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Determination of aspartame and phenylalanine in diet soft drinks by high-performance liquid chromatography with direct spectrofluorimetric detection

Kazimierz Wróbel*, Katarzyna Wróbel

Instituto de Investigaciones Científicas, Universidad de Guanajuato, L. de Retana No. 5, 36000 Guanajuato, Mexico

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Abstract

Application of spectrofluorimetric detection is proposed in this work for the determination of aspartame and its main hydrolysis product phenylalanine in diet soft drinks by high-performance liquid chromatography. Separation was achieved on a LiChrosorb RP18 column with the mobile phase methanol–acetonitrile–phosphate buffer (2:17:81), pH 4.3. Native fluorescence of the two analytes (λ_{ex}=205 nm, λ_{em}=284 nm) was used for detection. The detection limits for aspartame and phenylalanine were 0.06 mg l^{-1} and 0.01 mg l^{-1} and calibration graphs were rectilinear in the range 0.5–40 mg l^{-1} and 0.1–10 mg l^{-1}, respectively. The proposed method was applied to determination of aspartame and phenylalanine in soft drinks (Diet Pepsi, Diet Coke and Diet Sprite) and the results obtained using two different detectors (spectrofluorimetric and spectrophotometric) were compared and discussed.

Keywords: Soft drinks; Detection, LC; Food analysis; Aspartame; Phenylalanine; Artificial sweeteners

1. Introduction

Over the last few decades, the demand for dietary food has significantly increased. Aspartame (*N*-L-α-aspartyl-L-phenylalanine methyl ester) is a low-calorie sweetener commonly used in carbonated soft drinks and beverages [1]. Several analytical methods have been proposed for its determination in food products, titration with lithium methoxide being the official method [2]. More recently, spectrophotometric [3,4], enzymatic [5–7] and spectrofluorimetric [8] procedures have been reported.

On the other hand, aspartame was determined together with other food additives [9–11] and also with some of its decomposition products, as aspartic acid, diketopiperinaze, and aspartylphenylalanine [12–14]. Several authors have used reversed-phase high-performance liquid chromatography (HPLC) with spectrophotometric detection (205, 214, 215 nm) and with a mobile phase containing phosphate buffer and acetonitrile and/or methanol [12–16]. However, quantitation of phenylalanine in soft drinks has not been reported. It is well known that spectrofluorimetric detection allows not only better sensitivity but also is more specific than spectrophotometric detection. Hayakawa et al. [17] used pre-column derivatization of aspartame and its hydrolysis products to form the fluorescent 1-cyanobenz-[*f*]isoindole derivatives, which were separated by

*Corresponding author. Fax: (+52) 473 26252; e-mail <katarzyn quijote.ugto.mx>.

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HPLC with fluorescence detection (λ_{ex}=420 nm, λ_{em}=490 nm). García Sánchez et al. [10] also used pre-column derivatization (with fluorescamine) for the determination of aspartame and glutamate by reversed-phase HPLC. These authors compared two detection systems and obtained better analytical performance (precision, detection limit) using spectrofluorimetry than spectrophotometry. The clear drawback of the reported methods [10,17] is the need for a derivatization procedure.

In this work the spectrofluorimetric determination of aspartame and phenylalanine is proposed, which is based on HPLC separation with detection of the native fluorescence of the two analytes. The procedure was applied for the analysis of soft-drinks samples (Diet Coke, Diet Pepsi and Diet Sprite) and the results obtained were compared with those obtained using spectrophotometric detection.

2. Experimental

2.1. Apparatus

A Hewlett–Packard (Waldbronn, Germany) Series 1050 high-performance liquid chromatograph with a multiple wavelength spectrophotometric detector, spectrofluorimetric detector (HP 1046A) and ChemStation was used. A Merck LiChrosorb RP18 column (10 μm, 200×4.6 mm) was used at room temperature.

2.2. Reagents

The solvents were of HPLC-grade quality and all other chemicals were of analytical reagent-grade.

Aspartame, phenylalanine, saccharine, caffeine and benzoic acid were from Sigma. Their stock standard solutions (100 mg l^{-1}) in 25% acetonitrile and 20 mmol l^{-1} phosphate buffer were prepared weekly.

Phosphate buffer solution (20 mmol l^{-1}, pH 4.3) was prepared from respective sodium salts (SigmaUltra) with addition of acetonitrile (final concentration 34%) and methanol (4%). This solution was used for sample dilution. The mobile phase was obtained from this solution by dilution with water (1:1). Methanol and acetonitrile were from Baker.

The Carrez solutions [13] were prepared by dissolving the appropriate mass of potassium ferrocyanide (Merck) and zinc acetate (Sigma) to obtain final concentrations of 15% and 30%, respectively.

Diet Coke, Diet Pepsi and Diet Sprite for analysis were bought in a local supermarket.

Deionized water (Labconco) was used throughout.

2.3. Procedures

Six calibration solutions of aspartame and phenylalanine (final concentration between 0.5 and 50 mg l^{-1}) were prepared by diluting the adequate volumes of standard stock solutions with the mobile phase.

Soft-drink samples were degassed in ultrasonic bath (15 min). Then, 1 ml of degassed drink was mixed with 1 ml of two Carrez solutions and diluted with water to 25 ml. After precipitation, the samples were centrifuged (10 000 *g*, 10 min) and diluted (1:1) with phosphate buffer solution containing 34% of acetonitrile and 4% of methanol. The solutions were again centrifugated (10 000 *g*, 10 min) and filtered. Each analysis was repeated five times: for samples taken from one can and for samples taken from different cans. For recovery experiments, samples (from one can) were spiked with phenylalanine (25 and 50 mg l^{-1} with reference to the undiluted sample) and aspartame (200 and 400 mg l^{-1}) and prepared as described above.

A volume of 20 μl was injected onto the column and elution was carried out with phosphate buffer–methanol–acetonitrile (81:2:17) at pH 4.3 using the following flow gradient: 0–1 min, 0.7 ml min^{-1}; 1–2 min, 1.0 ml min^{-1}; 2–8 min, 1.0 ml min^{-1}. The two detectors were connected on-line: first in series was the spectrophotometric one (set for 214 nm, ref. 580 nm) and second was the spectrofluorimetric one (λ_{ex}=205 nm, λ_{em}=284 nm, cut-off filter 280 nm).

Quantitation was carried out using external calibration and peak height measurement mode (valley–valley).

3. Results and discussion

It was observed that, with excitation in the range 190–220 nm, phenylalanine and aspartame produced

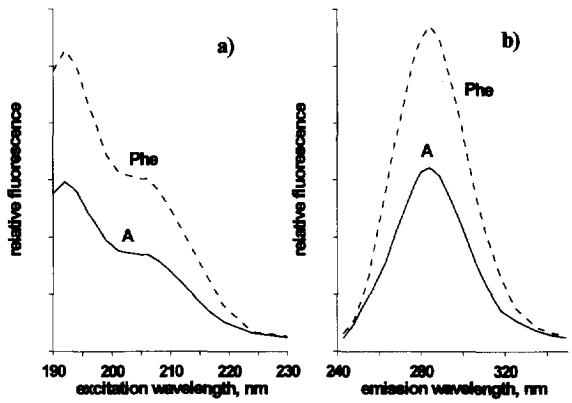

Fig. 1. Fluorescence spectra of: Phe – phenylalanine, 5 mg l^{-1}; and A – aspartame, 5 mg l^{-1}. (a) excitation spectra (λ_{em}=284 nm); (b) emission spectra (λ_{ex}=205 nm).

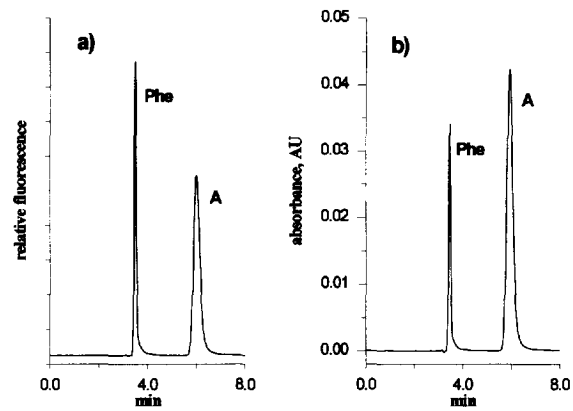

Fig. 2. Chromatogram of phenylalanine (5 mg l^{-1}) and aspartame (20 mg l^{-1}) in standard solution: (a) spectrofluorimetric detection (λ_{ex}=205 nm, λ_{em}=284 nm); (b) spectrophotometric detection (λ=214 nm).

native fluorescence emission with the maximum at 284 nm (spectra shown in Fig. 1). The two compounds contain the same fluorophore group (phenylalanine) and, as expected, spectra obtained were very similar with relative fluorescence signals corresponding to molar content of fluorophore. Using varying wavelengths for excitation the signal-to-noise ratios were compared for the two compounds as measured at 284 nm. The highest ratio values were obtained at 205 nm and this wavelength was selected for further studies. In these conditions, the effect of pH on the relative fluorescence signals was studied. The best sensitivity for the two compounds was obtained at pH 4 to 5 which corresponds to the approximate acidity of soft drinks. It is known that aspartame decomposition in aqueous solution is strongly pH dependent [18], thus for further experiments, pH 4.3 was selected and standards were prepared in 25% acetonitrile [16].

Chromatographic separation of aspartame and phenylalanine was achieved by reversed-phase HPLC with a mixture of phosphate buffer, acetonitrile and methanol as a mobile phase [12–16] and using a flow-rate gradient. In Fig. 2 the chromatogram of standard mixture is presented (selected conditions given in procedures) using spectrophotometric (Fig. 2b) and spectrofluorimetric (Fig. 2a) detection. As can be observed in these figures, the phenylalanine was eluted first, with retention time 3.46 min and aspartame at 5.97 min. Within-day precision of the retention times, measured as relative standard deviation for 10 injections, was below 0.1% for phenylalanine and 0.3% for aspartame. Between-days reproducibility of retention times, measured as relative standard deviation for 15 injections repeated during two weeks was 0.6% for phenylalanine and 1.4% for aspartame. Using two detection systems, the analytical characteristics for aspartame and phenylalanine determination were evaluated and the results are presented in Table 1. It can be observed that the application of spectrofluorimetric detection enabled a much better sensitivity and slightly better precision for the two analytes in standard solutions.

Diet soft drinks were treated with the Carrez [13] solutions: 1-ml aliquots of 15% potassium ferrocyanide and of 30% zinc acetate were added to 1 ml of degassed sample. After precipitation, samples were diluted in two steps. At first, water was added for dilution (25 times) and, after centrifugation, samples again were diluted (2 times) to equalize the composition of final solution to that of mobile phase. However, precipitation was observed during this second dilution, so the samples were again centrifuged and filtered before their introduction onto the column.

In Figs. 3 and 4, the chromatograms of real samples are presented, which were obtained using spectrofluorimetric (Fig. 3a, Fig. 4a) and spectrophotometric (Fig. 3b, Fig. 4b) detection. Using these same conditions, the chromatograms of blank (1 ml of deionized water, 1 ml of 15% potassium fer-

Table 1
Analytical characteristics for the determination of aspartame and phenylalanine using two detection systems

	Spectrofluorimetry (λ_{ex}=205 nm, λ_{em}=284 nm)		Spectrophotometry (λ=214 nm)	
	Aspartame	Phenylalanine	Aspartame	Phenylalanine
Correlation coefficient, r^2	0.9995	0.9994	0.9992	0.9993
Detection limit (mg l^{-1})	0.06	0.01	0.34	0.09
Linear range (mg l^{-1})	0.5–50	0.1–10	2–50	1–50
Precision at 2.5 mg l^{-1} (%)	4.2	2.1	5.1	2.1
Precision at 25 mg l^{-1} (%)	0.7	1.8	0.8	2.1

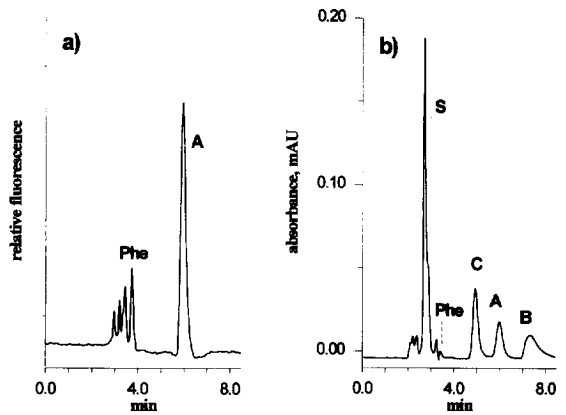

Fig. 3. Chromatogram of Diet Pepsi sample. The peaks correspond to elution of: S – saccharine and/or acesulfame; Phe – phenylalanine; C – caffeine; A – aspartame; B – benzoic acid. (a) Spectrofluorimetric detection (λ_{ex}=205 nm, λ_{em}=284 nm); (b) spectrophotometric detection (λ=214 nm).

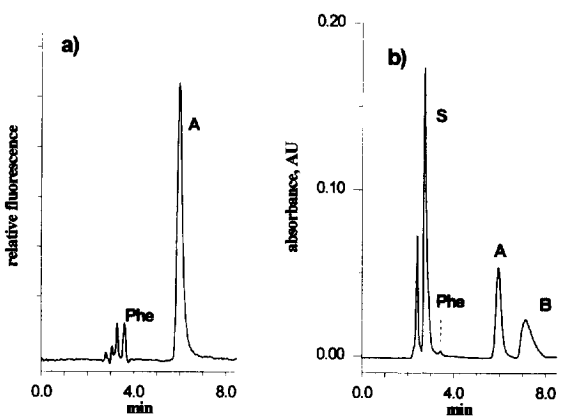

Fig. 4. Chromatogram of Diet Sprite sample. The peaks correspond to elution of: S – saccharine and/or acesulfame; Phe – phenylalanine; A – aspartame; B – benzoic acid. (a) Spectrofluorimetric detection (λ_{ex}=205 nm, λ_{em}=284 nm); (b) spectrophotometric detection (λ=214 nm).

rocyanide and 1 ml of 30% zinc acetate diluted as for real sample analysis) were obtained and it was observed that peaks appearing with a retention time lower that 3.2 min corresponded to elution of the components of Carrez solutions [13]. To identify other chromatographic peaks, their position was compared with the retention times of phenylalanine (3.46 min), aspartame (5.97 min), saccharine (2.70 min), caffeine (4.92 min) and benzoic acid (7.1 min), known to be the components of the soft drinks analysed. It should be mentioned that reproducibility or retention times for these compounds was very good (R.S.D. always below 1%) with the exception of benzoic acid (R.S.D. about 5.5%). Poor reproducibility of benzoic acid elution can be ascribed to the pH of the mobile phase (pH 4.3). As the dissociation constant of this acid is $6.5 \cdot 10^{-5}$, any change in pH of the mobile phase (e.g. due to changes of temperature) affects the polarity of this solute. Further identification of the chromatographic peaks was carried out by the standard addition technique. Also, using a diode-array spectrophotometric detector, spectra of eluted compounds were registered (in maximum of elution peak) and compared with spectra of phenylalanine, aspartame, saccharine, caffeine and benzoic acid standards, thus finally confirming identification of chromatographic peaks.

In Fig. 3, the chromatograms obtained for Diet Pepsi are presented (chromatograms for Diet Coke were very similar). Using a spectrofluorimetric detector (Fig. 3a), the elution of phenylalanine and aspartame can be observed, while using a spectrophotometric detector (Fig. 3b) more peaks appeared on the chromatogram. The elution of saccharine and/or acesulfame (2.70 min), phenylalanine (3.46

Table 2
Results of phenylalanine and aspartame determination in soft-drink samples (mean concentration, mg l^{-1} ±S.D., n=5) and literature data

Sample	Analyte	Spectrofluorimetric detector		Spectrophotometric detector		Literature
		A[a]	B[b]	A	B	
Diet Coke	Aspartame	492.5±5.4	494.3±9.7	495.7±6.2	496.3±11.1	460–570 [5,19]
	Phenylalanine	4.6±0.5	4.6±0.8	–	–	–
Diet Pepsi	Aspartame	494.1±6.7	492.7±2.0	497.3±7.8	495.1±13.3	454–480 [19]
	Phenylalanine	14.6±1.2	12.3±2.0	–	–	–
Diet Sprite	Aspartame	529.3±6.3	527.1±9.5	534.2±7.3	533.6±11.0	408–460 [3,19]
	Phenylalanine	6.9±0.5	7.3±0.8	–	–	–

[a] A=samples from one can.
[b] B=samples from different cans.

min), caffeine (4.92 min), aspartame (5.97 min) and benzoic acid (7.1 min) can be detected. In Fig. 3b, the peak corresponding to phenylalanine is much lower than in Fig. 3a, which clearly confirms the poor sensitivity of spectrophotometric as compared with spectrofluorimetric detection. The chromatograms of Diet Sprite are presented in Fig. 4 and using spectrofluorimetric detection (Fig. 4a), again the elution of phenylalanine and aspartame can be observed. With spectrophotometric detection (Fig. 4b) no peaks corresponding to elution of phenylalanine and caffeine were obtained.

The determination of phenylalanine and aspartame was carried out in Diet Coke, Pepsi and Sprite samples using external calibration (as indicated in procedures section) and the results obtained are presented in Table 2. The mean results presented were obtained in five repetitions of samples taken from one can (column A) and from different cans (column B). For aspartame, a good agreement was achieved between the results obtained using the two detectors. Slightly higher results for Diet Sprite were obtained as compared with the results reported by other authors [3,19], however our results correlated well with those reported by the manufacturer (500–600 mg l^{-1}) [3,5,19]. For phenylalanine, the quantitation was possible only using spectrofluorimetric detection and no bibliographic data corresponding to its concentration in soft drinks was found.

Finally, the recovery experiments were carried out in spiked samples of diet soft drinks using spectrofluorimetric detection. The obtained results are given

Table 3
Analytical results obtained from soft-drink samples spiked with aspartame and phenylalanine using spectrofluorimetric detection (mean concentration ±S.D., n=5)

Sample	Phenylalanineconcentration, mg l^{-1}		Aspartame concentration, mg l^{-1}	
	Spiked[a]	Found	Spiked	Found
Diet Coke	0	4.6±0.5	0	492.5±5.4
	25	28.4±1.8	200	682.5±6.9
	50	53.4±2.5	400	872.5±8.1
Diet Pepsi	0	14.6±1.2	0	494.1±6.7
	25	38.5±2.1	200	679.2±7.7
	50	64.0±2.7	400	875.2±9.0
Diet Sprite	0	6.9±0.5	0	529.3±6.3
	25	30.1±1.8	200	725.1±9.4
	50	53.9±2.4	400	949.8±12.8

[a] Spiked values given refer to spiked concentration of aspartame and phenylalanine in undiluted sample.

in Table 3, where it can be observed that quite good recoveries for the two analytes were obtained (95–98%).

4. Conclusions

The present work is the first report on simultaneous determination of aspartame and phenylalanine in soft drinks. It was shown that native fluorescence of these two compounds (λ_{ex}=205 nm, λ_{em}=284 nm) can be used for their detection in HPLC. Using such detection systems, it was possible to quantify phenylalanine in diet soft drinks. Moreover, much better sensitivity and slightly better precision was achieved for aspartame determination, as compared with the results obtained using spectrophotometric detection (Tables 1 and 2). Being more specific, the spectrofluorimetric detection allowed the determination of phenylalanine and aspartame in soft drink samples.

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