MA		$pK_1 = 4.1$ $pK_2 = 9.1$
Dimethylarsinate (dimethylarsinic acid)	DMA		pK = 6.3

Methylarsonite (methylarsonous acid)	MA(III)		
Dimethylarsinite (dimethylarsinous acid)	DMA(III)		
Trimethylarsine oxide	TMAO		pK = 2.7 (protonation of As=O) [12]
Arsenobetaine (Trimethylarsonio-acetate)	AB		pK = 2.1 [13]
Tetramethyl-arsonium ion	TETRA		
Arsenocholine	AC		
Trimethylarsonio-propionate	TMAP		
Trimethylarsonio-butanate	TMAB		
Dimethylarsinoyl-acetate	DMAA		pK = 4.0 [13]

Dimethyl-(5-ribosyl)arsine oxide + *R*	Arseno-sugars	
Glycerol sugar	*Gly*	
Phosphate sugar	*Phos*	
Sulfonate sugar	*SO3*	
Sulfate sugar	*SO4*	
Thioarsenicals (here: thio-dimethylarsinate)		
Arsenic containing fatty acids		

Usually, trivalent arsenic species and thioarsenicals are very reactive and easily oxidized [14]. Therefore, detection and reliable quantification of arsenite, other trivalent arsenic species and thioarsenicals are very difficult. A common approach is to oxidize all samples before measurement. Therefore, all arsenic species can be measured in their more stable pentavalent oxo-forms.

The arsenic toxicity is highly dependent on the arsenic species. In general, inorganic arsenicals (As(III) and As(V)) are more toxic than organic species, and trivalent forms are more toxic than pentavalent ones. This makes it important not only to determine the total

arsenic concentration but also to distinguish between the occurring compounds. Some LD_{50} values are summarized in Table 2.

Table 2: LD_{50} values for some arsenic species [15, 16]

Arsenic species	LD_{50} (oral) [mg/kg body mass]
Arsenite	15 - 145 (rat), 26 - 39 (mouse)
Arsenate	110 - 175 (rats)
MA	100 (rabbit), 1800 (mouse), 960 - 3200 (rat)
DMA	1200 - 1800 (mouse), 640 - 1400 (rat)
AB, AC, TMAO	> 10000 (mouse)
TETRA	600 - 900 (mouse)

The lethal dose for humans for arsenic trioxide is around 1 - 3 mg/kg body mass (bm), which means for a person with 70 kg a total amount of about 100 - 200 mg [17]. Still, cases have been reported where poisoning with 8 - 16 g sodium arsenite did not cause death [18].

Symptoms of acute arsenic poisoning are vomiting, diarrhea, cramps, edemas, heart problems and finally death [19]. Chronic exposure to arsenic can promote different forms of cancer (especially skin cancer) and enhance their lethality. It can also lead to skin and cardiovascular diseases and Blackfoot disease, is associated with diabetes mellitus and can even influence the health of a fetus in the mother's womb [1, 19]. Not only inorganic arsenic, also DMA has carcinogenic potential [16]. More recently it has been found out that the trivalent methylated metabolic intermediates MA(III) and DMA(III) are even more poisonous than inorganic arsenic [20].

2.1.3 Arsenic in the environment

In rivers and lakes, less than 2 µg As/L are usually found, and in groundwater the average As concentration is 1 - 2 µg/L [16]. Higher levels can occur, depending on the geological properties of the region. Also strongly reducing or oxidizing conditions might result in high arsenic concentrations in the water. Last but not least, anthropogenic arsenic pollution through mining or use of pesticides is possible.

In Austria, the arsenic concentration in groundwater is usually below 1 µg/L. Still, there are some regions with higher arsenic abundance, for example the Fischbacher Alps or the so called "Mittleres Ennstal" [21]. Figure 2 shows a map of the arsenic levels in Austrian groundwater, and in Figure 3 the arsenic concentrations in Austrian stream sediments are displayed. In Styria, higher values can be seen for example in the region around Bruck-Mürzzuschlag.

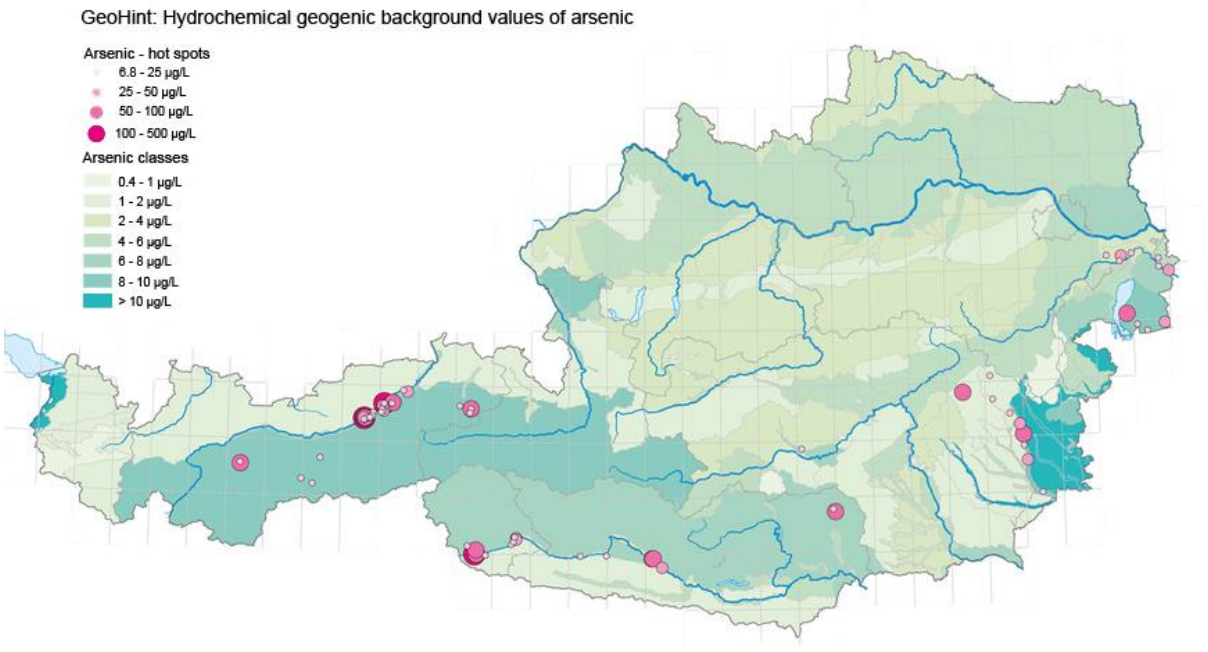


Figure 2: Hydro-chemical geogenic background arsenic concentrations in shallow groundwater in Austria [22]

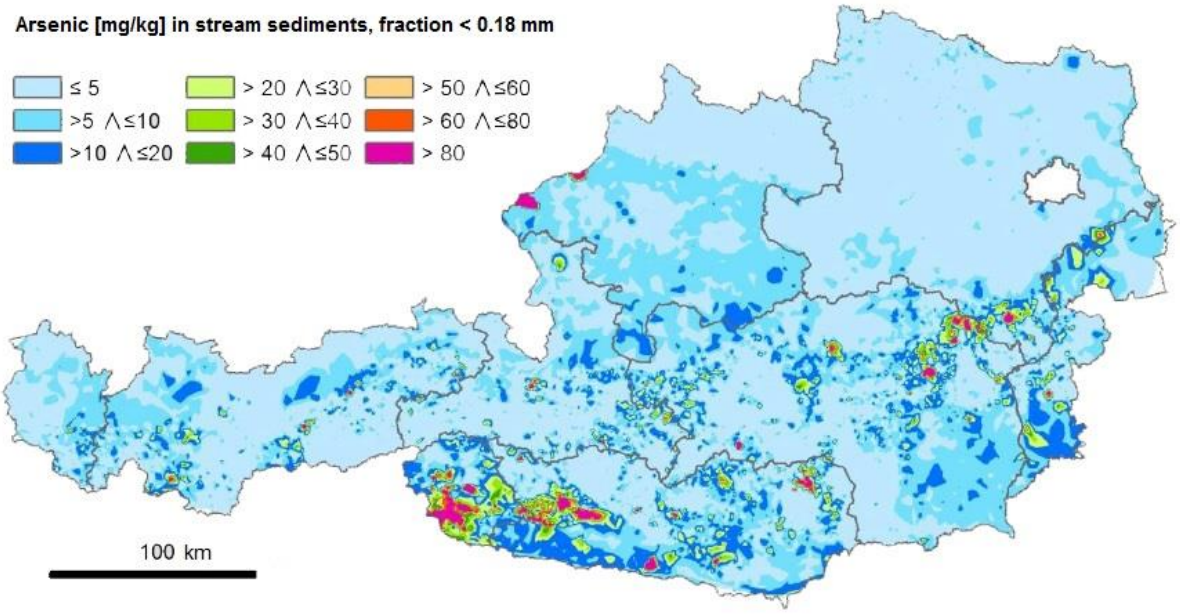


Figure 3: Arsenic in stream sediments in Austria [mg/kg] [21]

On a global level, Austrian water has low arsenic concentrations. There are some countries and regions that are suffering from extremely high arsenic concentrations in the groundwater, resulting in health problems of population and livestock. One dramatic example is an area in Bangladesh and northeast India, where more than 30 million people [23] and also cattle and poultry [24–30] are exposed to extremely high levels of arsenic in water and food.

Moreover, many parts of Latin America are well known to have high arsenic concentrations in the groundwater. One famous region is the Chaco-Pampean Plain in Argentina [31] (Figure 4). Already in 1913, Goyenechea described a connection between arsenic in the drinking water and skin lesions [32].

The illness was named “Bell Ville disease” after the town where it had been discovered for the first time. Later on, it became known in Latin America as HACRE (Hidroarsenisismo Crónico Regional Endémico), and high arsenic levels in the water were discovered in numerous areas not only in Argentina but all over Latin America [32].

In the Chaco-Pampean plain, groundwater arsenic concentrations can be as high as 5300 µg/L [33]. The geogenic reasons for this are discussed at length by Nicolli et. al. [31]. In brief, sediments in this area are consisting to a large part of volcanic deposits. The water is mostly oxidizing, and the pH varies from neutral to more than 9.0. These conditions are promoting dissolution, leaching and desorption processes of arsenic from minerals into water. Other elements are also affected, for example fluorine, boron, molybdenum, vanadium, uranium, selenium or antimony.

The Chaco-Pampean plain is the most important cattle farming region in Argentina and is responsible for a major part of the country’s dairy production. The transfer of arsenic from drinking water to cow milk has been examined in this area by Pérez-Carrera and Fernández-Cirelli [34, 35]. They found between 40 and 2600 µg As/L in the water and 0.3 to 10.5 µg As/L in milk. Overall, concentrations in water and milk were lower when the water source was a deep well and not phreatic groundwater. In another publication, Pérez-Carrera and Fernández-Cirelli reported arsenic concentrations in water, milk and bovine tissues from farms near Córdoba, Argentina [36]. Groundwater contained 230 - 2540 µg As/L. In milk, the arsenic concentration was between 2.8 and 11 µg As/L. Livers contained 90 - 160 µg As/kg dry mass (dm) and kidneys 120 - 365 µg As/kg dm.

Total arsenic concentration has been investigated in tissues, blood and excretes of numerous terrestrial animals all over the world [37]. Usually, arsenic concentrations in animal tissues are below 50 µg/kg wm [10], with liver and kidney tending to accumulate the highest amounts of arsenic. Table 3 summarizes arsenic concentrations in ruminants’ livers and kidneys that have been reported in literature.

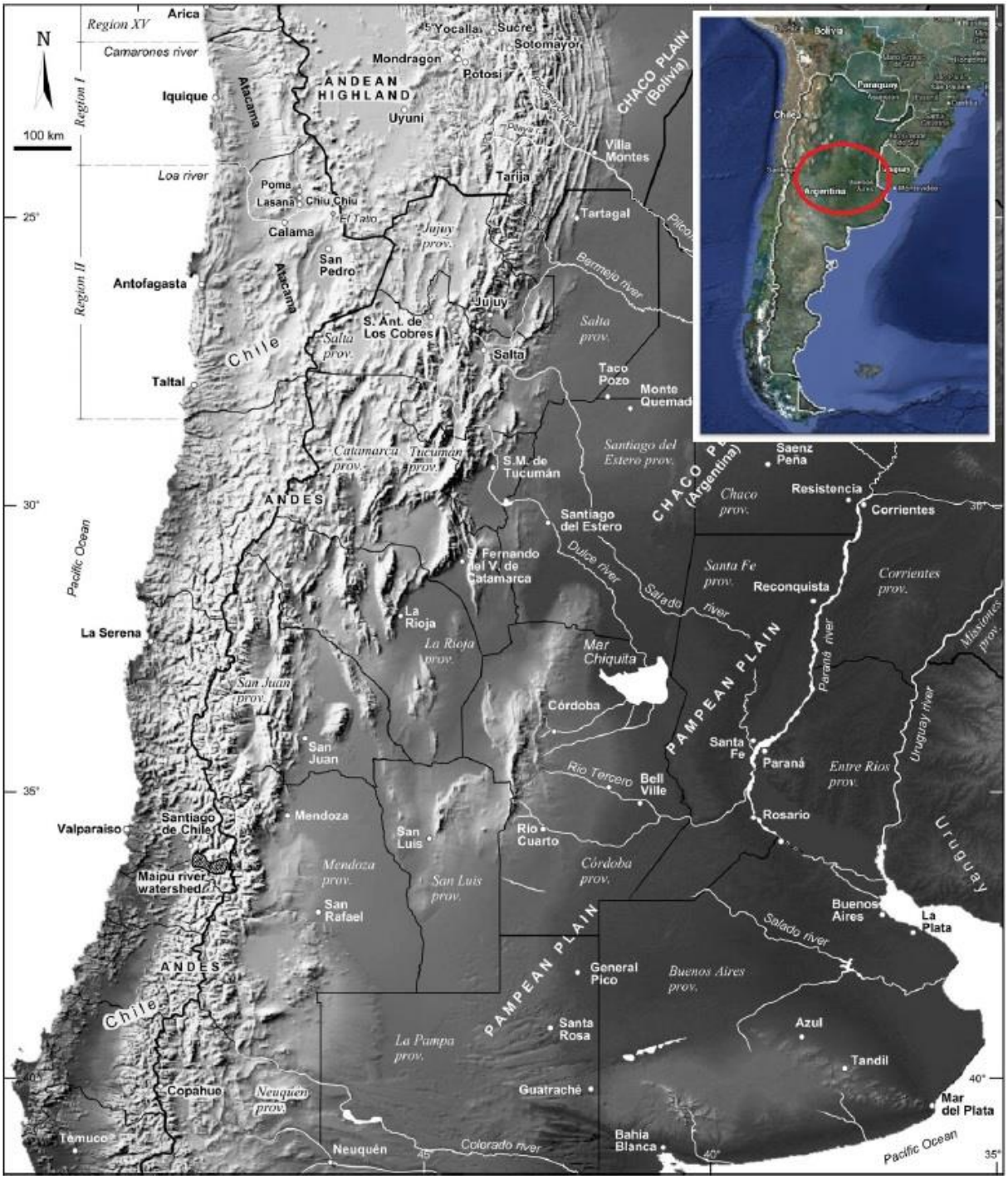


Figure 4: Big map: The Chaco-Pampean plain, Argentina, South America (from Bundschuh et al. [32]), small map: google maps, accessed 19th July 2013

Table 3: Total arsenic concentrations in ruminants' tissues in literature in µg/kg dry mass. (*concentrations were given on a wet mass basis, values were converted to dry mass assuming a dry mass content of 30 % in livers and 20 % in kidneys; *not a ruminant, but similar digestion system; n.a. = not available)

Animal	Origin	Number of samples	Liver [µg As/kg dm]			Kidney [µg As/kg dm]			Reference
			Mean	Median	Range	Mean	Median	Range	
Cattle	n.a.	>100	600	n.a.	n.a.	n.a.	n.a.	n.a.	[38]
Cattle	Australia	177	n.a.	n.a.	< 65 – 300*	150 ± 200*	n.a.	< 100 – 1000*	[39]
Cattle	Canada	n.a.	n.a.	n.a.	< 230 – 500*	n.a.	n.a.	< 350 – 550*	[40]
Cattle	Netherlands	118 (liver), 180 (kidney)	45 ± 30*	n.a.	< 3 – 260*	240 ± 160*	n.a.	10 – 900*	[41]
Cattle	USA	n.a.	500*	n.a.	n.a.	n.a.	n.a.	n.a.	[42]
Cattle	Sweden	68 (liver), 29 (kidney)	< 50*		< 50 – 2100*	75 ± 75*		< 75 – 325*	[43]
Cattle	Slovenia	3 (liver), 28 (kidney)	< 170*	n.a.	n.a.	< 250*	n.a.	n.a.	[44]
Cattle	Spain	494	n.a.	33*	< 25 – 1340*	n.a.	65*	< 35 – 2690*	[45–47]
Cattle	Spain	312	170*	n.a.	n.a.	330*	n.a.	n.a.	[48]
Cattle	Australia (former gold mine)	14	n.a.	n.a.	120 - 300	n.a.	n.a.	n.a. - 450	[49]
Cattle	Spain	120	< 55*	n.a.	n.a.	n.a.	n.a.	n.a.	[50]
Cattle	Jamaica	100	170*	n.a.	< 33 – 3700*	530*	n.a.	20 – 6800*	[51]

Animal	Origin	Number of samples	Liver [µg As/kg dm]			Kidney [µg As/kg dm]			Reference
			Mean	Median	Range	Mean	Median	Range	
Cattle	Croatia	86	n.a.	n.a.	n.a.	n.a.	10*	10 – 2500*	[52]
Cattle	Argentina	24	127 ± 30*	n.a.	90 – 160*	220 ± 85*	n.a.	120 – 365*	[36]
Cattle	Egypt	300	17 ± 3*	n.a.	n.a.	75 ± 10*	n.a.	n.a.	[53]
Cattle	Zambia	51	n.a.	n.a.	1 - 20	n.a.	n.a.	10 - 70	[54]
Cattle	Zambia	112	n.a.	7*	n.a. – 33*	n.a.	25*	20 – 140*	[55]
Cattle	Belgium	n.a.	n.a.	n.a.	n.a. – 100*	n.a.	n.a.	200 – 350*	[56]
Sheep	n.a.	450	n.a.	n.a.	< 10 - 150	n.a.	n.a.	< 10 - 150	[38]
Sheep	Netherlands	40	17*	n.a.	< 3 – 83*	55*	n.a.	< 5 – 220*	[57]
Sheep	Poland	100	< 1	n.a.	n.a.	n.a.	n.a.	n.a.	[58]
Sheep	Croatia	25	n.a.	n.a.	n.a.	n.a.	70*	35 – 1150*	[52]
Sheep	UK (seaweed eating)	11	292 ± 99	n.a.	145 - 477	565 ± 193	n.a.	281 - 916	[59]
Goat	n.a.	54	25 ± 16	n.a.	n.a.	28 ± 14	n.a.	n.a.	[60]

Animal	Origin	Number of samples	Liver [µg As/kg dm]			Kidney [µg As/kg dm]			Ref.
			Mean	Median	Range	Mean	Median	Range	
Moose	USA	81	500	n.a.	n.a.	n.a.	n.a.	n.a.	[61]
Moose	Canada	5	30	n.a.	n.a.	30	n.a.	n.a.	[62]
Reindeer	Norway	29	80*	45*	20 – 520*	n.a.	n.a.	n.a.	[63]
Deer	Canada	3	60	n.a.	n.a.	n.a.	n.a.	n.a.	[62]
Red deer	Spain	72	45 ± 6	n.a.	n.a.	n.a.	n.a.	n.a.	[64]
Hippo-potamus**	Zambia	16	n.a.	30	10 - 90	n.a.	n.a.	n.a.	[65]
Llama**	Argentina	14	n.a.	n.a.	570 – 1500*	n.a.	n.a.	n.a.	[66]

2.1.4 Arsenic metabolism

2.1.4.1 General aspects

The first mechanism for arsenic metabolism in mammalian organisms was proposed by Challenger in 1945 [67]. He proposed alternating reduction of pentavalent arsenicals to their trivalent forms and oxidative methylation of the trivalent compounds, starting from arsenate and ending at trimethylarsine (TMA, Me_3As, see Scheme 1).

$$As(V) \xrightarrow{2e^-} As(III) \xrightarrow{Me} MA(V) \xrightarrow{2e^-} MA(III) \xrightarrow{Me} DMA(V) \xrightarrow{2e^-} DMA(III) \xrightarrow{Me} TMAO(V) \xrightarrow{2e^-} TMA$$

Scheme 1: Arsenic metabolism scheme by Challenger, 1945

During the last decades, a lot of research has been devoted to the arsenic metabolism in mammals, mainly humans and laboratory animals (mice, rats, rabbits, monkeys), and many reviews have been published. [1, 19, 23, 68–77] A common finding was that trivalent arsenicals form strong bonds to proteins and thiols, particularly glutathione (GSH, Figure 5). S-adenosylmethionine (SAM, Figure 6) serves as methyl donor. Furthermore, arsenic methyltransferase (Cyt19, also called As3MT) was identified to play an essential role in the arsenic metabolism.

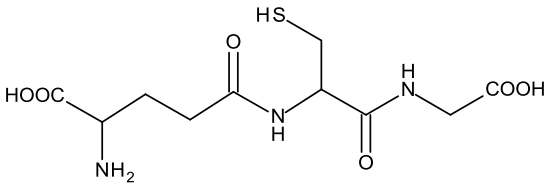


Figure 5: Structure of glutathione (GSH)

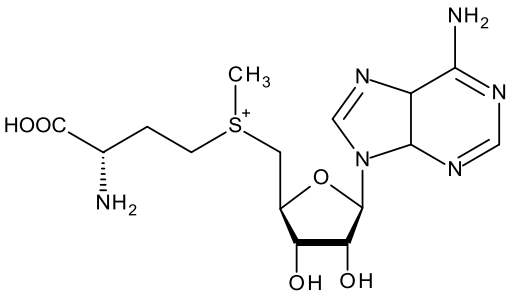
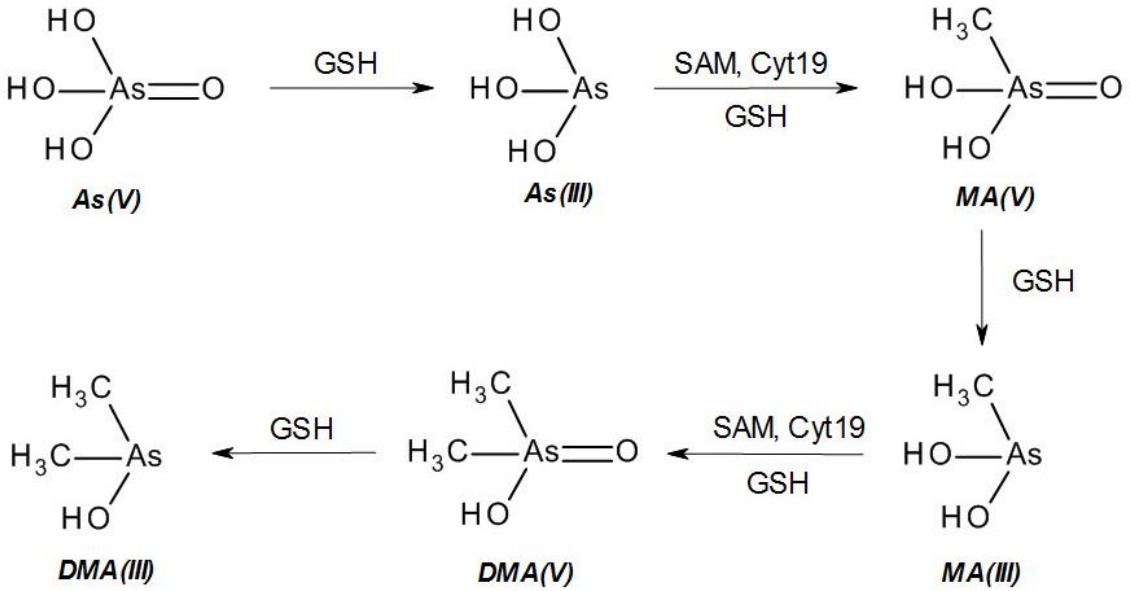


Figure 6: Structure of S-adenosylmethionine (SAM)

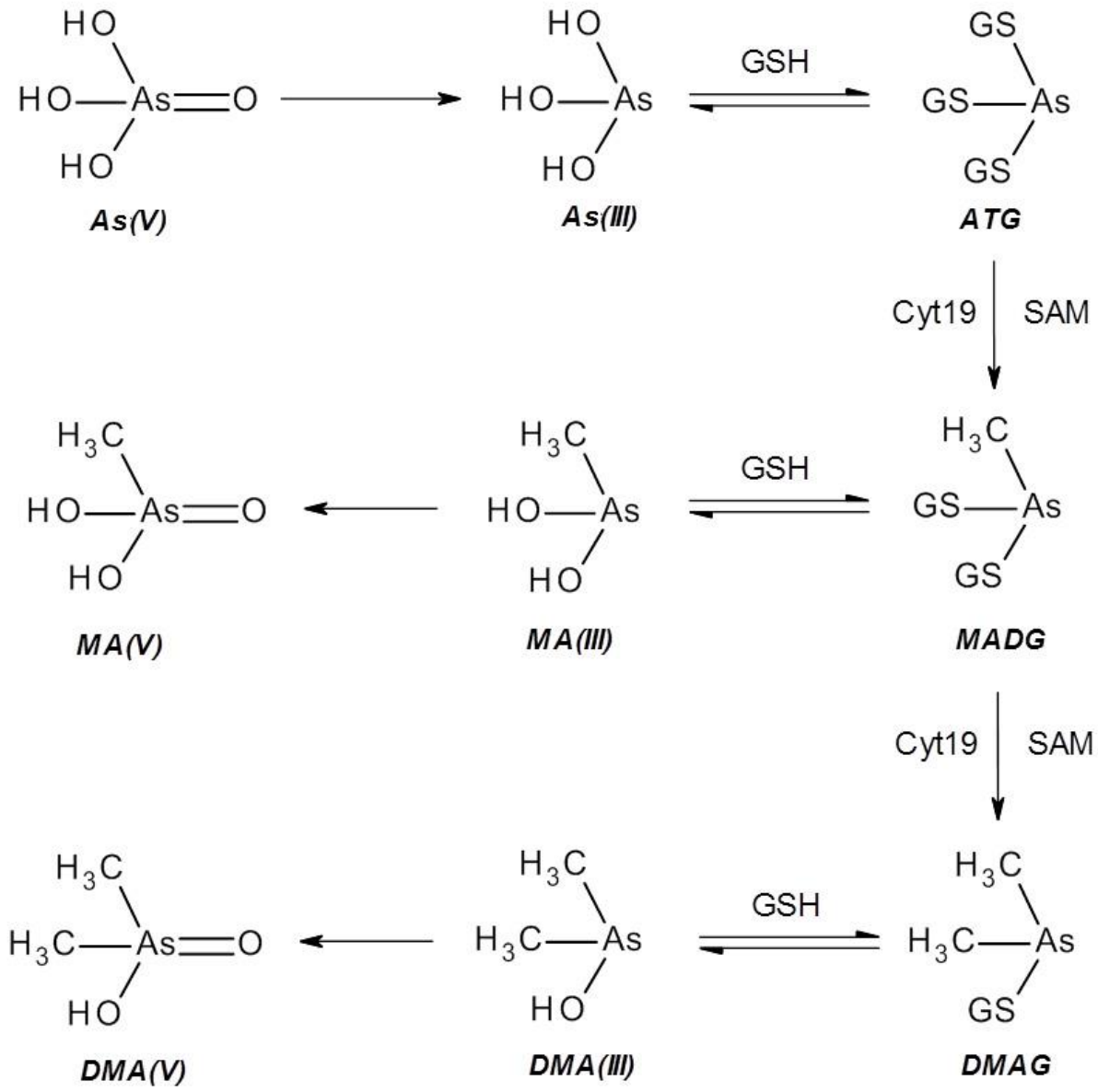
Generally, two different pathways of the arsenic metabolism in mammals have been suggested: The first one, the “classical” pathway, adapts the original ideas of Challenger and is shown in Scheme 2. In the presence of GSH, As(V) is reduced to As(III), then gets methylated to MA(V), which is again reduced and then methylated to DMA(V). Further reduction leads to DMA(III).



Scheme 2: Classical metabolic pathway of inorganic arsenic

In 2005, Hayakawa et al. introduced a different mechanism (Scheme 3) [78]. Like in the classical pathway, the first step is the reduction of As(V) to As(III). Then As(III) reacts with GSH to form arsenic triglutathione (ATG). Methylation of ATG yields methylarsonic diglutathione (MADG) and further on dimethylarsinic glutathione (DMAG). MADG and DMAG are in equilibrium with the corresponding trivalent forms of MA and DMA, which are finally oxidized to MA(V) and DMA(V).

The main organ for arsenic methylation is the liver [76]. It is generally accepted that the kidney is the major pathway of arsenic excretion. Therefore, especially urine, liver and kidney samples as well as isolated hepatocytes of different animal species have been subject of numerous investigations.



Scheme 3: Metabolic pathway of inorganic arsenic, as proposed by Hayakawa et al. [78]

2.1.4.2 Arsenic species in urine

One of the earliest publications on arsenic speciation in urine of terrestrial mammals was by Lakso and Peoples in 1975 [79]. They determined “inorganic” and “methylated” arsenic in urine of cows and dogs. Since then research focused almost entirely on laboratory animals (mice, rats, hamsters, rabbits, guinea pigs and monkeys/apes) and humans.

The major metabolites in the urine of these mammals are DMA, MA and inorganic arsenic. In the urine of unexposed humans the total arsenic concentration is usually between 5 and 10 µg/L [80], and the major arsenic metabolites are 60 - 80 % DMA, 10 - 20 % MA and 10 - 20 % inorganic arsenic [72]. For mice, rats, hamsters, rabbits and rhesus monkeys, the arsenic speciation in urine is similar to that of humans; only the amount of MA is significantly

lower [73]. However, some mammals, like guinea pigs, chimpanzees, marmoset monkeys, tamarin monkeys and squirrel monkeys, do not seem to have the ability to methylate inorganic arsenic [74, 81]. Hardly any MA or DMA can be found in their urine. A reason for this might be their lack of arsenic methyltransferase activity [75]. A possible explanation for this phenomenon is that those mammal species all have evolved from trypanosome endemic areas. Trypanosomes are organisms that are responsible for example for sleeping sickness or Chagas disease. Interestingly, some arsenicals like Melarsoprol have been or are being used against sleeping sickness.

Little is known about arsenic speciation in urine of other terrestrial mammals. Exceptions are the investigations of sheep in Northern Scotland, for example by Feldmann et al. [59]. The sheeps' diet consisted mainly of seaweed, which is known to contain large amounts of arsenosugars. Still, despite this unusual diet, the major arsenic species in the animals' urine was DMA. Other arsenicals like MA, inorganic arsenic, DMAE, TETRA and several unknown compounds were present in smaller amounts [82]. The urine of control sheep that were kept on grass only contained DMA and inorganic arsenic, other arsenicals were not detectable [59]. There is also a publication by Assis et al about arsenic species in samples of horses after intramuscular injection of MA [83]. Shortly after the administration of MA they only found MA in the horses' urine, but 5 days after the last injection, DMA was the main arsenical, accounting for 75 % of the urinary arsenic.

2.1.4.3 Arsenic species in liver and kidney

Although it is well known that DMA is the major metabolite that is excreted via the urine, little is known about the arsenic species that are present in tissues like liver or kidneys. It is thought that DMA is also the dominating arsenical in tissues [84], but evidence is scarce.

Most investigations on this topic included additional administration of different forms of arsenic. This only provides limited information about the natural occurrences of the arsenic species in the tissues. The result of the administration of high doses of As(V) or As(III) often was that inorganic arsenic was found to be the major arsenical in livers and kidneys [85–87]. Still, many publications identified DMA as the major metabolized arsenic compound in livers and kidneys of different mammals (sometimes ignoring the abundance of inorganic arsenic, the "not metabolized arsenic") [84, 88–92].

It has been reported that MA became more abundant than DMA in various tissues when larger amounts of As(III) were present [87, 93–95]. This was also observed in samples of free-living voles: While tissues of animals from an uncontaminated environment showed more DMA and inorganic arsenic than MA, samples of voles that had been living on strongly

contaminated sites contained mainly MA and inorganic arsenic, and only little DMA [96, 97]. Benramdane et al. observed that after acute poisoning with As(III), MA became more abundant than DMA in human tissues [17]. It has been reasoned that large amounts of As(III) inhibit the formation of DMA from its precursors [87].

2.1.5 Official limits

Although arsenic is the number one on the “Priority List Of Hazardous Substances” of the Agency for Toxic Substances and Disease Registry (ATSDR) [98], there are hardly any limits given by official authorities.

The World Health Organization (WHO) set up a guideline of a maximum of 10 µg/L for arsenic in drinking water (1993) [16]. The European Union [99], the U.S. Environmental Protection Agency (EPA) [100] and a number of other national agencies have adopted this value.

In 1989 the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a provisional tolerable weekly intake (PTWI) of 15 µg As/kg body mass [101]. This would mean that an average person of about 70 kg should not consume more than about 1 mg of arsenic per week via the food. However, this PTWI has been withdrawn in 2011 without replacement [102].

The Agency for Toxic Substances and Disease Registry (ATSDR) gives minimal risk levels (MRLs) for arsenic. An MRL is defined “as an estimate of daily human exposure to a substance that is likely to be without an appreciable risk of adverse effects (noncarcinogenic) over a specified duration of exposure” [15]. For inorganic arsenic, the oral MRLs are 5 µg/kg/day for acute-duration (up to two weeks) and 0.3 µg/kg/day for chronic-duration (more than one year).

In Europe, the total arsenic intake levels for humans are ranging from 0.45 to 4.3 µg/kg body mass per day [99]. Generally, people from landlocked countries like the Slovak Republic or Hungary only ingest very little arsenic (0.45 and 0.60 µg As/kg body mass for Slovak Republic and Hungary, respectively), whereas people from regions where fish and seafood account for a large part of the diet, for example Norway or Sweden, are consuming larger amounts of total arsenic (4.3 and 2.5 µg As/kg body mass for Norway and Sweden, respectively). This is mostly because of the high concentrations of organic arsenicals (mainly arsenobetaine) in marine food.

The European Food Safety Authority (EFSA) Panel on Contaminants in the food chain (CONTAM Panel) estimated that exposure to inorganic arsenic from water and food is between 9 and 40 µg As per day for an average consumer [99].

In 2013, the U.S. Food and Drug Administration (FDA) proposed an action level for inorganic arsenic in apple juice (10 µg/L) [103]. They assured that the As concentration in apple juice is usually very low and that all of the tested samples so far had been below this value.

There is also a regulation by the FDA for the “Specific Tolerances for Residues of New Animal Drugs” [104]. For total arsenic, up to 0.5 mg/kg in uncooked muscle tissue and up to 2 mg/kg in uncooked liver and kidney are accepted. This applies to chicken, turkey and swine. Other animals like cattle are not mentioned.

Roggeman et al. stated that 1 - 5 mg As/kg wet mass (wm) in bovine liver and kidney is classified as “high”, and above 5 mg As/kg wm, the concentration is considered poisonous [56].

Especially the different toxicity of the various arsenic compounds makes it difficult to agree on general limits for food. Still, there is an urgent need for worldwide maximum permissible total and species specific arsenic levels in food and beverages.

2.2 Schönbrunn Zoo

Schönbrunn Zoo (in German: Tiergarten Schönbrunn) is located on the grounds of Schönbrunn Castle in Vienna, Austria. The zoo was opened in 1752, which makes it the oldest still existing zoo of the world [108]. Since 2007 it is managed by Dr. Dagmar Schratter. It accommodates a great variety of animals which allowed us to obtain urine samples of many different terrestrial mammals in a very convenient manner.

2.3 Inductively coupled plasma mass spectrometry

Inductively coupled plasma mass spectrometry (ICPMS) is a powerful technique for the analysis of elemental concentrations. It is possible to measure most of the elements of the periodic table of elements “simultaneously”. Isotopic information, a high linear range (10^9, newest machines even up to 10^{11}) and excellent limits of detection (as low as < 1 ng/L, depending on element, sample matrix etc.) make it the method of choice for a lot of tasks from different areas, like semiconductor industry, pharmaceutical products or environmental chemistry. It is possible to analyze liquids as well as solids or gaseous samples; although in most cases liquid samples are analyzed.

2.3.1 Set-up

A scheme of an ICPMS can be seen in Figure 7.

Liquid samples are usually taken up by the autosampler needle and get pumped to the nebulizer. There, the liquid sample gets split into small droplets and forms an aerosol. Droplets that are larger than about 10 µm are removed in the spray chamber and pumped into the waste. Smaller droplets continue to the torch.

The torch consists of three concentric quartz tubes. The innermost one is for the carrier gas, which brings the sample aerosol from the nebulizer via the spray chamber to the plasma. The middle tube is the plasma gas which constitutes the plasma. The third gas is the auxiliary gas. In total, the gas flow in the torch is between 15 and 20 L/min. The ICPMS instruments that were used in the present work were all operated with argon.

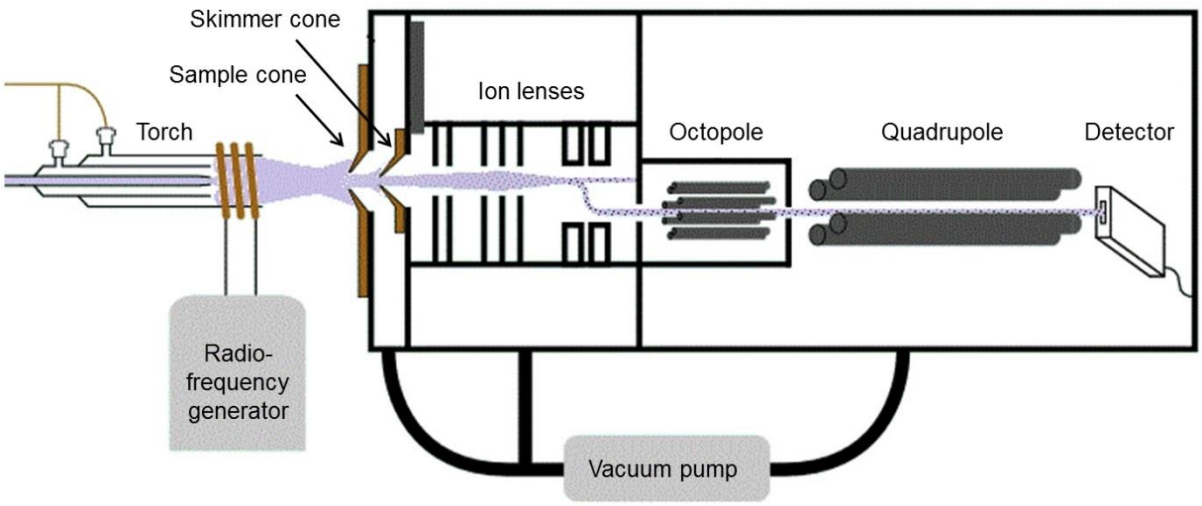


Figure 7: Scheme of an ICPMS, adapted from Dunnivant and Ginsbach [105]

In our setting, we introduced another gas flow, the so called optional gas (5 % CO_2 in Ar), between spray chamber and torch [106]. The reason for the addition of CO_2 is the so called carbon enhancement effect of arsenic (and other elements). It has been observed that a higher carbon concentration in the plasma can increase the arsenic signal up to a factor 5 [107]. When using samples of organic origin, the carbon load can vary a lot which leads to unsteady and not comparable arsenic signals. By adding CO_2, a continuously high carbon background is created which compensates for possible carbon concentration differences in the samples. Furthermore, the enhancement of the arsenic signal leads to better detection limits and allows the detection of even lower concentrations of the element.

At the end of the torch, a Tesla spark induces the plasma. It is then kept up by a high frequency coil around the torch. A plasma is an ionized gas, often called the forth state of matter. In our case, the plasma in the ICPMS consists of Ar^+ ions and reaches temperatures of around 6000-10000 K.

Due to the high temperatures in the plasma, and because most elements have a lower first ionization potential than argon (15.75 eV), the introduced sample gets dried, evaporated, atomized and finally ionized. Ideally, all ions generated in the plasma are single charged cations. Still, some elements get ionized more easily and can occur as doubly charged cations. Also, recombinations of ions can take place, one of the most prominent interference classes being oxides.

The ions are transported from the plasma to the interface (Figure 7). It consists of sample cone and skimmer cone, which are round metal plates (for example: Ni, Cu, Pt) with small round holes. The interface leads the ions from the plasma (atmospheric pressure) to the mass spectrometer (room temperature and high vacuum).

The ion lenses are focusing the ion beam and remove photons, electrons and uncharged particles.

The next part is the collision/reaction cell. It is used to remove interferences from the ion beam. The Agilent version consists of an octopole; 8 rods with applied current keep the ions on track. Depending on the operating mode, a collision gas (for example He) or a reaction gas (for example H_2) is introduced into the cell with about 4 mL/min.

A collision gas simply collides with the ions and reduces their kinetic energy. Polyatomic ions are hit more often, because they have a larger cross section than the analyte ions. An energy barrier between octopole and quadrupole excludes all ions that have too little kinetic energy from analysis. This mechanism is called Energy Discrimination (ED). Sometimes, collisions

can lead to dissociation of polyatomic ions, which is called Collision Induced Dissociation (CID).

The collision gas mode plays an important role when analyzing arsenic. As^+ and ArO^+ both have 75 m/z. To avoid the ArO^+ interferences, the collision gas mode is used with He as collision gas.

All ions that pass the collision/reaction cell enter the mass filter. Different types are possible, but in this work we were using a quadrupole. It is made up by 4 rods that are arranged in a square. Direct and alternating currents are applied and form a field in which only ions of one mass to charge (m/z) ratio can continue on a sTable path to the detector. By alternating the current settings, the allowed m/z is changed.

At the end, the detector transforms the arriving ion beam into an electric signal which can then be monitored on a computer.

2.4 High performance liquid chromatography

A common technique for the separation of different chemical substances is high performance liquid chromatography (HPLC).

In principle, the sample is transported in a mobile phase (“eluent”) through a stationary phase (“column”). Interactions between stationary and mobile phase result in separation of the analytes. Depending on the types of interaction, we can distinguish between three different separation mechanisms. They are adsorption, partition and size exclusion. We will briefly go through the typical setup of an HPLC system and then explain the separation techniques that have been used in this work.

2.4.1 Setup

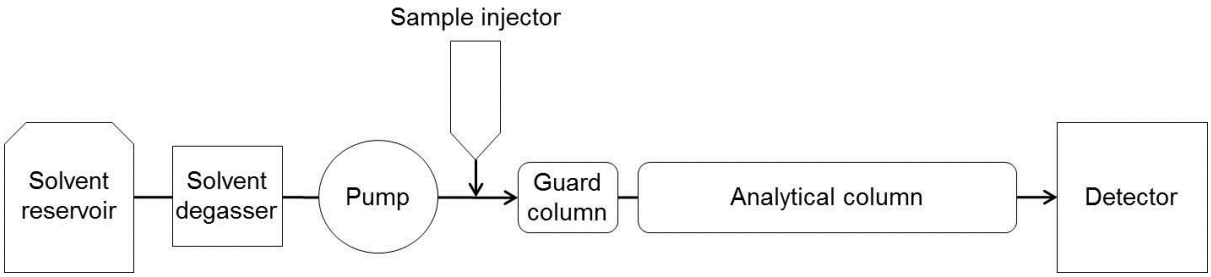


Figure 8: Scheme of an HPLC system

The mobile phase first gets pumped through a solvent degasser. Then, a small amount of the sample is injected. The mobile phase, loaded with the sample, passes through the column where separation takes place. Usually, a guard column is installed in front of the analytical column to protect the latter one. After the column, a detector registers the arriving substances, and a chromatogram (time resolved signal) is recorded.

Depending on the application, different detectors are at hand. Universal but unselective types are for example photometric, conductivity or refraction index detectors. On the other side there are molecule-selective (for example electrospray ionization mass spectrometry) and element-selective (for example ICPMS) detectors. A scheme of an HPLC can be seen in Figure 8.

2.4.2 Separation techniques

The stationary phase consists of small particles with a diameter of only a few µm (about 2 – 10 µm). Materials used for these particles are typically either silica or organic polymers. These particles are also referred to as the “backbone”. The surface of the particles can be

modified by attaching different functional groups, depending on the type of the column. Interaction usually only takes place between the mobile phase and the functional groups. Still, in some cases the analytes can react with the column backbone as well. In this thesis, we used ion-exchange and reversed-phase (RP) chromatography.

2.4.2.1 Ion-exchange chromatography

The stationary phase is modified on the surface with charged molecules. Anion-exchange columns have a positively charged stationary phase (for example, amines or quaternary ammonium groups) and are used to separate anionic compounds whereas cation-exchange chromatography uses a negatively charged stationary phase (usually modified with sulfonic acids or carboxylic acids) to separate cations in the mobile phase.

The mobile phase typically is an aqueous solution; different buffers are often used. Ionic compounds in the mobile phase (“counter ions”) are interacting with the ions on the stationary phase and form ionic bonds of different strength. The ions from the solvent are competing with the analyte ions for the free positions on the stationary phase. Separation can be influenced by varying different parameters like the pH or the ionic strength.

2.4.2.2 Reversed-phase (RP) chromatography

RP stationary phases consist of particles that are modified with long alkyl chains and are therefore nonpolar (hydrophobic). Prominent column types are C8 or C18. As mobile phase, polar solvents like water, methanol or acetonitrile are used. In principle, nonpolar analytes are retained much longer than polar compounds. The retention is primarily based on adsorption forces. RP is by far the most commonly used type of chromatography.

2.4.3 Preparative HPLC

Preparative HPLC is used to separate one or more compounds from a sample. Its functioning is the same as in analytical HPLC, but with larger volume dimensions. After passing through the column and an optional detector, the mobile phase can be collected in fractions. Usually, fractions that contain a compound of interest are used for further work (purification, analysis, synthesis, etc.).

3 Experimental

3.1 Instruments

3.1.1 ICPMS and HPLC

Agilent Technologies (Waldbronn, Germany):

7500ce ICPMS, equipped with an ASX-500 autosampler (Cetac, Nebraska, USA), a MicroMist nebulizer, a Scott-type spray chamber and a collision/reaction cell

7700x ICPMS, equipped with an Integrated Autosampler, a MicroMist nebulizer, a Scott-type spray chamber and a collision/reaction cell

1200 Series HPLC, equipped with a degasser, a quaternary pump, a thermostatted autosampler and a thermostatted column compartment

1260 Infinity HPLC, equipped with a degasser, a binary pump, a thermostatted autosampler and a thermostatted column compartment

1200 Series Preparative HPLC, equipped with a degasser, a preparative pump, a preparative autosampler, a VWD and a preparative fraction collector

MassHunter Workstation Software for ICPMS, G7201B, Version B.01.02

ChemStation Software for LC systems, Rev. B.04.05

3.1.2 HPLC columns

Hamilton (Bonaduz, Switzerland):

Anion-exchange: PRP-X100, 150 x 4.6 mm, 5 µm, serial numbers (SN): 601 and 872

Anion-exchange: PRP-X100, 150 x 2.1 mm, 5 µm, SN: 53

Guard column: PRP-X100, PEEK, 8 x 3 mm, 10 µm

Agilent Technologies (Waldbronn, Germany):

Anion-exchange: Zorbax SAX Analytical 150 x 4.6 mm, 5 µm, SN: USK003325

Guard column: Zorbax SAX Analytical, 12.5 x 4.6 mm, 5 µm

Cation-exchange: Zorbax 300-SCX Analytical, 150 x 4.6 mm, 5 µm, SN: USSD004438

Guard column: Zorbax SCX Analytical, 12.5 x 4.6 mm, 5 µm

Reversed-phase: Zorbax SB-C8 STable Bond Analytical, 150 x 4.6 mm, 5 µm, SN: USSH011476 and USSH011598

Preparative: PrepHT SB-C8, 150 x 21.2 mm, 5 µm, SN: USJH001047

3.1.3 Other intstruments

Balances: Denver Instrument®, SI-234, (Goettingen, Deutschland)

Pipettes:

Acura 825 autoclavable, 100 - 1000 µL, 10 - 100 µL, Socorex (Ecublens, Switzerland)

Bibby Sterilin, 100 µL and 500 µL, Bibby Scientific Limited (Staffordshire, United Kingdom)

Lyophilization: Christ® Gamma 1-16 LSC, Martin Christ Gefriertrocknungsanlagen GmbH (Osterode, Germany)

Liquidizer: Multipro FP920 002, calibrated glass, 1.5 liters, Kenwood Electronics Deutschland GmbH (Bad Vilbel, Germany)

Ultra Centrifugal Mill: Retsch® ZM200, 1100 W, 6000 rpm, 12 teeth rotor, ring sieves: 4 mm, 0.25 mm; titanium, Retsch GmbH (Haan, Germany)

Microwaves:

Ultraclave III, Software: easyCLAVE 5.25, MLS GmbH (Leutkirch, Germany)

Ultraclave IV, Software: Terminal 1640, MLS GmbH (Leutkirch, Germany)

Centrifugal vacuum concentrator: Maxi dry Lyo, Heto Lab Equipment (Allerød, Denmark), equipped with a Vacuum pump unit, PC 2001, Vario, 2.0 mbar, max 1.6 m^3/h, vacuubrand GmbH&Co.KG (Wertheim, Germany)

Centrifuge: Rotina 420 R, Hettich Lab Technology (Tuttlingen, Germany)

Total Solids Refractometer: TS400, Serum Protein 6.54, Leica Microsystems Inc. (Buffalo, USA)

Ultrasonic bath: Transsonic T 700/H, Elma GmbH&Co.KG (Singen, Germany)

pH-meter: Orion 5 Star, Thermo Scientific (Cambridgeshire, United Kingdom)

3.2 Materials

PP-tubes: polypropylene, Cellstar®, graduated, conical bottom, blue screw cap, sterile, 15 and 50 mL, Greiner bio-one (Kremsmuenster, Austria)

PS-tubes: polystyrene, GLKL, 12 mL Greiner bio-one (Kremsmuenster, Austria)

Centrifuge tubes for preparative HPLC: cylindrical, polypropylene, 31 mL, 24 x 93 mm, Kartell s.p.A. (Noviglio, Italy)

Filters: 25 mm, bright blue, membrane: Nylon (PA), 0.2 µm, Markus Bruckner Analysentechnik (Linz, Austria)

Syringes: Injekt®Solo, Single-use, 2-piece, 2 mL and 10 mL, B. Braun Melsungen AG (Melsungen, Germany)

Parafilm®: 4In.x125FT.Roll, PM-996, Benis Flexible Packaging (Neenah, USA)

HPLC-vials:

microvials PP, 0.7 mL with snap ring, VWR International (Vienna, Austria)

250 µL PP, with snap ring, Agilent Technologies (Waldbronn, Germany)

HPLC-caps: snap ring cap, 11 mm transparent, soft version, red rubber/PTFE, 45° shore A, 1.0 mm, VWR International (Vienna, Austria)

3.3 Chemicals

3.3.1 Standards

CertiPUR® ICP multi-element standard solution VI, for ICP-MS (30 elements in dilute nitric acid), UN2031, Merck KGaA (Darmstadt, Germany)

Single-Element ICP-Standard-Solution Roti®Star, Carl Roth GmbH+Co.KG (Karlsruhe, Germany):

Ca: 10 000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2503.1
Mg: 10 000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2524.1
Na: 1000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2439.1
Fe: 1000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2412.1
Zn: 10 000 mg/L ± 0.2 % in 3 % HNO_3, Art.No.: 2576.1
Cu: 1000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2426.1
Sb: 1000 mg/L ± 0.2 % in 20 % HCl, Art.No.: 2398.1
Ge: 1000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 1419.1
In: 1000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 26123.1
Se: 1000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2461.1

Peak Performance, Single Element Standard, CPI International (Santa Rosa, USA):

K: 10 000 ± 30 µg/mL in 1 % HNO_3, P/N S4400-10M411
Rb: 1000 ± 3 µg/mL in 2 % HNO_3, P/N S4400-1000451
Mn: 1000 ± 3 µg/mL in 2 % HNO_3, P/N S4400-1000321
Hg: 1000 ± 3 µg/mL in 2 % HNO_3, P/N S4400-1000331
As: 1000 ± 3 µg/mL in 2 % HNO_3, P/N S4400-100031
Lu: 10 ± 0.05 µg/mL in 2 % HNO_3, P/N S4400-010031

Prepared in-house (Analytical Chemistry, University of Graz):

Arsenate (As(V)): 1000 mg/L, from Na_2HAsO_4*7 H_2O, Merck KGaA (Darmstadt, Germany)

Arsenite (As(III)): 1000 mg/L, from $NaAsO_2$, Merck KGaA (Darmstadt, Germany)

Methylarsonic acid (MA): 1000 mg/L, from $NaAsO_2$ and MeI (Meyer reaction) [14]

Dimethylarsinic acid (DMA): 1000 mg/L, from sodium dimethylarsinate, Fluka (Buchs, Switzerland)

Arsenobetaine bromide (AB): 1000 mg/L, from trimethylarsine and bromoacetic acid according to McShane (PhD thesis, 1982, cited by Goessler and Pavkov [109])

Trimethylarsinoxide (TMAO): 1000 mg/L, synthesized according to Merijanian and Zingaro [110]

Arsenocholine (AC): 1000 mg/L, synthesized according to Irgolic et al. [111]

Tetramethylarsonium iodide (TETRA): 1000 mg/L, from trimethylarsine and methyliodide according to McShane (PhD thesis, 1982, cited by Goessler and Pavkov [109])

Dimethylarsinoylacetate (DMAA): 6.4 mg/L, synthesized according to Francesconi, Edmonds and Stick, 1989 [112]

Dimethylarsoniopropionate (DMAP): 10 mg/L synthesized according to Rumpler [113]

Dimethylarsinobutanate (DMAB): 100 µg/L, synthesized according to Francesconi, Edmonds and Stick, 1992 [114]

Arsenosugars: 4 anions: 2.5 µg/L, Gly: 28.85 µg/L, Phos: 25.8 µg/L, SO3: 295 µg/L, SO4: 518 µg/L, synthesized according to Madsen et al. [115]

3.3.2 Certified reference materials

NIST (Gaithersburg, USA):

Standard Reference Material® 1643e, Trace Elements in Water

Standard Reference Material® 1640a, Trace Elements in Natural Water

Standard Reference Material® 2669, Arsenic Species in Frozen Human Urine

NRC-CNRC (Ottawa, Canada):

DOLT-3, Dogfish Liver Certified Reference Material for Trace Metals

DORM-2, Dogfish Muscle Certified Reference Material for Trace Metals

Seronorm™ Trace Elements, Urine, BLANK, Lot FE1113**,** Sero AS (Billingstad, Norway)

Certified Reference Material No.18 “Human Urine”, NIES (Tsukuba, Japan)

3.3.3 Other chemicals

Ultrapure water ("milli-q"): resistance: 18.2 MΩ*cm, Millipore (Bedford, USA)

Orthophosphoric acid: 88.85 %, Fisher Scientific (Loughborough, United Kingdom)

Carl Roth GmbH+Co.KG (Karlsruhe, Germany):

Nitric acid: ≥65 % p.a., Rotipuran®, subboiled in-house (Analytical Chemistry, University of Graz)

Hydrogen peroxide: 30 % p.a., Rotipuran®, stabilized

Ammonia solution: Rotipuran® ≥ 25 %, p.a

Maleic acid: ≥ 99 % Ph.Eur., BP, Art.No.: 12304.2

Trifluoroacetic acid : ≥ 99.9 % for (peptide) synthesis

Methanol: Rotisolv® HPLC, ≥ 99.5 %

Merck KGaA (Darmstadt, Germany):

Acetic acid: 96 %, pro analysis

Pyridine: ≥ 99.5 %, pro analysis

Sodium sulfide: pro analysis, about 35 % Na_2S, Na_2S*xH_2O (x=7-9), M= 78.05 g/mol

Messer (Gumpoldskirchen, Österreich):

Argon: 5.0

Helium: 6.0

Optional gas: CO_2 (1 % v/v), 4.5 in Ar 5.0

3.4 Software

Calculations: Microsoft© Excel 2010

Figures: SigmaPlot 12.3

Writing: Microsoft© Word 2010

3.5 Sampling

3.5.1 Water

We collected samples from 4 different groundwater wells at pastures and one sample from a river (Río Luján) in the area of Mercedes, Buenos Aires Province, Argentina, on 6th April 2013 (Table 4, Figure 10). We collected samples ME01, 02 and 04 directly from the pipe that transports the ground water to the ponds (Figure 9). The well ME05 was out of order, so we took the samples from the water in the pond where a lot of algae were growing. Another sample was taken from the Lake Capri in the region of mount Fitz Roy which is said to have very good drinking water quality. All samples were collected and stored in PP-tubes in a refrigerator at about 4°C for most of the time, except the transport.

Table 4: Location of the wells of the water samples from Argentina

Original name	New sample name	GPS data
ME01	Well 1	S 34° 35.785', W 59° 28.442'
ME02	Well 2	S 34° 36.160', W 59° 28.435'
ME03	River	S 34° 37.971', W 59° 26.016'
ME04	Well 3	S 34° 42.354', W 59° 32.581'
ME05	Well 4	S 34° 42.261', W 59° 31.971'
Lake Capri	Lake Capri	Around S 49°, W 73°

Figure 9: Water sampling in March 2013

3.5.2 Bovine tissues

3.5.2.1 Tissue samples from Argentina

We obtained 13 liver and 6 kidney samples of Holstein cattle from four different areas in Argentina (Figure 10), slaughtered at local slaughterhouses. The areas were Ballesteros, (Córdoba Province, “BA”), Bell Ville (Córdoba Province, “BV”), Morrison (Córdoba Province, “MO”) and Buenos Aires (Capital Federal, “CF”). Each tissue sample had a code for identification (Table 5). The samples were freeze-dried at the University of Buenos Aires before we obtained them. They were present as rough granulates and stored in 15 mL PP-tubes at room temperature.

Table 5: List of kidney and liver samples from Argentina

Sample code	Type	Origin	Amount [g]
BAR02R1	Kidney	Ballesteros	2.4
BAR04R1	Kidney	Ballesteros	3.1
BAR07R1	Kidney	Ballesteros	3.1
BAR09R1	Kidney	Ballesteros	2.8
BVR01R1	Kidney	Bell Ville	3.2
BVR02R2	Kidney	Bell Ville	2.6
BVR07R1	Kidney	Bell Ville	2.9
BVR20R1	Kidney	Bell Ville	2.2
BVR23R1	Kidney	Bell Ville	3.3
BVR25R1	Kidney	Bell Ville	3.4
BVR26R1	Kidney	Bell Ville	3.6
BVH05R1	Liver	Bell Ville	4.0
BVH06X1	Liver	Bell Ville	3.9
BVH18R1	Liver	Bell Ville	3.5
CFR01R1	Kidney	Buenos Aires	3.3
CFR02R1	Kidney	Buenos Aires	3.7
CFH03R1	Liver	Buenos Aires	3.9
MOH03X1	Liver	Morrison	3.4
MOH04R1	Liver	Morrison	4.3

Figure 10: Sampling locations in Argentina. Red = tissues, yellow = water (Google Earth, 19th December 2013)

3.5.2.2 Tissue samples from Austria

We obtained liver and kidney samples from 10 cattle from the slaughterhouse in Graz, Norbert Marcher GmbH, on 11th June 2013. They were kept in small plastic jars for transport, and the amount of each sample was about 100 g. The date of slaughtering was 10th June 2013. Information about the samples is given in Table 6. The origins of the samples are indicated in Figure 11.

Table 6: Kidney and liver samples from Austria

Number	Origin	Date of birth	Earmark
1281	8784 Trieben	25th August 2011	AT287228619
1283	8904 Ardning	8th May 2011	AT312837618
1287	8940 Weissenbach	30th December 2009	AT857777917
1288	8913 Weng	2nd November 2010	AT505217518
1300	8913 Weng/Gesäuse	21st December 2003	AT953511872
1306	8943 Aigen	13th July 2005	AT321564409
1319	8614 Breitenau/H.	9th July 2012	AT988708418
1320	8642 St. Lorenzen/Mzt.	11th July 2012	AT486970322
1322	8652 St. Lorenzen/Mzt.	14th July 2012	AT281290919
1324	8680 Muerzzuschlag	8th October 2012	AT981035519

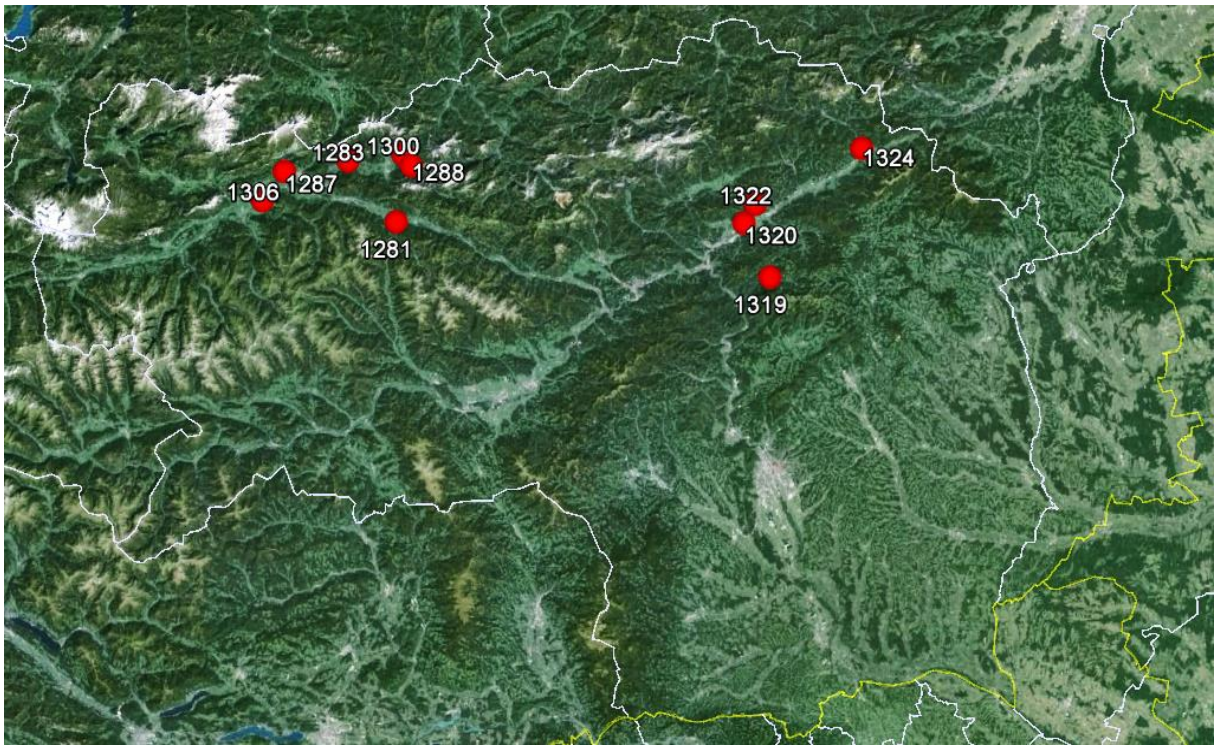


Figure 11: Origins of bovine tissues in Styria, Austria (Google Earth, 19th December 2013)

3.5.3 Urine

3.5.3.1 Cattle

We collected cows' urine samples at two farms in Styria, Austria. One is located in Eichberg near Ilz, Eastern Styria (Fasshold family), and one near Knittelfeld, Upper Styria (Goessler family). From the farm in Eastern Styria, we were able to obtain samples from 4 different cows on 09th June 2013 and later (23rd June 2013) again from 6 different cows, see Table 7. Both times one of the samples was from a calf ("Fanni"). At the farm in Upper Styria, we collected urine samples from 5 different cows (15th June 2013, Table 8). All animals were dairy cows.

We collected the urine in the barns directly from the urinating cows into 50 mL PP tubes. We were able to collect between 25 and 50 mL of fresh urine per cow. All samples were stored at 4°C until analysis.

Table 7: Cows of which urine samples were collected at the farm in Eastern Styria

Cow	Additional information	Collection dates
Emma	3 years old, Fleckvieh cattle	9th June, 23rd June
Hermine	9 years old, Fleckvieh cattle, pregnant	9th June, 23rd June
Frau Kainz	17 years old, Fleckvieh cattle	9th June, 23rd June
Betti	13 years old, Fleckvieh cattle	23rd June
Ermi	12 years old, Fleckvieh cattle	23rd June
Fanni	6 months old, hybrid Fleckvieh-Belgian Blue	9th June, 23rd June

Table 8: Cows of which urine samples were collected at the farm in Upper Styria

Cow	Additional information	Collection date
Bliala	4 years old, Fleckvieh cattle	15th June
Bira	6 years old, Fleckvieh cattle	15th June
Begi	6 years old, Fleckvieh cattle	15th June
Berle	6 years old, Fleckvieh cattle	15th June
Mora	4 years old, Fleckvieh cattle	15th June

3.5.3.2 Animals from the Schönbrunn Zoo

We collected urine samples from various terrestrial mammals at the Schönbrunn Zoo Vienna in two sampling periods. The first one took place between February 2013 and April 2013. During this time we were able to get 22 urine samples from 18 different animals of which 12 were ruminants. The second sampling period was in July 2013 and was used to obtain another two samples from the bison and a second sample from the water buffalo. The zookeepers were equipped with 2 mL syringes and 15 mL PP-tubes. They were told to collect urine samples from the ground, whenever possible. However, the actual sampling procedures were only reported at the second sampling period for the bison and the water buffalo. All samples were stored in labeled 15 mL PP tubes at around 4°C until analysis. The obtained samples are listed in Table 9 and

Table 10. Because of the big time span for collection, the samples were between 1 week and 3 months old when we analyzed them for the first time. The obtained amount of urine varied between 1 and 13 mL per sample.

Table 9: Urine samples of ruminants from the Schönbrunn Zoo

Animal	Additional information	Collection date	Volume [mL]
Bison I		6th March 2013	3
Bison II	“Meneva”, concrete floor with sawdust	5th July 2013	6
Bison III	“Meneva”, sandy ground	6th July 2013	1
Water buffalo I	female	10th March 2013	4
Water buffalo II	“Elfe”, female, “fresh”	15th July 2013	8
Tux cattle I	“Heidi”		5
Tux cattle II	“Hexe”		10
Pustertal pied	“Saliera”		13
Himalayan thar	Female		4
Reindeer I	Female	10th March 2013	2
Reindeer II		6th March 2013	6
Nilgai	Female	11th February 2013	4
Blackbuck			4
Barbary sheep	Female	14th March 2013	4
Pygmy goat		14th March 2013	4
Heidschnucke		8th April 2013	2
Giraffe	Male, 20 years old	7th February 2013	9

Table 10: Urine samples of other mammals (not ruminants) from the Schönbrunn Zoo

Animal	Additional information	Collection date	Volume [mL]
Barbary macaque	Male	6th March 2013	2
Vicuña I		13th February 2013	10
Vicuña II		13th February 2013	10
Collared peccary		7th March 2013	5
Damara zebra		24th April 2013	11
Indian rhinoceros	Female	11th February 2013	6
African elephant I	Male, “Tuluba”?	28th April 2013	12
African elephant II	Female, “Drumbo”	28th April 2013	10
African elephant III	Female, “Numbi”?	28th April 2013	11
African elephant IV	Female, “Tonga”	28th April 2013	12
African elephant V	Male, “Kibo”	28th April 2013	11
African elephant VI	Female, “Mongu”	27th April 2013	2

3.6 Sample preparation

3.6.1 Digestion in general

For determination of total element concentrations we used microwave assisted digestion with nitric acid. The procedure that is described below was applied for all samples. The utilized amounts of sample and nitric acid and the certified reference materials are given in the respective chapters of the different sample types.

We weighed each sample in triplicates into quartz tubes that had been cleaned three times. A certified reference material was prepared as well, which was treated like the other samples in all steps of sample preparation. We added nitric acid (the volume was dependent on the amount and type of the sample) and filled it up to 5 mL with ultrapure water, when necessary.

Every digestion batch also included 3 digestion blanks. They consisted of the same amount of nitric acid and ultrapure water as the samples, but did not contain any sample.

We closed the quartz tubes with PTFE caps and put them into the microwave oven which was equipped with an absorbing solution (300 g ultrapure water and 5 g sulfuric acid). We closed the microwave oven, filled it with 40 bar argon and started the temperature program. The operating power was 1000 W. The system was slowly heated up to 250°C, held there for

30 minutes and then cooled down again. Below about 80°C, the excess pressure was released from the system. Then, the samples could be removed from the microwave oven. We poured the digested samples into PP tubes, rinsed the quartz tubes two times with ultrapure water and then filled the digests to a final acidity of 10 % v/v (usually 10 or 50 mL)

3.6.2 Water

After the transport to Graz, Austria, we added nitric acid (final acidity: 1 % v/v) to the water samples from Argentina. Further on, we acidified all samples to 10 % v/v nitric acid (total dilution factor = 1.1) and then measured them in triplicates with ICPMS. We measured the total element concentrations with and also without filtration of the samples through 0.2 µm Nylon filters.

3.6.3 Bovine livers and kidneys

3.6.3.1 Argentinean samples

The liver and kidney samples from Argentina were already freeze-dried when we obtained them. We ground the granulates with agate mortar and pestle and used the resulting powder for digestion and extraction.

3.6.3.2 Austrian samples

Directly after collection we divided each sample into two parts. One was put into the freezer at -18°C for possible future investigations, and one was cut with a kitchen knife (stainless steel) into small pieces, put into plastic bags and then freeze-dried. For the freeze-drying, we froze the fresh tissue samples with liquid nitrogen and then put them into the freeze dryer. After about three to five days, the samples were dry. The storage area temperature was +14°C, the ice condenser temperature was -54°C, and the pressure was 0.180 mbar.

We calculated the water content by weighing the samples before and after freeze-drying.

Then, we carefully crushed the freeze-dried tissue samples into rough pieces with a hammer while they were still in the plastic bags and then ground them with the ultra centrifugal mill (12 teeth rotor, ring sieve with 0.25 mm holes, 6000 RPM). After each sample, we cleaned the mill with ultrapure water and isopropanol to avoid contamination.

3.6.3.3 Digestion

We weighed 250 mg of each Argentinean and Austrian sample into quartz tubes (triplicates) and digested them with 5 mL nitric acid. As certified reference material we used DOLT-3 (Dogfish liver). After the digestion, we diluted the digests with ultrapure water to a final volume of 50 mL and then measured the total element concentrations with ICPMS.

3.6.3.4 Extraction

We carried out tissue extractions using 0.02 mol/L trifluoracetic acid (TFA), containing 1 % v/v of a 30 % H_2O_2 solution, similar to the method described by Raber et al. [116].

We took 250 mg of tissue sample and added 5 mL of the extracting agent. It was important to be very careful to avoid loss of the samples, because they foamed up immediately after addition of the extractant. Samples were shaken and then sonicated for about 15 minutes at room temperature. They were centrifuged at 3300*g for 20 minutes, again at room temperature. We filtered the extract through 0.2 µm Nylon filters. We carried out the same procedure with a certified reference material (DORM-2) and a blank.

We also used a not oxidizing extraction method by not adding H_2O_2 to the 0.02 mol/L TFA. Thus, the samples were only extracted with TFA. They were shaken, sonicated, centrifuged and filtrated as described above. Only then we added 10 % v/v of a 30 % H_2O_2 solution to the filtrates and let them oxidize for at least one hour at slightly elevated temperatures (around 50°C) before analysis. We only used this method for the Austrian tissue samples and not for the Argentinean ones.

To get to know the extraction efficiencies, we digested 1 mL of the extracts with 1 mL HNO_3 and 3 mL ultrapure water. We filled the digests up to 10 mL with ultrapure water and measured the total arsenic concentration with ICPMS.

For chromatography we put the filtered extracts without further preparation into HPLC vials and then analyzed them with HPLC – ICPMS, using different methods (described later).

3.6.4 Urine of terrestrial mammals

We filtered all urine samples (from cattle as well as from animals of Schönbrunn Zoo) with syringes through 0.2 µm Nylon filters prior to analysis.

We measured the specific gravity of all samples with a total solids refractometer.

3.6.4.1 Digestion

For total element analysis, we took 1 or 0.5 mL of sample (depending on the total available amount) and digested with 1 mL nitric acid and 3 mL ultrapure water in triplicates. We also prepared a certified reference material (Seronorm™ Trace Elements Urine). We diluted the digests with ultrapure water to a total volume of 10 mL.

3.6.4.2 Sample preparation for chromatography

For anion-exchange chromatography, we usually oxidized the filtrated samples with 10 % v/v of a 30 % H_2O_2 solution and let them oxidize for about 1 - 2 hours at about 50°C. Additionally, we chromatographed some of the samples without oxidation.

For cation-exchange chromatography the filtered urine samples could be measured directly, without further sample preparation.

We used both oxidized and not oxidized samples for analytical reversed-phase chromatography. For preparative reversed-phase chromatography we only used not oxidized samples.

We used two different certified reference materials: SRM® 2669 (Arsenic Species in Frozen Human Urine) and CRM No. 18 (Human Urine, “NIES 18”). They were prepared according to their certificates and then treated like the animals’ urine samples.

3.7 Determination of total element concentrations

We measured the total arsenic concentration as well as the concentration of other elements of water, tissue and urine samples with ICPMS. Some elements were measured in nogas-mode, while others had to be analyzed with heliumas collision gas (4 mL/min). Selenium was the only element that was measured in reaction gas mode with hydrogen as reaction gas. More details are listed in Table 11.

We used external calibration for quantification. For most elements we took the Merck VI multi element standard as stock solution. Mercury and antimony had to be added from single element standards. The calibration range for most elements was either 0.01 - 1 or 0.1 - 10 µg/L (Table 11). However, some elements were present in the samples in very high concentrations (Na, Mg, K, Ca, Fe, Cu, Zn, Mn, Rb), which made it necessary to prepare a second set of calibration standards with higher concentrations. For most of the samples, these calibration ranges fitted very well. We prepared all calibration standards by diluting the

stock solutions with ultrapure water and nitric acid. The final solutions had an acidity of 10 % v/v.

For quality control we used certified reference materials. On the one hand, we had reference materials that were prepared like the samples (digestion or extraction, for example). On the other hand, we also always used SRM® 1643e or 1640a ("reference water"), which were diluted 1+9 with ultrapure water and nitric acid to a final acidity of 10 % v/v.

Another method for quality control was the use of so called drift standards. One of the calibration standards was re-measured every 10 - 15 samples to observe the stability of the run.

We also used an internal standard solution (200 µg/L germanium, indium and lutetium) which was added online (via a t-piece in front of the nebulizer) to all solutions that were measured. During the measurements, we followed the stability of the signals of the internal standards. A change in the intensity of these signals often indicates a greater or lower influence of the sample matrix. Therefore, the signals of the other elements were corrected by using the information of the internal standards. Which internal standard was used for which elements is listed in Table 11.

We optimized the configuration of the ICPMS before analysis to enhance the performance of the instrument.

Table 11: Settings for ICPMS measurements of different elements (m/z of internal standards: Ge = 74, In = 115, Lu = 175; *m/z was measured for control, but not used for quantification)

Element	m/z	Octopole mode	Internal standard	Calibration range
Ag	107	nogas	In	0.01 - 1 µg/L
As	75	He	Ge	0.1 - 10 µg/L
B	11	nogas	Ge	0.1 - 10 µg/L
Ba	137	nogas	In	0.01 - 1 µg/L
Be	9	nogas	Ge	0.1 - 10 µg/L
Bi	209	nogas	Lu	0.01 - 1 µg/L
Ca	43	nogas	Ge	0.1 - 10 mg/L
Cd	111 (114*)	nogas	In	0.01 - 1 µg/L
Co	59	He	Ge	0.01 - 1 µg/L
Cr	53 (52*)	He	Ge	0.01 - 1 µg/L
Cu	65	He	Ge	0.1 - 10 mg/L
Fe	56	He	Ge	0.1 - 10 mg/L
Ga	71	He	Ge	0.01 - 1 µg/L
Hg	201	nogas	Lu	0.01 - 1 µg/L
K	39	He	Ge	1 - 100 mg/L
Li	7	nogas	Ge	0.01 - 1 µg/L
Mg	24	nogas	Ge	0.1 - 10 mg/L
Mn	55	He	Ge	0.01 - 1 mg/L
Mo	98 (95*)	nogas	In	0.01 - 1 µg/L
Na	23	He	Ge	1 - 100 mg/L
Ni	60	He	Ge	0.01 - 1 µg/L
Pb	208	nogas	Lu	0.01 - 1 µg/L
Rb	85	He	Ge	0.01 - 1 mg/L
Sb	121	Nogas	In	0.01 - 1 µg/L
Se	78	H_2	Ge	0.1 - 10 µg/L
Sr	88	He	Ge	0.01 - 1 µg/L
Te	125	nogas	In	0.01 - 1 µg/L
Tl	205	nogas	Lu	0.01 - 1 µg/L
U	238	nogas	Lu	0.01 - 1 µg/L
V	51	He	Ge	0.01 - 1 µg/L
Zn	66	He	Ge	0.1 - 10 mg/L

3.8 Arsenic speciation analysis

We used two systems for arsenic speciation, depending on the availability: HPLC 1260 Infinity coupled to ICPMS 7500ce and HPLC 1200 coupled to ICPMS 7700x. The temperature of the HPLC autosampler was always set at 4°C. Stock solutions for the calibration standards were stored in the refrigerator at 4°C. We prepared all eluents for arsenic speciation analysis in 2 L plastic bottles to avoid contamination from glass bottles.

3.8.1 Quantification of the major arsenic species

We used the anion-exchange chromatographic method described by Scheer et al. [14] for quantification of inorganic arsenic, DMA, MA and also for an estimation of the sum of cationic arsenicals in all urine and tissue samples. We prepared the standards by mixing As(III), As(V), DMA, MA and AB and diluting them with ultrapure water and 10 % v/v of a 30 % H_2O_2 solution. We let them oxidize for about 1 hour at about 50°C. The concentrations of the calibrations standards were from 0.1 to 20 µg As/L for each arsenical.

We prepared the mobile phase by diluting 2.31 g (20 mmol) orthophosphoric acid in 1 L ultrapure water and slowly added NH_3 to adjust the pH to 6.0.

Eluent and stationary phase as well as the other conditions of the method can be found in Table 12, and a typical chromatogram of a calibration standard containing AB, DMA, MA and inorganic arsenic (As(III) was oxidized to As(V)) is shown in Figure 12. Especially the signal of inorganic arsenic is susceptible to shifts to shorter retention times when the column has been in use for a longer time.

Table 12: Conditions of the anion-exchange chromatography for the quantification of the main arsenicals

column	PRP-X100 (150 x 4.6 mm, 5 µm)
guard column	PRP-X100 (8 x 3 mm, 10 µm)
mobile phase	20 mM aqueous ammonium phosphate, pH 6.0
flow rate	1 mL/min
injection volume	20 µL
injection/draw speed	100 µl/min
column compartment temperature	40°C
typical pressure (roughly)	80 - 140 bar

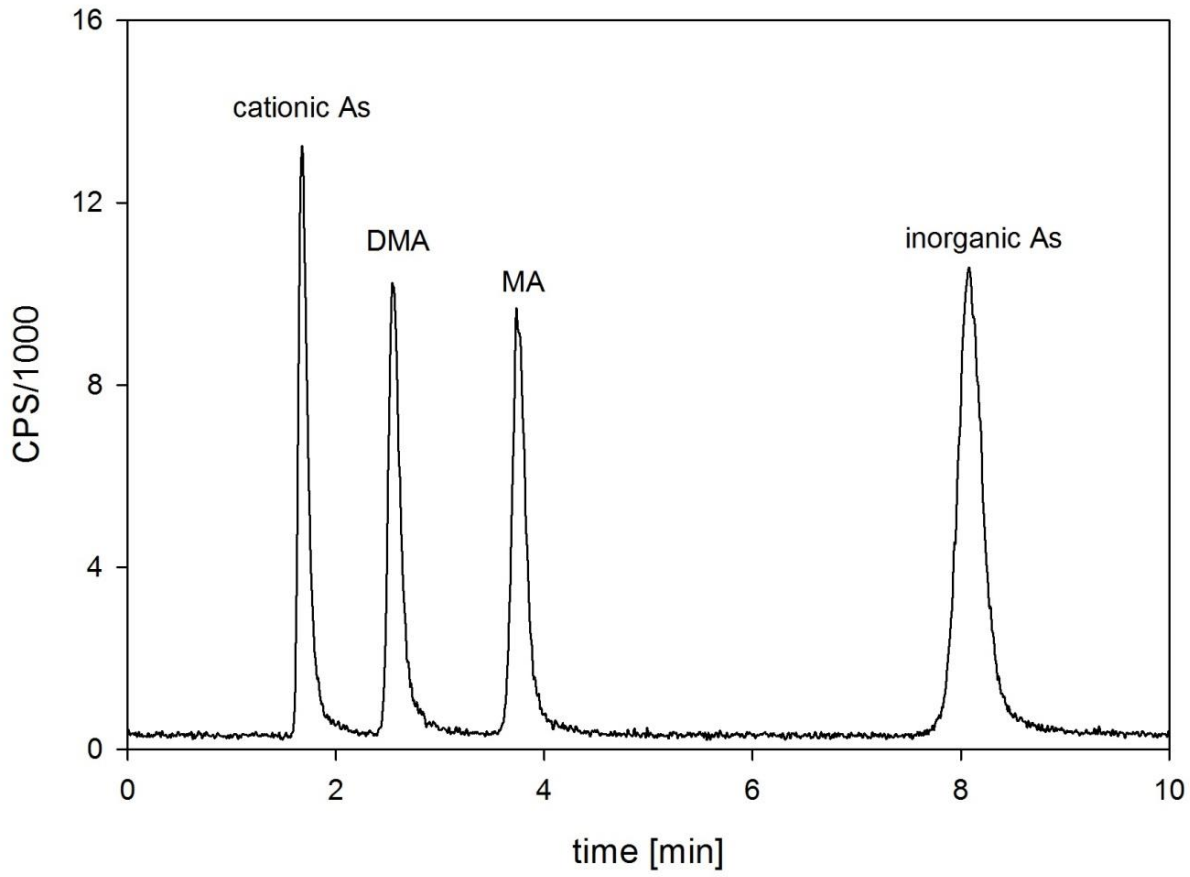


Figure 12: Anion-exchange chromatogram of a standard solution (10 µg As/L of AB, DMA, MA, As(III) and As(V), oxidized), with an aqueous solution of 20 mM ammonium phosphate, pH 6.0, as mobile phase and a PRP-X100 column as stationary phase

3.8.2 Determination of cationic arsenicals

We analyzed some naturally occurring cationic arsenic compounds in some of the urine samples with cation-exchange chromatography as described by Scheer et al. [14]. The compounds were AB; AC, TMAO and TETRA. We prepared the calibration standards by mixing the same amounts of solutions of the respective arsenicals and then diluting the mix solutions with ultrapure water to concentrations of 0.1 – 10 µg As/L of each arsenic compound.

We used a 10 mM aqueous pyridine buffer as mobile phase. We diluted 800 mg pyridine in 1 L ultrapure water and adjusted the pH to 2.3 with formic acid.

The conditions of the method can be found in Table 13, and a typical chromatogram of a calibration standard is shown in Figure 13.

Table 13: Conditions of the cation-exchange chromatography for analysis of some cationic arsenicals

column	Zorbax 300-SCX (150 x 4.6 mm, 5 µm)
guard column	Zorbax SCX (12.5 x 4.6 mm, 5 µm)
mobile phase	10 mM aqueous pyridine, pH 2.3
flow rate	1.5 mL/min
injection volume	20 µL
injection/draw speed	100 µl/min
column compartment temperature	30°C
typical pressure (roughly)	100 - 120 bar

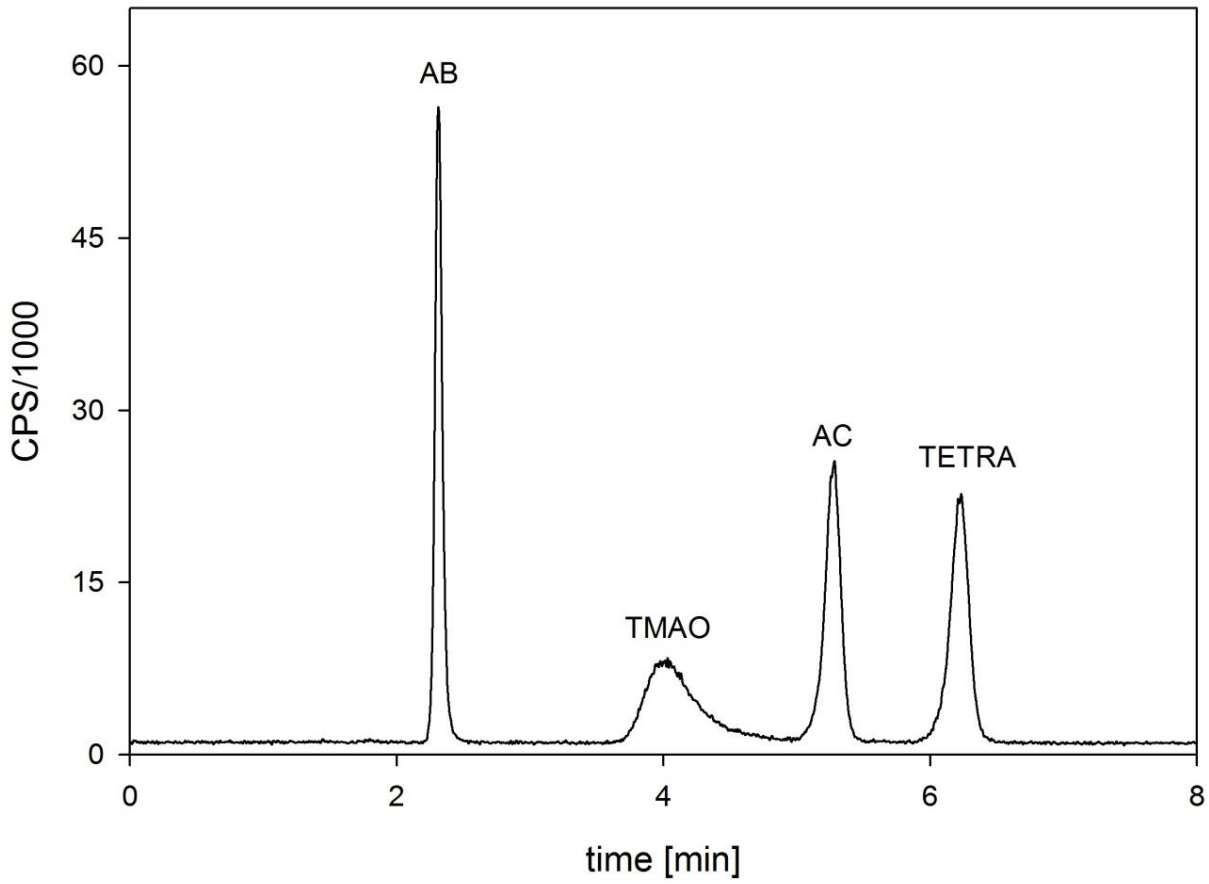


Figure 13: Cation-exchange chromatogram of a standard solution (10 µg As/L AB, TMAO, AC and TETRA) with 10 mM pyridine, pH 2.3, as mobile phase and a Zorbax 300-SCX column as stationary phase

3.8.3 Unknown arsenic compounds in ruminants' urine

3.8.3.1 pH dependency on the PRP-X100 anion-exchange column

We varied the anion-exchange chromatographic method described above (Table 12) by changing the pH of the mobile phase to 4.0, 5.0, 6.0, 7.0 and 8.0. Instead of the PRP-X100,

150 x 4.6 mm, we used the thinner PRP-X100 column with 150 x 2.1 mm dimensions and a flow rate of 0.25 mL/min. All other settings stayed the same.

For this experiment we utilized a standard solution with AB, DMA, MA and inorganic arsenic (As(III) and As(V)), oxidized with H_2O_2. As a representative sample we used one of the cows' urine samples with high arsenic concentrations ("Betti"), also oxidized with H_2O_2.

3.8.3.2 Anion-exchange chromatography with other mobile phases

For further investigations, we used an aqueous acetate buffer as well as an aqueous solution of maleic acid instead of aqueous ammonium phosphate as mobile phase on a PRP-X100 column. The conditions are listed in Table 14.

To obtain a 30 mM aqueous acetate buffer, we diluted 1.88 g acetic acid in 1 L ultrapure water. The pH was adjusted with NH_3. For the 5 mM aqueous maleic acid solution, we dissolved 0.58 g maleic acid in one liter ultrapure water and adjusted the pH with NH_3 to 7.0.

We also looked at the influence of organic solvents by introducing 10 % v/v methanol to the mobile phase (aqueous ammonium acetate, pH 6.0, and aqueous ammonium phosphate, pH 7.0).

Again, we used the oxidized bovine urine sample "Betti" and an oxidized standard solution that contained AB, DMA, MA and inorganic arsenic.

Table 14: Conditions for anion-exchange chromatography with different mobile phases

column	PRP-X100 (150 x 4.6 mm, 5 µm)
guard column	PRP-X100 (8 x 3 mm, 10 µm)
mobile phase 1	30 mM aqueous ammonium acetate, pH 5.0
mobile phase 2	*90 % v/v 30 mM aqueous ammonium acetate, pH 6.0 and 10 % v/v MeOH*
mobile phase 3	5 mM aqueous maleic acid, pH 7.0
mobile phase 4	*90 % v/v 20 mM aqueous ammonium phosphate, pH 7.0 and 10 % v/v MeOH*
flow rate	1 mL/min
injection volume	20 µL
injection/draw speed	100 µl/min
column compartment temperature	40°C
typical pressure (roughly)	80 - 130 bar

3.8.3.3 Anion-exchange chromatography with a different column type

We replaced the PRP-X100 anion-exchange column with the Zorbax SAX column. While the PRP-X100 has a polymer-based backbone, the backbone of the Zorbax SAX is based on silica. This way we investigated possible interactions of arsenic compounds in the samples with the column backbone. We used aqueous solutions of 20 mM ammonium phosphate, pH 6.0 and 20 mM ammonium acetate with different pH values as mobile phases. Details of the methods are listed in Table 15.

To obtain the desired concentration of 20 mM buffer solution, we used 1.25 g acetic acid and 2.31 g orthophosphoric acid per liter ultrapure water, respectively.

We used the urine sample of cow “Betti” (oxidized and not oxidized) and a standard solution with AB, DMA, MA and inorganic arsenic, oxidized with H_2O_2.

Table 15: Settings of the anion-exchange chromatography experiments with the Zorbax SAX column

column	Zorbax SAX Analytical (150 x 4.6 mm, 5 µm)
guard column	Zorbax SAX Analytical (12.5 x 4.6 mm, 5 µm)
mobile phase 1	20 mM aqueous ammonium phosphate, pH 6.0
mobile phase 2	*20 mM aqueous ammonium acetate, pH 4.0, 5.0 and 6.0*
flow rate	1 mL/min
injection volume	20 µL
injection/draw speed	100 µl/min
column compartment temperature	35°C
typical pressure (roughly)	65 - 75 bar

3.8.3.4 Cation-exchange chromatography with other mobile phases

We slightly varied the cation-exchange method described earlier by changing the pH of the buffer from 2.3 to 4.3. In another experiment we added 2.0, 5.0 and 10 % v/v methanol to the pyridine buffer. The column and the other settings were the same as in the original method (Table 13).

We used an aqueous standard solution with AB, AC, TMAO and TETRA as well as the cattle urine sample “Betti”, which was not oxidized for these experiments.

3.8.3.5 Reversed-phase chromatography (analytical)

For the separation and quantification of typical urinary arsenic metabolites, reversed-phase chromatography is usually not well suited. Nevertheless, we were able to use it for the

separation of some of the unknown compounds in our samples. The stationary phase was a Zorbax SB-C8 column. The mobile phase consisted of ultrapure water and methanol. We experimented with the amounts of water and methanol to optimize the separation for the next step, the preparative chromatography. The details of the analytical reversed-phase chromatographic methods are summarized in Table 16.

We used oxidized and not oxidized samples of the cow's urine "Betti", and as reference we first used an oxidized standard solution of AB, DMA, MA and inorganic arsenic. Later, we only utilized As(V) for comparison.

We collected the fraction that contained an unknown compound using ultrapure water with 10 % methanol as mobile phase at a flow rate of 1 mL/min. We did this by collecting the mobile phase from 2.0 to 2.8 minutes runtime manually after the column, before the sample could enter the ICPMS. This was carried out with 6 injections of 50 µL injection volume each.

We put the collected fractions in the vacuum concentrator for about 24 hours to concentrate the isolated arsenic compound. This solution served as reference in further experiments to identify this specific unknown compound from the unknown signals.

Table 16: Settings for reversed-phase chromatography

column	Agilent Zorbax SB-C8 (150 x 4.6 mm, 5 µm)
guard column	-
mobile phase	ultrapure water + 0 - 15 % v/v methanol (finally 7 %)
flow rate	1 or 1.5 mL/min (finally 1.5 mL)
injection volume	20 µL (50 µL for fraction collection)
injection/draw speed	100 µl/min
column compartment temperature	Room temperature
typical pressure (roughly)	50 - 160 bar, depending on the amount of methanol

3.8.3.6 Preparative reversed-phase chromatography

We transferred the optimized analytical reversed-phase method with ultrapure water and 7 % v/v methanol as eluent, flow rate 1.5 mL/min, to the preparative equivalent. The up-scaled settings are given in Table 17.

First, we chromatographed a blank, which was ultrapure water, and then we injected the not oxidized cow urine sample "Betti". One run was 8.5 minutes long. With the fraction collector we collected fractions from 2.5 to 8.5 minutes, with a time span of 0.5 to 0.75 minutes per

fraction. After the first experiments we exchanged the glass tubes that are usually utilized for fraction collection with 31 mL polypropylene tubes (Kartell s.p.A.) to avoid contamination.

We put the fractions and some blanks into the vacuum concentrator to remove the solvent. Then, we assimilated each residue in a small volume of the eluent (0.1 to 0.5 mL), shook it well and sonicated it for 15 minutes at room temperature. Then, we analyzed the samples for arsenic species with anion-exchange or reversed-phase chromatography.

Table 17: Settings for preparative reversed-phase chromatography

column	PrepHT SB-C8, 150 x 21.2 mm, 5 µm
mobile phase	ultrapure water + 7 % v/v methanol
flow rate	31.25 mL/min
injection volume	400 µL
column compartment temperature	Room temperature
fraction collection	2.5 – 8.5 min runtime, 0.5 – 0.75 min/fraction
typical pressure (roughly)	220 - 240 bar

3.8.3.7 Synthesis of thioarsenates

We tried to synthesize at least one of the four possible thioarsenates (mono-, di-, tri- and tetrathioarsenate) according to the procedures described by Maher et al. [117]. We dissolved 3.0 g Na_2S * xH_2O in 1 L ultrapure water in an ultrasonic bath for about 30 minutes and prepared a 10 mg As/L solution, using an As(III) standard. From these stock solutions we prepared a solution that contained 0.3 mg/L Na_2S * H_2O (1.335 µmol S/L) and 100 µg As/L (1.335 µmol As/L). We also prepared a similar solution that contained 100 times more Na_2S * H_2O (133.5 µmol S/L), but still 1.335 µmol As/L. We shook both solutions well. According to Maher et al, the first mixture (As:S = 1:1) should result in monothioarsenate, and the second mixture (As:S = 1:100) should yield di-, tri- and tetrathioarsenate.

We analyzed both solutions with anion-exchange chromatography (Table 12). We also tried to enhance the formation of the thioarsenates by applying around 50°C to the solutions for 2 hours and then analyzed them again with chromatography.

4 Results and Discussion

4.1 Quality control

4.1.1 Water from a "not polluted" area in Argentina

We measured the certified reference material NIST SRM® 1643e (Trace Elements in Water) to check the trueness of the measurements. For the elements that will be discussed later on (except uranium, which was not certified in the reference material), the trueness was between 93 and 96 % (Table 18).

Table 18: Trueness of SRM® 1643e

Element	Measured concentration [µg/L]	Certified concentration [µg/L]	Trueness [%]
As	58 ± 1	60.45 ± 0.72	96 ± 2
V	36 ± 2	37.86 ± 0.59	96 ± 5
Mo	116 ± 7	121.4 ± 1.3	96 ± 5
B	150 ± 10	157.9 ± 3.9	93 ± 6

4.1.2 Livers and kidneys of cattle from Austria and Argentina

4.1.2.1 Total elemental concentrations

We measured the SRM® 1643e (Trace Elements in Water) once per batch. The trueness for the measurements of the bovine tissue samples is displayed in Figure 14. The trueness of arsenic was 96 ± 4 %. For most of the other elements, the trueness was also very good, namely between around 90 and 115 %. However, we detected 145 ± 30 % of the certified value for potassium. It is very likely that this deviation was occurring because the lowest calibration standard for potassium was much higher than the concentration in the reference material. Therefore, this was not considered to be a cause for concern.

Another item for quality control was the certified reference material DOLT-3 (Dogfish Liver). With each digestion batch, DOLT-3 was digested in triplicate. The results for the measurements of the Austrian and Argentinean bovine tissues were very similar. The trueness of DOLT-3 for the measurement of the Austrian tissues can be seen in Figure 15. The trueness of arsenic when measuring the Austrian tissues was 87 ± 6 and 97 ± 9 % and around 85 – 88 % when measuring the Argentinean samples. The results were also very good for all other elements.

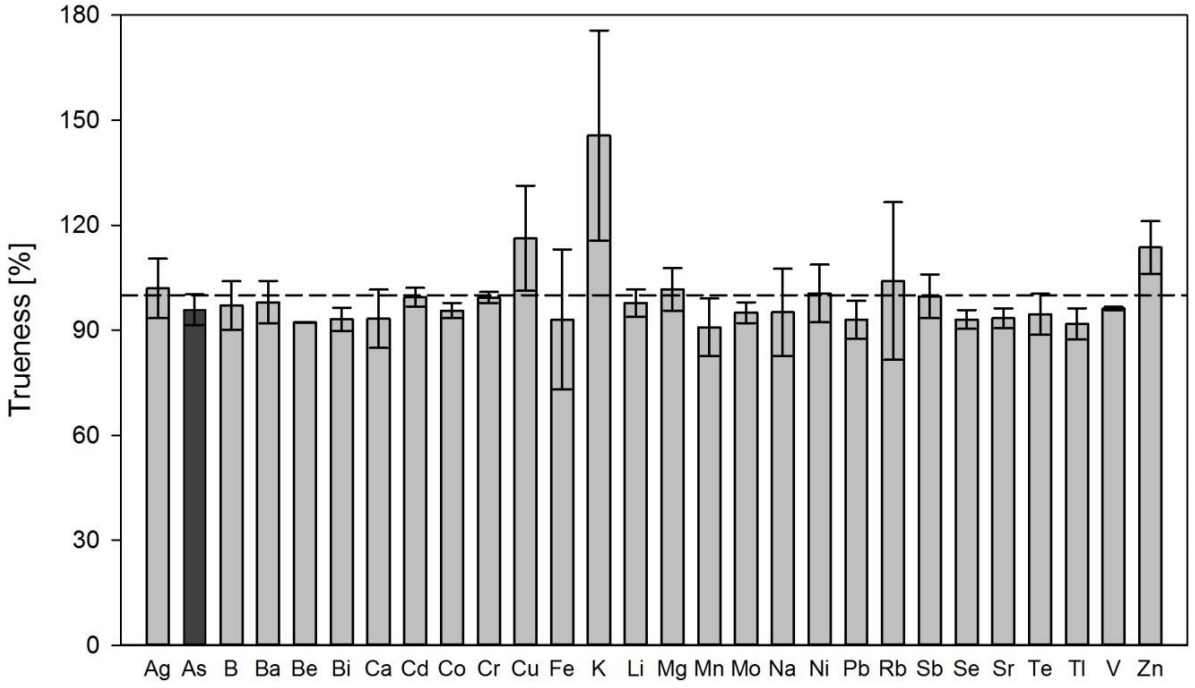


Figure 14: Trueness of element concentrations in SRM® 1643e (Analyses of tissue samples. Dashed line = 100 %)

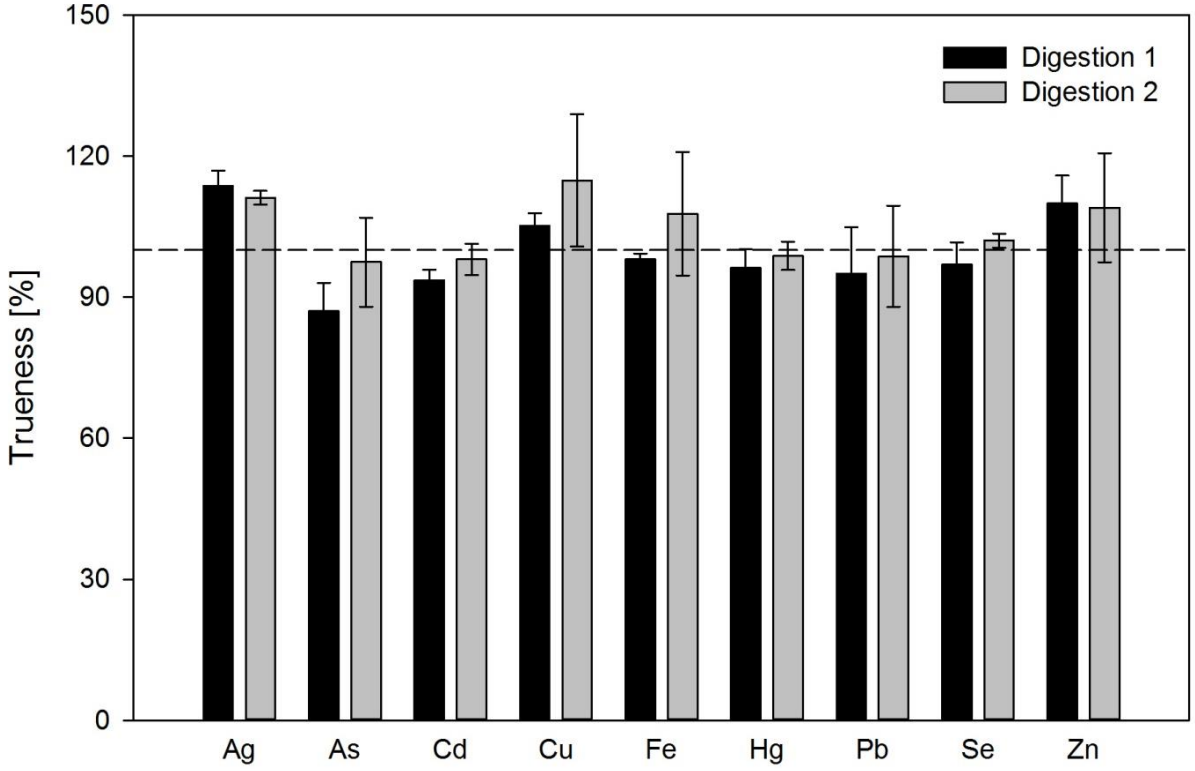


Figure 15: Trueness of element concentrations in DOLT-3 (Analysis of Austrian bovine tissue samples. Dashed line = 100 %)

Another quality control parameter was the measurement of internal standards (ISTD). We used germanium, indium and lutetium for this purpose and spiked them online to all of the samples and standards. The stability of the internal standards during the analysis of the Austrian tissue samples is shown in Figure 16. We also measured so called “drift standards” (one of the calibration standards) after every 10th to 15th sample to check the stability of the measurements. Overall, there were no drifts detectable, the detected concentrations stayed constant within every batch.

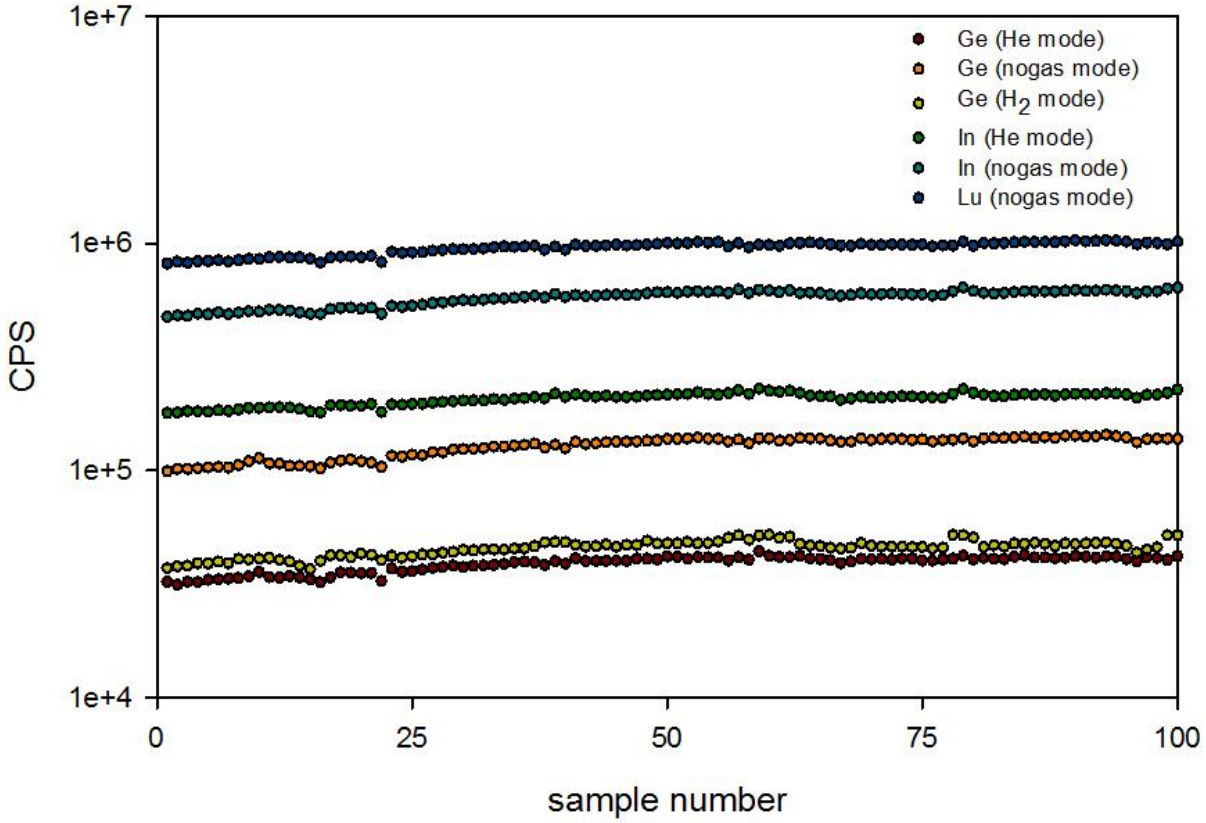


Figure 16: Signal intensity of the ISTD (analysis of Austrian tissues, CPS in logarithmic scale)

4.1.2.2 Arsenic species

We used SRM® 1640a (Trace Elements in Natural Water) and DORM-2 (Dogfish muscle) as a reference material for the arsenic speciation analysis of the bovine tissues. The total arsenic concentration as well as the concentrations of AB and TETRA are certified for DORM-2. Since we did not distinguish between AB and TETRA when using anion-exchange chromatography, we combined the given concentrations of the two arsenicals to “cationic arsenic”. The trueness for the speciation analysis of both Argentinean and Austrian tissue sample extracts was very good for both certified reference materials (Table 19).

Table 19: Trueness [%] of total arsenic and arsenic species in certified reference materials of the arsenic speciation analysis of Austrian and Argentinean tissue sample extracts

	Argentinean tissue extracts	Austrian tissue extracts
DORM-2, total arsenic	91	94
DORM-2, cationic arsenic	98	107
SRM® 1640a, total arsenic	105	89

4.1.3 Urine of terrestrial mammals

4.1.3.1 Total elemental concentrations

We checked the trueness of our measurements by using SRM® 1643e (Trace Elements in Water) and Seronorm™ Trace Elements (Urine). The results of SRM® 1643e for the measurement of the cows' urine samples are displayed in Figure 17 and discussed exemplarily for the measurements of the different urine samples.

The trueness of most of the elements was very good. We detected 99 ± 4 % of the certified total arsenic concentration, and the other elements were between 90 and 110 %.

We used the reference material Seronorm™ Trace Elements (Urine) mainly to check the trueness of arsenic, which was our major element of interest. The certified concentration of arsenic was 15.0 ± 1.1 µg/L, which was determined with hydride generation atomic absorption spectrometry. This analysis technique is not suited to analyze total arsenic concentrations, since compounds like AB do not form hydrides and are therefore not included in the analyzed total arsenic concentrations. Earlier unpublished analyses with ICPMS at the Analytical Chemistry department of the University of Graz showed that the total arsenic concentration in Seronorm™ was rather 90 ± 3.6 µg/L. In our measurements we detected between 80 and 98 µg As/L, which is in good accordance with this corrected value.

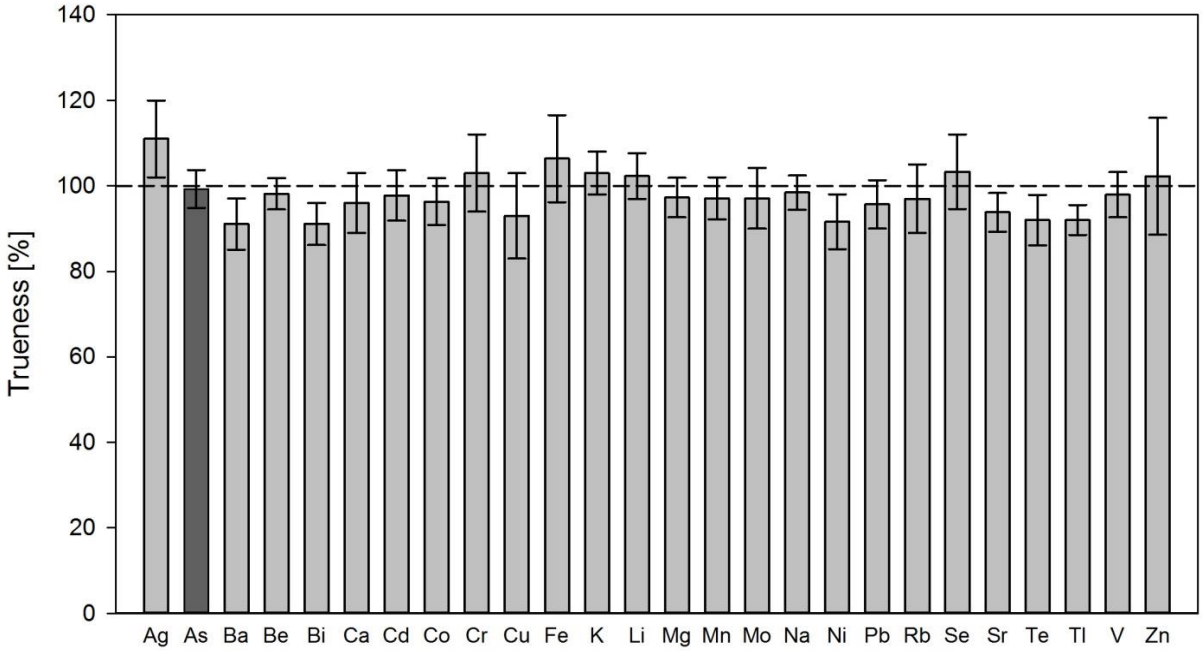


Figure 17: Trueness of element concentrations in SRM® 1643e (Analyses of urine samples. Dashed line = 100 %)

4.1.3.2 Arsenic species

For the quantification of the main arsenic species in urine, we used the certified reference materials SRM® 2669 (Arsenic Species in Frozen Human Urine) and CRM No. 18 (Human Urine, "NIES 18"). The trueness of the different arsenicals (mean ± standard deviation of the different measurements) is shown in Figure 18. Overall, the trueness was good for all four arsenic compounds. The standard deviation of MA was a bit high, which is partly because it was present in very low concentrations, which made it more difficult to correctly integrate the peaks.

We also chromatographed SRM® 1643e or SRM® 1640a and compared the obtained total arsenic concentration (more or less only inorganic arsenic) with the certified value, which fitted well all the time (around 90 – 105 %).

To check for possible drifts we re-measured one of the calibration standards after every 10th to 15th sample. We did not observe any significant differences between the signals of the different "drift standards" of one batch.

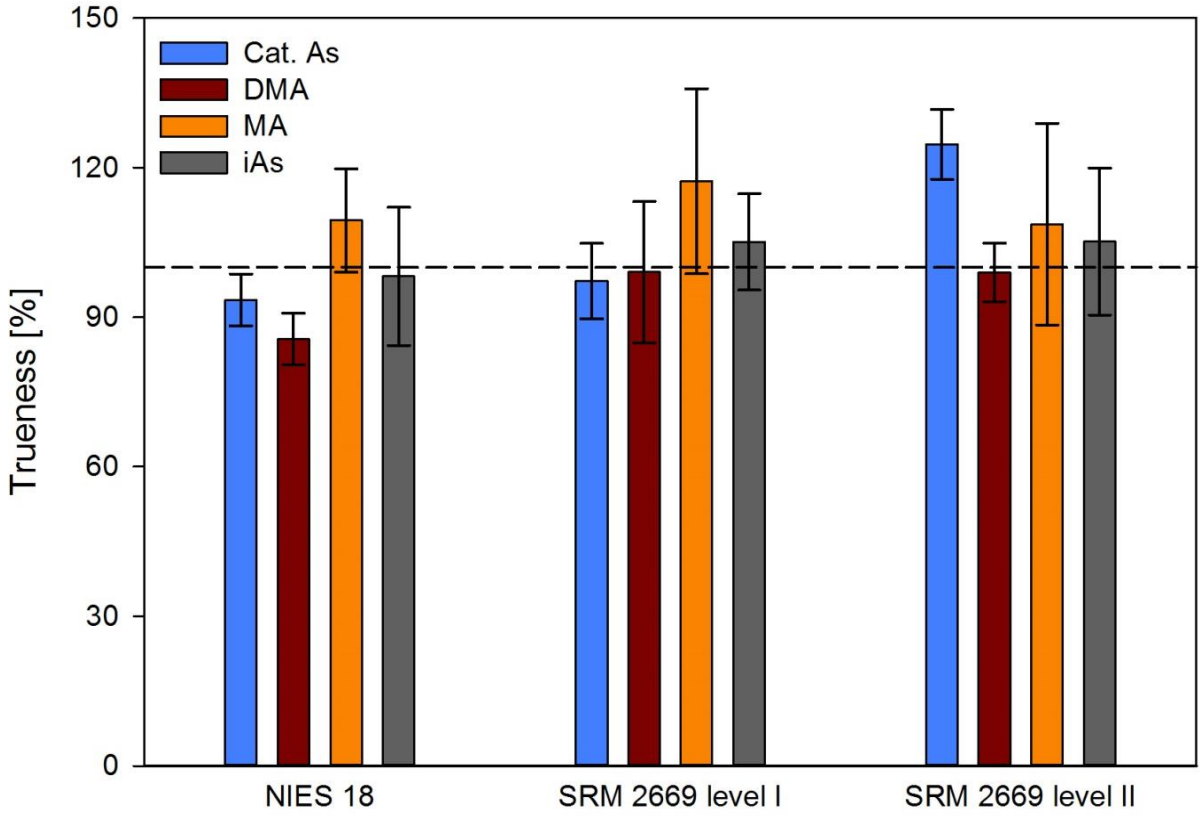


Figure 18: Trueness of arsenic species concentrations in certified reference materials for urine samples (Dashed line = 100 %)

4.2 Water from a "not polluted" area in Argentina

All studied water samples from Mercedes, Argentina, had concentrations of 10 or more µg As/L, although they are located in areas that are said to be "not polluted". According to the recommended maximum limit for total arsenic in drinking water (10 µg/L), none of the wells would be allowed as drinking water. Unfortunately we do not have any samples from cattle that had been drinking from these wells and therefore could not measure the arsenic uptake by the cattle via the water. The concentrations of arsenic and other elements of the 4 wells and the river and also of the lake Capri in Patagonia are displayed in Table 30 and Figure 19. The lake in Patagonia, which is said to have a very good water quality, had indeed a much lower arsenic concentration (0.3 µg/L) than the wells in Mercedes.

It is known that elements like boron, vanadium, molybdenum and uranium can occur together with arsenic in water from regions like the Chaco-Pampean plain [31]. We observed this correlation also in our samples (Figure 20).

According to literature, typical **vanadium** concentrations in drinking water from uncontaminated sites are between 1 and 6 µg/L [118]. Compared to this, the vanadium concentrations of 98 - 174 µg/L in the wells 1-3 and the river in Mercedes are rather high. However, there are similar and even higher vanadium concentrations reported for this area in literature: Farías et al. found up to 670 (median: 35) µg V/L and up to 593 (median: 43) µg As/L in groundwater from the Pampean plain [119]. Nicolli et al. reported < 5 – 590 (median: 130) µg V/L and 15 – 780 (median: 71) µg As/L in groundwater samples from the Santa Fe province, Argentina [31].

Boron levels in groundwater are varying a lot around the world, between less than 0.3 mg/L and even more than 100 mg/L [120]. The WHO guideline value for boron in drinking water is 2.4 mg/L [121]. The boron concentrations we detected in the Argentinean water samples were all below this limit.

The WHO threshold value for **uranium** it is 30 µg/L, and the concentrations in drinking water are usually below 1 µg/L [121]. Although we measured higher concentrations in four out of six of the water samples (5.7 - 19 µg/L), none of them exceeded the limit of the WHO. However, the legislation of Germany recommends an upper limit of 2 µg U/L for mineral water and especially water that is used to prepare food for infants [122]. All wells except number 4 exceeded this value. To avoid health risks, their water should not be given to children and babies.

The **molybdenum** concentration in drinking water is typically less than 10 µg/L [121]. Three of the water samples contained slightly more molybdenum, namely 14 ± 1, 38 ± 3 and 40 ± 1 µg/L. Molybdenum is an essential nutrient for mammals. The recommended daily intake is 75 – 250 µg Mo for adults and 15 – 40 µg Mo for infants [10]. Therefore, special care has to be taken when the water of the investigated wells is given to small children.

It has to be mentioned that a loss of analytes could have occurred between sampling and analysis, because nitric acid was only added to the water a couple of days after the samples had been taken.

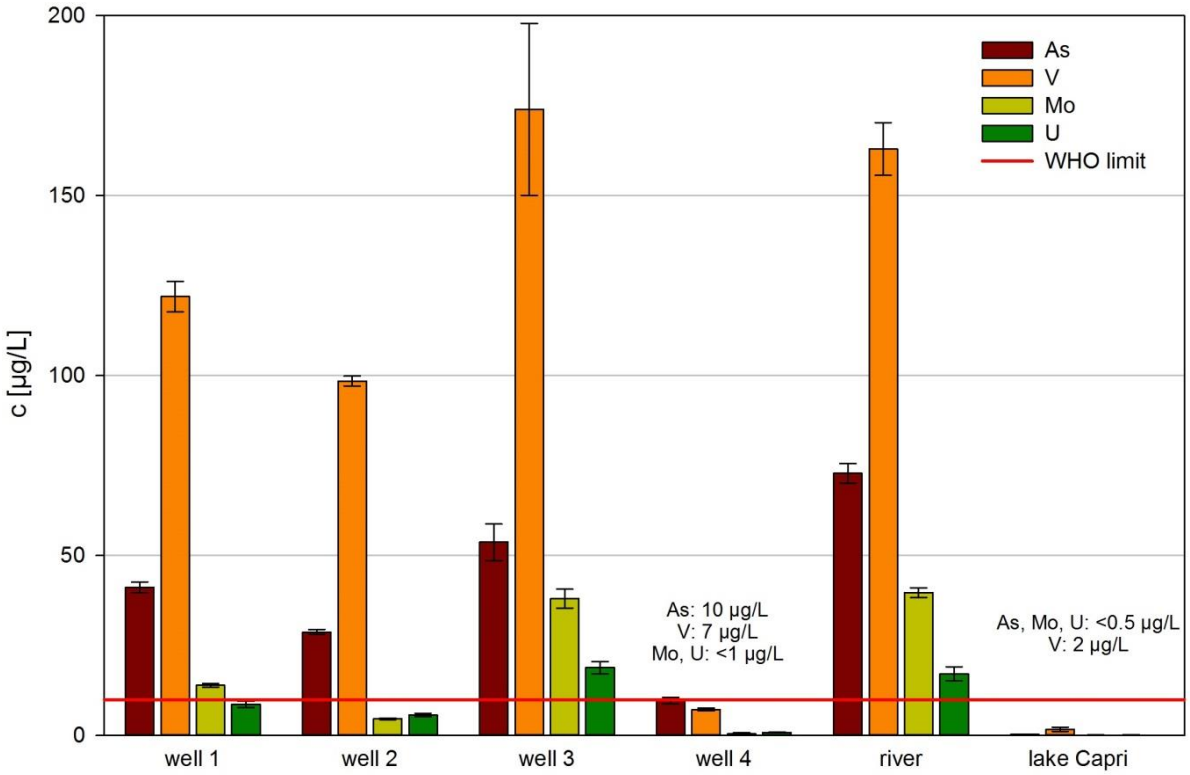


Figure 19: As, V, Mo and U concentrations in water from Mercedes, Argentina, from lake Capri, Patagonia, Argentina, and the WHO limit for arsenic in drinking water (10 µg/L)

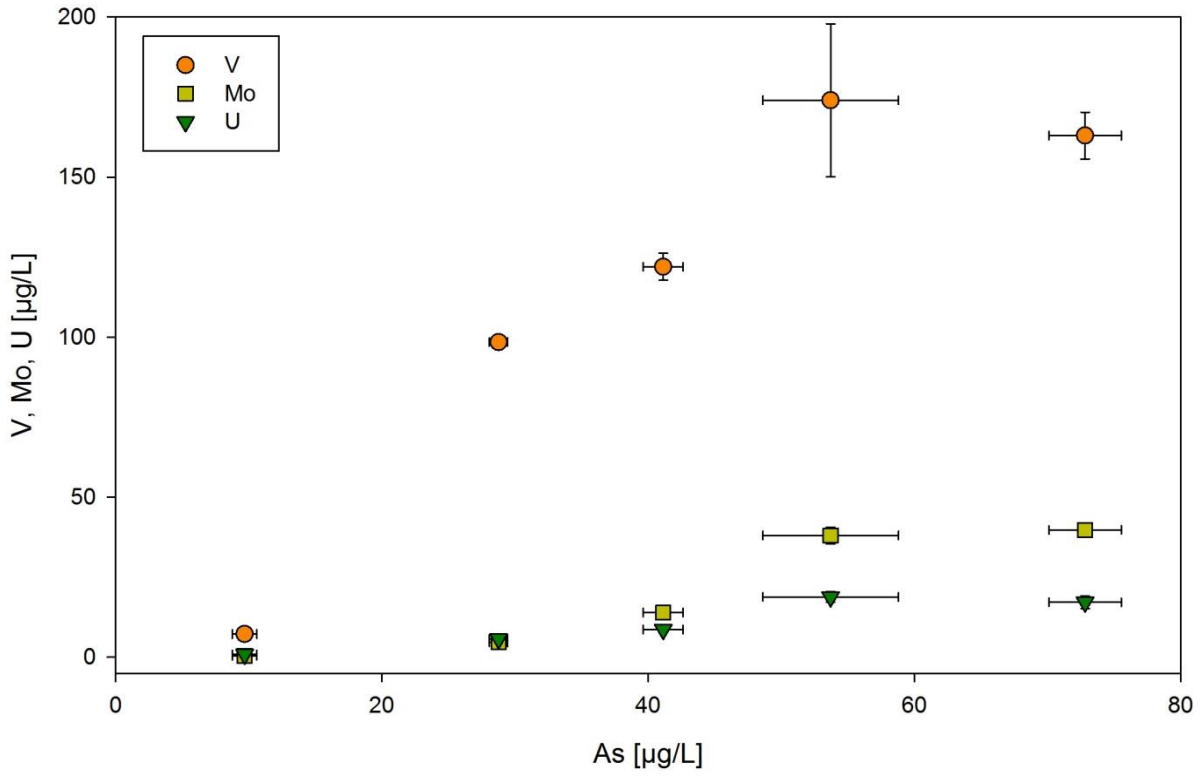


Figure 20: Correlation of As, V, Mo and U concentrations in water from Mercedes, Argentina (boron is not displayed because of its high concentrations compared to the other elements)

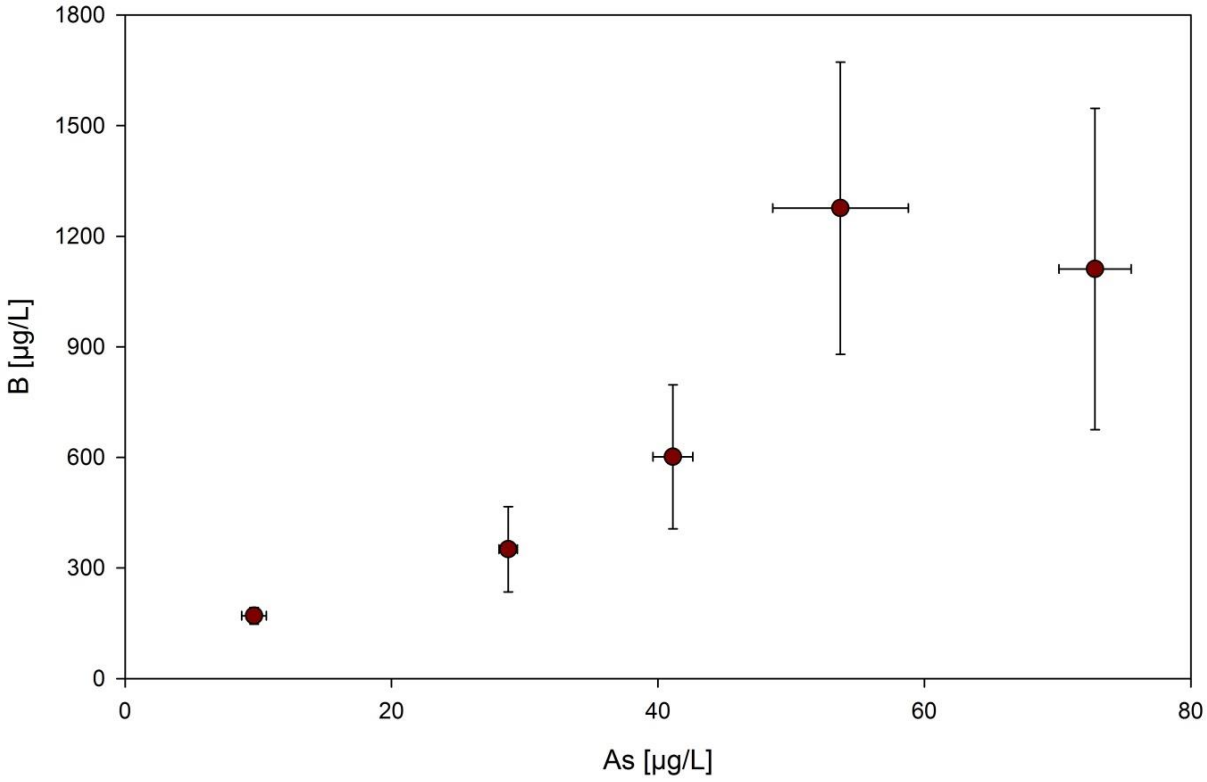


Figure 21: Correlation of As and B concentrations in water from Mercedes, Argentina

4.3 Livers and kidneys of cattle from Austria and Argentina

4.3.1 Total arsenic concentrations

Table 20 shows the total arsenic concentrations of the freeze-dried Austrian and Argentinean liver and kidney samples in µg/kg dry mass (dm). The dry mass of bovine livers was 30 ± 1 % and accounted for 20 ± 1 % in bovine kidneys. Using these values, we converted the dry mass arsenic concentrations to wet mass concentrations (Table 21), to make comparisons with literature more easily, because some authors are presenting their results for fresh and others for dried tissues. The results are visualized in Figure 22. The results of each sample are listed in the appendix (Table 31 and Table 32).

Table 20: Total arsenic concentrations in Austrian and Argentinean cattle tissues in µg/kg **dry mass** (values for Austria without sample No. 1320, *concentration of No.1320)

		Mean	Median	Range
Liver	Austria	45 ± 20	38	20 - 75, 170*
	Argentina	*100 ± 50*	*110*	*25 - 150*
Kidney	Austria	260 ± 130	290	105 - 480, 1390*
	Argentina	*270 ± 120*	*240*	*85 - 500*

Table 21: Total arsenic concentrations in Austrian and Argentinean cattle tissues in µg/kg **wet mass** (values for Austria without sample No. 1320, *concentration of No.1320)

		Mean	Median	Range
Liver	Austria	14 ± 6	11	6 - 23, 51*
	Argentina	*30 ± 15*	*33*	*7.5 - 45*
Kidney	Austria	52 ± 26	58	21 - 96, 280*
	Argentina	*54 ± 24*	*48*	*17 - 100*

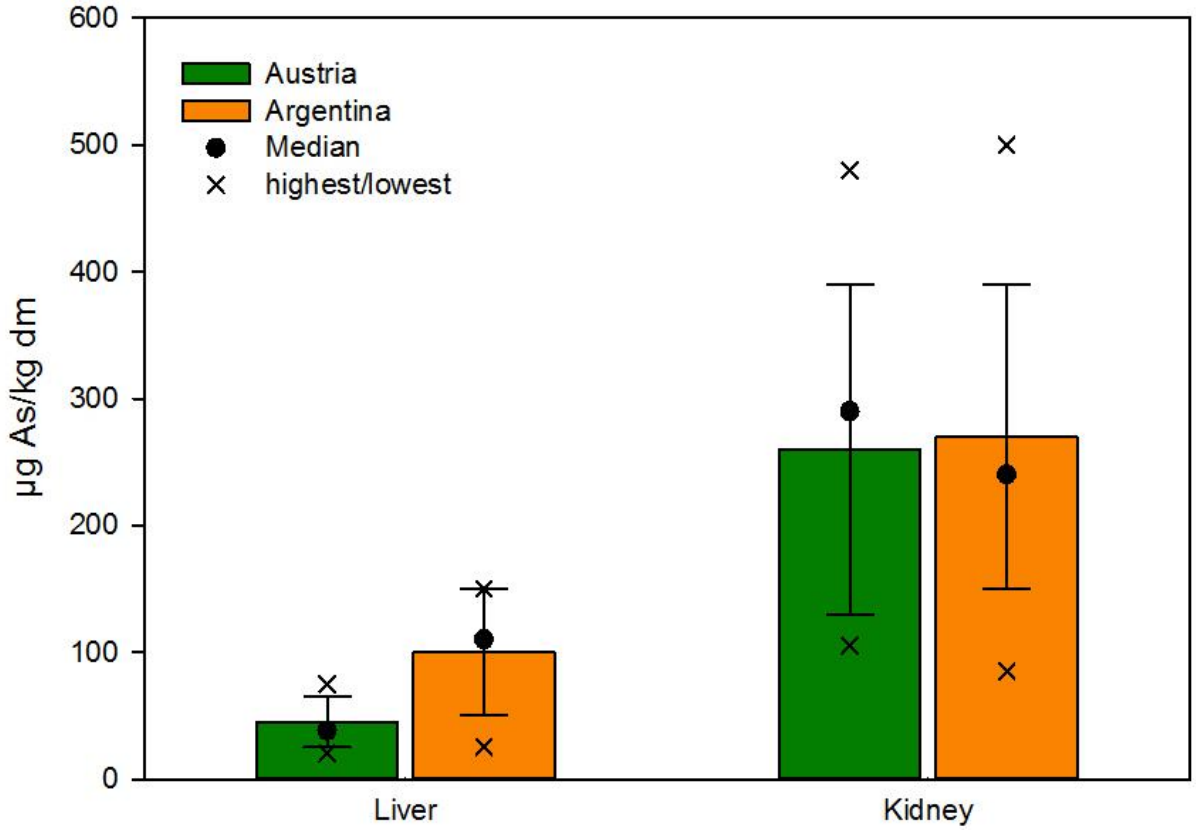


Figure 22: Mean ± standard deviation, median, highest and lowest arsenic concentration in Austrian and Argentinean livers and kidneys (Austrian tissues: without sample no. 1320)

4.3.1.1 Austrian tissues

The arsenic concentrations in 9 out of the 10 studied livers and corresponding kidneys ranged from 20 to 75 µg/kg dm and from 105 to 480 µg/kg dm, respectively (Table 20, Figure 22). We found exceptionally high arsenic concentrations in the 10th tissue pair (No. 1320), namely 170 µg/kg dm in the liver and 1390 µg/kg dm in the kidney. The origin of this sample pair was St. Lorenzen/Mürztal. Another sample pair (No. 1322) also originated from St. Lorenzen/Mürztal. Although the arsenic levels of No. 1322 were much lower than the ones of No. 1320, they were still the second highest values of the 10 sample pairs (75 µg/kg in the liver and 480 µg/kg dm in the kidney). Because we collected the tissues at the slaughterhouse in Graz, we don't have any information about the treatment of the animals. Possible arsenic sources are drinking water, feed and feeding supplements. Concerning the drinking water, a possible correlation can be found in Figure 3 (arsenic concentrations in Austrian stream sediments), where elevated arsenic values are drawn around the area the tissue samples of concern originated from.

Still, within this master thesis it remained an open question why one cattle had these extremely high arsenic concentrations in its liver and kidneys.

There is a strong linear correlation between the arsenic concentrations in livers and the corresponding kidneys (Figure 23). Investigated kidneys contained 4.0 - 8.4 times more arsenic than the livers.

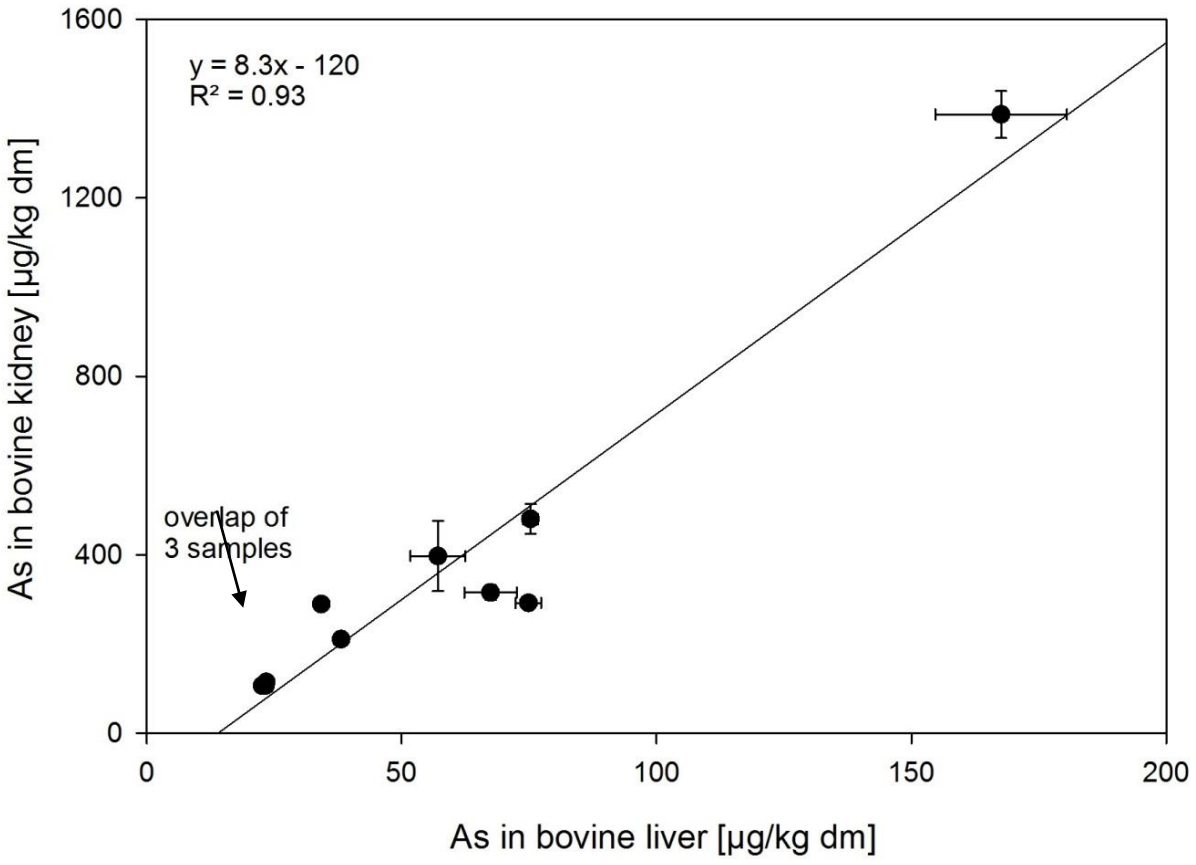


Figure 23: Correlation of total arsenic concentrations in 10 liver and kidney pairs of Austrian cattle

4.3.1.2 Argentinean tissues

In the 13 bovine kidney samples that we obtained from Argentina, we measured between 85 and 500 µg As/kg dm, and we found 25 - 150 µg As/kg dm in the 6 liver samples (Table 20, Figure 22). We did not observe any differences between the 4 investigated areas. We could not compare the liver with the kidney samples, because all tissues were from different animals.

Unfortunately, we were not able to obtain fresh water samples from the wells where the investigated animals had been drinking from. There is data from water of the Chaco-Pampean plain that has been published in 2005 by Pérez-Carrera and Fernández-Cirelli. They reported between 40 and 2600 µg As/L [34].

4.3.1.3 Comparison

We are assuming that the cattle from Argentina had been grazing on arsenic-contaminated pastures, whereas the Austrian cattle had been consuming uncontaminated feed and water. However, we were not able to obtain any information to confirm or contradict this.

Our results for the Argentinean samples are very similar to the findings of Pérez-Carrera and Fernández-Cirelli [36] who reported 38 ± 9 and 44 ± 17 µg/kg wm in Argentinean bovine livers and kidneys.

Our findings are also in good accordance with some already published total arsenic concentrations of ruminants' livers and kidneys from other parts of the world (Table 3), for example from Belgium [56], Australia [39] or Spain [48]. Some publications reported lower total arsenic values in ruminants' livers and kidneys, for example for tissues from cattle from Egypt (5 µg/kg wm in livers, 15 µg/kg wm in kidneys) [53] and Zambia (up to 10 µg/kg wm in livers, 4 – 28 µg/kg wm in kidneys) [55] or from sheep of the Netherlands (up to 25 µg/kg wm in livers, up to 44 µg/kg wm in kidneys) [57]. There were also cases where higher total arsenic concentrations had been found, for example 50 and 106 µg/kg wm in livers and kidneys of Jamaican cattle (mean values) [51] or 120 – 300 µg/kg dm in livers and up to 450 µg/kg dm in kidneys of Australian cattle that had been grazing near a former gold mine [49]. Moreover, tissues of seaweed eating sheep from Northern Scotland contained more arsenic than our bovine tissue samples from Austria and Argentina [59], but it has also to be kept in mind that arsenic concentrations in sheep and cattle might be different, anyways.

4.3.2 Other elements

Besides arsenic, we also analyzed a variety of other elements in the bovine tissue samples. All results of the multi element analysis of cattle tissues from both countries can be found in Table 22 (livers) and Table 23 (kidneys). Some of the results are worth mentioning:

Concerning **vanadium**, around 8 – 120 µg/kg dm have been reported in literature for beef liver [123, 124]. Our findings for the Austrian liver and kidney samples were in good accordance with this (2.2 – 20 and 7.9 -61 µg/kg dm), but the vanadium concentrations in samples from Argentina were significantly higher, namely up to more than 2 mg/kg dm. The median of vanadium was 15 times higher in Argentinean livers than in Austrian livers, and even 40 times higher in Argentinean kidneys than in Austrian kidneys (Figure 24). These high vanadium concentrations in the Argentinean tissue samples also reflect the results of our analyses of Argentinean well water, where the vanadium levels were uncommonly high as well (up to 174 µg/L).

Table 22: Median and range of different elements in bovine livers from Austria and Argentina (italics = mg/kg dm, underlined = g/kg dm, norma l= µg/kg dm), < 2 = below limit of detection)

Element	Austrian livers		Argentinean livers	
	median	Range	Median	Range
Ag [µg/kg dm]	11	3-83	50	27-180
As [µg/kg dm]	38	20-75 (170)	110	25-150
B [mg/kg dm]	*1.6*	*0.36-2.1*	*0.58*	*0.28-1.2*
Ba [µg/kg dm]	59	32-92	69	55-230
Be [µg/kg dm]	< 2	< 2	< 2	< 2
Bi [µg/kg dm]	< 2	< 2-14	< 2	< 2
Ca [mg/kg dm]	*160*	*110-180*	*130*	*110-160*
Cd [µg/kg dm]	110	39-350	73	48-460
Co [µg/kg dm]	230	170-330	140	110-230
Cr [µg/kg dm]	34	16-560	190	53-1100
Cu [mg/kg dm]	*37*	*6.0-290*	*22*	*3.8-75*
Fe [mg/kg dm]	*200*	*160-250*	*150*	*120-160*
Ga [µg/kg dm]	5.0	3.7-7.0	9	7.3-15
Hg [µg/kg dm]	< 2	< 2-5.6	2.6	< 2-65
K [g/kg dm]	13	12-14	10	8.9-11
Li [µg/kg dm]	18	5.9-48	35	22-48
Mg [mg/kg dm]	*710*	*650-820*	*620*	*570-640*
Mn [mg/kg dm]	*10*	*7.3-12*	*7.8*	*5.0-8.2*
Mo [mg/kg dm]	*4.3*	*3.4-4.8*	*3.1*	*1.3-3.6*
Na [g/kg dm]	2.4	2.1-2.7	2	1.9-2.6
Ni [µg/kg dm]	14	< 2-470	210	95-630
Pb [µg/kg dm]	140	28-870	100	25-770
Rb [mg/kg dm]	*45*	*14-180*	*11*	*3.7-18*
Sb [µg/kg dm]	6.3	2.2-22	< 2	< 2
Se [mg/kg dm]	*0.61*	*0.092-1.0*	*0.88*	*0.46-1.5*
Sr [µg/kg dm]	100	48-150	300	250-360
Te [µg/kg dm]	< 2	< 2-3.7	< 2	< 2
Tl [µg/kg dm]	< 2	< 2	< 2	< 2
U [µg/kg dm]	< 2	< 2	< 2	< 2
V [µg/kg dm]	6.5	2.2-20	100	63-2200
Zn [mg/kg dm]	*130*	*120-170*	*120*	*100-160*

Table 23: Median and range of different elements in bovine **kidneys** from Austria and Argentina (italics= mg/kg dm, underlined= g/kg dm, normal= µg/kg dm), < 2=below limit of detection)

Element	Austrian kidneys		Argentinean kidneys	
	Median	Range	Median	Range
Ag [µg/kg dm]	6	< 2-70	12	2.6-59
As [µg/kg dm]	290	105-480 (1390)	240	85-500
B [mg/kg dm]	*3.0*	*1.9-3.4*	*1.7*	*0.7-2.5*
Ba [µg/kg dm]	850	620-3900	1400	680-7300
Be [µg/kg dm]	< 2	< 2	< 2	< 2
Bi [µg/kg dm]	2.6	< 2-20	< 2	< 2
Ca [mg/kg dm]	*510*	*400-2800*	*420*	*320-1000*
Cd [µg/kg dm]	990	200-4700	450	110-2200
Co [µg/kg dm]	130	93-170	100	60-160
Cr [µg/kg dm]	130	24-620	700	440-1100
Cu [mg/kg dm]	*23*	*19-27*	*14*	*8.9-18*
Fe [mg/kg dm]	*290*	*200-550*	*230*	*120-270*
Ga [µg/kg dm]	3.3	2.4-4.8	7.2	4.7-9.0
Hg [µg/kg dm]	9.1	4.0-25	35	5.0-870
K [g/kg dm]	16	15-18	11	5.4-13
Li [µg/kg dm]	120	25-450	75	60-300
Mg [mg/kg dm]	*1000*	*1000-1600*	*720*	*500-800*
Mn [mg/kg dm]	*6.1*	*4.0-6.7*	*2.4*	*1.9-3.0*
Mo [mg/kg dm]	*2.7*	*3.1-4.6*	*1.8*	*1.3-2.7*
Na [g/kg dm]	9.9	8.7-13	6.7	3.7-8.2
Ni [µg/kg dm]	150	23-1200	380	230-780
Pb [µg/kg dm]	390	180-2400	360	80-510
Rb [mg/kg dm]	*41*	*12-130*	*4.7*	*2.2-11*
Sb [µg/kg dm]	3.5	2.0-8.2	< 2	< 2-5.2
Se [mg/kg dm]	*3.8*	*1.6-5.2*	*6.1*	*4.5-9.0*
Sr [µg/kg dm]	380	300-3300	1400	800-4100
Te [µg/kg dm]	3.7	2.2-8.1	4.6	2.2-18
Tl [µg/kg dm]	10	3.1-32	7.9	2.6-20
U [µg/kg dm]	< 2	< 2	12	3.2-31
V [µg/kg dm]	12	7.9-61	500	100-1500
Zn [mg/kg dm]	*110*	*100-160*	*87*	*73-150*

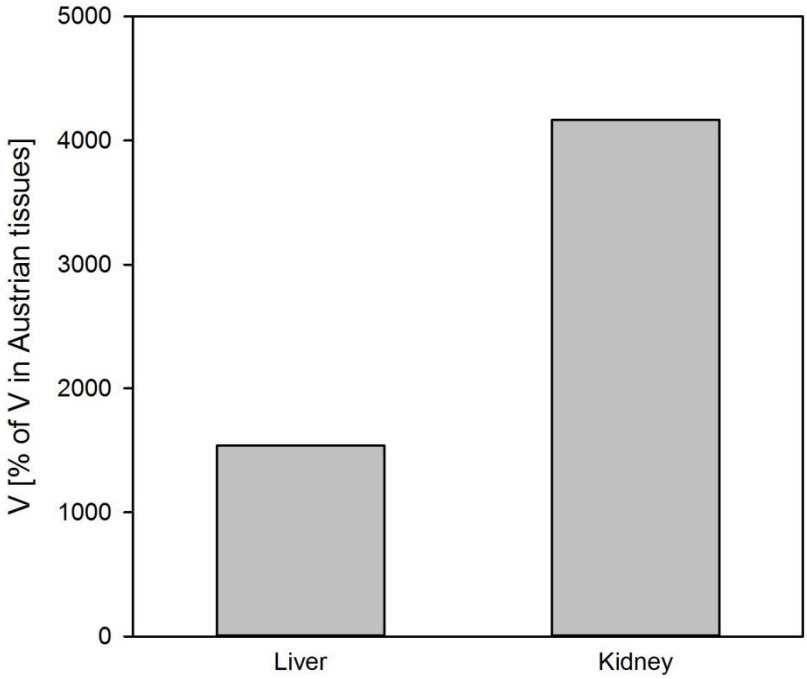


Figure 24: Vanadium in Argentinean bovine tissues (median), in % of vanadium in Austrian samples (median)

The essentiality of vanadium has been shown for animals, but still needs to be proven for humans [125]. It has great potential in the therapy and prevention of various types of cancer [126] as well as in the treatment of diabetes [127]. However, vanadium can also be toxic for mammals. Symptoms of acute poisoning are weakness, vomiting, headache, green discolorization of the tongue, anemia, etc., and industrial exposure is reported to cause irritation of the respiratory system and asthma-like symptoms [128].

Vanadium concentrations in blood and urine of unexposed humans are usually below 1 µg/L [129, 130]. The daily oral intake is estimated to be around 10 – 30 µg vanadium [125]. For example, the daily dietary intake for citizens of the United States was calculated to be around 16 – 18 µg vanadium [131]. The daily vanadium exposure in Northern Italy was between 3.3 and 17.9 µg [132] and slightly higher in Catalonia, Spain (28.9 µg V/day) [133].

Regularly drinking water with high vanadium levels, like the samples we analyzed, and also consuming beef products of cattle with such elevated vanadium concentrations, would lead to much higher vanadium intake levels and maybe also health problems of chronic vanadium exposure, although little is known about such effects.

Big differences between Austrian and Argentinean samples can also be seen regarding **mercury**: While Austrian tissue samples contained not more than 25 µg/kg dm, kidney samples from Argentina showed slightly higher concentrations, the median value being 35 µg/kg dm. Kidney sample CFR02R1 contained even 870 ± 40 µg/kg dm. According to the

EFSA, food other than fish and seafood generally contains less than 50 µg Hg/kg [134]. This is in good accordance with our results, except the one Argentinean kidney sample CFR02R1. The European Commission has established a maximum level for total mercury, but only for fish. Depending on the type of fish, this value is 0.5 or 1.0 mg/kg wm [135]. The outlier kidney sample from Argentina would still be below this limit, but for a tissue sample of a terrestrial mammal, its concentration is high.

We detected much more **chromium** in Argentinean tissues than in Austrian ones, with median concentrations of 34 and 190 µg/kg dm in Austrian and Argentinean livers and 130 and 700 µg/kg dm in the kidneys. Other elements that were more abundant in tissues from Argentina were **strontium** and **nickel**. The median values for strontium were 100 and 380 µg/kg dm in Austrian livers and kidneys and 300 and 1400 µg/kg dm in Argentinean livers and kidneys. The median concentrations of nickel were 14 and 150 µg/kg dm in Austrian livers and kidneys and 210 and 380 µg/kg dm in the corresponding Argentinean samples. Mean ± standard deviations of those elements are displayed in Figure 25. The high amounts of both nickel and chromium could be an indication of a contamination from tools that were used at the Argentinean slaughterhouses (for example, chromium-nickel steel). However, we did not obtain any information about the materials of the knives etc. that are used at the slaughterhouses to confirm this.

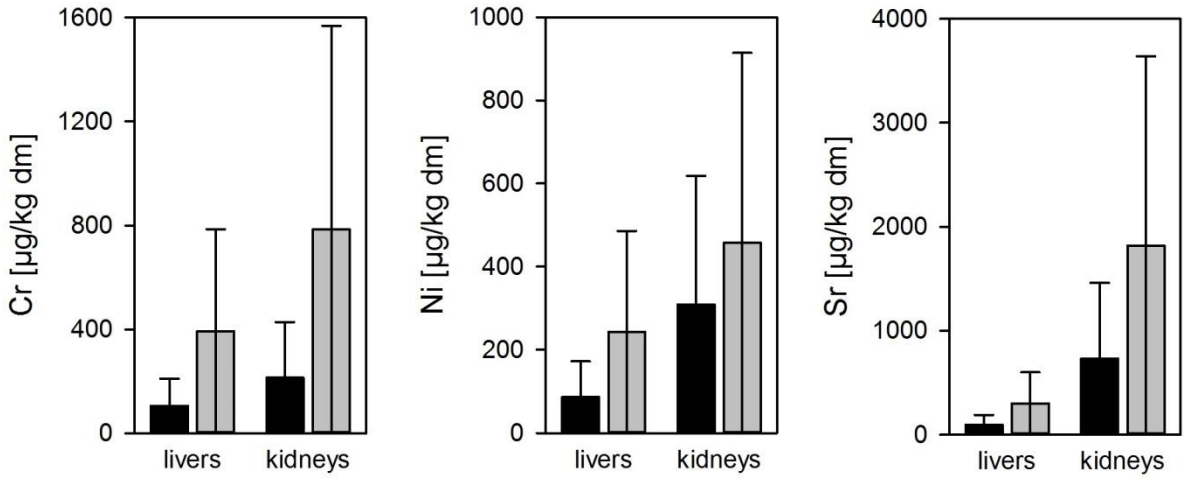


Figure 25: Chromium, nickel and strontium concentrations (mean ± standard deviation) in livers and kidneys from Austria (black) and Argentina (grey)

The **cadmium** concentrations in both Argentinean and Austrian kidneys were rather high. The median in Austrian kidneys was 990 µg/kg dm, and one sample (No.1306) even contained 4.7 ± 1.4 mg/kg dm. The European Comission set maximum levels for cadmium in bovine livers (0.5 mg/kg wm) and kidneys (1 mg/kg wm) [135]. Converted to dry mass concentrations, this means a maximum limit of 1.7 mg/kg dm in livers and 5 mg/kg dm in

kidneys. Of the investigated samples, kidney sample no. 1306 exceeded these limits. However, the standard deviation is unpleasantly high (almost 30 %, which is a result of the different concentrations of the triplicate digests: 3.0, 5.7 and 5.3 mg/kg dm), and the measurement would have to be repeated to give a safe statement whether the sample exceeded the maximum allowed level or not.

Austrian liver and kidney samples contained much more **rubidium** than the Argentinean samples. While the rubidium concentrations in Austrian kidneys ranged from 12 to 130 mg/kg dm, the highest value in Argentinean kidneys was 11.2 ± 0.1 mg/kg dm. The rubidium concentration in Argentinean livers ranged from 3.7 to 18 mg/kg dm and from 14 to 180 mg/kg dm in Austrian livers.

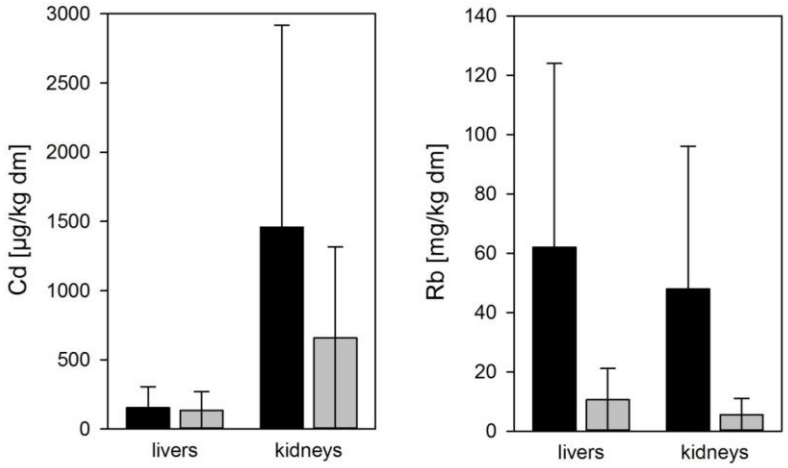


Figure 26: Cadmium and rubidium concentrations (mean ± standard deviation) in livers and kidneys from Austria (black) and Argentina (grey)

We found a correlation between the arsenic and **selenium** concentrations in the livers and kidneys from Argentina (Figure 27), but not in Austrian samples.

Arsenic was highly linearly correlated to **iron** in Austrian samples, and more vaguely correlated in Argentinean samples (Figure 28). It is possible that the iron concentration indicated the amount of hemoglobin (and therefore blood) was still left in the tissue samples, but we cannot say this for sure. However, if this argument should actually be true, the correlation of arsenic and iron could suggest that the arsenic in the blood was somehow coordinated to the red blood cells. Some of the iron could also be a contamination from knives and other tools used in the slaughterhouses.

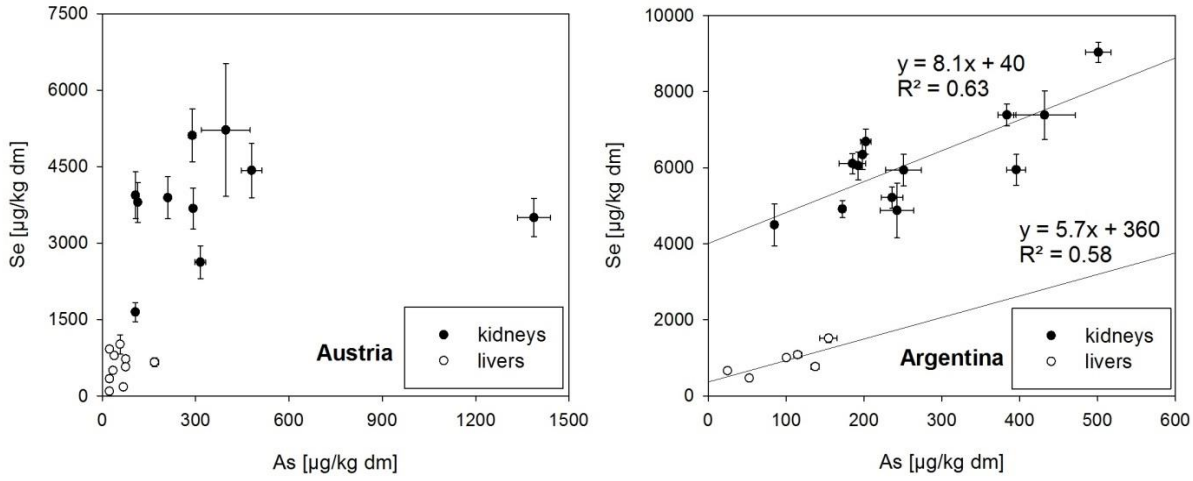


Figure 27: As and Se concentrations in Austrian and Argentinean kidney and liver samples. Correlation parameters are not given for the Austrian samples because no correlation was apparent.

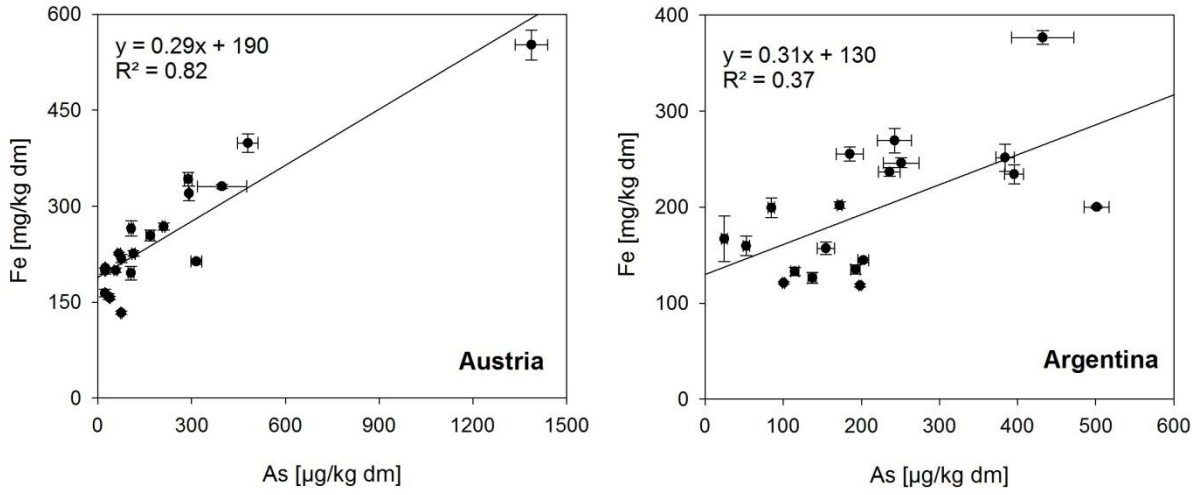


Figure 28: Correlation of As and Fe concentrations in Austrian and Argentinean cattle tissues (Correlation of the Austrian samples without the highest concentration pair: y = 0.44x + 170, R^2 = 0.74)

In addition to arsenic, we also found linear correlations between the elemental concentrations in Austrian liver and corresponding kidney samples for **lithium**, **vanadium**, **cadmium** and **rubidium** (Figure 29). Especially the correlation of the rubidium concentrations in livers and kidneys is remarkable. We did not find any liver-kidney correlation of the other alkali elements sodium or potassium.

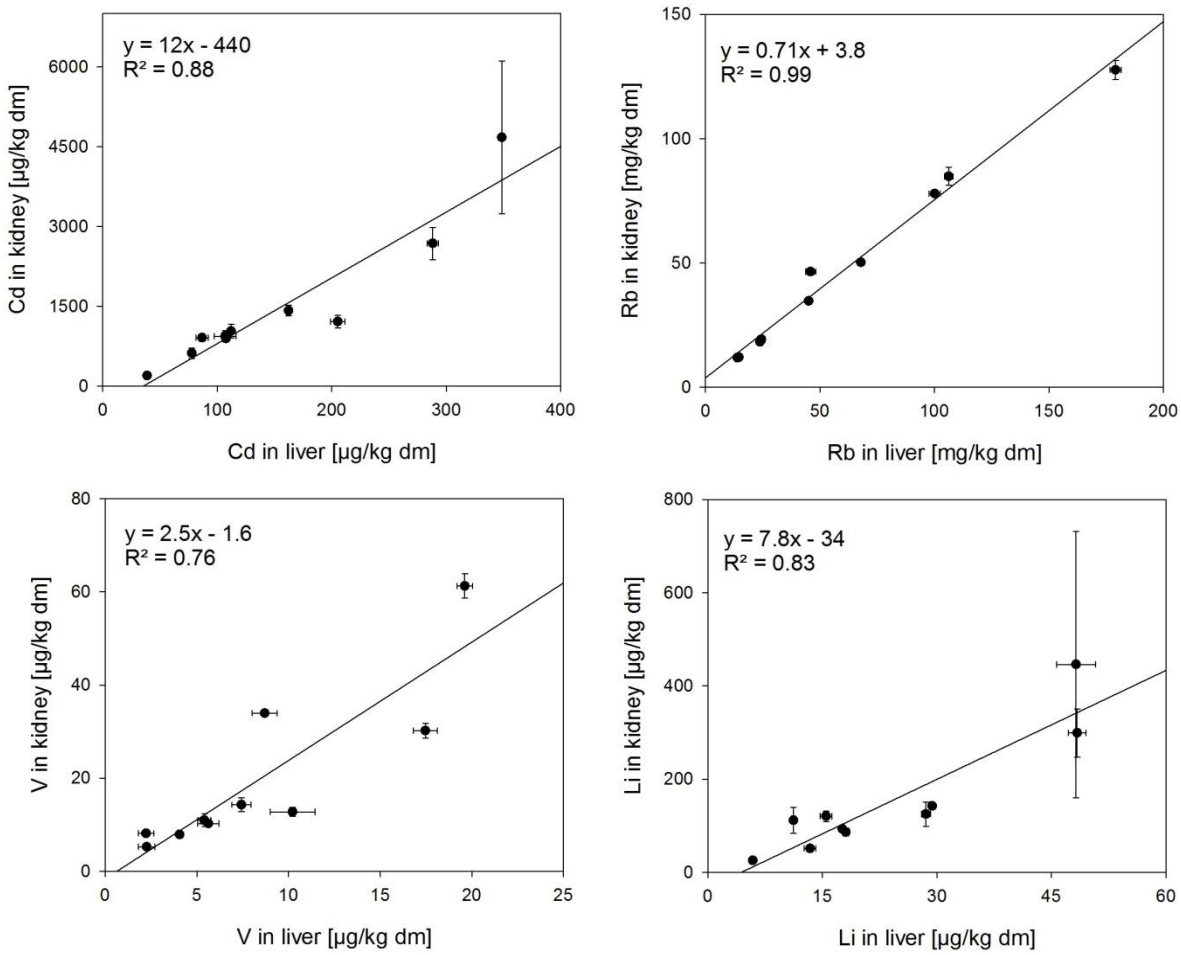


Figure 29: Correlation of elemental concentrations in Austrian bovine livers and corresponding kidneys: cadmium (Cd), rubidium (Rb), vanadium (V) and lithium (Li)

4.3.3 Arsenic speciation

4.3.3.1 Quantification of the major arsenic species

We analyzed the arsenic speciation in extracts of all Austrian and Argentinean tissue samples using anion-exchange chromatography with the conditions that are described in chapter 3.8.1, with a PRP-X100 column as stationary phase and an aqueous 20 mM ammonium phosphate solution at pH 6.0 as eluent.

There was no obvious difference between the arsenic speciation in Austrian tissue samples and the one in Argentinean tissue samples. Figure 30 shows chromatograms of the extracts of two kidney samples. According to the retention time, the major arsenic compound in all but two liver and kidney extracts was MA. The abundances of the different arsenic metabolites are summarized in Table 24 and visualized in Figure 33. In livers, 35 - 65 % and in kidneys, 30 - 70 % of the extractable arsenic was MA. Further on, about 15 - 40 % in livers and 10 - 35 % in kidneys were DMA. There was 5 - 30 % inorganic arsenic in most bovine liver and

kidney extracts. As far as we could find out with anion-exchange chromatography, the liver extracts contained hardly any cationic arsenic species (< 3 %), and also in kidney extracts the cationic arsenic compounds made up only 1 to 10 % of the extractable arsenic. The difference of the retention time of AB in the standard solution and the cationic compound(s) in the extracts indicated that the cationic arsenic species in the liver and kidney extracts was probably something else than AB.

Table 24: Abundance of different arsenicals in bovine tissue samples, in % of the extractable arsenic

Compound	Liver	Kidney
MA	35 - 65	30 – 70 (2 x 20)
DMA	15 - 40	10 – 35 (2 x < 10)
Inorganic As	5 - 30	5 - 30 (2 x 60)
Cationic As	< 3	1 – 10
Unknown A	1 x 5, 1 x 9	7 x 4, 2 x 20
Unknown B and C	-	3 x 3, 1 x 9

In two Argentinean kidneys (CFR01 and BVR20) inorganic arsenic turned out to be the major arsenical, accounting for 63 and 68 % of the extractable arsenic. This is not correlating with especially high total arsenic concentrations since we only measured 85 and 185 µg As/kg dm in the digests of those tissues. The extraction efficiencies of those two samples were 35 and 50 %, which makes a contamination during sample preparation unlikely. The other species in those two samples were around 20 % MA, 2.0 and 7.4 % DMA and roughly 8 % cationic arsenic. We have not found an explanation of this unusual arsenic speciation in the two samples.

In some of the extracts we were able to detect three unusual arsenic compounds that did not coelute with any of the arsenic standards that were available to us. Unknown A eluted close to MA, sometimes before and sometimes right afterwards, depending on the chromatographic conditions. We found this signal in 7 kidneys with an abundance of about 4 %, and in another 2 kidneys it accounted for even 20 % of the extractable arsenic. We also found the same compound in 2 of the liver extracts (5 and 9 %). Unknown B and C were retained on the column for about 7 - 8 minutes, in close proximity to inorganic arsenic. We only detected them in 4 kidney extracts where they made up about 3 % (one time even 9 %) of the extractable arsenic. We did not find them in any of the liver extracts.

In Austrian tissue samples, MA concentrations were 6 - 8 times higher in kidneys than in the corresponding livers, and we found 2.5 - 4 times more DMA in kidneys than in the corresponding livers (Table 25). The ratio of MA to DMA in livers as well as in kidneys gave a

linear correlation for Austrian as well as Argentinean samples (Figure 31), although the correlation was a little bit better established in Austrian samples. Although MA was the major compound in livers as well as in kidneys, its abundance compared to DMA was much lower in livers compared to kidneys, especially in Austrian samples.

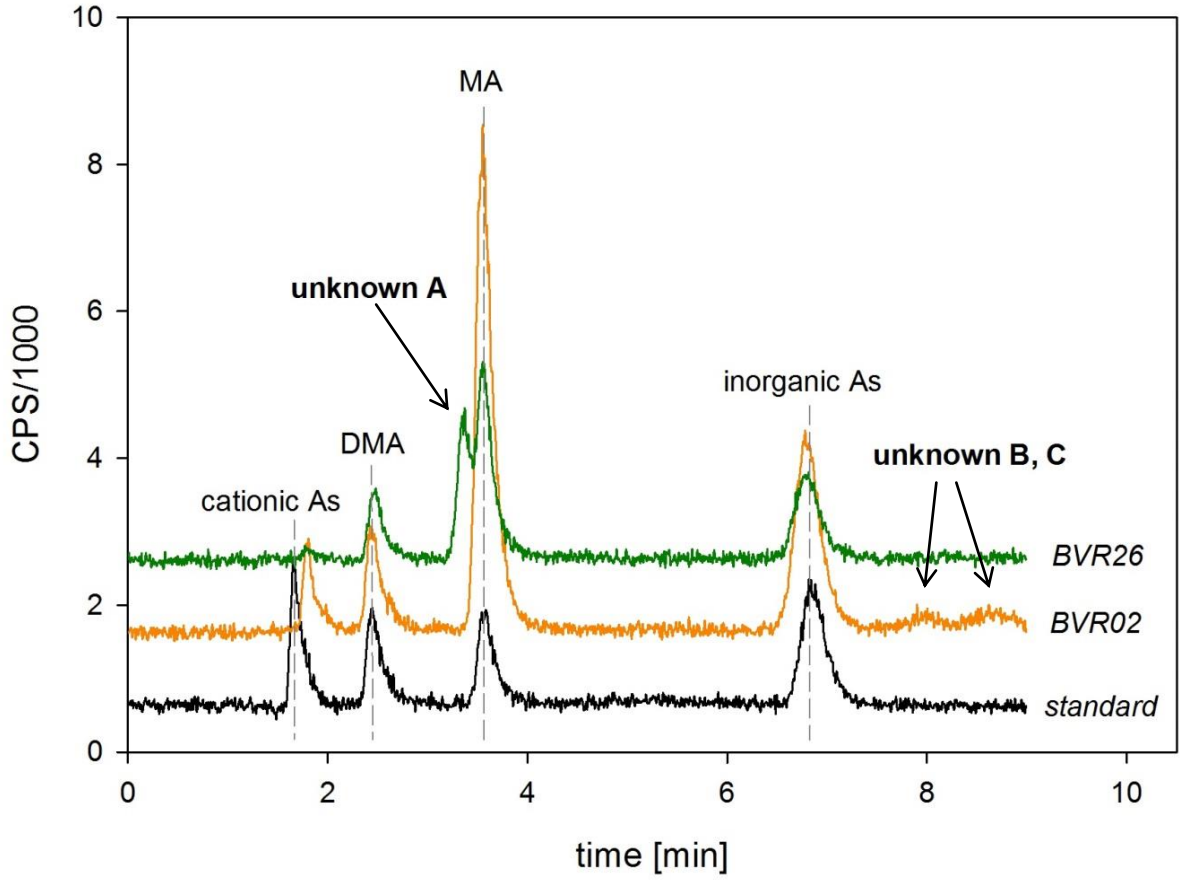


Figure 30: Chromatograms of extracts of the Argentinean kidney samples BVR02 (offset: +1000 CPS) and BVR26 (offset: +2000 CPS) and an oxidized standard solution with 1 µg As/L of AB, DMA, MA, As(III) and As(V)

Table 25: Different molar ratios of MA and DMA in bovine liver and kidney samples

	Austria	Argentina
MA (kidney)/MA (liver)	6 - 8	-
DMA (kidney)/DMA (liver)	2.5 - 4	-
MA (liver)/DMA (liver)	1.5 – 2.5	1.5 – 3 (1 x 6)
MA (kidney)/DMA (kidney)	4 – 5 (1 x 7)	1 - 5 (1 x 13)

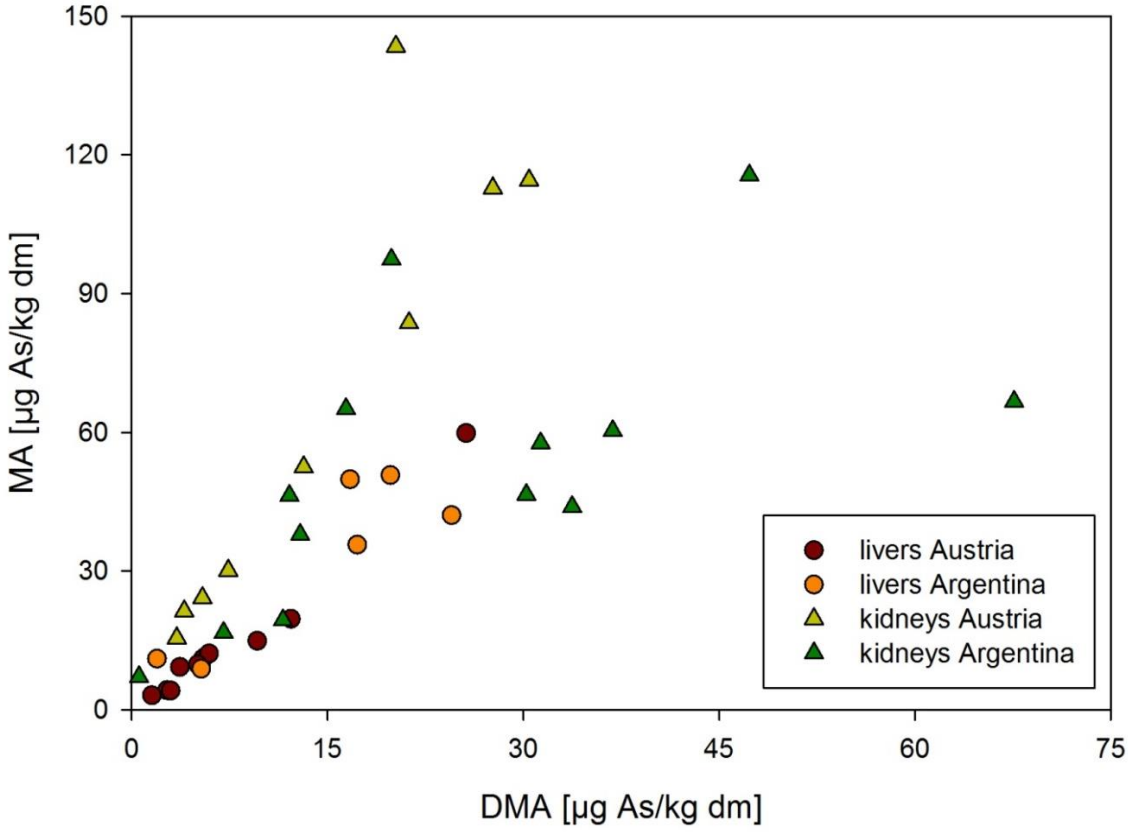


Figure 31: Correlation of MA and DMA in extracts of bovine livers and kidneys (Austrian kidney sample number 1320 is not displayed: MA = 470 µg As/kg dm, DMA = 110 µg As/kg dm)

4.3.3.2 Comparison of the two extraction methods

It is well known that trivalent arsenic species as well as thioarsenicals are labile and very easily oxidized to their pentavalent oxygenated analogues. A common approach to avoid uncertainties about the grade of oxidation during analysis is the complete oxidation of the arsenicals with hydrogen peroxide (H_2O_2), followed by analysis of the stable forms of the arsenicals. This is why we always oxidized the liver and kidney extracts at some point before the measurement, either directly during extraction or right afterwards.

Regardless which extraction method was used, the samples foamed heavily when H_2O_2 was added. It was added. It was important to work very carefully to avoid sample loss. The efficiency of the oxidizing extraction method with H_2O_2 in the extracting solution was between 20 and 70 % for liver as well as for kidney samples. Extraction of Austrian samples without H_2O_2 resulted in significantly lower extraction efficiencies (15 - 40 %). Chromatograms of extracts of the same kidney sample after application of the two different extraction conditions are shown in Figure 32. The concentrations of the arsenicals in the extracts of 5 individual livers and kidneys under oxidizing and not oxidizing extraction conditions are listed in Table 26 and

Table 27. The difference in the outcome of the two extraction methods is depicted in in Figure 33. In order to be able to compare the species distribution of the different samples with each other, all concentrations were normalized on the MA concentration of the individual extracts of the oxidizing extraction method before calculating the mean and standard deviation.

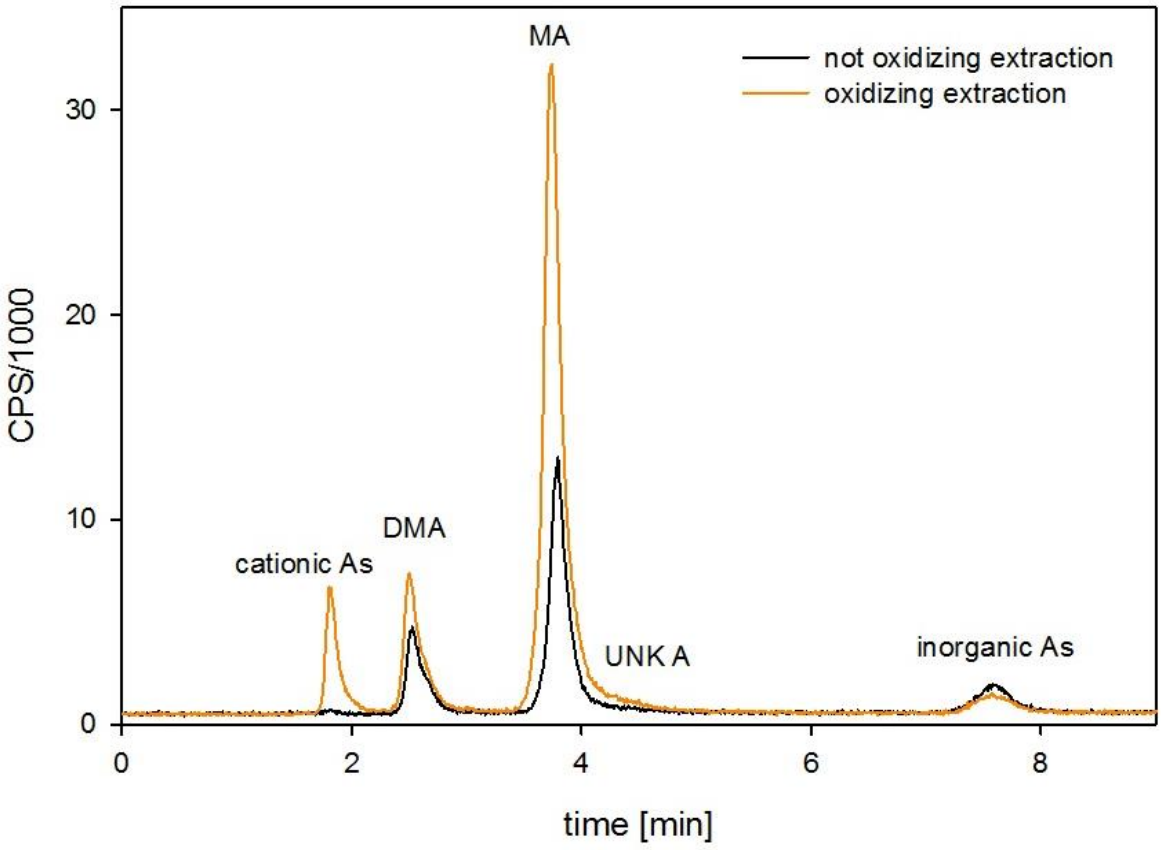


Figure 32: Comparison of the chromatograms of extracts of an Austrian kidney sample under oxidizing and not oxidizing extraction conditions

Table 26: Concentrations [µg/kg dm] of the arsenic species in extracts of 5 Austrian liver samples under oxidizing and not oxidizing extractions conditions (UNK = UNK A-C)

Liver number	Not oxidizing extraction conditions					Oxidizing extraction conditions				
	DMA	MA	iAs	cat. As	UNK	DMA	MA	iAs	cat. As	UNK
1283	2.7	4.2	3.5	-	-	6.2	12	7.5	-	-
1288	1.9	5.7	4.5	-	-	5.9	12	5.8	-	2.4
1306	5.1	10	5.8	-	-	4.6	14	6.3	-	-
1320	4.2	12	5.3	-	-	26	60	7.7	4.6	6.2
1324	11	18	5.0	-	-	12	20	4.6	-	-

Table 27: Concentrations [µg/kg dm] of the arsenic species in extracts of 5 Austrian kidney samples under oxidizing and not oxidizing extractions conditions (UNK = UNK A-C)

Kidney number	Not oxidizing extraction conditions					Oxidizing extraction conditions				
	DMA	MA	iAs	cat. As	UNK	DMA	MA	iAs	cat. As	UNK
1283	7.4	30	4.4	-	-	13	39	9	3.4	-
1288	17	25	18	-	-	21	84	19	6.0	13
1306	17	33	16	-	-	20	140	20	12	9.7
1320	61	180	40	-	-	110	470	36	65	42
1324	21	36	13	-	-	30	115	14	8.7	14

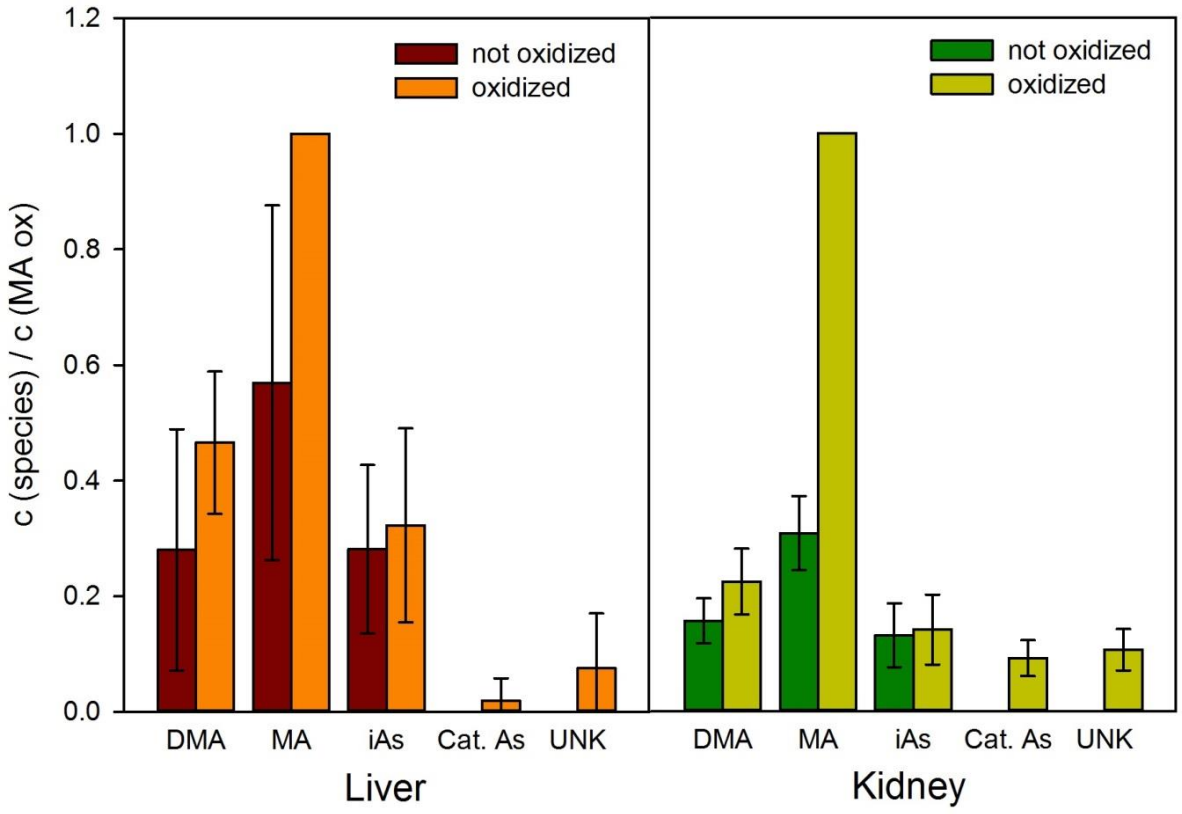
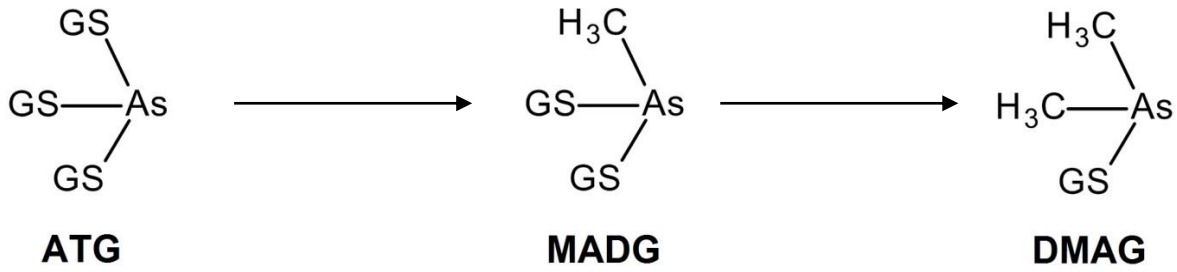


Figure 33: Mean ± standard deviation of arsenic species concentrations in Austrian tissue extracts under oxidizing and not oxidizing extraction conditions, normalized on the individual concentrations of MA in the oxidizing extracts (UNK = Unknown A, B and C)

It can easily be seen that the oxidation step massively affected MA, especially concerning the kidney samples. In some samples, up to 4 times more MA was present in the extracts with hydrogen peroxide than in the extracts without oxidizing agent. The abundance of DMA and inorganic arsenic was far less influenced by the extraction conditions. The concentration of inorganic arsenic was almost the same in both extractions. We were able to extract a bit more DMA from the liver samples under oxidizing extraction conditions than under not oxidizing conditions, but there was far less difference in the DMA concentrations of the

different extracts of the kidneys samples. The cationic arsenicals as well as the unknown compounds UNK A-C were only extractable under oxidizing conditions.

It is known that trivalent arsenicals are readily binding to proteins and thiols (for example GSH) and are released upon oxidation to their pentavalent equivalents. This provides an explanation for the different extraction efficiencies of the oxidizing and the not oxidizing extraction method: Large parts of the arsenicals had probably been present in the tissue samples as GSH-complexes (Scheme 4).



Scheme 4: GSH complexes present in the arsenic metabolism as proposed by Hayakawa et al. [78]

Application of hydrogen peroxide released the arsenicals from the GSH and oxidized them to their pentavalent forms, which could then be extracted from the samples. This process mainly affected MA, or rather its precursor MADG. It looks as if only small amounts of ATG and DMAG, but much larger amounts of MADG had been occurring in the organs, especially in the kidneys.

Hayakawa et al proposed that DMAG was not stable and therefore rapidly converted to DMA(V) [78]. Supposing that DMA was no longer bound to proteins and instantly transported into the urine, our findings that MA was much more abundant than DMA and inorganic arsenic are supporting the metabolic pathway of inorganic arsenic proposed by Hayakawa et al. (see Scheme 3 in chapter 2.1.3). The idea of a rapid release of DMA is also reinforced by our findings that DMA did not become much more abundant upon oxidation, which could mean that DMA was mostly present in bovine livers and kidneys as actual DMA and not as its precursor DMAG.

The unknown arsenicals A, B and C were only detectable upon extraction with H_2O_2. This could suggest that they had also been occurring in the tissues as trivalent arsenicals, bound to proteins or GSH like the main arsenicals. Still, we do not know yet if this assumption is justified or not. It could also be possible that the presence of hydrogen peroxide lead to the formation of artefacts in the extracts. We do not know anything else about these unknown arsenic compounds.

As already discussed in the introduction (chapter 2.1.4.3), there are different opinions in the scientific literature about the arsenic speciation in terrestrial mammals' tissues. It has been generally thought that DMA is the major arsenic metabolite in livers and kidneys [84]. It was suggested that MA only becomes more abundant than DMA when the organisms had been exposed to high amounts of As(III) [87]. Our results are not supporting this hypothesis. We identified MA as the major arsenical and smaller amounts of DMA and inorganic arsenic in the tissue extracts, although at least the Austrian animals had not been exposed to excessive amounts of arsenic, and also the total arsenic concentrations, which were not exceptionally high in 9 out of 10 sample pairs. It has to be taken into account that all publications so far had been dealing with primates or rodents. Since the gut flora of these organisms is quite different to the one of ruminants, differences in the arsenic metabolism are possible. To verify this, we would have to apply our method of sample preparation to rodents' or primates' tissues, measure the extracts with HPLC-ICPMS and then compare the results with our data about the bovine tissues.

4.4 Urine of terrestrial mammals

4.4.1 General remarks

The median of the specific gravities of the cows' urine samples was 1.025, with the single values ranging from 1.003 to 1.032. The specific gravities of the urine samples from Schönbrunn Zoo ranged from 1.004 (African elephant VI) to 1.046 (blackbuck and vicuña II), with a median of 1.026. The specific gravities were not taken into account in the calculations of the elemental urine concentrations and speciation analysis.

Because the samples from Schönbrunn Zoo were not collected directly from the animals but from the ground, it is possible is that some elements were absorbed from the ground into the urine before collection. It was not possible to determine the influence of the ground for all samples, also because the different animals were urinating on different areas. In an experiment with human urine we found out that the arsenic concentration did not significantly increase when it was poured on concrete floor. Unfortunately, we were not able to determine this for other elements. If elements were absorbed from the ground, the most probable candidates would be the alkaline earth metals like magnesium, calcium, strontium or barium.

An indication about the absorbed amounts can be the comparison of the samples from the zoo with the samples from the farm animals, since the latter ones never reached the floor.

4.4.2 Total arsenic concentrations

4.4.2.1 Cattle

In the urine of cows from the farm in Upper Styria we detected 3 - 25 µg As/L, with a mean of 17 ± 7 µg As/L and a median of 19 µg As/L. Surprisingly, we found high arsenic concentrations in the cows' urine samples from the farm in Eastern Styria, namely up to 125 µg/L. The sample with the lowest concentration (7.1 µg As/L) also had a very low specific gravity (1.003). There were also two urine samples of one calf that had significantly lower arsenic concentrations than the samples of the adult animals (2.9 and 8.6 µg/L, specific gravities: 1.003 and 1.009). Excluding the samples of the calf, the mean total arsenic concentration of the urine samples from Eastern Styria was 60 ± 30 µg As/L. The median was 44 µg As/L. To evaluate the origin of those high urinary arsenic levels, we analyzed the cows' drinking water and the feed supplement that was given to them at the time of sampling (Garant Qualitätsfutter 89022 Rimin Hofmischung, containing minerals and vitamins, 150 - 250 g/cow/day).

The drinking water only contained about 0.5 µg As/L. The arsenic concentration in the feed supplement was 1.4 ± 0.2 mg/kg. When a cow is fed 250 g of the supplement, the animal ingests about 350 µg arsenic.

The amount of excreta produced by one cow during one day varies with the water and feed intake. In literature, around 40 kg excreta (urine and feces) and urine amounts between 6.75 and 16.2 kg per cow and day are reported [136, 137]. If all of the arsenic from the feed supplement was excreted via the urine during one day, this would mean a urinary arsenic concentration between 20 and 50 µg/L (the specific gravity of urine is about 1.0). If the excretion via the feces is also taken into account, the arsenic concentration arising from 250 g of the supplement would be even less than 10 µg/kg in the cow's feces and urine.

The difference between the samples of the two different farms is visualized in Figure 34. The mean total arsenic concentration in the urine samples of the cattle from Schönbrunn Zoo is also displayed there. Among the different animals' urine samples from Schönbrunn there were in total three samples of cattle: two of Tux cattle and one of a Pustertal pied. With a mean total arsenic concentration of 15 ± 3 µg/L, those samples were very similar to the ones from the cows of Upper Styria.

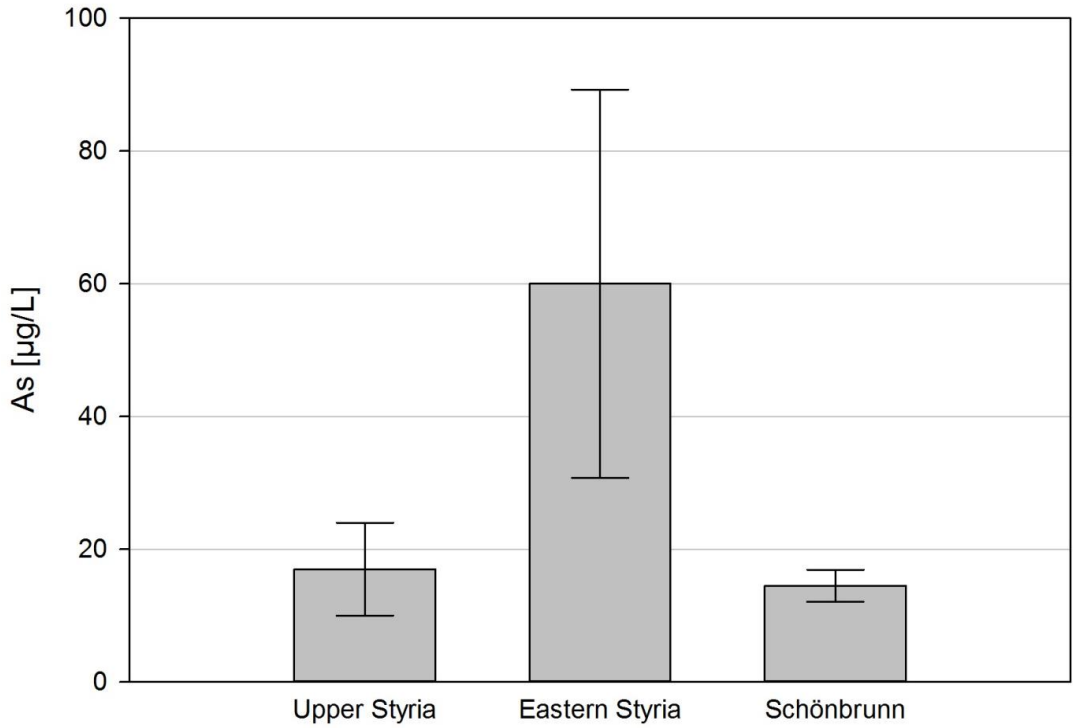


Figure 34: Total As concentrations in urine of cows from the farms in Upper Styria and Eastern Styria (without calf and heavily drinking cow) as well as of cattle from Schönbrunn Zoo (Tux cattle and Pustertal pied)

4.4.2.2 Animals from the Schönbrunn Zoo

The total arsenic concentrations in the urine samples of the different animals are displayed in Figure 35 (ruminants) and Figure 36 (other mammals), and they can also be found in Table 33 in the appendix. The standard deviations are from triplicate digestions of the samples. The concentrations ranged from 5.1 µg As/L (giraffe and African elephant VI) to 81 µg As/L (bison I). The other two bisons' and the water buffaloes' samples also contained high amounts of arsenic (50 - 77 µg/L), followed by the urine samples of blackbuck, Damara zebra, Indian rhinoceros and vicuñas. Additionally to the urine samples of the giraffe and African elephant VI, the samples of Himalayan thar, Barbary sheep, pygmy goat, Heidschnucke and Barbary macaque contained less than 10 µg As/L.

The bisons and the water buffaloes were provided with hay ad libitum. They were also fed pellets and carrots every day and every now and then some branches and green fodder, depending on the season. No obvious source of high amounts of arsenic was given to them.

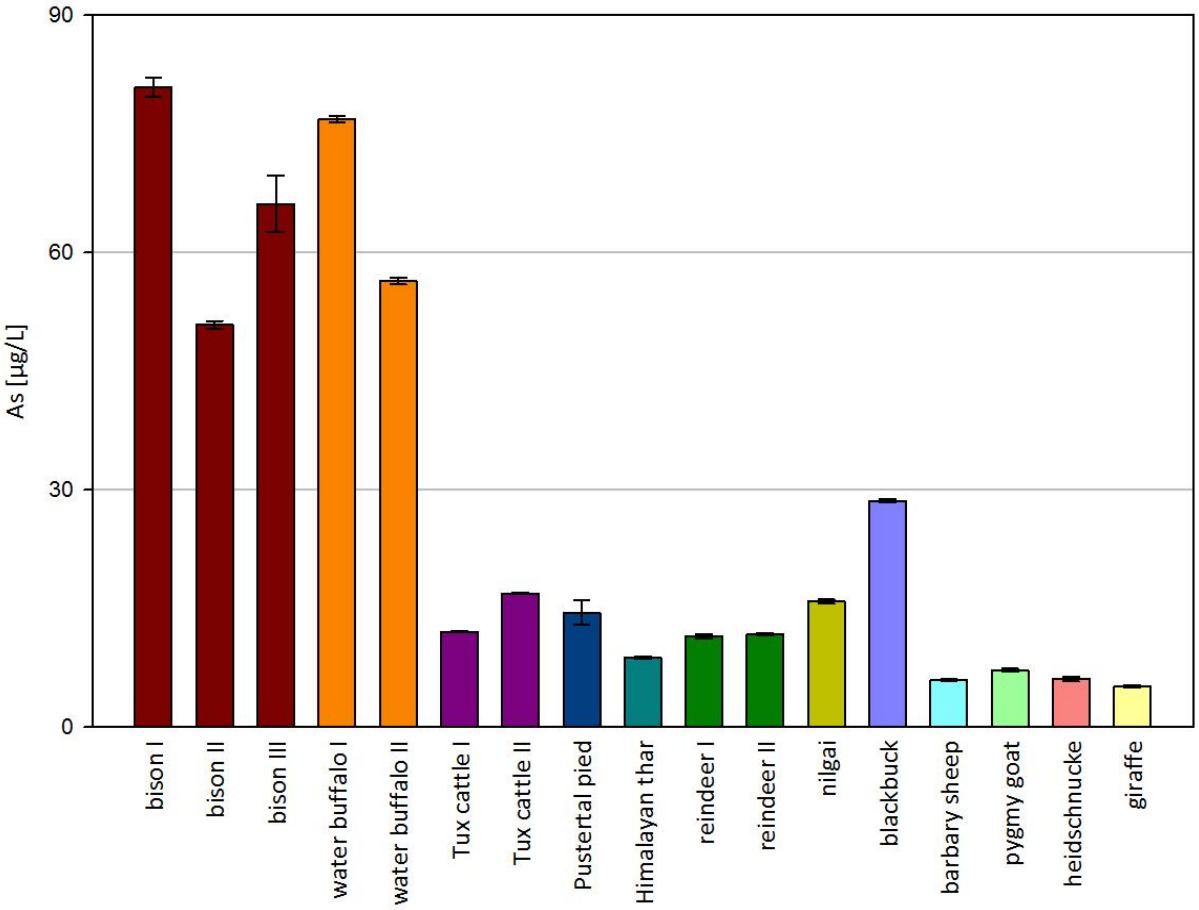


Figure 35: Total arsenic concentrations in urine of ruminants from Schönbrunn Zoo

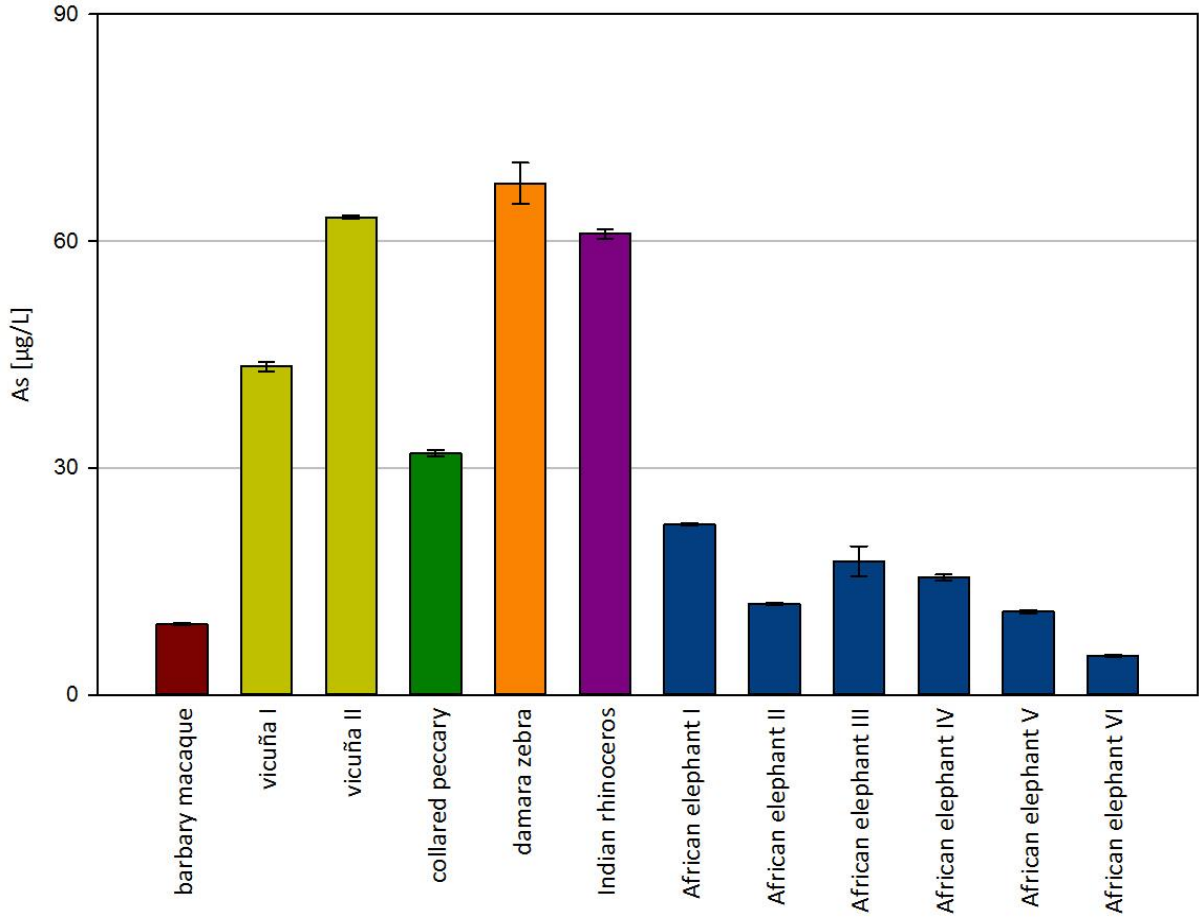


Figure 36: Total arsenic concentrations in urine of mammals from Schönbrunn Zoo (others than ruminants)

4.4.3 Other elements

We also determined the concentrations of various other elements in urine samples from Eastern Styria and from Schönbrunn Zoo. The levels of most elements were very heterogeneously distributed. This can be seen by the concentration ranges listed together with the median values in Table 28, where the results are grouped to three classes: cattle from the farm, ruminants from the zoo and other mammals from the zoo. Two of the reasons for this diversity are of course the great variety of animal species within the sample groups and that we did not consider the specific gravities of the urine samples for calculation. Still, some clear trends could be seen in the results:

The concentrations of some elements were significantly lower in urine samples of cattle from the farm in Eastern Styria than in samples from Schönbrunn Zoo, for example **cobalt, iron**, **manganese, molybdenum, sodium** and **zinc**.

Further on, the urine of ruminants from Schönbrunn Zoo contained more **chromium, rubidium** and **iron** than the other samples.

The urine samples of the African elephants had considerably higher **calcium** concentrations than urine of other animals. While all other samples contained less than 100 mg/L, the median of the six elephants' samples was 340 mg/L. Two of the samples even contained more than 1000 mg/L (1380 ± 10 and 1600 ± 20 mg/L).

All urine samples showed some correlation between the normalized concentrations of **barium**, **magnesium** and **strontium,** also the samples from the farm cows (see Equation 1 for the normalization and Figure 37 for the correlation). The median values of these elements were very similar for the cows and the zoo animals. This indicates that the samples from Schönbrunn did not accumulate high amounts of alkaline earth metals from the ground.

Equation 1: Normalization of elemental concentrations in urine on the specific gravity

$$conc._{corr} = \frac{conc.}{\frac{(specific\ gravity - 1)}{(mean\ specific\ gravity - 1)}}$$

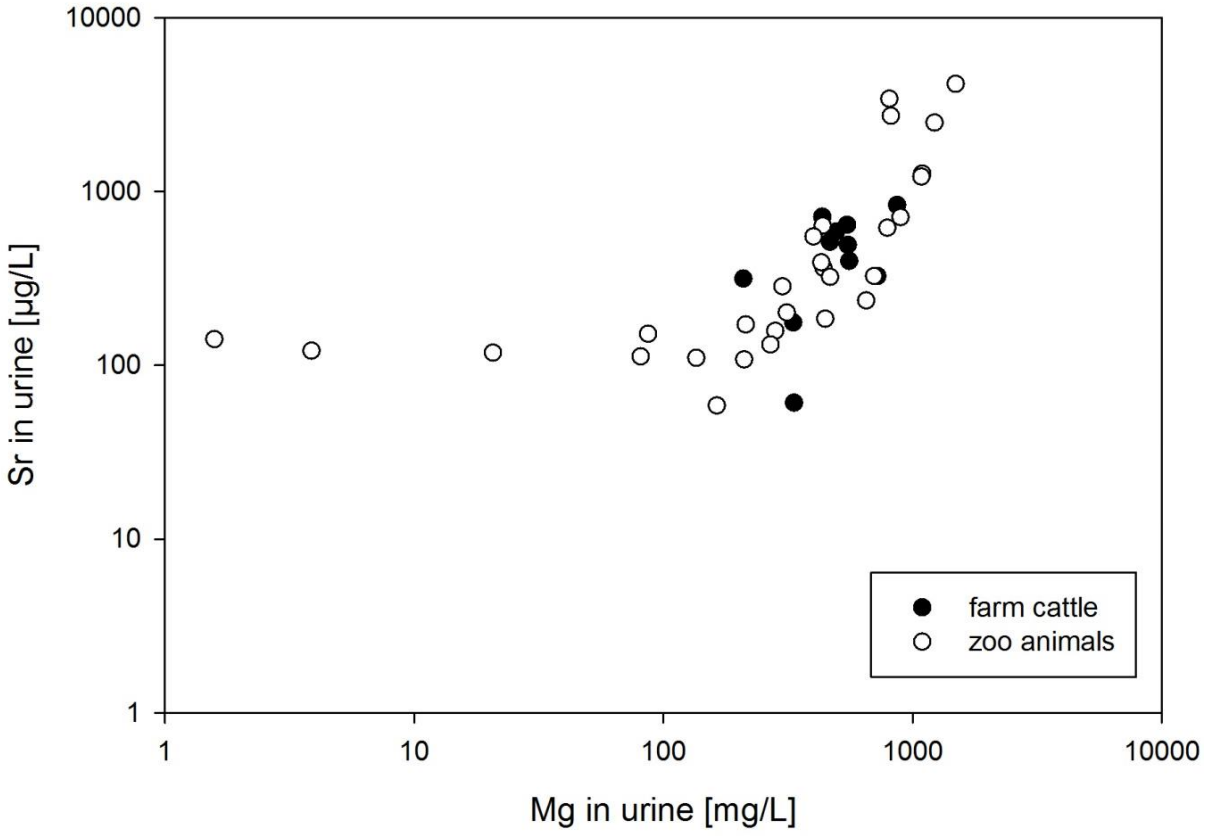


Figure 37: Correlation of magnesium and strontium concentrations in urine of cattle from farms and of animals from Schönbrunn Zoo, normalized on specific gravities, logarithmic scales

Table 28: Median and range of different elements in urine of cattle from Eastern Styria and animals from Schönbrunn Zoo (< 0.2 = below limit of detection, italics = mg/L, normal= µg/L)

	cattle (Eastern Styria)		ruminants (zoo)		other mammals (zoo)	
	median	range	median	range	median	range
Ag [µg/L]	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
B [mg/L]	*9.8*	*1.2 - 13*	8.2	4.8 - 14	5.9	2.4 - 18
Ba [µg/L]	240	26 - 370	170	26 - 680	38	4.0 - 360
Be [µg/L]	< 0.2	< 0.2	0.3	< 0.2 - 0.8	0.3	< 0.2 - 0.6
Bi [µg/L]	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
Ca [mg/L]	*24*	*1.1 - 240*	17	2.5 - 59	62	8.6 - 1600
Cd [µg/L]	0.3	< 0.2 - 2.0	0.6	< 0.2 - 4.4	< 0.2	< 0.2
Co [µg/L]	1.1	0.2 - 1.7	7.9	< 0.2 - 150	9.2	0.4 - 98
Cr [µg/L]	3.3	1.0 - 76	16	0.5 - 79	7.4	1.0 - 97
Cu [µg/L]	6.1	< 1 - 32	18.3	< 0.2 - 110	4.3	< 0.2 - 90
Fe [mg/L]	*0.021*	*0.0013 - 0.31*	1.3	0.1 - 34	1.1	0.03 - 4.7
Ga [µg/L]	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
Hg [µg/L]	< 0.2	< 0.2	0.3	< 0.2 - 0.7	0.3	< 0.2 - 1.6
K [mg/L]	*7000*	*1200 - 13000*	18000	4100 - 38000	8000	100 - 30000
Li [µg/L]	130	6.0 - 260	43	11 - 200	150	11 - 4100
Mg [mg/L]	*410*	*24 - 880*	420	2.8 - 1500	590	80 - 1300
Mn [µg/L]	0.2	<0.1 - 9.0	48	< 0.2 - 1200	0.5	< 0.2 - 550
Mo [µg/L]	0.6	0.1 - 15	65	5.6 - 300	26	11 - 150
Na [mg/L]	*3.4*	*0.4 - 86*	670	7.1 - 6400	220	11 - 2100
Ni [µg/L]	6.9	<1 - 89	12	< 0.2 - 1200	36	< 0.2 - 230
Pb [µg/L]	0.5	0.3 - 1.7	3.4	< 0.2 - 17	0.3	< 0.2 - 14
Rb [mg/L]	*4.1*	*0.43 - 12*	15	0.7 - 28	5.4	0.4 - 14
Sb [µg/L]	0.8	0.3 - 1.9	2.2	0.7 - 8.7	2.8	1.2 - 8.6
Se [µg/L]	13	2.7 - 56	50	6.4 - 72	50	2.3 - 140
Sr [µg/L]	330	20 - 650	300	51 - 1200	440	72 - 3200
Te [µg/L]	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
Tl [µg/L]	< 0.2	< 0.2	0.2	< 0.2 - 1.4	< 0.2	< 0.2
U [µg/L]	< 0.2	< 0.2	0.4	< 0.2 - 3.8	0.7	< 0.2 - 1.8
V [µg/L]	1.0	0.2 - 2.0	3.9	0.4 - 110	3.9	0.5 - 56
Zn [µg/L]	9.5	2.4 - 240	290	30 - 4100	190	< 0.2 - 1500

4.4.4 Quantification of the main arsenic metabolites

We determined the concentrations of the typical arsenic metabolites in all urine samples using the method described in chapter 3.8.1, with a PRP-X100 column as stationary phase and an aqueous 20 mM ammonium phosphate solution at pH 6.0 as mobile phase. The standard solutions were always oxidized with hydrogen peroxide. We also added H_2O_2 to the filtered urine samples and let them oxidize at about 50°C for about one hour.

Chromatograms of a cow's urine sample and a standard solution containing the main arsenic metabolites are shown in Figure 38. The shifts of MA and inorganic arsenic between urine and standard are probably because of matrix effects of the undiluted urine sample. We confirmed the presence of DMA, MA and inorganic arsenic with spiking experiments.

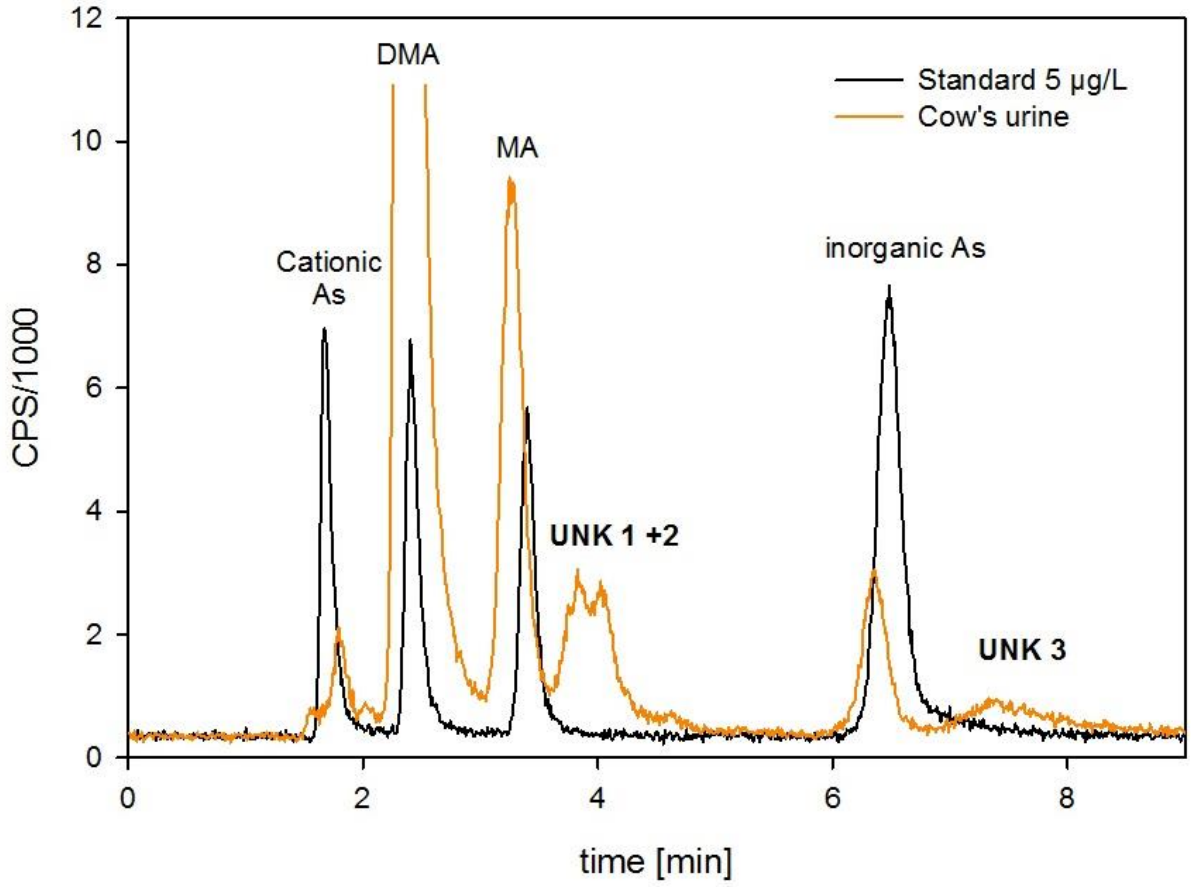


Figure 38: Anion-exchange chromatograms of a cow's urine (not oxidized) and an oxidized standard solution with 5 µg/L of AB, DMA, MA, As(III) and As(V). Shifts occurred due to matrix effects and/or aging of the column.

Table 29 lists the amount of arsenicals in the urine samples in percent of the total arsenic concentrations, also visualized in Figure 39. The major arsenic species in almost all investigated urine samples was DMA. It accounted for 35 to 75 % of the total arsenic. There were between 1 and 35 % inorganic arsenic and 2 to 15 % MA in these urine samples.

These results are in good accordance with the arsenic speciation that has been reported for urine of terrestrial mammals so far (see Introduction, chapter 2.1.4.2). One exception was the urine of the African elephants where we found slightly more inorganic arsenic than DMA, namely 35 ± 20 % inorganic arsenic and 30 ± 10 % DMA.

We were able to detect significant amounts of at least 3 unknown compounds in most of the urine samples, which we called UNK 1-3. UNK 1 and 2 eluted at around 3.8 to 4.2 minutes, right after MA, and were only poorly separated from each other. UNK 3 was much longer retained on the column, the signal appearing close to the one of inorganic arsenic, with a retention time somewhere between 6.5 - 9 minutes, depending on the condition of the column. Especially UNK 3 was much more abundant in the urine samples of ruminants than in the samples of other animals. It accounted for up to 28 %, and UNK 1 and 2 together accounted for up to 20 % of the total urinary arsenic. In some samples the unknowns could not be detected, which might be due to very low total arsenic concentrations (for example in the urine of pygmy goat, heidschnucke or giraffe).

Table 29: Arsenic species in urine of animals from Schönbrunn Zoo in % of the total arsenic concentrations Difference to 100 % = cationic arsenic, not listed. Where n > 2, mean ± standard deviation are given

	DMA	MA	inorganic As	UNK 1+2	UNK 3
Cattle (Upper Styria, n=5)	50 ± 10	8 ± 1	4 ± 2	20 ± 10	14 ± 9
Cattle (Eastern Styria, n=8)	63 ± 9	8 ± 2	6 ± 3	13 ± 6	5 ± 4
Bison (n=3)	50 ± 15	9 ± 7	25 ± 20	5 ± 3	7 ± 5
Water buffalo (n=2)	69	14	3	4	5
Tux cattle (n=2)	43	9	1	5	20
Pustertal Pied	45	11	7	6	21
Himalayan thar	56	4	3	< 1	28
Reindeer (n=2)	45	3	22	7	5
Nilgai	42	5	4	12	14
Blackbuck	36	5	21	7	8
Barbary sheep	62	6	9	< 1	< 1
Pygmy goat	36	8	17	< 1	3
Heidschnucke	40	4	37	< 1	< 1
Giraffe	62	8	< 1	< 1	< 1
Vicuña (n=2)	77	2	5	2	7
Collared peccary	77	9	3	3	< 1
Damara zebra	76	3	2	8	1
Indian rhinoceros	66	10	3	7	2
African Elephant (n=6)	30 ± 10	9 ± 7	35 ± 20	< 1 (1 x 15)	< 1

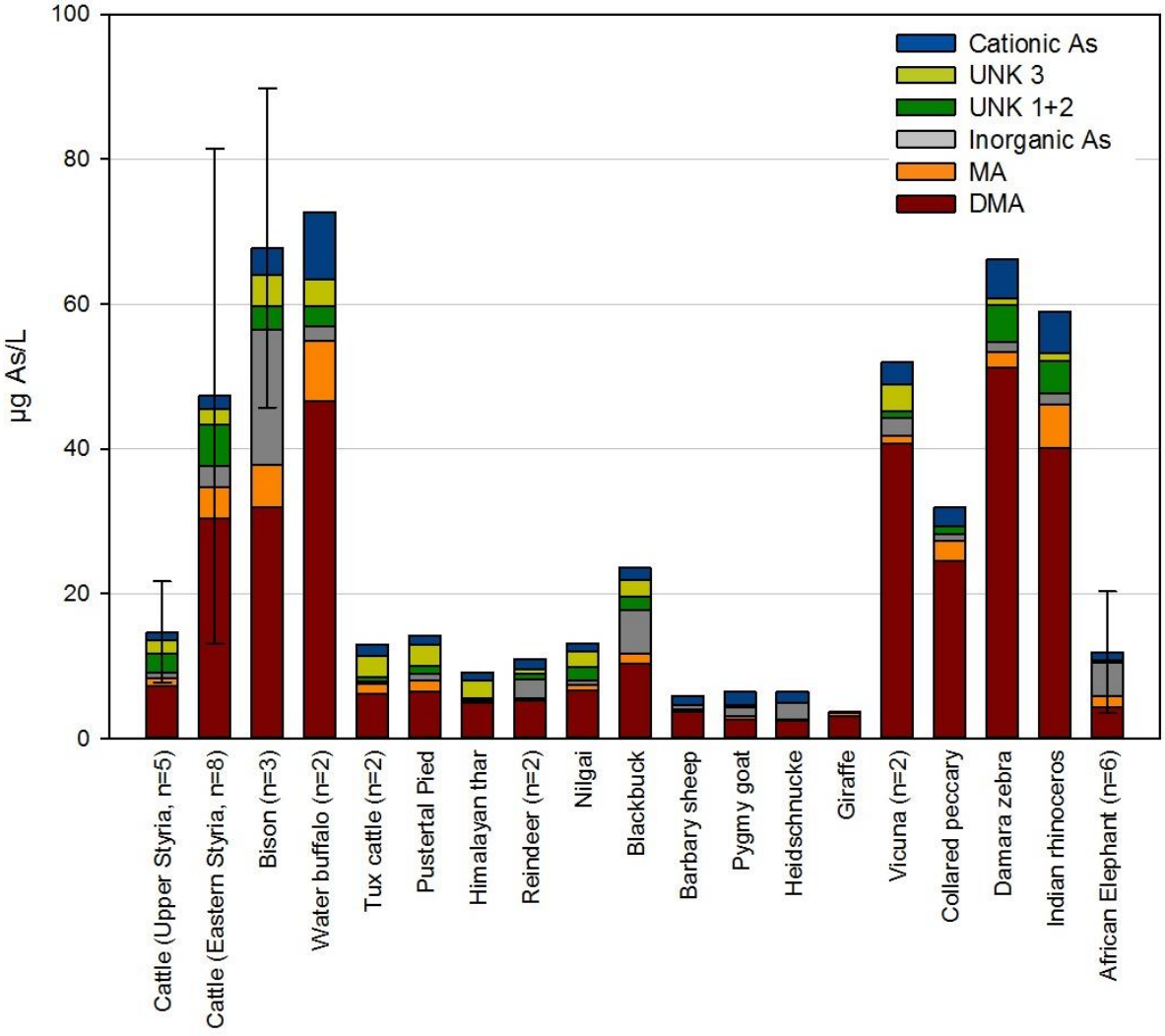


Figure 39: Arsenic speciation in the urine of the investigated animals of Schönbrunn in µg As/L

4.4.5 Influence of hydrogen peroxide

Oxidation with hydrogen peroxide revealed an interesting behavior of the unknown compounds. In Figure 40 the difference between the oxidized and the not oxidized form of a cow's urine sample ("Betti") is shown.

We usually let the urine samples oxidize after the addition of H_2O_2 for about one to two hours at slightly elevated temperatures (around 50°C). We compared of one of these "oxidized" samples with a not oxidized sample and a sample that had been oxidized some days before. This revealed that UNK 1 disappeared only after letting the samples react with hydrogen peroxide for about 12 to 24 hours at around 50°C. At the same time, the signal of UNK 3 increased. The decrease of UNK 1 and the increase of UNK 3 were about the same amount. We are assuming that UNK 1 gets oxidized to UNK 3, but only very slowly. This is unusual, since known trivalent and thio-arsenicals undergo oxidation much faster [14]. Unfortunately, our assumption that UNK 1 gets converted to UNK 3 implies that we do not know the actual

initial concentrations of UNK 1 and 3 in the samples. Further experiments with oxidized and not oxidized forms of all samples are needed to evaluate this.

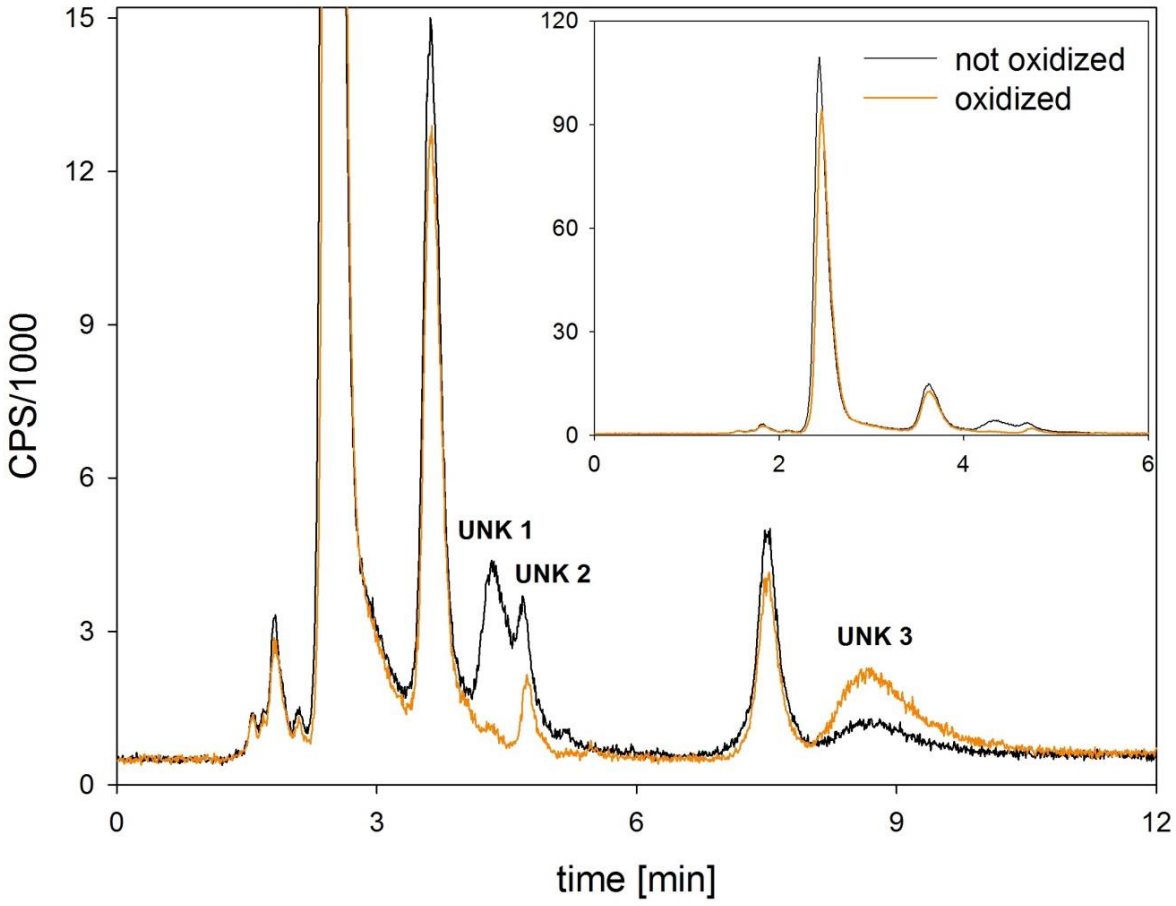


Figure 40: Comparison of the chromatograms of oxidized and not oxidized form of a cow's urine sample (dilution factor of the oxidation step: 1.1). Small insert: Same chromatogram as in the big frame, scaled to depict the whole DMA signal.

4.4.6 Cation-exchange chromatography

We used an already established method (Zorbax 300-SCX column and a pyridine buffer, pH 2.3, as described earlier in chapter 3.8.2) to determine the abundance of four common cationic arsenic species in the urine samples, namely AB, TMAO, AC and TETRA.

We did not oxidize the urine samples and the calibration standards for cation-exchange chromatography. We measured only some of the samples, which were from two cows from Eastern Styria ("Betti" and "Hermine") as well as samples from a bison and from a water buffalo from Schönbrunn Zoo.

In the investigated samples we were able to detect small amounts of AB (around 1 µg As/L) as well as TMAO (between 1 and 3.5 µg As/L). AC and TETRA were not detectable.

Additionally, there were two other signals appearing at higher retention times than the four cations of the calibration standards. Through spiking experiments we were able to identify the first one of the two peaks as UNK 3. It accounted for about 3 – 4 % of the total arsenic in the investigated samples. However, we could not find out anything about the identity of the second unknown signal, which made up between 5 and 10 % of the total arsenic. It is possible that it is UNK 1 or 2, but this remains to be confirmed.

Changing the pH of the 10 mM pyridine buffer from 2.3 to 4.3 prolonged the retention time of the unknown compounds enormously. While the signal of UNK 3 was shifted from 9.5 to about 23 minutes, the other unidentified signal was not visible even after 35 minutes.

We also varied the chromatographic method by adding 2, 5 and finally 10 % v/v methanol to the 10 mM pyridine eluent (pH 2.3). This did not affect the signals of AB or TMAO, but lead to shorter retention times of two unknown compounds. The influence of the methanol is displayed in Figure 41, and the chromatogram of one of the cows' samples with 10 % v/v methanol in the mobile phase is shown in Figure 42.

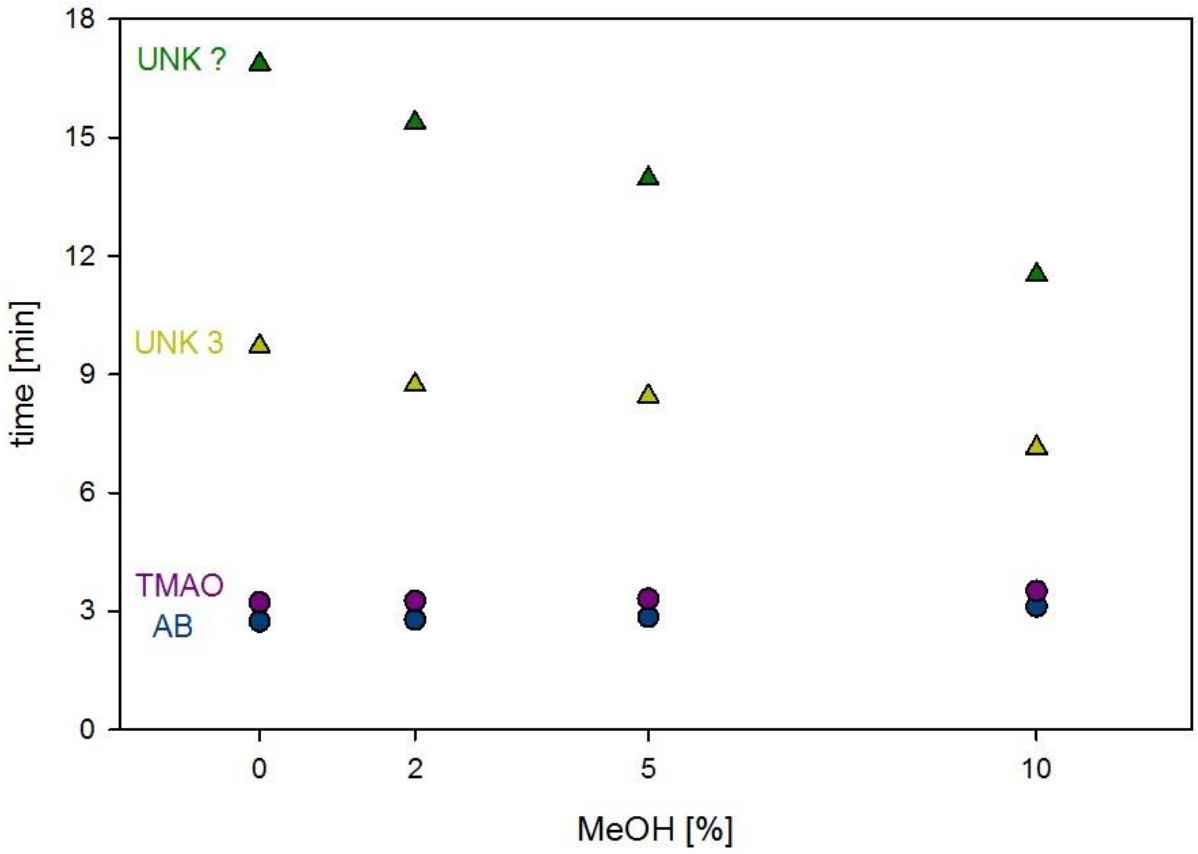


Figure 41: Behavior of the arsenicals in cow's urine on the cation-exchange column with varying amount of MeOH in the mobile phase

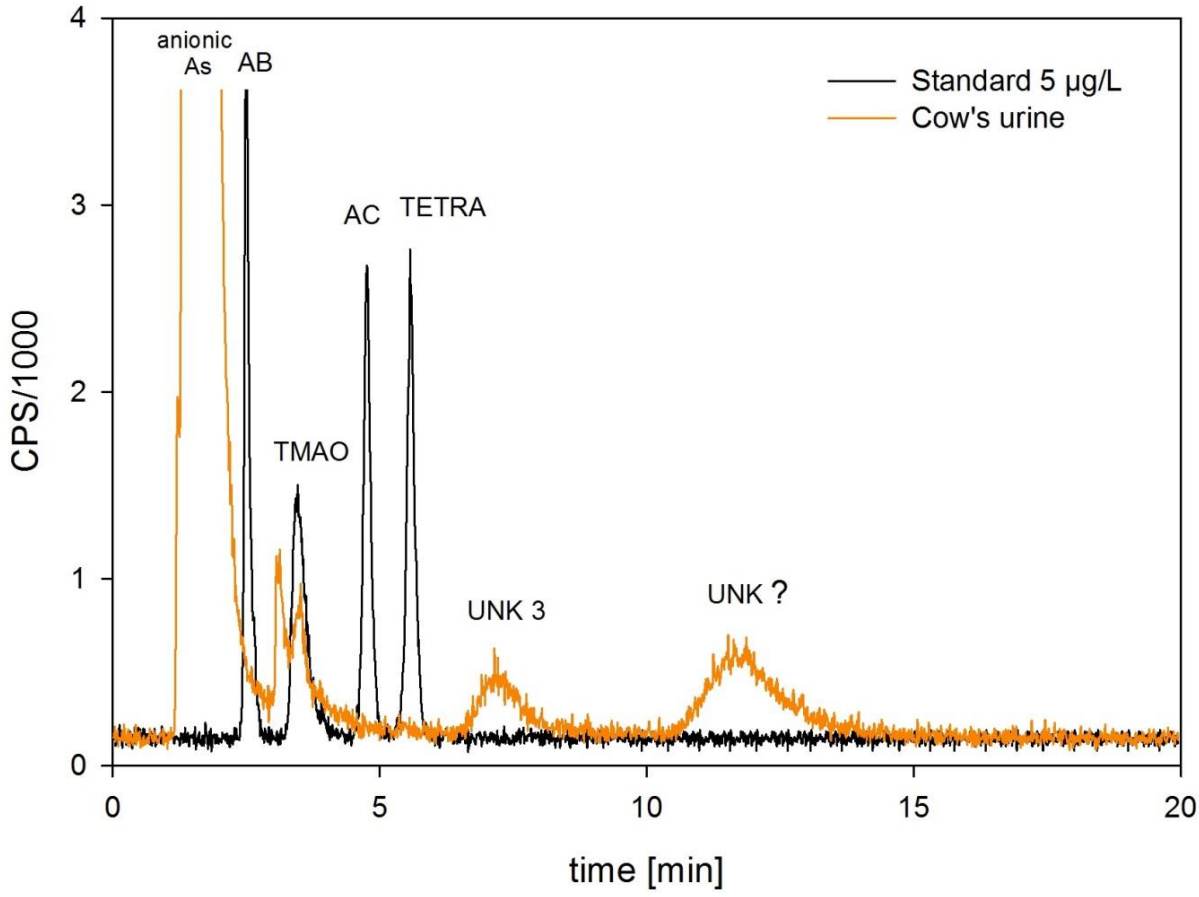


Figure 42: Cation-exchange chromatograms of cow urine (not oxidized) and a standard solution with 5 µg As/L AB, TMAO, AC and TETRA. 10 mM pyridine, pH 2.3, and 10 % v/v methanol as eluent.

The behavior of UNK 3 seemed quite unusual, since it was well retained both with anion- and cation-exchange chromatography. Normally, one would expect a compound with a long retention time on an anion-exchange column to be hardly retained on the cation-exchange column and vice versa. This indicates that probably other forces than simple interactions of the analyte with exchange sites of the stationary phase are taking place here, for example an interaction with the backbone of the column. For this reason we carried out experiments with an anion-exchange column with a different kind of backbone (chapter 4.4.12).

4.4.7 Comparison with different arsenic compounds

In addition to the main metabolites (DMA, MA, inorganic arsenic) and the most commonly investigated cationic arsenicals (AB, TMAO, AC and TETRA, chapter 4.4.5), we also performed experiments with some other arsenicals which had been reported to have a retention behavior similar to one of the unknown compounds. The candidates were DMAA [138], DMAB and DMAP [139], and the arsenosugars Gly, Phos, SO3 and SO4 [140].

Co-chromatography and spiking experiments with one of the urine samples (“Betti”) and standard solutions of the arsenicals revealed that none of these arsenic species had the same retention behavior as UNK 1-3 in the cow’s urine. DMAA eluted very close to UNK 1, but a spiking experiment showed that UNK 1 was a different compound (Figure 43).

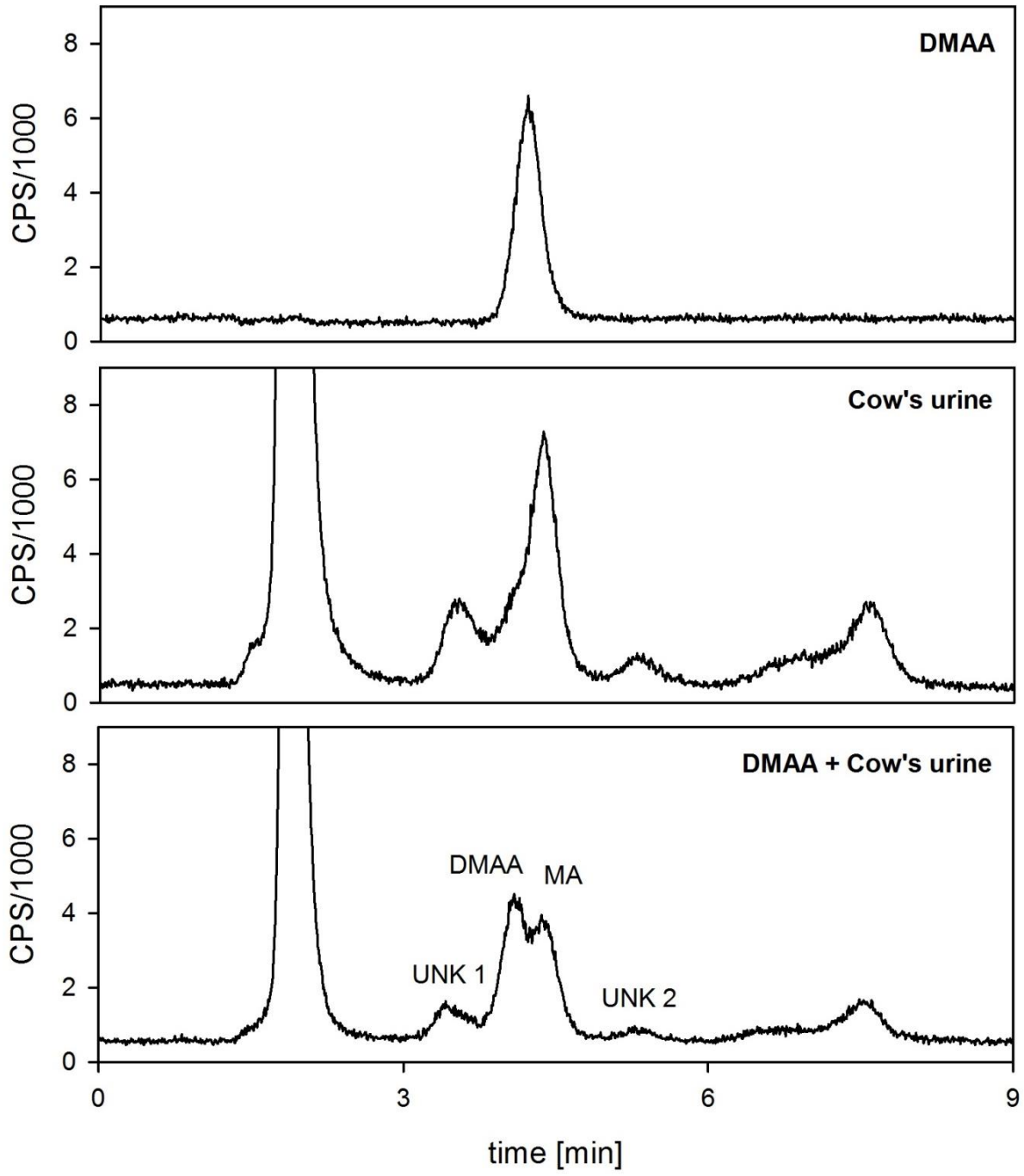


Figure 43: Comparison of chromatograms of a cow’s urine sample, DMAA and a spike containing DMAA and cow’s urine (1+1), on the PRP-X100 column with aqueous ammonium phosphate, pH 4.0, as eluent

As can be seen in Figure 44, DMAP and DMAB were not coeluting with any of the unknown arsenic species. DMAP was retained slightly longer than DMA, but shorter than MA, on the PRP-X100 column with an aqueous ammonium phosphate solution, pH 6.0, as mobile phase. The retention time of DMAB was between the retention times of UNK 1 and UNK 2.

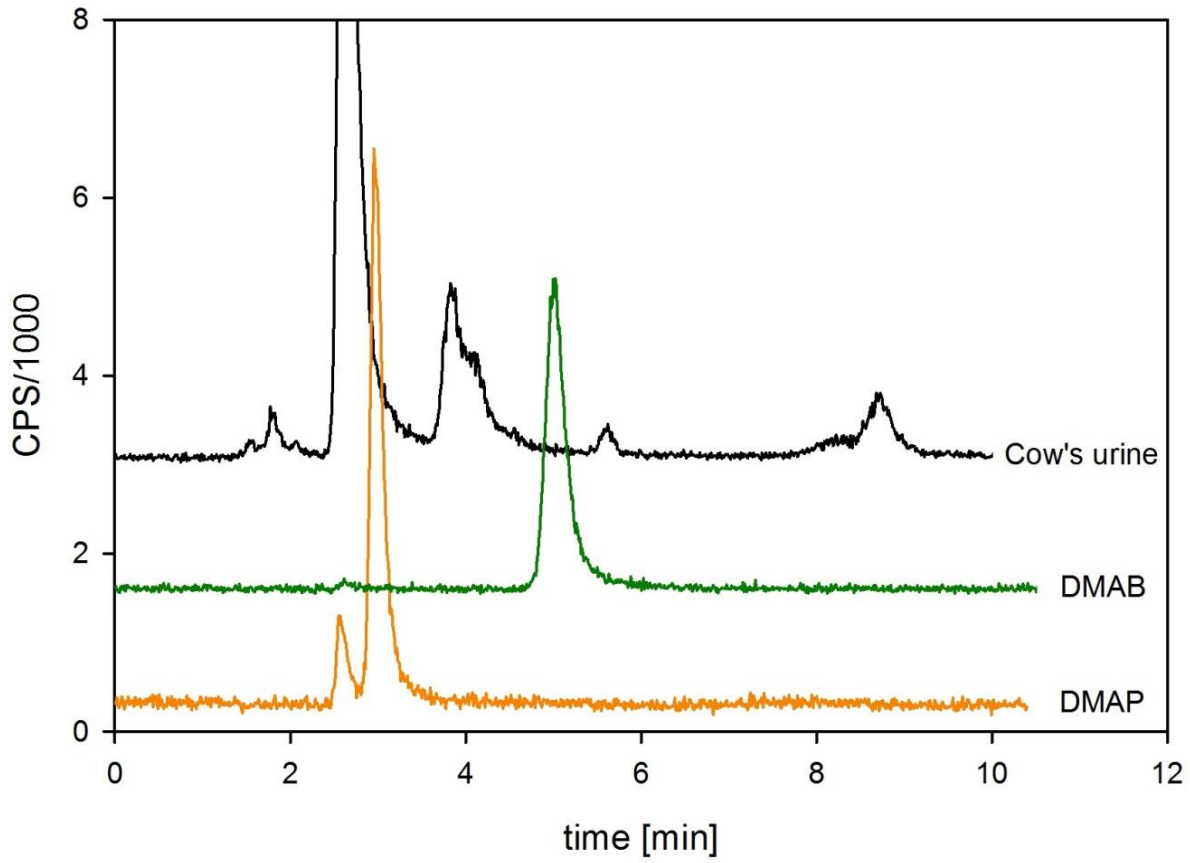


Figure 44: Chromatograms of a cow's urine (offset: + 3000 CPS) and standard solutions of DMAB (offset: + 1500 CPS) and DMAP (with DMA impurities)

We also analyzed a solution that contained the four arsenosugars Gly, Phos, SO3 and SO4 (and small amounts of MA, DMA and inorganic arsenic) with the same anion-exchange chromatographic method (Figure 45). Although the phosphate sugar eluted close to UNK 1, it was not the same compound. The sulfonate sugar had a similar retention time as inorganic arsenic and UNK 3, but it is very unlikely that UNK 3 was this arsenosugar.

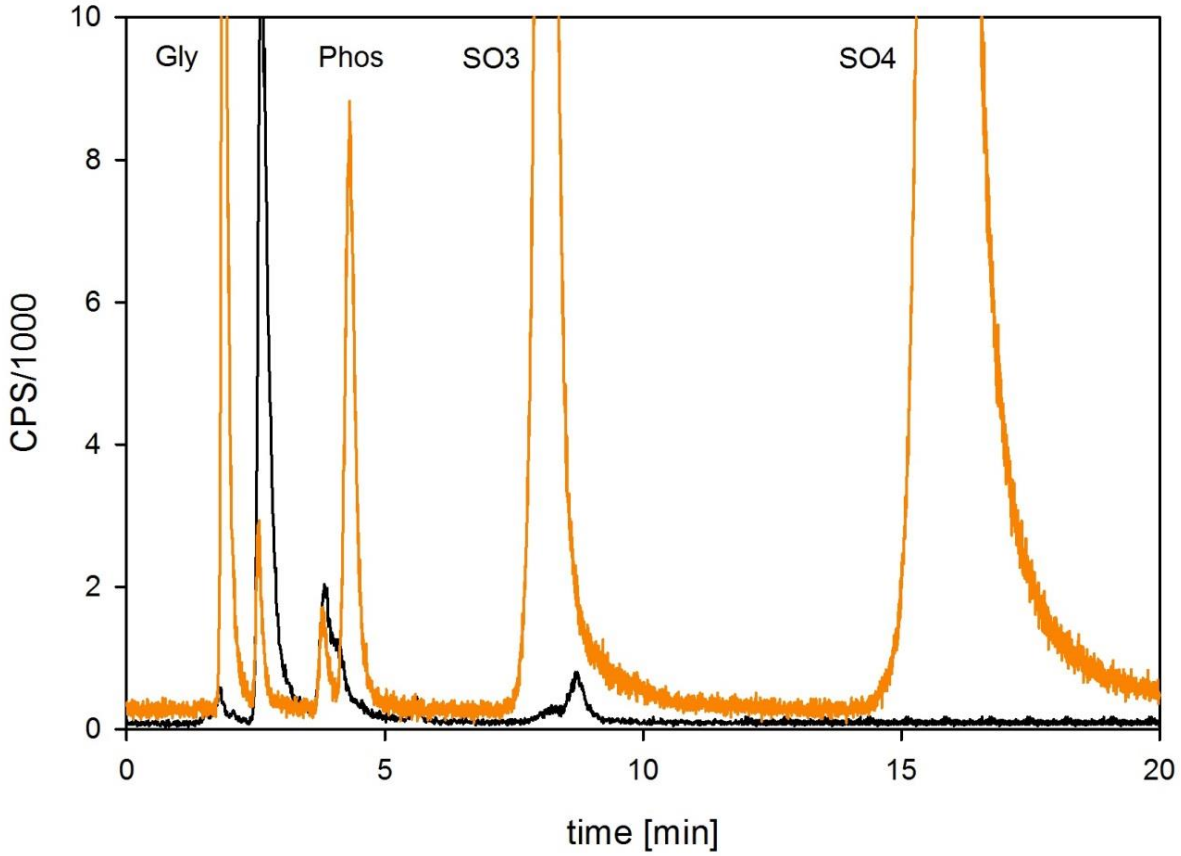


Figure 45: Chromatograms of a cow's urine (black) and a standard solution (orange) containing DMA, MA and inorganic As (2.5 µg As/L), Gly and Phos (25.8 µg As/L), SO3 (295 µg As/L) and SO4 (518 µg/L)

4.4.8 Behavior of the unknown arsenicals at different pH levels

To find out more about the unidentified arsenic species UNK 1-3, we carried out HPLC-ICPMS measurements with the same conditions as described earlier (PRP-X100 column, aqueous 20 mM ammonium phosphate as eluent), and varied the pH of the eluent between 4.0 and 8.0. Our findings are depicted in Figure 46. The behavior of the arsenicals DMA, MA, As(III) and As(V) had already been investigated some years ago by Gailer and Irgolic [141], and our findings were consistent with theirs.

The retention of compound UNK 1 was hardly influenced by the change of the pH. The retention time of UNK 3 decreased a little bit from pH 4.0 to pH 6.0, but then increased again at pH 7.0 and 8.0. Its retention time at pH 8.0 was about the same as it was at pH 4.0. The behavior of UNK 2 is more complicated. Its retention time decreased from pH 4.0 to pH 6.0 more or less parallel to MA. At pH levels below 6.0 it was separated quite well from UNK 1 and MA, but at pH 6.0 it almost coeluted with UNK 1. At higher pH levels its signal suddenly disappeared (Figure 47). However, another signal appeared at much higher retention times. We do not know for sure if this signal can be attributed to UNK 2 or not.

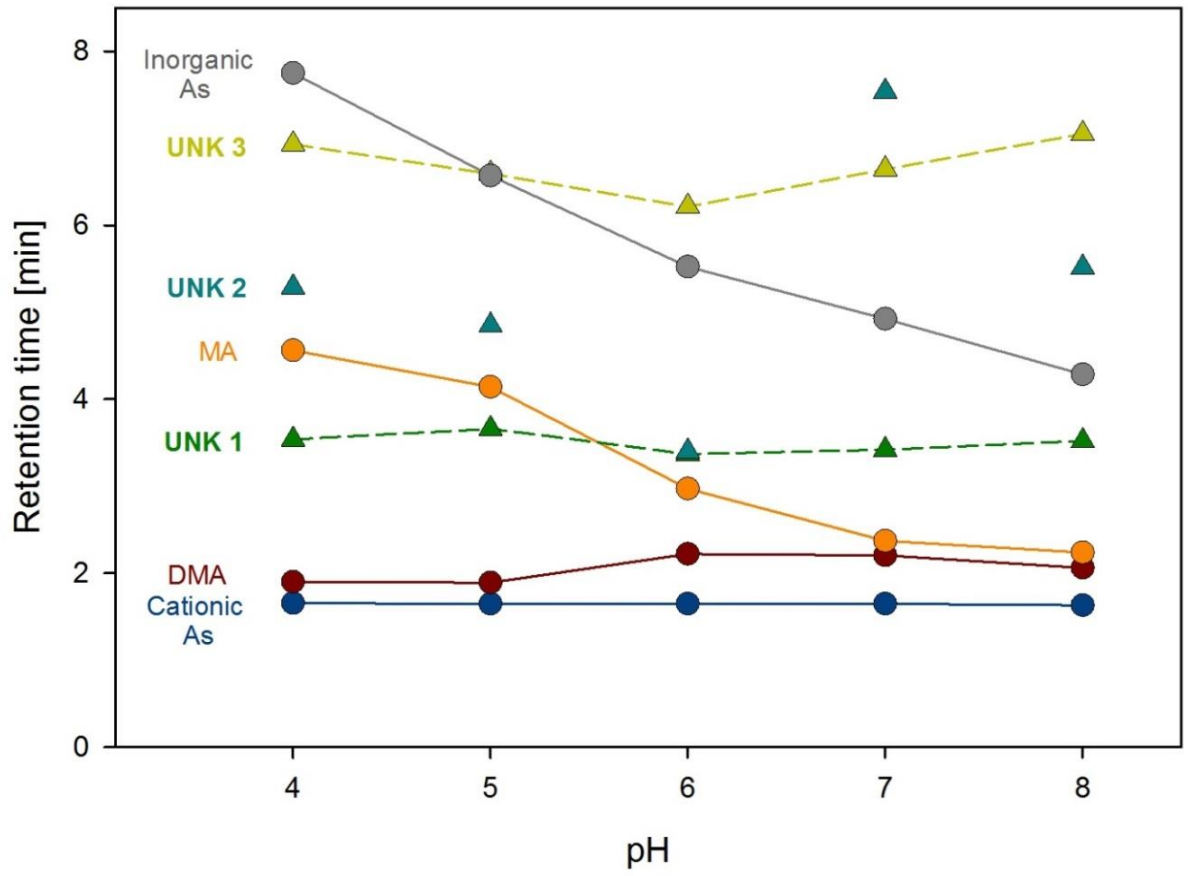


Figure 46: pH dependancy of the retention times of the different As species in cow urine on the PRP-X100 column, with an aqueous 20 mM ammonium phosphate solution as mobile phase

For this experiment and the interpretation of its results, some difficulties and possible sources of wrong deductions have to be mentioned: While we had already isolated UNK 3, which made it easier to identify the corresponding signal at the different chromatographic methods, we did not have such comparison possibility for UNK 1 and 2. Further on, UNK 1 and 2 were occurring in approximately the same concentrations in the investigated sample. These two circumstances combined made it impossible to distinguish between these two unknown arsenicals in the chromatograms of the different pH levels. Hence, there is a slight possibility that the attribution of UNK 1 and UNK 2 to the signals should actually be interchanged.

Another difficulty was the slow oxidation of UNK 1 to UNK 3, which we only discovered after the experiments at the different pH levels. At the time of these experiments we were assuming that oxidation of all arsenicals was complete after about one hour. Obviously, the oxidation of UNK 1 (probably to UNK 3) had not been complete when we carried out the experiment at pH 4.0 to 6.0, because a signal of UNK 1 was clearly visible. However, the lack of a signal for UNK 2 (or 1) at higher pH levels could be due to complete oxidation of

UNK 1. If this was true, there is still the open question remaining where the other signal at a higher retention time originated from.

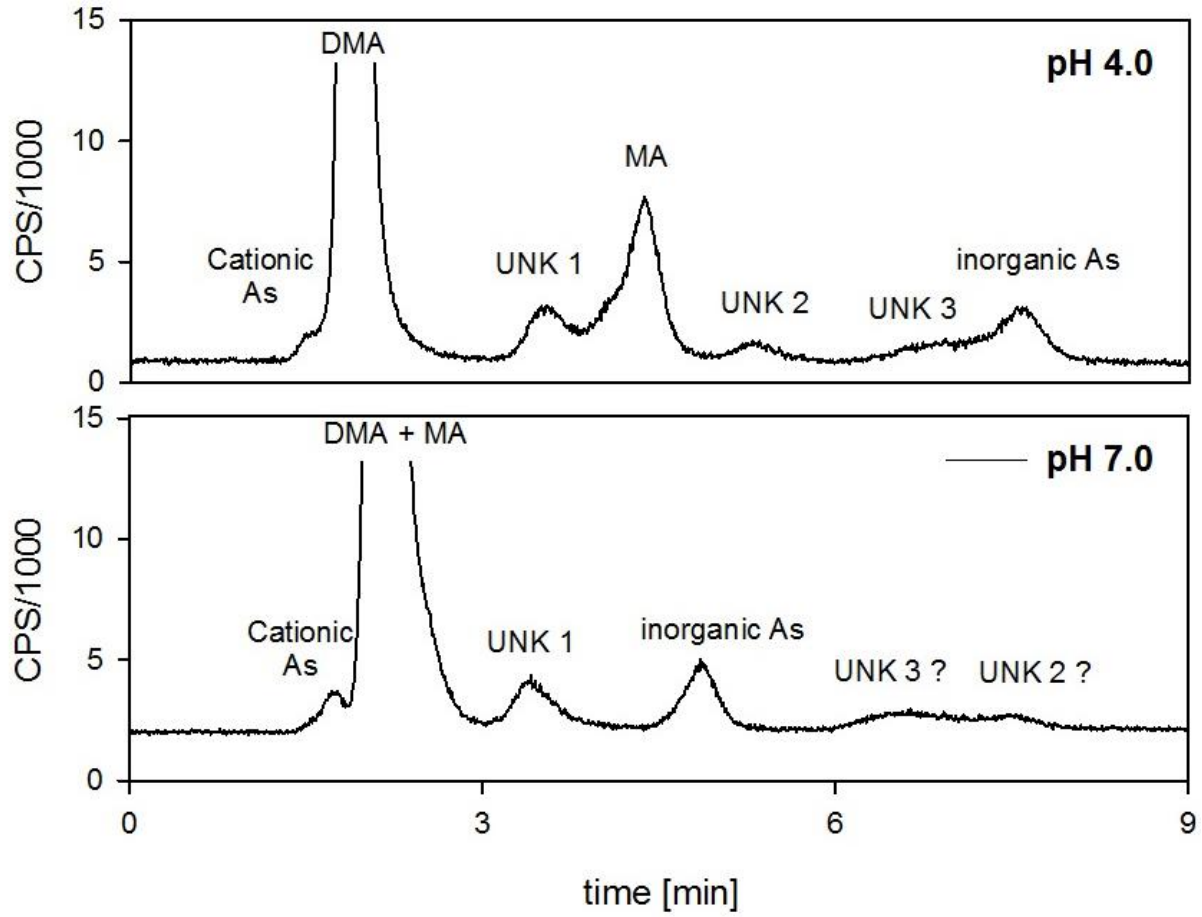


Figure 47: Chromatograms of a cow's urine at pH 4.0 and 7.0, both times on a PRP-X100 column with aqueous 20 mM ammonium phosphate as mobile phase

4.4.9 Influence of methanol in the mobile phase

We added 10 % v/v methanol to the mobile phase (aqueous 20 mM ammonium phosphate at pH 7.0) to see a possible influence of an organic solvent to the separation on the PRP-X100 column (Figure 48). DMA and MA were not affected. Both with and without MeOH in the mobile phase they were coeluting at around 2 – 3 minutes, because the pH was 7.0 (instead of the typically used pH 6.0). The signal of the inorganic arsenic was shifted from 4.9 to 5.7 minutes. UNK 1 was retained a bit shorter, which lead to a coelution with DMA and MA. It was not possible to properly distinguish between UNK 3 and the signal attributed to UNK 2 in neither of the chromatograms, but both signals were evidently retained much longer when MeOH was present in the mobile phase (from 6.6 and 7.5 to 8.9 and 11.2 minutes).

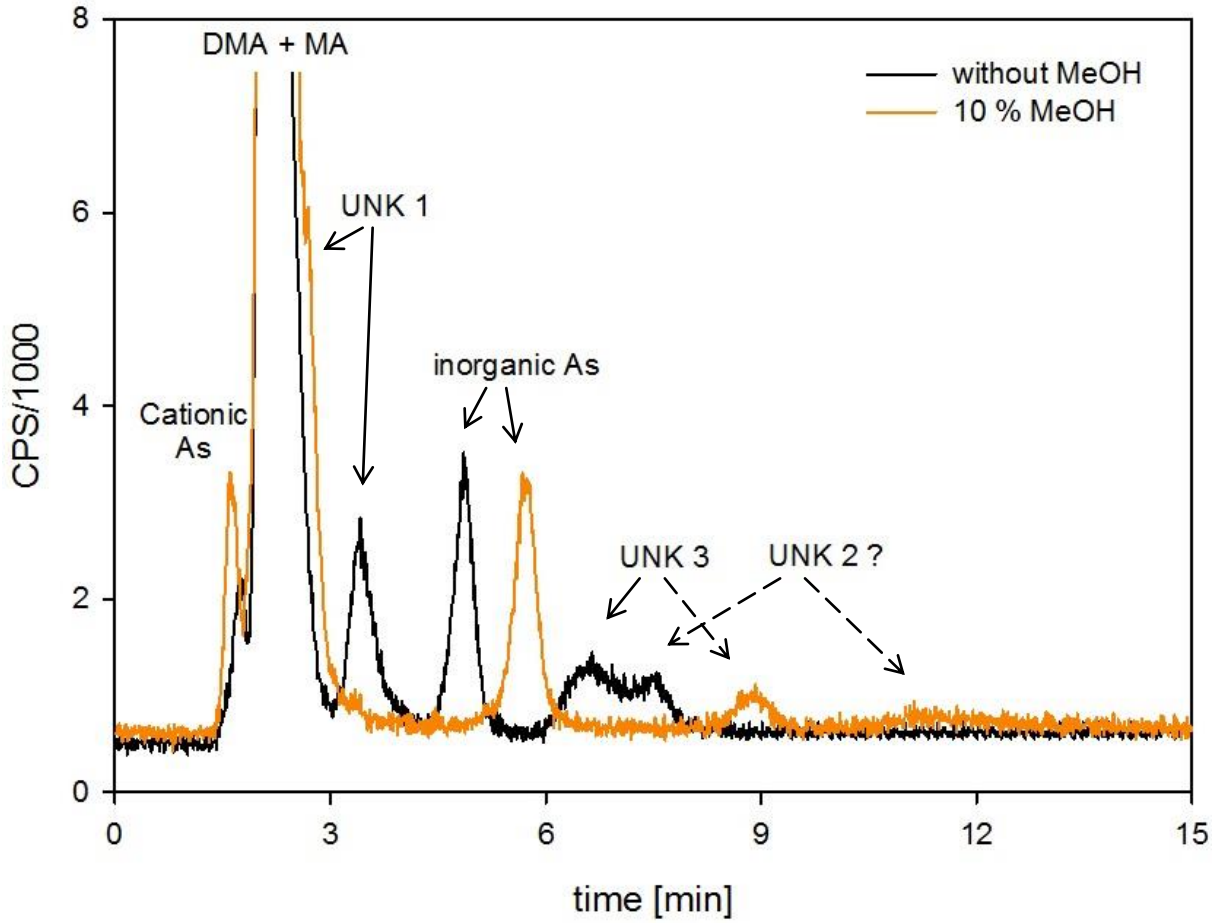


Figure 48: Anion-exchange chromatograms of a cow's urine sample with an aqueous 20 mM ammonium phosphate solution at pH 7.0 as mobile phase, with and without 10 % MeOH

4.4.10 Aqueous solutions of ammonium acetate as mobile phase

We replaced the aqueous ammonium phosphate buffer with an aqueous solution of 30 mM ammonium acetate and carried out experiments on a PRP-X100 column. First, we adjusted the pH of the acetate to 5.0. All other settings of the ammonium phosphate method remained the same. In Figure 49 a chromatogram of a cow's urine ("Betti") with ammonium acetate as mobile phase is compared with a chromatogram using ammonium phosphate as eluent. The pH was 5.0.

Changing the pH of the mobile phase to 6.0 led to an increase of the retention time of inorganic arsenic from 12 to 20 minutes. Also, DMA was retained slightly longer and MA a bit shorter than at pH 5.0. The unknown arsenicals were not visible very well, partly because there was a suppression of the signal between about 10 and 15 minutes (Figure 50). A possible reason for the suppression could be chloride species eluting at this time span, but we did not detect an increase of the argon chloride signal at m/z 77. We were not able to identify the actual reason for the suppression.

Addition of 10 % v/v methanol to the mobile phase strongly reduced the retention times of all compounds. The signal of inorganic arsenic appeared at around 7.5 minutes, and all other arsenicals were retained less than 2.5 minutes and were not properly separated from each other.

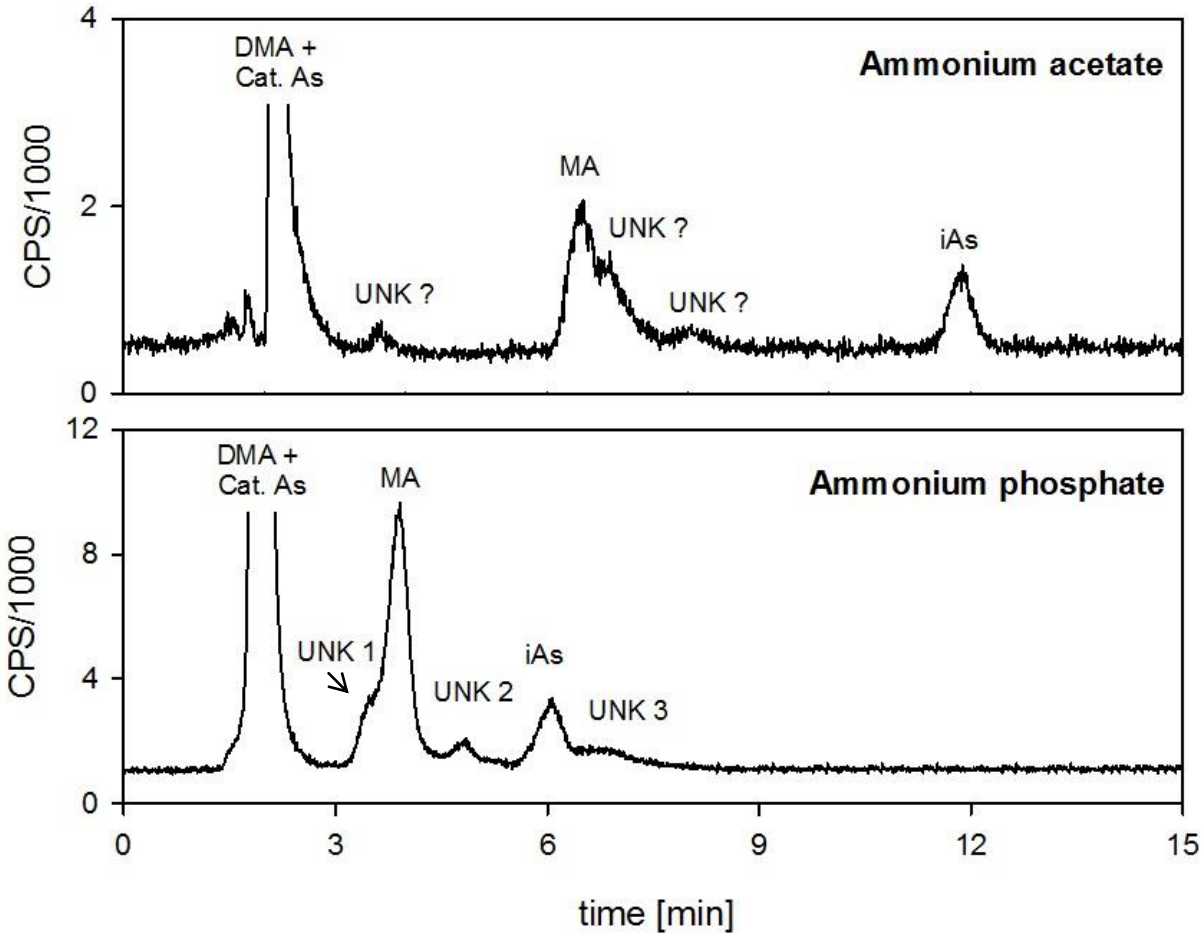


Figure 49: Chromatograms of a cow's urine sample (not oxidized) on a PRP-X100 column with an aqueous solution of 30 mM ammonium acetate and with a 20 mM aqueous ammonium phosphate solution, both times pH 5.0

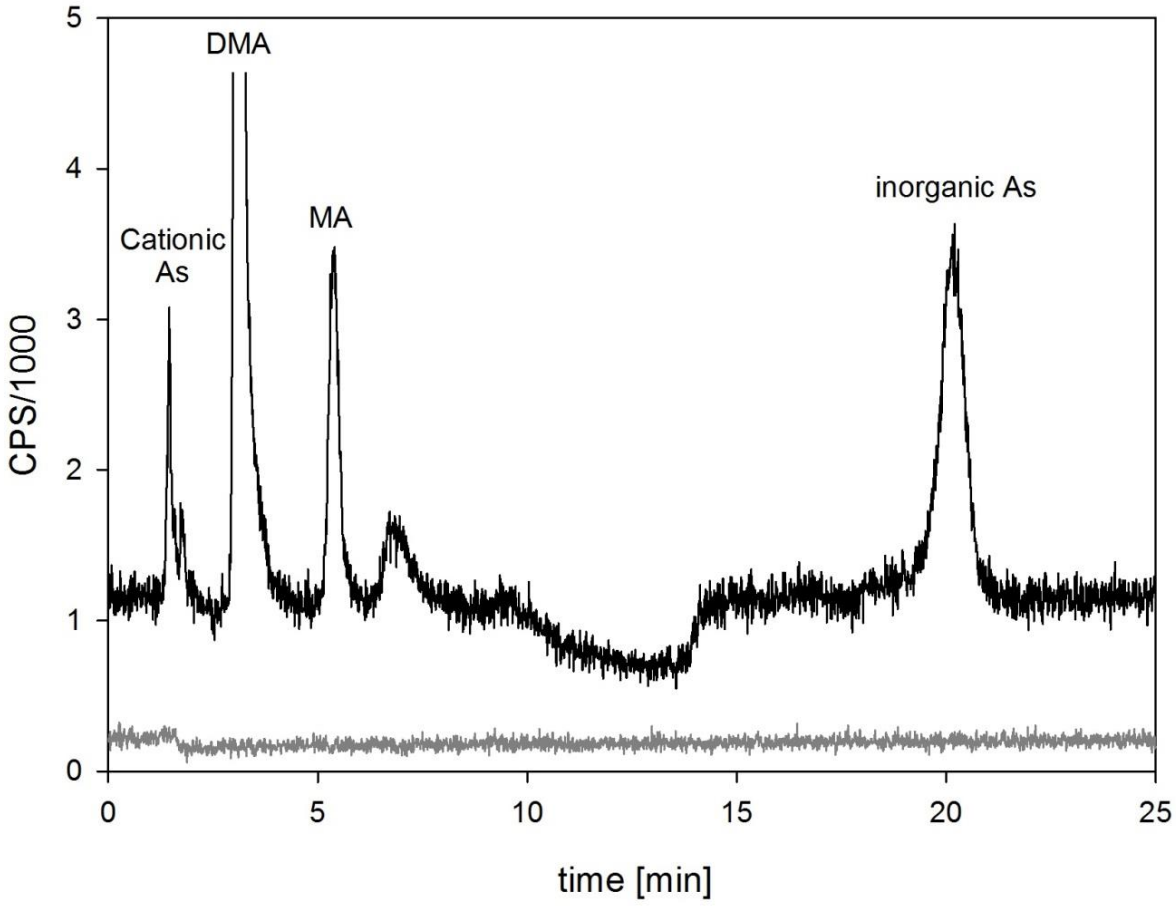


Figure 50: Chromatogram of a cow's urine on a PRP-X100 column with 30 mM ammonium acetate as mobile phase, at pH 6.0. Black = m/z 75 ("arsenic"), grey = m/z 77 ("argon chloride").

4.4.11 Maleic acid as mobile phase

Instead of aqueous 20 mM ammonium phosphate, we used a mobile phase consisting of ultrapure water with 5 mM maleic acid, pH 7.0. The stationary phase was the same (PRP-X100). Figure 51 displays chromatograms of a cow's urine sample ("Betti", not oxidized) and of a solution with 10 µg As/L of AB, DMA, MA, As(III) and As(V), oxidized with H_2O_2. It can easily be seen that DMA and MA were not separated well. In the cow's urine sample, MA completely disappeared beneath the dominating signal of DMA. As with the ammonium phosphate, we were able to detect three signals of unknown arsenicals. However, we could not determine to which of the compounds UNK 1 to 3 they corresponded to.

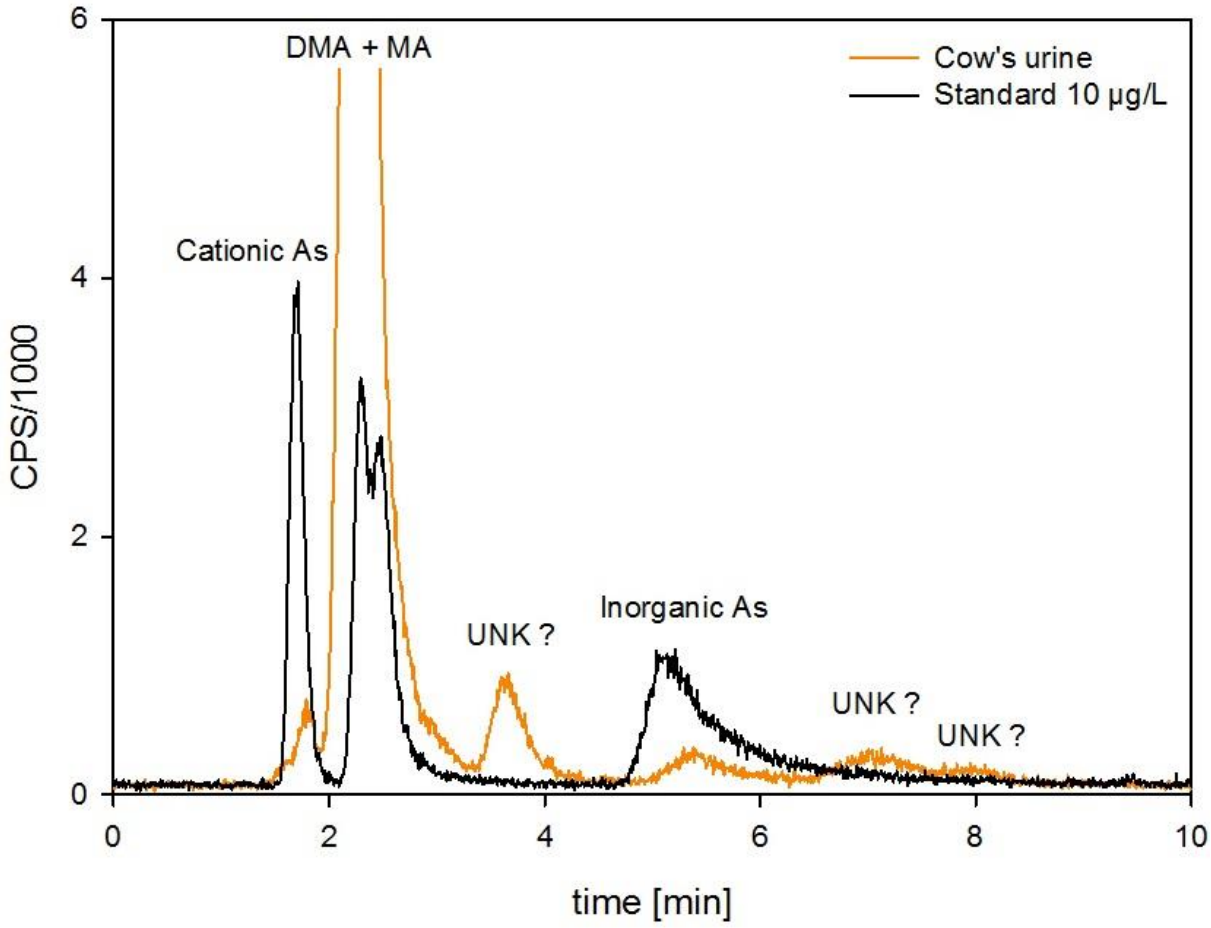


Figure 51: Chromatograms of a standard solution (10 µg As/L AB, DMA, MA, As(III), As(V), oxidized) and a cow's urine (not oxidized) on a PRP-X100 column with 5 mM maleic acid, pH 7.0, as mobile phase

4.4.12 Zorbax SAX column as stationary phase

4.4.12.1 Aqueous ammonium phosphate as mobile phase

The Agilent Zorbax SAX Analytical column has the same dimensions as the Hamilton PRP-X100 column. Both columns are strong anion-exchange columns, but they have different backbones. To determine if the arsenicals UNK 1-3 were interacting with the polymer-based backbone of the PRP-X100 column we carried out an anion-exchange chromatography of a cow's urine ("Betti") with the same conditions that we used for quantification of the three main species in urine of terrestrial mammals (DMA, MA and inorganic arsenic), but with the silica-based Zorbax SAX column instead of the polymer-based PRP-X100. The signals belonging to UNK 1 and 3 were identified via chromatography of a not oxidized and an oxidized sample of the same urine as well as co-chromatography with the already isolated UNK 3.

The results confirmed our idea of backbone interactions, since the retention time of the signal of UNK 3 was now much shorter than on the PRP-X100, as can be seen in Figure 52. Although the retention time of UNK 1 was almost the same on both columns, it eluted right in

front of DMA and MA on the Zorbax SAX, while it only eluted right after DMA and MA on the PRP-X100. UNK 2 was retained 4.0 minutes on the PRP-X100 and 6.2 minutes on the Zorbax SAX. However, it eluted shortly after MA on both columns.

Our findings also suggest that UNK 3 and maybe also UNK 1 probably have a more cationic than anionic character, at least up to pH 8.0.

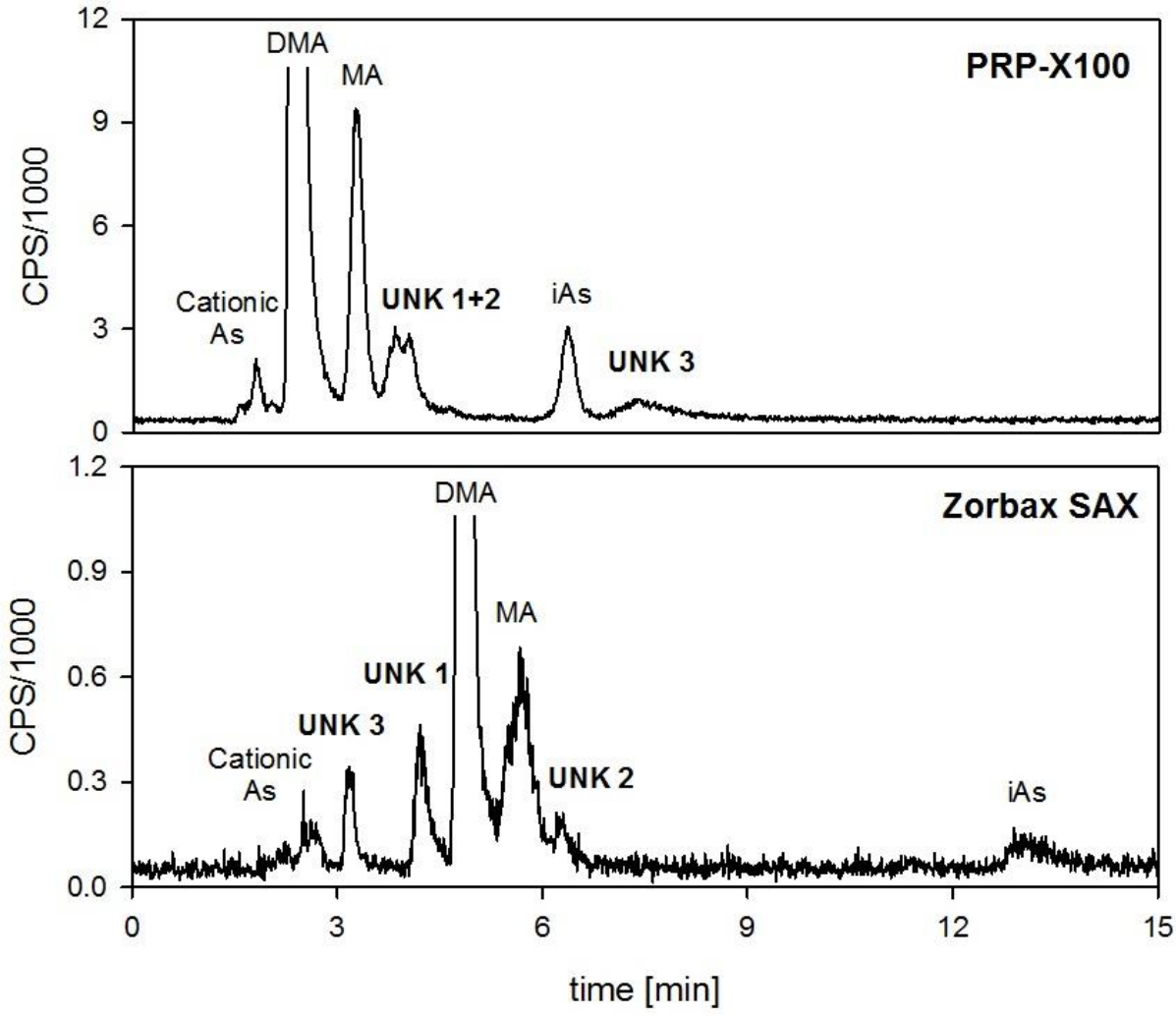


Figure 52: Chromatographic separation of the arsenicals in a cow's urine (not oxidized) on two different anion-exchange columns, both times with aqueous 20 mM ammonium phosphate, pH 6.0, as mobile phase

4.4.12.2 Aqueous solutions of ammonium acetate as mobile phase

We also chromatographed a cow's urine sample ("Betti") on the Zorbax SAX with an aqueous solution of ammonium acetate as mobile phase, at different pH levels. We adjusted the pH of the acetate with ammonia to pH 4.0, 5.0 and 6.0. The behavior of the different arsenic species is shown in Figure 53. At pH 4.0, MA and UNK 2 were retained about 33 and 34 minutes on the column. Their retention times were almost halved when the pH was raised to 5.0, and they probably coeluted with DMA at pH 6.0 (Figure 54), but this we do not know

for sure. The other arsenic compounds were not affected as much as UNK 2 and MA. The retention time of DMA increased slowly with increasing pH. The signal of UNK 3 appeared at almost the same spot at all three pH levels, with the retention time only slightly decreasing from pH 5.0 to 6.0. UNK 1 was retained 5.1 minutes on the column at pH 4.0 and a bit shorter at pH 5.0 (3.7 minutes). At pH 6.0 we were not able to definitely attribute a signal to UNK 1. It is possible that the signal that appeared at 6.4 minutes belonged to UNK 1, which would mean an increasing retention of this arsenical. Inorganic arsenic was too well retained on the column at pH 4.0 and 5.0 and did not appear within 40 minutes. Finally, at pH 6.0, there was a signal belonging to inorganic arsenic at 17 minutes.

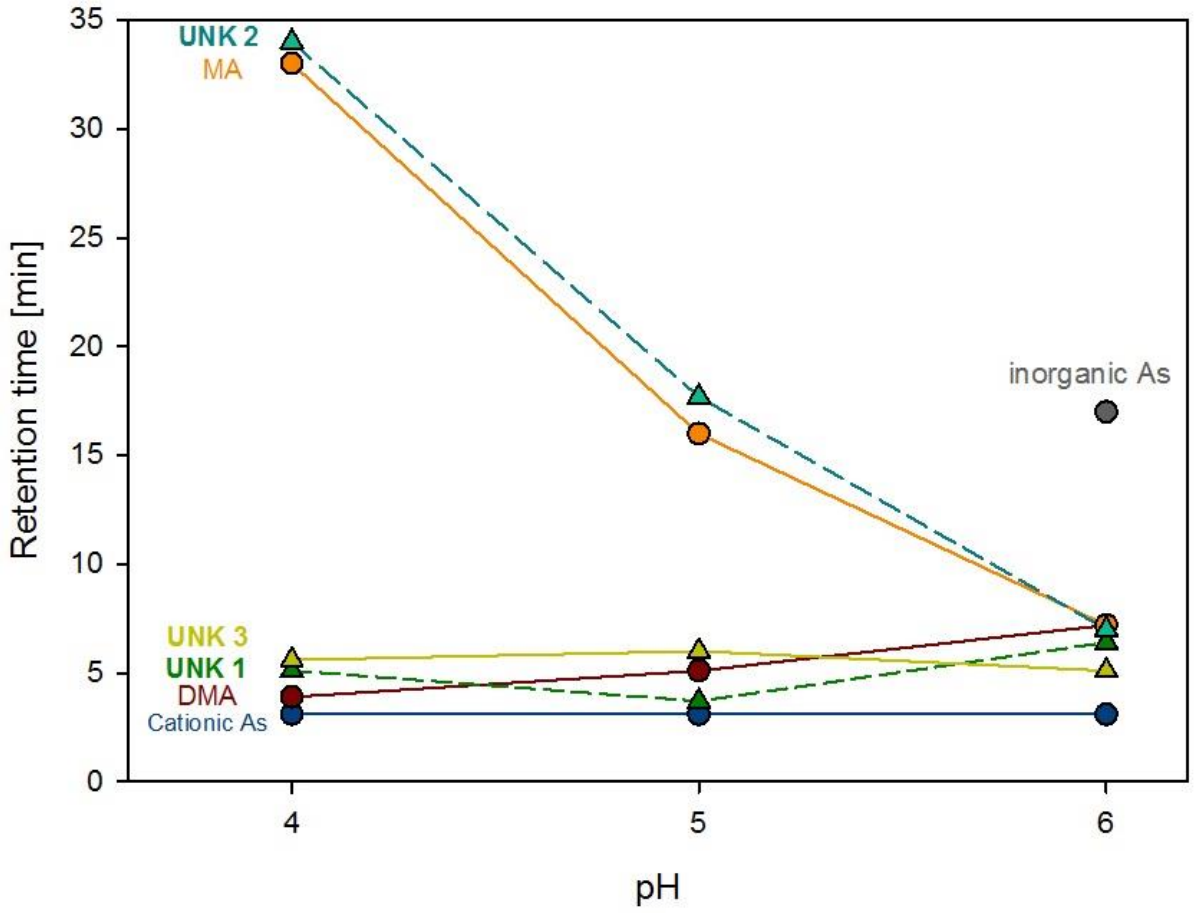


Figure 53: pH dependency of the retention times of the arsenic species in a cow's urine on the Zorbax SAX column with aqueous 20 mM ammonium acetate as mobile phase

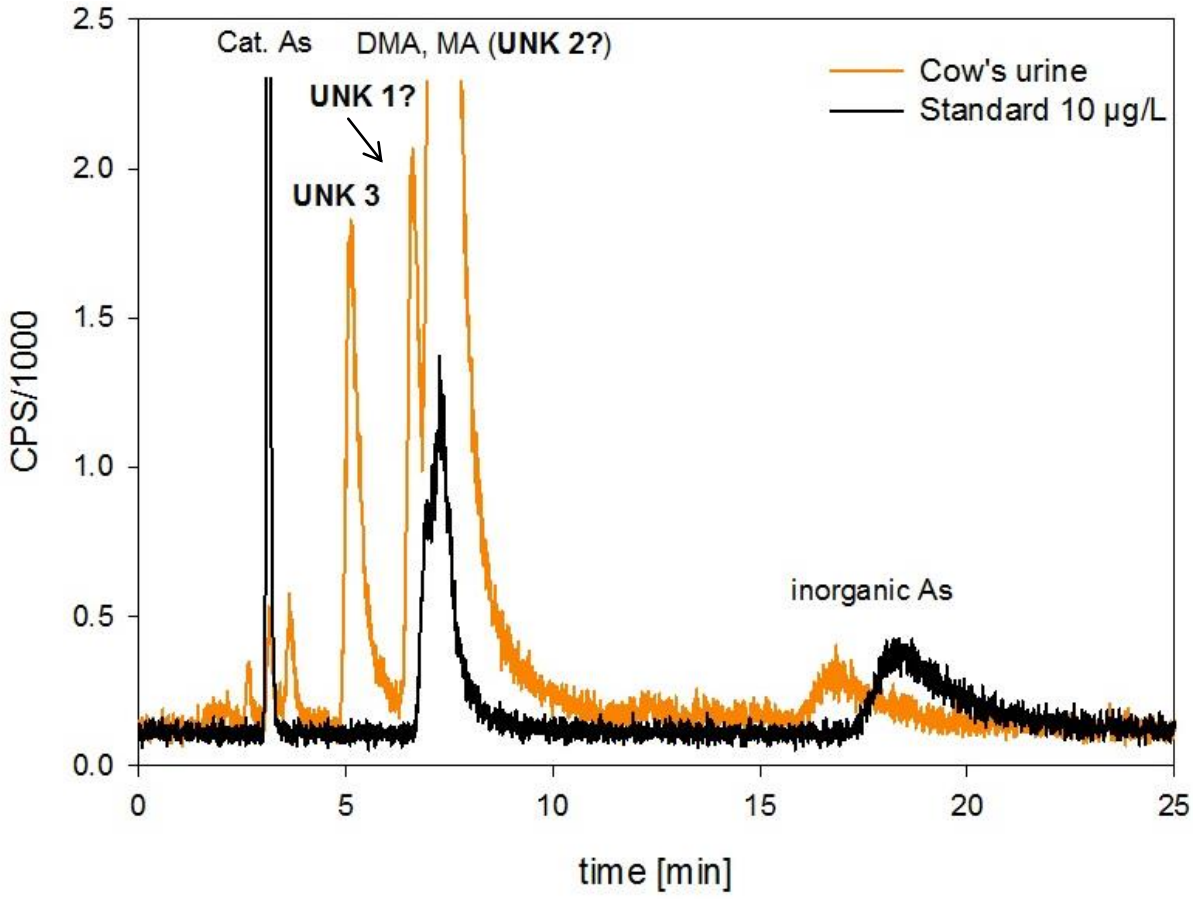


Figure 54: Chromatograms of a cow's urine (not oxidized) and an oxidized standard solution containing 10 µg As/L of AB, DMA, MA, As(III) and As(V) using a Zorbax SAX column and 20 mM aqueous ammonium acetate, pH 6.0, as mobile phase. The shift of the inorganic arsenic was probably due to matrix effects and/or aging of the column.

4.4.13 Reversed-phase chromatography

For investigations about the behavior of the unknown arsenic species on a RP column, we used a Zorbax-SB C8 column. A commonly used organic solvent in RP chromatography would be acetonitrile, but it is known to negatively affect the performance of ICPs, which results for example in large signal decreases [142]. The tolerance of the plasma is reported to be much higher towards alcohols, for example methanol [143]. For this reason we decided to use different mixtures of methanol and ultrapure water for our experiments.

We analyzed a cow's urine sample with 0, 2, 3, 5, 10 and 15 % v/v methanol in the mobile phase with a flow rate of 1.0 mL/min. The response of the different signals to the varying amount of organic solvent is shown in Figure 55. The sample was oxidized with H_2O_2 all the times. At 0, 5, 10 and 15 % v/v methanol we also analyzed a not oxidized version of the cow's urine to identify UNK 1 and 3. We did not do this at 2 and 3 % v/v methanol, which is the reason for the missing indication of UNK 1 in Figure 55.

The typical arsenicals (DMA, MA, inorganic arsenic) were eluting in the front and were hardly affected by methanol. UNK 1 and 3 were retained much longer. Without methanol in the mobile phase, their retention times were about 30 and 15 minutes, respectively. The addition of even small amounts of organic solvent led to a significant decrease of these retention times. At 15 % v/v methanol, they were almost coeluting with the other arsenicals at around 2 - 4 minutes. We could not identify a signal belonging to UNK 2.

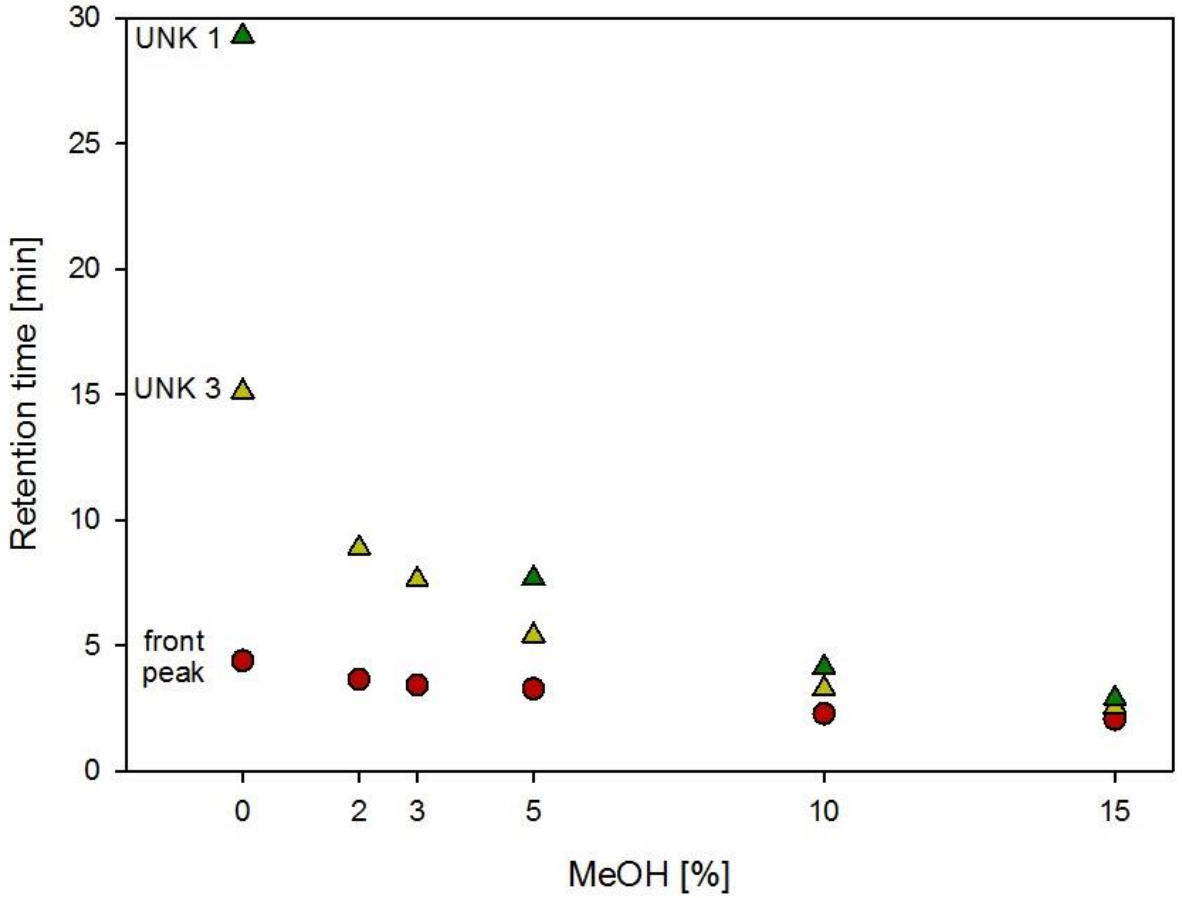


Figure 55: Chromatographic behavior of the arsenicals UNK 1 and 3 on a Zorbax C8 column with different amounts of MeOH in the mobile phase, Flow = 1.0 mL/min

We changed the flow rate from 1.0 to 1.5 mL/min and performed again chromatography with 5 % v/v methanol. UNK 1 and 3 were retained shorter, namely 5.2 instead of 7.7 minutes (UNK 1) and 3.7 instead of 5.4 minutes (UNK 3). Now, we added a bit more methanol to the mobile phase, leading to a total of 7 % v/v. The retention times of UNK 1 and 3 were 3.9 and 2.9 minutes (Figure 56). They were still separated quite well from the peaks in the solvent front (eluting after less than 2 minutes) and from each other. For this reason we decided to use these settings for preparative chromatography to isolate and concentrate UNK 1 and/or 3.

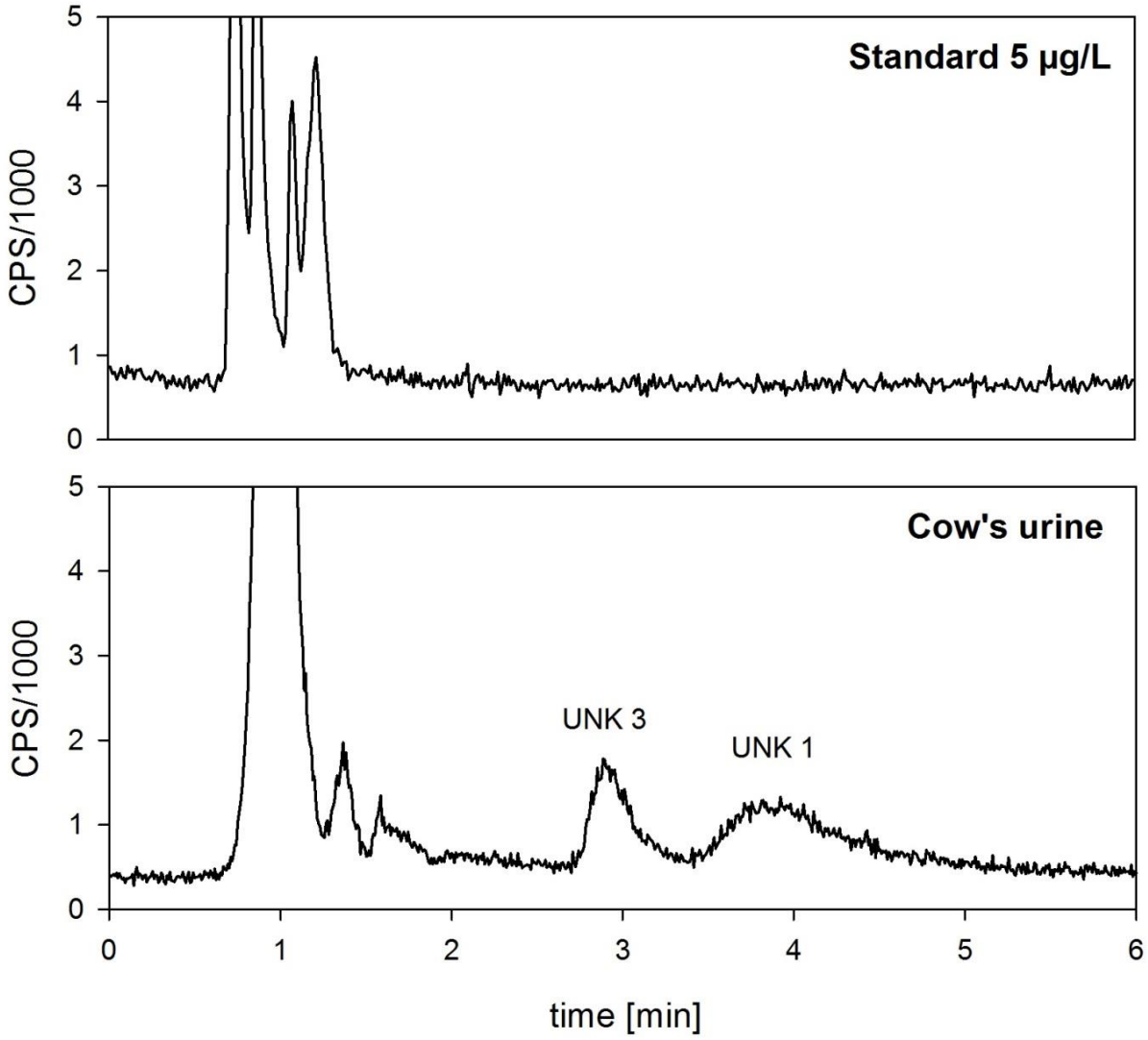


Figure 56: RP chromatograms of a standard solution containing 5 µg As/L of AB, DMA, MA and As(V) and of a cow's urine on a Zorbax SB-C8, 7 % MeOH, 1.5 mL/min

4.4.14 Preparative RP chromatography

We transferred the optimized method of the analytical reversed-phase column Zorbax SB-C8 with ultrapure water and 7 % v/v methanol as mobile phase, and a flow rate of 1.5 mL/min to the preparative reversed-phase column PrepHT SB-C8. The flow rate was 31.25 mL/min. We collected fractions starting from 2.5 minutes up to 8.5 minutes. After removing water and methanol with the vacuum concentrator we checked the fractions for total arsenic and arsenic species.

In the first experiments we used glass vials to collect the fractions. This resulted in total arsenic concentrations in the fractions that were up to 30 times higher than the total arsenic concentration of the whole cow's urine (Figure 57), although we expected to have excluded all arsenicals eluting before 2.5 minutes, which should have accounted for most of the

arsenic in the urine sample. To minimize possible contaminations from glassware, we replaced the glass vials for fraction collection by polypropylene tubes (Kartell s.p.A.). However, blanks only consisting of water and/or methanol still contained unusually high amounts of arsenic after removing most of the solvent(s) with the vacuum concentrator, no matter if they had been processed through the preparative column or not (up to 500 µg As/L) (Figure 57).

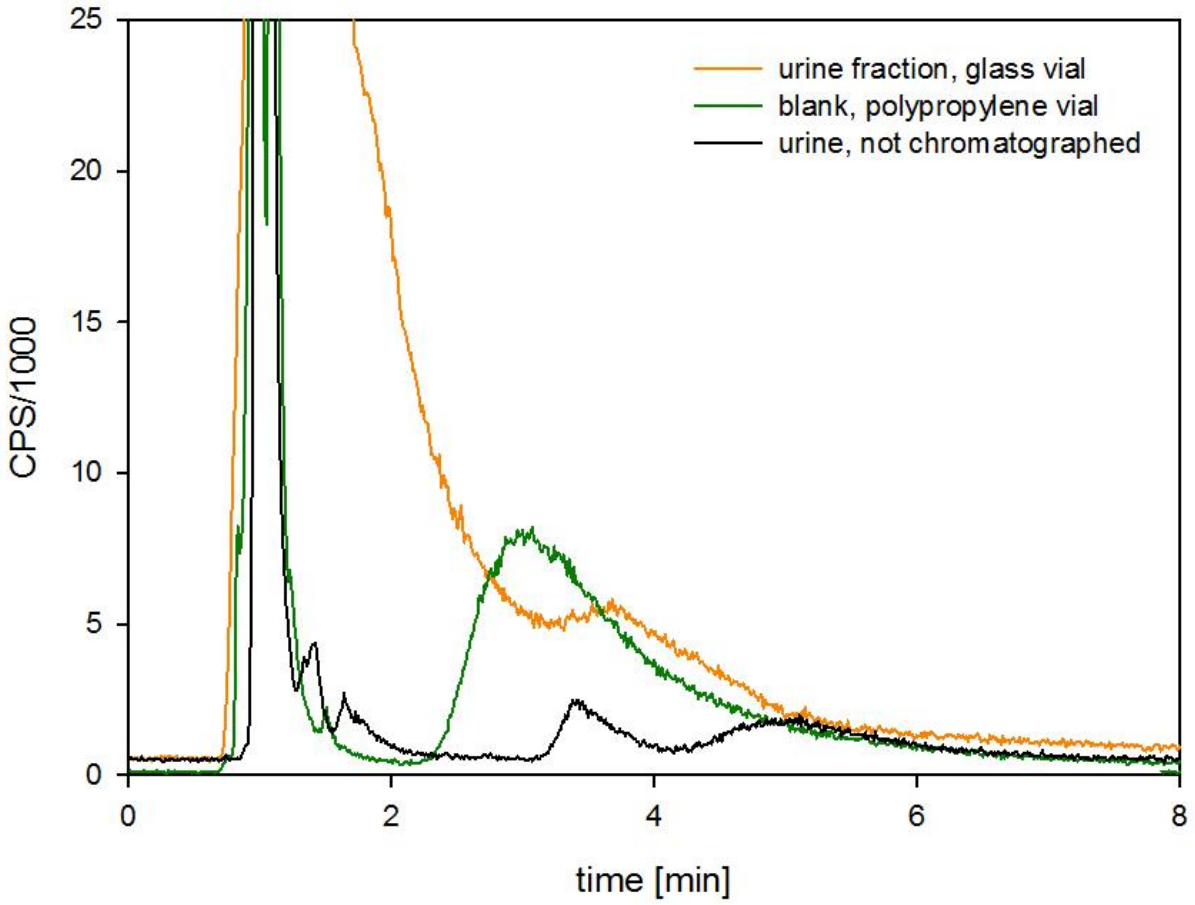


Figure 57: RP chromatograms of the fraction 6.0 - 6.5 minutes of a cow's urine (collected in a glass vial), a blank (ultrapure water + 7 % v/v methanol, without preparative chromatography) and cow's urine (without preparative chromatography)

As a next step we carefully cleaned all reachable parts of the vacuum concentrator with isopropanol and ultrapure water and replaced old tubings with new ones. Blanks now contained less than 0.5 µg As/L.

We injected again a urine sample into the preparative HPLC, collected the fractions, removed the mobile phase, resolved the residues in 0.5 mL eluent and checked the concentrations of the arsenic species with HPLC-ICPMS. The fractions 1, 3 and 4 (2.5 - 3.1 and 3.7 - 4.9 minutes) contained detectable amounts of UNK 3, besides other arsenicals. We combined the first four fractions, removed the solvent with the vacuum concentrator and dissolved the remaining sample again in 0.2 mL ultrapure water and 7 % v/v methanol. A

chromatogram of these combined fractions is compared with the chromatogram of a whole cow's urine sample in Figure 58. UNK 3 was slightly concentrated by this procedure. The cow's urine sample that we usually used for the experiments contained about 2.5 - 4.0 µg As/L of the unknown compound UNK 3, and UNK 1 and 2 together accounted for about 7 – 9 µg As/L. The investigated fractions were from 800 µL whole, not oxidized urine (2 injections, each 400 µL). Therefore, the total amount of UNK 3 in the combined fractions was expected to be between 2.0 and 3.2 ng As. We detected about 2.2 ng As of UNK 3 in the combined fractions 1-4, which is in good accordance with our prediction. However, we only found about 0.11 ng As of the combined UNK 1 and 2, which is only 1.5 - 2 % of the expected amount. It is possible that UNK 1 was oxidized to UNK 3, but then again, we would have detected too little UNK 3. With reversed-phase chromatography, the unknown compound UNK 2 was probably coeluting in the front of the chromatogram with the arsenicals DMA, MA and inorganic arsenic. This would explain its absence in the collected fractions, because we only started collecting at 2.5 minutes.

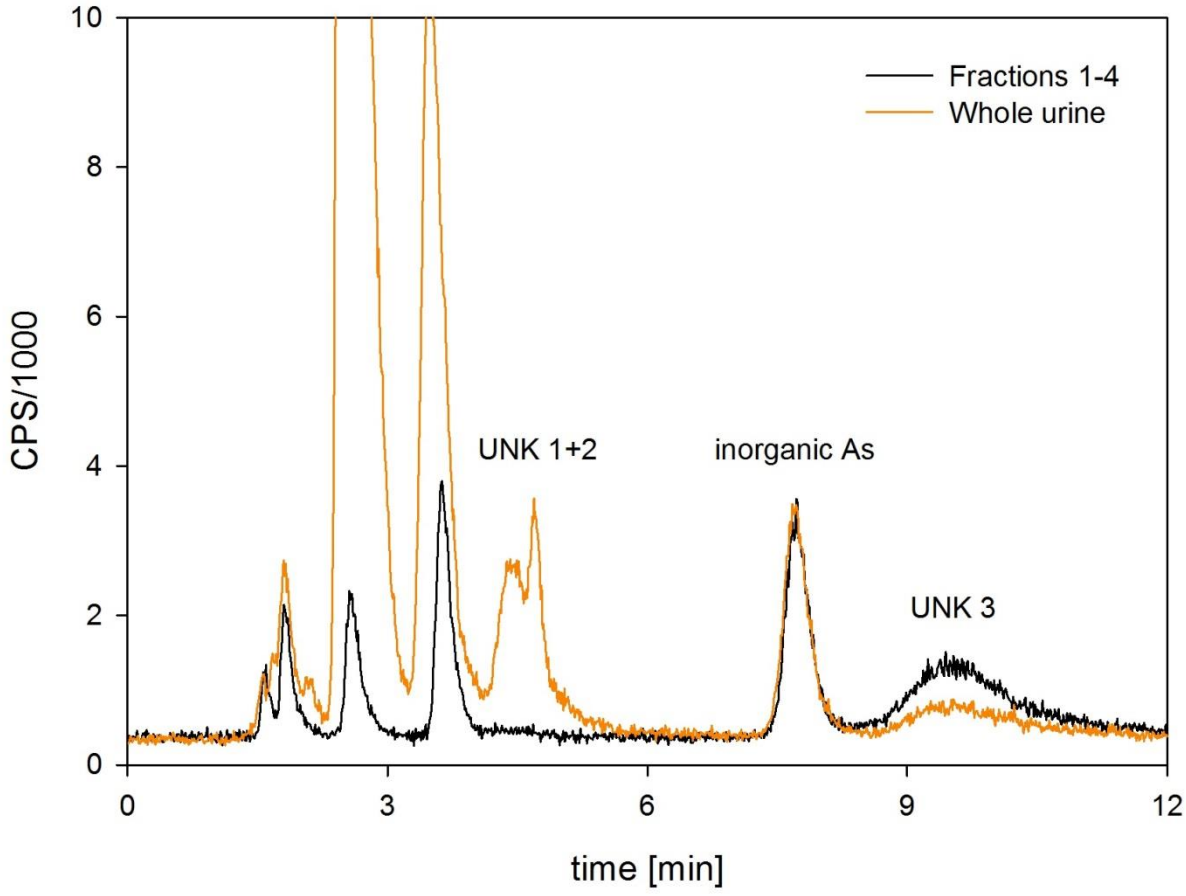


Figure 58: Anion-exchange chromatograms of the combined fractions 1-4 (2.5-4.9 minutes) after preparative chromatography of 800 µL cow's urine and of a whole, not oxidized cow's urine sample (offset: +1000 CPS)

DMA and MA were also present in the fractions, but in much smaller amounts than in the whole urine sample. However, the concentration of inorganic arsenic was not reduced during preparative HPLC. This was a surprising finding, since inorganic arsenic was always eluting right in the front when RP chromatography was applied (Figure 56). Therefore we expected to have excluded it from the fraction collection which only started after 2.5 minutes runtime. This leads to the suggestion that the inorganic arsenic in the collected fractions was actually formed during the separation and concentration processes. Maybe it was a degradation product of one of the unknown compounds. One of the simplest possible reactions would be the oxidation of a thioarsenate to (oxo-) arsenate. For this reason we decided to co-chromatograph a cow's urine sample with a standard solution of thioarsenates. See chapter 4.4.15 for further information.

The original idea of the preparative HPLC approach was to concentrate at least one of the unknown arsenicals to a concentration of at least 50 µg As/L in order to be able to detect it with molecular mass spectrometry. Unfortunately, the procedure described above was very time consuming. The main reason for this was the vacuum concentrator. It was not working properly most of the time, sometimes it took almost a week to remove the solvents, and the amount of samples that could be put into the vacuum concentrator was also very limited. Because of this we decided to discontinue our proceedings with the preparative chromatography for now.

4.4.15 Synthesis and co-chromatography of thioarsenates

Possible identities of UNK 1, 2 and especially 3 were thioarsenates. Because no standard solutions of them were available to us, we tried to synthesize them ourselves, according to the method described by Maher et al. [117] (see also experimental part, chapter 3.8.3.7). We chromatographed the obtained solutions and only found As(III) and a smaller amount of As(V). Even after allowing the solutions react at slightly elevated temperatures (around 50°C) for about 2 hours, we only detected As(III) and As(V) in the mixture that contained equal molarities of arsenic and sulfur. In the solution that contained 100 times more sulfur than arsenic, we were able to observe another signal at around 12 minutes retention time, as can be seen in Figure 59. It did not coelute with any of the unknown arsenic species in the cow's urine. The signal could belong to one of the thioarsenates, but to verify this, more experiments would be necessary. There is also another small signal right after As(III), but this was also visible in a solution that contained only As(III) and is therefore not a thioarsenical.

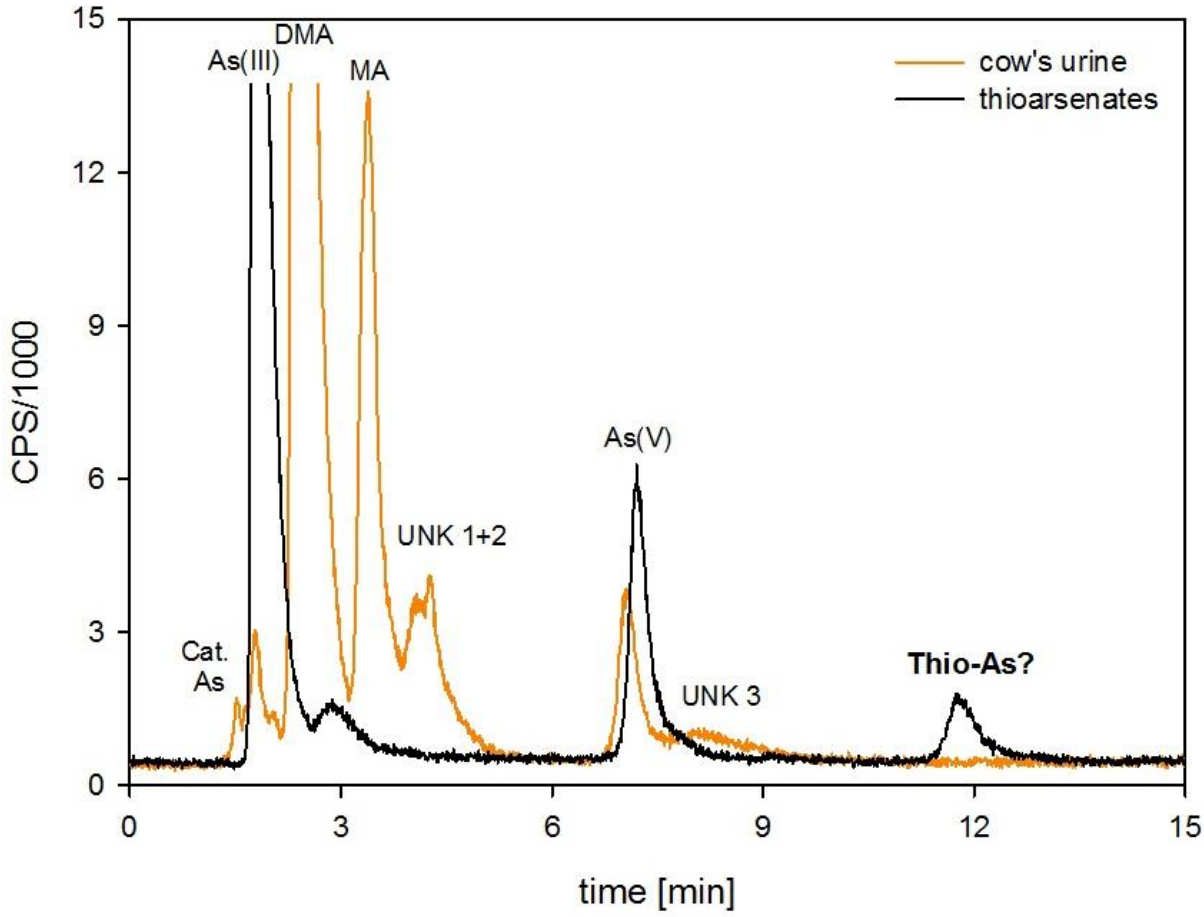


Figure 59: Anion-exchange chromatograms of a cow's urine (not oxidized) and a solution containing Na2S and As(III) ("thioarsenates")

Overall, the synthesis was not successful. One possible reason is that the Na_2S that we used was already very old. The color of the crystals was dark green to black. It was also very hard to dissolve the Na_2S in ultrapure water, and even after 30 minutes in the ultrasonic bath, it was not totally dissolved.

We are also not sure if the suggested synthesis is the best way to obtain thioarsenates. There are other routes described in literature, for example by Schwedt and Riekhoff, who were using elemental sulfur, As_2O_3 and NaOH to obtain thioarsenates [144]. Wilkin et al. synthesized thioarsenates by purging a solution of NaOH with H_2S gas and then mixing it with As(III) [145]. These possibilities remain to be tested.

5 Conclusions

5.1 Bovine livers and kidneys

Bovine kidneys generally contained more total arsenic than livers. There was a linear correlation between the total arsenic concentration in livers and corresponding kidneys from Austria. The kidney samples from Austria and Argentina had very similar total arsenic concentrations. Argentinean bovine livers contained slightly more total arsenic than Austrian ones. However, investigations of a larger amount of samples have to be carried out to determine whether cattle grazing on the Chaco-Pampean plain are accumulating arsenic from the contaminated groundwater or not. It would be necessary to obtain and analyze grass, fodder and drinking water in addition to the bovine tissue samples.

There was one Austrian kidney-liver sample pair with exceptionally high total arsenic concentrations. We do not know the actual reasons for these high values, but possible arsenic sources are drinking water, feed and feed supplements.

The total arsenic concentrations in both Austrian and Argentinean samples showed a linear correlation to the iron concentrations in the samples, but only in the tissues from Argentina we found a correlation between arsenic and selenium.

Water as well as bovine tissue samples from Argentina had extremely high vanadium concentrations. The Argentinean livers and kidneys also had higher concentrations of mercury, chromium, strontium and nickel than the Austrian samples. On the other side, rubidium was much more abundant in Austrian bovine livers and kidneys, and there was a linear correlation between the rubidium concentrations in livers and in the corresponding kidneys. This also applied for other elements like cadmium, vanadium or lithium.

The arsenic speciation was very similar for Austrian and for Argentinean bovine tissue extracts. Surprisingly, MA was the dominating arsenic compound in all bovine liver and all but two kidney extracts. DMA and inorganic arsenic were also fairly abundant. We detected hardly any cationic arsenic in the samples. There were traces of three unknown arsenic compounds in some of the kidney and two of the liver extracts. Inorganic arsenic was the major arsenical in two Argentinean kidney samples, followed by MA and DMA.

Hydrogen peroxide had a huge impact on the extraction yields. With H_2O_2 in the extracting solution, the total amount of extracted arsenic was higher than in extractions without hydrogen peroxide as oxidizing agent. Also, the abundance of MA compared to DMA and inorganic arsenic was even greater when the oxidizing extraction method was used.

To our knowledge, this is the first report on arsenic species in livers and kidneys of cattle. We were only able to compare our results with data on rodents and primates, but not ruminants. Since the digestive system of ruminants is quite different from that of primates and rodents, differences in the arsenic metabolism could be possible. When comparing our results with literature, other difficulties arose from the different ways of sample preparation, for example extraction methods, or from sample types that were used (whole tissues/homogenates and isolated cells). To find out if there are differences between the arsenic speciation in tissues different terrestrial mammal species, further investigations with samples of ruminants as well as of other terrestrial mammals are necessary.

5.2 How much beef liver or kidney is safe to eat?

At the moment, there is no official guideline for the maximum tolerable arsenic intake (see introduction for details). Still, we wanted to know how many Austrian or Argentinean bovine livers or kidneys we could eat without being poisoned by arsenic. We used two different approaches for this:

First, we took the former provisional tolerable weekly intake (PTWI) as a guideline (150 µg/kg body mass). Secondly, we took the approximate lethal dose for humans for As_2O_3 (300 mg) as an upper limit. We are aware that bovine tissues do not contain As_2O_3, but other arsenicals which have a different (lower) toxicity. Still, As_2O_3 was taken to simulate the “worst case”.

Bovine livers usually weigh about 5 kg, and kidneys about 0.6 kg. For the calculations, we used the mean wet mass arsenic concentrations of our liver and kidney samples from Table 21.

We found out that an average person of 70 kg can easily consume two fresh Austrian bovine livers (11 kg) or one fresh Argentinean bovine liver (5 kg) without exceeding the former PTWI for arsenic. Regarding the kidneys, one can eat 4 and a half fresh kidneys (Austrian or Argentinean), which is about 3 kg in total. Even if we use the concentration of the kidney sample with the exceptionally high arsenic concentrations (1390 mg/kg dm) for the calculation, one would still have to eat about 1 whole fresh kidney to exceed the former PTWI.

There is definitely no risk of being accidentally deadly poisoned by eating bovine livers or kidneys, regarding the lethal dose of As_2O_3.

It has to be mentioned that these calculations are only regarding arsenic and the consumption of bovine livers and kidneys. Other toxic elements and the intake of other food or drinks have not been taken into account here.

5.3 Urine of cattle and other terrestrial mammals

The total arsenic concentrations in cattle from a farm in Upper Styria as well as from Schönbrunn Zoo were around 15 µg/L, which is slightly higher than the typical value for urine of unexposed mammals. Higher intake of arsenic could occur from the feeding behavior of the animals. When grazing, small amounts of soil (usually containing higher arsenic concentrations than grass or hay) can also be ingested.

Urine samples from cattle from Eastern Styria contained significantly more arsenic, namely up to 125 µg/L. The reasons for that are yet unknown and subject of further experiments. Urine samples of other animals from Schönbrunn Zoo contained between 5.1 and 81 µg As/L.

Although the digestion system of ruminants is quite different compared to the one of primates or rodents, we found a very similar arsenic speciation in the urine. The dominant arsenical was DMA, which accounted for 35 – 75 % of the total arsenic, followed by inorganic arsenic (1 – 35 %) and MA (2 – 15 %). There were traces of AB and TMAO in the samples.

We also detected significant amounts of three unknown arsenic species (UNK 1-3) in most of the investigated urine samples. To our knowledge, they have not yet been reported in literature for any urine sample.

We carried out a number of experiments on HPLC-ICPMS with two different anion-exchange columns and various mobile phases to identify at least one of the unknown arsenicals. Upon oxidation, the signal of UNK 1 vanished. At the same time, the signal of UNK 3 increased by about the concentration range of UNK 1. A possible explanation is that UNK 1 is actually the unoxidized form of UNK 3. To proof this, more information has to be gained.

UNK 1 and 3 were well retained on the polymer-based anion-exchange column PRP-X100 as well as on the cation-exchange column Zorbax 300-SCX. However, their retention was much weaker on the silica-based anion-exchange column Zorbax SAX. Therefore, we are suggesting that these unknown compounds have a more cationic-like rather than anionic behavior, at least up to pH 8.0. Retention on the PRP-X100 was probably happening because of interactions with the backbone.

UNK 1 and 3 were also separated very well from the other arsenicals on the RP column Zorbax SB-C8. We also started to isolate and concentrate UNK 3 via preparative RP HPLC for analysis with molecular mass spectrometry.

6 Open questions and outlook

- Why were the total arsenic concentrations in the Austrian bovine liver-kidney pair no. 1320 so high?
- Is the arsenic speciation in ruminants' tissues different from the arsenic speciation in tissues of rodents and primates, or are the differences in the results (this work compared with literature) an effect of different types of experimental designs, sample preparations and analysis techniques?
- Do the high vanadium concentrations in water and bovine tissues from Argentina have any toxicological effect on grazing cattle and humans?
- What are the chemical structures of UNK 1-3? Is one of them a thioarsenate?
- What happens with the unknown arsenicals when heat is applied to the urine samples (with and without addition of H_2O_2)?
- Does application of hydrogen peroxide oxidize UNK 1 to UNK 3?
- What is the arsenic speciation in blood and rumen fluid of ruminants?

We will continue to separate and concentrate the unknown arsenicals UNK 3 and UNK 1 from a cow's urine to finally identify them with molecular mass spectrometry. The synthesis and co-chromatography of thioarsenates will be another approach to achieve this goal.

Future work will include the collection and analysis of total arsenic and arsenic speciation of additional tissue samples from cattle, from Argentina and Austria, especially from the area of origin of the Austrian tissue samples no. 1320, if possible from the same farm where the outlier tissues came from. This will be accompanied by collection and analysis of drinking water, feed, milk and urine of the investigated cattle. We will also try to obtain blood and rumen fluid of cattle to gain more information about the arsenic metabolism in ruminants.

To be able to compare the metabolism of arsenic of ruminants with the one of other mammals, we will also investigate the arsenic speciation in tissue samples of mice.

Further on, we will look at the vanadium concentrations and the vanadium speciation in different biological samples from Argentina and Austria to be able to assess a possible health risk for humans.

7 Abbreviations

dm	dry mass
GSH	Glutathione
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
ICPMS	inductively coupled plasma mass spectrometry
MeOH	Methanol
mM	$1*10^{-3}$ mol/L
n.a.	not available
SAM	S-adenosylmethionine
SD	Standard deviation
TFA	trifluoracetic acid
v/v (% v/v)	volume/volume (percent by volume)
wm	wet mass

For the abbreviations of the different arsenic species see Introduction, Table 1.

8 References

[1] Hughes, M. F., Beck, B. D., Chen, Y., Lewis, A. S., and Thomas, D. J. **2011**. Arsenic exposure and toxicology: A historical perspective. *Toxicol. Sci.* 123, 2, 305–332.

[2] Marsh, J. **1836**. Account of a method of separating small quantities of arsenic from substances with which it may be mixed. *Edinburgh Phil. J.* 21, 229–236.

[3] Bentley, R. and Chasteen, T. G. **2002**. Arsenic curiosa and humanity. *Chem. Educator* 7, 2, 51–60.

[4] Lykknes, A. and Kvittingen, L. **2003**. Arsenic: Not so evil after all? *J. Chem. Educ.* 80, 5, 497–500.

[5] Przygoda, G., Feldmann, J., and Cullen, W. R. **2001**. The arsenic eaters of Styria: A different picture of people who were chronically exposed to arsenic. *Appl. Organomet. Chem.* 15, 6, 457–462.

[6] Most, K. H. **1939**. *Arsen als Gift und Zaubermittel in der deutschen Volksmedizin mit besonderer Berücksichtigung der Steiermark*. Ph.D. Thesis, University of Graz.

[7] Nachman, K. E., Baron, P. A., Raber, G., Francesconi, K. A., Navas-Acien, A., and Love, D. C. **2013**. Roxarsone, inorganic arsenic, and other arsenic species in chicken: A U.S.-based market basket sample. *Environ. Health Persp.* 121, 7, 818–824.

[8] Stair, E. L., Kirkpatrick, J. G., and Whitenack, D. L. **1995**. Lead arsenate poisoning in a herd of beef cattle. *J. Am. Vet. Med. A.* 207, 3, 341–343.

[9] Neiger, R., Nelson, N., Miskimins, D., Caster, J., and Caster, L. **2004**. Bovine arsenic toxicosis. *J. Vet. Diagn. Invest.* 16, 5, 436–438.

[10] National Research Council. **2005**. *Mineral tolerance of animals*. National Academy Press, Washington, D.C.

[11] Uthus, E. O. **1992**. Evidence for arsenic essentiality. *Environ. Geochem. Health* 14, 2, 55–58.

[12] Larsen, E. H., Pritzl, G., and Hansen, S. H. **1993**. Speciation of eight arsenic compounds in human urine by high-performance liquid chromatography with inductively coupled plasma mass spectrometric detection using antimonate for internal chromatographic standardization. *J. Anal. At. Spectrom.* 8, 4, 557–563.

[13] Edmonds, J. S., Nomachi, M., and Morita, M. **2005**. Deuterium exchange in arsenobetaine and dimethylarsinoylacetic acid. *J. Inorg. Biochem.* 99, 3, 747–754.

[14] Scheer, J., Findenig, S., Goessler, W., Francesconi, K. A., Howard, B., Umans, J. G., Pollak, J., Tellez-Plaza, M., Silbergeld, E. K., Guallar, E., and Navas-Acien, A. **2012**. Arsenic species and selected metals in human urine: validation of HPLC/ICPMS and ICPMS procedures for a long-term population-based epidemiological study. *Anal. Methods* 4, 2, 406–413.

[15] ATSDR. **2007**. *Toxicological profile for arsenic*. http://www.atsdr.cdc.gov/ToxProfiles/tp2.pdf. Accessed 05th December 2013.

[16] World Health Organization (WHO). **2001**. *Environmental Health Criteria 224*. *Arsenic and arsenic compounds*. Second edition. World Health Organization, Geneva.

[17] Benramdane, L., Accominotti, M., Fanton, L., Malicier, D., and Vallon, J.-J. **1999**. Arsenic speciation in human organs following fatal arsenic trioxide poisoning - A case report. *Clin. Chem.* 45, 2, 301–306.

[18] Bartolomé, B., Córdoba, S., Nieto, S., Fernández-Herrera, J., and García-Díez, A. **1999**. Acute arsenic poisoning: Clinical and histopathological features. *Br. J. Dermatol.* 141, 6, 1106–1109.

[19] Ng, J. C. **2005**. Environmental contamination of arsenic and its toxicological impact on humans. *Environ. Chem.* 2, 3, 146.

[20] Mandal, B. K. and Suzuki, K. T. **2002**. Arsenic round the world: A review. *Talanta* 58, 1, 201–235.

[21] Wemhöner, U., Schartner, C., Loishandl-Weisz, H., Schubert, G., and Schedl, A. **2012**. *Metalle im Grundwasser in Österreich*. Karten und Erläuterungen. http://www.umweltbundesamt.at/umweltsituation/wasser/grundwasser/metallbericht/. Accessed 17th December 2013.

[22] Geologische Bundesanstalt. *Hydrochemischer geogener Hintergrundwert von Arsen*. https://www.geologie.ac.at/forschung-entwicklung/kartierung-landesaufnahme/geochemie/geohint-arsen/. Accessed 17th December 2013.

[23] Roy, D., Das, T. K., and Vaswani, S. **2013**. Arsenic: It's extent of pollution and toxicosis: An animal perspective. *Vet. World* 6, 1, 53–58.

[24] Bera, A. K., Rana, T., Bhattacharya, D., Bandyopadhyay, S., Pan, D., De, S., Samanta, S., Chowdhury, A. N., Mondal, T. K., and Das, S. K. **2010**. Ground water arsenic contamination in West Bengal, India: A risk of sub-clinical toxicity in cattle as evident by correlation between arsenic exposure, excretion and deposition. *Toxicol. Ind. Health* 26, 10, 709–716.

[25] Datta, B. K., Bhar, M. K., Patra, P. H., Majumder, D., Dey, R. R., Sarkar, S., Mandal, T. K., and Chakraborty, A. K. **2012**. Effect of environmental exposure of arsenic on cattle and poultry in Nadia district, West Bengal, India. *Toxicol. Int.* 19, 1, 59–62.

[26] Datta, B. K., Mishra, A., Singh, A., Sar, T. K., Sarkar, S., Bhatacharya, A., Chakraborty, A. K., and Mandal, T. K. **2010**. Chronic arsenicosis in cattle with special reference to its metabolism in arsenic endemic village of Nadia district West Bengal India. *Sci. Tot. Environ.* 409, 2, 284–288.

[27] Ghosh, A., Awal, M. A., Majumder, S., Mostofa, M., Khair, A., Islam, M. Z., and Rao, D. R. **2012**. Arsenic in eggs and excreta of laying hens in Bangladesh: A preliminary study. *J. Health Popul. Nutr.* 30, 4, 383–393.

[28] Ghosh, A., Awal, M. A., Majumder, S., Sikder, M. H., and Rao, D. R. **2012**. Arsenic residues in broiler meat and excreta at arsenic prone areas of Bangladesh. *Bangl. J. Pharmacol.* 7, 3.

[29] Ghosh, C. K., Datta, B. K., Biswas, S., Maji, C., Sarkar, S., Mandal, T. K., Majumder, D., and Chakraborty, A. K. **2011**. Chronic arsenicosis of cattle in West Bengal and it's possible mitigation by sodium thiosulfate. *Toxicol. Int.* 18, 2, 137–139.

[30] Ghosh, A., Majumder, S., Awal, M. A., and Rao, D. R. **2013**. Arsenic exposure to dairy cows in Bangladesh. *Arch. Environ. Con. Tox.* 64, 1, 151–159.

[31] Nicolli, H. B., Bundschuh, J., Blanco, M. D. C., Tujchneider, O. C., Panarello, H. O., Dapeña, C., and Rusansky, J. E. **2012**. Arsenic and associated trace-elements in groundwater from the Chaco-Pampean plain, Argentina: Results from 100 years of research. *Sci. Tot. Environ.* 429, 36–56.

[32] Bundschuh, J., Litter, M. I., Parvez, F., Román-Ross, G., Nicolli, H. B., Jean, J.-S., Liu, C.-W., Lopez, D. L., Armienta, M. A., Guilherme, L. R. G., Cuevas, A. G., Cornejo, L., Cumbal, L., and Toujaguez, R. **2012**. One century of arsenic exposure in Latin America: A review of history and occurrence from 14 countries. *Sci. Tot. Environ.* 429, 2–35.

[33] Pérez-Carrera, A. and Fernández-Cirelli, A. **2010**. Arsenic and water quality challenges in South America. In *Water and Sustainability in Arid Regions*. Springer, Netherlands, 275–293.

[34] Pérez-Carrera, A. and Fernández-Cirelli, A. **2005**. Arsenic concentration in water and bovine milk in Cordoba, Argentina. Preliminary results. *J. Dairy Res.* 72, 1, 122–124.

[35] Pérez-Carrera, A. and Fernández-Cirelli, A. **2009**. Transfer of arsenic from contaminated dairy cattle drinking water to milk (Cordoba, Argentina). In *Natural arsenic in groundwaters of Latin America*, J. Bundschuh, Ed. Arsenic in the environment 1. CRC Press, London.

[36] Pérez-Carrera, A. and Fernández-Cirelli, A. **2010**. Biotransference of arsenic from drinking water to bovine tissues and milk. In *Proceedings As 2010 3rd international congress arsenic in the environment*. *2010 May 17–21; Tainan, Taiwan.*, J.-S. Jean, J. Bundschuh and P. Bhattacharya, Eds. Balkema/CRC Press, Leiden, The Netherlands.

[37] Uneyama, C., Toda, M., Yamamoto, M., and Morikawa, K. **2007**. Arsenic in various foods: Cumulative data. *Food Addit. Contam.* 24, 5, 447–534.

[38] Doyle, J. J. and Spaulding, J. E. **1978**. Toxic and essential trace elements in meat-a review. *J. Anim. Sci.* 47, 2, 398–419.

[39] Kramer, H. L., Steiner, J. W., and Vallely, P. J. **1983**. Trace element concentrations in the liver, kidney, and muscle of Queensland cattle. *B. Environ. Contam. Tox.* 30, 5, 588–594.

[40] Korsrud, G. O., Meldrum, J. B., Salisbury, C. D., Houlahan, B. J., Saschenbrecker, P. W., and Tittiger, F. **1985**. Trace element levels in liver and kidney from cattle, swine and poultry slaughtered in Canada. *Can. J. Comp. Med.* 49, 2, 159–163.

[41] Vos, G., Hovens, J. P. C., and Delft, W. V. **1987**. Arsenic, cadmium, lead and mercury in meat, livers and kidneys of cattle slaughtered in the Netherlands during 1980-1985. *Food Addit. Contam.* 4, 1, 73–88.

[42] Nishizawa, K. **1991**. [Japanese]. *Syokuhin to Kaihatsu* 26, 48–51.

[43] Jorhem, L., Slorach, S., Sundstrom, B., and Ohlin, B. **1991**. Lead, cadmium, arsenic and mercury in meat, liver and kidney of Swedish pigs and cattle in 1984-1988. *Food Addit. Contam.* 8, 2, 201–212.

[44] Šinigoj-Gačnik, K. and Doganoc, D. Z. **2000**. Contamination of farm animals and fishes from Slovenia with heavy metals and sulfonamides. *B. Environ. Contam. Tox.* 64, 2, 235–241.

[45] López-Alonso, M., Benedito, J. L., Miranda, M., Castillo, C., Hernández, J., and Shore, R. F. **2000**. Arsenic, cadmium, lead, copper and zinc in cattle from Galicia, NW Spain. *Sci. Tot. Environ.* 246, 2-3, 237–248.

[46] López-Alonso, M., Benedito, J. L., Miranda, M., Castillo, C., Hernández, J., and Shore, R. F. **2000**. Toxic and trace elements in liver, kidney and meat from cattle slaughtered in Galicia (NW Spain). *Food Addit. Contam.* 17, 6, 447–457.

[47] López-Alonso, M., Benedito, J. L., Miranda, M., Castillo, C., Hernández, J., and Shore, R. F. **2002**. Cattle as biomonitors of soil arsenic, copper, and zinc concentrations in Galicia (NW Spain). *Arch. Environ. Con. Tox.* 43, 1, 103–108.

[48] Miranda, M., López-Alonso, M., Castillo, C., Hernández, J., Prieto, F., and Benedito, J. L. **2003**. Some toxic elements in liver, kidney and meat from calves slaughtered in Asturias (Northern Spain). *Eur. Food Res. Technol.* 216, 4, 284–289.

[49] Bruce, S., Noller, B., Grigg, A., Mullen, B., Mulligan, D., Ritchie, P., Currey, N., and Ng, J. C. **2003**. A field study conducted at Kidston Gold Mine, to evaluate the impact of arsenic and zinc from mine tailing to grazing cattle. *Toxicol. Lett.* 137, 1-2, 23–34.

[50] López-Alonso, M., Montaña, F. P., Miranda, M., Castillo, C., Hernández, J., and Benedito, J. L. **2004**. Interactions between toxic (As, Cd, Hg and Pb) and nutritional essential (Ca, Co, Cr, Cu, Fe, Mn, Mo, Ni, Se, Zn) elements in the tissues of cattle from NW Spain. *BioMetals* 17, 4, 389–397.

[51] Nriagu, J. O., Boughanen, M., Linder, A., Howe, A., Grant, C., Rattray, R., Vutchkov, M., and Lalor, G. **2009**. Levels of As, Cd, Pb, Cu, Se and Zn in bovine kidneys and livers in Jamaica. *Ecotox. Environ. Safe.* 72, 2, 564–571.

[52] Bilandžić, N., Dokić, M., and Sedak, M. **2010**. Survey of arsenic, cadmium, copper, mercury and lead in kidney of cattle, horse, sheep and pigs from rural areas in Croatia. *Food Addit. Contam. B* 3, 3, 172–177.

[53] Khalafalla, F. A., Ali, F. H., Schwagele, F., and Abd-El-Wahab, M. A. **2011**. Heavy metal residues in beef carcasses in Beni-Suef abattoir, Egypt. *Vet. Ital.* 47, 3, 351–361.

[54] Yabe, J., Nakayama, S. M., Ikenaka, Y., Muzandu, K., Ishizuka, M., and Umemura, T. **2011**. Uptake of lead, cadmium, and other metals in the liver and kidneys of cattle near a lead-zinc mine in Kabwe, Zambia. *Environ. Toxicol. Chem.* 30, 8, 1892–1897.

[55] Yabe, J., Nakayama, S. M., Ikenaka, Y., Muzandu, K., Ishizuka, M., and Umemura, T. **2012**. Accumulation of metals in the liver and kidneys of cattle from agricultural areas in Lusaka, Zambia. *J. Vet. Med. Sci.* 74, 10, 1345–1347.

[56] Roggeman, S., Boeck, G. de, Cock, H. de, Blust, R., and Bervoets, L. **2014**. Accumulation and detoxification of metals and arsenic in tissues of cattle (Bos taurus), and the risks for human consumption. *Sci. Tot. Environ.* 466-467, 175–184.

[57] Vos, G., Lammers, H., and van Delft, W. **1988**. Arsenic, cadmium, lead and mercury in meat, livers and kidneys of sheep slaughtered in The Netherlands. *Z. Lebensm. Unters. Fosch.* 187, 1, 1–7.

[58] Rudy, M. **2009**. The analysis of correlations between the age and the level of bioaccumulation of heavy metals in tissues and the chemical composition of sheep meat from the region in SE Poland. *Food Chem. Toxicol.* 47, 6, 1117–1122.

[59] Feldmann, J., John, K., and Pengprecha, P. **2000**. Arsenic metabolism in seaweed-eating sheep from Northern Scotland. *Fresen. J. Anal. Chem.* 368, 1, 116–121.

[60] Haenlein, G. and Anke, M. **2011**. Mineral and trace element research in goats: A review. *Small Ruminant Res.* 95, 1, 2–19.

[61] Custer, T. W., Cox, E., and Gray, B. **2004**. Trace elements in moose (Alces alces) found dead in Northwestern Minnesota, USA. *Sci. Tot. Environ.* 330, 1-3, 81–87.

[62] Laird, B. D. and Chan, H. M. **2013**. Bioaccessibility of metals in fish, shellfish, wild game, and seaweed harvested in British Columbia, Canada. *Food Chem. Toxicol.* 58, 381–387.

[63] Hassan, A. A., Rylander, C., Brustad, M., and Sandanger, T. M. **2012**. Level of selected toxic elements in meat, liver, tallow and bone marrow of young semi-domesticated reindeer (Rangifer tarandus tarandus L.) from Northern Norway. *Int. J. Circumpol. Health* 71, 0, 111.

[64] Reglero, M., Monsalve-González, L., Taggart, M., and Mateo, R. **2008**. Transfer of metals to plants and red deer in an old lead mining area in Spain. *Sci. Tot. Environ.* 406, 1-2, 287–297.

[65] Nakayama, S. M., Ikenaka, Y., Muzandu, K., Choongo, K., M'kandawire, E., Yasuda, J., and Ishizuka, M. **2012**. Metal and metalloid levels and bio-accumulation characteristics in soil, sediment, land plants and hippopotami (Hippopotamus amphibius L) from the South Luangwa National Park, Zambia. *Ecotox. Environ. Safe.* 80, 333–338.

[66] Ponce, R., Farías, S. S., Bovi Mitre, G., Vélez, D., and Montoro, R. **2006**. Determinación de arsénico total e inorgánico en carne y vísceras de camélidos (Lamma glama) autóctonos de la provincia de Jujuy, Argentina. *Rev. Fac. de Agronomía de la UBA (Buenos Aires, Argentina)* 26, 1, 105–109.

[67] Challenger, F. **1945**. Biological methylation. *Chem. Rev.* 36, 3, 315–361.

[68] Adair, B. M., Waters, S. B., Devesa, V., Drobná, Z., Stýblo, M., and Thomas, D. J. **2005**. Commonalities in metabolism of arsenicals. *Environ. Chem.* 2, 3, 161.

[69] Aposhian, H. V. **1997**. Enzymatic methylation of arsenic species and other new approaches to arsenic toxicity. *Annu. Rev. Pharmacol.* 37, 397–419.

[70] Healy, S. M., Wildfang, E., Zakharyan, R. A., and Aposhian, H. V. **1999**. Diversity of inorganic arsenite biotransformation. *Biol. Trace Elem. Res.* 68, 3, 249–266.

[71] Rehman, K. and Naranmandura, H. **2012**. Arsenic metabolism and thioarsenicals. *Metallomics* 4, 9, 881.

[72] Vahter, M. **1999**. Methylation of inorganic arsenic in different mammalian species and population groups. *Sci. Prog.* 82, 1, 69-88.

[73] Vahter, M. **2002**. Mechanisms of arsenic biotransformation. *Toxicology* 181-182, 211–217.

[74] Watanabe, T. and Hirano, S. **2013**. Metabolism of arsenic and its toxicological relevance. *Arch. Toxicol.* 87, 6, 969–979.

[75] Aposhian, H. V., Zakharyan, R. A., Avram, M. D., Sampayo-Reyes, A., and Wollenberg, M. L. **2004**. A review of the enzymology of arsenic metabolism and a new potential role of hydrogen peroxide in the detoxication of the trivalent arsenic species. *Toxicol. Appl. Pharm.* 198, 3, 327–335.

[76] Vahter, M. **1994**. Species differences in the metabolism of arsenic compounds. *Appl. Organomet. Chem.* 8, 3, 175–182.

[77] Kuehnelt, D. and Goessler, W. **2003**. Organoarsenic compounds in the terrestrial environment. In *Organometallic compounds in the environment*, P. J. Craig, Ed. J. Wiley, West Sussex, England, 223–275.

[78] Hayakawa, T., Kobayashi, Y., Cui, X., and Hirano, S. **2005**. A new metabolic pathway of arsenite: Arsenic-glutathione complexes are substrates for human arsenic methyltransferase Cyt19. *Arch. Toxicol.* 79, 4, 183–191.

[79] Lakso, J. U. and Peoples, S. A. **1975**. Methylation of inorganic arsenic by mammals. *J. Agr. Food Chem.* 23, 4, 674–676.

[80] Lindberg, A.-L., Goessler, W., Gurzau, E., Koppova, K., Rudnai, P., Kumar, R., Fletcher, T., Leonardi, G., Slotova, K., Gheorghiu, E., and Vahter, M. **2006**. Arsenic exposure in Hungary, Romania and Slovakia. *J. Environ. Monit.* 8, 1, 203–208.

[81] Vahter, M., Couch, R., Nermell, B., and Nilsson, R. **1995**. Lack of methylation of inorganic arsenic in the chimpanzee. *Toxicol. Appl. Pharm.* 133, 2, 262–268.

[82] Hansen, H. R., Raab, A., Francesconi, K. A., and Feldmann, J. **2003**. Metabolism of arsenic by sheep chronically exposed to arsenosugars as a normal part of their diet. 1. Quantitative intake, uptake, and excretion. *Environ. Sci. Technol.* 37, 5, 845–851.

[83] Assis, R. A., Kuchler, I. L., Miekeley, N., and Tozzi, M. B. **2008**. Intake and excretion of disodium monomethylarsonate in horses: a speciation study. *Anal. Bioanal. Chem.* 390, 8, 2107–2113.

[84] Vahter, M. and Marafante, E. **1983**. Intracellular interaction and metabolic fate of arsenite and arsenate in mice and rabbits. *Chem. Biol. Interact.* 47, 1, 29–44.

[85] Hirata, M., Mohri, T., Hisanaga, A., and Ishinishi, N. **1989**. Conversion of arsenite and arsenate to methylarsenic and dimethylarsenic compounds by homogenates prepared from livers and kidneys of rats and mice. *Appl. Organomet. Chem.,* 3, 335–341.

[86] Kenyon, E. M., Del Razo, L. M., and Hughes, M. F. **2005**. Tissue distribution and urinary excretion of inorganic arsenic and its methylated metabolites in mice following acute oral administration of arsenate. *Toxicol. Sci.* 85, 1, 468–475.

[87] Drobná, Z., Waters, S. B., Walton, F. S., LeCluyse, E. L., Thomas, D. J., and Stýblo, M. **2004**. Interindividual variation in the metabolism of arsenic in cultured primary human hepatocytes. *Toxicol. Appl. Pharm.* 201, 2, 166–177.

[88] Bertolero, F., Marafante, E., Rade, J., and Sabbioni, E. **1980**. Pharmacokinetics and intracellular distribution of inorganic arsenic in the rabbit. *Toxicol. Lett.* 6, SPEC.

[89] Sampayo-Reyes, A., Zakharyan, R. A., Healy, S. M., and Aposhian, H. V. **2000**. Monomethylarsonic acid reductase and monomethylarsonous acid in hamster tissue. *Chem. Res. Toxicol.* 13, 11, 1181–1186.

[90] Hughes, M. F., Kenyon, E. M., Edwards, B. C., Mitchell, C. T., Razo, L. M. D., and Thomas, D. J. **2003**. Accumulation and metabolism of arsenic in mice after repeated oral administration of arsenate. *Toxicol. Appl. Pharm.* 191, 3, 202–210.

[91] Naranmandura, H., Suzuki, N., Iwata, K., Hirano, S., and Suzuki, T. **2007**. Arsenic metabolism and thioarsenicals in hamsters and rats. *Chem. Res. Toxicol.* 20, 4, 616–624.

[92] Drobná, Z., Walton, F. S., Harmon, A. W., Thomas, D. J., and Stýblo, M. **2010**. Interspecies differences in metabolism of arsenic by cultured primary hepatocytes. *Toxicol. Appl. Pharm.* 245, 1, 47–56.

[93] Drobná, Z., Walton, F. S., Paul, D. S., Xing, W., Thomas, D. J., and Stýblo, M. **2010**. Metabolism of arsenic in human liver: The role of membrane transporters. *Arch. Toxicol.* 84, 1, 3–16.

[94] Stýblo, M., Del Razo, L. M., LeCluyse, E. L., Hamilton, G. A., Wang, C., Cullen, W. R., and Thomas, D. J. **1999**. Metabolism of arsenic in primary cultures of human and rat hepatocytes. *Chem. Res. Toxicol.* 12, 7, 560–565.

[95] Stýblo, M., Delnomdedieu, M., and Thomas, D. J. **1996**. Mono- and dimethylation of arsenic in rat liver cytosol in vitro. *Chem. Biol. Interact.* 99, 1-3, 147–164.

[96] Saunders, J. R., Knopper, L. D., Koch, I., and Reimer, K. J. **2010**. Arsenic transformations and biomarkers in meadow voles (Microtus pennsylvanicus) living on an abandoned gold mine site in Montague, Nova Scotia, Canada. *Sci. Tot. Environ.* 408, 4, 829–835.

[97] Saunders, J. R., Hough, C., Knopper, L. D., Koch, I., and Reimer, K. J. **2011**. Arsenic transformations in terrestrial small mammal food chains from contaminated sites in Canada. *J. Environ. Monit.* 13, 6, 1784–1792.

[98] ATSDR. **2011**. *2011 Priority List of Hazardous Substances*. http://www.atsdr.cdc.gov/SPL/index.html. Accessed 05th December 2013.

[99] EFSA Panel on Contaminants in the Food Chain (CONTAM). **2009**. Scientific Opinion on Arsenic in Food. *EFSA J.* 7(10):1351, 10, 1–199.

[100] U.S. Environmental Protection Agency (EPA). **2001**. National Primary Drinking Water Regulations. Arsenic and Clarifications to Compliance and New Source Contaminants Monitoring; Final Rule. *Fed. Reg.* 66, 6975–7066.

[101] FAO/WHO. **1989**. *Evaluation of certain food additives and contaminants*. *Thirty-third report of the Joint FAO/WHO Expert Committee on Food Additives*. World Health Organization technical report series 776. World Health Organization, Geneva.

[102] FAO/WHO. **2011**. *Evaluation of certain contaminants in food*. *Seventy-second report of the Joint FAO/WHO Expert Committee on Food Additives*. WHO technical report series 959. World Health Organization, Geneva.

[103] U.S. Food and Drug Administration (FDA). **2013**. *FDA Proposes "Action Level" for Arsenic in Apple Juice. Press announcement (12th July 2013)*. http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm360466.htm. Accessed 27th November 2013.

[104] U.S. Food and Drug Administration (FDA). **2013**. *Code of Federal Regulations*. *21 CFR 556.60*. http://www.fda.gov. Accessed 05th December 2013.

[105] Dunnivant, F. and Ginsbach, J. **2008**. *Scheme of an ICPMS*. http://people.whitman.edu/~dunnivfm/FAASICPMS_Ebook/CH4/Figures/F4_1_ICP-MS.gif. Accessed 07th January 2014.

[106] Kuehnelt, D., Juresa, D., Kienzl, N., and Francesconi, K. A. **2006**. Marked individual variability in the levels of trimethylselenonium ion in human urine determined by HPLC/ICPMS and HPLC/vapor generation/ICPMS. *Anal. Bioanal. Chem.* 386, 7-8, 2207–2212.

[107] Kovačevič, M. and Goessler, W. **2005**. Direct introduction of volatile carbon compounds into the spray chamber of an inductively coupled plasma mass spectrometer: Sensitivity enhancement for selenium. *Spectrochim. Acta B* 60, 9-10, 1357–1362.

[108] *Homepage of Schönbrunn Zoo*. http://www.zoovienna.at. Accessed 23rd October 2013.

[109] Goessler, W. and Pavkov, M. **2003**. Accurate quantification and transformation of arsenic compounds during wet ashing with nitric acid and microwave assisted heating. *Analyst* 128, 6, 796.

[110] Merijanian, A. and Zingaro, R. A. **1966**. Arsine oxides. *Inorg. Chem.* 5, 2, 187–191.

[111] Irgolic, K. J., Junk, T., Kos, C., McShane, W. S., and Pappalardo, G. C. **1987**. Preparation of trimethyl-2-hydroxyethylarsonium (arsenocholine) compounds. *Appl. Organomet. Chem.* 1, 5, 403–412.

[112] Francesconi, K. A., Edmonds, J. S., and Stick, R. V. **1989**. Accumulation of arsenic in yelloweye mullet (Aldrichetta forsteri) following oral administration of organoarsenic compounds and arsenate. *Sci. Tot. Environ.* 79, 1, 59–67.

[113] Rumpler, A. **2010**. *Synthetic adn analytical apporaches to naturally occuring arsenic and selenium compounds*. PhD Thesis, University of Graz.

[114] Francesconi, K. A., Edmonds, J. S., and Stick, R. V. **1992**. Arsenic compounds from the kidney of the giant clam Tridacna maxima: Isolation and identification of an arsenic-containing nucleoside. *J. Chem. Soc., Perkin Trans. 1,* 11, 1349–1357.

[115] Madsen, A. D., Goessler, W., Pedersen, S. N., and Francesconi, K. A. **2000**. Characterization of an algal extract by HPLC-ICP-MS and LC-electrospray MS for use in arsenosugar speciation studies. *J Anal At Spectrom* 15, 6, 657–662.

[116] Raber, G., Stock, N., Hanel, P., Murko, M., Navratilova, J., and Francesconi, K. A. **2012**. An improved HPLC–ICPMS method for determining inorganic arsenic in food: Application to rice, wheat and tuna fish. *Food Chem.* 134, 1, 524–532.

[117] Maher, W. A., Foster, S., Krikowa, F., Duncan, E., St John, A., Hug, K., and Moreau, J. W. **2013**. Thio arsenic species measurements in marine organisms and geothermal waters. *Microchem. J.* 111, 82–90.

[118] World Health Organization (WHO). **2000**. *Air Quality Quidelines for Europe - Second Edition*. *Chapter 6.12 - Vanadium*, Copenhagen, Denmark.

[119] Farías, S. S., Casa, V. A., Vázquez, C., Ferpozzi, L., Pucci, G. N., and Cohen, I. M. **2003**. Natural contamination with arsenic and other trace elements in ground waters of Argentine Pampean Plain. *Sci. Tot. Environ.* 309, 1-3, 187–199.

[120] World Health Organization (WHO). **1998**. *Environmental Health Criteria 204*. *Boron*. World Health Organization, Geneva.

[121] World Health Organization (WHO). **2011**. *Guidelines for Drinking-water Quality*. World Health Organization, Geneva.

[122] EFSA Panel on Contaminants in the Food Chain (CONTAM). **2009**. Scientific Opinion on uranium in foodstuffs, in particular mineral water. *EFSA J.* 1018, 10, 1–59.

[123] Frank, A., Madej, A., Galgan, V., and Petersson, L. R. **1996**. Vanadium poisoning of cattle with basic slag. Concentrations in tissues from poisoned animals and from a reference, slaughter-house material. *Sci. Tot. Environ.* 181, 1, 73–92.

[124] World Health Organization (WHO). **1988**. *Environmental Health Criteria 81*. *Vanadium*. World Health Organization, Geneva.

[125] Costigan, M., Cary, R., and Dobson, S. **2001**. *Vanadium pentoxide and other inorganic vanadium compounds*. Concise International Chemical Assessment Document (CICAD) no. 29. World Health Organization, Geneva.

[126] Bishayee, A., Waghray, A., Patel, M. A., and Chatterjee, M. **2010**. Vanadium in the detection, prevention and treatment of cancer: The in vivo evidence. *Cancer Lett.* 294, 1, 1–12.

[127] Thompson, K. H., Lichter, J., LeBel, C., Scaife, M. C., McNeill, J. H., and Orvig, C. **2009**. Vanadium treatment of type 2 diabetes: A view to the future. *J. Inorg. Biochem.* 103, 4, 554–558.

[128] Gummow, B. **2011**. Vanadium: Environmental pollution and health effects. In *Encyclopedia of environmental health*, J. O. Nriagu, Ed. Elsevier Science, Amsterdam, London, 628–636.

[129] Sabbioni, E., Kuèera, J., Pietra, R., and Vesterberg, O. **1996**. A critical review on normal concentrations of vanadium in human blood, serum, and urine. *Sci. Tot. Environ.* 188, 1, 49–58.

[130] Kucera, J., Byrne, A. R., Mravcova, A., and Lener, J. **1992**. Vanadium levels in hair and blood of normal and exposed persons. *Sci. Tot. Environ.* 115, 3, 191–205.

[131] Iyengar, G. V., Wolf, W. R., Tanner, J. T., and Morris, E. R. **2000**. Content of minor and trace elements, and organic nutrients in representative mixed total diet composites from the USA. *Sci. Tot. Environ.* 256, 2-3, 215–226.

[132] Turconi, G., Minoia, C., Ronchi, A., and Roggi, C. **2009**. Dietary exposure estimates of twenty-one trace elements from a total diet study carried out in Pavia, Northern Italy. *Br. J. Nutr.* 101, 8, 1200–1208.

[133] Bocio, A., Nadal, M., and Domingo, J. L. **2005**. Human exposure to metals through the diet in Tarragona, Spain: Temporal trend. *Biol. Trace Elem. Res.* 104, 3, 193–201.

[134] EFSA Panel on Contaminants in the Food Chain (CONTAM). **2012**. Scientific opinion on the risk for public health related to the presence of mercury and methylmercury in food. *EFSA J.* 10(12):2985, 10, 1–199.

[135] Commission of the European Communities. **2006**. Commission Regulation (EC) No 1881/2006 of 19 December 2006. setting maximum levels for certain contaminants in foodstuffs. *Off. J. Euro.Union,* L 364, 5–24.

[136] Aland, A., Lidfors, L., and Ekesbo, I. **2002**. Diurnal distribution of dairy cow defecation and urination. *Appl. Anim. Behav. Sci.* 78, 1, 43–54.

[137] Orr, R. J., Griffith, B. A., Champion, R. A., and Cook, J. E. **2012**. Defaecation and urination behaviour in beef cattle grazing semi-natural grassland. *Appl. Anim. Behav. Sci.* 139, 1-2, 18–25.

[138] Khokiattiwong, S., Goessler, W., Pedersen, S. N., Cox, R., and Francesconi, K. A. **2001**. Dimethylarsinoylacetate from microbial demethylation of arsenobetaine in seawater. *Appl. Organomet. Chem.* 15, 6, 481–489.

[139] Schmeisser, E., Rumpler, A., Kollroser, M., Rechberger, G., Goessler, W., and Francesconi, K. A. **2006**. Arsenic fatty acids are human urinary metabolites of arsenolipids present in cod liver. *Angew. Chem. Int. Ed.* 45, 1, 150–154.

[140] Raber, G., Francesconi, K. A., Irgolic, K. J., and Goessler, W. **2000**. Determination of 'arsenosugars' in algae with anion-exchange chromatography and an inductively coupled plasma mass spectrometer as element-specific detector. *Fresen. J. Anal. Chem.* 367, 2, 181–188.

[141] Gailer, J. and Irgolic, K. J. **1994**. The ion-chromatographic behavior of arsenite, arsenate, methylarsonic acid and dimethylarsinic acid on the hamilton PRP-X100 anion-exchange column. *Appl. Organomet. Chem.* 8, 129–140.

[142] Olesik, J. W. and Moore Jr. , A. W. **1990**. Influence of small amounts of organic solvents in aqueous samples on argon inductively coupled plasma spectrometry. *Anal. Chem.* 62, 8, 840–845.

[143] Boorn, A. W., Cresser, M. S., and Browner, R. F. **1980**. Evaporation characteristics of organic solvent aerosols used in analytical atomic spectrometry. *Spectrochim. Acta B* 35, 11-12, 823–832.

[144] Schwedt, G. and Rieckhoff, M. **1996**. Separation of thio- and oxothioarsenates by capillary zone electrophoresis and ion chromatography. *J. Chromatogr. A* 736, 1-2, 341–350.

[145] Wilkin, R. T., Wallschläger, D., and Ford, R. G. **2003**. Speciation of arsenic in sulfidic waters. *Geochem. Trans.* 4, 1–7.

9 Appendix

Table 30: Concentrations of selected elements in water from Mercedes, Argentina, from lake Capri, Patagonia, Argentina (mean ± standard deviation [µg/L])

	As	V	Mo	U	B
well 1	41 ± 1	122 ± 1	13.9 ± 0.5	8.6 ± 0.9	600 ± 200
well 2	28.7 ± 0.7	98 ± 1	4.6 ± 0.2	5.7 ± 0.5	350 ± 120
well 3	54 ± 5	174 ± 1	38 ± 3	19 ± 2	1300 ± 400
well 4	9.7 ± 0.9	7.2 ± 0.4	0.4 ± 0.4	0.9 ± 0.1	170 ± 20
river	73 ± 3	163 ± 7	40 ± 1	17 ± 2	1100 ± 400
lake Capri	0.3 ± 0.1	1.7 ± 0.6	0.1 ± 0.1	0.2 ± 0.1	< 10

Table 31: Total arsenic concentrations [mean ± SD in µg/kg dm] in bovine livers and kidneys from Austria

Sample number	Liver	Kidney
1281	38 ± 1	210 ± 4
1283	22.6 ± 0.3	106 ± 9
1287	23 ± 1	114 ± 5
1288	67 ± 5	315 ± 17
1300	23 ± 1	106 ± 8
1306	57 ± 5	400 ± 80
1319	34 ± 1	290 ± 10
1320	170 ± 10	1390 ± 50
1322	75 ± 2	480 ± 30
1324	75 ± 2	291 ± 2

Table 32: Total arsenic concentrations in bovine livers and kidneys from Argentina

Sample number	Tissue type	Mean ± SD
BAR02R1	Kidney	500 ± 20
BAR04R1	Kidney	172 ± 3
BAR07R1	Kidney	198 ± 2
BAR09R1	Kidney	400 ± 10
BVR01R1	Kidney	240 ± 10
BVR02R2	Kidney	430 ± 40
BVR07R1	Kidney	202 ± 7
BVR20R1	Kidney	190 ± 20
BVR23R1	Kidney	250 ± 20
BVR25R1	Kidney	380 ± 10
BVR26R1	Kidney	240 ± 20
BVH05R1	Liver	137 ± 5
BVH06X1	Liver	25 ± 4
BVH18R1	Liver	150 ± 10
CFR01R1	Kidney	85 ± 4
CFR02R1	Kidney	193 ± 6
CFH03R1	Liver	53 ± 4
MOH03X1	Liver	100 ± 10
MOH04R1	Liver	115 ± 10
BAR02R1	Kidney	500 ± 20

Table 33: Total arsenic concentrations in urine from animals of the Schönbrunn Zoo (mean and standard deviation from triplicate digestions of each sample) and their specific gravities

Animal	Total arsenic [µg/L]	Specific gravity
Bison I	81 ± 1	1.032
Bison II	50.9 ± 0.5	1.036
Bison III	66 ± 4	1.024
Water buffalo I	76.9 ± 0.4	1.045
Water buffalo II	56.4 ± 0.4	1.025
Tux cattle I	12.1 ± 0.1	1.038
Tux cattle II	16.9 ± 0.1	1.037
Pustertal pied	14 ± 2	1.025
Himalayan thar	8.8 ± 0.2	1.044
Reindeer I	11.5± 0.3	1.026
Reindeer II	11.7 ± 0.2	1.023
Nilgai	15.9 ± 0.3	1.039
Blackbuck	28.6 ± 0.2	1.046
Barbary sheep	5.9 ± 0.1	1.044
Pygmy goat	7.2 ± 0.2	1.044
Heidschnucke	6.1 ± 0.3	1.037
Giraffe	5.1 ± 0.1	1.012
barbary macaque	9.3 ± 0.2	1.016
Vicuña I	43.4 ± 0.6	1.034
Vicuña II	63.2 ± 0.2	1.046
Collared peccary	31.9 ± 0.4	1.012
Damara zebra	68 ± 3	1.02
Indian rhinoceros	60.9 ± 0.6	1.032
African elephant I	22.5 ± 0.2	1.024
African elephant II	12.0 ± 0.1	1.020
African elephant III	18 ± 2	1.023
African elephant IV	15.5 ± 0.4	1.027
African elephant V	11.0 ± 0.2	1.006
African elephant VI	5.1 ± 0.2	1.004

TU Graz
Graz University of Technology
Senat

Deutsche Fassung:
Beschluss der Curricula-Kommission für Bachelor-, Master- und Diplomstudien vom 10.11.2008
Genehmigung des Senates am 1.12.2008

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, am 31.03.2014 .....

.....
(Unterschrift)

Englische Fassung:

STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

31.03.2014
.....
date

.....
(signature)