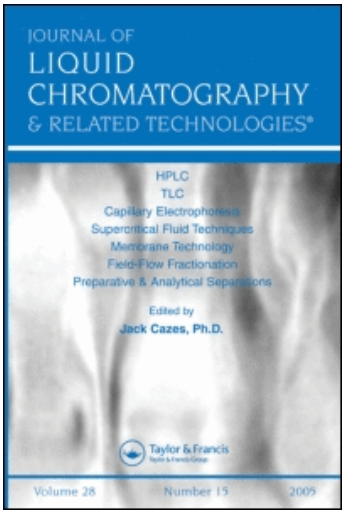

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DETERMINATION OF ORGANIC ACIDS IN APPLE AND CIDER BY LIQUID CHROMATOGRAPHY WITH ORDINARY AND NARROW-BORE COLUMNS. A COMPARATIVE STUDY

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ABSTRACT

Two narrow-bore columns packed with octadecylsilane of various particle sizes were used to compare their efficiency for the separation of organic acids in apple and cider with that of ordinary columns. The best simultaneous resolution of quinic, malic, shikimic, lactic, acetic, citric and succinic acid was accomplished by using a 100 x 2.1 mm ID, 3-μm Spherisorb ODS-2 column and a phosphate buffer as the mobile phase. This chromatographic system provided a separation efficiency comparable to that afforded by an ordinary 250 x 4.6 mm ID, 5-μm Spherisorb ODS-2 column, plus greater rapidity (30%) and economy, all of which allowed the accurate, precise determination (CV = 3%) of the above-mentioned compounds. Finally, the performance of an ordinary UV detector and that of a rapid spectral detector in this type of determination were critically compared.

INTRODUCTION

Developments in liquid chromatography based on columns of small diameters should basically be credited to the work of Scott (1–3), Novotny (4–7) and Ishii (8). These columns can classified into different categories according to their inner diameter, namely: open tubular capillary columns (ID between 3 and 50 μm), packed capillary columns (ID between 50 and 200 μm), microbore packed columns (ID of 0.5–1 mm) and narrow-bore packed columns (ID in the range 1–2.5 mm).

Some types of column, particularly microbore and narrow-bore columns can be used as such on commercially available chromatographs and offer some analytical advantages over ordinary columns with inner diameters between 2.5 and 9 mm. Thus, the lower mobile phase flow-rates typically required result in solvent savings, which can be of great interest in dealing with large numbers of samples in routine analyses.

The reduced inner diameters of narrow-bore columns also lower the minimal amount of analyte that can be determined. Small column bores have been shown to provide taller chromatographic peaks than wider-bore columns under identical working conditions (9); comparisons in this respect, however, are difficult to establish because of need to suit the geometry of the detector cell used to the column bore.

One of the most serious shortcomings of narrow-bore columns is that the variance of each peak, which determines its dispersion, is a function not only of intrinsic features of the column (efficiency, dimensions) but also of a series of external factors such as connecting tubes, frits, injection systems, etc., which contribute to the overall dispersion and ultimately to band broadening, thereby affecting resolution.

The external contributions to dispersion should always be taken into account and can be critical in using narrow-bore chromatographic columns, so much so that the resolution potentially provided by a column can be reduced by some of these factors. This compels users to minimize external contributions by optimizing the chromatographic set-up to be used in each instance.

We took account of all the above-mentioned advantages and disadvantages to develop a method for the separation and determination of quinic, malic, shikimic, lactic, acetic, citric and succinic acid by using narrow-bore columns (100 x 2.1 mm ID) packed with octadecylsilane.

The proposed method was applied to the determination of major acids in extracts from cider apples, the quality and nutritional value of which is conditioned to a significant extent by these compounds. The method can equally be used to monitor the evolution of the aforesaid organic acids in some industrial apple derivatives, particularly cider, as it includes acids that can occur in the samples as a result of fermentative processes in the musts.

The results obtained are compared below with those provided by ordinary chromatographic columns (4.6 mm ID) in the analysis of identical samples. The sensitivity afforded by an ordinary UV detector and a modern diode array detector as applied to this type of analysis is also critically compared.

EXPERIMENTAL

Apparatus and conditions

The instrumental set-up used consisted of a Hewlett-Packard HP 1090 liquid chromatograph, a Rheodyne 7010 injection valve with a 5-µl loop, a Hewlett-Packard

79881A filter photometric detector, a Hewlett-Packard HP-85B personal computer and a Hewlett-Packard 3390A integrator. Column effluents were monitored at 210 nm. Alternatively, detection was performed by using an LKB 2140 rapid spectral detector and an IBM data station. Analyses were done in the isocratic mode by using 0.01 M KH_2PO_4/H_3PO_4 at pH 2.25 and 25°C as eluent. The HPLC columns used were as follows: Hypersil ODS (100 x 2.1 mm ID, 5 μm), Spherisorb ODS-2 (100 x 2. 1 mm ID, 3 μm) and Spherisorb ODS-2 (250 x 4.6 mm ID, 5 μm).

Materials and reagents

Analytical-grade quinic, malic, lactic, acetic, shikimic, citric and succinic acid standards supplied by Sigma (St Louis, MO) and Merck (Darmstad, FRG) were used without further purification.

The mobile phases used were buffered solutions containing 0.01 M KH_2PO_4 and adjusted to different pH values with H_3PO_4. All solvents used were HPLC grade and were employed as supplied by manufacturers. High-purity water was obtained by distillation through a Millipore Milli-Q system.

Apple extracts were obtained according to the procedure of Richmond *et al.* (10) as modified by ourselves (11) to ensure extraction of other typical components of apples such as phenolic compounds. Prior to injection, both the apple extracts and ciders were filtered across a 0.45-μm Millex membrane to remove any impurities. Carbonated ciders were also degassed for 5 min in an ultrasonic bath.

All samples were analysed in triplicate to calculate average deviations in order to determine the reproducibility of the chromatographic measurements.

RESULTS AND DISCUSSION

We studied the effect of the elution conditions (pH, ionic strength, temperature and flow-rate) on the resolution of the acids concerned achieved with the normal and narrow-bore columns used by varying one parameter in turn while keeping all other constant. For this purpose we used similar octadecylsilane stationary phases of different particle sizes, namely 3 and 5 μm. The optimal working conditions were found to be as follows: pH 2.25; temperature 25°C; ionic strength 0.01 M KH_2PO_4; mobile phase flow-rate 0.5 ml/min (normal column) and 0.1 ml/min (narrow-bore column). UV detection was performed at 210 nm.

As can be seen in Fig. 1, which shows the chromatograms obtained with the three types of columns used, the narrow-bore column packed with 5-μm particles provided much poorer resolution than the other two (the peaks of malic and shikimic acid are completely overlapped). The higher efficiency of the narrow-bore column packed with 3-μm particles and that of the ordinary column packed with 5-μm particles enables adequate resolution of quinic, malic, lactic, acetic, shikimic, citric and succinic acid. Most of the acids present in the standard mixed solution featured baseline separation and eluted as sharp peaks.

Table 1 lists the detection limits obtained with the two types of column by using the ordinary filter detector and the diode array detector. As can be seen, the limits afforded by the narrow-bore column are slightly lower than those provided by the ordinary column, the sensitivity was also about twice. Analysts are using diode array detectors (DADs) increasingly in order to

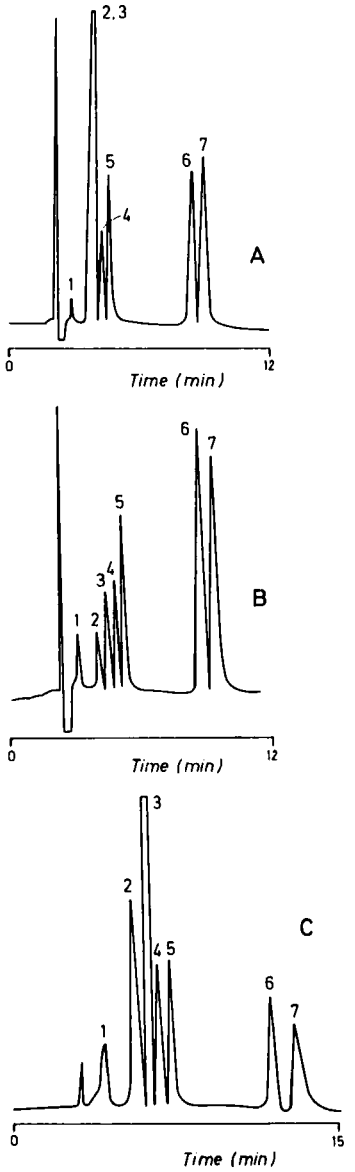

FIGURE 1. Chromatograms obtained from standard solutions of (a) quinic, (2) malic, (3) shikimic, (4) lactic, (5) acetic, (6) citric, and (7) succinic acid by using the following columns: (A) Hypersil ODS (100 x 2.1 mm ID, 5 µm), Spherisorb ODS-2 (100 x 2.1 mm ID, 5 µm), and (C) Spherisorb ODS-2 (100 x 2.1 mm ID, 3 µm). Mobile phase: 0.01 M KH_2PO_4/H_3PO_4 at pH 2.25. Flow-rate: (A) and (B) 0.1 ml/min; (C) 0.5 ml/min.

TABLE 1

Detection Limits (ng, *µg) of the Organic Acids Determined by Using Narrow-Bore and Normal-Bore Columns with Ordinary Photometric and Diode Array Detection.

Acid	Narrow-bore column Photometric detection	Normal-bore column Photometric detection	Normal-bore column Diode array detection
Quinic	34.5	45.0	2.2*
Malic	25.1	37.5	2.0*
Shikimic	0.4	0.6	33.3
Lactic	43.6	75.0	2.5*
Acetic	47.0	60.0	1.9*
Citric	29.0	50.0	2.4*
Succinic	58.0	108.0	5.0*

expedite and facilitate optimization of the separation and detection of analytes. However, as can be seen in Table 1, the detection limits provided by the DAD used were between 40 and 85 times higher than those provided by a good ordinary detector.

This is quite significant to the analysis of fruits and their industrial derivatives, which involves the determination of acids occurring in small amounts in the fruits (e.g. shikimic acid in apples) and evolving significantly during ripening, the evolution being quite difficult to monitor accurately with DADs, as is that of other compounds such as quinic acid, which occur in readily measurable quantities at some stage of the fruit ripening but in much smaller amounts by the end of the process. These problems are also encountered in determining organic acids in cider, from which some (e.g. quinic and malic acid) tend to disappear through fermentation, while others (lactic, acetic acid) appear as a result of the typical biochemical transformations involved in the fermentation of the juices and are rather

difficult to quantify at some stages of the process on account of their very low concentrations. These pitfalls can be circumvented to a great extent by using an ordinary detector connected with narrow-bore columns. In any case, diode array detectors are irreplaceable as regards the ease of optimization of detection conditions (12,13).

The time required for the simultaneous determination of the seven above-mentioned acids by using the narrow-bore column was 10 min, i.e. about 30% less than with the technique typically used for this purpose. In addition, the mobile phase flow-rate used (0.1 ml/min) was five times lower than that normally used with ordinary columns; this, together with the shorter overall analysis time, results in significantly decreased analytical costs.

The retention times achieved under the working conditions used were found to be highly reproducible. Six different analyses provided an average variation in the retention times of the organic acids in the standard synthetic mixture of 0.18% (0.05—0.31%) as CV.

The organic acids found to occur in appreciable concentrations in the apple extracts assayed were malic, quinic and shikimic acid. Citric acid could not be determined by either method because of its rather low concentration and was only detected when malic acid was present at higher concentrations because the two experience a similar evolution. The ciders resulting from fermentation of apple extracts normally contain succinic and lactic acid as major components, plus acetic acid resulting from anomalous fermentative processes.

Figures 2 and 3 illustrate the separation of major organic acids in an apple extract and a cider, respectively, under the above-described working

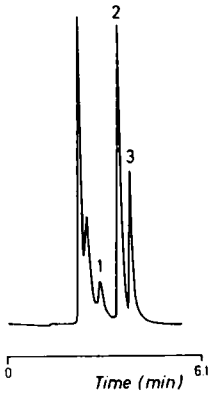

FIGURE 2. Typical chromatogram obtained from the organic acids in the apple extracts by using a Spherisorb ODS-2 (100 x 2.1 mm ID, 3 μm) column, 0.01 M KH_2PO_4/H_3PO_4 at pH 2.25 as mobile phase and a flow-rate of 0.1 ml/min. (1) Quinic, (2) malic, and (3) shikimic acid.

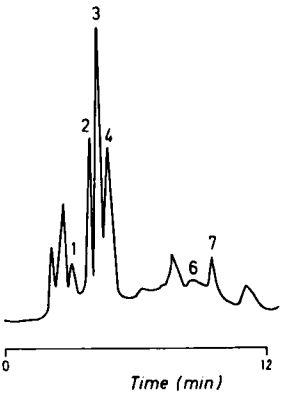

FIGURE 3. Typical chromatogram obtained from the organic acids in cider by using the same column and chromatographic conditions as in Fig. 2. (1) Quinic, (2) malic, (3) shikimic, (4) lactic, (5) citric, and (6) succinic acid.

TABLE 2

Results Obtained in the Determination of Organic Acids in Apples by Using a Narrow-Bore Column.

Apple variety	Quinic acid	Shikimic acid	Malic acid
Collaos	1.01 ± 0.3	30.0 ± 0.1	9.09 ± 2.8 (9.14 ± 1.9)
Durón Arroes	0.86 ± 0.3	52.1 ± 0.02	6.80 ± 1.0 (7.08 ± 0.5)
Raxao	0.34 ± 0.9	30.6 ± 0.04	10.34 ± 1.0 (10.33 ± 4.7)
Meana	0.98 ± 0.5	29.3 ± 0.02	10.50 ± 0.9 (10.44 ± 1.7)
Coloradona	0.58 ± 0.04	13.9 ± 0.01	3.05 ± 2.1 (3.08 ± 4.1)

The numbers in brackets are the malic acid concentrations obtained with a normal-bore column The concentrations of quinic and malic acid are expressed in mg/g; those of shikimic acid in µg/g.

conditions. The solutes in the apple extract were quantified by the external standard method. Standards were injected and the resulting integrator response factors were computed and then processed by the integrator to deliver the unknown concentrations. The injected volume used was always 5 µl and the amount of each organic acid present was directly obtained from the data module. Regular recalibrations were carried out in order to obtain new response factors.

In order to check the accuracy of the proposed method, we determined recoveries by analysing apple extracts as such and spiked with known amounts of the organic acid. All analyses were carried out in triplicate at two concentration levels. The average recoveries obtained ranged between 95 and 106% with the narrow-bore column and from 88 to 106% with the normal-bore column. These results testify to the accuracy of the proposed method.

The precision of the method was checked by analysing each sample in triplicate. The coefficients of variation thus obtained were always less than 3% for the narrow-bore column, i.e. much better than those provided by the ordinary column (5% on average).

Table 2 lists the results and standard deviations obtained in triplicate determinations of extracts from five apple varieties, namely Raxao (sharp), Collaos (mild sharp), Meana (bitter-sharp), Durón Arroes (sweet) and Coloradona (bitter-sweet), with a narrow-bore column. For comparison, the table also lists the results obtained for malic acid by using an ordinary column. As can be seen, the two methods provided consistent results.

CONCLUSIONS

Reversed-phase chromatography with narrow-bore columns packed with 3-μm particles and a buffered mobile phase to avoid solute ionization probably makes the most straightforward, rapid and economic alternative to the separation and determination of carboxylic acids.

Because of its brilliant performance, the proposed method is particularly suitable for determining the sensory properties of fruits and controlling industrial processes involved in the obtainment of juices and fermented beverages.

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