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SEPARATION OF PHENOLS, PHENOLIC ALDEHYDES, KETONES AND ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

EDUARD BURTSCHER, HANNO BINDER, ROLAND CONCIN and ORTWIN BOBLETER*
Institute of Radiochemistry, University of Innsbruck, Innrain 52a, A-6020 Innsbruck (Austria)
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SUMMARY

The high-performance liquid chromatographic separation of phenols, benzaldehydes, acetophenones, benzoic and cinnamic acids as well as some related compounds is described. An octadecyl silica column was used with a 0.01 *M* phosphate buffer–acetonitrile gradient as mobile phase. The retention times of the substances investigated depend on their polarity. An elution order depending on the basic functional groups and the substituents has been established.

INTRODUCTION

The analysis of phenols and related aldehydes, ketones and acids is frequently undertaken in clinical and forensic chemistry, biochemistry and wood chemistry, as well as in food control and environmental pollution examinations. However, a simple and fast method of analysis for phenolic degradation products with different functional groups is not available. Many separations have been achieved by paper chromatography[1–5], thin-layer chromatography[6–10] or gas chromatography[11–17], but they are time-consuming or need derivatization of the compounds.

Liquid chromatographic separations largely overcome these disadvantages. Roston and Kissinger[18] have briefly reviewed recent findings on the liquid chromatographic determination of phenolic acids of vegetable origin. Lange and co-workers[19,20] separated phenolic acids on an ion-exchange column using buffer solutions as eluent. Ion-pair chromatographic separation of sulphonic and carboxylic acids was achieved by Kraak and Huber[21] using long-chain aliphatic amines on Kieselguhr and dilute aqueous acids. Some years later Terweij-Groen and Kraak[22] investigated the influence of the type of amine, the temperature, the eluent composition and the concentration of the eluent acid. Hövermann *et al.*[23] used partition chromatography on Merckogel SI-150 columns for the separation of the *cis–trans* isomers of coumaric and ferulic acids. The separation of phenols on various normal- and reversed-phase chromatographic systems was described by Schabron *et al.*[24].

Many systems using octadecyl silica columns and water or alcohol (mainly methanol)–water mixtures with a small amount of acetic acid have been described for the analysis of phenolic acids and aldehydes[25–29]. Very good results were achieved by

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Horváth and co-workers[30,31] who separated over 100 urinary acids in less than 30 min using reversed-phase chromatography (RP-18) with 0.1 *M* phosphate buffer–acetonitrile (or methanol) gradient elution. Price *et al.*[32] reported a systematic study of the effect of the pH of the eluent on the separation of cinnamic acids with citrate buffer–methanol mixtures on a C-18 bonded phase. A similar phase was used by Charpentier and Cowles[33] for the analysis of phenolics with different solvent mixtures (water–acetonitrile–acetic acid). Roggendorf and Spatz[34] examined the selectivity effects of a water–methanol–tetrahydrofuran ternary system on the separation of phenolic aldehydes and cinnamic acids on a C-8 stationary phase. Vanhaelen and Vanhaelen-Fastré[35] selected an alkyl phenyl bonded phase because of its high selectivity for the analysis of aromatic acids and phenols.

The literature on high-performance liquid chromatographic (HPLC) separations mentioned above deals mainly with the analysis of phenolic acids. Little attention has been paid to the chromatographic behaviour of phenolic compounds with the same functional group (*e.g.*, aldehydes, ketones, acids) and different substituents of the aromatic nucleus and the side-chain. The need for a careful investigation of these effects arises from the fact that lignin degradation products show three basic units (*p*-hydroxyphenyl, 3-methoxy-4-hydroxyphenyl and 3,5-dimethoxy-4-hydroxyphenyl) with different functional groups and substituents at position 1 of the aromatic nucleus. Therefore, the aim of the present work was to establish a suitable method for the separation of this spectrum of phenolic compounds using an octadecyl silica column.

EXPERIMENTAL

Apparatus

A high-pressure liquid chromatograph (Spectra-Physics, Model SP 8000 B) with an integrated gradient programmer, column oven compartment and integrator and a variable-wavelength UV detector (Model SP 8400; Spectra-Physics, Santa Clara, CA, U.S.A.) was employed. The sample was injected by a sample loop valve fitted with a 25-μl loop (Valco Instruments Co., Houston, TX, U.S.A.). The separation was performed by gradient elution using an initial composition of 95% 0.01 *M* phosphate buffer, pH 2.0 (analytical grade KH_2PO_4; E. Merck, Darmstadt, G.F.R.) and 5% acetonitrile (analytical grade, Merck) and a flow-rate of 0.8 ml/min. The acetonitrile concentration was raised to 50% within 45 min. In order to purge the column, the acetonitrile content was further increased to 70% within 5 min and maintained for 9 min. To re-equilibrate the column, the initial conditions were adjusted by a 1-min step-gradient which was held for 10 min before the next injection was made. The column temperature was kept at 50°C.

Columns

Stainless-steel columns (No. 103.07; Knauer, Oberursel, G.F.R.), 250 × 4.6 mm I.D., with zero dead-volume fittings were used. Spherical totally porous silica gel with bonded octadecyl groups (Nucleosil 5 C_{18}, No. 712 130; Macherey, Nagel & Co., Düren, G.F.R.) with a particle diameter of 5 μm was utilized as packing material. The columns were filled by the slurry-packing technique[36,37] using a two-piston pump (Model 100; Altex, Berkeley, CA, U.S.A.) at 65 MPa with a flow-rate of 10 ml/min.

Materials

The reference substances were supplied by Fluka (Buchs, Switzerland), Ega-Chemie (Steinheim, G.F.R.), Loba-Chemie (Fischamend, Austria), Merck and C. Roth (Karlsruhe, G.F.R.). Some commercially unavailable compounds were obtained from Professor H. Griengl, Technical University of Graz. The samples were dissolved in methanol–water (50:50). The investigated compounds and their concentrations are listed in Tables I–III.

RESULTS AND DISCUSSION

As the polarity of the substances investigated differs only very slightly, a very selective mobile phase and an efficient stationary phase are necessary to achieve a good separation. Earlier studies demonstrated that Si 60 columns do not provide satisfactory results. The reproducibility was poor and some of the more polar substances such as phenolic acids were retained by the column. The use of octadecyl silica columns and water–acetic acid (9:1) as eluent allowed only separations with poor peak shapes[38].

Good results were obtained with the described gradient elution using 0.01 *M* phosphate buffer and acetonitrile. In contrast to acetic acid, the acidity of phosphoric acid is sufficient to prevent the dissociation of the phenolic acids, so that symmetrical peaks were achieved. The pH value of the buffer should be between 1.9 and 2.0; higher values lead to an equilibrium of the free acid and its conjugated base, resulting in irreproducible retention times and strong peak-tailing. On the other hand, the low molarity of the buffer allows high contents of acetonitrile without precipitation of the KH_2PO_4, so that less polar compounds are also eluted.

The flow-rate of the chromatographic system was optimized by calculation of the height equivalent to a theoretical plate, *H*, for 4-hydroxybenzaldehyde, vanillin,

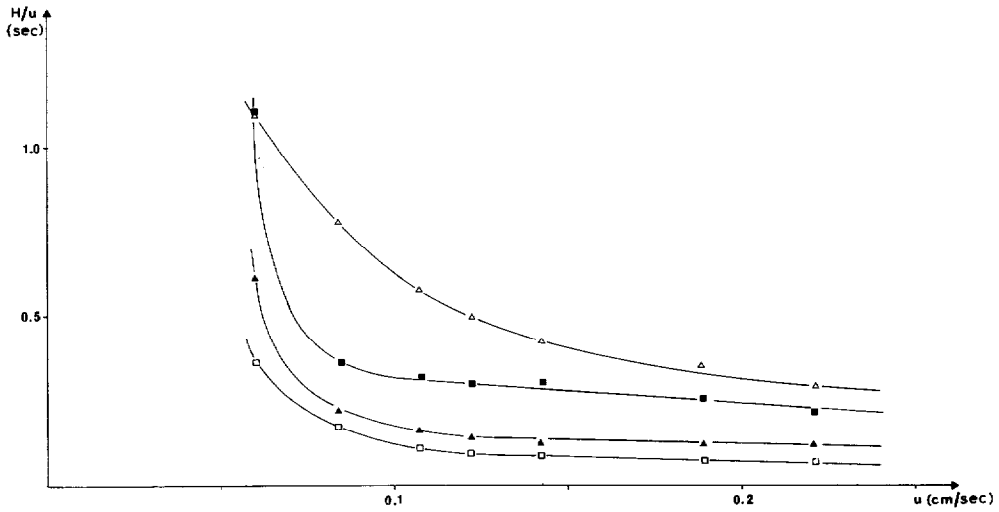

Fig. 1. Optimization of the flow-rate: graph of the term *H/u versus* linear velocity for 4-hydroxybenzaldehyde (△), vanillin (■), acetovanillon (▲) and veratraldehyde (□).

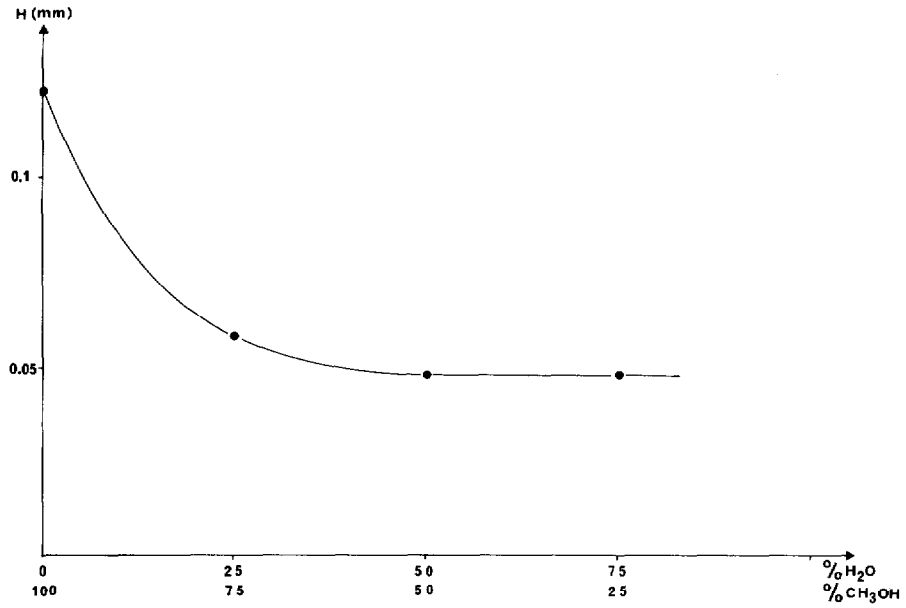

Fig. 2. Influence of the composition of the solvent on the height equivalent to a theoretical plate, *H*, for vanillin.

acetovanillon and veratraldehyde at different linear velocities, *u*. Fig. 1 shows the plot of *H*/*u versus* the linear velocity; *H*/*u* decreases very rapidly up to a linear velocity of about 0.11 cm/sec, corresponding to a flow-rate of 0.8 ml/min, used for all separations. Further increase of the flow-rate resulted only in a negligible gain in analysis time.

Most of the substances investigated have absorption maxima at approximately 280 nm; some of the phenolic acids absorb at lower wavelengths, and certain aldehydes such as syringaldehyde have a maximum at 308 nm. Therefore a wavelength of 280 nm was considered appropriate.

The solvent has a very strong influence on the quality of the separation, especially for the more polar compounds. In Fig. 2 the dependence of *H* on the composition of the solvent (methanol–water) is plotted for vanillin. At methanol contents greater than 50 % a strong pretailing is observed resulting in high *H* values, whereas at lower percentages of methanol a nearly constant *H* value is achieved. This can be explained by the fact that the equilibrium between the stationary and the mobile phases is disturbed by the higher methanol contents.

Using the optimized conditions described the separation of the following groups of substances was performed: phenols and aromatic ethers, phenolic aldehydes and ketones, phenolic acids. Fig. 3 shows a chromatogram of phenols and aromatic ethers (Table I). The influence of the substituents on the elution order is clearly demonstrated. If phenol is considered as the basic unit, a further hydroxyl group decreases the retention time (*e.g.*, phenol and resorcinol), whereas methyl, ethyl, methoxy and ethoxy groups increase the retention time. The influence of an alkyl group is shown in the series phenol, *p*-cresol, 4-ethylphenol and pyrocatechol, 4-

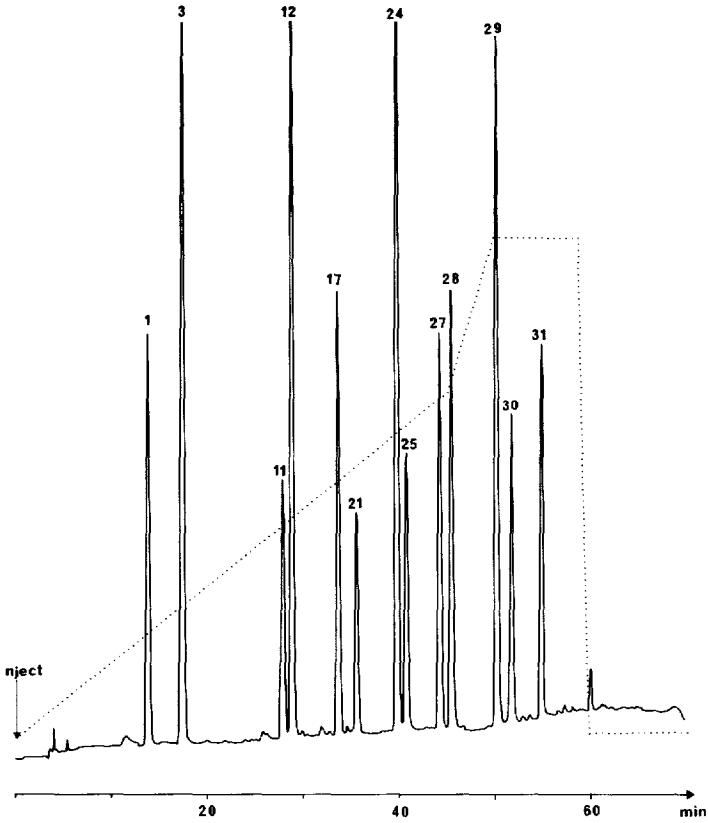

Fig. 3. Chromatogram of the separation of phenols and aromatic ethers. Column: 5 μm octadecyl silica, 250 × 4.6 mm I.D. Mobile phase: gradient elution with 0.01 *M* phosphate buffer (pH 2.0) and acetonitrile (·····). Temperature: 50°C. Flow-rate: 0.8 ml/min. UV-detection at 280 nm, 0.16 a.u.f.s. For peak identification see Table I.

homocatechol. The difference caused by an alkoxy group is demonstrated by pyrocatechol, guaiacol, guaethol and veratrole.

Steric effects also alter the retention time. *meta* and *para* substituents cannot be distinguished by the described method, but substituents in the *ortho* position, which hinder the interaction with the stationary phase (*e.g.*, veratrole and 1,3-dimethoxybenzene), accelerate elution, whereas a hindrance of the interaction with the mobile phase (*e.g.*, *p*-cresol and *o*-cresol or 4-ethylphenol and 2-ethylphenol) increases the retention time. An increase in the retention time is also caused by intramolecular hydrogen-bonding (disturbing the interaction with the mobile phase) and by *meta* substituents which hinder the interaction with the stationary phase (*e.g.*, resorcinol and pyrocatechol). With the exception of the steric effects, all these influences could be described by the Hammett equation. Further investigations, however, are necessary to obtain a complete set of data.

In Fig. 4 a chromatogram of phenolic aldehydes and ketones (Table II) is depicted. The basic units are 4-hydroxybenzaldehyde, 4-hydroxyacetophenone as

TABLE I

PEAK IDENTIFICATIONS, SAMPLE CONCENTRATIONS AND CAPACITY FACTORS OF PHENOLS AND AROMATIC ETHERS

Substance	*Peak No.*	*Sample concentration (mg/ml)*	*Capacity factor, k'*
1,3-Dihydroxybenzene (resorcinol)	1	0.2	2.56
1,2-Dihydroxybenzene (pyrocatechol)	3	0.2	3.47
Phenol	11	0.6	6.24
3,4-Dihydroxytoluene (4-homocatechol)	12	0.3	6.39
2-Methoxyphenol (guaiacol)	17	0.4	7.56
2,6-Dimethoxyphenol	21	0.6	8.10
3-Hydroxytoluene (*m*-cresol)	24	0.2	9.15
4-Hydroxytoluene (*p*-cresol)	24	0.2	9.15
2-Hydroxytoluene (*o*-cresol)	25	0.45	9.39
2-Ethoxyphenol (guaethol)	27	0.4	10.20
1,2-Dimethoxybenzene (veratrole)	28	0.6	10.48
1-Ethyl-4-hydroxybenzene (4-ethylphenol)	29	0.4	11.51
1-Ethyl-2-hydroxybenzene (2-ethylphenol)	30	0.25	11.87
1,3-Dimethoxybenzene	31	0.45	12.61

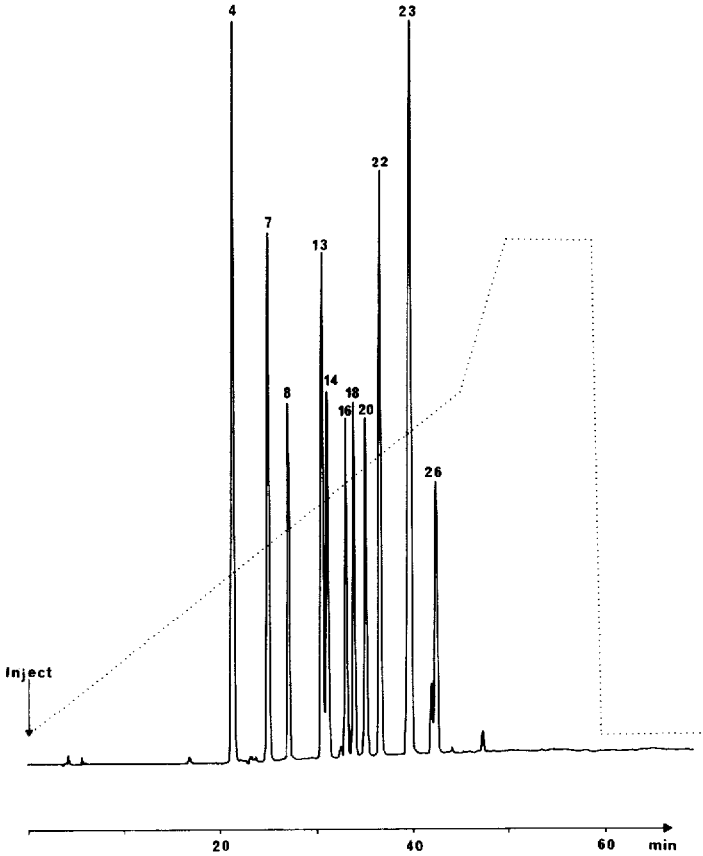

Fig. 4. Chromatographic separation of phenolic aldehydes and ketones. Separation conditions as in Fig. 3; for peak identification see Table II.

TABLE II

PEAK IDENTIFICATIONS, SAMPLE CONCENTRATIONS AND CAPACITY FACTORS OF PHENOLIC ALDEHYDES AND KETONES

Substance	*Peak No.*	*Sample concentration (mg/ml)*	*Capacity factor, k'*
3,4-Dihydroxybenzaldehyde	4	0.1	4.04
3,4-Dihydroxyacetophenone	7	0.1	5.08
4-Hydroxybenzaldehyde	8	0.1	5.56
4-Hydroxyacetophenone	13	0.1	6.60
4-Hydroxy-3-methoxybenzaldehyde (vanillin)	14	0.2	6.75
3,5-Dimethoxy-4-hydroxybenzaldehyde (syringaldehyde)	16	0.2	7.33
4-Hydroxy-3-methoxyacetophenone (acetovanillon)	18	0.1	7.61
3,5-Dimethoxy-4-hydroxyacetophenone (acetosyringone)	20	0.3	7.94
4-Hydroxypropiophenone	22	0.2	8.34
3,4-Dimethoxybenzaldehyde (veratraldehyde)	23	0.2	9.05
4-Hydroxy-3-methoxypropiophenone (propiovanillon)	26	0.1	9.79

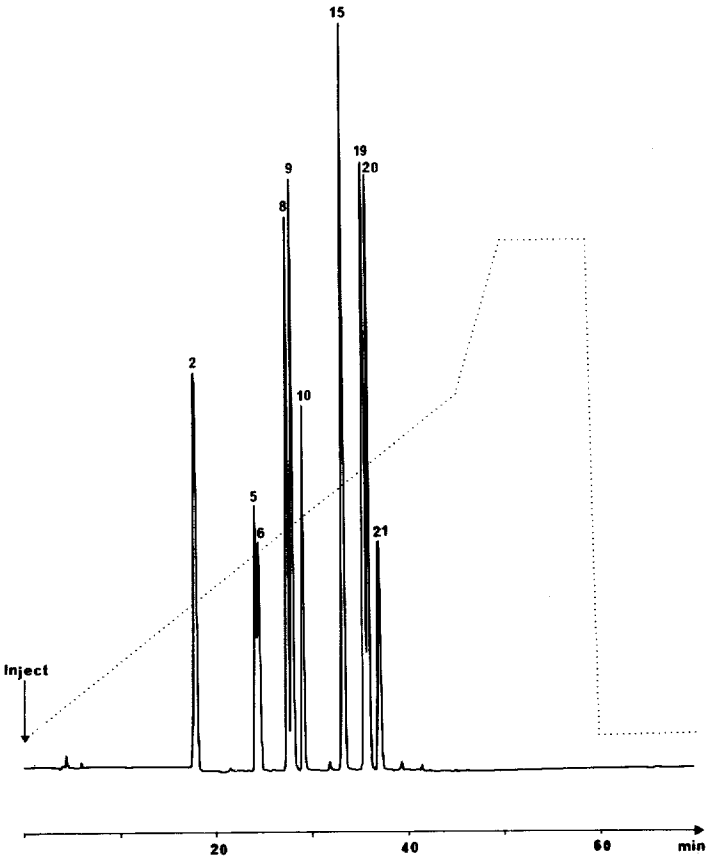

Fig. 5. Chromatogram of the separation of benzoic and cinnamic acids. Separation conditions as in Fig. 3; for peak identification see Table III.

TABLE III

PEAK IDENTIFICATIONS, SAMPLE CONCENTRATIONS AND CAPACITY FACTORS OF PHENOLIC ACIDS

Substance	*Peak No.*	*Sample concentration (mg/ml)*	*Capacity factor, k'*
3,4-Dihydroxybenzoic acid (protocatechuic acid)	2	0.6	2.90
4-Hydroxybenzoic acid	5	0.5	4.52
(4-Hydroxyphenyl)acetic acid	6	1.0	4.65
4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	8	0.2	5.56
(4-Hydroxy-3-methoxyphenyl)acetic acid (homovanillic acid)	9	0.6	5.66
3,4-Dihydroxycinnamic acid (caffeic acid)	9	0.3	5.66
3,5-Dimethoxy-4-hydroxybenzoic acid (syringic acid)	10	0.2	6.09
4-Hydroxycinnamic acid (*p*-coumaric acid)	15	0.3	7.12
4-Hydroxy-3-methoxycinnamic acid (ferulic acid)	19	0.3	7.80
3,5-Dimethoxy-4-hydroxycinnamic acid (sinapic acid)	20	0.3	7.94
Benzoic acid	21	1.4	8.10

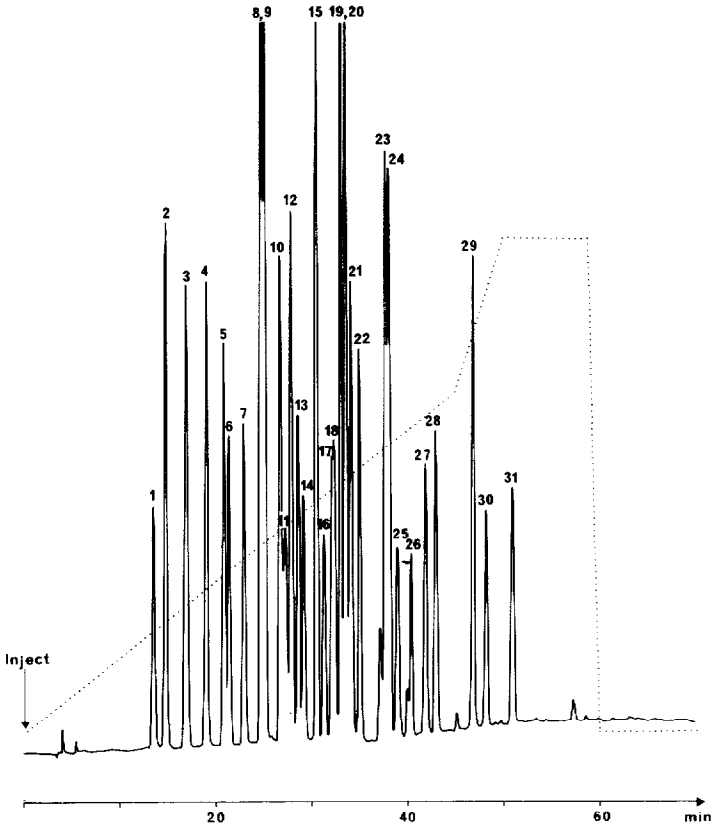

Fig. 6. Chromatogram of a mixture of phenols, aromatic ethers, benzaldehydes, acetophenones, propiophenones, benzoic and cinnamic acids. Chromatographic conditions as in Fig. 3; for peak identifications see Tables I–III.

well as 4-hydroxypropiophenone. All the effects discussed, such as the influence of a further hydroxyl group (3,4-dihydroxybenzaldehyde and 3,4-dihydroxyacetophenone) or a methoxy group (vanillin and syringaldehyde as well as the analogous acetophenones, acetovanillon and acetosyringone), are again found. In comparison to phenol, 4-hydroxybenzaldehyde has a shorter retention time because of its higher polarity. If 4-hydroxyacetophenone and 4-hydroxypropiophenone are considered, it can be seen that the stronger interaction with the stationary phase caused by the increase of the side-chain is the most important factor.

Fig. 5 shows a chromatogram of the phenolic acids (Table III). The reference substances used are substituted benzoic and cinnamic acids. The substituents influence the elution order as described above. The retention times of 4-hydroxybenzoic acid, (4-hydroxyphenyl)acetic acid and 4-hydroxycinnamic acid are determined by the lengths of the side-chains. The acids are eluted faster than the corresponding phenols and aldehydes because of their higher polarities. A separation of homovanillic acid and 3,4-dihydroxycinnamic acid was not possible under the chromatographic conditions chosen.

Finally, a mixture of all these compounds was chromatographed (Fig. 6). A complete separation was not of course attainable. In addition to the unresolved pairs homovanillic acid/3,4-dihydroxycinnamic acid and *m*-/*p*-cresol, also 4-hydroxybenzaldehyde/vanillic acid, sinapic acid/acetosyringone and 2,6-dimethoxyphenol/benzoic acid have the same retention times. The elution order of the basic units can be established as: benzoic acids < benzaldehydes < acetophenones < cinnamic acids. The influence of the substituents is as follows: 3,4-dihydroxy < 4-hydroxy < 4-hydroxy-3-methoxy < 3,5-dimethoxy-4-hydroxy.

This investigation shows that most of the monomeric reference substances can be separated in a relatively short time by HPLC and that this method is therefore suitable for the analysis of hydrothermally degraded lignins[39–41].

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