Bull. Pharm. Sci., Assiut University, Vol. 45, Issue 1, 2022, pp.269-279.

Bulletin of Pharmaceutical Sciences
Assiut University
Website: http://bpsa.journals.ekb.eg/
e-mail: bullpharm@aun.edu.eg

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

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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 R^2 >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&9].

Received in 5/2/2022 & Accepted in 8/3/2022

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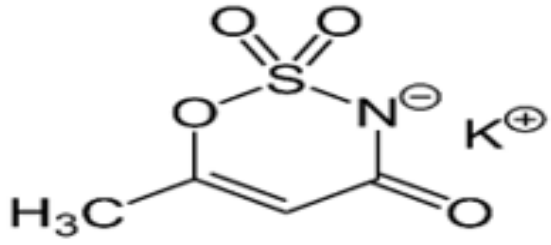
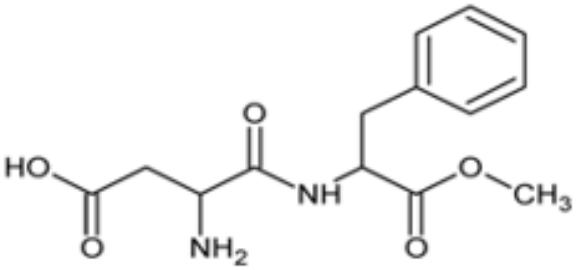


A (Acesulfame – k chemical structure)



B (Aspartame chemical structure)

Fig. 1: Chemical structure of analytes

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 guidelines[8].

Various analytical methods were conducted using HPLC in order to separate and determine the artificial sweeteners encompass: HPLC–CAD–UV/DAD[10], HPLC-MS/MS[11], reversed phase liquid chromatography[12], 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 1g 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

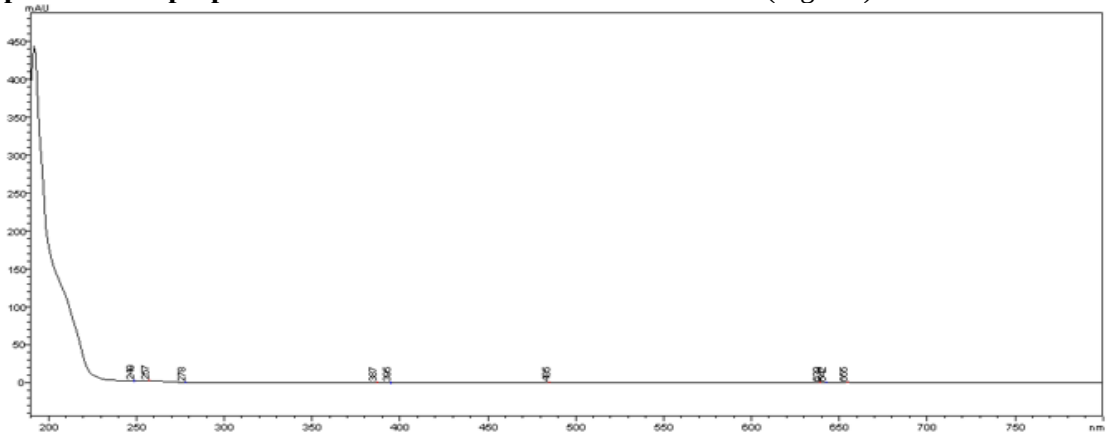
To prepare 2 mM of the phosphate buffer with pH= 4.5, 0.272 g of 98% pure potassium dihydrogen phosphate was transferred to a 1000 mL clean volumetric flask. This was filled to the calibration mark with deionized water.

RESULTS AND DISCUSSION

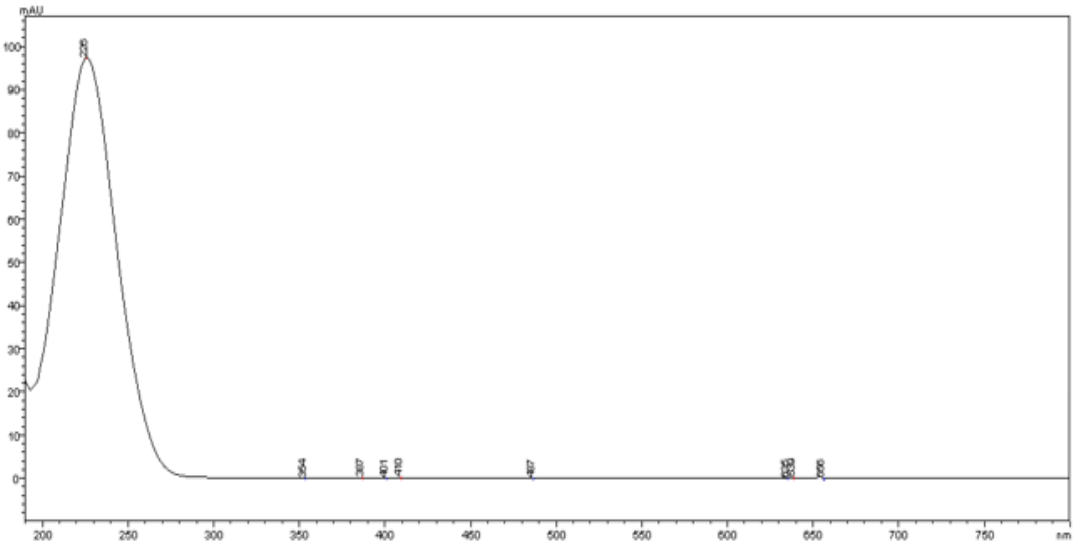
The optimal chromatographic conditions of analysis

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).



A: Aspartame UV-spectrum



B: Acesulfame-k UV-spectrum

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.

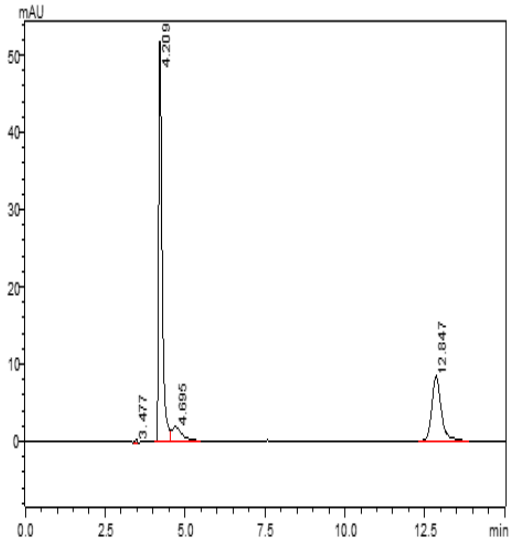
A rate of (85:15) phosphate buffer pH= 3.5 and acetonitrile was tested. Long retention time and tailing factor of Acesulfame-k was 2.3 (Fig. 3A).

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

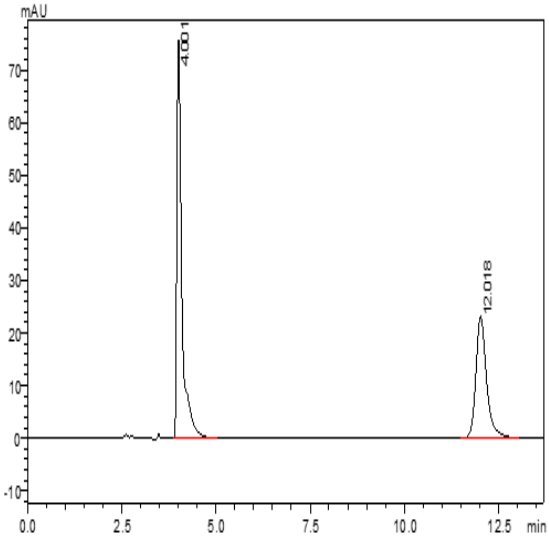
A rate of (70:30) phosphate buffer pH=3.5 and acetonitrile was tested. Bad resolution and tailing factor with superimpose peak (Fig. 3C).

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).

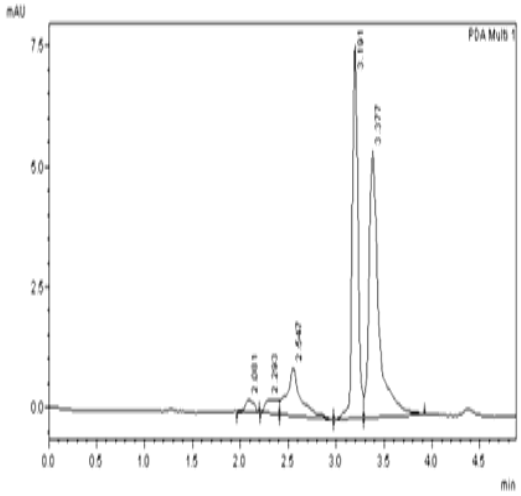
A rate of (80:20) phosphate buffer pH=4.5 and acetonitrile gave the optimum resolution with appropriate retention time and sharp symmetric peak shape. The tailing factor was 1.1 and 1.2 for Acesulfame-k and aspartame respectively (Fig. 4).



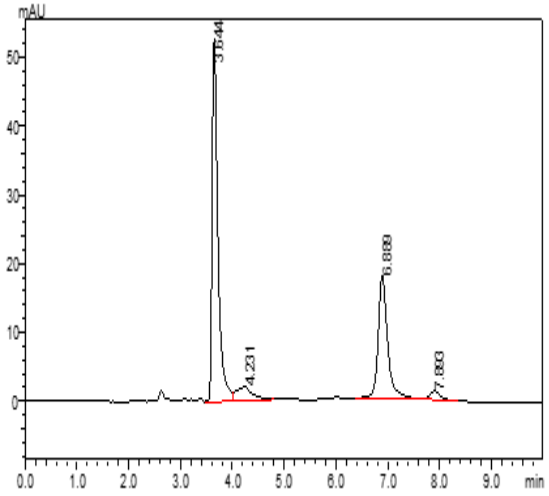
A. Acesulfame – k and Aspartame chromatogram using buffer pH= 3.5 and acentonitrile (85:15 v/v)



B. Acesulfame – k and Aspartame chromatogram using buffer pH= 3.5 and acentonitrile (80:20 v/v)

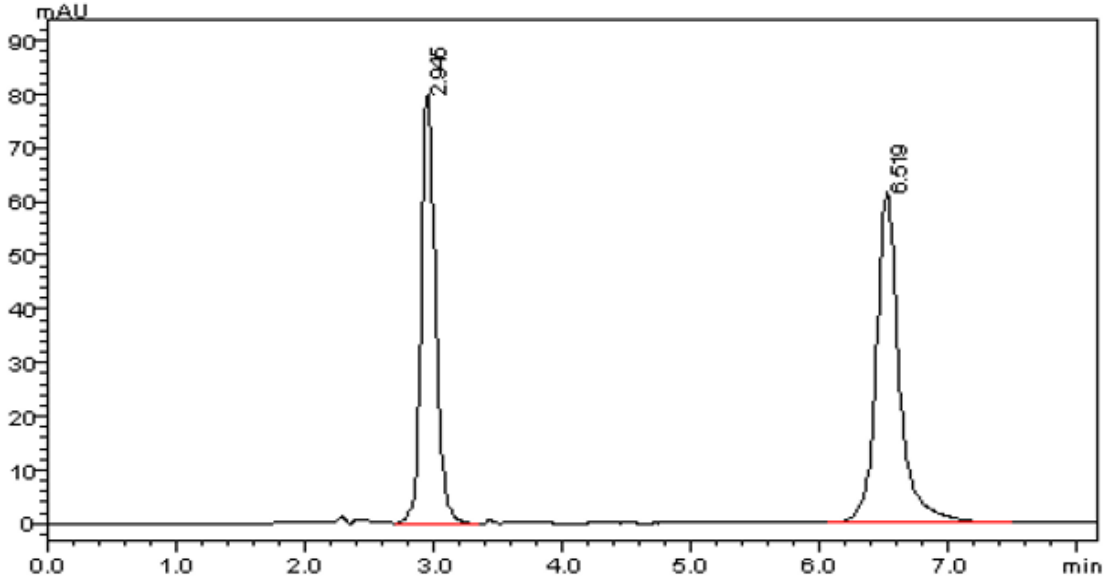


C. Acesulfame – k and Aspartame chromatogram using buffer pH= 3.5 and acentonitrile (70:30 v/v)

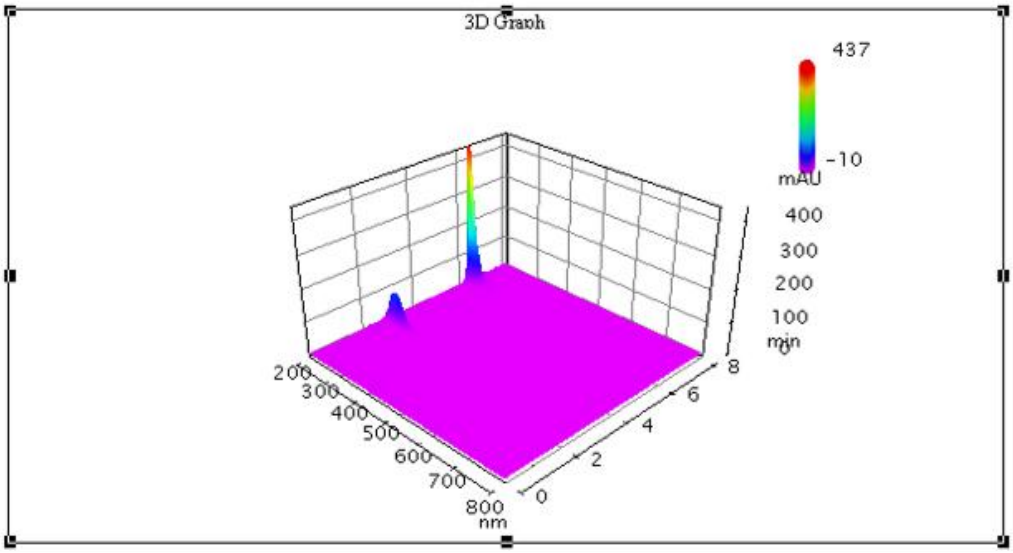


D. Acesulfame – k and Aspartame chromatogram using buffer pH= 2.5 and acentonitrile (80:20 v/v)

Fig. 3: Chromatogram of analytes with various ratios of phosphate buffer and acetonitrile



A: Acesulfame-k and Aspartame chromatogram by optimal conditions



B: 3D chromatogram of Acesulfame-k and Aspartame by optimal conditions

Fig. 4: Chromatogram of analytes by optimal conditions

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[15].

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[16]. 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	**Aspartame**			**Acesulfame-k**		
Theoretical concentration	**Con_1 (50)**	**Con_2 (100)**	**Con_3 (150)**	**Con_1 (10)**	**Con_2 (20)**	**Con_3 (40)**
Found concentration	50.68281	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	9.856606	19.91643	40.26898
Mean^n	50.37502	99.80035	149.8581	10.01414	20.23496	40.34226
Recovery%	100.75004	99.80035	99.90543	100.1414	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

Aspartame								
The spiked level%	**Amount of target compound (µg/mL)**	**Theoretical amount in matrix (µg/mL)**	**Area of the spiked material**	**Found Con 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
			34.1423	34.2186	99.47255			
			34.6597	34.7309	100.962			
100%	**23**	**23**	44.9144	44.8861	98.00462	99.36689	1.485577	1.495042
			45.4419	45.4085	99.14519			
			46.277	46.2355	100.9509			
150%	**23**	**34.5**	56.498	56.3573	98.01269	98.82341	0.70622	0.714628
			57.1599	57.0128	99.15265			
			57.2483	57.1003	99.3049			

Table 5: Spike Recovery Method of Acesulfame-k

Acesulfame-k								
The spiked level%	**Amount of target compound (µg/mL)**	**Theoretical amount in matrix (µg/mL)**	**Area of the spiked material**	**Found Con 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
			453548	13.53512	98.7965			
			454904	13.57755	99.10619			
100%	**9.15**	**9.15**	615687	18.60815	101.6839	101.3711	0.389613	0.384343
			614581	18.57354	101.4948			
			611305	18.47104	100.9347			
150%	**9.15**	**13.7**	762778	23.21035	101.6215	101.5696	0.070117	0.069034
			762603	23.20487	101.5975			
			761817	23.18028	101.4899			

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		Acesulfame-k	
	Con.(µg/mL)	Area	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[n]		pH	Peak Area[n]		λ [nm]	Peak Area[n]	λ [nm]	Peak Area[n]	FR mL/min	Peak Area[n]	
	AS	ACSK		AS	ACSK		AS		ACSK		AS	ACSK
26° C	1481595	353931	**4.3**	1482291	351303	**216**	1728982	**225**	352166	**0.9**	1644094	393178
	1481700	353553		1481608	351409		1729436		351760		1644557	393527
	1485431	354247		1483224	354203		1733576		352526		1646300	392717
30° C	1489750	354351	**4.5**	1489750	354351	**217**	1489750	**226**	354351	**1**	1489750	354351
	1489069	354039		1489069	354039		1489069		354039		1489069	354039
	1488942	353855		1488942	353855		1488942		353855		1488942	353855
34°C	1483859	352162	**4.7**	1489451	351569	**218**	1239577	**227**	353024	**1.1**	1347058	321003
	1486174	353367		1485939	359522		1239231		352400		1344904	321625
	1483617	352540		1487411	351508		1242141		353488		1351651	320571
RSD%	**0.212**	**0.214**		**0.219**	**0.740**		**14.282**		**0.258**		**8.615**	**8.775**

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 conformed with Syrian legislation[18].

Aspartame and Acesulfame-k chromatogram in peach beverage sample is shown in (Fig. 5).

Table 9: Assay data

n=3	Aspartame			Acesulfame-k		
Samples	**Area peak[n]**	**Found Amount mg/kg**	**RSD%**	**Area peak[n]**	**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		

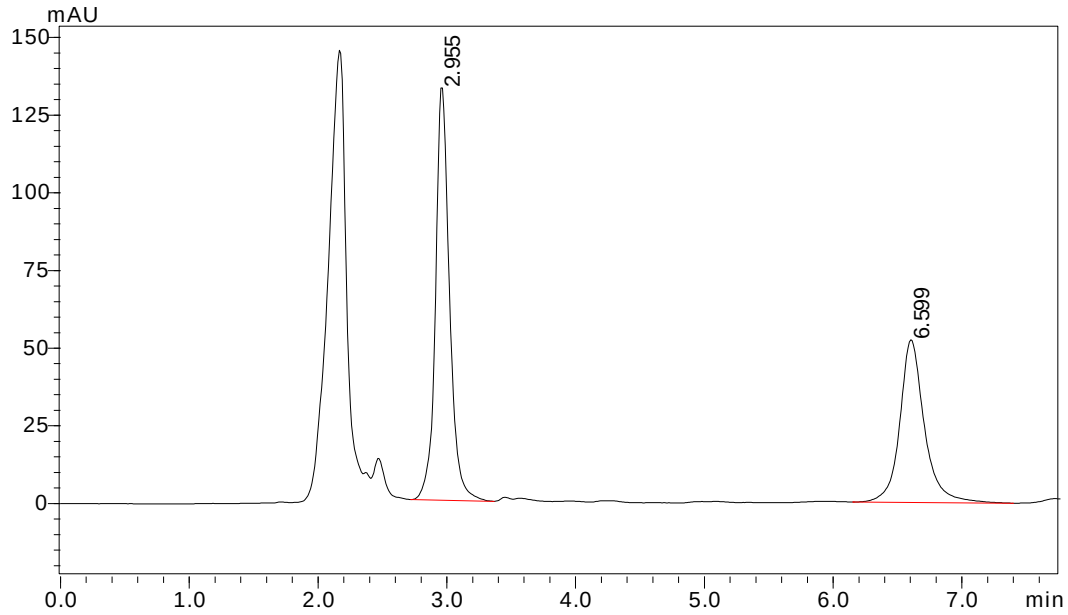


Fig. 5: Acesulfame-k and Aspartame chromatogram in peach beverage sample

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[3].

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.

Acknowledgements

- University of Aleppo, Faculty of Pharmacy.
- Julia & Marielle Woods.
- Editors & Reviewers efforts.

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Bull. Pharm. Sci., Assiut University, Vol. 45, Issue 1, 2022, pp. 269-279.

نشرة العلوم الصيدلية
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طريقة كروماتوغرافيا السائلة عالية الأداء متحقق من صحتها لفصل وتحديد كل من الأسبارتام والأسيسُلفام البوتاسيوم في المنتجات الغذائية

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طريقة عملية، اقتصادية، دقيقة، انتقائية، وسهلة التنفيذ لفصل وتحديد تركيز كُل من الأسبارتام وأسيسُلفام البوتاسيوم في المشروبات غير الكحولية والغازية.

تمت عملية الفصل باستخدام الكروماتوغرافيا السائلة عالية الأداء. العمود المستخدمWelchrom C18 ، بأبعاد(٤.٦×٢٥٠مم، ٥ مكم)، كانت درجة الحرارة ٣٠ م° خلال عملية الفصل، والكاشف المستخدم UV-Photo diode Array، حيثُ تم تحديد الطول الموجي الأعظمي عند ٢١٧–٢٢٦ نانومتر لكل من الأسبارتام والأسيسُلفام البوتاسيوم على التوالي.

يتكون الطور المتحرك المُستخدم في هذه الطريقة من: ٨٠% من الوقاء الفوسفاتي ثنائي الهيدروجين ذو pH= 4.5 و٢٠% من المحل العضوي اسيتونتريل. كان معدل التدفق بمقدار ١ مل/دقيقة، وحجم الحقنة ١٠ ميكرولتر.

كان زمن الاحتفاظ عند الشروط المُثلى لكل من الأسيسُلفام والأسبارتام هو ٢.٩٤ – ٦.٥١ على التوالي. زمن التحليل كان أقل من ١٠ دقائق.

أظهرتْ هذه الطريقة نتائج جيدة لكل من الخطية، الدقة، والصحة. طُبِقت بشكل فعّال لتحليل كل من الأسبارتام وأسيسُلفام البوتاسيوم.

كان المنحنى العياري للمادتين المدروستين خطي، حيثR2> 0.999 ، بينما كانت قيم الانحراف المعياري النسبي لاختبار الدقة أقل من ٢.

تراوحت متوسط قيم الإستردادية في اختبار الصحة لكل من المادتين المدروستين بين ٩٨.٩–١٠١.٥، مما يشير الى أن الطريقة دقيقة.

كانت المادتان المدروستان ذو متانة جيدة عند إجراء تغير طفيف في درجة الحرارة الحموضة، و أظهرأيضًا أسيسُلفام البوتاسيوم متانة جيدة عند إحداث تغيير بسيط في الطول الموجي.