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Ring trial for the simultaneous analysis of sweeteners and preservatives in soft drinks

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Abstract

In the present study a ring trial on the simultaneous determination of three artificial sweeteners (acesulfame-K, aspartame and saccharin) and two preservatives (sorbic and benzoic acid) in a soft drink is presented. High performance liquid chromatography was used for the analysis, according to the EN 12856:1999 method. Eleven laboratories participated in the ring trial. The method proved to be efficient for the simultaneous determination of the selected sweeteners and preservatives in one run analysis. The repeatability relative standard deviation was 2.26% for aspartame, 5.05% for acesulfame-K, 2.38% for sodium saccharin, 3.72% for benzoic acid, 7.93% for sorbic acid and the reproducibility relative standard deviation of the target analytes was 7.5, 6.3, 4.5, 11.0 and 7.2% respectively. The precision data for the three sweeteners were comparable to the respective values reported by the EN 12856:1999 according to inter-laboratory tests in orange juice beverage, orange flavoured beverage and orange juice.

Keywords: HPLC analysis, preservatives, ring trial, sweeteners

1. Introduction

According to statistics during the last decades, obesity has increased globally (Flegal *et al.*, 2010; World Health Organization, 2007). Even though there appears to be a levelling off of the prevalence of obesity, the rates of obesity remain at unacceptable high levels (Rokholm *et al.*, 2010). Hence low-calorie foodstuffs have been gaining a growing preference of the consumers. High-intense sweeteners, also known as non-nutritive sweeteners, can serve in weight management strategies and the treatment of obesity.

It is common that high intense sweeteners are used in combinations, in so called blends, so as to mask the unwanted side taste or aftertaste which some sweeteners impart to the foodstuff when they are used separately (Zhao and Tepper, 2007). In various foodstuffs, e.g. soft drinks, intense sweeteners are not the only additives used, since preservatives, such as sorbic and benzoic acid, are also added usually in small concentrations.

The content of the sweeteners as well as of preservatives in foodstuffs in the EU is established by Regulation No. 1333/2008 (European Parliament and the Council of the European Union, 2008), which provides the list of approved food additives and lays down the conditions of use of food additives in foods. In order to ensure that the additives are added only to the foodstuffs to which they are permitted, and that their amount is in accordance with the legislative requirements, their determination by efficient analytical techniques is imperative. More specifically, analytical methods for the simultaneous determination of sweeteners and preservatives are a valuable tool for the monitoring of the content of these additives.

Rapid methods for the determination of sweeteners and/or preservatives are highly required by the beverage companies, as it was identified in a research conducted within the MoniQA project. Furthermore, high-throughput methods can be a valuable tool for the food authorities as well.

Chromatographic methods are the most widely used for the multi-sweetener determination, with High Performance Liquid Chromatography (HPLC) being the most popular technique in this field. Capillary electrophoresis, flow injection analysis, gas chromatography, thin layer chromatography, ion chromatography, electroanalytical and spectroscopic techniques are also used for the determination of artificial sweeteners (Zygler *et al.*, 2009). Seperation of α-aspartame, sodium saccharin, acesulfame-K, vanilin and two preservatives (sorbic acid and benzoic acid) in cola drink was achieved with reversed phase HPLC-UV in 40 min, using 15% acetonitrile and ammonium acetate buffer (0.005 M) mobile phase at pH 4.0 and a YMC-ODS pack column (Demiralay *et al.*2006). Furthermore another method was proposed for the determination of sweeteners (aspartame, saccharin, acesulfame), preservatives (sorbic acid and benzoic acid) and dyes (ponceau 4R, sunset yellow and tartarazine) in soft drinks. This method involved a 10 m RP-18 column and a binary eluent consisting of an aqueous 0.1 M phosphate buffer at pH 4.0 added with methanol with a suitable gradient elution program. Separation time was less than 20 min (Dossi *et al.*, 2006). Lino and Pena (2010) validated a method for determination of benzoic and sorbic acid, caffeine and saccharin in soft drink and nectars using HPLC-UV, with a C18 column and a buffered mobile phase, KH_2PO_4 0.02 M/ACN (90:10)/phosphoric acid at pH 4.2 for chromatographic separation. Additionally a flow injection on-line dialysis high performance liquid chromatography (FID-HPLC) method for determination of acesulfame-K, saccharin, caffeine, benzoic and sorbic acid was developed, providing adequate precision (repeatability relative standard deviation (RSD_r) <5%) for all the additives (Kritsunankul and Jakmunee, 2011). Most of the published multi-additive (sweeteners and preservatives) determination methods include only single laboratory validation studies.

EN 12856:1999 specifies an HPLC method for the determination of acesulfame-K, aspartame and saccharin. It also allows the determination of caffeine, sorbic and benzoic acid in foodstuffs. The separation efficiency depends on the mobile phase and several alternative compositions are suggested in the method. The method has been validated through inter-laboratory tests for some sweeteners added alone in foods, in particular the determination of: (1) acesulfame-K in marzipan, yogurt, fruit yogurt, orange juice beverage, cola, cream, and jam; (2) aspartame in marzipan, fruit yogurt, orange juice beverage, orange flavoured beverage, cola, jam, and preparation for flan; and (3) sodium saccharin in marzipan, yogurt, fruit yogurt, orange juice, orange juice beverage, cola, cream, and jam. However no inter-laboratory tests have been done for the simultaneous analysis of the above-mentioned sweeteners and moreover, for preservatives.

The aim of the present study was to conduct a ring trial on multi-component analysis of several sweeteners and preservatives that are commonly used in foodstuffs. More specifically, the objective was to use the EN 12856:1999 method as a one run method for the determination of acesulfame-K, aspartame and saccharin as well as sorbic and benzoic acid in a soft drink. The selected sweeteners and preservatives are among the most commonly used in foodstuffs, and especially soft drinks. Eleven laboratories participated in the ring trial and lemonade was used as soft drink.

2. Materials and methods

Chemicals

All solvents were HPLC grade and were used as supplied by the manufacturer. Standards of aspartame, acesulfame-K, sodium saccharin, benzoic and sorbic acid were used for the preparation of stock solutions and test samples.

Chromatographic conditions

The analysis was performed by an HPLC equipped with a UV-Vis detector. According to the EN12856:1999 method an ODS C_{18} reversed phase chromatography column (250 × 4.6 mm, 5 µm) was used and absorbance was recorded at a wavelength of 220 nm. The mobile phase was a solution of phosphate buffer and acetonitrile at a ratio of 85:15. The buffer solution was prepared by dissolving 1.70 g of potassium dihydrogen orthophosphate in 800 ml of water. The pH was then adjusted to 3.5 by addition of phosphoric acid. Phosphoric acid had been prepared by pipetting 6 ml of phosphoric acid into a 100 ml volumetric flask already containing 80 ml water and diluting to the mark with water. The flow rate was 1.0 ml/min and the injection volume 20 µl.

Preparation of test samples

The test samples were prepared by one of the coordinating laboratories by the addition of the three sweeteners, namely aspartame (320 mg/l), acesulfame-K (150 mg/l) and sodium saccharin (170 mg/l), and two preservatives, namely benzoic acid (120 mg/l) and sorbic acid (160 mg/l) to soft drink (lemonade). The test samples were stored at approximately 4 °C throughout the ring trial. The timeframe of the completion of the ring trial was 60 days, so that the shelf life of the test samples would not be compromised. During the ring trial period plus 30 days (totally 12 weeks), the test samples were analysed periodically by the laboratory who prepared the samples to check the stability of each compound in the samples. During the first 6 weeks the test samples were analysed every week, every day in the 7th and 8th week and every week in the last four weeks.

Prior to the distribution of the test materials to the participants of the ring trial, the test materials were checked

for homogeneity. The homogeneity check was performed by the coordinating laboratory, which carried out the preparation of the test samples. Ten randomly selected test materials were analysed in duplicate for aspartame, acesulfame-K, sodium saccharin, benzoic and sorbic acid. The results are given with their statistical evaluation in Table 1. These data show sufficient homogeneity.

Since the homogeneity of the test material was assured, the test samples were distributed to the participants of the ring trial as blind duplicates, along with the instructions on the analytical procedure. Thus the participants proceeded with the preparation of the calibration curves and the analysis of the test samples.

Calibration curves

A mixed stock standard solution was prepared containing 1 g/l of each of the sweeteners and preservatives. Three standard solutions I, II and III containing respectively 100 mg/l, 50 mg/l and 10 mg/l of each sweetener and preservative were prepared by appropriate dilutions of the initial stock standard solution. The above concentration ranges were selected according to preliminary tests. The three standard solutions I, I and III were used for the preparation of calibration curves. In particular the analysis of each standard solutions was performed in three replicates, in order to prepare the calibration curve for each analyte. Linearity was obtained for all target compounds detected in the sample.

Analysis of test samples

The test samples were analysed according to the EN 12856:1999 method with slight modification. In particular the test sample (5 ml) was diluted to a final volume of 25 ml with the mobile phase solution instead of water. The solution was then filtered through a membrane filter with a pore size of 0.45 μm prior to injection, analysed at least twice and the mean of these two replicates was reported. The duplicate analysis was performed for eight individual test sample solutions. The quantification was performed according to the calibration curve and the contents of the sweeteners and the preservatives in the test material were calculated in mg/l.

3. Results and discussion

The analytical method proved efficient for the separation of the tested sweeteners and preservatives. A representative chromatogram of a test sample is presented in Figure 1.

Table 1. Homogeneity data of the detection of three sweeteners and two preservatives (mg/l) in lemonade test samples (two replicates).

	Aspartame (mg/l)		Acesulfame K (mg/l)		Sodium saccharin (mg/l)		Benzoic acid (mg/l)		Sorbic acid (mg/l)	
sample number	1	2	1	2	1	2	1	2	1	2
1	321.5	321.3	144.6	144.7	172.3	171.1	117.1	117.0	160.0	159.9
2	319.9	319.4	144.1	144.0	171.5	171.5	117.5	117.4	162.1	160.6
3	332.6	332.0	145.3	145.1	172.9	172.6	119.1	118.7	168.7	168.3
4	325.9	326.2	142.5	142.6	169.6	169.6	117.0	116.7	166.7	165.7
5	328.5	328.6	143.7	143.6	170.9	170.9	118.0	118.3	167.8	168.3
6	327.0	327.1	143.1	143.1	170.2	170.3	117.7	117.8	167.3	167.3
7	326.1	325.0	142.0	141.9	168.1	168.9	118.5	118.1	168.6	168.1
8	327.1	327.5	143.0	143.1	170.1	170.3	117.2	117.4	166.3	166.9
9	330.3	330.2	144.4	144.3	171.8	171.8	118.3	118.3	168.1	168.4
10	316.8	316.6	142.6	142.4	169.8	169.5	117.0	116.6	163.4	162.6
Mean	325.5		143.5		170.7		117.7		165.8	
Target standard deviation[a]	21.80		10.87		12.60		9.19		12.29	
Cochran's test (95%)[b]	0.56		0.37		0.59		0.26		0.44	
s^2_{sam}[c]	23.52		1.10		1.58		0.47		9.82	
Critical value (c)	80.51		20.00		26.96		14.31		25.81	
s^2_{sam} < c?	accepted		accepted		accepted		accepted		accepted	

[a] The target standard deviation was calculated using the Horwitz equation: for analyte concentrations ≥120 μg/kg and ≤13.8%, the target standard deviation = $(0.02xc^{0.8495})/mr$, where c is the concentration, expressed as a dimensionless mass ratio and mr is the dimensionless mass ratio (FAPAS, 2002).

[b] No need to remove any data according to Cohran's test.

[c] s^2_{sam} is the between-sample variance and is calculated form the equation: s^2_{sam} = (mean square between samples - analytical variance)/2 (FAPAS, 2002).

The retention time varied among laboratories ranging between 17 min and 27 min. When the retention time was short, an overlapping of acesulfame-K and sodium saccharin was observed (Figure 2). To obtain a better separation, the mobile phase solution was used as solvent for the preparation of the test sample solution. The better separation of acesulfame-K and sodium saccharin which was achieved is presented in Figure 3. Hence the calculation of peak areas and consequently the quantification was more accurate. It was also observed that the retention times were

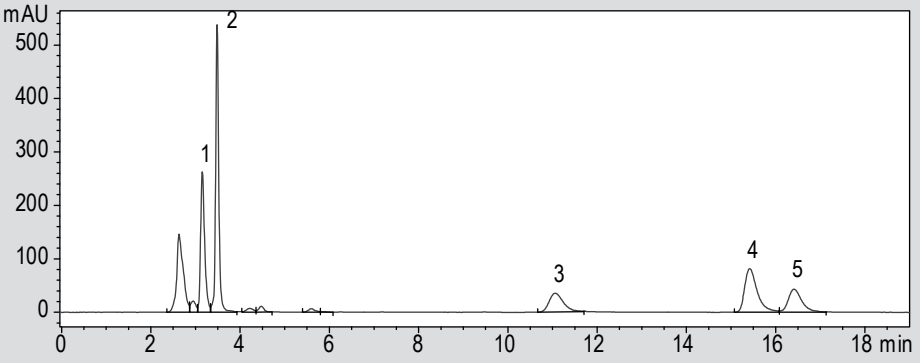

Figure 1. HPLC separation of a lemonade test sample using water as dilution solution. Numbers correspond to the following sweeteners and preservatives: 1 = acesulfame-K (retention time (Rt) 3.157 min); 2 = sodium saccharin (Rt 3.484 min); 3 = aspartame (Rt 11.062 min); 4 = benzoic acid (Rt 15.421 min); 5 = sorbic acid (Rt 16.412 min).

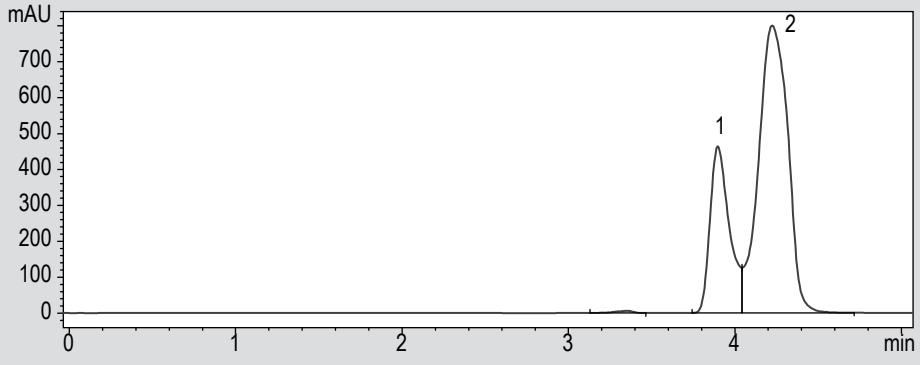

Figure 2. HPLC separation of a standard solution containing acesulfame-K and sodium saccharin using water as dilution solution. Numbers correspond to the following sweeteners: 1 = acesulfame-K (retention time (Rt) 3.897 min); 2 = sodium saccharin (Rt 4.224 min).

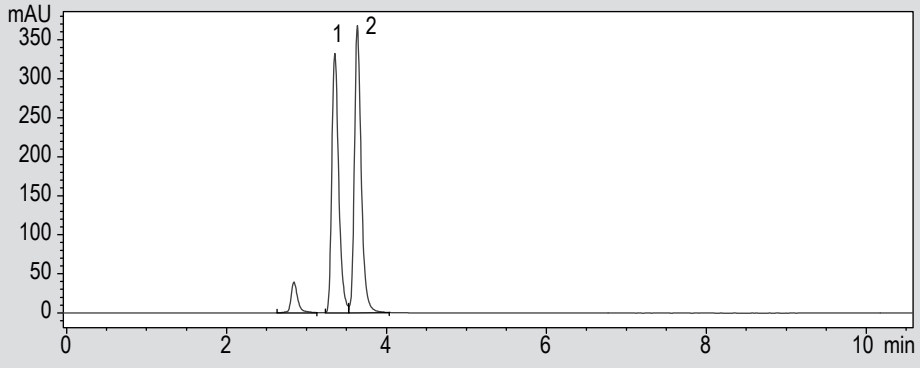

Figure 3. HPLC separation of a lemonade test sample using the mobile phase solution (phosphate buffer and acetonitrile at a ratio of 85:15) as dilution solution. Numbers correspond to the following sweeteners: 1 = acesulfame-K (retention time (Rt) 3.354 min); 2 = sodium saccharin (Rt 3.636 min).

lower when the mobile phase solution was used instead of water. In this case the mobile phase solution should be used as dilution solution for both the standards and the samples, in order to ensure that the final results are not manipulated.

The mean concentrations from the duplicate analysis for each one of the eight individual sample test solutions were reported by each participating laboratory. Based on these eight results reported by each participant, the mean concentration and the repeatability of each participating laboratory were calculated for each target compound (Table 2).

The repeatability levels of the analysis for each laboratory individually, as expressed by the repeatability relative standard deviation, ranged from 0.39 to 2.26% for aspartame, 0.41 to 5.05% for acesulfame K, 0.15 to 2.38% for sodium saccharin, 0.22 to 4.76% for benzoic acid and 0.43 to 7.93% for sorbic acid.

The results of the ring trial were based on the reporting received from nine of the eleven participating laboratories. Two participants were considered as outliers for not reporting their results according to the instructions and due to low repeatability, respectively. In addition, one of the participants

Table 2. Measurements of three sweeteners and two preservatives (mg/l) in lemonade test samples used for the repeatability analysis of each participating laboratory.

L no.		Aspartame	Acesulfame-K	Sodium saccharin	Benzoic acid	Sorbic acid
1	mean (mg/l)	306.2	166.3	176.0	117.6	153.2
	s_r (mg/l)	2.07	4.65	0.59	0.99	0.66
	RSD_r (%)	0.68	2.80	0.33	0.84	0.43
2	mean (mg/l)	326.1	143.6	170.7	117.8	166.0
	s_r (mg/l)	3.96	1.02	1.55	0.71	3.17
	RSD_r (%)	1.22	0.71	0.91	0.61	1.91
3	mean (mg/l)	307.3	162.1	172.9	120.9	-
	s_r (mg/l)	1.21	0.66	0.26	0.27	-
	RSD_r (%)	0.39	0.41	0.15	0.22	-
4	mean (mg/l)	294.3	147.6	157.8	131.2	160.5
	s_r (mg/l)	4.92	0.93	1.02	1.30	0.85
	RSD_r (%)	1.67	0.63	0.65	0.99	0.53
5	mean (mg/l)	290.9	150.3	162.1	110.2	147.4
	s_r (mg/l)	3.86	1.70	1.94	0.83	1.07
	RSD_r (%)	1.33	1.13	1.20	0.75	0.72
6	mean (mg/l)	342.3	136.3	156.8	157.3	147.5
	s_r (mg/l)	7.74	3.61	3.31	5.85	11.69
	RSD_r (%)	2.26	2.65	2.11	3.72	7.93
7	mean (mg/l)	335.8	144.9	171.6	141.4	138.0
	s_r (mg/l)	7.24	5.82	3.02	5.18	10.89
	RSD_r (%)	2.16	4.02	1.76	3.66	7.89
8	mean (mg/l)	262.0	146.0	157.0	113.3	140.5
	s_r (mg/l)	1.85	7.37	3.74	5.39	7.48
	RSD_r (%)	0.71	5.05	2.38	4.76	5.33
9	mean (mg/l)	314.9	151.1	163.3	120.9	166.0
	s_r (mg/l)	3.72	2.75	2.82	4.42	3.17
	RSD_r (%)	1.18	1.82	1.72	3.66	1.91
10	mean (mg/l)	2,137.4	176.6	247.1	150.5	150.8
	s_r (mg/l)	2,035.93	16.17	64.81	10.56	52.39
	RSD_r (%)	95.25	9.15	26.23	7.01	34.76
11[a]	result (mg/l)	345	127	227	263	149
	s_r (mg/l)	-	-	-	-	-
	RSD_r (%)	-	-	-	-	-

Abbreviations used: L no. = participating laboratory number; s_r = repeatability standard deviation; RSD_r = repeatability relative standard deviation (Horwitz, 1995).

[a] Only one measurement done.

did not perform the determination of sorbic acid, and was therefore considered as outlier only for this analyte.

The precision of the data for each target analyte are summarized in Table 3. The repeatability of the inter-laboratory results, as expressed by the repeatability relative standard deviation, was 2.26% for aspartame, 5.05% for acesulfame-K, 2.38% for sodium saccharin, 3.72% for benzoic acid and 7.93% for sorbic acid. The values of aspartame, acesulfame-K and sodium saccharin are lower than the respective repeatability values reported by the EN 12856:1999, according to inter-laboratory tests in orange flavoured beverage (3% for aspartame) and in orange juice (8% for acesulfame-K and 14% for sodium saccharin), but exceed the range in orange juice beverage (1.6, 1.2-3 and 2-2.4% respectively). The reproducibility of the inter-laboratory results, expressed as reproducibility relative standard deviation, was 7.5% for aspartame, 6.3% for acesulfame-K, 4.5% for sodium saccharin, 11.0% for benzoic acid and 7.2% for sorbic acid. The values of aspartame and sodium saccharin were lower than the respective values reported by the EN 12856:1999 according to inter-laboratory tests in orange juice beverage (12.1% for aspartame and 8-16.2% for sodium saccharin), in orange flavoured beverage (10.4% for aspartame) and orange juice (43% for sodium saccharin). The reproducibility of acesulfame-K was much lower than the one obtained in orange juice (50%) but slightly exceeded the range obtained in orange juice beverage (3-6%).

4. Conclusion

The data obtained from each laboratory demonstrate that the method has an excellent precision for each sweetener and preservative. The method presented here provides reliable and reproducible results in a rather short time of analysis. The findings are based on the results obtained from nine laboratories, which is an adequate number of participants in terms of the reliability of the ring trial outcome. Therefore, it can be concluded that the presented method allows an easy, fast and simultaneous determination of sweeteners and preservatives in soft drinks. Further inter-laboratory trials are needed for validation of the method.

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Table 3. Inter-laboratory reproducibility analysis based on measurements of three sweeteners and two preservatives (mg/l) in lemonade test samples.

Inter-laboratory analysis data	Aspartame	Acesulfame K	Sodium saccharin	Benzoic acid	Sorbic acid
L	11	11	11	11	11
E	9[a]	9[a]	9[a]	9[a]	8[a,b]
mean (mg/l)	305.7	150.6	165.1	123.4	153.8
s_r (mg/l)	7.74	7.37	3.74	5.85	11.69
RSD_r (%)	2.26	5.05	2.38	3.72	7.93
r (mg/l)	21.67	20.64	10.5	16.38	32.73
s_R (mg/l)	22.98	9.47	7.46	13.59	11.12
RSD_R (%)	7.5	6.3	4.5	11.0	7.2
R (mg/l)	64.34	26.52	20.89	38.05	31.14

Abbreviations used: L = number of participating laboratories; E = number of laboratories retained after eliminating outliers; s_r = repeatability standard deviation; RSD_r = repeatability relative standard deviation; *r* = repeatability limit (s_rx2.8); s_R = reproducibility standard deviation; RSD_R = reproducibility relative standard deviation; *R* = reproducibility limit (s_Rx2.8) (Horwitz, 1995).

[a] Two participants were excluded from the ring trial. One of them did not report the results according to instructions (no. 11 in Table 2). The other participant was considered as outlier according to the Kolmogorov-Smirnov test that was performed to test for normal distribution of the data. Next, Grubb's test was performed to find the outlier (no. 10 in Table 2).

[b] One of the participants (no. 3 in Table2) was the additional outlier, since it did not conduct the analysis of sorbic acid.

Technical University (ITU, Turkey) and Izmir Province Control Laboratory (IPCL, Turkey).

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