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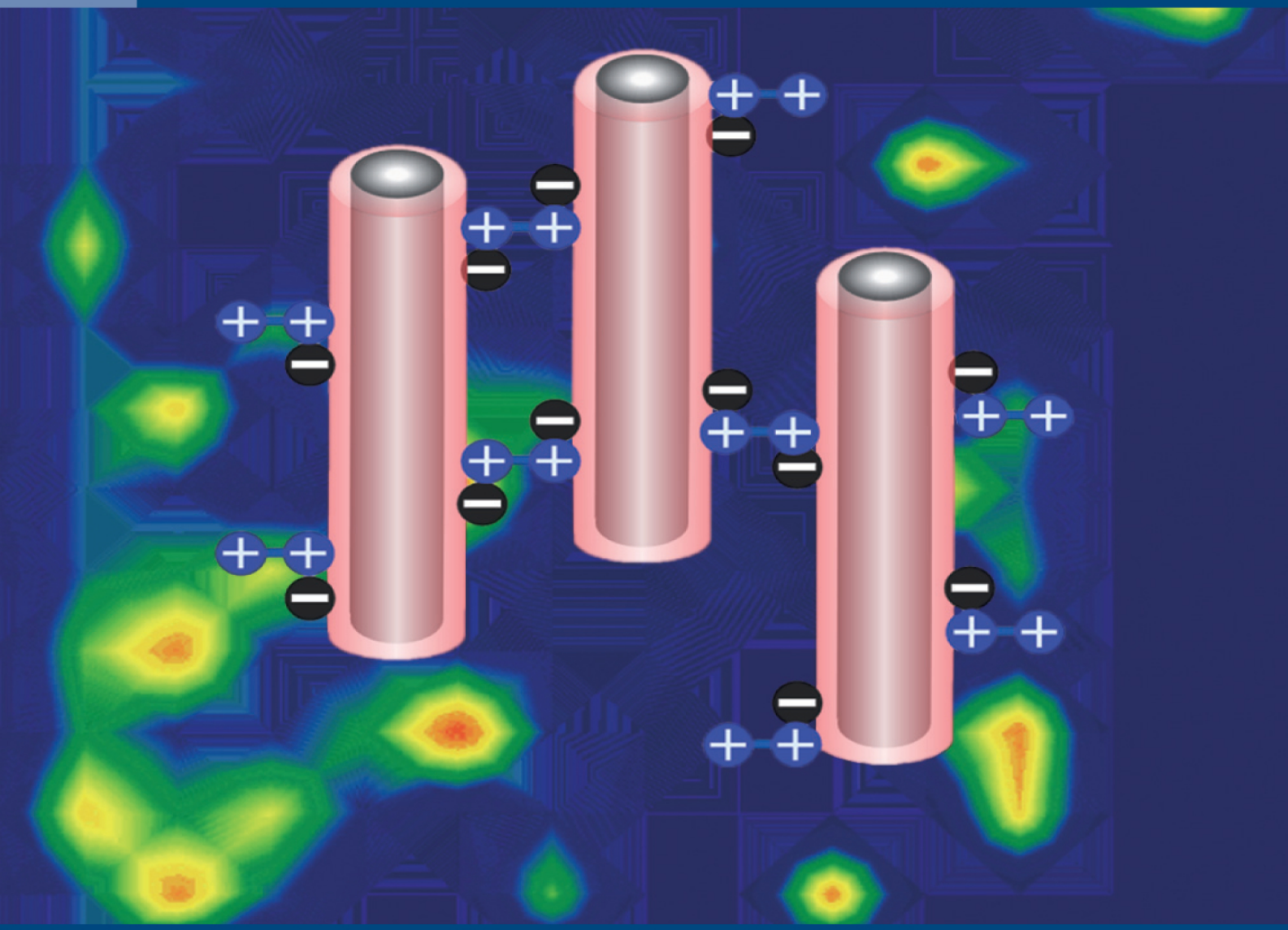

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Research Article

Ultra-high performance supercritical fluid chromatography of lignin-derived phenols from alkaline cupric oxide oxidation

Traditional chromatographic methods for the analysis of lignin-derived phenolic compounds in environmental samples are generally time consuming. In this work, an ultra-high performance supercritical fluid chromatography method with a diode array detector for the analysis of major lignin-derived phenolic compounds produced by alkaline cupric oxide oxidation was developed. In an analysis of a collection of 11 representative monomeric lignin phenolic compounds, all compounds were clearly separated within 6 min with excellent peak shapes, with a limit of detection of 0.5–2.5 μM, a limit of quantification of 2.5–5.0 μM, and a dynamic range of 5.0–2.0 mM ($R^2 > 0.997$). The new ultra-high performance supercritical fluid chromatography method was also applied for the qualitative and quantitative analysis of lignin-derived phenolic compounds obtained upon alkaline cupric oxide oxidation of a commercial humic acid. Ten out of the previous eleven model compounds could be quantified in the oxidized humic acid sample. The high separation power and short analysis time obtained demonstrate for the first time that supercritical fluid chromatography is a fast and reliable technique for the analysis of lignin-derived phenols in complex environmental samples.

Keywords: CuO oxidation / Humic acid / Lignin / Phenolic compounds / Ultra-high performance supercritical fluid chromatography
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1 Introduction

Lignin is a complex biopolymer that constitutes a large portion of the organic matrix of lignocellulosic biomass [1, 2]. It is built from methylated aromatic alcohols that are linked by ether and carbon bonds. Microbial degradation of dead plant matter, especially lignin, results in the formation of humic substances. Humic substances are classified into three subcategories: humin, humic acid, and fulvic acid, based on extractability at different pH values [3]. Lignin and HS play important roles in the global carbon cycle and have a wide occurrence in soils, sediments, and natural waters, due to their high resistance to biological and chemical degradations [4–6]. Furthermore, lignin is widely utilized as a tracer for terrestrial organic matter in geosciences because the composition of its phenols is an important indicator of the type of original plant tissues [7, 8].

Alkaline cupric oxide (CuO) oxidation is one of the mostly adopted methods for analyzing lignin in various complex environmental matrices such as soils and sediments [9]. Upon CuO oxidation, the lignin macromolecule is hydrolyzed into a series of methoxy phenyl and phenyl aldehydes, ketones, and acids [9]. The relative yields of these phenolic monomers give signatures of the vascular plant tissue types that have contributed to the total organic carbon in territorial samples [10].

Methods reported for the analysis of 11 major lignin-derived phenols generated by CuO oxidation are based on GC–MS, HPLC with diode array detection (DAD) and CE–DAD [11–15]. These methods all have different limitations: GC needs a derivatization process; the analysis time is relatively long for both GC and HPLC; CE suffers from a poor precision of retention times.

A low-viscosity mobile phase consisting of compressed carbon dioxide is used in SFC to achieve fast and efficient separation. For instance, phenolic compounds have been determined in a few applications using SFC [16–18]. Ultra-high performance supercritical fluid chromatography (UHPSFC) using columns with sub-2 μm packing can further improve the resolution of traditional SFC [19]. Compared with HPLC, the significantly higher diffusion coefficient and lower viscosity exhibited by the CO_2-based mobile phase lead to faster mass transfer and the possibility of using higher flow rates with high efficiency. In general, the retention mechanism of SFC is mainly decided by the stationary phase nature. However, the specific retention behaviors can also be largely impacted by other parameters, such as the addition of the modifier and the change of the mobile phase density [20]. SFC,

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Abbreviations: DAD, diode array detection; **UHPSFC**, ultra-high performance supercritical fluid chromatography

using carbon dioxide as mobile phase with nonpolar stationary phases, offers uniquely different selectivity from those of commonly used reversed-phase HPLC [21]. When used with polar stationary phases, SFC demonstrates normal-phase separation mechanism while offering better reproducibility of retention times [22]. Aside from the choice of stationary phases, SFC selectivity can also be fine-tuned by altering the density of the mobile phase, by changing the temperature and pressure and by addition of polar modifiers. In addition, faster separation of chiral compounds and easy removal of solvents are important advantages of SFC over HPLC, especially at the preparative scale [23]. The main advantages of SFC in comparison to GC are easier sample preparation for nonvolatile, polar, or adsorptive compounds, and the possibility to affect the separation by varying the composition of the mobile phase. In recent years, research on the use of SFC in pharmaceutical enantiomeric separations and food analysis has been abundant [24]. However, to the best of our knowledge, the use of SFC for the analysis of lignin-derived phenols has not been reported yet, despite the fact that the high separation efficiency possessed by SFC holds the potential of good and fast resolution of various components with similar molecular structures.

In the current study, we present a fast UHPSFC method for separation and quantification of lignin-derived phenols from alkaline CuO oxidation of humic acid with a diode array detector. Reproducible qualitative and quantitative analysis is enabled by the developed method.

2 Materials and methods

2.1 Chemicals

Lignin phenols (vanillin, acetovanillone, syringaldehyde, acetosyringone, vanillic acid, *p*-hydroxybenzaldehyde, *p*-hydroxyacetophenone, syringic acid, ferulic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid), ethylvanillin, CuO, and $Fe(NH_4)_2(SO_4)_3 \cdot 6H_2O$ were obtained from Sigma Chemical (St. Louis, Missouri, USA). Humic acid was obtained from Fluka (Buchs, Switzerland). Sodium hydroxide and hydrochloric acid (37%) were purchased from Acros Organics (Geel, Belgium). Methanol and 2-propanol were obtained from Scharlau (Barcelona, Spain). Ethyl acetate was obtained from Fisher Scientific (Waltham, MA, USA). Citric acid was obtained from Merck (Darmstadt, Germany). All organic solvents were of HPLC or higher grade. Water from a Milli-Q Water Purification System (Millipore) with a UV unit was used for solution preparation and washing purpose.

2.2 Cupric oxide oxidation and extraction

CuO oxidation of the humic acid sample was performed as described by Goñi and Montgomery with slight modification [25]. Three milliliters of 2 M NaOH solution (N_2 bubbling and ultrasound degassed) was added to a microwave reaction tube (10 mL, 10 mm inner diameter) under Argon flow. After continuously purging with Argon flow for 1 min, 50 mg humic acid, 50 mg $Fe(NH_4)_2(SO_4)_3 \cdot 6H_2O$ and 125 mg CuO were added to the tube and the tube was immediately sealed with a Teflon cap. The microwave assisted oxidation reaction was carried out in a Biotage Initiator Microwave Reactor (Uppsala, Sweden). The solution was oxidized at 155°C for 1.5 h.

After cooling to ambient temperature, 20 μL of internal standard (1.0 mM, ethylvanillin) solution was added to the mixture. After centrifugation at 3000 rpm for 10 min, the supernatant of the mixture was saved and the sediment pellet was washed with three portions of 3 mL 1 M NaOH (N_2 purged). The combined supernatants were acidified with 6 N HCl to pH 1, which caused a precipitation of insoluble fragments. The supernatant was saved and the sediment pellet washed with acidic water (pH 1) after centrifugation. The combined new supernatant was extracted three times with 10 mL portion of ethyl acetate. The ethyl acetate extracts were dried with Na_2SO_4. After rinsing the Na_2SO_4 with 10 mL ethyl acetate, the combined organic phases were saved and stored in the refrigerator until further processing.

The ethyl acetate extract was transferred quantitatively to a glass tube. The solvent was evaporated under a gentle N_2 flow. The residue was redissolved in 1 mL of 2-propanol. The 2-propanol solution was then stored in a freezer at –18°C until it was analyzed.

2.3 UHPSFC instrumentation

Chromatographic separation and detection of the lignin-derived phenols produced by CuO oxidation were performed with a Waters UltraPerformance Convergence Chromatography System (Milford, MA, USA) with a diode array detector (ACQUITY UPC^2 PDA Detector).

2.4 Column screening and tuning of the mobile phase

Four columns were screened for the separation of the target compounds: ACQUITY UPC^2 HSS C_{18} SB Column (1.8 μm, 3 mm × 150 mm), ACQUITY UPC^2 BEH 2-Ethylpyridine (2-EP) Column (1.7 μm, 3 mm × 150 mm), ACQUITY UPC^2 BEH Column (1.7 μm, 3 mm × 100 mm), and ACQUITY UPC^2 CSH Fluoro-Phenyl Column (1.7 μm, 3 mm × 100 mm). The mobile phase was composed of CO_2 and methanol as cosolvent. The elution strength of the supercritical CO_2 was tuned by changing the column temperature in the range 40–60°C and the pressure of the backpressure regulator in the range105–150 bar. To improve the peak shape of relatively more polar phenolic acid analytes, formic acid, TFA, and citric acid, were explored as additives to the mobile phase, respectively.

2.5 Chromatographic method

The temperature of the column was held at 60°C and the backpressure regulator was set to 135 bar. A binary gradient elution program was used with solvent A being supercritical CO_2 and solvent B consisting of methanol and 20 mM citric acid. The mobile phase gradient was starting with 98.5:1.5 (A/B vol.%), held for 2 min and then ramped up to 91:9 (A/B vol.%) at a rate of 2.5%/min of B, then held for 1 min and decreasing to starting composition in 1 min. The flow rate used during the whole separation process was 1.25 mL/min, the injection solvent was 2-propanol and the injection volume was 1 μL. The sampling rate for the detector was set to 20 points/s. Filter time was 0.1 s. Spectra were collected from 250 to 500 nm with a resolution of 1.2 nm. Signal data was collected at 280 nm with a resolution of 4.8 nm. For column screening, the column temperature was set at 45°C and backpressure at 125 bar. The mobile phase gradient was starting with 98:2 (A/B vol.%), and then ramped up to 75:25 (A/B vol.%) at a rate of 4.6%/min of B, then held for 1 min and decreasing to starting composition in 1 min. The flow rate used during the whole separation process was 1.20 mL/min. The columns were flushed and stored in CO_2 when not in use.

Lignin-derived phenols were identified not only by comparing retention times with those of chemical standards, but also by characteristics of absorption spectra between 250 and 500 nm. One of the benefits of using diode array detection lies in the fact that aliphatic products from CuO oxidation only marginally interfere with the UV detection, which enables good quantification of lignin-derived phenols [13]. Quantification of solutes was based on the integration results of the peaks at the wavelength of 280 nm, which simplifies data processing as all phenols have good molar absorptivity at this wavelength. The concentrations of the phenols and internal standard were derived from respective peak area versus nominal concentration regression equations. These results were then corrected by the internal standard recovery to provide the final concentrations used for calculating the phenol contents of the sample. The repeatability and reproducibility of the method were measured by six successive injections of one standard mixture and one oxidized HA sample respectively in one day and injections of the standard mixtures and sample in three nonconsecutive days. The LOD and LOQ of each phenol for both standard mixture and CuO oxidation sample were determined as three and ten times of the baseline noise height, respectively.

3 Results and discussion

The alkaline oxidation of lignin-containing environmental samples by CuO yields 11 major monomeric phenols with considerable similarities in molecular structure in a complex matrix (Fig. 1). The analysis of samples of this type poses a challenge on traditional chromatographic tools, as fast and efficient separation is difficult. In recent years, this issue has been repeatedly addressed by the development of chromatographic instrumentations. Advanced chromatography techniques, such as UHPLC and UHPSFC can provide significantly higher separation power than traditional HPLC and GC. The aim of this study was to investigate the suitability of SFC technique in the determination of major monomers in lignin alkaline CuO oxidation product samples.

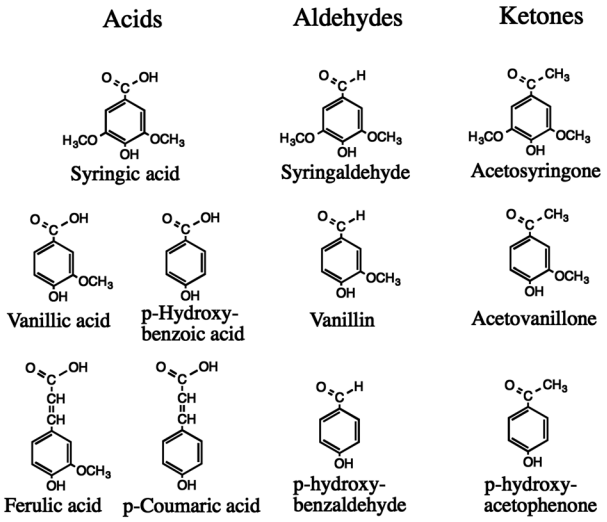

Figure 1. Molecular structures of lignin-derived phenols.

3.1 Column screening and mobile phase tuning

As can be seen in Fig. 2, all four columns screened provided near baseline separation for over half of the 11 analytes with narrow peaks, which demonstrated the high efficiency of SFC and its potential in rapid analysis of phenolic compounds. All four columns have similar general analyte elution profiles with comparatively stronger retention for acidic analytes compared with ketones and aldehydes. However, some specific changes in selectivity caused by the different stationary phases can be observed. For example, the C_{18} column

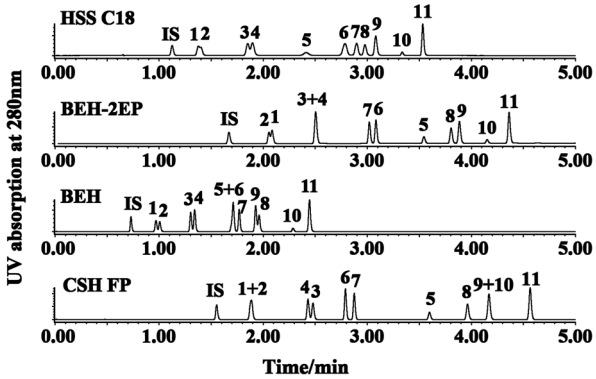

Figure 2. SFC chromatograms of column screening. For peak labels, see Table 1.

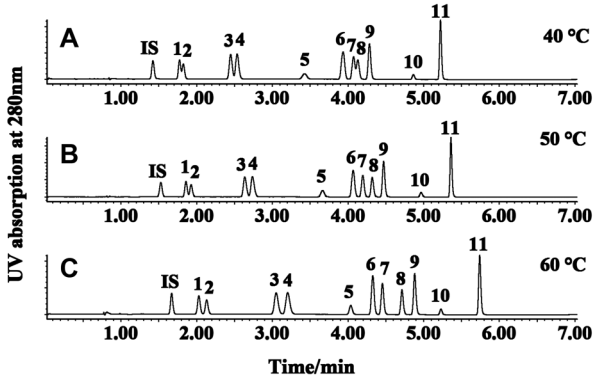

Figure 3. SFC chromatograms of (A) column temperature 40°C, (B) column temperature 50°C, and (C) column temperature 60°C, all at backpressure 135 bar. For peak labels, see Table 1. Chromatographic conditions: methanol (20 mM citric acid) 1.5%, 0–2 min; 1.5–9%, 2–5 min; 9%, 5–6 min; 9–1.5%, 6–7 min. Flow rate 1.0 mL/min. Detection at 280 nm.

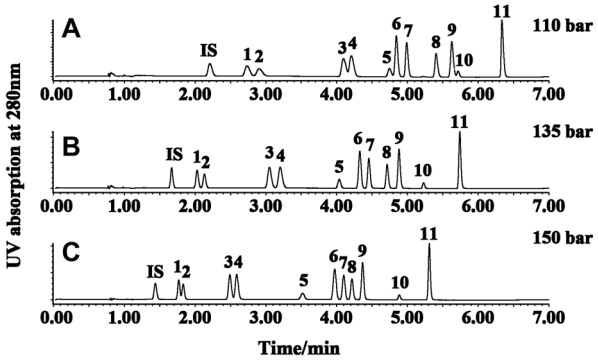

Figure 4. SFC chromatograms of (A) backpressure 110 bar, (B) backpressure 135 bar, (C) backpressure 150 bar, all at column temperature 60°C. For peak labels, see Table 1. Chromatographic conditions: methanol (20 mM citric acid) 1.5%, 0–2 min; 1.5–9%, 2–5 min; 9%, 5–6 min; 9–1.5%, 6–7 min. Flow rate 1.0 mL/min. Detection at 280 nm.

retained phenolic aldehydes more than phenolic ketones while it was the opposite with the 2-EP column. The C_{18} column was chosen due to a slightly better overall separation with a comparatively shorter analysis time. The tuning of column temperature and the backpressure showed that both of these two parameters had significant influence of the elution strength of the supercritical mobile phase. As can be seen in Fig. 3, at the same backpressure, with the increase of the column temperature, the density of the mobile phase decreased, which resulted in a decrease in elution power and longer elution times. Figure 4 showed that at the same column temperature, the density of mobile phase increased as the backpressure increased; the elution power of supercritical CO_2 increased accordingly and analysis time became shorter. The best resolution with regard to all phenols was achieved with 60°C and 135 bar. At the above column temperature and backpressure, different mobile phase flow rates in the range of 0.5–2.0 mL/min were tested, among which 1.25 mL/min gave the best overall separation of all peaks within relatively

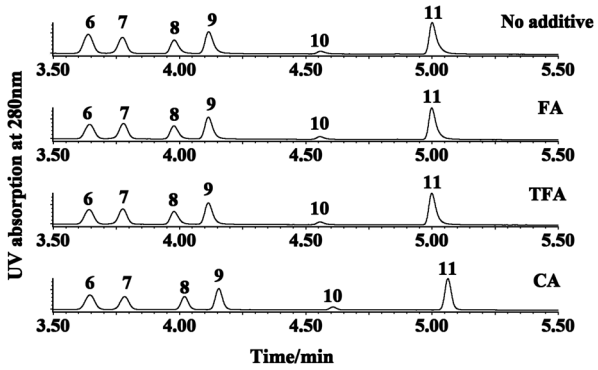

Figure 5. SFC chromatogram (from 3.5 to 5.5 min) of phenolic compound standard mixture using different additives in methanol cosolvent. For peak labels, see Table 1.

short analysis time (data not shown). To obtain good peak shape for the acidic analytes, formic acid, TFA and citric acid were tested as additives to the cosolvent. As Fig. 5 shows, neither the addition of 20 mM formic acid nor 20 mM TFA improved the shapes of tailing peaks compared with the chromatogram obtained with pure methanol as mobile phase. In contrast, phenolic acid peaks were narrower and more symmetrical with the addition of 20 mM citric acid in methanol. For example, the peak asymmetry factor (calculated as the ratio between the distance from the peak center line to the back slope and the distance from the peak center line to the front slope at 10% of the peak height) of *p*-coumaric acid was improved from 1.36 to 1.10 by the addition of citric acid, in comparison with 1.31 and 1.27 by the addition of formic acid and TFA, respectively. The advantage of citric acid over other acidic additives on improving peak shape for phenolic compounds proven were in accordance with other observations reported [17, 26, 27].

3.2 Performance of the UHPSFC method

An SFC chromatogram obtained with a standard lignin phenol mixture is shown in Fig. 6A. The 12 phenolic compounds (11 lignin phenols and ethyl vanillin as internal standard) were all well separated in less than 6 min with highly symmetrical peaks (peak asymmetry factors ranging from 0.98 to 1.10). Table 1 gives a summary of the chromatographic performances of this UHPSFC method. The resolution R_s of each pair of adjacent two peaks varied between 1.38 and 10.93, confirming that a near baseline separation was achieved for all the targeted phenols. All 11 lignin phenols were eluted between 1.5 and 5.1 min. The addition of ethylvanillin did not extend the separation to longer time than that needed for the 11 lignin phenols, as the ethylvanillin peak was detected at 1.3 min. Compared with a reversed-phase HPLC analysis of the same compounds [13], the SFC separation was about seven times shorter with significantly different elution order of the phenols. In general, phenolic acids eluted after the aldehydes and ketones. This can be attributed to the wide

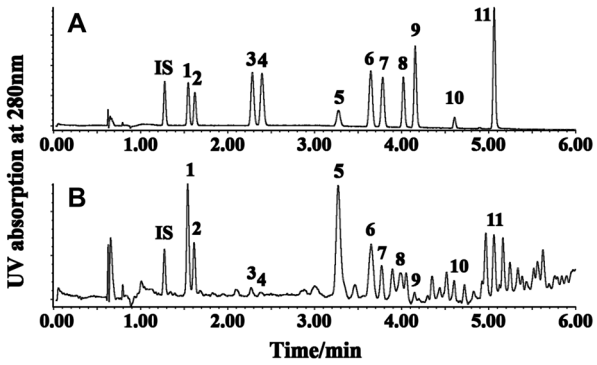

Figure 6. SFC chromatograms of (A) standard mixture and (B) humic acid sample. For peak labels, see Table 1. Chromatographic conditions: methanol (20 mM citric acid) 1.5%, 0–2 min; 1.5–9%, 2–5 min; 9%, 5–6 min; 9–1.5%, 6–7 min. Column temperature 60°C and backpressure 135 bar. Flow rate 1.25 mL/min. Detection at 280 nm.

presence of silanol groups on nonendcapped high-strength silica C_{18} stationary phase and carbon dioxide as the mobile phase, which lead to a normal-phase-like separation mechanism. Furthermore, SFC displays different elution orders from reversed-phase HPLC for compounds within the categories of aldehydes, ketones, and acids, as a combinational effect of different compound lipophilicity and hydrogen-bonding capability [13]. For a standard mixture, the calculated precisions of retention time and peak area for within-day continual injections demonstrated RSDs of ≤ 0.14 and ≤ 0.91%, and the corresponding interday RSDs were ≤ 0.23 and ≤ 1.56%, respectively. The within-day RSDs for oxidized HA samples were ≤ 0.30% (retention time) and ≤ 4.0% (area), and the interday RSDs were ≤ 0.95% (retention time) and ≤ 8.6% (area). The LOD for the phenols in the standard mixture ranges from 0.50 ± 0.20 to 2.5 ± 0.62 μM (1.6 ± 0.6 to 6.9 ± 1.7 μg/g) and LOQ from 2.5 ± 0.16 to 5.0 ± 0.50 μM (8.2 ± 0.7 to 13.8 ± 1.4 μg/g). The LOD and LOQ for the phenols in oxidized HA samples were estimated to be 6.1 ± 2.2 to 9.9 ± 3.2 μg/g and 12.2 ± 3.6 to 19.8 ± 4.4 μg/g, respectively. These results demonstrated that the complexity and matrix effect of real sample have significant negative effects on the detectability of the method. Linear relationship between absorption peak areas of all lignin phenols and concentration was found in the range 5.0 μM to 2.0 mM ($R^2 > 0.997$). In comparison with the HPLC–DAD [13, 14] and GC–MS [11] methods, the UHPSFC method exhibits relatively higher LOD. This can be attributed to the fact that despite the continuous evolvement of SFC instruments, SFC causes relatively noisier baseline than HPLC due to pressure fluctuation, mobile phase temperature effects, and modifier percentage [28]. In addition, smaller injection volume and the disadvantages of UV detection in terms of selectivity compared with MS detection also contribute to the lower S/N of SFC.

3.3 Analysis of humic acid sample

The applicability of the developed UHPSFC method to more complex samples was tested by the analysis of the phenol composition in an oxidized sample of a commercial Fluka humic acid sample. The oxidation process generates a complex sample matrix and the quantification of targeted phenols could be difficult if there were coeluting impurities of considerable amount. A representative SFC chromatogram of this study is shown in Fig. 6B. The purities of each phenol peak were checked by comparison of their UV absorption spectra with pure standard spectra. No major contaminant was found to coelute with the lignin phenols of interest (Fig. 6B). The average recovery for the internal standard ethyl vanillin after sample preparation steps involving ethyl acetate extraction was calculated to be 86.9%.

The analysis results (Table 2) showed a high content of vanillic phenols (vanillin, acetovanillone, and vanillic acid),

Table 1. Chromatographic parameters of lignin phenol standards (0.1 mM each)

Peak	Phenol	Retention Time/min	Retention factor	Resolution	Separation Factor	Peak area RSD[b] (%) (n[c] = 5)	LOD/μM	LOQ/μM
IS	Ethylvanillin (I.S.) [a]	1.278	1.03	5.28	1.42	0.76		
1	Vanillin	1.550	1.46	1.38	1.08	0.22	1.1	4.1
2	Acetovanillone	1.627	1.58	10.17	1.67	0.89	1.5	4.3
3	Syringaldehyde	2.292	2.63	1.49	1.07	0.52	0.9	3.7
4	Acetosyringone	2.402	2.80	10.93	1.50	0.49	0.9	3.5
5	Vanillic acid	3.278	4.20	4.43	1.14	0.68	1.8	4.5
6	p-Hydroxybenzaldehyde	3.649	4.79	1.69	1.05	0.42	0.8	3.5
7	p-Hydroxyacetophenone	3.785	5.00	3.09	1.08	0.58	0.9	3.8
8	Syringic acid	4.023	5.38	1.81	1.04	0.91	1.2	4.1
9	Ferulic acid	4.160	5.60	7.12	1.13	0.75	0.7	3.0
10	p-Hydroxybenzoic acid	4.611	6.31	7.76	1.11	0.89	2.5	5.0
11	p-Coumaric acid	5.068	7.04			0.69	0.5	2.5

[a] Ethylvanillin was added as internal standard.
[b] Relative standard deviation.
[c] Number of replicates.

Table 2. Quantified phenols from oxidation of humic acid (6 replicates, 95% confidence limits)

Lignin phenol	Amount (μg/g humic acid sample)	
	This study	Previous CE study [15]
Vanillin	129 ± 7	121 ± 4
Acetovanillone	87 ± 6	83 ± 5
Syringaldehyde	15 ± 4	n.q.
Acetosyringone	n.q.	n.q.
Vanillic acid	269 ± 10	189 ± 4
p-Hydroxybenzaldehyde	44 ± 3	49 ± 7
p-Hydroxyacetophenone	29 ± 2	104 ± 3
Syringic acid	49 ± 7	123 ± 9
Ferulic acid	16 ± 3	14 ± 6
p-Hydroxybenzoic acid	99 ± 6	104 ± 5
p-Coumaric acid	41 ± 4	49 ± 6

n.q.: not quantified.

which suggested that the humic acid originated partly from degraded vascular plant material. The characteristic syringyl phenols (syringaldehyde, acetosyringone, syringic acid) indicated that also angiosperms were part of the origin for the humic acid. The detection of cinnamyl phenols (ferulic acid and p-coumaric acid) can be correlated to the contribution of non-woody tissues [29]. Moreover, the high vanillic acid to vanillin ratio and the high acid to aldehyde ratio in general both suggest a comparatively intensive microbial alteration [30].

The dominance of vanillic acid over vanillin was also reported in a previous study on Fluka humic acid [15]. Acetosyringone and syringaldehyde were in both studies found at very low concentrations (Table 2). The differences in the quantification results were most probably a combinational result of batch variations and the different CuO oxidation procedures adopted, which have previously been reported to affect the degradation patterns [31, 32].

4 Concluding remarks

The UHPSFC method developed gives rapid and clear separation of the 11 major lignin-derived phenols from cupric oxide oxidation with resolutions between 1.4 and 10.9. No derivatization step is needed and the analysis time is less than 6 min—two major advantages over traditional GC and HPLC analysis methods. Application of this method in the quantitative study of humic acid sample proves its suitability and potential in the analysis of complex environmental samples. However, the LOD and LOQ of this method are not comparable with HPLC–DAD and GC–MS methods, which may limit its application in some fields, such as trace analysis. Research work is currently being performed on utilizing SFC coupled with MS to address the sensitivity issue and to analyze a wider range of lignin-derived phenols.

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The authors have declared no conflict of interest.

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