European Journal of Chemistry

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Application of multiple reaction monitoring for quantitation of sweeteners in food products

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RESEARCH ARTICLE

10.5155/eurjchem.10.3.263-266.1825

Received: 08 December 2018
Received in revised form: 15 May 2019
Accepted: 17 May 2019
Published online: 30 September 2019
Printed: 30 September 2019

KEYWORDS

UPLC
Aspartam
Stevioside
Sweeteners
Multiple reaction monitoring
Tandem mass spectrometric technique

ABSTRACT

A coupled UPLC-MS/MS method has been developed and validated for the simultaneous quantitation of food sweeteners stevioside (STV) and aspartame (ASP). Good chromatographic separation was achieved on a Hypersil gold 50×2.1 mm (1.9 µm) column, using a gradient flow of 10 mM ammonium acetate, pH = 2.9 adjusted with formic acid, and 10 mM ammonium acetate in acetonitrile:water (95:5, *v*:*v*) as the mobile phase. The eluate was introduced to ESI-Mass spectrometer and scanned using multiple reaction monitoring (MRM). The method was robust, reproducible and easy to use and was validated according to ICH guidelines for the accuracy and precision giving acceptable ranges. The utilization of multiple reaction monitoring improved the selectivity of detection. Method was linear in the ranges of 2-250 ng/mL for STV and ASP. Application of this method on laboratory mixtures of the selected sweeteners was successful. The using of mass spectrometry make the method selective and measurement did not affect the presence of impurities, additive and other ingredients of detection due to the simplicity and sensitivity of this method allows the utilization of method in quality control of STV and ASP.

Cite this: *Eur. J. Chem.* **2019**, *10(3)*, 263-266 **Journal website**: www.eurjchem.com

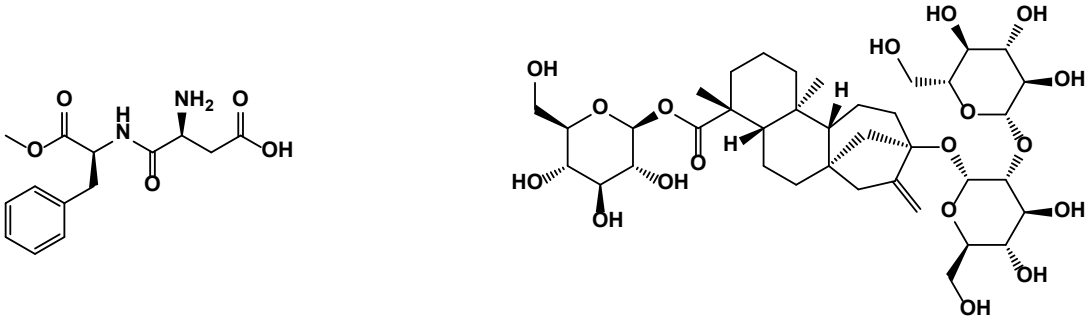
1. Introduction

Food additives are currently used in many infant, dietary and pharmaceutical formulations. They are used to improve the taste, color or appearance of a processed food. Food additives are defined as substances without or with little nutritive importance which are used in food industries during processing [1].

Sweeteners are among the most commonly used food additives. Stevioside (Figure 1) is regarded as the main component of stevia herbs with the following chemical name 13-[(2-o-beta-D-glucopyranosyl-alpha-D-glucopyranosyl) oxy] kaur-16-en-18-oic acid beta-D-glucopyranosyl ester. The other targeted sweetener is Aspartam (Figure 1), *N*-(L-α-aspartyl)-L-phenylalanine 1-methyl ester.

There are several methods of analysis of the selected sweeteners (STV and ASP) in food products using different analytical techniques either alone or in combination with other food additives. Because of the lack of chromophores in stevioside and aspartame, colorimetric assays are not used. Chromatographic techniques are the most commonly used for analysis of the selected food additives (STV and ASP) using different methods of detection for example; UV-visible, fluorescence and photodiode array detectors [2-5]. Recently in the last two decades, tandem mass spectrophotometry became the most useful and reliable technique to overcome the poor sensitivity and selectivity of other techniques. Also, the use of multiple reaction monitoring decreases the interference of the matrix. To our knowledge, there are few analytical methods using tandem mass spectrometry for the quantitation of the selected food additives (STV and ASP) [6-9]. Recently, the capillary electrophoresis with contactless conductivity used for the determination of stevioside and rebaudioside A in foods and beverages [10]. Multiple reactions monitoring is a very selective scan mode in tandem mass spectrometry. It improves method selectivity and sensitivity by decreasing the interference of matrix on interesting peak. The uses of naturally sweeteners have been increased in the last decade, however there is minimal published methods for the analysis of these food additive. The aim of this work is to provide a sensitive and selective ULPC-tandem mass spectrometry method for the analysis of STV and ASP.

ISSN 2153-2249 (Print) / ISSN 2153-2257 (Online)
http://dx.doi.org/10.5155/eurjchem.10.3.263-266.1825



(**a**) (**b**)

Figure 1. Chemical structures of (**a**) Aspartam and (**b**) Stevioside.

2. Experimental

2.1. Materials

All chemicals used were of analytical grade and solvents were of HPLC grade. Stevioside, aspartame, diphenhydramine (IS), methanol, acetonitrile, formic acid and ammonium acetate were purchased from Sigma-Aldrich, Germany. MiliQ water from Elga Labwater, Prima 7 (UK) was used during the experiment.

2.2. Instrumentations

The assay was done using a TSQ Quantum Access MAX triple stage quadrupole mass spectrometer, Thermo Scientific, New York, USA, equipped with an electrospray ionization (ESI) source and connected to Accela U-HPLC system which was composed of Accela 1250 quaternary pump and Accela Open Autosampler, New York, USA (operated at 25 °C). Acquisition and processing of the data were performed using Xcalibur software version 2.2.

2.3. Chromatographic and mass spectrometric conditions

Chromatographic separation was achieved on Hypersil-Gold column (C18-bonded ultrapure silica based column, 50×2.1 mm, 1.9 µm) using a gradient flow of 10 mM ammonium acetate, pH = 2.9 adjusted with formic acid, and 10 mM ammonium acetate in an acetonitrile:water (95:5, *v*:*v*) mobile phase (A) and an acetonitrile:water (5:95, *v*:*v*) mobile phase B. The flow rate was 0.3 mL/min, oven temperature was adjusted at 40 °C. Gradient programing started from: 20 % mobile phase B at zero time then ramped to 90 mobile phase B from 0.0-1.5 min, hold at 90% of mobile phase B till 3 min, back to 20% mobile phase B till the end of run. The injection volume was 2 µL and the total run time for each sample was 5 min. Mass spectrometer was run in positive-ion mode for all analyst and eluate was introduced to mass scanner using electrospray ionization (ESI). Quadrupole mass spectrometer used multiple reactions monitoring mode. The optimized parameters are: auxiliary gas of 5 psi, sheath gas of 25 psi, capillary temperature of 270 °C, turbo ion spray temperature of 400 °C and ion spray voltage of 3600 V. The transition of molecular ions to the product ions for STV 803.25 → 641.18 *m/z*, ASP 292.90 → 200.01 *m/z* and IS 256.20 → 167.16 *m/z*. The collision energies were 22, 24 and 19 V for STV, ASP and IS, respectively.

2.4. Standard solutions

Stock standard solutions of 1 mg/mL of each analyte and IS were prepared in methanol and stored at 4 °C. Appropriate dilution of each standard solution was done using methanol to obtain the required working standard solutions and were also stored at 4 °C.

2.5. Procedures

2.5.1. Linearity and calibrations range

Each calibrant was prepared from the working standard solution of each analyte using the required dilution. The concentration range of calibrants for STV and ASP is 2-250 ng/mL. IS (50 ng/mL) is added to each calibrant solution. A volume of 2 µL of each solution was injected into the LC-MS/MS system. The response of each calibrant is expressed as ratio of its peak area to IS peak areas and is plotted versus the corresponding concentrations.

2.5.2. Laboratory prepared mixtures

The working standard solutions of each analyte were mixed in different ratios to obtain binary solutions of STV and ASP in the concentration range of 2-250 ng/mL, using the same procedures in Section 2.5.1.

3. Results and discussion

3.1. Method development

STV contains hydroxyl group, however the formation $[M+H]^+$ is not easy and different voltage values were applied to obtain good instrument response. ASP contains aliphatic amino groups (NH_2, NH) which are easily ionized to form $[M+H]^+$. Different mass parameters were optimized to improve the intensity of peaks for example: optimizing sheath gas that facilitate the introducing of ions through the orifice of mass analyzer, however overusing of gas flow will decrease the intensity due to dispersion of the ions. Also, it was found that a spray voltage (The spray voltage is responsible for attracting the ion toward the cone of mass analyser) of less than 3600 V for the positive mode (in case of ASP and less than 3000 V for negative mode and in case of STV was not enough to attract the ions toward mass analyzer). Collision energy voltage (CE) is one of the critical parameters in MRM; by optimizing this parameters will improve the Signal/Nosie (S/N) to decreases ion suppression or enhancement of matrix. Under the optimized mass parameters, different mobile phases (Water with acetonitrile or methanol) containing neutral modifiers (ammonium formate and acetate) and acidic modifiers (formic acid and acetic acid) on HILIC, biphenyl and C18 columns were used to select the most efficient system.

Under the optimized mass and chromatographic parameters for each analyte mixture of STV and ASP were separated from each other with good resolution as shown in Figure 2. Different washing solvents were tested to avoid carryover. The addition of formic acid in the mobile phase helps to improve peak sharpness. Peak shape was satisfactory for quantitative work even at very low concentrations.

Table 1. Regression parameters for STV and ASP by the proposed LC-MS/MS method.

Item	STV	ASP
Linearity range (ng/mL)	2-250	2-250
Slope (b)	0.0172	0.002
Intercept (a)	0.0039	0.00046
LOD	0.8	0.7
LOQ	1.7	1.9
r^2	0.999	0.999

Table 2. Data of accuracy and precision obtained by the proposed method and the reported ones [3] for the analysis of STV and ASP in pure form *.

Item	STV	ASP
Mean±S.D.	99.57±0.76	101.23±0.64
% R.S.D.	7.6	6.4
n	5	5
% Error (% R.S.D./√n)	3.4	2.86
Intra-day precision	100.25±0.42	99.86±0.83
Inter-day precision	99.51±0.95	99.80±0.53

* S.D.: Standard deviation; %R.S.D.: Percent relative standard deviation.

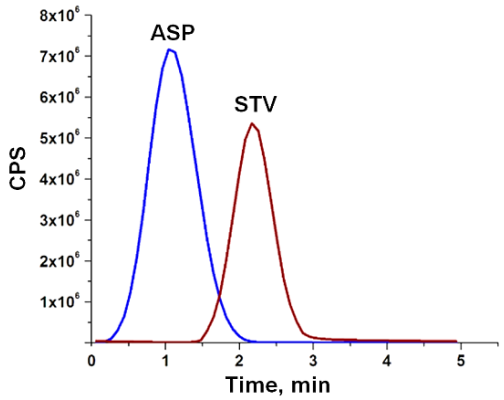


Figure 2. Chromatogram of ASP and STV at their upper limit of quantitation (ULOQ).

3.2. Method validation

Method was validated in terms of linearity, ranges, limits of detection, and limits of quantification, accuracy and precision. The method was validated according to ICH guidelines [11].

3.2.1. Linearity and range

Using the optimized mass and chromatographic parameters, the good linear relationships were obtained between concentration and peak area ratio for all analytes. The calibration curve was found to be linear in the concentration ranges of 2-250.0 ng/mL for ASP and STV. The regression parameters are listed in Table 1. Linear regression analysis of the data gave the following equations:

$y = 0.00390 + 0.0172\ C$ ($r^2 = 0.999$) for STV (1)

$y = 0.00046 + 0.0002\ C$ ($r^2 = 0.999$) for ASP (2)

where y is the peak areas ratio and C is the concentration of drug in ng/mL and r^2 is the regression coefficient. The high values of the correlation coefficients (>0.999) indicate good linearity of the calibration graphs.

3.2.2. Limit of quantitation and limit of detection

The limit of detection (LOD) is the lowest concentration of analyte that can easily detect, while the limit of quantitation (LOQ) is the lowest concentration of analyte that can be quantified by the method. Calculations of LOD or LOQ were done base on standard deviation (S.D.) of the response and slope of calibration curve (Table 1).

$LOD = 3.3\ \sigma/s$ (3)

$LOQ = 10\ \sigma/s$ (4)

where, s = Slope of calibration curve, σ = residual S.D. of response.

Residual (S.D.) of response could be calculated from S.D. of blank response or residual standard deviation of the regression line (y-residual) or S.D. of y-intercept of the regression line $S_{y/x}$, (Standard error of estimate) [12]. In the proposed method, calculation was done based on S.D. of the intercept. The results were listed in Table 1.

3.2.3. Accuracy

Evaluation of the accuracy of the proposed method was made by the analysis of five concentrations of the standard solution of each drug each concentration repeated three times. The recovery % was calculated and results of the proposed method were statistically tested for accuracy (Table 2).

3.2.4. Precision

Evaluation of the intra-day precision was made by replicate assay of the standard solutions of the studied drugs on the same day, while the inter-day precision was evaluated through replicate the assay of standard solutions of the studied drugs on three successive days (Table 2). The value of standard deviation was small what indicates that the repeatability of the proposed method is good.

Table 3. Results of system suitability of the proposed method.

Compound	Retention time (min)	Capacity factor (k)	Selectivity (α)	Resolution (Rs)	Tailing factor	Theoretical plates	HETP *
STV	1.1	0.96	-	-	1.54	4235	0.007
ASP	2.3	3.11	2.09	0.59	1.65	2896	0.045

* HETP: Height Equivalent to Theoretical Plat.

3.2.5. System suitability

System suitability applied to confirm the suitability of chromatographic system for analysis with high agrees of accuracy and precision. The suitability of method was done by determination of analytes concentration using external method (Table 3).

3.2.6. Robustness of the method

The robustness of an analytical method measures the capacity of the method to restrain minute but deliberate changes in method parameters [4]. Evaluation of the robustness of the proposed method was done for the chromatographic parameters as well as, the mass parameters, e.g. flow rate of mobile phase (±10 µL/min), vaporizer temperature or transfer capillary temperature (±5 °C), collision energy (±2 V) and sheath gas pressure (±5 psi). The changes in these parameters did not show significant changes in the values of peak areas.

3.3. Application of the proposed method

The proposed method was applied for analysis laboratory mixture of ASP and STV in different proportions (Table 4). Satisfactory results were obtained. The concentration of each drug was calculated from its regression equation.

Table 4. Determination of STV and ASP in laboratory prepared mixtures by the proposed LC-MS/MS method.

Concentration (ng/mL)		Concentration found (ng/mL)		Recovery percentage	
STV	ASP	STV	ASP	STV	ASP
5	5	4.96	4.93	99.23	98.63
25	25	24.67	25.31	98.67	101.25
50	50	48.39	48.28	96.77	96.55
100	100	98.97	100.06	98.97	100.06
200	200	194.14	194.64	97.07	97.32
400	400	395.48	396.96	98.87	99.24
Mean				98.30	98.80
S.D				1.10	1.70
% RSD				1.08	1.76

4. Conclusion

As conclusion, we developed and validated ULPC-MS/MS method for simultaneous determination of STV and ASP in dietary formulation. The utilization of µLC improves peak resolutions and separation in short time to save time and solvents. The method is simple, rapid, selective and sensitive. The proposed method was suitable for routine analysis and quality control testing of combined mixtures of all analytes.

Acknowledgement

Authors acknowledge The Pharmaceutical Services Centre and LC-MS Unit, Faculty of Pharmacy, Helwan University, Cairo, 11795, Egypt for affording the facilities during method development.

Disclosure statement

Conflict of interests: The authors declare that they have no conflict of interest.
Author contributions: All authors contributed equally to this work.
Ethical approval: All ethical guidelines have been adhered.
Sample availability: Samples of the compounds are available from the author.

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