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Determination of lipid-soluble arsenic species in seaweed-eating sheep from Orkney

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This work is part of an ongoing research study towards an understanding of the complete metabolism of arsenosugars in mammalian organisms when ingesting seaweed, using the North Ronaldsay (NR) sheep as a model organism. We focus on the analysis of only those arsenic species bound to the lipids of the feed (*Laminaria digitata*), faeces and the tissues of the NR sheep using a novel enzymatic hydrolytic method that is simple and reliable. This rare breed of sheep, found in the remote Orkney Islands in the north of Scotland, live the entire year on the beaches and eat seaweed that is washed ashore (up to 3 kg daily).

Previous studies on arsenic fractionation in muscle, kidney and liver tissues revealed that most of the arsenic is concentrated in the fat fractions of these tissues (muscle fat: 61%; liver fat: 66%; kidney fat: 25%) rather than in the non-lipid fractions. Hence, this study was undertaken in order to determine the arsenic species bound to lipids in the muscle, kidney and faeces of NR sheep and to compare these with the arsenic species bound to the lipids of the *L. digitata* consumed.

The enzymatic hydrolytic procedure has been successfully employed for the first time to cleave the arsenic species cleanly from the rest of the lipid structure. This makes the arsenic species water soluble and enables their direct determination by high-performance liquid chromatography coupled with inductively coupled plasma mass spectrometry. Dimethylarsinic acid (DMA(V)) and monomethylarsonic acid (MA(V)) were found to be the major hydrolysed arsenic species bound to the kidney and muscle lipids, whereas arsenosugar-1 was found to be the major hydrolysed arsenic species in *L. digitata* lipids. On the other hand, DMA(V) was found to be the major arsenical obtained after the enzymatic hydrolysis of the faeces lipids. These results seem to suggest that both direct absorption and biotransformation of the absorbed organoarsenicals are the likely reasons for their occurrence and accumulation in the NR sheep tissues.

KEYWORDS: arsenosugars; sheep; seaweed; enzymatic hydrolysis determination

INTRODUCTION

North Ronaldsay (NR) sheep are a unique breed of seaweed-eating sheep found in the most northern island of the Orkney Islands of Scotland. This rare breed of sheep feed for most of the year on seaweed that is washed ashore except during lambing time, when they are fed on grass for 3 to 4 months. It is well known that marine brown and red algae accumulate large amounts of elements such as arsenic, as much as 100 mg kg^{-1} dry weight. The majority of this arsenic is present as arsenofuranoribosides, commonly known as arsenosugars.[1–3]

Our previous studies have shown that the NR sheep receive, on average, a body burden of 35 mg arsenic daily, 85% of which is in the form of arsenosugars.[4] From a controlled feeding trial the excreted total arsenic in the faeces represented 13% of the total consumed, which means that more than 86% of the arsenic is retained and bioavailable. Total arsenic content in wool, blood, liver, kidney and urine were elevated by almost a factor of 100 when compared with non-exposed sheep.[5] This, however, does not pose any risk to consumers of the tissue, as the levels of arsenic do

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not reach the maximum allowed arsenic level in foodstuffs in accordance with the UK guideline (1 mg of arsenic per kilogram). Fractionation studies of arsenic in kidney, muscle and liver tissues showed that significant amounts of the arsenic were concentrated in the fat fraction (i.e. hexane extracts) of these tissues rather than in the non-lipid fractions (muscle fat: 61%; kidney fat: 25%; liver fat: 66%). Consequently, tissues with higher amounts of fat showed higher total arsenic concentration.[6] This fractionation of different classes of compounds by using different types of solvent, which is a standard approach in the identification of organic natural products, has, however, not often been used for arsenic speciation.[7]

In this study, therefore, we focus on the determination of the arsenic species bound to the lipids of feedstuff (seaweed) and two tissues of the NR sheep. Arsenic species in the lipids of kidney and muscle and in faeces were investigated and these were compared with the arsenic species present in the lipids of *Laminaria digitata*, the major algae consumed by the NR sheep.[8] This would indicate whether the organoarsenic compounds present in the NR sheep tissues are actually metabolized products (biotransformation pathway) or whether they are accumulated as such from the seaweed lipids (direct-absorption pathway).

Relatively little is known about lipid-soluble arsenic compounds. Certain strategies for their synthesis have been discussed[9–11] and their toxicity towards cancer cells was tested.[12] However, these are a relatively new class of arsenic compounds discovered amongst the host of other arsenicals that are water soluble and which already exist in the environment. So far, only two lipid-soluble arsenicals have been completely identified, namely phosphatidylarsenocholine (Fig. 1) and phosphatidylarsenosugar in marine animals,[13,14] as well as the latter in the brown alga, *Undaria pinnatifida*.[15] Recently,

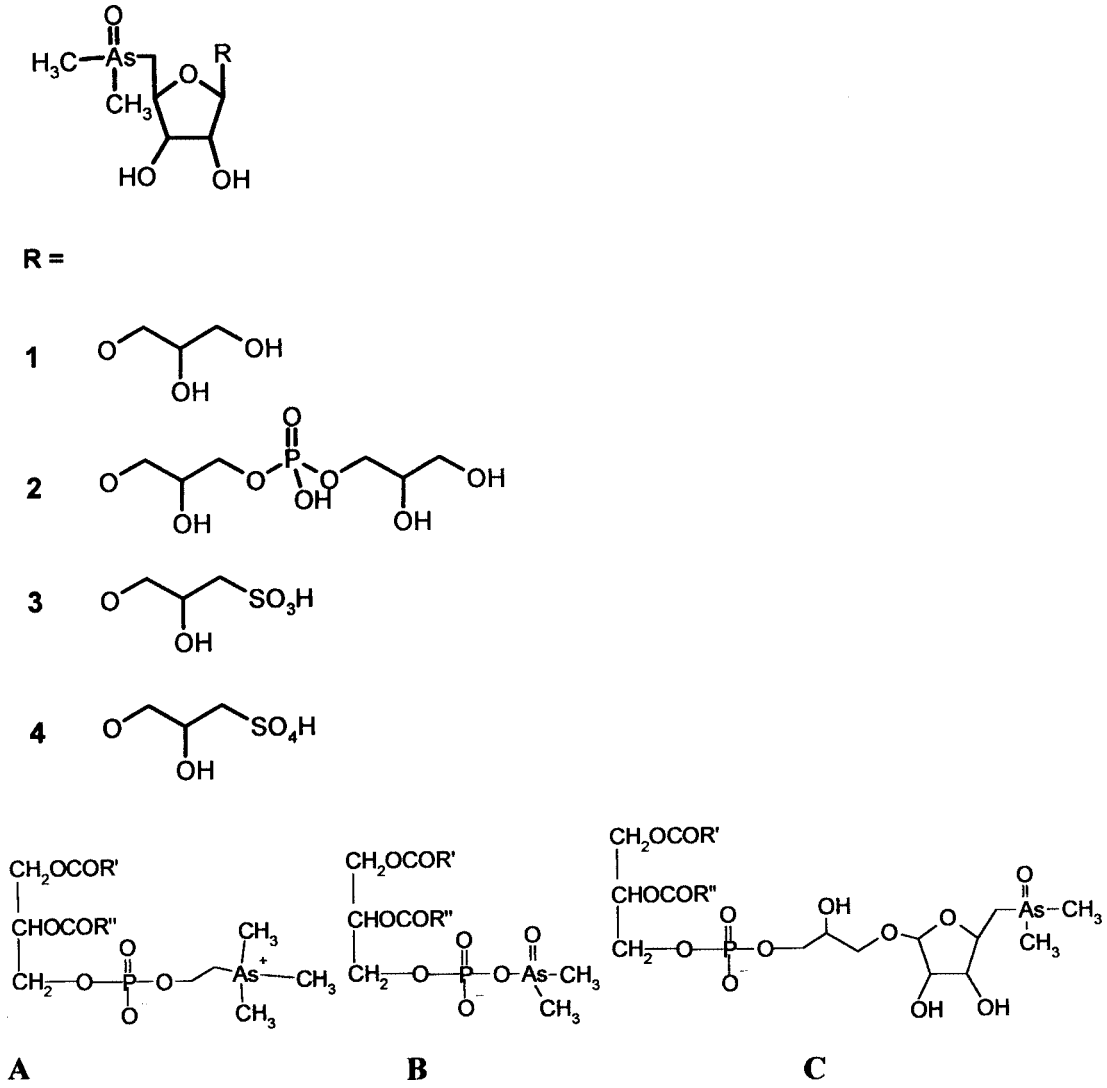

Figure 1. Structures of some arsenosugars (sugar-1, sugar-2, sugar-3, sugar-4) and three arsenolipids: **A**, phosphatidyl arsenocholine; **B**, phosphatidyl dimethylarsenic acid; **C**, phosphatidyl-arsenosugar.

dimethylarsinic acid (DMA(V)) has been identified in a star spotted shark as a major water-soluble part of a lipid-soluble arsenic-containing compound, which has been hydrolysed using mild alkaline conditions.[16] At present, the determination of the lipid-soluble arsenic compounds is a challenge because, being lipophilic, quantitative analysis of these compounds cannot be carried out directly by many of the routinely employed analytical techniques, such as high-performance liquid chromatography (HPLC) with an ion-exchange column using aqueous-based mobile phases coupled directly to inductively coupled plasma mass spectrometry (ICP-MS) as an arsenic-specific detector. In addition, no suitable standards of the lipid-soluble arsenic compounds are available yet. This necessitates the use of hydrolytic procedures on these compounds so as to make them water soluble for their analyses. Sequential chemical hydrolytic procedures have so far been employed for the hydrolysis and subsequent analysis of the arsenic lipids by HPLC–ICP-MS techniques. These procedures first involved a mild alkaline hydrolysis of the arsenic lipids, which deacylates the ester linkages at the 1- and 2-positions of the phospholipids. The glycerylphosphoryl arseno compounds thus obtained are water soluble. The glycerylphosphoryl arseno compounds were further subjected to strong acidic hydrolysis to obtain the arsenic species bound to them. Thus, by means of fractionation using sequential chemical hydrolytic procedures, the structure or at least the type of lipid to which the arsenic species are bound were determined.

In this study, we have for the first time used an enzymatic hydrolytic procedure successfully, using phospholipase D enzyme in order to cleave the arsenic species cleanly from the rest of the lipid structure to enable its subsequent analysis.

EXPERIMENTAL

Reagents and standards

All reagents used were of analytical reagent grade. Arsenic species stock solutions of 1000 ng ml^{-1} were prepared in deionized water. As the arsenosugar standard, the water-extractable fraction of the CRM material *Fucus* spp. (IAEA 140) containing mainly sugar-1, sugar-3, sugar-4 and DMA(V) was used; the quantitative results have been reported elsewhere.[17] Suitable aliquots of these solutions were diluted to obtain the working standards. The phospholipase D enzyme (2500 units activity) was purchased from Sigma. A 6 units ml^{-1} working solution was prepared, from which 2 ml was used each time for the hydrolysis of the arsenic lipids.

Samples

L. digitata was a stormcast seaweed collected from the shores of northeast Scotland, near Aberdeen, shortly after a storm. The seaweed was air dried and then homogenized with a mortar and a ball mill. The tissue samples were extracted from one NR sheep that lived its entire life, wild, on the shores of the island with its main diet as seaweed. The faeces were collected during the controlled feeding trial in which the sheep were fed only *Laminaria* spp.

Extraction and purification of lipids

Lipids from the kidney, muscle and faeces of the NR sheep were extracted by homogenizing the tissue sample two or three times with a 2:1 solvent mixture of chloroform/methanol. Various contaminants, such as amino acids, urea, sugars and salts, that extract along with the lipids need to be removed. Purification was carried out by shaking the combined solvent mixture with a quarter its volume of 0.88% KCl solution and allowing it to stand overnight for the complete separation of the two phases. The upper phase was discarded and the lower phase containing the purified lipids was evaporated to dryness in a rotavapour; this phase was weighed and stored in small amounts of chloroform at −20 °C until further analysis.[18] For the extraction of lipids from the algae (*L. digitata*), the sample was freeze-dried and powdered. A known weight of this powder was taken and the same procedure as above was followed, but this time using a sonication bath instead of a homogenizer to extract the lipids effectively into the organic phase.

Separation of lipids into simple and complex lipids

In order to determine whether the organic arsenic species belong to the category of simple lipids (neutral) or complex lipids (polar), the lipids of kidney and muscle were separated into the two fractions by a procedure involving adsorption on silicic acid followed by separate washings with chloroform and methanol to obtain simple and complex lipids respectively.[13] The total arsenic in each of the separated fractions was determined by ICP-MS analysis following careful digestion with an H_2SO_4–HNO_3–H_2O_2 acid mixture.[13]

Enzymatic hydrolysis of lipids

Since the arsenic lipids are not water soluble, they cannot be determined directly by the HPLC–ICP-MS technique. The arsenic species were therefore cleaved from the rest of the lipid molecule using phospholipase D enzyme solution for their subsequent analysis. The lipids extracted from each of the tissues were shaken vigorously for about 6 h with phospholipase D enzyme solution (12 units) in the presence of Ca^{2+} ions (0.25 mM) at pH 5.6 (0.125 mM NaOAc). The solution was then adjusted to pH 2.5 with glacial acetic acid. The resulting mixture was shaken with a 2:1 chloroform/methanol solvent mixture three or four times, or until the upper phase was clear, in order to remove phosphatidic acid and other organic substances. The aqueous phase was then evaporated to dryness in a rotavapour at room temperature, dissolved in small amounts of double-distilled water and analysed for the arsenic species by HPLC–ICP-MS.

Arsenic speciation methods

The arsenic species formed as a result of enzymatic hydrolysis of the lipid-soluble arsenicals were analysed by

HPLC–ICP-MS using both cationic and anionic exchange HPLC columns. The high-performance liquid chromatograph (Spectra Physica 400 p) was coupled directly to the ICP mass spectrometer (Spectromass 2000). A sample loop of 20 µl was used. The standard configuration of Spectromass 2000 was optimized for the detection of transient signals and m/z 75, 77 and 82 were monitored for arsenic, selenium and possible chloride interference. Chromatographic columns: PRP-X100 anion-exchange column (250 mm × 4.6 mm) with a Hamilton PEEK pre-column (11.2 mm, 12–20 µm) from Hamilton Company; Supelcosil LC-SCX cation-exchange column (250 mm × 4.1 mm). The mobile phase for anion-exchange chromatography was 30 mM phosphate buffer adjusted with aqueous ammonia to pH 6.0. The mobile phase for cation-exchange chromatography was a solution of 20 mM pyridine in water adjusted to pH 3.0. Chromatographic separations of relevant arsenic standards are shown in Fig. 2.

RESULTS AND DISCUSSION

The quantification of the total lipids shows variable proportions of extractable lipids in different samples (Table 1). The fatty inclusions from the neck muscle contain almost quantitative amounts of extractable lipids, whereas

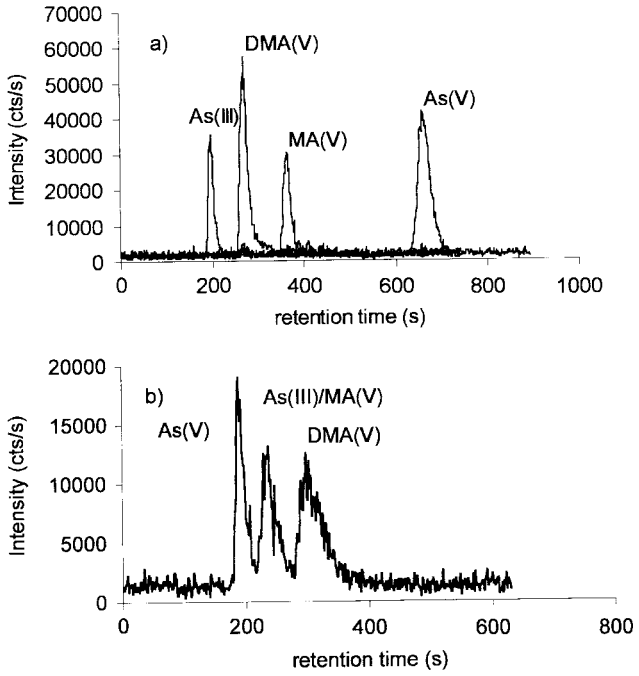

Figure 2. Arsenic standards measured by using (a) strong anion exchange (Hamilton PRP x-100, 30 mM ammonium phosphate, pH 6.0) chromatography coupled to ICP-MS and (b) strong cation exchange (Supelcosil, 20 mM pyridine, pH 3.0) LC–ICP-MS.

Table 1. Total extractable lipids from the different tissues in % of total mass ($n = 10$)

Tissue	Average amount extracted (%)	Standard error (%)
NR sheep kidney	5.0	1.0
NR sheep neck muscle (fatty inclusions)	89.3	9.4
NR sheep faeces	1.2	0.3
L. digitata	1.4	0.8

the kidney and, in particular, the seaweed and the faeces have only minor amounts of lipids.

An enzymatic hydrolytic procedure involving the use of a phospholipase D enzyme solution has been used in the present study to cleave the arsenic species from the rest of the lipid moiety for its subsequent analysis. Enzymatic hydrolytic procedures are commonly employed by lipid chemists for the structural identification of lipids.[19] Owing to the ability of lipolytic enzymes to distinguish between certain types of bonds in complex lipids and hydrolyse them selectively, it was envisaged that this could be used advantageously in our study for the determination of arsenic species bound to the lipids. Because the literature so far reports the findings of only arsenic species bound to the phospholipids, we have chosen phopholipase D enzyme in our present study. This is understandable because the arsenic species require a polar head to bind to the lipids which is available only in complex lipids and not in simple lipids. This enzyme also acts on another complex lipid, viz. the sphingolipids. Phosphatidic acid and arsenic species were obtained upon hydrolysis of the phospholipid using phospholipase D enzyme solution, as shown in Fig. 3. By this method, not only can the arsenic species be identified, but it can also be inferred that the corresponding lipid is a complex lipid (phospholipid or a sphingolipid), as phospholipase D enzyme acts selectively on only these lipids. This was confirmed for the muscle and kidney lipids by determining the total arsenic content in each of the separated fractions of simple (neutral) and complex (polar) lipids (Table 2). It can be seen from Table 2 that the complex lipids, which constitute less than 10% of the total lipid fraction, contain all the arsenic species. The results of the analysis of the arsenic species obtained after the enzymatic hydrolysis of kidney and muscle lipids are shown in Fig. 4.

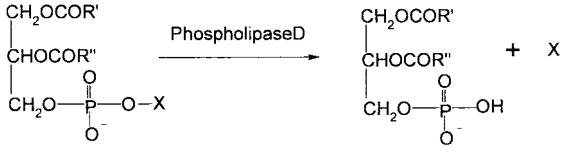

Figure 3. Enzymatic hydrolysis of phospholipid (X = arsenic species).

Table 2. Quantification of lipid-soluble arsenic species in the tissues of NR sheep in % of total lipid soluble arsenic

Lipid type	Proportion extracted (%)	Arsenic (μg kg^{-1})	
		Muscle	Kidney
Simple lipid	91.3 ± 0.4	<d.l.	<d.l.
Complex lipid	6 ± 5	6180	256

d.l.: detection limit (0.5 μg kg^{-1}).

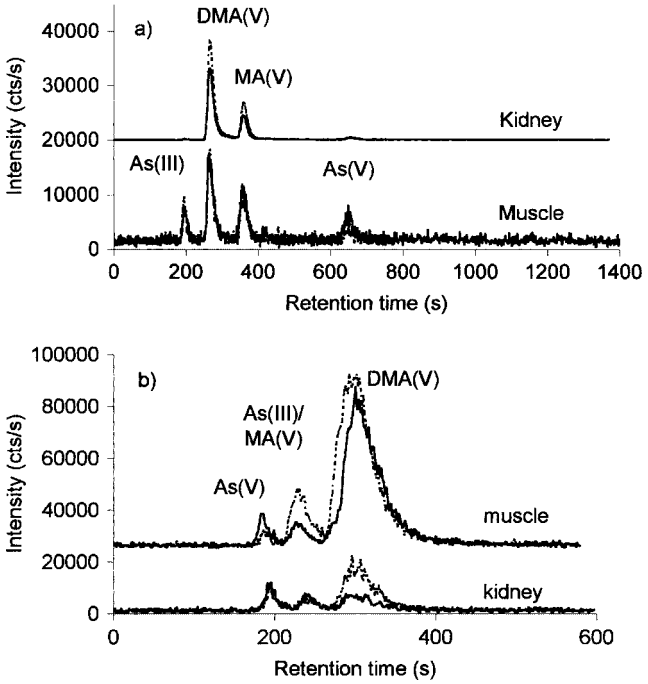

Figure 4. (a) Anion exchange HPLC–ICP-MS (*m/z* 75) and (b) cation exchange HPLC–ICP-MS of the hydrolysed complex lipids from the kidney and the fat inclusions of the neck muscle of the NR sheep. The dashed lines are the samples spiked with standards. Kidney was spiked with DMA(V) and MA(V), whereas the muscle extract was spiked with MA(V).

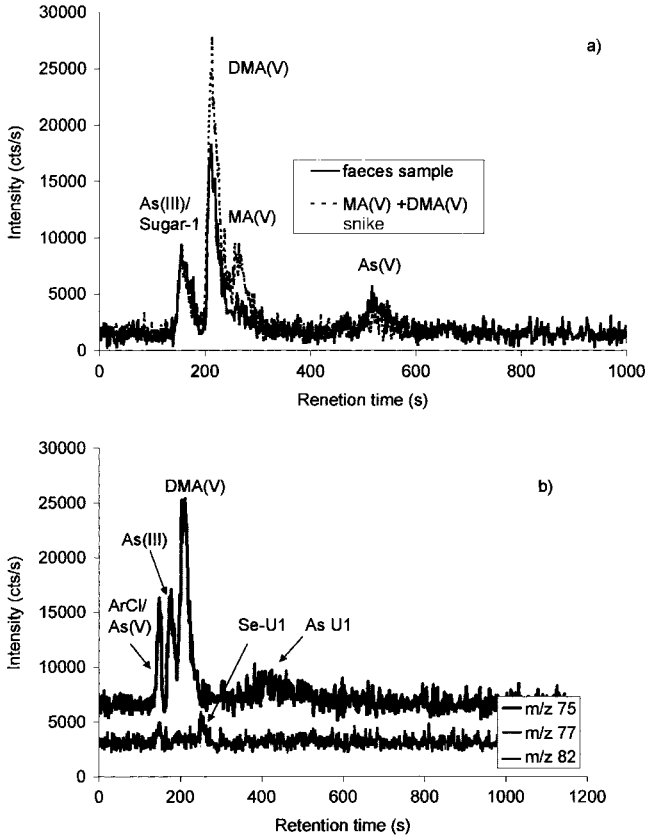

Figure 5. (a) Anion exchange HPLC–ICP-MS and (b) cation exchange HPLC–ICP-MS of the hydrolysed faeces of the NR sheep. Cation-exchange chromatogram of hydrolysed faeces lipid. Here, both arsenic (*m/z* 75) and selenium (*m/z* 77, 82) are shown in order to indicate the occurrence of a chloride interference and possibly a selenium-containing hydrolysed lipid.

The four peaks obtained with the anion-exchange column correspond to arsenic(III) and/or arsenosugar (sugar-1), DMA(V), methylarsonic acid (MA(V)) and arsenic(V), based on their matching retention times with those of standards and spiking studies (Fig. 3). The first peak corresponds to sugar-1 and/or arsenic(III), because they cannot be separated under these conditions; they elute within 10 s of each other. The cation-exchange chromatography, however, confirms the absence of sugar-1. In both tissues, traces of arsenate have been found.

From the analysis of chromatograms of both anion- (Fig. 5a) and cation-exchange (Fig. 5b) columns of the hydrolysed faeces lipids put together, it can be seen that DMA(V) is the main arsenic species. Only traces of an unknown species and arsenic(V) and arsenic(III) could be identified. Interestingly, there is a small signal for selenium in this phase, indicating that a lipid-soluble selenium compound is present in the faeces. This, however, needs further confirmation. In the hydrolysed lipid fraction of the main seaweed eaten by the NR sheep (Fig. 6), sugar-1 was found to be the major component, with DMA(V) being the minor component and traces of arsenic(V). It should be mentioned that the blanks, measured without the addition of the enzyme, do not contain sugar-1. Hence, sugar-1 has been bound to a lipid and is not a carry over from the water-soluble sugar-1 into the chloroform–methanol fraction.

Owing to the fact that the main seaweed consumed by the NR sheep contains DMA(V) bound to lipids, and since this was also found to be present in the lipids of kidney and muscle of the NR sheep, it is likely that these lipids have been absorbed directly by the gut and stored in the fatty tissues of the sheep. The sugar-1 bound to lipids has not been detected

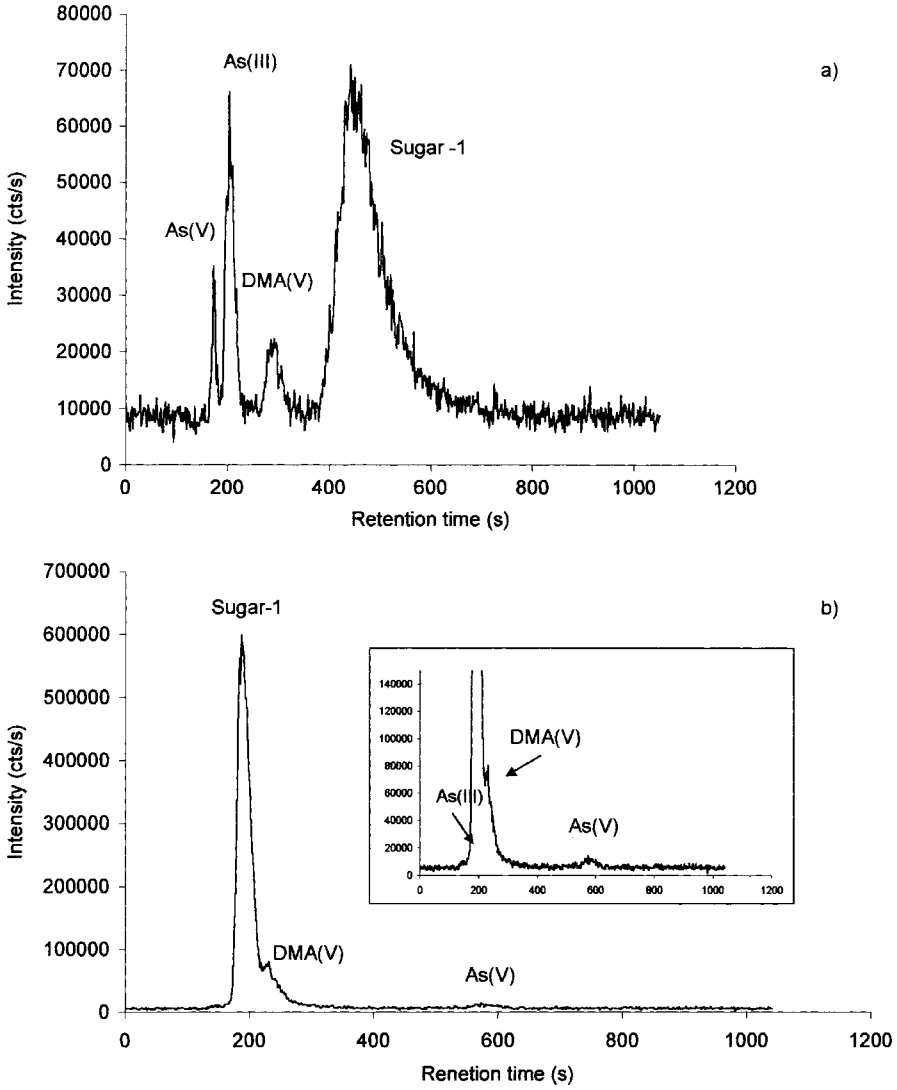

Figure 6. (a) Cation-exchange chromatogram of hydrolysed *L. digitata* lipids; (b) anion-exchange chromatogram using HPLC–ICP-MS. The trace shown is *m/z* 75.

in the lipids of the muscle and kidney or in the faeces of the NR sheep, although this was the major arsenic species in the hydrolysed lipid fraction of the seaweed. This means that this compound has been absorbed and metabolized. In contrast, however, DMA(V) bound to lipids has been found in large quantities in the faeces and to a lesser extent in the seaweed, but a mass balance cannot say whether the absorption of the DMA(V) bound to lipid is not as efficient as that of the sugar-1 bound to lipid, or whether biotransformation of the absorbed arsenic lipid is also taking place in the sheep body. This needs to be investigated in a detailed feeding experiment in a metabolic chamber. Further studies have to be carried out to determine the complete structure of the lipid part, including those of the fatty acid compositions. However, considering that sugar-1 was obtained as the major hydrolysed product in the lipids of *L. digitata* with the use of phospholipase D enzyme, which is highly selective to specific bonds in the complex lipids, the structure of the corresponding lipid part is most likely to be a phospholipid, as shown in Fig. 1. Similarly, the structure of the DMA(V) bound to lipids found as the major component in faeces lipids, and as a minor component in muscle, kidney and *L. digitata* lipids, is most likely to be a complex lipid, as shown in Fig. 1.

CONCLUSIONS

We report the findings obtained in the analysis of arsenic species bound to the lipids of NR sheep tissues as well as in the lipids of *L. digitata* using a novel enzymatic hydrolytic procedure that is both simple and reliable. Phospholipase D enzyme solution has been used to cleave the arsenic species from the lipid part for its subsequent analysis by HPLC–ICP-MS with both cationic- and anionic-exchange

columns complementary to one another. The arsenic species bound to the lipids of kidney, muscle and faeces of the NR sheep have been compared with those bound to the lipids of *L. digitata*, the major alga consumed by the NR sheep. Arsenosugar (sugar-1) was found to be the major arsenic species bound to the lipids of *L. digitata*, whereas DMA(V) was the major arsenic species bound to lipids in the kidney and muscle of the NR sheep and in their faeces. These studies seem to suggest that both biotransformation and direct absorption of the arsenic lipids are taking place in the sheep body. All the arsenic species were bound to the complex lipids. This study is important not only for understanding the metabolism of arsenosugars in the sheep body, but also in view of the growing concern due to the increasing number and toxicities of various arsenic species constantly being identified in the environment.

Acknowledgements

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REFERENCES

1. McSheehy S, Szpunar J. *J. Anal. At. Spectrom.* 2000; **15**: 79.
2. Pergantis SA, Wangkarn S, Francesconi KA, Thomas Oates JE. *Anal. Chem.* 2000; **72**: 367.
3. Francesconi KA, Edmonds JS. *Adv. Inorg. Chem.* 1997; **44**: 147.
4. Hansen HR, Raab A, Francesconi KA, Feldmann J. *Env. Sci. Technol.* 2003; **37**: 845.
5. Feldmann J, John K, Pengprecha P. *Fresenius J. Anal. Chem.* 2000; **368**: 116.
6. Feldmann J, Balgert T, Hansen H, Pengprecha P. In *Plasma Source Mass Spectrometry*, Tanner S, Holland G (eds). *Royal Society of Chemistry Special Publications*, Number 267. RSC: Cambridge, 2001; 380.
7. Francesconi KA. *Appl. Organmetal. Chem.* 2003; **17**: 682.
8. Hansen HR, Hector B, Feldmann J. *Anim. Feed Sci. Technol.* 2003; **105**: 21.
9. Kordalis NL, Ioannou PV. *Appl. Organmetal. Chem.* 2000; **14**: 273.
10. Ioannou PV. *Appl. Organmetal. Chem.* 2000; **14**: 261.
11. Fatouros D, Gortzi O, Klepetsanis P, Antimisiaris SG, Stuart MCA, Brisson A, Ioannou PV. *Chem Phys. Lipids* 2001; **109**: 75.
12. Gortzi O, Antimisiaris SG, Klepetsanis P, Papadimitriou E, Ionnou PV. *Eur. J. Pharm. Sci.* 2003; **18**: 175.
13. Hanaoka K, Kaise K, Irgolic JK. *Appl. Organometal. Chem.* 1999; **13**: 65.
14. Edmond JS, Shibata Y, Francesconi KA, Yoshinga J, Morita M. *Sci. Total Environ.* 1992; **122**: 321.
15. Morita M, Shibata Y. *Chemosphere* 1988; **17**: 1147.
16. Hanaoka K, Tanaka Y, Nagata Y, Yoshida K, Kaise T. *Appl. Organmetal. Chem.* 2001; **15**: 299.
17. Castelhouse H, Smith C, Raab A, Meharg AA, Feldmann J. *Environ. Sci. Technol.* 2003; **37**: 951.
18. Ways P, Hanahan DJ. *J. Lipid Res.* 1964; **5**: 318.
19. Christie WW. *Lipid Analysis*, 2nd edn. Pergamon Press: 1982; 155–166.

