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Doctor Forum

Determination of Lignin in Marine Sediment Using Alkaline Cupric Oxide Oxidation-Solid Phase Extraction-on-Column Derivatization-Gas Chromatography

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Abstract Lignin serves as one of the most important molecular fossils for tracing Terrestrial Organic Matters (TOMs) in marine environment. Extraction and derivatization of lignin oxidation products (LOPs) are crucial for accurate quantification of lignin in marine sediment. Here we report a modification of the conventional alkaline cupric oxide (CuO) oxidation method, the modification consisting in a solid phase extraction (SPE) and a novel on-column derivatization being employed for better efficiency and reproducibility. In spiking blanks, recoveries with SPE for the LOPs are between 77.84% and 99.57% with relative standard deviations (RSDs) ranging from 0.57% to 8.04% (n=3), while those with traditional liquid-liquid extraction (LLE) are from 44.52% to 86.16% with RSDs being from 0.53% to 13.14% (n=3). Moreover, the reproducibility is greatly improved with SPE, with less solvent consumption and shorter processing time. The average efficiency of on-column derivatization for LOPs is 100.8%±0.68%, which is significantly higher than those of in-vial or in-syringe derivatization, thus resulting in still less consumption of derivatizing reagents. Lignin in the surface sediments sampled from the south of Yangtze River estuary, China, was determined with the established method. Recoveries of 72.66% to 85.99% with standard deviation less than 0.01mg/10g dry weight are obtained except for p-hydroxybenzaldehyde. The lignin content Σ8 (produced from 10 g dry sediment) in the research area is between 0.231 and 0.587 mg. S/V and C/V ratios (1.028±0.433 and 0.192±0.066, respectively) indicate that the TOMs in this region are originated from a mixture of woody and nonwoody angiosperm plants; the high values of $(Ad/Al)_v$ suggest that the TOMs has been highly degraded.

Key words lignin; alkaline cupric oxide; SPE; on-column derivatization; marine sediments

1 Introduction

Lignin compounds are high molecular weight phenolic polymers composed of phenylpropane moieties linked by ether or carbon bond. As the specific component of vascular plants, lignin is one of the most important molecular fossils tracing Terrestrial Organic Matters (TOMs) because of its high natural abundance, single source, relative resistance to degradation, rich parametric information, *etc*.(Goñi *et al*., 1997; Hedges and Mann, 1979a; Hedges and Oades, 1997). It has therefore been widely accepted and applied since the 1970s in marine environment (Hedges and Parker, 1976; Hedges and Mann, 1979a, b). In recent years, lignin has found its extensive applications in the reconstruction of the input, transport and deposition of TOMs in different environmental settings, the inversion of paleoenvironmental and paleoclimate changes, *etc* (Hyodo *et* al, 2008; Rezende *et* al, 2010; Tareq *et* al, 2011). Lignin polymers are not amenable to direct chemical analysis without prior isolation and conversion. However, lignin can be characterized by chemical degradation to release small molecules carring 'embedded' source information. At present, thermochemolysis and mild oxidation are commonly used for chemical degradation of lignin. With its simplicity and rapidness, thermochemolysis has been accepted and applied for the analysis of lignin in marine sediments since the 1990s (Clifford *et al*., 1995; Hartog *et al*., 2004; Shadkami *et al*., 2010; Wysocki *et al*., 2008). However, it appears that thermchemolysis is more suitable for routine analyses of large number of samples rich in organic carbon (Stevenson, 1982). On the other hand, CuO oxidation (Bianchi *et al*., 2002; Creighton *et al*., 1944; Goñi and Hedges, 1992; Goñi and Montgomery, 2000; Hedges and Ertel, 1982; Kuo *et al*., 2008; Louchouarn *et al*., 2000; Louchouarn *et al*., 2010; Fu *et al*., 1995) which appears more consistent and accurate for identifying bulk sources, is appropriate for characterizing a smaller quantity of samples, especially for those with lower TOM content, such as marine sediments (Stevenson, 1982).

Alkaline CuO oxidation for the analysis of lignin was first established by Hedges and Ertel (1982). With CuO, instead of nitrobenzene, as the oxidant, blank and by-

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products were greatly reduced (Hedges and Ertel, 1982). Since then, several improvements have been made: ethyl vanillin as the recovery standard instead of radioactive ^{14}C-p-hydroxyacetophenone to avoid disposal of radioactive substances (Goñi and Montgomery, 2000; Louchouarn *et al*., 1997), ethyl acetate as the extraction solvent instead of ether to eliminate removal of peroxides each time (Goñi and Montgomery, 2000) and recently an increased sample throughput and reproducibility thanks to design improvements in reaction vessels and/or the use of microwave digestion (Goñi and Hedges, 1992; Goñi and Montgomery, 2000).

Kögel and Bochter (1985) was the first to use C_{18}-SPE technology for extraction of LOPs in lignin analysis of humus layer of forest, which is more convenient and quicker than the conventional LLE with even less solvent consumption. And then it was used to extract LOPs from natural waters and soils (Lima *et al*., 2007; Louchouarn *et al*., 2000). In these studies, recovery tests were made only for vanillin (97.6%) (Kögel and Bochter, 1985). However, it is very important to study the recoveries of different monomers in mixed solutions (Lima *et al*., 2007; Louchouarn *et al*., 2000) because of the existence of competition for adsorption sites on packing materials.

Derivatization of LOPs is another critical step for lignin analysis. In-syringe derivatization is one of the most important improvements. Studies show that in- syringe derivatization gives almost the same results as the previous in-vial derivatization while requiring 10 to 100 times less silylating reagent. In-syringe derivatization also allows analyzing freshly derivatized samples in each injection with less usage of sample and reagent (Goñi, 1992).

With the high decomposition efficiency, sensitivity and reproducibility (Hedges and Ertel, 1982; Kögel and Bochter, 1985; Fu *et al*., 1995), alkaline CuO oxidative decomposition combined with the usage of GC is most widely used for these types of samples. In this work, we report a modification of the conventional alkaline CuO oxidation combined with the usage of GC for the determination of lignin in marine sediments. We propose a Polar Enhanced Polymer-SPE (PEP-SPE) and a novel on-column derivatization of the LOPs for higher recoveries and better reproducibility. The established method was used for the determination of lignin in surface sediment samples from the south of Yangtze River estuary for method validation and evaluation.

2 Experimental Set-up

2.1 Extraction

A mixed stock solution of the recovery surrogates**,** ethyl vanillin (EVAL, 97%, Acros Organics) and trans-cinnamic acid (CiAD, 98%, Acros Organics), was prepared at a concentration of 1 μg μL^{-1} for each solution component. A mixed stock standard solution, in which all of the following reagents are contained with a concentration of 1 μg μL^{-1} for each, was also prepared. They are p-hydroxybenzaldehyde (99%, J&K Scientific, China); p-hydroxyacetophenone (99%, J&K Scientific, China); vanillone (99%, J&K Scientific, China); syringone (98%, J&K Scientific, China); p-hydroxybenzoic acid (99%, Acros Organics); syringaldehyde (98%, Acros Organics); vanillic acid (98.5%, Acros Organics); syringic acid (97%, Acros Organics); vanillin (98%, Acros Organics); p-coumaric acid (98%, Sigma-Aldrich); ferulic acid (99%, Sigma-Aldrich)**.** These stock solutions were made with acetonitrile (HPLC grade, Dikma Technologies, China).

It is reasonable to compare the recovery of each lignin monomer in a basic aqueous solution (similar to the reaction medium in lignin decomposition) for procedure optimization. Therefore, 10 μL of the recovery surrogate solution and 10 μL of lignin monomer standard solution were added into 30 mL of NaOH solution (1.5 mol L^{-1}); the mixed solution was then acidified to pH 1 with hydrochloric acid (6 mol L^{-1}) for subsequent extraction, derivatization and GC analysis.

SPE: A Cleanert SPE column (500 mg, Agela T-echnologies) was conditioned with 5 mL of ethyl actate, followed by the same amount of methanol and ultra-pure Milli-Q water. The above-mentioned mixed solution was passed through the cartridge in a flow rate of 1.5 mL min^{-1}, and then blown with air for 15 min. Subsequently, phenolic compounds (including lignin monomers and surrogates) were eluted with a certain volume of solvent. The eluate was blown under a gentle stream of N_2 and stored at 4℃ until derivatization and analysis. The type of SPE column, elution solvent and its volume were optimized by comparing the recoveries of the targeted compounds.

LLE: A known volume (8–10 mL) of ethyl acetate (treated with $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ to remove peroxide prior to use) was added into the above mixed solution. After thoroughly shaking and stratification, the organic phase was carefully transferred to a tube. This step was repeated three times to achieve sufficient extraction. After water removing with baked Na_2SO_4, the combined extracts were concentrated by vacuum rotary evaporation and transferred to a 2 mL vial for gentle nitrogen-blowing, and then stored at 4℃ until derivatization and analysis.

2.2 Derivatization and Gas Chromatography

LOPs generally need to be derivatized for GC analysis. For this purpose, bis-trimethylsilyl-trifluoroacetamide plus 1% trimethylchlorosilane (BSTFA+1%TMCS) is used as the derivatizing reagent to form trimethylsilyl derivatives. The derivatization efficiency is critical for accurate determination of lignin. The reaction temperature, time and vessel were then optimized for this step.

Immediately after the derivatization, the trimethylsilyl derivatives were quantified by a Shimadzu 2010 plus GC with a flame ionization detector (FID). For GC analysis, acetonitrile was used as solvent for the preparation of injection samples, and triisopropylbenzene (TIPB, 98.7%, Chem Service) as the internal standard for quantification. Samples were injected in a 'splitless' mode onto a RTX-1 capillary column (30 m × 0.25 mm × 0.25 μm) (J&K Scientific, China) with a column flow rate about 1.3 mL min^{-1}

of helium. The column temperature was programmed from 100℃ to 270℃ at 4℃ min^{-1}, and stayed at 270℃ for 10 min. Both injector and detector were maintained at 300℃.

2.3 Sample Collection and Analysis

Surface sediment samples were collected by using a grabber in the south of Yangtze River Estuary, as marked in Fig.1, during a cruise from April 8 to 26, 2010. Samples were stored at −20 ℃ immediately after collection and kept frozen until lignin analysis. Before analysis, samples were freeze-dried and ground to pass 80 mesh sieve in March, 2011 for further use.

Sample pre-treatment for lignin analysis was carried out according to the method established by Hedges and Ertel (1982) and improved by Goñi and Montgomery (2000) and Miliner and Emei (2000). Briefly, the oxidation was carried out in a Teflon reaction vessel charged with samples (containing 2–5 mg organic carbon), powdered CuO (pre-treated by using CH_2Cl_2 to eliminate lipid), and Fe $(NH_4)_2(SO_4)_2{\cdot}6H_2O$ (used as an O_2 scavenger) in aqueous NaOH solution (bubbled overnight with N_2 to remove dissolved oxygen). The vessel was capped without introduction of oxygen in a glove box with inert atmosphere and placed at 170℃ for reaction, which was terminated after 3 h, and was then immediately cooled down under running tap water.

After cooling and adding the recovery surrogates (EVAL and CiAD), the content was then transferred, centrifugated, washed and separated. Supernatant liquor was combined and acidified with concentrated HCl and extracted as described above. Procedural blanks (containing all reagents but no sediment sample) were also determined with the same procedure.

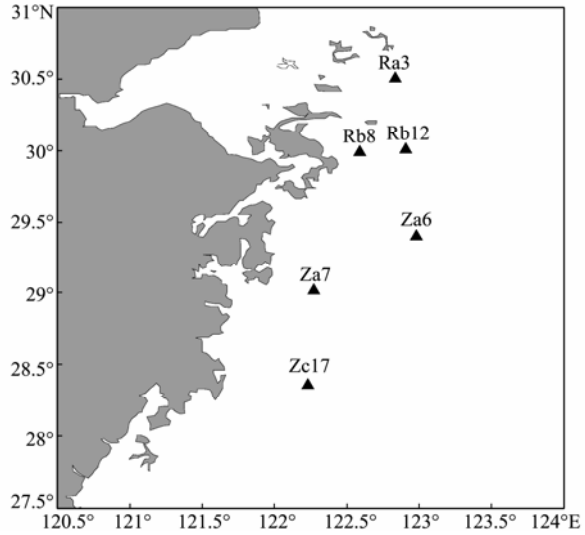


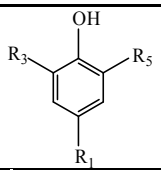
Fig.1 Stations for Surface Sediment Sampling in the South of Yangtze River Estuary, China.

3 Results and Discussion

3.1 Lignin Oxidation Products

Lignin is decomposed to 11 phenolic monomers (or LOPs) by alkaline CuO oxidation method. For further discussion, the structure of the 11 LOPs are shown in Table 1.

Table 1 Lignin oxidation products (LOPs) by CuO (Modified from Ref. 14)



A = CHO B = $COCH_3$ C = CO_2H
D = trans $CH{=}CHCO_2H$ E = H F = OCH_3

		Structure			Source plant [1]			
		R_1	R_3	R_5	G	g	A	a
p-hydroxyl（P）	p-hydroxybenzaldehyde (Pl)	A	E	E	*	*		*
	p-hydroxyacetophenone (Pn)	B	E	E	*	*		*
	p-hydroxybenzoic acid (Pd)	C	E	E	*	*		*
Vanillyl（V）	Vanillin (Vl)	A	E	F	*	*	*	*
	Acetovanillon (Vn)	B	E	F	*	*	*	*
	Vanillic acid (Vd)	C	E	F	*	*	*	*
Syringyl（S）	Syringealdehyde (Sl)	A	F	F			*	*
	Acetosyringone (Sn)	B	F	F			*	*
	Syringic acid (Sd)	C	F	F			*	*
Cinnamyl（C）	p-coumaric acid (Cd)	D	E	E		*		*
	Ferulic acid (Fd)	D	E	F		*		*

Notes:[1] G–gymnosperm woods; g–nonwoody gymnosperm tissues; A–angiosperm woods; a–nonwoody angiosperm tissues; *–main source of plants and tissues

3.2 Extraction

After decomposition of lignin, extraction of LOPs is needed for separation from the reaction medium. The extraction efficiency is one of the most critical influencing factors for accurate determination of lignin.

A preliminary study showed that the extraction efficiency of LLE was unsatisfactory, which intrigued us the use of SPE with a higher recovery and a better reproducibility. Reversed-phase packing is usually employed for extraction of LOPs from the sodium hydroxide reaction medium. Since only a few coverages have been found about C_{18}-SPE applied in lignin analysis (Knapp, 1979; Kögel and Bochter, 1985; Lima *et al*., 2007), we determined to optimize the procedure to apply SPE to lignin analysis in marine sediments. The elution solvent and the type of SPE column were optimized for higher recoveries of all the LOPs in a spiked NaOH solution.

The SPE column was first activated to produce active sites for adsorption and to rinse the adsorbed impurities on packing material, to eliminate any interference with the determination of LOPs. In order to prevent oxidation of aldehydes and isomerization of cinnamyl phenols, no methanol is used.(Lima *et al*., 2007) However, the polarity of ethyl acetate is much similar to those of LOPs, leading to a greater solubility of LOPs while with less co-elution of polar impurities and hence less interference. We therefore selected ethyl acetate as the eluting solvent in this work.

Three types of SPE columns, *i.e*. Cleanert C_{18} (end-capped, 500 mg), Cleanert ODS C_{18}-N (non-end- capped, 500 mg and Cleanert PEP (equivalent to Waters Oasis HLB, 150 mg, were screened based on a comparison between recoveries of lignin monomers and recovery surrogates in a spiked NaOH solution at a concentration level of 0.3 ng μL^{-1}. It was shown that the recoveries on PEP column were always the highest for any compounds (see Fig.2), and those on C_{18} were the lowest for most compounds. The recoveries on C_{18}-N column (averaged 60.5%) were generally higher than those on C_{18}, while they were too low for acid monomers (averaged 42.9%).

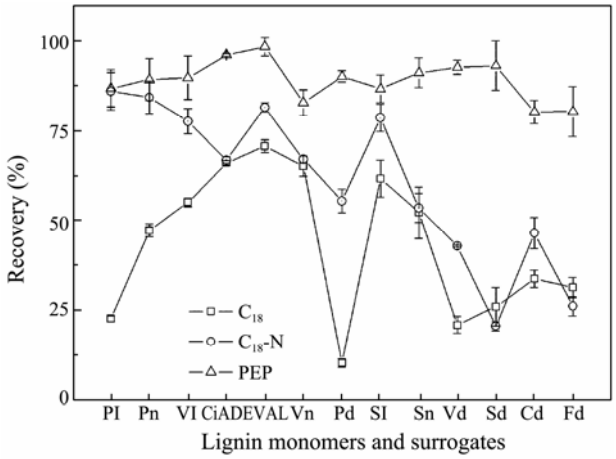


Fig.2 The recoveries obtained for lignin monomers by C-learnt C_{18}, C_{18}-N and PEP SPE columns (n=3) (CiAD: trans-cinnamic acid; EVAL: ethyl vanillin; Other abbreviations are as shown in Table 1).

Cleanert PEP is a co-polymer from hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene in a specific proportion, and thus with a balanced adsorption for various polar and non-polar compounds. The strongly hydrophilic compounds (such as acids in LOPs) are generally difficult to be retained on C_{18} column, but they are highly recovered on PEP column. The lowest recovery on PEP, which was observed for ferulic acid, still reached 77.8%. In contrast, the lowest recoveries on C_{18} and C_{18}-N, observed for p-hydroxybenzoic acid and syringic acid, were just 10.3% and 20.5%, respectively.

To determine the accuracy and reproducibility of PEP-SPE, we conducted three repeated trials using blank NaOH solutions spiked with 1, 10, 100 µg for each compound. The recoveries for most of the LOPs were higher than 80% (see Fig.3). The recovery of ferulic acid at any of the three concentrations was always relatively low, which might result from the adsorption loss of eluent during the process of nitrogen-blowing and/or the isomerization of cinnamyl phenols. The averaged RSDs for all of them were 6.59%, 4.56%, 1.26%, respectively, for spiking levels from low to high.

For comparison, we also used LLE to extract spiked NaOH solution (with 10 µg of each compound) by using ethyl acetate as the solvent for three times with 10mL each time. Results (Table 2) showed that the recovery of each LOP for spiked NaOH solution with SPE was always higher than that with LLE, especially for cinnamyl phenolic monomers. The RSDs of most of the monomers with SPE were less than 10%.

Comparison of the GC traces of sample Rb8 extracted with LLE and PEP-SPE (Fig.4) suggests that the purification of the sample is also improved with PEP-SPE, which is propitious to qualitative and quantitative analysis.

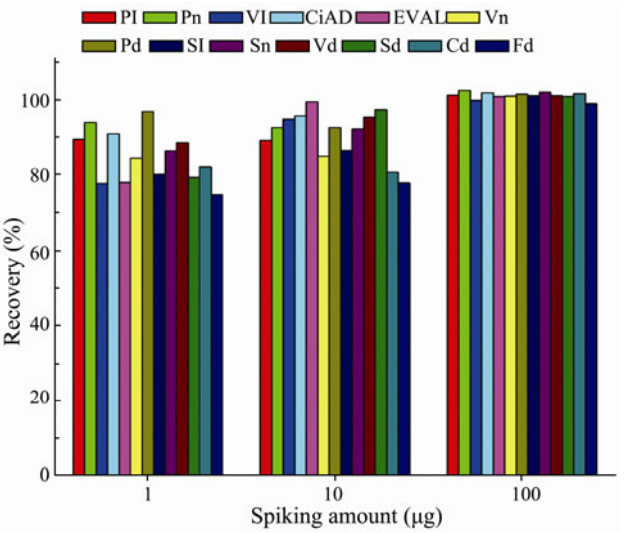


Fig.3 Recoveries obtained from repeated trials using blank NaOH solutions spiked with 1, 10, 100 μg for each compound (lignin monomers and surrogates) by PEP-SPE (Abbreviations are the same as in Fig.2).

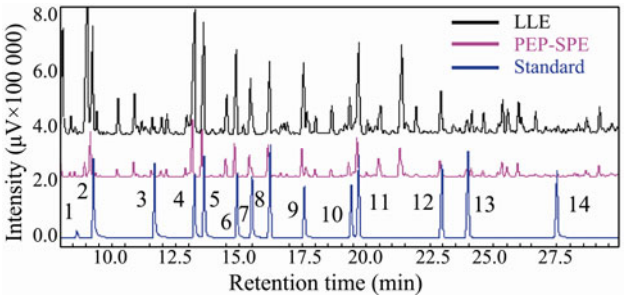


Fig.4 Gas chromatographic trace of a standard acetonitrile mixture of lignin monomers and surrogates after Me_3Si-derivatization, and those of the sediment sample Rb8 extracted by LLE and PEP-SPE (1. triisopropylbenzene (TIPB, internal standard); 2. Pl; 3. Pn; 4. Vl; 5. CiAD (surrogate); 6. EVAL (surrogate); 7. Vn; 8. Pd; 9. Sl; 10. Sn; 11. Vd; 12. Sd; 13. Cd; 14. Fd. Abbreviations are the same as in Fig.2).

Table 2 Comparison of recoveries and RSDs of LOPs for spiked NaOH solution (with 10 μg each, *n*=3) by using LLE and PEP-SPE

Targeted Compound [1]	LLE		SPE (Cleanert PEP)	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Pl	79.84	6.48	89.06	7.92
Pn	86.16	4.81	92.78	8.04
Vl	83.37	12.04	94.96	7.23
CiAD	78.47	0.53	95.86	0.57
EVAL	64.33	13.14	99.57	0.57
Vn	76.25	8.27	84.87	5.31
Pd	86.10	3.81	92.78	6.71
Sl	85.04	4.77	86.43	3.85
Sn	86.01	3.82	92.31	1.78
Vd	84.90	3.39	95.44	3.34
Sd	79.44	4.42	97.44	3.13
Cd	44.52	12.62	80.66	4.30
Fd	53.66	3.18	77.84	6.51

Notes:[1] CiAD: trans-cinnamic acid; EVAL: ethyl vanillin; Other abbreviations are as shown in Table 1

In summary, extraction of LOPs with Cleanert PEP-SPE gives higher efficiency, better reproducibility with better purification effect, and less solvent consumption. Furthermore, it avoids emulsification, which might occur in LLE process, and incomplete extraction caused by intersolubility of ethyl acetate and NaOH solution.

3.3 Derivatization

With their own characteristics of different polarity, low volatility and thermal instability, LOPs can not be detected directly by GC. A derivatization step is therefore needed and trimethylsilyl (Me_3Si) donors are particularly useful because they allow rapid, one-step derivatization of both phenolic and carboxyl functional groups (Li *et al*., 2002).

Traditionally, derivatization reaction is carried out in a sample bottle (in-vial derivatization), which needs more sample and expensive derivatizing reagents, or in a syringe (in-syringe derivatization).

In this work, we developed a novel on-column derivatization in which the sample and the derivatizing reagent were injected together into a GC, without a separate derivatizing step. The derivatization was completed instantaneously during the vaporization in the injection port.

To compare the derivatization efficiency, a mixed acetonitrile solution with standard lignin monomers and recovery surrogates at a concentration of 150 ng μL^{-1} for each was derivatized with different methods. For traditional in-vial and in-syringe derivatization, we also compared the efficiencies derivatized at different temperatures (room temperature (RT) and 70℃) for 10, 30 and 60 min. Overall, we obtained a similar efficiency averaged at 95% for both traditional methods, and a significantly higher efficiency of 100.8%±0.68% for on-column derivatization. Moreover, an improved reproducibility with RSD around 2.5% was observed (versus 4.6% and 6.5% for in-vial and in-syringe derivatization, *n*=7). In addition, it is obvious that on-column derivatization is time-saving, convenient to operate with less reagent consumption.

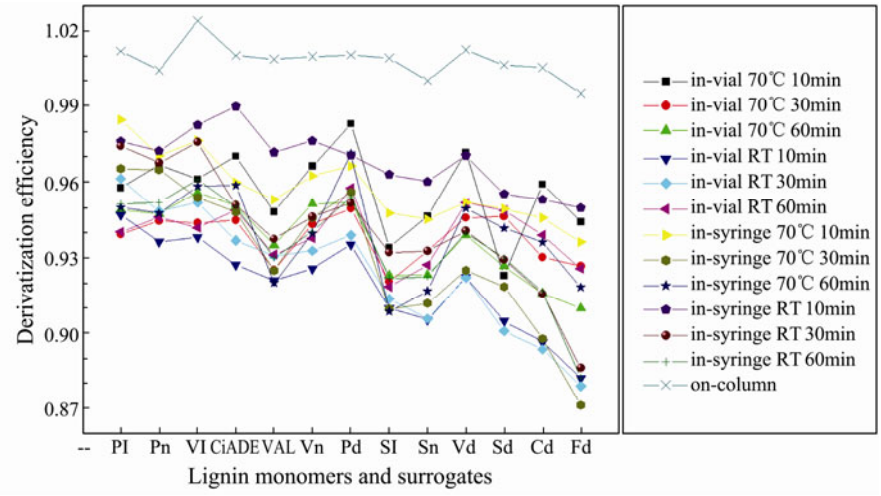


Fig.5 Comparison of the efficiency of on-column derivatization and those of in-vial and in-syringe derivatization at different temperatures for different time periods, for a standard acetonitrile mixture of lignin monomers and surrogates at a concentration level of 150 ng μL^{-1} for each. (Abbreviations are the same as in Fig.2).

3.4 Sample Analysis

We used this optimized method to determine lignin in surface sediment samples collected in the south of Yangtze River Estuary (shown in Fig.1). Two recovery surrogates (EVAL and CiAD) were added to evaluate the recoveries of targets with different structure and volatility. For quantification, a seven-point standard curve was obtained (concentration ranged from 1 ng μL^{-1} to 200 ng μL^{-1}), which show that there is a very good linear relationship between the peak area ratio and the concentration ratio for each of the LOPs, with correlation coefficients greater than 0.999. The averaged method detection limit (MDL, presented as three times signal-to-noise ratio) was 0.13 ng.

The recoveries of the surrogates in spiked samples were between 72.66% and 85.99%. The standard deviation for each monomer was less than 0.01 mg $(10\,g\,dw)^{-1}$ except for p-hydroxybenzaldehyde. Lignin yield (Σ8) was between 0.231 and 0.587 mg $(10\,g\,dw)^{-1}$. The ratio of syringic to vanillic monomers (S/V) was 1.028 ± 0.432 and that of cinnamic to vanillic monomers (C/V) was 0.196 ± 0.066, suggesting that these TOMs were originated from a mixture of woody and non-woody tissues of angiosperms (Hedges and Parker, 1976; Hedges and Mann, 1979a; b). High ratios of acid to aldehyde (greater than 0.4) in vanillic monomers indicate that the TOMs are highly degraded (Goñi and Hedges, 1992; Hedges and Mann, 1979a; b).

3.5 Quality Assurance and Quality Control (QA/QC)

Procedural blank: The whole process was completed under the optimized condition to assess the interference of experimental process and all reagents used. GC traces (not presented in this paper) showed that the procedure and reagents did not interfere with the determination of LOPs. Although some of the monomers have been detected with the content less than 0.4 μg $sample^{-1}$, the accuracy for quantification of lignin in sediment samples does not decrease in considering of errors related to other steps.

Accuracy, precision and reproducibility: For verification of the qualitative and quantitative accuracy and the reproducibility, five standard acetonitrile solutions with concentrations of 10, 50, 100, 150, 250 ng μL^{-1} for all LOPs were prepared. RSD (n=6) of the retention time was $0.18\% \pm 0.07\%$ for all monomers and surrogates with complete separation each other, and RSD (n=6) for content of each monomer was between 0.93% and 3.15%.

The accuracy and precision for the whole procedure of the proposed method were evaluated by repeated determination of a blank NaOH solution spiked with lignin monomers and surrogates with a concentration of 300 ng mL^{-1} for each. The recovery was $90.77\% \pm 6.61\%$ and the RSD was $4.56\% \pm 2.63\%$ (for n=3).

In addition, EVAL and CiAD were added to each sediment sample as surrogates to monitor the recoveries of targets with different structure and volatility. The averaged recovery of $79.32\% \pm 7.47\%$ with standard deviations less than 0.01 mg $(10\,g\,dw)^{-1}$ absolutely meets the requirement for detection of trace analytes in environmental samples (recovery of 40%–120% with RSD less than 30% for a surrogate (Li *et al*., 2002).

4 Conclusions

The modification of the conventional alkaline CuO oxidation method incorporates the use of PEP-SPE and a novel on-column derivatization to hydrolyze lignin phenols from macromolecular precursors in the marine sediments. In spiking blanks, recoveries with PEP-SPE for the LOPs are between 77.84% and 99.57% with RSDs ranging from 0.57% to 8.04% (n=3). In contrast to the traditional LLE method, PEP-SPE get better efficiency and reproducibility with less solvent consumption and shorter processing time. The average efficiency of on-column derivatization for LOPs($100.8\% \pm 0.68\%$) is significantly improved with still less consumption of derivatizing reagents.

The results of lignin in the sediment samples by the established method show that recoveries of 72.66% to 85.99% with standard deviation less than 0.01 mg $(10g)^{-1}$ dry weight except for p-hydroxybenzaldehyde absolutely meet the requirement for detection of trace analytes in environmental samples.

Acknowledgments

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