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Determination of monosaccharides in cider by reversed-phase liquid chromatography

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Abstract

A reversed-phase high-performance liquid chromatographic (HPLC) method is described for the simultaneous determination of aldoses and uronic acids in cider, previously derivatized with *p*-aminobenzoic ethyl ester (ABEE). Narrow-bore C8 columns are recommended as this alternative provides good separation efficiency, along with greater economy and sensitivity. Detection limits for aldoses (glucose, galactose, xylose, arabinose, ribose, fucose, and rhamnose) and uronic acids (D-glucuronic acid and D-galacturonic acid) range between 82 and 182 ng ml^{-1}. The ABEE derivatives are separated in 29 min. Recovery studies showed good results for all solutes (90–102%). The method is linear for all compounds over the concentration range tested, and precision was found to be satisfactory (R.S.D. < 5%). © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Monosaccharides; Uronic acids; *p*-Aminobenzoic ethyl ester; Cider

1. Introduction

Carbohydrates are among the most abundant compounds found in nature [1], and the analysis of sugars and sugar mixtures is of considerable importance to the food and beverage industry. The analysis of sugars in cider is of great interest, because sugars contribute to the flavor and to the sensory characteristics of ciders [2,3], and many ciders are obtained by mixing varieties of apples with different contents of sugars and other components such as organic acids and polyphenols. Saccharides are important for the nutritive value [1] and they can be used for the detection of adulteration, carried out by adding corn cane or beet sugar to apple juice. In addition, the analysis of sugars could be used to differentiate between ciders manufactured from apple juice obtained by fruit pressing or by liquefaction, since liquefaction increases aldoses and uronic acid contents [4].

A variety of chromatographic systems may be used to separate and analyze monosaccharides. Paper and thin-layer chromatography were the first chromatographic techniques used to separate individual sugars, but separations were limited to the number of recognized analytes [5], present poor resolution and are not always quantitative [6].

Gas–liquid chromatography with flame ionization detector or mass spectrometer has been used for the analysis of saccharides. This methodology is very powerful for structural analysis, but it needs very tedious derivatization steps before the analysis [7,8].

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A number of authors have studied and described the mechanisms of the separation of sugars by high-performance liquid chromatography (HPLC) with various stationary phases: silica gel [9,10], amine-bonded silica [11–14], polystyrene-based anion- [15–17] and cation- [3] exchange resins, and C18-bonded silica [18–24]. Each stationary phase presents certain advantages and drawbacks.

The lack of chromophores or fluorophores in the structure of monosaccharides limits the modes of detection. Refractive index detection and other related methods do not often meet the demands of modern trace level analysis with regard to sensitivity and/or selectivity. Chemical derivatization techniques are vital tools to circumvent this problem. Derivatization can be used to improve the chromatographic properties of the analyte of interest and to improve its efficient trace determination [25]. A large variety of reagents, UV active and fluorophore derivatization reagents have been suggested in the literature for this purpose, such as 3-methyl-1-phenyl-2-pyrazolin-2-one [24,26], 8-aminopyrene-1,3,6-trisulfonate [27], benzamidine [28], Fmoc-hydrazine [21], phenylisocyanate [19], 2-aminobenzoic acid [18], *p*-aminobenzoic ethyl ester (ABEE) [11,14,23,29], aminopyrazine [30], among others. The derivatization of reducing sugars with ABEE is easy and required no special equipment; moreover, the method showed higher sensitivity and elimination of the possible doublet that could be formed by mutarotation of the free reducing end of sugars. The separation of ABEE-derivatized monosaccharides was carried out on an amino-bonded vinyl alcohol copolymer gel column [14]. However, the peaks of fucose and rhamnose, and xylose and arabinose were overlapped. Likewise, it was not possible to separate the peaks of xylose, arabinose, and ribose on a C18 column [11], unless alkaline mobile phase (pH 9) was used [23].

The present paper is specifically concerned with the application of the HPLC method to the determination of the aldoses (glucose, galactose, xylose, arabinose, ribose, fucose, and rhamnose) and uronic acids (D-glucuronic acid and D-galacturonic acid) found in natural and sparkling ciders. The reducing sugars are derivatized with ABEE in the presence of sodium cyanoborohydride for UV detection, and separated on C8-bonded silica column.

2. Experimental

2.1. Reagents and standards

Aldoses and uronic acid standards, ABEE, sodium cyanoborohydride, sodium citrate, and citric acid monohydrate were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). Acetic acid was obtained from Probus (Badalona, Spain). HPLC-grade acetonitrile, methanol, chloroform, and tetrahydrofuran were purchased from Merck (Darmstadt, Germany). Milli-Q water (Millipore, Milford, MA, USA) was used throughout. All other chemicals and solvents were of analytical reagent or HPLC grade.

2.2. Derivatization procedure

The procedure employed for the derivatization of aldoses and uronic acids at their reducing end with ABEE was carried out according to the method of Wang et al. [10] modified by us.

To a sample solution containing 100 μmol sugars in 5 ml water was added 400 μl of 1.4 M $NaBH_3CN$ in distilled water, 400 μl of glacial acetic acid, and 2 ml of 0.6 M ABEE in methanol, and the mixture was heated at 80°C for 10 min. After cooling to ambient temperature, 2 ml of distilled water was added. The aqueous phase was extracted with 4 ml of chloroform to remove excess ABEE and the aqueous layer was subjected to HPLC analysis. Fig. 1 shows the equation of the derivatization reaction.

2.3. HPLC equipment and conditions

HPLC analyses were performed on a Shimadzu HPLC system equipped with two LC-10AD pumps, a UV–VIS SPD-M10AD photodiode array detector, a Sil-10AD automatic injector, and Gastor 150 LCD de gass on-line. A Tracer Extrasil ODS2 (Teknokroma, 150 mm × 2.1 mm i.d., 3 μm), Spherisorb C8 (Kromxpek, 150 mm × 4.6 mm i.d., 3 μm), discovery C16 amida (Sigma, 150 mm × 2.1 mm i.d., 5 μm), and Kromasil C8 (Teknokroma, 200 mm × 2.1 mm i.d., 3.5 μm) were used.

The analysis was monitored at 307 nm and the absorption spectra of compounds were recorded between 250 and 350 nm. The gradient conditions are given in the figure captions. The column was operated at 45°C.

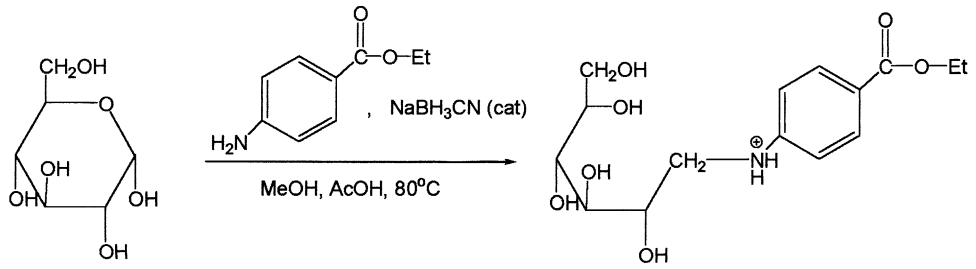

Fig. 1. Labeling reaction of glucose with *p*-aminobenzoic ethyl ester.

The sample injection volume was 5 μl. Identification of compounds was carried out by comparing their retention time values and UV spectra with those of standards previously synthesized separately and stored in the spectrum library. Quantitative determination was performed using the external standard method. All mobile phase solutions were filtered through a 0.45 μm membrane filter.

3. Results and discussion

3.1. Optimization of the derivatization procedure

A standard mixture of 100 μmol of sugar was used to evaluate the optimum reaction conditions of the derivatization procedure. The effects of concentration, temperature, and reaction time of the ABEE in the reaction mixture were examined. A 2 ml of solution of 0.6 M ABEE (200 mg) was found to be satisfactory for a quantitative derivatization of the sugars. The reaction time at which the reaction reached completion at 80°C was 10 min for all compounds, as can be seen in Fig. 2.

3.2. HPLC separation of ABEE–sugar derivatives

Separation of ABEE–sugar derivatives of selected monosaccharides was investigated by using various reversed-phase stationary and mobile phases. The aldoses and uronic acids derivatives were not separated on the C18 and C16 columns under any of the conditions tested due to the fact that the chromatographic peaks are too broad and asymmetric. The conventional C8 column (150 mm × 4.6 mm i.d., 3 μm) showed a good elution profile of derivatized solutes. Nevertheless, it was not possible to separate glucose from galactose, nor arabinose from xylose and ribose.

A substantial improvement in the separation of aldoses and uronic acids derivatives was obtained using a C8 micro-bore (200 mm × 2.1 mm i.d., 3.5 μm) column. To study the behavior of ABEE derivatives in this C8-bonded silica column, different mixtures of

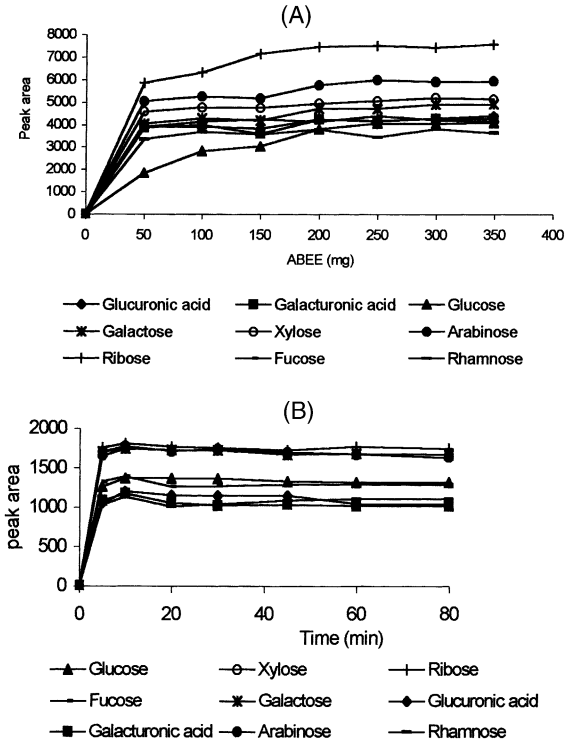

Fig. 2. Optimization of the conditions for the labeling reaction: (A) relationship between reagent amounts and relative peak area; (B) relationship between reaction time and relative peak area.

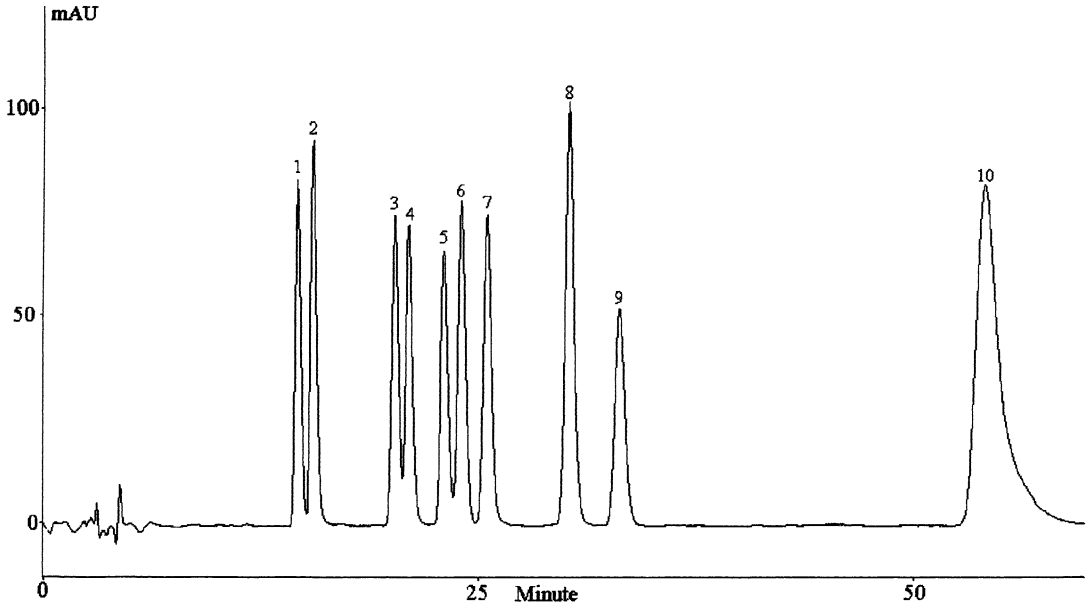

Fig. 3. Chromatogram of ABEE–sugar derivatives run in isocratic mode. Column, Kromasil C8 (200 mm × 2.1 mm i.d., 3.5 μm); mobile phase: solvent A (100 mM sodium citrate buffer, pH 5.5/THF = 88/12) and solvent B (acetonitrile) in a ratio of solvent A/solvent B = 99/1 (v/v%) at 45°C; flow rate, 0.15 ml min^{-1}, injection volume, 5 μl. Peaks: (1) D-glucuronic acid; (2) D-galacturonic acid; (3) glucose; (4) galactose; (5) xylose; (6) arabinose; (7) ribose; (8) fucose; (9) rhamnose; (10) excess ABEE.

aqueous buffers (at different ionic strengths and pH), methanol, acetonitrile, and tetrahydrofuran were used as mobile phases at different temperatures. As can be seen in Fig. 3, a good separation was achieved isocratically with solvent A (100 mM sodium citrate buffer, pH 5.5/THF = 88/12) and solvent B (acetonitrile) in a ratio of solvent A/solvent B = 99/1 (v/v%) at 45°C. For the purpose of decreasing the retention times, another approach is to use the gradient mode beginning with solvent A/solvent B (99:1) until 20 min, and then to increase the elution strength gradually with solvent B (20–28 min, 20% B), as shown in Fig. 4. The shape of the peaks was very sharp, and by using the narrow-bore column it was clear that less mobile phase solvent is consumed (flow rate of 0.15 ml min^{-1}), and thus the analytical cost is lower.

3.3. Validation

Standards linearity was verified by the analysis of duplicates of six points in the range of 10–150 μg of galactose, xylose, arabinose, fucose, rhamnose, ribose and D-glucuronic acid, and 10–500 μg of D-galacturonic acid and glucose. The calibration graphs for all the sugars showed a good correlation between the peak areas and sugar concentrations; the regression coefficients being >0.999 in all cases. The linearity of the calibration graphs was also checked using two different statistical tests (linearity and proportionality tests). For the linearity test, the values obtained from the Fisher test were always higher than the tabulated values ($\alpha = 0.05$), standard deviations of the slope and the response factor values were lower than 2 and 5%, respectively. In the proportionality test, the Student *t*-test values calculated for the intercept were always higher than the tabulated values ($\alpha = 0.05$), and the Student *t*-test values obtained for the slope were always lower than the tabulated ones for the same level of significance. Linearity was thus demonstrated.

The precision of the method was investigated using cider samples. Inter-day analysis was used to perform the validation. Total 12 repeated injections of a sample gave a relative standard deviation (R.S.D.) for instrumental repeatability that ranged between 0.54 and 1.58%. Six different samples injected in triplicate gave R.S.D. ranging between 1.68 and 3.48% for

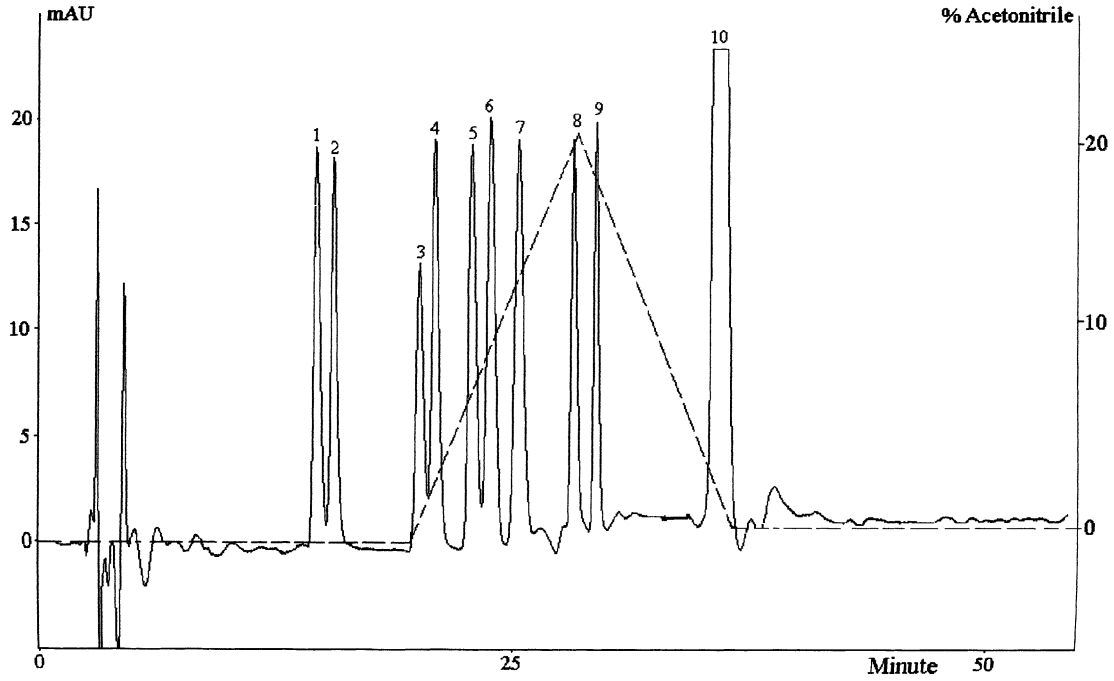

Fig. 4. Chromatogram of ABEE–sugar derivatives run in gradient mode. Column, Kromasil C8 (200 mm × 2.1 mm i.d., 3.5 μm); mobile phase: solvent A (100 mM sodium citrate buffer, pH 5.5/THF = 88/12) and solvent B (acetonitrile); gradient program: 1–20 min, 1% solvent B; 20–28 min, 20% solvent B; 28–36 min, 1% solvent B; temperature, 45°C; flow rate, 0.15 ml min^{-1}, injection volume, 5 μl. Peak numbers as in Fig. 3.

method repeatability. The inter-day reproducibility of the method was carried out by different analysts and was also very good for all sugars; the R.S.D. obtained ranged between 2.1 and 5.0%.

The detection limits are shown in Table 1. These were determined by injecting 5 μl of progressive dilutions of a concentrated standard mixture, each one derivatized as mentioned above in the derivatization procedure, followed by the preparation of calibration plots (peak area versus concentration injected), which were extrapolated to a signal-to-noise ratio (*S*/*N*) of 3, so as to assign the detection limit.

Table 1
Detection limits of aldoses and uronic acids

Sugars	Detection limit (ng ml^{-1})
D-Glucuronic acid	82
D-Galacturonic acid	65
Glucose	108
Galactose	124
Xylose	135
Arabinose	163
Ribose	182
Fucose	147
Rhamnose	174

Recovery experiments were performed in order to study the accuracy of the method. Known amounts of each solute were added to a variety of samples, and the resulting spiked samples were subjected to the entire analytical sequence. Each solute was spiked at three different concentrations and recoveries were calculated based on the difference between the total amount determined in the spiked samples and the amount observed in the non-spiked samples. All analyses were carried out in triplicate. The average recoveries obtained, which ranged between 90% for glucose and 102% for ribose, testify to the accuracy of the proposed method (Table 2).

3.4. Analysis of fermented beverages

In order to evaluate the applicability of the proposed method, commercial "natural" and sparkling ciders

Table 2
Recovery studies on saccharides added to the various cider samples

Sugars	Concentration in cider ($\mu g\,ml^{-1}$)	Concentration added ($\mu g\,ml^{-1}$)	Recovery (%)	R.S.D.
D-Glucuronic acid	0.3	1.0	93	1.6
		3.0	100	2.0
		5.0	90	1.3
D-Galacturonic acid	113	20	97	0.1
		35	98	2.5
		50	101	1.7
Glucose	0.7	4.0	90	1.5
		7.0	97	2.3
		10	95	2.2
Galactose	4.4	4.0	92	1.3
		7.0	94	0.8
		10	92	0.5
Xylose	4.1	4.0	99	2.4
		7.0	94	1.7
		10	97	0.6
Arabinose	4.3	4.0	99	1.8
		7.0	98	0.7
		10	90	1.6
Ribose	1.1	4.0	91	1.9
		7.0	95	1.0
		10	102	2.3
Fucose	2.0	4.0	97	1.9
		7.0	98	2.9
		10	92	2.2
Rhamnose	6.2	4.0	98	1.4
		7.0	97	0.8
		10	100	1.1

(5 ml) were analyzed. Fig. 5 shows a typical chromatogram obtained from a natural cider sample. As can be seen in Table 3, differences in the aldoses and uronic acid contents between natural and sparkling ciders were found. In general, sparkling cider is made from apple juice concentrate once this has been diluted and fermented with an appropriate starter of the genus *Saccharomyces*. However, natural cider is elaborated from fresh apple juice obtained by mechanical, hydraulic, or pneumatic pressing and spontaneous fermentation by yeasts and lactic and acetic acid bacteria. As we can see in Table 3, glucose, galacturonic acid, arabinose, xylose, rhamnose, and ribose contents presented the most important differences between the ciders analyzed. The higher glucose content for the sparkling cider can be explained by taking into

Table 3
Determination of aldoses and uronic acids ($\mu g\,ml^{-1}$) in natural and sparkling ciders[a]

Compound	Sparkling cider	Natural cider
D-Glucuronic acid	nd	0.31 ± 0.03
D-Galacturonic acid	4634 ± 8.4	113 ± 0.4
Glucose	17040 ± 13	0.71 ± 0.03
Galactose	nd	4.43 ± 0.22
Xylose	395 ± 5.4	4.06 ± 0.32
Arabinose	633 ± 3.9	4.32 ± 0.61
Ribose	20.9 ± 0.4	1.07 ± 0.10
Fucose	11.6 ± 0.4	2.04 ± 0.10
Rhamnose	66.4 ± 0.8	6.19 ± 0.13

[a] Results are expressed as mean ± S.D. ($n = 4$); nd, not detected.

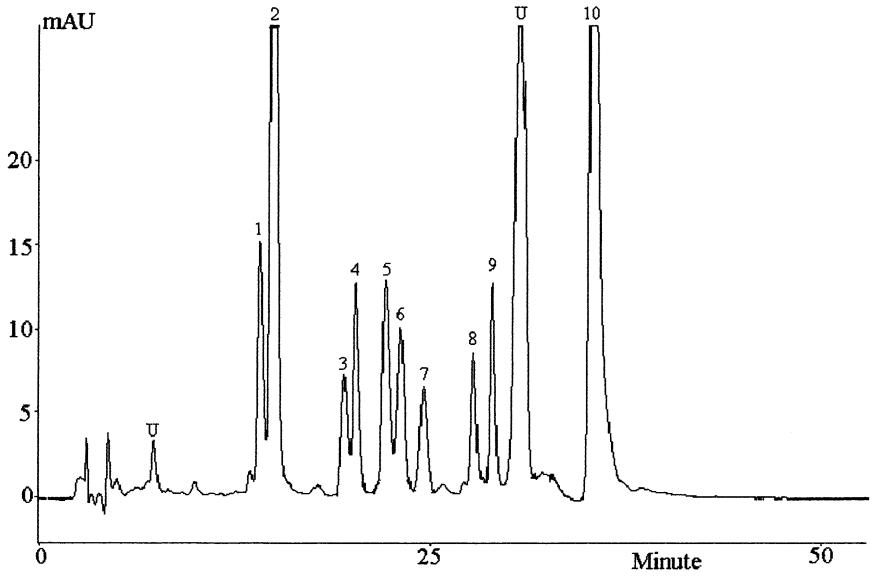

Fig. 5. Chromatogram of ABEE–sugar derivatives obtained from a natural cider sample. Chromatographic conditions as in Fig. 4; U, unknown peak.

account the fact that sucrose and glucose are added to increase the sweetness of sparkling cider. The concentrations of galacturonic acid, xylose, arabinose, ribose, and rhamnose were higher in sparkling than in natural cider. The use of liquefaction technology during the apple juice concentrate-making process could explain the differences detected for aldose and uronic acid levels between both cider types. Hemicellulases break xyloglucane linked to the cellulose microfibril and rhamnogalacturonan of the apple primary wall, and arabinases and pectinases hydrolyze the rhamnogalacturonan, producing arabinose, galacturonic acid, and rhamnose. These results also show that aldose and uronic acid composition could be employed for detecting the use of apple juice concentrate made by liquefaction in the cider-making process.

4. Conclusions

Reversed-phase HPLC with a C8 narrow-bore column provides a simple and economic alternative for the separation and determination of aldoses–ABEE and uronic–ABEE derivatives. The proposed method is particularly suitable for determining these compounds in cider and for detecting the use of apple juice concentrate in the cider-making process. It can also be applied to other samples, such as fruit juices, wines, brandies, etc.

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