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## Note

# Reversed-phase gradient high-performance liquid chromatography of procyanidins and their oxidation products in ciders and wines, optimised by Snyder's procedures

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The procyanidins of ciders and wines are based on a C-15 catechin structure examples of which are shown in Fig. 1 (ref. 1), and cover a range of molecular size from the monomeric to the heptameric. They are important to the sensory properties and browning potential of the beverage[2,3].

It has been shown previously[4] that procyanidins can be successfully separated under isocratic conditions by reversed-phase high-performance liquid chromatography (HPLC) using acidified aqueous methanol. In an attempt to improve resolution in mixtures of wide sample polarity, gradient elution was investigated using the procedures outlined by Snyder and co-workers[5,6] for optimising conditions.

The chromatographic behaviour of a solute in a mixed eluent (*e.g.* methanol–water) is described as follows[5]:

$$\log k' = \log k_w - S \cdot \varphi \tag{1}$$

where $k_w$ = capacity ratio ($k'$) in the weak solvent (water); $\varphi$ = fraction of the strong solvent (methanol) in the eluent and $S$ = a constant with a typical value of approximately 3.

By undertaking isocratic studies and by plotting $\log k'$ against solvent composition, the values of $\log k_w$ (intercept) and $S$ (slope) may be determined.

The optimal conditions for gradient elution have been described as follows[5]:

$$\varphi' = b/S \cdot t_0 \tag{2}$$

where $\varphi'$ = percentage increase in strong solvent per unit time (*i.e.* gradient steepness); $b$ = a parameter with an optimal value of 0.1–0.2 (argued by Snyder on theoretical grounds and justified by experimental study) and $t_0$ = retention time for an unretained solute.

In using these conditions to develop separations we have noted several ways in which the chromatographic behaviour of procyanidins differs from that of smaller solute molecules.

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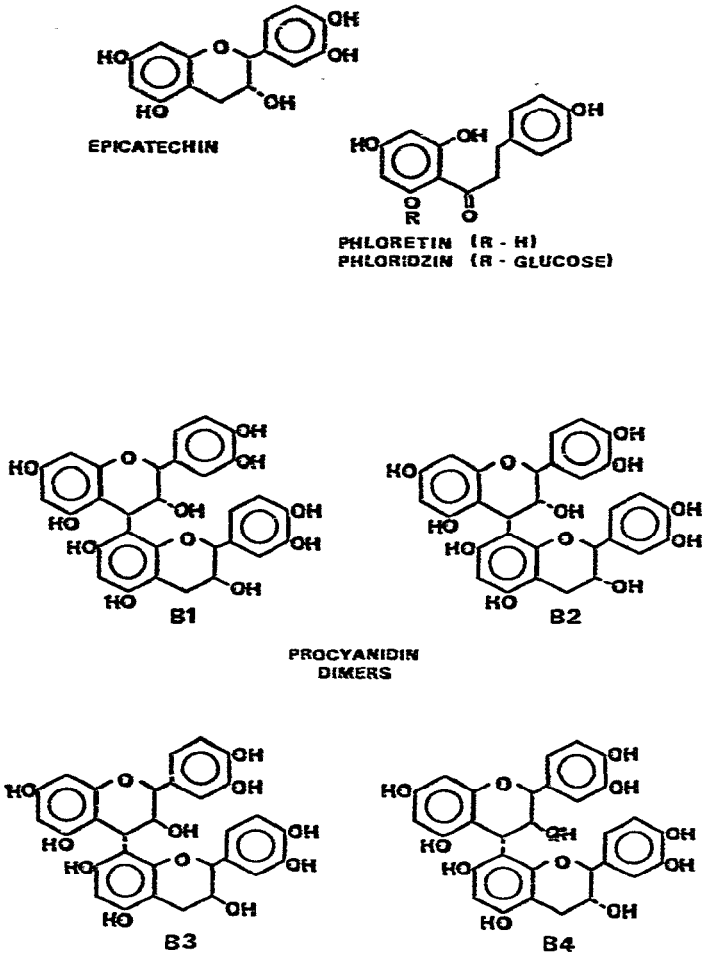


Fig. 1. Dimeric procyanidins of ciders and wines. Further polymers are built up from catechin or epicatechin in similar fashion. The phloretin glycoside, phloridzin, unique to apples, is also shown.

## EXPERIMENTAL

A Spectra-Physics SP8000 machine was used, with detection on a Pye Unicam LC3 spectrophotometer at 280 nm, 0.08 a.u.f.s. Samples were generally 10 $\mu$l of 0.1–0.4% aqueous solutions of fractions derived from wines and ciders by counter-current distribution[2,3] filtered through a 0.45-$\mu$m Millipore filter before use.

Reversed-phase columns, slurry packed in the laboratory were: LiChrosorb RP-8, 10 $\mu$m (250 × 4.6 mm); Spherisorb Hexyl, 5 $\mu$m (120 × 4.6 mm) and Hypersil SAS, 5 $\mu$m (120 × 4.6 mm).

Solvent A was water, prepared through an Elga de-ioniser and charcoal column, filtered through a 0.45-$\mu$m Millipore filter before use, and acidified to pH 2.0 or 2.5 by the addition of 0.1 or 0.01% perchloric acid, respectively. Solvent B was methanol, glass distilled from KOH, filtered through a 0.45-$\mu$m Millipore filter before use.

The water was changed daily to prevent microbial growth, and the columns and system were flushed through with methanol at the end of each working day.

All separations were carried out at 45 °C. $t_0$ was determined by injection of 0.1% uracil on to a column eluted with 80% methanol. Other conditions are noted in the text.

## RESULTS

### *Optimization of gradient*

The initial application of eqn. 2 to separation of procyanidins, using a typical value of $S = 3$, produced very poorly resolved chromatograms as in Fig. 2. Arbitrarily chosen shallow gradients improved the resolution but led to peak broadening and reduced detection sensitivity.

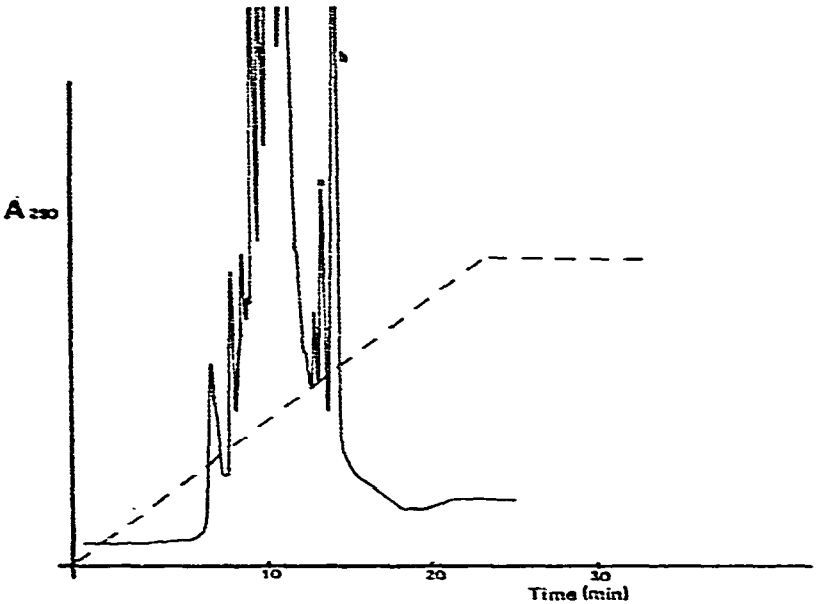


Fig. 2. Separation of a cider tannin extract ("Tremletts Bitter"). LiChrosorb RP-8. Solvent gradient (broken line) from 100% A to 100% B in 20 min. Flow-rate, 2 ml/min; $t_0$, 75 sec. a = Phloretin xyloglucoside, b = phloridzin.

To optimise conditions, therefore, isocratic studies of eqn. 1 were undertaken, typical results being shown in Fig. 3. These revealed that the value of $S$ for procyanidins on LiChrosorb RP-8, for instance, takes an average value of 8 rather than the value of 3 which is usually assumed for small molecules and which is typical of the *p*-hydroxybenzoates also shown in Fig. 3. Similar plots were also obtained for Spherisorb Hexyl. The gradient for optimal resolution from eqn. 2 becomes much shallower, therefore, typical results being shown in Figs. 4 and 5. Such conditions make it possible not only to separate the major classes of procyanidins from one another, but also to resolve the four stereoisomeric dimers B1–B4.

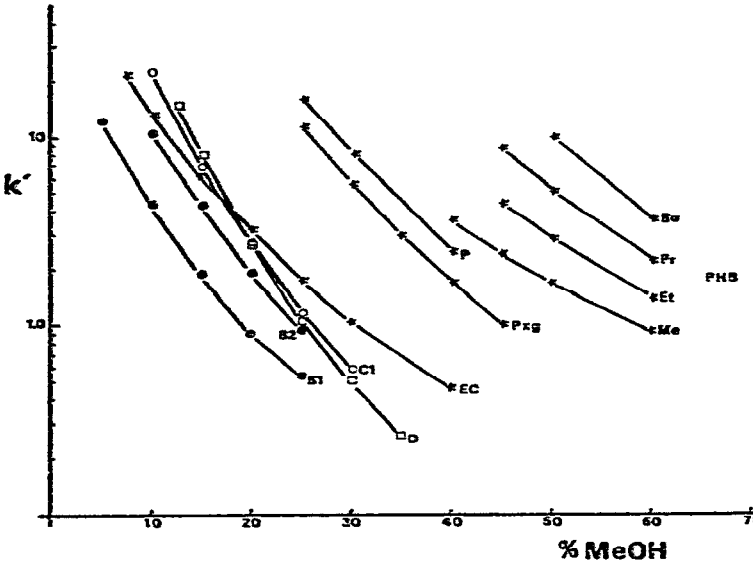


Fig. 3. Semi-log plots of $k'$ *vs.* per cent methanol (MeOH) on LiChrosorb RP-8. Me, Et, Pr, Bu PHB = methyl, ethyl, propyl, butyl *p*-hydroxybenzoates; P = phloridzin; Pxg = phloretin xyloglucoside; EC = epicatechin; B1, B2 = procyanidin dimers; C1 = procyanidin trimer; D = procyanidin tetramer.

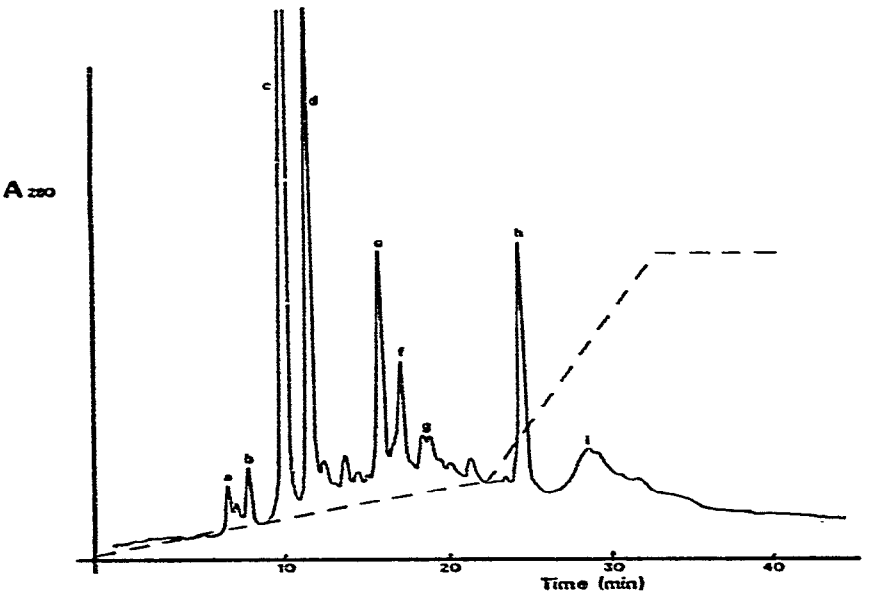


Fig. 4. Separation of a cider tannin extract ("Dabinett"). Spherisorb Hexyl. Solvent gradient (broken line) from 2% B to 25% B in 23 min, 25% B to 98% B in 10 min. Flow-rate, 1.5 ml/min; $t_0$, 47 sec. a = Procyanidin B3; b = procyanidin B1; c = epicatechin; d = procyanidin B2; e = procyanidin trimer C1; f = procyanidin tetramer(s); g = procyanidin pentamer(s); h = phloridzin; i = oxidised/polymeric procyanidins.

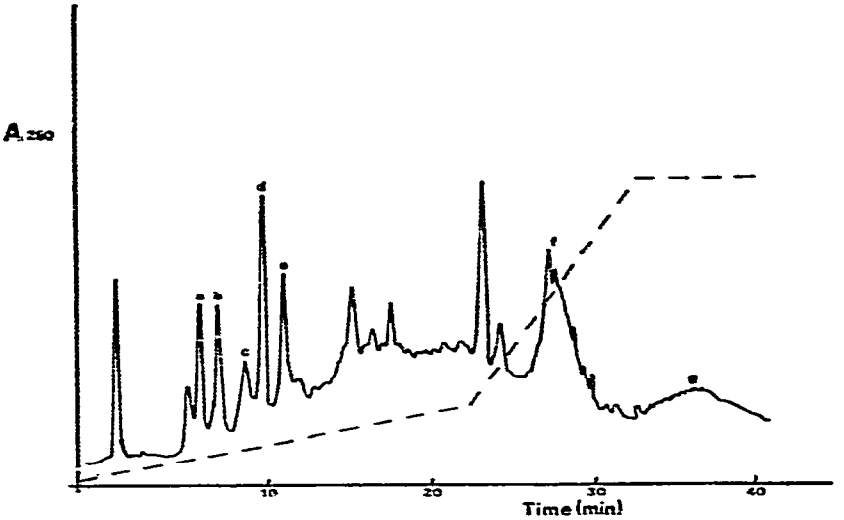


Fig. 5. Separation of a white wine tannin extract ("Müller-Thurgau"). Spherisorb Hexyl. Conditions as for Fig. 4. a = Procyanidin B3; b = procyanidin B1; c = procyanidin B4; d = epicatechin and catechin; e = procyanidin B2; f = oxidised/polymeric procyanidins; g = solvent impurities.

*Relationship between S and molecular weight*

Fig. 3 also shows that the value of $S$ increases with the procyanidin molecular weight, and it was of interest to examine this relationship. However, the plots in Fig. 3 are slightly concave and therefore it is difficult to know which particular value of $S$ should be used to characterise any particular solute. Although attempts have been made to replace eqn. 1 by a quadratic form to allow for this, it seemed that a simpler solution was presented by re-arranging Snyder's general gradient elution expression[6] into the following form:

$$S = \frac{\log(1 + 2.3 k_0 \varphi' t_0 S)}{\varphi'/(t_g - t_0 - t_d)} \quad (3)$$

where $k_0 = k'$ for a given solute in the starting composition of the gradient; $t_g$ = retention time of the solute in the gradient run and $t_d$ = delay time between gradient generator and column head.

Although no simple algebraic solution of this expression is possible, a programmable pocket calculator (Texas TI-51-III) was able to provide a solution using an iterative approximation routine.

From a single gradient run at an approximately optimal value of $\varphi'$, instantaneous values of $S$ could therefore be determined for a range of procyanidins from eqn. 3. When plotted against molecular weight on a semi-logarithmic scale, as in Fig. 6, a straight-line relationship was obtained. The intercept, for the hypothetical limiting case of a procyanidin with zero molecular weight, gave a value of $S = 3.3$ which corresponds very well with the values usually adopted for small molecules.

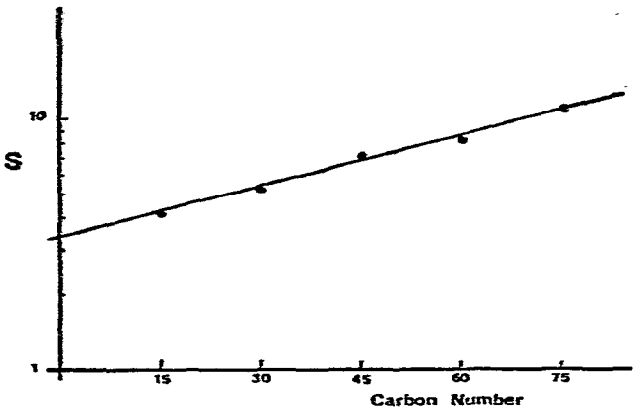


*Fig. 6.* Semi-log plot of *S vs.* molecular weight (carbon number) for procyanidins. 15 = Epicatechin; 30 = Procyanidin dimers, etc.

*Behaviour of oxidised procyanidins*

The chromatograms in Figs. 4 and 5 show a broad band which elutes after the sharp change in gradient steepness. Work with procyanidin samples from counter-current distribution which were progressively browner and more oxidised suggested that this band was associated with oxidation of procyanidins. It was further established that this band did not appear under isocratic conditions nor when operated with a continuous linear gradient. The explanation appears to be that the oxidation of procyanidins leads to an increase in ill-defined polymeric material, as has long been known[7]. Such polymeric materials do not elute with defined $k'$ values but tend instead to be spread out over the whole area of the chromatogram. As polymeric materials, however, their $S$ values are very high and so a rapid increase in solvent strength causes a marked depression in their $k'$ values. Hence they are eluted as a broad band near the "new" solvent front.

Confirmation of this effect was provided by running a sample of pure epicatechin, which displayed no oxidised band, whereas an identical sample which had been allowed to brown in solution for several weeks showed a strong oxidised band after the change in gradient steepness.

## DISCUSSION

It is obvious that reversed-phase gradient elution chromatography can be a powerful tool for the analysis of complex procyanidin mixtures, but the optimum conditions can only be determined with reference to studies of isocratic behaviour. Plots such as Fig. 3 also show the isocratic conditions under which certain separations are possible or impossible, and predict the reversal of elution order which may be observed when solvent strength is changed. Thus the isocratic elution order of procyanidins on Hypersil SAS in 20% methanol (see for instance, ref. 4), was in decreasing order of molecular weight, whereas by gradient elution starting at lower concentrations of methanol the order was generally reversed, as in Figs. 4 and 5. Incidentally, it was not possible to pursue detailed work on Hypersil SAS since this

particular packing is unstable below pH 3, whilst at higher pH values the procyanidins tail badly due to their slightly acidic nature. LiChrosorb RP-8 and Spherisorb Hexyl seem stable down to pH 2, however, and tailing is well supressed under such conditions.

The elution of polymeric or oxidised procyanidins as a defined band following a sharp change in gradient steepness may have considerable practical importance, since it now becomes possible to use this effect in studies of the oxidation and polymerisation of procyanidins in ciders and wines, work which has hitherto been hampered by a lack of suitable chromatographic techniques. The relationship between $S$ and procyanidin molecular weight may also have practical significance, since it is difficult to obtain reliable molecular weight estimations for procyanidins, and chromatographic data derived from eqns. 1 and 3 may therefore be useful in supplementing other measurements on samples where molecular weight is not known.

It is expected that a correlation should be shown between the elution order for procyanidins by reversed-phase chromatography and the elution order by countercurrent distribution between ethyl acetate and water. At first sight no such correlation is apparent but, by extrapolating the plots in Fig. 3 to high concentrations of methanol where adsorptive effects are minimised, the relative chromatographic values of $k'$ (hydrocarbon–aqueous methanol) become similar to those previously determined for the partition coefficient $K$ (ethyl acetate–water)[8], where the smaller procyanidins have the greater partition coefficients into the hydrocarbon phase.

ACKNOWLEDGEMENT

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