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FACULTY OF SCIENCE
DEPARTMENT OF CHEMISTRY

BIOGENIC COMPONENTS OF ESTUARINE
AND MARINE HUMIC MATERIALS

by

Valerie Singleton

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE
CHEMISTRY

Doctor of Philosophy

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The nature of the biogenic components in dissolved humic substances from the Beaulieu estuary (Hampshire, U.K.) and coastal waters (The Solent, Hampshire, U.K.) has been studied. Humic materials were extracted from the water using a macroreticular resin (Amberlite XAD-8), and carbohydrate, lipid and amino acid analyses were performed on the resulting samples.

A procedure was developed for the analysis of neutral sugars by on-column capillary gas chromatography, and this was applied to the determination of monosaccharides in aquatic humic substances. Glucose, galactose, arabinose, rhamnose and mannose were found to be the major sugars in these materials, with xylose and ribose detected at lower levels. Fucose and lyxose were also present. The estuarine distribution of these compounds supported a decreasing terrestrial and increasing planktonic input to these materials with increasing salinity.

Investigations on the lipid content of the humic material were performed by gas chromatographic analysis of their methyl ester derivatives. Despite significant contamination, certain unsaturated fatty acids, namely palmitoleic and oleic acids, were identified in several samples. Linoleic acid was also found in one instance.

Amino acids were studied by hydrolysis of the humic materials, followed by thin-layer chromatography. Quantitation was performed using an amino acid analyser. The amino acid content of the humic substances tended to increase in the summer months but no trends were noted in the patterns of the individual amino acids, either seasonally or between the three sampling sites. Glycine was found to be the major amino acid in most samples, with histidine, aspartic acid, glutamic acid and alanine contributing significantly in selected samples. Lower levels of arginine, cystine, leucine, isoleucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine and valine were also present.

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A B B R E V I A T I O N S

ASV	Anodic stripping voltammetry
BSTFA	Bis(trimethylsilyl)trifluoroacetamide
CPMAS	Cross-polarisation magic angle spinning
DOC	Dissolved organic carbon
DOM	Dissolved organic materials
DPASV	Differential pulse anodic stripping voltammetry
EDTA	Ethylenediaminetetraacetic acid
ESCA	Electron spectroscopy for chemical analysis
ESR	Electron spin resonance
FAB	Fast atom bombardment
FAME	Fatty acid methyl ester
FFAP	Free fatty acid phase
FID	Flame ionisation detector
FMOC	9-fluorenylmethyloxycarbonyl chloride
GC	Gas chromatography
GPC	Gel-permeation chromatography
HMDS	Hexamethyldisilazane
IR	Infrared
ISE	Ion selective electrode
LC	Liquid chromatography
MID	Multiple ion detection
MRT	Mean residence time
MS	Mass spectrometry
NOE	Nuclear Overhauser effect
NMR	Nuclear magnetic resonance
ODS	Octadecylsilane
OM	Organic matter
OPA	Ortho-phthalaldehyde
PAH	Polycyclic aromatic hydrocarbon
PITC	Phenylisothiocyanate
POC	Particulate organic carbon
R_f	Retention factor
RNA	Ribonucleic acid
RSD	Relative standard deviation
TFA	Trifluoroacetate
TIC	Total ion current
TLC	Thin-layer chromatography

TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
UV	Ultraviolet
WCOT	Wall-coated open tubular
XPS	X-ray photoelectron spectroscopy

L I T E R A T U R E R E V I E W

CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION AND HISTORY OF HUMIC MATERIALS

Humic substances are among the most widely distributed natural products on the earth's surface, occurring in soils, peat, leaves, decomposed municipal refuse, coal, lake and marine sediments, and fresh and sea waters (1,2,3). They even accumulate under aerobic conditions (4) due to their refractory (resistance to microbial and chemical decomposition) nature. They can be described as amorphous, hydrophilic, acidic polyelectrolytes, varying in colour from yellow to brown or black, with molecular weights ranging from a few hundred to tens of thousands of daltons.

The study of humic materials originated in the late 18th century from chemical investigations of soil organic matter. It was found that, in addition to containing recognisable plant and animal remains, and biogenic compounds, such as proteins, carbohydrates, amino acids, etc., an unidentified group of organic substances was also present. As they were thought to originate from decaying vegetation or humus, they were termed 'humic' substances.

Various historical reviews of research on soil organic matter have been published (e.g., (5)). Much of the early work consisted of attempts to isolate and fractionate the humic substances. Different humic fractions were thought to be individual chemical compounds and led to terms such as humic acid, humus acid, humus coal, humin, hylatomelanic acid, fulvic acid, ulmin, ulmic acid, crenic acid, apocrenic acid, glucic acid, apoglucic and chlorhumic acid. However, by the end of the 19th century, it became clear that humic substances were not individual chemical compounds, but complex mixtures. Numerous classification schemes for the isolation of these materials were proposed, one of the most important contributions being that of Oden (6), who divided humic substances into four categories, based on their solubility in various solvents (Table 1.1). This classification scheme is still used for the fractionation of humic materials, although two points should be noted:-

Table 1.1 Classification of humic substances (according to Oden (6))

Type ⁺	Solubility		
	Acid	Alcohol	Alkali
Fulvic acid	✓	✓	✓
Hymatomelanic acid	x	✓	✓
Humic acid	x	x	✓
Humin	x	x	x

✓ Soluble

x Insoluble

+ These are the current terms used for the various fractions - 'humus acid' and 'humus coal' were the names given by Oden to 'humic acid' and 'humin'.

- (i) All fractions are now thought to represent mixtures of compounds with similar molecular weights - Oden believed that humic and hymatomelanic acids were individual compounds.
- (ii) Alkali-soluble humic substances are now generally only classified into a lower molecular weight fulvic acid fraction and a higher molecular weight humic acid fraction. Hymatomelanic acids are not usually separated from the higher molecular weight fraction.

Following on from studies of soil humic materials, similar compounds were found in natural waters; these aquatic humic substances giving rise to the brown/yellow colouration of some of these waters. In the early 19th century, Berzelius isolated organic acids from mineral waters, noting that they were similar to soil humic substances, and were therefore probably washed from the soil (cited by Thurman (7)). However, most humic research up until the 1950's was performed on soil humic matter. Few studies were carried out on aquatic humus, and these have been summarised by Thurman (7). Since that time, the number of investigations of aquatic humic substances

has increased substantially. Initially, methods used in the study of soil humic matter were employed, but as new techniques, such as gel permeation chromatography, were discovered, these were also employed. In addition, health concerns over the formation of trihalomethanes during the chlorination of drinking water (8), and the development of improved methods for the isolation of aquatic humic substances, led to increased interest in the aquatic materials.

Initial studies of aquatic humic substances used coloured freshwater samples to obtain high yields of humic materials. The presence of humic materials in seawater was demonstrated by Kalle (9) who observed a blue fluorescence from seawater irradiated with ultraviolet radiation. This humic type material was given the name 'gelbstoff' due to the yellow colouration it often imparted to the water. Research on marine humic substances was hindered due to a lack of isolation techniques suitable for dealing with the low concentrations of these materials in seawater. The use of adsorption chromatography, in particular the advent of Amberlite XAD resin technology, however, enabled the extraction of sufficient quantities of the humic materials for analysis. Thus, the early 1970's saw the start of increased research interest in marine humic substances.

One of the problems with the isolation of aquatic humic substances is that the nature of the extracted material depends on the method of isolation. Therefore, in recent years, it has generally been accepted that aquatic humic materials may be defined as the fraction of dissolved organic carbon (DOC) isolated using Amberlite XAD resins. A problem with this definition, however, is that non-humic materials (e.g., identifiable organic compounds) may be co-extracted with the humic matter, and so interpretation of the data must take this into account.

1.2 ENVIRONMENTAL ROLE OF HUMIC SUBSTANCES AND THEIR USES

Interest in both terrestrial and aquatic humic materials arises from their involvement in many important environmental processes. In addition to enhancing the fertility of soils by improving their physical and chemical properties, they participate in a wide variety of chemical processes linked to the fate of trace components in the environment (10). They have found various uses in such applications

as soil fertilisers, in drilling muds for oil well rigs, as boiler-scale removers, and emulsifiers (10).

Complexation of trace metals by humic substances facilitates the mobilisation, transport, concentration and scavenging of these elements, which, in turn, alters their availability to plants and aquatic organisms (11,12). Beneficial increased solubility of essential micronutrients, such as iron, may be achieved by complexation. Under other circumstances, however, insoluble complexes may be formed when humic materials are bound to other soil constituents, such as oxides and clay minerals. In this case, a depleted supply of micronutrients results. In addition, the removal of uncomplexed metals from solution may decrease their availability to certain organisms. Humic substances may further modify the solubility of metal ions due to their ability to reduce oxidised forms of certain elements, such as iron, molybdenum, vanadium and mercury (13). Organic complexing agents may also contribute to the chemical weathering of rocks and minerals (13).

The toxic effects of metals are also affected by interactions with humic substances. Decreasing the free metal ion concentration may reduce its availability and toxicity to organisms (14), as may the formation of insoluble metal complexes (15). On the other hand, complexation may increase the toxicity of trace metals by facilitating their accumulation (16), or solubilising ions when they would otherwise be precipitated.

The interaction of organic chemicals with humic substances is of environmental importance as it affects the bioactivity, persistence, biodegradability, solubility and volatility of these chemicals. The inhibition or stimulation of various enzymes by humic materials has been noted (11). Binding of pesticides to humic matter improves their solubility, and this influences the leaching of these chemicals from the soil. In addition, the bound pesticide may no longer be active and pesticide application may therefore have to be increased (17). A further problem is the formation of stable chemical linkages between pesticide residues and soil organic matter, as this leads to greatly increased persistence of these residues (18). Humic substances may also promote the non-biological degradation of pesticides, such as the

chloro-s-triazines (19), and other organic chemicals.

Adsorption of organic pollutants by humic substances may either decrease their toxicity, due to the lower toxicity of the adsorbed compounds (11) or their decreased availability, or enhance it (12), presumably by increasing pollutant solubility. Interactions with humic materials have been used to explain changes in the rate of volatilisation of polychlorinated biphenyls, and the accumulation of polynuclear aromatic hydrocarbons in fish (cited by Malcolm (20)). Hydrocarbon solubility has been shown to increase in the presence of humic materials but that of the polynuclear aromatic hydrocarbons and phthalates was unaltered (21). The solubilisation of dialkyl phthalates has, however, been observed by other workers (22). Beneficial effects of the solubilisation of hydrocarbons were seen when oil from small boats in a Brazilian river was quickly dispersed by humic materials (23).

Photosensitising effects of humic substances may increase the rate of photodegradation of environmental chemicals (24). Humic materials may act as photosensitisers for the degradation of phenolic compounds, and this could aid the detoxification of phenolic effluents. Photodegradation of pesticides in the presence of humic substances has also been reported (25). Conversely, quenching by these materials may retard the photodegradation of environmental pollutants (24).

The formation of chlorinated organic compounds, such as the haloforms, during the chlorination of drinking waters (26) and industrial cooling waters (27), has caused concern in recent years. This has been related to low concentrations of humic substances in the water. Apart from the presence of several identified, chlorinated, organic compounds, many species remain unidentified, including a large proportion of the humic materials in the form of relatively large molecular weight chlorinated compounds whose potential health effects are unknown.

Further problems that the presence of humic substances present to man include the fouling of reverse-osmosis membranes (28) and ion exchange resins in water treatment plants. They also appear to survive municipal sewage treatment (29) and so toxic metal complexes

may not be destroyed.

Due to their ability to adsorb certain pesticides and metal ions, the use of native peat and chemically modified peat for removing these species from contaminated waters has been discussed (30).

1.3 DISSOLVED ORGANIC CARBON IN NATURAL WATERS

1.3.1 Content

Conventionally, the organic carbon in natural waters is divided into two main fractions - dissolved organic carbon (DOC) and particulate organic carbon (POC). The division between these fractions is arbitrary although most investigators consider that, on filtration of the water sample, POC is that material retained on a 0.45 μm filter, and DOC is that passing through it.

The DOC levels of aquatic systems have been reviewed (see, for example, (7)) and vary considerably, as shown in Table 1.2 which lists approximate DOC concentrations in a variety of natural waters.

Table 1.2 Approximate concentrations of dissolved organic carbon in natural waters (mg/l) (7.31)

Ice	0.2
Seawater	0.5
Groundwater	0.7
Precipitation	1.0
Oligotrophic lake	2.0
River water	5.0
Eutrophic lake	10.0
Marsh	15.0
Bog	30.0
Sediment pore water	50.0

The DOC data in Table 1.2, however, are only average concentrations - actual values show wide variations depending on environmental parameters. The DOC content of rivers, for example, varies with the size of the river, climate, season of the year,

pollutant input, degree of soil leaching, and surrounding vegetation. Data on the DOC content of several rivers from different environments and climates are given in Table 1.3. In contrast, the DOC concentrations in seawater are generally less variable. Values of around 1 mg/l are found in coastal waters, shallow seawater, and surface seawater, although increased concentrations may occur in coastal waters due to input of organic matter from rivers (the level of which is a subject of controversy as discussed later). For example, DOC concentrations of between 2.5 and 12.9 mg/l have been found off the coast of Georgia, U.S.A. (32). Deep open ocean water shows lower levels of DOC than surface water, ranging from 0.2 - 0.8 mg/l (33). Recent work by Sugimura and Suzuki (34), however, questions the accuracy of the methods used to determine the low levels of DOC in seawater, suggesting that they yield values lower than the true concentrations.

Table 1.3 Range of DOC of rivers from different environments and climates

<u>Environment</u>	<u>Range of DOC (mg/l)</u> <u>(Mean given in parentheses)</u>	<u>Reference</u>
Arctic and alpine	1 - 5 (2)	7
Subarctic	8 - 25 (10)	7
Cool temperate climate	2 - 8 (3)	7
Warm temperate climate	3 - 15 (7)	7
Arid climate	2 - 10 (3)	7
Wet tropical climate	2 - 15 (6)	7
Rivers draining swamps and wetlands	5 - 60 (25)	7
A polluted river (Tokyo, Japan)	30 - 320	35

As would be expected, estuaries have DOC concentrations ranging between those of rivers and coastal seawater. Polluted estuaries (e.g., (35)), however, are an exception to this in that increased levels of DOC may be seen at high salinities. In unpolluted systems, the riverine DOC is diluted with seawater of lower DOC on estuarine mixing. DOC in estuaries has been shown to be both conservative (36,37) and non-conservative (38,39). During estuarine mixing (40), DOC behaved conservatively but some removal of humic acid was

observed. Since humic acid is part of the DOC, this removal should have been observed in DOC versus salinity diagrams. It was suggested that the humic acid fraction removed was too small to be resolved by DOC measurements. The non-conservative behaviour observed by Sholkovitz (38) indicated that 3-11% of the riverine dissolved organic materials ($DOM \approx 2 \times DOC$) were removed on estuarine mixing. This value lies within the error limits of those experiments which demonstrated conservative mixing in estuaries (36,37). Therefore, it seems likely that some removal of humic acid may occur in estuaries, but it may sometimes go unnoticed if the percentage of humic carbon in the DOC is low.

In addition to the effect of the physical mixing process described above, chemical and microbiological processes may alter the DOC concentration within estuaries by, for example, biological conversion of POC. Specific environmental features may affect the removal of humic materials in the estuary. For example, the presence of minerals has been shown to lead to a reduction in the colour of fresh waters containing humic materials (41).

Variations in the DOC levels of other natural water systems, such as groundwater, interstitial water of soil and sediment, snow and glacial water, precipitation, and lake water have been reviewed elsewhere (7).

1.3.2 Composition

The DOC content of natural waters can be divided into three main categories:

- humic substances, which, in most cases, are operationally defined as the DOM fraction adsorbed onto Amberlite XAD resins at pH 2. Alternative extraction procedures, such as adsorption onto weak-base ion exchange resins, have also been employed (7,42).
- hydrophilic acids, which are those organic acids not retained by Amberlite XAD resin extraction at pH 2. Little is known of the structure of these materials although they are thought to contain both simple organic acids, such as volatile fatty acids, as well as complex polyelectrolytic acids. Leenheer (43) reported that the

infrared spectrum of this fraction showed strong hydroxyl and carboxyl character.

- specific identifiable organic compounds, which include lipids, carbohydrates, proteins, hydrocarbons, etc.

Humic substances generally constitute a large but variable (ca. 30-80% (20,44)) proportion of the total DOC in natural waters. Identifiable compounds account for only 10-15% of the DOC in seawater; the remainder consisting of humic substances and hydrophilic acids, of which the hydrophilic acids are estimated to account for approximately 50% of the total DOC (7).

The relative concentrations of the organic compounds in the identifiable fraction of the DOC vary between water systems but the main components generally remain the same. Trace components, such as pollutants, however, may vary. This fraction consists mainly of carbohydrates, fatty acids, amino acids and hydrocarbons, together with a selection of trace components, such as chlorophyll and other pigments, sterols, alcohols, aldehydes, ketones, phenols, organic bases, phosphorus compounds, organic sulphur compounds, and pollutants (e.g., plasticisers). A comprehensive review of these compounds in natural waters has been written (7).

1.3.3 Origin

The DOM in natural waters originates mainly from varying contributions of the following:-

- (i) *in situ* formation of organic compounds from aquatic organisms,
- (ii) leaching of soil organic matter,
- (iii) anthropogenic inputs.

These will be considered separately in the following sections. Other contributions to riverine DOM have also been suggested, including groundwater inputs and diffusion of DOM from the river bed during periods of high discharges (20). Experimental confirmation of these contributions, however, has yet to be obtained.

1.3.3.1 In situ formation

DOM can be released into the aquatic system by:

- excretion of metabolic products from aquatic organisms, such as fish, plankton, bacteria, etc.
- release of cellular components during grazing of phytoplankton by zooplankton.
- death and decomposition of the organisms.

The nature of the material excreted by phytoplankton has been summarised by Lancelot (45) and includes mono-, oligo- and polysaccharides, amino acids, proteins and polypeptides, fatty acids, glycollic acid, tricarboxylic acids, hydroxamic acids, and vitamins. Cell lysis and grazing of phytoplankton by zooplankton (46) also releases DOM into the water system. In addition, it is released directly by zooplankton (47). Production of polyphenolic compounds by algal communities has also been reported (48) together with the excretion of organic substances into water by a variety of marine organisms including copepods, anchovies, sea urchins and starfish (reviewed by Ehrhardt (49)).

Death and subsequent decomposition of plants and animals also releases DOM. In addition to containing labile compounds, such as amino acids, sugars, etc., a proportion of this material has been found to be resistant to biological decay (50).

1.3.3.2 Leaching of soil organic matter

Soil organic material is transported into rivers and lakes via direct leaching of soils or slow extraction after percolation through the soil. The DOM which results is a soluble fraction of soil organic matter and should therefore resemble this material. The nature of soil organic matter has been extensively reviewed (5,51) and similar material has been extracted from river (41) and lake water (52). It results from the degradation of plant material, consisting of both simple compounds, such as sugars, amino acids, and fatty acids, and compounds which are difficult to degrade, such as lignin, cellulose and humic substances. The study of aquatic organic matter resulting

from soil leaching must be performed in areas where there is a large degree of surface run-off, but low productivity, so that the contribution from *in situ* formation is low.

1.3.3.3 Anthropogenic inputs

Organic pollutants can enter natural water systems through a variety of routes including river run-off, discharge of industrial wastes, sewage (piped off-shore from land or direct input from ships), and oil pollution (either from leakage during off-shore mining or on transportation). Anthropogenic contributions to the total DOC content are generally small compared to the natural production, but some of them are resistant to biochemical degradation (e.g., alkylbenzene sulphonates, aromatic hydrocarbons, etc.) and thus may persist in the environment for long periods.

Hydrocarbons present in natural waters can be derived from both living organisms and pollution. The occurrence of marine hydrocarbons, both from biogenic sources and as a result of pollution, has been briefly reviewed by Parrish (53). Natural hydrocarbons are often derived from fatty acids, and, as naturally occurring fatty acids generally have an even number of carbon atoms and are unbranched, a predominance of odd-numbered n-alkanes results. By comparison, there is no preference for odd-numbered carbon atoms in pollutant hydrocarbons. In addition, they tend to have slightly lower molecular weights than biogenic hydrocarbons (54) although overlap does occur. Chlorinated hydrocarbons are also indicators of pollution (53).

Aromatic hydrocarbons result from oil pollution and, in addition, are products of fossil fuel combustion. Levels of marine alkylbenzenes have been correlated with the seasonal consumption of fossil fuels (55,56). Large concentrations of polynuclear aromatic hydrocarbons are also indicators of pollution as they are synthesised by very few organisms. They have been found in sewage, mussels and tap water (57), and in estuarine water (58).

Other hydrophobic pollutants occurring in the aquatic environment include polychlorinated biphenyls, pesticides and phthalic acid esters (59).

1.4 ORIGINS OF HUMIC SUBSTANCES IN RIVER AND SEAWATER

Humic substances are often a large proportion of natural water DOM. Much of the early work on aquatic humic materials was performed on highly coloured fresh waters, resulting from a high degree of soil leaching. As expected, these compounds possessed similar characteristics to soil organic matter (e.g., (60)). A comparison of the characteristics of soil, terrestrial aquatic, and algal aquatic humic substances (12) highlighted the similarities and differences of these sources. In general, the algal material was less coloured, had a lower phenolic content and a less negative carbon isotope ratio. Its aliphatic carbon content was approximately the same as the terrestrial aquatic material, but was higher than that of the soil humic matter. These differences indicate the complex nature of this material, in that, as well as there being differing contributions from leaching of soil organic matter and *in situ* production, possible changes in the soil organic matter may result from its journey into the water body. The additional presence of pollutants (32) may add to the problem.

The origin of DOM in seawater is a subject of controversy. Input of riverine DOM will depend on the extent to which DOC exhibits conservative or non-conservative mixing in estuaries (see section 1.3.1). In general, DOC has been shown to exhibit conservative mixing although removal of a large proportion of the high molecular weight humic acid (accounting for only a low percentage of the total DOC) has been noted (39).

Although the humic acid fraction of DOM seems to be removed in estuaries, the conservative nature of the DOC suggests that a large proportion of the organic material enters coastal areas. This fraction is likely to include the lower molecular weight fulvic acid fraction and the identifiable organic compounds. The conservative nature of dissolved organic nitrogen and combined amino acids has been shown (61). Studies have been carried out to investigate the presence of terrestrial organic materials in the marine environment. Several workers have performed isotopic composition experiments on terrestrial and marine organic matter. Nissenbaum and Kaplan (62) extracted humic substances from marine and non-marine sediments and soils. On

the basis of their carbon isotope ratios ($\delta^{13}\text{C}$ values), they suggested that the humic acids from marine (off-shore) samples were mainly of planktonic origin whereas those from coastal and littoral samples showed a more terrestrial nature. Gearing *et al.* (63) measured the relationship between the $\delta^{13}\text{C}$ content of sedimentary organic material and the distance from the mouth of the River Niger, and found that $\delta^{13}\text{C}$ showed a near linear relationship with the logarithm of the distance from the river mouth. Although this data, together with that of Nissenbaum and Kaplan (62), were obtained from sediment samples, they indicate the presence of terrestrial organic matter in coastal environments. Phenolic aldehyde indicators of terrestrially-derived organic material in sediments (64) also decreased with distance from the estuary. $\delta^{13}\text{C}$ measurements, by Stuermer and Harvey (65), on humic and fulvic acid from coastal seawater, and fulvic acid from surface seawater, supported *in situ* formation of marine humic substances. Harvey and Boran (66) have stated that marine humic substances are formed *in situ*, and a mixture of marine and terrestrially-derived materials are present in the coastal zone.

In summary, current evidence on the origin of marine DOM suggests that a high molecular weight fraction of the humic material (mainly humic acid) is removed in the estuary. Coastal DOM consists of material formed both *in situ* and of terrestrial origin, whereas open ocean DOM, far from any continental input, appears to be formed purely *in situ*.

1.5 AIM OF PRESENT STUDY

The aim of the present study was to analyse estuarine and marine humic materials to find evidence to support current theories of their origins. The contribution of biogenic material was investigated by analysing the humic substances for amino acids, carbohydrates, and fatty acids.

CHAPTER TWO

STRUCTURAL CHARACTERISATION OF HUMIC SUBSTANCES

Numerous methods have been employed in the characterisation of humic substances. The results of a selection of these are presented in this chapter.

2.1 ELEMENTAL ANALYSES

The elemental composition of humic materials varies with environment (Table 2.1). Fulvic acids generally have a lower carbon content and higher oxygen content than humic acids. In turn, soil-derived fulvic acids have a lower carbon and higher oxygen content than their riverine counterparts due to their higher carbohydrate content (67). However, these results must be compared with caution due to the fact that the different isolation procedures used in the studies of soil (direct solvent extraction) and aquatic (Amberlite XAD resin extraction) humic substances lead to differences in the composition of the extracted materials. For example, carbohydrates/amino acids may be co-extracted with soil humic substances, and ammonia may be incorporated into the aquatic materials (from the ammonium hydroxide eluent).

The H/C atomic ratio, a measure of the degree of saturation, is close to 1 for soil and river humic substances, but higher in sedimentary and marine humic materials. The latter material is therefore believed to be more aliphatic in character.

2.2 FUNCTIONAL GROUP ANALYSES

Functional group analyses on humic materials have included the determination of carboxyl, methoxyl, carbonyl, hydroxyl (total OH, phenolic OH, and alcoholic OH), quinone, hydroxamate, and ether groups, together with measurements on the total acidity. The methods employed (summarised in Table 2.2) have included both wet chemical methods, and infrared (IR) and nuclear magnetic resonance (NMR) studies.

Table 2.1 Elemental composition of humic substances from different environments

Sample	% C	% H	% N	% O	% S	% Ash	H/C ⁺	Ref.
Soil FA	46.94	3.48	2.31	47.27	ND	ND	0.89	68
	49.2	3.2	1.7	45.9	ND	4.5 ^x	0.78	69
Soil HA	56.69	4.94	2.47	35.90	ND	ND	1.05	68
	58.13	5.17	2.14	34.56	ND	ND	1.07	68
	52.3	5.6	3.7	38.4	ND	6.1 ^x	1.28	69
Sedimentary FA [*]	38.4	4.3	2.8	54.5	ND	11.5 ^x	1.34	69
	44.5	6.0	5.2	44.3	ND	9.4 ^x	1.62	69
	46.2	6.6	4.5	42.7	ND	10.6 ^x	1.71	69
	47.6	6.6	2.6	44.6	ND	13.0 ^x	1.66	69
Sedimentary HA [*]	48.7	6.1	3.4	41.8	ND	12.7 ^x	1.50	69
	52.8	6.6	5.6	35.0	ND	5.9 ^x	1.50	69
	55.7	7.0	4.9	32.4	ND	2.1 ^x	1.51	69
	54.1	5.7	5.6	34.6	ND	5.3 ^x	1.26	69
	50.2	5.6	3.6	40.6	ND	15.5 ^x	1.34	69
	55.0	6.2	4.9	33.9	ND	6.4 ^x	1.35	69
	53.8	6.5	3.8	35.9	ND	2.1 ^x	1.45	69
River FA	62.34	6.93	6.27	24.46	ND	ND	1.33	68
	51.96	4.32	1.04	39.80	3.05	<0.02	1.00	70
	53.32	4.51	1.20	38.90	1.77	0.03	1.02	70
	53.15	4.76	1.04	37.59	2.60	1.02	1.07	70
	51.47	4.51	1.63	40.78	1.14	0.34	1.05	70
River HA	51.1	3.62	1.13	ND	ND	1.0	0.85	71
	56.00	4.51	1.36	37.25	0.72	0.37	0.97	70
	55.29	4.53	1.26	37.08	1.78	0.25	0.98	70
	55.00	4.30	2.10	ND	0.8	ND	0.94	70
Lakewater FA	53.4	3.73	2.10	ND	ND	4.3	0.84	71
Lakewater HA	43.5	2.7	2.2	51.6	ND	ND	0.74	72
Marine FA	50.2	3.1	1.9	44.8	ND	ND	0.74	72
	49.98	6.76	6.40	36.40	0.46	3.4 ^x	1.62	65
	45.2	6.4	6.5	41.9	ND	ND	1.70	73 ^{**}

+ Atomic ratio

** Mean of 9 samples

* From marine sources

x Elemental composition corrected for ash

ND Not determined

FA Fulvic acid

HA Humic acid

Table 2.2 Methods used in the determination of functional groups in humic substances

Functional group	Method	Reference
Total acidity determination	Back titration of excess barium hydroxide remaining after reaction with sample	73,74
	Thermometric titration	75
Carboxyl	Reaction with calcium acetate and titration of released acetic acid	73,74
	Direct potentiometric titration in potassium chloride	76
	¹³ C NMR	67,77
	Tritium exchange of carboxyl protons in the sample, followed by methylation and liquid scintillation counting	78
Total hydroxyl	Acetylation with acetic anhydride, followed by hydrolysis of excess anhydride to acetic acid, which is then titrated with base	74
	¹³ C NMR	67,79
Phenolic hydroxyl	Calculated as total acidity - carboxyl groups	73,74
	¹³ C NMR	67,77
Alcoholic hydroxyl	Calculated as total hydroxyl - phenolic hydroxyl	74

Table 2.2 Methods used in the determination of functional groups in humic substances (continued)

Functional group	Method	Reference
Carbonyl	Reaction with an excess of hydroxylamine hydrochloride, and determination of the unreacted hydroxylamine by titration	73,74
	¹³ C NMR	67
Quinone	Reduction with either:	
	(i) alkaline tin (II) chloride	73
	or (ii) alkaline triethanolamine-iron (II), and back titration of excess reductant with dichromate solution	74
	Infrared spectroscopy	80

The functional group content of humic substances from various origins has been determined (Table 2.3). It can be seen that, in general, the total acidity of the fulvic acids is higher than that of the humic acids. The anomalous behaviour of the marine sedimentary fulvic acid was thought to be related to the loss of very low molecular weight fulvic acids during the purification processes (81). The increased percentage of carboxyl groups in fulvic acids is also apparent and has been noted by other investigators (e.g., (13) for soil and (7) for aquatic humic substances). A comparison of phenolic hydroxyls from terrestrial and aquatic environments suggests higher concentrations in the terrestrial samples in agreement with their probable lignin-based origin.

2.3 MOLECULAR WEIGHT AND SIZE DETERMINATIONS

The molecular weights of humic substances vary from a few hundred to tens of thousands. As humic substances are mixtures of molecules, molecular weight determinations yield 'average molecular weight' values, namely (82):-

Table 2.3 Functional group content of humic substances from different environments

Sample	Functional groups (meq/g)					Ref.
	Total acidity	Carboxyl groups	Phenolic hydroxyl	Carbonyl groups	Quinone groups	
Soil FA	ND	8.1	3.9	2.0	0.6	74
Soil HA	ND	4.4	3.3	2.2	1.0	74
Soil FA	9.0	3.5	5.5	5.0	ND	69
Soil HA	5.5	3.0	2.5	3.0	ND	69
Freshwater FA (n=3)	5.5-12.2 (8.9)	4.6-10.1 (6.8)	0.9-3.4 (2.1)	4.2-5.7 (5.0)	1.5-1.8 (1.6)	73
Marine FA (n=9)	5.3-13.4 (9.5)	4.4-8.9 (7.2)	0.9-4.5 (2.3)	1.3-6.5 (3.8)	0.9-3.9 (2.4)	73
Marine FA + (n=3)	2.0-2.5 2.2	1.0-2.0 1.5	0.5-1.0 0.7	3-6 4.3	ND ND	69
Marine HA + (n=9)	2.8-5.8 4.1	1.8-3.9 2.8	0.9-1.9 1.4	1.2-3.0 1.9	0.7-2.1 1.1	73
Marine HA + (n=4)	3.0-5.5 4.1	2.0-3.0 2.5	0.5-2.5 1.6	3-5 4.0	ND ND	69

ND Not determined

FA Fulvic acid

() Mean

HA Humic acid

+ Marine sedimentary humic substances

(i) Number-average molecular weight (\overline{M}_n) is generated by methods which determine the number of molecules present. These include cryoscopy (83), vapour pressure osmometry (84) and isothermal distillation.

(ii) Weight-average molecular weight (\overline{M}_w) may be determined using, for example, scattering of electromagnetic radiation, gel-permeation chromatography, and ultrafiltration (85). These techniques generally measure molecular size, and the molecular weight of the humic material is estimated by comparison with

standards of known molecular weight.

- (iii) z-average molecular weight ($\overline{M_z}$) can be measured by equilibrium ultracentrifugation. Humic samples are generally too polydisperse to yield reliable results, and meaningful data may only be obtained if prior molecular weight fractionation is carried out.

These values are method-dependent and are not always directly comparable either within or between methods (variations due to differences in isolation methods must, however, also be considered). Although $\overline{M_n} = \overline{M_w} = \overline{M_z}$ in a homogeneous system, humic materials constitute a polydisperse system, and, in this case, $\overline{M_n} < \overline{M_w} < \overline{M_z}$.

Numerous investigations have been performed on the molecular weight distribution of humic substances. Stevenson (51) reviewed molecular weight measurements on soil humic materials using a selection of the aforementioned methods. He concluded that a 'typical' soil fulvic acid had a molecular weight in the 500 - 2 000 range, whereas soil humic acids showed an average range of 50 000 - 100 000, with a few having values greater than 250 000. Thurman *et al.* (86) cited numerous molecular weight measurements on aquatic humic substances and compared them with their own values determined by small-angle X-ray scattering. Aquatic fulvic acids generally had molecular weights of less than 2 000, with most of the aquatic humic matter having values under 10 000 with a small fraction being greater than 100 000. In general, therefore, soil humic substances appear to have higher molecular weights and show a greater degree of polydispersity than freshwater humic substances. In addition, humic acids have higher molecular weights than fulvic acids.

Molecular weight and size measurements have also been performed using such methods as electrophoresis (87) and electron microscopy (88). Depending on pH, ionic strength, humic concentration, and sample drying conditions (88), humic substances can assume, amongst others, a spheroidal shape, or consist of elongated or coiled fibres, or exhibit a sheet-like structure. Thus, such investigations require stringent monitoring of the experimental conditions if they are to be compared to those of other workers.

2.4 SPECTROSCOPIC/SPECTROMETRIC STUDIES

2.4.1 Infrared spectroscopy

Infrared (IR) spectroscopy has been employed in the determination of the functional group content of both underivatised (89) and derivatised (90) humic materials, changes in structure following chemical (91) or physical treatment (e.g., (92)) and studies of exchangeable protons and complexation (89).

The characteristic feature of infrared spectra of humic materials is their apparent simplicity as they consist of a relatively few broad bands. This is due to the fact that a given functional group exists in a wide variety of environments due to the complex nature of the materials. Humic matter from different sources exhibit similar spectra but this only indicates that the net functional group content may be similar, rather than the actual structures (89).

The major infrared absorption bands of humic substances have been reviewed by MacCarthy and Rice (89) (Table 2.4). Various derivatisation techniques have also been employed in infrared analyses of humic materials, for the measurement of carboxyl, ester, aliphatic carbonyl, quinone and conjugated ketone, hydroxyl and acetal, ketal, furan, ether and peroxide groups (90).

2.4.2 Ultraviolet-visible spectroscopy

Ultraviolet (UV) and visible spectra of humic materials generally have little structure, with the optical density (absorbance) decreasing with increasing wavelength (10,89,90). A shoulder is sometimes present in the 260 to 300 nm region (51). It is interesting to note that lignosulphuric acid shows a peak at this wavelength (94) although the pH dependence of the spectrum casts some doubt over this assignment (70). The similarity of the UV-visible spectra of humic substances from various environments led earlier workers (10) to conclude that the materials had a similar basic structure. The apparent simplicity of the spectra, however, is now believed to be due to overlapping bands from a selection of chromophores in different molecular environments (89), and thus the basic humic structures need

Table 2.4 The major IR absorption bands of humic substances (89)

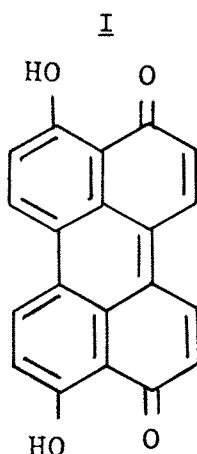
<u>Frequency (cm⁻¹)</u>	<u>Assignment</u>
3400	Hydrogen-bonded OH stretching
2860, 2920	Aliphatic C-H stretching in methyl and/or methylene units
1720	C=O stretching (mainly carboxyl)
1600-1650	Aromatic C=C conjugated with C=O and/or COO ⁻ [H ₂ O bending if sample not thoroughly dry] Hydrogen-bonded C=O stretching of quinones *
1510	Aromatic C=C stretching -NH ₃ ⁺ bending
1380-1440	Aliphatic C-H bending
1400	OH bending of alcohols or carboxylic acids
1220	C-O stretching or OH bending, due mainly to carboxyl groups
850	C-H bending in aromatic compounds *

* Assignments from reference 93

not be similar.

Absorption in the UV region by humic materials is postulated to result from CO₂H- and OH-substituted aromatics, α,β -unsaturated ketones, and dicarbonyls (e.g., quinones) (90). The presence of CO₂H and phenolic OH groups is supported by variations in the UV spectra with pH, caused by ionisation or protonation (90).

The source of absorption of visible radiation by humic substances is more uncertain. Sato and Kumada (95) proposed a structure related to 4,9-dihydroxyperylene-3,10-quinone (I). Alternative explanations have included light scattering, leading to apparent absorption of radiation (96), and the presence of charge-transfer complexes (97). However, these are probably minor contributors to the absorption because light scattering is not likely to be significant in the lower molecular weight fulvic acids, and there is little evidence for the formation of electron donor-acceptor complexes in humic substances (90).



The ratio of absorbances at 465 and 665 nm (E_4/E_6 ratio) has frequently been used to measure the extent of humification, with a low ratio indicating a more condensed aromatic structure of less recent origin (51,98). Thus fulvic acids yield higher E_4/E_6 ratios than humic acids. De Haan (99), however, found that, due to low fulvic acid concentrations, absorption at 465 and 665 nm was minimal and so he adopted the ratio of absorbances at 250 and 365 nm (E_2/E_3 ratio) as an index of humification.

UV-visible absorbance measurements at fixed wavelengths have been employed to estimate the concentration of humic materials/DOC, both of discrete samples (100,101,102) and in size-exclusion chromatography (103), in natural waters. Problems inherent in this method, however, include variations in the extinction coefficient between both humic fractions and materials from different origins (7), absorption by non-humic materials (94), and interference from inorganic constituents (104,105).

2.4.3 Fluorescence spectroscopy

This technique suffers from many of the limitations of UV-visible spectroscopy with regard to determining the functionality of humic materials. Additional problems inherent in the fluorescence method include the need to correct for light scattering and the variation of instrument sensitivity with wavelength (106) plus the possibility of fluorescence quenching if paramagnetic ions or molecules are present. The latter has been employed to advantage, however, in metal complexation studies on humic substances (107). As with UV-visible spectra, fluorescence intensities vary with pH (108). In addition,

changes in redox potential and temperature were found to affect the spectra (108).

In spite of these problems, however, several workers have performed fluorescence measurements on humic materials (MacCarthy and Rice (89) and Bloom and Leenheer (90)). Visser (108) has listed excitation and emission maxima for humic substances from various origins. Excitation spectra usually consist of one or more broad bands which occur over a wide region from about 250 to 500 nm. Emission spectra generally only exhibit one broad band over a narrower region, at ca. 400-550 nm. Sharper bands (and a different apparent absorption maximum) may occur as a result of Raman scattering (106) and so fluorescence spectra must be corrected to account for these. Excitation in the 300-450 nm range is most commonly employed, but for natural waters, which have a high absorbance at lower wavelengths, a greater fluorimetric response can often be obtained with low wavelength excitation. Stabel (cited by Steinberg and Muenster (12)) was able to differentiate between autochthonous DOM, DOM of terrestrial origin and DOM from saline lakes by using excitation at 230 nm.

Higher molecular weight material has been shown to fluoresce with a lower intensity and at lower excitation and emission wavelengths than lower molecular weight material (108,109). Suggested reasons for the lower fluorescence of humic acids have included the 'possible involvement of the fluorophores in cross-linking of the humic acid structure' (108) or increased quenching in the higher molecular weight molecules due to an increased density of aromatic chromophores (109).

Fluorescence spectroscopy has also been used to compare natural and synthetic humic substances. Synthetic marine humic materials, produced by allowing marine lipids and a diatom to autoxidise in seawater in the laboratory (110), showed the same broad fluorescence emission between 350 and 650 nm (350 nm excitation) as marine humic substances. The fluorescence spectra of synthetic phenolic and melanoidin polymers, however, were generally of lower intensity and had excitation maxima at a higher wavelength than natural humic substances (111).

Fluorescence measurements have found a number of other applications. Their use for the determination of DOC concentration (102) is considered by some to be unreliable (112). Fluorescence polarisation has been used to investigate the conformation of humic materials (113), and maximum excitation wavelength has been employed to provide information on the phenolic group content of humic substances.

2.4.4 Nuclear magnetic resonance spectroscopy

^1H and ^{13}C nuclear magnetic resonance (NMR) studies have been extensively employed in structural and quantitative studies of both underivatised and derivatised humic materials (114-117). Quantitative measurements by ^{13}C NMR, however, have inherent problems due to the variable relaxation times of carbons in different environments, and variable nuclear Overhauser effects (NOEs) necessitating a sufficient delay to be left between radiation pulses to allow all the nuclei to relax. In some cases, differences in relaxation times and variable NOEs can be overcome by the addition of paramagnetic relaxation reagents. Elimination of the NOE can also be obtained by turning off the proton irradiation during the delay period between pulses (inverse-gated decoupling).

The advent of cross-polarisation magic angle spinning (CPMAS) solid-state ^{13}C NMR overcomes relaxation and NOE problems - rapid relaxation occurs in the cross-polarisation experiment as the ^{13}C atoms relax at the faster time characteristic of protons. In addition, cross-polarisation enhances the signal up to a factor of four, sample solubility in NMR solvents is not required, and the sample is recovered in an uncontaminated state. However, there are still limitations to this method in that complete cross-polarisation may not occur and the sample cannot always be spun sufficiently fast at high fields to eliminate artefacts of the technique.

The general assignments of proton and ^{13}C resonances in humic materials are given in Tables 2.6 and 2.7. More detailed assignments for CPMAS ^{13}C NMR are documented by Malcolm (116). These are essentially the same as for solution-state ^{13}C NMR spectroscopy but may vary slightly (by a few units) due to solvent and temperature

Table 2.6 Major ^1H resonances of humic substances (117)

<u>Chemical shift (ppm)</u>	<u>Assignment*</u>
0.8 - 1.7	Alkyl protons attached to carbons removed from aromatic rings or polar functional groups
1.7 - 3.3	Protons of methyl or methylene groups α to aromatic rings or polar functional groups
3.3 - 5.0	Protons attached to carbon α to oxygen groups e.g., alcohol or ether protons, sugars of carbohydrates
6.5 - 9.0	Mainly aromatic protons, also quinones phenols and heteroaromatics
Variable	Exchangeable acidic protons of phenols and carboxylic acids

* For a more detailed assignment, see Wilson (117)

Table 2.7 Major ^{13}C resonances of humic substances (118)

<u>Chemical shift (ppm)</u>	<u>Assignment</u>
0 - 50	Alkyl carbon
50 - 90	Aliphatic carbon singly bonded to one oxygen atom ($-\text{C}-\text{O}-$)
90 - 112	Carbon singly bonded to two oxygen atoms ($-\text{O}-\text{C}-\text{O}-$)
112 - 160	Aromatic (and unsaturated) carbon
160 - 190	Carbon doubly bonded to one oxygen atom ($>\text{C}=\text{O}$)

effects. Except for minor details, most workers are in general agreement with the qualitative interpretation of these data. The degree of aromaticity of humic substances has long been a controversial subject. Early work, based on results from ^1H NMR experiments, generally indicated the absence (119) or low abundance of aromatic protons. This was thought to be caused by either the low solubility of the humic materials in the NMR solvent, a high degree of aromatic substitution, or by the spin of unpaired electrons affecting the relaxation of the nucleus (119). The introduction of ^{13}C NMR to the study of humic substances has enabled aromaticity to be determined more accurately although measurements may be potentially high due to the inclusion of olefinic carbon at similar chemical shift values. Hatcher *et al.* (120) obtained an aromaticity of 23% for a peat humic acid by ^1H NMR, but the same material analysed by ^{13}C NMR gave a higher value of 33%.

In general, terrestrial humic substances are more aromatic than those of marine origin (118,121). The aromaticity of soil humic material has been a subject of controversy between Ruggiero *et al.* (122), who found appreciable concentrations of aromatic protons in soil humic substances by ^1H NMR, and Wilson and Goh (123), who found that aromatic compounds were normally absent by ^{13}C NMR. In a later study, however, Wilson *et al.* also found aromatic carbon (124). Ruggiero *et al.* (122) concluded that aromatic structures are 'significant constituents' of soil humic substances but Wilson and Goh (125) suggested that soil humic materials from differing environments show a wider range of aromaticities.

The assignment of peaks in the aromatic region of the spectrum has also been challenged (126,127). Based on a comparison of the content of aromatic carbons determined by ^{13}C CPMAS NMR and oxidative degradation studies, Ikan *et al.* (126) concluded that the aromatic signal in the ^{13}C NMR spectrum could not be accounted for wholly by aromatic hydrocarbon rings. They suggested heterocyclic systems, such as hydroxy-furanone, pyrrole and furan, as the main building blocks of the polymeric core of humic substances. However, in contrast to these results, other investigators (128) have found that ^{13}C CPMAS NMR studies gave lower aromaticities than those previously calculated by chemical degradation methods. These assignments must therefore be

treated with caution.

The aliphatic component of humic material has also been investigated by NMR. It has been described by Hatcher *et al.* (129) as a 'major fraction of humic acid structures in both terrestrial and aquatic environments'. They suggested that the similarities of these complex, paraffinic structures in both terrestrial and marine humic substances indicate similar source material and/or formation mechanisms. They proposed that these structural units in marine humic substances originated from algal, or possibly bacterial, lipids. ^1H and ^{13}C NMR investigations by Wilson *et al.* (130) appeared to agree with this hypothesis as the NMR spectra of marine humic substances compared well with the extractable material from their phytoplanktonic precursors. The presence of polymethylene chains has also been noted by various workers (114) - their persistence probably being due to their low biodegradability.

The contribution of polysaccharides to humic structure has been studied by NMR but conclusions may be biased due to the co-extraction of non-associated carbohydrates with the humic material (67). Wershaw *et al.* (79) performed ^{13}C NMR studies on humic fractions methylated with ^{13}C -enriched reagents in order to differentiate between the different hydroxyl groups in the humic material. They demonstrated the presence of carbohydrate-like hydroxyl groups in 'Contech' fulvic acid, in addition to carboxyl and phenolic groups. Preston and Ripmeester (131) investigated humic substances and their hydrolysis products from four organic-rich soils. The fulvic acid spectra showed a very high carbohydrate content with little aromatic character, but the spectra of the hydrolysed materials had much higher aromatic contents with reductions in the signal due to carbohydrate moieties.

Malcolm (116) pointed out that care should be taken when assigning the 60-90 ppm region in ^{13}C NMR purely to carbohydrate resonances as it also covers other C—O signals. C—N is also sometimes observed in this region but humic substances generally have a low nitrogen content. He stated that independent analyses for simple hydrolysable sugars usually indicated a lower carbohydrate content than quantitation using the C—O resonance in the ^{13}C NMR spectrum. The presence of aliphatic polyethers in humic materials, as determined by

^{13}C NMR, has been suggested by Bayer *et al.* (132). They proposed that these groups occupied a dominant position as basic building blocks of humic substances. They did not mention, however, any contribution from carbohydrate moieties.

In summary, it can be seen that ^1H and ^{13}C NMR have made valuable contributions to the structural elucidation of humic substances. It is likely that in future, NMR spectroscopy of nuclei other than ^1H and ^{13}C will also prove useful in this field. ^{15}N and ^{29}Si NMR have already been employed in the study of derivatised fulvic acids (133).

2.4.5 Electron spin resonance spectroscopy

The application of electron spin resonance (ESR) spectroscopy to the study of humic substances has recently been reviewed by Senesi and Steelink (134). ESR spectra of humic substances consist of a single line and generally lack detailed hyperfine structure although a small degree of splitting has been observed by some workers (89,134). Fulvic acids usually have a lower spin content than humic acids. Spin content of humic substances are reported to decrease in the sequence:

Soil humic acid > Stream humic acid > Soil fulvic acid > Stream fulvic acid (135).

Low atomic H/C ratios and increased spin concentration have been related to a greater degree of molecular condensation and humification (136).

Physical and chemical influences on the ESR properties of humic materials (134) tend to support the presence of quinone/hydroquinone moieties. The quinone group is not, however, the only functional group contributing to the spin content of humic substances; factors such as the basicity of electron-donating groups (135) are also important. Metal complexes of humic acids have also been studied by ESR spectroscopy (137).

2.4.6 X-ray analyses

Humic substances have been studied using a variety of X-ray analyses including X-ray diffraction (10), X-ray photoelectron spectroscopy (also known as electron spectroscopy for chemical analysis (ESCA)) (90), and small-angle X-ray scattering (86). X-ray scattering is generally used to measure molecular size and weight (Section 2.3) and so will not be discussed further here.

X-ray diffraction of humic acids often shows broad bands around 3.5 Å, which have been attributed to layers of condensed aromatic rings (138). Fulvic acids, on the other hand, exhibit diffuse bands in the 4 to 5 Å region, thought to arise from an irregular arrangement of aromatic rings due to the presence of aliphatic side groups preventing closer packing. Kodama and Schnitzer (139) studied the X-ray diffraction pattern of a soil fulvic acid and observed a diffuse band at 4.1 Å and a few minor peaks. More detailed analyses led them to conclude that the fulvic acid carbon skeleton consists of a 'broken network of poorly condensed aromatic rings with appreciable numbers of disordered aliphatic or alicyclic chains around the edges of the aromatic layers'. These results are in agreement with other chemical and spectroscopic studies which suggest a more condensed aromatic structure in humic acids as compared to fulvic acids.

Few studies have been performed on humic materials using X-ray photoelectron spectroscopy (XPS). Desbene *et al.* (140) examined the composition of estuarine sedimentary humic substances and found that the elemental composition of humic acid by XPS compared well with elemental analysis for sulphur, but nitrogen and oxygen results differed by a factor of two or three. It was concluded that the surface composition of the samples was different from the mean bulk composition. The environments of the carbon, nitrogen and sulphur were similar in both fulvic and humic acids. Three forms of carbon were present - aliphatic or aromatic, C-O, and C=O; nitrogen had a slightly positive charge compared to quaternary ammonium ions; and two forms of sulphur were found - neutral sulphur atoms (e.g., RSH, RSSR, etc.) and positively-charged sulphur atoms (e.g., sulphones, sulphites, sulphates, etc.).

Other investigations have included a preliminary analysis of a soil humic acid, a soil fulvic acid, a fungal melanin and polymaleic acid (141). Carbon and nitrogen spectra were similar for humic acid and fungal melanin, and fulvic acid and polymaleic acid. Soil fulvic acid, however, appeared to show more C-O and C=O character, and less carboxyl character, than the polymaleic acid.

The binding of various cations to fulvic acid has also been studied (142). The data were consistent with salicylate-type coordination complexes. It has been suggested (90), however, that this technique is of limited use for obtaining structural information as it is not sensitive to subtle bonding differences.

2.4.7 Mössbauer spectroscopy

Relatively few Mössbauer studies have been performed on humic substances - a brief review is given by Bloom and Leenheer (90). Work on humic materials has generally focussed on iron as it is the most easily studied metal. The form of iron in humic and fulvic acids has been a subject of controversy between Dickson *et al.* (143) and Senesi (144). Dickson *et al.* (143) performed Mössbauer spectroscopy on humic and fulvic acid samples from natural sedimentary environments and stated that there was no evidence of any organically-bound iron in the materials studied. They indicated that the iron in the humic acid fraction was present in inorganic residues, such as silicate, and that the iron in the fulvic acid fraction was in the form of hydrated ferrous (Fe^{2+}) ions associated with the fulvic acid fraction. Senesi (144), however, criticised the use of Mössbauer spectroscopy alone without further spectroscopic/chemical evidence, and suggested that the iron occurs partly in the hydrated form and partly directly bound to the humic material. The occurrence of ferrous iron, both completely hydrated and associated with oxygen-containing functional groups in humic acid, has also been suggested by Goodman and Cheshire (145). Lakatos *et al.* (146) have concluded that both humic acid functional groups and water are coordinated to the iron (Fe^{2+}).

The presence of ferric (Fe^{3+}) ions in humic extracts has been reported (146) although it is thought likely that this was present as inorganic iron (147). The reduction of Fe^{3+} to Fe^{2+} in humic

substances at different pH values has been investigated (145) and was found to be favoured at lower pH.

2.4.8 Mass spectrometry

Mass spectrometry (MS) has been employed extensively in the identification of reaction products, often with prior gas chromatographic separation, in chemical degradation (e.g., (148)) and pyrolysis studies (e.g., (149)) on humic materials. However, few direct mass spectrometric studies on humic substances have been performed, mainly due to the complex appearance of the resulting spectra, coupled with instrumentation problems in analysing polymers.

Conventional electron impact mass spectra have been obtained on samples volatilised using a heated probe (150). This approach, however, leads to thermal decomposition of the sample with no evidence of high molecular weight ions as only volatilised pyrolysis products are ionised. Secondary reactions between pyrolysis products may also occur leading to incorrect structural interpretations. Field desorption mass spectrometry also applies thermal energy to the sample although larger molecular fragments can be obtained as the pyrolysis products are ionised *in situ* on the surface of the emitter and thus do not require vaporisation. Molecular fragments of humic materials up to $m/z = 670$ have been obtained using this technique (151).

Thermal decomposition of the sample can largely be avoided by using the fast atom bombardment (FAB) ionisation technique in which the sample, mounted in a suitable matrix, is ionised using a fast beam of atoms or ions (commonly argon atoms or, more recently, caesium ions). The source temperature is low and thus thermal decomposition is minimised. This technique generally yields an intense parent ion and few molecular fragments from a pure compound, and has frequently been employed in the analysis of high molecular weight polymers, such as proteins (152). The few FAB studies on humic materials have been relatively unsuccessful. Early investigations on a sedimentary fulvic acid fraction, during the developing stages of the FAB technique, showed no fragments above $m/z = 300$, although several low molecular weight ions were tentatively identified (153). Positive and negative ion FAB on a humic acid was recently performed by Andreux *et al.*

(151), who also found an abundance of low molecular weight fragments. Conversely, Thurman (7) reported the negative ion FAB mass spectrum of an aquatic fulvic acid with molecular fragments above $m/z = 600$. The spectrum was too complicated to obtain useful structural information.

FAB-MS therefore shows potential in the analysis of humic materials, generating complex spectra. Future work may involve analysing fractionated humic substances to simplify the resulting spectra, or performing further mass spectrometry on the initial complex spectrum (FAB-MS-MS). Both approaches are expected to yield valuable information on the structure of these materials (7).

2.5 DEGRADATIVE STUDIES

2.5.1 Oxidative degradation

Oxidative degradation of humic substances has been performed under a variety of conditions employing oxidants such as alkaline potassium permanganate (148), alkaline copper (II) oxide (160), nitric acid (154), alkaline nitrobenzene (155), periodic acid (156), hydrogen peroxide (157), sodium perborate (157), potassium persulphate (158), and sodium hypochlorite (67). Early work, mainly on soil humic materials, was reviewed by Schnitzer and Khan (10) although more recent surveys on oxidative degradation of both aquatic and soil humic substances have now been written (7,159,160).

Investigations using the oxidants listed above have generally yielded varying amounts of benzenecarboxylic acids and aliphatic mono-, di-, and tricarboxylic acids (often as methyl esters because the oxidation products are usually methylated prior to GC-MS analysis). In addition, hydroxybenzaldehydes have resulted from alkaline copper oxide and nitrobenzene oxidations (60,155), furancarboxylic acids and carboxyphenylglyoxylic acids from alkaline potassium permanganate (148), nitrophenols and nitro benzoic acids using nitric acid (154), and various chlorinated products from chlorination of aquatic humic substances (161).

Care should be taken in interpreting these results, however, as the products of oxidation have been shown to vary with both the

oxidation conditions (160,162) and the type of oxidant employed (157,160). Premethylation of the humic material has been adopted by some workers to protect phenolic moieties although it has been pointed out that side-reactions occurring during this process may alter the structure of the original sample (159,160). Reuter *et al.* (162) compared degradation products of aquatic humic substances using aqueous potassium permanganate oxidation of both unmethylated and methylated aquatic humic materials, and potassium permanganate oxidation of premethylated aquatic humic materials in an aprotic solvent. They found that, although similar reaction products resulted in all experiments, their relative concentrations varied dramatically. A predominance of benzenecarboxylic acids occurred without premethylation whereas oxalic acid dominated the oxidation products in the organic solvent. They suggested that oxidation in the organic solvent protected the primary reaction products from further oxidation as diprotic acids were insoluble in the reaction medium and hence precipitated as their potassium salts. A measure of the degree of oxidation was expressed by calculating the ratios of oxalic acid to benzenoid products, and phenolic to benzenecarboxylic acids. Oxidation in the organic solvent was the milder method. They concluded that the humic material studied contained a significant aliphatic component but warned that structural interpretation based on results from the more severe oxidation conditions would have favoured a more aromatic structure.

It appears, therefore, that a selection of oxidative degradation conditions should be employed to obtain a full picture of the structure of the original humic substances. Data should be compared with independent physical and chemical analyses of the materials, and, in order to compare humic substances of different origins, identical oxidation conditions should be employed. In addition, it must be remembered that the oxidation products are often only a minor portion of the original humic substances.

2.5.2 Reductive degradation

As in the case of oxidation studies, various procedures have been used for the reductive degradation of humic substances. These have included sodium amalgam reduction, zinc dust distillation and fusion (reviewed by Stevenson (163)), low (164) and high (165) pressure catalytic hydrogenation, degradation using sodium in liquid ammonia (166), phosphorus and hydriodic acid reduction (119,167), and cleavage by iodotrimethylsilane (164). Depending on the severity of the reaction conditions, different degradation products result. Milder conditions, such as sodium amalgam reduction, have produced phenols, phenolic acids and aliphatic components whereas those which are more drastic, such as zinc dust distillation and fusion, have yielded mainly polycyclic aromatic hydrocarbons (PAHs) (163).

The contribution of polycyclic aromatics to the overall humic structure is debatable. Drastic reduction conditions can lead to recombination of primary fragments and dehydrogenation, which can both yield these structures. It has been shown by Cheshire *et al.* (167) that furfural, dihydroxybenzoic acid and quinone polymers all generate polycyclic aromatic hydrocarbons from zinc dust distillation. The yield of dihydroxybenzoic acid was also found to vary with the distillation temperature (168). In addition, polycyclic aromatics have been determined in soil lipid fractions (51), and can be isolated using macroreticular resins (169). Thus, they may be co-extracted with both soil and aquatic humic substances. Adsorption of PAHs to humic materials has also been investigated (170). Considering the low levels of these compounds generally obtained by reductive methods, it is possible that they do not constitute the central 'core' of humic substances as has been suggested by various investigators (119,168), but are either co-extracted with, or loosely adsorbed to, these materials.

Catalytic hydrogenation, especially at high temperatures and pressures, also employs drastic reaction conditions. Stuermer and Harvey (165) performed exhaustive reduction of marine fulvic acid, using a multi-step reaction procedure, in an attempt to convert most functional groups to hydrocarbons. The distribution of the resulting hydrocarbons was used as an indicator of the origin of the humic

material. Some workers are critical of reduction by hydrogenation as they suggest that the high pressures and temperatures employed lead to molecular rearrangements and condensation reactions (163). Stuermer and Harvey (165), however, believe that the aromatic hydrocarbons obtained in their reduction products originated from the fulvic acid and were not products of cyclisation and aromatisation during reduction.

Due to the limited information resulting from severe degradation techniques, some workers have used milder conditions, which can lead to more specific bond cleavage. Sodium amalgam has been widely employed as a reducing agent for humic substances. It is thought to attack electron-rich areas of the molecules, such as ether linkages. Variable yields, often low, have resulted from this method, however, possibly due to differing reaction conditions. Piper and Posner (171) found that the yield of phenolic material changed as the reagent quantities were altered. It has also been shown that some phenolic degradation products may be further degraded in the reaction mixture (172).

More specific bond cleavage has been obtained by employing two different reducing agents. Michaelis *et al.* (164) performed reductive degradation using catalytic hydrogenation and cleavage with iodotrimethylsilane. In this method, alkyl ethers are easily cleaved by iodotrimethylsilane whereas hydrogenation degrades aryl ethers preferentially.

It can be concluded that results from reductive degradations must be viewed with caution as the yields of the various products may be dependent on the reaction conditions employed, and compounds may also be formed via secondary reactions in the reduction medium.

2.5.3 Hydrolysis

Hydrolysis of humic substances has been performed both as a pretreatment prior to further structural studies (both spectroscopic and degradative), and as a structural degradation procedure *per se*. Examples of these applications are included in a recent review by Parsons (173). The technique is a mild degradation procedure and is

thought to remove constituents loosely bound to or co-extracted with the humic macromolecule, such as carbohydrates and proteins, having little effect on the basic 'core' of the material. Hydrolyses have been carried out in acidic or basic solutions, in water alone, or, less frequently, using enzymes.

Hydrolysis in water alone provides a mildly acidic reaction medium which has yielded sugars, uronic acids, etc. (from polysaccharide structures), polypeptides (which gave amino acids on hydrolysis with hydrochloric acid (6 moles/l)) and simple phenolic compounds (119). On boiling the residue with hydrochloric acid (6 moles/l), more amino acids and phenols resulted, together with some inorganic material. The total material lost was 30 - 60% by weight although it is not unknown how much of the biogenic material was co-extracted with the humic substances. In addition to the aforementioned compounds, normal fatty acids and alkanes have also been found in aqueous hydrolysates of humic substances (174).

Acid hydrolyses have generally been performed using sulphuric or hydrochloric acids. Products derived from proteins and carbohydrates, together with phenolic compounds in some cases (e.g., (175)), have resulted (176). Determinations of carbohydrates and proteins in humic materials have usually employed an initial hydrolysis in acidic media. The classical procedure for protein hydrolyses involves hydrolysis in hydrochloric acid (6 moles/l) (e.g., (177)). Although tryptophan and sulphur-containing amino acids are degraded, this method is still widely used for amino analyses and has been employed for their determination in humic substances (e.g., (178,179)). For polysaccharide analyses, however, sulphuric acid hydrolysis is generally adopted as it causes less degradation of the resulting sugars than does hydrochloric acid (173). A problem with acid hydrolysis, however, can be the occurrence of secondary reactions between hydrolysis products.

Base hydrolyses of humic substances have most frequently been performed in sodium hydroxide. It is interesting to note, however, that extraction of both terrestrial and aquatic humic substances often involves dissolution in sodium hydroxide. Thus, it is possible that the extracted humic materials have already been subjected to mild

oxidative hydrolysis prior to subsequent analyses. Oxygen has been shown to be consumed during extraction using sodium hydroxide (180).

Aquatic humic material has been subjected to both sodium hydroxide hydrolysis, in the absence of oxygen, and alkaline potassium permanganate oxidation (148). In both instances, benzene carboxylic acids, furan carboxylic acids and aliphatic mono-, di- and tri-carboxylic acids (as their methyl esters due to methylation prior to analysis by gas chromatography (GC)) resulted. Lower yields occurred from the hydrolytic degradation and the identified percentage of GC peaks was lower in this case. It has been suggested (181) that the identification of more products following oxidative degradation of soil humic materials indicates that simpler products were formed by this process, compared with non-oxidative hydrolysis. The presence of (carboxyphenyl)glyoxylic acids (thought to be derived from polycyclic aromatic structures (148)) in the permanganate oxidation products, but not in the base hydrolysis medium, confirm the ineffectiveness of the latter procedure in degrading the central 'core' of the humic matter. Neyroud and Schnitzer (182) concluded that alkaline hydrolysis cleaved ether linkages and freed adsorbed compounds from humic substances, but did not degrade aromatic structures bound by C-C bonds.

Hydrolysis of lake sedimentary humic substances produced aliphatic hydroxy acids (thought to originate from carbohydrate moieties), aliphatic dicarboxylic acids, phenolic acids and long-chain fatty acids (183). Benzenepolycarboxylic acids were not detected in the sedimentary humic materials, in contrast to the lakewater humic substances analysed by Liao *et al.* (148). This may indicate the presence of more alkyl ring substituents or condensed aromatic structures in the sedimentary humic matter, with few oxygenated functional groups directly attached to the aromatic residues. The authors suggested that alkaline hydrolysis is superior to more oxidative degradation schemes as carbohydrate moieties yield aliphatic components characteristic of the original structure (CO_2 and H_2O result from oxidative analyses), and hydrolysis in the absence of oxygen preserves aliphatic structures, e.g., fatty acids.

A less popular hydrolysis method which has also been used is enzyme hydrolysis. This is discussed further in Section 2.5.5.

To conclude, hydrolysis can be seen to be milder than oxidation, generally cleaving C-O bonds and liberating materials loosely associated with the humic substances, but not breaking down the humic materials *per se* to any substantial degree.

2.5.4 Thermal degradation

Differential thermal analysis, thermogravimetry, differential thermogravimetry and isothermal heating have all been used to study the structure of humic substances (reviewed by Schnitzer and Khan (10)). Schnitzer and Hoffman (184) heated soil fulvic and humic acids to 540°C under air, sampling the solids at regular intervals. The carbon content increased with temperature, whereas the oxygen content decreased - at 540°C, no oxygen was found to be present although some stable nitrogen and sulphur still remained. The differential thermogravimetric curves both showed a well-defined peak above 400°C, which was assigned to decomposition of the 'core' of the materials. A peak at $\approx 280^\circ\text{C}$ occurred in the humic acid trace but was less prominent in that of fulvic acid. This was assigned to the removal of functional groups. Schnitzer and Hoffman (184) concluded that the reactions occurring during pyrolysis of the humic acid were dehydrogenation (up to 200°C), decarboxylation and dehydration (200 - 250°C), and dehydration (above 250°C). Dehydration was the main reaction in the pyrolysis of the fulvic acid.

Turner and Schnitzer (185) saw two main peaks in the differential thermogravimetric curves of another soil humic acid, but also found two smaller shoulders on these peaks. They concluded that the large peak at the lower temperature was due to cleavage of aliphatic and/or alicyclic structures, whereas the two shoulders and the main peak at higher temperatures were due to the break-up of aromatic structures.

Isothermal heating has also been employed in the analysis of humic materials. Kodama and Schnitzer (186) heated fulvic acid to 350°C to eliminate the functional groups, and then investigated the isothermal decomposition of the fulvic acid 'nucleus' at different temperatures between 370 and 390°C. They concluded that the main decomposition reaction occurring was dependent on a rate-determining diffusion process. Other isothermal heating studies have attempted to estimate

the aromaticity of the humic substances (92). After heating for extended periods of time (600 hours), complete oxidation of aliphatic and alicyclic structures occurred, leaving the aromatic core intact. Having corrected for the carboxylic carbon content (from the oxidised aliphatic and alicyclic groups), the remaining carbon and hydrogen is present in the aromatic core and so can be used to determine the aromatic content of the humic materials. It has been pointed out, however, that this may overestimate the aromaticity due to the production of aromatic artifacts produced during prolonged heating (187). Correction should also be made for proteins and carbohydrates in the materials.

By far the most popular thermal degradation method has been pyrolysis mass spectrometry (pyrolysis-MS), often employing GC separation of the pyrolysis products prior to MS analysis. This technique has become well-established for characterising polymers of various origins, e.g, biological, geochemical, synthetic (188). Due to the complex mixture of the products, direct mass spectrometric analysis is usually performed using softer ionisation techniques, such as field ionisation, to prevent excessive fragmentation. *In situ* pyrolysis has also been employed using field desorption MS (189). More recently, pyrolysis-GC-MS has become more popular as the mass spectra of individual pyrolysis products can be identified (conventional electron impact MS is generally used in this instance).

Pyrolysis studies of humic substances have generally fallen into two parts:-

- (i) Identification of pyrolysis products and their origin in the humic materials.
- (ii) Comparison of products resulting from the pyrolysis of humic materials from different environments, in an attempt to obtain further information on the origin of these materials.

The presence of biogenic materials, such as proteins, carbohydrates and lipids, has frequently been investigated in pyrolysis experiments on humic matter. Pyrolysis of individual pentoses and hexoses, and various polysaccharides, has recently been performed (190,191). Furaldehyde and anhydrosugars were found to be

produced by all the aldohexoses, ketohexoses and aldopentoses investigated. In addition, hydroxymethylfuraldehyde was detected in pyrolysates of hexoses but not pentoses. Positive identification of individual stereoisomers could be performed on the basis of the retention times of their distinctive anhydrosugars. The presence of anhydrosugars has not always been detected (192), probably due to the GC columns employed. Various carbonyl compounds and other furan derivatives have also been found in the pyrolysates of carbohydrates (192).

Pyrolysis of amino acids generally yields carbon dioxide and nitrile compounds, in addition to compounds unique to individual amino acids, such as pyrroles (proline, hydroxyproline), sulphur-containing compounds (cysteine and methionine), aldehydes (alanine, valine, nor-valine, leucine, iso-leucine) and aromatic residues (phenylalanine, tyrosine) (193). Determination of sulphur-containing amino acid residues has selectively been performed using a flame photometric detector (194). Polypeptides generally fragment to give the pyrolysis residues of the corresponding amino acids although dipeptides have been shown to yield products depending on the sequence of the acids in the peptide (193). Pyrolysates of lipids show less specificity for individual compounds - they yield series of saturated and unsaturated hydrocarbons (195), the maximum chain length being equivalent to the fatty acid present.

The presence of these biomolecules in humic preparations has been confirmed by pyrolysis. The polysaccharide contents of various soil fulvic acids have been determined by Martin (196). Removal of nearly 90% of the polysaccharides, however, was performed using gel-permeation chromatography. This indicates possible co-extraction of soil polysaccharides with the fulvic acid or their weak adsorption onto it. Saiz-Jimenez and de Leeuw (197), however, separated a soil fulvic acid fraction from a soil polysaccharide fraction by adsorption on Polyclar AT and stated that the fulvic acid sample was composed mainly of polysaccharides, in addition to considerable amounts of lignin moieties. This suggests that the carbohydrates form an integral part of this material, and are not co-extracted with the fulvic acid. Varying amounts of carbohydrates, peptides and lipids were found in fractions of soil organic matter using pyrolysis GC-MS

(198). Martin *et al.* (199) also investigated the pyrolysis of humic substances from various soils, which he separated into humic acid, fulvic acid and soil polysaccharide fractions. Major components of the humic acids were related to protein and polysaccharide moieties, and those of the fulvic acids to polysaccharide structures. Similarly, humic acids extracted from decaying aquatic plants, and various sediments (200) generally showed a predominance of peptidic compounds with low levels of carbohydrate compounds, whereas a sedimentary fulvic acid was almost entirely composed of sugars. It was suggested that the difference between the humic and fulvic fractions was, in fact, determined by the fractionation procedures, nitrogenous compounds precipitating at lower pH values whereas the carbohydrates remained in solution. The problem of co-extracted biogenic material as opposed to humic-bound material was not addressed in this instance. Components derived from proteins and carbohydrates have, however, also been determined by pyrolysis studies on aquatic humic substances isolated using Amberlite XAD-2 resin (198) which does not co-extract free polysaccharides or amino acids (201).

The removal of most of the protein, polysaccharide and lignin moieties was shown on pyrolysis of soil humic acid after acid hydrolysis (202). On methylation of the hydrolysed product prior to pyrolysis, several fatty acid methyl esters were among the major components. These were thought to originate from fatty acids of microbial origin, tightly-bound to the original humic material.

In addition to these biomolecules, other pyrolysis products, which have been found and are more characteristic of the origin of the humic materials, include phenolic compounds, and these often indicate the presence of lignin (terrestrial origin) (203). The occurrence of lignin pyrolysis products in various soil organic fractions has been reported (198). As lignin is lost during humification, the presence of its pyrolysis products in soil organic material can be used as an indication of the degree of humification (204). The absence of lignin in estuarine colloidal material (205), coastal humic substances and a marine diatom (206) has been noted. A pyrolysis GC-MS study of humic substances from decaying aquatic plants, indicated the presence of lignin in phanerograms but not algae (which were enriched in proteins) (200). It was also found that, when lignin is absent, algae can be an

alternative source of low levels of phenolic pyrolysis products from tyrosine residues. On the basis of their pyrolysis products, the origins of humic substances from various sedimentary environments were suggested. A comparison of terrestrial, freshwater, marine and planktonic humic materials using pyrolysis GC-MS (149) suggested the presence of lignin in terrestrial and freshwater humic substances, but not in those of marine or planktonic origin. This was based on the presence of methoxy phenol pyrolysis products. Care must be taken when assigning phenolic compounds to lignin precursors as they may also be pyrolysis products of other materials, such as proteins (207). However, they generally represent minor components in these cases as seen, for example, above (200). The presence of phenol and methylphenol was found in both the aquatic and terrestrial samples and was suggested as originating from marine vegetation. The authors did not mention, however, any possible contribution from the resin used to extract the humic materials. Contamination from Amberlite XAD-2 has been reported in a pyrolysis-mass spectrometric study (208).

In addition to these pyrolysis studies, other work has included the comparison of intracellular and extracellular material from a marine diatom with coastal humic substances (206). A close relationship was found between the extracellular material and the humic matter and it was suggested that algal exudates and their decomposition products are possible precursors of coastal humic substances. Seasonal variations in the composition of aquatic fulvic acids have also been studied (209) indicating a relatively high proportion of aromatics in the fulvic acids in winter compared to a dominance of carbohydrates in summer. Investigations on synthetic humic materials (210) and model humic compounds (197) have also been performed.

Recently, pyrograms of some hydrolysed soil humic acids, showing homologous series of alkanes, alkenes and alkadienes, were found to be similar to those of non-hydrolysable, highly aliphatic, biopolymers from higher plants and algal cell walls. Their occurrence has been summarised by Tegelaar *et al.* (211). It was suggested that this may form part of the 'core' of humic acids. However, further work is required, both on the structure of the biopolymer and its similarity to the humic core, to support this hypothesis as other biochemical /

geochemical structures (e.g., kerogen, lipids) yield similar series on pyrolysis (199)).

2.5.5 Biological degradation

Relatively few studies have used biodegradation methods in the structural analysis of humic substances. A brief review of less recent work is given by Schnitzer and Khan (10).

Burges and Lather (212) found a positive relationship between the ability of certain fungi to decolorise humic substances and their ability to reduce carboxylic acid groups in m-hydroxybenzoic acid. They concluded that carboxylic groups were reduced during the fungal degradation of the humic materials. This agrees with the results of Mathur and Paul (213) who found that humic acid degraded by *Penicillium frequentans* had a higher OH content than the original material. This was attributed to cleavage of ether bonds during degradation.

Less-coloured material also resulted from the high molecular weight fraction of fulvic acid when it was treated with benzoate-metabolising bacteria in the presence of benzoate (99). The product also showed more fluorescence and had a lower molecular weight than the original fulvic acid. Decomposition of humic compounds by soil anaerobic bacteria led to marked decreases in molecular weights and optical densities of the fractions investigated (214). Decarboxylation, decarbonylation and demethoxylation also occurred although, in contrast to the two studies cited above, the number of hydroxyl groups decreased.

Few compounds have been isolated from growth media after degradation. Salicylaldehyde, salicyl alcohol (213), p-benzoquinone and 2-methyl-1,4-naphthoquinone (215) were among the products resulting from degradations with *Penicillium frequentans* and a white rot fungus. However, a limitation of biological degradation procedures is that it is uncertain as to whether the isolated materials resulted from degradation of the humic substances or were produced by the organisms themselves (10). In addition, modification of primary products by the organisms may occur prior to their isolation.

More recent biodegradation studies have included the enzymatic hydrolysis of carbohydrates in aquatic fulvic acid, using a selection of enzymes of different origins (216). The results indicated that various glucose structures occurred in the fulvic acid investigated.

2.5.6 Photochemical degradation

Humic substances have been found to be involved in both direct and indirect photochemical reactions in the natural environment. Recent reviews have been written on both the photochemistry of natural water systems (217) and photochemical reactions in the environment involving organic material (218).

Direct photochemical degradation of humic substances has generally been studied using ultraviolet and fluorescence spectroscopy. Continued exposure of these materials, in solution, to ultraviolet radiation has been shown to lead to a decrease in their absorption intensity (41,218,219), although a corresponding decrease in the DOC content of water was not observed (41,218). This reduction in absorption intensity was accompanied by a drop in the intensity of electron spin resonance signals, indicating fewer long-lived radicals in the solutions (219). No decrease in the absorption intensity was found in the absence of light or oxygen (218), indicating the possible role of a photo-excited oxygen state in the degradation process.

Little work has actually been performed on the molecular structures involved in or resulting from these reactions. Visser (219a) suggested that irradiation of humic matter led to its partial oxidation and loss of some aromatic character, with older material being more resistant to degradation. In addition, he stated that fulvic acids were more susceptible to degradation than humic acids.

As well as direct photodegradation of humic matter, indirect photoreactions of these materials also occur and these have proved to be of environmental importance. Indirect photoprocesses involve photochemical reactions of compounds initiated through light absorption by the humic substances. Such reactions include the photodegradation of pesticides (25) and phenolic compounds (24) in the presence of humic materials. In contrast, certain photochemical

degradations are quenched by the presence of these materials (24). Other environmentally significant reactions involve the organically catalysed photoreduction of transition metals. Sunda *et al.* (220) demonstrated that insoluble manganese (IV) oxide was reduced in seawater containing humic material to soluble manganese (II) (thus increasing the bioavailability of the manganese) and that this reaction was greatly accelerated in sunlight.

Indirect photoprocesses are thought to occur via a variety of routes including electronic energy transfer, electron transfer, and free radical formation. These have been discussed in detail by Zepp (218). The nature and concentrations of excited states and reaction intermediates resulting from photoreactions of humic materials have been investigated using both steady state irradiation (220a) and laser flash photolysis (221). Fischer *et al.* (221) employed laser flash photolysis to examine transients created on absorption of light by humic substances in solution. Spectra of preconcentrated samples showed a maximum at 475 nm, which consisted of a strong short-lived (ca. 3 nanoseconds) and a long-lived (1-10 microseconds) absorbance. Dilute samples exhibited a long-lived transient at 700 nm. The effects of dissolved oxygen, transition metals, and pH on the transient species were monitored leading to the conclusions that the long-lived 475 nm transient was probably a triplet species (or a radical cation), and the transient at 700 nm behaved like a solvated electron.

Thus, it can be seen that data on photochemical degradations involving humic substances are limited at the present time, with little structural information on the decomposition products. Photoreactions may also play a part in the formation of humic substances as hypothesised by Harvey *et al.* (222). They proposed that the synthesis of marine humic materials occurs via an ultraviolet-catalysed, autoxidative cross-linking reaction of polyunsaturated fatty acids. Model studies on the autoxidation of pure marine lipids were also performed (110), which yielded material showing similar characteristics to marine fulvic acids, thus supporting the aforementioned hypothesis.

2.6 RADIOISOTOPE MEASUREMENTS

2.6.1 Radiocarbon dating

The age of humic substances has been determined by radiocarbon dating. The majority of ^{14}C measurements have been performed on soil humic materials. Typical values of the mean residence time (MRT - average age) for various surface soil organic materials have been listed by Stevenson (13) and vary between 250 and 3 280 years. Fractionated soil has also been studied (223) indicating that:-

- (i) fulvic acid is younger than humic acid. This is as expected since fulvic acid is generally considered to be a precursor of humic acid. Balesdent (224), however, found little difference between the ages of fulvic and humic acids.
- (ii) hydrolysable fulvic and humic acids had lower MRTs than the non-hydrolysable fractions. This is as anticipated due to the presence of non-refractory labile biogenic substances (e.g., carbohydrates) in the hydrolysable fraction. This is in agreement with the work of Balesdent (224).

Humic materials in buried soils (paleosols) are older than those in surface soils. Paleosol humic acids from a volcanic island off the coast of Italy were shown to range in age from about 6 000 to nearly 30 000 years (225).

Few studies have been performed on aquatic humic materials. Williams *et al.* (226) measured ^{14}C values on dissolved organic matter from deep (1900 m) open ocean water and found an average age of 3 400 years. By comparison, Suwannee River fulvic acid contained large amounts of 'bomb' ^{14}C and so is very recent (less than 30 years old) (67). The age of a groundwater fulvic acid has also been measured and found to be 660 (\pm 50) years old (227).

Humic substances can therefore be seen to exhibit a very wide range of ages. Further more detailed studies should be performed, for example, comparing the age of aquatic humic matter from both similar and diverse sources with other structural data, in order to obtain more information on the origins of these materials.

2.6.2 Isotope ratios

The study of isotopic compositions of organic matter can yield information on both the origin of the material and the processes involved in its formation. Carbon, hydrogen, nitrogen and sulphur isotope ratios have been determined although carbon is by far the most frequently measured. The percentage of ^{13}C in an organic material depends on its source of carbon, and so as terrestrial and marine organic substances generally arise from different origins, their percentages of ^{13}C are different. Carbon isotope ratios are measured mass spectrometrically and are expressed as:-

$$\delta^{13}\text{C} (\text{‰}) = \frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}} - (^{13}\text{C}/^{12}\text{C})_{\text{standard}}}{(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} \times 1000$$

A selection of data on the carbon isotope composition of various soil and aquatic humic substances is given in Table 2.8. Carbon isotope ratios of terrestrial and marine sediments have been listed by Nissenbaum and Kaplan (62) (including results of both their own work and those of other investigators). They found that the values of $\delta^{13}\text{C}$ for humic acids from marine sediments averaged between -20‰ and -23‰ , leading to the conclusion that the humic acids were formed *in situ* from plankton degradation products - values of $\delta^{13}\text{C}$ for plankton being around -19‰ (228,229). Humic acids from coastal and estuarine sediments generally had slightly lower ratios, in the -24‰ - -27‰ region, suggesting differing contributions from continental sources. Terrestrial humic acids were even more depleted in ^{13}C with the majority of values for sedimentary and soil materials in the range -25‰ - -28‰ , which is similar to those of higher plants (230). The data on dissolved aquatic humic substances (Table 2.8) can be seen to be in agreement with their sedimentary counterparts, with those of river and groundwater humic materials indicating a terrestrial origin, and marine humic matter, having higher $\delta^{13}\text{C}$ values, suggesting planktonic precursors.

Interesting variations were seen when Sigleo and Macko (231) compared $\delta^{13}\text{C}$ for particulate and dissolved colloidal estuarine organic material at different points in the estuary. The carbon isotope ratio of the particulate organic material became more enriched

Table 2.8 Stable carbon isotope ratios of aquatic and terrestrial humic substances

<u>Sample</u>		<u>$\delta^{13}\text{C}$ (‰)</u>	<u>Reference</u>
<u>Soil</u>			
Forest soil fulvic acid (Nova Scotia)		-26.3	62
Forest soil humic acid (Nova Scotia)		-26.2	62
Soil humic acid (Alberta, Canada)		-25.8	111
Minnesota Peat		-24.9	62
Forest soil fulvic acid (Saanich inlet)		-27.0	229
Forest soil humic acid (Saanich inlet)		-29.1	229
<u>Riverwater</u>			
Suwannee River fulvic acid		-31.4	67
Suwannee River humic material		-28.36	232
<u>Groundwater</u>			
Florida fulvic acid		-26.4	227
Colorado fulvic acid		-25.6	227
<u>Estuarine water</u>			
	(Date)		
Surface colloidal OM ⁺	7/83	-24.8 [0.6 ‰] ⁺⁺	231
		-25.0 [9.2 ‰] ⁺⁺	231
	11/83	-24.6 [0.7 ‰] ⁺⁺	231
		-24.7 [14.2 ‰] ⁺⁺	231
<u>Seawater</u>			
Marine fulvic acid (Gulf of Mexico)		-20.6; -21.5; -22.0	110
Coastal fulvic acid (Sargasso Sea)		-23.72	65
Coastal humic acid (Sargasso Sea)		-22.78	65
Coastal humic material (Baltic Sea)		-26.46 *	232
Off-shore humic material (Baltic Sea)		-26.13 *	232
Off-shore surface fulvic acid (Sargasso Sea)		-22.79	65

+ Discussed in text

++ Salinity

* Terrestrial input indicated

in ^{13}C as the salinity increased whereas that of the colloidal organic material remained constant in most samples. The data for the particulate organic material indicate the increasing influence of marine carbon with increasing salinity. The data for the colloidal matter is less easily explained, suggesting either that:-

- (i) there is a constant marine input to this fraction at salinities of 15 ‰ or less (unfortunately, measurements were not taken at higher salinities),
- or (ii) complex mixing/chemical processes are occurring in the estuary.

Care must be taken, however, when drawing conclusions on the source of carbon as anomalies may result. Decreased water temperatures, for example, have been shown to deplete ^{13}C in whole plankton (233), which may lead to incorrect assumptions regarding the origin of the material derived from this plankton. Materials from diverse origins may also yield misleading data, e.g., marsh fulvic and humic acids had values of -17.4 and -19.1 ‰ respectively due to the enrichment of ^{13}C in salt marsh plants (62).

In addition to obtaining information on the origin of humic substances, isotopic ratios have also been used to study the formation sequence of these materials. Nissenbaum and Kaplan (62) found that fulvic acids were, in general, isotopically heavier than their corresponding humic acids. In marine samples, the fulvic acid data were closer to that of their planktonic precursors than the humic acid data, and thus it was suggested that fulvic acids are an intermediate product in humic acid formation, as has been suggested by various other workers (229,234).

Other isotopes which have been less frequently studied include nitrogen, hydrogen and sulphur. $\delta^{15}\text{N}$ data, like $\delta^{13}\text{C}$ ratios, can provide information on the source of organic matter. Planktonic remains have $\delta^{15}\text{N}$ values of around +9 ‰ (235) whereas residual material from higher plants are more depleted in $\delta^{15}\text{N}$. Peters *et al.* (235) reported a good correlation between carbon and nitrogen isotope ratios. As with $\delta^{13}\text{C}$ values, $\delta^{15}\text{N}$ depends on the proportion of marine/terrestrial OM present, but anomalies may result under specific

environmental conditions. For example, some lake sedimentary $\delta^{15}\text{N}$ values, reported by Stuermer *et al.* (236), resembled atmospheric nitrogen rather than seawater nitrate (used by plankton to produce their cellular material) and it was suggested that the atmospheric nitrogen resulted from algae which utilised it.

Sulphur isotope ratios ($\delta^{34}\text{S}$) for humic compounds extracted from marine sedimentary materials and soils, have been determined by Nissenbaum and Kaplan (62). The marine samples had lower values than the terrestrial materials, with one exception which received detrital material from continental sources. The $\delta^{34}\text{S}$ values for the marine humic substances were similar to those obtained by Kaplan *et al.* (234) for free and organic sulphur in recent sediments, but differed from those of marine algae. The authors concluded, therefore, that the sulphur was introduced from external sources during diagenesis rather than occurring in the original source material.

Data on the hydrogen isotope ratio (δD) of humic substances are scarce. The usefulness of these ratios is yet to be established as factors such as deuterium exchange, plant transformations and precipitation all affect the deuterium content of organic material (236).

2.7 ELECTROCHEMICAL METHODS

2.7.1 Electrometric titrations

Electrometric titrations have been employed both to investigate the acid-base and metal-binding properties of humic materials. The application of these methods to the analysis of acidic functional groups in humic substances has been discussed by several workers (e.g., 10,51). Direct potentiometric titrations have generally yielded S-shaped titration curves (e.g., 20) similar to those of monoprotic acids although they are thought to represent overlapping curves of several acidic species in a polyprotic acid (51) or 'a mixture of polyprotic acids with a continuum of acid dissociation constants' (20), indicating the chemically disperse nature of the acid dissociating sites in fulvic acid. This shape is not unexpected as the dissociated protons in the two main acidic functional groups in

humic materials (carboxylic and phenolic OH) overlap, making it virtually impossible to distinguish between them by direct aqueous potentiometric titration (10).

Gamble (237) detected two endpoints in conductometric titration curves of a soil fulvic acid. He assigned the first one to carboxyl groups ortho to phenolic OH groups and the second to total carboxyl groups. Conductometric titration curves of Schnitzer and Skinner yielded one endpoint with sodium hydroxide but four with calcium hydroxide (238). Nonaqueous titrations with sodium aminoethoxide showed one inflection point when dimethylformamide was the solvent but two (corresponding to CO_2H and phenolic OH groups) when ethylenediamine was employed (cited by Schnitzer and Khan (10)).

Discontinuous titrations have been adopted to check attainment of equilibrium by the reaction mixture. Measurements, such as pH, are made at different times on aliquots of the sample containing increased amounts of the titrant. Several workers have noted a decrease in pH values with time in alkaline media (e.g., (239)). It has been suggested that this was due to base-catalysed side reactions yielding acidic components (240). Borggaard (241), in a different study, found that the pH decrease was due to bacterial activity and/or oxidation by air.

The metal-binding characteristics of humic substances have been investigated using potentiometric, conductometric and complexometric titrations. As the formation of a complex usually involves displacement of hydrogen ions from the humic materials, a pH drop is indicative of complex formation - the magnitude of which is related to the metal-binding tendency of the materials. The order of stabilities of various metal-humic complexes has been reported (242).

Titration of metal ions with base in the absence of humic substances shows inflections in the titration curves (238) due to the formation of metal hydroxides. When humic materials are titrated in the presence of low concentration of metal ions, the inflections characteristic of hydroxide formation are not present (238) indicating the formation of metal-humic complexes. If higher concentrations of metal ions are employed, however, hydroxide formation does occur

(238), indicating that all the metal-binding sites on the humic materials are taken. The number of protons released by the humic substances during titrations with no excess of metal ion can yield information on the stability of the complexes (243). Schnitzer and Skinner (238) found that, under identical conditions, the order of stability of fulvic acid-metal complexes, with increasing pH, was $\text{Al}^{3+} < \text{Fe}^{3+} < \text{Cu}^{2+} < \text{Ni}^{2+}$, the aluminium complex breaking up at pH 8 whereas the nickel complex remained intact at pH 10.

The conductometric titration of fulvic acid with sodium hydroxide shows a single minimum due to neutralisation of strongly acidic hydrogens (238). On titration with calcium hydroxide, however, three further inflections resulted which were interpreted as being due to strongly and weakly complexed calcium (238).

Finally, complexometric titrations have been used to compare copper-binding capacities of organic carbon from two water samples (243). Copper titrant ions (Cu^{2+}) were added to the samples and the solutions were analysed for remaining uncomplexed copper using differential pulse anodic stripping voltammetry (DPASV) and ion-selective electrodes (ISE). Both analyses indicated that one sample had a greater copper-complexing capacity than the other. Further studies employing ASV and ISE will be discussed in the next section.

The application of electrometric titrations to acid/base and metal-binding studies can, therefore, yield information on acid/base properties of humic materials and the ability of these materials to form metal complexes. In addition, the binding capacity and the stability of the resulting complexes can be studied using this method.

2.7.2 Other electrochemical techniques

Metal-binding studies on humic materials have also been performed using ion-selective electrode potentiometry, anodic stripping voltammetry, and pulse polarography. Studies using ion-selective electrodes are limited by sensitivity, and the range of divalent ions which can be measured (including Cu^{2+} , Pb^{2+} , Cd^{2+} , Ca^{2+} (13)) as only a few electrodes are commercially available. In addition, ISE determinations cannot be performed at very low ionic strength and so a

supporting electrolyte is often required.

Buffle *et al.* (244) measured the complexation properties of humic substances from two freshwater samples using lead and copper ion-selective electrodes. In addition to measuring the stability constants of the metal complexes, they also determined the mean molecular weight of the ligands and established the existence of both 1:1 and 1:2 metal:fulvic acid complexes.

Copper ion-selective electrode investigations have also been performed by Bresnahan *et al.* (245) and Buffle *et al.* (246), and additional lead-binding studies have been carried out by Saar and Weber (247). Bresnahan *et al.* (245) used a copper ion-selective electrode to determine the stability constants for the complexation of copper ions with water and soil fulvic acids. They compared these results with those obtained using differential pulse polarography (DPP), and found that the peak current in DPP was not proportional to the hydrated Cu^{2+} ion. They therefore employed the ISE method to determine the stability constants and number of binding sites per molecule. Two classes of binding sites were found (confirmed by electron spin resonance studies) with stability constants of soil and water fulvic acid complexes of around 1×10^6 and 8×10^3 respectively, and the number of sites per molecule increasing on increasing the pH from 4.0 to 6.0. The increase was attributed to conformational change with pH, rather than increased availability of donor atoms due to proton dissociation. Buffle *et al.* (246) investigated the influence of copper concentration, fulvic acid concentration, pH, and calcium concentration on the complexation properties of humic substances and then compared these properties for various water samples. They concluded that the complexation parameters varied little with source for similar water types. Lead binding to soil- and water-derived fulvic acids has been studied by Saar and Weber (247), indicating that the addition of even small amounts of lead caused aggregation of the fulvic materials. Stability constants increased with pH as for copper, yielding values of 1×10^4 at pH 4 and 2×10^6 at pH 6 for Pb^{2+} -soil fulvic acid complexes.

Gardiner (248), using a cadmium ISE, found that Cd (II) was bound by humic materials to a greater extent than by common inorganic

ligands, such as CO_3^{2-} , SO_4^{2-} , Cl^- , OH^- , particularly in waters where the humic ligands were predominant. As in the copper complexation studies of Bresnahan *et al.* (245), the degree of cadmium complexation was seen to increase with pH. Saar and Weber (249), investigating the complexation of Cd^{2+} by soil- and water-derived fulvic acid, found that conditional stability constants were dependent on the fulvic acid concentrations in contrast to their copper complexation studies. At pH 6, for example, K (soil fulvic acid- Cd^{2+}) decreased from 2.9×10^4 to 1.2×10^4 as the fulvic acid concentration increased from 19 to 360 mg/l. Other metals which have been studied using ISEs include sodium and potassium (250).

Differential pulse anodic stripping voltammetry (DPASV) has an advantage over ISE because it is more sensitive and can thus measure free metal ions at the levels found in natural waters without the requirement of preconcentration, thus reducing sample handling. In addition, minimal sample sizes are required (as little as 2 ml) and the method is non-destructive. However, it does have disadvantages in that, in common with ISE, a supporting electrolyte is required and its use is restricted to a limited number of metal ions. Also, the technique disturbs the equilibrium between free and complexed ions; dissociation often occurs during plating. Interpretation of results can additionally be difficult due to the adsorption of humic material onto the mercury electrode (107). These latter two problems have recently been overcome by Bhat *et al.* (251).

ASV is used to determine free and labile metal species in solution. Differentiation between complexed (non-labile) and free/labile metals has been performed by measuring the concentration of free/labile metal ions both before and after either acid digestion (252) or oxidation of organic matter (253). As the treatments are assumed to release all the bound metals, the difference between the two measured concentrations is taken as the concentration of complexed non-labile metal. The speciation of cadmium, copper and zinc in open ocean water at the natural levels of these metals has been performed using ASV on both acidified and unacidified samples (252). Addition of marine fulvic acid to the samples indicated little or no interaction of cadmium with these materials, strong interaction with zinc, and differing interactions with copper.

Other methods have involved employing ASV to determine the free/labile metal concentration during complexometric titrations. O'Shea and Mancy (254) developed such a method which differentiated between free metal and both labile and non-labile complexed metals by performing titrations with the metal and ligand as titrants and also measuring the peak current and potential during the titration. They studied the interactions between copper, thallium, cadmium and humic acid and found that copper formed strong, non-labile complexes whereas thallium produced mainly labile complexes, with cadmium showing intermediate behaviour.

Pulse polarographic investigations have proven less popular than ISE and ASV studies. Schnitzer and Khan (10) cited a polarographic study of reactions between humic acids in ammonium solutions and divalent metal ions. 10-15% of the added metal ions were complexed and the different metals (copper, nickel, cobalt) formed different numbers of complexes of varying stabilities. Lippolis *et al.* (255) investigated the pulse polarography of nitrosated fractions from gel-permeation chromatography, comparing the elution curves with those measured with an electrochemical detector and by atomic absorption measurements. Similarities between the elution curves measured by pulse polarography and those measured by the atomic absorption determination of iron led the authors to attribute the polarographic response to the reduction of an iron-nitroso complex. They suggested that the nitrosation technique followed by pulse polarography could be used to determine traces of iron in natural humic acids.

CHAPTER THREE

THE STRUCTURE AND FORMATION OF HUMIC SUBSTANCES

3.1 STRUCTURE

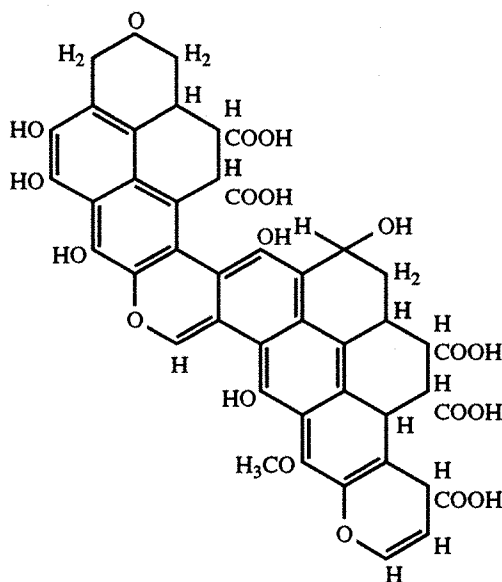
Despite the numerous independent approaches which have been used to study the structure of humic substances, the information arising from any one of these has been limited, and, in some cases, contradictory. In general, the resulting data only yield information on parts of the molecule (e.g., fluorescent chromophores, hydrolysable moieties) or confirm the presence of, for example, certain functional groups. They do not allow a complete molecular structure to be drawn as humic matter is a complex mixture of multifunctional molecules. Over the years, however, these studies have led to the continuing development of ideas on the general structures of humic substances and have led several workers to suggest 'class structures' for these materials which are generally consistent with certain experimental parameters. A selection of these ideas and class structures will be included here but the emphasis must be placed on the fact that any structures given are class structures and are not meant to represent the true (unknown) structures of these compounds. Caution must be exercised, however, as over-zealous analysis of these structures can be misleading, for example, structures derived from oxidative degradation studies are only modelling that limited portion of humic molecules susceptible to that form of degradation.

3.1.1 Soil humic substances

As stated in Chapter 1, early work on humic substances was dominated by investigations of soil humic matter. One of the earlier humic structures (Figure 3.1) was based on a model proposed by Fuchs (256) from his investigations on coal organic matter. However, present concepts of the low polycyclic aromatic content of humic substances together with a more flexible structure render this model obsolete.

As soil humic substances were thought to originate from lignin and lignin degradation products, they were generally considered to be

Figure 3.1 Structure of humic acid according to Fuchs (256)

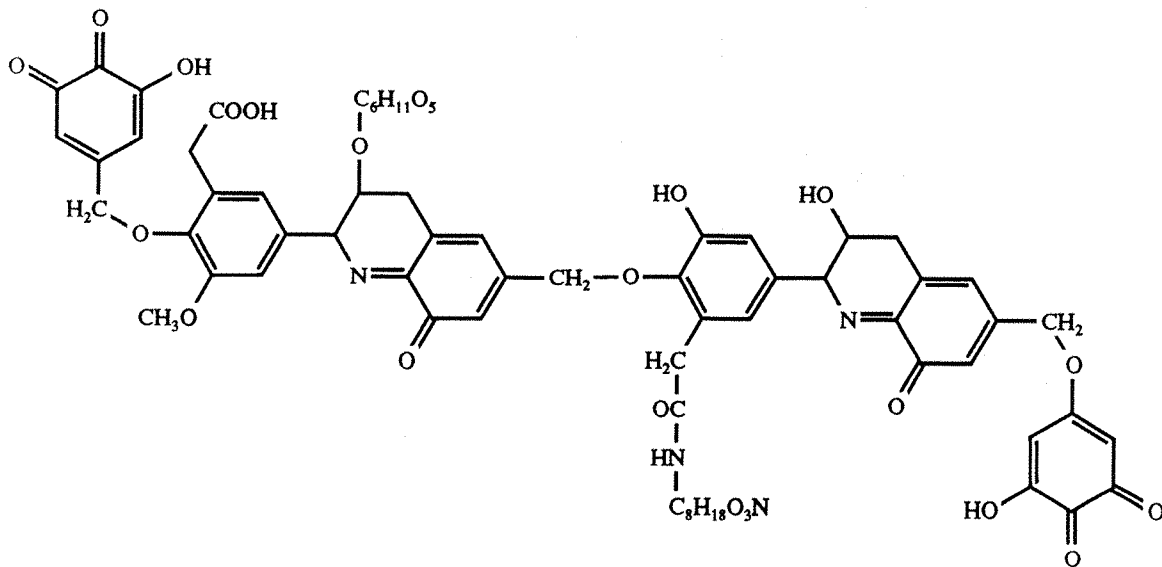


highly aromatic molecules, consisting of phenolic and benzenoid carboxylic acids. A selection of hypothetical structures of these materials is given in Figure 3.2. Each of these models possesses, to varying degrees, features in agreement with experimental studies on soil humic substances. All structures are highly aromatic and, in general, contain aromatic rings linked by aliphatic or hydrogen bridges. The models of Dragunov (cited by Kononova (5)), Flaig (257) and Felbeck (258) generally lack COOH groups, and those of Schnitzer (259) and Buffle (260) do not account for the presence of nitrogen in humic substances. The presence of quinone groups has been suggested by various workers (261) and occurs in the structures of Dragunov (cited by Kononova (5)), Flaig (257) and Stevenson (51). Tollin *et al* (261) proposed a humic acid model containing quinone moieties coexistent with semiquinone and quinhydrone structures.

Biogenic components are only incorporated into the structures of Dragunov (cited by Kononova (5)) and Stevenson (51). The contribution of these components to the overall structure of soil humic materials is difficult to judge as most soil extraction procedures also remove free carbohydrates, amino acids, etc. Both the carbohydrate and nitrogen contents of fulvic acids have been lowered on passing through gel filtration and cation exchange columns respectively, suggesting coextraction with or weak adsorption onto the humic materials (262).

Figure 3.2 A selection of structural models proposed for soil humic materials

(a) Structure of humic acid according to Dragunov (cited by Kononova (5))



(b) Hypothetical structure of humic acid according to Flaig (257)

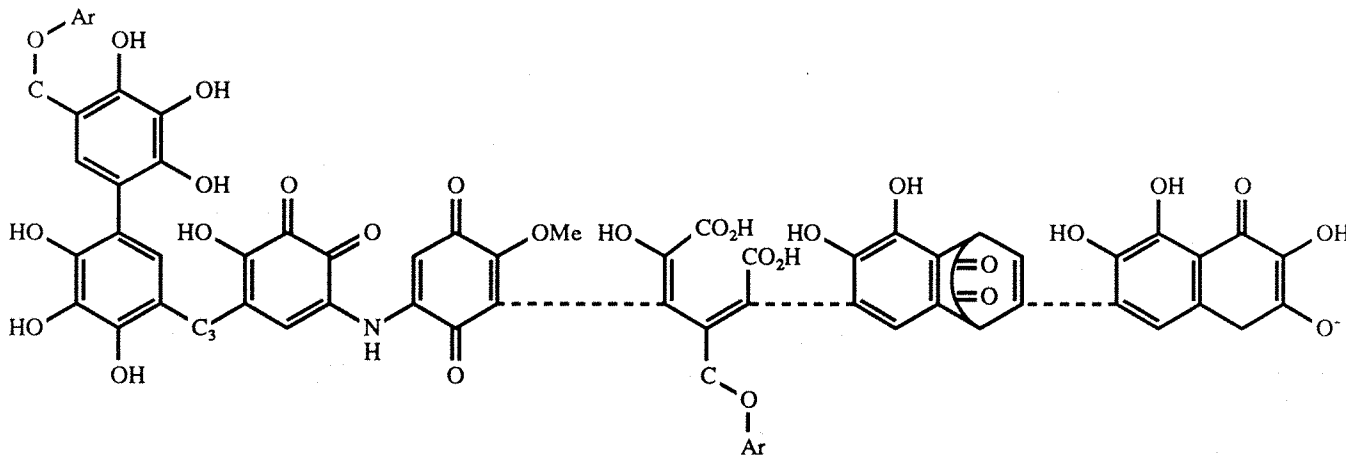
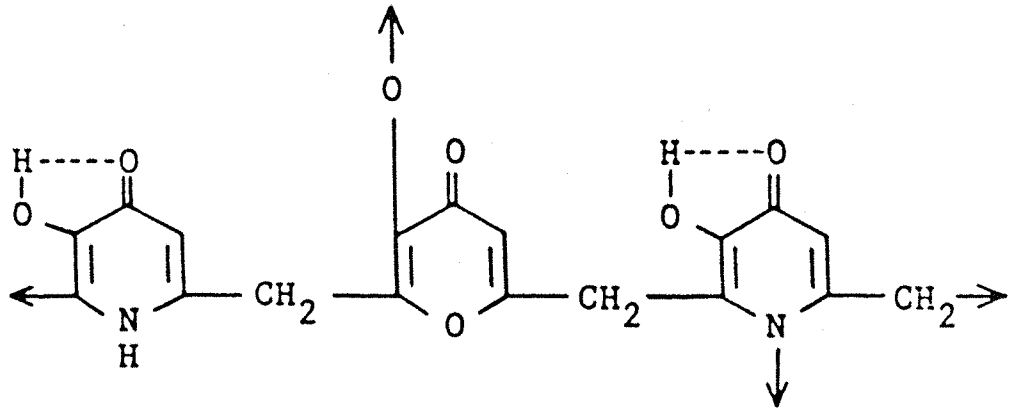


Figure 3.2 A selection of structural models proposed for soil humic (continued) materials

(c) Humic acid structure according to Felbeck's hypothesis (258)



(d) Structure of humic acid according to Schnitzer (259)

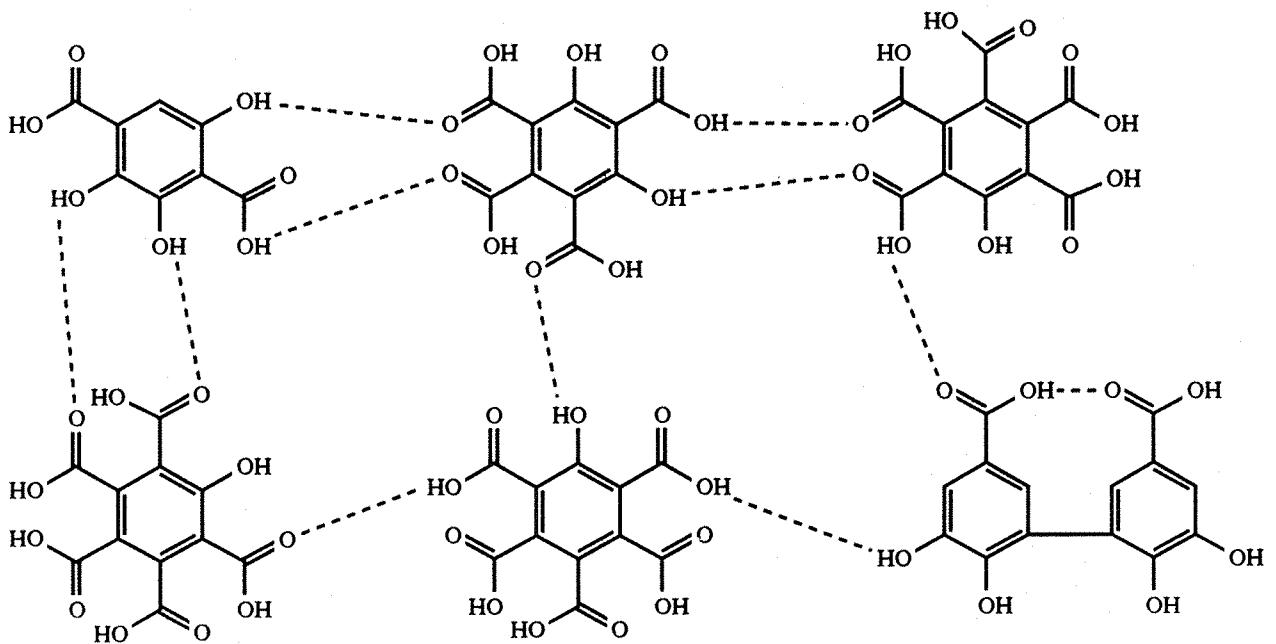
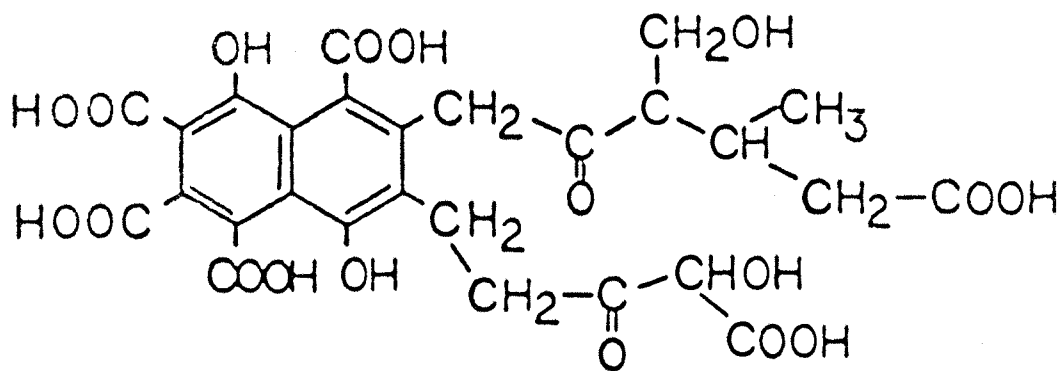


Figure 3.2 A selection of structural models proposed for soil humic (continued) materials

(e) Model structure of fulvic acid according to Buffle (260)



(f) Hypothetical structure of fulvic acid according Stevenson (51)

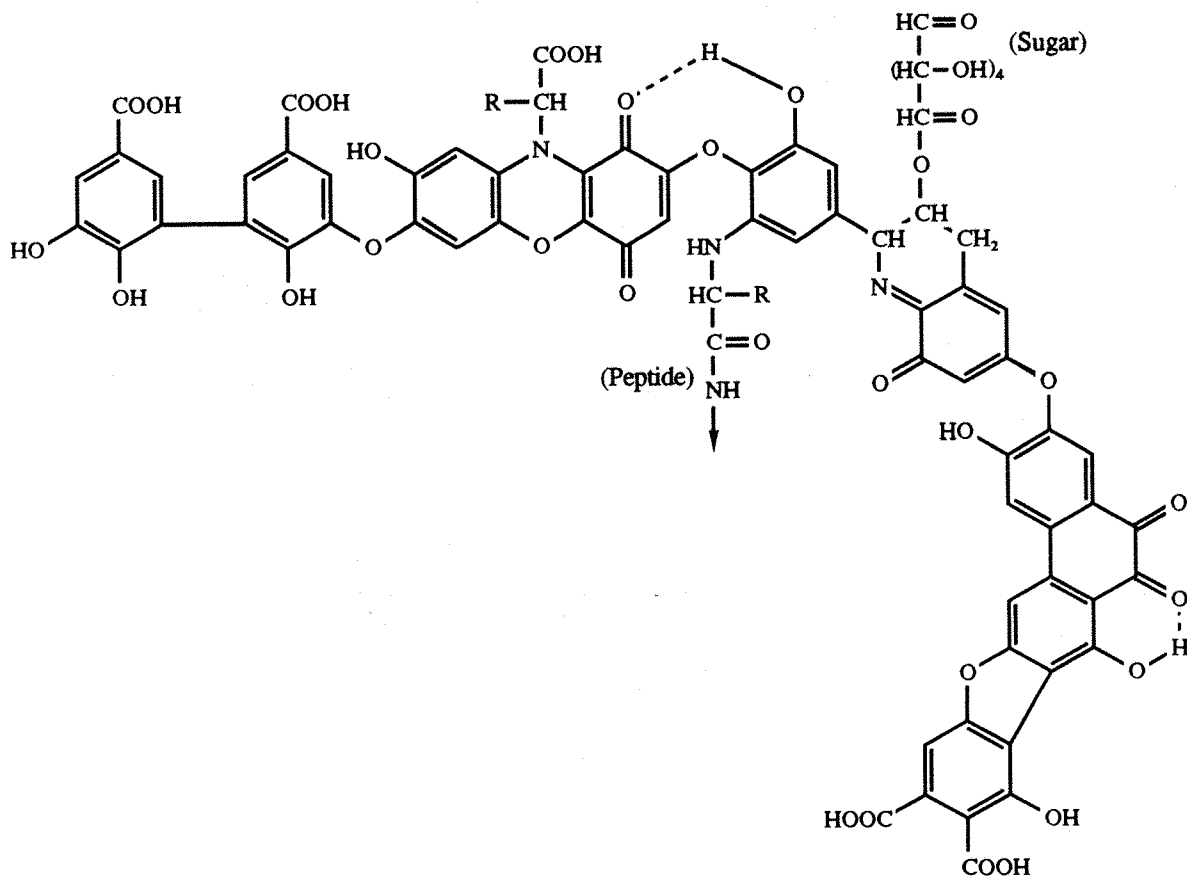
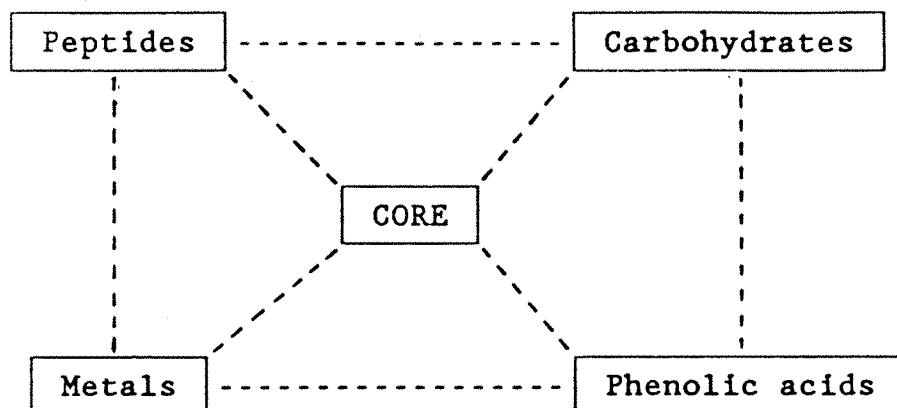


Figure 3.3 Diagrammatic representation of humic acid according to Haworth (119)



Both a diagrammatic representation (Figure 3.3) (119) and computer-generated models (263) have been used to represent possible structures of humic materials. Haworth (119) suggested that humic acid consisted of a complex condensed aromatic core with various groups, such as carbohydrates, proteins, simple phenols and metals, loosely attached to it. As mentioned above, however, loosely bound biogenic materials are more than likely co-extracted with the humic substances.

Computer-modelling was performed by Murray and Linder (263) to generate diagrams of different possible structures of fulvic acid for metal binding studies. Based on set elemental and functional group contents, together with selected degrees of aromaticity, random structures were generated. Numerous molecules can be generated in this way, however, and so this type of modelling, in its present form, has little use in the structural elucidation of humic substances.

More recent studies on soil humic materials, using NMR, have led to controversy over the contribution of aromatic moieties to the overall structure. As mentioned in Section 2.4.4, Ruggiero *et al.* (122) claimed that aromatic structures were significant constituents of soil humic and fulvic acids, whereas Wilson and Goh (125) stated that these materials show a wide range of aromaticities. Hatcher *et al.* (120) found that terrestrially derived humic acids had aromaticities ranging from 22 to 35% (based on ^{13}C NMR measurements), which is similar to these quoted by Malcolm (116). Soil fulvic acids

showed lower aromaticities, ranging from 10 to 26% (25% average on physical removal of polysaccharides) (116). Further work by Hatcher *et al.* (128) showed a wide range of aromaticities (35-92%) for soil humic samples from different climatic zones, in agreement with the conclusions of Wilson and Goh (125). More than 30% of aromatic carbon was found by Schnitzer and Preston (264) in both soil humic and fulvic acids, which increased to over 40% on hydrolysis (although the possibility of secondary reactions must not be forgotten). It is likely, therefore, that co-extraction of biogenic materials can lead to an under-estimate of the aromatic content of these materials. NMR investigators have also challenged the assignment of the aromatic region to benzenoid structures, suggesting a core for these materials based on heterocyclic derivatives (126). As stated in Section 2.4.4, however, the experimental evidence which led to this suggestion is debatable.

In addition to work on the aromatic moieties in soil humic materials, recent studies have also begun to look at the aliphatic structure in more detail. In many contemporary cases, this has been found to be the dominant fraction in these materials. Hatcher *et al.* (129) noted that the aliphatic signals in ^1H and ^{13}C NMR spectra of terrestrial humic acids were significant components of these materials. They suggested that these were equal in importance to the previously postulated aromatic 'building blocks' which probably dominated earlier studies as they survived chemical degradations. Other NMR investigators (124) have also noted the importance of alkyl chains in soil humic materials.

The presence of aliphatic moieties has also been implied from pyrolysis studies. Aliphatic pyrolysis products of the humic material were similar to those resulting from the pyrolysis of insoluble, non-hydrolysable aliphatic biopolymers found in both higher plants and algal cell walls, and so this material was proposed to form part of the core of humic acid (265). Tegelaar *et al.* (211) speculated that this biopolymer was a more likely precursor of the aliphatic moieties in soil humic acid than lipids or natural polyesters on the grounds of their resistance to chemical and biological degradation.

To conclude, therefore, earlier studies on the structure of soil

humic materials led to the proposal of models based on highly aromatic structures. More recent work, however, has suggested that, in some cases, aliphatic moieties may form the core of these materials. At present, it seems likely that soil humic substances can possess a wide range of aromaticities, depending on the climate, environment, etc.

3.1.2 Freshwater humic substances

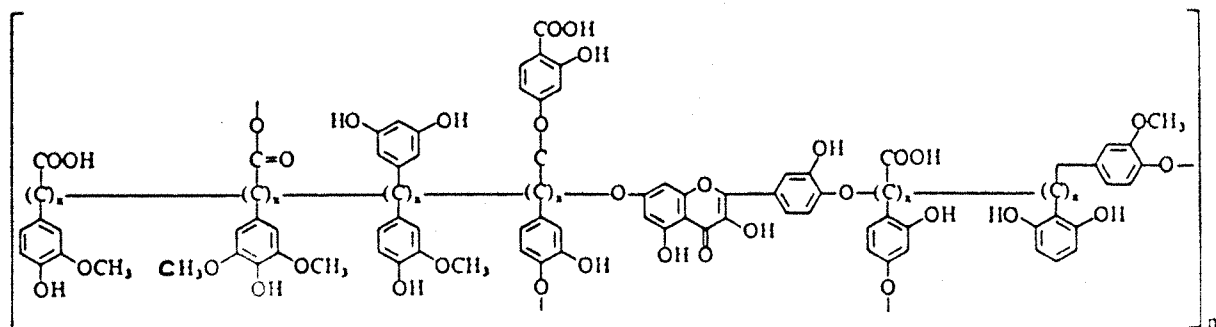
Prior to 1960, little work had been performed on the structure of freshwater humic substances. It was generally thought that these materials resulted from the leaching of soil organic matter into rivers, etc., during rainfall and thus their structure would resemble those of soil humic substances. The possible importance of the autochthonous material resulting from decomposition of plankton was not generally noted although Birge and Juday (266) suggested this source for refractory organic material in lake water.

Shapiro (267) isolated yellow organic acids from lakewater and investigated them using functional group analyses, molecular weight determination, infrared spectroscopy, and paper chromatography. He suggested that they were dicarboxylic, hydroxyaliphatic organic acids with molecular weights of about 450, and were probably unsaturated and apparently non-nitrogenous (in contrast to the majority of other workers who found evidence for the presence of nitrogen). He suspected that these materials originated from the soil due to similarities between lake coloured water and aquatic extracts of soil.

Christman and Ghassemi (60), on the other hand, described organic solids in a number of coloured waters as aromatic polyhydroxymethoxy carboxylic acids. On the basis of their oxidative degradation results, they proposed a hypothetical structure for these materials (Figure 3.4) which were thought to originate from soil due to their similarity to oxidation products of aquatic extracts of soil organic matter. However, as stated earlier, assignment of structures from degradation studies can be misleading as only a portion of the overall structure (in this case, a maximum of 50%) is studied.

Problems with these earlier studies, however, included the co-extraction of non-humic substances, leading to the isolation of

Figure 3.4 Hypothetical structure of macromolecules isolated from coloured waters according to Christman and Ghassemi (60)



extremely complex mixtures of materials. Attempts to extract aquatic humic matter using size-exclusion chromatography and ultrafiltration suffered from limitations such as interactions with either the resin or filter, and the co-extracted materials. Amberlite XAD resin technology, with its application to the extraction of humic substances, became popular in the mid 1970's, despite the often low overall recoveries. The ability to extract less contaminated aquatic humic materials combined with vast improvements in instrumental techniques, e.g., NMR, led to increased interest in this field from the mid 1970's.

Chemical similarities between aquatic and soil humic substances have been reported by Weber and Wilson (71). Wilson *et al.* (72) also suggested that lakewater humic substances largely consisted of terrestrial material originating from the surrounding watershed, based on an aromaticity of 41% obtained using ^{13}C NMR spectroscopy. Other more recent NMR studies by Malcolm gave aromaticities for stream fulvic acids of 21% (67), 17% (20) and 16-24% (116), and stream humic acids of 30% (20) and 30-35% (116), supporting predominantly terrestrial origins for these materials.

Pyrolysis studies have also indicated similarities between terrestrial and freshwater humic substances (149), and chemical degradation investigations have shown the presence of lignin-derived phenols in aquatic humic matter (164). In addition, seasonal variations in fulvic acids have been observed due to the introduction of water from different sources, confirming predominately allochthonous input (209).

Few studies have been performed on aquatic humic materials of mainly autochthonous origin. Gadel and Bruchet (200) isolated humic substances from decaying aquatic phanerograms (higher plants) and algae, and also from various sediments. After elemental analyses, infrared spectroscopy and pyrolysis GC-MS, they concluded that the humic acids extracted from decaying phanerograms showed terrestrial character, confirmed by the presence of lignin pyrolysis residues, whereas those from algae were enriched in proteins, fulvic acids being enriched in carbohydrates. The varying contributions of two types of aquatic plants to several sediments were suggested. Peschel and Wildt (268) compared allochthonous aquatic humic substances (from a peat bog) with autochthonous materials, isolated from riverwater, and materials of anthropogenic origin from a biological wastewater plant. Both the autochthonous and anthropogenic humic substances were similar, showing evidence of protein and carbohydrate moieties, whereas the terrestrial materials were generally more aromatic with few identifiable biogenic fragments.

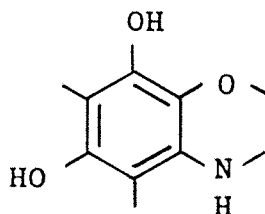
To summarise, therefore, most investigations on freshwater humic materials have been performed in areas where the influence of soil leaching appears to be significant. Consequently, it is not surprising that the resulting humic substances generally show evidence of aromatic lignin precursors with structural similarities to soil humic materials, suggesting a terrestrial origin. In areas where continental input is minimal, however, fewer aromatic moieties occur and the humic substances appear to be dominated by biogenic materials, being enriched in proteins and carbohydrates, probably indicating the much more recent origin of this material.

3.1.3 Marine humic substances

Yellow marine organic matter was first observed by Kalle (9) over fifty years ago due to the blue fluorescence it exhibited in seawater. However, until recently, few investigations had been performed on marine humic substances due to problems involved in the isolation of these materials, due, in turn, to their low concentrations in seawater and the presence of high concentrations of inorganic salts. Attempts to isolate them were performed using nylon stockings (269) and charcoal (270) but it was not until the introduction of Amberlite XAD

resins that investigations on marine humic substances began in earnest.

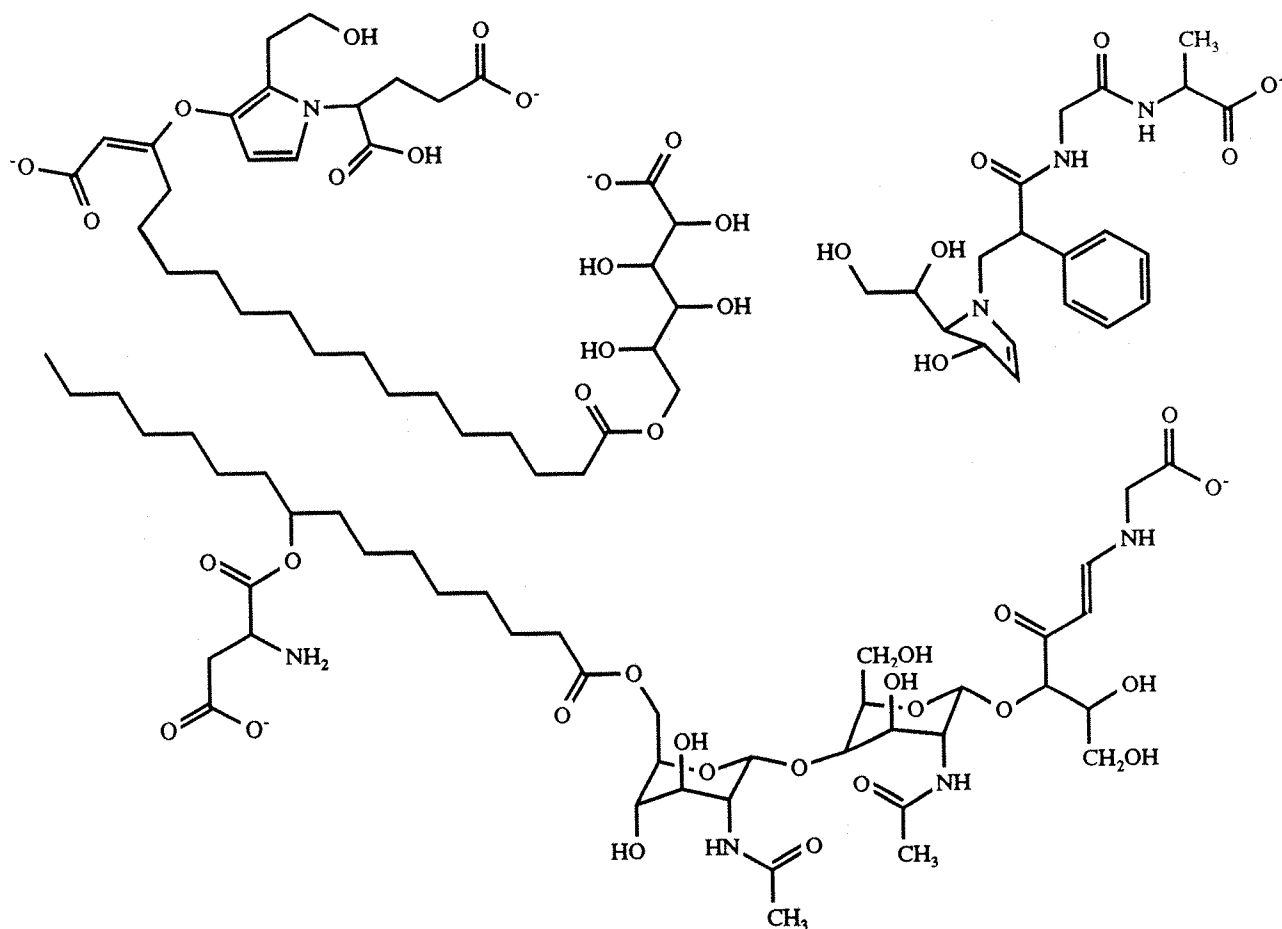
Thiele and Kettner (271) stated that humic matter and similar complex substances in seawater consist of three-dimensional anionic polymers containing substituted aromatic nuclei (substituted with, for example, phenolic, carboxylic, methoxyl, carbonyl groups) linked by atomic bridges (e.g., $-O-$, $=N-$, $-C-C-$, $-CH_2-$, or heterocyclic rings), for example,



Duursma (272) proposed that marine humic substances were analogous to complex organic substances in soil known as humus. This type of assumption, however, has hindered research into marine humic substances. Apart from the controversial contribution of terrestrial organic material to humic substances in coastal environments (see section 1.4), marine humic materials show few similarities to their terrestrial counterparts.

Gagosian and Stuermer (150) performed one of the first comprehensive studies on marine organic materials extracted using Amberlite XAD-2 resin. Physical properties and spectroscopic characteristics of these materials were measured in addition to performing chemical reduction and mass spectrometric studies, and determining their amino acid content. The results suggested a predominately aliphatic structure with little, if any, significant contribution from terrestrial organic materials. A hypothetical structure of typical humic substances was presented (Figure 3.5). It consists predominately of important biogenic precursors in the marine environment, such as sugars and amino acids (thought to originate from decaying algae/plankton, etc.), although other possible constituents, such as phenols and pigments, are not included. However, Gagosian and Stuermer point out that structures are 'type' structures and only represent their experimental results if various permutations of

Figure 3.5 A hypothetical structure of marine humic substances according to Gagosian and Stuermer (150)



different molecular weight and composition occur.

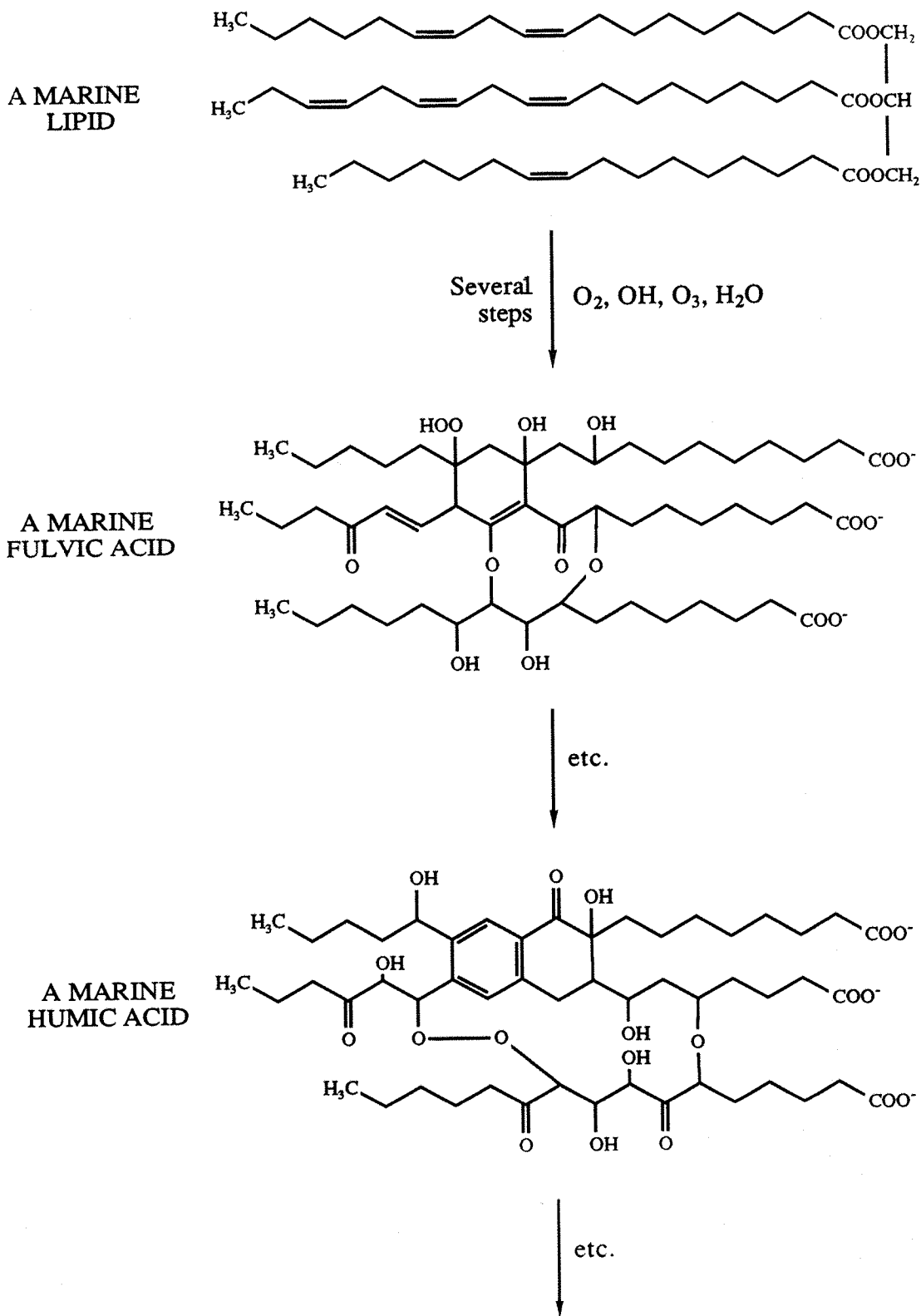
Recent NMR and pyrolysis studies are in agreement with a largely aliphatic structure for marine humic substances, originating from plankton degradation products. Wilson and co-workers (130,149,206) compared marine humic extracts with those from a marine diatom and found that both materials were similar, showing that carbohydrates, highly-branched alkyl chains, and, to a lesser extent, aromatic materials (hypothesised as originating from uronic acids) contributed significantly to their structure. The more aliphatic nature of marine humic substances, as compared to those of terrestrial origin, has also been shown in NMR studies of Dereppe *et al.* (118) and Stuermer and Payne (121). Malcolm (116), on the other hand, hypothesised, from his solid state ^{13}C NMR studies, that stream fulvic acids are a major

source of marine fulvic acids, stating that the aromaticity of marine humic materials is not low compared with that of stream humic substances. The similarity between, in particular, autochthonous stream humic matter and marine humic materials would not be unexpected as they are both thought to originate from decayed plankton. However, further work is required to support this hypothesis as the environmental conditions in which these formation reactions occur vary significantly between freshwater and marine environments. In addition, care must be taken when comparing structures or origins on the basis of aromaticities alone, because aromatic precursors of non-terrestrial origin, such as phenolic exudates of marine algae (48), aromatic amino acid residues, etc., may be present in the humic materials.

The most recent hypothesis on the formation and structure of marine humic substances was proposed by Harvey *et al.* (222). They suggested that these materials were water-soluble, aliphatic organic acids, formed by the UV-catalysed, autoxidative cross-linking of polyunsaturated marine dissolved lipids (Figure 3.6). Various aspects of these structures have been criticised (273) although model experiments, allowing pure marine lipids and a diatom to autoxidise in seawater in the laboratory, resulted in the formation of materials which were similar, in all respects studied, to the marine humic substances (110).

To conclude, having dismissed historical presumptions about the similarities between marine and terrestrially derived humic substances, investigations have tended to indicate a largely aliphatic structure for marine humic materials based on common biogenic precursors. The contribution of continental or riverine humic matter is, at present, unknown and may vary with changing environmental factors, such as local mineral type. It is obvious that more comprehensive studies, possibly incorporating particulate and sedimentary humic materials and describing environmental features in more detail, from a large number of sampling locations in a chosen area, must be performed if terrestrial contributions to marine humic structures are to be investigated fully.

Figure 3.6 Proposed pathway for the formation of marine humic substances by the UV-catalysed, autoxidative cross-linking of polyunsaturated marine lipids (222)



3.2 FORMATION

Over the years, a number of theories have been proposed for the formation of humic substances. These have generally fallen into two categories, namely those in which the humic substances are predominantly formed by direct degradation and chemical alteration of biopolymer molecules, retaining a degree of the structural integrity of these biomolecules, or those in which indirect formation occurs via repolymerisation of previously extensively degraded biopolymers. The theories arising from these two modes of formation have recently been discussed by Hedges (274), and Hatcher and Spiker (275), and are summarised below.

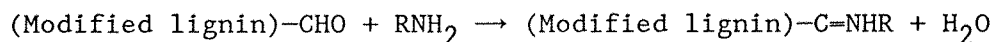
3.2.1 Soil humic substances

Several pathways have been suggested for the formation of soil humic materials. The three main theories which have emerged are:-

- (i) Lignin theory
- (ii) Polyphenol theory
- (iii) Melanoidin model

and these have been described, in detail, by Stevenson (51).

The lignin theory is the classical theory of humic matter formation. For many years, these materials were thought to be derived from lignin. The model was based on the evolution of humic substances from a lignin 'core', which had been modified by microorganisms. However, this hypothesis was not entirely accepted as the low nitrogen content of lignin did not account for the high nitrogen content of the resulting humic materials. Incorporation of further nitrogen into the structure was later suggested by Waksman (276) who proposed the introduction of protein moieties during modification of the lignin by such processes as Schiff base formation:-



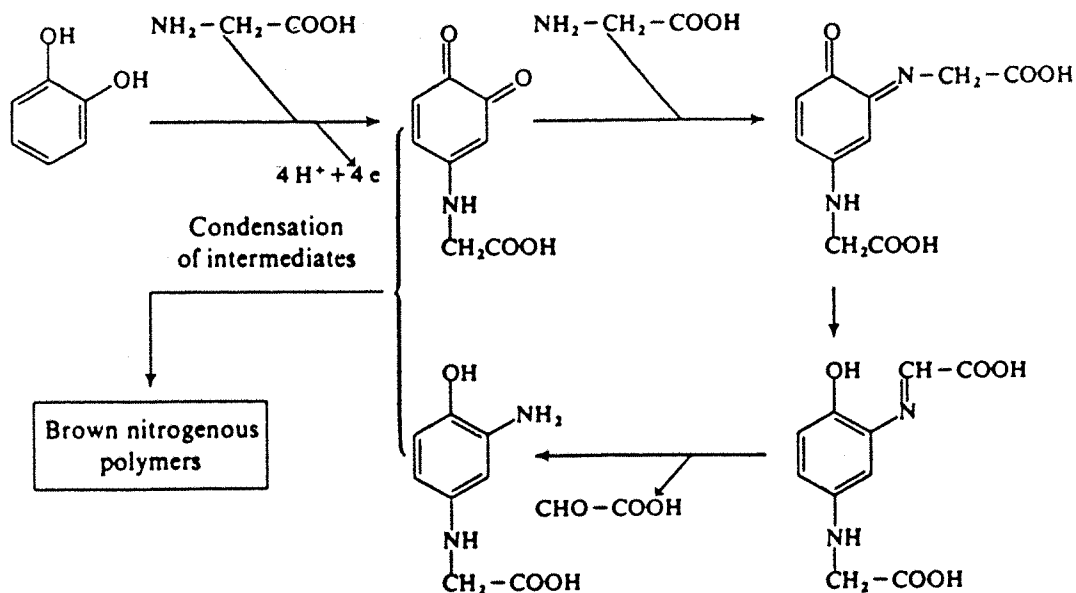
At the time, this model was widely accepted. However, in the late 1960's, a different theory began to emerge, based on the formation of

humic substances from simple phenols. Various experimental studies (e.g., (277)) supported this which led the lignin theory to gradually become obsolete.

Another theory based on a degradative humification pathway suggested that microbes synthesised high molecular weight humic-type polymers intracellularly and these were released, and subsequently degraded to humic acid, etc., on death of the organism. This mechanism has not been widely accepted, however.

The polyphenol theory is currently the most widely accepted mechanism for the formation of humic materials. It involves the oxidation (probably enzymatic) of polyphenolic precursors from, for example, microbial degradation of lignin or microbial synthesis (using moieties from non-lignin sources), followed by polymerisation, usually in the presence of amino compounds, to form humic molecules. An example of the condensation reaction between quinones and amino acids is given (Figure 3.7). The contribution of phenolic molecules from the various possible sources is unknown but is likely to depend on local environmental conditions. However, evidence for the presence of both lignin- and microbially-derived phenolic precursors has been cited (51).

Figure 3.7 An example of the reaction between quinones and amino acids, illustrated by catechol and glycine (51)



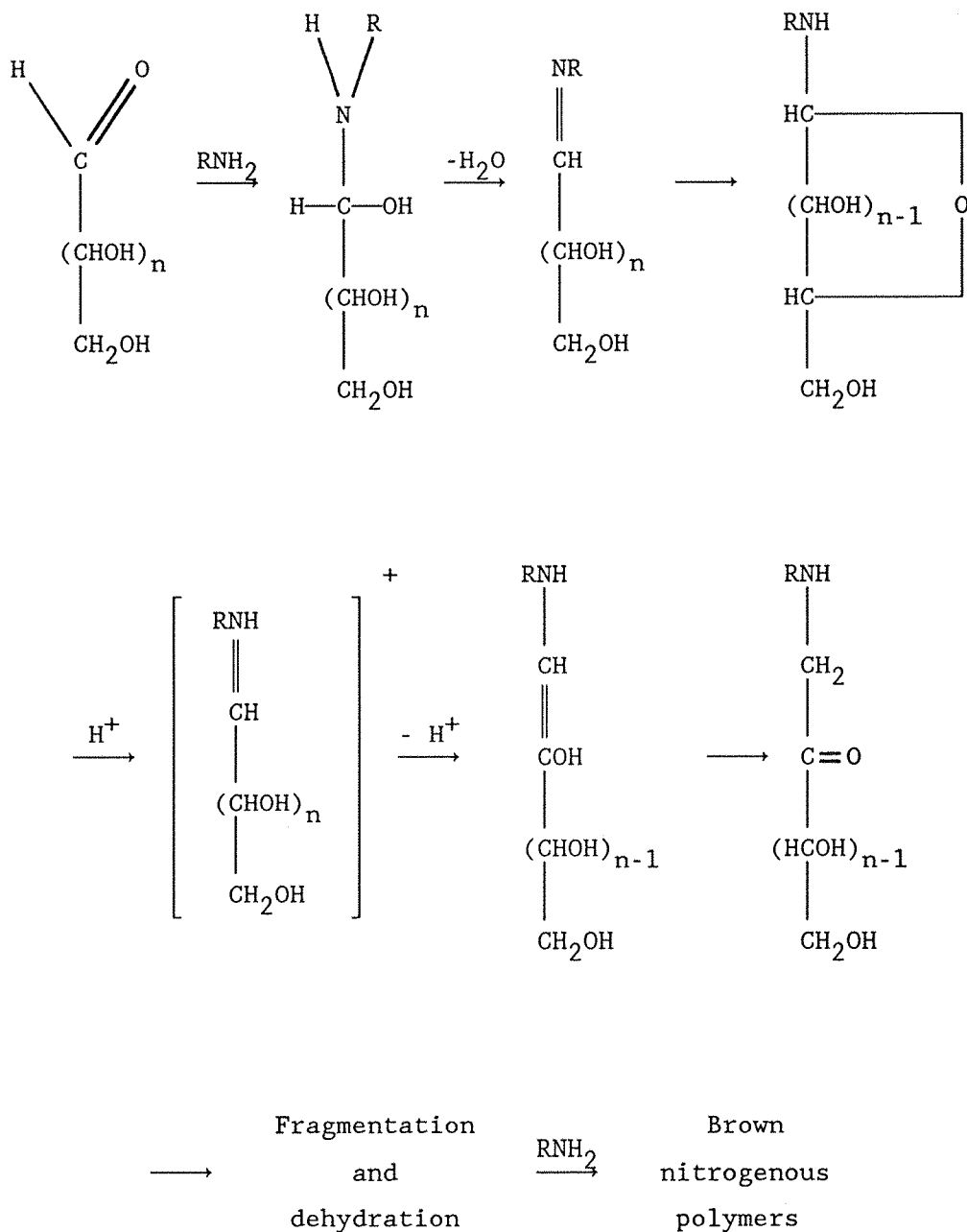
Advantages of this model are the ease of both the oxidation of phenols (51) and the polymerisation of quinones under environmental conditions (274). In addition, as aromatic materials are sometimes quite resistant to degradation by microorganisms, phenols are likely to persist at sufficient concentrations to be transformed to quinones (274). The formation of quinones from phenolic precursors by exoenzymes in the environment has also been noted (51). The large number of possible precursor molecules may also account for the heterogeneous nature of the resulting materials.

Finally, the third main theory for the formation of soil humic substances is that based on the melanoidin reaction. This involves the non-enzymatic polymerisation of sugars and amino acids to yield brown nitrogenous polymers (Figure 3.8).

An advantage of this model is that the two precursors (sugars and amino acids) are obviously abundant constituents of living organisms, and they will react under both oxidising and reducing conditions. A major problem, however, is that this reaction proceeds slowly at the temperature found in soil, and so, unless catalysed by other species present, it seems unlikely to occur to any significant degree.

As stated earlier, the currently accepted model is generally the polyphenol theory. However, as stated by Stevenson (51), it is possible that different mechanisms may dominate under different environmental conditions. It is also plausible that a combination of formation mechanisms may occur, for example, partial degradation of lignin followed by condensation reactions. Hatcher and Spiker (275) have recently revived the degradation theory, using this hypothesis to explain existing data. It is interesting to see that several studies gave results indicating that humic acid is a precursor of fulvic acid, which agrees with this hypothesis and contests the polyphenol theory. Obviously further investigations are required to check whether this previously rejected theory is, in fact, the mode of formation of soil humic substances.

Figure 3.8 Melanoidin theory of formation of humic substances (275)



3.2.2 Aquatic humic substances

Relatively few reports have been written on the formation of aquatic humic substances, mainly because, historically, they were thought to either originate from soil organic matter or be formed by similar processes to it. Also, as the origin, and therefore precursors, of these materials was not certain, it was difficult to suggest formation mechanisms.

Riverine humic substances may originate from various sources and different modes of formation may exist for each of these. Materials originating in the soil could arise directly from leached soil humic substances, and thus would be formed by the mechanisms suggested above. However, they may also be produced *in situ* from leached plant organic matter, possibly still by the formation mechanism of soil humic substances, or synthesised during the transportation of leached plant organic matter through the soil (7). These processes may involve those pathways mentioned in Section 3.2.1 but could also result from a completely different mechanism. Autochthonous formation introduces further possible pathways, including those mentioned above resulting from precursors present in riverwater, alteration of planktonic remains or unique humification processes. A problem which must be noted, however, as it is not generally evident in the formation of soil humic substances, is the somewhat unlikely process of condensation reactions occurring at the often low concentrations of precursors in riverwater. Hence it is possible that either a degradative pathway is occurring or intramolecular alterations are prevalent. Thus, due to the selection of possible origins of riverine humic substances, a variety of modes of formation may be occurring, so making constructive deductions on an already complex subject extremely difficult.

Marine humic materials in the open ocean, on the other hand, are now generally assumed to be formed autochthonously. Early work on this subject, however, still presumed similarities between the formation of these materials and soil humic matter (272). The polyphenol theory presents problems if extended to the synthesis of marine humic materials. Firstly, phenols are not an abundant component of marine plankton, and, although certain algae have been found to exude polyphenols (48), the concentrations of phenolic moieties in seawater are generally too low to envisage any considerable degree of intermolecular condensation reactions. In addition, the low aromaticity of marine humic substances suggests predominantly aliphatic precursors for these materials. Kalle (9) proposed the melanoidin pathway for the synthesis of marine humus. However, Gagosian and Stuermer (150) pointed out firstly that this reaction alone could not account for the presence of the observed long-chain aliphatic structures in marine humic substances, and

secondly, that the concentration of dissolved organic material in seawater is not conducive to condensation reactions. They suggested the formation of these materials via the melanoidin pathway in areas of concentrated organic matter in seawater, such as decaying organisms and the sea-surface microlayer, where intermolecular reactions may be possible. The incorporation of marine lipids, to account for the highly aliphatic structure, was thought to originate through their aggregation with humic substances, followed by condensation reactions. Further intramolecular reactions could then occur due to the proximity of the functional groups.

Even in areas of high concentrations of organic material in seawater, it seems unlikely that this type of reaction will occur to any significant degree because it is slow and the precursor molecules (free sugars and amino acids) have rapid turnover times, i.e., they do not persist for long enough for condensation reactions to occur (274). This led Harvey *et al.* (222) to suggest a mechanism involving purely intramolecular reactions. As stated in Section 3.1.3, they recently proposed a polyunsaturated lipid model for the formation of humic substances in seawater (Figure 3.6), which, although criticised (273), gave encouraging results when modelled in the laboratory (110). Advantages of this theory include the ubiquity of lipids, both freely dissolved in seawater (concentrated, in particular, in the sea-surface microlayer where ultraviolet light is most intense) and as components of marine organisms, and the proximity of the reactive groups. However, several problems must be resolved before this pathway can be accepted. Firstly, no nitrogen functionalities are incorporated into the given structure although Harvey *et al.* regarded them as 'appendages to the main structures'. Laane (273) suggested the presence of a Schiff base derivative to account for fluorescence phenomena although Harvey (278), in reply to Laane's criticism, discounted this on the basis of their instability under the environmental conditions, and the low concentration of precursor molecules in seawater. The present study suggests the incorporation of proteins/sugars by taking more complex precursor molecules such as lipoproteins or glycolipids. Although complex lipids have not been studied extensively in seawater/plankton, the presence of, for example, glycolipids in freshwater phytoflagellates has been reported (279). More work on this fraction of compounds in the marine

environment should be performed before any such formation schemes can be postulated.

A second problem with the mechanism proposed by Harvey *et al.* (222) concerns the stable isotope composition of the resulting materials. Hedges (274) commented that the proposed mechanism should result in little change in $\delta^{13}\text{C}$ between the precursor lipids and the final humic substances whereas both the model and natural marine humic substances showed a considerable fractionation between the model precursor ($-28.1^{\circ}/\text{oo}$) and the resulting materials (model $-19.7^{\circ}/\text{oo}$; natural -20.6 , -21.5 , $-22.0^{\circ}/\text{oo}$) (110). Hedges stated that the 'incompatibility' of the result with theory should be further tested since the $\delta^{13}\text{C}$ values of the humic substances more closely resemble those of the nonlipid components of marine plankton.

At the present time, therefore, it seems likely that marine humic materials are formed via a different process to their soil counterparts. The polyunsaturated lipid model is a possible pathway although further analyses are required to clarify the incorporation of nitrogen into the structure, and explain the large $\delta^{13}\text{C}$ fractionation between the precursor and humic molecules.

3.3 BIOGENIC COMPONENTS OF HUMIC SUBSTANCES

3.3.1 Carbohydrates

3.3.1.1 Soil humic materials

Carbohydrates have been determined in humic substances using colorimetric measurements for the assessment of total carbohydrate content (280), and hydrolysis followed by chromatographic separations for the quantitation of individual monosaccharides (e.g., (281)). Evidence of carbohydrate moieties has also been obtained using pyrolysis (196), IR (282) and NMR spectroscopy (79).

The total carbohydrate content of soil humic substances has generally not exceeded 10% but the comparison of data must be treated with caution due to the different extraction/hydrolysis/quantitation methods employed. Coelho *et al.* (281) determined the monosaccharides

resulting from the hydrolysis of humic acids from six Brazilian soil samples, and found percentages ranging from 1.3 to 2.9%. Similar values were obtained by Linhares and Martin (283) for some Californian soil humic acids (1.7 to 2.6%). Peat humic acid contained slightly less carbohydrate (0.4%). Ogner (284) reported the content of monosaccharides released by hydrolysis of soil humic and fulvic acids as 10.0 and 7.7% respectively, and Tsutsuki and Kuwatsuka (285) found values for soil humic acids ranging from 0.5 to 5.0%.

Arabinose, fucose, galactose, glucose, mannose, rhamnose and xylose were reported to be present in various soil humic acid hydrolysates (281). Traces of the sugar alcohol, inositol, were also detected in most samples. Glucose was the most abundant sugar (in agreement with other studies, e.g., (284)), accounting for 36 - 55% of the total monosaccharide fraction. Ribose has also been found to be present by Ogner (284) and Orlov *et al.* (286). Linhares and Martin (283) detected glucose, galactose, mannose, and, in one soil, xylose, using thin-layer chromatography (TLC), and Havrankova (287) additionally found arabinose in soil humic acids using this method. By analysing both non-methylated and premethylated humic samples, Ogner (284) concluded that the monosaccharides originated from branched polysaccharides in the humic and fulvic acids. Methylation of a whole soil sample, however, led Cheshire *et al.* (288) to conclude that a proportion of the sugars were substituted by non-sugar residues because the number of terminal sugar units was insufficient to account for purely carbohydrate branching units.

The origins of these monosaccharides are not clear. Cheshire (289) suggested that galactose, mannose, rhamnose and fucose are derived primarily from microbial sources, arabinose and xylose from plant material, and glucose can originate from both plants and microorganisms. Arabinose has also been found in synthetic fungal melanins (281). According to Stevenson (51), the major part of soil polysaccharides originate from bacterial and fungal sources as carbohydrates in plant remains are more or less decomposed by these species.

As mentioned previously (Section 2.1), soil humic fractions have generally included co-extracted free polysaccharides. In a recent

study by Malcolm (290), these materials were removed from the fulvic acid fraction by passing through Amberlite XAD-8 resin prior to monosaccharide analysis. The resulting sugars in the fulvic acid were still dominated by glucose (ca. 40% by weight) followed by rhamnose (26%). The monosaccharides in the untreated humic acid fraction showed a more uniform distribution of all the studied sugars (namely fructose, galactose, glucose, mannose, rhamnose, xylose), with glucose remaining the most abundant.

In addition to the common aldoses, several O-methyl monosaccharides have also been found in the soil humic and fulvic acids (284). These compounds have been reported in soil samples and may originate from a variety of sources including bacteria, fungi, algae or foliage from deciduous trees (291).

Apart from the determination of monosaccharides in humic matter by chromatographic methods, they have also been detected using other techniques. Tan and Clark (292) identified at least two kinds of polysaccharides, those from soil fulvic acid and those from humatomelanic acid, by infrared spectroscopy. NMR evidence for the presence of carbohydrate moieties in humic substances has also been reported (79,126), and pyrolysis studies of soil fulvic acids have yielded quantitative values for their carbohydrate content (196), ranging from ca. 5% to 34%, depending on the extraction procedure and origin. Spectroscopic studies have also been employed to confirm that the polysaccharides are not merely coextracted with the soil humic substances, but are chemically bonded to these materials. One example of this is the identification of a polysaccharide ester linkage in soil humic acids by IR spectroscopy (282).

To conclude, polysaccharides constitute 10% or less of soil humic materials. Simple aldoses, arabinose, fucose, galactose, glucose, mannose, rhamnose, ribose and xylose, and O-methyl monosaccharides have been detected, with glucose being the dominant sugar. They appear to originate primarily from microbial material and, to a greater or lesser extent, plant material, depending on soil type.

3.3.1.2 Aquatic humic materials

Whilst measurements have been made of the dissolved and particulate carbohydrates in natural waters, few studies have looked at their occurrence in the humic fraction.

The carbohydrate content of riverine fulvic acids from the Ogeechee, Shawsheen and Suwanee Rivers and Wellton-Mohawk Canal (U.S.A) were found to be approximately 4% (20,67,293,294). Thoreau's Bog fulvic acid yielded similar results (2.6%) (293) as did Como Creek fulvic acid (4%) (7) and a lake fulvic acid (2.9% in March and 3.9% in November) (216). Ogeechee river humic acid had a higher percentage of carbohydrate (ca. 10%) (20), in agreement with other data on aquatic humic substances (7).

Individual monosaccharides were determined in Thoreau's Bog fulvic acid (293). Arabinose, glucose, mannose and rhamnose were detected, with arabinose constituting 75% of the total hydrolysable carbohydrate. It was also the major sugar in the suspended material isolated from the bog. The authors noted that arabinose and xylose have been found in soils rich in plant remains. The monosaccharides in Como Creek fulvic acid were also dominated by arabinose (90%) but, in addition, some mannose and xylose were detected (7). Sweet and Perdue (295) carried out a comprehensive study of dissolved sugars in riverwater, determining free monosaccharides, humic-bound saccharides and polysaccharides. Arabinose, galactose, glucose, mannose and xylose were all detected in the humic-bound fractions. Smidsrød and Painter (296) determined the carbohydrates in the hydrolysate of humic acid from peat-bog water and identified galacturonic acid, galactose, glucose, mannose, arabinose, xylose and rhamnose. In most of these studies, the origins of the monosaccharides in the humic substances were not addressed although, like the humic materials themselves, they are likely to result from various contributions from both autochthonous and allochthonous sources.

A recent study by Malcolm (290) has compared the monosaccharide content of soil, river and marine humic materials. The saccharide content of soil humic substances was greater (approx. six times) than those originating from river and marine environments. Fructose and

glucose were the more abundant sugars in both riverine fulvic and humic acid whereas the distribution in marine fulvic acid was more uniform, galactose being the most concentrated sugar in this instance.

Other investigations have included the enzymatic hydrolysis of aquatic fulvic acid (216). By employing different enzymes, it was concluded that glucose occurs in a variety of structures in fulvic acid, including polysaccharides containing both α -1,4- and β -1,4-linkages. Pyrolysis studies have also been performed on lakewater fulvic acids, which exhibited seasonal variations in their carbohydrate content (209). Fulvic acids isolated during the summer were rich in carbohydrates whereas those extracted in the winter contained a relatively high proportion of aromatic compounds.

3.3.1.3 Sedimentary humic materials

Few investigations have been performed on the monosaccharide composition of the carbohydrates of sedimentary humic substances. Yamaoka has performed the most comprehensive work to date on humic and fulvic acids isolated from marine sediments (297,298). He studied the vertical distribution of carbohydrates together with the monosaccharide composition, of both fulvic and humic acid fractions isolated from coastal marine sediments. In general, he found an increased concentration of carbohydrates in the top few centimetres (ca. 200 - 450 μ g/g sediment - at least a factor of two greater than in deeper sediments), and a larger proportion of carbohydrates originating from fulvic rather than humic acid. This was explained as being due to a decrease in the fulvic acid carbohydrate with depth, indicating that the humic carbohydrate remained in the sediment due to its more refractory nature.

The monosaccharide composition of the sediments consisted of rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose. Their occurrence in plankton-derived organic material was noted (298). The major predominance of a sugar or sugars, as in, for example, dissolved carbohydrates in seawater (299), was not observed. In each fraction, similar concentrations of the different sugars resulted, with glucose, galactose and, in sediments from Suo Sound (298), rhamnose occurring in slightly greater abundance. Humic substances,

in particular humic acids, from Hiroshima Bay sediment samples, also showed the presence of slightly more xylose (297), possibly indicating the occurrence of more hemicellulose in these samples.

The monosaccharide composition of an estuarine sediment (300) was similar to those in the marine sediments studied by Yamaoka. Ribose was also detected in this sediment but at a lower concentration than the other sugars (ca. 10% of the other individual sugars).

Carbohydrates in humic substances from lake sedimentary materials have also been studied (301). As in the case of marine sedimentary humic substances, a higher percentage of carbohydrates was found in the fulvic acid compared to the humic acid and humin. The distribution of the monosaccharides in these fractions, however, was similar, with glucose, galactose and mannose being the most abundant. The seven monosaccharides detected by Yamaoka (297,298) were all present, in addition to ribose (least abundant). In his study, a predominance of glucose (31.9%) was seen over the other sugars (galactose - 16.7%; mannose - 15.4%; xylose - 9.5%; rhamnose - 9.2%; arabinose - 7.8%; fucose - 6.1%; ribose - 1.7%). It was noted that this distribution was similar to that of planktonic degradation products in lakewaters.

In summary, it can be seen that the monosaccharide composition of the humic fractions of sedimentary materials from both freshwater and marine environments appear similar and probably originate from planktonic remains.

3.3.2 Lipids

3.3.2.1 Soil humic materials

Lipids occur in humic fractions as a result of either physical or chemical associations with these substances. Loosely-bound lipids can be released by solvent extraction alone whereas those which are more tightly bound require chemical degradation prior to extraction.

Schnitzer and Neyroud (302) investigated the extraction of fatty acids from humic and fulvic acid after various pretreatments including

ultrasonication, hydrolysis with water, combined hydrolysis and ultrasonication, methylation, and alkaline saponification. The saponification method was found to be the most effective treatment, extracting nearly 100 mg/g from humic material; no pretreatment led to extraction of only ca. 1 mg, and methylation prior to extraction extracted less than 5 mg/g. Transesterification with boron trifluoride-methanol has also proved expedient, extracting over 150 mg/g from certain humic acids after prior removal of ca. 7 mg/g with chloroform (303). Schnitzer and Ogner (304) found that only 10% of the total fatty acids could be extracted from untreated fulvic acid using organic solvents. The remainder were only removed after methylation and adsorption on neutral aluminium oxide. Similar results were obtained for the extraction of normal fatty acids from humic acid, with less than 7% removed from the untreated material (305). By contrast, nearly 80% of branched or cyclic fatty acids were removed without pretreatment, suggesting that they were less tightly bound than the normal fatty acids.

The various treatments have generally yielded a range of fatty acids. Schnitzer and Ogner (304) found that ca. 95% of the isolated fatty acids were saturated, with the remaining 5% consisting of unsaturated and branched or cyclic acids. The fatty acids accounted for a maximum of 0.1% of the fulvic acid by weight. Normal fatty acids ranging from C_{14} to C_{36} , with an abundance of fatty acids with an even number of carbon atoms (even carbon-numbered), occurred in the major fraction, with C_{24} predominating. Two groups of fatty acids were evident, ranging from C_{14} to C_{26} and C_{27} to C_{36} , with abundances centred at C_{24} and C_{32} . Even carbon-numbered fatty acids in these ranges have been attributed to living organisms and the waxes of insects/plants respectively (306). Both microbial and higher plant contributions to these acids were therefore suggested (304). These origins have also been suggested by Khan and Schnitzer (305). Fatty acids extracted by Khan and Schnitzer (305) accounted for up to 0.47% of the initial humic acid; ca. 70% of these were normal fatty acids. Normal fatty acids in the unmethylated fraction (less tightly bound), ranged from C_{12} to C_{32} , with C_{12} and C_{16} dominating, whereas those resulting after methylation (more strongly bound) ranged from C_{14} to C_{38} , with C_{22} and C_{24} dominating. Again, even carbon-numbered fatty acids predominated. These results confirmed earlier findings (304)

that low molecular weight normal fatty acids are retained less tightly than higher molecular weight ones.

Grimalt and Saiz-Jimenez (307) studied the hymatomelanic (ethanol-soluble) fraction of some soil humic acids, both prior to and after methylation. In agreement with aforementioned investigations, they concluded that fatty acids constituted the main components of both fractions, with normal C_{14} to C_{20} ($n-C_{14}$ to $n-C_{20}$) saturated and unsaturated acids plus some branched components originating from microbial sources, and $n-C_{22}$ to $n-C_{30}$ saturated acids originating from higher plant contributions. The presence of α,ω -dicarboxylic acids and steroidal structures was also noted, thought to be derived from microbial oxidation of monocarboxylic acids and sterols, and free α -hydroxy acids were identified, suspected to come from vascular plant remains. After separation of the hymatomelanic fraction, more investigations were carried out on other solvent-extractable materials (308) which were subsequently saponified. This fraction was again dominated by fatty acids, mainly $n-C_{12}$ to $n-C_{20}$ even carbon-numbered homologues with $n-C_{16}$ (saturated) predominating. Branched and unsaturated components were present in low proportions, and ($\omega-1$) and ($\omega-2$) hydroxy acids, in addition to normal alcohols, were also detected.

Almendros and Sanz (303) identified compounds released from humic acids after transesterification with boron trifluoride-methanol. Prior extraction with chloroform released a maximum of 10% of the total material extracted after transesterification. The chloroform-extracted material consisted predominantly of fatty acids, C_{12} to C_{26} , including saturated, unsaturated and branched acids, which were thought to originate from microbial metabolism. After transesterification, aromatic acids were among the major products, including phenolic, dimethoxy benzene carboxylic, and benzene polycarboxylic acids. Phenylpropenic structures also resulted. Among the aliphatic compounds were α,ω -dicarboxylic acids (mainly C_{10} to C_{22}), unsubstituted (saturated, unsaturated and branched) and hydroxy fatty acids (C_8 to C_{20}), and fatty alcohols. These low molecular weight transesterification products amounted to about 17 mg/g of the lipid-extracted humic acid and it was speculated that they could originate from various sources including plant resistant biopolymers,

e.g., lignins (phenolic acids, C_6-C_3 compounds), natural polyesters of higher plants (ω -OH), and bacteria (β -OH). Products resulting from hydrolytic degradation followed by methylation of a soil fulvic acid also included normal fatty acids (identified as methyl esters) ($n-C_{16}$ and $n-C_{18}$), a dicarboxylic acid (succinic acid), benzene polycarboxylic acids and phenolic acids (identified as methoxybenzene polycarboxylic acid esters) (309).

Schnitzer and Neyroud (302) saponified soil fulvic and humic acids, identifying both fatty acids and phenolic acids in the resulting solutions. The fatty acids ranged from $n-C_{12}$ to $n-C_{38}$, with even carbon-numbered acids predominating, the majority being in the range C_{14} to C_{22} with $n-C_{16}$ and $n-C_{18}$ dominating. A microbial origin was suggested. The ratio of phenolic to fatty acids led the authors to suggest the presence of these structures in humic materials as fatty acid-substituted phenyl esters.

An additional technique which has proved successful for the removal of fatty acids from soil and soil humic substances is supercritical fluid extraction (310). Extracted fatty acids accounted for less than 0.2% of the humic material, with normal fatty acids predominating. Branched acids were also significant, and unsaturated, hydroxy and di-acids were additionally detected. The authors suggested that the major origin of these fatty acids was microbial as the majority had carbon chain lengths of less than C_{26} .

Little work has been performed to determine the lipid precursors of the fatty acids in humic substances. Although free fatty acids have been shown to occur, saponification/transesterification methods may break down more complex lipids whose identities have not generally been discussed. Lipids extracted from soil humic acids with an ethanol-benzene mixture (1:1) have been separated into acetone soluble and insoluble fractions shown to contain glycerol and phospholipids respectively (311). Further identification of these components, however, was not performed.

To conclude, therefore, it appears that fatty acids originating from both microbial and higher plant sources can occur in humic substances. In general, normal fatty acids have dominated with

branched structures occurring to varying degrees. Extraction with organic solvents appears to release loosely-bound aliphatic materials whereas harsher chemical treatments have yielded some aromatic moieties, in addition to aliphatic fatty acids. Glycerol and phospholipids have been detected in solvent extracts of humic acids although their component fatty acids were not identified.

3.3.2.2 Aquatic and sedimentary humic materials

Few studies have been performed on the lipid content of dissolved aquatic humic materials although a selection have been carried out on sedimentary materials. Aqueous sodium hydroxide hydrolysis of an aquatic humic material, followed by methylation with diazomethane, performed by Liao *et al.* (148), yielded various aliphatic mono-, di-, and tri-basic carboxylic acids in addition to a dominance of aromatic acids. Only short-chain acids (up to C₁₆) resulted, thought to result from the oxidation of aliphatic side-chains. The presence of fatty acid moieties in the original material was addressed.

The structure of marine humic material as proposed by Harvey *et al.* (222) should show evidence for the presence of unsaturated fatty acids, particularly in the early stages of formation. Methyl esters were detected by these workers following transesterification of marine humic materials with boron trichloride-methanol, although identification of the esters was not performed. The presence of fatty acids in marine humus has been confirmed by Stuermer and Harvey (165), following an extensive reductive degradation sequence. From the alkane distribution following reduction, the distribution of fatty acid moieties in the marine fulvic acid appears to differ from that in soil fulvic acids. The high molecular weight fatty acids originating from plant and insect waxes are not present in the marine humic matter, which shows fatty acids, ranging from C₁₂ to C₂₂, with C₁₈ and C₂₀ predominating, typical of marine organisms (312).

More comprehensive studies have been carried out on sedimentary humic substances. Ishiwatari (313) treated a humic acid fraction from a lake sediment with boron trifluoride-methanol which released long chain saturated (C₁₁ to C₃₄), unsaturated (C₁₆ to C₂₄) and branched (C₁₁ to C₁₇) fatty acids (n-C₁₆ dominating), amounting to 0.2 to 0.3%

of the original humic acid. As C₁₆ is a dominant acid in phytoplankton (314), it was suggested that these fatty acids resulted predominantly from this source. In addition, due to the quantity of the branched C₁₅ acid, bacteria were also suggested as contributors to the humic material.

Bourbonniere and Meyers (183) also found fatty acids thought to originate from phytoplankton, in alkaline hydrolysates of lake sediment humic matter. Among the resulting components were n-C₁₆ and n-C₁₈ fatty acids, together with some low molecular weight hydroxy aliphatic acids and some dicarboxylic acids. C₁₆ fatty acids were found to dominate the hydrolysate of a diatom present in the lake and were thus suspected to be the source of the fatty acids in the sediment.

Povoledo *et al.* (315) extracted lipids from Canadian lake sediments with aqueous acetone and found several low molecular weight (C₁₂ to C₁₈) saturated, unsaturated and branched fatty acids. They suggested that these were derived from micrororganisms, although planktonic input cannot be ruled out.

The contribution of algal lipids to lake sedimentary humic substances has also been supported by comparing potassium permanganate oxidation products of sedimentary lipids, humic substances and kerogen. The abundance of polymethylene chains in the humic acids, kerogen and algal lipids (316) led to the conclusion that these lipids were incorporated into kerogen and humic acid after the death of the algae.

In these limited investigations, therefore, it appears that phytoplanktonic lipids are the main contributors to lake sedimentary humic materials with no obvious contributions from higher plants. However, more sedimentary humic substances from a variety of environments should be studied to check for terrestrial inputs which are likely in rivers, etc., receiving input from the surrounding continent. It seems probable that further studies on the lipid content of dissolved aquatic humic material will also reveal input from aquatic organisms, in agreement with the work of Stuermer and Harvey (165).

Lipids extracted from a young marine sediment with chloroform-methanol (2:1) accounted for between 5 and 12% of a high molecular weight (> 300,000) humic acid fraction (317). Fulvic acids showed lower proportions (less than 5%) of lipids. However, the lipid components were not identified and the chloroform-methanol fraction would also be expected to extract hydrocarbons, etc., and so the fatty acid content of the humic material may be considerably lower.

3.3.3 Amino acids

3.3.3.1 Soil humic materials

Amino acids are among the main forms of nitrogen in humic substances. Other compounds contributing to the nitrogen content (which is of the order of a few percent - see Section 2.1) include amino sugars, ammonia, nucleic acid bases, and compounds containing amino groups.

Numerous investigations have been performed on the amino acids in soil humic substances. Care must be taken when comparing the data on individual amino acids due to different units employed by various workers, e.g., μmol nitrogen, μmol amino acid, mass amino acid. The amino acid contents of fulvic and humic fractions of three Italian soils were 1 - 9% and 12 - 24% respectively (318). Humic acid from an Indian soil had a value of 30% (179), and those from three Scottish soils had amino acid contents of 11 - 18% (319). It is possible, however, that high values resulted from co-extraction of free amino acids with the humic material. Representative soil fulvic and humic acid, with co-extracted amino acids removed by passing the soil solution through an Amberlite XAD-8 resin, yielded values of 6.4 and 10% respectively (290). Alternatively, variation in amino acid content may result from climatic differences between soils. A comparison of the amino acid nitrogen of humic substances formed under cool climates, and those from tropical soils, has indicated greater proportions of amino acid and amino sugar nitrogen in tropical soils due to higher microbial activity (320).

Despite variations in the amino acid content of humic materials, the major amino acids remain relatively constant, consisting of

aspartic acid, glutamic acid, glycine and alanine (Table 3.1). Glutamic acid, glycine and alanine are often the dominant amino acids in bacterial cell walls (321), indicating their possible microbial origin. The amino acid composition of fulvic and humic acids appear similar - slight differences among acidic and basic amino acids possibly originate from the extraction procedure. It is possible that some of the minor amino acids showed losses on hydrolysis (e.g., threonine, serine (322)) although corrections have not generally been made for these.

In addition to acid hydrolysis of humic materials, amino acids have resulted from enzymatic hydrolysis (323). Six proteolytic enzymes were tested for their ability to release amino acids from humic acid, and the amino acid yields were compared with those released using acid hydrolysis. Two of the enzymes (pronase and thermolysin) gave yields of amino acids about one-sixth of those resulting from acid hydrolysis. Subsequent acid hydrolysis of peptides resulting from thermolysin hydrolysis showed a three-fold increase in amino nitrogen. Papain showed slight activity but the other three enzymes did not react with humic acid.

Other enzyme hydrolysis studies (318), using pepsin and papain, failed to release amino acids. Proteolytic activity was observed for pronase, but for low organic matter:enzyme ratios, which was thought to be due to autodigestion. It was concluded that humic compounds deactivate pronase and, in the presence of excess enzyme, active pronase hydrolyses portions of the deactivated molecule.

Comparisons of the amino acid content of synthetic fungal melanins with those of soil humic materials have been made (324,325). The major amino acids in fungal melanins synthesised by Ortiz de Serra *et al.* (324) were aspartic acid, glutamic acid, alanine and glycine as with soil humic acids. However, the fungal materials generally contained lower proportions of aspartic acid, glycine and ornithine, but more cystine, methionine, tyrosine, lysine and arginine than their soil counterparts. The authors concluded that intact fungal melanins do not contribute substantially to soil humic acids but they may be present as degraded materials. Coelho *et al.* (325), obtaining similar results, commented that minor differences would be expected due to the

Table 3.1 Amino acid content of selected soil humic substances

Amino acid	Soil humic materials										
	FA ¹ (290)	HA ¹ (290)	HA ¹ (323)	HA ¹ (323)	HA ¹ (323)	HA ¹ (319)	HA ¹ (319)	HA ¹ (319)	HA ¹ (179)	FA ² (326)	HA ² (326)
<u>Acidic</u>											
Asp	100	110	34	112	86	130	223	153	534	15.6	10.2
Glu	120	81	34	113	85	77	140	106	365	9.6	8.9
<u>Basic</u>											
Arg	0.8	25	ND	ND	ND	27	38	26	19.5	1.4	2.4
His	13	54	ND	ND	ND	ND	ND	ND	ND	0.6	2.1
Lys	8.7	32	8	39	23	48	66	48	36.9	2.2	5.6
<u>Neutral</u>											
Phe	10	36	10	45	30	31	57	32	41.2	6.2	6.2
Tyr	3.7	20	3	11	9	10	17	9	63.5	6.8	6.6
Gly	66	100	43	125	94	121	208	145	741	13.8	14.9
Ala	50	86	34	123	89	85	155	98	329	9.2	8.9
Val	32	49	15	64	47	67	113	78	7.7	6.2	6.1
Leu	18	46	18	85	57	60	103	67	146	6.0	5.7
Ile	17	28	7	26	21	48	68	47	32.8	3.2	3.1
Ser	31	59	21	56	40	52	94	64	115	7.1	7.0
Thr	31	59	14	40	27	57	95	68	161	6.0	6.4
Pro	12	45	16	61	45	68	100	70	9.6	4.7	4.2
Hpro	ND	ND	ND	ND	ND	20	34	19	ND	ND	ND
Met	5.1	12	ND	ND	ND	ND	6	5	3.4	0.8	1.1
Cys	ND	ND	ND	ND	ND	ND	ND	ND	13.3	0.6	0.5
Cys-Cys	1.7	1.9	ND	ND	ND	10 ⁺	12 ⁺	13 ⁺	ND	Tr ⁺	Tr ⁺
% AA	6.4	10.5	ND	ND	ND	11.0	18.4	12.6	29.7	ND	ND

1 Amino acid concentrations in $\mu\text{mol/g}$

2 Relative molar distribution of amino acids (%)

ND Not determined

Tr Trace

+ Determined as cysteic acid

FA Fulvic acid

HA Humic acid

AA Amino acid

() Reference

combination of sources of humic acid in soil compared to the single source of the synthetic material. In addition, the synthetic materials are not subjected to microbial decomposition in the soil, and thus are not chemically altered in this way. These authors therefore concluded that fungal melanins play a significant role in the formation of soil humic acids.

Pyrolysis studies have also identified the presence of amino acids in soil humic materials. Pyrrole, acetonitrile and toluene were derived quantitatively from polypeptides in some soil humic acids (319).

Other investigations have attempted to isolate protein, rather than hydrolysed materials, from humic substances. Simonart *et al.* (327) isolated a protein fraction from soil humic acid, which yielded twenty amino acids on hydrolysis and possessed a small electronegative charge. Ramunni *et al.* (318), on the other hand, were unable to isolate proteinaceous material using electrophoresis.

Finally, amino acid composition can also be related to the age of geological materials. The rate of racemisation of amino acids has been employed as a means of dating these materials (328,329). Enantiomeric ratios of amino acids from humic-like substances extracted from fossils have been measured, indicating increasing racemisation with increasing fossil age (178). This method relies on the fact that recent fossil organic material consists almost solely of L-amino acids. These amino acids are abundant in living organisms, and so can be used as a means of dating material originating from these organisms. However, D-amino acids have been found in significant amounts in contemporary soils (330), and so application of this method to materials with possible terrestrial input may not be valid. Its application to dating humic materials therefore clearly requires further investigation.

A high correlation between age and amino acid/sugar content of humic substances from buried soils has also been suggested as a geochemical marker to estimate the age of organic matter (225). However, only a limited set of data was available in this instance, and further measurements, on both Paleosols and different soils, must

be performed to check this relationship.

3.3.3.2 Aquatic humic materials

Aquatic humic materials from various origins have been found to contain similar amounts of amino acids. Their concentrations are lower than in soil humic matter (7,290). As with soil humic substances, humic acids are enriched in amino acids compared to fulvic acids.

The amino acid contents of dissolved organic material from eight U.S.A. rivers varied from 3.3 to 5.5% (by weight) (93). Ohio river fulvic and humic acids had amino acid compositions of 0.7 and 3.7% respectively (290), whereas proteinaceous material in Mullica and Broadkill river humic acids accounted for 43 and 53% of the total organic extracts (40). Estuarine colloidal material in the Patuxent river (U.S.A.) had amino acid contents varying from 1.9% to 15.9% (331). Few studies have been performed on the amino acid composition of marine humic substances although two marine fulvic acids showed low percentages of 0.6% (150) and 0.8% (290), similar to the Ohio river fulvic acid mentioned above.

The individual amino acid distributions of aquatic humic substances from various origins (Table 3.2) show that, although aspartic acid, glutamic acid, glycine and alanine are among the major amino acids, as in soil humic substances, they do not dominate the amino acid fraction to such a degree as in their soil counterparts. Other significant components may also be present. For example, serine, which is a major constituent of diatom cell walls (332), is important in all the aquatic humic substances listed in Table 3.2. Additional major amino acids, such as leucine, valine, and proline, may also occur to varying degrees in different samples. Hydroxyproline was the major amino acid, along with glycine, in Shawsheen river fulvic acid (U.S.A.) (293), and was also detected, to a lesser degree, in both the suspended material and fulvic acid from nearby Thoreau's Bog. These are the dominant amino acids in collagen (321) suggesting their source as higher animals.

Seasonal variations in the amino acid composition of aquatic humic

Table 3.2 Amino acid content of selected aquatic humic substances

Amino acid	Aquatic humic materials								
	River					Estuarine		Marine	
	HS ⁺¹ (333)	FA ² (290)	HA ² (290)	HA ³ (40)	DOM ¹⁺⁺ (93)	Winter S=12.3% (331)	Summer S=9.0% (331)	FA ¹ (150)	HA ² (290)
<u>Acidic</u>									
Asp	13.6	9.2	27	420	14.0	17.1	14.1	11.4	7.6
Glu	9.6	4.9	48	250	8.0	16.9	12.6	10.1	7.2
<u>Basic</u>									
Arg	1.2	ND	6.7	-	0.1	7.7	4.8	ND	1.0
His	2.2	5.4	13	20	2.0	9.6	2.6	ND	3.2
Lys	3.0	1.3	5.8	50	3.4	8.6	4.4	ND	1.8
<u>Neutral</u>									
Phe	5.2	0.7	8.5	70	2.8	8.2	11.7	ND	1.8
Tyr	0.3	1.1	8.9	40	2.6	7.9	3.4	6.8	1.6
Gly	18.7	17	49	560	20.1	11.1	8.2	29.8	14
Ala	11.8	5.3	24	240	10.7	14.5	11.1	12.4	5.7
Val	4.7	2.9	16	140	5.4	9.1	5.7	4.6	1.0
Leu	4.7	2.3	17	140	4.1	12.9	7.8	2.2	3.5
Ileu	2.3	1.7	9.6	100	3.0	8.4	3.4	ND	2.3
Ser	9.5	4.5	24	280	9.0	8.7	9.1	16.7	5.3
Thr	6.1	4.1	19	-	8.8	11.0	8.2	ND	3.9
Pro	3.5	2.8	26	-	5.0	4.1	3.2	6.8	2.9
Met	3.6	0.4	2.8	-	0.8	3.4	3.0	ND	0.6
Cys	ND	ND	ND	ND	0.4	ND	ND	ND	ND
Cys-Cys*	ND	0.2	2.2	70	ND	ND	ND	ND	0.3
% AA	ND	0.7	3.7	ND	ND	15.9	11.3	0.6	0.8

1 Amino acid concentration in mol %

2 Amino acid concentration in $\mu\text{mol/g}$

3 Amino acid concentration in nmol/l

FA Fulvic acid

HA Humic acid

HS Humic substances

ND	Not determined	AA	Amino acid
DOM	Dissolved organic material	()	Reference
+	Average total concentration of amino acids in the Williamson river system over a two-year period. $\geq 96\%$ of the dissolved amino acids were associated with humic material.		
++	Mean of eight rivers.		
*	Determined as cysteic acid.		

substances have been studied by some workers. Lytle and Perdue (333) performed a monthly survey of the total amino acids in the Williamson river system over a two year period. The majority of the dissolved amino acids ($\geq 96\%$) were humic-bound. The five main constituents were glycine > aspartic acid > alanine > serine \approx glutamic acid, and this order did not vary significantly over the two year period. Minor amino acids, namely β -alanine, hydroxyproline, ornithine and tyrosine, in addition to those shown in Table 3.2, were also observed on a few occasions. Total amino acid concentrations did vary, however, being higher in winter and spring than in summer and autumn. As this correlated positively with humic carbon and discharge, it was suggested that the major source of humic carbon and amino acids was surface run-off, and that the amino acids were, in fact, associated with soil humic material and then carried into the stream.

Sigleo *et al.* (331) investigated the amino acid composition of estuarine colloidal material from five sites (fresh to brackish water) in both winter and summer. In winter, the amino acid content increased with increasing salinity whereas, in summer, the data were more variable. This appeared to be related to the ash content of the material which decreased with increasing salinity (and amino acid content) in winter, but was variable in the summer. The relative proportions of the individual amino acids were fairly constant both between sites and seasons, the main constituents, on a molar basis, being alanine \approx glycine > aspartic acid \approx glutamic acid > leucine. The amino acid composition of the colloidal material was compared with that of estuarine particulate organic material and that of a phytoplankton. The similarities between the amino acid contents of these materials led the authors to conclude that both the particulate and colloidal material were composed predominantly of planktonic remains. The absence of any significant terrestrial input was

confirmed by the absence of D-amino acid isomers, which have been found to be present in significant amounts in contemporary soils (329).

Seasonal variations have also been investigated using pyrolysis techniques. The composition of Tjeukemeer lakewater fulvic acids (209) showed a dominance of aromatic fragments in the June/July samples, thought to be derived from proteins resulting from the decline of a cyanobacterial bloom.

An interestingly low amino acid content (0.2%) was found in the filtered water obtained from a swampy pond (93). Unfiltered water from the same pond, however, showed a similar concentration to filtered riverwater (3.7%). Glycine and alanine were by far the major amino acids in the filtered sample with aspartic acid being depleted compared with nearby riverwater samples. The authors tentatively explained the absence of significant amino acid concentrations in the filtered water as being due to the slower breakdown of biopolymers to dissolved organic material at the low pH (pH 3.8) of the pond, hence their presence in the unfiltered sample.

Few investigations have been performed on the amino acid content of marine humic substances. Low levels of amino acids have been quantified in marine fulvic acid extracted using both Amberlite XAD-2 (150) and XAD-8 (290) resins. Bada *et al.* (334), however, found that amino acid levels were close to blank levels, suggesting that most of the amino acids arose from contamination rather than from the humic material itself. Clearly, further work is required in this area.

3.3.3.3 Sedimentary humic substances

The amino acid content of sedimentary humic materials is higher than that of their aquatic counterparts, being more similar to that of soil humic substances. Kemp and Mudrochova (335) isolated amino acids in humic materials extracted from Lake Ontario sediments. Total amino acids in the fulvic and humic acids accounted for 12.6% and 21.5% of the humic substances respectively. The amino acid contents of seven lake sedimentary fulvic and humic acids were 4-24% and 11-21% respectively (336). Amino acids in humic materials from carbonate

sediments were found to constitute 15-20% of the fulvic acids and 27-36% of the humic acids (on a dry weight basis) (337). Marine sedimentary humic acids, analysed by Romankevich (338), showed amino acid contents of 3.3 and 8.6%, based on humic carbon.

As in the case of both soil and aquatic humic substances, the lake sedimentary humic materials, as well as the total sedimentary amino acids, are dominated by aspartic acid, glutamic acid, glycine and alanine (336). The amino acid distribution in the humic acid from Lake Ontario sediments (335) resembled that of zooplankton and suspended sediment samples, supporting an autochthonous origin for these materials. The fulvic acid, however, showed low concentrations of basic amino acids, with the amino acid composition resembling that of combined amino acids in interstitial waters of the sediment.

Humic acids from marine sediments (338) were enriched in aspartic and glutamic acids. In comparison, total sedimentary amino acids contained glycine and α -alanine as the major components, with leucine and iso-leucine also being significant - lysine was important in one sample. Humic acids from marine sediments of San Diego Basin also showed a dominance of aspartic and glutamic acids, with leucine, proline and phenylalanine additionally contributing significantly (compared on a molar basis) (175). A comparison of the amino acid composition of both fulvic and humic acids in carbonate and non-carbonate sediments (337) showed that the major amino acid in carbonate sediments was aspartic acid, with glutamic acid and glycine also being significant. Glycine was more important in most of the non-carbonate samples and plant debris - glutamic acid, aspartic acid and alanine were also major contributors.

EXPERIMENTAL AND RESULTS

Practical points on experimental procedures

Various practical aspects of the experimental procedures in the following chapters now follow.

- (i) All glassware was soaked in chromic acid, rinsed with doubly distilled water and then oven-dried prior to use.
- (ii) Doubly distilled water was used for preparing sample solutions and cleaning glassware. The second distillation was initially from alkaline potassium permanganate although this was not found to be necessary in later work when a commercial apparatus for the preparation of doubly distilled water was employed.
- (iii) Reagent grade organic solvents were distilled in all-glass apparatus before use, apart from acetone (for chlorophyll 'a' analysis) and pyridine (analytical grade), and boron trifluoride in methanol and iso-propanol, which were employed without prior purification. The TLC solvents for amino acid analysis were also reagent grade (bar ammonia which was analytical grade) and were not purified further, with the exception of t-butanol. This was shaken with some dry cation exchange resin (Dowex 50W-X8, hydrogen form, 16-40 dry mesh, 10 g/100 ml) to remove impurities which may retain ammonia on TLC.
- (iv) Analytical grade inorganic solvents were employed. Hydrochloric acid for amino acid analysis was further purified by sub-boiling distillation.
- (v) Chemical reagents were reagent grade and were used without further purification unless otherwise stated. Anhydrous sodium sulphate (drying agent) and sodium chloride were cleaned by heating in a muffle furnace at 500°C for at least four hours, and stored in foil-lined, screw-capped glass jars. 2-hydroxypyridine for monosaccharide analysis was recrystallised from hot ethanol.
- (vi) Artificial seawater was prepared by the appropriate dilution of dehydrated seawater ('Tropic Marin', Aquarientechnik, Wartenberg, Germany) with doubly distilled water.
- (vii) Florisil, for the column chromatography of FAMES, was activated at 600°C overnight. This was performed at regular

intervals as prolonged exposure to the atmosphere has been shown to alter its chromatographic properties.

- (viii) Ion exchange resins (Dowex 50W-X8) in the hydrogen form, were prepared by washing with hydrochloric acid (ca. 2 moles/l) and then rinsing with doubly distilled water to pH 5. The anion exchange resin (Dowex 1X8-20) in the formate form, was prepared by washing with formic acid (ca. 2 moles/l) and rinsing as above.

CHAPTER FOUR

4.1 A REVIEW OF THE PROCEDURES USED FOR THE ISOLATION OF AQUATIC HUMIC SUBSTANCES

4.1.1 Methods of extraction

Due to the low concentrations of humic materials in natural waters, it is not possible, in most cases, to study them directly *in situ*. They must be isolated from the other species present, and concentrated. Methods used for this purpose, together with their advantages and disadvantages, have been reviewed (42,339). They include freeze concentration, lyophilisation, vacuum distillation, reverse osmosis, ultrafiltration, solvent extraction, precipitation, co-precipitation, centrifugation and adsorption methods. More recently, attempts have been made to isolate humic acid using supercritical fluid extraction (340). These failed, however, possibly due to precipitation of the humic acid.

The popularity of adsorption-based extraction techniques is due more to the difficulties inherent in the other methods than the suitability of adsorption. Other methods suffer from procedural difficulties such as the evaporation of large volumes of water (typically tens to hundreds of litres), are selective to specific classes of compounds, or give incomplete recoveries.

Various adsorbents have been used, both inorganic and organic. In the past, many workers have preferred inorganic adsorbents, such as alumina, silica gel, magnesia and calcium carbonate (341). However, these present problems of low recoveries due to inefficient adsorption and desorption steps. Alteration of the organic matter may also occur (42).

Higher recoveries can be obtained using organic adsorbents, such as activated carbon, nylon, polyamide (nylon) powder, ion exchange resins and macroporous sorbents (42). The extraction of organic compounds from water, using synthetic polymers, has been reviewed (342,343). Resins used include Amberlite XAD, Porapak, Chromosorb, Tenax, Spheron, polyurethane and Teflon. Of these, the Amberlite XAD

resins have been the most widely used for isolating humic materials from natural waters.

The main advantages of methods based on XAD resin adsorption are:

- the resins have high capacities.
- efficient desorption can be achieved.
- large quantities of organic extraction solvents are not employed as the humic materials are isolated directly from the water.
- they are simple, and capable of rapid processing of large volumes of water.
- the humic substances are separated from inorganic materials in the water.

They have the disadvantage, however, of a tendency to release organic matter, yielding high blank values. Careful resin cleaning is therefore required.

4.1.2 Extraction using Amberlite XAD resins

Amberlite XAD resins are non-ionic, macroporous copolymers, which possess large surface areas, have a high porosity, and are available in a variety of polarities and surface characteristics (344). They have been widely used to extract many different compounds, including pesticides, polychlorinated biphenyls, phenols, alkaloids and drugs of abuse from aqueous and biological media (169,342,345). The resins which have been the most frequently used for the extraction of trace organics from natural waters are XAD-1, XAD-2, XAD-4 (styrene-divinylbenzene copolymers), and XAD-7 and XAD-8 (acrylic esters).

The principal phenomenon of adsorption onto the resins involves Van der Waals forces, which bind the sorbate to a solid surface, although other interactions, such as hydrophobic bonding, dipole-dipole interactions and hydrogen bonding, are also thought to occur (346). The aqueous solubility of the solute and solution pH have been shown to determine the sorption of organic acids such as humic substances (346). In general, the sample is acidified, which protonates the weak acids leading to resin adsorption. The column is then eluted with a basic solution, in which the weak acids are ionised

and so desorption is favoured.

As humic substances contain both polar and non-polar groups, they can be removed from water using either polar or non-polar resins. Several studies have been carried out comparing the different XAD resins for their efficiency in extracting aquatic humic substances. Cheng (347) investigated the separation of humic acids from natural water using XAD-1, 2, 4, 7, and 8, and the less common XAD-11 and 12. He found XAD-12, a very hydrophilic resin with weak-base functional groups, to be the more efficient resin for the removal of humic acid from solution at pH 5. He did, however, add ethylenediamine-tetraacetic acid (disodium salt, EDTA) to the humic acid to complex any di- and tri-valent cations, which, he stated, may decrease the solubility of the humic acid. This may have affected the results and so should be remembered when comparing the data with that of other investigators.

The isolation of fulvic acid from aqueous solution, using XAD-1, 2, 4, 7 and 8, has been studied by Aiken *et al* (348). They found that XAD-7 and XAD-8 were more efficient than their styrene-divinylbenzene counterparts. This was thought to be partially due to the irreversible adsorption of a fraction of the fulvic acid onto the styrene-divinylbenzene resins, which was attributed to π - π bonding (charge-transfer complexation). In addition, the kinetics of sorption of fulvic acid onto these resins is slow compared with the acrylic ester resins (42). Of the two acrylic ester resins, XAD-8 was preferred (348) as it was found to exhibit less resin bleed.

Numerous studies have been carried out on humic matter in water from different sources using various XAD resins. The effective removal of humic substances from seawater, using XAD-1 (349) and XAD-2 (65,222) has been performed. XAD-2 has also been used to isolate dissolved organic materials from river water (35), as has XAD-7 (70,295) and XAD-8 (350). Humic substances have been extracted from lakewater, using XAD-2 (351), XAD-7 (70) and XAD-8 (148,216), and groundwater, using XAD-8 (350).

In the past, XAD-2 has been the most extensively used XAD resin for the extraction of humic materials from natural waters. However,

as XAD-8 has been shown to be more efficient than XAD-2 for this purpose (348,352), XAD-8 was chosen in the present study. Recent investigators have also favoured this resin (e.g., (216,290)) and a detailed scheme for the preparative isolation of aquatic humic substances, using XAD-8, has been devised (350).

The experimental procedure for extracting the humic substances consists of three main steps:

- Exhaustive cleaning of the XAD resin.
- Adsorption of the humic material onto the resin.
- Desorption of the material from the resin.

These will now be discussed in the following sections.

4.1.2.1 Cleaning the XAD resin

Due to the large amounts of impurities in XAD resins, the first step in any extraction scheme is an efficient clean-up procedure. The following are a selection of those which have been suggested.

- (a) Vacuum degassing and gas stream desorption, both at elevated temperatures (353).
- (b) The resin is washed successively with acetone, methanol and methylene chloride or chloroform. The last wash is concentrated and checked for interferences by GC (353).
- (c) Batch extraction of the resin in refluxing benzene is carried out until ammonium hydroxide, ethanol and methylene chloride eluents show no ultraviolet absorption after concentration (65).
- (d) The resin is rinsed twice with water to remove preservatives, and is then slurried in an organic solvent, such as methanol, acetone or iso-propanol. It is transferred to the column, washed with four bed volumes of solvent and allowed to stand for several hours under the solvent. It is then rinsed with distilled water, and backwashed with water to free entrapped air pockets and rid the bed of resin fines (344).
- (e) The resin is washed several times with water, then methanol, and is dried by filtration. It is then sieved to collect different ranges of mesh sizes, each of which is finally Soxhlet-extracted

- with diethyl ether (2 x 12 hours), and air-dried (353).
- (f) Several cycles of Soxhlet extractions, 24 hours each, with water, methanol and methylene chloride (353).
 - (g) The resin is Soxhlet-extracted with acetonitrile and several batches of benzene, and is then dried (354).
 - (h) The resin is washed free from chloride with distilled water, Soxhlet-extracted with acetone for two hours, rinsed with water and packed in a glass column. It is then washed with ten bed volumes of sodium hydroxide (0.5 moles/l) and finally distilled water again. For more than a few hours storage, it is treated with 0.02% sodium azide solution (355).
 - (i) The resin is decanted in methanol several times and is then sequentially Soxhlet-extracted, for eight hours each, with methanol, acetonitrile and diethyl ether. The purified sorbent is stored in a glass vessel, under methanol (169).
 - (j) The resin is washed with distilled water to remove fines, Soxhlet-extracted with acetone for more than eight hours, and rinsed thoroughly with distilled water. It is then packed into a glass column and washed with ten bed volumes of ammonium hydroxide (2 moles/l), before finally rinsing with a similar volume of distilled water (72).
 - (k) The large resin beads are sieved out and the fines are decanted from sodium hydroxide (0.1 moles/l), storing the remaining resin in sodium hydroxide (0.1 moles/l). Sequential Soxhlet extractions with acetone and hexane are performed for 24 hours each. The resin is then slurried in methanol, poured into the column and rinsed free of hexane with methanol. It is finally washed with distilled water, and rinsed with sodium hydroxide (0.1 moles/l), hydrochloric acid (0.1 moles/l), and distilled water, prior to sample application (43).

The clean-up procedure proposed by the manufacturer, (d), was found to be unsatisfactory (353) due to the considerable amount of monomeric material trapped interstitially in the porous structure of the resin, left by the manufacturing process. Sequential Soxhlet extractions for long periods, (f), gave better results, but were thought to be too time consuming (353). However, Soxhlet extraction has appeared to be the chosen method of the majority of workers. Various combinations of solvents have been employed (356). An

extension of method (i) above has been used extensively by Malcolm and co-workers (346,348,350,352), and has also been adopted by other investigators (148,216). With this approach, the resin is washed with sodium hydroxide (0.1 moles/l) before Soxhlet extraction (24 hours each solvent), and rinsed with alternating sodium hydroxide (0.1 moles/l) and hydrochloric acid (0.1 moles/l) solutions prior to sample application.

4.1.2.2 Adsorption of humic material

The percentage of humic matter adsorbed onto the column depends on several parameters, including pH of the water, flow rate, and column geometry (355). In most cases, the water is filtered and then acidified to approximately pH 2. The most efficient adsorption of humic substances has been found to occur at low pH (348,355). However, any possible alteration of the material in strongly acidic solution must be avoided. Therefore, a pH of 2 is generally chosen because it is high enough to prevent alteration of the humic matter but also low enough to result in efficient adsorption. Cheng (347), however, investigating humic acid, preferred adsorption at a higher pH (pH 5 for XAD-12) due to precipitation of humic acid at low pH. The greater proportion of humic material in natural waters though, is fulvic acid (according to Aiken (42), humic acid constitutes only about 5% of dissolved humic substances in water), which adsorbs best at lower pH values. For this reason, a pH of 2 was chosen for the present work.

Various flow rates have been employed for the isolation of humic substances using XAD resins (352,354,355). Mantoura and Riley (355) investigated the effect of flow rate on the retention of humic and fulvic acids on XAD-2, at pH 2.2, varying the rate from 5 to 130 bed volumes per hour. They found that acceptable adsorption (greater than 92% for humic acid and greater than 75% for fulvic acid) was obtained with flow rates of less than 35 bed volumes per hour. From their data, the loss in efficiency on going from 35 to 70 bed volumes per hour was only about 5%. Therefore, for speed of extraction, a rate of approximately one bed volume per minute was used in this work.

4.1.2.3 Desorption of humic material

Prior to desorption of the humic material, the column must be rinsed with either hydrochloric acid (pH 2) (222) or distilled water (354) to remove any salts. If distilled water is used, care must be taken to minimise the volume added as it will begin to elute the adsorbed organic acids. Therefore, a hydrochloric acid rinse was employed in this work.

The desorption efficiency of the humic substances depends on the nature of the eluent, its concentration and flow rate (355). Several solvents have been employed, the most common being sodium hydroxide (348,350,351) and methanolic ammonia (178,222,351). Other solvents which have been used include potassium hydroxide (349), ammonium hydroxide (354) and dioxane-water (1:1) (70). The percentage desorption of humic substances from XAD-2, using potassium hydrogen phthalate, tris(hydroxymethyl)aminomethane buffer, ammonium hydroxide, methanol, ethanol, acetone and methanolic ammonia, has been studied (355). The organic solvents showed incomplete desorption due to the low solubility of humic compounds in these solvents. The aqueous solvents were therefore preferable, the recoveries tending to improve with increasing pH. Maximum recovery was achieved using sodium hydroxide (0.2 moles/l). Disadvantages of this eluent, however, are the possibility of chemical alteration of the humic substances in the presence of sodium hydroxide (42), and the need to subsequently desalt the sample, which can result in the loss of low molecular weight material. The use of methanolic ammonia was found to yield slightly lower recoveries, but is advantageous in that it can be readily removed by rotary evaporation. A disadvantage, however, is the interaction of NH_4^+ with the humic substances, leading to erroneously high nitrogen contents in the resulting material (42,354). Due to the ease of removal, methanolic ammonia was chosen in the present study.

The flow rate for desorption must be as slow as is practical. It has been noted (355) that slightly greater recoveries can be achieved if the eluent is allowed to stand in the column overnight. This was therefore carried out in this work. Other investigators, however, have stated that this encourages bleed with acrylic ester resins and concluded that elution should be completed as quickly as is

feasible (348).

Fractionation of the humic substances on desorption has been performed using a pH-gradient or a selection of solvents. Mantoura and Riley (355) demonstrated the fractionation of humic and fulvic acids on molecular weight basis, by desorption from XAD-2 using a series of eluents having progressively greater pH values. Studies on XAD-8 by MacCarthy *et al.* (357) yielded two fractions after desorption using a pH gradient generated by titrating phosphorous acid with sodium hydroxide. Separation of the humic material into fulvic and humic acid fractions has been performed using two different eluting solvents (dioxane-water (11:1) and sodium hydroxide (0.01 moles/l)) (70). Comprehensive DOC fractionation schemes, yielding multiple fractions, have been employed, in which initial extraction with XAD-8 and elution with different solvents, was followed by further chromatography on a gel filtration column (44), on ion exchange columns (43) or on an ion exchange column followed by a carbon adsorbent (358).

Other fractionation methods have been employed following isolation of the humic materials, including molecular size or charge fractionation and precipitation methods (to separate the fulvic and humic fractions). Their application to aquatic humic substances has been reviewed (359). As the composition of the total humic fraction was of interest in the present work, fractionation of the resulting material was not performed.

4.2 EXPERIMENTAL

4.2.1 Area of study

The data presented in the present study are from samples collected from the River Beaulieu estuary and the Solent, Hampshire, England. The River Beaulieu is a small river, receiving a high input of terrestrial material from the drainage of New Forest heathland and bogs. The Solent receives discharge from the Beaulieu river, as well as the other main rivers in the region, the Test and Itchen, which flow into the estuary of Southampton Water. This, in turn, discharges into the Solent.

Previous studies on the Beaulieu river system have included the behaviour of dissolved organic material, iron, manganese, zinc (36,360) and arsenic (361) in the estuary; detailed investigations on the nature of the dissolved organic material have, however, not been performed. During estuarine mixing, substantial removal of iron has been found to occur (360) and removal of dissolved inorganic arsenic has also been shown (361). Dissolved organic carbon, manganese and zinc, on the other hand, have tended to exhibit more conservative behaviour (36,360).

The present study aims to take a more detailed look at the nature of the dissolved organic material in the Beaulieu estuary and the Solent in order to obtain information on its origin.

4.2.2 Sample collection

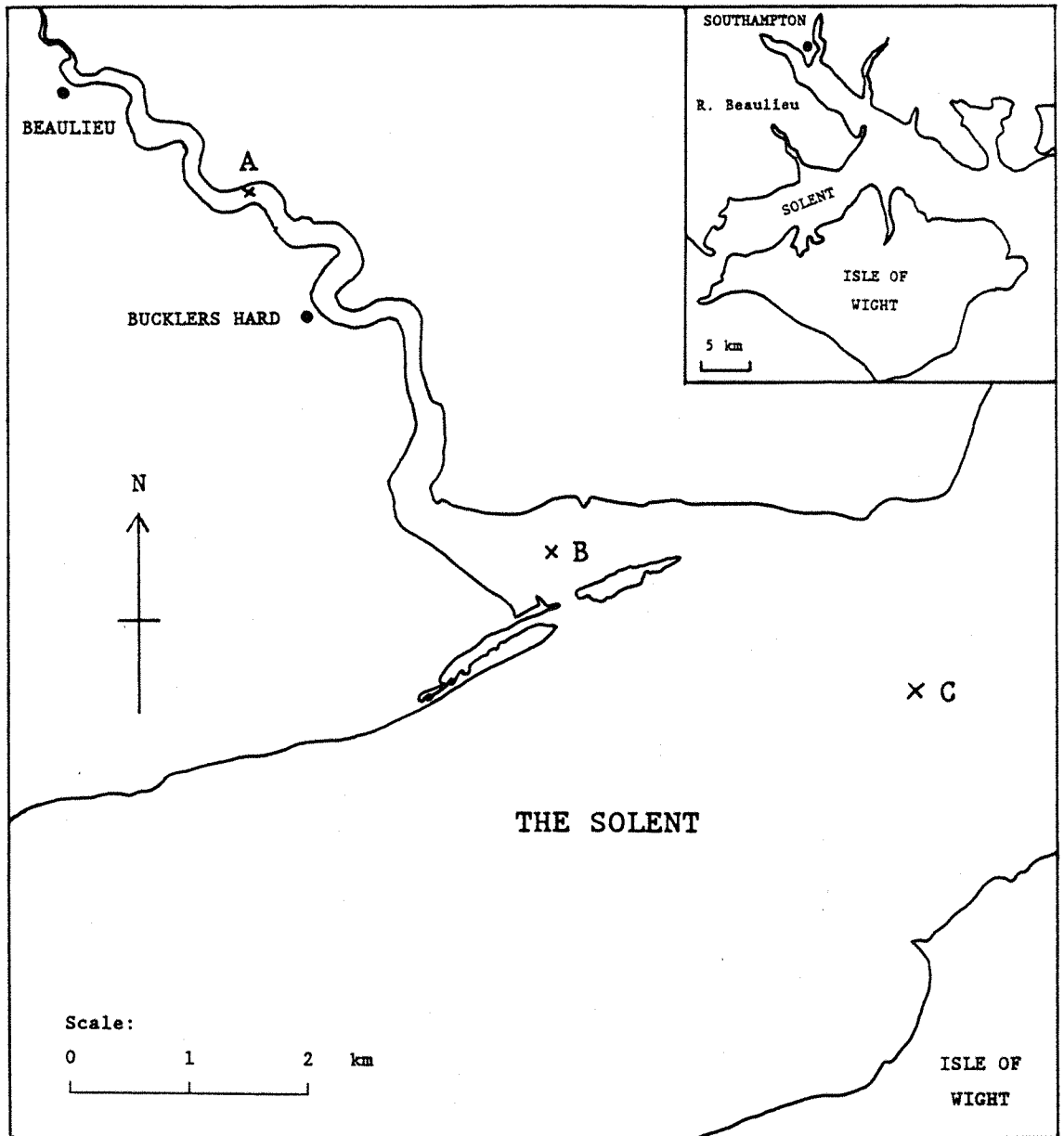
Water samples were collected from the Beaulieu estuary and the Solent on seven occasions during the period, August, 1985 to November, 1986. In each case, three sites were sampled (Figure 4.1 - approximate grid reference of site C was SZ 461969) from a motorised craft, commencing at approximately low water from the low salinity site.

Surface water samples were acquired at each site, using a galvanised iron bucket. On one occasion, samples were also taken ca. 1 m below the surface using an 'in house' constructed sampler, which passed through the water surface closed. Surface water temperatures and sample salinities were recorded.

Samples were stored in brown-glass Winchester bottles (chromic acid washed, and rinsed with the sample prior to use) with caps having aluminium foil inserts. They were filtered through heat-treated (450°C for at least four hours) Whatman glass microfibre filters (GF/C) as soon as possible after sampling (usually within 48 hours), and were stored in the dark, at 4°C, until extraction.

Filtered water samples for dissolved organic carbon (DOC) analysis (362) were stored in heat-treated (500°C for at least four hours) soda glass bottles with caps having pre-washed Teflon liners. Addition of

Figure 4.1 Location of sampling sites in the River Beaulieu estuary and the Solent



1.6% (w/v) mercury (II) chloride solution (2.5 ml/l sample) and storage in the dark, at 4°C, were carried out to prevent alteration of the samples. Chlorophyll 'a' and phaeo-pigments (chlorophyll degradation products) in the water were determined colorimetrically after extraction of the filters, according to published procedures (363).

4.2.3 Extraction of the humic materials

4.2.3.1 Cleaning the XAD resin

- New resin - The resin was washed free from salts with doubly distilled water. It was then cleaned by washing and soaking overnight with ca. sodium hydroxide (ca. 0.5 moles/l), rinsing with doubly distilled water to pH 7, and sequential Soxhlet extraction for 24 hours each, with methanol, acetonitrile and diethyl ether. Finally, it was rinsed free of diethyl ether with methanol, and stored under methanol, under nitrogen, in a glass vessel, until used.
- Regenerated resin - After use, the resin was stored under methanol, and then cleaned by initially washing with hydrochloric acid (ca. 0.5 moles/l) and then following the above procedure.

4.2.3.2 Adsorption

The purified resin was added to the chromatography columns as a methanol slurry, until a bed of polymer, ca. 16 cm high (diameter 2.5 cm, volume 80 ml), was obtained. The methanol was allowed to drain to the top of the polymer bed, and the resin was then washed with a minimum of five bed volumes of doubly distilled water acidified to pH 2 with concentrated hydrochloric acid.

The water samples were acidified to pH 2 (with concentrated hydrochloric acid) and passed through the resin, under gentle vacuum,

at a rate of less than one bed volume per minute. A blank analysis was performed on each sampling occasion, in which acidified doubly distilled water (pH 2) was substituted for the natural water. When all of the samples (25 l from each site) had passed through the columns, they were rinsed with acidified doubly distilled water (pH 2).

4.2.3.3 Desorption

The humic substances were eluted, under gravity, with at least four bed volumes of methanolic ammonia (1:1 (v/v) methanol : ammonium hydroxide (2 moles/l)) at a rate of less than 25 bed volumes per hour. The columns were allowed to stand overnight in the final bed volume. The eluent was concentrated to a small volume by rotary evaporation, at below 60°C, and was then lyophilised. The resulting humic material was stored in a deep-freezer, in the dark, over silica gel and under nitrogen.

4.3 RESULTS AND DISCUSSION

4.3.1 Water sample data

General data on the water samples, including temperature, salinity, DOC, chlorophyll 'a' and phaeo-pigments, are given (Table 4.1).

4.3.2 Comparing sampling using a bucket and a sub-surface sampler

On one occasion (27/11/86), water was sampled using both a galvanised iron bucket and an 'in-house' constructed sampler, which passed through the water surface closed. DOC analyses were performed on both sets of samples to see if any major differences were encountered. All samples were filtered, and poisoned (with mercury (II) chloride solution) for DOC analysis, on 28/11/86, with the exception of site A (bucket), which was filtered and poisoned on 27/11/86.

The results (Table 4.2) for sites B and C suggest no significant differences between the two sampling methods. Those from site A, however, indicate a possible variation but may not be significant as



Table 4.1 Data on water samples

Date	Site	Temp. (°C)	Salinity (‰)	DOC (mgC/l)	Chlorophyll 'a' (µg/l)	Phaeo-pigments (µg/l)
28/08/85	A	19.2	20.5	8.3	17.7	14.2
	B	17.8	31.3	2.9	6.5	6.2
	C	17.1	33.8	1.4	4.4	2.3
25/11/85	A	NA	NA	2.9	1.3	2.1
	B	NA	NA	1.3	1.3	2.3
	C	NA	NA	1.1	2.3	2.0
06/02/86	A	4.8	18.8	4.0	8.2	11.9
	B	5.4	27.3	1.9	4.7	9.0
	C	6.2	30.4	1.2	2.7	4.9
07/04/86	A	8.8	20.6 ⁺	3.6	23.0	12.9
	B	10.2	32.4 ⁺	1.5	6.2	4.8
	C	NA	33.1 ⁺	1.6	6.3	5.4
29/05/86	A	15.9	19.5	4.9	29.0	10.1
	B	15.3	27.5	2.9	15.1	4.0
	C	13.9	31.9	1.4	16.3	5.1
12/08/86	A	19.4	27.1	3.5	7.2	8.1
	B	18.7	32.5	2.1	6.0	4.9
	C	17.8	34.2	1.1	5.2	4.2
27/11/86*	A	10.6	10.9	8.2	6.5 (4.3)	4.6 (7.0)
	B	11.7	28.1	2.8	3.2 (3.6)	5.6 (2.9)
	C	12.0	33.0	1.2	1.6 (1.8)	1.1 (1.7)

+ Due to problems with the salinometer, these salinities were calculated from Mohr titration measurements of chlorinity (364), using the formula,

$$S(^{\circ}/\text{oo}) = 1.80655 \text{ Cl} (^{\circ}/\text{oo}).$$

* These samples were not extracted for humic substances - they were collected for storage experiments and a comparison of sampling methods only (Sections 4.3.2 and 4.3.3).

() Data in parentheses indicate results obtained using the sub-surface sampler (Section 4.3.2).

NA Data not available.

Table 4.2 A comparison of the DOC content of water sampled using a bucket and a sub-surface sampler

Site	DOC (mgC/l)	
	Bucket	Sampler
A	8.2	7.0
B	2.8	2.8
C	1.2	1.3

only one sample was taken.

To investigate smaller differences in DOC in the two sets of samples, a more comprehensive study could be performed, in which individual classes of dissolved organic material, such as monosaccharides, amino acids and fatty acids, are analysed.

4.3.3 Storage experiments

Storage experiments were conducted on water sampled with the sub-surface sampler from sites A and C. The filtered samples were left in a refrigerator (at 4°C), and aliquots were removed, at two day intervals, and poisoned for subsequent DOC analysis.

The results (Table 4.3) indicate that no obvious change in the DOC content of the filtered water samples occurred over a period of nine days, and, therefore, prior to extraction with XAD-8.

Table 4.3 Variation of DOC content with storage

Day	DOC (mgC/l)	
	Site A	Site C
0	7.0	1.3
1	6.7	1.3
3	6.8	1.3
5	6.9	1.2
7	4.0 *	1.3
9	7.2	1.2

Day 0 is taken as the day when the samples were filtered.

* Suspected to be erroneous.

4.3.4 Data on the resulting humic material

The masses of humic material (plus blank) recovered are given (Table 4.4), together with their elemental analyses. Carbon, hydrogen and nitrogen analyses were carried out, in duplicate, using an F and M Scientific Corporation, Model 185, Carbon Hydrogen Nitrogen analyser. The hydrogen data are not included, however, as they were not quantitative due to excessive tailing of the GC peaks. A selection of the samples were analysed in triplicate, to check the repeatability of the method.

Two important points to be noted from the resulting data are:

- (a) An appreciable quantity of material was recovered during the blank analyses.
- (b) N/C ratios have been calculated for humic substances from various origins, including soil, sediment, river water and coal (365). Under no circumstances were they greater than 0.1, indicating that compounds isolated in this study, were contaminated with non-humic matter of a nitrogenous nature, i.e., the material

Table 4.4 Mass of isolated humic material and its carbon and nitrogen content

Date	Site	Mass recovered (mg)	C (%) ⁺	N (%) ⁺	N/C ⁺⁺
28/08/85	A	188	31.7	7.9	0.21
	B	89	21.9	9.4	0.37
	C	54	10.3	11.9	0.99
	Blank	61	9.8	11.9	1.04
25/11/85	A	67	27.2	11.0	0.35
	B	Not available	15.5	17.2	0.95
	C	42	14.5	12.5	0.74
	Blank	29	4.4	17.6	3.43
06/02/86	A	115	32.9 (3.9)	8.6 (5.3)	0.22
	B	62	23.0	11.0	0.41
	C	44	16.5	13.7	0.71
	Blank	26	2.0	19.5	8.36
07/04/86	A	107	28.7	9.2	0.27
	B	55	14.8 (8.3)	12.4 (9.6)	0.72
	C	39	16.6	17.0	0.88
	Blank	42	1.2 (19)	15.6 (5.6)	11.1
29/05/86	A	143	34.1 (3.1)	9.6 (2.6)	0.24
	B	91	26.6 (6.8)	10.7 (5.8)	0.34
	C	36	16.1	15.6	0.83
	Blank	35	2.1	15.8	6.45
12/08/86	A	91	26.6	8.6	0.28
	B	71	19.3	9.6	0.43
	C	53	14.1	12.9	0.78
	Blank	39	3.4	16.3	4.11

+ Mean of two determinations.

++ Atomic molar ratios

Carbon and nitrogen data, in parentheses, indicate the relative standard deviation where more than two determinations were performed.

resulting after the blank analyses.

Therefore, to allow further conclusions to be drawn about the elemental composition of the humic substances, the amount of contamination and its nature was investigated.

4.3.5 Investigating the nature of the blank material

XAD resins, as delivered from the manufacturer, contain residual monomers and impurities associated with these monomers, artefacts of the resin manufacturing process, and chemical preservatives. They must, therefore, be subjected to extensive cleanup procedures prior to use (Section 4.1.2.1). Despite these, however, complete elimination of resin bleed is impossible. Various workers have studied the nature of these contaminants. Among those reported to occur in XAD-2 and XAD-4 are alkylbenzenes, divinylbenzenes, alkylstyrenes, alkyl-naphthalenes, biphenyl, ethyl acetate, benzoic acid, alkylbenzoates, alkanes, and acetophenone (343,366). In XAD-8, impurities which have been identified include methacrylic acid, aliphatic alcohols, benzaldehyde, phthalates, alkanes, alkylstyrenes, alkylbenzenes, divinylbenzene, naphthalene, and dichlorobenzophenone (356). The differing nature and quantities of the artefacts reported by the various investigators, may be due to the different resin cleanup procedures used or the elution solvents employed. Therefore, in a given analysis, identification of the bleed components, for that analysis, is essential.

In this work, the material resulting from the blank analyses, in which acidified doubly distilled water (pH 2) was substituted for the natural water, was a fluffy white solid. It seemed feasible that this contained ammonium chloride from the reaction of the acidified water with ammonium hydroxide. Ammonium carbonate may also have been present. To check if this was so, or whether it was an artefact of the XAD resin, or it originated from impurities in the doubly distilled water or the methanolic ammonia, two further blank experiments were performed:

- (a) Clean XAD-8 resin, which had been rinsed with four bed volumes of doubly distilled water (acidified to pH 2 with hydrochloric acid), was eluted with four bed volumes of methanolic ammonia. This was subsequently concentrated by rotary evaporation, at less than 60°C, and lyophilised.
- (b) Methanolic ammonia (400 ml) was concentrated to a small volume by rotary evaporation, and then lyophilised.

In both cases, fluffy white solids resulted, 51 mg from (a) and 59 mg from (b). Their elemental compositions, compared to the sample extraction blanks, are shown (Table 4.5).

Table 4.5 Carbon and nitrogen content of the sample extraction, XAD-8 elution and methanolic ammonia blanks

Blank	Date	C (%)	N (%)	N/C ⁺
Sample extraction	28/08/85	9.8	11.9	1.04
	25/11/85	4.4	17.6	3.43
	06/02/86	2.0	19.5	8.36
	07/04/86	1.2	15.6	11.1
	29/05/86	3.8	15.8	6.45
	12/08/86	3.4	16.3	4.11
XAD-8 elution	-	0.9	11.1	10.6
Methanolic ammonia	-	4.3	0	0

+ Atomic molar ratios.

The following points can be noted:

- (i) Although the resulting solids were similar in appearance, their elemental compositions show that they are not identical.
- (ii) The presence of a white solid in (b), in the absence of acid, indicates that the XAD-8 elution blank does not solely contain ammonium chloride. A further possibility was the presence of

ammonium carbonate but the absence of nitrogen in the methanolic ammonia blank material does not support this assignment.

In an attempt to aid identification, mass spectrometric analyses were carried out to obtain mass spectra of:

- (a) Ammonium chloride.
- (b) The sample extraction blank from 07/04/86.
- (c) The XAD-8 elution blank.
- (d) The methanolic ammonia blank.

Analysis of the XAD-8 elution blank dissolved in methanol was also performed by gas chromatography-mass spectrometry (GC-MS).

The results (Table 4.6) indicate that the main component (detectable by mass spectrometry) in the sample extraction and XAD-8 elution blanks was ammonium chloride. It is interesting to note, however, that the presence of ammonium chloride has not been mentioned by other investigators (222). The solid resulting from the concentration of methanolic ammonium hydroxide appears to be a carbonate or bicarbonate.

These results tend to indicate that the bulk of the extraneous material was inorganic in nature. Although this could be removed by, for example, ion-exchange chromatography or ultrafiltration, losses of humic substances by adsorption onto the resin/membrane can occur. As inorganic material should not interfere with subsequent analyses, further purification was not performed to prevent loss or alteration of the samples.

In order to assess the contribution of the blank material to the sample, the DOC contents of aqueous sample extracts of known concentrations were estimated by UV absorption measurements.

Table 4.6 Results from the mass spectrometric analyses of the blank materials

Blank	Appearance	Mass spectrum
(a) Ammonium chloride	1 wide peak (ca. 400 scans)	Major ions: NH ₃ - 17,16,15 HCl - 36,38,35,37
(b) Sample extraction	1 narrow peak (100 scans)	Ammonium chloride Major ions: NH ₃ - 17,16,15 HCl - 36,38,35,37
(c) XAD-8 elution	Wider peak than (b) (200 scans), with a shoulder	Ammonium chloride Major ions: NH ₃ - 17,16,15 HCl - 36,38,35,37
(d) Methanolic ammonia	Main peak - 1 narrow peak (150 scans)	Two dominant ions, m/e 44 and 18. Thought to be due to CO ₂ and H ₂ O, probably from a bicarbonate or a hydrated carbonate.
(e) XAD-8 elution - GC-MS	One narrow and one overloaded peak	Ammonium chloride (overloaded), and an unknown compound ⁺ .

+ The unknown compound was identified by computer search as 2,4-pentadienenitrile. It is possible that other components were hidden under the wide ammonium chloride peak.

4.3.6 Estimating the contribution of the blank material to the samples

The UV absorption of natural water samples has long been used as an indicator of organic carbon content/pollution (e.g., (367,368)). A plot of absorbance versus DOC generally reveals a linear graph with the slope depending on the nature of the sample (369). Variable non-zero intercepts on the DOC axis may also result, due to the presence of organic carbon which does not absorb at the wavelength of interest (e.g., (367)).

Several workers have proposed relationships between absorbance and DOC (Table 4.7) although their ability to yield accurate DOC values for varied samples is questionable. In addition, increases in absorbance can also result from interfering species such as iron (104), nitrate, and bromide (105), and so it is advisable to correct for these ions if present in significant concentrations.

A selection of the humic extracts (April, 1986 - sites A to C and blank; February, May and August, 1986 - sites A and B) were dissolved in doubly distilled water (ca. 1 mg/ml) and the UV/visible absorption spectra were recorded between 200 and 800 nm on a Shimadzu double-beam UV-190 spectrophotometer.

The majority of the spectra showed a maximum at about 380 - 390 nm with a shoulder at 350 nm. Exceptions to these were the sample from site C, April, 1986, which had the maximum at 350 nm with the shoulder at 380 nm, and the XAD blank extract, April, 1986, which consisted of one peak with two maxima at 350 and 380 nm, plus a small shoulder at ca. 260 nm. The presence of material in the blank showing a strong UV absorbance is thus obvious in all of these samples as the UV-visible spectra of pure humic substances are generally featureless, with absorptivities decreasing as the wavelength increases (89,90).

Due to the presence of UV-absorption by blank material, estimations of DOC levels using equations given in Table 4.7 are not feasible for wavelengths above 220 nm. However, the blank absorbance at 220 nm was zero and so DOC might have been estimated roughly as $50E_{220}$ mg/l (105). This yielded values of ca. 1-3 mg/l (absorbance

Table 4.7 Relationships between organic carbon and UV absorbance as found by various workers

Water sample	Relationship	Reference
Humic lake Tjeukemeer, The Netherlands. +	DOC = 18.9E ₂₅₀ + 5.7	102
Finnish lakewater	DOC = 24.7E ₂₅₀ + 2.7	370
Seawater organic matter	DOC = 30.5E ₂₅₀	370
New Zealand rivers	DOC = 50E ₂₂₀	105
Lake Hakojärvi, Finland	DOC = 59.6E ₃₆₀ + 1.9	101
- Colloidal humic	OC = 0.141(1000E ₄₂₀ - 0.025c _{Fe}) + 1.851	100
- Dissolved fulvic	With iron correction: OC = 0.909(1000E ₄₂₀ - 0.025c _{Fe}) + 0.0679	100
	Without iron correction: OC = 299E ₄₂₀ + 0.349	100

DOC = DOC concentration (mg/l)

c_{Fe} = Iron concentration (µg/l)

+ = Sampled at different times.

corrected for turbidity measured at 750 nm) compared to the initial sample concentration of ca. 1 mg/ml, suggesting that the organic carbon accounted for only 0.1% of the total extract. Estimation of the expected mass of extracted material from DOC measurements on the water samples (Table 4.1), however, indicated that this percentage was clearly erroneous.

It was therefore decided that the blank content of the samples should be estimated by subtracting the mass of material resulting following the extraction of doubly distilled water on each sampling occasion (25 l) (Table 4.8). The concentration of ammonium chloride resulting should resemble that of the sample extracts. This data is clearly only semi-quantitative but yields a closer estimation of the humic content of these extracts than uncorrected data.

Table 4.8 Estimated percentages of humic substances in sample
extracts corrected for the contribution of the blanks

Date	28/08/85	25/11/85	06/02/86	07/04/86	29/05/86	12/08/86
Site A	67.6	56.7	77.4	60.7	75.5	57.1
Site B	31.5	NA	58.1	23.6	61.5	45.1
Site C	-	31.0	40.9	-	2.78	26.4
Blank	0	0	0	0	0	0

- Less material resulted from sample extraction than blank extraction.

NA Data not available.

CHAPTER FIVE

ANALYSIS OF MONOSACCHARIDES IN ESTUARINE AND MARINE HUMIC MATERIALS

This chapter describes the modification of a method, developed by Cowie and Hedges (371), for the determination of neutral sugars in natural samples by capillary GC. The method was adapted for GC analysis using cold on-column injection, and was then applied to the analysis of monosaccharides in humic materials.

5.1 A REVIEW OF METHODS EMPLOYED IN THE ANALYSIS OF MONOSACCHARIDES

Thin-layer, gas and liquid chromatography have all been employed in the analysis of monosaccharides. More recently, however, gas and liquid chromatography have generally been more popular than thin-layer chromatography, due to the ease of quantitation combined with short analysis times. Supercritical fluid chromatography has also shown potential in the analysis of oligosaccharides and polysaccharides (372), but the formation of non-polar derivatives is still required as the mobile phases in common use are all relatively non-polar. For the analysis of monosaccharides, it currently offers little advantage over capillary GC, and so will not be discussed further.

5.1.1 Thin-layer chromatography

Whilst TLC can be employed quantitatively (373), it is most useful as a qualitative method of analysis. Various compound classes have been separated including monosaccharides, oligosaccharides, amino sugars, aldonic acids, aldaric acids, uronic acids, sugar alcohols, methyl glycosides, sugar phosphates, and also simple sugar derivatives (e.g., ozones, hydrazones and methyl ethers) (374). A number of adsorbents have been employed (374,375), but silica gel, Kieselguhr and cellulose are the most commonly used. Silica gel and Kieselguhr can be impregnated with buffers to enhance resolution (376). Various solvent mixtures have also been suggested for elution (reviewed in (374,375)), including both neutral and acidic/basic systems, such as chloroform:methanol (377), n-butanol:acetic acid:water (378), and n-butanol:pyridine:water (379).

Detection of carbohydrates on the TLC plate can be carried out non-selectively and destructively by charring or spraying with potassium permanganate (378). Alternatively, non-destructive methods include the formation of fluorescent derivatives (380), the use of an adsorbent containing a fluorescent indicator, or the application of various non-destructive spray reagents, such as iodine (375) (prolonged exposure of the sample to iodine, however, may result in its alteration). Specific visualisation techniques (375) range from the more general reducing sugar and glycol reagents through to more specific reagents, which can distinguish between different carbohydrate classes or target individual sugars.

5.1.2 Gas Chromatography

GC analysis of monosaccharides requires the formation of volatile derivatives. Early work involved the formation of fully or partially methylated methyl glycosides (381), their derivatives (382), or derivatives of methylated alditols (382). However, the sugars exist in solution in more than one anomeric form and multiple peaks therefore result from single sugars, which may be difficult to resolve. This also occurs with trimethylsilyl (TMS) ether (383) and trifluoroacetate (TFA) ester (384) derivatives. Alditol acetates (385) and alditol TMS ethers (386) generally yield only one peak for each sugar, thus overcoming this problem, but include a lengthy sample preparation procedure. They are also unable to differentiate between neutral and alcohol sugars, and certain sugar isomers. In addition, ketoses yield more than one alcohol. Other derivatives which have been successfully employed include TMS-oximes, aldonitrile acetates, acetals and ketals, dithioacetals and boronates. These have been reviewed by Blau and King (387).

Despite problems with peak multiplicity, the formation of TMS ethers has remained one of the most popular derivatives for monosaccharide analysis. The chromatographic behaviour of these compounds has recently been studied in detail (388,389). The derivatives are prepared by allowing the sugars to reach mutarotation equilibrium prior to silylation. By determining the equilibrium isomer distribution of individual sugars, their quantitation, in a complex mixture, can be performed by measuring a single, resolved peak

for each sugar. It is not always necessary to resolve a single peak for every sugar because the area of a peak can often be obtained by difference if the contribution of co-eluting peaks is known (390).

Equilibration has usually been performed in aqueous solution, pyridine or N, N-dimethylformamide (383,391,392). Aqueous solutions have the disadvantages of requiring the removal of water prior to silylation. However, equilibration in pyridine is slow at low temperatures and, at higher temperatures, a degree of epimerisation occurs (393). Therefore, in order to use pyridine at low temperatures, catalysts have been employed to accelerate this process. Bethge *et al.* used lithium perchlorate in pyridine (0.2% (w/v) in pyridine, at 40°C, for two hours) (393). The pyridine was found to tail severely on GC analysis and the TMS ethers were therefore extracted into hexane prior to analysis. Cowie and Hedges (371), however, successfully analysed sugar derivatives in pyridine containing 0.2% (w/v) lithium perchlorate. Equilibrium has also been achieved using the bifunctional catalyst, 2-hydroxypyridine (392). The sugar TMS ethers were analysed without any extraction procedure, and the samples could be injected directly into the GC as the sugar TMS ether of 2-hydroxypyridine is volatile and does not interfere with the chromatography.

A further method of GC analysis is pyrolysis GC, which has the advantage that it does not require the formation of carbohydrate derivatives, but the disadvantage of yielding complex chromatograms. Recent studies by Budgell *et al.* (190,191) have indicated the potential of this technique for the analysis of carbohydrates. They identified pentoses and hexoses by the retention times of their distinctive anhydrosugar products (with mass spectral confirmation), and then extended their study to identify the composition of several oligo- and polysaccharides. Other pyrolysis GC investigations have included the analysis of polysaccharides in soil (197), and red algae (394).

5.1.3 Liquid chromatography

The advantage of liquid chromatography over gas chromatography is that mono- and oligosaccharides can be determined without prior derivatisation. Three basic liquid chromatographic procedures have been applied to the analysis of carbohydrates: partition chromatography on ion-exchange resins, ion-exchange chromatography of sugar-borate complexes, and high performance liquid chromatography (HPLC).

Partition chromatography has been performed on both anion (chloride and sulphate forms) and cation (potassium, sodium and lithium forms) exchange resins (395). The carbohydrates are generally eluted with an ethanol-water mobile phase (396), and detected colorimetrically with, for example, orcinol-sulphuric acid (395), tetrazolium blue (397), or copper-biconchinate (398).

Chromatography of the borate complexes has an advantage over the partition method in that sample clean-up and desalting steps are not often required. Separations are performed on strong anion-exchange resins in the borate form, with borate-buffer eluents (399). Both colorimetric (399) and fluorimetric (400) detection methods have been employed.

HPLC of underivatised mono- and oligosaccharides has been carried out on several types of column, including both strong and weak cation-exchange, and reversed-phase (octadecylsilane-bonded (ODS) silica) columns (401). The amino-bonded and ODS silica columns have been the most commonly used. The amino phase columns generally employ acetonitrile-water eluents whereas ODS silica columns have the advantage of achieving good separations using only water (402).

Detection of the carbohydrates is normally performed using a refractive index detector but this has limited sensitivity and cannot be employed with gradient elution. UV detection at low wavelengths is more sensitive but more susceptible to interferences (401). In addition, both pre-column and post-column derivatisation methods have also recently been employed (403), which improve sensitivity and selectivity.

5.2 A METHOD FOR THE DETERMINATION OF MONOSACCHARIDES IN ENVIRONMENTAL SAMPLES

A method was required for determining the monosaccharide content of aquatic humic materials. Various procedures have previously been adopted for both the qualitative and quantitative analysis of carbohydrates in humic samples. These have included colorimetric (for total carbohydrate content) (293), nuclear magnetic resonance (79), and pyrolysis (196) studies. However, the more quantitative work has generally employed hydrolysis, often followed by desalting, and then chromatographic analysis, usually by GC (293,297). TLC and liquid chromatography have also been used, for example, in the analysis of sediments (294,299).

Mopper (299) evaluated the various steps in the extraction techniques for sugar analysis, i.e. hydrolysis, deionisation, volume reduction. Hydrolysis of sediments with 72% (w/w) sulphuric acid, for two hours, prior to dilution and further hydrolysis, was found to give maximum yields. In addition, neutralisation and desalting could be performed in a single step by ion-exchange chromatography, and volume reduction should be carried out by rotary evaporation, with the addition of glycerol to prevent losses due to sugar condensation reactions. This method was modified by Cowie and Hedges (371), who found that neutralisation was required prior to ion-exchange chromatography due to the larger volume of acid employed. They also stated that rotary evaporation could be performed without the addition of glycerol.

It was decided that the method of Cowie and Hedges (371) should be adopted in the present study. In this method, the free sugars are equilibrated in pyridine containing lithium perchlorate catalyst, derivatised, and analysed as their sugar TMS ethers by GC. In preliminary experiments, lithium perchlorate was found to interfere with the on-column injection GC analysis employed in the present work and an alternative catalyst, 2-hydroxypyridine, which had previously been proven for the analysis of monosaccharides by packed column GC (392), was therefore adopted.

5.2.1 Standards

The following standard sugar solutions were prepared in pyridine:

- a mixed sugar standard (100 ng/ μ l) containing
 - adonitol (50 ng/ μ l) lyxose
 - arabinose mannose
 - fructose rhamnose
 - fucose ribose
 - galactose sorbitol (50 ng/ μ l)
 - glucose xylose

Successive dilutions of this mixture were performed to obtain 12.5, 25 and 50 ng/ μ l solutions.

- individual solutions of all the above sugars (200 ng/ μ l).
- a solution of internal standards, containing adonitol and sorbitol (100ng/ μ l); these sugar alcohols are not naturally occurring and yield single peaks on chromatography on their TMS ethers.

The TMS ethers of these sugars were formed after equilibration and derivatisation as detailed in Section 5.2.2.3. The following standard solutions resulted:

- mixed sugar TMS ether standard; 5, 10, 20 and 40 ng/ μ l.
- individual sugar TMS ether standards; 10 ng/ μ l.
- internal standard, containing the TMS ethers of adonitol and sorbitol; 5 ng/ μ l.

All standard solutions were stored frozen and new sugar TMS ether standards were prepared monthly.

5.2.2 Method

5.2.2.1 Hydrolysis

Solid samples were accurately weighed and transferred to 50 ml Pyrex boiling tubes with ground glass joints, containing 72 wt % sulphuric acid (2 ml). Samples in solution were added directly to the sulphuric acid. The mixture was stirred for two hours at room

temperature, diluted to 1.2 moles/l with doubly distilled water (20.5 ml) and refluxed for a further three hours. The hydrolysis was then halted by placing the tubes in an ice bath. At this stage, the internal standard, containing adonitol and sorbitol (50 μ l), was added, the solution mixed, and transferred to a glass beaker (50 ml). Finely ground barium hydroxide (ca. 8 g) was used to neutralise the hydrolysates (pH 6.5 (\pm 0.2)) - precipitation of barium sulphate being aided by stirring and ultrasonic vibration. The mixture was then centrifuged in a polyethylene centrifuge tube for ca. 15 minutes at 2500 rpm (700 g) and the supernatant was decanted off.

5.2.2.2 Deionisation

The resulting solution was deionised using a combined cation and anion exchange resin consisting of a mixture of Dowex 50W-X8(H) cation exchange resin (hydrogen ion form, 20-50 dry mesh) and Dowex 1-X8 anion exchange resin (formate form, 100-200 dry mesh). The sample was passed through the ion exchange column (volume of resin = 15 ml) at less than 2 ml/min. The column was rinsed with at least 15 ml of doubly distilled water, and the combined eluents were transferred into a Kuderna Danish flask (500 ml), fitted with a boiling tube (5 ml). The solution was evaporated to dryness in a water bath, at less than 60°C, using a rotary evaporator.

5.2.2.3 Equilibration and Derivatisation

The sample was dissolved in pyridine (0.4 ml) and transferred to a round-bottom reaction vial (3 ml). An equal volume of 2-hydroxypyridine (0.2 moles/l) was added and the vial was then sealed with an open-top screw cap, containing a Teflon-faced silicone disc. The sample was left to equilibrate in an aluminium heating block, at 45°C, for 12 hours. Sugar TMS derivatives were formed by adding bis(trimethyl-silyl)trifluoroacetamide (BSTFA) containing 1% (v/v) trimethylchloro-silane (TMCS) (0.2 ml) to the solution. The mixture was then heated for a further ten minutes, at 45°C, to ensure complete derivatisation.

5.2.2.4 Gas Chromatography

Capillary gas chromatography (GC) was performed on all of the samples, using a Pye Series 104 gas chromatograph, fitted with a flame ionisation detector (FID).

The following operating conditions were used:

GC column	:	10 m, 0.3 mm internal diameter, immobilised methylsilicone (1.0 μm film thickness) wall-coated open tubular (WCOT), fused silica capillary column
Retention gap	:	1 m deactivated fused silica capillary
Carrier gas	:	Oxygen-free nitrogen (1.5 - 2.0 ml/min)
Injection system	:	Cold on-column injection with nitrogen purge (30 ml/min)
Temperature programme	:	130°C (1 min) \rightarrow 4°C/min \rightarrow 250°C (10 mins)
Amplifier attenuation	:	10 or 20
FID	:	Hydrogen : ca. 25 ml/min Air : ca. 200 ml/min

5.2.3 Results and discussion

5.2.3.1 Gas chromatography of the sugar TMS ether derivatives

5.2.3.1.1 Peak identification

Peak assignments and the GC trace of the mixed sugar TMS ether standard are shown in Figure 5.1. Complete resolution of an anomer was not always possible and, in some cases, quantitation was carried out by subtracting the contribution of the co-chromatographing sugar. The peaks which were routinely used for quantitation are denoted by an asterisk.

5.2.3.1.2 Chromatographic repeatability

Chromatographic repeatability was assessed by measuring peak heights and relative peak heights (versus two internal standards, adonitol and sorbitol) obtained in one day from repeated

Figure 5.1(a) GC trace of the mixed sugar TMS ether standard

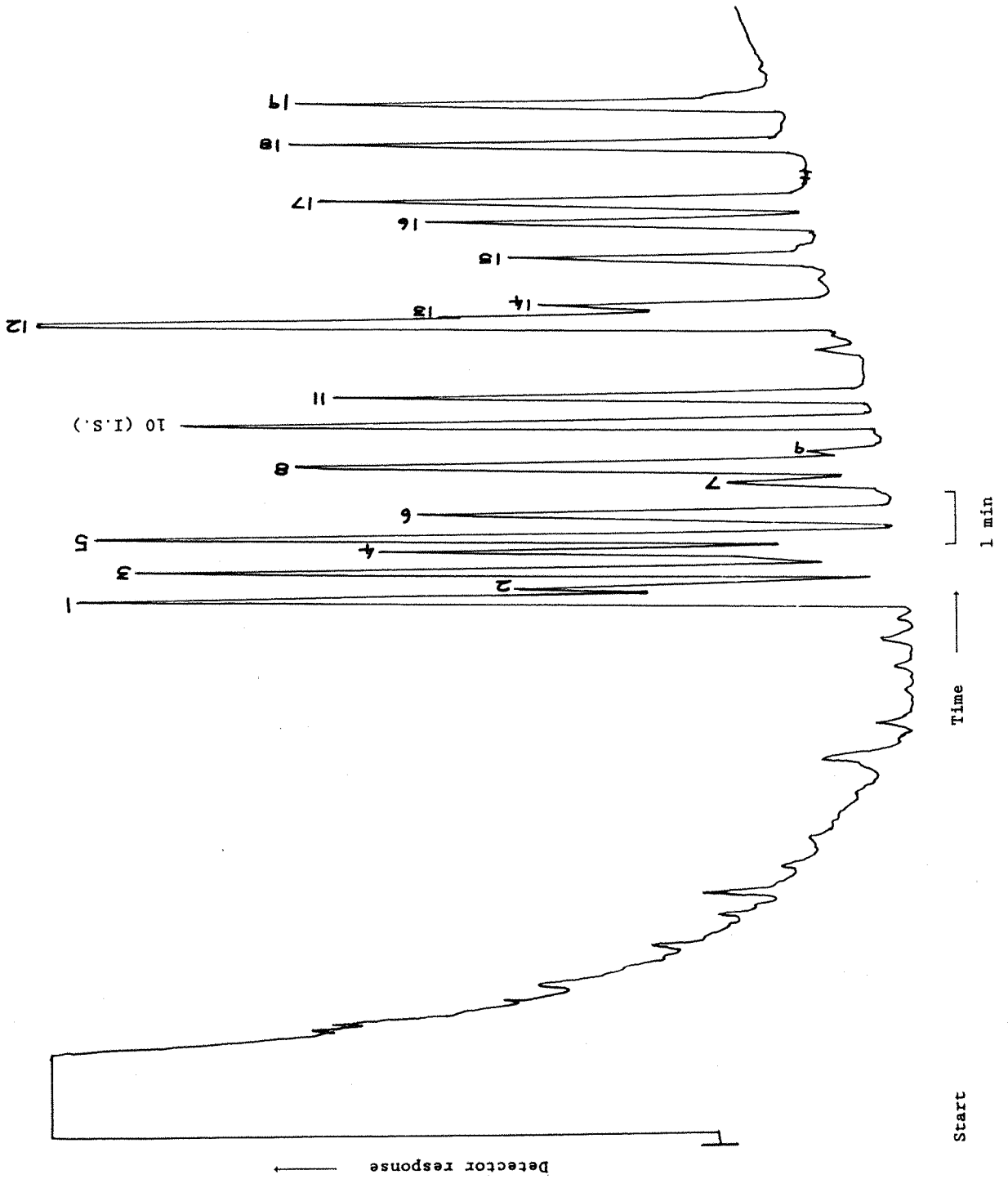


Figure 5.1(b) Peak assignments from the GC trace of the mixed sugar TMS ether standard

<u>Peak label</u>	<u>Sugar</u>	<u>Peak number</u>	<u>Peak routinely used for quantitation</u>
1	Lyxose	L ₁	*
2	Arabinose (shoulder)	A ₁	
	Arabinose	A ₂	*
3	Rhamnose	Rh ₁ , (X ₁) ⁺	*
4	Fucose, lyxose, ribose	F ₁ , L ₂ , Ri ₁ , (X ₂) ⁺	
	Arabinose, ribose	A ₃ , Ri ₂	
5	Lyxose, ribose	L ₃ , Ri ₃	* (-L ₃)
6	Arabinose, fucose, ribose	A ₄ , F ₂ , Ri ₄	
7	Rhamnose	Rh ₂	
8	Fucose, xylose	F ₃ , X ₃	* (-X ₃)
9	Fucose, rhamnose	F ₄ , Rh ₃	
10	Adonitol	Ad	*
11	Xylose	X ₄	*
12	Fructose, mannose	Fr ₁ , M ₁	* ⁺⁺
13	Fructose	Fr ₂	
14	Fructose, galactose	Fr ₃ , G ₁	
15	Galactose	G ₂	*
16	Glucose	Gl ₁	*
17	Galactose, mannose	G ₃ , M ₂	
18	Sorbitol	S	
19	Glucose	Gl ₂	

+ The contribution of X₁ and X₂ to peaks 3 and 4 was minimal and was ignored unless the concentration of xylose in a sample greatly exceeded that of the other sugars.

++ If fructose was present in the sample, M₂ (-G₃) was used for quantitation due to the variable anomeric distribution of fructose (Section 5.2.3.1.3).

chromatography of a mixed sugar TMS standard (5 ng/ μ l; injection volume 2 μ l). Two main points can be noted from the resulting data (Table 5.1):

- (i) Peak heights measured relative to the internal standards show better repeatability than absolute peak heights due to the elimination of variations in injection volume.
- (ii) There is little difference in repeatabilities measured relative to adonitol and sorbitol. Due to its central position in the GC run, adonitol was therefore chosen as the internal standard for quantitation.

In order to obtain information on the long-term repeatability (i.e. the reproducibility) of the GC data, peak heights relative to adonitol were noted for several GC determinations run over a period of five months. The results (Table 5.1) show a marked increase (especially towards the end of the trace) in the %RSD over the one-day repeatability. This was thought to be due to variations in the performance of the GC column, leading to different peak widths and degrees of resolution. Therefore, for quantitative determinations, experimental data were always compared to gas chromatograms of the mixed sugar TMS ether standard recorded on the same day.

Multiple GC injections of solutions containing the individual sugar TMS ether standards (10 ng/ μ l) and the adonitol TMS ether internal standard (5 ng/ μ l) were also performed, and peak heights and retention times relative to adonitol were determined (Table 5.2). These results highlighted the irreproducibility of the fructose data due to the variable resolution of the three anomer peaks. Quantitative analysis of fructose based on a single anomer peak was therefore not attempted. The excellent repeatability of the retention time data relative to adonitol can also be noted.

Table 5.1 Repeatability data for multiple GC determinations of the mixed sugar TMS ether standard

Peak number	n	SAME DAY REPEATABILITY						LONG-TERM REPEATABILITY	
		Peak height						Peak Height	
		Absolute (cm)		Relative to adonitol		Relative to sorbitol		Relative to adonitol	
		Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
1	10	15.6	5.6	1.24	2.0	1.54	3.1	1.21	5.5
2	11	8.2	7.4	0.65	6.2	0.79	6.1	0.62	18
3	11	13.8	8.7	1.09	5.0	1.33	4.0	1.12	5.7
4	11	9.6	7.0	0.75	3.5	0.92	2.5	0.78	5.6
5	11	15.0	5.5	1.18	3.5	1.45	3.2	1.19	6.2
6	11	8.8	7.4	0.69	2.8	0.85	3.2	0.72	6.1
7	11	2.9	5.2	0.23	5.7	0.28	7.1	0.23	3.3
8	11	13.1	4.2	1.03	3.5	1.26	5.3	0.92	13
9	10	1.7	6.5	0.14	9.3	0.17	8.8	0.15*	33
10	11	12.7	5.8	1.00	0	1.23	3.7	1.00	0
11	11	10.0	6.1	0.79	2.7	0.97	3.5	0.77	7.1
12	4	15.9	3.3	1.31	5.8	1.63	3.4	1.28	3.4
13	10	8.6	8.4	0.68	4.3	0.83	3.6	0.62 ⁺	6.1
14	11	6.9	11	0.54	8.5	0.67	6.4	0.42	29
15	11	6.2	6.3	0.49	4.3	0.60	3.2	0.44	8.4
16	11	8.4	6.4	0.66	2.1	0.81	2.8	0.62	16
17	11	11.5	5.8	0.90	3.4	1.11	3.2	0.76	17
18	11	10.4	7.7	0.82	3.9	1.00	0	0.72	15
19	11	11.4	6.3	0.90	5.1	1.10	6.2	0.74	26

* n=11 in this case

+ n=7 in this case

Table 5.2 Peak height and retention time data for GC determinations of the individual sugar TMS ether standards

Sugar	Peak number	Peak height relative to adonitol +			Retention time relative to adonitol			
		n	Mean	%RSD	n	Mean	%RSD	
Lyxose	L ₁ [*]	4	1.32	2.9	3	0.75	0.77	
	L ₂		0.051	1.9		0.81	0.72	
	L ₃		0.29	4.0		0.83	0	
Arabinose	A ₁	6	0.37	8.7	2	0.77	0.92	
	A ₂ [*]		0.60	8.6		3	0.77	0
	A ₃		0.66	6.5			0.82	0.71
	A ₄		0.17	9.4			0.87	0.67
Rhamnose	Rh ₁ [*]	3	1.10	5.1	3	0.79	0.73	
	Rh ₂		0.23	2.5			0.92	0
	Rh ₃		0.015	51			0.96	0.60
Ribose	Ri ₁	4	0.094	12	2	0.81	0.88	
	Ri ₂		0.25	2.3		3	0.82	0
	Ri ₃ [*]		0.97	4.2			0.83	0.70
	Ri ₄		0.30	5.0			0.87	0.67
Fucose	F ₁	3	0.18	6.4	2	0.81	0	
	F ₂		0.54	1.1		3	0.87	0.67
	F ₃ [*]		0.73	4.0			0.94	0
	F ₄		0.12	9.6			0.96	0.60
Xylose	X ₁	3	0.026	36	1	0.76	-	
	X ₂		0.022	7.0		1	0.79	-
	X ₃		0.65	5.3		3	0.94	0.62
	X ₄ [*]		0.75	4.6			1.04	0
Mannose	M ₁ [*]	4	1.30	5.1	3	1.13	0.51	
	M ₂		0.24	3.4			1.31	0.92
Fructose	Fr ₁	3	0.26	6.7	3	1.14	0.51	
	Fr ₂		0.51	14			1.15	0.50
	Fr ₃		0.16	63			1.16	0

Table 5.2 (continued)

Sugar	Peak number	Peak height relative to adonitol ⁺			Retention time relative to adonitol		
		n	Mean	%RSD	n	Mean	%RSD
Galactose	G ₁	3	0.25	4.0	3	1.16	0
	G ₂ [*]		0.49	4.7		1.23	0
	G ₃		0.77	8.5		1.32	0.44
Glucose	G ₁ [*]	4	0.67	4.5	3	1.28	0.45
	G ₂		0.90	7.1		1.44	0.40
Sorbitol	S	5	0.83 ⁺⁺	4.5	7	1.39	0.94

* Peak routinely used for quantitation

+ 10 ng sugar corresponding to 5 ng adonitol

++ 5 ng sugar corresponding to 5 ng adonitol in this case

5.2.3.2 Equilibration and Derivatisation

As the equilibration of free sugars in pyridine, using 2-hydroxypyridine, had not been employed previously with capillary GC, conditions for its use in this project were investigated.

5.2.3.2.1 Equilibration time

Conditions for mutarotation equilibrium of free sugars, using pyridine or N,N-dimethylformamide in the presence of 2-hydroxypyridine, have been studied by Reid *et al.* (392). Using pyridine as the solvent, they selected the following conditions:

Concentration of 2-hydroxypyridine : 0.1 moles/l
 Incubation temperature : 40°C
 Equilibration time : 6-24 hours

Equilibration, in the presence of 2-hydroxypyridine (0.1 moles/l) in pyridine, at 45°C, was chosen in the current study. The mixed

sugar standard was incubated at 45°C for varying periods of time, after which the sugar TMS ethers were formed.

The results (Table 5.3) show no obvious difference between equilibrating for 2 hours or 24 hours. Therefore, for convenience, an equilibration time of 12 hours (i.e., overnight) was chosen in the present work.

5.2.3.2.2 Equilibrium isomer distributions

The percentage composition of sugars under different equilibrium conditions has been investigated (392). Equilibrium isomer distributions have been shown to be dependent on the composition of the solvent used, and they are also temperature dependent (371). Therefore, in order to obtain accurate results, mutarotation equilibrium must be carried out under carefully controlled conditions. The standard conditions chosen for this study were:

Solvent	:	Pyridine (AnalaR)
Concentration of 2-hydroxypyridine	:	0.1 moles/l
Incubation temperature	:	45°C
Equilibration time	:	12 hours

Equilibrium isomer distributions under these conditions are shown in Table 5.4.

5.2.3.2.3 Quantity of silylating agent

Several silylating agents are available and differing quantities have been used in the formation of sugar TMS derivatives. Sweeley *et al.* (404) investigated the optimum proportions of hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) necessary for maximum yield of TMS derivatives, and adopted the following standard conditions: HMDS (0.2 ml) and TMCS (0.1 ml) in pyridine (1 ml) (i.e. 23% silylating agents). Reid *et al.* (392) employed the same ratio of HMDS to TMCS but used a larger percentage of total silylating agents (60%). Cowie and Hedges (371) formed sugar TMS derivatives by adding [bis-(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% TMCS] (0.1 - 0.25 ml) to pyridine (1 ml) containing 0.2% w/v lithium perchlorate

Table 5.3 The effect of varying the equilibration time on peak height

The values in the following table correspond to peak height relative to adonitol.

Peak label	Equilibration time				
	2 hours	4 hours	6 hours	12 hours	24 hours
1	1.14,1.19	1.22,1.28	1.27, -	1.16,1.18,1.33	1.23,1.15,1.18
2	0.62,0.61	0.67,0.68	0.67,0.67	0.63,0.65,0.66	0.63,0.62,0.65
3	1.09,1.11	1.13,1.14	1.14,1.13	1.09,1.05,1.17	1.12,1.10,1.11
4	0.75,0.76	0.77,0.82	0.76,0.75	0.73,0.77,0.80	0.77,0.76,0.73
5	1.19,1.19	1.22,1.25	1.21, -	1.10,1.12,1.24	1.17,1.21,1.13
6	0.70,0.74	0.73,0.75	0.71,0.67	0.66,0.70,0.75	0.69,0.67,0.69
7	0.22,0.23	0.23,0.24	0.23,0.22	0.22,0.23,0.24	0.23,0.22,0.24
8	1.04,1.02	1.08,1.07	1.05,1.07	0.98,1.06,1.11	1.10,0.98,1.01
9	0.12,0.12	0.12,0.12	0.12,0.12	0.12,0.14,0.14	0.12,0.12,0.14
10	1.00,1.00	1.00,1.00	1.00,1.00	1.00,1.00,1.00	1.00,1.00,1.00
11	0.79,0.78	0.81,0.82	0.83,0.80	0.77,0.80,0.81	0.78,0.84,0.79
12	- -	- -	- -	- - -	- - -
13	ND 0.58	ND ND	ND 0.55	0.57,0.64,0.69	ND 0.67,0.64
14	0.29,0.30	0.31,0.31	0.28,0.30	0.30,0.30,0.39	0.28,0.32,0.31
15	0.48,0.44	0.50,0.50	0.50,0.50	0.44,0.49,0.50	0.49,0.50,0.48
16	0.66,0.65	0.68,0.66	0.70,0.72	0.67,0.70,0.72	0.69,0.68,0.67
17	0.91,0.90	0.92,0.94	0.89,0.93	0.89,0.92,0.91	0.93,0.89,0.95
18	0.63,0.80	0.67,0.78	0.67,0.80	0.78,0.84,0.85	0.73,0.79,0.78
19	0.78,0.85	0.78,0.86	0.86,0.91	0.89,0.99,0.94	0.85,0.86,0.95

- These peaks were off-scale in the GC traces.

ND Not determined - these peaks could not be resolved from peak 12.

Table 5.4 Equilibrium isomer distributions after 12 hours equilibration

Sugar	Peak number	n	% Total	
			Mean	% RSD
Arabinose	A ₁	6	20.5	3.29
	A ₂ [*]		33.3	3.66
	A ₃		36.7	4.90
	A ₄		9.63	7.19
Fructose	Fr ₁	3	28.2	22.3
	Fr ₂		55.1	5.61
	Fr ₃		16.7	49.0
Fucose	F ₁	5	11.0	5.74
	F ₂		34.7	2.74
	F ₃ [*]		46.5	2.34
	F ₄		7.75	8.19
Galactose	G ₁	6	16.2	2.55
	G ₂ [*]		32.5	1.81
	G ₃		51.3	1.51
Glucose	Gl ₁ [*]	6	43.1	3.50
	Gl ₂		56.9	2.65
Lyxose	L ₁ [*]	4	79.5	0.608
	L ₂		3.06	2.12
	L ₃		17.5	3.01
Mannose	M ₁ [*]	4	84.3	0.534
	M ₂		15.7	2.87
Rhamnose	Rh ₁ [*]	4	81.6	1.12
	Rh ₂		17.0	2.32
	Rh ₃		1.39	47.1
Ribose	Ri ₁	6	6.07	11.1
	Ri ₂		15.2	2.78
	Ri ₃ [*]		59.9	1.08
	Ri ₄		18.9	1.01

Table 5.4 (continued)

Sugar	Peak number	n	% Total	
			Mean	% RSD
Xylose	X ₁	5	1.75	27.4
	X ₂		1.47	7.82
	X ₃		45.5	3.05
	X ₄ *		51.3	1.97

* Peak routinely used for quantitation

(i.e., 9 - 20% silylating agent).

As the method employed in the present study is based on that of Cowie and Hedges (371), BSTFA + 1% TMCS was chosen as the silylating agent. An investigation into the effect of varying the percentage of the reagent was carried out by preparing the following solutions:

Percentage silylating agent	Silylating agent	Volume 2-hydroxypyridine (0.1 moles/l)	Mixed sugar standard
10%	0.1 ml	0.85 ml	50 μ l
20%	0.2 ml	0.75 ml	50 μ l
50%	0.5 ml	0.45 ml	50 μ l

The results (Table 5.5) indicate no significant difference on changing the concentration of silylating agent. A 20% solution was adopted.

Table 5.5 The effect of varying the quantity of silylating agent

Peak label	Absolute peak height (cm) ⁺		
	10% silylating agent	20% silylating agent	50% silylating agent
1	12.1, 12.0	11.3, 12.0	11.1, 12.1
2	5.9, 5.6	5.7, 5.6	5.5, 6.2
3	10.4, 10.9	10.5, 11.2	10.4, 11.1
4	8.1, 7.7	7.1, 8.0	7.1, 8.0
5	12.1, 12.1	10.8, 11.4	10.8, 12.2
6	7.3, 7.2	6.7, 6.8	6.2, 7.0
7	2.6, 3.0	2.1, 2.1	2.0, 2.4
8	9.7, 9.4	8.8, 8.7	8.4, 9.1
9	1.3, 1.4	1.4, 1.2	1.1, 1.1
10	10.1, 10.5	9.2, 9.9	9.3, 10.2
11	8.9, 8.4	7.6, 8.0	7.3, 7.9
12	14.7, -	13.2, 14.3	13.1, 13.9
13	6.9, 6.9	6.9, 6.6	5.6, 6.4
14	3.0, 3.3	4.8, 3.1	3.0, 3.0
15	5.4, 5.6	4.8, 4.7	4.4, 5.0
16	7.3, 7.2	6.5, 6.6	6.2, 7.2
17	9.0, 9.6	7.9, 8.5	8.0, 8.4
18	9.1, 9.2	8.4, 8.0	8.2, 8.4
19	9.8, 10.0	9.7, 8.9	8.1, 8.7

+ Absolute peak heights were measured as changes would be expected to affect all sugars in a similar manner. They may not be observed if relative peak heights were monitored.

5.2.3.3 Preliminary experiment on the determination of glucose in cellulose and starch

Having established conditions for the use of 2-hydroxypyridine as an equilibration catalyst, preliminary assessment of the complete experimental procedure was carried out using two simple polysaccharides - cellulose and starch. The method (Section 5.2.2) was slightly modified in that cellulose and starch standards were prepared in 72 wt % sulphuric acid (due to their low solubility in water) and stirred for two hours at room temperature. An aliquot of each solution (0.5 ml, equivalent to 10 μg cellulose and 11.8 μg starch) was then diluted to 1.2 moles/l sulphuric acid and refluxed for three hours, as in the original procedure.

The results (Table 5.6) show that:-

- (i) absolute recoveries of glucose, based on peak height measurements, were low, especially for cellulose.
- (ii) the blank 'glucose' peaks accounted for a significant proportion of the sample peaks.
- (iii) recoveries of glucose, based on peak height ratios to adonitol, were ludicrously high. This was thought to be due to variations in the large blank peaks, combined with low experimental yields.

Therefore, in order to improve the procedure, investigations were carried out to attempt to:

- minimise experimental losses.
- minimise the procedural blank.

Table 5.6 Recovery of glucose from cellulose and starch

Sample	% recoveries based on absolute peak heights			% recoveries based on peak heights relative to adonitol
	Glucose + Not Blank Corrected	Glucose + Blank Corrected	Adonitol	Glucose + Blank Corrected
Cellulose	21	4.8	6.3	240
	28	12	7.8	270
	19	3.0	6.0	230
Starch	38	24	20	110
	31	17	19	85

+ Quantitation based on the height of Gl₁

These data were based on several GC traces from single analyses of both cellulose and starch.

5.2.3.4 An investigation into the reduction of sample losses and procedural blanks

5.2.3.4.1 Location of losses

Location of losses was performed by starting the experiment at various stages in the procedure (Section 5.2.2):

- (a) The mixed sugar standard (100 µl) was spiked into 1.2 moles/l sulphuric acid (22.5 ml), and then neutralised with barium hydroxide, etc.
- (b) The mixed sugar standard (100 µl) was added to doubly distilled water (ca. 35 ml) and passed through the ion-exchange resin, etc.
- (c) The ion-exchange resin in (b) was rinsed with a further 30 ml doubly distilled water, which was evaporated to dryness, etc.
- (d) The mixed sugar standard (100 µl) was equilibrated in 2-hydroxypyridine (0.1 moles/l) and derivatised. This was taken

to be a 100% recovery.

The results (Table 5.7) show that:

- (i) Approximately 20% of the standard was lost during the neutralisation and centrifugation stages.
- (ii) Approximately 25% of the standard was lost on passing through the ion-exchange column and evaporating to dryness.
- (iii) A possible 10% improvement in recovery may result from increasing the volume of doubly distilled water used in rinsing the ion-exchange column. Therefore, the resin wash was increased to 60 ml in future work.
- (iv) There appears to have been a preferential loss of sorbitol at all stages in the procedure. Cowie and Hedges (371) successfully used sorbitol as a second internal standard. Due to the lower concentrations employed in the present study, however, it is possible that selective physical sample losses may have occurred. Therefore, sorbitol was not used as a second internal standard in this work.

Attempts to reduce the losses at the different stages were therefore investigated. As the loss of adonitol appeared to follow the losses of the other sugars, percentage recoveries in some cases, were monitored using adonitol only, in order to facilitate the calculations.

5.2.3.4.2 Improvement of rotary evaporation stage

Addition of glycerol

There have been conflicting reports on the reduction of sugar losses during the evaporation stage. Mopper reported that the addition of glycerol (50 μ l) at this stage was necessary because it reduced losses by inhibiting wall-induced sugar condensation reactions and aided redissolution of the sample prior to analysis (299,405). Cowie and Hedges, however, found glycerol to be unnecessary in neutral solutions, and suggested that it was previously found to be important because hydrolysates were not completely neutralised by the ion-exchange technique (371).

Table 5.7 Recovery data for the location of losses

Peak label	Experiment				
	(a)		(b)		(c) ⁺
	% recovery	Mean	% recovery	Mean	% recovery
1	55, 52	54	72, 85	79	9.7
2	53, 51	52	70, 78	74	12
3	58, 58	58	80, 82	81	9.3
4	56, 54	55	72, 84	78	9.3
5	49, 47	48	69, 81	75	8.8
6	67, 65	66	84, 93	89	18
7	59, 59	59	78, 84	81	13
8	59, 54	57	71, 80	76	9.7
9	71, 65	68	79, 76	78	15
10	58, 54	56	71, 80	76	8.5
11	48, 45	47	64, 72	68	8.5
12 ⁺⁺	- -	-	- -	-	-
13	44, 43	44	51, 83	67	12
14	49, 54	52	62, 82	72	10
15	52, 49	51	64, 80	67	8.9
16	58, 50	54	69, 82	76	9.5
17	57, 48	53	68, 78	73	9.2
18	33, 29	31	51, 59	55	6.6
19	61, 55	58	69, 82	76	9.1

+ Not combined with resin wash from (b).

++ This peak was off-scale in all of the GC traces.

The effect of glycerol on losses during evaporation was investigated in the present study. Doubly distilled water (50 ml) was spiked with:

- adonitol (50 μ l) and 50% glycerol in ethanol (100 μ l).
- adonitol (50 μ l) only.

The solutions were evaporated to dryness, and subjected to equilibration and derivatisation.

It was found that glycerol interfered with the GC analysis. This may have been due to there being insufficient silylating agent present to silylate all of the glycerol and ethanol. As increasing the quantity of silylating agent would necessitate a decrease in the volume of pyridine used for redissolution of the sample, addition of glycerol was not carried out in the present study.

Silylation of glassware

The effect of silylating the glassware on losses during rotary evaporation was studied. Doubly distilled water was spiked with adonitol (50 μ l) in both a silylated (silylated with 10% trimethylchlorosilane in cyclohexane) and a non-silylated Kuderna Danish flask fitted with a boiling tube. The solutions were taken to dryness, and subjected to equilibration and derivatisation.

Recoveries were found to be lower using the silylated glassware (45% and 21%, compared to 61%, 61% and 69% using non-silylated glassware), and therefore, silylation of glassware was not performed.

5.2.3.4.3 Removal of deionisation step

The effect of removing the deionisation step was investigated on the reagent blank analysis. Experiments were carried out:

- (i) including the deionisation step. A reagent blank was processed using the complete experimental procedure (Section 5.2.2).
- (ii) excluding the deionisation step. A reagent blank was hydrolysed (Section 5.2.2.1) and, after centrifuging, was immediately

transferred to a Kuderna Danish flask for subsequent evaporation. Equilibration and derivatisation were then performed (Section 5.2.2.3).

The resulting chromatograms (Figures 5.2(a) and (b)) show a reduction in the reagent blank on removal of the deionisation step. However, loss of adonitol appeared to be greater in this instance, possibly suggesting that sugar losses may occur if this step was omitted.

The general increase of contamination with time was also noted in these experiments. This was thought to result from the presence of slowly silylating impurities. Therefore, to minimise this, gas chromatograms were recorded as soon as possible after the derivatisation.

To see if the removal of the deionisation step was feasible in the study of an environmental sample, humic material (site A, May, 1986; 6 mg) was analysed as above ((i) and (ii)). Removal of the deionisation step was found to interfere with the analysis, resulting in a wide, tailing solvent peak and loss of sugars.

Therefore, it was concluded that inclusion of the deionisation stage is necessary in the analysis of humic samples.

5.2.3.4.4 Reducing the quantities of reagents

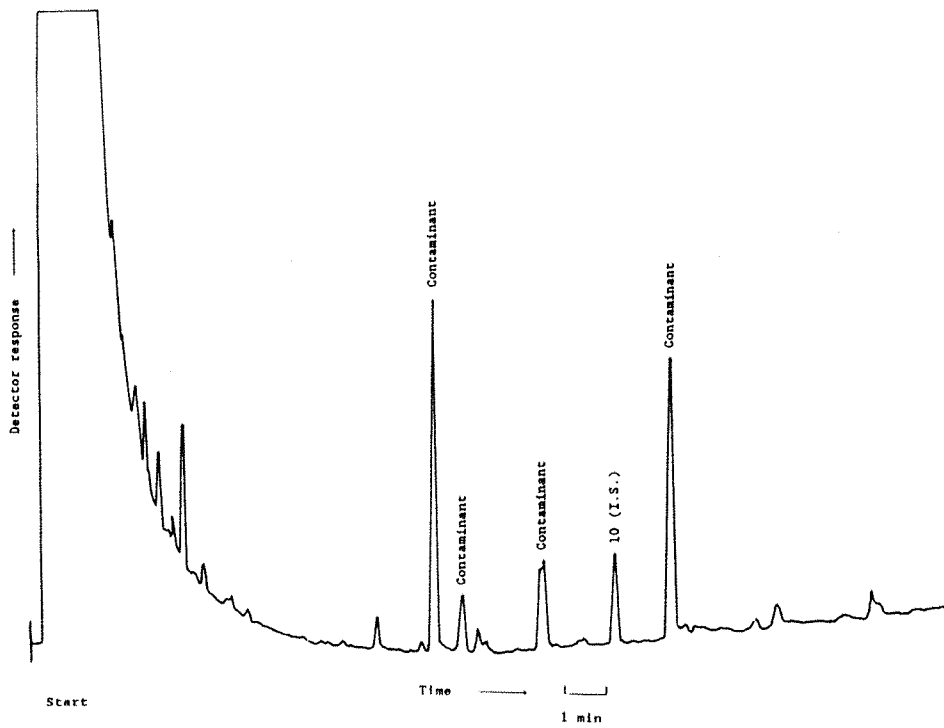
In an attempt to reduce the procedural blank, the experiment was scaled-down by halving the quantities of all the reagents employed. The mixed sugar standard (100 μ l) was analysed in duplicate.

A comparison of the results (Table 5.8) with experiment (a), Section 5.2.3.4.1 (Table 5.7), indicates a possible small decrease in the percentage recoveries on halving the reagent quantities although it is probable that this difference was purely due to experimental variation.

As in Section 5.2.3.4.1, preferential loss of sorbitol appears to have occurred. In addition, however, the recovery of adonitol was

Figure 5.2 GC traces of the reagent blank processed with and without the deionisation step

(a) With deionisation step



(b) Without deionisation step

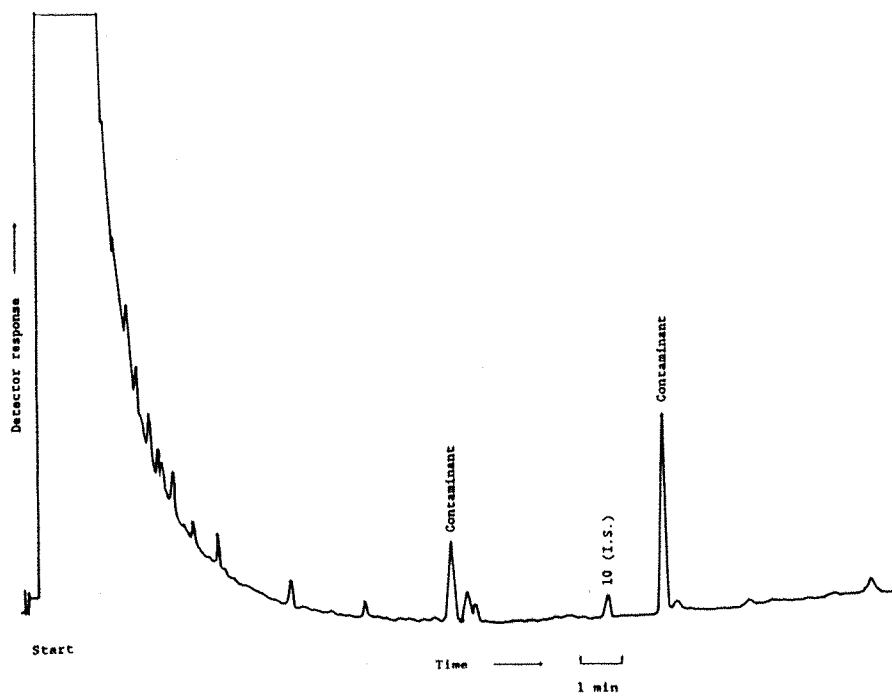


Table 5.8 Recovery of mixed sugar standard on halving the reagent quantities

The analysis was carried out in duplicate (experiments 1 and 2), performing two GC determinations in each analysis.

Peak number	Experiment 1		Experiment 2	
	% recovery	Mean	% recovery	Mean
1	38, 41	40	46, 46	46
2	46, 46	46	45, 52	49
3	46, 47	47	50, 56	53
4	49, 53	51	52, 56	54
5	33, 35	34	25, 27	26
6	61, 70	66	68, 66	67
7	55, 55	55	65, 61	63
8	57, 62	60	59, 60	60
9	63, 69	66	63, 56	60
10	41, 47	44	31, 38	35
11	43, 46	45	47, 53	50
12	45, 51	48	44, 51	48
13	28	28	32, 43	38
14	44, 50	47	45, 51	48
15	46, 51	49	39, 49	44
16	45, 55	50	43, 59	51
17	47, 53	50	43, 46	45
18	12, 18	15	4.2, 8.9	6.6
19	48, 57	53	38, 58	48

generally lower than the other sugars, especially in experiment 2, and also appeared to be variable as seen by differences between the results of experiments 1 and 2. This was thought to be due to the rotary evaporation stage and preferential losses of adonitol at this stage were therefore checked.

Doubly distilled water (50 ml) was spiked with the mixed sugar standard (100 μ l) and the solution was evaporated to dryness. Equilibration and derivatisation were then performed and the sample was subjected to GC analysis. Peak heights relative to adonitol (Table 5.9) indicated that preferential loss of adonitol also occurred in this instance.

To summarise, it appears that:

- a slight decrease in percentage recoveries resulted on halving the reagent quantities although the significance of this should be tested by performing multiple analyses. To minimise sample manipulation and reagent blank contributions, however, this procedure was adopted in future analyses.
- irreproducible preferential loss of adonitol occurred during the analyses. This was thought to take place during the rotary evaporation step. Therefore, despite inevitable procedural losses, it was decided that semi-quantitative analyses on the environmental samples would be performed on absolute peak height measurements rather than peak heights relative to adonitol.

To conclude, investigations into the reduction of sample losses and procedural blanks showed that:-

- (i) improved recoveries should result from increasing the volume of doubly distilled water used for rinsing the ion-exchange column.
- (ii) contaminant peaks generally increased with time and so gas chromatograms should be run as soon as possible after derivatisation.
- (iii) reduced reagent quantities showed a slight decrease in percentage recoveries but are preferable to minimise sample manipulation and reagent blank contributions.

Table 5.9 A comparison of peak heights relative to adonitol in an untreated mixed sugar standard with those after rotary evaporation of the mixed sugar standard in doubly distilled water

Peak label	Peak height relative to adonitol					
	After rotary evaporation			Mixed sugar standard		
	n	Mean	%RSD	n	Mean	%RSD
1	2	2.04	-	1	1.37	-
2	3	1.00	6.1	4	0.66	2.9
3	3	1.66	1.6	4	1.15	1.6
4	3	1.30	1.6	4	0.83	1.7
5	3	1.44	1.8	4	1.26	4.9
6	3	1.10	1.6	4	0.74	4.3
7	4	0.37	2.6	4	0.24	4.0
8	4	1.40	4.4	4	0.90	5.5
9	4	0.18	5.2	4	0.12	13
10	4	1.00	0	4	1.00	0
11	4	1.21	4.8	4	0.83	2.1
12	3	1.92	1.7	1	1.38	-
13	3	0.87	3.7	4	0.61	4.7
14	4	0.43	7.7	4	0.30	5.6
15	4	0.65	3.4	4	0.44	2.8
16	4	0.91	1.3	4	0.60	3.2
17	4	1.09	3.2	4	0.76	2.5
18	4	0.16	14	4	0.63	0.79
19	4	1.07	4.5	4	0.68	4.3

A revised experimental procedure (Figure 5.3), based on the method in Section 5.2.2, was therefore adopted in future work.

5.2.3.5 Determination of glucose in cellulose using the revised experimental procedure

A cellulose standard was prepared in 72 wt % sulphuric acid, as in Section 5.2.3.3. It was then sonicated for five minutes to ensure complete dissolution of the sample. After stirring for two hours, an aliquot of this solution (0.25 ml; equivalent to ca. 10 μ g cellulose) was diluted to 1.2 moles/l of sulphuric acid with doubly distilled water (2.5 ml) and subjected to the revised experimental procedure.

Two sets of experiments were performed:-

- (a) one aliquot of the cellulose standard and a reagent blank were analysed.
- (b) two aliquots of the same cellulose standard were taken and subjected to the analysis - no blank experiment was performed in this instance.

The results (Table 5.10) show that:

- better absolute recoveries of glucose, based on peak height measurements (compared to Section 5.2.3.3), were seen using the revised method. Similar recoveries (ca. 30%) were obtained in both instances in experiment (b) although large variations between replicate GC analyses (RSD > 15%) occurred due to deterioration of the GC capillary column.
- preferential loss of adonitol occurred, confirming that quantitation, using peak height ratios relative to adonitol, would be unsatisfactory.

Lower sample blanks were also obtained.

Therefore, although absolute peak height measurements showed low percentage yields of glucose, they were more reliable and so were adopted in future work.

Figure 5.3 Revised experimental procedure for the determination of monosaccharides

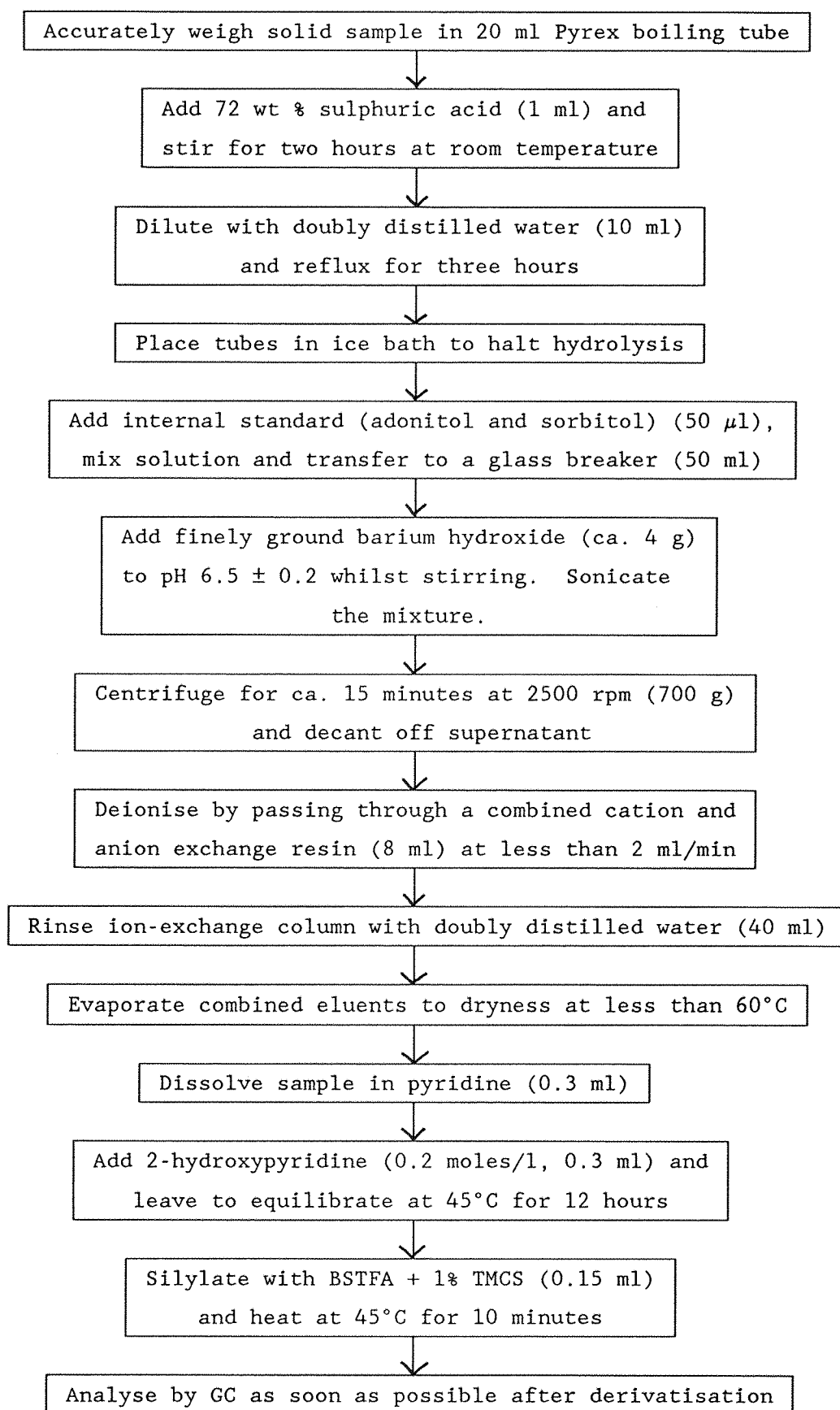


Table 5.10 Recoveries of glucose from cellulose using the revised experimental procedure

Experiment	Percentage recoveries based on absolute peak heights	
	Glucose (Gl ₁)	Adonitol
(a) ⁺ Standard*	49	32
Blank	10	33
(b) ⁺ Standard	33	19
Standard	31	23

+ Multiple GC determinations were performed in each case on single experiments.

* Not blank corrected.

Although the experimental yield was low, it was decided that the feasibility of the method for the analysis of environmental samples should be assessed.

5.2.3.6 Retention of carbohydrates by XAD-8

Prior to the determination of monosaccharides in the humic samples, it was necessary to confirm that any measured sugars were not free carbohydrates co-extracted with the humic materials. Thurman and Malcolm (44) found that free sucrose and glucose were not absorbed by XAD-8.

Sweet and Perdue (295), investigating the concentration and speciation of dissolved sugars in river water using XAD-7 resin, considered humic-bound saccharides, which remained on the resin, and monosaccharides and polysaccharides, which were not retained. However, Bertino *et al.* (216) reported that the presence of a small percentage of free glucose in non-hydrolysed fulvic acid extracted with XAD-8 resin, which indicated that either the free glucose was co-

extracted with the humic substances, or it was loosely bound to them. As the retention of free sugars by XAD-8 still appears contentious under the conditions employed in this work, the adsorption of free monosaccharides by XAD-8 resin, both in the presence and absence of humic substances, was investigated.

5.2.3.6.1 Experimental

Artificial seawater (100 ml) was spiked with:

- (i) a mixed sugar standard (50 μ l), containing arabinose, fructose, fucose, galactose, glucose, lyxose, mannose, rhamnose, ribose and xylose (200 ng/ μ l each sugar in pyridine).
- (ii) a mixed sugar standard (as in (i); 50 μ l), and humic substances (0.5 g of humic extract) extracted from site A (May, 1986).

A blank analysis was performed using unspiked artificial seawater (100 ml). Sugars were extracted both in the presence and absence of humic material as its presence has been known to alter the adsorption of certain compounds (406).

Each sample was passed through a scaled-down extraction procedure (Section 4.2.2), using 7 ml of XAD-8 resin. An internal standard solution (50 μ l), containing adonitol and sorbitol (100 ng/ μ l each sugar alcohol in pyridine), was added to the methanolic ammonia after elution, and the mixture was then evaporated to dryness. The residue was dissolved in 2-hydroxypyridine (0.1 moles/l, 0.8 ml) and analysed by GC as in Section 5.2.2.3.

The artificial seawater eluting from the XAD-8 resin was also analysed for its sugar content. An aliquot (25 ml) of the eluent was spiked with the internal standard solution (50 μ l), and desalted using a combined cation and anion exchange resin (Dowex 50W-X8(H) cation exchange resin (hydrogen form, 20-50 dry mesh) and Dowex 1-X8 anion exchange resin (formate form, 100-200 dry mesh); 50ml). The column was rinsed with doubly distilled water (150 ml), the combined eluents were evaporated to dryness, and the residue was dissolved in 2-hydroxypyridine (0.1 moles/l, 0.8 ml). Equilibration of the sugars, derivatisation and GC analysis were then performed as above.

5.2.3.6.2 Results and discussion

The results (Table 5.11) show that no free sugars were recovered in the methanolic ammonia eluent in the absence of humic substances. Furthermore, some sugars were detected in the artificial seawater eluting from the XAD-8, which suggests that the free sugars were not adsorbed onto the resin in the absence of humic substances. These results are in agreement with those of Thurman and Malcolm (44) cited above. Quantitative measurements on the artificial seawater eluent were not performed, and so it is not therefore possible to say whether all of the sugars passed through the resin. Some may have been adsorbed, but not eluted using methanolic ammonia.

Due to interferences in the GC analysis, the detection of sugars, in the presence of humic substances, was not possible in the methanolic ammonia eluent. As noted above, they were, however, detected qualitatively in the artificial seawater eluting from the resin, suggesting that free sugars were not absorbed onto XAD-8 in the presence of humic substances. This is in agreement with the work of Sweet and Perdue (295) cited above.

Adsorption of polysaccharides onto XAD-8 was not investigated in this instance. Further studies, using artificial seawater spiked with a selection of polysaccharides, should be performed to confirm that free polysaccharides also pass through the resin, as indicated by Sweet and Perdue (295).

5.2.3.6.3 Conclusions

There is currently no evidence to suggest that free monosaccharides are co-extracted with the humic samples.

Therefore, it is probable that any sugars detected in the humic matter extracted in the present work, are either an integral part of the material or are loosely bound to it - they have not been co-extracted with the humic substances.

Table 5.11 Retention of monosaccharides by XAD-8

	Eluent and spike					
	Methanolic ammonia			Artificial seawater		
	(i)	(ii)	(iii)	(i)	(ii)	(iii)
Detection of sugars	x	?	x	✓	✓	x

(i) Mixed sugar standard.

(ii) Mixed sugar standard plus humic substances.

(iii) Unspiked.

✓ Monosaccharides present.

x Monosaccharides absent.

? The humic substances were found to interfere with the GC analysis and so detection of sugars was not possible.

5.3 DETERMINATION OF MONOSACCHARIDES IN HUMIC SUBSTANCES

The monosaccharide content of humic samples, after hydrolysis, was determined by GC of the sugar TMS ether derivatives. Identification of the sugars was performed by GC-MS analysis.

Dominant peaks in the mass spectra of sugar TMS ether derivatives include ions at m/e values of 73, 147, 191, 204, and 217 (407). The structures of these ions is given (Figure 5.4). In general, the molecular ion is very weak or not seen at all.

Figure 5.4 The structures of the main ions in the mass spectra of sugar TMS ether derivatives (407)

<u>m/e</u>	<u>Structure</u>
73	Me_3Si^+
147	$\text{Me}_3\text{Si} - \overset{+}{\text{O}} = \text{SiMe}_2$
191	$\text{Me}_3\text{Si} - \text{O} - \overset{+}{\text{C}}\text{H} - \text{O} - \text{SiMe}_3$
204	$[\text{Me}_3\text{SiO} - \overset{+}{\text{C}}\text{H}]_2$
217	$\text{Me}_3\text{SiO} - \text{CH} = \text{CH} - \text{CH} - \text{OSiMe}_3$

5.3.1 Method

5.3.1.1 GC analysis

Humic samples (Table 5.12) were accurately weighed and transferred to 20 ml Pyrex boiling tubes. 72 wt % sulphuric acid (1 ml) was added, and the mixtures were sonicated for one minute, prior to stirring at room temperature for two hours. They were then treated according to the revised experimental procedure (Figure 5.3).

5.3.1.2 GC-MS analysis

The above samples (Section 5.3.1.1) were subjected to capillary GC-MS analysis to confirm the presence of monosaccharides. Initially, the samples from August, 1986 (site A and C, plus the XAD blank) were analysed using a Hewlett Packard 5890A gas chromatograph directly coupled to a VG TS-250 mass spectrometer. The instrument was operated

Table 5.12 Masses of humic material analysed for monosaccharides

Sampling date	Site	Mass of sample (mg) ⁺	Estimated mass of humic material in sample (mg) [*]
November, 1985	A	10.7	6.1
	B	-	-
	C	7.8	2.4
	Blank	2.9	0
February, 1986	A	13.0	10.1
	B	11.7	6.8
	C	10.2	4.2
	Blank	5.9	0
May, 1986	A	6.6	5.0
	B	5.6	3.4
	C	7.1	0.20
	Blank	4.7	0
August, 1986	A	11.4	6.5
	B	11.1	5.0
	C	10.2	2.7
	Blank	6.1	0

+ This consists of humic material plus blank material.

- No sample available.

* Calculated using the estimated blank content of the samples from Section 4.3.5.

in the full scan mode, scanning at 0.6 seconds/decade, and the mass spectra were processed using a Digital PDP-11 data system. The operating conditions were as follows:

Gas Chromatograph

GC column : 25 m, 0.25 mm internal diameter, BP1 WCOT fused silica capillary column
Carrier gas : High purity helium (at 1 ml/min)
Temperature programme : 100°C (5 mins) → 5°C/min → 265°C (5 mins)

Interface

Type : Direct inlet at 250°C

Mass Spectrometer

Ionisation mode : Electron impact (positive ion)
Ion source temperature : 230°C
Electron energy : 70 eV

At a later date, GC-MS was performed on the samples from November, 1985, February, 1986, and May, 1986 using a Finnigan MAT 9610 gas chromatograph directly coupled to a Finnigan MAT 4500 quadrupole mass spectrometer. In this instance, the instrument was operated in multiple ion detection (MID) mode, monitoring the ions characteristic of sugar TMS ether derivatives, i.e. 73, 147, 191, 204 and 217 (407). The advantage of MID is that (i) it can be used to target a given species, (ii) it eliminates all contaminant peaks not containing any of the required ions, and (iii) it enhances the sensitivity of the technique. Mass spectra were recorded using a Finnigan MAT data system with INCOS III software.

The following operating conditions were employed:

Gas Chromatograph

GC column : 25 m, 0.22 mm internal diameter, CP-SIL5CB (0.12 µm film thickness) WCOT fused silica

capillary column
Carrier gas : High purity helium (at ca. 1 ml/min)
Injection system : Splitless injection (275°C), with a splitless time of 1 minute, and a split rate of 30 ml/min
Temperature programme : 90°C (1 min) → 6°C/min → 300°C (5 mins)

Interface

Type : Direct inlet at 250°C

Mass Spectrometer

Ionisation mode : Electron impact (positive ion)
Ion source temperature : 200°C
Electron energy : 70 eV

GC peaks were labelled according to Figure 5.1(b). Increased resolution of these peaks occurred in the early part of the chromatograms in Figures 5.10 and 5.11 due to the use of different chromatographic conditions. The assignment of peaks 4 - 6 was therefore based purely on comparisons with those of Cowie and Hedges (371).

5.3.2 Results

5.3.2.1 GC analysis

The monosaccharide content of the humic samples (corrected for the contribution of the blank material) is given (Table 5.13 and Figure 5.5). Example chromatograms of the samples from February and August, 1986 are also presented (Figures 5.6 and 5.7). It can be seen that the XAD blank from February showed more contamination than the blank from August. Therefore, quantitation of the sugars in the February samples, in particular for site C, was more difficult.

Figure 5.5 shows no obvious site or seasonal variations. The relative abundance of the individual monosaccharides were also variable. Glucose, galactose, arabinose, rhamnose and mannose were

Table 5.13 The monosaccharide content of the humic materials
(presented as % ($\times 10^{-3}$) monosaccharide in the humic
sample)

Sugar	November, 1985			February, 1986		
	Site A	Site B	Site C	Site A	Site B	Site C
Arabinose	27	NA	ND	58	60	4.7
Galactose	60	NA	28	40	9.4	13
Glucose	NA*	NA*	NA*	> 84	61	>110
Lyxose	ND	NA	ND	ND	ND	ND
Mannose	26	NA	ND	40	27	30
Rhamnose	58	NA	17	35	38	7.7
Ribose	5.1	NA	4.8	3.4	6.1	4.1
Xylose	24	NA	1.8	25	25	4.2

Sugar	May, 1986**			August, 1986		
	Site A	Site B	Site C	Site A	Site B	Site C
Arabinose	80	130	300	56	64	24
Galactose	60	110	930	98	74	78
Glucose	28	80	660	250	15	ND
Lyxose	NA ⁺	NA ⁺	NA ⁺	ND	ND	ND
Mannose	41	120	1100	78	50	37
Rhamnose	38	62	850	66	49	43
Ribose	NA ⁺⁺	NA ⁺⁺	NA ⁺⁺	14	18	18
Xylose	13	20	460	44	25	25

* Quantitation not possible as this peak was off-scale in the blank.

** The data from site C were thought to be erroneous due to the high percentage of extraneous blank material in the sample.

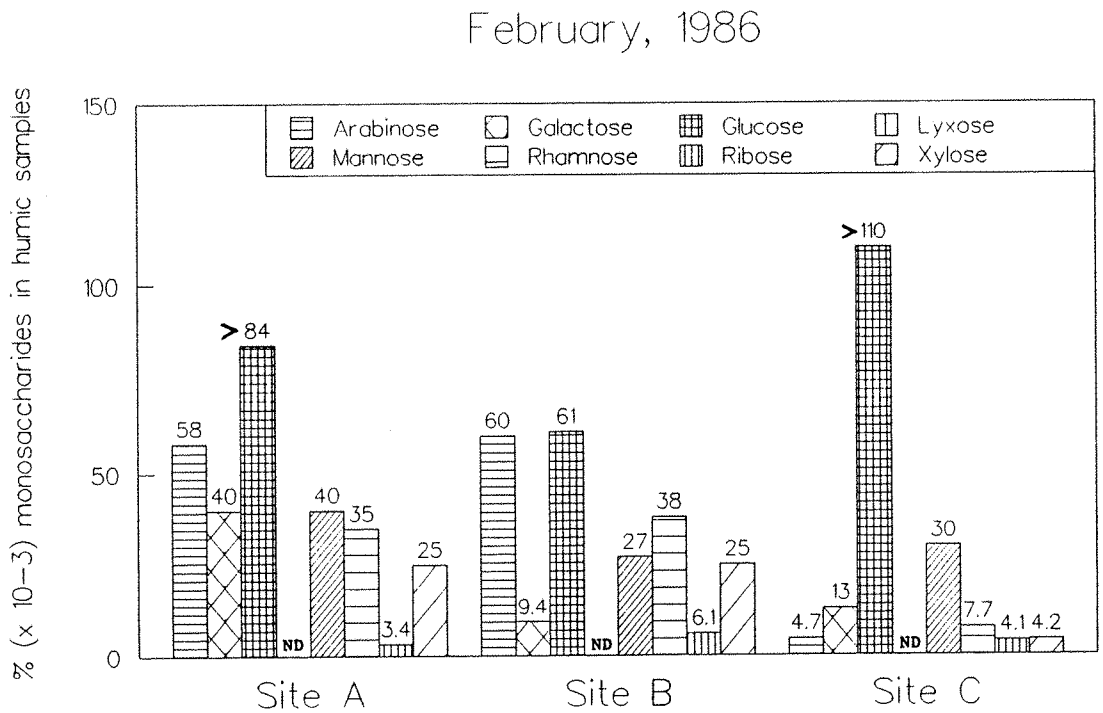
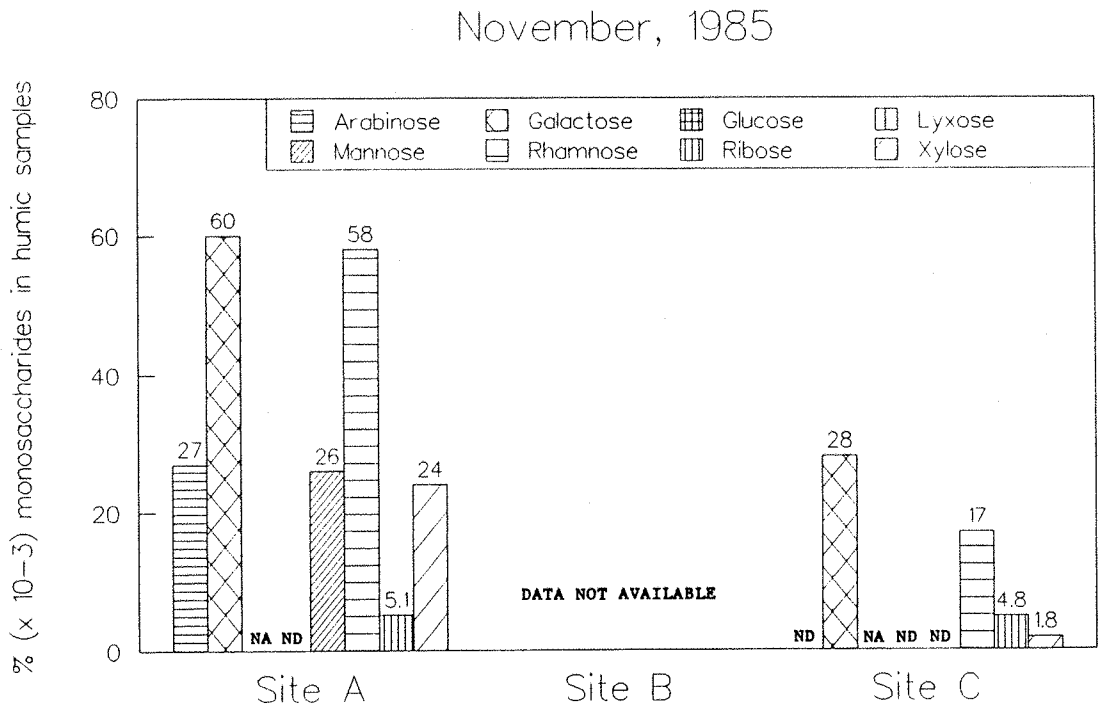
+ The contribution of contamination to the lyxose peak was too variable for quantitation.

++ Not calculated as the contribution from lyxose was not available.

NA Data not available.

ND Not detected.

Figure 5.5 The monosaccharide content of the humic samples

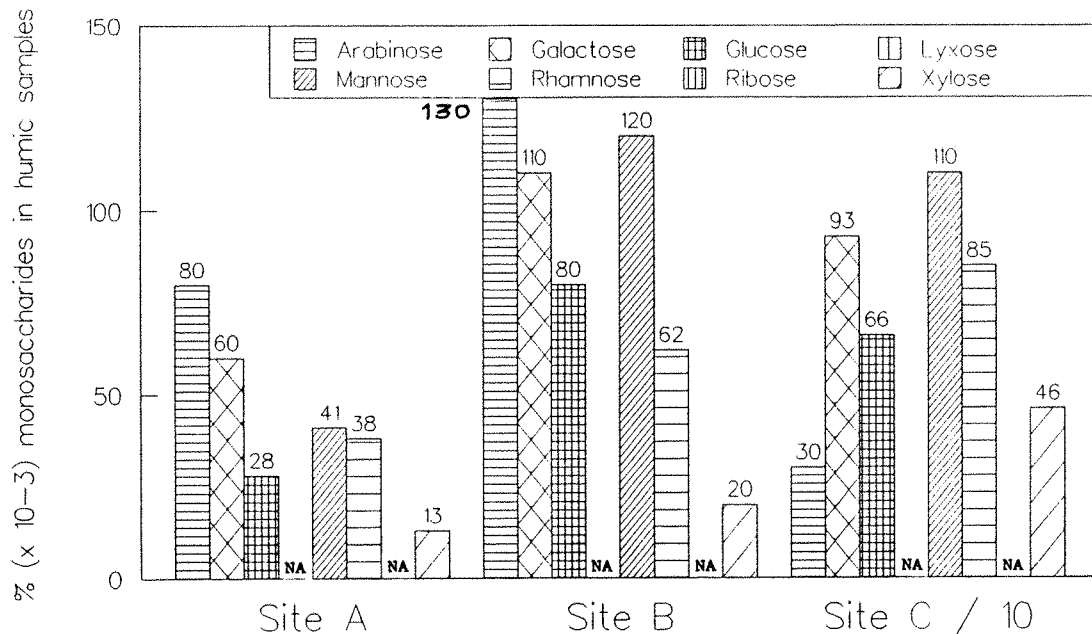


ND Not detected

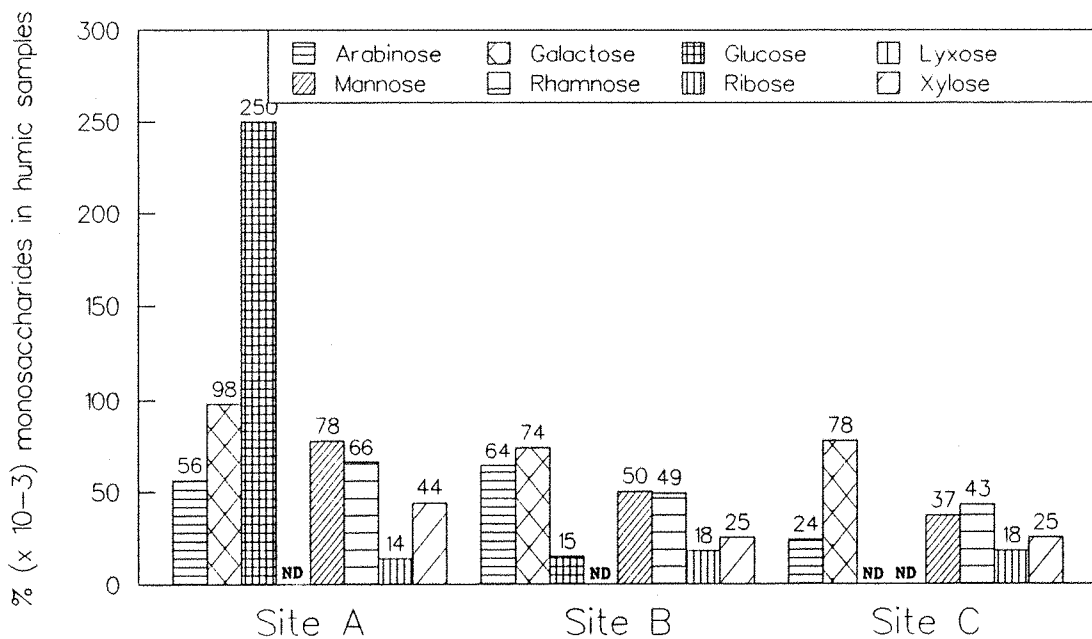
NA Data not available

Figure 5.5 The monosaccharide content of the humic samples
(continued)

May, 1986



August, 1986

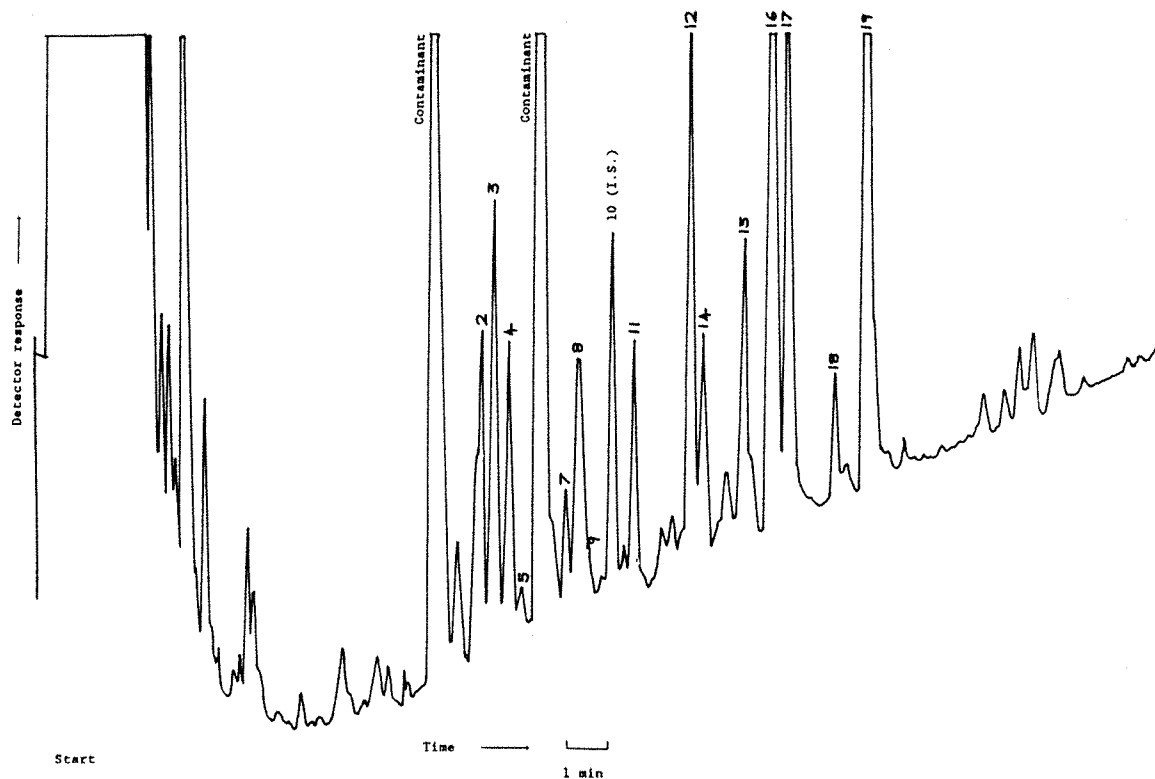


ND Not detected

NA Data not available

Figure 5.6 GC trace of sugar TMS ether derivatives of hydrolysed humic samples from February, 1986

(a) Site A



(b) Site B

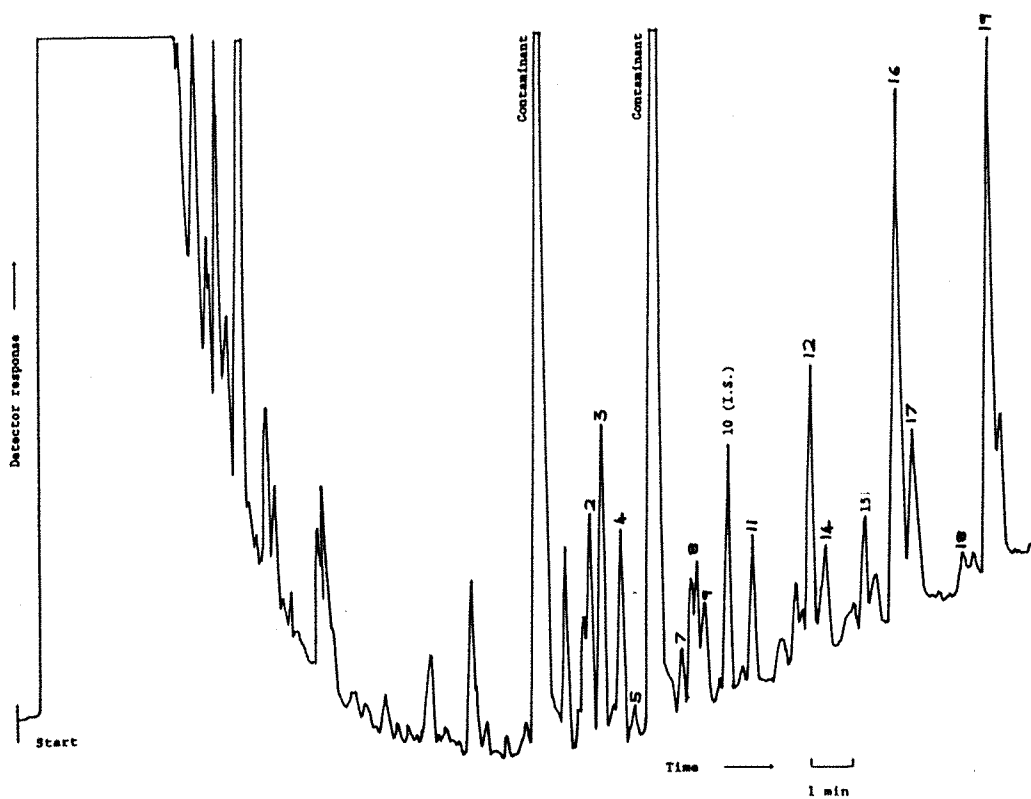
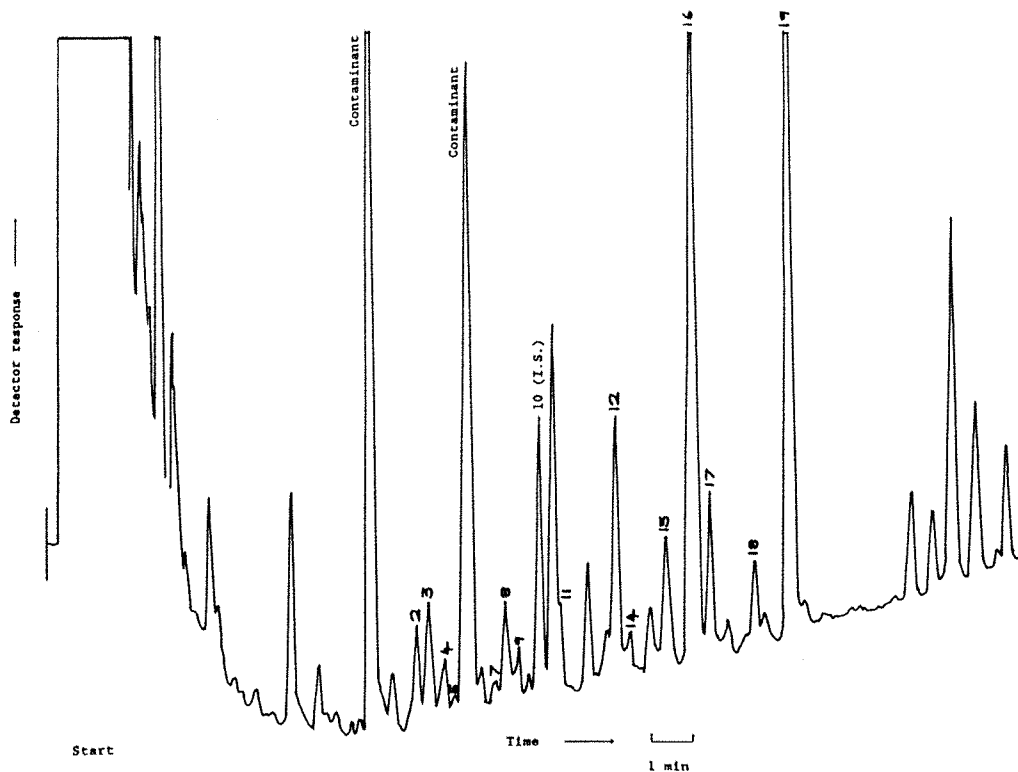


Figure 5.6 GC trace of sugar TMS ether derivatives of hydrolysed humic samples from February, 1986

(c) Site C



(d) XAD blank

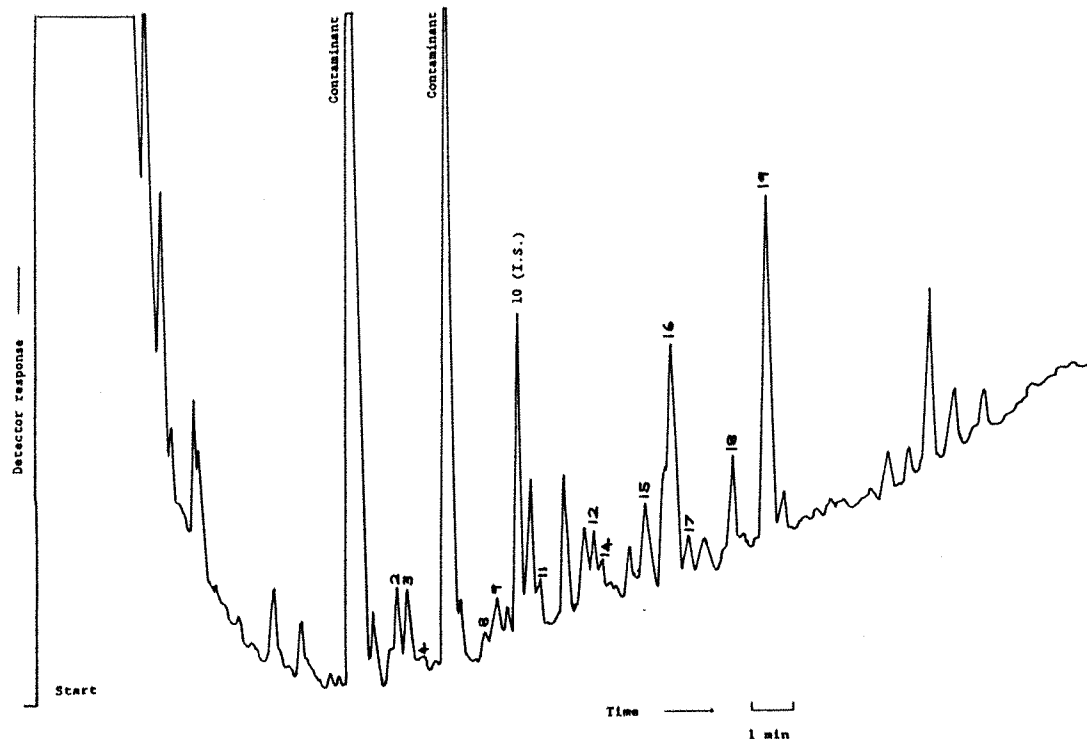
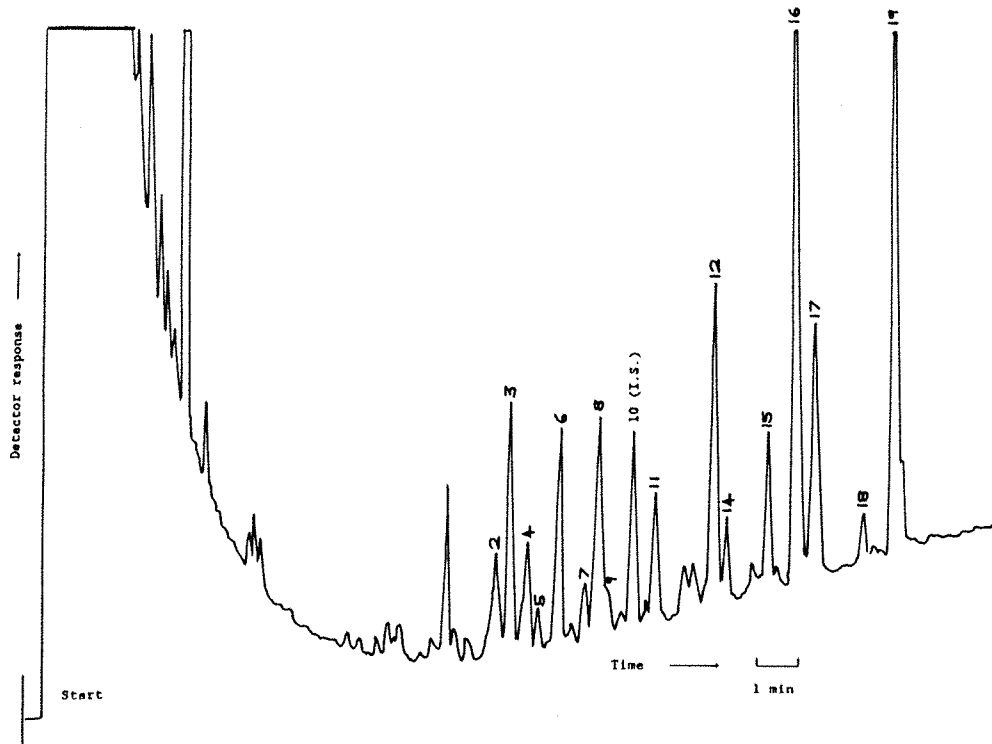


Figure 5.7 GC trace of sugar TMS ether derivatives of hydrolysed humic samples from August, 1986

(a) Site A



(b) Site B

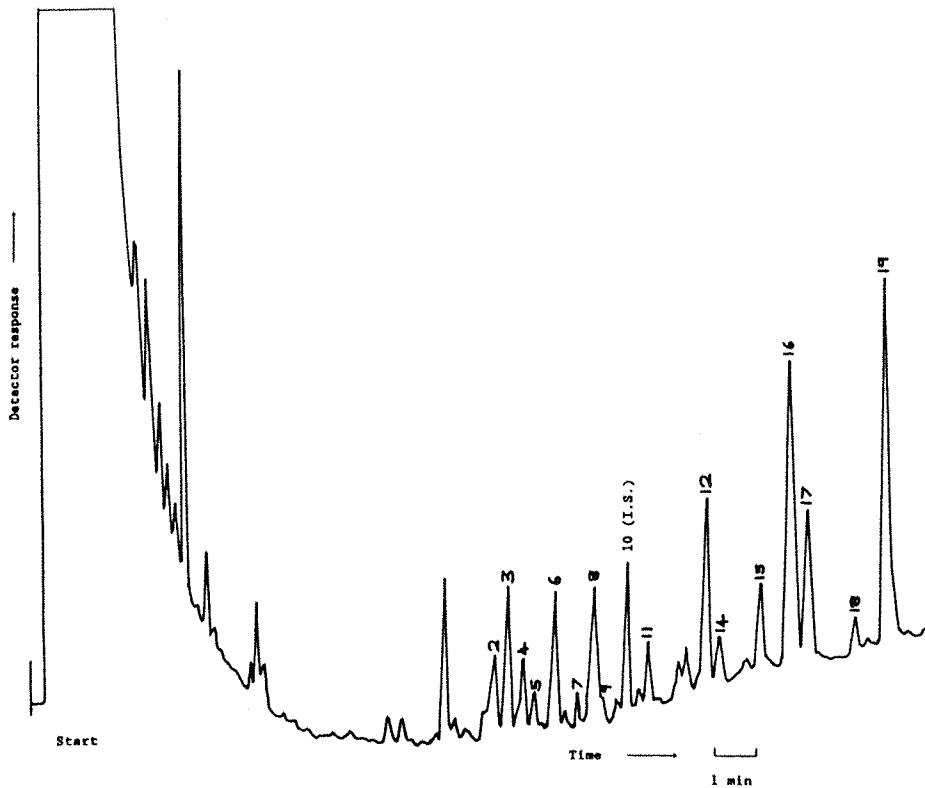
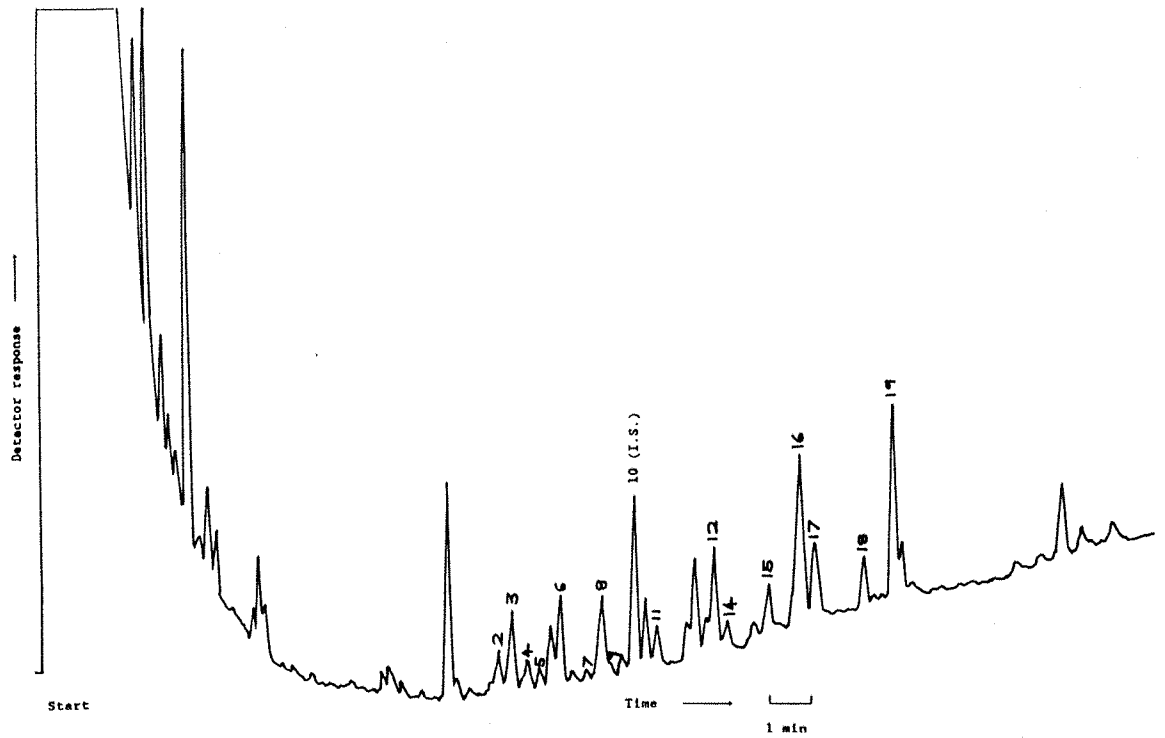
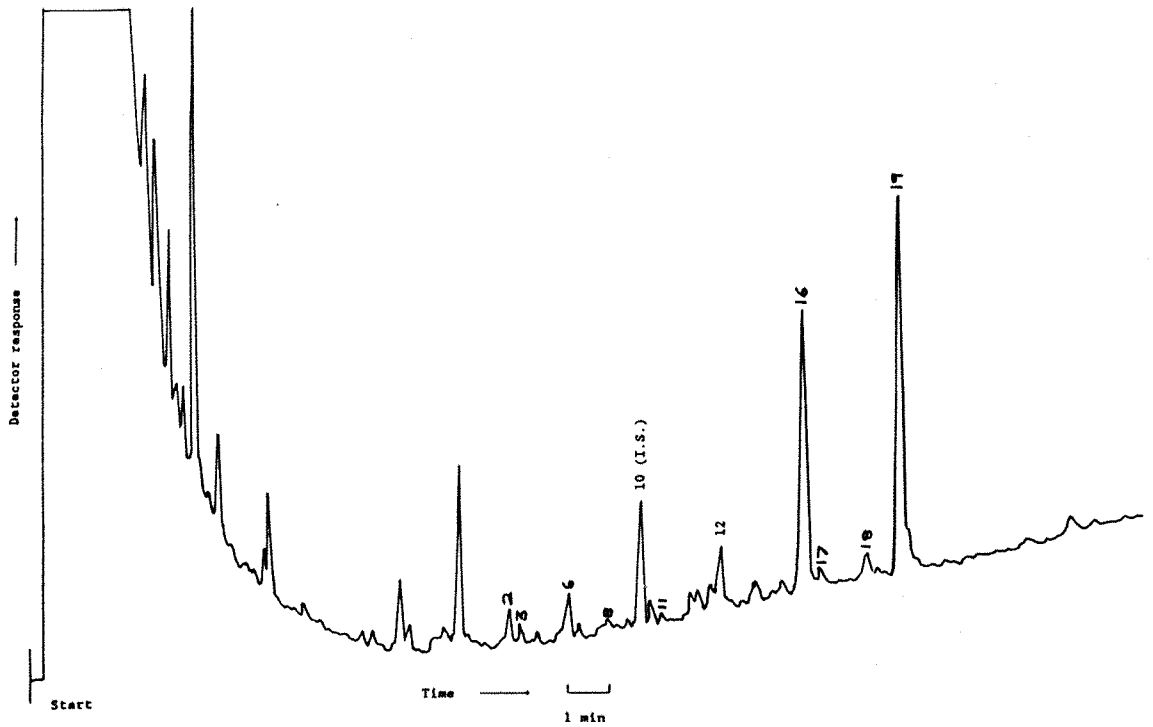


Figure 5.7 GC trace of sugar TMS ether derivatives of hydrolysed humic samples from August, 1986

(c) Site C



(d) XAD blank



more abundant than xylose and ribose, with glucose and galactose generally tending to dominate slightly. The increased importance of arabinose in sites A and B, compared to site C, can also be noted. Quantitation of fucose was not possible due to variable overlap with the xylose peak. However, its presence was noted.

5.3.2.2 GC-MS analysis

Figures 5.8 and 5.9 show the total ion current (TIC) traces for the mixed sugar TMS ether standard, and samples from August, 1986. Analysis of the samples from November, 1985, February, 1986, and May, 1986, at a later date, showed few peaks, suggesting that the sugar TMS ether derivatives had been lost during prolonged storage. Therefore, three samples from sites A and B, May, 1986, and an XAD blank from April, 1986 (no XAD blank was available for May) were treated again according to the method in Section 5.3.1.1, and immediately analysed by GC-MS. Figures 5.10 and 5.11 show the total ion current traces (for the five ions monitored) resulting in this instance. As some of the early eluting peaks were better resolved than in the original standard, these were identified by comparison with the GC results in the work of Cowie and Hedges (371). In addition to sugars found by comparison with the sugar TMS standard, several additional unidentified peaks were also observed.

A summary of the sugars present in each of the samples can be seen in Table 5.14. It can be seen that the sugars found by GC analysis have been confirmed by GC-MS. However, the presence of ribose in sites A and C, from August, 1986, and xylose in site C, from August, 1986, were not detected. It was thought that they were below the detection limit for the GC-MS analysis. In addition, a small amount of lyxose, not previously detected in the GC analysis, was observed in the August, 1986 samples.

Figure 5.8 TIC trace from the GC-MS analysis of the mixed sugar TMS ether standard

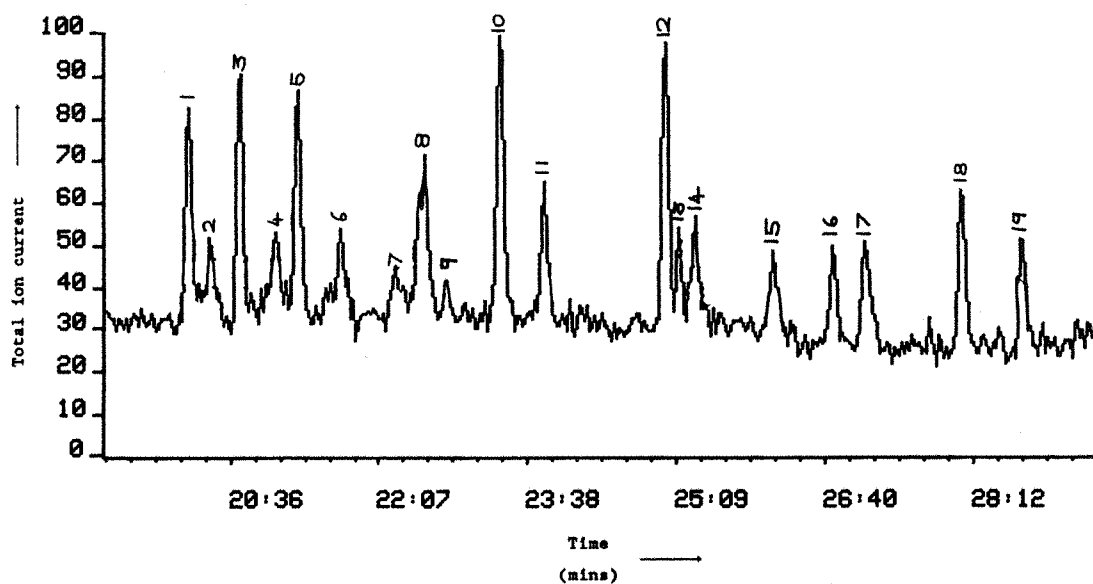


Figure 5.9 TIC trace from the GC-MS analysis of the hydrolysed humic samples from August, 1986

(a) Site A

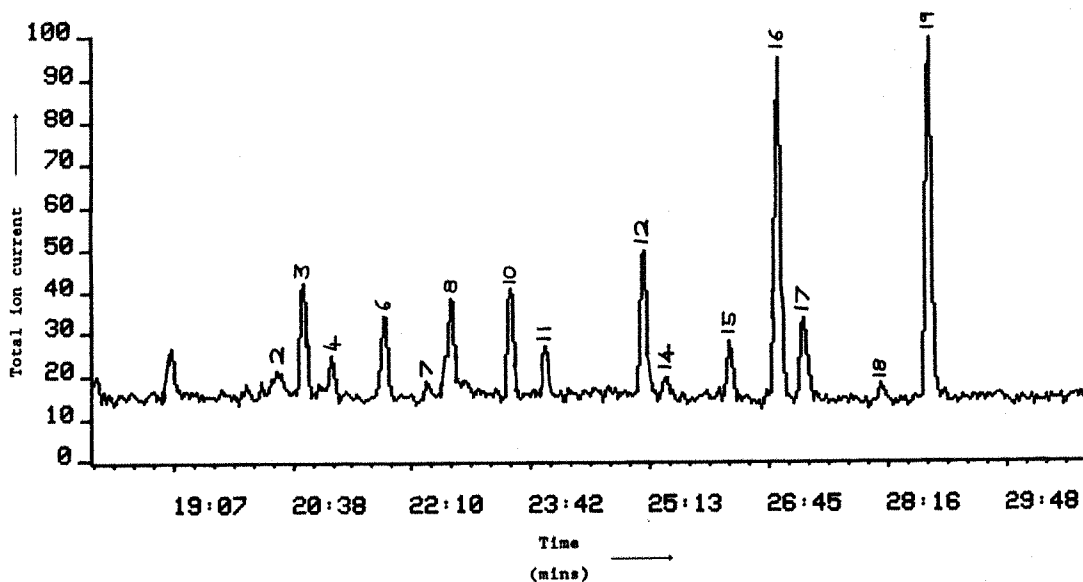
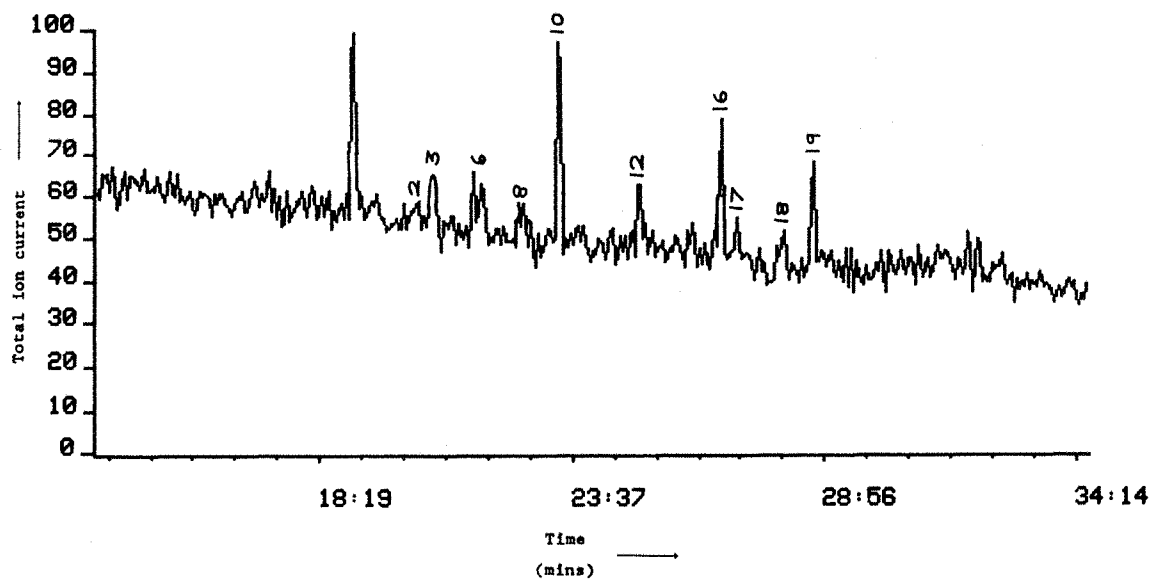


Figure 5.9 TIC trace from the GC-MS analysis of the hydrolysed humic samples from August, 1986

(b) Site C



(c) XAD blank

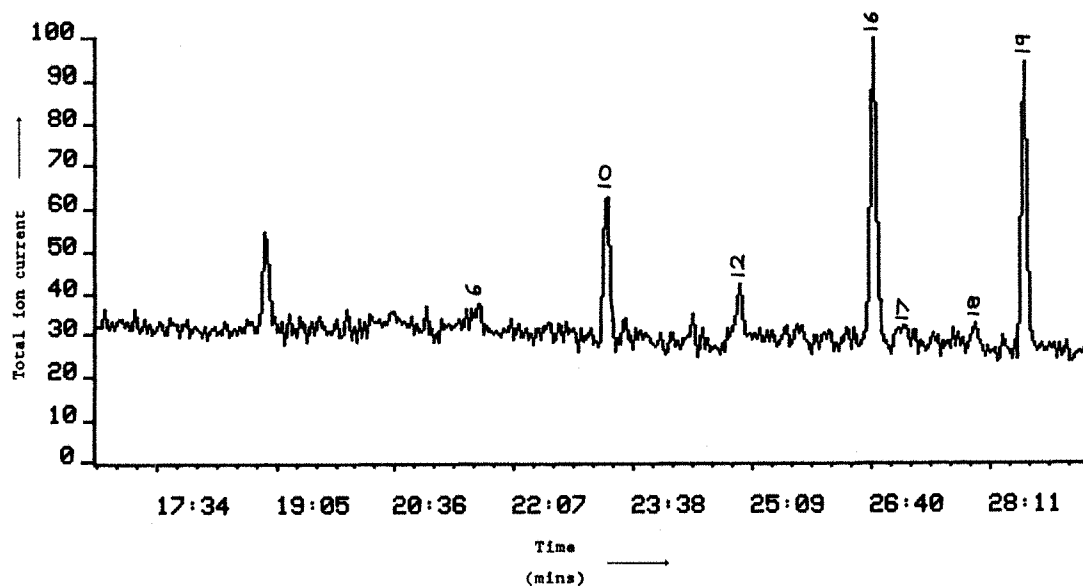


Figure 5.10 TIC trace for the ions, 73, 147, 191, 204, and 217, from the GC-MS analysis of the mixed sugar TMS ether standard

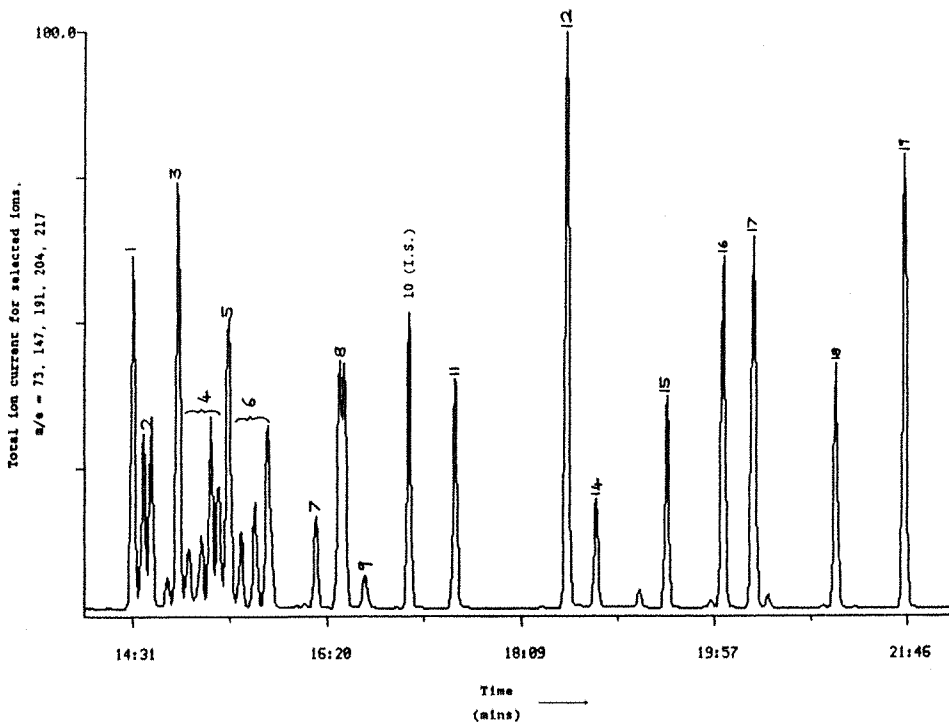


Figure 5.11 TIC trace for the ions, 73, 147, 191, 204, and 217, from the GC-MS analysis of hydrolysed humic samples

(a) Site A, May, 1986

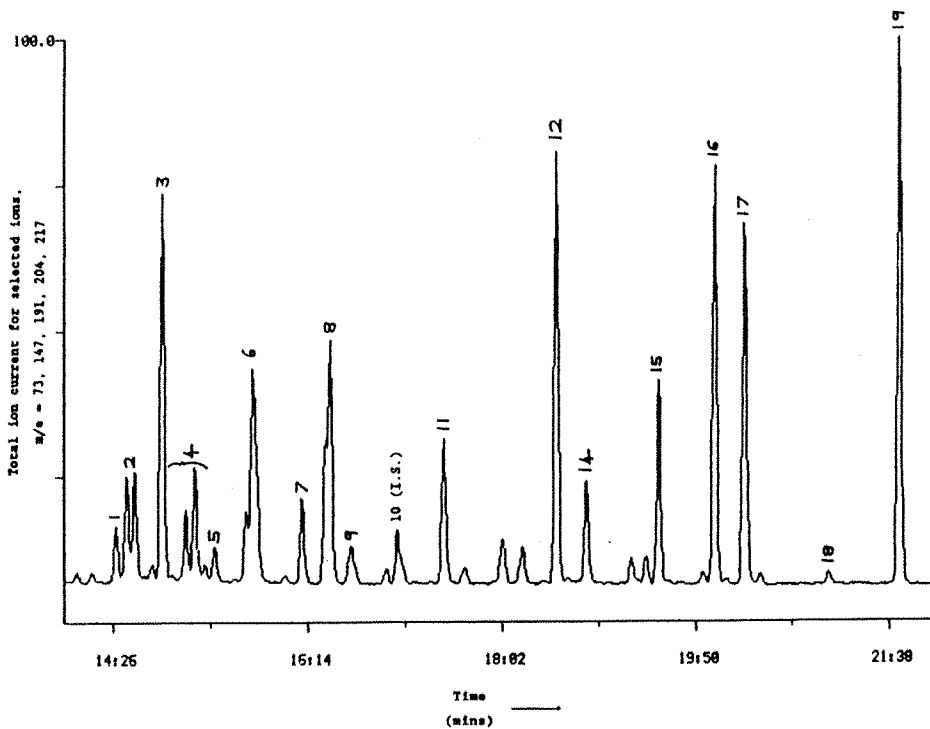
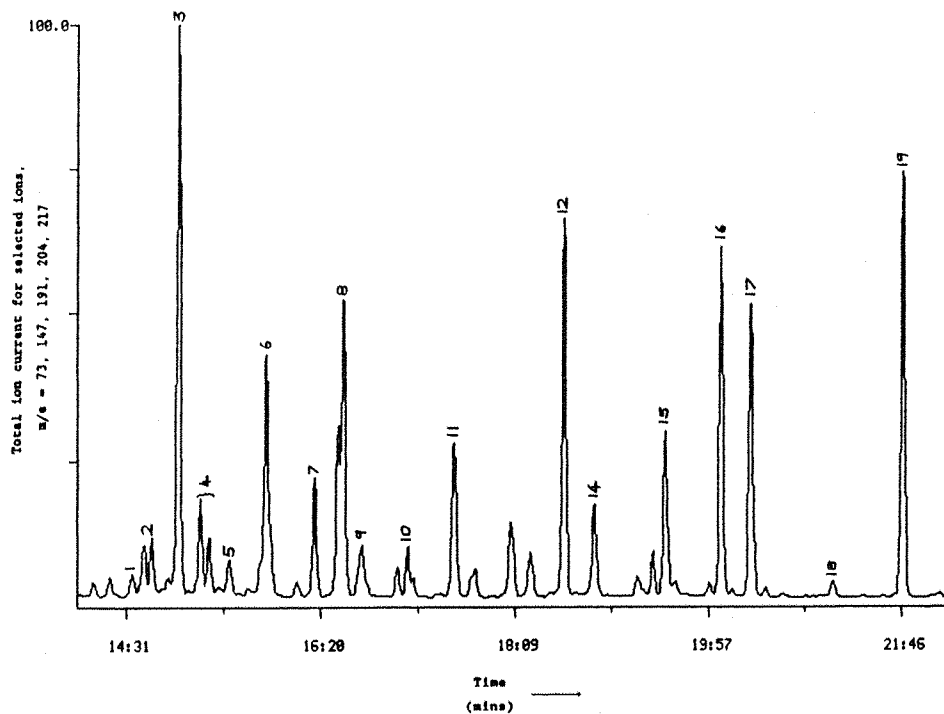


Figure 5.11 TIC trace for the ions, 73, 147, 191, 204, and 217, from the GC-MS analysis of hydrolysed humic samples

(b) Site B, May, 1986



(c) XAD blank, April, 1986

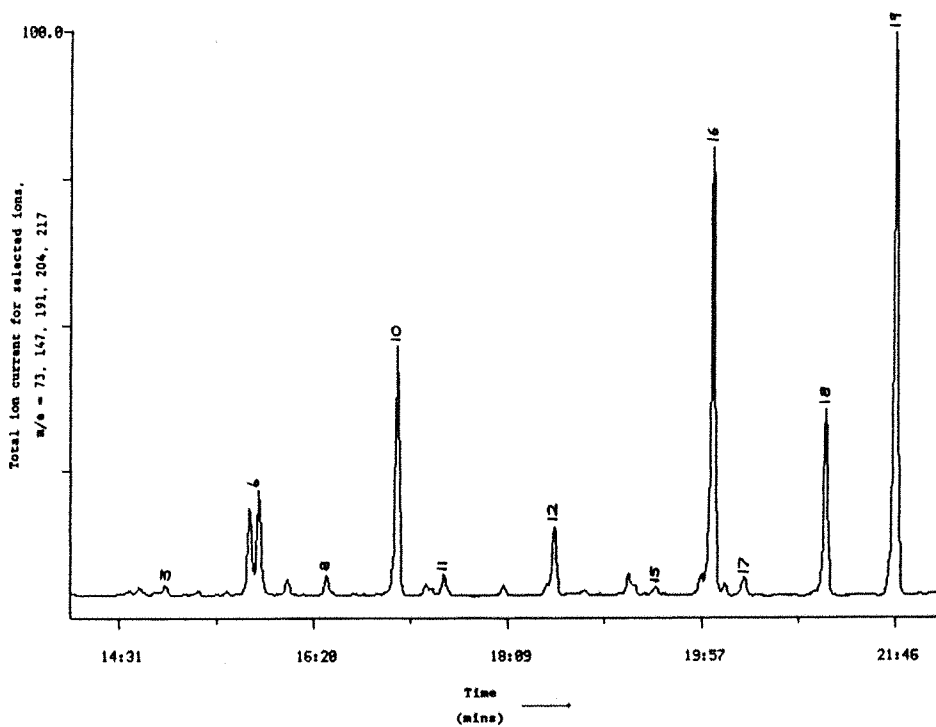


Table 5.14 Monosaccharides confirmed to be present in the humic samples by GC-MS

Sugar	May, 1986		April, 1986	August, 1986		
	Site A	Site B	XAD blank	Site A	Site C	XAD blank
Arabinose	✓	✓	x	Tr	Tr	x
Fucose	✓	✓	x	✓	✓	x
Galactose	✓	✓	x	✓	Tr	x
Glucose	✓	✓	✓	✓	✓	✓
Lyxose	✓	Tr	x	x	x	x
Mannose	✓	✓	✓	✓	✓	✓
Rhamnose	✓	✓	x	✓	✓	x
Ribose	Tr	Tr	x	x	x	x
Xylose	✓	✓	Tr	✓	x	x

✓ Present

Tr Trace suspected

x Not detected

5.3.3 Discussion

Prior to suggesting origins for the sugars detected in the aquatic humic materials in the present study, possible precursors for both terrestrial and aquatic humic substances will be discussed.

5.3.3.1 Origin of carbohydrates in soil humic substances

The monosaccharide content of soil humic substances has been reviewed earlier (Section 3.3.1.1). Sugars in soil humic materials have been found to be dominated by glucose (e.g., (281,284)). This is not surprising as cellulose and its derivatives can constitute up to 15% of the total organic material in soil (51). Cellulose is the major component of the cell walls of higher plants and is thus abundant in soils rich in undecayed or only partially decayed plant

remains. Arabinose and xylose, also major components of these soils (51), probably result from xylans - hemicelluloses which are structural components of plant cell walls. These consist of a xylose backbone with side-chains of other sugars, often arabinose (321). Glucose may additionally originate from storage polysaccharides in plants (starch), animals (glycogen) and bacteria (dextrans). This ubiquitous nature restricts its use as a specific biomarker.

In soils where the plant material has been decomposed by bacteria and fungi, the monosaccharide composition will reflect those carbohydrates synthesised by these microorganisms rather than those originating from higher plants. Cheshire (289) concluded that fucose, galactose, mannose and rhamnose were predominantly derived from microbial sources, and that arabinose and xylose originate from higher plants. Glucose resulted from both sources. Work on synthetic fungal melanins (281) has suggested that arabinose can also be derived from lower plant sources. Lowe (408), on the other hand, suggested that galactose and mannose, as well as glucose, may result from either source, and Folsom *et al.* (409) suggested that ribose, as well as fucose and rhamnose, originated from microorganisms.

5.3.3.2 Possible precursors to aquatic humic substances

Live phytoplankton constitute a significant portion of the particulate organic matter in the sea, with detritus (mainly dead organisms - both plant and animal) contributing to varying degrees (410). Thus plankton are likely to be the main precursors to marine humic substances, with freshwater materials liable to include both terrestrial and planktonic matter.

The carbohydrate content of various species of phyto- and zooplankton, and algae has been reviewed by Romankevich (338), indicating differences in chemical composition between species. Brown and red algae contain large quantities of carbohydrate (up to 74% of the organic matter); planktonic algae less, and zooplankton, even smaller amounts (a few percent of the organic matter). The levels in zooplankton, however, may reach 20% or more during periods when protein and lipid synthesis is slow. The monosaccharide composition of different species of diatoms is variable but glucose is often the

dominant sugar (reviewed in (338,411)). This results from glucan, the major reserve polysaccharide in phytoplankton, and cellulose, a constituent of planktonic and algal cell walls. Other monosaccharides occurring in significant amounts include mannose, galactose and rhamnose, with fucose and xylose also being major contributors in some species. Various precursors for these sugars can be suggested. The carbohydrates of algal polysaccharides have been reviewed by Percival (412), who divided them into reserve, structural and sulphated polysaccharides. The reserve polysaccharides consist mainly of glucose monomers from, for example, starch, although laminaran, a β -(1-3)-linked-D-glucan, contains a few percent of D-mannitol. Amongst the structural polysaccharides are cellulose, D-mannans, D-xylans, alginic acid, and pectins (polymers of galacturonic acid), and the sulphated polymers include fucans, galactans and polysaccharides containing various combinations of glucose, xylose, rhamnose, arabinose, mannose, and galactose.

In addition to the release of polysaccharides on death of the plankton, extracellular release of these materials from live plankton has also been documented, in particular in times of nutrient deficiency (413). Variations in the dissolved free and combined carbohydrates during a phytoplankton bloom have been investigated (413). Large amounts of combined carbohydrates were released but the dissolved free sugar fraction was thought to result mainly from *in situ* hydrolysis of combined polysaccharides rather than excretion of monosaccharides themselves. The rapid decrease in combined carbohydrates after the phytoplankton bloom indicated the labile nature of these materials and thus they are probably of little importance as contributors to humic substances.

Other contributors to aquatic humic substances will be the bacteria which degrade/alter aquatic organisms. They are the predominant group of marine microorganisms and are most abundant in areas of high planktonic population or where much photosynthesis is occurring. In some marine areas, they have been shown to approach the quantities of plankton in biomass (414). The carbohydrates of bacteria have been reviewed by Barker and Somers (415) and can be seen to be relatively diverse. The cell walls generally contain glycopeptide structural units, which, on acid hydrolysis, yield

glucosamine and amino acids, resulting from N-acetyl glucosamine (chitinous structures) and N-acetyl muranic acid (derivatives of N-acetyl glucosamine). Many bacteria are also rich in deoxy sugars and this has been used as an indicator for their origin in the marine environment (416). Ribose may additionally be a major sugar (416), resulting from ribonucleic acid (RNA) or other nucleotides. Ribitol residues, originating from teichoic acids, which are phosphate polymers accounting for up to 50% dry weight of the cell walls of many gram-positive bacteria, may also be present.

Concerning contributions from free dissolved and particulate carbohydrates in natural waters, the carbohydrate composition of particulate organic material would be expected to be similar to that of planktonic material. Similarities have been found between polysaccharides resulting from the particulate organic and planktonic materials (417), with the deeper water particulate matter tending to closely resemble structural polysaccharides of the plankton indicating the more refractory nature of these polymers compared to the reserve polysaccharides. This was confirmed by the fact that β -1,3-glucan, a laminaran-type reserve polysaccharide, decayed rapidly as the particulate organic matter settled. This was shown by a rapid decrease in the D-glucose concentrations. β -glucans have also been isolated from marine dissolved and particulate polysaccharides by Sakugawa and Handa (418). β -1,3-glucans, thought to originate from reserve polysaccharides (due to their labile nature), and β -1,4-glucans, probably from cell wall materials (due to their greater stability), were detected. In addition, another more refractory heteropolysaccharide (418) was found - similarities between the particulate and dissolved hetero-polysaccharides led the authors to conclude that the former was the precursor of the latter. As with the reserve polysaccharides, the free dissolved monosaccharides found in natural waters (reviewed by Thurman (7)) are generally dominated by glucose with varying contributions of other sugars. Fructose is also dominant in some seawater samples (419), and mannose, in several rivers (7). These would be expected to be rapidly metabolised by microorganisms and not to contribute to the formation of humic substances.

5.3.3.3 Origin of carbohydrates in aquatic humic substances

The present study indicates that glucose, galactose, arabinose, rhamnose, and mannose are the major sugars in humic materials from estuarine and coastal environments. Their relative abundances were variable. The presence of fucose was confirmed by GC-MS but it was not determined quantitatively.

Due to the lability of free monosaccharides and reserve polysaccharides, as stated above, it would be feasible to suggest that the main contributors to humic saccharides from plankton/bacteria would be structural carbohydrates. However, glucans likely to originate from reserve polysaccharides have been detected in aquatic fulvic acids. α -1,6-linked and β -1,3-linked glucans, possibly indicative of bacterial and algal sources respectively, have been found in aquatic fulvic acids in minor amounts (216). Reasons for their preservation are not clear. The major polysaccharides were normally structural α -1,4-linked and β -1,4-linked glucans and are likely to originate from cellulose and starch. Whilst it is a labile reserve polysaccharide, stable starch 'grains' have been reported in the marine environment (338).

Other studies on aquatic humic materials have generally concentrated on their monosaccharide composition (Section 3.3.1.2), and have yielded a variety of results. Arabinose was the dominant sugar in aquatic fulvic acid from Thoreau's Bog, as well as in the suspended material isolated from the bog (293), suggesting this to be a possible precursor of the humic matter. Arabinose was also the major sugar in Como Creek fulvic acid (7), and, although the distribution of the monosaccharides in riverwater studied by Sweet and Perdue (295) were variable, arabinose and xylose showed a slight predominance. It has been suggested that arabinose may be indicative of soil organic matter (289) although its presence in fungal melanins (281) and certain diatoms (332) cannot rule out an autochthonous source.

Arabinose was found to be a major constituent of the samples extracted in the current study, in particular in May, 1986. This was the main sugar in both bogwater and creekwater fulvic acid (7,293),

resulting predominantly from terrestrial input of plant material. The present work supports a terrestrial origin for this sugar as arabinose is important in the estuarine material (sites A and B) but less so in the coastal matter (site C). In agreement with this, a marked concentration gradient of dissolved organic materials has been shown to occur in the Beaulieu estuary, resulting from the mixing of water from the heathland and bogs of the New Forest with coastal seawater (36).

Glucose is dominant to varying degrees in these samples and is likely to originate mainly from cellulose. The large proportion of glucose in the coastal water sample from February, 1986 is unexplained. It is possible that it resulted from the flushing of soil organic matter into the estuary during a period of heavy rain although the absence of high DOC levels for this site (Section 4.3.2) suggest that this was not a recent event.

Galactose and mannose are present in significant concentrations, particularly in May and August. These can originate from both terrestrial sources or from diatom cell walls (332). Galactose has additionally been found to occur in significant quantities in water-insoluble polysaccharides in marine particulate organic matter (420). The predominance of galactose and mannose in May and August may indicate planktonic input - the February sample, a time of low planktonic input, agrees with this, having smaller contributions of these sugars.

Ribose and fucose have been employed as indicators of plankton/bacterial activity as they are present in greater levels in these organisms than in higher plant tissues (416). The percentage of ribose in vascular plants was found to be around 1%, whereas that of phytoplankton varied between 4 and 15%, zooplankton between ca. 15 and 18%, and bacteria was greater than 18%. Comparing the percentage of ribose in the samples from February and August, 1986, the material isolated in February showed values of < 1.2%, 2.7%, and < 2.4% ribose for sites A to C respectively, whereas that isolated in August gave values of 2.3%, 6.1%, and 8.0% for sites A to C respectively. Therefore, the winter samples indicated little planktonic input at all sites whereas those isolated in August indicated increased planktonic

input as salinity increased. A lower planktonic input in February than August is in agreement with the trends in the galactose and glucose data described above.

Finally, rhamnose is an important component of all samples. This has been found in cell walls of marine diatoms (e.g., (421)) but also as a major constituent of certain bacteria (416). Its ubiquitous presence in all samples from all seasons may indicate its bacterial rather than planktonic origin.

5.3.4 Conclusions

Glucose, galactose, arabinose, rhamnose, mannose, fucose, xylose, and ribose were found to be present in the humic samples from estuarine and coastal environments. A trace of lyxose was also detected in the May samples by GC-MS analysis.

The presence of significant quantities of arabinose in sites A and B but not at site C suggest terrestrial influence from soil humic material at these two sites, with lower input in November. The dominance of glucose in some samples is suspected to result from cellulose material from the same source. A significant increase in the percentage of glucose in the sample from site C, February, 1986, may be indicative of increasing terrestrial input due to a period of heavy rain.

Planktonic input is thought to contribute to a greater extent in the May and August samples as evidenced by increased concentrations of galactose and mannose, significant components of diatom cell walls. An increased percentage of ribose in August compared to February, indicating increased planktonic input, is in agreement with this. An increase in ribose with increasing salinity for the August samples suggests greater planktonic input at the coastal site at this time.

Thus, by considering the monosaccharide content of aquatic humic materials, it can be concluded that site A humic substances consist of material of a largely terrestrial origin and those from site B also contain a significant terrestrial input but, in addition, originate from a degree of planktonic input. Site C humic matter contains minor

contributions from terrestrial sources with planktonic input dominating.

These results indicate that this method is suitable for detecting monosaccharides in humic samples. Prior to further more quantitative analyses, a suitable internal standard should be found. Increased sample size would also improve the accuracy and precision of the data.

CHAPTER SIX

ANALYSIS OF LIPIDS IN ESTUARINE AND MARINE HUMIC MATERIALS

This chapter describes the modification of a GC method for lipid analysis and an attempt to determine the hydrolysable lipid fraction of estuarine and marine humic materials.

6.1 A REVIEW OF METHODS USED IN LIPID ANALYSIS

A comprehensive review of the analysis of lipids, including their isolation, separation and identification, has been written by Christie (422). A mixture of chloroform and methanol (2:1 (v/v)) has generally been employed to extract simple lipids (422), although certain complex lipid classes, have often required alternative systems, e.g., water-saturated n-butanol for lyso-phospholipid extraction (423).

Removal of non-lipid contaminants from a chloroform-methanol extract can be achieved by washing it with water or a dilute salt solution (424). Class separation of lipids may be performed by TLC or liquid chromatography or the lipids may first be hydrolysed (saponified) with dilute aqueous alcoholic alkali (422), or transesterified to obtain simpler components. These are then analysed by thin-layer, liquid or gas chromatography, the latter generally being the most popular method. This section reviews the application of these techniques to lipid analysis.

6.1.1 Thin-layer chromatography

TLC can be applied to both the fractionation of complex mixtures into individual lipid classes and to the separation of components within these classes (422,425,426).

Class fractionation has generally been performed on silica gel layers using non-polar solvents, such as hexane or petroleum ether, containing varying amounts of more polar solvents, such as diethyl ether and, in some cases, acetic acid. Numerous solvent systems, both for one- and two-dimensional TLC of lipid classes, have been documented by Kirchner (426). Within-class separations of the more

polar lipids, such as phospholipids and glycolipids, have also been performed on silica gel layers (sometimes containing 10% of, for example, ammonium sulphate or sodium acetate (427)), generally using more polar solvent systems, such as chloroform-methanol-water (e.g., (427)). Non-polar lipids within the same class, on the other hand, have frequently been separated on impregnated silica gel or Kieselguhr.

Reversed-phase TLC, in which the silica gel (or Kieselguhr) has been silanised or impregnated with, for example, silicone oil, paraffin or a high molecular weight alkane (such as tetradecane), has been used for separating various lipids in the same class. Those separated have included triglycerides, wax esters, free fatty acids and fatty acid methyl esters, generally employing solvent systems containing acetic acid or acetonitrile and water (425). Argentation TLC, in which silica gel is impregnated with silver nitrate, has been applied to the analysis of, for example, cholesteryl esters, triglycerides, free fatty acids and fatty acid methyl esters, generally using hexane/ether solvent systems (425). This technique separates lipids according to the number, configuration and, to some extent, position of double bonds due to the ability of silver compounds to reversibly form polar complexes with double bonds in aliphatic lipid molecules. It is, therefore, particularly useful in separating cis-trans isomers, which is not always possible using other techniques. Its application to lipid analysis has been reviewed by several workers (e.g., (425,428)).

Detection of lipids following TLC has frequently been performed by (422):

- exposure to iodine vapour.
- spraying with 2',7'-dichlorofluorescein in ethanol or an aqueous solution of Rhodamine 6G, and inspecting the plate under ultraviolet light - the lipids are revealed as yellow and pink spots, respectively.
- spraying with 50% sulphuric acid, and heating the plate to char the lipids - aqueous ammonium bisulphate (429) and 3% cupric acetate (430) in 8% orthophosphoric acid have also been employed, yielding less corrosive vapours on heating.

Both 2',7'-dichlorofluorescein and Rhodamine 6G are non-destructive reagents and so are suitable for preparative TLC investigations. A variety of alternative reagents have also been less frequently employed, some of which are mentioned in Kirchner's review (426). Concerning reversed-phase analyses, visualisation with iodine vapour may be performed (431), although charring is inappropriate with layers impregnated with paraffin, etc. In addition, although 2',7'-dichlorofluorescein can be adopted for visualisation on Kieselguhr G impregnated with paraffin, it is not suitable for use on impregnated silica gel G layers (431). Detection following argentation chromatography can be carried out with the fluorescein reagent or by charring organic compounds with sulphuric acid (432).

Quantitation of the lipids can be achieved in several ways, including charring followed by photodensitometry, fluorescence measurements after spraying with a fluorescent dye, or determination of the fatty acid content after extraction of the lipid from the TLC plate, derivatisation, and GC analysis (422). TLC with flame ionisation detection has also been performed (433) but presently offers no advantages in accuracy or reproducibility over the conventional methods (422).

6.1.2 Gas chromatography

A review of advances in capillary GC applied to lipid analysis has recently been published (434). Although low molecular weight free fatty acids have been analysed by gas chromatography (GC) on columns such as FFAP (free fatty acid phase) and Carbowax 20M (435), lipids are generally too polar or too involatile to analyse directly by this technique. Hence, they must be converted to non-polar and/or volatile derivatives prior to analysis, the most common being fatty acid methyl ester (FAME) derivatives. These have mainly been analysed on polar (436) capillary columns although non-polar columns have been tested (436).

The formation of methyl esters in lipid analysis has been comprehensively reviewed (422,437,438). The majority of investigators have adopted acid- or base-catalysed transesterification methods. These are by far the most convenient techniques for preparing methyl

esters, as prior saponification is not required. Acid-catalysed transesterifications (and esterifications of free fatty acids) are performed by heating the lipids in anhydrous methanol in the presence of an acidic catalyst such as anhydrous hydrogen chloride, concentrated sulphuric acid, boron trifluoride or boron trichloride (422). Boron trifluoride in methanol has proved a very popular reagent despite its limited shelf-life and the production of artefacts (e.g., (439)). Christie (422) preferred methanolic hydrogen chloride as a general-purpose esterifying agent, suggesting that boron trifluoride in methanol has been overrated. Sheppard and Iverson (438) reviewed the literature on the esterification of free fatty acids and concluded that selected methods using all the aforementioned reagents (except boron trichloride, which was not considered) were satisfactory for high molecular weight fatty acids. Formation of heavier derivatives, e.g., butyl esters (440), however, was recommended for low and medium molecular weight fatty acids. A new transesterification procedure employing aluminium chloride in methanol, has recently been developed by Seguro (441). No yield of methyl esters resulted from esterification of free fatty acids, as is the case of base catalysis.

Base-catalysed transesterification reactions have generally been used less than their acid counterparts, possibly because free fatty acids are not esterified simultaneously. Sodium methoxide in anhydrous methanol (0.5 moles/l) is the most popular reagent (442), but potassium methoxide and potassium hydroxide have also been used. Although not esterified with sodium methoxide, free fatty acids react with N,N-dimethylformamide dimethyl acetal in the presence of pyridine (443) to yield FAMES. Alternatively, quaternary ammonium salts of the free fatty acids are formed and these yield FAMES on thermal degradation when injected into the heated injection port of a GC (444).

Diazomethane may also be used to prepare methyl esters from the corresponding free fatty acids (445). The need for prior saponification of fatty acid esters, together with the toxicity and potentially explosive nature of diazomethane, however, indicate that this method is best avoided if at all possible. Other esterification reactions have also been employed, including the formation of methyl

esters of fatty acids by reaction of their silver salts with methyl iodide (446), and esterification of free fatty acids by heating them in a basic medium with dicyclohexylamine and dimethylsulphate (436). The former method is time-consuming and tedious, however, offering few advantages over the aforementioned procedures, and the latter has not been tested for a range of fatty acids (437).

The volatility of short-chain fatty acids has been reduced by forming butyl (440) or benzyl (447) esters. The methods described can often be adapted merely by substituting a suitable alcohol for methanol, e.g., ethyl esters have been prepared using sodium ethoxide in ethanol (448), propyl esters, using sodium propoxide in propanol (448), and butyl esters, using boron trifluoride-butanol (440). TMS esters of fatty acids have been prepared for GC analysis (449) although Christie (437) stated that they are not as useful as the alkyl esters, as the less polar silicone stationary phases required do not have such good resolving powers as the polar phases used for alkyl ester analysis. TMS derivatives have also been formed where hydroxyl functionalities are present, e.g., in partial glycerides (450).

As in the analysis of carbohydrates, pyrolysis GC of lipids can also be performed. Although pyrolysis of fats and waxes results in relatively simple chromatograms, yielding series of alkenes and alkanes (207), these products are not limited to lipid pyrolysates which restricts the use of this technique for targeting lipidic components in complex mixtures. Despite this limitation, pyrolysis studies have been performed in lipid analysis. For example, alkenes from the pyrolysates of a desert soil have been attributed to lipid precursors (207) and a pyrolysis method has been employed involving thermal decarboxylation of long-chain fatty acid salts, for the analysis of lipophilic groups in commercial soap (451).

6.1.3 Liquid chromatography

Various liquid chromatographic methods have been employed in lipid analysis, such as adsorption, normal and reversed-phase partition, gel-permeation and ion-exchange chromatography. Their applications have been extensively reviewed (422,452), and so only a summary of the systems employed will be given here.

Adsorption chromatography has been performed as both a low resolution preparative procedure, or as a higher resolution analytical procedure (HPLC). The most popular adsorbent for low resolution column chromatography is silicic acid with the components being removed from the column by stepwise elution with solvents of increasing polarity. Other adsorbents which have also been employed include Florisil (453), which has been used to separate hydrocarbons, cholesterol esters, triglycerides, free sterols, diglycerides, monoglycerides, and free fatty acids (453), and in the separation of normal and hydroxy FAMES (454). Extensive alteration of lipids has been documented with alumina (422). Modified adsorbents, such as those impregnated with silver nitrate (argentation chromatography) (455), have also been adopted in the liquid chromatography of lipids.

Adsorption chromatography has generally been applied to separations of lipid classes. This is because adsorption depends on the polarity of the molecules and so, for non-polar lipids such as fatty acids, for example, adsorption depends more on the number of fatty acid groups rather than the hydrocarbon chain-length (unless polar functional groups are present). If similar classes vary in the polar functional group content, however, separation can be achieved, e.g., separation of normal and hydroxy FAMES on Florisil (454).

As stated above, both adsorption and partition modes can operate in HPLC analyses. The favoured adsorbent for adsorption normal-phase HPLC is silicic acid with gradient elution usually being preferred. Both normal-phase and reversed-phase partition HPLC have been widely used for the analysis of lipids although reversed-phase currently seems to be the most popular method. Chromatography is performed on reversed-phase C₁₈-based columns using either isocratic or gradient elution of polar solvent systems containing such solvents as methanol,

acetonitrile, water, etc. Partition HPLC methods, in addition to being suitable for class separations (e.g., (456)), can also be employed to separate individual components within classes, for example, steroids (457) and FAMES (458).

Other liquid chromatography methods employed in lipid analysis include gel-permeation chromatography (GPC) (e.g., in the analysis of waxes (459)) and ion-exchange chromatography (e.g., (460)). These are not as widely used as the aforementioned techniques. GPC separates the lipids according to size and so is generally a class separation method. Ion-exchange chromatography, employing diethylaminoethyl cellulose, has been used for the separation of complex lipids (460). Although not completely understood, the principle is thought to be a combination of ion-exchange chromatography of ionic lipidic moieties and adsorption of highly polar, non-ionic moieties (422). For true ion-exchange to occur, the lipids must contain acidic or basic functional groups.

Detection of lipids following liquid chromatography can cause problems. In preparative liquid chromatography (LC), aliquots of eluent can be removed and determined spectrophotometrically (by determining the decrease in absorbance of potassium dichromate on addition of the lipid) (461) or fractions can be checked by TLC (422). On-line monitoring with conventional HPLC detectors proves difficult as lipids, unless unsaturated, are generally transparent to ultraviolet radiation. Formation of UV-absorbing derivatives has been attempted (452), but this loses one of the major advantages of LC over GC (i.e., analysis of underivatized samples). In addition, a separation technique is required for the derivatives if pre-column derivatization is carried out, and a loss in separation efficiency may result from mixing with post-column derivatization (452). Refractive index detectors can be used if isocratic elutions are carried out, which is generally true in GPC or if the lipids are closely related (462), but are not suitable in gradient elution experiments. Transport flame ionisation detectors (transport FIDs) have been used (423) in which a portion of the eluent is deposited onto a moving wire, the solvent is removed and then the sample transported into an FID. Several commercial instruments were apparently unsuccessful and are no longer available. Various other detectors have been used to a

greater or lesser degree, including IR, MS, radiochemical, a selection of which have been discussed by Aitzetmüller (452).

It can be concluded, therefore, that a selection of LC methods are applicable to lipid analysis. However, due to the problem of finding a suitable quantitative detection system, their application has not been as wide-spread as it could have been. GC analysis often remains the method of choice despite the need for derivatisation.

6.1.4 Supercritical fluid chromatography

Supercritical fluid chromatography has also been employed for the analysis of free (463) and esterified (464) fatty acids, in addition to glycosphingolipids (465) but is not, as yet, used for routine analyses and so will not be discussed further here.

6.2 DEVELOPMENT OF A METHOD FOR LIPID ANALYSIS

Transesterification using the boron trifluoride-methanol reagent was chosen for the present study. This reagent has been widely employed in lipid analyses. It was found to be an efficient esterification catalyst as early as 1931 (466) and was first used by Metcalfe and Schmitz (467) to prepare FAMES for GC analysis.

Several variants of the original method have been adopted over the years. Transesterification of a selection of lipids, including fatty acids, was studied by Morrison and Smith (468), who found that reaction of fatty acids, mono- and diglycerides, and a selection of phospholipids, was complete within 30 minutes. Due to the low solubility of triglycerides and sterol esters in methanol, however, the reaction of these lipids may not go to completion in this time. The conversion of glycerol tripalmitate to methyl palmitate has been shown to take between 50 and 400 minutes, depending on the percentage of boron trifluoride present (ca. 50 minutes for 14% (w/v) boron trifluoride in methanol) (468). Reaction times were reduced by adding a solvent (benzene) to solubilise the lipids.

The prolonged transesterification time of certain lipids led Metcalfe *et al.* (469) to perform saponification (to produce free fatty

acids) followed by esterification with boron trifluoride-methanol. A modified extraction scheme was also adopted. Some mixtures have only been extracted using an organic solvent, such as pentane (468), hexane (470), petroleum ether (467), and water, but Metcalfe *et al.* (469) washed the reaction medium with a saturated solution of sodium chloride prior to extraction with petroleum ether, which was shown to improve recoveries of short-chain FAMES over the previously employed extraction procedure.

A selection of problems have been encountered with transesterification using boron trifluoride-methanol. In addition to losses during aqueous extraction procedures, incomplete recoveries of short-chain fatty acid esters have been shown to result on refluxing the esterification medium (437). Losses of unsaturated esters, production of artefacts and differences between reagent lots have also been observed to varying degrees by different workers (439,468,470,471), as have variations with both the sample size (472) and the amount of reagent employed (471).

Despite these problems, transesterification with boron trifluoride-methanol has been used extensively for lipid analyses. A similar procedure to that of Metcalfe *et al.* (469) was initially adopted in the current work and subsequently modified in an attempt to increase sample yields from microgram amounts of lipid material.

6.2.1 Standards

The following lipid standards were prepared:

Lipid standard A (100 ng/ μ l)

Solvent : Diethyl ether

Myristic acid (free fatty acid)
Methyl palmitate (saturated FAME)
Methyl palmitoleate (unsaturated FAME)
Cholesterol stearate (cholesteryl ester)
Glycerol trioleate (triglyceride)
Erucic acid (free fatty acid)

Lipid standard B (100 ng/ μ l)

Solvent : Propan-2-ol

DL-dilauroyl- α -lecithin (phospholipid)

L-diarachidoyl- α -lecithin (phospholipid)

Lipid standard C (200 ng/ μ l, diluted to give a 40 ng/ μ l solution)

Solvent : Chloroform

Lauric acid (free fatty acid)

L-dimyristoyl- α -lecithin (phospholipid)

Palmitic acid (free fatty acid)

Methyl palmitoleate (unsaturated FAME)

Cholesterol stearate (cholesteryl ester)

Glycerol trioleate (triglyceride)

L-dibehenoyl- α -lecithin (phospholipid)

Lipid standard D (100 μ g/ μ l)

Solvent : Chloroform

Palmitic acid (free fatty acid)

Glycerol trioleate (triglyceride)

The following FAME standards were prepared in cyclohexane:

FAME standard A (100 ng/ μ l, diluted to give a 20 ng/ μ l solution)

Methyl laurate (12:0)

Methyl γ -linolenate (γ -18:3)

Methyl myristate (14:0)

Methyl arachidate (20:0)

Methyl palmitate (16:0)

Methyl eicosenoate (20:1)

Methyl palmitoleate (16:1)

Methyl eicosadienoate (20:2)

Methyl heptadecanoate (17:0)

Methyl eicosatrienoate (20:3)

Methyl stearate (18:0)

Methyl behenate (22:0)

Methyl oleate (18:1)

Methyl erucate (22:1)

Methyl linoleate (18:2)

Methyl tricosanoate (23:0)

Methyl linolenate (18:3)

FAME standard B (100 ng/ μ l, diluted to give 5, 10 and 20 ng/ μ l solutions)

Methyl palmitate (16:0)

Methyl oleate (18:1)

FAME standard C (40 ng/ μ l)

Methyl myristate (14:0)

Methyl palmitoleate (16:1)

Methyl heptadecanoate (17:0)

FAME standard D (10 ng/ μ l)

Methyl heptadecanoate (17:0)

Methyl linoleate (18:2)

Methyl erucate (22:1)

Two internal standards (200 ng/ μ l) were also prepared, one containing methyl pentadecanoate (15:0) and the other containing methyl heptadecanoate (17:0).

All standard lipid solutions were stored frozen, in the dark, and under an atmosphere of nitrogen to minimise autoxidation of the unsaturated lipids.

6.2.2 Method

6.2.2.1 Transesterification

Lipid standards A and B (50 μ l of each) were placed in a 50 ml Pyrex boiling tube, and taken to dryness under a stream of nitrogen. A reagent blank was also analysed simultaneously. 4 ml of methanolic sodium hydroxide (0.5 moles/l) were added to the mixture which was heated under reflux for 30 minutes. 14% (w/v) boron trifluoride in methanol (1 ml) was then added and the solution heated under reflux for a further five minutes. This was then shaken with 10 ml of a saturated solution of sodium chloride. The mixture was extracted twice with cyclohexane (10 ml), the combined organic layers were washed with saturated sodium bicarbonate solution (10 ml), and dried

over anhydrous sodium sulphate. The cyclohexane extract was then decanted into a glass sample vial and concentrated to ca. 1 ml under a stream of nitrogen. This was transferred to a round-bottom sample vial (1 ml), evaporated to dryness, and redissolved in cyclohexane (100 μ l).

6.2.2.2 Gas Chromatography

The FAMES were analysed by capillary GC, using a Pye Series 104 gas chromatograph, fitted with a flame ionisation detector. Initially, chromatographic separations were performed on 15m, 0.32 mm internal diameter, wall-coated open tubular (WCOT), fused silica capillary columns, dynamically coated, 'in-house', with free fatty acid phase (FFAP). Stationary phase was removed from the first meter of the columns to serve as a retention gap. Later analyses were carried out using a 10 m, 0.32 mm internal diameter, immobilised Carbowax 20M (0.5 μ m film thickness) WCOT fused silica capillary column. In this instance, 1 m deactivated fused silica capillary column was used as a retention gap.

In both cases, the following operating conditions were used:

Carrier gas	:	Oxygen-free nitrogen	:	FFAP - 1 ml/min CW20M - 2 ml/min
Injection system	:	Cold on-column injection with nitrogen purge		
Temperature programme	:	FFAP - 90°C (3 mins) \rightarrow 12°C/min \rightarrow 250°C (40 mins) CW20M - 90°C (1 min) \rightarrow 12°C/min \rightarrow 200°C (45 mins)		
Amplifier attenuation	:	typically 20		
FID	:	Hydrogen	:	ca. 40 ml/min
		Air	:	ca. 175 ml/min

6.2.2.3 Gas Chromatography-Mass Spectrometry

Peak identification in the case of environmental samples was confirmed by capillary GC-MS. This was carried out using a Perkin-Elmer 8500 gas chromatograph coupled to a Finnigan MAT ion trap detector. The instrument was run in full-scan mode, scanning from 40 to 400 a.m.u. every second. Mass spectra were recorded using an IBM PC-AT computer.

The following operating conditions were employed:

Gas Chromatograph

GC column : 12 m, 0.22 mm internal diameter, bonded phase BP1 (0.25 μ m film thickness) WCOT fused silica capillary column

Carrier gas : High purity helium with a flow rate of ca. 0.3 ml/min

Injection system : Splitless injection (300°C), with a splitless time of 20 seconds

Temperature programme : 100°C (2 mins) \rightarrow 10°C/min \rightarrow 270°C (45 mins)

Interface

Type : Open-split interface connecting the end of the GC column to a flow restrictor (1.2 m, 0.15 mm internal diameter, DB-5 fused silica capillary column). The transfer line (open-split interface and flow restrictor) was maintained at 275°C

Mass Spectrometer

Ionisation mode : Electron impact (positive ion)

6.2.3 Results and discussion

6.2.3.1 Gas chromatography of the fatty acid methyl esters

The FAMES in standard A were separated on FFAP and Carbowax 20M capillary columns. Similar chromatograms resulted - a typical chromatogram on Carbowax 20M is shown in Figure 6.1(a). GC-MS analyses were performed using a BP1 capillary column - a chromatogram of FAME standard A on this column is presented in Figure 6.1(b).

Multiple GC analyses of FAME standard A were performed, within one day, to assess short-term chromatographic precision. Chromatograms were run on both FFAP and Carbowax 20M capillary columns, and peak heights and relative peak heights (versus an internal standard, 17:0) were measured. The resulting data (Tables 6.1 and 6.2) show that measurements of peak heights relative to the internal standard are preferable to those of absolute peak height. Long-term precision measurements (over a period of six months) of FAME standard A on Carbowax 20M indicate that relative peak heights show a slight increase in the % RSD compared to the short-term precision data (Table 6.2). FAME standard A was therefore run on the same day as the samples for quantitative analysis.

6.2.3.2 Development of a modified method for trace level lipid analysis

Application of the aforementioned method to lipid standards A and B yielded essentially no FAMES. A number of modifications to the method were therefore performed.

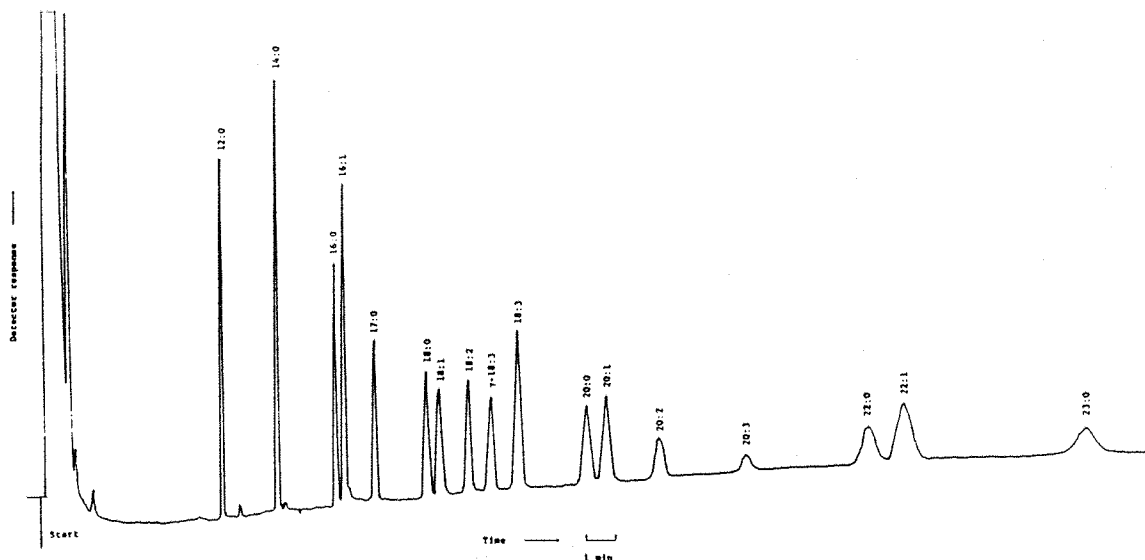
6.2.3.2.1 Reaction time

Incomplete recoveries of FAMES may result from:

- incomplete reaction of lipids with the boron trifluoride-methanol reagent.
- prolonged refluxing of the esterification medium.
- losses onto glassware.

Figure 6.1 GC traces and peak assignments of the mixed FAME standard

(a) On Carbowax 20M



(b) On BP1

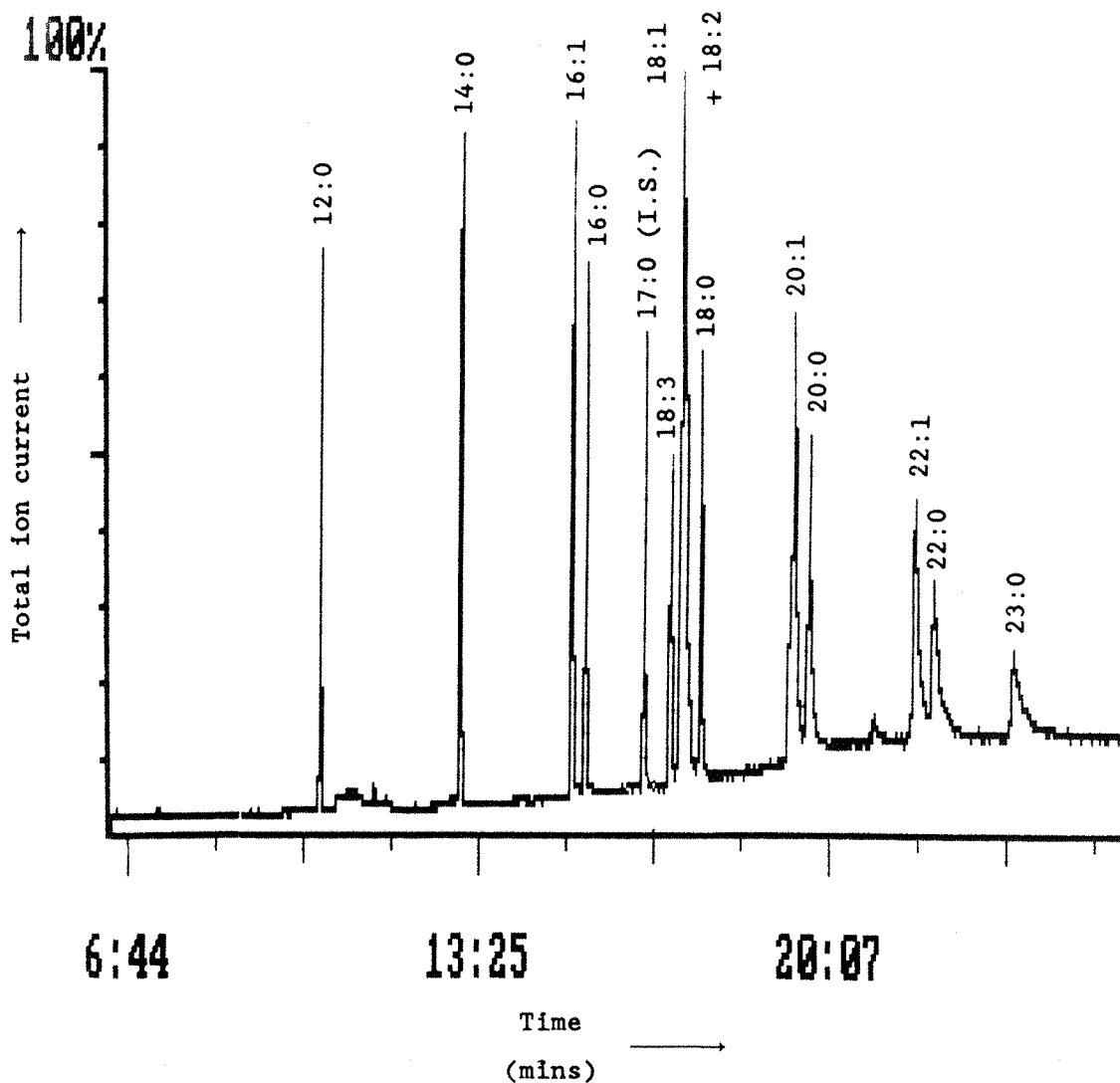


Table 6.1 Repeatability data for multiple GC determinations of the mixed FAME standard using the FFAP column

Fatty acid methyl ester	n	REPEATABILITY (SAME DAY)			
		Peak height			
		Absolute (cm)		Relative to to 17:0	
		Mean	%RSD	Mean	%RSD
12:0	4	13.9	7.6	1.97	2.9
14:0	2	15.2	NC	1.76	NC
16:0	4	8.9	8.3	1.34	1.1
16:1	3	11.7	13	1.19	1.3
17:0	4	6.7	9.3	1.00	0
18:0	4	5.4	8.7	0.82	2.2
18:1	4	5.0	7.5	0.43	2.9
18:2	4	5.3	13	0.73	4.4
γ -18:3	4	4.6	13	0.50	7.5
18:3	4	8.2	11	0.57	3.0
20:0	4	4.1	11	0.55	1.7
20:1	4	4.6	11	0.51	1.9
20:2	4	2.4	14	0.35	4.8
20:3	4	0.9	12	0.28	7.0
22:0	4	2.4	8.4	0.36	4.2
22:1	4	3.5	11	0.30	8.1
23:0	4	1.8	12	0.28	6.8

NC %RSD was not calculated

Table 6.2 Repeatability and reproducibility data for multiple GC determinations of the mixed FAME standard using the immobilised Carbowax 20M column

Fatty acid methyl ester	REPEATABILITY (SAME DAY)					REPRODUCIBILITY (LONG-TERM)				
	n	Peak height				n	Peak height			
		Absolute (cm)		Relative to 17:0			Absolute (cm)		Relative to 17:0	
		Mean	%RSD	Mean	%RSD		Mean	%RSD	Mean	%RSD
12:0	4	12.0	9.0	2.18	4.6	3	13.6	10	2.13	1.8
14:0	4	14.5	6.7	2.05	3.0	2	15.4	NC	2.00	NC
16:0	4	7.4	9.1	1.43	2.6	3	8.7	14	1.45	3.9
16:1	4	9.8	8.6	1.29	1.9	3	11.4	13	1.30	2.3
17:0	4	5.2	7.7	1.00	0	4	7.6	43	1.00	0
18:0	4	4.1	7.1	0.79	2.6	4	6.0	46	0.79	2.2
18:1	4	3.4	6.4	0.38	3.4	4	5.0	47	0.37	3.8
18:2	4	3.5	7.9	0.63	1.3	4	5.5	49	0.66	5.1
γ -18:3	4	2.8	8.3	0.40	2.0	4	4.7	59	0.44	14
18:3	4	4.9	7.4	0.45	1.3	4	8.1	55	0.46	12
20:0	4	2.5	3.7	0.44	6.1	4	3.6	45	0.42	2.7
20:1	4	2.8	6.5	0.40	6.2	4	4.0	50	0.39	5.7
20:2	4	1.3	6.5	0.25	4.6	4	2.0	52	0.26	8.6
20:3	4	0.5	5.1	0.19	4.3	4	0.8	58	0.20	11
22:0	4	1.2	3.5	0.22	8.1	4	1.7	52	0.22	7.9
22:1	4	1.7	5.0	0.19	5.1	4	2.6	51	0.18	6.9
23:0	4	0.8	5.1	0.16	11	4	1.2	51	0.16	8.3

NC %RSD was not calculated

To check the time required for complete transesterification, both base hydrolysis and esterification were carried out for one hour. Another approach was tried on 100 μ l each of lipid standards A and B to see if prolonged refluxing was causing losses. The reaction times were reduced - the hydrolysis to two minutes and the esterification to one minute. In addition, the reaction mixture was only extracted with 1 x 10 ml cyclohexane.

Extended transesterification gave no recovery of lipids but reducing the reaction time resulted in a low yield of FAME's (less than 20%). Attempts were therefore made to increase the yield of the transesterification method with the reduced reaction time (method 2).

6.2.3.2.2 Cyclohexane extraction

Lipid standards A and B (100 μ l of each) were analysed using method 2 but extracting twice with cyclohexane (2 x 10 ml). Increased yields did not occur as a result of the dual extraction. In fact, they were lower which was suspected to be due to the increased time required to concentrate 20 ml solvent under a stream of nitrogen. Thus, a single cyclohexane extraction was maintained.

6.2.3.2.3 Silylation of glassware

Recoveries of lipid standards A and B (50 μ l of each) were compared using both silylated and non-silylated glassware. No obvious improvement in the yield resulted on using the silylated glassware and so silylation was not adopted.

6.2.3.2.4 Removal of base hydrolysis

Direct transesterification of lipids without prior saponification has been carried out by several workers (467,468). As a reduction in the hydrolysis time improved the recovery of the FAMES (Section 6.2.3.2.1), the possible elimination of this step was investigated. The following experiments were therefore performed:

- (i) Lipid standard solution C (50 μ l) was taken to dryness, 14% (w/v) boron trifluoride in methanol (2 ml) was added, and the

mixture was heated under reflux for five minutes. It was then extracted and washed as in method 2 except that a smaller aliquot of cyclohexane (5 ml) was used.

- (ii) Sodium hydroxide in methanol (0.5 moles/l; 2 ml) was added to the dried lipid standard solution C (50 μ l) and heated under reflux for three minutes. 14% (w/v) boron trifluoride in methanol (1 ml) was added to this and the solution was heated under reflux for a further minute. It was then extracted and washed as above.

The results (Table 6.3) indicate a slight improvement on removal of the hydrolysis stage, particularly for the shorter-chain FAMES. As minimal sample manipulation is preferable, it was decided to omit this step in subsequent analyses (method 3).

It was also noted that improved recoveries with saponification resulted, compared with the original data from method 2 (Section 6.2.3.2.1). This was thought to be due to a reduction in the volume

Table 6.3 A comparison of the approximate yield of FAMES from a standard lipid solution with and without prior saponification

Lipid	FAME resulting	Approximate % yield	
		With saponification	Without saponification
Lauric acid	12:0	14, 3.1 ⁺	24
L-dimyristoyl- α -lecithin	14:0	33, 28	48
Palmitic acid	16:0	36, 45	66
Methyl palmitoleate	16:1	37, 41	49
Cholesterol stearate	18:0	58, 66	66
Glycerol trioleate	18:1	46, 52	66
L-dibehenoyl- α -lecithin	22:0	37, 46	47

+ This was thought to be low due to the volatility of lauric acid methyl ester

of cyclohexane extractant used in this experiment, leading to a reduction in the time required for concentration under a stream of nitrogen. This supports conclusions in Section 6.2.3.2.2.

6.2.3.2.5 Simplification of the extraction procedure

Various extraction schemes have been employed to recover the FAMES following transesterification (detailed in the introduction to Section 6.2). The following experiments were undertaken to determine the effects of such procedures:

14% (w/v) boron trifluoride in methanol (2 ml) was added to the dried lipid standard C (50 μ l) and heated under reflux for five minutes. It was then:

- (i) shaken with saturated sodium chloride solution (5 ml) and extracted with cyclohexane (1 x 5 ml). The organic layer was washed with saturated sodium bicarbonate solution (5 ml), dried over anhydrous sodium sulphate and taken to dryness.
- (ii) extracted with cyclohexane (1 x 5 ml), and washed with doubly distilled water (1 x 5 ml). The organic layer was removed and taken to dryness.

Qualitative analysis (Table 6.4) suggests little difference between the two methods for the longer-chain FAMES, i.e., 16:0 and above. However, there appears to be increased loss of the short chain acids when 'salting out' is not employed, in agreement with other workers (469). This data must be treated with care, however, as variable losses of 12:0 and 14:0 FAMES were observed in earlier experiments (Table 6.3) when 'salting out' was performed. This suggests possible irreproducible losses of the short-chain acids, either on transesterification, or on concentration under a stream of nitrogen. Preferential losses of short-chain fatty acids have also been shown to occur as sample size is reduced (472). Therefore, at the present time, quantitation can only be performed on the longer-chain FAMES (16:0 or greater).

As the two extraction methods yielded similar recoveries for long-chain FAMES, extraction with cyclohexane and doubly distilled water

Table 6.4 The effect of two different extraction procedures

Lipid	FAME resulting	Approximate % yield	
		NaCl, cyclohexane, and NaHCO ₃ extraction ⁺	Cyclohexane and water extraction
Lauric acid	12:0	24	3.3
L-dimyristoyl- α -lecithin	14:0	48	32
Palmitic acid	16:0	66	66
Methyl palmitoleate	16:1	49	44
Cholesterol stearate	18:0	66	85
Glycerol trioleate	18:1	66	53
L-dibehenoyl- α -lecithin	22:0	47	59

+ Results taken from Section 6.2.3.2.4

was adopted in subsequent analyses in order to minimise sample manipulation (method 4). In addition, as lipid recoveries were still not quantitative, it was decided that the procedure should be scaled-up, using a more concentrated standard, as sample size has been shown to affect the recovery of a selection of FAMES from triglycerides using the boron trifluoride-methanol transesterification method (472).

6.2.3.2.6 Testing different organic extraction solvents

A variety of organic solvents have been used to recover the FAMES from the transesterification mixture, including pentane (468), hexane (470) and petroleum ether (467). Cyclohexane was currently employed instead of petroleum ether as it is easier to purify. A comparison of pentane, hexane and cyclohexane as extractants was performed.

The concentrated lipid standard D (50 μ l) was taken to dryness and 14% (w/v) boron trifluoride in methanol (5 ml) added. The mixture was heated under reflux for a few minutes and then extracted with

either:

- (i) cyclohexane (5 ml) and doubly distilled water (5 ml).
- (ii) pentane (5 ml) and doubly distilled water (5 ml).
- (iii) hexane (5 ml) and doubly distilled water (5 ml).

The organic layer was removed and the extraction repeated twice more. The resultant extracts (3 x 5 ml) were taken to dryness separately and redissolved in cyclohexane as before.

The results (Table 6.5) indicate:

- (a) The extraction solvent did not affect the recovery of palmitic acid (16:0) and glycerol trioleate (18:1).
- (b) Recovery of palmitic acid was quantitative (greater than or equal

Table 6.5 A comparison of cyclohexane, pentane and hexane as extraction solvents

Solvent	Extraction number	Percentage yield		Total extracted (%)	
		16:0	18:1	16:0	18:1
Cyclohexane	1	93	70	100	74
	2	6.5	4.4		
	3	0.6	0		
Pentane	1	91	72	95	74
	2	4.4	2.1		
	3	ND	ND		
Hexane	1	90	71	98	76
	2	7.1	4.8		
	3	0.6	0		

ND Not determined

to 95% in all cases).

- (c) The recovery of glycerol trioleate was better than in the previous investigations but it was less than palmitic acid. Losses of unsaturated esters have been observed by other workers (468,470).

As the identity of the solvent did not affect the recoveries of the FAMES, the use of cyclohexane was continued.

6.2.3.2.7 Repeatability of experimental method

Having achieved more acceptable recoveries, the repeatability of the method was checked by performing four analyses.

Lipid standard D (10 μ l) was taken to dryness, 14% (w/v) boron trifluoride in methanol (2 ml) was added, and the mixture was heated under reflux for two hours. This was then extracted three times with cyclohexane and doubly distilled water (5 ml) as in Section 6.2.3.2.6. In experiments 1 and 2, the three organic extracts were individually made up to 50 ml with cyclohexane, whilst in experiments 3 and 4, the combined extracts were made up to 50 ml.

Overall, the means of the four analyses (Table 6.6) gave acceptable recoveries and repeatabilities, the spread being largely due to poor yields in experiment 2.

Therefore, a preliminary investigation was carried out on the lipid content of humic material.

6.3 TREATMENT OF HUMIC MATERIAL WITH BORON TRIFLUORIDE IN METHANOL

Three samples and an XAD blank (November, 1985) were analysed. 14% boron trifluoride in methanol (3 ml) was added to the humic material (ca. 10 mg) and the mixture was sonicated for 20 seconds before heating under reflux for three hours. The solution was then extracted three times with cyclohexane (3 x 5 ml) and washed with doubly distilled water (3 x 5 ml). The combined organic layers were dried over anhydrous sodium sulphate and the solvent was decanted off. The extract was then evaporated under a stream of nitrogen, and the

Table 6.6 Percentage yields from four analyses of the concentrated lipid standard D

Experiment	Extraction number	% yield		Total % extracted	
		16:0	18:1	16:0	18:1
1	1	106.5	95	108	95
	2	1.5	0		
	3	0	0		
2	1	76	74	79	76
	2	3	2		
	3	0	0		
3	Total extracted	103	98	103	98
4	Total extracted	89	92	89	92
Mean				95	90
% RSD				14	11

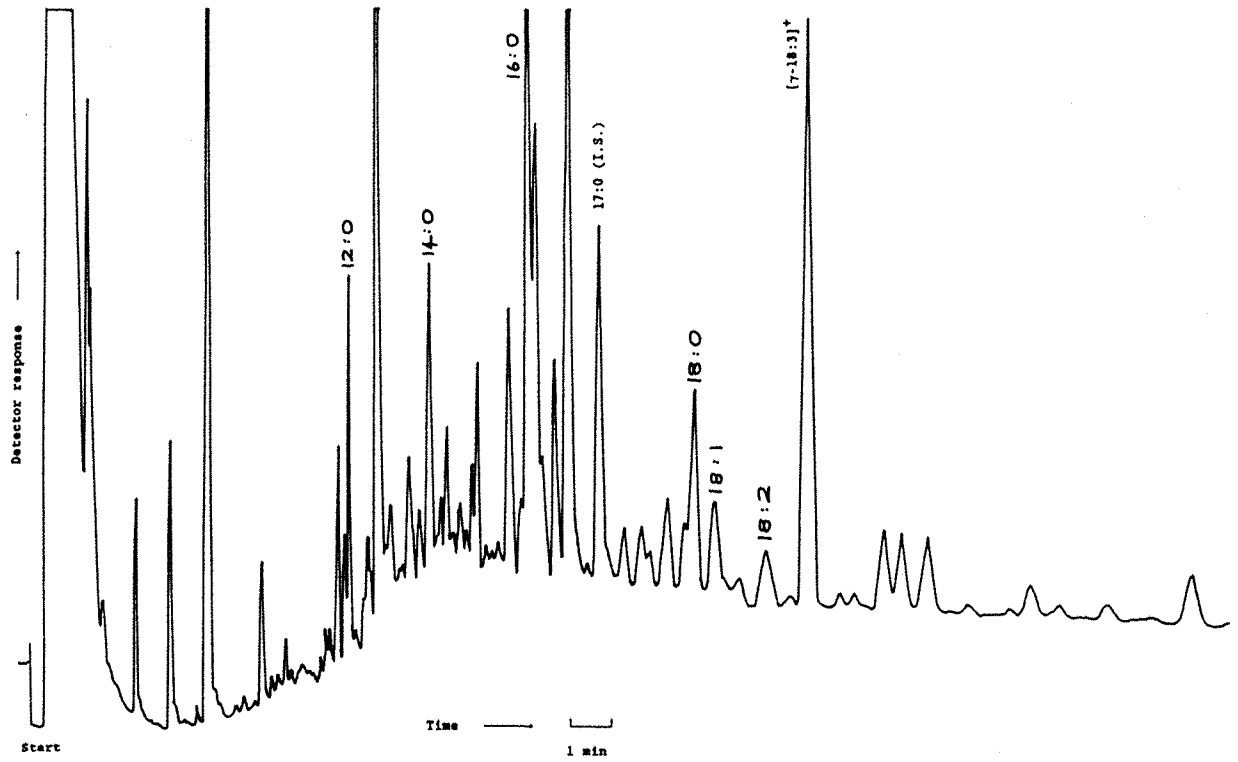
residue was redissolved in cyclohexane (50 μ l, containing the internal standard, 17:0, at 20 ng/ μ l).

The resulting chromatograms (Figures 6.2(a) - (d)) show that any sample present was swamped by large blank peaks although peaks with the same retention times as methyl esters of palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0) (in all samples) and eicosadienoic acid (20:2) (site C only) were present. Prior to quantitation, however, it was necessary to reduce the blank levels and so was decided that:-

- (i) attempts should be made to clean up the samples using thin-layer or column chromatography.
- (ii) the origin of the contaminated blank should be investigated.

Figure 6.2 Cyclohexane extract of humic material from November, 1985,
after treatment with boron trifluoride in methanol

(a) Site A



(b) Site B

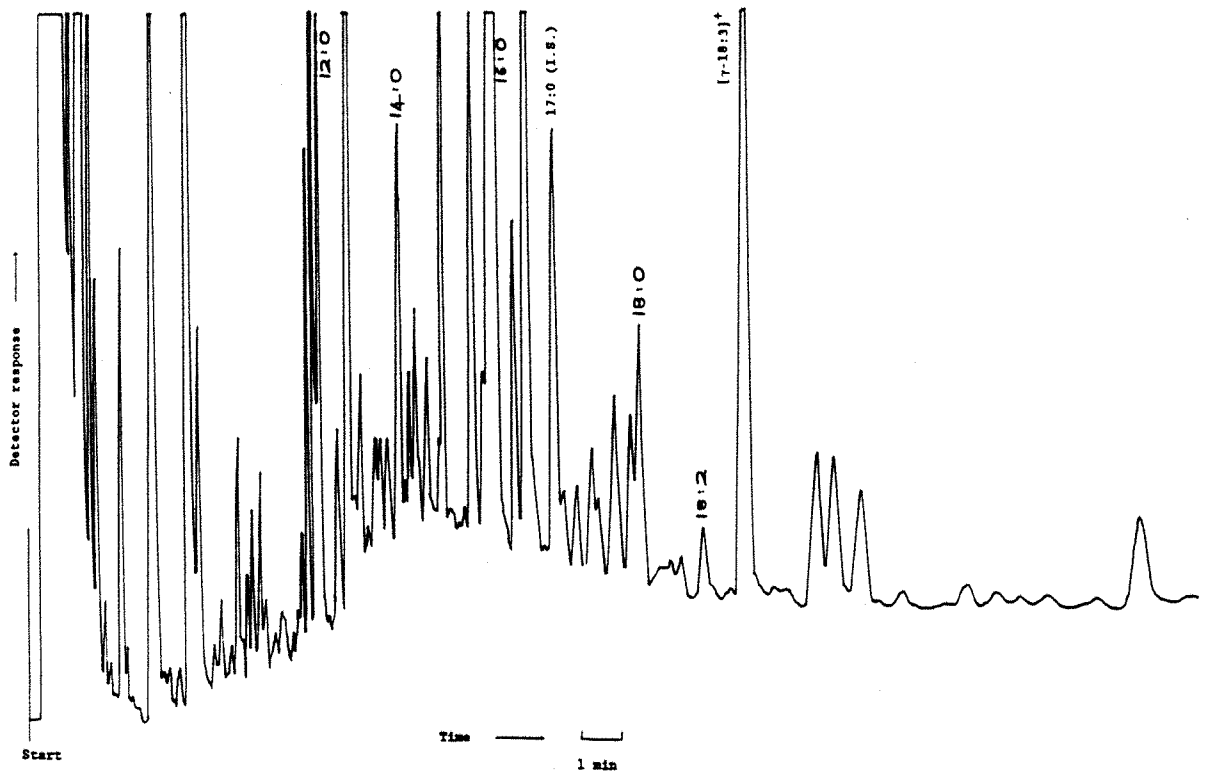
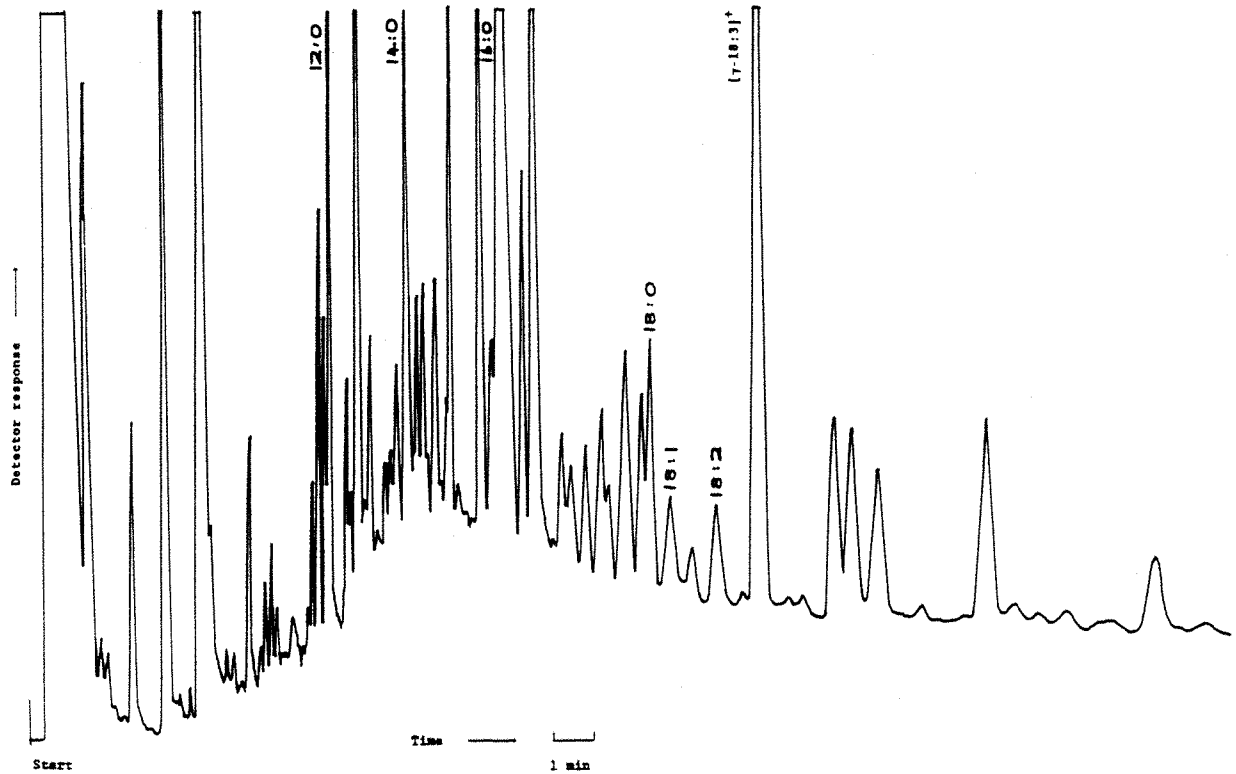
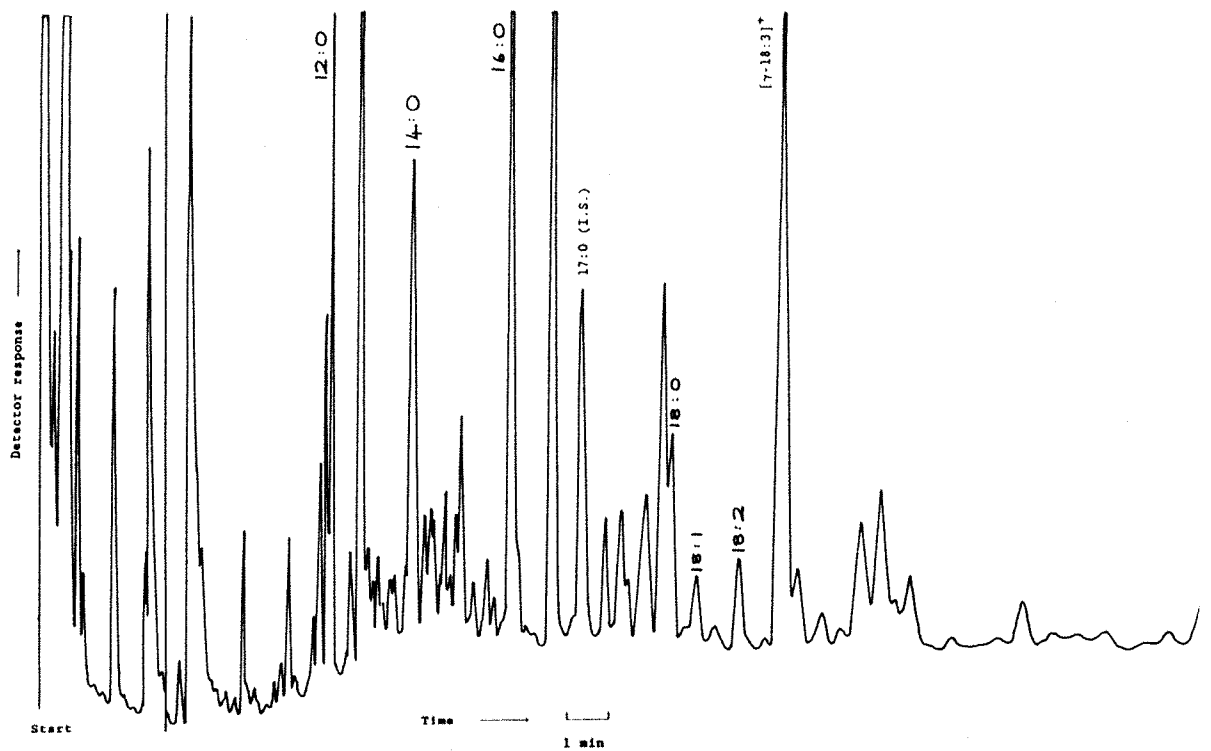


Figure 6.2 Cyclohexane extract of humic material from November, 1985,
(continued) after treatment with boron trifluoride in methanol

(c) Site C



(d) XAD blank



6.4 ATTEMPTED CLEAN-UP OF THE SAMPLES USING THIN-LAYER AND COLUMN CHROMATOGRAPHY

6.4.1 Development of thin-layer chromatographic method

As the nature of the contamination was not known, it was decided that a solvent system, which had been proven for the separation of lipids by TLC, should be tried. Diethyl ether in a non-polar solvent has been commonly employed (422). A cyclohexane/diethyl ether solvent system was chosen in this study.

6.4.1.1 Choice of solvent system

A variety of mobile phases were evaluated for the chromatography of FAMES on aluminium-backed silica gel TLC plates:-

- (i) Cyclohexane
- (ii) 5% (v/v) diethyl ether in cyclohexane
- (iii) 10% (v/v) diethyl ether in cyclohexane
- (iv) 50% (v/v) diethyl ether in cyclohexane
- (v) Diethyl ether

A solution (ca. 1 μ l) containing several FAMES (ca. 4 mg/ml total) was applied to the TLC plate, which was then eluted, with the solvent (ca. 20 ml), in a screw-capped jar, to a height of ca. 5 cm. After air-drying at room temperature, the FAMES were visualised with iodine. All the solvent systems resulted in a single spot, but only 5% and 10% (v/v) diethyl ether in cyclohexane gave the required retention factor (R_f) of approximately 0.5 (Table 6.7). The latter solvent was adopted for further work.

6.4.1.2 Trial TLC of humic sample

In order to assess whether the derivatised humic material could be purified by TLC, derivatised samples (November, 1985 - Site A and XAD blank; 1 μ l each) were analysed as above.

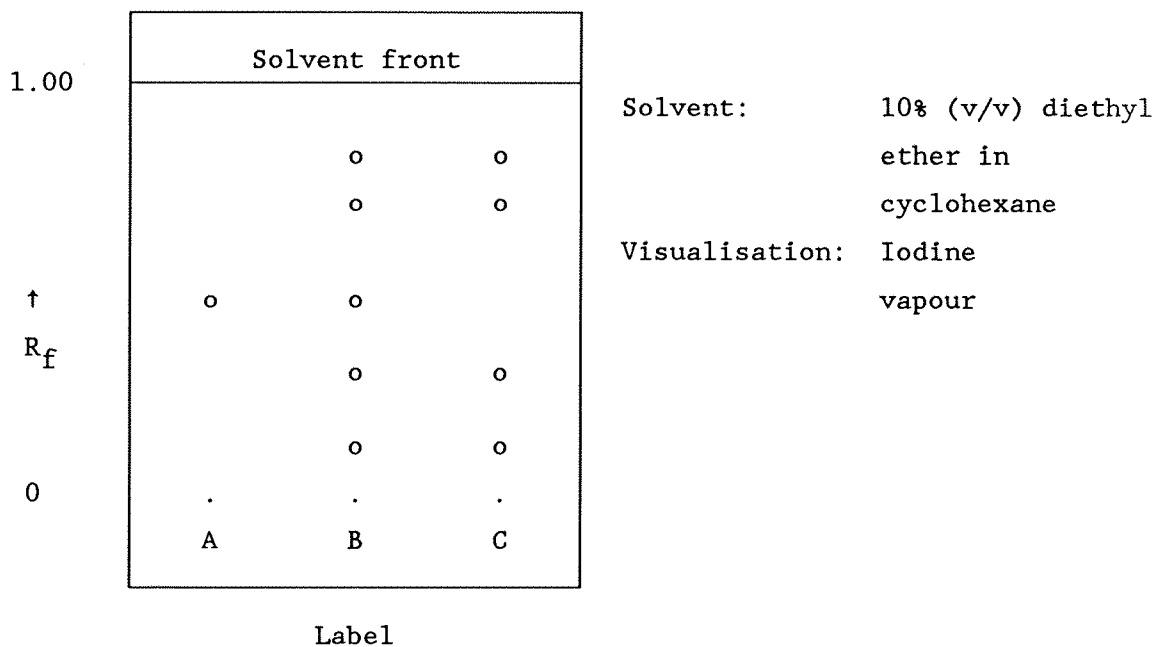
It can be seen (Figure 6.8) that, assuming the spot with an R_f value of 0.55 is due to FAMES, the TLC method successfully separated

the contamination from the FAMES. Preparative TLC was therefore attempted to recover the FAMES after separation.

Table 6.7 R_f values of FAME spot using different quantities of diethyl ether in cyclohexane

Percentage of diethyl ether (by volume)	R _f
0%	0.12, 0.12
5%	0.37, 0.37
10%	0.62, 0.64
50%	0.82
100%	0.91, 0.94

Figure 6.3 Diagrammatic representation of small-scale TLC of the November, 1985, derivatised samples (site A and XAD blank) compared to a solution containing FAMES



<u>Label</u>	<u>Solution</u>	<u>R_f</u>
A	FAMES	0.58
B	November, 1985, Site A	0.18, 0.35, 0.55, 0.80, 0.87
C	November, 1985, XAD blank	0.18, 0.36, 0.80, 0.88

6.4.1.3 Preparative TLC of FAME standard

For preparative TLC, glass plates were coated 'in house' with silica gel G and activated at 110°C for two to three hours. They were then pre-eluted with diethyl ether and dried at room temperature prior to analysis.

The TLC plate was scored vertically down the centre to separate it into two halves, and FAME standard C (100 μ l) was streaked (few cm) onto each half. The plate was then placed in a glass TLC tank, lined with filter paper, containing ca. 200 ml of 10% (v/v) diethyl ether in cyclohexane (previously equilibrated for 30 minutes). The solvent was allowed to ascend to a height of 15 cm, and the plate was then removed and air dried at room temperature.

To prevent possible alteration of the sample by exposure to iodine vapour, the plate was turned through 90°, and one half was visualised by rapidly eluting it in a chromatography tank containing 1% iodine to indicate the position of the FAMEs. The silica corresponding to this position on the unexposed side of the plate was then removed, transferred to a Pasteur pipette (plugged with glass wool at the narrow end of the pipette), and the FAMEs were then eluted with diethyl ether. Several experiments were performed, varying the volume of the eluting solvent and the diethyl ether was then evaporated to dryness under a stream of nitrogen. The residue was redissolved in cyclohexane (100 μ l) and subjected to GC analysis.

The results (Table 6.8) showed that:

- no obvious trend occurred on increasing the volume of the elution solvent, indicating that the minimum volume of 2 ml was sufficient to elute the FAMEs.
- the unsaturated FAME showed consistently lower yields than the saturated FAMEs.

It appears that, although 10% (by volume) diethyl ether in cyclohexane is a suitable solvent system for separating the contamination from the humic samples, the preparative TLC data showed possible preferential losses of unsaturated FAMEs, probably due to

oxidation on the TLC plate (473). Therefore, purification of the sample by an alternative method was attempted.

Table 6.8 Percentage recoveries of FAME standard C after preparative thin-layer chromatography

Total elution volume (ml)	Peak height ⁺			Peak height relative 17:0		Percentage recoveries		
	14:0	16:1	17:0	14:0	16:1	14:0	16:1	17:0
2	7.4	1.7	5.8	1.28	0.29	87	21	83
5	4.3	2.8	3.7	1.16	0.76	51	35	53
5	10.3	2.7	7.2	1.43	0.38	120	34	100
10	9.0	8.1	7.4	1.22	1.09	110	100	110
15	7.4	2.3	7.8	0.95	0.29	87	29	110
20	5.7	4.1	4.6	1.24	0.89	67	51	66
Standard* (%RSD) (n=3)	8.5 12	8.0 13	7.0 13	1.21 2.4	1.13 2.3	100 -	100 -	100 -

+ Blank-corrected peak heights based on one GC analysis only.

* Peak heights from GC analysis of FAME standard C - taken as 100% for comparison.

6.4.2 Development of column chromatographic method

Investigations were carried out to assess the recovery of a FAME standard by column chromatography on Florisil.

6.4.2.1 Variation of chromatographic parameters

Preliminary experiments were performed in which the bed volume of the column, and the volume and composition of the elution solvents were investigated. It was found that a Pasteur pipette column, containing ca. 1 g Florisil, gave acceptable recoveries, with reduced contamination, compared to a larger column (8 x 1 cm bed volume, ca. 4 g Florisil). Various elution schemes for column chromatography using Pasteur pipette columns were therefore evaluated (Table 6.9).

All three elution schemes yielded acceptable recoveries. The scheme employing the lowest percentage of diethyl ether in cyclohexane (20%) was chosen for future work to minimise elution of diethyl ether soluble contaminants in this fraction.

At this stage, clean-up of a derivatised humic sample (site A, November, 1985) was attempted.

6.4.2.2 Trial clean-up of a derivatised humic sample

Prior to column chromatography, frozen samples (derivatised humic sample from site A, November, 1985, and the derivatised XAD blank) were analysed by GC to check for losses on storage. As peak heights relative to the internal standard (previously added prior to GC analysis - see Section 6.3) had not changed significantly, these samples were subjected to column chromatography on Florisil (ca. 1 g in a Pasteur pipette). They were eluted with:

Cyclohexane	1 x 10 ml
20% diethyl ether in cyclohexane	1 x 10 ml
Diethyl ether	1 x 10 ml

The separate fractions were evaporated under a stream of nitrogen, redissolved in cyclohexane (50 μ l), and analysed by GC.

Table 6.9 Recovery of FAMES following column chromatography using various elution schemes

Column dimensions	:	Pasteur pipette containing ca. 1 g Florisil																																	
Flow rate	:	ca. 1ml/min or less																																	
FAME standard	:	FAME standard D (200 μ l)																																	
Elution schemes	:	<table> <tr> <td><u>A</u></td> <td>Cyclohexane</td> <td>1 x 10 ml</td> </tr> <tr> <td></td> <td>20% diethyl ether in cyclohexane</td> <td>1 x 10 ml</td> </tr> <tr> <td></td> <td>Diethyl ether</td> <td>1 x 10 ml</td> </tr> <tr> <td><u>B</u></td> <td>Cyclohexane</td> <td>1 x 10 ml</td> </tr> <tr> <td></td> <td>30% diethyl ether in cyclohexane</td> <td>1 x 10 ml</td> </tr> <tr> <td></td> <td>Diethyl ether</td> <td>1 x 10 ml</td> </tr> <tr> <td><u>C</u></td> <td>Cyclohexane</td> <td>1 x 5 ml</td> </tr> <tr> <td></td> <td>30% diethyl ether in cyclohexane</td> <td>1 x 5 ml</td> </tr> <tr> <td></td> <td>Diethyl ether</td> <td>1 x 5 ml</td> </tr> <tr> <td><u>D</u></td> <td colspan="2">Blank experiment - no FAME standard added</td> </tr> <tr> <td></td> <td colspan="2">Elution solvents as in B above</td> </tr> </table>	<u>A</u>	Cyclohexane	1 x 10 ml		20% diethyl ether in cyclohexane	1 x 10 ml		Diethyl ether	1 x 10 ml	<u>B</u>	Cyclohexane	1 x 10 ml		30% diethyl ether in cyclohexane	1 x 10 ml		Diethyl ether	1 x 10 ml	<u>C</u>	Cyclohexane	1 x 5 ml		30% diethyl ether in cyclohexane	1 x 5 ml		Diethyl ether	1 x 5 ml	<u>D</u>	Blank experiment - no FAME standard added			Elution solvents as in B above	
<u>A</u>	Cyclohexane	1 x 10 ml																																	
	20% diethyl ether in cyclohexane	1 x 10 ml																																	
	Diethyl ether	1 x 10 ml																																	
<u>B</u>	Cyclohexane	1 x 10 ml																																	
	30% diethyl ether in cyclohexane	1 x 10 ml																																	
	Diethyl ether	1 x 10 ml																																	
<u>C</u>	Cyclohexane	1 x 5 ml																																	
	30% diethyl ether in cyclohexane	1 x 5 ml																																	
	Diethyl ether	1 x 5 ml																																	
<u>D</u>	Blank experiment - no FAME standard added																																		
	Elution solvents as in B above																																		

The following table gives recoveries of FAMES in the second fraction (diethyl ether in cyclohexane):-

Elution scheme	Peak height (cm)			Percentage recovery		
	17:0	18:2	22:1	17:0	18:2	22:1
A	6.2	4.2	2.3	98	92	106
	5.8	3.7	2.0	91	82	96
B	5.6	3.8	1.8	89	85	85
	5.7	3.9	1.9	90	85	88
C	5.8	4.1	2.2	91	90	105
	5.4	3.8	1.9	85	84	88
D	ND	ND	ND	ND	ND	ND
Standard ⁺	6.3	4.5	2.1	-	-	-
(%RSD)	(9.2)	(7.3)	(11)	-	-	-

Analyses performed in duplicate, recording two GC runs of each solution

ND Not detected

+ Peak heights from the GC analysis of FAME standard D - taken as 100% for comparison

The resulting chromatograms (Figures 6.4, 6.5 and 6.6) show that:

- (i) little contamination was eluted in the cyclohexane fractions.
- (ii) similar peaks occurred in both the sample and blank diethyl ether/cyclohexane fractions, with the same retention times as the following FAMES - 12:0, 14:0, 16:0, 17:0 (internal standard), 18:0, and γ -18:3. In addition, the sample contained additional peaks at the same retention times as 16:1, 18:1, and 18:2. It is possible, therefore, that the blank contained saturated FAMES (the peak with the same retention time as γ -18:3 was thought to be a non-lipid contaminant as it also occurred in the diethyl ether fractions).
- (iii) the diethyl ether fractions eluted some contaminants. The presence of FAMES in this fraction was not suspected.

As the diethyl ether/cyclohexane fraction was still contaminated, it was decided to attempt to reduce it by lowering the percentage of diethyl ether in cyclohexane.

6.4.2.3 Further experiments on the column chromatography of FAMES

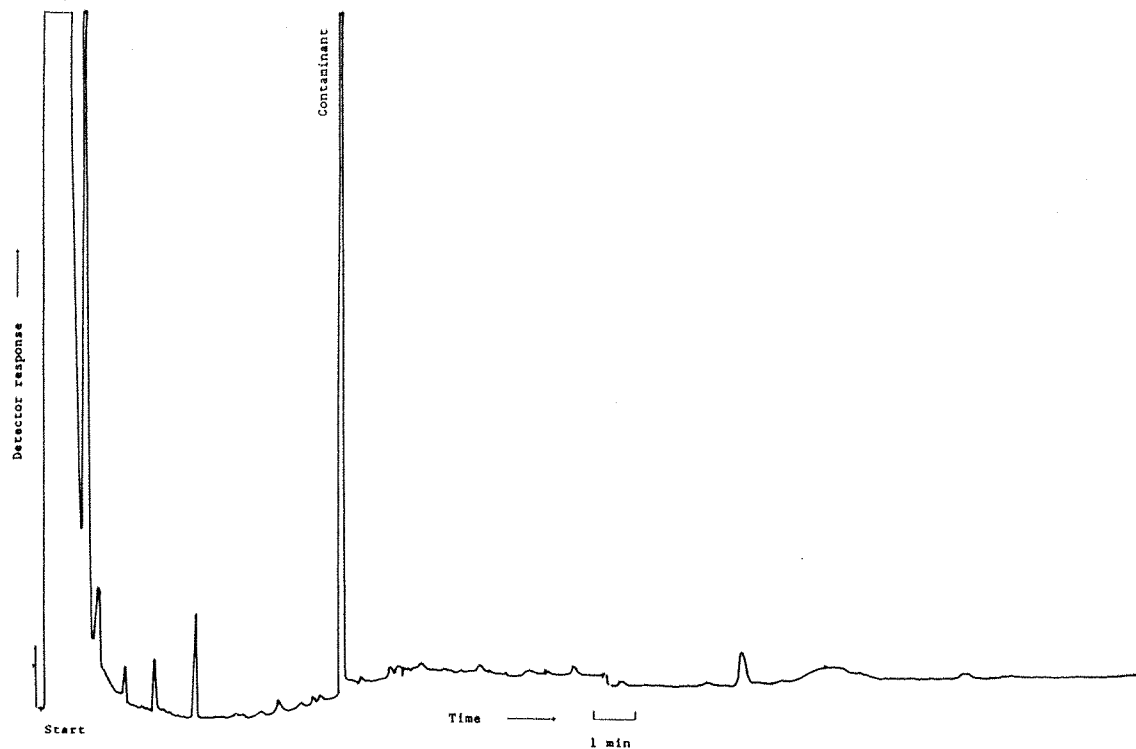
The effect of reducing the percentage of diethyl ether in cyclohexane on the recoveries of the FAMES was investigated. FAME standard D (200 μ l) was subjected to column chromatography using 5% and 10% (by volume) diethyl ether in cyclohexane, and the diethyl ether/cyclohexane fractions were concentrated and analysed by GC.

The results (Table 6.10) show that:

- acceptable recoveries were obtained using both elution schemes.
- in most cases, the percentage extracted in the second aliquot was less than 10% of the total extracted. It was therefore decided that a volume of 10 ml would be sufficient to extract the bulk of the sample.

Figure 6.4 Cyclohexane fractions resulting after column chromatography of the derivatised (a) humic material from Site A and (b) XAD blank, from November, 1985

(a)



(b)

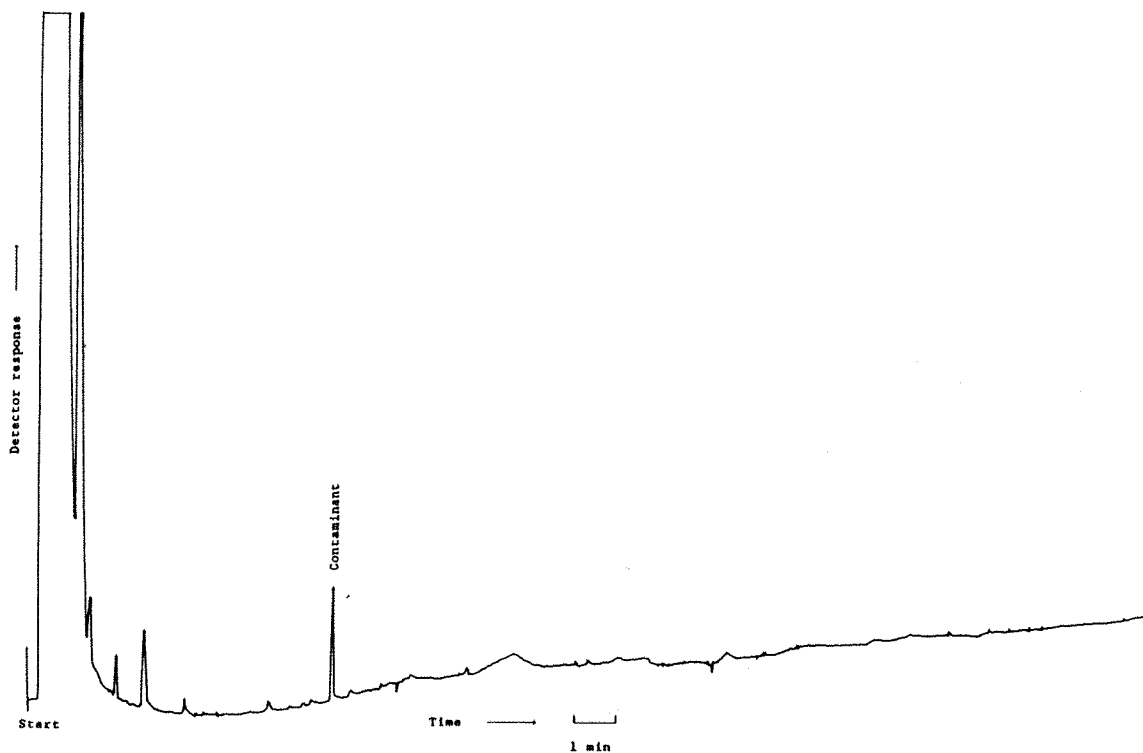
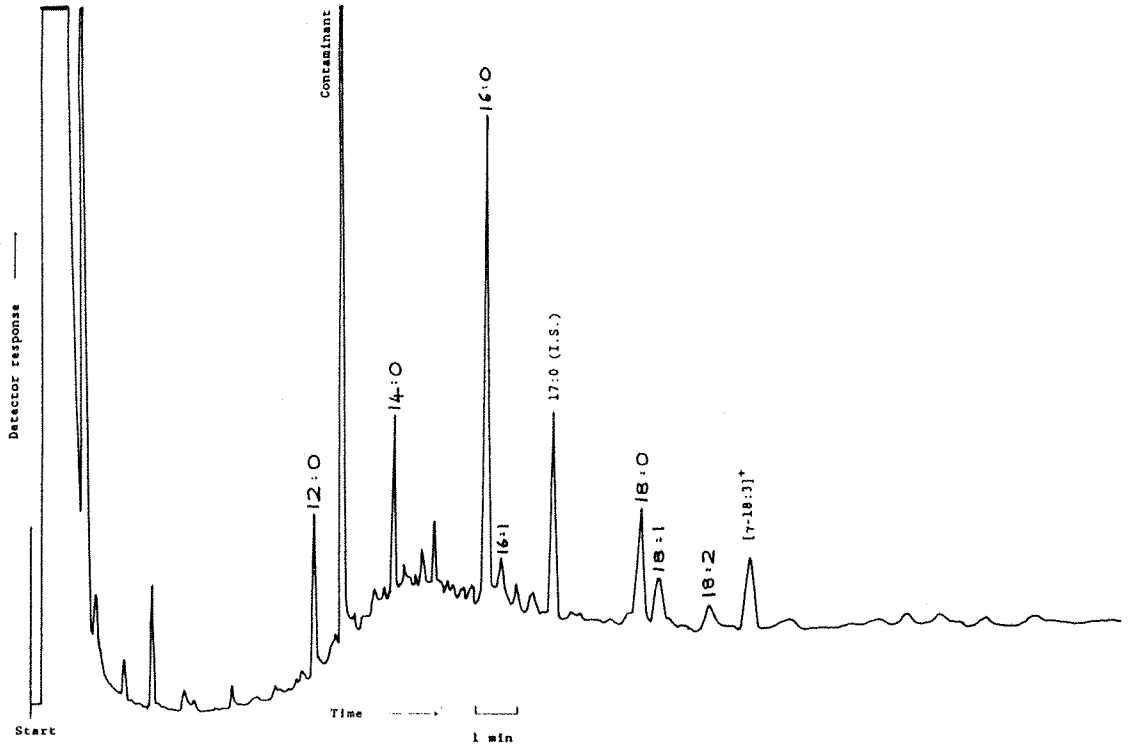


Figure 6.5 Diethyl ether/cyclohexane fractions resulting after column chromatography of the derivatised (a) humic material from Site A and (b) XAD blank, from November, 1985

(a)



(b)

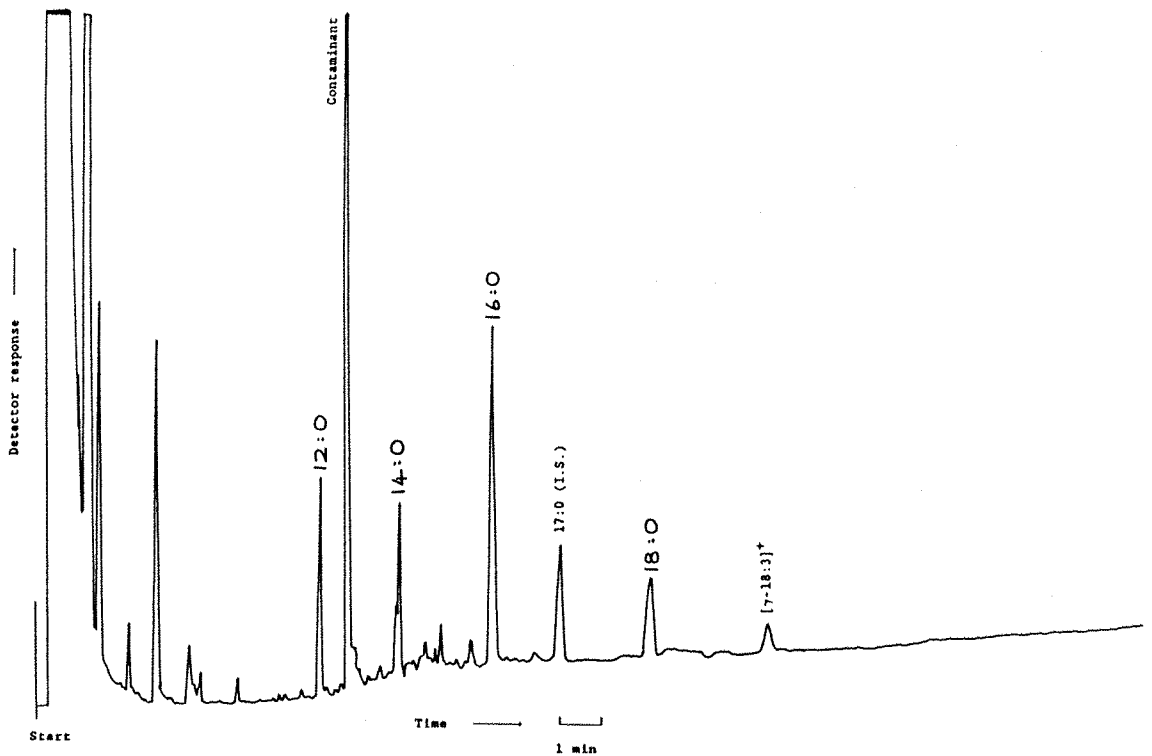
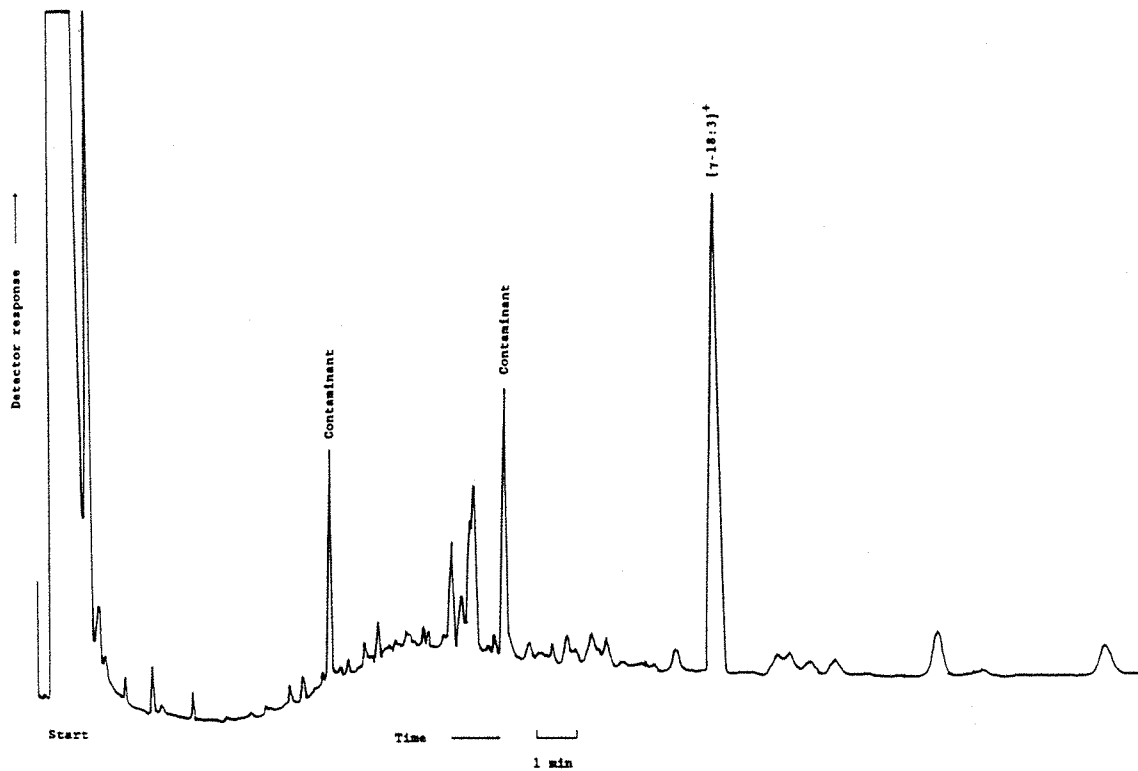
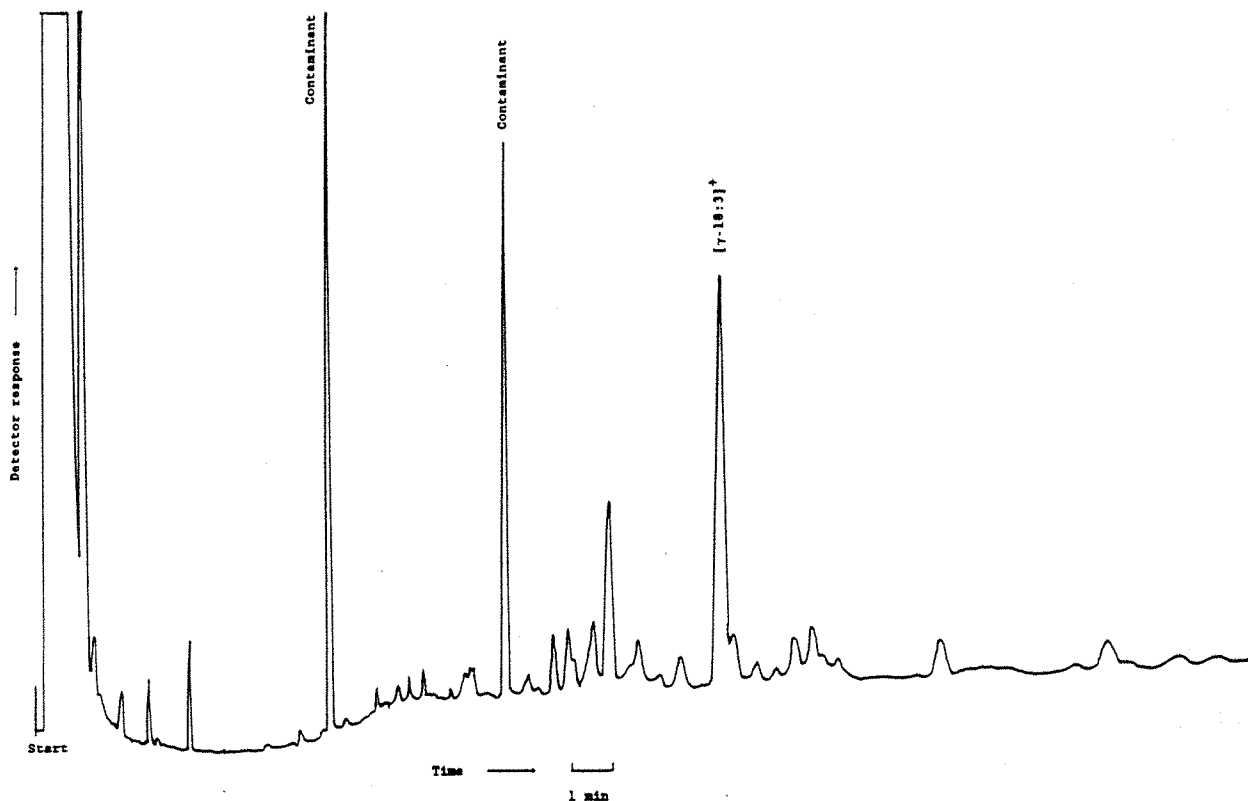


Figure 6.6 Diethyl ether fractions resulting after column chromatography of the derivatised (a) humic material from Site A and (b) XAD blank, from November, 1985

(a)



(b)



To attempt to minimise contamination, therefore, the lower percentage of diethyl ether in cyclohexane (5%, 10 ml) was adopted in future work.

Table 6.10 Recovery of FAMES following column chromatography using reduced percentages of diethyl ether in cyclohexane

Column dimensions : Pasteur pipette containing ca. 1 g Florisil
 Flow rate : ca. 1 ml/min
 Elution schemes : A Cyclohexane 1 x 10 ml
 5% diethyl ether in cyclohexane 2 x 10 ml
 Diethyl ether 1 x 10 ml
B Cyclohexane 1 x 10 ml
 10% diethyl ether in cyclohexane 2 x 10 ml
 Diethyl ether 1 x 10 ml

Recoveries of the FAMES in the diethyl ether/cyclohexane fractions are given below:-

Elution scheme	Peak height (cm)			Percentage recovery			
	17:0	18:2	22:1	17:0	18:2	22:0	
A 1st fraction	5.2	4.0	1.8	100	100	99	
	5.5	4.1	1.9	110	105	105	
	2nd fraction	0.6	0.3	0.2	11	7.6	11
		0.5	0.3	0.3	9.6	7.7	17 ⁺
B 1st fraction	5.1	3.8	1.6	98	98	89	
	5.5	4.1	1.8	105	105	100	
	2nd fraction	0.2	0.2	0.1	3.9	3.9	2.8
		0.3	0.2	0.1	4.8	5.2	5.6
Standard (n=2) ⁺⁺	5.2	3.9	1.8	100	100	100	

+ Only one GC run was recorded. (Analyses were normally performed in duplicate, recording two GC runs of each solution)

++ Peak heights from the GC analysis of FAME standard D - taken as 100% for comparison

6.5 TREATMENT OF HUMIC SUBSTANCES WITH BORON TRIFLUORIDE IN METHANOL FOLLOWED BY PURIFICATION BY COLUMN CHROMATOGRAPHY

6.5.1 Method

Three samples and an XAD blank (May, 1986) were analysed. 14% (w/v) boron trifluoride in methanol (4 ml) was added to the humic material (ca. 10 mg), the mixture was sonicated for 30 seconds, and then heated under reflux for four hours. It was subsequently extracted with cyclohexane (5 ml) and washed with doubly distilled water (5 ml). This was repeated twice more, then the organic layers were combined and dried over anhydrous sodium sulphate. After decanting off the solvent and evaporating to dryness, the residue was dissolved in cyclohexane (100 μ l, containing 15:0 at 10ng/ μ l) and the resulting solutions were analysed by GC.

Column chromatography was then performed on these solutions, using the following eluents:

Cyclohexane	1 x 10 ml
5% diethyl ether in cyclohexane	1 x 10 ml
Diethyl ether	1 x 10 ml

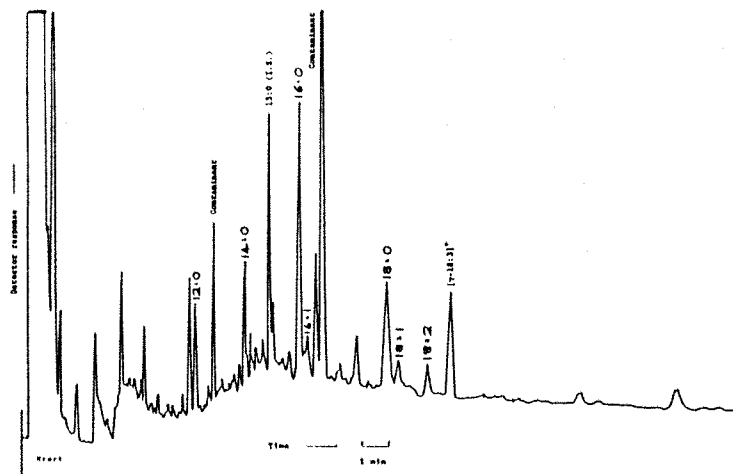
Pasteur pipette columns (containing ca. 1 g Florisil) were employed and eluted at a flow rate of ca. 1 ml/min. It should be noted that, during the cyclohexane elution of the sample from site B, the column was inadvertently allowed to become dry and so lower overall recoveries were expected in this case. The resulting solutions were taken to dryness under a stream of nitrogen, and the residues were redissolved in cyclohexane (100 μ l) then subjected to GC analysis.

6.5.2 Results

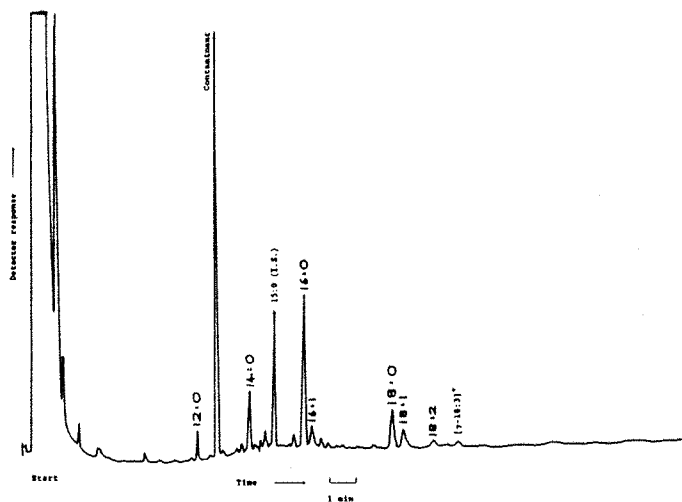
The gas chromatograms, both prior to and after column chromatography, are given (Figures 6.7 - 6.10). In all cases, the cyclohexane fraction (after column chromatography) only showed the presence of one peak and so these GC traces are not included.

Figure 6.7 Treatment of humic material from Site A, May, 1986, with boron trifluoride in methanol

(a) Extract before column chromatography



(b) Diethyl ether/cyclohexane fraction after column chromatography



(c) Diethyl ether fraction after column chromatography

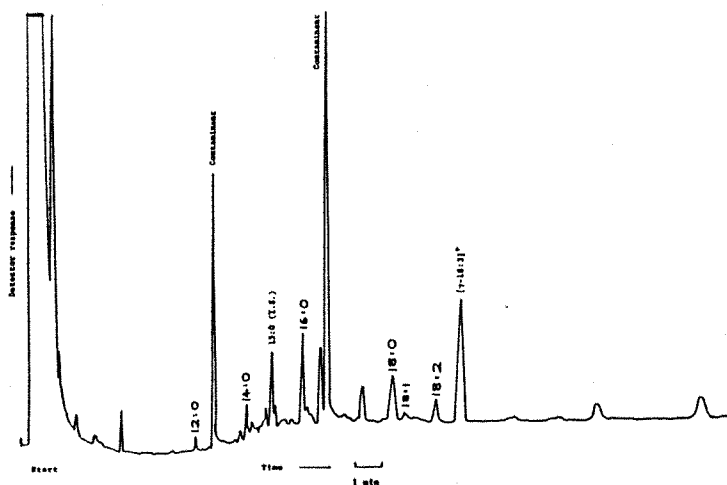
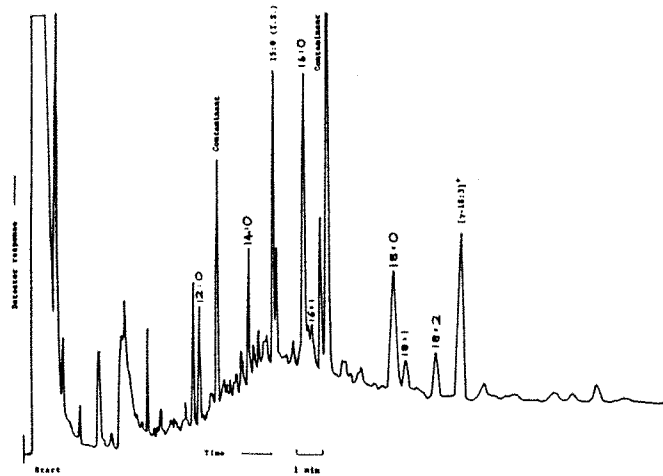
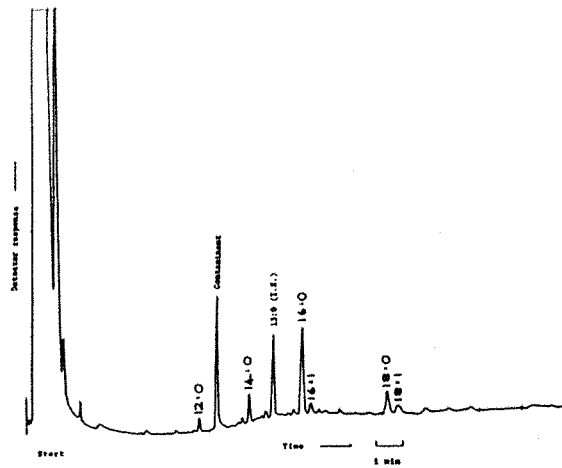


Figure 6.8 Treatment of humic material from Site B, May, 1986, with boron trifluoride in methanol

(a) Extract before column chromatography



(b) Diethyl ether/cyclohexane fraction after column chromatography



(c) Diethyl ether fraction after column chromatography

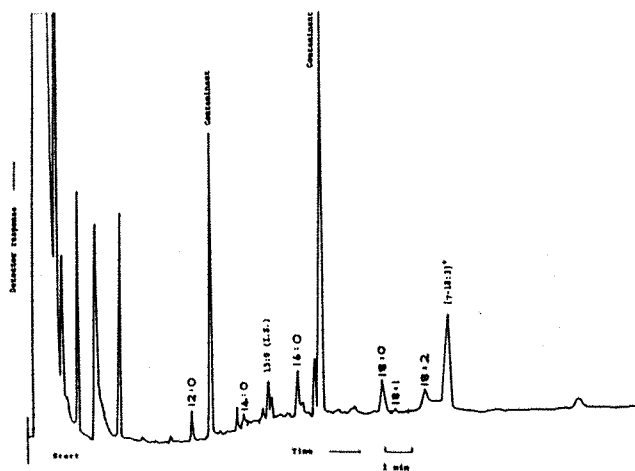
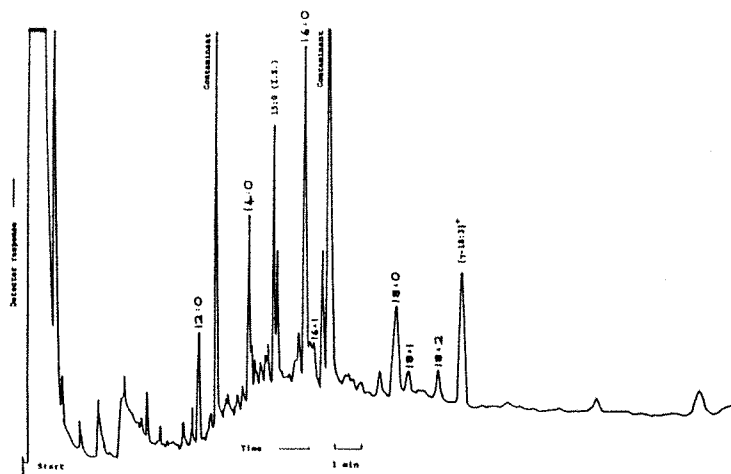
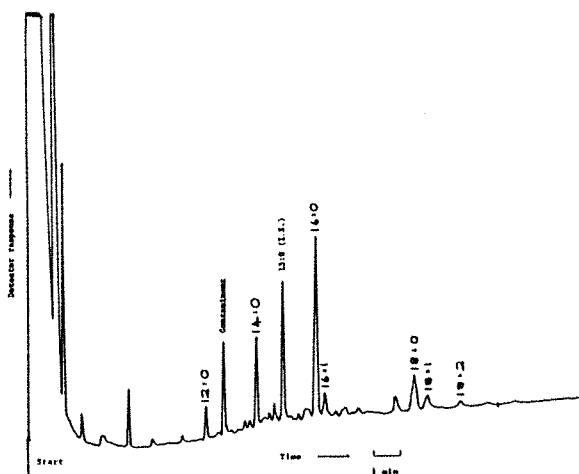


Figure 6.9 Treatment of humic material from Site C, May, 1986, with boron trifluoride in methanol

(a) Extract before column chromatography



(b) Diethyl ether/cyclohexane fraction after column chromatography



(c) Diethyl ether fraction after column chromatography

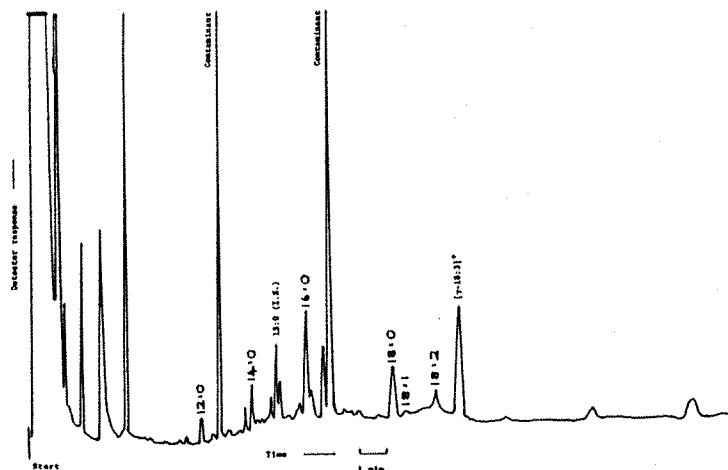
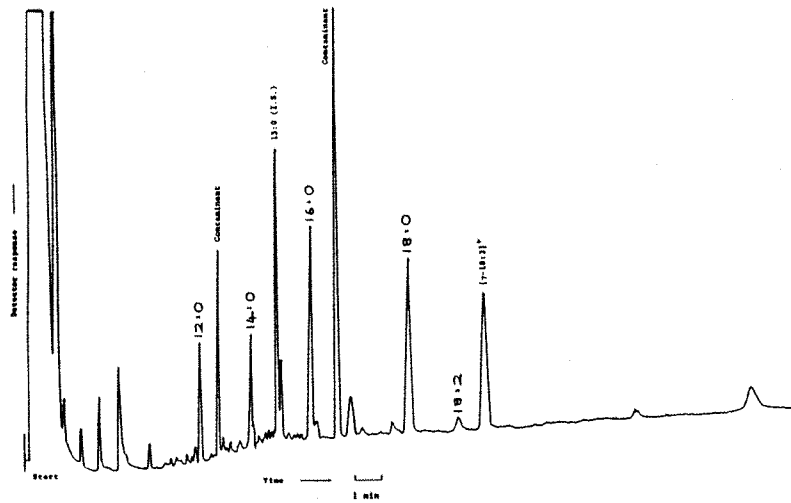
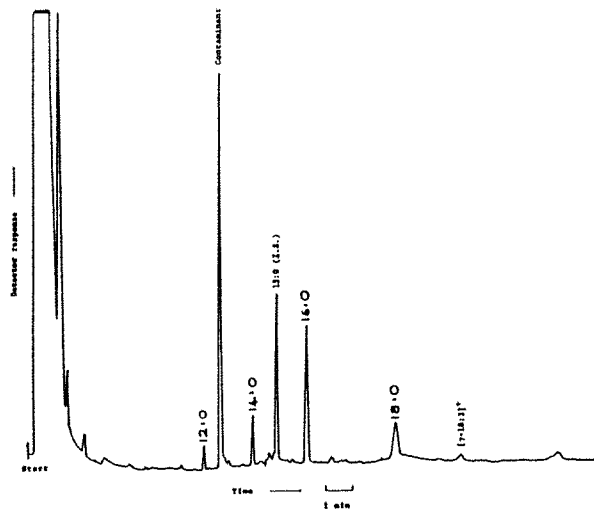


Figure 6.10 Treatment of XAD blank from May, 1986, with boron trifluoride in methanol

(a) Extract before column chromatography



(b) Diethyl ether/cyclohexane fraction after column chromatography



(c) Diethyl ether fraction after column chromatography

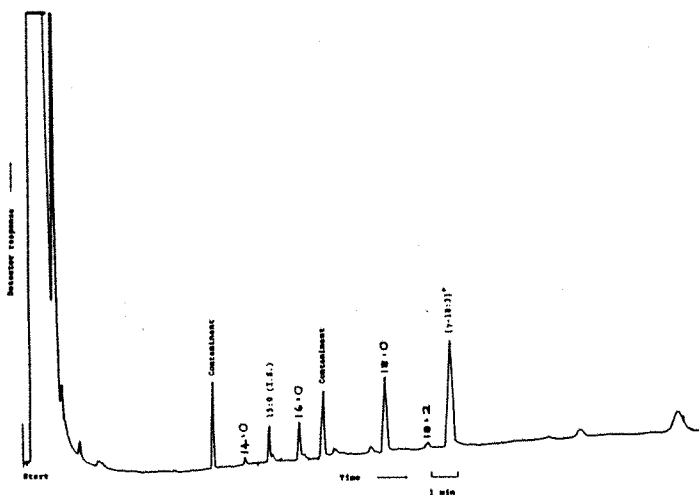


Table 6.11 summarises the tentative identification of the components of the various chromatograms, identified purely on the basis of their retention times.

The following points can be made from the results:

- (i) Some similar peaks occurred in both the diethyl ether in cyclohexane and the diethyl ether only fractions. Although

Table 6.11 Peaks occurring in chromatograms of derivatised humic substances from May, 1986, after column chromatography

Description	Fraction											
	Cyclohexane				5% diethyl ether				Diethyl ether			
	A	B	C	Bk	A	B	C	Bk	A	B	C	Bk
12:0	x	x	x	x	✓	✓	✓	✓	✓	✓	✓	x
Contaminant seen in all 3 fractions	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
14:0	x	x	x	x	✓	✓	✓	✓	✓	✓	✓	✓
15:0 (I.S.)	x	x	x	x	✓	✓	✓	✓	✓	✓	✓	✓
16:0	x	x	x	x	✓	✓	✓	✓	✓	✓	✓	✓
16:1	x	x	x	x	✓	✓	✓	x	x	x	x	x
In diethyl ether fraction only	x	x	x	x	x	x	x	x	✓	✓	✓	✓
18:0	x	x	x	x	✓	✓	✓	✓	✓	✓	✓	✓
18:1	x	x	x	x	✓	✓	✓	x	x	x	x	x

A = Site A; B = Site B; C = Site C; Bk = XAD blank

✓ = Present

x = Absent

I.S. = Internal standard

these could be due to co-eluting compounds, their predominance in the samples suggests the presence of FAMES not completely eluted in the diethyl ether/cyclohexane fractions.

- (ii) Saturated FAMES appear to occur in the XAD blank analysis (12:0, 14:0, 16:0 and 18:0) in agreement with previous results (Section 6.4.2.2). The peak at the same retention time as γ -18:3 was present in the diethyl ether fraction rather than the diethyl ether in cyclohexane fraction, confirming that it was not a FAME. In addition, the three samples in the present analysis also contained extra peaks corresponding to 16:1 and 18:1, as did the November, 1985 sample.
- (iii) Several small peaks, with different retention times to any FAMES in the standard, were seen in the samples but not in the blank. These were possibly branched-chain FAMES, positive identification of which could be performed by GC-MS on a more concentrated sample.

6.5.3 Conclusions

The XAD blank analysis appeared to be contaminated with saturated FAMES. Only unsaturated FAMES (16:1 and 18:1) could be identified in all the samples but not in the blank. Other minor components were also detected in the diethyl ether/cyclohexane fractions of the samples although these were not identified.

6.6 CONTAMINATION IN THE XAD BLANK ANALYSIS

The origin of the saturated FAMES in the XAD blank analysis was investigated.

6.6.1 Reagent blank

Over the period of this study, it was noticed that the reagent blank contained varying amounts of contamination, which was thought to be due to differing reaction conditions or possibly the age of the boron trifluoride reagent. Therefore, prior to investigating the complete blank XAD extraction procedure, the transesterification reagent blank was re-checked.

6.6.1.1 Origin of contamination

To trace the origin of any contamination in the reagent blank, the analysis was broken down into steps and each step checked. The following experiments were carried out:

- (i) Cyclohexane (10 ml) was taken to dryness under a stream of nitrogen, and redissolved in cyclohexane (100 μ l) containing an internal standard (15:0, 10 ng/ μ l).
- (ii) Doubly distilled water (5 ml) was extracted with cyclohexane (5 ml). The organic layer was removed, and the water extracted with a further aliquot of cyclohexane (5 ml). On shaking, more water was added (5 ml), and the combined organic layers were then dried over anhydrous sodium sulphate. The solvent was evaporated under a stream of nitrogen and the residue was redissolved in cyclohexane (100 μ l, containing 15:0 at 10 ng/ μ l).
- (iii) 14% (w/v) boron trifluoride in methanol (2 ml) was heated under reflux for 30 minutes. It was subsequently extracted with cyclohexane (5 ml) and washed with doubly distilled water (5 ml), and the organic layer was dried over anhydrous sodium sulphate. It was then treated as in (ii).

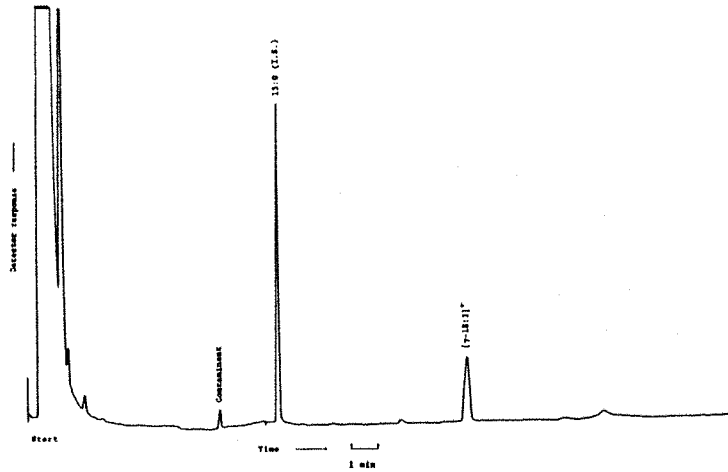
The resultant solutions were subjected to GC analysis.

The results (Figure 6.11 (a) and (b)) showed that the reagent blank was contaminated, with the complete transesterification step containing significantly more contaminants than the aqueous extraction alone. Peaks with the same retention times as 14:0, 16:0 and 18:0 were detected in the full blank analysis ((iii)). A further experiment, increasing the volume of boron trifluoride in methanol to 10 ml, suggested that this reagent was the main source of the contamination.

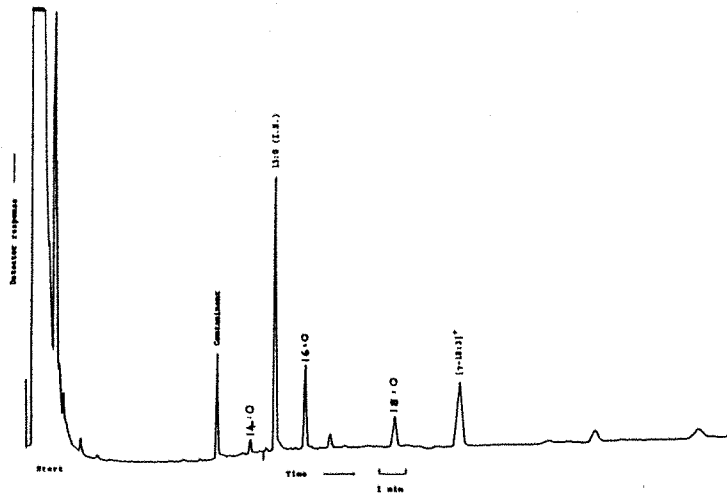
Blank reaction mixtures, resulting from transesterification with boron trifluoride in methanol, have been shown to contain extraneous peaks, which seemed to vary with reagent lot (470). Therefore, the use of boron trifluoride in methanol solutions from sealed ampoules was investigated.

Figure 6.11 GC traces of the reagent blank

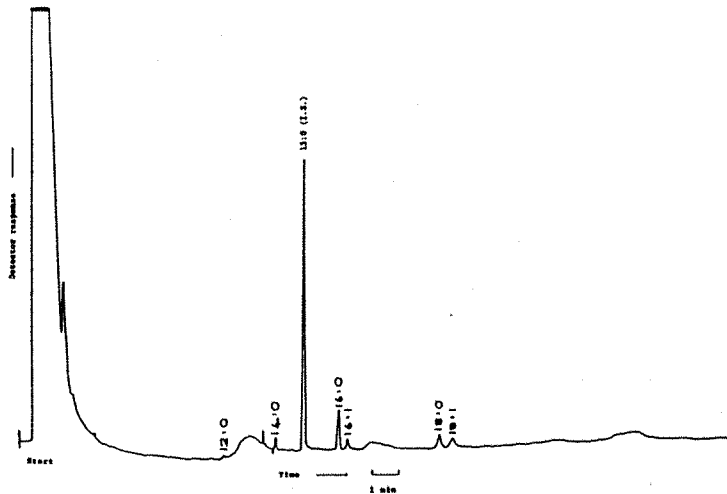
(a) Aqueous extraction step



(b) Transesterification and aqueous extraction steps



(c) Improved reagent blank using boron trifluoride in methanol from sealed ampoules



6.6.1.2 Reagent blanks using boron trifluoride in methanol from sealed ampoules

Two reagent blank experiments were performed using 14% (w/v) boron trifluoride in methanol from sealed ampoules:

- (i) The boron trifluoride (1 ml) was heated under reflux for 30 minutes, and was then extracted twice with cyclohexane (2 x 2 ml) and washed with doubly distilled water (2 x 2 ml). The combined organic layers were dried, evaporated under a stream of nitrogen and the residue was redissolved in 50 μ l cyclohexane (containing the internal standard, 15:0).
- (ii) Prolonged refluxing, combined with sonication, were employed in this instance. The boron trifluoride in methanol (1 ml) was sonicated for ten seconds, prior to heating under reflux for two hours. It was extracted with cyclohexane (1 ml), washed with doubly distilled water (1 ml), and, on removal of the organic layer, extracted with a further aliquot of cyclohexane (1 ml). The combined organic layers were then treated as in (i).

A similar level of contamination to that resulting from the use of boron trifluoride in a screw-capped bottle was seen in experiment (i) although some different contaminant peaks resulted (possibly due to contamination from the amber ampoules).

Improved blank levels were observed in experiment (ii) (Figure 6.11 (c)), although minor peaks at the same retention times as 14:0, 16:0, and 18:0 were still present. Additional minor peaks were also seen at the same retention times as 16:1 and 18:1, as in experiment (i). Possible reasons for the improved blank levels were:

- losses of impurities due to extended refluxing.
- incomplete extraction of impurities due to smaller volume of cyclohexane used.
- variations in purity of boron trifluoride between the ampoules.

To determine whether losses or incomplete extraction were occurring, a lipid standard (C, 15 μ l equivalent to 600 ng each lipid) was analysed using this method (experiment (ii)).

The resulting data (Table 6.12) showed low recoveries of the lower molecular weight FAMES (12:0 and 14:0) and incomplete recoveries of all FAMES (as in Section 6.2.3.2.5). Semi-quantitative recoveries of trace levels of lipids (ca. 600 ng/component), however, should be possible using this method. Improved blank levels resulted, and so ampouled boron trifluoride in methanol was employed in future work, using this procedure (method 5).

Table 6.12 Yield of FAMES from lipid standard C analysed using experiment (ii) (method 5)

Lipid	FAME resulting	Percentage yield (Blank corrected)
Lauric acid	12:0	38, 26
L-dimyristoyl- α -lecithin	14:0	43, 48
Palmitic acid	16:0	70, 90
Methyl palmitoleate	16:1	57, 64
Cholesterol stearate	18:0	88, 130
Glycerol trioleate	18:1	92, 120
L-dibehenoyl- α -lecithin	22:0	110, NA

6.6.2 XAD blank

Having reduced the contamination in the reagent blank, the complete XAD blank analysis was checked. Two experiments were performed (as in Section 4.3.5):

- (a) Clean XAD-8 resin, which had been rinsed with four bed volumes of doubly distilled water (acidified to pH 2 with concentrated hydrochloric acid), was eluted with four bed volumes of methanolic ammonia (1:1 (v/v) methanol : ammonium hydroxide (2 moles/l)). This was subsequently concentrated by rotary evaporation, at less than 60°C, and lyophilised.
- (b) Methanolic ammonia (1:1 (v/v) methanol : ammonium hydroxide (2 moles/l); 400 ml) was concentrated to a small volume by rotary evaporation, and then lyophilised.

The resulting white solids (ca. 10 mg) were subject to trans-esterification using method 5 (Section 6.6.1.2).

The chromatogram from experiment (a) (Figure 6.12) showed similar peaks and contamination levels to that from experiment (b). Peaks with the same retention times as 12:0, 14:0, 16:0, 16:1, 18:0, 18:1, and, possibly, 18:2 were detected. The presence of these peaks in the derivatised concentrated methanolic ammonia residue (experiment (b)) indicates that they were not introduced during the XAD extraction step.

A sample XAD blank (August, 1986) was also derivatised by the above method, and subjected to column chromatography. The chromatograms (Figures 6.13 (a) and (b)) show that:

- before column chromatography, the sample XAD blank was more contaminated than the elution only XAD blank in (a) above, suggesting that extraneous material was introduced during the aqueous extraction stage. This probably originated from the XAD resin itself, which has been known to release contaminants (356), even after extensive cleaning.
- after column chromatography, much cleaner samples resulted, indicating the success of the column chromatography in cleaning up the sample, although peaks were still seen with retention times corresponding to 12:0, 14:0, 16:0 and 18:0 as in experiment (a) and (b) above.

6.6.3 Conclusions

The main source of the contaminant peaks originally appeared to be the boron trifluoride-methanol reagent although, on derivatising both a methanolic ammonia extract and a sample XAD blank after improving the reagent blank levels, significant contaminants with the same retention times as saturated fatty acids were still present.

Two possible sources of contamination can be suggested:-

- (i) As the contaminants appeared to increase (compared to the improved blank) on including the methanolic ammonia

Figure 6.12 Derivatised XAD 'elution only' blank

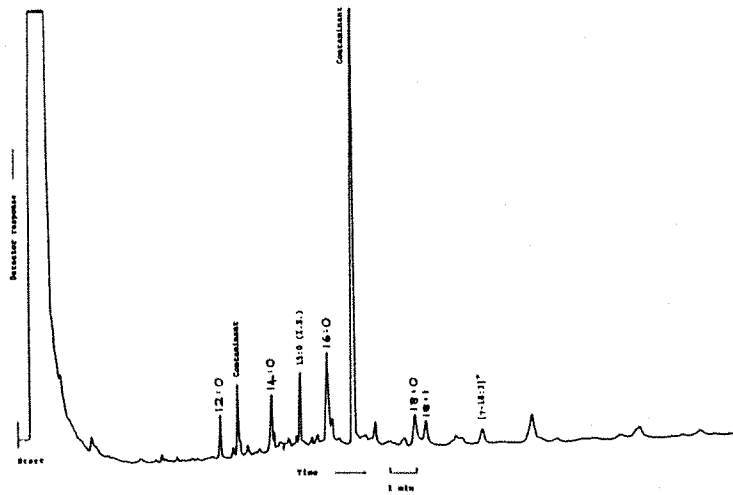
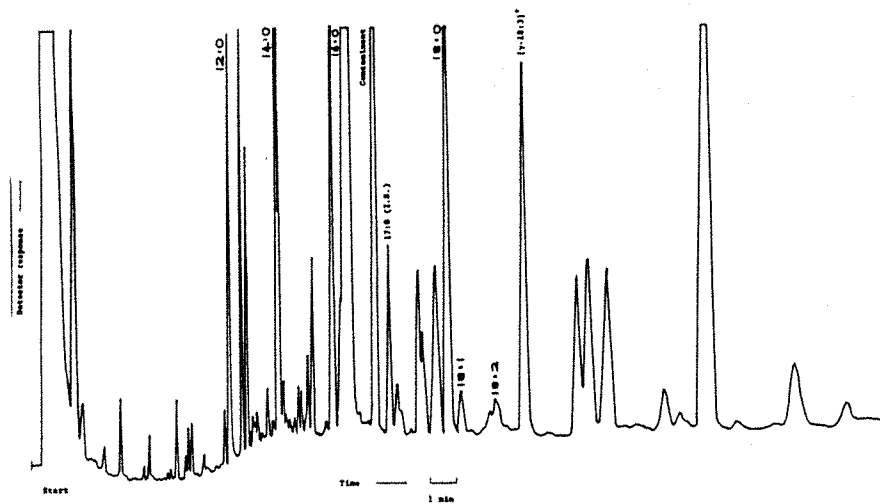
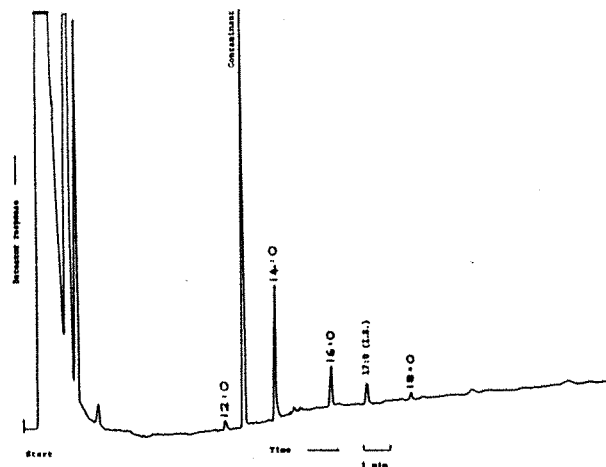


Figure 6.13 Derivatised sample XAD blank (August, 1986)

(a) Before column chromatography



(b) After column chromatography



concentration step, they could either result from the solvents (mainly derivatisation of involatile impurities) or the lyophilisation step (possibly incorporated by using equipment not dedicated to trace organic analysis).

- (ii) Variations in impurities in the boron trifluoride-methanol reagents between ampoules are possible - it was noted that some solutions were slightly coloured (thought to originate from the amber ampoules).

Further studies on the blank analysis, such as comparisons of different boron trifluoride-methanol ampoules and further purification of the methanolic ammonia reagent, should be performed in an attempt to remove these contaminants.

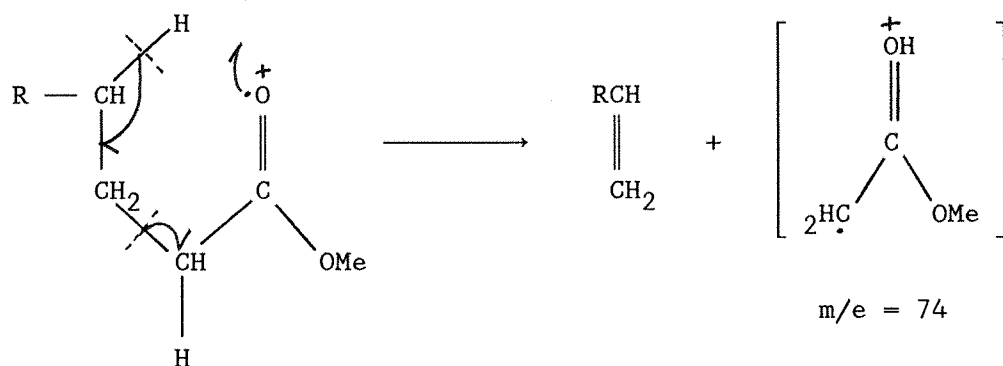
6.7 GAS CHROMATOGRAPHY-MASS SPECTROMETRY OF HUMIC SAMPLES AFTER TRANSESTERIFICATION WITH BORON TRIFLUORIDE IN METHANOL FOLLOWED BY COLUMN CHROMATOGRAPHY

In an attempt to identify the contaminant peaks, two further extracts were analysed by GC-MS.

6.7.1 Mass spectrometry of FAMES

The most characteristic peak in the spectra of FAMES is due to the McLafferty rearrangement (Figure 6.19). This is the base peak ($m/e = 74$) in the mass spectra of straight-chain methyl esters from C_6 to C_{26} (474).

The alkyl chain in straight-chain FAMES shows cleavage at each C - C bond to give an alkyl ion, $C_nH_{2n+1}^+$ (m/e 29, 43, 57,....), and an oxygen-containing ion, $C_nH_{2n-1}O_2^+$ (m/e 59, 73, 87,....), yielding hydrocarbon clusters at intervals of 14 mass units, with prominent peaks at $C_nH_{2n-1}O_2^+$. The peak at an m/e value of 87 ($CH_2CH_2COOCH_3^+$) is always more intense than its homologues although the reason for this is not obvious (474).

Figure 6.14 McLafferty rearrangement of normal FAMES (474)

The molecular ion peak of a straight-chain FAME is usually distinct, which aids in its identification. Another diagnostic peak is at M-31, due to $\text{R} - \overset{+}{\text{C}} = \text{O}$.

6.7.2 Transesterification and purification of humic samples

A humic sample (site C) and an XAD blank (November, 1985) were transesterified as in method 5 (Section 6.6.1.2), and then purified by column chromatography. As complete elution of the FAMES did not appear to result in the previous analysis of humic samples (Section 6.5.2), the percentage of diethyl ether in cyclohexane was increased to 10%. The columns (Pasteur pipette columns, containing ca. 1 g Florisil) were eluted sequentially with cyclohexane (1 x 10 ml), 10% diethyl ether in cyclohexane (2 x 10 ml), and diethyl ether (1 x 10 ml). Each fraction was taken to dryness, and redissolved in cyclohexane (50 μl).

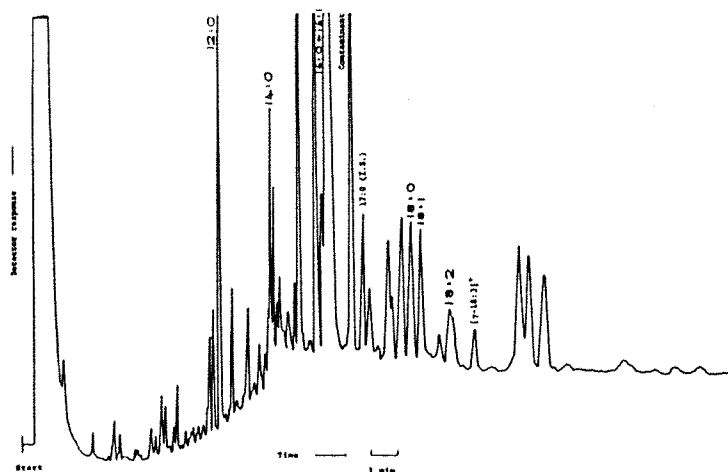
GC analyses were performed both prior to and following column chromatography, with the first diethyl ether in cyclohexane fractions (10 ml) being analysed by GC-MS.

6.7.3 GC and GC-MS analysis of the transesterified humic extracts

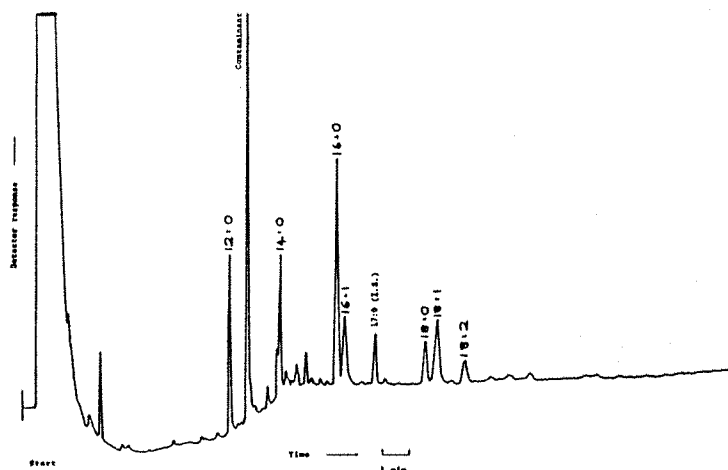
Chromatograms of the samples both prior to and following column chromatography are shown (Figures 6.15 and 6.16). Traces of FAMES in the second diethyl ether/cyclohexane fraction indicated that over 75% of the FAMES in both samples (site C - 78%; XAD blank - 86%; based on the height of 17:0) eluted in the first fraction. Figures 6.15 and

Figure 6.15 Derivatized humic material from site C, November, 1985

(a) Extract before column chromatography



(b) First diethyl ether/cyclohexane fraction after column chromatography



(c) Diethyl ether fraction after column chromatography

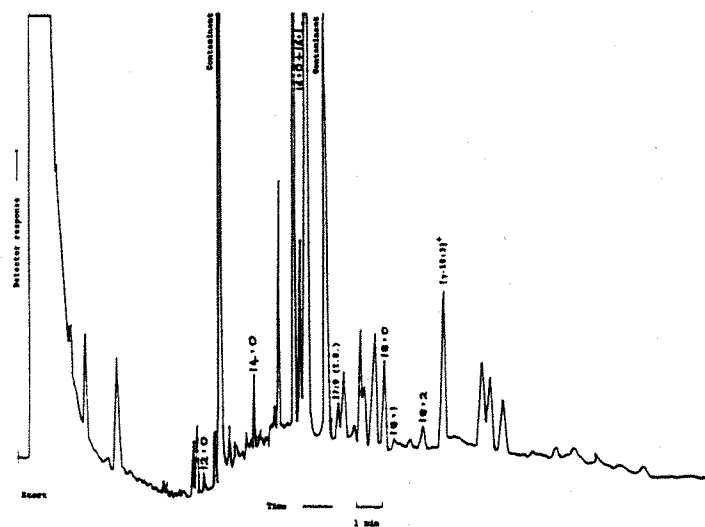
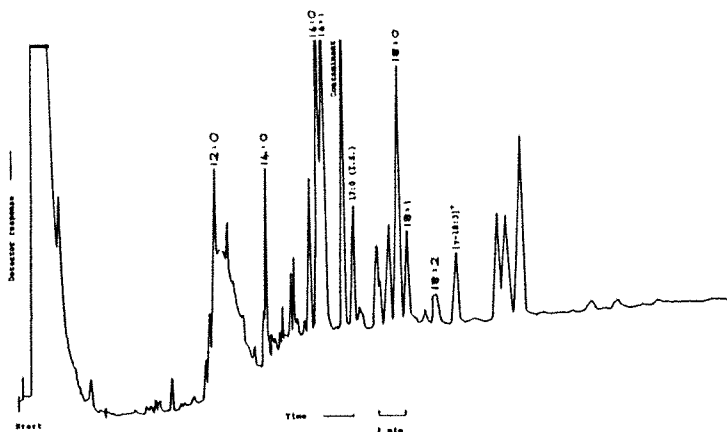
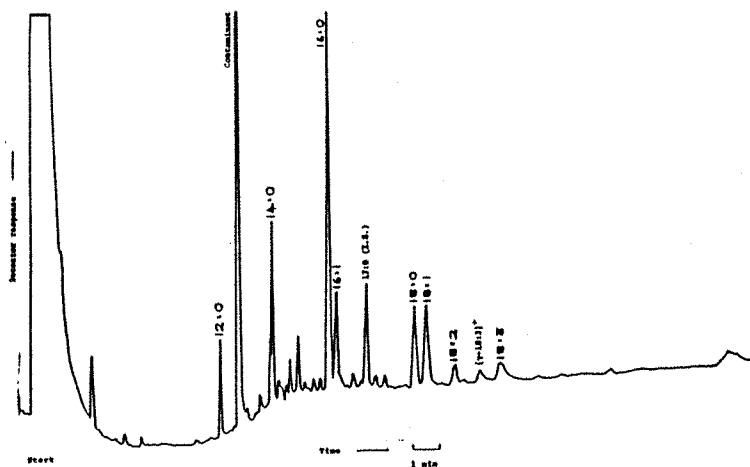


Figure 6.16 Derivatized XAD blank material from November, 1985

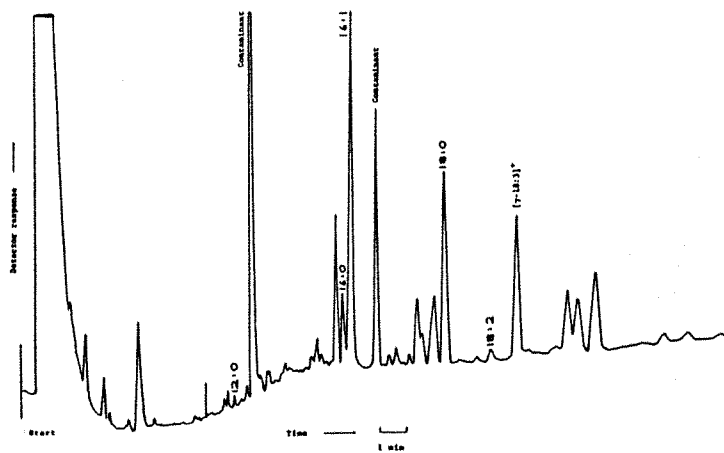
(a) Extract before column chromatography



(b) First diethyl ether/cyclohexane fraction after column chromatography



(c) Diethyl ether fraction after column chromatography



6.16 show that:

- (i) a large proportion of the contamination was removed by column chromatography, as required.
- (ii) in both samples, peaks in the diethyl ether/cyclohexane fraction occurred at the same retention times as 12:0, 14:0, 16:0, 16:1, 17:0 (internal standard), 18:0, 18:1, and 18:2. Both chromatograms were also similar in the minor peaks present.

To confirm the identity of the contaminant peaks, GC-MS analysis was carried out on the first diethyl ether/cyclohexane fractions (Figures 6.17 and 6.18). As normal FAMES show intense peaks at m/e values of 74 and 87 (474), single ion traces at these two m/e values were also recorded. The chromatograms show that all the major contaminant peaks, except for the most intense one, contained the ions at $m/e = 74$ and 87, suggesting that they were FAMES. Their mass spectra confirmed this. The main peak, which was not a FAME, yielded a mass spectrum with major ions at m/e of 71, 43 and 72. An attempt to match this with any library spectra (43 000 compounds) yielded 1-propoxypentane as the best fit (although a good match was not obtained).

Therefore, it can be concluded that, in this instance, the majority of the contaminant peaks were FAMES.

Having confirmed the dominance of FAMES in the samples/blank, their concentrations were estimated (Table 6.14). It can be seen that no component exceeded 1 μg in the final solution (i.e., in 50 μl cyclohexane).

Figure 6.17 GC-MS analysis of the diethyl ether/cyclohexane fraction from site C, November, 1985, after column chromatography

Selected ion chromatograms - (a) Total; (b) m/e = 74; (c) m/e = 87

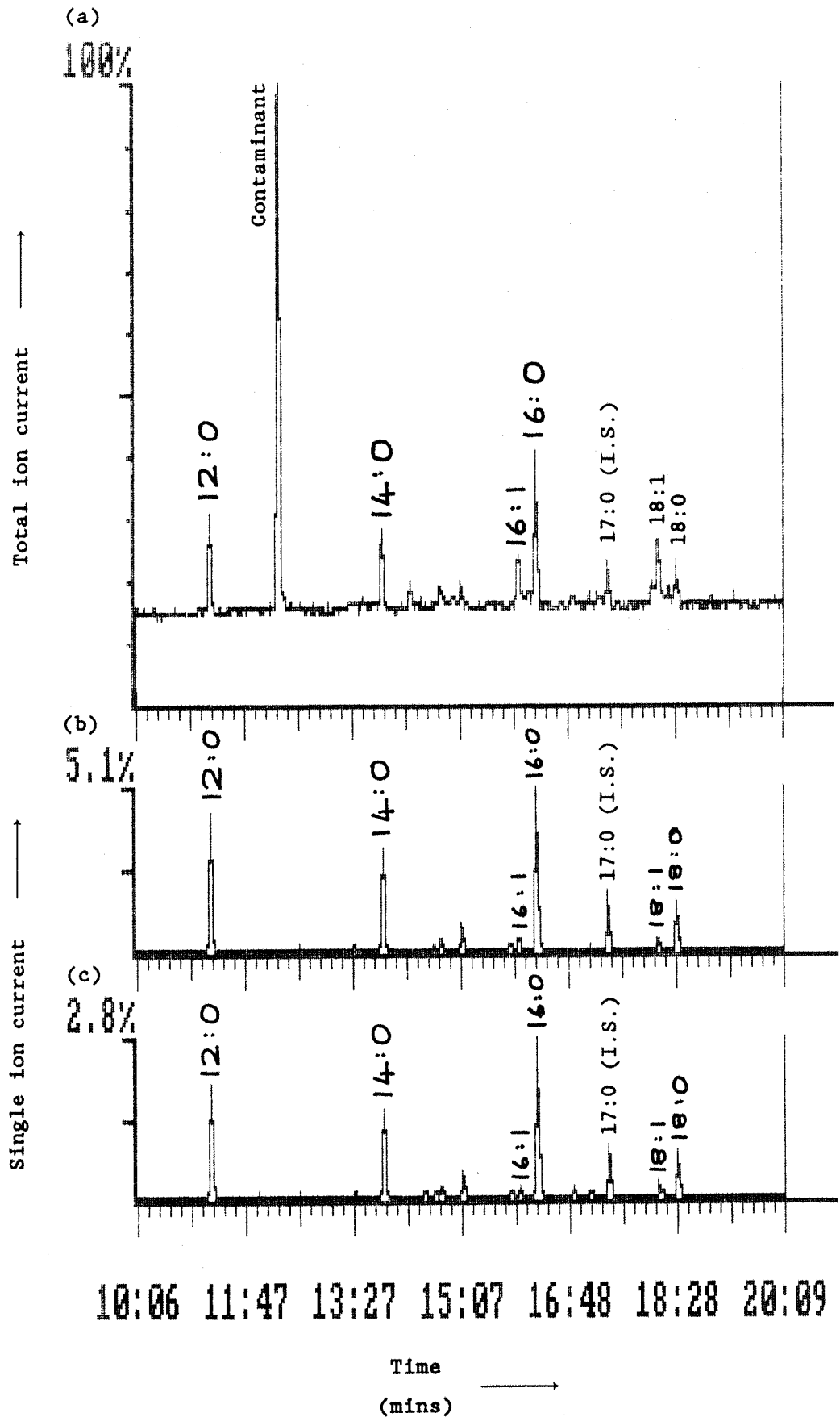


Figure 6.18 GC-MS analysis of the diethyl ether/cyclohexane fraction of the XAD blank from November, 1985, after column chromatography

Selected ion chromatograms - (a) Total; (b) m/e = 74; (c) m/e = 87

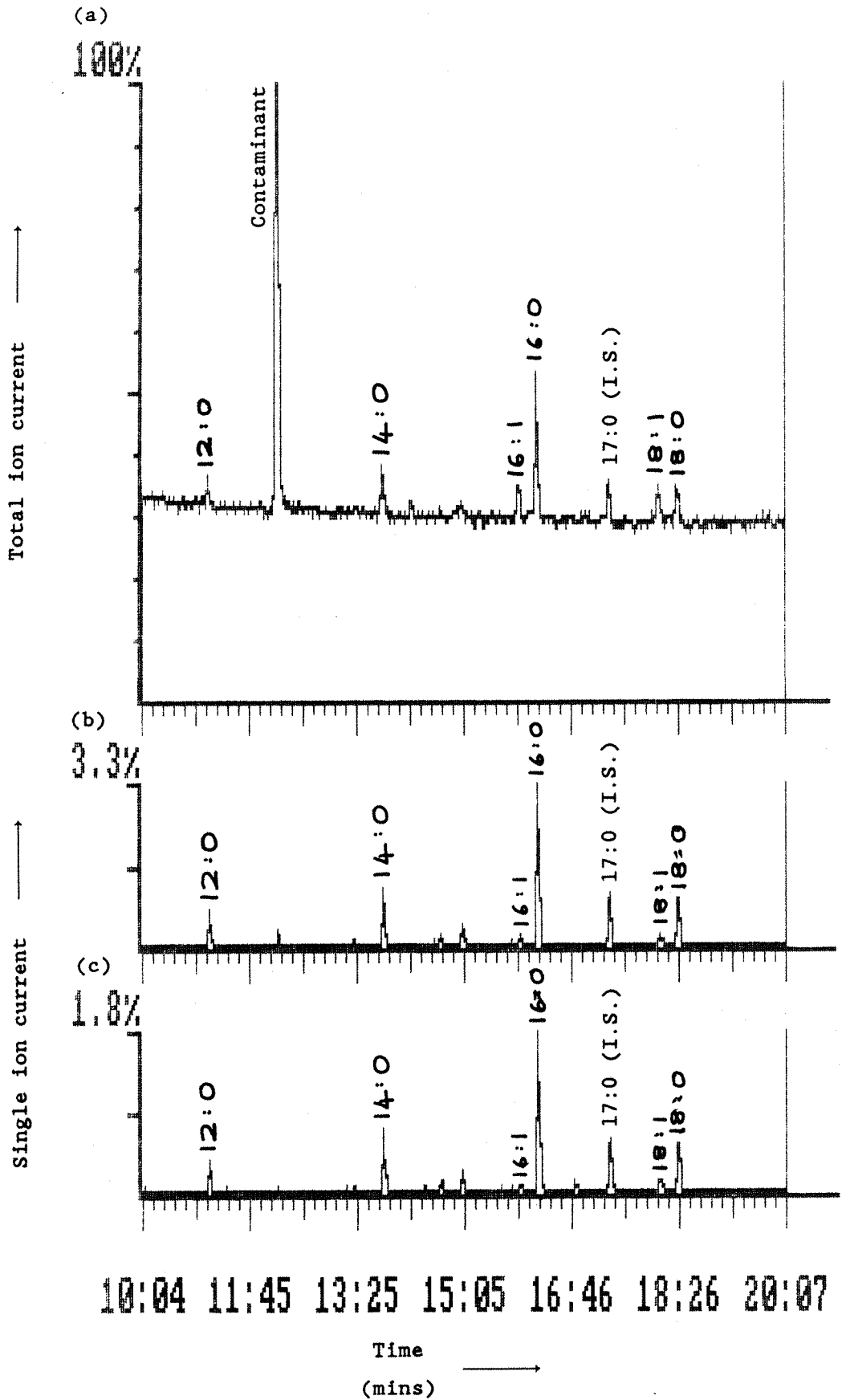


Table 6.14 Concentrations of FAMES in the sample from site C, November, 1985, and the XAD blank

Sample	Total mass in final solution (μg) *						
	12:0	14:0	16:0	16:1	18:0	18:1	18:2
Site C	NC	NC	0.9	NC	0.3	0.9	0.2
XAD	NC	NC	0.7	NC	0.3	0.6	0.09

NC Not calculated

* Total mass (μg) in final solution (50 μl)

6.8 DISCUSSION

6.8.1 Transesterification method

Although semi-quantitative yields of the FAMES were achieved from ca. 0.5 μg of selected lipids (containing the fatty acids, 16:0, 18:0, 18:1 and 22:0), recoveries of the lower molecular weight FAMES (12:0 and 14:0), and of 16:1, were less acceptable (Section 6.6.1.2).

Low yields of unsaturated esters have been shown to occur by Lough (475). Morrison and Smith (468), however, stated that this was due to the high concentration of boron trifluoride (50% (w/v)) employed, and found that, using 14% (w/v) boron trifluoride in methanol, acceptable yields of these esters were obtained, showing no greater loss than the other reagents studied. On the other hand, Fulk and Shorb (439) produced results supporting the findings of Lough, using the reagent with a concentration of 14% (w/v). Isobe *et al.* (471) observed differences between analysed values of unsaturated fatty acid esters on altering the amounts of reagent, and between reagent lots. In the current study, recovery of 18:1 appeared acceptable even though that of 16:1 was low. Further studies should be performed to check whether this result is reproducible, and, if so, how yields of unsaturated FAMES vary with carbon number and degree of unsaturation.

Losses of short-chain fatty acids have been found to result:-

- (i) on refluxing the transesterification medium (437).
- (ii) on aqueous extraction (467).
- (iii) as sample size is reduced (472).

In the present study, the removal of the saponification step led to a slight improvement in the yield of short-chain esters as did washing with saturated sodium chloride rather than water. However, variability between the results suggested additional alternative irreproducible losses which were possibly related to the concentration step under a stream of nitrogen. Recoveries of FAME standards, on removal of the solvent under a stream of nitrogen followed by redissolution in cyclohexane, indicated irreproducible yields of 12:0 and 14:0. When the solutions were not taken to dryness the yields improved although accurate solvent volumes are then difficult to determine. Further work on this aspect must be attempted.

Reduced recoveries of short-chain fatty acids due to the small sample size may also be resulting. Solomon *et al.* (472) found acceptable yields of 12:0, 14:0, 16:0, 18:0, 18:1, 18:2 and 18:3 FAMES from triglycerides (350 mg each component, tested separately). However, individual triglycerides of 50, 100 and 200 mg were also transesterified and showed lower overall recoveries as sample size decreased, as well as preferential loss of the shorter-chain acids (12:0 and 14:0). Interestingly enough, on testing recoveries from a composite mixture of the seven triglycerides (50 mg each component, to yield a total sample weight of 350 mg), however, acceptable yields ($\geq 95\%$) were obtained. This led the authors to specify that a minimum of 350 mg total lipid extract must be taken to achieve acceptable recoveries of FAMES.

In the present investigation, a lipid standard was taken corresponding to 0.6 μg each component in the transesterification medium, i.e., a total of ca. 4 μg lipid material. The data (Table 6.12) showed a similar trend to that of Solomon *et al.* (472) using 50 μg of individual lipids. They did not investigate the origin of these losses, however.

Therefore, the transesterification method must be investigated further to attempt to find the origin of, and to reduce, the losses, so that determinations of the very low levels of lipids necessary for this work can be performed quantitatively using the boron trifluoride in methanol reagent.

6.8.2 Fatty acids in humic substances and their likely origins

In the present study, saturated FAMES could not be detected in the humic samples due to contamination. Peaks corresponding to 12:0, 14:0, 16:0, and 18:0 occurred in all analyses including the blanks. Earlier analyses suggested the presence of 16:1 and 18:1 in humic samples from sites A to C, May, 1986, and 16:1, 18:1 and 18:2 in the sample from site A, November, 1985. The presence of these unsaturated acids (in a sample from site C, November, 1985 plus the XAD blank material) in a subsequent analysis, using a different batch of boron trifluoride in methanol reagent, was noted, however, and so the integrity of the earlier results requires confirmation.

The occurrence of unsaturated fatty acids in aquatic humic materials is in agreement with the work of Harvey *et al.* (222) on marine humic substances. Polyunsaturated triglycerides were suggested by these workers as precursors for the formation of marine humic substances and so transesterification of humic materials in the early stages of formation should show evidence of unsaturated FAMES as seen in the current work. The presence of unsaturated lipids would be expected in aquatic humic materials because they are among the major lipids in plankton (314) as well as other marine organisms (312). Long-chain, polyunsaturated acids (in particular 20:5 and 22:6) have been used as planktonic markers by various workers (476) due to their dominance in planktonic material and their absence in terrestrially-derived lipids.

Darley (411) reviewed the fatty acid composition of various diatoms and found no obvious differences between those from freshwater and marine environments. The major fatty acids were 16:0, 16:1 and 20:5, with 16:1 accounting for between 20 and 45% of the total fatty acid content of the diatoms. Unlike the amino acid composition of phytoplankton, which differs little from one species to another (477),

the fatty acid distribution was found to vary between species, and so may possibly be used to differentiate between them (314). For example, *cryptophyceae* are enriched in the fatty acid, 20:1, compared to the other species studied. The lipids of other marine organisms, such as marine invertebrates, fish and selected species of whale, have also been shown to contain significant amounts of unsaturated acids (312). Thus, both plankton and higher animals are possible sources of these unsaturated acids in aquatic humic substances.

Possible relationships between dissolved and particulate aquatic lipids and humic materials must be considered. The fatty acid composition of the particulate lipids in seawater would be expected to resemble that of the plankton biomass. A comparison of dissolved and particulate fatty acids with that of the planktonic material in Villefranche Bay, France (478) showed palmitic acid to be the major component in most samples. The importance of stearic acid, myristic acid and C₁₈ unsaturated acids plus, in selected samples, C₁₆ unsaturated acids was also noted. The similarity between planktonic and particulate matter was confirmed by the greater abundance of polyunsaturated acids in these materials. The decrease in unsaturated acids in the dissolved fraction was thought to be due to their photolytic/oxidative decomposition.

The C₁₄ to C₁₈ acids have generally been found to dominate the dissolved and particulate fraction of seawater. Blumer (479) reported the concentrations of dissolved fatty acids in Buzzard's Bay, U.S.A. and found C₁₆ and C₁₈ were the major fatty acids with C₁₄ also being significant. He did not separate saturated and unsaturated materials. Slowey *et al.* (480) found palmitic and stearic acids to be the main fatty acids in the seawater from the Gulf of Mexico and palmitic and oleic acids were found to dominate the average composition of the DOM during a plankton bloom in the Northern North Sea (481) although proportions of the individual acids varied at different stages of the bloom.

The major fatty acids in the dissolved and particulate material in the sea-surface microlayer were also myristic, palmitic and stearic acids (482). The significance of oleic acid (18:1) and, in certain samples, arachidic acid (20:0), together with a slight enrichment in

the unsaturated acids, was also noted in the particulate material. Thus, it appears that the dissolved and particulate material in seawater is dominated by saturated fatty acids, mainly C₁₄ to C₁₈, although the particulate organic material shows greater evidence of planktonic input due to increased concentrations of unsaturated acids. It has been suggested that these acids are decomposed *in situ* in the case of DOM (478) and so their incorporation into humic materials from this source is less likely.

In comparison to marine organic materials, mainly of planktonic origin, river water DOM material would be expected to contain varied inputs from soil material in addition to varying contributions from freshwater plankton. The fatty acids of soil lipids (originating mainly from fats, waxes and resins of plant or animal origin) consist mainly of normal saturated acids, ranging from C₁₂ to C₃₆ (51). As with other naturally occurring fatty acids, they normally show a predominance of even numbers of carbon atoms over odd. As reviewed by Kvenvolden for normal fatty acids in sediments (306), the lower molecular weight fatty acids (C₄ to C₂₆) occur in natural fats whereas the higher molecular weight acids (C₂₆ to C₃₈) are found mainly in insect and plant waxes. Thus normal fatty acids of higher molecular weight often indicate material originating from higher plants. Similar fatty acids have been found in soil humic materials (304) - the presence of two 'groups' of fatty acids, with maxima at C₂₄ and C₃₂, resulted, which was explained by proposing a dual origin (microbial and plant) for these materials.

The presence of branched-chain fatty acids in varying amounts has also been reported in soil humic substances (303), and may have a bacterial origin. Their presence in dissolved (483) and particulate (476) marine organic matter has been used as an indicator of bacterial activity.

Few comprehensive studies have been carried out of fatty acids in riverwater. Hullett and Eisenreich (484) studied non-volatile fatty acids in the Mississippi river by both GC and HPLC. Both free and bound fatty acids were analysed but only the results for the free fatty acid fraction were documented. The different chromatographic methods yielded different distributions of the individual fatty acids

although palmitic acid was the main component in both instances, accounting for over one third of the total free fatty acids isolated. In addition, major acids identified by HPLC were 13:0, 14:0, 17:0, 18:0 and 18:2. By GC, 13:0 was not detected but 14:0 (iso), 16:1, and 22:0 (iso) were also found. The lack of long-chain fatty acids (> C₂₀) was noted in both the free and bound fatty acid fractions, indicating the predominantly autochthonous nature of the material.

Preliminary work in this study showed that the major dissolved fatty acid in the Beaulieu river system at sites A and B (April, 1986) was palmitic acid. The importance of myristic acid and stearic acid, together with the low level of unsaturated material, was noted at site A. By comparison, site C showed a lower fatty acid content with the appearance of 20:1, and site B also showed increased amounts of unsaturated material compared to site A in addition to significant quantities of myristic and stearic acids. This data suggests the decreased terrestrial input and increased planktonic input on going from site A to site C.

Combining information on both the fatty acids in soil humic substances and those dissolved in riverwater, an abundance of saturated material would be expected in the aquatic matter if it originated from a predominantly allochthonous source. Although limited, data on the fatty acid content of humic substances in the Beaulieu river system indicate the presence of unsaturated fatty acids at all sites in May, 1986, and at site A in November, 1985. Due to the high level of unsaturated materials in planktonic and other marine organisms, the data in the present work indicates a predominantly autochthonous input to these humic substances. Despite significant blank levels for saturated fatty acids, major terrestrial input should have resulted in levels of saturated lipids greatly exceeding those of the unsaturated matter.

This result may seem surprising considering the distribution of dissolved fatty acids in April, 1986, suggesting increased terrestrial input on going towards the sea. However, it may be explained by a possible predominance of plankton-derived humic materials due to a recent spring plankton bloom (May) or periods of low terrestrial input at these sampling times. Unfortunately the plankton content of the

samples was not monitored.

Aside

Due to the presence of contaminants in the XAD blank extraction procedure, it was not possible to investigate whether free fatty acids were co-extracted with the humic materials in this instance. However, extraction of fatty acids using XAD-8 has been studied by other workers. Concentration and fractionation of hydrophobic organic acid constituents from natural waters, using XAD-8, has been performed by Thurman and Malcolm (44). Model compounds tested included heptanoic acid (7:0), octanoic acid (8:0), nonanoic acid (9:0), decanoic acid (10:0), palmitic acid (16:0) and stearic acid (18:0), all of which were adsorbed onto XAD-8, at pH 2. They were eluted with sodium hydroxide (0.1 moles/l) (heptanoic acid and octanoic acid), then deionised water (nonanoic acid and decanoic acid), and finally methanol (palmitic acid and stearic acid). Giabbai *et al* (358) also extracted stearic acid using XAD-8, at pH 2.

Elution of fatty acids from XAD-8, using methanolic ammonia, has not been documented. According to Aiken (485), long chain fatty acids are co-eluted with the humic material when organic solvents, such as methanol, are employed for desorption. This also occurs, to a lesser extent, if gradient elution of the humic substances is performed. Therefore, it is likely that the present extraction procedure co-extracts free fatty acids and possibly other lipids, along with the humic material. It has been suggested (485) that this could be overcome by extracting the water sample prior to acidification to pH 2, on an XAD pre-column, to remove the fatty acids as the sorption of fatty acids on XAD resins was said to be pH independent. The humic substances could then be adsorbed, at pH 2, on the analytical XAD column. This method is questionable, however, as the pH dependence of the adsorption of fatty acids onto XAD-2 has been reported (169). Alternatively, lipid material could be extracted from the resulting humic matter, using methylene chloride (222).

It cannot, therefore, be concluded that the unsaturated lipids extracted in this instance were an integral part of the humic substances.

6.9 Summary of results and conclusions

A transesterification method based on saponification followed by methylation was modified in an attempt to reduce sample losses in trace level lipid analysis. Application of this method to the analysis of lipids in aquatic humic substances resulted in chromatograms in which any sample was swamped by numerous blank peaks. Therefore, attempted clean-up was performed using TLC and column chromatography. Column chromatography was found to be more satisfactory due to losses of unsaturated acids during TLC. Various elution schemes were evaluated, and the clean-up was then tested on previously derivatised humic samples. After slight modifications to the chromatographic solvents, humic materials from May, 1986 were subjected to the complete analysis.

Due to the persistence of the contamination, the XAD extraction / reagent blanks were checked, revealing variations between different sources of boron trifluoride in methanol, in addition to contributions from both the XAD extraction (partial clean-up possible by column chromatography) and the methanolic ammonia concentration steps.

Despite these problems, palmitoleic (16:1) and oleic (18:1) acids were found at all sites during May, 1986. In addition, linoleic acid (18:2) was detected at site A, in November, 1985. This may suggest significant planktonic input to these materials. To the author's knowledge, no comprehensive study has previously been performed in order to identify individual fatty acid components of aquatic humic materials by transesterification procedures. However, due to the detection of unsaturated fatty acids in a subsequent blank analysis using a different batch of boron trifluoride in methanol, these results must be treated with caution. In addition, possible coextraction of fatty acids with the humic substances must not be discounted. The predominance of unsaturated fatty acids in recently formed aquatic humic substances, however, would add weight to the hypothesis of Harvey *et al.* (222), and hence, further work is required to confirm the results of the present study.

CHAPTER SEVEN

ANALYSIS OF AMINO ACIDS IN ESTUARINE AND MARINE HUMIC MATERIALS

This chapter describes the determination of the amino acid content of estuarine and marine humic materials, using TLC to screen for amino acids, and an amino acid analyser for their quantitation.

7.1 A REVIEW OF TECHNIQUES USED IN AMINO ACID ANALYSIS

Early work on amino acids employed classical gravimetric procedures and microbiological determinations (briefly reviewed by Eveleigh and Winter (486)). These were superseded, however, by the introduction of chromatographic techniques. Separations were initially performed using paper chromatography (487), but this has now largely been replaced by TLC due to its speed and greater sensitivity. Concurrently, column chromatographic techniques were also investigated, using solids such as aluminium oxide, silica gel, activated charcoal and starch (486). The introduction of ion-exchange chromatography improved both the resolution and speed of column-based techniques, with the sulphonated resins proving the most popular (488). By the late 1950's, an automatic amino acid analyser, based on ion-exchange chromatography of the amino acids with post-column ninhydrin derivatisation, had been designed (489). Variants of these amino acid analysers are now used routinely for the quantitative determination of amino acids. Further developments in column chromatography of amino acids have employed pre-column derivatisation methods, followed by reversed-phase HPLC.

Gas chromatography has also been used (since the early 1960's) in the analysis of amino acids, requiring the formation of volatile derivatives. More recently, supercritical fluid chromatography has additionally been employed, particularly in the field of enantiomeric separations of derivatised amino acids (490). Of the available methods, TLC is often used for qualitative applications whilst samples which require quantitation are generally analysed by either liquid or gas chromatography.

7.1.1 Thin-layer chromatography

Separations of amino acids, both underivatized and derivatized, have been performed on various adsorbents including starch, cellulose, Kieselguhr, aluminium oxide, ion-exchangers, and silica gel (491). Of these, silica gel and cellulose are the most commonly used for underivatized amino acids.

Numerous solvent systems have been employed for the separation of amino acids (e.g., (491)). Microcrystalline cellulose, which was chosen for the present study, can be eluted with a variety of solvent systems (Table 7.1). A typical two-dimensional system, using both acidic and basic solvent systems (492), employs t-butanol:methyl ethyl ketone:ammonia:water (t-BuOH:MEK:NH₃:H₂O) for elution in the first dimension, followed by a double development in the second dimension, using n-butanol:acetone:acetic acid:water (n-BuOH:acetone:acetic acid:H₂O). This system was assessed for application in the current study.

The location of the amino acids on the chromatograms can be performed using either general reagents, such as ninhydrin, fluorescamine and o-phthalaldehyde, which react with all amino acids, or more specific reagents, such as iodo-platinate (for sulphur-containing amino acids), which react with a specific functional group (492). Ninhydrin was adopted in the present work.

7.1.2 Liquid chromatography

There are basically two liquid chromatographic methods for the separation of amino acids, and these have been reviewed by several workers (493,494). They involve either separating the underivatized amino acids by cation-exchange chromatography, followed by a post-column derivatization reaction, or derivatizing the amino acids prior to chromatography, and then separating them by reversed-phase HPLC. Post-column detection of the derivatives is generally either spectrophotometric or fluorimetric although mass spectrometry is also becoming more popular with the advent of improved interfaces.

Table 7.1 A selection of solvent systems for the TLC of amino acids on cellulose

	<u>Solvent System</u>	<u>Solvent ratios</u>	<u>Ref.</u> ⁺
Acidic :	n-butanol:acetic acid:water	3:1:1	495
	n-butanol:acetone:acetic acid:water	70:70:20:40	492
	iso-propanol:formic acid:water	40:2:10	496
	n-butanol:formic acid:water	480:6:314	496
		75:15:10	497
	phenol:water	8:2	497
	iso-propanol:citric acid:water	40:2:10	496
	Basic :	ethanol:ammonia(0.88):water	180:10:10
pyridine:methyl ethyl ketone:water		15:70:15	497
		15:60:25	496
pyridine:methanol:water		4:80:20	497
pyridine:n-butanol:water		3:10:3	496
n-butanol:ethanol:diethylamine:water		10:10:2:5	496
n-butanol:acetone:diethylamine:water		10:10:2:5	496
sec-butanol:methyl ethyl ketone:		10:10:2:5	496
dicyclohexylamine:water			
sec-butanol:ethanol:		100:25:15:35	496
dicyclohexylamine:water			
tert-butanol:methyl ethyl ketone:		50:30:10	492
25% ammonia:water			
Neutral :	tert-amyl alcohol:methyl ethyl ketone:water	120:40:40	492
	n-butanol:ethanol:water	10:10:5	496
	tert-amyl alcohol:iso-propanol:water	100:40:55	496
	sec-butanol:tert-butanol:methyl ethyl ketone:water	25:25:25:25	498

+ References taken from a selection of reviews and original articles.

The separation of underivatised amino acids is performed on strongly acidic, usually sulphonated, polystyrene-based, cation-exchange resins. It is carried out with a gradient of acidic buffers, the more acidic amino acids eluting first. Detection of underivatised amino acids can be performed at short wavelengths (around 200 nm), but many other compounds absorb in this area and so the risk of artifacts is high. Therefore, amino acid derivatives are formed and detected spectroscopically. The two most popular derivatisation reagents are ninhydrin (derivatives detected spectrophotometrically), and ortho-phthalaldehyde (OPA) (494) (fluorescence detection of derivatives performed). Other fluorescent reagents which have been employed include fluorescamine (499) and 4-fluoro-7-nitrobenzo-2,1,3-oxadiazole (500).

The separation of amino acid derivatives following pre-column derivatisation is generally carried out using reversed-phase C₁₈-based systems. A variety of solvent systems have been employed, the organic components of which mostly contain methanol, acetonitrile and, less often, tetrahydrofuran. Detection of compounds eluting from the column is usually performed fluorimetrically, using reagents such as ortho-phthalaldehyde, phenylisothiocyanate (PITC), 9-fluorenylmethyl-oxycarbonyl chloride (Fmoc), dansyl chloride (1-dimethylamino-naphthalene-5-sulphonyl chloride), dabsyl chloride (4-dimethylamino-azobenzene-4'-sulphonyl chloride) or 4-fluoro-nitrobenzo-2,1,3-oxadiazole (493,494). OPA, PITC and Fmoc are the more popular reagents.

The present work employed ion-exchange chromatography of the underivatised amino acids, followed by spectrophotometric detection of the ninhydrin derivatives. Although the OPA-derivatisation technique shows improved sensitivity over that of ninhydrin (an increase of ten-fold or more), secondary amines, such as proline and hydroxyproline, do not react with OPA and so must be oxidised before reaction (494). Therefore, ninhydrin is advantageous if analyses are only required in the low nanomole region.

7.1.3 Gas chromatography

Prior to the gas chromatographic analysis of amino acids, volatile derivatives must be formed. A variety of methods have been used due to the multifunctional nature of amino acids, and reviews of these have been written (e.g., (501)). The most popular methods have employed the formation of esterified derivatives including N-trifluoroacetyl n-butyl esters and n-propyl, iso-butyl and iso-amyl esters of N-heptafluorobutyryl derivatives (502).

The use of the TMS group (503) has been adopted as an attractive alternative to the aforementioned derivatives as it employs a single functional group to block all active protons, and thus only one derivatisation reagent is required. However, the resulting TMS derivatives are very unstable and so produced irreproducible GC results for many of the amino acids. More recently, the more stable tert-butyldimethylsilyl group has been tested for amino acid analysis (504,505) and the resulting derivatives have been reported to be more stable (505). If mass spectral analysis is to be carried out, these derivatives have the added advantage of yielding a relatively simple mass spectrum, dominated by a unique, unambiguous [M-57] fragment ion (505).

A further method of GC analysis, which has the advantage of not requiring the formation of amino acid derivatives, is pyrolysis GC. Typical pyrolysis products of proteins and amino acids are pyrroles, indoles and nitriles, which are generally identified by mass spectrometry. However, the disadvantage of this technique is the complexity of the resulting chromatograms, which may consist of 50 or more peaks. This problem may be reduced if certain amino acids are targeted, for example, sulphur-containing amino acids have been analysed using pyrolysis-GC with a flame photometric detector (194). Despite the complexity of the chromatograms, however, many investigators have employed pyrolysis-GC-MS for both the determination of the amino acid content of proteins (193), and for the detection of amino acids in more complex matrices, such as soil (207).

7.1.4 Hydrolysis

Although some studies only require the determination of the free amino acid content of a sample, many call for the analysis of amino acids in proteins or peptides, or bound to other matrices. Therefore, the sample must be hydrolysed prior to amino acid analysis. Methods which have been employed include acid, base or enzymatic hydrolyses (486), but unfortunately no single method gives satisfactory results for all amino acids.

The most widely used procedure involves heating the sample in hydrochloric acid (6 moles/l), at 110°C, for 24 hours (e.g., (177)), although a variety of hydrolysis times and temperatures have been employed, including 105°C overnight (492), 110°C for 20 or 70 hours (506) and 155°C for 20 minutes (507). The hydrochloric acid is then removed by rotary evaporation, lyophilisation, or evaporation under vacuum over sodium hydroxide or phosphorus pentoxide. Eveleigh and Winter (486) preferred rotary evaporation because they stated that concentration of the sample to a syrup in a vacuum desiccator can lead to losses by esterification and oxidation, and removal of the hydrochloric acid by lyophilisation is slow due to its low melting point. However, losses of amino acids during rotary evaporation have also been reported (405).

The disadvantages of the hydrolysis method include oxidation of sulphur-containing amino acids (although this is minimised by carrying out the hydrolysis under vacuum, or partially evacuated in the presence of nitrogen), partial decomposition of several amino acids, such as serine, threonine, cystine and tyrosine, total decomposition of tryptophan, and slow hydrolysis of proteins containing valine and iso-leucine residues. Tryptophan decomposition can be avoided by performing alkaline hydrolysis, using barium hydroxide. Some amino acids, such as cystine, serine, threonine (492) and arginine (486) can be lost, however, and certain amino acids racemise with this method (486). Its use is therefore mainly limited to the determination of tryptophan.

The problems of decomposition of amino acids can, in some cases, be overcome by the use of enzymatic techniques (508), but complete

degradation of a protein to its constituent amino acids is difficult; the majority of suitable enzymes hydrolysing some peptide links and not others.

As the nature of the amino acids present was not known in this work, the widely employed acid hydrolysis technique was used.

7.2 THIN-LAYER CHROMATOGRAPHY OF AMINO ACID STANDARDS

A two-dimensional TLC method was developed, based on acidic and basic solvent systems suggested by Errser and Smith (492). The chosen solvent systems were assessed by one-dimensional TLC of amino acid standards, and the optimum systems were then combined in two-dimensional TLC. Both systems were applied to the analysis of amino acids in aquatic humic materials.

7.2.1 Standards

The following standard solutions, each containing two amino acids (ca. 1 mg/ml each amino acid), were prepared in 10% (v/v) iso-propanol in doubly distilled water. The more sparingly soluble amino acids, tyrosine and cystine, required the addition of hydrochloric acid (not exceeding a final concentration of 1%) for complete dissolution.

- 1 L-arginine monohydrochloride (arg) and glycine (gly)
- 2 L-arginine monohydrochloride and L-proline (pro)
- 3 DL-aspartic acid (asp) and DL-methionine (met)
- 4 DL-aspartic acid and L-cysteine hydrochloride (cys)
- 5 L-cystine (cys-cys) and DL-2-amino-n-butyric acid (anba)
- 6 L-cystine and DL-iso-leucine (ile)
- 7 DL-threonine (thr) and L-histidine monohydrochloride (his)
- 8 DL-threonine and DL-serine (ser)
- 9 L-glutamic acid (glu) and DL-nor-leucine (nor)
- 10 L-glutamic acid and L-hydroxyproline (hpro)
- 11 DL-tryptophan (try)
- 12 DL-tryptophan and DL-alanine (ala)
- 13 L-tyrosine (tyr) and L-lysine monohydrochloride (lys)
- 14 L-tyrosine and DL-ornithine monohydrochloride (orn)

15 L-leucine (leu) and DL- β -phenylalanine (phe)

16 L-leucine and DL-valine (val)

A standard solution containing all 23 amino acids (ca. 1 mg/ml) was also prepared in 10% (v/v) iso-propanol in doubly distilled water.

All standard solutions were stored in a refrigerator.

7.2.2 One-dimensional TLC method development

7.2.2.1 Method

One-dimensional TLC of the amino acid standards was performed on aluminium-backed microcrystalline cellulose layers (layer thickness 0.1 mm), using the basic and acidic solvent systems described below (Section 7.2.2). Each amino acid standard (ca. 1 μ l) was applied to the plate with a 5 μ l syringe, at a distance of 2 cm from its edge. The solvent was allowed to evaporate completely between applications.

The required solvent system (200 ml) was placed in the chromatography tank, and the walls were lined with filter paper to ensure uniform saturation with the solvent vapours. The tank was left to equilibrate for 30 minutes before inserting the TLC plate. The solvent was allowed to ascend to a height of 12 cm, and the plate was then removed and air-dried at room temperature.

The amino acids were visualised by spraying the dried chromatographic plate with ninhydrin (0.5% (w/v) in acetone). The plate was then air-dried in a fume cupboard, and warmed at 30°C for 30 minutes. The amino acids generally appeared as blue/violet/grey spots although proline and hydroxyproline were yellow.

7.2.2.2 Results and discussion

7.2.2.2.1 Basic solvent system

The separation of 23 amino acids was investigated using the basic solvent system, t-butanol:methyl ethyl ketone:ammonia:water (t-BuOH:MEK:NH₃:H₂O). Solvent ratios (by volume) employed were:

t-butanol : methyl ethyl ketone : ammonia (0.88) : water				
50	30	7.1	12.9	(492)
50	30	12.6	12.4	(509)
50	30	2.9	17.1	

The R_f values of the amino acids using these solvent ratios are given (Table 7.2). It can be seen that, with these systems, only one half of the available plate area was used, and work was therefore carried out to find a system giving a greater spread. The 23 amino acid standard (ca. 1 μl) was applied to the plate and the length of the amino acid band was measured.

The optimum system (Table 7.3) appears to be approximately t-butanol:methyl ethyl ketone:ammonia:water, 50:30:12:30. A system containing 40:25:10:25 was therefore used to separate the binary standards. Figure 7.1 shows that the amino acids were more evenly spread over the plate, and good spot shapes, with little tailing, resulted. This system was therefore chosen for the present work.

7.2.2.2.2 Acidic solvent system

Separation of the amino acids, using the solvent system n-butanol:acetone:glacial acetic acid:water, 35:35:10:20 (492), was carried out but poor chromatography and distorted spot shapes resulted. A similar system, n-butanol:glacial acetic acid:water (n-BuOH:acetic acid:H₂O), 60:15:25 (492), was also assessed. This produced good chromatography, with symmetrical spots and little tailing (Figure 7.2) and was therefore adopted.

Table 7.2 R_f values of amino acids in the solvent system,
t-BuOH:MEK:NH₃:H₂O

Amino acid	Solvent ratio ⁺		
	(i)	(ii)	(iii)
DL-alanine	0.10	0.13	0.10
DL-2-amino-n-butyric acid	0.15	0.18	0.15
L-arginine monohydrochloride	0.03	0.05	0.03
DL-aspartic acid	0.01	0.02	0.02
L-cysteine hydrochloride ⁺⁺	0.01	0.02	0.02
L-cystine	0.01	0.02	0.01
L-glutamic acid	0.01	0.02	0.01
Glycine	0.07	0.10	0.07
L-histidine monohydrochloride	0.09	0.12	0.09
L-hydroxyproline	0.07	0.11	0.07
L-leucine	0.39	0.41	0.38
DL-iso-leucine ⁺⁺⁺	0.35, 0.40	0.36, 0.41	0.34, 0.38
DL-nor-leucine	0.41	0.42	0.40
L-lysine monohydrochloride	0.04	0.09	0.03
DL-methionine	0.27	0.30	0.27
DL-ornithine monohydrochloride	0.04	0.07	0.03
DL- β -phenylalanine ⁺⁺⁺⁺	0.39	0.41	0.38
L-proline	0.14	0.19	0.13
DL-serine	0.12	0.15	0.11
DL-threonine	0.37	0.37	0.34
DL-tryptophan	0.35	0.38	0.35
L-tyrosine	0.17	0.17	0.20
DL-valine	0.24	0.28	0.22

+ The solvent ratios, t-BuOH:MEK:NH₃:H₂O, (by volume) were:

(i) 50 : 30 : 7.1 : 12.9

(ii) 50 : 30 : 12.6 : 12.4

(iii) 50 : 30 : 2.9 : 17.1.

++ Co-chromatography of cysteine and aspartic acid was suspected.

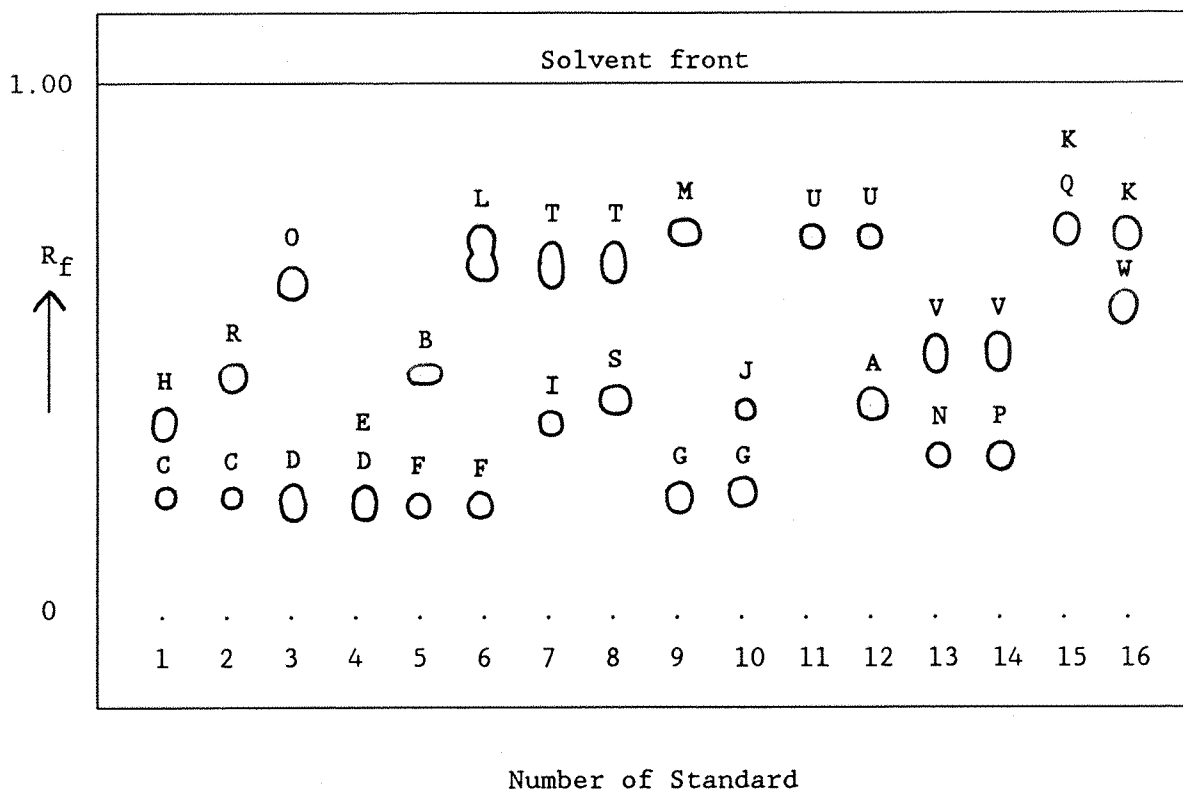
+++ Contaminant present in standard.

++++ Co-chromatography of phenylalanine and leucine was suspected.

Table 7.3 The spread of the amino acids using various solvent ratios

Ratio (t-BuOH:MEK:NH ₃ :H ₂ O)	R _f			Remarks
	Start	Finish	Range	
50 : 30 : 7 : 113	0.54	0.93	0.39	Too near solvent front/ flattened spots
50 : 30 : 12 : 20	0.03	0.59	0.56	Too near base
50 : 30 : 12 : 30	0.13	0.70	0.57	* Chosen *
50 : 30 : 12 : 40	0.28	0.83	0.55	Flattened spots near solvent front
50 : 30 : 12 : 60	0.43	0.89	0.46	Flattened spots near solvent front
50 : 40 : 12 : 20	0.02	0.54	0.52	Too near base
60 : 30 : 12 : 20	0.01	0.57	0.56	Too near base

Figure 7.1 Thin-layer chromatogram and R_f values of amino acids in the solvent system, t-BuOH:MEK:NH₃:H₂O, 40:25:10:25



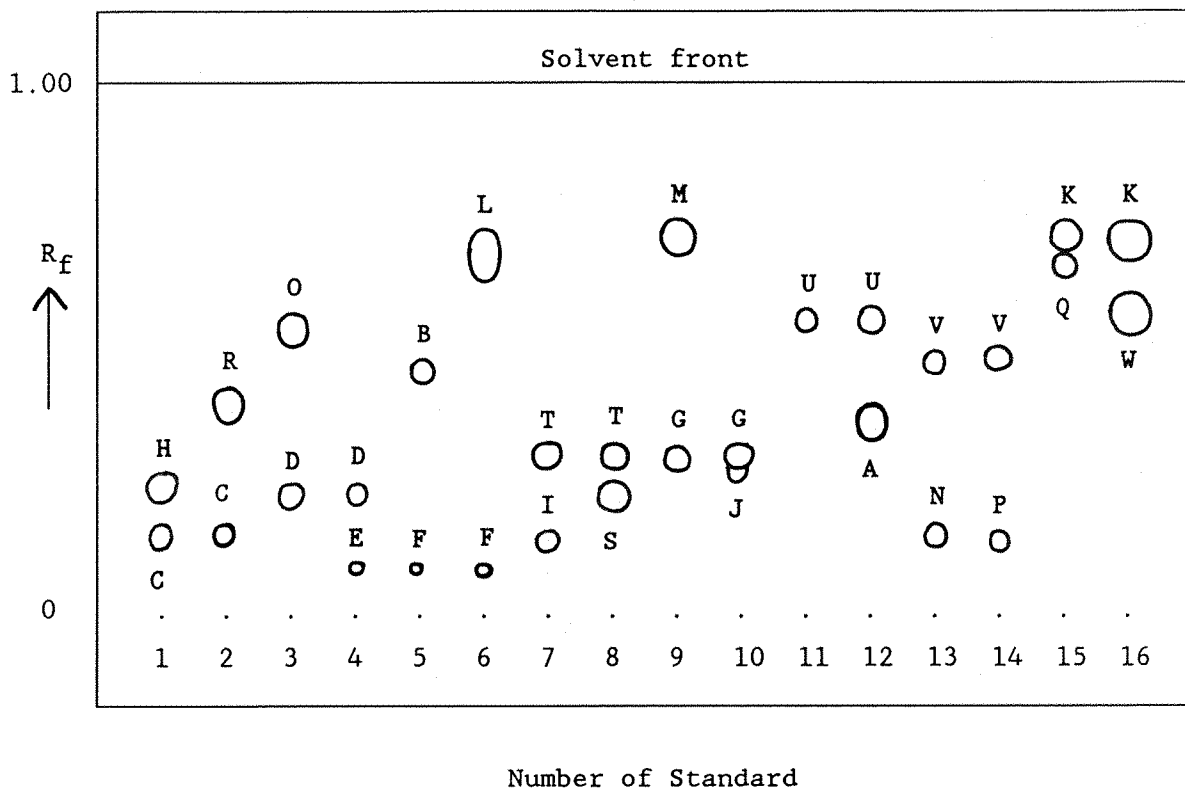
<u>Label</u>	<u>Amino acid</u>	<u>R_f</u>	<u>Label</u>	<u>Amino Acid</u>	<u>R_f</u>
A	DL-alanine	0.39	M	DL-nor-leucine	0.72
B	DL-2-amino-n-butyric acid	0.45	N	L-lysine monoHCl	0.30
C	L-arginine monoHCl	0.23	O	DL-methionine	0.62
D	DL-aspartic acid	0.23	P	DL-ornithine monoHCl	0.29
E	L-cysteine HCl ⁺	0.23	Q	DL-phenylalanine ⁺⁺⁺	0.72
F	L-cystine	0.21	R	L-proline	0.45
G	L-glutamic acid	0.22	S	DL-serine	0.41
H	Glycine	0.36	T	DL-threonine	0.66
I	L-histidine monoHCl	0.37	U	DL-tryptophan	0.71
J	L-hydroxyproline	0.38	V	L-tyrosine	0.49
K	L-leucine	0.72	W	DL-valine	0.58
L	DL-iso-leucine ⁺⁺	0.66,0.70			

+ Co-chromatography of cysteine HCl with aspartic acid was suspected.

++ Contaminant present in standard.

+++ Co-chromatography of DL-phenylalanine with L-leucine was suspected.

Figure 7.2 Thin-layer chromatogram and R_f values⁺ of amino acids in the solvent system, n-BuOH:acetic acid:H₂O, 60:15:25



<u>Label</u>	<u>Amino acid</u>	<u>R_f</u>	<u>Label</u>	<u>Amino Acid</u>	<u>R_f</u>
A	DL-alanine	0.35	M	DL-nor-leucine	0.71
B	DL-2-amino-n-butyric acid	0.45	N	L-lysine monoHCl	0.15
C	L-arginine monoHCl	0.16	O	DL-methionine	0.54
D	DL-aspartic acid	0.22	P	DL-ornithine monoHCl	0.14
E	L-cysteine HCl	0.08	Q	DL-phenylalanine	0.65
F	L-cystine	0.08	R	L-proline	0.39
G	L-glutamic acid	0.29	S	DL-serine	0.22
H	Glycine	0.24	T	DL-threonine	0.29
I	L-histidine monoHCl	0.14	U	DL-tryptophan	0.55
J	L-hydroxyproline	0.27	V	L-tyrosine	0.48
K	L-leucine	0.71	W	DL-valine	0.56
L	DL-iso-leucine ⁺⁺	0.66, 0.69			

+ These data are the mean of two determinations.

++ Contaminant present in standard.

7.2.3 Two-dimensional TLC method development

7.2.3.1 Method

Two-dimensional thin-layer chromatography was performed, using the optimum basic and acidic solvent systems, t-BuOH:MEK:NH₃:H₂O - 40:25:10:25, and n-BuOH:acetic acid:H₂O - 60:15:25. The 23 amino acid standard (ca. 1 μ l) was applied to the bottom left-hand corner of the TLC plate (aluminium-backed micro-crystalline cellulose layers, as above), at a distance of 2 cm from both edges of the plate. Chromatography was performed in the first dimension, in a solvent tank saturated with the basic solvent system. When the solvent had ascended to a height of 12 cm, the plate was removed and air-dried at room temperature. It was then turned through 90°, and chromatography was performed in the second dimension, using the acidic solvent system. On completion, the plate was air-dried and the amino acids were visualised with ninhydrin.

Additionally, two-dimensional chromatography was carried out using the acidic solvent system in the first dimension, followed by the basic system in the second.

7.2.3.2 Results and discussion

Better chromatography was found to result using the acidic solvent system in the first dimension. It is interesting to note that Ersser and Smith (492), performing two-dimensional chromatography with t-BuOH:MEK:NH₃:H₂O and n-BuOH:acetone:acetic acid:H₂O, suggested that the basic solvent system should be employed in the first dimension. It is possible that this may be so when natural samples, containing salts, are analysed.

The two-dimensional TLC, using the acidic solvent system in the first dimension, is shown (Figure 7.3), together with the 'theoretical' TLC (Figure 7.4), calculated using the one-dimensional TLC R_F values from Section 7.2.1.3. The spots were tentatively identified by comparison with the one-dimensional TLC data (Figure 7.4). It was thought that hydroxyproline was not observed as it was too pale to detect, and cysteine was oxidised to, and thus

Figure 7.3 Two-dimensional TLC of the 23 amino acid standard

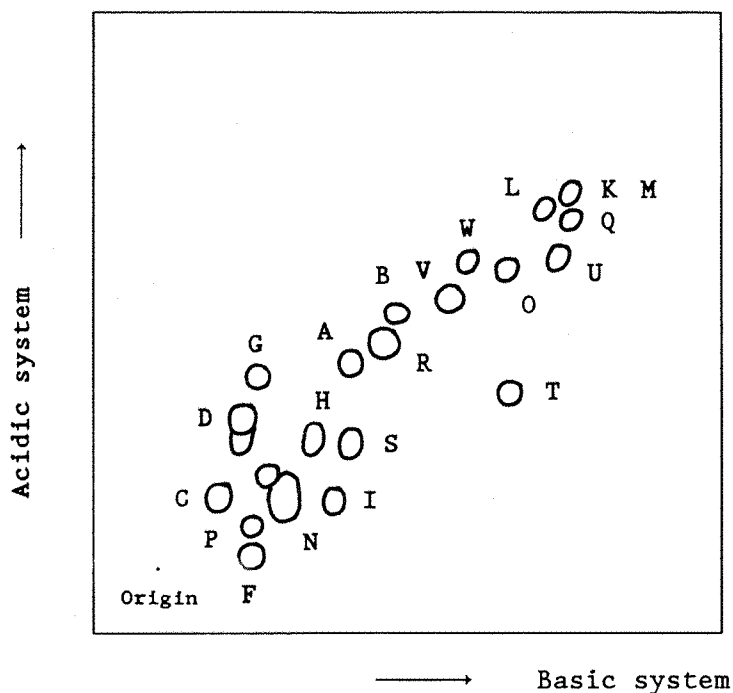
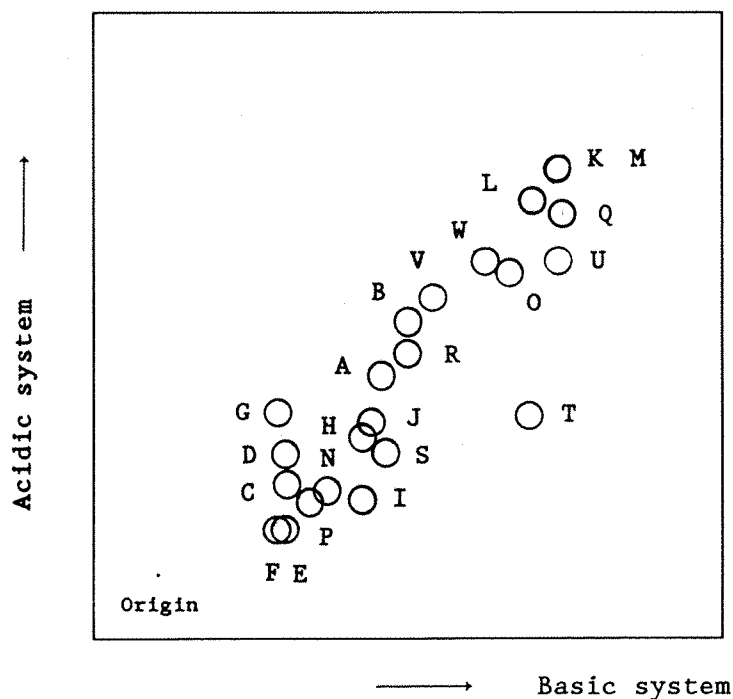


Figure 7.4 Theoretical two-dimensional TLC of the 23 amino acid standard +



+ Based on R_f values from the one-dimensional TLCs of the individual solvent systems.

Labelled as in Figures 7.1 and 7.2

detected as, cystine. Co-chromatography of leucine and nor-leucine was found to occur.

7.2.4 Retention of amino acids by XAD-8

Before any conclusions could be drawn about the amino acid content of the humic materials, it was necessary to check whether free amino acids in the water samples could be extracted by the XAD-8 extraction procedure.

7.2.4.1 Introduction

Few studies have been performed on the extraction of amino acids, using XAD resins, either in the presence or absence of humic substances. Lytle and Perdue (333) studied free, proteinaceous, and humic-bound amino acids in river water, using XAD-7, and indicated that neither the free amino acids nor proteins were adsorbed by the resin in the presence of humic materials. In agreement with this, glycine has been shown to pass through XAD-8 in a fractionation study using model compounds (358). However, contrary to the work of Lytle and Perdue, proteins have been isolated from fermentation broths using polymeric adsorbents (510), although the specific resins employed were not stated.

Therefore, whilst it seems unlikely that free amino acids will be adsorbed onto the XAD-8 resin, the adsorption properties of proteins are less well known.

7.2.4.2 Experimental

Artificial seawater (100 ml) was spiked with the 23 amino acid standard (500 μ l). This sample (analysed in duplicate) and an unspiked sample (reagent blank) were subject to a scaled-down extraction procedure (original procedure given in Section 4.2.2), using 7 ml of XAD-8 resin. The methanolic ammonia eluents were evaporated to dryness, and the residues were dissolved in 10% (v/v) iso-propanol in doubly distilled water (250 μ l). They were then analysed by two-dimensional TLC (as in Section 7.2.2) to detect any amino acids present.

The water eluting from the resins was also analysed by evaporating it to dryness, and redissolving the residues in 10% (v/v) iso-propanol in doubly distilled water (250 μ l). The samples were not desalted by ion-exchange chromatography, to ensure that no amino acids were lost during this step. The resulting solutions were analysed by two-dimensional TLC as above.

7.2.4.3 Results and discussion

The resulting chromatograms (Figure 7.5) show the presence of amino acids in the artificial seawater eluting from the column. DL-2-amino-n-butyric acid, iso-leucine, leucine, phenylalanine, tyrosine and valine were all tentatively identified by comparison with the two-dimensional TLC of the 23 amino acid standard. Poor chromatography resulted at lower R_f values, probably due to the presence of salts in the sample, and this prevented the identification of amino acids in that region.

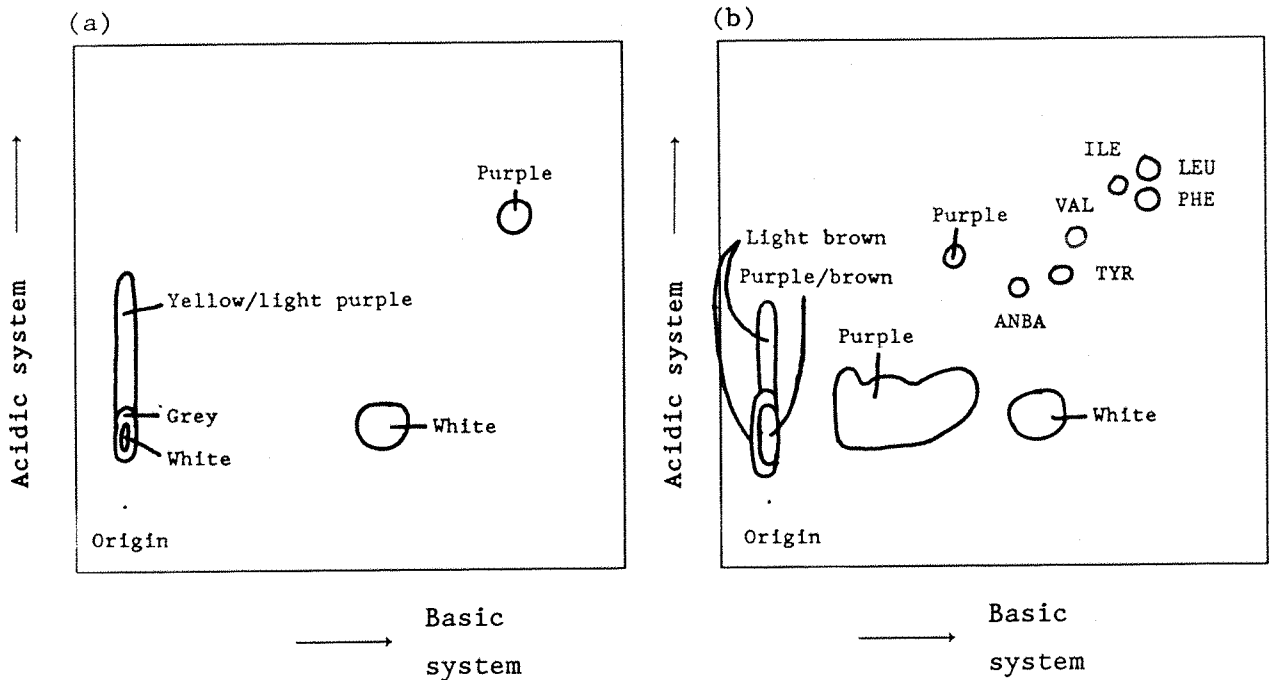
Only one purple spot was detected in the methanolic ammonia eluent. This was thought to be another ninhydrin-positive compound, e.g., an ammonium compound or an impurity from the XAD extraction procedure, and not an amino acid. This could be confirmed by amino acid analysis using column chromatography.

7.2.4.4 Conclusions

A ninhydrin-positive compound (suspected non-amino acid) was detected in the methanolic ammonia eluent but no amino acids were observed. They were seen, however, in the artificial seawater eluent. This suggests that no amino acids were retained by the XAD-8 resin, and agrees with previous work on XAD-7 (333).

Therefore, it can be concluded that any amino acids detected in the humic samples originated from the humic materials themselves, and were not free amino acids co-extracted with them. An alternative possibility, namely that adsorbed humic substances, in turn, take up the amino acids, was not investigated.

Figure 7.5 Two-dimensional TLC of (a) the methanolic ammonia and (b) the artificial seawater eluents



7.2.5 Application of two-dimensional TLC to the analysis of amino acids in humic materials

Having successfully separated most of the amino acids in the standard, the two-dimensional TLC method was applied to the analysis of amino acids in some humic samples.

7.2.5.1 Experimental

Prior to the analysis of amino acids in humic materials, bound amino acids must be freed by hydrolysis. Initially, humic samples from August, 1986 (sites A and C plus the XAD blank; ca. 5 mg each sample) were dissolved in a few drops of doubly distilled water, and then hydrochloric acid (6 moles/l; 0.5 ml) was added. The mixtures were sonicated for five minutes and partially evacuated under an atmosphere of nitrogen - excessive bubbling occurred if complete evacuation was attempted. They were then heated at 115°C, for 12 hours. On cooling, the acid was removed by leaving the solutions evacuated, over potassium hydroxide and silica gel, overnight. Attempts to remove the acid by rotary evaporation failed due to

excessive bumping. The residues were then dissolved in 10% (v/v) iso-propanol in doubly distilled water (50 μ l).

Further humic samples (February, 1986 - sites A, B and C; and August, 1986 - site A; ca. 15 mg each sample) were analysed, using a slightly modified method. The materials were dissolved in doubly distilled water (0.5 ml) and concentrated hydrochloric acid was added (0.5 ml) to give a solution with a concentration of 6 moles/l. After sonication for one minute and partial evacuation, the samples were heated at 120°C overnight. On cooling, they were transferred to plastic centrifuge tubes, and centrifuged for ten minutes. The supernatant was removed, taken to dryness as above, and the residues were redissolved in 10% (v/v) iso-propanol in doubly distilled water. These modifications were adopted for the following reasons:-

- (i) Dissolution of the humic materials in acid was found to be easier if it was dissolved in a small amount of doubly distilled water first. In the original method, however, this resulted in a dilution of the hydrochloric acid (originally 6 moles/l). Therefore, the modified method employed the addition of concentrated hydrochloric acid to yield a final concentration of 6 moles/l.
- (ii) A slightly higher hydrolysis temperature was used to try and ensure complete hydrolysis of bound amino acids.
- (iii) The hydrolysed solutions contained some particulate matter, which may have interfered with the chromatography. Therefore, solutions were centrifuged to remove any particulate matter in the modified method.

Both sets of samples (5 μ l of each) were then subjected to two-dimensional TLC, using the solvent systems employed in Section 7.2.3.

7.2.5.2 Results and discussion

The TLCs of all the samples are shown (Figures 7.6-7.11), together with tentative identification of the amino acids present (assigned by comparison with the two-dimensional TLC of the 23 amino acid standard - Section 7.2.3.2) (Table 7.5). The samples from February, 1986 showed poor chromatography, with large, non-discrete areas of purple coloration, indicating the presence of amino acids or other ninhydrin-positive compounds which could not be identified. The samples from August, 1986, however, showed discrete purple spots and identification of individual amino acids was possible. Compounds which interfered with the chromatography, were therefore thought to be present in the humic extracts from February, 1986, but not in those from August, 1986.

The sample from site A, August, 1986 was analysed by both methods, and, although some similar amino acids were found (i.e., aspartic acid, alanine, glutamic acid and leucine), some different ones were also detected (i.e. valine, iso-leucine and phenylalanine, in the original method, and glycine, cystine and histidine, in the modified method). This may have been due to the different conditions employed in the modified method, or the possible irreproducibility of the analyses due to the low level of amino acids in the samples. Further analyses are required to check this.

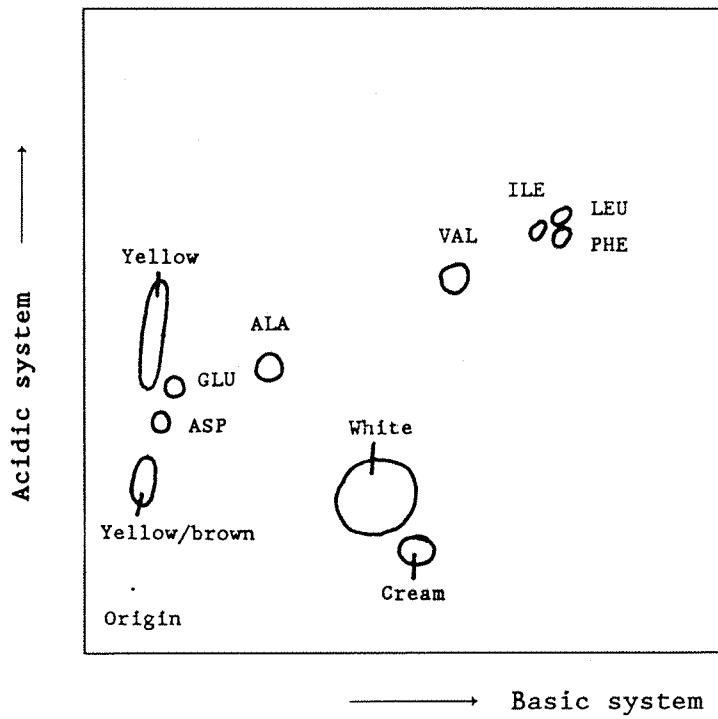
7.2.5.3 Conclusions

Two-dimensional chromatography of the hydrolysed humic materials showed the presence of amino acids but, due to poor chromatography, identification of amino acids could only be performed on two samples. The following amino acids were tentatively identified as being present in the samples:

- Site A, August, 1986 :	alanine	histidine
	aspartic acid	iso-leucine
	cystine	leucine
	glutamic acid	phenylalanine
	glycine	valine

Figure 7.6 Two-dimensional TLC of hydrolysed humic materials from site A, August, 1986

(a) Original method



(b) Modified method

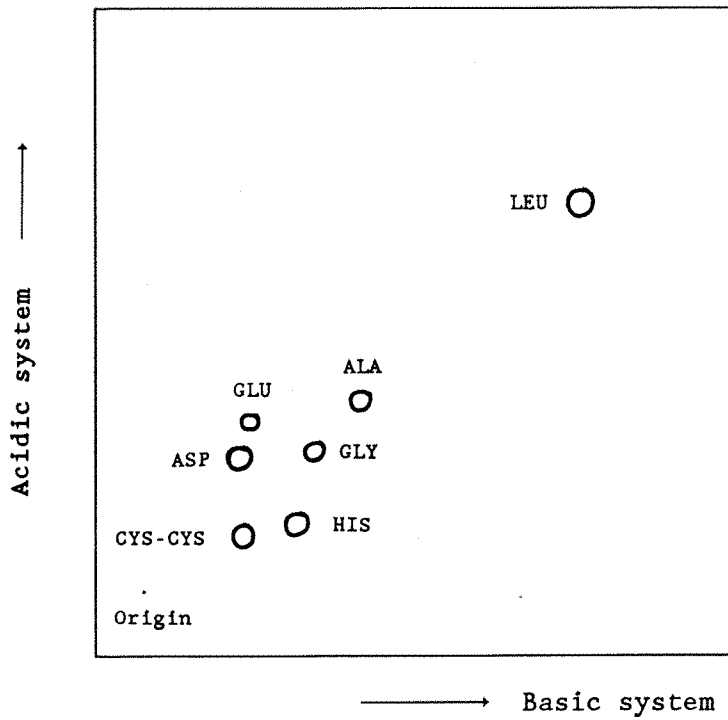


Figure 7.7 Two-dimensional TLC of hydrolysed humic materials from site C, August, 1986

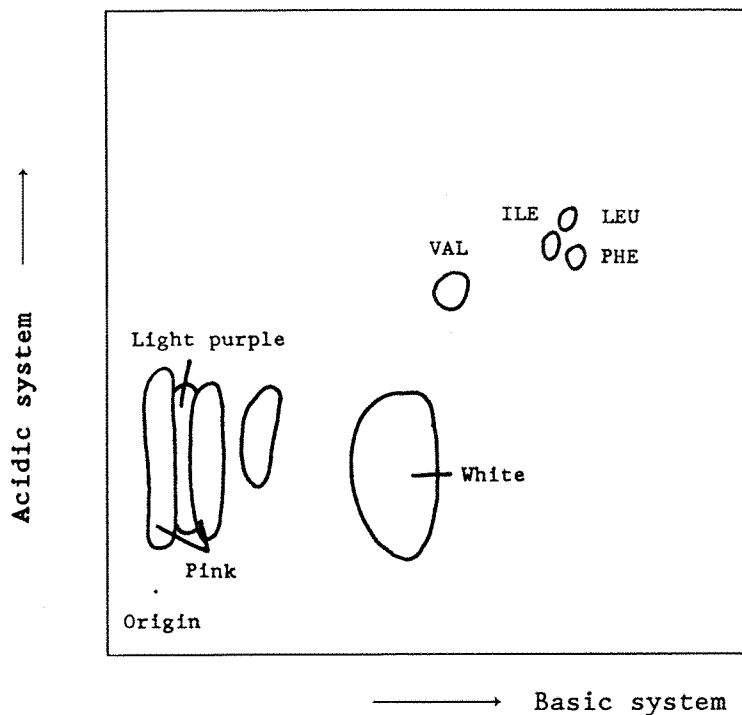


Figure 7.8 Two-dimensional TLC of hydrolysed XAD blank material from August, 1986

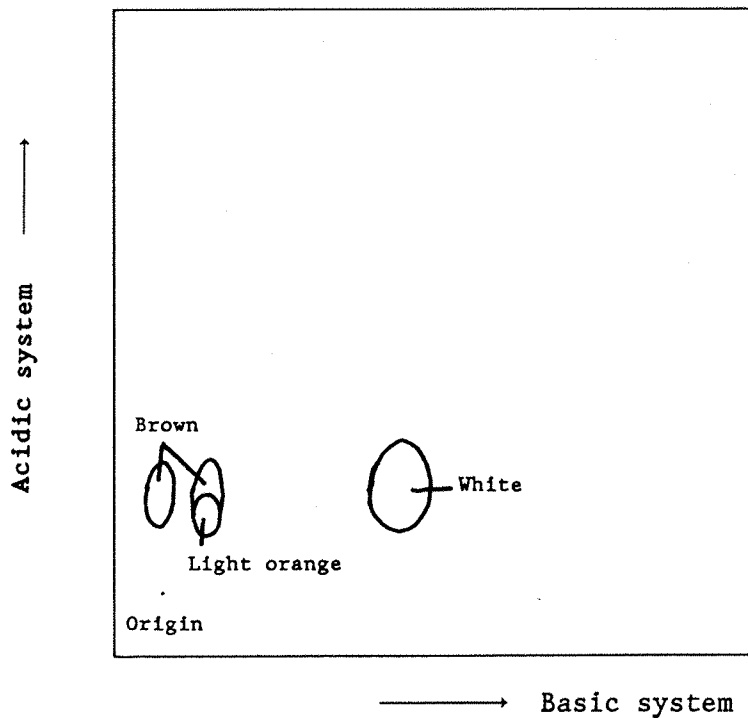


Figure 7.9 Two-dimensional TLC of hydrolysed humic materials from site A, February, 1986

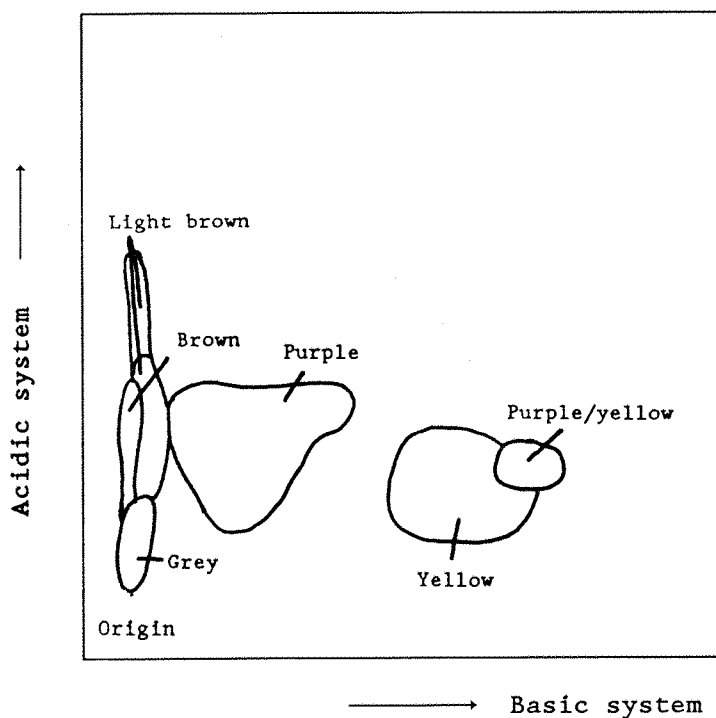


Figure 7.10 Two-dimensional TLC of hydrolysed humic materials from site B, February, 1986

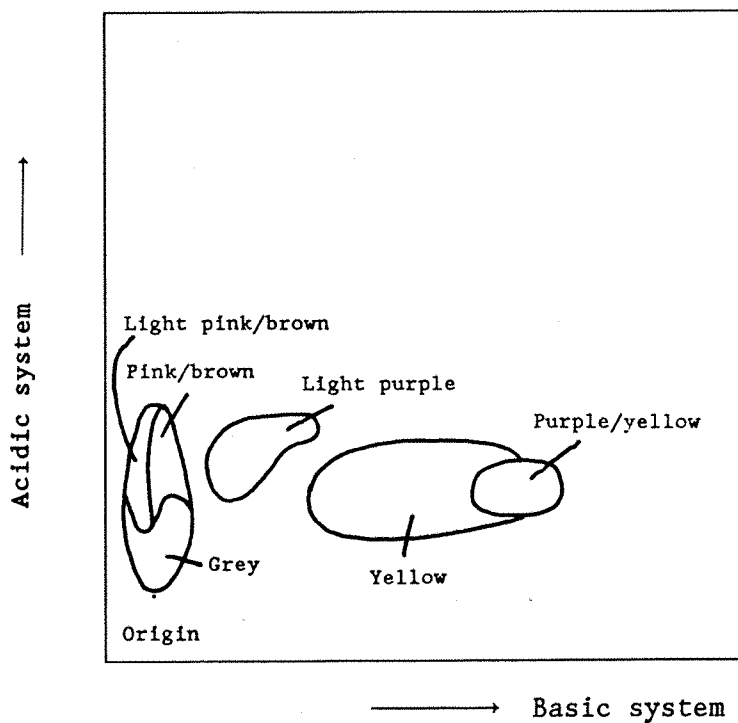


Figure 7.11 Two-dimensional TLC of hydrolysed humic materials from site C, February, 1986

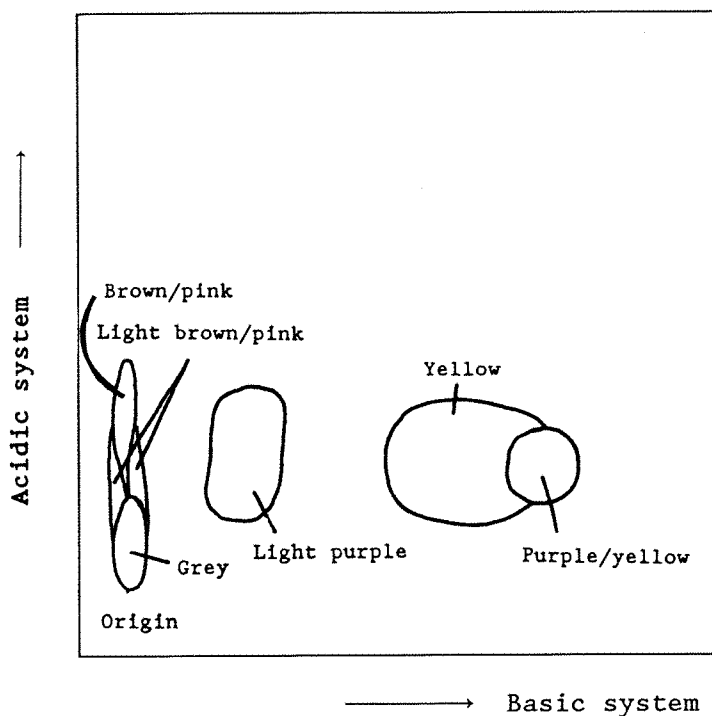


Table 7.5 Amino acids identified in hydrolysed humic materials after two dimensional TLC

<u>Sample</u>	<u>Tentative assignment of amino acids</u>
<u>Original method</u>	
August, 1986 : Site A	Alanine, aspartic acid, glutamic acid, iso-leucine, leucine, phenylalanine, valine
Site C	Iso-leucine, leucine, phenylalanine, valine
XAD blank	No amino acids were detected
<u>Modified method</u>	
February, 1986 : Site A	Amino acids may be present +
Site B	Amino acids may be present +
Site C	Amino acids may be present +
August, 1986 : Site A	Alanine, aspartic acid, cystine, glutamic acid, glycine, histidine, leucine
+ Identification was not possible.	

- Site C, August, 1986 : iso-leucine phenylalanine
 leucine valine

In order to confirm the presence of these amino acids in the humic materials, the samples were subjected to amino acid analysis, using ion-exchange chromatography with post-column ninhydrin-based colorimetric detection.

7.3 COLUMN CHROMATOGRAPHY OF AMINO ACIDS

7.3.1 Experimental

The humic samples (1 mg) were hydrolysed with hydrochloric acid (6 moles/l), at 110°C, for 24 hours. An internal standard, nor-leucine (200 nmoles/ml), was added prior to hydrolysis. This amino acid has frequently been employed as an internal standard in amino acid analysis (e.g., (511)) due to its rarity in biological materials. The samples were then taken to dryness and redissolved in hydrochloric acid (10 millimoles/l) for analysis.

The amino acids were analysed by Mrs. K. Platt (Boldrewood Medical School, Southampton University), using a Rank Hilger, Chromaspek amino acid analyser. Post-column derivatisation was performed with ninhydrin, and the amino acids were detected spectrophotometrically at a wavelength of 570 nm. Proline was determined at 440 nm.

7.3.2 Results

The amino acid contents of the humic samples are given in Table 7.6. The data are corrected for the contribution of the blank material (see Section 4.3.6). One sample (February, 1986, site A), analysed in duplicate, showed considerably divergent results, suggesting that either (i) the technique was not reproducible, or (ii) the sample was not homogeneous. In addition, the sample from site C, May, 1986, gave erroneously large values due to its high content of blank material (leading to an inaccurate estimation of the percentage of humic substances present).

Comparison of data, both seasonally and from different sampling

sites, was therefore difficult. Overall, there was generally an increase in the percentage of amino acids in the humic materials in the summer, and the sample from site C, February, 1986, showed a high amino acid content compared to other samples.

Despite reproducibility problems between samples, it was possible to compare the distribution of amino acids within each sample. The most abundant amino acid was generally glycine, with histidine, aspartic acid, glutamic acid and alanine also significant to varying degrees.

Comparing the data obtained by TLC with that obtained using amino acid analyser, it can be seen that a greater number of amino acids were detected using the column chromatographic technique, probably due to its lower detection limit. The TLC data from site C, August, 1986, however, did not compare well with the column chromatographic data, with the main amino acids detected by column chromatography not occurring in the TLC. It is obvious that both methods need further evaluation to check their reproducibility at the detection limits required in the present study.

7.3.3 Discussion

7.3.3.1 Amino acids in the aquatic environment

Prior to suggesting the sources of the amino acids in aquatic humic materials, the amino acid content of possible precursors will be discussed. Amino acids may originate from both allochthonous (e.g., terrestrial) or autochthonous (e.g., planktonic) sources and thus both soil and planktonic amino acids may be incorporated into aquatic humic materials to varying degrees. In addition, the possible incorporation of dissolved and particulate amino acids (from the water system) into these materials must be considered.

The predominant amino acids (on a molar basis) in both whole soils (51) and soil humic fractions (Table 3.1) are aspartic acid, glycine, glutamic acid and alanine, present mainly in combined rather than free forms. These are likely to originate from microorganisms as they are often the main amino acids in bacterial cells, along with lysine and

Table 7.6 The amino acid content of estuarine and marine humic materials

(a) Percentage of amino acids in humic materials by weight
(mg amino acid/mg humic sample x 100)

Amino acid	1185A	286A1	286A2	286B	286C	486A	586A	586B	586C	886A	886B	886C
Ala	0.04	0.10	-	0.04	1.13	0.13	0.40	0.13	-	0.21	0.20	0.15
Arg	-	0.43	-	-	2.17	0.20	0.30	-	-	-	-	-
Asp	0.07	0.45	0.05	0.17	1.35	0.57	0.22	0.13	1.32	0.39	0.55	0.16
Cys	-	-	-	-	2.84	Tr	-	-	-	0.04	0.10	-
Glu	0.11	0.29	0.07	0.10	1.79	0.24	0.33	0.24	1.00	0.35	-	0.05
Gly	0.29	0.56	0.12	0.28	1.20	0.52	0.96	0.34	3.26	0.45	0.22	0.17
His	0.22	0.32	0.12	0.18	1.81	0.20	1.65	0.15	4.82	0.20	0.31	1.24
Leu	-	0.08	-	-	1.60	0.19	0.19	-	-	0.10	0.12	-
Iso	-	0.05	-	-	1.32	0.09	0.13	-	-	0.08	0.12	-
Lys	0.09	0.19	-	-	2.36	0.12	-	0.20	-	0.11	0.24	-
Met	-	-	-	-	0.52	Tr	0.36	-	-	-	0.52	-
Phe	-	0.06	-	-	1.78	0.08	0.18	-	-	0.03	0.03	-
Ser	-	-	-	-	0.93	-	0.41	-	-	Tr	-	-
Thr	-	0.04	-	-	1.31	0.11	0.85	-	-	0.04	0.52	-
Try	-	0.18	-	-	-	0.24	-	-	-	-	-	-
Tyr	-	0.07	-	-	2.08	0.09	0.12	-	-	0.01	0.03	-
Val	-	0.12	-	0.02	1.29	0.17	0.41	0.08	-	0.11	0.11	0.18
Total	0.82	2.94	0.36	0.79	25.5	2.95	6.51	1.27	10.4	2.12	3.07	1.95

For the list of sampling site abbreviations, see after Table 7.6 (b).

Tr = Trace

Table 7.6 The amino acid content of estuarine and marine humic materials

(b) Molar percentage of amino acids in relation to the total amino acid content (moles amino acid/total number of moles of amino acids x 100)

Amino acid	1185A	286A1	286A2	286B	286C	486A	586A	586B	586C	886A	886B	886C
Ala	5.8	4.9	-	6.7	7.0	6.1	8.2	12.3	-	12.5	10.7	11.3
Arg	-	10.3	-	-	6.8	4.7	3.2	-	-	-	-	-
Asp	7.3	14.1	10.7	16.9	5.6	17.6	3.0	8.2	10.9	15.7	19.6	8.1
Cys	-	-	-	-	6.5	Tr	-	-	-	0.8	1.9	-
Glu	9.9	8.1	14.8	8.7	6.7	6.8	4.1	14.0	7.4	12.5	-	2.4
Gly	50.6	31.4	49.6	49.3	8.8	28.4	23.4	39.4	47.7	31.8	13.9	14.8
His	18.6	8.6	24.9	15.7	6.4	5.4	19.6	8.2	34.0	6.9	9.4	53.3
Leu	-	2.7	-	-	6.7	6.1	2.7	-	-	3.9	4.3	-
Iso	-	1.6	-	-	5.5	2.7	1.8	-	-	3.3	4.4	-
Lys	7.8	5.4	-	-	8.9	3.4	-	11.8	-	3.9	7.7	-
Met	-	-	-	-	1.9	Tr	4.4	-	-	-	1.6	-
Phe	-	1.6	-	-	5.9	2.0	2.0	-	-	1.1	0.8	-
Ser	-	-	-	-	4.9	-	7.1	-	-	0.2	-	-
Thr	-	1.6	-	-	6.0	4.1	13.0	-	-	1.8	20.7	-
Try	-	3.8	-	-	-	4.7	-	-	-	-	-	-
Tyr	-	1.6	-	-	6.3	2.0	1.2	-	-	0.3	0.8	-
Val	-	4.3	-	2.7	6.0	6.1	6.4	6.1	-	5.2	4.3	10.1

Sampling site abbreviations

1185A November, 1985, site A
 2861A February, 1986, site A
 286A2 February, 1986, site A
 286B February, 1986, site B
 286C February, 1986, site C
 486A April, 1986, site A

586A May, 1986, site A
 586B May, 1986, site B
 586C May, 1986, site C
 886A August, 1986, site A
 886B August, 1986, site B
 886C August, 1986, site C

the 'non protein' amino acids, ornithine and diaminopimelic acid (51). The detection of diaminopimelic acid in soils supports a bacterial origin as this amino acid is found almost exclusively in bacteria (512). Cell walls of gram-positive bacteria consist of a rigid structure of parallel polysaccharide chains cross-linked by short peptide chains containing L-alanine, D-alanine, D-glutamic acid and diaminopimelic acid or its decarboxylation product, L-lysine (321). Cross-linking of these chains can be achieved with, for example, glycine (321). Bacterial cell walls also contain several other components, which differ between species; these are mainly teichoic acids, polysaccharides or proteins. Teichoic acids consist of phosphodiester bridges linking glycerol and ribitol molecules, containing ester-linked alanine (321).

Autochthonous contributions of amino acids to the aquatic environment can result from both extracellular release from live plankton (46) and cell lysis following their death, in addition to contributions from higher animals. The amino acid content of both phytoplankton (513) and zooplankton (514) has been determined. Chau *et al.* (513) investigated the combined amino acids of 25 phytoplankton species and found that the principal amino acids were glutamic acid, alanine, leucine, aspartic acid and glycine (on a molar basis) with alanine generally dominating. The major amino acids of several diatom species were glycine, alanine, and serine with glycine predominating (477). The average amino acid composition of diatom cell walls was dominated by glycine and serine (332). Thus it appears that increased importance of glycine and serine may serve as an indication of the presence of diatoms over other phytoplankton. Fowden has reviewed the amino acid content of several algae (515) which showed varying amounts of the main amino acids, alanine, aspartic acid, glycine, glutamic acid, leucine and valine.

Investigating the amino acid composition of a marine copepod, *Calanus*, (a species of zooplankton), Cowey and Corner (514) found the principal amino acids were glycine, alanine, glutamic acid and aspartic acid with glycine being the main component. It has been noted that the amino acid composition of zooplankton was similar to that of the phytoplankton on which they fed. For example, the marine diatom, *Skeletonema costatum* (known to be utilised by *Calanus*), showed

the same principal amino acids as the copepod (514).

In addition to release of amino acids following the death of planktonic organisms (both in the free and combined forms), extracellular release of both amino acids and proteins from live plankton can also occur (46). Investigations into the heterotrophic utilisation of DOM by phytoplankton indicated that the smaller molecules were rapidly utilised whereas the larger ones, comprising the bulk of the excreted DOM, had longer residence times in seawater, requiring exoenzymatic hydrolysis before absorption by microbial organisms (45).

As well as originating from extracellular release by plankton, dissolved free amino acids may also result from hydrolysis of combined amino acids. The concentrations of the combined amino acids in seawater are more variable than those of the free amino acids, due to the fact that they may be difficult to metabolise, requiring hydrolysis, which may be slow, followed by bacterial consumption. Numerous studies have been performed on the dissolved amino acid content of natural waters (7). In seawater, the concentrations of dissolved combined amino acids (50 - 200 $\mu\text{g}/\text{l}$) are five to ten times greater than those of dissolved free amino acids (10 - 40 $\mu\text{g}/\text{l}$) (7). Constant concentrations of free amino acids have been found with distance from the shore and depth, and this has been suggested as evidence for their rapid turnover rates (516). Early work indicated that the composition of the dissolved free amino acid fraction was dominated by glycine and serine (e.g., (175)). More recent studies, however, not requiring preconcentration, have indicated that valine may be present in larger amounts and that aspartic and glutamic acids may dominate in many surface waters (517).

The presence of significant concentrations of ornithine combined with the absence of arginine has been attributed to the microbial degradation of arginine (518) which is converted to ornithine and urea by the action of the enzyme, arginase (321). Data on the dissolved amino acids in the Mediterranean Sea (511) support microbial degradation of arginine. In the free amino acid fraction, arginine was present at low levels all year round but ornithine was only detected in the summer months when increased microbial activity

occurs. In the combined fraction, ornithine was again only detected in the summer months and, in this instance, arginine tended to show a decrease in concentration at this time.

In the dissolved combined amino acid fraction, serine and valine may be less important than previously thought (517). Degens (519) stated that the combined amino acid fraction showed similarities to both the free dissolved and particulate fractions. In some cases, it may be intermediate between the two.

Concerning estuarine waters, dissolved free amino acids were again the minor fraction in the Patuxent estuary, U.S.A. (231), consisting mainly of serine, glycine and alanine with glutamic acid and phosphoserine contributing significantly to selected samples. The dissolved combined amino acids were dominated by glutamic acid and serine in summer surface samples, whereas both summer deep water samples and late autumn samples showed a greater abundance of the neutral amino acids, glycine, alanine and valine. In the Tamar estuary, serine and glycine were the main dissolved free amino acids (520).

The dissolved amino acids in 12 different rivers have been documented by Ittekkot *et al.* (521). The major acids included glutamic acid, glycine, aspartic acid and alanine. Thurman (7), reviewing selected data on riverine amino acids, reported that the major compounds were glutamic acid, glycine, aspartic acid and serine. Their relative abundances varied with location and it was suggested that each system reflected the source of the organic matter.

Total dissolved amino acids have been monitored during a phytoplankton bloom (522) - differentiation between dissolved and combined amino acids was not attempted. Variations related to phytoplanktonic activity were observed, suggesting release of amino acids during the early stages of the bloom followed by a decrease immediately after it indicating rapid utilisation of a labile amino acid fraction.

In comparison to the dissolved amino acids, consisting of rapidly metabolised free amino acids and a more stable combined fraction,

likely to originate from alteration of particulate material or extracellular release from phytoplankton, particulate amino acids often resemble the composition of planktonic material in the surrounding water body. Particulate material from Plymouth Sound was found to closely resemble that of the diatom, *Skeletonema costatum*, although serine was enriched in the particulate material (514), possibly due to selective preservation of planktonic cell wall components after death of the plankton. A comparison of the amino acid composition of zooplankton material and particulate material from Plymouth Sound (514) indicated the constant composition of *Calanus* over a yearly survey whereas the particulate material was more variable, possibly due to selective utilisation of specific amino acids after death of the plankton. Selective utilisation of glutamic acid by bacteria, for example, has been shown by Williams and Yentsch (523), and the increase in this amino acid in the particulate matter during the winter months (514) would be in agreement with lower heterotrophic activity during these months. The major amino acids in both planktonic materials and particulate matter were glycine, alanine, glutamic acid, and aspartic acid (with serine also dominating in the particulate material) confirming the similarity between these materials.

Data obtained by Degens (519) supported his statement that the particulate organic material in surface waters is dominated by plankton. The presence of ornithine, citrulline, urea and aminobutyric acids, however, suggested that a degree of degradation of planktonic material had also occurred. Amino acid concentrations of particulate material in the Patuxent estuary, U.S.A., were compared to those of both colloidal matter and cultured laboratory siliceous diatoms (*Amphora sp.*) (331). It was suggested that similarities between these three fractions indicated that the colloidal organic matter was derived primarily from productivity within the estuary. Comparing the particulate organic matter with that of the diatom, the dominant amino acids in both materials were glycine, alanine, glutamic acid and aspartic acid. Glutamic acid and arginine were enriched in the diatom - lower concentrations of arginine in the estuarine particulate material suggest its breakdown in the water column. Traces of ornithine were observed in some of the colloidal samples supporting this suggestion.

Concerning the particulate amino acids in rivers, Ittekkot *et al.* (521) found that this fraction was generally dominated by glutamic acid, glycine, alanine and aspartic acid with other amino acids, such as serine and lysine being significant in selected rivers. They stated that the particulate amino acid composition indicated a predominant input of soil organic matter but, in times of primary production, planktonic input may be significant, with the particulate amino acid composition resembling that of plankton.

To summarise, therefore, it is likely that the particulate fraction and part of the dissolved combined amino acid fraction, either originating from primary production or terrestrial input, are available for incorporation into aquatic humic substances. The dissolved free amino acids are rapidly metabolised and so are of minimal importance in the formation of humic materials. Additionally, a labile fraction of the combined dissolved amino acids may also be degraded fairly quickly and thus does not contribute to the formation of humic substances.

7.3.3.2 Origin of amino acids in aquatic humic materials

The percentage of amino acids in the humic substances studied in this work varied considerably, probably due to inaccurate estimations of the blank material in the samples. However, there appeared to be a general increase in the amino acid content of the humic matter in the summer, and this is in agreement with the work of other investigators (524).

Comparing the composition of individual amino acids over the year, no seasonal variation was observed. Lytle and Perdue (333) also found no significant variation in the patterns of the amino acids in the Williamson river system on sampling monthly over a two-year period.

The major amino acid in most samples was found to be glycine, with histidine, aspartic acid, glutamic acid and alanine also significant to varying degrees. Glycine has been found to be the dominant amino acid in the DOM fraction of various rivers (93), as well as riverine (290) and marine (150,290) humic substances and some soil humic acids (323,326). Thus, it is not a suitable biomarker *per se*. However, if

present with significant amounts of serine, it may be indicative of a diatomaceous origin, as glycine and serine are the main components of diatom cell walls (332). If it occurs as the major component with proline/hydroxyproline, it is likely to originate from higher animals (collagen (321)).

Serine was not found as a major amino acid in any samples in the current study, although it was significant in the material from site A, May, 1986, possibly indicating some planktonic input. It was generally a minor amino acid, however, suggesting either the absence of diatomaceous contributions to any of the samples or possibly removal of serine from the river system. Dehydration of serine to racemic alanine has been suggested as the reason for high D/L alanine ratios in deep ocean water (525). The kinetics of this reaction in shallow coastal waters have not been investigated but metal ions, for example, are known to catalyse dehydration reactions of free serine and threonine (526). The presence of high concentrations of iron in the Beaulieu estuary may be significant in this respect but further work is required in this area. Measurement of the D/L ratios of alanine would also indicate if dehydration was occurring as ratios of less than one generally result due to the dominance of L-alanine in most organisms (apart from bacteria).

The other major amino acids, aspartic acid, glutamic acid and alanine, are dominant to varying degrees in both soil and aquatic humic materials (Tables 3.1 and 3.2), and thus cannot be used as specific biological markers. The presence of histidine in significant quantities has not generally been noted by other workers. It was slightly enriched in the combined dissolved amino acid fraction of surface waters sampled in December by Daumas (511), and in both the Elliot soil humic acids and Ohio river fulvic acids reported by Malcolm (290), although reasons for this were not given by these investigators. It was also noted in significant concentrations in the total dissolved amino acids during the maximum of a phytoplankton bloom (522) where it was suggested as being a decay product of phytoplankton. The ability of histidine to bind metals such as copper has been reported (527), and thus it is possible that the high concentrations of iron and manganese in the Beaulieu estuary may stabilise this amino acid. Further studies on metal-organic

interactions in the Beaulieu estuary would have to be performed to investigate this.

The sample from site C, February, 1986, contained a much higher concentration of amino acids than the other samples. No reason for this was indicated by the data on the nitrogen content of the extracted material, or the DOC and chlorophyll 'a' data of the water sample (Section 4.1). It could be explained by the presence of a greater proportion of soil-derived humic materials after a period of heavy rainfall although the DOC and chlorophyll 'a' data do not indicate that this was a recent event. A significant terrestrial input to this coastal material is supported by the increased concentration of glucose observed earlier (Section 5.3.2) at this site.

Although amino acids have been used as indicators of the age of geological materials (Section 3.3.3.1), quantitation of individual stereo-isomers was not performed in the present study, and thus dating cannot be carried out by the measurements of D/L ratios. However, the applicability of this technique to dating materials with terrestrial input is questionable due to the presence of D-isomers in both soils (330) and bacteria (321). Amino acid / amino sugar content has also been shown to correlate negatively with the age of Paleosol humic acids (226) but this relationship has not been checked for other humic materials. Amino sugars were not, however, determined in this study.

The presence of the non-protein amino acids, ornithine, β -alanine and γ -aminobutyric acid, may serve as indicators of microbial activity and hence relate to the 'freshness' of the organic material. These amino acids, which were not monitored in the present study, result from the decomposition of arginine, aspartic and glutamic acids respectively. Sigleo *et al.* (331) found traces of ornithine in estuarine colloidal organic material which indicated that some degradation of proteins begins in the water column. The presence of significant quantities of arginine can thus be related to the recent nature of the organic material. Variable concentrations of this amino acid were found in the present study although its importance in the sample from site A, February, 1986 may be noted. The recent origin of this material would tend to agree with a recent terrestrial input of

'fresh' organic material as indicated above for site C, February, 1986.

7.3.4 Conclusions

The amino acid content of the humic materials tended to increase in the summer months. The composition of the individual amino acids, however, showed no seasonal variations. In addition, no significant differences were observed between the three sampling sites although an increased input of terrestrial material was possibly indicated in February, 1986.

The major amino acid was generally glycine, with histidine, aspartic acid, glutamic acid and alanine also important in various samples. Due to the ubiquity of glycine, aspartic acid, glutamic acid, and alanine in both terrestrial and aquatic environments, conclusions cannot be drawn on the origin of these amino acids in the humic samples. The origin of histidine is uncertain but it may indicate planktonic influence. The low level of serine, a dominant amino acid in diatom cell walls, was noted at most sites.

C O N C L U S I O N S

CHAPTER EIGHT

GENERAL CONCLUSIONS AND FURTHER WORK

8.1 CONCLUSIONS

8.1.1 Extraction procedure

Humic substances were isolated from estuarine water and coastal seawater using Amberlite XAD-8 resin, and were subsequently recovered from the resin by elution with methanolic ammonia. Elemental analyses (CHN) were performed on the resulting materials. Exceptionally high N/C ratios combined with recoveries of significant quantities of material from blank extraction experiments indicated that the extracted humic substances were contaminated. Attempts to characterise the contamination revealed the presence of both ammonium chloride and an unidentified compound (probably a bicarbonate/hydrated carbonate). Determination of the contribution of this material to the samples proved unsuccessful due to the presence of UV-absorbing impurities. The percentage of humic materials in the samples was therefore estimated by subtracting the mass of material extracted in the blank analyses.

On subjecting standard amino acid/carbohydrate mixtures to the extraction procedure, it was found that free amino acids and carbohydrates were not adsorbed by the XAD-8 resin. Therefore, the amino acids and carbohydrates isolated in the current study were an integral part of the humic substances and not co-extracted with them (unless the presence of the humic materials assisted in their uptake). The possible influence of the humic substances on the adsorption of these compounds, however, was not investigated in this work. In the case of fatty acids, however, co-extracted materials may be isolated. Although investigations into the extraction of free fatty acids by XAD-8 could not be performed in the present work due to the contamination problems encountered, other workers have recovered these materials using this resin. Further work on this aspect, together with an investigation into procedures to remove any co-extracted fatty acids, would be valuable.

8.1.2 Methods for the analysis of biogenic compounds in aquatic humic materials

8.1.2.1 Carbohydrates

A method for the analysis of neutral sugars in a variety of solid natural samples was assessed for its application to the determination of monosaccharides in aquatic humic substances. Following hydrolysis and subsequent neutralisation and deionisation, free monosaccharides were equilibrated in the presence of a catalyst (lithium perchlorate) and then subjected to GC analysis as their trimethyl silyl ether derivatives. The equilibration catalyst employed in this method, however, was found to interfere with the on-column injection GC analysis used in the present study. An alternative reagent, 2-hydroxypyridine, previously proven for packed column GC work, was therefore evaluated for on-column injection capillary GC analysis. Assessment of the complete experimental procedure on two simple polysaccharides indicated low experimental yields and considerable contributions from the reagent blank analyses. Minor modifications to the method led to slight improvements in the experimental yield, and lower reagent blanks. The use of an internal standard to account for procedural losses proved unsuccessful, due to preferential loss of the standard. Despite low experimental yields, however, the method was successfully applied to the semi-quantitative determination of monosaccharides in aquatic humic substances.

8.1.2.2 Fatty acids

A transesterification method for the analysis of saponifiable lipids was modified in an attempt to reduce sample losses in trace level lipid analysis. Contamination problems were encountered in the analysis of the humic samples, and so clean-up was attempted using TLC and column chromatography. Due to losses of unsaturated acids using the TLC clean-up, column chromatography was chosen. Various elution schemes were therefore evaluated. Subsequent analysis of the humic samples still indicated the presence of contamination. Blank analyses were therefore checked, revealing varying contributions from different sources of boron trifluoride in methanol in addition to contamination resulting from the complete XAD extraction procedure (although the

latter could generally be removed by column chromatography) and the methanolic ammonia concentration step. The derivatised contaminants in the humic samples and the extraction blanks (after column chromatographic clean-up) were identified as FAMES and so clearly originated from materials of a lipid nature.

8.1.2.3 Amino acids

One-dimensional TLC, using both acidic and basic solvent systems, was assessed for the separation of a mixed amino acid standard. Optimum solvent systems were then combined in a two-dimensional method. Application of this method to the separation of amino acids in hydrolysed humic substances was promising although, in some samples, poor chromatography, thought to be due to the presence of interfering compounds in the extracts, resulted in the inability to identify the amino acids. Quantitation of the amino acids was subsequently performed using an amino acid analyser.

8.1.3 Biogenic components of estuarine and marine humic materials

8.1.3.1 Carbohydrates

Quantitation of several sugars in estuarine and marine humic substances was successfully performed. Glucose, galactose, arabinose, rhamnose and mannose were found to be the major sugars in these materials. Xylose and ribose were also detected at lower levels and the presence of fucose was confirmed by GC-MS although it was not determined quantitatively. A trace of lyxose was detected in the May samples by GC-MS analysis.

Although no major site or seasonal variations in the monosaccharide content were noted, the following points can be made:

- Significant quantities of arabinose in samples from sites A and B compared to site C suggest increased terrestrial influence at the former sites, with lower input in November.
- Planktonic input probably contributed to a greater extent in the May and August samples as shown by increased concentrations of galactose and mannose.

- Increased planktonic input was indicated by an increased percentage of ribose on going from site A to site C in August, 1986.

The sugar content of the humic substances therefore suggests decreasing terrestrial and increasing planktonic input to these materials on approaching the sea.

8.1.3.2 Fatty acids

Contamination problems prevented any low level determination of saturated fatty acids. However, unsaturated fatty acids were detected (as their methyl esters) in both sets of samples analysed. It is not known whether they were an integral part of the humic materials or co-extracted with them. Palmitoleic (16:1) and oleic (18:1) acids were found in all samples from May, 1986 and in the single sample analysed from site A, November, 1985. Linoleic acid (18:2) was additionally detected in the November sample. The presence of significant amounts of unsaturated fatty acids suggests mainly planktonic input to the humic substances at all sites. These results must be treated with caution, however, due to the appearance of unsaturated fatty acids in subsequent blank analyses performed using a different batch of boron trifluoride in methanol reagent.

8.1.3.3 Amino acids

Quantitation of amino acids in estuarine and marine humic materials, using an amino acid analyser, indicated the dominance of glycine in most samples. In addition, histidine, aspartic acid, glutamic acid and alanine were present in significant amounts, and twelve other protein amino acids were detected at lower levels in selected samples. No trends in the pattern of individual amino acids were observed, either seasonally or between the three sampling sites, although the overall amino acid content of the humic materials tended to increase in the summer months.

Few conclusions can be drawn regarding the origin of these amino acids due to the dominance of glycine, aspartic acid, glutamic acid and alanine in both terrestrial and aquatic environments. The importance of histidine may indicate planktonic influence although the

absence of serine, a major component of diatom cell walls, should be investigated further.

8.1.3.4 Summary

The carbohydrate content of the aquatic humic materials suggests significant terrestrial input at site A with planktonic input dominating to a greater extent at site C. However, the fatty acids determined in this work indicate predominant planktonic input at all sites (May, 1986) with little evidence of terrestrially-derived materials. No conclusions on origin could be drawn from the amino acid data. Further work is obviously required, therefore, to explain this anomaly, particularly with regards to checking the data from the fatty acid analyses.

8.2 FURTHER WORK

Numerous areas of further work could be suggested. A selection of these are given.

8.2.1 General

A more comprehensive survey of the area would be interesting. In addition to extracting the dissolved humic materials from various sites in the estuary, humic substances should be isolated from other areas of the region, including soil from the drainage area, Beaulieu riverwater (above the salt intrusion), sedimentary material and also suspended particulate organic matter. Planktonic species present should be identified and monitored, and other general parameters on the water content, such as dissolved nutrients and bacterial levels, should be measured. In addition, significant meteorological events, such as prolonged periods of rainfall, should be recorded. In this way, relationships may be found between humic substances from different origins in the chosen area and other parameters.

8.2.2 Method development

8.2.2.1 Extraction

As the isolation of humic materials in the present study yielded unacceptable blank extraction levels, an alternative procedure would be preferable. Over the last few years, a 'standard' procedure for the isolation of humic substances from the aquatic environment has developed, based on that employed by Thurman and Malcolm (350). It involves extraction of the humic materials onto XAD-8 resin, followed by elution with sodium hydroxide and protonation by ion-exchange chromatography. It has come to be widely accepted for the isolation of humic substances and so should be adopted in any further work.

Further investigations should be performed on the co-extraction of biogenic materials with the humic substances. In addition to investigating the isolation of free fatty acids by XAD-8, studies on the extraction of various lipid classes, such as glycerides, phospholipids, etc., should be performed, in addition to studying the extraction of carbohydrate and proteinaceous materials. The effect of the presence of humic substances on these extractions should also be considered.

8.2.2.2 Carbohydrates

Further work should be performed to improve experimental yields in this analysis. In addition, alternative internal standards, which do not occur naturally or show preferential losses, should be found.

8.2.2.3 Fatty acids

Attempts to achieve lower, less variable blank levels in the reagent blank analysis should be carried out. Reagent blanks using other transesterification techniques should be investigated. In addition, the effect of sample size on the recovery of the lower molecular weight fatty acids should be studied and other transesterification methods should be checked to see if this effect is ubiquitous.

The possibility of adopting a rapid GC method, using short capillary columns, should be considered for the analysis of the higher molecular weight fatty acids (as their methyl esters) resulting from plankton and soils.

8.2.2.4 Amino acids

Work in this area could include clean-up of hydrolysed humic extracts so they are amenable to TLC analysis, and subsequent quantitative analysis by TLC. These results could then be compared with those obtained by column chromatography, and, if necessary, gas chromatographic analysis. Due to the variability between the amino acid content of identical samples in the current work, the repeatability of the column chromatographic analysis, in addition to the homogeneity of the samples, should be checked. Methods to differentiate between D/L isomers should also be investigated in order to obtain more information on both the origins and age of these materials.

8.2.3 Studies on biogenic materials

In all cases, the biogenic components of the aquatic humic materials should be studied alongside surveys of the dissolved and particulate free and combined amino acids, carbohydrates and fatty acids in the aquatic environment. These materials should also be analysed in the planktonic species present, and in humic substances extracted from other areas of the region, e.g., soil (as suggested in Section 8.2.1).

Additionally, attempts should be made to relate the amino acids, monosaccharides and fatty acids to their likely polymeric precursors in the humic substances. Problems in this area will probably include the extraction of the polymers without their breakdown, followed by quantitative isolation after separation.

Concerning the analyses of the individual biogenic compounds in aquatic humic substances, the following are suggestions:

- Amino acids : Determination of non-protein amino acids should be

carried out as these can give evidence on the 'freshness' of the organic materials. In addition, differentiation between D and L isomers should be performed to obtain clues on the terrestrial input to these substances. Finally, the absence of serine in the current study should be examined in more detail to see if losses occurred due to its degradation during hydrolysis or its dehydration in the environment. In addition, the origin of the significant amounts of histidine should be investigated.

- Fatty acids : The analyses should be repeated when the experimental yields have been improved and blank analyses are acceptable, in order to confirm, or otherwise, the presence of predominantly unsaturated fatty acids in the aquatic humic substances. In particular, the presence of plankton-derived fatty acids, namely 20:5 and 22:6, should be targeted.
- Carbohydrates : The current work should be repeated to give more quantitative results when a suitable internal standard has been found. It would be interesting to extend this study of neutral sugars to include related sugars such as uronic acids, amino sugars, etc., in addition to investigating the possible existence of glycoproteins and glycolipids. Their occurrence in plankton, soil and the aquatic environment could also be studied.

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