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Optimization of Ion Pair Reversed Phase Liquid Chromatography Separation of α-aspartame and Breakdown Products

Ebru Çubuk Demiralay* and Güleren Alsancak

Süleyman Demirel University, Faculty of Science and Literature, Department of Chemistry, Isparta, Turkey

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Abstract

In this work, the proportion of organic modifier of acetonitrile–water mixtures used as mobile phases were optimized in order to separate the α-aspartame and six breakdown compounds by using the relationships between the retention parameters of the compounds and the percentage of organic modifier. The separation was accomplished under ion pair reversed phase conditions using an YMC ODS AM (250x4.6 mm I.D., 5 μm) column. Optimum mobile phase for the chromatographic separation is composed of acetonitrile-water (20:80, v/v). Concentration of heptanesulfonic acid is 2.10^{-3} M at this mobile phase and the pH of the mobile phase is 2.60. Chromatographic hydrophobicity parameter and chromatographic hydrophobicity index were also calculated.

INTRODUCTION

Aspartame, a chemically synthesized dipeptide, was developed in 1965 and introduced to USA in 1983 as an artificial sweetener in food industry under the brand name ‘‘NutraSweet’’.

α-aspartame (L-α-aspartyl-L-phenylalanine methyl ester) is one of the most widely used artificial sweeteners (180–200 times sweeter than sucrose). This compound routinely consumed in low-calorie meals, soft drinks (i.e., carbonated and fruit-juice beverages), candy, chewing gums, and frozen desserts for dietary reduction of sugars and calories.

* Correspondence to: Ebru Çubuk Demiralay,

Department of Chemistry, Faculty of Science and Literature, Süleyman Demirel University, Isparta, Turkey

Tel: +90 246 211 41 67 Fax: +90 246 237 11 06
E-mail: ebru@sdu.edu.tr

The stability of α-aspartame in dry-product applications is relatively good. However, α-aspartame has limited stability in aqueous solutions. When added to food or soft drinks, the aspartame stability is subject to the influence of pH, temperature and light intensity within the matrix, which in turn causes the decomposition and thus raises the safety concerns [1]. The degradation products of α-aspartame are L-α-aspartyl-L-phenylalanine, derived from the hydrolysis of the methyl ester, and diketopiperazine, which is produced by the intramolecular aminolysis of the methyl ester linkage. Both L-α-aspartyl-L-phenylalanine and L-phenylalanyl- L-aspartic acid can be further hydrolyzed to give the individual amino acids, aspartic acid and phenylalanine [2,3].

The study reported previously has indicated that α-

aspartame is most stable at pH 4.0 [4]. Lowering the pH value to 3.1 at room temperature decreases the sweetness to two-thirds of the original level. However, the degradation is observed at pH >6.0 or <1.0 [5,6]. The effect of temperature on aspartame degradation has also been evaluated [7]. Not only α-aspartame can lose its sweetness but also shelf lifes of the food products also decreases depending upon these factors.

Because of α-aspartame can lose its sweetness and shelf lifes of the food products under several conditions, determination of α-aspartame and also breakdown products is extremely important for diet foods quality. In recent years, high performance liquid chromatography based on reversed stationary phase is one of the most powerful techniques for detection and quantification of α-aspartame and analysis of its breakdown products [6,8-12]. In these studies, the effects of the concentration of organic modifier and the ion-pair type, concentration and carbon chain length on the capacity factors of α-aspartame and its decomposition products were investigated. However there is only one study has been published on the optimization strategy related with the chromatographic separation of these compounds [13] .

The objective of this work is to develop of a simple, sensitive and fast isocratic liquid chromatography method for the simultaneous determination of α-aspartame and its breakdown products such as L-α-aspartyl-L-phenylalanine, L-phenylalanine methyl ester, L-aspartic acid, L-phenylalanine, diketo-piperazine and β-aspartame (L-β-aspartyl-L-phenylalanine methyl ester). Theoretical models describing the dependence of the retention factor, *k*, on the percentage of acetonitrile in the mobile phase, using reversed-phase sorbents, were derived by considering proportion of organic modifier in the mobile phase.

EXPERIMENTAL

Chemicals and Reagents

Stock standard solutions of 1000 µg/mL were prepared for all compounds (α-aspartame, β-aspartame, diketopiperazine, L-aspartyl-L-phenyl-alanine, L-phenylalanine methyl ester, L-phenyl-alanine and L-aspartic acid (Sigma, USA)) by dissolving the solid reagents in the mobile phase. HPLC grade heptanesulfonic acid sodium salt (Fluka, Steinheim, Switzerland) was used as an ion pair reagents. HPLC grade acetonitrile (Merck, Darmstadt, Germany) was used.

Apparatus

The HPLC analysis was carried out on a Shimadzu class LC-VP HPLC system with class LC-VP software, a pump (LC-10 ADVP), an autosampler (SIL-10ADVP) and a diode-array detector (SPD-M 10A VP). A 5 mm YMC ODS-Pack AM column (250x4.6 mm I.D., 5 µm) was used for the analysis.

pH measurements of the mobile phase were done with a Mettler Toledo using Hanna combination pH electrode. Potassium hydrogenphthalate (Merck, Darmstadt, Germany; dried at 120°C before use) was used as referance value standard for the standardization of this apparatus in acetonitrile-water binary mixtures in accordance with IUPAC rules [14].

Chromatographic Conditions

The chromatographic conditions were as follows: flow rate: 1.0 mL min^{-1}; volume injected: 10 mL; column temperature: 30°C; detection: 195 nm.

RESULTS AND DISCUSSION

In this study the retention behavior of α-aspartame and decomposition products has been modeled in water–acetonitrile mobile phases, in order to

optimize the chromatographic resolution between these compounds. Although liquid chromatography (LC) is a routine method for solving many practical analytical problems, separations are still being developed in a non-systematic manner, often by trial-and-error, which involves several disadvantages. The most evident disadvantage is the long development time that is required to select experimental conditions that are not necessarily the optimum ones, and often they are not as good as might be expected from this powerful technique [15].

The most important aspect of method development in LC is the achievement of sufficient selectivity. Usually, the methods are focused on optimizing the concentration of organic modifier in the mobile phase. Hydroorganic solvent mixtures are widely used in many branches of chemistry, ranging from reaction media for synthesis to electrochemistry, and from hydrometallurgy to liquid chromatography as mobile phases**.** Among these, acetonitrile–water mixtures have excellent solvent properties [16,17]. In order to predict solute retention from solvent composition and to find maximum resolution and minimum analysis time, it will be very important to know the effects of mobile phase compositions of binary solvents on the reversed phase chromatographic behavior. If the reverse phase liquid chromatography (RPLC) method development is unable to provide an adequate separation due to poor band spacing, ion-pair chromatography (IPC) provides an important additional selectivity option [18]. The use of ion-pair reversed phase chromatography to enhance retention of the compounds is an alternative approach for separation of α-aspartame decomposition products. The addition of alkyl sulfonic acid as an ion pair reagents to the mobile phase generally increased the selectivity of the chromatographic process. Ion-pair and RP-HPLC share several features. The column and mobile phase used for these separations are generally similar, differing mainly in the addition of an ion-pairing reagent to the mobile phase for IPC. There are several models explaining the mechanisms of processes occurring in ion association systems [19-21]. The ion-pair model assumes an association between the sample ion and an oppositely charged ion-pairing reagent in a liquid polar mobile phase before its adsorption on the hydrophobic stationary phase [22-24]. The ion-pair associate has a greater affinity for the non-polar stationary phase, which causes a stronger retention of the analyzed compound, adsorbing as an uncharged ion-pair.

The optimization of HPLC separations requires an accurate modeling of the relationship between the retention behavior of solutes and mobile phase properties. Solvent composition and pH are two important parameters, which have great effects on the resolution of ionogenic compounds. Great efforts were made on the retention modeling and optimization of pH and solvent composition and several methods have been proposed [15,25]. Generally, these methods can be divided into two categories: empirical and theoretical methods. The chief difference between them lies in how to describe the relationship between retention factor and pH value of mobile phase. The experimental parameter describing LC retention, used for a quantitative correlation with the structures of the analytes, is definitely the capacity factor (k). As k is usually determined in the chromatogram according to relationship,

$$k = t_r - t_o / t_o \quad (1)$$

where t_r and t_o are the retention times of the substance under investigation and a nonretained compound, respectively. Several researchers discuss the use of capacity factors and the theoretical number of plates as optimization criteria in HPLC methods development. The capacity factor and number of plates are based on fundamental

chromatographic theory and describe the degree to which each solute of the feed slug is resolved by the chromatography system. The capacity factor, k_i' for a given solute *i* is defined as the ratio of the amount of solute *i* bound to the stationary phase to the amount of solute *i* in the mobile phase. Note that capacity factors account only for the retention times of the various solutes and do not include a penalty for excessively wide peaks or for overlapping peaks which are both highly undesirable. Thus, the use of the capacity factor alone does not supply enough information to allow for adequate optimization of the system parameters the retention and selectivity in chromatograpic separation [26]. If the capacity factors vary as a function of the parameters to be optimized, criteria should be selected that allow the simultaneous optimization of the retention and the selectivity.

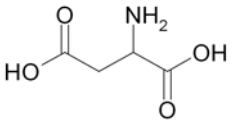
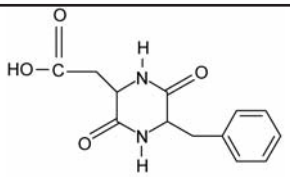
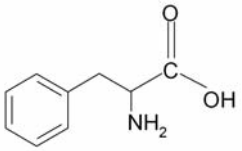
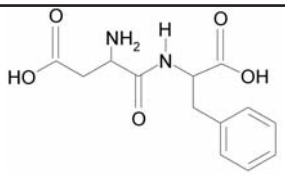
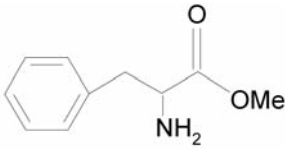
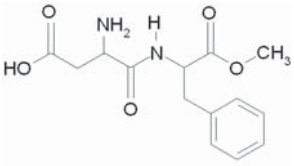
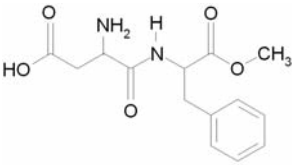
In this study, chromatographic behaviour of aspartame and its breakdown products (Table 1) was examined using mobile phases containing acetonitrile - water at various ratios (18:82; 20:80; 26:74; v/v*).* The addition of heptansulfonic acid to the mobile phase enabled a better separation and better peak symmetry was obtained as well. However content of heptane sulphonic acid in the mobile phase and pH of the eluent had lower influence than organic modifier content on investigated system responses, so in further experiments, it was kept constant at 2.10^{-3} M and 2.60.

The concentration of organic solvent in the mobile phase is the important factor in RPLC. It has not only a large impact on both the retention and selectivity, but is also a very convenient property, owing to the flexibility and accuracy in the implementation of changes. The retention behavior can be modeled accurately by establishing a quadratic relationship between the logarithm of the retention factor, *k*, and the volume fraction of organic solvent in the aqueous-organic mobile phase,φ:

$$\log k = c_o + c_1\varphi + c_2\varphi^2 \qquad (2)$$

where c_i are regression coefficients with characteristic values for a given solute and column /solvent system.

Table 1. Structures of α-aspartame and its breakdown products

Compounds number	Structures	Name
1		L-aspartic acid
2		Diketopiper-azine
3		L- phenylala-nine
4		L-α-aspartyl-L-phenylalanine phenylalanine
5		L- phenylala-nine methyl ester
6		β- aspartame
7		α- aspartame

This model can be simplified for narrow ranges of organic solvent to a linear relationship:

$$\log k = \log k_w - S\,\varphi \qquad (3)$$

The intercept of the fitted straight-line refers to the extrapolated log *k* for water as the mobile phase, and the slope indicates the sensitivity of retention to changes in elution strength (Table 2).

In this study, the criteria are applied to mobile phases containing a varying concentration of modifier, in order to observe the dependence of the retention time on the mobile phase composition. The variation of logarithm of capacity factor as the function of acetonitrile percentage for all breakdown products are shown in Table 2. This table show that the retention of solutes in the stationary phase is significantly decreased by increasing the amount of acetonitrile in the binary mixed solvent.

Table 2. Equation of log k obtained on YMC ODS AM vs. the volume fraction of acetonitrile, φ_{ACN}

Compounds	Equation	r	φ_0
diketopiperazine	log k = -0.46 - 1.00 φ	0.993	0.46
L-aspartic acid	log k = 0.46 - 4.57 φ	0.997	0.10
L-phenylalanine	log k = 1.56 - 6.52 φ	0.994	0.24
L-α-aspartyl-L-phenylalanine	log k = 2.11- 7.73 φ	0.998	0.27
L-phenylalanine methyl ester	log k = 2.42 - 8.49 φ	0.995	0.29
β-aspartame	log k = 2.55 - 8.80 φ	0.995	0.29
α-aspartame	log k = 2.84 - 9.41 φ	0.998	0.30

The intercept log k_w corresponds to the retention in pure water as a mobile phase and represents the commonly employed chromatographic hydrophobicity parameter. S is a solute-dependent solvent strength parameter specific to the organic modifier on the stationary phase under consideration [27,28].

In this study the retention parameters log k_w was obtained by linear extrapolation of the log k retention to pure water as the mobile phase. Table 2 shows the log k_w and S parameters of Eq. 3 obtained for the acetonitrile–water eluent system. It can be seen that, as expected, the relationships between the log k_w retention values and the φ acetonitrile concentrations were found to be linear and statistically highly significant for α-aspartame and breakdown products YMC ODS AM column. It is apparent from the data in Table 2 that the constant log k_w and the absolute value of S increase with increasing hydrophobicity of the α-aspartame and breakdown products.

Another retention-related parameter has been introduced recently, the isocratic chromatographic hydrophobicity index, φ_o [29]. The φ_o value represents the volume fraction of the organic solvent in the mobile phase for which the amount of solute in the mobile phase is equal to that in the stationary phase, i.e. the capacity factor is 1 (log *k* = 0):

$$\varphi_o = \log k_w / S \qquad (4)$$

where φ_o is equal to the ratio of the intercept and slope of Eq. 4. The isocratic chromatographic hydrophobicity index of the compounds studied were shown in Table 2. It is shown that the value of φ_0 is characteristic for a compound and depends only on the type of organic modifier. In addition, the relationships between the retention constants log k_w and S for the compounds studied were investigated and a linear relationship for all compounds were obtained Figure 1. The relationship can be expressed by the following equation:

$$\log k_w = 0.406\ S - 1.058 \qquad r = 0.991$$

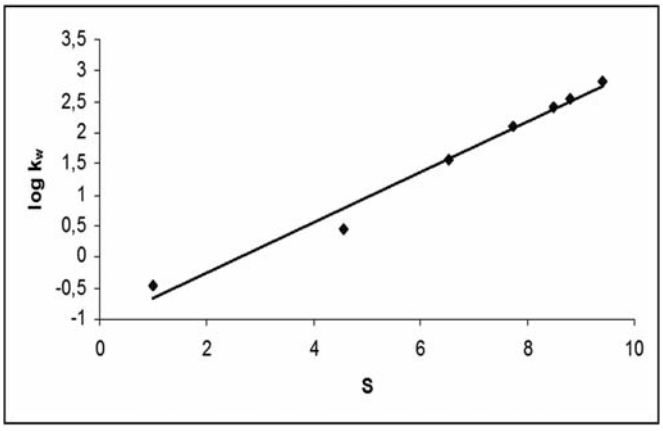


Figure 1. Plots of log k_w vs. S (Eq. [3]).

The experimental region was selected in a such way that the capacity factors of α-aspartame and breakdown products would stay within the limits 1< *k* < 10. These limits have been provided when the organic modifier content of the mobile phase was in the range of 20 -18 % (v/v). After performing the experiments, R_S values were calculated for all peak pairs according to equation 5. The chromatographic data obtained is illustrated in Table 3. The total number of peak pairs detected was six: (1) L-aspartic acid/diketopiperazine; (2) L-phenylalanine / L- aspartic acid; (3) L-α-aspartyl-L-phenylalanine / L-phenylalanine; (4) L-phenylalanine methyl ester / L-α-aspartyl-L-phenylalanine ; (5) β-aspartame / L-phenylalanine methyl ester; (6) α-aspartame /β-aspartame.

Table 3. The capacity factors, selectivity and separation factor values for the compounds studied

Compounds	% ACN	α	$\alpha-1/\alpha$	k_2	k_2/k_2+1	$1/4\sqrt{N}$	R_s
L- aspartic acid/diketopiperazine	18	1.80	0.44	0.45	0.31	31.60	4.36
L-phenylalanine / L- aspartic acid	18	5.67	0.82	2.55	0.72	27.10	16.03
L-α-aspartyl-L-phenylalanine / L-phenylalanine	18	2.11	0.53	5.38	0.84	29.94	13.28
L-phenylalanine methyl ester / L-α-aspartyl-L-phenylalanine	18	1.55	0.35	8.35	0.89	30.84	9.77
β-aspartame / L-phenylalanine methyl ester	18	1.20	0.17	10.0	0.91	34.24	5.19
α-aspartame /β-aspartame	18	1.47	0.32	14.71	0.94	33.96	10.17
L- aspartic acid/diketopiperazine	20	1.62	0.38	0.34	0.25	22.91	2.22
L-phenylalanine / L- aspartic acid	20	4.88	0.80	1.66	0.62	25.34	12.57
L-α-aspartyl-L-phenylalanine / L-phenylalanine	20	2.07	0.52	3.43	0.77	28.85	11.55
L-phenylalanine methyl ester / L-α-aspartyl-L-phenylalanine	20	1.41	0.29	4.82	0.83	29.03	6.99
β-aspartame / L-phenylalanine methyl ester	20	1.18	0.15	5.69	0.85	30.05	3.90
α-aspartame /β-aspartame	20	1.49	0.33	8.49	0.90	29.43	8.66
L- aspartic acid/diketopiperazine	26	1.10	0.09	0.19	0.16	20.35	0.30
L-phenylalanine / L- aspartic acid	26	3.90	0.74	0.74	0.43	17.83	5.64
L-α-aspartyl-L-phenylalanine / L-phenylalanine	26	1.70	0.41	1.26	0.56	15.45	3.55
L-phenylalanine methyl ester / L-α-aspartyl-L-phenylalanine	26	1.33	0.25	1.67	0.63	22.99	3.57
β-aspartame / L-phenylalanine methyl ester	26	1.13	0.12	1.89	0.65	29.06	2.19
α-aspartame /β-aspartame	26	1,33	0,25	2,52	0,72	28,33	5,03

For estimation of the system response the resolution between peak pairs was chosen. As known, achieving a good resolution between all of the working compounds is the main goal of chromatographic separation. In terms of fundamental chromatographic parameters the resolution, R_S, is affected by three independent variables :

$$R_S = (1/4)\sqrt{N} \quad [\alpha\text{-}1/\alpha] \quad [k_2/k_2+1] \qquad (5)$$

Efficiency Selectivity Retention

where *N* is the number of theoretical plates. Although the selectivity term [(α−1)/α] is generally regarded as the most important in LC, full attention must be given to all of the terms in Eq. 5. Achieving a good resolution between all the analytes of interest is the main goal of any chromatographic separation. Variation in resolution versus organic modifier percentage of mobile phases was shown in Figure 2.

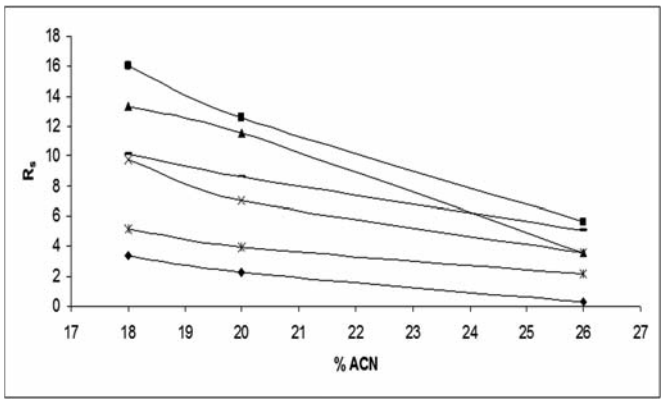


Figure 2. Plot of resolution, R_S, vs. percentage of ACN for pairs of compounds studied: ♦: L-aspartic acid / diketopiperazine, ■: L-phenylalanine / L-aspartic acid, ▲: L-α-aspartyl-L-phenylalanine/ L-phenylalanine, x: L-phenylalanine methyl ester /L-α-aspartyl-L-phenylalanine ∗ : β-aspartame / L-phenylalanine methyl ester – : α-aspartame/ β-aspartame.

We have obtained the chromatograms corresponding to of a mixture of the seven compounds in these three different experimental conditions. Figure 3 shows the chromatograms obtained at pH 2.60 and 18% acetonitrile (Figure 3a), 20% acetonitrile (Figure 3b), and 26% acetonitrile (Figure 3c). While IP RPLC is most useful for separating nonpolar, nonionic compounds, the analysis of acidic and basic compounds such as aspartic acid and phenyalanine is difficult due to poor resolution. As seen Figure 3a, insufficient resolution was observed between the peaks for the first two eluting compounds. It can be observed that the best result corresponds to a mobile phase containing 18% acetonitrile at pH 2.60, in which all solutes are well separated in an analysis time of about 35 min. Note that in chromatogram Figure 3a there is a good separation, but the time needed is much higher, and that chromatogram Figure 3c has

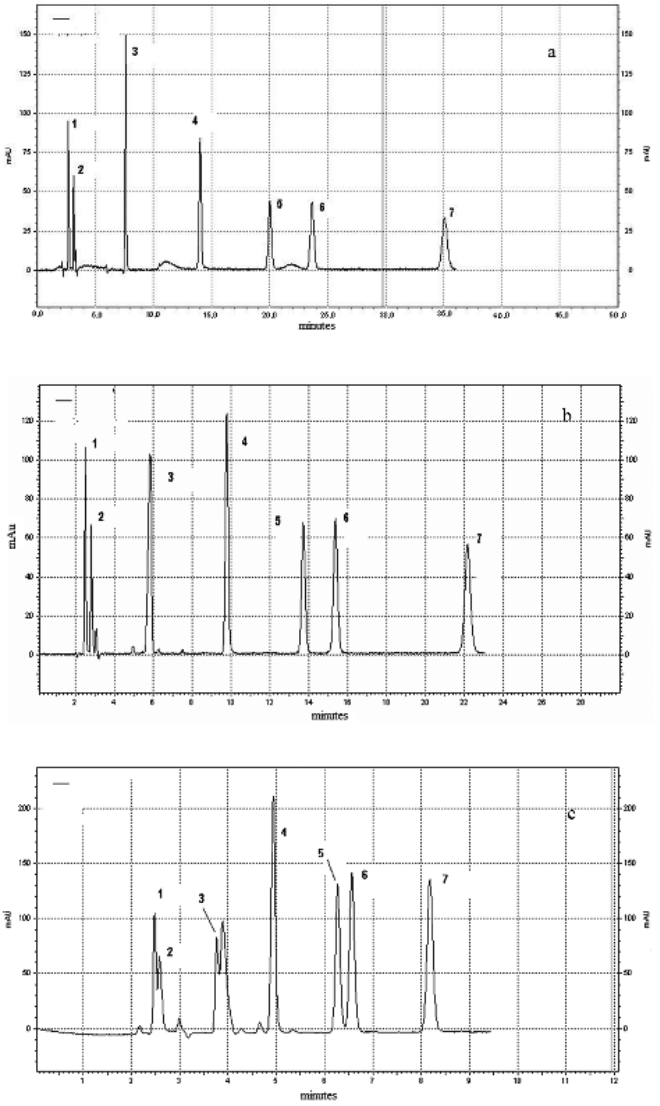


Figure 3. Chromatograms of a standard mixture of α-aspartame and breakdown products studied. 1: diketopiperazine, 2: L- aspartic acid, 3: L-phenylalanine, 4: L-α-aspartyl-L-phenylalanine, 5: L-phenylalanine methyl ester, 6: β-aspartame, 7: α-aspartame.

low resolution. This can be expected, as an increase of organic modifier concentration leads to lower retention times, and therefore lower resolution. The results show that the coverage of the sorbent surface by ion pair reagent decreases when the polarity of the eluent increases [30]. According to the experimental results, it was obvious that relavitely small changes of organic modifier content have great influence on the separation. The best result, which corresponds to high values of the R_S was obtained using a mobile phase composition of acetonitrile-water (20:80, v/v) at a pH of 2.60. A representive chromatograms of the aspartame and its breakdown products are presented in Figure 3. All respective compounds were clearly separated and their corresponding peaks were sharply developed. Separation was obtained in 22 min.

CONCLUSION

In this study, a simple isocratic ion pair reversed phase LC procedure was developed for the separation of α-aspartame and its breakdown products. The chromatographic separation of these compounds was carried out using a reverse phase YMC ODS AM C18 column (250 mm x 4.6 mm I.D., 5 mm) with a mobile phase of acetonitrile-water (20:80, v/v) containing 2.10^{-3} M heptane sulfonic acid and UV detection at a wavelength of 195 nm. The pH of the mobile phase was adjusted to pH 2.60 with HCl. The results obtained using this methodology propose clearly shows that it was possible to simultaneously, systematically optimize the influence of organic modifier content on the separation of the subtances investigated. Organic modifier content had the largest influence on resolution factor, yielding sufficient resolution and short analysis time in the range 18% and 26% acetonitrile. Increasing acetonitrile content leads to decreased analysis time, but also decreases the resolution between peaks.

The developed isocratic RP HPLC method permits simultaneous determination of α-aspartame and its related compounds spesified as decomposition products due to good separation and resolution of the chromatographic peaks and robutness towards reoanable changes in chromatographic parameters. The method is a simple, rapid, and robuts assay for decomposition assay. The method allows determination of α-aspartame and breakdown products in food samples such as powder, diet cola and ice-cream.

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