Scientific Papers of the University of Pardubice, Series A; Faculty of Chemical Technology
27 (2021) 33–47.

UNIVERSITY OF PARDUBICE FACULTY OF CHEMICAL TECHNOLOGY

HILIC-MS analysis of common artificial sweeteners in food and beverages

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Received: July 23, 2021; Accepted: August 16, 2021

The five most common artificial sweeteners were analysed in various samples of food and beverages and other products using hydrophilic-interaction liquid chromatography coupled with mass spectrometry. The optimised method employed Hypersil Gold HILIC analytical column combined with a binary mobile phase of 50 mmol/L ammonium acetate in water (pH 5; A)/acetonitrile (B) operating at a gradient program of 90–65 % B for 10 min. The individual sweeteners were quantified by the calibration curve method and the data obtained compared with the data declared on the packaging.

Keywords: Artificial sweeteners; HPLC-MS; Hydrophilic interaction liquid chromatography (HILIC); Foodstuff

Introduction

Nowadays, various sugar substitutes are increasingly used, either due to the association of sugar with obesity and diabetes, tooth decay, hepatic steatosis, and other diseases, or for extending the shelf life of food and reduction of its price. Sweeteners are legal and commercially available food additives used in a wide range of products [1,2]. The health safety of sweeteners has always been a disputable task causing controversy, especially because of their potential toxicity. Their E-numbers, use, maximum levels in different kinds of foodstuffs, and acceptable daily intakes are controlled by the national legislation of each country.

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Sweeteners can be sorted into the two main groups, non-nutritional and nutritional. Non-nutritional sweeteners include natural substances obtained from plants and fruits (thaumatin and stevioside), chemically modified natural substances (neohesperidine dihydrochalcone, aspartame, and sucralose), and fully synthetic substances (saccharine and acesulfame-K). The latter show much greater sweetness than that of sugar, providing no or low energy, and not affecting blood glucose level (see Table 1). However, most of them are not metabolized in the body and thus become momentous pollutants in the environment, where they subsequently accumulate due to their high persistence. For this reason, nutritional sweeteners, including especially sugar alcohols (e.g. sorbitol, mannitol, maltitol, erythritol, isomalt, and lactitol), are preferred. These substances have the sweetness comparable to sugar, but with a much lower energy intake and the ability to be partially metabolized [2–8].

Table 1 Caloric value, acceptable daily intake (ADI), and sweetness potency of selected sweeteners (according to [8])

Sweetener	Caloric value [Cal/g]	ADI [mg/kg of BW per day]	Potency [times sweeter than sucrose]
ACE-K	0	15	200
CYC	0	*	30
SAC	0	Permitted for use under an interim regulation	300
ASP	4**	50	180
SUC	0	5	300

*not clearly defined because of different metabolization of each one;
**quantity of aspartame is low in food and drinks and thus its caloric contribution is negligible
NOTES: ACE-K, acesulfame-K; ASP, aspartame; BW, body weight; CYC, cyclamate; SAC, saccharine; and SUC, sucralose

A control of sweeteners concentration in food and beverages plays a key role in ensuring the consumer's safety. Many publications dealing with their determination by thin layer chromatography [9, 10], gas chromatography [4, 11], electromigration [12–16] and electrochemical methods [17–19], flow injection analysis [20, 21], or spectrometric techniques [22–26] have already been published. However, high performance liquid chromatography (HPLC) in various separation modes combined with diverse detection systems has become the most common technique of all [14,27–35].

Due to the different physicochemical properties of the individual sweeteners, their mixtures cannot usually be determined by a single analytical method. Most of them are highly polar, non-volatile compounds without chromophoric, electrophoric or fluorophoric centres in their structure.

For these reasons, a separation of a mixture of sweeteners is very complicated using the traditional reverse-phase HPLC. However, their analysis can be advantageously performed by hydrophilic-interaction liquid chromatography (HILIC; [36,37]). A HILIC method combines polar stationary phases with even more polar eluents and can thus be applied in the separation of highly polar substances, such as sweeteners [36,38].

The aim of this work was to verify the presence of the most common sweeteners in selected products with their subsequent quantification. Therefore, 25 commercially available food and beverages and one oral hygiene supplement with declared addition of various sweeteners were subjected to the sample pre-treatment procedure followed by analysis using hydrophilic-interaction liquid chromatography coupled with mass spectrometry (HILIC-MS).

Experimental

Chemicals and reagents

Standards of sweeteners, namely, acesulfame-K (purity ≥99 %), aspartame (>98 %), sodium cyclamate (≥99 %), saccharine (≥98 %), and sucralose (≥98 %), together with acetonitrile (p.a.), ammonium acetate (≥98 %), ammonium formate (≥99 %), ammonium phosphate (98%), and potassium hydrogen phosphate (≥99 %), were purchased from Merck (Darmstadt, Germany). Other chemicals, namely, acetic acid (99%), formic acid (98%), phosphoric acid (85%), and sodium acetate trihydrate (p.a.), were purchased from Lach-Ner (Neratovice, Czech Republic). High purity water was prepared in Milli-Q purification system (Merck Millipore, Billerica, MA, USA).

Instrumentation

An HPLC-DAD instrumentation was equipped with an SPD-M30A diode-array detector, a RID-10A refractometric detector, two LC-20AD XR binary gradient pumps, a DGU-20A$_5$ degassing unit (all Shimadzu, Kyoto, Japan), an LCO 102 single column thermostat (Ecom, Prague, Czech Republic), and a six-port 7725i injection valve with 5 μL external loop (Rheodyne, Pompton Plains, NJ, USA). For HPLC-MS measurements, a QTRAP 4500 mass spectrometer (AB SCIEX, Framingham, MA, USA) coupled with binary gradient HPLC system consisted of two LC-20AD binary gradient pumps, a DGU-20A$_5$ degassing appliance, a SIL-20A HT autosampler (all Shimadzu), and an LCO 102 single column thermostat (Ecom) were used. Ascentis Express C18 (150 × 3.0 mm, 5 μm) and Ascentis Express C8 (150 × 3.0 mm, 5 μm) columns (both Supelco, Bellefonte, PA, USA) as well as Luna NH_2 100Å (50 × 4.6 mm, 3 μm) column (Phenomenex, Torrance, CA, USA) were used for RP-HPLC experiments.

Finally, Ascentis Si (150 × 4.6 mm, 5.0 μm) and Supelcosil LC-NH_2 (150 × 3 mm, 3.0 μm) columns (both Supelco) as well as Hypersil Gold HILIC (150 × 3 mm; 3.0 μm) column (Thermo Scientific, Waltham, MA, USA) were used for the HILIC experiments.

Standards and samples

Calibration solutions of standards of acesulfame-K (ACE-K), aspartame (ASP), cyclamate (CYC), sucralose (SUC), and saccharine (SAC) were prepared in water at nine concentration levels (0.005–10 mg/L). Each calibration solution was always measured three times (n = 3).

In total, 26 samples purchased in local stores were analysed. There were representatives of flavoured sparkling and still mineral waters (No. 1–5), a multivitamin juice (No. 6), cola type carbonated soft drinks (No. 7–9), a fruit energy drink (No. 10), other non-alcoholic sparkling beverages with lemon and orange flavour (No. 11 and 12), low-sugar beer (No. 13), liquid table-top sweetener (No. 14), effervescent beverage powder (No. 16), cocoa pudding powder light (No. 17), dried sugar-free cranberries (No. 18), fruit compotes (canned apricots, No. 19 and plums, No. 20), tomato ketchup without added sugar (No. 21), a low-calorie yoghurt (No. 22), fruit lollipop without sugar (No. 23), biscuits (No. 24–26), and a mouthwash (No. 15).

Sample pre-treatment

The liquid samples (No. 1–15) were degassed for 20 min. in an ultrasonic bath (if necessary), filtered, and diluted 100 times with deionized water. Liquid part of compote samples (No. 19 and 20) was filtered and diluted 100 times with deionized water. According to the recommended dilution indicated on the package of the effervescent beverage powder (No. 16), the whole dose (6 g) was dissolved in 1 L of deionized water and 1 mL of this solution was processed as the previous samples. A volume of 20 mL and 10 mL of water/methanol mixture (1:1; v/v) were added to 20 g (samples No. 18, 21, and 22) and 2 g (samples No. 23–26) of the sample, respectively. The mixture was degassed for 20 min. in an ultrasonic bath and then centrifuged for 10 min. at 2500×g. The liquid portion was collected and filtered through 0.45 μm PTFE filter. An amount of 2 g of sample No. 17 was mixed with 40 mL of water and processed as the previous samples. All samples were further diluted as needed, centrifuged for 15 min. at 5000×g, and analysed.

Spectrophotometric analysis

First, the absorption spectra of sweetener standards in the wavelength range of 200–400 nm were measured using a UV-2450 spectrophotometer (Shimadzu) in a 1 cm quartz cuvette (Fisher Scientific, Pardubice, Czech Republic).

Chromatographic analysis

At optimised separation conditions, a Hypersil Gold HILIC analytical column tempered at 40 °C was used in combination with the binary mobile phase composed of 50 mmol/L ammonium acetate (pH 5, A) and acetonitrile (B); operated at a flow rate of 1.0 mL/min and with a gradient program of 90–65 % B for 10 min. The injection volume depended on the instrumentation used and was 25 μL (HPLC-DAD) or 10 μL (HPLC-MS). The DAD detection was performed in the wavelength range of 200–400 nm. The HPLC-MS analysis employed electrospray ionization in the negative mode of selected ions monitored with an event time of 0.1 s. Optimised conditions of the MS experiments with direct infusion to acquire MRM (Multiple Reaction Monitoring) transitions were as follows: flow rate of the sample, 5–10 μL/min; ion spray voltage, −4500 V; input potential, −10 V. Flow rates of the gases involved in the analysis were then as follows: curtain gas, 10 psi; ion source gas 1, 20 psi; ion source gas 2, 0 psi; collision gas, medium. The value of the declustering potential depending on the compound ranged from −15 V to −165 V. For HPLC-ESI-MS measurements, the values of ion-spray voltage, input potential, and collision gas were set as those for the direct infusion. The other parameters were as follows: flow rate of eluate, 1.0 mL/min; temperature, 650 °C; curtain gas, 25 psi; ion source gas 1, 60 psi; and ion source gas 2, 60 psi. The mass scan range was set in the range of *m/z* 100–700. Details of MS parameters are given in Table 2.

Table 2 Parameters optimised for MS analysis

Sweetener	Q1 [*m/z*]	Q3 [*m/z*]	DP [V]	CE [V]	CXP [V]
ACE-K	161.7	81.9	−15	−18	−7
CYC	178.8	79.8	−85	−48	−7
SAC	181.8	105.9	−25	−24	−9
ASP	292.8	260.9	−90	−16	−7
SUC	394.7	259.2	−90	−48	−5

NOTES: ACE-K, acesulfame-K; ASP, aspartame; CE, collision energy; CXP = collision cell output potential; CYC, cyclamate; DP, declustering potential; Q1, precursor ion in the first quadrupole (*m/z* = ratio of weight to charge); Q3, product ion in the third quadrupole; SAC, saccharine; and SUC, sucralose

Chromatographic and MS data were collected and evaluated using Lab Solution (Shimadzu), Analyst (AB Sciex), and Clarity (DataApex, Prague, Czech Republic) software.

Data processing

Quantification of target compounds was performed by the calibration curve method. The regression diagnostic of the compounds quantified was performed using QC Expert 2.9 program (TriloByte, Staré Hradiště, Czech Republic). A significance of regression parameters was tested using Student's t-test. All the analyses of samples and standards were made in three replicates (n = 3) and the final results calculated and then presented as the confidence intervals $\bar{x} \pm st_{1-\alpha}$, where $\bar{x}$ is the arithmetic mean, s the standard deviation, and $t_{1-\alpha}$ the critical value of Student's t-distribution for three (2.353) repetitions at a significance level α of 0.05 (with 95% probability). The regression parameters with standard deviations and coefficients of determination are given in Table 3.

Table 3 Regression parameters of the analysed standards of sweeteners

Sweetener	Slope	Intercept	R^2
ACE-K	$(151.3 \pm 1.1) \cdot 10^5$	$(20.3 \pm 6.3) \cdot 10^3$	0.9994
SAC	$(365.7 \pm 0.9) \cdot 10^3$	*	0.9992
ASP	$(366.3 \pm 1.3) \cdot 10^3$	$(1.6 \pm 0.4) \cdot 10^3$	0.9997
SUC	$(175.4 \pm 0.8) \cdot 10^2$	83 ± 28	0.9993
CYC	$(218.1 \pm 0.5) \cdot 10^3$	*	0.9976

*The intercept is not given when was statistically insignificant
NOTES: ACE-K, acesulfame-K; ASP, aspartame; CYC, cyclamate; R^2, coefficient of determination; SAC, saccharine; and SUC, sucralose

Results and discussion

Selection of detection system

The absorption maxima of aspartame, acesulfame-K, and saccharine were found to be at 210 nm, 225 nm, and 230 nm, respectively. The absorption of cyclamate and sucralose is negligible (above the wavelength of 200 nm) and hence, the spectrophotometric detection is not very suitable for their quantitative analysis. Due to this, the suitability of refractometric detection was tested; however, this instrumentation detected the sweeteners at very high concentrations only, atypical for real samples. For this reason, all subsequent analyses were performed using the liquid chromatograph coupled to the mass spectrometer. The MRM transitions

of the individual sweeteners were examined by the direct infusion of the corresponding standard solutions into the mass spectrometer. Then, the most intensive MRM transitions were selected for each substance and the mass spectral analysis parameters were further tuned for optimal detection. Optimised MS parameters of each substance are shown in Table 2.

Optimisation of separation conditions

First, HPLC separations in the reverse-phase mode were performed using alkylsilica gel type columns (C18 and C8), each combined with various types and concentrations of buffers with different pH (5–50 mmol/L ammonium formate, potassium phosphate or sodium and ammonium acetate with pH 3.0–5.0) mixed with organic modifier (acetonitrile or methanol). Regardless of the separation conditions, most of the sweeteners analysed had eluted close to the dead volume of the column. In addition, the peaks of the analytes were often cleaved, tailed, and non-uniform, the baseline showed significant instability, and the noise frequently fluctuated. For these reasons, the unmodified silica gel column, as well as two silica gel columns with chemically bound aminopropyl, each combined with various compositions of mobile phase, were tested for both HILIC and RP-HPLC separations. However, the sweeteners did not retain and giving rise to asymmetrical and cleaved peaks again. Due to the low retention of sweeteners in such separation systems, the retention characteristics of the standards on the dedicated HILIC silica gel column with chemically bonded polyethyleneimine, again combined with acetate, phosphate, and formate buffers, were further investigated. The less polar component of the mobile phase for this HILIC separation was either 100% acetonitrile or its mixture with buffer in the ratio of 95:5 (v:v). The isocratic elution experiments with a mobile-phase ratio ranging from 5:95 to 30:70 (A:B; v:v), as well as gradient elution with different slopes, durations, and initial concentrations of component B were performed. Unfortunately, the problems with non-uniform and slightly co-eluted peaks occurred also in this case. This might be associated with a relatively complex equilibrium system in HILIC chromatography, especially for acid-base analytes. However, the mass spectrometer monitores only the unique MRM transitions corresponding to specific sweeteners, avoiding thus potential interferences in the chromatogram. Compared to the previous columns, the retention of the sweeteners was significantly higher in this column. The retention of highly ionized substances (saccharine and acesulfame-K) practically did not depend on the content of acetonitrile in the mobile phase up to 85 %. The increase of retention occurred only above this limit. Finally, the best separation of sweeteners was achieved using a binary linear gradient of 50 mmol/L ammonium acetate at pH 5 (A) and acetonitrile (B) with the gradient program 90–65% B in 10 min (Fig. 1).

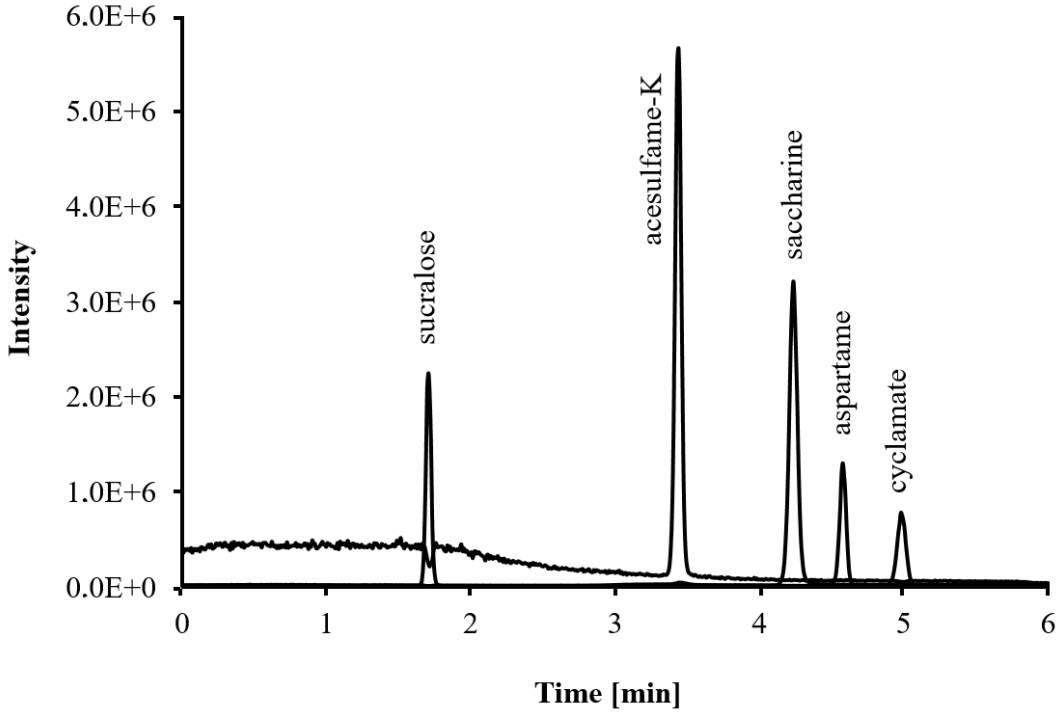

Fig. 1 HILIC separation of sweeteners standards with MRM-MS detection
Separation conditions: column: Hypersil Gold HILIC (150 × 3 mm, 3 μm), MF A: 50 mmol/L ammonium acetate at pH 5, MF B: acetonitrile, gradient: 90 % – 65 % B/10 min, flow rate: 1 mL/min, temp.: 40 °C, inj.: 10 μL, MS parameters in MRM mode (multiple reaction monitoring) according to the Table 2

Calibration measurements and analysis of samples

Calibration solutions of the standards in the concentration range of 0.005–10 mg/L, as well as 26 real samples were analysed using the optimised HILIC-MS method specified in the previous section. An example of a SIM chromatogram (selected ion monitoring) of diluted sample No. 1 is shown in Fig. 2. The quantification of analytes was performed using the calibration curve plotting the peak area *vs*. the corresponding concentration. The intercepts of the saccharine and cyclamate calibration equations were found to be statistically insignificant, and therefore, not included in the calculation. For the standards, the coefficients of linearity (R^2) were higher than 0.998, indicating a good linearity.

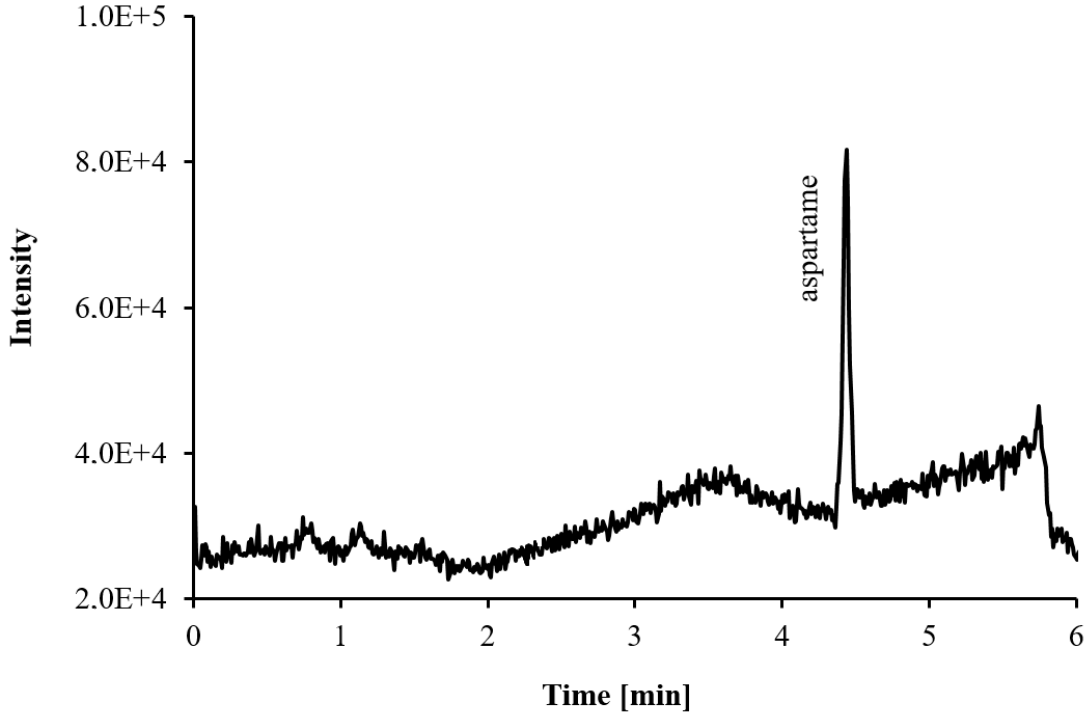

Fig. 2 MRM-MS record of aspartame in 1000× diluted sample No. 1

Separation conditions: column: Hypersil Gold HILIC (150 × 3 mm, 3 μm), MF A: 50 mmol/L ammonium acetate at pH 5, MF B: acetonitrile, gradient: 90 % – 65 % B/10 min, flow rate: 1 mL/min, temp.: 40 °C, inj.: 10 μL, MS parameters in MRM mode (multiple reaction monitoring) according to the Table 2

The concentrations of the individual sweeteners occurred in liquid (No. 1–15) and solid (No. 16–26) samples given in units of mg/L and mg/100g, respectively, are summarized in Table 4. The sum of sweeteners present in the individual samples is displayed in Table 4 as well. In general, their content was lower in the samples of flavoured waters and, surprisingly, also in biscuits. A special case was sample No. 13 (diabetic beer) that did not contain any sweetener, which is also consistent with the information declared by the manufacturer. On the other hand, high concentrations occurred in typically sweet beverages (cola type drinks and energy drinks), table-top sweetener, and, surprisingly, also in the sample of mouthwash and light pudding. In total, acesulfame-K was determined in nineteen samples (No. 1–12, 14, 16, and 22) in the concentration range of 2.4–412 mg/L (mg/100 g). Fifteen samples contained aspartame (No. 1, 3–12, 17, 18, 21, and 22) in concentrations ranging from 1.2 mg/L to 1120 mg/100 g. Sucralose was detected in samples No. 2, 10, 16, and 25 with a concentration of 160 mg/L, 9.5 mg/L, 266 mg/L, and 18 mg/100 g, respectively. Saccharine was found in eight samples (No. 5, 12, 14, 15, 19, 20, 24, and 26) at the concentration range of 5.9–17100 mg/L. Cyclamate should be present in six samples, but analysis had revealed its presence in five samples only; namely, samples No. 5, 8, 9, 11 and 14, with the concentration of 51 mg/L, 281 mg/L, 284 mg/L, 298 mg/L and 94000 mg/L, respectively.

Table 4 Concentrations of the individual sweeteners found in the samples and their total amount determined

Liquid Sample No.	Sweetener found	*c* [mg/L]	Total amount [mg/L]
1	ACE-K	140 ± 1.0	222
	ASP	82 ± 4.0	
2	SUC	160 ± 2.0	160
3	ACE-K	141 ± 2.0	206
	ASP	65 ± 1.0	
4	ACE-K	50 ± 1.3	98
	ASP	47.5 ± 0.4	
5	ACE-K	2.4 ± 0.2	61
	ASP	1.24 ± 0.06	
	SAC	5.9 ± 0.2	
	CYC	51 ± 1.0	
6	ACE-K	233 ± 1.0	311
	ASP	78 ± 7.0	
7	ACE-K	185 ± 1.0	405
	ASP	220 ± 2.0	
8	ACE-K	138 ± 1.4	526
	ASP	107 ± 3.0	
	CYC	281 ± 4.2	
9	ACE-K	141 ± 2.0	550
	ASP	124.2 ± 0.9	
	CYC	284.4 ± 0.3	
10	ACE-K	220 ± 7.5	400
	ASP	170.7 ± 0.5	
	SUC	9.51 ± 0.04	
11	ACE-K	56 ± 1.0	432
	CYC	298 ± 7.8	
	SAC	**78 ± 3.3**	
12	ACE-K	23.7 ± 0.6	103
	ASP	19.5 ± 0.4	
	SAC	60 ± 1.1	
13	–	–	–
14	SAC	17100 ± 330	91120
	CYC	74000 ± 4600	
	ACE-K	**20 ± 1.8**	
15	SAC	1040 ± 0.0	1040

NOTES: ACE-K, acesulfame-K; ASP, aspartame; CYC, cyclamate; SAC, saccharine; and SUC, sucralose
The samples that have not complied with the packaging are marked in bold

Table 4 Concentrations of individual sweeteners found and their total amount determined in samples (continued)

Solid Sample No.	Sweetener found	c [mg/100 g]	Total amount [mg/100g]
16	ACE-K	412 ± 5.0	678
	SUC	266 ± 4.0	
17	ASP	1120 ± 15	1120
18	ACE-K	14.1 ± 0.9	59
	ASP	45 ± 1.1	
19	SAC	101 ± 3.0	101
20	SAC	47 ± 1.0	47
21	ACE-K	14.2 ± 0.2	31
	ASP	16.8 ± 0.2	
22	ACE-K	4.5 ± 0.2	12
	ASP	7.4 ± 0.2	
23	ACE-K	49 ± 1.07	49
24	SAC	5.9 ± 0.2	6
25	ACE-K	12.91 ± 0.03	31
	SUC	18 ± 1.9	
26	SAC	2.12 ± 0.06	11
	ACE-K	**9.0 ± 0.5**	

NOTES: ACE-K, acesulfame-K; ASP, aspartame; CYC, cyclamate; SAC, saccharine; and SUC, sucralose
The samples that have not complied with the packaging are marked in bold

Sample No. 14 contained the highest concentration of sweeteners (over 90 g/L in total), representing a table-top sweetener which is generally not intended for direct consumption. The presence of cyclamate was not proven in sample No. 26. However, this sample contained acesulfame-K, although its use was not declared by the manufacturer. Other discrepancies were found in samples No. 11 and 14. Sample No. 11 containing saccharine in addition to the declared cyclamate and acesulfame-K, and sample No. 14 with acesulfame-K in addition to the declared saccharin and cyclamate. All other samples contained sweeteners corresponding to the information given on the product label. Unfortunately, the legislation does not require the specification of their concentrations.

Conclusions

This work has dealt with chromatographic analysis of the five most common artificial sweeteners added to food and other products and with comparison of their presence with the data declared by the manufacturer, thus helping to reveal possible misleading of the consumer.

Due to a very polar character of some sweeteners, the hydrophilic interaction liquid chromatography was found to be the technique of choice for their effective determination. The separation and detection parameters had to be carefully optimised. Because of unsuitability of both refractometric and UV/VIS detections, mass spectrometry was employed for this purpose. Six different analytical columns were tested in combination with mobile phases based on various types of buffers with different concentrations and pH values. The best separation has been achieved in the HILIC separation mode using the silica gel column with chemically bonded polyethyleneimine stationary phase, a binary mobile phase formed by 50 mmol/L ammonium acetate buffer of pH 5 (A) and acetonitrile (B) with a linear gradient elution of 90–65% B for 10 min.

In total, 26 commercially available products were analysed by the optimised method. Cyclamate should be present in six samples; however, its presence was confirmed in just five ones. Acesulfame-K should be present in seventeen samples, but its low content was additionally found in other two samples. Aspartame, saccharine, and sucralose were ascertained in fifteen, eight, and four samples, respectively, which was compliant with the information on the packaging.

Acknowledgement

A support received from the Faculty of Chemical Technology, the University of Pardubice (project No. SGS-2021-001) is gratefully acknowledged.

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