Molecular studies of organic residues preserved in ancient vessels

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Chapter 4

Identifying Biomolecular Origins of Solid Organic Residues using Direct Temperature - resolved Mass Spectrometry and Multivariate Analysis

Although several selective analytical techniques have been applied to the analysis of specific classes of compounds, such as extractable lipids, waxes, terpenoids and protein fragments, a non-selective analytical technique is required to characterise and categorise complete solid organic residues. In this study, Direct Temperature-resolved Mass Spectrometry (DTMS) is used for the characterisation of 34 solid residues. Sample preparation is limited to grinding very small samples (5 - 10 μg) and suspending them in 15 - 20 μl water. DTMS analysis of aliquots of this suspension (1 - 2 μl) gave information about a broad range of organic compounds, such as lipids, polynuclear aromatic hydrocarbons, markers for residual proteins and polysaccharides, and for newly formed complex condensed polymers. Multivariate analysis of the DTMS spectra identified five different chemotypes: groups of residues with comparable chemical characteristics. The biomolecular origin of each of these chemotypes is identified by comparison with experimentally charred reference materials. The chemotypes A_1 and A_2 consist of charred residues identified as starch-rich foods (mixed with animal or plant products), chemotype C consists of protein-rich charred animal products without starch, chemotype B contains smoke condensates from wood fires, and chemotype D consists of special protein-rich foods or non-food products containing little or no lipid fraction.

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1. Introduction

1.1. Organic residue analysis in ceramic study

The chemical characterisation of organic residues found in association with ancient ceramic vessels can provide archaeologists with valuable information about the daily lives of prehistoric people. Organic residue analysis can provide information about foods prepared, stored and served in ceramic vessels, some of which may not be detectable by any other means.
The chemical analysis of organic residues - both of solid surface residues and of residues absorbed into the ceramic of the vessel - has taken place as early as the 1920s, but has greatly expanded in the last two decades due to improvements in analytical instrumentation and an increasing interest in the functional aspects of pottery in ceramic studies (Rottländer & Schlichtherle 1980; Evershed *et al.* 1992; Heron & Evershed 1993; Evershed *et al.* 1999). Many analytical techniques have been applied to the study of specific classes of compounds such as solvent extractable lipids (Evershed *et al.* 1990; Evershed *et al.* 1999); waxes (Heron *et al.* 1994; Evershed *et al.* 1997d; Regert *et al.* 2001); terpenoids (Gianno *et al.* 1990; Charters *et al.* 1993a; McGovern *et al.* 1996; Dudd & Evershed 1998; Eerkens 2002) and amino acids (Evershed & Tuross 1996). The most recent developments include the use of gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) to identify isotope ratios of individual fatty acids absorbed in prehistoric and medieval ceramics (Evershed *et al.* 1994; Evershed *et al.* 1997b; Mottram *et al.* 1999) and the detection of the most abundant protein in milk (α-casein) using immunological methods (Craig & Collins 2000; Craig *et al.* 2000; Craig *et al.* 2005). Although these studies have facilitated the identification of a whole range of compounds in archaeological residues, and have provided plausible identifications for particular groups of residues, they are limited to specific compound classes. A non-selective technique is required to characterise the overall chemical composition of residues and categorise them accordingly.

1.2. Pyrolysis mass spectrometry in the study of ancient organic residues

Pyrolysis mass spectrometry (PyMS) is a rapid micro-analytical technique particularly suited for the identification of a wide range of natural organic polymers and other complex organic materials (Boon 1992; Moldoveanu 1998). Pyrolysis is the thermal dissociation of large molecules or polymers in an inert atmosphere that produces structurally specific and chemically characteristic fragments (also called 'pyrolysis products' or 'pyrolysates').
In Curie-point PyMS, or flash-pyrolysis, the sample is heated very rapidly to a set temperature dependent on the Curie-point of the metal filament (commonly between 500 ºC and 800 ºC). The resulting mass spectrum is a chemical 'fingerprint' indicative of the overall chemical composition of the residue. In the first Curie-point PyMS study of solid organic residues from ancient ceramics, indicative markers for a broad range of bioorganic moieties were detected and

subsequently identified using Curie-point PyGC/MS (Oudemans & Boon 1991). Protein remains indicative of severely denatured peptide chains could be detected, as well as polysaccharide remains in the form of various furans, phenols and benzenes. Lipids were detected in the form of fatty acids, and additional compounds formed from lipids under carbonising conditions could be identified. Multivariate studies (Chapter 2) of Curie-point PyMS measurements of a large group of residues showed five distinct groups of residues with specific chemical composition (Oudemans & Boon 1996).

1.3. DTMS – Direct Temperature-resolved Mass Spectrometry

New developments in analytical pyrolysis mass spectrometry have resulted in Direct Temperature-resolved Mass Spectrometry (DTMS) instrumentation. Commercially available DTMS instruments can measure much larger fragments (up to m/z 1000) then the locally developed Curie-point PyMS instrumentation (up to m/z 220). Additionally, DTMS gives temperature-resolved information. By gradually raising the temperature of the sample, a physical separation is achieved between low molecular weight compounds in the evaporation phase, and the cross-linked fraction of a sample in the pyrolysis-phase. The temperature-resolved information facilitates the interpretation of the results.
A collection of solid organic residues was analysed using DTMS in order to determine the chemical composition and identify the original biomaterials involved in their formation. Multivariate analysis was used to classify samples into groups of residues with similar chemical composition, so called 'chemotypes'. For each 'chemotype' identifications of the original biological materials involved are made by detailed comparison with experimentally charred modern reference materials.

2. Materials and Methods

2.1. Settlement

A large-scale excavation at Uitgeest-Groot Dorregeest in the Netherlands (Woltering 1982, 1983) uncovered habitation remains dating back to the Late Iron Age, the Roman Iron Age and the Medieval period. The indigenous settlement at Uitgeest-Groot Dorregeest (1 – 300 AD), was situated about 50 kilometre north of the Roman – German border, on top of a small, relatively dry sandy ridge formed by the remains of a coastal barrier and a sandy deposit from the Dunkirk I period (see also Appendix 1 for further description of the settlement site).

2.2. Samples and sample treatment

The ceramic assemblage of Uitgeest-Groot Dorregeest was studied extensively by Abbink (1985, 1999) and contains primarily simple, wide mouthed, globular or ellipsoid jars with short rim and neck and a maximum diameter equal to, or slightly larger than, the rim diameter. Many of the vessels contain visible surface residues of different kinds (Appendix 1, Table 3). In the assemblage of 147 partial vessels with identifiable morphological vessel type, soot residues occurred most commonly (45%); charred residues occurred on about every third vessel (32%); and other residues such as 'pigment' residues (5%) and 'creamy layers' (3%) occurred occasionally. Archaeological residues from all four categories were chosen for analysis (Table 1). Some of the residues originated from the group of partial vessels with identifiable morphological type (14), while others were taken from shards (20). In order to avoid selective sampling, residues recovered from different types of sediments were selected. All ceramics were washed with tap water immediately after excavation. The residue samples were scraped from the ceramic surface with a scalpel, after removal of the outermost 0.5 mm of residue.
In addition to the archaeological samples, a number of biomaterials were chosen as reference materials. Potato amylose (mw over 150000, Janssen Chimica) and a crystallised bovine blood albumin (BDH Biochemicals) were heated from 20 °C up to 250 °C (in 30 minutes) and subsequently progressively charred at 250 °C for 2 to 16.5 hours under a constant flow of nitrogen (100 ml/min). These charring conditions were chosen for two reasons: they were estimated to reflect conditions in cooking vessels on an open fire, and because earlier CuPyMS and CuPyGC/MS studies showed that heating at 250 °C for 2.5 hours were the minimum conditions needed for the formation of a condensed polymeric char network commonly observed in archaeological chars (Oudemans & Boon 1991; Pastorova *et al.* 1993b).
Sample preparation is limited to grinding 5 – 10 μg of residue with a small glass mortar and pestle, and suspending them in 15 – 20 μl ultra pure water (Millipore Q® grade). Multiple DTMS measurements (4) were performed on aliquots (1 – 2 μl) of each suspension over a period of three days.

2.3. Analytical procedures and instrumental conditions

DTMS measurements were carried out on a JEOL DX-303 double focussing (E/B) mass spectrometer equipped with a JEOL DA-5000 data system. The sample suspension was applied to the filament (Pt/Rh 9:1, 100 μm) of a direct insertion probe, which is resistively heated at 1 A/min in vacuo to a maximum temperature of ca. 800 ºC. Ions were generated under low voltage EI conditions (16 eV) in an ionisation chamber kept at 180 ºC and accelerated to 3 kV before being measured over a range of m/z 20 – 1000 at 1 s full range cycle time. Additional analytical work was performed on a selected number of residues using a JEOL JMS-SX/SX 102A with a JEOL MS-MP 9020D data system. In this mass spectrometer, the same filament was heated at a slower rate of 0.5 A/min, and the ions were generated under similar conditions in an ionisation chamber kept at 190 ºC and before being accelerated to 8 kV. Measurements were performed over a mass range m/z 20 – 1000 at a 3 s full range cycle time and post-accelerated to 10 kV. The resolution used was 1000.

Table 1: Archaeological residues and their chemical classification

Nr [a]	Find number	Sediment	Vessel Nr [b]	Residue Type	Residue colour	Pos.[c]	Chemo type [d]
1	7-7	Organic clay	7.07	Char	Brown	In	A_1
2	8-1	Organic clay	8.01	Pigment	Red brown	Ex	C
3	8-2	Organic clay	8.02	Char	Brown	In	A_2
4	8-5	Organic clay	8.05	Cream coloured	Beige	In	A_1
5	14-6-4.2	Sandy		Char	Brown	In	A_2
6	14-6-4.3a	Sandy		Char	Brown	In	D
7	14-6-4.3b	Sandy		Soot	Black	Ex	B
8	14-6-4.4	Sandy		Char	Brown	In	A_1
9	14-6-4.5	Sandy		Char	Brown	In	A_1
10	18-3-2a	Organic clay		Char	Brown	In	D
11	18-3-2b	Organic clay		Soot	Black	Ex	B
12	18-7	Organic clay	18.07	Char	Brown	In	A_2
13	19-7-90.2a	Organic clay	19.18	Char	Brown	In	A_2
15	20-4	Sandy	20.04	Char	Brown	In	A_1
16	20-4-157	Sandy		Char	Brown	In	A_2
17	30-12-3	Organic clay	30.02	Char	Brown	In	A_2
18	31-4a	Organic clay	31.04	Char	Brown	In	D
19	31-4b	Organic clay	31.04	Soot	Black	Ex	B
20	32-6-18	Peat		Char	Brown	In	A_2
22	33-5-2b	Peat		Char	Brown	In	A_1
23	33-8-2a	Peat		Char	Brown	In	A_2
24	33-8-2b	Peat		Char	Brown	In	A_2
25	34-0-12	Organic clay		Char	Brown	In	A_1
26	34-0-30	Organic clay	34.12	Char	Brown	In	C
28	34-7-95b	Organic clay		Soot	Black	Ex	E
29	34-11-3	Organic clay		Char	Brown	In	A_2
30	35-5-120	Sandy	35.33	Char	Brown	In	A_1
31	35-7-28	Sandy		Cream coloured	Beige	In	D
32	35-20	Sandy	35.20	Cream coloured	Beige	In	D
33	35-21	Sandy	35.21	Char	Brown	In	D
34	14-6-4.2b	Sandy		Char	Brown	In	C
35	14-6-4.3c	Sandy		Char	Brown	In	A_1
36	34-7-95a	Organic clay		Char	Brown	In	A_1
37	33-5-2ab	Peat		Char	Brown	In	A_1

[a] = Number corresponds to Chapter 2 (Oudemans & Boon 1996). Samples 14, 21 and 27 were excluded from this study due to limited sample size.

[b] = Vessel numbers are given when profile is is of identifiable morphological type, shards have no number (Abbink 1999).

[c] = The position of the residue on the ceramic vessel: in = interior of the vessel; ex = exterior of the vessel.

[d] = Chemotype defined according to DTMS analysis, see sections 4.1. through 4.7.

The summarised intensity of all ions as a function of time is called the Total Ion Current (TIC). The mass spectrum of each scan was registered separately, giving temperature resolved information. The mass spectra can be averaged over the entire heating time to give an "average mass spectrum" or "overall mass spectrum" which represents the overall chemical composition of the sample and is used as input for the multivariate comparison of samples. An average mass spectrum is calculated prior to normalisation by adding up the mass spectra in the selected scan range and averaging them.

2.4. Multivariate Analysis

The overall mass spectrum of each measurement was included in a dataset that was numerically analysed by Discriminant Analysis (DA) and Complete-linkage Cluster Analysis (CLCA) using the FOMpyroMAP multivariate analysis programme, a modified version of the ARTHUR multivariate analysis package produced by Infometrix Inc. (Seattle, USA, 1978 release). This procedure was used to reveal the main chemical similarities and dissimilarities between the samples and to group them according (Windig *et al.* 1982; Hoogerbrugge *et al.* 1983).
The validity of this kind of classification cannot be estimated easily for there is no cluster "validity measure" for hierarchical clustering techniques. Although theoretically cluster analysis is the most suitable multivariate technique (because no prior knowledge is available about the form or number of expected groups), no objective indicator exists to determine the quality of the proposed partitioning of the data (Lavine 2000). The stability of the combined DA and CLCA results is therefore established by performing a series of 34 test runs on the total sample set, minus one residue. New DFs were defined each time and a new CA was done with the excluded residue in the test set. The consistency of the classification of the residues was indicated by how often the excluded residue was subsequently classified in its original cluster and by the percentage of samples that were classified in their original cluster.

3. Results - Reference Materials

DTMS analysis of modern foods results in very specific mass spectra characteristic for the chemical composition of the original biomaterials. However, residues from archaeological context are often heated or (partially) charred and thus produce very different DTMS spectra. In order to understand the chemical changes during thermal degradation and produce reference materials, two modern foodstuffs (amylose and albumin) were experimentally heated and analysed using DTMS.

3.1. Amylose - Untreated and Experimentally Charred

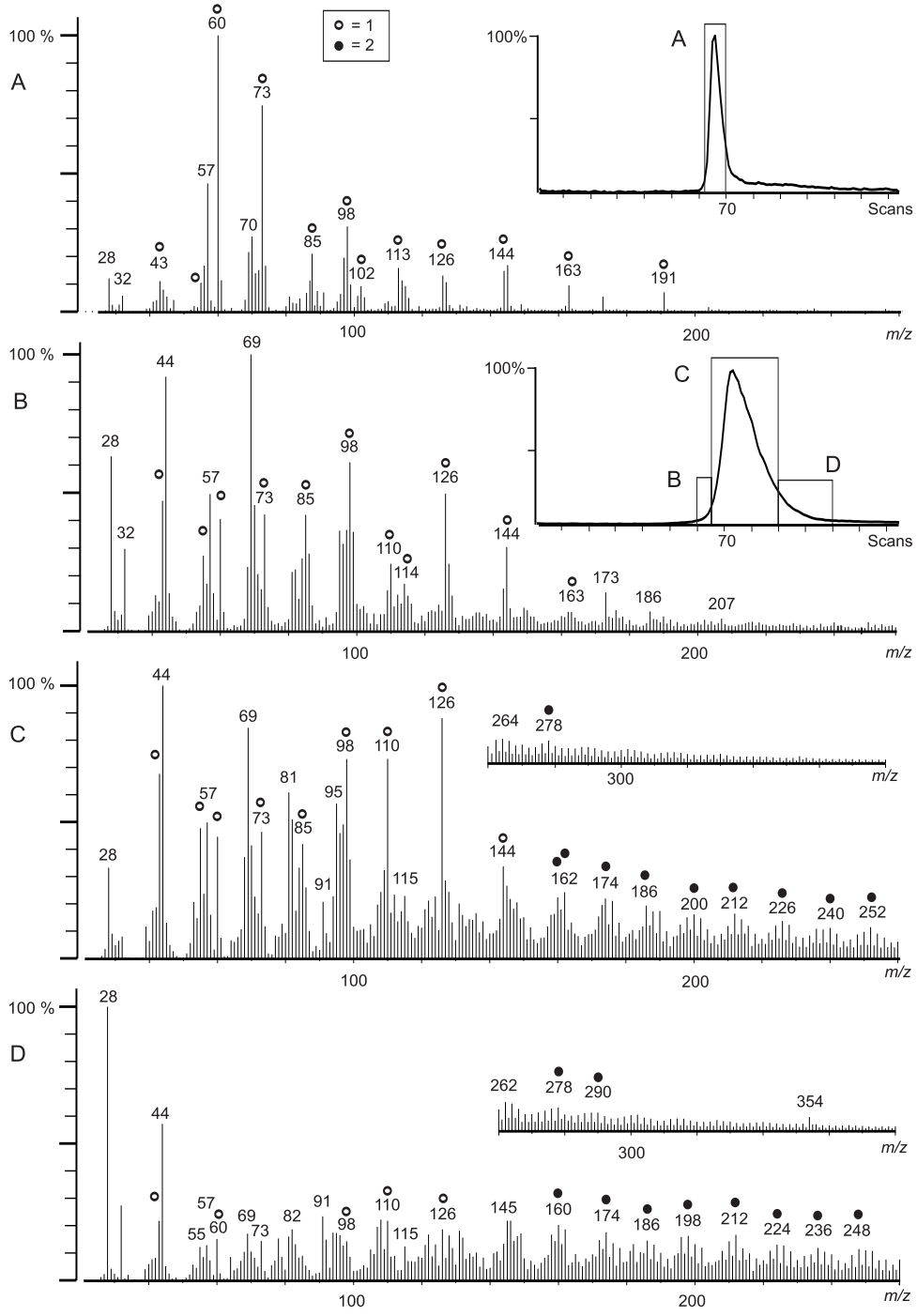
Amylose, one of the major macromolecular components of starch (Wong 1989; Davídek *et al.* 1990), was selected as a reference material to study the chemical effects of polysaccharide charring.

The DTMS spectrum of untreated amylose (Fig. 1A) shows all of the indicative markers for hexose polymers (Table 2) as identified in earlier studies using analytical pyrolysis (Meuzelaar *et al.* 1982; Pouwels *et al.* 1989; Boon 1992; Pastorova *et al.* 1993a). The absence of fragment ions in the higher mass area is indicative of a complete fragmentation of the polymer into smaller fragments and a lack of further ionisation induced fragmentation of the pyrolysis products. The given fragments are all formed around the same temperature (scans 60 – 72) indicating a structurally homogeneous sample.

In general, DTMS results of experimentally charred amylose (Fig. 1B – D and Table 2) are comparable to earlier studies of microcrystalline cellulose heated under anaerobic conditions up to 2.5 hours up to temperatures ranging from 190 to 390 ºC (Pastorova *et al.* 1993a; Boon *et al.* 1994; Pastorova *et al.* 1994). Some markers for intact hexose polymers are released from the amylose char (Fig. 1B) at slightly lower temperatures (scans 50 – 65) than in untreated amylose (Fig. 1A scans 60 – 72), which indicates fragmentation of high molecular-weight oligosaccharides into smaller units during the experimental charring. The high temperature part of the TIC (Fig. 1C – D) shows a typical higher mass fraction indicative of the condensed polymeric structure formed during heating of polysaccharides. The apex of the TIC profile has shifted to a higher temperature (from scan 67 to scan 73) and has broadened substantially, confirming the formation of a more thermally stable network polymer.

3.2. Albumin - Untreated and Experimentally Charred

Bovine serum albumin (BSA), a simple protein previously studied using Curie-point PyGC/MS (Munson & Fetterolf 1987), was selected to study the chemical effects of experimental protein charring. Detailed pyrolysis studies of amino acids performed in the 1980s and 1990s (Meuzelaar *et al.* 1982; Tsuge & Matsubara 1985; Chiavari & Galetti 1992) have identified specific pyrolysis products for many individual amino acids (Table 3). However, non-specific complex mixtures of primary and secondary fragmentation-products occur regularly in pyrolysis studies of proteinaceous materials and can vary extensively in composition depending on the pyrolysis conditions (Moldoveanu 1998, 373-397). Pyrolysis of polypeptides or intact proteins commonly reveals two groups of products: i) "amino acid markers" similar to those obtained from pyrolysis of single amino acids, and ii) "peptide & protein markers" indicative of two or more amino acids occurring in their original chain structure. These peptide markers originate from cyclisation of two amino acids prior to pyrolytic cleavage and may include diketopiperazines (DKPs) and their secondary fragmentation products (Smith *et al.* 1988; Stankiewicz *et al.* 1996); substituted 2,4-imidazolindiones (Munson & Fetterolf 1987;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Stankiewicz *et al.* 1996); and di-substituted 3-alkenyl-5-alkyl-pyrrolidin-2,4-diones and their corresponding 2,4-dialkyl-3,5-diketopyrroline isomers (Boon & De Leeuw 1987).

Table 2: Markers for hexose polymers in DTMS (16 eV EI) spectra.

Pyrolysis markers	m/z values	reference
Markers for Intact Hexose Polymers		
hexose polymers (amylose, cellulose, amylopectines)	31, 43, 55, 60, 73, 85, 97/98, 102, 110, 113, 114, 126, 144, 163, 191	[1,2,3,4]
Markers for Charred Hexose Polymers (250 – 310 °C)		
alkylated benzenes	92, 106, 120, 134, 148, 162, 176, 190	[4,5]
alkylated benzofurans	132, 146, 160, 174, 188	[4,5]
dibenzofurans	168, 182, 196, 210, 224, 238	[4,5]
alkyl-benzene aromatics	92, 104	[4,5]
alkyl-phenols	108, 122	[4,5]
Markers for Charred Hexose Polymers (over 310 °C)		
highly condensed aromatic structure	160, 162, 174, 176, 186, 188, 190, 198, 200, 202, 212, 214, 224, 228, 236, 238, 240, 248, 250, 252, 276, 278, 288, 290	[6]
CO and CO_2	28, 44	[6]

[1] = Meuzelaar et al. 1982; [2] = Pouwels et al. 1989; [3] = Boon 1992; [4] = Pastorova et al. 1993a; [5] = Boon et al. 1994; [6] = Pastorova et al. 1994.

DTMS results for untreated BSA (Fig. 2A - B) show both groups of pyrolysates (Table 3). Fragments are generated over a narrow temperature range (scans 62 - 80) indicating a structurally homogeneous sample. Intact peptide and protein markers are primarily generated at the beginning of the TIC curve (Fig. 2A), while amino acid markers are liberated over the entire temperature range (Fig. 2A - B).

Earlier DTMS studies of whole peas and of Pisane HD (pea protein isolate) heated under anoxic conditions up to temperatures ranging from 130 to 700 ºC for a maximum of 2 hours (Braadbaart 2004; Braadbaart *et al.* 2004a), showed markers for many protein chars (Table 3). The DTMS results for bovine serum albumin heated at 250 °C for up to 17 hours (Fig. 2C - D)

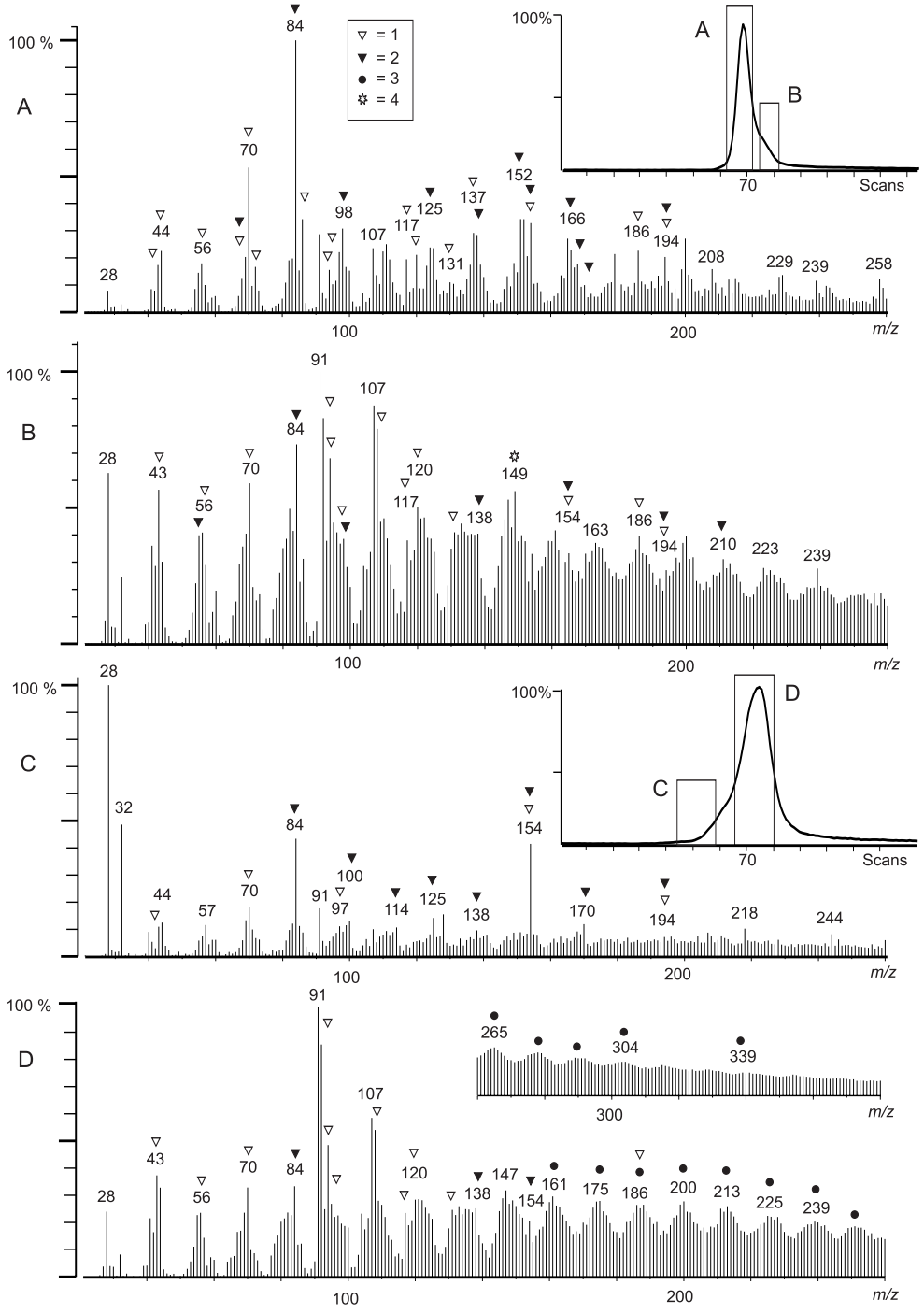
Figure 1 (on facing page): DTMS results for Amylose.
A: Mass spectrum for untreated potato amylose shows markers for (1) intact hexose polymers.
B – D: Mass spectra for potato amylose experimentally heated for 17 hours at 250 °C. B: The partial mass spectrum for low temperatures still includes many markers for (1) residual oligosaccharides. Partial mass spectra for higher temperature phases C and D are increasingly characterised by the presence of (2) a newly formed, thermally stable polymeric structure.

Table 3: Markers for proteins, polypeptides, amino acids in DTMS spectra

Pyrolysis markers	m/z values	ref.
Markers for Amino Acids		
Arginine	69, 114	[1]
Asparagine	97, 99	[1]
Cysteine	34, 93, 103, 127	[1,2]
Glutamine	67, 83, 97	[1]
Hydroxy-proline	67, 69, 95, 113, 160, 174, 186	[1]
Leucine and Isoleucine	69, 70, 83, 86, 87, 153, 155, 226	[1,3]
Lysine	69, 128	[1]
Methionine	34, 48, 74, 90, 105, 145	[1, 2]
Phenylalanine	92, 104, 106, 117, 121, 182	[1,2,3]
Proline	69, 71, 194	[1]
Tryptophan	117, 131, 143, 145, 156, 160, 170	[1,2,3]
Tyrosin	94, 108, 120, 122, 137	[1,2,3]
Valine	56, 73, 69, 72, 85, 99, 125, 127, 154, 198	[1,3]
Markers for Peptides and Proteins		
Diketopiperazines (DKP) (and additional smaller fragments)	154, 166, 168, 170, 182, 192, 194, 196, 198, 210 (58, 67, 68, 69, 70, 85, 87, 92, 99, 114, 124, 125, 138)	[4,5]
Substituted 2,4-imidazolidindiones (and additional smaller fragments)	100, 114, 142, 156 (29, 45, 57, 72)	[6,7]
3-alkyl-5-alkyl-pyrrolidine-2,4-diones & 2,4-dialkyl-3,5-diketopyrrolines isomers	55, 69, 84, 98, 124, 138, 152, 166, 180, 181, 195, 209	[5,6]
Markers for charred Proteins		
Alkylphenols/alkylbenzenes	91, 92, 94, 105, 107, 108, 119, 122, 131	[8,9]
N-containing heterocyclic compounds	117, 131, 133, 147, 161, 175, 189, 199, 213, 227	[9]
Benzene and Aromatic compounds	78, 146, 160, 174	[9]
HCN	27	[9]
$CO/CO_2/SO/SO_2$	28, 44, 48, 64	[9]
Benzene/Toluene	78, 92	

[1] = Chiavari & Galetti 1992; [2] = Meuzelaar et al. 1982; [3] = Tsuge & Matsubara 1985; [4] = Smith et al. 1988; [5] = Stankiewicz et al. 1996; [6] = Boon & De Leeuw 1987; [7] = Munson & Fetterolf 1987; [8] = Moldoveanu 1998; [9] = Braadbaart 2004.

Figure 2 (on facing page): DTMS results for Bovine serum albumin.
A – B: Partial mass spectra show a mixture of markers for (1) amino acids; (2) peptides and proteins, including markers for Diketopiperazines (DKPs); 3-alkyl-5-alkyl-pyrrolidine-2,4-diones and their matching 2,4-dialkyl-3,5-diketopyrrolines isomers and (4) a contaminant.
C – D: Bovine serum albumin experimentally heated for 17 hours at 250 °C. C: Partial mass spectrum still includes many markers for (1) peptides and proteins. D: Higher temperature partial mass spectrum also includes markers for (3) a newly formed, thermally stable polymeric structure.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are similar to those from heat-treated Pisane HD and most closely resemble mild thermal degradation products (Braadbaart *et al.* 2004a).
In charred BSA, part of the indicative peptide and protein markers (*m/z* 100, 114, 154, 170) are still present at temperatures slightly lower than in untreated BSA (Fig. 2C), indicating breaking of peptide bonds and formation of smaller peptides during experimental charring. At higher temperatures (Fig. 2D) the mass spectrum contains few markers for peptides and intact proteins and is dominated by amino acid markers and a wide spectrum of masses indicative of a condensed thermostable polymer. This polymer is characterised by the presence of odd numbered masses (*m/z* 117, 131, 133, 147, 161, 175, 189, 199, 213, 227). The apex of the TIC profile has shifted to a higher temperature (from scan 68 to scan 75) and has broadened substantially, confirming the formation of a more thermally stable condensed structure.

4. Results - Archaeological Residues

4.1. Multivariate Analysis

DTMS data of archaeological residues do not show such specific or immediately recognisable peak patterns. In order to identify significant combinations of chemical characteristics in DTMS spectra of archaeological residues, multivariate techniques were applied. Discriminant analysis was used to illustrate the differences within the set of samples, and to identify the mass values that determine those differences. Six Discriminant Factors (DFs), explaining 42.4%, 19.9%, 9.3%, 7.6%, 5.4% and 3.9% of the total relevant variance in the dataset respectively, were considered important for the chemical composition of the residue material. The map of discriminant functions DF_1 and DF_2 visualises the chemical similarity and dissimilarity between the residues (Fig. 4a). In the reduced six dimensional discriminant space defined by DF_1 through DF_6, clusters of chemically similar residues were determined using hierarchical complete-linkage cluster analysis (CLCA). A dendrogram representing the similarity matrix of all 132 mass spectra shows six groups of samples - clusters A_1, A_2, B, C, D and E - with similarity values of 0.70 or more (Fig. 3). Cluster A_1 and A_2 are named as subdivision of what used to be one cluster in Chapter 2 (Oudemans & Boon 1996), but are of the same level as the other clusters. Multiple measurements of the same residue gave similarity values of 0.9 or more (with the exception of sample 35).
The stability of the combined DA and CLCA results is established by performing a series of test runs that showed the consistency of the classification of the residues (see Table 4). The overall figures showed how often the test residue was classified within its original cluster and what percentage of total number of residues, were classified in their own cluster. The overall numbers are above 80% and indicate the relative consistency of the classification as defined by this method. Larger clusters are more consistent than the smaller clusters.

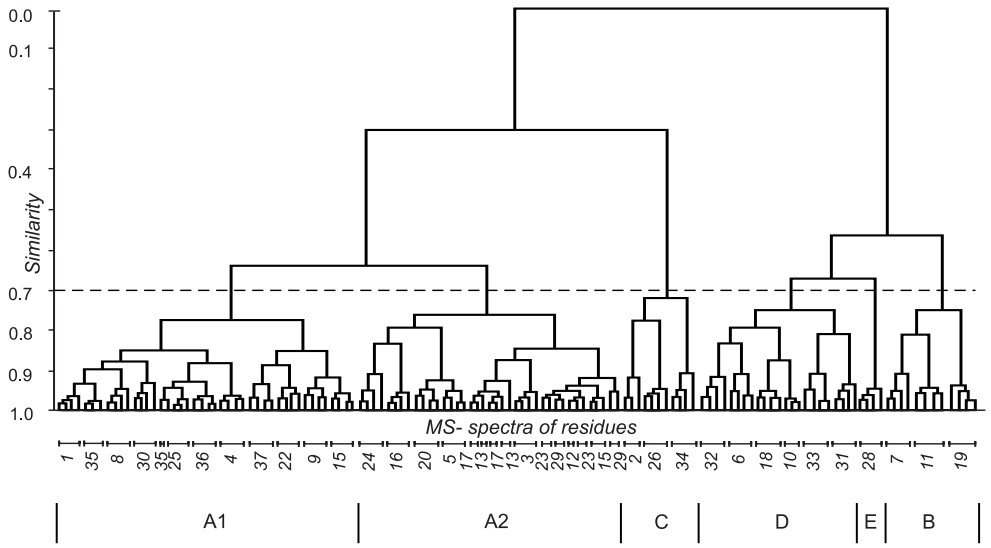


Figure 5: Complete-linkage dendrogram of the measured mass spectra of organic residues.
Residues are indicated with their sample number (see Table 2). Chemotypes show a similarity of 0.70 or higher. Similarity of multiple measurements of one residue is > 0.90 (except residue 35). Although multiple measurements mostly cluster with one another first, exceptions can been seen (residues 35, 17, 13, 23, 29 and 12).

Table 4: Chemotypes and their statistical consistency

Chemotype	A_1	A_2	B	C	D	E	Total
N	11	10	3	3	6	1	34
Residues	1, 4, 8, 9, 15, 22, 25, 30, 35, 36, 37	3, 5, 12, 13, 16, 17, 20, 23, 24, 29	7, 11, 19	2, 26, 34	6, 10, 18, 31, 32, 33	28	
Consistency of classification [a]	100%	93%	33%	100%	67%	0%	82%
Cluster Stability [b]	94%	96%	80%	88%	76%	76%	89%
Residues that shift position [c]	4, 25, 35, 36	16, 24	11	26, 34	6, 31, 32, 33	28	

[a] Consistency of classifications: percentage of residues from a cluster that classify in the same cluster during their test run.

[b] Cluster stability: percentage of residues in a cluster that do not shift position (averaged over 34 test runs). When a test divides a cluster in two subgroups, the cluster was defined as the main group containing the majority of the samples.

[c] Residues that shift position: sample numbers of residues that shift position three or more times during 34 test runs.

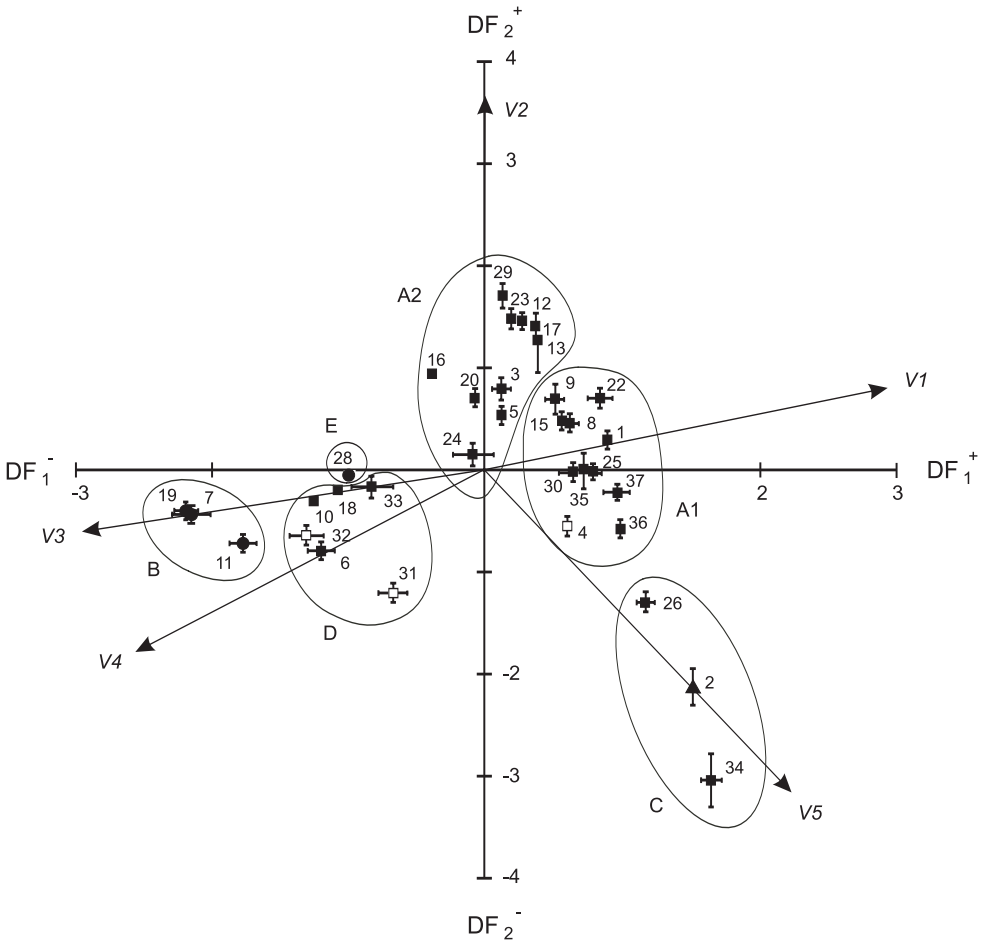
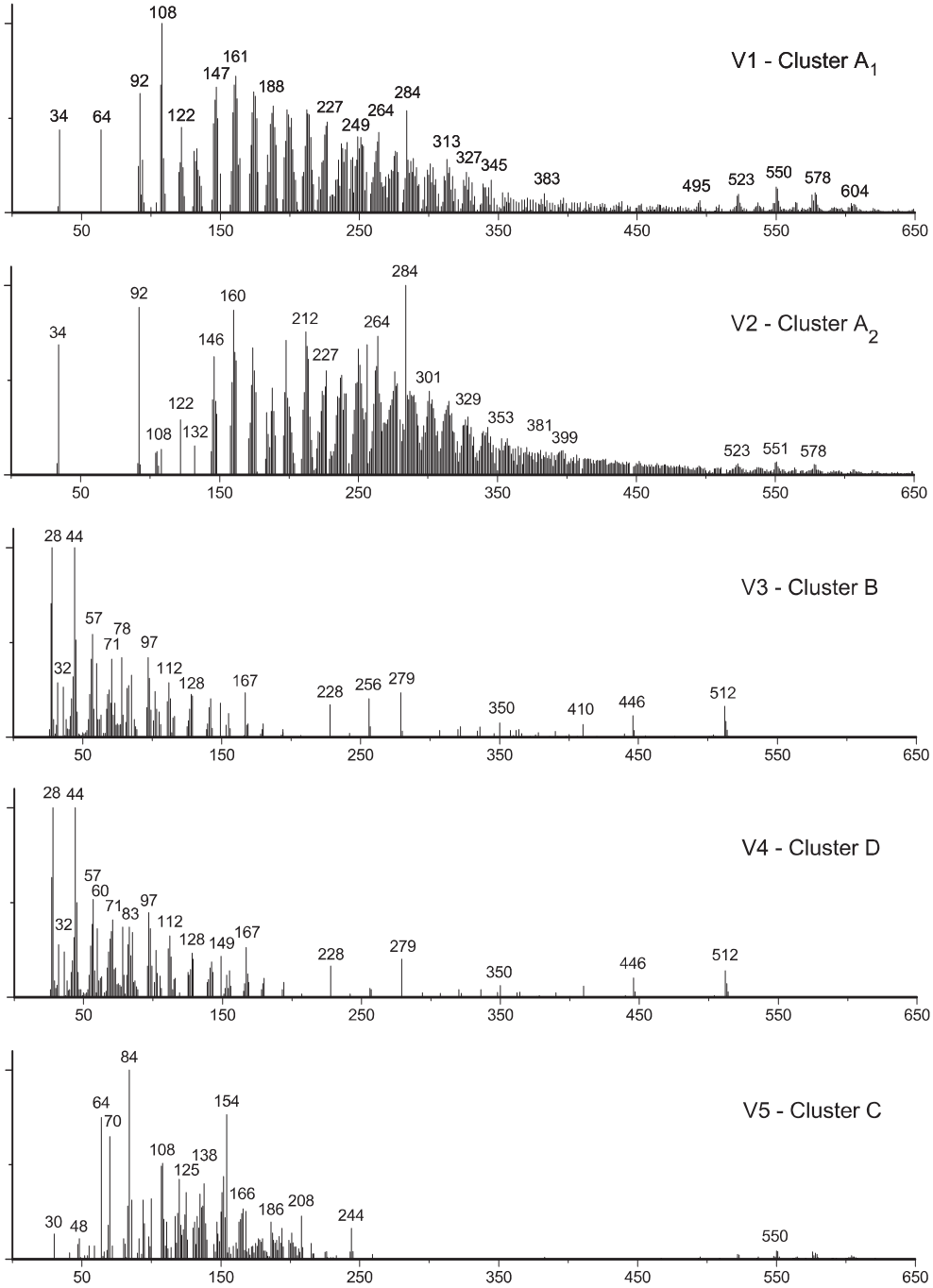


Figure 4: Discriminant map with chemotype clusters (A_1 through E).
A: In the discriminant map of DF_1 versus DF_2 the relative difference in chemical composition between residues, is expressed by their Euclidian distance. Multiple measurements are indicated by standard deviation error-bars. Clusters of samples with a similarity value S>0.70 have been indicated. Each cluster represents a particular chemotype. Chars on vessel interior (■); black residues on vessel exterior (●); red-brown residues (▲); and cream coloured residues (□).
B (on facing page): Vectors representing the typical chemical characteristics of clusters A_1 through D. Each chemotype is characterised by a set of correlated m/z values depicted in the rotational vectors 1 through 5. The vector spectra depict variations with respect to the zero-point spectrum. Chemotype E is not represented due to it similarity to chemotype B.


V1 - Cluster A1
108
161
147
92
188
227
284
34
64
122
264
249
313
327
345
383
495
523
550
578
604
V2 - Cluster A2
284
92
160
212
264
34
146
227
301
122
329
353
381
108
132
399
523
551
578
V3 - Cluster B
28 44
57
78
71
97
32
112
128
167
228
256
279
350
410
446
512
V4 - Cluster D
28 44
57
60
71
83
97
32
112
128
149
167
228
279
350
446
512
V5 - Cluster C
84
64
154
70
108
138
125
166
186
208
30
48
244
550
50
150
250
350
450
550
650

4.2. Chemotypes

In Figure 4a each cluster represents a particular chemotype: a group of residues with a recurring combination of chemical characteristics. Each chemotype is characterised by a set of correlated m/z values as represented by the DF scores in the rotational vectors 1 through 5 (Fig. 4b). Chemotype E is not depicted because it is determined primarily by contaminants such as phthalates and elementary sulphur (see also section 4.6.). The vector spectra depict variations with respect to the zero-point spectrum and are defined through graphical rotation (Windig *et al.* 1982). Mass values that appear in all samples will not be shown as typical characteristic for either direction. When studying the complete chemical composition, the original mass spectra must always be considered.

4.3. Chemotype A_1

Chemotype A_1 (Fig. 4b, vector 1) is primarily characterised by: i) a lipid profile with markers for intact lipids, such as diacylglyceryl-fragments and acylium ions (see also Table 5); ii) amino acid markers (Table 3); and iii) an envelope of higher molecular weight compounds including odd numbered compounds indicative of a condensed polymeric aromatic structure (Table 2 and 3). The DTMS data for charred residue 1 (nr. 7-7), a representative sample for cluster A_1, show an evaporation range (Fig. 5A) with intense mass peaks for diacylglyceryl-fragments (DAG 14 - 36) and acylium ions (C12:0 – 18:0). Even numbered DAGs are visible as m/z values for $[M\text{-}RCOOCH2]^+$ ions, although their intensity suggests an additional contribution from fragment ions $[M\text{-}RCOO]^+$ and $[M\text{-}RCOOH]^+$ of odd numbered DAGs (DAG 15 - 35). The presence of unsaturated triacylglycerides is indicated by $[M\text{-}RCOO]^+$ and $[M\text{-}RCOOH]^+$ fragment ions with m/z values 2, or 4 points lower then the saturated ones (DAG 36:1, 36:2, 34:1, 34:2, 32:1, 30:1, 28:1, 26:1, 24:1 and 22:1). Saturated free fatty acids (FA 10:0 - 18:0), unsaturated free fatty acids (C18:1) and cholesterol can be observed in the spectrum.
The pyrolysis range (Fig. 5B) shows few markers for peptides and intact proteins (m/z 138, 154, 168) and is dominated by amino acid markers and a wide range of masses indicative of a condensed polymeric structure. The spectrum shows the presence of both even and odd numbered peaks in the mass range m/z 140 – 300, indicating the incorporation of nitrogen containing compounds into the condensed aromatic material.

4.4. Chemotype A_2

Chemotype A_2 (Fig. 4b, vector 2) is primarily identified by: i) a lipid fraction with some markers for intact lipids, high amounts of free fatty acids and small amounts of phytosterols; and ii) an envelope of higher molecular weight compounds similar to the one in cluster A_1 but with relatively more even numbered mass peaks.

Table 5: Markers for lipids in DTMS spectra.

Pyrolysis markers	*m/z* values	Ref.
Markers for Triacylglycerides		
M^+ (TAG 34 – 36)	610, 638	[1]
M-18 (TAG 34 – 38)	592, 620, 648	[1]
$[M\text{-}RCOOH]^+$ even: DAG 16 – 36	326, 354, 382, 410, 438, 466, 494, 522, 550, 578, 606	[1]
$[M\text{-}RCOOH]^+$ odd: DAG 15 – 35	312, 340, 368, 396, 424, 452, 480, 508, 536, 564, 592	[1]
$[M\text{-}RCOO]^+$ even: DAG 16 – 36	327, 355, 383, 411, 439, 467, 495, 523, 551, 579, 607	[1]
$[M\text{-}RCOO]^+$ odd: DAG 15 – 35	313, 341, 369, 397, 425, 453, 481, 509, 537, 565, 593	[1]
$[M\text{-}RCOOCH2]^+$ DAG 22 – 36	425, 453, 481, 509, 537, 565, 593	[1]
$[RCO + 128]^+$ C12:0 – 18:0	311, 339, 367, 395	[1]
$[RCO + 74]^+$ C10:0 – 18:0	229, 257, 285, 313, 341	[1]
$[RCO]^+$ C12:0 - 18:0	183, 211, 239, 267	[1]
$[RCO\text{-}1]^+$ C10:0 - 18:0	154, 182, 210, 238, 266	[1]
Markers for Monoacylglycerides		
$[M\text{-}CH2OH]^+$	187, 215, 243, 271, 299, 327	[1]
$[RCO]^+$ C12:0 - 18:0	183, 211, 239, 267	[1]
Free Fatty Acid Markers		
M^+* unbranched FA12:0 – 28:0	200, 228, 256, 284, 312, 340, 368, 396, 424	[2]
M-OH = $[RCO]^+$ FA12:0 - 18:0	183, 211, 239, 267	[2]
$[(CH2)nCOOH]^+$ Up to FA20:0	73, 87, 101, 115, 129, 143, 157, 171, 185, 199, 213, 227, 241	[2]
$[M\text{-}H2O]^+$ FA16:1 – 18:1	236, 264	[2]
Markers for Sterols		
Cholesterol (C27) animal source	386, 368, 275	
Brassicasterol (C28) plant source	398	[3]
Ergosterol (C28) fungal source	396, 363, 253	
Campesterol (C28) plant source	400, 382	[3]
?-Sitosterol (C29) plant source	414, 396, 399, 329, 303	[3]
Stigmasterol (C29) plant source	412, 394	[3]
Alkylated steradienes (C27, C28, C29)	368, 382, 396	[4]
Markers for Natural Waxes		
Long-chain FA (FA20:0 –28:0)	312, 340, 368, 396, 424	[5,6,7]
Monoesters ME38 – ME52	536, 564, 592, 620, 648	[5,6,7]

[1] = Odham & Stenhagen 1972b; [2] = Odham & Stenhagen 1972a; [3] = Knights 1967; [4] = Braadbaart 2004; [5] = Nelson & Blomquist 1995; [6] = Kolattukudy 1976; [7] = Bianchi 1995.

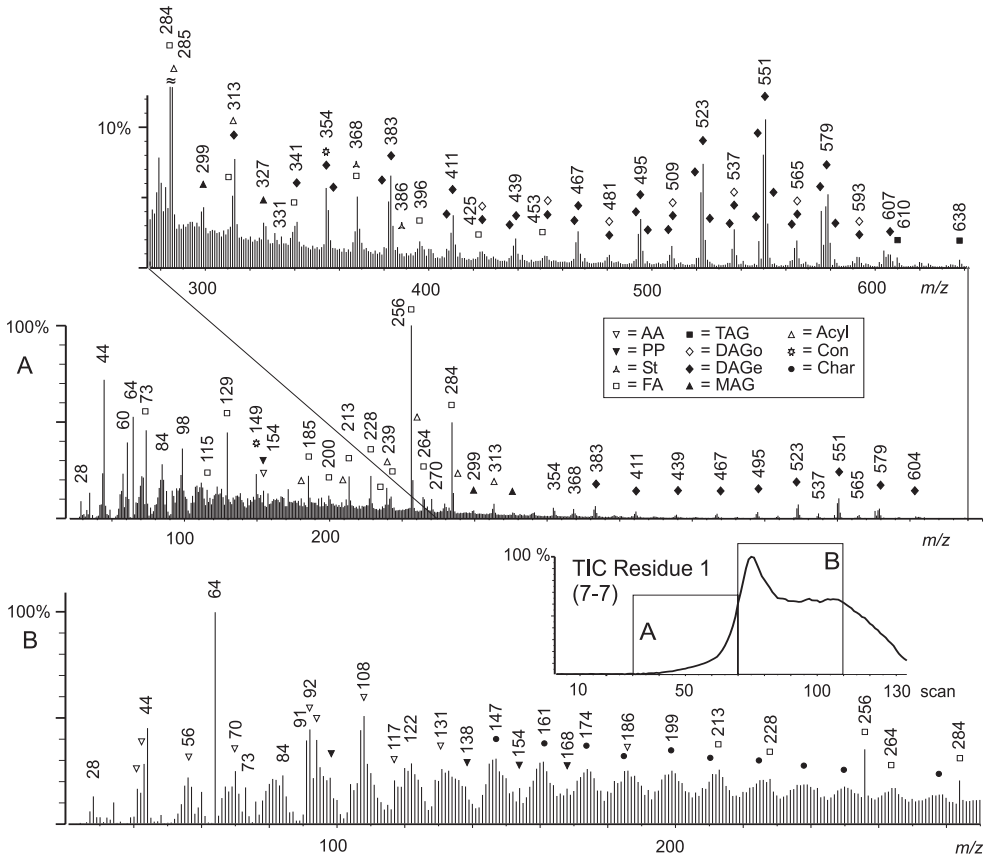


Figure 5: Cluster A_1 - DTMS results for charred residue 1 (nr. 7-7)
A: Partial mass spectrum of the low temperature range shows a mixture of lipid indicators including (FA) fatty acids and their fragments; (TAG) triacylglycerides M^+ ions; (DAGe) even numbered diacylglyceryl-fragments; (DAGo) odd numbered diacylglyceryl-fragments; (Acyl) acylium-ions; (MAG) Monoacylglycerides; and (St) cholesterol. Some markers can be seen for (PP) peptides and proteins and (AA) amino acids and an occasional contaminant (Con).
B: High temperature partial mass spectrum clearly shows markers for (Char) thermally stable condensed polymeric network.

DTMS results for a representative charred residue 23 (nr. 33-8-2a) are shown in Fig. 6. The lipid profile of charred residue 23 (Fig. 6A) shows high mass peaks for saturated free fatty acids (C10:0 – 28:0); some markers of DAG fragments (DAG 30 – 36) and small amounts of unsaturated free fatty acids (such as *m/z* 236, 264 for C16:1 and 18:1). An additional series of minor peaks (*m/z* 312, 326, 340, 354, 368, 382/384, 394/396/398, 410/412/414/416, 424/426/428) indicates the presence of what might be markers for sterols (*m/z* 368, 380, 382, 386, 394, 396, 400, 412, 414). At the high end of the mass spectrum an occasional peak is visible for what could represent the molecular ion of C42 and 44 wax esters (*m/z* 620, 648).

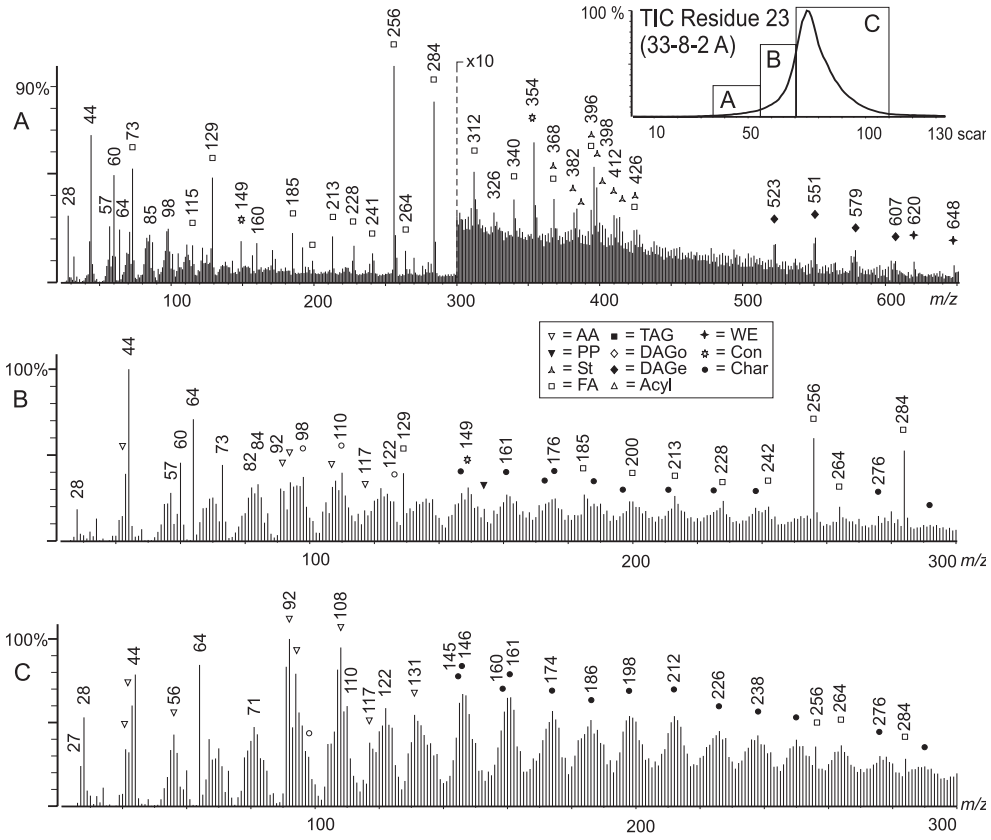


Figure 6: Cluster A_2 - DTMS results for charred residue 23 (nr. 33-8-2a)
A: Partial mass spectrum of the low temperature range shows a mixture of lipid indicators including (FA) free fatty acids; (DAGe) even numbered DAGs; (Acyl) acylium-ions; (St) various sterols including plant sterols and cholesterol; (WE) some markers for wax esters; and (Con) an occasional contaminant. B: At slightly increased temperatures, additional markers can be seen for (PS) residual oligosaccharides; and (Char) a condensed polymeric network. C: The high temperature mass spectrum contains mostly markers for (AA) amino acid and the (Char) thermally stable condensed polymeric network.

In the medium temperature range (Fig. 6B), some markers for intact polysaccharides, functionalised furans and alkyl-furans (m/z 98, 110), for intact peptide or proteins (m/z 154) and for residual fatty acid (m/z 256, 284) can be seen. The pyrolysis range (Fig. 6C) shows series of m/z values indicative of a highly condensed aromatic material. Peaks with both odd and even values are present. The peak for m/z 64 (SO_2) is very prominent in the higher temperature range, which suggests the presence of sulphur-containing inorganic compounds.

Table 6: Markers for other compounds in DTMS spectra.

Pyrolysis markers	*m/z* values
Markers for Aromatic Hydrocarbons	
Alkylated Benzenes	91, 92, 115, 119
Alkylated Naphthalenes	128, 142, 156, 170
Alkylated Phenantrenes/Antracenes	178, 192, 206, 220, 234
Alkylated Pyrenes	202, 216, 230, 244, 258
Alkylated Chrysenes	228, 242, 256, 270, 284
Markers for other compounds	
Aliphatic compounds	55, 56, 57, 69, 70, 71, 83, 84, 85, 97, 98, 111, 112, 113, 125, 126, 139, 140, 153, 154, 167, 168, 181, 182, 195, 196
CO and CO_2	28, 44
SO and SO_2	48, 64
H_2S	34
Elementary Sulphur	32, 64, 96, 128, 160, 192, 224, 256
Markers for Contaminants	
Phthalates	149, 167, 279
Squalene	410
Santovac 5 (pentaphenylether)	354, 446

4.5. Chemotype C

Chemotype C (Fig. 4b, vector 5) is characterised by: i) markers for peptides or intact proteins; and ii) amino acid markers.

DTMS results for representative charred residue 26 (nr. 34-0-30) show a lipid profile (Fig. 7A) comparable to that of residue 1 (nr. 7-7), including high peaks for saturated free fatty acids (C8:0 - 18:0) and small amounts of unsaturated free fatty acids (C16:1 and 18:1). Intact lipids are indicated by markers for acylium ions (C10:0 - C18:0); DAG - fragments both saturated (DAG 14 - 36) and unsaturated (DAG 36:1, 36:2, 34:1, 34:2, 32:1, 30:1, 28:1, 26:1, 24:1 and 22:1) as well as odd numbered DAGs (DAG 15 - 35), monoacylglyceryl-fragments (MAG 16:0 - 18:0) and cholesterol.

The pyrolysis range (Fig. 7B) shows residual markers for peptides and intact proteins such as diketopiperazines (DKPs) and alkylated pyrrolidinediones and their fragmentation products (*m/z* 98, 138, 154, 168) and relatively intense mass peaks for amino acid moieties (*m/z* 91, 92, 94, 107, 108, 117, 131) indicative for a protein fraction comparable to the experimentally charred BSA (Fig. 2D).

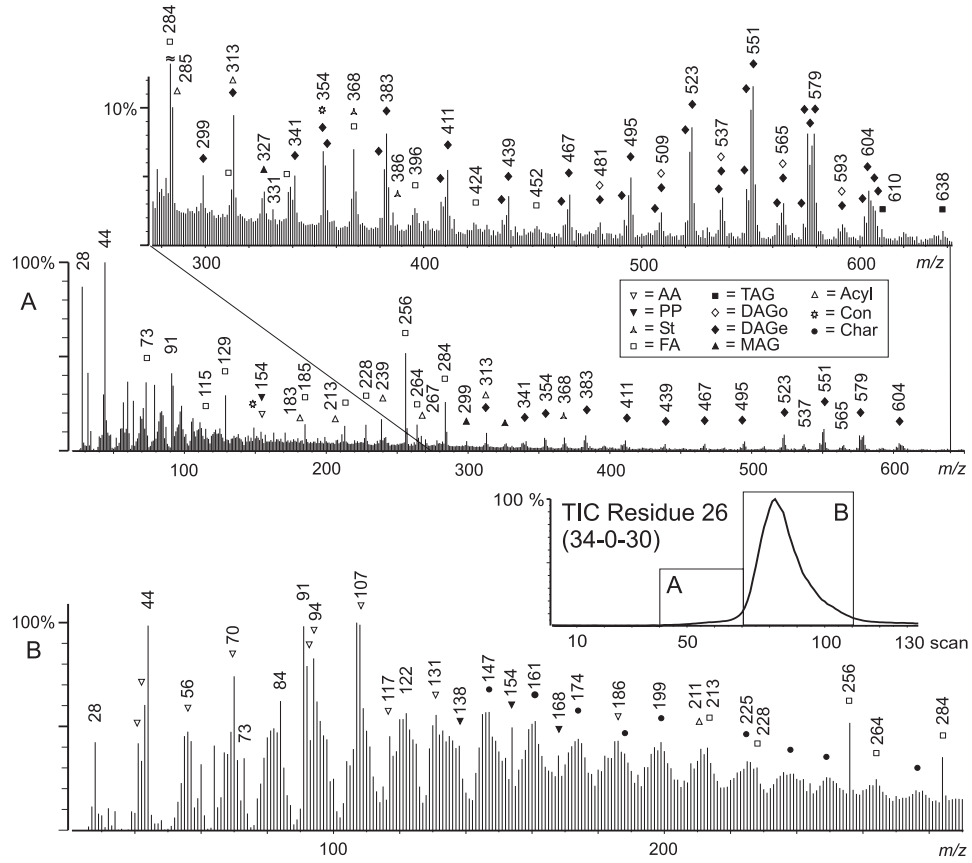


Figure 7: Cluster C - DTMS results for charred residue 26 (nr. 34-0-30)
A: The low temperature mass spectrum shows a mixture of lipid indicators including (FA) free fatty acids and their fragments; (TAG) triacylglycerides M^+ ions; (DAGo) odd numbered and (DAGe) even numbered DAGs and their fragments; (Acyl) acylium-ions; (MAG) MAGs; and (St) cholesterol. Some markers can be seen for (PP) peptides and proteins and (AA) amino acids and an occasional contaminant (Con). B: The high temperature mass spectrum also (Char) markers for a thermally stable condensed polymeric network.

4.6. Smaller Chemotypes B, D and E

The masses characteristic for the clusters B (vector 3), D (vector 4) and E (not depicted) are very similar, which is to be expected from their close association within the DF plot (Fig. 4a). The vectors generally depict the same characteristics: i) high peaks for m/z values 28, 32 and 44 throughout the measurement indicative of the presence of some air background in the ionisation chamber; ii) markers for short chain aliphatic compounds; iii) markers for alkylated

aromatic compounds; iv) some markers for sulphur-containing compounds (*m/z* 32, 34, 64, 66, 128, 256); and v) a small number of markers for contaminants visible in the low temperature range and the high temperature range (Table 6). All these characteristics indicate a low organic content for these residues, which causes the presence of small amounts of air and contaminants to play a role in the spectra and thus in the determination of their chemotype. The distinction between the smaller chemotypes can be seen more fully when looking at individual measurements in more detail.

Chemotype B

Soot residue 19 (nr. 31-4b) shows *m/z* values for sulphur-containing compounds and short chain aliphatic compounds in the evaporation range (Fig. 8A). No lipids can be detected. In the pyrolysis range (Fig. 8B) some amino acid markers can still be detected, although the majority of the peaks belong to alkylated aromatic compounds and the overall mass distribution suggests one or more homologous series with varying degrees of saturation such as alkylated benzenes and alkylated naphthalenes. As the pyrolysis-temperature increases (Fig. 8C), only markers for alkylated polyacenes can be seen ranging from naphthalene and phenanthrene to chrysene, in combination with some markers for small fragments (*m/z* 27, 28, 44, 64).

Chemotype D

Cream coloured residue 32 (nr. 35-20) primarily shows markers for sulphur-containing compounds and a peak representing a nitrogen-containing aromatic compound of unknown origin (*m/z* 173) in the evaporation range (Fig. 9A). No lipid markers can be detected. At slightly increased temperatures (Fig. 9B) aliphatic compounds become more prevalent. In the pyrolysis range (Fig. 9C) markers for a relatively intact protein fraction become evident. Some markers for peptides or intact proteins could be detected in minor amounts (*m/z* 138, 152, 154, 170, 194), and many markers for amino acids could be detected in relatively high intensities (*m/z* 70, 91, 92, 94, 98, 107, 108, 117, 131).

Chemotype E

Soot residue 28 (nr. 34-7-95b) primarily shows *m/z* values for sulphur-containing compounds and aliphatics. No lipids can be detected, but phthalates are present. This residue contains so little organic material, that it is not further discussed.

4.7. Comparing to CuPyMS

In general, the classification in five major chemotypes is consistent with earlier CuPyMS results in Chapter 2 (Oudemans & Boon 1996). The primary difference is the division of CuPyMS Cluster A, into two Chemotypes A_1 and A_2. This separation is primarily due to a better transmission of larger fragments that enables the detection of relatively large amounts of diacylglyceryl-fragments in residues of chemotype A_1 and larger pyrolysis products from

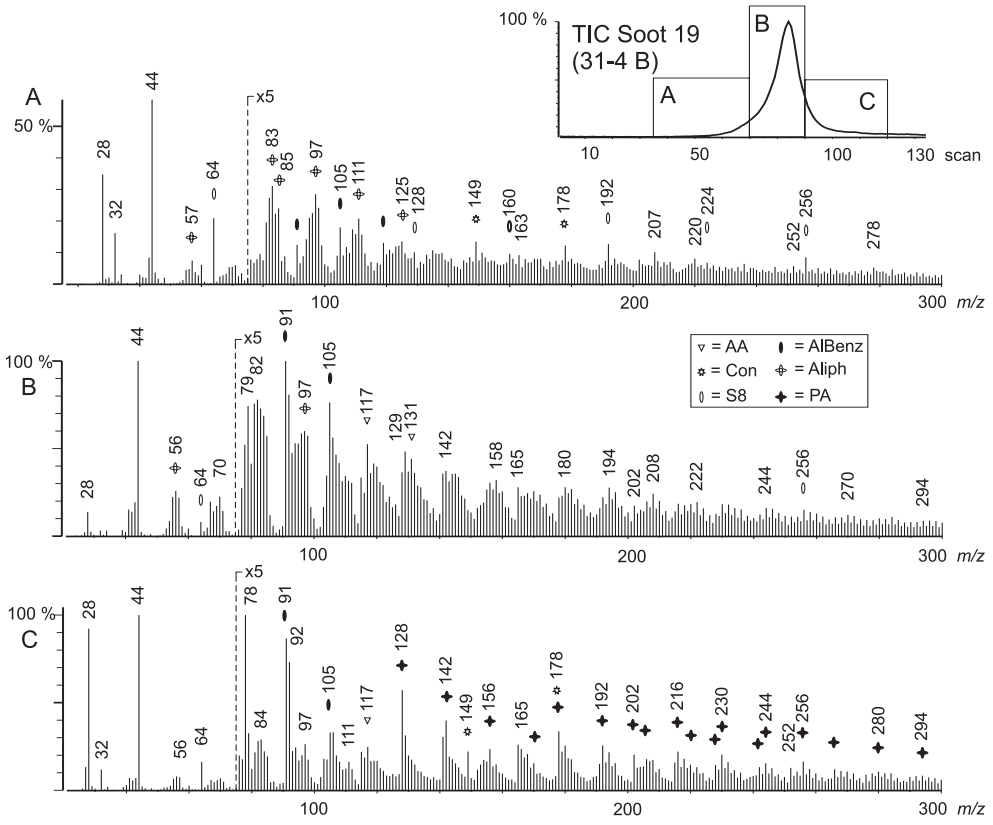


Figure 8: Cluster B - DTMS results for soot residue 19 (nr. 31-4b)
A: The low temperature results show a mixture of (S8) elementary sulphur; (AlBenz) alkylated benzenes; and (Aliph) aliphatic components and (Con) an occasional contaminant. B and C: At higher temperatures, mass spectra also include (AA) a few amino acid markers and (PA) many markers for polyacenes.

polysaccharide chars in chemotype A_2. The chemical characteristics for chemotype C are more or less consistent, and slight differences can be noted when comparing chemotypes B, D and E to the earlier clusters. These chemotypes are relatively less strictly defined and characterised primarily by a low organic content.

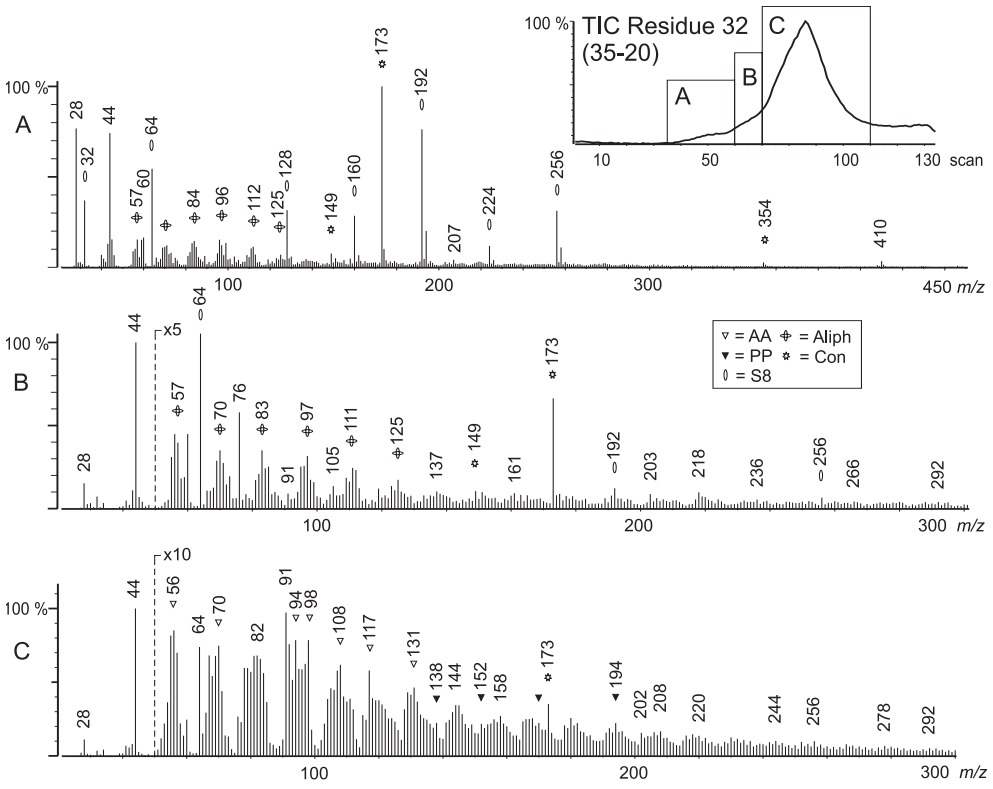


Figure 9: Cluster D - DTMS results for cream coloured residue 32 (nr. 35-20)
A and B: At the lower temperature ranges MS results show a mixture of (S8) elementary sulphur; (Aliph) aliphatic components and (Con) an occasional contaminant. B: The high temperature mass spectrum mostly shows (AA) amino acid markers and (PP) peptide and protein markers.

5. Discussion

5.1. Formation processes

The ability to disseminate the use of particular biomaterials in prehistoric times through organic residue analysis is to a large extent determined by a correct understanding of the formation processes that influence the chemical composition of the remaining residues.

Transformation processes include processes in the original prehistoric context or "systemic context" the so called C_1-tranforms (cultural transforms), and processes in the post-depositional context or "archaeological context" including the so called N-transforms (natural transforms) as well as the C_2-transforms (cultural transforms) that can take place during and after excavation (Schiffer 1972, 1983). The formation processes most significant for organic residue analysis are the C_1-transforms "mixing" and "thermal degradation" and the N-transforms "biodegradation" and "exchange or organic compounds between soil and residue" (see also Chapter 1). The way in which these transformations take place is partially determined by cultural phenomena specific to the given culture, and partially the result of chemical processes. The chemical processes that might have been significant in the formation and preservation of solid organic residues are discussed in the following subsections.

5.2. Thermal degradation

The thermal degradation that takes place during heating of foods is of great importance to the study of solid organic residues. Charring is one of the most important formation processes that results in preservation of solid organic residues on ceramic vessels. DTMS results of many charred residues show the presence of thermostable polymers with varying degrees of condensation (Oudemans & Boon 1991, 1996; Oudemans *et al.* 2005).
An earlier model for cellulose char formation (Boon *et al.* 1994; Pastorova *et al.* 1994) gives some insight into the way the preservation of biomolecular characteristics might work. The model proposes the formation of a condensed thermostable network polymer consisting of furanoid and (hydroxy) aromatic skeletons, hydrocarbon side chains and a large number of carboxyl and carbonyl groups. The polymer is formed at temperatures between 250 and 310 ºC and consists of a slowly growing three dimensional network that combines larger, non-volatile residual structural elements, such as anhydroglucose cores extended with glycol-aldehyde side chains, and small volatile compounds, such as enolic furans and glycol-aldehydes, trapped after their release inside the char (Boon *et al.* 1994). In chars heated over 310 ºC, a disproportionation of the furan dominated polymer occurs by loss of CO and CO_2 leading to the formation of a purely aromatic polymer with ever increasing amounts of C=C bonds and loss of hydroxyl groups (Boon *et al.* 1994; Pastorova *et al.* 1994). DTMS results (Fig. 1 A - D) indicate that the chemical mechanisms that take place during charring of amylose are comparable.
Earlier DTMS studies of protein chars have led to the definition of three thermal degradation phases in whole peas and Pisane HD (a pea protein isolate) heated under anoxic conditions up to temperatures ranging from 130 to 700 ºC for a maximum of 2 hours (Braadbaart 2004; Braadbaart *et al.* 2004a). The original protein markers could still be observed up to 270 ºC. The mild thermal degradation phase - up to temperatures ranging from 270 ºC to 310 ºC - results in markers for alkylated phenols and benzenes (m/z 91, 92, 94, 105, 107, 108, 119, 122), as well as odd numbered markers that can tentatively be attributed to N-containing hetero-cyclic compounds (m/z 117, 131, 133, 147, 161, 175, 189, 199, 213, 227), indicating the formation of a material with mainly aromatic compounds. The medium thermal degradation phase -

temperature range from 310 ºC up to a transitional stage from 400 to 440 ºC - results in similar spectra but was signified by a decrease in odd numbered markers and an increase in even numbered masses (*m/z* 146, 160, 174). This shift suggests a loss of nitrogen from the newly formed aromatic material, a phenomenon that becomes more pronounced at higher temperatures. The severe thermal degradation phase - temperatures over 440 ºC - results in DTMS spectra with ever decreasing yields of pyrolysis products and remaining mass peaks at *m/z* 27 (HCN), 28 (CO), 44 (CO_2), 48 (SO), 64 (SO_2), 78 (benzene), and 92 (toluene).

Although no model has been formulated for the formation of protein chars, it is clear that both protein- and polysaccharide-chars form condensed thermostable polymers at temperatures between 270 and 310 ºC. At these temperatures it is still possible to distinguish pure polysaccharide chars from chars that have a protein component. Pure polysaccharide chars show a predominance of even numbered *m/z* values and may contain some residual sugar characteristics in the lower temperature range of the DTMS spectrum (Fig. 1 C and D). Protein containing chars often show some residual markers for peptides and amino acids (*m/z* 154, 117) and show equal intensities for both even and odd numbered *m/z* values (Fig. 2D). Although the presence of a protein fraction in the original material can thus be identified, it is impossible to exclude the presence of a polysaccharide fraction in charred materials at this time.

As the heating temperature increases to the range between 310 - 400 °C, the homologous series of DTMS markers show distinct similarities between chars originating from proteins or polysaccharides, and masses representing alkylphenols and alkylbenzenes are present in both. An increasingly similar chemical composition is observed, suggesting that the remaining polymer becomes an increasingly C-enriched aromatic product without any other significant functionalities (Boon *et al.* 1994). At such temperatures it becomes increasingly difficult to determine the original biomaterials involved in the char formation. However, under the cooking/charring conditions to be expected from prehistoric cooking practices in ceramic vessels over open fires, temperatures higher than 300 °C are probably not reached inside the vessel.

5.3. Exchange of organic compounds between soil and residue

Although the N-transforms can have serious effects on organic residues, it is significant to notice that the chemical classification in clusters A_1 through D is not related to the kind of sediment in which the residue was buried. Residue classification shows no correlation with the sediment type surrounding the residue (Table 1). An earlier PyMS study compared the chemical composition of organic residues with that of two peat samples from the excavation in Uitgeest-Groot Dorregeest. In Chapter 2, peat samples clustered together and away from all archaeological residues and were characterised by markers absent from archaeological residue samples such as markers for lignins and intact polysaccharides (Oudemans & Boon 1996).

More detailed study through PyGC/MS in Chapter 3, confirmed the absence of such peat compounds in the archaeological residues (Oudemans & Boon 1991). PyMS and DTMS results give no indication that significant amounts of organic material was exchanged between soil and residue and no significant difference in degradation could be determined between different

Table 7: Chemotypes and their Biomolecular Origin

Chemo type	n	Residues	Example	Chemical Characteristics	Original Biomaterials
A_1	11	1, 4, 8, 9, 15, 22, 25, 30, 35, 36, 37	Charred residue 1 (7-7)	- Short chain FA and DAGs - Odd numbered DAGs - Cholesterol - Protein/peptide markers - Amino acid markers - Charred protein/polysaccharide	Charred animal product (most likely milk), possibly in combination with a starch. Cooked dairy product or cereal gruel.
A_2	10	3, 5, 12, 13, 16, 17, 20, 23, 24, 29	Charred residue 23 (33-8-2a)	- Short chain FA and DAGs - Unsaturated FA (C16:0 &18:1) - Plant sterols - Waxes (plant leaf wax) - Residual polysaccharide markers - Some protein/peptide markers - Charred polysaccharide/protein	Charred combination of (leafy) vegetables and grain, possibly in combination with an animal meat or fat.
B	3	7, 11, 19	Soot residue 19 (31-4b)	- Aliphatic compounds - Sulphur-containing compounds - Alkylated polyacenes	Low organic content – Soot or smoke condensate.
C	3	2, 26, 34	Charred residue 26 (34-0-30)	- Markers for intact peptides - Amino acid marker - Short chain FA and DAGs - Odd numbered DAGs - Cholesterol	Mildly charred animal product without starch (possibly milk).
			Red brown residue 2 (8-1)	- Markers for intact peptides - Amino acid marker - No lipids	Special protein-rich, lipid-free non-food product (used for decoration).
D	6	6, 10, 18, 31, 32, 33	Cream coloured residue 32 (35-20)	- Markers for intact peptides - Amino acid markers - Sulphur containing compounds - Aliphatic compounds - No lipids	Low organic content - Special protein-rich, lipid-free food (egg white), or non-food product (bone or skin glue).
E	1	28	Soot residue 28 (34-7-95b)	- Sulphur-containing compounds	Low organic content.

sediments within Uitgeest-Groot Dorregeest. This is in agreement with a study of extractable lipid in ceramics from Great Britain (Heron *et al.* 1991). At this stage it cannot be excluded that a small amount of organic compounds (short chain fatty acids, or water soluble polysaccharides) were exchanged between soil and residue, but the chemical classification is not affected by any such exchanges.

5.4. Biomolecular Origin of Residues

In spite of the broad range of original biomaterials involved, and the unique series of transformation processes each residue has undergone, tentative identifications of the original biomaterials can be made in many cases (Table 7).

Chemotype A_1 – Charred residue 1 (nr. 7-7)

The low temperature range of charred residue 1 (nr. 7-7) contains a relatively well-preserved lipid fraction with short chain lipids (both in fatty acids and in intact acylglycerides), cholesterol and acylglycerides with odd numbers of carbons. The biomolecular origin of such a lipid profile could be found in ruminant milk fats which commonly contain relatively high amounts of lower saturated acids (Breckenridge & Kuksis 1967, 1968; Marai *et al.* 1969; Christie 1981; deMan 1999). The presence of acylglycerides with an odd number of carbons seems in agreement with a milk fat origin (Murata 1977; Mottram *et al.* 1997; Mottram & Evershed 2001), although a post-depositional bacterial origin cannot be excluded at this time. The condensed polymeric component of the residue (Fig. 5B) is very comparable to charred BSA (Fig. 2D), and shows some residual peptide or intact protein markers as well as a higher mass envelope including both odd and even numbered mass peaks. Either a pure protein char or a protein/polysaccharide mixture could render such results. Residue 1 (nr. 7-7) can be identified as a relatively well-preserved animal product (most likely a ruminant milk) possibly cooked in combination with a starch. A cooked dairy product or a cereal-gruel seems to be the most likely origin of this residue.

Chemotype A_2 – Charred residue 23 (nr. 33-8-2a)

The lipid profile of charred residue 23 (Fig. 6A), shows high peaks for saturated free fatty acids (C10:0 – 28:0), a small amount of unsaturated C16:1 and C18:1 and some markers for DAG fragments in the range DAG30 - DAG36. The origin for the very long chain fatty acids can probably be found in the presence of plant waxes. Common components of either plant epicuticular wax (Kollattukudy 1976; Bianchi 1995) are long chain even numbered fatty acids ranging from C20:0 through 28:0 (m/z 312, 340, 368, 396, 424); markers for wax esters in the range of C38 – C52 (m/z 536, 564, 592, 620, 648); odd numbered long chain n-alkanes (C21 - C35), and even numbered primary alcohols in the C22 - C34 range. Although no indications could be seen for long chain alcohols or alkanes, minor peaks could be seen for wax esters C42 - 44. Plant leaf waxes have been detected before in organic residues preserved in association with ceramic vessels (Evershed *et al.* 1991; Charters *et al.* 1995). The additional series of minor peaks in the range m/z 396 through m/z 424 indicates the presence of what might be markers for a sterol mixture. Dehydrated sterols such as C27 (m/z 368), C28 (m/z 382) and C29 (m/z 396) have been shown to survive charring at 250 ºC for 120 min (Braadbaart 2004), and many studies have shown the survival of cholesterol (Evershed *et al.* 1990; Oudemans & Boon 1991; Evershed *et al.* 1995b) in ancient ceramics. However, plant sterols have only been identified in

soils and coprolites from archaeological context. The medium temperature spectrum (Fig. 6B) shows a mixture of incidental residual polysaccharide markers and markers for intact polypeptides and amino acids. The high temperature spectrum (Fig. 6C) shows some residual polysaccharide markers and a high mass area with an emphasis on the even mass values, indicative for a considerable polysaccharide component. Residue 23 is a mildly charred starch (with a minor protein component) with a partially hydrolysed lipid fraction including plant waxes. A combination of (leafy) vegetables with grain could be the origin of this residue. A small amount of animal material (meat or meat fat) could also have been included.

Cluster B - Soot residue 19 (nr. 31-4b)

Residue 19 (nr. 31-4b) is a residue most clearly characterised by the absence of markers for edible biomaterials. The presence of sulphur-containing compounds is most likely caused by a contamination of the sample with a small amount of ceramic material from the vessel wall (accidentally included when the sample was scraped from the vessel surface during sampling), but may also originate from the soil in which the ceramic lay buried. The presence of intense mass peaks m/z 28 and 44 in the early part of the temperature range, indicating decarboxylation of organic compounds and the presence of alkylated aromatic compounds and long-chain aliphatic compounds in the higher temperature ranges (Fig. 8B and 8C) suggests a wood smoke condensate or soot, an origin consistent with the situation of the residue on the exterior of the vessel. In the evaporation range (Fig. 8A), markers for short chain aliphatic compounds can be seen, indicating their origin from evaporation rather then pyrolysis. This origin contrasts with earlier interpretation that these compounds originate from pyrolysis of a polymeric networks (Oudemans & Boon 1996). It is possible that the aliphatic compounds are a minor component of the smoke condensate. However, considering the low organic content of the residue, the inclusion of a minor internal source in the mass spectrometer cannot be excluded.

Cluster C - Charred residue 26 (nr. 34-0-30)

The lipid profile of residue 34-0-30 contains a lipid fraction (Fig. 7A) not unlike the lipid fraction in residue 1 (nr. 7-7). The protein fraction shows a high degree of preservation (Fig. 7B) with markers for intact peptides such as diketopiperazines (DKPs) and alkylated pyrrolidinediones and relatively high mass peaks for amino acid moieties. A mildly heated protein source such as a charred milk product might render such a pattern easily, due to the thermal stability of casein. But a similar spectrum was obtained in the heating experiments with BSA (Fig. 2D), so the exact origin of the protein material cannot be established. Although the presence or absence of a starch source cannot be proven at this stage, it is likely that starch is either absent or is only a minor component of the original material. No positive indication for the presence of residual polysaccharide-characteristics can be seen, contrary to what one would expect in a residue with such limited thermal degradation. The origin for residue 34-0-30 is probably a lightly charred animal product, most likely milk.

Cluster C – Red-Brown "pigment" residue 2 (nr. 8-1)

Not all residues in cluster C are charred: residue 2 (nr. 8-1) is a red brown material situated on the exterior of the vessel. The placement of the material suggests the use of an organic material to decorate the vessel. Visually similar materials were seen applied to the exterior of various vessels in the Uitgeest-Groot Dorregeest assemblage in dots, stripes or small patches (Abbink 1999, 233 & 289). It is significant to note, that residue 2 contains absolutely no chemical evidence for the presence of lipids. Its placement in cluster C is based on the presence of markers for a well-preserved protein fraction. The absence of lipids in this residue probably indicates the use of a special protein-rich and lipid free material (possibly mixed with a pigment or other colourant) for decoration without heating the ceramic. It is possible that the material was applied to the surface of the vessel immediately after firing while the vessel is still warm. Most likely biomolecular origin of the residue would be egg white or bone glue (gelatin). The thermal stability of gelatin (Wong 1989) might render a well preserved protein profile even after some minor heating.

Cluster D - Cream coloured residues 32 (nr. 35-20) and 31 (nr. 35-7-28)

The residues 31 and 32 do not only differ from the other residues in visual appearance, they are also rather unusual in their chemical composition. The absence of lipids and the presence of a relatively well-preserved protein profile make these residues very similar in chemical composition to residue 2 (nr. 8-1) in cluster C. A low organic content has placed these residues in cluster D. Residues 31 (nr. 35-7-28) and 32 (nr. 35-20) may be the result of the preparation of a special kind of food (egg whites) or non-food product (bone or skin glue). It is obvious from the absence of markers for a polymeric network structure in the high temperature range (Fig. 9 C) that the residues have not undergone severe charring or may even have been kept in their vessel without heating.

6. Conclusions

Direct temperature resolved mass spectrometry in combination with multivariate analysis is a unique micro-analytical strategy for the characterisation and classification of solid organic residues from archaeological context. Information about the overall chemical composition is combined with a high sensitivity for a wide range of compound classes as diverse as lipids, waxes, polynuclear aromatic compounds, oligosaccharides, small peptides and protein fragments, and a variety of thermally stable (more or less condensed) polymeric char structures. The lack of any sample preparation prior to analysis makes DTMS an optimal technique to analyse extremely small amounts of solid organic mixtures. DTMS can be applied in archaeology in the study of organic residues of any kind, be it organic residues of food or adhesives in ceramics; paint residues from cave paintings, classical statues or ancient wooden objects; hafting residues on stone or bone tools or metal objects; or remainders of

mummification materials, inks, seals or other complex mixed organic materials. Within organic residue analysis in ceramic studies, DTMS is primarily unique in its capacity to rapidly determine overall chemical composition of solids without losing sensitivity for a wide range of compound classes. It prevents the misguided focus on the extractable part of the residues and opens up a wide range of biomolecular sources (such as protein and polysaccharide remains and polymerised lipids) that would otherwise remain undiscovered (especially if denaturation through charring or oxidation would prevent extraction).

The use of DTMS in combination with multivariate analysis, confirmed many of the earlier results obtained using Curie-point pyrolysis mass spectrometry (Oudemans & Boon 1991, 1996), and has resulted in a more detailed classification due to the measurement of a much wider range of masses. Charred residues have been better classified based on the identification of intact lipids and fragments for various condensed aromatic polymers. The temperature-resolved information obtained in DTMS measurements facilitates the interpretation of the mass spectrometric data in terms of chemical structure and illustrates that classifying characteristics are related to particular charred biomaterials. Experimentally "charred" modern foodstuffs (starch and protein) are used as reference materials to identify the biomolecular characteristics preserved throughout the thermal degradation that might take place during cooking and charring in ceramic vessels.

A combination of marker components and temperature-resolved information from the DTMS profile, give indication for the origin of each cluster of residues. Cluster A_1 contains charred animal products (most likely milk), possibly in combination with a starch. Cluster A_2 contains mildly charred (leafy) vegetable and grain mixtures. Cluster B contains only smoke condensates (soot) from the wood fires used for cooking. Cluster C contains a group of fairly diverse residues that represent both residues of mildly charred animal products without starch; and residues of special protein-rich, lipid-free non-food products (possibly used for decoration of vessels). Cluster D contains mildly heated residues of protein-rich, lipid-free foods or non-food products (bone or skin glue).

Although many molecular characteristics of the original foods have been lost as a result of extensive thermal degradation during cooking, some specific characteristics have been preserved within the newly formed, condensed polymeric char-material. Although the level of interpretation remains limited to general food groups, it is the interpretation of these specific "signature" characteristics that can render unexpected and exciting information about the origin of solid organic residues from archaeological context.