Capillary Zone Electrophoresis of Coniferyl Alcohol Oxidation Products

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Capillary zone electrophoresis (CZE) was developed for the quantitative determination of dimers obtained by horseradish peroxidase-catalyzed oxidation of coniferyl alcohol. The influence of pH, electrolyte concentration, applied voltage, and temperature on CZE performance was investigated, resulting in an efficient and rapid separation. Coniferyl alcohol-derived dimers were directly analyzed from their reaction mixtures, without any extraction or derivatization step. In addition, these dimers were analyzed within 14 min, a substantially shorter time than is required for the HPLC method or the conventional capillary gas chromatography of their silylated derivatives. Standard deviations between injection replicates were in the 0.4−0.7% range for migration times and in the 1.8−5.1% range for relative normalized peak areas. The method could therefore be successfully applied to follow the peroxidase-catalyzed oxidation of coniferyl alcohol.

Keywords: *Capillary electrophoresis; coniferyl alcohol; dehydropolymerization; horseradish peroxidase*

INTRODUCTION

Peroxidases (EC 1.11.1.7) catalyze the oxidation of various organic compounds using hydrogen peroxide as the ultimate electron acceptor (*1*). They are involved in various metabolic steps such as auxin metabolism (*2*), cross-linking of cell wall polymers by isodityrosine (*3*) or diferulic (*4*) bridges, and oxidation of *p*-hydroxycinnamyl alcohols (monolignols) prior to their polymerization during lignin and suberin formation (*5*, *6*). This polymerization, proceeding by coupling of phenoxy radicals, is currently viewed as a process without strict enzymatic control over the distribution of structural units. In the 1950s, Freudenberg and co-workers studied monolignol coupling mechanisms in vitro using various oxidative enzymes as polymerization initiators (*7*). The in vitro oxidation of coniferyl alcohol (**1**) with H_2O_2 in the presence of a peroxidase gives a dehydrogenative polymerisate with some similarities to gymnosperm lignin (*8*). In addition, some dehydrodimers are obtained, which are representatives of the main interunit bonds formed by in vitro oxidation of coniferyl alcohol. These are (±)-dehydrodiconiferyl alcohol (**2**), (±)-guaiacylglycerol *β*-*O*-4 coniferyl alcohol ether (**3**), and (±)-pinoresinol (**4**) (Figure 1). These dehydrodimers are usually extracted and then analyzed by HPLC or by GC-MS (*9*). Their determination is essential for a better understanding of the coupling mechanisms in order to highlight the physicochemical factors affecting the structure of natural or synthetic lignins.

During the past decade, capillary zone electrophoresis (CZE) has become a suitable method for the separation of charged and uncharged species (*10*−*14*). It offers the possibility of rapid quantitative and automated analysis of complex mixtures with high accuracy and relatively short analysis times. These properties prompted us to examine the potential of CZE for the analysis of coniferyl alcohol and its main dehydrodimers obtained by peroxidase-catalyzed oxidation. In the present study, we optimized this analysis with regard to electrolyte solution pH, electrolyte concentration, temperature, and voltage.

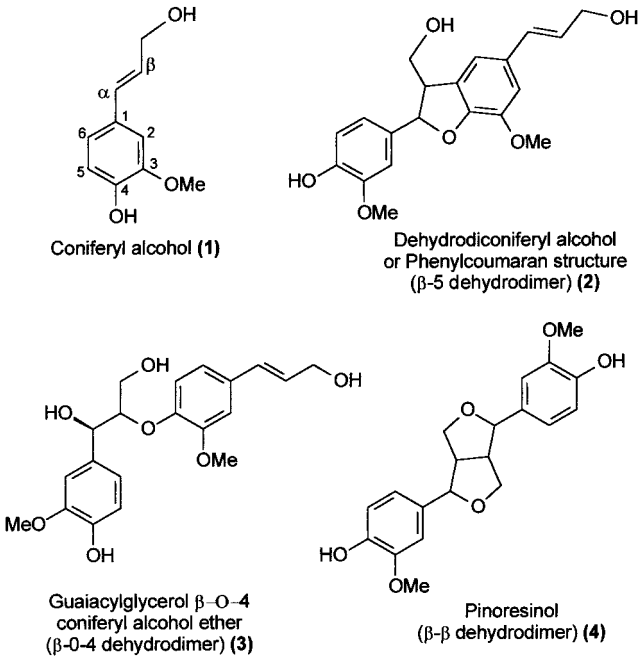

Figure 1. Structures of coniferyl alcohol and its main dimers obtained by peroxidase-catalyzed oxidation. Dimer **3** occurs as two (*erythro*/*threo*) diastereoisomers.

MATERIALS AND METHODS

Chemicals. All reagents were commercial products: coniferyl alcohol (Aldrich Chemical Co., Milwaukee, WI), horseradish peroxidase type II (200 purpurogallin units/mg of solid; Sigma Chemical Co., St. Louis, MO), 3,4,5-trimethoxybenzoic acid (Fluka, Buchs, Switzerland), hydrogen peroxide (35wt %

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solution; Acros Organics). Electrolyte solutions were prepared by dissolving boric acid and sodium dihydrogenphosphate (analytical reagents; Prolabo) in ultrapure water with a conductivity of 18 MΩ from a MilliQ system (Millipore, Bedford, MA) to a final concentration of C mM in phosphate and 2 × C mM in boric acid, with C varying between 8.3 and 50. A solution of 10 N NaOH was used to adjust the pH of the electrolyte solutions.

Oxidative Coupling of Coniferyl Alcohol. All of the oxidative coupling experiments run just prior to CZE were carried out at a microscale level according to a modified version of Wallace and Fry's method (*15*). An internal standard of 2 mM 3,4,5-trimethoxybenzoic acid (100 μL) in 25 mM NaH_2PO_4−Na_2HPO_4, pH 6, buffer was added to 200 μL of 2 mM coniferyl alcohol in the same buffer. A solution of 4 mM hydrogen peroxide (100 μL) in ultrapure water was then added before the addition of 200 μL of a 50 ng/mL solution of horseradish peroxidase type II in the 25 mM NaH_2PO_4−Na_2HPO_4, pH 6, buffer. A reference was made by replacing the peroxidase solution by 25 mM NaH_2PO_4−Na_2HPO_4, pH 6, buffer. Mixtures were kept at 30 °C for 30 min, and the oxidation reaction (600 μL) was then stopped by adding 1 reaction volume (600 μL) of MeOH and by placing mixtures in an ice bath prior to CZE.

Preparation of Dimer Standards. For the preparation of dimer standards, an oxidative coupling of coniferyl alcohol was performed as described above, but using 20 times larger reagent volumes. The final oxidation reaction (12 mL) was acidified with 6 N HCl to pH 2−3 before extraction with methylene chloride (1 × 50 mL, then 1 × 20 mL). The organic extract was dried over Na_2SO_4 and evaporated to dryness. Extracted oxidation products were redissolved in about 1 mL of methylene chloride and stored at 4 °C before thin-layer chromatography (TLC). These products (200 μL) were then spotted as an 8 cm line on TLC aluminum sheets precoated with silica gel 60 F_{254} (Merck). The sheets were developed with a chloroform/methanol mixture (9/1 v/v) according to the procedure of Okusa et al. (*16*). The compounds were detected as dark spots when illuminated under UV light (254 nm). They were scraped from the sheet and eluted with an AcOEt/CH_2Cl_2 mixture (1/1 v/v). The recovered organic extracts were washed with water slightly acidified with HCl, dried over Na_2SO_4, and evaporated to dryness. Residues were dissolved in methylene chloride (about 1 mL) prior to GC-MS analyses, then re-evaporated to dryness and redissolved in a solution of 25 mM NaH_2PO_4−Na_2HPO_4 pH 6 buffer and methanol (1/1 v/v) prior to capillary electrophoresis.

For identification by GC-MS, extracted oxidation products in methylene chloride (10 μL) were trimethylsilylated in a 200 μL silylation vial with 50 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 5 μL of GC grade pyridine for 1 h at room temperature. The silylated solution (2 μL) was then injected onto a poly(dimethylsiloxane) capillary column (SPB1, Supelco, 30 m × 0.25 mm i.d., 0.25 μm film thickness), with the temperature program mode held at 40 °C for 1 min, then raised to 180 °C at 30 °C/min, and finally to 260 °C at 2 °C/min, with helium as the carrier gas and with a splitless injector (260 °C; valve closed for 1 min). The GC3400 gas chromatographic system was coupled to a SATURN2000 ion trap mass spectrometer (Varian, Les Ulis, France) operating in the electron impact positive ion mode (70 eV), with an interface at 250 °C and a scanning range of m/z 50−650. Molecular ions [except that of trimethylsilylated dimers (**3**) due to the spectrometer limitation in m/z upper value] and prominent fragments of the different trimethylsilylated dimers were as follows: β-5 dimer (**2**), 574 (20), 484 (100), 454 (20), 394 (15), 103 (10), 73 (100); β-*O*-4 dimer (**3**), 324 (30), 297 (100), 209 (10), 103 (10), 73 (70); β-β dimer (**4**), 502 (100), 487 (25), 237 (55), 223 (100), 209 (55), 193 (40), 131 (25), 73 (100). Note that dimer **3** gave a single spot on TLC, whereas GC-MS analysis of the product revealed that it consisted of *erythro* and *threo* diastereoisomers.

Capillary Electrophoresis. Separations were carried out on a P/ACE 5000 system (Beckman) equipped with a fused-silica capillary (57 cm × 50 μm i.d.). The analyte zones were detected with on-capillary UV detection (50 cm from the inlet; 50 μm × 200 μm aperture) at 214 nm. Samples were introduced at the anodic end of the capillary by hydrodynamic injection during 2 or 5 s.

The capillary was daily conditioned by washing first with 0.1 M NaOH (10 min), followed by deionized water (10 min) and finally with the electrolyte solution (10 min). A good reproducibility of migration times was achieved by washing the capillary with 0.1 M NaOH (1 min), deionized water (1 min), and electrolyte solution (1 min) between consecutive analyses. All NaOH and electrolyte solutions as well as reaction mixtures were filtered through a 0.22-μm filter (Millex-GV, Millipore) prior to CZE analyses.

The electrophoretic mobility $\mu(i)$ (cm^2/V/s) of the solute i was calculated from eq 1

$$\mu(i) = \frac{L_d L_t}{V}\left(\frac{1}{t_m(i)} - \frac{1}{t_m(\mathrm{EOF})}\right) \quad (1)$$

with L_d and L_t the length (cm) of the capillary from the inlet to the detector (L_d) and the total length of the capillary (L_t), V the applied voltage (V), $t_m(i)$ the migration time (s) of the solute i, and t_m(EOF) the migration time of the electroosmotic flow (MeOH).

Electrophoretic mobility $\mu(i)$ of an ion can also be expressed by the Debye−Huckel−Henry theory (*17*)

$$\mu(i) = \frac{q}{6\pi r\eta} \quad (2)$$

where q is the charge on the particle, r is the Stokes' radius of the analyte, and η denotes the viscosity of the background electrolyte. In the employed configuration, $\mu(i)$ values are negative for anions. Therefore, for separation of a hypothetical mixture of ions having different charges and sizes, the smaller and more highly charged anions will be detected last because ion apparent mobility is given by eq 3:

$$\mu_{app}(i) = \mu(i) + \mu(\mathrm{EOF}) \quad (3)$$

Resolution R_s between two consecutive peaks was calculated using eq 4

$$R_s = 2\left[\frac{t_m(j) - t_m(i)}{w(j) + w(i)}\right] \quad (4)$$

with $t_m(i)$ and $t_m(j)$ the migration times (s) of the solutes i and j and $w(i)$ and $w(j)$ the base peak widths (s) of the solutes i and j. Two consecutive peaks are resolved when $R_s > 1$.

The number of theoretical plates N was calculated using eq 5:

$$N = 16\left[\frac{t_m(i)}{w(i)}\right]^2 \quad (5)$$

Due to different migration velocities and therefore different residence times in the detection aperture, peak areas were normalized by dividing each integrated peak area $A(i)$ by the migration time $t_m(i)$ corresponding to the solute i (*17*):

$$\mathrm{NA}(i) = A(i)/t_m(i) \quad (6)$$

Relative normalized peak areas RNA(i) were calculated from the normalized peak areas NA(i) and from that of the internal standard NA(IS):

$$\mathrm{RNA}(i) = \mathrm{NA}(i)/\mathrm{NA}(\mathrm{IS}) \quad (7)$$

RESULTS AND DISCUSSION

Optimization of Electrophoresis Conditions. The electrolyte solution used was a borate−phosphate solution because both borate and phosphate have a low UV-background signal that allows detection at 214 nm.

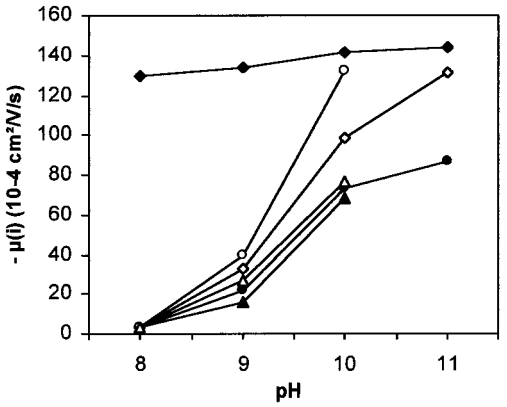

Figure 2. Influence of electrolyte solution pH upon the electrophoretic mobility of internal 3,4,5-trimethoxybenzoic acid standard (◆), coniferyl alcohol (**1**) (◇), *β*-5 dimer (**2**) (●), *β*-*β* dimer (**4**) (○), and *erythro* (▲) and *threo* (△) forms of *β*-*O*-4 dimer (**3**). Separation conditions: fused-silica capillary, 57 cm × 50 *μ*m; temperature, 25 °C; voltage, 30 kV; electrolyte solution, 150 mM borate−phosphate (e.g., C = 50 mM as described under Materials and Methods); UV detection at 214 nm; hydrodynamic injection, 2 s. The resulting currents were 95, 123, 170, and 205 *μ*A at pH 8, 9, 10, and 11, respectively. For compound structures, see Figure 1.

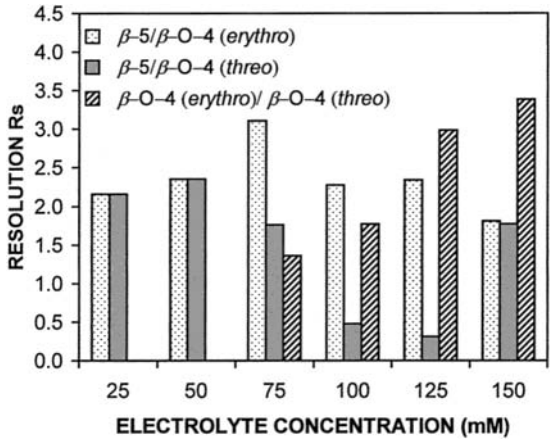

Figure 3. Influence of electrolyte concentration upon the resolution of the *β*-5 dimer (**2**) and *β*-*O*-4 dimers (**3**) (*erythro* and *threo* forms) outlined in Figure 1. Separation conditions: fused-silica capillary, 57 cm × 50 *μ*m; temperature, 25 °C; voltage, 30 kV; electrolyte solution, borate−phosphate, pH 10; UV detection at 214 nm; hydrodynamic injection, 5 s.

Borate−phosphate solutions have previously been used for capillary electrophoresis of natural products such as flavonoids in black tea or phenolic compounds in alcoholic beverages (*10*). Figure 2 shows the electrophoretic mobility $\mu(i)$ of coniferyl alcohol, 3,4,5-trimethoxybenzoic acid (internal standard), and the four major dimers as a function of the borate−phosphate solution pH. The different peaks were identified with pure internal standard and coniferyl alcohol solutions and with the dimer standards previously prepared. Identification of both *β*-*O*-4 diastereoisomers was deduced from their relative electrophoretic mobilities and from the literature. On the basis of eq 2 and using a tridimensional chemical structure software (data not shown), the larger *erythro* form was assumed to elute first. This was in accordance with the results of Okusa and co-workers (*16*), who observed by ^{13}C NMR that the *β*-*O*-4 product consisted of *erythro* and *threo* diastereisomers in a molar ratio of about 2:1, which corresponds to the area ratio observed between the first and second peaks of the *β*-*O*-4 compound (data not shown).

Coniferyl alcohol and oxidation products were not separated at pH 8 because these compounds exhibited no electrophoretic mobility. This is probably due to the fact that more alkaline conditions are required to deprotonate the phenolic groups of these compounds. The pK_a value of coniferyl alcohol (**1**) and the pK_{a1} and pK_{a2} values of pinoresinol (**4**) have recently been reported to be 9.53, 9.01, and 9.76, respectively (*18*). On this basis and not unexpectedly, a satisfactory separation of these compounds could still not be achieved at pH 9. Electrophoretic mobilities of the *β*-5 and *β*-*O*-4 dimers could not be determined at pH 11 because the low signal to noise ratio observed with the high corresponding current (205 *μ*A) impeded their detection. Finally, a borate−phosphate, pH 10, solution was shown to give the best separation even though the *β*-5 dimer (**2**) and *β*-*O*-4 diastereoisomers (**3**) still had very close $\mu(i)$ values.

The influence of electrolyte concentration (from 25 to 150 mM) was then investigated to improve the separation of these three compounds. As shown in Figure 3, *β*-*O*-4 diastereoisomers **(3)** were not resolved at low borate−phosphate concentrations (25 and 50 mM). At 100 and 125 mM borate−phosphate concentrations, the *β*-5 dimer and the *threo*-*β*-*O*-4 diastereoisomer were also not completely resolved. Only the 75 and 150 mM levels gave satisfactory R_s values (>1) for all compound pairs.

The effect of voltage upon $\mu(i)$ values and resolution R_s of the three products with close migration times [*β*-5 dimer (**2**) and *β*-*O*-4 diastereoisomers (**3**)] was studied using both 75 and 150 mM borate−phosphate solutions at pH 10. Both the electrophoretic mobilities of each compound and the electroosmotic flow slightly decreased when the applied voltage was reduced, so that a lower applied voltage did not significantly improve R_s values (data not shown). Nevertheless, an applied voltage of 20 kV with the 150 mM borate−phosphate electrolyte solution led to R_s values systematically >1.8 (versus 1.3 at 75 mM concentration). This was due to the numbers of theoretical plates obtained with the 150 mM electrolyte concentration at 20 kV, which were higher (from 190000 to 260000) than those obtained with the 75 mM concentration (from 70000 to 230000).

With respect to temperature, we observed that a run at 25 °C led to the best separation. Higher temperatures gave higher resulting currents and lower signal to noise ratios, without any improvement in the number of theoretical plates and, above all, with a dramatically decreased resolution of *β*-5 dimer (**2**) and *threo*-*β*-*O*-4 dimer (**3**) (data not shown). In conclusion, the best conditions for the CZE of the peroxidase-catalyzed oxidation products of coniferyl alcohol were found to be a 150 mM borate/phosphate, pH 10, electrolyte solution, an applied voltage of 20 kV, and a temperature of 25 °C. With these conditions, the resulting current was 93 *μ*A and the analysis time was 14 min (Figure 4).

The different migration times observed for the electropherogram in Figure 4 can be evaluated on the basis of eqs 2 and 3. At pH 10, the internal 3,4,5-trimethoxybenzoic acid standard was detected last because this compound is both small and singly charged. The singly charged coniferyl alcohol (**1**) was detected after the singly charged *β*-5 dimer (**2**) and *erythro* and *threo* forms of *β*-*O*-4 dimers (**3**) because of its lower radius. Finally, although the *β*-*β* dimer (**4**) is a larger molecule than coniferyl alcohol, it eluted later because of its two ionized phenolic groups.

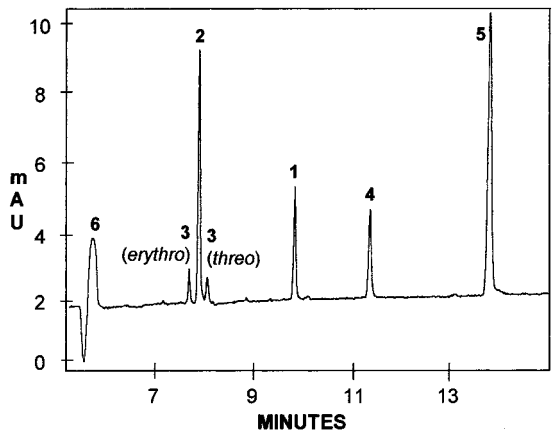

Figure 4. CZE analysis of peroxidase-catalyzed oxidation products of coniferyl alcohol: residual coniferyl alcohol (**1**); β-5 dimer (**2**); *erythro*- and *threo*-β-*O*-4 diastereoisomers (**3**); β-β dimer (**4**); internal standard (3,4,5-trimethoxybenzoic acid) (**5**); electroosmotic flow (MeOH) (**6**). Optimized separation conditions: fused-silica capillary, 57 cm × 50 μm; applied voltage, 20 kV; temperature, 25 °C; electrolyte solution, 150 mM borate−phosphate, pH 10; UV detection at 214 nm; hydrodynamic injection, 5 s. For compound structures, see Figure 1.

Table 1. Relative Standard Deviation (Percent) of Migration Times $t_m(i)$, Peak Areas $A(i)$, Normalized Peak Areas NA(i), and Relative Normalized Peak Areas RNA(i)

compound	$t_m(i)$	$A(i)$	NA(i)	RNA(i)
coniferyl alcohol (**1**)	0.4	13.8	14.6	2.1
internal standard	0.7	13.1	14.4	0
β-5 dimer (**2**)	0.4	13.0	14.0	1.8
β-β dimer (**4**)	0.6	14.5	15.3	2.4
β-*O*-4 dimer (**3**)				
erythro	0.4	13.9	14.8	2.4
threo	0.4	16.1	17.1	5.1

Quantitative Analyses. Ten consecutive injections of the extracted oxidation products were performed to evaluate the repeatability of migration times $t_m(i)$, peak areas $A(i)$, normalized peak areas NA(i), and relative normalized peak areas RNA(i). Table 1 reports for each compound the relative standard deviation (RSD; percent) of these different parameters. Whereas RSDs of migration times were found to be satisfactory (<1%) for all compounds, those of peak areas and normalized peak areas were not (>13%). In contrast, a good repeatability was observed for relative normalized peak areas because the RSD was lower than 2.5% for all compounds except for the *threo* form of the β-*O*-4 dimer (**3**). For this last compound, the higher RSD obtained (5.1%) could be explained by the small peak area and by a moderate resolution from the peak corresponding to β-5 dimer (**2**) ($R_s = 1.86$). This means that quantitative analyses have to be carried out on RNA(i) values only. These results highlight the benefit of using an internal standard in the reaction medium even though the choice of this internal standard has to be carefully considered. In our case, 3,4,5-trimethoxybenzoic acid was selected because of its solubility, small size, and carboxylate group, which ensured a high migration time avoiding any interference with other compounds. In addition, this internal standard could be added at the onset of the oxidation reaction without any interference on the reaction course or the resulting products.

Horseradish Peroxidase-Catalyzed Oxidation of Coniferyl Alcohol. A microscale oxidation reaction was conducted to evaluate the performance of the optimized CZE method. Aliquots of 30 μL were taken regularly, mixed with 30 μL of MeOH to stop the reaction, and placed in a ice bath prior to direct CZE analysis. As shown in Figure 5, under such reaction conditions, coniferyl alcohol was totally oxidized within 20 min, which consistently coincided with the maximum amount of each dimer. After the disappearance of coniferyl alcohol (between 20 and 60 min of reaction time), a decrease was observed for all dimers. This decrease most likely resulted from the peroxidase-catalyzed oligomerization of the dimers previously formed. Syrjänen and Brunow (*9*) showed that horseradish peroxidase-catalyzed oxidation of *p*-hydroxycinnamyl alcohols together with dimeric lignin model compounds yielded cross-coupling products (trimers) when initial phenols exhibited similar oxidation potential. From the results of Figure 5, it can be assumed that horseradish peroxidase can also catalyze the dimerization of dimers because no more coniferyl alcohol was available to lead to trimer formation.

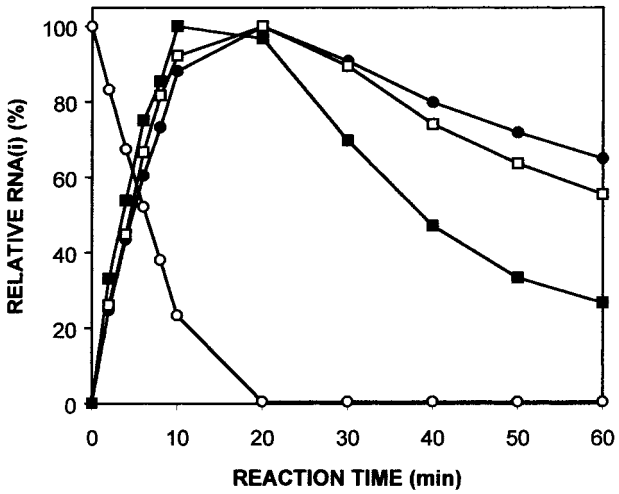

Figure 5. CZE monitoring of horseradish peroxidase-catalyzed oxidation of coniferyl alcohol: (○) coniferyl alcohol (**1**); (●) β-5 dimer (**2**); (□) total β-*O*-4 diastereoisomers (**3**); (■) β-β dimer (**4**). For compound structures, see Figure 1. For each compound, 100% corresponds to the maximum amount observed during the experiment.

CZE can be successfully applied for the on-line monitoring of coniferyl alcohol oxidation with a substantial time benefit compared to GC or HPLC methods. Using optimized separation conditions (150 mM borate−phosphate, pH 10; applied voltage = 20 kV; temperature = 25 °C), analysis of the oxidation products of coniferyl alcohol takes <15 min instead of 70 min for our conventional GC method and 25 min for the HPLC method (*15*). As for the HPLC, the CZE method requires neither extraction nor derivatization before analysis. In contrast, the HPLC method used by Wallace and Fry (*15*) implements a linear gradient and thus requires a time-consuming rinsing step. In conclusion, the screening capabilities of CZE make it a promising tool to explore the reaction mechanisms underlying the peroxidase-catalyzed oxidation of coniferyl alcohol in order to gain insights into lignification, a major biological event in the plant kingdom.

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