DEVELOPMENT AND VALIDATION OF AN HPLC METHOD FOR SIMULTANEOUS QUANTIFICATION OF ACESULFAME-K, SACCHARIN, ASPARTAME, CAFFEINE AND BENZOIC ACID IN COLA SOFT DRINKS

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Received: 17/03/2009
Accepted after revision: 18/05/2009

Abstract: A simple, selective, and precise reversed-phase HPLC method has been developed for the simultaneous quantification of acesulfame-K, saccharin, aspartame, caffeine and benzoic acid in no-added sugar cola soft drinks. The chromatographic separation was performed by using potassium dihydrogen orthophosphate buffer (*pH* = 4.3) and acetonitrile (88:12, v/v) as mobile phase, a DS Hypersil C 18.5 μm column (250 mm × 4.6 mm) and "diode array" detection at λ = 227 nm for acesulfame-K, λ = 265 nm for saccharin and λ = 217 nm for aspartame, caffeine and benzoic acid. The analysis time was less than 40 min. The calibration curves showed good linearity over the concentration range of 0-100 mg/L. The validation of the analysis method was attempted in terms of sensitivity, linearity range, reproducibility, repeatability, analytical recovery and robustness.

Keywords: *acesulfame-K, saccharin, aspartame, caffeine, benzoic acid, HPLC, "diode array" detection, light cola*

INTRODUCTION

Edulcorants with high sweetening power are used to reduce or, possibly, remove the ingestion of glucides or simply for technological reasons. The use of high sweetening edulcorants allows the obtaining of sweet tasting foods for diabetics. Nevertheless, the most significant market share of high sweetening edulcorants is found in low calorie food products in many countries. Their weight in people's diet in some European and North American countries is quite high. This is the result of the general trend of reducing high calorie foodstuffs and keeping body weight under control.
Given the latest results, high sweetening edulcorants allow the significant reduction of the energy value of several food products, soft drinks mostly [1].
Synthetic edulcorants were practically considered toxic free, but the latest research studies have shown their carcinogenic potential leading to vesicle cancers through less discovered mechanisms. This fact has caused the limitation of their use. At European level, the use of high sweetening edulcorants is regulated by the European Parliament and Council Directive 94/35/EC on sweeteners for use in foodstuffs, as well as by Directive 2003/115/EC of December 2003 amending Directive 4/35/EC.
Methods that have been developed to determine some of these edulcorants in foodstuffs include liquid chromatography (HPLC) [2, 3] and thin-layer chromatography (TLC) [4].
According to Directive 95/2/EC [5], article 1, paragraph 3(a) pointing that "preservatives are substances which prolong the shelf life of foodstuffs by protecting them against deterioration caused by micro-organisms", benzoic acid (E 210), sodium benzoate (E 211), potassium benzoate (E 212) or calcium benzoate (E 213) belong to the category of conditionally permitted preservatives.
The toxicity of benzoic acid, as well as of its derivatives, is low. In high recurrent doses, it produces irritations of the digestive mucous membrane and decreases the digestive usage coefficient by depressing some enzymes (pepsin, trypsin, polypeptides, D-amino-acid oxidase) [6].
People voiced their concern on the possible interaction of benzoic acid and its salts with ascorbic acid within certain drinks, by forming small quantities of benzene which is a carcinogen or cancer producing agent. The major ingredients leading to benzene formation are ascorbic acid and any of the benzoates products, especially sodium benzoate, potassium benzoate or calcium benzoate [6]. Given the fact that the European Union directives regulate the applicability fields and the maximum quantities for each of these preservatives, it is required that methods should be elaborated and validated for their quantitative determination.
Methods that have been developed to determine benzoic acid in foodstuffs include gas chromatography (GC) [7], high pressure liquid chromatography (HPLC) [8, 9], micellar electrokinetic chromatography (MECC) [10], spectrophotometry [11], high performance thin layer chromatography (HPTLC) [12] and potentiometry [9].
Caffeine is one of the most comprehensively studied ingredients in the food supply, with centuries of safe consumption in foods and beverages. In 1959, the U.S. Food and

Drug Administration (FDA) designated caffeine in cola drinks as "Generally Recognized as Safe" (GRAS). The FDA considers caffeine safe for all consumers, including children. In 1987, following extensive review, the FDA "found no evidence to show that the use of caffeine in carbonated beverages would render these products injurious to health". More than 140 countries have specifically considered the safety of caffeine and allow its use in beverages at various levels.

Consistent with federal regulations, beverage companies list caffeine on product labels when it is added as an ingredient. There is no requirement to list the precise amount of caffeine present. According to Directive 2000/13/EC, quinine and/or caffeine used as a flavoring in the production or preparation of a foodstuff must be mentioned by name in the list of ingredients immediately after the term "flavoring". In addition, according to Directive 2002/67/EC of 18 July 2002, drinks containing caffeine in excess of 150 $mg.L^{-1}$ must also provide a warning message on the label followed by an indication of the caffeine content such that: "High caffeine content (X mg/100 mL)".

All these considerations have lead to increased interest in the development of reliable methods for the evaluation and the quantitation of caffeine in food products. Many analytical methods have been developed for the determination of caffeine and the quality control of products containing caffeine including titrimetry, voltammetry [14], NIR-spectroscopy, derivative spectrophotometry [15], polarography, GC [16], and HPLC [17, 18].

Operational parameters leading to the development of a new analysis method of acesulfame-K, saccharin, aspartame, caffeine and benzoic acid in no-added sugar cola soft drinks have been studied in this paper by using liquid chromatography and "diode array" detection. The validation of the analysis method was attempted in terms of sensitivity, linearity range, reproducibility, repeatability, analytical recovery and robustness. The method has been developed according to the specifications of the SR EN1 2856/2001 standard [19], which, although developed for the determination of acesulfame-K, aspartame and saccharin, it also allows the determination of the benzoic acid and caffeine.

EXPERIMENTAL PART

The analytes in the test solution sample 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 227 nm wavelength for acesulfame-K, 265 nm for saccharin and 217 nm for aspartame, benzoic acid and caffeine, and are quantified by using a calibration graph.

Certified reference materials have been used, produced by Supelco such as acesulfame-K (99.9%, 47134), saccharin (99.0%, 47839), aspartame (99.0%, 47135) and benzoic acid (99.9%, 47508-U). All the other reagents, acetonitrile (Baker, 8257), potassium dihydrogen orthophosphate 98% (Alfa Aesar, A 12142), phosphoric acid 85% (Merk, 1805), caffeine 99.0% (Alfa Aesar, A 10431) were of analytical purity or for chromatographic use.

The water used was ultrapure, Basic TWF. The stock solutions and the corresponding dilutions were made in ultra-pure water and were stored in dark places at low temperature (+4 °C) between experiments.

HPLC was performed by using a Surveyor Thermo Electron system including vacuum degasser, Surveyor Plus LCPMPP pump, Surveyor Plus ASP auto sampler, "diode array" detector with 5 cm flow cell, DS Hypersil C18.5 μm column (250 mm × 4.6 mm) and Chrom Quest 4.2 software.
The mobile phase was filtered through a polyamide membrane (0.2 μm) and degassed with an ultrasonic bath DK 102p Bandelin.
The test solutions were filtered through nylon syringe filter (0.45 μm) before injection.

RESULTS AND DISCUSSION

The optimization of the analytes separation process

Considering the fact, which is common knowledge in chromatography, that the retention time of analytes depends upon the mobile phase ratio, the separation optimization was chosen according to this ratio. In order to have proper stability, the isocratic elution was chosen.
For this purpose several mobile phase ratios were used out of those recommended by the SR EN 12856 standard, namely the phosphate buffer solution : acetonitrile = 84:16 (a); 86:14 (b); 88:12 (c); 90:10 (d).
In the case of a 90:10 phase ratio, one may notice that although analytes are well separated, very high retention times result because of the high mobile phase polarity. The total time length of the chromatographic analysis in this particular case is 55 minutes.
The resulted chromatograms show that there is the possibility of decreasing the mobile phase polarity by increasing the acetonitrile weight in the mobile phase.
In the case of an 84:16 mobile phase ratio one may notice that acesulfame-K and saccharin can no longer be separated, as these two analytes have relatively close retention times. The total time length of the chromatographic analysis in this case is 26 minutes.
In the case of a 86:14 phase ratio, a proper separation of analytes takes places and the time length of the analysis is 34 minutes but, due to the fact that acesulfame-K and saccharin have very close retention times, for safety reasons it was decided that a 88:12 phase ratio should be used, as it offered the most efficient separation in the shortest time, i.e. 40 minutes for the separation of the whole five analytes. As result, mobile phase (c) was chosen as being optimal for isocratic determinations.
The determinations were made under isocratic conditions, at 20 °C, by using a mobile phase made of 88% phosphate solution (0.02 $mol.L^{-1}$ KH_2PO_4), adjusted to pH = 4.3 with phosphoric acid 5%, filtered through a polyamide membrane (0.2 μm) and 12% acetonitrile.
The analytes were determined at wavelengths 217 nm (caffeine, aspartame, benzoic acid), 227 nm (acesulfame-K) and 265 nm (saccharin). The column was equilibrated for one hour before samples injection. The volume injected was 5 μL and the flow rate of the mobile phase was 1 $mL.min^{-1}$.
After a complete series of analysis, the HPLC system was cleaned using an adequate washing method. The mobile phase in this method is made of water and acetonitrile, using a moderate gradient to attain the suitable conditions for the column keeping.

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

To test linearity, standard solutions of 10 mg.L^{-1}, 25 mg.L^{-1}, 50 mg.L^{-1}, 75 mg.L^{-1} and 100 mg.L^{-1} 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, the correlation coefficients and the selected wavelengths for the analytes detection are shown in table 1.

Table 1. *Equations of calibration graphs and correlation coefficients (r^2) for the five analytes*

Analyte	λ, [nm]	Equation of the calibration graph	r^2
Acesulfame- K	227	y = 1.38137e-005x – 1.04665	0.9999
Saccharin	265	y = 0.000104801x + 0.826302	0.9992
Caffeine	217	y = 1.23545e-005x – 0.791620	0.9999
Aspartame	217	y = 5.04373e-005x + 0.891074	0.9996
Benzoic acid	217	y = 1.47759e-005x + 1.49733	0.9997

The linearity range was 1–100 mg.L^{-1} (y = peak area in mAU×s; x = concentration in mg.L^{-1})[1].

To test peak area and retention time reproducibility, Chrom Quest software allows the calculation of the relative standard deviations (RSD) for the retention time of the analytes for all levels of the calibration graph and for peak area at each calibration level. The relative standard deviations (RSD) for the retention time were between 0.013% (acesulfame K) and 0.291% (caffeine) therefore, in standard solutions, the HPLC method developed for the chromatographic separation of the five analytes provides stable retention times. The calculation of peak areas led to RSD between 0.041% (aspartame) and 0.966% (acesulfame K). Moreover, the calculated relative standard deviations also prove stability in terms of peak height and asymmetry.

In order to establish the method traceability on real samples, a sample of cola light available for sale was taken for analysis, including four of the five analytes (acesulfame-K, aspartame, benzoic acid and caffeine). The sample was degassed, filtered, diluted in water and analyzed according to the developed method.

Five increasing addition levels of all the five analytes were added to this sample. Thus, 1, 2, 3, 4 and 5 mL of stock solution having a concentration of 1g.L^{-1} of each analyte were added in the 100 mL volumetric flask and the solution was then diluted up to the mark with light cola softdrink. The final concentrations of the five analytes in the addition test solutions were calculated. The five addition samples and the witness were chromatographically analyzed with three injections, according to the developed method, and for each analyte samples were treated in a way similar to a six point-calibration graph, with the calculated concentrations on the abscissa axis, as pointed above, and with the peak areas corresponding to the analytes in the addition samples on the ordinate axis. The calibration graphs resulted is linear and have six calibration levels, the first level representing the addition free test solution witness. Figures 1, 2 and 3

[1] mAU = mili absorbance units

show the chromatograms resulted for one of the injections of the addition sample (addition level 3).
The equations of these graphs, the correlation coefficients and the wavelengths selected for detection of the analytes are shown in table 2.
The relative standard deviations (RSD) for the retention time was between 0.0% (saccharin and acesulfame-K) and 0.303% (caffeine), therefore, on real samples, the HPLC method developed for the separation of the five analytes provides stable retention times. The calculation of peak areas led to very good RSD values below 2%, i.e. 0.067% (acesulfame K) and 0.665% (aspartame). Furthermore, the relative standard deviations also prove stability in terms of peak height and asymmetry.

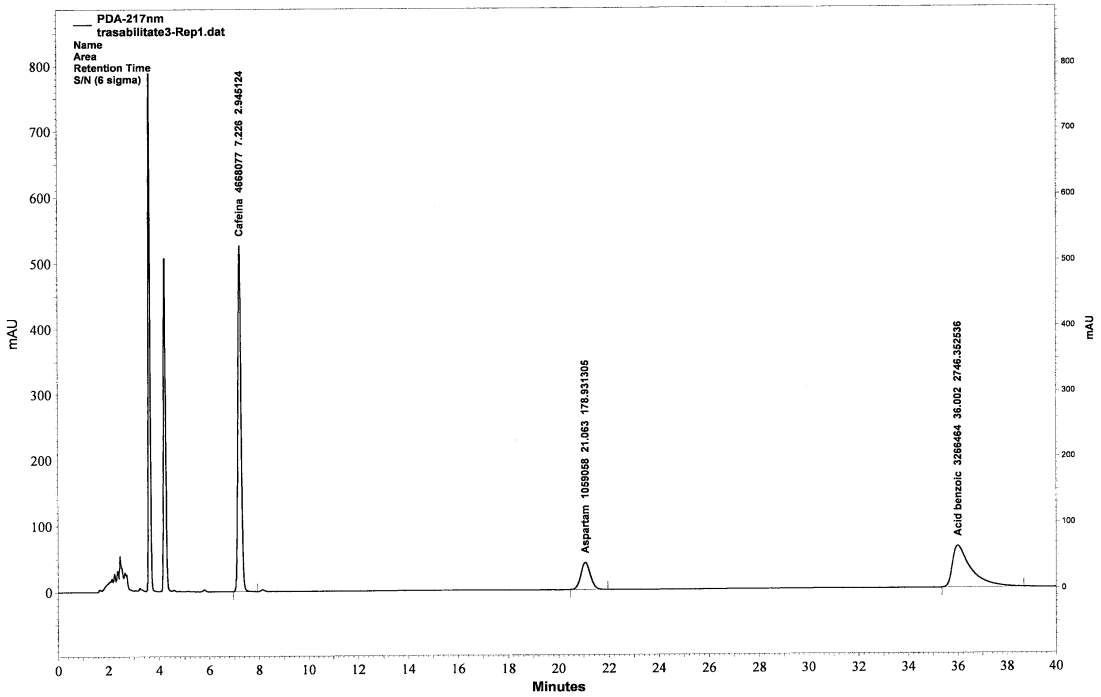

***Figure 1**. HPLC at λ = 217 nm of a light cola soft drink with addition (level 3)*

***Table 2**. Equations of calibration graphs and correlation coefficients (r^2) of analytes for real samples with addition*

Analyte	λ, [nm]	Equation of the calibration graph	r^2
Acesulfame- K	227	$y = 1.37837e\text{-}005x - 1.31120$	0.9987
Saccharin	265	$y = 0.000106957x + 0.826302$	0.9993
Caffeine	217	$y = 1.22475e\text{-}005x - 0.683213$	0.9989
Aspartame	217	$y = 5.19923e\text{-}005x + 0.194536$	0.9994
Benzoic acid	217	$y = 1.48840e\text{-}005x + 1.58806$	0.9995

In order to verify the reproducibility, the standard solution of 50 $mg.L^{-1}$ was analyzed by 10 repeated injections.
For the peak areas, the relative standard deviations (RSD) were between 0.103%, (saccharin) and 0.265% (benzoic acid), which shows very good reproducibility of the

method developed. The repeatability of the method was verified through the analysis of a light cola sample with addition, which was fully processed ten times, every time preparing the sample and chromatographically analyzing it, according to the developed method.
By using Chrom Quest software, relative standard deviations were calculated for retention times, heights and peak areas of each analyte for the ten replicates.
For peak areas, the RSD values varied between 0.030% (benzoic acid) and 1.249% (saccharin), fact which shows a good repeatability of the method developed.

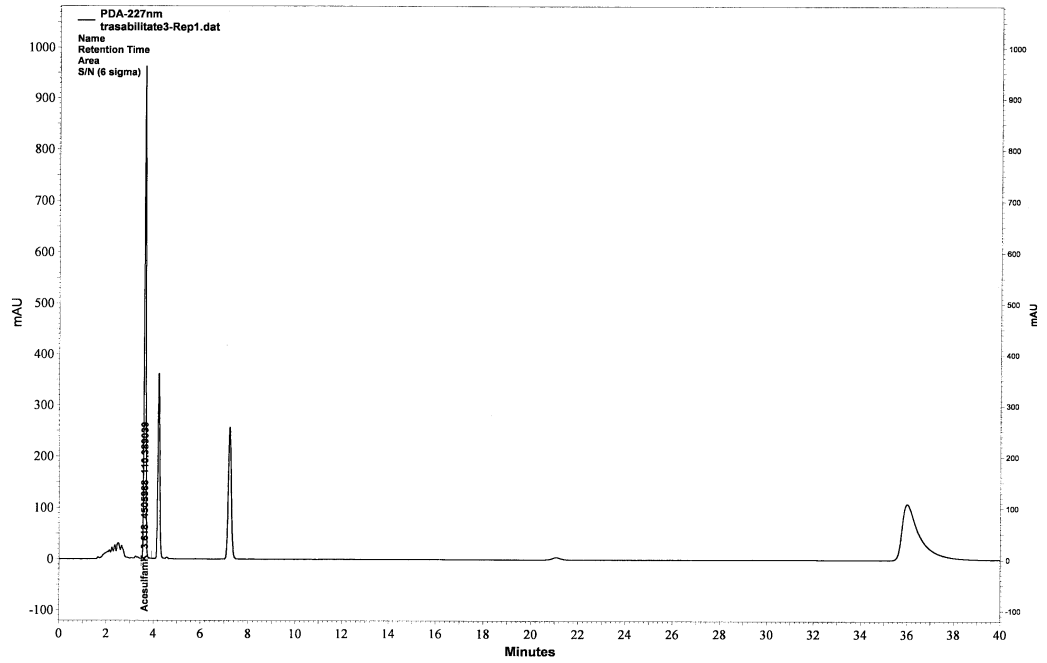

***Figure 2**. HPLC at λ = 227 nm of a light cola soft drink with addition (level 3)*

Robustness directly investigates the sensitivity of the method toward a certain parameter. This is achieved by a robustness test where the effect of a parameter change is noticed [20]. We choose the sample test injection volume as a parameter, proceeding in the following way: a sample of light cola was analyzed by the method developed, but using different injection volumes for the chromatographic analysis. Four levels of the sample injection volume were used, i.e. 2.5 μL; 5 μL; 7.5 μL and 10 μL.
The injection volume variation should determine a variation of the concentration resulted in direct proportion with the injection volume practiced. As result, we proceeded with the analysis and the calculation of concentrations in the four experimental variants and drawing a dependence graph of analytes concentration, according to the injection volume used.

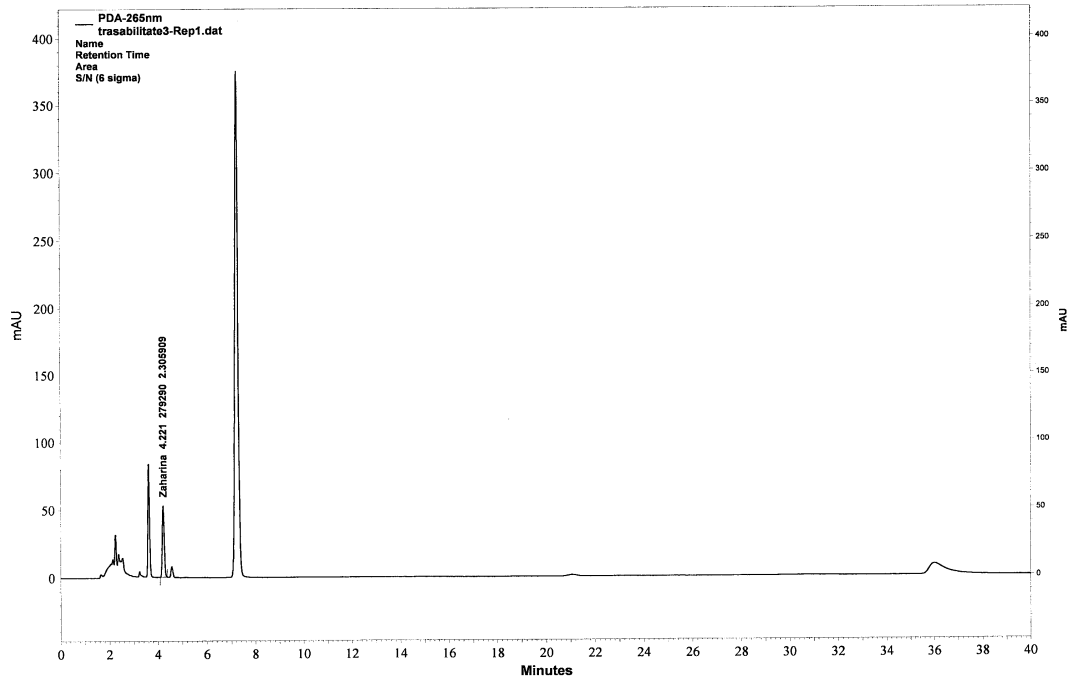

***Figure 3**. HPLC at λ = 265 nm of a light cola soft drink with addition (level 3)*

The equations of the dependence of the five analytes concentrations on the injection volume and the correlation coefficients (r^2) are shown in table 3. The high values of the r^2 prove a good sensitivity of the method to the injection volume parameter.

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

Analyte	Equations of the dependence graph	r^2
Acesulfame- K	$y = 10.108v + 0.4227$	0.9994
Saccharin	$y = 4.0195v + 0.4473$	0.9999
Caffeine	$y = 9.2591v + 0.2583$	0.9996
Aspartame	$y = 9.0827v – 0.5678$	0.9998
Benzoic acid	$y = 8.0668v – 0.9465$	0.9999

The bias of an analytical method is usually determined by study of relevant reference materials or by spiking studies. Bias may be expressed as analytical recovery (value observed divided by value expected) [20]. The average recovery values resulted were somewhere between 93.5% in case of aspartame and 104.9% in case of saccharin.
The detection limits were determined starting from the principle that a peak, to be detected, must have a signal-to-noise ratio > 3. The detection limits determined for the five analytes and the corresponding values of the signal-to-noise ratio are shown in table 4.

Table 4. *Detection limits for acesulfame-K, saccharin, aspartame, caffeine and benzoic acid*

Analyte	Concentration [mg.L^{-1}]	Signal-to-noise ratio S/N (6 sigma)	Detection limit [mg.L^{-1}]
Acesulfame-K	0.03	2.5048	0.05
	0.05	4.3809	
Saccharin	0.50	0.8684	1.00
	1.00	3.9027	
Caffeine	0.005	2.6660	0.01
	0.01	4.2532	
Aspartame	0.05	2.2226	0.08
	0.08	3.0001	
Benzoic acid	1.00	34.0979	1.00

CONCLUSIONS

Nowadays, low-calorie sweeteners and benzoic acid are widely used in foodstuffs and soft drinks. Investigations on the toxicity of these compounds have raised questions as to whether they are safe to consume or not. 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, saccharin, aspartame, caffeine and benzoic acid in cola soft drinks. The method provides stable retention times and detection limits between 0.01 mg/kg for caffeine and 1 mg/kg for saccharin and benzoic acid for a signal-to-noise ratio > 3. The five analytes in the sample test solution are separated by reversed-phase chromatography, detected by absorbance at wavelength of 227 nm for acesulfame-K, 265 nm for saccharin and 217 nm for aspartame, caffeine and benzoic acid and quantified with a calibration graph.

The method was validated in terms of sensitivity, linearity range, reproducibility, repeatability, analytical recovery and robustness. Average recovery values were between 93.5% for aspartame and 104.9% for saccharin. The proposed HPLC method for the simultaneous quantification of acesulfame K, saccharin, aspartame, caffeine and benzoic acid in cola soft drinks was found to be simple, precise, reproductible, sensitive and accurate.

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