Journal of Pharmaceutical and Biomedical Analysis 127 (2016) 184–192

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

# Comparison of hydrophilic interaction and reversed phase liquid chromatography coupled with tandem mass spectrometry for the determination of eight artificial sweeteners and common steviol glycosides in popular beverages

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ARTICLE INFO

*Article history:*
Received 26 October 2015
Received in revised form
31 December 2015
Accepted 2 January 2016
Available online 7 January 2016

*Keywords:*
HILIC–MS/MS
Artificial sweeteners
Steviol glycosides
HILIC method development

ABSTRACT

Hydrophilic interaction liquid chromatography (HILIC) coupled with tandem mass spectrometry (MS/MS) was used to separate artificial and natural sweeteners approved for use in European Union (EU). Among three tested HILIC columns (BlueOrchid PAL-HILIC, Ascentis Express Si and Acclaim™ Trinity™ P2) the last one was selected for the development of HILIC method due to the best results obtained with it. Early eluting and coeluting compounds in HILIC (acesulfame-K, saccharin, cyclamate, sucralose and aspartame) were successfully separated by the HILIC-based approach for the first time. The developed HILIC method allows for determination of all high potency sweeteners in one analytical run. The calibration curves for all analytes had good linearity within the tested ranges. The limits of detection and quantitation were in the range 0.81–3.30 ng/mL and 2.32–9.89 ng/mL, respectively. The obtained recoveries used for trueness and precision estimation were from 98.6% to 106.2% with standard deviation less than 4.1%. Sample preparation was reduced to a necessary minimum and contained only proper dilution and centrifugation. More than twenty samples of beverages were analyzed with the developed HILIC method. Finally, the chromatographic parameters of peaks (reduced retention time, width at baseline, width at 50% of peak height, tailing factor and efficiency) obtained in HILIC mode and in RPLC mode were compared. Developed HILIC method along with RPLC method can be applied for rapid evaluation of sweeteners' content, quality and safety control.



## 1. Introduction

The sugar substitutes known as artificial and natural sweeteners or substances with high sweetening power are commonly used by food producers. The possibility of the use of these food additives in food products has many benefits, including extended shelf-life, elevated quality and sweet taste. Among the available artificial sweetening substances the most popular are acesulfame-K, saccharin, cyclamates, aspartame, sucralose, alitame, neohesperidin dihydrochalcone (DC) and neotame [1,2]. New class of sweeteners known as steviol glycosides was added to this group in 2014 by the European Union (EU). These complex molecules are built of steviol and different simple sugars [3,4]. The most desired steviol glycosides, and with the highest sweetening power, are stevioside and rebaudioside A. Other minor glycosides are dulcoside A, steviolbioside, rubusoside and rebaudioside C, D, E, and F.

The use of high potency sweeteners is governed by the Regulation of the European Parliament and Council Regulation No. 1333/2008 [5], as amended by regulation No. 1129/2011 establishing a list of food additives [6]. For steviol glycosides another regulation was established [7]. Since April 2013, neohesperidin DC and one of the steviol glycosides (rebaudioside A) have been approved for use as flavoring substances by regulation No. 872/2012 [8].

All of the above mentioned sweeteners were successfully separated by reversed phase liquid chromatography (RPLC) [9]. Many other methods based on RPLC coupled with mass spectrometry or UV–vis detection are known and well described [1,10–21]. Due to the rapid development of the HILIC technique it was decided to check whether it can provide results similar to those obtained with RPLC-based methods. Theoretically, the HILIC mode allows for achieving better sensitivity when using a mass spectrometer (MS) as a detector. Furthermore, there is no method based on the HILIC technique that allows the separation of all EU-authorised high

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http://dx.doi.org/10.1016/j.jpba.2016.01.006
0731-7085/

potency sweeteners. In most cases only a few representatives of sweeteners highly soluble in water are chosen for the HILIC-type separation methods [22–24]. In some cases HILIC mode separations were eliminated in preliminary studies [10,25] as providing insufficient resolution and undesirable peak shapes. Other methods suffer from the coelution of acesulfame-K with saccharin and cyclamate with sucralose, as well as poor peak shape for aspartame [26]. The coelution of sucralose and neohesperidin DC was also observed [27]. In fact, in HILIC-type methods acesulfame-K, cyclamate and saccharin tend to elute close to the void time, despite the high organics content in the mobile phase. Nevertheless, the separation of water-soluble steviol glycosides can be achieved in the HILIC mode, and symmetrical peaks are observed [23,28–30].

The main objective of this research was to develop a method for the determination of natural and artificial sweeteners with the use of the HILIC technique coupled with tandem mass spectrometry detection (MS/MS). The other objectives included separation of early eluting compounds in the HILIC mode (acesulfame-K, cyclamate) and obtaining symmetrical peak shapes, comparable to those attained by RPLC methods. Finally, the chromatographic parameters (reduced retention time, width at baseline, width at 50% of peak height, tailing factor at 10% of height, efficiency and plate height) of peaks obtained in HILIC separation mode were compared to those obtained with the use of the previously described RPLC method [9]. The developed HILIC method allows the quantification of fourteen compounds during one analytical run with low limits of quantification (LOQ) values, recoveries close to 100% and good repeatability. The performance of the method was checked during the analysis of more than twenty samples of popular soft and alcoholic beverages.

## 2. Materials and methods

### 2.1. Chemicals

The following standards of artificial sweeteners and steviol glycosides were acquired: acesulfame-K, from Nutrinova (Frankfurt am Main, Germany), saccharin, sucralose and neohesperidin DC, from Sigma–Aldrich (St. Louis, USA), aspartame, from Ajinomoto Foods Europe (Nesle, France), cyclamate, from Merck KGaA (Darmstadt, Germany), alitame, from Frapp's Pharma (Hong Kong, China), neotame, from CHEMOS (Regenstauf, Germany), and rebaudioside A, stevioside, rebaudioside C, dulcoside A, steviolbioside, and steviol, from LGC Standards (Łomianki, Poland). The internal standard (IS) was sodium *N*-(2-methylcyclohexyl) sulfamate [16] synthesized on site. Acetonitrile (ACN) was purchased from Merck KGaA (Darmstadt, Germany). Ammonium acetate ($NH_4Ac$) was obtained from Sigma–Aldrich (St. Louis, USA). Acetic acid (AA) was purchased from POCH (Gliwice, Poland). Ultrapure water was produced by the HLP5 system from Hydrolab (Wiślina, Poland).

### 2.2. Samples

Twenty-one samples of alcoholic and non-alcoholic beverages, and three instant drink powders were purchased from local shops. Many of the bought products were labelled as containing steviol glycosides, although some of them contained artificial sweeteners as well. Three of them were free from any sweetener.

### 2.3. Preparation of standards and calibration solutions

Individual stock solutions of all sweeteners and IS were prepared by dissolving a proper amount of them in a mixture of ACN:$H_2O$ (60 + 40). The final concentration of each standard was around 50 ng/mL. Calibration solutions were prepared by mixing and dilution of the stock solutions with mobile phase component B (ACN 0.01% v/v AA). Two different calibration ranges were chosen for artificial and natural sweeteners. For acesulfame-K, saccharin, neohesperidin DC, aspartame, sucralose, cyclamate, alitame and neotame the concentrations of calibration solutions were 5, 20, 50, 100, 200, 400 and 800 ng/mL of each. For rebaudioside A, stevioside, rebaudioside C, dulcoside A, steviolbioside and steviol the concentrations were as follows: 5, 20, 100, 300, 600, 1000, and 1600 ng/mL. In all calibration solutions the concentration of IS was maintained at 50 ng/mL. Stock solutions and calibration solutions were stored in a refrigerator at 4 °C, and every month new solutions were made.

### 2.4. Sample preparation procedure and spiked samples

All samples of beverages were degassed in a sonic bath for 15 min. Powders of instant drinks were prepared according to the labels on them. An aliquot of a sample was placed in a volumetric flask together with appropriate amount of IS solution and diluted one hundred times with mobile phase component B (ACN 0.01% v/v AA). This dilution was enough to fit all results into the calibration curves ranges. The concentration of IS in diluted samples was equal to 50 ng/mL. Next, a solution of the sample was placed in an eppendorf tube and centrifuged for 5 min at 7000 rpm. Supernatant was collected and analyzed directly. The procedure for preparation of spiked samples was described in the previous publication [9].

### 2.5. MS/MS conditions

All analyses were done using a Shimadzu LC–MS–MS system (LCMS-8050, Shimadzu, Japan) with an ESI source in the polarity switching mode. Multiple reaction monitoring mode (MRM) was employed for quantitation purposes. Conditions of ion transitions were chosen separately for the HILIC mode and for the RPLC mode [9]. The parameters of the ion source were the same for both methods. The parameters of ion transitions and conditions of the ESI source for a method based on HILIC are presented in Table S1 (Supplementary material). For most of the compounds the negative mode of ionisation was chosen, except for aspartame, alitame and neotame. For these three compounds higher intensity was observed in the positive mode. In the case of sucralose, acetic acid adduct (454.85) produced much higher intensity of ion transition than fragmentation of the pseudomolecular ion (395.05). The steviol molecule does not produce any observable fragment ions, either in the negative or positive mode. For this compound the pseudo-transition in the negative mode was chosen (317.30 → 317.40).

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2016.01.006.

### 2.6. Separation conditions

The chromatographic separation was done using the UPLC Nexera X2 system (Shimadzu) consisting of the following components: degasser DGU-20A5R, controller CBM-20A, binary pump LC-30 AD, autosampler SIL-30AC and thermostated column oven CTO-20AC.

Among the available HILIC columns three were chosen: BlueOrchid PAL–HILIC 100 mm × 2 mm, 1.8 μm (Knauer), Ascentis Express Si 150 mm × 2.1 mm, 3 μm (Supelco) and Acclaim™ Trinity™ P2 100 mm × 2.1 mm, 3 μm (Thermo Fisher Scientific). A further discussion of the results obtained with all three columns is presented in Section 3.1. For the final HILIC method the Acclaim™ Trinity™ P2 column was chosen. Separation conditions for HILIC and RPLC methods are presented in Table 1.

## 3. Results and discussion

### 3.1. Separation of analytes

The main objective was to separate all sweeteners together with steviol as the main building block of steviol glycosides.