Research Article

Gas chromatographic mass spectrometric detection of dihydroxy fatty acids preserved in the 'bound' phase of organic residues of archaeological pottery vessels

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A methodology is presented for the determination of dihydroxy fatty acids preserved in the 'bound' phase of organic residues preserved in archaeological potsherds. The method comprises saponification, esterification, silica gel column chromatographic fractionation, and analysis by gas chromatography/mass spectrometry. The electron ionisation mass spectra of the trimethylsilyl ether methyl ester derivatives are characterised by fragment ions arising from cleavage of the bond between the two vicinal trimethylsiloxy groups. Other significant fragment ions are $[M-15]^{+\cdot}$, $[M-31]^{+\cdot}$, m/z 147 and ions characteristic of vicinal disubstituted (trimethylsiloxy) TMSO- groups ($\Delta^{7,8}$, $\Delta^{9,10}$, $\Delta^{11,12}$ and $\Delta^{13,14}$: m/z 304, 332, 360 and 388, respectively). The dihydroxy fatty acids identified in archaeological extracts exhibited carbon numbers ranging from C_{16} to C_{22} and concentrations varying from 0.05 to 14.05 µg g^{-1}. The wide range of dihydroxy fatty acids observed indicates that this approach may be applied confidently in screening archaeological potsherds for the degradation products of monounsaturated fatty acids derived from commodities processed in archaeological pottery vessels. Copyright © 2011 John Wiley & Sons, Ltd.

Fragments of ceramic containers, namely potsherds, are common artefacts found at archaeological sites, with their inorganic porous ceramic matrix providing a medium conducive to the preservation of biomolecules and their degradation products derived from commodities processed in the vessels, particularly fats, oils, waxes and resinous materials.[1–5] Commonly occurring organic compounds absorbed in potsherds are preserved either as free components, retained via strong absorptive forces trapped within molecule-sized pores, or as chemically bound components, e.g. linked via ester bonds within a polymeric organic or organic-ceramic network.[6] Free compounds are readily extracted from the ceramic potsherd matrix using organic solvents (e.g. CHCl₃/MeOH, 2:1 v/v), while the bound components can be released from the potsherd, following solvent extraction, by strong base hydrolysis (e.g. 0.5 M KOH in MeOH/H₂O; 9:1 v/v).[7,8] To date, the majority of archaeological studies have focused on freely extractable lipid components and have resulted in the characterisation of organic residues for an impressive range of commodities from around the world.[9] In contrast, complex, 'bound' fatty acid oxidation products have been only recently been used as biological marker compounds (biomarkers) for plant oils and terrestrial or marine oils and fats.[3,10,11]

A wide range of oxygenated degradation products has been detected in potsherds as 'bound' residues including: fatty acids, mono- and dihydroxy fatty acids, ketoacids and α,ω-dicarboxylic acids.[8] However, thus far, vicinal dihydroxy fatty acids have shown the most promise as biomarkers in 'bound' residues, since the position of the additional hydroxyl groups reflects the original double-bond position of the monounsaturated fatty acid precursors present in the original processed commodities.[3,11,12]

The complex mixtures of oxygenated compounds released by base hydrolysis require a subsequent clean-up procedure prior to analysis and full characterisation by gas chromatography/mass spectrometry.[11] Presented herein is an effective analytical protocol based on saponification, esterification, flash silica gel column fractionation, and gas chromatography/electron ionisation mass spectrometry for the detection of vicinal dihydroxy fatty acids preserved as polymeric or adsorbed 'bound' residues in potsherds from the archaeological record.

EXPERIMENTAL

Archaeological potsherd samples (n = 12) were supplied by Museu Universitário Professor Oswaldo Rodrigues Cabral – Universidade Federal de Santa Catarina (Florianópolis, Brazil) and corresponded to Itararé tradition vessels associated with the pre-colonial Jê population (ca. 1000 AD). The vessels originated from sites located on the coastal islands of Santa Catarina State (São Francisco do Sul and Santa Catarina Islands), south Brazil.

A thin surface layer of potsherd (ca. 0.5 mm) was removed initially, using an electric hand drill, to avoid traces of external

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contamination. Sub-samples of finely ground sherds (2 g) were then extracted with CHCl₃/MeOH solution (2:1 v/v, 10 mL, 2 × 15 min sonication) to remove 'free' lipids. A solvent-extracted potsherd (1 g) containing insoluble residues was then saponified with 0.5 M NaOH (MeOH/H₂O solution, 9:1 v/v, 5 mL, 70 °C, 1 h) to liberate 'bound' components. After cooling, the mixture was centrifuged (2500 rpm, 15 min) and the supernatant removed and acidified to pH 3 with 3 M HCl. Solvent-extracted H₂O was added (1 mL) and the hydrolysed lipids extracted with CHCl₃ (3 × 3 mL). The solvent was then evaporated under a gentle stream of nitrogen gas until completely removed. The hydrolysed acid components were methylated using BF₃·MeOH (14% w/v, 100 μL, 70 °C, 1 h), followed by addition of 2 mL of solvent-extracted H₂O from which the methyl esters were then extracted with CHCl₃ (3 × 2 mL).

The methylated extracts were fractionated using a glass column (200 × 5 mm i.d.) incorporating a porous glass sinter to retain the packing of 1 g of activated silica gel (120 °C, 24 h). Packing was achieved by applying a slurry of the silica gel in n-hexane using a positive flow of nitrogen gas. Excess solvent was eluted until the solvent level was 1 mm above the top of the silica gel bed. The methylated extracts were quantitatively transferred onto the column using 1 mL of n-hexane, which was again run to a level of 1 mm above the silica gel bed. The extracts were separated by applying a positive pressure with nitrogen gas to give an elution rate of 15 mL min⁻¹, using the elutropic series of the following four solvent systems: n-hexane (6 mL), n-hexane/dichloromethane (DCM) (9:1 v/v, 2 mL), DCM (6 mL), DCM/MeOH (1:1 v/v, 5 mL). The dihydroxy fatty acids eluted in the DCM/MeOH system. All fractions were collected and solvent removed under a gentle stream of nitrogen.

The dihydroxy fatty acid fraction was derivatised by the addition of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)

containing 1% of trimethylchlorosilane (TMCS) (30 μL, 70 °C, 30 min). Excess reagent was removed under nitrogen. Internal standard was added (10 μg, n-tetratriacontane) and the samples redissolved in n-hexane (200 μL) for analysis by GC/MS.

GC/MS analyses were performed using a ThermoFinnigan Trace GC/MS system (Thermo Fisher Scientific, Hemel Hempstead, UK) fitted with a programmable temperature vapourising (PTV) GC injector at 290 °C, and incorporating a quadrupole mass spectrometer. Helium was the carrier gas and the GC oven temperature programme was: 50 to 100 °C at 10 °C min⁻¹ then to 300 °C at 4 °C min⁻¹, isotherm for 10 min; and the column had a ZBI stationary phase (60 m × 0.32 i.d. × 0.1 μm film thickness; Phenomenex, Macclesfield, UK). The mass spectrometer was operated in electron ionisation (EI) mode with the following parameters: transfer line temperature 300 °C, electron energy of 70 eV, emission current of 300 mA, ion source temperature 170 °C, with the mass analyser scanning the range m/z 50–600 with a cycle time of 1.0 s.

RESULTS AND DISCUSSION

Figure 1 depicts a partial gas chromatogram showing the distribution of lipids typically observed for the DCM/MeOH fraction. The major lipid oxidation products are hydroxy fatty acids and α,ω-dicarboxylic acids. Vicinal dihydroxy fatty acids were identified by virtue of a specific cleavage between the two carbon atoms bearing the TMSO groups, resulting in fragment ions highly diagnostic of either a specific compound or an isomer bearing the same number of carbon atoms (Scheme 1). For example, the fragment ions at m/z 215 and m/z 287 are generated by derivatised 11,12-dihydroxyarachidic acid, and those at m/z 243 and m/z 259 by derivatised 9,10-dihydroxyarachidic acid (Figs 2A and B, respectively).
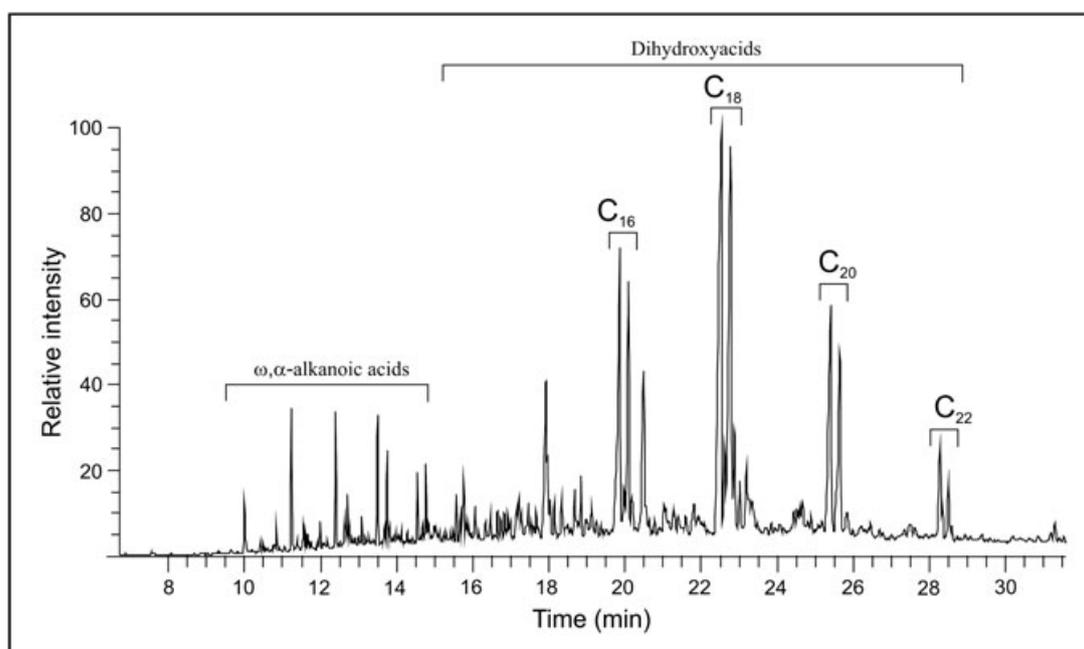
Figure 1. Total ion current ion (TIC) chromatogram showing the characteristic range of compounds observed in the DCM/MeOH fraction eluted from a flash silica gel column of an alkaline extract of the polymerised 'bound' phase of an archaeological potsherd.

		m/z	
		a	e
7,8-dihydroxypalmitic acid	C_{16}	215	231
9,10-dihydroxypalmitic acid	C_{16}	187	259
11,12-dihydroxypalmitic acid	C_{16}	159	287
9,10-dihydroxystearic acid	C_{18}	215	259
11,12-dihydroxystearic acid	C_{18}	187	287
13-14-dihydroxystearic acid	C_{18}	159	315
9,10-dihydroxyarachidic acid	C_{20}	243	259
11,12-dihydroxyarachidic acid	C_{20}	215	287
13,14-dihydroxyarachidic acid	C_{20}	187	315
11,12-dihydroxybehenic acid	C_{22}	243	287
13,14-dihydroxybehenic acid	C_{22}	215	315
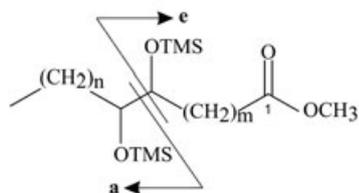
Scheme 1. Characteristic ions, diagnostic of the position of hydroxylation, arising from the fragmentation of vicinal dihydroxy fatty acids identified in the 'bound' phase of archaeological potsherds.
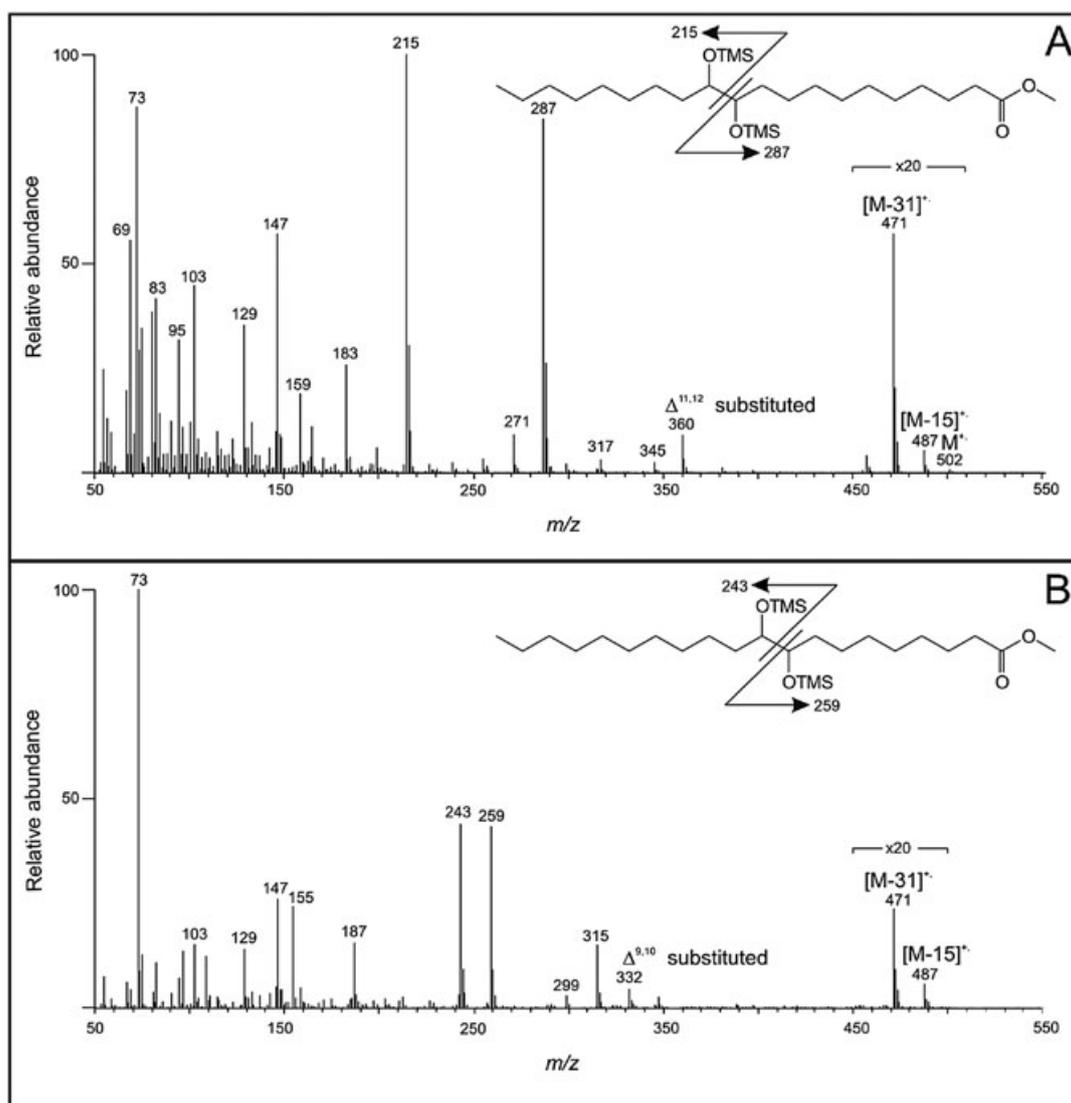
Figure 2. EI (70 eV) mass spectra of methyl ester TMS derivatives of *threo*-11, 12-dihydroxyarachidic acid (A) and *threo*-9,10-dihydroxyarachidic acid (B), extracted from the 'bound' phase of a potsherd sample ME17.

The GC resolution is challenging for isomers bearing the same number of carbon atoms (Fig. 1); thus, the identification of compounds is only possible through the characteristic fragment ions depicted in Scheme 1. The sum of these fragment ions yields the molecular weight, which may be corroborated by the presence of [M–15] and [M–31] fragments (for methyl ester-TMS derivatives; Figs. 2 (A) and 2(B)). Other characteristic fragment ions are those observed at m/z 147 and for vicinal dihydroxy acids substituted at $\Delta^{7,8}$, $\Delta^{9,10}$, $\Delta^{11,12}$ and $\Delta^{13,14}$, at m/z 304, 332,
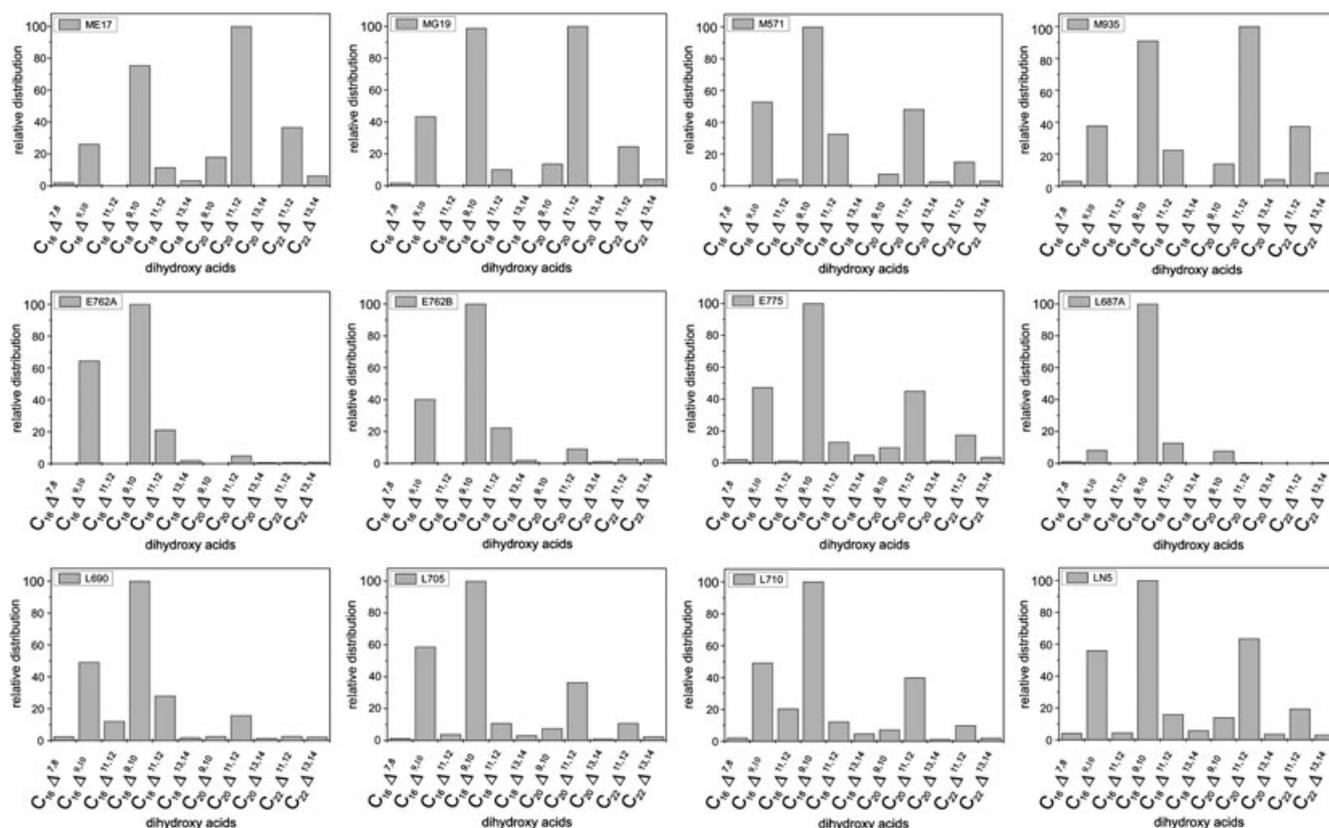
Figure 3. Normalised relative abundances of individual *threo*-dihydroxyacids extracted from the 'bound' phase of archaeological potsherds. The distributions were established considering the area of fragment ion 'e' mass chromatogram peak (Scheme 1). $C_x \Delta^{y,z}$ refers to dihydroxyacids, in which x is the number of carbon atoms and y,z are the Δ dihydroxy substitution positions in the carbon chain.

Table 1. Semi-quantitative concentrations and maximum and minimum concentrations of the single dihydroxy fatty acid isomers detected

Acids Sherds	C_{16} 7,8-	C_{16} 9,10-	C_{16} 11,12-	C_{18} 9,10-	C_{18} 11,12-	C_{18} 13,14-	C_{20} 9,10-	C_{20} 11,12-	C_{20} 13,14-	C_{22} 11,12-	C_{22} 13,14-
E775 (μg g^{-1})[a]	0.18	4.11	0.12	8.69	1.13	0.42	0.84	3.91	0.12	1.52	0.3
L687A (μg g^{-1})	0.15	1.15	nd	14.05	1.79	nd	1.09	0.09	nd	Nd	0.05
ME17 (μg g^{-1})	0.16	2.14	nd	6.18	0.94	0.28	1.47	8.18	nd	3.01	0.51
MG19A (μg g^{-1})	0.05	1.18	nd	2.67	0.27	nd	0.37	2.71	nd	0.67	0.11
M571 (μg g^{-1})	nd	4.46	0.34	8.45	2.76	nd	0.62	4.06	0.23	1.27	0.26
M935 (μg g^{-1})	0.05	0.69	nd	1.67	0.42	nd	0.26	1.84	0.07	0.69	0.15
range (μg g^{-1})		0.05–4.46			0.27–14.05			0.07–8.18			0.05–3.01
R^2		0.9897			0.9942			0.998			0.995

[a]Dihydroxy fatty acid concentrations were calculated based on the areas of fragment ion 'e' mass chromatogram peak (Scheme 1) of the *threo*-diastereoisomer and the *m/z* 71 mass chromatogram of the internal standard added before GC/MS analysis (10 μg, *n*-tetratriacontane).

360 and 388, respectively.[13] The Δ-substituted fragments are rearrangements of mid-chain vicinal dihydroxy TMS methyl esters, with their formation involving a cleavage of the bond between the two vicinal TMSO groups and migration of the eliminated TMS to the ester group.[13] These characteristic fragment ions are shown in Figs. 2(A) and 2(B) for the $\Delta^{11,12}$- and $\Delta^{9,10}$-dihydroxyarachidic acids, respectively. The EI mass spectra of the α,ω-dicarboxylic acids were readily characterised by the presence of fragment

ions at *m/z* 98, [M–31]$^+$ and [M–73]$^+$ (for methyl ester derivatives).[14]

Vicinal dihydroxy fatty acids may be observed in extracts of all sherds with different mixtures of isomers and chain lengths, ranging from C_{16} to C_{22}, varying between sherds (Fig. 3). *Threo* and *erythro* pairs of diastereoisomers of vicinal dihydroxy fatty acids are present in all the chromatograms (Fig. 1), their mass spectra being almost identical. α,ω-Dicarboxylic acids are present in most of the sherds with

the distributions ranging from C_7 to C_{14} with the C_9, C_{10} and C_{11} components predominant. The α,ω-dicarboxylic acids and the vicinal dihydroxy fatty acids have both been shown to be associated with the oxidation of unsaturated fatty acids. The α,ω-dicarboxylic acids are formed through a complex sequence of reactions involving hydration, oxidative cleavage, ω-oxidation, and further bond cleavages.[15] Vicinal dihydroxy fatty acids are probably formed through secondary reactions between unsaturated fatty acids and hydroperoxides or peroxide radicals via epoxide intermediates.[16]

Importantly, the position of the hydroxyl substituents provides a direct indication of the original position of unsaturation in the precursor fatty acid.[3,11,16] In spite of the fact that unsaturated fatty acids are poorly preserved in potsherds, due to their susceptibility to oxidation,[6] the detection of these dihydroxy fatty acids preserved as part of a 'bound' or polymeric phase in potsherds may be used to infer the processing of commodities containing high abundances of mono-unsaturated fatty acids, e.g. plant and/or fish oils, in archaeological pottery vessels.

Eleven vicinal dihydroxy fatty acids may be observed in the alkaline extracts, with 9,10- and 11,12-dihydroxystearic isomers and 9,10-dihydroxypalmitic acids being detected in all the potsherds analysed; 11,12-dihydroxyarachidic acid is present in all but one potsherd (Fig. 3). 9,10-Dihydroxystearic acids are detected in relatively high abundance in all sherds, but the sherds can be separated into three groups according to their *threo* 11,12-dihydroxyarachidic acid distribution. One group shows a high abundance of 11,12-dihydroxyarachidic acids (sherds: ME17, MG19, and M935), a second group with a medium abundance (sherds: M571, E775, L705, and LN5) of this component, and the third group with a low abundance or absence of 11,12-dihydroxyarachidic acid (sherds: E762A, E762B, L687A, and L690). Another diagnostic dihydroxy fatty acid is 9,10-dihydroxypalmitic acid, which is observed in high abundance in all but one of the sherds (L687A). Indeed, the extract of sherd L687A is distinct from the others, since dihydroxybehenic acid is undetectable and only a low concentration of 9,10-dihydroxyarachidic acid is evident (Fig. 3). The varying compositions of the various sherds may be explained by: (i) processing of different marine commodities during the use of archaeological pottery vessels (e.g. fish, mammals, and shellfish); (ii) geographical and seasonal effects on the mono-unsaturated fatty acid distributions of marine animal tissues before their capture, e.g. North Sea species have higher abundances of $C_{20:1}$ and $C_{22:1}$ mono-unsaturated fatty acids during winter than summer;[17] and (iii) the processing of mixtures of either different marine products or marine and terrestrial commodities, such as plant oils and animals fats. The narrow range of vicinal dihydroxy fatty acids and their positional isomers present in the lipid extract of sherd L687A suggests that this residue did not arise from the processing of marine products (Fig. 3).

Semi-quantitative analyses performed on six sherds show the dihydroxy fatty acids concentrations to vary widely: from 0.05 to 14.05 μg g^{-1} (Table 1). The C_{18} vicinal diol isomers are observed at highest concentrations, with the lowest concentration detected being 0.27 μg g^{-1}. The distribution of variables (concentration × area) for components with the same carbon number appears to be normal within the range; a strong linear relationship (R^2 >0.98) is seen for all isomers of the same carbon number (Table 1). As already discussed

above such a wide range of vicinal dihydroxy fatty acids bearing C_{16} to C_{22} carbon atoms and the different positional isomers seen for a given homologue is typical of that expected to arise from the monounsaturated fatty acids commonly observed to occur in marine oils and fats. This is consistent with the coastal locations from which the potsherds were recovered and the other archaeological finds, i.e. the remains of marine fauna.[11,18,19]

The experimental approach detailed above has considerable potential value in the analysis of any plant, and marine or terrestrial animal oil/fats likely to contain significant concentrations of monounsaturated fatty acids. Since dihydroxy fatty acids are the direct degradation products of mono-unsaturated fatty acids, the detection of vicinal dihydroxy fatty acid distributions is a useful means of inferring the original fatty acid composition and thus which commodities were processed in archaeological pottery vessels. ω-(o-Alkylphenyl)alkanoic acids have also been used for detecting the processing of marine oil/fats in archaeological vessels; however, the formation of these compounds is reliant on protracted periods of vessel heating.[20] In the case of vicinal dihydroxy fatty acids, heating is unnecessary since oxidation of monounsaturated fatty acids occurs spontaneously even at room temperature.[16] Thus, their presence in archaeological pottery vessels is expected to be more common than that of ω-(o-alkylphenyl) alkanoic acids. Finally, such is the purity of the dihydroxy fatty acid fraction isolated by saponification and silica gel column chromatography that, assuming there is not a large quantity of other dihydroxy fatty acids present, e.g. isomers with ω-hydroxy functionalities, the vicinal dihydroxy fatty acids pattern could be, in principle, determined using GC alone.

CONCLUSIONS

An effective GC/MS-based protocol for the analysis of vicinal dihydroxy fatty acids preserved as polymerised/'bound' residues in archaeological potsherds was reported. The method comprises saponification, esterification, 'flash' silica gel column chromatography, and identification using GC/MS. A total of eleven vicinal dihydroxy fatty acids were detected with carbon numbers ranging from C_{16} to C_{22} with vicinal dihydroxy fatty acid diastereoisomers present in all instances. The wide range of dihydroxy fatty acids observed indicates that this approach may be applied confidently in screening archaeological potsherds for the degradation products of monounsaturated fatty acids derived from commodities processed in archaeological pottery vessels.

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