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Article

Compound-specific stable carbon isotope analysis of lignin phenols by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS)

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Abstract

This study demonstrates the stable carbon isotope analysis of individual lignin phenols based on off-line degradation and derivatization by tetramethylammonium hydroxide (TMAH) thermochemolysis, semi-purification by high-performance liquid chromatography (HPLC), compound-specific isotope analysis (CSIA) by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS), and isotope effect correction by isotope mass balance calculation. In GC/C/IRMS, we obtained the following optimal conditions to accurately and precisely determine the isotopic composition: combustion temperature of 1000℃, He carrier flow rate of 1.6 mL min^{-1}, and injection amount of lignin phenols of 30–140 ng. HPLC semi-purification using a normal-phase silica column with a column oven (at 30℃) can achieve baseline-separation of individual lignin phenols on the GC chromatogram without isotopic fractionation. Isotopic fractionation during TMAH derivatization can be corrected by the logarithmic correlation between the isotopic composition of the incorporated carbon during TMAH derivatization and the ratio of relative abundance between lignin phenols and TMAH reagents. By using this method, the carbon isotopic composition of individual lignin phenols can be determined within a standard deviation (1σ) of 0.2‰–0.9‰, making this method suitable for molecular isotope studies in the field of organic geochemistry.

1. Introduction

Lignin phenols are monolignol units of lignin (polyphenolic biochemicals) unique to vascular plants. They are chemically classified into four types (*p*-hydroxyl, vanillyl, syringyl, and cinnamyl phenols; Fig. 1) whose distribution varies among plant classes and tissues (Hedges and Parker, 1976; Hedges and Mann, 1979a; 1979b). In general, angiosperms are characterized by the dominance of syringyl and vanillyl phenols, whereas gymnosperms contain relatively few syringyl phenols. Within the angiosperms, non-woody tissues (e.g., leaf and pollen) and herbaceous plants are characterized by abundant cinnamyl phenols. Accordingly, in previous studies, the relative distribution of lignin phenols in terrestrial vascular plant has been employed as a tool for qualitative assessment of variations in the input into soils and sediments of angiosperms relative to gymnosperms, and of trees relative to herbaceous angiosperms (e.g., Hedges and Mann, 1979b; Goñi and Hedges, 1992; Ishiwatari et al., 2006).

Compound-specific stable isotope analysis (CSIA)

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by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) yields the isotopic composition of individual compounds in natural samples such as soils and sediments, and has been employed as a powerful tool for tracing the sources and delivery of lipid biomarkers in geographical samples and for reconstructing the paleoenvironment (e.g., Chikaraishi, 2007; Chikaraishi and Oba, 2008). In addition, a comparison of the isotopic compositions of biosynthetically related compounds (e.g., precursor and product) provides detailed information on the biochemical process and flux related to production or degradation of the compounds (Chikaraishi et al., 2004; 2009). An analysis of the isotopic compositions of individual lignin phenols enables a quantitative assessment of the properties of plant matter inputs into geochemical samples. In particular, their carbon isotopic composition would indicate the relative contributions of C3 and C4 plants. However, analytical limitations mean that little progress has been made in the CSIA of lignin phenols within natural samples, as explained below.

First, isotopic fractionation during the degradation of lignin and the derivatization of produced lignin phenols (e.g., the degradation of alkaline CuO and TMAH (tetramethylammonium hydroxide) thermochemolysis; Goñi and Eglinton, 1996; Pulchan et al., 1997; Beramendi-Orosco et al., 2006) remain poorly understood. Cleaving of the ether bond of the complex structure of lignin during CuO oxidation could be a non-quantitative reaction, which is a candidate for isotopic fractionation. Moreover, CuO oxidation requires great skill in wet chemistry. In fact, Goñi and Eglinton (1996) noted that the processes of CuO oxidation may have isotopic fractionation effects associated with the chemical bond scission of lignin. The tetramethylammonium hydroxide (TMAH) method is an alternative method that can simultaneously and quantitatively induce the thermochemolysis of lignin and the methylation of produced lignin phenols (e.g., Challinor, 2001). In fact, the TMAH method is frequently employed as a rapid and simple method for both on-line and off-line gas chromatography/mass spectrometry (GC/MS) analysis (e.g., Hatcher and Clifford, 1994; Martin et al., 1994; Clifford et al., 1995; McKinney et al., 1995; Ishiwatari et al., 2006), even for the CSIA of lignin phenols (Pulchan et al., 1997; Beramendi-Orosco et al., 2006). However, isotopic fractionation during the TMAH method has yet to be investigated.

Second, GC/C/IRMS requires base-line separation between compound peaks on chromatograms to accurately determine the isotopic compositions of the compounds of interest (e.g., Chikaraishi and Oba, 2008). However, it has proved difficult to analyze lignin phenols because the degradation products of lignin are generally a complex mixture of not only lignin phenols but also sugars (e.g., saccharide) and lipids (e.g., carboxylic acids). The GC chromatographs of lignin phenols invariably show overlapping of chromatographic peaks with high background, resulting in

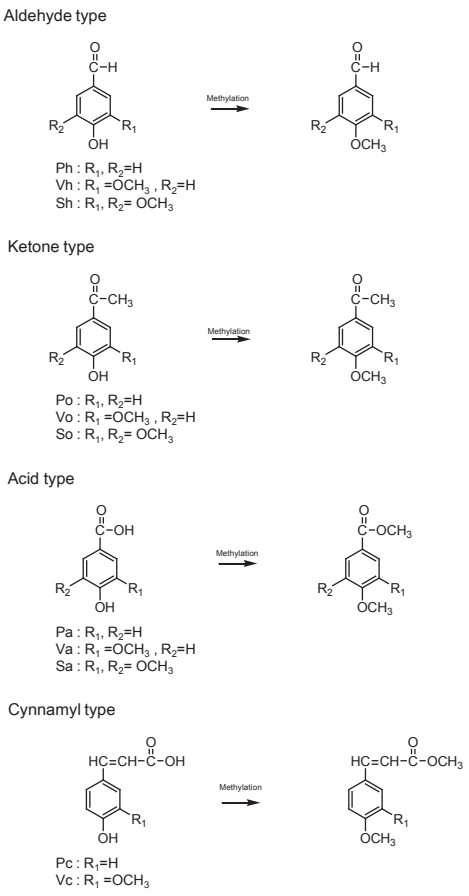

Fig. 1. Molecular structures of lignin phenols and their TMAH derivatives.

significant uncertainty regarding the observed isotopic compositions (Goñi and Eglinton, 1996; Pulchan et al., 1997; Beramendi-Orosco et al., 2006). Thus, isotopic fractionation and chromatographic separation are major issues to be addressed in the CSIA of lignin phenols.

In the present study, we demonstrate the stable carbon isotope analysis of individual lignin phenols based on off-line degradation and derivatization by the TMAH method, semi-purification by high-performance liquid chromatography (HPLC), CSIA by GC/C/IRMS, and isotope effect correction by isotope mass balance calculation.

2. Materials and methods

2.1. Reagents and standards

Lignin phenol standards and TMAH reagent were purchased from Sigma-Aldrich Inc. We used 11 lignin phenols: *p*-hydroxyl (Ph: 4-Hydroxy-benzaldehyde, Po: 4-Hydroxyacetophenone, and Pa: 4-Hydroxybenzoic acid), vanillyl (Vh: 4-Hydroxy-3-methoxybenzaldehyde, Vo: 4-Hydroxy-3-methoxy acetophenone, and Va: 4-Hydroxy-3-methoxybenzoic acid), syringyl (Sh: 4-Hydroxy-3,5-dimethoxybenzaldehyde, So: 4-Hydroxy-3,5-dimethoxyacetophenone, and Sa: 4-Hydroxy-3,5-dimethoxybenzoic acid), and cinnamyl (Pc: 3-(4-Hydroxyphenyl)-2-trans-propenic acid, and Vc: 3-(4-Hydroxy-3-methoxyphenyl)-2-trans-propenoic acid). The purity of these standards and reagent were 97%–99%.

The carbon isotopic composition of lignin phenols was determined by elemental analysis/isotope ratio mass spectrometry (EA/IRMS) using an 1110CHN EA (CE Instruments) coupled to a Delta V advantage IRMS via a Confro III interface (Thermo Fisher Scientific). The carbon isotopic composition is expressed as δ notation against Vienna PeeDee Belemnite (VPDB), and mean values with one standard deviation (1σ) are reported, derived from triplicate analyses. The analytical error (i.e., accuracy) of the isotope measurement was better than 0.3‰.

Three types of admixtures of lignin phenol standards (Table 1) were prepared, with different molecular compositions of individual lignin phenols; e.g., STD-1 had a higher abundance of Ph, whereas STD-2 had a higher abundance of Vh. These standards were dissolved with methanol and stored at –4℃ until analysis.

2.2. TMAH derivatization

The TMAH derivatives of lignin phenols were prepared by the following procedure. Standard solution was mixed with 10–150 μl of TMAH reagent (25% w/w, in methanol or distilled water) in a 10 ml ampule. After removal of methanol (and distilled water in some cases) by nitrogen stream, the ampule was vacuumed and sealed, and then heated at 300℃ for 30 min. After cooling to room temperature, the TMAH derivatives were extracted with 1 ml of ethyl acetate by triplicate. The combined solution was dried under reduced pressure and dissolved in 100 μl of ethyl acetate.

2.3. Semi-purification by HPLC

The ethyl acetate solution was filtered through a GL Chromatodisc (I.D., 0.45 μm 4N; GL Science Inc.), and the TMAH derivatives of lignin phenols were isolated by HPLC (JASCO Inc.) using a normal-phase column (Inertsil SIL 100 Å, silica column, 4.6 × 150 mm; GL Science Inc.) with a column oven (CO-965; JASCO Inc.) at 30℃. The injection loop size was 100 μl. We used a solvent gradient program consisting of *n*-hexane (H) and ethyl acetate (Ea), as follows: H/Ea (v/v) was 100/0 for 0–10 min, 98/2 for 10–20 min, 95/5 for 20–30 min, and 50/50 for 30–40 min. A differential refractometer (RI-2031 plus; JASCO Inc.) was used for the analyses.

Table 1. Comparison of the carbon isotopic composition (‰) determined by GC/C/IRMS among three admixtures of lignin phenol standards.

Lignin phenols	Concentration (mg/ml)			GC/C/IRMS (‰)			STD-(1 to 3)	
	STD-1	STD-2	STD-3	STD-1	STD-2	STD-3	Av.	S.D.
Ph	0.96	0.12	0.48	-31.6	-31.5	-32.0	-31.7	0.1
Pa	0.10	0.35	0.28	-38.3	-38.8	-38.3	-38.5	0.1
Vh	0.40	1.17	0.19	-30.8	-30.7	-31.1	-30.9	0.1
Va	0.47	0.38	0.69	-35.0	-35.4	-35.1	-35.2	0.1
Sa	0.13	0.51	0.26	-41.4	-41.9	-41.7	-41.7	0.1
Pc	0.65	0.22	0.42	-37.7	-38.3	-38.2	-38.1	0.1

2.4. Identification by GC/MS

TMAH derivatives of lignin phenols were identified by GC/MS using an HP 6890 coupled to an HP MSD 5973N (Agilent Technologies). The GC was fitted with a DB-5 capillary column (30 m long, 0.25 mm i.d., 0.25 μm film thickness). Samples were injected in split/splitless mode with helium (He) as carrier gas. The oven temperature was programmed as follows: initial temperature of 60℃ for 2 min, ramp up at 6℃ min^{-1} to 310℃, and dwell for 20 min.

2.5. Isotope analysis by GC/C/IRMS

CSIA of the TMAH derivatives of lignin phenols was carried out by GC/C/IRMS (Thermo Fisher Scientific) using a Trace GC coupled to a Delta V advantage IRMS via a GC combustion III interface. The standard solution of TMAH derivatives was injected with a split/splitless injector into an HP-5MS capillary column (30 m long, 0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies). Helium (He) was employed as carrier gas. The oven temperature was programmed as follows: initial temperature of 60℃ for 2 min, ramp up at 6℃ min^{-1} to 310℃, and dwell for 20 min. Isotopic compositions are expressed in δ notation relative to VPDB, and mean values with one standard deviation (1σ) are reported, derived from triplicate analyses. To ensure the optimal conditions for isotope analyses by GC/C/IRMS, the combustion temperature, carrier flow rate, and injection amount were controlled to 850–1000 ℃, 0.8–1.6 mL min^{-1}, and 7–140 ng, respectively.

3. Results and discussion

3.1 Instrumental conditions for GC/C/IRMS

In GC/C/IRMS, the combustion temperature, carrier flow rate, and injection amount are important factors in the quantitative combustion of organic compounds to CO_2 gas and in the accurate determination of the isotopic composition (e.g., Chikaraishi and Oba, 2008). In fact, the δ^{13}C values of TMAH derivatives of lignin phenols, as determined by GC/C/IRMS in the present study, are sometimes different from those independently determined by EA/IRMS, particularly for a combustion temperature of 850℃ at a carrier flow rate of 0.8 or 1.5 mL min^{-1} (Fig. 2), or for a sample amount of less than 14 ng (Fig. 3). Consequently, for all

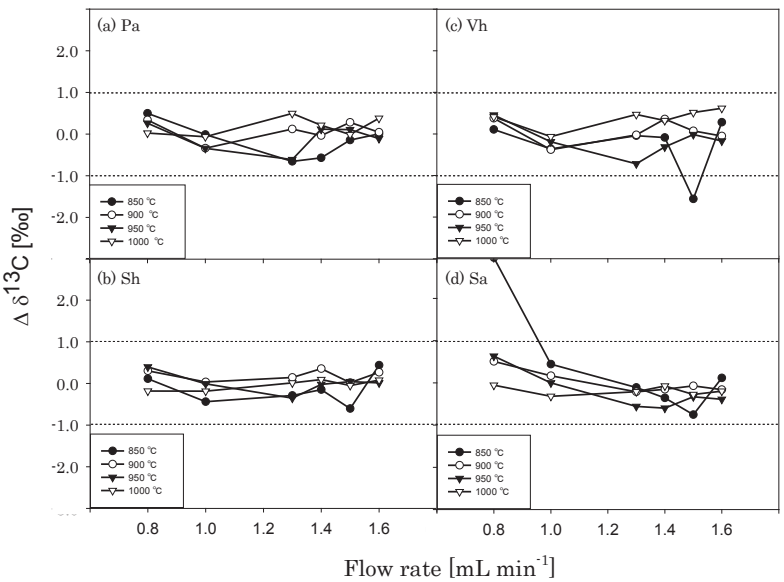

Fig. 2. Variations in the $\Delta\delta^{13}$C ($\delta^{13}C_{EA} - \delta^{13}C_{GC}$) values of lignin phenol standards (a) Pa, (b) Sh, (c) Va, and (d) Sa with respect to He carrier gas flow rate and combustion temperature. Dashed line represents the measurement error (±1.0‰).

analyses in the present study, we fixed the combustion temperature, carrier flow rate, and injection amount to 1000℃, 1.6 mL min^{-1}, and 30–140 ng, respectively, to ensure the accurate and precise determination of the isotopic composition of lignin phenols. Under these settings, the potential analytical error is less than 1.0‰.

3.2 Isotopic fractionation during TMAH derivatization

Hydroxyl and carboxyl groups of lignin phenols are replaced by methoxy and methyl ester groups, respectively, during TMAH derivatization (Fig. 1) performed simultaneously and quantitatively (e.g., Challinor, 2001). Although the specific mechanism (i.e., chemical pathway) of this derivatization remains poorly understood, it probably involves kinetic carbon isotope fractionation because the carbons in the derivative groups may act as a reaction-center during the derivatization.

In general, the isotopic composition of the derivative group (δ_{Me}) is calculated by isotope mass balance, using the isotopic composition of the underivative (δ_{LP}) and derivatives (δ_{LPMe}), as follows:

$$n_{Me} \times \delta_{Me} = n_{LPMe} \times \delta_{LPMe} - n_{LP} \times \delta_{LP}$$

where n is the number of carbon atoms, and the subscripts Me, LPMe, and LP indicate the derivative group (i.e., methoxy and methyl ester), lignin phenol TMAH derivatives, and underivative lignin phenol, respectively.

If isotopic fractionation is negligible or does not occur at all, the calculated δ_{Me} value is constant regardless of the analytical conditions. However, as shown in Fig. 4, the present δ_{Me} values are correlated using the relative molar ratio between the TMAH reagent and total lignin phenols (i.e., TMAH:TLP). Moreover, the values vary for the different structures of lignin phenols. Although the reasons for the observed isotopic fractionation remain unclear, these results clearly indicate that a significant isotopic fractionation occurs during the TMAH derivatization of lignin phenols, and the δ_{Me} values are controlled by the relationship between the TMAH reagent and lignin phenols (Fig. 4), for which the slope and intercept of the linear regression are summarized in Table 2.

We also investigated whether the molar ratio between lignin phenols in the samples had an effect on

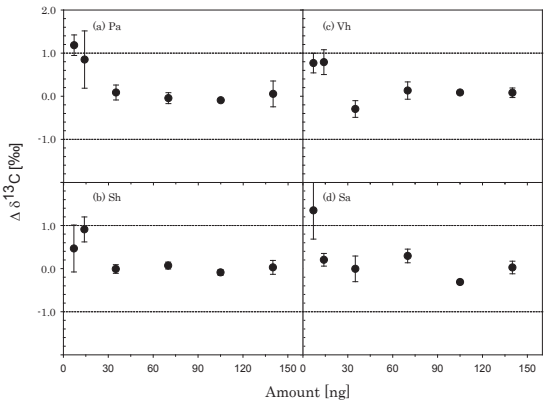

Fig. 3. Variations in the $\Delta\delta^{13}C$ ($\delta^{13}C_{EA} - \delta^{13}C_{GC}$) values of lignin phenol standards (a) Pa, (b) Sh, (c) Va, and (d) Sa determined by GC/C/IRMS, with respect to injected amount. Dashed line represents the measurement error (±1.0‰). Error bars indicate one standard deviation (1σ) from the mean value, as derived from triplicate analyses.

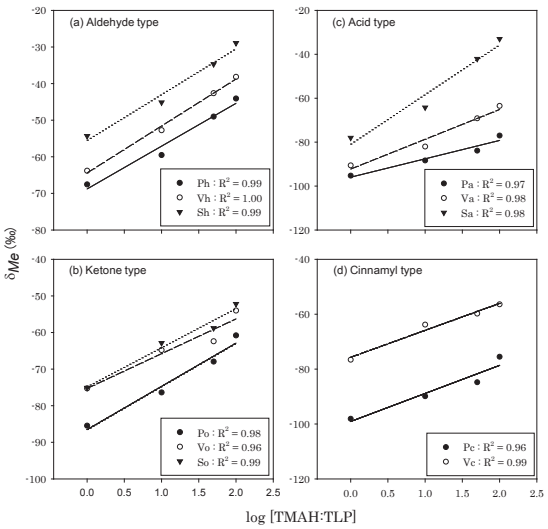

Fig. 4. Relationship between the carbon isotopic composition of the incorporated carbon during TMAH derivatization (δ_{Me}) and the relative abundance ratio between TMAH reagents and total lignin phenols, for the following phenol types: (a) aldehyde, (b) ketone, (c) acid, and (d) cinnamyl.

the measured δ_{Me} values, by using standards 1, 2, and 3, which have different compositions of each lignin phenol. However, as summarized in Table 1, the determined isotopic composition of TMAH derivatives of lignin phenols is identical among the three standards. These results clearly indicate that the degree of isotopic fractionation does not depend on the molar ratio between lignin phenols in samples.

Thus, for isotope analyses of lignin phenols, it is necessary to obtain both the δ_{Me} values (i.e., to obtain the slope and intercept of the correlation line in Fig. 4) and the TMAH:TLP ratio; however, not all previous studies reported this information (e.g., Pulchan et al., 1997; Beramendi-Orosco et al., 2006), meaning that uncertainty may exist regarding the isotopic compositions and related interpretations provided in earlier works.

3.3 Effect of solvent (methanol vs. H_2O)

The TMAH reagent is generally stored in methanol, which gives rise to the potential for transmethylation (i.e., exchange of the methoxy group) between the reagent and the solvent methanol (Ishida et al., 1995). Therefore, we investigated the effect of solvent type (methanol vs. H_2O) on the TMAH reagent. The isotopic composition of the TMAH derivatives of lignin phenols is consistent for both solvents (the difference in $\delta^{13}C$ between the solvents is just ～0.3‰), indicating that the solvent has a negligible effect on the measured isotopic composition.

Table 2. Table 2. Slope and intercept of the correlation line between δ_{Me} and log [TMAH:TLP] for the mass balance correction of the isotope effect during TMAH derivatization of lignin phenols.

Lignin phenols	EA/IRMS (‰)		Carbon number		Derivative carbon (n_{Me})	*δ_{Me}= a x log[TMAH:TLP] + b	
	$\delta^{13}C_{LP}$	S.D.$_{LP}$	underivative	derivative		a**	b**
Aldehyde type							
Ph	–26.7	0.07	7	8	1	11.7	–68.8
Vh	–28.5	0.01	8	9	1	12.8	–64.4
Sh	–30.0	0.04	9	10	1	12.5	–55.5
Ketone type							
Po	–31.1	0.06	8	9	1	11.8	–86.5
Vo	–26.0	0.03	9	10	1	9.5	–75.3
So	–37.9	0.03	10	11	1	10.7	–74.8
Acid type							
Pa	–26.6	0.02	7	9	2	8.4	–96.0
Va	–27.0	0.03	8	10	2	13.6	–92.2
Sa	–40.1	0.04	9	11	2	22.6	–80.9
Cinnamyl type							
Pc	–28.7	0.02	9	11	2	10.2	–99.1
Vc	–27.6	0.01	10	12	2	9.8	–75.7

*Corrected value (δ_{Me}) calculated by isotopic mass balance between derivatized and underivatized lignin phenols.
**a and b were calculated from the regression line in Fig. 4

3.4 Isotopic fractionation during HPLC semi-purification

Base-line separation of lignin phenol derivatives on GC chromatograms is necessary to accurately determine the isotopic composition of lignin phenols obtained from natural samples. However, the products after TMAH derivatization are a complex mixture, resulting in the overlap of peaks on a GC chromatogram. To overcome this problem in the present study, we followed TMAH derivatization with HPLC semi-purification prior to GC/C/IRMS isotope analysis.

Although the TMAH derivatives of lignin phenols cannot generally be found from the optical chromatogram on the RI detector in our HPLC system (Fig. 5a), based on the GC/MS analysis for each fraction collected every 1 min, they are eluted into three fractions: Pa and Pc are mainly eluted in Fraction (Fr.) 1 (17–22 min) and Fr. 2 (22–28 min); Ph and Po are found in only Fr. 2; Va, Sa, Vc, and Sh, are eluted mainly in Fr. 2 and Fr. 3 (28–34 min); and Vh, Vo, and So are eluted mainly in Fr. 3 (Fig. 5b). When the three fractions are injected into GC, one at a time, the peaks of individual lignin phenols can be separated because the baseline resolution is sufficient to accurately determine the isotopic composition.

However, as summarized in Table 3, after HPLC semi-purification of the TMAH derivatized lignin phenol standards, the occurrence of isotopic fractionation in the HPLC means that the isotopic composition of the same lignin phenols differs among fractions. For example, the $\delta^{13}C$ value of Vo from Fr. 2. is –31.3‰, which is much smaller than the original value for Vo (–28.8‰), whereas the $\delta^{13}C$ value of Vo from Fr. 3 is –26.1‰, which is much larger than the original

value. The weighted mean average of the $\delta^{13}C$ value is –28.7‰ for both fractions (i.e., for Frs. 2 and 3), which is consistent with the original value of the standard. Thus, the $\delta^{13}C$ value, as a weighted mean average, is identical to the original value before HPLC separation. Consequently, the HPLC separation demonstrated in this study is useful in the CSIA of lignin phenols.

4. Summary

We demonstrated the stable carbon isotope analysis of individual lignin phenols based on off-line degradation and derivatization by TMAH thermochemolysis, semi-purification by HPLC, CSIA by GC/C/IRMS, and isotope effect correction by isotope mass balance calculation. By using this method, the isotopic composition of individual lignin phenols can be accurately and precisely determined within a standard deviation (1σ) of 0.2‰–0.9‰ (Fig. 6); thus, this method is useful in molecular isotope studies in the field of organic geochemistry.

Acknowledgements

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Table 3. Carbon isotopic composition (‰) of derivatized lignin phenols in fractions separated by HPLC.

Compound	I: Before seperation	II: After seperation				Δ I–II
	Ave. ± S.D.	Fr. 1	Fr. 2	Fr .3	Ave. ± S.D.	
Ph	–30.6 ±0.1		–31.5 ±0.1		–31.5 ±0.1	0.9
Pa	–37.8 ±0.2	–37.5 ±0.4	–38.0 ±0.0	–36.1 ±0.6	–37.2 ±0.2	0.6
Vh	–31.1 ±0.0		–32.0 ±0.0	–30.7 ±0.1	–31.4 ±0.1	0.3
Vo	–28.8 ±0.0		–31.3 ±0.1	–26.1 ±0.1	–28.7 ±0.1	0.1
Va	–34.3 ±0.1	–36.9 ±0.2	–35.0 ±0.1	–31.3 ±0.2	–34.4 ±0.1	0.1
Sh	–31.3 ±0.0	–34.2 ±0.1	–32.3 ±0.0	–29.7 ±0.1	–32.1 ±0.1	0.8
So	–39.2 ±0.0		–41.5 ±0.1	–37.5 ±0.1	–39.5 ±0.1	0.3
Sa	–40.8 ±0.1	–43.2 ±0.1	–42.0 ±0.1	–39.8 ±0.2	–41.7 ±0.1	0.9
Pc	–33.2 ±0.2	–32.5 ±0.6	–31.7 ±0.0	–34.1 ±0.1	–32.8 ±0.2	0.4
Vc	–31.4 ±0.1	–33.0 ±1.0	–31.2 ±0.1	–29.5 ±0.1	–31.2 ±0.3	0.2

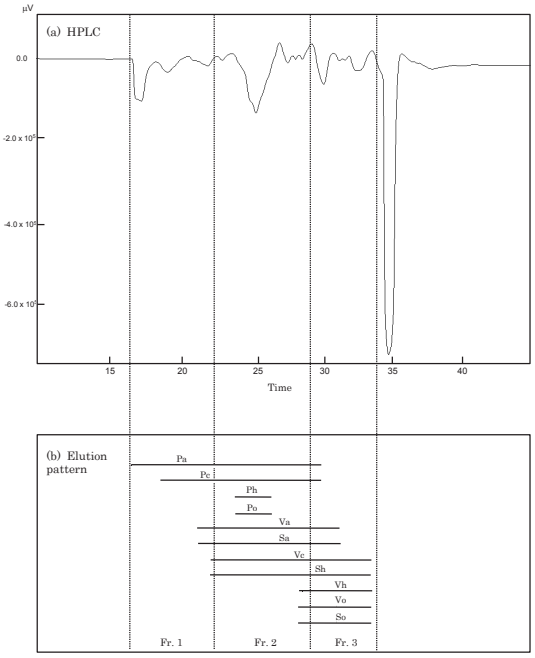

Fig. 5. HPLC elution of lignin phenols: (a) RI chromatogram, and (b) the elution pattern of individual lignin phenols. The elution pattern was obtained from GC/MS analyses for each fraction collected every 1 min.

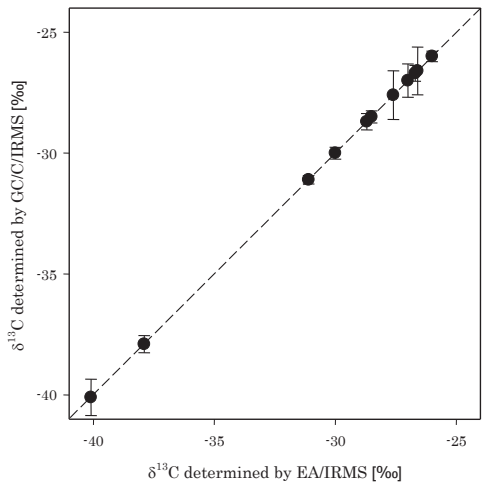

Fig. 6. Relationship between the $\delta^{13}C$ values of lignin phenol standards determined by GC/C/IRMS and EA/IRMS. Error bars indicate one standard deviation (1σ) from the mean value, as derived from triplicate analyses. The dashed line represents the 1:1 line.

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