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EIMS Fragmentation and MRM quantification of autoxidation products of 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5-ene in Arctic sediments

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RATIONALE:

Some highly branched isoprenoid (HBI) alkenes are commonly used as proxies for palaeoceanographic reconstructions. However, there is a real need to identify compounds that are sufficiently stable and abundant to be used as tracers of HBI oxidation in sediments. 2,6,10,14-Tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5(*Z/E*)-en-4-ols resulting from 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5-ene autoxidation appear to be useful for a purpose.

METHODS:

Comparison of EIMS mass spectra and retention times with standards allowed formal identification of autoxidation products of 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5-ene. EIMS fragmentations of TMS ethers of the main oxidation products (2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5(*Z/E*)-en-4-ols) were deduced by low energy CID-GC/MS/MS and accurate mass measurements. These compounds were then quantified in Arctic sediment samples in MRM mode using transitions based on the main fragmentation pathways elucidated.

RESULTS:

2,6,10,14-Tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5(*Z/E*)-en-4-ols were identified after autoxidation of the HBI alkene 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5-ene. CID-MS/MS analyses and accurate mass measurement allowed EIMS fragmentations of their TMS derivatives to be elucidated. Some specific fragment ions and chromatographic retention times were also useful for further characterization. As an application of some of the described fragmentations, TMS derivatives of these metabolites were characterized and quantified in MRM mode in Arctic sediments.

CONCLUSIONS:

Due to: (i) their production in high proportion during autoxidation of their parent HBI diene, (ii) their apparent stability in sediments, and (iii) their specific EIMS fragmentations, (*Z* and *E*) 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5-en-4-ol TMS derivatives appeared to be useful tracers of HBI autoxidation in sediments.

RUNNING TITLE: Mass fragmentations of autoxidation products of an HBI diene.

KEYWORDS: Autoxidation; HBI diene; (*Z* and *E*) 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5-en-4-ol TMS derivatives; EIMS fragmentation; Sediments.

1. INTRODUCTION

Highly branched isoprenoid alkenes (HBIs), which are biosynthesized by a relatively small number of diatom taxa belonging to the *Haslea*, *Navicula*, *Pleurosigma*, *Berkeleya*, *Rhizosolenia* and *Pseudosolenia* genera,¹⁻⁸ are nonetheless common constituents of marine and lacustrine sediments.⁹⁻¹¹ Due to their source specificity and relative stability within the geological record, some HBIs are now commonly used as proxies for palaeoceanographic reconstructions, especially in the Polar Regions.¹²

Application of such proxies requires careful consideration of alteration and preservation between their source and sedimentary environments. According to the number, the position and the ionization potential of their double bonds, HBIs may be affected more or less intensively by photooxidation in the euphotic layer of oceans^{13,14} and by autoxidation in oxic environments such as the water column and surficial sediments.^{14,15} Although some oxidation tracers of individual HBIs have been identified and characterised,¹⁵⁻¹⁷ they are often either too susceptible to secondary oxidation (e.g. for tri-unsaturated HBIs with *bis*-allylic double bonds¹⁵) or are produced in too low proportion (e.g. for mono- and di-unsaturated HBIs^{16,17}) to permit meaningful quantification. There is thus a real need to identify HBI oxidation products that are sufficiently stable and abundant to estimate the impact of oxidative degradation processes on the preservation of these lipid biomarkers in marine environments.

The present work focuses on the oxidation of 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5-ene (**1**). This HBI diene has been reported in diatom cultures,^{7,18} and sediments from various regions.^{7,8,18-20} It may also result from isomerisation of 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-6(17)-ene (IPSO₂₅) (**2**) (Scheme 1),²¹ a common constituent of Arctic and Antarctic sea ice and surface sediments.^{5,12,22,23} Electron ionization (EI) fragmentation pathways of trimethylsilyl (TMS) derivatives of (*Z* and *E*)

2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5-en-4-ols (**3** and **4**) arising from autoxidation of HBI **1** (Scheme 1) were elucidated by using low-energy collision-induced dissociation (CID) GC/MS/MS and accurate mass measurements. These compounds were then quantified in Arctic sediment samples in multiple reaction monitoring (MRM) mode using transitions based on the main fragmentation pathways elucidated and proposed as valuable tracers of HBI autoxidation in sediments.

2. EXPERIMENTAL

2.1 Chemicals

A small-scale sample of HBI **1** was obtained from a sediment extract described previously.⁸ The sediment extract was further purified here using silver-ion chromatography (Supelco, 250 mg, DCM:Acetone (3:1, v/v), 5 mL) to provide a few micrograms of HBI **1** (>93%; GC–MS). HBI **2** (IPSO₂₅) (containing approximately 4% of HBI **1**) was obtained from a culture of the marine diatom *Haslea ostrearia* as described previously.²²

Oxidation of HBI **1** using RuCl₃ and *tert*-butyl hydroperoxide in cyclohexane at room temperature for 16 h²⁴ and subsequent NaBH₄-reduction in ether-methanol (4:1, v/v) produced (*Z* and *E*) 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5-en-4-ols (**3** and **4**) in low yield.

Treatment of HBI **1** with a stoichiometric amount of perchloroperbenzoic acid in dry dichloromethane (4 h at 50 °C) afforded 5,6-epoxy-2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadecane (**5**).

The synthesis of the highly structurally related 2,6,10,14,18-pentamethylnonadec-5-en-4-ol (**8**) employed as a standard required two steps: (i) oxidation of (*E*)-phytol (Sigma

Aldrich, St. Quentin Fallavier, France) with CrO_3 /pyridine in dry dichloromethane (DCM),²⁵ and (ii) condensation of the resulting (*E*)-phytenal with isobutyl magnesium bromide (Sigma Aldrich, St. Quentin Fallavier, France) in dry diethylether.

2.2 Autoxidation of HBI 1

Autoxidation experiments were performed under an atmosphere of air in 15 mL screw-cap flasks containing HBI **1** (amount too low to be weighed) or a mixture of HBIs **1** and **2** (1 mg), *tert*-butyl hydroperoxide (300 ml of a 6.0 M solution in decane), di-*tert*-butyl nitroxide (1.2 mg) and hexane (2 mL). After stirring, the flask was incubated in the dark at 65 °C. Aliquots (200 μL) were withdrawn from the reaction mixture after incubation for different times. Each sub-sample was evaporated to dryness under a stream of nitrogen and analyzed by gas chromatography–electron ionization mass spectrometry (GC–EIMS) after NaBH_4 reduction and silylation.

2.3 Reduction of oxidation products

Hydroperoxides resulting from HBI oxidation were reduced to the corresponding alcohols by reaction with excess NaBH_4 in diethyl ether:methanol (4:1, v/v) at room temperature (1 h). After reduction, a saturated solution of NH_4Cl (10 mL) was added cautiously to remove any unreacted reducing agent; the pH was adjusted to 1 with dilute HCl (2 N) and the mixture shaken and extracted with hexane:chloroform (5 mL, 4:1, v/v; x3). The combined extracts were dried over anhydrous Na_2SO_4 , filtered and evaporated to dryness under a stream of nitrogen.

2.4 Sampling and treatment of sediments

Our sampling location for sediment material corresponds to Barrow Strait (Station 4, 74°16'12"N, 31°46'12"W) in the Canadian Arctic. Box cores were collected, sectioned on

board, with sub-samples (1-cm resolution) then freeze-dried before storage ($< 4^{\circ}\text{C}$) prior to analysis. Sediment sub-samples from sectioned box cores were placed in methanol (MeOH) (15 mL) and hydroperoxides were reduced to the corresponding alcohols with excess NaBH_4 (70 mg, 30 min at 20°C). Following the reduction step, water (15 mL) and KOH (1.7 g) were added and the mixture saponified by refluxing (2 h). After cooling, the contents of the flask were acidified (HCl, to pH 1) and extracted three times with DCM (30 mL). The combined DCM extracts were dried over anhydrous Na_2SO_4 , filtered and concentrated to give the total lipid extract (TLE). Since HBI oxidation product content was quite low relative to other lipids, accurate quantification required further separation of the TLE using column chromatography (silica; Kieselgel 60, 8×0.5 cm). HBIs were obtained by elution with hexane (10 mL) and their oxidation products by subsequent elution with DCM (10 mL).

2.5 Silylation

DCM eluates of sediments, reduced subsamples of autoxidation experiments (evaporated to dryness) and standard alcohol **8** were derivatized by dissolving them in 300 μL pyridine/bis-(trimethylsilyl)trifluoroacetamide (BSTFA; Supelco; 2:1, v/v) and silylated (50°C , 1 h). After evaporation to dryness under a stream of N_2 , the derivatized residue was dissolved in ethyl acetate/BSTFA (to avoid desilylation) and analysed by mass spectrometric methods.

2.6 Gas chromatography/electron ionization tandem mass spectrometry

GC/EIMS and GC/EIMS/MS experiments were performed using an Agilent 7890A/7000A tandem quadrupole gas chromatograph system (Agilent Technologies, Parc Technopolis - ZA Courtaboeuf, Les Ulis, France). A cross-linked 5% phenyl-methylpolysiloxane (Agilent; HP-5MS-Ultra Inert) ($30 \text{ m} \times 0.25 \text{ mm}$, $0.25 \mu\text{m}$ film thickness) capillary column was employed. Analyses were performed with an injector operating in

pulsed splitless mode set at 270°C and the oven temperature programmed from 70°C to 130°C at 20°C min⁻¹, then to 250°C at 5°C min⁻¹ and then to 300°C at 3°C min⁻¹. The pressure of the carrier gas (He) was maintained at 0.69 x 10⁵ Pa until the end of the temperature program and then programmed from 0.69 x 10⁵ Pa to 1.49 x 10⁵ Pa at 0.04 x 10⁵ Pa min⁻¹. The following mass spectrometric conditions were employed: electron energy, 70 eV; transfer line, 300°C; source temperature, 230°C; quadrupole 1 temperature, 150°C; quadrupole 2 temperature, 150°C; collision gas (N₂) flow, 1.5 mL min⁻¹; quench gas (He) flow, 2.25 mL min⁻¹; mass range, 50-700 Dalton; cycle time, 313 ms. Collision induced dissociation (CID) was optimized by using collision energies at 5, 10, 15 and 20 eV.

Due to the very low amounts of HBI **1** available, compounds **3** and **4** could not be produced in sufficient amounts to permit quantification, although comparison of their mass fragmentations and retention times with compounds detected in sediments allowed their unambiguous identification. Quantification of TMS ethers of alcohols **3** and **4** was thus carried out with a standard of the highly structurally related 2,6,10,14,18-pentamethylnonadec-5-en-4-ol (**8**; TMS derivative) in multiple reaction monitoring (MRM) mode. A correction factor that took into account the proportion of the selected precursor ion (*m/z* 379 for compounds **3** and **4** and *m/z* 367 for compound **8**) in the EIMS of each compound (Fig. 1) and that of the selected MRM transition in each CID-MS was employed.

2.7 Gas chromatography/electron ionization quadrupole time of flight mass spectrometry

Accurate mass measurements were carried out in full scan mode with an Agilent 7890B/7200 GC/QTOF System (Agilent Technologies, Parc Technopolis - ZA Courtaboeuf, Les Ulis, France). A cross-linked 5% phenyl-methylpolysiloxane (Macherey-Nagel; Optima-5MS Accent) (30 m × 0.25 mm, 0.25 µm film thickness) capillary column was employed.

Analyses were performed with an injector operating in pulsed splitless mode set at 270°C and the oven temperature programmed from 70°C to 130°C at 20°C min⁻¹ and then to 300°C at 5°C min⁻¹. The pressure of the carrier gas (He) was maintained at 0.69 x 10⁵ Pa until the end of the temperature program. Instrument temperatures were 300°C for transfer line and 230°C for the ion source. Nitrogen (1.5 mL min⁻¹) was used as collision gas. Accurate mass spectra were recorded across the range *m/z* 50-700 at 4 GHz with the collision gas valve opened. The QTOF-MS instrument provided a typical resolution ranging from 8009 to 12252 from *m/z* 68.9955 to 501.9706. Perfluorotributylamine (PFTBA) was utilized for daily MS calibration.

3. RESULTS AND DISCUSSION

3.1 Autoxidation of HBI 1

Comparison of retention times and EI mass spectra with synthesized standards allowed identification of isomeric (*Z* and *E*) 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5-en-4-ols (**3** and **4**) and 5,6-epoxy-2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadecane (**5**) after incubation of HBI **1** in hexane in the presence of *tert*-butyl hydroperoxide (radical enhancer) and di-*tert*-butyl nitroxide (radical initiator)²⁶ at 65 °C and subsequent NaBH₄-reduction and silylation (Figs. 1A and 1B). Allylic hydrogen abstraction and addition of peroxy radical to the double bonds generally compete during the autoxidation of alkenes.²⁷ Formation of alcohols **3** and **4** results from hydrogen abstraction at the allylic carbon 4 of HBI **1**, and epoxide **5** following peroxy radical addition to the C5-C6 double bond (Scheme 1). The lack of hydrogen abstraction at the allylic carbon 7 results likely from steric hindrance during hydrogen abstraction by the bulky *tert*-butylperoxy radicals employed during the incubation.¹⁷

Comparison of autoxidation rates of the two isomeric HBIs **1** and **2** shows that HBI **1** is oxidized 1.4 time faster than IPSO₂₅ (**2**). While autoxidation of IPSO₂₅ (**2**) mainly afforded

1,2-epoxy-2-(4-methylpentyl)-3-(3-methylpent-4-enyl)-6,10-dimethylundecane (**7**) and, to a lesser extent, 6-methylidene-2,10,14-trimethyl-7-(3-methylpent-4-enyl)-pentadecan-5-ol (**6**) (ratio **6/7** = 0.1)¹⁷ (Scheme 1), allylic hydrogen abstraction appeared to be more favoured during autoxidation of HBI **1** (ratio (**3+4**)/**5** = 1.2). This difference in reactivity of allylic carbon 4 of HBI **1** and carbon 5 of IPSO₂₅ (**2**) towards hydrogen abstraction is in good agreement with EPR spectroscopy results obtained previously by Camara et al.²⁸

Due to the well-known lability of epoxides in sediments¹⁷ and during their treatment,²⁹ the production of a higher proportion of allylic alcohols **3** and **4** during autoxidation of HBI **1** strengthens the tracer potential of autoxidation products of this alkene.

3.2 EIMS fragmentations of 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5(*Z/E*)-en-4-ol TMS ether derivatives (**3** and **4**)

TMS ethers of the *Z* and *E* isomers **3** and **4** exhibited the same EI mass spectra (Fig. 1A), with weak peaks at *m/z* 436 and *m/z* 421 corresponding to the molecular ion (**a**⁺) and [M – CH₃]⁺ (**b**⁺), respectively. Intense and interesting peaks were also observed at *m/z* 379, *m/z* 289, *m/z* 199, *m/z* 163, *m/z* 143 and *m/z* 109. α-Cleavage relative to the ionized ether group affords fragment ion **c**⁺ at *m/z* 379, which may lose a neutral molecule of trimethylsilanol (TMSOH) after 1,5-hydrogen shift from C-7 to the ionized ether group. Subsequent 1,4-cyclization yields ion **d**⁺ at *m/z* 289 (Scheme 2), which can then be cleaved to the fragment ion **e**⁺ at *m/z* 163 following 1,5-hydrogen shift from C-10 to the charged cyclobutyl ring and concerted ring extension (Scheme 2). Cleavage of ion **f**⁺ at *m/z* 379 (mesomer of ion **c**⁺) involving a 1,3-hydrogen shift from C-8 to the carbocation may be at the origin of the formation of the strongly stabilized ion **g**⁺ at *m/z* 143 (Scheme 2).

Ionization of TMS ethers of the *Z* and *E* isomers **3** and **4** can also take place at the 5-6 double bond affording ion **h**⁺ at *m/z* 436, which can be cleaved to the strongly stabilized ion

$\mathbf{i}^+$ at m/z 199 after a 1,3-hydrogen shift from C-4 to the ionized tertiary C-6 and subsequent cleavage of the C6-C7 bond (Scheme 2). Ion $\mathbf{i}^+$ can then either lose a neutral molecule of TMSOH after a 1,3-hydrogen shift from C-17 and subsequent 1,4-cyclization to produce the fragment ion $\mathbf{j}^+$ at m/z 109, or be converted to ion $\mathbf{g}^+$ at m/z 143 following a 1,3-hydrogen shift from tertiary C-2 and cleavage of the C3-C4 bond (Scheme 2).

The proposed fragmentation pathways are supported further by the results of CID analyses of fragment ions $\mathbf{a}^{+\bullet}$ and $\mathbf{h}^{+\bullet}$ at m/z 436, $\mathbf{c}^+$ and $\mathbf{f}^+$ at m/z 379, $\mathbf{d}^+$ at m/z 289 and $\mathbf{i}^+$ at m/z 199 (Table 1). Moreover, the accurate masses of ions $\mathbf{a}^{+\bullet}$ - $\mathbf{j}^+$ showed only minor deviations (ranging from 0.7 to 5.7 ppm) from the calculated theoretical masses (Table 2), thus confirming the elemental composition of the fragment ions in each case.

3.3 MRM quantification of compounds **3** and **4** in sediment samples

The mass spectral transitions employed for the quantification of oxidation products were m/z 379 $\rightarrow$ 143 for TMS ethers of compounds **3** and **4**, and m/z 367 $\rightarrow$ 143 for the TMS ether of the standard **8**. Transitions m/z 379 $\rightarrow$ 163 and m/z 379 $\rightarrow$ 289 were used as qualifiers. It may be noted that transitions resulting from the cleavage of ion $\mathbf{i}^+$ at m/z 199 appeared to be insufficiently specific for the analysis of sediment extracts and were thus discarded.

The limit of quantification (150 pg) was determined according to a signal-to-noise ratio greater than 3. The linear range was determined using values that met the standard analysis criteria of less than 15% deviation across the concentration range. Linear responses were obtained over 2 to 3 orders of magnitude. Despite the relatively weak concentration of HBI **1** in sediments from Barrow Strait (Table 3), compounds **3** and **4** could be readily detected and quantified (Table 3, Fig. 2). The proportion of such allylic alcohols relative to the parent HBI (up to 26%) (Table 3) appeared to be two orders of magnitude higher than that

of the alcohol **6** relative to the parent IPSO₂₅ (**2**) in the same sediments (up to 0.2%)¹⁷. The decrease of the ratio **3/4** observed in sediments relative to the autoxidation experiment (Fig. 2) was attributed to allylic rearrangement of the corresponding hydroperoxides to 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-4(*E*)-en-6-hydroperoxide (**9**) (Scheme 1) inhibited by high concentration of *tert*-butyl-hydroperoxide during the autoxidation experiment²⁶ but not in sediments. Indeed, it was previously observed that rearrangement of *E*-allylperoxyls was reversible but this was not the case for *Z*-allylperoxyls³⁰ (Scheme 1).

4. CONCLUSIONS

The main autoxidation products of the HBI diene 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5-ene (**1**) were identified as (*Z* and *E*) 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5-en-4-ols (**3** and **4**) and 5,6-epoxy-2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadecane (**5**). CID-MS/MS and accurate mass measurements allowed the EI mass fragmentations of TMS ethers of alcohols **3** and **4** to be elucidated. On the basis of these fragmentations, some MRM transitions were selected and applied to lipid extracts of Arctic sediments. These compounds appeared to be present in quite high proportions relative to the parent HBI (**1**) (up to 26%). The apparent stability of compounds **3** and **4** in sediments, their production in high proportion during autoxidation of HBI **1** and the potential isomerization of the widely distributed IPSO₂₅ (**2**) to their parent HBI **1** under environmental conditions supports the use of these alcohols as tracers of HBI autoxidation in sediments.

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FIGURE AND SCHEME CAPTIONS

Figure 1. EI mass spectra of 5,6-epoxy-2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadecane (**5**) (A), 2,6,10,14-tetramethyl-7-(3-methylpent-4-enyl)-pentadec-5(*E*)-en-4-ol TMS derivative (**4**) (B) and 2,6,10,14,18-pentamethylnonadec-5-en-4-ol TMS derivative (**8**) (C).

Figure 2. MRM chromatograms (m/z 379 $\rightarrow$ 143, m/z 379 $\rightarrow$ 163 and m/z 379 $\rightarrow$ 289) of silylated HBI **1** autoxidation products **3** and **4** (produced during autoxidation experiment) (A) and DCM fraction obtained from the 2-3 cm (B) and 10-11 cm (C) layer of the core sediment from Barrow Strait.

Scheme 1. Autoxidation and interconverting of HBI dienes **1** and **2**.

Scheme 2. Proposed fragmentation mechanisms of alcohols **3** and **4** TMS derivatives.

Table 1. CID analyses of the different fragment ions.

Code	m/z	Collision energy (eV)	Product ions
i⁺	199	5	199(100), 181(8), 157(5), 143(41), 129(7), 109(19), 99(9), 87(6), 75(38), 73(36)
d⁺	289	5	289(100), 199(23), 171(24), 163(45), 149(33), 127(14), 121(26), 107(29), 97(13), 95(26), 83(19), 81(39)
c⁺ and f⁺	379	5	379(100), 289(17), 269(11), 163(49), 149(14), 143(40), 129(17), 121(11), 109(22), 107(18), 103(14), 95(25), 81(18), 69(15)
a⁺ and h⁺	436	7	436(100), 379(37), 295(4), 275(6), 253(45), 225(30), 199(75), 129(23), 69(5)

Table 2. High-accuracy mass spectral data for ions **a⁺**-**j⁺**

Code	Formula	<i>m/z</i> calculated	<i>m/z</i> observed	Δ (ppm)
j⁺	C ₈ H ₁₃	109.1012	109.1011	-0.9
g⁺	C ₇ H ₁₅ OSi	143.0887	143.0886	-0.7
e⁺	C ₁₂ H ₁₉	163.1481	163.1485	+2.4
i⁺	C ₁₁ H ₂₃ OSi	199.1513	199.1516	+1.5
d⁺	C ₂₁ H ₃₇	289.2890	289.2896	+2.1
c⁺ and f⁺	C ₂₄ H ₄₇ OSi	379.3391	379.3401	+2.6
b⁺	C ₂₇ H ₅₃ OSi	421.3890	421.3866	-5.7
a⁺ and h⁺	C ₂₈ H ₅₆ OSi	436.4095	436.4102	+1.6

Table 3. Concentration of HBI **1** and its autoxidation products **3** and **4** in sediments from the Arctic station 4 (Barrow Strait)

Depth (cm)	HBI 1 ($\mu\text{g g}^{-1}$)	Compound 3 (ng g^{-1})	Compound 4 (ng g^{-1})	Total (3 + 4) (ng g^{-1})	(3 + 4)/ 1 (%)
1-2	0.19	4.0	15.6	19.6	10.3
2-3	0.32	2.0	19.6	21.6	6.8
4-5	0.31	4.6	43.4	48.0	15.5
6-7	0.27	3.8	42.6	46.4	17.2
8-9	0.16	Tr*	9.6	9.6	6.0
10-11	0.22	16.2	41.2	57.4	26.1

* Traces (< LOD)