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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF *E*- AND *Z*-MONOLIGNOLS AND THEIR GLUCOSIDES

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SUMMARY

Photoirradiation of the *E*-monolignols, *E*-*p*-coumaryl, coniferyl and sinapyl alcohols gave mixtures of the corresponding *E*/*Z* monolignols. Similar treatments of the glucosides, *E*- and *Z*-coniferin, *Z*-isoconiferin and *Z*-syringin afforded comparable *E*/*Z* mixtures. Without derivatization, separation of the individual *E*- and *Z*-monolignols, and the *E*- and *Z*-monolignol glucosides could only be obtained by high-performance liquid chromatography. This development now permits the long-awaited facile analysis of plant extracts for *E*/*Z* monolignol and corresponding glucoside composition.

INTRODUCTION

Lignins are complex polyphenolic polymers present in terrestrial vascular plants, and which have essential structural and defense functions. For some time, it has generally been accepted that they are formed exclusively via random dehydrogenative polymerization of the three *E*-monolignols, *p*-coumaryl (**1**), coniferyl (**2**) and sinapyl (**3**) alcohols[1].

In woody angiosperms and gymnosperms, the exact mechanism of transport of the *E*-monolignols **1**–**3** from the cytoplasm into the lignifying cell wall has not yet been unequivocally demonstrated. It is, however, thought that they are transported into the wall as glycosidic conjugates, such as *E*-coniferin (**5**) and *E*-syringin (**6**). (Interestingly, the glucoside **4** has never been isolated.) Action of a β-glucosidase in the cell wall regenerates the *E*-monolignols **1**–**3**, and lignification then proceeds in a reaction requiring H_2O_2 and peroxidase for initiation of the free-radical polymerization[1]. The importance of the metabolic pathway to lignins cannot be underestimated as lignins are, next to cellulose, the most abundant organic substances in nature.

In spite of lignin's abundance in plant tissue, the precursor monolignols and glucosides are often found only in trace quantities. In most studies, their identification relies solely upon thin-layer chromatographic comparison with authentic (*E*) standards.

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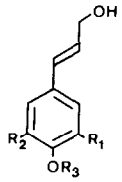

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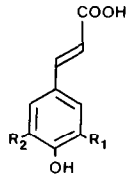

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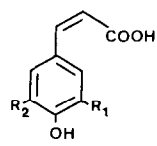

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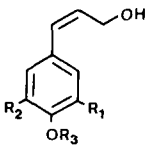

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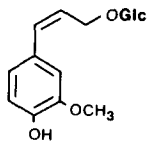

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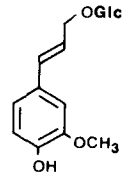

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In contrast to woody plants, grasses and herbaceous plants contain significant amount of cell-wall-bound hydroxycinnamic acids, *e.g.*, **7**–**10**[2–12]. These acids can be covalently linked to both polyoses (hemicelluloses)[8,10,11,13–16] and lignin[17–20]. Interestingly, these bound acids, *e.g.*, *p*-coumaric and ferulic acids, exist as mixtures of *E*- (**7**,**8**) and *Z*- (**11**,**12**) isomers, respectively[10,21–23]. All current evidence leans towards a photochemical mechanism for isomerization[23]. This is because (1) such acids are rapidly photochemically interconverted *in vitro*[10,21–23] and *in vivo*[21,23] and (2) plants grown in the presence of light contain mixtures of *E* and *Z* isomers, whereas etiolated plants contain only *E* isomers[21,23].

Unlike the hydroxycinnamic acids, it has been generally assumed that monolignols exist only in the *E* configuration. It was, therefore, rather surprising to discover that American beech (*Fagus grandifolia*) bark contained significant quantities of *Z*-monolignols, *Z*-coniferyl (**14**) and *Z*-syringyl (**15**) alcohols[24], and the glucosides, *Z*-coniferin (**16**) and *Z*-syringin (**17**)[25]. Importantly, the corresponding *E* isomers

could not be detected in this tissue. A third glucoside, *Z*-isoconiferin (**18**) was also isolated[25,26], where glucosylation had occurred at the allylic hydroxyl group; this compound had previously been described as faguside in European beech (*Fagus sylvatica*)[26].

In this paper, we describe a high-performance liquid chromatography (HPLC) method suitable for the separation of *E*- and *Z*-monolignols and their corresponding glucosides. This method allows us to rapidly quantify the amounts of both *E* and *Z* isomers in plant tissue. In this regard, it should be noted that most silica gel thin-layer chromatographic methods give only partial or no separation of these *E*/*Z* isomers.

MATERIALS AND METHODS

Instrumentation

The instrumentation in these experiments employed two Waters Model 510 solvent delivery systems fitted with a Model 721 system controller, a WISP Model 710B automatic injection module and a Model 990 photodiode array detector equipped with a NEC Power Mate 2, a Waters 990 Plotter and a NEC pinprinter CP6. Chromatographic separations used Waters Novapak C_{18} (150 mm × 3.9 mm, stainless steel) columns. For the separation of the *E* (**1–3**) and *Z* (**13–15**) monolignols, the column was eluted with degassed, filtered (0.45 μm) methanol–water (15:85, v/v). The flow-rate was 1.3 ml min^{-1}, and detection at 262 nm. Separation of the *E*- and *Z*-glucosides (**5,6,16–19**) employed two Novapak columns in series, with a mobile phase consisting of degassed, filtered (0.45 μm) acetonitrile–water (15:85, v/v) at 0.7 ml min^{-1}, and detection at 257 nm. ^1H NMR spectra were separately obtained in both $C^2H_3O^2H$ and $(C^2H_3)_2CO$ solutions, using tetramethylsilane (TMS) as an internal standard. Instrumentation employed a 270-MHz Bruker WP-270SY spectrometer.

*Preparation of E- and Z-coniferyl alcohols (**2,14**)*

Z-Coniferyl alcohol (**14**) was prepared as described previously[24]. E-Coniferyl alcohol (**2**) was obtained in 52% yield, via direct reduction of *E*-methyl ferulate using "ATE" complex[27] [diisobutylaluminumhydride (DIBAL-H) and *n*-butyllithium, *n*-BuLi] in anhydrous tetrahydrofuran (THF)[24], and then recrystallized from peroxide-free diethyl ether–light petroleum (b.p. 37–54°C) (1:1).

*Photoisomerization of E- and Z-coniferyl alcohols (**2,14**)*

E-Coniferyl alcohol (**2**) (5.99 mg, 33.3 μmol) was predissolved in methanol (0.665 ml) in a quartz tube (4 mm O.D., 2.5 mm I.D.), which was then purged with nitrogen. The resulting 0.05 *M* solution of coniferyl alcohol (**2**) was irradiated for 15 min, at a distance of 30.5 cm, with a Lifeguard mercury arc lamp (Philips, 400 W) whose outer glass shell had been removed. The energy fluence rate of the light below 320 nm, as determined by an UVX digital radiometer equipped with a short wave sensor (UVP, San Gabriel, U.S.A), varied from 48.6 to 113.0 mW cm^{-2}, depending upon the experiment. HPLC analysis of the resulting reaction mixture, with detection at 262 nm, showed the presence oı two components having elution volumes of 17.15 and 20.64 ml, and identical to those of authentic *E*- and *Z*-coniferyl alcohols (**2,14**), respectively. The UV spectrum of each component was also obtained during HPLC

separation, using a Waters 990 photodiode array detector: *E*-coniferyl alcohol (**2**) (λ_{max} 211, 262 nm); *Z*-coniferyl alcohol (**14**) (λ_{max} 211, 256 nm). Final product verification was obtained by ^{1}H NMR analysis of the reaction mixture, where integration of the olefinic resonances showed an *E*/*Z* ratio of 1.2:1. Following irradiation, the total recovery of *E*- and *Z*-coniferyl alcohols was 35.2%.

*Preparation of E- and Z-p-coumaryl (**1**,**13**) and sinapyl (**3**,**15**)alcohols*

Both *E*-*p*-coumaryl (**1**) and *E*-sinapyl (**3**) alcohols were prepared in a manner analogous to that described for *E*-coniferyl alcohol (**2**), *i.e.*, via reduction of the methyl esters of the corresponding hydroxycinnamic acids[24,27].

Photochemical isomerization of 0.05 *M* solutions of monolignols **1** and **3** could readily be achieved by 15 min irradiation as before. For *E*-*p*-coumaryl alcohol (**1**), irradiation afforded a mixture of two components having elution volumes of 11.73 and 14.00 ml, corresponding to *E*-*p*-coumaryl (**1**) and *Z*-*p*-coumaryl (**13**) alcohols, respectively. Analysis of the UV spectrum of each product was obtained during HPLC separation: *E*-*p*-coumaryl alcohol (**1**) (λ_{max} 205, 259 nm); *Z*-*p*-coumaryl alcohol (**13**) (λ_{max} 202, 253 nm). Again final verification of product identity was carried out by analysis of the ^{1}H NMR spectrum of the mixture; integration of the olefinic resonances gave an *E*/*Z* ratio of 1:1.1. Following irradiation, recovery of *E*/*Z* alcohols was 79.5%.

In a similar manner, irradiation of *E*-sinapyl alcohol (**3**) gave two components having elution volumes of 23.11 and 29.42 ml, corresponding to *E*- and *Z*-sinapyl alcohols (**3** and **15**), respectively. UV (λ_{max}): *E*-sinapyl alcohol (**3**) (220, 271 nm); *Z*-sinapyl alcohol (**15**) (217, 265 nm). Product confirmation was again obtained by analysis of the ^{1}H NMR spectrum; integration of olefinic resonances gave a 1.4:1 *E*/*Z* ratio. Recovery of *E*/*Z* sinapyl alcohols after photoirradiation was 33%.

*Preparation of Z-coniferin (**16**), Z-isoconiferin (**18**), Z-syringin (**17**) and E-coniferin (**5**)*

Glucosides **16**–**18** were isolated and purified to homogeneity exactly as described[25]. *E*-Coniferin (**5**) was synthesized in 50% yield, via "ATE-complex" reduction[27] of the condensation product of *E*-methyl ferulate and acetobromoglucose.

*Photoisomerization of E- and Z-coniferins (**5**,**16**)*

E-Coniferin (**5**) (17.38 mg, 50.8 μmol) was dissolved in methanol (1.015 ml) in a quartz tube under nitrogen as before. The solution was then irradiated for 15 min with an open-face mercury arc lamp (energy fluence rate of 94.2–138.0 mW cm^{-2}) at a distance of 30.5 cm. HPLC analysis of the resulting reaction mixture, monitored at 257 nm, showed the presence of two components having elution volumes of 3.44 and 3.98 ml, and corresponding to *E*- and *Z*-coniferins (**5**,**16**), respectively. The UV spectrum of each component was obtained during HPLC separation. UV (λ_{max}): *E*-coniferin (**5**) (212, 257 nm) and *Z*-coniferin (**16**) (212, 254 nm). Final product identification employed ^{1}H NMR analysis of the reaction mixture; integration of olefinic resonances gave a 2:1 *E*/*Z* ratio. Recovery of *E*/*Z* coniferins (**5**,**16**) after photoirradiation was 98.6%. In a similar manner, *Z*-coniferin (**16**) was irradiated to afford a 1:2 mixture of *E*/*Z* coniferins (**5**,**16**). Recovery of *E*/*Z* coniferins (**5**,**16**) after photoirradiation was 77.5%.

*Photoisomerization of Z-syringin (**17**) and Z-isoconiferin (**18**)*

Z-Syringin (**17**) (18.1 mg, 48.6 μmol) in methanol (0.972 ml) was irradiated with an open-face mercury arc lamp (energy fluence rate of 94.2–138.0 mW cm^{-2}) for 30 min at a distance of 30.5 cm, to give a mixture of *E*/*Z* isomers (**6**,**17**) in a 1:4.5 ratio as evidenced by ^{1}H NMR. (This represented the best conversion that we were able to effect; longer irradiation times resulted in severe losses of sample.) The HPLC elution volumes of *E*- and *Z*-syringins (**6**,**17**) were 3.65 and 4.34 ml, respectively, as evidenced by absorption at 257 nm. UV (λ_{max}): *E* isomer **6** (221, 263 nm); *Z* isomer **17** (215, 257 nm). Recovery of *E*/*Z* glucosides (**6**,**17**) was 99.5%. In an analogous manner, *Z*-isoconiferin (**18**) (16.08 mg, 47.0 μmol) in methanol (0.939 ml) was irradiated for 20 min, to afford a mixture of *E*/*Z* isoconiferins (**19**,**18**) in a 1:3.5 ratio, as evidenced by ^{1}H NMR. Elution volumes were 5.06 and 5.75 ml, respectively. UV (λ_{max}): *E* isomer **19** (206, 266 nm) and *Z* isomer **18** (212, 257 nm). Recovery of *E*/*Z* isoconiferins (**19**,**18**) was 19.4%.

RESULTS AND DISCUSSION

Until now, there have been no reports of the photochemical interconversions of *E*- and *Z*-monolignols and their glycosidic conjugates. This is in direct contrast to the situation for hydroxycinnamic acids **7**,**8**,**10**, whose facile photoisomerization with UV-A light (320–400 nm) has been well documented[10,21,-23].

Examination of the occurrence of hydroxycinnamic acids **7**–**10**, *Z*-monolignols **14**,**15** and their glucosides **16**–**18** in plant material reveals some rather interesting differences. In etiolated plants, only the *E* isomers of hydroxycinnamic acids, *e.g.* **7**,**8** are known to occur, whereas in light-grown plants mixtures of both *E* and *Z* isomers are present because of light-triggered reactions. The situation in beech bark is rather different, since only the *Z*-monolignols **14**,**15** and their glucosides **16**–**18**, and not the corresponding *E* isomers are found. To account for their formation, appropriate radiolabelling experiments with both *E* and *Z* precursors suggested that *Z*-coniferyl alcohol (**14**) formation occurred via direct isomerization of the corresponding *E* isomer **2**[28]. However, since these experiments were conducted in the absence of light, this suggested that the isomerization of the monolignols was not photochemically induced. This is in direct contrast to the *E*/*Z* isomerization of the hydroxycinnamic acids.

Since an enzyme capable of isomerizing *E*- and *Z*-monolignols has not been isolated to date, we sought to identify the conditions required for the photochemical interconversion of *E*- and *Z*-monolignols and their glucosides. Initial experiments were conducted with pure samples of *E*- and *Z*-coniferyl alcohols (**2**,**14**), respectively. Chromatographic conditions for their separation were established using a Waters Novapak C_{18} column eluted with methanol–water (15:85, v/v).

We next investigated the effects of photoirradiation of *E*- and *Z*-coniferyl alcohols (**2**,**14**) using, as the light source, a mercury arc lamp whose outer glass shell had been removed. For *E*-coniferyl alcohol (**2**), rapid photochemical isomerization was observed within 15 min to afford a 1.2:1 ratio of *E*- and *Z*-isomers as evidenced by ^{1}H NMR integration of the olefinic resonances (see Materials and methods section). A similar treatment with the *Z*-monolignol **14** afforded *E*/*Z* monolignols in a 1:2 ratio.

The elution volume, detected at 262 nm, and the UV spectrum of each mono-

lignol was recorded during chromatographic separation, using a Waters 990 photodiode array detector, and compared to that of authentic standards. Thus, the component eluted at 17.15 ml had a UV spectrum (λ_{max} 262, 211 nm) and an elution volume identical to that of *E*-coniferyl alcohol (**2**). In a similar manner, the component eluted at 20.64 ml (λ_{max} 256, 211 nm) was identical to *Z*-coniferyl alcohol (**14**).

Having demonstrated that individual photochemical treatment of *E*- and *Z*-coniferyl alcohols (**2**,**14**) resulted in *E*/*Z* isomerization, we next synthesized *E*-*p*-coumaryl (**1**) and *E*-sinapyl (**3**) alcohols via direct reduction of the methyl esters of the corresponding hydroxycinnamic acids **7** and **10**. Reduction was carried out using "ATE" complex, produced from DIBAL-H and *n*-BuLi[24,27]; recrystallization of each monolignol gave *E*-*p*-coumaryl (**1**) and *E*-sinapyl (**3**) alcohols in 60 and 45% yields, respectively.

In a similar manner to that employed for *E*/*Z* coniferyl alcohols **2**,**14**, samples of pure *E*-*p*-coumaryl (**1**) and *E*-sinapyl (**3**) alcohols were irradiated in solution for 15 min. For *E*-*p*-coumaryl alcohol (**1**), two components were present in the reaction mixture in an approx. 1:1.1 ratio, as evidenced by ^{1}H NMR integration of the olefinic resonances and HPLC analysis. The first component eluted had an elution volume (11:73 ml), UV (λ_{max} 259, 205 nm) and ^{1}H NMR spectra identical to that of *E*-*p*-coumaryl alcohol (**1**). On the other hand, the component eluted at 14.00 ml had a ^{1}H NMR spectrum consistent with that of *Z*-*p*-coumaryl alcohol (**13**), *i.e.*, the olefinic resonances at 5.67 and 6.42 ppm had a coupling constant of J = 11.7 Hz indicating a *cis* configuration. Additionally, its UV spectrum (λ_{max} 253, 202 nm) corresponded to that expected for the *Z*-isomer **13**. Photoirradiation of *E*-sinapyl alcohol (**3**) also produced a mixture of two components in approximately 1.4:1 ratio from ^{1}H NMR integration of olefinic resonances and HPLC analyses. The first component had an elution volume (23.11 ml) and UV spectrum (λ_{max} 271, 220 nm) identical to that of *E*-sinapyl alcohol (**3**), whereas the second had an elution volume (29.42 ml), UV spectrum (λ_{max} 265, 217 nm) and olefin ^{1}H NMR coupling constant, J = 11.7 Hz consistent for *Z*-sinapyl alcohol (**15**).

Fig. 1 shows the HPLC profile of a mixture of all six *E*- and *Z*-monolignols (**1**–**3**,**13**–**15**). It is noteworthy that the separation of the isomers follow the sequential order of *p*-coumaryl (**1**): coniferyl (**2**): sinapyl (**3**) alcohols due to the effect of the bulky methoxyl groups. Additionally, the *Z* isomers were also less polar than the

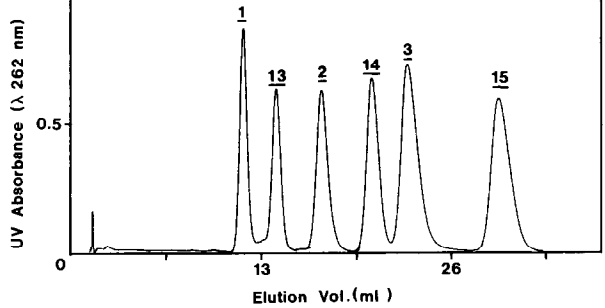

Fig. 1. HPLC elution profile of *E*/*Z* monolignols **1**–**3**,**13**–**15**. Elution details: Waters Novapak C_{18} column eluted with methanol–water (15:85, v/v) at a flow-rate of 1.3 ml min^{-1}. Numbers refer to structures previously shown.

corresponding *E* isomers, presumably due to an increased electronic interaction of the allylic hydroxyl group with the aromatic ring, and also due to a lower degree of delocalization of π-electrons in the molecular framework.

We next turned our attention to the photochemical isomerism of the *E* and *Z* isomers of coniferin (**5**,**16**). *E*-Coniferin (**5**) was prepared in 50% yield via direct reduction of the condensation product of *E*-methyl ferulate and acetobromoglucose with "ATE" complex[27], whereas *Z*-coniferin (**16**) was isolated from beech bark[25]. Chromatographic conditions for the separation of the individual glucosides required two Novapak C_{18} columns in series, which were eluted with acetonitrile–water (15:85, v/v) with detection at 257 nm. Photochemical irradiation of the *E* isomer **5** resulted in formation of both *E* and *Z* isomers in approximately 2:1 ratios, as evidenced by ^{1}H NMR analysis of the reaction mixture. Product identification of the two components again relied upon elution volumes, UV and ^{1}H NMR spectra. Thus *E*-coniferin (**5**) (λ_{max} 257, 212 nm) had an elution volume of 3.44 ml, whereas the corresponding *Z* isomer **16** (λ_{max} 254, 212 nm) was eluted at 3.98 ml. In a comparable fashion, *E*- and *Z*-mixtures of both syringin (**6**,**17**) and isoconiferin (**19**,**18**) were prepared by photoirradiation of the corresponding *Z* isomers, obtained from beech bark as before.

Fig. 2 shows the HPLC chromatogram of the six glucosides *i.e.*, *E*/*Z*-coniferin (**5**,**16**), syringin (**6**,**17**) and isoconiferin (**19**,**18**). Again, separation of the *E*/*Z* glucosides, coniferin and syringin, followed the general trend already noted for the *E*/*Z* monolignols.

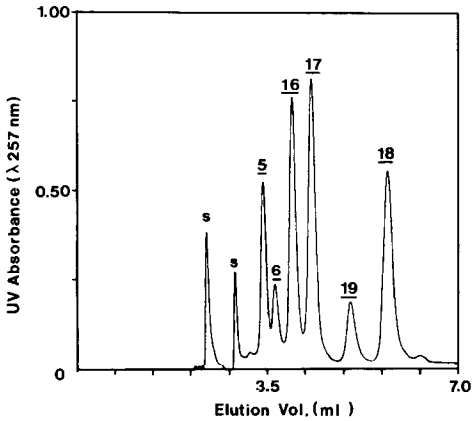

Fig. 2. HPLC elution profile of *E*/*Z* monolignol glucosides **5**,**6**,**16**–**19**. Elution details: two Waters Novapak C_{18} columns in series, eluted with acetonitrile–water (15:85, v/v) at a flow-rate of 0.7 ml min^{-1}. Numbers refer to structures previously shown; s = absorbance from solvents used in sample preparation.

CONCLUSIONS

This HPLC technique now permits the facile and rapid analysis of plant extracts, not only for the determination of *E*-monolignol and glucoside contents, but also for the corresponding *Z* isomers. Using this method, more meaningful chemotaxonomical studies of plants for these components can now be obtained, *i.e.*, the general significance of both *E*- and *Z*-monolignols and their glucosides, in various plants can now be determined.

This study also demonstrates that under suitable photochemical conditions, facile *E*/*Z* isomerization can be induced. However, unlike the hydroxycinnamic acids **7**,**8** which exist as mixtures of both *E* and *Z* isomers in graminaceous plants, only the *Z* isomers are detectable in beech bark tissue. If a simple photochemical process was in effect for the isomerization of *E*/*Z*-monolignols, then it would be expected that both isomers would accumulate in the bark. The underlying reasons for the exclusive accumulation of *Z* isomers in beech bark tissue needs to be determined.

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