DEVELOPMENT AND VALIDATION OF AN HPLC METHOD FOR THE DETERMINATION OF ACESULFAME K AND SACCHARIN IN CONFECTIONERY WITH NO ADDED SUGAR

Violeta Nour[1*], Ion Trandafir[2], Mira Elena Ionică[1]

[1]University of Craiova, Faculty of Horticulture, Str. A.I.Cuza nr.13, Craiova, Romania
[1]University of Craiova, Faculty of Chemistry, Bd.Calea Bucuresti, no.107I, Craiova, Romania

*Corresponding author: vionour@central.ucv.ro

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Abstract: A simple, selective, and precise reverse phase high performance liquid chromatographic method has been developed for the simultaneous determination of acesulfame K and saccharin in the confectionery with no added sugar (Solano candies). For analysis, the sample is extracted or diluted with water, and the solution of the sample with the two sweeteners is purified by an extraction with Carrez reagents. The chromatographic separation was achieved with potassium dihydrogen orthophosphate buffer (pH = 4.3) and acetonitrile (98:2, v/v) as mobile phase, a DS HYPERSIL C18 5 μm column (250 mm × 4.6 mm) and diode array detection at 220 nm. The analysis time was less than 20 min.
The calibration curves showed good linearity over the concentration range of 0-40 mg/L. The correlation coefficients were ≥ 0.999914 in each case. The method was validated with respect to sensitivity, linearity range, reproducibility, repeatability, recovery and robustness. There have been

obtained average recovery values of 93.0325% for acesulfame K and 93.7067% for saccharin for an addition level of 5 mg/L in the sample solution (the final concentration in sample 227 mg/kg acesulfame K and 234 mg/kg saccharin). The method proved high fidelity and stability, and the detection limit was 1 mg/kg for acesulfame K and 0.4 mg/kg for saccharin for a signal to noise ratio of 3.

Keywords: *HPLC, diode array detection, acesulfame-K, saccharin, sugar free confectionery*

INTRODUCTION

Low-calorie sweeteners are the only means of giving food a sweet taste without increasing its calorie content. Many consumers now use low-calorie sweeteners regularly to sweeten food or drinks, or they buy finished products prepared with low-calorie sweeteners.

The rate of the enhanced sweeteners for diets in some countries in Europe and North America is quite high as a consequence of the general trend to stop the ingestion of food rich in calories and also of the efforts to control the body weight [1]. The artificial sweeteners have been considered as non-toxic, but the research made recently proved their oncogene potential, leading to bladder cancer by mechanisms that have not been cleared up, which led to a restraint of their usage. At a European level, the usage of the sweeteners with a high sweetening capacity is regulated by Directive 94/35/EC. For confectionery with no added sugar it is allowed the usage of maximum 500 mg/kg acesulfame K, 1000 mg/kg aspartame and 500 mg/kg saccharin and its Na, K and Ca salts.

Methods that have been developed for determination of saccharin in foodstuffs include sublimation [2], gravimetry, differential pulse polarography [3], liquid chromatography [4, 5], qualitative thin layer chromatography [6]. For the liquid chromatographic method, sample preparation depends strongly on the matrix to be analyzed.

Within this work, the operational parameters that shall lead to the development of a new analysis method of acesulfame K and saccharin in the confectionery with no added sugar (Solano candies) have been studied, the technique used is the reverse phase liquid chromatography with diode array detection. It has been tried to validate the analysis method settled, in terms of sensitivity, linearity range, reproducibility, repeatability, recovery and robustness. The method has been developed according to the specifications of the standard SR EN 12856/2001 [7].

EXPERIMENTAL PART

Mainly, the sample is extracted or diluted with water. The solution of the sample with the two analytes is purified by an extraction with Carrez reagents. The analytes from the sample test solution, respectively acesulfame K and saccharin are separated by reversed phase chromatography on a 250 mm × 4.6 mm i.d., 5 μm particle DS HYPERSIL C18

column, detected by absorbance at the wavelength of 220 nm and quantified with a calibration graph.
Certified reference materials have been used: acesulfame-K (99.9% purity) and saccharin (99.0% purity) produced by SUPELCO. All the other reagents were of analytical purity or for chromatographic use. The stock solutions and the corresponding dilutions were made in ultra-pure water and were stored in dark places between the experiments, at low temperature (+4°C).
For preparing the sample, 2.5 g of homogenized sample are weighed, to the nearest 1 mg, into a 50 mL volumetric flask, 30 mL water are added and the flask is placed in an ultrasonic bath at 40°C for 20 minutes. 2 mL Carrez solution no. 1 is added (15 g of $K_4[Fe(CN)_6]\cdot 3H_2O$ in water diluted at 100 mL), it is mixed and 2 mL Carez no. 2 solution is added (30 g zinc sulfate ($ZnSO_4\cdot 7H_2O$) are diluted in water at 100 mL). The solution is shaken and it is kept at the room temperature for 10 minutes. The clarified sample mixture is centrifuged for 10 minutes at at least 1400 g before filtering it quantitatively into the 50 mL volumetric flask. The settled matter is washed twice with water and it is centrifuged again, each of the supernatants is collected in the 50 mL volumetric flask and the solution is then diluted to the mark with water. The test solution is filtered through a membrane filter (0.45 μm) before injection.
HPLC was performed with a Surveyor Thermo Electron system comprising vacuum degasser, Surveyor Plus LCPMPP pump, Surveyor Plus ASP autosampler, diode array detector with 5 cm flow cell and Chrom Quest 4.2 software.
The determinations were made in isocratic conditions, at 25°C, using a mobile phase made of phosphate solution ($C_{KH_2PO_4}$ = 0.02 mol/L), adjusted to pH = 4.3 with phosphoric acid ($C_{H_3PO_4}$ = 5%) filtered through a polyamide membrane (0.2 μm) and acetonitrile (98:2, v/v). The volume injected was 5 μL and the flow rate of the mobile phase was 1mL/min.

RESULTS AND DISCUSSION

Validation of the method. Determination of the performance parameters of the developed method

To test linearity, standard solutions of 5, 10, 20, 30 and 40 mg/L acesulfame K and saccharin were prepared and analyzed with three replicates and the results processed with Chrom Quest 4.2 software. The calibration graphs are linear, with five calibration levels. The equations of the calibration graphs and the correlation coefficients for the two analytes are presented in table 1.

Table 1. *Equations of the calibration graphs and the correlation coefficients (r^2) for acesulfame K and saccharin*

Analyte	Equation of the calibration graph	r^2
Acesulfame K	y = 1.46123e-005x – 0.194247	0.999917
Saccharin	y = 1.17821e-005x – 0.160696	0.999914

The calibration curves showed good linearity over the concentration range of 5 – 40 mg/L (y = peak area in mAU; x = concentration in mg/L).
To test peak areas and retention times reproductibility, Chrom Quest software allows the calculation of the relative standard deviation (RSD) for the retention time of the analytes for all levels of the calibration graph and the calculation of the relative standard deviations (RSD) for peak areas at each calibration level.
The relative standard deviations (RSD) for the retention time were of 0.143% for acesulfame K and 0.175% for saccharin, therefore, on standard solutions, the HPLC method developed for the chromatographic separation of the two analytes from Solano candies provides stable retention times. The calculation of peak areas led to RSD between 0.021% and 0.156% for acesulfame K and between 0.008% and 0.271% for saccharin, values less than 2%. Besides, the relative standard deviations prove stability from the point of view of the peaks height and asymmetry.
In order to establish the method traceability on real samples, a sample of Solano candies commercially available was taken for analysis, confectionery with no added sugar which contain acesulfame K and saccharin. Applying the developed method, the two sweeteners in the Solano candies were determined in three replicates.
At this sample, five increasing addition levels of the two analytes were added. Thus, 0.25, 0.5, 1.0, 1.5 and 2.0 mL of stock solution with the concentration of 1g/L of each analyte were added after weighing, water dilution and cooling, so before Carrez clarification. This might correspond to an addition of 5, 10, 20, 30 and 40 mg/L respectively in the sample test solution.
The final concentrations of acesulfame K and saccharin in the addition test solutions were calculated, depending on the sample weight and on the addition level.
The five addition samples and the witness were chromatographically analyzed with three replicates according to the developed method and for each analyte, the samples were treated in a way similar to a six points calibration graph, with the calculated concentrations in the abscissa, as pointed above, and with the peak areas corresponding to the analytes in the addition samples in the ordinate. The calibration graphs achieved are linear, with six calibration levels, the first level being the test solution of the witness with no addition. The equations of these graphs and the correlation coefficients are presented in table 2.

Table 2. *Equations of the graphs and the correlation coefficients (r^2) of the analytes in samples with additions for establishing the method traceability on real samples*

Analyte	Equation of the calibration graph	r^2
Acesulfame K	y = 2.07391e-005x – 3.53049	0.999137
Saccharin	y = 1.59383e-005x – 2.85971	0.999451

In figure 1, it is shown the chromatogram achieved for one of the injections of the witness sample.
As it proceeded with the determination of the method traceability for standard solutions, we tested the reproductibility of the peak areas and of the retention times and for traceability for real samples. The relative standard deviations (RSD) for the retention time were of 0.365% for acesulfame-K and 0.534% for saccharin, so, on real samples the HPLC method developed for the chromatographic separation of acesulfame-K and saccharin from Solano candies provides stable retention times. The calculation of the

peak areas led to a RSD between 0.104% and 0.379% for acesulfame K and between 0.044% and 0.436% for saccharin, values less than the 2% limit.

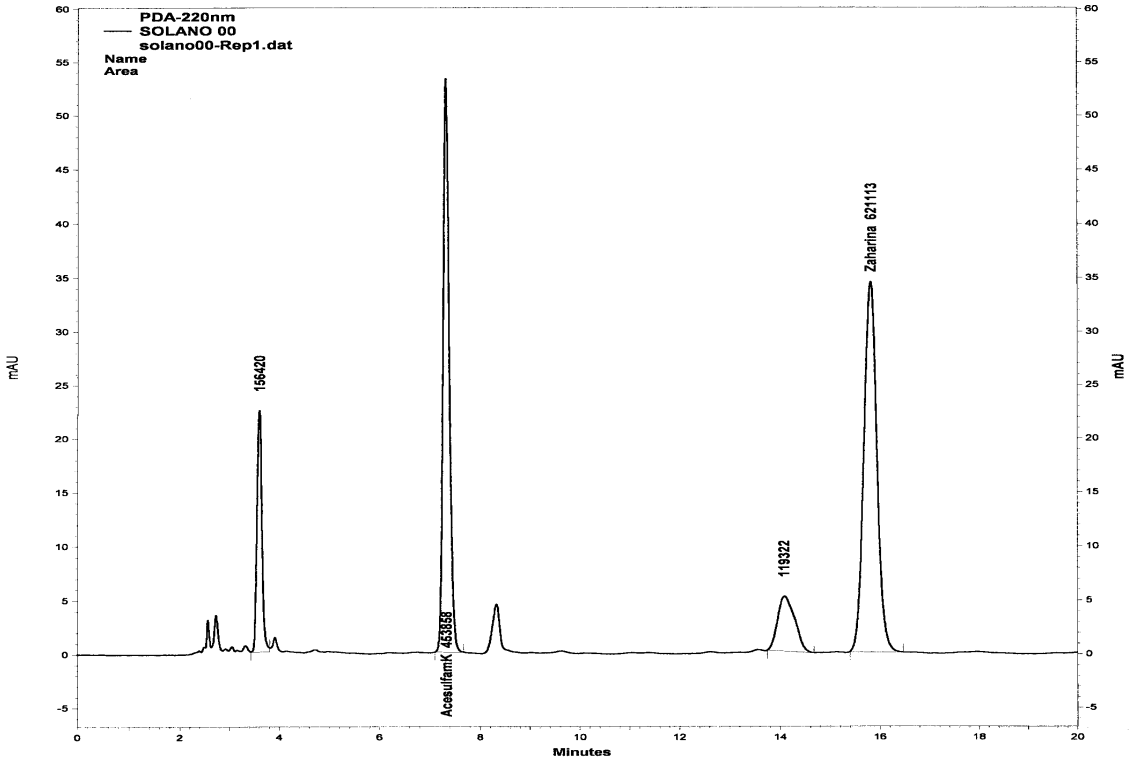

Figure 1. *HPLC of a test solution obtained from the witness sample of Solano candies*

In order to determine the standard reproductibility deviation, the standard solution of 40 mg/L was analyzed by 10 repeated injections. The chromatogram achieved for one of the ten replicates is shown in figure 2.

By the help of Chrom Quest software, there were calculated the relative standard deviations for retention times, heights and peak areas of the two analytes and for the ten replicates. For the peak areas, the relative standard deviations (RSD) were between 0.103% and 0.265% for acesulfame K and between 0.156% and 0.436% for saccharin, value less then the 2% limit, which proves a very good reproductibility of the method developed.

For determining the standard repeatability deviation, a sample of Solano candies with addition was integrally processed ten times, every time preparing the sample (dilution, Carrez clarification, centrifugation, filtration) and chromatographically analyzing, according to the developed method. By the help of Chrom Quest software there were calculated the relative standard deviations for retention times, heights and peak areas of each analyte for the ten replicates.

For the retention times, the RSD values were of 0.109 % for acesulfame K and 0.256 % for saccharin, while for the peak areas, the RSD value were of 0.444 % for acesulfame K and of 0.596 % for saccharin, values which proves a good repeatability of the method developed. The fidelity noticed will be an essential component of the measurement uncertainty of the method developed [8].

The robustness directly investigates the sensibility of the method toward a certain parameter. This is achieved by a robustness test where the effect of a parameter change is noticed [8].

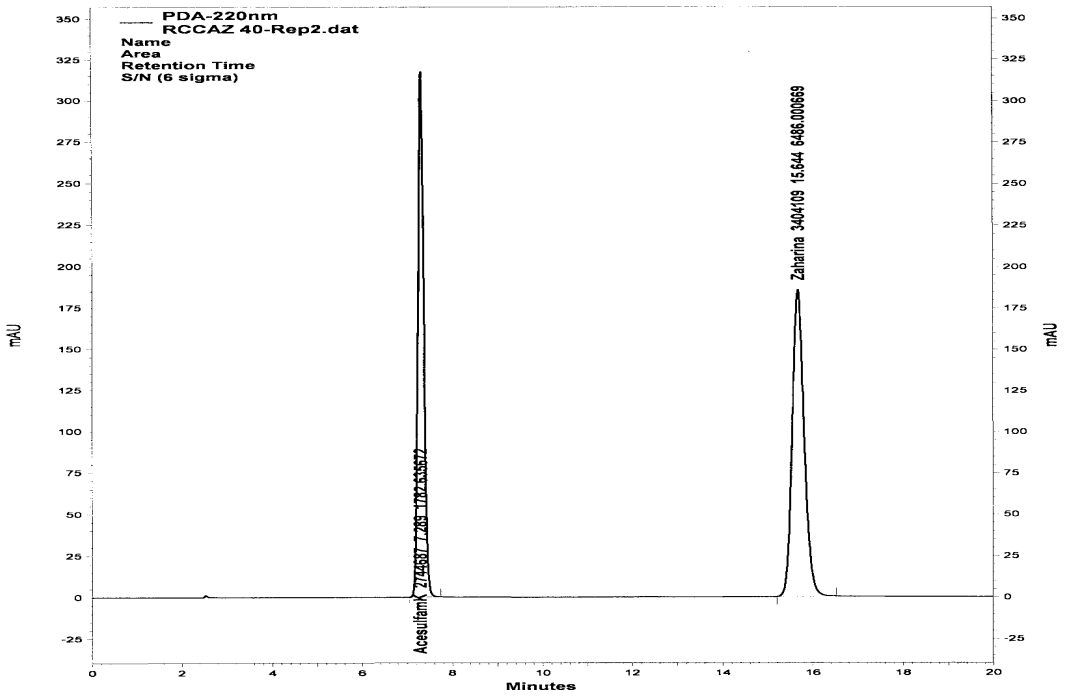

Figure 2. *HPLC of the standard solution with the concentration of 40 mg/L*

We choose the sample test injection volume as a parameter, proceeding in the following way: a sample of Solano candies was analyzed by the method developed, but using different injection volumes for the chromatographic analysis.

Four levels of the sample injection volume were used: 2.5, 5.0, 7.5 and 10 μL, with three replicates for each variant.

The samples were analyzed and the concentrations were calculated using the calibration graphs achieved, which were elaborated using a sample test injection volume of 5 μL. The injection volume variation should determine a variation of the concentration achieved in direct proportion with the injection volume practiced. As a result, we proceeded with the analysis and the calculation of the concentrations in the four experimental variants and drawing a dependence graph of each analyte concentration depending on the injection volume used.

The equations of the dependence of the two analytes concentrations on the injection volume and the correlation coefficients (r^2) are presented in table 3. The high values of the r^2 prove a good sensibility of the method toward the injection volume parameter.

Table 3. *Equations of the dependence of the two analytes concentrations on the injection volume and the correlation coefficients (r^2)*

Analyte	Equations of the dependence graph	r^2
Acesulfame K	$y = 1.48872v - 0.26048$	0,99994
Saccharin	$y = 1.56776v - 0.238$	0,99974

The method recovery can be expressed as value observed divided by value expected and it was determined on the samples with analytes addition. There have been obtained average recovery values of 93.0325% for acesulfame K and 93.7067% for saccharin for an addition level of 5 mg/L in the sample solution (the final concentration in sample 227 mg/kg acesulfame K and 234 mg/kg saccharin).
The method proved high fidelity and stability, and the detection limit determined was of 1 mg/kg for acesulfame K and 0.4 mg/kg for saccharin for a signal to noise ratio of 3.

CONCLUSIONS

Nowadays, low-calorie sweeteners are widely used in foods and soft drinks. Investigations of the toxicity of these compounds have raised questions as to whether they are safe to consume. As a result, their concentration in foods and beverages is regulated through legislation in order to prevent excessive intake.
An HPLC method with diode array detection was developed for the quantitative determination of acesulfame K and saccharin in confectionery with no added sugar. The method provides stable retention times and a detection limit of 1 mg/kg for acesulfame K and 0.4 mg/kg for saccharin for a signal to noise ratio of 3.
For analysis, the sample is extracted or diluted with water and the solution is purified by an extraction with Carrez reagents. The two sweeteners in the sample test solution are separated by reversed phase chromatography, detected by absorbance at the wavelength of 220 nm and quantified with a calibration graph.
The method was validated in terms of sensitivity, linearity range, reproductibility, repeatability, recovery and robustness. Average recovery of the acesulfame K was found to be 93.0325% and that of the saccharin was 93.7067%. The proposed HPLC method for the simultaneous estimation of acesulfame K and saccharin in Solano candies was found to be simple, precise, reproductible, sensitive and accurate.

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