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Note

Reversed-phase high-performance liquid chromatography of procyanidins and other phenolics in fresh and oxidising apple juices using a pH shift technique

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The principal phenolic constituents of apple juice and ciders are phenolic acids, catechins, phloridzin and procyanidins[1,2]. Typical examples are shown in Fig. 1. These compounds, particularly procyanidins, are important for their contribution to flavour and to oxidative browning, but until recently they have been very difficult to quantify in apple juices or ciders without pre-treatment. The method described here enables the direct determination of these components, which greatly assists the study of rapid enzymic browning.

Separation of apple phenolics

Although it is possible to separate the procyanidins, epicatechin and phloridzin in apple juice and cider extracts by reversed-phase high-performance liquid chromatography (HPLC) in acidified water–methanol gradients with detection at 280 nm[3,4], analysis of complete juices or ciders is not possible without modifying the method. This is because large amounts of phenolic acids are eluted in a non-ionic form at similar retention times to procyanidins of interest. Fig. 2 demonstrates the interference caused by phenolic acids at pH 2.5.

The pK_a of chlorogenic acid[5] is 3.5 and its retention time is thus pH-dependent in the range pH 1.5–5.5. It is therefore possible to take advantage of a pH shift during the run to improve the resolution of procyanidins and so to permit the analysis of complete cider or juices. By operating the column initially at pH 7.0 the phenolic acids are quickly eluted in an ionised form, but by reverting to pH 2.5 for the remainder of the run the procyanidins are eluted free from interference. Fig. 3 shows the improvement which is possible by this technique. For example, *ca.* 50 ppm of procyanidin B2 may now be detected in a Cox apple juice even in the presence of 300 ppm of chlorogenic acid. Since the phenolic acids are eluted close to the void volume with other non-phenolic materials, it is advisable to re-run the sample at pH 2.5 throughout if it is necessary to quantify these acids, and in this case to monitor at 320 nm where interference from catechins and procyanidins is effectively nil.

It has been found that buffer salts must be kept to a minimum if good procyanidin peak shape is to be maintained. The pH of the neutral eluent is not critical so long as it is above pH 5.5, which can be maintained by adding a trace of ammonia to

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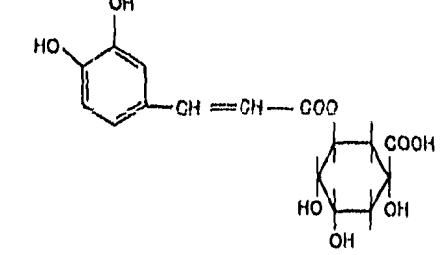
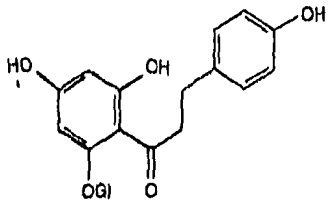
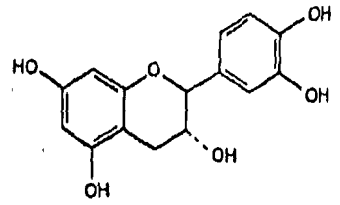


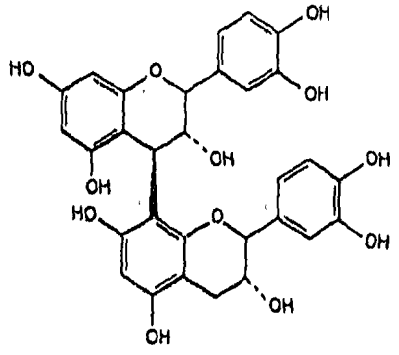
Chlorogenic acid



Phloridzin



(−) Epicatechin



Procyanidin B2

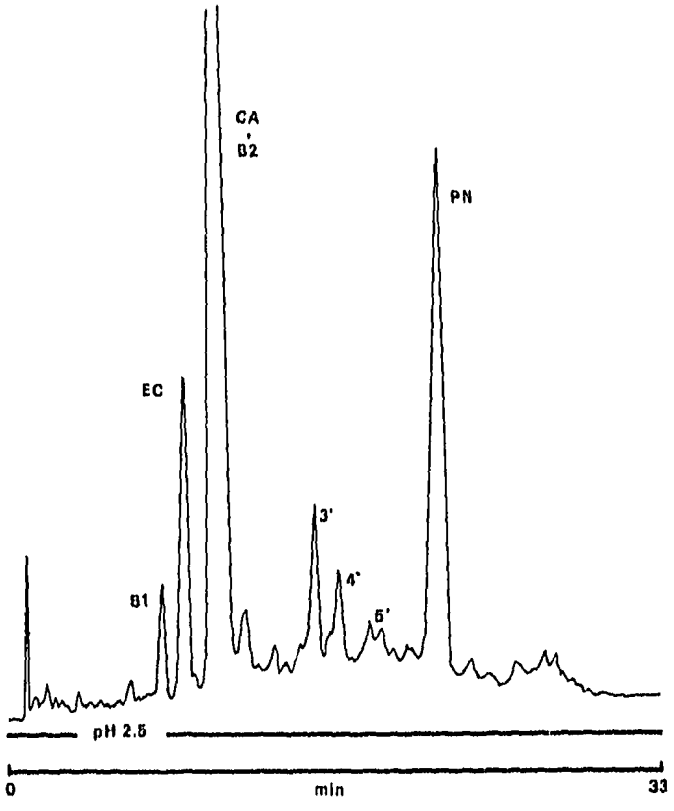


Fig. 1. The principal phenolic constituents of apple juice.

Fig. 2. Separation of Dabinett cider phenolics at pH 2.5. Peaks: CA = chlorogenic acid; EC = epicatechin; B1 and B2 = procyanidin dimers; 3°, 4° and 5° = procyanidin oligomers; PN = phloridzin.

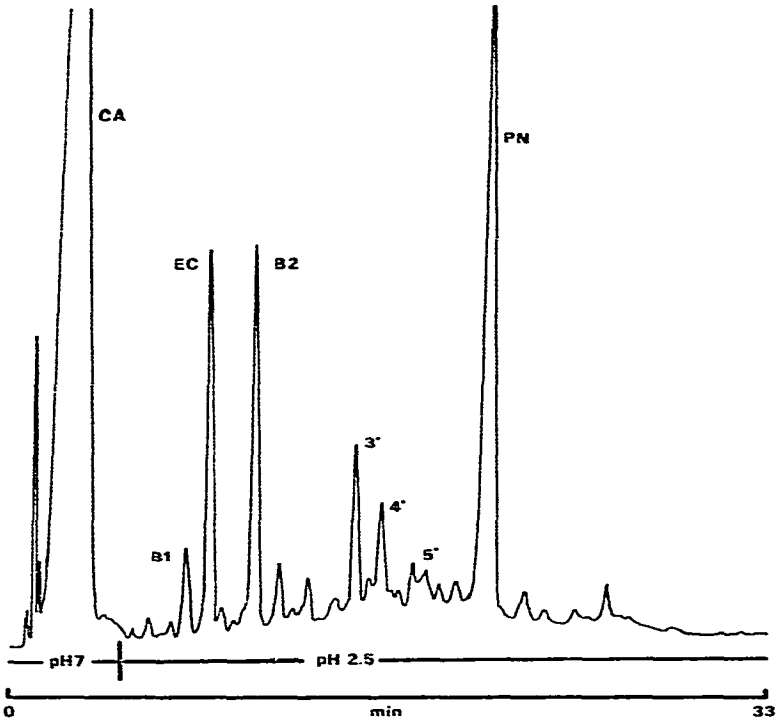


Fig. 3. Separation of Dabinett cider phenolics by the pH shift technique. Peaks as in Fig. 2. For conditions see text.

distilled water in equilibrium with aerial CO_2. Operating much above pH 7.0 may lead to solution of the silica-based packing and decrease the resolution of procyanidins. The shift from neutrality to pH 2.5 is essential for good peak shape during the remainder of the run, so it is important to allow sufficient equilibration time for neutral conditions to be restored at the beginning of the next run. Although this method was developed for the study of ciders, it is equally applicable to similar problems in red or white wines.

Study of oxidising juices

The system described above now permits detailed study of phenolic changes in oxidising apple juices, particularly when dual wavelength monitoring is used. Fig. 4 shows the separation of a totally unoxidised apple juice monitored both at 280 nm and 420 nm. Fig. 5 shows an analysis of the same juice after natural enzymic oxidation for 1 h. All the native phenolic compounds have diminished to differing extents and several new coloured peaks have appeared. Amongst these is the broad peak (Y), which is believed to consist largely of procyanidin polymers whose chromatographic behaviour following a sharp change in gradient steepness has been discussed previously[3]. Thus the new method provides a powerful tool for investigating the detailed mechanism of colour formation in apple juice.

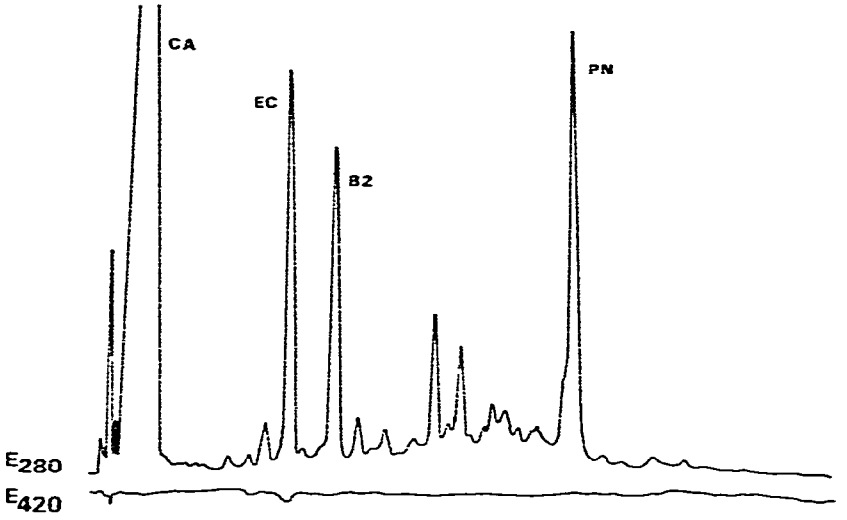


Fig. 4. Separation of phenolics in unoxidised Dabinett apple juice by the pH shift technique. Conditions as in Fig. 3.

EXPERIMENTAL

Chromatography

A Spectra-Physics SP 8000A machine was used, with detection at 280 nm supplemented by a variable-wavelength LC3 detector (Pye Unicam). The column was 100 × 5 mm I.D. (Shandon) packed in this laboratory with Spherisorb 5 Hexyl

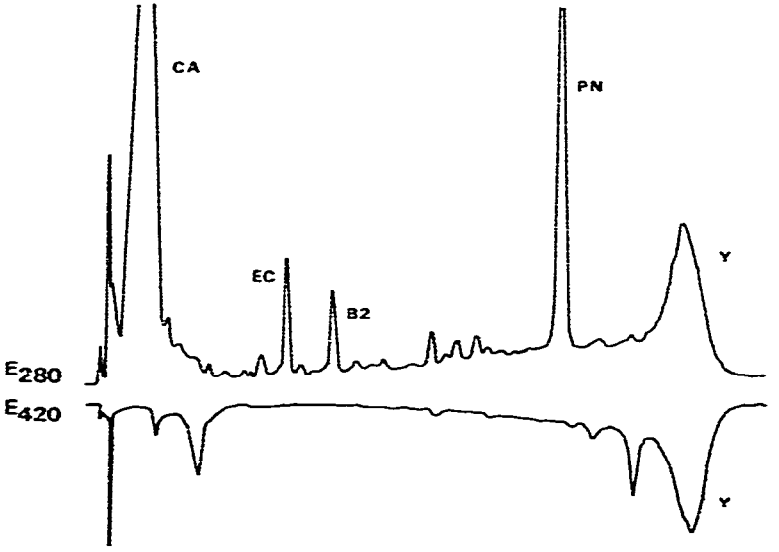


Fig. 5. Separation of phenolics in oxidised Dabinett apple juice by the pH shift technique. Conditions as in Fig. 3.

TABLE I

PERCENTAGE COMPOSITION OF THE SOLVENT

Time (min)	*A (water, pH 2.5)*	*B (methanol)*	*C (water, pH 7.0)*
0	0	2	98
3	0	5	95
3.1	95	5	0
23	75	25	0
33	2	98	0
35.9	2	98	0
36	0	98	2
40	0	98	2
45	0	2	98

(Phase Separations) and operated at 45°C. Sensitivity was 0.02–0.16 a.u.f.s. depending on the sample, and 20 or 50 μl of juice or cider was injected. Solvent A was charcoal-filtered and de-ionised (Elga) water brought to pH 2.5 with *ca.* 0.1% perchloric acid; solvent B was methanol redistilled from KOH; solvent C was water neutralised to pH 6.5–7.0 with ammonia. The flow-rate was 1.5 ml/min and the solvent gradient was as shown in Table I.

After the run, a further 30 ml of starting solvent was pumped to ensure complete neutrality. All solvents and samples were filtered through 0.45-μm HATF membranes (Millipore) before use (FHUP for methanol).

Preparation of apple juice

Typically, one apple of the cider variety "Dabinett" was chilled to 4°C for several hours and then extracted in a domestic juice extractor consisting of a grater plate and basket centrifuge (Moulinex). Part of the juice was collected into a helium-filled tube, centrifuged briefly, and filtered through a 0.45-μm membrane before direct injection into the machine. Since the cider apple polyphenoloxidase is tightly membrane bound[6], this prevented any oxidation and resulted in a water-white juice. The remainder of the juice was allowed to brown naturally in the presence of its solids and was centrifuged and filtered for analysis after 1 h.

ACKNOWLEDGEMENT

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