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Separation of phenolic aldehydes, ketones and acids from lignin degradation by capillary zone electrophoresis

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Abstract

Capillary zone electrophoresis (CZE) was investigated for the separation of eleven phenolic aldehydes, ketones and acids which are main components of lignin. The influence of the buffer pH, electrolyte composition, acetonitrile amount, temperature and voltage has been investigated to determine the best separation conditions. Using optimized buffer [55 m*M* borate–phosphate (pH 9.1) /acetonitrile (91.5–8.5, v/v)], efficient separations occur in less than 15 min with a good repeatability of the electropherograms, giving a relative standard deviation of each peak of less than 1%. The method was applied to the analysis of phenolic compounds produced by the CuO oxidation of foliar organs of gymnosperm and angiosperm trees. Results are in agreement with data obtained by other chromatographic methods.

Keywords: Ketones; Aldehydes; Phenolic acids; Phenolic compounds; Lignin

1. Introduction

Capillary zone electrophoresis (CZE) is becoming a commonly applied analytical tool for the resolution of mixtures of ionic natural compounds. Extensive applications have been demonstrated for complex substances such as nucleotides, amino acids and other ionizable organic compounds. Various types of phenolic carboxylic acids also have been separated by CZE, which is well adapted to the ionic nature of the analyzed compounds [1–3] or by micellar electrokinetic capillary chromatography (MECC), which is generally used to resolve neutral molecules [4] or to enhance the separation of charged species [5,6].

The family of natural phenolic compounds is widespread in the vegetal kingdom [7]. The major occurrence of phenolic compounds deals with lignins that are polyphenolic biochemicals only present in vascular plants [8] and thus produced almost exclusively in land environments [9]. The various phenolic components of the lignin have been first applied to study the pathways of the lignin biosynthesis [10] and, as the substitution patterns may indicate specific plant sources [7,8], geochemical applications were found to elucidate the domain of the sedimentary organic matter. Thus the distribution of lignin monomers in plants [8,11] and in marine or lacustrine sediments [11–14] has been assessed to trace the

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planktonic or terrestrial origin of the sedimentary organic matter.

The analyses of phenolic compounds of plant, soil or sediment lignin requires preliminary oxidative hydrolysis with nitrobenzene [8] or CuO [11] followed by extraction of lignin-derived phenols with an organic solvent, generally ethyl ether. Techniques commonly used for the characterization of lignin-derived phenols are either gas capillary chromatography on fused-silica columns [15] or high-performance liquid chromatography [16,17].

Recent progress in capillary electrophoresis instrumentation encouraged us to test this technique for approaching the problem of the pertinence of lignin monomers as soil biomarkers specific for constrasting land-use environments. To this end, a method was developed for the collection of ketones, aldehydes and acids composing the lignin polymers or resulting from their partial degradation (Fig. 1). First assays were performed with MECC, however, the best and the easiest separations obtained were by CZE. The development of this latter method was achieved by studying the effects of electrolyte composition and physical parameters on the migration time, peak area, peak efficiency and resolution between various phenolic compounds.

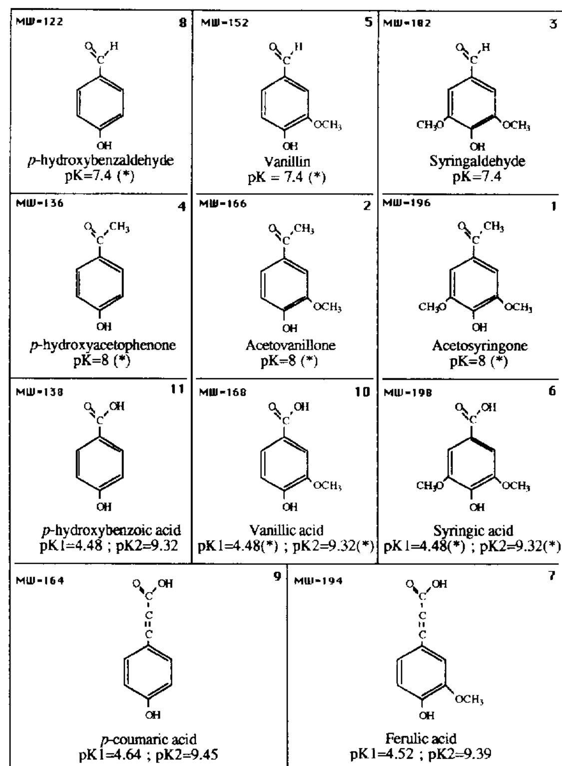

Fig. 1. Major phenolic constituents of lignin. The number at the top right refers to the peak of the corresponding molecule on following electropherogram.

2. Experimental

2.1. Samples and reagents

The investigated phenolic compounds are listed in Fig. 1 with some physico-chemical characteristics. Whereas the pK_a of the carboxylic groups of the phenolic acids can be found in the literature [18], those of the phenolic groups of ketones and aldehydes were computed from the Hammet correlation method which involves electronic and steric effects of structural moieties to predict the pK_a values of acid and base functions in organic molecules [19].

Pure phenolic compounds were purchased from Sigma or Aldrich (St. Louis, USA) and dissolved in methanol to obtain 0.01 *M* stock-solutions. Finally, standard solutions of the mixture of individual compounds in the 10×10^{-3} to 5×10^{-3} *M* concentration range were prepared after addition of 2,4,5-trimethoxybenzoic acid as an internal standard. Methanol has been used for the determination of the electroosmotic flow. Methanol and acetonitrile (analytical grade), used as solvent modifier, were purchased from Prolabo (Paris, France).

Stock solutions (0.1 *M*) of reagent-grade sodium tetraborate and sodium dihydrogen-phosphate (Aldrich) were prepared with water purified by reverse osmosis (Milli-Q system from Millipore, St. Quentin, France), then filtered through a 0.22-μm pore size filter (Millipore).

Electrolytes of different pH values were obtained by adding increasing volumes of 0.1 *M* sodium phosphate to 0.1 *M* sodium tetraborate solution up to the fixed pH. The buffer concentration varied from 30 to 65 m*M* to get the required pH (7.0–9.2). In experiments using acetonitrile and requiring pH 9.1, acetonitrile was added to 0.1 *M* sodium tetraborate solution before adding 0.1 *M* sodium phosphate to

get, finally, an electrolyte buffer–CH_3CN (91.5:8.5, v/v) mixture. All buffer solutions were filtered through a 0.22-μm filter.

2.2. Plant lignin hydrolysis

The extraction of compounds derived from vascular plant lignin was achieved according to the method described in Ref. [15], which involves the CuO oxidation of lignin. Twenty five milligrams of ground plant samples were placed in an airtight teflon vial protected by a steel bomb fitted with a screw cap. Cupric oxide (1 g), 50 to 100 mg of ammonium iron (II) sulfate hexahydrate [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$] and 7 ml of 8% hydroxide sodium were added to the sample, and the teflon vial and the bomb were sealed under nitrogen atmosphere in a glovebox.

The CuO oxidation was carried out by heating the bombs at 150°C for 4 h in an oven. After cooling and adding the internal standard, the content of the teflon vial was transferred to a 50-ml centrifuge tube and the vial was washed with 30 ml of 4% NaOH. Then, the washings and the reaction mixture were combined and centrifuged. The supernatant was saved and the sediment was washed with 10 ml of 4% NaOH. The combined basic supernatants and washings were acidified to pH 1 with 6 *M* HCl and extracted for three successive times with ethyl ether previously treated with a saturated solution of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ to remove peroxides [15]. After evaporation of the ethyl ether, the solid residue was dissolved in 5 ml of methanol. The methanolic solution was filtered through a 0.22-μm filter and kept in closed vials, under N_2, at 4°C in darkness, to avoid the oxidation of aldehydes [8] and the isomerization of ferulic and *p*-coumarique acids [20].

2.3. Apparatus

Phenol separations were carried out on a P/ACE 5510 system (Beckman, Fullerton, CA, USA) using PC GOLD-3M40 software (Beckman) and equipped with a fused-silica capillary (57 cm×50 mm). UV detection was performed at 214 nm through the capillary at 50 cm from the inlet. Samples were introduced at the anodic end of the capillary by hydrodynamic injection during 1 s.

The capillary was daily conditioned by washing first with 1 *M* NaOH (5 min), followed by 0.1 *M* NaOH (5 min), deionized water (5 min) and finally with the electrophoretic buffer (5 min). Good reproducibility of retention times was observed after washing the capillary with 0.1 *M* NaOH (2 min), deionized water (2 min) and buffer (2 min) between consecutive analyses. In CZE, the electrophoretic mobility [$m_{ep}(i)$] of solute *i* has been calculated from the following relationship:

$$m_{ep}(i) = \frac{L_d \cdot L_t}{V} \left(\frac{1}{t_m(i)} - \frac{1}{t_o} \right) \qquad (1)$$

where L_d and L_t are the length of the capillary from the inlet to the detector and the total length of the capillary (cm), respectively, V is the applied voltage (volts), $t_m(i)$ is the migration time (seconds) of the solute i and t_o the migration time of the electroosmotic flow (methanol).

The resolution (R_s) between two consecutive peaks has been calculated using the equation:

$$R_s = 2\left(\frac{t_{m2} - t_{m1}}{w_{b2} - w_{b1}} \right) \qquad (2)$$

where t_{m2} and t_{m1} are the migration times and w_{b2}, w_{b1} the base peak widths of the two solutes 2 and 1, respectively.

In contrast to chromatographic methods, solutes have different migration velocities and consequently different residence times in the detection region. We must correct for velocities by dividing integrated peak area by migration time to get the normalized peak area $A(i)$ of the solute i. Relative normalized peak areas [$RA(i)$] of the solute i was calculated from the normalized peak area [$A(i)$] and from that of the internal standard [$A(\text{I.S.})$] according to the relationship:

$$RA(i) = \frac{A(i)}{A(\text{I.S.})} \qquad (3)$$

3. Results and discussion

3.1. Method optimisation

3.1.1. Influence of buffer pH

Fig. 2 shows the electrophoretic mobility of the phenolic compounds as a function of the borate–phosphate buffer pH. The solute groups corre-

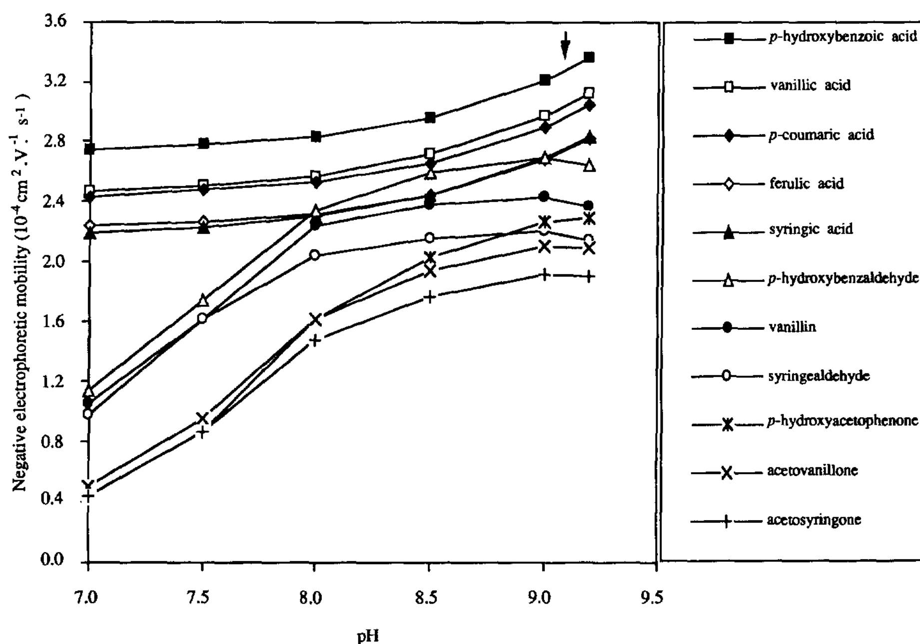

Fig. 2. Influence of buffer pH upon the electrophoretic mobility of several phenolic compounds. Separation conditions: silica capillary: 57 cm×50 μm; temperature: 25°C; voltage: +30 kV; buffer: borate–phosphate; UV detection at 214 nm; hydrodynamic injection: 1 s; sample: methanolic solution containing 0.5 m*M* of each compound.

sponding to acids, aldehydes and ketones exhibit different behaviours. At pH 7, acids (syringic, vanillic, *p*-hydroxybenzoic, ferulic and *p*-coumaric acids) show high mobility values due to the ionization of the acid groups. However, at higher pH values, the electrophoretic mobilities of aldehydes (syringealdehyde, vanillin and *p*-hydroxybenzaldehyde) and ketones (acetosyringone, acetovanillone and *p*-hydroxyacetophenone) strongly increased with buffer pH because of the ionization of phenol groups. At last, the electrophoretic mobility of aldehydes is rather constant for pH values higher than 8, whereas for ketones an apparent stability is reached at pH values close to 9. This fact depicts the difference of phenol group pK_a values between phenolic acids, aldehydes and ketones as reported in Fig. 1. We selected to work at pH 9.1 because *p*-hydroxybenzaldehyde was separated from syringic and ferulic acids and because syringealdehyde and *p*-hydroxyacetophenone were better separated.

3.1.2. Influence of buffer ionic strength

As expected, the electrophoretic mobility of each phenolic compound slightly increases with increasing ionic strength (from 20 m*M* to 70 m*M*) at constant pH value (pH 9.1). Fig. 3 reports the effect of buffer ionic strength on the resolution of the two phenolic compound couples rather difficult to resolve. At low borate–phosphate concentration (20 and 30 m*M*), these two couples of compounds, i.e. 2,4,5-trimethoxybenzoic acid–acetovanillone and syringealdehyde–*p*-hydroxyacetophenone comigrated, they could be resolved with 55 m*M* buffer concentration.

In contrast, the resolution values between two consecutive compounds were always higher than 2.0 for a borate–phosphate concentration higher than 55 m*M* (Table 1). However, working at higher borate–phosphate concentration may induce too high Joule heating, and we decided to work with 55 m*M* buffer concentration. For these experimental conditions, the peak efficiencies varied from 115 000 to 200 000 theoretical plates for *p*-hydroxybenzoic acid and *p*-hydroxyacetophenone, respectively (Table 1).

3.1.3. Effect of organic modifiers

The effects of organic solvent added to the buffer on the resolution were evaluated with 55 m*M*

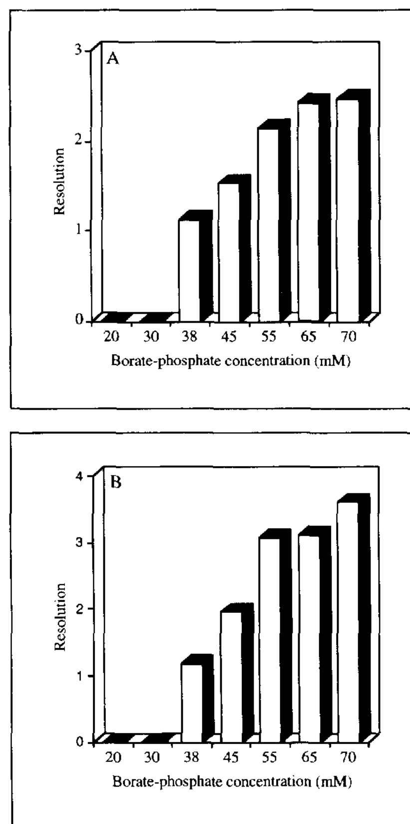

Fig. 3. Influence of the buffer ionic strength on the resolution: (A) 2,4,5 trimethoxybenzoic acid–acetovanillone, (B) syringealdehyde–*p*-hydroxyacetophenone. Separation conditions as in Table 1.

borate–phosphate buffer (pH 9.1). Acetonitrile was preferred to isopropanol to reduce electroosmotic flow. The electrophoretic mobility of phenolic acids decreases more than that of other phenolic compounds at pH 9.1 with an increase in CH_3CN amount. Without acetonitrile, *p*-hydroxybenzaldehyde, syringic acid and ferulic acid have the same migration time. For a 10% CH_3CN concentration, these two acids are well separated (R_s = 1.99), whereas *p*-hydroxybenzaldehyde and *p*-coumaric acid are not totally resolved (R_s = 1.59). Nice separations were achieved with 7.5 or 8.5% acetonitrile added to the aqueous buffer. In these conditions, resolutions between two consecutive peaks are always higher than 2.0 (Table 2). We chose to work with 8.5% of acetonitrile because only three couples have resolutions ranging from 2 to 3, instead of five couples with 7.5%.

3.1.4. Effect of applied voltage and temperature

The effect of voltage upon resolution has been studied using optimum buffer composition. An increasing voltage between +10 and +30 kV results in a shorter analysis time and an improvement of the efficiency. At +30 kV, the overall resolution values do not significantly change from 20 to 30°C, whereas the analysis time at 30°C is shorter (20%) than at 20°C. However, heat might not be totally dissipated from the capillary at 30°C and the separation might be altered by the probable evaporation of the organic modifier (acetonitrile). So, the separation has been achieved at +30 kV and 25°C.

Fig. 4 illustrates the separation of a standard mixture of eleven phenolic compounds with 2,4,5-trimethoxybenzoic acid as internal standard (peak I.S.) under optimized experimental conditions [55 m*M* borate–phosphate (pH 9.1)–acetonitrile (91.5:8.5, v/v)].

3.1.5. Quantitative study

Ten consecutive injections of a standard mixture of phenolic compounds were made to evaluate the repeatability of migration times and normalized peak areas of each compound. The concentration of each phenolic compound was 0.5 m*M*.

Table 3 reports for each compound the relative standard deviation (%) which is the ratio of standard deviation over mean value. The repeatability of the migration times of each compound is acceptable since the relative standard deviation is always less than 0.8%, with a mean value of 0.45%. The relative standard deviation value of the corrected area of each peak is less than 4% except for two compounds [acetovanillone (8.3%) and *p*-hydroxyacetophenone (7.0%)]. The highest value (8.3%) of the relative standard deviation for acetovanillone may be explained by the moderate resolution (R_s=2.15) between the internal standard and this solute (Fig. 4). No obvious reason seems to explain the high relative standard deviation (7.0%) of the *p*-hydroxyacetophenone peak.

As summarized in Table 3, the relative standard deviations were (i) slightly improved by using relative normalized peak areas (2.5%) rather than normalized peak areas (2.9%), and (ii) much better than peak area (4.7%). Thus, these parameters define a good repeatability of the measurements.

The calibration curves in the 0.0625–1.25 m*M*

Table 1
Variation of resolution (Part A) and peak efficiency (Part B) as a function of the borate–phosphate buffer concentration

	Borate–phospate concentration (mM)						
	20	30	38	45	55	65	70
Part A: Resolution							
Acetosyringone/internal standard	4.11	5.20	5.50	5.12	4.32	5.56	6.34
Internal standard/acetovanillone	0.00	0.00	1.12	1.53	2.15	2.42	2.47
Acetovanillone/syringaldehyde	2.82	3.40	4.83	4.15	3.42	4.45	4.93
Syringaldehyde/*p*-hydroxyacetophenone	0.00	0.00	1.18	1.96	3.08	3.11	3.61
p-Hydroxyacetophenone/vanillin	5.29	7.22	6.99	7.03	6.27	7.65	8.61
Vanillin/syringic acid	1.37	1.18	2.33	3.28	4.75	4.45	4.11
Syringic acid/ferulic acid	1.16	1.10	1.53	1.85	2.14	2.29	2.72
Ferulic acid/*p*-hydroxybenzaldehyde	2.52	2.97	3.31	3.38	3.12	3.71	4.27
p-Hydroxybenzaldehyde/*p*-coumaric acid	1.30	0.84	2.27	3.18	5.11	4.23	3.57
p-Coumaric acid/vanillic acid	1.39	1.58	1.79	1.95	2.02	2.16	2.14
Vanillic acid/*p*-hydroxybenzoic acid	0.00	8.42	10.20	11.32	13.33	13.57	14.80
Overall resolution ($\times 10^6$)	0.00	0.00	0.12	0.64	2.67	6.67	12.96
Part B: Efficiency							
No.							
1 Acetosyringone	153 415	198 670	185 915	197 750	197 125	194 980	186 110
I.S. Internal standard	125 280	127 020	230 310	174 770	179 280	196 630	177 780
2 Acetovanillone	N.d.	N.d.	293 080	179 685	195 190	196 415	188 720
3 Syringaldehyde	N.d.	N.d.	204 060	189 380	175 115	192 955	185 855
4 *p*-Hydroxyacetophenone	N.d.	N.d.	158 475	167 550	201 845	179 715	173 035
5 Vanillin	211 805	203 320	179 585	189 175	180 935	181 515	166 225
6 Syringic acid	255 930	221 310	176 130	187 410	190 750	170 320	172 415
7 Ferulic acid	316 480	150 135	145 430	150 325	173 285	165 985	170 465
8 *p*-Hydroxybenzaldehyde	210 090	146 895	177 065	185 810	172 335	204 495	136 305
9 *p*-Coumaric acid	171 625	176 830	171 440	182 460	173 575	170 730	148 275
10 Vanillic acid	152 625	146 615	158 440	156 880	155 285	157 705	128 730
11 *p*-Hydroxybenzoic acid	148 580	142 215	139 940	138 735	114 545	154 335	99 180

Separation conditions: silica capillary: 57 cm×50 mm; temperature: 25°C; voltage: +30 kV; buffer: borate–phosphate (pH 9.1)/acetonitrile (91.5:8.5, v/v); UV detection at 214 nm; hydrodynamic injection: 1s; sample: methanolic solution containing 0.5 mM of each compound.

Table 2
Variation of resolution as a function of acetonitrile amount (% v/v) added to the 55 mM borate–phosphate buffer (pH 9.1)

	Acetonitrile amount (% v/v)					
	0	2.5	5.0	7.5	8.5	10.0
Acetosyringone/internal standard	2.48	3.88	4.34	5.51	4.32	6.69
Internal standard/acetovanillone	0.00	2.99	2.57	2.08	2.15	1.10
Acetovanillone/syringaldehyde	2.74	3.30	3.68	4.34	3.42	4.69
Syringaldehyde/*p*-hydroxyacetophenone	3.63	3.20	2.90	2.64	3.08	1.82
p-Hydroxyacetophenone/vanillin	4.81	5.33	4.94	7.56	6.27	8.49
Vanillin/syringic acid	0.00	7.33	5.35	4.30	4.75	1.88
Syringic acid/ferulic acid	0.00	1.69	1.84	2.09	2.14	1.99
Ferulic acid/*p*-hydroxybenzaldehyde	0.00	0.52	1.19	2.86	3.12	4.47
p-Hydroxybenzaldehyde/*p*-coumaric acid	20.16	6.56	5.50	3.92	5.11	1.59
p-Coumaric acid/vanillic acid	3.48	2.62	2.39	2.15	2.02	1.96
Vanillic acid/*p*-hydroxybenzoic acid	10.69	11.89	12.32	12.75	13.33	12.67
Overall resolution ($\times 10^6$)	0.00	0.86	1.11	2.74	2.67	0.35

Separation conditions as in Table 1 except for acetonitrile amount.

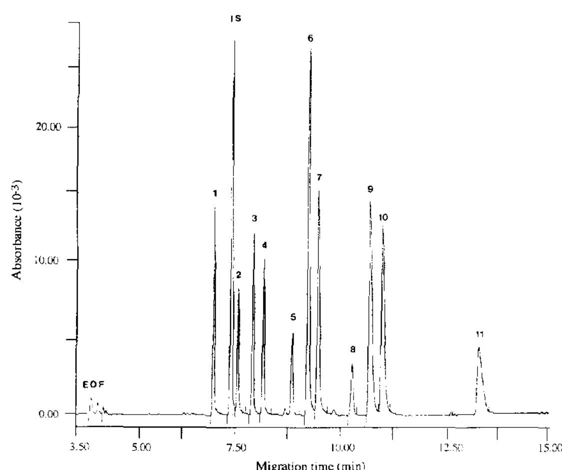

Fig. 4. Separation of a standard mixture of eleven phenolic compounds by CZE under optimized conditions. Separation conditions: silica capillary: 57 cm×50 μm; temperature: 25°C; voltage: +30 kV; buffer: 55 m*M* borate–phosphate (pH 9.1)–acetonitrile (91.5:8.5, v/v); UV detection at 214 nm; hydrodynamic injection: 1 s; sample: methanolic solution containing 0.5 m*M* of each compound. Peak assignement: 1 = acetosyringone, IS = internal standard, 2 = acetovanillone, 3 = syringealdehyde, 4 = *p*-hydroxyacetophenone, 5 = vanillin, 6 = syringic acid, 7 = ferulic acid, 8 = *p*-hydroxybenzaldehyde, 9 = *p*-coumaric acid, 10 = vanillic acid, 11 = *p*-hydroxybenzoic acid.

concentration range were performed for each phenolic compound under optimized conditions. So, in such electrophoretic conditions, the normalized peak areas vary linearly with the concentration of the phenolic compound and the correlation coefficient was always greater than 0.992 for each compound (Table 4).

Table 4
Parameters of linear calibration curves (correlation coefficient and slope) for eleven phenolic compounds

No.	Phenolic compound	Correlation coefficient	Slope (mM^{-1})
1	Acetosyringone	0.999	3.384
2	Acetovanillone	0.999	2.504
3	Syringealdehyde	0.998	2.290
4	*p*-Hydroxyacetophenone	0.996	1.445
5	Vanillin	0.993	1.577
6	Syringic acid	1.000	5.458
7	Ferulic acid	0.995	4.504
8	*p*-Hydroxybenzaldehyde	0.992	0.602
9	*p*-Coumaric acid	0.999	2.840
10	Vanillic acid	0.998	3.965
11	*p*-Hydroxybenzoic acid	0.994	1.715

3.2. Application to lignin monomers

Two natural samples were chosen to evaluate the performance of this analytical method. Fig. 5 shows the electrophoregrams of lignin monomers produced by CuO oxidation of green needles from *Pseudotsuga douglasei* (Fig. 5A) and of brown senescent leaves of *Fagus sylvatica* (Fig. 5B).

Table 3
Relative standard deviation of migration times, peak areas (normalized and relative normalized)

No.	Phenolic compound	Relative standard deviation (%)			
		Migration time	Area	Normalized area	Relative normalized area
1	Acetosyringone	0.46	7.7	3.4	2.1
I.S.	Internal standard	0.53	2.5	2.3	0.0
2	Acetovanillone	0.35	8.8	8.3	7.5
3	Syringealdehyde	0.23	5.0	3.8	3.4
4	*p*-Hydroxyacetophenone	0.76	8.8	7.0	6.7
5	Vanillin	0.68	4.6	3.6	3.3
6	Syringic acid	0.46	4.1	2.3	2.3
7	Ferulic acid	0.58	4.6	2.8	2.7
8	*p*-Hydroxybenzaldehyde	0.38	3.2	1.9	1.9
9	*p*-Coumaric acid	0.39	3.6	2.8	2.7
10	Vanillic acid	0.40	7.9	3.0	2.9
11	*p*-Hydroxybenzoic acid	0.50	4.4	3.2	1.8
	Mean value	0.45	4.7	2.9	2.5

Separation conditions as in Fig. 4.

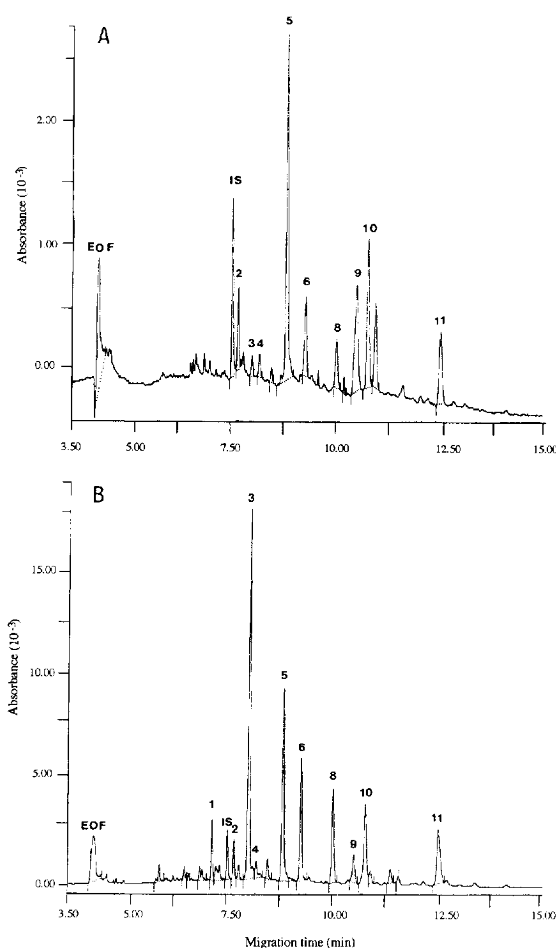

Fig. 5. Capillary electropherogram of CuO reaction products of lignin in *Pseudotsuga douglasei* needles (A) and *Fagus sylvatica* brown leaves (B). Separation conditions and peak assignement as in Fig. 4.

The electrophoregram of phenolic compounds deriving from *P. douglasei* shows a large predominance of vanillyl units over other phenols (Fig. 5A and Table 5). Cinnamyl (ferulic and *p*-coumaric acids) and syringyl phenols are obtained only in small amounts, as is well known for gymnosperm tissues [8–11], especially for fresh foliar materials [11]. Vanillin dominates over vanillic acid and acetovanillone. Confirming previous analyses [21], the aldehydic compound was found to be the equivalent of 65% by weight of the total vanillyl units. Laboratory tests demonstrated that the lower detection limit for syringic acid was 6.4×10^{-6} mol l^{-1} corresponding to a 1-s injection of 25 μg g^{-1} of a natural sample.

Table 5
Distribution of the four main phenolic units of lignin in green needles of *Pseudotsuga douglasei* (tsuga) and senescent leaves of *Fagus sylvatica* (beech)

Plant	Phenolic unit of lignin (% of total phenols)				V_{al}/V
	V	S	H	C	
Pseudotsuga douglasei	57	9	24	8	0.65
Fagus sylvatica	34	36	21	6	0.76

V: vanillic unit; S: syringic unit; C: ferulic and *p*-coumaric acids; H: *p*-hydroxybenzoic unit. V_{al}/V: vanillin over vanillic unit.

Fig. 5B shows the electropherogram of leaf litter of *Fagus sylvatica*. In agreement with the literature on angiosperm lignin [8], our results (Table 5) demonstrate that syringyl units are present in similar amounts as vanillyl units (39 and 34% by weight of the total phenols, respectively) and largely dominate the cinnamyl phenols.

4. Conclusions

The described CZE method allows fast, easy and inexpensive determinations of various phenolic compounds (ketones, aldehydes and acids). These compounds are generally those found in methanolic extracts of lignin degradation and CuO oxidation experiments. It seems therefore very easy and convenient to analyze crude extracts by CZE. Using optimum separation conditions [55 m*M* borate–phosphate (pH 9.1)–CH_3CN (91.5:8.5, v/v)], the analysis of the phenolic mixtures takes less than 15 min. Relative normalized peak areas must be employed to improve repeatability and linearity.

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