

Analytical difficulties for determination of acesulfame K in chocolate products

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Abstract

Sweeteners are substances used as a dietary supplement to replace sugar. Consumers are concerned about the high levels of sugar, calories and cariogenicity in confectionery products, which is why the popularity of the so-called. „Light“ products and „sugar-free“ products. Acesulfame K is a synthetic sweetener about 200 times sweeter than sugar. In the present work, an analysis of acesulfame K in cocoa and chocolate products was performed. For the determination of sweeteners acesulfame K, saccharin and aspartame in foodstuffs, a standardized reverse phase high performance liquid chromatography method with UV detection was used. A cocoa matrix-specific compound was observed in all chocolate products analyzed for acesulfame K. Interference did not correspond to acesulfame K on the UV spectrum and could not be removed by two-step purification. The comparison of the spectral characteristics allowed to avoid a misleading result for the presence of acesulfame K in chocolate and cocoa products.

Keywords

chocolate and cocoa products, E 950, HPLC – UV, synthetic sweeteners

Introduction

The use of sweeteners in the food industry is widespread. Sweeteners are also substances used as a dietary supplement to replace sugar. Sweeteners can be divided into two groups: natural and synthetic. Natural sweeteners are completely absorbed by the body and, like refined sugar, provide energy. Synthetic sweeteners do not contain calories and are not absorbed by the human body. They are significantly sweeter than sugar (30 to 700 times), which is why a very small amount of them is needed to achieve the required sweetness. Synthetic sweeteners are authorized for use in cocoa and chocolate products, soft drinks, flavored dairy products, preserved fruit and vegetable products in accordance with Regulation (EC) №1333/2008 on food additives.

The most commonly used synthetic sweeteners are saccharin, aspartame, cyclamate and acesulfame K.

Acesulfame K (E 950) is rapidly absorbed from the intestine and is found unchanged in the urine, as it is not metabolized or accumulated in the body. Some epidemiological studies have shown that synthetic sweeteners are suitable for low-calorie diets and for those suffering from glucose intolerance and type 2 diabetes (Gardner et al. 2012). However, in recent years, the available evidence suggests that the consumption of synthetic sweeteners can disrupt human metabolism, especially glucose regulation (Pepino and Bourne 2011; Suez et al. 2014). Synthetic sweeteners, such as acesulfame K, have been found to cause glucose intolerance and/or metabolic syndrome and may therefore lead to weight gain (Pfeffer et al. 1985; Dhingra et al. 2007; Fowler et al. 2008; Brown et al. 2010).

These findings suggest that synthetic sweeteners may increase the risk of obesity. However, the specific mechanism of disruption of human metabolism in the consumption of synthetic sweeteners remains unclear.

Although the toxicity data reported so far are considered insufficient (Karstadt 2010), some previous studies have found that acesulfame K is genotoxic and may inhibit glucose fermentation by intestinal bacteria (Bandyopadhyay et al. 2008; Genc et al. 2008). Also, acesulfame K, similar to saccharin and cyclamate, belongs to the group of sulfonamides associated with antimicrobial activity (Brown et al. 2010). A recent study in the United States (Frankenfeld et al. 2015), based on four days of food intake, found that healthy people who did not consume and those who consumed synthetic sweeteners (acesulfame K and aspartame) showed a difference in bacterial diversity. It is not yet known the exact mechanism of action of acesulfame K on intestinal microbiota.

Today's consumers are concerned about high levels of sugar, calories and cariogenicity in confectionery products, so the popularity of so-called „light“ products and „sugar-free“ is rising. Making sugar-free chocolate is a challenge as sugar needs to be replaced. Therefore, a thorough understanding of the applicability and safety of synthetic sweeteners as ingredients in the manufacturing of chocolate without sugar is important for both producers and consumers (Aidoo et al. 2013).

Chocolate is a thick suspension of solid particles containing 60–70% sugar and skimmed cocoa. Chocolate is rarely produced without sugar because of the multifunctional properties of sugar – to provide sweetness, volume and texture. In the initial stage of production of chocolate and related products (Nikoleli et al. 2012), sugar could be replaced by acesulfame K not only for the purpose of creating products for people with special dietary requirements, but also for economic reasons.

Acesulfame K (E 950) (Fig. 1) was discovered in 1967 in Germany. It is 200 times sweeter than sugar, about as sweet as aspartame, and half as sweet as saccharin. Acesulfame K is extremely thermostable with a wide pH range (from 2.5 to 9) (Coiffard et al. 1999).

Various methods for determining sweeteners are available in the literature using mainly liquid chromatography with different detectors depending on the available equipment (Tighrine et al. 2019). A standardized method

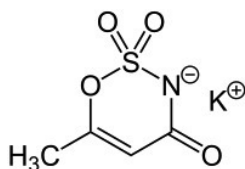


Figure 1. Structural formula of acesulfame K.

BDS EN 12856:2001 Foodstuffs – Determination of acesulfame K, aspartame and saccharin – High performance liquid chromatographic method has been used in the present work. This method was been verified and was used for analysis of sweeteners acesulfame K, saccharin and

aspartame in various matrices, in which sweeteners are most often contained – soft drinks, boza, various confectionery, flavored dairy products, canned fruits and vegetables. Our results from the application of the standardized method have been reported in a number of publications (Petrova et al. 2017, 2018). The method was accredited and has shown parameters that meet the requirements of the legislation and ISO 17025:2019. The method was used to analyze sweeteners in chocolate products for the first time. It was found that the positive results obtained for the presence of sweeteners contradicted the description of the labels. No descriptions of difficulties in identifying sweeteners in chocolate products have been found in the literature. In this regard, attention was focused on the need to confirm the results obtained, and as it was subsequently found, led to the need to eliminate analytical difficulties.

The **aim** of the present work is to analyze acesulfame K in cocoa and chocolate products and to overcome the analytical difficulties associated with its identification.

Materials and methods for analysis of sweeteners

Description of samples

Identical products from different companies, purchased from the Bulgarian and European markets, were studied in order to establish the compliance of the label with the content: candies – hazelnuts in caramel and nougat cream and chocolate; chocolates; milk chocolate; Swiss milk chocolate; milk chocolate with whole hazelnuts; dark chocolate; hazelnut cocoa cream; sponge cake with chocolate filling and milk filling; latte macchiato.

Analytical method

A standardized method BDS EN 12856: 2001 for the determination of sweeteners acesulfame K, saccharin and aspartame in foodstuffs by reverse phase high performance liquid chromatography with UV detection was used. The chromatographic analysis according to the method allows the simultaneous determination of the three sweeteners at a UV detection wavelength of 210 nm. The working range for each sweetener was from 5.0 to 100.0 mg/kg. The method was verified at three points in the working range and a limit of quantification (LOQ) was established for each of the sweeteners – 5.0 mg/kg.

Reagents:

- Acetonitrile, for HPLC
- Potassium dihydrogen orthophosphate (KH_2PO_4)
- Potassium hexacyanoferrate trihydrate $\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$
- Zinc sulfate heptahydrate $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$
- Phosphoric acid (H_3PO_4), w (H_3PO_4) = 5%
- Deionized water
- 0.45 μm Syringe filter NYLON

- Cartridges Strata C18-E (55 μm , 70 \AA), 1 g/, 6ml Tubes for Solid phase extraction (SPE), for single use

Standards:

- Acesulfame potassium, 99.4% purity, LGC Standards
- Saccharin, 99.6% purity, LGC Standards
- Aspartame, 97.7% purity, LGC Standards

The following solutions for extracting sweeteners from the complex chocolate matrix were prepared:

- 0.02 M Phosphate buffer (pH 3.5) was prepared by weighing 2.72 g of KH_2PO_4 (potassium dihydrogen phosphate), which was dissolved to 1 L with deionized water. The pH of the buffer was adjusted by adding drops of phosphoric acid (H_3PO_4);
- Carrez solution I was prepared by dissolving 15 g $\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$ in distilled water and diluted to 100 mL; the resulting solution was degassed in an ultrasonic bath at 30 °C;
- Carrez solution II was prepared by dissolving 30 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in distilled water and dilute to 100 mL; the resulting solution was degassed on an ultrasonic bath at 30 °C.

Preliminary preparation of samples

Samples of chocolate and cocoa products were purified, filtered and analyzed for content of synthetic sweeteners using HPLC.

First step: Purification of the samples by Carrez solutions.

Each product was aggregated, ground and homogenized well. About 5 g of the bulk sample was weighed into an Erlenmeyer flask, 20 ml of deionized water was added and the sample was treated in an ultrasonic bath at 40 °C for 20 minutes. The sample was left at room temperature until cool. Exactly 1 ml of Carrez solutions I and 1 ml of Carrez solutions II were added. The sample was transferred to a 50 ml volumetric flask and brought to the mark with deionized water (10-fold dilution).

After re-treatment in an ultrasonic bath, the diluted samples were filtered through a 0.45 μm Syringe filter and injected into the liquid chromatograph.

Equipment:

An Agilent HP type 1050 apparatus was used for the analysis of synthetic sweeteners.

- Chromatographic column: RP-18 (5 μm), Purospher;
- Mobile phase: Phosphate buffer (pH 3.5) : acetonitrile = 90:10; flow rate: 0.700 ml/min;
- UV detection $\lambda = 210 \text{ nm}$;
- Injection volume: 50 μl ;
- The integration of the obtained peaks was performed by area using Agilent 1100 software;
- The sweeteners are identified by comparing the retention times with those of standard solutions. The standardized method allows acesulfame K, saccharin and aspartame to be analyzed simultaneously. The Retention Times (RT) was as follow: RT (acesulfame K) = $3.0 \pm 0.15 \text{ min}$, RT (saccharin) = $3.66 \pm 0.14 \text{ min}$, RT (aspartame) = $13.35 \pm 1.01 \text{ min}$.

Verification of the standard method

The verification of the standard method was performed in 3 points from the working range from 5 to 500 mg/kg of food. In the verification, real food samples were used according to the following scheme (for acesulfame potassium):

- I (6.60 ± 0.40) mg/kg – diluted sample
- II (33.00 ± 2.10) mg/kg – real sample
- III (164.50 ± 8.8) mg/kg – real sample with spike

Similar schemes have been used for other sweeteners, which were not discussed here.

The method used was characterized by the following analytical parameters for acesulfame K as a result of the verification (Table 1):

- Limit of detection (LOD) was 1.0 mg/kg
- Limit of quantification (LOQ) was 5.0 mg/kg

Liquid chromatographic analysis of samples of chocolate and cocoa products

The obtained chromatograms of all analyzed products showed a poor signal-to-noise ratio (Fig. 2), which did not allow the identification of acesulfame K and required the introduction of a second step in the preliminary preparation of the samples by solid phase extraction.

Second Step: Additional purification by solid phase extraction.

Chocolate products are a complex food matrix to analyse (Nikoleli et al. 2012). Additional purification of the

Table 1. Analytical parameters from the verification of the method for the determination of acesulfame K.

Samples	Precision, %	Bias, %	RSD, %	Repeatability limit, mg/kg	Limit of reproducibility, mg/kg	Uncertainty, mg/kg
I – diluted sample	98.48	-1.52	4.48	0.89	0.99	0.25
II – real sample	100.3	0.30	4.89	4.53	5.61	1.30
III – real sample with spike	98.78	-1.22	4.51	20.51	22.87	6.40

The sweeteners saccharin and aspartame were similarly verified.

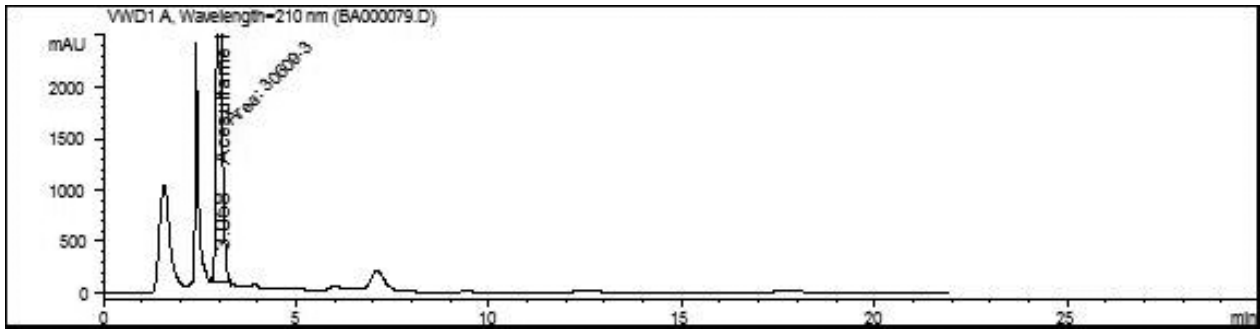


Figure 2. Chromatogram of a chocolate sample before solid phase extraction.

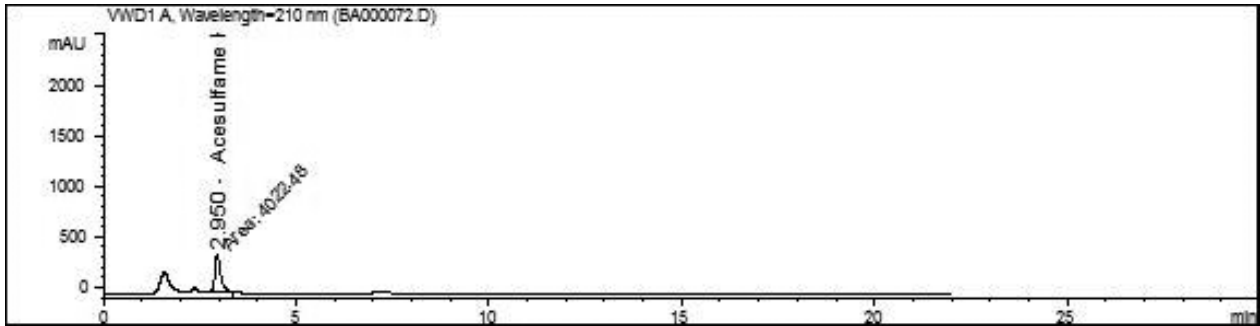


Figure 3. Chromatogram of a chocolate sample after solid phase extraction.

filtrate is required to remove the matrix interference. The solution obtained after the first stage of sample preparation was filtered through a pleated filter. An aliquot of 2.5 ml of the filtrate was purified by solid phase extraction (SPE) using a cartridge Strata C18-E (55 μm , 70 \AA), 1 g/, 6ml Tubes. The sorbent in the cartridge was activated with 6 ml methanol and 40 ml deionized water. Then, the sample was eluted through the cartridge using 20 ml of mobile phase (phosphate buffer : acetonitrile = 90:10). The filtrate was transferred to a 25 ml flask and brought to the mark with the mobile phase (10-fold dilution).

After sonication, the diluted samples were filtered through a 0.45 μm Syringe filter and injected into the liquid chromatograph.

Results

The chromatograms obtained showed an improvement in the signal-to-noise ratio compared to the results obtained after the first purification step (Fig. 3).

Chromatograms of all tested chocolate products showed signals at a retention time (RT) corresponding to that of acesulfame K standard solution (Fig. 4).

The results obtained showed that the analyzed products should contain acesulfame K. At the same time, these results did not correspond to the content of the products described in the labels stating that they contain sugar.

The complex matrix of chocolate products, despite the two-stage purification of the samples, can lead to misinterpretation of the chromatograms in identification only

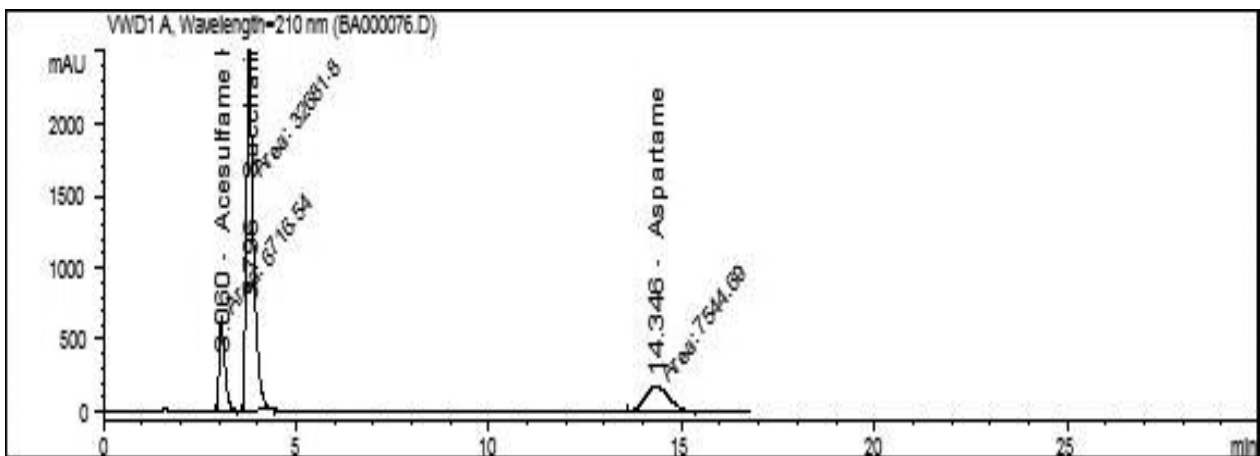


Figure 4. Chromatogram of a standard solution of sweeteners – acesulfame K (RT = 3.060 min), saccharin (RT = 3.796 min) and aspartame (RT = 14.346 min).

according to the retention times. To resolve this, additional steps of identification of acesulfame K are required.

Third Step: Additional identification of acesulfame K using a liquid chromatograph (Agilent HP type 1200) with UV-Vis (DAD) detector

- Chromatographic column: RP-18 (5 µm), Purospher;
- Mobile phase: phosphate buffer pH 3.5: acetonitrile = 90:10; flow rate: 1.000 ml/min;
- UV-Vis (DAD) detection λ = (210.30 ÷ 360.10) nm;
- Injection volume: 50 µl.

Fig. 5 shows a chromatogram of a standard solution of sweeteners – acesulfame K, saccharin and aspartame, demonstrating the retention times of each of them. The spec-

trum of acesulfame K from the standard solution shows a maximum absorption at a wavelength of 226 nm (Fig. 6), which corresponds to the literature data on the UV spectrum of acesulfame K (Fig. 7) (Coiffard et al. 1999).

The retention times of the sample and the standard sweetener solution were compared by superimposing the chromatograms (Fig. 8). The retention time of acesulfame K was found to match that of an unknown substance, but its spectrum was not characterized by the same absorption maximum at a wavelength of 226 nm as acesulfame K (Fig. 9).

This is observed in all chocolate products analyzed for acesulfame K. Therefore, this compound is probably characteristic of the chocolate matrix, cannot be removed by purification and is also not acesulfame K.

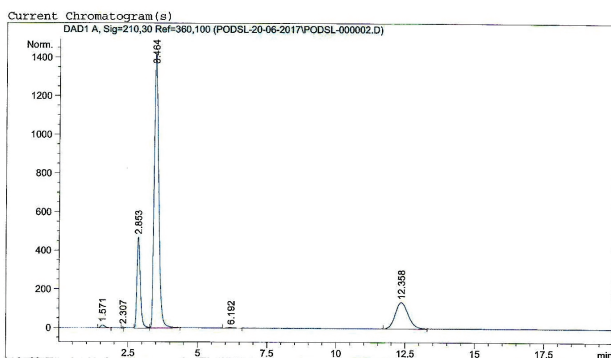


Figure 5. Chromatogram of a standard solution of sweeteners – acesulfame K (RT = 2.853 min), saccharin (RT = 3.464 min) and aspartame (RT = 12.358 min).

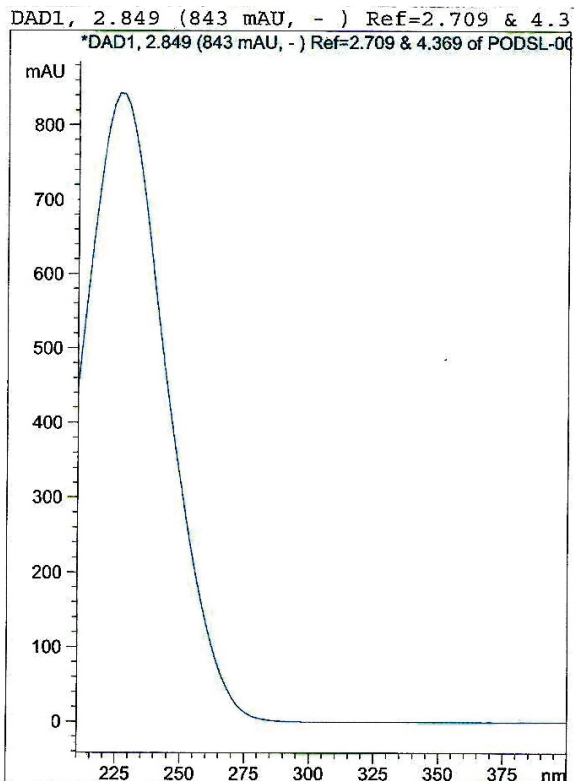


Figure 6. Spectrum of acesulfame K standard solution.

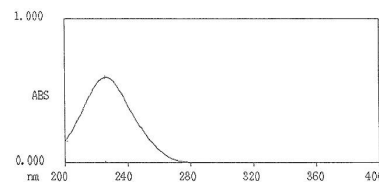


Figure 7. UV spectrum of acesulfame K (λ max = 226 nm) (Coiffard et al. 1999).

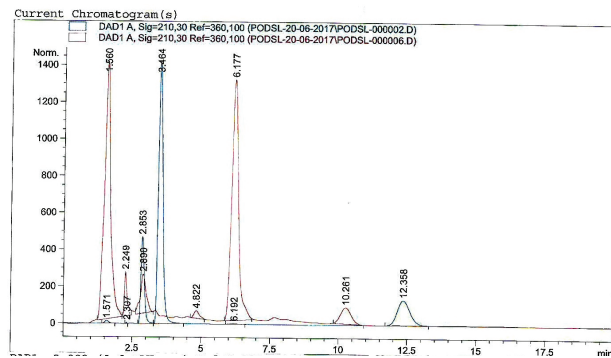


Figure 8. Chromatogram of standard solution of sweeteners and spiked sample.

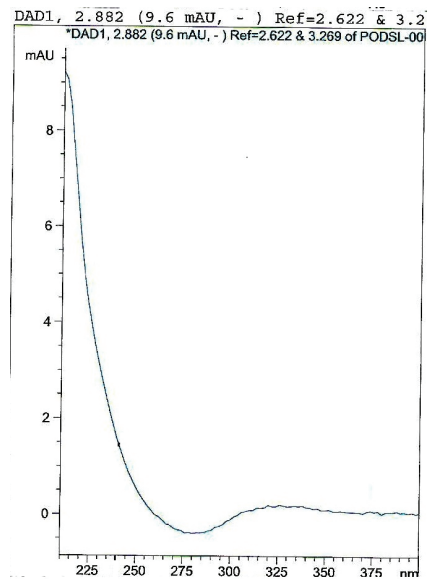


Figure 9. Spectrum of a sample to identify acesulfame K.

Conclusion

Chocolate products are characterized by a complex food matrix. For the determination of sweeteners acesulfame K, saccharin and aspartame in foodstuffs, a standardized method BDS EN 12856: 2001 with reverse phase high performance liquid chromatography with UV detection was used. In all chocolate products analyzed for acesul-

fame K, a compound characteristic of the matrix was observed, which could not be removed by the two-stage purification and did not correspond to acesulfame K in the UV spectrum. The comparison of the spectral characteristics allowing to avoid a misleading result for the presence of Acesulfame K in chocolate and cocoa products, was obtained using the standardized method BDS EN 12856: 2001 with UV detection.

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