A VALIDATED HPLC METHOD FOR SEPARATION AND DETERMINATION ASPARTAME AND ACESULFAME-K IN FOOD PRODUCTS

Nermeen Mahmoud Barakat*, Mohammed Al-Azem¹, Nazira Sarkis² and Saleh Trefi³

¹Department of Food Engineering Technology, Faculty of Technical Engineering, University of Aleppo, Syria
²Department of Analytical and Food Chemistry, Faculty of Pharmacy, University of Aleppo, Syria
³Department of Pharmaceutical Chemistry and Quality Control, Faculty of Pharmacy, University of Aleppo, Syria

A pragmatic, economical, precise, straightforward to execute, and selective method for separation and determination of Aspartame and Acesulfame-K concentrations in non-alcoholic beverages and soft drinks. The separation by HPLC was implemented using a Welchrom C18 column (4.6 x 250 mm, 5µm) at 30°C temperature and a SHIMADZU UV-photo diode array detector at 217, 226 nm for Aspartame and Acesulfame-K respectively. The mobile phase composition consisted of potassium dihydrogen phosphate pH=4.5 and acetonitrile (80:20 v/v) with a flow rate of 1mL/min and an injection volume of 10 µL. The retention time of Acesulfame-K and Aspartame were 2.94, 6.51 respectively. The analysis time was less than 10 minutes. This method demonstrated appropriate results of linearity, precision, and recovery. It was applied efficiently to analyze Aspartame and Acesulfame-K. The calibration curve was linear with R² >0.999. The precision values of percentage relative standard deviation were less than 2% RSD <2. The mean recovery of analytes has ranged between 98.9-101.5, so the method is accurate. The studied analytes were robust at the temperature and pH, whereas Acesulfame-K was robust at the wavelength.

Keywords: Analytical Chemistry; chromatography; HPLC; Aspartame; Acesulfame-K.

INTRODUCTION

Artificial high-intensity sweeteners, also known as nonnutritive sweeteners, are additives widely used in the food industry to impart a sweet flavor, without the caloric downsides of table sugar. Common examples of these include aspartame, acesulfame-K, neotame, stevia, saccharin and sucralose. Because these artificial sweeteners are many times more potent than glucose, they can be used in much smaller concentrations to elicit the same gustatory effect. Due to these low concentrations, even calorie-containing artificial sweeteners amount to no-to-low calories when used in food and beverages. This is popular among health-conscious consumers, especially in the context of a global obesity epidemic.¹,²

Acesulfame-K (ACS-K) and aspartame (ASP) are two artificial sweeteners commonly used in foods, beverages, and confectionery products.³

Acesulfame-K

Acesulfame-K was originally developed in Germany in 1967. Its chemical structure is shown in (Fig.1A); note that the “K” in the name references the potassium in the chemical structure. Not only is it approximately 200 times more sweet than table sugar, but it is also heat-stable and quite stable in the solid state. Additionally, it maintains its integrity at a pH of 3 or greater for long periods of time.⁴,⁵
Similarly, Aspartame (Fig. 1B) (N-L- aspartyl-L-phenylalanine methyl ester), is an artificial sweetener roughly 150-200 times sweeter than table sugar,5.

The most common apparatus to determine artificial sweeteners is high performance liquid chromatography (HPLC), due to its quantitative and qualitative, analytical uses with respect to each substance within a sample, as they relate to precision, sensitivity (for small concentrations), and versatility or even applicability.6,7.

This study aims to find a cheap and suitable approach using HPLC for the sake of separation and determination of aspartame and Acesulfame –k without performing an extraction for the analytes in the food samples, prior to analysis. In addition, this work was done with the intent of monitoring the Syrian food products that contain aspartame and Acesulfame-k and making sure that concentrations conform with Syrian legislation. The validation was conducted in accordance with ICH guidelines8.

Various analytical methods were conducted using HPLC in order to separate and determine the artificial sweeteners encompass: HPLC–CAD–UV/DAD10, HPLC-MS/MS11, reversed phase liquid chromatography12, high performance liquid chromatography (HPLC) coupled with electrospray ionization mass spectrometric detection (ESI-MS)13, and high-performance capillary electrophoresis (HPCE)14.

MATERIALS AND METHODS

Materials

A working standard of aspartame was purchased from Asia industries, Aleppo-Syria, whereas the working standard of Acesulfame-k was purchased from Niutang, Changhai-China. Acetonitrile (HPLC-grade) was obtained from Biosolve, France. Potassium dihydrogen phosphate 98% was obtained from Alpha Aesar.

Instrumentation

In this study, the chromatographic system used was Shimadzu LCsolution (1.25 version) with a degasser DGU (3 channels), the employed pump was LC-20AT with dual reciprocating plunger, and a CTO-20A column oven. The UV detector was photo diode array model SPD-M20A.

The ultrasonic bath was a power sonic 405 manufactured in Hwashin, Korea. Sartorius analytical balance 0.0001 g model ENTRIS124-1S. Crison pH meter model TitroMatic 1S.

Solution Preparation

Standard Solution Preparation

The stock solution of Aspartame and Acesulfame-k were prepared individually by weighing 0.05g of Aspartame and 0.01g of Acesulfame-k. These were then transferred into100 mL glass volumetric flasks; the flasks were filled with ultrapure water to the line marked.

The working solution was created by drawing up a particular volume of stock solution, placing it into 10 mL volumetric flasks, and diluting it with purified deionized water to the calibration mark, thereby achieving the desired concentration. The standard solution of Aspartame was sonicated for 30 min in 40°C, while the standard solution of Acesulfame-k was sonicated for 10 minutes in 25°C. Both were filtered through a 0.45µm nylon syringe filter.
Sample Preparation
Preparation of the beverages was conducted by weighing 1 g of each sample and placed into 50 mL volumetric flasks. They were diluted to 50 mL with deionised water and degassed in an ultrasonic bath for 15 min in 25°C room temperature, as to guarantee the total dissolution of the solid samples.

Soft drinks were degassed for 5 min to remove dissolved gases using an ultrasonic bath. 5 mL volume was taken from the sample and diluted to 50 mL with deionised water. Soft drinks and beverages products solutions both were filtered through a 0.22 µm nylon syringe filter beforehand injecting the samples into the HPLC.

Phosphate buffer preparation

To prepare 2 mM of the phosphate buffer with pH= 4.5, 0.272 g of 98% pure potassium dihydrogen phosphate was transferred to a 1000 mL clean volumetric flask. This was filled to the calibration mark with deionized water.

RESULTS AND DISCUSSION

The optimal chromatographic conditions of analysis
Selection of wavelength detection
The two compounds were analyzed in a spectrophotometer. The wavelength of maximum absorbance was 217 nm for aspartame (Fig. 2A) and 226 nm for acesulfame-k (Fig. 2B).

Fig. 2: UV-spectrum of analytes
Mobile phase composition
Various ratios of phosphate buffer and acetonitrile were tested to obtain the ideal chromatography peak. The optimum rate and pH were chosen after conducting many experiments in order to get symmetrical sharp peak with efficient separation, high resolution, and an appropriate retention time.

A rate of (85:15) phosphate buffer pH= 3.5 and acetonitrile was tested. Long retention time and tailing factor of Acesulfame-k was 2.3 (Fig. 3A).

A rate of (80:20) phosphate buffer pH=3.5 and acetonitrile was tested. Asymmetric peak and long retention time (Fig. 3B).

A rate of (70:30) phosphate buffer pH=3.5 and acetonitrile was tested. Bad resolution and tailing factor with superimpose peak (Fig. 3C).

A rate of (80:20) phosphate buffer pH=2.5 and acetonitrile was tested. tailing factor was 2.1 and 1.4 for Acesulfame-k and aspartame respectively (Fig. 3D).

A rate of (80:20) phosphate buffer pH=4.5 and acetonitrile gave the optimum resolution with appropriate retention time and sharp symmetric peak shape. The tailing factor was 1.1 and 1.2 for Acesulfame-k and aspartame respectively (Fig. 4).
**HPLC Analysis**

The separation and determination were done using a Welchrom C18 column (4.6 × 250 mm, 5µm) at 30°C temperature. The mobile phase composition consists of potassium dihydrogen phosphate pH= 4.5 and acetonitrile (80:20 v/v) with a flow rate of 1mL/min and an injection volume of 10 µL. The analysis time was less than 10 minutes and the wavelength of analytes was measured at 217 nm for aspartame and 226 nm for Acesulfame-k.

The working solutions were injected under these chromatographic conditions and the retention time was 2.94 for Acesulfame-k and 6.51 for aspartame.

The tailing factor was 1.1, 1.2 for Acesulfame and aspartame respectively which is satisfactory. The theoretical plates number were 3060 for Acesulfame and 7235 for aspartame. In this study the plates number requirement of N >2000 was met. The equilibration time of column was done for 40 minutes before injection.

**Method validation**

**Linearity**

The linearity was tested by preparing seven concentrations from the Aspartame stock.
solution and six concentrations from Acesulfame-k stock solution.

The analysis was done by taking suitable volume from the stock solution and diluting up to 10 mL volumetric flasks to get the required concentrations of 10, 25, 50, 100, 125, 150, and 175 μg/mL. The same steps were done for Ascesulfame-k to get the entailed concentrations of 1, 5, 10, 20, 40, and 80 μg/mL. The prepared solutions were filtered through a 0.45 μm nylon syringe filter and each one of the standard solutions was injected three times into the column under the optimal conditions. The correlation coefficient of Aspartame and Acesulfame-k are 0.9995 and 0.9999 respectively which is satisfactory. Results were recorded in Table 1.

### Table 1: The Regression equation and Correlation coefficients of analytes

|                | Aspartame | Acesulfame-k |
|----------------|-----------|--------------|
| Correlation coefficient | 0.9995    | 0.9999       |
| Regression equation       | $y = 1.0098x - 0.4116$ | $y = 31961x + 20952$ |

### System suitability test

It is a procedure that must be conducted prior to the analysis process in order to verify the convenience of the validated chromatographic method for application in this study\(^6\). Results were displayed in table 2.

### Table 2: System suitability parameters

| Parameters              | Acesulfame-k | Aspartame |
|-------------------------|--------------|-----------|
| Retention Time          | 2.94         | 6.51      |
| Tailing Factor          | 1.1          | 1.2       |
| Theoretical Plates Number | 3060         | 7235      |
| Resolution              | -            | 13.7      |
| RSD% of Peak Area (n=6) | 0.126        | 0.423     |

### Accuracy

Concentrations of 50, 100, 150 μg/mL of aspartame and concentrations of 10, 20, 40 μg/mL of Acesulfame-k were studied to test accuracy with three replicates of each concentration. The table 3 shows the mean recovery percentage of aspartame and Acesulfame-k was between (98%-102%), so the method is accurate.

Another common way to study accuracy is with the spike recovery method which adds a known particular concentration of the spiked substance to the sample matrix and measures recovery of its response\(^17\). Results are listed below in tables (4, 5).

### Table 3: Accuracy data

| Substance μg/mL | Theoretical concentration | Aspartame | Acesulfame-k |
|-----------------|---------------------------|-----------|--------------|
|                 | Con₁ (50) | Con₂ (100) | Con₃ (150) | Con₁ (10) | Con₂ (20) | Con₃ (40) |
| Found concentration | | | | | | |
| 50.6821 | 101.397 | 151.2554 | 10.01433 | 20.29771 | 39.95182 |
| 50.68142 | 101.2515 | 150.9983 | 10.13175 | 20.27127 | 40.09246 |
| 50.32739 | 101.0278 | 151.2948 | 10.08345 | 20.0515 | 40.11746 |
| 50.88889 | 98.84393 | 149.2846 | 10.09042 | 20.55127 | 40.78167 |
| 50.7042 | 98.81115 | 149.313 | 10.12409 | 20.56071 | 40.65567 |
| 50.61052 | 98.60349 | 149.3026 | 10.13376 | 20.58452 | 40.72382 |
| 50.11626 | 99.81907 | 149.0826 | 9.836645 | 19.9526 | 40.19333 |
| 50.02505 | 99.34027 | 149.149 | 9.8562 | 19.92863 | 40.29511 |
| 49.33868 | 99.10893 | 149.043 | 9.85606 | 19.91643 | 40.26898 |

| Mean\(\bar{X}\) | 50.37502 | 99.80035 | 149.8581 | 10.01414 | 20.23496 | 40.34226 |
| Recovery % | 100.75004 | 99.80035 | 99.90543 | 100.1474 | 101.1748 | 100.8556 |

| Mean recovery % ±SD | 100.1519 ±0.52 | 100.7239 ±0.53 |
| RSD% | 0.519 | 0.525 |

n=9
Table 4: Spike Recovery Method of Aspartame

| The spiked level% | Amount of target compound (µg/mL) | Theoretical amount in matrix (µg/mL) | Area of the spiked material | Theoretical amount in matrix (µg/mL) | Found Conc of the spiked material (µg/mL) | Spike Recovery% | Mean Recovery% | SD | RSD% |
|-------------------|----------------------------------|-------------------------------------|----------------------------|-------------------------------------|------------------------------------------|----------------|----------------|----|-----|
| 50%               | 23                               | 11.5                                |                            | 34.2824                             | 34.3573                                  | 99.87587       | 100.1035       | 0.770382 | 0.769586 |
| 100%              | 23                               | 23                                  |                            | 44.9144                             | 44.8861                                  | 98.0462        | 99.36689       | 1.485577 | 1.495042 |
| 150%              | 23                               | 34.5                                |                            | 56.498                              | 56.3573                                  | 98.01269       | 98.82341       | 0.70622  | 0.714628 |

Table 5: Spike Recovery Method of Acesulfame-k

| The spiked level% | Amount of target compound (µg/mL) | Theoretical amount in matrix (µg/mL) | Area of the spiked material | Theoretical amount in matrix (µg/mL) | Found Conc of the spiked material (µg/mL) | Spike Recovery% | Mean Recovery% | SD | RSD% |
|-------------------|----------------------------------|-------------------------------------|----------------------------|-------------------------------------|------------------------------------------|----------------|----------------|----|-----|
| 50%               | 9.15                             | 4.6                                 |                            | 454207                              | 13.55574                                 | 98.94701       | 98.9499        | 0.154862 | 0.156506 |
| 100%              | 9.15                             | 9.15                                |                            | 615687                              | 18.60815                                 | 101.6039       | 101.3711       | 0.389613 | 0.384343 |
| 150%              | 9.15                             | 13.7                                |                            | 762778                              | 23.21035                                 | 101.6215       | 101.5696       | 0.070117 | 0.069034 |

Precision

Intermediate Precision

Three concentrations were chosen within the linearity for both Aspartame and Acesulfame-k in order to determine the intra-day precision with three injections of each concentration. The inter-day precision was determined with the same three concentrations that were determined in the intra-day, but do it in the next day with an entirely new preparation.

The results have shown that the intermediate precision method is valid and accurate with RSD% less than 1% of intra-day and %RSD less than 2% of inter-day. Results were recorded in Table 6.

Table 6: Intermediate Precision

| Substance       | Conc. Range (µg/mL) | RSD% Intra-day (n=3) | RSD% Inter-day (n=6) |
|-----------------|---------------------|----------------------|----------------------|
| Aspartame       | 50                  | 0.785                | 0.623                |
|                 | 100                 | 0.35                 | 1.432                |
|                 | 150                 | 0.213                | 0.977                |
| Acesulfame-k    | 10                  | 0.210                | 0.720                |
|                 | 20                  | 0.140                | 0.243                |
|                 | 40                  | 0.067                | 0.535                |

Repeatability

The repeatability was confirmed by picking one particular concentration within the linearity of Aspartame as well as Acesulfame-k. This concentration was injected into the column six times. The relative standard deviation percentage (%RSD) was less than 1% which is precise. Results were displayed in Table 7.

Robustness

Some chromatographic conditions were altered to assess the robustness of this method such as temperature, pH, wavelength, and flow rate. Every condition was tested individually. Results were displayed in table 8.

The results demonstrated the percentage relative standard deviation (%RSD) does not exceed 5%. Therefore, the method is robust at the temperature and pH for both studied compounds, and at the wavelength for Acesulfame-k.
Table 7: Repeatability

| Number of injection times | Aspartame Con.(μg/mL) | Area | Acesulfame-k Con.(µg/mL) | Area |
|---------------------------|-----------------------|------|--------------------------|------|
| 1                         | 125                   | 1231910 | 80                      | 2807895 |
| 2                         |                       | 1228759 | 2796795                |
| 3                         |                       | 1224918 | 2803195                |
| 4                         |                       | 1221340 | 2801538                |
| 5                         |                       | 1220184 | 2801905                |
| 6                         |                       | 1218781 | 2801840                |
| RSD%                      | 0.423                 | 0.126 |

Table 8: Robustness.

| Temp | Peak Area* | pH | λ [nm] | Peak Area* | λ [nm] | FR ml/min | Peak Area* |
|------|------------|----|--------|------------|--------|-----------|------------|
| 26° C| 1481595    | 4.3| 1482291| 353931     | 353103 | 1.1       | 1483298    |
|      | 1481700    |    | 1481608| 351409     | 352180 | 0.9       | 1483298    |
|      | 1485431    |    | 1483224| 352033     | 352526 | 1         | 1483298    |
| 30° C| 1487970    | 4.5| 1489750| 354351     | 354315 | 1.1       | 1489750    |
|      | 1489069    |    | 1489069| 354039     | 354039 | 1         | 1489069    |
|      | 1488942    |    | 1488942| 353855     | 353855 | 1         | 1488942    |
| 34° C| 1483859    | 4.7| 1489451| 351569     | 353240 | 1.1       | 1489451    |
|      | 1486174    |    | 1485939| 355522     | 355522 | 1         | 1485939    |
|      | 1483617    |    | 1487411| 351508     | 351508 | 1         | 1487411    |
| RSD% | 0.212      |    | 0.214  | 0.219       | 0.740  | 0.258     | 8.615      |

n= 3 the number of injections, AS= Aspartame, ACSK= Acesulfame-k, λ= Wavelength, FR= Flow Rate.

Assay

The beverages and soft drinks were purchased from the local shops, Aleppo- Syria. The samples solutions were injected under the optimal conditions. The concentrations of the samples were calculated by the linearity equation of Aspartame and Acesulfame-k. The results are listed in table 9.

All the studied samples were found to be conform with Syrian legislation\textsuperscript{18}. Aspartame and Acesulfame-k chromatogram in peach beverage sample is shown in (Fig. 5).

Table 9: Assay data

| Samples         | Area peak\textsuperscript{a} | Found Amount mg/kg | RSD% | Area peak\textsuperscript{a} | Found Amount mg/kg | RSD% |
|-----------------|-------------------------------|--------------------|------|-------------------------------|--------------------|------|
| Peach beverage  | 49.75253                      | 2485               | 0.959| 304760                        | 440                | 1.794|
| Mango beverage  | 54.50087                      | 2700               | 0.677| 261693                        | 375                | 0.703|
| Yosuf beverage  | 25.0005                       | 1250               | 0.848| 1026846                       | 1570               | 1.764|
| soft drink- Pepsi | 33.488                       | 1675               | 0.269| 158480                        | 215                | 1.484|
| soft drink- Seven up | 30.35743                   | 1525               | 0.340| 265435                        | 380                | 1.596|
| Maximum permitted level mg/kg | 5500                  |                    |      | 2000                          |                    |      |
Conclusion

Food additives are ubiquitous. With such widespread use comes responsibility: the industry must ensure that these products are safe for consumption and do not have adverse effects on human health. In order to fulfill this responsibility, reliable and accurate methods for measuring additive concentration are essential. In the case of artificial sweeteners, this means ensuring that concentrations conform with industry standards and do not exceed the Acceptable Daily Intake (ADI) set by FAO/FDA.

The method detailed in this paper has numerous advantages for the food industry. It is precise, valid, economical, and safe. Because it uses a low rate of acetonitrile in the mobile phase composition, this method is also an environmentally friendly approach.

The present study took into consideration the low buffer concentration which maintains the quality of the column from the residues and occlusions during the analysis operation.

A low buffer concentration helped to overcome these shortcomings, reduced the applied pressure to the column, and improved the flow capacity of the mobile phase.

This method is straightforward to execute, easily scalable, and compatible with routine analyses commonly employed to determine Aspartame and Acesulfame-k concentrations.

Competing Interests

Authors declare that there are no competing interests.

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أهمية كروماتوغرافيا السائلة عالية الأداء محققة من صحتها لفصل وتحديد كل من الأسيتام والليستامين البوتاسيوم في المنتجات الغذائية

نورين محمود بركات 1 - محمد العظم 2 - نظيرة سركيس 3 - صالح طريفي 3

1 قسم تقانات الغذاء التكنولوجيا، كلية الهندسة التقنية، جامعة حلب، سوريا
2 قسم الكيمياء التحليلية والغذائية، كلية الصيدلة، جامعة حلب، سوريا
3 قسم الكيمياء الصيدلية والمراقبة الدوائية، كلية الصيدلة، جامعة حلب، سوريا

طريقة عملية، اقتصادية، دقيقة، انتقائية، وسهولة التنفيذ لفصل وتحديد تركيز كل من الأسيتام والليستامين البوتاسيوم في المشروبات غير الكحولية والغازية.

تمت عملية الفصل باستخدام الكروماتوغرافيا السائلة عالية الأداء، العوام المستخدم بتعداد (2.64×100.5 مم، 5 كم). كانت درجة الحرارة 30° م خط لعملية الفصل، وكشف المستخدم UV-Photo diode Array pH= 4.5. ناونومتر لكل من الأسبرامين والليستامين البوتاسيوم على التوالي.

بكون الطور المتحرك المستخدم في هذه الطريقة من: 50% من الوعاء الفوسفات ثنائي الهيدروجين ذو 4.5 pH و 20% من المحلول العضوي استونتريل. كان معدل الهدف بمقدار 1 مل/ دقيقة، وحجم الجنة 10 ميكروتريل.

كان زمن الاحتفاظ عند الظروف المثلى لكل من الأسيتام والليستامين هو 2.94 - 2.51 على التوالي. زمن التحليل كان أقل من 10 دقائق.

أظهرت هذه الطريقة نتائج جيدة لكل من الخطية، الدقة، والصحة. طبقة بشكل فعال لتحليل كل من الأسبرامين والليستامين البوتاسيوم.

كان المنحنى العددي للنوات عبرين في اختبار الصحة لكل من الأسبرامين والليستامين: حيث 0.999 R2، بينما كانت قيمة الانحراف المعياري النسبي لاختبار الدقة أقل من 2.

تراوحت متوسط قيم الاستردادية في اختبار الصحة لكل من الأسبرامين والليستامين بين 0.98-0.915، مما يشير إلى أن الطريقة دقيقة.

كانت المناخات المنوية ذات مثانية جيدة عند إجراء تغير طفيف في درجة الحرارة الحموضة، و أظهر أيضًا أسيلام البوتاسيوم مثانية جيدة عند إحداث تغيير بسيط في الطول الموجي.