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Determination of terrestrial markers in marine environments by gas chromatography–mass-selective detection compared to high-performance liquid chromatography–fluorescence detection

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Abstract

Vanillic acid, syringic acid and often *p*-hydroxybenzoic acid, were found to be appropriate terrestrial markers in marine sediments, and were analyzed as their *n*-butyl/acetate esters by gas chromatography with mass-selective detection. The high sensitivity of the method allows terrestrial inputs remote from the source to be measured. Input from angiosperms was the principal terrestrial source in marine sediments. Terrestrial inputs to the outer Great Barrier Reef and the New Guinea Trench were confirmed. The HPLC–fluorescence method is an alternative to the gas chromatography method, providing significantly better sensitivity for syringic acid. Its principal advantage is higher reproducibility.

Keywords: Marine sediments; Sediments; Organic acids; Humic acids

1. Introduction

Marine environments and especially coastal areas, are influenced by terrestrial runoff that includes nutrients, sediments, pollutants and various organic compounds [1–6]. The sediments can form deltas and fill deep ocean trenches [7,8], and the terrestrial organic matter can influence early diagenesis in marine sediments [1,9]. Nutrients enhance the growth of microorganisms in sediments, but can be deleterious to coral growth in tropical areas when inputs are excessive [10]. Quantifying the terrestrial contribution has been an important but difficult task. Lignin oxidation products have been used as terrestrial markers in marine environments, but there are serious drawbacks to their use. It has been found that the severe oxidation conditions used to degrade lignins give aromatic artefacts that can be derived from marine humic acids, not lignins (unpublished data). Further, lignin degradation and workup is difficult to control, and reproducibility is poor [11,12]. The use of multiple GC capillary columns has been necessary for lignin-poor samples because other organic compounds interfere with the separation [13], and for open ocean waters, extensive preconcentration has been required [14]. In this study, naturally occurring lignin degradation products (vanillic and syringic acids; *p*-hydroxybenzoic with precautions) were found to be suitable markers, overcoming the need for severe oxidation. Mass-selective detection (MS) overcame the use of multiple capillary columns. Reproducibility was similar to that reported for other methods [11,12]. The method is extremely sensitive and can be used to measure

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terrestrial influences in small samples remote from a terrestrial source, without the need for preconcentration. Published GC methods and the GC–MS method of this study give low reproducibility for the terrestrial marker acids. In some of our work, small changes in marker acid concentrations were measured. To be confident that the changes were significant, a more reproducible method was required. We therefore developed an HPLC method in which the humic acid interference was minimized. Our results confirmed that the New Guinea Trench is being filled in by sediments from the Fly River and other Papua New Guinea (PNG) rivers, and that terrestrial influence extends to the outer central Great Barrier Reef (GBR), but is negligible beyond the shelf.

2. Experimental

2.1. GC–MS instrumentation

A Hewlett-Packard (Palo Alto, CA, USA) 5790 gas chromatograph with a split/splitless injector, 5970A mass-selective detector, 9825B controller and 2671G integrator was used. The injector was used in the splitless mode (30 s delay), and for quantifying marker acids the detector was operated in the selective ion monitoring (SIM) mode. The column was a 50 QC3/BP5 capillary column (0.33 mm I.D.; 0.5 μm film thickness) from SGE (Ringwood, Australia) cut to 25 m. Carrier gas was He at a total flow of 60 ml min^{-1} and column flow of 6 ml min^{-1}. The injector and interface temperatures were 250°C and 280°C, respectively. The column temperature was held at 40°C for 2 min, ramped to 280°C at 20°C min^{-1}, and finally held at 280°C for 6 min. Allowing for cooling and column equilibration, samples could be injected each 35 min. The EM voltage was 2000 V, and the detector was turned on between 8 and 16 min. The butyl/acetate esters of the marker acids were monitored at 138 u (*p*-hydroxybenzoic acid), 168 u (vanillic acid) and 198 u (syringic acid).

Sample components were identified at higher resolution when the column temperature was ramped at 4° C min^{-1} to 280°C, and held at this temperature for 10 min. MS was operated in the total ion mode and scanned between 50 and 450 u at 380 u sec^{-1}. The EM voltage was 1800 V, and the detector was turned on between 16 and 60 min. Other conditions were as for the SIM operation. Mass spectra were scanned with BenchTop/PBM software from Palisade (New York, NY, USA) containing the Wiley Registry of Mass Spectral Data, 5th edition, to obtain tentative component identification. Authentic standards were then run and unknowns were confirmed by a comparison of retention times and mass spectra.

2.2. HPLC instrumentation

An LKB (Bromma, Sweden) Model 2150 HPLC pump and injector with a Hitachi (Tokyo, Japan) F-4000 fluorimeter and Brownlee RP-18 (Santa Clara, CA, USA) Spheri-10 (250×4.6 mm I.D.) 10-μm particle size silanized column were used. The mobile phase consisted of 70% methanol (BDH, Poole, UK) and 30% Super-Q water (Millipore, Bedford, MA, USA). The flow-rate was 0.8 ml min^{-1}, with fluorescence detection at excitation wavelengths of 260 and 275 nm and an emission wavelength of 355 nm (10 nm slit widths).

2.3. Chemicals and solvents

n-Butanol, dichloromethane (DCM) and acetic anhydride were obtained from BDH (Poole, UK), NaOH and concentrated HCl were from Ajax (Auburn, Australia), and concentrated H_2SO_4 was from Merck (Darmstadt, Germany). *p*-Hydroxybenzoic, vanillic, syringic, phthalic, *p*-coumaric, ferulic, cinnamic, adipic and malic acids, *n*-decane, *n*-dodecane, *n*-tetradecane and pyridine were from Sigma–Aldrich (St. Louis, MO, USA). All chemicals and solvents were high-purity and water was distilled in glass.

2.4. Sample collection and preparation

Sediments were collected across the central GBR shelf about 200 km North of Townsville, Queensland, Australia. Deep-sea sediments were collected off the coasts of Australia and PNG in the western and northern Coral Sea. Soil, river water and both living and senescent plant samples were collected within 500 km of Townsville. Humic acids were extracted from local soils. Samples were collected and prepared as previously described [1,9,15,16].

Briefly, GBR sediments were taken in 20 cm lengths using a 0.027 m^2 modified Bouma boxcorer, oven-dried at 80°C for 16–24 h and milled. Deep-sea sediments were taken with a 0.2 m^2 Smith–McIntyre grab using precautions to keep the sediment-water interface intact, freeze-dried and milled. Soils were air-dried at ambient temperature for 2 weeks and ground to a fine powder. Humic acids were extracted from soils with 0.1 *M* NaOH, precipitated with concentrated HCl, filtered and dried. Plant matter was cut into small segments, freeze-dried for 40 h, and extracted with NaOH. River water (20 ml) was evaporated to near-dryness on a hotplate with the aid of an air stream (freeze-drying is an alternative), transferred to a 4-ml glass vial with distilled water and dried at 105°C.

To extract the marker acids, between 100 and 250 mg of sediment and soil, 5 mg of humic acid and 10 mg of plant matter were placed in a 4-ml glass vial, 3 ml of 0.1 *M* NaOH was added and the samples were heated at 80°C for 1 h with regular shaking [17]. If necessary, samples stood at 4°C for up to 3 days to allow fine particles to settle. Then 2 ml supernatant was removed and placed into another 4-ml glass vial with the addition of 100 μl of concentrated HCl. To the evaporated river water, 2 ml of 0.1 *M* NaOH and 100 μl of concentrated HCl were added. Samples were evaporated to dryness at 105°C with the aid of an air stream. Butylation was achieved by adding 2 ml of *n*-butanol and 40 μl of concentrated H_2SO_4, and heating at 105°C for 24 h [18]. The samples were washed with 2 ml-aliquots of distilled water until the washings were a neutral pH. This usually took 8 washings with 1 ml of *n*-butanol added after the 5th washing. The *n*-butanol was evaporated by heating the samples at 105°C under an air stream. Acetylation for the GC–MS method was achieved by adding 250 μl of acetic anhydride and 25 μl of pyridine to the residue and heating at 70°C for 1 h. The samples were again evaporated to dryness at 105°C with the aid of an air stream. For the GC–MS method, samples were dissolved in 100 μl of DCM containing 10 μg ml^{-1} of *n*-dodecane (internal standard). Samples with extremely low concentrations of marker acids were evaporated to 10–25 μl prior to injection. A volume of 3 μl was injected into the GC system. For the HPLC method, samples were dissolved in 500 μl of methanol, centrifuged to settle particles, 400 μl aspirated and evaporated. They were redissolved in 70 μl of methanol plus 10 μl of 1% (w/v) sodium dodecyl sulphate in water, and 50 μl were injected into the HPLC system. Appropriate external standards and blanks were prepared and run in the same manner.

3. Results and discussion

3.1. Appropriate markers

Terrestrial plant lignins degrade to methoxy-substituted phenols such as vanillic and syringic acids, and *p*-hydroxybenzoic acid is often reported as a lignin oxidation product [19]. Since lignins are absent in phytoplankton and algae, these acids are specific terrestrial markers in marine sediments. Together with phthalic acid, these acids were extracted in this study with dilute NaOH from plants, wood-rotting fungi, soils and marine sediments (Fig. 1), similar to a previous study [12]. The acids were absent in seagrasses and non-wood-rotting fungi, consistent with the lack of a lignin source. However, *p*-hydroxybenzoic acid is also a tyrosine oxidation product and can be obtained from the oxidation of non-vascular plants, including marine algae [12,19]. The origin of phthalic acid is unknown, but could be a humic acid oxidation product (unpublished data), and is also difficult to analyze accurately because di-*n*-butyl phthalate is a contaminant plasticizer in solvents and apparatus. Therefore phthalic acid was rejected as a marker. Vanillic and syringic acids were found in all plant, soil and marine sediment samples surveyed, hence they were chosen as appropriate terrestrial markers. *p*-Hydroxybenzoic acid was also found in all samples, but can only be used as a terrestrial marker if it is known that tyrosine is not a precursor. Their *n*-butyl/acetate derivatives (Fig. 2) were used in the GC–MS analysis. The base peaks of their mass spectra (Fig. 3) are separated by 30 u, reflecting the difference in the number of their methoxy groups. The base peaks occurred at M minus 98 u, probably due to loss of *n*-butyl plus acetyl groups followed by hydrogen rearrangement, and only the vanillic acid *n*-butyl/acetate ester gave a molecular ion (at 266 u). Other methoxy-substituted phenols such as vanillin that are obtained from

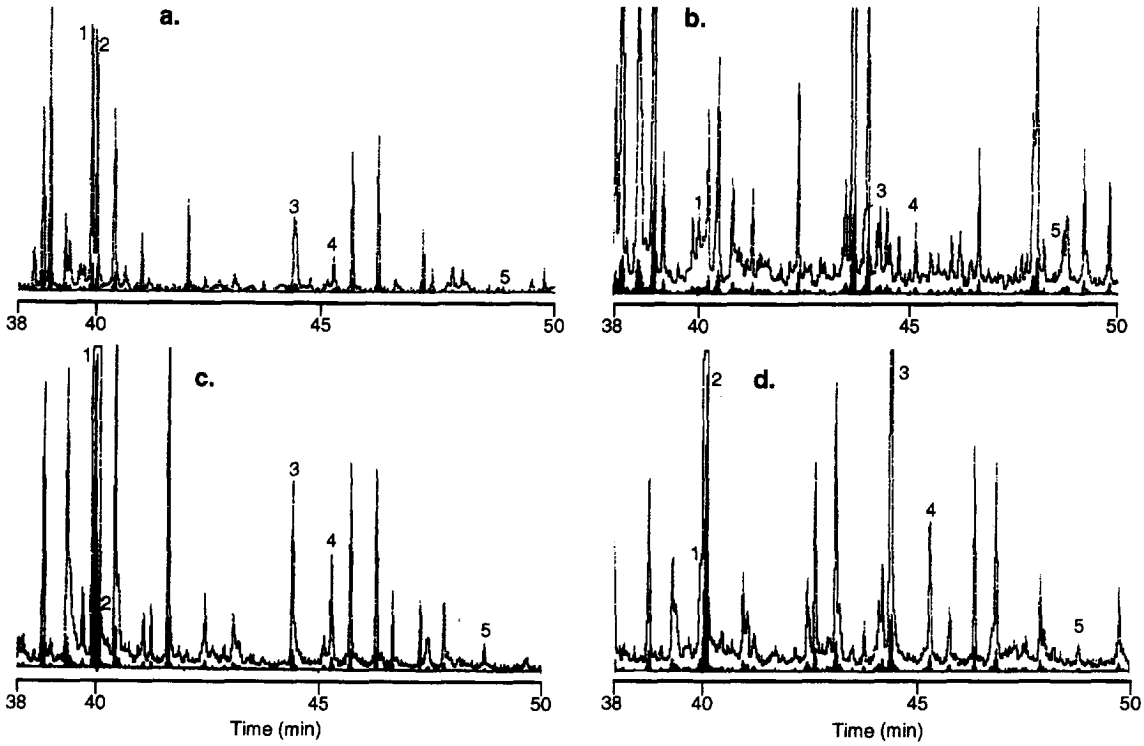
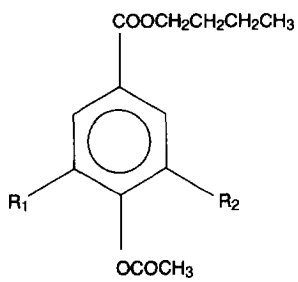


Fig. 1. Typical GC–MS total ion chromatograms of extracts from: (a) soil; (b) marine sediment; (c) senescent grass; (d) living wood-rotting fungi. Labelled components are: (1) *p*-hydroxybenzoic acid, *n*-butyl/acetate ester; (2) malic acid, di-*n*-butyl/acetate ester; (3) vanillic acid, *n*-butyl/acetate ester; (4) phthalic acid, di-*n*-butyl ester; (5) syringic acid, *n*-butyl/acetate ester.

the CuO oxidation of lignins were not detected in the samples, consistent with a previous study [12]. Some plant and soil samples also contained *p*-coumaric, cinnamic, ferulic and 3,4-dihydroxybenzoic (protocatechuic) acids, hence are potential markers [14], but they were not detected in our marine sediment samples.



	R_1	R_2
(a)	H	H
(b)	H	OCH_3
(c)	OCH_3	OCH_3

Fig. 2. Molecular structures of the *n*-butyl/acetate esters of: (a) *p*-hydroxybenzoic acid; (b) vanillic acid; (c) syringic acid.

It is difficult to separate the marker acids from other organic components, especially in marine sediments (Fig. 1b). In other studies multiple columns were used [13], but we investigated the use of MS in the SIM mode to overcome this problem. For example, *p*-hydroxybenzoic acid *n*-butyl/acetate ester was unresolved from adipic acid di-*n*-butyl ester, and vanillic and syringic acid *n*-butyl/acetate esters at low concentrations were unresolved from unknown organic components. Mass spectra of coeluting *p*-hydroxybenzoic acid and adipic acid esters show some peaks that are unique to *p*-hydroxybenzoic acid (Figs. 3 and 4), including the 138 u peak that was chosen to analyse *p*-hydroxybenzoic acid. For vanillic and syringic acid esters, mass

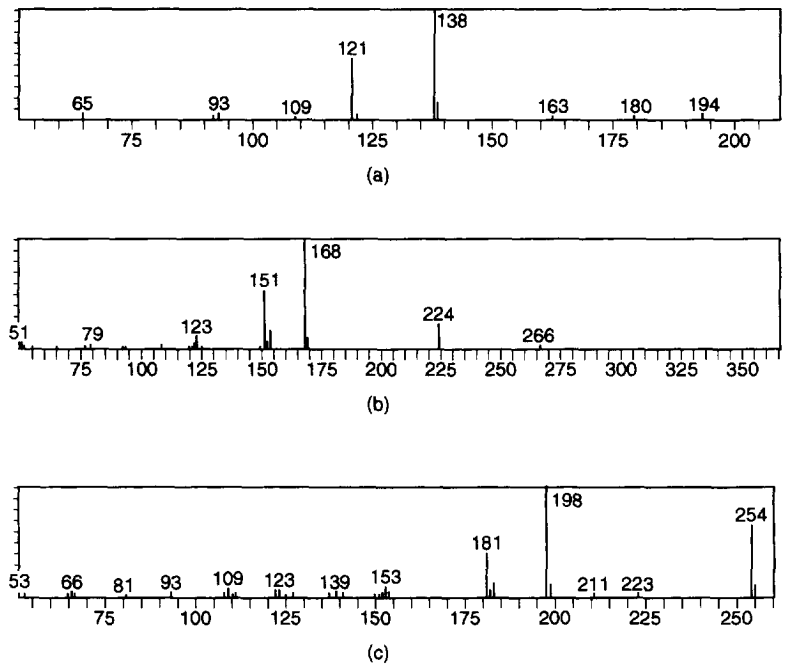


Fig. 3. Mass spectra of the *n*-butyl/acetate esters of: (a) *p*-hydroxybenzoic acid; (b) vanillic acid; (c) syringic acid.

spectra on either side of the peak maxima were obtained and compared to the mass spectra of the authentic standards. In this way unique peaks for these markers could be chosen by rejecting peaks that were common to the interfering components. The peaks at 168 and 198 u for vanillic and syringic acid esters respectively (Fig. 3), were chosen. All peaks chosen for SIM analysis were the most abundant peaks, optimizing the sensitivity of the method. The marker acids are well resolved from other organic components using SIM, in both soils and marine sediments (Fig. 5a,b).

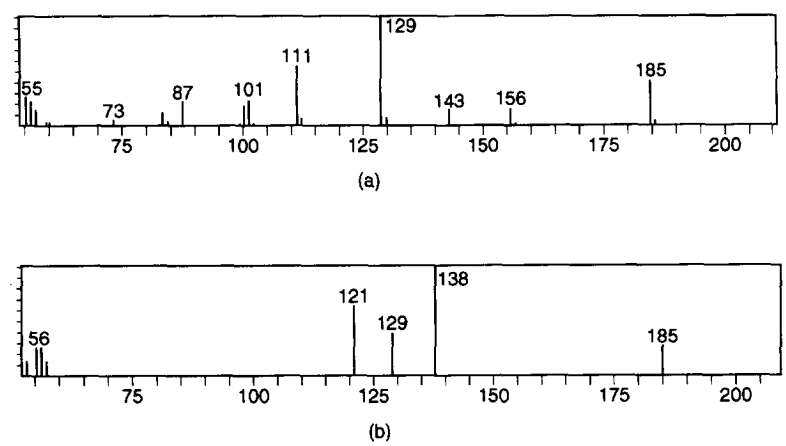


Fig. 4. Mass spectra of: (a) adipic acid, di-*n*-butyl ester; (b) coeluting adipic acid, di-*n*-butyl ester and *p*-hydroxybenzoic acid, *n*-butyl/acetate ester in a soil extract.

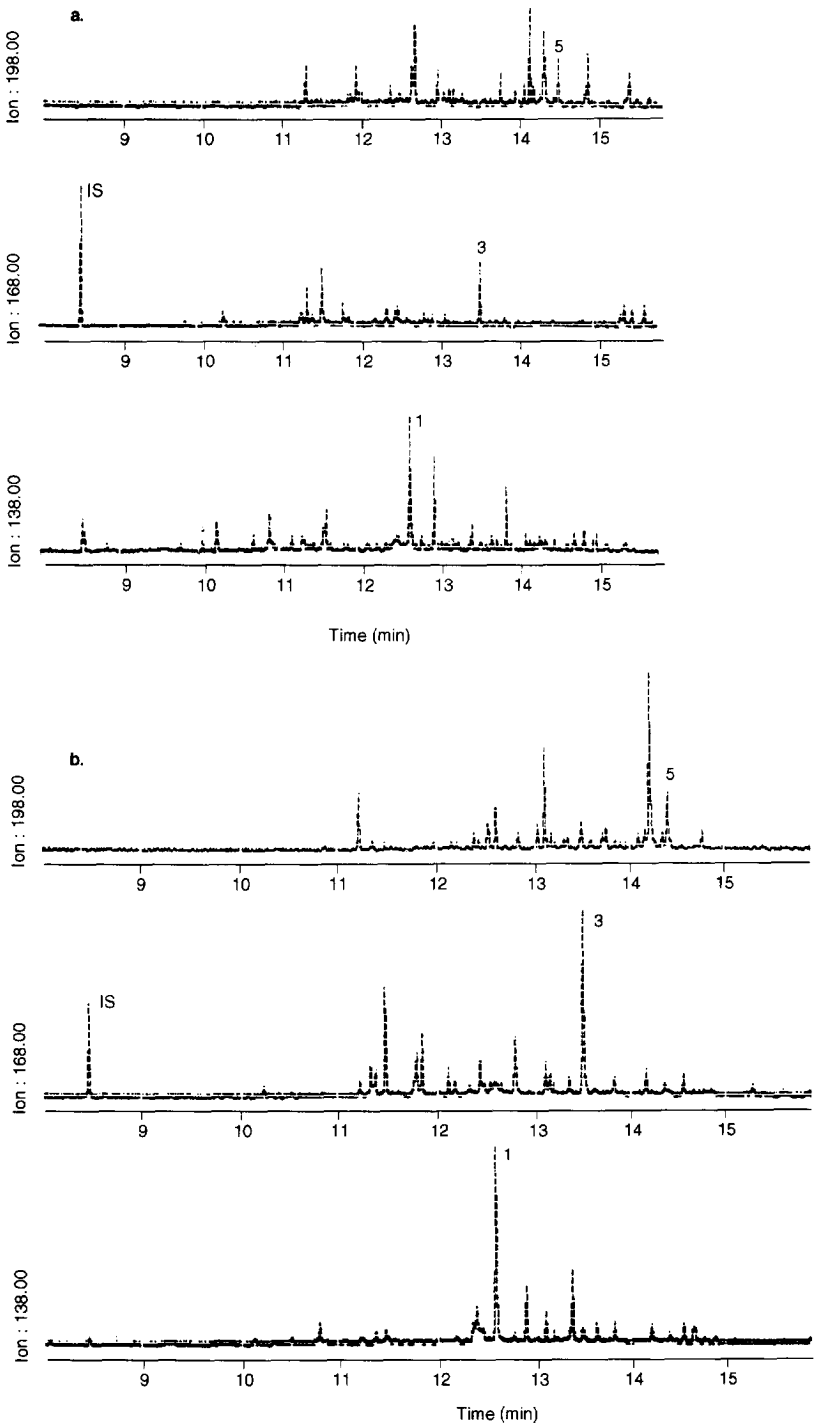


Fig. 5. Typical selected ion chromatogram obtained for the analysis of the marker acids. Ions were monitored concurrently at 198, 168 and 138 u for a: (a) soil extract; (b) marine sediment extract. Labelled components are as in Fig. 1. I.S. = *n*-dodecane internal standard.

3.2. GC–MS optimum conditions

Marine sediments contain humic acids, which are known to retain hydrophobic organic compounds [20]. Humic acids were coextracted with the marker acids and were found to bind extensively with organic compounds such as proteins. Further, the marker acids are far less abundant than humic acids, with soil humic acid samples containing <200 μg g^{-1} of these markers (Table 1). Humic acids were deposited on the sides of the injector glass liner and interfered with the elution of the marker acid derivatives onto the capillary GC column. The problem was minimized by cleaning the glass liner regularly. Oxidation of the humic acids with 0.1 *M* NaOH/H_2O_2 reduced the necessity for frequent glass liner cleaning, but because total oxidation could not be achieved, reproducibility of the marker analysis was not improved.

Reaction conditions were optimized by measuring SIM responses after varying the relevant condition. The optimum butylation temperature was >100°C, hence 105°C was used, the maximum for the heating block available. The optimum concentrated H_2SO_4

Table 1
Concentrations (μg g^{-1}) of marker acids in terrestrial and marine samples

Sample	Source	pOHBA[a]	VA[a]	SA[a]
Mangrove (macerated)[b]	Near Townsville	24	94	54
	Near Townsville	150	230	210
	Near Townsville	6.1	22	80
	Near Townsville	67	140	80
Soil	Patch rainforest	10	22	71
	Eucalypt forest	0.40	1.3	<0.5
	Island rainforest	6.5	15	<1.5
Soil humic acid	Grassland	30	39	124
	Sclerophyll forest	38	72	<8
Water (ng ml^{-1})	River No. 1[c]	4.0	1.9	6.2
	River No. 1	<0.4	1.6	<5
	River No. 2	0.56	1.9	11
	Stagnant pool	0.25	0.71	<4
Marine sediment	Central GBR[d]	140	580	20
	Central GBR	5.3	27	26
	Central GBR	1.4	7.0	8.4
	Central GBR	0.90	4.4	6.8
	Central GBR-off shelf[e]	0.034	<dl	<dl
		0.028	<dl	<dl
	PNG-off Fly River[e]	0.95	1.4	1.5
		0.83	1.5	1.2
	PNG Trench[f]	1.5	0.68	<dl
	PNG Trench	1.4	1.8	0.64
	PNG Trench	0.76	0.67	0.34
	PNG Trench[e]	0.051	0.093	<dl
		0.052	0.27	<dl
	PNG-Woodlark Basin[e]	0.32	0.36	<dl
		0.13	0.20	<dl

[a] pOHBA = *p*-hydroxy benzoic acid; VA = vanillic acid; SA = syringic acid.
[b] Stems, leaves and seeds from different species of mangroves.
[c] No. 1 was sampled on different days; No. 2 was from an industrial city.
[d] In order of increasing distance from the coast.
[e] 2 samples from this site.
[f] In order from the trench top.
dl = detection limit.

concentration was about 2% (v/v), and the optimum butylation time was about 24 h for vanillic and syringic acids, and >6 h for *p*-hydroxybenzoic acid, so 24 h was used. The optimum concentration of the extractant (NaOH) was 0.1–1.0 *M*, so the lower value was used. The optimum acetylation time was about 1 h. Recoveries fell significantly if the amount of humic acid in the marine sediment was >0.5 mg. For a humic acid concentration of 2000 μg g^{-1} (concentrations above this are rare), sample masses of 250 mg could be used. The presence of NaCl (from the neutralization of the extractant with concentrated HCl) significantly increased the recovery, probably because it increases the butylation rate of humic acids (unpublished data) and subsequent release of the marker acids. Recoveries of vanillic and *p*-hydroxybenzoic acids increased markedly while the recovery of syringic acid decreased slightly with increasing sample mass during the extraction process, hence a sediment mass of between 100 and 250 mg was chosen as a compromise. Although the marker acids are very soluble in methanol and hot water, these solvents were poor extractants compared to NaOH, probably because the acids bind to clays or other minerals [21].

Benzoic, *p*-coumaric and 2,5-dihydroxybenzoic (gentisic) acids were added as internal standards to the marine sediment prior to extraction at a concentration of 200 μg per 100 mg of sediment. They were not recovered, probably being irreversibly bound to clays in the sediment [21]. Higher concentrations were not tried to avoid possible interference with the marker acid recovery. Either *n*-decane or *n*-dodecane were added as internal standards prior to injection to standardize the amount injected. *n*-Dodecane allowed the detector to be switched on later than when *n*-decane was used. *n*-Tetradecane coeluted with some peaks and could not be used as an internal standard. The 168 u ion used for detecting the vanillic acid *n*-butyl/acetate ester could be used for measuring *n*-dodecane (Fig. 5a,b). Appropriate blanks were run and calculations were based on an external standard.

The detection limit (2×noise level) for the maximum sample mass of 250 mg was 1.6 ng g^{-1} for *p*-hydroxybenzoic acid; 3.0 ng g^{-1} for vanillic acid and 16 ng g^{-1} for syringic acid. For water samples where 20 ml were used, the respective detection limits are 0.02, 0.04 and 0.2 ng ml^{-1}. Such low detection limits allows the marker acids to be measured in samples far removed from their terrestrial source, such as in open ocean sediments and waters. For a marine sediment the relative standard deviation was 8% for *p*-hydroxybenzoic acid; 12% for vanillic acid and 16% for syringic acid (Table 2). This compares favourably with other published methods, although still somewhat high, probably due to humic acid interferences.

3.3. HPLC method development

Esterification does not adversely affect the fluorescence efficiency of vanillic and syringic acids. *p*-Hydroxybenzoic acid fluorescence is too weak to be useful, hence only vanillic and syringic acids were chosen as markers. The marker acid derivatives were well separated from interfering components (Fig. 6), but could not be separated from each other, although a number of ODS columns and a phenyl column were tried with methanol, tetrahydrofuran and acetonitrile in the mobile solvent. Because the vanillic and syringic acids have different fluorescence excitation spectra (Fig. 7), they could be quantified without separation by measuring fluorescence at two wavelengths. By injecting various ratios of a standard mixture of the acids (*n*-butyl ester), empirical relationships were derived for excitation measurements at 260 and 275 nm, respectively, with emission at 355 nm. The derived equations are:

$$VA = (0.706F_1 - 0.504F_2) \cdot 10^{-5}$$

$$SA = (0.741F_2 - 0.290F_1) \cdot 10^{-5}$$

where: F_1 and F_2 = fluorescence excitation at 260 and 275 nm, respectively, VA = mg vanillic acid; SA = mg syringic acid.

It is likely that these equations will vary for different instruments and will need to be derived empirically.

3.4. Method application

The marker acids were found in plant, soil, humic acid, fresh water and marine sediment samples (Table 1). In different mangrove species, the abso-

Table 2
Summary of the methods[a]

System	Conditions
GC–MS (SIM)	
Column	50QC3/BP5, 25 m×0.33 mm I.D.; 0.5 μm film
Oven temperature	2 min 40°C; 20°C per min to 280°C; 6 min 280°C
Injector temperature	250°C
Interface temperature	280°C
He flow	Total 60 ml min^{-1}; column 6 ml min^{-1}
EM voltage	2000 V
Selected ions	pOHBA, 138 u
	VA, 168 u
	SA, 198 u
Detector on	8–16 min
Injected amount	3 μl
Sediment amount	100–250 mg
Extractant	3 ml 0.1 *M* NaOH
Butylation time	24 h
Acetylation time	1 h
Detection limit (sediment)	pOHBA, 1.6 ng g^{-1}
	VA, 3.0 ng g^{-1}
	SA, 16 ng g^{-1}
R.S.D. ($n=6$)	pOHBA, 8%
	VA, 12%
	SA, 16%
HPLC	
Column	RP18, 25 cm, 10 μm pore size
Mobile solvent	Methanol–water (70:30)
Flow-rate	0.8 ml min^{-1}
Fluorescence	ex. 260/275 nm; em. 355 nm
Detection limit	VA, 5.7 ng g^{-1}
	SA, 6.0 ng g^{-1}
R.S.D. ($n=5$)	3.0% (at 1.8 μg g^{-1})

[a] Symbols as in Table 1.

lute and relative concentrations of the marker acids varied considerably. The reason for this is not known, but may reflect differences in their lignin structures. Consistent with their terrestrial source, concentrations of the marker acids decreased across the central GBR and PNG coast, supporting earlier observations of plant input based on the manual extraction of large (>0.5 mm) plant pieces in these samples [9]. Concentrations off the continental shelf of the central GBR and in the Woodlark Basin off PNG were very low, consistent with little transport off the GBR shelf, and the lack of major rivers near the Woodlark Basin, respectively. The marker acids were detected in river waters, and in marine sediments close to the Fly River of PNG, but their concentrations decreased markedly in deep-sea sediments (to a depth of 4350 m) from the PNG Trench, off-shore from the Fly River. These data demonstrate the usefulness of the method in determining terrestrial inputs from shallow coastal to abyssal areas. Marine sediments taken in close proximity to mangrove forests contained a higher concentration of the marker acids than continental soils, reflecting the high detrital export from mangrove forests.

For the sediment samples from the central GBR, *p*-hydroxybenzoic acid concentrations correlated with vanillic acid concentrations ($r^2=1$), confirming that *p*-hydroxybenzoic acid is derived from lignin and not tyrosine. *p*-Hydroxybenzoic acid is therefore a valid marker in these samples. Vanillic acid did not correlate with syringic acid ($r^2=0.14$) for these samples, due to the atypical concentration of the inshore sample (Table 1). The difference between the inshore sample and the other GBR samples

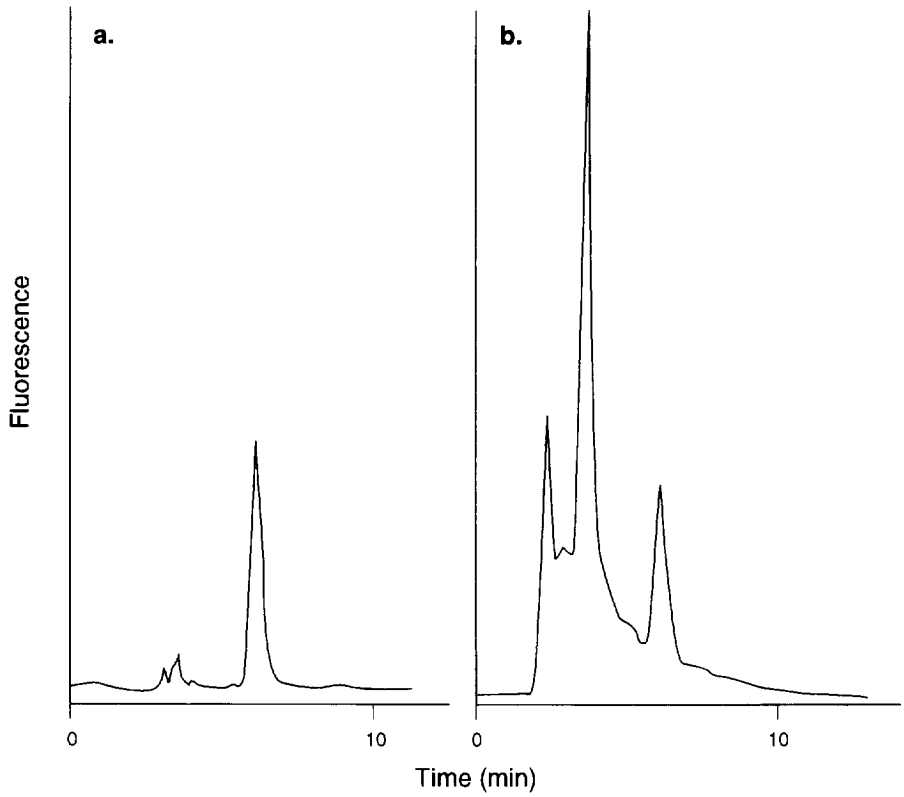


Fig. 6. HPLC trace with fluorescence detection of: (a) a standard vanillic/syringic acid *n*-butyl ester; (b) a GBR marine sediment sample. The components eluting before the vanillic/syringic acid *n*-butyl ester are due to humic acids.

probably reflects different populations of microorganisms selectively degrading *p*-hydroxybenzoic and vanillic acids in the off-shore sediments (vanillic acid-degrading bacteria are known [22]), although more data would be needed to confirm this. Hence the markers may be useful for site differentiation. The lack of cinnamyl phenols (e.g., cinnamic and ferulic acids) in the marine sediments indicates the lack of input from non-woody tissues, and the presence of syringyl phenols indicates input from angiosperms as the major source [14].

Like the GC–MS method, the HPLC–fluorescence method is extremely sensitive and selective (Table 2), and can therefore be used for samples of low terrestrial input such as open ocean sediments, with minimal sample manipulation. Detection limits for vanillic and syringic acids are 5.7 and 6.0 ng g^{-1}, respectively (sample mass of 250 mg). The syringic acid detection limit is substantially enhanced and the vanillic acid detection limit is slightly poorer than for the GC–MS method. The advantage of the HPLC method is the increased reproducibility, essential for comparing samples with similar concentrations of marker acids. The superior reproducibility occurs because interference from humic acids is minimized. The mobile solvent elutes most of the humic acids from each injection, so they cannot interfere with subsequent injections, and the sodium dodecyl sulphate in the sample matrix improves peak shape, probably by minimizing binding of the marker acids by humic acids [15]. The R.S.D. for consecutive injections ($n=5$) of a single sample is 3.0% for a vanillic acid concentration of 1.8 μg g^{-1}. For a vanillic acid concentration of 0.11 μg g^{-1}, just 3

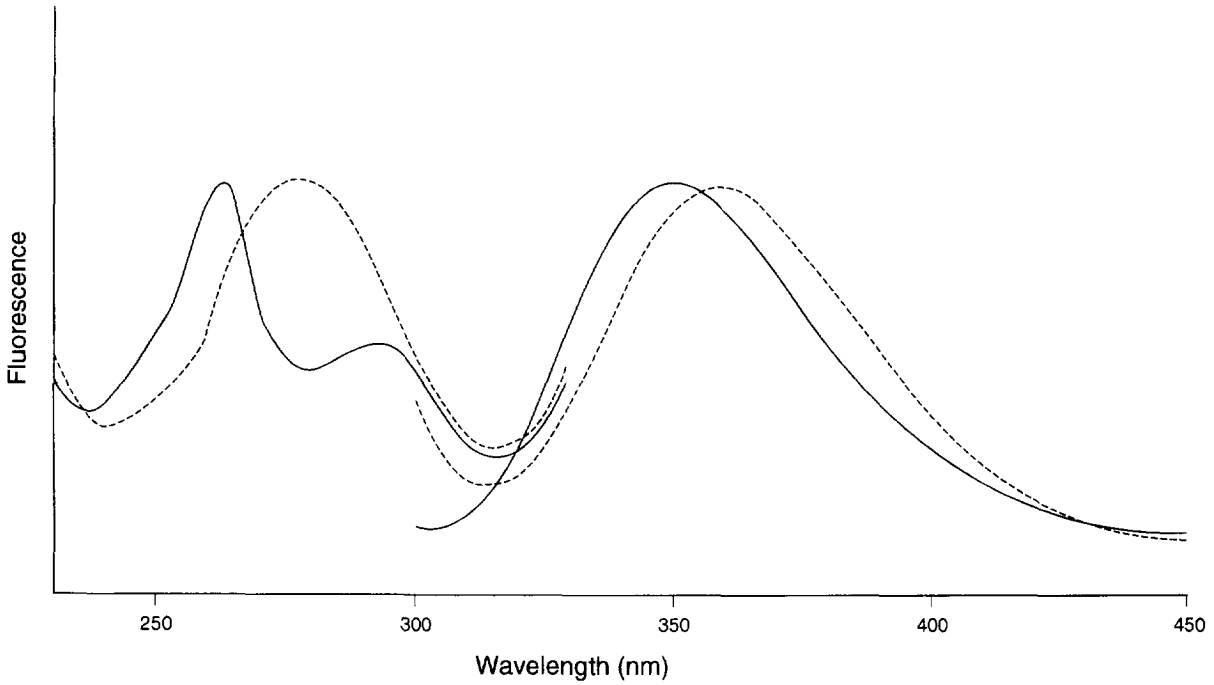


Fig. 7. Fluorescence excitation and emission spectra for the butyl esters of vanillic (solid line) and syringic (dashed line) acids.

times the level of blanks, 5 sub-samples of a GBR sample gave a R.S.D. of 18%, which is adequate at such low concentrations.

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