

Monitoring the Arsenic and Iodine Exposure of Seaweed-Eating North Ronaldsay Sheep from the Gestational and Suckling Periods to Adulthood by Using Horns as a Dietary Archive

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Trace elements often accumulate in keratin-rich tissues. Hair, nails, and horns grow steadily but once formed are metabolically inactive and provide an archive of trace element exposure when analyzed in segments. Here we demonstrate the use of laser ablation ICP–MS for the high-resolution monitoring of trace elements in the horns of seaweed-eating sheep from North Ronaldsay, which live on grass only during lambing time. Due to this peculiar husbandry/dietary pattern and the fact that seaweed is rich in arsenic and iodine, we hoped to use iodine and arsenic as markers for seaweed ingestion. Cross sections and scans along the growing axis (representing the first 8–10 months of the sheep's life) revealed that these elements were not homogeneously distributed in the horn, with arsenic representing the amount of seaweed intake. The scans show the periods in which the lambs were fed on milk and grass and the change to seaweed ingestion with the successive replacement of milk with seaweed; this was supported by the carbon and nitrogen isotope signatures ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of the horn and the arsenic speciation in the horn. The period of low arsenic accumulation in the horn had terrestrial isotope signatures and accumulated arsenic of mainly inorganic origin. The period of high arsenic accumulation was characterized by isotope signatures of marine origin, and the majority of accumulated arsenic in the horn was the main arsenosugar metabolite dimethylarsinic acid. Although we have investigated only four different horns of individual sheep, this study shows that arsenic is not significantly transported with milk. However, the high concentration of arsenic in the oldest part of the horn, which was formed in utero, points to a relatively high placental transport of arsenic while the ewe was eating seaweed. In contrast to arsenic, iodine is transported

not only through milk ingestion but also through the placenta in large quantities.

Introduction

Monitoring environmental exposure to toxic elements such as arsenic is often done by measuring the arsenic concentration in blood or urine of mammals or measuring the elemental concentrations in organs. All these measurements are however snapshots and are only representative of one moment in time. There is however an urgent need for a tool which could be used as a historic archive of the chronological exposure to trace elements. Hair has often been proposed as a record keeping tissue but suffers from external contamination, which cannot be removed by any sample preparation process (1). Here we propose to use horns for the monitoring of arsenic and iodine exposure in sheep. The horn is mainly made of keratin, and arsenic is supposed to accumulate in keratin, a sulfhydryl-rich tissue. The accumulation of iodine in keratin tissues is unknown. The keratin fiber is formed in the epidermal cells, and during this time there is the possibility of arsenic or iodine inclusion, transported through the blood. Moreover, the horn growth starts before birth and grows continuously over the years, generating horn material characteristic of the metabolic status of the animal at the time of keratin formation.

As proof of this concept we studied the exposure of lambs from seaweed-eating sheep from Orkney, Scotland to arsenic and iodine during gestation, suckling, and into early adulthood. This breed of sheep, a primitive breed, lives entirely on a seaweed-based diet. They roam the beaches of North Ronaldsay, the most northerly island of the Orkney Archipelago, and eat large amounts of seaweed, approximately 0.5 kg (d.w.) daily (2). The seaweeds consumed are mainly brown kelp (*Laminaria spp.*) (3), which contains on average 74 ± 4 mg/kg arsenic (2) and 5700 mg/kg iodine (4). Arsenic occurs mainly as arsenosugars (5), while iodine is mainly in the form of iodide in the *Laminaria spp.* (4). During the last weeks of gestation, the ewes are taken inland and set on grass. After birth the lambs are exposed to ewe's milk and grass for the next 5 months, until they are taken to the beach where the grass is replaced by the only food available—seaweed.

Here we provide the first evidence that horns can be used as an archive of trace element exposure in sheep. In particular we could identify that arsenic and iodine is transported through the placenta when the ewes are exposed to the seaweed, while no significant amount of arsenic is transferred to the lambs through milk in contrast to iodine. This knowledge has been acquired by using a series of state-of-the-art complementary analytical methods: laser ablation ICP–MS, arsenic speciation analysis using HPLC–ICP–MS, and C and N stable isotope ratio analysis. Laser ablation ICP–MS can be used as a microscale mapping tool for trace elements in biological samples (6), in order to identify the time-dependent accumulation of these elements with high temporal resolution in the horn. Since the growth rate and pattern of the horn is unknown, the time scale and events cannot be determined directly. Due to the previous knowledge that the ingested arsenosugars in seaweed are bioaccessible and metabolized to several organoarsenicals, mainly to arsenic species containing a dimethylarsenic moiety (5, 2, 7, 8), those species can be expected to accumulate in horns in a similar way as they did in the wool of the NR sheep.

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FIGURE 1. Inner surface of one of the North Ronaldsay Sheep's horn, used for laser ablation. Two different materials appear, in the cortex (A) and in the center (B) of the horn. The solid arrows indicate the scanned lines along the growth axis, while the dotted lines are the scanned cross sections.

Thus, they could be used as indicators for seaweed ingestion (9). Arsenic speciation analysis using gentle extractions of the arsenic species from the horn with the subsequent HPLC-ICP-MS analysis has been used successfully to give information about the accumulated arsenic species in the different part of the horn and could identify the arsenic coming from seaweed ingestion. Plants of marine origin have different C and N isotope signatures to terrestrial plants, which can result in a different C and N signature being carried over into the grazing animals. Such signatures can be recognized in the hair (10). Furthermore, the C and N stable isotope ratio has been used to characterize the dietary history of beef (11). Since these sheep change between marine seaweeds and terrestrial grass, so the change of diet should easily be identified in the horn as well. These different analytical approaches enabled us to assign the different phases of development and maturity and identify the extent and the pathway of the exposure to toxic arsenic and iodine.

Materials and Methods

Chemicals and Standards. The arsenic standards were prepared from sodium arsenite (Merck), sodium arsenate (Merck), dimethylarsinic acid, DMA(V) (Sigma chemicals), and methylarsonic acid, MA(V) (ChemService MC, West Chester UBA). Dimethylarsinothioic acid (DMAS) and the S-analogue of MA(V) were prepared as described in ref 12. The mobile phase was prepared from ammonium carbonate (BDH Chemicals). Human hair (NCS ZC 81002b) certified reference material was obtained from China National Analysis Center for Iron and Steel, China.

For the speciation analysis ammonium carbonate (GPR Grade, BDH Chemicals) and TMAH (tetramethyl ammonium hydroxide 25%, Aldrich) were used. 1000 mg/L high purity Standard (Charleston, SC) was used for iodine, while tellurium and arsenic were purchased as Aristar grade (BDH Chem.). Only deionized water (Elga, UK) was used throughout the experiment.

Description of Horn Samples. The horn is a complex matrix. It is formed by epidermal cells that create among others a protein called keratin. The keratinization process is purely a degenerative process through drying of degenerated and dead epithelial cells (13). By looking at the inner surface of the horn, two different materials clearly appear. The middle of the horn is made of a white soft and porous substance, while the cortex, the outer part of the horn, is made out of a brown strong and dense material (Figure 1). Although only

the tip of the horn was used, which should only contain dead cells and no connective tissue and blood vessels, the horn was scanned along the growth layer in the center (B) of the horn as well as at the cortex (A). The growth rate of the horn is in the first year under optimal nutritional conditions approximately between 19 and 25 cm at the outer curvature for rams and 11–12 cm for the ewes. Only the tips of the horns from individual ewes were used in this study which represent the first 8 months of their lives.

Horn Samples for Laser Ablation. Three horn samples from three different North Ronaldsay (NR) sheep, which lived in the traditional manner on NR (Orkney Archipelago, Scotland) were used in this study, and as a control one horn from a Pyrenees sheep (Pau, France), which has not been exposed to seaweed. In order to analyze the horns with the laser, the horn's surface had to be smooth and flat. Hence, the horns were cut in two equal parts lengthwise with a diamond saw and then in pieces of 2–3 cm in order to be analyzed by laser ablation using the standard ablation cell from CETAC Technologies (Omaha, U.S.A.). The inner surface was used for the laser ablation and was smoothed over with a microtome (Bright OTF Cryostat), using a carbon/tungsten blade.

Qualitative Arsenic and Iodine Determination by LA-ICP-MS. Laser Ablation. The horn samples were analyzed by laser ablation coupled to an inductively coupled plasma mass spectrometer (ICP-MS). The laser used was a Nd:YAG laser (CETAC LSX 200 +) with wavelength quadrupoled to 266 nm. After optimization for the best arsenic and iodine sensitivity, the analyzing conditions used for the laser were as follows: the energy level was set to 100% which is a fluence of 12.2 J/cm² at a pulse repetition rate of 20 Hz and a spot size of 200 μm if not stated otherwise. It was decided to use a scan in which the sample was constantly moved while the laser was pulsing at a constant rate, instead of a spot raster with definite ablation spots since the sensitivity and the time resolution as well as the analysis time was superior for the scan (14). The scan speed used was 50 μm/s for the analysis. In order to clean the surface, which will be analyzed, and remove the contaminants adsorbed at the surface, a pre-ablation scan at 200 μm/s was done the same way prior to the analysis. The ICP-MS used was an Agilent 7500c. In order to account for polyatomic interferences, additional to *m/z* 75 for arsenic and *m/z* 127 for iodine, *m/z* 77 and 82 for selenium and the polyatomic argon cluster ArCl⁺ were monitored. Carbon (*m/z* 13) was used as an internal standard to cancel out variations in ablation, transport, and ionization efficiency.

Mapping the Horn. A 2 D mapping is a very laborious process of these large samples. Thus, certain 1 D scans were performed in order to characterize the time and material dependent accumulation of trace elements in the horn. Scans in length from the tip of the horn (the oldest keratin fibers, corresponding to the first horn formation in the lamb's life, which occurs in utero before birth) to the bottom of the horn (corresponding to the newest growth layers, the last days of the sheep's life) will represent the time axis—two scans in the cortex of the horn (on the left part and on the right part) and one scan in the middle of the horn (Figure 1). Cross section scans from the outer surface on the left to the outer surface on the right of the horn through the middle of the horn will give a good account for the differences in the material.

Quantitative Arsenic and Iodine Determination. One of the three horns from the NR sheep was cut into pieces to determine total arsenic and iodine concentrations. Each piece was ground into powder by a ball mill and digested by adding 1 mL of TMAH to 0.1 g of horn in polypropylene vials. As a control, a piece of the Pyrenees sheep horn was digested in the same way. Then each solution was diluted with water,

and tellurium was added as an internal standard to a concentration of 10 $\mu\text{g/L}$. Solutions of external standard containing As, I, and Te were prepared in TMAH. Quantitative recovery was found for arsenic in the human hair CRM (NCSZC 81002b) with 0.20 ± 0.01 mg/kg and a certified value of 0.198 ± 0.023 mg/kg.

Arsenic Speciation Analysis in the Horn. Seaweed contains mainly arsenosugars, and these arsenic compounds are bioavailable and metabolize mainly to a series of arsenicals with the dimethylarsenic moiety in common. Our previous studies (9, 15) revealed that hot water extraction of wool and hair with subsequent analysis using ion exchange HPLC-ICP-MS can conserve this moiety. Thus, arsenic speciation analysis in horn can be used for the determination of arsenic metabolites from seaweed consumption. Briefly, horn extracts were prepared by using between 0.05 and 0.50 g (depending on the availability of the samples) added to 10 mL of deionized water and then placed in a microwave digestion unit (CEM, Mars5, U.S.) with a temperature-controlled heating program as follows: 35 °C at 600 W for 10 min, then 75 °C at 600 W for 10 min, and finally 95 °C at 600 W for 60 min. The extracts were filled into PP vials and stored at -20 °C until analysis. Prior to injection on the HPLC-ICP-MS the extracts were filtered through 0.45 μm membrane filter. The strong anion exchange column PRP-X100 (4.6 \times 250 mm) (Hamilton) was used and coupled online to the ICP-MS (SpectroMass2000, Spectro Analytics, Germany). The ICP-MS was run under normal operating conditions at a flow rate of 1 mL/min and was tuned with an arsenic standard solution before the HPLC was coupled to the nebulizer. As a mobile phase 3.50 g of ammonium carbonate in 1 L of deionized water was made up daily. A mixture of As(III), DMA(V), MA(V), As(V), and DMAS, the S-analogues of DMA(V), was used for quantification and species identification.

^{13}C and ^{15}N Stable Isotope Analysis. Stable isotope signatures of carbon and nitrogen are characteristic of certain plants and animals. The major differences can be seen in C3/C4 plants and plants of marine and terrestrial origin. Five samples of the North Ronaldsay sheep's horn, from the top to the bottom, a sample from the Pyrenees sheep as a control, and a sample of seaweed (*Laminaria hyperborean*) usually eaten by the sheep were cut and ground. ~1 mg samples of horn were used for continuous flow-isotope ratio mass spectrometry (CF-IRMS) analysis using a Europa Scientific ANCA-NT 20-20 Stable Isotope Analyzer with ANCA-NT Solid/Liquid Preparation Module (Europa Scientific Ltd., Crewe, U.K.). For these samples, containing around 10% N, the continuous flow-isotope ratio mass spectrometry (CF-IRMS) was operated in the dual isotope mode, allowing $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ measurements on the same sample. The analytical precision (SD, $n = 5$) was 0.2‰ for both N and C, estimated from standards analyzed along with the samples. Working standards were 1 mg of leucine prepared by freeze drying 50 μL of a 20 mg/mL stock solution into tin cups and calibrated against 'Europa flour' and IAEA standards N1 and N2. For seaweed, containing less N, a single isotope analysis for $\delta^{15}\text{N}$, using larger samples, was required. The methods are fully described elsewhere (16). The isotope ratios are expressed in the standard delta notation (17):

$$\delta^{13}\text{C} = \left(\frac{(^{13}\text{C}/^{12}\text{C})_{\text{smp}}}{(^{13}\text{C}/^{12}\text{C})_{\text{std}}} - 1 \right) * 1000\text{‰}$$

$$\delta^{15}\text{N} = \left(\frac{(^{15}\text{N}/^{14}\text{N})_{\text{smp}}}{(^{15}\text{N}/^{14}\text{N})_{\text{std}}} - 1 \right) * 1000\text{‰}$$

Results and Discussions

Total Element Concentration in the Horn. Before the element distributions in the horns were measured, the horn

TABLE 1. Arsenic and Iodine Concentration (mg/kg) for Several Samples of the North Ronaldsay Sheep Horn and the Pyrenees Sheep Horn Measured by ICP-MS After TMAH Digestion^a

sample	arsenic (mg/kg)	iodine (mg/kg)
Pyrenées horn	0.07 \pm 0.01	0.4 \pm 0.6
NR horn 10-20 mm	0.16 \pm 0.01	43.8 \pm 0.1
NR horn 20-25 mm	0.19 \pm 0.01	48.9 \pm 0.1
NR horn 25-30 mm	0.28 \pm 0.01	51.0 \pm 0.8
NR horn 40-50 mm	2.2 \pm 0.1	52.9 \pm 0.2

^a The distance is given in mm from the tip oldest part of the horn.

samples were analyzed for their total element concentration using TMAH extraction and ICP-MS analysis in order to get a low-resolution distribution pattern (Table 1). Arsenic and iodine in the Pyrenees sheep were significantly lower than the five different horn samples from the NR sheep ($p < 0.001$, two-tailed Student's *t*-test). However, samples from the different parts of the horns from NR sheep showed a large variability. In the older part of the horn the arsenic levels were generally lower (0.16-0.28 mg/kg) than in the newer part of the horn (2.2 mg/kg), while the iodine level was much higher than that of arsenic but did not change dramatically (44-53 mg/kg). A horn from another NR sheep was analyzed at 80-90 mm and gave 4.9 ± 0.1 mg/kg arsenic and 16.6 ± 0.3 mg/kg iodine.

The low concentrations of arsenic correspond to the earlier stages of the lamb's life, while still grazing on a grass pasture. The middle section of the horn is characterized by an increase in arsenic to about 2.2 mg/kg and further to 4.9 mg arsenic per kg that might represent the gradual increase in seaweed-eating. For iodine the distribution pattern shows not much variation over the same time period in one horn (approximately 5 months); however, it shows lower levels of iodine in the newest part of the horn from another sheep. Whether these values indicate elevated levels or not cannot be determined since no reference values of arsenic and iodine in horn have been found and only one control sheep was used. The concentrations of both elements in the control sheep indicate that those elements are elevated in the horn of the NR sheep. However, it can be concluded that high-resolution microscale analysis is needed in order to utilize the horn as an archive for the trace element status of the lamb.

Mapping of the Horn. Internal Standardization. The efficiency of the ablation process during LA-ICP-MS measurements depends on the sample's interaction with the laser and the surface roughness as well as sample density. Therefore the data have to be normalized by using a continuous internal standard in order to normalize the intensities for the entire scanned surface. The concentration of the internal standard must not vary across the sample, so that the resulting intensity during the ablation/detection process can be used as an internal standard. Due to the abundance of carbon in biological samples, the minor isotope ^{13}C has often been used successfully as an internal standard for mapping purposes in soft tissue (6), while calcium is often used for hard tissues such as bones (18, 19). Since the ionization of carbon in the plasma of the ICP is expected to be different from that of the analytes it would be unwise to assume it can be used to completely cancel out any surface roughness or change of materials. However, when the ablation process is varied as indicated in the ^{13}C trace for the marked events in Figure S1 (Supporting Information), the resulting variability of arsenic, which is not due to the variability of the element concentration in the horn, can be cancelled out by normalization using the ^{13}C signal. However,

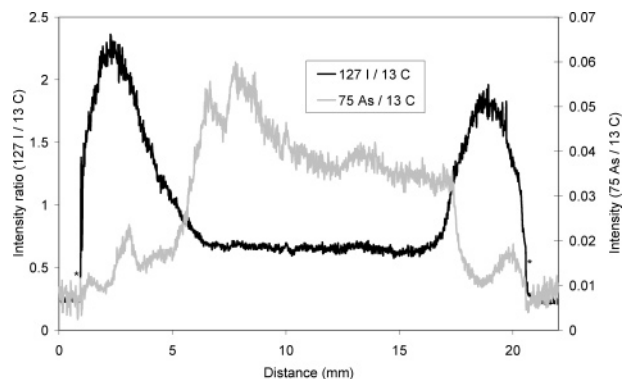


FIGURE 2. LA-ICP-MS scan of the horn orthogonal to the growth axis, iodine, and arsenic intensity (cps) rationed by the simultaneously acquired ^{13}C as a function of the distance in the horn (mm). Operation of the laser marked with (*).

if the sample is prepared properly, then a stable trace of ^{13}C throughout the scan can be observed with an RSD of 1.8% over a scanning length of several centimeters (Supporting Information Figure S2).

Cross Section of the Horn. The cross sections of the horn samples were ablated from the outer cortex through the middle section to the inner cortex (see Figure 1). The outer surface of the horn was not ablated in order to exclude the effect of surface contamination at the outside of the horn. The horizontal profiles have been made in order to check whether iodine and arsenic are homogeneously accumulated in the center and the cortex of the horn. The signals of the horn sample from the control sheep were about 0.0017 ± 0.0002 (cts/cts) for $^{75}\text{As}/^{13}\text{C}$ and 0.0030 ± 0.0003 for $^{127}\text{I}/^{13}\text{C}$ and relatively homogeneously distributed (Figure S3). Both elements in the horns of the NR sheep showed signals orders of magnitude above those of the control sheep from the Pyrenees. However, as shown in Figure 2 the two elements were not homogeneously distributed in the cross sections. While iodine shows higher concentrations in the cortex than in the center, arsenic shows the opposite behavior.

These results can be interpreted in two different ways. First, since the material in the middle section, which is also dead tissue, differs from that in the cortex, the elements may accumulate differently in the different constituents in the horn. Using ^{13}C as an internal standard, the difference in material density and the differences in laser interaction can be excluded. The second possibility is that the growth layers of the horn may not be horizontal but form stacked cuplike growth layers giving a slanted profile. This has been observed in other horns (20) and would fit to the horn's visual appearance.

High-Resolution Scans along the Growth Axis. Several scans along the main growth axis at the cortex and in the middle section should reveal whether the assumption that the horn growth is like stacked cups. Due to the size of the ablation chamber the horn samples were divided into 3–5 samples which were ablated one after another and then reconstructed to represent the entire horn starting at the tip of the horn, i.e., the oldest part (Figure 3). The profiles shown in Figure 3 are representative for the other NR sheep horns. At least three different domains appear in the horn. For arsenic there is at the very tip of the horn a slight peak, which is more obvious in the center scan than in the cortex scan. This peak is very narrow and is characterized by a steep drop to a very low arsenic intensity where it stays for a few centimeters. This period is then followed by a gradual increase of the arsenic signal, starting in the center scan earlier than in the cortex. The general trend of low concentration in the older part of the horn to the high concentration in the new parts is substantiated by the total digest analysis (Table 1). However,

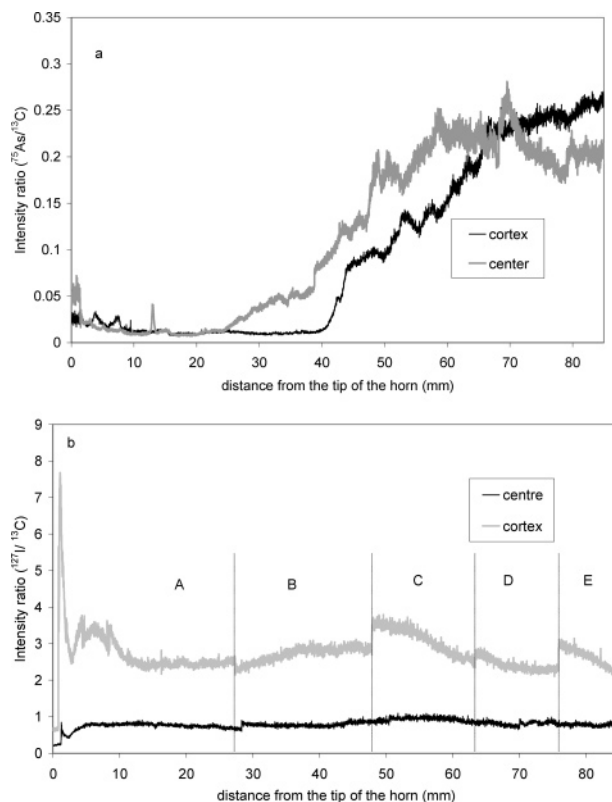


FIGURE 3. a: LA-ICP-MS scan along the growth axis of a NR sheep horn. The specimen represents the first 8 months of the lamb. Intensity ratios ($^{75}\text{As}/^{13}\text{C}$) are shown as a function of scanning distance from the tip of the horn, i.e., the oldest part of the horn. One scan represents the center and the other a cortex scan on the same specimen. b: LA-ICP-MS scan along the growth axis of a NR sheep horn. Intensity ratios (I/C) are shown as a function of distance. One scan represents the center and the other a cortex scan on the same specimen starting at the tip of the horn, i.e., the oldest part. This horn was divided into 5 pieces A (old part) to E (younger part), and the scan was reconstructed.

the total digests cannot pick up the first peak of the scans. This can be explained by the dilution effect; when the first 10 mm of the horn are taken, homogenized, and analyzed, the narrow band of higher arsenic within the low arsenic concentration does not increase the arsenic concentration significantly. Only high-resolution microscale analysis such as the laser ablation ICP-MS can identify such short events. The length scan confirms the theory that the horn grows in a stacked cuplike structure (Figure 1). For example, a cross section taken at 25–60 mm of the horn would give the arsenic profile seen in Figure 2.

Figure 3b shows the intensity of iodine in one horn of NR sheep from the older (0 mm) to the younger part (50 mm) of the horn, in the center and the cortex, for the different pieces of the horn, from the pieces A to E. The iodine in the center is lower than in the cortex by approximately a factor of 3, which correlates well to the cross section scans (Figures 2 and 3b). Since the concentration in the cortex is strongly dependent on the distance from the outer horn surface, it explains the small offsets between the different pieces A–E used in the reconstruction of the horn. Although, the scan along the growth axis confirmed the distribution pattern gained from the total digests of the horn samples, that the iodine concentration is less variable over the entire growth period, the origin of the low iodine concentration in the newest section of the horn indicated from the total digests could not be confirmed, and its cause is unknown to the authors. It appears that the signal is flat and constant in the

TABLE 2. Arsenic Speciation in the Hot Water Extract of the Horn Using HPLC-ICP-MS Analysis

sample	% as from speciated arsenic						methyl-As (%)	inorg As (%)	total (mg/kg)	% extraction efficiency
	As(III)	DMA(V)	MA(V)	As(V)	DMAS	U1 ^a				
10–20 mm	21	23	3	49	1	2	26	70	0.19	> 100
50–70 mm	5	73	3	5	13	1	89	10	2.20	45
90–100 mm	2	80	2	2	13	1	95	4	4.90	41
CRM	19	15	3	61	2	< 1	20	80	0.20	49

^a Unknown species (see Figure 5a).

entire horn, except at the tip, where a narrow iodine peak shows up. Similarly as for arsenic, it must represent a very short period of time, which dramatically ends since it shows a steep decrease of iodine concentration. In order to characterize these events arsenic speciation analysis and stable isotope ratio analysis were performed on sections of one horn.

Arsenic Speciation Analysis. For the speciation, three samples representing three different sections of the horn were prepared and analyzed in addition to blanks and a CRM hair sample. The amount of sample available varied, hence, the sample dependent detection limits. The approximate detection limits are between 0.01 and 0.02 mg/kg for the different arsenic species in the horn when a 0.5 g sample was used. For the sample (10–20 mm) only 0.05 g was used, which results in high detection limits. This explains the low precision in the extraction efficiency of that sample. The other samples however reveal similar extraction efficiency between 41 and 49%. Although half of the accumulated arsenic is in a form, which cannot be identified, the analysis of the arsenic extract gives crucial information about the main arsenic species in the horn that might indicate the source of arsenic (Table 2 and Figures S4 and S5 in the Supporting Information). It becomes obvious that the old part of the horn, characterized by low levels of arsenic, consists mainly of inorganic arsenic, while the horn sections with the higher arsenic levels accumulate mainly dimethylated arsenic. That means arsenic was in the extract in the form of DMA(V) and its sulfur-analogue DMAS, the latter one has often been misidentified as DMA(III) (21). This is not necessarily the form the arsenic was stored in the horn, since weak arsenic sulfur bonds can be broken or even formed during the extraction process as reported before (15), but it indicates that the arsenic is mainly accumulated as dimethylated arsenic. Since many dimethylated arsenic species such as DMA(V), DMAA, DMAE, and DMAAS are excreted as arsenosugar metabolites in the sheep’s urine, it can be assumed that the accumulation into the horn follows a similar process as in the wool (9). Hence, the increase in the proportion of dimethylated arsenic can be used as an indicator of seaweed intake. The proportion of methylated arsenic increases with the age of the sheep from 26% to more than 95% of the total extracted arsenic. Therefore, it can clearly be identified that the elevated arsenic is from the exposure to arsenosugars, i.e., from seaweed-ingestion, while the period of low arsenic concentration seem to be characterized by food intake of different sources which do not contain elevated levels of arsenosugars or other dimethylated arsenic species. This arsenic—mainly of an inorganic nature—may originate from the grass or from the milk but certainly not from seaweed ingestion. It would be useful to have arsenic speciation analysis from the tip of the horn in which an elevated arsenic peak had been identified by using the high-resolution LA-ICP-MS. Unfortunately, not enough sample material for arsenic speciation analysis was available.

$\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ Determination. In order to characterize the very tip of the horn in relation to the rest of the horn,

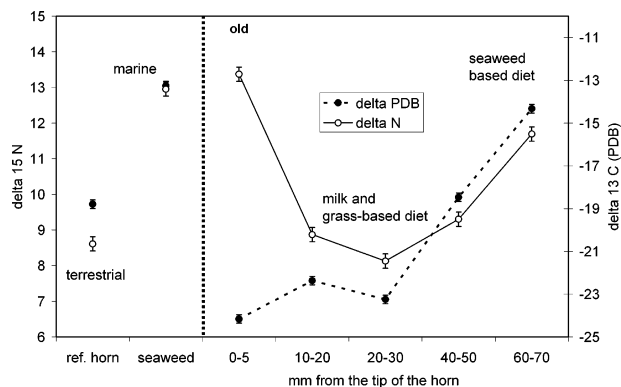


FIGURE 4. Stable isotope ratios expressed as $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ for several samples from the North Ronaldsay sheep horn, Pyrenees sheep horn, and seaweed.

stable isotope ratio measurements have been performed for which only 1 mg of sample is necessary. The control samples show very distinctive ^{13}C and ^{15}N values (Figure 4). While seaweed has -13.25‰ for $\delta^{13}\text{C}$ and $+12.95\text{‰}$ for $\delta^{15}\text{N}$, a marine signature, the horn of the Pyrenees sheep shows a characteristic terrestrial signature (-18.79‰ for $\delta^{13}\text{C}$ and $+8.61\text{‰}$ for $\delta^{15}\text{N}$). The $\delta^{13}\text{C}$ values of the five NR horn samples vary from marine to terrestrial C_3 plant signatures, while the $\delta^{15}\text{N}$ values show smaller variability. According to a recent study (22), the terrestrial $\delta^{13}\text{C}$ of grass on the Orkney islands is about -27‰ , while the marine $\delta^{13}\text{C}$ is about -13‰ according to the data for *Laminaria hyperborea*. Looking at the ^{13}C ratios, the ratios change with increasing age of the sheep from -24.16 to -14.33‰ indicating that the feed of the lambs changes dramatically from terrestrial to marine origin. This would be in line with the assumption that the lambs are getting their food mainly from milk with increasing amounts of grass; while after 5 months the grass is then instantaneously replaced by the marine macroalgae (seaweed), when the lambs are taken from the field to the beach. The $\delta^{13}\text{C}$ value for the very young NR lambs is lower than the control sheep from the Pyrenees. This may be due to the intake of different plants by the control sheep from the Pyrenees, which can result in geographical differences as observed in a stable isotope ratio study on the dietary history of beef (11). In particular C_3 plants mainly found in temperate climates such as grass have a lower $\delta^{13}\text{C}$ (-20 to -30‰), while some plants in hotter climates show a higher $\delta^{13}\text{C}$ (-10 to -14‰). These so-called C_4 plants have a different carbon fixing process during photosynthesis.

The ^{15}N ratios confirm for most samples the ^{13}C ratio trend with the exception of the very tip of the horn. The tip of the horn shows the very same $\delta^{15}\text{N}$ as the seaweed. Due to the fact that the lambs are not exposed to the seaweed in the first 5 months, this could only indicate that the lambs were exposed to other feed than milk and grass. This suggests that the first keratin was probably formed during the late gestation period, when the ewes were still on a seaweed-based diet. Therefore the trace elements in the very tip of the horn must

be transported through the placenta (23). This means that arsenic and iodine concentrations in the very tip of the horn represent placental uptake, while the period of low arsenic concentration represents the period of trace element uptake through milk and grass. Since the lambs are taken to the beach after 5 months, where the lambs right away replace grass with seaweed, this rapid process is not mirrored in the arsenic trace of the horn. The arsenic concentration rises steadily until a plateau is reached instead of changing at once. This can be explained by the eating habits of the young lamb. They still rely on milk, and the grass or the seaweed is not their main feed. However, the seaweed replaces the milk with an increasing time at the beach until it is the sole feed for the NR sheep.

In order to estimate the arsenic exposure at every stage of the lamb's development which has been monitored by the microscale laser ablation ICP-MS mapping of the horn, the previously published data (2) for the daily arsenic uptake per kg body weight (b.w.) of 1.5 ± 0.3 mg arsenic per kg b.w. can be used. Assuming a linear accumulation model the $^{75}\text{As}/^{13}\text{C}$ intensity ratio of the LA-ICP-MS trace of the newest part of the horn, which represents the period at which the sheep have matured and are based on a seaweed only diet, can be used to estimate the placental uptake. Approximately 0.32 ± 0.15 mg/kg b.w. ($n = 3$) can be calculated using the horns from three different NR sheep. This is still approximately 20% of the arsenic intake when seaweed is consumed. For the milk/grass eating period an uptake of 0.080 ± 0.068 mg/kg b.w. ($n = 3$) has been estimated, which is less than 5% of the seaweed-based diet, but is still significantly higher than that for the control sheep from the Pyrenees (0.011 mg/kg b.w.).

From the arsenic speciation and the stable isotope analysis, it is possible to establish good markers for the different periods of the lamb's first year. In contrast to arsenic, the iodine concentration in the horn is however rather stable (Figure 4b), which indicates that a milk/grass diet contains similar amounts of iodine to the seaweed-based diet. Since we do not see any dramatic variation when the grass is replaced by seaweed, it can be concluded that the milk of the ewes must contain similar concentrations of iodine to the seaweed. These high concentrations of iodine in the milk of the lactating ewe, which is fed on low iodine feedstuff, can only be explained by the elimination of iodine from iodine-rich tissues. The ewes have been exposed to enormous iodine concentrations due to their year round seaweed-based diet with an estimated daily intake of 124 mg iodine/kg b.w. (4), which is not fully homeostatically regulated. Hence, the sheep accumulate iodine in all tissues, i.e., liver, kidney, and muscle. When the ewes are on grass during lambing time, iodine is released from the body, in particular via milk, which is a well-reported phenomenon (24). However, it appears that the placental transport of iodine and thyroid hormones is still unknown (25). The horn analysis indicates that placental iodine transport is approximately a factor of 1.5 higher than iodine uptake (190 ± 106 mg/kg b.w. ($n = 3$)) through seaweed eating, while iodine transport through milk/grass is only slightly lower 74 ± 19 mg/kg b.w. ($n = 3$). The iodine transport through milk is still extremely high, although the ewes are on a low iodine-based diet-grass.

There is growing evidence that the uptake and metabolism for arsenosugars are similar for the NR sheep and humans, since the very same arsenic metabolites have been identified in their urine (26, 27). From this study, it might be encouraging to know that arsenic is not significantly transported via the milk but that elevated placental arsenic transport should be looked at more closely, in particular when pregnant women are or have been exposed to large amounts of arsenic either through seaweed-eating or arsenic in other food or drinking water. The enormous placental transport of iodine is certainly

a point of concern. Exposure to large amounts of iodine during pregnancy might trigger hypothyroidism and goiter formation (28).

Acknowledgments

We thank the Sheep Court at North Ronaldsay for permission to study their sheep and the Chemistry Department of the University of Aberdeen for permitting and financially supporting these experiments. Additionally we thank EPSRC (GR/R50837/01 and GR/S98689/01) for providing the financial means for the equipment used. S.O. thanks the Royal Thai Government Fund, and G.C. thanks the Socrates Exchange Programme.

Supporting Information Available

LA-ICP-MS scan of a nonideal horn surface (Figure S1), scan of the horn from NR sheep (Figure S2), cross section scan of the horn of the control sheep (Figure S3), and HPLC-ICP-MS analysis of the horn extract representing the low arsenic intake from grass and milk (Figure S4) and the medium arsenic intake from seaweed and milk (Figure S5). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Received for review September 20, 2006. Revised manuscript received January 10, 2007. Accepted January 25, 2007.

ES062241Y