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Note

Chromatography of natural phenolic cinnamate derivatives on Sephadex LH-20 and G-25

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Extracts from higher plants contain mixtures of phenolic cinnamic acid derivatives, often of the chlorogenic acid type. In the course of efforts to apply chromatography on Sephadex to separation of these compounds from natural samples, difficulties were encountered with peak shape, purity and variability. Substances of the chlorogenic acid type were adsorbed from water and were generally well separated by continued elution with water from preceding and following impurities lacking the typical ultraviolet (UV) absorption spectra of cinnamates. Individual chromatographic peaks were obtained for cinnamate derivatives, but they were often slow to reach maximal absorbance with an abrupt decline after the maximum. When rechromatographed, peak fractions expected to be pure were contaminated by cinnamates concentrated in other peaks and, depending upon conditions, multiple peaks in various quantitative proportions could be produced from the same fraction.

Reasons for such behaviour have been clarified by this research and the value of Sephadex chromatography to study *cis–trans* isomerism in these compounds illustrated.

EXPERIMENTAL

Sephadex LH-20 or G-25, as obtained from Pharmacia (Uppsala, Sweden), was swelled in water for at least one hour and slurry-packed into an LKB 4200 precision column, 12 × 300 mm, under a flow-rate of about 0.5 ml/min produced by an LKB varioperpex 12000 peristaltic pump to give a packed length of about 240 mm. Effluents were monitored at 280 nm with an LKB Uvicord II 8300 having a 3-mm light-path flow cell and recorded by an LKB type 6520-6 recorder, but converted to absorbance by an Infometrics PSU 15/50 Linalog converter giving 0.5, 1.0 or 2.0 A full scale.

A repurified commercial sample of 5-chlorogenic acid and 4-*p*-coumaroylquinic acid isolated from apples[1] were used as the test compounds. UV irradiation

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was by a 125-W Hanovia medium pressure Hg arc lamp. Absorption spectra were obtained with a Unicam SP-800B spectrophotometer.

RESULTS AND DISCUSSION

Fig. 1 shows the elution chromatograms obtained when equal portions of the same solution of 4-*p*-coumaroylquinic acid were developed on Sephadex LH 20 with water as the eluent. The freshly dissolved crystalline material (*trans*) in A and the isomerized (*cis* + *trans*) in B. The characteristically skewed curves are shown plus evidence of partial separation of the *cis* and *trans* isomers. Addition of 0.2% (v/v) of glacial acetic acid in the eluting solvent gave the comparable chromatograms shown in Fig. 2. Note that the *trans* derivative now produces a slower eluting, Gaussian-shaped peak (Fig. 2A) and the *cis* form produced by irradiation is completely separated (Fig. 2B, baseline between) from the *trans*. These columns, the exit tubing and the receivers were completely darkened during the run by covering with aluminium foil. That the earlier peak is the *cis* form and the later the *trans* was verified by the absorption spectra (*cis* maximum 5 nm shorter wavelength and extinction about 50% of the *trans*[1,2]) and paper chromatography in 2% acetic acid of the separated fractions.

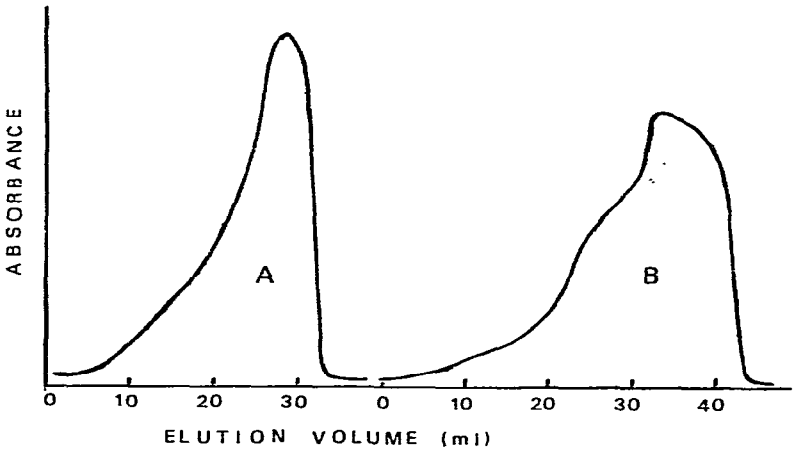

Fig. 1. 4-*p*-Coumaroylquinic acid chromatographed in the dark on Sephadex LH 20 developed with water. A, *trans*-4-*p*-coumaroylquinic acid (2 mg/ml); B, an equal portion of the same solution as in A UV irradiated during 30 min.

The exposure of the *trans*-4-*p*-coumaroylquinic acid in dilute solution in ordinary laboratory light for 9 h (on a cloudy day and with only about 2 h of artificial lighting) gave apparently complete isomerization as indicated by a chromatogram very nearly identical to Fig. 2B and by the lack of further change in the absorption spectrum of the solution upon UV irradiation.

The effect of the acetic acid in the eluting solvent is believed to be primarily to suppress ionization of the quinic acid carboxyl, a principle well established in chromatography. However, the results indicate that the carboxy anion form is considerably less strongly adsorbed on Sephadex LH 20 than is the un-ionized carboxyl.

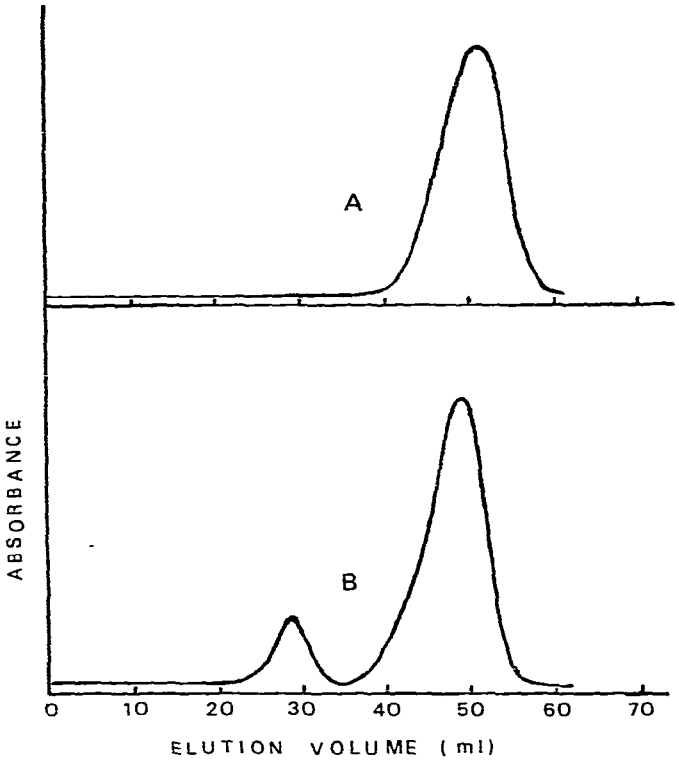

Fig. 2. 4-*p*-Coumaroylquinic acid, (A) *trans* and (B) *cis* + *trans*, chromatographed in the dark on Sephadex LH-20 and developed with 0.2% acetic acid in water.

A hydrogen-bonding mechanism is suggested with the acidic carboxyl hydrogen forming an especially strong bond, presumably with the oxygen H-bond acceptors in the Sephadex. The effect of the acetic acid is not limited to this, however, because it should also suppress the conversion of the *trans* forms to the *cis* forms. It has been shown that in the cinnamate series the equilibrium favours the *trans* form more at low pH than at pH 5–7[3]. This can be a factor even during a column run if the column is not protected from light. Fig. 3 shows that an isomerized *cis–trans* mixture did not resolve when the column was continuously irradiated but rather the continued isomerization gave a broader, flatter peak representing the average mobility of the rapidly intraconverting two forms.

A third benefit of the acetic acid should be the conversion of any salts of the phenolic quinates and related compounds to the free acids. In fact, we have found with certain plant extracts that acetic acid appears insufficiently acidic to quickly displace all the complexes and therefore recommend addition only to the first void-volume of the eluent of 0.05 *N* HCl or other strong acid. Without this the major peaks are unaffected, but more rapidly moving (highly water-soluble), cinnamate-bearing peaks may occur which appear to be base-salts of the acid function of the cinnamoyl ester (quinate, etc.) rather than additional parent cinnamate derivatives.

Similar ability to resolve the *cis* and *trans* forms of 5-chlorogenic acid was found and Fig. 4 shows a chromatogram of a mixture of the *cis* and *trans* isomers of

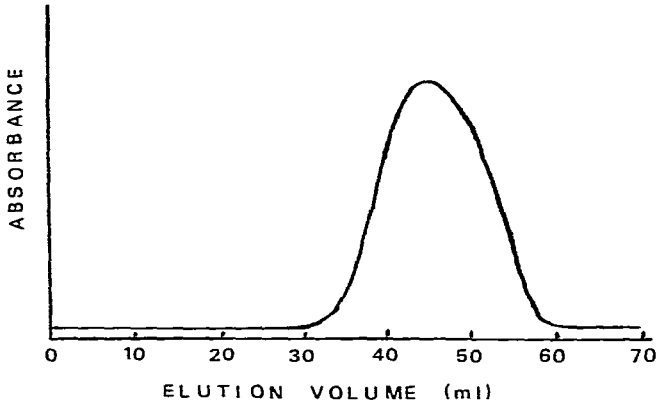

Fig. 3. Chromatogram of 4-*p*-coumaroylquinic acid under conditions identical to Fig. 2B except that the column was continuously irradiated with UV light during the run.

4-*p*-coumaroylquinic acid and 5-chlorogenic acid. Note that the separation is more greatly affected by *cis* or *trans* rather than by an additional phenolic OH-group with the result that the elution sequence was the two *cis* forms followed by the two *trans* forms. It thus becomes evident why a peak fraction from a chromatogram not protected from light and especially if run without suppression of carboxyl ionization often contains other members of the phenolic cinnamoyl family of compounds; the *cis* or *trans* form of homologues may overlap, equilibria can occur on the column and the slowly rising leading edge can cause overlap during a considerable elution volume. This isomerization in the sample or on the column by light may also help explain observed difficulties in reproducing chromatograms from the same plant extract sample.

Chromatograms run on Sephadex G-25 of similar samples showed similar effects —the *cis* forms preceded and were well separated from the *trans* forms, the *cis–trans* effect on separation was larger than the effect of one more phenolic OH, the

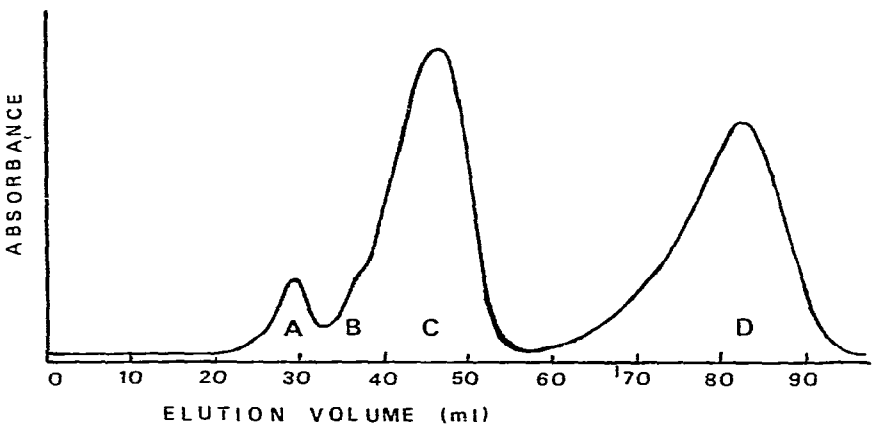

Fig. 4. Chromatography in the dark on Sephadex LH-20 with 0.2% acetic acid in water of a mixture of (A) *cis*- and (C) *trans*-4-*p*-coumaroylquinic acid and (B) *cis*- and (D) *trans*-5-chlorogenic acid.

sequence of elution with dilute acid was similar and with both adsorbents the free acids were held tighter than the quinate esters; *e.g.*, caffeic followed chlorogenic acid.

Chromatography on Sephadex LH 20 or G 25 with proper attention to the effects of light and acidic aqueous eluents can be, therefore, not only a very satisfactory technique for isolation and purification of chlorogenic-like compounds, but clearly it can also be used to study the *cis* and *trans* isomerization. We have been able to produce separate crystalline *cis* and *trans* derivatives of *p*-coumaroyltartaric acid by separation on LH 20 and concentration etc. in the dark. As far as we are aware this has not been done before since crystallization from an equilibrating mixture produced the *trans* forms of these compounds. Other systems, of course, will separate *cis* and *trans* forms of such compounds, but none seems to be as effective and as simple. Paper chromatography in aqueous solvents is low in capacity and perhaps more difficult to protect from light. Silica gel chromatography does not seem to separate certain pairs[4] and gas chromatography requires derivative formation plus smaller samples[5].

The method can be used to get very pure individual compounds by taking advantage of their *cis–trans* isomerization. For example, *cis*-chlorogenic acid isolated in the dark as a simple peak can be isomerized and rechromatographed to separate the *trans* form. Since the newly formed *trans* isomer would now fall in a portion of the chromatogram known to have been "base-line empty", it should be free of contamination.

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REFERENCES

1 G. C. Whiting and R. A. Coggins. *Phytochemistry*, 14 (1975) 593.
2 G. Kahnt, *Biol. Zentralbl.*, 85 (1966) 545.
3 G. Kahnt, *Phytochemistry*, 6 (1967) 755.
4 K. R. Hanson and M. Zucker, *J. Biol. Chem.*, 238 (1963) 1105.
5 R. D. Hartley and E. C. Jones *J, Chromatogr.*, 107 (1975) 213.