Journal of Chromatography, 303 (1984) 256–262
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,071

Note

Isocratic liquid chromatography method for the simultaneous determination of aspartame and other additives in soft drinks

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(Received July 18th, 1984)

In connection with our studies on the reaction of L-phenylalanine-N-L-α-aspartyl-1-methyl ester (aspartame) with pyridoxal-5′-phosphate (vitamin B_6), a simple, sensitive method for the determination of aspartame was required. Very few methods have been reported so far for the determination of aspartame. Aspartame has been measured using amino acid analysis[1,2,3], by a fluorimetric method[4] and with the aid of gas liquid[5] or high performance liquid chromatography (HPLC)[6,7,8]. With the exception of the recently reported HPLC methods[7,8], the other methods are either not sensitive enough or specific enough.

Aspartame, a dipeptide, was recently cleared by the Food and Drug Administration for use as a low-calorie artificial sweetener for soft drinks. Currently, in the United States, aspartame is replacing only part of the saccharin in low-calorie carbonated beverages. Thus, these beverages contain, usually, saccharin, aspartame, benzoic acid (preservative), and caffeine.

In this work the development of a simple, sensitive, fast isocratic liquid chromatography method for the simultaneous determination of aspartame and the other three additives in soft drinks is reported.

EXPERIMENTAL

HPLC

The liquid chromatograph used was a Waters Assoc. system (Milford, MA, U.S.A.) with an M-45 pump, a Model U6K injector, a Model 441 detector equipped with a zinc lamp and a 214 nm filter. The detector was connected to a Model A-25 strip-chart recorder (Varian Aerograph, Walnut Creek, CA, U.S.A.) and to a Model 3390A reporting integrator (Hewlett-Packard, Avondale, PA, U.S.A.). A 250 × 4.60 mm I.D. stainless-steel Partisil-10SCX (Whatman, Clifton, NJ, U.S.A.) cation-exchange column was used. A silica gel saturation column and a 2-μm column inlet filter (Rheodyne, Cotati, CA, U.S.A.) were used. The mobile phase, 0.1 *M* ammonium dihydrogen phosphate ($NH_4H_2PO_4$) in glass distilled water, was passed through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.). The column was operated at ambient temperature, and the flow-rate was 1 ml/min.

0021-9673/84/$03.00

Reagents

Saccharin, aspartame, caffeine, and adenine were purchased from Sigma (St. Louis, MO, U.S.A.). Benzoic acid was from Eastman Kodak (Rochester, NY, U.S.A.), and the sample used had been purified by sublimation. All reagents were analyzed by HPLC under the same conditions as for the determinations and found to contain no interfering impurities except for saccharin which contained an impurity at approximately 0.6% concentration (detector response at 214 nm). Saccharin was not purified.

Standard solutions

Solutions of the reference compounds (1 mg/ml) and the internal standard, adenine sulfate, (0.2 mg/ml) were made in mobile phase solution and kept in the refrigerator.

Standard curves

The internal standard plot method was used. A series of standard mixtures containing various amounts of the four additives, and the same amount of the internal standard (4 μg/ml) were analyzed by HPLC. Replicate injections (six) of 10 μl from each standard solution were used to construct linear regression lines (peak area ratios of the four additives over adenine versus the corresponding ratios of their respective weights). The injected quantities of the compounds were in the linear range of the detector, and the correlation coefficients of the straight-line graphs of all four additives were better than 0.999. The quantities of the four compounds injected were in the following ranges: saccharin (100–400 ng), benzoic acid (60–300 ng), aspartame (50–470 ng) and caffeine (50–220 ng). Of course, the larger figures in the ranges do not mean that they are the upper limits of the linear range of the detector response.

Recovery studies

Quantities of the four additives at three levels each were added into 100-ml volumetric flasks that contained 400 μg of internal standard and 10 ml of "caffeine-free" commercial carbonated beverage and made to volume with mobile phase. Recoveries of the individual additives were calculated from the linear regression lines of the standard curves.

Preparation of samples

Samples were bought at the local market. Carbonated beverages were decarbonated by agitation. Into 100-ml volumetric flasks containing 400 μg of internal standard were added 10 ml of the beverage and made to volume with mobile phase. After mixing, 10 ml aliquots were removed into vials. To three of these vials was added 0.05, 0.075 and 0.1 ml of standard benzoic acid solution, respectively, and to another three, 0.05, 0.1 and 0.15 ml of standard sodium saccharin solution, respectively. Replicate injections (six) of 10 μl from each of the vials were used to calculate the concentrations of saccharin and benzoic acid by the method of standard additions.

A sugar-free soft drink mix containing particulate matter was reconstituted according to the instructions on the package. A 2-ml aliquot was added into a 100-ml volumetric flask that contained 400 μg of internal standard and made to volume

with mobile phase. An aliquot was filtered through a 0.45-μm Millipore filter and 10 μl injected into chromatograph. This dilution (2:100) is very high, and the filtration step is not necessary.

RESULTS AND DISCUSSION

To separate the vitamin B_6 compounds, we have been using a column with a strong cation exchanger (Partisil-10 SCX) and 0.1 *M* $NH_4H_2PO_4$ as mobile phase[9]. A series of experiments in which the pH of the mobile phase buffer 0.1 *M* $NH_4H_2PO_4$ was varied from 3 to 5.5 showed that the best separation of the four additives (saccharin, benzoic acid, aspartame and caffeine) was achieved when the pH was 4.5. A 0.1 *M* $NH_4H_2PO_4$ solution has a pH very close to that. The changes in the pH of the buffer had an effect, mainly on the retention times of benzoic acid and aspartame. At pH 4.5 all four additives showed base-line separations.

Initially the wavelength was set at 254 nm; however, at that wavelength the response of aspartame was low. Since aspartame is a dipeptide, the 214 nm wavelength was tried, and fortunately, there was no interference from other compounds present in soft drinks for the determination of aspartame and caffeine. However, there is small interference for the determination of saccharin and benzoic acid.

Attempts to quantify aspartame and the other three additives by the external standard method were unsuccessful in our system. The relative standard deviation of all four compounds was at times in the range of 5–8%, too high for quantitative work. Various compounds that could be used as internal standards were tried, and adenine was found appropriate giving base-line separation with the additives that are eluted close to it (Fig. 1). In addition, no other compound present in the soft drinks examined (Fig. 2) was eluted in the area where adenine appeared. The small peak between those of saccharin and benzoic acid in the standard mixture (Fig. 1) represents the impurity present in saccharin.

To study the recovery of the four additives from soft drinks, a sample of "caffeine-free" carbonated beverage was used (Fig. 2) in which the only additive (out of the four) present was a very small amount of caffeine. The recoveries of the additives were examined at three levels (mg/ml): saccharin, 0.15, 0.225 and 0.30; benzoic acid, 0.06, 0.12 and 0.18; aspartame, 0.05, 0.10 and 0.20; caffeine, 0.05, 0.10 and 0.15. Due to interference from compounds in the "caffeine-free" soft drink, the recovery of saccharin was, as expected, high at the lower level of addition (Table I), and as the level of addition increased, the percent of recovery decreased. For benzoic acid, due again to interfering compounds having close retention times to that of benzoic acid, at the low level of addition the percent recovery was high; however, at the two higher levels of addition the percent recovery was low mainly due to a compound that elutes a little after benzoic acid. At small concentrations of benzoic acid, the small peak after benzoic acid was resolved, but at higher concentrations the peak was not resolved and the intergrator terminated the area of the peak under benzoic acid prematurely. For aspartame the recovery was almost 100% at all levels of addition. For caffeine, as expected again, for low levels of addition the recovery was a little high due to the presence of a small quantity of caffeine in the "caffeine-free" soft drink. As the level of addition increased, the percent recovery decreased.

Figs. 3, 4 and 5 show chromatograms of the three low-calorie soft drinks exam-

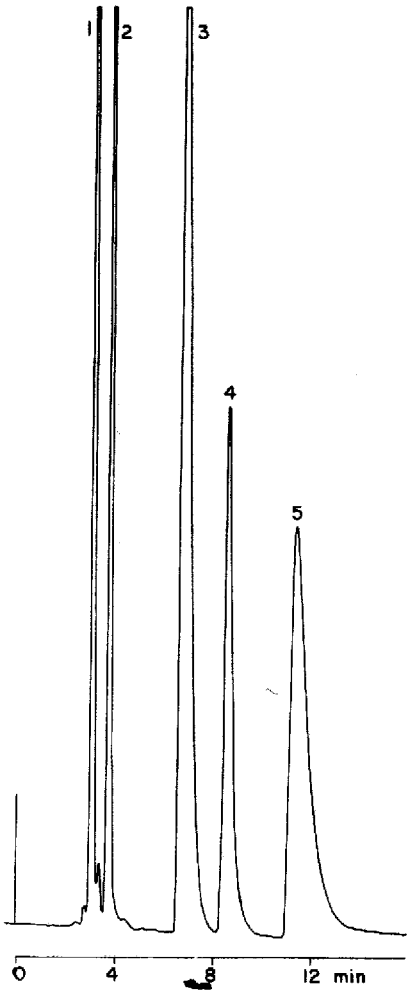


Fig. 1. Separation of standards on Partisil-10 SCX (250 × 4.6 mm I.D.). The mobile phase was 0.1 *M* $NH_4H_2PO_4$ at a flow-rate of 1 ml/min and detection at 214 nm. Peak identification: 1 = saccharin; 2 = benzoic acid; 3 = aspartame; 4 = adenine; 5 = caffeine.

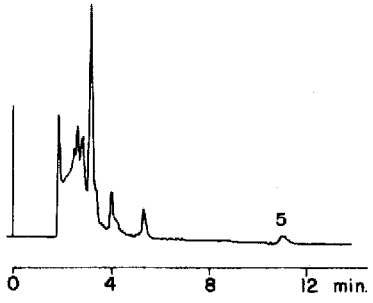


Fig. 2. Chromatographic tracing at 0.05 a.u.f.s. of 1 μl from a "caffeine-free" carbonated beverage. Chromatographic conditions and peak identification as in Fig. 1.

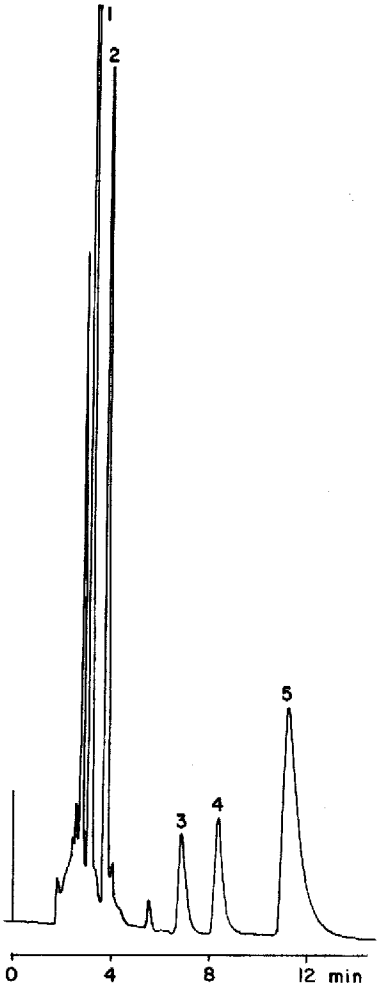
TABLE I

RECOVERY (%) OF THE FOUR ADDITIVES FROM A "CAFFEINE-FREE" CARBONATED DRINK

Additive	*Quantity added (mg/ml)*	*Recovery (%) ± S.D.**
Saccharin	0.15	113.17 ± 0.96
	0.225	109.34 ± 0.98
	0.30	106.18 ± 1.21
Benzoic acid	0.06	108.48 ± 2.06
	0.12	97.48 ± 1.74
	0.18	97.48 ± 1.12
Aspartame	0.05	99.24 ± 0.63
	0.10	99.51 ± 1.02
	0.20	99.33 ± 0.86
Caffeine	0.05	104.60 ± 1.08
	0.10	102.75 ± 1.11
	0.15	102.50 ± 1.56

* Standard deviation for six 10-μl injections.

ined after the addition of the internal standard. It is clear from the chromatograms in Figs. 2, 3 and 4 that due to the presence of interfering compounds in these carbonated beverages, an accurate determination of small quantities of saccharin and benzoic acid is not possible. However, this problem can be overcome by using the method of standard additions. To test the feasibility of the proposed method for the simultaneous determination of the four additives, two brands of commercially available low-calorie carbonated beverages were used. The quantities of the four additives determined by this method are reported in Table II. It can be seen from that table that the quantity of saccharin is overestimated while that of benzoic acid is underestimated. Although the "caffeine-free" carbonated beverage used for the recovery studies was a product of brand "A", the quantity of saccharin found by the internal standard method of brand "A" low-calorie carbonated drink was not much higher, contrary to what was expected, than the quantity calculated by the standard method of additions. This finding could mean that the quantities of the compounds interfering with the determination of saccharin are different in the "caffeine-free" than those in the low-calorie beverage of that brand. It is believed that the values for saccharin and benzoic acid determined by the method of standard additions are closer to the correct quantities present in these soft drinks. The quantities of the four additives reported in Table II might differ a little from the true ones, since the standards used in this method might not have been 100% pure or the amount of moisture might have been different than the one stated on the reagent bottle. It must be noted that a can of brand "A" low-calorie carbonated soft drink was bought and analyzed by this method two months before the one reported in Table II, and the quantity of benzoic acid was found to be close to 34 mg per 12 fluid ounces (Fig. 4), while the quantities of saccharin, aspartame, and caffeine were very similar to the ones reported in Table II. It is obvious from the chromatograms in Figs. 3 and 4 that the quantity



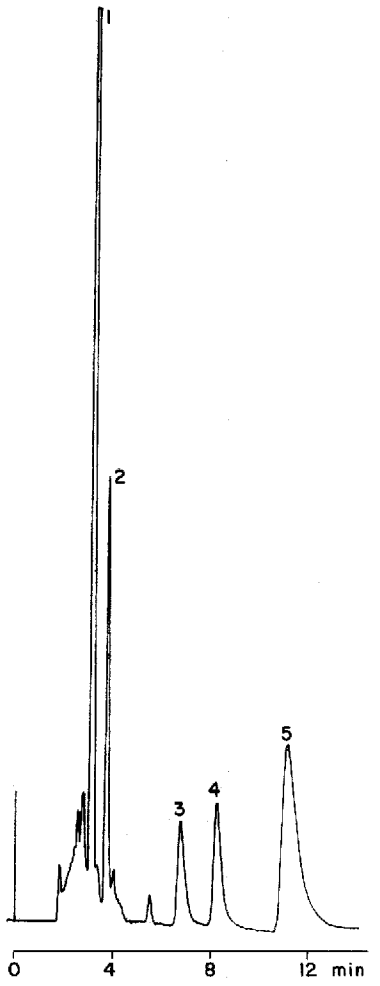


Fig. 3. Chromatographic tracing at 0.05 a.u.f.s. of 10 μl from a 1:10 dilution of brand "B" low-calorie carbonated beverage after addition of adenine. Chromatographic conditions and peak identification as in Fig. 1. Peaks represent the following quantities in ng: 1 = 203; 2 = 190; 3 = 86; 4 = 40; 5 = 124.

Fig. 4. Chromatographic tracing at 0.05 a.u.f.s. of 10 μl from a 1:10 dilution of brand "A" low-calorie carbonated beverage after addition of adenine. Chromatographic conditions and peak identification as in Fig. 1.

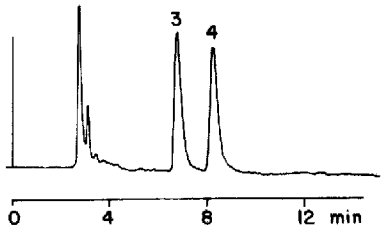


Fig. 5. Chromatographic tracing at 0.05 a.u.f.s. of 10 μl from a 1:50 dilution of a sugar-free soft drink mix after addition of adenine. Chromatographic conditions and peak identification as in Fig. 1.

TABLE II

DETERMINATION OF ADDITIVES IN LOW-CALORIE SOFT DRINKS (mg/12 fluid ounces)

Carbonated beverages	*Additives*					
	Saccharin	*Saccharin by method of standard additions*	*Benzoic acid*	*Benzoic acid by method of standard additions*	*Asparatame*	*Caffeine*
Brand "A"	65.40 ± 0.72*	64.08 ± 1.20	22.89 ± 0.57	23.89 ± 0.33	30.41 ± 0.26	38.09 ± 0.77
Brand "B"	75.00 ± 1.68	72.12 ± 0.72	61.13 ± 0.55	67.34 ± 1.29	30.60 ± 0.35	43.90 ± 0.53
Sugar-free soft drink mix (mg/l)					576.41 ± 4.32	

* Standard deviation for six 10-μl injections.

of benzoic acid and caffeine is higher in brand "B" than those of brand "A". In both cases 10 μl was injected from a 1:10 dilution. By considering the amounts of caffeine added to "caffeine-free" carbonated soft drink for the recovery studies, as quantities added for the determination of caffeine in the "caffeine-free" soft drink by the standard method of additions (although too large for this purpose), the quantity of caffeine in the decaffeinated soft drink was calculated to be 0.62 mg per 12 fluid ounces. The quantity of aspartame found in a sugar-free soft drink mix was 576 mg/l (Table II). However, it must be mentioned that the net weight of the dry mix package was higher than what was reported on the package. The smallest amount of aspartame injected was 50 ng used for the construction of part of the standard curve and was detertimed with a precision of less than 1% R.S.D. No attempt was made to find the limit of its detection.

When the flow-rate of the mobile phase was increased to 2 ml/min, the analysis time was reduced in half without sacrificing in resolution.

ACKNOWLEDGEMENT

This study was a part of Project No. 50-0330 of the Agricultural Experiment Station, College of Agriculture, University of Illinois at Urbana-Champaign, IL, U.S.A.

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